Stem Cells International

Environmental Influences on Stem Cell Behaviour

Lead Guest Editor: Margherita Maioli
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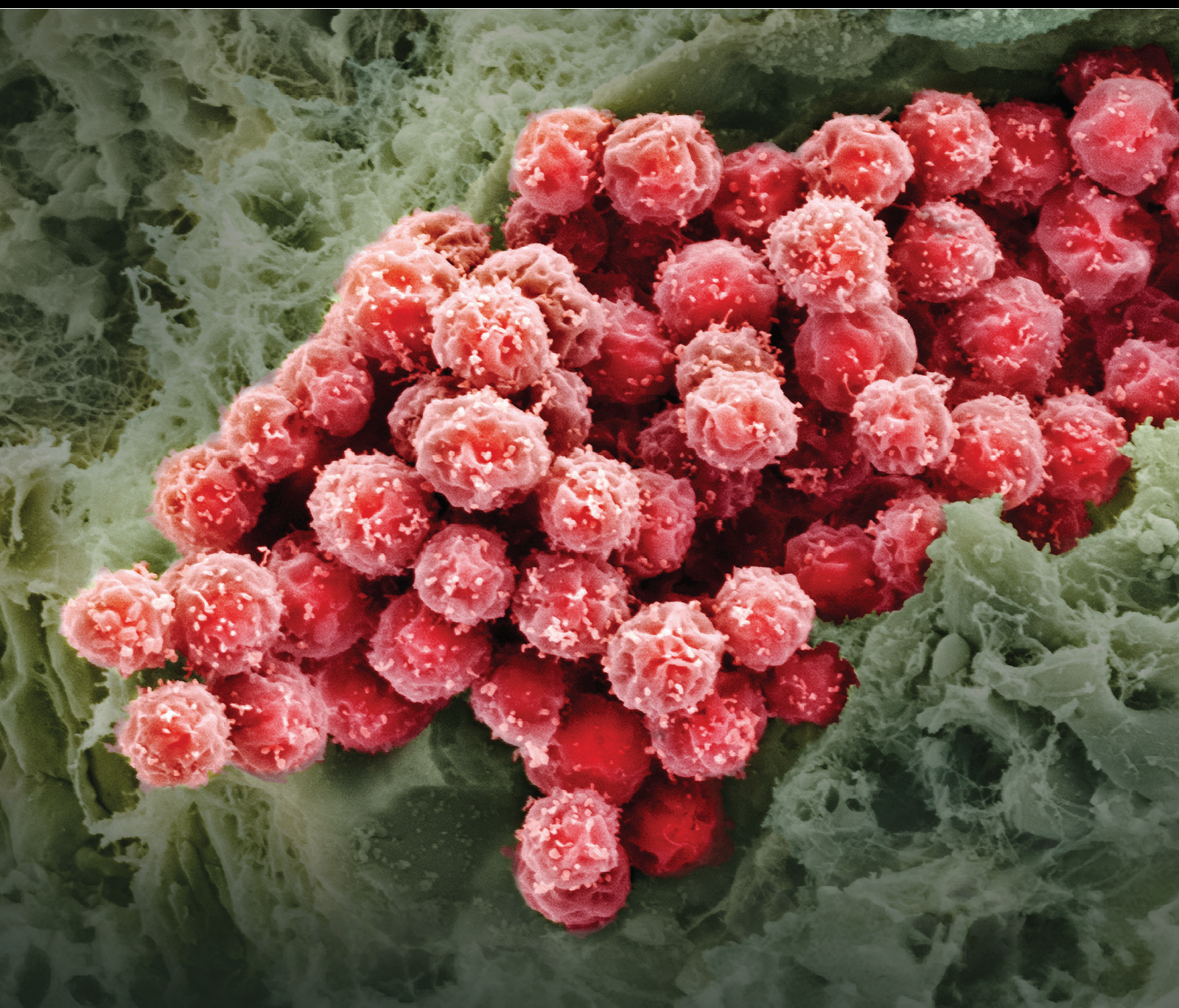

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Editorial Board

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Hindawi
Stem Cells International
Volume 2018, Article ID 7415460, 2 pages
https://doi.org/10.1155/2018/7415460

Editorial

Environmental Influences on Stem Cell Behavior

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Received 9 October 2018; Accepted 9 October 2018; Published 10 December 2018

Stem cells are unique elements capable of acquiring a specific phenotype under the exposure of specific stimuli. Within this context, regenerative medicine represents a novel branch of Medicine mainly focused on stem cell-based cellular therapies. For years, scientists developed different kinds of molecules in the attempt to convince stem cells to assume a specific phenotypic identity. These compounds are both natural molecules, for example, melatonin or vitamin D [1, 2], or mixtures of physiological molecules able to act as epigenetic regulators of stem cell fate [3, 4]. This special issue brings together 5 papers to highlight recent developments in the field. Within this landscape, the review by L. Huang and G. Wang perfectly summarizes the effects of different factors used in the past years to orchestrate neural stem cell proliferation and differentiation. This manuscript represents a source for future translational applications of nerve tissue engineering in regeneration after CNS injury. Extracellular vesicles (EVs) are emerging as novel actors for intercellular communication and as a potential diagnostic tool in human diseases. Different cells under physiological and pathological conditions, including tumor cells, produce EVs. The review by I. Laurenzana et al. describes the involvement of EVs in bone marrow-derived stem cell communication, also underlying their role during hematological malignancies as a part of the communication among the niche, HSCs, and MSCs. Recently, studies described the use of physical stimuli in vivo, to induce the patient's own regenerative capabilities, based on stem cell recruitment and fate modulation. Enhanced endogenous response based on posttreatment rehabilitation is driving a new area of regenerative rehabilitation [5–7]. The potential to enhance regenerative processes using physical energies is supported by the ability of electromagnetic fields and mechanical vibrations to drive an efficient reprogramming of the differentiation and regenerative potential of our endogenous stem cells [6–8]. The manuscript by F. Facchin et al. perfectly summarizes these findings, by describing the effect of different kinds of electromagnetic fields and of sound vibrations on stem cell proliferation and differentiation. Stem cell behavior can also be influenced by oxygen concentrations. Upon stimulation, stem cells migrate to more oxygenated areas to heal damaged tissue. Adult stem cells remain in a state of quiescence in their specialized niche until external signals induce a metabolic shift towards an oxidative metabolism [9]. As reported in the research article by A. Menon et al., hypoxia, through the activation of a specific factor, plays a crucial role in preserving stem cells in an undifferentiated state within tissue "hypoxic

niches." The research article by A. Banerjee et al. perfectly fits with this finding, viewing that mesenchymal stromal cells (hAMSCs) from the amniotic membrane can be influenced in their differentiation behavior by high oxygen tension (20%), a condition able also to activate mitochondrial function and induce the production of IL6. We hope that this special issue will introduce novel concepts in understanding stem cell behavior, not only by defining a wide panel of chemical but also physical players in regenerative medicine.

Margherita Maioli
Heinz Redl
Martin J. Stoddart

Conflicts of Interest

The authors declared no conflicts of interest.

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Hindawi
Stem Cells International
Volume 2018, Article ID 9502451, 11 pages
https://doi.org/10.1155/2018/9502451

Research Article

Oxygen Tension Strongly Influences Metabolic Parameters and the Release of Interleukin-6 of Human Amniotic Mesenchymal Stromal Cells *In Vitro*

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Received 12 February 2018; Revised 18 May 2018; Accepted 6 September 2018; Published 28 October 2018

Academic Editor: Hannele T. Ruohola-Baker

The human amniotic membrane (hAM) has been used for tissue regeneration for over a century. *In vivo* (*in utero*), cells of the hAM are exposed to low oxygen tension (1–4% oxygen), while the hAM is usually cultured in atmospheric, meaning high, oxygen tension (20% oxygen). We tested the influence of oxygen tensions on mitochondrial and inflammatory parameters of human amniotic mesenchymal stromal cells (hAMSCs). Freshly isolated hAMSCs were incubated for 4 days at 5% and 20% oxygen. We found 20% oxygen to strongly increase mitochondrial oxidative phosphorylation, especially in placental amniotic cells. Oxygen tension did not impact levels of reactive oxygen species (ROS); however, placental amniotic cells showed lower levels of ROS, independent of oxygen tension. In contrast, the release of nitric oxide was independent of the amniotic region but dependent on oxygen tension. Furthermore, IL-6 was significantly increased at 20% oxygen. To conclude, short-time cultivation at 20% oxygen of freshly isolated hAMSCs induced significant changes in mitochondrial function and release of IL-6. Depending on the therapeutic purpose, cultivation conditions of the cells should be chosen carefully for providing the best possible quality of cell therapy.

1. Introduction

Oxygen is the element of highest electronegativity after fluorine, and even molecular oxygen is still a highly reactive and therefore, toxic molecule. Photosynthesis by organisms in early evolution led to the "oxygen explosion" in the earth's atmosphere 2.3 billion years ago, causing mass extinction of anaerobic species [1, 2]. By hypothesis, only the entering of an oxygen-utilizing prokaryote into a preeukaryote allowed surviving an oxygen-containing atmosphere [3]. This process of endosymbiosis, marking the birth of mitochondria, changed the course of evolution [3] (reviewed in [4]).

Tissue oxygen tension is a measure of the oxygen partial pressure in the interstitial (extravascular) space reflecting the balance between oxygen supply and demand (reviewed in [5]). Oxygen tension in cellular microenvironment can strongly influence cellular processes (reviewed in [6]). In the course of evolution, organisms developed numerous strategies to cope with the potentially toxic effects of oxygen, such as antioxidant systems and enzymes. Exposure to

oxygen in tissues is therefore highly regulated by the level of vascularization (reviewed in [5]). Mammalian tissues have highly specific oxygen tensions which can range from 16% oxygen in alveolar air (reviewed in [5, 7]) down to almost anoxic calculated oxygen tension in the bone marrow hematopoietic compartment [8, 9]. Therefore, different cells of the body are exposed to different oxygen tensions. Adult stem cells, such as mesenchymal stem cells, neural stem cells, and hematopoietic stem cells, maintain their stem cell state in niches of very low oxygen tensions (reviewed in [10, 11]). An increasing number of publications have demonstrated energy supply via glycolysis in embryonic [12, 13] (reviewed in [14]) and mesenchymal stem cells [15–18] and even induced pluripotent stem cells (iPSCs) [19]. According to current models, stem cells remain quiescent in their specialized niche [20] (reviewed in [21, 22]) until external signals induce a metabolic shift. Stem cells then switch their metabolism from primarily glycolytic to oxidative phosphorylation and start to differentiate towards progenitor and precursor cells (reviewed in [23]). Therefore, it is now widely accepted that maintenance of a stem cell state requires maintenance of their highly specific microenvironment.

An attractive source of adult stem cells is the human amniotic membrane (hAM) [24]. This fetal membrane consists of an epithelial layer, formed by a monolayer of human amniotic epithelial cells (hAECs), and a collagen-rich mesenchymal layer, in which the human amniotic mesenchymal stromal cells (hAMSCs) are embedded. In clinics, the hAM has been used decellularized or denuded for tissue regeneration purposes for over a century [25, 26]. Transplanted hAM does not cause rejection reactions in the patient (reviewed in [27]), and furthermore, the hAM and the cells thereof have anti-inflammatory [28–36] and immunomodulatory properties [37–39]. Additionally, cells of the hAM can differentiate into cells of all three germ layers *in vitro* and *in vivo* [40–44]. Therefore, the use of cells of the hAM for tissue regeneration has moved into the focus of many research groups.

