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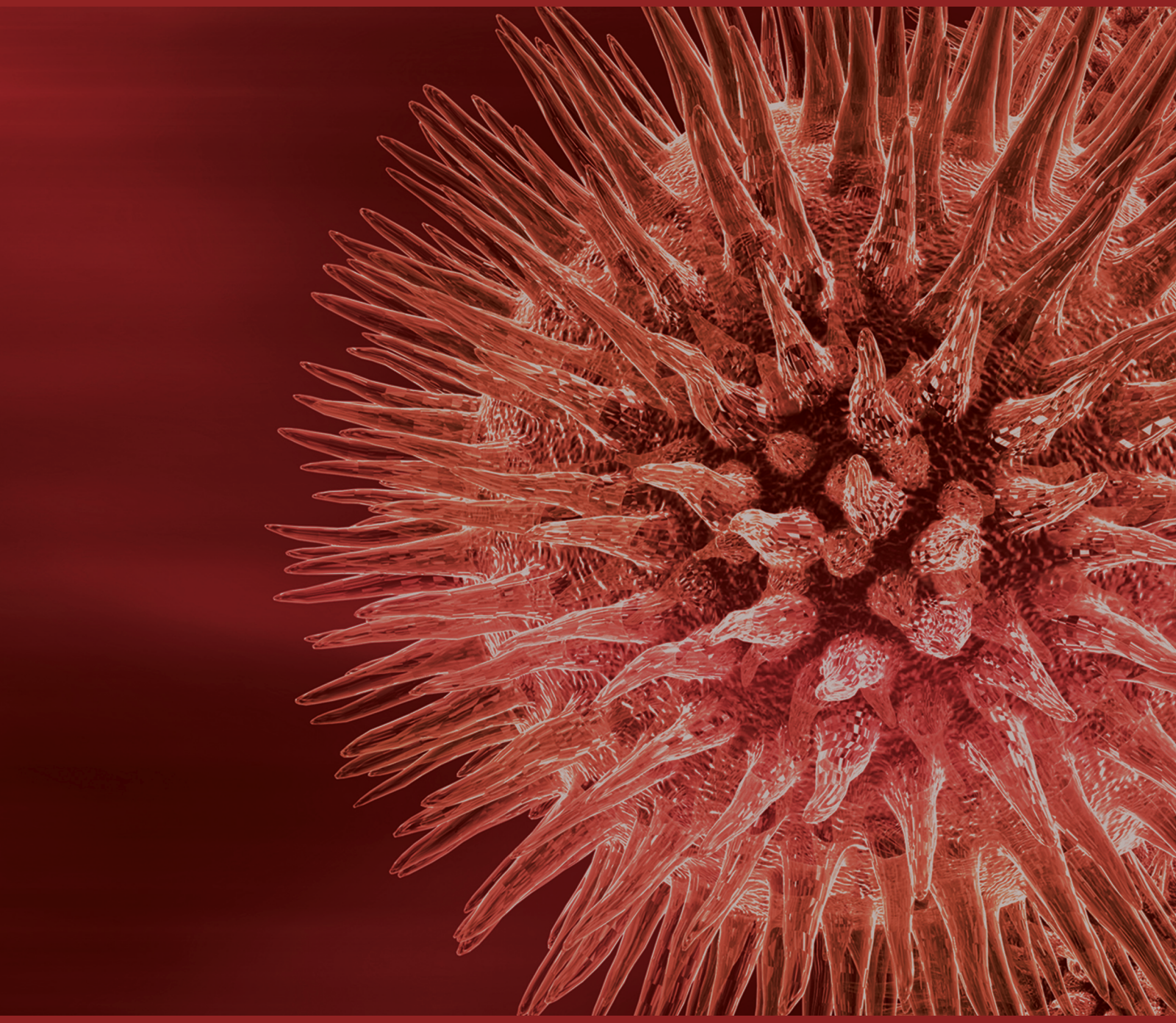
Stem Cells in Reproductive Tissues: From the Basics to Clinics

Guest Editors: Irma Virant-Klun, Thomas Skutella, Deepa Bhartiya, and Xuan Jin



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Contents

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Editorial

Stem Cells in Reproductive Tissues: From the Basics to Clinics

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Twenty-one articles were accepted for publication in this special issue. All these contributions confirmed that human and animal reproductive tissues represent a very interesting and heterogeneous source of stem cells: from ovarian and testicular stem cells to embryonic (J. M. Campbell et al.; S.-J.Park et al.), trophoblast (M. Weber et al.), endometrial decidual (E. Rossignoli et al.), and umbilical cord stem cells, and induced pluripotent stem (iPS) cells. Moreover, recent research shows that, like in other adult organs and tissues, small embryonic-like stem cells (VSELs) are present in human reproductive tissues and may play an important role in the reproductive biology, as reviewed in the article "*Very small embryonic-like stem cells (VSELs): implications in reproductive biology*" by D. Bhartiya et al.

There is more and more evidence that human spermatogonial stem cells are fact and not fiction. It is possible to short-term and long-term culture the human and animal spermatogonia (L. A. Martin and M. Seandel), and moreover, it is a time to critically stress the possibility of restoring fertility in sterile childhood cancer survivors by autotransplanting spermatogonial stem cells (R. B. Struijk et al.). An efficient clinical grade cryopreservation protocol for human testicular tissue and cells including spermatogonia is proposed by J. Pacchiarotti et al. In spite of the critical scientific debate if human testicular germ stem cells expressing a degree of pluripotency really exist, there is some further confirmation of their real existence. In the article "*In vitro culture-induced pluripotency of human spermatogonial stem cells,*" J. J. Lim et al. provided strong evidence that human germ stem cells from adult testis show similarities with embryonic stem cells being able to generate real teratomas after injection into SCID mice. Therefore, the natural plasticity of adult human spermatogonial stem cells could enable a clinical perspective in contrast to artificial iPS cells which are created by overexpression of transcription factors. In spite of that, iPS cells are a perfect model to study the *in vitro* spermatogenesis, as confirmed in the mouse model. The paper by P. Li et al. explored the differentiation potential of mouse iPS cells towards male germ cells; the expression of marker proteins, including MVH, CDH1, and SCP3, was remarkably increased, and mRNA expression of *Stra8*, *Odf2*, *Act*, and *Prm1* was upregulated in iPS cells by retinoic acid or testosterone induction. Interestingly, there is also some new evidence that even mesenchymal stem cells may play an important role in restoration of fertility. In the very interesting research article "*Recovery of fertility in azoospermia rats after injection of adipose tissue derived mesenchymal stem cells: Sperm Generation*" by C. Cakici et al., the authors argue for the provocative idea of the *in vivo* transition and differentiation of adipose-derived mesenchymal stem cells into the direction of sperm after transplantation into the testes of sterilized rats.

Putative stem cells from adult human ovaries are an exciting and promising research subject which might lead to the clinical practice in the future. I. Virant-Klun et al. confirmed the development of oocyte-like cells from putative stem cells scraped from the ovarian surface epithelium of

women with severe ovarian infertility-premature ovarian failure. These cells expressed several genes related to pluripotency and oocytes, as revealed by single-cell gene expression profiling, but were still more stem cells than real oocytes. Additionally, in the review paper "*Gene expression profiling of human oocytes developed and matured in vivo or in vitro*" by I. Virant-Klun et al., the authors present new knowledge about oocyte quality and oogenesis *in vivo* and *in vitro* in terms of gene expression profile, which might be introduced into clinical practice in the future. But the ovarian surface epithelium is far to be the only potential source of stem cells in adult human ovaries. K. C. Kossowska-Tomaszczuk and De Geyter reviewed the existence of stem cells in somatic compartments of the ovaries, including granulosa cells. In addition to the reproductive potential, there is still another important aspect of ovarian stem cells: the manifestation and potential new treatment of aggressive ovarian cancer. In their review article, J. Pasquier and A. Rafii exposed the importance of the microenvironment in ovarian cancer stem cell maintenance. Ovarian stem cells may be a new target for cancer therapy and more individualized treatment in the future, as proposed by Zhan et al.

Interestingly, there are also some other sources of stem cells which may be interesting for reproductive and regenerative medicine in the future. D. Zhang et al. were able to show in their article "*Estradiol synthesis and release in cultured female rat bone marrow stem cells*" that female bone-marrow-derived stem cells can synthesize and release estradiol and may contribute to autologous transplantation therapy for estrogen deficiency in reproductive medicine. Additionally, fetal stem cells might represent an important support to *in vitro* culturing and differentiation of stem cells. In the article by J. Xi et al., the authors describe an erythroid liquid culture system starting from cord blood-derived hematopoietic stem cells for the homogeneous erythroid cells cultured *in vitro*. The large number and purity of erythroid cells and red blood cells produced from cord blood make this method useful for fundamental research in erythroid development. J. Li and G. Lepski reviewed different sources of stem cells which can be used for spinal cord injury treatment in the future, including mesenchymal stem cells from human umbilical cord which have already been successfully differentiated into Schwann-like cells *in vitro* and grafted into the lesion sites of spinal cord injury rats; a partial recovery of motor function was reported. Last but not least, adipose-derived stem cells are an important future prospect for regenerative medicine. X. Zhu et al. successfully established a lentiviral vector encoding human hepatocyte growth factor (hHGF) and infected human adipose-derived stem cells. In this way they produced cells that overexpressed hHGF, which may provide a new strategy for the treatment of ischemic heart disease and other ischemic diseases in the future.

The human reproductive tissues provide an extremely interesting source of stem cells from both the basic and clinical views, which are more natural than iPS cells and also avoid several dilemma related to human embryonic stem cells. It was shown that the donation of surplus embryos for research is a worldwide problem. In the article of X. Jin et al. "*Patients' attitudes towards the surplus frozen embryos in China*," it was found that 58.8% of the infertile couples included in the *in vitro* fertilization programme preferred to dispose surplus frozen embryos rather than donate them to research, mostly citing a lack of information and distrust in science as significant reasons for their decision.

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Research Article

Isolation, Characterization, and Transduction of Endometrial Decidual Tissue Multipotent Mesenchymal Stromal/Stem Cells from Menstrual Blood

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Mesenchymal stromal/stem cells (MSCs) reveal progenitor cells-like features including proliferation and differentiation capacities. One of the most historically recognized sources of MSC has been the bone marrow, while other sources recently include adipose tissue, teeth, bone, muscle, placenta, liver, pancreas, umbilical cord, and cord blood. Frequently, progenitor isolation requires traumatic procedures that are poorly feasible and associated with patient discomfort. In the attempt to identify a more approachable MSC source, we focused on endometrial decidual tissue (EDT) found within menstrual blood. Based also on recent literature findings, we hypothesized that EDT may contain heterogeneous populations including some having MSC-like features. Thus, we here sought to isolate EDT-MSC processing menstrual samples from multiple donors. Cytofluorimetric analyses revealed that resulting adherent cells were expressing mesenchymal surface markers, including CD56, CD73, CD90, CD105 and CD146, and pluripotency markers such as SSEA-4. Moreover, EDT-MSC showed a robust clonogenic potential and could be largely expanded *in vitro* as fibroblastoid elements. In addition, differentiation assays drove these cells towards osteogenic, adipogenic, and chondrogenic lineages. Finally, for the first time, we were able to gene modify these progenitors by a retroviral vector carrying the green fluorescent protein. From these data, we suggest that EDT-MSC could represent a new promising tool having potential within cell and gene therapy applications.

1. Introduction

Mesenchymal stromal/stem cells (MSCs) are adult progenitor cells isolated from several human adult and perinatal tissues [1, 2]. While the acronym MSC seems to equate the biological properties of these useful progenitors, the possibility to isolate these cells from different tissue has been outlining common features combined with source-specific peculiarities that are still under investigation.

MSC demonstrated a positive impact in several pathological conditions [1, 3, 4], exerting their therapeutic functions on damaged tissues through different mechanisms, including differentiation into mature cells and largely obscure paracrine effects [5]. Moreover, MSCs have also been investigated for

their possible use to deliver wild-type or gene modification-induced bioactive molecules with promising, but still undefined, influence in cancer models [6–9]. In the light of these findings, it appears reasonable to propose that a tissue source of MSC could be deemed relevant and useful by determining if it provides cells with varied differentiation potential and distinct cytokine profiles that may indicate an advantageous role in the interaction with tumors [1, 10]. Therefore, understanding these features from distinct MSC tissue sources shall be a primary objective to efficiently translate these cells into different clinical applications.

One most recognized source of MSC has been the bone marrow obtained from iliac crest and more recently, MSC progenitors have been isolated from lipoaspirates and other tissue sources including teeth, bone, muscle, placenta, liver, pancreas, umbilical cord, and cord blood [11]. In the majority of these cases, especially the autologous sources, tissue isolation requires traumatic procedures sometimes linked with patient's discomfort.

In the attempt to identify a different and more approachable MSC source for cell and gene therapies, we focused on endometrial decidual tissue (EDT) obtained from menstrual blood. Previous cloning studies of isolated human endometrial cells provided early evidence of rare clonogenic mesenchymal cells, representing approximately 1% of endometrial cells suspension obtained by uterine tissue digestion after hysterectomy [12]. These endometrial stromal elements demonstrated properties similar to bone marrow and adipose tissue MSC including substantial self-renewal ability *in vitro*, high proliferative potential, and multilineage differentiation [13, 14]. However, obtaining these cells directly from endometrium either after hysterectomy or by biopsy still implies invasive procedures.

In order to evaluate the possibility of obtaining multipotent cells from the uterus by a non-invasive and reproducible manner, researchers started to analyze shed menstrual blood and tissue in the attempt to identify menstrual blood-derived MSC [15, 16]. These pilot studies confirmed what was already known for endometrial cells obtained from hysterectomy, suggesting that endometrial stem/progenitor cells may be shed in menstrual blood.

Following these early findings and by different isolation steps, this study characterizes EDT as a novel tissue source of MSC with regards to possible applications in regenerative medicine and gene therapy. MSCs from different sources have been investigated for their possible uses as tumor-specific delivery vehicles for suicide genes, oncolytic viruses, or secreted therapeutic proteins [8]. The mechanisms through which MSCs are considered to home to tumors are not completely clear, but seem to be dependent on biological properties of tumor microenvironment as well as the native tropism of selected MSC and also on the experimental procedures used [10, 17]. Thanks to genetic manipulation techniques, this natural tropism has been recently and convincingly exploited to transform MSC in "bullets" capable to deliver oncolytic viruses or various anticancer agents directly into tumor sites [8]. The possibility of having noninvasive procedures to obtain autologous progenitor cells, although with a gender limitation, paves the way to a more intense research activity aimed to deeply characterize MSC from a different source for selected biomedical applications.

2. Materials and Methods

2.1. Cell Procurement and Processing. Menstrual blood was collected from healthy female volunteer donors ($n = 3$) during the first few days of the cycle. Written consent was obtained from each donor and the Local Ethical Committee approved cells donation for research purposes. Each donor has been endowed with a menstrual cup (DivaCup, Diva International, San Francisco, CA, USA) to collect blood, which was transferred in phosphate buffered saline (PBS, PAA Laboratories, Pasching, Austria) with 1% penicillin/streptomycin (10,000 U/mL Penicillin, 10 mg/mL Streptomycin in 0.9% NaCl solution, PAA Laboratories), 35 mg/mL fluconazole (Diflucan, Pfizer, New York, NY, USA) and heparin (500 U/mL, Sigma, St. Louis, MO, USA). Samples were maintained at 4°C for 24–48 h after procurement until reaching the processing laboratory.

The endometrial tissue, if present, was discarded, and the remaining blood was homogenized by 20 passages through a 19G needle using a 10 mL syringe. Cell suspension was then cultured as below reported. In addition, two alternative isolation protocols involving separation of the corpuscular fraction of blood through normal or density-gradient centrifugations were initially introduced. However, these latter two approaches were discontinued because of poor isolation efficiency (data not shown).

2.2. Cell Culture. Isolated cells were seeded into culture flasks to obtain an adherent fraction by adding αMEM (Gibco), 1% L-glutamine (200 mM in 0.85% NaCl solution, Lonza Verviers, Belgium), 1% penicillin/streptomycin (PAA Laboratories), and 10% fetal bovine serum (FBS; PAA Laboratories). In addition, a serum-deprived medium (Quantum 333) with 1% penicillin/streptomycin was also introduced (all from PAA Laboratories). Cells were cultured for 7 days, washed with PBS (PAA Laboratories) to remove the nonadherent fraction, and fresh medium was added. At confluence, cells were detached by trypsin (trypsin 0.05% EDTA 0.02% in PBS, EuroClone, Milan, Italy) and subcultured at a density of 6000 cells/cm^2 until functional assays. All flasks were incubated at 37°C, 5% CO_2, and medium was changed every other day.

2.3. Clonogenic Assay. Adherent cells, out of passage 1 (P1) or passage 2 (P2), were seeded at clonal density of 100 cells/cm^2 in αMEM (Gibco), 1% L-glutamine (Lonza), 1% penicillin/streptomycin (PAA Laboratories), and 10% FBS (PAA Laboratories). Colony formation was monitored daily. On day 10, cells were fixed with cooled absolute methanol for 2 minutes and stained for 5 minutes with 1% crystal violet aqueous solution (Sigma). Colonies with more than 50 cells were then counted, as originally described for marrow MSC [18]. Each assay was repeated in triplicate, and cloning efficiency (E) was calculated as $E\% = (\text{n. clones/cells seeded}) \cdot 100$.

2.4. Proliferation Assay. Adherent cells were seeded at a density of 6000 cells/cm^2 in αMEM (Gibco), 1% L-glutamine (Lonza), 1% penicillin/streptomycin (PAA Laboratories), and 10% FBS (PAA Laboratories) in different flasks for the following time points: 24, 48, 72, and 96 hours. At each time point, cells were trypsinized and counted. Data obtained were plotted as number of harvested cells (y) against hours of culture (x), and the exponential growth curve was generated using GraphPad Prism software (GraphPad Software Inc., version 5.00). The doubling time (T) was obtained from the growth constant (k) of the exponential equation $y = a \cdot e^{kx}$, where $k = \ln 2/T$.

2.5. Senescence-Associated β-Galactosidase Staining. To assess cell senescence, the activity of β-galactosidase (β-gal) at pH 6 was evaluated. β-gal expression is a feature of senescent cells [19]. According to Senescence β-galactosidase Staining Kit (Cell Signaling Technology, Beverly, MA, USA), growth medium was removed, and cells were washed with PBS (PAA Laboratories). Cells were then incubated for 15 minutes with a fixative solution (20% formaldehyde, 2% glutaraldehyde in 10x PBS) and washed twice with PBS (PAA Laboratories). Color development was obtained by incubation overnight at 37°C with the provided staining solution (40 mM citric acid/sodium phosphate pH 6.0, 150 mM NaCl, 2 mM $MgCl_2$, 5 nM potassium ferricyanide, and 1 mg/mL X-gal in DMSO). Plates were then observed by microscopy for the development of blue color.

2.6. FACS Analyses. Adherent cells were harvested for surface antigen analysis. Briefly, cells were detached from plastic support by trypsin (EuroClone), counted, and aliquoted in FACS analyses polypropylene tubes ($0.5–1 \cdot 10^6$ cells/tube) (VWR, Milan, Italy). EDT-MSCs were subsequently incubated in blocking buffer (100 μL each $0.5–1 \cdot 10^6$ cells) containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco), 10% FBS (PAA Laboratories), and 0.1 M sodium azide and human immunoglobulin G (both from Sigma) and incubated for 20′ on ice. After a PBS (PAA Laboratories) washing step, cells were resuspended in PBS (PAA Laboratories) with 0.5% bovine serum albumin (BSA, Sigma) and stained on ice and in the dark for 30′ with the following monoclonal antibodies: APC-anti-CD45, FITC-anti-HLADR, PE-anti-CD34, PE-anti-CD14, and FITC-anti-CD56 (all from Becton Dickinson, Franklin Lakes, NJ, USA); PE-anti-CD31 (BioLegend); APC-anti-CD90, PE-anti-SSEA-4 (both from eBioscience, San Diego, CA, USA); FITC-anti-CD105, PE-anti-CD73 (all from BD Pharmingen); APC-anti-CD146 (Miltenyi Biotec). In all the experiments, the corresponding isotype-matched antibodies were used as negative controls (BD Pharmingen and Becton Dickinson). Data were collected using a FACS Aria III flow cytometer (BD Biosciences) and analyzed on FACS Diva software (BD Biosciences).

2.7. Multilineage Differentiation Assays. To assess *in vitro* differentiation capacities, adherent cells after P2 were cultured in specific induction media. Media were changed every other day and undifferentiated controls were concurrently cultured in αMEM (Gibco), 1% L-glutamine (Lonza), 1% penicillin/streptomycin (PAA Laboratories), and 10% FBS (PAA Laboratories) for the same incubation time. Each assay was performed in triplicate.

Adipogenic, osteogenic, and chondrogenic differentiations were performed as previously reported [20]. Briefly, for osteogenic differentiation, cells were seeded at the density of 10000 cells/cm^2 and maintained in the growth medium until confluence. Culture medium was then substituted with the induction one, composed by αMEM (Gibco) supplemented with 10 nM dexamethasone (Sigma), 10 mM β-glycerol phosphate (Sigma), 0.1 mM L-ascorbic acid-2-phosphate (Sigma), 10% defined FBS (Hyclone, Logan, UT, USA), 1% L-glutamine (Lonza), and 1% penicillin/streptomycin (PAA Laboratories). Induction was maintained for 14 days and, from the seventh day, 100 ng/mL BMP-2 (Peprotech, Rocky Hill, NJ, USA) was added. Confirmation of osteogenic differentiation was performed through Alizarin Red staining combined by real-time qPCR analysis (described below). At the end of the induction period, culture wells were washed briefly with a buffer solution containing 20 mM Tris-HCl and 150 mM NaCl in water. Cells were then fixed and dried with cooled absolute methanol for 2 minutes. After a washing step with ddH_2O, cells were stained by 1.5% Alizarin Red aqueous solution pH 4.0–4.2 (Sigma) for 5 minutes and washed again first with ddH_2O and then with PBS (PAA Laboratories) for 15 minutes. The last step consisted of dehydration using cooled absolute ethanol for 2 minutes followed by microscopic observation.

For adipogenic differentiation, EDT-MSCs were seeded at the density of 10000 cells/cm^2 and maintained in the growth medium until confluence. The medium was then substituted with the induction one, composed of αMEM (Gibco) supplemented with 1 μM dexamethasone (Sigma), 60 μM indomethacin (Sigma), 10 μM insulin (Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma), 10% rabbit serum (EuroClone), 5% horse serum (EuroClone), 1% L-glutamine (Lonza), and 1% penicillin/streptomycin (PAA Laboratories). Induction lasted 10 days and confirmation of differentiation was achieved through Oil Red O staining, as follows. Culture wells were washed briefly with PBS (PAA Laboratories). Cells were fixed with vapors of 37% formaldehyde (Sigma) for 10 minutes and then washed with water for 2 minutes. Staining was obtained adding an Oil Red O solution (10 mg/mL Oil Red O in ethanol 70% and acetone; all from Sigma) into the wells for 3 minutes. Excessive stain was removed by washing with water and cells were then counterstained by Harris hematoxylin (Bio-Optica, Milan, Italy) for 30 seconds.

Finally, for chondrogenic differentiation $2–5 \cdot 10^5$ EDT-MSCs were aliquoted in a 2 mL tube and then centrifuged at 1200 rpm for 10 minutes. Cells were maintained pelleted in the growth medium at 37°C with the plug opened. After 2 days of incubation, tubes were centrifuged again, and the medium was substituted with the induction one, composed by αMEM (Gibco) supplemented with 100 nM dexamethasone (Sigma), 200 μM L-ascorbic acid-2-phosphate (Sigma), 100 μg/mL sodium pyruvate (Biochrom AG, Berlin, Germany), 40 μg/mL proline (Sigma), 1x ITS+ premix (BD Biosciences, San Jose, CA, USA), 10 ng/mL TGF-β (Peprotech), 0.5 μg/mL BMP-6 (Peprotech), 1% L-glutamine (Lonza),

Table 1: Primer sequences.

Gene	Primer sequence	Amplified length
β-actin	5′-ACC TTC TAC AAT GAG CTG CG-3′ (sense) 5′-CCT GGA TAG CAA CGT ACA TGG-3′ (antisense)	148 bp
ALP	5′-GAT GTG GAG TAT GAG AGT GAC G-3′ (sense) 5′-GGT CAA GGG TCA GGA GTT C-3′ (antisense)	142 bp
COL1A2	5′-AGG ACA AGA AAC ACG TCT GG-3′ (sense) 5′-GGT GAT GTT CTG AGA GGC ATA G-3′ (antisense)	146 bp
OC	5′-CAG CGA GGT AGT GAA GAG AC-3′ (sense) 5′-TGA AAG CCG ATG TGG TCA G-3′ (antisense)	144 bp

ALP: alkaline phosphatase, COL1A2: collagen 1A2, OC: osteocalcin.

and 1% penicillin/streptomycin (PAA Laboratories). Before and after each medium change, tubes were centrifuged at 1200 rpm for 10 minutes. During the incubation period, cells remained as pellet with the tube plugs opened. Induction lasted 21 days, and specimens were fixed for 1 hour in 10% formaldehyde (Sigma) and then dehydrated by serial passages into ethanol at increasing concentrations, from 70% to 100%. Samples were then included into paraffin blocks and cut in slices on microscope slides for Alcian Blue staining. Slides were deparaffinized with the Histo-C cleaning agent (Celltech, Turin, Italy) and rehydrated through passages into a decreasing concentration alcoholic ladder (from 100% ethanol to 70% ethanol). Sample sections were then incubated with a 0.5 mg/mL Hyaluronidase (Sigma) in buffer phosphate solution (8 g/L NaCl, 2 g/L NaH_2PO_4, and 0.3 g/L Na_2HPO_4) with 10 mg/mL BSA (Sigma). Slides were washed in water for 5 minutes and then immersed in a 3% acetic acid solution for few seconds. Staining with 10 mg/mL Alcian Blue solution in 3% acetic acid (pH 2.5, Sigma) lasted 30 minutes, and after a washing step in water, samples were counterstained for 5 minutes with nuclear fast red solution (Sigma) and then washed in water.

2.8. Real-Time qPCR. Total cellular RNA was isolated from osteogenic committed and uncommitted cells, using Trizol reagent method (Invitrogen) according to manufacturer's instructions. Samples were concentrated by ethanol precipitation and suspended in RNase-free water. RNA quantity was assessed by spectrophotometry (DU730 UV/VIS Spectrophotometer; Beckman Coulter, Milan, Italy). A 2 μg aliquot was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas) by oligo $(dT)_{18}$ primers in a final volume of 20 μL. Reactions were performed at 42°C for 1 h with a final step at 70°C for 5 minutes. cDNA was then used to determine the osteoblast-associated gene expression by quantitative real-time PCR technique using Step One Real-Time PCR System Thermal Cycling Block (Applied Biosystems, Foster City, CA, USA). Each sample was compared with noninduced control for the expression of alkaline phosphatase, collagen 1A2, and osteocalcin. β-actin was used as a reference gene. All primers were purchased from Integrated DNA Technologies, and sequences are reported in Table 1. PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems) which uses AmpliTaq Fast DNA Polymerase, SYBR Green I dye to detect double-stranded DNA, and ROX dye as a passive internal reference. Reaction proceeded through an initial step at 95°C for 20″, followed by 40 cycles of denaturation (3″ at 95°C), annealing, and extending (30″ at 60°C). The final stage comprises the analysis of the melt curve through a denaturing step (15″ at 95°C) followed by annealing (1′ at 60°C) and ramping to 95°C with 0.3°C increment/step. Levels of mRNA for tested genes were quantified using ΔΔCT method and normalized against human β-actin as a housekeeping gene. Data have been analyzed by StepOne software (version 2.1, Life Technologies Corporation, Carlsbad, CA, USA).

2.9. Gene Modification of EDT-MSC. Cultured cells were infected by a bicistronic murine stem cell virus-derived retroviral vector (pMIGR1) encoding for green fluorescent Protein (GFP). Retrovirus production was performed by the FLYRD packaging cell lines, as published by Marx et al. [21]. FLYRD cells were seeded in a T175 flask with 10 mL of medium composed by DMEM (Gibco) supplemented with 10% defined FBS (Hyclone). Cell supernatant was collected and filtered (PES) with a 0.45 μm filter, and EDT-MSC were incubated for 6 hrs with 5 mL culture medium, composed by: αMEM (Gibco), 1% L-glutamine (Lonza), 1% penicillin/streptomycin (PAA Laboratories), and 10% FBS (PAA Laboratories) with the addition of 5 mL viral particles-containing supernatant and 6 μg/mL polybrene (Sigma). Cells were then washed by PBS (PAA Laboratories) and culture medium was changed. The infection step was repeated for three consecutive days at which time cells were evaluated at FACS Aria III flow cytometer (BD Biosciences) for GFP protein expression. 7-amino-actinomycin D (7AAD) staining was also performed to evaluate mortality after transduction. Cells were evaluated by FACS Aria III (BD Biosciences), and data were analyzed using FACS Diva software (BD Biosciences).

2.10. Statistics. Data are expressed as average values, and analyses were performed by GraphPad Prism software. *t*-test was considered as significant with P value <0.05.

3. Results

3.1. EDT-Derived Cells Are In Vitro Heterogeneous but Retain Predominant MSC Features. Adherent cells isolated from menstrual blood initially displayed *in vitro* fibroblast shape morphology in both αMEM with 10% FBS and Quantum 333

(Figures 1(a)-1(b)). However, prolonged cultures in αMEM with 10% FBS showed a significant better growth performance, and this medium was then preferentially used for all subsequent functional analyses. Adherent elements had typical mesenchymal aspect being fibroblastoid-like, spindle-shaped cells with an elongated cytoplasm. Within this population, it was also possible to identify more infrequent cell clusters with a distinct morphology. These appeared as endothelial-like sometimes binucleated cells, forming a monolayer with polygonal shape (Figure 1(c)). However, we did not confirm the nature of this population, because their presence disappeared after very early passages and because they were not the subjects of this study.

3.2. EDT-Derived Cells Show Robust Clonogenic and Proliferative Potential. Having observed the fibroblast shape of EDT isolated cells, we then focused on their clonogenic and proliferative potential. We observed a high clonal efficiency with an average of 14.1% (10.8–17.9%) of the seeded cells able to generating colonies. This result indicates that the menstrual-derived cell population contains a large fraction of actively cycling cells with signficant clonogenic potential (Figure 2(a)). Of interests, colonies did not appear homogenous, and we were able to identify at least two kinds of morphologies. On the one hand, densely populated clones constituted by small size cells (Figures 2(b)-2(c)) and on the other, smaller cell clusters with elements having a large cytoplasm with an evident cytoskeleton (Figures 2(d)-2(e)).

Isolated and expanded cells also demonstrated surprisingly low doubling time, with an average value of 27.6 hours (21.9–33.0) (Figure 3). The high number of passages that these cells were able to reach, further supported this remarkable growth property. The β-galactosidase staining, performed to evaluate senescent cells, showed that cells cultured in Quantum 333 underwent senescence at passage 17, while those cultured in αMEM supplemented with 10% FBS reached passage 26 before growth arrest (Figures 4(a)–4(d)). Together these data suggest how plastic-adherent, fibroblast shaped cells from EDT retain a strong proliferative potential comparable or even superior to MSC from other sources.

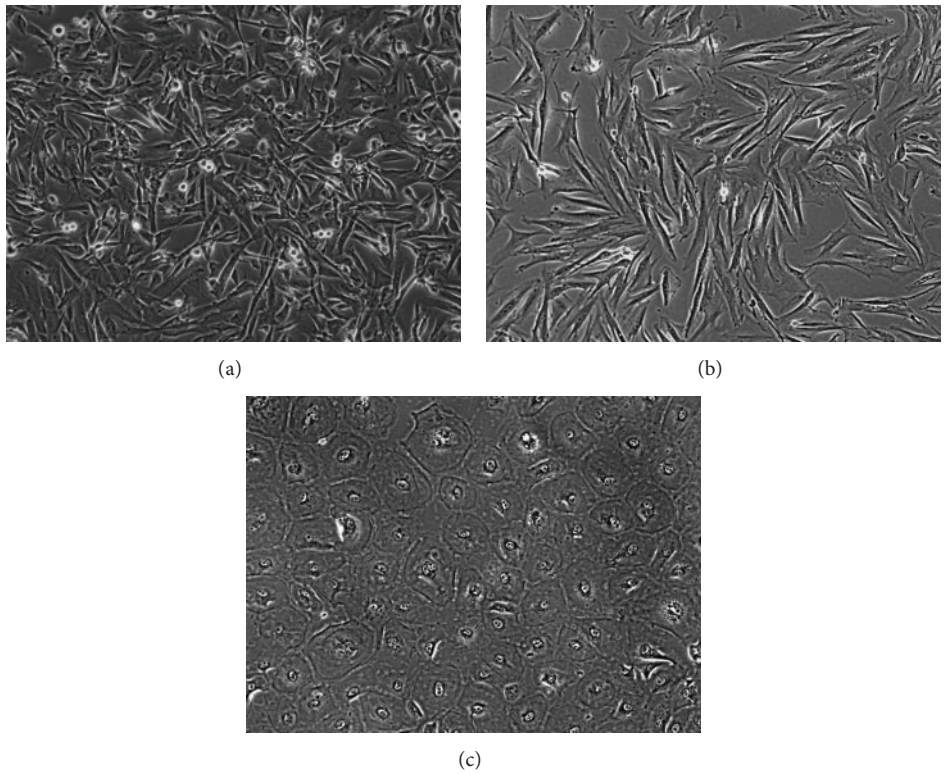
3.3. EDT-Derived Cells Display MSC Phenotypic Features. Having evaluated the proliferation potential and to more carefully define the MSC nature of isolated and expanded cells, we assessed their antigen expression profile. EDT-derived cells express typical MSC markers (Figures 5 and 6), and in particular more than 90% of tested cells were positive for CD90 and CD73, constituting main features of MSC from other sources [22]. We also observed that more than 80% of cells expressed CD146, an adhesion molecule related but not restricted to MSC and also expressed by endothelial cells [1]. The levels of CD45, HLADR, CD31, and CD14, assessed early in culture and commonly used to distinguish MSC from hematopoietic and endothelial cells, were below 2% in most cases. In sample 1, we observed a slightly increase in the $CD45^+$ fraction, suggesting the presence of hematopoietic elements which might have been isolated together with EDT-MSC. Other markers were also considered, such as CD56 whose positivity was extremely variable by up to 45%. Collectively, EDT-MSC from different donors showed the same overall trend in markers expression (Figure 6), despite for some markers, such as for CD56, CD105, and CD146, the variability has been considerably high. The phenotypic heterogeneity was also confirmed by analysis of physical parameters by FACS. Forward scatter versus side scatter plot was very dispersed, and it was impossible to identify consistent cell groups with similar physical parameters. Interestingly, we have to report a consistent small fraction (>1.0%) of SSEA-4 positive cells that reached 19.4% suggesting the expression of a pluripotency marker in this MSC type.

3.4. EDT-MSCs Are Precursors of Three Mesenchymal Tissues In Vitro . FACS analyses were then followed by assays aiming to assess EDT-MSC multipotency. We first focused on adipose commitment and, after 10 days of adipogenic inducing cocktail, EDT-MSCs were able to differentiate into vacuole-producing elements. The Oil Red staining confirmed the lipid content of those cells indicating the fat-producing ability of isolated and induced cells (Figure 7(a)). Osteogenic medium was then applied for 14 days, and cells underwent to osteoblastic commitment, forming a compact calcified matrix with calcium deposits confirmed by specific Alizarin Red staining (Figure 7(b)). Finally, after 21 days of chondrogenic induction, pellets of EDT-MSCs were included into paraffin blocks, and Alcian Blue highlighted the sulfated proteoglycans expression typical of the cartilaginous matrix, while Fast Red staining revealed the nuclei of resident chondrocytes derived from EDT-MSC (Figure 7(c)). Since a main focus of our research group is bone regeneration, we then coupled the cytochemical assay of osteogenic induced EDT-MSC with qPCR to further confirm bone commitment. As seen in Figure 8, all tested genes demonstrated an increased expression in induced samples versus noninduced controls. In particular, in osteoblast-induced cells the expression of alkaline phosphatase resulted 45 times higher ($P < 0.0001$), while more modest increases were observed for collagen 1A2 (2.9 times; $P < 0.05$) and osteocalcin (2.1 times; $P < 0.05$). These findings, together with positive Alizarin staining, reinforced the evidence for commitment of induced EDT-MSC to functional osteoblasts.

3.5. EDT-MSC Can Be Efficiently Gene Modified. To explore whether *ex vivo* expanded EDT-MSC could be genetically manipulated for future gene delivery approaches, cells were incubated with supernatants containing retroviral particles carrying the GFP gene. As shown in Figure 9, RD114 pseudotyped retroviral particles were able to efficiently and stably transduce EDT-MSC with levels greater than 80%, suggesting how this MSC type could be suitable for gene delivery approaches.

4. Discussion

Several MSC types have been obtained starting from different sources; here, we have isolated a population of mesenchymal progenitors from menstrual EDT and characterized them both at morphological and molecular levels investigating



(a) (b)

(c)

Figure 1: *In vitro* adherent cells from decidual tissues. (a) Representative photomicrograph of spindle-shaped adherent cells isolated by αMEM and 10% FBS. (b) Cells isolated *in vitro* by serum-deprived medium (Quantum 333) at early passages. (c) Another population of cells was *in vitro* isolated contextually with EDT-MSC. These elements share similarities with endothelial cells forming a monolayer of polygonal, sometimes binucleated cells. Original magnification 100x.

their ability to proliferate, to differentiate, and to be gene modified. EDT-MSCs appear as a heterogeneous cells population that can be safely and easily isolated by noninvasive manner, providing an expandable source of cells. In culture, they show mesenchymal morphology and, although some studies suggest they could derive from bone marrow [23], EDT-MSCs are generally considered to originate from the shedding of endometrial stem/progenitor cells which are mainly resident both in the basalis layer and, partially, in the functionalis area of endometrium [24].

The presence of a uterine population of progenitor cells would be consistent with the high tissue turn-over after each menstrual cycle. However, it is still uncertain how many subpopulations of progenitors are present in the endometrium and what are their specific properties. Therefore, in this pilot study, we began by addressing the existence of mesenchymal progenitors in the decidual endometrial tissue.

The initial *in vitro* approach was performed to evaluate the clonogenic potential of these cells. EDT-MSC seeded at clonal density revealed the presence of two distinct colony types which closely resembled the stromal cell clones isolated by Chan et al. from endometrium after hysterectomy [12]. One was composed of smaller and densely packed cells, and the other was composed of larger and sparser elements. This finding seems peculiar for EDT-MSC and differs from colonies established from BM-MSC that appear larger and characterized by a central nucleus of cells surrounded by a crown of more sparse ones [18]. Next to qualitative evaluations of the colonies, our data suggest an average EDT-MSC clonogenic efficiency of 14%. This value is higher than what is described for BM-MSC and more similar or even superior to what reported for AT-MSC that retained around 10% of clonogenic precursors [25] (Table 3). In addition, our EDT-MSC population seemed to have a higher clonogenic efficiency compared to those described by other groups dealing with endometrial stromal cells whose values ranged from 1 to 11% [12, 24, 26]. While this may be due to donor-related issues such as age, *in vitro* technical aspects including harvesting and preservation procedures that may also drive these differences. Dimitrov et al. observed a tendency for these cells to decrease in clonal efficiency with the increase of seeding density [26]. Similarly, other culture conditions may generate better performing EDT-MSC. In this pilot study, we tested two different growth media, such as αMEM with 10% FBS and a serum deprived medium. Although these cells apparently did not shown particular nutritional requirements, serum-containing medium proved to be the best choice, ensuring a better morphological appearance and an improved longevity. Previous studies reported the use of different media such as DMEM/F-12 and Chang complete media often supplemented with calf serum, hormones, and growth factors [12, 14, 26, 27]. This wide range of culture conditions is likely to impact the

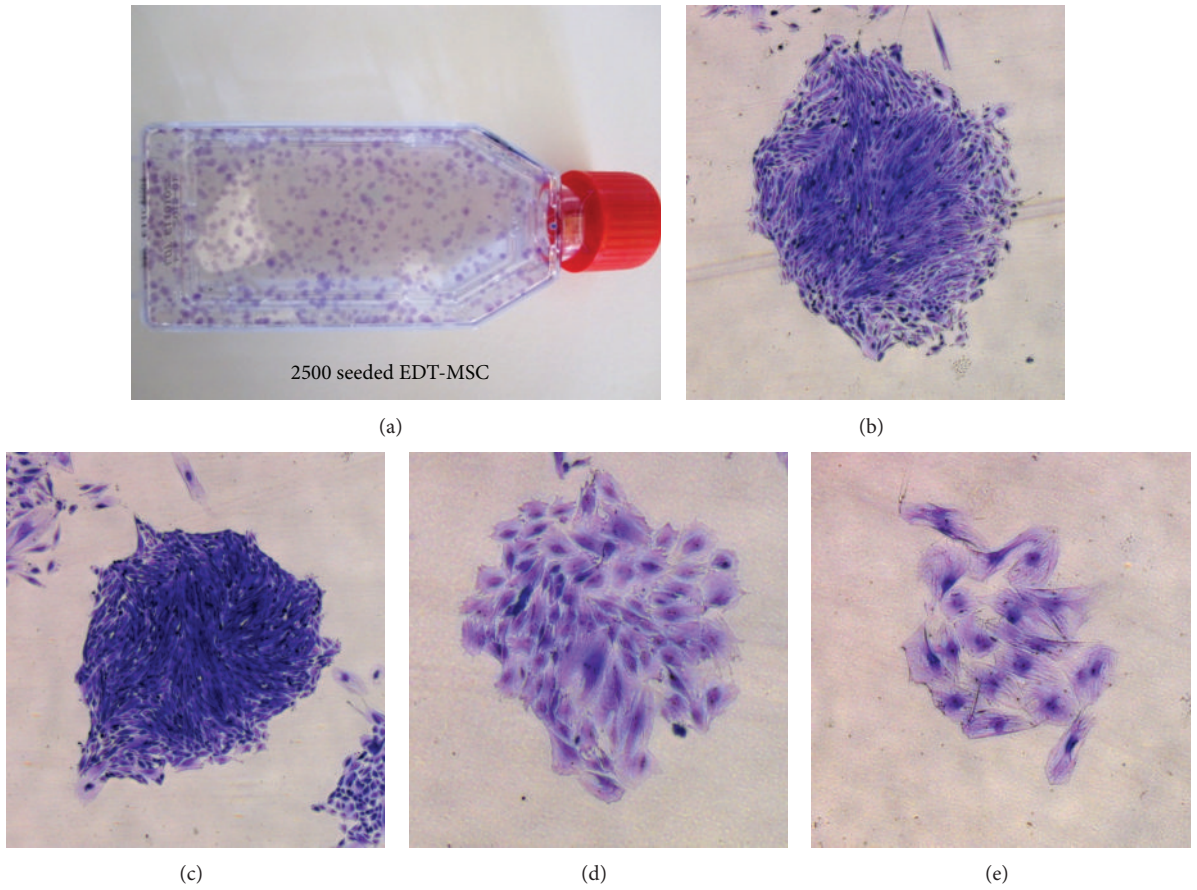


Figure 2: EDT-MSC clonogenic precursors. (a) A representative T-25 culture flask with crystal violet-stained EDT-MSC colonies. (b) and (c) EDT-MSC compact clones with small size cells. (d) and (e) EDT-MSCs could also generate less populated colonies with larger cellular elements. Original magnification 50x.

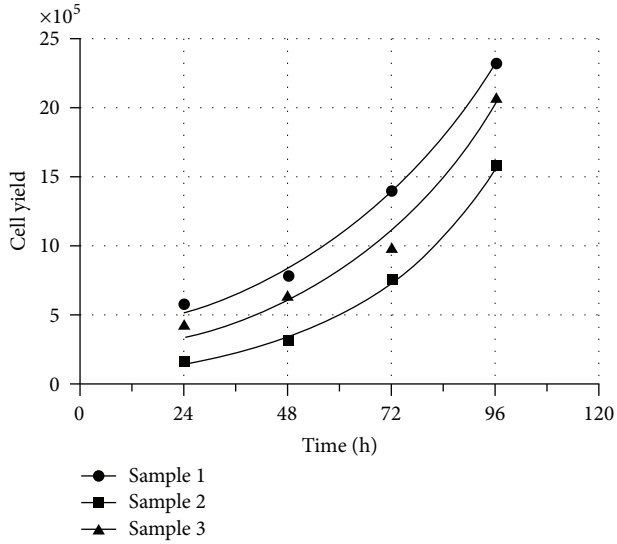


Figure 3: EDT-MSC proliferation. Cell proliferation assays performed to assess EDT-MSC doubling time. Doubling time for sample 1 was 33.0 h, for sample 2 was 21.9 h, and for sample 3 was 27.9 h.

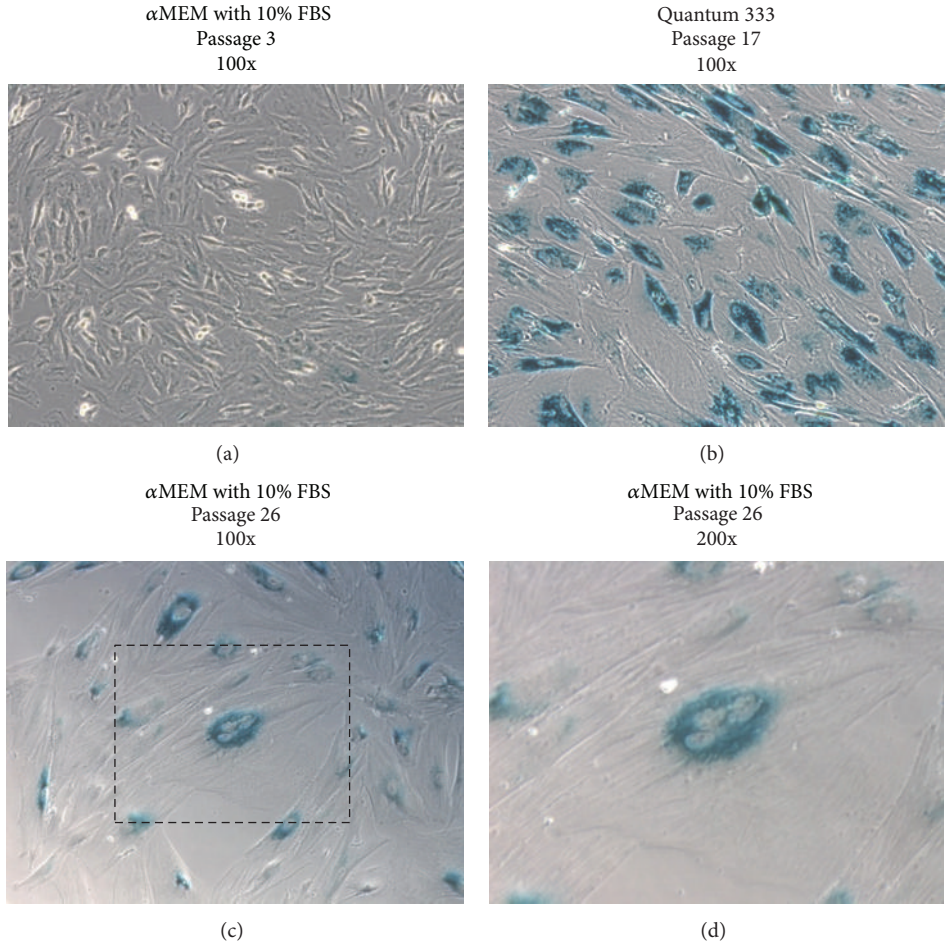


Figure 4: β-galactosidase staining. (a) Activity of β-galactosidase at pH 6 was assessed on passage 3 EDT-MSC cultured in FBS resulting almost negative for the presence of typical blue senescent cells. The conspicuous number of mitotic event is also visible, highlighting an intense proliferative activity. (b) Cells grown in Quantum 333 underwent senescence at passage 17 and resulted in deep blue staining within most of the amplified EDT-MSC. (c) Cells cultured in FBS were capable to reach passage 26 before showing senescence features, such as blue staining, large cytoplasm, multinucleated elements, and evident cytoskeleton. (d) A detail of a senescent cell. Original magnification 100x.

in vitro behavior, leading to different performance in *ex vivo* pivotal parameters.

Using a FBS-based medium, EDT-MSC demonstrated a remarkable proliferation rate rapidly doubling their number in less than 28 hrs. This result agrees with other similar studies, which reported a doubling time between 18 and 36 h for EDT-MSC [15, 28]. As seen in Table 3, similar attitude has been also reported for dental pulp stem cells (DPSCs) and umbilical cord (UC) MSC that duplicate in approximately 30 hrs, considerably faster versus both BM-MSC and AT-MSC [29–31].

Beside this robust proliferative attitude, we also observed that plastic adherent fraction from shedded DT could be expanded for more than 25 passages before reaching senescence, a result once again higher than what reported for MSC from other sources. As summarized in Table 3, BM-MSC and AT-MSC senescence has been reported from passages 7 and 8 [32], while UC-MSC grow longer in culture, until passage 16 [31]. These findings reinforce the hypothesis that EDT-MSC can duplicate for long time before senesce and are consistent with reports asserting 25–30 population doublings for uterine-derived precursors [15, 28, 33]. This unique feature fit well with the high rate of renewal retained *in vivo* by the endometrium, leading to the conclusion that this new MSC source could have a tremendous potential for large-scale clinical applications in regenerative medicine.

In this work, EDT-MSC showed other similarities with MSC from the other sources and, in particular, under the immunophenotypical point of view satisfying recognized phenotypic criteria for MSC [22]. The biomarker profile, as compared in Table 2, shows a close resemblance of EDT-MSC with other sources MSC, expressing high levels of CD73 and CD90 and being negative for CD14, CD45, CD31, and HLA-DR. Other markers have been additionally evaluated. CD56, also known as neural cell adhesion molecule (NCAM), is a glycoprotein expressed by neuroectodermal-derived cells such as neurons and glia but also by skeletal muscle, NK cells and activated T cells [34]. Among our EDT-MSC, CD56 expression has been extremely variable (1.3–45%), and this may support the observation made by others

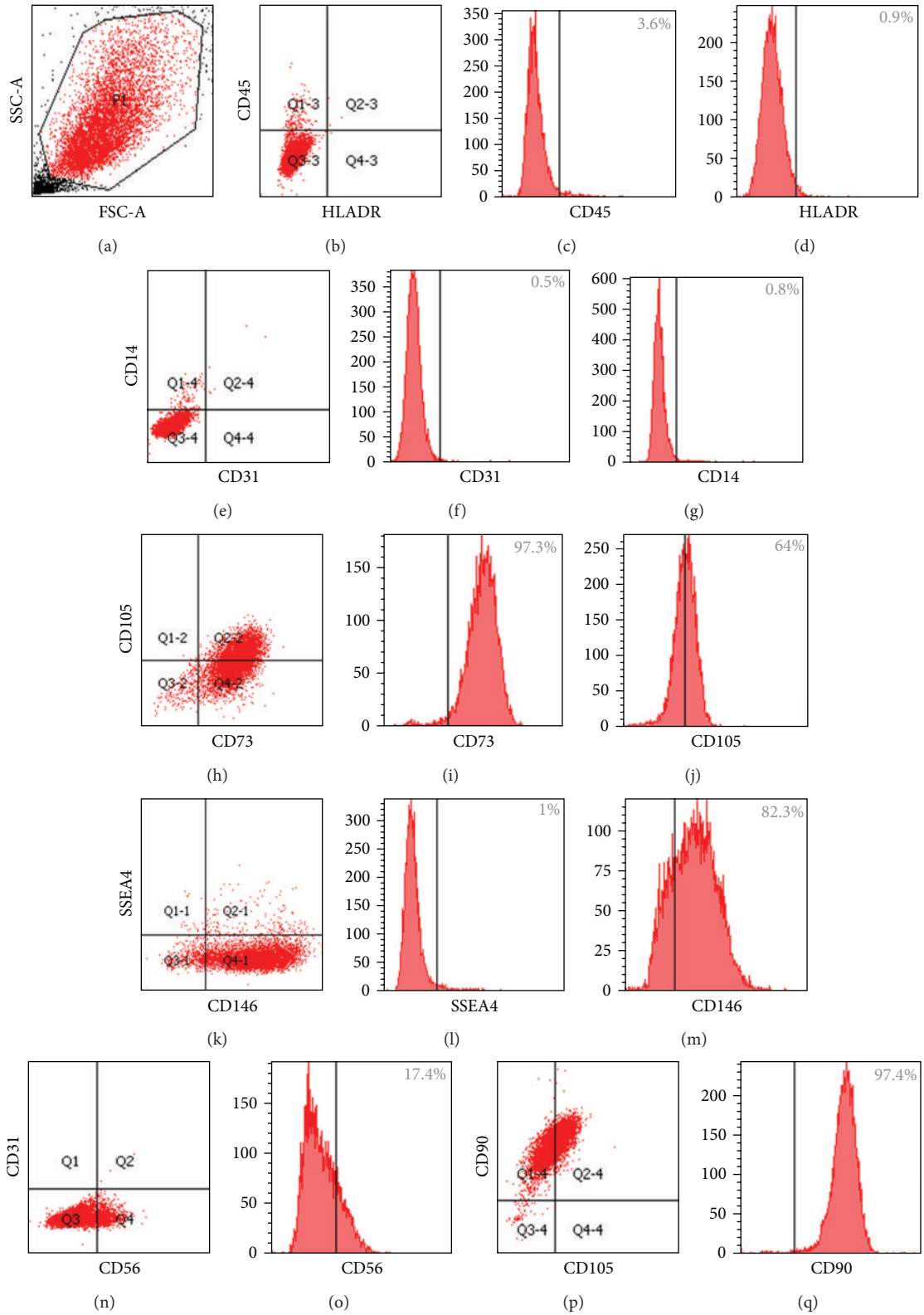


Figure 5: EDT-MSCs express typical MSC markers. Representative FACS analyses of EDT-MSC immunophenotype. Percentages are referring to the average positivity for each marker. Cells were gated out of 7-AAD positivity to exclude nonviable elements.

Table 2: Immunophenotype of MSC from different sources.

Marker	CD14	CD45	CD73	CD90	CD105	CD146	CD31	HLA-DR	References
BM-MSC	−	−	+++	+++	+++	++	−	−	[43, 44]
PB-MSC	−	−	N/A	+++	++	N/A	−	−	[45]
DPSC	−	−	+++	+++	+++	N/A	−	−	[30]
UC-MSC	−	−	+++	+++	+++	N/A	−	−	[2, 43]
AT-MSC	−	−	+++	+++	++	N/A	−	−	[2, 43]
EDT-MSC	−	−	+++	+++	++	+++	−	−	‡

Comparison between positive and negative MSC surface markers from different sources, including EDT-MSC. Variable expression is highlighted with ±. BM-MSC: bone marrow MSC; PB-MSC: peripheral blood MSC; DPSC: dental pulp stromal cells; UC-MSC: umbilical cord MSC; AT-MSC: adipose tissue MSC; EDT-MSC: decidual tissue MSC. ‡Original data from this study, references.

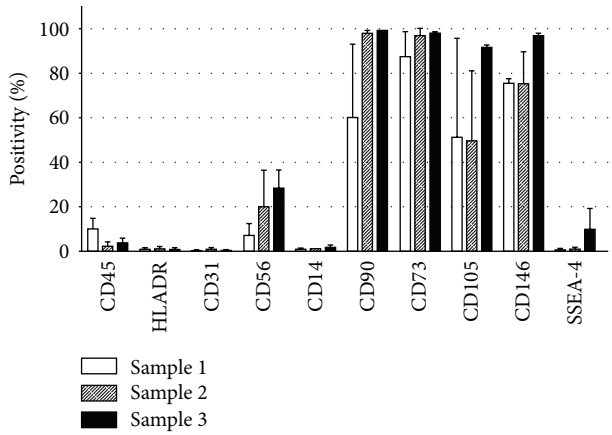


Figure 6: Overall EDT-MSC antigens expression after passage 1 *in vitro*. Marker expressions are here reported as average percentage (±standard deviation) of each tested sample.

exploiting CD56 expression to discriminate distinct marrow MSC subpopulations [35, 36]. Moreover, we evaluated the expression of SSEA-4, a glycolipid recently proposed to be a novel marker for BM-MSC isolation and that is expressed by teratocarcinoma cells and embryonic stem cells [37]. EDT-MSC demonstrated a noticeable expression of SSEA-4 that, while reaching 19.4% appeared far from the 90% positivity reported by others on decidual-derived cells [15]. However, different laboratories reported discrepancies on EDT-MSC phenotype [15, 28, 38], and this could be likely due to the heterogeneity of menstrual blood cell population, to differences in cell selection and, as stated, to culture conditions [13]. In addition, the relatively short time from the early evidence of these progenitors does not help in the phenotypical standardization and certainly will require deeper and multicentric studies.

Multipotency is another key feature of MSCs and all EDT-MSC lines showed the *in vitro* capacity to differentiate into three mesodermal lineages, such as osteoblast able to generate mineralized matrix, lipid vacuole-containing adipocyte and matrix-producing chondrocytes. Additional experiments will be necessary to confirm this ability, prompting their broad introduction in regenerative medicine applications. However, we have here begun to assess the capacity of these cells for robust osteogenic commitment confirmed by the differentiation data obtained by staining for mineralized matrix, we observed a relevant increase in the expression of typical osteoblast-associated genes such as alkaline phosphatase, osteocalcin, and collagen 1A2.

Regenerative therapies based on stem cells, and more specifically on MSC infusion, may counteract tissue-losing states and may also be useful to substitute the congenital abnormal stem cell compartment in order to regenerate healthy functional cells. In this perspective, ease of supply, wide window of harvesting, and availability of EDT-MSC would allow autologous or, ultimately, allogeneic EDT-MSC transplantation. These properties have already been exploited, and EDT-MSC have demonstrated a therapeutic effect in a murine model of muscular dystrophy [38] and a neuroprotective effect in an experimental stroke model [27]. Moreover, endometrial-derived cells seem to have robust anti-inflammatory and immunomodulatory properties similarly to other MSCs [27, 39]. For this reason they were intravenously and intrathecally transplanted in 4 patients with multiple sclerosis, notably without arising immunological reactions or other adverse effects and with a clinical benefit that shall be carefully assessed [40].

Another original result we here report is the high transduction efficiency of EDT-MSC by a GFP-carrying retroviral vector. The procedure generated exogenous gene expression in more than 80% of the cells and, to our knowledge, this is one of the first reports of such highly efficient genetic manipulation of EDT-MSC by a single GFP-carrying vector providing a basis for use of these cells in gene therapy approaches, including targeted cancer treatment or their use as cell vectors for peptides or trophic factors. Having extensively characterized EDT-MSC, we presume that these cells may be useful for a more efficient delivery of proapoptotic molecules thanks to the high transduction efficiency and the rapid proliferation. This may allow a more targeted approach and better persistence of the cells in the tumor microenvironment.

Starting from these findings, future studies shall focus on the characterisation of the heterogeneous cell populations identifiable in menstrual decidual tissue, investigating the existence of different progenitor subtypes as well as understanding the origin of these cells. More reliable techniques to isolate and cultivate pure populations of EDT-MSC should be developed and standardized in parallel to allow a rapid introduction of this promising therapeutic tool into the clinic.

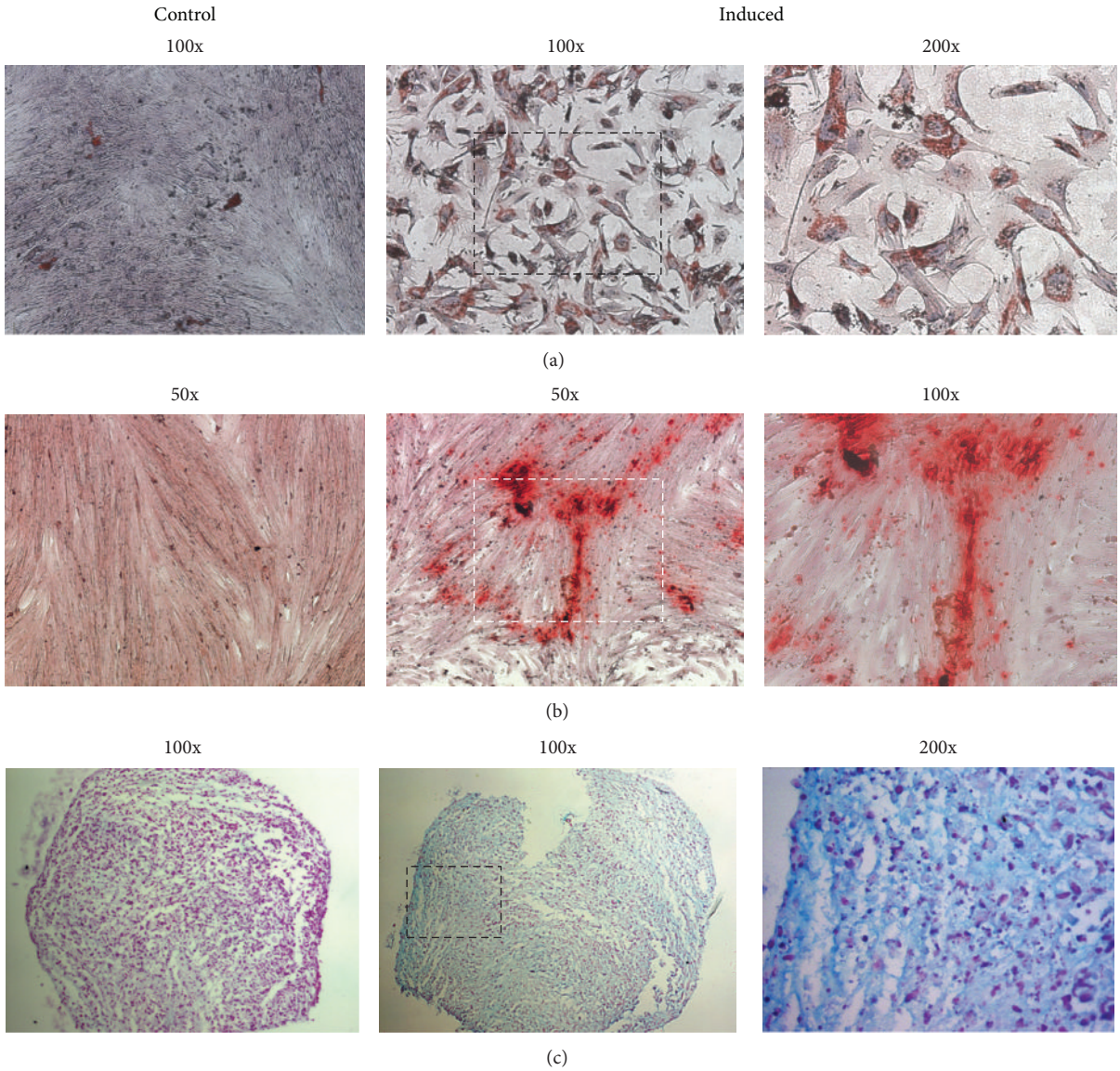


Figure 7: EDT-MSCs differentiate into committed mesenchymal tissues. (a) Oil Red O staining in adipogenic induced and control EDT-MSC. The red-brown vacuoles demonstrate differentiation into fat-producing cells. Original magnification 100x. (b) Alizarin Red staining showing calcium deposits in induced cells when compared with noninduced controls. Original magnification 50x. (c) Alcian Blue staining outlining sulfated proteoglycans expression suggesting differentiation into a chondrogenic tissue. Original magnification 100x.

Table 3: Growth characteristics of MSC from different sources.

Cell type	Source	Isolation yield	Doubling time	Clonogenic efficiency	Senescence passage
BM-MSC	Bone marrow aspirate	60–600 cells from 1 mL of blood [41]	61.2 h [29]	3.9%*	7 [32]
PB-MSC	Peripheral blood	1 to 13 MSC from 1 million of mononuclear cells [11]	N/A	N/A	N/A
DPSC	Deciduous and adult teeth	N/A	30 h [30]	N/A	N/A
UC-MSC	Umbilical cord of newborns	$5 \cdot 10^4$–$5 \cdot 10^5$ cells from 1 cm^3 of umbilical cord tissue [42]	24 h [31]	N/A	16 [31]
AT-MSC	Liposuction, lipectomy, and lipoplasty	$5 \cdot 10^3$ cells from 1 g of tissue [11]	45.2 h [29]	10% [25]	8 [32]
EDT-MSC	Decidual tissue	Variable‡	27.6 h‡	14.4%‡	26‡

Comparison between growth parameters of MSC from different sources. BM-MSC: bone marrow MSC; PB-MSC: peripheral blood MSC; DPSC: dental pulp stromal cells; UC-MSC: umbilical cord MSC; AT-MSC: adipose tissue MSC; EDT-MSC: decidual tissue MSC. *Unpublished data; ‡original data from this study, references.

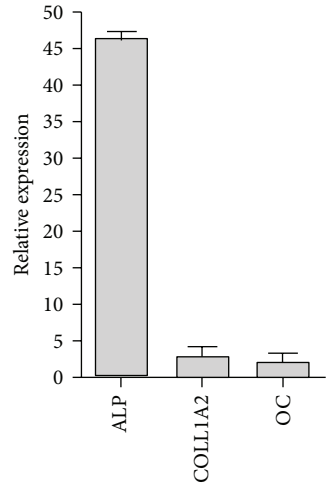


Figure 8: qRT-PCR for osteoblast-associated genes. The relative quantity of osteoblast-associated transcripts in induced EDT-MSC versus noninduced controls revealed an increased expression of the considered markers. All values were statistically significant ($P < 0.05$). ALP: alkaline phosphatase, COLL1A2: collagen 1A2, and OC: osteocalcin.

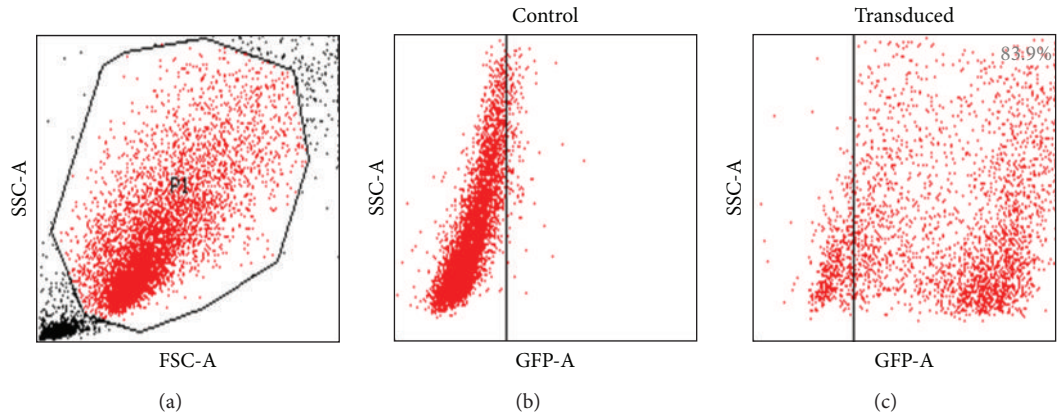


Figure 9: GFP expression of transduced and wild-type EDT-MSC. Representative FACS analyses assessing GFP positivity of wild-type control and transduced EDT-MSC.

5. Conclusions

This work highlights crucial features of a novel and still not completely understood population of mesenchymal progenitors isolated form endometrial decidual tissue. Cells grown *in vitro* were characterized by a rapid proliferation, a high clonogenic potential and a long-term survival. Advantages in comparison with MSC from other sources include a greater ease of supply and the protracted availability during a woman's lifetime with the additional benefit of deriving stem cells from a waste tissue, thus avoiding critical ethical issues. In the light of their capacity to differentiate into mesenchymal tissues and their propensity to undergo genetic manipulation, EDT-MSCs hold promise for novel therapeutic approaches for still incurable and highly disabling diseases.

Conflict of Interests

The authors do not have direct financial conflicts of interest.

Authors' Contribution

F. Rossignoli and A. Caselli are equally contributing authors.

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Research Article

HTR8/SVneo Cells Display Trophoblast Progenitor Cell-Like Characteristics Indicative of Self-Renewal, Repopulation Activity, and Expression of "Stemness-" Associated Transcription Factors

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Introduction. JEG3 is a choriocarcinoma—and HTR8/SVneo a transformed extravillous trophoblast—cell line often used to model the physiologically invasive extravillous trophoblast. Past studies suggest that these cell lines possess some stem or progenitor cell characteristics. Aim was to study whether these cells fulfill minimum criteria used to identify stem-like (progenitor) cells. In summary, we found that the expression profile of HTR8/SVneo (CDX2+, NOTCH1+, SOX2+, NANOG+, and OCT-) is distinct from JEG3 (CDX2+ and NOTCH1+) as seen only in human-serum blocked immunocytochemistry. This correlates with HTR8/SVneo's self-renewal capacities, as made visible via spheroid formation and multi-passagability in hanging drops protocols paralleling those used to maintain embryoid bodies. JEG3 displayed only low propensity to form and reform spheroids. HTR8/SVneo spheroids migrated to cover and seemingly repopulate human chorionic villi during confrontation cultures with placental explants in hanging drops. We conclude that HTR8/SVneo spheroid cells possess progenitor cell traits that are probably attained through corruption of "stemness-" associated transcription factor networks. Furthermore, trophoblastic cells are highly prone to unspecific binding, which is resistant to conventional blocking methods, but which can be alleviated through blockage with human serum.

1. Introduction

The master regulatory networks of human embryonic stem cell (hESC) transcription factors, OCT4, SOX2, and NANOG, as well as other cell fate determining transcription factors that are implicated in stem cell self-renewal capacities, such as NOTCH1 and STAT3, are expressed not only by embryonic stem cells, but also by a number of cancers [1]. Some of these factors are also expressed in choriocarcinoma (gestational trophoblastic disease) [2]. This has led to the thought that choriocarcinoma may also represent a group of tumors, in which hESC transcription factor deregulation has led to their transformation into cancer stem cells.

In mammalian development, the first cell differentiation step segregates trophoblast and embryonic cell lineages, thus resulting in the formation of the blastocyst's outer lining, the trophectoderm (TE), and its inner cell mass (ICM). The trophectoderm consists of trophoblast stem cells that express CDX2, a homeobox transcription factor, which is required for the emergence of these cells [3]. Physiological invasion is seen during blastocyst implantation, which is mediated through the trophectoderm. Interestingly, both CDX2 and SOX2 deficiency lead to implantation failure of the blastocyst secondary to trophoectoderm differentiation problems [4–6].

The trophectoderm also differentiates into several trophoblast subsets in order to create the placenta of the first trimester pregnancy. Of these subsets, the cytotrophoblast is considered a putative "progenitor cell," which replenishes the outer layer of the villous (syncytiotrophoblast), but which is

also able to invade the decidua in a cancer-like manner when necessary and desirable (extravillous trophoblast) [7]. This behaviour is often believed to be driven by hypoxia, and it is a well-orchestrated and closely controlled process, mostly through a network of interaction between the invading trophoblast, the decidua, the maternal endothelium, and the maternal immune system; the detailed description of which would tax the scope of this introduction [8]. The first trimester placenta is especially ample with invasive (cyto)trophoblast, while the term placenta trophoblast loses this capability [8].

The uniqueness of this situation, in which physiologic, spatially (limited to the decidua, first third of the myometrium, and the invasion into maternal spiral arteries), and temporally (limited to the first trimester of pregnancy) regulated invasion (by the trophoblast) and pathologic, deregulated, and malignant invasion (by choriocarcinoma) are set so close together, has drawn the attention of cancer researchers worldwide [8]. However, since isolation of primary trophoblast and choriocarcinoma cells is often cumbersome, in recent years, several trophoblastic cell lines have been utilized as imperfect models for the invasive trophoblast(ic) cell. Some of the most popular cell lines used constitute the immortalized first trimester trophoblast cell line, HTR8/SVneo, and the choriocarcinoma cell line JEG3. HTR8/SVneo cells are often considered a closer model of trophoblast cells, because the HTR8/SVneo cell lines were established by immortalizing a physiologic extravillous trophoblast cell via transfection with a plasmid containing the simian virus 40 large T antigen (SV40) [9], while the JEG3 cell line was cloned from a primary choriocarcinoma strain [10].

Our own recently published data, however, demonstrate that the miRNA profiles of these two cell lines are quite differing, surprisingly with JEG3 encompassing an miRNA profile that is closer to primary first trimester trophoblast cells than that of the HTR8/SVneo cell lines [11]. Villous cytotrophoblast and HTR8/SVneo cells have interestingly also been implicated in producing a "side population" that either demonstrates long-term repopulating properties or expresses classical hESC markers [12, 13].

Following the idea that both JEG3 and HTR8/SVneo are transformed cells and have been proposed to produce cancer stem cell or progenitor (side population) cell populations, we aimed to characterize the putative cancer and trophoblast stem/progenitor cell traits of HTR8/SVneo and JEG3 cells on the basis of general minimum recommendations for identifying cancer stem cells or progenitor cells [14, 15]. This is accomplished first by assessing the capacity of these cells to form spheroid bodies, second by determining the expression of various transcription factors related to progenitor or to cancer stem cell development, and finally by investigating the cells' ability to repopulate trophoblast tissue in a near in vivo model.

For these studies, we phrase SOX2, OCT4, and NANOG as core "stemness-" associated transcription factors [16] and CDX2 [3] as a trophoblast stem/progenitor cell transcription factor. NOTCH1 is included as an often abused, prominent cell-fate transcription factor associated with both cancer stem cells and hESC [17–20] and is, henceforth, termed a cell-fate determining transcription factor.

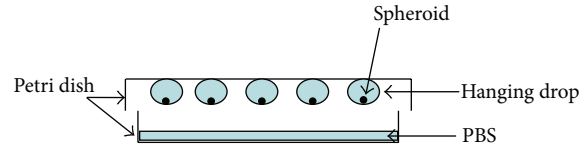

Figure 1: Schematic diagram of spheroid formation.

2. Methods

2.1. Spheroid Formation with Hanging Drops. We chose the hanging drops protocol as reviewed by Kurosawa [21].

Briefly, 20 000 cells per 30 μL drop supplemented RPMI (as described later in Section 2.2) were plated onto the lid of two Petri dishes in regular arrays (20 drops/Petri lid). The lid was inverted over the bottom of the PBS-filled Petri dish (see schematic representation Figure 1). The Petri dish with the hanging drops were cultured under standard conditions (37°C, 5% CO_2, humidified atmosphere) for 48 hours. A schematic image of the hanging drop principle is seen in Figure 1. The experiment was carried out in the same manner for HTR8/SVneo cells and for JEG3 cells (40 drops per cell line).

2.2. Cell Culture. HTR-8/SVneo cells (a kind gift from Professor Charles Graham of the Department of Anatomy and Cell Biology at Queen's University, Kingston, ON, Canada) were cultured in RPMI (PAA) and JEG3 cells in F12 Medium. Both media were supplemented with 10% fetal bovine serum (FBS; SIGMA, St. Louis, USA) and 1x penicillin/streptomycin (PAA Laboratories; Pasching, Austria). Cell cultures were maintained under standard culturing conditions (37°C, 5% CO_2, humidified atmosphere).

2.3. Immunocytochemistry (ICH). Cells were trypsinized, centrifuged, and resuspended in 1 mL respective medium. Slides (SuperFrost/Plus slides; Menzel, Germany) were washed and sterilized with ethanol, coated with cells (200 μL), and incubated over night at 37°C. The cells were fixed on the next day with ethanol/methanol and consequently used to perform immunocytochemistry. To inhibit endogenous peroxidise activity, the cells were incubated in methanol/H_2O_2 for 5–10 min and washed for 5 min in phosphate-buffered saline (PBS, pH 7.4), followed by incubation firstly with and without 5% human AB serum (PAA), which corresponds to an approximate Fc-concentration of 0.6 mg/mL, in order to further eliminate the possibility of Fc-receptor cross-reactions (as described in [22]), and secondly with goat serum at room temperature for 20 min (Vector Laboratories) to eliminate regular nonspecific background staining.

Samples were then incubated with the primary antibodies (please refer to Table 1) for 60 min at room temperature. Antibodies were diluted in DAKO Antibody Diluent with Background Reducing Components (DAKO, Denmark).

Table 1: List of antibodies.

		Immunohistochemistry (IHC)		
Antibody	Clone	Isotype	Concentration IHC	Source
Cdx2	—	Polyclonal Rabbit	1 : 200	Cell Signaling
Sox2	D6D9	Polyclonal Rabbit	1 : 100	Cell Signaling
Notch1	D6F11	Polyclonal Rabbit	1 : 200	Cell Signaling
Nanog	—	Polyclonal Rabbit	1 : 400	Cell Signaling
Oct4A	C52G3	Polyclonal Rabbit	1 : 300	Cell Signaling
Isotyp control	DA1E	Polyclonal Rabbit	1 : 100	Cell Signaling
ABC Elite kit (rabbit IgG)				Vector Laboratories (Lörrach, Germany)

In the next step, our samples were incubated with the biotinylated secondary antibody (Vector Laboratories) for 30 min at room temperature. For a listing of antibodies, please refer also to Table 1. Following incubation with the secondary antibody, an incubation period with ABC-complex (avidin-biotinylated peroxidise; Vector Laboratories) again for 30 min at room temperature was completed. Between each step, all samples were washed profusely with PBS. The peroxidase reaction was achieved with DAB (diaminobenzidine/H_2O_2; 1 mg/mL; DAB; Dako) and after 5 min discontinued with water. Hematoxylin staining was used for cell nuclei staining (2 min). Finally, slides were dehydrated by an ethanol-to-xylene treatment, covered with Histofluid (Paul Marienfeld, Lauda-Königshofen, Germany), and analysis was completed with the Axioplan 2 microscope (Carl Zeiss, Jena, Germany).

A negative control was prepared by replacing the primary antibody with DAKO Antibody Diluent only. Isotype controls were prepared in the same manner as the primary antibody.

Analysis of staining intensity and gross estimation of stained cell numbers was accomplished by eye and by two blinded investigators (criteria similar to standard immunoreactive scoring).

2.4. Immunofluorescence Staining. The cells were cultured on SuperFrost/Plus slides (Menzel, Germany) over night with serum-free media. Cells were fixed on the subsequent day with ethanol/methanol. To reduce nonspecific background staining, all samples were incubated either with goat serum or with 5% human AB serum (PAA, as recommended by [22]) at room temperature for 20 min (Vector Laboratories). Samples were incubated with the primary antibodies (please refer to Table 1 for company names and concentrations used) overnight at 4°C. Antibodies were diluted in DAKO Antibody Diluent with Background Reducing Components (DAKO, Denmark). On the next day, they were incubated with the secondary antibody labeled with Cy3 for 1 h at room temperature. Between each step, all samples were washed profusely with PBS. Sections were counterstained Vectashield Mounting Media with DAPI (VECTOR Laboratories) and then cover slipped.

A negative control was prepared by replacing the primary antibody with DAKO Antibody Diluent only. Isotype controls were prepared in the same manner as the primary antibody. All samples were analyzed with an AxioPlan2 microscope (Carl Zeiss, Jena, Germany).

Assessment was accomplished by eye and by two investigators only in terms of positive or negative expression and pattern of expression.

2.5. Placental Explant Cultures and Confrontation Cultures. Two biopsy-sized "explants" (2 mm diameter) each from villous tissue of five healthy human term placentae (after elective caesarian section) were collected. An approval by the local ethical committee exists. Prior to confrontation cultures, all spheroids were stained by application of 10 nM Mito Tracker dye (fluorescent green; Invitrogen) for 30 min at 37°C and then intensively washed in PBS (Biochrom, Germany). Commencing from the time that the placental "biopsies" or "explants" are placed in culture, these are termed villous explant cultures.

Each explant was then confronted in culture with one spheroid within the respective hanging drop for 48 h. Subsequently, the confronted tissues were incubated with a solution of 10% nonfat milk in PBS containing 1% Triton (AppliChem) to permeabilize cell membranes and to block nonspecific binding sites for 1 h. Following intensive washing step, tissues were incubated with a rat anti-human CD31 (anti-PECAM1; Millipore, Germany) for 2 h, followed by incubation with a goat anti-rat IgG-Cy5 conjugate (Milipore) for 90 min, all within the previously described solution (nonfat milk/PBS/Triton). After staining, descriptive analyses with the tissues and spheroids were accomplished on a confocal laser scanning microscope (Carl-Zeiss, Jena, Germany).

3. Results

3.1. HTR8/SVneo Cells Have a High and JEG3 Cells a Low Propensity to Form Spheroid Bodies Within Hanging Drops. The first step to confirm putative cancer stem/progenitor cell status is to confirm their capacity for self-renewal, which is often accomplished by propagation of these cells as spheroids in stem cell culturing conditions [14]. Cells with self-renewing potential can be disaggregated from the spheroids and passaged multiple times with retention of spheroid-forming ability [15].

Of the 40 hanging drops experiments per cell line, 100% of the incubated HTR8/SVneo cells and only 50% of the incubated JEG3 cells were able to form spheroids (data not demonstrated). The developed HTR8/SVneo spheroids regularly measured a diameter of approximately 700–750 μm,

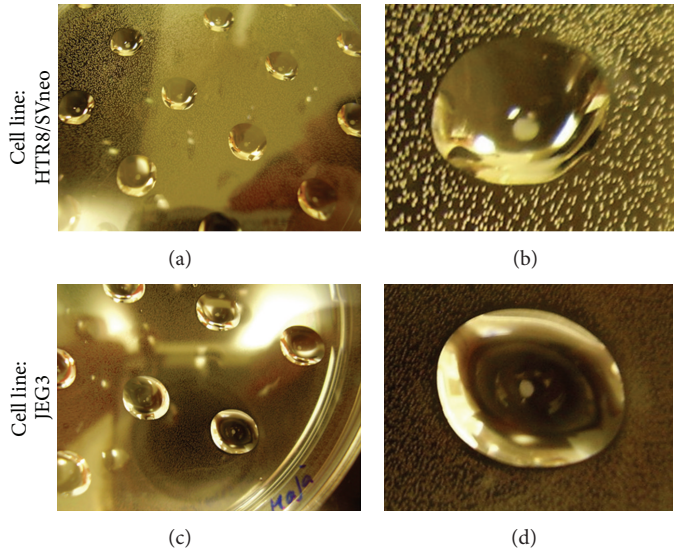

Figure 2: Spheroid formation of HTR8/SVneo and JEG3 cells via hanging drops. Forty hanging drops per cell line were produced with 20 000 cells per 30 μL drop. The environment of the hanging drop delivers the prerequisite for spheroid formation. After an incubation period of 48 h, 100% of HTR8/SVneo-containing drops formed visible spheroids (a, b), while only 50% of JEG3-containing drops formed spheroids (c). JEG3 spheroids appeared smaller in circumference, less globular (oblong shapes), and rather on the verge of disaggregation (d).

which was also visible by the "naked" eye (Figures 2(a) and 2(b)). On the other hand, JEG3 spheroids were much smaller (as visible even by the "naked" eye) and irregularly shaped (Figures 2(c) and 2(d)). The JEG3 spheroids were also unstable and were disaggregated easily and thus could not easily be pipetted into the shortened tip of a pipette for transportation into, for example, a new hanging drop.

In order to verify that spheroid formation is not secondary to cell aggregation, we proceeded to disaggregate the spheroids, split these cells, and propagate them again in hanging drops to assess their continued ability to reform spheroids (as recommended in [15]). Under this experimental setting, we passaged the HTR8/SVneo spheroids multiple times (5 passages and continuing) without their loss of spheroid-forming abilities. As little as 5000 HTR8/SVneo cells are able to form a spheroid (splitting ongoing). Please note that we have not yet tried to form spheroids with less cells than the mentioned. Only 50% of the initial JEG3 spheroids were able to reform spheroids following disaggregation and splitting; further passages could not be maintained.

3.2. HTR8/SVneo and JEG3 Cells Express the Trophoblast Stem Cell Marker CDX2. CDX2 is a transcription factor that is necessary for the first differentiation of hESC into the trophoblast stem cell (reviewed in [3]). Loss of CDX2 activity disables the blastocyst to implant correctly during murine pregnancy [23]. Following the hypothesis that HTR8/SVneo or JEG3 cells recapitulate features of a cancer stem cell or a trophoblast(ic) stem or progenitor cell, we sought to investigate whether the classic trophoblast stem cell "marker" is expressed in both cell lines.

Since it has been described that nonspecific binding in trophoblast(ic) cells is extreme and often cannot be alleviated through conventional blocking procedures [22], we initially blocked cell lines with normal goat serum and further blocked them with or without human serum. According to immunofluorescence staining results, both cell lines seem to stain positive for CDX2. However, since there was no alteration in the pattern of staining before and after application of human serum, and since it is rather unlikely that CDX2 is so prominently expressed in the cytoplasm [24, 25], we conclude that unspecific binding is still too high in immunofluorescence stainings to be reliable.

In contrast, ICH results show that HTR8/SVneo cells express CDX2 in the nucleus, although the trophoblast marker expression appeared lower, especially in terms of number of nuclei, after blockage with human serum (Figure 3(b) without human serum versus Figure 3(a) with human serum). In JEG3 cells, a low-intensity CDX2 signal was observable even after blocking with human serum with numbers of positive-stained nuclei rather unchanged (Figures 4(a) and 4(b)). Via immunofluorescence staining procedures, CDX2 expression is made visible, and the signal patterns are again not affected by blocking procedure; thus, this procedure is again deemed unreliable (Figures 3(c), 3(d), 4(c), and 4(d)).

3.3. HTR8/SVneo Cells Express NOTCH1, NANOG, and SOX2, but Not OCT4 according to ICH Analysis. In theory, cancer stem cells have probably reacquired properties similar to stem cells during transformation into a malignancy [1, 2, 26]. We analyzed the expression of the classic or core "stemness-" associated transcription factors OCT4, NANOG, and SOX2 (reviewed in [16]). We also chose to assess the expression of the cell fate determining transcription factor NOTCH1 due to its recent suggestion as a maintainer of "stemness," as well its ever-emerging role in the invasion potential of tumors (reviewed in [17, 20, 27, 28]).

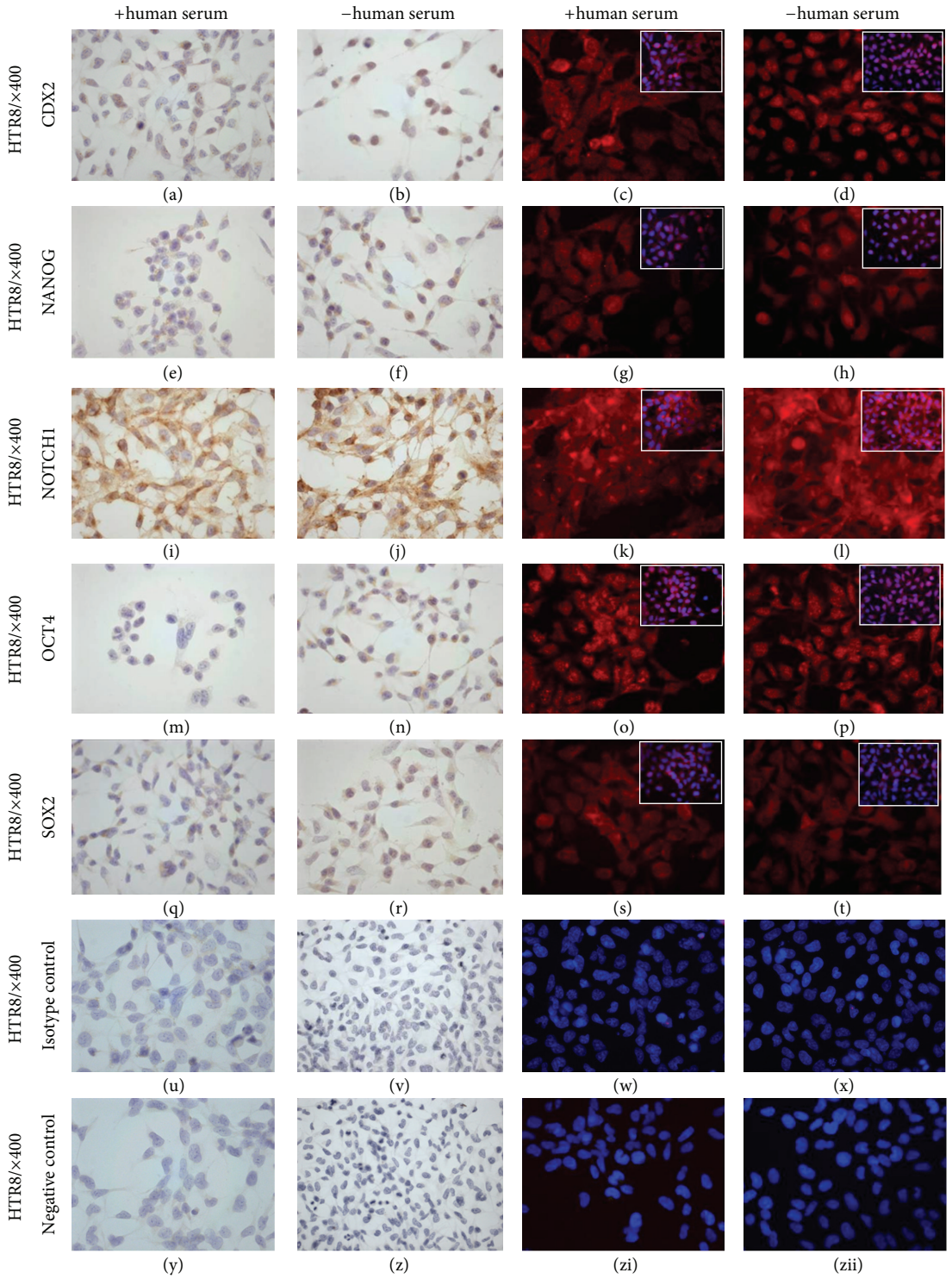

Figure 3: Expression of trophoblast (CDX2), cell fate determining (NOTCH1), and core "stemness-" associated (OCT4, NANOG, and SOX2) stem cell transcription factors in HTR8/SVneo cells. HTR8/SVneo cells express CDX2, NOTCH1, NANOG, and SOX2 ((a), (e), (i), and (q)). OCT4 signaling is lost after blocking the samples with human serum ((m) versus (n)). Human serum is needed to further block exceptional, unspecific binding on trophoblastic cells that cannot be eliminated via conventional blocking measures. Immunofluorescent staining seems less specific with or without blockage with human serum ((c), (d), (g), (h), (k), (l), (o), (p), (s), and (t)).

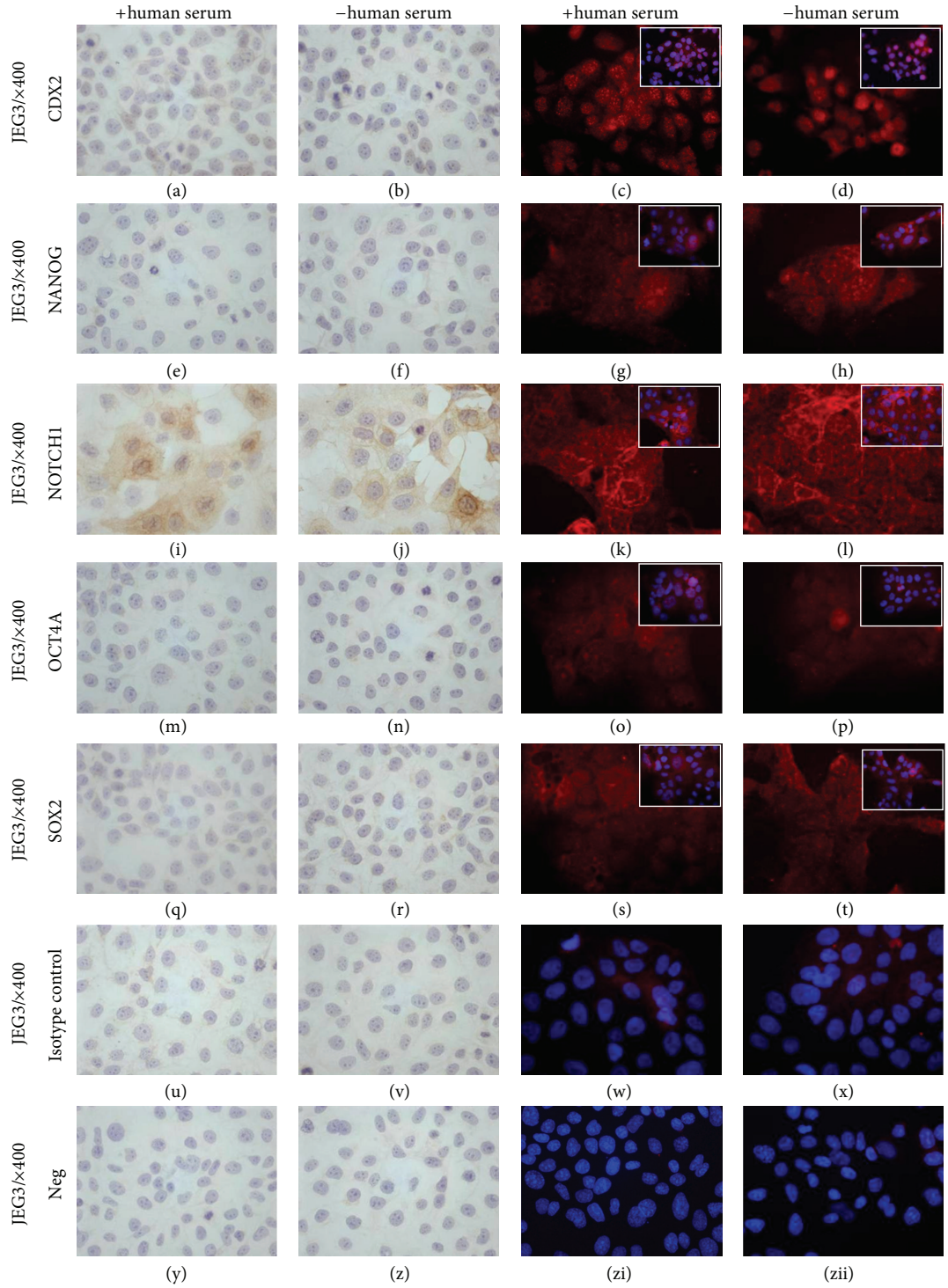

Figure 4: Expression of trophoblast (CDX2), cell stypo determining (NOTCH1), and core "stemmness-" associated (OCT4, NANOG, and SOX2) stem cell transcription factors in JEG3 cells. JEG3 cells express CDX2 and NOTCH1 ((a) and (i)). OCT4, NANOG and SOX2 signaling are not visible (with human serum: (e), (m), and (q); without human serum (f), (n), and (r)). Immunofluorescent staining seems less specific with or without blockage with human serum (all positive signals: (c), (d), (g), (h), (k), (l), (o), (p), (s), and (t)).

Since it has been described that nonspecific binding in trophoblast(ic) cells is extreme and often cannot be alleviated through conventional blocking procedures [22], we initially blocked cell lines with normal goat serum and further blocked them with or without human serum. Upon blocking of HTR8/SVneo cells with human serum, the positive signals for all investigated transcription factors were reduced, especially in the nuclei, or, as in the case of OCT4a, disappeared altogether (Figure 3: (f), (j), (n), and (r) without human serum versus (e), (i), (m), and (q) with human serum). The expression intensity was highest for NOTCH1, and this expression was only slightly altered after blockage with human serum (Figures 3(i) and 3(j)). In contrast to peroxidase staining, we detected all stem cell markers via fluorescence staining, and there were no visible differences in staining pattern between the blocking procedures (Figure 3, for NANOG: (g) and (h); for NOTCH1: (k) and (l); for OCT4: (o) and (p); for SOX2: (s) and (t)).

Taking the recommendations of Honig et al. [22] into consideration (meaning that we now deem the immunofluorescence procedure unreliable for staining of these transcription factors in these cells), we surmise that HTR8/SVneo cells express all of the investigated transcription factors except for OCT4.

3.4. JEG3 Cells Express NOTCH1, but Not NANOG, SOX2, and OCT4 according to ICH Analysis. NANOG, OCT4, and SOX2 were not detectable in JEG3 cells regardless of blocking methods (Figure 4: (e), (f), (m), (n), (q), and (r)) after ICH analysis.

JEG3 cells express NOTCH1, and, as in HTR8/SVneo cells, NOTCH1 was detectable with both blocking methods (Figures 4(i) and 4(j)). Interestingly, the NOTCH1 staining signal in JEG3 cells appears less intensive than that in HTR8/SVneo cells (Figure 3(i) versus Figure 4(i)).

Similar to HTR8/SVneo cells, the fluorescent staining of JEG3 cells shows positive signals for NANOG, NOTCH1, OCT4, and SOX2, and it makes no difference in staining pattern if the cells were blocked with or without human serum (Figure 4: (g), (h), (k), (l), (o), (p), (s), and (t)).

3.5. HTR8/SVneo Spheroids Contain Chorionic Villi-Covering Cells Indicative of Repopulation. To demonstrate repopulation capacity of cancer stem/progenitor cells, the progenitor cell candidates are usually injected in an immunocompromised mouse in the general vicinity of the target organ in which prior elimination of the target population has taken place (as the progenitor cells are supposed to replace them) [29]. If the progenitor cell candidates (e.g., mammary gland stem cell) possess repopulating potential, then they will be found in the stead of the original target cell population (e.g., mammary gland).

It has been observed before that altering in vitro culture standards can cause a side population of HTR8/SVneo cells to differentiate into trophoblast subpopulations, as made visible by ICH analysis of differentiation markers [12]. The HTR8/SVneo capacity to actually repopulate the villous in an in vivo setting has not yet been demonstrated.

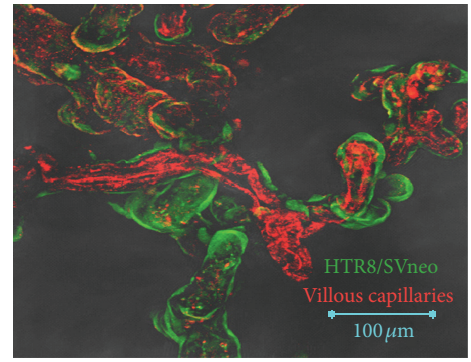

Figure 5: HTR8/SVneo spheroid cells repopulate chorionic villi of placental biopsies. HTR8/SVneo spheroids were confronted with placental biopsies derived from healthy pregnancies after elective, ceasarian section. After 48 h, HTR8/SVneo spheroid cells (green) have covered chorionic villi (endothelium of fetal capillaries within the villi are depicted in red).

The syncytiotrophoblast layer within villous explant cultures is known to fully degenerate following 4 h of culture initiation [30].

We sought to determine whether the cells present in the HTR8/SVneo spheroids could repopulate the entire chorionic villous, which according to the previous citation is now replete of the syncytiotrophoblast layer, following confrontation with placental villous explants in an effort to remain in a near in vivo model of the placenta.

We excluded JEG3 from this experiment as the previous investigations suggest that JEG3 cells possess only low prominent cancer stem cell characteristics. Furthermore, as JEG3 spheroids were unstable and easily disaggregated, it was not possible to transfer them to new hanging drops in which coculture experiments were performed. In coculture of HTR8/SVneo with placental explants, spheroids are completely disaggregated after 48 h, and all retrievable HTR8/SVneo cells cover the chorionic villous (Figure 5; HTR8/SVneo spheroid cells in green; endothelium of fetal capillaries within chorionic villi in red). Ten individual confrontation cultures have been performed with qualitatively similar results.

Assuming that the syncytiotrophoblast layer of placental explants truly do degenerate following 4 h cultivation, then the previous situation suggests that the HTR8/SVneo spheroid cells contain the progenitor cell characteristic of repopulating activity in a near in vivo model.

4. Discussion

HTR8/SVneo and JEG3 cells are highly popular transformed cell lines often used as an imperfect model of the trophoblast, with HTR8/SVneo often deemed as closer to the physiologic setting. Both cell types have been proposed to possess "stem-like" or "progenitor-like" characteristics [2, 12].

One aim of this study was to characterize stem/progenitor cell traits of these cell lines according to general minimum standards used to identify putative cancer stem cells

[14, 15]. Another aim of this study was to characterize the HTR8/SVneo and JEG3 cell lines for their expression pattern of various transcription factors associated with "stemness" (OCT4, NANOG, and SOX2), cell fate (NOTCH1), and trophoblast stem cells (CDX2) and to correlate this to their intrinsic progenitor/stem cell capacities, because their transformed character is likely to contribute to altered transcription factor expression, which in turn is likely to be responsible for stem/progenitor-like behavior. This is a first step in defining a cell population as a progenitor or cancer stem cell.

In our hands, HTR8/SVneo cells have a high propensity to form spheroid bodies, while expressing virtually all investigated transcription factors (specifically CDX2, NOTCH1, SOX2, and NANOG, and not OCT4). Furthermore, HTR8/SVneo spheroid cells demonstrate behavior reminiscent of self-renewal and replenishing properties. In contrast, JEG3 cells have only a limited ability to form spheroid bodies, and although they express the trophoblast stem cell marker CDX2 and a cell fate determinant NOTCH1, they did not express the investigated hESC cell markers or so-called "stemness-" associated transcription factors. Taken together, this is indicative of the fact that HTR8/SVneo cells are transformed in a manner that might make them closer in phenotype to a trophoblast progenitor cell than to a differentiated trophoblast cell, such as a syncytio- or extravillous trophoblast. Our experiments reveal traits that speak for JEG3 cells being a form of cancer stem cell as previously proposed.

As mentioned earlier, our own recent results indicate that HTR8/SVneo cells have less in common with the primary extravillous trophoblast cell than JEG3 cells [11]. GeneChip analyses of the expression signatures of primary trophoblast versus choriocarcinoma cell lines (including JEG3) and versus extravillous trophoblast derived cell lines (including HTR8/SVneo) have revealed that all three groups cluster distinctly [31]. Furthermore, the genes that are similarly expressed between EVT and choriocarcinoma cell lines are related to cell motility, signaling, vasculature and tissue development (all functional signs of differentiation), while HTR8/SVneo and EVT similarities are restricted to those genes regulating RNA transport and metabolism (housekeeping characteristics) [31]. Classic hallmark characteristics for primary, differentiated trophoblast cells are its expression of cytokeratin 7 and negative expression of vimentin [32, 33]. HTR8/SVneo cells show an expression profile that is just the opposite [12, 31]. In this aspect, it is interesting that HTR8/SVneo cells express N-cadherin, while their JEG3 counterparts do not [34], meaning that HTR8/SVneo express at least two known epithelial-mesenchymal transition (EMT) markers (N-cadherin and vimentin), which is also a sign of partial dedifferentiation (reviewed in [27]). Finally, HTR8/SVneo cells express HLA-G, a typical extravillous trophoblast differentiation marker [33], only weakly or not at all [12] and secrete β-HCG, a hallmark characteristic of a syncytiotrophoblast cell (as reviewed in [35]), weakly, but more than primary cytotrophoblast, which do not secrete hCG at all [9].

In our study, we also demonstrate the self-renewal properties of HTR8/SVneo cells propagated as spheroids under the same culture conditions as human embryonic stem cell embryoid bodies. HTR8/SVneo cells not only survive multipassages after spheroid formation, but are continually able to reform spheroids after each passage even with fairly low cell numbers. This is also in line with a recent observation that HTR8/SVneo cells form a side population that displays self-renewal characteristics [12]. We found it somewhat puzzling that all HTR8/SVneo cells incubated in hanging drops formed spheroids in our experiments, while Takao et al. describe only a very exclusive side-population that demonstrates self-renewal properties [12]. However, since we excluded the possibility that HTR8/SVneo spheroids are mere cell aggregates, we propose that the hanging drop environment induces HTR8/SVneo cells to alter its phenotype towards that of a progenitor-like cell.

We have not proven in our own studies that the syncytiotrophoblast layer of placental explants has actually degenerated during the 48 h cultivation period we chose; however, other studies have described that this occurs after 4 h [30]. Following coculture of our placental explants with HTR8/SVneo cells for 48 h, we reveal for the first time that HTR8/SVneo cells have the propensity to "renew" or at least cover villous tissue in a model near to the in vivo situation. We speculate that the syncytiotrophoblast layer is lost, as described, and that HTR8/SVneo cells answer a distress call during the cultivation period, which allows the HTR8/SVneo cells to migrate to this area and cover or indeed replace the villous with syncytiotrophoblast-like cells. This is an indication for a certain degree of plasticity within the HTR8/SVneo cells, which we have not corroborated here with syncytiotrophoblast-specific expression of surface molecules. We believe that this is likely though, since Takao et al. have been able to demonstrate that a side-population of HTR8/SVneo cells is able to differentiate into syncytiotrophoblast and other trophoblast lineages [12]. Future studies are needed in order to unravel which transcription factor or other signal is responsible for regulating this migratory and replenishing characteristic.

Our investigations characterize for the first time the expression of "stemness-" associated cancer and trophoblast stem cell transcription factors in the HTR8/SVneo cell line. The fact that both cell lines produce CDX2, while only HTR8/SVneo shows progenitor cell capacities, could indicate that CDX2 is rather a sign of trophoblast lineage derivation instead of a trophoblast stem cell differentiation alone. However, CDX2 has been shown to be expressed only in first trimester trophoblast, while term placentae lose this capacity. Furthermore, the same study identified that CDX2+ELF5+ cells within the placentae characterize cytotrophoblast populations [36]. This in turn suggests that JEG3 cells have retained a certain cytotrophoblastic identity, which we have not been able to visualize as a progenitor-like function in this study.

The regulatory network between CDX2 and the "stemness-" associated transcription factors is tight and complicated; reiterating this in detail goes beyond the scope of this investigation. Briefly, CDX2 and the "stemness-" associated transcription factors are thought to reciprocate or mutually antagonize each other, also because this is the expression pattern found during blastocyst formation. At least in the mouse,

CDX2 is known to downregulate OCT4 and NANOG [37, 38]. However, SOX2 and CDX2 are known to cooperate during trophectoderm formation [4]. In our analysis, while JEG3 cells did not express any of the so-called "stemness-" associated transcription markers, HTR8/SVneo cells expressed most of them (OCT4a^{neg}, NANOG^{weak}, and SOX2^{weak}). Interestingly, a recent characterization of trophoblast progenitor cells derived from first trimester placentae reveals that undifferentiated trophoblast progenitor cells that form embryoid bodies and that are capable of multipassage also display OCT4^{neg}, NANOG^{weak}, and SOX2^{weak} phenotypes [39]. Due to the association of this transcription marker expression profile with the presence of progenitor-like functions in HTR8/SVneo cells and in trophoblast progenitor cells, it is enticing to conclude that NANOG and SOX2 are responsible for the observed functions. Further studies with gain/loss-of-function analyses would, however, be necessary to finalize that conclusion. Furthermore, since CDX2 is coexpressed in HTR8/SVneo cells with certain hESC transcription factors, it is most plausible that the "stemness-" associated transcription factor regulatory network has been corrupted, probably through alterations secondary to transfection of HTR8 parent cells with a plasmid containing the simian virus 40 large T antigen (SV40).

We were surprised to see that NANOG was not expressed in JEG3 cells as described in a recent publication, in which NANOG was especially detected in the nuclear fraction of JEG3 cells [40]. Currently, we cannot explain this discrepancy other than in methodology differences, and further analyses on the mRNA and protein level are under way.

The NOTCH1 signal was remarkably visible in both cell lines. NOTCH1 has been associated with "stemness" properties, as well as with the differentiation of cancer stem cells (tumor stem-like cells) into endothelial progenitor cells [18, 19, 27]. Furthermore, NOTCH1 expression is linked with trophoblast, as well as with malignant types of invasion [17, 20, 28]. In the physiologic placenta, NOTCH1 expression is thought to be vital for placental angiogenesis, while defective NOTCH signaling is thought to contribute in the pathogenesis of preeclampsia (reviewed in [41]). With our current information, it is as yet impossible to conclude whether NOTCH1 expression is a sign of stem-like properties, of EMT or invasion potential. Further functional analyses would be helpful in unraveling this aspect.

In summary, though, all of the previously analyzed characteristics of HTR8/SVneo are in line with the idea that HTR8/SVneo cells share more similarities to a trophoblast progenitor cell, perhaps the cytotrophoblast, than a primary extravillous trophoblast. Our investigation, together with that of Takao et al., is first step in defining HTR8/SVneo progenitor cell characteristics; however, the final step of in vivo testing per xenotransplantation into immuncompromised mice is as yet pending (but seems likely to be successful, since xenografts of human placental explants into immuncompromised mouse is highly successful [42]).

In all finality, we hypothesize that transfection of the HTR8/SVneo cell line has altered the original (extravillous) trophoblast cell in a manner leading to development of the characteristics described in these investigations; thus, investigators should use caution when using the popular HTR8/SVneo cell line as a model for primary extravillous trophoblast cells.

We wish also to direct investigators to the expression alterations (or lack thereof) seen in our immunocytochemistry and immunofluorescence results before and after blockage with human serum. Our results suggest, as with [22], that trophoblastic cells are prone to unspecific binding, and caution should be exercised when interpreting immunostaining results.

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Research Article

Expression of Pluripotency and Oocyte-Related Genes in Single Putative Stem Cells from Human Adult Ovarian Surface Epithelium Cultured *In Vitro* in the Presence of Follicular Fluid

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The aim of this study was to trigger the expression of genes related to oocytes in putative ovarian stem cells scraped from the ovarian surface epithelium of women with premature ovarian failure and cultured *in vitro* in the presence of follicular fluid, rich in substances for oocyte growth and maturation. Ovarian surface epithelium was scraped and cell cultures were set up by scrapings in five women with nonfunctional ovaries and with no naturally present mature follicles or oocytes. In the presence of donated follicular fluid putative stem cells grew and developed into primitive oocyte-like cells. A detailed single-cell gene expression profiling was performed to elucidate their genetic status in comparison to human embryonic stem cells, oocytes, and somatic fibroblasts. The ovarian cell cultures depleted/converted reproductive hormones from the culture medium. Estradiol alone or together with other substances may be involved in development of these primitive oocyte-like cells. The majority of primitive oocyte-like cells was mononuclear and expressed several genes related to pluripotency and oocytes, including genes related to meiosis, although they did not express some important oocyte-specific genes. Our work reveals the presence of putative stem cells in the ovarian surface epithelium of women with premature ovarian failure.

1. Introduction

From the literature it is known that oocyte-like cells expressing different oocyte-specific genes can be developed *in vitro* from mouse embryonic stem cells (mESCs) [1–8], human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) [9–11], stem cells from human amniotic fluid [12], from porcine fetal skin [13, 14], and even from rat pancreatic stem cells [15].

Although *in vitro* oogenesis from animal and human ESCs could represent a model to study the mechanisms of oogenesis and their pathologies, the potential oogenesis from the autologous ovarian stem cells would be of great advantage because it may be realistically applied in human medicine in the future. Ovarian stem cells may play an important role. More studies have already confirmed the presence of pluripotent/multipotent stem cells in neonatal and adult ovaries of mice [6, 16, 17] and proposed human ovarian surface epithelium (OSE) as an important source of stem cells in human [18–22] and other mammalian species, such as sheep and monkey [22]. Moreover, White et al. have recently published the existence of rare mitotically active cells—germline stem cells—with a gene expression profile that is consistent with primitive germ cells, which can be purified from adult human ovarian cortical tissue by fluorescence-activated cell sorting-based protocol [23]. They have proven that these cells can

be expanded for months *in vitro* and can spontaneously be developed into haploid oocyte-like cells with diameters of up to 35–50 μm. When marked with green fluorescence protein (GFP) and transplanted into human ovarian cortical biopsies, the follicles containing GFP-positive oocytes were formed 1-2 weeks after the xenotransplantation into immunodeficient female mice. They concluded that the ovaries of reproductive-age women possess rare mitotically active germ cells that can be propagated *in vitro* and can generate oocytes *in vitro* and *in vivo*.

Premature ovarian failure (POF) is one of the most serious indications of female infertility resulting in nonfunctional ovaries without mature follicles and oocytes before the age of 40 years [24]. It is characterized by high levels of gonadotropins in the blood and amenorrhea. These women have no mature oocytes and do not conceive and cannot bear their own child. Any potential to regenerate the nonfunctional ovaries in these women would be of the greatest importance in reproductive medicine.

The aim of this study was to culture *in vitro* putative stem cells from the OSE of nonfunctional ovaries in the presence of donated follicular fluid, rich in substances important for oocyte growth and maturation to trigger their growth and the expression of genes related to human oocytes. Because the genetic status of oocyte-like cells developed *in vitro* from stem cells is still poorly understood, these cells were analyzed by detailed single-cell gene expression profiling in comparison to human embryonic stem cells, oocytes at different stages of maturity, and somatic fibroblasts to elucidate their genetic status. In this way we made some steps further from our previous work. The primitive oocyte-like cells developed in this study expressed several genes characteristic of pluripotent stem cells and oocytes, including some genes related to meiosis, but were more "stem cells" than "oocytes" at this stage.

2. Materials and Methods

In five women with premature ovarian failure (POF) and with no naturally present mature follicles or oocytes the putative ovarian stem cells were retrieved by OSE brushing. The mean female age was 34 years (range: 21–39 years). Each woman donated a part of her ovarian tissue for the purpose of research after having the study explained in detail and then provided written consent to participate. All women were characterized by irregularities in their menstrual cycle, elevated levels of gonadotropins (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) in their blood serum, and a thin endometrium, as can be seen in Table 1. The molecular status of oocyte-like cells developed *in vitro* was compared to hESCs (H1 cell line, WiCell Research Institute, Madison, WI, USA) and nonfertilized oocytes from the *in vitro* fertilization programme, donated for the purpose of research with the written consents of the donating women. There was no financial recompense to the donors of oocytes. This research was approved by the Slovenian Medical Ethical Committee (Ministry of Health of the Republic of Slovenia, No. 110/10/05).

2.1. Ovarian Stem Cell Retrieval. In each of the five women with POF the putative ovarian stem cells were retrieved by soft OSE brushing of the whole ovary and small ovarian cortex biopsy (approximately 0.3 mm^3), retrieved by the usual diagnostic laparoscopic procedure. A part of the ovarian cortex biopsy was sent to the histopathological service lab to evaluate the presence of follicles or oocytes after haematoxylin-eosin (HE) staining, common in everyday clinical practice; another part of the ovarian cortex tissue was used to set up a cell culture. The ovarian cortex biopsy was put in a volume of 2.5 mL of warmed and preincubated supplemented DMEM/F-12 culture medium (composition described below). The OSE layer was mechanically scraped several times using a sterile surgical blade (Swann-Morton, Sheffield, United Kingdom, ref. 0501), which was washed in the surrounding culture medium to retrieve a scraped suspension of cells to set up cell cultures.

