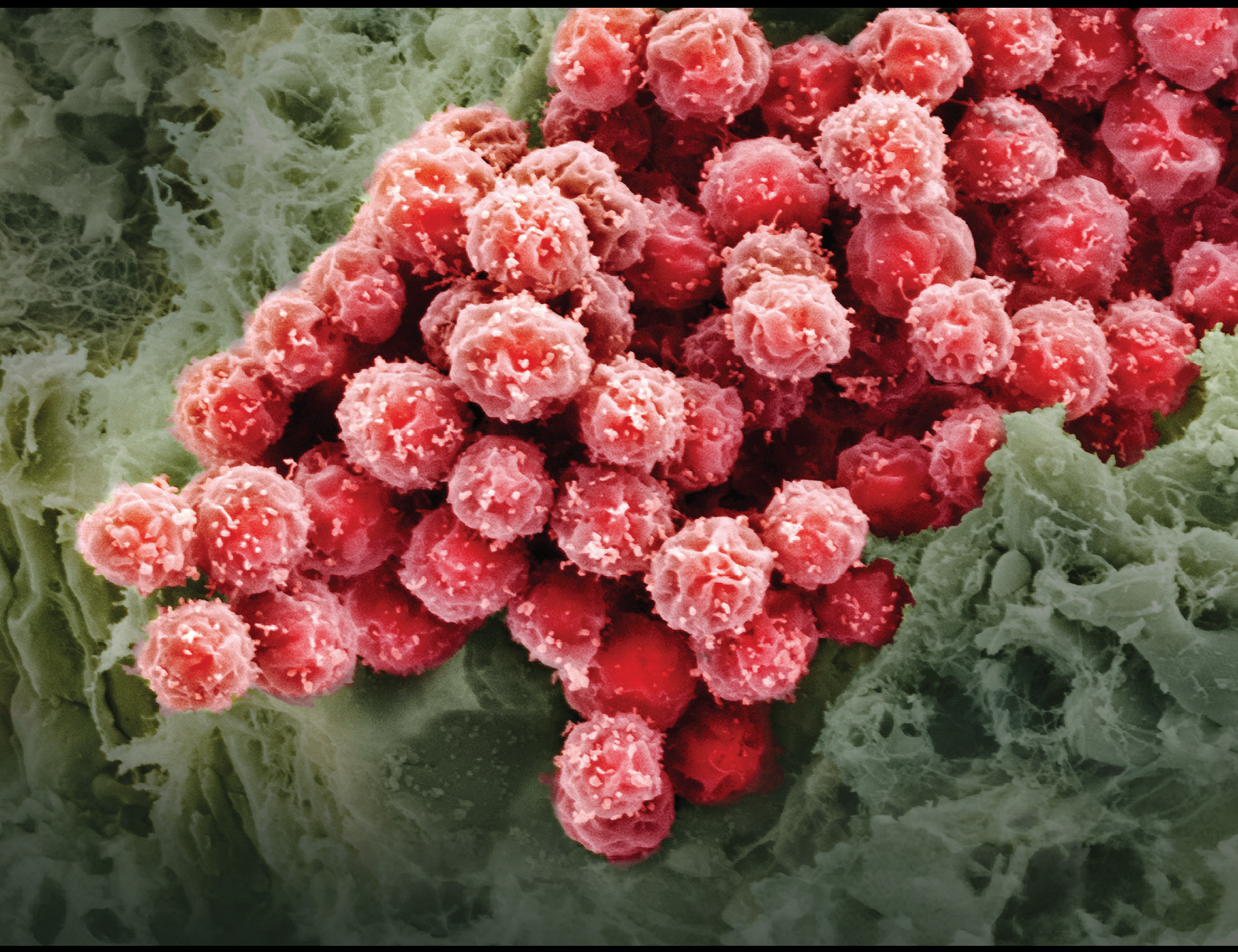
Stem Cells International

Mesenchymal Stromal Cells: Novel Methods for Characterization, Understanding Differentiation, and Function

Guest Editors: Vivek Tanavde, Mohan Vemuri, and Radhika Pochampally



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Editorial Board

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Editorial

Mesenchymal Stromal Cells: Novel Methods for Characterization, Understanding Differentiation, and Function

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MSCs are increasingly being used to treat a wide variety of disorders. These include diseases of the musculoskeletal system, wound healing, and vascular disorders. The tissue sources for MSCs are also growing. In addition to the traditional bone marrow and adipose tissue, many adult tissues have resident MSC populations that contribute to the renewal and replacement of cells in that tissue. Tissues like umbilical cord/Wharton's jelly, dental pulp, peripheral muscle, and so forth are also gaining acceptance as viable sources of MSCs.

The International Society for Cellular Therapy (ISCT) adopted a definition for MSCs in 2006. MSCs defined by these markers are not a homogenous cell population and exhibit tissue specific differences. Increasingly, MSCs are being used in a variety of veterinary applications using autologous transplantation. Therefore, there is an urgent need for complete characterization of the animal MSCs. Similar to human MSCs, animal MSCs clinical trials are hindered by lack of well-defined antibodies in different animal species for isolation and identification of those cells. Therefore, it is very important to develop new methods for MSC characterization for veterinary use. Thus, the challenges of comprehensively defining MSCs from different tissues and different species remain. The main hurdles to overcome for routine therapeutic use of MSCs have been in vitro expansion of cells (1) while retaining multipotentiality, (2) while maintaining the epigenetic and genetic stability of cells, and (3) while obtaining therapeutically effective numbers of cells efficiently.

Although MSCs have been reported to differentiate into a wide variety of different cell types in vitro, it has been a challenge to generate enough differentiated cells from MSCs for therapeutic applications. This is especially true since MSCs are differentiating into lineages other than bone, cartilage, or fat. Thus, it is important to generate protocols for the massive expansion of native MSCs that can be differentiated efficiently into different lineages or protocols that achieve massive expansion in cell number during differentiation into a specific lineage. The articles in this special issue address some of these challenges while providing some interesting solutions.

Transcriptome analysis of MSCs from different sources is increasingly revealing the heterogenous nature of MSCs derived from different sources. Although these MSCs are all based on surface marker expression, their gene expression profiles are quite different. The paper by J. Bosch et al. reports similar findings when comparing gene expression profiles of bone marrow versus cord blood MSCs. These results may have important implications in defining markers for MSC. It is possible that we need a different set of gene markers for MSCs based on their origin.

Genetic and epigenetic stability of MSCs during culture has been another concern during expansion of cells to obtain the high number of cells for therapeutic applications. The review article by A. Bentivegna et al. comprehensively summarizes the knowledge of genomic and epigenomic stability of MSCs during extended culture periods. The authors summarize that MSC genome is extremely stable during extended periods of culture, perhaps aided by methylation of specific gene promoters.

Another challenge for therapeutic use of MSCs is efficiently generating large numbers of MSCs for specific applications. In their article, P. Prasajak and W. Leeanansaksiri describe a highly efficient protocol for hepatocyte differentiation of Wharton's jelly derived MSCs in just 18 days. Using hypoxic conditions, the authors were able to achieve

80% efficiency of conversion of MSCs to hepatocytes which is quite remarkable. The review by C. M. Raynaud and A. Rafii further elaborates on the characterization necessary for clinical use of MSCs. The authors raise pertinent questions like what is the definition of cells being used for therapy, whether MSCs from different tissues are equivalent, what is the context in which MSCs should be used for clinical trials and so forth. It is important to answer these questions in a systematic manner before widespread use of MSCs in the clinic becomes accepted.

The last two articles in this issue focus on the use of surface markers in isolation of MSCs from different animal species. The review article by Y. Mabuchi et al. summarizes the marker expression of murine and human MSCs. They propose the use of PDGF-Rα and Sca-1 as markers for prospective isolation of murine MSCs. The article by C. Adamzyk et al. describes the effect of culture media on sheep MSCs. They conclude that variation in culture media combined with biological variation between different animals introduces huge variations in the gene expression profiles of MSCs. This makes it difficult to identify reliable biomarkers for sheep MSCs from gene expression data. Such variations in culture conditions may also have important implications for the downstream function of MSCs.

We hope these latest original research articles in the field of MSC research would promote and enable better understanding of the basic biology of MSC that is critical to the successful use of these cells in patient specific translational therapies.

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Review Article

DNA Methylation Changes during *In Vitro* Propagation of Human Mesenchymal Stem Cells: Implications for Their Genomic Stability?

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Mesenchymal stem cells (MSCs) hold great promise for the treatment of numerous diseases. A major problem for MSC therapeutic use is represented by the very low amount of MSCs which can be isolated from different tissues; thus *ex vivo* expansion is indispensable. Long-term culture, however, is associated with extensive morphological and functional changes of MSCs. In addition, the concern that they may accumulate stochastic mutations which lead the risk of malignant transformation still remains. Overall, the genome of human MSCs (hMSCs) appears to be apparently stable throughout culture, though transient clonal aneuploidies have been detected. Particular attention should be given to the use of low-oxygen environment in order to increase the proliferative capacity of hMSCs, since data on the effect of hypoxic culture conditions on genomic stability are few and contradictory. Furthermore, specific and reproducible epigenetic changes were acquired by hMSCs during *ex vivo* expansion, which may be connected and trigger all the biological changes observed. In this review we address current issues on long-term culture of hMSCs with a 360-degree view, starting from the genomic profiles and back, looking for an epigenetic interpretation of their genetic stability.

1. Properties of Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are multipotent adult stem cells with a great therapeutic potential in tissue engineering, regenerative medicine, autoimmune diseases, and pathologies characterized by chronic inflammatory processes [1, 2]. MSCs from bone marrow (BM-MSCs) are the best characterized adult stem cells but MSC-like populations have been isolated from several tissues such as adipose tissue, umbilical cord blood, skin, skeletal muscle, and also from dental tissues as dental pulp, exfoliated deciduous teeth, and periodontal ligament [3, 4]. Compared with other stem cell types, such as embryonic stem cells (ESCs) and neural stem cells, MSCs have several advantages and no ethical concerns limit their use. MSCs can be easily isolated, have a capacity for extensive proliferation and self-renewal, present a low risk of tumorigenicity, and can be used autologously. Moreover MSCs are considered immunoprivileged because they express low level of MHC-I molecules but not MHC-II and costimulatory molecules CD80, CD86, and CD40 [5]. The therapeutic effect of MSCs is mainly based on some key properties: (1) MSCs are able to differentiate not only into mesodermal lineages (osteogenic, adipogenic, and chondrogenic lineages) but also towards endodermal or ectodermal derivatives; (2) MSCs can exert strong anti-inflammatory and immunosuppressive effects; (3) MSCs can secrete many bioactive molecules affecting local cellular environment [6]. Finally, the capacity of MSCs to migrate preferentially to injured places, site of inflammation, and lymphoid organs allows different routes of administration [7].

A major problem for MSC therapeutic use is represented by the very low amount of MSCs which can be isolated from different tissues (e.g., in bone marrow the MSC population is 0.001–0.01% of the total cell number). To provide sufficient cell number for MSC clinical applications, after isolation an *in vitro* expansion phase is required. Differences in isolation

methods, culture conditions, and seeding density greatly affect stem cell yield and properties [3, 8]. Different parameters are evaluated to optimize MSC expansion such as culture surface substrates, oxygen tension, medium composition, pH condition, and substitution of serum with plated-rich plasma [9, 10]. Furthermore the 3D expansion of MSCs on microcarriers could represent an interesting alternative to the conventional 2D monolayer culture method [9, 11]. Regardless of the culture conditions it is crucial that during the *in vitro* expansion MSCs retain their peculiar properties unchanged and no genetic alterations occur.

2. hBM-MSCs: Really Stable at the Genomic Level?

Despite the clinical prospective of stem cell-based therapy, a few potential risks were recently described as the "risk profile" by Herberts et al. [12]. The hazard arises from the need of *in vitro* expansion and/or differentiation of human BM-MSCs (hBM-MSCs) before administration to a patient, and the malignant transformation is undoubtedly the more debated risk. In fact, the high proliferation rate in an artificial cell culture environment could favor the occurrence of genetic and epigenetic alterations. Since every cell division has a small chance of introducing deleterious mutations, it is generally known that chromosomal aberrations accumulate with age. In addition, numerous studies on tumour genotyping reported that genomic alteration is a hallmark of tumorigenesis [13, 14]. The main concerns are for autologous transplant applications in which the immune system is less efficient in eliminating potentially transformed cells. However, few publications reported spontaneous transformation of both adipose tissue and bone marrow-derived MSCs, after long-term *in vitro* culture expansion [15–17]. By contrast, other researchers supported the genomic stability of human MSCs (hMSCs) derived from different tissues [18–22]. On the other hand, genomic instability after long-term *in vitro* culture has been widely described in mouse and rat BM-MSCs [18, 19, 23–25], and it has been also associated with spontaneous malignant transformation [18, 19, 23, 24]. However, some reports on behalf of spontaneous transformation of hMSCs have been subsequently retracted from the same authors, since the results derived from contaminating tumor cell lines [26–28].

In this context, we had recently reported a general chromosomal stability of hBM-MSCs, though the occasional existence of transient clonal aneuploidies in two out of seven hMSCs samples [22]. In particular, in one case at least 52% of metaphases at passage 9 (P9) presented trisomy of chromosome 7; at P12 the same karyotype was found in 50% of the metaphases; moreover, in 11% of cells there was a loss of one chromosome X, so the total number of chromosomes was 46. In the second case, two equally represented subpopulations were evidenced at P4: a normal one, and a second with karyotype 49,XX,+5,+7,+9. However, for this sample, further analysis at later passages failed to reveal any clonal abnormalities, probably due to *in vitro* negative selection of the aneuploid clone. Moreover, a general stability of the genomic profile has been confirmed by array comparative genomic hybridization (a-CGH) analysis [22]. Similarly, Tarte et al. had revealed nonrandom aneuploidy in 5 of 20 hBM-MSC cultures, including recurring trisomy of chromosome 5 with occasional trisomy of chromosomes 8 and 20 [29]. Interestingly, 3 of 5 abnormal cultures were derived from the same donor, who provided two separate BM samples cultivated in either fetal calf serum and fibroblast growth factor or platelet lysate. These data suggest that recurring chromosomal alterations are not related to the specific culture conditions and could be donor-dependent. Once again, the abnormal karyotype did not persist on prolonged culturing demonstrating that all hBM-MSCs, with or without chromosomal alterations, showed progressive growth arrest and entered senescence without evidence of transformation either *in vitro* [22] or even *in vivo* [29]. Also Binato et al. demonstrated chromosome variability after passage 4 in nine cultures of hBM-MSCs using conventional cytogenetic analysis [30]. They showed that seven of the nine cultures presented random aneuploidy, but the abnormalities were lost by the next passage. Nevertheless, in one culture, a clonal abnormality was identified from passage 6 to passage 8. However, at the molecular level, changes were observed from passage 5 onwards, indicating initiation of differentiation, reduction in proliferation, and potential induction of senescence in all analysed samples, including even those with karyotypic abnormalities. Therefore, these genetic alterations are not associated with a selective growth advantage *in vitro*; indeed they conferred a growth disadvantage to abnormal cells, probably linked to DNA damage-associated senescence [31] or through a not yet well-defined internal mechanism of self-regulation.

In the literature a link between ageing/senescence and genomic stability is often reported [32], as well as between hypoxia and ageing/senescence [33], due to a compromised DNA repair gene activity. Furthermore, experimental data have indicated that hypoxia causes downregulation of DNA mismatch repair (MMR) genes and genomic instability in stem cells via specific epigenetic events [34]. For these reasons, we should not neglect the effects of hypoxia on long-term culture of hMSCs, although it is not the central focus of this review, addressing the topic on genomic stability. Whereas many authors agree that hypoxia enhances proliferation, inhibits senescence, and maintains stem cell properties of hMSCs [35–38], data on the effect of hypoxic culture conditions on genomic stability are few and contradictory. Some authors argue that hypoxic hMSCs maintains normal chromosome karyotype and intact genetic integrity [37], and others argue the exact opposite [39] claiming that amplification of hMSCs in a low-oxygen environment facilitated chromosomal instability via repeated cell division. In addition, a high frequency of detected chromosomal abnormality breakpoints corresponded to common fragile sites (CFSs), in analogy with tumorigenesis [40, 41]. Considering these conflicting data, the question is still open and particular attention should be given to the use of low-oxygen environment, through continuous monitoring of the chromosomal stability in addition to the proliferative capacity and differentiation of hMSCs. Finally, it should be remembered that the impact

of culture condition on epigenetic properties of pluripotent stem cells and preimplantation embryos, for example, has already been established [42].

3. How Measure Cell Ageing?

As anticipated in the previous section, culture expansion of hBM-MSCs is limited and after a certain number of cell divisions they enter a senescent state and ultimately stop proliferating. This phenomenon, the "Hayflick limit" [43], also known as replicative senescence, restricts the life span *in vitro* of all primary mammalian somatic cells. Senescent cells are mitotically arrested, and thus they are not dead and remain metabolically active. In this condition, the majority of cells acquire a characteristic large and flat fried egg morphology [22]. Since the first discovery of the "Hayflick limit" several studies have shown an inverse relationship between donor age and the replicative life span *in vitro* for MSCs [44, 45], proving that the age of an organism can have an influence on MSC proliferation. However, there is a high variation between different donor samples [22, 46].

It is hard to predict at which passage or number of cell divisions MSCs are approaching replicative senescence. First of all it would be necessary to identify a standardized system to track long-term culture [47]. Although many groups provide the number of passages as an indicator for cellular ageing, this approach is largely dependent on number of cells that have been seeded as well as confluence at the time of harvesting [48]. Population doublings (PDs) may provide a more accurate measure for cellular ageing and were calculated as quotient of the number of cells harvested divided by the number of cells that have been initially seeded [49]. However PDs do not include apoptosis or necrosis, which affect cell number. Nevertheless, despite standardized culture methods, there is considerable variation between different donor samples [48]. Even if the formulae can be modified to take into account the cell culture time, the population doubling time (PDT) still has the same limits [22]. So far, the only well-established method to quantify the amount of senescent cells is the senescence-associated β-galactosidase (SA-β-gal) staining [50]. Although this enzyme is active only in senescent hMSCs, unfortunately this staining does not facilitate absolute quantification of the senescent state.

In conclusion, there is no golden standard in the measurement of cell ageing and a more specific molecular marker would be necessary in order to grade the level of senescence of hMSC preparations.

4. Telomere Length and Differentiation Ability: Two Sides of the Same Coin

Telomeres consist of a repeated sequence located at each end of each chromosome. This repeated sequence is required for chromosomal stability and integrity, functions closely connected with for both cancer and ageing [51]. It has been proposed that the progressive shortening of the telomeres is the main trigger for replicative senescence, because it functions as an internal clock and the number of telomere repeats decreases at every cell division [48]. However, it is still being debated whether telomere shortening is really the initiating mechanism or whether it is instead an effect of replicative senescence [52–54]. Telomeric loss results in a variety of consequences such as inhibition of mitosis, genotoxic damage due to accumulation of free radicals, and chromosomal rearrangement, which may trigger a DNA damage response leading to senescence and cell apoptosis [55, 56]. The length of telomeric ends is controlled by telomerase, a ribonucleoprotein complex whose RNA and protein components were both essential for activity [57]. Telomerase is constituted by a catalytic unit with reverse-transcriptase activity (Tert) and RNA component (Terc) that serves as template for telomere extension [58]. Pluripotent cells, such as germ line cells, embryonic stem cells, and induced pluripotent stem cells, can bypass the barrier of senescence by telomerase expression. On the contrary, telomerase activity and hTERT transcripts were not expressed in cultured MSCs [20, 59], and progressive shortening of the telomeres has been demonstrated during *ex vivo* expansion of MSCs derived from human and non-human primate [22, 60–62]. On the other hand, the transformed hBM-MSCs described by Wang et al. exhibited telomerase activity [16]. Even though cancer cells have been shown to have increased levels of telomerase activity [63], constitutive expression of TERT by itself does not generate malignant conditions as it does not cause growth deregulation [64]. Thus, the barrier of senescence may be advantageous for hMSCs since it reduces the risk of oncogenic transformation upon prolonged *in vitro* culture [65]. On the other hand, the senescence may be disadvantageous for hMSCs since it may impair their differentiation capability. Indeed, long-term culture has a significant impact on differentiation capacity of hMSCs, especially towards adipogenic lineage [46, 60, 66–68].

It has been recently demonstrated that ectopic expression of telomerase can immortalize hMSCs maintaining the differentiation potential *in vitro* toward the osteoblastic and adipogenic lineages [69, 70]. The generation of a hMSC line expressing TERT that exhibits enhanced cell proliferation and stability in cell culture could be a new strategy for both basic and applied tissue engineering studies of bone development and repair [70]. Finally, the progressive shortening of the telomeres seems to be a self-regulating mechanism able to reduce the risk of oncogenic transformation of MSCs in culture, since it can limit expansion of potentially malignant cells.

5. Epigenetic Program and Differentiation Potential in hMSCs

Gene expression potential in stem cell renewal and differentiation is regulated by epigenetic mechanisms that alter the transcriptional permissiveness of chromatin, of which DNA methylation (DNAm) is the best characterized component [71]. DNAm consists in the addition of a methyl group to the carbon 5 of the cytosine into CpG contexts and it is involved in development and cellular differentiation [72]. However, DNAm does not work alone, since histone

modifications and noncoding RNA regulation collaborate in controlling chromatin plasticity. It is commonly accepted that DNAm silences gene expression. Actually, gene expression depends on promoter CpG content, with methylated high-CpG content promoters being usually inactive, while methylated low-CpG content promoters can be active or inactive [73]. Thus, the "open" chromatin, that is, "global DNA hypomethylation" and abundance of transcriptionally active chromatin marks, such as trimethylated H3K4 (H3K4me3) and acetylation of histone H4, correlates with the ability to activate a wide range of cell type-specific genes during the differentiation programs [71]. The maintenance of the pluripotency state in ESCs is given by development-associated transcription factors, such as *OCT4*, *NANOG*, and *SOX2*, which activate genes of self-renewal at their unmethylated promoters [74]. Differentiation of ESCs is due to methylation of these pluripotency genes such as *OCT4*, determining their downregulation [75]. MSC epigenetic profiles reflect a more limited differentiation potential as compared to ESCs (that is why MSCs are better classified as multipotent than pluripotent), but numerous epigenetic modifications occur concomitantly during both osteogenic and adipogenic differentiation [76]. In adipose tissue stem cells (ASCs) and BM-MSCs *OCT4* is silenced by promoter hypermethylation, whereas *NANOG* and *SOX2* are unmethylated despite the repressed state of the genes [77], indicating the implications of other chromatin-based mechanisms, such as post-translational histone modifications. Epigenetic studies from the laboratory of Collas suggested a model of epigenetic commitment or preprogramming of MSCs toward particular lineages. They affirmed that post-translational histone modifications on promoters contribute to establishing a permissive state of differentiation but cannot predict transcriptional activation outcome [78]. In this issue several studies evidenced the role of histone H3K9Ac and H3K9Me2 modifications (associated to gene activation and gene silencing, resp.) in regulation of MSC fate commitment and ultimately predict cell fate. Tan et al. identified several differentially expressed genes regulated by acetylation of H3K9 (H3K9Ac) and/or dimethylation of H3K9 (H3K9Me2), implicating their role in hMSC osteogenic differentiation [79]. Similarly, Li et al. showed the role of histone H3 acetylation in regulating MSC ageing and spontaneous osteogenic differentiation [80]. Interestingly, they demonstrated that the basic fibroblast growth factor (bFGF) promoted MSC proliferation and suppressed its spontaneous osteogenic differentiation, modulating histone H3 acetylation in the *OCT4* gene. Very recently, Wang et al. showed that low concentrations of trichostatin A (TSA), a histone deacetylase inhibitor, prevented the spontaneous differentiation of human umbilical cord MSCs during long-term culturing, delaying their ageing [81]. In conclusion, the crucial role of post-translational histone modifications in regulating the differentiation potential of MSCs provides a system for their selective manipulation in order to hinder their ageing *in vitro*.

6. Epigenomic Modifications during hBM-MSCs *In Vitro* Expansion: Random Fluctuations or Thin Autoregulation?

More specific studies have recently addressed the relationship between epigenetic changes acquired during culture of MSCs and their functional changes. Wagner et al. speculated that replicative senescence and ageing might be regulated by similar mechanisms [82]. DNAm patterns were overlapping and maintained throughout both long-term culture and ageing, and highly significant differences were observed only at specific CpG sites, associated with promoter regions, especially in homeobox genes and genes involved in cell differentiation [83]. In this context, the group of Wagner defined the "Epigenetic-Senescence-Signature" as the senescence-associated DNAm (SA-DNAm) changes, which were related to age-associated modifications in MSCs from young versus elderly donors and could be used to monitor senescence for quality control [84]. Schellenberg et al. [85], analyzing functional, genetic, and epigenetic sequels of long-term culture of hMSCs demonstrated that the DNAm profiles differed markedly in MSCs from adipose and bone marrow, also confirming the data on gene expression profiles of other studies [86, 87]. Furthermore, Schellenberg et al. evidenced that senescence-associated hypermethylation and hypomethylation were often localized to regions with repressive histone marks, such as abundance of H3K9me3, H3K27me3, and targets of the histone methyltransferase EZH2 [85]. Interestingly, EZH2, a component of the polycomb-repressive complex 2 (PRC2), has previously been implicated in replicative senescence: its levels are downregulated in senescent cells, so the program of differentiation is permitted [88]. On the contrary, EZH2 overexpression is associated with several cancers [89, 90]. Furthermore, it was found that genes which are targets of the polycomb group proteins (PCG) undergo hypermethylation with age, hindering cell differentiation [91]. Thus, age may contribute to carcinogenesis by irreversibly silencing genes that are suppressed in stem cells and by stabilizing stem cell features.

Two recent studies compared the methylation profiles during *in vitro* expansion of MSCs. Choi et al. [92], comparing differential methylation patterns between early and late passages of hBM-MSCs, evidenced that hypermethylation increases at genes related to DNA replication, cell cycle, and adipogenic differentiation, due to long-term culture.

In our study [22] we performed a gene ontology (GO) analysis on genes with a change in the methylation status from early to late passages in hBM-MSCs. We identified several correlations between the functional changes and the change of methylation profiles of hBM-MSCs both acquired during culture. As an example, the categories "cell signaling" and "apoptosis and cell death", including genes that have essential functions for the viability and functionality of MSCs, were unmethylated at early passages and remained so even at late passages; thus, they should not be turned off. Among the "methylated gene promoters", which could be inactivated with increasing passages, we found several metabolic processes such as genes for lipid and fatty acid metabolic process.

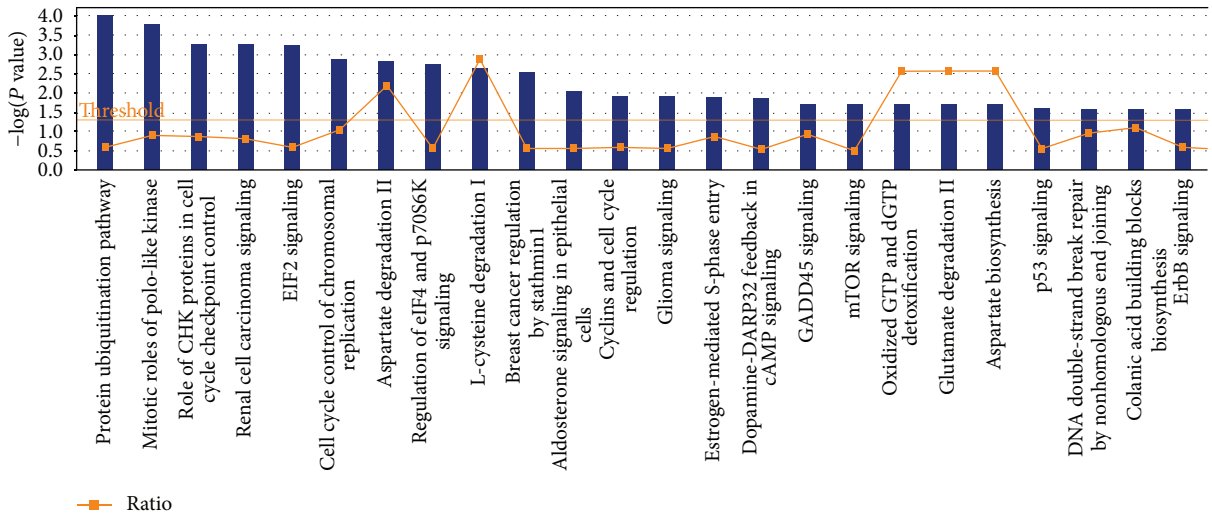

Figure 1: This graph shows the most statistically significant pathways involved in methylation variations of gene promoters between early and late passages of hBM-MSCs, obtained by Ingenuity Pathway Analysis (IPA). Blue bars indicate −log(*P* value), while the orange squares indicate the ratio of input list genes that map to the considered pathway divided by the total number of genes involved in this specific pathway.