While common cell culture conditions derive originally from cultivations of chicken fibroblasts at 20% oxygen, other cells, such as stem cells, need a more specialized oxygen microenvironment. Changes in the oxygen microenvironment particularly affect mitochondria, also designated as the "main sink of oxygen" [45]. Oxygen, with its high standard redox potential, is the final electron acceptor in the mitochondrial electron transport chain for the generation of adenosine triphosphate (ATP) via oxidative phosphorylation. This metabolic process also releases superoxide, a reactive oxygen species (ROS), predominantly produced by mitochondrial complexes I and III [46, 47]. ROS, formerly considered as mere damaging byproducts, came recently into focus for their signalling function (reviewed in [48]). Therefore, it does not come as a surprise that mitochondrial function plays a critical role in maintaining stemness [49], orchestrates cell fate (reviewed in [23]), and also plays a critical role for tissue regeneration [50].

In vivo, cells of the hAM are exposed to low oxygen tension (1–4%; [51]) while *in vitro* cultivation or storage is usually performed at 20% oxygen. As changes in the microsurroundings of hAMSCs in culture may impact cellular processes, we tested the influence of low (5%) and high (20%) oxygen tensions on mitochondrial function of freshly isolated hAMSCs after 4 days in culture. As we found different mitochondrial activities in reflected and placental amnion biopsies in a former study [52], we separately investigated hAMSCs from placental (P-hAMSCs) and reflected amnion (RA-hAMSCs). Furthermore, as the anti-inflammatory properties of the hAM represent a potentially crucial function in a clinical transplantation situation, we also measured parameters linked to inflammation. The results of this study could support the possibility of specific selection and preparation of amniotic cells according to clinical requirements.

2. Material and Methods

2.1. Preparation of the Human Amniotic Membrane. Placentae were obtained from planned caesarean sections from healthy patients at full term. The patients had signed informed consent with approval of the local ethics committee, in accordance to the Declaration of Helsinki. Placentae were transported within 4 hours of delivery, in 500 mL Ringer solution. Placentae from caesarean sections of premature deliveries, emergency caesarean sections, and placentae with detached amniotic membranes were excluded from the study. The reflected and placental regions of the hAM were separated from each other as previously described [52].

2.2. Isolation of Human Amniotic Mesenchymal Stromal Cells. Isolation of hAMSCs was performed as previously described [33]. Briefly, reflected and placental amnions were cut into 2×2 cm pieces, incubated in 1 mg/mL collagenase solution, and shaken for 2 h at 37°C. Digestion was stopped with cold PBS, and the cell suspension was filtered through 100 μm cell strainers and centrifuged at 4°C for 9 min at 400 g. The cell pellet was resuspended in DMEM, supplemented with 10% FCS, 2 mM L-glutamine, and 1% penicillin/streptomycin (medium and supplements from Sigma-Aldrich, USA). Cell yields of approximately 15×10^6 cells for reflected amnion and 10×10^6 cells for placental amnion were reached per donor, depending on the size of the hAM. Donors with more than 10% dead cells (staining positive for trypan blue) were excluded from the study.

2.3. Cultivation of Human Amniotic Mesenchymal Stromal Cells at 5% and 20% Oxygen. Freshly isolated hAMSCs of both amniotic regions, reflected and placental, were cultured for 4 days at 37°C, humidified atmosphere, 5% CO_2, and 5% or 20% O_2. The cell culture medium, DMEM, was supplemented with 10% FCS, 1% penicillin/streptomycin, 1% L-glutamine (medium and supplements from Sigma-Aldrich, USA), and 20 mM HEPES (Gibco™, USA). Prior to incubation at 5% O_2, the cell culture medium was purged with medicinal N_2, in order to replace the oxygen dissolved in the medium. The decrease of O_2 level was confirmed with Blood Gas Analyzer Radiometer ABL800 Flex (Radiometer, Denmark). Samples for all measurements described below were taken at day 0 or after 4 days incubation at

5% or 20% O_2 without additional passaging. Therefore, all cells were measured directly in passage 0 or after detachment of passage 0.

2.4. Measurement of Mitochondrial Activity. Mitochondrial respiration of isolated hAMSCs was measured with high-resolution respirometer (Oxygraph-2k, Oroboros Instruments, Austria). At day 0, freshly isolated hAMSCs were seeded with a density of 20,000–30,000 cells/cm^2 and incubated at oxygen tensions of 5% or 20%. At day 4, the cells were detached and counted. For measurement of ROUTINE respiration, 4×10^6 hAMSCs were resuspended in DMEM at pH 7.2 and 37°C. For measurement of LEAK respiration, ATP synthase was inhibited with 1 μM oligomycin (Sigma-Aldrich, USA). Maximum electron transfer system capacity was measured by titration of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma-Aldrich, USA) in steps of 0.5 μM. Phosphorylation-related respiration was calculated by subtraction of LEAK respiration from ROUTINE respiration (Supplemental Figure 1). Analysis of the data was performed by calculating the slopes of the oxygen concentration curves with Microsoft Excel (Version 14.0.7190.5000 (32 bit)). Sample numbers (biological replicates) $n = 5 - 7$.

2.5. Measurement of Lactate Concentrations. Lactate concentrations were quantified in the cell culture supernatants of 100,000 cells/mL after 4-day incubation of reflected and placental hAMSCs with Blood Gas Analyzer Radiometer ABL800 Flex (Radiometer, Denmark). Sample numbers (biological replicates) $n = 4$.

2.6. Adenosine Triphosphate (ATP) Measurement. The samples for measurement of ATP were taken either from freshly isolated hAMSCs or from hAMSCs after cultivation for 4 days at 5% or 20% oxygen. 100,000 cells were pelleted, snap frozen in liquid nitrogen, and stored at −80°C. The cells were homogenized in Precellys tubes with ceramic beads (Keramik-Kit 1.4 mm Peqlab VWR, USA) in a ball mill (CryoMill MM301, Retsch, Germany) with 500 μL of Tris-HCl Buffer (20 mM Tris, 135 mM KCl, pH 7.4). To 100 μL homogenate, 400 μL of boiling 100 mM Tris/4 mM EDTA Buffer (pH 7.75) was added and incubated for 2 min at 100°C and centrifuged at 1000g for 2 min. ATP was determined by ATP Bioluminescence Assay Kit CLS II (Roche, Switzerland) using luciferase reagent with Luminat LB 9507 (Berthold, Germany). Sample numbers (biological replicates) $n = 3 - 5$.

2.7. Determination of Mitochondrial DNA (mtDNA) Copy Number. Cellular DNA was extracted from a pellet of 10,000 hAMSCs using the Tissue & Cell Genomic DNA Purification Kit in accordance with the manufacturer's protocol (GMbiolab Co., Taiwan). The ratio of mtDNA to nDNA was determined as an estimate for the number of mitochondrial genomes per cell using quantitative PCR assays against the single-copy nuclear gene *MYC* and the gene *MT-ND1* representing the minor arc of the mitochondrial genome [53] (Supplemental Table 1). Sample numbers (biological replicates) $n = 5$. The Cq values measured by quantitative PCR were transformed into copy numbers using digital PCR [53].

2.8. Reactive Oxygen Species. Electron paramagnetic resonance (EPR) spectra from frozen samples (100,000 cells) were recorded by Miniscope MS200 EPR spectrometer (Magnettech Ltd., Germany) at −196°C (modulation frequency 100 kHz, microwave frequency 9.429 GHz, microwave power 30 mW, modulation amplitude 5 G) as previously described [53]. Intensities of oxidized cyclic hydroxylamine 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride CP-H (3-CP, Noxygen, Germany) signals were recorded at 3359 ± 200 G and quantified by single integrating the low field peak of the 3-CP signal, as previously described [54]. Sample numbers (biological replicates) $n = 4$.

2.9. Nitric Oxide Concentration in the Supernatant. Total nitric oxide (NO) levels in the cell culture supernatants of 100,000 cells/mL were analyzed with Sievers 280i-NO Analyzer (General Electrics) as previously described [55]. Briefly, plasma samples were injected through a septum into the glass vessel, where NO species were converted by VCl_3 to $NO_{(g)}$. A subsequent chemiluminescent reaction with O_3 caused photon emission, which was converted and displayed as the voltage signal after detection with photomultiplier. Sample numbers (biological replicates) $n = 5$.

2.10. Release of Immunoactive Substances. Interleukin- (IL-) 1β, IL-6, IL-10, and hepatocyte growth factor (HGF) concentrations were detected in the cell culture supernatant of 100,000 cells/mL after 4-day incubation of hAMSCs at 5% O_2 and 20% O_2 with the immunoassay ProcartaPlex™ Human Basic Kit (Thermo Fisher Scientific, Invitrogen, USA), using antibody-coated magnetic beads (Luminex™). Measurement was performed according to the manufacturers' protocol, and absorption was measured with Bio-Plex® 200 instrument (Bio-Rad Laboratories, USA). Sample numbers (biological replicates) $n = 6$.

2.11. Statistical Analysis. Data was analyzed using GraphPad Prism software (GraphPad Software, USA). For analysis of day 0 versus day 4, one-way ANOVA was used, followed by the Bonferroni post hoc test in normally distributed data and Kruskal-Wallis combined with Mann-Whitney test in groups showing a non-Gaussian distribution. The Wilcoxon matched pairs test was used to analyze differences in mtDNA copy numbers. Paired t-test was used to analyze differences between amniotic regions (reflected versus placental amnion) and oxygen tensions (5% versus 20% oxygen). In all tests, n (sample size) represents the number of biological replicates (donors). Results are presented as mean ± SD for normally distributed data. Copy numbers of cellular mtDNA are presented as scatter dot plots. Level of significance was set at 0.05 and is indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Measurement of Mitochondrial Activity and Glycolysis. To assess the impact of different oxygen tensions on the

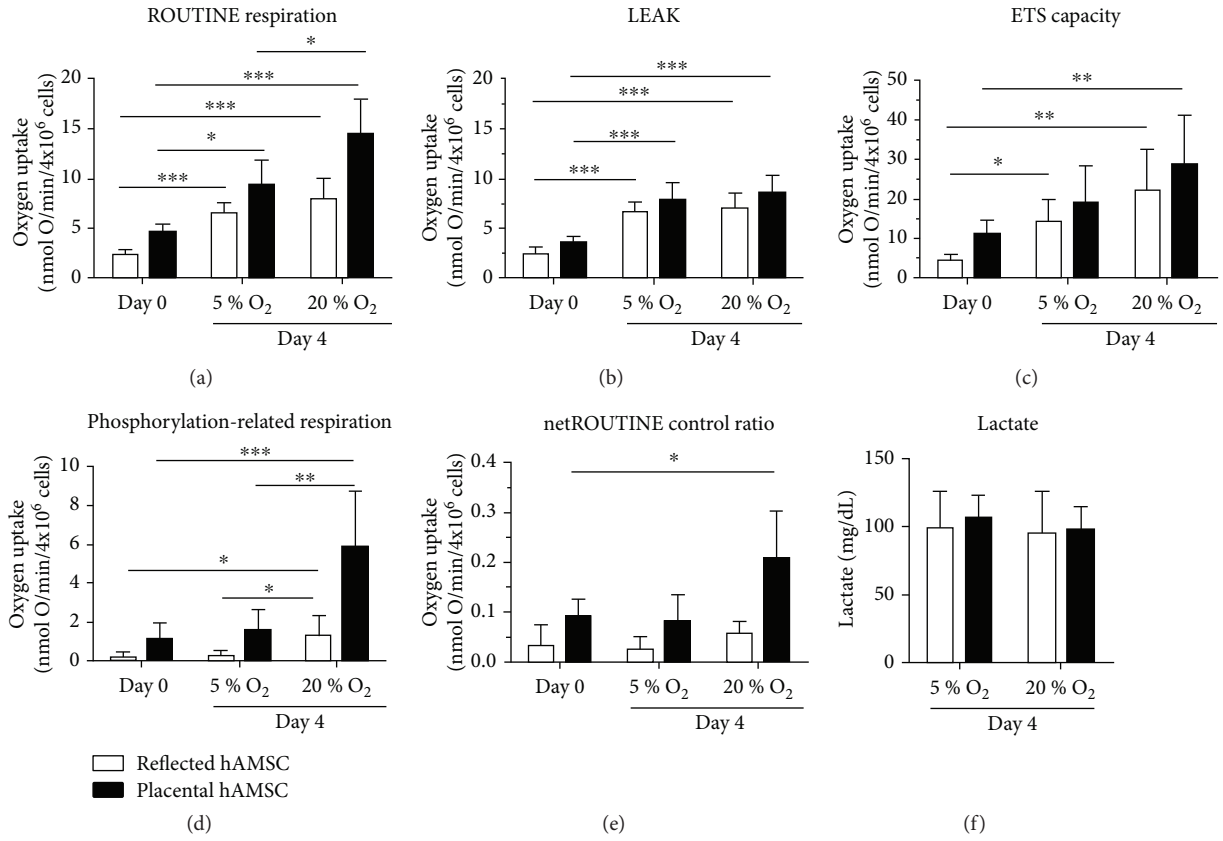

Figure 1: Mitochondrial activity and glycolysis. Mitochondrial respiration was measured in freshly isolated hAMSCs (day 0) and after 4-day incubation at 5% and 20% oxygen (a)–(e). Lactate in the cell culture supernatant was measured at day 4 (5% and 20% oxygen) (f). Cultivation of hAMSCs at 5% and 20% oxygen for 4 days led to elevated ROUTINE respiration (a), LEAK (b), and ETS capacity (c) for both oxygen concentrations but no difference in lactate production (f). Phosphorylation-related respiration was significantly increased only in samples incubated at 20% oxygen (d). This switch to oxidative phosphorylation in hAMSCs incubated at 20% oxygen was confirmed by the netROUTINE control ratio (e) which increases upon stimulation of oxidative phosporylation. $n = 5 - 7$ (biological replicates), mean ± SD. Abbreviations: ETS: electron transfer system; hAMSCs: human amniotic mesenchymal stromal cells; O_2: oxygen.