2.2. Ovarian Surface Epithelium Cell Culture. The OSE cell cultures were set up using OSE brushings of cortex biopsies. In each patient the OSE cell culture was set up by dropping five drops of the cell suspension from brushed OSE into the DMEM/F-12 culture medium, prepared as follows: Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12 Ham with L-glutamine, phenol red, and 15 mM HEPES (Sigma Aldrich), supplemented with 3.7 g/L $NaHCO_3$, 1% penicillin/streptomycin, and 0.5% gentamycin, and with the pH adjusted to 7.4 with 1 M NaOH; this culture medium was supplemented with 20% (v/v) donated follicular fluid from the *in vitro* fertilization programme. In each patient the cell culture was set up in 12 IVF Multidish Four well Nunclon dishes (Nunc, Roskilde, Denmark, ref. 144444). Each well was filled with 350 μL of preincubated culture medium to which drops of the cell suspension from brushed OSE were added. The cells were cultured for two months in a CO_2 incubator (Heraeus 6000, Heraeus Holding, Hanau, Germany) at 37°C and 6% CO_2 in the air and monitored daily under a heat-staged inverted microscope (ECLIPSE 2000-S, Nikon, Tokyo, Japan) equipped with a Digital Sight camera (Nikon, Tokyo, Japan).

2.3. Preparation of Follicular Fluid. The follicular fluid, retrieved after the written consent of the young donor who had normal ovarian function, was used immediately after the removal of the oocytes (to be fertilized *in vitro*) in order not to coagulate. It was centrifuged for 10 minutes at 2,500 rpm (349 ×g). The supernatant was filtered through a sterile Sartorius Minisart 0.45 μm filter to remove all the cells. The filtered supernatant was heat inactivated at 56°C for 45 minutes. Then it was aliquoted and stored at −20°C until its use. When it was used, an aliquot of prepared follicular fluid was thawed at the room temperature and added to the culture medium. This fluid contained substances important for oocyte growth and differentiation and was added to provide an ovarian-like growth milieu.

2.4. Analyses of Reproductive Hormones in Culture Medium. In the samples of culture medium with added follicular

Table 1: Clinical data of all five patients with POF included into this study, which were documented at the Department of Obstetrics and Gynecology, University Medical Centre Ljubljana. All patients had increased serum levels of gonadotropins FSH (normal: <11.3 IU/L) and LH (normal: <11.6 IU/L) and thin endometrium.

Clinical data	Patients				
	P1 (R.A.)	P2 (G.K.)	P3 (M.S.)	P4 (G.A.)	P5 (P.R.A.)
Age (years)	39	40	21	31	39
FSH (IU/L)	89.1	65.8	11.8	162.0	67.8
LH (IU/L)	23.3	23.4	10.1	59.0	37.8
Prolactin (mg/L)	12	4.6	16.4	9.3	/
Estradiol (nmol/L)	<0.073	0.08	0.2	/	/
Inhibin B (ng/L)	<10	<10	52.4	>10	/
S-AMH (mg/L)	0.00	0.00	0.89	0.00	/
Karyotype	Abnormal (mosaic 45X, 47XXX, 48XXXX, 46XX)	Normal (no FMR1-fragile X mutation)	Normal (no FMR1-fragile X mutation)	Normal (no FMR1-fragile X mutation)	Normal (no FMR1-fragile X mutation)
Ovarian cortex histology	Inclusion cysts, corpora albicantia, focal ovarian surface epithelium, no follicles or oocytes	Inclusion cysts, corpora albicantia, simple or stratified ovarian surface epithelium, no follicles or oocytes	Simple columnar ovarian surface epithelium, several primordial follicles	Simple cuboidal ovarian surface epithelium, corpus luteum in regression, no follicles or oocytes	Simple cuboidal ovarian surface epithelium, no follicles or oocytes
Antiovarian antibodies	No	No	Yes	Yes	No
Premature ovarian failure (POF)	Secondary (previous birth of a child), irregularities of menstrual cycles, thin endometrium	Primary (no children), irregular menstrual cycles, thin endometrium	Primary (no children), irregular menstrual cycles, thin endometrium	Primary (no children), amenorrhea, small left ovary, thin endometrium	Secondary (pregnancy ended in spontaneous abortion), amenorrhea, thin endometrium

fluid which were taken from two different OSE cell cultures after 15–17 days of culturing the concentrations of estradiol, progesterone, androstenedione, and testosterone were measured in comparison with the same medium with added follicular fluid and without cell culture and the same medium without follicular fluid and without cell culture. Estradiol was measured by a chemoluminescent, competitive, immunochemical method by reagents (Immulite, Siemens, USA) which included alkaline phosphatase-conjugated estradiol and highly specific polyclonal rabbit antiestradiol antibodies on paramagnetic particles. Progesterone was measured by a chemoluminescent, noncompetitive, immunochemical method by reagents (Immulite, Siemens, USA) which included alkaline phosphatase-conjugated progesterone and highly specific monoclonal rabbit antiprogesterone antibodies on paramagnetic particles. Both hormones were analyzed by Immulite analyzer (Siemens, USA). Androstenedione was measured by a specific double antibody RIA using 125 I-labeled hormones (Diagnostic Systems Laboratories, Webster, TX, USA). Testosterone level was determined by RIA (DiaSorin and DPC, Los Angeles, CA, USA). All procedures were performed according to standardized procedures at the Institute of Nuclear Medicine, University Medical Centre Ljubljana.

2.5. Single-Cell Gene Expression Analyses Using the Fluidigm BioMark System. Gene expression analyses of single oocyte-like cells in comparison with single hESCs and groups of five, ten, and twenty hESCs of H1 line (positive controls), single nonfertilized oocytes from the *in vitro* fertilization programme (positive controls), and single human fibroblasts and groups of five, ten, and twenty fibroblasts of F161 line (negative controls) were performed using the BioMark Real-Time quantitative PCR (qPCR) system (Fluidigm, San Francisco, CA, USA). In all oocyte-like cells, mechanically removed from the cell cultures expressions of 56 genes, 21 genes characteristic of pluripotent stem cells (*KIT*, *KIT LIG*, *OCT4A*, *NANOG*, *MYC*, *KLF4*, *SOX-2*, *UTF1*, *TDGF1*, *LIN28B*, *TERT*, *CD9*, *LIN28*, *NANOS*, *CDH1*, *STAT3*, *REX1*, *MEST*, *CRKRS*, *STELLA*, and *GDF3*) and 34 genes typical of oocytes (*VASA*, *DAZL*, *STELLA*, *GFRa1*, *KIT*, *KIT LIG*, *DNMT3B*, *DNMT1*, *BMP15*, *ZP1*, *ZP2*, *ZP3*, *ZP4*, *SCP1*, *SCP2*, *SCP3*, *BUB1*, *BUB3*, *NOBOX*, *MSH5*, *NLRP5*, *FMN2*, *HIFOO*, *MLH1*, *ZAR1*, *REC8*, *PRDM1/BLIMP1*, *FIGLA*, *STAG3*, *DMC1*, *SMC1*, *CD9*, *BNC1*, and *CCNB1*) and of the housekeeping gene *GAPDH* were analyzed. The inventoried TaqMan assays (20x, Applied Biosystems, Life Technologies, Carlsbad, CA, USA) were pooled to a final concentration of 0.2x for each of the 56 assays. The cells to be analyzed were harvested directly into

9 μL RT-PreAmp Master Mix: 5.0 μL CellsDirect 2x Reaction Mix (Invitrogen, Life Technologies, Carlsbad, CA, USA); 2.5 μL 0.2x assay pool; 0.2 μL RT/Taq Superscript III (Invitrogen, Life Technologies); 1.3 μL TE buffer. The harvested cells were immediately frozen and stored at −80°C. Cell lysis and sequence-specific reverse transcription were performed at 50°C for 15 minutes. The reverse transcriptase was inactivated by heating to 95°C for 2 minutes. Subsequently, in the same tube cDNA went through limited sequence-specific amplification by denaturing at 95°C for 15 seconds and then annealing and amplification at 60°C for four minutes for 14 cycles. These preamplified products were diluted 5-fold prior to analysis with the Universal PCR Master Mix and inventoried TaqMan gene expression assays (ABI) in 96.96 Dynamic Arrays on a BioMark System. Each sample was analyzed in two technical replicates. Ct values obtained from the BioMark System were transferred to the GenEx software (MultiD) to analyze the gene expressions.

2.6. Analyses of Single-Cell Gene Expressions by GenEx Software (MultiD). Ct values were obtained from the BioMark System and transferred to the GenEx software (MultiD Analyses, Göteborg, Sweden). Missing data in the BioMark System were given a Ct of 999. These were removed in GenEx. Also Ct values larger than 25 were removed (cut-off value > 25), since samples with such high Ct values in the BioMark 96 × 96 microfluidic card were expected to be negative, and these readings were unreliable. Technical repeats were then averaged. Missing data were then replaced by the highest Cq + 1 for each gene. This corresponded to assigning a concentration to these samples that was half of the lowest concentration measured and was motivated by sampling ambiguity. There was also a need to handle missing data for downstream classification with multivariate tools. Linear quantities were calculated relative to the sample having lowest expression, and data were converted to $\log_2$ scale. Because of single-cell gene expression analyses, normalization to the housekeeping genes was not performed. The data were now prepared for multivariate analysis to classify the samples based on the combined expression of all the genes. Heatmap clustering (Ward's Algorithm, Euclidean Distance Measure), hierarchical clustering (Ward's Algorithm, Euclidean Distance Measure), and principal component analysis (PCA) were performed. In addition, descriptive statistics were calculated individually for the genes using a 0.95% confidence level, and groups were compared using 1-way ANOVA (Tukey-Kramer's pairwise comparison) and unpaired 2-tailed t-test. According to Dunn-Bonferroni, corrected statistical significance was set at $P < 0.00244$ for genes of pluripotency or $P < 0.00151$ for oocyte-specific genes to account for false positives due to multiple testing.

2.7. DAPI Staining of Cell Nuclei. To monitor the cell nuclei, single oocyte-like cells were isolated from the cultures and put into a drop of Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Peterborough, United Kingdom) and observed under a fluorescence microscope (ECLIPSE E-600 with a Digital Sight camera, magnifications of 200/400x, Nikon, Tokyo, Japan) after 20 minutes of incubation at the room temperature and in the dark.

3. Results

3.1. Histopathology of Ovarian Tissue. A part of each ovarian cortex biopsy was sent to the histopathological routine service. In four of them no follicles or oocytes were observed in their ovarian cortex, and in one biopsy only a few primordial follicles were found after haematoxylin-eosin (HE) staining (Figure 1). Cytokeratin (CK7) staining revealed the presence of OSE in all five ovarian biopsies.

3.2. In Vitro Culture of Scraped Ovarian Surface Epithelium. Ovarian fibroblasts were the first cells to attach to the bottom of the dish. They were observed from approximately the second day of culture as elongated cells spreading the dish bottom. After five days, the first large, round cells with different diameters appeared in the culture (Figures 2(a) and 2(b)). Most of them grew attached to the autologous ovarian fibroblasts (Figure 2(c)). The largest cells had diameters of 50–60 μm and had different granularity to their cytoplasm. Some of them had low-granular cytoplasm and resembled primitive oocytes (Figures 2(d) and 2(e)). Henceforth, we refer to them as primitive oocyte-like cells. Rarly they developed zona pellucida-like structures (Figure 2(f)). Some of them developed into parthenogenetic blastocyst-like structures, as can be seen in Figure 3 (OLC18). The primitive oocyte-like cells that developed showed no signs of degeneration during extended cell culturing of up to two months and retained the round shape.

3.3. Concentrations of Reproductive Hormones in Culture Medium. All samples of culture medium with added follicular fluid were characterized by very high concentrations of estradiol and progesterone in comparison with reference serum values during different phases of female menstrual cycle (Table 2). They also contained significant concentrations of androstenedione and testosterone in comparison with culture medium without added follicular fluid. In samples of culture medium from ovarian cell cultures the concentrations of all hormones were lower than in samples with added follicular fluid, but without ovarian cell culture; these hormones were depleted/converted by ovarian cell cultures. Also a pure culture medium without added follicular fluid contained a low level of estradiol thus reflecting the presence of phenol red, which is known to express weak estrogenic activity in tissue culture media [25].

3.4. Characterization of Individual Primitive Oocyte-Like Cells Differentiated In Vitro by Expression Profiling of Pluripotent Stem Cell and Oocyte-Specific Genes. In samples from all patients who had no follicles or oocytes in their ovarian cortex, 18 primitive oocyte-like cells differentiated *in vitro* (Figure 3) and cultured for up to one month (from 5 to 31 days) were mechanically removed from the culture with a glass pipette. Expression of 56 genes was measured in each

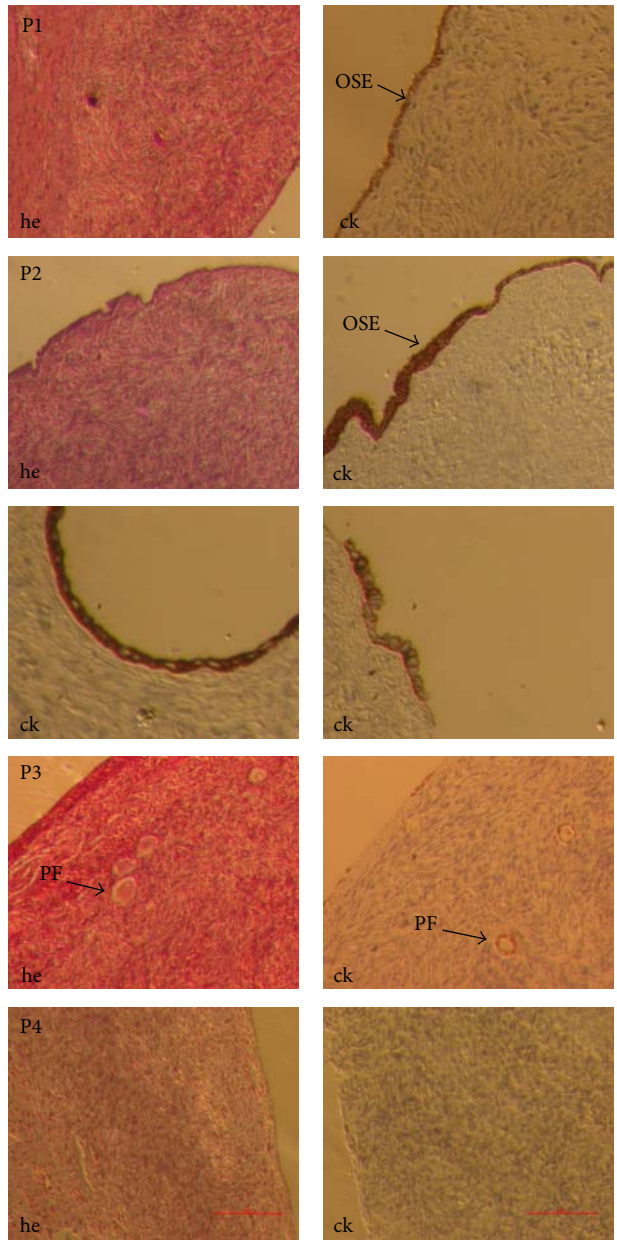


Figure 1: Histology of ovarian sections after haematoxylin-eosin (he) and cytokeratin (ck) stainings in patients (P1, P2, P3, and P4) with POF. It confirmed no naturally present follicles or oocytes in their ovarian cortex, except for primordial follicles in P3. In P5 the histology has been performed in another medical institution. (inverted microscope, Hoffman, magnification 40/100x.) Legend: OSE: ovarian surface epithelium (brown stained), PF: primordial follicle.

primitive oocyte-like cell. Twenty-one genes were characteristic of pluripotent stem cells, 34 genes were typical of oocytes, and one gene was housekeeping gene (see Section 2). Nineteen individual nonfertilized oocytes from the *in vitro* fertilization programme collected at different stages of maturation (six germinal vesicle(GV), four metaphase I-MI, four metaphase II-MII, and five *in vitro* matured(IVM) oocytes) and hESCs of the H1 line (three single cells, a group of five cells, a group of ten cells, and a group of twenty cells) were used as positive controls, and human adult fibroblasts from the F161 line (three single cells, a group of five cells, a group of ten cells, and a group of twenty cells) were used as negative controls.

3.4.1. Comparison of Gene Expression Profiles for All Analyzed Genes in All Analyzed Types of Cells. Different types of cells express different genes, and when comparing the expression among the cells we analyze, we expected to see significant differences (Figure 4). There is substantial natural variation in the transcript levels of individual cells of the same kind because of temporal fluctuations caused by transcriptional bursting [26, 27]. The cells of different kinds cannot be

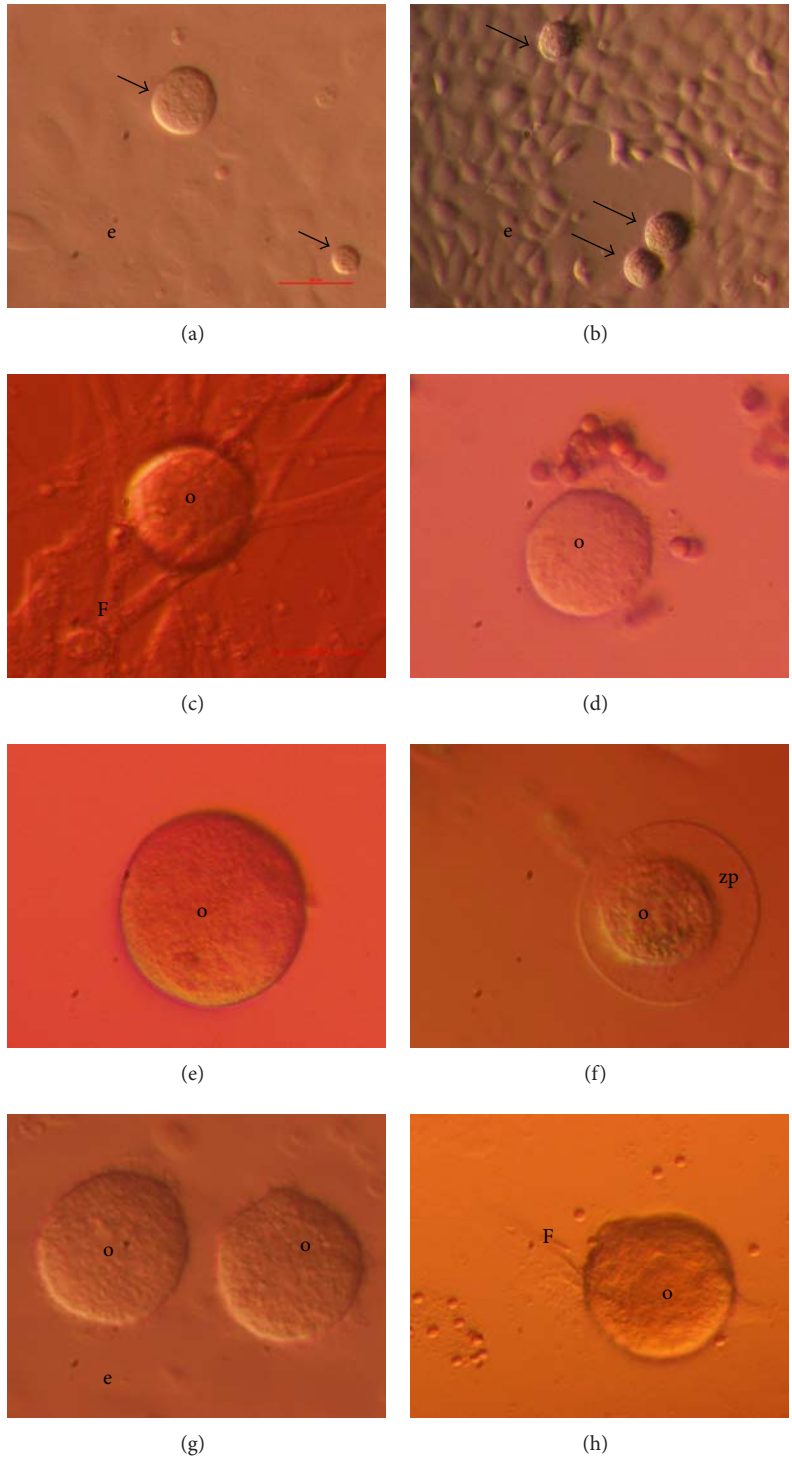


Figure 2: Primitive oocyte-like cells (arrows) developing in ovarian surface epithelium cell cultures set up by ovarian cortex biopsy scrapings: (a, b) among epithelial cells, (c) attached to autologous ovarian fibroblasts, (d, e) oocyte-like cells, (f) growing primitive oocyte-like cell with zona pellucida-like structure, (g) oocyte-like cells, and (h) primitive oocyte-like cell attached to fibroblast. Scale bars: (a, b) 100 μm, (c–h) 50 μm. Legend: e: epithelial cells, F: fibroblast, o: oocyte-like cell, and zp: zona pellucida-like structure.

Table 2: The mean concentrations of reproductive hormones in samples of culture medium (DMEM/F-12) with added follicular fluid (FF). Retrieved from two different ovarian cell cultures (OSE) with developing oocyte-like cells after 15–17 days of culturing in comparison with the same culture medium with added follicular fluid (without OSE cell culture) and the same culture medium (without added follicular fluid and OSE cell culture).

	Concentrations of hormones in culture medium			
	Estradiol* (nmol/L)	Progesterone** (nmol/L)	Androstenedione (nmol/L)	Testosterone (nmol/L)
DMEM/F-12 + FF + OSE (min.–max.)	175.5 (149.0–202.0)	1473.5 (3999.0–1052.0)	81.4 (82.5–80.3)	2.55 (2.4–2.7)
DMEM/F-12 + FF (min.–max.)	310.5 (377.0–244.0)	4783.5 (5528.0–4039.0)	91.4 (90.1–92.8)	4.15 (3.9–4.4)
DMEM/F-12 (min.–max.)	0.73 (0.66–0.81)	<0.64	0.015 (0.01-0.02)	1.1 (1.0–1.2)

*Reference serum values in women. Follicular phase: 0–0.59 nmol/L; follicular phase, day 2-3: 0–0.31 nmol/L; at ovulation: 0.12–1.47 nmol/L; luteal phase: 0.1–0.9 nmol/L; nontreated postmenopausal: 0–0.11 nmol/L; treated postmenopausal: 0–0.34 nmol/L; at oral contraception: 0–0.37 nmol/L. Reference serum value in men: 0–0.21 nmol/L.

**Reference serum values in women. Follicular phase: up to 3.6 nmol/L; follicular phase, middle: up to 3.1 nmol/L; at ovulation: 1.5–5.5 nmol/L; luteal phase: 3.0–68 nmol/L; luteal phase, middle: 19–76 nmol/L; postmenopausal: up to 3.2 nmol/L; at oral contraception: 1.1–2.9 nmol/L; pregnancy, first quarter: 29.6–106 nmol/L; pregnancy, second quarter: 93.8–159 nmol/L; pregnancy, third quarter: 264–509 nmol/L. Reference serum value in men: 0.86–2.9 nmol/L.

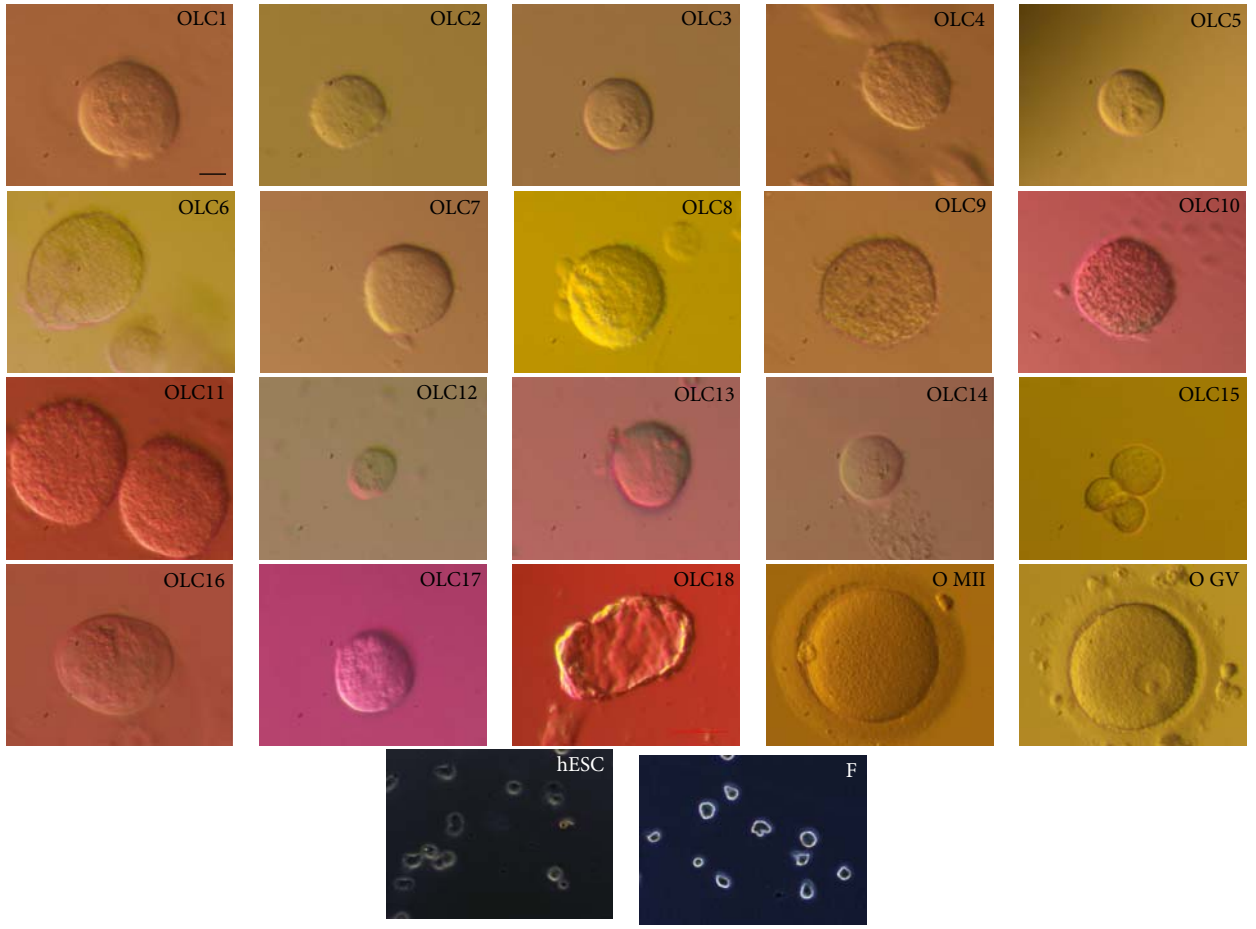


Figure 3: Single cells analyzed on expression of genes: eighteen single primitive oocyte-like cells (OLC1–18), developed *in vitro* (OLC18 further developed in a parthenogenetic blastocyst-like structure) and cultured for up to one month (OLC1–8 and OLC16–18 for 15 days, OLC9–11 for 30 days, and OLC12–15 for 5 days), one mature (O MII) and one immature (O GV) oocyte (inverted microscope, Hoffman, scale bar: 10 μm), and human embryonic stem cells (hESCs) and fibroblasts (F) under a phase-contrast microscope (magnification 200x).

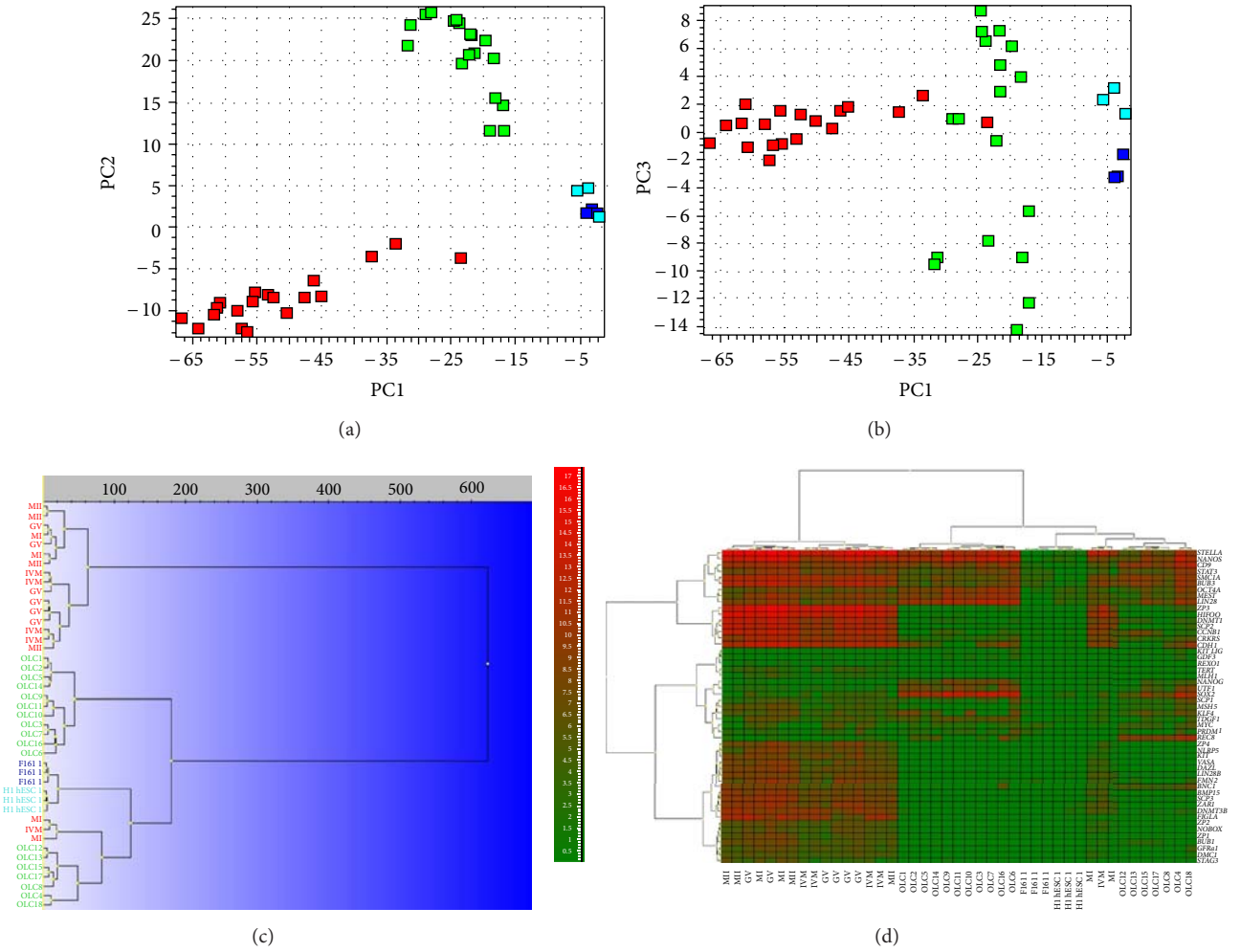


FIGURE 4: Single-cell gene expression profile of all analyzed genes in primitive oocyte-like cells developed *in vitro* (OLCs). Comparison with human embryonic stem cells (H1 hESCs), nonfertilized oocytes from the *in vitro* fertilization programme (O) and fibroblasts (F161) revealed the expression of several genes related to pluripotent stem cells and oocytes. (a) Principle component analysis (PC1 versus PC2). (b) Principle component analysis (PC2 versus PC3). (c) Hierarchical clustering. (d) Heatmap clustering. Legend of analyzed cells:OLC(1–18): primitive oocyte-like cells developed *in vitro*; O: oocytes; H1 hESC: human embryonic stem cells of line H1 (1: one cell, 5: five cells, 10: ten cells, and 20: twenty cells); F161: fibroblasts of F161 line (1: one cell, 5: five cells, 10: ten cells, 20: twenty cells); MII: mature, metaphase II oocytes; IVM: immature, *in vitro* matured oocytes; MI: immature, metaphase I oocytes; GV: immature, germinal vesicle oocytes from the *in vitro* fertilization programme. Cell group colours: red: oocytes from the *in vitro* fertilization programme, green: primitive oocyte-like cells, developed *in vitro*, aquamarine: hESCs, and dark blue: fibroblasts.

unambiguously distinguished based on the expression of any individual gene; it is rather more effective to separate them based on the correlated expression of the panel of genes [28]. The most common and powerful tools to categorize samples based on the expression of multiple genes are the principle component analysis (PCA) and hierarchical clustering [29]. PCA identifies the combinations of genes that account for most of the variation in the measured data and can be used to visually cluster samples based on similar expression profiles in a scatter plot with axes that represent those optimal linear combinations of genes. Those optimum combinations are called principal components (PCs). Figure 4(a) shows all the analyzed single-cell samples clustered in a PC1 versus PC2 scatter plot.

In this study most of cells were either oocytes or primitive oocyte-like cells, and therefore the mathematical model developed is dominated by features that distinguish between oocytes and oocyte-like cells. As a consequence, the fibroblasts and hESCs do not distinguish themselves appreciably in this comparison. In cases like this, biologically relevant information may be hidden in higher order PCs. The oocyte-like cells, hESCs, and fibroblasts were all found at high PC1 values, which reflects a shared high expression of the genes behind PC1 among them. Along PC3 the fibroblasts and hESCs clearly separated (Figure 4(b)). This suggests that they have different expression among the genes defining PC3. Moreover, primitive oocyte-like cells also separated along PC3 (Figure 4(b)). This suggests there are two kinds of

oocyte-like cells that share features either with the hESCs or with the fibroblasts. Eight primitive oocyte-like cells shared features with the fibroblasts and were more "somatic," possibly, whereas ten primitive oocyte-like cells shared features with the hESCs and were putative stem cells. Primitive oocyte-like cells sharing features with the hESCs also shared features with three oocytes: two immature MI oocytes and one *in vitro* matured(IVM) oocyte, as seen in Figures 4(c) and 4(d).

In hierarchical clustering (heatmaps), sixteen oocytes group together, while three oocytes (two immature MI oocytes and one *in vitro* matured(IVM) oocyte) cluster with seven primitive oocyte-like cells developed *in vitro* (Figure 4(d)). hESCs and fibroblasts cluster together mainly due to low expressions of the chosen genes.

3.4.2. Expression of Genes Characteristic of Pluripotent Stem Cells. Oocyte-like cells developed *in vitro* expressed 17 out of the 21 analyzed genes characteristic of pluripotent stem cells (Figure 5(a)). Primitive oocyte-like cells expressed *KIT-LIG*, *OCT4A*, *NANOG*, *MYC*, *KLF4*, *SOX2*, *UTF1*, *TDGF1*, *CD9*, *LIN28*, *NANOS*, *CDH1*, *STAT3*, *MEST*, *CRKRS*, *STELLA*, and *GDF3* and did not express *KIT*, *LIN28B*, *TERT*, and *REX1*. Most of the 13 cells clustered together with the groups of 10 and 20 hESCs, as revealed by hierarchical clustering (dendrogram) (Figure 5(b)) and PCA (Figure 5(c)). Only five cells clustered with three outstanding oocytes (Figure 5).

Primitive oocyte-like cells developed *in vitro* expressed *LIN28*, *NANOG*, *SOX-2*, and *UTF1* to significantly a greater extent and *STELLA*, *CDH1*, and *CRKRS* to significantly lesser extent than oocytes, as revealed by two-tailed Mann-Whitney test (Figure 6(a)). To keep the overall risk of false positive at 5%, a *threshold* value of $P < 0.00244$ was used to indicate significance. The P values are presented in Table 3. *KIT-LIG* was the only gene not expressed in the oocytes. Primitive oocyte-like cells developed *in vitro* expressed *CD9*, *NANOS, STELLA*, *STAT3*, *UTF1*, and *LIN28* to significantly greater extents than the hESCs (Figure 6(b)). hESCs did not express *KIT-LIG* and *REX1*. Different from the primitive oocyte-like cells and hESCs, fibroblasts did not express *KIT*, *KIT-LIG*, *GDF3*, *NANOG*, *UTF1*, *TDGF1*, *LIN28B*, *TERT*, *CDH1*,and *REX1*,which are characteristic of pluripotent stem cells (Figure 6(c)). Further, they did express *NANOS*, *LIN28*, *STELLA*, *CD9, SOX-2*, *OCT4A*, *MEST*,and *STAT3* at a very low level, substantially less than the primitive oocyte-like cells (Figure 6(c); P values in Table 3). Comparing all groups of cells statistically significant differences (Table 3) were found in the expression of *KIT*, *STELLA*, *OCT4A*, *LIN28*, *NANOG*, *SOX-2*, *UTF1*, *TDGF1*, *LIN28B*, *TERT*, *CD9*, *NANOS*, *CDH1*, *STAT3*, *MEST*, and *CRKRS* based on the One-Way ANOVA.

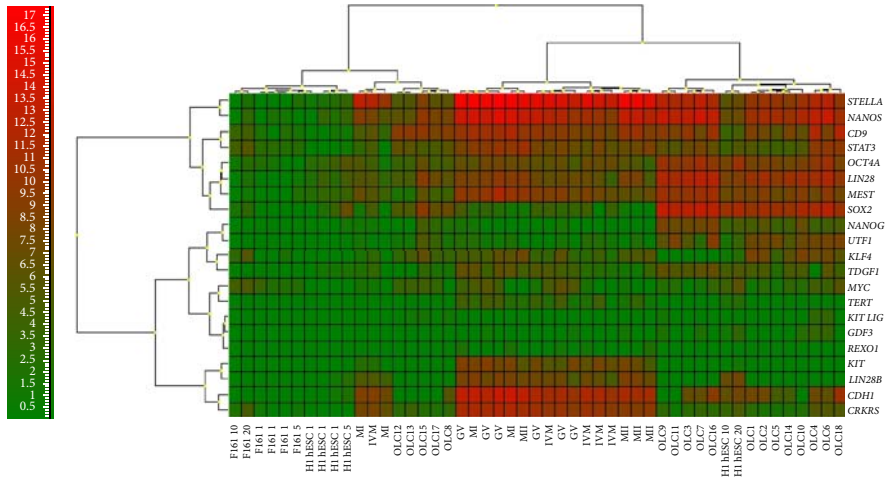
3.4.3. Expression of Oocyte-Specific Genes in Comparison with Nonfertilized Oocytes from the In Vitro Fertilization Programme. Primitive oocyte-like cells developed *in vitro* expressed 22 out of the 34 analyzed oocyte-specific genes (Figure 7(a)): *GFRAa1*, *KIT-LIG*, *DNMT3B*, *DNMT1*, *ZP3*, *SCP1*, *SCP2*, *SCP3*, *CCNB1*, *FMN2*, *HIFOO*, *DMC1*, *BUB1*, *BUB3*, *STELLA*, *STAG3*, *SMC1A*, *BNC1*, *REC8*, *MSH5*, *PRDM1/BLIMP1*, and *CD9* and did not express the twelve important oocyte- (germline-) specific genes: *VASA*, *DAZL*, *KIT*, *BMP15*, *ZP1*, *ZP2*, *ZP4*, *NOBOX*, *NLRP5*, *MLH1*, *ZAR1*, and *FIGLA*. As revealed by hierarchical clustering (Figure 7(b)) and PCA (Figure 7(c)), the oocytes form an independent cluster evidencing a distinct expression profile with the exception of two immature MI oocytes and one IVM oocyte. Positive control oocytes from the *in vitro* fertilization programme expressed all the oocyte-specific genes except *KIT-LIG*. They expressed *BNC1* at a significantly higher level than *in vitro* developed oocyte-like cells (Table 3) according to the Mann-Whitney test (two-tailed). To keep the risk of false positive at 5%, a *threshold* value of $P < 0.00151$ was used. At this very conservative *threshold* differential expression of other genes was not significant.

Focusing on 13 oocyte-specific genes that are directly or indirectly involved in meiosis (*SMC1A*, *SCP1*, *SCP2*, *SCP3*, *CCNB1*, *BUB3*, *MSH5*, *FMN2*, *HIFOO*, *MLH1*, *REC8*, *STAG3*, and *DMC1*) we find that the primitive oocyte-like cells developed *in vitro* expressed twelve out of the 13 genes. Only the *MLH1* gene was not expressed (Figure 7(a)). In the primitive oocyte-like cells the meiotic gene *MSH5* was expressed at a significantly lower level than in the nonfertilized oocytes from the *in vitro* fertilization programme, while *REC8* was expressed at a significantly higher level (two-tailed Mann-Whitney test, significance *threshold* $P < 0.00394$; P values presented in Table 3). Differential expression of other genes was not statistically significant based on this conservative *threshold*. The gene expression levels (Ct values) of the primitive oocyte-like cells and of the oocytes matched very well, particularly for *SCP1*, *SMC1A*, and *MSH5*, whereas for the hESCs and the fibroblasts they did not (Figure 8). Although the primitive oocyte-like cells and the oocytes separate two distinct groups of cells based on the overall gene expression profile, as revealed by hierarchical clustering (Figure 7(b)), there are substantial similarities. The hESCs and the fibroblasts express meiotic genes at very low level. Seven genes (*SCP2*, *SCP3*, *MSH5*, *FMN2*, *HIFOO*, *MLH*, and *STAG3*) were not expressed at all in the hESCs, and another set of seven meiotic genes (*SCP2*, *SCP3*, *MSH5*, *HIFOO*, *MLH1*, *STAG3*, and *DMC1*) was not expressed in the fibroblasts (Figure 7(a)).

3.5. DAPI Staining the Cell Nuclei. Sixty-eight oocyte-like cells developed *in vitro* and collected from all patients were monitored after DAPI staining: 67 (98.5%) cells were normal, mononuclear, and one cell (1.5%) had two nuclei (Figure 9).

4. Discussion

This study confirms the existence of cells expressing several markers of pluripotency—putative stem cells—in the OSE layer of women with POF. In this study the putative ovarian stem cells were scraped from the ovarian surface epithelium of women with nonfunctional ovaries and cultured *in vitro* in the presence of follicular fluid from the *in vitro* fertilization programme. The added follicular fluid triggered development of stem cells into primitive oocyte-like cells, which expressed some markers of pluripotency and oocytes.



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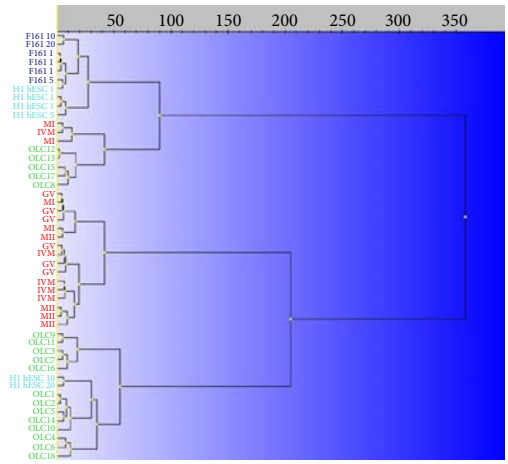

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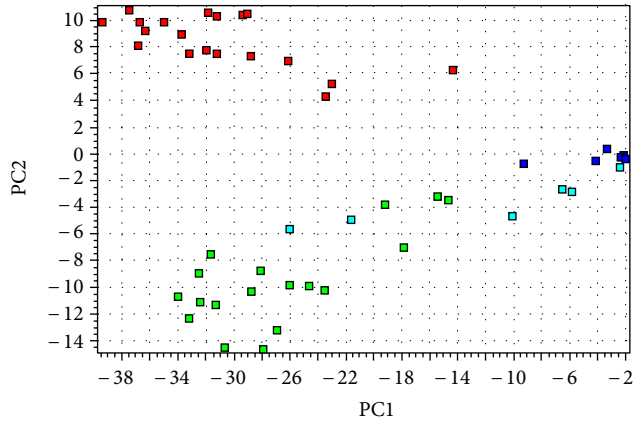
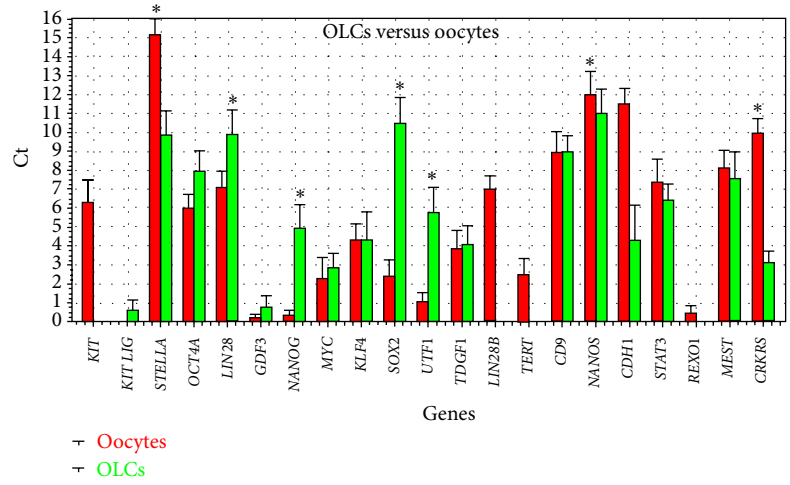


(c)

Figure 5: Single-cell gene expression profile of genes related to pluripotency in primitive oocyte-like cells (OLCs) developed *in vitro*. Comparison with human embryonic stem cells (H1 hESCs), nonfertilized oocytes from the *in vitro* fertilization programme (O) and fibroblasts (F161) revealed the expression of several genes related to pluripotency in oocyte-like cells. (a) Heatmap clustering. (b) Hierarchical clustering. (c) Principle component analysis (PC1 versus PC2). Legend of analyzed cells:OLC(1–18): oocyte-like cells developed *in vitro*; H1 hESC: human embryonic stem cells of line H1 (1: one cell, 5: five cells, 10: ten cells, and 20: twenty cells); F161: fibroblasts of F161 line (1: one cell, 5: five cells, 10: ten cells, and 20: twenty cells); MII: mature, metaphase II oocytes; IVM: immature, *in vitro* matured oocytes; MI: immature, metaphase I oocytes; GV: immature, germinal vesicle oocytes from the *in vitro* fertilization programme. Cell group colours: red: oocytes from the *in vitro* fertilization programme, green: oocyte-like cells, developed *in vitro*, aquamarine: hESCs, and dark blue: fibroblasts.



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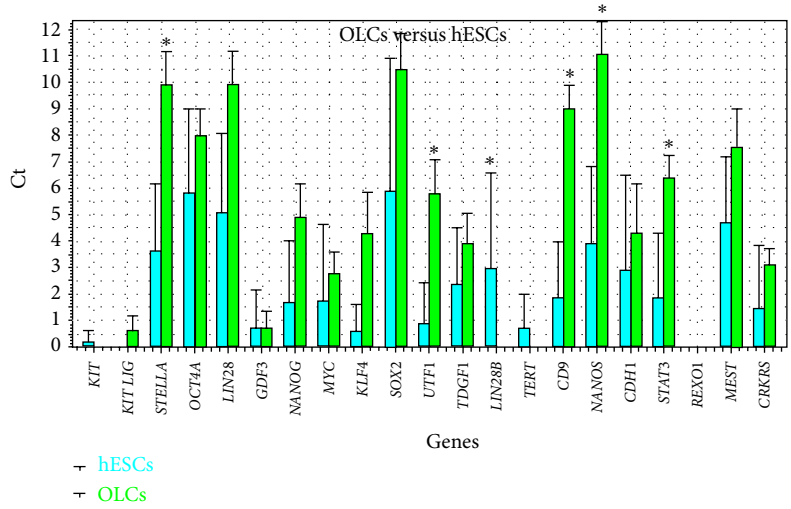

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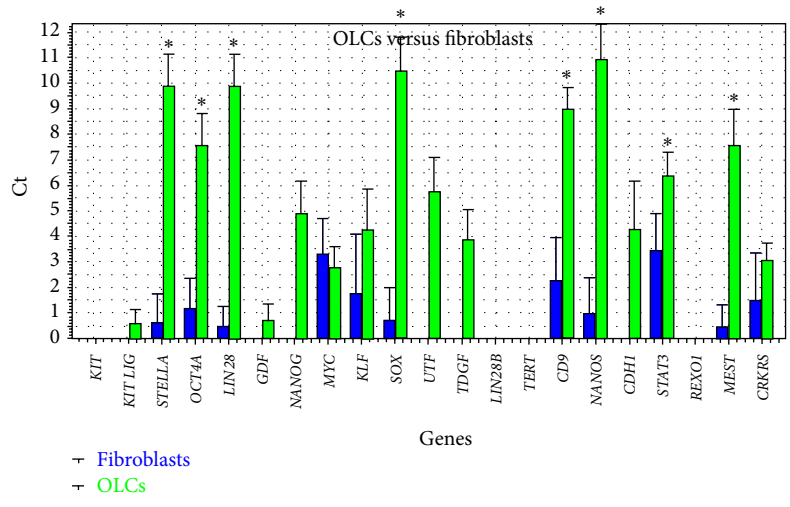
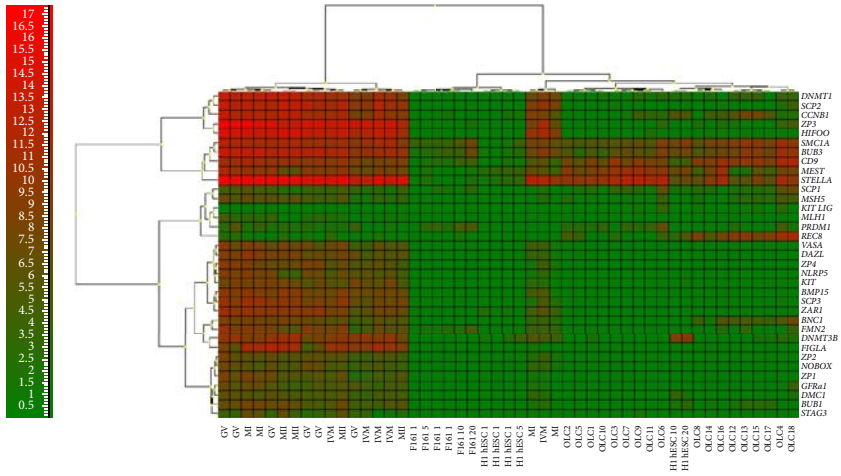


(c)

Figure 6: Descriptive statistics of the expression of genes (Ct values), characteristic of pluripotent stem cells in primitive oocyte-like cells (OLCs), developed *in vitro*. (a) Comparison with oocytes. (b) Comparison with hESCs. (c) Comparison with fibroblasts. Cell group colours: green: oocyte-like cells, developed *in vitro*, red: oocytes from the *in vitro* fertilization programme, and dark blue: fibroblasts. Legend: *Statistically significant difference at $P < 0.00244$ (Dunn-Bonferroni correction), as revealed using the two-tailed Mann-Whitney test.



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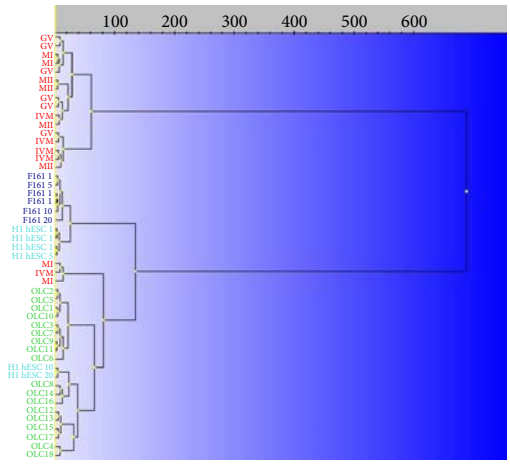
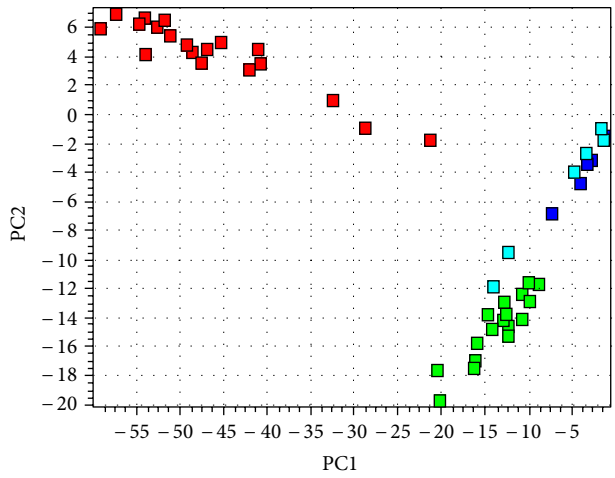


(b)



(c)

Figure 7: Single-cell gene expression profile of oocyte-specific genes in primitive oocyte-like cells developed *in vitro* (OLCs). Comparison with human embryonic stem cells (H1 hESCs), nonfertilized oocytes from the *in vitro* fertilization programme (O) and fibroblasts (F161) revealed the expression of several oocyte-specific genes in oocyte-like cells. (a) Heatmap clustering. (b) Hierarchical clustering. (c) Principle component analysis (PC1 versus PC2). Legend of analyzed cells:OLC(1–18): oocyte-like cells developed *in vitro*; O: oocytes; H1 hESC: human embryonic stem cells of line H1 (1: one cell, 5: five cells, 10: ten cells, and 20: twenty cells); F161: fibroblasts of F161 line.

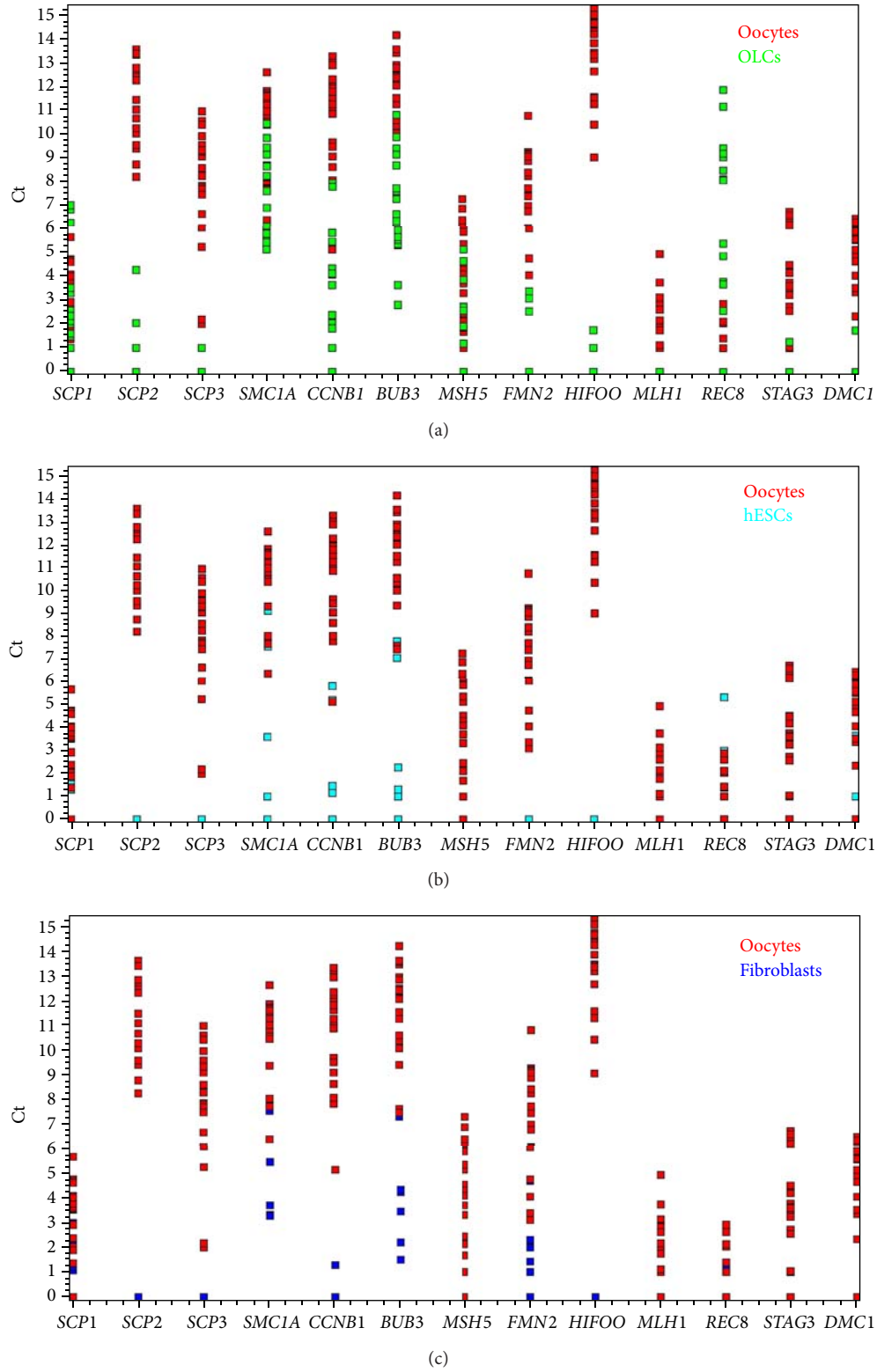


Figure 8: Expression of meiosis-related genes in oocyte-like cells developed *in vitro*. Primitive oocyte-like cells expressed the most, 12 out of 13 meiosis-related genes. In oocyte-like cells gene expressions fitted with oocyte gene expressions while in hESCs and fibroblasts they did not. Expression of meiosis-related genes (Ct values) presented in 2D plots: (a) oocytes (red) and oocyte-like cells (green); (b) oocytes (red) and hESCs (aquamarine); (c) oocytes (red) and fibroblasts (dark blue).

Table 3: *P* values retrieved by statistical analyses of single-cell gene expressions in analyzed groups of cells.

Statistical test	Compared cells	*P* value
	Genes of pluripotency	
Two-tailed Mann-Whitney test	OLCs versus oocytes	*LIN28* ($P = 0.00078$), *STELLA* ($P = 1.0766 \times 10^{-6}$), *NANOG* ($P = 8.5203 \times 10^{-6}$), *SOX-2* ($P = 2.2075 \times 10^{-7}$), *UTF1* ($P = 1.1288 \times 10^{-5}$), *CDH1* ($P = 1.0766 \times 10^{-6}$), *CRKRS* ($P = 2.2075 \times 10^{-7}$)
Two-tailed Mann-Whitney test	OLCs versus hESCs	*CD9* ($P = 0.00036$), *NANOS* ($P = 0.00097$), *STELLA* ($P = 0.00154$), *STAT3* ($P = 0.00154$), *UTF1* ($P = 0.00154$), *LIN28* ($P = 0.00242$)
Two-tailed Mann-Whitney test	OLCs versus fibroblasts	*NANOS* ($P = 0.00036$), *LIN28* ($P = 0.00036$), *STELLA* ($P = 0.00036$), *CD9* ($P = 0.00036$), *SOX-2* ($P = 0.00036$), *OCT4A* ($P = 0.00036$), *MEST* ($P = 0.00060$), *STAT3* ($P = 0.00242$)
One-Way ANOVA	All types of cells	*KIT* ($P \leq 1 \times 10^{-8}$), *STELLA* ($P \leq 1 \times 10^{-8}$), *OCT4A* ($P = 6.86 \times 10^{-6}$), *LIN28* ($P \leq 1 \times 10^{-8}$), *NANOG* ($P = 1.3 \times 10^{-7}$), *SOX-2* ($P \leq 1 \times 10^{-8}$), *UTF1* ($P = 1 \times 10^{-8}$), *TDGF1* ($P = 0.00167408$), *LIN28B* ($P \leq 1 \times 10^{-8}$), *TERT* ($P \leq 1 \times 10^{-8}$), *CD9* ($P \leq 1 \times 10^{-8}$), *NANOS* ($P \leq 1 \times 10^{-8}$), *CDH1* ($P \leq 1 \times 10^{-8}$), *STAT3* ($P = 4.67 \times 10^{-6}$), *MEST* ($P = 5.7 \times 10^{-7}$), *CRKRS* ($P \leq 1 \times 10^{-8}$).
	Oocyte-specific genes	
Two-tailed Mann-Whitney test	OLCs versus oocytes	*BNC1* ($P = 0.00011$)
	Genes of meiosis	
Two-tailed Mann-Whitney test	OLCs versus oocytes	*MSH5* ($P = 0.00204$), *REC8* ($P = 0.00166$)

Legend: OLCs: oocyte-like cells; hESCs: human embryonic stem cells.

Follicular fluid retrieved at the ultrasound-guided oocyte aspiration in the *in vitro* fertilization programme contains several substances important for oocyte growth and maturation including high levels of hormones [30]: estrogens, progesterone and FSH, androgens, proteins [31], and amino acids [31], a high concentration of lipids including free cholesterol and meiosis-activating sterol (FF-MAS) [32, 33], and growth factors [34]. Many of these substances are important for oocyte growth and maturation.

The culture medium with added follicular fluid used in this study was characterized by very high concentrations of estradiol and progesterone. The high concentrations of these reproductive hormones in follicular fluid were normal and resulted from the ovarian hormonal stimulation protocol, which was routinely used in a patient to retrieve oocytes for *in vitro* fertilization. The OSE cell cultures including oocyte-like cells depleted/converted these hormones from the culture medium. It has already been found that follicular fluid can

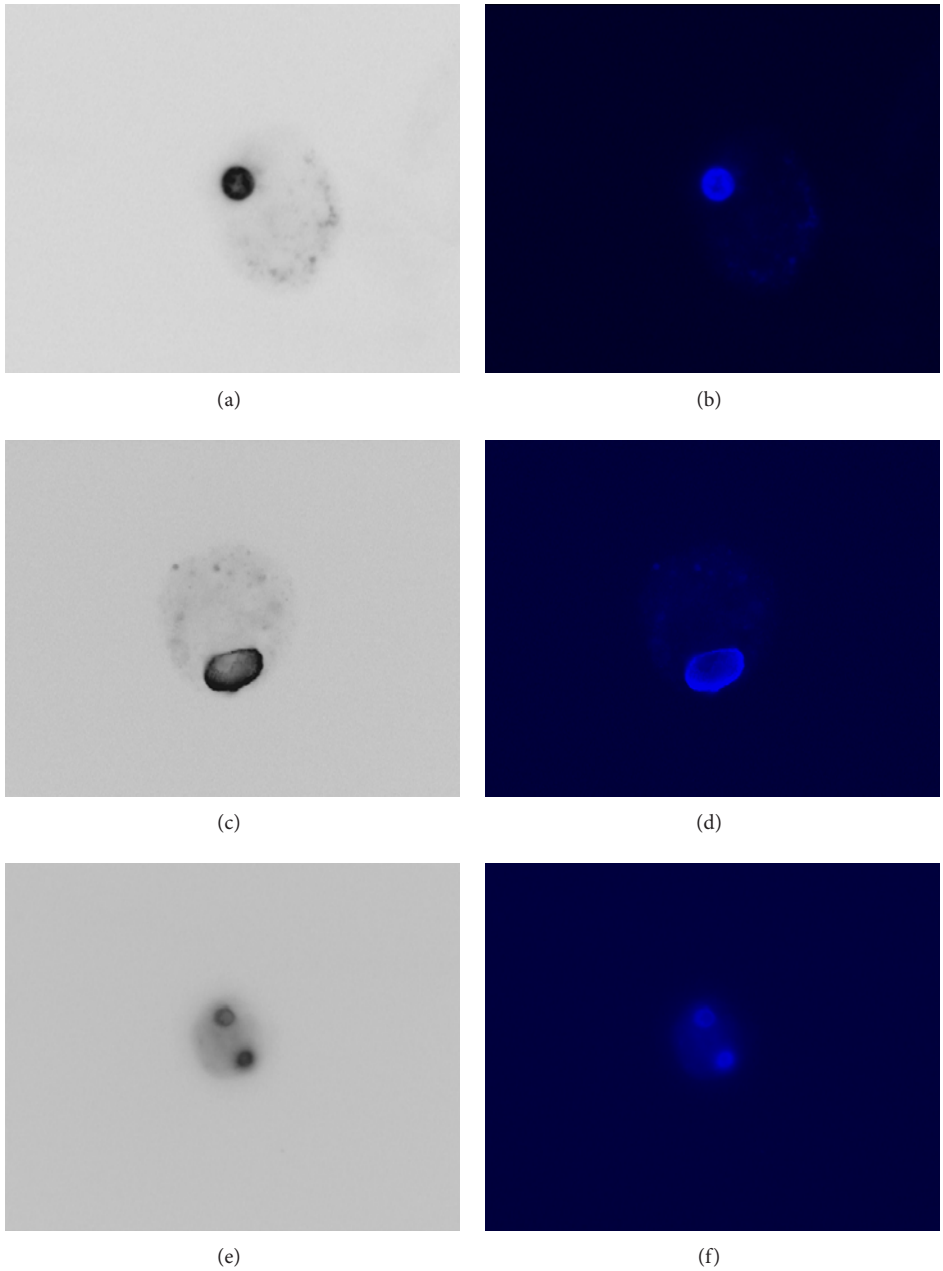


Figure 9: Nuclei of *in vitro* developed primitive oocyte-like cells monitored after DAPI staining. DAPI staining confirmed mononuclear and binuclear cells: ((a)–(d)) two normal mononuclear primitive oocyte-like cells. ((e), (f)) One abnormal primitive oocyte-like cell with two nuclei. (Light and fluorescence microscope, magnification 400x.)

induce OSE cell proliferation, but estradiol and progesterone did not induce the cell proliferation; it was concluded that follicular fluid directly stimulates OSE cell proliferation by nonsteroidal mitogens [35]. On the other hand several studies confirmed that estradiol has a positive impact on the human oocyte growth and maturation *in vivo* and *in vitro*. It has already been published that oocyte-like cell development *in vitro* can be triggered by weak estrogenic activity of phenol red in the cell culture medium [18, 25]. The estradiol can directly influence the quality of maturing human oocytes by steroid action on the cell surface via Ca^{2+} as a second messenger and contribute to the oocyte potential for fertilization and early postfertilization development [36]. In the *in vitro* fertilization programme estradiol and testosterone levels in follicular fluid may be used as predictive parameters of oocyte maturity [37]. In the follicular phase of menstrual cycle, follicles which contained a healthy but not degenerative oocyte had a significantly higher level of estradiol in the follicular fluid [38]. The oocytes which gave rise to successful pregnancies were obtained from follicles which contained greater concentrations of estradiol than did oocytes from which pregnancy did not result [39]. Moreover, transient

estradiol supplementation improved the oocyte *in vitro* maturation rate and subsequent developmental competence in porcine [40] and white-tailed deer oocytes [41]. Similarly, estradiol and progesterone improved *in vitro* cytoplasmic maturation and developmental competence of oocytes from unstimulated prepubertal and adult rhesus monkeys [42]. Based on all this knowledge, it is not excluded that estradiol alone or in combination with other substances from the follicular fluid was involved in *in vitro* development of primitive oocyte-like cells in this study.

The majority of primitive oocyte-like cells developed *in vitro* were normally mononuclear and expressed several genes characteristic of pluripotent stem cells and of oocytes based on single-cell gene expression profiling. They express most of the genes of pluripotency tested and several oocyte-specific genes, including genes related to epigenetic regulation (e.g., *DNMT1*, *DNMT3B*), zona pellucida structure (*ZP3*), and late meiotic genes (e.g., *SCP1, SCP2,* and *SCP3*). Although these genes were expressed at a significantly lower extent than in nonfertilized oocytes from the *in vitro* fertilization programme, there was a subgroup of immature or *in vitro* matured oocytes that clustered together with oocyte-like cells and not with other oocytes based on their gene expression profiles.

Although primitive oocyte-like cells less expressed a majority of the analyzed genes involved in meiosis (*SCP1*, *SCP2*, *SCP3*, *SMC1A*, *CCNB1*, *BUB3*, *MSH5*, *FMN2*, *HIFOO*, *MLH1*, *REC8*, *STAG3*, and *DMC1*), they still expressed *REC8* at a significantly higher level than the oocytes. *REC8* plays an important role in sister chromatid cohesion during meiosis [43]. In spite of that, oocyte-like cells did not express some important oocyte-specific genes, such as *VASA, DAZL*, and *FIGLA*, and therefore exhibited the characteristics of pluripotent stem cells more than of competent oocytes. As shown in the model of human and mouse ESCs, the expression of germ cell-specific genes (including *VASA*) in oocyte-like cells developed *in vitro* can be regulated by different culture conditions [7, 10], for example, by the addition of retinoic acid into the culture medium that regulates germ cell differentiation through a Smad-dependent pathway [44]; therefore the possibilities of *in vitro* "oogenesis" in this study were far from being exhausted. As previously proposed, oocyte-like cells developed *in vitro* could also be matured *in vivo* by transplantation into other organs, tissues, or ovarian biopsies [5, 23]; it may be possible to autotransplant the oocyte-like cells to *in vivo* direct their growth, maturity, and function or perhaps to "awaken" the ovaries in women with severe ovarian infertility, if sure that teratoma would not form.

The results of this study are in accordance with findings of White et al. [23], who published the existence of rare mitotically active cells—stem cells—with a gene expression profile that is consistent with primitive germ cells in ovarian cortical tissue of women in reproductive period of age. These cells were able to generate oocyte-like cells *in vitro*.

The results of our study show that the adult OSE in women with nonfunctional ovaries may be an important source of putative stem cells.

5. Conclusions

The addition of donated follicular fluid, rich in substances for oocyte growth and maturation, to the culture medium triggers development of putative stem cells from the ovarian surface epithelium of women with nonfunctional ovaries into the direction of primitive oocyte-like cells that express several genes related to pluripotency and oocytes, but are more "stem cells" than real "oocytes" at present.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

I. Virant-Klun was a leader and coordinator of the research project. She performed all cell cultures, cultured oocyte-like cells, and wrote this paper. T. Skutella provided single-cell gene expression analyses by the Fluidigm system and read this paper. M. Kubista performed biostatistics of single-cell gene expression profiling and read this paper. A. Vogler surgically retrieved ovarian tissue to be researched. J. Sinkovec performed all histopathological evaluations of ovarian tissue in patients included into this study. H. Meden-Vrtovec included her patients with premature ovarian failure into this study.

Acknowledgments

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Review Article

Gene Expression Profiling of Human Oocytes Developed and Matured *In Vivo* or *In Vitro*

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The quality of the human oocyte determines the success of fertilization and affects the consequent embryo development, pregnancy and birth; it therefore serves as a basis for human reproduction and fertility. The possibility to evaluate oocyte quality in the *in vitro* fertilization programme is very limited. The only criterion which is commonly used to evaluate oocyte quality is its morphology. There is a mass of oocytes in the *in vitro* fertilization programme which are not fertilized in spite of normal morphology. In the past, several attempts focused on oocyte gene expression profiling by different approaches. The results elucidated groups of genes related to the human oocyte. It was confirmed that some factors, such as oocyte *in vitro* maturation, are detectable at the molecular level of human oocytes and their polar bodies in terms of gene expression profile. Furthermore, the first genetic evaluations of oocyte-like cells developed *in vitro* from human stem cells of different origin were performed showing that these cells express some genes related to oocytes. All these findings provide some new knowledge and clearer insights into oocyte quality and oogenesis that might be introduced into clinical practice in the future.

1. Introduction

A viable quality human oocyte is a prerequisite for successful fertilization, implantation, and development of a new human being. Although an enormous development of techniques of *in vitro* fertilization has arisen in recent years and several indications of infertility became treatable, the general implantation and pregnancy rates after the embryo transfer are still low [1]. A mass of embryos are developed in the *in vitro* fertilization programme which do not implant. This is mainly due to a lack of reliable criteria on oocyte quality and the selection of embryos with sufficient developmental potential. The quality of oocytes obtained under ovarian stimulation for *in vitro* fertilization varies considerably and depends on several factors. A large proportion of oocytes are capable of being fertilized, but only approximately half of fertilized oocytes fully complete their preimplantation development and still fewer implant [2]. More studies have evidenced that some gene expression levels could serve as oocyte quality markers. Additionally, recent studies also confirmed that oocyte-like cells expressing some oocyte-specific genes can be developed *in vitro* from different types of stem cells. These include human embryonic stem cells (hESCs) [3–7], human induced pluripotent stem cells (hiPSCs) [6, 7], putative stem cells from adult human ovaries of women with no naturally present follicles or oocytes [8–10], and human amniotic fluid stem cells (hAFSCs) [11]. Therefore it is also important to know the gene expression profile of these cells to evaluate their quality and safety. The aim of this paper is to summarize the new knowledge on the gene expression profile of human oocytes developed and matured *in vivo* and *in vitro*.

2. Human Oocyte

An oocyte is a female germ cell involved in reproduction. It is one of the largest cells in the body with a diameter of at least 100 μm when mature. The oocyte is rich in cytoplasm

that contains yolk granules to support the cell's growth, maturation, and the early development after fertilization. *In vivo* it is produced in the ovarian follicle, a functional unit of the ovary, during the process of oogenesis/folliculogenesis [12]. The oogenesis then starts with the development of oogonia. Each oogonium inside the fetal ovaries divides and enters the initial stage of meiosis (meiosis I) to become the *primary oocyte*. The diploid *primary oocyte,* however, does not complete meiosis I but is stopped at the first meiotic prophase stage, called dictyate. At this stage the oocyte nucleus is called the *germinal vesicle* (GV), as this stage refers to the GV stage of maturity. GV oocytes are localized within the primordial follicle, also consisting of a flattened and condensed layer of surrounding mesenchymal granulosa cells. By the end of the fetal period, all *primary oocytes* have formed and are stopped at the dictyate stage. Although meiosis was arrested, the dictyate chromosomes continue to synthesize large amounts of mRNA and rRNA, which are later used to generate a bulk of essential proteins needed for oocyte maturation and further development of the fertilized oocyte and embryo [12]. Then the *primary oocytes* are maintained for years, until puberty (menarche). Upon puberty the *primary oocytes* finish meiosis I, and a *primary oocyte* divides into two daughter cells: a haploid *secondary oocyte* and an extruded nonfunctional polar body. *During* the menstrual cycle only a few of *primary oocytes* are recruited, and only one matures and is ovulated. When this secondary oocyte enters meiosis (meiosis II), it does not finish but is arrested again and held at the metaphase II (MII) stage until fertilization. The MII-stage oocyte has the potential to be fertilized. When the oocyte is fertilized, the process of meiosis is terminated and the second polar body is extruded. During the menstrual cycle only 15–20 early antral follicles/oocytes are recruited and only one dominant follicle matures fully and is ovulated. Each oocyte develops and matures in the functional unit of the ovary—the follicle. The *primary oocytes* grow and mature during the development and maturation of primordial, primary and secondary follicles, whereas the secondary oocytes develop along with the tertiary and preovulating Graafian follicle. This process is regulated by hormones and other substances.

In the *in vitro* fertilization programme oocytes are retrieved after hormonal stimulation of patient's ovaries and by ultrasound guided aspiration. Oocytes at different stages of maturity can be retrieved. Most oocytes are mature metaphase II (MII) oocytes, while a relatively small proportion of oocytes are immature metaphase I (MI) oocytes and prophase I (PI) or germinal-vesicle- (GV-) stage oocytes. The MII oocytes are characterized by a round shape, zona pellucida, and an extruded polar body; MI oocytes by a round shape, zona pellucida, and the absence of a polar body; GV-stage oocytes by a round shape, zona pellucida, and the presence of a germinal vesicle (for morphology see Figure 1). In the *in vitro* fertilization programme only mature MII-stage oocytes can be fertilized and develop into an embryo; therefore, the immature oocytes need to be matured *in vitro* or discarded in daily clinical practice.

Because the main role of an oocyte is to be fertilized and to grow into a fully functional organism, it has to be able to regulate many different cellular and developmental processes, such as the regulation of the cell cycle progression and cellular metabolism, fertilization, activation of zygotic transcription, embryo development, activation of the embryonic genome, and formation of body axes. During oocyte growth a variety of maternally transcribed mRNAs are supplied which represent the maternal contribution to the oocyte and, consequently, the newly fertilized oocyte, zygote, and early embryo. These mRNAs can be stored in message ribonucleoprotein (mRNP) complexes and then translated when needed [13]; additionally, they can be localized within a specific region of the cytoplasm or dispersed within the cytoplasm of the entire oocyte. Maternally synthesized proteins can also be localized or ubiquitous throughout all the oocyte's cytoplasm.

3. Gene Expression Analyses of Human Oocytes

The first gene expression analyses in human oocytes were performed by reverse transcription-polymerase chain reaction (RT-PCR; see Table 1) and were followed using microarray technology (Table 2). Microarrays enable gene expression profiling, which gives the researcher the ability to monitor and quantify the expression of thousands of known genes at the same time (e.g., whole genome microarray). This methodological breakthrough has the potential to provide detailed insight into cellular processes involved in the regulation of gene expression in different cell types, including oocytes and cumulus cells.

3.1. Human Oocyte-Specific Genes. More studies elucidated the oocyte gene expression profile from different aspects; most of these studies used the microarray technology and are listed in Table 2. In the study by Bermúdez et al. [14], microarray methods were used to examine the expression of linearly amplified RNA from individual oocytes and groups of five oocytes. In this study the amplification strategy consistently enabled a complex representative cDNA population. With this methodology a catalogue of 1,361 different transcripts expressed in human oocytes was identified; 406 of them have been independently confirmed by other methods. According to their function, the expression of several genes was related to apoptosis, cell cycle, circadian rhythms, cytoskeleton, secretory pathways, exocytosis, endocytosis, kinases, membrane receptors, ion channels, mitochondria, structural nuclear proteins, phospholipases, protein degradation, protein synthesis, secreted proteins, signaling pathways, DNA, chromatin, RNA, transcription, and others. The results of this study highlighted the potential of microarray analyses in reproductive medicine.

Using microarray analysis, Kocabas et al. analyzed the transcriptome of non-fertilized mature MII oocytes from the *in vitro* fertilization programme [15]. They analyzed three groups of 10 oocytes from three young donors, each less than 35 years of age, reproductively healthy with regular ovulatory cycles, with the male factor as the only cause of infertility, and a considerable number of developing follicles. The transcriptome of these oocytes was compared to 10 different normal human tissues, including skeletal muscle, kidney, lung, colon, liver, spleen, breast, brain, heart, and stomach. Compared with reference samples, there were 5,331

Table 1: Analyses of human oocyte gene expression by RT-PCR.

Oocyte gene expression analyses by RT-PCR				
Analyzed oocytes	Maturity of oocytes	Expression of genes	Functions of analyzed genes	Study
Four oocytes, forty-two embryos	MII, non-fertilized	High expression of *BRCA1*, *BRCA2*, *ATM*, *TP53*, *RB1*, *MNAD2*, *BUB1*, *APC*, *beta-actin* in oocytes, but low in embryos	Genes related to the preimplantation development	Wells et al., 2005 [17]
Four single oocytes, five embryos	MII, non-fertilized	*LDH-A*, *LDH-B*, *LDH-C* expression in oocytes and embryos *SUMO-1*, *SUMO-2*, *SUMO-3* expression in oocytes and embryos	Genes encoding lactate dehydrogenase isozymes Small ubiquitin-like modifier isoforms	Li et al., 2006 [18]
Oocytes from follicles	From primordial follicles to MII oocytes	*NOBOX* expression from primordial follicles to MII oocytes	Homeobox genes encoding transcription factors	Huntriss et al., 2006 [19]
	From primordial to early primary follicles	*HOXA10* expression from primordial to early primary follicles		
	From primordial follicles to GV oocytes	*HOXB7* expression from primordial follicles to GV oocytes		
	GV oocytes	*HOXA7* expression in GV oocytes		
	MII oocytes	*HOXA1*, *HEX* expression in MII oocytes		
	Cumulus cells	*HOXC9*, *HOXC8*, *HOXC6*, *HOXA7*, *HOXA5*, *HOXA4* expression in cumulus cells		

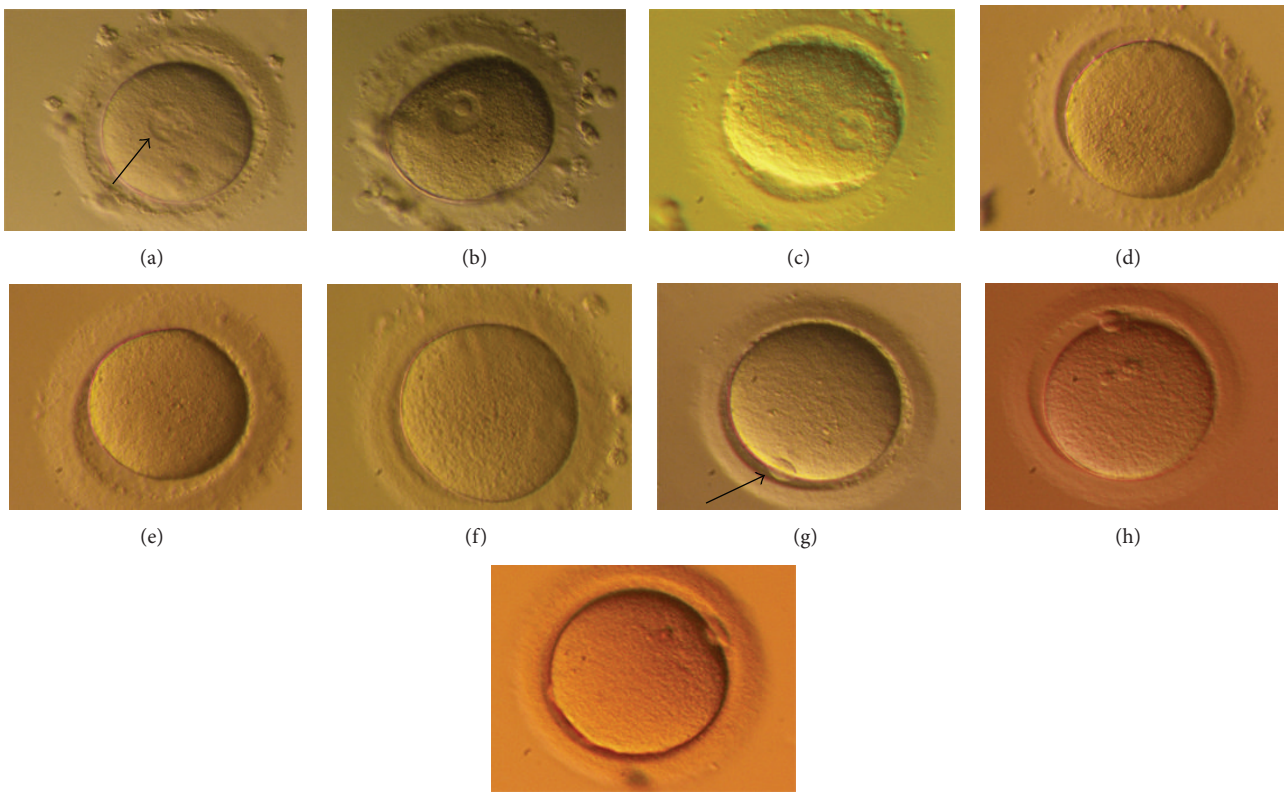

Figure 1: Human oocytes from the *in vitro* fertilization programme (a)–(c): immature germinal vesicle (GV) oocytes with a germinal vesicle (arrow) and without a polar body; (d)–(f): immature metaphase I (MI) oocytes without a polar body; (g)–(i): mature metaphase II (MII) oocytes with a polar body (arrow).

Table 2: Gene expression profiling of human oocytes using microarrays.

Analyses of oocyte gene expression by microarrays			
Analyzed oocytes	Expression of genes	Functions of analyzed genes	Study
Seventy seven *in vivo* matured oocytes at different stages of maturity: 20 GV oocytes (7 patients), 20 MI oocytes (7 patients), 37 MII oocytes, cumulus cells	Identification of new potential regulators and marker genes involved in the human *in vivo* oocyte maturation	Transcription regulation, DNA repair, cell cycle checkpoint	Gasca et al., 2007 [2]
Individual MII oocytes and groups of 5 MII oocytes	1,361 transcripts expressed in oocytes	Apoptosis, cell cycle, circadian rhythms, cytoskeleton, secretory pathways, exocytosis, endocytosis, kinases, membrane receptors, ion channels, mitochondria, structural nuclear proteins, phospholipases, protein degradation and synthesis, secreted proteins, signaling pathways, DNA, chromatin, RNA, transcription, and others	Bermúdez et al., 2004 [14]
Groups of 10 MII oocytes from women aged <35 years in comparison to 10 different normal human somatic tissues	5,331 transcripts significantly up-regulated and 7,074 transcripts significantly down-regulated in human oocytes	Up-regulated TGF-β pathway, DNA, RNA and protein metabolism, transcription regulation, chromatin modification	Kocabas et al., 2006 [15]
Groups of 20 GV, 20 MI, and 16 MII oocytes	Oocytes expressed in average 8,728 genes. The lowest number of expressed genes in MII oocytes (5,633) and highest in GV oocytes (10,892)	Genes specifically expressed in germinal cells and oocytes, meiosis, components of the maturation-promoting factor (MPF), spindle checkpoint, transforming growth factor-beta superfamily, chromatin remodeling	Assou et al., 2006 [16]
Nine MII and GV oocytes, preimplantation embryos	Human oocytes are low RNA template samples and an amplification step is required to provide sufficient labeled RNA as a microarray target (PCR and serial analysis of gene expression SAGE, microarrays)		Neilson et al., 2000 [20] Dobson et al., 2004 [21]
Seven individuals: 5 GV (primary) and 2 MII (secondary) oocytes and 15 preimplantation embryos	Down-regulation of genes in preimplantation embryos in comparison with oocytes	Oocyte maturation and embryo development	Dobson et al., 2004 [21]
Single and pooled GV oocytes	Oocytes need to be pooled for the starting template for each array and sufficient microarray experiments performed to minimize the variance associated with processing		Jones et al., 2007 [22]
Four individual MII oocytes and four 4-cell and three 8-cell embryos	A total of 631 genes exhibited differential expression in oocytes and embryos. In oocytes 184 genes were expressed more than twofold above the median value. Only two genes were at least twofold below the median value	Interconversion of lactate and pyruvate, lactate dehydrogenase, oocyte maturation, embryo development	Li et al., 2006 [18]
Immature oocytes from primordial, intermediate, and primary follicles	A total of 6,301 unique genes were significantly expressed; extraordinary high expression levels of *TMEFF2*, *OPHN1* and *ATP6*; expression of oocyte- or germline-specific genes	RNA binding, translation initiation structural molecule activity, BMP receptors, activin receptors, IGFI receptor, fibroblast growth factors, different enzymes	Markholt et al., 2012 [23]

Table 2: Continued.

Analyses of oocyte gene expression by microarrays			
Analyzed oocytes	Expression of genes	Functions of analyzed genes	Study
Seventy six GV oocytes from 55 donor patients, hESCs, and human foreskin fibroblasts	10,183 genes were expressed in GV oocytes including oocyte-specific genes. Distinct sets of genes were detected in oocytes, hESCs and fibroblasts	In GV oocytes 4 signaling pathways—MOS-MPF, transforming growth factor-beta, Wnt, and Notch, oocyte maturity, embryo development	Zhang et al., 2007 [24]
GV and MII oocytes, hESCs, somatic tissues	Identified a common oocyte/hESC gene expression profile	Cell cycle, enzymes involved in general cell metabolism, nucleoside synthesis, DNA repair, cell cycle regulatory machinery, regulation of the topologic state of DNA, mitotic spindle assembly checkpoint, pluripotency, chromatin remodelling, transcription factors, ubiquitination, and proteasome pathways	Assou et al., 2009 [25]
GV oocytes, MII oocytes matured *in vivo* and *in vitro*	GV, *in vivo* matured MII oocytes, and *in vitro* matured MII oocytes expressed 12,219, 9,735, and 8,510 genes. There was an extensive overlap among the all three groups of oocytes, but also some significant differences. There were some immature GV oocyte patterns of gene expression, which still persisted in *in vitro* matured oocytes	Nuclear maturity, cytoplasmic functions expressed in an immature manner, cellular storage and homeostasis	Wells and Patrizio, 2008 [26]
GV oocytes, MII oocytes matured *in vivo* and *in vitro*	More than 2,000 genes were expressed at more than 2-fold higher levels in oocytes matured *in vitro* than those matured *in vivo*	Transcription, the cell cycle and its regulation, transport and cellular protein metabolism	Jones et al., 2008 [27]
Fresh, slowly frozen, and vitrified MII oocytes	Oocyte slow freezing and vitrification negatively affected the gene expression profile of human oocytes in comparison with fresh controls	Chromosomal structure maintenance, cell cycle regulation, genes of the ubiquitination pathway	Monzo et al., 2012 [28]
Thirty nine MII oocytes with total fertilization failure and control oocytes	Misexpression of several genes, characterized by important fold changes in oocytes with total fertilization failure	Meiosis, cell growth, and apoptosis control	Gasca et al., 2008 [29]
Fifteen GV oocytes which matured to MII stage overnight and their polar bodies	Transcripts that were present in greater abundance in the single oocytes were also detected in qPCR replicates from single polar bodies, except oocyte-specific *H1FOO*		Klatsky et al., 2010 [30]
Single MII oocytes and single polar bodies after biopsy	Human polar bodies reflected the oocyte transcript profile. 5,256 mRNAs, or 97%, including miRNAs were expressed in both oocytes and polar bodies		Reich et al., 2011 [31]
MII oocytes of younger (<32 years) and older women (>40 years)	Found that the global gene expression profiles in oocytes are related to female age. Genes were down-regulated in older women	Cell cycle regulation, cytoskeletal structure, energy pathways, transcription control, and stress responses	Steuerwald et al., 2007 [32]
Single MII oocytes of younger (<34 years) and older women (37–39 years)	7,470 genes (10,428 transcripts) were expressed in oocytes; 342 genes were expressed at significantly different expression levels between the two age groups of patients	Cell cycle regulation, chromosome alignment, sister chromatid separation, oxidative stress and ubiquitination, the signaling network of genes for cell cycle and organism development	Grøndahl et al., 2010 [33]
Seven MII oocytes (three normal and four aneuploid) and their polar bodies after biopsy	At comparative genomic hybridization 327 genes were differently expressed in both groups of oocytes; the relation between mRNA transcript numbers and female age	Meiotic spindle assembly, chromosome alignment, production of cell surface, or excretory molecules.	Fragouli et al., 2010 [34]

transcripts significantly up-regulated and 7,074 transcripts significantly down-regulated in the human oocytes. Genes up-regulated in oocyte samples included most of the well-known germ cell-specific genes. The authors confirmed the presence of pathways previously described in the mouse oocytes, especially the TGF-β. Among genes up-regulated in oocytes, 1,430 were of unknown function. A group of 66 genes up-regulated in human oocytes was identified by intersecting significantly up-regulated genes in human oocytes with those from the mouse oocytes and from human and mouse embryonic stem cells. Additionally, microarray results were validated using RT-PCR analysis for a selected set of oocyte-specific genes. The genes up-regulated in human oocytes were related to RNA, DNA, protein metabolism, and chromatin modification. The overexpression of genes associated with RNA metabolism is in agreement with the fact that oocytes store a great amount of RNA to support the processes of fertilization, early embryonic development, and activation of the embryonic genome. The up-regulation of genes related to DNA metabolism, transcription regulation (zinc finger proteins), and chromatin modification is in agreement with the oocyte need to remodel the sperm chromatin after fertilization. The human oocyte is proposed to be transcriptionally silent at the MII stage of maturity but is very active in transcription and translation during the growth phase and must be prepared to initiate transcription during embryonic genome activation at the 4- to 8-cell embryo stage. The authors concluded that further understanding of the biological role of genes up-regulated in mature human oocytes may extend the knowledge on the meiotic cell cycle, fertilization, chromatin remodeling, lineage commitment, pluripotency, tissue regeneration, and morphogenesis. Moreover, Assou et al. found 1,514 genes which were up-regulated in human oocytes in addition to already known genes, such as *DAZL*, *BMP15,* or *GDF9*. Among them were the meiosis-related genes *PTTG3* (securing) and *AURKC* (Aurora kinase), previously unreported growth factors such as *TNFSF13*/*APRIL*, *FGF9*, *FGF14* and *IL4*, as well as transcription factors, including *OTX2*, *SOX15,* and *SOX30* [16]. The most specific and significantly up-regulated oocyte genes, summarized from the perviously mentioned manuscripts, are listed in Table 3. In spite of some complex gene expression profilings of human oocytes, the relation of expressed genes to the fertilization process, embryo development, and conceiving is still poorly understood and needs to be elucidated in the future.

Further attempts were made to optimize a microarray-based approach for deriving representative gene expression profiles of human oocytes. Since the human oocyte is a low RNA template sample which contains approximately 55 to 100 pg of polyA+ mRNA [20, 21], it was proposed that an amplification step is required to provide sufficient labeled RNA as a microarray target. Jones et al. tried to optimize a protocol for deriving reproducible and representative gene expression profiles by microarrays from very rare samples of human oocytes, available for research purposes [22]. In their study cRNA was generated from both single and pooled immature human GV-stage oocytes. The amplification products were used as a microarray target to optimize a protocol of gene expression profiling. They found that linear amplification and exponential amplification were both capable of generating sufficient products for hybridization to the microarrays, even from the low amount of template mRNA present in a single human oocyte. The results of this study showed that the majority of the variance associated with amplification and hybridization procedures resulted from the molecular processing; therefore, oocytes need to be pooled for the starting template for each array and sufficient independent microarray experiments need to be performed to minimize the variance associated with molecular processing. In this study the hierarchical cluster analysis of the five immature GV-stage oocytes and the two MII-stage oocytes showed significant deviation in branch distances despite the fact that the oocytes were retrieved from women of similar age and indication of infertility.

Our own preliminary data show that single human oocytes from the *in vitro* fertilization programme can also be successfully analyzed by the Biomark Real-Time Quantitative PCR (qPCR) System (Fluidigm). This methodology enables qualitative analyses of single cells after preamplification. The advantage of the methodology is that many genes can be analyzed at the same time. In our preliminary work 19 single human oocytes at different stages of maturity (6 GV, 4 MI, 5 *in vitro* matured (IVM), and 4 mature MII oocytes) were analyzed on 56 genes related to pluripotent stem cells and oocytes by the Fluidigm system after the preamplification procedure. The analyzed genes were selected to elucidate the relation of human oocytes to hESCs and to better understand the molecular status of oocyte-like cells developed *in vitro* from stem cells of different origins, including pluripotent hESCs and iPSCs. The inventoried TaqMan assays (Applied Biosystem) were pooled to a final concentration of 0.2× for each assay. The cells to be analyzed were harvested directly into RT-PreAmp Master Mix. The harvested cells were immediately frozen and stored at −80°C. Cell lysis and sequence-specific reverse transcription were performed at 50°C for 15 minutes. The reverse transcriptase was inactivated by heating to 95°C for 2 minutes. Subsequently, cDNA went through limited sequence-specific amplification by denaturing at 95°C for 15 seconds and annealing and amplification at 60°C for four minutes for 14 cycles. These pre-amplified products were diluted 5-fold prior to analysis with the Universal PCR Master Mix and inventoried with TaqMan gene expression assays in 96.96 Dynamic Arrays on a BioMark System. Ct values obtained from the BioMark System were transferred to the GenEx software (MultiD) to analyze the gene expressions. The results confirmed that a small proportion—three outstanding oocytes (two immature MI oocytes and one *in vitro* matured oocyte IVM)—differed at the molecular level from other oocytes and did not cluster with them, as revealed by heatmap, hierarchical clusterings, and principal component analysis (Figure 2). Although no statistical differences in gene expression were found between them, we concluded that a higher number of single oocytes at each stage of maturity would have to be analyzed using this methodology in the future.

3.2. Genes Related to Preimplantation Development. Still little is known about gene expression in human oocytes and during

Table 3: Some of genes significantly overexpressed in oocytes according to [15, 16] and GeneCards data.

Genes significantly overexpressed in oocytes		
Gene symbol	Gene title	Chromosome
	Gamete markers	
DAZL	Deleted in azoospermia-like	3
DDX4/VASA	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	5
DPPA3/STELLA	Developmental pluripotency associated 3	12
	Embryogenesis, pluripotency, self-renewal, proliferation, development	
ZAR1	Zygote arrest 1	4
PUM1	Pumilio homolog 1 (*Drosophila*)	1
PUM2	Pumilio homolog 2 (*Drosophila*)	2
NANOS1	Nanos homolog 1 (*Drosophila*)	10
NANOG	Nanog homeobox	12
SOX2	SRY (sex determining region Y)-box 2	3
SALL2	Sal-like 2 (*Drosophila*)	14
KLF4	Kruppel-like factor 4 (gut)	9
LIN28B	Lin-28 homolog B (*C. elegans*)	6
	Maturation promoting and related factors	
CCNB1	Cyclin B1	5
CCNB2	Cyclin B2	15
CDC2/CDK1	Cell division cycle 2, G1 to S and G2 to M	10
CDC25A	Cell division cycle 25A	3
CDC25B	Cell division cycle 25B	20
CDC25C	Cell division cycle 25C	5
	Spindle checkpoint	
BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog	2
BUB1B/BUBR1	BUB1 budding uninhibited by benzimidazoles 1 homolog beta	15
CENPA	Centromere protein A	2
CENPE	Centromere protein E	4
CENPH	Centromere protein H	5
MAD2L1/MAD2	MAD2 mitotic arrest deficient-like 1	4
	Cytoplasmic receptors	
NALP5/NLRP5/MATER	NLR family, pyrin domain containing 5	19
	APC/C complex, securin, cohesins	
ANAPC1/APC1	Anaphase promoting complex subunit 1	2
ANAPC10/APC10	Anaphase promoting complex subunit 10	4
CDC20	CDC20 cell division cycle 20	1
PTTG1	Pituitary tumor-transforming 1	5
PTTG3	Pituitary tumor-transforming 3 (meiosis)	8
STAG3	Stromal antigen 3 (meiosis)	7
	Epigenetic remodeling	
DNMT1	DMA (cytosine-5-)-methyltransferase 1	19
DNMT3B	DMA (cytosine-5-)-methyltransferase 3 beta	20
HDAC9	Histone deacetylase 9	7
H1FOO	H1 histone family, member O, oocyte-specific	3
HCAP-G	Chromosome condensation protein G	4
	Meiosis, miscellaneous	
AKAP1	A kinase (PRKA) anchor protein 1	17
MCM3	MCM3 minichromosome maintenance deficient 3	6
MOS	v-mos Moloney murine sarcoma viral oncogene homolog	8
REC8	REC8 homolog (yeast)	14
STAG3	Stromal antigen 3	7
FMN2	Formin 2	1
SYCP1	Synaptonemal complex protein 1	1
SYCP2	Synaptonemal complex protein 2	20
SYCP3	Synaptonemal complex protein 3	12
SMC3	Structural maintenance of chromosomes 3	10

Table 3: Continued.

Gene symbol	Gene title	Chromosome
	Genes significantly overexpressed in oocytes	
SMC1B	Structural maintenance of chromosomes 1B	22
STRA8	Stimulated by retinoic acid gene 8 homolog (mouse)	7
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (*E. coli*)	3
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)	22
PELO	Pelota homolog (*Drosophila*)	5
WEE2	WEE1 homolog 2 (*S. pombe*)	7
SGOL2	Shugoshin-like 2 (*S. pombe*)	2
PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme	5
SPAG16	Sperm associated antigen 16	2
TUBB4Q	Tubulin, beta polypeptide 4, member Q	4
FBXO5/EMI1	F-box protein 5	6
AURKC	Aurora kinase C	19
	Extracellular matrix, growth factors, cell surface, signaling	
BMP15	Bone morphogenetic protein 15	X
BMP6	Bone morphogenetic protein 6	6
GDF9	Growth differentiation factor 9	5
FGFR2	Fibroblast growth factor receptor 2	10
FGF9	Fibroblast growth factor 9 (glia-activating factor)	13
FGF14	Fibroblast growth factor 14	13
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	4
IL4	Interleukin 4	5
TNFSF13/APRIL	Tumor necrosis factor superfamily, member 13 v-erb-a erythroblastic leukemia viral oncogene	17
ERBB4	Homolog 4	2
FZD3	Frizzled homolog 3	8
GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	7
GPR39	G protein-coupled receptor 39	2
GPR51	G protein-coupled receptor 51	9
GPR126	G protein-coupled receptor 126	6
GPR143	G protein-coupled receptor 143	X
GPR160	G protein-coupled receptor 160	3
ZP1	Zona pellucida glycoprotein 1 (sperm receptor)	11
ZP2	Zona pellucida glycoprotein 2 (sperm receptor)	16
ZP3	Zona pellucida glycoprotein 3 (sperm receptor)	7
ZP4	Zona pellucida glycoprotein 4	1
SLC5A11	Solute carrier family 5 (sodium/glucose cotransporter), member 11	16
SOCS7	Suppressor of cytokine signaling 7	17
	Transcription factors, oogenesis, folliculogenesis	
FIGLA	Folliculogenesis specific basic helix-loop-helix	2
POU5F1	POU class 5 homeobox 1	6
NOBOX	NOBOX oogenesis homeobox	7
BNC1	Basonuclin 1	15
GCNF/NR6A1	Nuclear receptor subfamily 6, group A, member 1	9
SOX15	SRY (sex determining region Y)-box 15	17
SOX30	SRY (sex determining region Y)-box 30	5
OTX2	Orthodenticle homolog 2 (*Drosophila*)	14
FOXR1	Forkhead box R1	11
JARID2	Jumonji, AT rich interactive domain 2	6
	Chromatin reprogramming	
NPM2	Nucleophosmin/nucleoplasmin 2	8
	Postreplicative DNA mismatch repair system	
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*)	2
	Oolema receptor (oocyte-sperm adhesion)	
ASTL	Astacin-like metalloendopeptidase (M12 family)	2

Table 3: Continued.

Gene symbol	Gene title	Chromosome
	Genes significantly overexpressed in oocytes	
	Imprinted genes	
MEST	Mesoderm specific transcript homolog	7
	Apoptosis	
BNIP1	BCL2/adenovirus E1B 19 kDa interacting protein 1	5
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)	17
BCL2L10	BCL2-like 10 (apoptosis facilitator)	15

early embryo development because of the rare availability of materials to be researched however some studies have tried to elucidate this (see Tables 1 and 2).

Wells et al. quantified the expression of nine different genes *BRCA1*, *BRCA2*, *ATM*, *TP53*, *RB1*, *MAD2*, *BUB1*, *APC,* and *beta-actin* in four mature human oocytes and forty-two embryos by RT-PCR [17], as can be seen in Table 1. These genes are known to play key roles in processes important for preimplantation development, such as cell cycle regulation, DNA repair, signaling pathways, construction of the cytoskeleton, and apoptosis. The authors found that the analyzed oocytes showed relatively high levels of mRNA transcripts, while 2-3-cell preimplantation embryos were found to contain very little mRNA from any of the genes analyzed. The recovery of gene expression levels was not seen until the 4-cell stage embryo or later, which may be related to the activation of the embryonic genome. During preimplantation development of embryos some genes displayed great increases in expression at the stage of 4–8 cells, but for most genes the maximal expression was not achieved until the blastocyst stage. From these results it may be concluded that it is possible to define characteristic gene expression profiles for each stage of human preimplantation development. The identification of genes expressed at different preimplantation phases of development may provide the link to the cellular pathways which are activated at defined stages of oocyte development. The expression of genes related to DNA repair pathways in cleavage stage embryos indicated that DNA damage may be a common feature at this stage. It was suggested that specific profiles of gene expression may be indicative of embryo development and implantation potential. In another study Li et al. analyzed the gene expression of human lactate dehydrogenase isozymes (*LDH-A*, *LDH-B*, and *LDH-C*) and small ubiquitin-like modifier isoforms (*SUMO-1*, *SUMO-2*, and *SUMO-3*) in four single MII-stage oocytes, two 4-cell and three 8-cell embryos using the reverse transcription-polymerase chain reaction [18]. The mRNAs for SUMO-1, SUMO-2, SUMO-3, and LDH-B (heart) were detected in all oocytes and 4- and 8-cell embryos. The mRNA for LDH-A (muscle) was detected in two of four oocytes and in one of three 8-cell embryos. Lactate and pyruvate are the crucial nutrients for the cleavage stage embryos of many mammalian species, including humans, as confirmed also by this study. The interconversions of lactate and pyruvate are catalyzed by lactate dehydrogenase (LDH), which was expressed in oocytes and embryos in this study. Small ubiquitin-like modifier (SUMO) proteins in humans, expressed in oocytes and embryos in this study, are involved in protein trafficking and targeting during posttranslational modification. The mRNA for testis-specific LDH-C was not detected in any sample in this study.

Using microarray technology Dobson et al. analyzed gene expression in single human oocytes and preimplantation embryos to elucidate the gene expression profile during human preimplantation development from the oocyte to the 8-cell stage embryos [21]. The results of this study provided the first global analysis of the human preimplantation embryo transcriptome and demonstrated that RNA can be successfully amplified from single oocytes and embryos for analysis by cDNA microarray technology. To identify the genes whose transcript levels changed throughout the first 3 days of preimplantation development, they analyzed gene expression in single oocytes (five primary oocytes and two secondary oocytes) and embryos (seven day 1 embryos, three day 2 embryos, and five day 3 embryos), each compared to a control primary oocyte. They found that a specific pattern of gene expression exists with most genes that are transcriptionally modulated during the first three days following fertilization being not up-regulated but down-regulated in comparison with oocytes. They also observed that the majority of genes which showed differential expression during preimplantation development were of unknown identity and function and that embryonic transcriptional programs were clearly established by day 3 following fertilization, even in embryos that arrested prematurely at 2-, 3- or 4-cell stages. For the first time it was indicated that the failure to activate transcription is not associated with the majority of human preimplantation embryo loss in the *in vitro* fertilization programme. In the study of Li et al. the cDNA microarray from single oocytes and 4- and 8-cell embryos were used to elucidate the differential expression profiles [18]. In oocytes 184 genes were found to be expressed more than twofold above the median value, but only two genes were at least twofold below the median value. In 4-cell embryos 29 genes were expressed more than twofold above the median value, but 98 genes expressed at least twofold below the median value. In 8-cell embryos 65 genes were found to have a value more than twofold above the median value, and 287 genes were expressed at least twofold below the median value of all genes expressed in oocytes and embryos. This indicated that the expression of some zygotic genes had already occurred in 4-cell embryos.

3.3. Gene Expression Profile during Oogenesis/Folliculogenesis. Huntriss et al. studied the expression of the *NOBOX* gene in human ovarian follicles and oocytes and presented the first cDNA cloning and transcript expression analysis of the human *NOBOX* gene using RT-PCR [19], as can be seen in

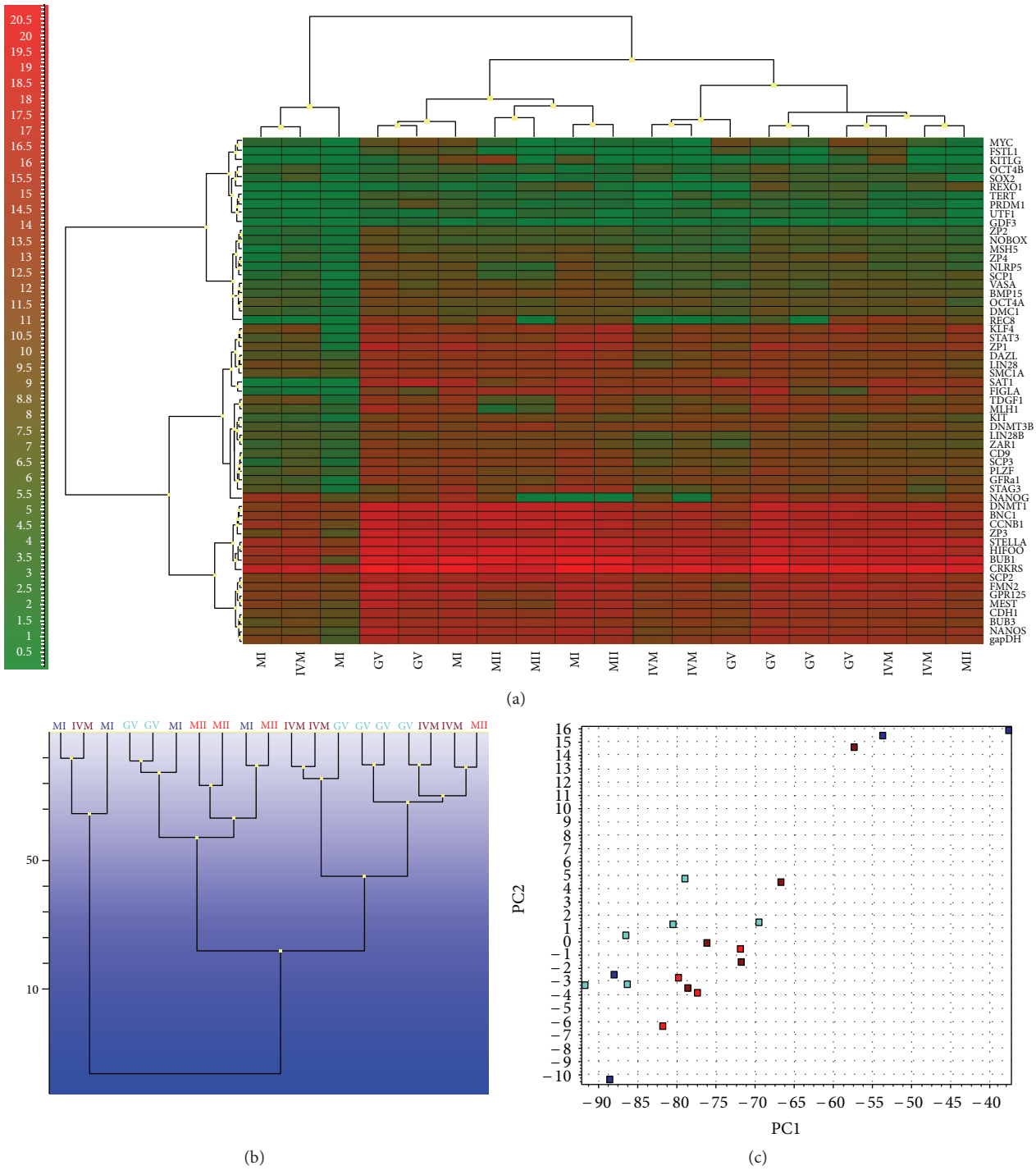

Figure 2: Analyses of nineteen human oocytes at different stages of maturity on the expression of fifty-six genes related to pluripotent stem cells and oocytes using a Fluidigm Real-Time system confirmed three outstanding oocytes (two MI and one IVM oocyte). (a) heatmap clustering (Ward's Algorithm, Euclidean Distance Measure), (b) hierarchical clustering (Ward's Algorithm, Euclidean Distance Measure), (c) principal component analysis (PCA) *legend for* (a) red—expressed, green—nonexpressed; *legend for* (b) *and* (c) aquamarine—GV oocytes, blue—metaphase I oocytes, red—MII oocytes, dark red—IVM oocytes. Analyzed genes: *VASA*, *GPR125*, *DAZL*, *KIT*, *KIT-LIG*, *STELLA*, *GFRa1*, *PLZF*, *OCT4B*, *OCT4A*, *LIN28*, *GDF3*, *NANOG*, *MYC*, *KLF4*, *SOX2*, *UTF1*, *TDGF1*, *DNMT3B*, *LIN28B*, *TERT*, *CD9*, *NANOS*, *CDH1*, *STAT3*, *REX01*, *DNMT1*, *BMP15*, *ZP1*, *ZP2*, *ZP3*, *ZP4*, *SCP1*, *SCP2*, *SCP3*, *SMC1A*, *FSTL1*, *CCNB1*, *BNC1*, *BUB1*, *BUB3*, *NOBOX*, *MSH5*, *NLRP5*, *FMN2*, *HIFOO*, *MEST*, *CRKRS*, *MLH1*, *ZAR1*, *REC8*, *PRDM1*, *SAT1*, *FIGLA*, *STAG3*, and *DMC1*.

Table 1. *NOBOX* is a homeobox gene that is preferentially expressed in the oocytes and ovarian follicles and was first identified in the mouse. When analyzing adult human tissues, they found that the expression of this gene is limited to the ovary, testis, and pancreas. The *NOBOX* gene expression within the ovary was oocyte-specific and was found from the primordial stage of ovarian follicles through to the mature MII oocytes. Additionally, Huntriss et al. analyzed the expression profiles of 14 additional homeobox genes during human oogenesis and early development. Using RT-PCR they found that the expression of *HOXA10* was limited to primordial and early primary follicles. The gene *HOXB7* was expressed from primordial and early primary stage follicles to the GV-stage oocytes. The homeobox genes gastrulation brain homeobox 1 (*GBX1*) and *HOXA7* were predominately expressed by GV oocytes and *HOXA1* and *HEX* by MII oocytes. It was concluded that the homeobox gene transcripts detected in ovarian follicles and oocytes were distinct from those expressed in human embryos at the blastocyst stage (*HOXB4*, *CDX2,* and *HOXC9*) and in granulosa cells (*HOXC9*, *HOXC8*, *HOXC6*, *HOXA7*, *HOXA5,* and *HOXA4*).

Markholt et al. have isolated pure populations of oocytes from the early follicles at different stages of maturity—primordial, intermediate, and primary follicles—by laser capture microdissection methodology and analyzed them by whole-genome microarray analysis [23]. The microarray data were also confirmed by qPCR for selected genes. Their results confirmed that a total of 6,301 unique genes were significantly expressed representing categories of genes related to "RNA binding," "translation initiation," and "structural molecule activity". Several genes were also found to be associated with early oocyte development, where some were identified with extraordinarily high expression levels, such as the antiproliferative transmembrane protein with an epidermal growth factor-like and two follistatin-like domains (*TMEFF2*): the Rho-GTPase-activating protein oligophrenin 1 (*OPHN1*) and the mitochondrial-encoded ATPase6 (*ATP6*). The genes *TMEFF2* (also known as *Tomoregulin-2*) and *OPHN1* were among the most expressed genes overall. In oocytes from primordial/primary follicles the mitochondria-related gene *DC48* was also strongly expressed. In the oocytes from the primordial/primary follicles the genes related to ovarian steroidogenesis were not expressed or were only weakly expressed. In terms of steroid receptors the androgen receptor (*AR*), estrogen receptor 2 (*ER2*), and the progesterone receptor membrane component 1 + 2 were expressed at a low level, while there was no expression of the follicle-stimulating hormone receptor (*FSHR*) and luteinizing hormone receptor (*LHR)*. Among transcription factors several genes from the POU and Forkhead families were expressed in oocytes from early follicles: *POUF3F2* (*OCT-3*), *POU4F2*, *POU2F1* (*OCT-1*), *POU5F1* (*OCT-4*), and *FOXO1*, *FOXO3*, *FOXR1*, *FOXP1*. Among TGFβ family of growth factors and other growth factors were several genes which were not expressed: bone morphogenetic protein (*BMP15*), anti-Mullerian hormone (*AMH*), *AMH* receptor 2 (*AMRH2*), activin/inhibin subunits, insulin-like growth factor I (*IGFI*), platelet-derived growth factor (*PDGF*) and its receptor, and epidermal growth factor (*EGF*) and its receptor (EGFR) were not expressed in appreciable levels, while in contrast a number of BMP receptors (*BMP1A* and *BMP1B*), activin receptors (*activin 1B*, *2A*, and *2B*), *IGFI* receptor, fibroblast-growth factor (*FGF*)-*9*, *FGF14*, *FGF* receptor 4, and transforming growth factor alpha (*TGF*α) were consistently expressed. In oocytes from early follicles several genes of signal transduction were expressed, such as genes for enzymes phosphatidylinositol 3-kinase (*PI3K*), *PI4K*, and *PTEN* that regulate the levels of phosphatidylinositol 3,4,5-triphosphate and genes for phosphodiesterases (*PDE4D*, *PDE8A* + *B,* and *PDEI2*) and for the mitogen-activated protein kinase I (*MAPKI*). In oocytes from primordial follicles some oocyte or germline-specific genes were found to be expressed, such as the factor in the germline alpha (*FIGLA*), zona pellucida glycoproteins *ZP1*, *ZP2*, *ZP3*, *ZP4*, spermatogenesis and oogenesis-specific basic helix-loop-helix I (*SOLHI*), *SOLH2*, the newborn ovary homeobox (*NOBOX*) gene, the maternal embryonic leucine zipper kinase (*MELK*), and oocyte-specific gene (*MATER*). The authors proposed a technique of laser capture microdissection combined with transcriptome analysis of the rare material of human oocytes from the early follicle stages as an important tool to gain better understanding of early human folliculogenesis and oogenesis. These results indicated some genes which might be related to the oocyte maturation process.