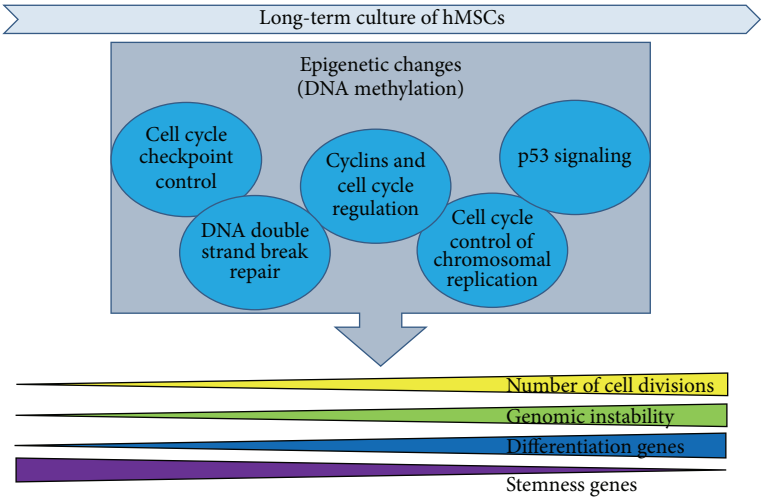

Figure 2: Epigenetic changes (in particular DNA methylation) at specific gene promoters during long-term culture of hMSCs may regulate processes like senescence and proliferation but also genomic stability. Many statistically significant pathways involved in methylation variations between early and late passages of Figure 1 concern cell cycle regulation, DNA repair, and cancer (light-blue circles).

These data well correlate with the decreased adipogenic differentiation potential during long-term culture. Furthermore, we analyzed our DNA methylation data by Ingenuity Pathway Analysis (IPA). IPA software examines functional relationship within an input list of genes and identified which pathways, from the IPA library of canonical pathways, are most significantly associated to the data set [93]. In our study, the input list was represented by genes whose promoters modify their methylation status between early and late passages (unpublished data). The most statistically significant pathways involved in methylation variations of gene promoters between early and late passages of culture are shown in Figure 1, and among these numerous concern cell cycle regulation, DNA repair, metabolism, and cancer.

7. Functional Consequences in hMSCs during Long Term-Culture: Everything Changes around, but an Apparent Genomic Stability Remains

Long-term *in vitro* expansion alters the biology of adult MSCs and induces tightly regulated epigenetic modifications. However, the genome of hMSCs appears to be relatively stable and so far malignant transformation upon hMSC transplantation has not been observed in clinical trials [29]. One might speculate that the genomic stability is somehow guaranteed in hMSCs during *in vitro* long-term culture. As mentioned above, the abnormal karyotype generally did not persist on prolonged culturing, probably due to DNA

damage-associated senescence. Epigenetic changes might therefore antagonize some genetic alterations arisen during long-term culture of hMSCs.

Izadpanah et al. have provided data supporting this hypothesis, analyzing the transcriptome of both ASCs and BM-MSCs, at early and late passages, in human and rhesus macaque [94]. All MSCs have altered cell cycle progression, resulting in both cellular crisis and senescence. In addition, hMSCs underwent an increase in the frequency of cells in the S phase at P20 and higher. However, extended culture of hMSCs failed to reveal any chromosomal alterations, whereas all rhesus MSCs (rMSCs) displayed an aneuploidy karyotype. Gene ontology analysis indicated that genes involved in protein metabolism, protein catabolism, and regulation of pol II transcription were over-represented in rASCs, whereas those involved in the regulation of cell cycle and regulation of IκB/nuclear factor-κB (NFκB) cascade were over-represented in hBM-MSCs. These data showed a correlation between the observed differences in karyotype changes and gene expression changes between rMSCs and hMSCs. Thus, hMSCs during *in vitro* expansion could trigger a specific program in order to protect the integrity of the genome, preventing genetic instability via arrest in S phase and involving p53 and NFκB pathways, both expressed in hBM-MSCs but not in rASCs [94]. Similarly, our IPA analysis of methylation changes of gene promoters between early and late passages of hBM-MSC cultures evidenced the implication of several anticancer pathways, suggesting that the genomic stability observed in hBM-MSCs during long-term culture may be determined by the methylation changes at specific gene promoters (Figure 2).

8. Conclusion

The intimate correlation between DNA methylation, stem cell renewal and differentiation and between stem cell culture condition, genomic instability, and cell proliferation is now evident. The study of the mechanisms for the genomic integrity maintenance could be useful not only for standardization and safety of hMSCs for therapeutic applications but also for cancer prevention, risk prediction, detection, prognosis, and therapy.

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Research Article

Different Culture Media Affect Proliferation, Surface Epitope Expression, and Differentiation of Ovine MSC

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Orthopedic implants including engineered bone tissue are commonly tested in sheep. To avoid rejection of heterologous or xenogeneic cells, autologous cells are preferably used, that is, ovine mesenchymal stem cells (oMSC). Unlike human MSC, ovine MSC are not well studied regarding isolation, expansion, and characterization. Here we investigated the impact of culture media composition on growth characteristics, differentiation, and surface antigen expression of oMSC. The culture media varied in fetal calf serum (FCS) content and in the addition of supplements and/or additional epidermal growth factor (EGF). We found that FCS strongly influenced oMSC proliferation and that specific combinations of supplemental factors (MCDB-201, ITS-plus, dexamethasone, and L-ascorbic acid) determined the expression of surface epitopes. We compared two published protocols for oMSC differentiation towards the osteogenic, adipogenic, and chondrogenic fate and found (i) considerable donor to donor variations, (ii) protocol-dependent variations, and (iii) variations resulting from the preculture medium composition. Our results indicate that the isolation and culture of oMSC in different growth media are highly variable regarding oMSC phenotype and behaviour. Furthermore, variations from donor to donor critically influence growth rate, surface marker expression, and differentiation.

1. Introduction

Mesenchymal stem cells (MSC) are a well characterized and highly adaptable cell source for regenerative medicine and tissue engineering. In 1968, Friedenstein and coworkers described for the first time that plating bone marrow cells in serum-containing medium results in the formation of colonies of fibroblast-like adherent cells, capable to differentiate into osteoblasts [1–3]. Today, the role of MSC in clinical applications has been explored in phase I/II clinical trials involving, for example, autoimmune disorders, treatment of acute graft versus host diease (GvHD), and engraftment of hematopoietic cells [4, 5]. Other applications of MSC, especially in combination with biomaterials for the repair of damaged tissues like cartilage and bone, have raised cautious optimism for future therapies. However, novel biomaterials designed for clinical applications are usually tested in several preclinical studies involving animal models. Sheep are a convenient large-animal model for orthopedic research because of their availability, ease of handling and housing, cost, and ethical acceptance [6, 7]. In particular, mature sheep are considered as a valuable model for human bone turnover and remodeling activity, due to the fact that animals of 7–9 years of age show similar bone structure and composition.

Furthermore, they possess a body weight comparable to that of adult humans and long bone dimensions enabling the use of human implants [6, 8–10]. Therefore, in orthopaedic research, sheep are frequently employed for critical-size bone defects, which are then treated with different biomaterials combined with (predifferentiated) MSC [8, 9, 11–17]. Despite a considerable number of reports employing oMSC in tissue engineering, characterization of ovine oMSC or proof of multipotency *in vitro* is limited to few studies only [18–23]. In these studies, there is widespread variation in the composition of growth and differentiation media, as well as for analyzing the expression of surface antigens. Growth media for oMSC typically are based on α-MEM, DMEM, or DMEM combined with MCDB201. The serum content varies between 2% [24] and 20% [18, 23] of autologous or fetal calf serum (FCS), and other supplements such as ITS-Plus premix, dexamethasone, L-ascorbic acid, or growth factors like epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) may be applied [24]. As a consequence, considerable variances in the cultured oMSC population, and their behaviour in tissue engineering applications are presumed.

For human MSC (hMSC), systematic research has uncovered donor-dependent heterogeneity in proliferation and differentiation [25–28]. Because of varieties in characterization, nomenclature (e.g., bone marrow stromal cells, multipotent adult progenitor cells, and mesenchymal stem cells), and culture media, the *Mesenchymal and Tissue Stem Cell Committee* of the International Society for Cellular Therapy proposed the introduction of a standardization for the phenotypic characterization of hMSC, as stated in their position papers of Horwitz et al. in 2005 [29] and Dominici et al. (2006) [30]. Despite large donor-dependent variations, the differentiation of hMSC towards adipocytes, osteoblasts, and chondrocytes is nowadays standardized according to protocols and media originally published by Pittenger in 1999 [31]. In contrast, no standardized protocols for the characterization and differentiation of oMSC are available so far.

Here we undertook a systematic study of oMSC cultured in eight different growth media of defined composition. The first medium that was tested is one described several times for the culture of oMSC [21, 22, 32, 33] and consisted of DMEM low glucose containing 10% FCS. The second medium is often used for hMSC culture [34–36] and contained only 2% FCS, but MCDB-201, ITS-Plus, dexamethasone, L-ascorbic acid (summarized abbreviated in the following as "S" for supplements) and 10 ng/mL epidermal growth factor (EGF). Results for differentiation after preculture in the two media were irreproducible and varied strongly for each donor. Furthermore, lots of lipid droplets, indicative for adipogenic differentiation could be observed in the experimental controls, when investigating the trilineage differentiation potential. We therefore decided to break down the oMSC multipotency more systematically and had a detailed view on the expansion, differentiation, and surface marker expression of oMSC, when grown in eight different culture media containing varying FCS and EGF combinations, with or without supplements as described previously. Our results indicate that the proliferation of oMSC is strongly dependent on the FCS content, while EGF and S play only minor roles. Furthermore, the surface epitopes CD73 (5′-ecto-nucleotidase), CD90 (Thy-1), and CD105 (Endoglin) were inducible depending on the FCS but not on the EGF concentration in the culture medium. Finally, the differentiation of oMSC is donor dependent, protocol dependent, and culture medium dependent.

2. Materials and Methods

2.1. Isolation and Expansion of Ovine MSC. All animal experimentation was carried out according to ILAR and FELASA rules and was approved by an Animal Experimentation Ethics committee appointed by the State of North-Rhine Westfalia. Ovine MSC were isolated out of 40 mL bone marrow aspirate from Rhoen sheep iliac crest that was supplemented with 2 mL 1.107% Di-Sodium-AEDTA (Alleman Pharma, Rimbach, Germany) to prevent coagulation. Mononuclear cells were harvested by Ficoll density gradient centrifugation (density 1.077 g/mL; Biochrom, Berlin, Germany) and subsequently seeded in 25 cm^2 tissue culture flasks in the growth media detailed as follows.

The next day, nonadherent ovine cells were removed by medium change. Further medium changes were performed every 3-4 days. At 80–90% confluence, stem cells were trypsinized with stem cell trypsin (Lonza, Walkersville, USA) and reseeded in a density of 500.000 cells per 75 cm^2 tissue culture flask.

2.2. Growth Media. The growth curves and systematic surface antigen analysis were performed with oMSC grown in eight different media containing varying combinations of FCS (F), supplements (S), and EGF. The media composition is summarized in Table 1.

The growth curve and doubling time were determined by counting the cell numbers after each passage, using a Casy cell counter (Merck, Darmstadt, Germany), for 1–5 passages and using the exponential growth formula:

$$N_t = N_0 \bullet e^{(\mu * t)}, \tag{1}$$

where N_t is the number of cells at given time point, N_0 is the initial number of cells, μ is the growth rate [/day], and t is the given time period [days].

The doubling time T_D was calculated by converting the previous formula to (see [37])

$$T_D = \frac{\ln 2}{\mu}. \tag{2}$$

2.3. Differentiation. Cell differentiation was induced using cells isolated and cultured in F10 or in F2-S-EGF medium. F10 is analogous to one standard medium used for oMSC in previous studies [22, 33, 38, 39], and F2-S-EGF is a standard medium routinely used for hMSC [34–36, 40].

Differentiation of oMSC was analyzed in passage 2-3, after preculture in F10 or F2-S-EGF. The differentiation was induced by culture for 21 days in induction media according to published protocols for hMSC (protocol 1) [31, 35] and

Table 1: Composition of growth media for culture of oMSC.

100 mL medium	F2	F10	F2-EGF	F10-EGF	F2-S	F10-S	F2-S-EGF	F10-S-EGF
DMEM 1 g/L glucose [mL][1]	97	89	97	89	56.4	51.6	56.4	51.6
L-Glutamine penicillin streptomycin (LGPS) [mL][1]	1	1	1	1	1	1	1	1
F								
FCS [mL][2]	2	10	2	10	2	10	2	10
S								
MCDB-201 [mL][3]	—	—	—	—	37.6	34.4	37.6	34.4
ITS + Premix 1x [mL][3]	—	—	—	—	1	1	1	1
Dexamethasone[3], stock 100 mM [μL], final conc. 1 nM	—	—	—	—	1	1	1	1
L-Ascorbic acid[3], stock 500 mM [mL], final conc. 100 μM	—	—	—	—	2	2	2	2
EGF								
EGF [ng/mL][3]	—	—	10	10	—	—	10	10

[1]PAA, Coelbe, Germany.
[2]Pan Biotech, Aidenbach, Germany.
[3]Sigma-Aldrich, Steinheim, Germany.

Table 2: Osteogenic differentiation media according to standard protocols

Osteogenic differentiation	Protocol 1 [31]	Protocol 2 [21]
DMEM 1 g/L glucose		
FCS [%]	10	10
Dexamethasone [μM]	0.1	0.1
Sodium-ß-glycerophosphate [mM]	10	10
L-ascorbic acid [mM]	0.05	0.05
Cell density [/cm^2]	31.000	2.000

Table 3: Adipogenic differentiation media according to standard protocols.

Adipogenic differentiation	Protocol 1 [31]	Protocol 2 [21]
Adipogenic induction medium		
DMEM 4.5 g/L glucose		
FCS [%]	10	10
Dexamethasone [μM]	1	1
Indomethacin [μM]	0.2	200
Insulin [mg/mL]	0.01	0.01
3-Isobutylxanthin [mM]	0.05	0.5
Cell density [/cm^2]	80.000	30.000
Adipogenic maintenance medium		
Serum [%]	10	
Insulin [mg/mL]	0.01	

oMSC differentiation (protocol 2) [19, 21]. The composition of the tested media can be taken from Tables 2, 3, and 4; all media contained 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all PAA, Coelbe, Germany).

Alizarin red staining (40 mM) was used to identify calcium phosphate accumulations. Cell cultures were fixed in 4% formaldehyde for 15 min and washed twice in distilled water. Cells were stained for 20 min, washed in distilled water, and photographed.

Following protocol 1, adipogenic induction medium was first added after 24 h; afterwards, adipogenic induction and maintenance medium were added alternately. For protocol 2, only adipogenic induction medium was added for 21 days. Media were changed three times a week.

After 30 min of fixation in 50% ice-cold ethanol, 10 min staining in Oil red O staining solution (0.02% w/v dissolved in 3/4 100% methanol and 1/4 1 M NaOH) was used to identify lipid-laden fat cells. Cultures were counterstained with Mayer's Haematoxylin (Sigma, Steinheim, Germany) and photographed.

After 21 days of culture, pellet aggregates were fixed in 4% formaldehyde, paraffin embedded, and histologically stained with safranin red (0.01% w/v in 40% ethanol) for 2 min. After treatment in ascending ethanol series, slides were mounted and photographed.

2.4. Analysis of Surface Epitope Expression by Flow Cytometry. For flow cytometry, cells were trypsinized and approximately 2×10^5 cells per sample were washed twice in PBS. Antibodies frequently used to stain human or ovine MSC (Table 5) were incubated with the cells for 45 min on ice. The samples were either fixed in 1% formalin or measured directly with the FACS-Canto (BD Bioscience, Walkersville, Texas). A decrease of fluorescence by fixation in formalin within 7 days was excluded in previous experiments (data not shown). Unstained cells served as a control for background fluorescence. At least 1×10^4 events were measured per sample. All cells were analyzed in passage 1.

2.5. Statistical Analysis. Data analysis was performed using GraphPad Prism 6 software. Comparison between groups was performed via analysis of variance (ANOVA) models with Tukey's multiple comparison tests. Differences with $P < 0.05$ were considered statistically significant. The data were obtained from independent samples (n = 3-4) and are expressed as the mean ± standard deviation.

Table 4: Chondrogenic differentiation media according to standard protocols.

Chondrogenic differentiation	Protocol 1 [31]	Protocol 2 [19]
DMEM 1 g/L glucose		
Serum depleted		
Dexamethasone [μM]	1	1
L-Ascorbic-acid-2-phosphate [mM]	0.17	0.17
Sodium pyruvate [μg/mL]	100	100
Proline [mM]	0.05	0.05
ITS-Plus Premix [mL]	5	5
Human transforming growth factor-ß$_3$ (TGF-ß$_3$)	10 ng/mL	
Human transforming growth factor-ß$_1$ (TGF-ß$_1$)		10 ng/mL
Bone morphogenic protein-7 (BMP-7)		100 ng/mL
Cell density [/pellet]	1.000.000[1]	1.000.000

[1]The original cell number of 250.000 hMSC per pellet [31] was adapted to 1.000.000 oMSC per pellet, as oMSC are considerably smaller than hMSC.

Table 5: Antibodies used for flow cytometry analysis of oMSC to detect surface epitopes.

Antibody	Alternate name	Reactivity	Identical amino acid residues between man and sheep [%][3]	Volume [μL] per test (in 100 μL PBS)
CD29[1]	Integrin beta 1	PE rabbit anti-human	93	10
CD44[2]	H-CAM, PGP-1	FITC mouse anti-sheep	—	5
CD45[1]	Leukocyte common antigen	FITC mouse anti-sheep	—	10
CD73[1]	NT5E, 5′ nucleotidase, E5NT, eNT, NT, NT5	APC mouse anti-human	81–89	3
CD90[1]	Thy-1	FITC mouse anti-human	—	3
CD105[1]	Endoglin, ENG, END, HHT1, ORW, ORW1	PE mouse anti-human	70–75	3
CD166[1]	ALCAM	PE mouse anti-human	90	10

[1]BD Biosciences, Beckton Dickinson, Franklin Lakes, USA.
[2]Serotec, Oxford, England.
[3]Protein BLAST sequence alignment, ncbi database (including transcript and splicing variants).

3. Results

3.1. Morphology. In general, the morphology of oMSC appeared flat and fibroblast-like in all growth media. However, minor changes in morphology could be observed. Ovine MSC grown in media containing 10% FCS (F10) and/or supplements (S) were smaller with initially bigger colony size in P0. In P1, cells cultured in media containing EGF showed less differentiated morphology with more diffuse boundaries. Overall, oMSC grown in media without S and EGF showed an initially smaller colony size, less cell-cell contacts and a rough shaped morphology (Figure 1). Furthermore, cells grown in F2-S and F2-S-EGF medium rapidly formed large cell aggregates in passage numbers > 1 (pictures not shown).

3.2. Cell Proliferation. Figure 2 shows the initial colony density in P0, growth characteristics, and doubling times for each medium. oMSC cultured in media containing 10% FCS (F10) showed initially higher cell density and larger colony sizes, compared to media containing 2% FCS (F2). However, low FCS content media with addition of S (F2-S and F2-S-EGF) showed higher cell density and larger colony sizes, compared to media without S (F2 and F2-EGF).

Cells cultured in F2 and F2-EGF medium took about 60 days to pass from P0 to P1 and about 125 days to pass to P2. These media were therefore estimated as not useful for oMSC culture. The growth curves from F2-S-EGF revealed a flattened shape for two of four donors, where the proliferation decreased after passage 3. Importantly, S and EGF both decreased the doubling time in F2 medium from 31.9 ± 14.3 to 17.9 ± 5.3 (F2-S) or 16.7 ± 2.3 (F2-EGF), but the combination of these two factors decreased the doubling time significantly to only 5.9 ± 3.7. All F10 culture media promoted a rapid proliferation of oMSC with an average doubling time of approximately 2 days. For cells cultured in F10 media, S and EGF again decreased the doubling time from 2.5 ± 0.6 to 1.5 ± 0.4 (F10-S) 1.6 ± 0.3 (F10-EGF), but the combination revealed an increase in doubling time to 5.84 ± 0.4 (F10-S-EGF).

3.3. Surface Antigen Expression. We observed large variations from donor to donor in passage 1 (Figure 3(a)), ranging from completely negative to highly positive values (CD105,

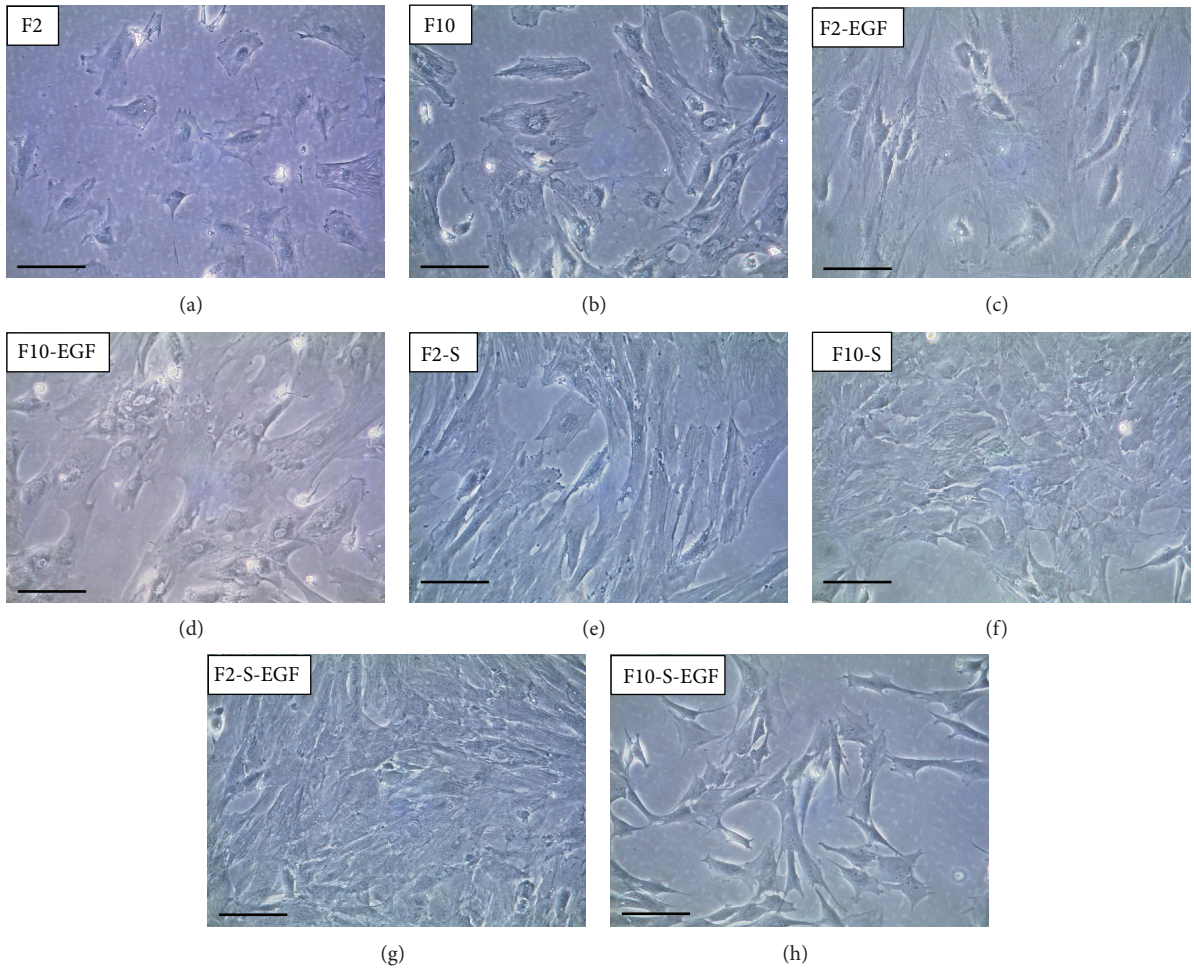
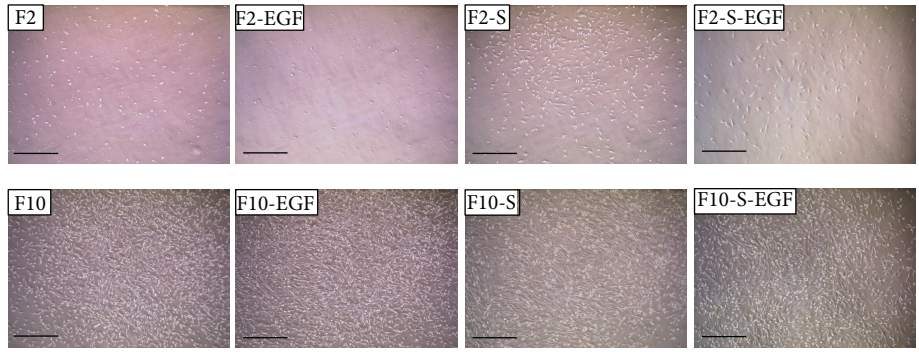


Figure 1: Representative pictures of oMSC in P1, cultured in media of varying composition (FCS (F), supplements (S), and epidermal growth factor (EGF)). All cells showed typical flat and spindle-shaped morphology. Ovine MSC grown in media containing EGF showed less differentiated morphology. oMSC grown in media without S and EGF showed lower density, less cell-cell contacts, and more flat and roughly shaped morphology. Scale bars 100 μm.

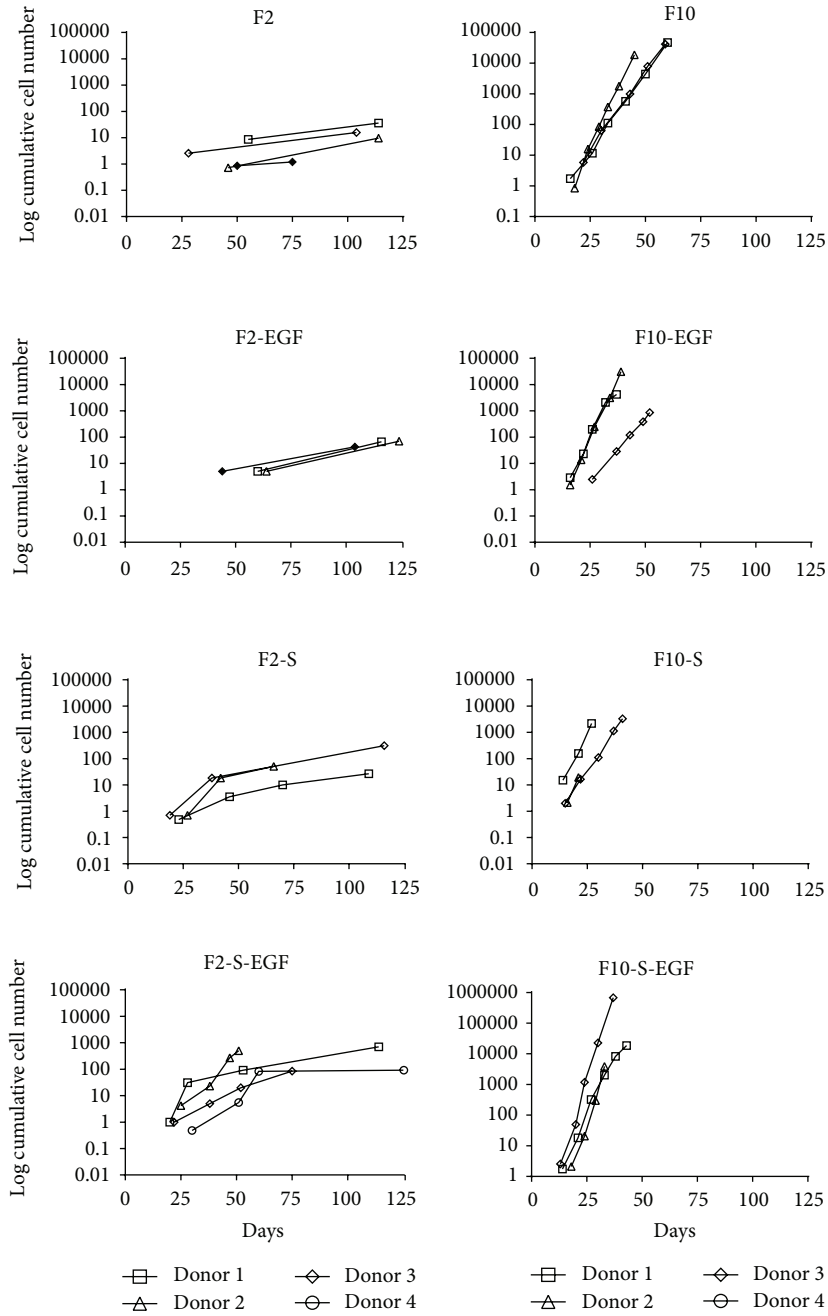
F2-S-EGF: 0–70%). The heatmap in (Figure 3(b)) shows the summarized surface epitope expression for up to five passages. CD44 was consistently detected in each medium, although to a higher degree in the F10 media. CD45 was overall low, but instead, in F10-S-EGF it was even more decreased. The expression of MSC associated surface epitopes CD73, CD90, and CD105 was increased in F2 media. EGF led to a downregulation of those markers. However, in combination with S, the downregulation was decelerated. In each of the F10 media, the addition of S had a more prominent effect on the downregulation of the surface markers, while EGF alone had nearly no influence on those markers at all. Interestingly, the addition of EGF led to a downregulation of all of those markers, both in F2 and F10 media. Furthermore, the heatmap demonstrates that the addition of S in F10 media led to an overall decrease in the detection of CD45 and the hMSC associated markers CD73, CD90, and CD105. CD45 was particularly high in medium F2, where all epitopes seemed to be expressed to a higher level than in the other media. The addition of EGF and S reduced the expression of CD45 and CD166 in both, F2 and F10 media. F10 media showed overall lower expression of CD73, CD90, and CD105. Remarkably, the result did not change much with ongoing passages, especially for measurement of CD29, CD44, and CD166.

