Stem Cells International

Stem Cells and Nuclear Reprogramming

Guest Editors: Fuliang Du, Mark G. Carter, Giorgio A. Presicce, Shinn-Chih Wu, and Perng-chih Shen

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Editorial Board

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doi:10.4061/2011/584686

Editorial

Stem Cells and Nuclear Reprogramming

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Received 18 August 2011; Accepted 18 August 2011

Stem cells are found in all multicellular organisms, including two broadly defined cell types: embryonic stem cells (ESC) (C. Y. Cheong and T. Lufkin; and N. Lifantseva et al.) that are derived from the inner cell mass of blastocyst-stage embryos and adult stem cells that are present in adult tissues (C. M. Teven et al., R. Chung et al., and A. C. Wilber et al.). Nuclear reprogramming refers to the erasure and remodeling of epigenetic marks, which is a part of normal mammalian development. This reprogramming is likely required for totipotency of the newly formed embryo and erasure of acquired epigenetic changes (Felici). Advances in stem cells including induced pluripotent stem (IPS) cells (D. Dey and G. R. D. Evans; and P. Noisa and R. Parnpai) and nuclear reprogramming (C. M. Teven et al.) will provide new insights into the mechanisms of cellular differentiation, during embryonic development (N. Lifantsevaa et al.) as well as in adult tissues (C. M. Teven et al.), and their pluripotency (A. C. Wilber et al.), which may lead to cell-based therapies (R. Eggenschwiler et al.) for several human diseases (R. Chung et al.).

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Volume 2011, Article ID 619583, 11 pages
doi:10.4061/2011/619583

Review Article

Generation of Induced Pluripotent Stem (iPS) Cells by Nuclear Reprogramming

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Received 16 March 2011; Revised 18 June 2011; Accepted 22 June 2011

Academic Editor: Mark G. Carter

During embryonic development pluripotency is progressively lost irreversibly by cell division, differentiation, migration, and organ formation. Terminally differentiated cells do not generate other kinds of cells. Pluripotent stem cells are a great source of varying cell types that are used for tissue regeneration or repair of damaged tissue. The pluripotent stem cells can be derived from inner cell mass of blastocyst but its application is limited due to ethical concerns. The recent discovery of iPS with defined reprogramming factors has initiated a flurry of works on stem cell in various laboratories. The pluripotent cells can be derived from various differentiated adult cells as well as from adult stem cells by nuclear reprogramming, somatic cell nuclear transfer and so forth. In this review article, different aspects of nuclear reprogramming are discussed.

1. Introduction

Adult human beings cannot regenerate organs as the regeneration has silenced during evolution to protect from tumorigenesis. However, in human, part of the liver can regenerate. In lower animals, regeneration of organs or part of organs are very common. Song birds' brain tissue that controls the singing of birds dies after every breeding season and that lost neuron is again are replaced at the advent of next breeding season [1]. Reptiles like lizards lose their tail to deceive predators and the lost tail regenerates. Terminal differentiated cells like fibroblasts are thought to be unable to convert to other cell types. However, cloning experiments in amphibians and later in mammals proved that indeed they have the potential to reprogram and generate other cell types [2, 3].

Human adult neurons, heart tissues never thought to regenerate but in the late 90's it was found that in occasional cases human adult neurons divide [4].

It is now established that terminal differentiation of cells is a reversible process that has generated a lot of interest in reversion of cell differentiation and reprogramming to iPS. Stem cells can divide unlimitedly and can give rise to its own and different kinds of adult cells. It holds the promise for the treatment of several neurological and other debilitating diseases like Parkinson, Alzheimers, ischemic heart failure, diabetes mellitus, Huntington disease, and sickle cell anemia [5].

2. Sources of Pluripotent Cells

Several methods of pluripotent cell derivation exist. (a) Embryonic stem (ES) cells can be harvested directly from the inner cell mass (ICM) of preimplantation blastocyst. (b) Somatic cell nuclear transfer (SCNT) is a therapeutic technique where adult somatic nuclei are microinjected into enucleated eggs. The egg, now containing the nucleus of a somatic cell, is stimulated with a shock and the somatic cell nucleus is reprogrammed by the host egg cell and forms a blastocyst. (c) Cellular hybridization where pluripotent hybrid cells are formed via somatic cell fusion with an ES cell. (d) Induced pluripotent stem (iPS) cells are developed from-patient-specific somatic cell reprogrammed to an ES cell-like state. For practical purpose, hES cells have faced difficulties because of ethical issues, potential immuno-incompatibility, and an increase of MHC molecule

Table 1: Nuclear reprogramming: various cell types that can be reprogrammed are shown in Table 1. During reprogramming several cell markers for pluripotency or differentiation are upregulated or downregulated.

Phenomena	Factors
Starting cell types	Keratinocytes, t-cells, fibroblasts, Adipose-derived stem cells (ADSCs), mesenchymal stem cells (MSCs), dental pulp stem cells, germ line stem cells, neural stem cells, cord blood stem cells, hair follicle, retina, skeletal muscle.
Factors Upregulated/ Downregulated	(a) Embryonic development—upregulated (Oct4, Sox2, Nanog, Lin-28, NR5A2, TBX3, STAT3 and ZIC3).
	Downregulation of differentiation specific genes (PAX6, ATBF1 and SUZ12).
	(b) Proliferation—upregulated (Cyclin D1, c-Myc, KLF4, Rem2).
	Downregulated (p53, p21, $p16^{INK4A}$).
	(c) Epigenetic—downregulated (DNMT1, HDAC, Histone demethylase).
	(d) Signalling pathway—downregulated (TGFβ, Wnt/β catenin, PI3/AKT).
	(e) Chromatin—opened (by SWI/SNF).
	(f) Miscelleneous—to express (Vitamin C, hypoxia, TERT, E-cadherin).

expression during differentiation [6]. SCNT for humans are difficult to achieve and mired with ethical issues related to egg destruction. Similarly, the utilization of ES cells has been difficult due to ethical reasons. As an alternative to ES, the establishment of iPS cells has raised more interesting potential as they are created from adult cells [7]. In his seminal work on iPS, Yamanaka et al. used 4 reprogramming factors (they are also transcription factors) such as Oct3/4, Sox2, Nanog, and c-Myc to reprogram mouse somatic cells. Later, reprogramming factors (RFs) delivery were done by retroviral and lentiviral vectors and other nonviral delivery systems (Table 1). The minicircle (MC) delivery system is designed to deliver TFs episomally to avoid the unintended consequences of viral use in the clinical setting [8]. Recently, however, mRNA with 4 reprogramming factors added directly to the cell demonstrated a high efficiency of reprogramming of adult skin cells [9]. Reprogramming efficiency has also improved with the use of various chemical compounds and growth factors (such as Wnt and TGF-β signaling pathways) in addition to 4 reprogramming factors (Figure 1).

3. ES and iPS Cells

ES and iPS cells have the ability to produce almost any types of adult cells. However, use of ES cells for clinical purpose is controversial. Additionally, cells derived from ES cells can be rejected by the host immune system as they are autogenously derived. To overcome the ethical and immune rejection issues, iPS cells are developed from adult differentiated cells by reprogramming factors. This iPS, which is morphologically and characteristically close to ES, can be differentiated to various types of tissues and replenish host tissue loss in neurodegenerative and other diseases. Direct reprogramming of cells by iPS is more convenient and reliable than generation of pluripotent cells from ICM of blastocyst [10]. However, different studies have shown that iPS cells are not as superior as ES cells.

Before starting any reprogramming, we need to understand genetic and epigenetic changes at the molecular level that causes reprogramming from a differentiated cell to a pluripotent one. There is a gulf between lab bench success on iPS and clinical application of this technology as still characterization is taking place. For the safety concerns need to be addressed before any clinical consideration. The bottlenecks of iPS are inadequate cell number, immune rejection, and tumor (teratoma) formation. All the pluripotent cells (hESC, iPS, and NTSC) are potentially tumorigenic. More research is needed to overcome this problem [11].

4. Cell Types for Reprogramming

Before starting any reprogramming, information about the plasticity of the starting cell is helpful as cellular reprogramming is an interplay between plasticity and environmental factors like epigenetic modifications. Both adult stem cells and terminally differentiated cells can be reprogrammed but efficiency, time period, and extent of reprogramming varies among the starting cells. The derivation of functional neurons, cardiomyocytes, pancreatic islet cells, hepatocytes, and retinal cells prove that it is possible to derive cells from many sources of tissues by reprogramming, thus facilitating the various treatments. The starting cells should not be necessarily from the organ that will require repair. As an example, regeneration of insulin-producing cells can be done from primary source (in this case pancreatic β cells), insulin-producing cells can be also derived from other alternative sources like ES, adult stem cell, mesenchymal and hematopoietic stem cells. This was also achieved by various means like nuclear reprogramming and transdifferentiation. Bone marrow tissues are a source of adult mesenchymal stem cells but are collected by more invasive and painful surgery. Adipose-derived stem cell (ADSC) is the most abundant and can be easily isolated from the patient by local anesthesia. Interestingly fat tissues contain about 100-fold adult stem cells more than bone marrow making it an attractive source for adult stem cells. Human ADSCs are a heterogeneous group of multipotent progenitor cells and it has certain advantages; they can be collected autogenously in high numbers (100 mL of human adipose tissue yields about 1×10^6 cells) with minimal morbidity [12]. Additionally, ADSCs express 3-4 times endogenous Klf4 compared to human ES cells and 1.3 times higher expression of c-Myc both are reprogramming agents. Reprogramming of ADSCs to

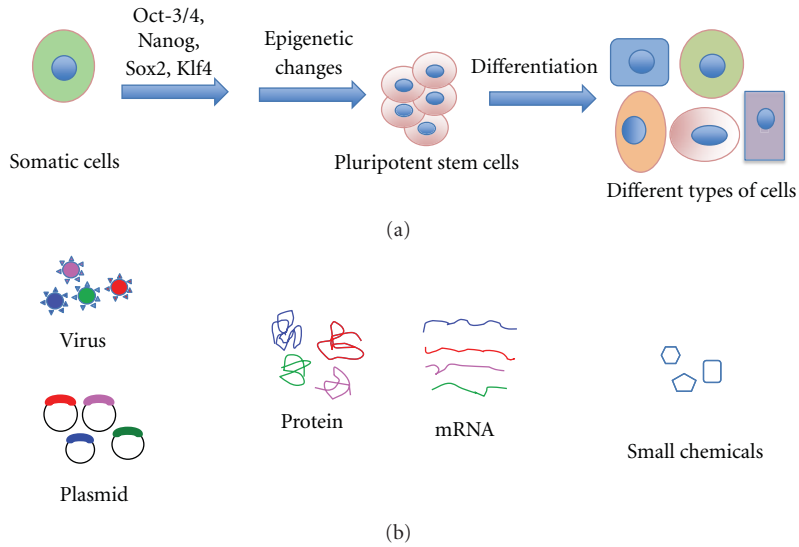

Figure 1: A simplified version of reprogramming of adult cells to pluripotent cells by 4 factors. (a) Any adult source of cells like epithelial, muscle, fibroblast, and adipose cells is isolated and cultured in appropriate medium. Then cells are transfected, nucleofected, or transduced by 4 reprogramming factors. These factors may be Oct3/4, Sox2, Nanog, and Klf4/c-Myc. The reprogramming paths are not well understood yet, requiring expression of several genes as well as epigenetic modification of the genome. (b) Reprogramming factors (transcription factor genes) can be delivered by various methods. In case of viral gene delivery, genes are integrated with the host chromosome randomly. Methods of gene deliveries like plasmids, mRNAs, proteins, genes are expressed transiently and diminished the expression with the concomitant increase in expression of pluripotent markers.

iPS and subsequent neuronal differentiation is an attractive alternative to hES. ADSCs can be induced to differentiate into osteogenic [13], chondrogenic [14], adipogenic [15], hepatic [15], cardiogenic [16], neurogenic [17], islet-like insulin secreting cells [18], and hematogenic (mouse) lineages [19].

5. The Reprogramming Vectors

5.1. Reprogramming by Retroviral Vectors. The reprogramming with gene delivery system that remains as episome (like adenovirus, mRNA, and minicircle) has great advantage over the integration of genes into the host chromosome (hantavirus, retrovirus). The reprogramming factors are expressed transiently and then degraded or diminished. This primes and drives the host cell to a choreography of downstream reactions (pathways are not yet well understood) that ultimately convert the cell to pluripotency. For DNA-based (viral vector) reprogramming, 4 proteins trigger reprogramming but DNA remains altered due to viral integration into the chromosome. Nevertheless, the efficiency of retroviral reprogramming is at very low frequency (less than 0.1%). In the original reprogramming method a retrovirus (MMLV) was used; the RNA virus carried the transgene and reverse-transcribed inside the host cell. This DNA was integrated into the host chromosome and was a constant source of transgene protein. The retroviral promoter was inactivated probably by histone modification (methylation) [20]. Reprogramming of adult cells by viral delivery of 4 reprogramming factors (Oct4, Sox2, Nanog, and c-Myc) have been used successfully for iPS generation. Takahashi et al. first developed mouse iPS and one year later established human iPS [7, 21]. These iPS cells are supposed to be equivalent or comparable to ES cells in morphology, gene expression, and epigenetic status and can give rise to 3-germ layers. There are some disadvantages of retroviral vectors. It integrates randomly in host chromosome making leaky expression and transforms the cell into tumor. Besides, the random integration also makes cells heterogeneous and iPS cells should be screened for various clones (efficacies and safety). Using c-myc as an agent of reprogramming factors makes cell more tumorigenic as the role of myc has been well documented in connection with tumor. Myc is an immediate early gene and reactivation of c-Myc gave rise to transgene-derived tumor formation in chimeric mice [22]. Chimeric mice produced with c-myc-free iPS did not develop any tumor until 6-month observation period compared to control mice. Besides c-Myc, various tumors also express OCT3/4, SOX2, and KLF4 the other 3 reprogramming factors. Overexpression of these 3 factor-derived iPS causes tumor formation. Hopefully, there are some refinements to the above procedures. Some labs have minimized the number of integration into the host genome by putting all reprogramming factors in one vector. This is technically/practically accomplished by putting IRES sequence between reprogramming genes or a self -cleavage 2A peptide sequence. This way iPS cells are produced with only single insertion in the genome [23].

5.2. Reprogramming by Nonviral Vectors. Until now, there have been several nonviral vectors designed for TFs delivery. A partial list of them is shown in Table 2. Minicircle (MC) vectors have been developed by Dr. Joseph Wu's team at

Table 2: Nuclear reprogramming factor delivery. List of reprogramming factors delivery by various methods shown in Table 2.

Mode of delivery	Vectors
Viral delivery	MMLV retrovirus, lentivirus, adenovirus, hantavirus, transposon, retrotransposon.
Episomal delivery	PiggyBack, plasmid, minicircle, synthetic mRNA, IVT mRNA, protein, cell fusion (ES + adult), cell fusion (tumor + adult), protein delivery by gag protein,
Small molecule (chemical)	ALK5 inhibitor, GSK3 inhibitor, and MEK inhibitor.

Stanford University [8]. Minicircles (MC) are circular nonviral DNA elements that are generated by an intramolecular (cis-) recombination from a parental plasmid (PP) pLGNSO (Lin28, GFP, Nanog, Sox2, and Oct3/4) mediated by ϕC31 integrase. Thus delivering only the minicircles to cells lengthens the expression of the transgene over traditional transient transfection of plasmids. For dividing cells, expression of the minicircles lasts up to 14 days. For nondividing cells, expressions of the minicircles drop slightly after the 1st week, but can continue the expression of transgenes for months. The beauty of minicircle is that MC possesses higher ectopic expression and less inactivation by cellular machineries. The plasmid contains a single cassette of 4 reprogramming factors and GFP coding sequences are linked by "2A" peptide sequences [24]. The minicircle when used to transfect the adult cell, gradually over time becomes ES type morphology without changing the global gene expression. Inside the cell, the MC is not integrated in the genome, but instead it is transcribed as a whole single long mRNA containing all the 5 genes (4 RFs & GFP). This is translated as a single protein where individual proteins are processed by self-cleavage peptide 2A. In case of synthetic RNA-based reprogramming RNA is translated into protein in the cytoplasm and host DNA remains unaltered. So, synthetic RNA-based reprogramming is clean, safe, and fast and iPS cells are genetically identical to their source cells. The reprogramming process takes a little over 2 weeks with 4% of the cells being reprogrammed. This is about 100-fold more efficient than reprogramming by gene transfer technique [9]. They named it RNA-induced pluripotent stem (RiPS) cells. However, it is difficult to synthesize long mRNA chemically and researchers have not been able to make large mRNAs. Needless to say mRNA is prone to degradation and needs very rigorous quality control in the lab. The cells have a defense system against RNA virus infection. That is an old defense mechanism present in both plants and animals. The host cell perceives foreign RNA as a viral invasion and degrades RNA in small fragments by RISC machinery that is available inside the cells. This cell-mediated RNA degradation is known as RNA interference (RNAi) [25]. To circumvent that problem, Dr. Rossi's team chemically synthesized mRNA with some modified bases (nucleotides) at intervals so it is not recognized as an outsider RNA and protected from degradation. Yakubov et al. also confirmed reprogramming by mRNA of 4 TFs. However, they did not use synthetic RNA instead they used mRNA which was synthesized by in vitro transcription (IVT) from the DNAs of 4 genes. For continuous expression, they transfected a total of five times and established iPS that is alkaline phosphatase positive and expressed several pluripotent markers [26].

5.3. Model Reprogramming of Cells: hADSCs. Human ADSCs are a heterogeneous group of multipotent progenitor cells that can be collected autogenously in high numbers [27]. ADSCS are more abundant than bone marrow by a factor of 100. Nucleofector (Amaxa, Germany) is used for nucleofection with 4 reprogramming factors. Nucleofection is a poorly explained method where DNA materials are directly delivered to the nucleus. All cells are used for reprogramming are within early passage. Transfected hADSC cells are then seeded onto a Mitomycin C treated MEFs feeder layer or on Matrigel-coated tissue culture dishes (ES qualified, BD Biosciences). The feeder layer provides nutrition for the cells that are being reprogrammed. Mitomycin C or radiation treated cells are alive but they do not divide as the cell cycle is arrested by DNA damage response pathway. On days 4 and 6, hADSCs are transfected again with minicircles using lipofectamine type transfection reagent which is less toxic to the cells than nucleofection. Cells were switched to human ES cell culture medium prepared using DMEM-F12+ 10% knockout serum and 100 ng/mL of bFGF (FGF-2). Colonies with morphologies similar to human ES cell colonies are expected to be visible in 3-4 weeks after transfection. Gradual loss of GFP expression due to dilution of minicircles followed by activation of endogenous Oct4 expression will be observed on successive cell proliferative cycles. During this reprogramming, cells divide and form round/circular compact colony of cells with a clear margin. In mouse cell reprogramming, leukemia inhibitory factor (LIF) is also used in the media to keep them in a pluripotent state while avoiding any differentiation.

The pluripotency of the iPS cells is determined by immunofluorescence for ES markers like Oct4, Sox2, Nanog, and so forth. For clinical applications, Southern blots are done to check any genomic integration of reprogramming genes. The isolated colonies look morphologically similar to hES. Immunostains for pluripotent markers and alkaline phosphatase will be positive. iPS cells typically express SSEA4, TRA-1-60, and Nanog but not SSEA1.

5.4. Some Less Used Reprogramming Methods. Various Gram-negative bacteria have type III secretion system (T3SS) that injects virulent proteins (regulated by a variable secretion signal sequence) into the eukaryotic cell cytoplasm. Bichsel et al. used *Pseudomonas aeruginosa* exotoxin ExoS to translocate Cre recombinase containing a Cre-NLS signal [28]. Upon infection, bacteria delivered Cre-NLS to undergo LoxP mediated chromosomal DNA recombination. This resulted in iPS and establishes the use of T3SS for the delivery of TFs in cellular reprogramming. Retroviruses assemble polymer of Gag protein which is proteolytically cleaved before entering

the cell through receptor binding. This property of retrovirus was used to deliver the reprogramming proteins into the cells. Proteins were incorporated with nuclear localization signal (NLS) and tagged/attached to the retroviral protein. After cell entry, proteins were cleaved by retrovirus protease and the active TFs proteins were translocated inside the nucleus where it reprograms the cells. This expression is transient as proteins will be degraded after their intended job [29]. In routine iPS generation, germ-line transmission and live birth from iPS cells are produced by tetraploid complementation. It has been shown that addition of Tbx3 improves the quality of iPS cells. iPS cells derived with Tbx3 and Oct 4, SOX2, KLF4 are more successful in germ cell contribution [30].

Dermal papilloma (DP) cells are reprogrammed more efficiently than skin and embryonic fibroblasts. DP inherently express higher levels of SOX2 and c-Myc, so these DPs can be easily reprogrammed with only Oct4 and KLF4 [31]. Among the 4 reprogramming factors, Oct4 is a very important TF for iPS generation. Oct4 cannot be replaced with other TF whereas SOX2, KLF4 and c-Myc can be replaceable. However, Ng has shown that orphan (ligand is not known for the receptor) nuclear receptor Nr5a2 can be used for mouse somatic cell reprogramming with greater efficiency than Oct4. Genomewide expression analysis showed that Nr5a2 shares many target genes with SOX2 and KLF4 indicating they work together in concert [32]. The nuclear receptor superfamily has 48 genes. This nuclear receptor superfamily maintain various aspects of stem cell-like regulation of stemness, reprogramming of terminally differentiated cells [33]. An Oct-4 promoter-based reporter system has been developed in mouse and pig and is a useful tool for monitoring the differentiating status of porcine cells both in vivo and in vitro [34]. A recent work by Sugii et al. have improved the iPS efficiency with or without the feeder layer [35]. They also demonstrated that adipose stem cells (ASC) can be grown on ASC feeder layer instead of the MEF feeder layer. They reduced the iPS programming 1.5 and 2.5 weeks for mouse and human iPS, respectively. Terminally differentiated mature B lymphocyte has been reprogrammed with basic reprogramming factors plus the addition of C/EBPalpha [36]. Generally 4 reprogramming factors are added in equal ratio for iPS generation. However, increasing the concentration of OCT3/4 enhances reprogramming efficiency but increasing the concentration of SOX2, KLF4, and c-Myc reduces reprogramming efficiency [37]. The time required for reprogramming also varies among the starting cell types, source, and so forth. Mouse fibroblasts can be reprogrammed in 3 weeks whereas human fibroblasts with the same reprogramming factors require 4 weeks to reprogram. Interestingly, 4-factor reprogramming repressed cardiac differentiation due to prolonged expression of OCT4 and Fgf4. Contrary to that, 3-factor reprogramming (excluding c-Myc) enhances the expression of precardiac (CXCR4, Flk-1, and Mesp1/2) and cardiac-specific (Nkx2.5, Mef2c, and Myocardin) genes. The differentiation of cells showed continuous beating activity in a petri dish. So, reprogramming devoid of c-Myc TF has a tendency of preferentially generating cardiac tissues [38].

Human iPS induction was also done by adding several other factors like TBX3, mirna-291-3p, miR-294, and miR295. The adult newt can regenerate lens from pigmented epithelial cells (PECs) through dedifferentiation. It is like reprogramming iPS *in vivo* without any exogenous factors. The authors were interested in seeing which genes are being activated/expressed during the dedifferentiation procedure. They isolated mRNAs and constructed a cDNA library. Upon analysis, they found the expression of apoptosis and cancer-related genes and concluded that cancer and apoptosis-related genes expression may be a hallmark during dedifferentiation in newt [39]. Vitamin C enhances the generation of iPS in mouse and human somatic cells. It may play an auxilliary role to reverse senescence and promotes pre-iPS cells to complete programming of cells [40]. Lineage reprogramming has been an important tool for studying cell fate choice during differentiation. Several TFs that can drive cells from one lineage to another [41]. Induced expression of leukemic oncogene AML1-ETO in embryonic zebrafish reprogram hematopoietic progenitor cells from erythroid to myeloid.

5.5. Small Chemical Molecules. Small chemicals molecules that target enzymes of cell reprogramming pathways have been identified that control the cells fate like stem cell maintenance, reprogramming, and differentiation [42]. By using small synthetic chemical molecules a pluripotent state can be induced which is known as a chemically induced pluripotent cell (CiPSs) [43]. Li et al. has suggested that small molecules should not only be able to reprogram cells in vitro, but they can also be delivered into the body as conventional therapies to target a patient's own tissue for the treatment of degenerative diseases, injuries, and cancer (to target cancer stem cells) [44]. Cancer cells get resistant to chemotherapeutic drugs. It has been shown that reprogramming of GI cancer cells by the ectopic expression of TFs leads to reprogramming. The induced pluripotent cancer (iPC) cells were sensitized to chemotherapeutic drugs and also responded to differentiation-inducing treatment in short term cell culture [45]. Reprogramming of somatic cells is also possible by fusing ES cells with adult cells. In case of fusion-induced reprogramming, OCT4 reactivation starts within 1-2 days after ES cell and somatic cell fusion [46]. Reprogramming by fusion was achieved by the fusion of MEFs with mouse ES cells by using hemagglutinating virus of Japan envelope (HVJ-E). Microsatellite analysis of the derived stable cell line showed that they possess genes from both ES and MEF. The fused cells were tetraploid and positive for Alkaline Phosphatase (AP) and stem cell markers (OCT3/4, NANOG, SOX2) but not fibroblast cell marker (Col1a1 and Col1a2) [47]. Fusion between intestinal epithelial cells and macrophages in a cancer context results in nuclear reprogramming [48]. The structure of telomeric chromatin is dynamic and changes during cell transformation to cancer. Telomere is shortened in every cell cycle until it reaches a crisis stage where it goes to apoptosis [49]. Telomere expression is silenced in most adult somatic tissues except the adult stem cell compartments.

During reprogramming telomeric chromatin is remodeled and telomeres are elongated by telomerase [50], although there are some heterogeneity during the reprogramming process with respect to telomere length [51].

5.6. Reprogramming by Transdifferentiation. Transdifferentiation is defined as the conversion of one cell type to another. Eberhard et al. used synthetic glucocorticoid dexamethasone and reprogrammed pancreatic cells to hepatocytes [52]. Cobaleda C dedifferentiated adult B cells into multipotent progenitor cells and afterward reprogrammed to alternative lineages T cells and macrophages [53]. Mak et al. used mammalian affinity purification and lentiviral expression (MAPLE) to deliver several protein complexes involved in transcription (RNA polymerase II associated factor, negative elongation factor, positive transcription elongation factor b, SWI/SNF complexes). They showed that TF KLF4 facilitates chromatin remodelling with the SWI/SNF complex [54].

The generation of animals by SCNT has shown that epigenome of differentiated cells can be reset to a pluripotent state. The nucleus from the somatic cell is inserted into an enucleated egg and can transform it into an embryonic stage (pluripotent). The cytoplasm of the egg is enough to reset the epigenome and DNA sequence remains unaltered. Compatibility of mitochondrial DNA haplotypes between donor cells and host oocytes improves reprogramming efficiency possibly by epigenetic modification. To increase the SCNT viability, treatment with chemicals that causes chromatin modification like Trichostatin-A (TSA) decreases expression of histone deacetylase (HDAC1 and HDAC2) and DNA methylation (DNMT3a and DNMT3b) while increases the expression of histone acetylation (P300 and CBP), pluripotency (OCT4 and NANOG) genes [55]. *Histone deacetylase inhibitor valproic acid (VPA) enabled reprogramming of human fibroblasts with only Oct4 and SOX2 [56]. Wnt signalling results in inhibition of GSK-3 and stabilization of cytoplasmic β-catenin. Small molecule inhibitors of GSK-3 can mimic the activation of wnt signalling and maintain the pluripotency of mES* [57–59]. An immune system protein, activation-induced cytidine deaminase (AID) helps in reprogramming by DNA methylation. Thus important reprogramming genes Oct4 and nanog are induced. Li et al. has been able to reprogram somatic cells without the TF Sox2. TFs Oct4, and KLF4 alone can reprogram MEF when cultured with glycogen synthase kinase-3 (GSK-3) inhibitor CHIR 99021. They conclude that GSK-3 inhibitor may replace TF SOX2 in both mouse and human reprogramming. Additionally, CHIR 99021 in combination with parnate, TFs Oct4, and KLF4 are sufficient for reprogramming of human primary keratinocytes [60].

6. In Vitro Differentiation of iPS Cells to Differentiated Cells

Pluripotent iPS is like a hub from where differentiation to various lineage types is possible. For generation of mature neuron cells from iPS, cells are induced with culture media containing differentiation factors. Expression of differentiation marker is determined by the presence of α-smooth muscle actin, α-fetoprotein, and β-III tubulin. If viability of iPS cell is a problem, treatment of iPS cells with ROCK inhibitor Y-27632 before harvesting to increase their viability is possible. Recently cells can be induced to transdifferentiation by treatment with several transcription factors and transform cell from one lineage (fibroblast) to another (functional neuron) bypassing the conversion of pluripotent stem cell-like stage [61].

7. Developing of Embryo, Pluripotent Stem Cells and Cancer Cells

Embryo, pluripotent stem cells and cancer cells have some similarity in behavior. They are actively dividing and proliferated. Many genes which are silent (unexpressed) in the differentiated adult tissue are reactivated during cellular reprogramming (iPS) and cancer as well. Back in time, the first pluripotent cells were derived from teratocarcinoma (a germline tumor). When (explanted) grown in tissue culture, teratocarcinoma generated embryonic carcinoma cells. This happens due to reprogramming of cancer cell to a pluripotent state [62]. Therefore, it needs careful observation to limit the growth of iPS towards tumors/malignancy.

The success of iPS in clinical setting will be determined by safety, genomic integrity, programming efficiency, and so forth. The Rb-p53-p16 (INK4a) is a very important pathway for cell cycle progression, cell proliferation, and scenescence. At the molecular level, cancer of all kinds converges in p53 pathway. Due to mutation of Rb, p53 or p16 cell cycle progresses uncontrollably and untimely. p53 is a tumor suppressor gene and is dubbed as the guardian of the genome. Around 50–80% tumors of various kinds have p53 sporadic mutation [63]. The protein is quickly degraded (half life is around 30 mins). However, this protein is quickly activated (stabilized) during viral infection, untimed cell cycle progression, in DNA damage, and other stress [63]. After DNA damage the protein is phosphorylated by ATM/ATR kinases and stabilized. The protein upregulates the expression of the p21 gene that brakes the cell cycle progression from G1 to S phase. Among many genes, it also upregulates bax expression, a proapoptotic gene. During the cell cycle blockage, p53 assesses the degree/extent of DNA damage. If it is repairable, then it recruits/upregulates the DNA repair proteins and restores the integrity of the genome. But if the DNA damage is significant, then it directs the cells towards apoptosis. As ES and iPS cells also proliferate rapidly, the intact p53 pathway is an impediment of efficient reprogramming. Cell cycle and apoptosis act as a rate-limiting step during reprogramming. Anything that accelerates cell division, helps in faster reprogramming. It can be done if p53 is knocked down temporarily by p53 or p21 specific short hairpin (shRNA). This may facilitate faster reprogramming but also increases the chance of tumor development. Several labs have used temporary p53 knockdown for faster reprogramming, and then/later on

restoring p53 pathway after iPS generation helps to guard against the development of any cancer [64].

Rem 2 is a suppressor of the p53 pathway and is expressed higher in hESC. Rem2 works by accelerating the cell cycle and simultaneously protecting from apoptosis through an effect on cyclin D1. Rem 2 is a major player of hESC pluripotency and self-renewal. Rem 2 is as efficient as c-Myc and both enhance reprogramming efficiency by eightfold from human somatic cells to iPS.

8. The Epigenetical Modification

The Epigenetical modification has been widely observed in cancer, during embryogenesis and cellular reprogramming. During the mammalian development genomewide epigenetic reprogramming takes place in vivo but in vitro epigenetic reprogramming is not so efficient. That is why it takes a long time to reprogram. Epigenetic changes that modify DNA and DNA packaging protein (called histones) alters gene expression patterns and regulate cell identity [65]. To reprogram the cells to iPS we need to understand the cellular reprogramming process as well as epigenetic changes of the genome that follows the reprogramming steps. Epigenetics is defined as heritable changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence. The epigenetic mechanism to be considered as another dimension/language other than DNA. Reprogramming is regulated not only by 4 RFs but also by epigenetic modification. Epigenetic modification can happen at the DNA level by DNA methylation of promoter and methylation/acetylation of proteins (histone) that wrap the DNA; gene may be expressed or silenced. The protein acetylation facilitates the RNA synthesis machinery to access the promoter. Tumor suppressor gene silencing has been reported in various cancers by epigenetic mechanism resulting in unrestricted cell growth. In case of SCNT aberrant DNA methylation (epigenetic) pattern has resulted in an inability to reach its pluripotent state during development into blastocyst in cattle [66]. In such cases, histone deacetylase inhibitor trichostatin A (TSA) treatment increases the efficiency of development to term of SCNT embryos [67]. The promoters of Oct3/4 and nanog are highly methylated and silent in starting fibroblast cells but demethylated and active in iPS cells. iPS efficiency can be increased by the treatment with epigenetic modification drugs. These may be DNA methyltransferase inhibitors like 5′-Azacytidine and RG108, histone deacetylase inhibitors such as valproic acid and TSA, and histone methyltransferase inhibitor BIX-01294. It was expected that iPS cells may have the same epigenetic modification as ES cells. Later it was identified that there are 71 differential methylation regions (DMRs) between ES cells and iPS cells [68]. iPS cells are not as equal as ES cells in some aspects and iPS cells have some memory about the tissue of origin. This was evidenced by comperative gene expression profiling of ES and iPS cells. Kang H and Roh S showed that although murine SCNT embryos activated to reprogram with or without TSA (a strong inhibitor of histone deacetylase), TSA (treated 11 h) showed higher blastocyst rate (21.1%) compared to nontreated embryos (3.4%). TSA-treated embryo showed decreased expression of histone deacetylase (both HDAC1 and HDAC2) and DNA methylation genes (DNMT3a and DNMT3b) where expression levels increased for expression of histone acetyltransferase (P300 and CBP) and pluripotency markers (OCT4 and NANOG). Thus chemicals like TSA can be used to compensate for the shortcomings of iPS [55]. Epigenetic changes happen when differentiation takes place from pluripotency and *vice versa* [69]. The iPS cells that fulfill pluripotent criteria may contain heterogeneous profiles that affect lineage-specific differentiation. Moreover, cells reprogrammed by iPS may contain a residual memory persistent from the original parental source as well as some remnants of the programming process itself which leads to biased potential to differentiate into tissues like cardiac tissues [70]. By enforced expression of TFs, it has shown that although distinct iPS colonies morphologically looks like ES cells, at the molecular level they are very heterogenous in expressing various stage-specific in differentiation potential. They found only one type of cells that represents true iPS cells and others are reprogramming intermediates. Expression of cellular markers like TRA-1-60, DNMT3B, and REX1 can be found in fully reprogrammed cells. Contrary to our understanding AP, SSEA-4, GDF3, hTERT, and NANOG are insufficient markers. Pluripotency is maintained by an open chromatic state of cells. It may be achieved in 2 ways (a) factors that keep chromatin open globally to facilitate the entry of transcription machineries to the gene promoters, and (b) factors that act locally to silence the lineage specific genes until the initiation of differentiation [71]. Bioengineered stem cells can be used for replacement tissue for a number of diseases and has shown therapeutic benefits upon transplantation in animal models [72]. Tursun et al. was able to convert various neuron types (glutamatergic, cholinergic, and GABAergic) by the ectopic expression of only one transcription factor in *C. elegans*. This was achieved by the removal of the histone chaperone LIN-53 (homologous to human RbAp46/48) that works as histone remodeling and modifying complex. It can be mimicked by chemical inhibition of histone deacetylases [73].

9. Molecular Mechanism of Reprogramming

The detailed molecular mechanism of cellular reprogramming is not known yet. Several of the reprogramming factors work with protein-protein interaction. These genes bind with promoters of several genes. Epigenetic modification facilitates enzymes/proteins access to the promoter. There are 2 sets of genes with opposing functions: upregulation of genes that are involved in stemness like STAT3 and ZIC3 [74] and downregulation of genes that are responsible for differentiation like PAX6, ATBF1, and SUZ12 [75]. During reprogramming of MEF, induction of SSEA-1 and repression of Thy-1 gene are noticed at the early stage of reprogramming [76]. More late stage endogenous pluripotent markers OCT3/4 and SOX2 as well as telomerase are expressed that add telomere at the end of chromosomes.

Fully reprogrammed cells are positive for pluripotent marker SSEA-4 and negative for TRA-1-60 and fibroblast marker, CD13. They are also supposed to have inactivated retroviral promoter. iPS has been produced from various source of adult cells like mouse liver [77], pancreatic beta cells [78]. Work on signaling pathway shows that modulation of Wnt/β-catenin, MAPK/ERK, TGF-β, and PI3K/AKT signaling pathway increases the likelihood of somatic cell reprogramming [79].

Prigione and Adjaye 2010 have demonstrated that human ES and iPS both in the undifferentiated state and all stages of differentiation have similar mitochondrial properties which are distinct from those of fibroblasts. This was done by global transcriptional profiling and suggests that mitochondrial profile remains similar upon differentiation [80].

10. Incomplete Programming

Incomplete programming of mouse colony tends to express lineage-specific genes. CD13 markers are lost in the early stages of iPS followed by diminished expression of GFP and increased expression of SSEA4 and TRA-1-60. Hoechst staining is also diminished at the late stage of reprogramming as stem cells tend to pump out this nuclear staining dye. In summary, starting with CD13+ human fibroblasts, at the 1st and 2nd stages of dedifferentiation, both have GFP+, Hoechst bright and TRA-1-60 negative. But at the 2nd stage SSEA-4 positive which is negative in the 1st stage. Stages 2 and 3, both have CD13-, SSEA4+; GFP is negative at stage 3 but positive at stage 2; TRA-1-60 is positive at stage 3 and negative at stage 2; Hoechst is dim in stage 3 but bright at stage 2. However, the above-mentioned markers are not always enough as cells expressing these markers sometimes fail to expand. Together with these markers and growth properties they help to identify the real iPS cell lines. *The incompletely reprogrammed cells may self-renew due to the presence of c-Myc. Removal of c-Myc from reprogramming factors has shown significantly decreased number of incompletely reprogrammed colonies in the mouse and human [10]. For that reason, Hanna et al. used a soluble wnt3a that promotes iPS regeneration in the absence of c-Myc. Wnt-3a conditioned media can reprogram MEF cells [36]. Wnt signalling results in inhibition of GSK-3 and stabilization of cytoplasmic β-catenin.*

Li et al. has created iPSC from somatic cell by genetic transduction that remains homogeneous. They developed a method where both human and rat iPSCs (riPSCs) can be maintained by LIF and a cocktail of ALK5 inhibitor, GSK3 inhibitor, and MEK inhibitor [60].

11. Determination of Cell Integrity after iPS

Before using iPS for lineage-specific differentiation, the quality and genetic integrity of iPS cells are determined. Cells are karyotyped to rule out any chromosomal aberrations and cells should be confirmed as diploid, normal. Expression of exogenous pluripotency markers and nonintegration of LGNSO genes to host chromosome is determined by RT-PCR and standard Southern blotting, respectively. To examine the coexpression of Lin28, Nanog, Sox2, and Oct4, transfected cells are subjected to Western blot or immunofluorescence analysis. Pluripotency is determined *in vitro* and *in vivo*. EB formation is one of the hallmarks for *in vitro* differentiation of ES cells. Teratoma formation *in vivo* is a test for pluripotency. Spontaneous differentiation of MC-iPS cells into mesoderm, endoderm, and ectoderm lineages is detected by immunofluorescence and RT-PCR of genes that are specific for differentiation. To examine the *in vivo* developmental potential of human iPS cells generated through reprogramming, cells are injected into the dorsal flank of 6-week-old immune-compromised SCID-beige mice. After eight weeks, teratoma formation is evaluated.

12. Conclusion

Although iPS cells are easy to create in the lab ES cells are the gold standards. The ES cells are far more efficient in deriving other types of tissues than iPS. However, using ES cells is not easy due to ethical reasons. Since the establishment of iPS as an alternative to ESC, a new avenue has opened in stem cell research. Now cells can be autologously reprogramed to pluripotency for therapeutic applications. Any adult cells like skin cells, muscle cells, and adipose tissue cells have been used for reprogramming purposes. The original viral transduction method has evolved to reprogramming methods where reprogramming factors can be delivered without viral vectors thus genome remained unperturbed. This has been done by plasmid, protein, mRNA transfection, and minicircle nucleofection. Different cell types have different levels of reprogramming potency and different level of requirements of reprogramming factors. This may suggest that there is still undiscovered factors remained that are needed for efficient and quick generation of iPS. On the other hand, some cells express few factors abundantly than other kind of cells thus obviating the necessity of the reprogramming factor for that cell type. Teratoma formation from iPS is a drawback and needs careful screening of iPS that gives rise to teratoma *in vivo*. Direct reprogramming (iPS) is in nascent stage. Further research will facilitate the possibility of iPS in wide ranging of tissue engineering and regenerative medicine making the technology more available for therapeutic purposes.

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Stem Cells International
Volume 2011, Article ID 924782, 11 pages
doi:10.4061/2011/924782

Research Article

Hepatic Differentiation of Murine Disease-Specific Induced Pluripotent Stem Cells Allows Disease Modelling *In Vitro*

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Received 17 March 2011; Revised 12 July 2011; Accepted 13 July 2011

Academic Editor: Shinn-Chih Wu

Direct reprogramming of somatic cells into pluripotent cells by retrovirus-mediated expression of OCT4, SOX2, KLF4, and C-MYC is a promising approach to derive disease-specific induced pluripotent stem cells (iPSCs). In this study, we focused on three murine models for metabolic liver disorders: the copper storage disorder Wilson's disease (toxic-milk mice), tyrosinemia type 1 (fumarylacetoacetate-hydrolase deficiency, $FAH^{-/-}$ mice), and alpha1-antitrypsin deficiency (PiZ mice). Colonies of iPSCs emerged 2-3 weeks after transduction of fibroblasts, prepared from each mouse strain, and were maintained as individual iPSC lines. RT-PCR and immunofluorescence analyses demonstrated the expression of endogenous pluripotency markers. Hepatic precursor cells could be derived from these disease-specific iPSCs applying an *in vitro* differentiation protocol and could be visualized after transduction of a lentiviral albumin-GFP reporter construct. Functional characterization of these cells allowed the recapitulation of the disease phenotype for further studies of underlying molecular mechanisms of the respective disease.

1. Introduction

To date, more than 200 liver-based defects are identified, which can cause hepatic and extrahepatic diseases, and such inborn errors of liver metabolism account for 15% to 20% of liver transplantation indications for children. Hence, the elucidation of the molecular pathways of liver regeneration and extensive preclinical cell transplantation experiments in animals have led to the application of hepatocyte transplantation in a number of patients with hereditary metabolic liver disease and acute liver failure [1–4]. In these first clinical studies, hepatocyte transplantation has been considered either as a full-treatment option, or, in more severe situations as a bridge to transplantation [5]. Furthermore, it was shown that cryopreserved hepatic cells from one organ could be applied for multiple children [6].

The generation of iPS cells from adult somatic cells by retrovirus-mediated expression of pluripotency-associated genes in mice [7–10] and in humans [11–13] offers a unique tool to generate disease-specific iPS cells for pathophysiological studies (Figure 1). As a proof-of-principle, it was demonstrated in recent publications that iPS cells from a murine sickle cell anaemia model as well as from Fanconi Anaemia patients can be generated, repaired, and used for the correction of the disease by transplantation [14, 15]. However, in order to use those iPS derivatives for studies of the disease, suitable differentiation protocols need to be applied to get a disease-specific cell phenotype. In the past, we and others have generated hepatic precursor cells from human and mouse embryonic stem-cell lines [16–19]. With the existing differentiation protocols, a primitive hepatic phenotype with foetal gene expression patterns can be induced in the majority of the embryonic stem cells [16, 20]. Transplantation of these cells, however, have so far resulted only in scattered formation of hepatocytes or were reported to form small hepatocyte clusters in major urinary protein promoter- (Mup-) driven urokinase-type plasminogen activator (uPA) and $FAH^{-/-}$ mice [21–23]. Nevertheless, hepatic cells suitable for pharmacological testing have been described [24], and in a more recent publication, hepatic cell differentiation of human ES cells was refined [25] achieving

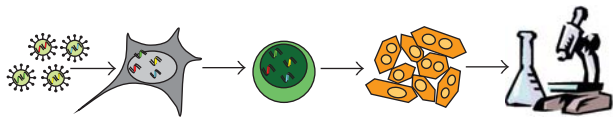

Figure 1: iPS cells as model to study metabolic liver diseases. Induced pluripotent stem cells (iPSCs) can be generated using retro- or lentiviral constructs expressing the canonical reprogramming factors Oct4, Klf4, and Sox2 with or without c-Myc. This technique allows the generation of pluripotent stem cells from diseased individuals. Subjection of these iPS cells to hepatic differentiation protocols results in an easily accessible source of disease-specific hepatic cells. Importantly, this technique might pave the way for in depth study of corresponding liver diseases.

transplantable hepatic cells, which functionally engrafted in livers of immunodeficient mice.

In our studies, we aimed on generating disease-specific iPS cell from mice carrying genetic defects for three clinically relevant metabolic liver diseases. As murine model of the copper storage disorder Wilson's disease, we chose the so-called toxic milk mice, which carry a point mutation in the ATP7b gene [26] and closely represent the disease phenotype in patients [27]. As model for acute tyrosinemia type I, we studied fumarylacetoacetate hydrolase-deficient ($FAH^{-/-}$) mice [28], which also serve as a well established liver regeneration model for studies on hepatic cell transplantation mechanisms. Finally, we investigated a transgenic mouse strain, which expresses the mutated isoform of the human α1-antitrypsin variant (PIZ) and which depicts liver damage related to accumulation of the misfolded proteins [29].

2. Materials and Methods

2.1. Mice, Animal Tissue Samples, and Cell Sources. C3HeB/FeJ-Atp7btx-J/J (common name: toxic-milk mice) were obtained from Jackson Lab (Bar Harbor, Me, USA). $FAH^{-/-}$-mice were provided by Arndt Vogel (Hannover Medical School, Germany), and tissue samples (ear cuts and tail tips) from adult PiZ mice were obtained from Robert Bals (University of Homburg/Saar, Germany). All animals were maintained and handled in accordance with institutional guidelines with free access to food and water. Murine embryonic stem cells carrying an Oct4 promoter-driven eGFP transgene derived from OG2 mice [30] as well as embryonic stem cells derived from C3H mice (Charles River) were provided by the Max Planck Institute for Molecular Biomedicine (Münster, Germany).