3.4. Gene Expression Profiles in Human Oocytes and Human Embryonic Stem Cells. The real origin of hESCs is still a matter of debate. Thus far it has been believed that they most closely resemble pluripotent primitive ectoderm cells derived from the blastocyst inner cell mass. However, differences between ESCs and primitive ectoderm cells have opened the question whether ESCs really have an *in vivo* equivalent or whether their properties mostly reflect their artificial culture environment. Some authors have proposed that they might be related to the germinal lineage [35]. Early human preimplantation embryo development is characterized by the induction of totipotency, followed by pluripotency. The understanding of this very complex process could be implicated among *in vitro* fertilization methodology and regenerative medicine. Human mature MII-stage oocytes and hESCs have some common features: both are able to achieve cell reprogramming towards pluripotency in different ways (e.g., by somatic cell nuclear transfer and cell fusion). The comparison of the transcriptome of these two types of cells may highlight some genes that are involved in the pluripotency initiation. Zhang et al. confirmed the expression of distinct sets of developmentally regulated genes that are expressed by both the human oocytes and hESCs [24]. The main point of their study was to identify genes that were expressed differently during final oocyte maturation and early embryonic development in humans. Using the microarrays and RT-PCR methodology they compared gene expression profiles of 76 human GV-stage oocytes from 55 donor patients included in the *in vitro* fertilization programme, hESCs, and human foreskin fibroblasts. In their study 10,183 genes were expressed in human GV oocytes, and 45% of these genes were still unclassified by their biological function. Four oocyte-specific genes—*MATER*, *ZAR1*, *NPM2,* and

FIGLA—were for the first time detected in human GV-stage oocytes. Additionally, the components of 4 signaling pathways—MOS-MPF, TGF-β, Wnt, and Notch—were also found to be expressed in human GV oocytes. Distinct sets of genes were detected by comparison of gene expression profiles between human GV oocytes, hESCs, and human foreskin fibroblasts, and these gene sets may be involved in the processes of oocyte maturation and early embryonic development. Furthermore, Assou et al. compared the gene expression profile of mature MII oocytes from the *in vitro* fertilization programme and hESCs to that of somatic tissues [25]. They identified a common oocyte/hESC gene expression profile, which included several genes involved in the cell cycle, such as those encoding enzymes involved in general cell metabolism (*METAP2*, *SHMT2*, etc.), nucleoside synthesis (*DHFR*, *TYMS*, *RRM2*, *PPAT*, etc.), DNA repair including mismatch repair (*MSH2* and *MSH6*) or base excision repair (*UNG* and *PCNA*), main components of the cell cycle regulatory machinery (*CCNB1* and *2*, *CCNA*, *CCNE*, etc.), regulators of the topologic state of DNA (*TOP1* and *TOP2A*) and components of the mitotic spindle assembly checkpoint (the centromere constituents *CENPE*, the securin *PTTG1*, and *MAD2L1*, *BUB1B*, *BUB3*), genes related to pluripotency (e.g., *LIN28* and *TDGF1)*, large chromatin remodeling network genes (e.g., *TOP2A, DNMT3B, JARID2, SMARCA5, CBX1, and CBX5*), 18 different zinc finger transcription factors, including *ZNF84*, and several genes which are still poorly understood, such as *KLHL7*, *MRS2*, or the selenophosphate synthetase 1 (*SEPHS1*). Additionally, a large set of genes was also found to encode proteins involved in the ubiquitination and proteasome pathways. After differentiation of hESCs into embryoid bodies, the transcription of these gene pathways was found to decline. These data indicated the important relation of oocytes to hESCs but also some important oocyte-specific differences.

3.5. Oocyte-Like Cells Developed In Vitro. There were also several successful attempts to culture oocyte-like cells (OLCs) from hESCs [3–7], hiPSCs [6, 7], hOSCs [8–10], and hAFSCs [11]. These OLCs expressed some oocyte-specific genes, as revealed by RT-PCR. The data are summarized in Table 4. West et al. found that the enrichment and differentiation of human germ-like cells were mediated by mouse embryonic fibroblast (MEF) feeder cells and basic fibroblast growth factor (bFGF) [3]; these OLCs significantly up-regulated genes related to premigratory/migratory processes, as well as some meiosis-related genes, as can be seen in Table 4. Furthermore, they cultured OLCs from hESCs and confirmed that endogenous BMP expression caused germ-like (DDX4 (VASA)+ POU5F1+) cell differentiation, and the inhibition of this pathway resulted in a significant decrease of germ cell-related gene expression and the number of germ-like cells [4], as also presented in Table 4. Additionally, they confirmed that the loss of KITL in the culture system resulted in a significant down-regulation of germ cell genes and a 70.5% decrease of germ-like cells. Their results indicated that both the BMP and KITL expressions are important for *in vitro* development of hESC-derived germ-like cells and that they may play an important role in human oogenesis.

Richards et al. [5] cultured embryoid bodies from hESCs in six different culture conditions (mitotically inactivated porcine ovarian fibroblasts, 100% conditioned medium from porcine ovarian fibroblasts, 50% conditioned medium from porcine ovarian fibroblasts, forskolin, transretinoic acid RA, and forskolin and RA) and in all cultured conditions confirmed the development of OLCs, which expressed several oocyte-specific genes, such as *VASA*, *DAZL*, *GDF3*, *GDF9*, *MLH1*, *SCP1*, *PUM1*, *PUM2,* and *POU5F1* (see Table 4). Among all culture conditions, porcine ovarian fibroblasts were proven to be the best system for initiating germ cell differentiation *in vitro*.

Medrano et al. [6] found that with overexpression of *VASA* and/or *DAZL* genes, both the hESCs and iPSCs were differentiated into primordial germ cells, and maturation and progression through meiosis was enhanced. These cells expressed several genes related to early germ cells (*VASA*, *DAZL*, *FITM1*, *PRDM1A*, *GCNF*, *GDF3*, *CKIT*, *PELOTA*) and late germ cells and meiosis (*SCP3*, *MLH1*, *DMC1*, *GDF9*, *ZP4*), as can be seen in Table 4. Moreover, Eguizabal et al. [7] have recently reported that hESCs and hiPSCs were successfully differentiated into OLCs and then successfully passed through the process of meiosis, were haploid, and expressed several genes related to early and late germ cells as well as the process of meiosis (Table 4). This was the first report indicating that OLCs developed *in vitro* from stem cells might pass through the process of meiosis and finally differentiated in haploid cells.

The OLCs and parthenogenetic-like structures were developed from the putative human ovarian stem cells from the ovarian surface epithelium of women with no naturally present oocytes and follicles—postmenopausal women and women with premature ovarian failure—as reported by our group [8–10]. These *in vitro* developed OLCs expressed some oocyte-specific genes [8, 9] and a variety of genes related to pluripotent stem cells [9], listed in Table 4. A big advantage of these cells is that they can be developed from autologous ovarian stem cells, even in women with severe ovarian infertility.

Recently, Cheng et al. successfully developed stem cells from human amniotic fluid (hAFSCs) into OLCs [11]. After 15 days of cell culture and differentiation, OLCs with a diameter of 50–60 μm and zona-pellucida- (ZP-) like structures were observed. They were trying to elucidate if the bone morphogenetic protein 15 (*BMP15*) gene was activated during the differentiation of human amniotic fluid stem cells into OLCs. When OLCs were analyzed by RT-PCR, *BMP15* was activated from approximately day 10 of cell differentiation. Additionally, the green-fluorescent-protein- (GFP-) BMP15 was transfected into the differentiating human amniotic stem cells, and its expression was positive in the OLCs. The RT-PCR analysis showed that the oocyte-specific genes, such as *ZP1*, *ZP2*, *ZP3*, and *c-kit,* were expressed in the differentiating hAFSCs, and the immunofluorescence assay confirmed that the ZP2 was detected in the OLCs. Using quantitative RT-PCR they found that that *ZP2* and *ZP3* expressions were significantly elevated in the differentiating stem cells. They concluded that the *BMP15* could be used as an important marker of oogenesis to follow the differentiation of amniotic fluid stem cells and other stem cells into the OLCs.

Table 4: Expression of genes in oocyte-like cells developed *in vitro* from human stem cells of different sources.

Gene expression of oocyte-like cells developed *in vitro* from stem cells in humans			
Source	Critical culture condition	Expression of genes (method of detection)	References
Human embryonic stem cells (hESCs)	bFGF, feeder layer (mouse embryonic fibroblasts)	Premigratory/migratory genes related to pluripotency: *IFITM3*, *DAZL*, *NANOG*, *POU5F1*, postmigratory genes: *PIWIL2*, *PUM2* and genes related to meiosis: *SYCP3* and *MLH1* (RT-PCR).	West et al., 2008 [3]
hESCs	bFGF, feeder layer (mouse embryonic fibroblasts)	Germ cell-related gene *DDX4* (*VASA*) and pluripotency-related *POU5F1* (*OCT4*). (RT-PCR)	West et al., 2010 [4]
hESCs	Ovarian fibroblasts	*VASA*, *DAZL*, *GDF3*, *GDF9*, *MLH1*, *SCP1*, *PUM1*, *PUM2*, *POU5F1* (RT-PCR)	Richards et al., 2010 [5]
hESCs Human-induced pluripotent stem cells (hiPSCs)	Culture medium with glutamine, 2-mercaptoethanol and bFGF	Early germ cell markers:*VASA*, *DAZL*, *IFITM1*, *PRDM1A*, *GCNF*, *GDF3*, *CKIT*, *PELOTA* Late germ cell and meiotic markers: *SCP3*, *MLH1*, *DMC1*, *GDF9*, *ZP4* (Biomark 96.96 microfluidic qPCR chip Fluidigm).	Medrano et al., 2012 [6]
hESCs hiPSCs from keratinocytes and umbilical cord blood	Culture medium with retinoic acid, forskolin, and bFGF	*OCT4*, *NANOG*, *SOX2*, *KLF4*, *C-MYC*, *DPPA4*, *DNMT3B*, *REX1*, *SALL2*, *LIN28, VASA*,and *STRA8* (RT-PCR) Haploid cells confirmed by fluorescence in situ hybridization for chromosomes X, Y, and 18	Eguizabal et al., 2011 [7]
Stem cells from adult ovarian surface epithelium (hOSCs)	Culture medium with phenol red (weak estrogenic stimuli)	*OCT4A*, *OCT4B*, *c-KIT*, *VASA*, and *ZP2* (RT-PCR)	Virant-Klun et al., 2008 [8]
Stem cells from adult ovarian surface epithelium (hOSCs)	Culture medium with added follicular fluid	*OCT4A*, *SOX-2*, *NANOG*, *NANOS*, *STELLA*, *CD9*, *LIN28*, *KLF4*, *GDF3*, and *MYC* (Single-Cell Gene Expression Analyses by Fluidigm BioMark System)	Virant-Klun et al., 2011 [10]
Amniotic fluid stem cells (hAFSCs)	Culture medium with glutamine, beta-mercaptoethanol, and porcine follicular fluid	*BMP15*, *ZP1*, *ZP2*, *ZP3*, *c-kit, VASA* (RT-PCR) BMP15 was proposed as an important marker of oogenesis	Cheng et al., 2012 [11]

The results of all these studies showed that it is possible to trigger the development of OLCs from different types of stem cells by the addition of certain substances to the culture medium, such as bFGF, retinoic acid, forskolin or human/animal follicular fluid, or by using ovarian fibroblasts or mouse embryonic fibroblasts (MEFs) as a feeder layer (Table 4). The oocyte-like cells mostly had a diameter of around 60 μm, and some of them expressed a zona-pellucida-like structure. In spite of some promising new knowledge, the existing results of genetic analyses showed that the OCLs developed *in vitro* are still far from real, fully competent human oocytes. They mostly failed to progress through the whole process of meiosis in spite of the complex *in vitro* maturation medium; therefore, the transplantation of these cells to mature them *in vivo* was proposed as a more reliable approach which may lead to potential clinical practice in the future [36]. Development of oocytes *in vitro* would be of great importance for the treatment of severe ovarian infertility (e.g., premature ovarian failure). Yet, in general, the OLCs which developed *in vitro* from human stem cells have not been genetically analyzed in greater detail, including the microarrays. This reveals the need to analyze the OLCs genetically and epigenetically in more detail in comparison with mature oocytes from the *in vitro* fertilization programme to evaluate their quality and safety in the future. At present the most important task is to better elucidate the expression of oocyte-specific genes (see Table 3).

3.6. Gene Expression Profile and Oocyte In Vivo and In Vitro Maturation. The maturity of the human oocyte is one of the crucial features to sustaining a successful pregnancy to birth. It is known that immature human oocytes at the GV stage and MI stage of maturity cannot be fertilized. Human oocyte maturity is related to nuclear and cytoplasm maturity. Gasca et al. analyzed a pool of immature oocytes (20 GV oocytes in 7 patients and 20 MI oocytes in 7 patients), 37 non-fertilized mature MII oocytes, and cumulus cells by microarrays [2]. In all samples the gene expression level of *BRCA1* and *2*, *ATM*, *TP53*, *RB1*, *BUB1*, *MAD2*, *APC,* and *ACTB* was analyzed. The expression of *ACTB* and *MAD2*

Table 5: Genes which were significantly up-regulated in human oocytes at different stages of maturity.

Up-regulated genes at different stages of oocyte maturity			
GV (immature)	MI (immature)	MII (mature)	References
RBBP8, *ATR*	*BRAP*, *BUB1B*, *BUB3*	*RBBP7*, *RBL2*	Gasca et al., 2007 [2]
GBX1, *HOXA7*		*HOXA1*, *HEX*	Huntriss et al., 2006 [19]
Molecular functions of differently expressed genes (GV versus MII oocytes)			
GV Up-regulated genes (number)		GV Down-regulated genes (number)	References
(i) Nucleic acid binding (149)		(i) Nucleic acid binding (235)	
(ii) Zinc finger transcription factor (58)		(ii) Ribosomal protein (101)	
(iii) KRAB box transcription factor (41)		(iii) Receptor (39)	
(iv) Ligase (31)		(iv) Translation factor (22)	
(v) Chaperone (25)		(v) Ribonucleoprotein (15)	Wells and Patrizio, 2008 [26]
(vi) Synthase and synthetase (25)		(vi) Translation elongation factor (14)	
(vii) Receptor (22)		(vii) Signaling molecule (13)	
(viii) Other RNA-binding factors (19)		(viii) G-protein coupled receptor	
(ix) mRNA processing factor (17)		(ix) Tubulin	

was present in all samples. Gene *RB1* was down-regulated in oocytes, while the genes *BUB1*, *BRCA1* and *2,* and *MAD2* were down-regulated in cumulus cells. The expression of *BRCA1* and *2* was absent in cumulus cells, while the expression of *TP53* was absent in MI oocytes and *RB1* expression in MII oocytes. They identified new potential regulators and marker genes involved in the human *in vivo* oocyte maturation, such as *BARD1*, *RBL2*, *RBBP7*, *BUB3*, or *BUB1B* (Table 5). In the *RB1* group of transcription factors the expression of *RBBP8* was highest in the GV oocytes, *RBBP4* in MI oocytes, and *RBBP7* and *RBL2* in MII oocytes. The high expression levels of *RBBP7*, *RBBP4,* and *RBL2* in MI and MII oocytes suggested that this transcription regulation pathway could be functional during oocyte maturation (Table 5). The expression of the gene *RBL1* was highly restricted for cumulus cells, indicating that this gene may be involved in the regulation of transcription in these cells. DNA repair markers *ATM* and *ATR* were differentially expressed during oocyte maturation; the expression of *ATR* mostly appeared in immature GV oocytes. Also the DNA repair marker *BARD1* was expressed during oocyte maturation. The cell cycle checkpoint markers *BUB3* and *BUB1B* were expressed in immature MI oocytes, and the expression of gene *BRAP* was reduced in MII oocytes (see Table 5). Furthermore, Assou et al. [16] analyzed the total cRNA which was synthesized from pools of GV-, MI- or MII-stage oocytes, then labeled, and hybridized to pan-genomic oligonucleotide microarrays. Oocytes expressed on average 8,728 genes. The lowest number of genes was found to be expressed in MII oocytes (n = 5,633) and the highest number in GV oocytes (n = 10,892). On the other hand, in MII oocytes 444 genes were overexpressed, while in GV oocytes only 104 genes were overexpressed; in the transient MI oocytes only 4 genes were overexpressed. The expression variations between GV, MI, and MII oocytes were low. Similarly, Wells and Patrizio [26] found that GV oocytes expressed the highest number of genes, and *in vivo* matured MII oocytes the lowest number. Most of the differences between GV-stage oocytes and other stages were the consequence of a reduction in the number of mRNA transcripts occurring during progression to MII. This suggested that relatively little new gene expression occurred after the GV stage of maturity, with degradation of mRNA transcripts leading to the differences in transcript numbers observed at later stages of maturity in spite of the fact that the post-GV oocytes were not completely quiescent in terms of gene transcription, as revealed by the detection of some transcripts absent at the GV stage. Differently expressed genes were classified according to their molecular functions and biological processes. In Table 5 we can see the molecular function of most up-regulated genes in GV-stage oocytes in comparison to *in vivo* matured MII oocytes. For example, a number of genes for storage proteins displayed up-regulation in GV oocytes compared with *in vivo* matured MII oocytes.

In vitro maturation of human oocytes is an attractive strategy for *in vitro* fertilization treatment, especially in women with polycystic ovarian syndrome (PCOS) and with a risk of ovarian hyperstimulation after gonadotropin administration for oocyte retrieval. There is also a proportion of immature oocytes which are discarded in daily medical practice because of their immaturity. However, current *in vitro* maturation protocols unfortunately produce oocytes with a poor clinical outcome shown by poor embryo development and early pregnancy loss [37]. This is also reflected at the molecular level of *in vitro* matured oocytes. Wells and Patrizio analyzed *in vivo* matured oocytes at GV or MII stages of maturity and compared the gene expression profiles between MII oocytes matured *in vivo* and *in vitro* [26]. The non-fertilized oocytes from the *in vitro* fertilization programme

Table 6: Genes which were differently expressed in human oocytes matured *in vitro* in comparison with oocytes matured *in vivo* at a cut-off value of 10-fold or higher [26, 27].

Genes differently expressed in oocytes matured *in vitro* or *in vivo* [27]	
Significantly up-regulated genes in oocytes matured *in vitro*	Significantly down-regulated genes in oocytes matured *in vitro*
HNRNPA2B1, *TCEB1*, *HINT1*, *PNPLA1*, *PAOX*, *NLRP12*, *KIF23*, *PRPF38B*, *UBA52*, *GEM*, *MBD4*, *MORF4L2*, *ATF2*, *AFF4*, *ZCCHC14*, *ZNF669*, *CCNG2*, *NFE2L2*, ***MSH2*** *, *ZNF610*, *C21orf66*, *CDC37L1*, *UPF3B*, *TAOK3*, *GPR64*, *HSPA14*, *C18orf24*, *TAF1A*, *MAP2K7*, *ZNF571*, *PFKFB2*, *CUL1*, *CLDN10*, *CLK1*, *RCOR1*, *ZPBP2*, *GTF2H2*, *TSG101*, *ARHGAP11A*, *HPS3*, *GRHL1*, *EIF4G2*, *RFC4*, *VPS26A*, *PIK3C2A*, *GDI2*, *RAB23*, *WDSOF1*, *PLCL1*, *DCDC2*, *MINPP1*, *PTPN12*, *METAP2*, *LHX8*, *LOC91664*, ***SYCP2*** *, *ATP5L*, *ICK*, *FUCA1*, *SMARCAD1*, *BRD7*, *TBPL1*, *ABCD3*, *SSR3*, *FEZ2*, *FHOD3*, *AMD1*, ***DAZL*** *, *ZMYM2*, *FANCL*, *SLCO1A2*, *NUFIP1*, *STAU2*, *PCF11*, *PPP1R3C*, *POLI*, ***SGOL2*** *, *PBLD*, *CCNL1*, *SLBP*, *MRPS30*, *TMOD2*, *FBXW11*, *CDK7*, *HNRNPR*, *CFDP1*, *DEPDC7*, *CTSL2*, *DYNC1I2*, *NCKAP1*, *TRPA1*, *DCLRE1A*, *RAB18*, *GTDC1*, *LOC283514*, *CLUL1*, *ZNF131*, *SP3*, *ZNF302*, *MAD2L1*, *TRAPPC6B*, *C10orf137*, *MTPN*, *ZNF313*, *PLEKHA3*, *ZCCHC14*, *RTTN*, *ZNF136*, *ADH5*, *CCDC88A*, *CPEB2*, *SPATA5L1*, *UGP2*, *UGP2*,*MFSD1*, *TMEM27*, *ZNF443*, *UAP1*, *MIB1*, *MUT*	*HTRA1, CCDC69, FHL2*
Molecular functions of differently expressed genes (*in vitro* versus *in vivo* matured oocytes) [26]	
Significantly up-regulated genes in oocytes matured *in vitro* (number)	Significantly down-regulated genes in oocytes matured *in vitro* (number)
(i) Nucleic acid binding (62)	(i) Nucleic acid binding (75)
(ii) Zinc finger transcription factor (13)	(ii) Ribosomal protein (37)
(iii) Other RNA-binding factors (10)	(iii) Translation factor (8)
(iv) Ligase (9)	(iv) Translation elongation factor (6)
(v) KRAB box transcription factor (8)	(v) Storage protein (6)
(vi) mRNA processing factor (8)	(vi) Receptor (5)
(vii) Chaperone (7)	(vii) Tubulin (4)
(viii) Receptor (7)	(viii) Signaling molecule (3)
(ix) Signaling (7)	(ix) Ribonucleoprotein (2)
(x) Ribonucleoprotein (7)	(x) Defense/immunity protein (1)

*Genes related to germ cells and meiosis.

were analyzed for more than 29,000 genes with RNA amplification and microarray technology. It was found that GV oocytes expressed 12,219 genes, *in vivo* matured MII oocytes 9,735, and *in vitro* matured MII oocytes 8,510. It is interesting that *in vivo* matured MII and *in vitro* matured MII oocytes shared very similar patterns of gene expressions, but they also noted some significant differences (see Table 6). It is interesting that some germ cell-specific genes (e.g., *DAZL*) and genes related to meiosis (e.g., *SYCP2*, *SGOL2*, and *MSH2*) were also among genes which were up-regulated in oocytes matured *in vitro*. In Table 6 we can see the molecular functions of genes which were expressed differently in oocytes matured *in vivo* versus *in vitro*. Some immature GV oocyte patterns of gene expression still persisted in MII oocytes matured *in vitro*. Although the *in vitro* matured MII oocytes closely resemble the *in vivo* matured oocytes, especially for genes related to nuclear maturity, there were several genes related to cytoplasmic functions which were expressed in an immature manner. It seems that the cytoplasmic maturation may be a crucial point regarding the worse clinical outcome obtained by *in vitro* matured MII oocytes. *In vivo* matured MII oocytes also expressed some genes associated with cellular storage and homeostasis differently.

In the important further study by Jones et al. on global gene expression profiling using microarrays and bioinformatics a molecular basis for differences in the developmental competence of oocytes matured *in vitro* in comparison with *in vivo* matured oocytes was elucidated [27]. They found that more than 2,000 genes were identified as expressed at more than 2-fold higher levels in oocytes matured *in vitro* than those matured *in vivo*, and 162 of them were expressed at 10-fold or greater levels in oocytes matured *in vitro* (see Table 6). Many of these genes up-regulated in oocytes matured *in vitro* are involved in transcription, the cell cycle and its regulation, transport, and cellular protein metabolism. They came to an important conclusion that the overabundance of transcripts identified in immature GV-stage oocytes retrieved from gonadotropin stimulated cycles and matured *in vitro* in the programme of *in vitro* fertilization is probably due to deregulation in either gene transcription or posttranscriptional modification of genes. Each of the two proposed mechanisms would result in an incorrect temporal

utilization of genes which may culminate in developmental incompetence of any embryos derived from these oocytes, as experienced in the clinical programme of *in vitro* fertilization. All these data indicated that the oocyte *in vitro* maturation procedure is far from optimal and needs to be further researched in the future. The relation between changed gene expression profile and the quality of oocytes is still poorly understood. Some attempts were made to relate the gene expressions to developmental competence of oocytes. O'Shea et al. [38] performed a meta-analysis on previously published microarray data on various models of oocyte and embryo quality and identified 56 candidate genes associated with oocyte quality across several species, including human. Moreover, they found that twenty-one potential biomarkers were associated with increased oocyte competence, and thirty-five potential biomarkers were associated with decreased oocyte competence (see Table 7). The up-regulation of METAP2 and the decrease of multiple genes linked to mRNA and protein synthesis in models of competence highlight the importance of *de novo* protein synthesis and its regulation for successful oocyte maturation and subsequent development. Also, the expression of ATRX and several other transcription factors were linked to decreased competence in oocytes (Table 7). These genes could potentially be used also as biomarkers of oocyte quality after the *in vitro* maturation procedure. The molecular genetic analyses of oocytes are an extremely important tool to provide a more efficient and safe procedure of oocyte *in vitro* maturation in the future.

3.7. Gene Expression Profiles in Human Oocytes and In Vitro Fertilization Methods. Oocytes matured *in vitro* can be cryopreserved or fertilized in the *in vitro* fertilization programme. Cryopreservation is now considered to be an efficient way to store human oocytes to preserve fertility in young cancer patients before oncotherapy [39–41] (i.e., chemo- and radiotherapy) or in patients with no sperm on the day of *in vitro* fertilization [42]. There are two different principles in the preservation of human oocytes: stepwise—slow freezing and thawing in a liquid nitrogen vapour [43, 44]—and vitrification—the immediate plunge of oocytes into a vitrification solution with a high concentration of cryoprotectant and into liquid nitrogen [45–47]. There is a big debate over which method is of clinical preference. But in general, the clinical results of *in vitro* fertilization obtained by frozen-thawed MII oocytes are for the most part significantly worse than in fresh MII oocytes. In spite of the fact that these procedures are already in the clinical practice, the effects of these technologies on the oocyte gene expression are little known. Monzo et al. [28] studied the effect of these two different cryopreservation procedures, slow freezing and vitrification, on the gene expression profile of human MII oocytes. The gene expression profiles and associated biological pathways in slowly frozen/thawed and vitrified oocytes were compared with those of fresh control oocytes. It was found that both cryopreservation procedures negatively affected the gene expression profile of human oocytes in comparison with fresh controls. It is interesting that slowly frozen and vitrified oocytes displayed distinct gene expression profiles: slow oocyte freezing was associated with the down-regulation of genes related to chromosomal structure maintenance (e.g., *KIF2C* and *KIF3A*) and cell cycle regulation (e.g., *CHEK2* and *CDKN1B*) that may lead to a reduction in the oocyte developmental competence, while in vitrified oocytes many genes of the ubiquitination pathway were down-regulated, including those of the ubiquitin-specific peptidase family and subunits of the 26S proteasome. Such inhibition of the oocyte degradation machinery might stabilize the maternal protein content which is necessary for oocyte developmental competence. They concluded that the low pregnancy rates achieved by frozen-thawed human MII oocytes could be explained by the alterations of the oocyte gene expression profile.

Not only were the procedures of oocyte cryopreservation confirmed to be related to the changed oocyte gene expression profile, but so was the total fertilization failure (no fertilization of oocytes) in the *in vitro* fertilization programme. In patients with male factor infertility the total fertilization failure may be explained by the bad sperm quality, while in patients without male factor infertility the lack of identifiable criteria poses the question of the reliability of the clinical management. Gasca et al. analyzed the gene expression profiles of MII oocytes after total fertilization failure in a 30-year-old patient who had experienced three successive total fertilization failures (39 oocytes) and fertile control patients diagnosed with tubal or male infertility [29]. Transcriptional analysis of unfertilized MII oocytes revealed a total fertilization failure-altered gene expression profile with misexpression of genes related to meiosis, cell growth, and apoptosis control, all characterized by important fold changes. These results confirmed that even morphologically normal, high-grade oocytes in the *in vitro* fertilization programme may express some molecular abnormalities at the gene expression level, especially those related to the failure of MII oocyte activation. The authors proposed the microarray approach to improve the clinical therapeutic treatment and to offer informed counseling to the patients about alternate therapeutic solutions. These studies need to be performed with a higher number of oocytes to make a real conclusion.

3.8. Polar Body Gene Expression Profiling. In the *in vitro* fertilization programme some improved methods are needed to reliably evaluate oocyte quality prior to fertilization and to choose the right embryos to implant. Recent evaluation of oocyte quality is based only on the evaluation of morphology, but this is very limited, and also oocytes with high-grade morphology sometimes do not fertilize or develop into the embryo. mRNA and proteins produced during the oogenesis support embryonic development until the zygotic transition. Polar body gene expression profiling might be an important safe tool to verify the molecular status of *in vivo* or *in vitro* matured oocytes before the *in vitro* fertilization procedure. The first polar body is extruded from a mature oocyte before fertilization and can be biopsied without damaging the oocyte. The polar body transcriptome was proposed as a potential tool for the evaluation of oocyte quality in the *in vitro* fertilization programme [30, 31]. Klatsky et al. tested the hypothesis that mRNA originating from the expression

Table 7: Transcription factors associated with developmental competence of oocytes found by meta-analysis of previously published microarray studies comparing *in vivo* and *in vitro* matured oocytes in bovine and monkey, human healthy and polycystic ovaries (PCOS-polycystic ovary syndrome), and mouse young and aged ovaries [38].

Transcripts associated with developmental competence of oocytes			
Increased developmental competence		Decreased developmental competence	
CPD	Carboxypeptidase D precursor	PRKG1	cGMP-dependent protein kinase 1, alpha isozyme
SH3BGRL	SH3 domain binding glutamic acid-rich-like protein	IGFBP3	Insulin-like growth factor binding protein 3
CDC123	Cell division cycle 123	DUSP1	Dual specificity phosphatase 1
AQP1	Aquaporin 1	NDRG4	Ndr4
GNB5	Guanine nucleotide binding protein beta 5	ATOX1	Copper transport protein
METAP2	Methionine aminopeptidase 2	TMSB10	Thymosin beta-10
UBE2E3	Ubiquitin-conjugating enzyme E2E3	ATRX	Alpha thalassemia/mental retardation syndrome X-linked
USP6NL	USP6 N-terminal-like protein	SHMT2	Serine hydroxymethyltransferase 2
HMGA1*	**High Mobility group AT-hook1**	**TGFBR3***	**Transforming growth factor beta receptor III**
TCTEL1	TCTEL1 protein	FANK1	Fibronectin type 3 and ankyrin repeat domains 1 protein
SNRPD3	Small nuclear ribonucleoprotein D3 polypeptide	PTPN1	Protein tyrosine phosphatase, nonreceptor type 1
MTG1	Mitochondrial GTPase 1 homolog	DDX55	ATP-dependent RNA helicase DDX55
GKAP1	G kinase anchoring protein 1	**SFRP1***	**Secreted frizzled-related protein 1**
UFM1	Ubiquitin-fold modifier 1	BMP4	Bone morphogenetic protein 4
MITD1	Mitochondria interacting transport domain 1	HIST1HBG	Histone H2B type 1-C/E/G
MRPL3	Mitochondrial ribosomal protein L3	SEC61G	Protein transport protein Sec61 subunit gamma
KIF23	Kinesin family member 23	**FOXM1***	**Forkhead box protein M1**
DDX52	DEAD box polypeptide 52	NDUFA1	NADH dehydrogenase 1 alpha subcomplex subunit 1
		TUSC4	Tumor suppressor candidate 4
		JMJD1C	Jumonji domain containing 1C
		POL2RL	DNA-directed RNA polymerases I, II, and III subunit RPABC5
		MED1	Mediator complex subunit 1
		SLC39A14	Solute carrier family 39 member 14
		CALM2	Calmodulin 2
		RPS18	40S ribosomal protein S18
		INVS	Inversion
		RBMS1	RNA binding motif single stranded interacting protein 1
		PLXNC1	Plexin C1
		BTF3l4	Basic transcription factor 3-like 4
		MAP3K12*	**Mitogen-activated protein 3 kinase 12**
		PXMP4	Peroxisomal membrane protein 4
		EG216818	Ubiquitin
		JAM2	Junctional adhesion molecule 2
		IFITM1	Interferon-induced transmembrane protein 1
		LRRC28	Leucine rich repeat containing 28
Transcription regulation			
		Associated with decreased competence in oocytes	
		E2f4 65	
		Sp3 61	
		Gata-1 62	
		C/ebp β 62	
		Rela (P65 NF-Kb Subunit)	

*Potential biomarkers of oocyte developmental competence.

in the meiotic MII oocyte from the *in vitro* fertilization programme is present and detectable in a single polar body prior to fertilization [30]. In their study immature oocytes from the intracytoplasmic sperm injection (ICSI) programme were cultured overnight and checked on the following day for their maturity. In all MII oocytes polar body biopsy was performed and followed by reverse transcription without RNA isolation. Sibling oocytes were prepared similarly to polar bodies. Then the complementary DNA from all samples was preamplified over 15 cycles for candidate genes using selective primers. Single-cell real-time PCR was performed to detect and quantify relative gene expression. It was important that polar body mRNA was detected for 11 of 12 candidate genes (*BCL2L10*, *ODC1*, *DPPA3*, *PADI6*, *DDX4*, *AGO2*, *DROSHA*, *GAPDH*, *EIF6*, *PABP*, *DICER*); interestingly, the oocyte-specific gene *H1FOO* was not expressed in any single polar body. Transcripts that were present in greater abundance in the single oocytes were also detected in qPCR replicates from single polar bodies. They concluded that preamplification of cDNA synthesized without RNA isolation can facilitate the quantitative detection of mRNA in single human polar bodies. This study was followed by the study by Reich et al. [31] who performed the first gene expression of microarray analyses of polar bodies. They performed a polar body biopsy on mature MII oocytes followed by the single-cell transcriptome analysis of the oocytes and their sibling polar bodies. They then compared over 12,700 mRNAs and miRNAs from the oocyte samples with the 5,431 mRNAs from the sibling polar bodies (5,256 shared mRNAs or 97%, including miRNAs). Their results showed that human polar bodies reflected the oocyte transcript profile. The valuable conclusion from their work is that mRNA detection and quantification through high-throughput quantitative PCR or microarrays could result in the first molecular diagnostics for gene expression profile of MII oocytes. The gene expression profiling of a polar body could enable the molecular diagnostics of oocyte quality in the programme of *in vitro* fertilization and might become a very important safe tool to evaluate the molecular status of *in vitro* matured oocytes and improve the clinical outcome of oocyte *in vitro* maturation in the future.

3.9. Oocyte Gene Expression Profile, Female Age, and Meiotic Aneuploidies. It is known that female fertility and development competence of human oocytes decline with increasing female age and that the proportion of oocytes with genetic abnormalities increased with female ageing. Steuerwald et al. found that the global gene expression profiles in human oocytes are related to female age [32]. Their results confirmed that the expression of oocyte genes related to major functional processes and features, including cell cycle regulation, cytoskeletal structure, energy pathways, transcription control, and stress responses depends on female age. Similarly, Grøndahl et al. analyzed the single mature MII non-fertilized oocytes from two groups of patients—younger (<36 years) and older (37–39 years)—in the *in vitro* fertilization programme [33]. Based on 15 independent replicates of single MII oocytes, 7,470 genes (10,428 transcripts) were identified in the MII oocytes. Among these genes, 342 were expressed at a significantly different expression level between the two age groups of patients. These genes were found to be involved in cell cycle regulation, chromosome alignment (e.g., MAD2L1 binding protein), sister chromatid separation (e.g., separase), oxidative stress, and ubiquitination. The top signaling network of genes, which was proven to be affected by female age, was "cell cycle and organism development" (e.g., *SMAD2* and activin B1 receptor). It was concluded that these genes may be associated with the ageing process and decreased fertility. Advanced female age may be also related to oocyte aneuploidy (the abnormal number of chromosomes). Fragouli et al. confirmed the link between mRNA transcript numbers in oocytes and female age [34]. They combined a comprehensive cytogenetic investigation of 21 oocytes with a detailed assessment of their transcriptome. The first polar body was removed from each oocyte and aneuploidy assessed using comparative genomic hybridization. Then the mRNA transcript data were produced using microarrays for seven oocytes, three normal and four aneuploid. The results showed that 327 genes were differently expressed in both groups of oocytes at statistical significance, and they provided the list of these genes. Ninety-six of these genes were further assessed by RT-PCR. The results confirmed that oocyte aneuploidy was associated with altered transcript levels affecting a subset of genes. They concluded that different transcript levels in normal and aneuploid oocytes may have an impact on cellular pathways, such as meiotic spindle assembly, chromosome alignment, production of cell surface or excretory molecules, and might potentially serve as targets for noninvasive oocyte aneuploidy assessment. This may explain the decline of female fertility and increase in spontaneous abortion with age.

When talking about the oocyte *in vitro* maturation procedure, it is more appropriate to be applied in younger women because of a higher risk of oocyte genetic abnormalities in older women. Increased female age may be an important contraindication for the oocyte *in vitro* maturation procedure.

All these new findings provide some new knowledge and better insights into human oocyte quality and oogenesis that might be introduced in clinical practice and might also lead to successful *in vitro* oogenesis in women with no naturally present oocytes in the future.

Conflict of Interests

The authors declare that there is no financial or other conflict of interests related to this paper.

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Research Article

Recovery of Fertility in Azoospermia Rats after Injection of Adipose-Tissue-Derived Mesenchymal Stem Cells: The Sperm Generation

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The recent reports on the treatment of azoospermia patients, in which spermatozoa could not be traced in their testes, are focused more on the potential use of adult stem cells, like mesenchymal stem cells (MSCs). The aim of this study was to demonstrate the potential use of MSCs derived from adipose tissue in the treatment of azoospermia using rat disease models. After busulfan application, the rats ($n = 20$) were injected with the GFP^+ MSCs into left rete testes. After 12 weeks, the testes with cell injection (right testes) were compared to control (left testes) after dimensional and immunohistochemical analyses. Testes treated with MSCs appeared morphologically normal, but they were atrophic in rats without stem cell treatment, in which the seminiferous tubules were empty. Spermatogenesis was detected, not in every but in some tubules of cell-treated testes. $GFP^+/VASA^+$ and $GFP^+/SCP1^+$ cells in testes indicated the transdifferentiation of MSCs into spermatogenetic cells in the appropriate microenvironment. Rats with cell treatment were mated to show the full recovery of spermatogenesis, and continuous generations were obtained. The expression of GFP was detected in the mesenchymal stem cells derived from adipose tissue and bone marrow and also in the sperms of offspring. In conclusion, MSCs might be studied for the same purpose in humans in future.

1. Introduction

The self-renewal and the multilineage differentiation capacities of adult stem cells (ASCs) show great promises for regenerative medicine. Despite of the greater differentiation potential of embryonic stem cells (ESCs) compared to ASCs, ethical concerns and governmental restrictions are the main obstacles of the ESCs standing in the way of their clinical applications [1]. On the other hand, bone-marrow-derived MSCs (BM-MSCs) are among the mostly studied ASCs, and their potential to treat a wide variety of diseases, including erectile dysfunction and male infertility, was demonstrated. Alternatively, adipose-tissue-derived MSCs (AT-MSCs) could be used in future clinical applications instead of bone marrow stem cells due to their comparable differentiation and therapeutic potential, but AT-MSCs are easier and safer to obtain [1–18].

The stem cells were relatively lately adapted in andrology researches on erectile dysfunction and infertility as potential therapeutic agents. The studies related in this area showed that ESC could participate in spermatogenesis by forming functional male germ cells *in vitro* or by supporting the maturation of primordial germ cells into haploid male gametes [19–21]. Nayernia et al. reported germ cell line formation

from pluripotent teratocarcinoma cells in 2004, and after two years, the generation of offspring mice from ESC-derived germ cells was succeeded for the first time [22, 23]. The milestone in adult stem cell research to treat the infertility was the murine BM-MSC differentiation into male germ cells that was succeeded by the same group in 2006 [24]. The differentiation of BM-MSCs into germ cells, Sertoli cells, and Leydig cells was demonstrated in busulfan-treated infertile mice [25, 26]. MSCs derived from human fetal lung and umbilical cord were also shown to differentiate into sperm like cells [27, 28]. Due to their germ cell formation capacity *in vitro*, they suggested that those cells could be the base for a treatment of male infertility. These recent studies reveal that the treatment of diseases, like male infertility and testosterone deficiency, was possible by ASCs.

In this study, the mesenchymal-stem-cell-based therapy for azoospermia was aimed. For this purpose, allogenic AT-MSCs were injected into testicles of azoospermia rat models to recover the infertility. A strategy was herewith proposed for the treatment of azoospermia by intratesticular transfer of adult stem cells.

2. Materials and Methods

2.1. Animals. Animals were housed in the Laboratory Animal Care Center (Kobay A.S., Ankara, Turkey). All animal procedures were approved by the local ethical animal research committee. Male Wistar rats ($n = 32$) aged 8–12 weeks were housed in temperature-controlled rooms (20–22°C) under 12 h light/dark cycle. Later, female Wistar rats ($n = 24$) aged 8–16 weeks were housed for mating. The rats were fed with standard commercial chow diet *ad libitum*.

2.2. Experimental Design. MSCs were isolated from rat ($n = 8$) adipose tissue and labeled with GFP. The rest of male rats ($n = 24$) were sterilized with busulfan. After assessing the infertile status by analyzing the testes of rats ($n = 4$), the right testis of each rat ($n = 20$) was injected with MSCs. The other testis was left as control. After twelve weeks, testes of four animals were removed for dimension analysis. For immunohistochemical analyses, four additional rats were excised. The remaining male rats ($n = 12$) were mated with female rats ($n = 24$). Cells from offspring were analyzed for GFP expression.

2.3. Isolation and Culture of Rat Adipose-Tissue-Derived Mesenchymal Stem Cells (rAT-MSCs). Rats ($n = 8$) were anesthetized by injection of 10 mg/kg Xylazine and 75 mg/kg Ketamine. 1-2 cm^3 of preperitoneal adipose tissue was removed. Tissue samples were washed several times with Hanks' balanced salt solution supplemented with 5% antibiotic-antimycotic solution (Gibco Life Technologies, Paisley, UK), and vascular structures were removed. The yellowish white tissue was minced and enzymatically digested in MEM medium (Gibco Life Technologies) containing 0.075% collagenase 2 (Sigma, St. Louis, MO) at 37°C for 60 min. The cell suspension was filtered with 70 μm sieve (Becton Dickinson Labware, Franklin Lakes, NJ). The cells were resuspended in MEM medium supplemented with 1% penicillin/streptomycin and 15% FBS (standard culture medium). After the centrifugation at 1200 rpm for 10 min, the cells were cultured in standard culture medium in 25 cm^2 culture flasks. After 7 days, the medium was replaced with fresh medium and subsequently replaced twice a week. Erythrocytes and other non adhesive cells were removed from the culture. After reaching 70–80% confluence, the cells were harvested with 0.025% trypsin-EDTA for 3 min, collected by centrifugation, and subcultured at 1 : 3-1 : 4 ratio. The cells were counted by Trypan blue (Biological Industries, Kibbutz Beit Haemek, Israel). The blue staining of cells after mixing (1 : 1) was used as indicator of cell death.

2.4. Flow Cytometry. Undifferentiated rAT-MSCs were subjected to flow cytometry analysis. After passage 3 (P3), stem cells were harvested. Flow cytometry was performed using a FACS Calibur (BD Biosciences, San Jose, CA). Immunophenotyping analysis was performed against the following antigens: CD29, CD45, CD54, CD90, and CD106 (BD Biosciences).

2.5. Immunostaining of rAT-MSCs. For immunofluorescence staining, samples were rinsed briefly in PBS and fixed in ice-cold methanol for 10 min. After permeabilization with 0.025% Triton X-100 (Merck, Darmstadt, Germany), the cells were incubated with 1.5% blocking serum (Santa Cruz Biotechnology, Heidelberg, Germany) in PBS for 30 min at 37°C followed by incubation overnight at 4°C with the primary antibody. After three PBS washes, cells were incubated with secondary antibodies for 25 min. The samples were mounted with mounting medium containing DAPI (Santa Cruz Biotechnology).

Immunohistochemical (IHC) analyses were performed using the streptavidin-peroxidase method (UltraVision Plus Detection System, Thermo Scientific, Chesire UK). Cultured cells were fixed in ice-cold methanol with 0.3% hydrogen peroxide (Carlo Erba) for 15 min. Cells were incubated with Ultra V Block for 5 min and incubated overnight at 4°C with the primary antibodies and for 15 min with secondary antibody at room temperature. After treatment with streptavidin peroxidase for 15 min at room temperature, signals were detected by the AEC kit (Zymed Laboratories, Inc., San Francisco, CA). The cells were counter-stained with hematoxylin (Santa Cruz Biotechnology). The list of primary antibodies was given in Table 1.

2.6. In Vitro Differentiation. To induce adipogenic differentiation, cells were seeded onto 6-well plates (P3; 3000 cells/cm^2) and cultured with Mesencult MSC Basal Medium supplemented with 10% adipogenic supplement (Stem Cell Technologies Inc., Vancouver, BC, Canada) and 1% penicillin/streptomycin for 3 weeks. The medium was refreshed every 2–4 days. Intracellular lipid droplets indicate adipogenic differentiation confirmed by Oil Red O staining (0.5% in methanol; Sigma-Aldrich).

For osteogenic differentiation, cells (P3; 3000 cells/cm^2) were seeded onto collagen I precoated cover slips in 6-well

Table 1: Immunocytochemical properties of rAT-MSCs.

Antibody/marker	Dilution	Source	Detection
CD 26	1 : 50	Santa Cruz Bio.	+
CD 34 (C-18)	1 : 150	Santa Cruz Bio.	Ø
CD 45 (H-230)	1 : 150	Santa Cruz Bio.	Ø
CD 71 (K-20)	1 : 150	Santa Cruz Bio.	Ø
CD105/Endoglin (M-20)	1 : 100	Santa Cruz Bio.	+
c-Fos (4)	1 : 50	Santa Cruz Bio.	+
Collagen II (2B1.5)	Predilute	Thermo Scientific	+
Collagen Ia1 (D-13)	1 : 50	Santa Cruz Bio.	+
β-tubulin	1 : 50	Santa Cruz Bio.	+
Nestin (Rat-401)	1 : 50	Santa Cruz Bio.	+
Vimentin (C-20)	1 : 100	Santa Cruz Bio.	+
Fibronectin (EP5)	1 : 100	Santa Cruz Bio.	+
ASMA	1 : 800	Thermo Scientific	+
Myogenin (F5D)	Predilute	Thermo Scientific	+
MAP 2a, b (AP20)	Predilute	Thermo Scientific	+
GFAP	Predilute	Thermo Scientific	+
Osteocalcin (FL-100)	1 : 50	Santa Cruz Bio.	+
Osteonectin (SPARC)	1 : 50	Millipore	+
Osteopontin (AKm2A1)	1 : 50	Santa Cruz Bio.	+
Ki67	1 : 300	Thermo Scientific	+
PCNA	1 : 200	Thermo Scientific	+
Tenascin-C	1 : 50	Santa Cruz Bio.	+
Cytokeratin 18	1 : 50	Santa Cruz Bio.	Ø

+: positive; Ø: lack of marker expression.

plates. The differentiation medium (MEM supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 0.05 μM ascorbate-2-phosphate (Wako Chemicals, Richmond, VA, USA), 10 mM β-glycerophosphate (Sigma-Aldrich), 1% antibiotic/antimycotic, and 10% FBS) was replaced twice a week. After four weeks, osteogenic differentiation was assessed by Alizarin red staining. For Alizarin red staining, cells were fixed for 5 min in ice-cold 70% ethanol. The cells were stained with Alizarin red solution (2%, pH 4.2) for 30 s. Stained cells were dehydrated in pure acetone, fixed in acetone-xylene (1 : 1) solution, and cleared with xylene.

To induce neurogenic differentiation, cells (P3) seeded on collagen-I-coated cover slips were cultivated until 70% confluency. Cells were cultured for 3–5 days in differentiation medium (MEM supplemented with 0.5 mM isobutylmethylxanthine (IBMX), 10 ng/mL brain-derived neurotrophic factor (BDNF), 10 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast growth factor (bFGF), 20% neural stem cell proliferation supplements (Stem Cell Technologies Inc.) and 1% penicillin-streptomycin). Differentiation was assessed by immunofluorescence staining of GFAP (SC-71141), HNK (SC-49034), Neurofilament (SC-12980), beta-tubulin (SC-9935), beta3-tubulin (SC-69965), c-Fos (SC-52), Nestin (SC-23927), and Eno2 (SC-59538). All antibodies were obtained from Santa Cruz Biotechnology.

2.7. In Vitro Tube Formation Assay. To show the angiogenic potential of rAT-MSCs, tube formation was induced by culturing on Matrigel (Basement Membrane Matrix, LDEV-Free, BD Biosciences, Cat. No. 354234). 100 μL of Matrigel was spread on the prechilled surface of each 24 well of the plate and incubated in standard culture medium (1 : 1) at 37°C for at least 30 min for polymerization. Cells suspended in serum supplemented medium were seeded on Matrigel-coated wells to a final density of 2.5×10^4 cells/cm^2. The formation of tube-like structures was observed after 24 h.

2.8. GFP Labeling of rAT-MSCs. The plasmid was supplied from Clontech (Palo Alto, CA), amplified in *E. coli* strain XL-1, and purified by Endofree PlasmidMaxi kit (Qiagen, Hilden, Germany). GFP coding gene was located downstream of the CMV (murine leukemia virus) constitutive promoter on vector. The cells were transfected by Neon Transfection System (Invitrogen Life Technologies, Carlsbad, CA) with the following parameters: 990 V, 40 ms, and 2 pulses. 2×10^5 cells were mixed with 1 μg plasmid DNA in 10 μL transfer buffer. The transformed cells were cultured in MEM-medium supplied with 15% FBS. After 48 h, the cells were selected for antibiotic resistance toward G418 (400 μg/mL) for 6 weeks. Then, the GFP stability of cells was monitored by continuous culturing for 4 passages, and the number of GFP$^+$ cells was counted in flow cytometer. The integration of GFP gene into genome was checked by Real-Time PCR, and the copy number of integration in chromosome was determined.

2.9. Busulfan Treatment of Rats and Cell Transplantation. For long-term infertility, rats ($n = 24$) were injected with alkylating agent, busulfan (15 mg/kg; Sigma-Aldrich), twice with 14 days of interval to disrupt spermatogenesis. Once every 4 weeks, gonadotropin-releasing hormone (GnRH) agonist, leuprolide acetate (Lucrin, Abbott AS, Istanbul, Turkey), was administered subcutaneously (1.5 mg/rat) for 12 weeks until the animal was analyzed, according to the previous reports [29]. The effectiveness of this process was determined first by measuring testis size and weight. To confirm the effect, testes of 4 rats were removed, fixed in Bouin's fluid (9% formaldehyde, 5% acetic acid, 0.9% picric acid in water) and embedded in paraffin. To evaluate the spermatogenetic activity in tubules by histological analysis, the sections were stained with hematoxylin.

AT-MSCs' suspension was mixed with sterile toluidine blue (1 : 1, v/v). Rete testis was identified by using stereomicroscope (Olympus, KL1500LCD), and these cells were injected into the lumen of the seminiferous tubules of recipient rat testis ($n = 20$), as described before [30]. 100 μL of AT-MSCs' mixture (10^6 cells) was injected into the rete testis of the left testicle (Figure 4) under stereomicroscope (Olympus, SZX7) using FemotoJet semiautomatic microinjector (Brinkmann Instruments Inc., Westbury, NY). The toluidine blue served as a marker to monitor the success of the injection. The untreated right testicle was served as control.

2.10. Analysis of Recipient Rats. Twelve weeks after cell transplantation, the testes of rats ($n = 4$) were analyzed dimensionally. The volume of testis was estimated by Cavalieri's principle. To localize the GFP-tagged rAT-MSCs in testes, rats ($n = 4$) were sacrificed for immune staining. Testis tissue samples were removed, fixed in formalin (10%, pH 7.0–7.6) for 24 h, and embedded in paraffin. Transversal serial sections (4 μm thick) were taken. GFP labeled rAT-MSCs, used for cell tracking after injection, were double-stained on sections for GFP, and antigen of interest. Slides were deparaffinized with two changes of xylene for 5 min each and rehydrated in a series of graded alcohol solutions. Sections were antigen retrieved using a steamer-citrate buffer antigen retrieval method. Endogenous peroxidase was inhibited by incubation with fresh 3% H_2O_2 in PBS buffer. Nonspecific staining was blocked with the mixture of two different serums (with respect to the type of the antibodies used for blotting) in 1.5% PBS for 30 min at room temperature. Afterwards, the sections were incubated in the mixture of two primary antibodies in a pairwise fashion against GFP (SC-9996 or SC-5385) and vimentin (SC-7557), VASA (SC-67185), or SCP1 (SC-20837) for 1 h at RT. The sections were incubated in a mixture of two appropriate fluorescent-conjugated secondary antibodies and were mounted with mounting medium containing DAPI (Santa Cruz).

2.11. Mating the Rats. Female rats ($n = 24$) were mated during the proestrus phase with male rats ($n = 12$) with rAT-MSCs' transplantation. Every male rat was cohabitated with two female rats in polycarbonate cages until evidence of mating, vaginal plug, was observed.

2.12. Detection of GFP Gene in Rat Chromosomal DNA. To determine whether these injected cells contributed in spermatogenesis and support the formation of offspring, GFP gene was traced in the genome of rat offspring. For this purpose, chromosomal DNA was isolated by QIAamp DNA Blood Mini Kit (Qiagen) from the blood samples of rat offspring according to the kit procedures. The GFP gene was amplified first by conventional PCR using the following primer pairs: 5′-cttgttgaattagatggtgatg/5′-ctgttacaaactcaagaaggacc. Template DNA-free PCR reaction was used as negative control. The gene copy number in DNA samples was estimated by real-time PCR using the same primer pairs and Power SYBR Green Master Mix (Applied Biosystems Life Technologies) in amplification reaction. Sox2 gene was amplified as reference for each DNA sample with the following primer pairs: 5′-atgtacaacatgatggagacg/5′-tcacatgtgtgagaggggcagtg. The GFP gene in genome was further confirmed by Southern blot hybridization assay [31]. 5 μg of rat chromosomal DNA was incubated with XbaI and XhoI restriction enzymes (10 U/each; Fermentas, Vilnius, Lithuania) for 16 h at 37°C, blotted on positively charged nylon membrane (Roche) by capillary action, and detected by DIG (digoxigenin) Nucleic Acid Detection Kit (Roche) according to the protocol by manufacturer. Oligonucleotide probe for detection was synthesized by random labeling of GFP gene with DIG-labeled nucleotides. The detection of chemiluminescence was performed by DNR Bio-Imagining Systems (MF-ChemiBIS 3.2, Jerusalem, Israel).

2.13. Detection of GFP Expression in Offspring's rBM-MSCs and rAT-MSCs. To observe the GFP expression in MSCs of offspring, both adipose-tissue- and bone-marrow-derived MSCs were analyzed. rAT-MSCs were isolated and characterized by the methods described previously (Sections 2.3–2.6). rBM-MSCs were isolated with the following procedure. Under sterile conditions, both rat femur and tibiae were excised, and the bone marrow was extruded by flushing with standard culture medium using 21-gauge needle. Marrow plug suspension was dispersed by pipetting, successively filtered through 70 μm mesh nylon filter (BD Biosciences, Bedford, MA), and centrifuged at 200 xg for 10 min. The bone marrow was diluted to 1 : 3 with PBS and layered over a Ficoll-histopaque gradient (1.077 g/mL, Sigma). The low-density mononuclear cells were collected, washed twice with PBS, counted, and plated in tissue culture flasks at a density of 1.4×10^5 cells/cm^2 in standard culture medium. The MSCs were isolated based on their ability to adhere on plastic. By replacing the culture medium with fresh medium, the unattached cells were removed from the culture, and the culture was allowed to grow further. Cells at 70–80% confluency were harvested with 0.25% trypsin-EDTA solution after washing with Ca^{2+}-Mg^{2+}-free PBS. Both rBM-MSCs and rAT-MSCs from offspring were cultured to passage 2 (P2), and immunofluorescence staining was performed as previously described in Section 2.5. To detect the GFP expression in stem cells, anti-GFP antibody was used.

Both rBM-MSCs and rAT-MSCs from offspring were cultured to passage 2 (P2), and immunofluorescence staining

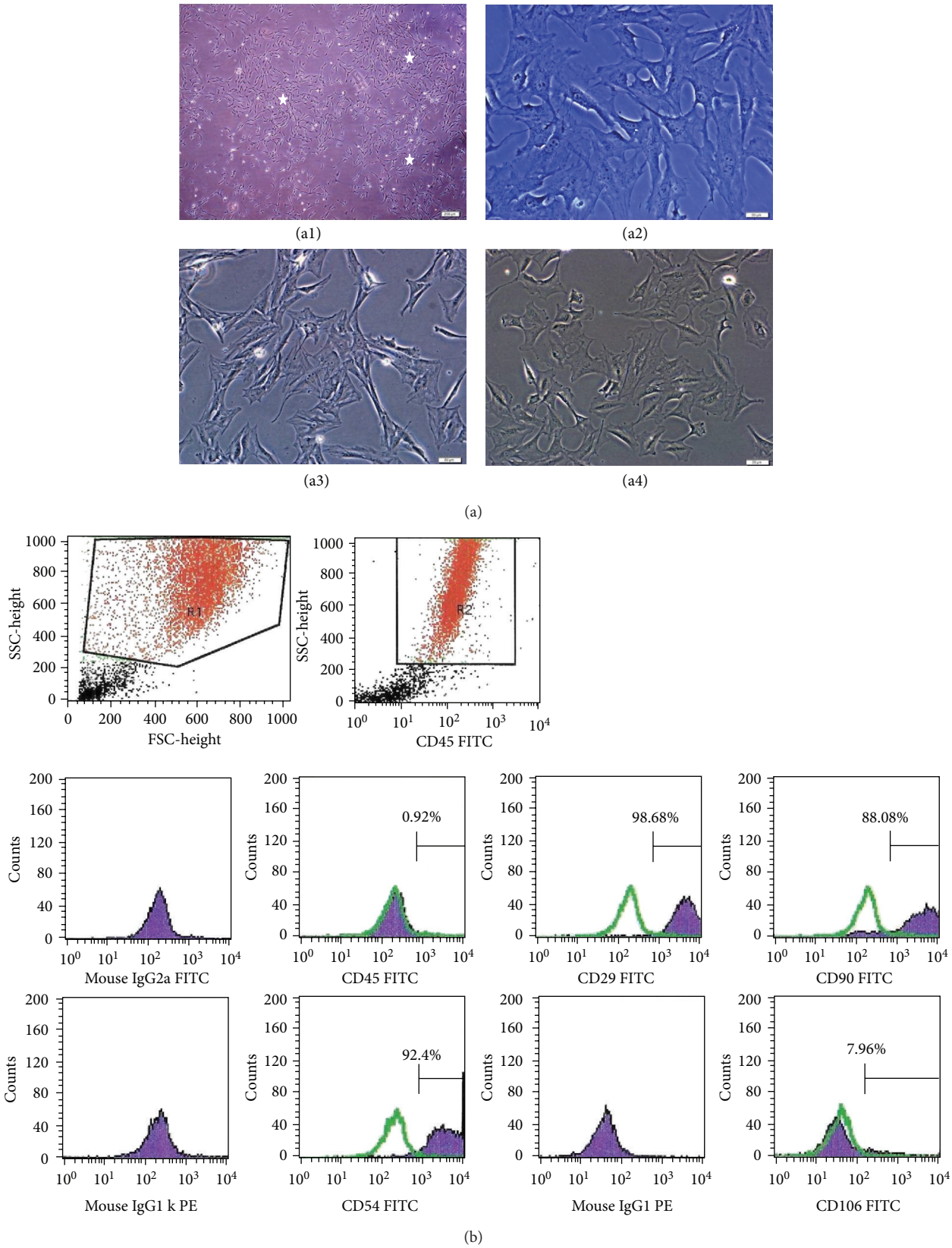


Figure 1: Morphological and phenotypic characteristics of rAT-MSCs. During the onset of culture (a1: P0-5th day), the isolated cells from rat adipose tissue formed single-cell-derived colonies (arrows). After the next days and passages, most of these SCs exhibited large, flattened or fibroblast-like morphology (a2: P0-7th day, a3: P1-2th day, and a4: P3-1th day). (b) A representative flow cytometry analysis of cell-surface markers of rAT-MSCs at P3; cells were labeled with antibodies against hematopoietic (CD45) and MSC markers (CD29, CD54 and CD90) and with vascular cell adhesion protein 1 (CD106). (green line: histogram of isotype control immunoglobulin).

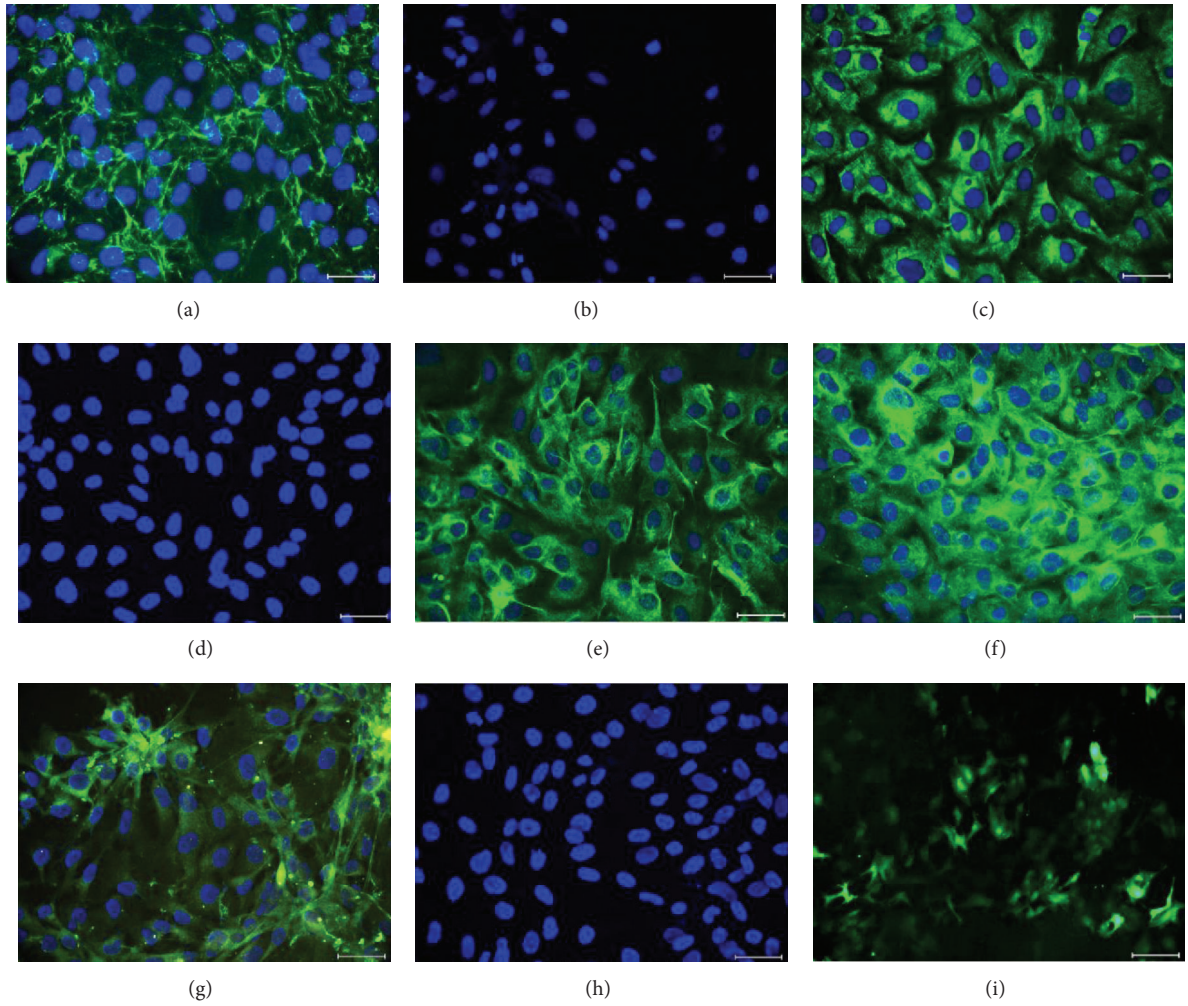


Figure 2: Immunofluorescence staining of rAT-MSCs for fibronectin (a), CD34 (b), GFAP (c), CD45 (d), Nestin (e), Vimentin (f), Map2a, b (g), and Cytokeratin 18 (h). Staining pattern was cytoplasmic for fibronectin, GFAP, nestin, and vimentin; and both membranous and cytoplasmic for Map2a, b. After transfection, rAT-MSCs showed GFP^+ immunostaining (i). Nuclei were labeled with DAPI (blue). Scale bars: 50 μm.

was performed as previously described. To detect the GFP expression in stem cells, anti-GFP antibody was used.

To show the GFP^+ sperms, the testes of offspring (1st generation) were removed and cut in small sizes in Ca^{2+}-Mg^{2+}-free Hank's Balanced Salt Solution (HBSS with, Gibco Life Technologies) with 0.35 g/L sodium bicarbonate and 1 g/L D-glucose. Large tissue particles were removed, and sperm cells were collected on glass slides in the cytocentrifuge (1500 rpm, 10 min). Slides were stained with antibody to GFP (Santa Cruz, sc-5385). The nuclei were labeled with DAPI.

2.14. Statistical Analysis. A computer program (SPSS 10.0) was used for statistical analysis. The results were expressed as means ± standard deviation (SD). Two-tailed paired t-test was used for the comparison of the groups. Differences between the groups were considered as statistically significant when $P < 0.05$ and highly significant when $P < 0.01$.

3. Results

3.1. Culture of rAT-MSC. MSCs attached to the culture flasks sparsely and displayed a fibroblast-like, spindle-shaped morphology during the initial days of incubation. Following 3-4 days of incubation, proliferation started and the cells gradually grew into small colonies. During culture, adjacent colonies interconnected with each other, and a monolayer confluence was obtained after 12–15 days of incubation. In later passages, MSCs exhibited large, flattened fibroblast-like morphology (Figures 1(a1)–1(a4)) and did not change throughout 25 passages. Tests for bacterial and mycoplasma contamination were negative. The viability of cells was higher than 95%, determined by Trypan blue staining of cells. rAT-MSCs expressed CD29, CD54, and CD90, but not CD45 and CD106 (Figure 1(b)) and maintained their phenotype in the following passages.

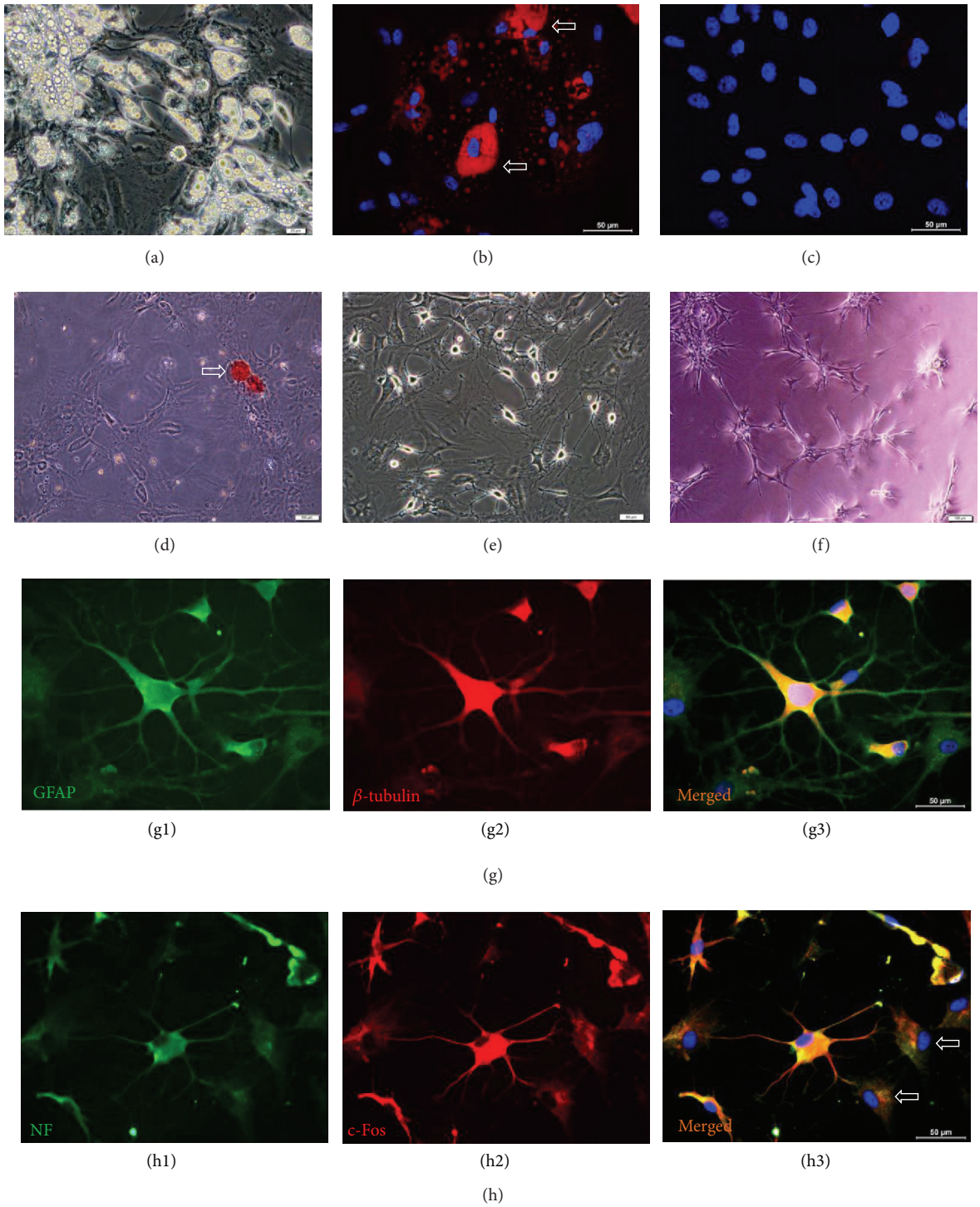


Figure 3: Microscopic images of rAT-MSCs differentiated into adipocytes (a, b), osteoblasts (d), neuron-glial like cells (e), and endothelial cells (f). The arrows indicate the neutral lipid vacuoles stained with Oil Red O (b). rAT-MSCs were undifferentiated in standard culture medium (c). Phase contrast microscope images of the rAT-MSCs differentiated into osteogenic lineage, where calcified nodules (arrow) were stained with Alizarin red S (d). Differentiation of rAT-MSCs to neuron-glial like cells after 3 days (e). Endothelial tube formation by rAT-MSCs on Matrigel (f). Immunostaining of cells for GFAP (green) and beta-tubulin (red) differentiated cells (g1–g3). Increased cytoplasmic and nuclear staining of differentiated cells for c-Fos (red) and NF (green; cytoplasmic and membranous) was observed (h1–h3).

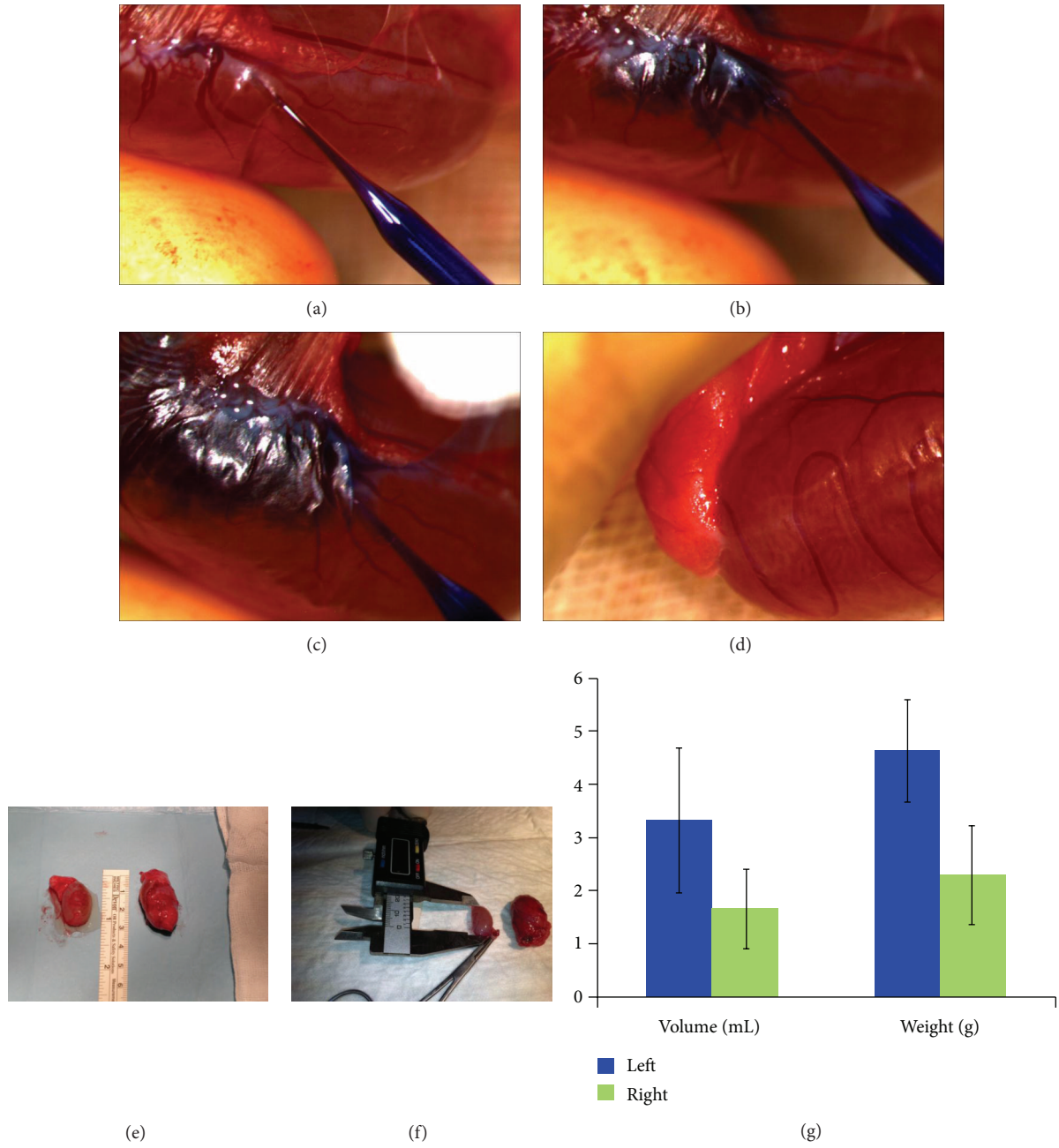


FIGURE 4: Cell transplantation into rete testis and morphological analyses of testes after 12 weeks. 1×10^6 cells were mixed with toluidine blue (1 : 1, v/v), and the mixture was injected into the rete testis of the left testicle of rats (a–c). The right testicle of rats was left untreated after busulfan (d). After 12 weeks, the atrophy in right testicles (f) was significant, when compared with the left testicles with rAT-MSCs treatment (e). There were significant differences in both volume and weight of testicles: $P = 0.0074$ and $P = 0.0015$, respectively (g).

3.2. Immunostaining of rAT-MSCs. Immunohistochemical studies were performed to characterize the progeny of the rAT-MSCs by using antibodies specific to known antigens of MSCs. Immune reactivity profile for rAT-MSCs was shown in Table 1. Under standard culture conditions, fibronectin (Figure 2(a)), GFAP (Figure 2(c)), Nestin (Figure 2(e)), Vimentin (Figure 2(f)), Map2a, b (Figure 2(g)), CD105, Collagen type-I, Collagen type-II, beta-tubulin, ASMA, Myogenin, Osteopontin, Osteocalcin, Osteonectin, Ki67, PCNA, and Tenascin (data not shown) were expressed. Surface markers including CD34 (Figure 2(b)), CD45 (Figure 2(d)), Cytokeratin 18 (Figure 2(h)), and CD71 (Table 1) were not expressed by rAT-MSCs.

3.3. Differentiation Potential of rAT-MSCs. rAT-MSCs (P3) were differentiated within 3 weeks in the adipogenic differentiation medium. In cells, lipid droplets enlarged and invaded

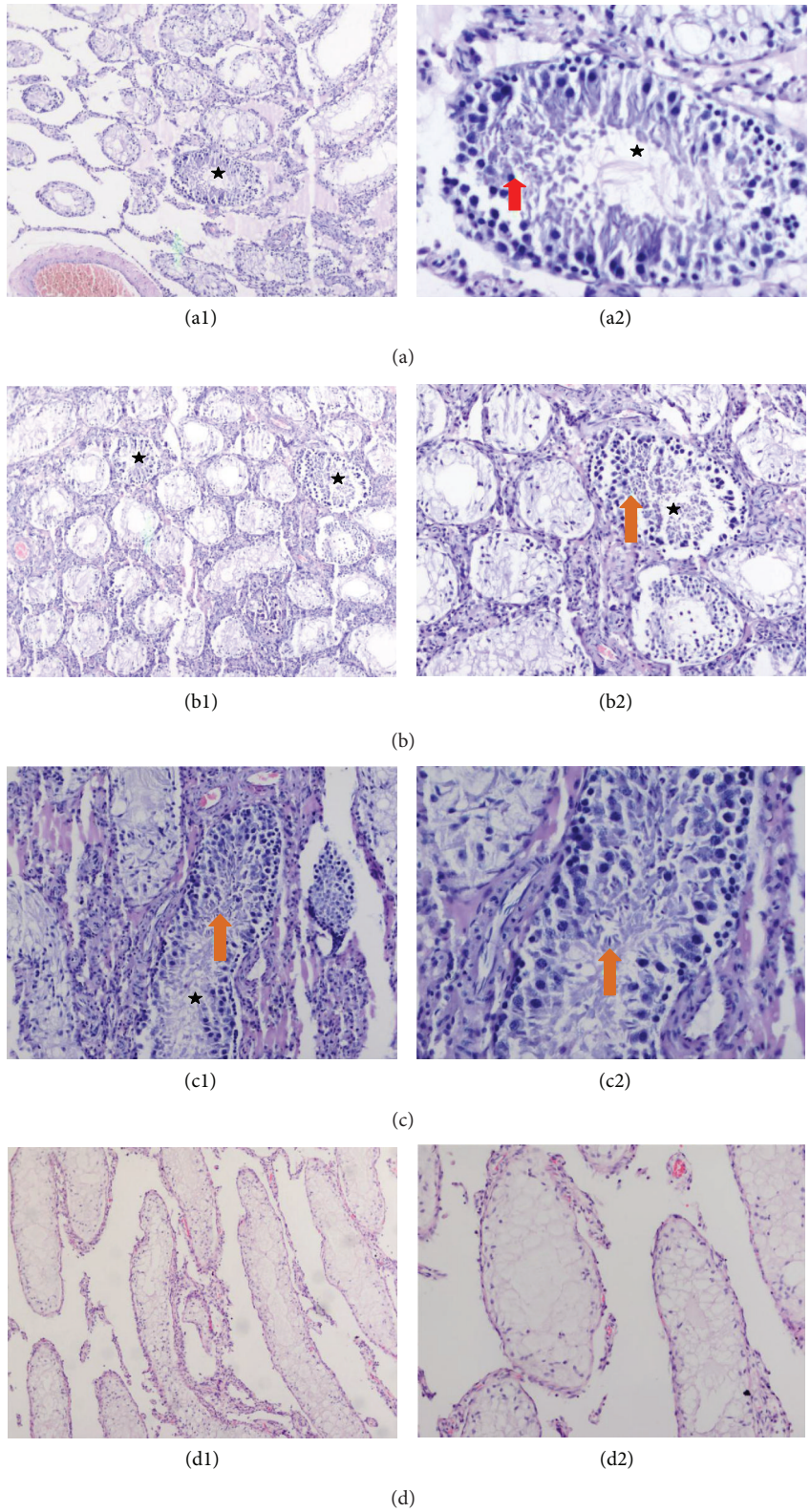


Figure 5: Sections of treated testes with rAT-MSCs. Most of the seminiferous tubules were empty indicating the absence of spermatogenesis. Only a few tubules appeared to be filled with spermatogenetic cells up (asterisks). Spermatogenetic cells were shown in these tubules (red arrow) (a2). Spermatozoa were also shown with arrows (orange arrows) (b1, b2, and c2). Sections of busulfan-treated testis (right), which are not transplanted with stem cells, demonstrate empty seminiferous tubules that indicate no spermatogenetic activity (d1, d2). The higher magnification of the slides (a1, b1, c1, and d1) was shown on the right of the same sections (a2, b2, c2, and d2). Original magnifications: a1, b1, d1-X48, c1, b2, d1-X100, a2, c2-X200.

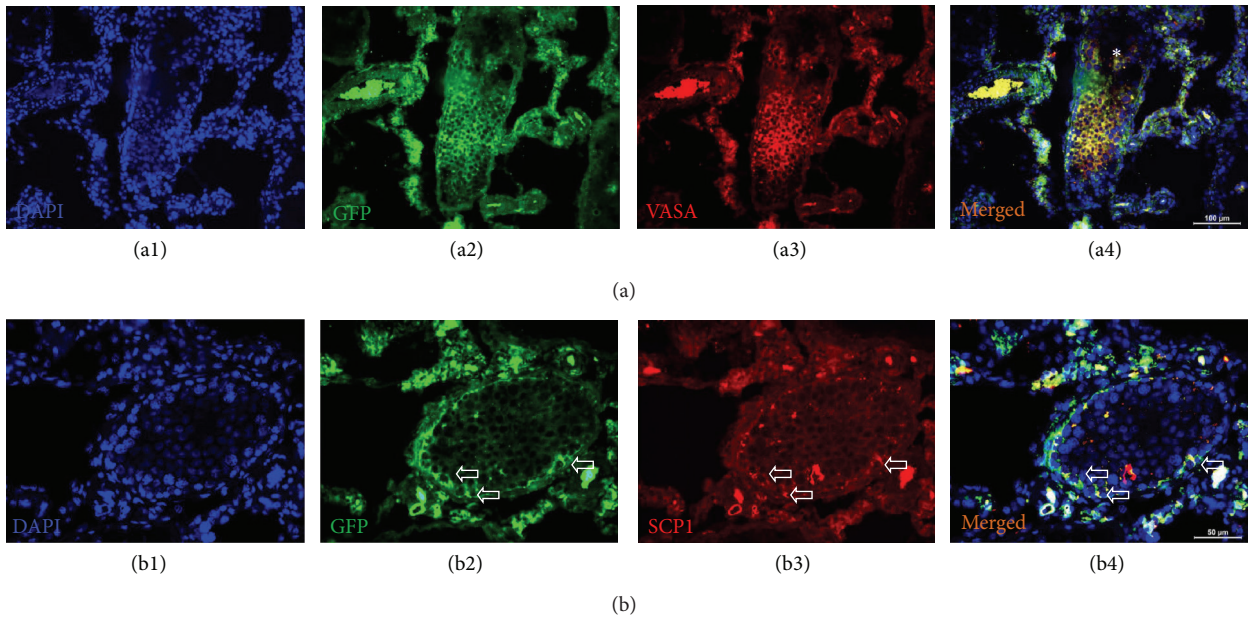


Figure 6: Immunostaining of GFP$^+$/VASA$^+$ and GFP$^+$/SCP1$^+$ cells in busulfan-treated testis with rAT-MSCs injection. GFP$^+$/VASA$^+$ cells were localized in the seminiferous tubules of testis (white star), but most of the tubules were empty (blue asterisk) (a1–a4). The expression of meiosis marker SCP1 in GFP$^+$ cells might indicate the transdifferentiation of MSCs into spermatogenic cells (white arrows) (b1–b4).

the entire cytoplasm (Figures 3(a) and 3(b)). The lipid droplet formation was not observed in undifferentiated rAT-MSCs (Figure 3(c)).

In the osteogenic differentiation, cells proliferated and reached almost complete confluency after 8–10 days of incubation. Later, the cellular aggregates were observed in differentiated cultures and the gradually increased. The aggregates were characterized by the presence of amorphous material deposits. These nodular aggregates in osteogenic cultures were stained with Alizarin red S after 28 days, demonstrating that the amorphous deposits were actually calcium deposits (Figure 3(d)).

rAT-MSC-derived neuron-like cells displayed distinct morphologies, ranging from extensively simple bipolar to large, branched multipolar cells (Figure 3(e)). Within 24 h, these stem cells formed tube-like structures after culturing on Matrigel (Figure 3(f)). For characterizing their neuronal character further, differentiated rAT-MSCs were stained for neuron and glial cell specific markers including GFAP (Figures 3(g1) and 3(g3)), beta-Tubulin (Figures 3(g2) and 3(g3)), Neurofilament (Figures 3(h1) and 3(h3)), c-Fos (Figures 3(h2) and 3(h3)), Map-2a,b, beta3-Tubulin, Eno2, and HNK (data not shown).

3.4. Testicular Size and Spermatogenesis. The difference in dimensions of the left (treated with rAT-MSCs) and the right testicles (without cell transplantation) were analyzed, and mass increase of almost 50% was measured in testes with rAT-MSCs (Figures 4(e), 4(f), and 4(g)). It was observed that the average volume of the testicles with rAT-MSCs was higher than the ones with no transplantation and atrophy was not seen in testicles with rAT-MSCs injection (Figures 4(e) and 4(f)). The volume increase of busulfan-treated testes was only observed significantly in the test subjects with MSCs transplantation (Figure 4(g)). A significant increase of size in other interstitial tissues was not noticed.

3.5. Histological Assessment of Spermatogenesis. Formalin-fixed and paraffin-embedded testis tissue sections were stained with hematoxylin-eosin. These samples from stem-cell-injected tissues were examined under light microscope for any spermatogenic activity. After generating the infertility in rat with double injection of 15 mg/kg of busulfan, the testes of the control animals were thoroughly analyzed for any spontaneous recovery of spermatogenesis, and no sign was observed for reinitiated spermatogenesis. The scanning of sections showed atrophy, complete, and incomplete spermatocytic arrest and Sertoli cell-only appearance for samples without rAT-MSCs. After the treatment with busulfan, spermatogenesis process was blocked (Figures 5(d1) and 5(d2)). However, the presence of spermatogonium in the tissues with stem cell transplantation was noted (Figures 5(a1)–5(c2)). The seminiferous tubules of the controls, which were not treated with MSCs, were empty and indicated the disruption of spermatogenesis (Figures 5(d1) and 5(d2)). On the other hand, the tubules appeared to be filled up with spermatogenetic cells in the sections of cell-treated tissues, but with low rate. Spermatozoa were observed (Figures 5(a1)–5(c2)).

3.6. Detection of Spermatogenic Cells and Markers for Meiosis. The rAT-MSC-injected testis sections were positive for both GFP and VASA (Figures 6(a1)–6(a4) and 7). VASA-positive staining pointed to the presence of spermatogenic cells in

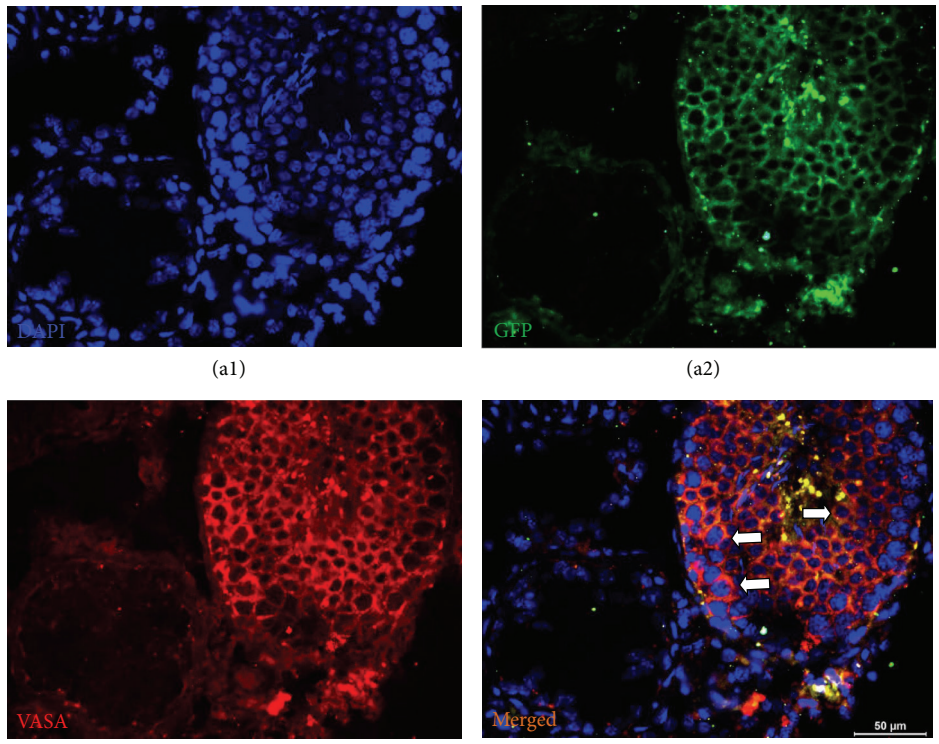
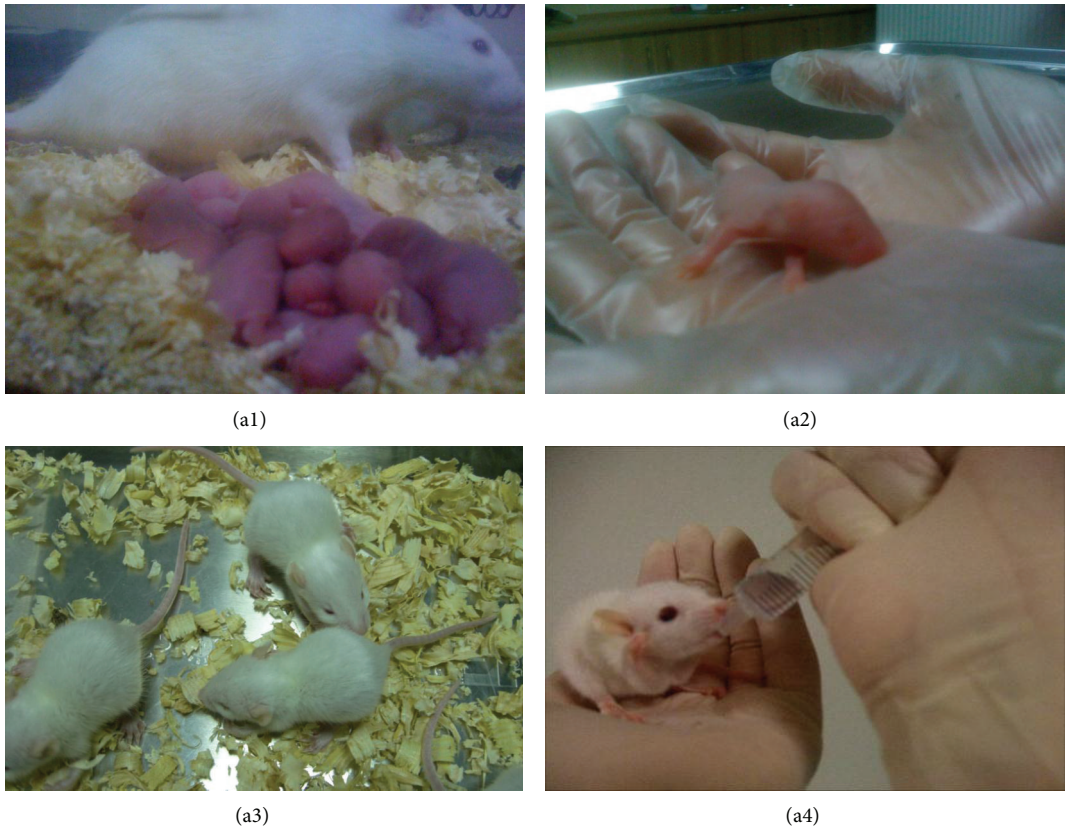


Figure 7: GFP$^+$ cells in seminiferous tubule. MSC-received testis was fixed and stained for GFP (green) and spermatogenic cell marker, VASA (red). The cells in seminiferous tubule with dual staining were shown by arrows (a4). The adjacent tubules showed no staining for VASA, which indicated the absence of spermatogenic activity.

tubules, and the GFP staining designated the origin of cells to be the rAT-MSCs used in transplantation. Interestingly, not all GFP$^+$ cells were expressing VASA (germ cell marker). However, the GFP$^+$ and VASA$^+$ cells (yellow) might indicated that those cells possibly underwent in the sperm formation process in tubules. The expression of meiosis marker, SCP1, also supported the evidence of participation of rAT-MSC in spermatogenesis (Figures 6(b1)–6(b4)). Both markers were not expressed in the undifferentiated state of rAT-MSC in cell culture *in vitro* (see in Supplementary Material available on line at http://dx.doi.org/10.1155/2013/529589. Data 1).

3.7. Restoration of Male Fertility and Birth of Offspring. After mating and successful pregnancy periods following pairing of female rats with male rats with GFP-labeled rAT-MSCs, 9 viable offspring were obtained (Figures 8(a1)–8(a4)). These male rats were further mated with other females, and the preserved fertility was observed in stem-cell-transplanted animals by obtaining the next offspring. To determine whether the offspring were originated from stem-cell-treated males' spermatozoa, 0.4 mL of blood of each animal was collected for DNA isolation and detection of GFP gene in genome. GFP gene was detected by PCR and Southern blot hybridization (Figures 9(a) and 9(b)). The fragment of expected size (634 bp) was amplified in conventional PCR. Exogenous gene integration into genome of offspring was also shown by Southern blot hybridization for GFP (Figure 9(b)). Because the parental rats lack the GFP gene, the only possible origin of this foreign gene in offspring would be the GFP gene used to label rAT-MSCs prior to the injection in testes. The copy number of GFP gene was estimated to be equal to the reference gene, Sox2, by real-time PCR. The gene copy was also estimated in rAT-MSCs before transplantation to be the same as Sox2 gene (Figure 9(c)). The MSCs were isolated from the bone marrow and adipose tissue of the offspring (Figures 10 and 11). The immunostaining of those cells with GFP antibody gave positive result, meaning that both types of MSCs inherited their GFP gene from the paternal rats and were functionally expressed in their cytoplasm like the rAT-MSCs injected in testes. The location of the GFP expression was observed in cytoplasm, but very close to the nuclei. The same staining pattern was also observed in the cell culture of GFP$^+$ rAT-MSCs (see Supplementary Data 2). The only possible source of this nonmammalian gene could be the GFP labeled rAT-MSCs, injected in infertile male rats, it could be considered as a significant evidence for the maturation of functional sperms from injected rAT-MSCs. In the analysis of GFP$^+$ cells, MSCs were deliberately selected because of the complexity of mammalian gene expression regulation and being aware of GFP was previously expressed in MSCs. In addition, the presence of GFP was also detected in the sperms of offspring (see Supplementary Data 3). The sperm cells were stained with GFP antibody to increase the intensity of



(a1) (a2)

(a3) (a4)

Figure 8: Newborns (offspring) after pairing healthy females with rAT-MSC-injected males with busulfan-treated testes. Offspring after birth (a1-a2) and after 10 days (a3-a4).

green light and their nuclei were shown by DAPI. The sperm cells were observed with GFP accumulation overlying the nuclei. This transgenic rat line has been propagated to the third generation by inbreeding without silencing of transgene expression.

4. Discussion

The development of stem-cell-based therapies currently represents one of the major challenges of medical research. Adipose-tissue-derived mesenchymal stromal cells could be obtained from the stromal vascular fraction [32] under *in vitro* culture conditions. These cells are characterized by fibroblast-like morphology, adherence to plastic surfaces, continuous cell proliferation over long culture periods and multilineage differentiation capacity. They could transdifferentiate into variety tissue cells including endothelial, epithelial, muscle, Schwann cells, hepatocytes and neurons *in vitro* [15, 32–42].

In infertility and sterility, stem cell therapy promises to be a potential source of male and female germ cells. Not only ESCs but also fetal porcine skin stem cells, human fetal lung-MSCs, bone marrow, and umbilical cord MSCs were the candidate for the germ cell differentiation *in vitro* [20, 23, 25, 28, 43–46]. On the other hand, stem cells experimentally derived from bone marrow have been recently used in experimental busulfan-treated infertility rodent models. Nayernia et al. [23, 24] showed for the first time that murine BM-MSCs could differentiate into male germ cells. Yazawa et al. [47] proved that MSCs have the capacity to differentiate into steroidogenic cells, such as Leydig cells, both *in vivo* and *in vitro* [46]. More recently, Lue et al. [26] showed that BM-MSCs, transplanted into testis of a busulfan-treated infertility mouse model, appeared to differentiate into germ cells, Sertoli cells, and Leydig cells [26]. This finding raises the possibility of using MSCs to treat male infertility and testosterone deficiency. The general consensus on AT- and BM-MSC is that they are virtually identical in cell surface marker profile, gene expression profile, and differentiation potential [12]. This issue has been confirmed in a preclinical study in which both cells were found equally effective in treating a porcine model of cardiac infarction [17]. Whereas bone marrow could only be obtained in limited quantity, the adipose tissue is usually available in abundance. Therefore, the difference in the clinical application potential of AT-MSC and BM-MSC is quite obvious [1]. Numerous studies conducted to date have indicated that stem cells derived from adult human tissues could be reprogrammed to differentiate into different cell types. However, no progress or evidence has been reported so far in the isolation and

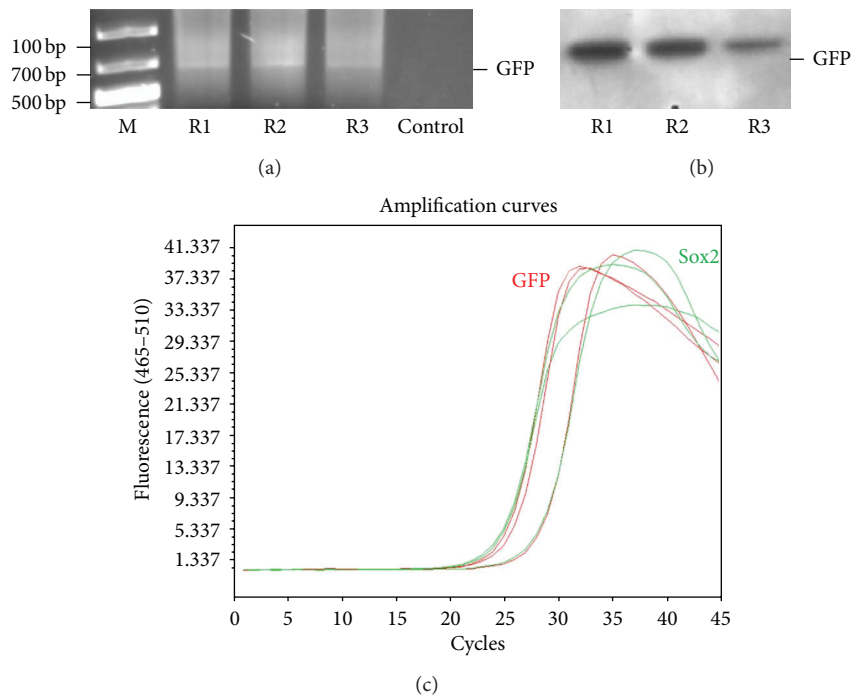
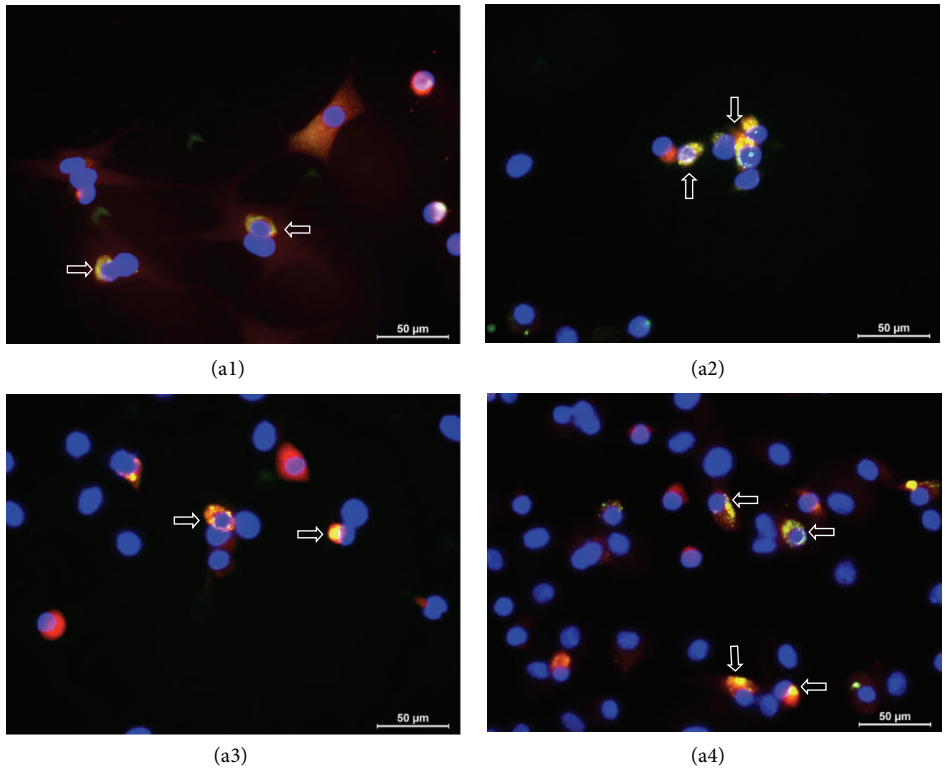


Figure 9: Representative analysis of chromosomal DNA for exogenous gene (GFP) insertion for three offspring (R1–R3). DNA isolated from blood samples were analyzed for GFP gene by PCR amplification (a) and southern blot hybridization (b). Gene copy number of GFP and Sox2 in genome was estimated to be same by Real-Time PCR (c). The GFP gene in chromosomal DNA of rat supported the evidence for transdifferentiation of MSCs into functional sperms.

characterization of AT-MSCs to differentiate into functional germ cells. This study showed the differentiation potential of clonally expanded MSCs into germ cells or sperm-like cells. The transplantation of AT-MSCs was successfully achieved into testis through rete testis of busulfan-treated infertility rat models. Later, there was observed the recovery of the fertile status of those males. The analyses also supported the evidences of the functional spermatogenesis progress in testes.

The expression of GFP and the meiosis marker, SCP1, by these cells in tubules might indicate the involvement of rAT-MSC in spermatogenesis. The function of MSCs in spermatogenesis might be direct (transdifferentiation), or they interact with niche of testis tissue. Reprogramming might be achieved by changing the cellular microenvironment (niche), in which the cells grow, to provide signals that might activate appropriate metabolic pathways. Successful experiments were performed *in vivo* by using the microenvironment of the target cell type. When the conditions were provided similar to the *in vivo* microenvironment, differentiation of stem cells into the targeted cell types with full functionality could be obtained also *in vitro* [48–58]. In the study by Kim et al., it was indicated that the niche might include some factors that promote the genetic and epigenetic status of stem cell self-renewal *in vitro* [27]. Most importantly the niche was pointed out for serving as a cellular platform for the expansion of stem cells that could potentially be exploited for therapeutic tissue/organ neogenesis.

The entire process of spermatogenesis from spermatogonial stem cells (SSCs) to spermatozoa takes place in the seminiferous tubules. SSCs involve into regeneration and maintenance of spermatogenesis and are located in the basal compartment, also recognized as niche. This site was defined as specialized microenvironment necessary for maintenance of stem cells. This concept was first proposed by Schofield in 1978 for the hematopoietic cell system [59]. According to this hypothesis, stem cells cannot survive long enough to function as SSCs outside of the basal compartment niche. Therefore, a stem cell needs this niche to proliferate in controlled manner and to execute its role in the body. There might be several important players in SSC niche formation: Sertoli cells, the basement membrane, peritubular myoid cells, and undefined signals external to the seminiferous tubules. Sertoli cells might be the most important component, as they provide growth factors for SSCs and have been described to insinuate themselves between all of the neighboring germ cells, leaving very few regions with evident contact between germ cells. Recent studies have demonstrated that Sertoli-cell-derived growth factor, glial-cell-line-derived neurotrophic factor, plays a key role to promote SSC survival and self-renewal, thereby stimulating SSC proliferation *in vivo* and *in vitro* [60–64]. rAT-MSCs, injected in the testes, were localized both in and out of the seminiferous tubules, from which SSCs were removed by busulfan treatment. As soon as the migrated MSCs had contact with the niche in the seminiferous tubules and with the cytokines secreted by



(a1) (a2)

(a3) (a4)

Figure 10: GFP$^+$/Vimentin$^+$ mesenchymal stem cells derived from bone marrow (P0) from offspring. rBM-MSCs were isolated from the offspring (a1–a4), and some vimentin- (red) positive cells showed the GFP (green) expression (arrows). The expression of nonmammalian gene GFP in rBM-MSCs indicates the transdifferentiation of GFP-labeled rAT-MSCs in testes.

the Sertoli cells, they might transdifferentiated into SSCs and initiate the spermatogenesis. This specific microenvironment could provide a unique and excellent condition for meiosis and functional sperm formation to MSCs. Therefore, the rAT-MSCs demonstrated a better differentiation into functional sperm compared to the *in vitro* differentiation studies.

The main handicap of the busulfan treatment in rat models was the possibility of severe destruction of niche, especially Sertoli cells in testes. This damage could be the explanation of why the spermatogenesis was not started in all seminiferous tubules, but the activity was limited only in a couple of tubules. The destruction of Sertoli cells by busulfan treatment caused the damaged of niche, such that it did not have the capacity to support the transdifferentiation of migrated rAT-MSCs anymore. It was considered that the niche might play a vital role in differentiation of rAT-MSCs into functional sperm.

The differentiation of insufficient number of cells and the high number of cells in the phase of incomplete meiosis are considered the two main drawbacks of germ-like cell derivation of stem cells [64]. In those studies, small number of cells after busulfan treatment was positive for specific germ cell markers, like VASA and SCPs. The differentiation took place not in all but in some tubules. In our study, the busulfan treatment was proven to be effective in elimination of cells in tubules, but the overexposure might also cause the damage of niche. The spermatogenesis activity was only observed to start in a few tubules, but it was sufficient to initiate successful pregnancy.

The male rats recovered their fertility after receiving the injection of AT-MSCs. The stem cells could affect either by maintaining the preexisting SSCs leading to reinitiate the spermatogenesis or by transdifferentiation into SSC-like cells to form spermatocytes. To explain this event, the AT-MSCs were transferred with GFP gene before injection in testes, and the integrity of this gene into genome was assured. Following mating and pregnancy, the first generation of offspring was obtained. The sperms recovered from the offspring were GFP$^+$. MSCs isolated from the tissues of offspring were analyzed, and GFP was shown to be expressed in cells by immunostaining. Although this exogenous gene was integrated into genomic DNA, GFP expression was not observed in all cells of offspring. The GFP gene was transferred for reporting purpose and not for generating a transgenic animal. The vector was designed to express GFP gene in cells under the control of CMV promoter. Hypermethylation of the viral promoter sequences by de novo DNA methylation in host cells might cause silencing of the transgene in some cells and in next generations [65]. But the important point is that the only origin of GFP could be the rAT-MSCs injected into the testes. The results indicated that these cells transdifferentiated into functional sperm in seminiferous tubules, and these sperms later involved in delivery of GFP gene to offspring genome. As the sperm formation was not observed *in vitro*

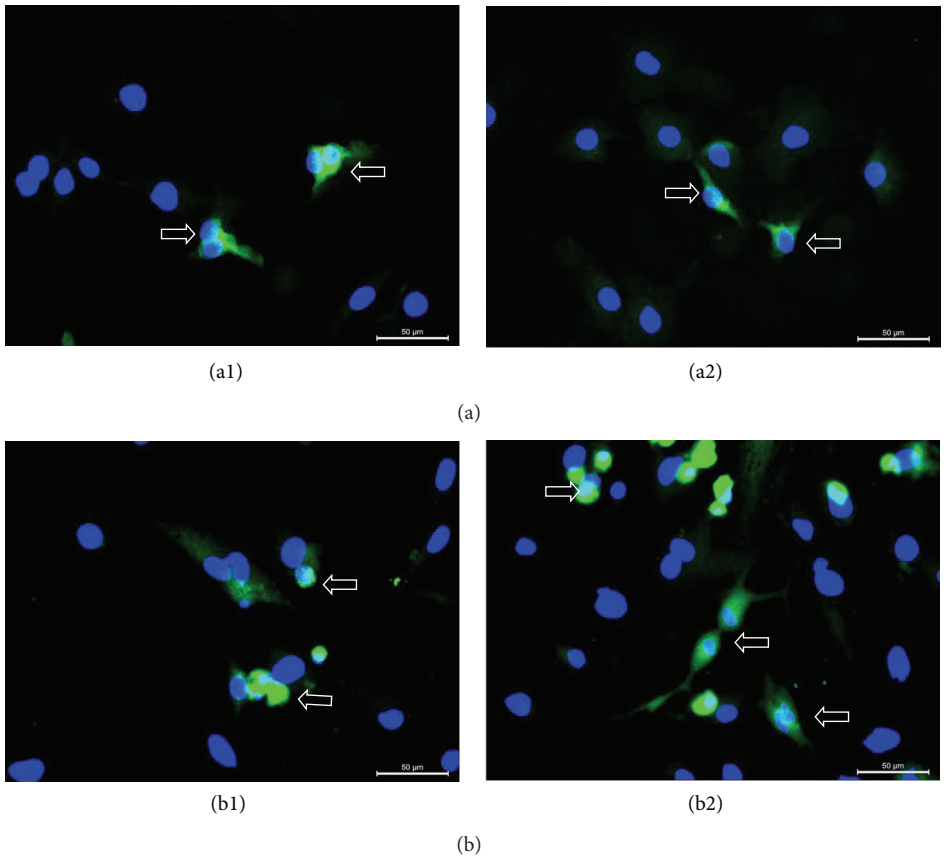


Figure 11: GFP$^+$ rBM- and rAT-MSCs (P2) derived from offspring. MSCs were isolated and characterized from the bone marrow (a1-a2) and adipose tissue (b1-b2) of offspring. The GFP-gene could only be originated from the paternal rat injected with GFP labeled rAT-MSCs.

and also in severely damaged tubules, it might point to the significance of the well-preserved niche for the differentiation MSCs into sperm. There is also another possibility, which should be taken into account, that the MSCs had the characteristics to fuse with other cells spontaneously [66]. From the early studies with MSCs, it was well known that these cells could fuse with somatic cells, albeit rarely. It was found that the frequency of fusion between MSCs and differentiated cells increased in the presence of TNF-alpha and/or IFN-gamma [67]. The apoptosis was induced in testes by busulfan, and these cytokines were highly expressed, consequently. The increase in the fusion events was expected in testes. It should be noticed that there was a period of 12 weeks between the busulfan treatment and MSCs injection, and the inflammatory factors were reduced during this period. For spermatogenesis activity, MSCs should form hybrid cell with existing SSCs. However, the severe damage by busulfan eliminated almost all SSCs in tubules, and the only cells left were Sertoli cells. Although they are very important for niche, they could not have the capacity for spermatogenesis. Therefore, the fusion could not have played a significant role in the recovery of infertility.

On the other hand, the expression of pluripotency markers by rAT-MSCs might have an effect on sperm generation. Although the mesenchymal stem cells are generally classified as multipotent with respect to their differentiation capacity, the expression of these pluripotency markers could enhanced their differentiation potential into various tissues [68]. Obviously, it was not enough for sperm generation from MSCs in cell culture. Some additional factors might be required that could be provided by niche. The expression levels of some pluripotency genes (Oct4, Sox2, Rex1, and FoxD3) were analyzed for the same rAT-MSC line (unpublished data). The expression for Sox2 and FoxD3 could not be detected, but there was relatively strong expression of Rex1 and weak expression of Oct4. The expression of Rex1 is known to preserve the undifferentiated state of stem cells. This gene was also suggested to be important in spermatocytes, and it might play a role in meiosis [69]. Beside the effect of niche, the intrinsic factors, like expression of Rex1, might also have direct or indirect effects on sperm generation.

In conclusion, the fertile status of busulfan treated-male rats was recovered by rAT-MSCs transplantation in this study. The GFP$^+$ cells were found both outside of the basal compartment and in the seminiferous tubules, supporting the idea that MSCs might have functioned in reestablishment of spermatogenesis by two ways: MSCs' differentiation into sperm, or maintenance of SSCs. These results showed the rAT-MSCs could be both rich and functional source for the infertility treatment. The most important issue of this

study was the achievement of first successful results in spermatogenesis by endogen reprogramming using adult stem cells. If this protocol was also proven to be functional in human, the possibility to treat the males with azoospermia would arouse.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Very Small Embryonic-Like Stem Cells: Implications in Reproductive Biology

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The most primitive germ cells in adult mammalian testis are the spermatogonial stem cells (SSCs) whereas primordial follicles (PFs) are considered the fundamental functional unit in ovary. However, this central dogma has recently been modified with the identification of a novel population of very small embryonic-like stem cells (VSELs) in the adult mammalian gonads. These stem cells are more primitive to SSCs and are also implicated during postnatal ovarian neo-oogenesis and primordial follicle assembly. VSELs are pluripotent in nature and characterized by nuclear Oct-4A, cell surface SSEA-4, and other pluripotent markers like Nanog, Sox2, and TERT. VSELs are considered to be the descendants of epiblast stem cells and possibly the primordial germ cells that persist into adulthood and undergo asymmetric cell division to replenish the gonadal germ cells throughout life. Elucidation of their role during infertility, endometrial repair, superovulation, and pathogenesis of various reproductive diseases like PCOS, endometriosis, cancer, and so on needs to be addressed. Hence, a detailed review of current understanding of VSEL biology is pertinent, which will hopefully open up new avenues for research to better understand various reproductive processes and cancers. It will also be relevant for future regenerative medicine, translational research, and clinical applications in human reproduction.