quantity of mitochondrial activity, we measured ROUTINE respiration, reflecting the aerobic metabolic activity. Cultivation of hAMSCs at 5% O_2 and 20% O_2 for 4 days lead to a significant increase of ROUTINE respiration for both oxygen concentrations. Values were always higher in placental amnion hAMSCs, and the effect was most pronounced in P-hAMSCs incubated at 20% O_2 (Figure 1(a)).

To determine changes in the quality of mitochondrial activity, we first measured the LEAK state, reflecting proton pumping of the electron transport chain without producing ATP. We observed increased LEAK respiration after 4-day incubation, independent of the oxygen tension and the amniotic region (Figure 1(b)).

Determination of the maximum capacity of the mitochondrial electron transfer system (ETS) showed a similar picture as ROUTINE respiration. The increase in maximum capacity was oxygen-dependent, reaching the highest values in P-hAMSCs incubated at 20% O_2 (Figure 1(c)).

Calculation of the phosphorylation-related respiration showed a drastic increase in cells incubated at 20% O_2, and this effect was most pronounced in hAMSCs of placental amnion (Figure 1(d)).

To see which fraction of ETS capacity is utilized to drive the phosphorylation of ADP to ATP, we calculated the "net ROUTINE control ratio" by dividing the phosphorylation-related respiration by the ETS capacity. The strongest increase was observed in P-hAMSCs cultivated at 20% O_2, indicating that a higher proportion of ETS capacity is utilized to drive ATP synthesis in these cells (Figure 1(e)).

Concentrations of lactate in the cell culture supernatants were measured at day 4, which were neither influenced by the oxygen tension nor the amniotic region (Figure 1(f)).

3.2. Measurement of ATP. ATP concentrations in hAMSCs after 4 days in culture increased compared to day 0 with both oxygen concentrations (Supplemental Figure 2).

3.3. Counting the Cellular mtDNA Copy Numbers. To estimate if the increase in mitochondrial respiration is due to an increase of the cellular mitochondrial content, the mtDNA copies per cell were counted. We observed a trend to an increasing mtDNA copy number in RA-hAMSCs (Figure 2(a)) and P-hAMSC (Figure 2(b)) incubated in 20%, but this increase was not significant.

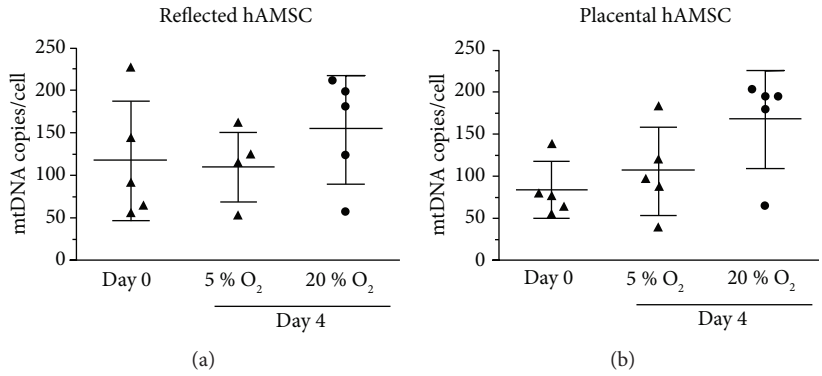

Figure 2: Cellular mitochondrial DNA copy number. The mtDNA copy number per cell was measured by digital PCR in freshly isolated human amniotic mesenchymal stromal cells (day 0) and after 4 days (5% and 20% oxygen) (a) and (b). We observed a trend to an increasing mtDNA copy number in samples incubated at 20% oxygen, but this increase was not significant. $n = 5$ (biological replicates), mean ± SD.

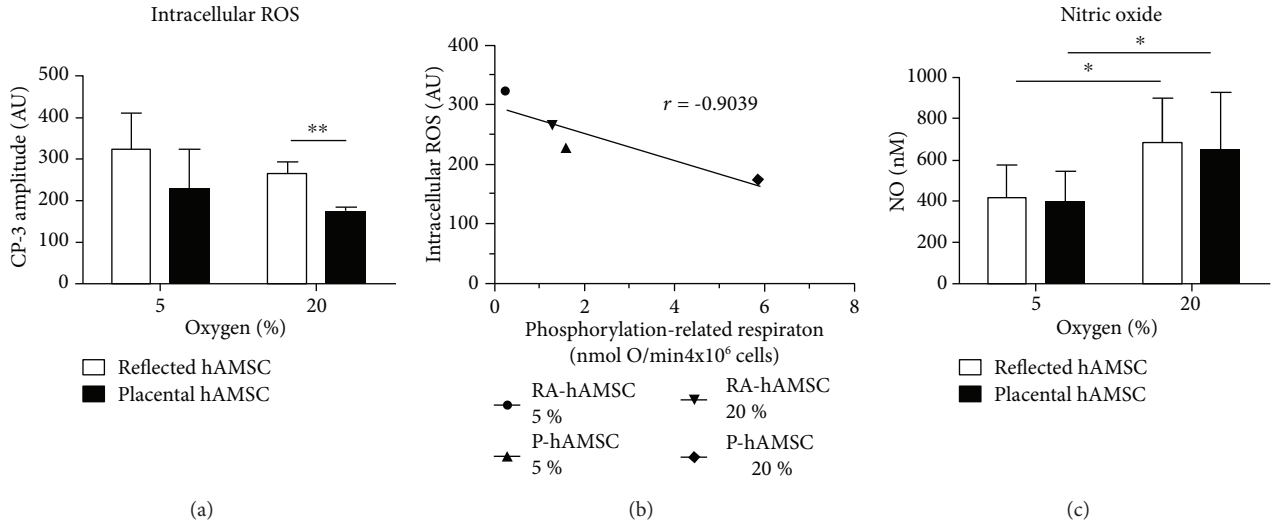

Figure 3: Reactive oxygen and nitrogen species. Intracellular reactive oxygen species levels and nitric oxide levels were measured after 4 days incubation at 5% and 20% oxygen (a)–(c). No significant differences in ROS levels between 5% and 20% oxygen samples were observed (a). hAMSCs from placental amnion showed significantly lower levels of intracellular ROS (a). Calculation of correlation coefficient showed a strong negative association ($r = -0.9039$) between intracellular ROS levels and phosphorylation-related respiration (b). Increased nitric oxide release was detected in cell culture supernatants of samples incubated at 20% oxygen (c). $n = 5$ (biological replicates), mean ± SD. Abbreviations: hAMSCs: human amniotic mesenchymal stromal cells; ROS: reactive oxygen species; RA: reflected amnion; P: placental amnion.

3.4. Generation of Reactive Oxygen and Nitrogen Species. Against our expectations, EPR measurement showed a trend to lower levels of intracellular ROS in hAMSCs incubated at higher oxygen tension. Furthermore, at 20% oxygen, we detected significantly lower levels of intracellular ROS in P-hAMSCs compared to RA-hAMSC (Figure 3(a)). Calculation of correlation between levels of intracellular ROS and phosphorylation-related respiration revealed a strong negative correlation ($r = -0.9039$) between these parameters (Figure 3(b)).

Levels of nitric oxide in the supernatant of the cells showed significant differences between 5% and 20% O_2. However, within the same oxygen tension, no differences were found between cells of reflected and placental amnions (Figure 3(c)).

3.5. Release of Immunoactive Substances. Measurement of immunoactive substances after 4 days showed significantly higher concentrations of IL-6 in the cell culture supernatant of hAMSCs of placental amnion compared to hAMSCs from reflected amnion (Figure 4(a)). Oxygen tension impacted P-derived hAMSCs to secrete higher levels of IL-6 when incubated at 20% O_2 compared to 5% O_2 (Figure 4(a)). A similar pattern was observed when measuring IL-10 release, but significant differences between reflected and placental amnions were only found when cells were incubated at 20% oxygen (Figure 4(b)). Oxygen tension did not impact the release of hepatocyte growth factor (HGF); however, hAMSCs from placental amnion released significantly more HGF compared to hAMSCs from reflected amnion (Figure 4(c)). In cells of both regions, we detected

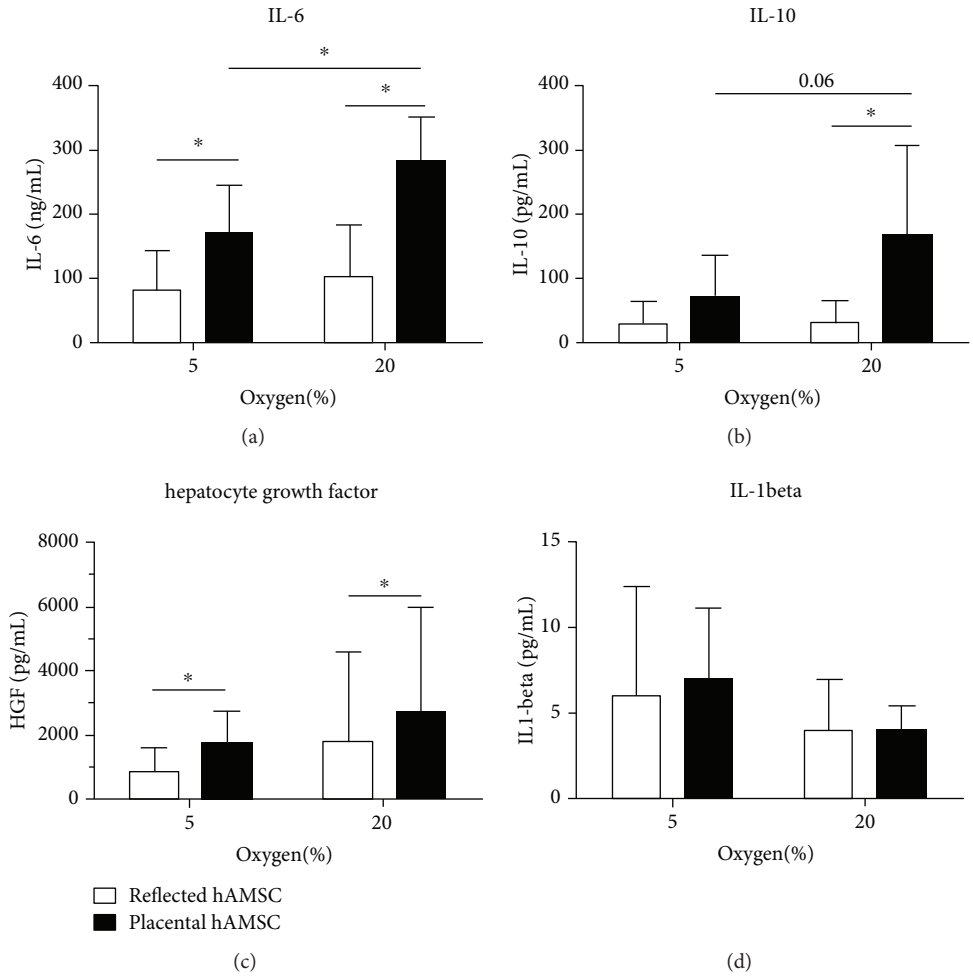

Figure 4: Release of immunoactive substances. Release of immunoactive substances in the cell culture supernatant of hAMSCs of reflected and placental amnion was measured after 4 days (5% and 20% oxygen) incubation (a)–(d). Higher oxygen tension (20%) lead to significantly higher secretion of IL-6 (a) and a trend to higher IL-10 release (b) in hAMSCs from placental amnion and had no effect on the release of hepatocyte growth factor (c) and IL1-beta release (d) compared to lower oxygen tension (5%). Cells from placental amnion released more IL-6 (a), IL-10 (b) and hepatocyte growth factor (c) when compared within cultures at same oxygen concentrations (5% and 20% oxygen). $n = 6$ (biological replicates), mean ± SD. Abbreviations: hAMSCs: human amniotic mesenchymal stromal cells; IL: interleukin.

very low concentrations of IL-1beta independent of the applied oxygen tension (Figure 4(d)).