2.2. Isolation and Reprogramming of Adult Mouse Fibroblasts. Fibroblasts were isolated foetuses of toxic-milk and $FAH^{-/-}$ mice (ED 13.5) and ear cuts of adult PiZ mice, respectively. Cells were maintained in DMEM low glucose (PAA, Austria) supplied with 10% FBS (PAA), 1% Penicillin/Streptomycin + L-Glutamine (PAA) and 100 μM β-Mercaptoethanol (Gibco, Germany). One day before transduction, fibroblasts were seeded at 100,000 cells per well of a 6-well plate (TPP, Switzerland). Next day, toxic-milk and $FAH^{-/-}$ fibroblasts were transduced with 3 μL each of concentrated retroviral reprogramming vectors hOCT4, hSOX2, hKLF4, and hC-MYC, whereas PiZ fibroblasts were transduced with 3 μL each of concentrated lentviral hOCT4, hSOX2, and hKLF4. Cells were cultured for 24 days in mouse ES cell medium supplemented with leukemia inhibitory factor (LIF) and replated on a 6 cm dish of C3H mouse embryonic fibroblast (MEF) feeder cells. 6 days later, colonies were picked and subcloned.

2.3. Production of Lentiviral and Gamma-Retroviral Vectors. For lentiviral vectors production, one day before transfection, HEK 293T cells were seeded at 3×10^6 cells per 10 cm dish (TPP) in DMEM complete (DMEM high glucose, PAA) supplied with 10% FBS (PAA) and 1% Penicillin/Streptomycin + L-Glutamine (PAA). On the day of transfection, medium was exchanged with 8 mL DMEM complete supplemented with 25 μM Chloroquine (Sigma-Aldrich, Germany). Plasmids encoding for lentiviral gag/pol (pCDNA3.GP.CCCC, 10 μg), RSV-Rev (pRSV-Rev, 5 μg), VSV-G (pMD2.G, 2 μg), and packaging plasmid encoding for respective transgene (SFFV-hOCT4, SFFV-hSOX2, SFFV-hKLF4, SFFV-hC-MYC, and Alb-GFP, 10 μg) were mixed in 400 μL of ddH_2O and 100 μL of 1.25 M $CaCl_2$. The plasmids-$CaCl_2$ mixture was added dropwise to 2xHBS and observed until precipitates became visible in phase-contrast microscope and then added to HEK cells. 7 hours later, medium was exchanged with 10 mL DMEM complete, and 36 hours later, supernatant was collected, passed through 0.45 μm filter, and centrifuged at 14,000 $\times$g for 8 h. Virus pellet was resuspended in PBS (PAA) in 0.5% of volume of collected supernatant (200-fold concentration). Gamma-retroviral vectors were produced as previously reported [31].

2.4. Pluripotent Stem-Cell Culture. Murine iPSC and OG2 (Oct4-GFP transgenic) embryonic stem (ES) cells were cultivated on mouse embryonic fibroblasts on gelatinized dishes (Falcon BD) in culture medium I (Knock Out DMEM, Invitrogen) supplemented with 15% FCS (selected batches) and additives: 0.2% 50 mM β-Mercaptoethanol, 1% Pen/Strep/L-Gln, 1% nonessential amino acids (all PAA) and 10 ng/mL recombinant human leukemia inhibitory factor (LIF, Chemicon).

2.5. Immunocytochemistry. Cells were seeded on C3H feeder cells three days prior to cytochemistry staining at a density of 50 cells per well in a 24-well dish (TPP). Cells were fixed with 4% PFA for 20 min at 4°C, washed twice with TBS, and permeabilized with 0.1% Tween 20 and 0.05% NP-40 in TBS for 30 min at room temperature (RT). Fixed cells were blocked with 5% BSA in TBS for 30 min at RT, and washed three times with TBS. Primary antibody was diluted in TBS 1 : 75 for Nanog (Santa Cruz, sc-30328) and 1 : 100 for Oct4 (Santa Cruz, sc-5279), incubated for 1 h at RT and washed three times. Secondary antibody was diluted 1 : 400 in TBS (Invitrogen: Nanog Alexa Fluor 647A21447 and for Oct4 Alexa Fluor 488 A11001) and incubated for 45 min at RT. Cells were washed twice and stained with 0.2 μg/mL DAPI (Invitrogen D3571) in TBS for 1 min at RT. Cells were

washed twice and analysed using Olympus IX71 and Cell P software.

2.6. Alkaline Phosphatase (AP) Staining. AP staining was performed using the Chemicon Alkaline Phosphatase Detection Kit (Millipore, USA) according to manufacturer's protocol.

2.7. cDNA Synthesis and Taqman-Based qRT PCR. cDNA synthesis was performed using the SuperScriptTM III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, USA) with Random Hexamers. Taqman-based qRT PCR assays were performed on the StepOne Plus Cycler (Applied Biosystems) using the standard settings. Taqman probes were ordered from Applied Biosystems for murine β-Actin (Mm00607939_s1), Oct4 (Mm00658129_gH), Sox2 (Mm00488369_s1), Nanog (Mm02019550_s1), Albumin (Mm00802090_m1), Afp (Mm00431715_m1), Ck18 (Mm01601706_g1), Abcc2 (Mm00496899_m1), Ttr (Mm00443267_m1), Hnf4a (Mm00433964_m1) and human SERPINA1 (Hs01097800_m1), OCT4 (Hs_03005111_g1), SOX2 (Hs_01053749_s1), KLF4 (Hs_00358836_m1), and C-MYC (Hs_01570247_m1). Analyses of exogenous retrovirally expressed murine reprogramming was performed using the following primer/probe combinations: pMXs-Oct4 for: TGGTACGGGAAATCACAAGTTTG, rev: GTCATAGTTC CTGT TGGTGA AGTTCA, probe:6FAM-CTTCACCATGCCCCTCA-MGB; pMXs-Sox2 for: G T G T G G T G G T A C G G G A A A T C A C, rev: TTCAGCTCCGT CTCC ATCATG, probe: 6 F A M - TGTACAAAAAAGCAGGCTTGT-MGB; pMXs-Klf4 for: GTGTGGTGGTACGGGAAATCA, rev: CGCGAACGTGG - AGAA GGA, probe: 6FAM-CTTCACCATGGCTGTCAG-MGB; pMXs-cMyc for: TGGTACGGGAAATCACAAGTT-TG, rev: GTCATAGTTCCTGTTGGTGAAGTTCA, probe: 6FAM-CTTCACCATGCCCCTCA-MGB.

2.8. Teratoma Assays. For teratoma formation assays, 1×10^6 cells were transplanted subcutaneously into NOD/SCID mice and grown for 2-3 months. Mice were sacrificed, and teratomas were fixed in 10% formalin and embedded into paraffin. Sections were stained with Hematoxylin and Eosin and evaluated for the formation of the three germ layers.

2.9. Hepatic Differentiation Protocol. iPSCs were differentiated with a modified version of the hanging drop method as previously published by Kania et al. [17]. Briefly, iPSCs were trypsinized and preplated on gelatin-coated 6 cm dishes (TPP) for 45 min for separation from feeder-cells. iPS cell containing supernatants were centrifuged at 250 ×g for 4 min and resuspended in mouse ES cell culture medium without LIF to 30000 cells/mL. From this cell suspension, $50 \times 20\,\mu$L hanging drops were put on the inside of the lid of a 10 cm dish and grown at 37°C in 5% CO_2 incubator for embryoid body (EB) formation. At day 5, EBs were collected and kept in IMDM with 20% FBS, 1% Pen/Strep/L-Gl n, 1% nonessential amino acids, 0.2% 50 mM β-Mercaptoethanol, 0.1% 450 mM α-Monothioglycerol (all PAA) on gelatin-coated 6-wells (25 EB's per well). 9 days later, cells were trypsinized and transferred on rat tail collagen type I- (Roche Diagnostics) coated 6-wells and kept in IMDM for overnight attachment. Next day, cells were washed once with PBS and kept on HCM with SingleQuots (Lonza) for further hepatic differentiation for 14–20 days. In case of transduction with lentiviral Alb-GFP reporter construct, $1 \times 10e6$ lentiviral particles (MOI ~ 3–5) were added to 2 mL HCM in each well of a 6-well at day 5 + 9 + 3 and incubated for 48 h before medium exchange. This protocol yields in transduction efficacy close to 100% in murine hepatoma cells (Hepa 1–6) with on negligible expression in nonhepatic cells, such as fibroblasts. Hepatic differentiation efficiency was estimated by FACS analysis for GFP-positive cells at the end of differentiation on a BD FACS Calibur (BD Biosciences).

2.10. Albumin ELISA. Cell supernatant was harvested after three days from day 5 + 9 + 14 hepatic differentiated cells. Supernatant of Hepa 1–6 cells served as a positive control and was diluted 1 : 1 with fresh media. The ELISA was performed according to manufactures protocol (Mouse albumin ELISA Kit; Bethyl Laboratories) using 100 μL supernatant per reaction. The primary antibody was diluted 1 : 1,000, the secondary antibody 1 : 10,000 and TMB ELISA Substrate was added (Thermo Scientific Pierce, USA). Secreted albumin was calculated per 1,000 cells and normalized to time.

2.11. Urea Production. Urea production was analysed using the diacetyl monoxime method [32]. Supernatant was harvested after three days from day 5 + 9 + 14 hepatic differentiated cells. For the colour reaction, 100 μL supernatant was mixed with 1400 μL reagent (distilled water, mixed acid reagent, and mixed colour reagent in the ratio 1 : 1 : 1) and boiled for 15 min at 98°C. Absorbance was detected using a 540 nm filter in a single point measurement. The results were normalized 10,000 cells and 24 h.

2.12. Cytochrome P450 Activity. Activation of cytochrome P450 subtype 1A1 was determined using the EROD assay [33]. Fresh media containing 10 μM dicumarol and 8 μM 7-ethoxyresorufin was added for two hours on day 5 + 9 + 14 hepatic differentiated cells. 75 μL of cell supernatant was mixed with 25 μL 0.1 M Na-Ac solution and incubated with 10 units of β-glucuronidase/arylsulfatase for 2 h at 39°C. Reaction was stopped by adding 200 μL EtOH, and resorfurin was analysed photometrically using 535 nm excitation and 595 nm emission filters. Results were normalized to 10,000 cells and 2 h.

2.13. Karyotype Analysis. Cells were treated with 10 μm/mL Nocodazole for 8 hours to arrest cells in metaphase. These cells were then collected after trypsinization and lysed in 0.56% KCl before fixation in methanol : acetic acid (3 : 1). The cell nuclei were then dropped on glass slides from a height of 1.5 meters and further stained with DAPI for chromosomal count analysis.

3. Results and Discussion

3.1. iPSC from the Murine Model of Wilson's Disease. As first murine model of hepatic liver diseases, from which we generated iPSCs, we chose Jackson toxic-milk mice. These mice reflect the human disorder Wilson's disease leading to fibrotic changes, inflammatory infiltration, and copper accumulation in the mice's liver [27]. Toxic-milk mice-derived iPSCs were generated using foetal fibroblasts cultured under adherent monolayer conditions. Cells were transduced with gamma-retroviruses encoding for the mouse Oct4, Sox2, Klf4, and c-Myc cDNAs [7, 31] and maintained under normal feeder cell culture conditions. After six days, the transduced cells were split and seeded on irradiated feeder cells applying standard mouse embryonic stem-cell culture conditions. About two weeks later colonies with different morphologies emerged from the initially transduced cells (Figure 2(a)). Those colonies, which morphologically most closely resembled normal embryonic stem cells, were mechanically picked and subcloned, prior to further cultivation for derivation of individual iPSC lines (Figure 2(b)). For further characterisation, the iPSCs were seeded on cover slips for immunohistochemical staining (Figures 2(c)–2(f)). Analyses of the two most important pluripotency-associated markers Oct4 (Figure 2(c)) and Nanog (Figure 2(d)) clearly depicted the colocalisation of these markers with the DAPI stained nuclei (Figure 2(e)) of toxic-milk iPSC colonies. Furthermore, the pluripotent status of the toxic-milk iPSC colonies was confirmed by alkaline phosphatase (AP) activity as depicted in Figure 2(g), which is a functional hallmark of reprogrammed pluripotent stem cells. Our findings were further supported by quantitative analyses of the gene expression profile by qRT PCR (Figure 2(i)). The derived toxic-milk iPSC line shows a high expression level of the pluripotent markers Oct4, Nanog, and Sox2 referred to silent transcriptional activity in the starting murine embryonic fibroblasts. Compared to control embryonic stem cells (ESCs), derived from Oct4-GFP transgenic (OG2) mice, the gene expression of these markers was slightly reduced, but the proportions between the different markers reflected the expression pattern of murine embryonic stem cells. Toxic-milk iPSCs were able to form teratomas when transplanted subcutaneously into NOD/SCID mice (Figures 2(j)–2(l)), and, moreover, the reprogramming transgenes were shown to be silenced in the fully reprogrammed iPSCs (Supplementary Figure 1(A) which is available online at doi: 10.4061/2011/924782). Karyotype analysis revealed a diploid set of 40 chromosomes (Figure 2(h)). To summarise, our toxic-milk iPSC line exhibited the most important pluripotent stem cells' characteristics suggesting a well-established pluripotency network and a fully pluripotent status of these reprogrammed cells.

For the hepatic differentiation of toxic-milk iPS cells we used a protocol based on the generation of cell aggregates in embryoid bodies [17, 21]. The embryoid bodies were first cultured in "hanging drops" and then plated in media containing α-monothioglycerol supporting endodermal specification, before maturation in hepatocyte culture medium (HCM), which supports differentiation of hepatic precursor cells. At the final stage of this protocol, we obtained cells exhibiting a polygonal morphology, which resembled definitive endoderm-derived hepatic cells (Figure 3(a)). For further analyses, the differentiated cells were transduced with a lentiviral reporter construct expressing enhanced green fluorescent protein (eGFP) under transcriptional control of the Albumin promoter/enhancer cassette [21]. This assay identified the polygonal-shaped differentiated cells as eGFP-expressing hepatic cells (Figure 3(b)) obtained at the final step of the differentiation protocol. However, the total amount of eGFP-positive cells was below 10%, and, therefore, the putative hepatic cells were purified by fluorescence-activated cells sorting (FACS) prior to gene expression analyses by quantitative RT-PCR (Figure 3(c)). The toxic-milk iPSC-derived hepatic cells expressed the adult hepatic marker albumin (Alb), the progenitor marker α-fetoprotein (Afp), cytokeratin 18 (Ck18), which is a marker distinguishing hepatic cells from cholangiocytic cells, transthyretin (Ttr), and hepatocyte nuclear factor 4α (Hnf4α). Furthermore, expression of the apical conjugate export pump MRP2 (AbcC2) was detectable, which is a hallmark of polarized hepatocytes, exhibiting a (sinusoidal) basolateral and (canalicular) apical membrane domain. Next, we applied 250 μM copper sulphate during the last 7 days of hepatic differentiation and evaluated ATP7B-mediated copper transport in toxic-milk iPSC-derived hepatic cells in comparison to hepatic cells from controls (C3H mice-derived iPSCs). The number of hepatic cells derived from the control C3H-iPSCs increased 1.8-fold when 250 μM copper sulphate was added, due to the toxic effect of copper on nonhepatic cells in the bulk fraction of differentiating cells (Figure 3(d)). Remarkably, hepatic cells derived from the toxic-milk iPSCs were not protected during the copper sulphate challenge, which confirms the ATB7B-deficient phenotype of these cells.

3.2. iPSC From FAH-Deficient Mice to Study Tyrosinemia Type 1. As a second murine model of hereditary metabolic liver diseases, we investigated fumarylacetoacetate hydrolase knockout ($FAH^{-/-}$) mice [28], which serve as a model for acute tyrosinemia type 1, resulting in a lethal neonatal liver dysfunction phenotype [34]. We have generated iPSCs from these mice by transduction of $FAH^{-/-}$-MEFs with retroviral vectors encoding mouse Oct4, Sox2, Klf4, and c-Myc. 9–12 days after initial transduction first iPSC-like colonies were observed, which were subcloned as individual $FAH^{-/-}$-iPSC lines on day 16 (Figure 4(a)). The expression of endogenous pluripotency markers in these reprogrammed cells was confirmed by immunofluorescence staining against murine Oct4 and Nanog (Figures 4(b) and 4(c)). Fully reprogrammed $FAH^{-/-}$-iPSCs cells showed effective silencing of reprogramming transgenes (Supplementary Figure 1(B)), retained a normal karyotype (Figure 4(d)) and activated the endogenous pluripotency network with strong expression of Oct4, Nanog, and Sox2 (Figure 4(e)). Furthermore, 5 weeks after subcutaneous injection of 1×10^6 undifferentiated $FAH^{-/-}$-iPSCs into NOD/SCID mice we could analyse teratomas, which contained various cell types from all three germ layers as depicted in Figures 4(f)-4(g). Next, we applied

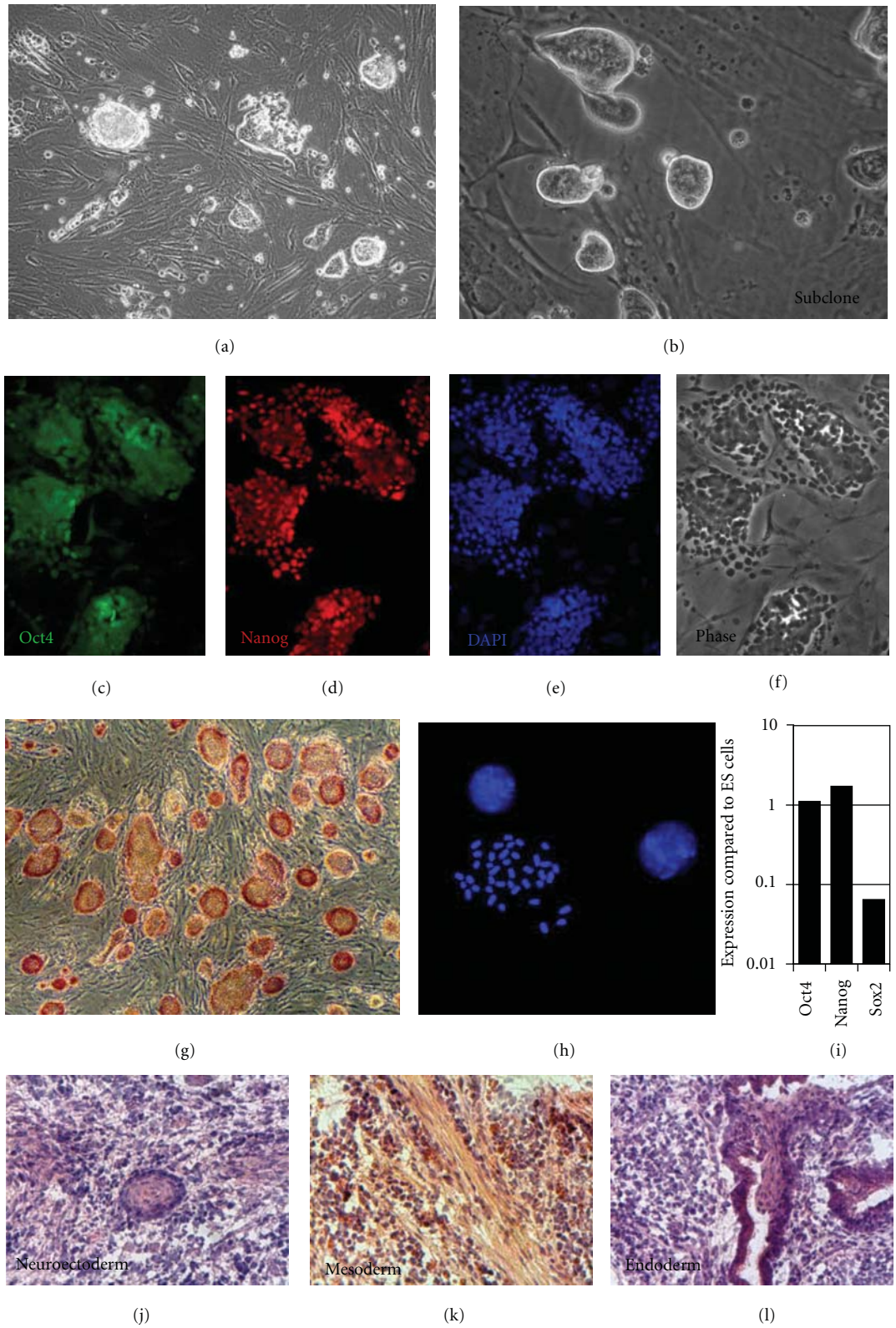

Figure 2: Generation & characterization of toxic milk iPS cells. (a) iPSCs generated from fibroblasts isolated from toxic-milk mice using retroviral vectors expressing human OCT4, KLF4, SOX2 and c-MYC. (b) iPSCs were subcloned to obtain better colony morphology. (c)–(f) Subcloned iPSCs stained positive for murine Oct4 and Nanog, whilst DAPI was used to stain the nuclei. (g) Staining for alkaline phosphatase expression of toxic-milk iPSC subclones. (h) Karyotype analysis of DAPI stained metaphase spreads. (i) qRT PCR analysis for endogenous Oct4, Nanog and Sox2 expression in toxic-milk iPSCs and OG2 ESCs compared to mouse embryonic stem cells. (j)–(l) Teratoma sections depicting formation of neuroectoderm, mesoderm (connective tissue), and endoderm.

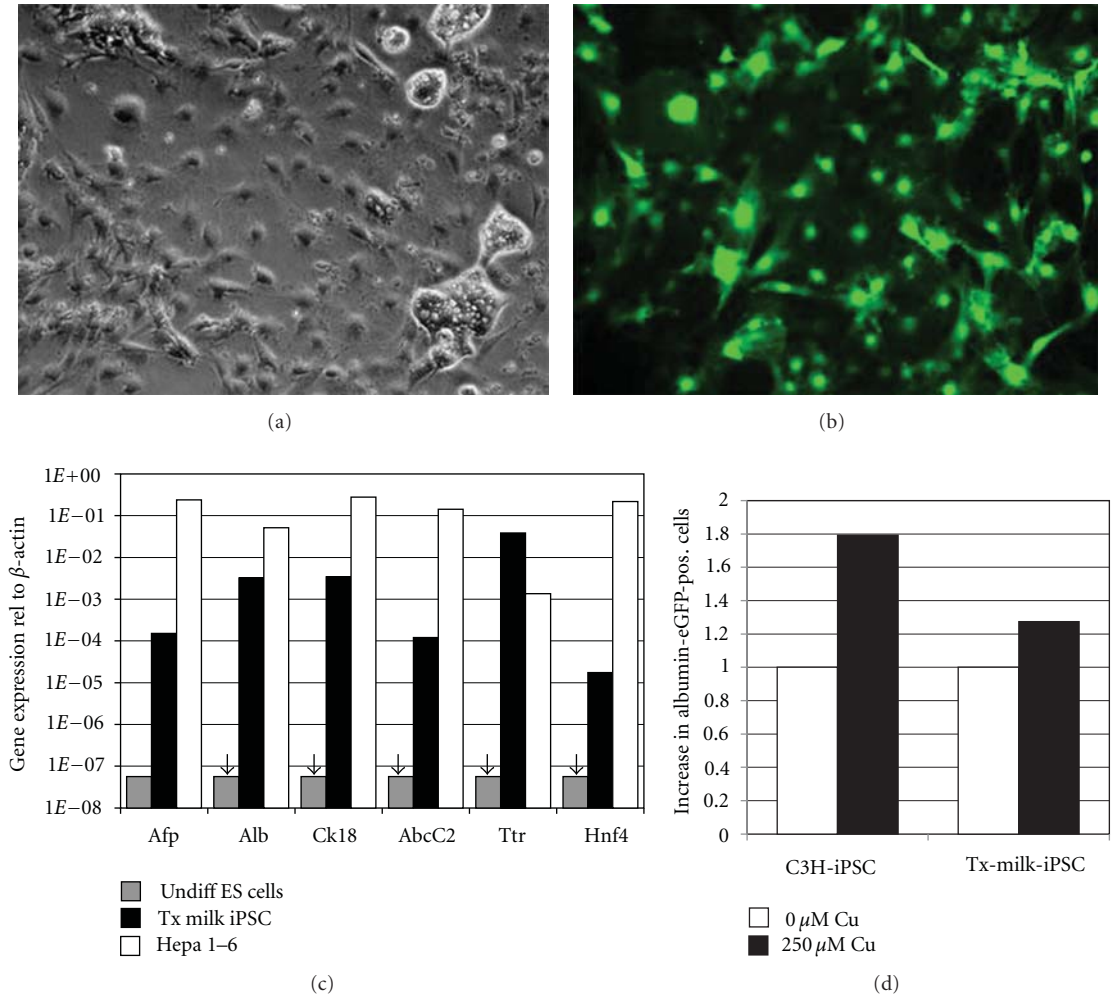

Figure 3: Hepatic differentiation of toxic milk iPS cells. (a, b) Toxic-milk iPS were subjected to hepatic differentiation using hanging drop method and transduced with lentiviral Alb-GFP expression vector. (c) qRT PCR analysis for hepatic marker genes Afp, Alb, Ck18, Abcc2, Ttr, and Hnf4α. Undifferentiated cells served as negative control, in which no hepatic gene expression was determined until cycle 45 (↓). (d) During the last seven days of differentiation, the cells were challenged with copper. The toxic-milk mice-derived cells could not export copper, and, therefore, no enrichment of Alb-positive cells was achieved. However, C3H-derived control cells were able to "detoxify" copper after hepatic specifications and, therefore, Alb-eGFP-positive cells were enriched.

the previously described hepatic *in vitro* differentiation protocol also to the FAH$^{-/-}$-iPSCs. After differentiation, FAH$^{-/-}$-iPSCs showed expression of a set of characteristic hepatic markers (Afp, Alb, Ck18, AbcC2, Ttr, Hnf4) as shown in Figure 4(i). The amount of hepatic cells derived from the FAH$^{-/-}$-iPSCs was estimated by the proportion of eGFP-positive cells (Figures 4(j)-4(k)), at the end of the differentiation period (day 5 + 9 + 20), three days after transduction with the lentiviral reporter construct expressing eGFP driven by the Albumin promoter/enhancer [21]. Because neonatal FAH$^{-/-}$-mice can be rescued from hepatic failure by supplementation with the compound 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione (NTBC) we also added this substance during the last 10 days of the *in vitro* differentiation protocol to a subset of our experiments (Figures 4(l)-4(m)). Fluorescence microscopy at the end of the differentiation protocol revealed that the treatment with 20 μg/mL of NTBC supports the viability of hepatic cells derived from FAH$^{-/-}$-iPSCs, as we could observe a higher proportion of Alb-eGFP^{pos} cells in the differentiated population of cells receiving NTBC (Figure 4(l)) compared to differentiated cells from untreated cells (Figure 4(j)).

3.3. PiZ Mice-Derived iPSCs for Studying α1-Antitrypsin Deficiency. As a third murine model of a hereditary metabolic liver disease, we investigated PiZ-mice, which express a mutated form of human α1-antitrypsin and acquire a human disease-specific liver phenotype [27, 29]. The PiZ α1-antitrypsin protein corresponds to a point-mutation (E342K) in the human *SERPINA1* gene [35] and is termed proteinase inhibitor Z-variant or PiZ, which is prone to polymerization and accumulation in the endoplasmic reticulum of hepatocytes and can lead to liver cirrhosis and hepatocarcinoma [36]. To generate iPSCs from such

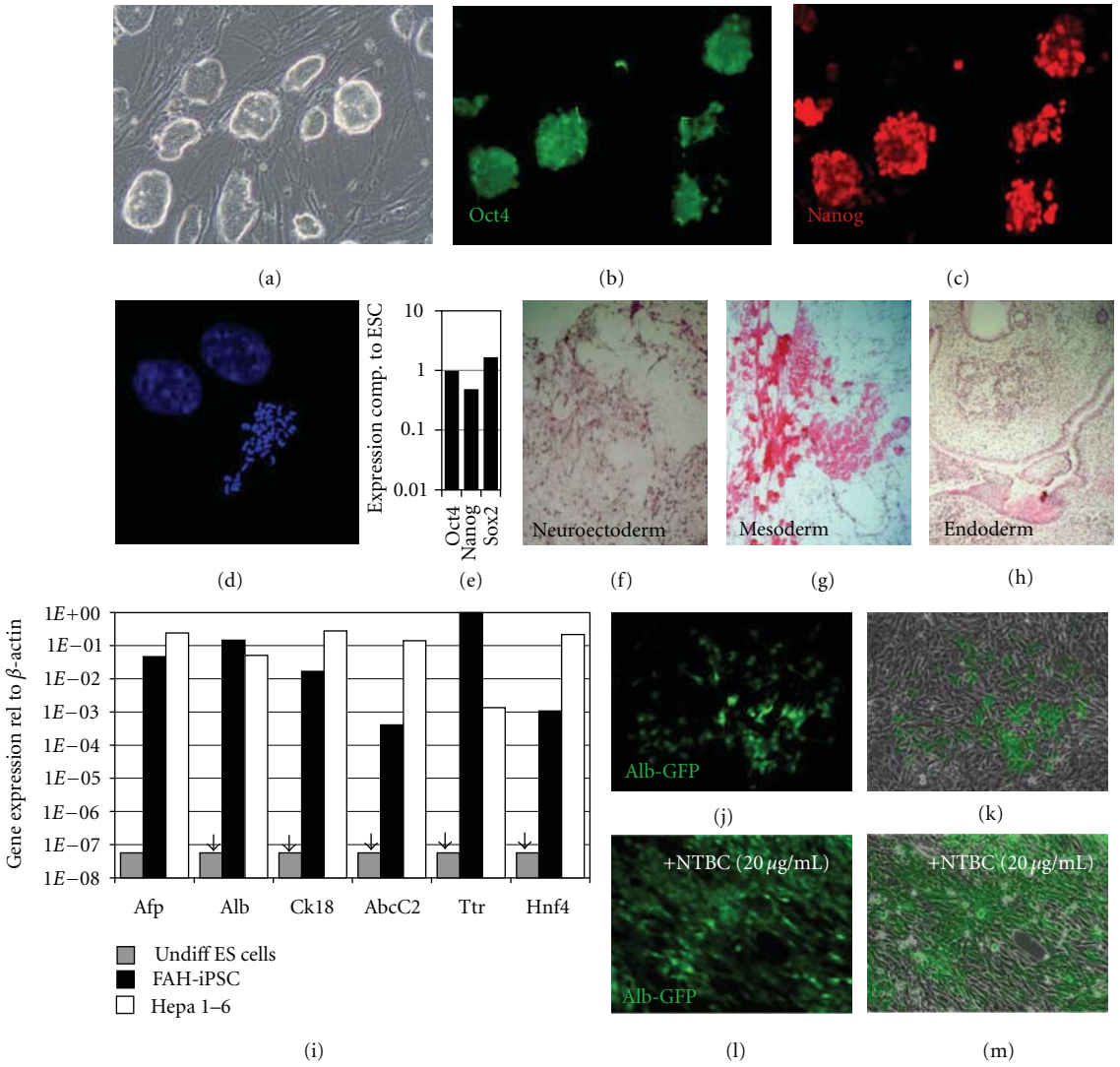

Figure 4: FAH$^{-/-}$-iPS cells. (a)–(c) iPS generated from fibroblasts isolated from FAH$^{-/-}$-mice using retroviral vectors expressing human OCT4, KLF4, SOX2, and c-MYC were stained for murine Nanog and Oct4. (d) Karyotype analysis of DAPI stained metaphase spreads. (e) Expression of endogenous Oct4, Nanog, and Sox2 determined by qRT PCR compared to ES cells. (f)–(h) FAH$^{-/-}$-iPSCs were able to form tissues from all three germ layers when transplanted subcutaneously into NOD/SCID mice. (i) qRT PCR analysis for hepatic marker genes Afp, Alb, Ck18, Abcc2, Ttr, and Hnf4α. Undifferentiated cells served as negative control, in which no hepatic gene expression was determined until cycle 45 (↓). (j)-(k) FAH$^{-/-}$-iPSCs differentiated using the hanging drop method and transduced with lentiviral Alb-eGFP expression vector. (l)-(m) FAH$^{-/-}$-iPSCs differentiated with the same method but with NTBC treatment. The NTBC-treatment rescued the lethal phenotype of hepatic cells, and more Alb-eGFP positive cells were detectable.

PiZ mice, we transduced ear fibroblasts from PiZ-mice with lentiviral vectors encoding human OCT4, SOX2, and KLF4, respectively. 14 days after transduction iPSC-like colonies were picked and subcloned to derive individual PiZ-iPSC lines. These iPSCs depicted all morphological features of murine pluripotent stem cells (Figure 5(a)) with a compact colony shape and a clear shining borderline to the surrounding feeder cells. Moreover, PiZ-iPS cells stained positive for alkaline phosphatase (Figure 5(b)) and possess a diploid karyotype consisting of 40 chromosomes (Figure 5(c)). The characteristic gene expression of endogenous pluripotency markers Oct4, Nanog and Sox2 was in the same range as control murine embryonic stem cells (Figure 5(d)), whereas exogenous human OCT4 was silenced when compared to freshly transduced PiZ ear fibroblasts (Supplementary Figure 1(C)). Pluripotency of these PiZ-iPSCs was further confirmed by teratoma formation after subcutaneous injection into NOD/SCID mice resulting in tumours with various cell types of all three germ layers (Figures 5(e)–5(g)). Finally, we investigated if hepatic derivatives of PiZ-iPSCs express the mutated human α1-antitrypsin variant. To this end, we subjected PiZ-iPSCs to our hepatic differentiation protocol. At day 5 + 9 + 3 after the start of differentiation, we transduced the cells using our lentiviral Alb-eGFP-expressing reporter construct, and we could observe morphologically distinct clusters of

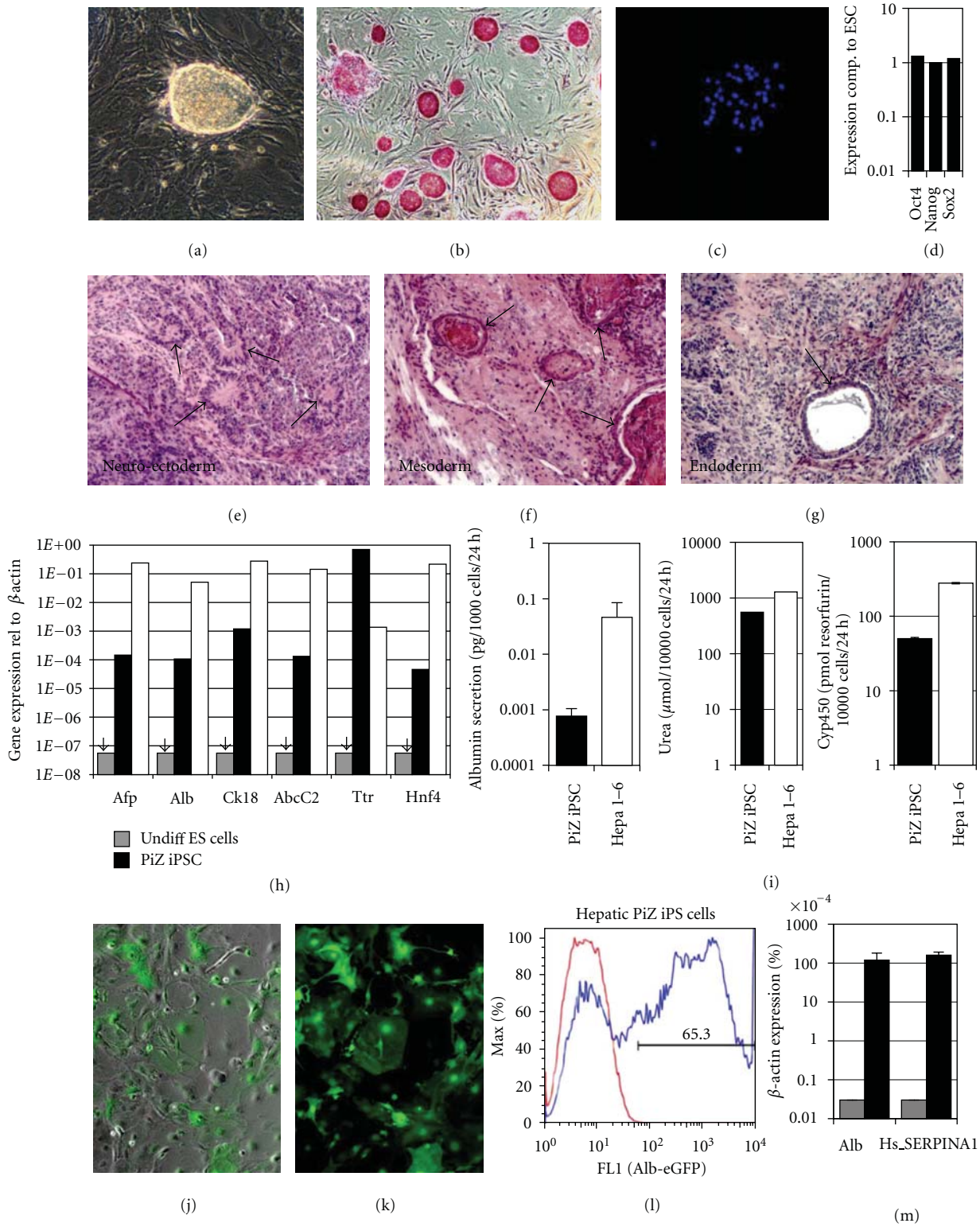

Figure 5: PiZ iPS cells. (a) iPSCs generated from ear fibroblasts isolated from PiZ mice using lentiviral vectors expressing human OCT4, KLF4, and SOX2. (b) Staining for alkaline phosphatase expression of PiZ-iPSCs. (c) Karyotype analysis of DAPI stained metaphase spreads. (d) qRT PCR analysis for endogenous expression of pluripotency markers Oct4, Nanog and Sox2 in PiZ-iPS and OG2 ES. (e)–(g) PiZ iPS were able to form tissues from all three germ layers when transplanted subcutaneously into NOD/SCID mice. (h) qRT PCR analysis for hepatic marker genes Afp, Alb, Ck18, Abcc2, Ttr, and Hnf4α. Undifferentiated cells served as negative control, in which no hepatic gene expression was determined until cycle 45 (↓). (i) Functional analysis of iPSC-derived hepatic cells: albumin secretion detected by ELISA, urea production, and CYP450 activity assay. (j)-(k) Day 5 + 9 + 14 hepatic differentiated PiZ-iPSCs transduced on day 5 + 9 + 3 with a lentiviral Alb-eGFP reporter construct (l) FACS-analysis on day 5 + 9 + 14 of hepatic differentiation for Alb-GFP positive cells. (m) qRT PCR analysis for expression of human SERPINA1 and Albumin on d 5 + 9 + 14 of cytokine-based differentiation, compared to undifferentiated PiZ-iPSCs (grey bars).

eGFP-positive hepatic cells derived from differentiated PiZ-iPSCs on day 5 + 9 + 14 (Figures 5(j)-5(k)). Differentiated and transduced cells were also analyzed by flow cytometry for Alb-GFP positive cells on day 5 + 9 + 14 (Figure 5(l)) demonstrating that the majority of cells (65.3%) exhibited a hepatic phenotype. When we applied Taqman-based qRT PCR analysis to these hepatic PiZ-iPSC-derivatives, we were able to confirm the hepatic phenotype by showing expression of a set of characteristic hepatic markers, such as Afp, Alb, Ck18, AbcC2, Ttr, Hnf4 (Figure 5(h)), and, more importantly, we detected expression of the disease-causing PiZ variant of the human SERPINA1 transgene, which was as strong as albumin expression (Figure 5(m)). Functionality of differentiated PiZ iPSCs was characterized by measuring albumin secretion by ELISA, urea production, and CYP450 activity (Figure 5(i)).

Combining two recent breakthroughs in stem-cell biology, the generation of patient-derived pluripotent stem cells [37] and the generation of hepatic cells from uncommitted pluripotent stem cells [16, 18–20, 25], new therapeutic approaches for various metabolic liver diseases may emerge. With respect to metabolic liver diseases, two recent publications support the feasibility of generating such disease-specific iPS cells from murine [38] and human individuals [39]. Those cells offer a unique opportunity for studies of the pathophysiology of the respective disease on a cellular level. To this end, we investigated whether hepatic progenitors depicting the pathophysiological hallmarks of the respective disease can be generated from iPSCs derived from murine models of the copper storage disorders Wilson's disease [26], of acute tyrosinemia type I [28], and of α1-antitrypsin deficiency [29]. All three murine disease models cover the relevant characteristics of the hepatic disease phenotypes in patients and were well-studied animal models.

Although recent publications suggest alternative approaches for iPSC generation such as use of nonintegrating viral vectors [40–43], protein transduction [44, 45], plasmid transfection [46], or stabilized mRNA application [47], we chose the well-established gamma-retroviral vector system [7, 31] for delivery of the reprogramming factors to MEFs from toxic-milk mice and $FAH^{-/-}$-mice and a recently improved lentiviral delivery system for iPSC generation from PiZ-mouse fibroblasts [48]. In the light of recent publications discriminating between partially and fully reprogrammed iPSCs and differences in the epigenetic memory in iPSCs [49–51], we assume that the robust viral delivery method is the most stable reprogramming system. This conclusion is supported by the fact that fully iPSC-derived mice were so far only generated using iPSCs derived by these viral techniques for complementation of nondeveloping tetraploid embryos [49, 52–54]. The molecular and functional data from our experiments provides strong evidence that our iPSC from the various disease models are fully reprogrammed as we observed a stable ES-like morphology for multiple passages, strong expression of pluripotency markers and, most importantly, unimpaired differentiation capabilities into hepatic cells, applying *in vitro* differentiation protocols.

Even if the embryoid body-based hepatic differentiation protocol [17, 21] does not use the extrinsic supplementation of instructive cytokines as Activin A or BMP4 for induction of endodermal differentiation, we robustly obtained a proportion of around 10% of the cells, which acquired a hepatic phenotype. Furthermore, these cells functionally correlated to the respective diseases, such as sensitivity to excess copper administration in toxic-milk hepatic cells or response to NTBC treatment in $FAH^{-/-}$-hepatic cells. When we analyzed the α1-antritrypsin related iPSCs from PiZ mice, we chose a recently established cytokine-based differentiation protocol, which is based on a monolayer cell culture in serum-free media conditions [16]. Preliminary results suggested that the hepatic cells obtained with this protocol are more mature, but the overall reproducibility of this protocol needs further attention. Most probably, the optimal cytokine application schedule is strongly dependent on cell proliferation and density as well as on other factors during the time course of differentiation, and, therefore, the robustness of this protocol is not easy to achieve. Nevertheless, we chose this protocol for the hepatic differentiation of PiZ-iPSCs, because we aim to further elaborate on the α1-antitrypsin-defiency with patient-derived iPSC in the future, which only is possible with cytokine-based protocols acting on monolayer human iPSC cultures.

4. Conclusions

Here, we successfully generated iPSC from three highly relevant murine metabolic liver disease models, and we established *in vitro* differentiation into disease-specific hepatic cells, which exhibit the pathophysiological phenotype of the clinical condition. Such cells will allow future studies on new drug targets or gene repair strategies for the respective metabolic liver disorder.

Acknowledgments

The authors are very grateful to Holm Zaehres and Axel Schambach for generation of and support with viral constructs (pMX retroviruses for the generation of toxic-milk iPSC and FAH iPSC (H.Z.), lentiviral vectors (A.S.) for the generation of PiZ iPSCs). Furthermore, they thank Martina Radstaak for excellent technical assistance. Parts of this work were funded by the Federal Ministry of Education and Research (Grants nos. 01GN0812, 01GM0854) and by the German Research foundation (DFG EXC 62/1).

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 907961, 7 pages
doi:10.4061/2011/907961

Review Article

Technical Challenges in the Derivation of Human Pluripotent Cells

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Received 15 March 2011; Accepted 25 April 2011

Academic Editor: Perng-chih Shen

It has long been discovered that human pluripotent cells could be isolated from the blastocyst state of embryos and called human embryonic stem cells (ESCs). These cells can be adapted and propagated indefinitely in culture in an undifferentiated manner as well as differentiated into cell representing the three major germ layers: endoderm, mesoderm, and ectoderm. However, the derivation of human pluripotent cells from donated embryos is limited and restricted by ethical concerns. Therefore, various approaches have been explored and proved their success. Human pluripotent cells can also be derived experimentally by the nuclear reprogramming of somatic cells. These techniques include somatic cell nuclear transfer (SCNT), cell fusion and overexpression of pluripotent genes. In this paper, we discuss the technical challenges of these approaches for nuclear reprogramming, involving their advantages and limitations. We will also highlight the possible applications of these techniques in the study of stem cell biology.

1. Introduction

Pluripotent cells can give rise to any fetal or adult cell types, over 200 specific cell types. Those in contrast to progenitor cells that are able to differentiate into a limited number of cell fates are described as multipotent cells, such as hematopoietic progenitor cells. The first pluripotent human embryonic stem cells (hESCs) were derived from the isolation and culturing of the human inner cell mass (ICM) [1]. The methodology for deriving hESCs has remained the same as the original protocol for the derivation of pluripotent mouse ESCs [2]. According to the first described protocols detailing the propagation of hESCs, the blastocyst's outer trophecodermal layer is first removed by immunosurgery and the ICM is subsequently plated onto gamma-irradiated or mitomycin C-treated mouse embryonic fibroblasts (MEFs) in the presence of high serum concentration. After several days in culture, hESC colonies begin to form [1, 3]. Undifferentiated colonies of most hESCs show a compact morphology with a high nucleus-cytoplasm ratio and retain pluripotent ability in both *in vitro* and *in vivo* experiments. They are able to form embryoid bodies which lead to spontaneous differentiation into three embryonic germ layers [4]. hESCs can also form teratomas when implanted into SCID mice [5, 6], which reflects their *in vivo* differentiation capability. Such teratoma contribute cellular regions representative of all three embryonic germ layers, including gut and glandular epithelium (indicative of endoderm), cartilage, bone and smooth muscle (indicative of mesoderm), and neural epithelium and embryonic ganglia (indicative of ectoderm) [1, 3]. However, due to ethical restrains, cannot be tested in human system. The major limitations of hESC establishment are the availability of donated IVF embryos and ethical restriction in some countries. This has brought about the development of alternative nuclear reprogramming approaches to obtaining human pluripotent cells that are closely resemble to hESCs. It was long thought that when cell differentiates, it loses their plasticity and permanently inactivated gene that is no longer need. Recent findings demonstrated three approaches for nuclear reprogramming which are, (1) somatic cell nuclear transfer, (2) cell fusion, and (3) direct reprogramming of somatic cells

by overexpression of hESC transcription factors. However, to improve the success rate of derivation of human pluripotent cells, it is essential to understand the key regulatory network of pluripotency because this knowledge will improve the derivation proficiency and culture conditions of human pluripotent cells. Therefore, this paper intend to describe the basic pluripotency network of human pluripotent cells which is followed by the discussion of technical challenges of the aforementioned reprogramming approaches.