1. Introduction

Stem cells have the capacity to self-renew as well as give rise to differentiated progeny. They have generated a lot of interest amongst the general public as well as the scientific fraternity because of their potential for regenerative medicine. Although this field of research has been associated with a lot of hype, it definitely holds a lot of hope when applied to reproductive health. Considerable research has gone into the differentiation of embryonic stem cells [1, 2] and even induced pluripotent stem cells [3] to generate synthetic gametes. The idea of generating gametes *in vitro* has tremendous applications in treatment of infertility and understanding gametogenesis and also as a source of gametes for therapeutic cloning and regenerative medicine. However, although male gametes generated from mouse embryonic stem cells *in vitro* resulted in the birth of pups, most of them suffered epigenetic defects [4]. Similar issues may surface when stem cells isolated from ovaries of reproductive age women [5] are used to generate oocytes. It appears to be a major shortcoming and one wonders if this research will find translation in the clinics. Other applications of stem cells in the field of reproductive health have also been reviewed including the treatment of reproductive diseases [6].

Recently few groups have succeeded in deriving pluripotent ES-like cultures using adult testicular biopsies of mice [7–9] and humans [10–13]. These pluripotent stem cells are autologous, embryo-free, patient-specific, and potentially safe for regenerative medicine with no associated sensitive ethical issues as compared to embryonic stem cells. Emerging literature suggests that it may be possible to derive similar ES-like cultures from ovarian tissues of mice [14], humans [15, 16], and other higher mammalian species including rabbits, monkeys, and sheep [17]. Zou et al. [18] successfully cultured female germline stem cells derived from both neonatal and adult ovary for several months *in vitro,* which when transplanted in busulfan treated mice led to the birth of normal pups. This demonstrated supremacy of the gonadal stem cells differentiated by the *in vivo* cues over *in vitro* manipulated ES cells to generate synthetic gametes. White et al. [5] recently showed that DDX4 expressing cells isolated from adult mouse and reproductive age women can be

used to generate oocytes *in vitro* as well as *in vivo* after xenotransplantation in immunodeficient mice.

It was postulated that spermatogonial stem cells (SSCs) undergo dedifferentiation and result in ES-like colonies *in vitro* [13], but recent studies from our group demonstrated the presence of pluripotent, very small embryonic-like stem cells (VSELs) with high nucleocytoplasmic ratio and nuclear Oct-4 in adult human testis [19] and ovary for the first time [17]. We propose that rather than dedifferentiation of SSCs as earlier postulated, it may be possible that the VSELs *per se* expand to give rise to ES-like colonies *in vitro* [20]. Their presence in few numbers in adult gonadal tissue biopsies may explain the poor success of ES-like colonies derivation *in vitro* from gonadal tissue biopsy.

VSELs are the primordial germ cells that migrate into the gonadal ridge during early embryonic development and persist into adulthood, as also suggested by de Felici [21]. However, there is a disparity in the size of migrating PGCs (15–20 um) and VSELs (1–3 um); thus, more studies are needed to better understand whether the VSELs are similar or more primitive to PGCs. According to the existing school of thought, PGCs may give rise to pluripotent stem cells *in vitro* but they do not behave as stem cells *in vivo*, and later on during fetal development the true stem cell population of SSCs appears in the testis that divides throughout life giving rise to waves of spermatogenesis [22]. Similarly, Byskov et al. 2011 [23] have also suggested that ovary may have cells with stem-like characteristics which may be provoked to enter differentiation pathway into oocytes, at least *in vitro*. As evident a lot of misperception exists on our basic understanding of gonadal stem cells.

An introduction to gonadal stem cells, namely, VSELs and their possible role during premeiotic expansion of germ cells during gametogenesis and their relevance to reproductive and cancer biology are the focus of the present paper. In conclusion, we will summarize the possible translational applications of this emerging and exciting field of research.

2. Very Small Embryonic-Like Stem Cells (VSELs)

Pluripotent VSELs ($Oct4^+$, $SSEA1^+$, $Sca1^+$, Lin^-, $CD45^-$) were first reported by Ratajczak and group in adult mice tissues [24, 25], the highest numbers being in brain, kidneys, muscles, pancreas, and bone marrow [26]. These are diploid cells with high telomerase activity, express other pluripotent (Rex-1, Nanog, SSEA, and Klf-4) and germ cell (Mvh, Stella, Fragilis, Nobox and Hdac-6) markers, and decrease in numbers with age [27]. Like embryonic stem cells, they do not express MHC class I and HLA-DR antigens and are also negative for mesenchymal stem cell markers like $CD90^-$, $CD105^-$, and $CD29^-$. They are very small in size (3–5 um) and have a large nucleocytoplasmic ratio, large nuclei with abundant euchromatin, and an open chromatin structure for Oct-4 and Nanog promoter [28]. Oct-4 expression at mRNA and protein level in VSELs has been confirmed using sequence specific primers. VSELs have the ability to differentiate into three germ layers *in vitro*; however, unlike ES cells, VSELs neither complement during blastocyst development nor form teratomas in immunodeficient mice [29]. Attempts have been made to propagate them on feeder layers, but they do not self-renew as easily as the established embryonic stem cell lines possibly do because of altered methylation status of some developmentally crucial genes. Similarly VSELs have also been isolated from human umbilical cord blood, mobilized peripheral blood, and adult bone marrow by flow cytometry as $CD133^+$, lin^-, $CD45^-$ [30] and also by the differential centrifugation method [31, 32].

VSELs are descendants of epiblast stage pluripotent stem cells. They get deposited in various body organs including the gonads in early stages of development, as a quiescent stem cell population which possibly serves as a back up to the tissue committed stem cells (TCSCs). These two populations of stem cells (VSELs and TCSCs) together are responsible in bringing about tissue renewal, homeostasis, and regeneration after injury throughout life and decrease in number with age. The coexistence of two stem cell populations (the more primitive being quiescent and the progenitor being more rapidly dividing) has been recently proposed by Li and Clevers [33]. VSELs are the DNA label-retaining (e.g., BrdU), quiescent stem cells with a lower metabolic state whereas the tissue committed stem cells divide actively and do not retain DNA label over time. They are highly mobile, respond to the SDF-1 gradient, and enter into circulation in case of any injury to bring about regeneration and homeostasis. They are also considered as a missing link to support the germline hypothesis of cancer development [34, 35]. The clinical potential of VSELs, isolated from cord blood or bone marrow by flow cytometry, is just beginning to emerge. In various disease models like myocardial infarct [36, 37], stroke [38], skin burn injury [39], neural regeneration [40], and so forth, these cells get mobilized into circulation within 24 hours. For myocardial regeneration, the VSELs are very efficient to improve LV ejection fraction and attenuation of myocardial hypertrophy [37]. As they become scarce with age, regeneration becomes inefficient resulting in age-related disease manifestations.

3. Localization of VSELs in Mammalian Gonads

Our group has demonstrated the presence of VSELs for the first time in their natural somatic niche *in situ* in adult testicular and ovarian tissue collected from prostate cancer patients and perimenopausal women, respectively. These VSELs were localized in the basal layer of cells adjacent to the basement membrane in seminiferous tubules [19] and were found interspersed with the ovarian surface epithelial cells [17]. Similarly VSELs have also been observed in adult mice gonads [20], whereas the ovarian VSELs have been detected in scraped ovarian surface epithelium in rabbits, sheep, and monkey [17] and also in mouse ovary [41] by our group. Thus, the presence of VSELs in gonadal tissue appears to be evolutionarily conserved.

3.1. Oct-4 as a Pluripotent Marker to Study VSELs. Oct-4, also designated as Oct-3 or POU5F1, is present as a maternal transcript in mature oocytes and besides being the

gatekeeper in the beginnings of mammalian development [42] and pluripotency of inner cell mass in blastocysts, it is also a cell fate instructor through gene dosage effect [43] and is essential for primordial germ cell survival [44]. Oct-3/4 expression has also been associated with germ cell tumors and gonadoblastoma. Oct-4 gene is located on chromosome 6 and has five exons. It encodes two main variants by alternative splicing, namely, Oct-4A and Oct-4B which differ from each other in that exon 1 is present only in Oct-4A. The two transcripts give rise to 360 aa and 265 aa, respectively, of which 225 aa of C-terminal are identical. In contrast to Oct-4A, Oct-4B is not responsible for pluripotency [45].

Published literature on Oct-4 in somatic stem cells has confused stem cell researchers [46–48] because of the presence of several pseudogenes and alternatively spliced transcripts [46, 49]. Thus, a careful designing of primers for RT-PCR analysis and proper selection of antibodies becomes essential to detect specific transcripts. Also, a careful selection of Oct-4 antibodies is essential to detect pluripotent stem cells [47]. We used a polyclonal Oct-4 antibody that enabled the simultaneous identification of VSELs with nuclear Oct-4 and tissue committed stem cells, namely, SSCs and OGSCs with cytoplasmic Oct-4. In addition, careful selection of primers for Oct-4A and total Oct-4 for Q-PCR studies has helped us generate interesting results [17, 19, 20, 41].

Presence of Oct-4 positive VSELs in adult gonads and other body tissues contradicts the earlier views proposed by Jaenisch's group [50, 51] that an active pluripotency Oct-4 network exists only in embryonic and induced pluripotent stem cells. No abnormalities in homeostasis or regeneration were observed by them even after silencing Oct-4 gene in various tissues like intestine, bone marrow, hair follicle, liver, CNS, and so forth in 8-week-old mice. On the basis of the results, they proposed that Oct-4 is dispensable for functions of somatic cells. Berg and Goodell [52] authored a commentary on their work and had speculated that it may be possible for stem cells that were not directly tested in the experiments to have brought about the regeneration. In agreement with their view, it is felt that the regeneration may have occurred by the VSELs which get mobilized from the bone marrow into the circulation, in response to the injury. Thus, although the tissue specific Oct-4 was deleted, normal regeneration and homeostasis were observed in the young 8–10-week-old mice. It would be interesting to carry out similar studies in old mice (>12–14 months) having probably reduced number of VSELs and to observe whether regeneration occurs or not.

Indeed presence of VSELs have confused biologists in several other instances as well; for example, Tilly's group [53] and Nayernia et al. [4] concluded that bone marrow could be a possible source of female and male germ cells, respectively—leading to a flurry of scientific debate in the literature. Similarly, cells with early cardiac markers have been reported to be present in the bone marrow [54]. All these results are easily explained on basis of VSELs which are pluripotent stem cells and can differentiate into any kind of differentiated progeny depending on the body's need.

3.2. VSELs in Adult Testicular Tissue. We have documented that an adult testis harbors a novel population of pluripotent VSELs (with nuclear Oct-4A) which are more primitive to A_{dark} SSCs (with cytoplasmic Oct-4B). The VSELs possibly give rise to A_{dark} SSCs which in turn undergo clonal expansion as evident by the presence of cytoplasmic bridges between the rapidly dividing cells [19]. Oct-4 is not immunolocalized in more differentiated male germ cells.

The characteristic dark stained nuclei in A_{dark} SSCs is easily explained on the basis of stem cell biology. VSELs have abundant open euchromatin and the differentiated cells that arise by asymmetric cell division undergo extensive reprogramming and compaction of chromatin (by DNA methylation) which may result in a dark nuclear appearance, a characteristic of the A_{dark} SSCs [19]. Chromatin compaction occurs by DNA methylation wherein cytosine gets methylated and enables DNA to maintain similar sequence but genes get silenced or activated. This process can be studied using a simple immunolocalization procedure. More direct evidence and multicolor colocalization studies need to be carried out to prove this hypothesis but the preliminary immunolocalization study carried out using monoclonal 5-methyl cytosine antibody (source: Calbiochem, Merck, Millipore) has yielded interesting results (Figure 1(a)). Staining was predominantly observed in A_{dark} SSCs indicating on extensive nuclear reprogramming in the progenitor stem cells which arise by asymmetric cell division from the pluripotent VSELs (with abundant euchromatin).

3.3. VSELs in Adult Ovarian Tissue. Careful scraping of ovarian surface epithelium in rabbits, sheep, monkey, and perimenopausal women resulted in the detection of VSELs (1–3 um) and also ovarian germ stem cells (OGSCs; 5–7 um). The VSELs were smaller than RBCs, had high nucleocytoplasmic ratio, abundant euchromatin, nuclear OCT-4, cell surface SSEA-4 and other pluripotent markers [17]. Interestingly, H & E staining of the stem cells in scraped OSE resulted in the visualization of OGSCs with dark stained nuclei [17], possibly signifying similar stem cells biology like A_{dark} SSCs and exhibited nuclear staining for 5-methyl cytosine (Figures 1(b)–1(d)). Three-week culture of these stem cells gave rise to putative oocyte-like structures, embryo-like structures, neuron-like structures, ES-like colonies, and embryoid-like bodies, signifying the pluripotent to totipotent nature of the stem cells [17].

4. Role of VSELs during Gametogenesis

Gametogenesis, a process by which haploid gametes are produced from diploid germ cells in the gonads, ensures transmission of genetic information from generation to generation and thus the continuation of species. The primordial germ cells (PGCs) of epiblast stage embryo colonize into the gonadal ridge in the undifferentiated gonad and differentiate into female or male germ cell precursors. These PGCs possibly persist as VSELs in adult gonads and undergo asymmetric cell divisions throughout life to self-renew and give rise to tissue committed gonadal stem cells, namely, A_{dark} SSCs in the testis and OGSCs in the ovary.

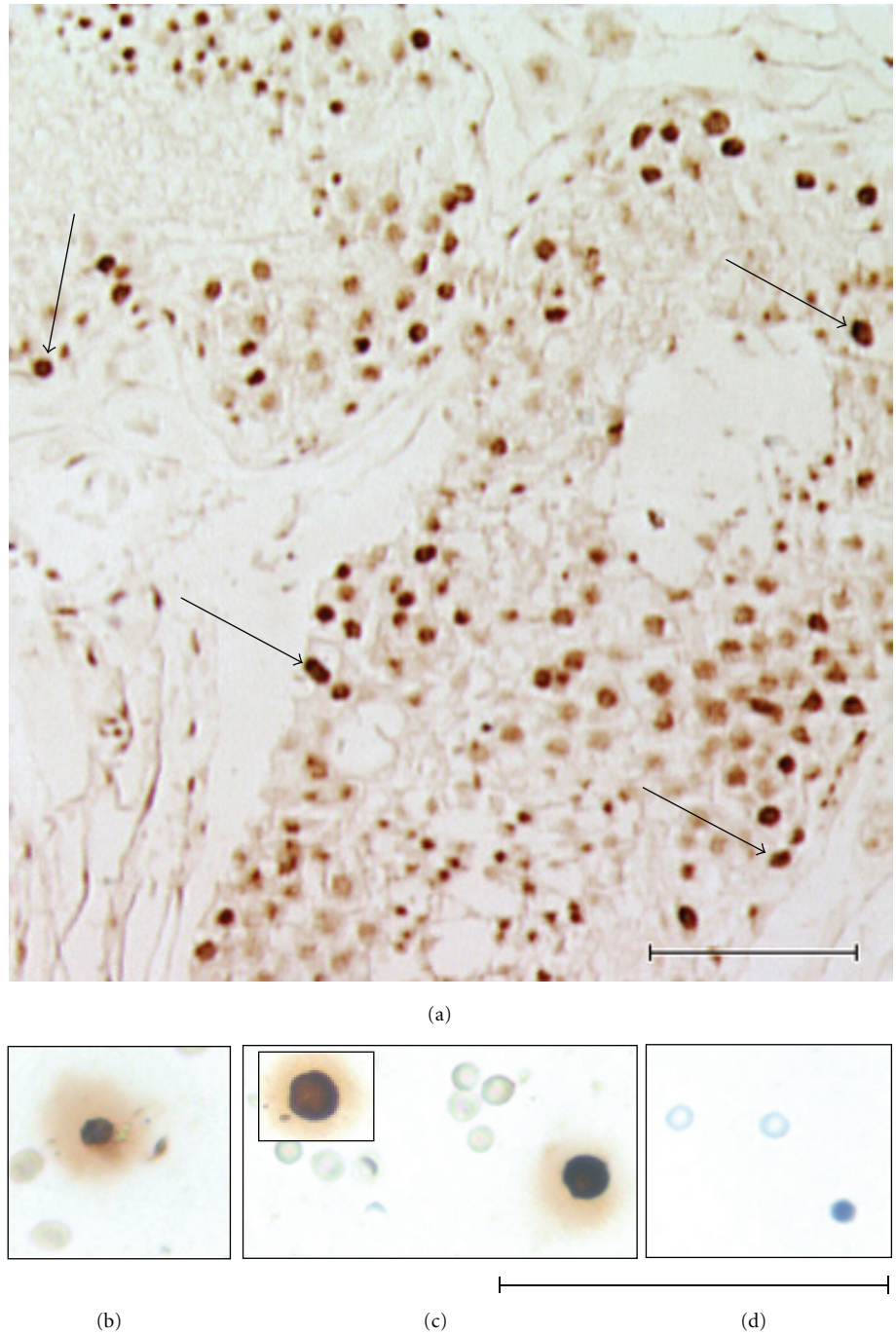

Figure 1: Immunolocalization of 5-methyl cytosine on adult human testicular section and peri-menopausal ovary surface epithelium smear (using standard protocol published earlier, [55]). Note dense staining in the spermatogonial stem cells (SSCs, arrow, (a)) while the spermatocytes showed minimal staining. In few tubules spermatids showed positive staining. Similarly the ovarian germ stem cells (OGSCs) stain positive ((b) and (c)) Negative control (d). The results indicate that A_{dark} SSCs in testis and OGSCs in ovaries, derived by asymmetric cell division of VSELs undergo nuclear reprogramming associated with extensive methylation—suggesting that similar basic stem cell biology exists in both the sexes. Scale bar = 20 μm.

4.1. Spermatogenesis. Undifferentiated SSCs maintain a stable diploid population of germ cells and produce differentiating spermatogonia, which finally enter meiosis and give rise to spermatocytes which differentiate and produce sperm throughout life. A comprehensive review on various aspects of self-renewal, proliferation, and differentiation of SSCs was recently published [56, 57] highlighting the dearth of our present knowledge.

Difference of opinion exists in our understanding of kinetics of proliferation of SSCs in the humans based on the

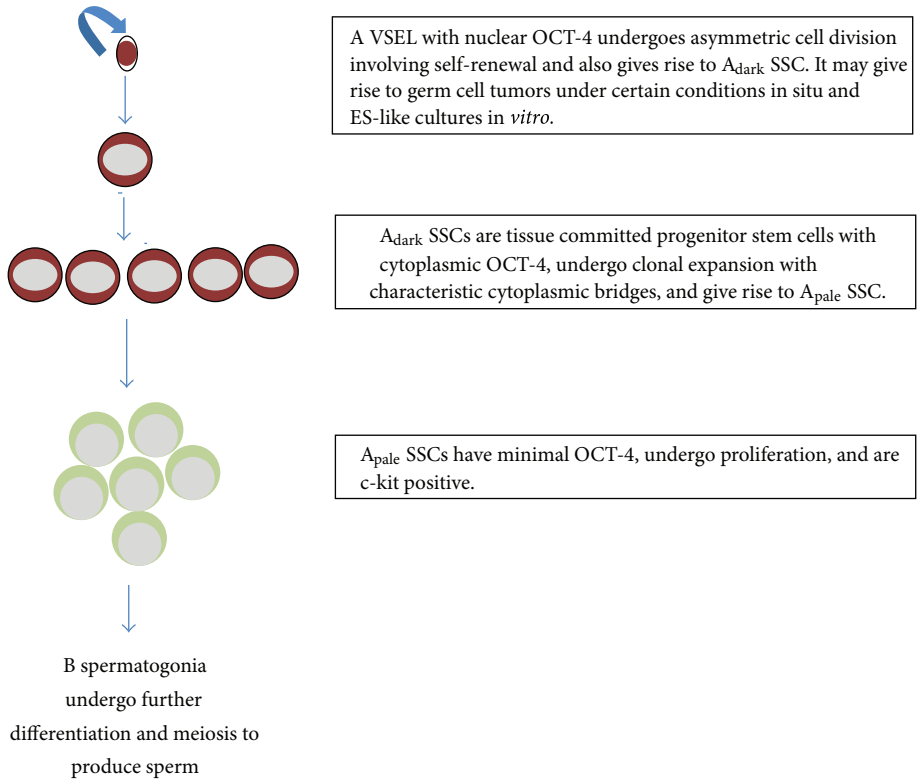

Figure 2: VSELs are implicated during human spermatogenesis. The relationship between VSELs and SSCs during premeiotic expansion of germ cells is depicted.

earlier reports of Clermont [58] and the recently proposed scheme by Ehmcke and Schlatt [56]. Clermont suggested that the A_{dark} SSCs undergo regular mitotic divisions whereas A_{pale} spermatogonia divide only once whereas Ehmcke and Schlatt propose that spermatogenesis starts with the division of a pair of A_{pale} spermatogonia and A_{dark} SSCs divide very rarely. They further suggest that A_{dark}, A_{pale} and B spermatogonia do not form mixed pairs or chains and none of the premeiotic germ cell types undergo unequal divisions. Detection of VSELs in the adult testis adds another dimension to this current school of thought since they are implicated during pre-meiotic expansion of testicular germ cells. Various events like asymmetric cell division, self-renewal, clonal expansion, and proliferation as they occur during spermatogenesis have been further clarified (Figure 2). Till recently, the description of SSCs in primates has been based on studies conducted on histological sections, whole mount preparations, and so forth. An urgent need is felt to study the expression and localization of various growth factors and cytokines in the testicular compartment with respect to various stages of proliferation and differentiation. This kind of stage-specific analysis of germ cell markers approach will help define and dissect out mitotic, meiotic, and postmeiotic germ cell processes leading to improved translational opportunities as suggested earlier also [59].

4.2. Oogenesis. Even after decades of research, reproductive biologists are still confused whether the ovary has a fixed number of follicles at birth which diminish with age and menopause is associated with a dramatic decline in number of follicles or there is a continuous renewal of follicles throughout adulthood just like sperm in testis (recently reviewed in favor of postnatal oogenesis by Woods and Tilly [60] and in favor of a fixed number of eggs by Notarianni [61]).

VSELs have been reported in the scraped surface epithelium of rabbit, sheep, monkey, and perimenopausal human ovary [17]. It is interesting to mention here the work published by Szotek and group [62] which showed the presence of a population of stem cells in mice OSE that retains label for more than four months—indicating quiescence with asymmetric label retention. In addition to the VSELs in the scraped OSE, slightly bigger cells with more cytoplasm that express cytoplasmic Oct-4 and minimal SSEA-4 also exist—which are the progenitor ovarian germ stem cells (OGSC), comparable to A_{dark} SSCs in the testis. Later the OGSCs get surrounded by pregranulosa cells that develop by epithelial mesenchymal transition of epithelial cells, resulting in PF assembly as suggested recently by us [17].

A recent report by Byskov and group [23] found no evidence for the presence of oogonia in the adult human ovary after their initial clearance in first two years of postnatal life. However, the archived tissues that were used to arrive at this conclusion were fixed in formaldehyde and 30–40 um sections were used for immunostaining. Such an approach will never detect the VSELs (being 3–5 um in size) and

could have resulted in negative results [63]. The choice of fixative and its effect on immunolocalization results has been discussed earlier [19, 64]. In contrast to their results, confocal microscopy studies on scraped surface epithelium from rabbit, monkey, sheep, and human ovaries [17] demonstrated the presence of a distinct population of stem cells with Oct-4 (both nuclear and cytoplasmic) and cell surface SSEA-4. Similarly, Zhang et al. [65] generated experimental evidence that no mitotically active female germline progenitors exist in adult mouse ovaries. However, here the choice of cell surface marker DDX1 to isolate the progenitors is an issue [66, 67]. Basically, using DDX-1 as a marker they used 10–15 μm cells for their study, whereas VSELs range between 3–5 μm in size. Thus, rather than using DDX-1, SSEA-1 in mouse (SSEA-4 in humans) may be a better cell surface marker to isolate pluripotent ovarian stem cells.

Intriguingly Byskov et al. [23] in their paper discussed the results of Liu et al. [68] who also found no evidence for the presence of oogonia in normal adult human ovaries, neither early meiosis-specific or oogenesis-specific mRNAs nor immunohistochemical markers for oogonia or meiosis. But a closer scrutiny of the published results of RT-PCR and immunolocalization studies [68] shows Oct-3/4, DMC1, and SCP3 in adult ovary (although much less as compared to fetal samples)—which cannot be ignored. RT-PCR data also fail to discriminate between cells having a low level of expression compared to a scenario where few cells exist with high expression. In the light of these concerns, the existence of stem cells and oogonia in adult human ovary needs to be re-evaluated. These discrepancies and ambiguous biological conclusions based on technological limitations need to be resolved and the concept of presence of stem cells and postnatal oogenesis in adult ovary should be understood soon.

5. Effect of Aging on VSELs in Mammalian Gonads

The stem cells exist in a specialized microenvironment provided by the somatic cells termed as the "niche". This term was first coined by Schofield in 1978 for the mammalian hematopoietic system [69] and now is discussed in context to various tissues. The niche ensures normal functioning of stem cells and regulates specific properties like self-renewal, pluripotency, quiescence, and ability to differentiate. It gets compromised with age and results in reduced homeostasis and regeneration ability of stem cells [70].

5.1. Effect of Aged Niche in Males. An age related reduction in both quality and quantity of sperms in mice and men is possibly because of compromised niche rather than the reduction of stem cell potential [71, 72]. When SSCs from young, fertile male mice are transplanted into 1-and 2-year-old atrophied testis, only 1-year-old testis showed regeneration, thus indicating age-related alterations in somatic cells can impair spermatogenesis. The impaired ability of Sertoli cells to respond to FSH and reduced production of GDNF with age [73] explains the age-related decline in fertility.

5.2. Effect of Aged Niche in Females. Menopause is the age-related cessation of ovarian function indicating the end of fertile phase of a woman's life. The mammalian ovary is believed to be endowed with a fixed number of eggs and a sudden loss of PF results in menopause in women [74, 75] and infertility in mice [76]. However, the proponents of postnatal oogenesis in females propose that menopause may actually be the result of compromised somatic niche (comprised of ovarian epithelial cells), which does not allow stem cells to undergo self-renewal, differentiation, and follicular assembly to form PF *in situ* [77, 78]. OSE stem cells from anovulatory postmenopausal ovaries have the capacity to differentiate *in vitro* into oocytes [79] and the immune system could be responsible for termination of follicular renewal *in vivo* [80]. Estradiol secretion by the ovarian tissue is reduced *in vitro* after being exposed to oncotherapy and also if collected from aged ovaries [77] indirectly indicating a compromised somatic environment which may restrict stem cells to undergo follicular assembly and thus result in menopause. Lee et al. [81] reported that BMT can restore long term fertility in preclinical mouse model of chemotherapy induced premature ovarian failure. Niikura [82] have shown that ovarian stem cells from aged mice ovary into a young host result in the resumption of oogenesis. The three-week culture studies of stem cells collected by scraping OSE of menopausal ovary suggest that once the *in vivo* inhibitory cues are withdrawn, the stem cells differentiate into oocytes-like structures, embryos, ES-like colonies, and EB-like structures *in vitro*, demonstrating their pluripotent nature. This has been a consistent observation not only in case of humans but also in other mammals like rabbit, sheep, and monkey [17].

6. VSELs and Cancers

VSELs are the possible precursors to cancer stem cells [34, 35]. It is a well-known fact that incidence of malignant tumors increases with old age [83], in both animals and humans. A change in the aged somatic niche disrupts the stem cell biology and VSELs possibly undergo a symmetric cell division resulting in tumor rather than their quiescent nature and asymmetric cell divisions under normal conditions in a younger niche as has been suggested earlier also [84]. Oct-4 is a well-established marker for diagnosis of carcinoma *in situ* (CIS), neoplastic gonadoblastoma, and invasive germ cell tumors in adults [85, 86]. Cools et al. [87, 88] concluded that gonadoblastomas, gonadal maturation delay, and early germ cell neoplasia in patients with under-virilization syndromes, have Oct 3/4 positive germ cells in dysgenetic gonads.

The connection of stem cells (VSELs) with ovarian cancers is based on the published literature and circumstantial evidence but is not yet well accepted by the scientific community. 90% of ovarian cancers (most lethal amongst the gynecological malignancies) arise from OSE (which also houses the VSELs)! Incessant ovulation hypothesis suggests that continuous ovulation (without associated apoptosis) subjects OSE to transformation events and damaged cells are retained leading to cancer [89]. Overexpression of FSHR is

also observed in OSE in cancer tissues as compared to normal OSE—that may activate oncogenic pathways leading to cancer [90]. Chen et al. [91] suggested that neither incessant ovulation nor FSHR present in OSE is required for inducing ovarian tumors. They argued that FORKO mice have high circulatory levels of FSH and LH, ovarian androgens are elevated, estrogens are very low, no FSHR and still have high incidence (>90%) of ovarian tumors by 12 months of age. These mice have endocrine profile similar to postmenopausal women, are infertile, never ovulate, and have no FSHR but still develop cancers. Thus, the only consistent observation is that ovarian cancers are more frequent in menopausal women, where possibly the VSELs residing in a compromised somatic niche may be implicated in tumor growth. It will be interesting to study VSELs in FORKO mice and especially age related changes in the somatic niche that triggers uncontrolled proliferation of these stem cells that lead to cancer.

7. VSELs as Autologous Source of Pluripotent Stem Cells for Regenerative Medicine

Derivation of ES-like cultures using adult gonadal tissue from mice and humans has recently been reported. This has resulted in a lot of excitement since adult gonads may be a novel "autologous", non-embryonic source of pluripotent stem cells in contrast to human embryonic stem cells where issues regarding immune rejection exist during cell-based therapies in future. Moreover, they may also be superior to induced pluripotent stem cells (iPS) since they are derived from a very quiescent stem cell population and are thus "young" cells with long telomeres that could be isolated from an aged body, in contrast to iPS cells which are derived from terminally differentiated somatic skin fibroblasts (with shortened telomeres) that tend to accumulate DNA mutations over time. This is in accordance with the "disposable soma" theory proposed by Kirkwood et al. [92], which suggests that investments into maintenance are higher in germline cells and get down regulated as "too costly" in somatic cells.

However, extensive research needs to be undertaken to establish technology to obtain *in vitro* expansion of VSELs similar to embryonic stem cells. Attempts in the field have resulted in successful ES-like culture from testicular tissue in mice [7–9] and men [10–13]. These cultures have been characterized using various pluripotent markers and exhibit some differences compared to embryonic stem cells. They have the ability to differentiate into three germ layers but result in small teratoma formation [10, 11] after injecting more number of cultured ES-like cells into immuno-compromised mice. This may actually be good reason to prefer these cells over embryonic stem cells for regenerative medicine [13] and could be because of the epigenetic differences between VSELs (from which ES-like cultures were derived during testicular cultures) and embryonic stem cells especially related to insulin growth factor [93].

8. Future Perspectives

Application of VSELs to improve reproductive health needs to be established. We have recently studied the differential effect of busulfan on the relatively quiescent VSELs versus rapidly dividing germ cells in adult mice gonads (unpublished results). The VSELs were found to be resistant to the treatment and this opens up newer and exciting avenues for fertility preservation. The VSELs are localized in OSE and several investigators have reported extensive proliferation in OSE in response to PMSG [94] ; we need to understand these published results in context of stem cells. Also a better understanding of VSELs will help manage menopause, infertility, and reproductive diseases. How these stem cells are implicated in PCOS and POF patients and so forth is altogether a new field of research with direct bearing on a woman's health that requires further investigation.

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Review Article

Use of Insulin to Increase Epiblast Cell Number: Towards a New Approach for Improving ESC Isolation from Human Embryos

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Human embryos donated for embryonic stem cell (ESC) derivation have often been cryopreserved for 5–10 years. As a consequence, many of these embryos have been cultured in media now known to affect embryo viability and the number of ESC progenitor epiblast cells. Historically, these conditions supported only low levels of blastocyst development necessitating their transfer or cryopreservation at the 4–8-cell stage. As such, these embryos are donated at the cleavage stage and require further culture to the blastocyst stage before hESC derivation can be attempted. These are generally of poor quality, and, consequently, the efficiency of hESC derivation is low. Recent work using a mouse model has shown that the culture of embryos from the cleavage stage with insulin to day 6 increases the blastocyst epiblast cell number, which in turn increases the number of pluripotent cells in outgrowths following plating, and results in an increased capacity to give rise to ESCs. These findings suggest that culture with insulin may provide a strategy to improve the efficiency with which hESCs are derived from embryos donated at the cleavage stage.

1. Introduction

The embryo begins as a single totipotent cell it then undergoes multiple rounds of division coupled with differentiation until it forms a blastocyst and has the potential to implant in the uterus. The pluripotent epiblast of the inner cell mass (ICM) then undergoes further division and differentiation to develop into the fetus and eventually a fully developed organism. Epiblast cells can be isolated and cultured in conditions which allow embryonic stem cell (ESC) lines to be derived. ESC lines, especially hESC lines, hold considerable promise in the fields of drug discovery, developmental biology and regenerative medicine. However, the efficiency of hESC derivation is low. This paper brings together work by our group which used a mouse model to develop a strategy for improving the efficiency of hESC derivation. As most embryos donated for human ESC derivation were cultured in relatively simple media, now known to perturb development, before being frozen at the precompaction stage up to 10 years earlier, we examined the hypothesis that the period where they are subsequently cultured to the blastocyst stage could be exploited to improve the efficiency with which cell lines could be derived. In particular, our work has focussed on adding insulin to culture media to increase epiblast cell number.

In initial studies [1], we showed that in vitro culture of embryos during the precompaction stage in a simple medium that was designed to model the culture conditions that human embryos available for hESC derivation were previously exposed to reduces embryo quality; as highlighted by reduced developmental rates, decreased epiblast cell number and altered gene expression in outgrowths compared with that seen for embryos culture in modern G1 medium. Some aspects of this reduction in quality could be restored after compaction by culture in a medium designed to support postcompaction embryo development in vitro (G2), including epiblast cell number. However, epiblast cell number was only partially improved compared with embryos cultured in G1/G2. The findings of [2, 3] as well as our own later studies

[4] suggest that increased epiblast cell number correlates with an increased capacity to give rise to ESCs. Culture in G2 medium postcompaction also increased the proportion of embryos which reached the hatched blastocyst stage which was subsequently shown in [4] to be correlated with an increased capacity to give rise to primary ESC colonies. Together, these findings highlight that subsequent culture in modern culture systems can improve the efficiency of ESC derivation from embryos initially cultured in simple media.

Numerous growth factors are known to influence embryo development. However, these are not routinely included in embryo culture media. In contrast, growth factors such as leukaemia inhibitory factor (LIF) are routinely used for the isolation and maintenance of ESCs. Based on these observations, we hypothesised that addition of a growth factor to embryo culture media could further improve blastocyst development, epiblast cell number, outgrowth formation rate, and ESC derivation. In particular, we investigated whether insulin could be used to increase ESC derivation efficiency because it has previously been shown to increase ICM cell number when added to embryo culture media [5, 6].

The results from these studies showed that the inclusion of insulin in postcompaction culture medium increased the number of pluripotent cells in blastocysts. In a series of experiments we showed that insulin acted via the PI3K/GSK3 p53 pathway to shift the balance of differentiation versus pluripotency within the ICM to increase epiblast number and proportion [7]. This resulted in an increase in their capacity to give rise to outgrowths with more pluripotent cells, as well as an increase in capacity to give rise to primary ESC colonies [4]. These findings suggest that the inclusion of insulin in embryo culture medium postcompaction could be used to improve the quality of cryopreserved or fresh human embryos donated at or near compaction.

2. Impact of Culture Conditions on Epiblast Cell Number and Pluripotency

The relatively low efficiency with which hESCs can be derived has been attributed to the reduced quality of human embryos donated for this purpose [8–10]. In vitro culture of embryos is typically associated with reductions in embryo quality and viability [11]. Furthermore, human embryos for hESC derivation have often been cryopreserved for 5–10 years prior to their donation [12, 13]. As a consequence many were cultured in media now known to perturb viability, and which supported only low levels of blastocyst development. This necessitated cleavage stage transfers for the majority of IVF cycles performed.

In order to model this system, mouse embryos were cultured in relatively simple medium for the first 48 h to the 8-cell stage to approximate the culture period commonly used in human IVF experiments [1]. These experiments demonstrated that culture of embryos in simple medium retarded the development of blastocysts and significantly reduced the number of epiblast cells, which was consistent with previous studies in human and mouse [14–19]. These experiments also demonstrated that there was some capacity to improve blastocyst development and epiblast cell number by transferring embryos to the more complex G2 medium for culture from the 8-cell stage. Despite this, initial culture in relatively simple medium had a lasting negative impact on subsequent development. Furthermore, we found that embryos cultured in simple medium were less likely to contain an epiblast and therefore lacked the capacity to generate an ESC line, irrespective of the culture medium used for the second 48 h. Additionally, assessment of outgrowths generated from these blastocysts showed that the perturbing conditions of a simple medium had lasting effects on the gene expression of the outgrowths, with altered gene expression of *Atrx* and *Nanog*.

Human embryos donated for hESC derivation are likely to have been exposed to conditions such as simple style culture medium, examples of which include HTF, Earle's, and T6, which were widely used in IVF and can still be found in use. Collectively, the findings of [1] therefore indicate that these embryos are likely to have a reduced capacity to give rise to hESCs. This in turn suggests that the characteristics of hESC lines could be affected by predonation embryo culture conditions.

As many human embryos, historically and presently, are cryopreserved at the cleavage stage [20–22] and therefore donated for ESC generation at this stage, they must be further cultured to the blastocyst stage before ESC derivation. This additional culture period represents a window where the pluripotency of embryos which have previously been exposed to perturbing culture conditions can be improved. The results of [1] demonstrated that while the quality of mouse embryos could be improved by culturing them in modern complex medium purpose designed to support embryo development from the 8-cell stage, additional interventions are necessary to fully exploit the cleavage to blastocyst culture period.

3. Insulin Stimulation of Pluripotency in Postcompaction Embryos

The inclusion of select growth factors in embryo culture media has previously been shown to be capable of improving embryo development and viability [5, 23]. However, growth factors are not routinely included in culture media commercially available for human embryo culture [24–28]. To further examine how interventions to the culture medium for the postcompaction stage embryo may affect epiblast cells and pluripotency, the growth factor insulin was added to the culture medium. Insulin has previously been shown to increase the ICM cell number of embryos [29] and was selected as the most promising candidate from a panel of growth factors previously used to improve embryo culture.

The findings of [7] demonstrated that 1.7ρM insulin increased epiblast cell number without affecting ICM cell number. This resulted in a significant increase in the proportion of ICM cells which were epiblast as opposed to primitive endoderm. This novel finding suggested that insulin was acting to shift the balance of differentiation within the ICM towards more pluripotent cells, rather than acting as a general mitogenic factor and stimulating overall cell growth.

This was further highlighted by the finding that total cell number and trophectoderm cell number were also unaffected by the addition of insulin. However, as in previous studies [5, 23], there was a threshold concentration where the effect of insulin was maximal, above which further increases led to the loss of the increase in epiblast cell number. If this strategy is implemented in the human, it may be necessary to repeat these dose response experiments to establish an optimal dose in terms of epiblast cell number increases. While this work shows that insulin is able to maintain pluripotency in the ICM and direct differentiation, other growth factors may also have beneficial effects, and it is possible that a combination of growth factors may produce a synergistic effect and improve blastocyst quality and epiblast cell number.

4. Molecular Mechanism of Action of Insulin on Pluripotency in the Blastocyst

Having demonstrated that the culture of postcompaction stage embryos with insulin increases epiblast cell number, further experiments were undertaken to determine the signalling pathways behind this effect [7]. At the concentration identified as increasing epiblast cell number and proportion, insulin is known to activate the insulin receptor [29]. One of the primary second messengers of the insulin receptor is PI3K, which has previously been shown to be integral for maintaining pluripotency in ESCs [30]. Using inhibitors, it was demonstrated that PI3K activity was necessary for insulin to increase epiblast cell number (Figure 1). One target of PI3K is GSK3, which is phosphorylated by active PI3K, inactivating it. When active, GSK3 is capable of phosphorylating many second messengers which converge to reduce Nanog transcription, which is important for the retention of pluripotency [31–37]. Inhibiting GSK3, theoretically reproducing the effect of culture with insulin and active PI3K, increased epiblast cell number. Furthermore, activating GSK3 blocked insulin's ability to increase epiblast cell number without affecting the epiblast cell number of embryos cultured without insulin, replicating the effect of PI3K inhibition. These results suggest that the inactivation of GSK3 is an important component of the insulin signalling pathway in relation to increasing epiblast cell number.

The pro-apoptotic protein p53, which is also regulated by PI3K, specifically via PI3K activated ubiquitinase MDM2 [38–40], causes cell death and differentiation when active and binds to the *Nanog* promoter region to repress Nanog transcription [41]. As with GSK3, inhibition of p53 increased epiblast cell number, while activation blocked insulin-mediated epiblast increases, strongly suggesting that p53 is involved in insulin-mediated increases to epiblast cell number. Interestingly, there are multiple points of cross-reactivity between GSK3 and p53 [42–46]; however, no additional epiblast increases were found for co-inhibition of the two factors. This suggests that the potential of GSK3 inhibition to cause the accumulation of p53 [45, 46] did not have a confounding effect in these experiments. In conclusion, the results of these studies demonstrated that insulin increased the epiblast cell number via the activation of PI3K (most likely via its interaction with the insulin receptor), which subsequently inactivates the second messengers GSK3 and p53, to increase Nanog transcription and therefore promote pluripotency and the epiblast (Figure 1).

5. Expression of OCT4 and Nanog in Blastocysts

The localisation of OCT4 and Nanog in blastocysts on day 4 (early blastocysts), day 5 (predominantly expanded blastocyst), and day 6 (hatching blastocysts) was determined by immunohistochemistry [4]. Of note, it was found that at the stage of development where the literature sources suggested that OCT4 and Nanog would be restricted to the ICM and epiblast, respectively [47, 48], both were still widely expressed. A comparison of methodologies suggested that this difference is likely the result of collecting and beginning embryo culture at the zygote stage in this study, rather than at the 2-cell stage or later.

Human embryos are ubiquitously cultured from the zygote stage following in vitro fertilisation. As such, this finding suggests that future researchers who use the mouse blastocyst to model the in vitro development of human embryos should culture embryos from the zygote stage, as the discrepancy appears to produce a meaningful difference particularly with regards to epiblast development. Future work in this area should include the direct comparison of OCT4 and Nanog expression of in vitro and in vivo grown mouse embryos as well as the characterisation of Oct4 and Nanog expression in human blastocysts.

6. Effect of Insulin in Embryo Culture Medium Persists in Outgrowths

Despite the increase in Nanog positive cell number due to culture with insulin, we found that when blastocysts were plated before the transcription factor was restricted to the epiblast, outgrowths from insulin-treated embryos contained no more epiblast cells than outgrowths from control embryos. Further, our results showed that despite earlier stage blastocysts possessing more OCT4 and Nanog positive cells than later stage blastocysts, they gave rise to outgrowths with significantly fewer epiblast cells. As hESCs have been shown to be most efficiently derived from blastocyst where Oct4 has been restricted to the ICM [49], this finding supports the use of mouse blastocysts in modelling human embryo development and hESC derivation. The important finding from these experiments, however, was that when embryos were allowed to develop until Nanog was restricted to the epiblast and OCT4 was restricted to the ICM before plating, culture of embryos in insulin postcompaction resulted in the generation of outgrowths which were more likely to contain an epiblast and which contained a larger number of epiblast cells.

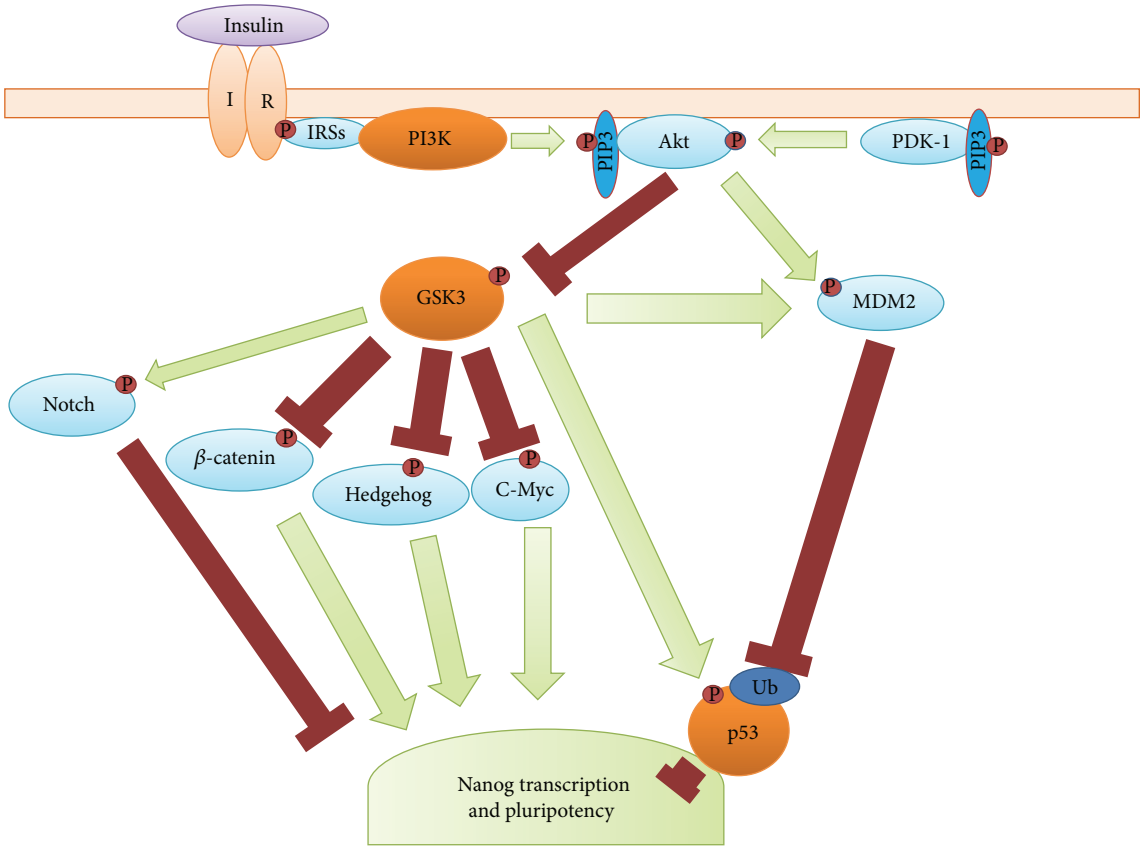

Figure 1: Schematic of insulin signalling and its regulation of Nanog expression and pluripotency. Green arrows indicate reactions with a stimulatory effect on their target, and red closed bars indicate reactions with a retarding effect on their target. P marks reactions where phosphorylation occurs, and Ub marks reactions where ubiquitination occurs. Insulin binds the insulin receptor (IR), a tyrosine kinase which is then able to phosphorylate the IRSs. PI3K is able to bind to the phosphorylated IRSs by its SH2 domains, resulting in activation. PI3K phosphorylates the phospholipid PIP2, producing PIP3, which can be bound by the pleckstrin homology domains of PDK-1 and Akt. Results in [7] show that activation of PI3K is necessary for insulin to increase the number of Nanog positive epiblast cells during embryo culture. When PDK-1 and Akt are colocalised to the cell membrane, PDK-1 is able to phosphorylate and activate Akt. Active Akt can phosphorylate GSK3, inactivating it. When active, GSK3 is able to phosphorylate β-catenin, Hedgehog, and c-Myc; all factors which safeguard pluripotency through interactions with other second messengers. Additionally, active GSK3 phosphorylates and protects the intracellular domain of Notch, promoting differentiation. Further, inactivation of GSK3 is necessary for insulin to increase the number of Nanog positive epiblast cells during embryo culture [7]. Akt is also able to phosphorylate and activate MDM2 which ubiquitinates the proapoptotic factor p53, causing its inactivation and removal from the nucleus, where it would bind to the *Nanog* promoter and suppresses its expression. Inactivation of p53 is necessary for insulin to increase the number of Nanog positive epiblast cells during embryo culture [7]. GSK3 and p53 are able to form a dimer, resulting in the phosphorylation of p53 and the increased activity of both factors. GSK3 is also able to phosphorylate and activate MDM2. However, despite these outcomes, the interaction of GSK3 and p53 do not have a significant effect on Nanog positive epiblast cell number during embryo culture [7].

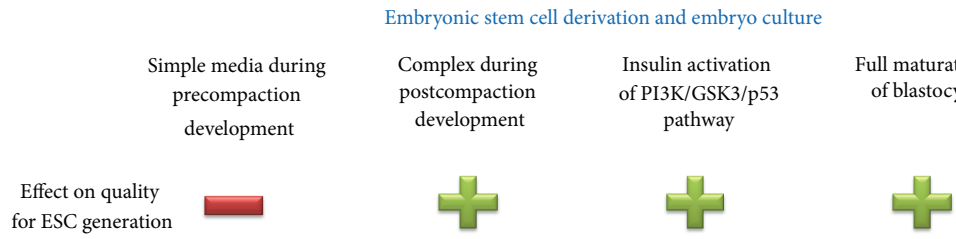

Figure 2: Summary of the culture effects examined in this paper and their observed effect on the retention of pluripotency towards ESC derivation.

7. Insulin in Culture Media and the Effect of ESC Colony Generation

Insulin- and control-treated blastocysts were plated on day 6, outgrown, trypsinised, and replated, and primary cell colonies with an ESC morphology were stained for OCT4 and Nanog expression to confirm pluripotency [4]. Blastocysts were twice as likely to give rise to primary ESC colonies if they were cultured with insulin for the postcompaction stage. Interestingly, examining how the inclusion of insulin interacted with this process led to the novel observation that culture of embryos with insulin increased the proportion of embryos which, at the point of plating, were at the most advanced morphological stage (hatched) and also increased the proportion of those hatched blastocysts which gave rise to ESCs. As such, hatched insulin-cultured blastocysts are more plentiful and more likely to give rise to ESCs than hatched control-cultured blastocysts. This result demonstrates that insulin improves ESC isolation through mechanisms beyond simply improving morphology, which has previously been linked to increased ESC derivation rates. It is likely that the improved capacity of insulin-cultured blastocysts with the highest morphological quality to give rise to primary ESC colonies is the result of the increased epiblast cell numbers demonstrated in both [4, 7].

Modelling of the experimental outcomes enabled conclusions to be made around the most significant characteristics that an embryo must contain to generate a primary ESC colony. The greatest predictor of a control-cultured blastocyst giving rise to a primary ESC colony was it cavitating on day 4, whereas for blastocysts cultured with insulin, the greatest predictor was being hatched on day 6. For day 4, this observation is likely the result of insulin increasing the rate of cavitation and thereby making the marker less selective. The finding on day 6 is suggestive that in the control group, hatched blastocysts, which have shown the best development, have no more epiblast cells than their more slowly developing counterparts. Both of these observations warrant further investigation.

The results of this work demonstrate that the addition of insulin to embryo culture medium from the cleavage stage to the blastocyst stage improves the efficiency with which ESCs can be generated from these embryos. As human embryos are most often donated at the cleavage stage and ESC derivation is most often attempted at the blastocyst stage, the application of this strategy has the potential to improve hESC derivation efficiency. Due to the limited availability of human embryos for ESC derivation, improving efficiency is a matter of key importance.

Future work which would be necessary to validate these findings is the expansion of mESC colonies from control- and insulin-cultured embryos to fully characterised mESC lines and the reproduction of these experiments in the human. Further, our work has shown that the effect of insulin persists beyond embryo culture through the outgrowth phase and into ESC derivation. This suggests that during embryo culture-insulin may have a permanent positive effect on cell properties and that ESC lines derived from embryos cultured with insulin may have altered characteristics. As such, future work should include not just the characterisation of ESC lines from control and insulin-cultured embryos for pluripotency and self-renewal, but also more in depth characterisation including metabolic profile, an assessment of DNA methylation and acetylation, and gene expression, to provide a more detailed and precise picture of the quality and differentiation status of ESC lines, with a view towards investigating whether the culture of embryos with insulin results in the derivation of higher quality ESC lines.

8. Conclusion

In conclusion, the results presented in this paper show that while culture in simple medium during the cleavage stage decreases pluripotency, the inclusion of insulin in embryo culture medium from the compaction stage stimulates pluripotency supporting pathways to increase the number of epiblast cells in the fully developed blastocyst, resulting in an increased capacity to generate ESCs (Figure 2). This strategy is of particular relevance for hESC derivation where embryos are most often donated at the cleavage stage and of reduced quality.

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Review Article

Ovarian Cancer Stem Cells: A New Target for Cancer Therapy

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Ovarian cancer is a highly lethal disease among all gynecologic malignancies and is the fifth leading cause of cancer-related death in women. Although the standard combination of surgery and chemotherapy was initially effective in patients with ovarian cancer, disease relapse commonly occurred due to the generation of chemoresistance. It has been reported that cancer stem cells (CSCs) are involved in drug resistance and cancer recurrence. Over the past decades, increasing studies have been done to identify CSCs from human ovarian cancer cells. The present paper will summarize different investigations on ovarian CSCs, including isolation, mechanisms of chemoresistance, and therapeutic approaches. Although there are still numerous challenges to translate basic research to clinical applications, understanding the molecular details of CSCs is essential for developing effective strategies to prevent ovarian cancer and its recurrence.

1. Introduction

Ovarian cancer, the fifth leading cause of cancer-related death in women, is a highly lethal disease among all gynecologic malignancies. It is estimated that 22,280 women are diagnosed with ovarian cancer and 15,500 women will die of this disease in 2012 in the United States. From 2005 to 2009, the median age at diagnosis for ovarian cancer in women was 63 years. Based on incidences from 2007 to 2009, one in seventy-two women will be diagnosed with ovarian cancer during their lifetime. The overall five-year relative survival rate was 43.7% from 2002 to 2008 [1].

Ovarian cancer is a heterogeneous disease composed of different types of tumors [2]. Based on different histological features, most tumors of the ovary contain three major types of cells: surface epithelial stromal cells, sex cord stromal cells (including granulose, theca, and hilus cells), and germ cells (oocytes) [3]. Epithelial ovarian carcinoma (EOC) is the major form of the disease and accounts for about 90% of ovarian tumors [4]. According to distinctive morphology and molecular genetic background, epithelial ovarian cancer can be further categorized into eight subtypes, including serous, mucinous, endometrioid, clear cell, transitional cell tumors (Brenner tumors), carcinosarcoma, mixed epithelial tumor, and undifferentiated carcinoma [5]. Various subtypes of epithelial ovarian cancers can be also simply divided into two groups named type I and type II by Kurman and Shih in 2010 [6]. Type I tumors are clinically indolent and genetically stable, including low-grade serous, low-grade endometrioid, clear cell, and mucinous and transitional (Breener) carcinomas. Type II tumors are more aggressive and genetically unstable, including high-grade serous, high-grade endometrioid, carcinosarcoma, mixed epithelial tumor, and undifferentiated carcinomas [7].

Over the past decades, the combination of surgery and platinum-based chemotherapy was the standard treatment for advanced ovarian cancer [8]. Although numerous molecular targeting agents have been developed due to deeper understanding of the disease progression, recurrence still commonly occurs in 70% of patients who underwent the first-line treatment within 18 months. The five-year survival rate of those patients with advanced ovarian cancer is only 30.6% [9, 10]. Thus it is crucial to develop effective strategies to attack cancer cells that become resistant to current chemotherapy.

Recently, scientists have proposed that the existence of cancer stem cells was one of the reasons for disease relapse [11, 12]. Traditional chemotherapy can kill the majority of cancer cells, while failing to target cancer stem cells. Moreover, initial treatment increased the proportion of drug-resistant cancer stem cells, resulting in recurrence of disease [13]. In

this paper, we will summarize the studies on ovarian CSCs, including the isolation, their roles in chemoresistance, and the therapeutic approaches.

2. Cancer Stem Cells of Ovaries

The terms cancer stem cells (CSCs) or cancer initiating cells (CICs) are a very small subgroup of tumor cells with the ability to self-renew, differentiate, and form secondary/tertiary tumors after serial xenotransplantation into immune-compromised animal models [14, 15]. Actually, the reason for 90% of tumors arising from ovary surface epithelium is that stem cells reside in the area. In early stage of ovarian cancer, the number of EOC stem cells can be used to predict progression of the disease [16].

Understanding the origin of cancer cells may have clinical significance. It has been reported that both luminal and basal epithelial cells are cells of origin for prostate cancer [17, 18]. In the case of CSCs, it originated not only from adult stem cells that underwent oncogenic transformation, but also from downstream progenitor or differentiated cells with acquired stem cell-like characteristics [19]. However, limited evidence suggested that adult stem cells were the originator of ovarian cancer. Tumors arising from CSCs usually contain a mixed population of cells due to asymmetric division of CSCs. Such cell division can produce one daughter cell that retains the feature of parent cell and another that continually divides to form the bulk of tumor [20].

In 1997, Bonnet and Dick first isolated the cancer stem cells in leukemic cells expressing stem cell marker CD34 [21]. Later, many other types of CSCs were also identified, including ovarian CSCs [22]. The first evidence of ovarian stem cells was the isolation of the ascites from a patient with ovarian cancer [23]. One of the properties for stem cells is to exclude harmful dyes, thus containing less cytoplasmic dyes compared to the rest of the cell population by fluorescence-activated cell sorting (FACS) analysis [24]. Ovarian cancer stem cells can be successfully isolated via distinctive efflux of the DNA binding dye Hoechst 33342. These ovarian CSCs are also called "side population" (SP) stem cells that have the capacity of self-renewal and differentiation in comparison with the non-SPs [25]. However, there is no universal single marker for ideally isolating the ovarian CSCs. In 2009, Gao and his colleagues have isolated SP cells from OVCAR-3, a human ovarian cancer cell line. However, these cell fractions only accounted for 0.9% of the total cell populations [26]. Another study successfully established stable SP cells and ALDH1A1 positive cell populations from the ovarian cancer cell line A2780. Those SP cells exhibited partial resistance to the chemotherapy drug platinum. Nevertheless, it should be noted that a cancer stem cell population may not be a group of cells with a single feature, but may contain overlapping cell fractions with mixed stem-like markers [27].

3. Chemoresistance of Ovarian CSCs

Although the standard combination of surgery and chemotherapy can effectively reduce tumor mass, most patients with residual ovarian CSCs eventually acquire chemoresistance. Hence, recurrent cancer is inevitable in the vast majority of cases [28, 29]. Such phenomenon attracts researchers' attention to decipher the molecular mechanisms involved in escaping the chemotherapy for cancer stem cells.

3.1. Glutathione (GSH) System. The GSH system can suppress oxidative stress and maintain cellular redox homeostasis [30]. The contribution of GSH and GSH-related enzymes to chemoresistance has been demonstrated in different types of tumor, including ovarian cancer and brain tumor [31, 32]. GSH is also involved in the detoxification of various xenobiotics [33]. Upon metabolism of chemotherapeutic agents, the enzymes of glutathione-S-transferase (GST) family could prompt the formation of GSH-drug conjugates. Many chemotherapeutic agents have been shown to conjugate with GSH, including chloroethylnitrosoureas (CENUs), platinum compounds, and other alkylating agents. The resulting GSH-drug conjugates are more water soluble and less active than the compounds themselves. They are thus exported from the cell via the transporter-mediated system [34]. These findings reasonably support the application of antioxidant inhibitors, in combination with standard chemotherapy in patients.

3.2. Overexpression of Bmi-1. Bmi-1, a member of the polycomb group (PcG) family, participates in the self-renewal and maintenance of CSCs [35]. As an oncogene, Bmi-1 could enable cancer cells to escape apoptosis by modulating multiple growth signaling pathways [36]. Thus, its overexpression in cancer cells could be used as a survival marker. The role of Bmi-1 in chemoresistance has been addressed recently. For example, Bmi-1 could allow the resistance of glioma cells to chemotherapy drug such as doxorubicin and bischloroethylnitrosourea (BCNU) [37]. It can also prompt chemoresistance, invasion and tumorigenesis in pancreatic cancer cells [38]. For ovarian cancer cells, silencing of Bmi-1 gene could promote sensitivity to cisplatin and induction of apoptosis [39].

3.3. Loss and Localization of p53. The tumor suppressor gene p53 plays a critical role in cell proliferation and apoptosis by controlling several signaling pathways. Loss of p53 function could cause multidrug resistance in many types of tumors, including ovarian cancer [40]. In addition, the control of intracellular localization of p53 is also associated with the regulation of apoptosis and chemosensitivity in human ovarian cancer cells [41]. The p53-associated Parkin-like cytoplasmic (PARC) protein is critical for p53 subcellular localization and function. It has been demonstrated that a low level of PARC could increase p53 accumulation in nucleus, thus inducing apoptosis [42]. Downregulation of Ca^{2+}-dependent PARC could enhance cisplatin-induced apoptosis in chemosensitive but not in chemoresistant human ovarian cancer cells [43]. The detailed molecular mechanism affecting PARC/p53 interaction between chemosensitive and chemoresistant cancer cells remains to be determined. However, it is vital to note that p53 is not an absolute indicator for the resulting response to

chemotherapy because not all drugs induce cell death via p53 in cancer cells [44].

3.4. Drug Effluxion. The development of multidrug resistance is also associated with the failure of drug uptake. The export of drugs is mediated by transmembrane polysubstrate efflux pumps, which prevent drugs from entering their intracellular targets [45]. These drug transporters are composed of four domains, including two nucleotide-binding domains (NBD) and two transmembrane domains (TMD). The TMD recognizes and translocates substrates, while the NBD is required for conformational changes [46].

Mammalian P-glycoprotein is a transmembrane transporter related with resistance of hydrophobic anticancer drugs. It belongs to one of the ATP-binding cassette (ABC) transporter families [47]. For decades, other efflux transporters in the ABC transporter family have been also identified. For example, ABBC2 encoding for MRP2 (multidrug resistance protein) was involved in effluxion of cisplatin-derived compound in ovarian cancers [48]. ABCG2 (breast cancer resistance protein or BCRP) permitted effluxion of cellular DNA-binding dye Hoechst. Thus, Hoechst can be used to isolate stem-like cells in a variety of tissues, including bone marrow, skeletal muscle, mammary epithelium [49, 50], and ovarian carcinomas [51]. Moreover, ABCG2/BCRP was considered as a drug-resistant marker, which involved in transport of substances and cellular products by using ATP as energy source [52]. In addition to the ABC family, some other transporters have been described such as copper transporter proteins (CTR), organic cation transporters (OCTs), copper-transporting ATPases, and multidrug and toxin extrusion (MATE) [53].

Wender and his colleagues recently conjugated a known drug (Taxol) to oligoarginine, which is a guanidinium-rich molecular transporter responsible for delivery of attached molecule into cells. Such Taxol-oligoarginine conjugates may overcome drug efflux-based resistance through prolonging the half-life of the drug and increasing the drug stability in human ovarian carcinoma cells [54].

3.5. Quiescence of Ovarian CSCs. Mammalian adult stem cells are known to maintain in a quiescent, nondividing, or G0 state [55]. CSCs also demonstrated the similar property. This is also one of the reasons for their resistance to chemotherapy since most anticancer drugs preferentially target dividing cancer cells. Thus, intensive understanding of quiescent mechanism of CSCs is important to improve clinical outcome for cancer patients.

Recent studies have suggested that several genes played key roles in maintaining quiescence of normal stem cells and CSCs. For example, p53 expression was increased and could promote quiescence in hematopoietic stem cells (HSCs) [56]. Necdin, a growth-suppressing protein, as well as a p53 target gene, has been recently identified to improve hematopoietic stem cells quiescence [57]. Nonetheless, the loss of zinc-finger repressor Gfi-1 (growth factor independent 1) enabled HSCs high proliferation [58]. Cited2, a transcriptional modulator, could maintain HSCs quiescence via both HIF-1 (a negative regulator) dependent and independent pathways. Deletion of Cited2 could improve HSCs apoptosis and loss of quiescence. Moreover, its deletion could increase cycling in conditional knockout mice [59]. In addition, the reduced miRNAs (miR-31 and let-7) were demonstrated to keep the balance between lung cancer stem-like side population (SP) cells and nonside population (non-SP) cells. Inhibition of let-7 could prompt growth of both SP and non-SP cells by accelerating G1 to S phase transition, while repression of miR-31 could cause cell cycle arrest in G0/G1 phase in both of SP and non-SP cells [60].

4. Therapeutic Approaches of Ovarian CSCs

The elimination of ovarian CSCs has been challenging in part due to heterogeneity. Thus the efficacy of any single drug was limited for cancer patients. Combined treatments that target CSCs will be a new direction in the future. Nevertheless, drug treatment for CSCs may increase the risk of toxicity since CSCs share common features with normal stem cells. The current therapeutic strategies in ovarian CSCs are discussed below.

4.1. Cell Surface and Nonsurface Markers. Cell surface markers (i.e., CD molecules, short for cluster of differentiation) have been widely used to isolate putative CSCs through flow cytometry. Most types of CSCs share the identical biomarkers, including ovarian cancer stem cells. To activate the immune system to clear cancer cells in patient body, antibody-based therapy for cancer has been developed for decades. Moreover, the strategy of antibody-drug conjugates has achieved considerable success in recent years [61]. Indeed, development of specific therapies that target biomarkers of ovarian CSCs could improve clinical outcome and patient's survival [62].

4.1.1. CD133. CD133, a transmembrane glycoprotein, is one of the most widely described ovarian CSCs markers [63]. Its expression level is higher in advanced serous ovarian cancer than that in normal ovaries and benign tumors [62]. Tumor cells carrying CD133 marker (often abbreviated as $CD133^+$) displayed greater resistance to chemotherapy [63]. In addition, $CD133^+$ ovarian CSCs have hyperactivity in migration and invasion due to the activation of chemokine (c-c motif) ligand 5 (CCL5) [64]. In 2009, Baba and his colleagues found that methylation in promoter region could regulate the expression of CD133 in ovarian cancers, implying that epigenetic modification might be involved in the induction of stemness of tumor [65]. In addition, the combination of a murine derived anti-human CD133 antibody and a cytotoxic drug (monomethyl auristatin F, MMAF) significantly inhibited the cell growth in hepatocellular and gastric cancers [66].

4.1.2. CD44. CD44, another CSC surface transmembrane glycoprotein, is a receptor for hyaluronic acid (HA) involved in cell-cell and cell-matrix interactions. It will ultimately affect cellular growth, differentiation, and motility [67, 68]. CD44 is highly expressed in many types of cancer, including

ovarian CSCs. The $CD44^+/CD24^-$ ovarian cancer cells were correlated with invasion and chemoresistance [69]. Several antibodies against isoforms of CD44 have been developed, and some of them have entered into clinical trials for the patients with head and neck squamous cancers [70]. VFF18 and BIWA-1 were two murine IgG1 monoclonal antibodies that recognized human CD44 variant exon 6 (CD44v6). They were evaluated for their targeting potential in squamous cell carcinoma (SCC) and head and neck SCC (HNSCC), respectively. To avoid human anti-mouse antibody response in patients, humanized forms of such antibodies were developed, such as BIWA-2, BIWA-4 and BIWA-8 [71]. The phase I clinical trial of BIWA-4 (bivatuzumab) has been carried out to evaluate its safety, tumor-targeting potential, pharmacokinetics, and immunogenicity in patients with HNSCC [72]. However, these clinical outcomes still need further confirmation. Except for antibody-based therapy, scientists also proposed other approaches in recent years. Casagrande and his colleagues reported that a toxin called clostridium perfringens enterotoxin (CPE) could eradicate chemoresistant $CD44^+$ ovarian CSCs in mouse xenograft model [73]. In addition, the conjugate of hyaluronic acid to paclitaxel has been also tried for the treatment of ovarian cancer [74].

4.1.3. CD24. CD24 is a glycosylphosphatidylinositol-linked cell surface protein expressed in various solid tumors [75]. Gao et al. have successfully isolated $CD24^+$ CSCs from ovarian tumor specimens and identified CD24 as a putative CSC marker in ovarian cancer [76]. Expression of CD24 affected metastasis and represented poor prognosis in ovarian cancer [77]. A study demonstrated that CD24 could localize in the cytoplasm of ovarian serous tumors, while normal epithelium and serous cystadenomas expressed CD24 marker in the apical membrane. Thus, the cytoplasmic expression of CD24 could be used as a specific marker to predict the survival rates and recurrence of cancer [78]. The depletion and over-expression of CD24 could regulate the phosphorylation of STAT3 and FAK by affecting Src (nonreceptor tyrosine kinases) activity. SWA11, an antibody against CD24 reduced tumor size in xenograft mice transplanted by lung cancer cells A549 and pancreatic cancer cells BxPC3 [79]. In 2009, Su and his colleagues successfully applied short hairpin RNA (shRNA) to reduce CD24 expression. The knockdown of CD24 decreased cell viability by activation of apoptosis in ovarian cell line SKOV3 in vitro and also suppressed tumor growth in nude mice bearing ovarian cancer in vivo [80]. Therefore, CD24 inhibition may be considered as an effective approach for cancer therapy.

4.1.4. CD117. CD117, known as c-kit, is a type III receptor tyrosine kinase involved in cell signal transduction. It involved in various cellular processes, including apoptosis, cell differentiation, proliferation, and cell adhesion [81]. High expression level of CD117 was observed in ovarian cancers [82]. Luo and his colleagues further demonstrated that as few as 10^3 $CD117^+$ ovarian cancer cells had the ability to self-renew, differentiate, and regenerate tumor in xenograft model [83]. It has been also suggested that CD117 in ovarian carcinoma was associated with poor response to chemotherapy [84]. The activation of Wnt/β-catenin-ATP-binding cassette G2 pathway was required for cisplatin/paclitaxel-based chemoresistance caused by CD117 in ovarian CSCs [85]. A potent CD117 specific inhibitor (Imatinib Mesylate) has been used in the clinical trials for the treatment of many types of cancer, including persistent epithelial ovarian cancer [86]. Patel and his colleagues demonstrated that Imatinib Mesylate involved in complex cellular processes, including metabolic pathways, cell cycle, cell proliferation, apoptosis, and signal transduction through mass spectrometry-based proteomics method in human ovarian cancer cell line A2780 [87].

4.1.5. EpCAM. The epithelial cell adhesion molecule EpCAM is a glycosylated membrane protein. It is highly expressed in different tumor types, including colon, lung, pancreas, breast, head and neck, and ovary [88]. EpCAM was found to be hyperglycosylated and frequently associated with cytoplasmic staining in carcinoma tissues [89, 90]. EpCAM is comprised of an extracellular domain (EpEX), a single transmembrane domain and a short 26-amino acid intracellular domain (EpICD). Among them, EpEX is required for cell-cell adhesion [91]. Downregulation of EpCAM could cause loss of cell-cell adhesion and promote epithelial mesenchymal transition (EMT). Metastasis thus occurred in carcinomas [92]. EpCAM positive cells also have tumor-initiating potential, making it a potential target for cancer therapy. Catumaxomab, a monoclonal antibody against EpCAM is a trifunctional antibody, which can bind three different cell types, including tumor cells, T cells, and accessory cells (dendritic cell, macrophages, and natural killer cells) [93]. It is now used in phase III clinical trials in patients with malignant ascites [94]. The investigation of its efficacy and safety was also entered in phase II clinical trials on advanced ovarian cancer patients who had experienced complete chemotherapy. Based on both preclinical and clinical outcomes, EpCAM may be served as a possible therapeutic target against epithelial ovarian cancer.

4.1.6. Aldehyde Dehydrogenase (ALDH) Isozymes. ALDH proteins are a superfamily containing 19 enzymes that protect cells from carcinogenic aldehydes [95]. ALDH1A1 was identified as a putative cancer stem cell marker, and it was associated with chemoresistance in the ovarian CSC [96]. Besides ALDH1A1, other ALDH isozymes such as ALDH1A3, ALDH3A2, and ALDH7A1 also had high expression level in ovarian tumors when compared to normal ovarian tissues [97]. The dual positivity of ALDH and CD133 ovarian cells had higher ability to regenerate tumor in mice than single $ALDH^+$ or $CD133^+$ ovarian cancer cells [98]. These findings suggest that ALDH can be used as a reliable marker to study ovarian cancer stem cells.

Recently, clinical trials have been initiated using disulfiram (an ALDH inhibitor). The combination of disulfiram with a drug named gemcitabine had a synergistic effect on cytotoxicity in glioblastoma multiforme cells [99]. A novel class of ALDH inhibitor (Aldi) discovered recently could

endow lung cancer cell line A549 with higher sensitivity to mafosfamide [100].

Other two stem cell markers, Lin28 and Oct4, are also served as new molecular targets due to their roles in the maintenance of pluripotency in ovarian cancer [101]. In addition, high expression of the Müllerian inhibiting substance (MIS) type II receptor has been reported in ovarian cancer cell lines [102]. MIS could significantly inhibit the cell population with stem-like characteristics in ovarian cancer cell lines [103].

4.2. Differentiation of Ovarian CSCs. Current methods to eliminate CSCs cannot be successfully applied in all clinical situations. One way to eradicate CSCs is to induce their differentiation, resulting in loss of their stemness property [104]. Thus, the understanding of regulation of differentiation processes is necessary for designing new agents to eliminate CSCs. In 2012, Yin and his colleagues observed that TWIST-1 (a basic helix-loop-helix transcription factor) played a key role in triggering differentiation of epithelial ovarian cancer (EOC) [105]. Jain et al. recently reported that p53 capable for regulating molecular networks can activate two miRNAs (miR-34a and miR-145). These miRNAs were then shown to prompt differentiation of human embryonic stem cells [106]. Indeed, emerging evidence indicated that miRNAs were involved in self-renewal and differentiation of normal and cancer stem cells. It is suggested that such miRNAs should be a new therapeutic target for cancer treatment [107].

Retinoic acid (a vitamin A metabolite) and its analogs are the most common differentiation agents. They are also the only agents used in clinical trials [108]. The all-trans-retinoic acid (ATRA) can inhibit the proliferation and induce the differentiation via inhibition of Wnt/β-catenin pathway in head and neck squamous carcinoma CSC [109]. The clinical study of ATRA has shown an increased survival rate of patients with acute promyelocytic leukemia. However, successful cases are limited in solid tumors [110]. Recently, Whitworth and his colleagues effectively reduced the growth of ovarian CSC via a drug (Carboplatin) combined with three novel retinoid compounds [111]. In addition, specific unsaturated fatty acids (palmitoleic, oleic, and linoleic acids) can trigger adipocyte-like differentiation in many types of cancer cells, including ovarian cancer cell line SKOV3 [112]. However, more detailed regulation of differentiation remains to be determined.

4.3. Niches of CSCs. Niches are microenvironments where CSCs reside, containing cell-cell, cell-extracellular matrix, and soluble factors that support the growth, progression, and metastasis of CSCs [113]. Bone-marrow-derived mesenchymal stem cells (MSCs) are known to form fibroblast and myofibroblast populations in the tumor-associated stroma. Recently, evidence has been demonstrated that MSC and derived cell types could secrete prostaglandin E2 and release various cytokines, which is vital for the formation and progression of a tumor [114]. Furthermore, MSC affected metastatic ability and chemoresistance in two ovarian cancer cell lines: OVCAR3 and SKOV3 [115]. Katz et al. reported that tumorigenic ability of ovarian tumor cells was dependent on niches derived from human embryonic stem cells [116]. The hypoxic niches were beneficial for acquirement of stem-like properties of ovarian cancer cells [117].

These findings highlight the vital role of CSCs niches, which represent a promising therapeutic target for eradicating CSCs in the future. Indeed, disrupting components in the niches may yield better outcomes without noncytotoxic effect, when compared with that of removing the CSCs [118].

4.4. MicroRNAs (miRNAs). MiRNAs are a group of small noncoding RNAs with 20–28 nucleotides in length. They could regulate gene expression at posttranscriptional level. Thus, miRNAs are involved in diverse biological processes, such as development and tumorigenesis [119]. The expression profile of miRNAs was different between normal stem cells and CSCs [120, 121]. MiR-214 was highly expressed in ovarian CSCs and endowed the property of self-renewal and chemoresistance in ovarian CSCs via repressing p53-Nanog pathway [122]. MiR-199a significantly rescued the sensitivity of ovarian CSCs to some chemotherapy agents, including cisplatin, paclitaxel, and Adriamycin. Moreover, miR-199a prevented tumorigenesis in xenograft model via downregulating expression of CSCs marker CD44 [123]. In addition, the expression of miR-200a could reduce migrating ability of $CD133^+$ ovarian CSCs. This was because miR-200a inhibited E-cadherin and ZEB2, two genes critical for migration process [124]. However, some miRNAs own oncogenic property, such as miR-125, miR-9, miR-30, miR-21, and miR-215 [125, 126]. In conclusion, miRNAs have become a potential target for ovarian cancer treatment.

5. Conclusion

Understanding the roles of CSCs in cancer therapy may markedly improve the survival rate of ovarian cancer patients. However, it is impossible to cure patients with advanced ovarian cancer in all cases. One possible reason is the heterogeneity of ovarian CSCs, which leads to different sensitivities to the therapy used for one subset of CSCs. Thus combinative therapy will be the major direction for ovarian cancer treatment in the future. In addition, personalized medicine dependent on different genomic background of individuals will become a more effective therapeutic method. Current technological advances, such as next-generation DNA sequencing and mass spectrometry- (MS-) based proteomics, would facilitate implementation of personalized medicine. The establishment of comprehensive gene/protein network from cancer patients could provide more accurate platform for clinical prognosis [127, 128]. In 2012, Vathipadiekal and his colleagues have reported the gene expression profile in ovarian cancer stem cells by affymetrix microarray and identified the activation of Notch signaling pathway, as well as several other genes unique to ovarian CSCs [129].

In brief, we have highlighted the recent advances on ovarian CSCs, including isolation, mechanisms of chemoresistance, and therapeutic strategies. It is easy to imagine that understanding of the CSCs will be helpful to guide medical decision. Basic research is also fundamental to develop new

agents for patients. It is our hope that therapies that target ovarian CSCs will result in better clinical outcomes.

Abbreviations

CSCs: Cancer stem cells
EOC: Epithelial ovarian carcinoma
SP: Side population
GSH: Glutathione
PARC: p53-associated Parkin-like cytoplasmic
ABC: ATP-binding cassette
MRP: Multidrug resistance protein
BCRP: Breast cancer resistance protein
HSCs: Hematopoietic stem cells
HA: Hyaluronic acid
SCC: Squamous cell carcinoma
HNSCC: Head and neck SCC
CPE: Clostridium perfringens enterotoxin
EpCAM: Epithelial cell adhesion molecule
EMT: Epithelial mesenchymal transition
ALDH: Aldehyde dehydrogenase
MSC: Mesenchymal stem cells.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

In Vitro Large Scale Production of Human Mature Red Blood Cells from Hematopoietic Stem Cells by Coculturing with Human Fetal Liver Stromal Cells

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In vitro models of human erythropoiesis are useful in studying the mechanisms of erythroid differentiation in normal and pathological conditions. Here we describe an erythroid liquid culture system starting from cord blood derived hematopoietic stem cells (HSCs). HSCs were cultured for more than 50 days in erythroid differentiation conditions and resulted in a more than 10^9-fold expansion within 50 days under optimal conditions. Homogeneous erythroid cells were characterized by cell morphology, flow cytometry, and hematopoietic colony assays. Furthermore, terminal erythroid maturation was improved by cosculturing with human fetal liver stromal cells. Cocultured erythroid cells underwent multiple maturation events, including decrease in size, increase in glycophorin A expression, and nuclear condensation. This process resulted in extrusion of the pycnotic nuclei in up to 80% of the cells. Importantly, they possessed the capacity to express the adult definitive β-globin chain upon further maturation. We also show that the oxygen equilibrium curves of the cord blood-differentiated red blood cells (RBCs) are comparable to normal RBCs. The large number and purity of erythroid cells and RBCs produced from cord blood make this method useful for fundamental research in erythroid development, and they also provide a basis for future production of available RBCs for transfusion.

1. Introduction

Erythropoiesis defines the process of differentiation and proliferation from hematopoietic stem cells (HSCs) to mature red blood cells (RBCs). In adult humans, erythroid differentiation produces about 2×10^{11} red cells per day. Erythroid lineage development requires a delicate balance between the opposing effects of proliferation promoting factors and differentiation-inducing factors. The two most important cytokines are erythropoietin (EPO) and stem cell factor (SCF) [1]. EPO protects erythroid progenitor cells from apoptosis by activating antiapoptotic proteins, and it also stimulates hemoglobin (Hb) synthesis and is essential for terminal differentiation [2]. SCF acts synergistically with this lineage-specific factor by promoting proliferation of erythroid progenitor cells [1]. A variety of other factors, such as insulin-like growth factor 1 (IGF-1), insulin, and glucocorticoids, have been proposed to have a supportive effect on human RBCs development. In this way, IGF-1 enhances proliferation as well as terminal differentiation, by which it promotes nucleus condensation and enucleation [3]. Glucocorticoids, such as dexamethasone (Dex), seem to have a direct influence on the proliferation of erythroid progenitor cells [4]. *In vivo*, both renewal and maturation of human erythroid progenitors proceed in parallel in the bone marrow. It thus has been difficult to assess the contribution of particular signaling pathways and their deregulation during erythroid cell development.

Despite this knowledge about RBC development, most of the mechanisms underlying erythroid differentiation and maturation both at cellular and molecular levels still remain unknown. In particular, the mechanisms of enucleation, a unique phenomenon of human erythropoiesis, are still unknown. Thus, the availability of an *in vitro* erythropoiesis model, which exhibits all stages of RBC development, would

be a powerful tool for investigating the factors and molecular mechanisms involved in proliferation and differentiation of human erythroid cells under normal and pathophysiologic conditions. Now, most information was obtained from established cell lines and primary animal cell models of chicken and mouse in defined media [2, 5].

Most of the commonly available *in vitro* assays of erythropoiesis are based on cell lines, such as murine MEL or human K562, HEL, and UT-7 cell lines. However, cell lines usually do not recapitulate the entire process of erythropoiesis, as many regulatory pathways have been altered during the transformation process that led to their immortalization. Therefore, for a long time, efforts have been made to establish *in vitro* unilineage differentiation of human primary erythroid cells.

Cord blood has received a great deal of attention as a source for HSCs as an alternative to bone marrow stem cells in transplantation medicine [6, 7]. Furthermore, cord blood is highly enriched in committed hematopoietic progenitors, including those of the erythroid lineage [8]. We addressed this problem by *in vitro* large scale production of erythroid cells from umbilical cord blood derived HSCs.

Decades ago, most *in vitro* experiments were performed under semisolid culture conditions. This approach had the disadvantage that only immature differentiation stages were generated and the relatively small cell numbers derived in colonies severely limited subsequent investigations. In addition, the options for investigating the influence of specific growth hormones were restricted. To overcome these limitations, liquid cultures (LCs) have been developed over the past few years. Most LCs, however, showed only moderate proliferation or an absence of terminal differentiation and enucleation [9–11]. These methods are difficult to test the effects of various factors at different maturation stages since it is difficult to add or subtract components to/from the culture. However, the use of liquid cultures is also limited either by the production of mixed erythroid and myeloid cells or by a weak or absent terminal enucleation [12].

Several reports suggest that hematopoietic niche cells may promote the terminal enucleation of erythroid cells *in vitro* [13, 14]. Recently, Isern, et al. report that the fetal liver (FL) provides a previously unrecognized developmental niche for the maturation and enucleation of primitive erythroid cells (EryP). These results demonstrate that the FL is a niche for maturation of primitive erythroid cells [15]. And the FL is also a major site for the development of definitive erythroid cells (EryD), which matures within erythroblastic islands (EBIs) [16]. EBIs, first identified in bone marrow and later in FL and spleen, are morphologically distinct 3D structures comprising a central macrophage surrounded by EryD at various stages of maturation [17]. Studies have revealed macrophage extensions that surround peripheral erythroblasts, providing intimate membrane contact between these cells. The central macrophages of the EBIs are thought to function as nurse cells during erythropoiesis [17–19]. Therefore, we hypothesized that unknown growth proteins are produced as yet unidentified populations of fetal liver stromal cells that stimulate the maturation of erythroid progenitor cells.

Here, we thoroughly describe optimized long-term serum-free culture conditions that allowed expansion into mass cultures of highly homogeneous human erythroid progenitors derived from umbilical cord blood. Cells could be routinely expanded for more than 50 days, undergoing up to 35 population doublings. The mature red blood cells could be produced by coculturing erythroid progenitors with human fetal liver stromal cells (hFLSCs). With this procedure, it is possible to reproduce different steps of human normal erythropoiesis. The large number and purity of erythroid cells produced from a small amount of peripheral blood make this method useful for studying either normal or pathological erythropoiesis. In addition, production of mature red blood cells from cord blood derived HSCs is of great potential and of importance in practice for it could eventually become an alternate source of cell for transfusion.

2. Materials and Methods

2.1. In Vitro Culture and Characterization of Erythroid Cells from Cord Blood HSCs. After informed consent had been obtained, cord blood from healthy volunteers was collected into sterile heparinized tubes. Mononuclear cells were obtained by centrifugation on Lymphoprep density gradient. The mononuclear cells were then enriched for HSCs by positive selection using anti-CD34-tagged magnetic beads using Mini-MACS columns (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. Purity was more than 95%; as determined by flow cytometry. CD34 positive cells were cultured as described previously [20, 21]. Briefly, for initial expansion, 1×10^6 cells/mL were cultivated in serum-free medium (StemSpan; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with Epo (2 U/mL; Sigma-Aldrich, St. Louis, MO), the synthetic glucocorticoid Dex (1 μM; Sigma), IGF-1 (40 ng/mL; R&D Systems, Minneapolis, MN), SCF (100 ng/mL; R&D Systems), and iron-saturated human transferrin (400 μmg/mL; Sigma-Aldrich).

Homogeneous cultures of erythroid progenitors established after several days were kept in the same medium at 2×10^6 cells/mL by daily partial medium changes. As the cultured erythroblasts being over 5×10^7 (about 20 wells of 24-well plate), we purified the erythroblasts by density centrifugation (Ficoll, 1.077 g/mL). And then only about 2×10^6 cells (about 20 wells of 24-well plate) were continued to be cultured. And the cells lost by density centrifugation were corrected when calculating the proliferation of erythroid cells. Proliferation kinetics of cells were daily monitored using an electronic cell counter (Vi-cell XR, Beckman Coulter, Germany); cumulative cell numbers were calculated as previously described [22]. Cumulative cell numbers were calculated and plotted against time.

Cell morphology was analyzed by light microscopy on cytocentrifuged smears stained with Wright-Giemsa. Hematopoietic colony assays were performed on Day 21. 5000 cells were plated in 35 mm plastic culture dishes containing methylcellulose-based medium (Methocult T GF H4435, StemCell Technologies). Cells were incubated for

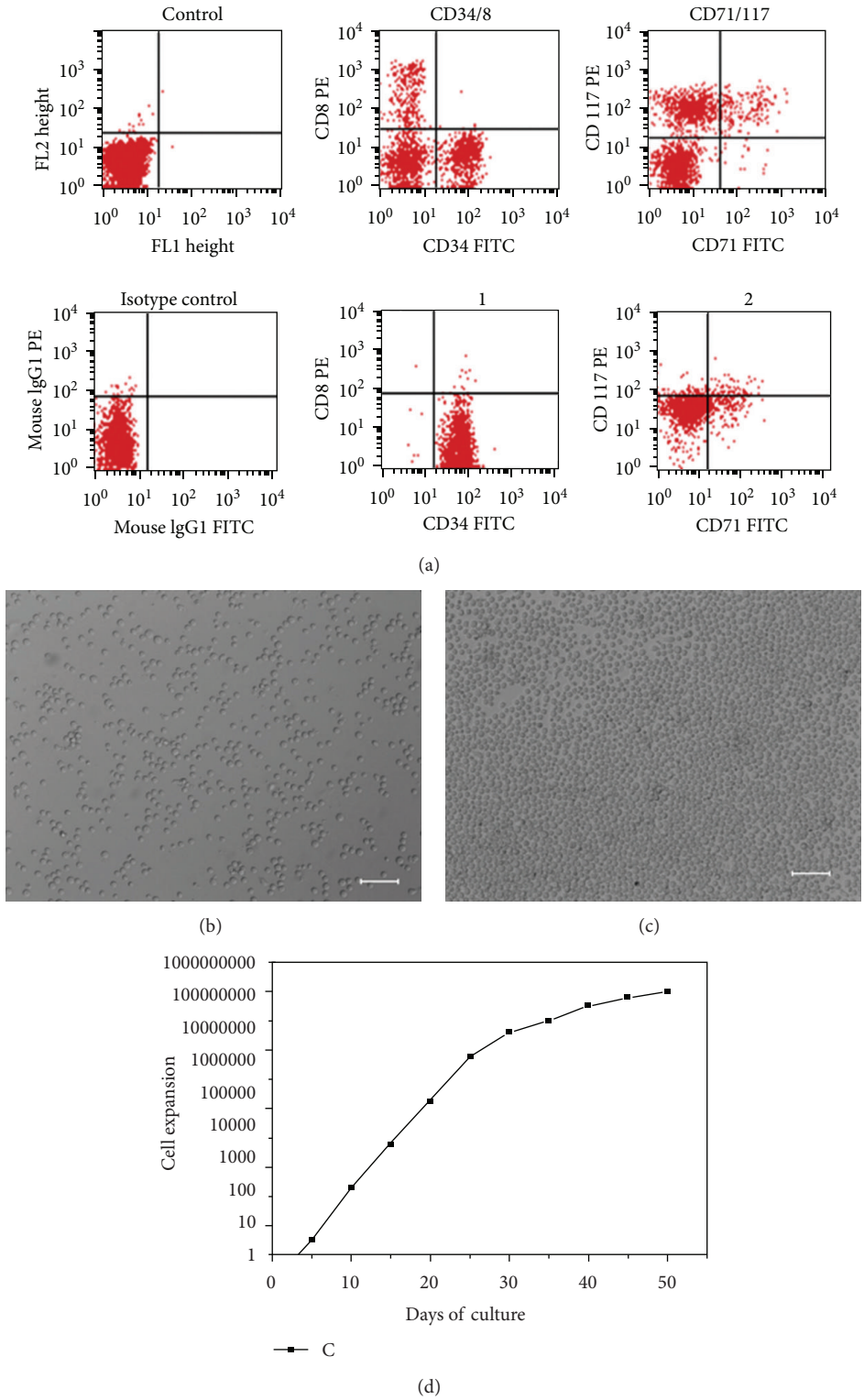

Figure 1: Long-term expansion of human erythroid progenitors from cord blood HSCs. (a) The purity of cord blood derived HSCs was analyzed by flow cytometry. The expression of CD8, CD34, CD71, and CD117 was shown. ((b) and (c)) Morphology of the cultured erythroid progenitor cells in different density. (d) Proliferation kinetics were determined by daily measurements in an electronic cell counter. Cumulative cell numbers were calculated as described in Section 2. Results of one optical conditions are shown.

14 days at 37°C and 5% CO_2. Colonies were enumerated by microscopy.

The surface marker expression of the cultured erythroid progenitor cells were analyzed by flow cytometry. Cells was harvested on days 7, 14, and 21. All of the conjugated antibodies and the corresponding isotype controls were purchased from BD Pharmingen, Heidelberg, Germany. The antibodies against human cluster of differentiation (CD) molecules we used were CD3-allophycocyanin (APC), CD4-fluorescein isocyanate (FITC), CD8-Phycoerythrin (PE), CD13-FITC, CD14-APC, CD19-PE, CD33-FITC, CD34-FITC, CD38-PE, CD41-FITC, CD45-APC, CD71-FITC, CD117-PE, and glycophorin A (GPA)-PE. Erythroid cells were collected and washed twice in phosphate-buffered saline (PBS) with 0.1% BSA and stained in accordance with the manufacturer's suggested concentration of conjugated antibody for 30 min at 4°C. The stained cells were then washed 2× in PBS + 0.1% BSA and fixed with the wash buffer supplemented with 1% paraformaldehyde. The samples were then analyzed using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cell populations were analyzed with the CellQuest program (Becton Dickinson).

2.2. Isolation, Culture, and Characterization of hFLSCs. hFLSCs were obtained from 24-week human fetal liver tissues as described previously [23]. Human tissue collection for research purpose was approved by Research Ethics Committee of Beijing Institute of Transfusion Medicine. Pregnant women gave written consent for clinical procedure and research use of the embryonic tissues in accordance with the Declaration of Helsinki. hFLSCs were cultured in a medium comprising 45% Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich), 45% DMEM/F12 (Sigma-Aldrich), and supplemented with 10% FCS (fetal calf serum; Gibco, Grand Island, NY). Briefly, hFLSCs were prepared using a ceiling culture method. Human fetal liver tissues were minced into 1 mm^3 pieces and transferred onto human gelatin-coated 25-cm^2 flasks. 4 mL of hFLSCs culture medium was added onto the ceiling surface, and after 5 h the flasks were turned back over. The tissue pieces were incubated undisturbed for 7 days at 37°C, allowing the hFLSCs to migrate and adhere to the gelatin-coated surface. Once the primary hFLSCs reached confluency, the hFLSCs were digested with 0.25% Trypsin (Sigma-Aldrich) for 5 min at 37°C. After adding hFLSCs medium, cultures were pipetted into single-cell suspensions, centrifuged, resuspended, and replated onto new flasks.

The surface marker expression of hFLSCs was analyzed by flow cytometry. Stained cells were analysed on a FACS Calibur using Cell Quest software (Becton Dickinson). All of the conjugated antibodies and the corresponding isotype controls were purchased from BD Pharmingen, Heidelberg, Germany. The antibodies we used were CD11b-PE, CD29-PE, CD34-FITC, CD44-FITC, CD45-FITC, CD90-FITC, CD105-PE, and CD144-PE.

2.3. Enucleation of Cord Blood-Derived Erythroid Cells In Vitro. To induce terminal maturation, proliferating erythroid progenitor cells were cocultured with irradiated (40 Gy) hFLSCs at 2×10^6 cells/mL in StemSpan supplemented with Epo (10 U/mL, Sigma-Aldrich) and iron-saturated human transferrin (400 μg/mL; Sigma-Aldrich). Differentiating erythroblasts were maintained at 2 to 4×10^6 cells/mL for 8 days.

Table 1: Flow cytometry analysis of cell surface markers during erythroid progenitor cells differentiation from cord blood derived HSCs (mean ± SD of triplicate assays).

Marker	7 day (%)	14 day (%)	21 day (%)
CD3*	1.78 ± 0.23	0.14 ± 0.08	0.42 ± 0.26
CD8*	1.14 ± 0.19	1.76 ± 0.40	2.31 ± 0.98
CD14*	6.20 ± 1.48	1.4 ± 0.29	2.11 ± 1.20
CD33*	81.23 ± 2.17	66.02 ± 1.93	59.41 ± 4.08
CD38*	30.65 ± 4.42	3.28 ± 0.85	0.84 ± 0.31
CD45	97.15 ± 1.43	96.28 ± 1.78	96.31 ± 0.80
GPA*	1.15 ± 0.51	7.49 ± 1.32	14.92 ± 2.12
CD4*	1.39 ± 0.28	1.16 ± 0.59	3.60 ± 1.27
CD13*	26.40 ± 1.86	4.72 ± 1.51	8.20 ± 1.26
CD19	1.21 ± 0.22	0.08 ± 0.04	0.04 ± 0.03
CD34	0.53 ± 0.11	0.50 ± 0.41	0.64 ± 0.25
CD41*	4.24 ± 1.21	3.18 ± 0.76	2.55 ± 1.57
CD71*	73.46 ± 2.89	96.32 ± 1.44	94.44 ± 0.67
CD117*	63.56 ± 7.05	83.57 ± 3.13	88.72 ± 2.63

The cell surface markers expression of cultured erythroid progenitor cells significantly changed. The expression of CD4, CD8, CD71, CD117, and GPA significantly increased, while the CD3, CD13, CD14, CD33, CD38, and CD41 significantly decreased (* P value < 0.01, using Student's t-test).

2.4. Functional Analysis of Hemoglobin. Cocultured erythroid cells collected at 8 days were used to characterize the function of hemoglobin. Oxygen equilibrium curves were determined using a Hemox-Analyzer, Model B. The gas phase gradients were obtained using nitrogen and room air, and the curves were run in both directions.

2.5. Reverse Transcription-Polymerase Chain Reaction Analysis. Total RNA was extracted by TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 1 μg RNA was then reverse-transcribed into cDNA by Avian Myeloblastosis Virus (AMV) reverse transcriptase (Takara Bio, Shiga, Japan). PCR was performed with rTaq polymerase (TaKaRa). An aliquot of PCR products was analyzed on 1.5% ethidium bromide-stained agarose. β-actin was used as a housekeeping gene to evaluate and compare quality of different cDNA samples. The following primer pairs were used for the amplification of target mRNAs [24]: ζ globin (400 base pair (bp)) forward primer 5′-CCA AGA CTG AGA GGA CCA TCA TTG and reverse primer 5′-AGG ACA GAG GATACGACC GATAGG; ε globin (212 bp) forward primer 5′-AAG ATG AAT GTG GAA GAG GCT GG and reverse primer 5′-TTA GCAAAG GCG GGC TTG AG; β-globin (394 bp) forward primer 5′-GGG CAG GTT GGT ATC AAG GTT AC and reverse primer 5′-GGG GAA AGA AAA CAT CAA GCG; GATA-1 (378 bp) forward primer 5′-TCAATT CAG CAG

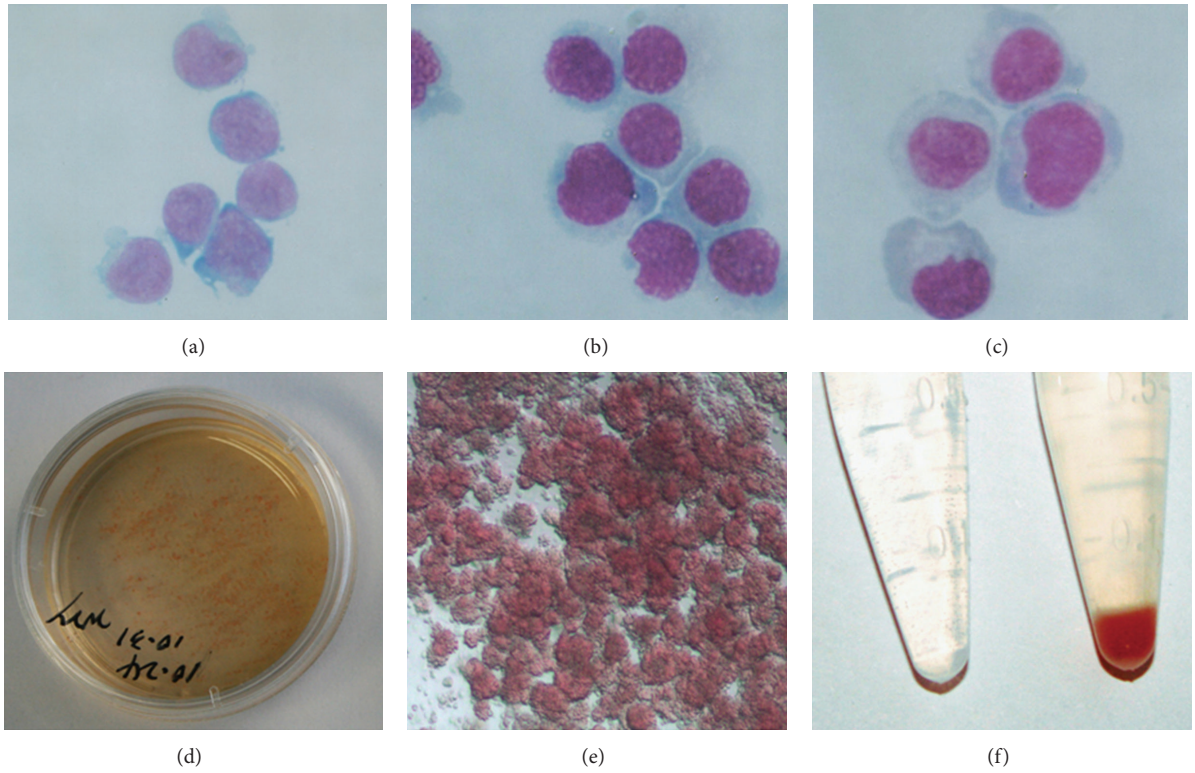

Figure 2: Characterization of cord blood-derived erythroid progenitor cells. ((a)–(c)) Photographs of cytospin preparations of the erythroid progenitor cells stained by Wright-Giemsa. $CD34^+$ cells (a), and day 10 (b) and day 15 (c) erythroid progenitors were cytospined on a polylysine-coated slide and stained with Wright-Giemsa reagents. Original magnification: ×400. ((d) and (e)) Methyl-cellulose assays of 21 days erythroid cells. (f) Erythroid progenitor cells (pellet) cultured for 21 days *in vitro*. Left is PBS as control.

CCT ATT CC and reverse primer 5′-TTC GAG TCT GAA TAC CAT CC; PU-1 (600 bp) forward primer 5′-CGA CCA TTA CTG GGA CTT CC and reverse primer 5′-TTC TTC TTC ACC TTC TTG ACC; SCL/TAL-1 (356 bp) forward primer 5′-ATG GTG CAG CTG AGT CCT CC and reverse primer 5′-ATA TAC TTC ATG GCC AGG CGG; β-actin (222 bp) forward primer 5′-GAT CCA CAT CTG CTG GAA GG and reverse primer 5′-AAG TGT GAC GTT GACATC CG.

2.6. Karyotype Analysis. Karyotype analyses of hFLSCs were carried out at passages 20 using the standard G-banding procedure. The karyotype analyses were done at the Beijing Institute of Radiation Medicine, Cytogenetics Laboratory (Beijing, China).

3. Results

3.1. In Vitro Large Scale Production of Human Erythroid Progenitors. The purity of the isolated $CD34^+$ HSCs was always greater than 95% (Figure 1(a)). $CD34^+$ cells derived from cord blood were induced to erythroid progenitors in serum-free medium (StemSpan) by using a combination of Epo, SCF, Dex, and IGF-1. From day 7 on, morphologically homogeneous erythroid progenitors could be expanded into mass cultures in a rapid time (Figures 1(b)-1(c)), which proliferated exponentially for over 50 days, demonstrating a clear capacity for long-term self-renewal (Figure 1(d)). At regular intervals, more mature or apoptotic cells were removed by density gradient centrifugation. After 50 days, the majority of progenitors was undergoing gradual proliferation arrest before eventually undergoing apoptosis. During the entire expansion, an apparently overall increase in cell number of 10^8-fold to more than 10^9-fold was obtained ($n = 5$). Under optical conditions, the erythroid cells resulted in a more than 10^9-fold expansion within 50 days (Figure 1(d)).

3.2. Characterization of Cord Blood-Derived Erythroid Progenitors. Morphologically, the erythroid progenitors obtained using the above protocol were nucleated and substantially larger than definitive erythocytes with an average diameter of approximately 10 μm. Giemsa-Wright staining showed the morphology of the cultured erythroid progenitor cells (Figures 2(a)–2(c)). In order to characterize the morphological changes of the cultured cells through the stages of erythropoiesis, cell morphology was assessed by microscopic examination, at day 14, greater than 95% of the cells in culture were erythroid cells, and less than 5% of cells morphologically appeared to be promyelocytes and monocytes.

Our protocol for proliferative induction only targeted erythroid progenitors, namely, Burst Forming Unit-Erythroid (BFU-E), Colony Forming Unit-Erythroid

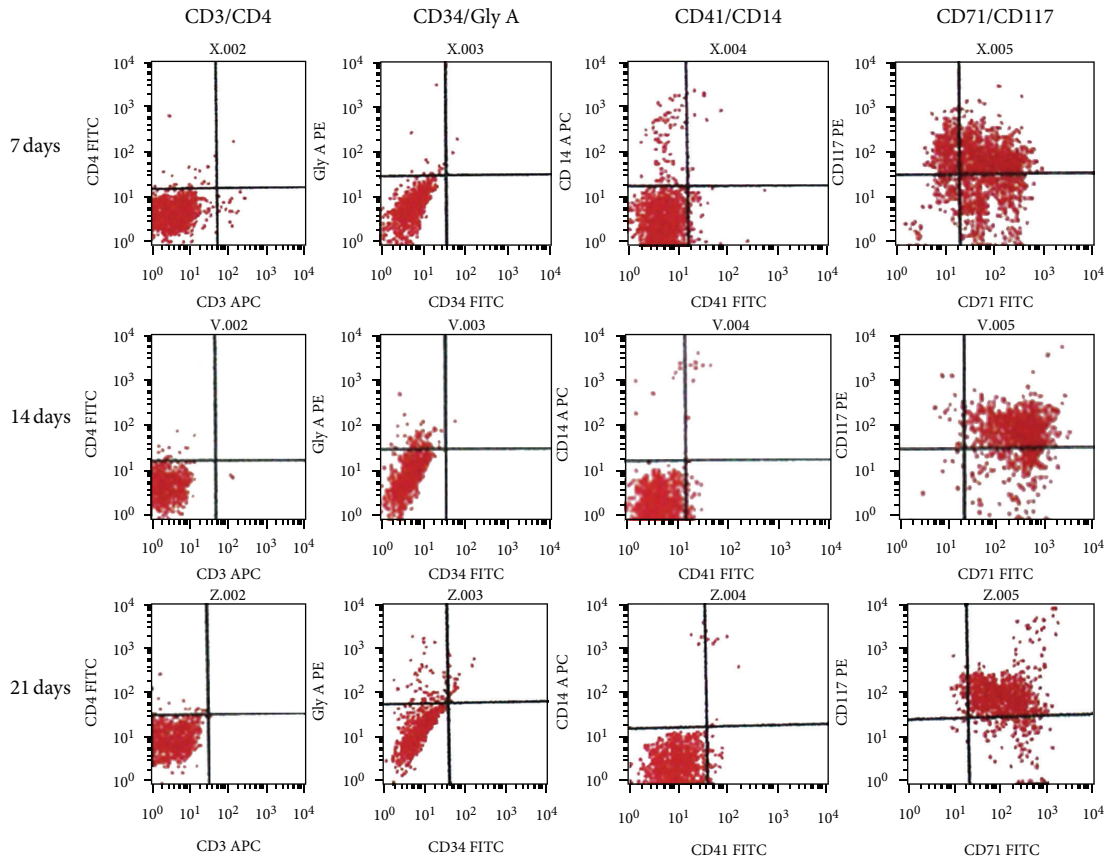

Figure 3: Progression of cell-surface marker expression in developing erythroid progenitor cell cultures. Aliquots of erythroid cells were harvested at the times indicated, stained with combinations of fluorescently labeled antibodies against markers characteristic for different hematopoietic lineages and stages of development, and subjected to flow cytometry.

(CFU-E) but not granulomacrophagic progenitors, namely Colony Forming Unit Granulomacrophagic (CFU-GM) (Figures 2(d) and 2(e)). Hematopoietic colonies formation assay showed that most induced cells of our system are erythroid progenitors.

3.3. Flow Cytometric Analysis of Differentiation into Erythroid Progenitor Cells. Differentiation over time of $CD34^+$ cells was documented by FACS analysis of cell surface markers (Figure 3 and Table 1). At day 21, 95% were CD71 positive, 90% were CD117 positive, and 15% of the cells expressed GPA, whereas the majority of the cells did not express myelomonocytic or megakaryocytic or lymphatic antigens (1% of cells expressed CD14; 2.4% of cells expressed CD41; 3% of cells expressed CD4; 2.5% of cells expressed CD8) and progenitor antigens (0.7% cells were positive for CD34; 0.5% cells expressed CD38) (Table 1).

$CD34^+$ cells rapidly declined, to be quite absent on day 14 (Figure 3). This is expected since CD34 is a marker of early progenitor cells and, in the erythroid lineage, is not expressed after the BFU-E stages. In contrast, GPA, which is a marker of more mature erythroid cells, first appears at the basophilic erythroblast stage and its expression increases throughout the rest of erythroid differentiation [16]. As expected, GPA expression increased almost linearly by the culture time.

3.4. Isolation and Characterization of hFLSCs. Because fetal liver is an important hematopoietic organ for erythroid development, we hypothesized that hFLSCs might produce protein(s) that support *in vitro* terminal maturation of erythroid progenitors. We isolated and cultured hFLSCs from 24-week human fetal liver. After one week culture, the stromal cells migrated out the border of the tissue clumps (Figures 4(a) and 4(b)), and it took two weeks for the cells to become confluent. After the primary and three subsequent passages, hFLSCs were adherent to be fusiform shape and highly uniform in morphology (Figure 4(a)). As hFLSCs are propagated in culture for more than 30 passages, there are no obviously cell morphology changes. Flow cytometry analysis was used to characterize cell surface markers of hFLSCs, showing that the hFLSCs were positive for some stromal progenitor markers, such as CD29, CD44, CD71, CD90, and CD105, but negative for CD34 and CD45 (Figure 4(c)). hFLSCs maintained normal karyotypes after 20 passages (Figures 4(d) and 4(e)).

3.5. Enucleation and Maturation of Cord Blood-Derived Erythroid Cells In Vitro. A critical scientific and clinical issue is over whether cultured erythroid cells can be matured *in vitro* to generate enucleated erythrocytes. We tried to induce erythroid progenitors to terminal maturation by coculturing

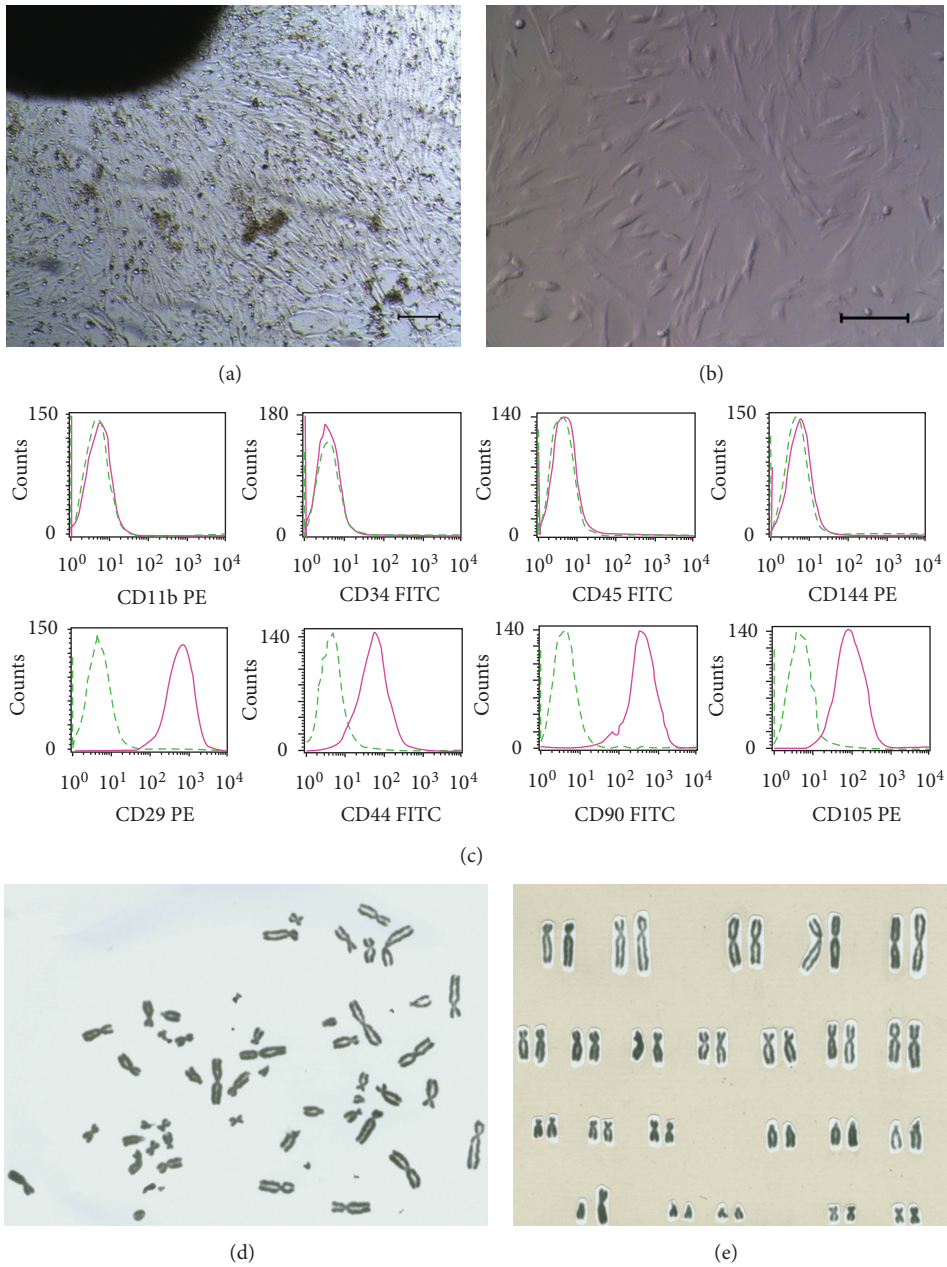

Figure 4: Isolation and characterization of human fetal liver stromal cells (hFLSCs). ((a) and (b)): hFLSCs derived from 24-week fetal liver showed morphology of flattened fibroblast-like cells when grown to confluency. Bars = 100 μm. (c) Surface marker of hFLSCs analyzed by flow cytometry. ((d) and (e)) Normal 46, XY karyotype of hFLSCs for 20 passages.

with hFLSCs. Erythroid progenitor cells cultured in this condition with hFLSCs to induce terminal maturation. And the cells expanded one or more times when cocultured with hFLSCs (Figures 5(a) and 5(b)). Approximately 60–80% of erythroid cells were enucleated when these cells were transferred to hFLSCs layers from nonstromal cultures and cocultured from days 8 (Figures 5(c) and 5(d)). Erythroid progenitor cells kept in liquid conditions (without transfer to hFLSCs) could enucleate 10%–30% suggest that enucleation could not be achieved completely in feeder-free condition.

To further investigate the events associated with enucleation, we examined cell surface marker expression related to the process of erythrocyte maturation. We observed a progressive decrease of CD117, an early erythroblast marker, on day 8 and decreased their expression over time. And they showed low to negligible level of GPA protein, a mature erythrocyte marker, at the beginning, but increased their expression dramatically with their maturation (Figure 5(e)).