3.4. Differentiation in F10 and F2-S-EGF Medium. As stem cells are characterized by their ability to differentiate, we tested the response of oMSC after preculture in F2-S-EGF and F10 media to osteogenic, adipogenic, and chondrogenic stimuli. Differentiation was assayed according to published protocols for hMSC and oMSC (Table 2).

3.4.1. Osteogenic Differentiation of oMSC. Figure 4 summarizes the results of osteogenic differentiation. oMSC precultured in F2-S-EGF and F10 media both showed differentiation into the osteogenic lineage for at least one of three donors. Cells of donor 1 did not show any differentiation in both protocols but a prominent formation of aggregates. Cells of



(a)



(b)

Figure 2: Continued.

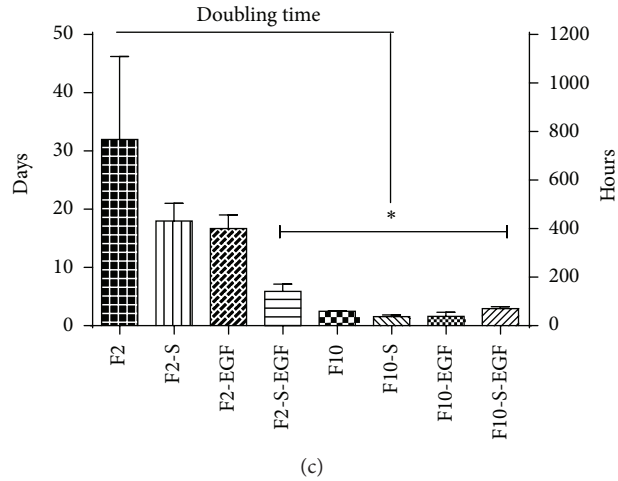


Figure 2: (a) Representative pictures of oMSC colony size and cell density in P0, after 9 days of culture in media of varying composition (FCS (F), supplements (S), and epidermal growth factor (EGF)). (b) Cumulative cell number of oMSC determined for up to five consecutive passages. (c) Doubling times calculated from exponential growth curves in (days) (left ordinate) or (hours) (right ordinate). Significant differences are marked with an asterisk ($P < 0.01$).

donor 2 were able to differentiate towards osteoblasts using either protocol 1 or protocol 2, when precultured in F2-S-EGF medium but not when precultured in F10 medium. Instead, donor 3 could be differentiated successfully into the osteogenic lineage with protocol 1 for both, preculture in F2-S-EGF medium and F10 medium. Thus, variation in osteogenic differentiation is influenced by donor variations, protocol dependent variations, and preculture dependent variations.

3.4.2. Adipogenic Differentiation of oMSC. Adipogenic differentiation of oMSC was performed using two different protocols (Table 3) and two preculture conditions (F2-S-EGF medium versus F10 medium). When investigating the adipogenic fate of oMSC, the presence of small lipid droplets in all control culture conditions (protocol 1 and 2, F10 and F2-S-EGF preculture) was noticeable (Figure 5). No outstanding lipid droplet formation was achieved by differentiation culture following protocol 1 or 2, when oMSC were precultured in F10 medium. However, preculture in F2-S-EGF resulted in prominent lipid droplet formation for all three donors, when using protocol 2 adipogenic differentiation (Figure 5). In contrast to the osteogenic differentiation, as each of the donors differentiated using protocol 2, we did not detect donor variations for adipogenic differentiation but a strong medium dependent differentiation.

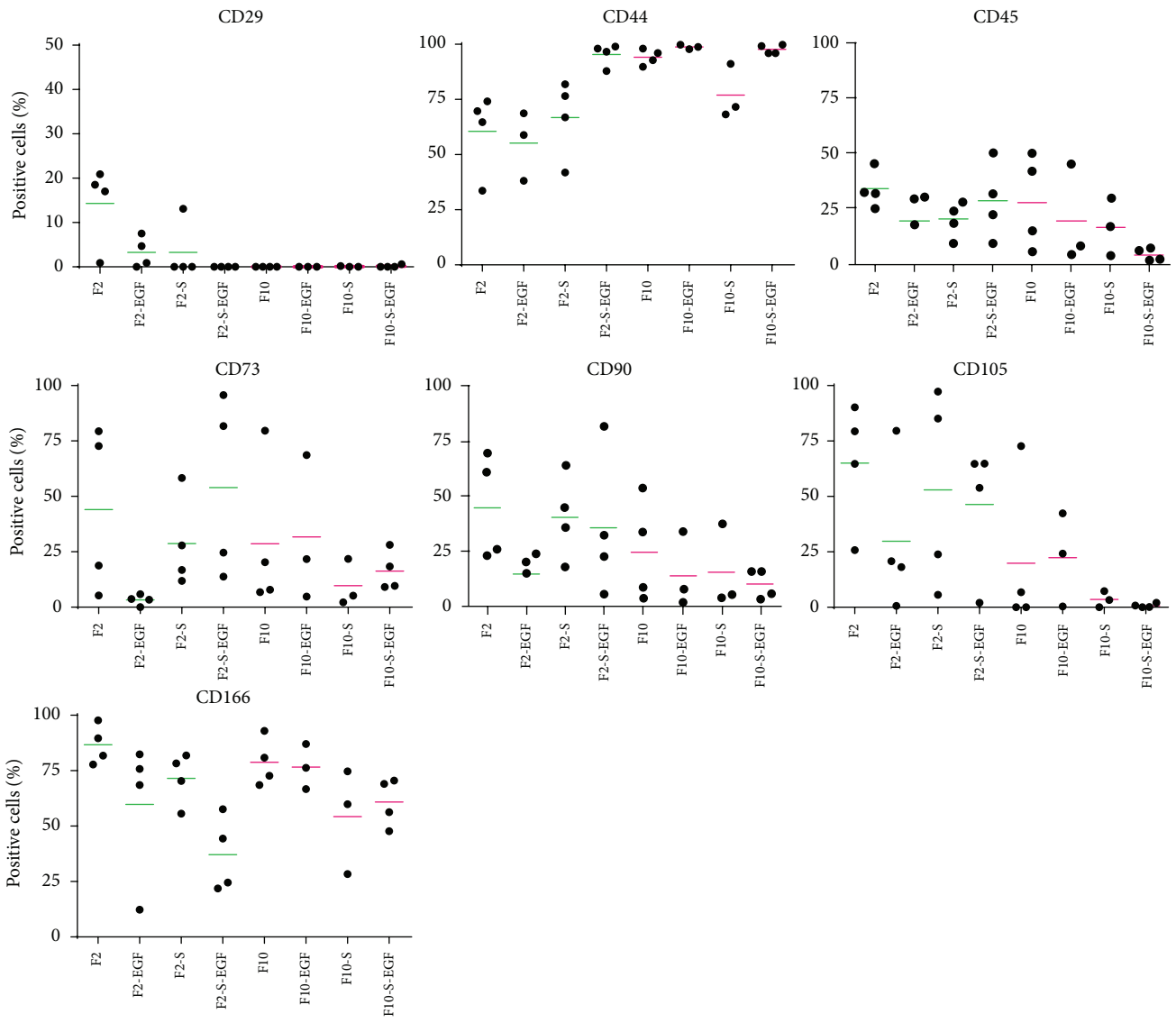
3.4.3. Chondrogenic Differentiation of oMSC. After osteogenic and adipogenic differentiation, we followed two different protocols (Table 4) to induce chondrogenic differentiation of oMSC. Chondrogenic differentiation was performed by using either TGF-β3 (protocol 1) or TGF-β1 in combination with BMP-7 (protocol 2). Figure 6 summarizes the results for chondrogenic differentiation of oMSC. While pellets were considerably larger when cells were precultured in F10 medium and induced following protocol 2, cells precultured in F2-S-EGF medium and induced via protocol 1 showed more efficient differentiation, as indicated by pellet morphology and cell structure. In 2 control conditions no pellet could be obtained during culture, indicating a low overall robustness of the pellets. Spontaneous differentiation was also observed in the controls of all three donors, when precultured in F2-S-EGF medium. Again, we found the differentiation of oMSC to be donor dependent, protocol dependent, and preculture dependent.

4. Discussion

Recently, numerous studies were published employing sheep stem cells from bone marrow, referred to as ovine mesenchymal stem cells (oMSC) [19–21, 23, 33, 41, 42]. Since there is no consent on expansion (varying culture media) and characterization of oMSC (varying differentiation protocols and surface epitope analyses), we here investigated the impact of variations in culture conditions on proliferation, differentiation, and expression of surface epitopes associated with MSC.

The oMSC culture in media of different compositions resulted in morphological variances, as previously described for hMSC and other cell types [43–45]. However, it is not clear if and how these morphological changes are related to cell functions or differentiation potential.

The fastest colony formation and proliferation were observed in media containing 10% FCS. An increased proliferation depending on the FCS content has already been described for MSC of human and other species origin, as well as for many other cell types [46–49], underlining the importance of serum content for growth media. The addition of EGF or S (MCDB-201, dexamethasone, L-ascorbic acid, and ITS-plus) alone resulted in a reduced doubling time for both high and low serum content media, while the combination of EGF and S resulted in an even more reduced



(a)

oMSC	F2	F2-EGF	F2-S				F2-S-EGF				F10					F10-EGF					F10-S					F10-S-EGF				
Passage	P1	P2	P1	P1	P2	P3	P4	P1	P2	P3	P4	P1	P2	P3	P4	P5	P1	P2	P3	P4	P5	P1	P2	P3	P4	P5	P1	P2	P3	P4	P5
CD29																															
CD44																															
CD166																															
oCD45																															
CD73																															
CD90																															
CD105																															

>90% | 60–90% | 50–60% | 30–50% | 10–30% | <10%

(b)

FIGURE 3: Surface epitope expression analysis of oMSC, cultured in media of varying composition (FCS (F), supplements (S), and epidermal growth factor (EGF)). (a) Percentage of positive stained cells in passage 1, detected by flow cytometry. (b) Heatmap of corresponding values of positive stained cells for up to 5 passages.

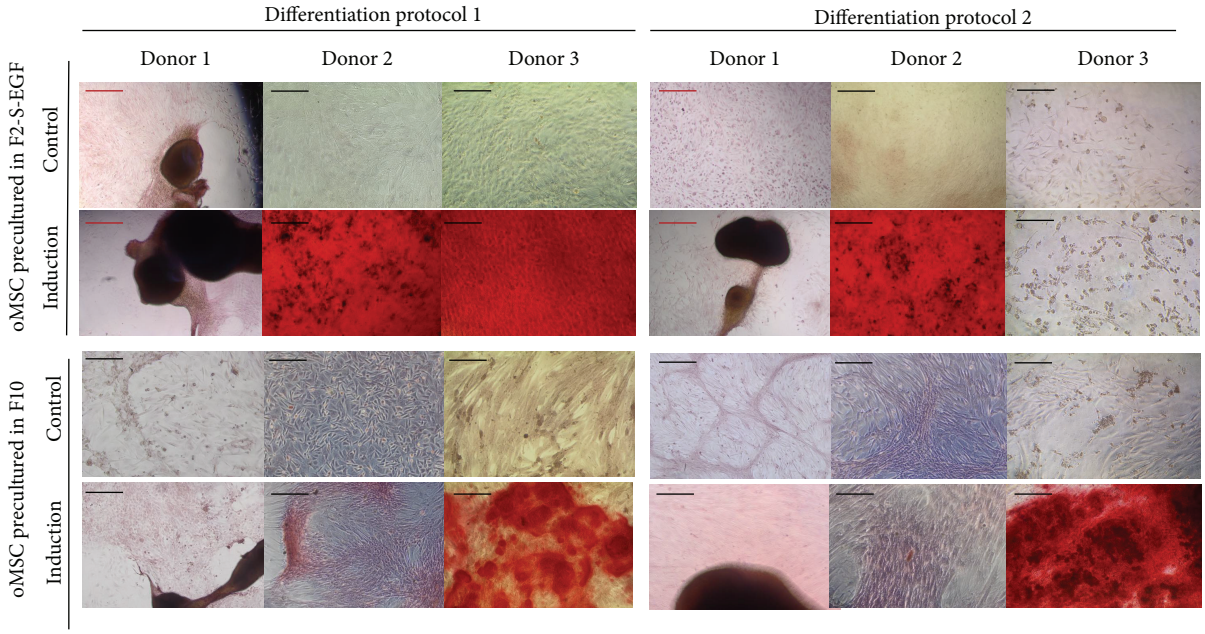


Figure 4: Alizarin red staining after osteogenic differentiation of oMSC precultured in F10 or F2-S-EGF medium and subsequently differentiated following two established protocols (varying in induction media and cell density) for 21 days. $n = 3$, red scale bars 500 μm and black scale bars 100 μm.

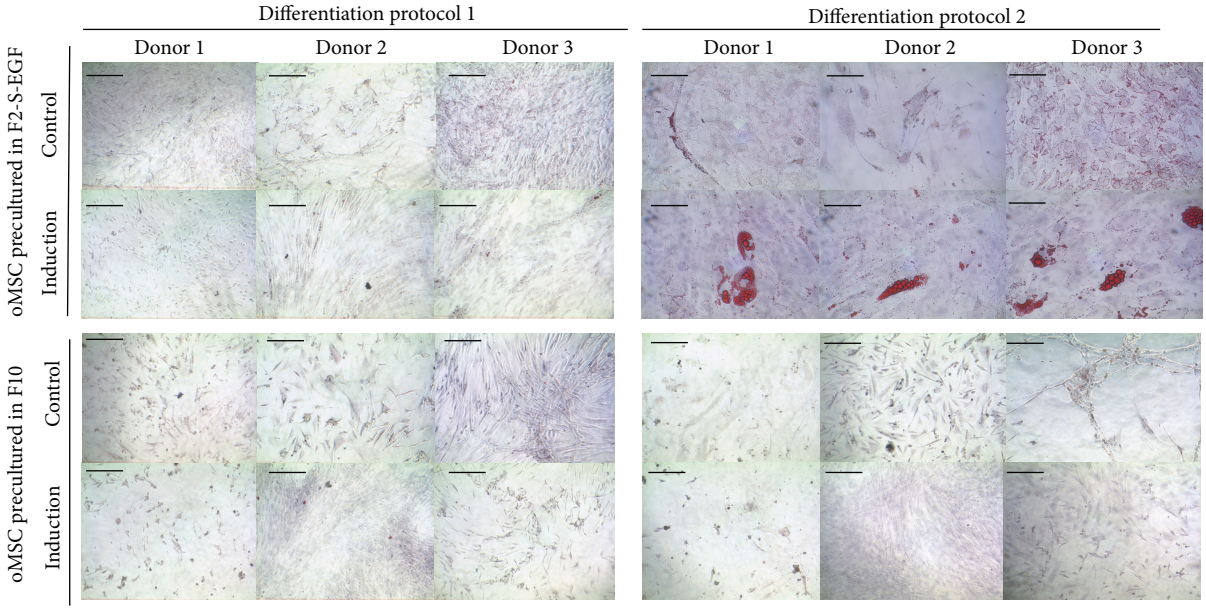


Figure 5: Oil red O staining after adipogenic differentiation of oMSC precultured in F10 or F2-S-EGF medium and subsequently differentiated following two different established protocols (varying induction media and cell density) for 21 days. $n = 3$. Scale bars 100 μm.

doubling time for F2 media. In F10, no further reduction of the doubling time could be achieved by the combination of these factors. It has been described for hMSC that the proliferation rate is significantly enhanced by culture with certain growth factors such as EGF, fibroblast growth factor (FGF), or platelet-derived-growth-factor- (PDGF-) BB [47, 50, 51]. However, the addition of EGF to a low serum content medium resulted in a nonfeasible culture of oMSC with more than 65 days from P0 to P1. oMSC were able to proliferate in combination with other supplements, and the doubling time was remarkably reduced in comparison to medium containing 2% FCS alone. Therefore, not only one but several factors work synergistically for fast or slow proliferation of oMSC.

In F2-EGF medium the average expression of most of the surface epitopes was at the lowest rate, while in F2 medium it

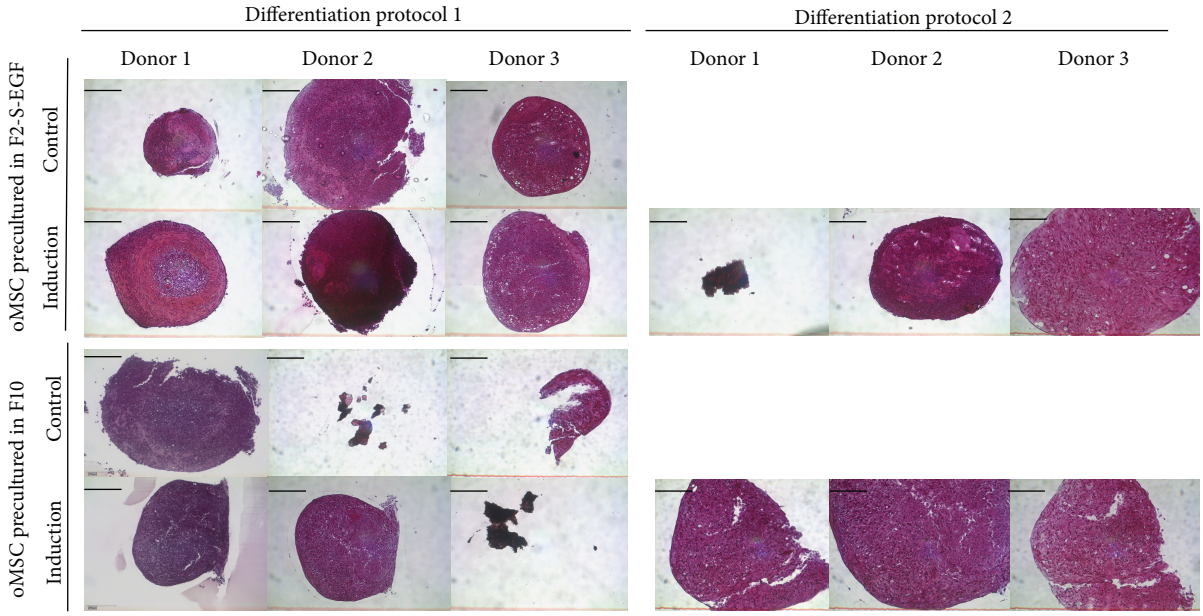


Figure 6: Safranin red staining after chondrogenic differentiation of oMSC precultured in F10 or F2-S-EGF medium and subsequently differentiated following two different established protocols (TGF-β3 or TGF-β1/BMP-7) for 21 days. Proteoglycans in the extracellular matrix are stained in deep red. In 2 control conditions no pellet could be obtained during culture, indicating a low overall robustness of the pellets. $n = 3$. Scale bars 200 μm.

was at the highest rate (Figure 3(a)). This context indicates that growth factors play a key role for the expression or depression of surface epitopes associated with stem cells. For hMSC, soluble EGF has been shown to trigger ERK and Akt/PKB signalling, which can lead to phosphorylation of surface proteins, by activation of the EGF receptor [47]. The connection between EGF and surface marker depression of oMSC still needs to be elucidated, especially when considering the foreign species origin of EGF used in this study (lack of availability of sheep growth factors). The appropriateness of human growth factors and fetal calf serum for culture of ovine cells may be worthy of discussion. Although there are studies employing sheep autologous serum for oMSC culture [17], our study aimed at comparing media with standard components frequently used in cell culture. There may be of course differences in response to autologous growth factors; nevertheless, our results add to basic knowledge on oMSC behaviour in different culture media. Since anti-human antibodies do not necessarily cross-react with ovine cells, the use of anti-human antibodies, although frequently applied in previous studies [19–21, 23], is an imperfect methodology, which further emphasizes the need for established surface markers for oMSC characterization.

Interestingly, the surface epitope profile did not change considerably with ongoing passages. This has also been shown before for hMSC [52]. However, hMSC lose their potential to differentiate with passage 5-6, and the surface markers commonly investigated for MSC might therefore not be connected to differentiation, especially as some types of mature cells like fibroblasts exhibit the same set of MSC markers [52].

It is likely that the high expression of CD45 in F2 media indicates differences in isolation of cocultured cells like macrophages that are known to frequently contaminate MSC cultures [30, 53], depending on the media composition (FCS concentration).

Taken together, the analysis of surface marker expression revealed remarkable differences depending on FCS content, media supplementation, addition of growth factors, and donor-dependent differences.

As mentioned before, there is large variation in the characterization protocols for oMSC. For surface marker expression, Rentsch et al. report positive immunofluorescence staining for CD9, CD44, CD54, CD73, CD90, CD105, and CD166, as well as successful trilineage differentiation with the afore mentioned protocol 2. The cells were previously cultured in medium, which was analogous to our F10. Our results, however, do only partly agree with those obtained by Rentsch et al., regarding the positive expression of CD73, CD90, and CD105, which we found to be inconsistent in our study. This fact may be due to the differences in surface marker detection, as well as differences in the applied antibodies (species specificity were not mentioned).

Flow cytometry results by McCarty et al. also described oMSC to be highly positive for CD44 and CD166, but in contrast to Rentsch et al., to be negative for anti-human CD90 and CD105, which coincides with our results. In the same study, stimulatory effects on proliferation by EGF on oMSC were also confirmed and were described to be even more prominent than in media with 10% FCS only. Spontaneous adipogenic differentiation or large donor variations were not described in these studies.

Although we did not observe a decisive impact on the differentiation, the addition of growth factors to oMSC culture should be carefully considered, as the measurement of the tested surface marker set was decreased by EGF addition. Otherwise, the FCS content of 10% without EGF led to a significant reduction in the doubling time and retainment in the expression of most of the surface markers, but it did not promote the adipogenic differentiation in our study. Considerable variations in FCS content and additions of supplements or growth factors are also usual for hMSC culture. However, standardized protocols to measure surface epitopes and perform differentiation in osteogenic, adipogenic, and chondrogenic lineage have been established [31].

Considering the various sources of influence, it is very likely that the culture conditions for oMSC from sheep bone marrow might have an influence on their response to chemical, physicochemical, or even topographical factors in tissue engineering approaches. A separation by binding of antibodies to specific surface epitopes is routinely implemented for the isolation of some cell types (hematopoietic stem cells, mouse MSC). We propose that for oMSC, donor variations might be reduced when preselection of surface epitopes [23] such as CD44 in combination with CD166 would be performed, as these markers are frequently reported to be highly positive for oMSC [19–21, 23]. Despite the overall strong expression of CD44, there is still no standard for surface epitopes that should be expressed by oMSC. Studies investigating the multipotency of oMSC vary in the selection of surface epitopes and even testing the same epitopes resulted in different outcome (anti-human markers were found to be positive and negative depending on the study) [17, 19–21, 23].

Whereas for hMSC, a loss of surface epitope expression by growth factor supplemented culture had no effect on their ability to differentiate [47]; this context still needs to be clarified for oMSC. Although it has been shown that oMSC are also able to transdifferentiate into cells with neuronal phenotype, this goal was only achieved with variable success [18]. Additionally, in the same study, large donor variations were reported. Together with the spontaneous differentiation observed in our study, it is possible that multipotency of oMSC might not be stable under recently accepted cell culture conditions. Furthermore, the only selection factor for oMSC is plastic adherence with fibroblast-like morphology. Thus, different progenitor stages might be isolated that are either inducible or noninducible by culture media [3, 54]. It has already been described for hMSC that "cells in such cultures differ markedly to the point that some have progenitor properties and multipotent differentiation capacity, whereas others are devoid of this ability and are not unlike what is commonly called fibroblasts" [3]. We here propose a similar heterogeneity for culture of oMSC.

5. Conclusion

This study adds to several previous studies that demonstrated the influence of the choice of expansion media not only on growth characteristics, but also on the surface epitope phenotype and differentiation potential of MSC [55]. We here compared these characteristics for oMSC, following culture in expansion media with varying FCS content, EGF, and/or other supplementation. The oMSC behavior and phenotype is dependent on the donor, the medium of preculture, and the differentiation protocol. We therefore conclude that the conditions of oMSC culture will also affect the composition of oMSC population and therefore the outcome of oMSC-based tissue engineering strategies. Hence, a standard for oMSC culture conditions and minimal criteria for their characterization, analogous to the position papers by Horwitz et al. [29] and by Dominici et al. [30], is obligatory. Next steps should focus on eliminating protocol- and medium-related variations to receive reproducible results when using oMSC for tissue engineering studies using sheep as a large animal model.

Conflict of Interests

The authors indicate no potential conflict of interests.

Acknowledgment

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Research Article

Comparing the Gene Expression Profile of Stromal Cells from Human Cord Blood and Bone Marrow: Lack of the Typical "Bone" Signature in Cord Blood Cells

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With regard to the bone-regenerative capacity, bone marrow stromal cells (BMSC) can still be termed the "gold standard." Nevertheless, neonatal stromal cells from cord blood (CB) feature advantages concerning availability, immaturity, and proliferation potential. The detailed gene expression analysis and overexpression of genes expressed differentially provide insight into the inherent capacity of stromal cells. Microarray and qRT-PCR analyses revealed closely related gene expression patterns of two stromal cell populations derived from CB. In contrast to the CB-derived cell types, BMSC displayed high expression levels of *BSP*, *OSX*, *BMP4*, *OC*, and *PITX2*. Lentiviral overexpression of *BSP* but not of *OSX* in CB-cells increased the capacity to form a mineralized matrix. *BMP4* induced the secretion of proteoglycans during chondrogenic pellet culture and extended the osteogenic but reduced the adipogenic differentiation potential. BMSC revealed the typical osteogenic gene expression signature. In contrast, the CB-derived cell types exhibited a more immature gene expression profile and no predisposition towards skeletal development. The absence of *BSP* and *BMP4*—which were defined as potential key players affecting the differentiation potential—in neonatal stromal cells should be taken into consideration when choosing a cell source for tissue regeneration approaches.

1. Introduction

With respect to the regeneration of cartilage or bone after tumor resection, accidents, or due to diseases affecting the skeleton, there is, great need for tissue-engineered bone. The cellular component of these approaches has been in the focus of interest for many years.

The first described [1] and therefore the best studied non-hematopoietic stromal cell type derives from bone marrow (BM). The *in vivo* bone forming potential—including recruitment of hematopoietic cells of recipient origin—of these bone marrow stromal cells (BMSC) after transplantation on a hydroxyapatite scaffold was reported by several groups [2, 3]. The potential risks associated with the bone marrow donation made other sources of stromal cells, for example, adipose tissue or peripheral blood, attractive alternatives. Due to its immaturity compared to adult bone marrow, neonatal cord blood (CB), which can be collected noninvasively and without ethical concerns, can be regarded as a proper source of neonatal stromal cells with potential clinical relevance in the future. Cord blood contains at least two distinct populations of nonhematopoietic stromal cells with comparable proliferative potential [4], which were termed unrestricted somatic stromal cells (USSC) and cord blood-derived stromal cells (CBSC). So far, USSC and CBSC cannot be isolated prospectively but can be distinguished on the basis of cell surface antigens, differentiation potential, and gene expression. In flow cytometric analyses, CBSC revealed a stronger expression of CD146 (MCAM, melanoma adhesion molecule) compared to USSC [4]. During *in vitro* differentiation assays, CBSC but not USSC possess the potential to differentiate into adipocytes [5]. Former results indicated a correlation

of the absent adipogenic potential and the expression of *DLK1* (delta, *Drosophila* homolog-like 1) in USSC, since USSC but not CBSC express *DLK1* [5]. Recent results suggested that *DLK1* might not be the sole factor responsible for the inhibition of *in vitro* adipogenesis in USSC [6]. In microarray and PCR analyses, the expression of *HOX* (homeobox) genes was defined as additional distinguishing feature: USSC completely lack *HOX* gene expression, while CBSC are *HOX* positive [7]. Furthermore, USSC can be discriminated from CBSC on the basis of their higher hematopoiesis-supporting capacity in coculture experiments [6].