2. Pluripotency: The Regulation Mechanisms of Human Embryonic Stem Cells (hESCs)

Self-renewal of hESCs is regulated by both intrinsic and extrinsic factors. Intrinsic factors are transcription factors that are essential for maintaining hESC identity. The best studied intrinsic factors are OCT4, NANOG and SOX2, which play essential roles in both mouse and human ESCs. OCT4, encoded by the POU5F1 locus, is a homeodomain transcription factor of the POU family. OCT4 is necessary for pluripotency, as defined by gene knockout and transgenic experiments in mice [7]. Knockingdown OCT4 by RNAi in hESCs forced them to differentiate into extraembryonic endoderm lineages [8]. Studies have defined several target genes of OCT4. Genes dependent on OCT4 activity for their expression include FGF4 [9], REX1 [10], and Lefty-1 [11] while human chorionic gonadotropin (HCG) is repressed by OCT4 activity [12]. Nanog and SOX2 are also highly expressed in hESCs and dramatically downregulated upon cell differentiation [3, 13, 14]. Like OCT4, NANOG expression appears to be crucial for the maintenance of ICM and hESCs; removal of NANOG results in ICM cells adopting a visceral and parietal endoderm fate while overexpression retards differentiation of hESCs and forces the maintenance of undifferentiated phenotype [15]. Similar to OCT4, SOX2 is important to maintain pluripotent state of hESCs. The deficiency of SOX2 mediated by RNAi is able to cause hESC differentiation toward the trophectoderm [13]. The significant roles of SOX2 in pluripotency have been confirmed by the ability to reprogram human fibroblasts to become pluripotent cells by expression of SOX2 along with OCT4, KLF4, and c-Myc [16].

Extrinsic factors, such as growth factor signaling pathways, are also very important for regulating self-renewal of hESCs. However, unlike intrinsic factors, the signaling pathways required for maintaining self-renewal of mouse and human ESCs seem very different. Although both ESCs were originally isolated and maintained by coculture on mitotically inactivated MEF feeder cells, they may require different signals from the feeder cells for retaining their undifferentiation status. Whereas the derivation of mESCs and their propagation in an undifferentiated state requires LIF [17], hESC self-renewal requires FGF2 [3]. One possible reason for this difference may result from the different growth factor receptor expression profiles in mouse and human ESCs. mESCs express leukemic inhibitory factor (LIF) receptor/gp130 receptor complexes, which bind LIF and mediate pluripotency through downstream activation of STAT3 [18]. In contrast, hESCs do not express LIF receptors or gp130 receptors [19]. FGF signaling is thought currently to be the predominant mechanism by which hESC pluripotency is maintained in culture. It was found that undifferentiated hESCs express FGFR1, the cognate receptor for FGF2, more abundantly than differentiated cells [19]. Other FGFRs, including FGFR2, FGFR3, and FGFR4, also appear to be enriched in the undifferentiated hESCs [20]. In addition to FGF signaling, activin/nodal pathway also maintains pluripotency of hESCs through mechanisms in which FGF2 acts as a competence factor [21]. It is hoped that, as the different mechanisms underlying pluripotency of hESCs are unrevealed, essential growth factors, cytokines, and signaling molecules will be discovered, and this could improve the maintenance and derivation efficiency of human pluripotent cells in the simplified, but optimal, systems for prolonged periods of time.

3. The Birth of Human Pluripotent Embryonic Stem Cells (hESCs)

In 1998, Thomson et al. reported the first establishment of hESCs from blastocysts of donated IVF embryos [1]. Growth of hESCs on inactivated MEFs or in the presence of MEF-conditioned media introduces the potential of transferring xenobiotic pathogens from the mouse to human tissues. Thus, later, the derivation of hESC lines using human feeder and human serum in order to avoid potential contamination with xenoproteins and xenogenic tissues was reported [22]. These experiments show that new hESC lines can be derived in the xeno-free system, thus permitting their therapeutic application in the future. However, it was an issue of using feeder cells such as the variation of feeder cells, the inconvenience in large-scale production, and the suspicion of molecules produced by feeder cells. Significant progress has been made very recently in understanding how to maintain hESCs in feeder-free environments by virtue of supplementation by various cytokines, either FGF2 alone [23] or in combination with either noggin [24] or activin A [25, 26]. To further apply hESC for clinical lines, it is obviously a priority to eventually refine hESC culture system in order to reach GMP standard, which is including culture conditions, physical environment, facility construction, equipment, and maintenance [27]. One of the key issues causing hESC technology to be useful for cell and tissue therapy in human is the histocompatibility [28]. Recent data support the concept that hESCs and their differentiated progeny possess immune-privileged properties, suggesting that cells derived from hESCs may provide a potential tool for induction of immunotolerance [29]. However, there are only few available hESC lines, which are derived as clinical grade and there is a concern over the genetic stability of hESCs after long-term amplification *in vitro* [30]. More importantly, the ethical debate over the destruction of human embryos has largely prohibited the derivation of large number of hESC lines. The discovery of hESCs opened up the possibility for the application of human pluripotent cells in transplantation therapy, drug screening, and toxicology studies. However,

the previously mentioned obstacles must be overcome before such potential can be realized. In another scenario for which the term "personalized pluripotent cells" has been coined, people could use their own somatic cells to be reprogrammed back to the pluripotent cell state. The following message emphasizes on the challenges of the creation of human pluripotent cells by various approaches.

4. Somatic Cell Nuclear Transfer (SCNT): The Classical Approach of Nuclear Reprogramming

When a nucleus from differentiated somatic cells, such as skin cells, is transplanted into an enucleated oocyte, nuclear reprogramming is initiated, leading to the generation of an entire individual, which is genetically identical clone of the original somatic cells. Generation of pluripotent cells by SCNT has been well documented in mouse model [31, 32]. It is noted that the process of SCNT is comprised of multiple stages and technical demanding. Principle technique of nuclear transfer involves a somatic donor cell and unfertilized, enucleated oocytes. The nucleus from the somatic donor cells is transplanted into the enucleated oocytes by micromanipulator, leading to union of both components. This reconstructed cell is stimulated to progress embryonic development. Stimulation could be performed by either electrical pulse or chemical agents [33]. The unifying aspect of the reprogramming of somatic nuclei following their transfer into the egg is that the biochemical changes establishing constrains on genetic potential are reversed. The efficiency of this reversal most probably determines the subsequent developmental success of the nuclear transfer embryos. It was realized that nuclear reprogramming by nuclear transfer to enucleated oocytes is an inefficient process. While there are sporadic reports of high efficiency, the overall rate of development to offspring is of the order of 1–3% [34]. The oocytes to use in SCNT could be either *in vivo* or *in vitro* matured cells, but the *in vivo* matured oocytes seem to give a better rate of blastocyst development [35]. The explanation for this event is that oocytes from sexually mature animals are more developmentally competent because they have a better supply of factors that will remodel the nucleus when it is transferred to the nucleus. In addition to the source of oocytes, the origin of donor cells also affects the quality of SCNT-derived embryos. The relatively less differentiated donor cells result in the better outcome of blastocyst development when compared to more differentiated cells [34]. In addition, the longer culturing of donor cells *in vitro* generally causes the decreased development to blastocysts [36]. The possibility for this reason is that the nuclei from less differentiated cells are more plastic and more readily able to remove and replace proteins that affect transcription than nuclei of more differentiated cells. This also correlates with the progressive stabilization of various repressive chromatin structures that assembles as development proceeds [37]. In addition to all those considerations, SCNT-derived embryos contain maternal mitochondrial DNA (mtDNA) in oocytes. The mutation of mtDNA could cause cellular dysfunction, cancer, and diseases [38–40], limiting the potential uses of pluripotent cells obtained from this imperfect oocytes. the success of the replacement of mitochondrial genome in mature non-human primate It was recently reported [41]. This approach was performed by transferring spindle chromosomal complex from one egg to an enucleated, mitochondrial replete egg. The reconstructed oocytes with the mitochondrial replacement were capable of supporting normal fertilization, embryo development, and healthy offspring. Even this technique is skillful; it could be soon applied to human oocytes in which mutations of mtDNA are found.

Reprogramming by nuclear transfer technique has not been extensively demonstrated in human system since the access to a source of human oocytes is not only a rare opportunity, but also an ethical concern of the moment [42]. Apart from low availability, this procedure also depends on voluntary donation of these oocytes and the success rate of this technique is considerably low [43, 44]. More recently, somatic nuclei were transplanted into enucleated zygotes leading to the generation of cloned embryonic stem cells and mice. In this technique, mouse zygotes were temporarily arrested in mitosis using drug nocodazole. The resulting embryos developed into mice, thus supporting somatic nuclear reprogramming [45]. Application of this process toward human system may be applicable; however, recently, the translation of this procedure is restricted in human. Interspecies cloning, transplanting human somatic cell nucleus into enucleated animal oocytes, has shed light on surmounting the limitation of availability of human oocytes. In 2007, there was an introduction of controversial proposal in the UK for permitting the creation of interspecies embryos that was hoping to obtain a sufficient number of hybrid hESC lines for research [46]. But this had brought a massive argument toward the possibility of human clone generation, and in some countries this option had been completely banned. Moreover, due to the development of alternative approaches of nuclear reprogramming, human interspecies cloning is no longer in focus by scientists.

5. Heterokaryon: The Generation of Hybrid Pluripotent Cells

Another method to generate human pluripotent cells is cell fusion between somatic cells and hESCs [47] which is leading to the birth of "heterokaryon." The term heterokaryon means a cell that contains multiple genetically different nuclei. In contrast to enucleated oocytes, ESC cytoplasm lacks the ability to reprogram somatic cells, but does the ESC nuclei [48]. A conceptually related approach to the reprogramming of somatic cell nuclei after cell fusion is the dominant genotype/phenotype of hESCs to nuclear reprogram of somatic cell after hybridization. The hybrid cells established by cell fusion show remarkably similar characteristics to hESCs. It was postulated that the cellular contents of hESCs comprised of reprogramming factors that could influence the epigenetic status of the nucleus of somatic cells back to pluripotent state [49, 50]. These reprogrammed cells expressed key pluripotent genes, Oct4, Sox2, and Nanog [47],

and also could generate all three embryonic germ layers both *in vitro* and *in vivo* [51]. As this process involves the fusion of both somatic and embryonic stem cells, the reprogrammed cells consist of chromosomal components from both cells. It was found that neural stem cells were cocultured with ESCs suggesting that the acquisition of pluripotency by the adult neural stem cells may be mediated by spontaneous cell fusion with the pluripotent cells. These cells were found to fuse and retain both adult markers and pluripotential [52, 53]. The pluripotency of such a fusion hybrid cell may not be derived entirely from the somatic genome, as the somatic genome may be controlled by key regulatory genes of pluripotent cell genomes.

However, it is challenging that since hybrid cells originate from the fusion of somatic cells and hESCs, the reprogrammed cells contain chromosomal materials from both cell types as well as the cells exhibit chromosomal tetraploid. Up to date, there has been no reported of the success to remove genetic contents of hESCs from the hybrids cells. It was hoping that if the hESC genomes were successfully removed from the hESC-somatic cell hybrids, it could be possible to obtain immune-matched pluripotent cells from patient's own cells. The use of hESC cytoplasm nucleated from the metaphase stage is another possible option [54]. However, with all these possibilities, there is still one note of caution that has to be seriously taken into account. Although the hybrids could contribute to all three embryonic germ layers, further investigations regarding their epigenetic anomaly are necessary. It needs to be clarified whether differentiated progeny from hybrid cells are functional and do not present any genetic/epigenetic aberrations which could subsequently result in cell transformation. Finally, the factors responsible for the regulation of somatic nuclei in fusion hybrids must be characterized before they find their usefulness in therapeutic approaches.

6. Induced Pluripotency by Transcription Factors: The Modulation of the Core Gene Regulatory Network

The breakthrough in iPSC research was due to the initial cloning experiment of Dolly the sheep [55]. Then, some studies revealed that reprogramming factors in ovular cytoplasm and ESCs are able to induce pluripotency in somatic cells [56]. In 2007, it was discovered that overexpression of 4 key transcription factors, Oct4, c-Myc, Sox2, and Klf4, of hESCs could reprogram human fibroblasts to hES-like cells, called human induced-pluripotent stem cells (hiPSCs) [16]. The success of iPSCs was confirmed by latter studies from multiple groups, including a combination of different factors, Nanog and Lin28 [57]. iPSCs have passed the most stringent examinations for gene expression profile, pluripotency, self-renewal and germ layer differentiation both *in vitro* and *in vivo* [16, 58], confirming their remarkable similarity to hESCs. The development of hiPSC technology and their characteristics have opened for the new hope for regenerative medicine. However, the recent report discovered that hiPSCs still retained epigenetic memory of the origin cell [59] and this could cause differentiation propensity of iPSCs toward the lineage of starting cells. This issue seems to limit further application of hiPSCs, especially cell-based therapy.

In the past few years, major focuses for the derivation of human pluripotent stem cells are centered at iPSCs. Although the process of reprogramming by overexpression reprogramming factors sounds simple, there are currently two main problems with the iPSC technology: (1) the efficiency of hiPSC is substantially low which is less than 0.1% of fibroblasts become hiPSCs [16, 58, 60, 61] and (2) the use of virus as a vector can result in the random integration of viral DNA into the host-cell genome. Compared to other strategies, the use of viral vector, retrovirus, and lentivirus is considered as a high-efficient tool for gene transfer; thus, viral transfection is becoming the most popular choice for the expression of pluripotency-inducing transgenes [16]. However, the transfer of reprogramming genes by viral vectors appears to be a disadvantage of iPSCs in human clinical setting, such as transplantation, because the integration of viral DNA into the host-cell genome could alter the normality of host-cell genome and lead to cell transformation [62]. There are many studies to find alternatives to using viral vectors for reprogramming somatic cells. These techniques include the use of plasmid transfection and the piggyBac transposition system [63]. An alternative non-viral vector transfection method is using a single multiprotein expression vector comprised of coding sequences of c-Myc, Klf4, Oct4, and Sox2 linked with 2A peptides to reprogram both mouse and human fibroblasts [61]. Once reprogramming was achieved, the transgene could be removed using transient Cre expression. The piggyBac system is a transposon to deliver genes in mammalian cells. The piggyBac system is able to deliver large genetic elements without significant reduction in efficiency. This system was used to reprogram human fibroblasts to iPSCs [60]. Studies also explored the application of modified mRNA and proteins, encoded by those reprogramming genes [64, 65] and showed the success of reprogramming process by using these molecules. Nevertheless, compared to viral vectors, the proficiency of these alternatives significantly reduced the number of reprogrammed cells and the production of these reprogramming molecules requires specific laboratories. Despite many challenges, there are potentially multiple advantages of hiPSCs over hESCs, as the former can be generated from individual, maintaining one's genetic constitution and identity. In addition, iPSCs represent a source of differentiated cell types genetically identical to the person of origin that may be useful for screening drugs for individual forms of pathology and for being a source of transplantable tissues.

7. Future Challenges

It is a long and amusing history of nuclear reprogramming in which increasingly sophisticated technologies have became accessible. A comparison of pluripotent cells derived from these three different approaches exhibits certain common properties, including gene expression profile and differentiation capability. In addition, the elongation of telomere

and reactivation of human telomerase reverse transcriptase are commonly found in these reprogrammed cells. However, for all nuclear reprogramming strategies, some somatic cells are more readily reprogrammed than others. Thus, these features can be differentially exploited to investigate the principle mechanisms underlying pluripotency. Each of the three approaches is distinct and has its own pros and cons. For instance, SCNT is characterized by rapid reprogramming process [66] and is ideally appropriated to elucidating the fundamental principles of early embryonic development and reproductive biology, as well as yielding a sufficient number of hESCs for therapeutic purposes. In contrary, cell fusion is technically simple. When cell fusion is used to form mixed-species heterokaryons, which do not proliferate, pluripotency genes is activated quickly and with high efficiency [67]. This approach is therefore particularly well suited to demonstrating molecular mechanisms that control the onset of nuclear reprogramming, but it does not yield clinical grade cells. On the other hand, forced expression of pluripotent transcription factors could provide such cells. Due to the ease of iPSC technology, it is now worldwide studied in laboratories all over the world. In addition, iPSCs offer a tool to study human diseases [68] and drug discovery [69]. In the near future, it is much possible that the novel approach of nuclear reprogramming will emerge which could present as the safer strategy and with higher efficiency than those currently available.

Abbreviations

hESC: Human embryonic stem cell
ICM: Inner cell mass
MEF: Mouse embryonic fibroblast
SCNT: Somatic cell nuclear transfer.

Conflict of Interests

The authors declare no conflict of interest.

Acknowledgments

The authors apologize to those whose works is not cited because of space constraints. This work was supported by Suranaree University of Technology.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 560261, 8 pages
doi:10.4061/2011/560261

Review Article

Alternative Splicing in Self-Renewal of Embryonic Stem Cells

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Received 10 February 2011; Accepted 7 April 2011

Academic Editor: Perng-chih Shen

Much of embryonic stem cell biology has focused on transcriptional expression and regulation of genes that could mediate its unique potential in self-renewal or pluripotency. In alignment with our present understanding on the genetic, protein, and epigenetic factors that may direct cell fate, we present a short overview of the often overlooked contribution of alternative splice variants to regulatory diversity. Progressing beyond the limitations of a fixed genomic sequence, alternative splicing offers an additional layer of complexity to produce protein variants that may differ in function and localization that can direct embryonic stem cells to specific differentiation pathways. In light of the number of variants that can be produced at key ES cell genes alone, it is challenging to consider how much more multifaceted transcriptional regulation truly is, and if this can be captured more fully in future works.

1. Introduction

Embryonic stem cells (ESC or ES cells) are unique in their ability to self-renew and differentiate into specialized lineages representative of the three germ layers of an organism [1, 2]. First isolated from the inner cell mass (ICM) of a mouse blastocyst and subsequently in other species including human [3], ES cells have provided insight into the fundamental workings of otherwise inaccessible early developmental stages. Because of the pluripotent nature of ESCs, their potential to regenerate specific cell types is also of therapeutic interest. In this respect, strides have been made with the discovery of protein factors that were able to reprogram somatic cells into induced pluripotent stem cells (iPS cells) types that bear much similarity to ES cells [4, 5]. These reprogramming studies together with work in ES cells have provided a vast amount of insight into the pathways, mechanisms, and key transcription factors involved in pluripotency. In addition to transcriptional regulation, epigenetic mechanisms such as chromatin modifications are now known to aid in the activation/repression of developmental stage-specific genes [6–9]. Reasonably, much of our present understanding of ES cells has and will continue to arrive through comparative studies of pluripotent cells to its differentiated counterparts, though these comparisons require further refinement in light of the multiple mechanisms and subtle differences in protein complexes used in transcriptional regulation. Less widely understood are the complementary mechanisms that refine the transcriptional profile of ES cells, going beyond bulk gene expression levels to look at the transcript variants and alternative splicing (AS) that occurs at each gene.

More than 74% of human genes are known to undergo alternative splicing [10, 11], a phenomenon that can result in a combination of exons and/or untranslated 5′ and 3′ regions (UTRs) that differ from the canonical transcript. Alternatively spliced products can have implications on protein translation and RNA regulation [12], and aberrant splicing is responsible for up to 15% of human genetic disease caused by point mutations [13]. Correctly spliced, alternative transcripts can increase the diversity of the proteome through multiple splicing permutations, in a manner that does not require a concomitant increase in gene sequence.

Mouse and human ES cells share many similarities including their dependence on key ES cell transcription factors Oct4, Sox2, and Nanog for pluripotency [14–17], existence

of "bivalent" chromatin marks that repress differentiation-specific genes [18] and importance of Polycomb repressor complexes in transcriptional repression [19]. However, there are still discrepancies in the signaling pathways and requirements for specific transcription factors or microRNAs between mouse and human ES cells, the most well-documented being that of the LIF/Jak-STAT pathway signaling required for mouse but not human ES cells [20–22]. In somatic cell reprogramming, a related set of factors were found to be necessary for the generation of iPS cells. While OCT4/Oct4 and SOX2/Sox2 were deemed necessary for both mouse and human ES cells, additional mouse factors originally required were Klf4 and c-Myc, whereas the combination included NANOG and LIN28 in the case of human ES cells [4, 5]. Plausibly, the prevalence, yet underestimated impact of AS can serve to fine-tune our understanding of these species-specific differences, seen in the light of the increased functional dimensions presented by alternative transcripts.

In this paper, we highlight works related to alternative splicing of key ES cell transcription factors. Because of the paucity of AS variant data in ES cells, AS findings of these factors in other developmental stages may also give insights into the previously unexplored potential of these factors in ES cells. This paper also demonstrates the importance of examining splice variants despite their apparently subtle sequence differences.

2. Functional Implications of Alternative Splicing

Through expressed sequence tag (EST) databases for multiple tissues and species, a large percentage of mammalian genes (35–60%) were found to be alternatively spliced, by alignment of these EST sequences to the cDNA reference [23–25]. Though AS can occur anywhere across the transcribed sequence, the majority of splicing occurs within the coding region and can serve to increase the repertoire of proteins that may be utilized in the cell [24, 26, 27]. While an average of 2–5 AS transcripts are produced for each human gene involved in AS [12], an extreme example demonstrates the vast complexity allowed by AS; the *Drosophila* gene Dscam generates a potential 38000 isoforms, separated by developmental time and space [28].

Through the inclusion or exclusion of exons, protein domains are altered. This can result in changes to binding affinities, catalytic activity, stability, localization, or even posttranslational modifications [12].

Yet not all exon changes result in a new protein—some result in the degradation of the mRNA instead. Nonsense-mediated decay (NMD) of the mRNA occurs if a stop codon is encountered at a distance more than 50 base pairs from the 3′ most splice junction, due to the accumulation of NMD-associated Upf proteins at the 3′ end that are not removed by ribosomes as they traverse the mRNA sequence [29, 30]. While these unproductive splice products are unstable and not well understood, they are not uncommon. Approximately 30% of alternative exons can introduce frameshifts and stop codons into mRNA sequences, resulting in a substantial amount of what is perceived as inefficient transcription [26, 31, 32]. Surprisingly, a phylogenetic study of unproductive splice variants among primate species using the DNA polymerase POLB gene, suggests that these occur at varying frequencies among primates and are largely not conserved, yet the extent of AS variants found correlate closely with life expectancy, as well as age at first reproduction [33]. Through a mechanism as yet unknown, these noncoding protein splice forms may serve to regulate transcription, and its levels may also be tuned to reflect the amount of transcriptional control required [34, 35].

The means of AS resulting in alternative promoter usage at the 5′ and 3′ UTRs are also not well understood. However, a recent study of AS in mouse ES cells found that 12% of genes that they examined for alternative exon usage were mapped to alternative promoter sites [36]. Given the function of the 5′ region in transcriptional initiation, and the poly(A) tract in the 3′ end for mRNA stability, it is likely that the use of alternative UTRs can alter the half-time of an mRNA strand, and by extension, the amount of protein production from a functional sequence [37, 38].

3. Evolutionary Impact of Alternative Splicing

While genome-wide comparisons between human and mouse demonstrate high-sequence conservation (~90%) at canonical exons of orthologous genes [39, 40], only about 25% of alternative exons were conserved. However, when such AS exons were identified in an EST database of one species, the corresponding exon was likely to be found in the other species as well [40]. The authors attributed the inclusion of a particular exon in an EST database as indicative of a minimum expression level and found this congruent with a finding that ancient conserved regions correlated with more highly expressed genes [41]. It appears that the ~75% remainder of alternative nonconserved exons are expressed at lower levels and segregate in a tissue-specific manner in the species of origin, suggesting that these exons arose after divergence from a common ancestor. The large proportion of alternative exons with such features emphasizes the significance of AS in promoting genetic variation and possibly, speciation along with increasing organismal complexity. Extending this to the variation offered by AS transcripts between for tissue specificity, a separate study comparing mouse ES to hematopoietic stem cells found that ~30% of splice variants were unique to the mouse ES cell profile, although the functionality of these variants remains to be seen [42]. In line with the widely held view that strongly conserved sequences are indicative of core functions [43], ubiquitously expressed genes such as heterogeneous nuclear ribonucleoproteins (hnRNPs) show little AS and are located in ultraconserved regions (defined as 200 base pairs or greater of sequence with 100% identity between human, mouse, and rat) [44, 45]. In contrast, tissue-specific genes are likely to exhibit a larger number of AS variants with little cross-species homology [42] and face much less selection pressure against the insertion of Alu and other repeat elements that can lead to sequence "exonisation" that is permissive for species adaptation [46, 47].

4. Alternative Transcripts Can Direct ES Cell Differentiation

4.1. OCT4. In both mouse and human, the OCT4/Oct4 protein is a well-described transcription factor containing a POU domain that is able to bind a consensus octamer sequence on DNA [48–50]. Belonging to a group of closely related proteins of the Oct family, OCT4 is related to multiple pseudogenes, derived from earlier retrotransposition events into other chromosomes [51–54], as well as AS transcripts arising directly from the *Oct4/Pou5f1* locus [55, 56]. While OCT4 pseudogenes are expressed in human hematopoietic stem cells, it is uncertain if these pseudogenes are involved in ES cells [53, 54]. However, alternative splicing of *OCT4* is evident in ES cells, where *OCT4* is most highly expressed [15, 48]. The most commonly described transcript is *OCT4A*, which is translated into a full-length nuclear-localized OCT4 protein with an N- and C-terminal transactivation domain separated by a POU DNA-binding domain [48]. Interestingly, the primary shorter transcript, *OCT4B*, still contains the same downstream sequence as *OCT4A*, albeit with a shorter N-terminal domain that results from a skipped exon 1, but an extended 5′ end of exon 2 [55, 56]. Though a putative nuclear localization signal appears to be retained in the translated OCT4B protein, it is cytoplasmically located, in contrast to OCT4A [57].

Recently, a novel third transcript, *OCT4B1*, was identified as an ES cell-specific transcript of OCT4 and considered as a possible stemness marker, given its significant correlation with *NANOG* expression in ES and differentiated cell lines [56, 58]. While it is still uncertain if *OCT4B1* functions primarily as a transcript, or is translated into protein products, preliminary evidence from multiple groups suggests that *OCT4B1* can be spliced into the same products as *OCT4B*, all of which are cytoplasmically located [59, 60].

Furthering the diversity of output from a single locus, *OCT4* not only undergoes AS of mRNA transcripts, but goes a step further with different translations from a single mature mRNA form. Unusually, *OCT4B* and *OCT4B1* mRNA both contain 2 possible start codons, as well as an internal ribosomal entry site (IRES) formed in part by the extended 5′ end of exon 2 in OCT4B/B1 [55, 56, 61, 62]. This results in proteins of 265, 190, and 165 amino acids (a.a.) from *OCT4B/B1* although it cannot be excluded that the 164 a.a. long version is also translated from *OCT4A*, which also contains the downstream in-frame ATG codon in the 3′ end of exon 2. Structurally, the shorter OCT4 variants produced do not contain an N-terminal transactivation domain, but more importantly, lack the POU-S portion of the complete POU domain [50, 63]. Functionally, it appears that OCT4A and OCT4B variants may serve differently, since they localize to different cellular regions. OCT4A is found in the nucleus and is responsible for the well-known activities of OCT4 in ES cell self-renewal and pluripotency [64–66]. Conversely, the full OCT4B isoform (or OCT4B-265) containing an N-terminal transactivation domain was not able to reside in the nucleus, nor bind to the consensus OCT4-binding site on DNA, due to the altered configuration of the N terminal [64]. The role of OCT4B-265 in ES cells is therefore unclear, since its different intracellular localization precludes it from acting as a negative regulator of OCT4A activity. A recent study that analyzed changes to *OCT4B1* transcript levels in a gastric cancer cell line however, points to a likely role for *OCT4B1* as an antiapoptotic factor, since cells deprived of *OCT4B1* take on a giant cell morphology or undergo apoptosis directly [60]. In ES cells, the prevalence of *OCT4B1* may also serve to manage the rapid cell cycling characteristic of this proliferative cell type.

Interestingly, a mainly cytoplasmic pyruvate kinase, Pkm2, has previously been described as an Oct4 interactor [67] and may well be one of many Oct4B partners. Pkm2, itself one of two alternative transcripts from the *Pkm* gene, is the primary transcript at early embryonic stages [68] and was shown to bind to the POU domain of Oct4 [67]. As the large body of protein interaction data may not distinguish between Oct4 isoforms as baits, these data sets should be more carefully examined for clues that suggest functionality for OCT4B-265/Oct4B in the cytoplasm. Still, this cytoplasmic Oct4 variant might just make it into the nucleus after all—Kpna2, a protein involved in nuclear import was found to bind to the Oct4 POU domain [69]. While all Oct4 protein variants contain a nuclear localization signal, this is necessary but not sufficient for actual nuclear localization [57]. With the possibility of Oct4B transferred to a nuclear locale, albeit at lower levels than in the cytoplasm, this could serve as a self-generated negative feedback loop for the sequestration of active Oct4A, through its heterodimerization with Oct4B.

The shorter OCT4B-190 and OCT4B-164 proteins are not typically expressed in ES cells, yet an increase in OCT4B-190 was observed on heat shock of human ES cells, and presumed to take on a protective role against apoptosis [64], thereby highlighting an additional means by which alternative transcripts function in developmental time and function, though directed from the same locus.

Correspondingly, there is evidence that a number of Oct4 splice variants are also found in mice, although no present findings support the presence of a mouse homolog to OCT4B1 [70]. Intriguingly this may be attributed in part to the increasing evidence that suggests the nonequivalence of human and mouse ES cells. Because of the differences in growth factors and trophectoderm differentiation potential of these two cell types, it is believed that human ES cells are representative of a later epiblast stage of the developing blastocyst than mouse ES cells [71–73]. As such, it is of note that the lack of Oct4B1 in mouse ES cells might demonstrate the narrowly defined window of function for OCT4B1 in human ES cells, as supported by its possible role as a stemness marker [58]. Species-specific differences in alternative splicing may also be a contributing factor, suggesting that it is prescient to more carefully consider the model systems and likelihood of alternative splice variants in future studies.

4.2. Sall4. SALL4/Sall4 is a known ES cell-specific transcription factor that interacts with OCT4 and Nanog and also regulates stem cell pluripotency [74, 75]. In both mouse and human, SALL4/Sall4 exist as two splice isoforms, *Sall4a* and *Sall4b* that differ by the inclusion or exclusion of part

of exon 2 and results in a different number of zinc finger domains [76, 77]. *Sall4* −/− mice fail to develop an inner cell mass (ICM), demonstrating the essentialness of one or both isoforms in early embryonic development [74, 78].

While most other studies have not distinguished between isoforms, *SALL4* mutations that affect both isoforms are implicated in the human Duane-Radial Ray syndrome and acute myeloid leukemia (AML) [76, 79, 80]. Interestingly, a transgenic mouse with human *SALL4B* recapitulated the features of AML, suggesting that the truncated isoform is sufficient to initiate disease.

Chromatin immunoprecipitation experiments for Sall4a and Sall4b were informative for target genes of each of these isoforms in mouse ES cells. In line with evidence that hetero- or homodimerization of isoforms are both possible, Sall4a/b show shared as well as distinct targets in ES cells [77]. Shared targets were enriched for genes involved in developmental processes and organ morphogenesis including the essential ES cell factors Oct4, Nanog and Sox2, and Sall4a alone was targeted to a specific niche of genes involved in olfaction and sensing [77]. However, Sall4b was targeted to a larger group of genes, enriched for transcription and gene expression. In conjunction, Sall4a/b and Sall4b target genes alike were found with activating chromatin marks (H3K4me3 and H3K36me3), whereas the repressive H3K27me3 was enriched at target genes of Sall4a [77]. In the transgenic *SALL4B* mouse with AML, SALL4B expression was most evident in the initiating cancer stem cell population, but not in the chronic disease cells [76]. Sall4b is also required for proper ICM development and the maintenance of pluripotency [77, 81]. Through these animal models, it is evident that the normal contribution of this shorter isoform in development is to perpetuate self-renewal in the ICM, and in aberrant development to sustain a tumor initiating population of stem cells. Conversely, the longer Sall4a isoform appears to regulate transcription of differentiation specific genes, with bivalent chromatin marks representing active or poised genes, present only at loci that were cobound by other pluripotency factors such as Oct4 [77]. A separate Sall4a/b ChIP demonstrated that Sall4a/b binding was evident at 27% of known genes with bivalent domains—a subset of these would include Sall4a-only binding [18, 82].

Given the conservation of exons and alternative exons between human and mouse, it is likely that both Sall4 isoforms are also highly conserved across other species. Certainly the discovery of Sall4 through its homology to the *Drosophila spalt* gene [74, 83] suggests its ancestral function in organogenesis and sensory development has been retained in mammals, though this has been expanded in vertebrates through the existence of multiple Sall family genes. While exon exclusion in evolution occurs at <20% frequency in a representative human gene set, it is interesting to observe that the truncation of Exon 2 in Sall4b might have conferred additional capabilities to Sall4b for transcriptional regulation and its essential role in early development, in addition to its conserved function in cell fate differentiation with Sall4a. Despite these differences in the target genes of Sall4 brought about by an exon truncation that resulted in the loss but not ablation of all zinc finger domains, it is evident that subtle changes to domain structure through alternative splicing is able to directly impact protein function.

4.3. Tcf3. Tcf3 is a more recently described transcription factor that forms part of the core regulatory network present in ES cells [84–86]. As a transcriptional repressor and the effective end of the Wnt pathway, Tcf3 is able to translate extracellular stimuli into a directive for transcription through its binding to beta catenin. Genome-wide analysis of Tcf3-binding sites identified its frequent co-occupancy with some transcription factors that promulgate self-renewal, namely, Oct4 and Nanog [84–86]. Through this, both transcriptional activator(s) and repressor(s) are locked in a close counter-balancing relationship that can more sensitively regulate genes according to its cell fate requirements. While the repressive activity of human TCF3 can be attenuated through its phosphorylation [87], the gene itself also undergoes AS. Available in 2 known variants, the shorter Tcf3 (Tcf3(s)) lacks 14 a.a. present in the Groucho-binding domain of its longer twin, Tcf3(l), though both are expressed in mouse ES cells [36, 88, 89]. Through variant-specific knockdowns of Tcf3, Salomonis et al. categorized the response of 34 genes anticipated as Tcf3 target genes as defined by the available literature and looked to see if their responses differed. Sharing much similarity in domain structure, both Tcf3 variants showed overlaps in gene targets. However, despite the apparently small 14 a.a. change, it appears that Tcf3(s) and Tcf3(l) can also regulate mutually exclusive sets of genes associated with different downstream pathways. While Tcf3(s) target genes were involved with lineage differentiation, Tcf3(l) targets were more directed towards cardiac and neural development. Clearly, AS can increase the dimensionality of a protein's function, while still retaining its original purpose. Though comparisons of beta-catenin binding to each of the Tcf3 variants has not been described, it is very probable that both variants are still responsive to beta catenin, since such interactions in a domain separate from the affected Groucho binding region [90, 91].

Intriguingly, a recent study derived rat and human-induced pluripotent stem cells that better resembled the characteristics of mouse ES cells, including an ability to contribute to rodent chimera models when introduced into preimplantation embryos [92]. This involved the use of a GSKβ inhibitor in the culture media, suggesting a strong role for Tcf3, as part of the Wnt pathway, in regulating pluripotency. It is plausible that the splice variants of Tcf3 observed in mouse ES cells facilitate the interconversion of ES cells between different "metastable" levels of pluripotency that ES cells are able to adopt, depending upon the available ES cell factors [93].

5. Conclusion

Amplifying the complexity involved in decoding a gene into a functional RNA or protein product, alternative splicing of mRNAs occurs as a post-transcriptional regulatory process that adds to the repertoire of known mechanisms for

organismal complexity. Through weak associations of the mRNA with generic splicing factors, and possibly tissue-specific splicing factors, a newly transcribed mRNA may be transformed into a variety of protein and RNA products that differ in domain content, structure, and functional value, depending upon the tissue of interest. Although gene expression studies often seek to derive tissue-specific gene expression profiles as a hallmark of each tissue, these studies may not fully consider the implications of sequentially similar but alternative transcripts whose individual expression levels may not be resolved.

In this paper, we have considered the possible functional implications of alternative splicing. As a post-transcriptional regulatory mechanism, this can result in the inclusion or exclusion of amino acid residues that can affect protein function. Because of the large number of ways in which protein function may be regulated, disruptions can occur through alternate posttranslational modifications, domain content and binding site affinities among others. RNA stability and regulation may precede or preclude the translation of such a protein, especially through the introduction of new promoter or translation start sites. Powerfully, AS is observed through evolution and first understood as a means of increasing signal nodes and complexity despite the relative similarity of DNA content between species of recent divergence. While approximately 3/4 of all known genes undergo AS, only a third of these are estimated to show cross-species conservation. A study that considered the extent of AS for a single gene between primate species suggests that the frequency of such events is not conserved and instead may correlate with life expectancy. While such correlations may be more case appropriate than prescriptive for the diverse spectrum of known genes, it is interesting to consider how transcript variety can have seemingly profound implications on a species' life cycle and the extent of an individual's environmental interactions. Additional studies demonstrate that tissue-specific genes are subject to AS more often than ubiquitously expressed "housekeeping genes". In view of the common developmental pathways shared between mammals, embryonic stem cell biology has been instrumental in providing insight into early developmental stages. Here, we highlight 3 key ES cell transcription factors in human and mouse that are essential for self-renewal and also show that these factors undergo AS. While transcription factors such as Oct4 are generally perceived to be expressed specifically in the ICM, and to a lesser extent in primordial germ cells or their *in vitro* equivalents, it is increasingly evident that our previous notions of what constitutes "expression" needs to be more clearly distinguished, and that low levels of alternative transcripts may be present in alternative cell types, but remain unknown. These transcripts can serve to discriminate one species from the next. Although the minority of AS products are conserved between species, such products are often associated with biologically important, core pathways [43].In this paper, a number of these AS variants appear to show such behavior, in association with their essential role in ES cell renewal. At this point, much research in ES cell biology has not delved significantly to consider the impact of alternative transcripts in modulating or expanding the function of key ES cell factors. We anticipate that future studies that examine the possibility of splice variants in more detail might bring forth new evidence to distinguish ES cell mechanisms and species differences more clearly.

Abbreviations

ESC/ES Cells:	Embryonic Stem Cells
ICM:	Inner Cell Mass
iPS Cells:	Induced Pluripotent Stem Cells
UTR:	Untranslated Region
AS:	Alternative Splicing
ChIP:	Chromatin Immunoprecipitation
miRNA:	Micro-RNAs
NMD:	Nonsense mediated decay.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 795239, 13 pages
doi:10.4061/2011/795239

Research Article

Expression Patterns of Cancer-Testis Antigens in Human Embryonic Stem Cells and Their Cell Derivatives Indicate Lineage Tracks

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Received 14 March 2011; Accepted 25 April 2011

Academic Editor: Perng-chih Shen

Pluripotent stem cells can differentiate into various lineages but undergo genetic and epigenetic changes during long-term cultivation and, therefore, require regular monitoring. The expression patterns of cancer-testis antigens (CTAs) MAGE-A2, -A3, -A4, -A6, -A8, -B2, and GAGE were examined in undifferentiated human embryonic stem (hES) cells, their differentiated derivatives, teratocarcinoma (hEC) cells, and cancer cell lines of neuroectodermal and mesodermal origin. Undifferentiated hES cells and embryoid body cells expressed MAGE-A3, -A6, -A4, -A8, and GAGEs while later differentiated derivatives expressed only MAGE-A8 or MAGE-A4. Likewise, mouse pluripotent stem cells also express CTAs of Magea but not Mageb family. Despite similarity of the hES and hEC cell expression patterns, MAGE-A2 and MAGE-B2 were detected only in hEC cells but not in hES cells. Moreover, our analysis has shown that CTAs are aberrantly expressed in cancer cell lines and display low tissue specificity. The identification of CTA expression patterns in pluripotent stem cells and their derivatives may be useful for isolation of abnormally CTA-expressing cells to improve the safety of stem-cell based therapy.

1. Introduction

Two approaches for pluripotent stem cell line production have been developed. The traditional way consists in the isolation of pluripotent cells from preimplantation embryos or the conversion of embryonic germ line cells into pluripotent stem cells [1–4]. Another approach is experimental genome reprogramming of somatic cells to change their differentiation potential. There are three technologies of reprogramming: somatic cell nuclear transfer, fusion of pluripotent and somatic cells, and induction of pluripotency in somatic cells by introduction of pluripotency-related genes or proteins [5–7]. Despite different origin, all pluripotent stem cell lines display considerable similarity of the basic biological properties: high self-renewal rate and ability of in vitro and in vivo differentiation into a wide variety of cell types. On the other hand, comparative analysis of numerous derived human embryonic stem (hES) cell lines demonstrated that they differed in cell growth rate, gene expression profiles, gene methylation profiles, and microRNA profiles [8–10]. These differences may be due to the genetic background of pluripotent embryonic cells initiating hES cell lines, different culture systems used for their maintenance as well as stochastic events during long-term in vitro cultivation which lead to genomic alterations [11–14]. Moreover, induced pluripotent stem (iPS) cells derived from different somatic cells differ in their differentiation and tumorigenic potentials [15–17]. The variation of transcriptional and gene methylation profiles of human ES and iPS cell lines has been widely discussed [18–24].

Numerous studies have shown that long-term cultivation leads to the accumulation of different genetic aberrations and abnormal epigenetic changes by the pluripotent stem cells, and such changes can contribute to genomic instability, cell

transformation, and cancer development [11, 13, 25, 26]. Furthermore, most iPS cell lines were generated by overactivation of cell oncogenes C-Myc and Klf4 that might enhance their spontaneous uncontrolled expression in undifferentiated pluripotent stem cells and in differentiating progenitor cells, and, therefore, these cells may be transformed to cancer stem cells [27]. Taken together, all these data indicate that utilized pluripotent stem cell lines require regular monitoring of genetic and epigenetic integrity. In addition, large-scale searching of new gene markers is required for identification of the cells that underwent tumorigenic transformation.

Cancer-testis-associated antigens (CTAs) may be considered as potential gene candidates specific for transformed cells because they are frequently expressed in different types of cancers but have very restricted expression patterns in normal tissues [28]. All CTAs have been shown to be expressed in male gonads, some of them are expressed in the trophoblast, placenta, and developing central nervous system [29–33]. Several gene families are ubiquitously expressed in somatic and germ cells as well as in cancer cells [30]. In addition, certain CTAs were detected in the mesenchymal stem cells and differentiated hES cells [29, 34]. However, cell functions of most CTA families which include more than 100 genes remain enigmatic. Recent studies have demonstrated that CTAs are involved in the regulation of transcription [35], cell cycle and proliferation [36–39], apoptosis [40], and susceptibility to cytokines in cancer cells [41]. Moreover, the expression of some CTAs including MAGEA, SSX, and NY-ESO families is regulated epigenetically by promoter methylation and histone acetylation mechanisms [42–44]. A subset of CTA proteins has been found to elicit spontaneous humoral and cytotoxic T-cell-mediated immune responses in cancer patients, and, therefore, these antigens could be potential cancer vaccine targets [28, 45, 46].

On the other hand, CTAs may be involved in the specification of the early embryonic lineages. MAGEA and GAGE families have been demonstrated to be expressed specifically in the human germ line during the development of male and female reproductive systems [47–50]. In order to clarify the possible role of CTAs in lineage determination during early development and specificity of CTA expression during pathological tissue development, we have examined CTA expression patterns of MAGE A, B, D, and GAGE families in the pluripotent stem cells, their spontaneously differentiated cell derivatives, and cancer cell lines derived from tissues of neuroectodermal and mesodermal origin.

2. Materials and Methods

2.1. Cell Lines. We utilized hES cell lines SC5, SC7, and SC3a derived from blastocysts (Institute of Cytology, Russian Academy of Sciences, St. Petersburg) [51]. Mouse ES cell R1 line was kindly provided by A. Nagy (Mount Sinai Hospital, Toronto, Canada), mouse embryonic germ cell (EG) line EGC-10 derived from E10.5 embryos was provided by A. McLaren (WTCR Institute of Cancer and Developmental Biology, Cambridge, UK). All cancer cell lines were obtained from Russian Cell Culture Collection (http://www.rccc.cytspb.rssi.ru/). The following groups of cancer cell lines were used: (1) human teratocarcinoma PA-1 and mouse teratocarcinoma F9, (2) human neuroblastomas IMR-32 and SK-N-MC, (3) human glioblastomas A-172, GL-6 (U-251MG), T-98G, (4) human rhabdomyosarcoma A-204 v and embryonal rhabdomyosarcoma RD, and (5) human osteosarcoma HOS (TE85, clone F5), MG-63, U-2 OS.

2.2. Human Tissue Specimens. The human tissue samples of testes and brains were obtained from adult males during postmortem examination in Forensic Medical Examination Bureau, Moscow Department of Public Health. The procedures of human tissue sampling were carried out according to the Funeral Law of Russian Federation and were approved by the Institutional Ethics Committee. After autopsy, the tissue samples were immediately transferred to TRIzol Reagent (Invitrogen).

2.3. Mouse Embryos and Tissues Sampling. C57Bl/6 mice at the age of 2-3 months were obtained from the Animal Breeding Facility-Branch "Pushchino" (Institute of Bioorganic Chemistry, Russian Academy of Sciences). Animal keeping and experiments were approved by the Institutional Ethics Committee. Total RNAs were extracted from E 7.5 mouse embryos with removed placenta, fetal gonads associated with attached mesonephros of E11.5 embryos, and male fetal gonads from E14.5 embryos. In addition, total RNAs were isolated from testicle and brain tissue samples of adult mouse males.

2.4. Cell Line Maintenance. Human ES cell lines were maintained on human embryonic fibroblast feeder cells (Russian Cell Culture Collection of Vertebrates at the Institute of Cytology) inactivated by mitomycin C treatment (10 ug/mL, Sigma). ES cells and their differentiated cell derivatives were cultivated in KnockOut Dulbecco's modified Eagle's medium (KDMEM) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 10 ng/mL basic fibroblast growth factor bFGF (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and 15% Knockout Serum Replacement (Gibco/Invitrogen). Colonies of undifferentiated hES cells were manually detached from the feeder cells, divided into cell clusters and transferred into low adhesion culture plates for spheroid generation or into new plates with feeder cells for following expanding. After isolation from the feeder cells, the hES cell clusters formed embryoid bodies (EBs) within one day of cultivation into low adhesion plates (Greiner Bio-one). At day 10 of cultivation in nonadherent culture, EBs were collected and used for RT-PCR analysis.

The early cell derivatives of hES SC5 or SC7 cells have been isolated from spontaneously differentiated adherent ES cell cultures maintained without feeder cells for 10–14 days in the hES cell media. Uniform cell outgrowths of extraembryonic endoderm, early neuroectodermal-like rosette structures and mesenchymal-like cells were isolated manually, propagated under feeder-free conditions, and collected for RT-PCR and immunohistochemical analyses.