3.6. Gene Expression during Erythropoiesis. Erythroid progenitors cultured at day 10 and 15 and enucleated cells at day 4 and 8 were analyzed for erythroid gene expression (ζ, ε, and β globins, GATA1, PU-1, and SCL/TAL-1) (Figures 6(a) and 6(b)). The expression level of transcription factor GATA1

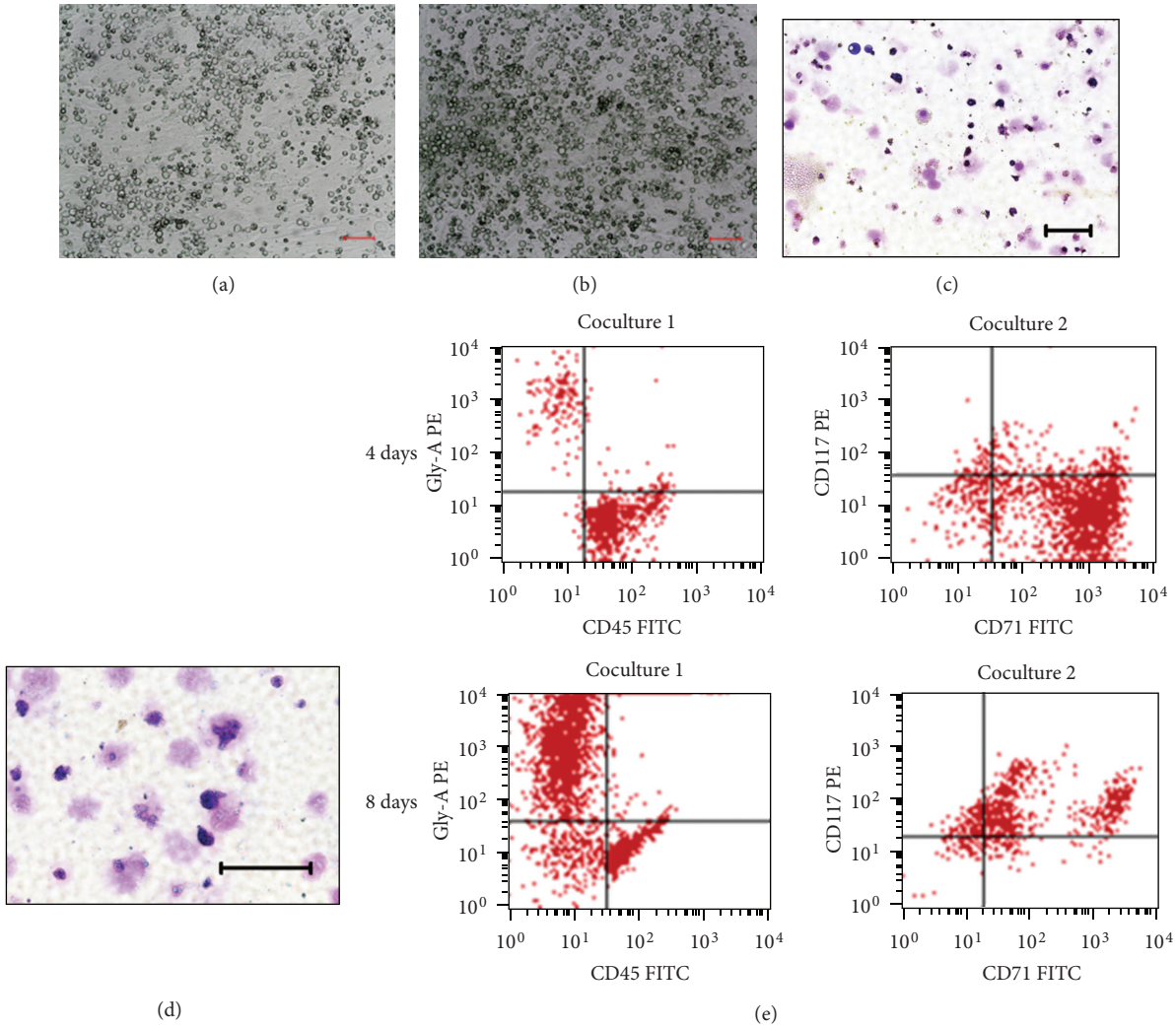

Figure 5: Enucleation of cord blood HSCs-derived erythroid cells *in vitro*. ((a) and (b)) Erythroid progenitors cocultured with hFLSCs for 4 days (a) and 8 days (b). Bars = 100 μm. ((c) and (d)) Erythroid cells derived from human cord blood HSCs were cocultured with hFLSCs for 8 days. On day 4 (c) and 8 (d), cells were cytospun and stained with Wright-Giemsa dye. Bars = 100 μm. (e) Maturation of cord blood-derived erythroid cells mimic erythroid development. Expression of GPA, a mature erythrocyte marker, increases with time and CD71, an immature red blood cell marker, shows a decrease in expression over time.

mRNA was upregulated during the early stages of erythroid differentiation and then downregulated when cocultured with hFLSCs. At an early stage of differentiation, ζ and ε-globin mRNA was hardly expressed; during maturation the expression level of β-globin mRNA increased. At cocultured day 8, the enucleated cells only expressed the β-globin.

3.7. Oxygen Equilibrium Analysis. The oxygen equilibrium curves of the cord blood-derived erythroid cells (cultured with hFLSCs for 8 days) were either very similar to or somewhat rightward shifted (Figure 6(c)), relative to those of human cord blood and normal adult RBCs.

4. Discussion

The expansion of hematopoietic progenitors from umbilical cord blood *ex vivo* is of great interest, both for basic research and the exploitation of clinical potential. This work described a method which enabled the purification and *in vitro* expansion of large numbers of erythroid progenitors from cord blood-derived HSCs. This system could be used to reproducibly generate large numbers of erythroid cells under serum-free conditions.

In the presence of Epo, SCF, IGF-1, and Dex added after 2 week of culture, almost all the cells were erythroid progenitor cells positive to erythroid specific cell surface markers. After long time culture, the culture conditions resulted in a great cell expansion and accumulation of erythroid cells. So, Epo, SCF, IGF-1, and Dex represented the most potent factors required for inducing proliferation of erythroid progenitor cell [1, 20, 21, 25]. The complete erythroid differentiation and maturation were documented by the morphological analysis and by changes in surface markers expression. More

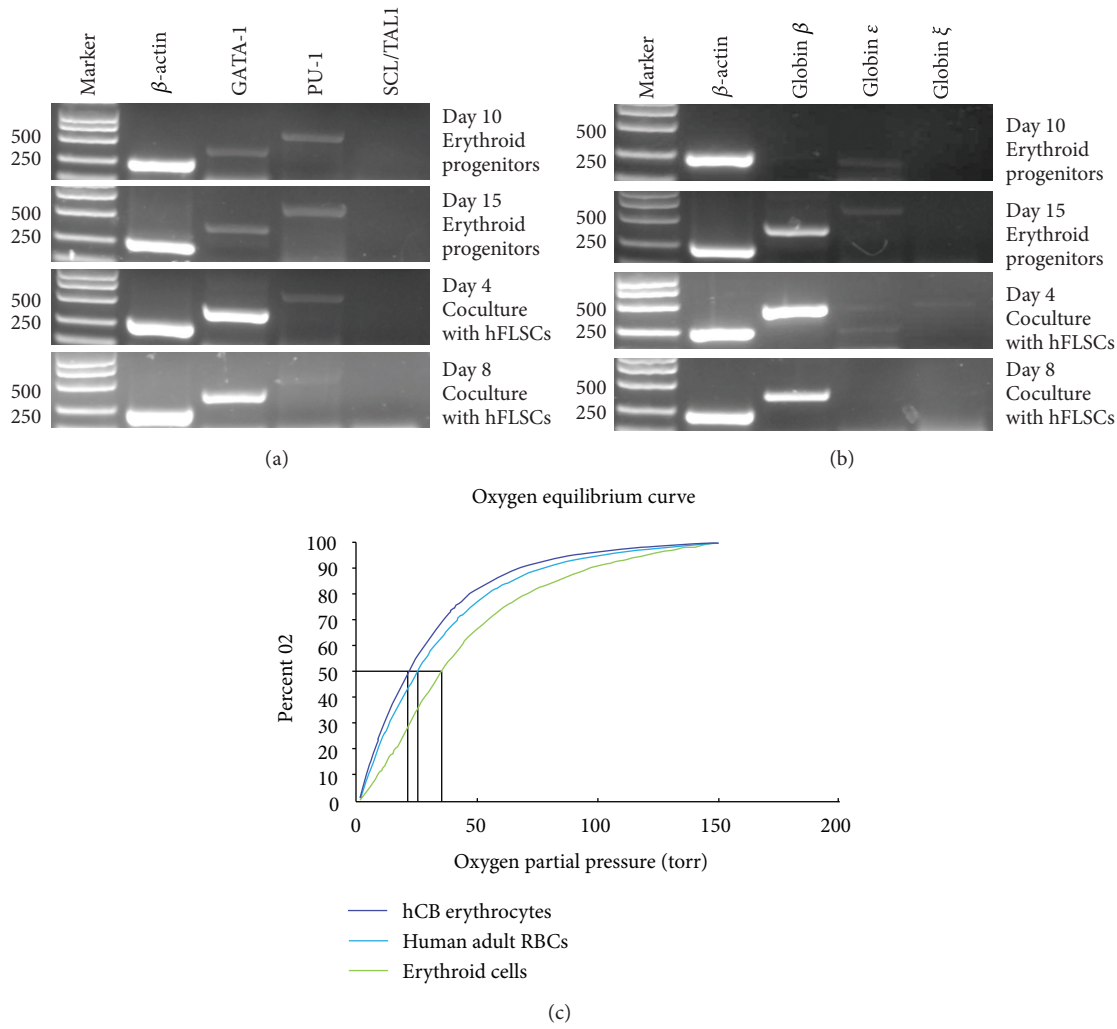

Figure 6: Characterization of cord blood HSCs-derived red blood cells. (a) RT-PCR analysis for erythroid specific transcriptor gene expression. (b) RT-PCR analysis for globin gene expression. Gene expression was normalized to the internal control, β-actin gene, for each data point. (c) Functional characterization of cord blood HSCs-derived erythroid cells. Oxygen equilibrium curves of normal human adult and cord blood RBCs and cord blood-derived RBCs by coculturing with hFLSCs for 8 days.

than 90% of the cells expressed GPA after 8 days of being cocultured with hFLSCs.

CD71 is highly expressed on both proliferating cells of various cell types and early erythroid cells with high iron uptake for Hb synthesis [26, 27]. After day 14 in the erythroid progenitor cells expansion conditions, CD71 expression is up to 97%. And on the last culture days with hFLSCs almost all cells expressed GPA. Furthermore, most GPA^+ cells became $CD71^-$, which is known as a sign of cell cycle arrest and terminal maturation to erythrocytes [28].

Previously used procedures failed to yield long-term proliferating but immature erythroid progenitors, due to massive spontaneous differentiation [12, 29]. Using serum-free media together with Epo, SCF, IGF-1, and Dex we could expand human erythroid progenitors from cord blood derived HSCs for more than 50 days. This yielded mass populations with an *in vitro* lifespan of approximately 30–35 generations, almost absent from spontaneous differentiation largely facilitating future biochemical and molecular characterization. Importantly, optimization of differentiation conditions allowed induction of synchronous terminal maturation, resulting in enucleated cells virtually indistinguishable from erythrocytes of peripheral blood.

In our hands, the serum-free medium used here was critical for generating long-term cultures. Other serum-free media proved to be less efficient. Compared with other current procedures, it should also be noted that our methods did not require negative selection (depletion) against lymphoid or granulocytic cells [30]. Routinely, a (calculated) 10^8–10^9-fold erythroid cells expansion was obtained, in some cases even a (calculated) 10^{10}-fold increase was obtained (about 35 generations). Thus, the procedure described here enables

the production of large numbers of homogeneous primary erythroid cells for detailed molecular characterization of mechanisms.

In vivo, stromal cells form a complex microenvironment for HSCs that controls their multiple fates, including apoptosis, migration, and the cell divisions that lead to formation of lineage-committed progenitors [31, 32]. Different types of stromal and other cell types can have either positive or negative effects on fates of HSCs. In previous studies, terminal differentiation and enucleation of erythrocytes *in vitro* were reported to be dependent on macrophages presence in cultures [33, 34]. Giarratana et al. [13, 35] recently described a protocol to achieve *in vitro* terminal erythroid maturation (90%–100% of enucleated erythrocytes) using the murine stromal cell lines MS5 or human stromal cell lines to reproduce *ex vivo* the microenvironment existing in vivo. In our model, terminal erythroid maturation was satisfactorily achieved in coculturing with hFLSCs, making the culture system more safe.

However, the underlining mechanism of the hFLSCs during erythroid differentiation was not fully explained in the present study. There are some other studies showed that the mouse and human FLSCs can promote the *in vitro* generation of erythroid cells from different sources of stem cells [36–38]. All these studies suggested that the fetal liver stromal cells might mimic the hematopoietic supportive environment of the fetal liver *in vitro* through many different pathways [39].

Erythropoiesis at molecular level is driven by a combination of transacting factors that act in concert to direct the genes expression for erythroid-specific proteins [40, 41]. We established that culture conditions of cord blood-derived HSCs allow to reproduce normal erythroid differentiation confirmed also at the molecular level. Genes required for primitive erythropoiesis were expressed at high levels in early days of cultures. Globin gene expression in the maturing erythrocytes *in vitro* recapitulated expression patterns which occur in adult hemoglobin expression well [42, 43]. A more recent study has used the *in vitro* culture model to study the molecular mechanisms of erythropoiesis. The results show that KLF1 is a critical activator of the BCL11A gene, encoding a suppressor of HbF expression [44]. And, little has been known so far about enucleation mechanism of erythroblasts. Our methods also provided a new model to investigate molecular and cellular mechanisms involved in the enucleation of human RBCs.

We demonstrated here that erythroid cells derived from cord blood HSCs possessed oxygen equilibrium curves comparable to normal cord blood and adult RBCs. This finding demonstrated that the cord blood-derived RBCs have oxygen carrying properties that are comparable to those of normal adult erythrocytes. Another critical issue for clinical utilization of cord blood-derived RBCs is about whether they can be enucleated *in vitro*. hFLSCs coculture improved efficiency of enucleation, but even without these cells enucleation could not be achieved for a completely feeder-free system. We showed that 60%–80% of the RBCs underwent multiple differentiation events, including a progressive decrease in size and increase in GPA expression (a mature RBC marker), which is similar to normal RBCs. However, further study will be necessary to investigate cord blood-derived erythroid cells in vivo function.

Taken together, culture method described here could facilitate extensive biochemical analyses of erythropoiesis, which were technically demanding so far due to lack of material. Our system also provided a useful basis for future production of available RBCs for transfusion.

Acknowledgments

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Research Article

Establishment of a Lentiviral Vector Encoding Human HGF and the Infection of Human ADSCs

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The delivery of adipose-derived stem cells (ADSCs) for promoting tissue repair has become a potential new therapy, while hepatocyte growth factor (HGF) is an important growth factor with angiogenic, anti-fibrotic, and anti-inflammatory benefits. In this paper, hADSCs were separated, cultured and identified based on the expression of cell surface antigens and multiple differentiation potential. We successfully generated a lentiviral vector encoding human HGF, infected hADSCs with this vector and examined the protein expression pattern. Finally we found that the hHGF lentiviral vector was successfully generated, and the lentiviral vector was able to safely infect hADSCs with high infection efficiency, thereby producing cells that overexpressed hHGF, which may provide a new strategy for the treatment of ischemic heart disease (IHD) and other ischemic diseases.

1. Introduction

Ischemic heart disease (IHD) remains the leading cause of death in modern society. The pathologic mechanism of IHD is characterized by the irreversible loss of functional cardiomyocytes followed by myocardial fibrosis and ventricular remodeling due to the decreased number of vessels, which diminishes the blood supply [1]. Therapeutic angiogenesis is currently a popular focus of study in IHD research involving growth factor protein therapy, cell transplantation, and gene therapy [2]. With the development of vascular tissue engineering, stem cell technology has been widely used and represents the latest advances in this field. Mesenchymal stem cells (MSCs) are a group of heterogeneous multipotent cells that can be isolated from many tissue types. Many studies have focused on MSCs isolated from bone marrow. However, there are issues associated with the clinical use of MSCs derived from bone marrow, including pain, morbidity, and low numbers of harvested cells. In contrast, adipose tissue contains an abundance of adult stem cells (termed adipose-derived stem cells, ADSCs), and these ADSCs are easy to isolate and differentiate into osteogenic, chondrogenic, myogenic, endothelial, and neurogenic lineages [3, 4]. Animal studies have shown that ADSCs display potential beneficial effects for therapeutic angiogenesis [5, 6].

HGF, which was originally identified and cloned for hepatocyte, has been reported to exert mitogenic, angiogenic, antiapoptotic, and antifibrotic activity in various cell types [7]. Several studies have revealed that HGF is an endogenous cardioprotective factor, as it protects cardiomyocytes from acute ischemic death during acute myocardial infarction (AMI) and enhances the survival of cardiomyocytes exposed to oxidant stress [8, 9]. Recent study showed that intramyocardial injection of HGF and microbubbles (MBs) in combination with insonation enhanced neovascularization and reduced ventricular remodeling and infarct size [10].

Therefore, the angiogenic effects of ADSC transplantation combined with HGF expression directed into the infracted heart area may exert more beneficial effects than either gene therapy or stem cell therapy alone. In the present study, we generated a lentiviral vector encoding human HGF, infected ADSCs with this vector, and examined the protein expression pattern to provide a new strategy for the treatment of IHD.

2. Materials and Methods

2.1. Identification and Sequencing of the pcDNA3.0-hHGF and hHGF PCR Amplification Products. The pcDNA3.0

plasmid encoding human HGF (pcDNA3.0-hHGF) was a kind gift provided by Dr. Y. S. Zhou at the Chinese Academy of Military Medical Sciences. The pcDNA3.0 plasmid and hHGF open reading frame (ORF) sequences were obtained from the Invitrogen and NCBI websites. Based on the genomic sequences, we selected the Hind III, Xba I, BamH I, and Not I restriction enzyme sites, and we sequenced the DNA. The hHGF mRNA primers were designed using Primer 3 software. The forward primer (5′-GATCCGCTAGCGCTACCGGTCGCCACC *ATGTGGGTGACCAAACTCC*-3′) and reverse primer (5′-TCACCATGGTGGCGACCGGTAG*TGACTGTGGTACCT TATATGTTA*-3′) both contained an Age I restriction enzyme site. The length of the amplified segment was 2218 bp.

The PCR products were assessed via electrophoresis using a 1% agarose gel. The PCR products were isolated from the gel using a viltaLight lamp. The target gene PCR products were isolated and purified using a gel extraction kit.

2.2. Cloning the hHGF Genomic Fragment into the pGC-E1 Vector. The purified hHGF fragment and pGC-E1 gene plasmid were separately digested with Age I. Using an In-Fusion Kit, the hHGF fragment was ligated into the pGC-E1 expression vector, which was previously digested with Age I. The total ligation reaction volume was 20 mL (2 mL of 100 mg/mL vector DNA, 2 mL of 100 ng/mL hHGF fragment, 1 mL of 10x In-Fusion exchange enzyme buffer, 0.5 mL of In-Fusion exchange enzyme, and 13.5 mL of ddH_2O). Two control groups, either without vector DNA or without hHGF, were included in the reaction system. The reaction was run at 23°C for 15 min followed by 42°C for 15 min, and the DNA cloning ligation system was then prepared.

After transforming the DNA into DH5α competent bacteria cells, which were grown in LB medium, the positive clones were identified via PCR to verify the successful insertion of the hHGF fragment into the lentiviral shuttle plasmid, and the PCR products were subsequently analyzed via DNA sequencing by Beijing GeneChem Co. The final plasmid was termed pGC-E1-hHGF.

2.3. Production and Titration of the Recombinant Lentiviral Vector. The recombinant pGC-E1-hHGF vector and two packaging components (pHelper1.0 and pHelper2.0) were extracted from the positively transformed bacteria. The plasmid DNA concentration was measured using UV A260/A280 absorption values (normally between 1.8 and 2.0). The recombinant lentiviral vector, Lenti-hHGF, was generated by cotransfecting 293T cells with 20 g of pGC-E1-hHGF, 15 g of pHelper1.0, and 10 g of pHelper2.0 in 10 cm dishes with Lipofectamine 2000 (Invitrogen, USA). The 293T cells were then cultured in DMEM (Gibco, Invitrogen) containing 10% heat-inactivated fetal bovine serum FBS (Gibco, Invitrogen). The culture supernatants were collected every 24 h for 3 days, filtered through a 0.45 um pore size filter, and concentrated twice via ultracentrifugation at 50,000 xg at 20°C for 120 min. The viral supernatants were concentrated 1,000 times by ultracentrifugation, resuspended in sterile phosphate-buffered saline (PBS), and then stored at −80°C until use. The virus titers were determined via a one-in-one whole dilution.

2.4. Isolation, Culture, and Identification of ADSCs

2.4.1. ADSC Isolation and Culture. Human adipose tissue derived from patients undergoing selective section-assisted lipectomy was collected, after obtaining informed consent from the patients, according to procedures approved by the Ethics Committee at Chinese Academy of Medical Sciences and Anhui Provincial Hospital of Anhui Medical University. This tissue collection procedure has been described previously [1]. Briefly, adipose tissue was extensively washed with D-Hanks' solution (Gibco Life Technologies, Paisley, UK) to remove contaminating debris and red blood cells, cut into small pieces, and then digested with 0.2% collagenase II (Sigma, St. Louis, MO, USA) at 37°C for 30 min with gentle agitation. The collagenase was inactivated with an equal volume of DMEM/10% FBS. The cells were washed twice and plated in T-75 tissue culture flasks at a density of 2×10^6 cells/mL. The expansion medium contained 90% DMEM, 10% FBS, 10 ng/mL epidermal growth factor (EGF, Sigma), 10 ng/mL platelet-derived growth factor BB (PDGF-BB, Sigma), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco, Invitrogen). The medium was changed 24 h later to remove the nonadherent cells. Once the adherent cells were more than 90% confluent, the cells were separated and cultured.

2.4.2. Immunophenotype Analysis of ADSCs. The third passage of ADSCs was adopted for immunophenotype analysis. Cells (2×10^5) were resuspended in 200 μL of PBS containing 0.5% BSA and incubated (in the dark) for 30 min at 4°C with fluorescence-labeled antibodies against human CD14-phycoerythrin (PE), CD29-fluorescein isothiocyanate (FITC), CD31-FITC, CD34-PE, CD44-FITC, CD45-peridinin chlorophyll protein (PerCP), CD71-PE, CD86-PerCP, CD106-PE, CD117-PE, HLA-DR-FITC, or the appropriate isotype controls, which were obtained from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). The cells were analyzed via flow cytometry using an ELITE flow cytometer with WinMDI2.9 software (Beckman Coulter, Fullerton, CA,USA).

2.4.3. The Multiple Differentiation Ability of ADSCs. For osteogenic differentiation, cells at passage 5 were incubated in DMEM medium containing 10% FBS, 20 nM dexamethasone, 100 U/mL penicillin, 100 mg/mL streptomycin, 2.5 mg/mL amphotericin, 10 mM b-glycerophosphate, and 0.05 mM L-ascorbic acid-2- phosphate. Control cultures were fed only DMEM containing 10% FBS and antibiotics. After 21 days of culture, the osteogenic differentiation of stem cells was confirmed via the positive alizarin red staining of the mineralized matrix.

For adipogenic differentiation, the cells were incubated with DMEM medium containing 3% FBS, antibiotics, 33 mM biotin, 17 mM pantothenic acid, 1 mM insulin, 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX),

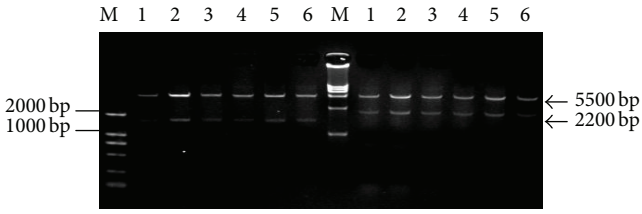

Figure 1: The pcDNA3.0-hHGF vector double digested, first with Hind III, Xba I and BamH I (left) and then with Not I (right). 1–6: Clone 1–6; M: DNA marker. Clones 1–6 all tested positive.

5 mM rosiglitazone, and 5% rabbit serum for 3 days. The cells were then treated with inducing medium without rosiglitazone and IBMX. After 19 days of culture, the cells were fixed with 10% formalin and incubated for 20 min with Oil-Red O to visualize lipid droplets.

2.5. Infection of ADSCs with Lentiviral Vectors. On the day of infection, the cells were plated at a density of 4×10^4 cells/well in 96-well plates along with lenti-hHGF or lenti-GFP at different multiplicities of infection (MOI) in serum-free growth medium containing 5 μg/mL polybrene. Serum-containing growth medium was added after 4 h and replaced after 48 h. Reporter gene expression was examined using fluorescent microscopy on day 4 or 5 after infection. The ideal MOI for the formal experiment was selected. The infected cells were passaged, and the percentage of GFP^+ cells was assessed via flow cytometry.

2.6. Detection of hHGF Protein in Target Cells. The hHGF protein expression levels in the ADSCs were assessed via western blot analysis. Briefly, uninfected ADSCs and ADSCs infected with either lenti-hHGF or lenti-GFP were harvested, and a specific volume of ice cold 2x lysis buffer was applied to the cells. The supernatant was collected, and the protein concentration was assessed using a Bradford assay. Sample buffer (2x) was then added to the samples (the volume of buffer depended on the concentration of the protein sample), and the proteins were denatured at 100°C for 5 minutes. The protein samples were then separated via 12% SDS-PAGE electrophoresis (25 μg/pore). The proteins were transferred to PVDF membranes at 120 V for approximately 2 hours. Subsequently, the membrane was blocked with a 5% milk TBST solution overnight. The membranes were then incubated with primary antibody (1 : 1000 dilution) at room temperature for 2 hours, washed three times with TBST (3×10 min), incubated with secondary antibody (1 : 1000 dilution) at room temperature for 2 hours, and washed again. Finally, ECL reagent was added to visualize the protein bands. The film was exposed after a 5-minute incubation with ECL reagent, and the film was then developed and fixed. Actin was used as a control to assess the relative expression levels of the proteins in each group.

To detect the expression of hHGF protein in supernatant, ELISA was used. Cell culture supernatants were collected from the culture media of uninfected ADSCs and ADSCs infected with either lenti-hHGF or lenti-GFP at the time points of 1, 3, 5 and 7 days after infection seperately. Concentrations of hHGF protein in the cell supernatant were determined by ELISA (Human HGF ELISA kit; R&D) according to the manufacturer's protocols.

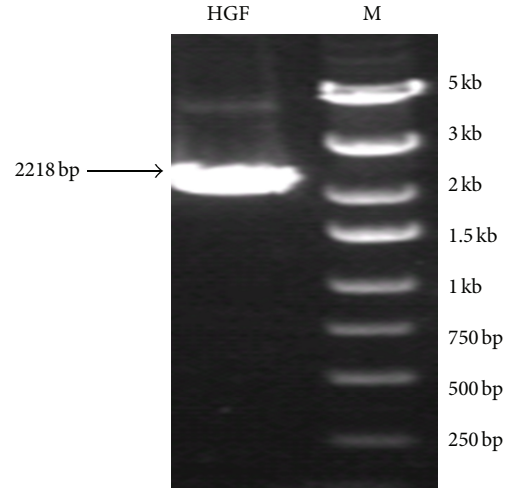

Figure 2: hHGF amplification product (PCR).

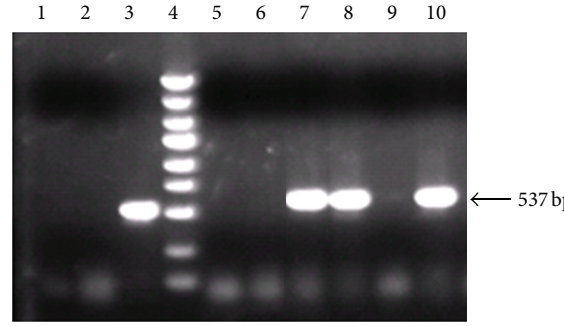

Figure 3: Verification of pGC-E1-hHGF expression in bacterial clones via PCR. Lane 1: negative control (ddH_2O); lane 2: negative control (pGC-E1 empty vector); lane 3: positive control (pcDNA3.0-hHGF); lane 4: marker (5 kb, 3 kb, 2 kb, 1.5 kb, 1 kb, 750 bp, 500 bp, and 250 bp); lanes 5–10: six pGC-E1-hHGF-transformed clones. Clones 7, 8, and 10 tested positive.

2.7. Statistical Analysis. SPSS 16.0 for windows was used for all statistical analysis. Variables were presented as mean ± standard deviation. Comparisons between 2 groups were made by Student's t-test. For $\geqslant 3$ groups, one-way analysis of variance with a post hoc test of LSD test was used for the statistical analysis. A P value of <0.05 was considered significant.

3. Results

3.1. Identification and Sequencing of the pcDNA3.0-hHGF and hHGF PCR Amplification Products. Six pcDNA3.0-hHGF clones were double digested, first with Hind III, Xba I, and BamH I and then with Not I. Based on the sequences after

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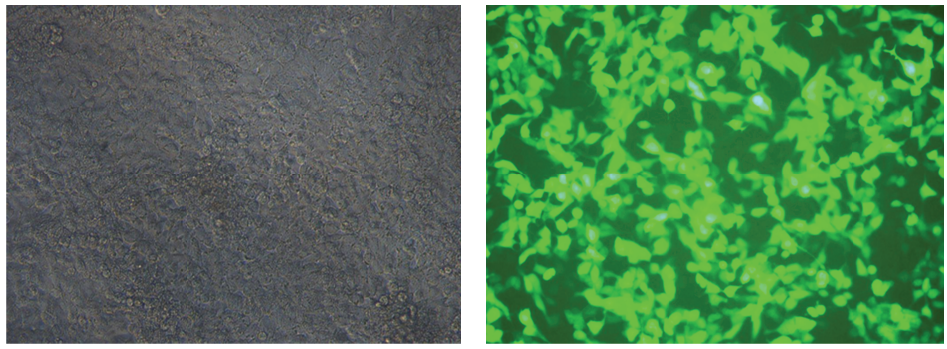
Query    1  TGACTGTGGTACCTTATATGTTAAAATAATTTTGTGTATCCATTTTGCATAATATGCTAC 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2334 TGACTGTGGTACCTTATATGTTAAAATAATTTTGTGTATCCATTTTGCATAATATGCTAC 2275

Query   61  TCGGAC-AAAATACCAGGACGATTTGGAATGGCACATCCACGACCAGGAACAATGACACC 119
           ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2274 TCGGACAAAAATACCAGGACGATTTGGAATGGCACATCCACGACCAGGAACAATGACACC 2215

Query  120  AAGAACCATTCTCATTTTATGTTGCTCCCAAACAAGTGGGCCACCATAATCCCCCTCACA 179
           |||||||||||||||||||||||||  ||||||||||||||||||||||||||||||||
Sbjct  2214 AAGAACCATTCTCATTTTATGTTGCTCACAAACAAGTGGGCCACCATAATCCCCCTCACA 2155