To date, the proof of the ability of CB-derived stromal cells to form true bone and to recruit hematopoietic cells after transplantation in standardized *in vivo* assays is still missing. Before performing such assays, the identification of potential differences on molecular level between CB-cells and the "gold standard" BMSC is mandatory. With respect to their immunophenotype, CB- and BM-derived cells are barely different. A potential cell surface marker to distinguish these cell types quantitatively by flow cytometric analyses is CD146 [4], but this antigen was also described to be expressed on pericytes, regardless if they are osteogenic or not [3]. On transcriptome level, differences in the gene expression were described for cell types of distinct origin [8]. In the present study, further genes expressed differentially in BM- and CB-derived cell populations were examined to find potential candidate genes influencing the *in vivo* regenerative potential. Special attention was paid to genes regulating the formation of the skeleton by endochondral or intramembranous ossification during fetal development.

Chondrogenesis is precisely adjusted by extracellular matrix and growth factor signals as well as by intracellular signaling pathways and gene transcription in a temporal-spatial manner [9]. Essential regulatory pathways involved in fetal chondrogenesis are FGF, hedgehog, BMP, or WNT signaling [9, 10]. BMPs—in particular *BMP2*, *4*, and *7*—are known to act during early (chondroprogenitor cell determination and differentiation) and late stages (terminal differentiation to hypertrophic chondrocytes) of chondrocyte maturation [9]. Furthermore, *BMP4* is also involved in the regulation of osteoblast maturation [11]. During endochondral ossification, the cartilaginous matrix is replaced by bone matrix synthesized by osteoblasts. One of the most important and earliest transcription factors controlling this process is the runt-related transcription factor 2 (*RUNX2*) which, for instance, binds the promotor of the osteoblast-specific hormone osteocalcin (*OC*) [12]. Beside *RUNX2*, the transcription factor SP7 (osterix, *OSX*) is essential for the differentiation of osteoblasts in mice: inactivation of *Osx* leads to a failure in bone formation [12, 13]. *OSX* is located downstream of *RUNX2*, as evidenced by its absence in *Runx2*-deficient mice [13]. In the later stages of bone formation, the newly formed bone matrix mineralizes through accumulation of hydroxyapatite, collagens, and noncollagenous proteins. The secreted phosphoprotein *BSP* (integrin-binding sialoprotein) constitutes the main part of the noncollagenous proteins of the human bone extracellular matrix [14]. An essential role for *Bsp* regarding the *in vivo* bone forming potential has been reported for murine BMSC: only clonal cell lines expressing *Bsp* revealed an *in vivo* osteogenic potential, whereas the *Bsp*-negative cell lines were nonosteogenic [15].

In the present study, genes differentially expressed in stromal cells from cord blood (USSC and CBSC) and bone marrow (BMSC), which potentially affect the *in vivo* bone forming capacity, were identified by microarray data analyses and quantitative RT-PCR. *BMP4*, *BSP*, and *OSX* were stronger expressed in BM- compared to CB-derived stromal cells and were selected for overexpression experiments to assess the gene function during the regulation of differentiation. Further analyses indicated an osteosupportive role for *BMP4* and *BSP*, whereas *OSX* seemed to have a negative effect on the bone forming capacity *in vitro*.

2. Materials and Methods

2.1. Isolation and Expansion. The ethical review board of the Medical Faculty of the University Düsseldorf granted the ethical approval to isolate the different cell types (Study nos. USSC/CBSC: no. 2975, BMSC: no. 3240).

USSC and CBSC were isolated using the same protocol. To discriminate the cell types, the adipogenic differentiation potential as well as the *DLK-1* [5] and *HOX* gene expression [7] was determined in passage 4 or 5. The immunophenotype and growth potential of both cell types were compared in a previous study [4].

The cell isolation was conducted as published before [5, 16]. In brief, human CB was collected from the umbilical cord vein with written informed consent of the mothers. The mononuclear cell fraction (MNC) was obtained by ficoll gradient separation (Biochrom AG) followed by ammonium chloride lysis of red blood cells. 5–7 $\times$ 10^6 MNC/mL were cultured in Dulbecco's Modified Eagle Medium (DMEM) low glucose (Lonza) with 30% fetal calf serum (FCS, Hyclone), 10^{-7} M dexamethasone (Sigma-Aldrich), and 1% penicillin/streptomycin/Lglutamine (PSG, Lonza). Single colonies were detached with trypsin (0.25%) using cloning cylinders (Merck Millipore) and expanded in the same medium without dexamethasone.

BMSC were isolated using bone marrow aspirated from the iliac crest of healthy donors as described previously [17].

All cell types were cultured at 37°C in a humidified atmosphere with 5% CO_2 until reaching 80% confluence. USSC and CBSC were detached with 0.25% trypsin, while BMSC were detached with 0.25% trypsin/EDTA (both Lonza).

2.2. Microarray Gene Expression Analyses. Cell lines in passage 5 were used for microarray gene expression analyses. Total RNA was extracted according to the RNeasy Mini Kit protocol (Qiagen). RNA preparations were checked for RNA integrity by Agilent 2100 Bioanalyzer. All samples in this study showed high-quality RNA integrity numbers (RINs) of 10. RNA was quantified by photometric Nanodrop measurement. Synthesis of cDNA and subsequent biotin labeling of cRNA was performed according to the manufacturers' protocol (3' IVT Express Kit; Affymetrix, Inc.). Briefly, 100 ng of total RNA was converted to cDNA, followed by *in vitro* transcription and biotin labeling of aRNA. After

fragmentation, labeled aRNA was hybridized to Affymetrix PrimeView Human Gene Expression Microarrays for 16 h at 45°C, stained by streptavidin/phycoerythrin conjugate, and scanned as described in the manufacturers' protocol. Data analyses on digitized fluorescence signal intensities were conducted with GeneSpring GX software (Vers. 12.1; Agilent Technologies). Probes within each probeset were summarized by RMA after quantile normalization of probe level signal intensities across all samples to reduce interarray variability [18]. Input data before processing was concluded by baseline transformation to the median of all samples. After grouping of samples according to their respective experimental conditions (USSC, CBSCs and BMSC, three replicates each), a given probeset had to be expressed above background (i.e., fluorescence signal of a given probeset was detected within the 20th and 100th percentiles of the raw signal distribution of a given array) in at least two of the three replicates in every single one of the three experimental groups. The resulting cell-type-specific gene expression profiles was compared by separating overlapping from cell type specific gene lists (Venn diagram analysis). Global similarity of gene expression profiles were determined by principal component analysis (PCA). Expression values were mean centered and scaled to unit standard deviation. Pruning options within GeneSpring GX software were set to a fixed number of principal components (numPrincipalComponents = 3).

Those genes expressed uniquely in one cell population (Venn diagram) were grouped using the "Functional Annotation Cluster Tool" provided by DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/) [19–21].

2.3. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). RNA isolation was performed using RNeasy Kits (Qiagen). RNA of differentiated cells was isolated using Tri Reagent (Sigma-Aldrich) according to the manufacturer's instructions followed by DNA digestion with DNase I (Life Technologies). Prior to RNA isolation, chondrogenic pellets were incubated at 37°C in pronase E (Merck) for 1 h followed by incubation in collagenase P (Roche) for 24 h. Reverse transcription (RT) was performed with SuperScriptIII (Life Technologies) according to the supplier's protocol. Complementary DNA (cDNA) which approximated 50 ng of RNA was used for subsequent qRT-PCR with Power SYBR Green PCR Mastermix (Life Technologies). The primer sequences and corresponding annealing temperatures are listed in Table S1 available online at http://dx.doi.org/10.1155/2013/631984. To screen the genes associated with the *WNT* pathway, WNT signaling pathway PCR arrays (PAHS-04, SA Biosciences, Qiagen) were applied (the gene expressions of *MYC* and *PITX2* are presented in this paper). After comparison of different potential housekeeping genes regarding the gene expression stability during the differentiation process (data not shown), we decided to use human ribosomal protein L13a (*RPL13A*) as reference gene for normalization. For *SOX9*, a TaqMan Gene Expression Assay in combination with the TaqMan 2x Universal PCR Master Mix No Amp Erase UNG (all Life Technologies) was used. All reactions were run in duplicates on an ABI Step One Plus Detection System using the following standard program: 95°C 10 min; 95°C 15 s, 55/60/65°C 1 min (40 cycles). Relative changes in gene expression were calculated following the ΔΔCt-method.

2.4. Statistical Analysis. Data are presented as arithmetic means with standard deviation of at least three different cell lines. Unpaired *t*-tests were conducted with GraphPad Prism Version 5.01. *P* values lower than 0.05 were considered significant ($^{*}P = 0.01$ to 0.05; $^{**}P = 0.001$ to 0.01; $^{***}P < 0.001$).

2.5. In Vitro Differentiation. For induction of adipogenic differentiation, cells were plated at 8.3×10^3 cells/cm^2 in 6-well plates until reaching 70% confluence. Adipogenic differentiation media were changed twice a week for 21 days, alternating induction and cultivation medium. The former consisted of DMEM high glucose (Lonza) supplemented with 10% FCS, 1% penicillin/streptomycin/Lglutamine (PSG), 10^{-6} M dexamethasone, 0.2 mM indomethacin, 0.1 mg/mL insulin, and 1 mM 3-isobutylmethylxanthine (all Sigma-Aldrich); the latter was made up of DMEM high glucose, 10% FCS, 1% PSG, and 0.01 mg/mL insulin. As a negative control, the cells were cultured in DMEM low glucose, 10% FCS, and PSG. The differentiated cells were fixed with formaldehyde and stained with Oil Red O (Sigma-Aldrich) to visualize lipid vacuoles. The stained lipid vacuoles were quantified using the ImageJ Java-based image processing software for Windows. A minimum of 3 pictures for each experiment were analyzed. The stained area was calculated and the corresponding negative control was subtracted.

For induction of osteogenic differentiation, cells were plated at 8.3×10^3 cells/cm^2 in 6-well plates. When reaching 70% confluence, the osteogenic differentiation medium containing DMEM low glucose supplemented with 30% FCS, 1% PSG, 10^{-7} M dexamethasone, 50 μg/mL ascorbic acid, and 10 mM beta-glycerolphosphate (all Sigma-Aldrich) was added. As a negative control, the cells were cultured in DMEM low glucose, 10% FCS, and PSG. Osteogenic differentiation was performed for 14 days; the medium was changed twice a week. To detect mineralization, a staining with silver nitrate ("Von Kossa") or Alizarin Red S applying standard protocols was performed. For Von Kossa staining, the cells were fixed in cold ethanol (70%, 10 min), incubated in silver nitrate (Roth, 5%, 30 min) followed by sodium thiosulfate pentahydrate (Merck, 1%, 1 min). Nuclear fast red aluminium sulfate solution (Merck, 0.1%, 30 min) was applied for counterstaining. Distilled water was used to wash the cells between the steps of the staining procedure. For Alizarin Red S-staining, the fixation was performed in cold ethanol (70%, 10 min), followed by incubation in Alizarin Red S (Sigma-Aldrich, 2%, 10 min) and 5 washing steps with distilled water. After the staining procedure, the amount of Alizarin Red was quantified. 800 μL of acetic acid was added and incubated for 30 min under permanent shaking. The cell layer was detached with a cell scraper, vortexed, and incubated first at 85°C for 10 min, then on ice for 5 min. After a centrifugation step (24500 g, 15 min), 500 μL of the supernatant was mixed with 200 μL of ammonium hydroxide (10%) and was analyzed

photometrically (plate reader, Bio-Tek Instruments Inc.) at 405 nm. Values of the negative control were subtracted from those of differentiated cells. Each sample was measured in triplicates.

To induce chondrogenesis, aliquots of 2×10^5 cells were centrifuged at 150 g for 7 min in 15 mL polypropylene conical tubes. The pelleted cells were incubated for 21 days in DMEM high glucose supplemented with 1% PS, 100 nM dexamethasone, 35 μg/mL ascorbic acid-2-phosphate, 1 mM sodium pyruvate (all Sigma-Aldrich), Insulin-Transferrin-Selenium (1/100 dilution) (Gibco), and 10 ng/mL TGF beta1 (MACS, Miltenyi Biotec). The media were changed three times a week. For Safranin O/Fast Green staining, the pellets were embedded in Tissue Freezing Medium (Jung, Leica) and cut into sections of 6 μm using a cryotome. The slides were fixed with cold ethanol (70%), stained with Safranin O for 30 min and Fast Green (both Waldeck) for 5 s. After washing in distilled water, the slides were incubated in ethanol (96%) and xylol. Entellan (Merck) was used as mounting medium.

2.6. Lentiviral Overexpression. To isolate DNA containing the gene of interest, specific primers with restriction sites for the restriction enzymes were designed (Table S1). The genes fragments were inserted into the pCL6IEGwo vector (Figures S1 and S2). *E. coli* TOP 10 (Life Technologies) was used for transformation. To verify correct gene delivery, the constructs (pCL6*BMP4*, pCL6*BSP*, and pCL6*OSX*) were sequenced using the BigDye Terminator Cycle Sequencing Kit (Life Technologies). Lentiviral particles were produced using FuGENE transfection reagent (Roche) to transfect HEK293T cells with the envelope plasmid pALF-GALV, the helper plasmid pCD/NL-BH, and the expression vector pCL6IEGwo containing eGFP and the cloned gene sequence (Figure S2). HEK293T transfection was accomplished according to the following protocol. Day 1: HEK293T were plated in DMEM (high glucose), 10% FCS, and 1% PSG (5×10^5 cells/cm^2) on 10 cm plates. Day 2: HEK293T transfection: DMEM (high glucose), 5 μg of each plasmid, and 45 μL FuGENE were mixed and incubated for 15 min at room temperature and added to HEK293T in DMEM (high glucose), 5% FCS. Day 3: target cells were plated (1×10^5 cells/60 cm^2). HEK293T culture medium was changed (DMEM (high glucose), 5% FCS, 1% PSG). Day 4: infection of target cells: HEK293T supernatant containing virus particles was sterile filtered (0.45 μm filter) and added to target cells (diluted if necessary). For control cells ("Mock"), medium without virus particles (DMEM (high glucose), 5% FCS, 1% PSG) was applied. Days 5 and 6: medium change of target cells (DMEM (low glucose), 30% FCS, and 1% PSG). To ensure a high transfection efficiency of the target cells, the eGFP expression was measured via flowcytometric analysis. Fluorescence-activated cell sorting (FACS) was accomplished in the Core Flow Cytometry Facility of the Heinrich-Heine-University Medical Center Düsseldorf, Germany.

3. Results

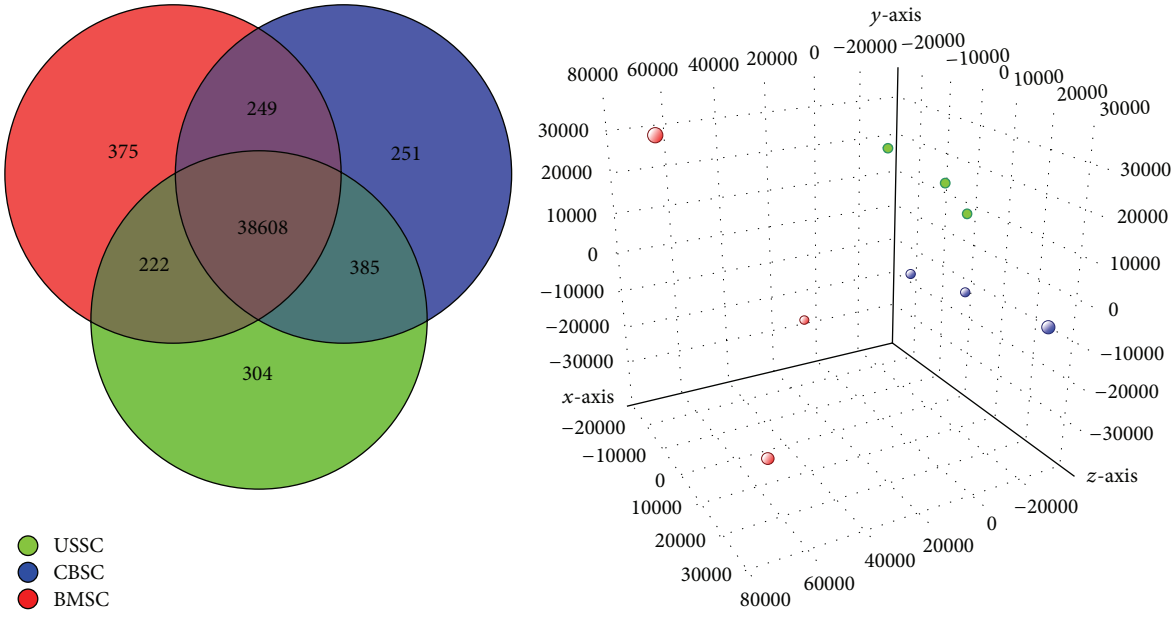
3.1. The Gene Expression Profiles of Neonatal USSC and CBSC Are More Similar to Each Other Than to Adult BMSC. To get an overview of the gene expression profiles of USSC, CBSC, and BMSC (three replicates each), microarray gene expression analyses were performed. The results were depicted in a Venn diagram and a principal component analysis (PCA, Figures 1(a) and 1(b)).

The Venn diagram illustrates the count of genes expressed by one or by more cell types. The vast majority of genes was expressed in common by all three cell types (38608 genes, Figure 1(a)). Among those genes not expressed by all cell types, USSC and CBSC expressed 385 genes which were absent in BMSC. 249 genes were present in CBSC and BMSC but not in USSC, while the expression of 222 genes was shared by USSC and BMSC. 304 genes were expressed uniquely in USSC, 251 in CBSC and 375 in BMSC (Figure 1(a)). For detailed analyses, those genes were assigned to biological functions (gene ontology (GO) terms) using the functional annotation cluster tool of the DAVID Bioinformatics Resources website. In Table 1, those genes expressed uniquely in USSC, CBSC, or BMSC associated with the process of osteogenesis are listed. USSC and CBSC exhibited the unique expression of only three "bone-related" genes in three GO terms each. On the contrary, BMSC expressed 13 genes grouped in ten GO terms that the CB-derived cell types did not express (Table 1).

The principal component analysis presents the correlation between the three replicates of one cell type depicted as spheres in a three-dimensional space. Those spheres which display a high gene expression similarity are positioned closer to each other. The analysis of the three bone marrow cell lines revealed a scattering in the three-dimensional space, whereas the triplicates of USSC and CBSC are closer related to each other (Figure 1(b)).

To summarize, the analysis of the microarray data suggests a more similar gene expression pattern of USSC and CBSC compared to that of BMSC (Figure 1(a)). In addition, BMSC expressed more osteogenesis-related genes in unique than USSC or CBSC, indicating an inherent "osteogenic signature" of the bone-marrow-derived cells (Table 1). Furthermore, the BMSC cell lines exhibited a stronger biological variance compared to USSC or CBSC cell lines (Figure 1(b)).

3.2. Differentially Expressed Genes Were Evaluated by Quantitative RT-PCR: BMSC Exhibited an Osteogenic Signature. After genome wide microarray analyses, a more detailed insight was gained by qRT-PCRs to assess genes expressed differentially in the cell populations. After interpretation of the microarray gene expression data, special focus was placed on genes associated with the process of bone formation. The gene expression was analyzed in at least three different cell lines per cell type to compensate the biological variance. Integrin-binding sialoprotein (*BSP*), osterix (*OSX*), bone morphogenetic protein 4 (*BMP4*), osteocalcin (*OC*), and paired-like homeodomain transcription factor 2 (*PITX2*) revealed a stronger expression in BMSC compared to the CB-derived cell types (Figure 2, unpaired t-test: *BSP*: USSC/BMSC, $P = 0.004$, CBSC/BMSC, $P = 0.02$; *OSX*: USSC/BMSC, $P = 0.03$, CBSC/BMSC, not significant (n.s.); *BMP4*: USSC/BMSC, $P = 0.02$, CBSC/BMSC, n.s.; *OC*:



(a) (b)

Figure 1: PrimeView human gene expression array. The gene expression of USSC (green), CBSC (blue), and BMSC (red, each in triplicate) was analyzed. (a) Venn-diagram illustrating common and unique expression of genes. The gene expression was filtered for each cell type: a probeset had to be expressed above the background (20th and 100th percentiles of the raw signal distribution) in at least two out of three replicates. This resulted in 39519 transcripts for the "group USSC," 39493 transcripts for CBSC, and 39454 transcripts for BMSC which were compared. (b) principal component analysis (PCA) to depict the correlation of the single replicates of each cell type. Analyzed were those probesets that were expressed above the background in at least two out of three replicates in at least one out of three cell types which resulted in 40394 transcripts. The first three principal components accounted for 62.9% of the total variance (component 1 (x-axis): 31.6%; component 2 (y-axis): 18.3%; component 3 (z-axis): 13.0%).

USSC/BMSC, $P = 0.01$, CBSC/BMSC, $P = 0.03$; *PITX2*: USSC/BMSC, $P = 0.046$, CBSC/BMSC $P = 0.047$). Homolog of muscle segment homeobox *Drosophila* 2 (*MSX2*) was stronger expressed in USSC and BMSC, whereas CBSC revealed a reduced gene expression (Figure 2, unpaired t-test: USSC/CBSC, $P = 0.02$; CBSC/BMSC, $P = 0.02$). The qRT-PCR analysis of V-Myc avian myelocytomatosis viral oncogene homolog (*MYC*) revealed the strongest expression in USSC followed by CBSC. BMSC expressed *MYC* only slightly (Figure 2, unpaired t-test: USSC/BMSC, n.s.; CBSC/BMSC, $P = 0.02$).

BMSC showed a high expression level of the osteogenesis-related genes *BSP*, *OSX,* and *BMP4*. Taken into account that bone-marrow-derived stromal cells can still be denoted as the most reliable source for *in vivo* bone regeneration [22], these genes are potential candidate genes for the regulation of the osteogenic potential of a cell type. Thus, overexpression experiments of these genes were performed to assess the gene function in neonatal stromal cells.

3.3. Overexpression of BSP Resulted in Increased Calcification. With regard to the distinct expression pattern of *BSP* in CB-derived cell types compared to BMSC (Figure 2), lentiviral overexpression experiments were performed in two *BSP*-negative USSC cell lines. The transfection efficiency was proved via qRT-PCR (Figure 3(a), USSC1: 215927-fold stronger expression in relation to Mock control-cells, USSC2: 4185-fold stronger). The potential to differentiate towards adipocytes was not affected by the overexpression (data not shown). In contrast, the osteogenic *in vitro* differentiation potential was improved after *BSP* overexpression as analyzed by Von Kossa and Alizarin Red S staining (Figure 3(b)) with subsequent quantification (Figure 3(c)). Von Kossa staining revealed an intensified brown/black staining due to stronger calcification after overexpression of *BSP* compared to the nontransfected cells (Mock). The light microscopic photos of Alizarin Red S-stained cells after differentiation did not allow an interpretation of the amount of calcification; therefore, the quantity of bound Alizarin Red S dye was measured (Figure 3(c), USSC1: $P = 0.0977$, n.s.; USSC2: $P = 0.0165$, significant).

Overexpression of *BSP* supported the *in vitro* osteogenic differentiation of both USSC cell lines transfected. Thus, the expression of *BSP* should be taken into consideration when assessing the osteogenic potential of a cell type.

3.4. Overexpression of OSX Led to Decreased Osteogenic Differentiation Potential. Comparable to the expression of *BSP*, *OSX* was almost absent in USSC while a minimal expression was detected in CBSC (Figure 2). Thus, overexpression of *OSX* was performed in two USSC cell lines. Figure 4(a) illustrates the gene expression after *OSX* transfection (USSC1 Mock/pCL6*OSX*: 2831-fold increased expression, USSC2 Mock/pCL6*OSX*: 1223-fold). Due to the *OSX* overexpression,

Table 1: Osteogenesis-related genes expressed uniquely in USSC, CBSC, or BMSC. After Venn diagram analyses (Figure 1(a)), the genes expressed uniquely in one cell population (USSC: 304, CBSC: 251, and BMSC: 375) were assigned to gene ontology (GO) terms using the "Functional Annotation Cluster Tool" provided by DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/). Only those GO terms associated with "bone formation" are presented.

	Term	Genes
USSC	GO:0001503~ossification	*CHRDL2, TNFRSF11A, FGF9*
	GO:0060348~bone development	*CHRDL2, TNFRSF11A, FGF9*
	GO:0001501~skeletal system development	*CHRDL2, TNFRSF11A, FGF9*
CBSC	GO:0001503~ossification	*AMBN, MMP13*
	GO:0060348~bone development	*AMBN, MMP13*
	GO:0001501~skeletal system development	*AMBN, MMP13, HOXD10*
BMSC	GO:0001649~osteoblast differentiation	*IGF1, IGFBP5, SPP1*
	GO:0010810~regulation of cell-substrate adhesion	*BCL2, TDGF1, SPP1*
	GO:0001952~regulation of cell-matrix adhesion	*BCL2, TDGF1*
	GO:0060348~bone development	*BCL2, IGF1, IGFBP5, SPP1*
	GO:0001501~skeletal system development	*HOXC8, BCL2, IGF1, TFAP2A, COL12A1, IGFBP5, SPP1*
	GO:0030155~regulation of cell adhesion	*BCL2, TDGF1, SPP1*
	GO:0001503~ossification	*BCL2, IGF1, IGFBP5, SPP1*
	GO:0043062~extracellular structure organization	*NRCAM, COL14A1, CADM1, PCDHB16, PCDHB14, COL12A1*
	GO:0030199~collagen fibril organization	*COL14A1, COL12A1*
	GO:0030198~extracellular matrix organization	*COL14A1, COL12A1*

AMBN: ameloblastin (enamel matrix protein); *BCL2*: B-cell CLL/lymphoma 2; *CADM1*: cell adhesion molecule 1; *CHRDL2*: chordin-like 2; *COL12A1/14A1*: collagen type XII alpha 1/type XIV alpha 1; *FGF9*: fibroblast growth factor 9; *HOXC8/D10*: homeobox C8/D10; *IGF1*: insulin-like growth factor 1; *IGFBP5*: insulin-like growth factor binding protein 5; *MMP13*: matrix metalloproteinase 13; *NRCAM*: neuronal cell adhesion molecule; *PCDHB14/16*: protocadherin beta 14/16; *SPP1*: secreted phosphoprotein 1 (Osteopontin); *TDGF1*: teratocarcinoma-derived growth factor 1; *TFAP2A*: transcription factor AP-2 alpha; *TNFRSF11A*: tumor necrosis factor receptor superfamily, member 11a.

the expression of *RUNX2* (runt-related transcription factor 2) was down regulated (Figure 4(a), USSC1: −2.3-fold compared to Mock cells, USSC2: −1.7-fold), whereas the expression of *BSP* and *BMP2* (bone morphogenetic protein 2) increased (Figure 4(a), *BSP*: USSC1: 15.1-fold increased expression compared to Mock cells, USSC2: 1.8-fold; *BMP2*: USSC1: 2.1-fold, USSC2: 1.5-fold). The adipogenic differentiation potential was not affected by the overexpression of *OSX* (data not shown). In contrast, the mineralization during osteogenic differentiation was reduced in *OSX*-transfected USSC which was analyzed using Von Kossa and Alizarin Red S staining and quantification (Figures 4(b) and 4(c)). Von Kossa staining revealed a slight decrease of calcification in the cell line USSC1 after overexpression of *OSX* which was confirmed by quantification of the bound Alizarin Red S dye (Figures 4(b) and 4(c), $P = 0.0059$, very significant). A more pronounced decrease of calcification after overexpression was detected in the cell line USSC2. In Von Kossa as well as in Alizarin Red S staining, almost no calcification was measured which was confirmed by Alizarin Red S quantification (Figures 4(b) and 4(c), $P < 0.0001$, extremely significant).

OSX was described to be essential for bone formation [13]. Nevertheless, in the present study a reduced *in vitro* osteogenic capability of USSC after *OSX* overexpression associated with a decreased expression of *RUNX2* was detected. The upregulation of *BSP* and *BMP2* after overexpression did not support the osteogenic potential.