4. Discussion

Cultivation of stem cells poses a great challenge, since maintenance of stemness requires niches with very low oxygen tensions. Common cell culture laboratories are usually set up for cultivation at 20% oxygen. Mitochondria are the main oxygen consumers, thereby providing energy via oxidative phosphorylation. Of note, stem cells acquire energy through ATP generation via glycolysis, and the metabolic switch from glycolysis to oxidative phosphorylation changes stem cell fate and cell function (reviewed in [23]).

Many research groups have experimented with bone marrow-derived or adipose tissue-derived mesenchymal stem cells under low or atmospheric oxygen tensions [17, 56–58]. Yet, to our knowledge, the influence of low and high oxygen tensions on hAMSCs under cell culture conditions and especially on their mitochondrial function has not been investigated. Furthermore, as the anti-inflammatory properties of the hAM could be critical in a clinical transplantation situation, we also measured parameters linked to inflammation.

Our data show that the mitochondrial metabolism of freshly isolated hAMSCs in culture is highly sensitive to surrounding oxygen levels. ROUTINE respiration (=LEAK state + phosphorylation-related respiration), reflecting total mitochondrial oxygen consumption, was increased in cultures of reflected amnion-derived hAMSCs (RA-hAMSCs) and placental amnion-derived hAMSCs (P-hAMSCs) in an oxygen-dependent manner. This increase came on one hand from an increase in LEAK state. On the other hand, incubation at 20% oxygen accelerated energy production via oxidative phosphorylation which was demonstrated by phosphorylation-related respiration and the netROUTINE control ratio. Interestingly, this effect was more pronounced in hAMSCs derived from placental amnion. This is in line with previous reports, suggesting the need for low oxygen cell culture conditions, similar to the physiological stem cell niche [59].

The idea to use mitochondrial parameters such as respiratory function as an index for stem cell competence was postulated previously [60, 61]. Our findings confirm that mitochondria-linked considerations should be taken into account in the proceedings of cell therapy in regenerative medicine to ensure quality of therapeutic cells.

We found that the LEAK state, reflecting proton permeability of the inner mitochondrial membrane without producing ATP, increased after 4-day incubation, independent of the oxygen tension and the amniotic region. Such an increase could be caused by different capacities of the electron transfer system (complex I–IV) in different microenvironments. This can be excluded in our study, as stimulation of maximal ETS capacity showed similar increases. Increased ROS production [62] is also unlikely, as we found lower levels of ROS. Interestingly, in stem cells, uncoupling proteins prevent mitochondrial glucose oxidation in response to high substrate concentration and thereby prevent ROS accumulation [63]. Therefore, our results can be explained by the high substrate concentration in common cell culture media compared to *in vivo* conditions. These results indicate that not only nonphysiological oxygen tensions but also nonphysiological substrate concentrations can change the cellular metabolism. We will focus on this matter in future studies.

Lactate concentrations in the supernatants were not influenced by oxygen tension, neither in reflected nor in placental amnion. This is not surprising since 5% oxygen is considered to be the physiological oxygen tension for amniotic cells. In addition, lactate concentrations are more often influenced by other factors than oxygen levels (reviewed in [64]).

Similarly, ATP concentrations are also influenced by many factors. We measured ATP concentrations of freshly isolated hAMSCs, as well as after cultivation at 5% and 20% oxygen tensions for 4 days. We found an increase of ATP concentrations after 4 days of culture, compared to freshly isolated cells. However, the net cellular ATP concentrations represent a steady state between synthesis and consumption. Therefore, ATP concentrations do not reflect actual mitochondrial activity (reviewed in [65, 66]).

We further found a trend to increasing mtDNA copy numbers in P-hAMSCs incubated at 20% oxygen, which is similar to an observation by Chen et al., who found increasing mtDNA copy numbers concomitant with an increasing oxygen consumption rate in the early phase of osteogenic induction [67]. In our study, the data suggest that the higher phosphorylation-related respiration is not due to a higher cellular mitochondrial density but to a higher coupling efficiency between the mitochondrial electron transfer system (complex I–IV) and the ATP synthase.

Surprisingly, although mitochondrial oxidative phosphorylation was increased in hAMSCs incubated at high oxygen tension, we observed a trend to lower levels of intracellular ROS in these cells. Calculation of correlation between phosphorylation-related respiration and intracellular ROS revealed a strong negative correlation between these parameters. Such findings have also been found with bone marrow-derived hMSCs [67]. The authors observed an increased oxygen consumption rate concomitant with decreased intracellular ROS levels. In addition, they detected upregulation of two mitochondrial antioxidant enzymes, manganese superoxide dismutase (Mn-SOD) and catalase [67], and could show that especially Mn-SOD was concurrently upregulated to prevent the accumulation of intracellular ROS.

Intracellular ROS such as superoxide ($O_2^{\cdot -}$) could also be inactivated by nitric oxide ($NO^{\cdot}$), which, beside its vasodilatory and signalling functions, has also antioxidant effectiveness [68]. In our study, independent of the amniotic region, $NO^{\cdot}$ showed higher levels at 20% oxygen compared to 5% oxygen. This was not surprising, since $NO^{\cdot}$ is predominantly generated in the presence of oxygen [69], except enzyme-independent generation of nitrite occurring in tissues under ischemic conditons [70]. However, in order to develop its full antioxidant capacity, $NO^{\cdot}$ has to be present in excess compared to $O_2^{\cdot -}$ [71]. In contrast, equimolar concentrations of $NO^{\cdot}$ and $O_2^{\cdot -}$ or excess of $O_2^{\cdot -}$ can induce oxidative damage [71]. Thus, modulation of the balance between $O_2^{\cdot -}$ and $NO^{\cdot}$ can be critical and may impact clinical applications.

For wound healing, increased $NO^{\cdot}$ generation could also be beneficial, as it has been shown to increase angiogenesis and improve neovascularization (reviewed in [72]). However, it can also activate matrix metalloproteinase- (MMP-) 9 (reviewed in [72]), which has been shown to play an important part in the spontaneous rupture of fetal membranes [73], possibly by its extracellular matrix-degrading activity. We suggest taking this fact into consideration while handling or cultivating the hAM under common cell culture conditions at 20% oxygen.

It is also important to note that excessive production of $NO^{\cdot}$ can cause inhibition of mitochondrial respiration, by irreversibly binding to complex I [74] or reversibly binding to complex IV [75]. Such excessive generation of $NO^{\cdot}$ occurs in response to inflammatory stimuli, which cause the upregulation of iNOS via nuclear factor- (NF-) κB or signal transducers and activators of transcription- (STAT-) dependent pathways [76].

Regarding parameters linked to inflammation, high oxygen tension showed the strongest effect on P-derived hAMSCs, which secreted significantly more IL-6, when incubated at 20% oxygen without any additional inflammatory challenge, compared to 5% oxygen. Similar results were found in a recent study with fetal membranes [77], which however, did not analyze reflected and placental amnions separately. IL-6 has been shown to inhibit proliferation of T cells [78] and, interestingly, also human amniotic epithelial cells [79]. Furthermore, Kumar et al. found proinflammatory cytokines in amniotic fluid to induce collagen remodelling, apoptosis, and weakening of cultured human fetal membranes [80]. The differences between the amniotic regions are more pronounced than the influence of the oxygen tension. Placenta-derived hAMSC secreted more IL-6, IL-10, and HGF compared to hAMSC of the reflected region. These data corroborate previous studies, where we and others already showed evidence of regional differences of the hAM [52, 53, 81–85]. In our latter report, we determined for the first time different mtDNA copy numbers in cells of the same type taken from different anatomical regions of the same individual, thus demonstrating that even at a normal physiological state, the cellular mtDNA copy number is tightly controlled not only in a cell type-specific but also in

a region-specific manner [53]. Epigenetic regulation of the nuclear and/or mitochondrial genomes represents one of the putative factors that should be studied with regard to these regional alterations of the cellular mtDNA content considering that epigenetic marks are cell-type specific [86, 87] and that demethylation of specific mtDNA sites may cause an elevation of its copy number [88].

We hypothesize that the different anatomical locations of the amniotic regions, one covering the placenta, the other opposite of it, may be the cause for the differences observed. The two different areas may carry out different biological tasks during pregnancy. Furthermore, placental amnion could be supplied with different amounts of oxygen and different patterns of nutrients than reflected amnion. It is, however, not entirely known, how the avascular amniotic membrane is provided with nutrients and oxygen. If oxygen is transferred by diffusion, then, *in utero*, more oxygen might diffuse to placental amnion compared to reflected amnion. This hypothesis is corroborated by the behaviour of mitochondria in human amniotic mesenchymal cells and human amniotic epithelial cells. Compared to reflected amnion, placental amniotic cells show higher phosphorylation-related respiration, a parameter strongly linked to aerobic energy generation [53].

In this study, we found placental amniotic cells to respond to oxygen more readily than reflected amniotic cells. This was seen not only with mitochondrial parameters but also with the release of $NO^{\cdot}$ and IL-6. Similar to $NO^{\cdot}$, IL-6 can also act in a dual role (reviewed in [89]). As recently shown, beside its pro-inflammatory action, IL-6 can attenuate inflammation [90]. Thus, cultivation under a "nonphysiological" oxygen tension may also have beneficial consequences for cell-based therapy. Hence, we propose that amniotic regions and the influence of oxygen should be taken into account for the cell cultivation for clinical applications of the hAM.

5. Conclusion

Taken together, we could show that even short-time cultivation (4 days) at common cell culture conditions (20% oxygen) of freshly isolated hAMSC induced significant changes in mitochondrial function and the release of IL-6. Furthermore, the impact of high oxygen tension can be different, depending on the amniotic region, reflected or placental. Investigation of some selected parameters already showed that cultivation conditions can strongly influence cell physiology.

Mitochondrial activity and immunoactive factors are tightly interconnected with fundamental cellular processes involved in tissue regeneration. These distinctly different properties should be taken into consideration for clinical applications of amniotic cells. Depending on the therapeutic purpose, cultivation conditions of the cells should be chosen carefully for providing the best possible quality of cell therapy.