Mouse ES and EG cells were maintained on mouse embryonal fibroblast feeder cells inactivated by mitomycin C or in a feeder-free system in media containing leukemia inhibitory factor (LIF, 10 ng/mL). Mouse ES and EG cells, mouse teratocarcinoma F9 and human teratocarcinoma PA-1 cells were cultivated in DMEM supplemented with 2 mM L-glutamine, 1% nonessential amino acids, (HyClone), 0.1 mM β-mercaptoethanol (Sigma), and 15% Characterized Fetal Bovine Serum (HyClone). For EB generation R1, EGC-10, F9, and PA-1 cells were placed in "hanging drops" (500 cells per drop) for 3 days. After formation, EBs were collected and cultured for 10 days in low adhesion plates.

Neuroblastoma, glioblastoma, rhabdomyosarcoma, and osteosarcoma cell lines were maintained as recommended for these cell lines previously (see http://www.rccc.cytspb.rssi.ru/).

2.5. Teratoma and Teratocarcinoma Assay. For teratoma and teratocarcinoma to be obtained, we used as recipients immunodeficient nude mice from the Animal Breeding Facility-Branch "Pushchino". All animal study protocols were approved by the Institutional Bioethical Committee. Mouse ES, EG, and EC cells and human ES and EC cells were injected subcutaneously into nude mice (10^6 cells per mouse). After tumor development, the animals were sacrificed, teratomas and teratocarcinomas were isolated and fixed by 10% paraformaldehyde (Sigma), dehydrated according to the standard method, and embedded into paraffin for sectioning. Histological preparations were stained by hematoxylin and eosin and examined under a Leica DMRXA2 microscope.

Karyotyping. To prepare metaphase chromosomes, 0.1 μg/mL colcemid (Karyomax, Gibco, USA) was added to the culture media 4 h prior to cell fixation. After dissociation, the cells were treated with hypotonic solution of 0.075 M KCl and 1% sodium citrate and then fixed by glacial acetic acid (3 : 1). Metaphase spreads were prepared by dropping cells onto glass slides followed by air drying. Preparations of metaphase spreads were stained by Giemsa solution. For the karyotype analysis, routine G-banded technique were applied. No less than 30 metaphase spreads for SC5, SC7, and SC3a were analysed. The hES cell karyotypes were studied by an Axio Imager M1 microscope (Carl Zeiss, Germany) with Ikaros4 Karyotyping System (MetaSystems, Germany) and described according to the International System for Human Cytogenetic Nomenclature.

2.6. Detection of Alkaline Phosphatase Activity (ALP) and Immunostaining. Human and mouse ES, EG, and EC cells and EBs were fixed by 2% paraformaldehyde in phosphate-buffered solution, PBS, pH 7.0 within 15 min. ALP activity was detected after incubation in a solution containing 10 mL 0,02 M Tris-HCl buffer (pH 8,7), 1 mg Naphtol-AS-B1-phosphate, and 5 mg Fast Red dye Texas Red (all from Sigma) at 37°C for 1 h.

For immunofluorescence analysis, cells fixed in 4% paraformaldehyde in PBS for 1 h were washed and permeabilized with 0.5% Triton X-100 (Sigma). Nonspecific reaction was blocked by 10% chicken serum (Gibco/Invitrogen). Cells were incubated in a solution of primary antibodies in PBS-Tween 20 at 4°C overnight. Primary antibodies rabbit anti-Oct4, goat anti-GATA4 (Santa Cruz Biotechnology), goat anti-Nanog (R&D Systems), mouse anti-Nestin (Abcam), anti-α-Actinin (Sigma), and rabbit anti-BRY (Abcam) were used in dilution 1 : 100. Secondary chicken antirabbit, donkey antigoat, and chicken antimouse antibodies conjugated with Alexa Fluor 594 and Alexa Fluor 488 (Molecular Probes) were diluted at 1 : 900 in blocking buffer and applied to cells for 3 h at room temperature. DAPI (Molecular Probes) was applied for nuclear staining for 20 min. Cells were mounted and examined under a Leica DMRXA2 fluorescent microscope. For negative controls, primary antibodies were omitted and the same staining procedure was used.

2.7. RNA Isolation and RT-PCR Analysis. Total RNAs were extracted from all cell lines, human and mouse tissues, and mouse embryonic samples using TRIzol Reagent (Invitrogen) according to the manufacturer recommendations. Each sample was treated with TURBO DNase (Ambion/Invitrogen) to avoid DNA contamination, and 1 μg of total RNA from each sample was reverse transcribed using RevertAid M-MuLV revertase and random hexamer oligonucleotide primers (Fermentas) for cDNA synthesis. PCR reaction mixtures were prepared according to the manufacturer protocol for Taq polymerase (Silex). Probes were denaturated at 94°C for 5 min and cycled at 94°C for 45 s, at 58°C for 45 s, and at 72°C for 45 s followed by final extension at 72°C for 5 min after the completion of 30 cycles. The expression of housekeeping genes (human RPL19 and mouse Hprt genes) was used for the normalization of PCR reaction. Primer sequences and size of their expected products are represented in Tables S1 and S2 (see Supplementary Material available online at doi: 10.4061/2011/745239). Primer pair for GAGE1, 2, 10, 12, 13 was borrowed from [29].

3. Results

3.1. Expression of CTA Genes in Adult Human and Mouse Testicular and Brain Tissues. At first, we tested the primers for the detection of CTA expression by RT-PCR in normal human and mouse tissues used as positive and negative controls. The expression of MAGE-A2, -A3, -A6, -A4, -A8, MAGE-B2, MAGE-D1, -D2, and several members of the GAGE family (GAGE-1, -2, -10, -12, -13) was detected in human testes whereas only MAGE-D1 and MAGE-D2 were expressed in human brain samples (Figures 1(a)–1(c)). Likewise, we detected the expression of Mage-a4, Mage-a 1, 2, 3, 5, 6, 8, Mage-b 1-3, Mage-b3, Mage-d1, -d2 in mouse testes and Mage-d1, -d2 in brains correspondingly (Figures 1(d) and 1(e)). Our analysis has demonstrated that all primers detected only PCR sequences of expected size and did not detect additional nonspecific sequences. In addition, the expression of all CTAs studied was detected only in the

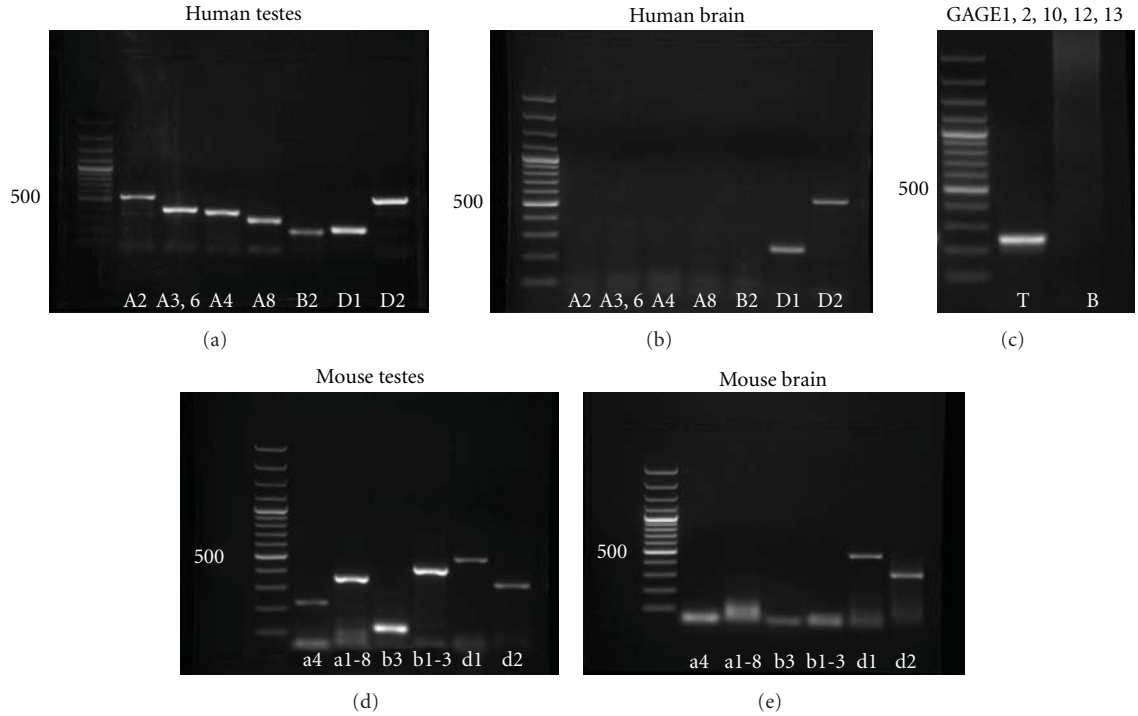


Figure 1: CTA expression in adult human and mouse testicular and brain samples. Designations: T: human testes, B: human brain.

testes whereas in normal somatic tissue (brain samples) only MAGE-D1, -D2/Mage-d1, -d2 have expressed as expected.

3.2. CTA Gene Expression in Human ES and EC Cells. Human ES cell lines SC5, SC7, and SC3a were recently derived and characterized as pluripotent stem cells by standard in vitro and in vivo assays [51] and (Figures 2(a)–2(c)). The cytogenetic analysis of these cell lines has shown that they retained normal diploid karyotypes during at least 30 passages: 46, XX for SC5 and SC3a, and 46, XY for SC7 (Figure 2(c)). However, the differentiation potential of these lines has been found to be different. Human ES cell lines SC5 and SC7 formed teratomas with derivatives of three germ layers (Figure 2(b)) while SC3a cells were more prone to differentiation in vitro and had restricted capacity to grow in teratomas.

In our experiments, undifferentiated SC5, SC7, and SC3a cells and EB cells expressed key pluripotency genes OCT4 and NANOG and displayed a high ALP activity (Figures 2(a) and 2(d)). In differentiating 10-days EBs, the expression of OCT4 and NANOG was downregulated, and ALP activity was diminished (Figures 2(a) and 2(d)). Analysis of CTA expression has shown that in undifferentiated hES cell lines SC5, SC7, and SC3a only MAGE-D1, -D2 were expressed at a high level while other CTA genes were either not expressed or expressed very weakly (Figure 2(d)). It is plausible that the low level of expression could be due to few differentiated cells which sometimes contaminate undifferentiated cell cultures. On the other hand, these CTAs may be expressed in both undifferentiated and early differentiated hES cells. In two hES cell lines, SC5, and SC7, the expression of MAGE-A3, -A6, -A4, -A8 and GAGEs was detected in undifferentiated hES cells and in 10-day EBs formed by these lines. However, the undifferentiated hES cells and EBs of SC3a line expressed MAGE-A3, -A6, -A8, and GAGEs but did not express MAGE-A4. The expression of MAGE-A2 and MAGE-B2 was not detected in any lines studied. In addition, the expression profile of each hES cell line remained stable during passages (data not shown).

Nullipotent teratocarcinoma PA-1 cells are the malignant counterpart of pluripotent stem cells that lost completely the ability of differentiation (Figures 2(a) and 2(b)). EC PA-1 cells and EBs have expressed high level of OCT4 and NANOG and almost all CTAs studied, MAGE-A2, -A3, -A6, -A8, MAGE-B2, MAGE-D1, -D2, and GAGE family genes. Interestingly, like hES SC3a cells, PA-1 cells did not express MAGE-A4 (Figure 2(d)). Our analysis of hEC PA-1 cells and hES cells has shown that their CTA expression profiles were very similar, but, at the same time, they differed in the expression of two CTAs.

3.3. CTA Gene Expression in Differentiated Human ES Cell Derivatives. We studied three types of early differentiated cell derivatives of human ES SC5 and SC7 cells that predominated during spontaneous hES cell differentiation in vitro. These cells were easily distinguished from other cells in morphology and could be easily separated from other cells in cell outgrowths (Figure 3(a)). To identify cell types, we analyzed specific gene and protein expression in the studied samples. The expression of specific marker genes for pluripotent (OCT4, NANOG), extraembryonic endoderm (GATA4, AFP), neuroectodermal (NESTIN) and

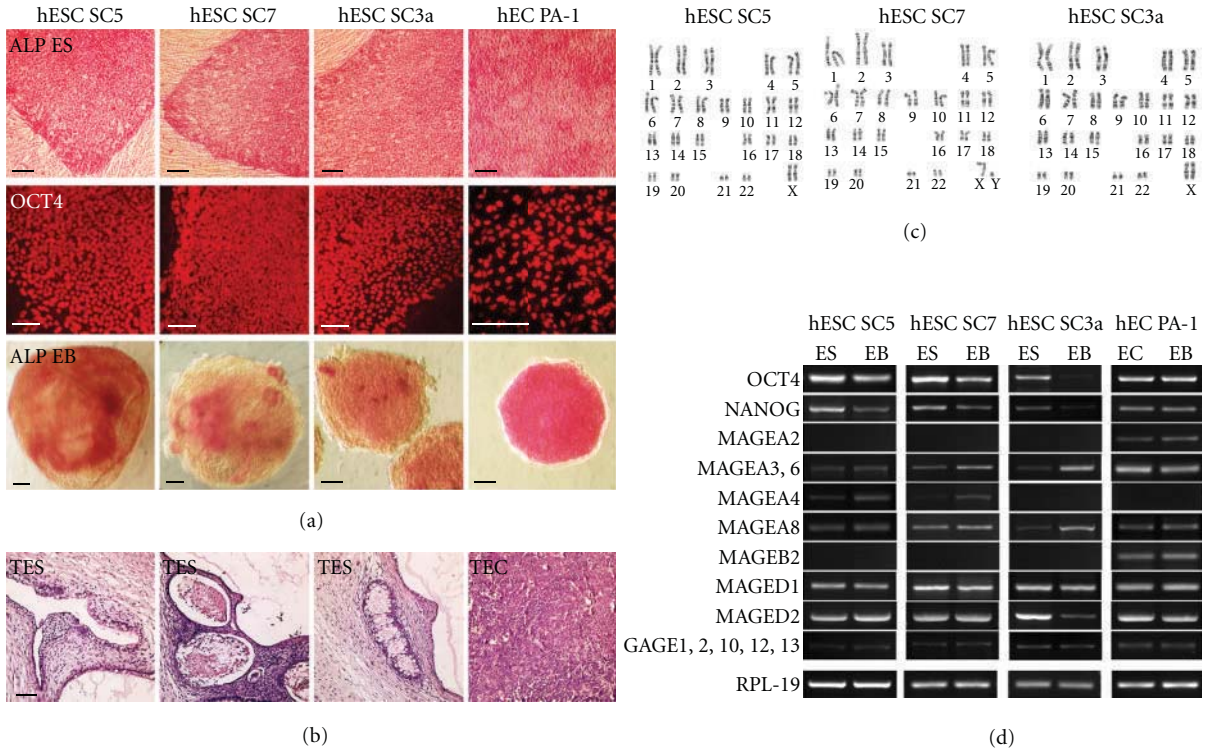


Figure 2: CTA expression patterns in hES SC5, SC7, and SC3a cells and hEC PA-1 cells. (a) Activity of alkaline phosphatase and OCT4 in undifferentiated hES and hEC cells and in EBs formed by these cell lines. Scale bar = 100 μm. (b) Histological sections through teratomas and teratocarcinomas formed by hES and hEC cell lines. Cell derivatives of three germ layers were found in teratomas (TES) formed by hES cells and entirely cancer cells in teratocarcinomas (TEC). Scale bar = 100 μm. (c) Normal diploid karyotypes of hES SC5, SC7, and SC3a cells: SC5—46, XX; SC7—46, XY; SC3a—46, XX. (d) Expression of CTAs in hES SC5, SC7, and SC3a cells, hEC PA-1 cells and EBs formed by these cells.

mesenchymal-like (BRY and α-Actinin) cells was tested by RT-PCR (Figure 3(b)) and immunohistochemical staining (Figure 3(a)). Analysis of CTA expression in these selected cell derivatives has shown that the extraembryonic endoderm cells expressing high levels of GATA4 and AFP and low level of OCT4 express MAGE-A8 and MAGE-D1, -D2 as well (Figure 3). Mesenchymal-like cells that were α-Actinin positive but BRY-negative had CTA expression profile similar to that of extraembryonic endoderm cells (Figure 3). In contrast, the neuroectodermal cell derivatives expressed NESTIN and MAGE-A4 (Figure 3). Moreover, all three cell types expressed MAGE-D1, -D2 genes and did not express GAGE genes.

3.4. Expression of CTAs in Human Cancer Cell Lines of Neuroectodermal and Mesodermal Origin. In order to determine whether CTA-specific expression patterns were associated with histological origin or with other characteristics of tumors, we tested cell lines which were derived from the embryonic (neuroblastoma, embryonal rhabdomyosarcoma), childhood (rhabdomyosarcoma, osteosarcoma), and adult (glioblastoma) cancers of mesodermal and neuroectodermal origin. In addition, the expression of OCT4 and NANOG was studied in all cancer cell lines (Figures 4(a) and 4(b)).

In the neuroblastoma cell lines IMR-32 and SK-N-MC, CTA expression patterns were markedly different. SK-N-MC cells expressed all MAGEs tested while IMR-32 cells only two (Figure 4(c)). However, the expression of MAGE-B2 and different levels of MAGE-A8 were detected in both cell lines. All three glioblastoma cell lines, GL-6, A-172, T-98G, expressed MAGE-A8 and very weak level of MAGE-B2. Two of three cell lines, GL-6 and T-98G, expressed MAGE-A3. Thus, most cancer cell lines from tissues of neuroectodermal origin expressed MAGE-A8 and MAGE-B2 mRNAs but embryonic tumors (neuroblastomas) expressed significantly higher level of MAGE-B2. The expression of GAGEs was not detected in any of neuroectodermal cancer cell lines (Figure 4(c)).

Analysis of CTA expression profiles of cancer cell lines from the tissues of mesodermal origin has shown that all of them have expressed variable levels of MAGE-A8 and MAGE-A4. Two lines, embryonal rhabdomyosarcoma RD and osteosarcoma U-2 OS, had very similar CTA profiles, except MAGEB 2, and expressed almost all CTAs studied including GAGEs (Figure 4(d)).

As mentioned above, the expression of OCT4, NANOG, NESTIN, and BRY was tested in cancer cell lines also. Interestingly, mRNAs of OCT4 and NANOG were detected by RT-PCR in all cancer lines while the proteins were not revealed by immunostaining. Likewise, low expression of

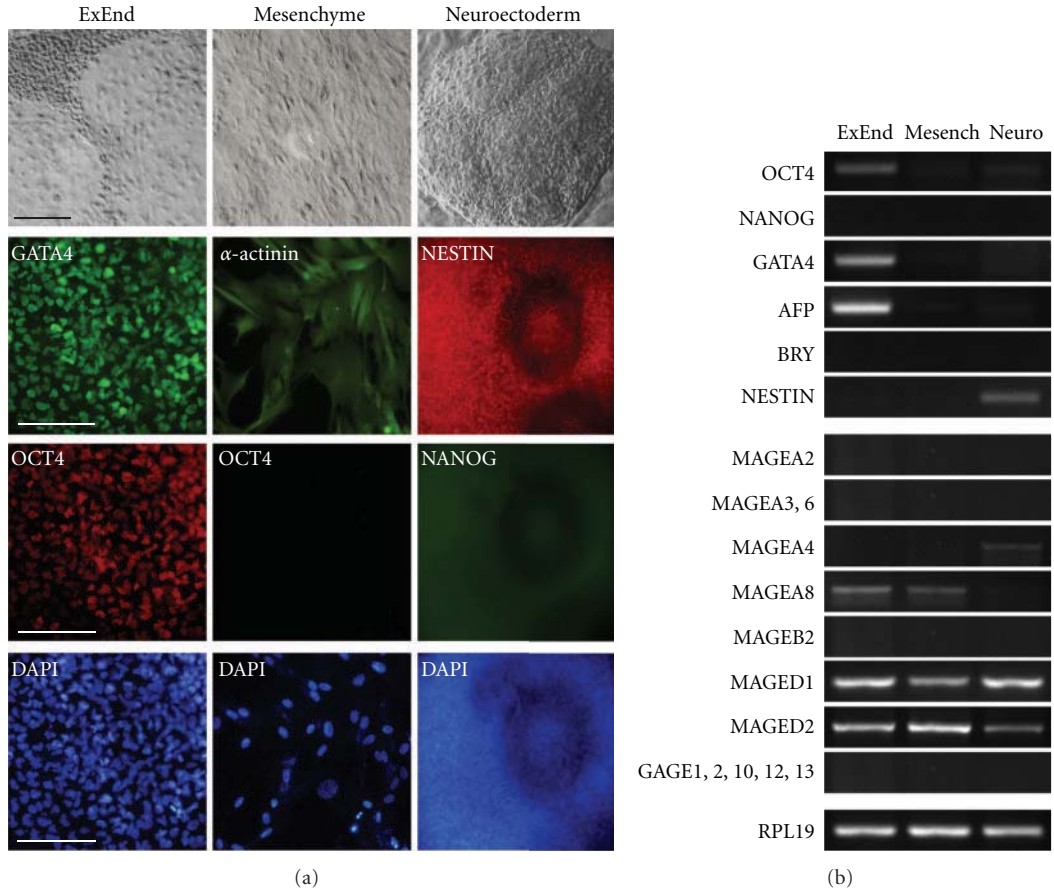


Figure 3: Expression of CTAs and lineage-specific genes in differentiated hES cell derivatives. (a) Morphology and immunostaining of extraembryonic endoderm derivatives by antibodies against GATA4 and OCT4, mesenchymal cells against α-ACTININ and OCT4, neuroectodermal cells against NESTIN and NANOG. Scale bar = 100 μm. (b) Expression profiles of CTAs and lineage marker genes in hES cell derivatives.

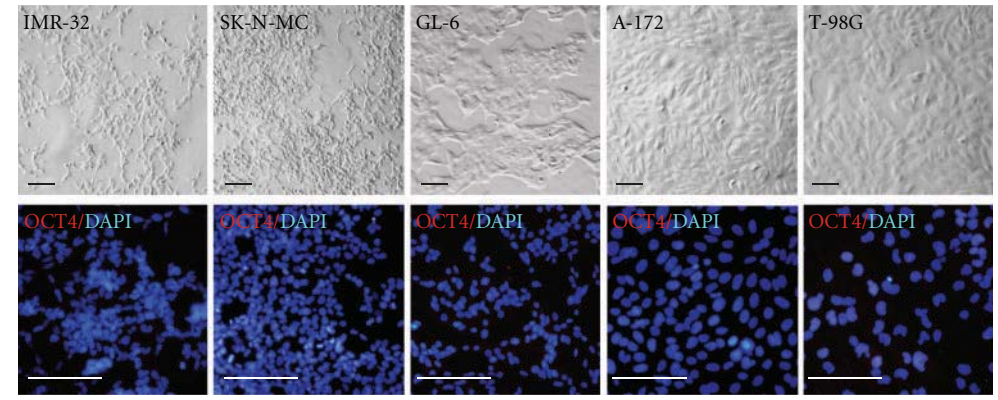
BRY has been found at mRNA levels in rhabdomyosarcomas, and NESTIN in neuroblastomas and glioblastomas but not at the protein level (Figure 4, and data not shown). We suggest that OCT4 and NANOG transcripts detected in all cancer lines studied may be relevant to the pseudogenes and have no functional meaning. Similarly, the expression of BRY and NESTIN at mRNA level may be activated aberrantly in these cells as often observed in cancer cells.

3.5. CTA Gene Expression Profiles in Mouse ES, EG, and EC Cells and in Mouse Germ Line Cells. The murine Mage genes, like their human homologues, are expressed in a wide variety of tumors, in fetal and adult male gonads, and in several embryonic and extraembryonic tissues [52–57]. In order to determine whether specific expression patterns of Mage-a and Mage-b genes can be attributed to pluripotent stem cell differentiation, CTA expression profiles were examined in mouse pluripotent stem cells (ES and EG cells) and embryonal teratocarcinoma cells (Figures 5(a) and 5(b)). Moreover, we compared them with the profiles of mouse primordial germ cells at the critical stages of germ line development: epiblast cells at early gastrulation stage, post-migratory primordial germ cells just after their occupation of developing genital ridges and gonocytes of male embryonic gonads. Our results suggest that the pluripotent ES and EG cells, nullipotent teratocarcinoma EC cells and their EB cells expressed Mage-a1, 2, 3, 5, 6, 8 and Mage-d1, -d2 but did not express CTAs of Mage-b family. Furthermore, the expression of Magea-4 gene was detected in EGC-10 and EC F9 cells but not in ES R1 cells (Figure 5(b)).

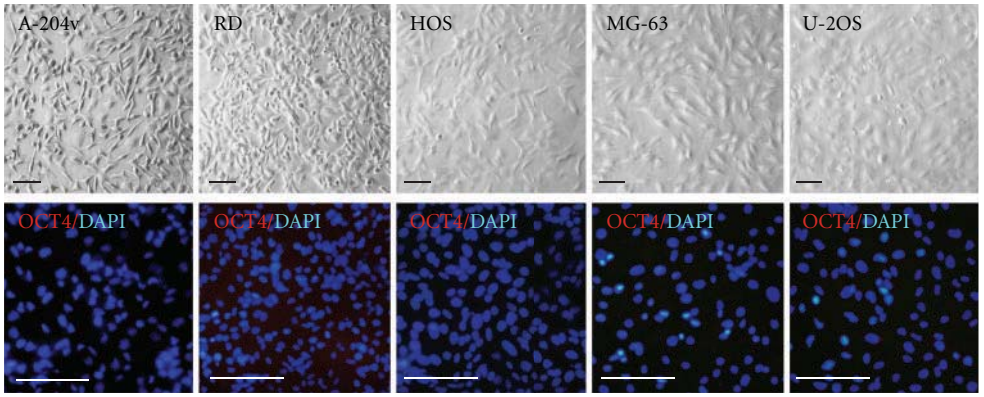
The profiles of embryonic germ line cells at the studied stages were very similar because all CTAs tested, except Mage-a4, were expressed in all types of germ line cells (Figure 5(c)). Interestingly, in contrast to other CTAs, Mage-a 4 was expressed in adult mouse testes but not in the early primordial germ cells and gonocytes. Thus, the CTA expression profiles of mouse ES, EG, and EC cells significantly differed even from those in the early epiblast cells. On the other hand, mouse and human embryonic germ line cells expressed similar CTA families except GAGE antigens which were not identified in mice.

4. Discussion

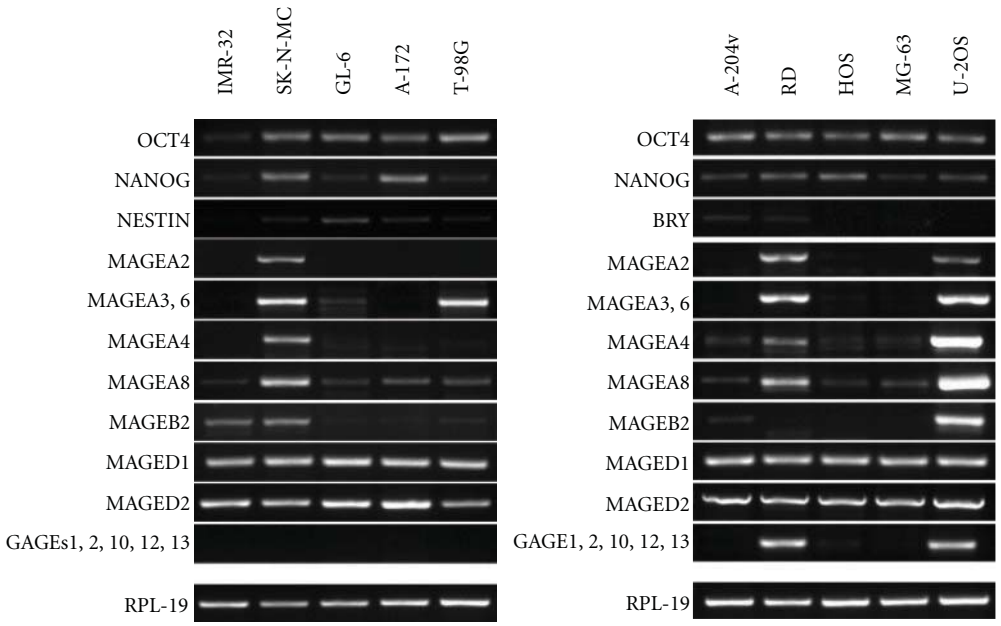
The expression of most CTA genes in the normal tissues is restricted to adult testicular germ cells but is aberrantly



(a)



(b)



(c) (d)

Figure 4: CTAs in cancer cell lines derived from tissues of neuroectodermal and mesodermal origin. (a) Cell morphology of neuroblastomas and glioblastomas and immunostaining of these cell lines by antibody against OCT4. Scale bar = 100 μm. (b) Immunocytochemical analysis of OCT4 expression in rhabdomyosarcoma and osteosarcoma cell lines. Scale bar equal 100 μm. (c) CTA expression profiles of cancer cell lines of neuroectodermal and mesodermal lineages.

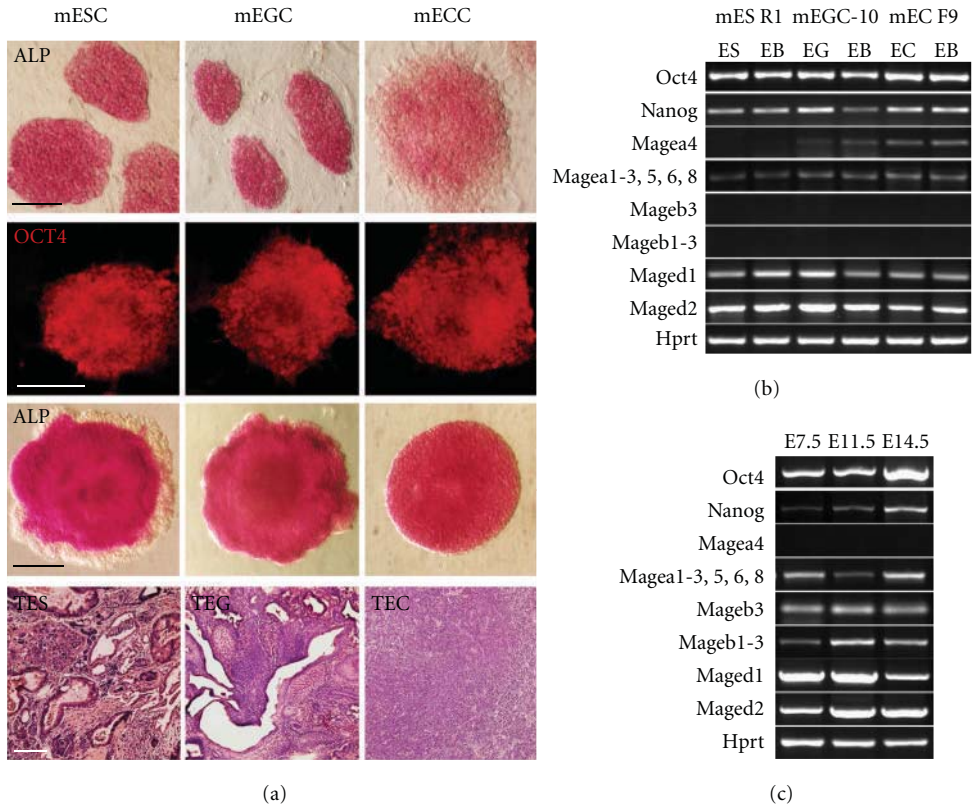


Figure 5: Gene expression profiles of CTAs in mouse ES, EG, and EC cells and in mouse germ line cells. (a) Pluripotent ES and EG cells and nullipotent EC cells express alkaline phosphatase and Oct4 and develop EBs; ES and EG cells differentiate into derivatives of three germ layers in teratomas (TES, TEG) while EC cells grow in vivo as malignant tumors (TEC). Scale bar equal 100 μm. (b) Expression of CTAs, Oct4, and Nanog in mouse ES, EG, and EC cells and EBs. (c) CTA expression profiles of germ line cells isolated from embryos of E 7.5, E 11.5 and E 14.5 stages.

activated in various types of cancers. Moreover, CTAs have been found to be expressed in embryonic germ and somatic cells as well as in the extraembryonic structures [29, 50]. We hypothesized that unique expression pattern of CTAs in germ cells may be a part of the developmental program which includes the restriction of germ line from somatic cells during the early gastrulation stages. On the other hand, deviations from the normal lineage specification can be associated with disturbances of the typical CTA expression patterns. We investigated the CTA expression patterns during ES cell differentiation in vitro using them as a model of normal lineage specification and compared CTA profiles of undifferentiated ES cells, their differentiated cell derivatives, and cancer cell lines of neuroectodermal and mesodermal origin in order to identify coincident and discriminating gene subsets.

Our results suggest that undifferentiated pluripotent hES cells expressed low mRNA levels of several CTAs, MAGE-A3, -A6, -A4, -A8, and GAGEs. Note that MAGE-A4 was not expressed in hES SC3a cell line that differed from SC5 and SC7 cells in their growth and differentiation potential [51]. However, unlike undifferentiated cells, the early hES cell derivatives expressed only one gene of MAGEA family and did not express CTAs of GAGE family. It can not be ruled out that MAGE-A4 and -A8 expression may be referred to both undifferentiated hES cells and extraembryonic endoderm, mesenchyme-like and neuroectoderm cells also residing in EBs whereas MAGE-A3, -A6, and GAGEs were expressed exclusively in pluripotent cells.

Previously, Gjerstorff et al. [29] also detected MAGE-A2 and MAGE-A6 expression in EBs of KMEB1 and KMEB2 hES cell lines and cell derivatives of these lines in teratomas but not in undifferentiated hES cells. However, only 3 of 6 hES cell lines studied expressed CTAs during in vitro and in vivo differentiation. Moreover, the expression of GAGEs was not detected either in hES cells or in EBs. In our experiments, the same primer pairs were used for GAGE expression analysis but low levels of GAGE 1, 2, 10, 12, 13 transcription were detected in both undifferentiated hES cells and EBs in all cell lines studied. Conversely, we did not observe the expression of MAGE-A2 in any hES cell lines or their differentiated cell derivatives but identified MAGE-A2 mRNA in EC PA-1 cells. As mentioned above, SC5 and SC7 hES cell lines had very similar CTA expression profiles and similar growth and differentiation potentials, and, moreover, each hES cell line has retained its specific CTA profile during long-term cultivation. We suppose that interlinear variations in CTA expression patterns may be characteristic for a certain hES cell line state.

Mouse pluripotent ES and EG cells also expressed several genes of Mage-a family, but, surprisingly, Mage-a4 was expressed only in mouse EG and EC cells. Furthermore, the expression of Magea4 was not detected in mouse primordial germ cells of E 11.5 and E14.5 embryos as well, although Mage-a4 was expressed in adult male gonads. Mouse embryonic and adult germ cells also expressed Mage-b1, 2, 3 genes unlike mouse pluripotent stem cells. Similarly, the expression of MAGE-B2 was found in adult human testes but not detected in human ES cells or their cell derivatives. Thus, the CTA expression profiles of mouse ES, EG, and EC cells significantly differed even from the early epiblast cells as well as from primordial germ cells. Hence, the pluripotent stem cells in vitro may not be quite equivalent to any of these cell types in embryos. Nevertheless, the similarity of CTA expression profiles of human and mouse pluripotent stem cells suggests their similar cell state and origin.

Little is known about CTA expression during the development of the germ and somatic lineages in mammals. Previous studies have shown that during human primordial germ cell determination, differentiation, and gonad maturation, the CTA expression patterns undergo significant changes. Firstly, GAGE proteins were expressed in the male and female primordial and mature germ cells, from 5–8 weeks of gestation until adulthood [29, 49, 50]. The expression of MAGE-A1, MAGE-A4, and NY-ESO-1 in the fetal testes and ovaries is initiated later than GAGE expression and terminated at different development stages in male and female fetal gonads [47, 48, 50]. MAGE-A4 has been found to be expressed firstly in single cells of the fetal testes at the age of 17 weeks, and, thereafter, the number of MAGE-A4-positive gonocytes and spermatogonia progressively increased until 28 weeks. MAGE-A1 and NY-ESO-1 proteins were also detected in the fetal testes at the age of 9 weeks and in ovaries at the age of 13 weeks but these proteins displayed differential expression patterns through male and female germ cell development. Our data about the Mage-a, Mage-b, and Mage-d expression patterns in the mouse primordial and fetal germ cells also demonstrate for the first time the CTAs implication in an early mouse germ line development as well as similarities and dissimilarities of CTA expression patterns in mouse and human embryonic cells.

Apart from germ cells, the MAGE-A family members were expressed in somatic lineages, in particular, in human developing central nervous system and peripheral nerves as well as in myotome and myoblasts at the early stages (from 5 to 8 weeks) but no MAGE-A expression was detected in the neural structures of 17- and 23-weeks old fetus or in the adult brain [29]. Moreover, these authors reported that GAGE proteins were expressed in the early ectodermal and neuroectodermal cells but their expression disappeared in the later differentiated cells of these lineages. In addition, MAGE-A protein expression did not correlate with the GAGE expression pattern in the neuroectodermal cells as opposed to germ cells. Our findings that MAGE-A4 and MAGE-A8 are expressed in the early mesenchymal and neuroectodermal cells derived from hES cells are consistent with the previous observations on human fetal tissues and differentiated hES cells in teratomas [29]. On the other hand, we did not find GAGE expression in the above mentioned differentiated cell types. Therefore, further detailed examination of CTA expression pattern in different cell types derived from pluripotent cells during in vitro differentiation and in embryonic tissues can clarify this discrepancy.

The expression of CTAs was also detected in extraembryonic tissues. We identified MAGE-A8, MAGE-D1, -D2 expression in extraembryonic endoderm cells derived from hES cells. Previously, in immunohistochemical study of more than 50 human placenta samples of different gestational ages, the high level of MAGE-A3 and MAGE-A4 expression was revealed while the expression of NY-ESO-1 and GAGEs was sporadic [31]. Genome-wide analysis of CTA expression in the normal and cancer tissues displayed the MAGE-A 2–6, 8–11, and XAGE expression in placenta [30]. Human MAGEL2 gene and its mouse homologue Magel2 were detected in human and mouse placenta [56]. Taken together, all these data demonstrate that CTAs are involved in germ line and somatic lineage development of different mammals. Specific spatio-temporal patterns of CTAs expression may be associated with the determination and specification of different lineages both in the embryo and during pluripotent stem cell differentiation (Figure 6).

Another aspect of our work was the analysis of CTA expression profiles of teratocarcinoma cell lines that are malignant counterparts of pluripotent stem cells and cancer cell lines derived from the tissues of neuroectodermal and mesodermal origin in order to find the specificity of CTA expression during normal and pathological tissue development. Initially, we compared CTA profiles of undifferentiated hES cells, hEC cells, and EBs formed by these cell lines. The hES and hEC cell CTA profiles were very similar, but hEC cells expressed two additional genes, MAGE-A2 and MAGE-B2, which were not expressed in the pluripotent stem cells. Moreover, hEC PA-1 cells did not express MAGE-A4 like hES SC3a cell line. Interestingly, the CTA profiles studied of mouse ES cells differed from those of mouse EC cells only in Mage-a4, and, moreover, mouse EC F9 cells expressed Mage-a4 as opposed to human EC PA-1 cells. Nevertheless, Mage-a4 was also expressed in the normal mouse EG cell line. Thus, MAGE-A4/Mage-a4 expression pattern is variable in murine and human pluripotent and teratocarcinoma cells, and the question of specificity of this expression in pluripotent cells remains open. Recent studies of normal testes and different types of germ cell tumors have shown that normal spermatogonia and seminoma cells specifically expressed MAGE-A4 while anaplastic seminoma and nonseminoma germ cell tumors were negative for this antigen [47]. On the other hand, the expression of MAGE-A2, -A3, -B1, and -B2 was also found in most seminomas studied while MAGE-A2 and MAGE-A4 were expressed in pure embryonal carcinoma tumors, as well as in a half of pure yolk sac tumor samples [33]. In addition, in our study hEC PA-1cells expressed MAGE-A2 and MAGE-B2 that were not detected in hES cells, and, therefore, these CTAs may be considered as gene candidates for their further investigation as early markers of transformed pluripotent cells.

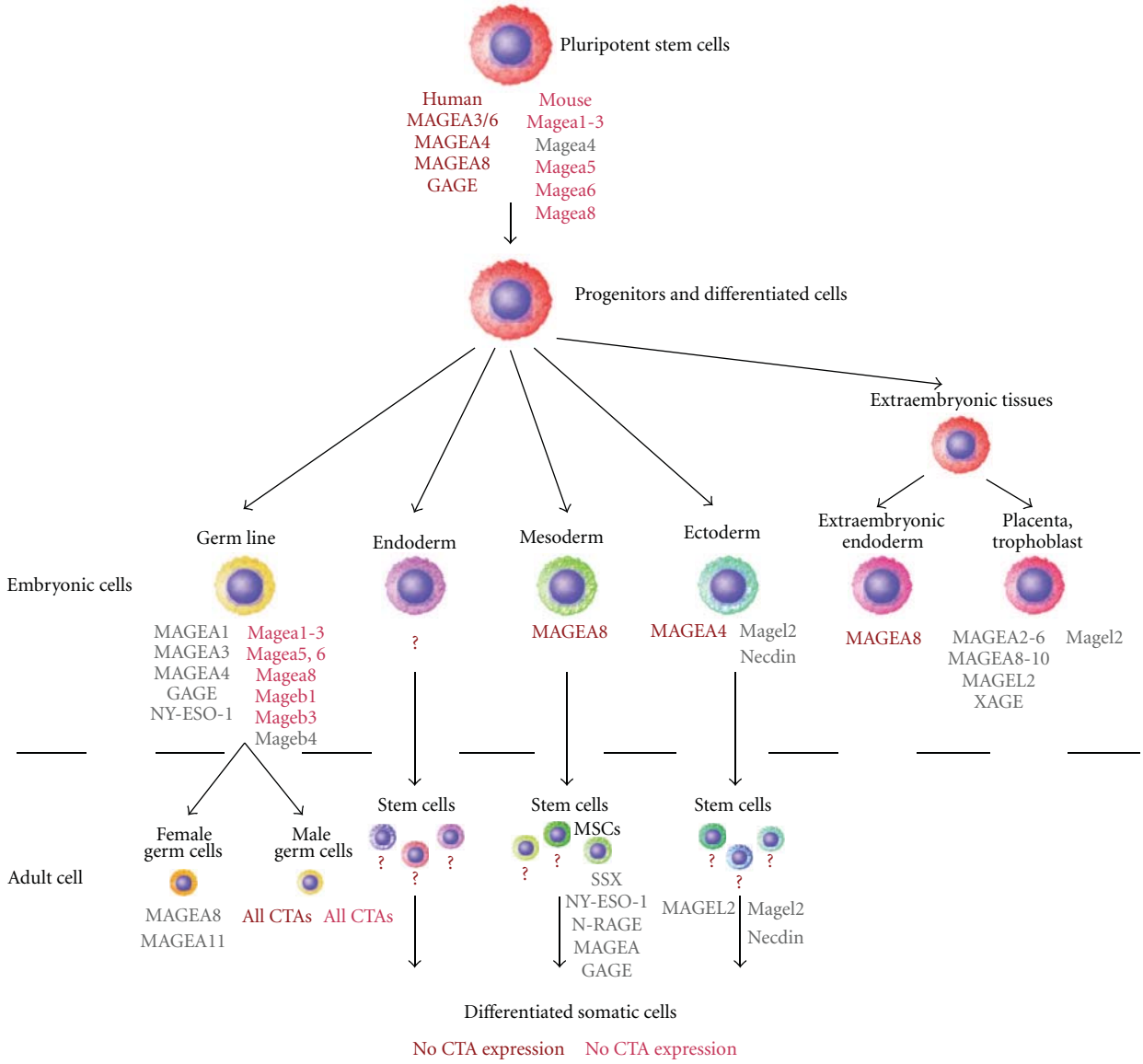


Figure 6: CTAs expression in pluripotent cells and derivatives of different lineages. Data are summarized from present study (marked by brown and red) and [29–31, 34, 47–50, 52–57]. Human CTAs are marked with capital letters.

Analysis of CTA expression in the neuroectodermal cancer lines has shown that MAGE-A8, MAGE-B2, and MAGE-A3, -A6 together with MAGE-D1, -D2 are expressed in most cell lines tested whereas the early neuroectodermal cells derived from hES cells expressed only MAGE-A4 and MAGE-D1, 2. Thus, no common genes of MAGE-A, MAGE- B, and GAGE families were revealed in the normal and cancer cells of this lineage. On the other hand, the frequencies of MAGE-A1 and MAGE-A3 expression in different astrocytomas and glioblastomas have been demonstrated to vary in range from 0 to 30% [58–60]. Hence, CTA expression patterns in brain tumors may have low tissue specificity. Also, we determined MAGE-A8 and MAGE-A4 as common CTAs in expression profiles of rhabdomyosarcoma and osteosarcoma lines, and MAGE-A8 was also found in the mesenchymal derivatives of hES cells. Interestingly, the cancer cell lines of both neuroectodermal and mesodermal origin expressed MAGE-A8 while normally it was expressed in the mesenchymal hES cell derivatives only.

Relatively recently it was found that several CTAs, including SSX, NY-ESO-1, and N-RAGE, were also expressed in undifferentiated mesenchymal stem cells (MSCs) derived from adult bone marrow or embryonic liver, but their expression was downregulated after osteocyte and adipocyte differentiation [33]. These important data require further investigations since it is unclear whether CTA expression is characteristic of MSCs or is activated during their cultivation in vitro likewise in cancers.

Overall, the frequency of CTA expression is highly variable among different tumor types [28, 30, 60]. For instance, melanoma, ovarian, liver, and lung cancers are "CTA-rich" tumors because they have high frequency of CTA

expression while hematopoietic, colon, renal, and pancreas cancers have low frequency of CTA expression. Moreover, the cancers of higher histological grade and later clinical stage as well as metastatic tumors display higher frequency of CTA expression than the primary tumors. The frequency of CTA expression correlates with the worst prognosis. Thus, it is plausible that CTAs are aberrantly activated and expressed in different cancers and have low lineage specificity. However, examination and systematization of CTA expression patterns in embryonic and adult normal cells as well as cancer cells of different types may illuminate whether CTAs are implicated in normal and pathological lineage development.