3.5. Overexpression of BMP4 Improved Chondro- and Osteogenesis In Vitro but Reduced the Ability to Form Adipocytes. The bone morphogenetic protein 4 (*BMP4*) was strongly expressed in BMSC, while the CB-derived cell populations exhibited a weaker expression (Figure 2). The lentiviral gene delivery of *BMP4* was accomplished in two USSC and two CBSC cell lines as proved by qRT-PCR (Figure 5(a), USSC2: 9381-fold stronger as Mock-cells, USSC4: 34598-fold stronger, CBSC1: 890-fold stronger, and CBSC3: 5714-fold stronger). The overexpression caused a reduced expression of *RUNX2* while *LRP5* (low-density lipoprotein receptor-related protein 5), which is associated with the WNT signaling pathway, showed an upregulation (Figure 5(a), *RUNX2*: USSC2: −1.7-fold compared to Mock cells, USSC4: −3.1-fold, CBSC1: −2.1-fold, CBSC3: −2.1-fold; *LRP5*: USSC2: 1.3-fold, USSC4: 2.8-fold, CBSC1: 1.6-fold, and CBSC3: 3.1-fold). The influence on the potential to differentiate into adipocytes, osteoblasts, or chondrocytes was assed via *in vitro* differentiation assays. The lack of the ability of USSC to differentiate into adipocytes [5] was not influenced by the overexpression of *BMP4*. On the contrary, the potential of CBSC to differentiate towards the adipogenic lineage was diminished after overexpression of *BMP4*, as assessed by Oil Red O staining and ImageJ-based quantification of the staining (Figures 5(b) and 5(c), unpaired *t*-test: CBSC1 Mock/pCL6*BMP4*, $P = 0.06$; CBSC3 Mock/pCL6*BMP4*, $P = 0.01$). *BMP4*-transfected USSC and CBSC secreted more proteoglycans during chondrogenic

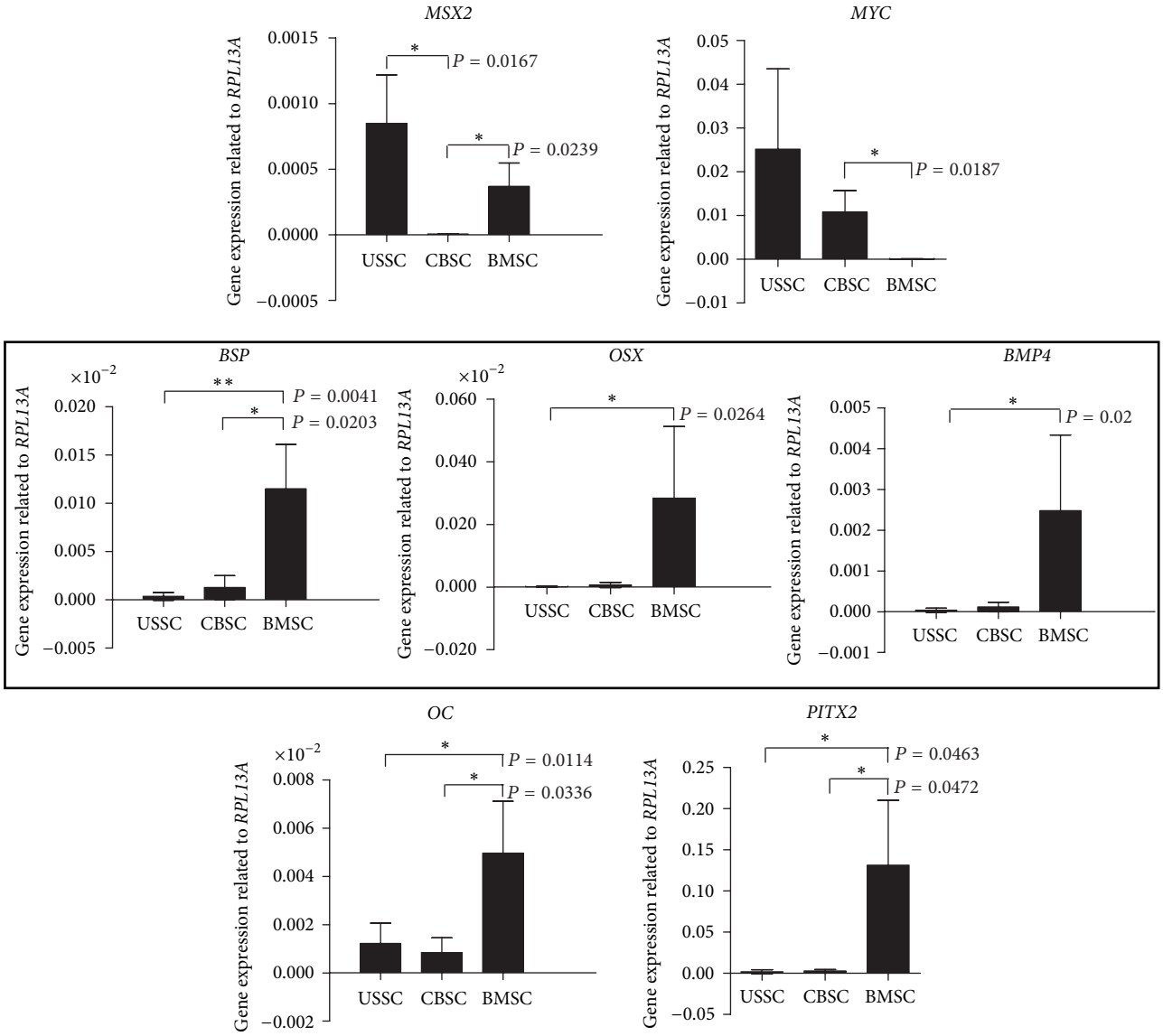


Figure 2: Quantitative RT-PCR analyses of genes expressed differentially in USSC, CBSC, and BMSC. Illustrated are the arithmetic means and standard deviations of at least 3 different cell lines per cell type. $^{*}P = 0.01$ to 0.05, significant; $^{**}P = 0.001$ to 0.01, very significant (unpaired t-test). *RPL13A* was used as housekeeping gene. Genes selected for overexpression experiments are highlighted. MSX2: homolog of muscle segment homeobox Drosophila 2, MYC: V-Myc avian myelocytomatosis viral oncogene homolog, BSP: integrin-binding sialoprotein, OSX (SP7): osterix, BMP4: bone morphogenetic protein 4, OC (BGLAP): osteocalcin, PITX2: paired-like homeodomain transcription factor 2, and RPL13A: ribosomal protein L13A.

differentiation in pellet culture illustrated by the intensified purple/red Safranin O staining (Figure 5(f)). Likewise, during osteogenic differentiation, transfected cells showed an enhanced calcification which was proved by Von Kossa and Alizarin Red S staining with subsequent quantification (Figures 5(d) and 5(e), USSC2: $P < 0.0001$, extremely significant; USSC4: $P = 0.0003$, extremely significant; CBSC1: $P = 0.0073$, very significant; CBSC3: $P = 0.0002$, extremely significant).

In summary, the overexpression of *BMP4* in two USSC and two CBSC cell lines resulted in a "switch" of the cell capability: the adipogenic differentiation potential was reduced, while the chondrogenic and osteogenic potentials were improved.

4. Discussion

The evaluation of the multilineage *in vivo* differentiation capacity of distinct stromal cell types is of particular importance, for example, in the field of bone tissue engineering regarding the applied cell source. Basis for these *in vivo* assays is the detailed analysis of the gene expression profile in combination with the *in vitro* differentiation potential to gain insight into the inherent capacity of a stromal cell and

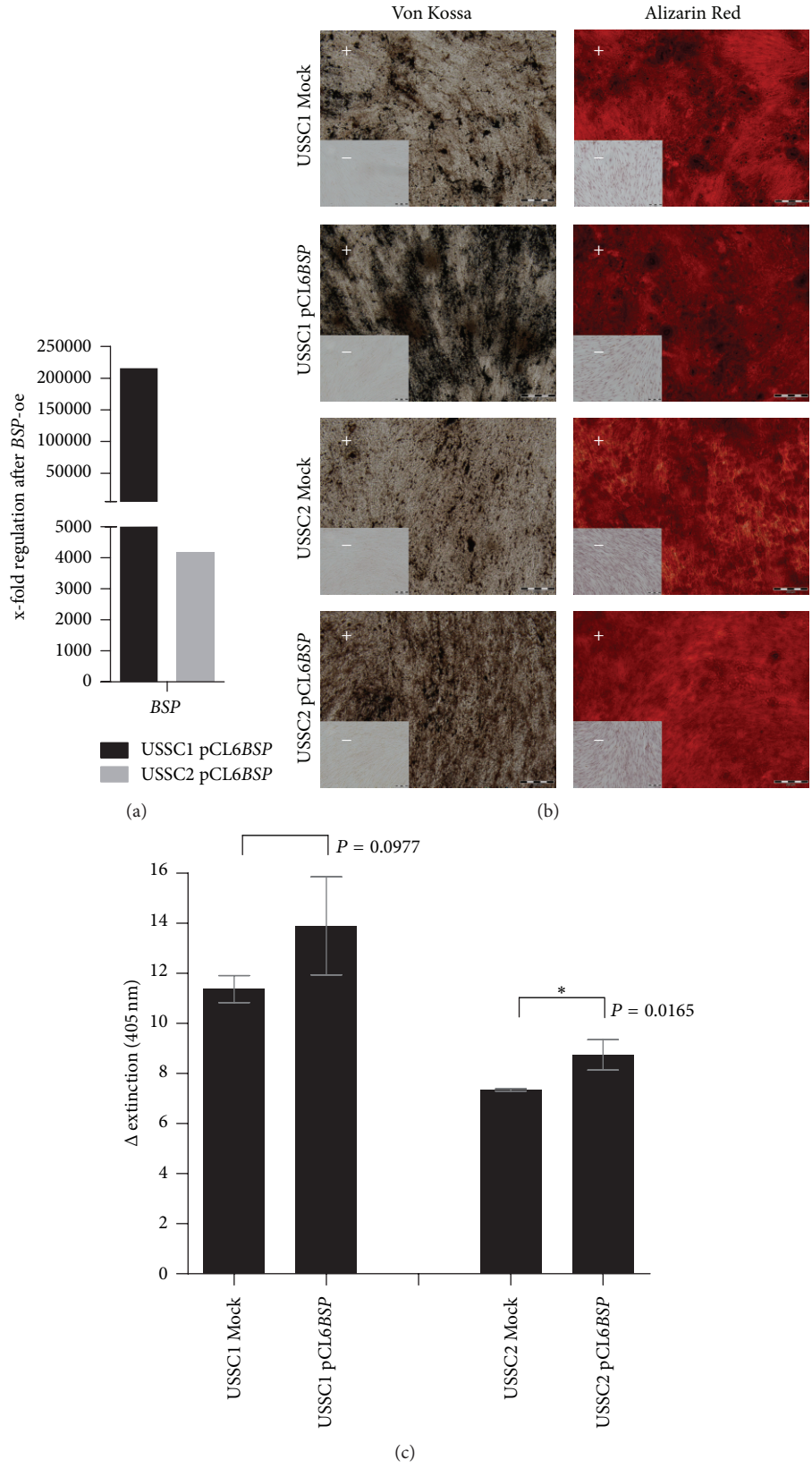
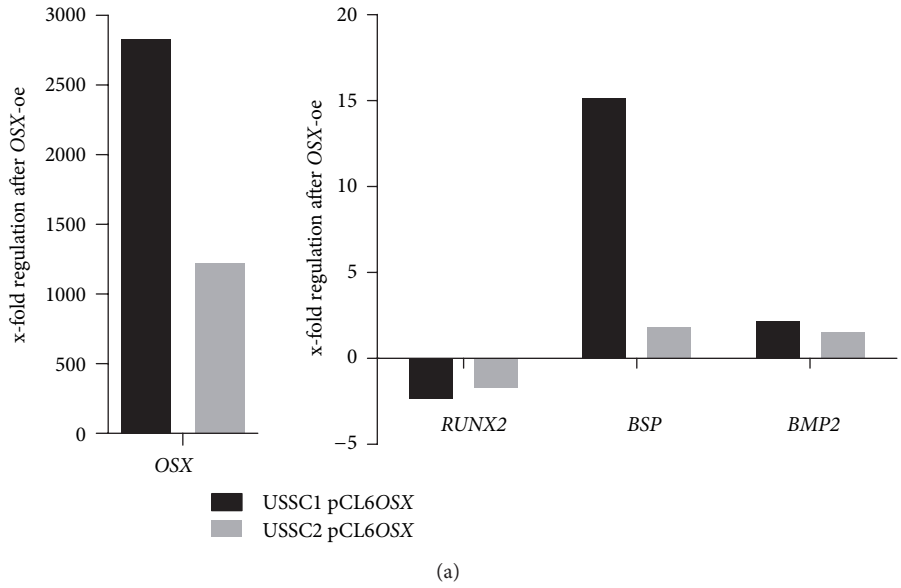
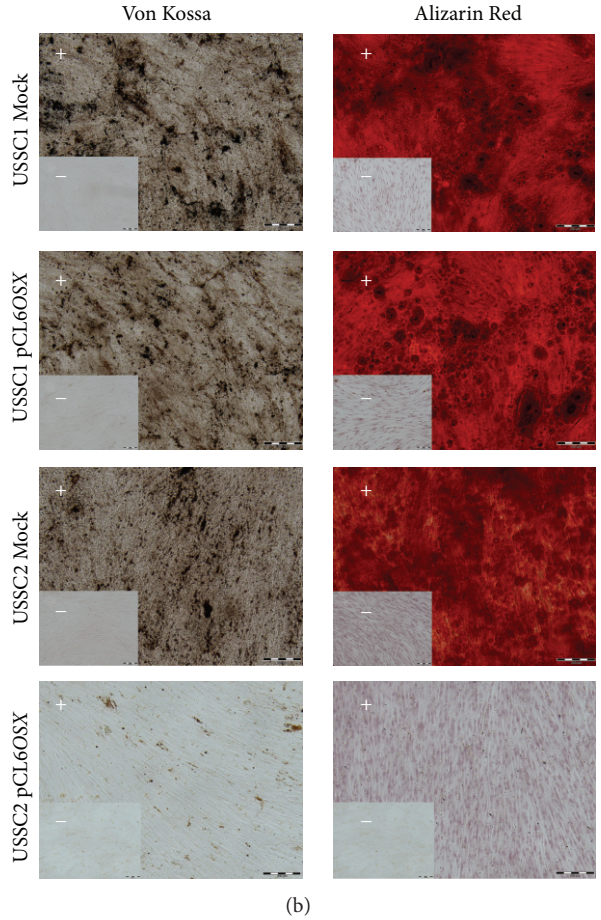


Figure 3: Lentiviral overexpression of *BSP* in two USSC cell lines. (a) Quantitative RT-PCR analysis of *BSP* gene expression (in undifferentiated cells) after overexpression in relation to the control cells (Mock). *RPL13A* was used as housekeeping gene. (b) Osteogenic differentiation experiments in overexpressed and Mock cells. After 14 days of differentiation, Von Kossa and Alizarin Red S staining were performed in the induced (+) and noninduced (−) cells. Mineralized areas are stained in brown/black or red, respectively. Scale bar: 200 μm. The results were confirmed in independent experiments; representative histological staining and the subsequent quantification of the bound Alizarin Red S-dye (c) are depicted. $^{*}P = 0.01$ to 0.05, significant (unpaired t-test). *BSP*: integrin-binding sialoprotein *RPL13A*: ribosomal protein L13A.



(a)



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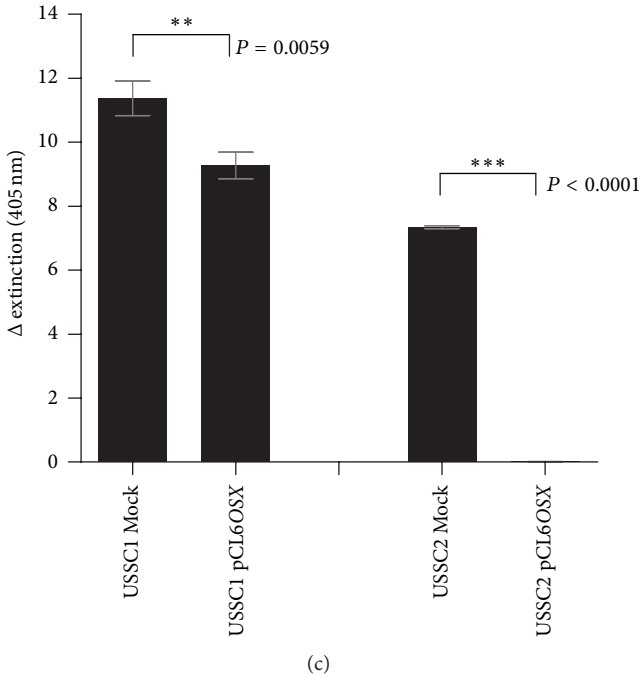

(c)

Figure 4: Lentiviral overexpression of *OSX* in two USSC cell lines. (a) Quantitative RT-PCR analysis of gene expression (in undifferentiated cells) after overexpression in relation to the control cells (Mock). *RPL13A* was used as housekeeping gene. (b) Osteogenic differentiation experiments in overexpressed and Mock cells. After 14 days of differentiation, Von Kossa and Alizarin Red S staining were performed in the induced (+) and non-induced (−) cells. Mineralized areas are stained in brown/black or red, respectively. Scale bar: 200 μm. The results were confirmed in independent experiments; representative histological staining and the subsequent quantification of the bound Alizarin Red S-dye (c) are depicted. $^{**}P = 0.001$ to 0.005, very significant; $^{***}P < 0,001$, extremely significant (unpaired t-test). *OSX (SP7)*: osterix, *RUNX2*: runt-related transcription factor 2, *BSP*: integrin-binding sialoprotein, *BMP2*: bone morphogenetic protein 2, and *RPL13A*: ribosomal protein L13A.

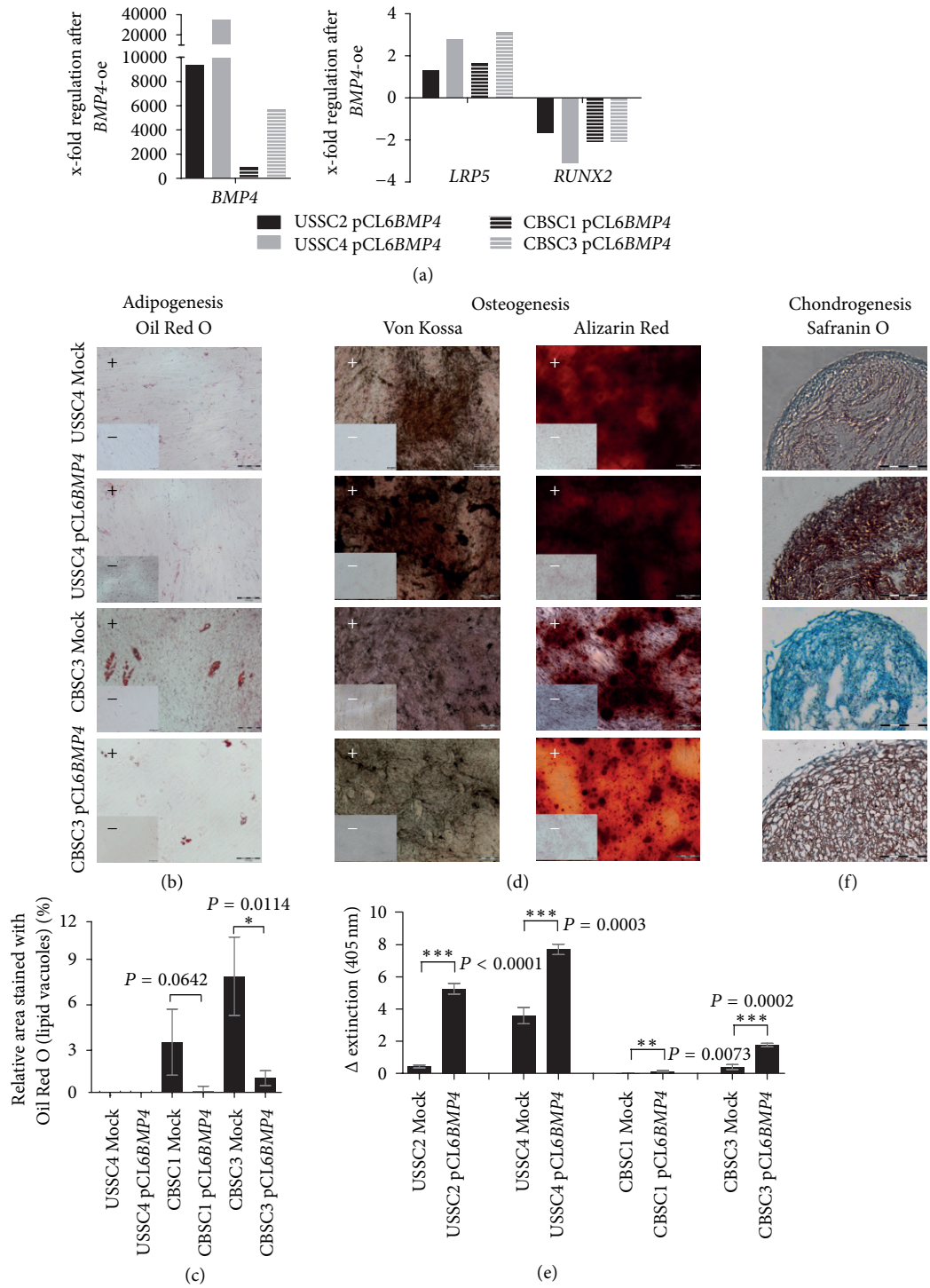

Figure 5: Lentiviral overexpression of *BMP4* in two USSC and two CBSC cell lines. With regard to the staining, only one representative cell line per cell type is shown. (a) Quantitative RT-PCR analysis of gene expression (in undifferentiated cells) after overexpression in relation to the control cells (Mock). *RPL13A* was used as housekeeping gene. (b): Representative illustration of the Oil Red O staining after adipogenic differentiation (21 d) of *BMP4*-transfected and control-cells. +: induced cells; −: non-induced cells. Lipid vacuoles are stained red. Scale bar: 100 μm. (c) ImageJ-based quantification of Oil Red O staining. $^{*}P = 0.01$ to 0.05, significant (unpaired t-test). (d) Osteogenic differentiation experiments in overexpressed and Mock cells. After 14 days of differentiation, Von Kossa and Alizarin Red S staining were performed in the induced (+) and non-induced (−) cells. Mineralized areas are stained in brown/black or red, respectively. Scale bar: 200 μm. (e) Subsequent quantification of the bound Alizarin Red S-dye. $^{**}P = 0.001$ to 0.005, very significant; $^{***}P < 0,001$, extremely significant (unpaired t-test). (f) Safranin O staining of transfected and control cells after chondrogenic differentiation in pellet culture for 21 days. Proteoglycans are stained purple/red. Scale bar: 200 μm. *BMP4:* bone morphogenetic protein 4, *LRP5*: low-density lipoprotein receptor-related protein 5, *RUNX2:* runt-related transcription factor 2, and *RPL13A*: ribosomal protein L13A.

to define genes relevant to the regulation of differentiation. Therefore, microarray gene expression analyses were performed in the present study to compare stromal cells from bone marrow (BMSC) and cord blood (USSC and CBSC). In a second step, genes expressed differentially in the cell populations were validated by quantitative RT-PCR analyses. Finally, three genes associated with cartilage/bone-formation (*BSP, OSX,* and *BMP4*) were overexpressed to assess their function in cord blood-derived stromal cells.

Microarray analyses (Figure 1) revealed a closer related gene expression pattern of USSC and CBSC reflecting their common cord blood origin. In contrast to the CB-derived cell types, the three BMSC cell lines revealed a higher variance in their expression profile (Figure 1(b)) which may be due to variances regarding donor age or sex. An "osteogenic signature" was determined for BMSC following microarray and qRT-PCR analyses (Figures 1 and 2) according to the *in vivo* role of these cells creating the endosteal niche which regulates the self-renewal and differentiation of hematopoietic stem cells [23]. In accordance with this biological function, BMSC expressed high levels of *PITX2* (Figure 2) which—beside other functions—were described to affect the hematopoietic supportive capacity of bone marrow stromal cells [24]. The osteogenesis-related genes *BSP*, *OSX*, *BMP4,* and *OC* revealed a stronger expression in BMSC compared to USSC and CBSC (Figure 2) which reflects the more immature status of the neonatal cord blood-derived stromal cells. Unlike BMSC, USSC and CBSC had a reduced predisposition towards skeletal development.

The phosphoprotein *BSP* is part of the human bone extracellular matrix [14]. *Bsp* expression has been described to be essential for the *in vivo* bone forming potential of murine BMSC [15]. *Bsp* knockout mice ($BSP^{-/-}$) displayed an impaired bone growth and mineralization associated with reduced bone formation [25]. In the present study, *BSP* was described as a gene expressed discriminatively between BMSC and the CB-derived cell types (Figure 2). *BSP* can be regarded as potential key player in the regulation of bone formation which was confirmed by the enhanced calcification of *BSP* overexpressing USSC in an *in vitro* osteogenesis assay (Figure 3). Furthermore, the increase in calcification corresponds to the study by Hunter and Goldberg, reporting that *BSP* initiates hydroxyapatite crystal formation during bone formation [26].

In contrast to *BSP*, the overexpression of the transcription factor *OSX* (*SP7*) in USSC resulted in a decreased *in vitro* osteogenic capacity (Figure 4), although *OSX* is commonly described as an essential regulator for bone formation [13]. *Osx* null mice display normal cartilage with mature hypertrophic chondrocytes but fail to form bone which highlights the specific role for *Osx* in the differentiation of osteoblasts [13, 27]. In the study herein, the overexpression of *OSX* caused a downregulation of upstream-located *RUNX2* in a negative feedback loop (Figure 4(a)). The enhanced expression of *BSP* and *BMP2* after transfection of *OSX* into USSC (Figure 4(a)) did not support the mineralization. Comparable results were presented in a study by Kurata et al. in human primary fetal stromal cells. Overexpression of *OSX* did not result in extracellular calcium crystals [28]. Likewise, Yoshida and coworkers overexpressed *Osx* in murine primary osteoblasts and reported a reduced mineralization at a late stage of osteoblast differentiation. Furthermore, *Osx* transgenic mice exhibited a reduced bone mineral density (osteopenia) [29]. In contrast, other overexpression studies in murine-adipose-tissue-derived stromal cells [30] or murine BMSC [31] reported an improved osteogenic potential. These controversial results may be due to the different cell types used for overexpression experiments which potentially reveal a distinct expression pattern of cofactors required to induce differentiation. One of these is *NFAT* (nuclear factor of activated T cells) which forms a complex with *OSX* to control osteoblastic bone formation [32].

BMP4 is a secreted signaling molecule that plays an essential role during embryogenesis [9, 33]. Regarding limb development, a threshold level of BMP signaling is required for early chondrogenic processes. The loss of both *Bmp2* and *Bmp4* during murine knockout experiments resulted in impaired osteogenesis [11]. In the present study, overexpression of *BMP4* supported the chondro- and osteogenic but reduced the adipogenic differentiation of human cells (Figure 5) which corresponds to previous studies in mice. Kan et al. described *Bmp4* transgenic mice which developed a phenotype characterized by progressive heterotopic bone formation [34]. Another report by Duprez and colleagues addressed the influence of ectopic retroviral overexpression of *BMP4* in developing chick limbs. The overexpression resulted in an increase in the volume of cartilage elements caused by an extended amount of matrix [35]. In the study herein, *BMP4* overexpression in human neonatal cells led to a reduced expression of *RUNX2* (Figure 5(a)) which suggests a *RUNX2*-independent stimulation of osteogenic differentiation. In contrast, the expression of *LRP5*, which functions as coreceptor of the Frizzled (Fzd)-receptors during canonical WNT signaling, was enhanced after *BMP4* overexpression (Figure 5(a)), indicating a role of WNT signaling in the *BMP4*-caused promotion of mineralization. Due to the fact that osteoblasts and adipocytes share a common progenitor, some differentiation factors, such as *PPARγ* (peroxisome proliferator-activated receptor gamma), are essential not only in the cell fate decision by induction of adipogenesis but also in suppression of osteogenic developmental processes [36, 37] or vice versa. The interpretation of the data demonstrated in the present paper indicates a potential role of *BMP4* in the cell fate decision of precursor cells promoting the differentiation towards the chondro-/osteogenic direction and suppressing the adipogenic differentiation.

In summary, the overexpression experiments suggested a supportive role for *BSP* and *BMP4* during *in vitro* osteoblast differentiation; *BMP4* additionally promoted the chondrogenic but diminished the adipogenic differentiation potential which indicates a role for *BMP4* in the cell fate determination towards the osteoblast lineage. In contrast, *OSX*-overexpression did not support mineralization.

Beside the *DLK1*- and *HOX*-gene expressions described by our group in former studies [5, 7], *MSX2* is a further gene expressed differently between USSC and CBSC (Figure 2). The expression was significantly stronger in USSC compared

to CBSC. Mice deficient in the homeobox gene *MSX2* displayed defects in endochondral bone formation [38]. Ichida and colleagues reported a promotive effect of *MSX2* on the differentiation of mesenchymal cells towards osteoblasts. In contrast, *MSX2* inhibited the expression of *PPARγ* resulting in a diminished adipogenic differentiation potential [39]. These data are consistent with our results reporting that USSC, which exhibit a strong osteogenic potential *in vitro* but are not able to differentiate into adipocytes, exhibited a high expression level of *MSX2*. In contrast, CBSC, which have a diminished potential to differentiate towards the osteogenic lineage *in vitro* but are able to form adipocytes, did not express *MSX2* (Figures 2–5 and [4]).

Contrary to *MSX2*, both CB-derived cell types strongly expressed *MYC* while BMSC exhibited only a slight expression (Figure 2). *MYC* encodes the transcription factor C-MYC which activates or represses genes involved in cell growth or cell cycle control. For example, C-MYC represses the expression of the growth arrest gene *GAS1,* hereby promoting cell proliferation [40]. This correlates with the extended growth potential of USSC and CBSC compared to the reduced growth potential of BMSC [4].