Abbreviations

ATP: Adenosine triphosphate
CP-H: Cyclic hydroxylamine 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride
EPR: Electron paramagnetic resonance
ETS: Electron transfer system
hAM: Human amniotic membrane
hAMSCs: Human amniotic mesenchymal stromal cells
HGF: Hepatocyte growth factor
IL: Interleukin
iNOS: Inducible nitric oxide synthase
MMP: Matrix metalloproteinase
Mn-SOD: Manganese superoxide dismutase
mtDNA: Mitochondrial DNA
NF: Nuclear factor
P-hAMSCs: Placental amnion-derived hAMSCs
RA-hAMSCs: Reflected amnion-derived hAMSCs
ROS: Reactive oxygen species
STAT: Signal transducers and activators of transcription.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

We acknowledge Philipp Steindorf (VetCore, Vetmeduni Vienna) and Mohammad Jafarmadar for technical assistance. This work was partly supported by the European Union's Horizon 2020 research and innovation program "Arrest Blindness" (grant number 667400) and Österreichische Forschungsförderungsgesellschaft (FFG, grant number 854180).

Supplementary Materials

Supplemental Table 1: oligonucleotide sequences of quantitative PCR and digital PCR. Supplemental Figure 1: mitochondrial activity measurement. Representative oxygen consumption trace of placental hAMSCs incubated for 4 days at 20 % oxygen (P, blue), and reflected hAMSCs incubated for 4 days at 5 % oxygen (RA, magenta). 1 = ROUTINE respiration, 2 = LEAK, 3 (D1-2) = phosphorylation-related respiration. Abbreviations: hAMSCs, human amniotic mesenchymal stromal cells. Supplemental Figure 2: adenosine triphosphate concentration (ATP). ATP concentration wasmeasured in 100 000 freshly isolated hAMSCs at day 0, and after 4 days incubation at 5 % and 20 % oxygen. The cells were homogenized and ATP was determined using luciferase reagent with Luminat LB9507. n = 3-5 (biological replicates), mean ± SD, $^{*}p < 0.05$ vs day 0. Abbreviations: hAMSCs, humanamniotic mesenchymal stromal cells; O2, oxygen. *(Supplementary Materials)*

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Hindawi
Stem Cells International
Volume 2018, Article ID 7412035, 8 pages
https://doi.org/10.1155/2018/7412035

Review Article

Tissue Regeneration without Stem Cell Transplantation: Self-Healing Potential from Ancestral Chemistry and Physical Energies

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Received 28 December 2017; Accepted 20 June 2018; Published 3 July 2018

Academic Editor: Andrea Ballini

The human body constantly regenerates after damage due to the self-renewing and differentiating properties of its resident stem cells. To recover the damaged tissues and regenerate functional organs, scientific research in the field of regenerative medicine is firmly trying to understand the molecular mechanisms through which the regenerative potential of stem cells may be unfolded into a clinical application. The finding that some organisms are capable of regenerative processes and the study of conserved evolutionary patterns in tissue regeneration may lead to the identification of natural molecules of ancestral species capable to extend their regenerative potential to human tissues. Such a possibility has also been strongly suggested as a result of the use of physical energies, such as electromagnetic fields and mechanical vibrations in human adult stem cells. Results from scientific studies on stem cell modulation confirm the possibility to afford a chemical manipulation of stem cell fate *in vitro* and pave the way to the use of natural molecules, as well as electromagnetic fields and mechanical vibrations to target human stem cells in their niche inside the body, enhancing human natural ability for self-healing.

1. Introduction

The human body continuously regenerates due to the peculiar properties of its resident stem cells.

These cells possess the unique ability to self-renew and differentiate, and the balance between these two processes defines the stem cell fate and their primary role in tissue regeneration [1].

Regeneration is the recovery of the organ structure and function after injury and it is at the basis of our self-healing potential and therefore of the preservation of human health. Such a process exhibits remarkable grading in the way it is fashioned in living organisms, since, within the same species, the regenerative potential is different among the various organs [2].

To rescue damaged tissues and restore functional organ mass, huge efforts have been made in the growing field of regenerative medicine, engaging scientific research in the understanding of the molecular mechanisms through which the regenerative potential of stem cells (as human mesenchymal stem cells - hMSCs) may be unfolded into a clinical application [3]. Stem cells in fact have the capability to differentiate into a wide range of adult cells and the discovery and isolation of them paved the way to new hopes in the regenerative field.

On the other hand, many aspects of the cell-based therapy prevent the use of stem cells to regenerate organs and tissues: among them, a large amount of stem cells is required and the senescence process occurs during primary cell expansion. Moreover, it is not easy to isolate stem cells and to

commit all of them toward a specific phenotype, since they can differentiate in all types of mature cells, including cancer cells. Therefore, a proper set up of *in vitro* MSC expansion, cryopreservation, and banking should be necessary to establish safety and efficacy in transplanted patients.

In addition, most of the applications of stem cells directed on patients are still under the phase of experimental trials, except for some procedures actually used in clinical practice, as the bone marrow transplantation in hematology [4].

Even tissue engineering, one of the branches of the regenerative medicine based upon tissue regeneration from cells with the aid of biomaterials and growth factors, still is facing several problems. In fact, the regenerated tissues usable by patients are still very limited, as skin, bone, cartilage, capillary, and periodontal tissues [5]. Moreover, the engineered artificial tissue still exhibits some limitation correlated to the dimensions of the construct that cannot be used for the recovery of serious defects. Actually, the only amenable engineered tissues with a tridimensional structure are vases, cave structures like the trachea [6], or tissues which are not physiologically scattered, since the viability of cells seeded on a scaffold gradually decreases with thickness. Even the use of growth factors alone or in association with 3D constructs is still considered as being not completely safe since the resulting influence on recipient's environment remains in part to be established. Finally, other hurdles remain, such as finding the best scaffold, the most suitable bioreactor, and the optimal solution for seeding different cell populations in order to have a relevant mature material implantable on patients.

All these issues need to be addressed before cells or engineered constructs can be used routinely in the clinical setting. Therefore, multiple studies have long been running to address the modulation of relevant physiological traits known to be involved in tissue homeostasis and in the activation of the stem cell niches. To this end, besides investigating the effects of synthetic molecules, many researchers have also focused their attention on the effects elicited by natural molecules and physical energies. Their findings are reported below.

2. Natural Molecules

The ability to regrow body parts is common to a lot of animal species, although the regenerative potential varies among taxa [7]. Some phyla are able to rebuild every part of the body, while others cannot regenerate internal organs [8].

Danio rerio (zebrafish) is among the organisms capable of amazing regenerative processes, prompting the needs for uncovering the underlying regeneration strategies. Zebrafish is since recently commonly used as an animal model of organogenesis and regeneration, owing to its ability to regenerate complex organs, like the heart, the central nervous system, and the limbs, at an extraordinarily higher efficacy than humans [2, 3, 9–16]. Another species exhibiting astonishing regenerative potential is the Mexican axolotl (*Ambystoma mexicanum*), which can make self-copies regenerating a missing limb, tail, or parts of the brain, heart, and lower jaw. Other creatures in the spotlight for their regenerative capabilities are the salamanders, as well as several frogs [17], or the tunicates [18]. Despite their evolutionary distance, as in the case of zebrafish which is separated by about 450 million years from humans, our stem cells can still sense ancestral microenvironmental cues from these species, as shown by the finding that human cord blood $CD34^+$ cells are recruited into early vasculogenesis upon transplantation in pre-gastrulation, but not post-gastrulation zebrafish embryos [19]. Akin to this view is the finding that conserved transcriptional responses have been discovered among the differentiation of hMSCs, Xenopus embryogenesis, and axolotl regeneration, identifying common networks across model species that are associated with depolarization (changes in cellular resting potential) [20].

On the whole, these findings and the deployment of comparative biology into the analysis of conserved evolutionary patterns in tissue regeneration may lead to the identification of natural molecules capable to extend their regenerative potential from ancestral species to human tissues through the manipulation of common/similar mechanisms in their resident stem cells.

Investigation of the role of natural molecules in stem cell biology is becoming a growing area of inquiry. Psoralidin, for example, a natural phenolic compound found in the seeds of *Psoralea corylifolia*, has been seen to inhibit NOTCH1 in breast cancer stem cells and in breast cancer cells, leading to a growth arrest and inhibition of epithelial to mesenchymal transition (EMT) [21]. Moreover, two herbal extracts (*Tithonia diversifolia* leaf extract and *Momordica foetida* extract) led to a decrease of the adipogenesis and accumulation of lipid droplets in human adipose-derived stem cells (hADSCs) [22, 23]. Two natural compounds, honokiol (a low-molecular-weight polyphenol isolated from the genus *Magnolia*) and hyperoside (a flavonoid compound extracted from *Hypericum perforatum*), were shown to potentially induce the differentiation into neurons in the murine embryonic carcinoma cell line P19 [24]. Synthetic compounds created by the assembly of natural molecules have also been proven effective in the modulation of stem cell biology *in vitro* and *in vivo*. To this end, mixed esters of naturally occurring molecules, such as hyaluronan mixed esters with butyric and retinoic acids (HBR), have been shown to remarkably increase cardiogenesis and vasculogenesis in mouse embryonic stem cells [25] and hMSCs [26], enhancing the ability of term placenta hMSCs of promoting the regeneration of infarcted myocardium *in vivo* in both small (rat) and large (pig) animal models with post infarct heart failure [27, 28]. Intriguingly, in the myocardium of infarcted rats, HBR itself acted through the intracellular release of its natural grafted molecules to afford significant decrease in infarct size, and apoptotic myocytes, leading to reverse myocardial remodeling, normalization in myocardial contractility, and increase in vital myocardial mass and metabolism, through the enhancement/recruitment of the number of endogenous stro-1 (a mesenchymal stem cell marker)-positive stem cells, the increase in the number of local elements with pericyte identity and important revascularization processes [29]. This finding shows the feasibility of

chemical targeting damaged organs to afford tissue survival and repair without stem cell transplantation. Consonant with these results, a simple cocktail of hyaluronic, butyric, and retinoic acids was able to improve islet graft revascularization and function by adipose tissue-derived hMSCs in diabetic rats [30].

The addition of melatonin to this mixture of natural molecules was able to shift the commitment of hMSCs towards an osteogenic fate, indicating the feasibility of creating a multicomponent, multitarget ensemble of natural agents to chemically redirect the multilineage repertoire of hMSCs [31].

A major breakthrough in the effort of using natural arrays of molecules to drive cellular fates under normal and pathological conditions came by the discovery that extracts from zebrafish embryos obtained at different developmental stages were able to counteract the proliferation rate of several cancer cell lines [32–35]. Extracts from the beginning, intermediate and final embryonic development stages led to an evident increase in p53 expression in association with the growth reduction [33]. In some cancer cell lines, such as kidney adenocarcinoma, the proliferation decrease was associated with changes in pRb phosphorylation, a cell cycle modulator [34]. Moreover, in colon adenocarcinoma cells, an activation of the p73-dependent apoptotic pathway was observed [35]. A mixture of early, middle, and late developmental stage zebrafish extracts was also able to enhance cell survival to toxic stimuli, as shown by the reduction in mortality observed in cells from mouse hippocampal slices (CA1 area) that had been subjected to serum deprivation or NMDA (N-methyl-D-aspartate) treatment [36]. These findings and previous observations showing that embryonic microenvironment is able to suppress tumor development during cell differentiating processes [37, 38] led us to further investigate whether zebrafish embryonic factors may also be exploited in a developmental stage manner to control essential features in stem cell dynamics. To this end, we successfully used early-stage developmental zebrafish extracts (obtained from 5.15 hours post fertilization embryos) on early-passage hADSCs to enhance the stem cell expression of multipotency, and the transcription of *TERT*, encoding the catalytic subunit of telomerase, as well as the gene expression of *BMI1*, a chromatin remodeler acting as a major telomerase-independent repressor of senescence [39].

On the whole, the above mentioned studies, showing the possibility to afford a chemical manipulation of stem cell fate *in vitro*, may pave the way to the use of natural or synthetic chemistry to target human stem cells where they are already resident in all body tissues. This would lead to the development of a regenerative medicine executed without the needs for (stem) cell or tissue transplantation.