Query  180  TGGTCCTG 187
           ||||||||
Sbjct  2154 TGGTCCTG 2147
```

Figure 4: Gene sequence results of the hHGF insert in the pGC-E1-hHGF expression vector (after the positive clones were selected).



(a) (b)

Figure 5: GFP expression of the hHGF recombinant lentiviral plasmid in 293T cells 48 h after transfection (100x). Left: dark field; right: bright field.

digestion with Hind III and Xba I, four bands (5.4 kb, 1.7 kb, 440 bp, and 113 bp) were identified. After digestion with BamH I and Not I, a vector band (approximately 5.5 kb) and an hHGF band (approximately 2.2 kb) were observed. According to the results, there were six positive clones with two bands (approximately 5.4 kb and 1.7 kb) after digestion with Hind III and Xba I and two bands (approximately 5.5 kb and 2.2 kb) after digestion with BamH I and Not I (Figure 1). The third clone was submitted for sequencing analysis. The base sequence of the inserted gene in the pcDNA3.0 plasmid matched the genomic sequence of hHGF provided by GenBank. As predicted, the hHGF product generated by the two primers ran at approximately 2218 bp on a gel (Figure 2).

3.2. pGC-E1-hHGF Plasmid Construction and Sequencing. The purified hHGF fragment and the pGC-E1 gene plasmid were digested separately with Age I, and the pGC-E1-hHGF vector was generated via In-Fusion enzyme ligation. DH5α competent bacteria cells were transformed, and three positive bacterial clones containing the hHGF coding sequence were identified via PCR. The product size was 537 bp (Figure 3). The recombinant pGC-E1-hHGF plasmid was confirmed via sequencing, and the sequence was consistent with the hHGF gene sequence provided by GenBank (Figure 4).

3.3. Production and Titration of the Recombinant Lentiviral Vector. After cotransfection of 293T cells with the three plasmids, GFP expression was assessed using a fluorescence microscope to ensure that the lentiviral vectors were properly generated (Figure 5). The virus titers were determined via a one-in-one whole dilution, and the final virus titers were 1×10^8 TU/mL after concentration.

3.4. Immunophenotype Analysis of ADSCs. The freshly isolated ADSCs displayed adherence and expansion in culture, and they assumed a fibroblast-like morphology when observed using a light microscope. After the third passage, a FACS analysis revealed that the ADSCs were positive for CD29, CD44, and CD71, but the ADSCs assayed negative

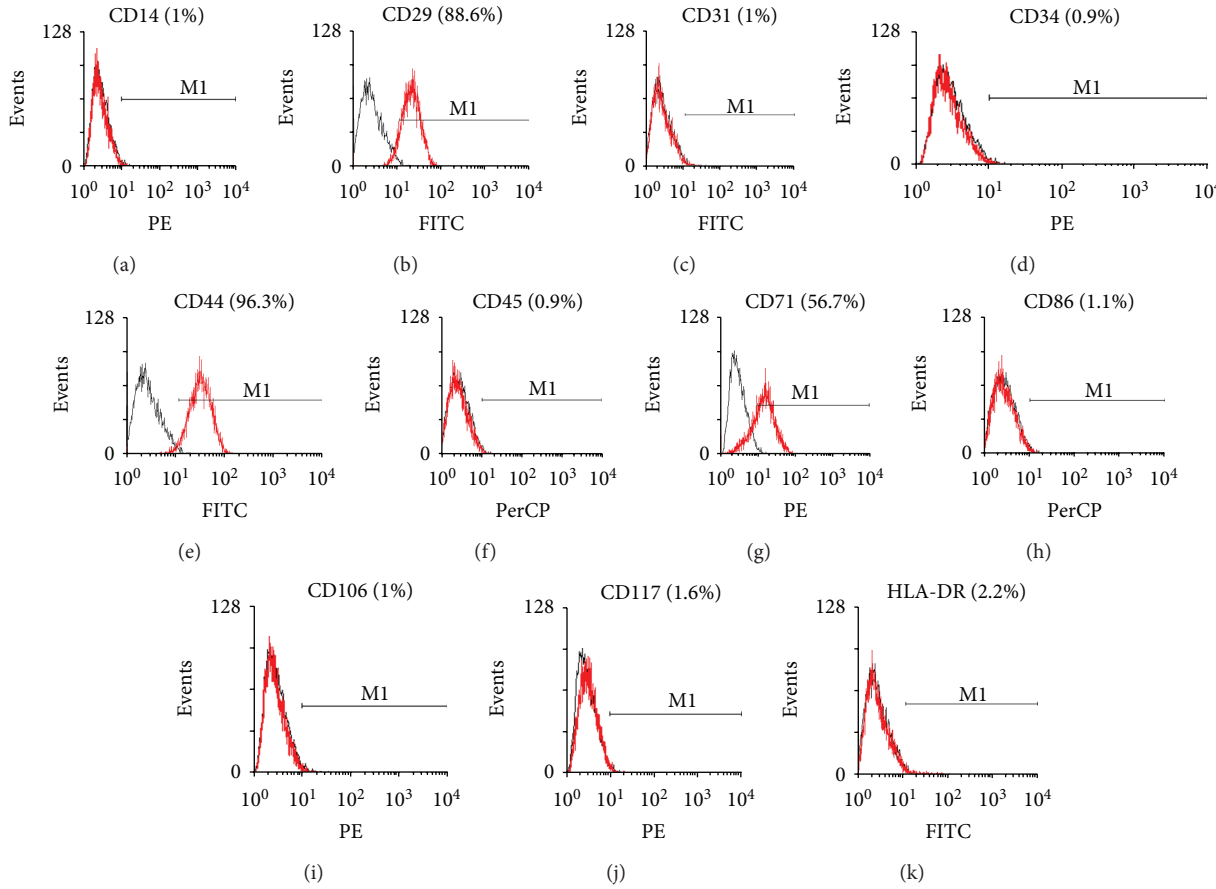


Figure 6: Human ADSC phenotypes at the third passage, as assessed via flow cytometry analysis. The red lines represent the specific fluorescence-labeled antibodies, and the black lines represent the isotype controls.

for hematopoietic and endothelial lineage markers, including CD31, CD34, CD45, CD106, and CD117, resulting in a phenotype similar to that of bone marrow MSCs. Moreover, immunogenicity markers, including CD14, CD45, CD86, and HLA-DR, were not observed (Figure 6).

3.5. Osteogenic and Adipogenic Differentiation of ADSCs. The osteogenic differentiation of human ADSCs at passage 5 was confirmed via alizarin red staining (Figure 7(a)). After feeding the ADSCs with osteogenic-inducing media, dark red mineralized bone matrix (bone nodules) was observed within the alizarin red-stained section.

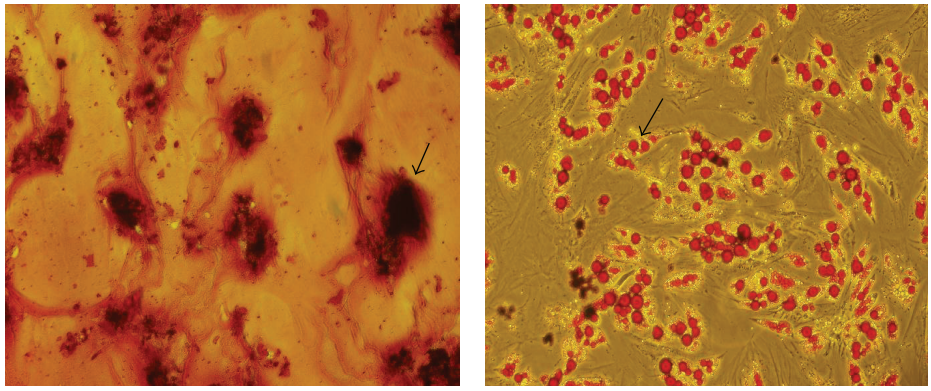
The adipogenic differentiation of human ADSCs was confirmed via Oil Red-O staining. After feeding the ADSCs with adipogenic-inducing media for 21 d, oil droplets were observed in the cytoplasm (Figure 7(b)).

3.6. The Infection Efficiency of ADSCs with Lentiviral Vectors. On the day of infection, hADSCs were infected with either lenti-hHGF or lenti-GFP at an MOI of 100, or 200. GFP expression was observed via fluorescent microscopy (Figure 8), and the infection efficiency rate was assessed via flow cytometry. The results indicated that the efficiency rate of infection with an MOI of 200 was (53 ± 15)%, which was higher than (36 ± 9)%, the efficiency rate of infection with an MOI of 100.

3.7. hHGF Protein Expression in ADSCs. The protein lanes corresponding to the uninfected ADSCs and the ADSCs infected with either lenti-hHGF or lenti-GFP are shown in Figure 9(a). The actin protein ran at 42 kDa, and the HGF protein ran at 83 kDa. Both the HGF gene-infected ADSCs and the uninfected ADSCs expressed HGF, but the expression level observed in the lenti-hHGF-infected group represented the highest level of expression of the three groups. ELISA found that both gene infected and uninfected ADSCs produced hHGF protein, but the concentrations were significantly higher in hHGF infected group at different time points after infection ($P < 0.001$). And the infected cells secreted the peak level of hHGF at 5 days after infection (Figure 9(b)).

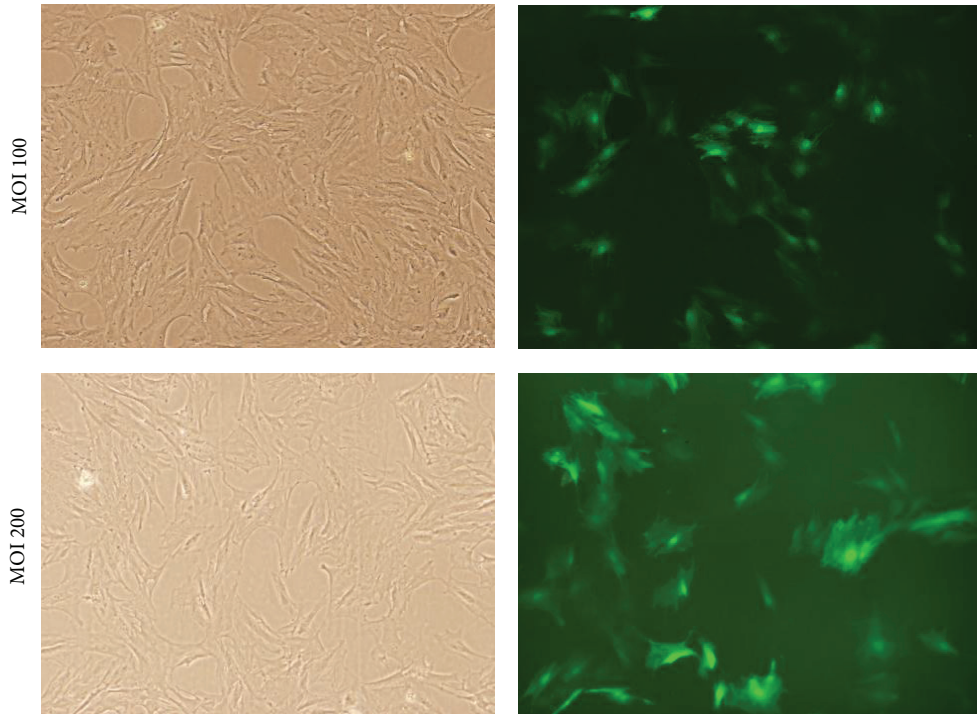
4. Discussion

Stem cells possess the ability to self-renew and differentiate into multiple cell types. Due to their reproducibility and multipotency, they have a significant role in many clinical and preclinical fields [11]. Stem cells can be harvested from



(a) (b)

Figure 7: Osteogenic and adipogenic differentiation of human ADSCs. (a) ADSCs treated with osteogenic media for 21 d (stained with alizarin red). The arrow indicates the mineralized matrix produced by osteoblasts (100x). (b) ADSCs treated with adipogenic media for 21 d (stained with Oil Red-O). The arrow indicates oil droplets (100x).



(a) (b)

Figure 8: Infection of ADSCs with lenti-hHGF at an MOI of 100 or 200 (100x). GFP expression was observed via either light (a) or fluorescence microscopy (b).

various mesenchymal sources, such as bone marrow, peripheral blood, cord blood and adipose tissue. The most common source of stem cells is bone marrow. However, harvesting stem cells from bone marrow causes pain and discomfort to patients, and only a relatively small number of cells can be harvested. Currently, an increasing number of researchers are focusing on ADSCs because they are multipotent, immune-privileged, abundantly harvested, and easily expanded *ex vivo*. In the present study, ADSCs were isolated from adult human adipose tissue and subsequently amplified in culture. The cultured ADSCs were positive for CD29, CD44, and CD71 expression, but they did not express hematopoietic and endothelial lineage markers (CD31, CD34, CD45, CD106, and CD117). The phenotype of these cells was similar to that of the MSCs derived from bone marrow with the exception that MSCs express CD106. Additionally, the expression of immunogenicity markers (CD14, CD45, CD86, and HLA-DR) was not observed, which demonstrated that the human ADSCs were immune privileged. The osteogenic- and adipogenic-induced differentiation experiments confirmed

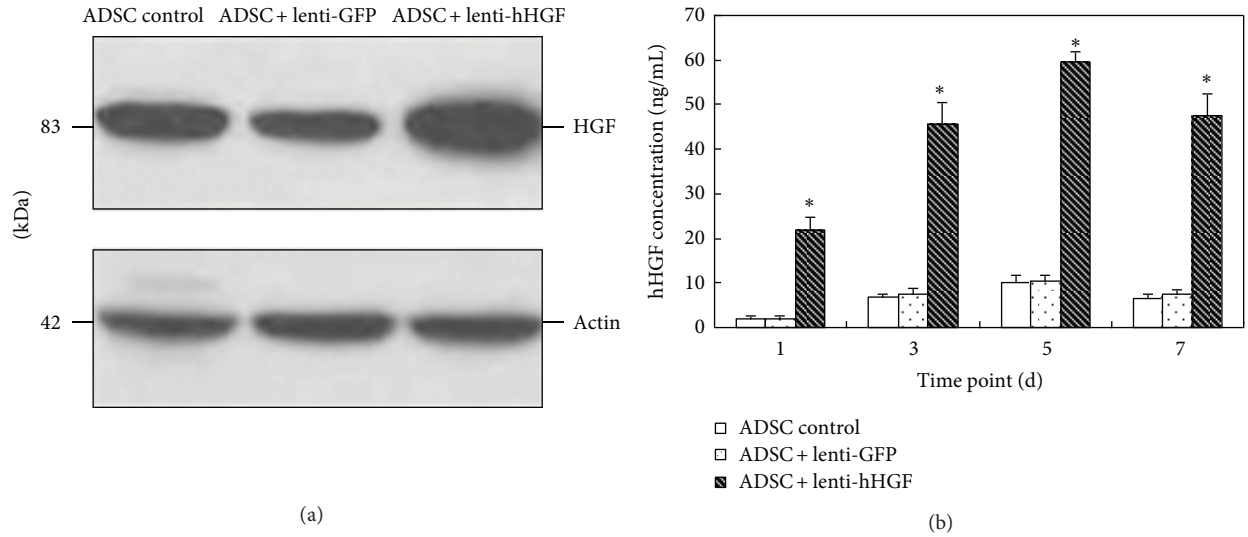


Figure 9: hHGF protein expressed in ADSCs. (a) Expression of hHGF as assessed by Western blot; (b) Enzyme linked immunosorbent assay of hHGF in the cell supernatants at different time points after infection. The results were expressed as mean ± standard deviation. $^{*}P < 0.001$ for ADSC + lenti-hHGF versus ADSC control or ADSC + lenti-GFP.

that the obtained ADSCs possessed multiple differentiation abilities.

Single-stem cell therapy is characterized by some deficiencies, such as the instability of stem cells, low cell numbers, and low activity. Therefore, stem cells combined with cytokines are more effective, and this combination has become the new therapy for IHD. HGF, which was originally identified and cloned as a potent for hepatocyte, has been reported to possess mitogenic, angiogenic, antiapoptotic, and antifibrotic activity in various cells. Many studies have shown that acute myocardial infarction, ischemia reperfusion injury, and congestive heart failure induce the expression of HGF in the heart. HGF gene therapy can improve cardiac function via the induction of angiogenesis, reduction of fibrosis, and recruitment of stem cells derived from bone marrow, which affects myocardial regeneration [12]. To express HGF in the target cells, we selected a lentiviral vector for the gene therapy study because lentiviral vectors have several attractive gene delivery vehicle properties as follows: (i) sustained gene delivery via stable vector integration into the host genome; (ii) the capability of infecting both dividing and nondividing cells; (iii) broad tissue tropisms, including important types of gene- and cell-targeted therapy; (iv) no viral protein expression after vector transduction; (v) the ability to deliver complex genetic elements, such as polycistronic and intron-containing sequences; (vi) a potentially safer integration site profile; (vii) a relatively easy system for vector manipulation and production [13].

In this study, we constructed a HGF recombinant lentiviral expression vector. Using molecular cloning techniques, such as PCR and DNA sequencing, the human HGF lentiviral expression plasmid was successfully constructed, and high titer viral particles were obtained after plasmid transfection into packaging cells. The HGF-GFP expression levels reached more than 60% when human ADSCs were infected. The western blot and ELISA results showed that HGF gene-infected ADSCs overexpressed HGF. These results demonstrated that the lentiviral vector successfully delivered the human HGF gene into ADSCs and mediated high levels of HGF expression in ADSCs. In the future, ADSCs infected with the recombinant human HGF lentiviral vector will be used to study the function of the target gene in combination with stem cells in IHD and other ischemic diseases.

Conflict of Interests

The authors declare that there is no conflict of interests with the trademarks mentioned in their paper.

Authors' Contribution

X. Zhu and L. Xu contributed equally to this paper and should be considered co-first authors.

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Review Article

Cell Transplantation for Spinal Cord Injury: A Systematic Review

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Cell transplantation, as a therapeutic intervention for spinal cord injury (SCI), has been extensively studied by researchers in recent years. A number of different kinds of stem cells, neural progenitors, and glial cells have been tested in basic research, and most have been excluded from clinical studies because of a variety of reasons, including safety and efficacy. The signaling pathways, protein interactions, cellular behavior, and the differentiated fates of experimental cells have been studied *in vitro* in detail. Furthermore, the survival, proliferation, differentiation, and effects on promoting functional recovery of transplanted cells have also been examined in different animal SCI models. However, despite significant progress, a "bench to bedside" gap still exists. In this paper, we comprehensively cover publications in the field from the last years. The most commonly utilized cell lineages were covered in this paper and specific areas covered include survival of grafted cells, axonal regeneration and remyelination, sensory and motor functional recovery, and electrophysiological improvements. Finally we also review the literature on the *in vivo* tracking techniques for transplanted cells.

1. Introduction

During the last 20 years research on spinal cord injury (SCI) conducted in basic neuroscience research centers and neurology clinics has steadily increased. Researchers have investigated the issue from several angles, ranging from the design of novel therapeutic agents to elucidating the basic mechanisms underlying axon regeneration, remyelination, and inflammation; all with the aim of eventually promoting functional recovery in humans. Recent research has significantly advanced our understanding of SCI and has provided a few potential therapies. However, many questions remain unanswered and more continue to emerge. There has been a recent trend in the field to move towards combinatorial therapies, in an effort to synergize and boost the therapeutic effects of single therapies [1, 2]. Likewise there has also been increased interest in the use of pluripotent stem cells capable of differentiating into multiple cell types. Stem cell therapy for SCI is based on a strategy to treat the injuries and to restore lost functions by replacing lost or damaged cell populations [3].

Stem cells are several large series of immature and multipotential cells which can be found in all multicellular organisms. Self-renewal and multipotential differentiation are the two main characteristics of stem cells, and embryonic stem cells and adult stem cells are the two major categories [4, 5]. In 1903, Maximow proposed the hypothesis of stem cells at the congress of hematologic society in Berlin for the first of time [6]. Eighty nine years after the scientific use of the term of "*Stem Cell*", neural stem cells were successfully cultured *in vitro* in 1992. These multipotential cells were generated from mammalian neural crest as neural spheres [7].

2. Spinal Cord Injury

Spinal cord injury (SCI) is caused by direct mechanical damage to the spinal cord that usually results in complete or incomplete loss of neural functions such as mobility and sensory function [8]. Motor vehicle accidents (40.4%), falls (27.9%), and acts of violence (15%) are the most frequent causes of SCI, and people with the average age of 40.7 years

are most at risk [9]. The annual incidence of SCI is 40 cases per million population in the United States [10]. An estimated 12000 cases of paraplegia and quadriplegia are caused by SCI in the United States in each year, and approximately, 4000 patients die on the way to hospital and 1000 die during their hospitalization [11]. About 16% SCI patients have to live with life-long tetraplegia which is caused by high-level spinal cord injury [9].

The pathophysiological processes that underlie SCI comprise the primary and secondary phase of injury [10, 12]. The primary injury refers to the mechanical trauma to the spinal cord injury. In this phase, spinal cord tissue is disrupted by the force imparted by the primary injury mechanism. The most common injury mechanism is contusion of the spinal cord at the moment of injury and the prolonged compression caused by vertebral bony structures and soft tissues that have become dislodged [13]. During the injury process, the spinal cord might be hyper-bent, over-stretched, rotated, and lacerated [14], but the white matter is usually spared [15]. Although serious impairment of neural functions can be caused by the direct damage to the spinal cord tissue within the primary phase, the pathophysiological mechanisms involved in the secondary phase are an important determinant of the final extent of neurological deficits [8, 16].

Secondary damage occurs following the initial spinal cord trauma. The posttrauma inflammatory response plays a core role in the whole period of secondary phase after SCI though the modulation of a series of complex cellular and molecular interactions [17]. After spinal cord trauma, the blood-spinal-cord barrier, that protects and separates the spinal cord parenchyma from peripheral circulation, is broken down due to hemorrhage and local inflammation [18]. Increased production of chemokines (8–14 kDa polypeptides) and cytokines of the IL-1 family, which mediate the activation and recruitment of inflammatory cells t, is one of the triggers of SCI-induced inflammation [19]. The activation and recruitment of peripheral and resident inflammatory cells that include microglial cells, astrocytes, monocytes, T lymphocytes, and neutrophils further promotes the development of secondary damage following spinal cord injury [20]. The secondary phase of injury can be subdivided into the acute-phase (2 hours–2 days), the subacute phase (days–weeks), and the chronic phase (months–years) [13, 15, 16]. The pathophysiological changes that occur within these different phases are distinct. (1) Acute phase: edema, ischemia, haemorrhage, reactive oxygen species (ROS) production and lipid peroxidation, glutamate-mediated excitotoxicity, ionic dysregulation, blood-spinal-cord barrier permeability, inflammation, demyelination, neuronal cell death, and neurogenic shock. (2) Subacute phase: macrophage infiltration, microglial activity, astrocyte activity and scar formation, and initiation of neovascularization. (3) Chronic phase: Wallerian degeneration, glial scar maturation, cyst and syrinx formation, cavity formation, and schwannosis. The end of spontaneous post-SCI changes is identified as a pathophysiological phenomenon with solid glial scar formation, syrinx formation, and neuronal apoptosis. There is retraction and demyelination of spared axons which may induce permanent loss of sensorimotor functions that is unresponsive to treatment [21]. In order to select the best time-point for therapeutic cell transplantation, an understanding of the timeline of secondary damage cascades is important [22]. In order to promote functional recovery, stem cell transplantation must suppress the inflammatory response, inhibit neuronal apoptosis and necrosis, enhance neuronal regeneration, and promote axon regeneration and remyelination [23].

3. Cell Transplantation for Neural Regeneration

Cell transplantation may promote neural regeneration and rescue impaired neural function after spinal cord injury by means of (1) parasecreting permissive neurotrophic molecules at the lesion site to enhance the regenerative capacity; (2) providing a scaffold for the regeneration of axons; (3) replacing lost neurons and neural cells [24]. An early stem cell transplantation study in humans was reported as a one patient case report by a Korean research team in 2005. Multipotent adult stem cells from umbilical cord blood were directly injected into the lesion site of a SCI patient who had been nonstanding disabled for years, and the ability to walk was reported to be restored [25]. Then, a Chinese surgeon performed highly controversial experiment in China and claimed that about hundreds SCI patients who accepted direct injection of olfactory ensheathing cells, isolated from aborted fetuses, into the spinal cord were cured without complications [26, 27]. These studies were received with skepticism and general concern about the ethicality about the research [28, 29]. In recent years, the advancement of stem cell therapy for SCI has been encouraging and inspiring [30–32]. Nandoe Tewarie et al. explained the strategies of stem cells therapy for SCI [33], and several animal studies and clinical studies have demonstrated cellular regeneration and functional recovery using stem cells [30–32, 34, 35]. Although promising results for the treatment of subacute injury have been obtained, functional recovery still remains a challenge in the treatment of chronic injury [36].

4. Embryonic Stem Cells and Induced Pluripotent Stem Cells

Embryonic stem cells (ESCs) are a kind of pluripotent stem cells that can be derived from the inner cell mass of the early embryo [5]. Compared with the adult stem cells (ASCs) found in adults, ESCs are able to replicate indefinitely and to differentiate into all three primary germ layers cell lines and eventually generate all cell types in the body [37]. In contrast, the generated cell types from ASC differentiation were limited [38]. By introducing Sox2, Klf4, Oct3/4, and c-Myc, induced pluripotent stem cells (iPSCs) can be produced from cultured fibroblast with fewer ethical issues and reduced risk of immunological rejection and therefore may be more useful in clinical regenerative therapies [39]. According to the report from Miura et al., the iPS cells are capable of generating three main neural cell types *in vitro*, which are electrophysiologically functional neurons, astrocytes, and oligodendrocytes [40]. Recently, ESCs and iPSCs have been

investigated to verify their therapeutic efficacy and their safety *in vivo* after SCI [41] (Table 1). Bottai et al. directly injected one million undifferentiated ESCs through the tail vein within 2 hours after the lesion. In this straightforward experiment, a significant improvement of BBB scores was confirmed in the experimental group when compared with the vehicle treated mice. In addition, an unexpected effect on the postinjury inflammatory response was also observed. The authors reported a greatly reduced number of invading macrophages and neutrophils. The authors speculated that the transplanted ESCs may improve lesion site preservation through this inflammation inhibition effect [42].

Since the ESCs and iPSCs have the capability to differentiate into all cell types, the most common strategy in rescuing the neural function after SCI is not the direct application of these cells, but the transplantation of various derived cell lines from ESCs or iPS cells. Several scientists have tried to generate neural progenitor/stem cells, motor neurons, oligodendrocyte progenitor cells, and olfactory ensheathing cells *in vitro*, and then transplant these cells into various animal models in order to verify the capability of neural function restoration *in vivo*. The derived cells that were injected into the animal models were restricted to one specific cell lineage, therefore reducing the risk of tumorigenesis when compared with directly applying ESCs or iPS cells [41]. Stem cell-derived neural stem/progenitor cells (NS/PCs) are currently considered a promising option of various cell replacement strategies for the treatment of spinal cord injury. However, these derived NS/PCs may possess variable characteristics depending on different derivation protocols. Using their own neurosphere-based culture system, firstly, Kumagai et al. [43] generated two different kinds of neurospheres, primary neurospheres (PNS) and passaged secondary neurospheres (SNS), these two kinds of neurospheres exhibit neurogenic and gliogenic potentials, respectively. Then, they transplanted PNS and SNS into rodent subacute SCI model. Interestingly, the positive results which included axonal growth promotion, remyelination, angiogenesis, and significant locomotor functional recovery were not obtained in the PNS group, but in the SNS group. This phenomenon might be induced by the neurotrophic parasecretion from gliogenic neurospheres transplantation, even though, this report still suggests that ESC-derived neurospheres are effective in promoting functional recovery after SCI *in vivo* [43]. Lowry et al. developed a novel coculture protocol with endothelial cells for treating mouse ESCs in the expansion phase with sonic hedgehog (Shh) and retinoic acid (RA) to generate motor neurons. The significant recovery of sensory and motor function in adult mouse SCI model was attained after transplantation of these motor neurons [44]. On perfecting the derivation protocol for the generation of consistent character NSCs from several different lines of ESCs and iPSCs, Koch et al. and Falk et al. presented a novel protocol which can produce a pure population of long-term self-renewing rosette-type ESC/iPSC-derived neural stem cells (lt-ESC/iPSC-NSCs) [113, 114]. This kind of lt-ESC/iPSC-NSCs exhibit consistent characteristics such as continuous expandability, stable neuronal and glial differentiation ability, and the capacity of generating functional mature neurons in monolayer culture. In order to verify the long-term ability of promoting functional recovery, Fujimoto et al. [45] transplanted lt-iPSC-NSCs into the lesion site of mouse. These grafted cells were observed not only to enhance remyelination and axon regeneration, but also to support the survival of endogenous neurons. More importantly, at the ninth week after transplantation, the previously attained motor function recovery was reduced significantly by means of the ablation of transplanted lt-iPSC-NSCs via the introduction of Diphtheria toxin.

It is precisely because of the efficacy of neuronal regeneration and neuronal function promotion, a variety of ESC/iPSC-NSCs application strategies for SCI has been reported by several groups. The neural cell adhesion molecule L1 was thought to be able to promote the survival of grafted cells in the lesion site of central nervous system, and to favor axonal growth *in vivo* [46]. Based on this, Cui et al. transplanted L1-overexpressing substrate adherent embryonic stem cell-derived neural aggregates (SENAs) into a mouse SCI model. Eventually, an increased number of surviving cells, enhanced neuronal differentiation, reduced glial differentiation, and increased tyrosine hydroxylase expression was confirmed when compared with wild type SENAs transplanted group [47].

Neurogenins are a family of bHLH transcription factors involved in specifying neuronal differentiation. As a marker of neuronal differentiation, Neurogenin-2 (Ngn2) is essential for the development of CNS tissue as well, especially for the dentate gyrus [115]. By utilizing Ngn2 expressing ESC-derived NPCs, Perrin et al. and Shapiro et al. fully restored weight support and significantly improved functional motor recovery of rats after severe spinal cord compression injury. In addition, the expression of serotonin 5HT1A receptor, which is expressed in the raphespinal tract which plays a major role in locomotion and is particularly affected after SCI, was partially restored [48, 116]. In another report, Hatami et al. injected human embryonic stem cell-derived NPCs (hESC-NPCs) with collagen scaffolds into hemisection rat model. As reported, the grafted hESC-NPCs successfully differentiated into neurons and glia *in vivo*, and promoted hindlimb locomotor recovery and sensory responses with observed migration of transplanted stem cells toward the lesion site [49]. The collagen scaffolds were believed to support the survival of transplanted cells at the initial phase of transplantation *in vivo*. In a recent report, cotransplantation of hESC-NPCs and Schwann cells (SCs) was applied by Niapour et al. They wanted to take advantage of a cotransplantation strategy to overcome the low rate of neuronal differentiation of individual NPC transplantation [50]. Based on their results, significant motor function recovery was observed in all engrafted groups (NPCs, SCs, NPCs + SCs) when compared with the control group. Moreover, in comparison with the two individual transplantation groups, the greatest functional recovery was observed in the hESC-NPCs/SCs transplanted group with significantly increased expression of TUJ1 and MAP2, and decreased expression of GFAP at the fifth week after transplantation. Their study suggested that the cotransplantation of hESC-NPCs with SCs might be a feasible strategy to provide a sufficient synergistic effect to enhance neuronal differentiation and

Table 1: *In vivo* transplantations of ESCs and iPSCs.

First author	Year	SCI Models	Main graft	Transplantation methods	Pre-differentiation or pretreated	Combination	Tissue sparing	Neuronal regeneration	Axonal regeneration /remyelination	Sensory function	Motor function	Cavity and/or scare formation	Inflammation	Others
Bottai, [42]	2010	T8, contusion -mice	Mouse ESCs	i.v.	—	—	—	—	Imp.	—	Imp.	—	Decreased nr. of macrophages and neutrophils	—
Kumagai, [43]	2009	T10, contusion -mice	Mouse ESCs	Lesion epicenter i. medu.	Neurogenic neurospheres	—	Imp.	No Imp.	No Imp.	—	No Imp.	—	—	No angiogenesis
			Mouse ESCs	Lesion epicenter i. medu.	Gliogenic neurospheres	—	Imp.	Imp.	Imp.	—	Imp.	—	—	Enhanced angiogenesis
Lowry, [44]	2008	T8, dorsal hemisection-mice	Mouse ESCs	Lesion site and rostral i. medu.	Endothelial cells/hedgehog /retinoci acid pre-treated	—	—	Imp.	Imp.	Imp.	Imp.	—	—	—
Fujimoto, [45]	2012	T10, contusion -mice	Human iPSCs	Lesion epicenter i. medu.	Neuroepithelial-like stem cells	—	Imp.	Imp.	Imp.	—	Imp.	—	—	—
Chen, [46]	2005	T8, compression -mice	Primed-hNSCs	Rostral and caudal i. medu.	L1-transfection	—	Imp.	Imp.	Imp.	—	—	Imp.	—	—
Cui, [47]	2011	T9, compression -mice	Mouse ESCs	Rostral and caudal i. medu.	Neuronal differentiation and L1 expression	—	Imp.	Imp.	Imp.	—	Imp.	—	Decreased microglial reaction	—
Perrin, [48]	2010	T9, compression -rats	Human ESCs	Lesion site, rostral and caudal i. medu.	Ngn2-transfection	—	No Imp.	Imp.	Imp.	—	Imp.	—	—	—
Hatami, [49]	2009	T10, lateral hemisection-rats	Human ESCs	Lesion site, i. medu.	Differentiated to NPs	Collagen I scaffold	—	Imp.	—	Imp.	Imp.	—	—	—
Niapour, [50]	2012	T9, contusion-rats	Human ESCs	Lesion epicenter i. medu.	Differentiated to NPs	NPs + rat SCs	—	Imp.	Imp.	—	Imp.	—	—	—
Rossi, [51]	2010	C5-C6, contusion -rats	Human ESCs	ventral horn, rostral and caudal i. medu.	Differentiated to MPs	—	—	Imp.	Imp.	—	Imp.	—	—	—
Kim, [52]	2010	T13, lateral hemisection-rats	Mouse ESCs	s. i.	GABAergic differentiation	—	—	—	—	Imp.	—	—	—	Increased evoked activity of WDR neurons
Keirstead, [53]	2005	T10, contusion -rats	Human ESCs	Rostral and caudal i. medu.	OPCs differentiation	—	No Imp.	Imp.	Imp.	—	Imp.	—	—	—

Table 1: Continued.

First author	Year	SCI Models	Main graft	Transplantation methods	Pre-differentiation or pretreated	Combination	Tissue sparing	Neuronal regeneration	Axonal regeneration /remyelination	Sensory function	Motor function	Cavity and/or scare formation	Inflammation	Others
Kerr, [54]	2010	T9, contusion-rats	Human ESCs	Lesion site, i. medu.	OPCs differentiation	—	—	Imp.	Imp.	—	Imp.	Imp.	—	Improved electrophysiological activities
Sharp, [55]	2010	C5, contusion-rats	Human ESCs	Rostral and caudal i. medu.	OPCs differentiation	—	Imp.	—	Imp.	—	Imp.	Imp.	Suppression of acute inflammation	—
Erceg, [56]	2010	T8, complete transection-rats	Human ESCs	Rostral and caudal i. medu.	OPCs and MPs differentiation	OPCs + MPs	—	Imp.	Imp.	—	Imp.	—	—	Improved electrophysiological activities
Salehi, [57]	2009	T9, complete transection-rats	Human ESCs	Lesion site, i. medu.	MNs differentiation	MNs + OECs	Imp.	Imp.	Imp.	—	Imp.	Imp.	—	—

—: Not reported; Imp.: Improvement; i.v.: intravenously; i. medu.: intramedullary injection; s. i.: subarachnoid injection; SCI: spinal cord injury; NPs: neural precursors; OPCs: oligodendrocyte progenitor cells; MPs: motoneuron progenitors; MNs: motoneurons, SCs: Schwann cells; OECs: olfactory ensheathing cells.

to simultaneously suppress glial differentiation, ultimately promoting functional recovery.

Besides glial cell activation, motor neuron loss is considered as another major characteristic of spinal cord injury which contributes to motor functional deficits, especially at the cervical level. According to the review from Nogradi et al., they suggested that the application of ESC/iPSC-derived motoneuron grafts is an efficient way to replace missing motoneurons which result from spinal cord injury [35]. The grafted motoneurons may be able to reinnervate the denervated muscles by extending their axons along the entire length of reimplanted ventral root and reach the muscles to restore limb locomotion function, rather than partially restoring integrity of the lesion site with local neuron or axon regeneration [117]. In vitro, a typical electrophysiological action potential of ESC-derived motor neuron can be elicited, and several physiologically active growth factors can be expressed and secreted by ESC-derived motor neuron progenitor (MNP). These include neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), nerve growth factor (NGF), and vascular endothelial-derived growth factor (VEGF), can promote neurite branching and neuronal surviving [51]. *In vivo*, in order to test function and to observe the cellular behavior, Rossi et al. transplanted ESC-derived MNPs into the cervical lesion site of an adult rat model of SCI. When compared with vehicle control group, a series of significantly better results, that included enhanced sprouting of endogenous serotonergic (5-HT) projections, enhanced survival of endogenous neurons, enhanced gross tissue sparing, and decreased phosphorylation of stress-associated protein kinase which can result in apoptosis, immune activation, and inflammation were observed by them [51]. In addition to the recovery of motor function, the attenuation of tactile hypersensitivity and the recovery of general sensory function is another target which scientists want to achieve in research for a treatment for SCI. The upregulation of decreased GABAergic system activation after SCI was verified to have a role of relieving the pain-like response in rat hemisection SCI model [118]. For this reason, another kind of ESC/iPSC-derived neuron, ESC/iPSC-derived GABAergic neuron, was introduced into transplantation experiments for rescuing impaired sensory function post SCI by generating GABA around the lesion site during a long-term period [52]. Kim et al. evaluated the mechanical sensitivity of the hind paws by measuring paw withdrawal thresholds (PWTs) in the hemisection rat model on the application of a von Frey filament. After the intrathecal transplantation of ESC/iPSC-derived GABAergic neurons, a significant reversal of decreased PWTs was assessed beyond posttransplantation week 5 when compared with control group. Interestingly, the evoked response of wide dynamic range (WDR) neurons, which are responsive to all sensory modalities (thermal, chemical, and mechanical), to three different stimuli (brush, pressure, and pinch) in ESC/iPSC-derived GABAergic neuron transplanted group were significantly restored from a hypersensitive condition, to a level similar to the sham operation group. Importantly, all these phenomena could be blocked by the administration of GABA receptor inhibitors. Therefore, their study showed that a transplantation strategy using ESC/iPSC-derived GABA neurons may be a potential solution for the loss of sensory function after SCI.

Beyond the direct transplantation of derived neurons, transplantation of neural supporting cells remains attractive to scientists and ESC/iPSC-derived oligodendrocyte progenitors are one of the optimal options. In 2005, improved axon remyelination and motor function in a rat contusion SCI model by means of ESC-derived OPC transplantation was reported by Keirstead et al. [53]. Furthermore, ESC-derived OPCs demonstrated some distinctive immunological characteristics and were largely resistant to killing by human NK cells as well as to the lytic effect of antibodies [119]. With the capability to restore motor function via remyelination and specific immune-properties which suggest that these cells could be weakly immunogenic in nature and may not be rejected by the immune system, ESC-derived OPCs exhibit favorable properties for further development as a potential therapy for SCI. Kerr et al. derived OPCs from human ESCs and then injected these cells into a contusion SCI model in rats during the acute phase after injury. After eight days of transplantation, oligodendrocyte markers, including CNPase, GalC, Olig1, O4, and O1, were detected in the grafted ESC-derived OPCs. These grafted cells were reported to survive for a minimum of eight days and to migrate away from the injection sites to integrate into the injured spinal cord tissue. Some increased neurological responses were demonstrated in the transplanted group through behavioral and electrophysiological assessments compared to control groups [54]. In another study, Sharp et al. transplanted ESC-derived OPCs into a cervical contusion rat model to assess the restoration of forelimb motor function and to examine neural tissue protection from lesion pathogenesis. From the fourth week till the ninth week after transplantation, a significantly improved forelimb stride length was measured when compared with the control group. In the nontransplanted control group, a characteristic injury-induced cavity was observed with a lack of neural cells and axons in the border area surrounding the cavity. Moreover, perivascular cuffing and inflammatory infiltrates were identified in the lesion site which suggested dynamic, ongoing pathology. Meanwhile, in contrast to the control group, widespread white and gray matter sparing was observed in the lesion sites of the transplanted group, and most importantly, no injury-induced cavitation was identified. Furthermore, unlike the nontransplanted spinal cords, there was reduced demyelination and more oligodendrocyte remyelinated axons than schwann cell-remyelinated ones in the ESC-derived OPC transplanted spinal cords [55].

Although the transplantation of ESC/iPSC-derived neurons or OPCs has achieved promising results, combinatorial strategies have also been tested. Erceg et al. derived motoneuron progenitors (MPs) and OPCs from ESCs through different protocols, respectively, and then verified the efficacy of functional recovery promotion by MPs and OPCs, together, in a complete transection SCI rat model. As they expected, when compared with single-cell treatment and control group, the combined treatment group had significantly better BBB scores with significantly higher amplitude of motor-evoked potential (MEP) in electrophysiological evaluation

at the end of experiment [56]. A similar positive result was reported in another combinatorial study by Salehi et al, who transplanted olfactory ensheathing cells (OECs) and ESC-derived motoneurons (MNs) into contused SCI rats [57]. This cotransplantation strategy rescued a significantly greater percentage of spared spinal cord tissue from contused lesion, and successfully enhanced remyelination after injury. The survival of grafted ESC-derived MNs in cotransplanted rats was sufficiently supported by OECs and, the number of surviving ESC-derived MNs in the cotransplanted group was significantly higher than in the single cell type transplantation with ESC-derived MNs group. A significant recovery of hindlimb function was observed in rats in the cotransplanted groups, together with improved histopathology.

5. Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cell (MSCs) lineage is a kind of self-renewing and multipotent stem cell, which was initially identified from the bone marrow (BM) [120, 121]. In the adult human bone, the population of MSCs is rare, approximately 0.001%–0.01% of the total population of nucleated cells in the marrow [122]. However, human MSCs can be easily obtained from bone marrow by simple iliac crest puncture, and they are biologically safe and have been used extensively for transplantation in patients suffering from hematological cancer [23].

According to the statement of International Society for Cellular Therapy, the definition of multipotent MSCs must be fulfilled to a minimum criterion [123]. First, MSCs must be plastic-adherent when cultured in standard conditions. Second, MSCs must express CD105, CD73, and CD90, and lack the expression of CD45, CD34, CD14, or CD11b, CD79a, or CD19 and HLA-DR surface molecules. Third, MSCs must be able to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*. MSCs are able to be differentiated *in vitro* into osteoblasts, chondrocytes, adipocytes, neural cells, and even myoblasts [122, 124]. Within the field of regeneration research after CNS injury, MSCs are being advocated as a promising cell source for repair. The isolation of a population of multipotent stem-cells from human bone marrow [122], and demonstration of spontaneous neuronal differentiation of MSCs implanted into both irradiated mice [125, 126] and humans [127]; along with isolation of subtypes of nonhematopoietic MSCs capable of neuronal differentiation, have paved the way for their clinical use in neurorestorative approaches [124, 128, 129].

In stem cell therapy research for SCI, the application of MSCs is favored by some researchers because of the following excellent properties. First, the acceptance from the donor and the isolation from cryopreservation are relatively easy and simple [130, 131]. Second, the expansion of cells to clinical scales can be achieved in a relatively short period of time [132]. Third, the preservation of MSCs with minimal loss of potency can be performed conveniently [133]. Fourth, transplanted MSCs are capable of decreasing demyelination, reducing neural inhibitory molecules, of promoting axonal regeneration, and of guiding axon growth [134]. Lastly and importantly, there are no reports of adverse reactions to allogeneic versus autologous transplants, and allogeneic MSCs are well tolerated and do not elicit immediate or delayed hypersensitivity reactions [135, 136]. Carrade et al. injected equine allogeneic and autologous umbilical cord derived mesenchymal stem cells (UMSCs) twice into horses intradermally [135]. After the first injection, no adverse local and systemic responses within 7 days after injection were observed, except some minor wheal formations which were characterized as mild dermatitis and fully resolved by 48–72 hours. The second injection was 3-4 weeks later, and they reported no more significant physical and histomorphologic alterations compared with the first injection. This result indicated that neither the immediate, cytotoxic, immune-complex, and delayed hypersensitivity reactions, nor the graft-versus-host responses can be elicited by transplanted UMSCs.

Azizi et al. (1998) reported spontaneous differentiation of human bone-marrow-derived stromal cells into astrocytes following implantation into the striate body of adult rats [137]. These cells, however, did not transform into neurons. Shortly after, Mezey et al. [126] and Brazelton et al. [125] simultaneously described spontaneous acquisition of cells bearing neuronal antigens, from bone marrow cells infused intraperitoneally in rats which had migrated to the brain of host animals. Mezey et al. used male-rodent mesenchymal cells, implanting them into females with congenital bone marrow aplasia. They confirmed neuronal differentiation through NeuN expression by immunohistochemical staining and confirmed cells as being those of the donor by using in situ hybridization of the Y chromosome, a difficult to execute technique yielding substantial unspecific punctiform staining patterns, potentially misinterpreted as Y chromosome. Notwithstanding, they reported that 0.3 to 1.8% (depending on age of recipient) of neuronal cells in the host rat forebrain were derived from the donor. In the second study, the authors employed transgenic rats whose cells constitutively expressed green fluorescent protein (GFP). Bone-marrow-derived stromal cells were extracted from these animals and subsequently implanted intravenously into irradiated rats with no viable bone marrow. They reported immunostaining for NeuN and high-molecular-weight neurofilament protein (NFH) coexpressing GFP in different cell types from olfactory bulbs of the host rats.

Transplantation of MSCs in SCI animal models has been applied by several groups to promote sensorimotor function recovery and bladder function recovery via neural lineage differentiation, neurotrophic paracrine effects and posttrauma inflammation regulation (Table 2). As Nakajima et al. reported, the activation of macrophages in the post-SCI inflammatory environment can be regulated by the transplantation of MSCs [58]. After transplantation into the contusion epicenter, the undifferentiated MSCs significantly upregulated the level of IL-4 and IL-13, and downregulated the level of TNF-alpha and IL-6. These changes of inflammation factors resulted in the shifting of macrophage phenotype from M1 (iNOS- or CD16/32-positive) to M2 (arginase-1- or CD206-posistive). With the alteration of macrophage phenotype, more preserved axons, less scar tissue formation, and increased myelin sparing were observed, furthermore,

locomotion recovery in the MSCs transplantation group was confirmed. In another MSCs transplantation trial, Karaoz et al. claimed significant motor recovery in the MSCs implanted group, however, only Nestin+/GFAP+ astrocytic-like cells were observed at 4 weeks after transplantation [59]. By implanting human MSCs into the contusion rat model, more rapid restoration of hindlimb function was achieved when compared with other control groups, but significant differences of BBB scores and coupling scores among all groups were not obtained. More importantly, bladder function was not restored in either group [60]. In addition to motor function deficits and bladder dysfunction, neuropathic pain is also a common and debilitating symptom in SCI patients which is induced by abnormal neuronal activities in the spared tissue surrounding the lesion site. In order to clarify the relationship between chronic inflammation and the therapeutic effects of MSCs on sensory deficits, Abrams et al. evaluated chronic inflammation, posttrauma cyst formation, and mechanical and thermal sensation thresholds of contusion SCI rats treated with MSCs transplantation [61]. After MSC injection at three different sites (the lesion site, rostral and caudal to the lesion), the injury-induced sensitivity to mechanical stimuli was significantly attenuated, although no effect was observed on injury-induced sensitivity to cold stimuli. More importantly, GFAP + reactive astrocytes and ED1+ macrophages/microglia, assessed as a measure of the chronic inflammatory response, were significantly attenuated by MSCs administration. The improvement of locomotor function in SCI rats by means of MSCs transplantation was also reported.

However, the therapeutic *in vivo* application of MSCs for spinal cord injury might face a series of challenges which include low survival rate of grafted cells (5–10%), the lack of neural differentiation, glial scar formation, cystic cavity formation, the inhibitory cellular environment, the transplantation time point, and the graft/host immune responses [58, 64–66]. In addition, different transplantation routes can also bring different outcomes after MSCs transplantation. In a comparison experiment, Kang et al. compared the BBB motor scores of SCI rats between intravenously (IV) and intralesionally (IL) transplanted groups [62]. The fates of engrafted allogenic MSCs in two different groups were also investigated. Based on their results, the NeuN positive neural differentiation and CC-1 positive oligodendroglial differentiation of engrafted MSCs was observed in the IL group, and GFAP positive astrocyte differentiation was observed in the IV group. Meanwhile, the expression of both BDNF and NGF in the IL group was significantly higher than the IV group. This phenomenon was suggested to be related to the absolute number of the engrafted MSCs. Regarding motor function recovery, both MSC transplantation groups achieved significantly better outcomes than the control group (BBB scale 6.5 ± 1.8). The BBB scores in the IV group (11.1 ± 2.1) was significantly better than the IL group (8.5 ± 2.8). The authors suggested that the nonfavorable motor function improvement in IL group might be related to the additional injury during the transplantation in the intralesional injections. By means of intravenous transplantation of LacZ reporter gene transduced MSCs in the earlier postinjury infusion time, Osaka et al. reported significantly improved locomotor recovery in severe contusive SCI rats, and they suggested that the minimal invasive, intravenous cell administration is a prospective therapeutic approach in acute and subacute SCI [63]. Mothe et al. investigated the effects of another transplantation approach, intrathecal transplantation, with neural stem/progenitor cells (NS/PCs) and bone-marrow-derived mesenchymal stromal cells (BMSCs) [64]. Most of transplanted cells were showed to remain in the intrathecal space, and neither NS/PCs nor BMSCs migrated into the parenchyma of the injury site.

After implantation into the injured spinal cord, the neuronal differentiation of MSCs *in vivo* is not efficient and the lack of neuronal markers expression has been reported in some transplantation studies [64–66]. Without neuronal differentiation, the engrafted MSCs may generate a favorable environment for functional recovery through modulating the post-SCI inflammatory response and by having neurotrophic paracrine activity [58, 64–66, 138]. As Boido et al. reported, significantly reduced lesion volume and improved hindlimb sensorimotor functions were observed after mouse MSCs were transplanted into the lesion cavity of compression SCI mouse model, even though the engrafted MSCs were observed to be neuronally undifferentiated and astroglial and microglial activation was not altered [65]. Gu et al. also reported similar results, the reduced volume of post-SCI cavity and increased spared white matter were observed after transplantation of bone marrow mesenchymal stem cells into the epicenter of the injured spinal cord of rats [66]. Interestingly, despite the lack of expression of neuron, astrocyte, and oligodendrocyte cell markers, an increase in the number of axons in MSCs transplanted rats was confirmed via transmission electron microscopic examination. In the *in vitro* experiment of the same study, Gu et al. investigated the paracrine activity of MSCs by means of a MSCs and spinal neuron coculture system. Their results confirmed the expression of brain-derived neurotrophic factor (BDNF) and glia cell line-derived neurotrophic factor (GDNF).

The therapeutic effects of MSC transplantation on the sensorimotor deficits in animal SCI models have been clearly confirmed by a large number of studies [61, 63, 65, 67].

In order to overcome the potential problems associated with direct transplantation of undifferentiated MSCs, researchers have tested several modifications of transplantation strategies, such as pretransplantation neural differentiation, neurotrophic gene transduction, glial cell cotransplantation, and tissue engineering [67–75, 139–142]. The neural pretransplantation differentiation is the most commonly used strategy to promote the therapeutic effects of engrafted MSCs. Rodent MSCs are able to efficiently differentiate into neural precursors by culturing with basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and heparin [143]. One method of human MSC neural differentiation was described by Alexanian et al. in 2011 [67]. According to his method, human MSCs were exposed to histone deacetylases inhibitor (Trichostatin), DNA methyltransferase inhibitor (RG-108), biologically active form of cAMP, and phosphodiesterases inhibitor (Rolipram) in a medium consisting of NeuroCult/N2 supplemented with

Table 2: *In vivo* transplantations of Mesenchymal stem cells.

First author	Year	SCI Models	Main graft	Transplantation methods	Pre-differentiation or pretreated	Combination	Tissue sparing	Neuronal regeneration	Axonal regeneration /remyelination	Sensory function	Motor function	Cavity and/or scare formation	Inflammation	Others
Nakajima, [58]	2012	T9-T10, contusion-rats	Rats bMSCs	Lesion epicenter i. medu.	—	—	Imp.	—	Imp.	—	Imp.	Imp.	Shifting of macrophage phenotype	—
Karaoz, [59]	2012	T9–T11, contusion-rats	Rats bMSCs	Lesion epicenter i. medu.	—	—	Imp.	—	—	—	Imp.	—	—	Only Nestin+/GFAP+ astrocytic-like cells were observed
Park, [60]	2010	T9, contusion-rats	Rats bMSCs	Lesion epicenter i. medu.	—	—	—	No Imp.	—	—	more rapid restora-tion*	—	Decreased nr. of macrophages and monocytes	No bladder function improvement
Abrams, [61]	2009	T11-12, contusion-rats	Rats bMSCs	Lesion site, rostral and caudal i. medu.	—	—	—	—	—	Partially Imp.	Imp.	Imp.	Attenuated chronic inflammation	—
Kang, [62]	2012	T8–10, contusion-rats	Rats bMSCs	i.v.	—	—	—	No Imp.	—	—	Imp.	—	—	—
		T8–10, contusion-rats	Rats bMSCs	Rostral and caudal i. medu.	—	—	—	NeuN+ differentiation	—	—	Imp.	—	—	Expression of BDNF and NGF
Osaka, [63]	2010	T9, contusion-rats	Rats bMSCs	i.v.	—	—	—	—	—	—	Imp.	Imp.	—	—
Mothe, [64]	2011	T8-9, clip compression-rats	Rats bMSCs	i. thec.	—	—	—	No Imp.	Imp.	—	—	—	—	—
Boido, [65]	2012	T9, compressed-mice	Mice bMSCs	Lesion site, i. medu.	—	—	Imp.	No Imp.	—	Imp.	Imp.	—	—	—
Gu, [66]	2010	T9, contusion-rats	Rats bMSCs	Rostral and caudal i. medu.	—	—	Imp.	No Imp.	Imp.	Imp.	Imp.	Imp.	—	Expression of BDNF and GDNF
Alexanian, [67]	2011	T9, contusion-rats	Human bMSCs	Rostral and caudal i. medu.	Neural differentiation	—	Imp.	Imp.	—	No Imp.	Imp.	Imp.	—	—
Ban, [68]	2011	T9, contusion-rats	Rats bMSCs	Lesion epicenter i. medu.	—	MSCs + SCs	—	—	Imp.	—	Imp.	Imp.	—	—
Cho, [69]	2009	T9, contusion-rats	Rats bMSCs	Lesion epicenter i. medu.	Neural differentiation	—	—	Imp.	—	—	Imp.	—	—	Improvements in SSEPs and MEPs
Pedram, [70]	2010	T8-9, catheter compression-rats	Rats bMSCs	Rostral and caudal i. medu.	Neural differentiation	Differentiated and undiffer-entiated MSCs	—	Imp.	—	—	Imp.	—	—	—
Liu, [71]	2011	T9, contusion-rats	Rats bMSCs	Lesion epicenter i. medu.	bFGF-transfection	—	—	Imp.	Imp.	—	Imp.	—	—	—

Table 2: Continued.

First author	Year	SCI Models	Main graft	Transplantation methods	Pre-differentiation or pretreated	Combination	Tissue sparing	Neuronal regeneration	Axonal regeneration /remyelination	Sensory function	Motor function	Cavity and/or scare formation	Inflammation	Others
Zhang, [72]	2012	T9, ethidium bromide-induced demyelination-rats	Rats bMSCs	Lesion site, i. medu.	NT-3-transfection	—	—	Imp.	Imp.	—	Imp.	—	—	Improvements in SCEPs
Zeng, [73]	2011	T8, complete transection-rats	Human bMSCs	Lesion site, i. medu.	—	Scaffolds + MSCs	—	Imp.	Imp.	—	Imp.	Imp.	Decreased nr. of macrophages and microglial	Enhanced angiogenesis
Kang, [74]	2012	T8-9, complete removal of a 2-mm length of spinal cord	Human bMSCs	Lesion site, i. medu.	—	Scaffolds + MSCs	—	Imp.	Imp.	—	Imp.	—	—	Improvements in MEPs
Park, [75]	2012	L2-3 balloon catheter compression-dogs	Canine aMSCs	Lesion site, i. medu.	Neural differentiation	Matrigel + neural-induced MSCs	—	Imp.	—	—	Imp.	Imp.	Decreased expression of inflammation markers	Increased expression of neurotrophic markers
Guo, [76]	2011	T9, contusion-rats	Human uMSCs	Lesion epicenter i. medu.	Schwann-like cells	NT-3 + huMSC-derived Schwann-like cells	—	Imp.	—	—	Imp.	—	—	—
Shang, [77]	2011	T9, contusion-rats	Human uMSCs	Lesion epicenter i. medu.	NT-3-transfection	—	—	Imp.	Imp.		Imp.	Imp.	—	—
Lee, [78]	2011	L2-3 balloon catheter compression-dogs	Human uMSCs	Lesion site, rostral and caudal i. medu.	—	—	—	Imp.	Imp.		Imp.	—	—	Expression of BDNF and NT-4

*More rapid restoration of hindlimb function without significant differences compared with control groups; —: Not reported; Imp., Improvement; i.v.: intravenously; i. medu.: intramedullary injection; i. thec.: Intrathecal implantation; SCI: spinal cord injury; SCs: Schwann cells; bMSCs: bone-marrow-derived Mesenchymal stem cells; aMSCs: canine adipose-derived mesenchymal stem cells; uMSCs: umbilical-cord mesenchymal stem cells.

bFGF for two weeks before transplantation. Park et al., reported a new method to generate functional motor neuron (MN)-like cells from genetically engineered human MSCs [141]. They transduced motor neuron-associated transcription factor gene expression into the human MSC, then they treated the genetically engineered MSCs expressing Olig2 and Hb9 with optimal MN induction medium. By using an *ex vivo* model of SCI, they showed that these reprogrammed MSCs exhibited characteristics of MN-like lineage and are potentially therapeutic for autologous cell replacements.

Alexanian et al. injected neural modified bone-marrow-derived MSCs rostral and caudal to the T-8 lesion immediately after injury [67]. 12 weeks after SCI, locomotor function was significantly improved by the neurally modified MSCs, and the volume of lesion cavity and white matter loss were significantly reduced. However, the improvement of thermal sensitivity was not observed. Cho et al. transplanted neurally differentiated rat MSCs (NMSCs) into the epicenter of a contusive lesion, thereafter, the BBB scores, somatosensory evoked potentials (SSEPs) and motor evoked potentials (MEPs) were evaluated. Nine weeks after NMSCs transplantation, the recovery of motor function was reported, and significantly shortened initial latency, N1 latency and P1 latency of the SSEPs were observed [69]. Pedram et al. utilized a Fogarty embolectomy catheter to create a contusion lesion at T8-9 level of rats' spinal cord, then the autologous neural differentiated and undifferentiated MSCs were cotransplanted into the center of lesion cavity [70]. Five weeks after transplantation, the BBB scores in both cotransplantation group and predifferentiation group were reported to be significantly higher, when compared with undifferentiated group, respectively. However, no significant difference between cotransplantation and predifferentiation groups was observed.

In addition to neural predifferentiation, neurotrophic gene transfection has also been tested in some MSC *in vivo* studies. Liu et al. implanted bFGF transgene expressing rat MSCs into the SCI rat model and reported a significantly higher BBB score in the bFGF group when compared with control groups at 3 weeks after the injection. Furthermore, significantly more bFGF-positive neurons were observed in the bFGF group, and significantly higher optical density values of NF200-positive neurons and MBP-positive axons were also demonstrated in the bFGF group. Therefore, they suggested that the bFGF gene-modified MSCs might be effective in promoting axon regeneration and functional recovery after SCI [71]. In another *in vivo* study using gene modified MSCs, Zhang et al. investigated the therapeutic effects of Neurotrophin-3 (NT-3) gene modified MSCs in an ethidium bromide (EB)-induced demyelination SCI model of rats [72]. 21 days after the administration of NT-3 modified MSCs, locomotor function was improved, and similar to that in the saline injured control group. The improvement was significantly better than the other groups which include MSC group, LacZ gene modified group, and EB injured group. Similar improvements of spinal cord evoked potentials (SCEP) amplitude and SCEP latency were also achieved in the NT-3 modified MSCs group. Via immunostaining, significantly higher number of NG2- and APC-positive engrafted MSCs were observed in the demyelination site of the spinal cord after transplantation of NT-3 modified MSCs at the end of experiment.

In order to provide a favorable environment for neural regeneration and to support the survival of implanted cells and their neural differentiation, the use of biologic scaffolds has drawn increasing interest. Zurita et al. developed a biologic scaffolds system from blood plasma, called platelet-rich plasma (PRP) scaffolds. According to their report, most of the cocultured human MSCs demonstrated optimized capabilities of survivalg and neural differentiation after the administration of BDNF [142]. In 2011, a gelatin sponge (GS) scaffold system, which was constructed by ensheathing GS with a thin film of poly-(lactide-co-glycolide) (PLGA), was reported by Zeng et al. Based on their work, this GS scaffolds system was able to provide a favorable environment for seeded rat MSCs to adhere, to survive, and also to proliferate. After they transplanted GS scaffolds seeded with rat MSCs into the rat SCI model, a promising result which includes attenuated inflammation, promoted angiogenesis, and reduced cavity formation was reported [73]. In 2012, a combinatorial strategy using a similar PLGA scaffolds system and human MSCs was employed by Kang et al. to evaluate the therapeutic effects on motor function improvements. After PLGA scaffolds seeded with human MSCs were transplanted into a completely transected SCI rat model, significantly higher BBB scores were demonstrated. More importantly, the amplitude of motor-evoked potentials (MEPs) in the combinatorial strategy treated group was significantly higher than the other control groups. In addition, implanted cell survival, neural differentiation, and axon regeneration in the combinatorial strategy group were confirmed by immunohistochemical staining images [74]. In another study, a combination of Matrigel and neural-induced adipose-derived MSCs (NMSCs) was applied by Park et al. to investigate the therapeutic effects on functional recovery from SCI in dogs. 8 weeks after the administration of the combination of Matrigel and NMSCs, a significantly better functional recovery was observed as higher BBB and Tarlov scores. Meanwhile, the reduced fibrosis from secondary injury processes, decreased expression of inflammatory and astrogliosis markers, increased expression of neuronal and neurotrophic markers were also confirmed [75].

Although the bone marrow is the main source of MSCs, scientists have been seeking other sources because bone-marrow-derived cells are highly vulnerable to viral infection and the significantly increased cell apoptosis and the loss of differentiation capability that occurs in these cells with age [144]. Alternative sources of MSCs have been identified by researchers, such as, adipose tissue [140], amniotic fluid [145], placenta [145, 146], umbilical cord blood (UCB) [138, 147], and in several fetal tissues including liver, lung, and spleen [148]. Among all the substitutes for BM-derived MSCs, the UCB is the best choice with many advantages of UCB as compared to BM. The collection of cord blood units is more easier and noninvasive for the donor, the UCB units can be stored in advance and are rapidly available when needed, and the MSCs from UCB is more primitive than the MSCs collected from other sources [149, 150]. Importantly,

they are less likely to induce graft-versus-host reactivity due to their immaturity [151]. Ryu et al. investigated the effects of MSCs from different tissues on the regeneration of injured canine spinal cord, which are fat tissue, bone marrow, Wharton's jelly and umbilical cord blood [152]. Although the differences among four experimental groups were not detected in this study, more neural regeneration and anti-inflammatory activity were observed in the experimental group with umbilical cord blood derived MSCs.

Guo et al. [76] induced human umbilical cord mesenchymal stem cells (hUMSCs) into Schwann-like cells *in vitro* and grafted these cells into the lesion site of SCI rats. A partial recovery of motor function was reported. Furthermore, neurotrophin-3 (NT-3) administration combined with *in vivo* transplantation, significantly increased the survival of grafted cells and improved the behavioral test results compared to the cell transplantation only group. Meanwhile, Shang et al. [77] transplanted genetically modified NT-3-hUMSCs to the spinal cord injured rats, and the Basso, Beattie and Bresnahan (BBB) scores and grid tests were applied to evaluate the functional recovery at the end of 12 weeks after SCI. In addition to the promotion of transplanted cell survival, significantly better motor function recovery compared to hUMSCs group was achieved in the NT-3-hUMSCs group. This was associated with intensified 5-HT fiber sprouting, more spared myelin, and reduced cystic cavitation.

The pathological processes at the lesion site in SCI evolve over time, from acute phase, subacute to chronic phase, therefore transplantation at different times postlesion, may have varied effects. The comparison of three different transplantation times (12 hr, 1 week, and 2 weeks after injury) has been explored by Park et al., they injected 1×10^6 canine UMSCs into the balloon-induced compression lesion site of experimental dogs in different time groups [153]. The significant improvement of Olby and Tarlov scores, which were used to evaluate functional recovery of the hind limbs, was observed in the 1 week transplantation group, and the accompanying increase in the expression of neuronal markers and decreased expression of inflammation markers were measured as well. In addition, less fibrosis was demonstrated in the 1 week group compared to other groups. Therefore, it is reasonable to conclude that one week after SCI may be the best time point for the further development of therapeutic studies to obtain neuronal regeneration, reduced fibrosis, and eventual function improvement.

In most studies, assessing the long-term effects of treatments is technically difficult due to associated risks of weight loss, urinary infection, and sepsis in injured animals. However, a 3 year long-term effects study of hUMSC transplantation in dogs with SCI was reported by Lee et al. in 2011 [78]. The hUMSCs were transplanted into the balloon injured lesion site in seven experimental dogs. Despite two transplanted dogs dying within one month after transplantation, four of the five surviving experimental dogs survived for three years. These four dogs had restored the hind-limb motor functions (BBB scores) with significant improvement at three years after injury and deep pain recovery was detected from 5 days post injury. Immunohistochemical staining revealed remyelination with many myelin protein-zero positive axons which is the major structural protein of peripheral myelin.

6. Neural Stem/Progenitor Cells

Neural stem/progenitor cells (NS/PCs) were first demonstrated in the subventricular zone of the mouse in 1989 [154] and were isolated from the mouse striatal tissue and subventricular zone for the first time in 1992 [7, 155]. These cells were capable of self-renewal and generating the main phenotypes (neurons, astrocytes, and oligodendrocytes) of CNS cells *in vitro* and *in vivo* [156]. After transplantation into the injured spinal cord, NS/PCs generate mature neural phenotypes and provide neural functional recovery in some SCI models [156].

In vitro culture, NS/PCs can be maintained in a particular and unique living cluster shape to proliferate called a neurosphere. This neurosphere culture system is the main method of neural stem/progenitor cells' study, which was developed by Reynolds and Weiss [155]. Neurospheres are mainly composed of two sorts of cells, one which is an electron-dense and slowly dividing neural stem cell population and their progeny which are immunopositive to actin, weakly positive to vimentin, and nestin-negative, and another population of electron-lucent and fast-dividing progenitor cells which are actin, vimentin and nestin positive [155, 157]. Epidermal growth factor (EGF) and fibroblast growth factor (FGF) are two vital nutritional growth factors that can promote neural progenitor and stem cell growth *in vitro* and *in vivo* [158, 159]. The EGF and FGF receptors are widely expressed in the cytoplasm and nucleus of neural stem/progenitor cells. The amount of sphere component nestin-positive progenitors determines the neurosphere size, and changes in the different cellular populations within neurospheres can result in the alterations of the survival, proliferation, and differentiation capabilities of their neural stem/progenitor cells [160]. As Weible and Chan-Ling reported [161], with the presence of bone morphogenetic protein 4 (BMP4) and leukemia inhibitory factor (LIF) in medium, the portion of oligodendrocytes and neurons can be significantly decreased to 3% and 16%, respectively, and the portion of GFAP+ neural precursor cells is increased to 79%. Based on this study, the neurospheres culture system is able to provide a pure population of astrocytes, which have been extensively utilized for stem cell research. However, the neurospheres culture system cannot be used as a precise assay for assessing clonality, number and fate of stem cells due to the intrinsic dynamic property of neurospheres [162]. Thus, on the basis of proliferative potentials, Louis et al. developed a novel assay for neural stem cells research, the Neural Colony-Forming Cell Assay, which is capable of discriminating NSCs from various progenitor cells, and more accurate regulating of NSCs for specific applications in further experiments or therapeutic use [163].

The *in vivo* transplantation of neural stem/progenitor cells has been widely applied in the therapeutic study of SCI. Scientists have attempted to restore neural functions

via a number of different strategies including neuronal differentiation, axon regeneration, remyelination, and nutrient secretion (Table 3). The survival rate and cellular character alternations in a long period are vital to the transplantation therapy. The long-term properties of human spinal cord-derived neurospheres were examined by Åkesson et al. [79], they were successful in culturing neurospheres *in vitro* with EGF, bFGF, and CNTF for up to 25 passages for about 350 days. After 18 passages expansion *in vitro*, the differentiated neurons and neural cells were transplanted into the spinal cord lesion of rats. The minimum survival time for the majority of transplanted cells was 6 weeks, and the expression of neuronal and astrocytic phenotypic markers were observed in these surviving cells. Their results suggested that the neurospheres can be well maintained, expanded, and remain multipotent for a long period of time *in vitro*. The results demonstrated that these long-term cultured cells still have a promising survival rate and differentiate in the injured spinal cord *in vivo*, although most of them likely differentiate into astrocytes.

In most cases, *in vivo* transplanted NSCs have shown a preferential capability of differentiating into glial lineages, especially astrocytes [164]. The direct transplantation of NSCs or NPCs are not always efficient for functional recovery after SCI. Webber et al. [80] transplanted fetal NPCs, derived from fetal rats, into the dorsal column lesion site of adult rats. Although most of the grafted cells survived and remained around the lesion, only minor sensory function improvement was observed, and the motor function recovery was not restored. This result was probably a result of the high differentiation rate (40%) of grafted stem cells into glial cells, low neuronal differentiation, and the failure of axon regeneration beyond the lesion site. Tarasenko et al. treated hNSCs with bFGF, heparin, and laminin for priming before transplantation. Then, they transplanted these primed hNSCs into the contusion lesion of rats at the same day or 3 or 9 days postinjury. Compared with the unprimed group, the best results with optimized survival rate, neuronal and oligodendroglia differentiation, and improved trunk stability were obtained 3 months after the engraftment in the primed and 9 days postinjury transplantation group [81]. They claimed that human neural stem cell fate determination *in vivo* might be influenced by the predifferentiation treatment prior to grafting, and furthermore the functional improvement is related with the transplantation time point after injury, and the newly differentiated neurons and oligodendrocytes. Yan et al. [82] reported that the spinal cord microenvironment can probably change the differentiating fate of grafted NSCs. The centrally located NSCs appeared to differentiate into neurons, and the other cells located under the pia membrane tend to have an astrocytic phenotype. Moreover, the lesion microenvironment in the white matter of the spinal cord can markedly promote the differentiation of NSCs into astrocytes.

Grafted NSCs can also differentiate into neurons with certain pretreatments. Remyelination, and synaptic contact reformation is essential for the restoration of spinal cord circuitry which are the structural and physiological elements for functional recovery. Yasuda et al. transplanted shi-NS/PCs, which were obtained from myelin-deficient shiverer mutant mice, into the lesion site of rats in order to compare the capability of remyelination with wt-NS/PCs. At the end of experiment, they claimed that the remyelination capability of wt-NS/PCs was vital to motor and electrophysiological functional recovery [83]. Hwang et al. transplanted Olig2-NSCs, which were transfected by retrovirus with Olig2 transcription factor expression, into contused spinal cord [84]. They observed high proliferative activity of Olig2-NSCs in the experimental group by 7 weeks after transplantation, and the increased volume of spared white matter and reduced cavity volume were observed as well. Further, thickened myelin sheath was detected, which may have been induced by the differentiation of NSCs into oligodendrocytes. More importantly, significant locomotor recovery of the hindlimbs was also measured. Alexanian et al. isolated A2B5(+) NG2(+) NPCs from hNPC neurospheres, and then transplanted them into SCI rats. As a result, compared with NCAM(+) A2B5(+) group and NCAM(+) A2B5(+) group, the significantly improved locomotor and sensory functional recovery was obtained in the A2B5 (+) NG2 (+) group [85]. Both of the studies above indicated that oligodendrocyte differentiation from grafted neural stem/progenitor cells is vital to the functional recovery promoted by remyelination by oligodendrocytes in the CNS. Besides remyelination, synaptic contact reformation is also important for the reconstruction of neurofunctional circuitry. Yan et al. [82] grafted NSCs from human fetal spinal cord into the lumbar cord of adult nude rats. The large-scale differentiation into neurons, axon regeneration, and extensive synaptic contacts reformation with host motor neurons was observed. As they reported, the newly differentiated neurons integrated into the host neural circuits, which indicated the possibility of neural circuitry restoration in the traumatically injured spinal cord. In addition to the transplantation of single cell types, the combined dual-type or multitype strategies are also being actively pursued. Wang et al. [86] injected NSCs and olfactory ensheathing cells (OECs) into the spinal cord lesion of rats at 7 days post-SCI, and reported hindlimb locomotor functional recovery at twelve weeks post transplantation. Novel NF200 positive fibers which crossed through the injured region were observed by them, however, they did not examine the axon remyelination and synapse formation. The optimal time point for cellular transplantation after spinal cord trauma has not been established till now. The most common transplantation time window ranges from 7 d.p.i. to 10 d.p.i. However, the largest population of SCI patients is composed of chronically injured individuals. The inflammatory response, glial cell activation, and the inhibitory microenvironment that exists in the acute phase after trauma largely acts as a negative obstruction to any form of cellular therapy. On the other hand, the pathological alterations of the lesion site in a chronic patient may not be reversible due to the formation of a glial scar, the permanent demyelination/dysmyelination of spared axons, and the apoptosis of spared neurons. Therefore, the optimal transplantation time-window most probably lies in the subacute phase. The study of Salazar et al. demonstrated significantly improved locomotor recovery in an early chronic spinal cord injury mouse model after NSC transplantation [87]. More importantly, they showed that most of the

Table 3: *In vivo* transplantations of neural stem/progenitor cells.

First author	Year	SCI Models	Main graft	Transplantation methods	Pre-differentiation or pretreated	Combination	Tissue sparing	Neuronal regeneration	Axonal regeneration /remyelination	Sensory function	Motor function	Cavity and/or scare formation	Inflammation	Others
Åkesson, [79]	2007	T8, clamp compression-rats	human spinal cord-derived neurospheres	Lesion epicenter i. medu.	—	—	—	Imp.	—	—	—	—	—	—
Webber, [80]	2007	C4, dorsal hemisection-rats	Fetal NSCs-rats	Lesion site, rostral and caudal i. medu.	—	—	—	No Imp.	No Imp.	Partially Imp.	No Imp.	—	—	High glial differentiation rate
Tarasenko, [81]	2007	T9-10, contusion-rats	Human fetal NSCs	Lesion epicenter i. medu.	—	—	—	Imp.	Imp.	—	Imp.	—	—	—
Yan, [82]	2007	L4 and L5 roots avulsion—nude rats	Human fetal NSCs	Lesion epicenter i. medu.	—	—	—	Imp.	Imp.	—	—	—	—	Synaptic contact reformation
Yasuda, [83]	2011	T10, contusion-mice	Mouse fetal NSCs	Lesion epicenter i. medu.	—	—	Imp.	Imp.	Imp.	—	Imp.	—	—	—
Hwang, [84]	2009	T9-10, contusion-rats	Human fetal NSCs	Lesion epicenter and rostral i. medu.	Olig2-transfection	—	Imp.	Imp.	Imp.	Imp.	Imp.	Imp.	—	—
Alexanian, [85]	2010	T8, compressed-rats	Human fetal NPs	Rostral and caudal i. medu.	Differentiated to OPCs	—	—	—	Imp.	Imp.	Imp.	—	—	—
Wang, [86]	2010	3/4 lateral transection-rats	Rats fetal NSCs	Rostral and caudal i. medu.	—	NSCs + OECs	—	Imp.	Imp.	—	Imp.	—	—	—
Salazar, [87]	2010	T9, contusion-mice	Human fetal NSCs	Rostral and caudal i. medu.	—	—	No Imp.	Imp.	—	No Imp.	Imp.	No Imp.	—	—

—: Not reported; Imp.: Improvement; i. medu.: intramedullary injection; SCI: spinal cord injury; NSCs: neural stem cells; NPs: neural precursors; OPCs: oligodendrocyte progenitor cells; OECs: olfactory ensheathing cells.

transplanted NSCs had differentiated into oligodendrocytes and neurons and that astrocytic differentiation was rare. The authors also reported the integration of transplanted human NSCs with host cells.

7. Olfactory Ensheathing Cells

Olfactory Ensheathing Cells (OECs) are considered as a special class of glial cells which exist in both the PNS and CNS, and share certain features and functions with astrocytes as well as Schwann cells [165]. OECs are present in the olfactory epithelium, where neurogenesis occurs throughout adulthood. The olfactory epithelium (OE) is composed of two kinds of neural stem cells, which are the globose basal cells (GBCs) and the horizontal basal cells (HBCs). GBCs are the main resource for homeostatic neurogenesis that leads to the birth of neurons and other cellular populations such as OECs. Unlike the GBCs, HBCs are normally quiescent, but they can be activated to generate novel GBCs to reconstruct the cellular populations of OE after injury [166, 167]. OECs were identified as an elongated shape with thin laminar processes that ensheath olfactory nerves *in situ*, but the morphologies of cultured OECs are distinct, from flat shape to bipolar and tripolar, moreover, there are also various antigenic differences. These heterogeneities may be caused by the different origins of the olfactory tissue used, the age of donor, the method of isolation, and culture conditions, and can also be affected by extracellular and intracellular molecules [168]. This kind of property is thought to allow OECs to transform themselves within different morphological and antigenic types to exhibit different functions and to adapt various environments [168]. When OECs act as Schwann cells with the same bipolar appearance, they can produce similar axon growth molecules, although the remyelination ability is poorer than Schwann cells [169, 170]. When they are transformed to astrocyte-like flattened cell shapes, a GFAP positive cellular supporting structure can be detected [165]. Nevertheless, compared with Schwann cells, OECs are more likely to rescue neural function in the injured spinal cord by virtue of their cell-specific properties. The bridging effect of transplanted OECs on regenerated axons of from dissected dorsal root into spinal cord was reported by Li et al. [171]. Importantly, OECs were shown to be able to repress astrocyte proliferation and reactivity *in vitro*, activated astrocytes after injury are the main source of the glial scar [172].

On account of their neuronal regeneration-promoting potential and their ability to support axonal outgrowth, OECs have been tested in *in vitro* and *in vivo* experiments for their regeneration promoting effects in SCI [173, 174] (Table 4). Although *in vivo* functional recovery by means of OEC transplantation has been reported by several groups, the mechanism of the regeneration-promoting ability is still far from clear. A recent study reported electrophysiological evidence of the recovery of motor-evoked potentials and axonal regeneration after OEC injection into a complete transection lesion [88]. But other groups have shed doubt on the functional improvements induced by OECs grafts, and have suggested that they are caused by a trophic support mechanism and not the birth of new neurons, which means that the therapeutic potential of OECs after SCI may be limited [89, 90]. Lu et al. reported that no significant axon growth promoting effect was detected in the OECs transplanted group, and no bridge-crossing phenomenon of corticospinal axons was observed beyond a dorsal column lesion [89]. Collazos-Castro et al. transplanted OECs into cervical contusion injury model of rats, neither dorsal corticospinal tract axon regeneration nor locomotor deficits recovery was demonstrated [90]. Furthermore, the result from the olfactory tissue transplantation study of Centenaro et al. [91] and Aoki et al. [92] also suggest that OECs may be of limited use in promoting recovery after SCI. They transplanted tissue pieces of olfactory lamina propria (OLP) and respiratory lamina propria (RLP) into the transection lesion site of adult rats. After grafting, similar hindlimb motor improvement, comparable spinal cord tissue sparing and sprouting in the lesion site was observed between the OLP and RLP groups. In addition, only limited supraspinal axonal regeneration was shown by retrograde tracing, even though a large number of 5-HT positive fibers were found next to the grafts. Therefore, they suggested that the limited functional recovery and neural reparative effects may not be exclusively related to OECs [91]. Aoki et al. transplanted the whole-layer olfactory mucosa into the completed injured rats, and only observed limited functional recovery [92].

All these negative results above may be attributable to a number of factors, such as the nature of cell donor, the tissue source, the injury models, graft cells preparation, the time point of transplantation, and transplantation procedures. Compared with olfactory bulb-derived OECs (OB-OECs), Richter et al. reported reduced cavity formation, better axon regeneration, and remyelination after transplantation of lamina propria-derived OECs (LP-OECs) [93]. Zhang et al. reported that LP-OECs can indirectly promote tissue repair, axonal regeneration and remyelination, and shrink the cavity after scar ablation and lamina propria tissue transplantation. However, motor function recovery was not achieved [94, 95]. The result from Yamamoto indicated that olfactory mucosal cells were not able to promote CST axon regeneration, despite restoration of fore-paw motor function [96].

Concerning the "transplantation Time-window point", the inflammatory reaction and acute cellular response in the acute phase after injury is certainly antagonistic to neuronal regeneration, axonal extension, and grafted cell survivals surviving. *In vitro*, the apoptosis rate of OECs with the appearance of acute explants of spinal cord was demonstrated significantly higher than the chronic group [175]. The chronic lesion site was divided into three different histological zones from outside to center by Zhang et al. (1) Fibrotic zone, which consists of invading connective tissue. (2) Cellular zone, which is composed of invading Schwann cells. These Schwann cells might presumably migrate from the lateral dorsal roots. (3) Axonal zone, which is composed of spared and regenerated axons. After ablation of scars, the OECs from LP grafts increased the size of the cellular and axonal zones, more importantly, the absence of scar formation, the integration of repaired tissue with spared tissue, and remyelinated axons in the axonal zone were observed [94]. Muñoz-Quiles et al. [97] compared the motor function recovery

Table 4: *In vivo* transplantations of olfactory ensheathing cells.

First author	Year	SCI Models	Main graft	Transplantation methods	Pre-differentiation or pretreated	Combination	Tissue sparing	Neuronal regeneration	Axonal regeneration /remyelination	Sensory function	Motor function	Cavity and/or scare formation	Inflammation	Others
Ziegler, [88]	2011	T9, complete transection-rats	Rats OECs	Rostral and caudal i. medu.	—	—	—	—	—	—	Imp.	—	—	Improvements in MEPs
Lu, [89]	2006	C4, dorsal hemisection-rats	Rats OECs	Rostral and caudal i. medu.	—	—	—	No Imp.	Partially Imp.	—	—	—	—	—
Collazos-Castro, [90]	2005	C7, contusion-rats	Rats OECs	Rostral and caudal i. medu.	—	—	—	No Imp.	No Imp.	—	Partially Imp.	—	—	—
Centenaro, [91]	2011	T9, complete transection-rats	OLP and RLP-rats	Lesion site, i. medu.	—	—	—	—	Imp.	—	No Imp.	—	—	—
Aoki, [92]	2010	T10, complete transection-rats	Rats-whole-layer olfactory mucosa	Rostral and caudal i. medu.	—	—	—	—	Partially Imp.	—	Partially Imp.	No Imp.	—	—
Richter, [93]	2005	C4, dorsal lateral hemisection-rats	Mouse lamina propria-derived OECs	Lesion epicenter and rostral i. medu.	—	—	Imp.	—	Imp.	—	—	Imp.	—	Enhanced angiogenesis
Zhang, [94]	2011	T10, contusion-rats	OLP and RLP-rats	Lesion site, i. medu.	—	—	—	—	Imp.	—	—	—	—	Activation of host SCs
Zhang, [95]	2011	T10, contusion-rats	Rats lamina propria-derived OECs	Rostral and caudal i. medu.	—	Glial scar ablation + OECs	—	—	Imp.	—	No Imp.	Imp.	—	Activation of host SCs
Yamamoto, [96]	2009	T10, hemisection-rats	Rats OECs	Lesion epicenter i. medu.	—	—	—	—	No Imp.	—	—	—	—	—
Muñoz-Quiles, [97]	2009	T9, complete transection-rats	Rats OECs	Rostral and caudal i. medu.	—	—	—	—	Imp.		Imp.	Imp.	—	Best results in sub-acute transplantation group
Novikova, [98]	2011	C4, lateral hemisection-rats	Rats OECs	Rostral and caudal i. medu.	3 Weeks or 7 weeks pre-culture	—	—	Imp.	Imp.	—	—	—	—	Aged cells are less effective
Toft, [99]	2007	L3, lateral hemisection-rats	Rats OECs	Lesion site, i. medu.	—	—	—	—	Imp.	—	—	Imp.	—	Improvements in cord dorsum potentials and SEPs
Liu, [100]	2010	T11-12, catheter compression-rats	Rats OECs	Lesion epicenter i. medu.	—	—	—	Imp.	—	—	Imp.	—	—	Improvements in SEPs and MEPs
alinčík, [101]	2010	T4, complete transection-rats	Rats OECs	Lesion epicenter i. medu.	—	—	—	Imp.	—	—	—	—	—	Improvements of autonomic dysreflexia

Table 4: Continued.

First author	Year	SCI Models	Main graft	Transplantation methods	Pre-differentiation or pretreated	Combination	Tissue sparing	Neuronal regeneration	Axonal regeneration /remyelination	Sensory function	Motor function	Cavity and/or scare formation	Inflammation	Others
Tharion, [102]	2011	T10, contusion-rats	Rats OECs	Lesion site, rostral and caudal i. medu.	—	—	—	—	Imp.	—	Imp.	—	—	Improvements of MEPs
Stamegna, [103]	2011	C2, lateral hemisection-rats	Rats OECs	Lesion site, rostral and caudal i. medu.	—	—	—	—	Imp.	—	Imp.	Imp.	—	Improvements in diaphragm activities Improvements in phrenic nerve activities
Bretzner, [104]	2010	C4, dorsal lateral hemisection-rats	Mice OECs	Rostral and caudal i. medu.	—	cAMP infusion + OECs	Imp.	—	Imp.	Imp.	Imp.	Imp.	—	—
Ma, [105]	2010	T9, contusion-rats	Rats OECs	Lesion level, rostral and caudal s. i.	NT-3-transfection	—	—	—	Imp.	—	Imp.	—	—	—
Salehi, [57]	2009	T9, complete transection-rats	Rats OECs	Lesion site, i. medu.	—	MNs + OECs	Imp.	Imp.	Imp.	—	Imp.	Imp.	—	—
Amemori, [106]	2010	T8 balloon catheter compression-rats	Rats OECs	Rostral and caudal i. medu.	—	OECs + MSCs	Imp.	—	—	Partially Imp.	Partially Imp.	—	—	Partial improvements of MEPs

—: Not reported; Imp.: Improvement; i. medu.: intramedullary injection; s. i.: subarachnoid injection; SCI: spinal cord injury; MNs: motoneurons, SCs: Schwann cells; OECs: olfactory ensheathing cells; RLP: respiratory lamina propria; OLP: olfactory lamina propria; MSCs: mesenchymal stem cells.

after OB-OEC transplantation into completed transection injured rats among subacute (1 month after injury), chronic (4 month after injury), and nontreatment groups. At the seventh month after transplantation, all the treated rats had improved hindlimb motor function, and the improvement was significant when compared with nontreated rats. In addition, the final plateau of percentage of recovery in subacute transplantation group was reported 10% higher than the chronic group. Interestingly, they also showed the demonstration of regeneration of motor axons growing beyond into the lesion site, which indicates the lesion site-crossing phenomenon from rostral to caudal [97]. Based on these data therefore, we propose that the subacute or chronic cellular transplantation to bypass the acute phase after spinal trauma combined with scar ablation may be a potentially effective strategy. Considering the potential of secondary damage to clinical patients caused by scar ablation, cellular therapy during the subacute phase which occurs prior to the formation of the permanent glial scar may be more valuable and feasible for further clinical application.

After transplantation into injured spinal cord, the fate of grafted OECs can also be influenced by *in vitro* culture conditions. The survival of transplanted OECs and their properties of neuroprotection, neurotrophic factor expression, axon growth-promotion, and remyelination can be affected by the duration of pretransplantation culture and the purification methods used [98]. According to the report of Novikova et al, compared with the shorter preculture time (3 weeks), OECs with longer preculture time (7 weeks) are significantly less effective in protecting neurons and promoting axonal regeneration due to aging of the cells [98]. They also suggest that the differential cellular signal responses to disparate microenvironments within different purification methods used for preparation might induce distinct cellular behaviors after OEC transplantation.

Although several questions regarding the application of OECs have been raised, several recent studies support a protective/regenerative role [88, 102, 103, 176]. Electrophysiologically, OECs were confirmed to be able to preserve the function of circuitry with the evoked cord dorsum potentials and sensorimotor cortex potentials in the region of dorsal column lesion after transplantation [99]. In another study, Liu et al. comprehensively analyzed behavioral improvements, somatosensory and motor evoked potentials in rats after OECs transplantation. Significantly improved results were measured in OEC-treated rats compared with control groups despite the absence of retrograde labeling [100]. Furthermore, autonomic dysreflexia which can cause abnormalities in blood pressure, heart rate, and respiration in high level spinal cord injury was assessed in the study from Kalinčík et al. They reported the normalization of enlarged sympathetic preganglionic neurons by means of OEC transplantation in a transection SCI model [101]. This cellular morphological normalization was suggested to be meaningful for the recovery from autonomic dysreflexia although no effect on cardiovascular parameters was confirmed in the OEC-grafted group except a 25% shorter recovery time from hypertension [101]. Regarding motor function recovery, Tharion et al. assessed motor-evoked potentials and scores on the BBB scale after OECs transplantation. They reported significant improvement in the OEC-grafted group when compared with the control group. Furthermore, they reported one animal that was followed-up for 264 days after transplantation with the highest BBB score of 17 [102]. Improvements in respiratory function are vital for recovery post-high-level spinal trauma. By using a cervical contusion rat model, Stamegna et al. induced a persistent hemidiaphragmatic paralysis for assessing the therapeutic efficiency of OEC transplantation at 2 weeks postcontusion. At 3 months after transplantation, significant improvement of breathing movements, activities of the ipsilateral diaphragm and axonal sprouting in the lesion site was observed, suggesting that respiratory function was partially restored [103].

Although the application of only OECs has shown promise in the promotion of recovery after SCI, combinatorial approaches have also been utilized in order to boost efficacy. cAMP treatment [104], Neurotrophin-3 (NT-3) production via genetic modification [105], Laserpuncture [177], and cotransplantation with other cells [57, 106, 178] have been combined with OEC transplantation. The weak intrinsic neuronal growth response has been shown to contribute to the failure of neuronal regeneration after SCI. The cAMP pathway has been shown to be critical for increasing this intrinsic capacity in neurons [179]. Bretzner et al. transplanted lamina propria-derived OECs into dorsolateral funiculus crush lesion site with cAMP infusion treatments. The authors reported a significant decrease of GFAP expression and cavity formation, with remarkable axon regeneration and both sensory and motor function improvement. Their study indicates the feasibility and efficacy of a combined strategy of OEC transplantation and intrinsic cellular signal enhancement to promote recovery after SCI [104]. As a member of the neurotrophic superfamily, NT-3 can counteract pathological factors post-SCI and promote the survival of neurons after SCI [180]. NT-3 can also stimulate neuronal regeneration and neurite outgrowth [181]. Ma et al. transplanted NT-3 gene-modified OECs, which can express NT-3 efficiently, into the contusion lesion of rats in order to promote better morphological and functional recovery when compared with simple OEC transplantation [105]. Based on their results, both axonal regeneration, which was verified via HRP retrograde tracing, and motor function recovery, which was assessed by BBB scoring, were significantly better in the combination group compared to normal OECs and the control group.

In the development of therapeutic research for neural function recovery after SCI, distinct cellular functions specific to different transplanted cell lines have been identified in repairing damaged neural tissue. Theoretically, the combined application of different cell types may provide more benefits than single-celltype transplantation by means of synergistic effects. Salehi et al. reported significantly better recovery of rat hindlimb motor function, which was accompanied by significantly greater percentage of spared tissue, axon regeneration, and remyelination in the cotransplantation group of OECs and embryonic stem cell-derived motor neurons, when compared with the other single-celltype groups [57]. However, not all types of cells in combination confer a

therapeutic advantage. Amemori et al. tried to develop a cotransplantation strategy which included OECs and MSCs, and expected a significant synergistic effect in neural function improvement. Unexpectedly, no significant differences of BBB scores and plantar tests were assessed among all groups at the end of the experiment, although some improvements of motor function were observed within different time points [106].

8. Schwann Cells

In the SCI patients and large animal models of SCI, a cystic cavity usually forms after injury, and a glial scar formed wall separates this cavity from the surrounding spared rim of white matter. At the edge of the glial scar, the regenerated axons regularly terminate in dystrophic endings, which means the termination of axon regrowth [182]. In response to overcome this serious obstacle of regeneration, developing an efficient corresponding bridging countermeasure becomes more and more urgent. After the spinal cord injury, the injured neurons can demonstrate an intrinsic capability of growth cone formation and axon extension initially, but all these "regenerating behaviors" are soon suppressed by the inhibitory microenvironment. When transplanted these injured neurons from the spinal cord lesion site are transplanted into a peripheral neural environment, they can completely recover as normal neurons electrophysiologically and morphologically [183]. The Grafted peripheral nerve segments in the spinal cord were reported to be capable of improving the recovery of behavioral and electrophysiological function *in vivo*, via axons regeneration, reformation of functional synapses with host neurons, neurotrophic molecule secretion, and by providing a permissive PNS-like environment providing [184, 185] (Table 5). Schwann cells (SCs), the myelinating cells of the PNS, play important roles in postinjury nerve regeneration by contributing to the axon regeneration and remyelination and forming guidance bands, bands of Büngner, for regenerating axons [186]. After transplantation into a demyelinated spinal cord slice *ex vivo*, SCs can stimulate the survival and intrinsic regeneration ability of damaged neurons by producing a number of neurotrophic factors which include NGF, BDNF, and CNTF [186]. In addition, the grafted SCs can also generate a variety of cell adhesion molecules and extracellular matrix proteins to support axonal growth as well, such as integrins, N-cadherin, N-CAM, L1, contactin, laminin, and collagens [187, 188]. More importantly, in order to achieve the goal of neural functional recovery by means of SCs treatment, the remyelination of demyelinated axons or newly sprouted axons must occur, as has been observed and confirmed [107, 189].

Traditionally, SCs were commonly isolated from peripheral nerves, and proliferated in culture to generate a large number of cells. Recently, the SCs used in SCI research have been derived from several categories of stem cells or neural progenitors directly, including mesenchymal stem cells [186], adipose-derived stem cells [190], and skin-derived precursors [191]. In an *ex vivo* experiment [186], Park et al. examined the neurotrophic effects of MSC-derived SCs with Neuro2A cells in a lysolecithin-induced demyelinated organotypic coculture system. The significantly enhanced axonal outgrowth of Neuro2A cells was promoted by the two specific neurotrophic factors: hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), which were secreted by MSC-derived SCs. Concurrently, a dramatic decrease in lysolecithin-mediated cell death was confirmed via the assessment of average number of TUNEL-positive cells per slice, which was decreased by 30% and 50% when compared with the MSCs treated vehicle group and control group, respectively. Xu et al. generated neurospheres from adipose tissue collected from embryonic mesoderm, and then successfully differentiated into SCs [190]. They cultured SH-SY5Y cells in the conditioned medium (CM) collected from SCs for 3 days in order to evaluate the effects of soluble factors secreted from SC-like cells. Compared with the results of the control group, beta-tubulin III positive neurite outgrowth was detected in around 31% of SH-SY5Y cells in the CM treated group. After 14 days, the multilayer membranes composed of myelin structures were observed surrounding the PC12 cell neurites cocultured with SCs via electron microscopy. Biernaskie et al. developed an efficient protocol for generating Schwann cells from skin-derived precursors (SKPs) which can be isolated from the dermis of both rodent and human skin [191]. They transplanted SKP-derived SCs or SKPs into a murine contused model to examine their repair promoting abilities in the injured spinal cord [107]. Although both the SKPs and SKP-derived SCs contributed to the reduced size of contusion cavity and remyelinated axons in the lesion site at 12 weeks after transplantation, SKP-derived SCs provided more promising results when compared with the SKPs transplantation group, including lesion site bridging effect, increased size of spared tissue, and reduced reactive gliosis [107]. Functionally, a significant enhancement of locomotor recovery was achieved in the SKP-derived SCs transplantation group, although there was no restoration of sensory function. Agudo et al. assessed the therapeutic potential of Schwann cell precursors (SCP) in an acute SCI model by immediate cell injection into the lesion site after surgery [108]. Unlike the SCs, they reported that SCPs started to proliferate rapidly right after the transplantation to fill the site of cavity where an injury-induced cavity is present in the control group. Within the cystic cavity, SCPs induced angiogenesis which was verified by the appearance of typical immunostained blood vessels with the expression of smooth muscle actin (SMA). Instead of the proliferation, which had been reduced to less than 40% after 4 weeks after transplantation, the maturation of SCPs into S100b positive SCs was observed. 8 weeks after transplantation, the SCP-differentiated SCs group had significantly reduced glial scar formation with significant reduced expression of GFAP. More importantly, the grafted cells successfully integrated into the host tissue, and a robust bridging effect was observed extending rostrocaudally. The regenerated BDA-labeled CST axons, which successfully crossed the lesion site, were assessed using anterograde tracing. These regenerated axons were also confirmed to be remyelinated by P0 positive myelin. However, motor function

Table 5: *In vivo* transplantations of Schwann cells.

First author	Year	SCI Models	Main graft	Transplantation methods	Pre-differentiation or pretreated	Combination	Tissue sparing	Neuronal regeneration	Axonal regeneration /remyelination	Sensory function	Motor function	Cavity and/or scare formation	Inflammation	Others
Biernaskie, [107]	2007	T10, contusion-rats	Rats SKP-SCs	Lesion epicenter i. medu.	—	—	Imp.	Imp.	Imp.	—	Imp.	Imp.	—	—
Agudo, [108]	2008	C4, dorsal hemisection-rats	SCPs-postnatal 2d rats	Rostral and caudal i. medu.	—	—	—	—	Imp.	No Imp.	No Imp.	Imp.	—	Enhanced angiogenesis
Patel, [109]	2010	T9, contusion-rats	Rats SCs	Lesion epicenter i. medu.	—	Gelling matrix (laminin + collagen) + SCs	—	—	Imp.	—	Imp.	—	—	—
Olson, [110]	2009	T8-9, transection-rats	Rats SCs	Lesion epicenter i. medu.	—	Gelling matrix + NSCs + SCs	—	Imp.	Imp.	—	No Imp.	—	—	—
Ban, [68]	2011	T9, contusion-rats	Rats SCs	Lesion epicenter i. medu.	—	MSCs + SCs	—	—	Imp.	—	Imp.	Imp.	—	—
Fouad, [111]	2005	T8, complete removal of a 4-mm length of spinal cord-rats	Rats SCs	Lesion site, rostral and caudal i. medu.	—	Gelling matrix + MSCs + SCs	—	—	Imp.	Imp.	Imp.	—	—	—
Sharp, [112]	2012	T9, contusion-rats	Rats SCs	Lesion site, i. medu.	—	SCs + dbCAMP	—	—	Imp.	—	No Imp.	—	—	—

—: Not reported; Imp.: Improvement; i. medu.: intramedullary injection; SCI: spinal cord injury; SCs: Schwann cells; SKP: skin-derived precursors; SCPs: Schwann cell precursors; dbCAMP, dibutyryl CAMP; MSCs: mesenchymal stem cells.

was not significantly different between the SCP and vehicle group.

In order to overcome the limitations of one particular cell type and to maximally tap into the potential of SCs; the genetic modification, combined treatments, and cotransplantation have all been used in SCI research. Glial cell line-derived neurotrophic factor (GDNF) promotes the survival of dopaminergic and motorneurons and increases axonal regeneration [192]. Deng et al. examined the interaction between genetically modified GDNF overexpressing SCs (GDNF-SCs) and reactive astrocytes in a specific guidance channel culture system *in vitro* [193]. According to the results of their study, GDNF-SCs suppressed the expression of GFAP and CSPG of reactive astrocytes, and then induced robust migration of astrocytes into the GDNF-SC transplants with elongated processes which extended in parallel to the regenerated axons. Importantly these axons were remyelinated (SMI-31 positive) by GDNF-SCs. Overall, their work indicates a novel and attractive strategy to control the reactive astrocyte induced inhibitory environment and to promote greater axonal regeneration, remyelination, and functional recovery following SCI.

In addition to genetic modifications, combining SCs with different kinds of matrices and scaffolds have also been attempted in recent years [109, 110]. Pearse et al. suggested that the traditional cell injection media of DMEM, with low oxygen levels, and high levels of oxidative metabolites and inflammatory cytokines, may be responsible for the low survival rate of SCs after implantation [194]. In the interest of graft survival after transplantation, Patel et al. investigated whether transplantation of SCs within various injectable gelling matrixes as suspension cells could improve their long-term survival in the contused SCI model [109]. At the end of the experiment, the matrices which composed of laminin and collagen was reported to show significantly increased capability of improving SC survival, graft vascularization, and axonal in-growth over controls. And the SC transplantation within Matrigel from BD was reported to significantly enhance locomotor function as assessed by the BBB scale. In another study which using the biodegradable polymer scaffolds (polilactic acid and polyglycolic, PLGA), Olson et al. injected SCs within BD Matrigel into the multichannel of the PLGA scaffolds in order to assess the potential of this combined treatment to promote axon regeneration after SCI *in vivo* [110]. One month after transplantation, animals were sacrificed for immunohistochemical staining. This paper showed significant axonal regeneration, but the lack of behavioral improvements.. There were no significant differences in BBB scores between the SCs+scaffold group and the control group in the one month period after surgery.

The cotransplantation approach has also been tested for SCs in SCI research [195]. After obtaining promising results *in vitro*, Ban et al. transplanted SCs and MSCs together into the epicenter of injury [68]. Significantly more regenerated axons in the corticospinal tract, which surrounded and crossed through the posttrauma cavity, were observed in the cograft group. In addition, this group also had the smallest population of GFAP positive astrocytes in the epicenter of injury. Moreover, under electron microscopy, the completely reconstructed myelin sheaths were found in the cograft group. In addition to these histopathological improvements, hindlimb motor function in the cograft group was significantly improved as determined by increased BBB scores. In another co-transplantation experiment, instead of simply mixing different cell types together and injecting the mixture into the animal, Fouad et al. combined the bridging effect of SCs and the MSCs in an innovative way [111]. They seeded SCs into a scaffold with Matrigel, and then implanted this component into the lesion site to build up a bridge for the extension and reconnection of regenerated axons. Then they grafted OECs rostral and caudal to the lesion site, to test whether this approach had any therapeutic advantage. Axonal regeneration within the corticospinal and reticulospinal tracts was not observed through the SCs-scaffold bridge. There was significantly improved motor function as measured by BBB scores and forelimb/hindlimb coupling, which was accompanied by significantly increased numbers of remyelinated serotonergic axons through the bridge.

Not all results from combined treatment experiments have been positive and encouraging. In an *in vivo* experiment, Sharp et al. [112] repeated a previous experiment [196] which exhibited significant locomotor recovery enhancement after contusion injury by means of a combined treatment, which included SC transplantation, systemic delivery of cAMP level enhancer (Rolipram), and intraspinal injection of a non hydrolyzable analog of cAMP (dibutyryl). Almost completely contradictory results were obtained by Sharp et al. compared to the previous study. No significant differences, which include BBB scores, base of support, stride length, and paw rotation were replicated With regards to the anatomical assessments, although a reduction in mean cavity area at the lesion epicenter and remyelinated axons crossing the lesion site were observed in the two groups that received SCs, there were no significant differences between the groups. Bunge and Pearse responded to the replicated work of Sharp, and they provided more details of the original experiment in order to explain the differences [197]. Scott et al. identified some important variables that contribute to explain those different results between the original and replicated study, which included the experimental group arrangements, consistency of injury severity, appropriated statistics, and animal surgery [198].

9. Clinical Trials

With progress in *in vivo* studies, scientists and surgeons have been eager to conduct clinical trials to explore the therapeutic effects of cell transplantation on spinal cord patients. Various cell types, different administration strategies, and different kinds of SCI patients have been involved in clinical trials, however, several obstacles that are inherent to human studies including ethical issues differences in anatomy, and differences in underlying pathophysiological processes, has hampered progress. Until now, no promising cell therapies that are safe and effective for SCI patients have been achieved.

9.1. Clinical Trials of ESC/iPSC or ESC/iPSC-Derived Therapy. The expectation of clinical application by means of an ESC/iPSC or ESC/iPSC-derived therapy has been widely discussed in the media. There are several issues concerning the safety and the efficacy of these stem cell strategies, which may range from target population selection, long-term tumor genesis, to a series of ethical problems [199–201]. According to Aznar and Sánchez, the data available do not justify a clinical trial of stem cell-related therapies, more preclinical study should be carried out and repeated in large animal models of SCI (e.g., cat, dog, rabbit, or primate) [202]. Even though the concern of cyst formation after stem cell transplantation at the injury site was raised by scientists, the Geron Corporation was allowed to run the first clinical trial of stem-cell therapy for SCI in 2009. In the next year, Geron corporation initiated the first clinical trial (Phase I) to test the safety of human embryonic stem cell-derived OPCs, GRNOPC1, within patients who were suffering from complete thoracic level paraplegia with the loss of motor and sensory function [203]. GRNOPC1 was administered into the lesion site within 14 days of injury with a low dose of 2 million cells. To date, there are no serious adverse events in the long-term followup reported by them. Furthermore, they plan to test the safety in patients with a higher cell concentration with 20 million cells in the next step. In November 2011, Geron announced that it had ended its SCI stem cell research program largely due to financial reasons [204]. Based on the work that was completed in the Phase I clinical trial, no therapeutic improvements were reported, although Geron was looking mainly at the safety profile at this stage. So far, no further safety issues have emerged [204]. As this was a significant trial for stem-cell-based therapy for SCI, its premature end, the trial design and the safety results it generated have drawn much attention and interest from researchers around the world. Bretzner et al. proposed a comment to argue the target population selection in the clinical trial of GRNOPC1, and they suggest a more detailed criteria for selecting patients for different study purposes: (1) chronic complete SCI patients for a safety trial, (2) subacute incomplete SCI patients for an efficacy trial, (3) and perhaps primary progressive multiple sclerosis patients for a combined safety and efficacy trial [201]. They posed that the chronic completed SCI patients may be a more preferable target population than subacute complete SCI patients in the phase I clinical trial, because simultaneous recovery may occur in some subacute complete SCI patients and may confound results. In addition, the chronic complete lesion site may ensure a stable microenvironment after cell transplantation in which to assess the safety of transplanted cells [205]. The potential tumorigenicity of ESC-derived OPCs involved in the first clinical trial also concerned scientists, despite several studies reporting the absence of teratomas in rodent experiments [42, 206]. The teratoma-forming propensity was reported to be related with the persistence of undifferentiated cells, even in animal experiments, however, the direct transplantation of undifferentiated ESCs/iPSCs was rare [40]. Du et al. reported a formation of typical teratoma in all immunodeficient experimental mice after the transplantation of hESC-NPCs in spinal cords, but no tumor formation was observed in testis and subcutaneous tissue transplantation group [207]. Teratoma-formation can be suppressed through specific treatments. According to the report from Matsuda et al, the stage-specific embryonic antigen-1 (SSEA-1) expression, which is considered as a marker of teratoma formation, and some mRNA expression markers of undifferentiated ESCs, such as Oct3/4, Utf1, Nanog, Sox2, and Eras, were both significantly reduced in the coculture group of ESCs and bone marrow stromal cells (BMSCs) *in vitro*. The cocultured BMSCs induced undifferentiated ESCs to differentiate into neuronal like MAP-2 positive cells by synthesizing NGF, GDNF, and BDNF *in vitro*. No tumor development was observed after ESCs and BMSCs were grafted together into the mouse SCI model. In contrast, tumor-formation was identified in the solo ESC transplanted group, in which the behavioral improvement also ceased after 21 days of transplantation [208]. In conclusion, ESC/iPSC cell therapies offer promising therapeutic potential for SCI which at this stage waits further clinical testing.

9.2. Clinical Trials of Mesenchymal Stem Cells. Although the long-term safety of MSCs therapies has not been well established until now, clinical tests have proceeded. Although the transplantation of MSCs after SCI has shown some promising results in animal experiments, the therapeutic effects of MSC administration in human SCI still remains inefficacious and has had adverse side effects [34, 209, 210]. Ichim et al. reported a MSCs and CD34 cell combined cellular therapy protocol with a total of 13 intrathecal administrations and 2 IV injections in 3 cycles of treatment. In total, 4.05×10^7 CD34 cells and 1.0134×10^8 MSCs were injected into a 29-year old and ASIA scale type A classified patient within a period of 10 month [34]. Sensory function and lower limbs muscle strength recovery was assessed during the procedure, and significant improvements were measured at the end of treatment. The 10/10 pretreatment neuropathic pain was significantly relieved into occasional pain once a week at a level of 3/10. Six months after the end of treatment, this patient was finally categorized as ASIA type D. Moreover, they reported that neither the immunological reactions nor GVHD was noted. However, this case report did not show evidence from biochemical marker analysis or cell-tracking studies to defend their conclusion that the encouraging functional recovery was caused by the effect of grafted cells, and not spontaneously. Intrathecal administration of MSCs for chronic complete SCI patients, a population of 64 completely injured patients (ASIA Scale: A) who had a mean of 3.6 years rehabilitation therapies 3 times weekly was investigated by Kishk et al. [209]. Autologous MSCs were administrated monthly to forty-five patients for 6 months. 12 months after completing the therapy, a series of paralysis grading systems and a questionnaire of bladder and bowel control were used to evaluate the potential therapeutic effects of MSC administration. However, no differences between the MSCs group and control group were found. More importantly, neuropathic pain was observed in twenty-three MSCs-administrated patients. Therefore, the authors concluded that the safety, the side effects and the potential therapeutic effects

of MSCs should be carefully studied via preclinical models before launching clinical trials. Shortly after the report of Kishk et al., another clinical trial of MSCs administration for chronic complete SCI patients was reported by Bhanot et al. [210]. After a laminectomy, the autologous MSCs were administered at the lesion site of spinal cord. At the end of followup, only one patient demonstrated improvement in motor function, and other two patients showed inconsistent improvement in pin prick sensation below the level of injury. The outcome of MSCs therapy from this clinical study was therefore not successful. In 2012, Park et al. and Karamouzian et al. reported two clinical trials for spinal cord injury by using MSCs transplantation, even though some improvements were noticed in some patients, the therapeutic effects of MSCs transplantation have not been established in human SCI patients [211, 212]. In the study of Park et al., 10 traumatic cervical SCI patients with severe paralysis were involved (ASIA classification A or B) [211]. MSCs were administered three times during the course of the study. First of all, 8×10^6 autologous MSCs were directly injected into the intradural space, after 4 and 8 weeks, another 5×10^6 MSCs, each time, were injected into the spinal cord above the lesion cavity and into the cavity, respectively. With a 6-month follow-up, the motor power grade of the extremities, magnetic resonance imaging, and electrophysiological recordings were assessed. At the end of this study, improvements of daily living activities, increased motor power of the upper extremities, shrinked lesion cavity size, and electrophysiological improvement were observed in 3 patients. Various partially improvements were observed in the rest of 7 patients. And, they claimed that no permanent complication associated with MSCs transplantation was observed. Karamouzian et al. transplanted autologous MSCs into the cerebrospinal fluid via lumbar puncture for eleven SCI patients with complete thoracic injuries. As they observed, 5 of 11 patients in the MSCs transplantation group and 3 of 20 patients in the control group showed marked function recovery, however, the differences between the two groups were not significant. On the other hand, no adverse reaction and complications in both groups were experienced by patients, which may indicate the safety of intrathecal administration of MSCs in human patients.

9.3. Clinical Trials of Schwann cells. Any potential clinical trials of Schwann cells treatments will require addressing a number of questions and concerns. Similar to the ESC-derived OPCs clinical trial, the clinical experiment design must also fulfill the strictest criteria to ensure the safety of grafts and to protect the involved patients from the threat of tumorigenesis and any other serious side effects. Recovery was reported by Xian-Hu et al. within their 6 clinical cases after 5-years followup. The group tested three different transplantation protocols but provided no information on the the distribution of grafted SCs in the body of patients or the tumorigenesis assessment [213]. Furthermore, they provided no criteria for selecting their patient population. On assessment of their methods and results, several important questions arose such as, the ages of patients which range from 7 to 44 years old, the presurgery ASIA evaluations which ranges from A to C, and the transplantation time post-SCI which range from 1 week to 20 months. Importantly, here as in other clinical studies on SCI, the issues of spontaneous recovery and patient heterogeneity (in terms of age, clinical course, and severity) are central to deriving any meaningful information from these results. In another clinical trial report, Saberi et al. transplanted purified SCs which had been acquired from autologous sural nerve into four patients (22–43 years old) who were suffering from stable chronic SCI (28–80 months posttrauma) [214]. Transient paresthesia or increased muscle spasm after transplantation was found in all the four patients. After one year followup, only one patient with incomplete SCI showed some sort of improvement with extensive and continuous rehabilitation. And neither visible positive changes nor negative pathological findings were observed via magnetic resonance imaging. In 2011, Saberi et al. reported another 2-years followup clinical trial for the safety assessment of SCs transplantation therapy [215]. In this study, 33 patients who suffering from completed cervical or thoracic level paraplegia for at least 6 months were enrolled. According to their report, no case of permanent neurological worsening and no severe postoperative complications were found during the following up period. In addition, no new increment in syrinx size and tumor formation was observed via magnetic resonance imaging. To some extent, these reports might be able to suggest the safety of clinical trials for SC therapy, however, more replicable large animal experiments and phase I clinical trials following critical criteria are necessary before large-scale phase II clinical trial can be attempted.

10. *In Vivo* Tracking of Stem Cells

Although the cellular behavior of grafted cells *in vivo*, which include cell survival, migration, and differentiation, can be commonly assessed through various techniques of tissue slice staining, noninvasive real-time observations within living animals or patients are much more useful and informative regarding the fate of these cells *in vivo*. In pace with the development of general magnetic resonance imaging (MRI), cellular MRI techniques that visualize and track grafted cells in living organisms have also expanded considerably in recent years [216, 217]. With the utilization of *in vivo* tracking techniques, scientists can observe the grafted cells directly, and to evaluate important parameters of transplanted cells, such as, the survival, the distribution pattern, the route of migration, and the integration with host tissue [216]. In order to track grafted stem cells in the injured spinal cord by cellular MRI, the stem cells must be labeled with of magnetic particles prior to transplantation. Recently, several kinds of magnetic particles are available for labeling multiple-cell lines, for example, superparamagnetic iron oxide nanoparticles (SPION), magnetic CoPt nanoparticles, Gd-DTPA, and FDA-approved ferumoxytol [218–221].

Among all the label particles, magnetic labeling with SPION is the most widely used and developed method. Scientists have used a variety of improved methods to generate

several subtypes of SPION with distinct coating for promoting the uptake rate, extending the effect duration, enhancing the resolution of signal, and decreasing the toxicity. Lee et al. coated SPION with unfractionated heparin (UFH) as a novel negative contrast agent for tracking MSCs *in vivo*. The uptake efficiency of UFH-SPION by MSCs was reported to be improved by threefold when compared with dextran coated SPION. Moreover, no transfection agents were involved to help uptake by MSCs. They suggested that the UFH-SPION uptake was likely mediated by endocytosis, and internalized into the cytosol of MSCs to maintain the visualization for 28 days *in vitro*. After transplantation into nude mice, UFH-SPION remained detectable by T2-weighted MRI for one month [222]. According to Andreas et al., the low-labeling efficiencies and the need of potentially toxic transfection agents are the main obstacles to the utilization of commercial SPION [221]. Because of this reason, they coated SPION with citrate, and then compared the labeling efficiencies effects on stem cell functionality, and the *in vivo* MRI visualization of new citrate-coated SPION with commercial Endorem and Resovist SPION. The citrate-coated SPION presented significantly better uptake efficacy without the presence of transfection agents, and *in vivo* visualization by MRI in the comparison. Although the expression of MSC surface marker antigens and differentiation into the adipogenic and osteogenic lineages were not affected by citrate-coated SPION labeling, the chondrogenic differentiation were significantly impaired with increasing amounts of citrate-coated SPION incorporation [221]. The influence on neurogenic differentiation was however, not assessed.

Another experimentally coated SPION, chitosan-coated SPION, was verified by Reddy et al. for their labeling efficiency of MSCs. Interestedly, 100% labeling efficiency with no alterations in the surface markers expression and differentiation potential was reported. After transplantation of chitosan-coated SPION labeled MSCs into rabbit ischemic brain, the distribution and migration of labeled MSCs at day 16 was clearly visualized on T2-weighted images and susceptibility weighted images. In addition, the size of the ischemic area was significantly decreased at day 16 when compared with an early time point of day 4 [223].

To observe the fate of transplanted NSCs *in vivo*, Meng et al. labeled the cells with magnetic CoPt hollow nanoparticles (CoPt-NPs) for MRI detection. First the optimized nanoparticle concentration that had no negative impacts on cell viability and the effects on differentiation potential were assessed *in vitro*. In the second step, *ex vivo*, the CoPt-NPs labeled NSCs were transplanted into organotypic spinal cord slices. As they expected, a small number of labeled NSCs could be identified by MRI efficiently with enhanced image contrast [219]. Although iron oxide nanoparticle based tracking of stem cells is effective and reproducible, the intrinsic iron signal derived from erythrocytes may be able to mask target cells *in vivo* [217]. Liu et al. verified the safety and feasibility of applying gadolinium-diethylene triamine pentaacetic acid (Gd-DTPA) for T1W signal enhancement for MSC tracking in a rat SCI model. After obtaining promising results in the differentiation assay *in vitro*, the Gd-DTPA labeled MSCs were transplanted into the lesion site of SCI rat, and the positive signal enhancement of labeled cells on T1W images was detected in the duration from 3 to 14 days. Furthermore, the BBB scores in the Gd-DTPA labeled MSCs transplantation group were significantly higher than the control group [224]. However, the observation period of 14 days was less than the time required to evaluate the long-term effects of transplanted stem cells in the treatment for SCI animal. Additional approaches for long-term assessment of labeled cells were developed by scientists. Berman et al. labeled NSCs with SPION, transfected them with the luciferase bioluminescence reporter gene, and then transplanted these cells into the brains of mice. Over a long-term period of 93 days, the bioluminescence signal was able to be detected via 3D surface topography imaging device. In addition, the hypointensities from the SPIO label in T2W image were also detectable over the course of the experiment [225].

11. Conclusion

Taken all above together, in order to achieve the dream of saving the life of SCI patients, improving the life quality and curing the injured spinal cord completely, cellular replacement therapies have recently attracted a lot of attention and several recent publications have shed light on the mechanisms involved and potential hurdles that need to be overcome for the successful translation of this approach. Scientists have been trying all efforts to improve various experimental methods which contribute to the reconstruction of histologically impaired tissue structure, and to the restoration of neural function eventually. With the development of stem cell therapy research, the capability of promoting neuroregeneration of various stem cells is becoming gradually clear, and the obstacles have been overcome one by one. A multipronged approach may be the only effective way to improve functional recovery after SCI. Not all cell populations will have the same effects on each of these modalities, but the population(s) with the maximal effects may have eventual therapeutic benefit for SCI.

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Research Article

Developing a Clinical-Grade Cryopreservation Protocol for Human Testicular Tissue and Cells

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Recent work in preservation of female fertility as well as new information on the nature of spermatogonial stem cells has prompted an investigation into the possibility of an effective clinical-grade procedure for the cryopreservation of testicular cells and/or tissue. Clinical-grade reagents, validated equipment, and protocols consistent with cGTP/cGMP standards were used in developing a procedure suitable for the safe and effective cryopreservation of human testicular cells and tissues. These procedures were designed to be compliant with the relevant FDA regulations. The procedure proved to effectively cryopreserve both testicular cells and tissue. The cryopreservation of testicular tissue was comparable in most aspects we measured to the cryopreservation of isolated cells, except that the viability of the cells from cryopreserved testicular tissue was found to be significantly higher. On the other hand, cryopreservation of cells is preferred for cell analysis, quality control, and sterility testing. This study demonstrates that testicular tissue and cells from sexual reassignment patients can be successfully cryopreserved with a clinical-grade procedure and important cell populations are not only preserved but also enriched by the process. Further studies will determine whether these findings from hormone-treated patients can be generalized to other patients.

1. Introduction

Cryopreservation of reproductive cells and/or tissues has become an increasingly important methodology for fertility preservation [1–3]. Success of autologous, cryopreserved ovarian tissue transplantation in patients has shown the ability of transplanted tissue to restore fertility in women and has generated live births [4–6]. Currently, there are no methods for male patients that restore fertility or allow for future generation of new gametes in the event that their fertility is compromised due to testis damage. Cryopreservation of testicular cells and/or tissue prior to any fertility compromising condition or therapy may allow for future cell/tissue transplantation back to the autologous donor so that they may regain the ability to naturally conceive their own biological children [7]. Alternatively, these cells may be used to create new sperm outside the body through germ cell maturation protocols [8].

A procedure to preserve male fertility must be proven safe before it can be used in human. Regulations and guidance set up by agencies such as the Food and Drug Administration (FDA) describe the procedures and systems that must be put into place before a product can be deemed safe to use in humans. Investigational techniques for cryopreserving testicular tissue and cells have been tested and reported by several groups [9–12]; however, to our knowledge, a clinical-grade protocol for the cryopreservation of human testicular cells or tissue has not been previously described. All previous studies used protocols noncompliant with current Good Tissue Practice (cGTP) standards, nonclinical-grade reagents, and animal products that made them unfit for clinical use. Additionally, no sterility testing was reported in these studies to ensure the absence of microbial contamination. This study addresses and solves those concerns. The federal cGTP regulations in 21 CFR Part 1271 were used to determine what procedures and systems to put in place.

Moreover, at times this study followed the current Good Manufacturing Practices (cGMP) regulations—particularly in terms of equipment validation and documentation. Equipment used in these procedures was validated to ensure proper installation, operation, and performance. Critical points in the procedures were all documented to prove the proper adherence of SOPs.

Besides creating a clinically applicable procedure, this study directly compares the cryopreservation of cells isolated from fresh human testis with the cryopreservation of whole pieces from the same tissue. Cryopreservation can induce production of ice crystals from the water inside the cells, which can damage the cells' internal structure and cellular membrane—and lead to cell death [12, 13]. Studying the effect of cryopreservation on both cells and tissue will help to determine which method is most suitable and applicable for clinical use. The cells and tissue in this study were cryopreserved in a medium using cryoprotectants to prevent ice crystals from forming, thus improving the ability of the cells to survive freezing and thawing. While some studies have focused on the structural effect of cryopreservation on testicular tissue [13], this study focuses on an analysis of the isolated cells.

After viability assessment, there are three markers for which the cells will be analyzed that define three important populations of cells in the human testes. First, stage-specific embryonic antigen 4 (SSEA4) has been shown in nonhuman primates and humans to be an effective spermatogonial stem cell (SSC) marker [14, 15]. *In vivo*, SSCs require the support of Sertoli and Leydig cells as they go through spermatogenesis. An effective marker to identify and investigate Leydig cells is the luteinizing hormone receptor (LHR) [16], the second marker used in this study. Finally, VASA (also known as DDX4) is a specific germ cell lineage intracellular marker [17]. All of these cell types, in addition to many other cell types, comprise the heterogeneous mixture of testicular cells and are affected in contrasting ways by cryopreservation of testicular cells or tissue. The differences between cells isolated from fresh tissue will be compared to both the cryopreserved cells and cells from the cryopreserved tissue. Additionally, the differences between the cryopreserved cells and the cells from the cryopreserved tissue will be directly compared.

The cryopreservation of testicular cells and/or tissues is of particular importance to patients for whom sperm freezing is not an option. Prepubertal patients undergoing radiation and/or chemotherapy are at risk for fertility loss due to the cytotoxic effects of those therapies on the germinal epithelium—where SSCs are located [18, 19]. The direct toxic effects of chemotherapy and radiation exposure on the gonads are generally dose-dependent [20, 21] and the long-term effects of chemotherapy on the testes have not been well characterized. The survival rate among children with cancer has improved over the past several years; close to 80% are expected to survive [22]. Although there are no established options for prepubertal boys who are later found to be infertile, their preserved testicular cells or tissue may potentially be used to restore their fertility [23, 24].

Another population for who this process might be beneficial is men whom have begun the process of sexual reassignment. These patients undergo hormone treatment regimens, which last varying amounts of time and have devastating effects on spermatogenesis in the testes [25]. For this reason, it is usually difficult or impossible for these men to preserve sperm after a critical point in their treatments for use in assisted reproductive techniques. Without sperm, the only option for these men to preserve their fertility may be the preservation of their testicular cells or tissue. This cryopreserved material could then be used in germ cell maturation procedures to produce sperm for use in assisted reproductive techniques.

In this study, testicular tissue from male sexual reassignment patients was used. This was done for two reasons. First, the unavailability of normal human testicular tissue; Second, there are no previous studies for the cryopreservation of testicular cells or tissues from sexual reassignment hormone-treated patients and these patients might directly benefit from this paper. If this process is to be used for these patients, an investigation into the effectiveness of cell and/or tissue cryopreservation is required.

2. Materials and Methods

2.1. cGTP and cGMP Environment. This study was performed under cGTP guidelines for a product regulated under section 361 of the Public Health Service Act and was compliant with other relevant FDA regulations and guidance at an FDA-registered and inspected tissue processing facility. All critical protocols were performed in a certified clean room. Moreover, protocols were performed using validated equipment and clinical-grade reagents and supplies according to cGMP guidelines. Documentation was followed for quality assurance/quality control and compliance with quality standards and regulations.

2.2. Tissue Collection and Testicular Cell Isolation. Sexual reassignment patients included in this study (5) have been treated with hormones for a period of 6–12 months and their age varied between 25 and 40 years. All patients signed an informed consent form with the surgical facility agreeing for their tissue to be used for this study. Testes were surgically removed from the scrotum and washed in sterile cGMP-grade phosphate-buffered saline (PBS, Irvine Scientific, Irvine, CA, USA) before being placed in a sterile bottle of 4°C cGMP-grade PBS (Irvine Scientific). After removal, the testes were shipped overnight in a validated shipper (ThermoSafe, Arlington Heights, IL, USA) between 2°C and 8°C and arrived approximately 24 hours after being removed from the patient.

Upon arrival at the processing facility, the tissue was processed in the certified clean room. The seminiferous tubules were dissected by decapsulating the testes after removal of additional fat and membranes. The tissue was washed in cGMP-grade PBS (Irvine Scientific). A piece of the tissue was cut off, weighed, and placed in a sterile 50 mL conical tube with cGMP-grade PBS (Irvine Scientific) for cell isolation. Other pieces of tissue were cut, weighed, and used for tissue freezing (described below). Fresh or frozen/thawed

tissue was dissected by sterile tweezers to smaller strips for enzymatic digestion with Liberase (Roche Applied Science, Indianapolis, IN, USA). Liberase, a cGMP-grade mixture of enzymes, was added to each piece for a final enzymatic digestion concentration of 0.3 units/mL of Collagenase and 1000 units/mL of Thermolysin. The tissue was digested at 37°C on a reciprocating shaker at 110 RPM for 1.75 hours. Undigested tissue was removed from isolated cells by a sterile 100 μm cell strainer (BD, San Jose, CA, USA) before centrifuging the cells at 400 ×g for 5 minutes at 4°C. Cells were resuspended in a mixture of cold cGMP-grade PBS (Irvine Scientific) and 10% human serum albumin (HSA, SeraCare Life Sciences, Milford, MA, USA) and kept at 4°C for further processing.