5. Conclusions

In contrast to bone-marrow-derived stromal cells, the cord blood-derived cell types USSC and CBSC lacked a signature related to skeletal development as shown by microarray gene expression and quantitative RT-PCR analyses. After overexpression experiments, *BSP* and *BMP4*, which were absent in the CB-derived cells, were defined as potential key players affecting the differentiation potential. *BSP* influenced the calcification during osteogenic differentiation assays. *BMP4* reduced the adipogenic potential but enhanced the secretion of proteoglycans during chondrogenic as well as the calcification after osteogenic differentiation assays. Thus, *BMP4* seems to determine the cell fate towards the chondro-/osteogenic lineage.

Understanding the influence of different signaling pathways that control differentiation is essential to predict the applicability of a distinct cell population for regenerative therapy. Hence, BMSC seem to be a cell source more suitable for bone tissue engineering approaches compared to USSC or CBSC which exhibited a more immature signature.

Conflict of Interests

The authors indicate no potential financial conflict of interests.

Acknowledgments

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Review Article

The Necessity of a Systematic Approach for the Use of MSCs in the Clinical Setting

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Cell therapy has emerged as a potential therapeutic strategy in regenerative disease. Among different cell types, mesenchymal stem/stromal cells have been wildly studied *in vitro*, *in vivo* in animal models and even used in clinical trials. However, while clinical applications continue to increase markedly, the understanding of their physiological properties and interactions raises many questions and drives the necessity of more caution and supervised strategy in their use.

1. Introduction

Since the discovery of pluripotent embryonic stem cells (ESCs) derived from the inner cell mass of blastocysts of embryos, stem cells have been defined by two principal characteristics: self-renewal and ability to differentiate in various cell types. The interest in stem cell use for clinical therapy and regeneration has been growing due to their ability to differentiate into various functional cell types. Among stem cells, two classes can be distinguished: pluripotent stem cells such as embryonic stem cells and induced pluripotent stem cells (IPSCs) [1] and multipotent stem cells with more restricted differentiation capacities, often referred to as adult stem cells. The source of ESCs and the methods used to generate IPSCs [2] together with the risk of teratoma formation [3] raise ethical and safety issues for the clinical use of ESCs and IPSCs [4]. Among adult stem cells, mesenchymal stem/stromal cells (MSCs) are the main seed cells used in regenerative medicine and are an expanding area of research, over the past decade, due to their unique biological properties. These properties cover a large spectrum ranging from immune modulation, local signaling to differentiation abilities. It has been demonstrated *in vitro* that MSCs can differentiate into osteoblast, chondrocyte, adipocyte, and hepatocytes/cardiomyocytes-like cells. But the use of these cells in numerous preclinical trials raises multiple questions/dilemmas that we will try to address in this review.

(i) Are these cells sufficiently defined and are they true stem cells?

(ii) Should MSCs isolated from different tissues be considered as equivalent?

(iii) What are their major characteristics?

(iv) Can we use them in clinical trials and if so what should be the context?

2. How Do We Define Mesenchymal Stem Cells?

2.1. Mesenchymal Stem Cells: Is It Appropriate? Nonhematopoietic cells in the bone marrow were first isolated by Friedenstein et al. in 1968 [5] and defined as spindle-shaped, fibroblast-like multipotent cells capable of colony-forming unit-fibroblast (CFU-F). The studies in the following decade better defined these cells and mainly focused on their abilities to sustain hematopoiesis [6–8]. Pittenger et al. demonstrated

their *in vitro* capacity to differentiate into various mesodermal cell types defining them as mesenchymal stem cells [9]. The bone marrow MSCs represent approximately 0,001 to 0,01% of bone marrow nucleated cells. Therefore, due to the low number of cells and the invasive method required to isolate them, alternative sources of MSCs have been investigated. Cells with similar proprieties have been isolated from a broad range of tissues like skin [10, 11], peripheral blood [12], umbilical cord blood [13], muscle [14], adipose tissue [15], placenta [16, 17], dental pulp [18] or liver [19], and others. The differences in isolation protocols and tissues of origin lead to numerous definitions and use of various terms to refer to these cells such as mesenchymal stem cells, mesenchymal progenitor cells, or mesenchymal stromal cells. The confusion in the definition and proprieties of isolated cells prompted the International Society for Cellular Therapy (ISCT) to establish a standard definition of MSCs in 2006 [20]. To qualify as MSCs a cell must have the following characteristics:

(1) plastic adherence;

(2) possess a trilineage differentiation capacity into adipogenic, chondrogenic, and osteogenic cells;

(3) present a surface expression of CD105 (endoglin, END), CD73 (ecto-5′-nucleotidase), and CD90 (Thy1) and the absence of the hematopoietic markers CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR.

This definition was broadly accepted by the scientific community and allowed more reproducibility between publications. We can underline that these cells were labeled mesenchymal "stromal" cells rather than "stem" cells. At present, MSCs are usually defined as positive for the following markers CD73, CD90, CD105, CD166, CD44, and CD29 and negative for CD14, CD34, CD31, and CD45 [21]. The principal challenge in the definition remains the absence of a single specific marker.

2.2. Mesenchymal Stem Cells: Stromal Cells or Fibroblast? MSC isolation from various sources hinders a precise characterization. In the attempt to better characterize MSCs, most studies have focused on isolation and characterization of surface markers that would define MSCs. The MSCs marker is comprehensively reviewed by Mafi et al. [22]. For example, STRO-1 has been documented as a potential MSCs marker [23] of true multipotent cells. However it appears that while Stro-1 is a robust marker for isolation of such MSCs from tissue like bone marrow, it is not expressed in adipose tissue derived MSCs [22, 24]. Furthermore, *in vitro* culture of MSCs induces modification and alteration of surface marker and capacities [22].

It is now broadly accepted that MSCs cultures represent a mix of various cells with various degrees of stemness. Indeed, the "stemness" of MSCs has been previously documented by Lee et al. and Muraglia et al. [25, 26] showing, through limiting dilution, that only some clones displayed multilineage differentiation potential and self-renewal. The rest of the so-called MSCs displayed only limited proliferation potential or partial differentiation ability. The multiclonality of cultured MSCs and their potential modifications is an important factor to consider. Results obtained with MSCs isolated from tissue should be cautiously discussed as few authors actually confirmed rigorously the stemness of the isolated cells.

Another issue is the potential contamination with fibroblasts. The similar shape and the fact that the two cell types share common surface markers are cofounding factors. In a comprehensive review Hematti highlighted that *ex vivo* culture-expanded MSCs and fibroblasts are indistinguishable by morphology, cell-surface markers, differentiation potential, and immunologic properties [27]. These findings increase the uncertainty of the identity of MSCs. Other than being a semantic debate, this highlights the importance of using the MSCs terminology cautiously. It may be safer, even if not optimal, to refer in most cases to these cell populations as mesenchymal "stromal" cells, as per ISCT definition.

3. How Different Are MSCs from Different Tissues?

As mentioned previously, MSCs have been isolated from a broad range of tissues. Following ISCT definition, the necessity to differentiate them into the three main lineages is now a standard for publication. Though, despite the ability to direct differentiation into those different lineages multiple reports demonstrate that their ability to differentiate depends on the tissue origin. For example, we demonstrated that MSCs isolated from bone marrow display around 400 genes differently expressed (at least 2 fold difference) when compared to MSCs isolated from fetal membranes. Their capacity to differentiate (even in the 3 lineages defined by ISCT) is consequently affected by this transcriptional variability [17]. Similarly, numerous publications documented the variable characteristics, differentiation capacities, and therapeutic effects of MSCs isolated from different tissues [28–35]. To explain such differences observed between cells displaying similar phenotype and abilities, multiple theories have been proposed. First, the tissue of origin can induce tissue-specific epigenetic modifications. Indeed, it is easily understandable that in its native organ contexture, an MSC is more likely to differentiate into a certain cell type. This might be pre-determined via chromatin modeling, histone acetylation, methylation, and phosphorylation. The genes involved in the differentiation into a particular cell type can therefore be activated immediately, while forcing this cell into a different cell type requires chromatin remodeling. MSCs isolated from fetal tissues (cord blood, placenta, amniotic membrane, etc…) might be an exception. In this case, the early developmental stage still without tissue-specific epigenetic modification can lead to higher plasticity [32, 34, 36–39].

The studies showing that MSCs from different organs have different properties prompt caution when comparing different preclinical and clinical trials using MSCs. One could advocate for the necessity to develop a panel of standard tests to be used systematically for MSCs characterization in the therapeutic context. Indeed the scientific community

needs clear standard protocols that will allow increasing reproducibility and ability to compare different studies. This will allow us to meet, in the next decade, the stringent requirements of regulatory authorities.

4. What Are the Optimal Uses of MSCs?

4.1. Immunosuppressive Proprieties. One of the most interesting proprieties of MSCs is their immune-modulatory capacity [40–44]. This immune-modulation effect has been extensively studied and reviewed, but certain aspects remain yet to be elucidate [45–49]. As a quick overview, inhibition of TNF-alpha, interferon-gamma, IL-10 and IDO, and nitric oxide production has been proposed to explain the suppression of T-cell proliferation by MSCs [45, 50]. Similarly, inhibition of B-cell proliferation and differentiation might be caused via similar mechanisms [51]. Besides the inhibition of B cells and T cells, activation by MSCs of Foxp3+ regulatory T cells was recently proposed [52]. Reduction of IL-1, CD40, and TNF-alpha together with production of prostaglandin E2 (PGE2) was proposed to explain reduction of monocyte and dendritic cell maturation [53]. Finally, NK cells proliferation and cytotoxicity have been demonstrated as inhibited *in vitro* by MSCs via PEG2 [54].

MSCs have other roles in the immune/inflammatory context. Indeed MSCs were proven to be chemo-attracted to sites of inflammation and to release proinflammatory cytokines [55]. The presence of functional toll-like receptors (TLRs), in particular TLR3 and TLR4 at the surface of MSCs, has been previously well documented. Those TLRs allow the recruitment of MSCs at the site of inflamed and damaged tissues. The TLRs also induce activation of proinflammatory signals and prevent the suppression of T-cell proliferation [56]. This mechanism was proposed to be Notch ligand mediated [56–58].

This bipolarity in MSCs action leads Waterman et al. [58] to propose a paradigm where, in analogy with monocyte/macrophage M1 and M2, MSCs can act as MSC1 type (proinflammatory) or MSC2 type (immunosuppressive). Though, the identification of the factors influencing the balance between those two functions is still yet to be determined.

Overall, the complex multiple mechanisms surrounding the immune-modulation effect of MSCs remain unclear in many aspects and are still being investigated. The multiplicity of interacting immune cells type and the multitude of mechanisms involved necessitate *in vivo* analysis of the involved mechanisms. Though, the discrepancy between animal and human immune system together with their MSCs differences renders a direct animal/human comparison as difficult.

4.2. Clinical Applications: Diverse Range of Trials for a Broad Range of Proprieties. MSCs have been tested in a wide range of organ traumas or diseases such as liver failure, hematopoietic stem cells (HSCs) implantation, bone trauma, spinal injury, brain trauma, Crohn's disease lesions, immune disease, kidney injury, articular cartilage, and cardiac regeneration [35, 59–65].

The rationale of all these trials was based on different properties of MSCs. Nevertheless, all those different characteristics of MSCs are complementary, and the improvements observed are most often the result of these cumulative effects. The various reported effects of MSCs are represented in Figure 1.

4.2.1. Clinical Use of Their Immune-Modulatory Effect. The immune-modulatory effect remains the most intriguing aspect of MSCs biology. This propriety has been widely studied and reviewed [45]. This led to numerous clinical trials for treatment of immune diseases. The main example is for treatment of graft versus host disease (GVHD). Use of MSCs gave promising results in phase 1 and 2 of clinical trials [66–68]. Indeed, Le Blanc et al. first transplanted haploidentical MSCs in a child with severe treatment-resistant grade IV. They also documented striking clinical response with a patient 1 year after treatment. Subsequently, Ringdén et al. in 2006 treated eight patients, with steroid-refractory GVHD, with MSCs. Acute GVHD resolved completely in six of eight patients. Complete cure was seen in gut (6 patients), liver (1 patient), and skin (1 patient). Their survival rate was significantly better than control patients [68]. Kebriaei et al. recently reported that out of the 31 patients treated, 94% showed an initial response to MSCs and 77% had a complete response.

However, mixed results came out from a larger scale phase III clinical trial including 192 acute GVHD patients [45]. In this study, even if no differences were found with the placebo, an improvement in gastrointestinal and liver outcome of these patients was observed. Nevertheless, the dose and frequency of administration in those GVHD patients were not homogeneous and might have impaired conclusive results.

Similarly, promising results were also obtained in preclinical and clinical phase I trials for Crohn's disease [59]. Duijvestein et al. indicated that autologous bone marrow-derived MSCs improve the clinical condition and showed a significant decrease in Crohn's disease activity index, 6 weeks after-treatment in 3 of 10 Crohn's disease patients [59].

In experimental autoimmune encephalomyelitis MSCs injection was reported to improve both condition and histological severity of the disease in multiple trials [69–71]. In multiple sclerosis, disability scale score improvement was observed in 5 patients and stabilization in 1 patient out of 10 included in the trial [72].

The immune-modulatory role of MSCs appears to be of primordial importance in their ability to prevent allograft rejection and was therefore tested in various cell-based therapies. Indeed, their use as immune-modulatory adjuvant to other cell therapies has been broadly tested in various animal models of degenerative diseases [61, 63, 64, 73–75]. Trials for cord blood hematopoietic stem cell engraftment in mice showed that CD45+ cells detected 3 weeks after transplantation were significantly higher in mice cotransplanted with human MSCs. At late time points evaluation (6 weeks) human cells engraftment was higher in the group where MSCs were cotransplanted. Similarly, islets cotransplantation with MSCs in mice demonstrated a significantly lower average blood

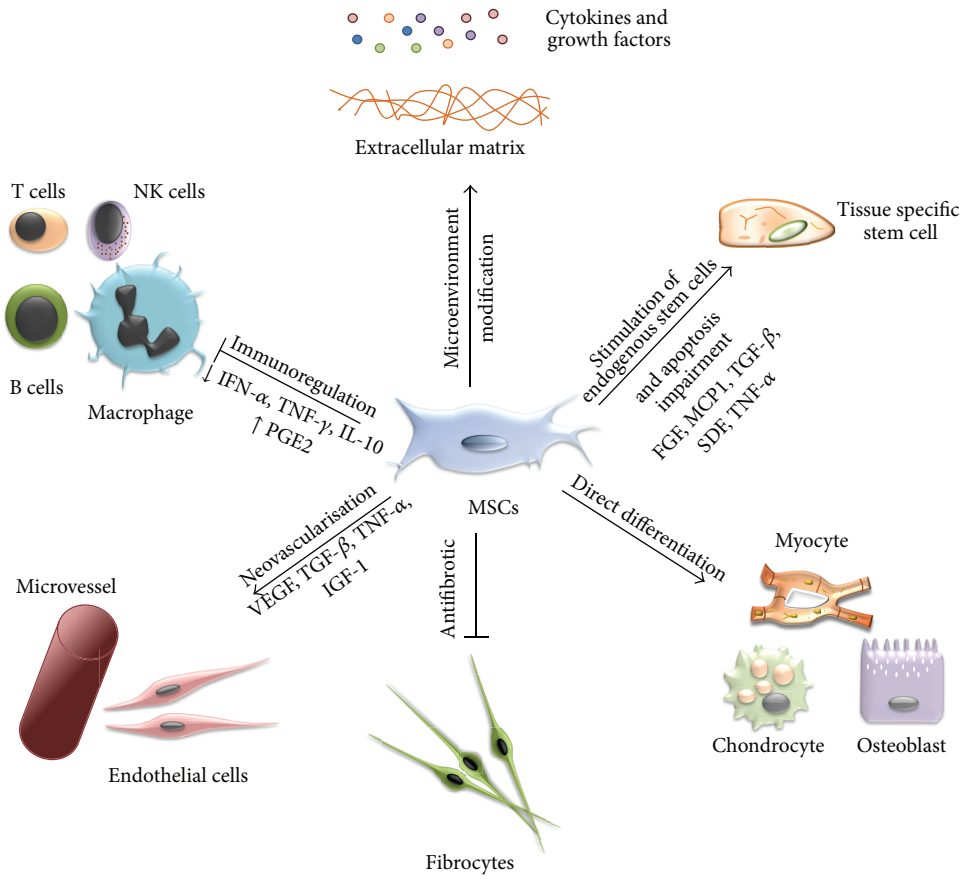

Figure 1: MSCs can enhance tissue regeneration via multiple mechanisms.

glucose concentration by 3 weeks. By week 6, 71% of the cotransplanted group was cured compared with 16% of the islet-alone group. All this work precluded the successful use of MSCs for cotransplantation assays with HSCs in clinical trials to prevent graft rejection [76–78]. For instance, in the first report, Muller et al. showed that a nine-year-old boy transfused with 2×10^6 cells/kg of MSC from the same HSCs donor. The patient remained alive and well three years later. Another fourteen-year-old girl received three doses of 0.4×10^6 cells/kg before the second HSCs transplant which engrafted properly, and she was disease-free two years later.

4.2.2. Regenerative Potential in the Clinical Setting. The main idea in the clinical use of MSCs remains the potential use of these cells in a regenerative context. Since MSCs were first isolated from bone marrow and their capacity to differentiate into osteoblasts is long known, MSCs turned naturally into a promising candidate in bone defect or trauma repair. Protocols for *in vitro* culture and differentiation into osteoblast have been perfected. Large bone area defects are usually repaired by scar tissue and often lead to complications such as nonunion. Different clinical trials showed that injection of MSCs alone is not sufficient [62, 79]. But a combination with scaffold demonstrated better outcomes in animal models and human preclinical trials [80–83]. For instance, four patients with large bone diaphysis defects were transplanted with ceramic scaffolds seeded with autologous bone marrow MSC. Complete fusion between the implant and the host bone was observed 7 months after surgery. All patients demonstrated a good integration of the implants in long-term followup. This study clearly established the advantage of a combined scaffold-cellular therapy as bone engineering approach. Proof of concept was also given when large portion of bones were to be replaced. In a clinical trial, three patients with loss of 4.0–7.0 cm bone segment were transplanted with MSC-seeded scaffolds. Abundant callus formation and good integration at the interfaces with the host bones were reported using radiography [81, 83].

The source of MSCs used played a critical role. In fact, it was demonstrated that MSCs isolated from bone marrow displayed greater capacity towards osteodifferentiation compared to MSCs isolated from adipose tissue [84, 85]. Similarly, we recently published that MSCs isolated from placenta better responded to osteoactivin (a potential adjuvant for bone reparation) stimulation for osteoblast differentiation than bone marrow derived MSCs [17]. In order to increase osteodifferentiation of injected MSCs, various promising components such as osteoactivin are tested and should be brought to preclinical and clinical trials [17, 86].

The other well-characterized ability of MSCs that demonstrates clinical potential is their ability to differentiate into

chondrocytes. MSCs are used as cellular treatment of cartilage defects [87, 88]. Preclinical and clinical trials mainly focused on treatment of osteoarthritis with MSCs alone or in combination with scaffolds or other additives [89–97] and demonstrated significant results, with *in vivo* chondrocyte differentiation of MSCs, motion improvement, pain relief, and promotion of cartilage repair after intra-articular injection [98].

Similarly, in liver failure, *in vitro* culture medium supplemented with growth factors is able to induce transdifferentiation of MSCs into functional hepatic cells producing albumin and urea with an ability to store glycogen [60, 99]. Clinical trial using MSCs for the treatment of fulminant hepatic failure, end-stage liver disease, cirrhosis, and inherited metabolic disorders also demonstrated encouraging results with restoration of hepatic function [100–103] and should be brought to larger scale trials.

In vitro and animal studies demonstrated that under an appropriate environment and/or stimulus, MSCs could differentiate into polynucleated myotubes, consistent with a myocyte lineage [104–108]. Animal models showed implantation and differentiation of MSCs in normal or post-myocardial infracted hearts. Successful engraftment was demonstrated by observing the MSCs implantation into scarred myocardium, as well as their expression of α-actin, tropomyosin, troponin T, myosin heavy chain, connexin-43, GATA-4, and Nkx2.5 [109–111]. Various procedures of administration of MSCs have been tested: intravenous, intracoronary, catheter-based intramyocardial, or direct intramyocardial injection. Based on those results, a broad range of clinical trials were performed for acute myocardial infarction, ischemic cardiomyopathy, or chronic ischemic left ventricular dysfunction [112–115] with marked improvement of cardiac function and patients' outcome. For instance, a randomized double-blind placebo controlled dose escalation study of MSCs administration after acute myocardial infarction in 53 patients demonstrated first the safety of MSCs injection and preliminary efficacy data [114] with reduced ventricular arrhythmias ($P = 0.025$) and improved pulmonary function ($P = 0.003$) in patients receiving MSCs. In a subset analysis, patients with an anterior acute myocardial infarct had improved ventricular function (ejection fraction) compared with the placebo cohort. It seems that more than a direct transdifferentiation into cardiomyocytes, the benefit observed in those studies relied on other MSCs properties.

4.2.3. MSCs Paracrine Effects. It is now accepted that a major indirect effect of MSCs after implantation is related to their so-called paracrine effect. Through a broad spectrum of cytokines and growth factors, MSCs were proposed to drive tissue recovery via stimulation of endogenous stem cells, apoptosis impairment, stimulation of neovascularization, and extracellular matrix modification, together with reduction of fibrosis and scar tissue formation [116–118].

Bone marrow derived MSCs have been described as important actors in HSCs niche in bone marrow [119, 120]. MSCs have been tested as adjuvant to HSCs engraftment. It is clearly established that cotransplantation of HSCs with MSCs decreases the risk of rejection and increases the long-term repopulation. More than direct interaction with injected HSCs, it is proposed that the large spectrum of released molecules is responsible for acute engraftment [61].

We previously discussed that restoration of hepatic function is achieved following MSCs injection; however, the rate of long-term implantation of the cells is low [121, 122]. Following these results, recent studies demonstrated that MSC-conditioned medium or MSC-derived molecules also demonstrated important positive results comparable to direct MSCs transplantation [102, 123].

Similarly to liver studies, it was demonstrated that MSCs conditioned media could improve cell survival and prognostic when injected into an infracted heart [124]. Additionally, more than direct implantation and differentiation of MSCs, the paracrine effect of MSCs has been postulated to contribute to improve endogenous cell survival, cardiogenesis stimulation of inner progenitor, and neovasculogenesis of infracted regions [65, 117, 124–126]. The scaring process of infracted tissue is tuned down after MSCs injection, most probably due to their capacity of extracellular matrix modification.

5. Limitations and Caution in Clinical Use of MSCs

Despite all the promising results published and reporting improvement following MSCs injection in various models, numerous area of uncertainty remains. First of all, data on long-term efficacy are still missing in many contexts. Mostly short-term followup has been published to date, and even long-term rodent studies are by nature limited.

In vitro culture of MSCs previous to all clinical trials engenders different risks. *In vitro* culture can modify cell characteristics. There is always a risk of viral, bacterial, or primal infections [127, 128]. Thus the requirement to develop standard procedures within highly regulated GMP laboratories.

Another poorly documented risk with MSCs injection is the migratory potential of MSCs. MSCs have been shown to display significant migration following stimulation with numerous factors such IL8, VEGF, and IGF [129]. For example, a study in rabbits showed that MSCs injected in the articulation could be later found in digestive tractus and thymus [130]. This ectopic implantation of MSCs has been shown to result in bone formation in rodent studies [131, 132]. Finally the inability to control the differentiation potential might lead to complications such as bone differentiation within ectopic tissue such as the heart in preclinical models [133].

Finally, many reports show a fundamental role of MSCs in tumor malignant transformation and progression [134, 135]. It was recently established that, even if limited, long-term culture of MSCs leads to chromosomal aberrations [136, 137] leading to the risk of injection of cells with carcinogenic potential [138].

6. Discussion

In summary, the past 5 to 10 years have been remarkably active for MSC studies. Even if MSCs are revealed to be a strong tool with various convenient properties and promising potential, additional initiatives should be undertaken to further accelerate the process of enhancing our understanding of MSC biology *in vivo*. Additionally, appropriately designed clinical trials with multicentric randomized trials should be achieved to clarify results and allow comparison of the various trials leaded. Unlike in animal models, followup of engraftment and MSCs persistence remains complex in human clinical trials and remains the point of focus of multiple technological developments. Creation of trial database with long-term followup would help in monitoring secondary deleterious effect of MSCs administration to patients.

Even if MSCs injection demonstrated encouraging improvement of patient's conditions, where classical treatment fails, monitoring long-term clinical outcome is of primary importance. Finally, acute understanding of molecular mechanism and factors involved in MSCs injection benefice may lead to a safer replacement of MSCs by controlled molecular therapy with similar outcomes.

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Research Article

Developing a New Two-Step Protocol to Generate Functional Hepatocytes from Wharton's Jelly-Derived Mesenchymal Stem Cells under Hypoxic Condition

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The shortage of donor livers and hepatocytes is a major limitation of liver transplantation. Thus, generation of hepatocyte-like cells may provide alternative choice for therapeutic applications. In this study, we developed a new method to establish hepatocytes from Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) cell lines named WJMSCs-SUT1 and WJMSCs-SUT2 under hypoxic condition. This new method could rapidly drive both WJ-MSCs cell lines into hepatic lineage within 18 days. The achievement of hepatogenic differentiation was confirmed by the characterization of both phenotypes and functions. More than 80% MSCs-derived hepatocyte-like cells (MSCDHCs) achieved functional hepatocytes including hepatic marker expressions both at gene and protein levels, glycogen storage, low-density lipoprotein uptake, urea production, and albumin secretion. This study highlights the establishment of new hepatogenic induction protocol under hypoxic condition in order to mimic hypoxic microenvironment in typical cell physiology. In conclusion, we present a simple, high-efficiency, and time saving protocol for the generation of functional hepatocyte-like cells from WJ-MSCs in hypoxic condition. The achievement of this method may overcome the limitation of donor hepatocytes and provides a new avenue for therapeutic value in cell-based therapy for life-threatening liver diseases, regenerative medicine, toxicity testing for pharmacological drug screening, and other medical related applications.

1. Introduction

Orthotopic liver transplantation has been shown to be an effective treatment for patients with end stage of liver dysfunction. However, this treatment is limited by the shortage of donor organs. Although hepatocytes transplantation has been shown to be successful treatment in some conditions such as liver-based metabolic disorders, the insufficient donor organs and hepatocytes remain obstacles for this technique [1]. Recently, stem cells are a promising tool for using as cell-based therapy because of their superior properties including self-renewal and broad differentiation potential into several cell types. To date, mesenchymal stem cells (MSCs) have been shown to obtain promising capacity not only multilineages differentiation potential but also immunomodulatory properties [2]. In addition, MSCs can be extensively expanded *in vitro*, due to effective cryopreservation and being easy to access from various sources such as bone marrow, adipose tissue, amniotic fluid, umbilical cord Wharton's jelly, and placenta [3–6]. These make MSCs become a good stem cell candidate for therapeutic purpose in clinical applications.

Umbilical cord Wharton's jelly is an enriched source of MSCs which has superior advantages over the other sources such as noninvasive collection, less ethical concern, and enrichment with MSCs [7]. Several studies have demonstrated that MSCs from various sources including Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) possess hepatogenic differentiation potential both *in vitro* and *in vivo* [8–13]. Interestingly, hepatocyte-like cells generated from adipose tissue-derived MSCs showed therapeutic effect on mice models with both acute liver failure and chronic liver

injury [14, 15]. Recent study also reported clinical improvement in patients with end-stage liver failure by chronic hepatitis C after transplantation with bone marrow-derived hepatocyte-like cells [16]. Based on these data, generation of hepatocyte-like cells from MSCs shows great potential in clinical use as regenerative medicine.

According to previous published protocols, several studies have shown expensive cost, time consuming, and multiple steps induction of MSCs into hepatic lineage [10, 17–21]. Therefore, a simpler method is needed for developing an effective protocol to generate functional hepatocyte-like cells from MSCs. In this study, we developed a new method to induce WJ-MSCs cell lines, WJMSCs-SUT1, and WJMSCs-SUT2, into hepatic lineage followed by characterization of the MSCs-derived hepatocyte-like cells (MSCDHCs) at both cellular and molecular levels. Here, we show the achievement of hepatogenic differentiation of WJ-MSCs by using our new induction protocol under hypoxic condition. The hepatocyte-like cells generated from WJ-MSCs offer an alternative source of functional hepatocytes which will provide great advantages in liver disease treatments, drug discovery including toxicological research, and other medical applications.