3. Physical Energies

The possibility of using physical energies to boost regenerative processes has been strongly suggested by the ability of electromagnetic fields and mechanical vibrations to drive efficient *in situ* reprogramming of the differentiating and regenerative potential of our endogenous stem cells.

We are in fact embedded in a wide variety of physical stimuli, including electromagnetic fields, light radiation, and mechanical oscillatory patterns. In this sense, our life which contains a seeming infinity of rhythmic oscillations, including calcium and pH intracellular oscillations [40–42], as well as the rhythmic expression of genes and proteins [43, 44], can be considered as a part of the vibratory nature of the universe.

It is now evident that our cells perceive and generate energies like magnetic fields and mechanical oscillations [45–47]. Cells contain a network of microtubules that, due to their electrical polarity and intrinsic vibration modes, is able to generate high-frequency electric fields with radiation features [48]. Applying scanning tunneling microscopy (STM) to microtubules growing onto a nanoelectrode array, within an artificial cell replica designed to pump electromagnetic frequencies, has shown the existence of resonance patterns between the tubulin dimers, or the whole microtubules, and the applied frequencies [49]. STM also provided evidence that such resonance patterns could be imaged as specific "tunneling current profiles" corresponding to the pumped electromagnetic frequencies [49]. The frequency region selectivity for engaging particular types of conformational modifications establishes that pure mechanical changes can be remotely managed in an atomically way by using electromagnetic fields.

The importance of the microtubule network as an *information-transporting-system* is also deduced by the finding of multilevel memory-switching properties in a single brain microtubule [50]. Even DNA, despite its role of storage and expression of genetic information, when considered as an electrically charged vibrational entity, may contribute to cell polarity, also by virtue of its constant assembly into different loops and domains that are an essential component of the nanomechanics and nanotopography imparted to this macromolecule by transcription factors and molecular motors. Accordingly, electromagnetic resonance frequency spectra have been revealed for DNA, which was found to exhibit electromagnetic resonances in the wide frequency range from KHz, MHz, GHz, to THz [51].

Recently, regenerative medicine has been focused on the use of biophysical stimuli to modulate cellular dynamics [52]. Physical factors in the cellular microenvironment, including matrix mechanics, cell geometry and shape, mechanical forces, and nanotopographical aspects of the extracellular matrix, can modulate the stem cell fate [53, 54]. There is evidence that this type of regulation is highly affected by coexisting insoluble, adhesive, mechanical, and topological cues contained and dynamically regulated within the stem cell niche [55, 56]. Biophysical stimuli can be sensed and transduced into intracellular biochemical and functional responses by stem cells, a process known as mechano-transduction [55]. The stem cell sensory machinery can at the same time perceive and integrate several signals from the niche and turn them into coherent responses affording downstream modulation of gene expression and stem cell fate [55, 57–59].

For years, scientists tried to drive stem cell fate by the aid of chemistry, increasing cell proliferation with growth factors

or fabricating 3D constructs derived from the combination of stem cells or mature adult cells, with natural or artificial polymers. Only in the last years, efforts have been made to interact with cells *in vivo*, directly on patients or on animal models, and *in vitro* on cell cultures. Recently, some research groups have shown the possibility to use physical stimuli directly on patients, tissues, and cells [60].

The idea behind the use of physical stimuli on tissues and body was already proposed in 1974 by Richard Nuccitelli who gained evidence on endogenous ionic current and interaction with electric field in multicellular animal tissues [61]. Nowadays, it is possible to explain changes in cellular behavior, following electromagnetic stimulation, considering an effect on cell polarity [62] and on the stem cell niche in the body [63].

The use of physical energies for therapeutic purpose is now well known, being approved by the Food and Drug Administration (FDA) and used on patients. Several devices based on different physical mechanisms have been designed, and the beneficial effects have been observed directly on patients. Ultrasounds have been used for medical purposes since the 1950 in some pathological situations, such as tendinitis or bursitis [64].

Even the use of extremely low-frequency electromagnetic fields (ELF-EMFs) with frequencies lower than 100 Hz, and magnetic field intensity spanning from 0.1 to 20 mT, became a useful therapy for soft tissue regeneration, fracture repair, and osteoporosis treatment [65]. The mechanisms of action of ELF-EMFs are not clear yet. However, it has been shown that electric currents can accelerate cell activation [66] and influence epigenetic remodeling. In particular, the use of 50 Hz ELF-EMF on GC-2 cells decreased genome-wide methylation and the expression of DNA methyltransferases [67] in neural stem cells (NSCs) isolated from the hippocampus of newborn mice. Moreover, the ELF-EMF irradiation at 1 mT, and 50 Hz, for 12 days enhanced NSC proliferation and neuronal cell fate specification through Cav1 channel-dependent regulation and histone modification [68]. These results show the feasibility of using physical stimuli to affect cell fate.

Within this context, we have first demonstrated the possibility to use ELF-EMFs to modulate the gene transcription of essential growth regulatory peptides in adult myocardial cells [60] and to enhance cardiogenesis and terminal differentiation into spontaneously beating myocardial cells in mouse embryonic stem (ES) cells [69]. Then, by the aid of a radio electric asymmetric conveyer (REAC), we found that properly conveyed radioelectric fields of 2.4 GHz could produce important biological effects in mouse ES cells and human adult stem cells. In both cell types, we showed that REAC-conveyed radioelectric fields elicited an increase of the expression of stemness-related genes, followed by the commitment towards neuronal, myocardial, and skeletal muscle lineages [70, 71]. The same differentiating outcomes were induced by REAC exposure in human skin fibroblasts [72]: for the first time, human non-stem somatic adult cells were committed to lineages in which they would never otherwise appear. This effect was mediated by a biphasic change in pluripotency gene expression, a temporary overexpression followed by a down regulation, and did not require the use of viral vector-mediated gene transfer technologies or cumbersome synthetic chemistry.

Noteworthy, REAC exposure of hADSCs was able to turn stem cell senescence, occurring after prolonged (up to 30 passage) *in vitro* expansion, into a reversible phenomenon, associated with a decrease in the expression of senescence-associated β-galactosidase and an increase in *TERT* gene expression and telomere length. The REAC action also enhanced the gene transcription of *BMI1* and that of stemness-related genes, establishing a telomerase-independent arm for senescence reversal [73]. These findings may have important biomedical implications, since senescent stem cells decrease their self-renewal and differentiation potential, reducing their ability for tissue regeneration *in vivo* and the possibility of a prolonged expansion *in vitro* prior to transplantation.

Compounding the wide-ranging biological effects of REAC stimulation is the observation that this technology was able to promote neurological and morphofunctional differentiation in PC12 cells [74], a rat adrenal pheochromocytoma cell line displaying metabolic features of Parkinson's disease. Cell response to the electromagnetic field was mediated by the transcriptional activation of neurogenic genes, as *neurogenin-1*, *β3-tubulin*, and *nerve growth factor (NGF)*, and was associated with a consistent increase in the number of cells expressing both β3-tubulin and tyrosine hydroxylase [74]. These findings open the new perspective of using physical energies in the treatment of neurodegenerative diseases and in the reprogramming of cancer (stem) cells into normal regenerative elements. More recently, we found that the REAC action could be significantly counteracted by stem cell treatment with 4-methylumbelliferone (4-MU), a potent repressor of type-2 hyaluronan (HA) synthase and endogenous HA synthesis [75]. This observation suggests that REAC-mediated responses may have occurred through an essential pleiotropic role of this glycosaminoglycan in regulating (stem) cell polarity.

Extracorporeal shock waves (ESW) represent another type of biophysical stimuli that is increasingly being applied in the field of regenerative medicine and that could be classified as "mechanotherapy" (*i.e.*, extracorporeal shock wave therapy, ESWT). In fact, ESW are "mechanical" waves, characterized by an initial positive very rapid phase, of high amplitude, followed by negative pressure, producing a "microexplosion" that can be directed on a target zone (body, tissue, or cells) in order to stimulate or modify the cells in their behavior. Shock waves are generated by an electrohydraulic device that produces underwater high-voltage condenser spark discharge, conveyed by an elliptical reflector on tissues or cells.

In the 1980s, shock waves were used in urology (lithotripsy) to disintegrate renal stones [76]. Then, ESW application has been extended to other fields, showing promising hopes for promoting tissue healing and the recovery from pathological disorders. One of the first applications was in the orthopedic field, in order to induce neovascularization and improve blood supply and tissue regeneration. Investigations on the use of this technology spread progressively, and

leading to its application in the treatment of musculoskeletal disorders [77], tendon pathologies [78], bone healing disturbances, and vascular bone diseases [79]. The use of ESW has also been extended to the field of dermatology for the wound healing disturbances and ulcers. However, to date, the exact mechanism through which cells convert mechanical signals into biochemical responses is not well understood yet. Emphasis has been placed so far into mechanisms mediated by ATP release and P2 receptor activation that may foster cell proliferation and tissue remodeling via Erk1/2 activation [80, 81], as well as PI-3K/AKT and NF-κB signaling pathways, and the implication of TLR3 signaling and subsequent TLR4. Several studies performed *in vitro* proved the effect of ESW on cell modulation through "mechano-transduction". Recently, ESW were found to activate ADSCs through MAPK, PI-3K/AKT, and NF-κB signaling pathways [82, 83] and to induce in HUVEC cells an overexpression of angiogenic factors and of caveolin-1, a constitutive protein of caveolae, implicated in the regulation of cell growth, lipid trafficking, endocytosis, and cell migration [84].

In addition, the ESWT effect on cell behavior proved to be a dose-dependent phenomenon. In a study published by Zhang and coworkers, cells exposed to low-energy ESW (0.04 e 0.13 mJ/mm^2) improved the expression of some angiogenic factors, such as eNOS, Ang-1, and Ang-2. On the other hand, at higher energy, ESW induced a reduction in angiogenic factor expression and an increase in apoptosis [85]. These findings suggest that the biological effects of shock waves strongly correlated with the intensity of applied energy and thus with the related mechanical forces.

Recently, the effects of shock waves have been characterized on the expression of IL-6, IL-8, MCP-1, and TNF-α in human periodontal ligament fibroblasts [86]. Following an early inhibition on the expression of pro-inflammatory mediators, shock waves elicited a dose-related increase in IL-6 and IL-8, while down-regulating TNF-α expression [86]. Most of the literature showed an anti-inflammatory effect of ESWT *in vivo* [59, 78, 79, 87, 88]. Nevertheless, the pro-inflammatory effect of ESWT partially observed on cells *in vitro* may suggest a pro-activator event mediated by cytokine and chemokine expression. It was supposed that the shock wave impulses on cells were able to create a pro-inflammatory milieu, mediated by mechano-transduction [80]. However, this mechanism may involve a more complex action on the whole niche architecture, with the embedded (stem) cells behaving as sensors and activators of the regenerative response.

In actual fact, mechanical vibration may represent a relevant modality to affect stem cell reprogramming *in vivo* without having to resort to transplantation procedures. In this regard, we have shown and patented for the first time the cell ability to exhibit "vibrational" (nanomechanical) signatures of their health and their multilineage repertoire [89]. Wide-ranging vital processes are fashioned around the nanomechanical features of subcellular structures, like the microtubular networks, imparting feature characteristic of connectedness and synchronization that can be transferred and recorded from the cell surface. Atomic force microscopy (AFM) can be used to gain insights on cellular nanomechanical properties [89, 90], providing the chance to identify vibrational signatures that can be used to drive lineage-specific commitments in different stem cell populations *in vitro* or even *in vivo* to promote endogenous rescue in diseased organs.

4. Conclusion

The emerging view of a (stem) cell biology governed by physical forces and influenced by ancestral natural molecules may lead us to reinterpret the way we envision the field of regenerative medicine for a near future.

In fact, due to the diffusive nature of electromagnetic fields and mechanical vibrations, the chance is emerging to target and reprogram the stem cells where they are, enhancing our natural ability for self-healing without the needs for (stem) cell transplantation which still shows remarkable limitations.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Authors' Contributions

Federica Facchin and Eva Bianconi contributed equally as co-first authors to this study.