2.3. Cell Count and Viability Assessment. Cells were counted on a validated hemacytometer with the addition of Trypan Blue (Life Technologies, Grand Island, NY, USA) to count the number of dead cells. Each sample was counted twice and an average was taken from the two counts. Viability was calculated by dividing the number of live (viable) cells by the total number of cells counted (live and dead) and displaying the number as a percentage where 100% represents a population of cells that is entirely alive and 0% represents a population that is entirely dead. In addition to Trypan Blue, viability of cells was confirmed using a flow Cytometry based assay by 7AAD staining (see the flow Cytometry analysis section). The number of cells obtained was normalized by the weight of the tissue and expressed as a ratio of viable cells per gram of tissue.

2.4. Cryopreservation. Freshly isolated cells were centrifuged as described above and resuspended in cold cryopreservation media (CM) of 10% HSA (SeraCare Life Sciences), 10%DMSO/1%Dextran (Origen Biomedical, Austin, TX), and cGMP-grade PBS (Irvine Scientific). One mL of cell suspension, containing 3–5 × 10^6 cells, was pipetted into 1.8 mL cryovials (Nunc, Rochester, NY, USA). Cells were cryopreserved by a validated Kryo-16 Controlled Rate Freezer (Planer, Middlesex, UK). The protocol for the Kryo-16 was as follows: vials were held at 4°C for 10 minutes before being cooled at a rate of −1°C/min to −80°C. The vials were further cooled at a rate of −50°C/min to −120°C. Vials were held at −120°C until they were quickly transferred to a validated MVE TEC 3000 Dewar and stored in the vapor phase of liquid nitrogen at ~−188°C.

Tissue pieces (120 to 500 mg) were cryopreserved in a similar manner. Tissue was placed in a cryovial with 1 mL of cold (4°C) CM and soaked for 30 minutes at 4°C prior to undergoing the cryopreservation procedure.

2.5. Flow Cytometry Analysis. Flow cytometry was conducted with a BD FACS Canto (BD) using unstained and secondary-antibody-only stained cells as controls. Cells from freshly isolated and thawed conditions were separately stained with Alexa-488 conjugated anti-human SSEA4 (Ebioscience, San Diego, CA), purified rabbit anti-human LHR (GeneTex, Irvine, CA, USA), and purified rabbit anti-human VASA (Abcam, Cambridge, MA, USA). VASA is an intracellular protein, therefore the cells stained for VASA were first fixed in 4% paraformaldehyde (EMS, Hatfield, PA, USA) overnight and washed in PBS + 0.01% Triton-X. All primary antibody dilutions were optimized at 1 : 200 and staining time was for 30 minutes at 4°C. For stains that required a secondary antibody, cells were first blocked in 10% goat serum for 15 minutes and labeled with a goat anti-rabbit Alexa 488 antibody (Invitrogen, Eugene, OR, USA) at 1 : 500 for 30 minutes at 4°C. All samples included 7AAD (BD Pharmingen, San Diego, CA, USA) to determine and exclude the dead cells during analysis.

For each marker from each sample, the percentage of viable cells positive for that marker was determined. The percentage was then multiplied by the number of total viable cells isolated per gram of tissue to determine how many viable cells positive for each marker were isolated per gram of tissue. These numbers were compared between fresh-tissue cell isolation and either cryopreserved cells or cryopreserved/thawed tissue to determine percent recovery of cells positive for each marker from each type of cryopreservation.

2.6. Cell and Tissue Thawing. Cryovials were removed from liquid nitrogen storage and immediately placed in a 37°C water bath. Vials were swirled in the water bath until a small piece of frozen cells remained (~2 minutes). Thawed cells were transferred into a 50 mL conical tube and diluted with 9 mL of 4°C cGMP-grade PBS (Irvine Scientific) and 10% HSA (SeraCare) over the course of several minutes to dilute the CM 1 : 10. The cells were centrifuged as described above and resuspended in PBS (Irvine Scientific) and 10% HSA (SeraCare) for counting as described above. For thawing tissue, the tissue/CM was thawed and the CM was diluted as described above. Instead of centrifugation, the tubes of thawed tissue were held at 0–4°C for 5–10 minutes to allow CM to dilute out of the tissue. Tissue was then transferred to PBS (Irvine Scientific) and placed on ice to await cell isolation. Cell isolation and counting was performed as described above.

2.7. Statistics. Average cell recovery was calculated by dividing the average number of cells after cryopreservation by the average number of cells isolated from fresh tissue. All other averages were calculated by dividing the summation of the values in the category by the sample size. Smith's Statistical Package was used for two sample-student t-test for statistical analysis and $P < 0.05$ was considered as significant. Standard error of the mean (SEM) was calculated by dividing the standard deviation by the square root of the sample size.

2.8. Sterility Testing. PBS used for transport of the tissue as well as samples of isolated and/or thawed cells for sterility testing were aseptically collected into sterile 1.8 mL cryovials (Nunc). The vials were shipped to a qualified and CLIA-approved laboratory for sterility testing. Samples were inoculated into Trypticase Soy Broth and Fluid Thioglycollate Medium to test for the growth of yeast, fungi, aerobic, and

Table 1: Viability of cells before and after cryopreservation.

Patient	Viability of cells		
	Fresh cells	Frozen cells	Frozen tissue
1	84.9%	38.1%	70.1%
2	91.9%	64.1%	81.8%
3	93.0%	53.8%	68.9%
4	90.6%	53.3%	75.9%
5	90.2%	52.8%	73.5%
Average	90.1%	52.4%[a]	74.0%[b]
SEM	1.3%	3.9%	2.2%

The viability of each cell population was determined by dividing the number of viable cells counted with the number of total cells counted (viable + dead). Two sample t test was used for statistical analysis and $P < 0.05$ was considered as significant. [ab]$P = 0.0019$.

anaerobic bacteria. Cultures were grown for 14 days. Any detected growth after 14 days was a condition for failure of the sterility test.

3. Results

3.1. Viability. The viability of the cells is important for determining the effectiveness of the cryopreservation and the condition of the cells after freezing and thawing. The average viability of the cells isolated from fresh testicular tissue was 90.1%. When the same cells were cryopreserved and then thawed, the average viability dropped to 52.4%. When tissue from the same testes was cryopreserved, thawed, and the cells isolated by the same procedure as the fresh tissue, the average viability of the cells was 74.0%—lower than the average viability of cells isolated from fresh tissue by only 16.1% and higher than the average viability of the cryopreserved cells by 21.6% (Table 1). The difference between the viabilities of the cryopreserved cells and the cells from cryopreserved tissue was statistically significant ($P = 0.0019$). This suggests that testicular cells have a better survival rate when frozen as tissue pieces as compared to freezing isolated cells.

3.2. Cell Recovery. When cells or tissue are cryopreserved and then thawed, some cells are naturally going to be lost due to cell damage and destruction. These lost cells are not accounted for when performing a simple live-dead count because they are only present as cellular debris. Therefore, comparing just the viability is insufficient. A comparison was made between the number of viable cells initially cryopreserved and the number that remained after thawing. This test was utilized to determine if cryopreserving tissue pieces or cryopreserving isolated cells is a better method: the superior method should yield more viable cells recovered after cryopreservation and thawing. An average of 42.5×10^6 viable cells were isolated per gram of fresh testicular tissue. Upon thawing, an average of 14.0×10^6 of those cells were recovered—a recovery rate of 32.9%. When tissue pieces were cryopreserved, thawed, and enzymatically digested in the same manner as fresh tissue, the number of cells isolated per gram of tissue on average was 37.4% (15.9×10^6 viable cells) of the number of cells isolated per gram of fresh tissue. This indicates that 4.5% additional cells may be recovered, on average, after cryopreservation of tissue pieces. However, this difference was not statistically significant ($P = 0.78$), and it should be noted that only 3 out of the 5 patients showed greater recovery of cells when cryopreserving tissue (Table 2).

3.3. Cell Marker Analysis. Immunolocalization of the cells positive for the specific markers used in this study is presented in Figure 1. Testicular cell isolations are a mixture of the various component cells of the testes, most importantly to fertility preservation are SSCs. The survival of these cells after cryopreservation is the key to fertility preservation or other therapeutic regenerative medicine techniques. The recovery and the percentage of the cells positive for each cell marker before and after cryopreservation are presented in Table 3 and Figure 2, respectively. For every gram of fresh tissue, on average 630,923 viable SSEA4+ cells were recovered. After cell thawing an average of 246,578 of those cells were recovered. This represented a recovery rate of 39.1%. By comparison, when tissue pieces were cryopreserved, thawed, and enzymatically digested, an average of 50.4% of the number of SSEA4+ cells isolated per gram of fresh tissue was recovered—higher by 11.3% (50.4%–39.1%) in absolute terms than when cryopreserving isolated cells. However, only 3 out of the 5 patients showed a higher recovery percentage when cryopreserving tissue rather than isolated cells. Although there is a trend that cryopreserving testicular tissue allows for more survival of the SSCs than cryopreserving isolated cells, the difference was not statistically significant ($P = 0.4254$).

Interestingly, the recovery rate of SSEA4+ cells was higher than the recovery rate of the total cell population. For cryopreserved cells, the average recovery of SSEA4+ cells was higher by 9.0% in absolute terms. For cryopreserved tissue, recovery rate of SSEA4+ cells was higher by 6.1% in absolute terms compared with the average recovery of the total cell population. The difference in recovery of SSEA4+ cells between cryopreserving cells and cryopreserving tissue was not significant ($P = 0.14$), although 4 out of 5 patients had higher recovery of SSEA4+ cells from cryopreserved tissue. The difference between the recovery of viable SSEA4+ cells isolated before and after cryopreservation of cells was not statistically significant ($P = 0.1484$), as was the difference between fresh tissue isolation and frozen tissue isolation ($P = 0.5432$). Even though cryopreservation leads to the loss of some SSEA4+ cells, it enriches the total population for SSEA4+ cells in the cell suspension.

Leydig cells produce testosterone and are important for the proper proliferation and differentiation of SSCs into functional gametes [16]. As such, their survival could be important for postcryopreservation maturation of SSCs. An average of 46.8% of Leydig cells as indicated by LHR was recovered after cryopreserving isolated cells. When cryopreserving tissue pieces, on average 138.4% of LHR+ cells were recovered—an indication that more LHR+ cells are able to be isolated by first cryopreserving the tissue than if the

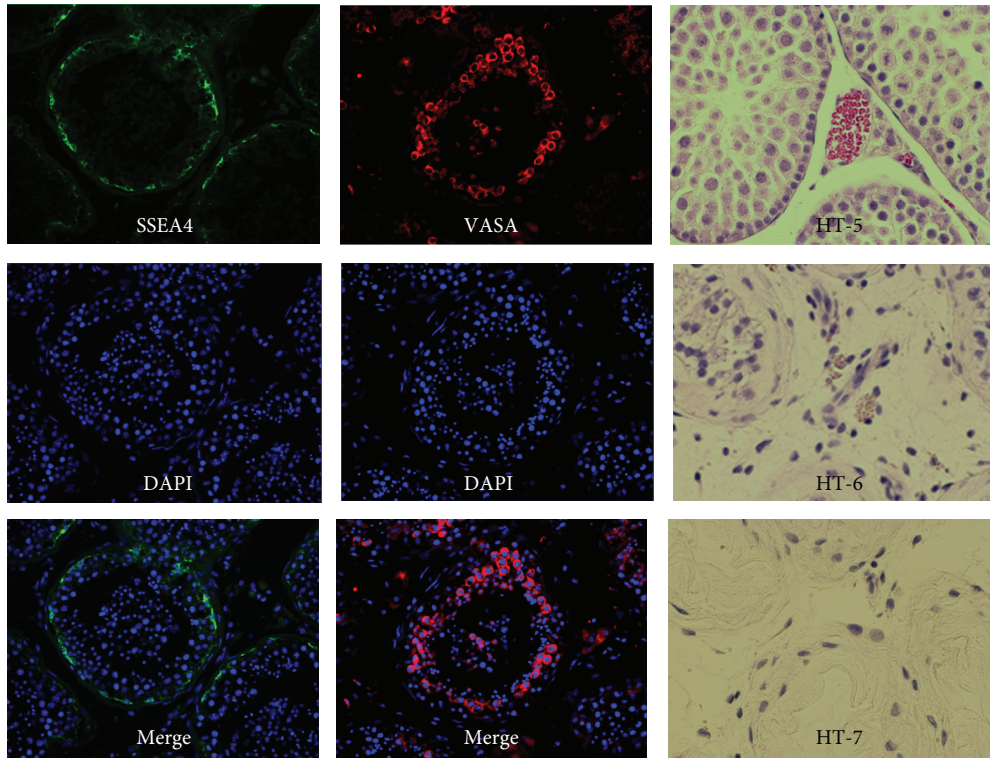

Figure 1: Immunolocalization of cell marker and representative images of testicular cross-sections from estrogen-treated patients. This figure shows localization of SSEA4 and VASA positive and various degrees of spermatogenesis in the human testicular tissue collected from sexual reassignment patients. SSEA4 only stains the cells along the basement membrane of the seminiferous tubules. VASA stains all the germ cells including those along the basement membrane and in the lumen of the seminiferous tubules. Note various degrees of spermatogenesis were found in testes collected from three sexual reassignment patients (HT-5, HT-6, and HT-7).

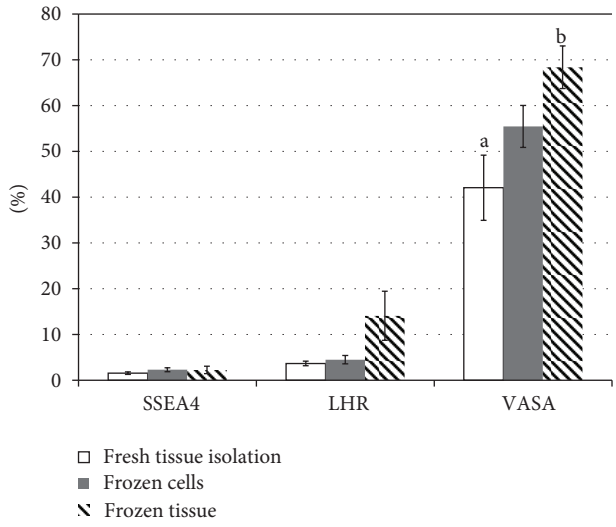

Figure 2: Flow cytometry analysis of the enrichment of cells positive for each marker before and after cryopreservation. The numbers of viable cells positive for each marker were determined by flow cytometric analysis and are expressed as a percentage of the total viable cells in each condition. VASA positive cells were fixed before staining. VASA positive cells were counted without information about which ones were viable or dead. This graph shows that the average percentage of cells positive for each marker is higher after cryopreservation of cells and tissue. Two-sample t-test was used for statistical analysis and $P < 0.05$ was considered significant. $^{ab}P = 0.0188$.

Table 2: Number of viable cells per gram of tissue and recovery of cryopreserved cells.

Patient	Viable cells per gram of tissue			Percent Recovery	
	Fresh cells	Frozen cells	Frozen tissue	Frozen cells	Frozen tissue
1	15,300,000	4,057,018	6,630,259	26.5%	43.3%
2	42,800,000	13,312,147	32,958,482	31.1%	77.0%
3	26,900,000	4,114,322	17,889,704	15.3%	66.5%
4	68,624,368	21,006,944	11,224,466	30.6%	16.4%
5	58,877,485	27,549,020	10,704,884	46.8%	18.2%
Average	42,500,371	14,007,890	15,881,559	33.0%	37.4%
SEM	9,313,258	4,390,103	4,390,059	4.8%	11.7%

The number of cells calculated per gram of tissue is compared between isolating cells from fresh tissue, cryopreserved cells, and cryopreserved tissue. For fresh cells, the weight of the tissue of each enzymatic digestion was determined. For frozen cells, the number of cells recovered from each frozen vial was used to calculate how many cells would have been recovered had the cells from 1 gram of tissue been frozen. For frozen tissue, the number of cells isolated from each piece after thawing was used in conjunction with the weight of the tissue before freezing. For all three, only viable cells were used for the calculations. Cell recovery was calculated by dividing the number of viable cells per gram of tissue from either the cryopreserved cell or cryopreserved tissue with the viable cells from fresh tissue cell isolation.

Table 3: Recovery of viable cells positive for cell marker per gram of tissue.

Patient	SSEA4+			LHR+			VASA+		
	Fresh cells	Frozen cells	Frozen tissue	Fresh cells	Frozen cells	Frozen tissue	Fresh cells	Frozen cells	Frozen tissue
1	382,500	133,392	126,144	459,000	222,571	523,697	10,128,600	2,894,214	5,642,505
2	642,000	271,081	540,099	1,883,200	378,955	5,003,870	8,089,200	5,820,763	24,075,066
3	215,200	141,845	215,335	1,102,900	67,546	1,069,848	10,733,100	1,939,856	11,733,700
4	829,602	259,954	108,069	3,402,562	1,201,910	644,282	28,455,915	11,992,882	6,595,656
5	1,085,311	426,618	599,564	1,141,806	1,865,490	3,814,563	25,813,056	15,991,324	6,356,519
Average	630,923	246,578	317,842	1,597,894	747,294	2,211,252	16,643,974	7,727,808	10,880,689
SEM	146,814	50,549	99,337	477,708	323,267	872,536	4,096,440	2,567,434	3,288,223
Percent recovery	N/A	39.1%	50.4%	N/A	46.8%	138.4%	N/A	46.4%	65.4%

The number of cells positive for each marker per gram of tissue isolated from fresh tissue cell isolation and recovered from either frozen cell or frozen tissue. Percent Recovery was calculated by dividing the average after cryopreservation by the average before cryopreservation. No significant differences were observed.

LHR+ cells were isolated from fresh tissue. Again, 4 out of the 5 patients had higher recovery of LHR+ cells from cryopreserving tissue compared with cryopreserving cells, although the results were not statistically significant ($P = 0.1225$).

Similar to SSCs, as indicated by SSEA4, cryopreservation enriched the population of supporting Leydig (LHR+) cells in the total cell population. The average recovery of LHR+ cells in the cryopreserved cells was higher by 16.7% in absolute terms than the average recovery of the total cell population. The average number of LHR+ cells isolated from cryopreserved tissue was higher by 94.1% in absolute terms than the recovery of the total cell population from the same tissue. Both cryopreserved cells and tissue had a higher rate of recovery of LHR+ cells than total cell population in 3 out of the 5 patients while at the same time neither enrichment was statistically significant ($P = 0.4136$ and $P = 0.0743$, resp.). The results suggest that LHR+ cells are enriched by both tissue and cell cryopreservation.

VASA is an intracellular transcription factor expressed in all germ cells [17] and was used as an indicator of the survival of the total germ cell population during cryopreservation. Thawed cells isolated from fresh tissue, on average contained 46.4% of the VASA+ cells that were cryopreserved. When tissue was cryopreserved, the number of VASA+ cells isolated per gram of tissue was on average of 65.4% compared with the number of VASA+ cells isolated per gram of fresh tissue. In 3 out of 5 patients, more VASA+ cells were recovered from cryopreserving tissue and in the other two cryopreserving cells preserved more VASA+ cells. However, the greater number of VASA+ cells recovered after tissue cryopreservation was not statistically different from cryopreservation of cells ($P = 0.0731$).

VASA+ cells were enriched when cryopreserving either cells or tissue. For cryopreserved cells, the average recovery rate of the VASA+ cells was higher by 16.3% in absolute terms than the average recovery rate of the total cell population. Similarly, for cryopreserved tissue, the average number of VASA+ cells isolated per gram of tissue was enhanced by 21.1% in absolute terms. Neither enrichment was statistically significant ($P = 0.2334$ and $P = 0.3052$, resp.) (Figure 2).

3.4. Sterility Testing. To ensure that there was no contamination of either the cells/tissue or the processing environment sterility testing was performed on the tissue transport PBS, on the cryopreserved cells, the thawed cells, and the air and surfaces in the clean room. For 3 out of the 5 patients, PBS

and cells were sent to a CLIA-approved and FDA-registered clinical microbiology laboratory. Each sample of PBS used to transport the tissue was found to be contaminated with several microorganisms. This was likely due to contamination during surgical removal of the tissue. In contrast, all three cryopreserved cell products and thawed cells were completely free from contaminating microorganisms. This indicates that not only the process was aseptic (in that it did not introduce contamination) but our cell processing procedure actually eliminated contamination that was present before the process. Additionally, the processing space where the cell isolation, cryopreservation, and thawing took place was tested for the presence of microbial contamination: samples from both the surfaces and air in the clean room were collected, tested, and found to be free of any viable microorganisms—an indication that the space remained aseptic from outside air and that nothing from the cells/tissue contaminated the working space.

4. Discussion

In the present study, the feasibility of a clinical-grade cryopreservation procedure for human testicular cells and tissue obtained from sexual reassignment patients was studied. Our results clearly show that cryopreservation of human testicular cells and tissue with a protocol compliant with cGTP and some cGMP requirements results in acceptable viability and cell recovery of different testicular cell subpopulations. Also our results clearly demonstrate that human testicular tissue processed and cryopreserved under the cGMP environment described here are free from microorganism and can be used for clinical applications.

Finding a clinical-grade method for preservation of testicular cells or tissue would be very helpful in allowing these cryopreserved biologics to be used for future regenerative medicine applications in humans. Without a validated procedure, regulatory agencies would not allow testicular cells/tissues to be transplanted back into the patient's body. Other methods have shown that it is possible to effectively isolate and/or cryopreserve testicular cells and/or tissues; however, these methods did not comply with cGTP or cGMP regulations or employ clinical-grade reagents/supplies or possess the rigorous documentation that would make the procedures permissible for a clinical study. In our protocol, cGTP and some cGMP conditions were complied with throughout. All 5 tissues were processed using clinical-grade and/or cGMP grade materials and reagents. All the tools and reagents that directly came into contact with the cells and tissue were sterile. The equipment was properly validated, the procedures codified in SOPs, proficiency assessed and ascertained, and the critical information for the process, equipment, and reagents/supplies (vendor, catalog numbers, lot numbers, serial numbers, expiration dates, certificates of sterility, conformity, and analysis) were documented.

Two major components of our freezing medium are DMSO and Dextran (a disaccharide). Both DMSO and disaccharides (e.g., sucrose) have been widely used for cryopreservation of testicular cells [11] and tissue [18]. In addition, the DMSO-Dextran cryopreservative has been frequently used for and validated in human cord blood [26], bone marrow [27] and peripheral blood stem cell freezing and other clinical applications. In this protocol fetal calf serum (FCS), used in previous studies, was replaced with HSA. FCS use should be avoided for clinical protocols because its animal origin increases the risk of contaminating the cells and/or tissue with bovine blood-borne pathogens. On the other hand, HSA is approved for human use and has been successfully used in other cryopreservation protocols including peripheral blood mononuclear cells [28] and ovarian tissue and follicles [29].

For cryopreservation, in addition to controlled rate freezing, we also tested manual freezing and found no effect on cell viability after thawing (data not shown). This is in agreement with a previous study in a bovine model [10] and our own unpublished data in the mouse and primate models indicating that manual freezing provides similar freezing conditions to the controlled rate freezing. However, controlled-rate freezing is preferred to ensure more reproducible conditions. Controlled-rate freezing also provides the ability to perform the cryopreservation in properly validated equipment with documentation detailing the cryopreservation process—in compliance with cGMP regulations. Our results give credence for the first time that this clinically applicable procedure can be used to effectively produce cells or tissue that are safe for potential use in humans.

We found that cryopreserving tissue may be a better method for preserving testicular cells using this clinical-grade procedure—especially regarding the viability of those cells. It should be noted that in this population of hormone-treated patients, the viable cell number recovery was not statistically significant, with the variance of results possibly affected by the variability of hormone treatment. In all categories, on average, cryopreserving tissue yielded more cells after cryopreservation than cryopreserving cells, though none of the comparisons yielded statistically significant comparisons in this sample size of variable tissue except viability. Of particular interest in the testicular cell population are the SSCs, represented in this and other studies by SSEA4+ cells [14, 15]. These cells have the potential to be transplanted back into the body to restore fertility, to be terminally differentiated into sperm for assisted reproductive techniques, or to be differentiated into different cell types for other regenerative medicine applications. Cryopreservation of testicular tissue generated greater recovery of SSEA4+ cells in this study. Enrichment of SSCs due to cryopreservation and thawing has also been reported by other investigators [30].

The same was true for the other cell population investigated in this study. Leydig cells are responsible for production of testosterone and maturation of SSCs into functional gametes. Cryopreserving both cells and tissue enriched the total cell population for cells positive for LHR (a Leydig cell marker) but when cryopreserving tissue, the LHR+ cells were even more enriched after cryopreservation and thawing. The fact that Leydig cells survive cryopreservation so effectively indicated that these cells perhaps are more resistant to cryodamage and could support the germ cells after cryopreservation. In an effort to preserve human testicular tissue, it has been shown that Leydig cells are more resistant to cryopreservation compared to spermatogenic cells [12]. This

Table 4: Pros and cons of cryopreservation of testicular cells and tissue for clinical application.

Material to Freeze	Pros	Cons
Tissue	(1) Requires less time and effort. (2) Less expensive. (3) Requires fewer equipment to keep validated.	(1) Cells have to be isolated first before transplantation or other procedure. (2) No information about the sterility of the cell product can be acquired at the time of transplantation. (3) No information about the cellular composition is collected. (4) No quality control and stability test can be done.
Cells	(1) A complete profile of the viability, sterility, and cellular composition has been collected before cryopreservation. (2) Small samples of cell product can be used for quality control and stability testing during long-term storage. (3) The cell product with the known quality can be safely stored and sent to the clinic prior transplantation.	(1) Requires more time and effort for cell dissociation. (2) More expensive. (3) Has to be done in a cGTP compliant cell processing facility.

is important as these cells could play a key role in maturation of SSCs to functional sperm. Further experiments are needed to investigate this hypothesis. Among all cell types present in the adult testes, more differentiated spermatogenic cells including spermatocytes, spermatids, and spermatozoa are the most abundant cell types in seminiferous tubules and perhaps the most susceptible to cryodamage. We used VASA as a general germ cell marker in this study and the majority of the VASA positive cells in the testes are the advanced germ cells. Surprisingly, VASA positive cells were also enriched after cryopreservation. This might be due to the fact that hormone treatment in these patients has eliminated the majority of the advanced germ cells and, in fact, what is left from the VASA positive cells are the early-stage germ cells including SSCs and differentiating spermatogonia. Deleterious effects of steroid hormones, normally used in sexual reassignment patients, on spermatogenesis has been reported [31]. Similar to SSCs and Leydig cells, VASA positive cells were less susceptible to damage when they were cryopreserved as part of whole tissue. In general, all three cell types that have been examined in this study were better preserved when the tissue was frozen as compared to cryopreservation in cell suspension.

There are many considerations that go into determining if cryopreservation of testicular cells or tissue is the most appropriate. The quality of the cells that result from either cell or tissue freezing is important—as measured here by the viability and the number of cells isolated (both the total viable population and the survival/enrichment of the SSCs, Leydig cells, and germ cells). Other considerations are also taken into account: cryopreservation of testicular tissue requires less time and effort, is less expensive, and requires less equipment. On the other hand, cryopreserving cells isolated from fresh tissue has its advantages. First, more information is known about the cells—such as the quality of the cells as explained above. Secondly, the process of isolation and cryopreserving the cells eliminates any contamination found in the transport PBS before processing. Thirdly, it is much easier to perform quality control and stability testing on the cryopresevered cells. Finally, the cryopreserved cells can be tested for sterility. Sterility testing in particular takes several weeks to obtain using the methods currently employed in CLIA-approved laboratories as indicated in this study. If testicular tissue is cryopreserved the results of sterility would not be obtained until far after the cells would be isolated and transplanted back into the body for fertility preservation. For these reasons, cryopreservation of testicular cells isolated from fresh tissue is favorable over cryopreservation of tissue pieces (Table 4).

To the best of our knowledge, this is the first study showing that testes of sexual reassignment patients contain sufficient amounts of SSCs, Leydig cells, and germ cells that can be effectively cryopreserved. However, there were some variations between tissues from different patients especially in the number of viable cells isolated per gram of tissue. In this respect, it can be speculated that these differences might be due to the effect and length of treatment of the estrogen and other hormones used in the sexual reassignment procedure, their effect on spermatogenesis, and individual patient susceptibility. The more estrogen is used and the longer it is administered, the more spermatogenic cells will be eradicated, the emptier the tubules will be, and the more volume of the seminiferous tubules will be filled with fluid rather than cells. This leads to an overall decline in the number of cells isolated per gram of tissue. Therefore, patients with less treatment will likely be less affected, with more cells isolated per gram of tissue. Because these patients had varying amount of estrogen treatment, it is more important to look at what percent of cryopreserved cells were recovered from cell and tissue cryopreservation to understand the effectiveness of the cryopreservation procedure. In that regard, cryopreserving tissue has a nonstatistically significant edge. In the future, we hope to expand our understanding of testicular tissue freezing and the applicability of either cryopreserved cells or tissue for use in a clinical setting. First, repeating this study on adult human testicular tissue that has not undergone hormone treatment. In particular, repeating

this experiment on testicular tissue from prepubertal boys will provide a clinically applicable procedure that would give young patients the possibility of restoring their fertility. Including patients with other conditions or therapies that threaten male fertility will expand the applicability of this procedure.

The potential application of cryopreserved testicular cells or tissue needs investigation to make clinical use of this material. A procedure for reimplantation of the testicular cells into a healthy testis needs to be developed. For cancer-survival patients, that kind of procedure presents a risk of reintroduction of cancer to their bodies. Those patients and others such as males who have completed the male-to-female sexual reassignment surgery, testicular torsion or varicocele patients for whom fertility restoration is not possible could use their cryopreserved cells for assisted reproductive techniques. A study describing the methods and effectiveness of these applications, including possible maturation of SSCs into functional gametes, would allow for the use of their cryopreserved material. Additionally, the results of this study would be improved by comparing controlled rate freezing and vitrification of human testicular tissue. Vitrification has shown a better success in cryopreservation of ovarian tissue [32, 33]. Similarly, vitrification has proven to preserve survival, development, integrity, and functionality of prepubertal testicular tissue in mouse [34], nonhuman primate [35] and human [36]. Therefore, in future studies we would like to compare the effectiveness of slow freezing and vitrification on cryopreservation of testicular tissue obtained from hormone treated as well as untreated patients.

5. Conclusions

A clinically applicable method successfully cryopreserved human testicular cells and tissue. We found for the first time that testicular tissue and cells from patients undergoing sexual reassignment can be successfully cryopreserved—and important cell populations can be enriched by the cryopreservation process. Sterility tests show that the cryopreserved cells and the thawed cells processed by our protocols under cGTP and some cGMP conditions were free from any microbial contamination. Cryopreservation of testicular cells seems to be more clinically applicable than cryopreservation of testicular tissue.

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Review Article

Restoring Fertility in Sterile Childhood Cancer Survivors by Autotransplanting Spermatogonial Stem Cells: Are We There Yet?

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Current cancer treatment regimens do not only target tumor cells, but can also have devastating effects on the spermatogonial stem cell pool, resulting in a lack of functional gametes and hence sterility. In adult men, fertility can be preserved prior to cancer treatment by cryopreservation of ejaculated or surgically retrieved spermatozoa, but this is not an option for prepubertal boys since spermatogenesis does not commence until puberty. Cryopreservation of a testicular biopsy taken before initiation of cancer treatment, followed by *in vitro* propagation of spermatogonial stem cells and subsequent autotransplantation of these stem cells after cancer treatment, has been suggested as a way to preserve and restore fertility in childhood cancer survivors. This strategy, known as spermatogonial stem cell transplantation, has been successful in mice and other model systems, but has not yet been applied in humans. Although recent progress has brought clinical application of spermatogonial stem cell autotransplantation in closer range, there are still a number of important issues to address. In this paper, we describe the state of the art of spermatogonial stem cell transplantation and outline the hurdles that need to be overcome before clinical implementation.

1. Introduction

Childhood cancer, defined as cancer occurring before the age of 14, is an increasingly prevalent disease that affects many children across the globe. More than 12.000 children in the USA alone are diagnosed with cancer each year [1]. In Europe, the incidence of childhood cancer is estimated to be 139 per million children [2]. Highly effective cancer treatments have led to a spectacular increase in life expectancy in these children, from a 60% 5-year survival rate in the late 1970s to an 80% 5-year survival rate in 2002 [3]. It is estimated that currently 1 in 250 young adults is a survivor of childhood cancer [4].

Given this success in pediatric oncology, long-term adverse side effects of cancer treatment have become of increasing importance [5]. One of the most prevalent long-term side effects of cancer treatment in boys is infertility. Cancer treatment regimens such as alkylating agents and radiation therapy [6, 7] destroy the small pool of spermatogonial stem cells (SSCs) in the prepubertal testis. SSCs are the progenitors of male gametes and thus critical for sperm production and the ability to father offspring. Already present at birth, SSCs reside on the basal membrane of the seminiferous tubules in the testes. Before puberty SSCs do not develop into sperm, but after onset of puberty they will maintain spermatogenesis throughout the rest of a man's life.

Loss of spermatogonial function impairs the generation of functional gametes thereby leading to infertility [8]. Rates of gonadal dysfunction in childhood cancer survivors are variable and depend on dose and type of treatment [9], ranging from a mean 17% azoospermia in patients after treatment of different types of tumors [10] to 82% after treatment for Hodgkin disease [11]. Prepubertal patients are regularly too young to fully understand the profound impact of therapy on their reproductive capacity, but two-thirds of parents whose prepubertal boy has been diagnosed with cancer would agree to freeze a testicular biopsy if a future therapy could lead to potential restoration of spermatogenesis [12, 13]. An interview among long-term childhood cancer survivors between 19–37 years old revealed that most of the

participants wish to have genetically own children in the future [14] and becoming infertile due to cancer treatment is a reduction in quality of life for these patients [15]. Not only does cancer treatment impose devastating effects on one's ability to have children, childhood cancer survivors also suffer from psychological effects due to their disease history and some even experience problems in attracting a partner because of being infertile [14].

Until cancer treatment can exclusively target tumor cells, infertility among these boys will remain an important long-term consequence. Oligozoospermic adult cancer patients may consider intracytoplasmic sperm injection (ICSI) of ejaculated sperm into an oocyte and azoospermic patients may theoretically benefit from testicular sperm extraction (TESE) [16] followed by ICSI if spermatozoa are found [17]. Those survivors who are completely sterile (i.e., when no spermatozoa are found upon TESE) have no way of achieving a pregnancy from their own genetic material. Men that develop cancer before adolescence do not have functional spermatozoa as spermatogenesis does not commence until puberty and they cannot be helped by TESE/ICSI either. Needless to say, there is substantial need for a technique that safeguards or restores fertility in these long-term cancer survivors.

SSC autotransplantation may be a way to restore the spermatogonial stem cell pool after cancer treatment, thereby leading to life-long spermatogenesis and the chance to achieve pregnancy. Transplantation of SSCs was first described in mice in 1994, generating full spermatogenesis in an otherwise infertile recipient mouse and functional sperm leading to donor-derived offspring [18]. This achievement boosted research on SSC functionality and has led to major advancements in unraveling SSC biology that will hopefully pave the way to future clinical implementation (see Table 1).

Based on the mouse transplantation model, the theoretical way to restore reproductive potential in human male childhood cancer survivors is to cryopreserve a testicular biopsy before cancer treatment and to transplant cells from the biopsy back into the testis when that patient is cured from cancer and expresses the wish to have children [19, 20]. Briefly, SSC transplantation can be achieved by ultra-sound guided needle injection of testis cell suspensions into the rete testis of a recipient as was shown to work in several large animal models and in human testis *ex vivo* [21–23] (see Section 5). Besides this proposed SSC transplantation therapy model (SSCT), other experimental technological approaches to tackle infertility include testis tissue grafting [24, 25], *in vitro* production of spermatozoa from SSCs [26], and derivation of male germ cells from induced pluripotent cells (iPS) [27], but these approaches are still in the very early experimental phase.

The most critical steps in bringing SSCT to the clinic involve *in vitro* propagation to increase the limited number SSCs from a small testis biopsy, assessment of genetic and epigenetic stability during SSC propagation *in vitro*, elimination of possible remaining malignant cells and investigation of the health of offspring generated after autotransplantation (summarized in Figure 1). In this review, we focus on the current state of the art of SSCT and we provide a stepwise description of what has been achieved concerning these matters. We will also outline the obstacles that need to be overcome before SSCT can be implemented in the clinic as a means to restore fertility in sterile childhood cancer survivors.

2. Proliferation of SSCs *In Vitro*

As is the case for stem cells in many tissues, the fraction of SSCs compared to surrounding somatic cells is relatively low. In mice, SSCs represent only around 0,03% of all testicular cells [28]. To obtain enough SSCs for transplantation, the few SSCs originating from prepubertal testis biopsies need to be expanded artificially to repopulate an adult testis. Clinicians would need to compensate for the larger testicular volume in which cells are transplanted back, especially considering that an adult human testis is approximately 60 fold larger than a prepubertal testis biopsy. Successful long-term *in vitro* proliferation of SSCs was first demonstrated in mouse [29] and more recently in adult men and prepubertal boys [30, 31]. When cultured for 64 days, the number of SSCs increased over 18,000 fold in a human testicular cell culture system [31]. After culture, human spermatogonia were still detectable as shown by the expression of markers for undifferentiated germ cells *PLZF, ITGα6,* and *ITGβ1* [30]. Upon transplantation in immunodeficient mice, these cells were able to migrate to the niche in the seminiferous tubule, as was shown by the presence of the human marker COT-1. Xenotransplantation of human SSCs to the mouse testis using cells of an early and late time point in culture shows that artificial propagation of SSCs is possible in men.

Expansion of SSCs in an *in vitro* culture system would ideally resemble the *in vivo* situation as closely as possible. In the *in vivo* situation, a complex niche environment exists where SSCs and somatic supporting cells interact to establish essential intracellular signaling. A number of factors have been identified that are required for stem cell maintenance (e.g., EGF, LIF, GDNF, and bFGF) [32]. Artificial mimicking of the niche environment is very difficult, because there are numerous factors that orchestrate the interaction between SSCs and somatic cells and most of them are only poorly characterized. Usage of a "feeder layer" (a layer of somatic cells, often inactivated mouse embryonic fibroblasts) is considered essential for successful propagation of SSCs [29, 33]. Growth of spermatogonia on a feeder layer will result in three-dimensional aggregates termed "clusters," that contain multiple cell types including SSCs [34]. In the mouse germ line stem cell culture systems, animal-derived serum, and a feeder layer are used to mimic the *in vivo* environment [29].

For future human clinical application, a clinical grade medium would preferably not contain any serum derived from animals due to possible zoonotic or xenotoxic effects. The use of somatic cells present in the testis biopsy might maintain SSCs and circumvent the use of exogenous feeder cells. On the other hand, one can imagine that culturing in media lacking (animal-derived) serum [41] or certain growth factors [42] might impact on SSC function, possibly leading to reduced germ line potential [43]. Interfering with culture

Table 1: Selected milestones in the history of spermatogonial stem cell research.

Year	Author	Highlighted findings	Species	References
1966	Clermont	Initial histological description of A_{pale} and A_{dark} spermatogonia	Human	[35]
1971	Huckins	Model for renewal and differentiation of spermatogonia and existence of "spermatogonial stem cells" (SSCs)	Rat	[36]
1994	Brinster and Avarbock	First successful transplantation of testis-derived cells from one mouse to another resulting in donor-derived F1 progeny	Mouse	[18]
1998	Nagano et al.	*In vitro* maintenance of SSCs for 4 months on a somatic feeder layer	Mouse	[37]
1999	Schlatt et al.	Xenotransplantation of primate testis cell suspensions from one primate into the testes of another	Macaque	[23]
2002	Nagano et al.	First report on successful colonization of mouse testes after xenotransplanting human SSCs	Human	[38]
2003	Kanatsu-Shinohara et al.	Prolonged *in vitro* propagation of SSCs using GDNF, without immortalization of the cells in culture	Mouse	[29]
2005	Keros et al.	Proof of successful cryopreservation of testicular biopsies without decreasing structural integrity	Human	[39]
2005	Kanatsu-Shinohara et al.	Long-term propagation of SSCs under serum free and feeder free conditions	Mouse	[40]
2009	Sadri-Ardekani et al.	Long-term propagation of adult SSCs *in vitro* with retainment of functionality	Human	[31]
2011	Sadri-Ardekani et al.	Long-term propagation of prepubertal SSCs with retainment of functionality	Human	[30]
2012	Hermann et al.	Production of functional sperm by infertile prepubertal macaques after autotransplantation, capable of fertilizing oocytes	Macaque	[21]

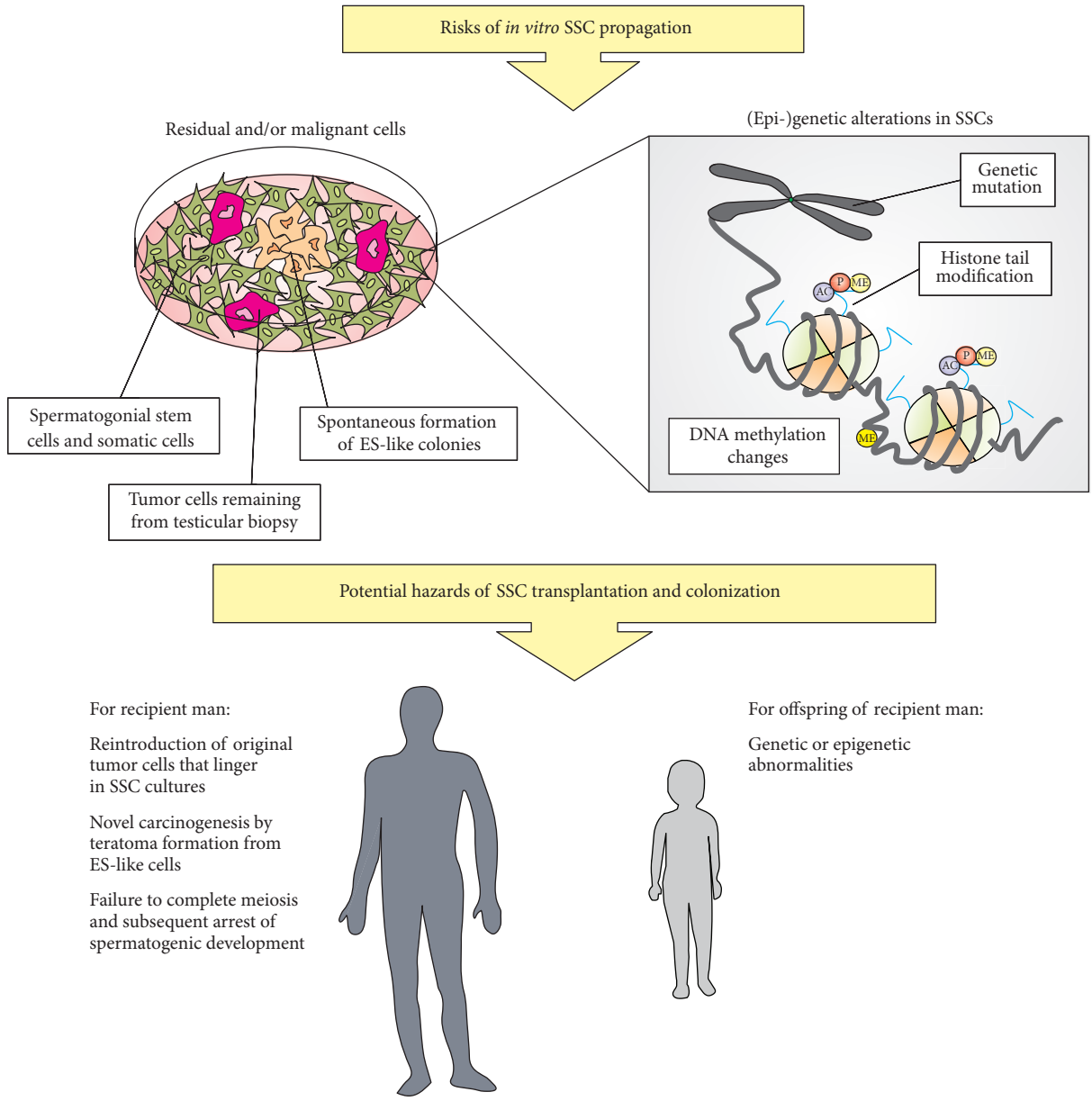

Figure 1: Potential risks of *in vitro* SSC propagation and subsequent SSC transplantation. In *in vitro* propagated SSCs cultures derived from a patient testis biopsy, there is the risk that unwanted cells, such as lingering tumor cells from the patient or spontaneously formed colonies of ES-like cells, are present in the material used for transplantation. Structural integrity of propagated SSCs might be affected due to culture conditions, either on the genetic or the epigenetic level. Alterations that arise *in vitro* can potentially influence the health of the recipient patient or the offspring of that patient.

conditions is a double-edged sword with on one hand the improvement of propagation efficiency by addition of certain factors and on the other hand the possibility of altering SSC functionality because of those same additions.

3. Genetic Stability of SSCs *In Vitro*

Since SSCs are the only stem cells in the adult male body capable of eventually transmitting information to a subsequent generation, it is crucial that these cells are genetically identical to their *in vivo* counterparts. Alterations to the genome are well known to change cellular phenotypes and can lead to a spectrum of genetic diseases [44–46]. These alterations, for example, translocations, small deletions or duplications, base pair mutations or copy number variations (CNVs) can be the direct result of an instable genome.

Artificial propagation of mammalian cells in an *in vitro* environment has been shown to cause instability of the genome [47, 48]. For instance, *in vitro* culture of murine hematopoietic stem cells, which normally reside in hypoxic bone marrow, induced chromosomal instability associated

with relatively high oxygen tension in culture [49]. Such a chemical stressor could cause DNA damage induced by reactive oxygen species and account for loss of genomic integrity. Another example of an external stressor influencing genome stability is temperature. For proper functioning, testicular tissue normally requires a slightly lower temperature of 2–4°C below body temperature [50]. Surprisingly, SSCs are typically propagated at 37°C [29–31], while it is known that elevated temperature of the testis is associated with decreased testis weight, decreased testis viability, and induction of DNA damage in spermatozoa [51]. How exactly the genome becomes instable is not known, but the mode of action is perhaps similar to the way carcinogenic events cause large chromosomal changes *in vivo* [52]. Rather than being driven by an active process, structural mutations may also arise spontaneously, as was shown for cultured hematopoietic stem cells [53]. Mutations that escape normal DNA repair are clonally expanded in an *in vitro* environment and will persist in every newly formed cell.

Cultures of mouse SSC show a normal euploid karyotype after 139 passages (~2 years of culture), indicating that they remain genetically highly stable even after prolonged exposure to *in vitro* culture conditions [54]. This suggests that SSCs possess a unique mechanism to prevent or repair genetic changes. However, in the same study, loss of telomeres has been observed. Although it would take many cell divisions before telomeres reach a critically short length so that cell senescence would be induced, senescent cells no longer divide, which might result in too few stem cells for transplantation in the case of SSCT. Conversely, it is known that telomere length is highly variable within a pool of male germ line stem cells and that germ cells are very tolerable to either high or low telomere lengths [55]. Whether genetic alteration or telomere shortening occurs in cultures of human SSCs has not been studied yet.

4. Epigenetics in Cultured SSCs

Besides changes in the genetic code itself, alterations in the epigenetic state of a cell can also occur as a result of environmental stressors [56, 57]. Epigenetics refers to the study of epigenetic traits, defined as *"stably inherited phenotypes resulting from changes in a chromosome without alterations in the DNA sequence"* [58]. One of the main functions of epigenetic modification is to establish differential gene expression by regulating transcription factor binding capacity to promoter regions, either via DNA methylation or chromatin modification. As it is well established over recent years, DNA methylation is closely intertwined with surrounding chromatin subunits and chromatin-related proteins [59] and distortion of the epigenetic landscape is associated with major diseases such as cancer [60, 61]. Some examples of *in vitro* aberrations of DNA methylation in stem cells come from studying cultured human mesenchymal stromal cells, which were shown to have significant changes in methylation when comparing a late passage to an early passage [62]. In this case, activation/repression of homeobox genes by changes in DNA methylation caused mesenchymal stromal cells to undergo senescence. Furthermore, altered DNA methylation in MSCs is correlated with repressive histone marks, which also leads to senescence [63].

In light of SSC transplantation, propagation of cells and transplantation procedures could serve as trigger for genetic and epigenetic changes, which may affect the health of SSC derived offspring (see Section 7). In a study comparing sperm derived from SSCs in grafts versus sperm derived from SSCT, no DNA methylation changes were found between these groups, but transplantation-derived sperm showed some variation in histone 4 acetylation [64]. Aberrant histone acetylation at this stage in development might have limited significance because in humans 85–95% of all histones are replaced by protamines to ensure proper packaging of DNA before delivery [65]. The small percentage of histones that do persist reside on HOX-gene promoters, miRNA genes, and imprinted genes. It has not been investigated if a change in histone modifications hampers functionality of the sperm. Culturing mouse testicular cells in medium containing GDNF and/or LIF does not alter methylation of the paternal imprinted *H19* locus, indicating that growth factors do not alter DNA methylation *per se* [66]. Long-term (>2 years) culture of mouse germ line cells also does not alter DNA methylation as was shown by combined bisulfite restriction analysis (COBRA) of five selected imprinted genes [54]. It should be noted that analysis of DNA methylation is often limited to a selection of imprinted genes, which may lead to a biased underestimation of epigenetic changes on the genome level. There is a need for experiments that will include all CpG sites in the genome and that will shed light on the true epigenetic status of a cell instead of a selected proportion of the genome. DNA methylation or histone modification has not been investigated in human cultures of SSCs.

5. Colonization of Cultured SSCs after Transplantation

It is essential that propagated SSCs maintain their ability to migrate to the niche and colonize the seminiferous tubules of a recipient testis upon transplantation. Nearly two decades ago murine testis-derived cells were transplanted in the testis of recipient mice and achieved colonization in 70% of the mice [18]. If a sufficient number of cells were transplanted, progeny could be generated harboring the same haplotype as the donor male mice. Since then, many groups have reported colonization of mouse SSCs and homing to a niche in the testes of mice [29, 67–71]. Others managed to perform successful homologous transplantations in pig [72], bull [73], non-human primate [21, 23] and recently zebrafish [74, 75]. Xenotransplantation to mouse recipients has been performed using dog [76], hamster [77], and bull [78–80] SSCs.

Building on the data gathered in animal models, several labs reported successful human SSC xenotransplantation using either uncultured cell suspensions [38] or *in vitro* propagated SSCs [30, 31]. Human SSCs can be cultured for long periods of time while maintaining their ability to migrate to their niche upon transplantation. However, xenotransplanted human SSCs cannot undergo spermatogenesis but

will rather divide a limited number of times and steadily decrease in number. An explanation why human SSCs cannot undergo full spermatogenesis in a mouse host environment is that postnatal primate SSCs, including human SSCs, are different from other species in terms of the expression of several spermatogonial markers like *POU5F1* (also known as *OCT-3/4*) [81] and *MAGE-A4* [82]. However, the spermatogenic arrest seen upon xenotransplantation could also be a result of phylogenic differences between the donor and recipient species [38, 83, 84] as is seen for many other non-primate species [76, 79]. Even so, xenotransplantation of SSCs is considered the only reliable bioassay at present to test for SSC functionality. In continuation of successful SSC xenotransplantation, homologous transplantation of primary SSCs has been demonstrated to initiate spermatogenesis in non-human primates [23]. In a recent publication, functional sperm was derived from both adult and prepubertal infertile rhesus macaques after autologous SSCs transplantation [21]. Not only was regeneration of spermatogenesis shown, but sperm derived from transplanted animals was also capable of fertilizing rhesus oocytes producing embryos ranging from four-cell stage to blastocyst with confirmed donor parental origin in 8,6% of embryos. There is a single report of SSC transplantation in humans in which a testicular cell suspension from cryopreserved testicular tissue was transplanted in 7 men [85]. Apart from this single study, other clinical attempts to reintroduce spermatogenesis in humans have not been described.

6. Remaining Tumor Cells in Testis Biopsy

Concerns have been raised about the potential presence of malignant cells in a biopsy taken from a patient that was diagnosed with cancer. Patients diagnosed with nonsolid tumors would be at high risk for this, because there is a chance that infiltrated tumor cells in the testis biopsy may linger in the *in vitro* culture and end up in the cell population used for transplantation. The most commonly diagnosed nonsolid tumor in prepubertal individuals is acute lymphoblastic leukemia (ALL), which infiltrates the testis in approximately 30% of cases [86]. For solid tumors that do not originate in the testis, the risk of nonintentional transplantation seems limited because solid tumors rarely metastasize to the testis [87].

Attempts to remove malignant cells from testicular cells have been limited. By sorting uncultured murine testicular cells mixed with leukemic cells for MHC-I$^+$/CD45$^-$, leukemic cells could be successfully separated from germ cells [88] and after transplantation of these cells to a recipient mouse, no leukemia was observed. Others have tried to reproduce these findings but did not succeed in completely removing malignant cells from the transplanted cell population [89]. Inoculation of T-lymphoblast cells with prepubertal primate testis cells still has a remainder of 0.1% of tumor cells after FACS sorting for CD90$^+$(THY-1$^+$) and CD45$^-$, and the remaining tumor cells were able to form tumors after transplantation to nude mice [90]. Testicular cells derived from a leukemic rat can transmit lymphoblastic cells and subsequently induce leukemia even when as few as 20 cells are transplanted in a recipient rat [91]. Hitherto, successful removal of malignant cell types is difficult but of utmost importance for the success of SSCT. It is important to note that all these studies examine uncultured SSCs. Sorting procedures might be different in cultured cells as compared to uncultured cells, because expression of certain cell membrane markers is lost upon culturing. A careful selection of membrane markers still present on cultured SSCs or alternatively on tumor cells is important for efficient removal of malignant cells.

Besides the danger of reintroducing lingering malignant cells, the SSC culture system could also lead to the spontaneous arising of embryonic stem-like (ES-like) cells that are potentially carcinogenic. Indeed during the culture of mouse germ line cells, colonies of ES-like cells arise as spontaneous by-products of testicular cell cultures [92–94]. These ES-like cells are pluripotent and when they are relocated to an *in vivo* environment, they can form teratomas. Induction of teratomas after transplantation of testis derived ES-like cells has been well described in mouse models. In humans, the presence of pluripotent ES-like cells in germ line cultures is not as uniformly accepted as compared to mouse. Generation of ES-like colonies from human testis has been reported by several groups [95–97] but only one showed formation of teratomas that could be differentiated into cell types of all three germ layers [98]. Apart from this report, no other group could reproduce the formation of teratomas, and rather show that these testis-derived cell colonies do not express pluripotent markers at high levels. These two features are considered essential to classify cells as being pluripotent. Moreover, it has recently been shown that ES-like colonies in human germ line cultures have mesenchymal potential and might thus be multipotent rather than pluripotent [99, 100]. This also argues in favor of the theory that the "ES-like" colonies found in human germ line cultures are not truly ES-like. Teratoma formation upon accidental transplantation of ES-like colonies present in human germ line cultures is therefore less likely and seems of less significance in humans as compared to mice.

7. Health of Offspring

Reports of SSCT-derived offspring mainly focus on the proof of concept that SSCT can generate offspring [29, 67–71, 101] while the general health of offspring is studied very minimally. Some studies perform no health analysis, while others only report basic variables such as weight, length [67] and fertility for a limited number of offspring [29, 68–70]. In some cases, growth abnormalities were observed in SSCT-derived offspring in mice [101]. It was shown that the karyotypes of first and second generation SSCT-conceived mice look normal as compared to naturally conceived mice [102]. The genome of F1 SSCT-conceived offspring was screened for genetic abnormalities by comparative genome hybridization (CGH) and no significant duplications or deletions were reported [70]. Remarkably, in some studies as many as 85–92% of all constructed embryos were lost

during embryonic development [54, 101]. Whether this loss can be attributed to poor health of the embryos or technical constraint induced during round spermatid injection (ROSI) is unclear. ROSI has been associated with cleavage arrest in the early human embryo and similar failure rates have been described for generation of mouse embryos using ROSI [103, 104]. This suggests that the loss of embryos is caused by the ROSI procedure rather than SSC culturing or transplantation itself. CGH has the disadvantage that it can only reveal large genetic changes and cannot distinguish smaller genetic alterations such as SNPs or small CNVs. Base pair mutations on the single nucleotide level in spermatogonia have been shown to cause severe disease phenotypes including congenital disorders such as craniosynostosis syndrome (e.g., Apert Syndrome) [105, 106]. Since genetic alterations are essentially irreversible, they can be transmitted to the next generation and cause such phenotypes in the offspring. It is therefore crucial that the genome of experimental SSC derived offspring is screened on the highest resolution possible to reveal potentially harmful genetic mutations that arise in cultured SSCs.

In recent years, it has become apparent that epigenetic alterations may also be transmitted to subsequent generations. Studies on transgenerational epigenetic inheritance show the influence of the intrauterine environment on the epigenome and the mechanisms that lay behind these processes. Well-known examples of how the environment may cause heritable epimutations in humans are the Dutch Famine studies [107, 108] and the Överkalix cohort studies [109], which give evidence that both prenatal exposure to famine and food restriction during childhood are associated with an increased susceptibility of the offspring to multifactorial diseases such as cancer, diabetes, and cardiovascular disorders [56]. Animal studies have pointed out that various prenatal and early-life dietary conditions, such as high fat diet, low protein diet, overfeeding and malnutrition induce differential methylation of genes that may lead to a range of pathological phenotypes [110–116].

Methylation levels of the *H19* promoter and *Snrpn* promoters in SSCT-derived mice were shown to resemble those of naturally conceived controls, suggesting that there were no apparent methylation defects present in the offspring [70]. Likewise, no differences in DNA methylation of imprinted genes *Igf2* and *Peg1* occur in SSCT-conceived mice compared to naturally conceived mice [67]. In contradiction, distorted DNA methylation of *H19* and *Snrpn* promoter and altered histone modification was reported in pups conceived through fetal germ cell transplantation, alterations that were transmitted vertically up to 4 generations [101]. The observed epigenetic changes might be explained due to the potentially immature DNA methylation status of fetal germ cells compared to that of SSCs at the moment of isolation. During normal development, nearly all methylation marks undergo demethylation at the time PGCs migrate to the embryonic genital ridge and are remethylated in a sex-specific manner starting around the onset of the gonocyte stage [117]. Fetal germ cells may have been disrupted in the critical step of epigenetic reprogramming during culture, leading to the observed DNA methylation changes.

Heritable epigenetic influence on the phenotype is not only seen in experimental settings, but also in daily clinical care. Some studies show that assisted reproduction technologies (ART), such as IVF and ICSI, are associated with an increased risk of imprinting disorders such as Beckwith-Wiedemann Syndrome (BWS) [118]. Imprinting disorders are caused by loss or gain of parental DNA methylation at imprinted loci which results in aberrant gene expression during development and thereby leads to severe, irreversible phenotypic changes [119, 120]. The association between ART and imprinting disorders still remains controversial. While some studies on BWS show a 4-fold increased incidence of 4,6% in children conceived by IVF or ICSI in comparison to the background incidence rate of 0,8% [121], others report no increase of BWS cases in children conceived by ART [122, 123]. Moreover, it remains questionable whether the relation found between ART and imprinting disorders is causative, as it has been suggested that underlying sub-fertility of the parents might play a role [124]. Although SSCT and IVF/ICSI are both techniques to restore fertility, one should keep in mind that SSCT and IVF/ICSI are different on many levels. In SSCT resulting embryos are not cultured in an artificial environment, and therefore the risk to imprinting disorders may be absent or of a different magnitude than in IVF/ICSI. Even though the above results indicate that SSCT-derived offspring are fairly healthy, one must realize that the offspring studied are very low in number, in some cases as limited as one or two per study.

8. Concluding Remarks

Ever since Brinster and Avarbock were able to obtain healthy offspring following SSCT in mice, many investigators have made efforts to translate this model into a clinical application. In this paper, we have discussed the current state of the art and hurdles that should be overcome (summarized in Figure 1) before SSCT can be implemented clinically. Many achievements have been made since the first successful transplantation in mice, and currently we are able to maintain and propagate human SSCs *in vitro* for long periods of time, without loss of expression of spermatogonial markers and with maintenance of their stem cell ability to migrate to the niche in the seminiferous tubules upon transplantation. These encouraging results make SSCT a potentially powerful therapeutic strategy to preserve and restore fertility in childhood cancer patients in the future.

Future research needs to focus on a way to ensure there is no chance of reintroducing malignant cells in an individual that has just been treated and cured from a cancer. The risk of reintroducing malignancy by transplanting lingering tumor cells along with SSCs seems present as long as we cannot utterly remove them from cultures. Studies on the epigenetic stability of SSCs in culture and posttransplantation are scarce and results are contrasting. Efforts should be made to dissect the precise changes on both the genetic and epigenetic level when SSCs are cultured in an artificial environment. Adding up to this, it is unclear whether SSCT and subsequent SSC development to sperm from (epi-)genetically altered SSCs

has any influence on the epigenome of the offspring. Arguably the most important factor is the health of offspring, and therefore more research should be started to assess general health of SSCT-conceived offspring in an adequate animal model with sufficiently large populations of animals, before a clinical trial in humans.

Modern next-generation sequencing techniques make it more and more feasible to map the entire (epi-)genome of a cell culture or cell population on the single nucleotide level [125–127] and there are already a number of publications available that describe genome-wide DNA methylation for a range of male reproductive cell types [128, 129]. These advances provide us with a powerful tool to generate much needed information on how SSCs react to an *in vitro* culture environment in terms of methylation and base pair alterations. Steady progress concerning SSCT techniques is ongoing and this is why many researchers and clinicians are becoming increasingly confident that SSCT is viable as a way to restore fertility in prepubertal cancer patients. All in all, SSCT is a promising technique that will be beneficial for many young individuals diagnosed with cancer in the near future.

Authors' Contribution

R. B. Struijk and C. L. Mulder contributed equally to this study.

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Review Article

Propagation of Adult SSCs: From Mouse to Human

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Adult spermatogonial stem cells (SSCs) represent a distinctive source of stem cells in mammals for several reasons. First, by giving rise to spermatogenesis, SSCs are responsible for the propagation of a father's genetic material. As such, autologous SSCs have been considered for treatment of infertility and other purposes, including correction of inherited disorders. Second, adult spermatogonia can spontaneously produce embryonic-like stem cells *in vitro*, which could be used as an alternative for therapeutic, diagnostic, or drug discovery strategies for humans. Therefore, an increasing urgency is driving efforts to understand the biology of SSCs and improve techniques to manipulate them *in vitro* as a prerequisite to achieve the aforementioned goals. The characterization of adult SSCs also requires reproducible methods to isolate and maintain them in long-term culture. Herein, we describe recent major advances and challenges in propagation of adult SSCs from mice and humans during the past few years, including the use of unique cell surface markers and defined cultured conditions.

1. Introduction

The spermatogonial stem cells (SSCs) of the adult testis in mammals possess both the extraordinary ability to self-renew, in order to maintain a near life-long pool of stem cells and the means to differentiate into lineage-committed germ cells. Adult SSCs, however, represent a unique model for a number of reasons. First, male fertility and genetic diversity of species both depend on continuous, normal spermatogenesis during reproductive life [1, 2]. Second, spermatogonia *in vitro* can spontaneously produce embryonic-like stem cells [3] and therefore can be used as a model to study mechanisms of reprogramming and maintenance of pluripotency or to develop strategies for regenerative therapy for humans similar to embryonic stem cells (ES) or induced-pluripotent stem cells (iPS). The use of spermatogonial-derived pluripotent stem cells could avoid ethical concerns over the use of ES cells and also obviate the need for exogenous pluripotent factors, such as those necessary to generate iPS from adult somatic cells [4]. Furthermore, the likelihood of immunological rejection by the host would be greatly reduced compared to ES-derived cells, since autologous pluripotent cells can potentially be isolated from the human testis [5, 6]. In addition, SSCs represent one of the few adult stem cell populations that can be maintained in a long-term *in vitro* culture system [7, 8] and for which a functional *in vivo* transplantation assay has been established [9, 10]. However, the identification and further characterization of the true stem cells among the spermatogonia remain challenging because of the absence of specific markers that would allow the isolation and further analysis of this population. This is particularly true in humans, because human germ cells tend to be technically difficult to study. Additionally, the unique features of adult SSCs as opposed to neonatal SSCs are important to consider, since spermatogonia undergo substantial changes in gene expression during the post-natal period (e.g., reduction of OCT4 expression) and also in function [11–13]. This review will focus on the theoretical and practical basis for long-term culture of adult mouse SSCs and recent efforts toward the development of human SSC cultures.

2. SSC Identity and the Spermatogonial Stem Cell Niche

Spermatogenesis consists of the differentiation of male germ cells into spermatozoa, the male gametes that carry genetic information to subsequent generations and occur within the seminiferous tubules [14, 15]. The seminiferous tubules

are highly structured convoluted tubules, consisting of a lumen, into which the spermatozoa are released, and the peripheral basement membrane. Two types of somatic cells are located on the periphery of the seminiferous tubules: (1) peritubular myoid cells covering the external side of the basement membrane and (2) Sertoli cells that form the epithelium on the inner surface of the basement membrane to nourish the male germ cells in various stages of maturity. Spermatogenesis in the adult testis relies on SSCs that are derived from prospermatogonia that themselves mature from gonocytes in the fetus. Gonocytes, in turn, are derived from primordial germ cells that migrate into the gonad during embryonic development.

While most recent studies have relied on molecular markers of spermatogonia, it is important to understand the morphological basis for subclassifying this group of undifferentiated germ cells [16]. The spermatogonial population is located along the basement membrane of the seminiferous tubules and has been grouped, based on morphological criteria, into A, Intermediate, and B subtypes. The A type spermatogonia is then divided into undifferentiated (A_{undiff}) and differentiated [17]. A_{undiff} represents the most primitive spermatogonia, and it is characterized by minimal heterochromatin condensation. In the case of rodents, A_{undiff} can be further classified into A_s (single), A_{pair} (cohorts of two cells), and $A_{aligned}$ (cohorts of 4, 8, and 16 cells) [18–20]. The A_s spermatogonia are thought to include the stem cell population, while A_{pr} and $A_{aligned}$ represent their progeny. $A_{aligned}$ continue to mature into differentiated spermatogonia to ultimately produce diploid spermatocytes. In primates, however, A_{undiff} spermatogonia are separated uniquely into two subtypes of A_s, A_d (dark) or A_p (pale), based on distinct levels of chromatin condensation [21–26]. Type A_d are considered the reserve stem cells, while A_p divide symmetrically to produce either new A_p or type B spermatogonia that will further differentiate to form spermatocytes and spermatids.

The stem cells represent a minor fraction of the undifferentiated spermatogonial pool. In rodents, it remains controversial whether stem cell capacity resides exclusively in the A_s pool, or a fraction thereof, or whether A_{pair} cells also retain stem cell activity. While we and others have previously referred to undifferentiated spermatogonia as "spermatogonial progenitor cells," this term is somewhat confusing due to the unintended implication that such cells may be even more primitive than SSCs. The identity of human SSCs is unknown, in part due to the challenge of maintaining them in long term in culture (to be discussed below) [27]. However, it is generally accepted that the human SSCs correspond to a minor fraction of the A_d or A_p spermatogonia. Recent studies suggest that there are two functional populations of SSCs in the mouse testis [28, 29]: self-renewing SSCs (referred to as actual stem cells) and another population that maintains the ability to self-renew but only under stressful conditions (referred to as potential stem cells) [30, 31]. These studies seem to support the idea of plasticity within the hierarchy of spermatogonial differentiation in the sense that SSCs comprise a heterogeneous population including cells with different degrees of stem cell potential, whereas certain cells that are committed to differentiation may switch back and self-renew in response to physiological or pathophysiological perturbation. If a similar paradigm applies to humans, then this may conflict directly with one of the central assumptions underlying classical models for the kinetics of maturation of early human spermatogonia; namely, differentiation is linear, unidirectional, and irreversible [24].

One of the most critical elements for stem cell maintenance and function is the associated microenvironment, or niche, that provides physical support and regulates fate decisions of stem cells [31–33]. The niche concept, first proposed by Schofield in 1978 [34], refers to distinct microanatomical locations where tissue-specific stem cells reside. The stem cell niche comprises several components, including resident cells that create essential structural features, provide the proper growth factor milieu to promote self-renewal and/or differentiation of the stem cells, and maintain the stem cell population without excessive proliferation [35]. Spermatogonia are in close contact with Sertoli cells, which are considered one of the most critical constituents of the SSC niche. Sertoli cells exhibit polarity and are connected through tight junctions that create a blood-testis barrier, dividing the epithelium into basal and adluminal compartments. While spermatogonia reside in the basal compartment, germ cells entering meiosis cross the tight junctions and occupy the adluminal zone where subsequent steps of spermatogenesis take place, until the spermatozoa are finally released to the lumen [1, 36–39]. Such subcompartmentalization enables differential exposure of the spermatogonia to signals either secreted by interstitial cells or elaborated by the vascular network, while differentiated germ cells, adluminal to the Sertoli cell tight junctions, are less exposed to such factors. Some data suggest that the vascular network and interstitial tissues also directly contribute to the stem cell niche in the testis [40, 41]. In particular, Leydig cells, best known for producing testosterone, and a subpopulation of peritubular myoid cells may contribute to the function of the SSC niche by secreting specific factors, such as cytokine colony-stimulating factor (CSF1) which potentiates self-renewal in mice cultured spermatogonia.

Some of the more tantalizing questions that arise are whether or not stem cells are immortal or long lived, and whether aging is due, in part, to the progressive cell-autonomous loss of stem cell self-renewal capability, the progressive deterioration of the supporting niche, or perhaps both. SSCs, like hematopoietic and hair follicle stem cells, can be used to address these questions, as such studies require functional assays that are available for only a handful of organ systems [31, 42, 43]. The work by Ryu et al. (2006) using SSC transplantation into a heterologous recipient environment (young or aged, busulfan-treated mouse testis) suggests that SSCs are potentially immortal, since the self-renewal capability of SSCs from an older donor was maintained in a young environment, while aged testes failed to support normal colony formation [44]. Whether extremely long replicative potential is exclusive to SSCs (perhaps because the germline is essential for survival of a species) or whether such longevity is a characteristic shared with other adult stem cells remains unclear. Recent work by Chakkalakal et al. (2012) on the muscle stem cell niche shows that quiescence

is essential to maintain stem cell function and that the aged niche disrupts quiescence state of the stem cells, promoting differentiation through an increase in FGF signaling [45]. This effect ultimately leads to depletion of the self-renewing population, supporting the idea that aging of the niche is the root cause of the loss of stem cell capacity in adult tissues.

3. Long-Term Culture of Adult SSCs

The studies discussed previously suggest that microenvironmental changes (i.e., niche deterioration) are critical for loss of stem cell maintenance or to drive differentiation over self-renewal and vice versa. The identification of such signals emanating from the SSC niche is therefore critical to establish long-term culture conditions of SSCs. An essential component of the spermatogonial niche is glial cell line-derived neurotrophic factor (GDNF), which is secreted by Sertoli cells [46]. Mutant mice deficient in GDNF exhibit disrupted spermatogenesis and loss of germ cells. In contrast, transgenic mice that overexpress GNDF accumulate undifferentiated spermatogonia, which ultimately lead to tumor-like structures composed of germ cells. Based on these data, preliminary studies defined specific factors and cell culture conditions that increased survival of male mouse germ cells *in vitro* [7, 47–49]. Such signals include GDNF and FGF2 (formerly bFGF); these were combined with SIM mouse embryo-derived thioguanine and ouabain-resistant (STO) feeders that were previously shown to support different stem cell survival [47, 50–52]. Using these tools, Kubota et al. (2004) developed a defined culture system that promoted long-term *in vitro* expansion on SSCs from mouse pup testis [53]. Additionally, two other factors facilitated the successful establishment of long-term cultures. First, donor testis cells were enriched for SSCs by means of surface markers. Second, a serum-free medium was developed, since previous studies had suggested that serum could induce apoptosis or differentiation of SSCs. In fact, this culture system was able to support the expansion of neonatal, pup, and even adult SSCs from several mouse strains, which was the main limitation in previous reports. To date, however, much of the data on SSC culture has been obtained using neonatal testis as the donor tissue as opposed to that of adults, likely because adult SSC lines have been challenging to derive.

Further studies demonstrated that the dependence of SSCs on GDNF signaling is conserved across species, like rat and rabbit, supporting the critical role of GDNF in mammalian SSCs [54–56]. However, GDNF alone is not sufficient to enable long-term culture of SSCs. Moreover, RET and GFRα1, the GDNF receptor complex, are expressed in spermatogonia but are not entirely restricted to the stem cells [57–59]. Several studies have shown that in adult mice the fraction of testicular cells expressing GFRα1 is not enriched for SSCs, opposite to what is seen in early stages of postnatal development [57]. Additionally, the RET-positive fraction of cells in postnatal mice is not enriched in SSCs. It has been suggested that GDNF is the main survival factor in spermatogonia, while other factors could be responsible for the fate decision during SSC division. Similarly, while FGF2 alone does not support maintenance of SSCs in culture, it does increase the proliferation rate in conjunction with GDNF. The role of FGF2 in the stem cell niche *in vivo* is still unclear, but it is known that various cell types in the testis produce FGF2, including Sertoli, Leydig and differentiating germ cells [60, 61]. Moreover, a recent study shows that FGF2 improves self-renewal of SSCs *in vitro,* activating the MAP2K1 signaling pathway that upregulates ETV and BCL6B, two critical transcription factors for SSCs survival [62, 63].

Other extrinsic factors have been shown to enhance the survival of SSCs [46–48, 53]. EGF and IGF-1, for example, seem to have similar effects to FGF2 [49]. On the other hand, CSF1 is expressed in Leydig, cells and a subset of leukocytes and, as mentioned previously, promotes self-renewal of mouse SSC in culture. Furthermore, the CSF1 receptor (CSF1R) is also highly expressed in undifferentiated spermatogonia in mouse testis [41]. However, CSF1 does not increase proliferation in cultures maintained in the presence of GDNF and FGF2, suggesting that CSF1 alters cell fate decisions in SSCs in culture [40]. Finally, although leukemia inhibitory factor (LIF) has a major role in maintaining pluripotency of ES cells and facilitates the establishment of germ cell colonies from the newborn testis, increased proliferation of mouse and rat SSCs was not seen with addition of LIF either to serum-containing media or to GDNF-dependent serum-free cultures [49, 54, 64, 65].

The development of systems that facilitate the expansion *in vitro* of SSCs rapidly spawned attempts to specifically culture adult SSCs. The justification for developing adult SSC cultures is several fold. First, the transmission of genetic information to offspring requires faithful spermatogenesis during adulthood and maintenance of a pristine stem cell pool. Also, the adult testis will ultimately be the primary source of human SSCs for *in vitro* genetic manipulation and potentially reparative therapies (i.e., germ line modification). While pluripotent stem cells had been successfully generated previously from neonatal mouse testis, it remained unclear until recently whether the same could be achieved for wild-type adult SSCs in long-term culture [66]. Some systems for derivation and long-term *in vitro* expansion of adult SSCs were inefficient. Moreover, previous methods required the initial enrichment of SSCs using immunoselection (with the caveat that specific markers for SSCs remain still unknown), similar to other adult stem cells, or required cryptorchid mice that contain a higher ratio of stem cells over other types.

In 2007, we developed a highly proliferative long-term culture system to expand adult SSCs, free of nongermline contaminants [3, 67]. The method used mitotically inactivated testicular stromal cells as feeders, based on the hypothesis that removal of somatic cells from the initial culture disrupts the stem cell niche; this approach allows the *in vitro* propagation of functional SSCs for over a year. SSCs cultured in such conditions self-renew and can reconstitute spermatogenesis after transplantation into busulfan-treated recipients. Furthermore, the *in vitro* milieu preserves the ability of adult SSCs, even after long term in culture, to generate pluripotent adult stem cells that can differentiate into derivatives of the three germ layers and contribute to

chimeric embryos. We also identified a novel putative surface marker, an orphan G-protein-coupled receptor (GPR125), which is expressed in the testis exclusively in undifferentiated spermatogonia and can be utilized to track spermatogonia within the mixture of testicular cells.

Recently, similar approaches utilizing other forms of testicular feeder cell cultures have been utilized to model niche-stem cell interactions [68, 69]. The Shinohara group provided evidence that CXCL12 and GDNF are chemotactic factors that promote homing of SSCs into their niche; this system reproduces the *in vitro* formation of cobblestone colonies growing underneath the stroma similar to previously described colonies in hematopoietic stem cell (HSC) cultures [70, 71]. The cobblestone formation assay has been utilized as an *in vitro* alternative to evaluate HSC potential, particularly useful in certain circumstances when direct transplantation is not possible. It is possible that a similar approach could be utilized with cobblestone colonies derived from germ cells. Nevertheless, the method allows the identification of molecules involved in proliferation and homing of SSCs that can possibly be extrapolated to the *in vivo* niche. For example, the addition of exogenous GDNF was not necessary, suggesting that GDNF is secreted from testis somatic cells, although exogenous GDNF and, to a lesser extent, EGF plus FGF2 enhanced cobblestone formation. Additionally, follicle-stimulating hormone (FSH), which has been reported to induce expression of GDNF in Sertoli cells, improved the formation of cobblestone colonies when used together with EGF plus FGF2. Interestingly, these findings suggest that other cytokines maintain the SSCs in the culture, since, in absence of exogenous FGF2, GDNF was able to increase the SSC population, likely due to FGF2 and/or additional FGF family molecules secreted by Sertoli cells.

A certain amount of controversy remains in the field regarding the true identity and functionality of the proliferating cells that are enriched in long-term cultures of SSCs with different methods [7, 53, 67, 72]. Although it has been reported that feeder-free conditions could be employed [48] and that the proliferation of mouse SSCs *in vitro* is dependent on LIF instead of GDNF [73], most studies have shown that the use of both, mitotically inactivated somatic feeders cells and GDNF are critical for maintaining long-term self-renewal of SSCs *in vitro* (Figure 1).

The protocols to maintain mouse SSCs for long term in culture, together with what has been learned about culture conditions for human ES cells, have allowed the development of parallel strategies to propagate human SSCs. However, attempts to establish long-term cultures of human SSCs have been problematic. Among other genes, $CD49f^+$, SSEA-4, GFRα1, GPR125, and PLZF are known to be expressed in human spermatogonia [5, 74–76]. Importantly, this gene expression information along with surface markers identified in spermatogonia from the primate testis and from other species has enabled the development of enrichment methods for putative human SSCs. For instance, human $CD49f^+$ (α_6 integrin) and $SSEA^+$ germ cells preserved the ability to repopulate busulfan-treated testis in immunodeficient mice, suggesting not only that both populations are enriched in self-renewing SSCs but also that the niche is at least somewhat compatible between human and mouse [77]. However, as will be discussed further, nor SSEA-4 neither CD49f are considered specific marker for human spermatogonia, since they are also expressed either in somatic cells or in differentiating germ cells that coexpress markers of differentiation as well (e.g., KIT) [65]. Nevertheless, Chen et al. (2009) were able to maintain $CD49f^+$ germ cells isolated from human fetal testes for two months using a combination of media containing a similar formulation to the one used to maintain mouse SSCs (GDNF, bFGF, and LIF) and human ES cell-derived fibroblast-like cells (hdFs) as feeder cells [78]. The human spermatogonial colonies derived in the culture expressed known markers associated with spermatogonia, like OCT4 [13, 49, 79].

A similar result was reported by Sadri-Ardekani et al. in 2009 for cells isolated from adult human testis from prostate cancer patients after orchiectomy [80]. The germline stem cell clusters that arose from testicular cell suspensions were cultured in media containing recombinant human epidermal growth factor (rhEGF), rhGDNF, and rhLIF. The SSCs were expanded up to four months, expressed spermatogonial markers, and were able to colonize recipient mouse testes [66]. In another study, the isolation of GPR125-positive spermatogonia from adult human testis resulted in an increase in undifferentiated cells after two weeks in culture in media containing human GDNF, LIF, EGF, and TGF and other factors that likely increase GDNF, FGF2, and TGFβ signaling (GFRα1-Fc, NUDT6 and Nodal, resp.) (Figure 1) [76]. Similar to mouse SSCs, MAPK1/3 signaling was increased in GPR125-positive germ cells after two weeks in culture, revealing one molecular mechanism that may be involved in proliferation of human spermatogonia. Remarkably, the spermatogonia were obtained from deceased organ donors, a reasonable source from which to obtain human SSCs from healthy donors. A recent report using testicular tissue from patients with azoospermia identified genes differentially expressed between proliferating putative human SSCs *versus* the senescent human SSCs that resulted from human SSC-like cells after five passages in culture [81]. This yielded a list of potential genes related to proliferation of human germ cells.

4. Molecular Markers of Mouse and Human SSCs

Long-term expansion of SSCs *in vitro* cannot be fully realized until (1) stem cells are validated and quantified by the transplantation assay and (2) the identity of the expanded SSCs is confirmed through the use of molecular markers that distinguish them from spermatogonia in other stages of differentiation. Moreover, molecular markers expressed in SSCs are commonly employed using a variety of different techniques (i.e., immunohistochemistry or cell sorting strategies) to characterize and identify spermatogonia prior to culture. This becomes particularly relevant in the case of human SSCs, since, as opposed to mouse SSCs, fibroblasts are more easily established than SSCs from human testicular cultures without prior enrichment for spermatogonia [82].

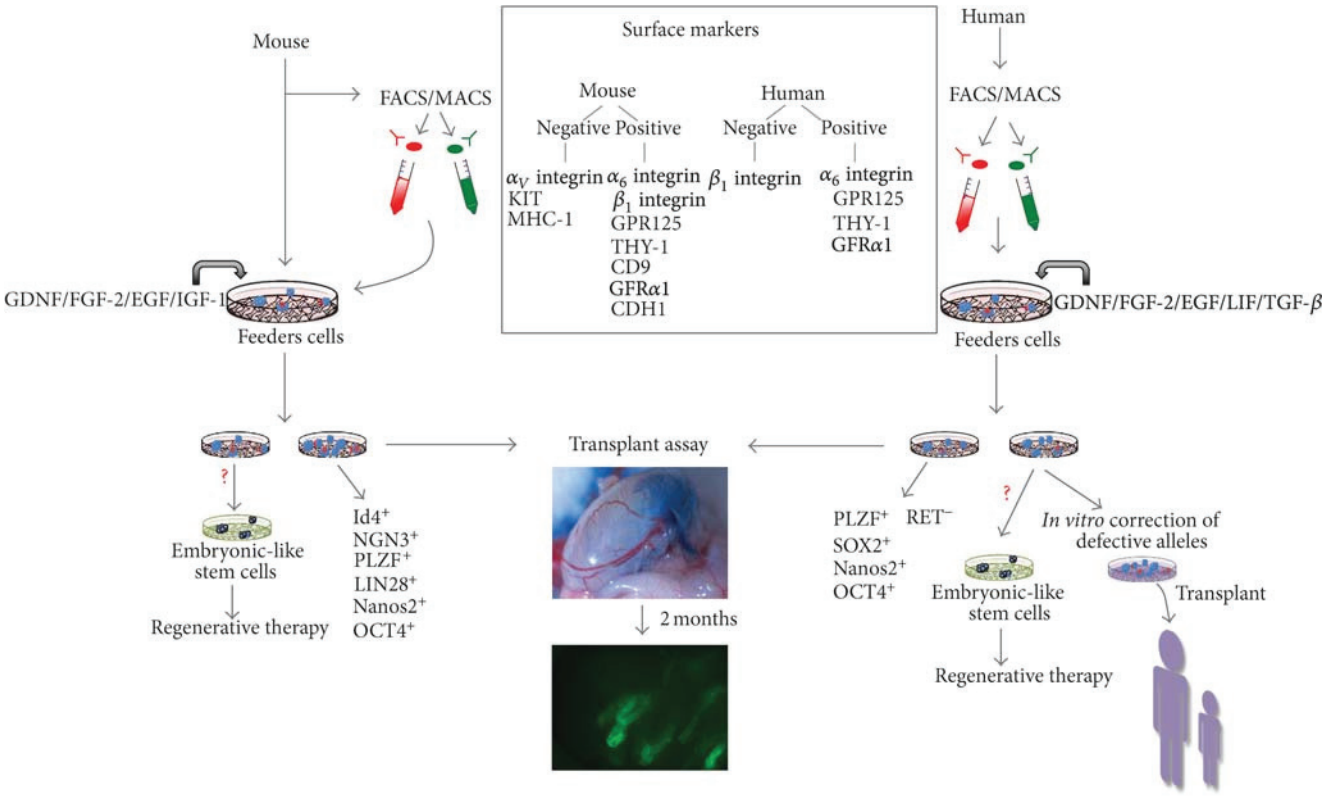

Figure 1: *In vitro* propagation of adult SSCs. SSCs are derived from the adult testis using somatic feeders and media containing diverse growth factors such as GDNF and FGF-2. In the case of human SSCs, a preenrichment sorting step (FACS or MACS) using previously identified surface marker, is critical for the successful expansion of SSCs. Mouse cultures established in such way can be maintained for over 1 year. Long-term expansion of SSCs *in vitro* is confirmed by analyzing the expression of molecular markers of spermatogonia. Furthermore, the number of stem cells expanded in the culture must be validated and quantified by an *in vivo* functional assay consisting of transplantation of SSCs into busulfan-treated recipient mouse testis. The fluorescent image corresponds to seminiferous tubules repopulated with donor GFP-positive cells. Potential clinical applications of SSCs include restoration of male fertility and/or *in vitro* correction of mutated alleles prior to transplant. Furthermore, *in vitro* SSCs can spontaneously reprogram to embryonic-like stem cells and could be used for regenerative therapy.

The greatest limitation relies on the fact that SSCs are presumed to be a very rare population in the human testis, and their unequivocal identification has been extremely difficult to achieve. In fact, there are currently no specific markers expressed in mouse or human spermatogonia that are completely restricted to the pool of stem cells, although Oatley et al. (2011) have reported recently that Id4 expression is limited to the A_s pool in mouse spermatogonia [83]. As discussed previously, however, it remains unclear whether the A_s cells represent the only stem cell pool on the testis. Nevertheless, the results of many studies together have yielded a list of genes and surface markers proven to be expressed on SSCs, inspite of being not restricted to them, representing extremely valuable tools in SSC research as recently reviewed elsewhere [84, 85]. It was established very early that β_1 integrin (CD29) and α_6 integrin (CD49f) are expressed in mouse SSCs, while the KIT receptor tyrosine kinase and α_v-integrin (CD51) are low or absent in undifferentiated spermatogonia and are considered to be markers of the transition to differentiating type A spermatogonia [86, 87]. Since β_1-integrin and α_6-integrin are located on the cell surface, they were promptly utilized in fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) to enrich mouse SSCs from testicular tissue. Similarly, other surface markers were progressively identified as either expressed or absent, defining a surface phenotype for mouse spermatogonia [88–94]. Positive markers include THY-1 (CD90), CD9, GFRα1 and CDH1, while α_v-integrin, KIT (CD117), MHC-1, and CD45 are negative or low. However, there are various technical reasons why surface expression does not guarantee that a given marker will be useful for stem cell enrichment (Figure 1).

Recently, Kanatsu-Shinohara (2011) showed that the CD9$^+$EPCAM$^{-/low}$ population is more enriched for SSCs [95]. GPR125 is also a marker for undifferentiated spermatogonia in mouse [3]. In addition to surface markers, the expression of intracellular factors, including PLZF, LIN28, NANOS2, and OCT4, correlates with undifferentiated spermatogonia, although, once again, these are not restricted to the stem cell pool (Figure 1) [74, 96–100]. The zinc-finger RNA-binding protein NANOS2, for instance, regulates SSC maintenance and is expressed in A_s,A_{pr}, and some

A_{al} [101]. Similarly, the promyelocytic leukemia zinc finger protein (PLZF) is a transcriptional repressor necessary for maintenance of germ cell lineage, generally associated with undifferentiated spermatogonia, including SSCs [102].

Several surface markers identified in mouse SSCs have been successfully tested in humans [75, 86]. For instance, THY-1 and GFRα1 were used to purify human spermatogonia by MACS. Similarly, He et al. (2010) employed GPR125 expression to isolate human spermatogonia and confirmed the expression of α_6 integrin, THY-1, GFRα1, and PLZF in the sorted population [76]. Interestingly, only one or two spermatogonia per seminiferous tubule were estimated to express GPR125. On the other hand, neither β_1 integrin, RET, nor NGN3 is considered to be markers for human spermatogonia [75, 85]. Additionally, molecular markers correlated with pluripotency (i.e., OCT4, NANOG, or SOX2) have been a detected in human spermatogonia, and it has been suggested that a subset of these cells exhibit the characteristics of pluripotent cells (Figure 1) [65, 103].

Other molecular markers in human spermatogonia have been recently identified and correlated with the different subpopulations established under the morphological criteria described earlier [104, 105]. For instance, only A_d spermatogonia express high levels of the exosome component 10 (EXOSC10), a feature linked to the immature state of the cell, while A_p and B spermatogonia share expression of Ki-67, which is associated with cell proliferation [106], and DMRT1, a protein that promotes differentiation-associated mitosis [107]. Furthermore, FGFR3 has been identified on the surface and in the cytoplasm of a subpopulation of rarely dividing type A_d spermatogonia that are also negative for Ki-67 and DMRT1. With such recently described molecular and functional markers have come newer theoretical models to explain the relationships between different subpopulations of human spermatogonia. For instance, it has been suggested that the nuclear differences observed in the A_p and A_d populations reflect stem cells in different stages of the cell cycle rather than spermatogonia in different stages of differentiation [26, 108].

5. Conclusions and Remarks

While a variety of research applications and clinical uses of SSCs can be envisioned, the ability to manipulate SSCs *en masse* in the culture dish is a critical if not essential tool for most of such endeavors. Cell transplantation is a conceptually straightforward use of SSCs. For example, the *in vitro* correction of defective genes prior to transplant could be used either to restore male fertility or to prevent transmission of mutant alleles associated with genetic diseases. Pre pubertal human SSCs would be useful for chemotherapy-induced infertility arising from childhood cancers (Figure 1) [109]. However, it is also apparent that propagation of aberrant SSCs can lead to human diseases [110]. Therefore, it will be critical, in the future, to assess the potential risks and benefits associated with SSC-based therapy, including the possibility of propagating genetic or epigenetic abnormalities. With the proper knowledge base in place, including a good grasp of the intricacies of SSC self-renewal, only then can clinical strategies move forward successfully for the benefit of patients and their families.

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Research Article

Differentiation of Induced Pluripotent Stem Cells into Male Germ Cells *In Vitro* through Embryoid Body Formation and Retinoic Acid or Testosterone Induction

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Generation of germ cells from pluripotent stem cells *in vitro* could have great application for treating infertility and provides an excellent model for uncovering molecular mechanisms controlling gametogenesis. In this study, we explored the differentiation potential of mouse induced pluripotent stem (iPS) cells towards male germ cells. Embryoid body formation and retinoic acid/testosterone induction were applied to promote differentiation of mouse iPS cells into male germ cells *in vitro*. Quantitative RT-PCR and immunoflourescence were performed to characterize the iPS cell differentiation process, and notably there were different temporal expression profiles of male germ cell-associated genes. The expression of proteins, including MVH, CDH1, and SCP3, was remarkably increased. mRNA expression of *Stra8*, *Odf2*, *Act*, and *Prm1* was upregulated in iPS cells by retinoic acid or testosterone induction, whereas *Oct-4* transcription was reduced in these cells compared to the controls. Hormones were also measured in the EB medium. DNA content analysis by flow cytometry revealed that iPS cells could differentiate into haploid cells through retinoic acid or testosterone treatment. Collectively, our results suggest that mouse iPS cells possess the potency to differentiate into male germ cells *in vitro* through embryoid body formation and retinoic acid or testosterone induction.

1. Introduction

Male germ cells play a critical role in transmitting genetic information to the offspring by combining with the female germ cells through the unique process of fertilization. Gametogenesis is a process in which a diploid precursor becomes a haploid germ cell. Any error at any stage of the gametogenesis process results in subfertility or infertility, which is a major public health issue affecting about 10–15% of couples [1]. As an example, azoospermia is observed in 1% of the general population and in 10–15% of infertile men [2, 3]. Furthermore, nonobstructive azoospermia, resulting from a testicular failure, affects about 10% of infertile men and is diagnosed in 60% of azoospermic men [2, 4]. However, little is known about molecular mechanisms underlying gametogenesis due to the lack of an efficient and reproducible model representing gametogenesis.

Recently, considerable progress has been made in the derivation of germ cell from embryonic stem cells (ESCs), which are regarded as a desirable experimental model for elucidating mammalian germ cell development and potential strategies for producing haploid germ cell. In mice, Hübner et al. first reported the successful derivation of gametes from mouse ESCs *in vitro* [5]. This is a significant breakthrough,

and it has important impact on the study of germ cell development. Furthermore, Nayernia et al. showed the first live offspring of mice from intracytoplasmic sperm injection (ICSI) using sperm induced from ESCs *in vitro* [6]. Mouse ESCs can be induced to generate motile and tailed sperm by ectopic expression of Dazl [7]. In human, differentiation of germ cell from human ESCs has also been demonstrated [8–14]. Undoubtedly, ESCs possess the capacity to differentiate into sperm. Generally, there are two methods to produce sperm from the ESCs *in vitro*, namely, the monolayer differentiation and the embryoid body (EB) formation [15]. EB is a three-dimensional cellular aggregate including a mixture of cells from the endoderm, the ectoderm, and the mesoderm when the ESCs are cultured in a condition without any differentiation inhibitors.

However, there are ethical problems in obtaining ESCs which results in a limited resource of ESCs. One of the exciting breakthroughs in stem cell biology is establishment of the induced pluripotent stem (iPS) cells from somatic cells by transferring pluripotent genes, including *Oct4*, *Sox2*, *c-Myc*, and *Klf4* [16]. Notably, iPS cells have advantages over ESCs in the following aspects: (1) there is no ethical issue using human somatic cells; (2) the source of human somatic cell is abundant. The iPS cells can give rise to all types of cells including the germ cells [16]. Significantly, iPS cells were able to generate viable, live-born offspring through tetraploid complementation [17], demonstrating that iPS cells also have a similar developmental pluripotency with ESCs. Mouse iPS cells could differentiate into hematopoietic precursor cells that have been used to rescue the mice with sickle cell diseases [18]. Recent studies have demonstrated the feasibility of *in vitro* differentiation systems for germ cell derivation from iPS cells. The iPS cells derived from mouse adult hepatocytes were able to be induced into primordial germ cells [19]. Park et al. also reported that human iPS cells could differentiate into primordial germ cells when cocultured with human fetal gonadal cells [20], and mouse iPS cells could differentiate into epiblast-like cells that further generate primordial germ cell-like cells by treatment with BMP4 [21]. However, it is not yet known whether iPS cells derived from fibroblast cells could spontaneously produce male germ cells or with retinoic acid (RA) treatment. Fibroblast cells could be obtained easily, and patient-derived iPS cells could be used for patient-specific therapy without immune rejection. Therefore, we explored the capability of fibroblast-derived iPS differentiation into male germ cell *in vitro* using embryoid body formation and retinoic acid/testosterone induction. Our data suggest that iPS cells can differentiate into spermatogonial stem cells and late stages of male germ cells, which could provide an ideal platform to uncover the mechanisms regulating spermatogenesis and open novel possibilities for using male germ cells derived from patient-derived iPS cells in treating male infertility in future.

2. Materials and Methods

2.1. Mouse iPS Cells and Culture. Mouse iPS cell line (Tg-GFP-miPS11.1; 40, XY; [22]) was a kind gift from Professor Ying Jin (Shanghai Jiao Tong University School of Medicine). These iPS cells originated from MEF cells by retroviral transduction of *Oct4*, *Sox2*, *c-Myc*, and *Klf4*. Mouse iPS cells were cultured with the high-glucose DMEM supplemented with 15% FBS (Thermo Scientific HyClone, South Logan, UT, USA), 0.1 mM nonessential amino acids, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 100 U/mL penicillin, 100 mg/mL streptomycin (Invitrogen, San Diego, CA, USA), and LIF (Millipore, Billerica, MA, USA), on feeder cells in gelatinized dishes. The cells were passaged every 2-3 days from passages 16 to 30.

2.2. Spontaneous Differentiation of iPS Cells and Retinoic Acid or Testosterone Induction. After being digested with trypsin-EDTA, $\sim 1 \times 10^6$ iPS cells were transferred to 10 cm Petri dishes containing 10 mL of the above culture medium without LIF. EBs were formed using the handing drop method and maintained in suspension. At day 3, half of the medium was changed every 2 days. In addition, 2 μM retinoic acid (RA, Sigma, St. Louis, MO, USA) or 1 μM testosterone (Sigma) or 2 μM RA and 1 μM testosterone combination was added into culture medium at day 5. Forty-eight hours later, the medium was changed, and the cells were cultured for two weeks.

2.3. RNA Isolation, cDNA Synthesis, and Quantitative PCR. The iPS cells and EBs (at days 0, 4, 7, and 14) were digested with trypsin and collected by centrifugation. Total RNA was extracted using Trizol (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. cDNA was generated from 2 μg of total RNA using random hexamer primers under standard conditions (Promega Biotech Co., San Luis Obispo, CA, USA). For quantitative PCR (qPCR), SYBR Green master mix (Life Technologies Corporation, Carlsbad, CA, USA) was added to each well of the PCR plate (10 μL of SYBR Green, 6 μL of water, 2 μL of primers, and 2 μL of cDNA), according to the following procedure: 40 cycles at 95°C for 30 s, 60°C for 45 s, and 72°C for 60 s. Samples were run in triplicates. qPCR data and relative quantification were analyzed by Graphpad Prism5 for windows. According to the delta-Ct method, the threshold of cycle values was normalized against the threshold value of mouse housekeeping gene *Gapdh*. The list of gene primer for the selected genes was from the [23] and shown in Table 1.

2.4. Western Blots. Total proteins were extracted from EBs using the RIPA lysis buffer supplemented with a mixture of PMSF, sodium orthovanadate, and protease inhibitors (Promega Biotech Co.). After 30 min lysis on ice, cell lysates were cleared by centrifugation at 12,000 rpm, and the concentration of protein was measured by the Bio-Rad Bradford assay with BCA as the standard. For Western blots, 30 μg of cell lysate from each sample was used for SDS-PAGE and transferred to nitrocellulose membranes. The membrane was probed with rabbit polyclonal to the MVH antibody (Catalog : ab13840, 1 : 100 dilutions, Abcam, Cambridge, CB, UN), and goat anti-mouse secondary antibodies were used. The antibody-antigen complexes in the membranes were visualized using an enhanced-chemiluminescent detection kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Table 1: Primers for q-PCR analysis of male germ cell markers.

Gene	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
Oct-4	CCCTCTGTTCCCGTCACTG	ACCTCCCTTGCCTTGGCT
Dppa3	TGTCGGTGCTGAAAGACCCTAT	TTTCCTTCGAGCCTTTTTTGTC
Piwil2	TGACCTGTGCATCCCCTTCT	TCCCCACAAGCTTCATATCCA
Tex14	GCGTATCGCAGTCGGCA	CCATGTGCAGCACTGGGA
Stra8	GTTTCCTGCGTGTTCCACAAG	CACCCGAGGCTCAAGCTTC
Dazl	AATGTTCAGTTCATGATGCTGCTC	TGTATGCTTCGGTCCACAGACT
Scp1	CGCTACAACCACATGCTTCG	GGAACGCTGCTTAGATCTCCTC
Scp3	ATGCTTCGAGGGTGTGGG	TTCCACCAGGCACCATCTTT
Msy2	CACCAAGGAGGATGTCTTTGTTC	CCAACACTCCGCAGAAACTTC
Odf2	CTGCCTTGTTAAGGTGTTGATGTC	TCATGGCCTTGAAGGATACCA
ACT	GTGTGCAGCCTGCACCAA	ACTGGCGGTCTTGAAAGCA
Akap3	ACGCCACTTTGACTTTGTAACCA	AAGACACCAATAAGGCTCATTCG
Prm1	AGGTGTAAAAAATACTAGATGCACAGAATAG	TTCAAGATGTGGCGAGATGCT
Gapdh	AGA ACATCATCCCTGCATCC	CACATTGGGGGTAGGAACAC

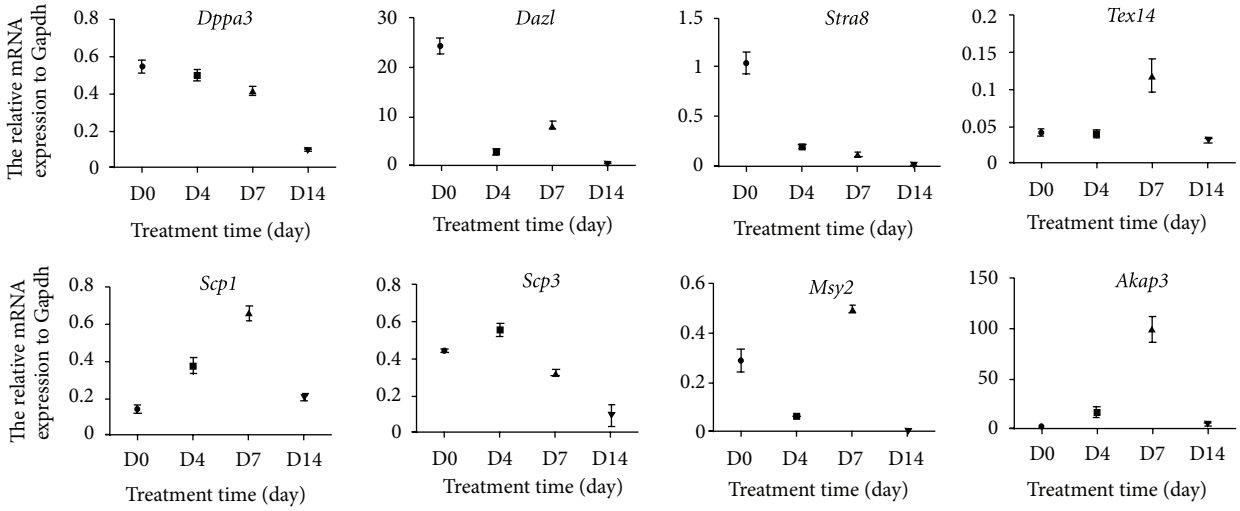

Figure 1: The expression profiles of germ cell-associated genes during EB formation from iPS cells. Real-time PCR was performed using cDNA from EB cultures maintained for 0, 4, 7, and 14 days (D0, D4, D7, and D14) to detect the relative mRNA expression of *Dppa3*, *Stra8*, *Tex14*, *Dazl*, *Scp1*, *Scp3*, *Msy2*, and *Akap3*. The mean normalized expression of each gene relative to that of *Gapdh* was shown.

2.5. Immunofluorescence Staining. EBs derived from iPS cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.4% Triton X-100 in PBS. After blocking, the primary antibodies against MVH (abcam), CDH1 (LifeSpan Biosciences Inc., Seattle, WA, USA), and SCP3 (abcam) were diluted at 1 : 100 and incubated for 2 hours at room temperature before staining with PE-conjugated secondary antibodies. Slides were examined under confocal fluorescence microscopy (Leica Microsystems CMS GmbH, Mannheim, Germany).

2.6. Measurement of Estradiol, Testosterone, and Gonadotropin from EB Medium. Three batches of EBs were cultured with EB medium. Half of medium was changed every 2 days. About 3 mL of the medium was collected after 4, 7, and 10 days of culture and stored at −20°C. The fresh EB medium was used as the control. The concentrations of testosterone, estradiol, and gonadotropin were determined by radioimmunoassay (RIA, courtesy of Dr. Kejia Gao, Department of Nuclear medicine, Central Hospital of Huangpu District, Shanghai, China).

2.7. Fluorescence-Activated Cell Sorting (FACS) Analysis. The iPS cell-derived EBs were digested by 0.25% trypsin-EDTA (Gibco) to obtain single iPS cell suspension. Cells were incubated with PE anti-mouse SSEA1 (Biolegend, San Diego, CA, USA) for 1 hour at room temperature. The cells were washed twice with PBS and analyzed with an FACS Calibur system (BD, Franklin Lakes, NJ, USA). The cells without primary antibody but with mouse IgG conjugated to PE were used as controls.

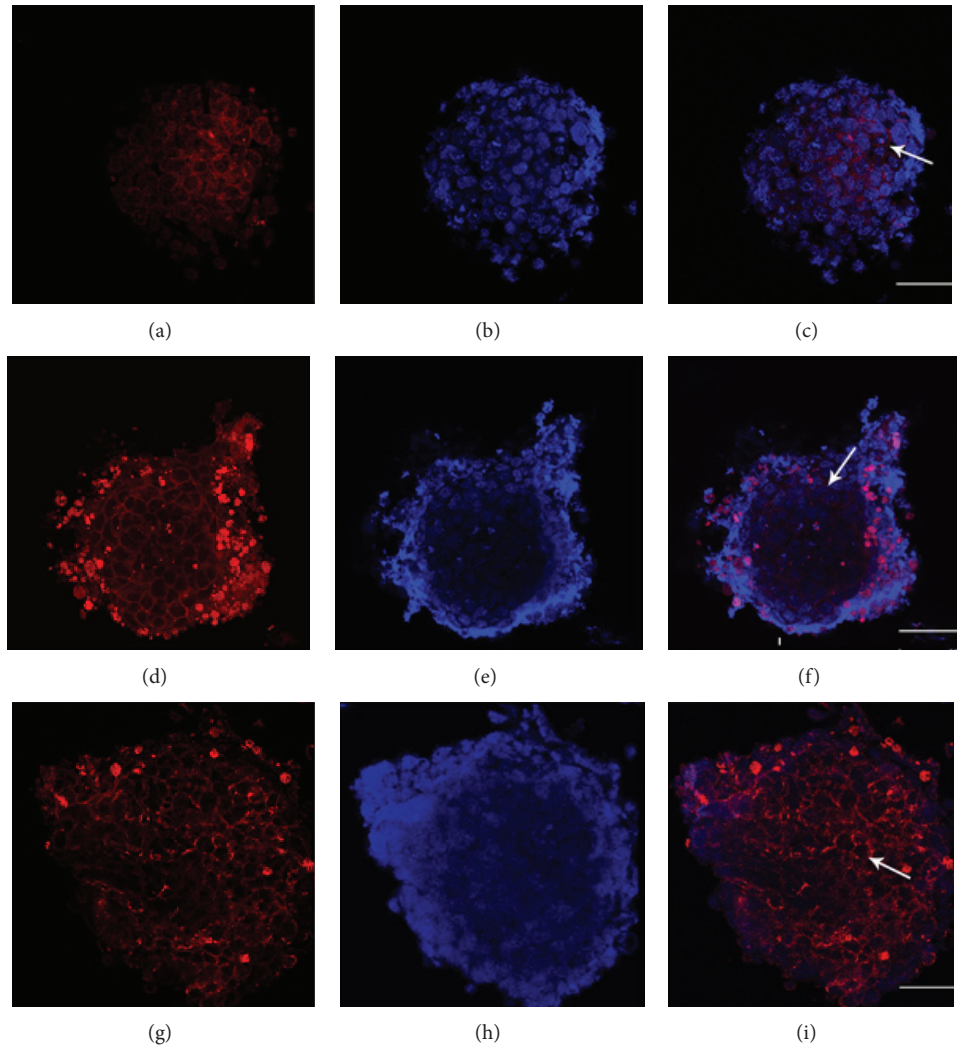

Figure 2: Expression of male germ cell markers in EBs derived from iPS cells. Expression of MVH (a), CDH1 (d), and SCP3 (g) in the EBs after cultured for 4–7 days was detected by immunofluorescent staining. DAPI (b, e, h) was used to indicate cell nuclei, and merged pictures (c, f, i) were shown. Arrows indicated the cells that were positive for MVH, CDH1, or SCP3. Scale bars = 50 μm.

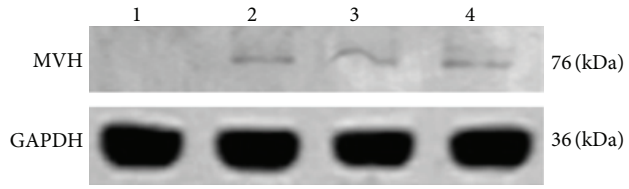

Figure 3: Western blot analysis of MVH (VASA) expression in iPS cells and EBs derived from iPS cells. Protein lysates from iPS cells (1), 4-day EBs (2), 7-day EBs (3) and 10-day EBs (4) were blotted and stained by MVH antibody.

2.8. DNA Content Analysis by Flow Cytometry. The cells derived from iPS cells were collected and fixed with 70% ethanol for 1 hour at room temperature. These cells were incubated with a staining solution 0.1% Triton X-100, 0.2 mg/mL of RNase A and 0.02 mg/mL of propidium iodide (Invitrogen) for 15 min at 37°C. The cell was resuspended, and the DNA profile was analyzed on a Becton-Dickinson FACS Calibur (BD) according to procedure as described previously [8].

2.9. Statistical Analysis. All values were presented as mean ± SEM from three independent experiments, and statistically significant differences ($P < 0.05$) were determined among various groups by ANOVA and Tukey posttest using SPSS 12.0 statistical software.

3. Results

3.1. Expression of Germ Cell-Associated Genes during Spontaneous Differentiation of iPS Cells into EBs. A total of 8 genes were analyzed during EB formation. Among them, *Dppa3* (also called *Stella*) is a marker for cell pluripotency, and *Dazl* (deleted in azoospermia-like) and *Tex14* (testis expressed 14) represent the state of premeiotic stage of the cells [23]. *Scp1* (synaptonemal complex protein 1) and *Scp3* (synaptonemal

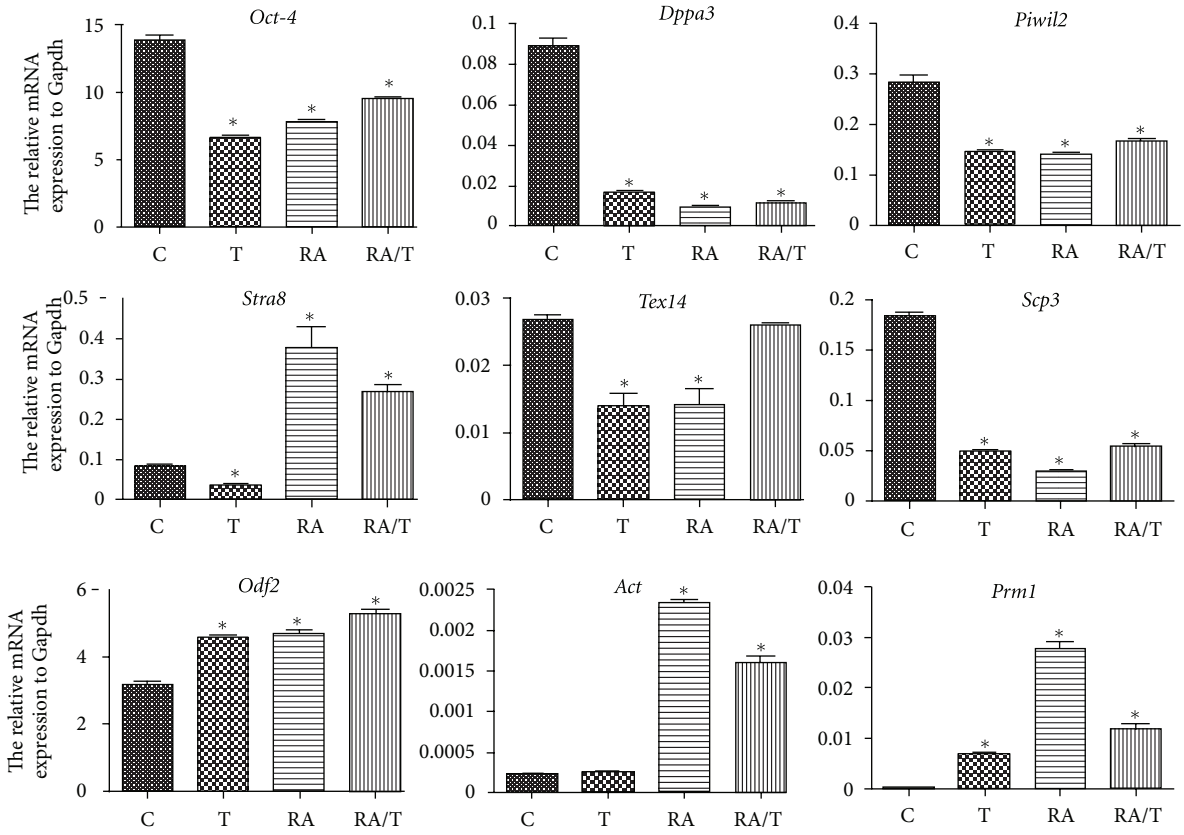

Figure 4: The expression profiles of germ cell-associated genes in iPS cells treated with RA or testosterone *in vitro*. qPCR was carried out using the cDNA of iPS cells in the presence or absence of RA and testosterone. The mean normalized expression of each gene relative to that of *Gapdh* was shown along the *y*-axis. C: the control group without retinoic acid or testosterone induction. T: the testosterone induction group. RA: the retinoic acid induction group. RA/T: the retinoic acid and testosterone induction group. Note: *indicated statistically significant differences ($P < 0.05$) in the mRNA expression between the RA- or testosterone-treated groups and the control.

complex protein 3) are markers of meiosis [15], while *Msy2* (also called Ybx2, Y box protein 2) and *Akap3* (A kinase anchor protein 3) are only expressed in the haploid germ cells [23]. *Stra8* (stimulated by retinoic acid gene) represents the ability of the cells' responding to retinoic acid which is accumulated in the premeiotic germ cells [15]. The expression patterns of these 8 genes were summarized in Figure 1. We found that, during EB formation, the expression of *Dppa3* and *Stra8* was decreased dramatically. In contrast, expression of *Scp1*, *Scp3* and transcripts of *Akap3* and *Msy2* were enhanced from day 4 to day 7 of EB formation. These results suggest that mouse iPS cells possess the potency differentiated into haploid male germ cells *in vitro* at day 7 after EB formation phenotypically.

3.2. Expression of the Germ Cell-Associated Proteins during Spontaneous Differentiation of iPS Cells into EBs. The expression of male germ cell-associated proteins (MVH, CDH1, and SCP3) was also analyzed during EB formation from iPS cells by immunofluorescence. MVH, also namely VASA, is encoded by *Ddx4* for a DEAD box polypeptide 4, and it is a marker for male germ cells. CDH1 is encoded by Cdh1 for e-cadherin, and it has been regarded as a maker for spermatogonial stem cells in mice. SCP3 is the product of *Scp3* which is synaptonemal complex protein 3. As shown in Figure 2, we revealed that the expression of male germ cell marker MVH, spermatogonial stem cell marker CDH1, and synapsis marker SCP3 was expressed in the EBs after cultured for 4–7 days. These data indicate that the iPS cells we used possess the potential to differentiate into male germ cells, spermatogonial stem cells, and spermatocytes. We further detected MVH expression of iPS cells and EBs at day 4, day 7, and day 10 by Western blots. As shown in Figure 3, we found that expression of MVH started at day 4 of EB formation and was maintained for 10 days.

3.3. Measurement of Estradiol, Testosterone, and Gonadotropin from EB Medium. We determined the concentrations of estradiol, testosterone, and gonadotropin of EB medium using radioimmunoassay. We found that level of estradiol was increased to 317 ± 57 pmol/mL in EB culture for 10 days (50% replacement of medium every 2 days; 7.5 mL medium per flask), while the estradiol was not detected in control medium. Neither testosterone nor chorionic gonadotropin was detected in culture or control medium. These results suggest that estradiol plays a role in the differentiation of PS cells into male germ cells.

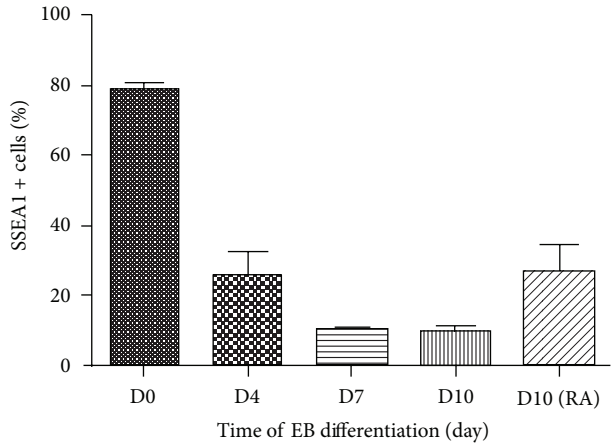

Figure 5: The percentage of SSEA1-positive cells during EB differentiation and RA induction. D0: the percentage of SSEA1-positive cells in iPS cell clones. D4: the percentage of SSEA1-positive cells in day 4 EBs. D7: the percentage of SSEA1-positive cells in day 7 EBs. D10: the percentage of SSEA1-positive cells in day 10 EBs. D10 (RA): the percentage of SSEA1-positive cells after RA induction.

3.4. Effect of RA/Testosterone on iPS Cell Differentiation towards Male Germ Cells. We further revealed that the expression of *Oct-4*, a marker for iPS cells, was significantly decreased by RA or testosterone induction (Figure 4). Conversely, the expression of *Stra8* was significantly increased by the addition of RA. Notably, the transcripts of haploid cell markers *Odf2*, *Act*, and *Prm1* [15] were enhanced significantly under RA or testosterone stimulation. There is a decrease for the expression of *Tex14* and *Scp3* in iPS cells in response to RA or testosterone alone. However, RA and testosterone combination induced an increase of *Tex14* expression, suggesting that RA and testosterone induce the differentiation of iPS cells into premeiotic male germ cells. SSEA is a marker for primordial germ cells, and interestingly we revealed that the percentage of SSEA1-positive cells was increased from 9% to 26% through RA treatment (Figure 5). Furthermore, flow cytometry showed that 2–8% of the cells were haploid cells after RA or testosterone induction (Figure 6). Collectively, these results indicate that iPS cells could differentiate into haploid male germ cells with RA or testosterone stimulation combined with EB formation.

4. Discussion

The iPS cells can be obtained through the introduction of defined factors into somatic cells. These cells are thought to resemble ESCs based on global gene expression analyses and possess the potential to differentiate into germ cells when placed in the proper environment *in vivo* [17, 24, 25]. However, few studies have tested the iPS cells' ability and efficiency in differentiation towards germ cells *in vitro*. Imamura et al. reported that iPS cells derived from mouse adult hepatocytes were able to be induced into primordial germ cells [19]. However, it is hard for the surgeons to obtain the hepatocytes of patients. Notably, fibroblasts are easy to obtain, and thus differentiation of iPS cells derived from fibroblasts into male germ cells is more convenient to patient-specific therapy. Hayashi et al. showed that mouse iPS could reconstitute the mouse germ cell specification pathway [21]. The iPS cell lines were generated by different somatic cells [26], and not all iPS cell clones can contribute to the germline [25, 27]. In the current study, two iPS cell lines (Tg-GFP-miPS11.1 and Tg-GFP-miPS4.1) were applied, and only the Tg-GFP-miPS11.1 iPS line showed the potential of differentiation into male germ cells. Thus, it is essential to select proper iPS cell line(s) with proven potential of contribution to germ cells.

Previous studies have demonstrated the upregulation of late meiotic germ cell markers in ESCs by differentiation induction [7, 15, 23, 28]. Our q-PCR data demonstrated that there were downregulation of *Dppa3* and upregulation of premeiotic germ cell marker (*stra8*), meiotic germ cell markers (*Scp1*, *Scp3*, and *Msy2*), and haploid cell marker (*Akap3*, *Act*, *Odf2*, and *Prm1*) [15] when iPS cells are differentiated. During iPS cell spontaneous differentiation period, the expression profiles of *Scp1* and *Scp3* were not identical, and they reached peak at different time point. The expression profile of *Scp3* was consistent with Clark et al.'s findings [10], but Silva et al. reported that *Scp1* and *Scp3* had opposite expression profiles [23]. Furthermore, Lin et al. reported that *Dazl* has an obligatory function upstream of *Stra8* expression [29], and thus, there are similar expression patterns of *Stra8* with that of *Dazl* during EB formation.

In this study, we revealed that both in the spontaneous and RA-induced differentiation, the iPS cells could enter different germ differentiation stages. We found that during 4–7 days of EB formation, some cells in the middle of EB expressed MVH (a germ cell marker), whereas iPS cell clones did not express MVH, which is similar to ESCs [30]. Some of the differentiated cells expressed CDH1, a specific spermatogonial stem cell protein [31], and other cells are positive for SCP3, a special marker for meiosis. Previous studies have shown that MVH is a specific marker for male germ cells from E10.5 to E17.5 [32] and from spermatogonia to the post-meiotic stage [19, 30]. MVH is considered as the most reliable germ cell-specific maker. Furthermore, Eguizabal et al. demonstrated that RA could promote the complete meiosis from human iPS cells, and the iPS-derived germ cells express MVH (VASA) [33]. Meiotic spreads were classified as punctate or elongated SCP3 staining patterns, corresponding to the early leptotene stage (punctate) and the later zygotene, pachytene, and diplotene stages (elongated) of meiotic prophase I [34]. In this study, most of cells were positive for SCP3, which indicates that these cells were the elongated male germ cells. This was verified by our q-PCR data that expression of *Tex14*, *Scp1*, *Akap3*, and *Msy2* reached peak at day 7 of EB formation. Qin et al. also proved that germ cell-associated markers were expressed in EBs from day 3 [35], and Toyooka et al. reported that about $0.4 \pm 0.2\%$ of the EB cells were MVH positive from day 5 to day 7 during spontaneous differentiation period [30]. All these data indicate that iPS cells could differentiate into male germ cells, and from day 4 to day 7 is the best period to add the inducer for iPS cell induction.

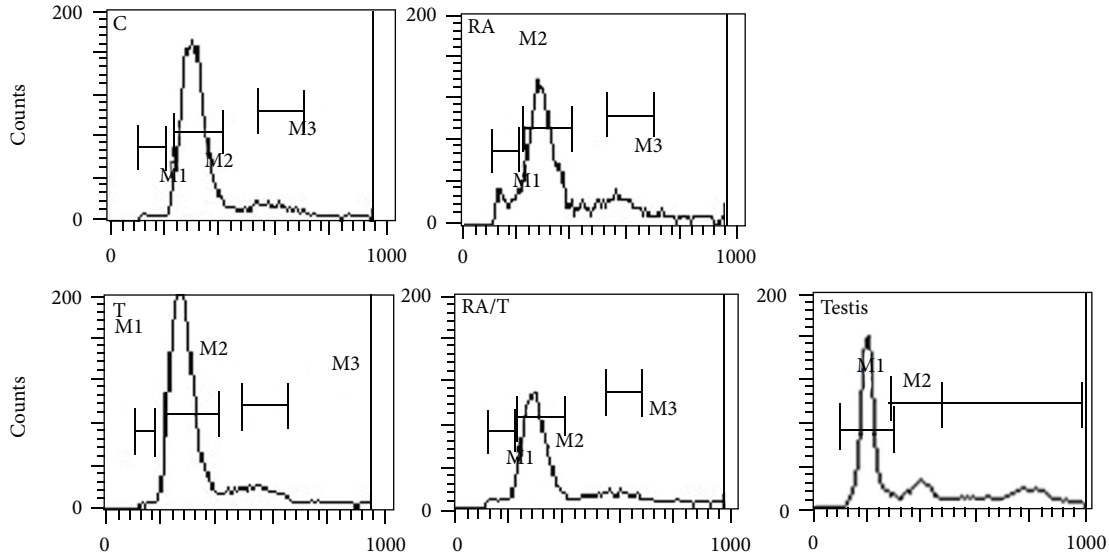

Figure 6: Flow cytometry showed DNA content in EBs derived from iPS cells with RA or testosterone *in vitro*. EBs were dispersed as single cells and stained with the DNA-binding dye Hoechst 33342 to reveal DNA content, and the cell debris was removed by setting the gates. M1, haploid cells; M2, diploid cells; M3, tetraploid cells. C: control group, M1 (1.15%), M2 (77.72%), and M3 (9.31%); RA: RA induction group, M1 (8.31%), M2 (61.69%), and M3 (12.98%); T: testosterone induction group, M1 (0.24%), M2 (81.27%), and M3 (10.17%); RA/T: RA and testosterone induction group, M1 (2.56%), M2 (74.49%), and M3 (8.86%); testis: testicular cells, M1 (59.67%), M2 (15.07%), and M3 (25.68%).

Significant efforts by numerous labs worldwide have established the roles for RA, SCF/kit, and BMPs to induce stem cells into germ cells [36]. RA can promote the progression of spermatocytes through early stages of meiosis and iPS cells towards primordial germ cells [20, 24, 25, 33], and thus, it was chosen as the inducing agent in this study. RA is a small and polar molecule that easily diffuses through tissues and acts by binding to nuclear RA receptors (RARs), which heterodimerize with nuclear retinoid X receptors (RXR) [37, 38]. RAR-RXR dimers bind to RA-response elements (RAREs) and thereby control the expression of RA-responsive genes [37]. It has been shown that exposure to RA controls whether mouse fetal germ cells enter meiosis or not [39, 40]. In our study, the final RA concentration is 2 μM, which was consistent with Silva et al.'s study [23]. RA could be applied to select SSEA1-positive germ cells from the EBs [15]. In our study, the percentage of SSEA1-positive cells was increased from 9% to 26% after 48 hours of RA treatment.

Testosterone secreted by the Leydig cells is required for spermatogenesis *in vivo*, and it acts on Sertoli cells to stimulate gene transcription and produce growth factors that promote germ cell differentiation [41]. Testosterone was not detectable above background in our study, but this hormone can be aromatized to estradiol, which is crucial for spermatogenesis. Level of estradiol was increased in medium to 317 ± 57 pmol/mL in EB culture for 10 days. Silva et al. reported that a combination of RA and testosterone could induce mouse ESCs into germ cells [23], and thus, testosterone was applied to induce iPS cells into germ cells. Testosterone significantly decreases the expression of iPS cell marker *Oct-4*, whereas the expression of haploid cell markers *Odf2*, *Act*, and *Prm1* was increased significantly. Testosterone may act on Sertoli cells to produce growth factors including stem cell factor (SCF) that is required for differentiation of spermatogonial stem cells into male germ cells [41].

Since *Stra8* is the target gene of RA, the expression of *Stra8* is suppressed in absence of RA, and with RA addition, *Stra8* is successfully stimulated in the study. Furthermore, the pluripotent stem cell marker *Oct-4* is suppressed. However, the response of premeiotic and the postmeiotic markers with RA stimulation is different. The postmeiotic markers (*Odf2*, *Act*, and *Prm1*) [6, 15] are stimulated markedly. It is suggested that RA exerts effects via targeting *Stra8* to promote the haploid germ cell formation during EB formation. Recent study has demonstrated that the iPS cells could be induced into primordial germ cells. However, there is so far not a perfect stimulation protocol for iPS cells towards functional gametes *in vitro* available. In the current study, about 2–8% of the EB cells were haploid cells after RA or testosterone induction, while West et al. reported that about 12% of the cells were haploid cells [15].

In summary, we have demonstrated that mouse iPS cells could differentiate into male germ cells, even haploid cells through EB formation and RA or testosterone induction. The ability to generate male germ cells from human iPS cells derived from fibroblasts provides an excellent paradigm for elucidating the mechanism of gametogenesis as well as for developing new approaches for treating male infertility.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

P. Li and H. Hu are contributed equally to this work.

Acknowledgments

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Research Article

Direct Effect of Chenodeoxycholic Acid on Differentiation of Mouse Embryonic Stem Cells Cultured under Feeder-Free Culture Conditions

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Chenodeoxycholic acid (CDCA), a farnesoid X receptor (FXR) ligand, is a member of the nuclear receptor family and is probably involved in regulating the cellular activities of embryonic stem (ES) cells. Recently, although it was reported that the FXR ligand can mediate differentiation, apoptosis, and/or growth arrest in several cell types, it is still not well known how CDCA mediates effects in ES cells. Therefore, we investigated the direct effect of CDCA on mES cells. Feeder-free mES cells were treated in a dose-dependent manner with CDCA (50, 100, and 200 μM) for 72 h, and then a 100 μM CDCA treatment was performed for an additional 72 h. We analyzed the morphology, cell growth, cell characteristics, immunocytochemistry, and RT-PCR. In CDCA-treated cells, we observed the disappearance of pluripotent stem cell markers including alkaline phosphatase, Oct4, and Nanog and a time- and dose-dependent increase in expression of nestin, PAX6, and α-smooth muscle actin, but not α-fetoprotein. The 100 μM CDCA-treated cells in their second passage continued this differentiation pattern similar to those in the controls. In conclusion, these results suggest that CDCA can guide mES cells by an FXR-independent pathway to differentiate into ectoderm and/or mesoderm, but not endoderm.

1. Introduction

Since the establishment of embryonic stem (ES) cell lines [1, 2], it has been known that ES cells have the capacity for self-renewal and pluripotency, with the ability to differentiate into multiple cell types *in vitro* and *in vivo*. These characteristics of ES cells make them a valuable model system for differentiation study and cell-based regeneration therapies.

Numerous reports have documented the differentiation of ES cells into specific cell types, such as neurons [3], cardiomyocytes [4], adipocytes [5], endothelial cells [6], hepatocytes [7], keratinocytes [8], and pancreatic cells [9] under the appropriate culture conditions. So far, ES cell differentiation required the formation of an embryoid body (EB) in most studies in general. However, alternative approaches have shown directed differentiation of ES cells into a desired lineage without going through EB formation [10, 11]. There are some problems in ES cell differentiation through EB formation. It may lead to uncontrollable complexity and to unwanted cell types [12], and some of the cells of the EB might not be terminally differentiated [10].

The farnesoid X receptor (FXR, NR1H4), meanwhile, may modulate the differentiation into myocyte [13] during myogenesis of tissue-specific stem cells. Therefore, the

differentiated cell population tends to be directed more uniform, and a larger number of precursors and more differentiated cells can be obtained using this pathway. The FXR, a member of the nuclear receptor superfamily, is highly expressed in liver, intestine, and kidney tissues [14]. FXR is known to be a key player in the control of multiple metabolic pathways including bile acid biosynthesis from cholesterol and lipid/glucose metabolism [15, 16]. In liver, especially, activated FXR induces liver regeneration by a homeostatic mechanism [17] and affects vascular remodeling [18]. In the intestine, it protects the tissue from bacterial-induced mucosal injury by bile acids [19]. It is also known that the FXR activators inhibit cell proliferation, trigger differentiation, and induce apoptosis. Bile acids reduce the growth of keratinocytes, human fibroblasts, and smooth muscle cells [20–22]. Additionally, activated FXR plays a critical role in regulating adipogenesis [23] and also induces apoptosis in cancer cells [24]. However, studies on the effects of activated FXR on proliferation or differentiation of ES cells are scarce.

Chenodeoxycholic acid (CDCA, 3α, 7α-dihydroxy-5β-cholanic acid) is a primary bile acid directly synthesized from cholesterol. It was shown to be the most potent activator of the FXR [25, 26]. It binds directly to FXR, which then regulates several known FXR target genes and induces bile acid-binding protein for bile acid transport [27]. Therefore, CDCA is not simply metabolic products but also regulates involving gene transcription and signaling of transduction pathway. Moreover, CDCA is involved in many cellular activities including cell proliferation, differentiation, and apoptosis [23, 28].

In this paper, we investigated the effects of the FXR ligand and CDCA on the differentiation of mouse ES (mES) cells without a feeder layer or EB formation. To examine whether CDCA mediates differentiation through FXR signaling, we checked the mRNA expression of FXR in CDCA-induced and differentiated mES cells. Additionally, direct differentiation was performed in the presence of LIF in order to determine the relationship between LIF signaling and CDCA-mediated cellular activities.

2. Materials and Methods

2.1. Mouse ES Cell Cultures. The E14TG2a (ATCC number CRL-1821) mouse ES (mES) cell line was routinely cultured on feeder layers of primary mouse embryonic fibroblasts (MEF) pretreated with mitomycin C. The cells were maintained with Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, Republic of Korea) supplemented with 15% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich Corp, St. Louis, MO, USA), 1% nonessential amino acids (NEAA; Gibco-Invitrogen, Carlsbad, CA, USA), 1% (v/v) penicillin-streptomycin (Gibco-Invitrogen), and 1,000 U/mL of recombinant mouse leukemia inhibitory factor (LIF; Chemicon, Temecula, CA, USA) in 5% CO_2 at 37°C. The mES cells were subcultured onto new feeders every 3 or 4 days using 0.05% trypsin-EDTA (Gibco-Invitrogen).

2.2. Treatment of mES Cells with Chenodeoxycholic Acid (CDCA). For direct differentiation of mES cells by CDCA, feeder cells were removed by plating on nongelatin coated dish for 1 h, which allowed the feeder cells to adhere, while most of the mES cells stayed in the suspension. The suspended mES cells were once transferred onto a new 0.1% gelatin-coated dish for propagation in the presence of 1,000 U/mL of LIF. The feeder-free mES cells were subcultured after 24 h. Then the cells were incubated under different conditions for 72 h. The cells were incubated in (i) basal medium (spontaneously differentiated control), (ii) basal medium supplemented with 1,000 U/mL of LIF (undifferentiated control), and (iii) basal medium supplemented with 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich Corp) in the absence of LIF (solvent control). Treated groups were cultured in basal medium supplemented with (iv) 50 μM, (v) 100 μM, and (vi) 200 μM of CDCA. After the treatment for 72 h, the control groups and 100 μM CDCA-treated cells were subcultured using 0.05% trypsin-EDTA (Gibco-Invitrogen) on new gelatinized dishes and treated with 100 μM CDCA for additional 72 h.

The cells were incubated in basal medium, basal medium supplemented with 1,000 U/mL of LIF, and basal medium supplemented with 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich) as a control. Treated groups were cultured in basal medium supplemented with 50, 100, and 200 μM of chenodeoxycholic acid (CDCA, Sigma-Aldrich) for 72 h. Then, the 100 μM CDCA-treated cells were subcultured and treated with 100 μM CDCA for an additional 72 h. The cell number was counted by trypan blue dye exclusion. The experiment was repeated three times.

2.3. Cell Viability Assay. Cell viability was assessed by a tetrazolium salt (WST-1)-based colorimetric assay. A commercial WST-1 kit (EZ CyToX; Daeil Lab, Seoul, Republic of Korea) was used. The absorbance was measured at 450 nm Bio-Rad microplate reader Model-550 (Bio-Rad, Hercules, CA, USA).

2.4. Alkaline Phosphatase Activity. The cells were fixed with a 4% paraformaldehyde solution and stained with Naphthol/Fast Red Violet Solution (Mix Fast Red Violet (FRV) with Naphthol AS-BI phosphate solution and water in a 2 : 1 : 1 ratio) at room temperature in the dark for 15 min. Later, the cells were rinsed with PBS and observed under a phase contrast microscope.

2.5. Immunocytochemistry. To detect pluripotent stem cell markers and three-germ layer-specific marker antigens, the cells were fixed in culture dishes with 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.01 M, pH 7.4) for 15 min. Endogenous peroxidase activity was blocked by hydrogen peroxide for 30 min at RT after a PBS wash. The cells were permeabilized with 0.1% Triton X-100 for 10 min and incubated with normal goat serum (Jackson Immunoresearch Laboratory, West Grove, PA) for 60 min to block nonspecific binding sites. The cells were incubated with the primary antibodies overnight at 4°C. The primary antibodies were Oct4 (Santa Cruz Biotechnology, Santa Cruz,

Table 1: Primer sequences and cycling conditions used for RT-PCR.

Gene	Primer sequence	Annealing temperature	Product size
Oct4	Forward 5′-GAAGCCCTCCCTACAGCAGA-3′ Reverse 5′-CAGAGCAGTGACGGGAACAG-3′	60°C	297 bp
Nanog	Forward 5′-CCCCACAAGCCTTGGAATTA-3′ Reverse 5′-CTCAAATCCCAGCAACCACA-3′	60°C	255 bp
Nestin	Forward 5′-TAGAGGTGCAGCAGCTGCAG-3′ Reverse 5′-AGCGATCTGACTCTGTAGAC-3′	60°C	170 bp
NCAM	Forward 5′-AGATGGTCAGTTGCTGCCAA-3′ Reverse 5′-AGAAGACGGTGTGTCTGCTT-3′	60°C	187 bp
α-SMA	Forward 5′-ACTGGGACGACATGGAAAAG-3′ Reverse 5′-CATCTCCAGAGTCCAGCACA-3′	60°C	240 bp
Desmin	Forward 5′-TGACAACCTGATAGACGACC-3′ Reverse 5′-TTAAGGAACGCGATCTCCTC-3′	60°C	180 bp
α-FP	Forward 5′-TGCACGAAAATGAGTTTGGGA-3′ Reverse 5′-TTGCAGCCAACACATCGCTA-3′	60°C	159 bp
Albumin	Forward 5′-TGCTGCTGATTTTGTTGAGG-3′ Reverse 5′-GCTCACTCACTGGGGTCTTC-3′	60°C	500 bp
FXR	Forward 5′-TTGCGACAAGTGACCTCCAC-3′ Reverse 5′-TGATGGTTGAATGTCCGGAG-3′	58°C	653 bp
GAPDH	Forward 5′-GTCATCATACTTGGCAGGTT-3′ Reverse 5′-GTCGTGGAGTCTACTGGTGT-3′	60°C	489 bp

NCAM: neural cell adhesion molecule; α-SMA: alpha-smooth muscle actin; α-FP: alpha-fetoprotein; FXR: farnesoid X receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

CA, USA) and Nanog (Abcam, UK) as pluripotent stem cell markers, nestin (Chemicon) and PAX6 (Chemicon) as ectoderm markers, α-smooth muscle actin (Sigma-Aldrich) as mesoderm marker, and α-fetoprotein (Santa Cruz) as endoderm marker. The cells were then probed with secondary antibodies (peroxidase-labeled goat anti-mouse IgG or goat anti-rabbit IgG (1 : 200); Jackson Immunoresearch Laboratory) for 1 h at RT. DAB (DAKO, Carpinteria, CA, USA) was used for visualization about 30 seconds.

2.6. Total RNA Isolation and RT-PCR. Total RNA was extracted from the cells using TRIzol (Gibco-Invitrogen) according to the manufacturer's instructions. After total RNA extraction, the cells were treated with DNAse I (Rnase free, Takara, Japan) for discarding genomic DNA contamination. The concentration and quality of isolated RNA were determined using an ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Complementary DNA was synthesized from 1 μg of total RNA using M-MLV Reverse Transcriptase Kit I (Bioneer, Daejon, Republic of Korea) with oligo-dT primers. One microliter of cDNA was used as template in the PCR reactions. Each PCR reaction mixture contained PCR buffer (2.0 mM $MgCl_2$), 2.5 mM of each dNTP, 10 pM of each mouse-specific primer sets, one unit of i-MAX II DNA Polymerase (Intron, Seoul, Republic of Korea).The primer sets and the PCR conditions are summarized in Table 1. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing 0.4 μg/mL ethidium bromide (Sigma-Aldrich Corp). Band intensities were quantified three times each by densitometry analysis using Bio1D software (Vilber Lourmat, Mame la Vallee, France).

3. Results

3.1. Morphological Changes and Viability of CDCA-Treated mES Cells. The attached feeder-free mES cells formed tightly packed colonies, same as cells on feeder layers. The nucleus shows irregularly shaped thin smooth nuclear membrane with prominent multiple nucleoli. The cytoplasm is scanty in volume and cytoplasmic molding in shape. Feeder-free mES cells still showed the characteristics of pluripotent stem cells, in terms of their alkaline phosphatase activity, Oct4, and Nanog expression (see Supplement 1 in Supplementary Material available online at doi:http://dx.doi.org/10.1155/2013/375076). This result indicates that mES cells can be maintained in an undifferentiated state in feeder-free culture conditions.

When LIF was discarded, the mES cells differentiated spontaneously. Moreover, the CDCA-treated cells showed remarkable changes. The differentiated cells showed decrease in nucleus size, abundant cytoplasm, and low nucleus/cytoplasm ratio, in a dose- and time-dependent manner. The prominent nucleoli are also decreased in size, and cytoplasmic pod extension was noted. We observed primarily this type of cells in the 50 μM CDCA-treated group and more stretched sharp-ended cytoplasmic cells in the 100 μM CDCA-treated group (Supplement 2). CDCA-treated cells became larger, flatter, and more elongated in a time-dependent manner. The high-dose CDCA-treated (200 μM)

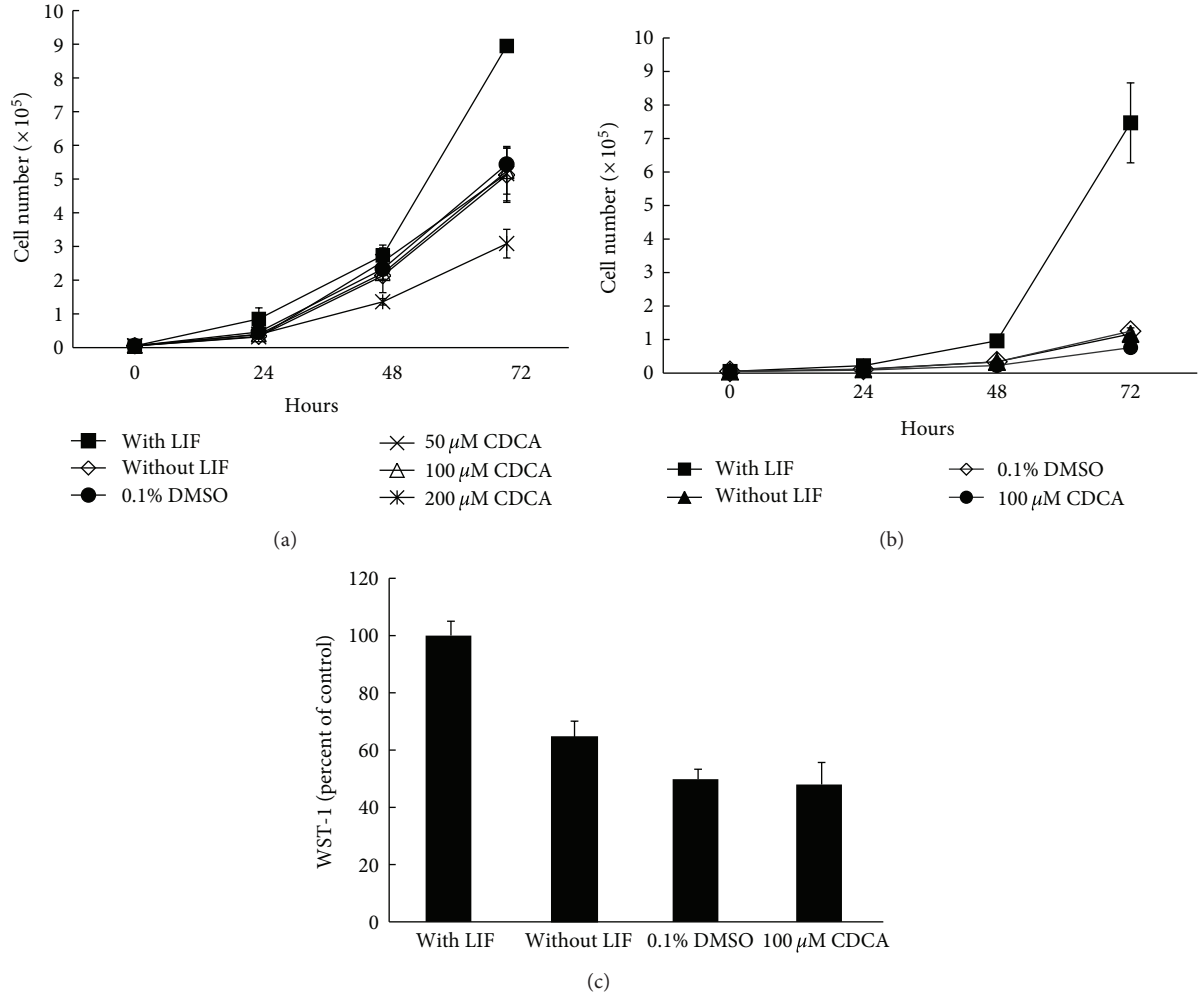