2. Materials and Methods

2.1. Cell Lines. Two human WJ-MSCs cell lines, WJMSCs-SUT1 and WJMSCs-SUT2, were established and well characterized by Dr. Wilairat Leeanansaksiri's laboratory (Suranaree University of Technology, Thailand). WJMSCs-SUT1 and WJMSCs-SUT2 were derived from the cultivation of WJ-MSCs in Dulbecco's modified Eagle's medium with 1.0 g/L glucose (DMEM-LG) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and embryonic stem cells conditioned medium (ESCM) at 37°C with 5% CO_2 and 5% O_2, respectively. Both cell lines express cell surface markers: $CD29^+$, $CD44^+$, $CD90^+$, $CD34^-$, and $CD45^-$, and contain capacity of differentiation into osteocytes, chondrocytes, and adipocytes as standard characteristics of mesenchymal stem cells.

2.2. Induction of Hepatogenic Differentiation In Vitro. The WJMSCs-SUT1 and WJMSCs-SUT2 were expanded until reaching 90% confluence and then subjected into hepatocytes differentiation processes by using our new two-step protocol (Figure 1). Briefly, the WJMSCs-SUT1 and WJMSCs-SUT2 were separately incubated in stage 1 differentiation medium containing serum-free DMEM-LG supplemented with 100 U/mL penicillin (Sigma, St. Louis, MO, USA), 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA), 2 μg/mL amphotericin B (Bristol-Myers Squibb, NY, USA), 20 ng/mL human hepatocyte growth factor (HGF) (Peprotech, Rocky Hill, NJ, USA), 10 ng/mL fibroblast growth factor 4 (FGF-4) (Peprotech, Rocky Hill, NJ, USA), and 5 mM nicotinamide (Sigma, St. Louis, MO, USA) for 7 days. Subsequently, these cells were further cultured in stage 2 differentiation medium to achieve hepatic maturation which had components as serum-free DMEM-LG supplemented with 100 U/mL penicillin (Sigma, St. Louis, MO, USA); 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA); 2 μg/mL amphotericin B (Bristol-Myers Squibb, NY, USA); 40 ng/mL oncostatin M (OSM) (Sigma, St. Louis, MO, USA); 2 μM dexamethasone (Sigma, St. Louis, MO, USA), and 20 μl/mL insulin, transferrin, and selenium (ITS + premix) (BD Biosciences, Franklin Lakes, NJ, USA) for up to 18 days. The differentiation medium was changed twice a week. The undifferentiated cells from both 2 cell lines were included as negative controls.

2.3. Immunofluorescence Staining. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 20 minutes. Nonspecific immunostaining was prevented by 30 minutes incubation of the cells in PBS solution containing 3% bovine serum albumin (BSA) (Invitrogen, Carlsbad, CA, USA) at room temperature. Cells were then incubated in blocking solution overnight at 4°C with primary antibodies as follows: rabbit polyclonal anti-human albumin (ALB) and mouse monoclonal anti-human cytokeratin 18 (CK-18) (all 1 : 100) (DakoCytomation, Glostrup, Denmark). Cells were washed 3 times with PBS and incubated for 1 hour at room temperature with the secondary antibodies FITC-coupled polyclonal goat anti-mouse immunoglobulin G (1 : 100) (BD Biosciences, Franklin Lakes, NJ, USA) or FITC-coupled polyclonal swine anti-rabbit immunoglobulin G (1 : 100) (DakoCytomation, Glostrup, Denmark). Nuclei was revealed by 3 minutes of staining with the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA). After 3 washes, cells were mounted with antifading solution (Vector Laboratories, Burlingame, CA, USA) and examined under fluorescence microscope (BX51) (Olympus, Japan).

2.4. Periodic Acid-Schiff (PAS) Staining. After 4% paraformaldehyde fixation, cells were incubated for 5 minutes in 1% periodic acid (Sigma, St. Louis, MO, USA) and washed with distilled water prior to incubation with Schiff's reagent (Sigma, St. Louis, MO, USA) for 15 minutes. After 5-minute wash in tap water, hematoxylin counterstain was performed for 1 minute. Cells were washed and visualized under light microscope (CKX41) (Olympus, Japan).

2.5. Uptake of Low-Density Lipoprotein Assay. The uptake of low-density lipoprotein (LDL) was detected with the Dil-Ac-LDL staining kit (Biomedical Technologies, Stoughton, MA, USA). The assay was performed according to the manufacturer's instructions. Briefly, cells were incubated in serum-free DMEM-LG containing 10 μg/mL 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-acetylated-LDL (Dil-Ac-LDL) for 4 hours at 37°C. Cells were then washed and visualized under fluorescence microscope (BX51) (Olympus, Japan).

2.6. Urea Assay. Cells were incubated with serum-free DMEM-LG containing 5 mM NH_4Cl (Sigma, St. Louis, MO, USA) for 24 hours. Urea concentrations in supernatants were measured by colorimetric assay as QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions with an absorbance reader

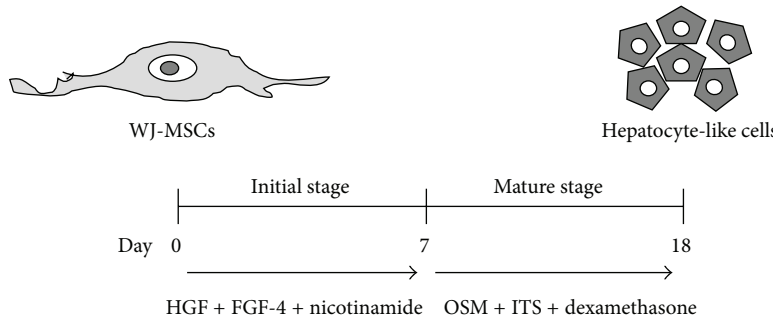

Figure 1: New method for hepatocyte-like cells generation from WJ-MSCs under hypoxic condition. This schematic figure represents two-step protocol for *in vitro* hepatogenic differentiation. In the first stage of induction, WJMSCs-SUT1 and WJMSCs-SUT2 were treated with the combination of HGF (20 ng/mL) + FGF-4 (10 ng/mL) + nicotinamide (5 mM) in DMEM serum-free medium for 7 days. To induce maturation, these cells were cultured in differentiation medium containing OSM (40 ng/mL) + ITS (20 μL/mL) + dexamethasone (2 μM) for a further 11 days.

Table 1: List of primer sequences used in this study.

Gene	Primer sequence (5′ to 3′)	Annealing Temp.	Size (bp)	Reference
AFP	GCTTGGTGGTGGATGAAACA TCCTCTGTTATTTGTGGCTTTTG	62	157	[22]
ALB	TGAGAAAACGCCAGTAAGTGAC TGCGAAATCATCCATAACAGC	62	265	[22]
CYP3A4	CCTTACAT TACACACCCTTTGGAAGT AGCTCAATGCATGTACAGAATCCCCGGTTA	62	382	[22]
HNF4α	GCCTACCTCAAAGCCATCAT GACCCTCCCAGCAGCATCTC	62	275	[22]
AAT	ACATTTACCCAAACTGTCCATT GCTTCAGTCCCTTTCTCGTC	56	183	[23]
TTR	GGTGAATCCAAGTGTCCTCTGAT GTGACGACAGCCGTGGTGGAA	61	352	[24]
G6P	GCTGGAGTCCTGTCAGGCATTGC TAGAGCTGAGGCGGAATGGGAG	56	350	[23]
TAT	CCCCTGTGGGTCAGTGTT GTGCGACATAGGATGCTTTT	56	345	[23]
GAPDH	AGCCACATCGCTCAGACACC GTACTCAGCGGCCAGCATCG	60	302	[25]

ALB: albumin; *AFP*: alpha-fetoprotein; *AAT*: alpha-1-antitrypsin; *CYP3A4*: cytochrome P450 type 3A4; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *G6P*: glucose-6-phosphatase; *HNF4α*: hepatocyte nuclear factor 4α; *TAT*: tyrosine aminotransferase; *TTR*: transthyretin; Temp.: temperature; bp: base pair.

(Benchmark Plus) (Bio-Rad Laboratories, Hercules, CA, USA). Undifferentiated cells (day 0) and human hepatocellular carcinoma cell line (HepG2) were represented as a negative control and positive control, respectively.

2.7. Albumin Assay. After accomplishment of the differentiation, albumin concentrations in supernatants were screened by ELISA assay as AssayMax Human Albumin ELISA Kit (Assaypro, St. Charles, MO, USA) according to the manufacturer's instructions with an absorbance reader (Benchmark Plus) (Bio-Rad Laboratories, Hercules, CA, USA). Cell culture supernatants from undifferentiated cells (day 0) and HepG2 were represented as a negative control and positive control, respectively.

2.8. RNA Extraction and RT-PCR. Cells were subjected to total RNA extraction by using Total RNA Mini Kit (Tissue) (Geneaid, Taiwan) and RNase inhibitor treatment (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cDNA was generated by RevertAid First Stand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol. The corresponding cDNA was added into PCR master mix containing 10X PCR buffer, 1 U Taq polymerase, 25 mmol/L $MgCl_2$, 10 mmol/L dNTP mixed, and 10 μmol/L of each primer set for the corresponding target gene. The used primer sequences were shown in Table 1. Amplification conditions were as follows: initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 56–62°C for 30 seconds (see Table 1 that refers to temperatures used), extension for 1 minute at 72°C, and a final extension at 72°C for 10 minutes. The samples were separated on a 2% agarose gel, stained with ethidium bromide, and photographed under UV light. Undifferentiated cells and HepG2

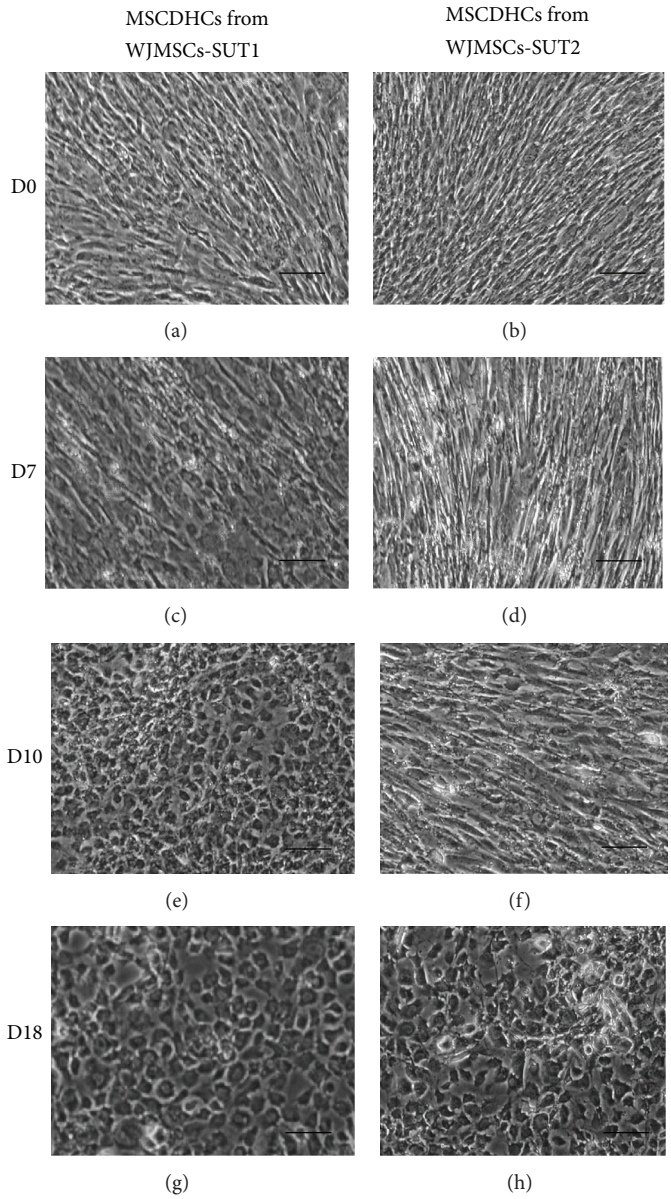

Figure 2: Hepatocyte-like cells feature of the differentiated cells during hepatogenic induction for 18 days. Morphological changing of MSCDHCs generated from WJMSCs-SUT1 ((a), (c), (e), and (g)) and WJMSCs-SUT2 ((b), (d), (f), and (h)) were observed following the differentiation period. Original magnifications: ×100 for all pictures. Bars indicate 100 μm.

were represented as a negative control and positive control, respectively.

2.9. Statistical Analysis. All data were represented as mean ± standard deviation (SD) calculation. Statistical analysis was calculated by statistical software SPSS17.0 (SPSS Inc., Chicago, IL, USA) and results were analyzed by Student's t-test with significance at $P < 0.05$.

3. Results

3.1. Morphology of MSCs-Derived Hepatocyte-Like Cells (MSCDHCs). Morphological changing of the cells was determined on day 0, 7, 10, and 18 following differentiation. Upon induction, we observed morphological changing from fibroblastic cells of WJ-MSCs into polygonal round cells of hepatocytes feature. More than 80% of both WJMSCs-SUT1 and WJMSCs-SUT2 could be induced into hepatocyte-like cells morphology at the end of induction. Moreover, WJMSCs-SUT1 changed morphology from fibroblastic shape into epithelial round shape earlier on day 10 of differentiation whereas WJMSCs-SUT2 showed this feature on day 12 (Figure 2). For the control, both 2 cell lines did not show spontaneous differentiation into hepatocyte-like cells during the culture periods (data not shown).

3.2. Expression of Hepatic-Lineage Markers. The mRNA expressions of hepatic-lineage markers were determined

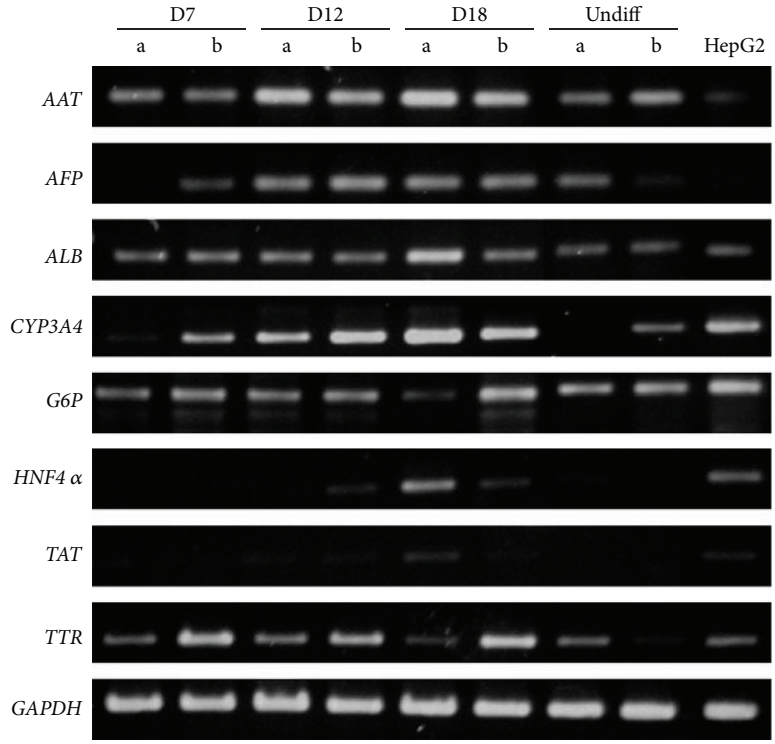

Figure 3: Hepatic-specific gene expressions of the differentiated cells after induction for 18 days. Total RNA was isolated from MSCDHCs generated from WJMSCs-SUT1 (lane a) and WJMSCs-SUT2 (lane b) following the differentiation period, and mRNA expressions of hepatic-specific genes were analyzed by RT-PCR. HepG2 was used as positive control. *GAPDH* was used as an internal control.

at different time points during differentiation periods. We found that undifferentiated stage of WJMSCs-SUT1 and WJMSCs-SUT2 could express low level of some hepatic-specific genes transcripts including *AAT*, *ALB*, *CYP3A4*, and *G6P* (Figure 3). Upon induction, MSCDHCs generated from both 2 cell lines showed the significant upregulation of mature hepatic markers, *AAT* and *CYP3A4*, in a time-dependent manner. Additionally, we observed correlation between the expressions of key hepatic transcription factors, *HNF4α*, and mature hepatic marker *ALB* in MSCDHCs generated from both 2 cell populations. Interestingly, in late stage of differentiation, the WJMSCs-SUT2 had faster response to hepatic-lineage induction than WJMSCs-SUT1 did. This result was supported by the early expression of *HNF4α* transcripts on day 12 while it was undetectable in MSCDHCs from WJMSCs-SUT1 on the same day. However, both the WJMSCs-SUT1 and WJMSCs-SUT2 could express all tested hepatic-lineage markers at mRNA level at the end stage of differentiation period (Figure 3).

We further analyzed more mature hepatic markers, albumin (ALB), and cytokeratin 18 (CK-18), at protein level. The low expression of CK-18 was observed in undifferentiated stage (day 0) of these 2 cell lines (Figures 4(i) and 4(m)). Following differentiation, more than 80% MSCDHCs generated from both 2 cell populations showed the significant upregulations of ALB (Figures 4(a)–4(h)) and CK-18 (Figures 4(i)–4(p)) in a time-dependent manner. These findings suggest that both WJMSCs-SUT1 and WJMSCs-SUT2 could also express hepatic-lineage markers at protein level in response to our induction medium in addition to the other hepatic mRNA expressions. Taken together, these results demonstrate that WJMSCs-SUT1 and WJMSCs-SUT2 could be induced into hepatocyte-like cells feature not only in a cellular phenotype aspect but also in hepatic marker expressions both at mRNA and protein levels.

3.3. Hepatocytes Functions of MSCs-Derived Hepatocyte-Like Cells. The biological functions of hepatocytes were evaluated in the differentiated cells at various stages upon differentiation. First, the capacity of glycogen storage was analyzed by PAS staining. The results showed low level of PAS staining in undifferentiated stage (day 0) of WJMSCs-SUT1 and WJMSCs-SUT2. However, upon induction, MSCDHCs generated from both 2 cell lines displayed significant positive staining of glycogen granules in their cytoplasm as early as on day 7 and more stronger staining on day 12 and the strongest at day 18 (Figure 5(a)). In addition, more than 80% of the differentiated cells from these 2 cell populations had capacity to accumulate low-density lipoprotein (LDL) inside the cells like functional hepatocytes in a time-dependent manner whereas undifferentiated cells (day 0) did not perform this ability (Figure 5(b)). Furthermore, albumin secretion and urea production were also determined in order to confirm the hepatocytes functions. We found that undifferentiated stage (day 0) of WJMSCs-SUT1 and WJMSCs-SUT2 did not secrete detectable levels of albumin even though they have been detected albumin expression both at gene and protein levels. However, during differentiation processes, MSCDHCs

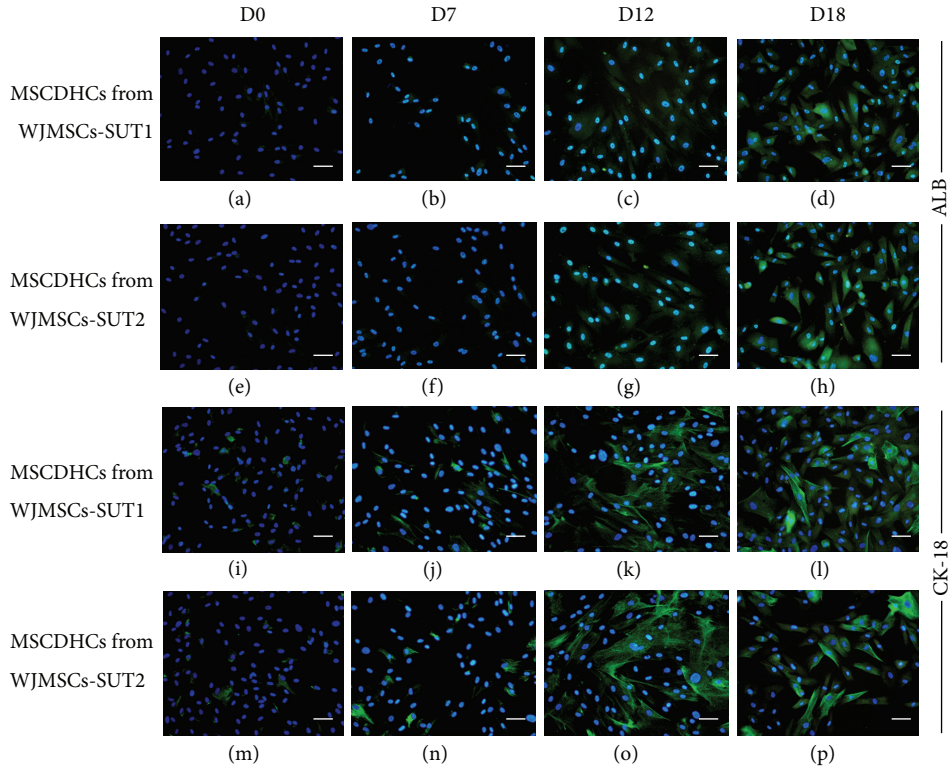

Figure 4: Hepatic-specific protein expressions of the differentiated cells after induction for 18 days. Albumin (ALB) expression analysis: MSCDHCs generated from WJMSCs-SUT1 ((a)–(d)) and WJMSCs-SUT2 ((e)–(h)) were stained with rabbit polyclonal anti-human albumin antibody following the differentiation period. For detection of cytokeratin 18 (CK-18) expression, MSCDHCs generated from WJMSCs-SUT1 ((i)–(l)) and WJMSCs-SUT2 ((m)–(p)) were stained with mouse monoclonal anti-human cytokeratin 18 antibody following the differentiation period. Cells nuclei were counterstained with DAPI. Original magnifications: ×200 for all pictures. Bars indicate 50 μm.

generated from both 2 cell lines continuously secreted albumin since entry into maturation step from day 12 to day 18 (Figure 6(a)). Finally, we also tested metabolic function of the differentiated cells to detoxify ammonia to urea which is a less toxic form. In consistency, the differentiated cells from WJMSCs-SUT1 and WJMSCs-SUT2 could produce urea in response to ammonia detoxification process at all points of differentiation period (Figure 6(b)). Taken together, these data suggest that both WJMSCs-SUT1 and WJMSCs-SUT2 could commit toward functional hepatocyte-like cells by our new hepatogenic induction protocol under hypoxic condition.

Furthermore, the percentage of differentiated cells has been evaluated based on both hepatic phenotypes (ALB-expressing cells, CK-18-expressing cells) and functions (LDL and PAS positive stained cells) by counting the numbers of positive stained cells of each marker against the total numbers of the cells as references by their stained cell nuclei from the same sample. For hepatic phenotypes, the induced cells highly expressed hepatic markers both ALB (89.00 ± 1.00% for WJMSCs-SUT1, 88.33 ± 1.53% for WJMSCs-SUT2) and CK-18 (85.00 ± 2.00% for WJMSCs-SUT1, 83.00 ± 2.00% for WJMSCs-SUT2). For hepatic functions, these cells could highly store glycogen inside the cells as shown by positive stained cells of PAS staining (82.33 ± 2.52% for WJMSCs-SUT1, 87.00 ± 2.00% for WJMSCs-SUT2) whereas more than 80% of LDL positive stained cells were observed in both 2 cell lines. Taken together, our developed protocol under hypoxic condition could generate high yield of functional hepatocytes ranging from 80 to 90% of the induced cells from both WJMSCs-SUT1 and WJMSCs-SUT2. Here, we successfully generated a new method to establish high yield and functional hepatocyte-like cells from WJ-MSCs which may be advantages in clinical applications, hepatic toxicity drug screening test including toxicological research, and other medical applications.

4. Discussion

The WJ-MSCs are expected as a promising tool for therapeutic purposes. This is mainly due to their wide range differentiation capacity, noninvasive collection, and low immunogenicity. They have immunomodulatory as inhibitory effect on stimulated T cells *in vitro* that would be an advantage to allogenic transplantation [26]. Previous study found that human umbilical cord matrix stem cells could be successfully xenotransplanted into immunocompetent rats with little or no host immune response [27]. These findings support low immunogenic property of WJ-MSCs *in vitro*. In addition, WJ-MSCs have broader differentiation potential into several cell types such as insulin-producing cells [28], nerve cells [29], and cardiomyocytes [30] including hepatocyte-like cells [17]. Furthermore, preclinical studies found that WJ-MSCs

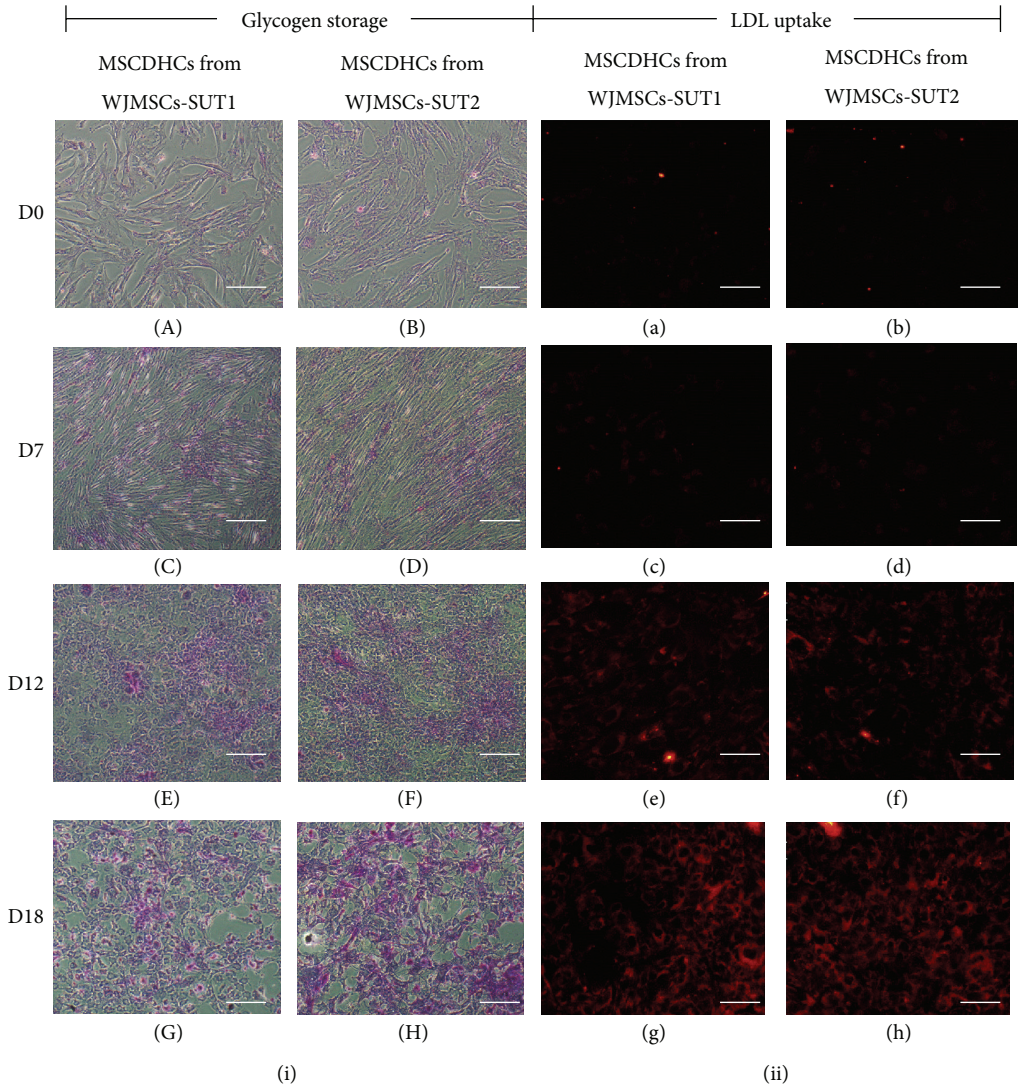

Figure 5: The capacities of the differentiated cells to store glycogen and accumulate LDL inside the cells after induction for 18 days. Glycogen storage of MSCDHCs generated from WJMSCs-SUT1 ((i): (A), (C), (E), and (G)) and WJMSCs-SUT2 ((i): (B), (D), (F), and (H)) were characterized by PAS staining following the differentiation period. LDL-uptake capacity of MSCDHCs generated from WJMSCs-SUT1 ((ii): (a), (c), (e), and (g)) and WJMSCs-SUT2 ((ii): (b), (d), (f), and (h)) were analyzed by LDL-uptake assay following the differentiation period. Original magnifications: ×100 for all pictures. Bars indicate 100 μm.

could improve the liver functions, restore the survival rate, and decrease liver fibrosis in animal model with liver injury [13, 31]. Although WJ-MSCs have not been applied in clinical trials related to liver diseases, recent pilot study demonstrated the safety and successful treatment with placenta-derived MSCs in type 2 diabetes patients [32]. Based on their advantages, WJ-MSCs seem to possess beneficial effect on liver disease treatment.