Acknowledgments

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: funded by Eldor Lab, Milan, Italy, and AMeC (Associazione Medicina e Complessità), Via Valdirivo 19, 34100 Trieste, Italy.

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Hindawi
Stem Cells International
Volume 2018, Article ID 9863194, 11 pages
https://doi.org/10.1155/2018/9863194

Review Article

Extracellular Vesicles: A New Prospective in Crosstalk between Microenvironment and Stem Cells in Hematological Malignancies

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Received 19 January 2018; Accepted 31 March 2018; Published 27 May 2018

Academic Editor: Margherita Maioli

The bone marrow (BM) microenvironment in hematological malignancies (HMs) comprises heterogeneous populations of neoplastic and nonneoplastic cells. Cancer stem cells (CSCs), neoplastic cells, hematopoietic stem cells (HSCs), and mesenchymal stromal/stem cells (MSCs) are all components of this microenvironment. CSCs are the HM initiators and are associated with neoplastic growth and drug resistance, while HSCs are able to reconstitute the entire hematopoietic system; finally, MSCs actively support hematopoiesis. In some HMs, CSCs and neoplastic cells compromise the normal development of HSCs and perturb BM-MSCs. In response, "reprogrammed" MSCs generate a favorable environment to support neoplastic cells. Extracellular vesicles (EVs) are an important cell-to-cell communication type in physiological and pathological conditions. In particular, in HMs, EV secretion participates to unidirectional and bidirectional interactions between neoplastic cells and BM cells. The transfer of EV molecular cargo triggers different responses in target cells; in particular, malignant EVs modify the BM environment in favor of neoplastic cells at the expense of normal HSCs, by interfering with antineoplastic immunity and participating in resistance to treatment. Here, we review the role of EVs in BM cell communication in physiological conditions and in HMs, focusing on the effects of BM niche EVs on HSCs and MSCs.

1. Introduction

Normal hematopoietic stem cells (HSCs) reside in bone marrow (BM) and are supported by specialized and strictly organized stem cell niches, like endosteal and vascular [1]. The communication with other BM cells, including mesenchymal stromal/stem cells (MSCs), is crucial for HSC self-renewal, survival, and behavior. This dialogue within BM cell populations takes place through numerous extracellular and intracellular factors including hematopoietic growth factors and their receptors, signaling pathways, and cell cycle signaling [2].

Genetic alterations in HSCs or progenitors are associated to several hematologic malignancies (HMs) such as myelodysplastic syndrome (MDS), myeloproliferative neoplasia, acute myeloid leukemia (AML), chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL), and acute lymphoblastic leukemia [3]. Following genetic alterations, HSCs or progenitors are transformed into leukemia stem cells (LSCs) that retain self-renewal capability and uncontrolled

differentiation into leukemic blasts [4]. LSCs reside in the same niche as healthy HSCs and, on one side, they benefit from BM niche support and, on the other side, they modify the BM niche in order to induce a favorable environment for leukemic growth hampering normal hematopoiesis [5]. In addition, the interactions between LSCs and the endosteal niche sustain their silent state and protect them from the cytotoxicity of conventional chemotherapy [6, 7].

Studying the crosstalk between HSCs, LSCs, hematological neoplastic cells, and the BM microenvironment will enhance our comprehension of some human diseases including several HMs and the discovery of new potential therapies.

Extracellular vesicles (EVs) are emerging as new players in the intercellular communication and as new potential biomarkers for diagnosis and prognosis of human diseases [8–12]. They are a heterogeneous group of cell-derived vesicles including exosomes (Exo) and microvesicles (MVs) with a size ranging between 15 nm and 10 μm in diameter and with diverse biogenesis [13]. Different cells in physiological and pathological conditions, including tumor cells, can secrete EVs [14]. They act both in short-range intercellular communication, for example in the medullary microenvironment or in coculture conditions, and in long-range communication when released into the bloodstream through which they can reach secondary sites and give rise to premetastatic niches [15–17]. EVs carry part of DNA, RNA, proteins, lipids, and metabolites of the origin cells. Since EVs are present in biological fluids such as blood, urine, and sperm, [18, 19] and are a representative part of the whole cell for their phenotype and content, they could be used as a diagnostic tool by mimicking a "liquid biopsy." These last characteristics make them excellent candidates as diagnostic and/or prognostic biomarkers in different diseases, especially in tumors, through noninvasive or minimally invasive procedures. In our recent study, we found that serum EV number and their specific *oncomiRNA155* are higher in HM patients than in healthy subjects and, more importantly, EVs exposed specific tumor-associated surface markers [20, 21].

Stem cells (SCs) from embryos [22, 23], from different adult tissues such as BM, liver, and adipose tissue, and from induced pluripotent SCs, release EVs [24, 25]. Moreover, embryonic SC-EVs deliver mRNAs of pluripotent transcriptional factors such as HoxB4, Nanog, Oct3/4, and Rex-1, and transfer them to recipient cells, supporting hematopoietic progenitor cell expansion [26]. In addition, SC-EV microRNAs (miRNA) downregulate cell adhesion molecule levels, contributing to hematopoietic progenitor cell mobilization [27]. In a tumor context, SCs secrete EVs, which act as a means of communication in the tumor microenvironment playing multiple roles in tumorigenesis, and both in tumor angiogenesis and metastasis [28]. Finally, in *in vivo* models, SC-EVs mainly exhibit an inhibitory effect on the immune system suppressing proinflammatory processes and reducing oxidative stress and fibrosis [29]. Remarkably, MSC-EVs promote tissue renewal by inducing a proregenerative environment allowing stem and progenitor cells to successfully maintain tissue homeostasis. Importantly, MSC-EVs were used in two human disease therapies. In the first study, the administration of MSC-EVs reduces graft-versus-host disease (GvHD) symptoms and reduces steroid doses in an allogeneic transplantation of patients suffering from steroid refractory GvHD [30]. In the second study, the MSC-EV therapy triggers the regeneration within the affected kidney in patients with chronic kidney disease [31].

Although much has been reported about the stem cell and MSC-EV role, less is known about the influence of BM-EVs on HSCs and MSCs in physiological conditions and in malignancy onset, progression, and therapy resistance. In this review, therefore, we will discuss the recent advances in the field of EVs as actors in communication between cells within the BM niche in physiological conditions and in HMs, underlining the role and the effects in the tumor microenvironment-stem cell crosstalk. In particular, we will focus on the effects of EVs from BM niche cells on HSCs and MSCs.

2. Stem Cells

2.1. Hematopoietic Stem Cells (HSCs). HSCs are the only cells into the hematopoietic system that possess the potential for both pluripotency and self-renewal [1]. Pluripotency is the ability to differentiate into all functional blood cells; self-renewal is the ability to generate identical daughter cells without differentiation [32]. Postnatally, the BM is the primary site of HSC maintenance and hematopoiesis, but hematopoietic stress reallocates the niche to the spleen and induces extramedullary hematopoiesis. Although HSCs comprise only about 0.005–0.01% of the BM cell population, each single HSC retains the capability alone to reconstitute the entire hematopoietic system [33].

In AML, leukemia initiating cells (also named LSCs) represent a rare cell population that self-renews and generates an immature progeny invading and perturbing normal hematopoietic tissues [34]. HSCs and LSCs physically and functionally interact with the BM niche [35]. It is demonstrated that both HSCs and LSCs can be extended *in vitro* for a long time either in environmental conditions that mimic BM support or in coculture with BM stromal cells. These observations reinforce the crucial role that the BM niche, in particular the stroma, plays in healthy and leukemic stem cell homeostasis [5, 36–38]. It is still controversial whether cell-cell contact between hematopoietic stem/progenitor cells (HSPCs) and stromal cells is necessary to promote the hematopoietic cell expansion [39–43]; it is indeed clear that the definition of niche components and how they regulate hematopoiesis will provide the opportunity to improve regeneration after injury or HSC transplantation and to understand how disordered niche function could contribute to diseases, in particular to HMs.

2.2. Mesenchymal Stromal/Stem Cells (MSCs). The International Society for Cellular Therapy reported the minimal criteria for MSC definition: (i) they adhere to plastic under standard culture conditions; (ii) they express CD73, CD90, and CD105; (iii) they lack the expression of CD45, CD34, CD11b or CD14, CD19 or CD79a, and HLA-DR; and (iv) they have the potential to differentiate into the osteogenic, chondrogenic, and adipogenic cell lineages [44, 45]. MSCs

may be isolated from BM, umbilical cord, liver, adipose tissue, and multiple dental tissues [46, 47]; here, we will focus on MSCs derived from BM. They maintain long-term, quiescent HSCs through the presentation of surface signals and the secretion of major stemness supportive cytokines such as leukemia inhibitor factor and IL-6 [48, 49].

On the contrary, MSCs from leukemic patients hamper *in vitro* hematopoietic cell expansion and differentiation. In particular, AML-patient MSCs significantly impair the expansion of human umbilical cord blood $CD34^+$ progenitors and limit their differentiation to maintain a stable pool of immature quiescent precursors ($CD34^+$ $CD38^-$) compared to healthy donor-derived MSCs (hereafter healthy MSCs) [50]. Remarkably, healthy MSCs maintain AML patient blasts in a quiescent state resulting in increased leukemic survival after treatment with cytarabine [51]. Overall, MSCs have a functional role in the regulation of the BM microenvironment, in particular by influencing the immune system and angiogenesis and in supporting hematopoiesis [52–55] and, consequently, they are widely used in allogeneic hematopoietic stem cell transplantation [56, 57].

However, much work remains in defining the relationship between MSCs, HSCs, and other niche cells, especially on how they interact with each other and how these interactions regulate the hematopoiesis. Uncovering how the microenvironment participates in normal and HM progression will enhance new approaches to hematological disorders.

3. Extracellular Vesicles

On the basis of biophysical properties (i.e., size and shape) and the mechanism of biogenesis, EVs are classified into Exo, MVs, apoptotic bodies, and oncosomes [58, 59].

Exo are the smallest EVs (20–150 nm) that are generated inside multivesicular bodies which are secreted after their fusion with the plasma membrane [60, 61]. They show a higher rigidity of their lipid bilayer compared with that of cell membranes, making them resistant to degradation and useful as vehicle of different biomolecules. The formation and the release of Exo take place through both endosomal sorting complex required for transport-dependent or -independent mechanisms [60, 61].

MVs enclose EVs with a more heterogeneous size (50–1000 nm) bud directly from the plasma membrane and, for this reason, their surface markers are largely dependent on the composition of the membrane from which they derive [59].

Apoptotic bodies are membrane blebs that are released during cell apoptosis [62] with a diameter ranging between 50 nm and 5 μm, contain DNA binding histones, and are depleted in glycoproteins [63, 64].

Lastly, oncosomes are the largest EVs (1–10 μm in size) produced by membrane protrusions of malignant cells that lug bioactive molecules involved in the progression of cancer [64, 65].

The release of EVs from donor cells can be constitutive or be induced in response to activation or stress signals [64], including glucose and intracellular Ca^{2+} concentrations, oxygen tension, and microenvironmental pH [66]. Interestingly, EVs contain cargos of diverse nature including nucleic acids (i.e., mRNA, noncoding RNA such as miRNA, transferRNA, and genomic and mitochondrial DNA), cytosolic and membrane proteins, lipids, cellular organelles like mitochondria [67, 68], and metabolites [69, 70]. Interestingly, some databases such as EVpedia, Vesiclepedia, and ExoCarta collect the currently known components of EVs [71–73].

Notwithstanding, the content of EVs generally reflects the nature and the status of the donor cell: EVs could be enriched or depleted of specific materials with respect to origin cells [64, 74]. Likewise, EV cargo nature and abundance are also influenced by the pathways that lead to the formation of different EV subtypes [75].