5. Conclusions

We have shown that several CTAs, such as MAGE-A3, -A4, -A6, -A8, and GAGEs, are expressed in the undifferentiated hES cells and early differentiated EB cells while only one gene of MAGE-A family was expressed in the later differentiated cell derivatives of hES cells, MAGE-A8 in the extraembryonic endoderm and mesenchymal cells and MAGE-A4 in the neuroectodermal progenitors. Like hES cells, mouse pluripotent cell lines, ES and EG cells, also expressed CTAs of Mage-a family but did not express Mage-b family genes unlike epiblast and primordial germ cells. Moreover, we detected different expression pattern of MAGE-A4/Magea4 in both human and mouse pluripotent and teratocarcinoma cells. Despite great similarity of CTA expression patterns in hES and their malignant counterparts, hEC cells, two of CTAs studied, MAGE-A2 and MAGE-B2, were detected only in hEC cells but not in hES cells. These CTAs may be considered as marker gene candidates of transformed pluripotent cells for further study. Comparative analysis of CTA profiles of cancer cell lines derived from the tissues of neuroectodermal and mesodermal origin and hES cell-derived progenitor cells of the similar lineages has shown that in most cases CTAs were aberrantly expressed in cancer cells and display low tissue specificity. Thus, further investigation and identification of CTA expression patterns in pluripotent and multipotent stem cells and their derivatives as well as different types of cancers is an important step towards understanding of CTA functions in normal and cancer cells and also may be useful for the isolation and removal of abnormally CTA-expressing cells to improve the safety of stem cell-based therapy.

Acknowledgments

The authors thank T. M. Nikonova for her assistance in histological section preparation. They are grateful to Prof. N. N. Mamaev and Dr. T. L. Gindina (SPb I. P. Pavlov Medical University) for a possibility to use Ikaros4 Karyotyping System for transmitted light (MetaSystems, Germany). They also thank Dr. Sergey Vasetskiy for his critical reading of the manuscript. This work was supported by the Russian Foundation for Basic Research, Grant 05-04-49185, 11-04-00379.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 201371, 18 pages
doi:10.4061/2011/201371

Review Article

Epigenetic Regulation of Mesenchymal Stem Cells: A Focus on Osteogenic and Adipogenic Differentiation

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Received 21 March 2011; Accepted 27 April 2011

Academic Editor: Giorgio A. Presicce

Stem cells are characterized by their capability to self-renew and terminally differentiate into multiple cell types. Somatic or adult stem cells have a finite self-renewal capacity and are lineage-restricted. The use of adult stem cells for therapeutic purposes has been a topic of recent interest given the ethical considerations associated with embryonic stem (ES) cells. Mesenchymal stem cells (MSCs) are adult stem cells that can differentiate into osteogenic, adipogenic, chondrogenic, or myogenic lineages. Owing to their ease of isolation and unique characteristics, MSCs have been widely regarded as potential candidates for tissue engineering and repair. While various signaling molecules important to MSC differentiation have been identified, our complete understanding of this process is lacking. Recent investigations focused on the role of epigenetic regulation in lineage-specific differentiation of MSCs have shown that unique patterns of DNA methylation and histone modifications play an important role in the induction of MSC differentiation toward specific lineages. Nevertheless, MSC epigenetic profiles reflect a more restricted differentiation potential as compared to ES cells. Here we review the effect of epigenetic modifications on MSC multipotency and differentiation, with a focus on osteogenic and adipogenic differentiation. We also highlight clinical applications of MSC epigenetics and nuclear reprogramming.

1. Introduction

Two characteristics distinguish stem cells from other cell types: the ability to self-renew and to differentiate into multiple lineages. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst during early embryogenesis [1, 2]. ES cells are unique in their ability to form all cell types in the human body and self-renew indefinitely and thus have been extensively investigated in the arena of regenerative medicine since their isolation 30 years ago [1, 2]. However, ethical considerations, technical challenges, and governmental regulations have hindered their use [3]. As a result, the study of somatic or adult stem cells, which does not generate the same ethical concerns, has increased dramatically.

Unlike ES cells, adult stem cells are characterized by a restricted differentiation potential and finite self-renewal. Adult stem cells have been localized to many tissues including mesenchymal [4], neural [5], gastrointestinal [6], hepatic [7], gonadal [8, 9], and hematopoietic [10]. Mesenchymal stem cells (MSCs) are multipotent adult stem cells that differentiate into osteoblastic, chondrogenic, myogenic, and

adipogenic lineages [11–13]. MSCs are found in large numbers in the adult human, primarily in bone marrow and adipose tissue and have been widely investigated for their potential role in treating human disease. While much knowledge has been garnered regarding the characteristics and clinical applications of MSCs [14], our understanding of their behavior is still limited. Given the therapeutic potential of MSCs for a variety of conditions including bone and cartilage defects, ischemic heart disease, and cerebral ischemia, it is important that we continue to elucidate the precise mechanisms that direct MSC fate.

Though stem cell behavior is largely mediated by DNA sequence, there are multiple levels of regulation apart from this genetic blueprint including posttranscriptional, translational, posttranslational, and epigenetic regulatory processes. Epigenetic regulation is based upon heritable changes in the pattern of gene expression that occur without a change in the primary nucleotide sequence [15]. These changes remain as cells divide mitotically and meiotically and often last for multiple generations. A fundamental example of epigenetic regulation occurs as cells terminally differentiate. For example, a terminally differentiated epithelial cell shares the same DNA sequence as its ES cell precursor. However, these two cell types differ significantly in behavior and function, and some regulatory process or processes must underlie this change in phenotype. In this case, epigenetic mechanisms are largely responsible for the variable activation and repression of specific genes at specific time points during the lifespan of the cell, allowing for the terminally differentiated phenotype. Major mammalian epigenetic mechanisms include DNA methylation and histone modifications, both of which have been tightly linked to gene regulation and other cellular processes including division and survival [16, 17].

In recent years, epigenetic regulation has also emerged as an important modulator of stem cell differentiation [18]. Moreover, the disruption of epigenetic regulation has been associated with human disease [19]. An example of this occurs in patients with Angelman's syndrome or Prader-Willi syndrome, where epigenetic deregulation of imprinted genes at the 15q11–13 loci on the maternal or paternal allele, respectively, produces the associated phenotype [20, 21]. Epigenetic deregulation has also been implicated in many malignancies, including MSC-derived tumors [22–28]. Given its association with various disease states, epigenetic regulation has become an important focus of potential therapy. The mechanism of action of many anticancer drugs involves the alteration of DNA methylation patterns or the modification of histone proteins [29]. The therapeutic potential of epigenetic manipulation is not limited to drug therapy, however. It is also under investigation as a therapeutic modality as it relates to the process of cellular reprogramming.

Investigation of the epigenetic regulation of cell fate determination has largely focused on ES cells. Recent studies have also elucidated epigenetic states responsible for lineage-specific differentiation of adult stem cells. While DNA methylation patterns are crucial for ES cell differentiation, histone modifications and other chromatin-based mechanisms may serve a larger role in MSC differentiation capacity [30]. Interestingly, DNA methylation profiles of MSCs suggest that, in contrast to ES cells, MSCs have a limited differentiation potential [31]. Presently, it is uncertain whether unraveling the epigenetic landscape of MSCs will lead to novel strategies to enhance their differentiation capacity. It is plausible that the heritability of gene expression in reprogrammed cells can be enhanced by the controlled manipulation of epigenetic alterations. In this paper, we summarize our current understanding of the epigenetic profile of MSCs, specifically highlighting signatures related to multipotency and differentiation into osteogenic and adipogenic lineages. We also focus on the reprogramming of MSCs and whether alterations of the MSC epigenome can enhance their therapeutic potential.

2. Major Epigenetic Mechanisms

Epigenetic mechanisms play a central role in the promotion of appropriate transcriptional pathways during both embryonic development and adult tissue maintenance. Regulation of gene expression at the epigenetic level occurs via modifications of chromatin architecture that alter the accessibility of genes to transcription factors and other modulators. Specifically, these modifications regulate gene expression by facilitating the opening of DNA (euchromatin) to permit transcription or the condensing of DNA (heterochromatin) to repress transcription. Loss of proper chromatin modifications during development and differentiation has been associated with embryonic lethality [32–34]. We will briefly summarize the major mechanisms underlying epigenetic regulation as they have been reviewed extensively elsewhere [35, 36].

2.1. DNA Methylation. Mammalian DNA methylation is unevenly dispersed over much of the genome in a pattern described as global methylation [36]. DNA methylation consists of the addition of a methyl group to position 5 of cytosine (m^5C) at cytosine-phosphate-guanine (CpG) dinucleotides and occurs symmetrically on both DNA strands. Regions dense in CpG dinucleotides, known as CpG islands, are found near promoters of many human genes [37]. In general, promoter DNA methylation is associated with repression of the corresponding gene [38, 39]. However, this association is not always straightforward. Genes associated with methylation-free CpG islands often remain silent while genes that correspond to methylated promoters occasionally undergo transcription. This relationship may depend on the content of promoter CpG dinucleotides, where methylation of high content CpG promoters usually represses transcription, while methylation of low content CpG promoters can either activate or repress transcription [40]. Occasionally, DNA methylation may require additional epigenetic events to occur concomitantly for transcription to be affected [41].

DNA methyltransferases (DNMTs) catalyze the methylation of CpGs. Two DNMTs, DNMT3a and DNMT3b, are responsible for de novo DNA methylation during embryonic development and cell differentiation [42]. During cell division, a third DNMT, DNMT1, recognizes hemimethylated DNA and ensures methylation profile fidelity by catalyzing

the methylation of its corresponding daughter strand [43]. DNA methylation is crucial for many processes including long-term gene silencing [41, 44], proper development [45–48], X chromosome inactivation [49], and genomic imprinting [50–53].

Though DNA methylation occurs in all cells, the unique pattern of methylation varies based on cell type [54]. Bibikova et al. [55] investigated the DNA methylation status of over 1500 CpG sites in 14 human ES cell lines and compared it to the methylation status of 38 non-ES cells lines. Using bead array and cluster analyses and methylation-specific polymerase chain reaction (PCR), the authors reported that, based on methylation profiles, human ES cells contain a unique epigenetic signature [55]. This finding may have implications on ES cell pluripotency and developmental potential. We have recently begun to uncover methylation patterns unique to MSCs as well (see below) [56, 57].

Currently, the "gold-standard" method to analyze DNA methylation patterns is bisulfite genomic sequencing [58]. This methodology consists of the bisulfite-mediated chemical conversion of unmethylated cytosine in CpG dinucleotides to uracil whereas methylated cytosines remain protected from chemical conversion [59]. PCR then substitutes uracils with thymidines and subsequent sequencing illustrates the methylation state of the original sequence. A quantitative assessment of the extent of methylation can be evaluated by bacterial cloning of the PCR products.

2.2. Histone Modifications. Chromatin, which is comprised of DNA and proteins, refers to the state in which DNA and these proteins are packaged within eukaryotic cell nuclei. As described above, chromatin can be packaged loosely as euchromatin, which facilitates gene transcription, or tightly as heterochromatin, which facilitates gene repression. The nucleosome is the fundamental unit of chromatin and is composed of 2 subunits of each of the four core histone proteins (H2A, H2B, H3, H4) around which 147 base pairs of DNA are wrapped. Histones are small basic proteins that are predominantly globular in nature other than their unstructured N-terminal "tails". Subsequent to histone protein translation, N-terminal tails can be covalently modified in numerous ways to regulate gene expression [35]. The most thoroughly investigated histone modifications are acetylation and methylation.

The *histone code hypothesis* suggests that "distinct modifications, on one or more tails, act sequentially or in combination to form a "histone code" that is read by other proteins to bring about distinct downstream events" [65–67]. Histone codes can be transient or stable; if stable, these codes constitute epigenetic regulation as they imply heritability [67, 68]. Epigenetic regulation mediated by histone modification is dynamic in nature and inherently complex. For example, the methylation of histone lysine residues, catalyzed by histone methyltransferases (HMTs), can correlate with either transcriptional activation and repression [69]. Trimethylation of lysine 4 of histone H3 (H3K4me3) marks euchromatin and gene activation. In contrast, H3K27me3 and H3K9me3 signal heterochromatin and gene repression. The H3K27me3 mark is thought to be critical to the "stemness" of stem cells [70, 71], as H3K27 demethylation triggers cellular differentiation [72–74]. Further adding to histone modification complexity, the ability of HMTs to methylate H3K9 in order to silence transcription often depends on the methylation status of adjacent lysine residues on H3 [18, 75]. HMTs and histone demethylases (HDMs) work in tandem to determine the level of histone lysine methylation found within a cell [76].

Histone acetylation is also a widely studied histone modification. The opposing activities of histone acetyltransferases (HATs) and histone acetyl-deacetylases (HDACs) are responsible for the level of cellular histone acetylation [76]. In general, acetylation of histone lysine residues correlates with transcriptional activation whereas histone lysine deacetylation silences gene transcription. Acetylation of H3K9 (H3K9ac) and acetylation of H4K16 (H4K16ac) are common marks found on euchromatin near genes that are actively being transcribed [56]. Although histone modifications mainly act by altering chromatin architecture, specific modifications (e.g., H3K4me3 and H3K9ac) also mediate gene regulation by recruiting and tethering transcriptional modulators to chromatin [77–81].

Chromatin immunoprecipitation (ChIP) assays, which were originally designed to study RNA polymerase II behavior [82–85], have allowed researchers to map the positioning of histone modifications within the genome or onto individual promoters [86]. A specific histone modification can be immunoprecipitated so that DNA sequences associated with it can be identified by PCR [86]. Researchers can also indentify histone proteins that are associated with a particular region of the genome using ChIP.

Various lines of evidence suggest that chromatin within undifferentiated ES cells is generally less compact, and thus more "transcription-permissive", compared with differentiated cells [87]. For example, pericentric heterochromatin progressively clusters as human and mouse ES cells differentiate [88, 89]. In addition, using fluorescence recovery after photobleaching (FRAP), a technique that measures the exchange rate of chromatin-associated proteins [90], Meshorer and colleagues [91] demonstrated that ES cells contain hyperdynamic chromatin proteins that loosely bind to chromatin. As ES cells begin to differentiate, these hyperdynamic proteins become immobilized on chromatin, which signal lineage commitment of these cells [91]. Indeed, the loose association of chromatin and its structural proteins may be an important marker of cellular pluripotency. Less well defined is the association of specific histone modifications to MSC cell fate. Collas et al. [56] have described the presence of bivalent histone marks (H3K4me3 and H3K27me3) on lineage-specific promoters in undifferentiated MSCs derived from adipose tissue. This finding, in addition to evidence that these same lineage-specific promoters are hypomethylated (see below), may suggest that adipogenic promoters in MSCs are preprogrammed for adipogenic stimulation [56].

Researchers investigating differences in histone modification patterns between epidermal stem cells and terminally differentiated cells of the epidermal lineage found that Myc-induced differentiation of adult stem cells correlates with

Table 1: Examples of clinical applications of mesenchymal stem cells.

Author	Year	Indication	Outcome
Bang et al. [60]	2005	Cerebral ischemia	Functional recovery after ischemic stroke improved in MSC-treated patients compared to control patients
Dill et al. [61]	2009	IHD	Intracoronary MSC administration improved LVF after STEMI
Horwitz et al. [62]	2002	Metabolic bone disease	5 of 6 OI patients showed accelerated bone growth velocity after IV infusion of allogeneic MSCs
Marcacci et al. [63]	2007	Critical size bone defect	Implantation of HA scaffolds seeded with MSCs into diaphysis defects resulted in fusion between implant and host bone
Wakitani et al. [64]	2007	Cartilage defect	Direct site transplantation of MSCs into articular cartilage defects resulted in clinical symptom improvement and defect repair

MSC: mesenchymal stem cell; IDH: ischemic heart disease; LVF: left ventricular function; STEMI: ST-segment elevated myocardial infarction; OI: osteogenesis imperfecta; IV: intravenous; HA: hydroxyapatite.

numerous chromatin modifications [92]. Specifically, quiescent epidermal stem cells were found to contain high levels of H3K9me3 and H4K20me3 and low levels of H4 acetylation and H4K20me1 (a modification generally associated with gene activation) [92]. As Myc-treated stem cells underwent differentiation, there was a corresponding increase in H4 acetylation as well as the silencing H3K9me2 and H4K20me2 marks [92]. These data suggest that a single transcription factor has the ability to induce widespread change in chromatin state, though it remains unclear how Myc-induced differentiation of epidermal stem cells induces an increase in chromatin modifications associated with both gene activation and gene silencing. More importantly, alterations in chromatin architecture, largely mediated by epigenetic phenomena, probably underlie numerous mechanisms that facilitate cell differentiation. By elucidating avenues to manipulate such phenomena, we can potentially improve our ability to attenuate pathologies associated with tissue degeneration by directing cell fate.

3. Mesenchymal Stem Cells: Epigenetic Characteristics and Potency

Though MSCs have attracted significant attention for their potential to regenerate tissue, we have yet to identify a cell marker specific to MSCs. In order facilitate a more consistent approach to the study of MSC biology, the International Society of Cryotherapy has proposed that human MSCs meet the following criteria: (1) plastic adherence of cultured cells in standard culture conditions; (2) expression of CD105, CD73, and CD90 and lack of expression of CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules; (3) the capacity to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [93]. Populations of multipotent cells derived from adipose tissue, bone marrow, and skeletal muscle have all been found to meet these defined criteria in vitro [94–98]. Not surprisingly, these differing populations of MSCs are closely related in various capacities. For example, MSCs derived from adipose tissue (adipocyte-derived stem cells; ASCs), as well as MSCs derived from bone marrow (bone marrow MSCs; BMMSCs), express similar gene expression profiles [99–101], surface markers [94, 98], and share a similar differentiation potential [98, 102]. Sorensen et al. [103] reported that DNA methylation profiles between MSCs isolated from human adipose tissue, bone marrow, and muscle are also similar. In contrast, MSC promoter methylation profiles are distinct from other cell types, including human ES cells, multipotent ES cell-derived mesenchymal cells, and hematopoietic stem cells (HSCs) [103, 104].

As phenotypic, transcriptomic, functional, and now, epigenetic evidence suggests that MSCs isolated from various tissues are related, it is plausible that MSCs originate from a common origin [112]. To this end, pericytes, which have been isolated within mesodermal tissues including fat, bone, and muscle, have been found to contain several characteristic features to MSCs [113–115]. As such, authors have hypothesized that MSC populations may be traced to a pericytic origin [112, 113].

3.1. Epigenetic Profile of MSCs in Culture. In the last two decades, MSCs have been isolated from many animal [116–122] and human tissues [123–131]. Excitement regarding their use for tissue engineering purposes in part stems from the finding that MSCs navigate toward injured tissue [132] and are considered MHC II negative cells, lacking the co-stimulatory molecules CD40, CD80, and CD86 [133]. As a result, they can be allogeneically transplanted without the need for immunosuppression of the recipient. Indeed, the therapeutic potential of MSC-based treatment for a variety of conditions has already been demonstrated in humans [60–64, 134–140]. Specifically, investigators have evaluated their efficacy in the treatment of critical size bone defects, cartilage degeneration, metabolic bone disease, ischemic heart disease, and cerebral ischemia (Table 1). Nevertheless, obstacles have limited the widespread use of MSCs. In general, it has proven difficult to harvest large quantities of MSCs from many tissues, especially that of bone marrow [4]. As a result, MSCs must be expanded ex vivo after their isolation to be used for therapeutic purposes. A potential concern regarding this strategy, however, stems from the finding that MSCs display variable proliferative and differentiation capacities in culture [141]. In contrast to early-passage MSCs, late-passage MSCs have a reduced differentiation potential [142].

Moreover, MSCs may undergo malignant transformation in vitro, though this finding is controversial [143–145]. Studies addressing such concerns have documented that late-passage MSCs display normal karyotypes [146–148] and genomic stability [149], yet their behavior change in culture implies alterations of some aspect of their regulation.

Epigenetic profiles of nonmesenchymal-derived cells occasionally display instability in culture [157–159]. To evaluate if similar phenomena occur in MSCs, Dahl and colleagues [57] examined CpG methylation patterns in human BMMSC cultures for 170 cell cycle- and cancer-related promoters. Nearly 90% of these genes maintained their methylation profile between early and late passage, indicating that MSC cultures derived from bone marrow have a stable CpG methylation status in vitro. Furthermore, the methylation profile of ASCs remains consistent up to at least 4 passages in vitro, which corresponds to 20 population doublings from a single cell [160]. Further studies are required to assess the methylation status of ASCs after longer periods in culture (i.e., after 15 passages), but it appears unlikely that alterations of promoter DNA methylation are responsible for the reduced differentiation capacity seen in late-passage MSCs [150].

In contrast, histone modification patterns have been implicated in variable cell behavior in culture. Under normal conditions, adipogenic and myogenic promoter regions in ASCs are associated with a bivalent combination of histone modifications. Specifically, promoter regions are enriched with H3K4me3 and H3K27me3, while lacking H3K9me3 and H3K9ac [161, 162]. As early passage ASCs differentiate, there is a corresponding rise in H3K9 acetylation and H3K27 demethylation, thereby relieving H3K4me3/H3K27me3 bivalency. In contrast, late-passage MSCs are associated with H3K27me3 maintenance and minimal H3K9 acetylation [161]. There is also global upregulation of the Polycomb repressor complex protein ExH2 (an enzyme that catalyzes the methylation of H3K27) and a global increase of H3K9 deacetylation in long-term cultured MSCs [161]. From this data, it appears that histone modification-mediated epigenetic alterations in late-passage MSCs may be responsible for a deceased ability to differentiate as cultured MSCs age. However, further studies are needed to more fully characterize this epigenetic variability on a global scale.

3.2. Are MSCs Pluripotent from an Epigenetic Standpoint?

Pluripotent cells have the ability to become cell types of all lineages in the body whereas multipotent cells differentiate into various cell types from one lineage. MSCs are often referred to as multipotent given their proclivity to form cell types within the mesodermal lineage. Recently, Jaenisch and Young [163] noted that it is unclear whether a truly pluripotent stem cell can be isolated from the adult animal. However, analyses have demonstrated that certain populations of MSCs have the ability to differentiate into cell types from all 3 germ layers [164–169]. Jiang et al. [170] localized a cell within human BMMSC cultures that, upon stimulation, could differentiate into mesenchymal, neuroectodermal, endodermal [171], and endothelial tissues [172, 173]. After injection into early blastocysts, the authors reported that this population of MSCs contributed to most or all adult cell types, thereby indicating pluripotency [170]. D'Ippolito and colleagues [174] also isolated a unique subpopulation of human bone marrow stromal cells, termed marrow-isolated adult multilineage inducible (MIAMI) cells, which could differentiate into mature cells of all 3 germ layers. Nevertheless, MSCs have not yet met more stringent criteria for pluripotency, including germline contribution or tetraploid complementation [163].

Presently, it is generally accepted that MSCs are confined to the mesodermal lineage, but under certain conditions can differentiate into most or all tissues. This notion of lineage-restriction has been supported by epigenetic studies from the laboratory of Collas et al. [103, 112, 160, 175]. MSC lineage-specific promoters are largely hypomethylated in MSCs. In contrast, the endothelial promoter for *CD31* is fully methylated in ASCs and BMMSCs, which correlates with a lack of CD31 expression in MSCs. Furthermore, using methylated DNA immunoprecipitation (MeDIP), it was found that hypermethylated genes in MSCs are often associated with regulation of development, transcription, signaling, and metabolic functions. Interestingly, many promoters of genes expressed in nonmesodermal derived cells remain hypomethylated in MSCs, even though MSCs generally do not differentiate into cells that express these genes. Thus, the Collas et al. laboratory has proposed that strong methylation of lineage specification and developmental promoters may restrict MSC differentiation capacity; however, hypomethylation of lineage-specification promoters is of little predictive value in differentiation capacity [112]. This is consistent with a lineage-priming molecular model of MSC differentiation capacity, which posits that MSCs express a subset of genes corresponding to differentiation pathways to which they can commit [176]. Furthermore, in ES cells, methylation occurs on pluripotency-associated loci as cells lose pluripotency (i.e., during differentiation) [159, 177]. Taken together, epigenetic data support the notion that MSCs are better classified as multipotent than pluripotent.

4. Epigenetic Control of MSC Cell Fate

Questions of how the epigenetic state of a cell influences fate determination have predominately focused on ES cells. In ES cells, for example, lineage-specific promoters that are associated with terminal differentiation are often DNA methylated [178]. This presumably impedes improper or premature differentiation toward a specific lineage, thereby preserving pluripotency. Recently, studies have found that some of these same promoter regions are unmethylated in MSCs [112], indicating that the epigenetic state of ES cells changes as they differentiate into MSCs. However, whether these epigenetic alterations are the cause or result of ES cell fate decisions remains unclear. Furthermore, the mechanisms underlying MSC differentiation toward a particular cell type within the mesodermal lineage have yet to be fully elucidated. Here, we review epigenetic regulation associated with osteogenic differentiation (Table 2) and adipogenic

Table 2: Epigenetic regulation of osteogenic differentiation of mesenchymal stem cells.

Epigenetic regulation	Target	Finding	Reference
DNA methylation	*OC*	Reduced promoter DNA methylation is associated with osteogenic differentiation	Villagra et al. [105]
DNA methylation	*OPN*	Mechanically induced promoter DNA demethylation is associated with accelerated osteogenic differentiation	Arnsdorf et al. [106]
DNA methylation	*Brachyury*	Promoter DNA methylation is associated with osteogenic differentiation	Dansranjavin et al. [107]
DNA methylation	*Trip10*	Promoter DNA methylation is associated with accelerated osteogenic differentiation	Hsiao et al. [108]
Histone modification	*OC*	Acetylation of H3 and H4 is associated with OC expression and osteogenic differentiation	Shen et al. [109]
Histone modification	*HOXA10*	HOXA10-mediated chromatin acetylation and H3K4 methylation promotes transcription of osteogenic genes	Hassan et al. [110]
Histone modification	*AP-2α*	H3K4 and H3K36 methylation is associated with AP-2α expression and subsequent osteogenic differentiation. Mutations in demethylation-related proteins (e.g., BCOR) are associated with the OFCD syndrome	Fan et al. [111]

OC: osteocalcin; OPN: osteopontin; Trip10: thyroid hormone receptor interactor-10; BCOR: BCL-6 corepressor; OFCD: oculo-facial-cardio-dental.

differentiation (Table 3) of MSCs, as these pathways have been the most widely investigated.

4.1. Osteogenic Differentiation. Osteogenic differentiation of MSCs is a complex process that is tightly controlled by numerous signaling pathways and transcription factors [11]. Runt-related transcription factor 2 (Runx2) is considered a master regulator of osteogenic differentiation and is expressed at many stages of bone development and maturation [179–181]. Runx2 transcriptional activity is itself subject to robust regulation, as demonstrated by its association with numerous coactivators and corepressors [181–184]. It is becoming increasingly clear that epigenetic regulation is also crucial to Runx2 activity and thus osteogenic differentiation. Epigenetic regulation generally results in structural changes in chromatin that alter the binding ability of Runx2 and other transcription factors to osteogenic promoter regions. The most thoroughly studied promoter of the osteogenic lineage is the promoter for *osteocalcin* (*OC*), which contains binding sites for many factors crucial to the activation of osteoblast-specific genes including Runx2 [105, 109, 185–188]. Acetylation of histones H3 and H4, as well as a decreased level of DNA methylation, increases accessibility of the *OC* promoter to osteo-inductive transcription factors [105, 109, 185]. Furthermore, HOXA10-mediated chromatin hyperacetylation and H3K4 Trimethylation induce chromatin structural changes that facilitate Runx2-mediated activation of genes that encode OC and other osteoblastic phenotypic markers [110]. In addition, CreMM/CHD9, a recently characterized member of the CHD chromatin remodeler family [189–191], has been detected in MSCs near newly formed adult bone [192]. CReMM/CHD9 binds to promoters for both *Runx2* and *OC* during osteogenic gene expression. Though CReMM/CHD9 is thought to alter chromatin architecture via DNA-dependent ATPase activity, the exact epigenetic mechanism linking CReMM/CHD9 to osteogenic differentiation is unknown [192].

Skeletal loading and loading-induced dynamic fluid flow are also key regulators of osteogenic differentiation [193–199]. A recent investigation addressed whether these regulators act via epigenetic modifications [106]. Mechanically induced differentiation is associated with a decreased level of DNA methylation at the promoter for *osteopontin* (*OPN*; an important factor for bone remodeling) as well as increased OPN expression and osteogenic differentiation. Similarly, biologically-induced osteogenic differentiation of MSCs (using growth media supplemented with β-glycerolphosphate, ascorbic acid, and dexamethasone) correlates with a decrease in *OPN* promoter methylation as well as increased OPN expression [106].

It is not surprising that modifications of epigenetic regulation at genes crucial to osteogenic differentiation occur as MSCs become osteoblasts. However, recent evidence suggests that alterations of epigenetic regulation may occur on a more global scale as MSCs differentiate toward bone. For example, methylation at the promoter region for the mesodermal transcription factor *Brachyury*, which silences brachyury expression, is associated with osteoinduction of MSCs [107]. In addition, Hsiao and colleagues [108] reported that thyroid hormone receptor interactor-10 (*Trip10*), an adaptor protein involved in diverse cellular functions, is epigenetically modified during human BMMSC differentiation. The authors elected to investigate whether variation of *Trip10* epigenetic regulation could alter MSC differentiation patterns because of its association with the H3K27me3 mark. Interestingly, after transfection of MSCs with in vitro-methylated *Trip10* promoter DNA, MSCs underwent progressive cytosine methylation of the endogenous *Trip10* promoter, which led to reduced Trip10 expression and accelerated MSC differentiation toward osteogenic and neuronal lineages [108]. In addition to demonstrating that Trip10 expression levels are associated with osteogenic differentiation, this study illustrated how manipulation of the MSC epigenome in a manner distinct from classic nuclear

Table 3: Epigenetic regulation of adipogenic differentiation of mesenchymal stem cells.

Epigenetic regulation	Target	Finding	Reference
DNA methylation	*PPARγ2*, *lep*, *fabp4*, *lpl*	Promoters for these 4 adipogenic genes are hypomethylated in ASCs	Noer et al. [150]
DNA methylation	*PPARγ*	Expression of PPARγ is regulated by promoter DNA methylation. Promoter methylation corresponds to a decreased expression of PPARγ and decreased adipogenic differentiation	Fujiki et al. [151]
DNA methylation	*Glut4*	Promoter DNA demethylation occurs as cells undergo adipogenic differentiation	Yokomori et al. [152]
DNA methylation	*Lep*	The *Lep* promoter region is highly methylated in preadipocytes but is unmethylated in terminally differentiated adipocytes	Melzner et al. [234]
DNA methylation	*Agouti*	Genistein-mediated DNA hypermethylation of a retrotransposon upstream of *Agouti* is associated with decreased obesity	Dolinoy et al. [153]
Histone modification	*ApM1*	H3 hyperacetylation and H3K4me3 at the *apM1* promoter region correlate with early adipogenic differentiation. Inhibition of H3K4 methylation results in decreased apM1 expression and decreased adipogenesis	Musri et al. [154]
Histone modification	Multiple gene promoters	Downregulation of HDACs is required for adipogenic differentiation	Yoo et al. [155]
Histone modification	*PPARγ* gene targets	Unphosphorylated RB recruits HDAC3 to promoters of *PPARγ* gene targets, which decreases adipogenic differentiation. Inhibition of HDAC3 activity results in *PPARγ* activation, and subsequent adipogenesis	Fajas et al. [156]

PPARγ: peroxisome proliferator-activated receptor-gamma; lep: leptin; fabp4: fatty acid-binding protein 4; lpl: lipoprotein lipase; ASC: adipose-derived stem cell; Glut4: glucose transporter type 4; ApM1: adiponectin; H3K4me3: Trimethylation of lysine 4 on histone 3; HDAC: histone deacetylase; RB: retinoblastoma.

reprogramming (see below) could be utilized as a therapeutic modality. However, further studies regarding the long-term effects of this type of epigenetic manipulation are necessary before it can be widely used in humans.

Support for the role of epigenetic regulation in MSC osteogenic differentiation has also come from reports of abnormal bony development. Oculo-facial-cardio-dental (OFCD) syndrome is characterized by teeth with excessively long roots and craniofacial, eye, and cardiac abnormalities [200–204]. Genetic studies have associated this X-linked dominant syndrome to mutations of the BCL-6 corepressor (BCOR) protein [204]. Under normal conditions, the repressive actions of BCOR are mediated by chromatin modifications via interactions with HDACs, HDMs, and H2A ubiquitin ligase [205–207]. MSCs have been isolated from dental and craniofacial tissues [208–210], which led Fan et al. [111] to investigate whether BCOR mutations enhance the osteo/dentinogenic potential of MSCs. Using gain- and loss-of-function assays, the authors demonstrated that the *AP-2α* -+, is largely responsible for the osteo/dentinogenic capacity of MSCs. The methylation of H3K4 and H3K36 at the *AP-2α* promoter is associated with gene activation [211, 212]. BCOR normally catalyzes the demethylation of H3K4me3 and H3K36me2 [213, 214], but fails to do so when mutated [111]. The resultant methylation impedes the binding of the BCL-6 repressor protein to the *AP-2α* promoter, leading to uncontrolled AP-2α expression. As such, a BCOR mutation that impairs its demethylating activity permits uncontrolled osteo/dentinogenic differentiation of MSCs in the OFCD syndrome [111].

4.2. Adipogenic Differentiation. The development of adipocytes during adipogenic differentiation of MSCs occurs in two phases [131, 215]. The first phase, determination, is the commitment of MSCs to the adipogenic lineage, which entails losing the ability to differentiate into another lineage. The second stage, differentiation, occurs as MSCs begin to express the phenotypic characteristics of a mature adipocyte. Similar to osteogenic differentiation, adipogenesis is a highly coordinated process that involves numerous transcription factors performing specific functions at various time points [216–218]. Just as Runx2 serves as a master regulator of osteogenic differentiation, the nuclear hormone receptor peroxisome proliferator-activated receptor-gamma (PPAR-γ) has a significant role in adipogenic differentiation [219, 220]. In addition, many coregulators and transcription factors central to adipogenesis have chromatin-modifying activities [221–223], supporting the role of epigenetic regulation during the differentiation of MSCs to adipocytes.

Noer et al. [160] examined the DNA methylation profile of both adipogenic and nonadipogenic genes in human ASCs using bisulfite genomic sequencing. The promoters for four adipogenic genes—*PPARγ2*, *leptin (lep)*, *fatty acid-binding protein 4 (fabp4)*, and *lipoprotein lipase (lpl)*—were found to be hypomethylated in freshly harvested human ASCs [160]. Interestingly, the CpG methylation profiles between and within donors were described as mosaic (i.e., they were not uniform) [160], which is consistent with stem cells found elsewhere in the body [224, 225]. Of note, mosaic CpG methylation is believed to stem from stochastic methylating events at various CpG sites due to environmental-, health-, and age-related factors [226–229]. Noer and colleagues

[160] also noted that promoter regions for housekeeping genes such as GAPDH and LMNB1 were unmethylated whereas nonadipogenic lineage-specification gene promoters (*Myogenin*, myogenic; *CD31/PECAM1* and *CD144/CDH5*, endothelial) were hypermethylated. These findings suggest that the commitment of MSCs to the adipogenic lineage may be reflected by a particular epigenetic signature in which adipogenic gene promoters are hypomethylated while nonadipogenic promoters are methylated. In vitro analyses have correlated the demethylation of various adipogenic promoters, including that of *PPARγ*, with adipogenic differentiation in murine cell lines as well [151, 230, 231]. However, the pattern of promoter DNA methylation in ASCs does not always correlate with protein expression [101, 160]. This indicates that additional regulatory layers are necessary for adipogenic differentiation.

Specific histone-mediated chromatin architecture modifications have been documented as multipotent MSCs become "preadipocytes" during determination [232]. H3K4me2, an active mark of transcription, has been identified at promoters of adipogenic genes including *adiponectin (apM1)*, *glut4*, and *lep* during determination [154]. As cells progress toward committed adipocyte precursors during differentiation, further characteristic epigenetic marks have been described. In addition to promoter DNA demethylation at *glut4* and *lep* [152, 233, 234], these promoters also undergo H3K9 demethylation, H3 acetylation, and H3K4 Trimethylation [154, 232], all of which are epigenetic marks of gene activation. Furthermore, the downregulation of cellular HDACs during differentiation appears to facilitate adipogenic lineage commitment, while its overexpression attenuates it [155]. Interestingly, unphosphorylated retinoblastoma (Rb) protein recruits HDAC3 to promoters of *PPARγ* gene targets, thereby inhibiting the transcription of their associated genes and thus repressing adipogenic differentiation [156, 235].

MSC differentiation is required for proper tissue development and repair, but it can be detrimental when it occurs excessively. A example of this lies in the obesity epidemic currently plaguing the United States and globally [236]. As epigenetic regulation has become an increasingly recognized programming factor in the process of adipogenesis, it could follow that epigenetic deregulation has a role in the development of obesity. In fact, induced methylation alterations have been linked to obesity in mice [153, 237]. It is hopeful that further study of the epigenetic regulation of adipogenic differentiation will provide insight into potential therapies for obesity and related metabolic disorders.

5. Nuclear Reprogramming of Mesenchymal Stem Cells

Direct epigenetic manipulation (e.g., by transfection of methylated DNA) has not been widely used in humans because the long-term effects of such therapies are unknown. The epigenetic program of a cell can be altered in other ways, however. Several strategies have been employed to reprogram somatic cells to a pluripotent embryonic state. We will briefly summarize these strategies as they have been extensively reviewed previously [163, 238, 239].

Somatic cell nuclear transfer (SCNT), also referred to as nuclear transplantation (NT), is the process by which the nucleus of a somatic donor cell is introduced into an enucleated oocyte. SCNT has been used to generate cloned animals including the cloned sheep Dolly [240]. SCNT also mediates the creation of genetically matched replacement cells. As such, SCNT is of great medical interest as it has the potential to circumvent immunologic incompatibility associated with cells donated from a source other than the patient. Moreover, nuclear reprogramming of MSCs has been most widely studied in the context of SCNT. Another cellular reprogramming strategy consists of fusing somatic cells with ES cells to produce a hybrid that demonstrates ES-like features including pluripotency. However, a shortcoming of this approach is the resultant tetraploidy of reprogrammed cells. A third strategy involves the transient incubation of somatic cells with extracts of ES cells devoid of their nuclei. This method has been utilized to enhance somatic cell pluripotency in vitro without creating cells with 4 sets of chromosomes. Finally, Takahashi and Yamanaka engineered a groundbreaking nuclear reprogramming strategy with the creation of induced pluripotential stem (iPS) cells in 2006 [241]. The authors successfully reprogrammed mouse embryonic/adult fibroblasts to pluripotent ES-like stem cells (iPS cells) by introducing the transcription factors Oct4, Sox2, c-Myc, and Klf4 into differentiated cells via viral-mediated transduction [242, 243]. The ectopic expression of these reprogramming factors in infected cells initiates a sequence of epigenetic events in endogenous genes critical for the maintenance of pluripotency and lineage specification of ES cells, thereby activating the pluripotential state of iPS cells [76, 163, 241, 244]. Using a combination of similar factors, authors have also isolated iPS cells from human fibroblasts [242, 245, 246]. However, the finding that mice derived from iPS cells often develop malignancies has considerably hindered the application of this technique [247]. Interestingly, the oncogenic transcription factor c-Myc may not be necessary to reprogram cells, though it facilitates a speedy and efficient reprogramming process [246, 248, 249]. The factor or combination of factors essential to reprogramming still remains unclear, as does the specific molecular circuitry underlying pluripotency. Nevertheless, because nuclear reprogramming carries the potential to create patient-specific cells allowing for customized therapy, it is currently of great interest to many investigators.

Within the last few years, research has revealed specific epigenetic modifications that occur during the processes of differentiation and reprogramming. For example, as ES cells commit to a particular lineage, the transcription factor Oct4, which is thought to be necessary to maintain ES cell pluripotency, is rapidly silenced. Epigenetically, *Oct4* silencing correlates with a loss of gene activity marks (H3K4me3, H3K7ac, and H3K9ac), as well as an increase of gene silencing marks (H3K9me3 and DNA methylation) at the *Oct4* promoter [163, 250]. In order to generate iPS cells from somatic cells, these silencing marks must be progressively removed from the *Oct4* promoter. Furthermore, lineage-specification genes must be repressed in order to *dedifferentiate* a cell during the reprogramming process.

iPS cells display chromatin modifications that prevent the transcription of genes encoding developmental regulators, thus maintaining pluripotency by repressing differentiation [163, 251, 252]. The promoters of pluripotency regulators also exhibit decreased DNA methylation patterns in iPS cells [247, 251, 252]. Taken together, these data indicate that epigenetic remodeling is an essential element to the nuclear reprogramming of somatic cells.

Although progress has been made in the field of nuclear reprogramming, there are still limitations to its therapeutic application. SCNT, for example, is an inherently inefficient process. Most clones die soon after implantation or are born with severe abnormalities due to faulty reprogramming [253, 254]. Some authors have hypothesized that, compared to terminally differentiated cells, less differentiated cell types may increase SCNT efficiency as they may be more easily reprogrammed. Faast and colleagues [255] examined if MSCs could increase the SCNT efficiency compared to terminally differentiated fibroblasts in a pig model. The use of MSCs did not increase cleavage rates compared to adult fibroblasts obtained from the same animal, but the percentage of embryos that developed to the blastocyst stage was almost doubled [255]. These findings were consistent with earlier studies that demonstrated improved SCNT efficiency using ES cells compared to differentiated somatic cells [256–260]. Jin et al. [261] also reported that compared to fetal fibroblasts, porcine MSCs have greater donor cell potential. In contrast, other investigators have noted that no significant difference exists between the number of MSCs that reach the blastocyst stage compared to fibroblasts after SCNT [262, 263]. Recently, Brero et al. [264] evaluated the efficiency of nuclear reprogramming by SCNT in MSCs and adult fibroblasts in a rabbit model by monitoring levels of histone modifications associated with transcriptionally active euchromatin (H3K4me2/3) or transcriptionally repressive facultative heterochromatin (H3K27me3). Subsequent to SCNT, H3K27me3 was found to be reprogrammed (i.e., largely undetectable) in both MSCs and adult fibroblasts, which was consistent with H3K27me3 patterns in control embryos. However, the reprogramming status of the H3K4me2/3 mark largely depended on cell type as it was inconsistent between MSCs, fibroblasts, and control embryos [264]. Based on the development of cloned embryos to the blastocyst stage as well as the level of reprogrammed histone modifications, the authors reported that MSCs were not better nucleus donors than adult fibroblasts [264]. It remains unclear why reports differ with respect to cloning efficiency using donor cells at different stages of development, but variations in methodology, technique, and species may be responsible. Further studies to address this issue will be required.

Because SCNT has proven to be an inefficient process regardless of the nuclear donor used, investigators have attempted to improve SCNT pharmacologically. The HDAC inhibitor Trichostatin A (TSA) has been shown to increase SCNT efficiency in mice [265, 266], pigs [267, 268], cattle [269, 270], and rabbits [271], though these findings are not universal [272, 273]. TSA reversibly binds to and inhibits the actions of HDACs, thereby causing acetylated histones to accumulate in cells [274]. TSA has also been shown to affect DNA methylation, DNMT expression levels, and heterochromatin remodeling [275–277]. Indeed, as epigenetic factors dynamically interact with one another, agents targeting epigenetic mechanisms have pleiotropic effects. To further evaluate the epigenetic factors modified by TSA, Martinez-Diaz and colleagues [278] assessed changes in epigenetic markers in pre- and postimplantation organism development after SCNT and TSA treatment using porcine bone marrow cells (BMCs; putative MSCs) and fetal fibroblasts. While TSA treatment increased the immunofluorescent (IF) signal of H3K14ac in embryos derived from both cell types, it did not increase the IF signal of H3K9me2. The authors also reported that TSA treatment accelerated the rate of development to the blastocyst stage for fibroblast-derived embryos but not for embryos derived from BMCs. Furthermore, embryos reconstructed from fibroblasts developed postimplantation with and without TSA treatment, whereas TSA treatment was necessary for postimplantation development in BMC-derived embryos [278]. This study partially clarified the epigenetic actions of TSA treatment during SCNT. However, it further demonstrated that the question of whether less differentiated cells (in this case BMCs) are more amenable to nuclear reprogramming than further differentiated cell types has yet to be solved.

6. Outlook

Stem-cell-based therapy may eventually serve as a potential remedy to many human pathologies previously thought to be incurable. MSCs in particular are promising for conditions requiring the regeneration of tissue such as bone and cartilage defects. However, before such treatments become readily available, we must further elucidate the mechanisms responsible for stem cell behavior. As we have discussed, MSCs are subject to many levels of control. Epigenetic phenomena have only recently been identified as important regulators of MSC fate. Numerous epigenetic modifications occur concomitantly during both osteogenic and adipogenic differentiation of MSCs. While much knowledge has been generated regarding the epigenetic modifications responsible for MSC differentiation, further investigation to this end will augment our ability to use MSCs therapeutically. Indeed, it is conceivable that manipulation of epigenetic signatures associated with multipotency and pluripotency, as well as modifications associated with lineage-specific differentiation, could direct patient-specific therapy. Identification of the factors necessary to reprogram mesenchymal-derived somatic cells to less differentiated states can also provide insight into the regulation of MSC fate determination.

Abbreviations

ApM1: Adiponectin
ASC: Adipose-derived stem cell
BCOR: BCL-6 corepressor
BMC: Bone marrow cell
BMMSCs: Bone marrow mesenchymal stem cell
ChIP: Chromatin immunoprecipitation

CpG: Cytosine-phosphate-guanine
DNMT: DNA methyltransferase
ES: Embryonic stem
fabp4: Fatty acid-binding protein 4
FRAP: Fluorescence recovery after photobleaching
Glut4: Glucose transporter type 4
H3K9ac: Acetylation of lysine 9 on histone 3
H3K4me3: Trimethylation of lysine 4 on histone 3
H3K27me3: Trimethylation of lysine 27 on histone 3
HAT: Histone acetyltransferase
HDAC: Histone acetyl-deacetylase
HDM: Histone demethylase
HMT: Histone methyltransferase
HSC: Hematopoietic stem cell
IDH: Hschemic heart disease
IF: Immunofluorescent
iPS: Induce pluripotential stem cell
lep: Leptin
lpl: Lipoprotein lipase
LVF: Left ventricular function
MeDIP: Methylated DNA immunoprecipitation
MSC: Mesenchymal stem cell
NT: Nuclear transplantation
OC: Ostecalcin
OFCD: Oculo-facial-cardio-dental
OI: Osteogenesis imperfecta
OPN Osteopontin
PCR: Polymerase chain reaction
PPARγ: peroxisome proliferator-activated receptor-gamma
RB: retinoblastoma
Runx2: runt-related transcription factor 2
SCNT: somatic cell nuclear transfer
STEMI: ST-segment elevated myocardial infarction
Trip10: thyroid hormone receptor interactor-10
TSA: Trichostatin A.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The authors apologize to the investigators whose original work was not cited due to space constraints. The reported work was supported in part by research Grants from the National Institutes of Health (RRR, HHL, RCH, and TCH), and the 863 and 973 Programs of the Ministry of Science and Technology of China (no. 2007AA2z400 and no. 2011CB707900 for J. Luo and T. C. He).