In this study, we aim to investigate the efficiency of a new established method for *in vitro* induction of WJ-MSCs into hepatic lineage by testing with our human WJ-MSCs cell lines as WJMSCs-SUT1 and WJMSCs-SUT2. Based on several hepatogenic induction methods, we developed a new two-step protocol to induce *in vitro* hepatogenic differentiation of WJ-MSCs under hypoxic condition which resembles the normal microenvironment in human physiology. Typically, the existing published protocols that have been used for hepatogenic induction of MSCs are performed in normal atmosphere of 20% O_2 and 5% CO_2. Here, we used hypoxic environment (5% O_2 and 5% CO_2) to accelerate hepatogenic differentiation potential of WJ-MSCs based on the superior advantages of this condition. In human ES cells, it has evidence that hypoxia is suitable for using in final hepatic induction to produce high yield of hepatocyte-like cells [33]. Moreover, hypoxia has been shown to activate hypoxia-inducible factor 1 (*HIF-1*) expression which has target gene asretinoic acid-receptor-related orphan receptor alpha (*Rorα*) transcripts in HepG2 [34]. The Rora upregulations are involved in controlling *ADIPOQ* and *G6PC* expressions which play roles in regulation of lipid and glucose metabolisms in hepatic cells, respectively [35]. From these data, hypoxia seems to provide appropriate environment

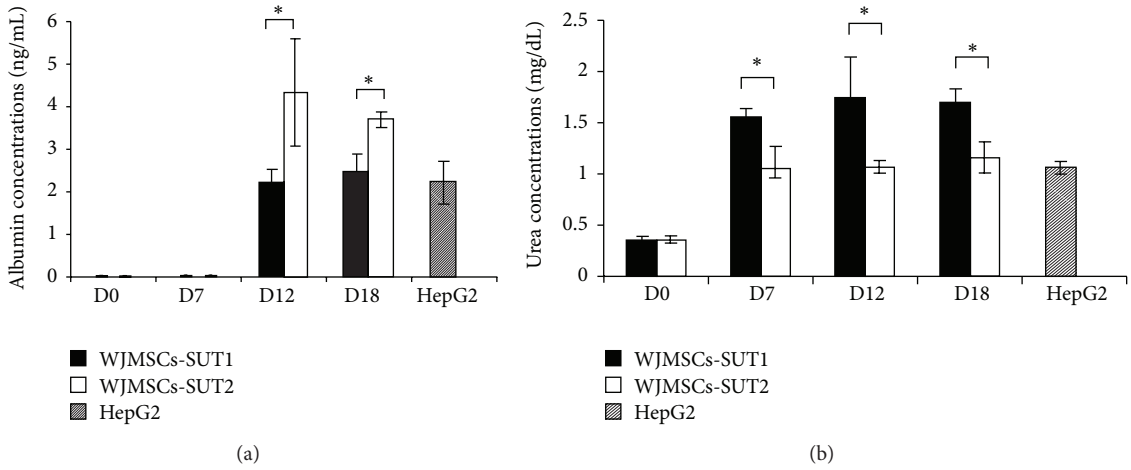

Figure 6: The capacities of the differentiated cells to secrete albumin and produce urea after induction for 18 days. Albumin secretion (a) and urea production (b) of MSCDHCs generated from WJMSCs-SUT1 and WJMSCs-SUT2 were determined by ELISA and colorimetric assay following the differentiation period, respectively. HepG2 was used as positive control. All data are presented as mean ±SD ($n = 3$). $^{*}P < 0.05$.

for maintaining physiological activities including hepatic-lineage differentiation in liver cells. In addition, hypoxic preculturing of MSCs has been shown to increase cell homing, migration, and engraftment efficiency both *in vitro* and *in vivo* studies [36]. Therefore, it has possibility that hypoxic condition also enhances these efficiencies of MSCDHCs in addition to the improvement of the hepatogenic differentiation. The hepatocyte-like cells generated from hypoxic condition may retain these promising abilities which provide a great benefit in therapeutic purposes.

According to developing a new protocol, the achievement of MSCDHCs generation was observed from both WJMSCs-SUT1 and WJMSCs-SUT2 by sequential treatment with our new cytokines-based cocktail medium. Sequential exposure of these cytokines was mimicked from the behavior of mouse embryonic liver development. In early stage of differentiation, we used FGF-4, HGF, and nicotinamide as main induction factors. In facts, FGFs expressed by cardiac mesoderm play a role in induction ventral foregut endoderm to initiate early liver development. Afterward, liver bud formation is occurred and rapid hepatoblast proliferation is stimulated by HGF that is a crucial factor for hepatogenesis. Nicotinamide is a water-soluble amide form of niacin or vitamin B3 which serves as primary precursor of nicotinamide adenine dinucleotide (NAD^+) and the phosphorylated derivative $NADP^+$ synthesis [37]. Actually, NAD^+ and $NADP^+$ play roles in maintaining energy for cellular functions including DNA repair and genomic stability. For hepatogenic differentiation, nicotinamide has been shown to enhance the proliferation of primary rat hepatocytes and formation of small hepatocytes colonies [38]. Based on these data, we also used nicotinamide in combination with our first stage hepatogenic induction medium. In late stage of differentiation, we utilized OSM, dexamethasone, and ITS as major factors. It has been reported that OSM secreted by hematopoietic cells is involved in the maturation fate of fetal hepatocytes [18]. In addition to these factors, it has been reported that dexamethasone is required for maintaining the expression of liver-enriched transcription factors which are essential for stimulating liver-specific genes transcription [39]. ITS is known as chemically defined supplement for supportive *in vitro* proliferation in various mammalian cells. For hepatogenic induction, ITS seems to maintain cell survival as monolayer during the induction period. Thus, we also used dexamethasone and ITS to induce more mature hepatocytes differentiation in our system.

For hepatic-lineage marker expressions, *HNF*4*α* is a crucial liver-enriched transcription factor that plays an important role in stimulating the expression of liver-specific genes during liver development [40]. During the hepatogenic differentiation processes, early expression of *HNF*4*α* was detected in MSCDHCs generated from WJMSCs-SUT2 on day 12 which correlated with the upregulations of mature hepatic markers such as *ALB*, *AAT*, and *CYP*3*A*4. Similarly, MSCDHCs generated from WJMSCs-SUT1 showed the compatibility of *HNF*4*α* and hepatic-specific gene expressions but delayed to day 18. These findings imply that WJMSCs-SUT2 had a trend to differentiate into hepatic lineage in response to our induction method faster than WJMSCs-SUT1 did. Although these hepatocyte-like cells could express all mature hepatic markers, the expression of *AFP* which is a marker of immature hepatocytes was still detected even at late stage of differentiation. These data suggest that the hepatocyte-like cells or MSCDHCs generated from both 2 cell lines contain some hepatoblasts or hepatic progenitor cells. Similarly, previous studies demonstrated that human placenta-derived multipotent cells [41], umbilical cord matrix stem cells [17], and mesenchymal stromal cells derived from umbilical cord Wharton's jelly [10] were not yet fully differentiated into mature hepatocytes because *AFP* expression was still detected in the differentiated cells throughout the induction period. Based on these results, MSCs isolated from primitive origin seem to be reserved the immature phenotype even though they have driven toward specific lineage.

In a late stage of differentiation, MSCDHCs generated from both WJMSCs-SUT1 and WJMSCs-SUT2 achieved

characteristics of functional hepatocytes which were confirmed by LDL uptake assay, PAS staining for glycogen storage, albumin secretion, and urea assay. In comparison to previous published protocols [10, 17–21], our method provided high yield production of hepatocyte-like cells from WJ-MSCs, approximately more than 80% of the induced cells. Our protocol does not need any extracellular matrix substances for coating the culture dish to enhance hepatogenic differentiation. Moreover, this method required a shorter differentiation period, approximately 2 weeks, as compared to the other protocols which performed longer, at least for 3 weeks. Here, we demonstrate a simple, high efficiency, and time saving protocol for the generation of functional hepatocyte-like cells from WJ-MSCs. Altogether, these findings provide a new alternative option for an *in vitro* hepatocytes generation which may overcome the limitation of liver cell donors for future use in clinical applications.

5. Conclusions

In summary, we report a simple, high efficiency, and time saving protocol for the generation of functional hepatocyte-like cells from WJ-MSCs. This method succeeds to drive WJ-MSCs into hepatic lineage under hypoxic condition. The achievement of hepatogenic differentiation was confirmed by both phenotypes and functions including hepatic marker expressions both at gene and protein levels, glycogen storage, low-density lipoprotein uptake, urea production, and albumin secretion. The achievement of MSCDHCs generation from this new method may provide greater potential in clinical applications and overcome the shortage of donor livers which is a major limitation of orthotopic liver transplantation. The implications of this work are therapeutic value in cell-based therapy for liver disease treatments and other regenerative medicine, hepatic toxicity drug screening in pharmacological approaches, and other medical applications in the future.

Conflicts of Interests

The authors have declared that no conflict of interests exists.

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Review Article

Prospective Isolation of Murine and Human Bone Marrow Mesenchymal Stem Cells Based on Surface Markers

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Mesenchymal stem cells (MSCs) are currently defined as multipotent stromal cells that undergo sustained *in vitro* growth and can give rise to cells of multiple mesenchymal lineages, such as adipocytes, chondrocytes, and osteoblasts. The regenerative and immunosuppressive properties of MSCs have led to numerous clinical trials exploring their utility for the treatment of a variety of diseases (e.g., acute graft-versus-host disease, Crohn's disease, multiple sclerosis, osteoarthritis, and cardiovascular diseases including heart failure and myocardial infarction). On the other hand, conventionally cultured MSCs reflect heterogeneous populations that often contain contaminating cells due to the significant variability in isolation methods and the lack of specific MSC markers. This review article focuses on recent developments in the MSC research field, with a special emphasis on the identification of novel surface markers for the *in vivo* localization and prospective isolation of murine and human MSCs. Furthermore, we discuss the physiological importance of MSC subtypes *in vivo* with specific reference to data supporting their contribution to HSC niche homeostasis. The isolation of MSCs using selective markers (combination of PDGFRα and Sca-1) is crucial to address the many unanswered questions pertaining to these cells and has the potential to enhance their therapeutic potential enormously.

1. Introduction

Bone marrow (BM) is comprised of hematopoietic stem cell (HSC) and nonHSC populations. Mesenchymal stem cells (MSCs) reside in the nonHSC fraction. HSCs form the cornerstone of therapy for many hematological diseases. MSCs, on the other hand, are nonhematopoietic cells initially identified in the BM [1–4] that can differentiate along various mesenchymal lineages to generate fat, bone, and cartilage. The hypothesized physiological function of MSCs is to support hematopoiesis and stromal tissue regeneration. Interestingly, these multipotent cells are found in a variety of fetal and adult tissues in addition to the BM, including umbilical cord blood [5, 6], dental pulp [7, 8], term placenta [9, 10], and adipose tissue [11, 12].

MSCs possess therapeutic potential for the repair and regeneration of damaged tissues of mesenchymal origin [13, 14]. Additionally, they have potent immunosuppressive properties and are currently utilized to treat a wide variety of autoimmune conditions [15–19]. Despite the large number of clinical studies now investigating the suitability of MSCs as therapeutic agents, conventional adherence to a plastic tissue culture substrate is still the most commonly employed method for their isolation. However, isolating MSCs in this way has several limitations. For example, such MSC populations frequently contain contaminating cells. Furthermore, the differentiation potential and proliferative ability of traditionally isolated MSCs (also termed colony forming unit-fibroblasts (CFU-Fs)) gradually diminish as the cells mature [20]. MSCs may also acquire chromosomal

abnormities that predispose them to malignant transformation [21]. Finally, prolonged culture on plastic dishes changes the surface marker expression of MSCs, making identification of selective makers difficult [22, 23]. For these reasons, little information exists concerning the *in vivo* identity and biological function of MSCs within the BM niche. Nonetheless, exciting progress has recently been made in terms of elucidating reliable murine and human MSC surface markers offering exciting experimental and therapeutic opportunity (Figure 1).

This review summarizes the historical identification of MSCs and important milestones in the evolution of MSC research. We focus on the identification of MSCs in mouse and human and describe the utilization of specific murine and human MSC surface markers to facilitate the *in vivo* localization and prospective isolation of these cells. Finally, we summarize the evidence supporting a physiological role for MSCs within the BM/hematopoietic niche.

2. Historical Perspective

Dr. Friedenstein initially identified BM-derived, plastic-adherent cells that generated CFU-Fs when plated as single cells *in vitro* [24, 25]. Dr. Friedenstein subsequently demonstrated that these cells were capable of osteogenic differentiation *in vitro*. The physiological function of MSCs was next elegantly demonstrated by Reddi and colleagues, who subcutaneously implanted biological matrices comprising the shafts of long bones into allogenic rodents [26]. Bone and cartilage formed on the implants after a period of time, and the resulting bony ossicle supported hematopoiesis *in vivo*. These data were the first to support the presence of stromal progenitors and to illustrate their biological significance. Largely based on these studies, the term "MSC" was coined in 1991 to describe stromal progenitor cells [27]. Although MSCs have since become the subject of intense research, very little was uncovered until recently in regard to their anatomical localization, physiological function, and stromal hierarchy [28].

3. Definition of MSCs

Traditionally, MSCs appear as spindle-shaped cells that form colonies (i.e., CFU-Fs) following the culture of whole BM on plastic substrates. The multilineage potential of these colonies is then examined after a period of culture in defined media that induces cell differentiation. Additionally, phenotypic analysis of MSCs is determined by their culture conditions. Therefore, MSC properties have historically been described for plastic-adherent cells after prolonged *in vitro* culture. Although conventionally cultured MSCs are not characterized by unique markers and probably denote a heterogeneous population, there is a consensus among the scientific community that they do not express hematopoietic markers. Hence, MSCs stand apart from HSCs. Furthermore, the expression levels of stromal antigens in MSCs can vary based on the culture conditions. The Tissue Stem Cell Committee of the International Society for Cellular Therapy thus proposed a set of minimum criteria that define human MSCs [29] as follows. The cell must be plastic-adherent when cultured under standard conditions and express the surface markers cluster of differentiation (CD) 73, CD90, and CD105, and not express CD45, CD34, CD14, CD11b, CD79, or CD19. Additionally, human MSCs must be capable of *in vitro* differentiation into osteoblasts, adipocytes, and chondrocytes.

While this statement somewhat clarifies the cellular characteristics of human MSCs, the situation remains unclear for murine MSCs. Until recently, specific surface markers for murine MSCs were lacking and murine MSCs were also defined by plastic adherence, spindle-shaped morphology, and trilineage differentiation. These definitions for MSC isolated from both species have however generated controversy. The classic definition of a stem cell requires that it possess unlimited self-renewal ability and plasticity. Experimentally, serial transplantation experiments demonstrating that infused stem cells give rise to terminally differentiated daughter cells, while maintaining their naïve phenotype, provide evidence of stemness. Such experiments were not historically performed with MSCs, leading researchers to consider that the term "MSC" had been inappropriately applied [30].

4. Identification of Specific Murine MSC Markers

The identification of specific murine MSC markers began with the observation that hematopoietic and mesenchymal lineage cells are derived from individual lineage-specific stem cells [31]. Based on the hypothesis that MSCs most likely reside in the endosteum, a detailed screening of candidate surface markers was initially performed in the BM and the collagenase-digested bone of mice. The surface markers, platelet-derived growth factor receptor-α (PDGFR-α), and stem cell antigen-1 (Sca-1) were significantly enriched in the digested fraction of the bone, and PDGFR-α^+Sca-1^+ (PαS) dual positive cells were isolated and characterized [32, 33]. Notably, the resultant PαS cells fulfill the basic requirements for the definition of MSCs in mice. These cells are capable of unlimited self-renewal and can differentiate into osteoblasts, chondrocytes, and adipocytes under appropriate conditions *in vitro* [33]. PαS cells proliferate almost without senescence when cultured on plastic, yielding more than 1×10^7 cells from an original 5,000 cells seeded onto the substrate, with a doubling time of 50.6 hours. Moreover, the CFU-F frequency of PαS cells is approximately 120,000-fold higher than that of unfractionated BM mononuclear cells.

PαS cells reside in the perivascular space adjacent to vascular smooth muscle in mice. They express angiopoietin-1 (Ang-1) and chemokine (C-X-C motif) ligand 12 (CXCL12), suggesting that these MSCs play a physiological role in the maintenance of the hematopoietic niche. Transplantation experiments in which freshly isolated PαS cells were intravenously injected into lethally irradiated recipient mice demonstrated the stemness of PαS cells. Specifically, the infused cells homed to their niche in the BM and continued to

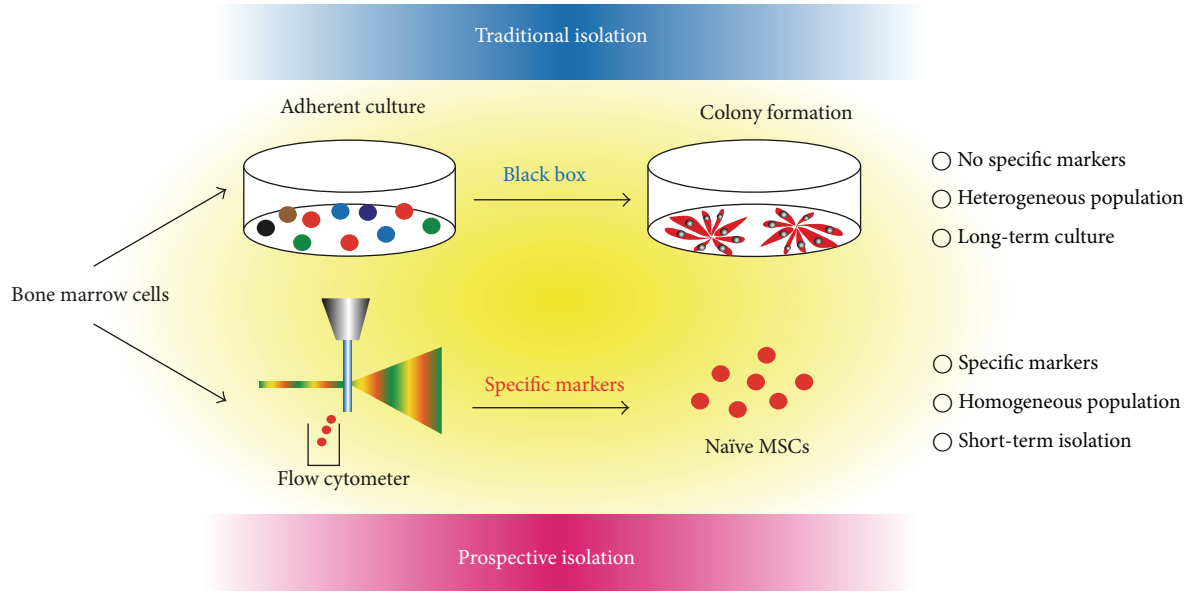

Figure 1: Prospective isolation of MSCs. Traditional MSC isolation by adherent culture on plastic tissue culture substrates (top). Following a period of prolonged culture, the majority of the contaminating cells are washed away or overgrown, enriching for CFU-Fs (colony formation). These MSCs have a spindle-shaped morphology and are capable of differentiating into adipocytes, chondrocytes, and osteoblasts *in vitro*. Prospective isolation of MSCs by using specific markers via flow cytometry (bottom). This method allows the isolation of a pure population of active, multipotent MSCs (naïve MSCs) and avoids cellular contamination.

express the hematopoietic niche factors Ang-1 and CXCL12, while also differentiating into osteoblasts and adipocytes *in vivo*. Sixteen weeks following cell transplantation, the mice were sacrificed, and the PαS cells were isolated. Notably, the isolated cells were still capable of both CFU-F formation and trilineage differentiation *in vitro*.

The identification of PDGFR-α as a selective MSC marker coupled with Cre/loxP-mediated lineage analysis [34] suggests that a subpopulation of adult BM MSCs might have a developmental origin in the murine neural crest [35, 36]. This is in agreement with a series of previously reported developmental studies in quail, chick, and rat [37, 38]. Murine and human MSCs are also an excellent cell source for the efficient generation of high-quality induced pluripotent stem cells [39, 40], which can in turn generate neural crest-like cells. More recently, a transgenic mouse reporter line expressing GFP under the control of enhancer/promoter of *nestin* gene, encoding an intermediate protein highly expressed in the neural stem/progenitor cells [41], was successfully used to identify and prospectively isolate murine MSCs in BM [42]. The *nestin*-GFP$^+$ cells in this transgenic mouse also expressed the intermediate filament protein Nestin and represented a small subset of nonhematopoietic stromal cells in the BM. These cells are anatomically located in the perivascular space, in close proximity to catecholaminergic nerve fibers and HSCs. In keeping with PαS MSCs, the *nestin*-GFP$^+$ cells express hematopoietic niche factors and are capable of trilineage differentiation both *in vitro* and *in vivo*. Nestin$^+$ MSCs also play an important functional role in maintaining the HSC niche. For example, the number of HSCs was dramatically reduced *in vivo* following the depletion of *nestin*-GFP$^+$ MSCs in mice, and the homing of transplanted HSCs back to their BM niche was significantly impaired in these animals following irradiation.

The studies discussed above are the first to identify specific markers that can be used for the *in vivo* localization and prospective isolation of MSCs. PαS and Nestin$^+$ MSCs have been analyzed in traditional stem cell assays (e.g., serial transplantation assays and clonogenic assays), which confirmed their properties of self-renewal and potency. Our own research group has also gained valuable insights into the importance of these cells in maintaining the HSC niche as well as the possibility of MSC subpopulations within the BM. For example, it is not entirely clear if Nestin$^+$ cells are the same as PαS cells. We know that Nestin$^+$ cells largely overlap with the PDGFRα^+CD51$^+$ population; however, this population also contains Sca-1$^+$ and Sca-1$^-$ cells (personal communication). These data suggest that the Nestin$^+$ population comprises both PαS and PDGFRα^+ cells. Notably, investigations using *nestin-Cherry* [43] and *nestin-GFP* [44] double transgenic mice detected *nestin-Cherry* expression around the larger blood vessels in the BM but not around the sinusoids, while *nestin-GFP* expression was detected around both structures [45]. Thus, different *nestin* promoter/enhancer-driven transgenes are apparently expressed by different subpopulations of perivascular stromal cells. Regardless, the identification and prospective isolation of PαS and Nestin$^+$ cells will provide indispensable information for ongoing research into the biological function, stromal hierarchy, and therapeutic potential of MSCs.

5. Identification of Specific Human MSC Markers

Numerous putative human MSC surface markers (i.e., CD49a [23], CD73 [1], CD105 [46], CD106 [47], CD271 [22], MSC antigen-1 [48], Stro-1 [49], and stage-specific embryonic antigen-4 [50]) have been identified thus far. These markers are used singly or in combination to enrich for CFU-Fs in human BM and avoid cellular contamination. Unfortunately, many of these markers are widely expressed in stromal cells and lack specificity, contributing to the significant heterogeneity among CFU-Fs derived from single isolations. The lack of specific MSC markers has thwarted attempts to uncover the true identity and function of these stem cells *in vivo*. Additionally, the traditional isolation of human MSCs by adherence to plastic substrates attenuates the differentiation potential and proliferative ability of CFU-Fs as the cells senesce, greatly reducing their therapeutic potential [51].

Various techniques, such as culture under hypoxic conditions, culture under nonadherent conditions, and supplementation of the culture media with growth factors, have been used in an attempt to avoid cellular senescence and enhance the therapeutic properties of MSCs. For example, human MSCs cultured as three-dimensional spheroids in a model of peritonitis acquired enhanced anti-inflammatory properties compared with those cultured under more conventional conditions [52]. The spheroid-associated cells were also smaller, allowing them to escape readily from the lung circulation and migrate to a variety of organs after intravenous administration to mice. Other investigators showed that long-term culture of MSCs under hypoxic conditions helps to keep the cells in an undifferentiated and multipotent state [53, 54].

As far as clinical applications are concerned, the number of clinical trials using *ex vivo* expanded stromal cell populations for therapeutic purposes is rapidly increasing (see http://www.clinicaltrials.gov/) [55, 56]. For example, MSCs have shown promise for the treatment of acute graft-versus-host disease, Crohn's disease, multiple sclerosis, osteoarthritis, and cardiovascular diseases. However, there is little consistency in the methods used to isolate MSCs for infusion, or in the media used to expand these cells in culture. Commercially available MSC medium frequently contains growth factors (required for cell expansion) that most likely influence the fate and therapeutic potential of the MSCs. These limitations further underscore the need to identify specific surface markers that can be used to probe the physiological functions and biological properties of human MSCs expeditiously. The prospective isolation and culture of such cells (with or without further manipulation) will certainly allow for safer and more effective clinical treatments in the future.

CD146 is one such marker that has helped discern the *in vivo* localization and function of human MSCs [57]. CD146 is found on the surface of adventitial reticular cells that reside in the endothelial space in human BM. These cells also express typical stromal markers (CD105, CD49a, CD73, CD90, and CD140b) and are capable of robust trilineage differentiation. Their physiological function was shown in immunodeficient mice following subcutaneous transplantation of human $CD146^+$ clonogenic cells seeded onto a scaffold (hydroxyapatite/tricalcium phosphate particles embedded in a fibrin gel). The transplanted human $CD146^+$ MSCs supported formation of bony ossicles and sinusoidal vasculature and finally established a functioning hematopoietic microenvironment. Immunohistochemical analysis demonstrated that a small proportion of the infused cells targeted the murine HSC niche, where they expressed Ang-1 and other supporting factors. The transplanted human $CD146^+$ MSCs were reisolated, cultured, and subsequently shown to form CFU-Fs capable of trilineage differentiation, demonstrating the self-renewal potency of these cells.

MSCs were initially thought to reside only within the BM, forming the stromal counterpart to HSCs. However, the utility of CD146 as a prospective marker for human MSCs is not limited to adult human BM, casting doubt on this assumption. Crisan et al. [58] used immunohistochemistry to examine various tissue types (e.g., adult and fetal human skeletal muscles, pancreas, adipose tissue, and placenta) and identified CD146, neuron-glial antigen 2 proteoglycan, and PDGFRα as specific pericyte markers [58]. With the aid of these markers, a pure population of pericytes was prospectively isolated from each tissue type via flow cytometry. The isolated pericytes expressed typical stromal markers (CD73, CD90, and CD105) and could be induced to differentiate into muscle, bone, fat, and cartilage by using standard MSC culture conditions and the appropriate differentiation factors. These data clearly identify CD146 as a specific surface marker of mesenchymal progenitor cells in a wide range of organs.

6. Role of MSCs *In Vivo*

The HSC niche provides a specialized microenvironment that promotes stem cell maintenance and function [59–63]. Several cells types, including osteoblasts, endothelial cells, and adventitial reticular cells, have been suggested to contribute to niche function [59, 64]. For many years, MSCs were surmised to be among these cells, although until recently, their participation has remained merely speculative. Our previous observations that PαS cells reside in the HSC niche (the perivascular space adjacent to HSCs) and express niche factors (Ang-1 and CXCL12) support the hypothetical involvement of MSCs in the regulation of the HSC microenvironment [33]. Indeed, $Nestin^+$ MSCs apparently play a critical role not only in the maintenance of HSCs within the niche, but also in the homing of transplanted HSCs back to the BM.

Although a significant proportion of the perivascular PDGFRα^+ cells described above express Nestin, the exact impact of each subpopulation of perivascular cells on the HSC niche remains to be elucidated. Recent data suggest that nonmyelinating Schwann cells participate in the maintenance of the HSC niche via activation of latent transforming growth factor-β [65]. It is noteworthy that these cells express Nestin, thus evoking some controversy in the research field as to their possible stemness. Recently, Ding et al. [45] confirmed the importance of perivascular cells in maintaining the HSC niche through the production of stem cell factor (Scf). HSC

frequency and function were not affected when Scf was conditionally deleted from hematopoietic cells, osteoblasts, or Nestin$^+$ cells. However, HSCs were eliminated from the BM when Scf was deleted from endothelial cells or Leptin receptor-expressing perivascular stromal cells (which were also positive for PDGFRα, PDGFRβ, CXCL12, and alkaline phosphatase expression). Clearly, much remains unknown about the complex microenvironment of the HSC niche and its regulatory factors. Nevertheless, the data suggest that one or more MSC subtypes critically contribute to HSC niche homeostasis.

7. Conclusions

The hypothesis that a rare population of multipotent stromal progenitor cells or MSCs, capable of generating all stromal cell subtypes, existed in the BM was greeted with almost universal approval in the scientific world. However, until recently little evidence supported the proposed physiological functions of MSCs, including maintenance of the HSC niche, replenishment of mesenchymal tissue, wound healing, and tissue repair. An absence of specific MSC surface markers proved to be a significant stumbling block to unraveling the biology and function of MSCs. Nonetheless, the field has lately taken a significant leap forward with the identification of such markers in the mouse and human, allowing the prospective isolation of MSCs for the first time. As a result, we can now convincingly assay and confirm the stem cell properties of MSCs and elucidate their biological functions (their role in the maintenance of the HSC niche). We suggest that the prospective isolation (e.g., combination of PDGFRα and Sca-1) of MSCs will also allow scientists to address the many unanswered questions related to these cells, and most importantly, to advance MSCs as a therapeutic agent.

Conflict of Interests

H. Okano is the scientific consultant of SanBio, Inc., Eisai Co., Ltd., and Daiichi Sankyo Co., Ltd. The remaining authors report no conflict of interests.

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