Figure 1: Growth rate and cell viability of CDCA-treated mES cells. Changes in mES cell number after CDCA treatment (a) and after the second round of treatment with 100 μM CDCA (b). The effects of 100 μM CDCA on the viability of subcultured mES cells after 72 h incubation, measured by the WST-1 assay. Data represent viability as a percentage of the control (1000 U/mL of LIF-treated cells) (c). Data are expressed as the mean ± SE ($n = 3$). LIF: leukemia inhibitory factor; DMSO: dimethyl sulfoxide; CDCA: chenodeoxycholic acid.

group shows abundant and thin cytoplasm with vacuolation, similar to the senescence phenomenon. Furthermore, the treated cells had inhibited cell proliferation and the cell number was decreased by 43.25% compared to the vehicle control (Figure 1(a)).

After subculturing, the mES cells treated with 100 μM CDCA changed their morphology according to the previously described pattern. Treatment with 100 μM CDCA reduced the cell number to the cell number seen in the negative controls (without LIF and 0.1% DMSO-treated group) compared to the positive control with LIF (Figure 1(b)). The cell viability of 100 μM CDCA-treated cells, as measured by MTT assay, was similar to that of the negative controls. These results indicate that 100 μM CDCA does not have a cytotoxic effect on mES cells grown under feeder-free conditions, even after a 2nd passage. In addition, adding LIF to the medium clearly stimulates the mES cells under feeder-free conditions (Figure 1(c)).

3.2. Characterization of CDCA-Treated mES Cells. The expression of ALP, Oct4, and Nanog was lower in LIF-withdrawn mES cells and DMSO-treated cells than in LIF-treated mES cells. CDCA treatment reduced the expression of ALP, Oct4, and Nanog in a concentration-dependent manner, even when compared to negative controls. ALP, Oct4, and Nanog expression in LIF control showed almost 100% positive cells. CDCA decreased the ALP expression about 3–5% positive cells. These phenomena were similar to Nanog expression. In contrast, decrease of Oct4 expression by CDCA was much slower than ALP and Nanog. 50 μM CDCA reduced Oct4-positive cells by 70% compared to LIF control. The decreasing effect showed CDCA dose-dependent manner (100 μM: 50%, 200 μM: 20%). Oct4 positive cells almost disappeared but still remained at the second passage of mES cells treated by 100 μM CDCA, respectively, (Figure 2). While the cells in the control groups were negative for all three germ layer markers, CDCA-treated mES cells

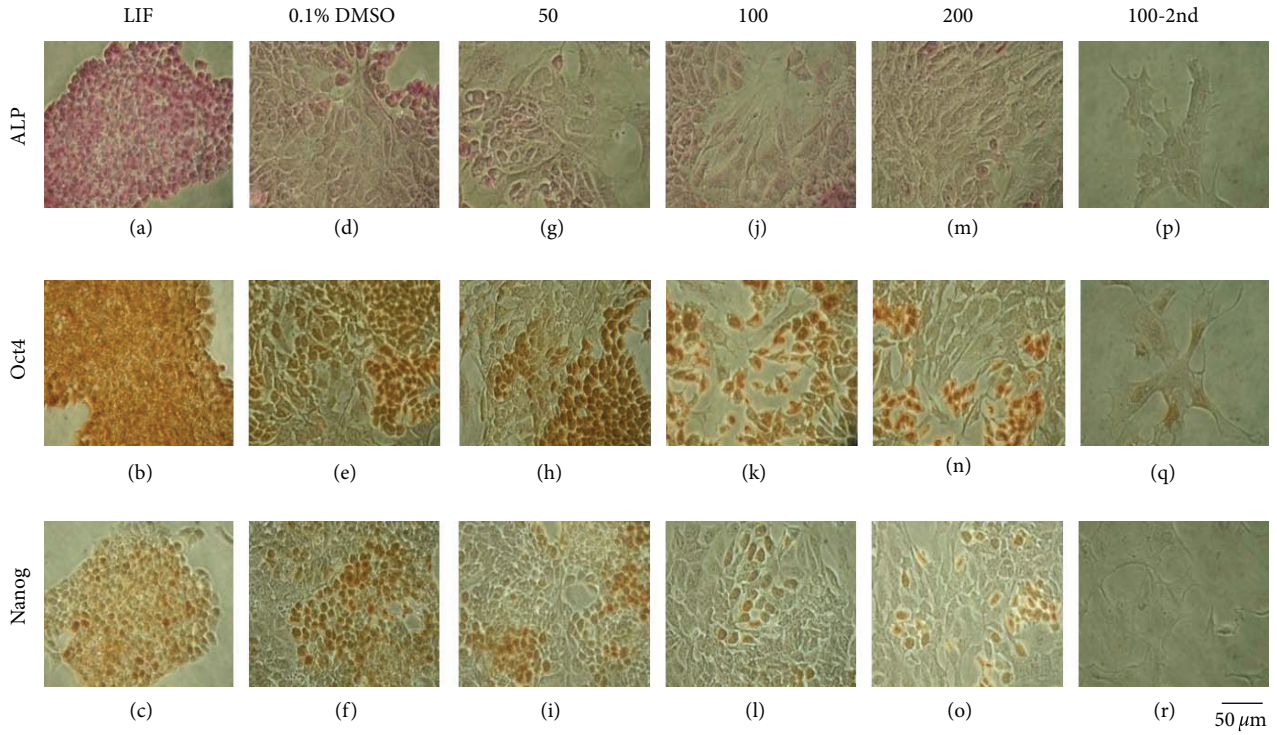

Figure 2: Changes of pluripoent markers in CDCA-treated mES cells. mES cells were cultivated with 50 μM, 100 μM, or 200 μM CDCA for 72 h. Subcultured mES cells were treated with 100 μM CDCA for another 72 h. The cells were probed with antibodies against pluripotent stem cell markers, alkaline phosphatase (ALP), Oct4, and Nanog. LIF: leukemia inhibitory factor; DMSO: dimethyl sulfoxide; CDCA: chenodeoxycholic acid

showed strong expression of nestin and α-smooth muscle actin. CDCA increased nestin expression in concentration-dependent manner (50 μM: 20%, 100 μM: 50%, 200 μM: 70%). The second passage of 100 μM CDCA showed almost 90% cells showing positive. CDCA increased Pax6 expression, but the pattern was much slower than nestin. At the second passage of 100 μM CDCA-treated mES cells showed positive cells by 50%, respectively. Subcultured mES cells were treated with 100 μM CDCA for another 72 h. CDCA induced the α-smooth muscle actin expression in lower concentration compared to nestin and Pax6. From 50 μM CDCA treatment, it showed strong expression. However, α-fetoprotein was not detected in any CDCA-treated groups (Figure 3).

The mRNA expression pattern was similar to the protein expression pattern. In the 200 μM CDCA-treated mES cells cultured for 72 h, Oct4 mRNA expression was downregulated and the phenomenon disappeared at 100 μM CDCA for the second passage (total 144 h incubation with CDCA). Nanog mRNA expression was maintained at a steady state, but it disappeared similar to Oct4 at 100 μM CDCA for the second passage. Cells treated with CDCA for 72 h expressed nestin, NCAM, α-smooth muscle actin, and desmin at 200 μM CDCA. The pattern was clearer at 100 μM CDCA for the second passage. However, α-fetoprotein and albumin were not expressed, which is consistent with the results of the immunocytochemical analysis (Figures 4(a) and 4(b)). FXR mRNA was not detected in any CDCA-treated cells or control group cells (Supplement 3).

4. Discussion

In this study, we investigated the effect of CDCA, an FXR ligand, on mES cell differentiation under feeder-free conditions. We focused on the effect of CDCA on the proliferation and differentiation of mES cells into specific lineages by analyzing the changes in mES cell characteristics, mRNA expression patterns, and cell physiology. First, we treated mES cells cultured without a feeder layer with different doses of CDCA in the culture medium for 72 h. Because 100 μM CDCA showed an effect on the differentiation of mES cells without changing cell viability, when compared to the controls, we treated the cells for an additional 72 h. The second round treatment of mES cells with CDCA resulted in ectodermal and mesodermal differentiation, but not endodermal lineage. During differentiation, the cell viability was similar to that observed for the negative controls (0.1% DMSO without LIF).

In our preliminary experiments, we changed the mES cell culture to feeder-free culture by adding 1,000 IU LIF. The cells were successfully maintained, but the margin of the colony was occasionally irregular. All of the ES cell characteristics were maintained, as previously reported elsewhere [29].

Since the regulation of the nuclear receptor, and especially FXR-dependent bile acid signaling, could contribute

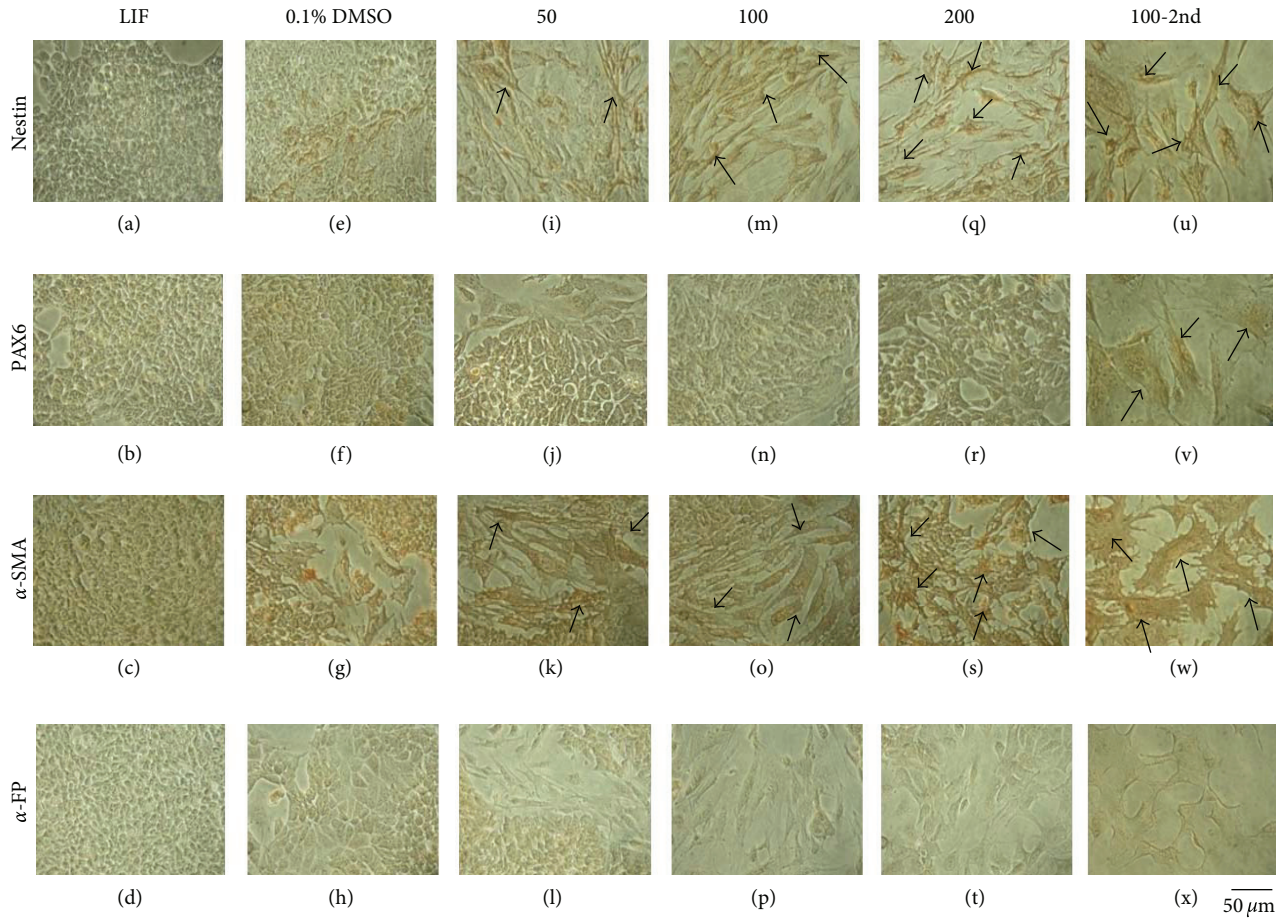

Figure 3: Characteristic changes in CDCA-treated mES cells. mES cells were cultivated with 50 μM, 100 μM, or 200 μM CDCA for 72 h. Subcultured mES cells were treated with 100 μM CDCA for another 72 h. Lineage-specific markers such as nestin and PAX6, α-smooth muscle actin (α-SMA), and α-fetoprotein (α-FP). LIF: leukemia inhibitory factor; DMSO: dimethyl sulfoxide; CDCA: chenodeoxycholic acid.

to endodermal organ and liver regeneration [17], there is a possibility that the FXR ligand, CDCA, could cause differentiation of mES cells. However, the direct differentiation of mES cells by treatment with CDCA without an EB step has not yet been studied. To determine the optimal concentration of CDCA for inducing differentiation of mES cells, we used 50, 100, and 200 uM CDCA to treat the mES cells for 72 h. In mES cells treated with 100 and 200 μM CDCA, the pluripotent signals were reduced as the cells differentiated into ectodermal and mesodermal lineages. Concomitant results were shown for mRNA expression analysis by RT-PCR, demonstrating that CDCA can induce the differentiation of mES cells. However, endodermal differentiation was not observed. Since 200 μM CDCA reduced the cell number as well as viability about 50% than 100 μM CDCA, we chose to use a 100 μM dose for the second passage. In the second round of treatment, differentiation of mES cells into ectodermal and endodermal lineages was more significant. The expression of pluripotent markers of ES cells such ALP, Oct4, and Nanog was almost absent at the protein level, and the mRNA levels of these markers were drastically reduced, while maintaining the cell viability.

Our results indicate that CDCA treatment of undifferentiated mES cells causes differentiation by a pathway other than the one involved in adult organ regeneration and does not involve the FXR receptor. CDCA is a primary bile acid synthesized directly from cholesterol in the liver and secreted via the bile into the small intestine that plays a key role in the digestion and absorption of dietary fats [30]. CDCA-activated FXR regulates expression of genes whose products are critically important for bile acid and cholesterol homeostasis in cultivated hepatocytes [31, 32] and liver slices [33]. Moreover, several studies have shown that the FXR ligand, CDCA, can induce differentiation, inhibit proliferation, and induce the apoptosis of several primary cell types including human fibroblast [21] and keratinocytes [20]. Furthermore, CDCA can regulate the differentiation of mouse preadipocytes into mature adipocytes [23] and can mediate apoptosis in vascular smooth muscle cells and breast cancer cells [22, 24].

It is not reported whether there is an endogenous expression in embryonic stage but there are several reports that human fetus in early gestation (weeks 13–19) produces CDCA [34] and porcine fetus also does in even earlier stage

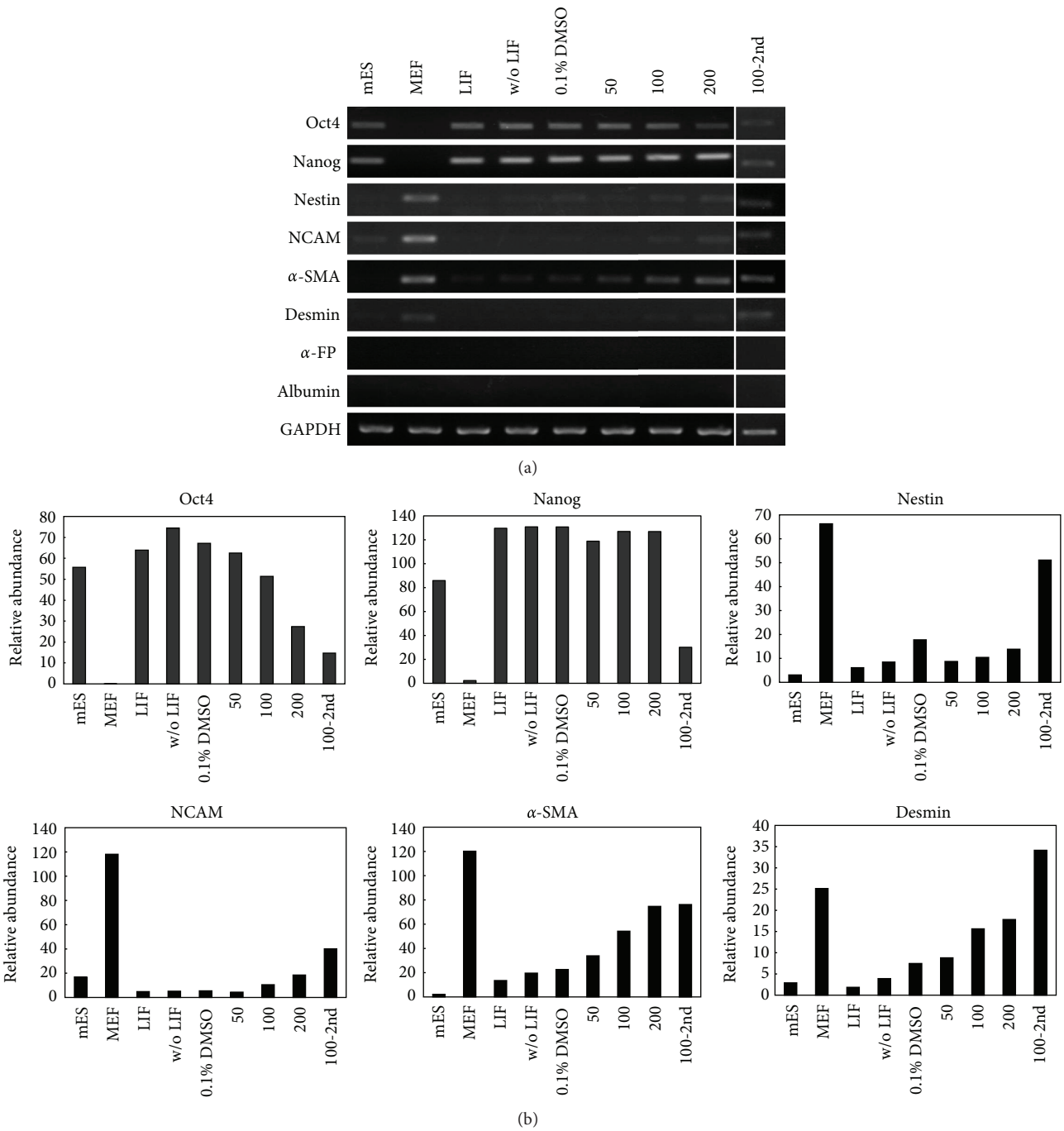

Figure 4: Characterization of CDCA-treated mES cells by RT-PCR analysis. (a) The mRNA expression of pluripotent stem cell markers and lineage specific makers were analyzed. The mES cells were cultivated with 50 μM, 100 μM, or 200 μM CDCA for 72 h. Sub-cultured mES cells were treated with 100 μM CDCA for another 72 h. MEF, mouse embryonic fibroblast, LIF, leukemia inhibitory factor; DMSO, dimethyl sulfoxide; CDCA, chenodeoxycholic acid. (b) Relative semi-quantitation of PCR signals by image analysis.

during gestation (weeks 4) [35]. Recently, FXR-deficient mice showed that FXR may control adipocyte differentiation via PPAR-γ and Wnt/β-catenin pathways [36].

There have been several reports that CDCA can mediate effects in an FXR-independent regulatory manner. CDCA can mediate the activity of PKC [37, 38], which plays a key role in the regulation of cell growth, differentiation, and apoptosis [39]. Moreover, CDCA can directly activate the growth regulatory gene, *cyclooxygenase-2* [40], and transcription factors such as *c-Fos* [41] and *activator protein-1 (AP-1)* [42], which are involved in the regulation of cell growth and differentiation. Recent reports suggest that bile acid-mediated apoptosis is dependent on death receptor signaling [43] or mitochondria dysfunction [44]. Through activation of

these diverse signaling pathways, CDCA can regulate several cellular activities.

Although the exact mechanism of CDCA-induced differentiation of mES cells has to be elucidated, we have demonstrated that CDCA directly induces the differentiation of mES cells into ectodermal and mesodermal cells in a dose-dependent manner but does not promote endodermal differentiation. It would be also necessary to investigate the CDCA-induced differentiation for longer period to prove determination of their lineages to the ectodermal/mesodermal not endodermal cells in the near future.

Furthermore, CDCA-induced differentiation of mES cells seems to be mediated by an FXR-independent mechanism. In conclusion, these results provide useful information concerning the role of CDCA in the cellular activities of mES cells. However, determination of the exact mechanisms of CDCA-mediated antiproliferation and differentiation of mES cells requires further research.

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Review Article

Cells with Stem Cell Characteristics in Somatic Compartments of the Ovary

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Antral follicular growth in the ovary is characterized by rapid expansion of granulosa cells accompanied by a rising complexity of their functionality. Within two weeks the number of human granulosa cells increases from less than 500,000 to more than 50 millions cells per follicle and differentiates into groups of cells with a variety of specialized functions involved in steroidogenesis, nursing the oocyte, and forming a functional syncitium. Both the rapid proliferation and different specialized functions of the granulosa cells can only be explained through the involvement of stem cells. However, luteinizing granulosa cells were believed to be terminally differentiated cells. Only recently, stem and progenitor cells with FSH-receptor activity were identified in populations of luteinizing granulosa cells obtained during oocyte collected for assisted reproduction. In the presence of the leukaemia-inhibiting factor (LIF), it was possible to culture a subpopulation of the luteinizing granulosa cells over prolonged time periods. Furthermore, when embedded in a matrix consisting of collagen type I, these cells continued to express the FSH receptor over prolonged time periods, developed globular formations that surrogated as follicle-like structures, providing a promising tool for reproductive biology.

1. The Dynamics of Ovarian Follicular Growth and Development

Ovarian follicular development is initiated from a pool of inactive primordial follicles. Each follicle contains a small nongrowing oocyte and a single layer of nondividing cells encapsulated by the follicular basal lamina [1]. As part of an ongoing process, primordial follicles become active, the oocyte starts to grow, and its surrounding granulosa cells start to become mitotic. As the granulosa cells divide, the number of layers of granulosa cells (called the membrane granulosa or follicular epithelium) around the oocyte increases, and the basal lamina expands [2, 3]. Primordial follicles give rise to primary follicles which transform into preantral (secondary follicles), then antral follicles (tertiary follicles), and finally preovulatory and Graafian follicles, in a coordinated series of transitions regulated by hormones and local intraovarian factors [1, 4, 5]. During the ultimate stages of follicular growth several millions of GCs [6] exert a multitude of specialized functions encompassing the function of the follicle, such as producing large amounts of estradiol, adapting its FSH, luteinizing hormone receptivity to the endocrine milieu, nursing the oocyte, and communicating both with the enclosed oocyte and the surrounding thecal cells. The signalling leading to ovulation results in luteinization of the remnants of the ovulated follicle. Luteinized GCs are considered to be terminally differentiated, being replaced in the midluteal phase of the menstrual cycle by small, luteinized cells originating from the surrounding theca [7].

The mammalian ovary produces mature oocytes capable of being fertilized and sustaining early embryonic development. Developmental competence of the oocyte correlates with follicular size, larger oocytes being more developmentally competent [8, 9]. The development of an oocyte ultimately capable of undergoing fertilization and embryogenesis depends on appropriate signalling from surrounding ovarian granulosa cells (GCs) and follicle-stimulating hormone (FSH) [10].

2. The Role of Surrounding Somatic Cells in Folliculogenesis

There are three ovarian functional somatic cell types involved in folliculogenesis: (1) the ovarian surface epithelium that surrounds ovary, (2) the theca, and (3) the granulosa cells, which essentially reside within the avascular space of the ovarian follicle.

Primordial follicles are not distributed uniformly in the ovary but are predominantly located in the ovarian cortex. The ovarian cortex is covered by a layer of irregularly shaped cells [11], commonly known as the ovarian "germinal" or surface epithelium, which is attached to the tunica albuginea. In functional human ovaries the surface epithelium is found in certain areas only, but in women with polycystic ovaries, the ovarian surface is completely covered with surface epithelium [12]. These observations indicate that the surface epithelium derived epithelial nests may represent primitive granulosa cells. They may either invade surface epithelium from adjacent structures and are extruded from the ovary [13].

The tunica albuginea is a thick fibrous subepithelial layer with cells embedded in connective tissue, which does not begin to form until the end of intrauterine life [13, 14]. In adult human females, mesenchymal cells in the ovarian tunica albuginea undergo a mesenchymal-epithelial transition into ovarian surface epithelial cells (OSE) [13, 15, 16], which may differentiate sequentially into primitive granulosa. These structures assemble in the deeper ovarian cortex may form new follicles to replace earlier primary follicles undergoing atresia [17–19], but this concept still needs verification.

Theca cells surround the developing follicle, form the two layers known as the theca externa and interna, and produce the androgens which are ultimately converted to estradiol by the GCs.

During the final stages of follicular growth, GCs are at the centre of ovarian function, as they not only direct the growth of the oocytes thereby inhibiting meiotic progress but also produce and secrete the hormones which prepare such processes as ovulation and endometrial proliferation. The antral growth, the proliferation, differentiation, and function of GCs are initially controlled by the follicle-stimulating hormone (FSH) alone, later by both FSH and luteinizing hormone (LH). FSH targets its receptor (FSHR) and induces the maturation of ovarian follicles through proliferation of GCs; induction of the LH-receptor (LHR) and formation of a functional syncytium [20–23] which surrounds and nurses the oocyte produce the bulk of steroids. Steroids are then secreted into blood circulation to manage successful ovulation, fertilization, and subsequent implantation of the embryo.

The membrana granulosa or follicular epithelium is more complex than most other epithelia for various reasons. At first it expands from a single to a multilayered epithelium, as the follicle grows. In the transition from a pre- to a postantral follicle, the shape of GC changes from nondividing flattened appearance to dividing cubical appearance. The epithelium also expands laterally with time, as the follicle enlarges. During the preovulatory phase the membrana granulosa becomes vascularised with capillaries sprouting from the surrounding theca interna. Finally, at ovulation GCs differentiate into luteal cells. It is the fate of 99% of all follicles to become atretic, and apoptosis among the GCs is one of the first indicators of follicular atresia [2, 9, 24].

3. Ovarian Surface Epithelial for Renewal of the Follicle Pool after Birth

Oogenesis has been demonstrated in cultured mouse embryonic stem cells [25], and mitotically active germ cells have been reported in ovaries of adult prosimian primates [26] and mice [27, 28]. Regarding follicular renewal in adult human females, reports provide evidence that the OSE could be a source of germ cells, and new primary follicles are formed by assembly of oocytes with nests of primitive granulosa cells in the ovarian cortex [18, 19]. Components for the new primary follicles, primitive granulosa and germ cells, are proposed to differentiate *de novo* from mesenchymal progenitor cells residing in the ovarian tunica albuginea. During differentiation into OSE cells the mesenchymal progenitor cells line either the ovarian surface or invaginated epithelial crypts. Mesenchymal progenitor cells would first contribute to the development of epithelial cells similar to granulosa cells, and these cells subsequently form epithelial nests descending into the deeper ovarian cortex. These cells may be a source of germ cells, which assemble together with nests of primitive granulosa cells to form primary follicles [17]. Oogenesis may follow later. These reports all represent challenges to established dogma on the fetal origin of mammalian follicles [29, 30].

Gene expression profiling of human ovarian surface epithelial cells suggests that some of these cells are multipotent [31] and these findings are consistent with the hypothesis that OSE expresses many genes involved in somatic stem cell maintenance. Pluripotent stem cells were also found in the OSE of patients with premature ovarian failure [15, 32, 33]. These cells were an integral part of the ovarian surface epithelium and displayed morphology of oocyte cells and expression pattern of pluripotent stem cells.

Two distinct populations of putative stem cells (PSCs) were also detected in scraped OSE [16]: embryonic-like PSCs were pluripotent and underwent spontaneous differentiation into oocyte-like structures, whereas epithelial cells, probably the tissue progenitor stem cells, transformed into mesenchymal phenotype by epithelial-mesenchymal transition.

4. Culture of Human Granulosa Stem and Progenitor Cells In Vitro

Though GCs are deeply involved in human ovarian function and its various dysfunctions, and little has been known, most likely due the impossibility to culture them over prolonged time periods *in vitro*. Most studies on ovarian functions have been carried out with subhuman primates and nonprimate animals [34] and result from short-term cultures *in vitro*.

Existing immortalized human granulosa cell lines, obtained from developing follicles or ovarian carcinomas, showed little steroid hormone biosynthesis and/or limited detectable expression of the genes characteristics for GCs markers [34–38]. Immortalized human GC lines are useful for study follicular and oocyte maturation *in vitro*; however, those lines are not physiological, as most of them were established from a primary human GC tumor or were established by transfection of luteinizing GC.

Only recently methods have been developed to culture luteinizing GCs over prolonged time periods [39]. These cells are available in large quantities, as then can be retrieved from infertile women undergoing controlled ovarian hyperstimulation for assisted reproduction. This source of GCs also carries the advantage, as the donors of these cells are usually well characterized.

The crucial difference between earlier trials was the use of LIF, a cytokine commonly used in culture media supporting the development and growth of stem cells. LIF promoted the long-term survival of luteinizing GC; whereas in the absence of LIF, these cells invariably became apoptotic. LIF is a glycoprotein with a remarkable range of biological actions in different tissues, such as long-term maintenance of mouse, but not human embryonic stem cells [40]. In a number of tissues LIF has been shown to be important for stem cell self-renewal, such as the brain [41], the gut [42], and bone marrow [43].

LIF has been detected both in fetal and adult human ovaries [44], is present in the follicular fluid, and may be involved in the transition of primordial to primary follicles [45].

In addition to the specific effects of the various components of the ECM, the latter allows the cells to grow in a 3D environment, which has been shown to be essential for sustaining the morphology of ovarian follicles, including cell-cell and cell-matrix interactions [4, 46], thereby promoting follicular growth and cell proliferation. In a 2D culture system, murine follicles fail to maintain their *in vivo*-like architecture [9, 47, 48] and typically fail to grow [49]. The short-term beneficial effects of culturing GC in a 3D environment have been demonstrated previously [50, 51], but not the long-term effects.

Alginate hydrogels, a widely used substitute of the ECM in tissue engineering and characterized by optimal biomechanical properties, have been used to promote the development of mouse ovarian follicles *in vitro*, and both oocyte maturation and life offspring have already been achieved with this method [48, 52]. However, alginate hydrogels are manufactured from brown algae and consist of a polymeric scaffold of polysaccharides, therefore being unphysiological for human ovarian tissue. If any cultured material is to be used for transplantation purposes, good manufacturing practice stipulates that all constituents of the culture system should not be of animal or plant origin.

Therefore, an alternative solution was offered by using collagen type I, which is a normal constituent of ovarian tissue. Collagen fibers are the most abundant protein constituents of the ECM, and various subtypes of collagens have been demonstrated in both the animal and human ovary [9, 53–55]. The ECM of the ovary is composed of a variety of molecules that are involved in a multitude of functional processes, including steroidogenesis and luteinization [51, 56, 57]. Whereas collagen type IV has been shown to be present in the basal membrane, separating the theca interna and the granulosa, and collagen type I and type III are present in the theca externa and type I between the individual granulosa cells [58]. Together with laminin, another important component of the basal membrane, collagen interacts with its neighbouring granulosa cells via integrins expressed on the membrane of GCs [48, 59].

It has now become possible to culture human GC over prolonged time periods in the presence of LIF, as set of experiments was designed to demonstrate that a significant subpopulation of human luteinizing GCs collected from mature ovarian follicles are able to maintain their functional characteristics over prolonged time period, when they are cultured in a 3D matrix made of collagen type I, and that they can become integrated into newly developing follicles after GC transplantation into the ovaries of immunoincompetent mice [60].

5. Granulosa Cells Phenotype in Accordance to Neofolliculogenesis

The 3D culture system of GCs coated with collagen type I not only extended cellular survival *in vitro* but also allowed GCs to maintain many of their morphological and functional characteristics such as FSHR, LHR, and P450 aromatase [60]. The demonstration of both FSHR and Coll IV in GCs cultured in 3D suggested that this culture system mimics physiological ovarian follicular development (Figure 1). Similar experiences were made earlier when whole ovarian follicles were cultured *in vitro* [9, 56, 61–64]. In a 3D culture system with intact murine follicles collagen type I promoted an increase in size of two-layered follicles but had no effect on multilayered follicles [9].

In the 3D model based on collagen type I in culture medium supplemented with LIF, Coll IV was found to surround patches of GCs containing the FSHR [60]. This is in accordance with the observation that in early antral follicles collagen type IV is localized specifically in the basal membrane [65], whereas in preovulatory follicles collagen type IV is also detected in more central layers of the granulosa [57]. The basal membrane influences GC proliferation and differentiation [62, 66–69], and above observation confirms previous results [55] by demonstrating that collagen type IV, a major component of the basal membrane and of the ECM, is produced by the GCs themselves.

Taking all these findings together, the GCs in the 3D culture system based on collagen type I and LIF display a development which is reminiscent to surrogate follicle-like structures. However, for ethical reasons the culture of human oocytes was not attempted systematically.

The proliferative potential of human GCs and their ability of long lasting when in culture with follicular fluid (FF) were recently confirmed [70]. There are many advantages of coculturing GCs with FF, which among other molecules

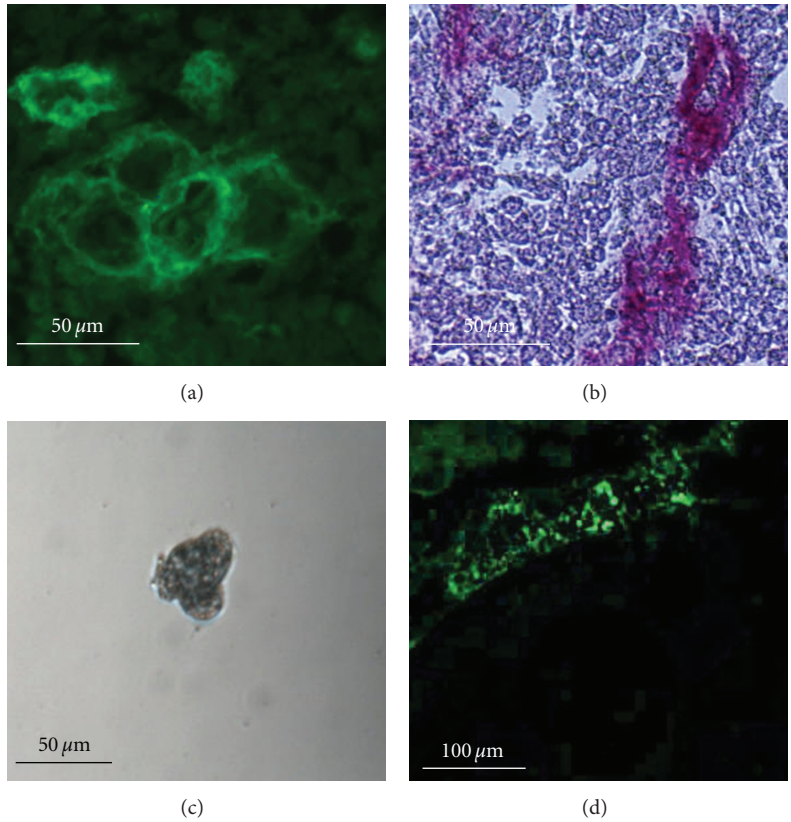

Figure 1: (a) Follicular cells cultured in 3D conditions together with type I collagen after 3 weeks (a) presented positive staining for FSHR (b) and positive staining for type IV collagen. (c) Clonogenic proliferation of follicular cells collected from mature ovarian follicles of infertile women treated with assisted reproduction and sorted with FACS based on the presence of the follicle-stimulating hormone receptor (FSHR). Clonogenic proliferation of a single follicular cell cultured for 12 days in a single well in the medium supplemented with leukemia-inhibiting factor (LIF). (d) Follicular cells cultured for 3 weeks in 3D conditions together with type I collagen were transplanted into the ovaries of immunodeficient mice. Immunostaining for HLA-ABC detected human cells after transplantation into immunodeficient mice observed in mice oviduct (data from previously published experiments in [39, 60]).

contain LIF. Being the natural environment for GCs, FF retained their morphology and intercellular connections, improves their attachment in culture and proliferation of primary culture.

Additional proof of proliferation potential of human granulosa cells is the clonogenic growth of single GCs to 3D colonies, when they are cultured in the presence of LIF (Figure 1) [60].

6. Somatic Stem Cells in the Ovary

In contrast to the ongoing controversy with regard to the possibility of ongoing renewal of oogenesis in the ovary and the possible existence of oocyte-producing stem cells in the adult ovary, the existence of stem cells sustaining the other compartments of the ovary has long been neglected.

Conventional thinking considers the ovarian follicle as an isolated structure, distinct both in space and time, either destined for early degeneration through atresia or for growth to a mature, Graafian follicle, ovulation, and formation of the corpus luteum. The cyclicity of follicular development, ovulation, and luteal function is seen as discrete phenomena, separated both in time and space. In recent years, however, experimental evidence has shed some doubt on this conventional thinking. Most notably, it has been demonstrated that mature and fully grown mouse oocytes are able to influence the development of preantral follicles in mouse ovaries [71], indicating the interdependency of the cyclic events occurring during subsequent menstrual cycles. In addition, other investigators were able to demonstrate that upon ovulation, the epithelioid granulosa cells redifferentiate into the mesenchymal cells of the corpus luteum [2].

GCs cultured *in vitro* invariably cease to proliferate already after two to three passages. Similar results have been obtained when culturing thecal cells. Recently, it has been shown that the four growth factors, bFGF, EGF, LIF, and IGF1, exhibited significant enhancing effects on colony growth of thecal cells, leading to the detection of thecal stem cells in the ovary [71, 72].

A subpopulation of mural granulosa cells has now been demonstrated to contain cells with multipotent stem cells potential, as they express the stem cell marker Oct-4 [39]. POU5F1 (Oct-4) is known to be expressed in human epithelia

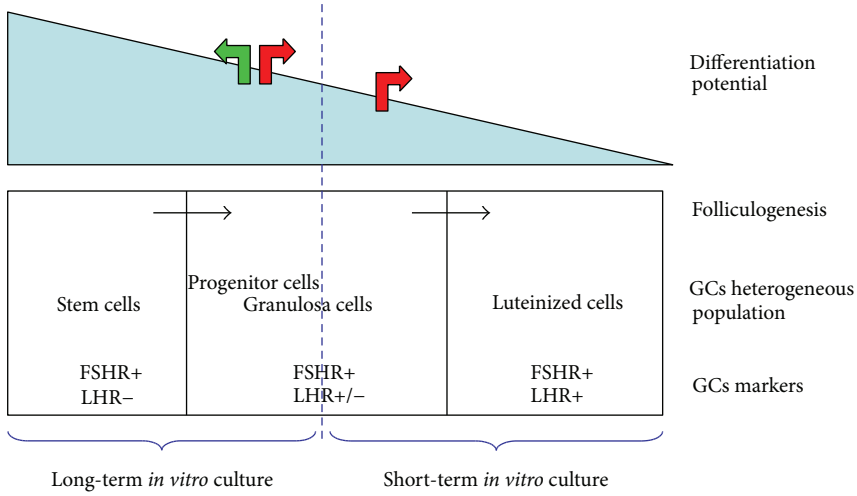

Figure 2: Hypothesis presenting several subpopulations of GCs within preovulatory follicle. GCs stem cells: positive only for FSHR. Progenitor GCs: positive for both FSHR and moderately for LHR markers. Luteinized GCs: positive for FSHR and LHR markers.

[73]. As GCs represent one of the most dynamic epithelium in the body, this could be as well true for GCs and explain its stable expression of Oct-4. Functional properties of stem cells in general include pluripotency, mitosis without differentiation, when confined to their niche, and by their ability to proliferate when isolated out off their niche [74]. The ECM is crucial for maintaining the pluripotency of stem cells through contact inhibition. Lack of contact inhibition occurs *in vivo* during growth of a follicle [75] particularly during the preantral stage of follicular development, when granulosa cells are in close physical contact to the ECM [3].

The hypothesis for the presence of stem and progenitor cells in the granulosa was first postulated, when it was demonstrated that the granulosa possesses some marked similarities to other epithelia in the body [2, 67]. The granulosa of ovarian follicles resides on a basal membrane, and the morphology of the cells highly differs in various regions of the granulosa. These authors concluded that there must be populations among the granulosa cells containing less differentiated cells and, at a distance from these, populations with highly differentiated cells.

Most adult tissues contain a heterogeneous population of cells with a hierarchy of multipotent stem cells, progenitor cells, and terminally differentiated cells [76]. We hypothesized that within preovulatory follicle there are several subpopulations of GCs with distinct characteristics. GCs expressing both FSHR and LHR, denominated luteinized GCs, will enter apoptosis during prolonged culture *in vitro*. GCs, positive for FSHR but not for LHR, may either become dedifferentiated to the progenitor GCs or differentiate to luteinized GCs (Figure 2).

It was suggested earlier that the cohort of granulosa cells in a human preovulatory follicle is derived from a clonal expansion of a small number (3 cells) of ovarian stem cells [77]. The presence of a subpopulation of GCs with multipotent stem cell characteristics explains why GCs, taken from preovulatory follicles and cultured under the appropriate conditions, can survive over prolonged time periods and can be differentiated into other tissue types, otherwise not present in the ovary. In addition, when cultured in 3D in an extracellular matrix similar to the ovary, the GCs seem to retain most of their characteristics including the FSHR and steroidogenesis [60].

In addition to the granulosa cells FSHR/LHR subgroups the substantial subpopulations of long cultured granulosa cells were attained by expression of mesenchymal lineage marker CD117 (c-kit). The results differed within patients (Kossowska-Tomaszczuk and coworkers; data unpublished).

GCs stained for both FSHR and CD117 (c-kit) established 3 populations: cells positive only for FSHR, cells positive for both markers, and cells negative for those markers. Our observation showed that women with good quality of oocytes and the one who got pregnant after the IVF therapy had higher amounts (25%–40% of total GCs) of cultured GC positive for both CD117 and FSHR markers than a population only positive for FSHR or with none. However, correlation between the number of those cells among fertile and subfertile women failed to indicate any role with respect to the likelihood of successful pregnancy.

c-Kit is the stem cell factor receptor and is expressed in human ovarian follicular development, and their interaction is required for the survival of follicles in long-term culture [78]. c-Kit was confined to the oocyte and granulosa cells in primary and secondary follicles and preovulatory granulosa cells [28, 79]. 50–70% of freshly isolated granulosa cells from patients undergoing assisted reproduction contained cells which stained positively for c-Kit [80]. c-Kit signalling is likely to control the survival of human ovarian follicles during early follicular development. Blocking the c-Kit receptor induces follicular atresia.

7. Conclusion: The Potential Significance of Granulosa Stem and Progenitor Cells

Together with the stem cells located in the theca [72, 81], the multipotent stem cells of the progenitor type in the granulosa

[39] may provide the niche, in which oocyte-producing stem cells may thrive [82]. The finding of granulosa cells displaying multipotency and having a prolonged lifespan, extracted from mature ovarian follicles, is likely to have a significant impact on evolving theories in ovarian physiology and reproductive biology, particularly with reference to folliculogenesis and the pathogenesis of ovarian endometriosis and ovarian cancer. Multipotent stem cells in ovarian follicles may be involved in the early origin of some forms of ovarian tumors, in particular granulosa cell tumors, as well as to the origin of ovarian endometriosis, which is considered to arise from undifferentiated, hitherto labeled as metaplastic cells in the ovary (Figure 1).

In female mammals, the normal and physiological production of good quality gametes relies upon the highly controlled growth and differentiation of the surrounding ovarian follicle. GC proliferation is maintained throughout folliculogenesis, providing not only a specialized microenvironment but also nutrients for oocytes growth. GC multipotency and the use of granulosa stem and progenitor cells in the newly developed 3D *in vitro* culture system may provide a promising technical tool for *in vitro* maturation of both human and animal ovarian follicles. Such culture systems can be used in reproductive toxicology, in drug targeting, and in assisted reproduction, respectively, breeding.

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Research Article

Patients' Attitudes towards the Surplus Frozen Embryos in China

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Background. Assisted reproductive techniques have been used in China for more than 20 years. This study investigates the attitudes of surplus embryo holders towards embryos storage and donation for medical research. *Methods*. A total of 363 couples who had completed in vitro fertilization (IVF) treatment and had already had biological children but who still had frozen embryos in storage were invited to participate. Interviews were conducted by clinics in a narrative style. *Results*. Family size was the major reason for participants' (dis)continuation of embryo storage; moreover, the moral status of embryos was an important factor for couples choosing embryo storage, while the storage fee was an important factor for couples choosing embryo disposal. Most couples discontinued the storage of their embryos once their children were older than 3 years. In our study, 58.8% of the couples preferred to dispose of surplus embryos rather than donate them to research, citing a lack of information and distrust in science as significant reasons for their decision. *Conclusions*. Interviews regarding frozen embryos, including patients' expectations for embryo storage and information to assist them with decisions regarding embryo disposal, are beneficial for policies addressing embryo disposition and embryo donation in China.

1. Introduction

According to a 2006 report by the International Committee Monitoring Assisted Reproductive Technology, more than 3 million babies worldwide were conceived through in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) [1]. The development of advanced techniques to cryopreserve embryos has made the cryopreservation of surplus embryos an integral part of IVF procedures, as a result improving pregnancy rates and reducing risks and costs. However, the number of embryos in storage around the world is steadily increasing, to the point that the increasing number of embryos in storage has become a burden for the centre of assisted reproduction. In some countries, legislation stipulating a maximum storage period of human embryos has come into effect. Storage limits vary between countries and between the states within some countries; most countries have determined that embryos can remain in storage for up to 5 years [2–9]. Such legislation prevents an unlimited accumulation of stored embryos. When surplus embryos approach the storage time limit, there are several options for patients. For example, in Australia, the embryos can be destroyed, donated to infertile couples, or donated to science (since 2003, following the enactment of federal legislation permitting embryonic research under some conditions) [4]. These three options also exist in the United States of America, following the 2010 removal of the ban on the use of federal funds for the creation or use of new cell lines generated from embryos [10]. In Switzerland, surplus embryos can either be disposed of or used for medical research under certain restricted conditions [2].

In China, since 1988 when the first baby was born by IVF, millions of infertile couples have had opportunities to conceive and give birth to their own children; by thawing and transferring cryopreserved embryos, patients who undergo IVF or ICSI have greater chances of becoming pregnant without undergoing new stimulation cycles. The legislation and guidelines in China specify that (1) two embryos can be transferred at the same time in patients under 35 years old and in their first stimulation cycles; (2) three embryos can be

transferred at same time in patients aged 35 years or older, or in patients younger than 35 years old but in their second stimulation cycles; (3) three frozen embryos can be transferred at the same time to a patient of any age, unless it is the first embryo transfer and she is younger than 35 years old, in which case two frozen embryos should be transferred [11, 12]. The surplus embryos that are not transferred are stored and used in subsequent treatment cycles to enable pregnancy. As a result, the number of frozen embryos accumulates constantly; the most difficult problems associated with a long duration of embryo cryopreservation are the increase of "unclaimed" embryos and the associated ethical, legal, and economic pressures. All of these problems require the centre of assisted reproduction to determine the fates of surplus frozen embryos. In China, no law has been adapted specifying a maximum storage limit of cryopreserved human embryos [11, 12]. This study is the first in China that assesses attitudes towards the disposition of surplus embryos and, involves a large representative sample of couples who already have children and still have cryopreserved embryos. The study includes two sections that (1) investigate attitudes towards the discontinuation of surplus embryo storage and (2) investigate attitudes towards the donation of surplus embryos for research.

2. Materials and Methods

2.1. Ethics and Recruitment. In China, IVF treatments can only be provided by licensed centres. This study was approved by the Human Research Ethics Committee at the First Affiliated Hospital of Nanchang University. Before cryopreservation, all patients at the IVF centre at the first affiliated hospital of Nanchang University signed a form allowing medical staff to freeze and store their supernumerary embryos (if the quality of these embryos was considered sufficient for cryopreservation).

Participants were recruited from the IVF centre at the first affiliated hospital of Nanchang University. All participants had biological children and still had surplus embryos stored at the centre; couples who did not have surplus embryos in storage or who had surplus embryos in storage but did not have biological children were excluded from the study. At the beginning of the procedure, 427 couples who had completed IVF treatment between 2001 and 2011 were included; the actual number of couples invited to the interview was 363 (85%). In 59 cases, the patients could not be reached due to changes in their contact information (that the clinic was not notified of), and in the other 5 cases, the patients refused the interviews. About 63% of the participants were living in Jiangxi province, while the rest lived in other provinces. On the basis of their children's ages, we separated the participants into three groups: 0–3 years old (group I), 3–5 years old (group II), and older than 5 years (group III).

2.2. Procedure. All in-depth interviews (in a narrative style) were conducted at the IVF centre by the researchers, either in person or by telephone, between January and April of 2012. Each interview was about 0.5 hours in duration. With the participants' permissions, the personal interviews were recorded and transcribed by an independent transcriber and checked for accuracy. All the names for persons were replaced by numbers (i.e., patient number 1). The interviews had two purposes: (1) to collect a range of participant decisions regarding the destinations of their surplus embryos and (2) to collect a range of information in relation to their decisions on the excess embryos, including the reasons and feelings behind their decisions, as well as the educational backgrounds of the participants.

An interview guide was used, couples were interviewed together. First, participants were asked what decisions they had made regarding their excess embryos (continuing or discontinuing storage). Second, the participants who chose to discontinue the storage of their embryos were asked about their thoughts regarding the fates of their unused embryos, which would be either donated to research or disposed of. Finally, the participants were asked to describe their reasons for choosing their preferred options and how they felt about the decision-making process.

It is worth noting that because no legislation permitting the use of surplus cryopreserved embryos for research has thus far come into effect in China, the discussion about donation to research was in response to a hypothetical scenario, rather than actual practice.

2.3. Analysis. The data were analysed using SPSS software (version 11.5). Associations were assessed using chi-square test; P values of <0.05 were considered to be statistically significant.

3. Results

3.1. Attitudes towards the Storage of Cryopreserved Embryos. Although there is one-child policy in China presently, storage of embryos is permitted in case an accident happened to the child and parent still have chance to get another child if their only one child was dead or had some disease which is under one more children born permission. Besides of that, to ensure the possibility of pregnancy, two embryos would be generally transferred into uterus each time, which caused the rate of twins higher by IVF than that by natural fertility. Table 1 shows couples' attitudes towards the storage of their cryopreserved embryos and towards the continuation or discontinuation of storage. In the group with children aged 0 to 3 years old (group I), 83.3% of the couples choose to continue storing their embryos, only 16.7% would discontinue their embryos' storage, and one person declined the interview. In the group with children aged 3 to 5 years old (group II), among the 131 participants, 26.7% wanted to continue storing their embryos; 73.3% discontinued storing their embryos, and two people declined the interview. In the group with children older than 5 years (group III), 25% wanted to continue storing their embryos, and 75% discontinued; this group was similar to group II. There were no differences in terms of attitudes towards the storage of

Table 1: The participants' attitudes towards the storage of their cryopreserved embryos.

Total participants	Respondents	Continue storage		Discontinue storage		Reject
N: 363	*n*	*n*	%	*n*	%	*n*
Group I Children 0–3 years old	72	60	83.3	12	16.7	1
Group II Children 3–5 years old	131	35	26.7	96	73.3	2
Group III Children >5 years old	160	40	25	120	75	2

Table 2: Participants' reasons to (dis)continue storage.

	Group I Children 0–3 years old		Group II Children 3–5 years old		Group III Children >5 years old	
Reasons to continue storage	*n* = 60		*n* = 35		*n* = 40	
	n	%	*n*	%	*n*	%
Worrying about the children health	30	50	17	48.6	12	30
More baby wish	50	83.3	26	74.2	40	100
Embryo is potential life	20	33.3	17	48.6	20	50
Reasons to discontinue storage	*n* = 12		*n* = 96		*n* = 120	
	n	%	*n*	%	*n*	%
Storage fee	12	100	57	59.3	60	50
Family is complete	12	100	86	89.6	80	66.7
Achieved natural pregnancy	0		5	5.2	0	
Worrying about frozen embryos quality	0		10	10.4	60	50

embryos between the provincial regions (data not shown in table).

3.2. Reasons to (Dis)continue Storage of Surplus Embryos

3.2.1. Continue Storage. As Table 2 shows, wanting more children was the major reason cited by most of the couples who wanted to continue storing their embryos; in the three groups, the percentages reporting this reason were 83.3%, 74.2%, and 100%.

> *Patient Number 1. We plan to have more babies in three years, so we want to continue the embryo storage for 3 years; maybe next time, we will have a boy.*

> *Patient Number 2. I know only one child is permitted per couple by Chinese law, but I still hope that maybe one day more children will be permitted in China; then, we will have a chance to have more children.*

> *Patient Number 3. We are from the countryside, so we should have a boy.*

Worrying about their children's health was another important reason reported within groups I and II, whose children were younger than 5 years old. This fear decreased when the children were older than 5 years; only 30% of participants worried about their children's health at that point, compared with about 50% in groups I and II.

> *Patient Number 4. Please keep storing our embryos, just in case; our baby is so young, after all, and we are not sure what will happen to him.*

In addition, the moral status of embryos was a reason named by couples who wanted to continue storing their embryos. In these cases, the storage decision was not always consistent with a desire for more children. However, few storage decisions were linked to religion(data not shown).

> *Patient Number 5. The embryos are still life. Although I have not made a decision about whether I will transfer them or not, they are still potential life.*

> *Patient Number 6. Yeah, they are our children who have not been born; we have tried so hard to have the embryos, so we will continue storing them.*

> *Patient Number 7. It is so hard for us to make this decision. Can we postpone the decision making? If not, we choose to continue the storage.*

3.2.2. Discontinue Storage. Among the reasons that couples chose not to store their embryos, family completion and storage fees were two common reasons.

> *Patient Number 8. The storage fee is a big financial burden for us. Since we have a baby, we won't pay the fee to continue the storage of the embryos.*

Table 3: Difference of desire for extra embryos storage.

	Group I (children 0–3 years old) $n = 60$		Group II (children 3–5 years old) $n = 35$		Group III (children >5 years old) $n = 40$	
	n	%	n	%	n	%
Women's desire	0		10	28.6	30	75
Men's desire	0		12	34.3	5	12.5
Couples' desire	60	100	13	37.1	5	12.5

Patient Number 9. We have two children from the last IVF treatment. This is enough for our family; we do not want more children, so the surplus embryos need not be stored anymore.

Concern about the quality of frozen embryos increased along with the duration of embryo storage; this was an important reason for the couples in group III who did not want to continue storage (50%, compared with 10.4% of group II and 0% of group I).

Five women achieved natural pregnancy; they all chose to discontinue storage.

3.3. Desire for Extra Embryos Storage. Table 3 shows the differences in men's and women's desires for surplus embryo storage. In group I, both the husbands and wives had strong desire to continue storing the surplus embryos. As the children got older, however, the men's desire to store embryos decreased correspondingly. In group III, when the children were older than 5 years, 75% of the embryo storage decisions came from the women alone, in contrast to 100% among the couples in group I.

Husband of Patient Number 5. About the destination of our frozen embryos, I do not care. It depends on my wife's attitude; whatever decision she makes, I agree.

3.4. Attitudes towards the Destination of Surplus Embryos. Table 4 shows the couples' attitudes towards the destinations of surplus embryos that they discontinue storing. More people were likely to discard their embryos than to donate them to research or therapy (58.8% versus 41.2%, resp.). In the approximately two-fifths of participants who supported embryo donations, about 28.7% considered donating to medical therapy (if available) but did not want to donate to medical research.

Patient Number 10. By donating the embryos to medical therapy, I felt they might save somebody. But with research, I cannot imagine what kind of research is done on the embryos; after all, they are like my unborn children.

Among all of the correlates entered into the model predicting couples' attitudes towards donation to medical research or therapy and disposal, educational level had no relationship with the decision regarding the destination of the embryos; of the participants, 68.1% of high school graduates and 31.9% of college and university graduates preferred donation, while 69.4% of high school graduates and 30.6% of college and university graduates chose to discard the embryos. The differences in couples' views on the moral status of embryos had a significant impact on the decision to discard or donate embryos; about 45.5% of the couples who choose to discard their embryos felt that an embryo is just a cluster of cells, while the 13.6% of couples choosing donation considered the embryo to be life or a potential child. For these couples, the donation decision was difficult, and they hesitated.

Patient Number 11. No, I disagree with donation. Actually, I care about how the embryos are disposed of. You know, they deserve at least some sort of respect.

Patient Number 12. No, I cannot imagine donating the embryos to research. I would donate my own body or organ when I die if it is needed, but donating my embryos, I cannot accept that.

Patient Number 13. Yes, I prefer donation of the embryos to medical research or therapy. Since my family is complete now, we will be happy that they could help others.

Patient Number 14. The embryos are our potential children. I disagree with donation.

Two hours after the end of the interview, patient Number 14 called the investigator, saying the following.

Patient Number 14. We, as a couple, discussed donation again. We prefer to donate our embryos to medical research or therapy; we hope that they can help others.

4. Discussion

This study consists of a first survey conducted in China using a relatively large representative sample of patients who have either experienced successful IVF/ICSI treatment or experienced natural pregnancy later but still owned cryopreserved embryos. Cryopreservation of embryos for later use is a routine procedure in China, as no strict legal regulations or deadlines for the destruction of cryopreserved embryos preserved for reproductive purposes have thus far

Table 4: Attitudes toward destination of surplus embryos and correlates associated with the decision.

	Donation						Discarding	
			n	%			*n*	%
Discontinue storage of embryos $n = 228$			94	41.2			134	58.8
	To medical research only		To medical therapy only		To medical research and therapy			
	n	%	*n*	%	*n*	%		
	0		27	28.7	67	71.3		
	Correlates associated with couples' decision							
			n	%			*n*	%
Education								
Elementary school			0				0	
High school			64	68.1			93	69.4
College and university			30	31.9			41	30.6
Participants' view of the moral status of embryos								
Cluster of cells			81	86.2			61	45.5
Life, potential child			13	13.6			73	55.5

been adopted. Maintaining the accumulating "unclaimed" embryos in infertility centres is an expensive and time-consuming task; this has become a common problem. In this paper, we address the influence and relevance of the external context, such as embryo holders' expectations for and perceptions of frozen embryos and storage fees.

Our survey showed that couples' storage decisions largely depended upon the ages of their children. When the children were aged between 0 and 3 years, more than four-fifths of the couples wanted to maintain their embryos; of those, some couples were not sure whether they would transfer the embryos. Conversely, when their children grew older (older than 3 years), 70% of the couples chose to discontinue the storage of their embryos. Of the reasons behind the storage decisions, the desire to have more children was the most important factor, as has been described in other studies [3, 13, 14]. Additionally, worrying about the children's health was a concern for couples when their children were younger than 5 years old. Another type of storage decision, aside from the desire for more children, concerned the moral status of embryos; this emotional reason, despite the absence, in some cases, of another purpose, could be a factor adding to the number of participants who were hesitant and wanted to postpone their decision making as long as possible. This finding is consistent with other studies [5, 15, 16]. No significant regional differences based on the reasons for storage were found.

We found that participants' attitudes towards storage fees generally varied with their stages of decision making. Most of them told us that without storage fees, they would have little motivation to make a disposal decision and would instead keep the embryos in storage indefinitely. Storage fees were one reason that some participants abandoned frozen embryos, which cannot be neglected [17–22]; this was especially true among residents from the countryside.

Family size was also found to be an important determinant. Because of the one-child policy in China presently, in our sample, couples who already had an average of two children were generally satisfied with their family sizes and felt that their families were complete. Other participants, however, reportedly would consider using surplus embryos in the future when the one-child policy changes. The importance of family size has also been found in other studies [5, 13, 23]. Besides of the family size, the preference for male children is one more reason for the couples to keep their embryos storage. In China, bearing progeny is generally considered as part of a stable marital nexus. Children, particularly sons, are regarded as a continuance of a whole family. Furthermore, sons are regarded as a source of income and security in old age for many lower income or lower educated people. The third reasonable one that the couples would consider is also the effect of China's one-child policy on aged couples. There are some aged couples, their only child died for some reason, they had to suffer the lonely life without children at the rest of their time, because wive's ovary is failure and no available oocytes can be used at that time. That is why some couples continue their embryos stored, just in case.

Another interesting finding in our survey was that the duration of embryo storage was strongly linked to patients' choices for their embryos' fates; nearly half of the participants opted to abandon their surplus embryos once they had been stored for more than 5 years, as they were not confident in the quality and integrity of their cryopreserved embryos.

In terms of attitudes towards embryo storage, women tended to be more preoccupied with the statuses of their embryos than their husbands were. In couples who did not desire more children, it was more difficult for the women to make decisions about the fates of their surplus embryos; deciding to dispose of their embryos was always emotionally fraught for them. In addition, women's opinions regarding

excess embryos were usually respected by their partners. The moral status of embryos plays an instrumental role in the symbolic meaning of the relationship between partners [16].

Donation to science is another option for the disposal of surplus embryos. Recent observations have shown that donation to medical research or therapy has become more popular; some participants who would probably donate to research described discarding embryos as wasteful or selfish [24–26]. Our findings are not entirely consistent with published reports; in fact, 41.2% of couples chose donation to research, versus 58.8% who preferred to discard their excess cryopreserved embryos. The decision not to donate was, for many couples, linked to their relationships with the embryos. The participants' views of embryos as potential life have direct implications with respect to their views of donation of embryos for stem cell research, which is consistent with a previous study [24]. For most of the individuals interested in donation, embryos were viewed as a cluster of cells.

In addition, we found that lacking information about what the research using embryos actually entailed was another significant factor for the patients who were more likely to discard their embryos. Because of the absence of specific information, the participants thought that discarding their embryos would prevent them from being misused. A minority of couples stated they would donate to medical therapy, but not to research. This distrust in science or scientists consequently affected donation decisions, as was also described in earlier papers [17, 18, 25, 27]. Moreover, the educational backgrounds of participants did not seem related to their decisions. A survey in the US reported that Asians who immigrated to the US were less likely to donate excess cryopreserved embryos for research use than Caucasians and Asians born in the US and that they were more likely to discard embryos; embryo disposition plans among Asians born in the US, on the other hand, were similar to those of all other ethnicities [28]. With more permissive regulations than those in the US, between 2001 and 2009, human embryonic stem cell research has been performed on a large-scale in China, Japan, South Korea, Singapore, India, and Taiwan [29]; thus, cultural bias against human embryonic research is unlikely a real reason. Many interviews suggested that acquainting participants with more information about the kinds of research being done or directing them to a specified research project would assist them in choosing a donation option. The feeling that parents still have some control over the type of research project for which their embryos would be used is definitely influential. Therefore, our study suggests that misunderstanding or not receiving adequate information about the disposal choices is a significant reason for many stem cell candidates to refuse donation; this conclusion is consistent with previous reports [30, 31]. It is interesting to note that 92% of couples preferred to donate their supernumerary embryos to stem cell research instead of discard them, according to a Swedish study; furthermore, the high level of interest in donation is due partly to the detailed and comprehensive oral and written information about the specific research project that is given to each patient [32]. In addition to adequate information, additional psychological support is also helpful, particularly for couples hesitating to donate to research. A few couples admitted that the decision to discard their embryos was made at the "last minute." In the US, studies performed in the 1990s indicated little willingness to donate embryos to science [33–35]. In contrast, donation to science has become an increasingly popular disposal option in recent years [24–26]. This apparent shift in patient attitudes may reflect increased public awareness of stem cell research in the U.S.

Another interesting finding from our survey is that religious consideration did not play a significant role in couples' storage decisions or donations to research. The influence of religious beliefs has yielded inconsistent results in previous studies [2, 4, 16, 36].

5. Conclusion

On the whole, maintaining large numbers of frozen embryos is currently quite a burden for IVF centres in China. In some countries, legislation limiting the amount of time that embryos can be stored has been established, resulting in large scale disposal of abandoned embryos [2–9]. In China, the one-child policy and its effect on couples' family planning decisions should also be considered to the rule of embryos storage setting up. Another strategy that we recommend to IVF clinics around the world is providing more detailed information regarding research, directed research options, and even psychological support groups to assist in decision making by embryo holders. Although it is a complex issue, it is still worth considering.

Conflict of Interests

All authors report no conflict of interests and have nothing to disclose.

Authors' Contribution

X. Jin and G. Wang contributed equally to the paper. X. Jin, as the first and corresponding author, was involved in the study design, implementation, execution, data collection and analysis and wrote and coordinated revision of the manuscript. G. Wang, as the first co-author, was involved in the study design, execution, and data analysis. S. Liu was involved in the data analysis and provided expert knowledge and critical discussion. M. Liu and J. Zhang were involved in the data collection. Y. Shi was involved in the paper drafts and provided expert knowledge on statistics.

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Research Article

Estradiol Synthesis and Release in Cultured Female Rat Bone Marrow Stem Cells

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Bone marrow stem cells (BMSCs) have the capacity to differentiate into mature cell types of multiple tissues. Thus, they represent an alternative source for organ-specific cell replacement therapy in degenerative diseases. In this study, we demonstrated that female rat BMSCs could differentiate into steroidogenic cells with the capacity for *de novo* synthesis of Estradiol-17β (E2) under high glucose culture conditions with or without retinoic acid (RA). The cultured BMSCs could express the mRNA and protein for P450arom, the enzyme responsible for estrogen biosynthesis. Moreover, radioimmunoassay revealed that BMSCs cultured in the present culture system produced and secreted significant amounts of testosterone, androstenedione, and E2. In addition, RA promoted E2 secretion but did not affect the levels of androgen. These results indicate that BMSCs can synthesize and release E2 and may contribute to autologous transplantation therapy for estrogen deficiency.

1. Introduction

Steroid hormones play important regulatory roles in female reproduction, in which estrogen is essential for folliculogenesis beyond the antral stage and is necessary to maintain the female phenotype of ovarian somatic cells [1–4]. Estradiol-17β (E2), a product of androgen aromatization, is the principal estrogen and is secreted in large amounts by the large preovulatory follicles in the ovary [5]. Although the ovaries are the principal source of systemic oestrogen in the premenopausal nonpregnant woman, a number of extragonadal sites of oestrogen biosynthesis, including mesenchymal cells of the adipose tissue and skin, osteoblasts, vascular endothelial, aortic smooth-muscle cells, and brain, become the major sources of oestrogen beyond menopause. However, the total amount of oestrogen synthesized by these extragonadal sites may be small. Within these sites, E2 is probably biologically active only at local tissue level in a paracrine or intracrine fashion without significantly affecting circulating levels [6–8].

The reduction of estrogen production in the ovary may cause menopausal symptoms. In addition, premature ovarian failure may be caused by any process which reduces the number of oocytes within the ovary [9]. For example, chemotherapy can reduce ovarian reserve and affect ovarian stromal function to produce less estrogen [10]. Although estrogen replacement therapy has been established and is recommended for postmenopausal women or patients with hypogonadism, due to its beneficial effects, follicular estrogen production is regulated by a complex set of signals that synergize to produce optimal steroidogenesis [11]. Still, it is difficult to provide an optimal therapeutic dose for long-term estrogen replacement therapy. Furthermore, it is associated with a substantial risk for cardiovascular disease and breast cancer [12]. For this reason, alternative therapies such as steroidogenic cell transplantation may have advantages over

HRT for hypogonadism. It should allow control of hormone levels in nature by hypothalamus-anterior pituitary axis.

Several earlier studies have suggested that stem cells can serve as an alternative source for various steroid hormones [13–17]. Bone marrow stem cells (BMSCs) are thought to be multipotent cells, which can replicate as undifferentiated cells and have the potential to differentiate into mature cell types of multiple tissues [18, 19]. In the present study, we investigated whether female rat BMSCs could produce steroidogenic cells with the capacity for the synthesis of E2.

2. Materials and Methods

2.1. Isolation and Culture of BMSCs. Female SD rats, weighing 80 to 100 g, were obtained from Center of Laboratory Animals of Nanchang University and used in accordance with a protocol approved by the Nanchang University Animal Care and Use Committee. The bone marrow cells were isolated from femurs and tibias of female rats by flushing the shaft with phosphate-buffered saline (PBS) using needles, and the cells were further dispersed several times by gentle, repeated pipetting with a sterile pipet. The dissociated cells were seeded in 75 cm^2 culture flasks for the primary culture in the high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM, Hyclone, Utah) supplemented with 10% fetal bovine serum (FBS, Stem Cell Tech Inc., Canada) and incubated at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. The nonadherent cells were removed by washing with PBS and replacing the fresh complete medium every 3 or 4 days. The adherent cells were passaged every 7 days by harvesting the cells with 0.25% trypsin/0.02% EDTA, and replating at a 1 : 4 dilution.

2.2. Induction of BMSC Differentiation In Vitro. Cultured cells at passage 3 were recovered and used in these experiments. BMSCs were reincubated in 12-well culture plates (Nunc, Denmark) at a density of 2×10^5/well in the high glucose (4.5 g/L) DMEM containing 10% FBS supplemented with or without 10^{-5} mol/L all-trans retinoic acid (RA, Sigma) for 4 days. Differentiated cells from the BMSCs were analyzed by immunocytochemical staining or RT-PCR analysis for expression of aromatase cytochrome P450 (P450arom). The levels of testosterone (T), androstenedione (ASD), and E2 in culture media were measured by radioimmunoassay (RIA).

2.3. Immunocytochemistry of P450arom. BMSCs were fixed in 4% acetone at 4°C for 15 minutes and washed 3 times with PBS. Endogenous peroxidase was quenched by incubating the fixed cells with 3% H_2O_2 in methanol for 20 minutes. After being washed with PBS for 15 min (5 min ×3 times), cells were incubated for 20 minutes with 10% normal goat serum; then with a rabbit polyclonal antibody to aromatase (Boster Co., Wuhan, China), they were diluted 1 : 100 in PBS overnight at 4°C. The negative control was prepared in an identical manner except that the primary antibody was replaced with normal serum. After washing with PBS, cells were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 hour at room temperature. After washing, the immunoreaction was detected by using DAB system.

2.4. Real-Time PCR Analysis. Total RNA was extracted from cultured BMSCs using Trizol reagent (Sigma, St. Louis, MO) and was reverse transcribed into cDNA using the First-Strand cDNA synthesis kit. Real-time PCR was performed to quantify the samples' cDNA copies using SYBR premix ExTaqTM fluorescent quantitation PCR kit (TaKaRa, Japan). The *CYP19* primers forward: 5′-GCTTCTCATCGCAGSGTAT-3′, reverse: 5′-CAAGGGTAAATTCATTGGG-3′. The β-actin primers forward: 5′-GGAAATCGTGCGTGACATTAAA-3′, reverse: 5′-TGCGGCAGTGGCCATC-3′. Conditions for PCR were 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds. The cycle threshold (Ct) was set up at the level that reflected the best kinetic PCR parameters, and melting curves were acquired and analyzed. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to estimate the copy numbers in *CYP19* gene.

2.5. T, ASD and E2 Measurement. Before culture (defined as time 0) and 1, 2, 3, or 4 days after culture, cell culture medium was centrifuged and collected, and the levels of T, ASD and E2 were measured by Beijing Sino-UK Institute of Biological Technology.

2.6. Statistical Analysis. The experiment was repeated a minimum of three times. All data were expressed as the mean ± SD and analyzed by ANOVA and Ducan's multiple range test using the SAS 8.0 software. $P < 0.05$ was considered significantly different.

3. Results

3.1. RT-PCR Analysis for CYP19 mRNA Expression. RT-PCR analysis showed that there was expression of aromatase gene *CYP19* in BMSCs cultured for 4 days in a high glucose DMEM, and the expression was significantly higher than that in 0 day cells. Furthermore, we investigated the effects of RA on BMSC differentiation in vitro. The result showed that the expression of *CYP19* mRNA was not further elevated by RA treatment at a concentration of 10^{-5} mol/L (Figure 1).

3.2. Immunocytochemical Analysis of P450arom. To elucidate the capacity of BMSCs to generate E2, we examined the expression of P450arom protein, the enzyme responsible for estrogen biosynthesis, by immunocytochemical staining. The results showed that P450arom was expressed in BMSCs cultured in a high glucose culture condition alone or in combination with RA treatment, with a positive labeling in part of the cells, and it was primarily immunolocalized in the cytoplasm (Figure 2).

3.3. RIA for E2. To evaluate E2 biosynthesis and release in cultured BMSCs, the levels of E2 in culture medium were

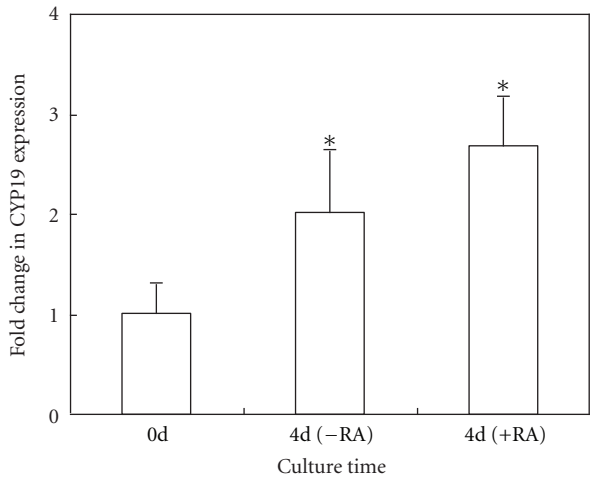


Figure 1: Real-time PCR analysis of CYP19 expression in rat BMSCs. The expression of CYP19 was determined relative to the β-actin expression. Expression data ($n = 5$) were reported as fold change ($2^{-\Delta\Delta Ct}$). BMSCs cultured in high glucose DMEM alone (control) or in combination with RA treatment (10^{-5} mol/L) for 4 days as compared with 0 day cells. The experiment was repeated three times. Statistical significance was determined by a t-test. $^{*}P < 0.05$, versus 0 day.

measured by RIA. Before incubation, the culture medium of BMSCs at passage 3 contained a low concentration of E2. After exposure to a high glucose condition for 1 day, E2 levels were increased significantly. However, E2 content was not obviously altered after prolonged culture time (2–4 days). Similar results were observed in cultured BMSCs that were given a combined treatment with high glucose and RA. The maximal effect of RA was observed in BMSCs cultured for 48 hours, and the release of E2 significantly increased compared with the high glucose medium alone (Figure 3).

3.4. RIA for T and ASD. To investigate *de novo* synthesis of E2, we measured the levels of T and ASD in medium by RIA. After BMSC culture for 4 days and 1 day in high glucose medium alone or together with RA, the release of T and ASD significantly increased, respectively. However, there were no obvious differences in the levels of T and ASD between the high glucose group and combination group (Figures 4 and 5). In addition, prolonged culture time (2–4 d) had no effect on the release of ASD (Figure 5).

4. Discussion

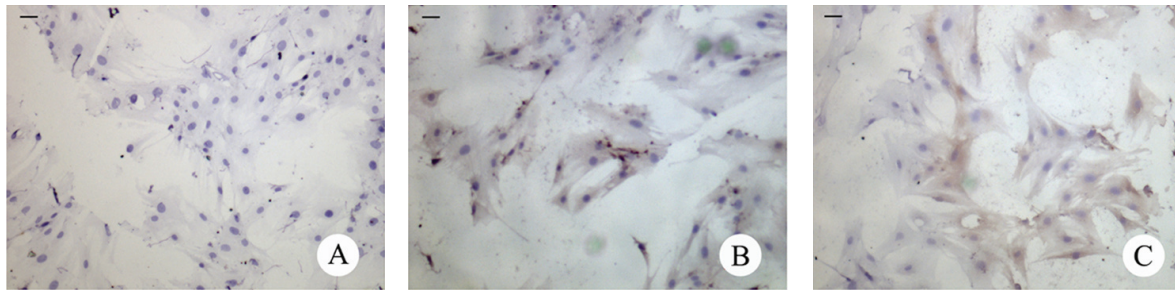
The high degree of stem cell plasticity provides a promising strategy for cell replacement therapy. During the past several years, a great deal of attention has been focused on the plasticity of BMSCs. Since BMSCs have tremendous differentiative potential, they can differentiate in vitro and in vivo into mature cells of the heart, liver, kidney, lungs, GI tract, skin, bone, muscle, cartilage, fat, endothelium, and brain. These BMSC-derived cells have been shown to contribute to clinical treatment of genetic disease or tissue repair [20–29]. In the present study, we investigated the ability of the BMSCs to generate steroidogenic cells and release E2 in vitro. Our study revealed that BMSCs cultured in high glucose DMEM with or without RA were capable of differentiating into cells that produced and secreted significant amounts of E2.

Under physiological conditions, E2 is produced *de novo* from cholesterol and synthesized by the ovary in a sequential manner. Steroidogenic granulosa and theca cells cooperate under gonadotropin control to produce estrogens by stimulating synthesis of steroidogenic enzyme messenger RNAs [11]. In the theca, under the influence of LH, cholesterol is converted to pregnenolone and metabolized through a series of substrates ending in androgen production. Theca cell-derived androgens transported to the granulosa cells of developing follicles, where they are aromatized to oestrogens by P450arom, the product of the *CYP19* gene, which is responsible for conversion of C19 steroids to estrogen [2, 6, 30]. In our culture systems, P450arom mRNA and protein were expressed in BMSCs, which also produced and released T and ASD. These results suggested that BMSCs could produce steroidogenic cells with the capacity for the synthesis of E2.

In recent years, some studies found that transfection of BMSCs from human and murine with steroidogenic factor 1 (SF-1, an essential factor for differentiation of the pituitary-gonadal axis) can transform BMSCs into steroidogenic cells, which produce various steroid hormones, including E2, and expressed mRNA for P450arom [16, 17]. When transplanted into immature rat testes, adherent marrow-derived cells were found to be engrafted and differentiated into steroidogenic cells that were indistinguishable from Leydig cells [17]. These results provided evidence that BMSCs were capable of differentiating into steroidogenic cells and represented a useful source of stem cells for cell transplantation therapy. In this study, without forced expression of SF-1, we demonstrated the ability of the BMSCs to spontaneously form steroidogenic cells and secrete E2 under a high glucose condition.

RA is well known as the biologically active form of vitamin A and has been shown to play an important role in normal embryonic development and maintenance of differentiation in the adult organism [31]. Previous studies showed that RA could induce BMSCs to differentiate into male germ cells [32] and stimulate E2 and T synthesis in rat hippocampal slice cultures [33]. Therefore, we examined the effects of RA on E2 and T biosynthesis in cultured female rat BMSCs. In the present study, BMSCs were incubated in the absence or presence of RA to investigate RA-induced differentiation of BMSCs to steroidogenic cells in vitro. Compared with high glucose medium alone, E2 secretion was stimulated by RA treatment without any increase in the levels of androgen, suggesting that RA, at least at a concentration of 10^{-5} mol/L, may promote the differentiation of BMSCs to estrogen-producing cells.

A great deal of efforts had been directed at understanding what role stem cells may play in the physiology and pathology of the mammalian female gonads [34]. Over the past few years, some studies found that bone marrow transplantation (BMT) generated immature oocytes and rescued long-term



(a) (b) (c)

Figure 2: Immunocytochemical staining of P450arom in rat BMSCs cultured for 4 days. (a), negative staining; (b), control group; (c), RA treatment group. P450arom was expressed in BMSCs cultured in a high glucose culture condition alone (control group) or in combination with RA treatment. Scale bar: 20 μm.

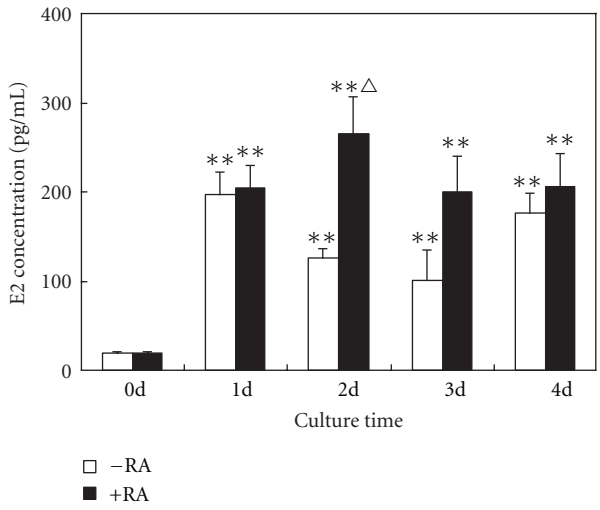


Figure 3: Measurement of E2 concentrations in culture media by RIA. Compared with 0 day cells, the release of E2 significantly increased in rat BMSCs cultured in a high glucose DMEM or in combination with RA for 1–4 days. The experiment was repeated three times. Values represent means ± SD ($n = 5$). $^{**}P < 0.01$, versus 0 day; $^{\triangle}P < 0.01$, versus high glucose DMEM.

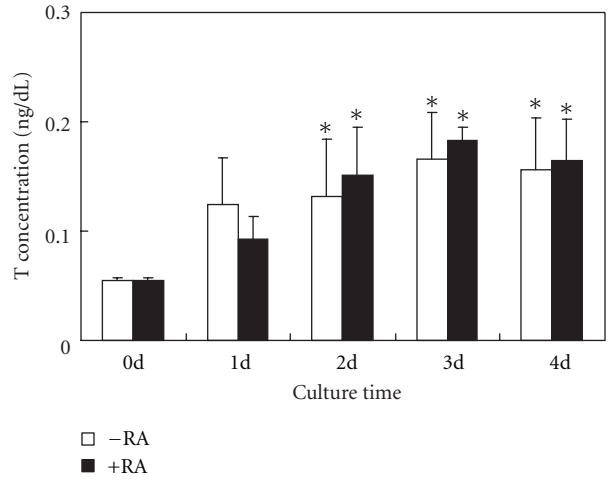


Figure 4: Measurement of T concentrations in culture media by RIA. Compared with 0 day cells, the release of T significantly increased in rat BMSCs cultured in a high glucose DMEM for 2–4 days. RA treatment did not obviously increase the levels of ASD. The experiment was repeated three times. Values represent means ± SD ($n = 5$). $^{*}P < 0.05$, versus 0 day; $^{**}P < 0.01$, versus 0 day.

fertility in a preclinical mouse model of chemotherapy-induced premature ovarian failure. Although all offspring were derived from the recipient germline, donor-derived oocytes were generated in ovaries of recipients after BMT [35]. Furthermore, MSC transplantation can improve ovarian function and structure damaged by chemotherapy, and the paracrine mediators secreted by MSC might be involved in the repair of damaged ovaries [36]. These results suggested that the potential of BMSCs for ameliorating female reproductive function was involved in reversal of both ovarian germline and somatic cell insufficiency.

In a previous report, mouse embryonic stem cells in culture developed into oogonia that could enter meiosis, recruit adjacent cells to form follicle-like structures, which expressed aromatase and secreted E2 [15]. In addition, mouse-induced pluripotent stem cells cocultured with ovarian granulosa cells in vitro could form granulosa cell-like cells and secret E2 [37]. BMSCs in our culture systems did not form follicle-like structures, nor did exhibit the morphology of mature ovarian cells. However, they expressed P450arom, suggesting that BMSCs have the ability to synthesize and to release E2, which may contribute to autologous transplantation therapy of BMSCs for hypogonadism.

5. Conclusion

In this study, we showed that female rat BMSCs cultured in high glucose DMEM with or without RA could express CYP19 and P450arom, and excrete T, ASD, and E2. These results indicated that the cultured BMSCs could produce steroidogenic cells with the capacity for E2 synthesis. This study would help to provide basis for clinical application of BMSCs in autologous cell transplantation therapy for patients with estrogen deficiency.

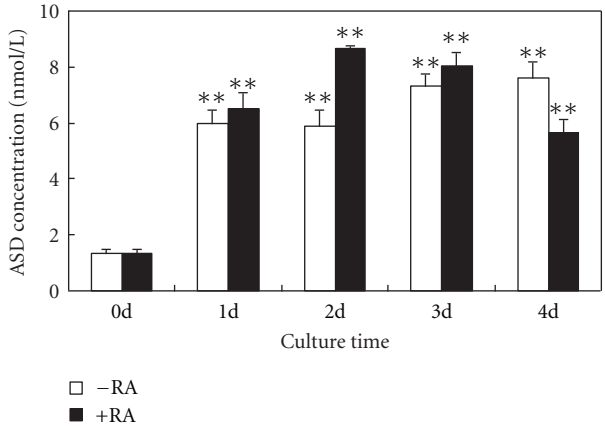


Figure 5: Measurement of ASD concentrations in culture media by RIA. Compared with 0 day cells, the release of ASD significantly increased in rat BMSCs cultured in a high glucose DMEM for 1–4 days. RA treatment did not obviously increase the levels of ASD. The experiment was repeated three times. Values represent means ± SD ($n = 5$). $^{*}P < 0.05$, versus 0 day; $^{**}P < 0.01$, versus 0 day.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

D. Zhang, B. Yang, and W. Zou equally contributed to this work.

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Review Article

Role of the Microenvironment in Ovarian Cancer Stem Cell Maintenance

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Despite recent progresses in cancer therapy and increased knowledge in cancer biology, ovarian cancer remains a challenging condition. Among the latest concepts developed in cancer biology, cancer stem cells and the role of microenvironment in tumor progression seem to be related. Indeed, cancer stem cells have been described in several solid tumors including ovarian cancers. These particular cells have the ability to self-renew and reconstitute a heterogeneous tumor. They are characterized by specific surface markers and display resistance to therapeutic regimens. During development, specific molecular cues from the tumor microenvironment can play a role in maintaining and expanding stemness of cancer cells. The tumor stroma contains several compartments: cellular component, cytokine network, and extracellular matrix. These different compartments interact to form a permissive niche for the cancer stem cells. Understanding the molecular cues underlying this crosstalk will allow the design of new therapeutic regimens targeting the niche. In this paper, we will discuss the mechanisms implicated in the interaction between ovarian cancer stem cells and their microenvironment.

1. Introduction

Ovarian cancer remains a challenging condition for both clinicians and scientists. Indeed, it often presents as an advanced metastatic disease; however most patients are treated with a combination of major debulking surgeries and chemotherapy to achieve complete cytoreduction (no tumor residue) [1]. The clinical course of patients with no residue at the end of the treatment remains unpredictable with a group of early recurrence (refractory patients) [2]. The clinical trials of targeted therapies (trastuzumab, imatinib, etc.) as well as dose intensifications or use of several agents have failed to significantly improve outcomes [3–6]. Finally, procedures such as intraperitoneal chemotherapy or hyperthermic intraoperative chemotherapy have only a slight effect on prognosis with significant increase in overall morbidity [7].

The biology of ovarian cancers also has striking features; over the last decade the heterogeneity of ovarian cancers among and within subtypes has been illustrated by transcriptomic and genetic profiling [8]. Many authors have presented prognosis signatures without a clear translation to the clinical setting [9]. Recently, a broad study by The Cancer Genome Atlas (TCGA) has demonstrated among other findings that serous ovarian adenocarcinoma could be clustered in 4 different subtypes without being able to relay them to prognosis [10]. The mutational spectrum of ovarian cancers seems to be limited with most genetic events happening at the copy number variation level. Metastatic lesions have a genetic profile different to primary lesions, again reflecting tumor heterogeneity [11]. However the specific biological features responsible for recurrences have not been clearly identified.

Recently, the concept of cancer stem cells (CSCs) has emerged as an alternative to the clonal theory of tumor evolution. Indeed among the heterogeneous populations constituting a tumor, a small proportion of cells (0.01% to 0.1%) have properties that mimic to certain extent normal stem

cell biology: (i) self-renewal with asymmetric and symmetric cell division; (ii) recapitulation of the tumor heterogeneity in immune-suppressed mice; (iii) ability to undergo serial passages *in vitro* and *in vivo* due to unlimited division potential [12]. The role and biology of ovarian cancer stem cells have been already illustrated in other comprehensive reviews [13, 14]. The tumor is now perceived as a complex structure where the tumor cells closely interact with the stroma, which provides protumoral and prometastatic cues [15]. Our group has demonstrated the role of mesenchymal stem cells in transferring multidrug resistance protein (MDR) or inducing a prometastatic phenotype of ovarian cancer cells [16, 17]. Thus, microenvironment might have a real role in the biology of ovarian cancer stem cells (OCSCs).

Here, we review the data about ovarian cancer stem cells and their interaction with the tumoral microenvironment. Understanding the molecular cues responsible for the crosstalk between the tumor and its stroma might help us design new therapeutic strategies aiming at disrupting specific prostemness tumor-stroma interaction rather than targeting tumor cells alone.

2. Ovarian Cancer Stem Cells

Genetic changes in regular stem cells might give rise to OCSCs [18, 19]. As the exact origin of ovarian cancer is still debated (ovarian surface epithelium versus fallopian tube) and its complexity is not limited to one subtype, characterization and definition of OCSCs have been really challenging. Besides, OCSCs can display different states (quiescent or proliferative) depending on the microenvironment and the cellular stresses such as chemotherapy which makes it more difficult to gather a unique definition [20, 21]. Currently surface markers or a particular phenotype (side population) are used to identify OCSCs.

The most commonly described marker is CD133. Different authors showed that $CD133^+$ from cell lines or primary xenografts had greater capacity to initiate tumors than $CD133^-$ [22, 23]. OCSCs were more comprehensively characterized by the combination of CD133 and the stem cells marker aldehyde dehydrogenase (ALDH) [24, 25]. Finally previously described CSCs markers CD44 and CD117 were used to characterize OCSCs. Cancer stem cells have the increased ability to be grown in 3D anchorage-independent culture setup as spheres (Figures 1(a) and 1(b)). The formation of primary and/or secondary sphere is currently routinely used to enrich and/or quantify the stem cell population [26]. The other striking feature of OCSCs is their chemoresistance and thus their potential role in residual and recurrent disease even if this has not been yet clinically demonstrated [22, 27, 28]. Indeed in ovarian cancer, $CD44^+CD117^+$ spheroids were resistant to chemotherapy and were able to initiate and propagate tumors in mice [22]. Similarly Luo et al. described that chemoresitsant $CD117^+$ cells isolated from xenografts displayed phenotypic feature of cancer stem cells such as serial transplantation and asymmetric division [29]. Recently, Gao et al. described that $CD24^+$ population expressed increased level of some stem cells genes such as *Nestin*, *β-catenin*, *Bmi-1*, *Oct4*, *Oct3/4*, *Notch1*, and *Notch4* compared to $CD24^-$ and displayed quiescence, chemoresistance, and tumor initiation [20].

One of the challenges is to determine the hierarchy of the different markers described. In mammary gland, a hierarchy of stem cells is described using the different breast cancer stem cells markers [30]. Such hierarchy is essential to understand and identify the factors which regulate CSCs self-renewal versus proliferation and differentiation. Recently, Burgos-Ojeda et al. proposed a hierarchy for the OCSCs where they hypothesized that a common ovarian cancer stem cell can undergo asymmetric division to give rise to two different early OCSC progenitors $ALDH^+CD133^+$ ($CD24^{+/-}$) or $CD44^+CD117^+$ ($CD24^{+/-}$). Each of these early OCSC progenitors can then produce intermediate progenitor cells by asymmetric division which could produce more differentiated tumors [14].

The molecular drivers of the hierarchy can represent potentially important therapeutic targets. Several studies described correlations between OCSC markers and patient prognosis. Zhang et al. reported a poor prognosis associated with CD133 expression from a tumor bank of over 400 ovarian cancers [31]. More recently, Steg et al. showed the presence of CD133, ALDH1A1, and CD44 at low number in primary samples. The same markers were increased on a sample collected after chemotherapy and reduced back to initial level in recurrent tumor samples, suggesting their role in recurrence [32]. Using all these stem cells markers as target in a clinical trial seems to be the logical next step. Unfortunately, while limited numbers of tumors express CD133 (34–40%) or CD177 (30–40%), CD24 and CD44 are expressed in numerous tumors but targeting these cells *in vivo* is limited by wide expression of these molecules in normal tissues [22, 25, 33].

Stemness relies on a very precise equilibrium between the stem cells and the components of the niche. Recently, stem cells and their niches were identified in mammalian tissue such as the nervous system, muscle satellite cells, and spermatogonial stem cells [34, 35]. Many studies described how the niche and the stem cell interact through tissue-specific molecular signaling in maintaining stemness and inducing expansion of the stem cell population. For example, recent studies have clearly demonstrated the role of the endothelial niche in the expansion and maintenance of stemness of hematopoietic stem cells [36]. Molecular cues from stromal cells or the extracellular matrix will provide the signaling to maintain and expand the stem cell phenotype.

The constitution of the tumor stroma brings another level of complexity. While for the sake of clarity we have separated different elements, most of them remain closely related and dependent.

3. Stromal Environment and Stemness

Cancer lesions are often perceived as never healing wounds with an inflammatory microenvironment. The infiltrating inflammatory cells include tumor promoting and tumor killing subtypes. Much molecular signaling can be hijacked

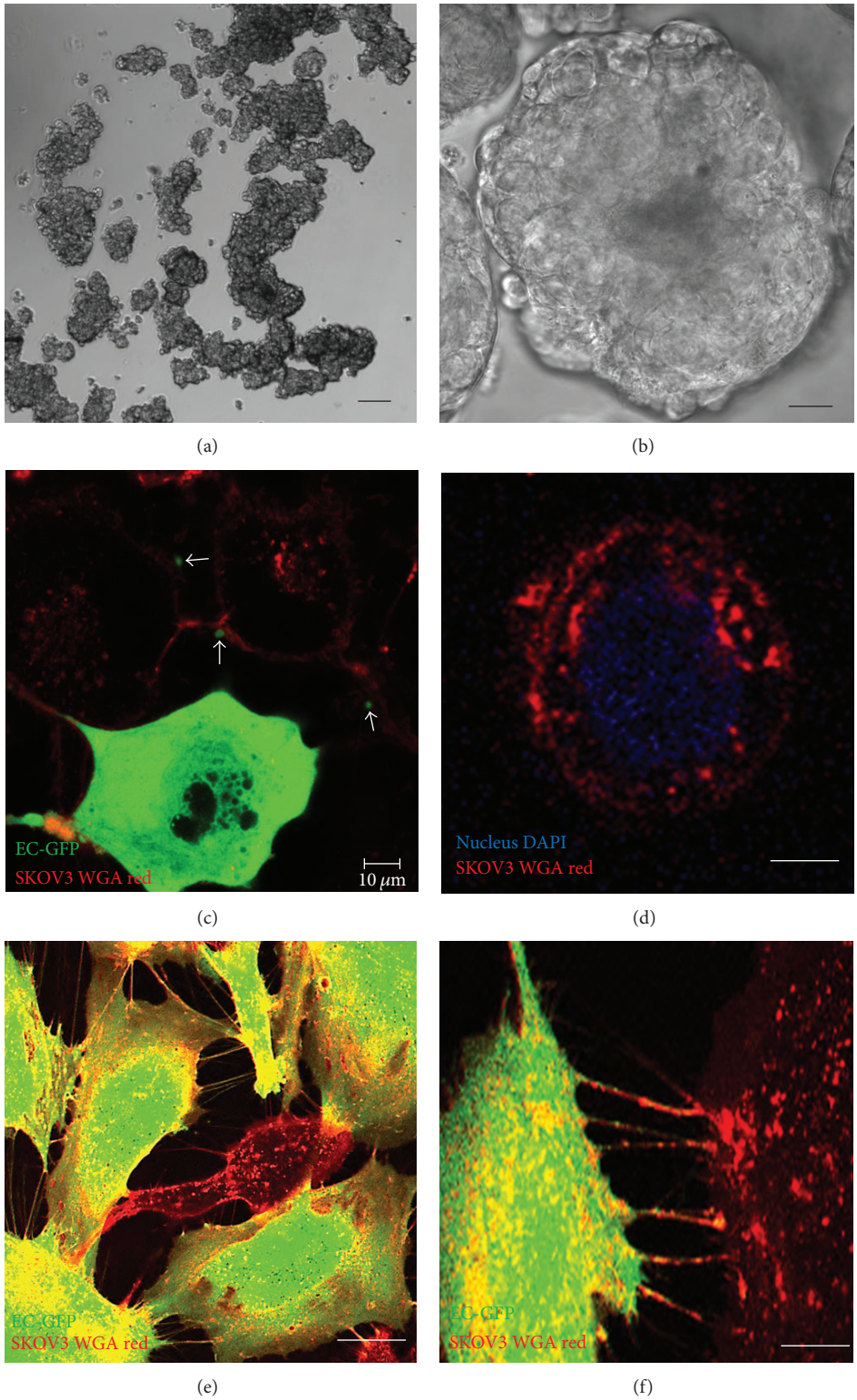

Figure 1: Ovarian cancer cells. (a) Ovarian cancer cell lines, SKOV3 in spheroid culture. Scale bar 100 μm. (b) Confocal imaging of an SKOV3 sphere. Scale bar 20 μm. (c) Coculture of endothelial cells- (ECs-) GFP (green) and SKOV3 (red). ECs secrete microparticles (arrows) which are uptaken by ovarian cancer cells. Scale bar 10 μm. (d) Microparticles from ECs were tagged with WGA-alexa fluor 594 and added to culture of SKOV3 during 6 hours. Ovarian cancer cells are able to uptake ECsmicroparticles. Scale bar 5 μm. (e)-(f) Coculture of ECs-GFP (green) and SKOV3 (red). Both cell types are interconnected by tunneling nanotubes. Scale bar 10 μm.

by cancer cells and enhance tumorigenesis and progression toward a metastatic phenotype [37]. The stromal inflammatory reaction constitutes an environment containing many bioreactive molecules such as proliferative and survival signaling (EGF, FGF, HGF, IGF, or IL-6) that could enable CSCs maintenance and expansion. Moreover, many of these cytokines have been implicated in the occurrence of epithelial to mesenchymal transition (EMT) in many tumor types [38]. Recent lines of evidences have linked EMT phenotype to stemness [39, 40]. Therefore, we could assume that an inflammatory microenvironment will enable a subfraction of the tumor cells to gain/maintain a mesenchymal phenotype permissive to maintenance of stemness.

The tumor cells also participate to the inflammatory stroma as the upregulation of inflammatory molecules has been documented in the literature [11]. Ovarian cancers overexpress LL-37 (leucine, leucine 37) which is a member of the cathelicidin family of antimicrobial polypeptides. While LL-37 does not act directly on ovarian cancer cells, it attracts mesenchymal stem cells (MSCs) into ovarian tumor xenografts. MSC treated by LL-37 secreted increased amount of IL-1 receptor antagonist, IL-6, IL-10, CCL5, and VEGF and had a proangiogenic effect [41]. Long et al. demonstrated that $CD133^+$ cells had increased expression of the chemokine CCL5 and its receptors, CCR1, CCR3, and CCR5, compared to $CD133^-$ [42]. CCR5 mediated nuclear factor κB (NF-κB) dependent MMP9 secretion. These studies demonstrate the complex crosstalk relying on multiple cytokines and resulting in a permissive niche that will provide all molecular cues for maintenance and expansion of ovarian cancer stem cells.

The tumor stroma constitutes a hypoxic microenvironment before the appropriate signaling cues are able to induce neoangiogenesis. Hypoxia maintains and even upgrades stem cell characteristics [43, 44]. Under anaerobic conditions, glycolysis is favored and only a small amount of the pyruvate will be directed toward the mitochondria. The glycolytic metabolism activates tumor suppressor genes and oncogenes such as p53, RAS, or MYC. These oncogenes can activate HIF1α and HIF2 associated with activation of pluripotency marker genes such as *OCT4, SOX3*, and *KLF4* [45]. Liang et al. studied the effect of hypoxia on ovarian cancer stem cells [46]. They demonstrated increased ability for OCC to form spheres and colonies. $CD44^{bright}$ displayed higher level of the stemness transcription factors OCT3/4 and Sox2 when cultured in hypoxic condition. In another study, CD44 and CD133 expression was increased through the Sox2 and OCT3/4 regulation in two different ovarian cancer cell lines (ES-2 and OVCAR3) [47].

4. Cellular Elements of the Stroma

The inflammatory stroma attracts other cell types such as mesenchymal stem cells and endothelial cells [48]. The protumoral and premetastatic roles of both cell types have been widely described in the literature. However few studies point out their interaction with cancer stem cells. There are many models of cellular interaction mediated by direct contact (tunneling nanotubes, synapses, trogocytosis) or microparticle mediation (Figures 1(c) to 1(f)). Their role in the maintenance of stemness remains to be clearly established. Mitsui et al. described increased expression of the $CD133^+$ and sphere formation when Yolk sac ovarian cancer stem cells were cocultured with peritoneal MSCs [49]. The $CD133^+$ cells displayed increased migration and invasion in culture with the MSCs. The crosstalk in this study seemed mediated through the SDF1/CXCR4 axis. In a more comprehensive approach, McLean et al. showed that cancer-associated MSCs (CA-MSC) had greater ability to increase tumor growth compared to normal MSCs [15]. They demonstrated that the CA-MSCs had abnormal BMP production. Treatment with recombinant BMP2 had the ability to increase ovarian cancer cell line stem cell population as defined by ALDH and $CD133^+$ (up to 60%). Similarly the treatment of primary derived spheres with BMP2 also induced a 3.2- to 4.4-fold increase of the $ALDH^+$ population.

As illustrated above, there is a strong interaction between cancer cells and the different elements of the niche. This crosstalk has a strong role in tumor biology as it participates to the plasticity of the tumor cells. Abelson et al. used the human embryonic stem cell derived experimental platform [50]. They isolated different clones from a single clear cell ovarian tumor. They showed that while some clones were not able to grow in a classical xenograft model their injection in a hESC-derived teratoma produced a tumor recapitulating the different cell populations of the primary tumor. More interestingly, they demonstrated that the microenvironment could switch the non-stem-cell $EPCAM^+CD44^-$ population to a stem cell $EPCAM^+CD44^+$ population.

The microenvironment-dependent phenotypic plasticity has great therapeutic implication. This could result in failure of treatments targeting a single stable self-renewing clone. One option might then be to use multimodal approach to balance the equilibrium between the self-renewing and rapidly proliferating populations. Maintaining a permanent low level of self-renewing cell population will allow having a chronic disease rather than a rapidly lethal tumor. Many questions need to be answered such as whether self-renewal is a durable state rather than a dynamic niche dependent which is supported by many findings in the literature [51, 52]. This might also be dependent on tumor type as, for example, the Morrison groups demonstrated that nonputative stem cell population could give rise to a tumor in xenograft models when the microenvironment was modulated [53]. These findings were however obtained in the melanoma model and mice malignant peripheral nerve sheath tumors both originating from the neural crest.

The role of endothelial cells in cancer stemness has not been yet clearly identified. Shank et al. have studied the action of metformin on ovarian cancer stem cells [54]. They demonstrated that metformin reduced $ALDH^+$ CSC *in vitro* and *in vivo* and inhibited the growth of ovarian tumor spheres. One of the action of metformin resulted in decreased microvascular density consistent with previous studies [55, 56]. The data in the literature demonstrates that CSCs are highly angiogenic [25, 57] and that endothelial cells participating to neoangiogenesis provide essential growth factors for OCSCs [58].

5. Cytokines Environment and Global Crosstalk

Cytokines play an essential role in intercellular communications as described above. Many of them regulate stem cell phenotype in a variety of contexts ranging from normal development to neoplasia. Cao et al. showed that TGFβ which is highly secreted in the ovarian cancer microenvironment induces tissue transglutaminase (TG2) expression and its enzymatic activity [59]. The treatment by TGFβ induced spheroid formation enabling peritoneal dissemination. They demonstrated that TG2 was responsible for an EMT-mediated increase of the $CD44^+CD117^+$ population. Interestingly the effect of TGFβ was greater when the ovarian cancer cells were cultured on fibronectin once again demonstrating the additive role of the different component of the microenvironment.

Among the different cytokines c-kit's role as a stem cell factor has already been described and plays a particular role. Indeed c-kit-mediated pathways are activated in cancer [60, 61]. Ovarian tumor abnormal expression of c-kit has been associated with poor prognosis. Chau et al. demonstrated increased expression of c-kit after enrichment for OCSC [62]. They showed that c-kit knockdown inhibited sphere formation. They displayed that hypoxia increased c-kit expression which in turn induced overexpression of the ABC drug ABCG2 transporter through the Wnt/β-catenin pathway, leading to chemoresistance in OCSC. One of the interesting findings of this group is that multiple rounds of chemotherapy seemed to enrich for OCSC harboring a high chemoresistance profile.

The crosstalk between cancer cells and the microenvironment has been illustrated in many other contexts. In a study by Ko et al., the authors showed a poor prognosis of tumors with HOXA9 expression [63]. *In vitro*, HOXA9 was not able to induce autonomous tumor cell growth. However ovarian cancer HOXA9 expression induced a cancer-associated fibroblasts phenotype for the peritoneal fibroblasts which stimulated OCC and endothelial cell growth. HOXA9 activated the transcription of TGFβ2 which acted in a paracrine manner on peritoneal fibroblasts which in turn upregulated the protumoral panel of cytokines (CXCL12, IL-6, and VEGF-A) expression. This study illustrates the promotion of a permissive microenvironment which will provide the optimal ground for tumor growth. In accordance with these data, Alvero et al. demonstrated a very intricate relation between OCSC and the microenvironment [64]. They demonstrated that ovarian cancer stem cells participated to blood vessels and acquired markers of endothelial cell such as CD34 and VE cadherin. Interestingly, the process was not relying on VEGF but IKKβ/NFκb. While these data need to be confirmed by more functional studies demonstrating the ability of these cells to act as endothelial cells, the participation of the ovarian cancer stem cells to blood vessels beyond underlying their crosstalk with the endothelium could suggest resistance pathways to anti-VEGF-based targeted therapies.

6. Epithelial to Mesenchymal Transition (EMT)

Ovarian cancer represents a heterogeneous group of tumors with distinct clinical features, genetic alterations, and tumor behaviors. The phenomenon of EMT has been widely studied in ovarian cancer. The authors have suggested that OCC can undergo EMT to detach and MET to develop a metastatic nodule. These data should therefore be investigated in the context of OCSCs. Indeed targeting EMT has been suggested, as a potential treatment [65]. Several studies in breast cancer have demonstrated that EMT induced an increase in the CSCs population defined as $CD44^+CD24^-$ [66–68]. However, in 2012, Sarrio et al. indicated that a mesenchymal-like phenotype did not correlate with the acquisition of global stem cells/progenitors characteristics in breast cancer [69]. Supporting these findings Celià-Terrassa et al. showed that the acquisition of mesenchymal features (correlated with the loss of their epithelial properties) by cancer cells occurred at the expense of their self-renewal potential, in prostate and bladder cancer [70]. The body of data suggests that stemness might be a plastic phenotype that could depend both on tumor type and global stromal context.

In ovarian cancer, only a few studies have focused on the link between EMT and OCSCs. Recently, Jiang et al. demonstrated that the ovarian cancer cell lines displayed a side population with mesenchymal traits and typical mesenchymal genes. Inhibition of EMT process by Snail1 silencing decreased this side population occurrence and affected its invasive capacity and tumorigenicity *in vivo* [71]. Dahl Steffensen et al. established a correlation between the percentage of epithelial OCSCs and survival in early-stage ovarian cancer (FIGO I/II) in a cohort of 117 patients [72]. Concordant with findings in other epithelial cancers [70], Yin et al. demonstrated the ability of the OCSCs to generate peritoneal metastasis in an *in vivo* model. Furthermore they showed that TWIST-1 (a major transcription factor implicated in EMT) is constitutively degraded by the proteasome in OCSCs [73]. They suggest that OCSCs could be a source of ovarian cancer metastasis through balance of EMT/MET.

7. Other Tumors

Ovarian cancer is not the only one to be maintained by a subpopulation of cells that display stem cell properties, mediate metastasis, and contribute to treatment resistance. A similar hierarchy governs many solids tumors, including breast [74], pancreas [75], glioblastoma [76], and prostate [77]. They are defined by different cell surface markers and characterized by specific phenotypic traits.

Several markers have been proposed in the literature to identify CSCs in many human cancers, but to date there is still no gold standard to define CSCs, leading to the hypothesis that the CSC phenotype might be dynamically switched [78]. Compared with the hematopoietic tumors, the properties of CSCs in solid tumors remained relatively undefined until recently. The first solid CSCs were identified in breast cancer by Al-Hajj et al. in 2003 with two surface markers $CD44^+/CD24^{-/low}$ [74]. Further characterization of breast CSCs was established using ALDH1 [79], mammosphere assay [80], and transplantation into immunodeficient mice [81]. For instance, CD133 has been described to be one of the most recurrent CSC markers in a number of solid malignancies, including brain tumor [82], prostate

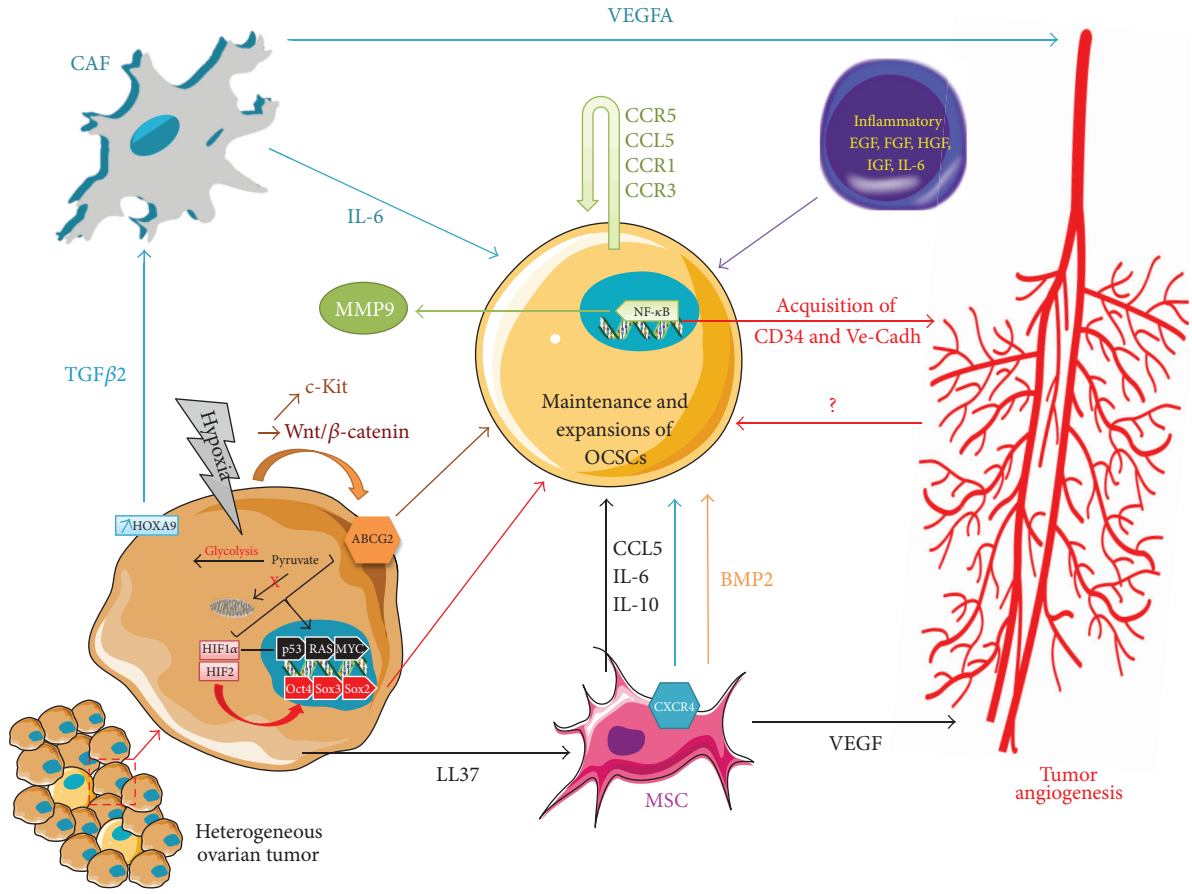

FIGURE 2: Maintenance and expansion of ovarian cancer stem cells by the tumor microenvironment. Schematic of the potential regulatory actors of the microenvironment in the maintenance of ovarian cancer stem cells.

carcinoma [83], hepatocellular carcinoma [84], colorectal cancer [85, 86], and lung cancer [87].

All those CSCs are regulated by, and in turn regulate, cells within the tumor microenvironment. Recently, an emerging area of research supports that CSCs may promote tumor angiogenesis. As mentioned in glioma CSCs by Bao et al., the VEGF expression in $CD133^+$ cells was 10–20-folds upregulated, combined with a significant increase in vascular density demonstrated by CD31 staining [57]. Furthermore, they described that therapy with VEGF antibody (bevacizumab) could reduce CSC-induced vascular endothelial cell migration and tube formation. They finally demonstrated, *in vivo*, that bevacizumab inhibited specifically the tumor growth of CSC-derived xenograft. Other studies support this finding that CSCs contribute to tumor vascular development in glioma [88, 89]. Cytokines produced by endothelial cells directly regulate CSCs contributing to their maintenance and their proliferation [90, 91]. This cross-regulation between CSCs and endothelial cells seems to be common in various solid tumor such as breast cancer [92], colon cancer [93], or brain tumor [94].

CSCs are also known to play a role in metastatic disease. Indeed, in breast cancer cells, CSCs can go through EMT via activation of Hedgehog (Hh), Wnt, Notch, or TGFβ (transforming growth factor-β) leading to the upregulation of a group of transcriptional factors that drive EMT, resulting in the transformation of epithelial-like CSCs into cells with aggressive mesenchymal-like phenotypes [95]. All these pathways are induced by extracellular factors related to tumor microenvironment such as matrix metalloproteinase (MMP) family proteins [96]. The involvement of CSCs mesenchymal transition in metastatic spread was described in many tumors including head and neck squamous cell carcinoma [97], colorectal cancer [98], prostate cancer [99], and pancreatic cancer [100].

Inside their complex microenvironment, CSCs are also in close interaction with MSCs or tumor-associated macrophage [101]; however their precise interaction remains to be elucidated.

8. Conclusion

The recent discovery of cancer stem cells in solid tumors mimicking leukemia has added another level of complexity to tumor heterogeneity. Indeed, the tumor now appears to be constituted by different cell populations harboring different phenotypes. Moreover, data presented above argue for a tremendous plasticity induced not only by clonal evolution but also by the interaction between the cancer cells and their microenvironment. It is difficult today to have a clear

perception of the essential molecular hubs as the number of studies per disease is still limited and many studies have addressed few molecules rather than broad pathways (Figure 2). Accumulating evidence reveals that the composition of tumor microenvironments may define CSCs role throughout the different steps of carcinogenesis.

These findings have several consequences for patients' management. Indeed, as the tumor is now perceived as a dynamic structure, new factors (stem cell fraction, presence of stromal elements, and immune infiltrate) might be useful to predict prognosis. More importantly, we might have to consider absence of chemosensitivity rather than chemoresistance. Indeed, in the clonal theory of tumor evolution, upon treatment clones were able to develop resistance. In a more global approach, we could consider that at a time point some tumoral cells might not be sensitive to chemotherapy protected by their stemness and/or their interaction with the tumor microenvironment. Obviously, clonal and stem cell theories are not mutually exclusive, and under selective pressure the tumor plasticity could shift through clonal selection.

Therefore, there is a great need to gain a comprehensive understanding of the networks governing tumor plasticity, in particular the interaction between the stem cell compartment and the stroma. This will drive the design of new therapeutic approaches disrupting the tumor-stroma interaction to reduce tumoral plasticity.

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Research Article

In Vitro Culture-Induced Pluripotency of Human Spermatogonial Stem Cells

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Unipotent spermatogonial stem cells (SSCs) can be transformed into ESC-like cells that exhibit pluripotency *in vitro*. However, except for mouse models, their characterization and their origins have remained controversies in other models including humans. This controversy has arisen primarily from the lack of the direct induction of ESC-like cells from well-characterized SSCs. Thus, the aim of the present study was to find and characterize pluripotent human SSCs in *in vitro* cultures of characterized SSCs. Human testicular tissues were dissociated and plated onto gelatin/laminin-coated dishes to isolate SSCs. In the presence of growth factors SSCs formed multicellular clumps after 2–4 weeks of culture. At passages 1 and 5, the clumps were dissociated and were then analyzed using markers of pluripotent cells. The number of SSEA-4-positive cells was extremely low but increased gradually up to ~ 10% in the SSC clumps during culture. Most of the SSEA-4-negative cells expressed markers for SSCs, and some cells coexpressed markers of both pluripotent and germ cells. The pluripotent cells formed embryoid bodies and teratomas that contained derivatives of the three germ layers in SCID mice. These results suggest that the pluripotent cells present within the clumps were derived directly from SSCs during *in vitro* culture.

1. Introduction

Embryonic germ cells (EGCs), which can be derived from fetal unipotent primordial germ cells (PGCs), are pluripotent and have expression patterns of cell surface and gene markers similar to those of embryonic stem cells (ESCs). These markers include alkaline phosphatase, OCT-4, SSEA-4, NANOG, TRA-1-60, and REX-1. Other important characteristics, such as multicellular colony formation, maintaining normal and stable karyotypes, the ability to proliferate continuously, and the ability to differentiate into all three embryonic germ layers, can be acquired during *in vitro* induction [1]. Although it has been suggested that PGCs are typically unipotent and are able to produce only germ cells [2], several studies have shown that a small number of PGCs express OCT4 and NANOG during various stages of prenatal development. These results provide evidence that there exists a population of multipotent PGC. In contrast to the induction of pluripotent stem cells (iPSCs), this type of induction was a solely culture-induced procedure and did not rely on the introduction of exogenous transcription factors.

Spermatogonial stem cells (SSCs) are derived from PGCs during the neonatal period and can self-renew and produce large numbers of differentiating germ cells that become spermatozoa throughout adult life. Recently, some groups reported that SSCs obtained from neonatal and adult mouse testes can be induced to form multipotent SSCs (mSSCs) or multipotent germline stem cells (mGSCs) during *in vitro* culture, and these cells may have a pluripotency similar to that of ESCs [3, 4]. In mice, mSSCs (mGSCs) are phenotypically similar to ESC/EG cells except with respect to their genomic imprinting pattern. These stem cells can differentiate into various types of somatic cells *in vitro* and can produce teratomas *in vivo* [3]. These multipotent cells

were isolated and established from adult mouse testes using genetic selection, and the rate of establishment of cell lines was about 27% [4]. Additionally, another group established a similar type of multipotent cells derived from $GPR125^+$ spermatogonial progenitor cells, and derivatives of the three germ layers (contractile cardiac tissues *in vitro* and formed functional blood vessels *in vivo*) have been generated [5]. These results suggest that stem cells in the germline lineage may retain the ability to generate multipotent cells [4].

Conrad et al. reported that human adult germ-line stem cells (haGSCs) from testicular tissue can be induced to form ESC-like cells that display multipotency *in vitro* [6]. Several researchers have also reported the establishment of human mSSC lines with different morphologies in previous papers [7–9]. Those studies were performed with various culture systems, and the findings for human adult testicular tissue remain questionable [10–12]. In 2010, Ko et al. reported that clusters of cells from human testicular fibroblasts (hTFCs) can be easily established from human testicular cultures. Those cells that were not pluripotent were found to be morphologically similar to haGSCs [13]. It was believed that the controversy regarding the characterization of human mSSCs was primarily due to a lack of a protocol for direct induction from well-characterized SSCs. Recently, our group reported that highly pure human SSCs were isolated using a gelatin/laminin-coated dish. These cells proliferated under exogenous feeder-free culture conditions, and then their functions were characterized [14]. The aim of the present study was to identify and isolate human pluripotent SSCs derived from the long-term *in vitro* culture of well-characterized human SSCs.

2. Materials and Methods

2.1. Patient Samples. Testicular tissues were donated from obstructive azoospermic (OA) patients subjected to multiple testicular sperm extraction (TESE)-intracytoplasmic sperm injection (ICSI) treatment. When sperm were found in the dissected samples, the testicular material remaining after clinical requirements was donated for this study after obtaining the patient's consent. This study was approved by the Institutional Review Board of the CHA Gangnam Medical Center, Seoul, Korea.

2.2. Isolation and In Vitro Culture of SSCs. The isolation and culturing of human SSCs was performed as described in our previous report [14]. Briefly, the testicular tissues of 18 OA patients were placed in 10 mL of enzyme solution A containing 0.5 mg/mL type I collagenase (Sigma-Aldrich, St. Louis, MO), 10 μg/mL DNase I (Sigma-Aldrich), 1 μg/mL soybean trypsin inhibitor (Gibco/Invitrogen, Grand Island, NY), and 1 mg/mL hyaluronidase (Sigma-Aldrich) in Ca^{++}/Mg^{++}-free PBS and incubated for 20 min at room temperature (~25°C). After the peritubular cells were removed in the washing step, the seminiferous tubules were re-dissociated in 10 mL of Enzyme Solution B containing 5 mg/mL collagenase, 10 μg/mL DNase I, 1 μg/mL soybean trypsin inhibitor, and 1 mg/mL hyaluronidase in Ca^{++}/Mg^{++}-free PBS and incubated for 30 min at 37°C. After incubation, the sperm in the dissociated testicular cell samples were removed using a modified two-gradient (35%–70%) Percoll method. The recovered testicular cells were then plated and incubated on uncoated dishes in Germ Cell Culture Medium I, which consisted of DMEM (Gibco/Invitrogen) containing 20% FBS (Gibco/Invitrogen), 10 μmol/L 2-mercaptoethanol (Gibco/Invitrogen), 1% non-essential amino acids (Gibco/Invitrogen), and 10 ng/mL rat GDNF (R&D systems), at 37°C under a humidified atmosphere of 5% CO_2 in air. Over the following 48 hours, unattached cells were harvested and replated on laminin-coated dishes in Germ Cell Culture Medium II, which consisted of StemPro-34 SFM (Invitrogen) supplemented with 6 mg/mL D(+)glucose, 5×10^{-5} M β-mercaptoethanol, 1 μM d(L)-lactic acid, 2 mM L-glutamine, 30 μM pyruvic acid, 10^{-4} M ascorbic acid, 60 ng/mL progesterone, 30 ng/mL β-estradiol (Sigma-Aldrich), 0.2% BSA (ICN Chemicals), 100 U/mL penicillin, 100 μg/mL streptomycin, 1 × insulin-transferrin-selenium (ITS) supplement, 1 × MEM vitamin solution, 1 × MEM non-essential amino acids, 20 ng/mL mouse EGF, 10 ng/mL human bFGF (Invitrogen), 1% KSR (Invitrogen), 10 ng/mL rat GDNF (R&D systems), and 10^3 U/mL LIF (Chemicon, Billerica, MA) (modified from [14]). To obtain highly pure SSCs, un-attached cells collected after a 4-h incubation were re-sorted by magnetic activated cell separation (MACS) using an anti-CD9 antibody. The isolated SSCs slowly proliferated and then formed slightly attached clump-like structures (≥10 cells) on the bottom of dishes 2–4 weeks after seeding. During culture, approximately 80% of the medium was changed carefully every other day under a stereomicroscope to avoid the loss of floating clumps. Only clumps were collected. The clumps were dissociated by trypsinization and then re-plated every 2 weeks using the same medium. After every passage, cells clumps were divided into two groups. One group was fixed or sampled for characterization, and the other group was passaged using the method previously described.

2.3. Characterization of SSCs from In Vitro Culture. To characterize isolated highly pure SSCs and to investigate the relative expression levels of multipotent markers in the SSC clumps, we performed immunocytochemistry using the SSC markers GFR α1 (Chemicon International) and CD9 (Chemicon international) and the pluripotent stem cell markers OCT-4 (Santa Cruz), SSEA-4 (Chemicon international), TRA-1-60 (Chemicon international), and TRA-1-81 (Chemicon international). The samples were washed three times in DPBS with 5% FBS and were then fixed in paraformaldehyde (4% v/v in DPBS) for 24 hours. For permeabilization, the cell clumps were incubated in 0.1% Triton X-100 in DPBS for 1 hour. After washing three times with DPBS, the nonspecific binding of antibodies was suppressed by incubating the cells in blocking solution (4% normal goat serum in DPBS) for 30 min at room temperature. After washing three times with PBS, immunocytochemical staining was performed by incubating the fixed samples with primary antibody diluted 1 : 200–1 : 500 with DPBS containing 0.1%

Tween-20 and 1% BSA for 60 min at room temperature or overnight at 4°C. Immunoreactive proteins were then detected using CY3- or FITC-conjugated secondary antibodies diluted 1 : 500 with DPBS for 60 min at room temperature. Finally, samples were counterstained with 1 μg/mL 4′,6′-diamidino-2-phenylindole (DAPI; Sigma). Following multiple washes, samples were mounted in Vectashield mounting medium (Vector laboratories, Burlingame, CA). The staining was viewed using an inverted confocal laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) with fluorescence at a 400x magnification. Micrographs were stored in LSM (Zeiss LSM Image Browser version 2.30.011; Carl Zeiss Jena GmbH, Jena, Germany).

Alkaline phosphatase activity was assessed by histochemical staining. Cells were fixed in 4% paraformaldehyde at room temperature for 1 min, washed twice with PBS and stained with an alkaline phosphatase substrate solution (10 mL FRV-Alkaline Solution, 10 mL Naphthol AS-BI Alkaline Solution; Alkaline Phosphatase kit, Sigma-Aldrich) for 30 min at room temperature. Alkaline phosphatase activity was detected colorimetrically (red) by light microscopy.

2.3.1. RT-PCR. RT-PCR was performed to assess the expression of multipotent marker genes, specifically, *OCT4, NANOG,* and *Integrin α6*, in clumps from SSCs. Total RNA was extracted from 100 colonies using the TRIzol method (Gibco). Amplification was performed in a 20 μL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 2 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.25 mmol/L dNTP, 3–5 pmol of each primer, and 1.25 IU Taq polymerase (Gibco). The following genes were amplified using the primers indicated in parentheses: *OCT-4* (F: 5′-GGA AAG GCT TCC CCC TCA GGG AAA GG-3′, R: 5′-AAG AACA TGT GTA AGC TGC GGC CC-3′, 460 bp, GenBank accession number NM002701); *NANOG* (F: 5′-CCC ATC CAG TCA ATC TCA-3′, R: 5′-CCT CCC AAT CCC AAA CAA-3′, 565 bp, GenBank accession number NM024865); *Integrin α6* (F: 5′-GGG AGC CTC TTC GGC TTC TC-3′, R: CAC ATG TCA CGA CCT TGC CC-3′, 286 bp, GenBank accession number NM000210) and 18S ribosomal RNA (F: 5′-TAC CTA CCT GGT TGA TCC TG-3′, R: 5′-GGG TTG GTT TTG ATC TGA TA-3′, 255 bp, GenBank accession number K03432). PCR was initiated with a denaturation step at 94°C for 5 min, followed by 35–40 cycles of 30 s at 94°C, 30 s at 55–60°C, and 30 s at 72°C. A final extension step for 10 min at 72°C completed the amplification reaction, after which the products were separated by 1.5% agarose-gel electrophoresis. Negative controls included mock transcription without mRNA and PCR with distilled deionized water.

2.4. Flow Cytometry. SSC clumps were dissociated in trypsin-EDTA and resuspended in PBS containing 2% FBS. Then, the cells were incubated with APC-conjugated antibody to SSEA-4 (BD/Pharmingen) for 60 min at 4C. Finally, the cells were placed in the flow cytometer (Becton Dickinson FACS IV San Jose, CA, USA) for analysis. Cells without antibody staining were used as negative controls.

2.5. Karyotype Analysis. Chromosome spreads were prepared as described [15]. Briefly, SSCs were treated with 0.06 μg/mL colcemid (Invitrogen) for 2–4 h, trypsinized, incubated in 0.075 M KCl for 10 min, and fixed in Carnoy's fixative. The chromosome number and banding patterns were analyzed with a 300–500 band resolution.

2.6. EB Formation from SSCs. After 5 passages, over 200–400 SSC clumps cultured in HEPES-buffered DMEM/F-12 (Gibco) supplemented with 10 μg/mL ITS (Gibco), 10^{-4} mol/L vitamin C (Sigma), 10 μg/mL vitamin E (Sigma), 3.3×10^{-7} mol/L retinoic acid (Sigma), 3.3×10^{-7} mol/L retinol (Sigma), 1 mmol/L pyruvate (Sigma), 2.5×10^{-5} IU recombinant human FSH (Gonal-F; Serono), 10^{-7} mol/L testosterone (Sigma), 1 × antibiotic-antimycotic (ABAM, containing penicillin, streptomycin and amphotericin B; Gibco), and 10% bovine calf serum (Hyclone), for spontaneous *in vitro* differentiation [6]. SSCs clumps were transferred to 1.0 mL of differentiation culture medium in a 24-well dish and were cultured for up to 4 weeks at 37°C in a humidified atmosphere of 5% CO^2 in air. The medium was replaced on alternate days. After culturing, the EBs were fixed in 10% neutral buffered formalin, embedded in paraffin, stained with hematoxylin and eosin (H&E) and examined immunocytochemically. The endoderm marker α-fetoprotein (Chemicon international), the ectoderm marker nestin (Chemicon international) and the mesoderm marker cardiac troponin I (Chemicon international) were used.

2.7. Teratoma Formation from Pluriotent SSCs. Pluripotency was determined by harvesting ~2,000 SSC clumps (~2×10^5 cells) and injecting them subcutaneously into the back of 4- to 8-week-old severe combined immunodeficient (SCID) mice (CB 17 strain; Jackson Laboratory, Bar Harbor, ME) using a sterile 26 G needle. After 12 weeks, the resulting tumors were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 5-μm serial sections, H&E-stained and immunocytochemically examined. The human specific markers, α-fetoprotein (Chemicon international), MAP-2 (Chemicon international) and STEM 121 (Stem-121, Stem Cells Inc., Cambridge, UK) were used.

3. Results

3.1. Morphology and Karyotype of In Vitro Cultured SSCs. Significant staining for pluripotent marker (SSEA-4) was detected in hESCs. But testicular tissue did not express this marker (Figure 1(a)). In the primary culture after enzyme treatment, seeding cells exhibited positive signal of GFR α1. However, SSEA-4, a pluripotent marker, was not detected in those cells (Figure 1(b)). After the selection procedure, the isolated and cultivated SSCs exhibited high expression levels of SSC marker, as described in our previous report [14]. Significant staining for SSC markers (CD9 and GFR α1) was detected at a high level in the SSC clumps (Figure 1(c)). SSCs were well maintained and proliferated in culture, ultimately forming small clumps (>10 cells), and were passaged by

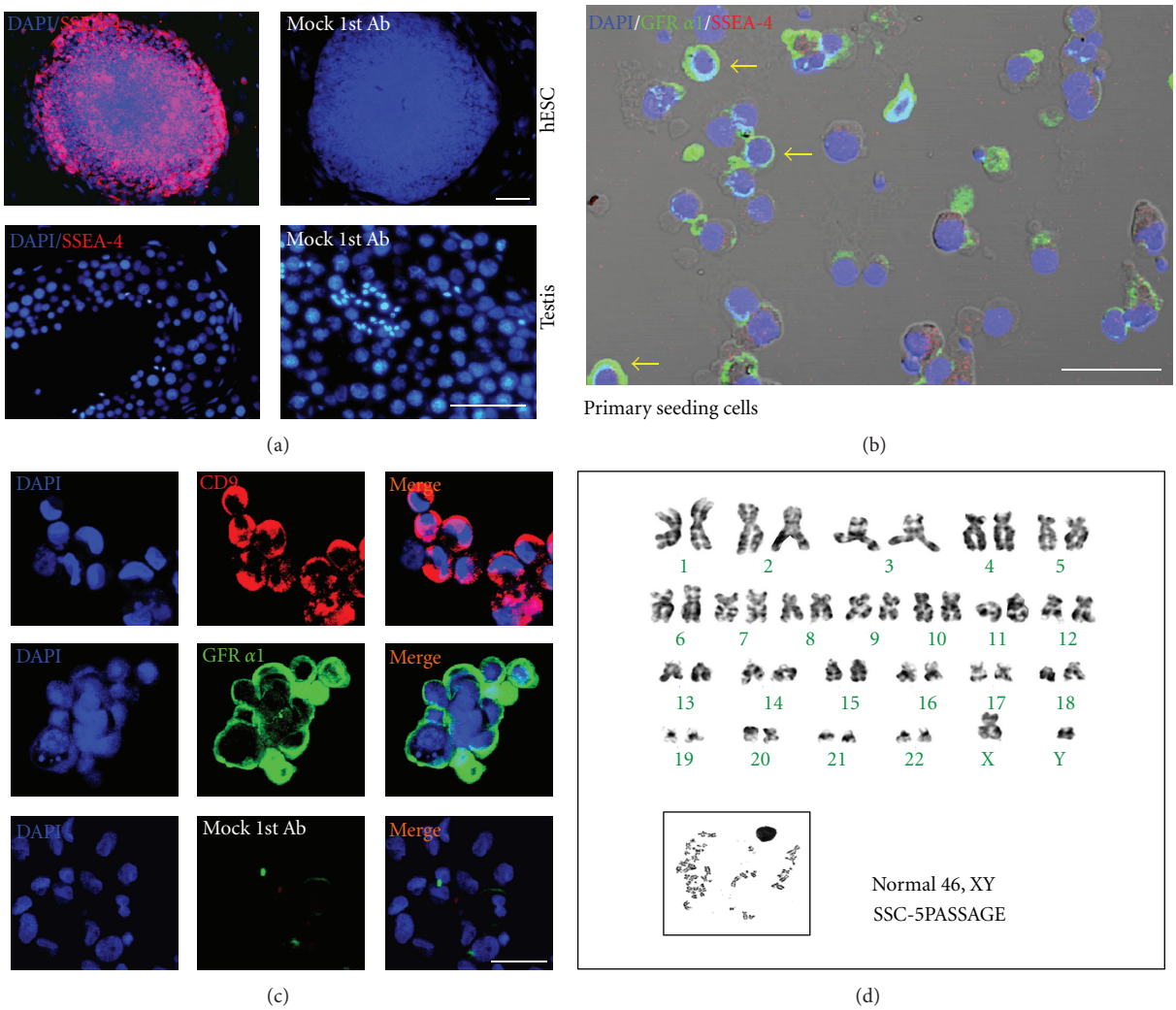

Figure 1: Characterization of spermatogonial stem cells (SSCs). (a) Expression of pluripotent stem cells marker (SSEA-4) in the hESCs and testis. (b) Expression of pluripotent stem cells (SSEA-4) and SSCs marker (GFR α1) in the primary seeding cells. The yellow arrows indicate a GFR α1-positive signals which were expressed in SSCs. (c) Localization of specific markers for SSCs (CD9, red, and GFR α1, green) in the cultured cell clumps (at passage 1). (d) Karyotyping of SSCs performed at passage 5. Note: Mock 1st Ab; cultured cells were stained with secondary antibody only as a negative control. Scale bars = 100 μm.

trypsin-dissociation and plating on new culture dishes with fresh medium containing GDNF. SSCs attached to the plate after incubation for 2-3 days and then proliferated by re-forming floating clumps. Somatic cells and differentiated cells attached to the dish. The passaging of floating clumps was repeated every two weeks. Dissociated SSCs continued to proliferate for more than 5 passages (>10 weeks) and re-formed floating clumps. Using this method, we successfully isolated SSCs and maintained proliferating SSC cultures from more than 83.3% (15/18) of OA patients.

To determine the chromosome stability of SSC clumps, karyotyping analysis was performed at passage 5. The results demonstrated that the SSC clumps had a normal karyotype (46, XY), and no indications of other cytogenetic abnormalities were detected (Figure 1(d)).

3.2. Immunocytochemical Staining. The morphology of SSC clumps was flattened and loosely associated at first (upper panel of Figure 2(a)) and then changed to tightly associated clumps (lower panel of Figure 2(a)). High levels of AP activity were associated with multicellular clumps *in vitro* (Figure 2(b)). Figure 2(c) summarizes the expression of pluripotent stem cell markers in the SSC clumps. At passages 1 to 5, immunostaining analysis showed that SSC clumps expressed pluripotent stem cell markers (Oct-4, SSEA-4, TRA-1-60, and TRA-1-81). The expression levels of these markers were slightly increased up to passage 5, but their relative amounts were still low. Additionally, in SSC clumps, some cells expressed the pluripotent stem cell marker SSEA-4, and most expressed a SSC marker, GFR α1. Interestingly, a few SSCs co-expressed SSEA-4 and GFR α1 (Figure 2(d)).

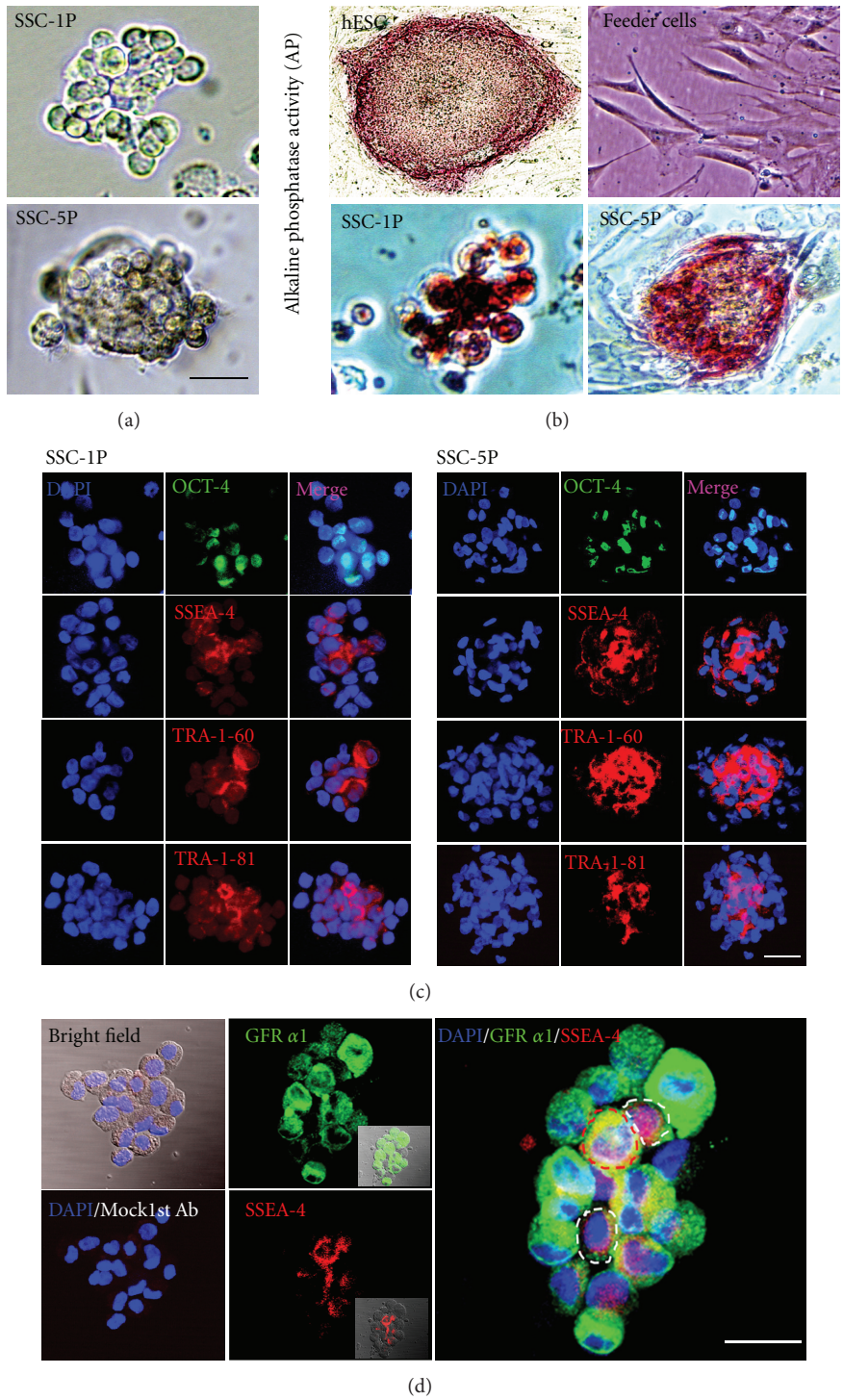

Figure 2: Characterization of pluripotent stem cells within spermatogonial stem cell (SSC) clumps. (a) Morphology of spermatogonial stem cell (SSC) clumps after *in vitro* culture (upper: passage 1, lower: passage 5). (b) Alkaline phosphatase activity in SSC colonies after culture (passage 1 and passage 5). CHA-hES4 cells (human embryonic stem cell line, hESCs) were used as a positive control, and feeder cells were used as a negative control. (c) Immunocytochemical analysis of pluripotent stem cell markers (OCT4, SSEA-4, TRA 1–60 and TRA 1–81) was performed with SSC clumps at passage 1 (left panel) and passage 5 (right panel). (d) Colocalization of specific markers for pluripotent stem cells (SSEA-4, red color) and SSCs (GFR α1, green color) in the cultured SSC clumps (at passage 1). The red circle indicates a SSC in which both markers were co-expressed. The yellow circles indicates a mSSC in which pluripotent stem cell-marker were only expressed. Note: Mock 1st Ab; cultured cells stained with secondary antibody only as a negative control. Scale bars = 50 μm.

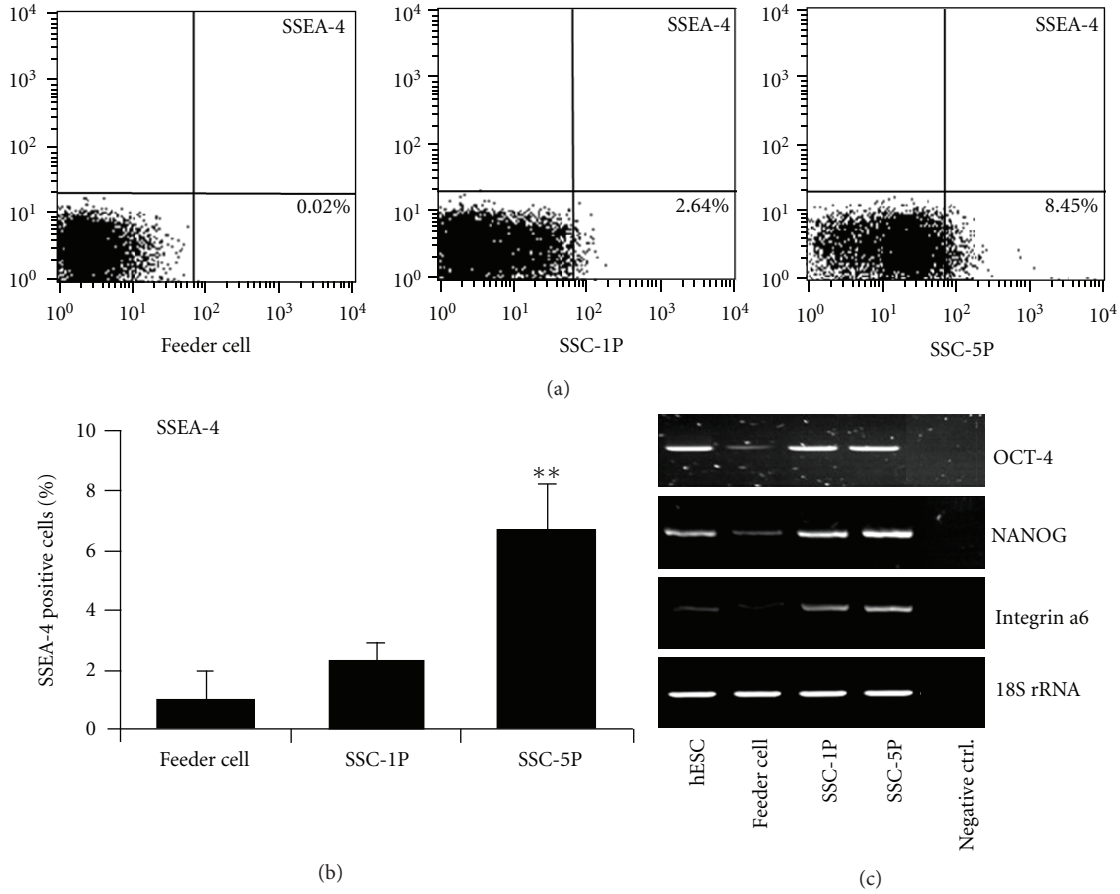

Figure 3: Identification of pluripotent stem cells within spermatogonial stem cell (SSC) clumps using flow cytometry and RT-PCR. (a) Flow cytometric analysis of the cultured SSC clumps using a pluripotent stem cell marker (SSEA-4). (b, c) Immunocytochemical analysis and RT-PCR analysis of pluripotent stem cell markers (*OCT-4*, *NANOG,* and *Integrin α6)* in the cultured SSC clumps. 18S ribosomal RNA was used as an experimental control.

3.3. Flow Cytometric and Gene Expression Analysis of Pluripotent Stem Cell Markers in SSC Clumps. The flow cytometric analysis indicated that the number of SSEA-4-positive cells in SSC clumps was greater at passage 5 (8.45%) than at passage 1 (2.64%). These results were similar to the immunostaining results (Figures 3(a) and 3(b)). The gene expression levels of the markers *OCT-4*, *NANOG,* and *Integrin α6* were confirmed by RT-PCR. ESC cells and SSC clumps (from passage 1 and 5) strongly expressed the mRNAs for *OCT-4*, *NANOG* and *Integrin α6*. In contrast, testicular feeder cells did not express or weakly expressed these genes (Figure 3(c)).

3.4. Spontaneously Differentiation In Vitro . To investigate the differentiation potential of the SSCs clumps *in vitro*, SSC clumps at passage 5 were spontaneously differentiated using the suspension EB-formation method in the absence of growth factors. After 10–14 days under these culture conditions, the SSC clumps consistently aggregated and formed EB-like structures (Figure 4(a)). Markers of ectodermal progenitor cells (nestin, which is present in neuro-epithelial cells) and endodermal lineage cells (α-fetoprotein, expressed in early and late hepatocytes) were detected in the EB-like structures [16, 17]. We also found that marker of mesoderm cells (cardiac protein, widely localized in cardiac muscle cells) was expressed in the EB-like structures and the expression of integrin α6, a marker of pluripotent stem cells, was remained after *in vitro* differentiation (Figure 4(b)).

3.5. Teratoma Formation Potential of SSC Clumps. To confirm the *in vivo* differentiation potential, we subcutaneously injected SSC clumps derived from OA patients into the dorsal skin of immune-deficient mice and examined the ability of these cells to form teratomas *in vivo*. Injected SSC clumps gave rise to teratomas in recipients 12 weeks after transplantation. However, wide-scale expansion to large teratomas was not observed, as is typically observed with hESCs (Figure 5(a)). However, the small teratomas contained derivatives of all three embryonic germ layers. Histological analysis revealed that a variety of cell types was present (Figure 5(b)). Using this assay, teratomas were formed in three of ten (30%) injected mouse. In our previous report, 80–90% density SSC-positive cells did not form teratomas or other tumours after transplanatation into SCID mice (data not shown) [14]. Teratomas from the cultured SSC were distinguished from

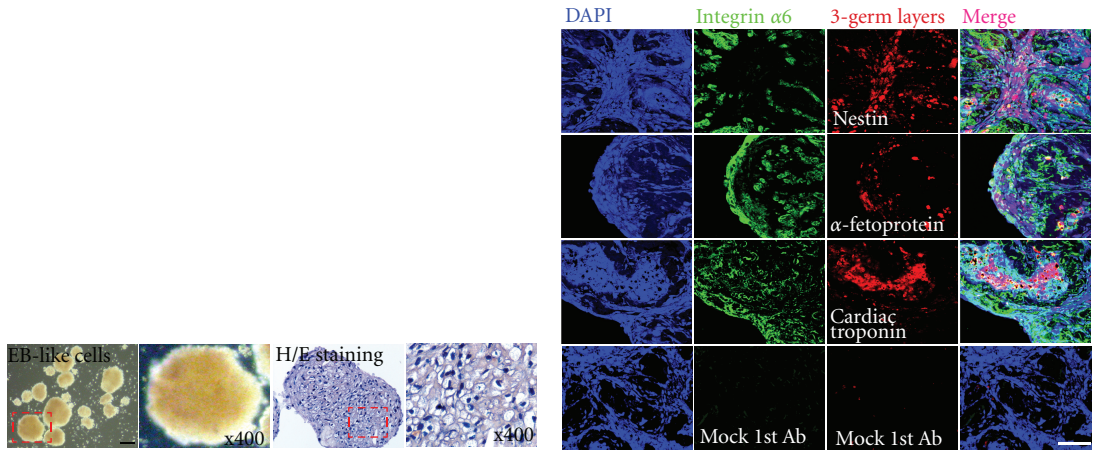

FIGURE 4: Spontaneous differentiation of spermatogonial stem cell (SSC) clumps *in vitro*. (a) Morphology of differentiated EB-like structured cells after plating onto non-coated dishes, and hematoxylin and eosin staining of EB-like structured cells. (b) Expression of three germ layer-specific markers in EB-like structured cells derived from SSC clumps. Markers of ectodermal progenitor cells (nestin), endodermal lineage cells (α-fetoprotein) and mesodermal cells (cardiac protein). Note: Mock 1st Ab; cultured cells stained with secondary antibody only as a negative control. Scale bars = 100 μm.

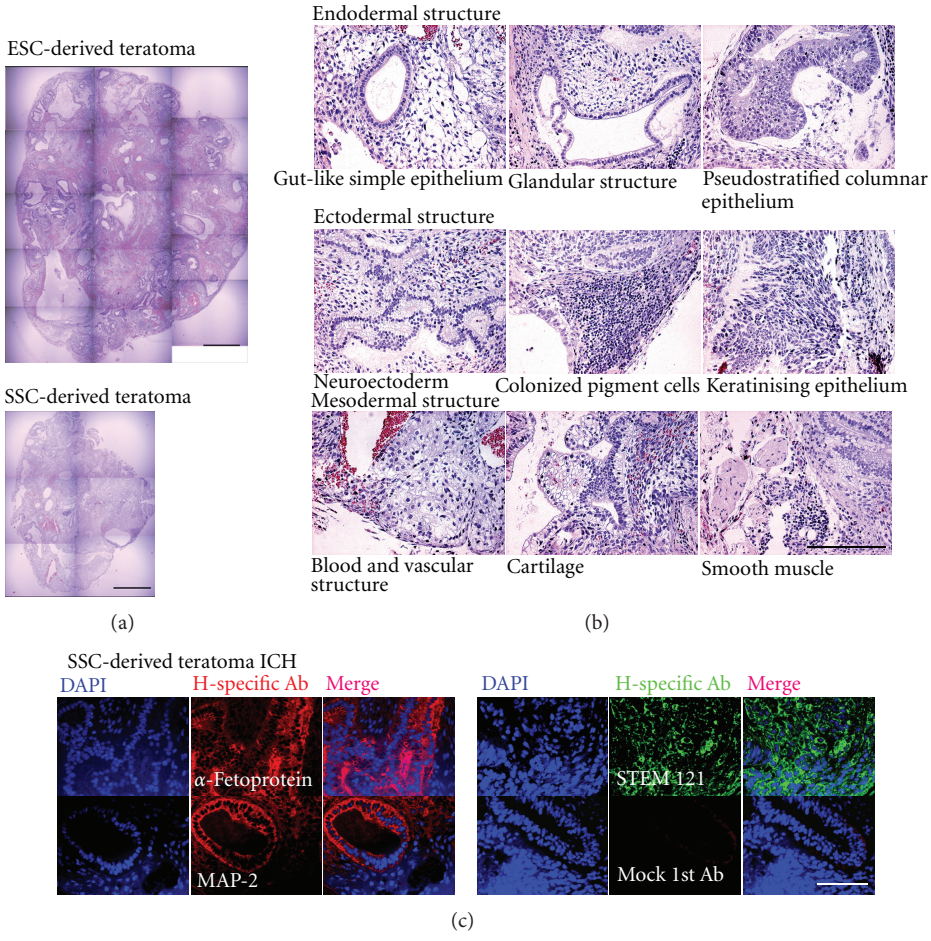

FIGURE 5: Teratoma formation from transplanted pluripotent stem cells within spermatogonial stem cells (SSCs) (a) Teratoma obtained from hESCs (CHA-hES4) and multipotent stem cells among SSCs. (b) Morphology of three germ layer-like structures obtained from multipotent stem cells within SSCs. (c) Immunohistochemical studies for human specific antibody. α-fetoprotein (ectoderm marker reacts with human), MAP-2 (mesoderm marker reacts with human) and STEM121 (cytoplasm marker reacts with human). Note: Mock 1st Ab; cultured cells stained with secondary antibody only as a negative control. Scale bars = 100 μm.

host SCID mouse tissues by human specific antibody (α-fetoprotein, MAP-2, and STEM121) (Figure 5(c)).

4. Discussion

Although the existence of pluripotent stem cells in the testis has been reported in human models [6–9, 18], the characteristics and origin of these cells have remained uncertain [10–13]. In contrast to mouse mSSCs [3], human pluripotent SSCs were isolated from heterogeneous testicular cells including SSCs and somatic cells that were not well-characterized. In the present study, we confirmed that human pluripotent SSCs with ESC-like characteristics can be derived from highly pure SSCs. These cells underwent culture-induced reprogramming without any genetic manipulation.

In 2003, Kanatsu-Shinohara and his colleagues succeeded in culturing SSCs obtained from postnatal mouse testes. SSCs formed uniquely shaped colonies when cultured in the presence of GDNF [19]. Even after 2 years of culture *in vitro*, the cells had the potential to produce normal offspring [20]. Recent studies also indicate that the self-renewal of these SSCs in rodents (and possibly all mammals) is dependent on bFGF and LIF [21, 22]. Based on these properties, we isolated human SSCs from testes and propagated these cells in a modified *in vitro* culture system based on that for mouse SSCs [14]. In the present study, the generation of pluripotent stem cells was observed in long-term cultures of purified human SSCs with a normal chromosome status, but immunocytochemistry and flow cytometric analysis revealed that the relative amount of these cells was disappointingly low (~10%). It has been suggested that the current culture conditions may not fully support the stable maintenance or propagation of pluripotent SSCs, and other factors provided by ESC culture medium or additional feeder cells may be required [18]. Generally, pluripotent stem cells such as ESCs are plated and cultured on mouse embryonic fibroblast feeder cell layers, and following a brief period of attachment and expansion, the resulting outgrowth is disaggregated and re-plated onto another feeder cell layer [23]. In our culture system, the feeder cells, mouse embryonic fibroblasts, did not affect the proliferation of human pluripotent SSCs (data not shown). Hence, it is important to identify suitable feeder cells for pluripotent SSCs.

The clump shape of SSCs was similar to that of ES cell colonies during long-term culture (Figure 2(a)). Additionally, very small numbers of cells co-expressed GFR α1 and SSEA-4, and some cells expressed SSEA-4 (Figure 2(d)). These results are very similar to our previous results in mice indicating that the intermediate state of SSCs has an expression profile more similar to that of pluripotent stem cells than to that of mSSCs, yet the expression of germ cell markers is preserved [24]. These results suggest that the morphological transformation of SSCs and the start of the expression of specific markers indicate that SSCs can be reprogramed during long-term culture under specific conditions.

Kerr et al. have provided some findings regarding the development and differentiation of human germ cells in the fetal testis, including a very small population of PGCs with a molecular signature including OCT4, NANOG, c-Kit, SSEA-1, SSEA-4, and alkaline phosphatase [25]. Our initial characterization showed that a small number of isolated SSCs expressed markers of undifferentiated stem cells such as OCT4, SSEA-4, Tra-1-60 and Tra-1-81 [26, 27]. The population of these cells was slightly larger after passaging (Figures 2(b) and 3) and was then maintained under defined culture conditions. Pluripotent stem cells have the potential to differentiate into nearly all cell types in the human body [28]. *In vitro* and *in vivo*, these cells are able to generate embryoid bodies or teratomas that express marker genes of all three germ layers and develop into different cell types. The capacity of pluripotent stem cells to differentiate into almost all of the cell types of the human body highlights the potentially promising role of these cells in cell replacement therapies for the treatment of human diseases [29]. If the pluripotent stem cells can be derived from existed/reprogrammed human testis cells, these cells will generate embryoid bodies in culture. In our system, pluripotent cells among SSCs were able to generate EB-like structures and exhibited some characteristics of pluripotency, differentiating into cells of the three germ layers (Figure 4). Additionally, these cells formed teratomas after injection into SCID mice (Figure 5).

5. Conclusion

This result revealed that pluripotent cells were induced from SSCs during *in vitro* culture and were present within the SSC clumps isolated from human adult testicular tissues. Additionally, no genetic modification was needed for this procedure, which can be compared to the generation of iPSCs from somatic cells [11]. The isolation and long-term proliferation of mSSCs from human testicular tissue may allow the development and use of individual cell-based therapies without ethical and immunological problems. In this study, we demonstrated that adult SSCs are able to develop into pluripotent stem cells in *in vitro* culture, which differentiate into cells of the three germ layers. However, before pluripotent stem cells derived from SSCs can be used in treatments, a highly efficient system to induce and propagate these cells on a large scale is required.

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