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 570125, 10 pages
doi:10.4061/2011/570125

Review Article

Preclinical Studies on Mesenchymal Stem Cell-Based Therapy for Growth Plate Cartilage Injury Repair

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Received 15 March 2011; Accepted 7 June 2011

Academic Editor: Shinn-Chih Wu

In the last two decades, there has been a strong interest in searching for biological treatments for regeneration of injured growth plate cartilage and prevention of its bony repair. Various means have been tried, including implantation of chondrocytes, mesenchymal stem cell (MSC), together with exogenous growth factor and scaffolds, and gene therapy. However, with the lack of success with chondrocytes, more research has focussed on MSC-based treatments. In addition to circumvent limitations with MSC-based treatments (including cell harvest-associated morbidity, difficulties/time/cost involved in MSC isolation and *ex vivo* expansion, and potential disease transmission), mobilising endogenous MSCs to the growth plate injury site and enhancing *in situ* regeneration mechanisms would represent an alternative attractive approach. Further studies are required to investigate the potential particularly in large animal models or clinical setting of the *ex vivo* MSC approach and the feasibility of the endogenous MSC *in situ* approach in growth plate regeneration.

1. Introduction

Situated at the ends of all long bones, the growth plate is solely responsible for the lengthening of long bones. However, being of a cartilaginous nature, the growth plate is highly susceptible to injuries. Depending on the severity and location, often these injuries are often repaired undesirably by bony repair tissue (also known as a bone bridge formation) which in turn often results in orthopaedic conditions such as limb length discrepancies and bone angulation deformities. As the current methods of correcting growth plate injury-induced bone growth defects are surgically based, highly invasive and not always successful, increasing interest has been shown towards the development of biological treatments which aim to promote growth plate cartilage regeneration and prevent the faulty bony repair. However, although a myriad of studies have investigated potential therapeutic effects of tissue-, chondrocyte-, growth factor-, or mesenchymal stem cells-(MSC-) based approaches in repairing injured growth plate with different degrees of success, currently there is not a biological therapy clinically available that can induce growth plate regeneration. This paper attempts to summarise previous and current research investigating therapeutic potentials of various biological materials or approaches with a particular focus on MSC-based therapies in attempt to induce growth plate cartilage regeneration.

2. The Growth Plate Cartilage

Children's long bones contain a large cartilaginous region known as the growth plate (epiphyseal plate) which is responsible for the longitudinal growth of that particular long bone, through chondrocyte proliferation, hypertrophy, apoptosis, cartilage matrix synthesis, mineralization, and vascularisation [1–3]. The area of this cartilaginous region significantly decreases as the young person gets older and it closes when the maximum growth of the long bone is achieved. The region directly under the growth plate is called the metaphysis which is where the mineralised

growth plate cartilage is being replaced by bone, a process called endochondral ossification [4]. Thus, endochondral ossification bone lengthening is via a two-step process that involves growth plate cartilage scaffold formation and the differentiation and function of bone-forming cells osteoblasts to initiate bone formation in the metaphysis [1, 5].

3. Growth Plate Injury and Current Treatments

Due to accidents in sports and play, skeletal fractures are common in children, with up to 50% children of 5–18 years old experiencing a bone fracture [6]. Since the growth plate is the least rigid region of the long bone, its injuries are common, and it has been estimated that around 20% childhood bone fractures involve growth plate [7]. The Salter-Harris classification system has been used to distinguish the different types of growth plate injuries and the relationship between the characteristics of the fractures and their prognoses (Figure 1) [1, 8–10]. Current literature indicates that the most common type of growth plate fractures occurring in the distal tibiae of younger children is type II (around 40%), which in most cases has a reasonably good prognosis as the cells responsible for interstitial growth of the growth plate as well as the epiphyseal blood supply remain undisturbed [10–12]. Other types of fractures, types III, IV, and V, however, may/will all result in bony formation at the injured site [13]. It has been estimated that in up to 30% of all children with growth plate-related injuries, undesirable bony repair, and bone bridge at the injury site hinder normal growth of the developing long bone in the affected limb [14, 15], which results in significant orthopaedic problems such as limb length discrepancy and bone angulation deformity [15, 16].

Due to the significant orthopaedic problems resulting from growth plate injuries, many previous studies have looked at different ways of correcting growth plate injury-induced defects as well as preventing the bony repair [17]. The type of treatment for growth plate injuries is largely dependent on the age of the patient as well as the severity and type of injury sustained [18]. Surgical intervention is usually needed only if the patient is quite young and significant growth remains. If the injury only results in a very slight length discrepancy, it is often fixed through the use of a shoe lift, and in most cases the patient must cease using the affected limb for a period of time in order to prevent orthopaedic problems, such as angular deformity, from occurring. An already established angular deformity is commonly corrected with a wedge osteotomy [19–21]. On the other hand, larger limb length discrepancies require bone lengthening or bone shortening procedures [22–24]. The most common way of correcting larger limb length discrepancies is through a surgical and lengthening procedure which surgically create a fracture at the diaphysis and then gradually lengthens the injured limb to match the growth of the unaffected limb using a large external frame (*Ilizarov* frame) placed around the affected limb [20, 23, 25]. As effective as this method of treatment is, the downside is that the procedure is highly invasive, painful and lengthy. As only a limited amount of lengthening can be done at a time, the patient often requires the procedure several times throughout adolescence until skeletal maturity is reached. Furthermore, complications arising from pin site infections, further fractures, dislocation, and compartment syndromes make this procedure even more difficult [26]. More recently, another technique has been introduced which can be used to lengthen the affected limb involving the use of an implantable and programmable distraction internal nail known as "Fitbone" for adolescents who have reached maximal growth [27]. Fitbone eliminates the need for an external fixator and hence has the potential to reduce pain and the risk of infections occurring within the treatment site.

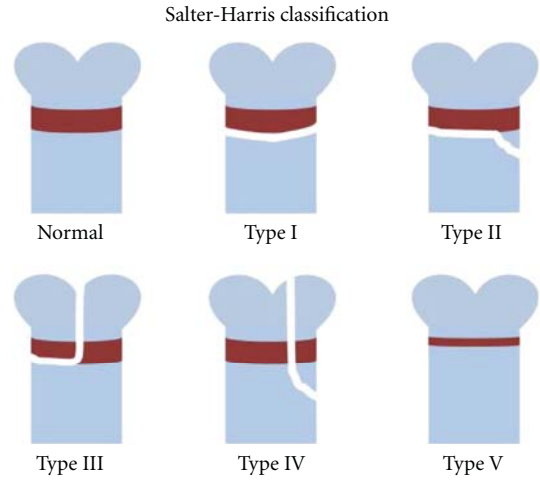

Figure 1: The Salter-Harris classification system. Types I and II fractures do not affect the epiphyseal blood supply. On the other hand, types III, IV, and V do disrupt the blood supply and will more than often result in undesirable bony repair tissue-causing problems of angulations and growth arrest.

Sometimes, an established bone bridge can be surgically removed for correction of growth defects. In order to prevent growth arrest and angulation deformity from recurring, the defect site can be filled with transplanted fat, muscle, polymeric silicone, bone wax, and bone cement as interposition materials [17]. This procedure is called the Langenskiold method [28]. However, all of these available treatments so far are extremely invasive, time consuming, and often ineffective. Currently, much interest has been drawn in finding a better treatment (particularly by a preventative biological approach) to prevent and/or correct problems associated with bony bridge formation. In particular, in more recent times, more research has focussed on utilising tissue engineering and the use of mesenchymal stem cells (MSC) for the regeneration of growth plate cartilage.

4. Previous Attempts with Chondrocyte/Cartilage Transplantation

This void or deficiency of a biological treatment for growth plate injuries has instigated many medical scientists and clinicians to find a potential biological therapy which is able to prevent the bony repair at the injured growth plate and

hence thwart the serious orthopaedic problems associated with this condition. Ideally, a successful therapy would have the ability to regenerate the growth plate cartilage so that the long bone is able to grow with minimal disruption minimising any angulation and/or growth arrest of the affected limb. However, as with any cartilaginous structures the transphyseal growth plate injuries are very hard to heal to the original state as chondrocytes are very difficult to regenerate [29, 30].

Allogeneic and autologous chondrocyte transplantations are one potential approach to overcome this problem, and both methods of chondrocyte transplantation have previously been utilised or trialled for articular cartilage or growth plate repair studies. Allogeneic chondrocyte transplantation involves the removal of healthy chondrocytes from one source followed by the *ex vivo* expansion and finally the replantation of the expanded chondrocytes into another individual (of the same species) [31]. However, the disadvantages of this procedure involve the risk of disease transmission between the two individuals. Alternatively, autologous chondrocyte transplantation involves the direct harvest of healthy chondrocytes (often from the knee) which are then cultured and expanded *ex vivo*; unlike the allogeneic approach, the chondrocytes are implanted back into the patient at the location of the defect, therefore, eliminating any risks of disease transmission [32]. Nevertheless, the disadvantage of this method is the time frame taken to collect, expand, and reimplant the chondrocytes, which has been estimated 3 weeks [33]—by which time, in the case of growth plate injury—a bone bridge has already started to form, thus eliminating this autologous chondrocyte transplantation approach being feasible for growth plate regeneration.

Although there have been many successful studies which have used the allogeneic and autologous chondrocyte transplantation approach for articular cartilage regeneration, very few studies have been performed on growth plate injury models. One earlier study, Bentley and Greer [34] found some success when allogeneic chondrocytes (collected from the growth plate) were delivered into the growth plate injury site of White New Zealand rabbits. This study reported that chondrocytes filled the defect and were able to form columns. In addition, although there were signs of endochondral ossification at the base of the injury site, no rejection of the implanted chondrocytes occurred [34]. However, one study, using a large animal (sheep) tibial growth plate injury model, attempted transplanting chondrocytes directly into the growth plate injury site and did not produce any successful outcomes in preventing the bony bridge formation [35]. Hence, this highlights the unlikelihood of achieving successful growth plate cartilage regeneration with this chondrocyte transplantation approach.

5. Recent Attempts with MSC-Based Growth Plate Cartilage Repair

Due to the limitations associated with chondrocyte transplantation including instability during expansion and donor tissue availability as well as outcome success [36], an alternative cell source, that has been heavily investigated, has been the stem cells. Being of an undifferentiated type, embryonic stem cells hold great potential in differentiation and successful tissue engineering; however, the myriad of ethical and potential health risks and dilemmas involved with their use deem them almost inaccessible [37]. On the other hand, adult mesenchymal stem cells (MSC) are renewable, undifferentiated pluripotent cells which are also capable of differentiating into many different cell types [38] such as cartilage, bone, and fat cells.

MSCs are abundant and have been successfully isolated from many sources including bone marrow [39, 40], periosteum [41–43], trabecular bone [44, 45], adipose tissue [46–48], skeletal muscle [49, 50], and synovium [51–53]. Due to their pluripotency, abundance and accessibility, bone marrow-derived MSCs have made a particularly attractive source for use in articular and growth plate cartilage regeneration [5, 22, 39, 54]. Additionally, an *in vivo* study done by Park et al. [55] showed that MSCs derived from bone marrow and perichondrium/periosteum were more successful at forming hyaline cartilage than from those MSCs derived from other sources such as adipose tissue [55].

Although bone marrow-derived MSCs make up a small proportion of total marrow nucleated cells, they can be easily isolated and expanded with high efficiencies [36]. A plethora of bone marrow-derived MSC related studies have demonstrated the ability of MSCs to differentiate *in vitro* into multiple cell lineages depending on defined culture conditions including differentiation into chondrocytes [40, 54, 56]—making them an ideal candidate for use in articular cartilage repair and potentially for growth plate cartilage repair. In addition, MSCs have also been documented as possessing unique immunosuppressive properties which are advantageous during procedures such as transplantation [57, 58]. Furthermore, it has been hypothesized that bone marrow-derived MSCs secrete various factors which are bioactive with the ability to inhibit scar tissue formation, suppress apoptosis, stimulate angiogenesis [59], as well as having immunoregulatory and regenerative properties [60–62] in comparison to MSC derived from other sources.

Using a growth plate injury model in rabbits, Chen et al. [63] successfully transplanted periosteum-derived MSCs into the growth plate defect and found that the high-proliferation rate of MSCs made them an excellent source for donor cells [63]. Similar to chondrocyte transplantation, two potential methods of delivering MSCs into the desired area of injury is via autologous or allogeneic transplantation. Autologous transplantation of MSCs involves the harvesting of patients' own MSCs and then reimplantation after *ex vitro* expansion. On the other hand, allogeneic transplantation of MSCs involves the use of MSCs taken directly from a cell bank. Planka et al. [64] compared the differences between autologous and allogeneic MSC transplantation and found that there were no major differences in the effect of these implanted MSCs on tibia length and potential angular deformities [64]. Furthermore, the implantation of these cells saw the formation of hyaline chondrocytes within the growth plate injury site [64]. This result was also seen when

allogeneic MSCs were transplanted into the site of growth plate injury in a guinea pig model [65].

It is not guaranteed that implanted MSC will change into the desirable chondrocytes. The differentiation of MSCs is highly dependent on cellular environment, hence, is heavily influenced by the presence of certain growth factors [66]. To optimise the expansion and chondrogenesis of MSCs for cartilage repair, certain growth/survival, and chondrogenic factors need to be present in order to stimulate the migration, growth, survival and chondrogenic potentials of MSCs or progenitor cells. There have been many previous studies which have identified some stimuli or signal molecules controlling their migration, proliferation (PDGF-BB, *FGF-2*), and chondrogenic differentiation (TGF-β1, IGF-I). PDGF-BB has long been found to be an important growth and survival factor of MSCs [54, 67], and *FGF-2* has been shown to enhance mitotic and chondrogenic potentials of human bone marrow-derived MSCs in culture [68]. TGF-β3 has been shown to stimulate chondrogenic differentiation in MSCs and expression of cartilage matrix molecules [69, 70]. In support, Anh et al. [71] found that in young New Zealand White rabbits with growth plate defects, gelfoam (porcine skin gelatin) with MSC, as well as TGF-β3 was found to have remarkably reduced the angular deformity following injury repair [71]. On the other hand, one more recent study which used similar methods in an ovine tibial growth plate injury model did not produce successful cartilage regeneration outcome at the injured growth plate (which is in contrast to the their rabbit model) [72]. However, the study found the addition of the MSCs/growth factor/gelfoam complex did not alter the rate of bony repair formation [72]. In addition, IGF-I has been found to be essential for the differentiation and maturation of growth plate chondrocytes, with important anabolic effects on matrix production for maintaining articular cartilage homeostasis. Furthermore, it has been shown that the structural, functional, and molecular properties of engineered cartilage can be modulated by sequential application of growth factors [54]. While TGF-β stimulates MSC chondrogenesis and IGF-I can enhance their extracellular matrix synthesis, the combined stimulatory effects of TGF-β1 and IGF-I may form a potentially valuable dual stimulatory effect on intrinsic or transplanted MSC function. Such combined stimulatory effects have been demonstrated in the chondrogenesis of periosteum MSCs *in vitro* [69]. Overall from the few studies which focus on combined effects of growth factors and MSC implantation, supplementation with an appropriate growth factor or combination of growth factors is important for a successful outcome for MSC-based growth plate cartilage regeneration. However, further studies are required exploring potential, more potent growth factors, their optimal delivery and formulation for enhancing success for MSC use in cartilage engineering.

Without the correct support and environment, studies have shown that newly injected MSCs were not able to be viable for a sufficient length of time [73]. Therefore, similar to chondrocyte transplantation, to increase longevity and activity, to encourage chondrogenesis as well as to direct the transplanted MSCs into the desired area, a supporting scaffold made from an appropriate material is needed. A myriad of natural and synthetically produced materials have been studied such as fibrinogen, collagen, collagen derivatives, as well as various man-made polymers and other synthetic biomaterials. Di Martino et al. [74] outlined several important qualities when developing the ideal scaffold including biocompatibility, bioabsorbability/biodegradability, appropriate pore size, as well as providing a stable foundation for new tissue formation—in particular suitable for MSC growth, proliferation, and chondrogenesis [74]. Currently, many studies which have reported success in MSC transplantation and differentiation use scaffolds of various types made of natural substances. Natural substances are biologically more compatible and biodegradable, and they provide a more natural microenvironment for the embedded MSCs [59]. Some of the commonly used natural materials are both protein and carbohydrate-based, including chitosan, collagens, fibrin gels, hyaluronan, and alginate [47, 75–80].

Some of the more commonly researched natural materials for use in cartilage regeneration in growth plate and articular cartilage studies include chitosan and fibrin gels. Planka et al. [64] embedded MSC into a scaffold of chitosan and collagen and placed the complex into the growth plate injury site of miniature pigs. The gel scaffold was able to be sealed with a bioceramic material to stop cells from deviating from the desirable area which resulted in some success in preventing growth arrest and angulation deformity [65]. Similarly, an earlier study conducted by Li et al. [81] reported their chitosan-MSC construct was able to restore large growth plate defects in immature rabbits [81]. Medrado et al. [82] also reported the benefits of a chitosan-gelatine construct *in vivo*, whereby the addition of MSCs and dexamethasone resulted in an increase of cell adhesivity, proliferation as well as cell viability. Interestingly, the addition of dexamethasone found an increase in the concentration of collagen-2a when combined with the chitosan-gelatine-MSC complex [82]. Apart from chitosan, a few studies have used materials such as agarose—a polysaccharide obtained from agar. Chen et al. [63] did a large growth plate defect study on a 6-week-old NZW rabbits using agarose with embedded MSCs harvested from the periosteum. Chen et al. [63] found that growth arrest and angular deformation and loss of length of tibia induced by the growth plate defect were corrected by the MSC-agarose treatment in comparison to agarose-only controls [63]. In addition to these naturally occurring substances, synthetic materials such as poly (lactic-co-glycolic acid) (PGLA) and poly (lactic acid) (PGA) have also been used for cartilage tissue engineering. Unlike chitosan and other natural substances, these synthetic counterparts allow modifications such as pore size, fibre diameter, and degradation properties to suit their specific use. Previous studies have found some success in using these synthetic materials for articular cartilage regeneration [83–85]. However, some of the limitations associated with their use include relatively poor cell adhesion properties as well as issues concerning their biocompatibility [86].

In recent times, the development of injectable hydrogels has become of great interest for cartilage repair and potentially growth plate cartilage regeneration. These are gel-like

substances which can have MSC embedded into them [87]. Hydrogels offer the administration of growth factors and/or cells into a cartilage defects more accessible and easier. Cho et al. [88] have developed alginate/polyvinyl alcohol (PVA) hydrogels which is able to gelatinize at a more controllable rate than solely alginate hydrogels. Future studies will reveal potential of MSCs alongside these natural and injectable scaffolds and the appropriate growth factors to regenerate articular and growth plate cartilage.

6. Combined MSC and Gene Therapy Approach for Cartilage Repair

Successful chondrogenic regeneration involves two key points—first, to encourage chondrogenesis, and second to form new cartilage. Although current studies have provided a myriad of different bioactive factors that have potential to greatly benefit the repair process, difficulties associated with their administration have slowed down any real progress. This explains why new techniques involving methods such as genetic engineering and gene transfer technology have become of interest. Although a majority of these studies were not done specifically for repairing growth plate cartilage, many of the techniques could potentially be applied for this use. Successful gene transfer can be achieved through a few different approaches: the direct vector administration to cells or surrounding cells within the injury site or alternatively and the transplantation of genetically modified chondrogenic cells into the affected area [89].

Direct modification of *ex vivo* chondrocytes has been well studied. Cultured chondrocytes were able to maintain the expression of certain transgene products after genetic modification with recombinant adenoviral of TGF-β [90, 91], BMP-7 [92], and IGF-I [91, 93]. Nixon et al. [93] found that *in vitro* experiments involving the adenoviral over expression of IGF-I in chondrocytes resulted in the stimulated expression of proteoglycans as well as collagen type 2 [93]. Proteoglycan and collagen type 2 synthesis were also stimulated when TGF-β1 was transduced adenovirally on a monolayer of chondrocytes [90, 91]. In more recent times, interest has been shown for the gene transfer of transcriptional factors such as Sox-9. Sox-9 is a known master regulator of chondrogenesis, hence when Sox-9 was retrovirally overexpressed, it resulted in increased collagen type 2 expression in a pellet culture [94].

Since treatment with growth factors is often not successful due to the short half-life of many growth factors, and since gene delivery is a better alternative to deliver growth factors because it is more stable and flexible than the protein itself [95], much interest has been drawn towards genetic modification of MSCs with growth factor genes for enhancing cartilage repair. This technique requires the *ex vivo* genetic modification of MSCs followed by the transplantation of the altered cells back into the affected area [59]. Since modification by any means is an alteration of the original, Hu et al. [96] questioned whether gene-altered MSCs were still capable of possessing their characteristic of multipotency [96]. They found that after retroviral transfection with human IGF-I, rat MSCs showed a greater ability to express IGF-I as well as an increased ability to proliferate and reduce apoptosis, and that modifications of MSCs could potentially affect the types of tissues they differentiate into [96]. In order to lengthen the time and versatility of MSCs, Song et al. [97] utilised gene therapy to transfect bone marrow-derived MSCs with the *FGF-2* gene, which showed an improvement in survival of MSC against hypoxic conditions *in vitro* [97]. In addition, another study also modified MSCs with angiogenin adenoviral vector which resulted in the enhancement of implanted cells against hypoxic injury [98].

Although there are many studies which have successfully transduced MSCs with variety of chondrogenic growth factors, an interesting study discussed some of the limitations of this MSC + gene therapy approach for cartilage repair. A study by Palmer et al. [99] showed that only a certain amount of gene expression was needed to induce chondrogenic differentiation of bone marrow-derived cells, and that overexpression by gene-induced transduction may have negative, opposing effect on chondrogenic differentiation [99]. In addition, a few other disadvantages of the *ex vivo* approach for gene therapy include high cost and being fairly laborious and time consuming. However, *ex vivo* gene therapy allows the safety testing and control of the cells before the reimplantation and hence minimising any risk of disease transmission [100]. Overall, although the potential of combining gene therapy techniques with MSCs has been more recently explored, not many have applied it to the regeneration of growth plate cartilage. Hence, further studies are needed to investigate whether this type of cartilage engineering is useful in growth plate cartilage regeneration.

7. Endogenous Stem Cell Possibility

Although a number of studies with rabbit growth plate injury repair models have shown that MSCs may have some potential in regenerating injured growth plate and prevent bone growth defects [63–65, 71]. However, recent work with a large animal model has questioned value of this *ex vivo* MSC approach [72]. While ovine bone marrow MSCs are multipotential and can form cartilage-like tissue *in vivo* [101], however, in a growth plate injury model in lambs, autologous bone marrow-derived *ex vivo*-expanded MSCs failed to promote growth plate regeneration [72]. In addition, currently, MSC-mediated cell therapies are limited by various difficulties and issues such as morbidity associated with cell harvest, difficulty in stem cell isolation, genetic and phenotypic instability associated with *ex vivo* expansion, difficult up-scaling, high costs, variability, and risks of disease transmission particularly with allogeneic MSC transplantation [54]. While transplantation of both allogeneic and autogenous MSC as well as modified MSCs offer many advantages in cartilage repair, a major problem associated with their use is the need for fetal calf serum during *ex vitro* expansion.

The existence of functional stem cells within the local environment and their migratory capacity represent an opportunity to circumvent limitations of *ex vivo*-based MSC therapy and to achieve *in situ* cartilage regeneration

by enhancing local reparative mechanisms and mobilising endogenous MSCs [52, 102]. MSCs express adhesion molecules [103] and can migrate to sites of injury healing [51, 97]. Indeed, synovial mesenchymal cells migrate to cartilage defects and may serve as a cell source for repair under specific growth factor stimulation [104], and marrow MSC migrate and contribute to cartilaginous formation during bone healing and contribute to articular repair [105].

Although present in small quantities during the fibrogenic infiltrate with the growth plate injury site, endogenous multipotent mesenchymal stromal cells were observed during growth plate injury repair [106]. These cells demonstrated their multipotency—differentiating into bone and cartilage tissues within the injury site [67, 106–110]. With infiltration of progenitor cells into growth plate injury site, it will be of particular interest to investigate whether these endogenous progenitor cells can be mobilised to enhance growth plate regeneration.

However, the main problem with accessing the endogenous cells for example from the bone marrow is that they may not be present in a density large enough to support adequate cartilage regeneration. Hence, to overcome this problem, a recent study has suggested that selected growth factors are needed to stimulate and enhance MSC migration and accumulation into the cartilage injury site [111]. Dar et al. [112] found the chemokine/receptor pair SDF-1/CXCR4 is present and functional in MSC population [112]. Kitaori et al. [113] found that inhibition or absence of this signaling resulted in absence of MSC in a bone fracture model [113]. In addition to the SDF-1/CXCR4 signalling, Schenk et al. [114] reported monocyte chemotactic protein (MCP-3) as another homing chemotactic signaling pathway for MSC migration. Schenk et al. [114] found that in myocardial infarction, the systemic infusion of this protein resulted in a MSC migration response. Furthermore, Ode et al. [115] studied roles and influences of different extracellular matrix components on MSC migration and behavior and found that the collagen family excluding collagen-V as well as adhesion proteins such as fibronection and vitronectin all influenced and encouraged the migratory and proliferatory behavior of MSCs [115]. More studies are required to study how endogenous MSCs can be regulated to enhance migration into and expansion within the injury site for cartilage regeneration.

8. Conclusion

Growth plate injuries are common in children and their "faulty" bony repair impairs bone growth and cause lifelong orthopaedic problems. Current treatments (surgical correction) for these problems are highly invasive and often requiring repeated surgeries, and thus there is a strong need for a biological treatment that can promote growth plate cartilage regeneration. Although the approach of using *ex vivo* expanded MSC has shown some promise in promoting growth plate repair in rabbit models, the efficacy of this approach has been questioned in a recent "translational study" using a large animal model. Further studies are required to define more potent chondrogenic growth factor(s) or matrix scaffold that will enhance growth plate regeneration using *ex vivo*-expanded MSC, and more studies are needed to investigate the therapeutic potential of MSCs for growth plate regeneration in large animal models. In addition, due to the time (around 3 weeks) required for MSC isolation and expansion, this *ex vivo* approach with autologous MSC may not be practical to treat a growth plate shortly after the fracture; further studies are required to investigate whether endogenous MSCs or progenitor cells within the local environment or bone marrow can be mobilised and local regenerative mechanisms be optimised to achieve *in situ* growth plate regeneration after a growth plate fracture so to circumvent limitations of *ex vivo*-based MSC therapy.

Conflict of Interests

All authors declare no conflict of interests.

Acknowledgments

This paper was funded by Bone Growth Foundation, Channel-7 Children's Research Foundation of South Australia, Australian National Health, and Medical Research Council (NHMRC).

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 717069, 14 pages
doi:10.4061/2011/717069

Research Article

Efficient Non-Viral Integration and Stable Gene Expression in Multipotent Adult Progenitor Cells

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Received 14 January 2011; Revised 1 June 2011; Accepted 15 June 2011

Academic Editor: Mark G. Carter

Non-viral integrating systems, PhiC31 phage integrase (ϕC31), and *Sleeping Beauty* transposase (SB), provide an effective method for *ex vivo* gene delivery into cells. Here, we used a plasmid-encoding GFP and neomycin phosphotransferase along with recognition sequences for both ϕC31 and SB integrating systems to demonstrate that both systems effectively mediated integration in cultured human fibroblasts and in rat multipotent adult progenitor cells (rMAPC). Southern blot analysis of G418-resistant rMAPC clones showed a 2-fold higher number of SB-mediated insertions per clone compared to ϕC31. Sequence identification of chromosomal junction sites indicated a random profile for SB-mediated integrants and a more restricted profile for ϕC31 integrants. Transgenic rMAPC generated with both systems maintained their ability to differentiate into liver and endothelium albeit with marked attenuation of GFP expression. We conclude that both SB and ϕC31 are effective non-viral integrating systems for genetic engineering of MAPC in basic studies of stem cell biology.

1. Introduction

Stem cells are capable of long-term self-renewal in culture and can be induced to form a variety of cell types. These characteristics provide a unique resource for genetic and developmental studies or for therapeutic use in the development of cell and gene therapies. Multipotent adult progenitor cells (MAPC) represent a class of stem cells derived from bone marrow (BM) and other adult mammalian tissues that can be expanded in culture [1–3] to generate multiple cell types *in vitro* and *in vivo* [4–8]. Several transplantation studies have demonstrated that human MAPCs are beneficial for functional recovery following vascular ischemia possibly via trophic effects [9–12] and possess contact-independent immunosuppressive potential [13, 14]. These properties suggest that MAPCs may be an effective source for potential use in immunomodulation and in the treatment of ischemic diseases. Further extension of its use in single-gene disorders and for use in creation of cell-based screening models requires methods to engineer MAPC by introduction of defined genetic elements.

Non-viral DNA-mediated gene transfer has been explored as a means of expressing new genes in a variety of different cell types *in vitro* and *in vivo*. However, the usefulness of naked DNA vectors is often restricted by the inability to provide sustained gene expression. Stable integration of

the plasmid-encoded sequence is inefficient, as it relies on random double-strand break-mediated recombination. One method of addressing this problem is to use non-viral vectors in conjunction with a recombinase that has the capability of integrating genetic cargo into cellular chromosomes.

Sleeping Beauty (SB) transposase and PhiC31 (ϕC31) integrase are plasmid-based systems that have been demonstrated to mediate stable gene transfer and expression in mammalian cells. SB is a member of the *Tc1/mariner*-like family of DNA transposons, which are found as inactive remnants throughout various genomes in all of the major kingdoms except bacteria and mediate gene transfer by a "cut-and-paste" mechanism [15] (Figure 1). The SB transposon system was generated by "repairing" an evolutionarily decayed *Tc1*-like sequence found in the genomes of salmonid fish [16]. The autonomous element consists of the catalytic transposase flanked by inverted terminal repeats (ITRs) each containing two direct repeats (DR), which are binding sites for the transposase and essential for transposition [16, 17]. For gene transfer applications, the transposase is separated from the transposon ends and replaced with any DNA cargo. The transposase can be supplied on the same (*cis*) or separate (*trans*) plasmid from the one encoding the transposon or as *in vitro* transcribed mRNA [18]. When expressed, the transposase excises the transposon from the donor plasmid and precisely inserts this genetic element into vertebrate chromosomes at a TA dinucleotide. In contrast to random recombination, insertion mediated by transposition occurs without altering the flanking chromosomal sequence. The SB transposon system has been used for stable genetic modification of multiple rodent and human cell lines [16, 17, 19] and primary cells including mouse liver [20–25], human skin cells [26], mouse lung [27–29], and human peripheral blood T-cells [30] as well as embryonic stem (ES) cells derived from mice [31, 32] and humans [33, 34]. Murine MAPC modified using an SB transposon engineered for expression of a dual reporter encoding DsRed2 and firefly luciferase have been used to study the homing pattern of MAPC via *in vivo* bioluminescence imaging after transplant into immunodeficient mice [35]. Furthermore, recent studies have demonstrated the usefulness of the SB transposon system for genetic modification of human $CD34^+$ hematopoietic progenitor cells isolated from cord blood [36, 37].

The ϕC31 integrase is a member of the serine recombinase family found in *Streptomyces* [38]. Under natural conditions, the two-component ϕC31 system mediates a unidirectional recombination event between the *attP* site of the phage genome and the *attB* site of the *Streptomyces* chromosome. For applications in mammalian cells, gene sequences on an *attB* containing plasmid are inserted into cellular chromosomes at sites having partial homology to the wild-type phage *attP* sequence ("pseudo-*attP*" sites) when codelivered with a source of the phage integrase enzyme [39, 40] (Figure 1). The frequency of PhiC31-mediated integration is about 10- to 100-fold higher when compared to the reversible recombinases (Cre and FLP) both of which require that the target recognition sequence be preinserted into the genome [41]. The utility of the ϕC31 system for mediating stable gene expression has been demonstrated in various cultured mammalian cell lines [42], and in primary cells, including mouse liver [43, 44], human skin cells [45], and muscle-derived stem cells [46] as well as for site-specific genomic insertion in mouse [47] and human ES cells [48].

Here, we directly tested these two non-viral integrating vector systems for the capacity to mediate stable gene transfer into primitive adult stem cells. An internally controlled, bifunctional plasmid was utilized to codeliver recognition sequences for both ϕC31 and SB integrating systems. We investigated the frequency of gene insertion, strength of gene expression, and the effect of genetic modification on the stem cell potential of MAPC. These results provide an assessment of the relative usefulness of these non-viral integrating systems for the purpose of *ex vivo* gene transfer and a means to generate integrated engineering platforms for non-viral delivery and expression of transgenes in stem cells.

2. Materials and Methods

2.1. Plasmid Construction. The integrating vector (pKT2/NAG) was constructed using T2 inverted terminal repeat sequences flanking the cargo [49]. For construction of pKT2/NAG, the neomycin phosphotransferase (Neo) cDNA was obtained by PCR using pT/Neo [50] as a template with primers Neo-F (5′-GCC ACC ATG ATT GAA CAA GAT GGA TTG C-3′) and Neo-R (5′-CGC TCA GAA GAA CTC GTC AAG AAG-3′), and subsequently cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif, USA) to form pTOPO-Neo. An *Eco*RI fragment containing the amplified Neo sequence was inserted into the polylinker between the PGK promoter and rabbit β-globin polyadenylation signal of pKT2/PGK to form pKT2/PGK-Neo. The *att*B sequence and EF1α regulated GFP coding sequence was created by introducing a 307-bp *Eco*R1 fragment containing the ϕC31 *att*B site from pTA-*att*B [39], kindly provided by Dr. Michele Calos, Stanford University) into the same site in plasmid pVITRO-GFP (Invivogen, San Diego, Calif, USA). A *Hin*dIII-*Xho*I fragment containing the *attB* sequence, EF1α promoter, GFP coding sequence, and SV40 polyadenylation signal was subsequently isolated and cloned upstream of the PGK promoter in pKT2/PGK-Neo between *Hin*dIII and *Sal*I.

The luciferase (pCMV-Luc) and SB10 transposase (pCMV-SB) expression vectors have been previously described [50, 51]. ϕC31 integrase was placed under transcriptional control of the CMV promoter by isolating a *Nhe*I-*Spe*I fragment containing the integrase coding sequence from pTOPO-Int (kindly provided by Dr. Michele Calos, Stanford University) and cloning it in place of luciferase in pCMV-Luc between *Nhe*I and *Xba*I. Plasmid DNA was prepared using an Endofree Maxi Prep Kit (Qiagen, Valencia, Calif, USA).

2.2. Cell Culture and Gene Transfer. Human fibrosarcoma HT1080 cells were maintained in complete growth medium consisting of Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% antimycotic-antibiotic (Invitrogen, Carlsbad, Calif, USA), and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

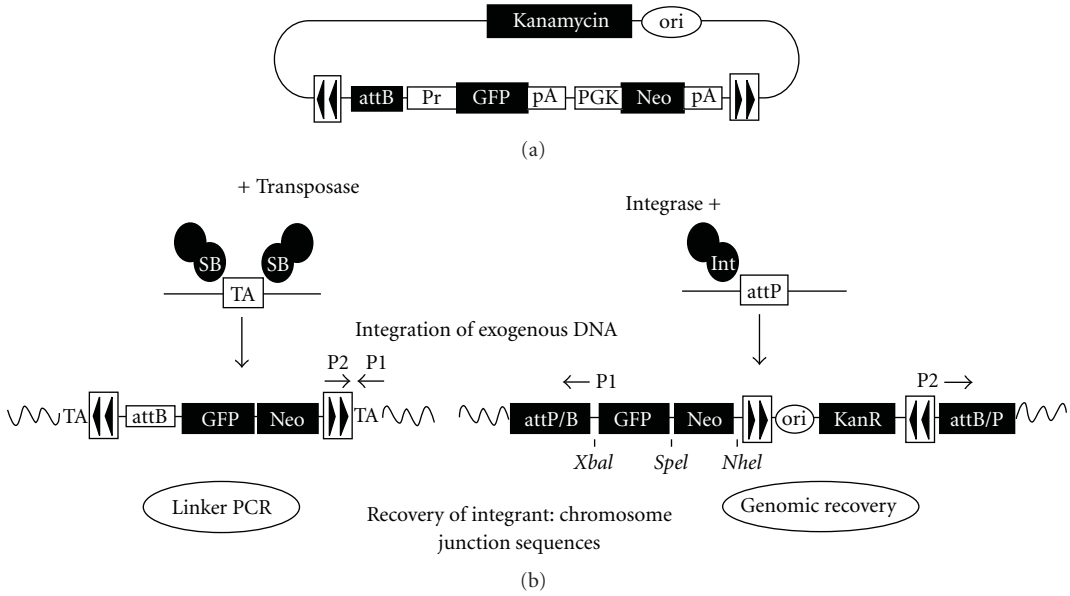

Figure 1: Schematic diagram of the internally controlled pKT2/NAG vector and integration products mediated by *Sleeping Beauty* (SB) transposase and by bacteriophage PhiC31 (ϕC31) integrase. (a) The integrating vector consists of (i) a ϕC31 integrase recognition site (*attB*); (ii) eF1α or CMV- (Pr-) regulated GFP expression unit; (iii) neomycin resistance gene (Neo) transcriptionally regulated by the PGK promoter; (iv) flanking T2 transposase binding sites (IR/DRs; boxes with double triangles) separated by; (v) a colE1 bacterial origin of replication and kanamycin resistance gene; (vi) pA, polyadenylation signal from the rabbit beta globin gene. (b) SB transposase-mediated integration (left): the SB transposase excises transposon sequences at IR/DR transposase binding sites and precisely inserts them into TA-dinucleotide targets in cellular chromosomes, which are subsequently duplicated. ϕC31 integrase-mediated integration (right): exogenous gene sequences on an *attB* containing plasmid integrate into mammalian genomes at "pseudo-*attP*" sites, chromosomal sequences having partial homology to the wild-type phage *attP* sequence.

The day before transfection, 4-5 $\times$ 10^5 cells were seeded into 6-cm tissue culture plates. Cells were cotransfected with pKT2/NAG (500 ng) plus pCMV-Luc, pCMV-SB, or pCMV-Int (150, 500, or 1500 ng) using SuperFect reagent (Qiagen, Valencia, Calif, USA) in a final volume of 1 mL complete growth medium for three hours before changing the medium. For stable gene transfer, cells were collected two days after transfection, stained with trypan blue, and counted. Viable cells (30,000) were plated into 100-mm dishes containing complete growth medium supplemented with 850 μg/mL G418 (Invitrogen, Carlsbad, Calif, USA). After 14 days of selection, cells were fixed and stained with a 70% methanol solution containing 1% crystal violet.

Rat MAPC (rMAPC) were maintained on fibronectin coated flasks or dishes using previously described conditions [52]. On the day of transfection, rMAPC were released from plates with trypsin, washed with PBS, and 0.1 to 0.5 $\times$ 10^6 viable cells (trypan blue negative) were resuspended in nucleofection solution V (Amaxa, Gaithersburg, Md, USA) with pKT2/NAG (5 μg) and an equal amount of either pCMV-Luc, pCMV-SB, or pCMV-Int, transferred into the supplied cuvette, and electroporated (Amaxa; setting A-23) as described earlier [52, 53]. The cells were immediately resuspended in prewarmed growth medium and seeded into 6 cm plates. For stable transfection, cells were grown in medium supplemented with G418 (400 μg/mL) 1 day after transfection.

2.3. Flow Cytometry. HT1080 cells or MAPC were harvested and rendered into single-cell suspensions for flow cytometric analysis. Live cells were identified and gated by exclusion of propidium iodide and then tested for expression of GFP on a FACSCalibur System (Beckton-Dickinson) using CellQuest analysis software (BD Biosciences, Heidelberg, Germany).

2.4. Southern Hybridization Analysis. Southern blotting was performed as previously described [44]. Briefly, 10 μg of genomic DNA isolated from several G418-resistant MAPC clones was digested overnight with *Bam*HI (SB-treated cells) or *Spe*I (ϕC31-treated cells), electrophoresed through 0.8% agarose gel, and then blotted onto nytran. An 835-bp fragment encoding the GFP sequence was isolated from the integrating vector (pKT2/NAG) by *Age*I-*Hin*dIII digest and ^{32}P radio-labeled using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, Calif, USA) to use as the DNA probe.

2.5. In Vitro Differentiation and Marker Expression. Undifferentiated rMAPC that were unmanipulated, had gone through random integration upon cotransfection with the luciferase expression vector or stable integration mediated by ϕC31integrase or SB transposase were harvested for RNA isolation and RT-PCR analysis was carried out as previously described [52]. The rMAPC were differentiated

into endothelium or hepatocytes as described [2, 6, 7]. For endothelial differentiation, transgenic MAPC were cultured in basal growth medium supplemented with 2% or 5% serum plus vascular endothelial growth factor (VEGF, 10 ng/mL). Liver differentiation was elicited by addition of 2% serum, hepatocyte growth factor (HGF, 20 ng/mL), and fibroblast growth factor-4 (FGF4, 10 ng/mL) to the growth medium. At days 9 and 14, cells were harvested for RNA isolation, and end-stage RT-PCR or qRT-PCR analysis was performed using primers for endothelium-specific transcripts vascular endothelial growth factor receptor-2 (Flk1), vascular endothelial growth factor receptor-1 (Flt1), and endothelial-derived gene-1 (Eg1) or hepatocyte-specific markers hepatocyte nuclear factor-3-beta (HNF3b), alpha-fetoprotein (AFP), and transthyretin (Ttr), where glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for RNA loading and integrity. Amplicons from end-stage RT-PCR were separated by electrophoresis through 2% agarose gel. For qRT-PCR, target gene expression was normalized to GAPDH and relative expression calculated by the $2^{-\Delta\Delta CT}$ formula. Primer sequences described in earlier studies were used [6, 7]. All experiments were performed at least in duplicate with technical replicates in each set.

2.6. Cytogenetic Analysis. Transgenic rMAPC were washed to remove dead cells and resuspended in 10 mL culture medium. Cells were sent to the University of Minnesota Cytogenetics Core Laboratory for analysis. Briefly, cells were treated with colcemid for 3 hours and then harvested according to standard cytogenetic protocol. Approximately 100 metaphases were evaluated by G-banding at a 400–425 band level resolution.

2.7. Recovery of Integration Sites. G418-resistant clones were isolated after cotransfection of each cell type with pKT2/NAG plus pCMV-Luc, pCMV-SB, or pCMV-Int. Genomic DNA (gDNA) was isolated using the Puregene DNA purification kit (Gentra Systems, Minneapolis, Minn, USA). A genomic recovery method was used for identification of PhiC31 integrase-mediated chromosomal insertions. High molecular weight DNA (2 μg) was digested with *Nhe*I, *Spe*I, and *Xba*I (which generate compatible 5′ termini; schematically represented in Figure 1) precipitated in 100% ethanol and the recovered DNA was ligated under dilute conditions (500 μL) with 4 units of T4 DNA ligase (New England BioLabs, Beverly, Mass, USA). The ligated DNA was precipitated with 100% isopropanol, pelleted by microcentrifugation and washed with 70% ethanol before being resuspended in 10 μL of sterile H_2O. Two microliters of this DNA was electroporated into DH10B electrocompetent *E. coli* (Promega, Madison, Wis, USA), allowing bacterial cells to recover in SOC media by incubation with agitation at 37°C/200 rpm for 1 hour before plating on Luria/Bertania agar containing 50 μg/mL kanamycin. Plasmid DNA was isolated and sequenced using primers that flank the *attB* site (shown in Figure 1(b)): *attB*-F (5′-TAG GGC GAA AGG AAG GG TGG-3′) and *attB*-R (5′-GGC TTC GAG ACC GTG ACC TA-3′). For SB-mediated integration events, a linker-mediated PCR technique was used to recover transposon-chromosome junction sequences as described [54] and schematically represented in Figure 1. Genomic DNA (2 μg) was digested with *Bfa*I and ligated to a linker. Primary PCR was performed with primers 5′-GTA ATA CGA CTC ACT ATA GGG C-3′ and 5′-CTG GAA TTT TCC AAG CTG TTT AAA GGC ACA GTC AAC-3′ under the following conditions: 94°C for 2 min, then 25 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 90 sec. The PCR products were diluted and nested PCR was carried out under the same conditions using primers 5′-AGG GCT CCG CTT AAG GGA C-3′ and 5′-GAC TTG TGT CAT GCA CAA AGT AGA TGT CC-3′. The products of the nested reaction were separated by electrophoresis through 2% agarose gel. Specific products were excised, purified using the Qiaquick gel extraction kit (Qiagen, Valencia, Calif, USA), and cloned into pGEM-T vector (Promega, Madison, Wis, USA) using ElectroMax DH10B (Invitrogen, Carlsbad, Calif, USA) for transformation. All recovered integration events were sequenced at the Advanced Genetics Analysis Center at the University of Minnesota and subjected to BlastN analysis against the rat genome using the ENSEMBL database.

3. Results

3.1. Gene Transfer and Stable Expression in Cultured Human Fibroblasts. The SB and ϕC31 recombinases each require their own unique recognition sequence and catalytic component to mediate the insertion of gene sequences into chromosomes. We directly compared the effectiveness of integration and long-term expression using a single two-component plasmid that is internally controlled for the integrating sequence (Figure 1(a)). The mode of integration into the host genome catalyzed by SB and ϕC31 is represented in Figure 1(b).

To functionally test our internally controlled vector (pKT2/NAG) when cotransfected with each recombinase, we performed a colony-forming assay in cultured human male fibrosarcoma HT1080 cells. This cell line was selected on the basis of a relatively normal karyotype by cytogenetic analysis (46, XY, with 5p+ and 11q+) [55]. The effect of the dose of the codelivered recombinase-encoding plasmid on stable gene transfer and expression was determined by G418-resistant colony formation and GFP fluorescence. pKT2/NAG (500 ng) was cotransfected in triplicate with 150, 500, or 1500 ng of either CMV-regulated luciferase (pCMV-Luc), transposase (pCMV-SB), or integrase (pCMV-Int) expression plasmids (Figure 2(a)). Flow cytometric analysis of GFP expression two days later demonstrated nearly equivalent transfection efficiencies (20%–25% of cells were GFP positive; data not shown). Similar to previous studies [20, 50, 56], our data revealed the inhibitory effect that is frequently observed with increasing doses of transposase-encoding plasmid. The lowest dose of pCMV-SB10 (150 ng) yielded the greatest increase (20-fold greater than the no transposase control) in G418-resistant colony formation, while the highest dose (1500 ng) produced only 5-fold more colonies than the no transposase control (Figure 2(b)). For

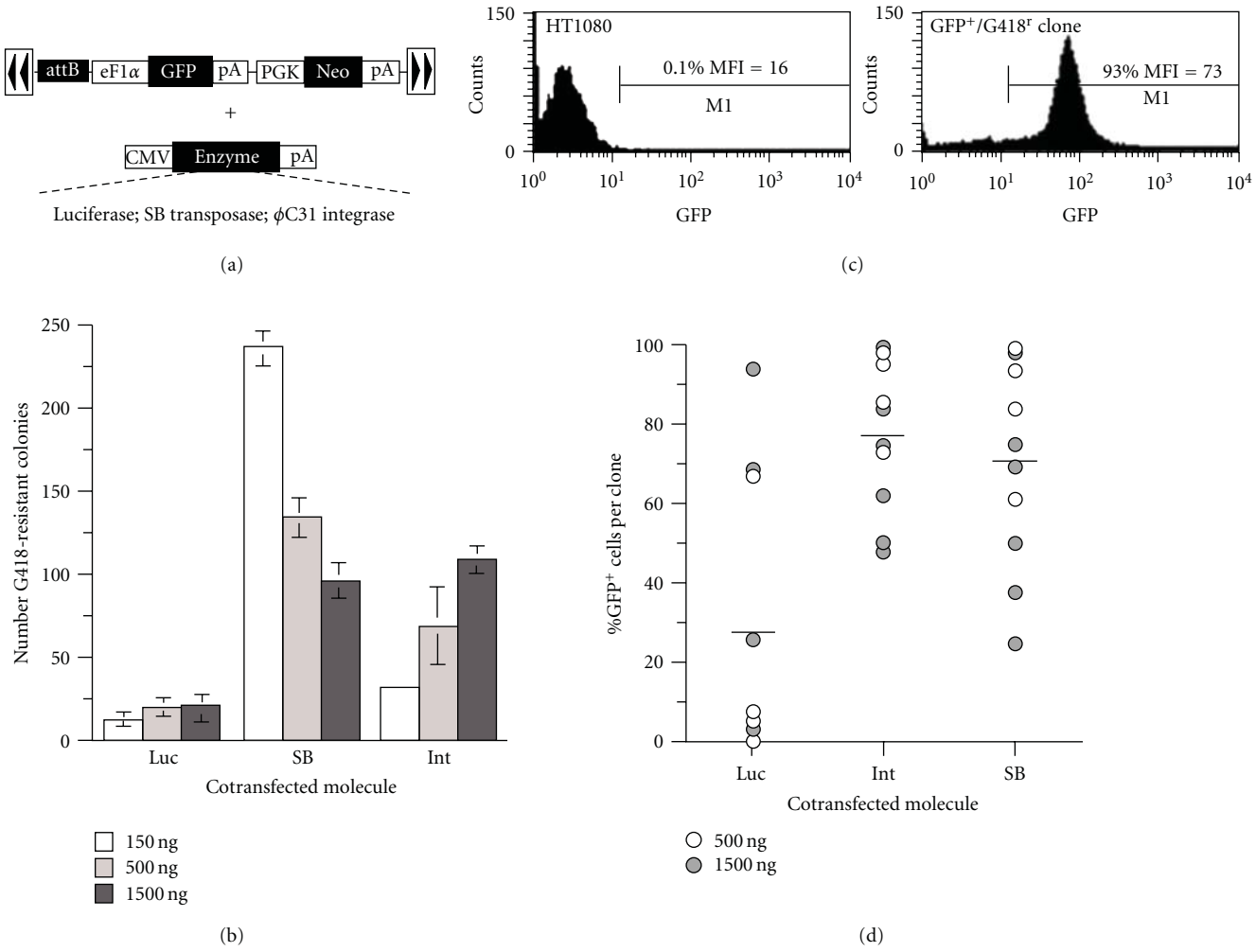

Figure 2: Stable expression mediated by *SB* transposase and ϕC31integrase in cultured human fibroblasts. (a) Schematic diagram of plasmids cotransfected into HT1080 cells in a colony-forming assay. The integrating vector (pKT2/NAG) is described in Figure 1. Recombinase-encoding plasmids are transcriptionally regulated by the cytomegalovirus immediate early promoter (CMV). The firefly luciferase encoding expression vector (Luc) serves as a control for colony formation resulting from random recombination compared to stable gene transfer mediated by SB transposase (SB) or ϕC31 integrase (Int). (b) Colony-forming assay for integration efficiency. HT1080 cells ($4\text{-}5 \times 10^5$) were cotransfected in triplicate with pKT2/NAG (500 ng) and 150 ng, 500 ng, or 1500 ng of Luc, SB, or Int encoding expression plasmids as described in Section 2. The number of G418-resistant colonies per 3×10^4 cells plated is shown for each group ($n = 3$). Values are reported as mean ± SE. (c) GFP expression in transgenic HT1080 cells. Examples of flow cytometry plots are shown for unmanipulated HT1080 cells (left panel; GFP negative) or an expanded G418-resistant clone (right panel; GFP positive). (d) Percentage GFP-positive cells determined by flow cytometric analysis of 10 independent G418-resistant clones expanded from HT1080 cells cotransfected with pKT2/NAG and 500 ng (open circle) or 1500 ng (filled circle) of Luc, SB, or Int expression vectors. Mean percentages of GFP-positive cells for each condition are indicated by solid lines.

ϕC31 integrase, increasing the concentration of pCMV-Int improved G418-resistant colony formation (from 3- to 7-fold) compared to the no integrase control pKT2/NAG (Figure 2(b)).

Ten G418-resistant clones generated by cotransfection with 500 ng or 1500 ng of SB transposase or ϕC31 integrase were independently expanded in G418-containing medium for an additional four weeks to characterize expression of the upstream GFP reporter by flow cytometry (Figures 2(c) and 2(d)). Three drug-resistant clones generated by random recombination (+ pCMV-Luc) showed expression of GFP (> 60%), but the majority failed to express GFP (6/10 <10% GFP positive). In contrast, GFP expression was maintained in all clones rendered drug-resistant by SB- or PhiC31-mediated integration but with increased efficiency in cells transfected with 500 ng versus 1500 ng of each recombinase. These results indicate that the single bifunctional construct in the presence of the appropriate recombinase can mediate integration into the host genome resulting in persistent transgene expression.

3.2. Non-Viral Integration Efficiency and Stable Gene Expression in MAPC. Bone marrow-derived stem cells with the capacity for self-renewal and proliferation in culture are

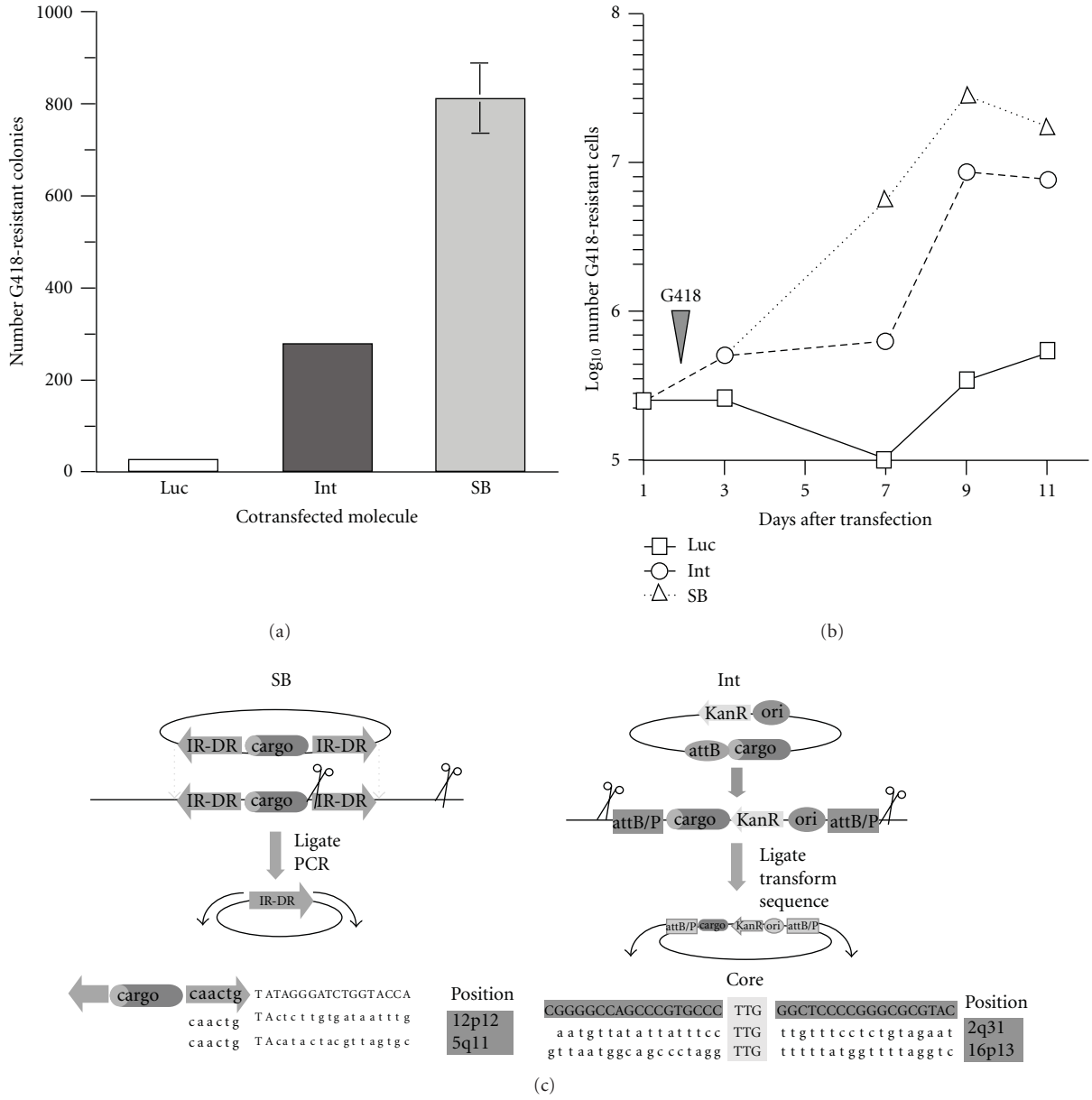

Figure 3: Non-viral integration efficiency in MAPC. Nucleofected rMAPC were plated and the medium was supplemented with G418 (400 μg/mL) one day later. (a) Colony-forming assay. After 10–12 days of growth under selective conditions, cells were fixed and stained to determine the frequency of G418-resistant colony formation. The number of G418-resistant colonies ($n = 3$) for each group is shown ± S.D. (b) Cells were harvested into a suspension and viable cell counts were performed by trypan blue exclusion on the indicated days and the total number of cells in culture is reported as mean ± SE. (c) Genomic DNA isolated from the individual clones obtained with SB and Int was subjected to restriction enzyme digestion, plasmid sequence rescue, and sequencing of the recovered fragment carried out to determine the genomic site of integration.

important biological tools for studying cell fate and differentiation. To determine the integration efficiency mediated by SB transposase (SB) and ϕC31 integrase (Int) in adult stem cells, we evaluated stable gene transfer in MAPC derived from the bone marrow of newborn rat (postnatal days 2–5). Nucleofection was used as the method of gene transfer based on previous studies demonstrating efficient gene transfer and low toxicity in these cells [52, 53].

The integrating vector pKT2/NAG was codelivered with a source of recombinase at a 1 : 1 mass ratio (a dose demonstrated to effectively render HT1080 cells resistant to G418 selection) into undifferentiated MAPC using Amaxa Nucleofector technology (described in Section 2.2), where transfection efficiency ranged from 15% to 20% by flow cytometric analysis of GFP positive cells 24 hours later (data not shown). After 7 to 10 days of growth in culture, we found

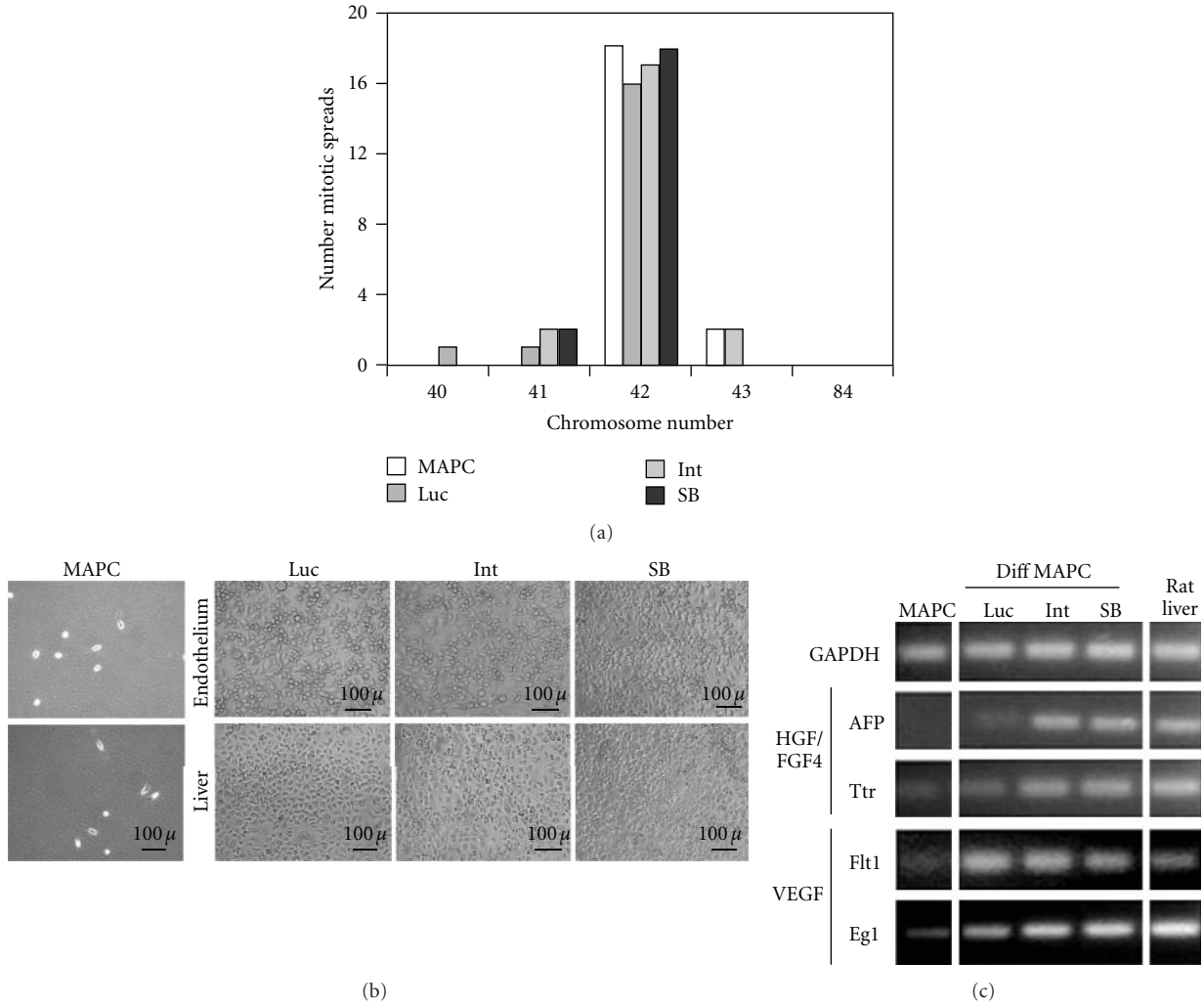

Figure 4: MAPC maintain their stem-cell character and the ability to differentiate following genetic engineering. (a) Karyotypic analysis of pooled G418-resistant cells; the number of mitotic spreads containing the number of chromosomes indicated on the abscissa is indicated. Absence of aneuploid or diploid cells indicates no gross karyotypic anomalies or aberrant cell-cycle activity in the analyzed cells. (b) MAPCs are small spindle-shaped cells that are cultured at very low density. Morphological changes following differentiation into liver and endothelium for two weeks as described in Section 2 show significant difference between cells LUC-, Int- or SB-treated cells. (c) RT-PCR analysis of the differentiated cells shows the presence of alpha-fetoprotein (AFP) and transthyretin (TTR) for liver differentiation, and vascular endothelial growth factor receptor-1 (Flt1) and endothelial-derived gene-1 (Eg1) for differentiation into endothelium. Rat liver was used as a positive control for liver and endothelial markers and undifferentiated MAPC used as the negative control.

that G418-resistance was increased over background by 30-fold for SB and 10-fold for Int when MAPC were allowed to form distinct colonies (Figure 3(a)) or when pooled drug resistant clones were subcultured to maintain the cell density between 100–500 cells/cm^2 (Figure 3(b)). These clones were expanded for further analysis including determination of genomic integration site (Figure 3(c)).

3.3. Transgenic MAPC Maintain the Ability to Differentiate. The effect of genetic engineering on the karyotype of MAPC was evaluated at the cytogenetic level for G418-resistant pooled clones that were maintained at a density of 100–500 cells/cm^2. The majority of the spreads (>80%) were diploid with a normal karyotype for unmanipulated rat cells as well as cells that had gone through random integration upon cotransfection with the luciferase expression vector or stable integration mediated by ϕC31integrase or SB transposase (Figure 4(a)).

MAPC are characterized by the capacity to differentiate into multiple cells types. Cells engineered for G418-resistance using the control Luc, SB transposase, or PhiC31 integrase were maintained in culture under reduced serum (2–5%) and in the presence of vascular endothelial growth factor (VEGF) or fibroblast growth factor 4 (FGF4) plus hepatocyte growth factor (HGF). Following 14 days of differentiation, distinct changes in the morphology of spindle-shaped MAPC were observed. No significant difference in the morphology between Luc, SB, and Int was apparent

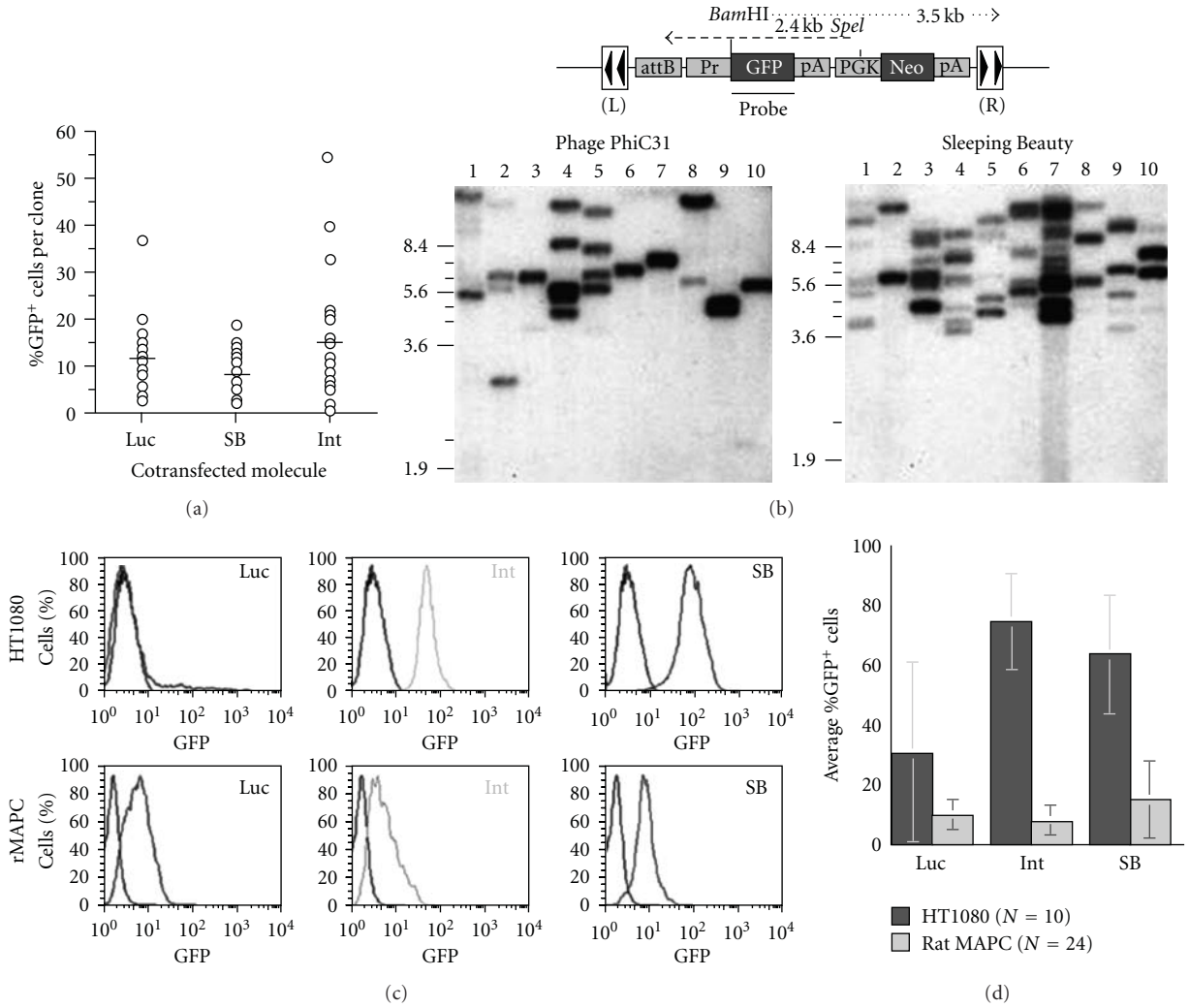

Figure 5: Stability of gene expression in MAPC. (a) Percentage GFP positive cells determined by flow cytometric analysis of 24 independent G418-resistant clones expanded from MAPC cotransfected with pKT2/NAG and Luc, SB, or Int expression vectors. Mean percentages of GFP+ cells for each condition are indicated by black bars. (b) Southern hybridization analysis of SB- and ϕC31-mediated integration events. Top shows a schematic representation of the 6709 bp circular plasmid nucleofected into rat MAPC and the location of the GFP probe. See legend of Figure 1 for explanation of sequence elements contained in the vector. *Spe*I and *Bam*HI restriction sites were used for digestion of high molecular weight genomic DNA isolated from each G418-resistant clone. The *Spe*I site is located 2.4 kb from the breakpoint in the *attB* site. The *Bam*HI site is located 3.5 kb from the right IR/DR. The bottom figure shows the hybridization image of genomic DNA digested with *Spe*I (ϕC31) and *Bam*HI (SB) hybridized with the GFP probe. Clone number is indicated across the top, and marker positions are indicated along the sides. (c) Flow cytometry of representative stable G418-resistant clones exhibiting GFP expression in HT1080 human fibroblasts and rat multipotent adult progenitor cells (rMAPCs) with integrating vector alone (left panel), integrating vector plus ϕC31 integrase (middle panel) and integrating vector plus SB transposase (right panel) compared to mock transfected cells of each type (black). (d) FACS analysis of individual clones was also measured and the average %GFP+ cells determined. Bar graph showing the average GFP% expression in HT12080 (dark bars) and rMAPC (light bars). Error bars represent standard deviation.

(Figure 4(b)). To further quantify and compare the differentiation levels between the different methods, both end point RT-PCR and qRT-PCR analyses of RNA isolated from these cells were positive for expression of endothelium (Flk-1, Flt1, and Eg1) or hepatocyte (HNF3b, AFP, and TTR) specific markers (Figure 4(c) and Table 1). These results demonstrate that MAPC genetically engineered using SB or ϕC31 maintain their stem-cell character and their ability to differentiate into multiple cellular lineages upon induction.

3.4. Strength of Gene Expression in MAPC. The strength of gene expression was determined for the GFP reporter. Twenty-four drug-resistant clones were randomly isolated for both SB and ϕC31 that had been expanded in G418-containing medium for an additional 3-4 weeks. The levels of GFP expression were determined for each independent clone by flow cytometry (Figure 5(a)). Although all of the clones were G418 resistant, GFP intensity of most clones was low with only a few percentage of the cells exhibiting

Table 1: qRTPCR analysis of differentiated rMAPC. Endothelium markers Flk1, Flt1, and Eg1 and liver markers HNF3b, AFP, and TTR were measured and Ct values normalized to GAPDH levels to determine ΔCt. Adult rat liver was used as the positive control and corresponding undifferentiated cells as negative control. Relative expression was carried out by determining ΔCt values and comparing expression levels to the positive control sample.

	FIk1			FIt1			Eg1		
	ΔCt	SD	Rel Exp	ΔCt	SD	Rel Exp	ΔCt	SD	Rel Exp
MAPC	20.13	4.95	0.00006	15.23	11.81	0.00071	4.46	0.7	0.23982
Luc	8.34	0.35	**0.22298**	2.42	0.69	**5.09824**	1.79	0.69	**1.52626**
Int	10.13	0.15	**0.0647**	2.61	0.34	**4.46915**	2.28	0.33	**1.08673**
SB	8.84	0.63	**0.15822**	2.46	0.06	**4.95883**	1.8	0.13	**1.51572**
Adult rLiver	6.18	1.23	**1**	4.77	0.45	**1**	2.4	0.1	**1**
	HNF3b			AFP			TTR		
	ΔCt	SD	Rel Exp	ΔCt	SD	Rel Exp	ΔCt	SD	Rel Exp
MAPC	3.27	0.21	1.38992	22.03	0.5	0	13.34	0.57	0
Luc	2.6	0.16	**2.21914**	16.42	0.32	**0.0002**	13.22	0.25	0
Int	2.51	0.14	**2.35381**	10.99	6.1	0.00867	3.95	1.96	**0.0003**
SB	1.78	0.35	**3.90413**	6.43	0.26	**0.20448**	1.95	0.2	**0.0012**
Adult rLiver	3.75	0.22	**1**	4.14	0.15	**1**	neg7.76	0.09	**1**

HNF3b: hepatocyte nuclear factor-3-beta; AFP: alpha-fetoprotein; TTR: transthyretin for liver differentiation, and Flk1: vascular endothelial growth factor receptor-2; Flt1: vascular endothelial growth factor receptor-1; Eg1: endothelial-derived gene-1 for differentiation into endothelium. Total RNA isolated from adult rat liver tissue (rLiver) was used as a positive control for liver and endothelial markers and undifferentiated MAPC (MAPC) used as the negative control.

expression above background. The clones were grouped into three categories based on percentage (%) of cells with GFP expression above background: low ≤5% GFP$^+$; intermediate: 10–20% GFP$^+$; and high ≥20% GFP$^+$. Using these criteria, we found that the majority of the G418-resistant clones contained a low or intermediate percentage of GFP-expressing cells, including 23/24 clones generated by random integration, 23/24 by PhiC31 integrase, and 18/24 by SB transposase, with only 1 of the SB-mediated clones exhibiting significantly higher levels of GFP expression.

To determine the effect of each recombinase on integrant copy number, we studied the average number of integrants per MAPC clone by Southern blot analysis. Genomic DNA isolated from 10 out of 24 randomly selected G418-resistant MAPC clones was digested with enzymes that cut once within the integrating vector sequence, generating GFP-hybridizing fragments of varying size for each integrant and providing an assessment of the number of stable insertions mediated by SB transposase or ϕC31 integrase (Figure 5(b)). The number of integrants among the SB-mediated stable clones was 6 ± 3, with one G418-resistant clone (number 7) showing at least 12 independent integrants. This value was 3-fold higher than previously described for SB10-mediated insertions in cultured human fibroblasts (1-2 transposon integrants per clone) [50, 56, 57]. Interestingly, human fibroblasts with 1-2 genomic integrants per clone exhibited stable gene expression, while MAPC with an increased number of integrants showed weak transgene expression. There were fewer integrants per clone in the case of ϕC31 integrase-mediated gene transfer (3 ± 1), likely resulting from the site-preferred character of this vector system.

Interestingly, transgene expression in the MAPC clones was significantly lower compared to clones obtained with the same construct in HT1080 (Figure 5(c)). Analysis of average GFP+ cells in individual clones obtained in HT1080 and rMAPC further confirm this observation (Figure 5(d)). These results suggest either low copy number integration, epigenetic suppression of the promoter-transgene elements [58], or integration at a genomic site that does not support active transcription.

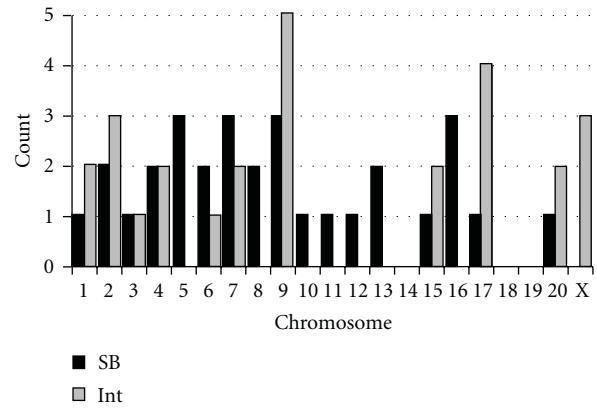

Figure 6: Chromosomal distribution of SB and ϕC31 integrants. Integrants with flanking sequence were recovered from the genome of MAPC by either linker-mediated PCR (SB; grey bars) or plasmid rescue (ϕC31; black bars); the frequency and distribution of independent events is displayed.

3.5. Molecular Analysis of Integration Sites in Isolated MAPC Clones. Several integrants were further characterized at the sequence level using either linker-mediated PCR (SB) or plasmid rescue (ϕC31) techniques (see Section 2.7). For SB,

Table 2: ϕC31 and SB chromosome junction sequences recovered from rat MAPC. Around 10 each of drug-resistant clones obtained with PhiC31 integrase and SB transposase were chosen at random to determine the site of genomic integration. Most clones showed more than one integration event, consistent with southern blot results. Exact genomic locations were determined by comparison of the chromosomal junction sequence with that of the rat genome.

(a)

	ϕC31 Integrase	
Clone	Chromosome band	Location
1	4q42	158791838
	9q22	51217205
2	2q44	236122145
	9q22	51217122
	15q11	58645720
	17q12.1	57593447
3	2q26	141365031
	3q21	51055488
	7q13	32562140
	15q12	66145915
	20q12	4538590
4	2q34s	199022700
	7q13	32562264
	9q22	51217198
	20q12	5244926
5	4q41	139277121
	6q12	11098254
	9q22	51217202
	Xq12	18543448
6	17q12.1	57594096
	Xq35	134407720
7	1q41	193493175
	17q12.1	57593343
	Xq35	134407720
8	1q41	193493175
	9q22	51217207
9	17q12.1	57593353

(b)

	SB Transposase	
Clone	Chromosome band	Location
1	2q33	171779390
	13q11	32001139
	13q11	39927168
	20q12	44445336
2	16p14	13906741
3	1p11	34991484
	7q32	94455924
	9q36	97344920
4	6q32	128228570
	8q24	59559434
	15q24	104110283
	16q12.1	57613884
5	5q35	128927150
	11q23	77290939

(b) Continued.

	SB Transposase	
Clone	Chromosome band	Location
6	3q35	102093005
	4q22	59940513
	4q23	71432455
	5q35	134761715
	16q12.2	60379119
7	6q13	23449543
	17q11	49437069
8	7q12	16815756
	8q24	77171774
	9q11	7186249
9	9q36	90439317
	12p12	2500679
10	2q11	6206693
	5q21	48519577
	7q13	28888887
	10q26	75143457

Table 3: Correlation of GFP expression and integration site for ϕC31 Integrase. Three clones were selected based on the expression level of GFP (high, medium, or low). Plasmid rescue was carried out to determine if the expression level correlated with integrant number or genomic location.

Clone	%GFP	Chromosome band	Position
Lo	<5%	5q12	18035560
	<5%	17q12.1	57593963
	<5%	Xq35	134407720
Med	10–20%	1q41	193493175
	10–20%	17q12.1	57594096
Hi	>20%	8q31	93775192
	>20%	10q31	87259340
	>20%	17q12.1	57594096
	>20%	Xq35	134407720

we recovered 30 unique transposon : chromosome junction sequences that mapped to positions on 17 independent chromosomes (Figure 6 and Table 2). We also obtained 27 distinct ϕC31 integrants that were distributed over 11 chromosomes (Figure 6 and Table 3). MAPC clones generated using SB exhibited a relatively random distribution of integration, while ϕC31 treated MAPC revealed an enrichment for specific sequences on chromosomes X, 9, and 17 (Tables 2 and 4). As these sites were represented in multiple independent clones, we decided to test three clones demonstrating high, medium, or low GFP expression with respect to integrant number and genomic location (Table 3). This analysis revealed that neither the number of integrants per clone nor the genomic location of the integrant was associated with GFP expression level. The clone exhibiting a higher percentage of GFP^+ cells had 4 integrants, while clones characterized as having medium or low levels of expression had 2 and 3 integrants each, respectively. Furthermore, the clone demonstrating increased expression of GFP (Hi) contained insertions on chromosomes X and 17 similar to the clone demonstrating reduced GFP expression

Table 4: Hotspots for ϕC31-mediated integration in the genome of rat MAPC. Nine drug-resistant clones and an additional three clones picked on the basis of GFP expression were analyzed for integrant number and genomic location. Of all the integration sites identified, the location on chromosome 17 (17q12.1) was found to be targeted with highest frequency (7 out of 12 clones) followed by chromosome locations 9q22 (5 out of 12 clones) and Xq35 (4 out of 12 clones). Exact genomic location of the integrant and the nearest gene to the site of integration is listed for each chromosome hotspot.

Chrom.	Clone	Chromosome band	Position	Nearest gene
17	2	17q12.1	57593447	
	6		57594096	
	7		57593343	
	9		57593353	ATI4B 59334046–59457079
	Lo		57593962	
	Med		57594096	
	Hi		57594096	
9	1	9q22	51217205	
	2		51217122	
	4		51217198	SDPR 47373044–47385061
	5		51217202	
	8		51217207	
X	6	Xq35	134407720	
	7		134407720	Xpnpep2 134474700–134501921
	Lo		134407720	
	Hi		134407720	

(Lo), while integration on chromosome 9 was not detected. Similarly, additionally analyzed clones with over 5 integrants per cell did not exhibit high levels of GFP expression. These data suggest that the number of integrants or the genomic location of integrants alone may not dictate persistence in expression of the transgene but a combination of these along with other epigenetic factors could play a key determining role.

4. Discussion

In this study, we used an internally controlled bifunctional plasmid to deliver expression cassettes encoding GFP or neomycin phosphotransferase along with both components of the ϕC31 and SB integrating systems into cultured human fibroblasts and rat multipotent adult progenitor cells (rMAPC). Both systems effectively mediated stable gene transfer resulting through recombinase-mediated integration of exogenous sequences. Genetically modified rMAPC maintained their stem cell features, demonstrated by the ability to differentiate into endothelium and liver tissues in the presence of specific cytokines. Silencing of GFP was observed for each system when G418-resistant rMAPC were expanded in culture. Southern blot analysis demonstrated that the number of integrants among the SB-transposed stable clones was 6 ± 3 while fewer independent integrants were detected for ϕC31 integrase engineered cell lines (3 ± 1).

Non-viral, DNA-mediated gene transfer has been explored as a means of potential gene therapy targeting a variety of different cell types *in vitro* and *in vivo*. One long-range goal is to test non-viral integrating vector systems for the capacity to mediate stable, DNA-mediated gene transfer in hematopoietic stem cells (HSC). Genetic engineering of HSC with the capacity to complete long-term repopulation of the hematopoietic system has previously been accomplished with the use of integrating viral vectors and has been reported using the SB transposon system [36, 37]. Unlike HSC, MAPC are capable of long-term self-renewal in culture and can be induced to differentiate with specific cytokines to form multiple cell types. These characteristics provide a unique resource for testing: (i) non-viral gene transfer in primitive stem cells, (ii) the effect of genetic engineering on maintenance of the stem cell character, (iii) stability of transgene expression following differentiation, and (iv) insertion site profiles on a genome wide scale, with the potential for functional screening in a primitive and differentiating cell system.

Therapeutic application of stem cells for the correction of genetic disorders will likely require long-term expression of newly introduced therapeutic genes, most effectively accomplished by integration of the transgene. However, along with the incorporation of exogenous elements into chromosomal DNA comes the potential for adverse effects resulting from insertional mutagenesis. Integration could lead to deregulated expression of tumor suppressor genes, oncogenes, or cell-cycle regulatory genes resulting in cancer [59–63]. As a result, genome-wide analyses of the insertion-site preferences for integrating vectors are being explored in a variety of target cells and tissues [60, 61, 64, 65].

SB transposons exhibit a relatively random integration pattern in mammalian genomes [66–68] and may be less likely to integrate into transcribed genes or transcriptional regulatory regions than other integrating vectors such as retroviruses and lentiviruses [66, 67, 69]. Having a comparable gene transfer efficiency, ϕC31 integrase displays preference for a seemingly limited number of insertion target sites in mammalian genomes, benefiting from sequence requirements of "pseudo *attP*" sites. In fact, analysis of several ϕC31-mediated integration events suggests that preferential sites for integration exist in both the mouse and human genomes consistent with at most 30–40% partial sequence homology to the wild-type phage *attP* target [20, 40, 42]. These data suggest that ϕC31 integrase-mediated DNA insertion is a relatively site-preferred approach for achieving persistent gene expression through integration.

A common limitation of viral or plasmid-based integration in undifferentiated cells is gene silencing that occurs during expansion or differentiation of the target population. This quenching of expression is likely caused by epigenetic effects such as methylation of transcriptional regulatory sequences or inhibition of transcription factor binding through chromatin condensation and genome remodeling.

Our results indicate that while SB transposase achieves multiple integrations per clone, ϕC31 integrase mediates an average of 2–4 integrations per clone. This is more than what has been reported for mouse and human cells (an average of 1 integration site per clone [42, 43]) and could either be due to the concentration of integrating plasmid used or the ratio of integrating plasmid to ϕC31 integrase. Alternately, rat cells in general or stem cells such as MAPC in particular might have a more open chromatin structure, thus exposing more "pseudo *attP*" sites for targeting by the PhiC31 integrase.

The only other study reporting successful use of PhiC31 integrase in rat cells was performed using the rat embryonic fibroblast cell line (Rat2) to first identify preferential sites of genomic integration. Based on these preferential sites, a PCR-based method was developed to characterize ϕC31-mediated integration events in retinal pigment epithelial cells following direct injection and subsequent *in vivo* electroporation [70]. From this methodology, three sites on chromosomes 12q16, 1q41, and 2q26 were determined to be preferential targets with each displaying a limited level of sequence homology to each other and with the ϕC31 *attP* site. Our results confirm the finding that preferential ϕC31 targets exist on 1q41 and 2q26 and extend this observation to identify three new targets which represented a higher frequency of integration events on chromosomes 17q12.1, 9q22, and Xq35. It is possible that these genomic hotspots for PhiC31 integration identified in rat MAPC are unique due to the nature of the chromatin in these undifferentiated cells.

5. Conclusions

We report here a relatively high efficiency of non-viral modification of MAPC using the SB transposon and the PhiC31 phage integrase, where SB can be used to achieve higher gene expression and PhiC31 for fewer integration sites but reduced expression levels. While both systems offer an alternative to viral methods of gene transfer into multipotent adult progenitor cells as well as other types of stem cells, still needed are comparative studies designed to characterize maintenance of gene expression after differentiation.

Acknowledgments

The authors thank the Masonic Cancer Center Cytogenetics Core facility at the University of Minnesota for performing cytogenetic analysis. This work was supported by grants from the Fanconi Anemia Research Foundation Grant (U. Lakshmipathy), Lymphoma and Leukemia society Translation Grant (U. Lakshmipathy), Arnold and Mabel Beckman Foundation (R. S. McIvor), NIGMS training Grant no. T32 GM08347, and University of Minnesota Doctoral Dissertation Fellowship (A. Wilber). A. Wilber and F. U. Montoya contributed equally to this work.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 425863, 15 pages
doi:10.4061/2011/425863

Review Article

Nuclear Reprogramming in Mouse Primordial Germ Cells: Epigenetic Contribution

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Received 2 March 2011; Accepted 11 July 2011

Academic Editor: Giorgio A. Presicce

The unique capability of germ cells to give rise to a new organism, allowing the transmission of primary genetic information from generation to generation, depends on their epigenetic reprogramming ability and underlying genomic totipotency. Recent studies have shown that genome-wide epigenetic modifications, referred to as "epigenetic reprogramming", occur during the development of the gamete precursors termed primordial germ cells (PGCs) in the embryo. This reprogramming is likely to be critical for the germ line development itself and necessary to erase the parental imprinting and setting the base for totipotency intrinsic to this cell lineage. The status of genome acquired during reprogramming and the associated expression of key pluripotency genes render PGCs susceptible to transform into pluripotent stem cells. This may occur *in vivo* under still undefined condition, and it is likely at the origin of the formation of germ cell tumors. The phenomenon appears to be reproduced under partly defined *in vitro* culture conditions, when PGCs are transformed into embryonic germ (EG) cells. In the present paper, I will try to summarize the contribution that epigenetic modifications give to nuclear reprogramming in mouse PGCs.

1. Introduction

Nuclear reprogramming is generally defined as the process reverting the nucleus of a differentiated cell to a pluripotent or totipotent state. The formation in culture of embryonic stem (ES) or epiblast embryonic stem (EpiES) cells from the inner cell mass (ICM) of the blastocyst or the epiblast of postgastrulating embryo, respectively, of embryonic germ (EG) cells from primordial germ cells and more recently of induced pluripotent stem (iPS) cells from differentiated somatic cells are examples of nuclear reprogramming *in vitro*. As far as I know, in mammals, physiological nuclear reprogramming leading to totipotency occurs only at the onset of embryogenesis when the genome of the zygote and subsequently of the early blastomeres acquires totipotency. This reprogramming requires a genome status of gametes that originates from early processes of nuclear reprogramming occurring at the beginning of gametogenesis in the primordial germ cells (PGCs), the embryonic precursors of the gametes. The identification of the timing and the underlying molecular mechanisms of this early process in PGCs offers precious information not only about gametogenesis and reproduction, but also on the secretes of stemness and clues for a number of pathologies including cancer development.

Nuclear reprogramming involves a variety of genetic and epigenetic modulators. This latter include DNA methylation and a variety of posttranslational histone modifications. The emerging small regulatory RNA molecules can be also regarded as epigenetic regulators but will not be discussed here (for reviews, see [1, 2]).

During the last decades, with the relevant contribution of the ideas and the inspiration of the late Anne McLaren, her work and that of her disciples, important advancements have been done on the basic principles and mechanisms governing nuclear reprogramming in PGCs, mainly in the mouse. The present review, is an attempt to summarize the emerging information relative to the contribution of epigenetic changes, in particular DNA methylation and histone modification, to nuclear reprogramming in mouse PGCs.

2. DNA Methylation

In mammals, methylation to the 5 position of the cytosine pyrimidine ring represents the major epigenetic modification of DNA (for reviews, see [3, 4]). It occurs predominantly in regions containing high frequency of the sequence cytosine phosphate guanine (CpG), termed CpG islands. Transcription repression is generally associated to methylation of CpG islands located into or near gene promoters (5′flanking region). In almost all cell types, tissue-specific genes generally undergo demethylation of CpG islands specifically in their tissue of expression. In contrast, housekeeping genes contain CpG islands unmethylated in all cells.

Mammalian genome encodes three DNA methylases (DNMTs): the maintenance methyltransferase DNMT1 and the *de novo* methyltransferases DNMT3a and DNMT3b. Moreover, DNMT3L, another member of the DNMT3 family, does not possess DNA methyltransferase activity, but it is required for DNMT3a and DNMT3b functions. Once imposed by *de novo* DNMTs, DNA methylation is transmitted to the cell progeny by DNMT1 as long as demethylation processes take place.

DNA methylation/demethylation can be divided into global (genome wide) and specific (when just specific DNA sequences are methylated/demethylated). While it is thought that global methylation state, termed methylome, is relatively stable in differentiated cells, dynamic changes of methylome occur during cell differentiation. Global demethylation occurs at two specific times during development, namely, at the onset of embryogenesis and during the PGC development and is associated to nuclear reprogramming; specific demethylation seems typical of somatic cells responding to particular signals.

DNA demethylation can be achieved passively by the failure of the maintenance methylation by DNMT1 during DNA synthesis at the S stage of the cell cycle or by the active removal of methyl groups from cytosine, independently of DNA replication. Active DNA demethylation can be achieved basically by (1) direct removal of methyl group, (2) the removal of entire DNA patch followed by filling with new nucleotides by nucleotide excision repair (NER), and (3) the removal of methylated base either by direct removal of methylcytosine, or through previous cytosine modification. This latter can occur for example by 5-meC deamination to produce thymine (T) or hydroxylation to produce 5-hydroxymethylcytosine (5-hmeC) followed by the removal of T by T/G mismatch or 5-hmeC by several ways and the insertion of unmethylated cytosine using base excision repair (BER) machinery (for a review, see [5]). As discussed later, in mammals, active DNA demethylation seems mostly to employ mechanisms referred in point (3).

3. Histone Modifications

Histone modifications provide an additional and complex source of epigenetic modification of the genome. Many enzymes that regulate histone modifications, mainly occurring in their amino terminal tail, have been identified. They include histone acetyltransferases (HATs), deacetylases (HDACs), methyltransferases (HMTs), and demethylases (HDMases). Phosphorylation, ADP ribosylation, sumoylation, and ubiquitination are other possible histone modifications, but they will not be discussed here since, as far as I know, there is no evidence about their involvement in PGC nuclear reprogramming.

Generally, the histones are acetylated and deacetylated on lysine (K) residues. These reactions are catalyzed by enzymes with histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity. In most cases, histone acetylation enhances gene transcription while histone deacetylation represses transcription. Histones may be methylated on either lysine (K) or arginine (R) residues by the addition of one, two, or three methyl groups. The process is catalyzed basically by three families of HMT enzymes: the protein arginine N-methyltransferase (PRMT) family, the lysine methyltransferases of the Su (var) 3–9, enhancer of zeste, trithorax (SET)-domain, or the disruptor of telomere silencing 1 (DOT1/DOT1L) protein families (for a review, see [6]). The regulative consequence of histone methylation on transcriptional activation or repression depends on the site and degree of methylation.

Among the best-characterized mediators of histone methylation are protein complexes of the polycomb (PcG) and trithorax (TRXG) groups containing a SET domain (for a review, see [7]). PcG and TRXG proteins form multimeric complexes that bind to DNA and direct post-translational histone modifications. They are critical regulators of gene expression, repressors (PcG), or activators (TRXG), necessary for cell fate specification and maintenance. PcG proteins catalyze preferentially two distinct histone modifications: trimethylation of lysine 27 of histone 3 (H3K27me3) by polycomb repressive complex 2 (PRC2) and mono-ubiquitination of lysine 119 H2A (H2AK119ub1) by PRC1. H3K27 is trimethylated by the enhancer of zeste 2 (EZH2), which is the catalytic subunit of PRC2 that includes noncatalytic subunits suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED). EEDs may also interact with HDACs and mediate repressive histone de-acetylation. Some TRXG proteins methylate histone H3 at lysine 4 (H3K4), a transcription permissive mark, while others posses demethylation or acetylation activities.

Elements of the histone code and some combinatorial pattern of histone marks are shown in Table 1. A particularly well-studied histone combination is the contemporary presence of the repressive H3K27me3 with the active H3K4me3, termed a "bivalent domain." Bivalent domains maintain genes in a state that is repressed but ready for activation and have been recently discovered in ES cells (see below). Genomic regions that are associated with gene silencing, including transposons and repetitive elements, frequently possess the heterochromatin marks H3K9me3 and H4K20me3.

Histone methylation was believed to be a quite stable modification. After the discovery of histone demethylases (HDMases), histone methylation is now considered

Table 1: Summary of the main histone code in mammalian cells.

Type of modification	Histone								
	H3K4	H3K9	H3K14	H3K27	H3K79	H4K20	H2BK5	H3K4 + H3K27	H3K9 + H4k20
Mono-methylation	Activation	Activation		Activation	Activation	Activation	Activation		
Dimethylation	Activation	Repression		Repression	Activation				
Trimethylation	Activation	Aepression		Repression	Repression			Bivalent	Heterochromatin
Acetylation		Activation	Activation						

a dynamic modification. Two kinds of histone lysine demethylases have been identified, including lysine specific demethylase 1 (LSD1) and Jumonji C (JmjC) domain family proteins. Peptidyl-arginine deiminase 4 (PAD4/PADI4) antagonizes methylation on arginine residues by converting mono-methyl arginine in histone H3 and H4 to citrulline.

4. Basic Principle/s of Nuclear Reprogramming in PGCs

Before entering into the specific themes of the present review, a brief description of the PGC development in the mouse embryo is needed. For more detailed information excellent reviews are available on this topic [8–12].

The PGC precursors (about 6 cells) are specified in the proximal epiblast in the posterior region of the embryo around 6.25 *days post coitum* (dpc) [13]. While proliferating, PGC precursors move through the posterior primitive streak into the extraembryonic mesoderm at the basis of allantois. Here, around 7.25 dpc, a founder population of about 40 PGCs is determined [14]. Thereafter, these PGCs migrate and enter the gonadal ridges from 10.5 dpc onwards. By 13.5 dpc, PGCs enter meiotic prophase in the female becoming primary oocytes and mitotic arrest in the male gonad becoming prospermatogonia. In the present review, we will refer as early or pregonadal PGCs between 7.5 and 10.5 dpc before entering into the gonads, and late PGCs between 10.5 and 13.5 dpc after arrival and colonization of the gonadal ridges.

Three basic principles govern nuclear reprogramming in PGC development: first, the need to inhibit somatic cell lineage pathways, second, the establishment of a transcription regulatory network necessary to maintain differentiation plasticity, and third, the resetting of the genome epigenetic status to eliminate epimutations and erase the parental imprinting. These conditions are likely to be interrelated and require both genetic and epigenetic modulators. Alltogether, they are intrinsic to the germ cell differentiation pattern itself and necessary for setting the germ cell genome towards totipotency.

The inhibition of all cell differentiation pathways, including the germ cell lineage, and the presence of transcription regulatory network for pluripotency are typical features of the embryonic stem (ES) cells, derived from the culture of the inner cell mass (ICM) of the blastocyst. In addition, ES cells posses self-renewal, the capability of dividing while maintaining their undifferentiated and pluripotent status for long periods. PGCs, however, at least in the mouse, do not possess a long-lasting self-renewal capability, are lineage determined and do not manifest pluripotency in conditions in which ES cells normally do (i.e., chimeras [15, 16]). This means that in PGCs, some stem cell characteristics coexist with specific differentiation pathways, a unique feature of the germ cell lineage.

Before their final differentiation into oocytes in females and prospermatogonia in males, in several mammalian species, including humans, PGCs can be induced to deviate their normal differentiation pathway and give rise to true stem cell lines similar to ES cells termed embryonic carcinoma (EC) cells *in vivo*, and embryonic germ (EG) cells *in vitro* (for a review, see [17]). This process super imposes a nuclear reprogramming on PGCs that basically inhibits their germline differentiation pattern, confers them the self renewal capability and allows their latent pluripotency to manifest in suitable environments. This process offers a formidable model to understand the origin of certain tumours from germ cells and perhaps from other cell types and interesting clues about important aspects of stemness that will be discussed in a separate section below.

As reported above, when PGCs finally differentiate into oocytes, a very specialized differentiation pathway centred on meiosis begins. During meiosis prophase and up to meiotic block at the diplotene stage around birth, the genome of the oocyte is organized in condensed chromosomes, and all players of the transcription regulatory network for pluripotency (i.e., *Oct4*, *Nanog*, and *Sox2* genes) are silenced. Thereafter, when the oocyte is included within a primordial follicle and meiosis is blocked, the genome remains relatively quiescent until the growing phase begins in selected primordial follicles. This signs the beginning of intense transcription activity and the reexpression of transcription factors typical of pluripotency (i.e., OCT4, SOX2). It is likely that during the oocyte growing phase, other genome changes complete nuclear reprogramming towards totipotency, but very little is known about. At the end of the maturation process, oocytes posses pluripotency as shown by their capability to give rise to teratomas, a kind of tumours composed of multiple cell types derived from one or more of the three germ layers [18].

In the male germ cell lineage, PGCs give rise to prospermatogonia, also called, gonocytes, which within the forming testicular cords of the fetal testis progressively undergo mitotic arrest. These cells remain quiescent until

after birth reenter a mitotic cell cycle. Through unknown processes, a subpopulation of gonocytes give rise to a true stem cell populations the spermatogonia stem cells (SSCs) able to self-renewal and to give rise to waves of proliferating spermatogonia that enter meiosis as spermatocytes. It is now clear, that SSCs not only posses self renewal but, like PGCs, have an intrinsic pluripotency. In fact, under certain culture conditions, different from those causing PGCs transformation into EG cells, SSCs obtained from prepubertal or adult testes can give rise to pluripotent stem cell lines termed germ-like stem cells (GSCs, [19]), multipotent adult germline stem cells (maGSCs, [20]), and multipotent adult spermatogonial-derived stem cells (MASCs, [21]). There is no information about the mechanisms responsible for the nuclear reprogramming of SSCs into such stem cell types.

5. Timing and Mechanisms of Nuclear Reprogramming in PGCs

During development, nuclear reprogramming in PGCs occurs at several steps: when their precursors are specified, during their determination, the migratory phase, and after their arrival into the gonadal ridges. It is not known if it takes place under the influence of the different microenvironments or if it occurs following the activation of an autonomous program or both. It is known, however, that it is associated to epigenetic changes involving wide progressive DNA demethylation and several histone modifications. Moreover, complex genome-wide transcription dynamics are strictly related to these epigenetic modifications.

It is to be pointed out that the study of epigenetic modifications in PGCs mainly during pregonadal stages is made difficult by the small numbers of available cells. For example, methods as immunoprecipitation of methylated DNA (MeDIP) and chromatin immunoprecipitation technologies (ChIP) cannot be used with pregonadal PGCs. Changes in DNA methylation in pregonadal PGCs have been so far analysed only by immunohistochemistry using antibodies against 5-meC. In late PGCs, both immunohistochemistry and bisulphite methods followed by PCR have been used.

5.1. DNA Demethylation in Pregonadal PGCs. Early studies indicated that mouse PGCs isolated from the gonadal ridges possess relatively under methylated DNA [22, 23]. It is now known that just after specification, PGCs have a relatively high genome methylation status similar to that of the surrounding epiblast cells and that during the subsequent stages, they undergo various rounds of passive and active DNA demethylation [24]. This means that PGC precursors do not escape the progressive *de novo* methylation of extraembryonic and embryonic lineages that follows the loss of genomic methylation occurred at the onset of embryogenesis between the zygote and blastocyst stages.

According to immunohistochemistry, a genome-wide DNA demethylation begins in a portion of PGCs at around 8 dpc, soon after their determination [24]. This seems to arise mostly passively. In fact, at this time, several conditions in PGCs favour passive demethylation. They are proliferating, and the expression of three main methyltransferases, the maintenance methyltransferase DNMT1, the *de novo* methyltransferase DNMT3a, and DNMT3b, is repressed [25, 26]. In addition, the expression of *Np95/Uhrf1*, one of the DNMT1's cofactors, is also repressed [27]. It appears that the key transcription factors governing the germ-cell specification, BLIMP1/PRDM1 and PRDM14 (for a review, see [9, 28, 29]), are directly or indirectly involved in this repression [27]. DNMT3L that at later stages will be essential for the establishment of the primary imprinting both in male and female germ cells and DNA methylation of transposons in meiotic male germ cells [30], is not expressed in PGCs [31].

Subsequently, between 8.5 and 9.5 dpc, when PGCs begin to migrate towards the gonadal ridges and become temporarily mitotic quiescent, demethylation extends to the most part of PGCs [25]. At this time, because mitotic quiescence and the DNMT1 reexpression, demethylation is likely to be mostly active [25, 27]. Accumulating evidence supports the possibility that active DNA demethylation in PGCs employs DNA repair-mediated pathways [32, 33]. Such evidence actually comes from studies carried out in gonadal PGCs (see below), and no information is available for pregonadal PGCs. In a recent review, Mochizuki and Matsui [34] speculated that the expression in pregonadal PGCs of the growth arrest and DNA-damage-inducible protein 45 (GADD45) [27] and the DNA deaminase (AID) [35], suggest their involvement in active DNA demethylation in such cells. In this model, it is assumed that deamination by AID converts 5-meC into thymine and gives rise to a T-G mismatch followed by the insertion of unmethylated cytosine using BER; GADD45 might recruit AID and the DNA glycosidase methyl-CpG-binding domain protein 4 (MBD4) to 5-meC [36].

In this context of passive and active DNA demethylation, imprinted genes, repetitive DNA elements including retrotransposons and satellite centromeric sequences and some germ-cell specific genes (i.e., *Vasa*, *Gcna1*, *Dazl*, *Scp3*), remain largely protected from demethylation. An important unresolved question is how such selective epigenetic modifications can occur. At the onset of embryogenesis, in early PGCs, DNMT1 and STELLA might be responsible for such protection [37, 38].

Which might be the function of DNA demethylation at these stages? The wide-genome demethylation starting in pregonadal PGCs might favor the maintenance of one of the major processes of PGC specification, the inhibition of differentiation pathway towards somatic cell lineages (for a review, see [9, 28, 29]). At the same time, demethylation might favour the expression of pluripotency-related and germ cell-specific genes occurring just after PGC specification. Many pluripotency-related genes (i.e., *Oct4*, *Nanog*, *Sox2*, *Rex1*, *Fbx15*) and some germ cell-specific genes (i.e., *Stella*, *Nanos3*) are expressed specifically in PGCs at fate determination [26, 27]. Hypomethylation of promoters characterizes the expression of three key pluripotency genes such as *Oct4*, *Nanog*, and *Sox2* in cell lines. Moreover, the suppression of *Oct4* and *Nanog* expression associated to hypermethylation of these CpG islands has been reported

in differentiating ES cells [39–41]. In the case of *Oct4*, *de novo* methylation of CpG islands mediated by DNMTs serves to stabilize the repression. In fact, during ES differentiation, the interaction of GCNF with the methyl-CpG-binding proteins MBD2 and MBD3 initiates *Oct4* repression. The lysine-9 trimethylation of histone H3 mediated by the G9a histone methyltransferase also contributes to such early repression [42–44]. Due to the limited cell numbers, no such information are available for PGCs. However, a recent report has showed CpG hypomethylation of the flanking region of *Oct4* during PGC-like cell induction from epiblast stem cells (EpiSCs) [45]. Similarly, the germ-like specific *Stella* and the pluripotency-related *Rex1* and *Fbx15* genes become hypomethylated in the CpGs of the flanking regions in PGC-like cells induced from EpiSCs in which hypermethylation of these CpG islands is associated to lack of expression of these genes [45].

Finally, demethylation events in female pregonadal PGCs might be involved in the initial reactivation of the inactive X. It is now known, that, contrary to the previous suggestions, reactivation of the inactive X already begins in nascent female PGCs and proceeds gradually [46]. The mechanism for X chromosome inactivation involves a nontranslated RNA transcript of the X-inactive specific transcript *(Xist)* gene located on the X chromosome inactivation centre (XIC) [47]. The control of *Xist* expression appears to be due to DNA methylation on the inactive X chromosome since the gene control region is unmethylated on the inactive X chromosome and methylated on the active X chromosome which does not express *Xist* [48]. X reactivation is accompanied by a progressive decrease of the *Xist* transcripts, reexpression of X-linked genes, and, quite surprisingly, by demethylation of the *Xist* promoter [49]. It seems, therefore, that reactivation of the inactive X chromosome is accompanied by epigenetic mechanisms other than *Xist* methylation.

5.2. DNA Demethylation in Gonadal PGCs. After 9.5 dpc, PGCs reenter the cell cycle and begin the colonization of the gonadal ridges. Around 13.5 dpc, in female, they enter meiosis as primary oocytes while in the male they undergo mitotic arrest in G2. During this period, PGCs undergo a further wave of DNA demethylation [24, 32]. The bulk of such demethylation takes place quite rapidly in about 24 h, mostly between 11.5 and 12.5 dpc, and despite the presence in the PGC nucleous of DNMT1. Thus suggesting that it mainly occurs by active demethylation. Such process preferentially affects single copy-imprinted, and nonimprinted genes, whereas demethylation of repetitive elements (especially of intracisternal A-particle (IAP) and long interspersed repeated (LINE-1) elements) is more protracted [49–51]. Genomic imprinting is a phenomenon by which certain genes are expressed in a parent-of-origin-specific manner. It is a typical epigenetic process that involves DNA methylation in order to achieve monoallelic gene expression (for a review, see [52, 53]). The erasure of imprinting in PGCs ensures the establishment of sex-specific new imprinting during the following stages of gametogenesis. On the other hand, the protracted demethylation of repetitive elements may be necessary to prevent dangerous transcriptional activation of the transposable elements, since this would increase the risk of germline mutations through deregulation of adjacent genes and through transposition.

When PGCs reach the gonadal ridges several, germline specific-genes are upregulated such as *Mvh*, *Dazl, Gcna1*, *Mageb4*, and *Scp3*. In *Dmnt1*-deficeint mice, however, premature expression of these genes occurs in pregonadal PGCs, indicating the possible importance of DNA demethylation for the expression of these genes [54].

Chromatin remodelling in the PGC nuclei seems another consequence of DNA demethylation. In fact, carefully observation by Hajkova et al. [55] showed that DNA demethylation precedes chromatin remodelling.

Recent studies have began to reveal the mechanisms possibly involved in the active demethylation in gonadal PGCs. Popp et al. [32], using unbiased sequencing of bisulphite-treated DNA by next generation sequencing (BS-Seq) method, investigated genome-wide DNA methylation in 13.5 dpc mouse PGCs. This analysis revealed that DNA from male and particularly female PGCs, was highly unmethylated (approximately 15% and 7% methylation of total DNA, resp.) compared to methylated DNA levels in sperm (about 85%), ES cells (about 80%), the foetus (about 75%), and placenta (about 45%). When this analysis was repeated with tissues obtained from mice depleted of the DNA deaminase (AID), it was found that while in most tissues examined, AID deficiency did not alter the level of DNA methylation; in PGCs a significant increase in methylation was observed (about 5% and 10%, resp.), thus, indicating a loss of DNA demethylation in these cells. In this model, deamination by AID converts C into T and gives rise to a glycosylase-media-ted T-G mismatch followed by the insertion of unmethylated cytosine by BER. These results support the notion that in PGCs active AID-mediated demethylation occurs but also that other factors are involved. Another study has shown that at the time of DNA demethylation in 11.5 dpc PGCs, there is an upregulation of transcripts of genes involved in BER, including *Parp1*, *Ape1*, and *Xrcc1*. This increase was asso-ciated to the presence in the PGC nuclei of XRCC1, a primary component of BER, together with PARP1 and APE1 [33]. The authors favour the possibility that BER is activated by the conversion of 5-mC to 5-hmC, for which the enzymes of the ten-eleven translocation (TET) family are responsible, followed by the excision of this latter by a specific glycosylase; *Tet1* expression was found indeed at high levels in 11.5 dpc PGCs [33].

The abundant presence of PARP1 and PAR polymers, a product of PARP1, in the PGC nuclei during DNA demethylation [33] (our unpublished observations) and the multifunctional actions of members of the PARP family on the genome (for a review, see [56]) suggest a role of this enzyme beyond than in BER. Surani and Hajkova [28] suggested that PARP1 might be responsible for the higher-order chromatin changes and the loss of chromocenters in PGCs. In addition, a direct involvement of PARP1 in DNA methylation is possible (for a review, see [57]). In this regard, high levels of PAR might inhibit DNMT1 during the about-five

cell cycles occurring in PGCs between 10.5 and 13.5 dpc [58], the period of the imprinting erasure.

5.3. Histone Modifications in Pregonadal PGCs. Using immunohistochemistry with antibody specific for methylated or acetylated histones, it has been shown that the staining patterns of the PGC precursors and PGCs around 7.5 dpc for the modifications H3K4me2, H3K4me3, H3K9Ac, H3K9me1, H3K9me2, H3K9me3, H3K27me2, and H3K27me3 were indistinguishable from those of their somatic neighbours. It is of note that the lineage-restricted PGC precursors in female embryos showed prominent accumulation of H3K27me3 in a single spot, most likely the inactive X chromosome [59, 60]. This suggests that the initiation of the X-inactivation process in the germline is similar to that in the somatic lineages. Chuva de Sousa Lopes et al. [61], using immunohistochemistry for H3K27me3 and an X-located green fluorescent protein (GFP)-carrying transgene, actually confirmed that BLIMP1-positive PGC precursors were subjected to X inactivation like their somatic neighbors.

Around 8.0 dpc, a number of global histone changes begin in PGCs. These include erasure of H3K9me2, a repressive mark with high stability, and upregulation H3K27me3, a repressive mark with apparent plasticity. The first modification occurs despite the presence of G9a (also known as EHMT2), a histone methyltransferase with a strong HMTase activity towards H3-K9. The absence of glucagon-like peptide-2 (GLP-2 or EHMT1) necessary to form the active G9a-GLP2 complex and/or competition with the activation mark H3K9ac probably prevent G9a action. On the other hand, the enhancement of H3K27me3 might be due to the action of the EZH2, a polycomb group enzyme [24, 25, 55]. Interestingly, Seki et al. [25] found that these histone changes occur progressively in migrating PGCs, most likely depending on their developmental maturation. Moreover, they observed that before or concomitant with the erasure of H3K9m2, PGCs enter G2 arrest, which continued until they acquire high levels of H3K27me3. Notably, although the overall levels of nuclear H3K27me3 were increasing, female PGCs gradually lose H3K27me3 mark on the silent X chromosome [61]. Following the onset of H3K9me2 erasure, PGCs show also repression of RNA polymerase II-dependent transcription that is gradually relieved after the release from the G2 arrest and the acquisition of high levels of H3K27me3. The precise significance of repression of RNAP II-dependent transcription and G2 arrest is currently unknown. Although both events seem to occur through mechanisms independent from the histone modification state of PGCs [25], they are likely to be necessary for their efficient epigenetic reprogramming.

Histone modifications in pregonadal PGCs include at least two other changes: the enrichment of H3K4me2 and H3K4me3, generally associated to actively transcribed euchromatin, and the symmetrical methylation of arginine 3 on histones H4 and H2A (H4/H2AR3me2s), a repressive mark conferred by a complex between the transcriptional repressor BlIMP1 and protein Arginine methyl transferase 5 (PRMT5). This latter mark is likely important for maintaining the PGC lineage during migration [62]. So far, only the DEAH (Asp-Glu-Ala-His) box polypeptide 38 (*Dhx38*) gene encoding the pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16 has been identified as target of this repressive mark [62]. Finally, another repressive mark H3K9m3 that specifically marks centromeric heterochromatin as well transposon and repetitive elements, is maintained relatively constant during this period up to 11.5 dpc. In female PGCs, the accumulation of repressive H3K27me3 gradually diminishes specifically on inactivated X [25]. This latter is accompanied by displacement of PcG repressor proteins EED and SUZ12 from the X despite the presence of high levels of these proteins in the nuclei of PGCs between 9.5 and 11.5 dpc [63]. As far as I know, no other information about the presence and role of PcG proteins in mammals PGCs are available.

These observations demonstrate that within euchromatin regions PGCs erase a repressive mechanism operated by H3K9me2 and replace it with H3K27me3 and H4/H2AR3me2s. The first modification may allow greater genome plasticity, the latter is possibly necessary to prevent differentiation towards somatic cell lineages. Transcriptionally permissive H3K4 methylations and H3K9ac are progressively increased. These marks might cooperate in events, discussed above, regarding DNA methylation, as the upregulation of pluripotency and germ cell-specific genes and might reflect the reprogramming of the PGC genome eventually necessary for totipotency at the onset of embryogenesis. Such chromatin status partly resembles that of ES cells and probably favours the transformation of PGCs into the pluripotent EG and EC cells (see below).

5.4. Histone Modifications in Gonadal PGCs. The first sign of histone modification in gonadal 11.5 dpc PGCs is a rapid loss of linker histone H1 accompanied by "loosening" of the chromatin and loss of chromocenters [55]. A concomitant downregulation of H3K9me3, H3K27me3, and H4/H2AR3me2s, and the disappearance or redistribution of factors are associated with facultative or constitutive heterochromatin, such as heterochromatin protein 1α (HP1α), HP1β, and HP1γ, the homologue of α-thalassaemia/mental retardation syndrome X-linked (ATRX) protein, and the polycomb-like protein M33 (also known as CBX2). Transcriptionally permissive H3K4 methylations and H3K9ac are also lost [24, 55]. Thus, it seems that the genome during PGC reprogramming undergos a phase of removing most epigenetic marks. It seems that this is achieved, at least in part, by histone replacement, potentially involving the histone chaperone and nucleosome assembly protein 1 (NAP1) [55].

In Figure 1, the main epigenetic changes accompanying mouse PGC development are schematically represented.

6. Comparing PGC and ES Cell Epigenetics

As reported above, some of the principles of nuclear reprogramming in pregonadal PGCs are common with those

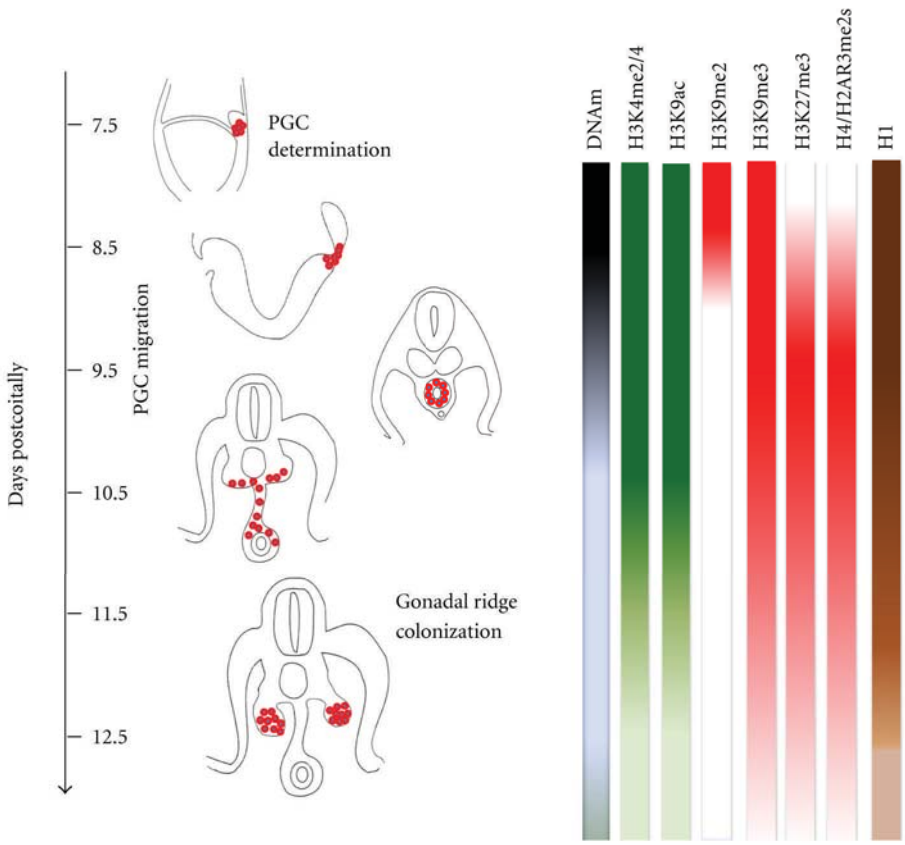

Figure 1: PGC development and associated epigenetic events in the mouse embryo. Changes in DNA methylation (DNAm) and histone modifications are represented by different shading intensity.

governing the formation and the maintenance of the undifferentiated state of ES cells, in particular the generalized inhibition state of somatic cell lineage pathways associated with the expression of key pluripotency genes. At the same time, however, PGCs show limited or absent self-renewal capability, and are committed to the germline lineage in which a unique DNA methylation resetting centred on the erasing of imprinting occurs. By considering the status of DNA methylation and the histone code in ES cells and how they are involved in the maintenance of pluripotency and self-renewal as well in the differentiation pathways of these cells, some clues about the contribution of these epigenetic changes and the underlying controlling mechanisms of the nuclear reprogramming in PGCs can be achieved.

Like somatic cells, ES cells show high global levels of DNA methylation, with about 60–80% of all CpG dinucleotides being methylated [64]. Although the global mCpG content is similar, the distribution of the mark is quite different from that of any other somatic cell type. In ES cells, promoters containing high level of CpG have low DNA methylation levels, whereas low CpG promoters are relatively high methylated [64–66]. CpG-rich promoters are almost invariably associated with the active H3K4me3 mark. Some of these promoters control constitutively expressed housekeeping genes, but others corresponding to developmental regulators also contain the repressive H3K27me3 mark. Methylated low CpG promoters are marked neither by H3K4me3 nor by H3K27me3 and are mostly repressed in ES cells [64–66]. Interestingly, the presence of two active XX chromosomes makes DNA globally hypomethylated both at repetitive and unique sequences [67].

From the information reported above on DNA methylation in PGCs, two main differences with ES cells are apparent. First, a progressive general demethylation was associated to PGC development in comparison to a more stable global methylation status of ES cells. Second, a DNA demethylation activity of PGCs was coupled to the erasure of the genome imprinting absent in ES cells. More detailed analyses of similarities and differences in DNA methylation have been performed only between ES cells and PGC-derived EG cell lines. Similarly to PGCs, EG cells derived from 11.5–12.5 dpc PGCs were shown to be grossly hypomethylated (mainly in EG cells with both active XX, [68]) and possess strong demethylation activity [69]. As for methylation of imprinted genes, EG cells derived soon after PGC specification (early EG cells) showed heterogeneous DNA methylation patterns in comparison with EG cells derived after PGC colonisation of the gonadal ridges (late EG cells) which were uniformly hypomethylated at these sites with the expected exception of *H19 locus* [68]. Direct comparison of the global methylation

of ES and EG cells performed with different methods and on different lines revealed distinct DNA methylation patterns at various CpG islands [70] while substantial similarity in the methylation patterns of promoters with intermediate level of CpG content (between 2% and 9%) in which most dynamics changes in methylation occur [71].

Taken together, these results indicate that cell pluripotency, except for hypomethylation of the promoter of key pluripotency genes, cannot be associated with a particular status of global DNA methylation. Methylation of different CpG sites may vary in pluripotent cells probably depending on the different characteristics of their pluripotency. In addition, in the germ cells, the dynamics of DNA methylation is unique because functional to other process discussed above such as the removal of epimutations and erasing of the parental imprinting.

All four DNMTs are highly expressed in ES cells. While DNMTs are essential for embryonic development [72], loss of DNA methylation by ablation of single or various DNMTs does not influence the ES cell viability and self-renewal but impairs their differentiation properties (for a review, see [73]). Demethylating agent, 5-azacytidine (5-AZA), was reported to reverse the differentiation status of ES cells forming embryoid bodies (EBs) back to undifferentiated ES cells [74]. Both *Oct4* and *Nanog* are devoid of CpG islands in promoter regions [75], whereas the promoter region of *Sox2* is CpG rich. The promoter regions of *Oct4* and *Nanog* are hypomethylated in ES cells but acquire significant methylation during cell differentiation process. However, the promoter region of *Sox2* gene is almost completely unmethylated in both ES cells and differentiated cells.

All together, these observations suggest that DNA methylation in ES cells is passively and actively maintained at high levels by DNMT1, but loci of key pluripotent genes are maintained unmethylated. DNA methylation is not sufficient to repress differentiation nor is crucial for self-renewal and pluripotency. Differentiation towards certain cell lineages requires that a portion of DNA methylation is maintained. None of these suggestions is incompatible with the wide-genome demethylation accompanying PGC development and in contrast with the principles of their nuclear reprogramming. Despite high DNA methylation level, ES cells show that an open chromatin structure and active chromatin domains are widespread [76].

The histone code is probably the main responsible for the unique genome state of ES cells. Analyses of genome-wide chromatin-state maps have recently shown that many genes in mouse ES cells, including several that are involved in differentiation, are characterized by an unusual combination of histone marks, termed the "bivalent domains." This includes H3K9ac and methylated H3K4, which are marks of active chromatin, and H3K27me3, which is typical of silent chromatin [77]. In most non stem cells, genes have either active or repressive marks, but not both. A similar epigenetic profile was also identified by others at genes encoding developmentally important transcription factors [78, 79]. These authors showed that loss of these epigenetic marks was correlated with differentiation and proposed that the presence of both active and repressive marks allowed differentiation-specific genes in ES cells to be repressed but also to be primed for activation when the right signals are received. Interestingly, about half of the identified bivalent domains in ES cells have binding sites for at least one of the three key pluripotency-associated transcription factors OCT4, NANOG, and SOX2.

As reported above, histone acetylation depends on the activities of several HATs or HDACs while histone methylation/demethylation is catalyzed by families of HMTs and HDMases, respectively.

In general, it appears that ES cell differentiation is accompanied by a global increase of histone acetylation except in specific loci of key pluripotent genes [80], followed by differentiation-specific deacetylation [81]. In line with this notion, increased level of acetylation, induced by the inhibition of HDACs by trichostatin (TSA), results in a rapid repression of pluripotent genes and the induction of differentiation-associated genes [82, 83]. A sustained inhibition of HDACs favours differentiation towards certain cell lineages such a cardiomyocytes and neurons [83].

Recently, a number of general global-wide analyses have identified large numbers of binding sites for the HMTs of the PcG family across the ES cell genome [84, 85]. The PcG proteins are essential for early mammalian embryo development [86–89], but not for maintaining ES cell pluripotency. PcG mutant ES cells can still self-renewal, maintain normal morphology, and express pluripotent genes [90–93]. In addition, although the PcG knockout ES cells do not differentiate efficiently into the three germ layers, they can still contribute to their formation [90, 92, 93]. However, loss of individual PRC proteins in ES cells results in increased expression of diverse lineage-associated genes and spontaneous differentiation [90, 92, 93], an effect that is more pronounced in ES cells carrying targeted deletions of both *Prc1* and *Prc2* genes [93]. Notably, the promoter regions that are occupied by PcG proteins in ES cells contain the "bivalent" H3K4me3 and H3K27me3 marks [76, 78]. These genes were also found to be generally transcriptionally silent. This suggests that PcG proteins help to maintain the silencing of these genes in undifferentiated ES cells. Because some results suggest that the repressive H3K27me3 mark can be heritably transmitted to daughter cells to maintain specific gene expression programs [94–96], the expression of developmentally regulated genes would necessitate the removal of the H3K27me3 mark. Demethylation of H3K27me3/me2 by the UTX and JMJD3 might be the mechanism by which PcG-repressed promoters are activated [96–98].

Alltogether, these results suggest that epigenetic regulation may be dispensable for maintaining ES cell identity. It appears that epigenetic mechanisms of gene silencing contribute to the overall stability of pluripotency but are downstream in this setting. ES cell characteristics might principally be regulated by transcription factors and intrinsic molecular pathways activated by exogenous signals. Epigenetic chromatin-based repressive and activating modifiers may serve transcriptional corepressor and coactivating functions in this process. On the other hand, the stability of a given cell state relies on the silencing of genes encoding

players of other cell states. In this context, the major ES cell transcription factors appear to activate both programs of self-renewal and pluripotency through an autoregulatory circuit. In some cases, they could possess also the ability to alter chromatin structure [99–102].

As in ES cells, the activity of key pluripotency transcription factors such as OCT4, NANOG, and SOX2 is likely crucial for nuclear reprogramming in the emerging PGCs. Before this, however, the first process governing nuclear reprogramming in PGCs seems exerted by the repressive action of the transcription factor BLIMP1/PRDM1 on somatic *Hox* genes [103]. This is closely followed by the expression of PRDM14 that together with BLIMP1 is critical for the activation of pluripotency genes [104]. In PGCs, these events, however, do not activate a long-lasting self-renewal capability. This likely requires the contemporary expression of other transcription factors (i.e., c-MYC, KLF4) and the activity of intracellular signalling pathways (i.e., STAT3) necessary for self-renewal. At the same time, in PGCs, the expression of genes and activities of molecular pathways specific of the germline could restrain the self-renewal circuit. BMPs, namely, BMP-4, BMP-2, and BMP-8b and probably adhesion molecules such as E-Cadherin and Fragilis are exogenous signals regulating such initial reprogramming (for review, see [8–12]). In this contest, as discussed above, epigenetic changes might favour and stabilize the germ cell differentiation programme. Moreover, in PGCs, they serve to begin the genome resetting for the sex-specific gamete imprinting and towards totipotency.

The first detectable epigenetic modification (the beginning of genome-wide DNA demethylation, erasure of H3K9me2, upregulation H3K27me3, H3K9ac, H3K4me2, and H3K4me3 and H4/H2AR3me2s), are seen around the period when PGCs are determined 7-8 dpc, suggesting that they exert as in ES cells, transcriptional corepressor, and coactivating functions. At least in part, these modifications appear to be directly or indirectly activated by the BLIMP1/PRMT5 complex and PRDM14 [27, 103, 104]. Interestingly, the pattern of histone marks established in pregonadal PGCs with the contemporary presence of H3K27me3 and H3K4 methylations and H3K9ac partly resembles the "bivalent domain" code of ES cells, thus, suggesting that this chromatin structure might represent the base for the maintenance of latent PGC pluripotency at this stage. How HATs or HDACs and HMTs and HDMases participate to the histone modifications in PGCs is not known. In fact, apart from the observations reported above that the progressive X reactivation in female PGCs is accompanied by displacement of PcG repressor proteins EED and SUZ12 and that high levels of these proteins are present in the nuclei of PGCs between 9.5 to 11.5 dpc [62], no other information about the presence and role of PcG proteins in mammals PGCs are yet available.

7. Reprogramming on Reprogramming: EG Cell Formation

During migration and for some time after the arrival into the gonadal ridges, PGCs from several mammalian species, including humans, can be induced *in vitro* to deviate from their normal differentiation pattern and transform or transdifferentiate into EG cells, cell lines showing self-renewal, and pluripotency characteristics very similar to ES cells (for a review, see [17]). After PGCs begin to differentiate into oocyte and prospermatogonia, around 12.5 dpc in the mouse, they lose this capability. As reported above, however, descendents of PGCs, the SSCs in male and the mature oocytes in female, show again the capability to give rise multipotent stem cells in culture and teratomas *in vivo*, respectively, indicating the presence of latent pluripotency like PGCs.

The formation of EG cells from PGCs means that a new nuclear reprogramming can be superimposed on the ongoing nuclear reprogramming until a certain developmental stages. This basically renders PGCs able to manifest their latent pluripotency and acquire self-renewal.

Early studies showed that a fraction of pregonadal mouse PGCs (around 2–20% of 8.5 dpc PGCs) transformed into EG cells when they were cultured *in vitro* onto cell monolayers in serum-supplemented medium and in continuous presence for 7–10 days of three exogenous growth factors, namely, the kit ligand (KL), leukemia inhibitory factors (LIFs), and basic fibroblast growth factors (bFGFs) [105, 106]. Each of the growth factors required for EG cell derivation activates unique signal transduction pathways partly overlapping in the downstream effectors. Several studies by us and others have established that KL and LIF act as antiapoptotic factors and comitogens to control PGC survival and proliferation whereas bFGF appeared mostly mitogenic for PGCs (for a review, see [107]). This latter notion was also supported by the observation that in the growth factors cocktail, bFGF can be replaced with potent PGC mitogens such as retinoic acid (RA) or agents that activate cAMP such as forskolin (FRSK) [108, 109]. More recently, it has been shown that bFGF and partly KL can be also substituted by hyperactivation of AKT [110], a potent survival and proliferative stimulus downstream phosphoinositide 3-kinase (PI3K). This can be activated by a number of growth factors including bFGF and KL. In line with this, EG cell colony formation was enhanced in PGCs depleted of phosphatase and tensin homolog (PTEN) which antagonizes the actions of PI3K [109, 111]. Thus, sustained PGC survival and proliferation in culture appear a prerequisite for EG cell formation.

At the same time, genetic and epigenetic events of nuclear reprogramming must take place. It has been proposed that following bFGF-binding upregulation of the FGFR-3 receptor and its translocation into the nucleus is a crucial event for inhibiting PGC differentiation PGCs and beginning their reprogramming in culture as EG cells [16]. Exposure of PGCs to bFGF for just the first 24 hours of culture is necessary and sufficient for EG cell formation providing that LIF is present in the medium after 1 day of culture [16, 31]. Since as reported above, bFGF can be substituted by RA, FRSK, or hyperactivated AKT, several signalling pathways in collaboration with LIF can induce the reprogramming of cultured PGCs.

A number of genetic events involved in such process have been identified. A key early event during the culture of PGCs

in the presence of bFGF is the downregulation of *Blimp1* and the consequent upregulation of its target genes *Dhx38*, *c-Myc*, and *Klf4* [31]. While *Dhx38* repression has been proposed to be necessary for maintaining early germ cells [62], the latter represents two of key transcription factors known to promote reprogramming of somatic cells to the pluripotent state of the iPS cells [112–115].

It has been proposed that the AKT promoting action on EG cell derivation is exerted through two targets: direct phosphorylation and inhibition of GSK-3 and inhibition of p53 activity mediated by GSK-3-dependent phosphorylation of MDM2, an E3 ubiquitin ligase for p53. In line with these, both inhibition of GSK-3 and absence of p53 favour the EG cell derivation [110, 116]. GSK-3 participates in various signaling pathways, such as WNT/β-catenin, Hedgeog proteins, Notch, and protein kinase A (PKA) signals. However, it seems unlikely that the activation of the WNT/β-catenin, usually a consequence of the GSK-3 inhibition, plays a role in nuclear reprogramming of PGCs in culture. In fact, the suppression of WNT/β-catenin signalling by GSK-3 appears to be necessary for normal PGC proliferation [117]. The p53 deficiency in the cultured PGCs, that only partly, however, substitute bFGF, might favour the acquisition of dedifferentiate state. Recent studies have identified the roles of p53 in suppressing pluripotency and cellular dedifferentiation. In this context, p53 suppresses the self-renewal of embryonic stem cells and blocks the reprogramming of somatic cells into iPSCs by inducing the expression of hundreds of genes, leading to cell cycle arrest, apoptosis, and senescence (for a review, see [118]). These new studies support the idea that molecules critically involved in genome stability function as not only guardians of the genome, but also barriers to pluripotency. It is interesting to note that, as reported above, in addition to p53 deficiency also that of PTEN, another important tumour suppressor, greatly facilitates EG cell derivation.

Whatever are the GSK-3 targets, its inhibition is alone not sufficient for PGC conversion. In fact, it requires the contemporary inhibition of the extracellular signal-regulated kinases (ERKs). Notably, EG can be obtained from 8.5 dpc PGCs in serum-free culture in 2i-LIF medium supplemented only with LIF and containing an inhibitor of GSK-3 and of the upstream kinase MAP ERK kinases (MEK1/2) [119]. Recently, it has been shown that the mouse ES cells can be self-renewal in basal medium if autocrine-activated ERK signalling is eliminated and GSK-3 activity reduced. Moreover, the 2i-LIF medium allowed efficient derivation and expansion of ES cells [119].

Finally, trichostatin (TSA), an inhibitor of HDACs not only replaced bFGF but it also accelerated and increased the efficiency of PGC reprogramming into EG cells [31]. This indicates that epigenetic modification is crucial for reprogramming PGCs in culture and suggests that it is the chromatin organization that prevents establishing the OCT4, NANOG, and SOX2 circuit of active pluripotency and prolonged self-renewal in such cells. These results are difficult to reconcile with a previous study by Maatouk and Resnick [120] in which the authors reported that under similar culture conditions TSA and 5-AZA as well accelerated the differentiation of 8.5 dpc PGCs as estimated by a more rapid downregulation of alkaline phosphatase and upregulation of GCNA1 after three days of culture. A possible explanation might be found in the fact that nuclear reprogramming leading to dedifferentiation actually occurs only in a subpopulation of PGCs, as reported above around 2–20% of 8.5 dpc PGCs, and/or that in the short period of culture analysed by Maatouk and Resnick [120], the drugs might induce temporary changes in the expression of some genes irrelevant for nuclear reprogramming.

In this context, it is likely that LIF action is necessary in the longer time for stabilizing and maintaining the full reprogramming process after the main reprogramming events are occurred. In fact, LIF is not required during day one of culture when bFGF or hyperactivated AKT exerted their action [16, 31, 108], but its presence is obligatory after this time in any EG cell derivation conditions. Notably, pregonadal PGCs do not express STAT3 ([121], our unpublished observation), the transcriptional activator that mediates the LIF-dependent self-renewal and maintenance of pluripotency in ES cells, but it appears after 4 days of culture [31], likely as a consequence of the previous nuclear reprogramming.

In Figure 2, the hypothetical multistep process of the nuclear reprogramming of PGCs in culture is shown.

8. Concluding Remarks

Nuclear reprogramming occurs progressively in PGCs during migration and gonadal ridge colonization. Epigenetic mechanisms of gene silencing and activation contribute to such nuclear reprogramming, but they are likely downstream genetic players. In this regard, PGC characteristics might principally be regulated by transcription factors whereas the most part of the epigenetic modifications are mainly associated with resetting the genome for subsequent parental imprinting on the gametes and regaining zygote/blastomeres totipotency. Nuclear reprogramming in nascent PGCs centred on BLIMP1/PRDM1- and PRMD14-dependent inhibition of somatic cell lineages and activation of core transcriptional circuits of pluripotency is activated by growth factors of the BMP family. Future studies will clarify whether epigenetic changes are also controlled by transcription factors and/or exogenous signals or occur by intrinsic regulators. Improvements of epigenomic analysis technologies are needed to identify the whole normal epigenomic setting of PGCs and epigenomic changes occurring in mutants and disease conditions. The possibility to apply single-cell gene expression analysis to PGCs (see, [103]) will probably facilitate such studies.

A further nuclear reprogramming can be superimposed on PGCs *in vivo* and *in vitro* leading to their transformation into pluripotent stem cells. Reprogramming of PGCs *in vitro* only requires the addition of exogenous growth factors and provides important insights into the molecular mechanisms regulating the stemness characteristics. The expression in PGCs of key pluripotency transcription factors such as OCT4, NANOG, and SOX2 is not sufficient to confer them

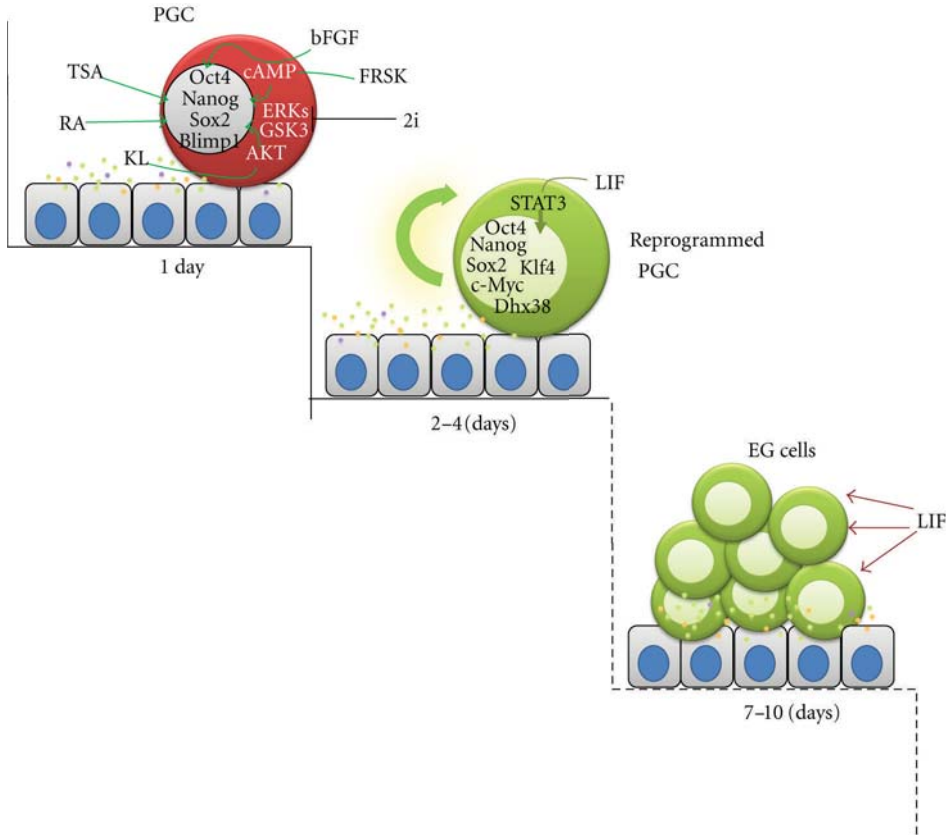

Figure 2: PGC transformation in EG cells. The process is schematically subdivided in to three main steps: rapid 1 day reprogramming processes involving TSA-induced epigenetic modifications and resetting of various gene and intracellular signalling pathways, a mid-time (2–4 days) establishment of a LIF/STAT3-dependent self-renewal circuit, and the formation of EG cell colonies requiring 7–10 days of culture. Various growth factors and compounds controlling each steps are reported; a cell monolayer producing KL and other not identified compounds is also represented.

a long lasting self-renewal capability and a manifest pluripotency. This might be a consequence of the lack of expression of other transcription factors (i.e., c-MYC, KLF4) and intracellular signalling pathways (i.e., STAT3) necessary for self-renewal. The derivation of EG cells *in vitro* from PGCs is actually triggered by downregulation of BLIMP1/PRDM1 and activation of *c-Myc* and *Klf4* as well the establishment of a LIF/STAT3 signalling. Interestingly, epigenetic modifications induced by a TSA-dependent DNA iper-acetylation or inhibition of ERK and GSK-3 pathways are able to replace the reprogramming action of growth factors on PGCs. Besides highlighting the crucial relevance of such processes for nuclear reprogramming, these results suggest that the development of the germline relies on the maintenance of a definite epigenetic state and the activity of certain intracellular pathways.

Acknowledgment

The author's research is supported by the Italian Ministry of the University and Research, Grant COFIN PRIN 2008.

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