The total cargo of human MSC-EVs is recently defined by next generation sequencing and proteomic analyses. They are enriched in proteins that support tumor (PDGFR-β, TIMP-1, and TIMP-2), lipids (sphingomyelin and diacyl-glycerol), metabolites (glutamic and lactic acid), several oncomiRNAs (*miRNA21* and *miRNA34a*) [76], critical surface markers, and signalling molecules characteristic of MSCs [77]. A recent work reports that BM-MSC-Exo are highly enriched in transferRNAs that represent more than 35% of the total small RNAs, while miRNAs account for only 2–5% [78]. This composition differs in MSC-Exo released from other tissues. In addition, BM-MSC-EVs contain a pattern of miRNAs essential for the metabolism, proliferation, differentiation, and homing of SCs [79]. Additionally, different chemokines, such as MCP-1, IP-10, and SDF-1, are found in BM-MSC-Exo in multiple myeloma (MM) [62]. These chemokines are important in supporting MM cell viability.

3.1. EV Uptake from Recipient Cells. Once released, EVs reach recipient cells where they exert pleiotropic effects through distinct signalling cascades via autocrine, paracrine, and juxtacrine feedback loops [80].

EVs can be internalized into recipient cells with different mechanisms including endocytosis, direct cell surface membrane fusion, and a lipid raft-mediated energy-dependent process, or they can remain permanently associated with plasma membrane [81].

Surface molecules, such as integrins or receptors, and microenvironment conditions control the EV uptake by regulating their specific cell tropisms, while EV cargo, released into target cells, alters their composition by inducing phenotypic, functional, and epigenetic modifications [17, 82].

In particular, the specific integrin-mediated adhesion of tumor Exo to specific cell types and organs induces the metastatic niche formation [83]. Similarly, BM dendritic cell Exo are preferentially internalized by splenic conventional dendritic cells, rather than by B-lymphocytes, macrophages, or splenic T cells [84]. Moreover, Exo from mantle cell lymphoma cells are preferentially taken up by themselves while only a minor fraction of Exo was internalized into T-cell leukemia and BM stroma cell lines [85]. The specific cell type uptake of EVs has also been observed *in vivo*. In fact, human MSC-EVs injected into the blood stream of mice primarily accumulated in the liver, spleen, and in sites of acute kidney injury, where they facilitated injury recovery [86]. Similarly, melanoma-derived Exo accumulated in the lungs, bone, liver,

and spleen and they increased the frequency of metastasis at these sites [87]. Finally, Parolini et al. reported that low pH favors Exo uptake by melanoma cells [88].

4. Role of EVs in Physiological BM Niche

As reported, MSCs are commonly studied as EV donor cells. EVs from BM-MSCs shuttle the selected molecular cargo to recipient cells targeting genes involved in organogenesis, cell survival and differentiation, tissue regeneration, immunomodulation, and angiogenesis [79, 89–91]. Nevertheless, the role of MSC as EV target cells must not be ignored. In fact, EVs derived from differentiated cells are able to modulate the MSC phenotype [92]. In particular, miRNA contained in EVs released from neuronal [93], endothelial [94–96], and kidney epithelial [97] cells induce proliferation, migration, and secretion of soluble factors in MSCs.

Immune cells, such as monocytes, use EVs to communicate with MSCs modulating their phenotype by upregulating osteogenic gene expression [98]. In fact, Ekström et al. demonstrated that both RUNX2 and BMP-2 expression is significantly increased in MSCs after monocyte-EV stimulation, whereas no significant difference is observed in osteocalcin [99], an osteoblastic gene regulated by BMP-2 via RUNX2 [100].

Regarding the hematopoietic system, Ratajczak et al. demonstrated that, besides coagulation, MVs derived from activated platelets play a role in important biological processes. In particular, these last enhance the chemotactic responsiveness of HSPCs, and increase their survival and proliferation by transferring specific mRNA and proteins [101]. In another study, the same authors reported that EVs released from embryonic SCs sustain HSPC stemness and multipotency by delivering specific "stemness" mRNAs [101].

More recently, it was demonstrated that mRNA and miRNA in mast cell EVs have been transferred to $CD34^+$ progenitors. In fact, Ekström et al. identified, by using miRNA microarray analysis, 116 miRNAs in Exo and 134 in donor mast cells. Furthermore, microarray experiments revealed the presence of approximately 1800 mRNA in Exo, which represent 15% of the donor cell mRNA content. Transfer experiments reveal that Exo could shuttle RNA between human mast cells and $CD34^+$ hematopoietic progenitor cells suggesting their role in cell communication [102].

A recent discovery showed that stromal cells release biologically active EVs which act on HSPCs. Specifically, two murine stromal cell lines, one with and the other without HSPC supportive capacity, produce different EV types in terms of size and of small RNA and mRNA signature. $Lin^-Sca1^+cKit^+$-HSPCs preferentially take up EVs produced by a supportive stromal line (suppEVs) but not those released by a nonsupportive one. SuppEVs transfer mRNA and miRNA in $Lin^-Sca1^+cKit^+$-HSPCs by modifying their gene expression profile. Importantly, suppEVs maintain the survival and clonogenic potential of $Lin^-Sca1^+cKit^+$-HSPC by inhibiting their apoptosis [103]. Collectively, these data assert that EVs constitute an important novel communication system in mediating the HSPC-supporting capacity of MSCs.

5. EV Role in BM Niche of Hematological Malignancies

It is now clear that BM cell populations, including malignant cells, influence the tumor microenvironment, via autocrine [104] or paracrine mechanisms through the secretion of soluble factors including EVs [105]. In HMs, neoplastic EVs promote tumor progression via an autocrine loop which includes interacting with their producing malignant cells, supporting autosustainability, and increasing aggressiveness [58, 105]. This relevant cross-talk mechanism is clearly demonstrated in MM [106], in pre-B acute lymphoblastic leukemia [107], in erythromyeloblastoid, and CML [108].

EVs from resistant neoplastic cells can transfer drug resistance to sensitive cells in AML [109, 110]. In particular, EVs from apoptosis-resistant AML cells modulate the expression of apoptosis-associated proteins in chemotherapy sensitive blasts [109]. A multiresistant AML cell line transfers, through EVs, chemoresistance to sensitive acute promyelocytic leukemia cells [110].

BM-MSC derived EVs induce survival, proliferation, and migration of MM cells *in vitro* and *in vivo* in a mouse model [111, 112]. Finally, Exo from AML MSCs and not from healthy MSCs protected a leukemic cell line carrying FLT3 internal tandem duplication from treatment with a specific FLT3 inhibitor [113].

HM-EVs exert also the immune modulation effects; malignant EVs inhibit natural killer cell cytotoxicity, promote T cell apoptosis, and enhance immunosuppressive activity of myeloid-derived suppressor cells *in vitro* and *in vivo*. These EV effects are reported in B and T cell lymphomas [114, 115], CLL [116], AML [117], and MM [62, 118, 119]. Overall these data support the idea that there is indeed a complex and intriguing EV-mediated cross talk between malignant cells and BM cells that defines a favorable neoplastic microenvironment. In this context, we summarize the role of HM niche EVs on SCs and MSCs in Figure 1.

5.1. HM Niche EVs versus SCs. Different studies reported that Exo released from AML cell lines impair hematopoiesis by suppressing HSPC clonogenicity and by reprogramming stroma [120, 121]. According to Razmkhah et al., BM-AML-MVs promote the survival of healthy HSCs by inducing leukemic molecular characteristics, like high level of *miRNA21* and *miRNA29* [122]. Interestingly, an essential role of VPS33B in Exo pathways in HSCs and in leukemia development at early stage was demonstrated. In fact, its deletion in an *in vivo* AML model impairs the maturation and secretion of Exo and delays the AML onset [123]. Interestingly, MVs released from LSCs enhance proliferation, migration, and inhibition of apoptosis of AML cells. LSC-MVs containing a high level of *miRNA34a* inhibits the effects of LSCs on AML cells [124, 125].

Muntion et al. suggested that MVs derived from MSCs of MDS patients modify $CD34^+$ cell properties, promoting their cell viability and clonogenic capacity and altering their miRNA and gene expression profiling [126].

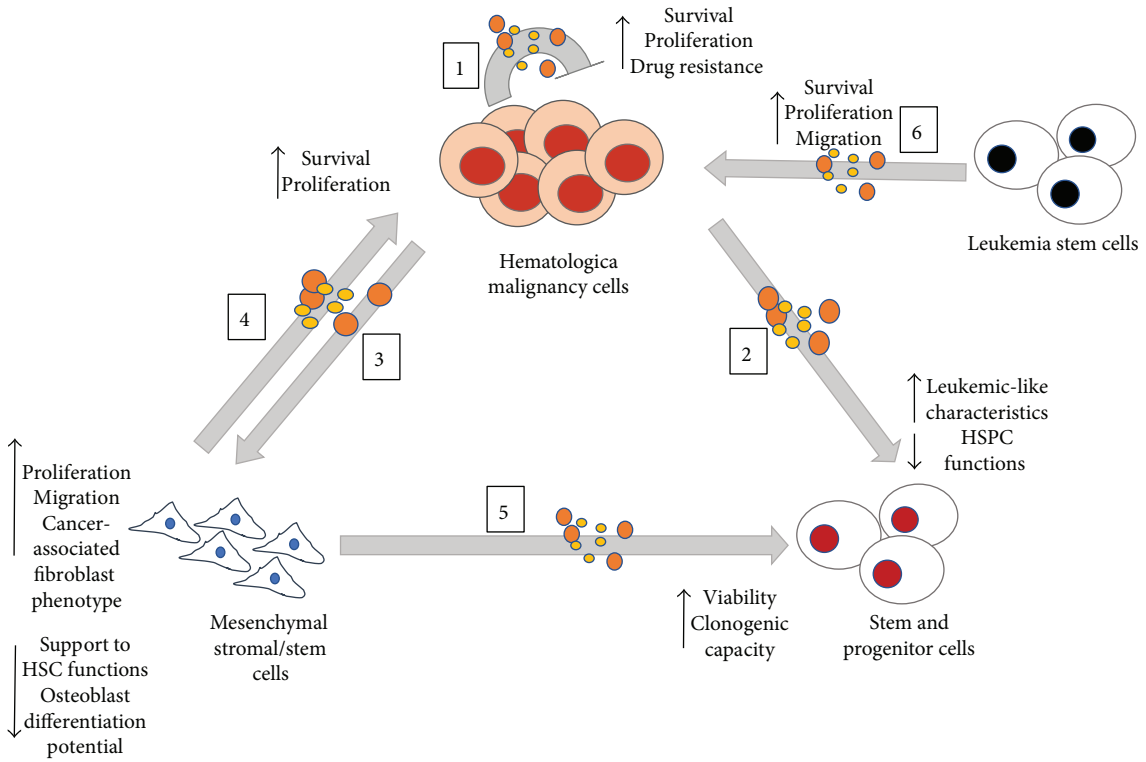

Figure 1: A schematic drawing of neoplasm EV effects in BM of HMs. Tumor EVs (colored balls) can (1) render malignancy more aggressive through autocrine mechanisms via (2) the induction of a suppression of hematopoietic stem/progenitor cell (HSPC) functions and a stem cell malignant transformation and (3) modification of mesenchymal stromal/stem cells (MSCs) reducing their HSC support. On the other hand, "reprogrammed" MSCs release EVs that (4) support the proliferation of malignancy cell proliferation and (5) promote HSPC viability and clonogenicity. In addition, leukemia stem cell EVs induce proliferation and migration of malignant cells (6). Arrows turned upwards (∧) and downwards (∨) indicate an increase and a reduction, respectively.

EVs released by myeloproliferative neoplastic MSCs, enriched in *miRNA155*, induce an increase of granulocyte colony forming unit number in neoplastic $CD34^+$ cells [127].

Collectively the reported studies show that the leukemia niche, in terms of LCSs and MSCs, is able to deregulate normal HSCs and neoplastic cells by EV-mediated communication.

5.2. HM Niche EVs versus MSCs. In the tumor context, MSC-EVs have a controversial role: they can promote or inhibit the tumor progression. These opposite effects of MSC-EVs can likely depend from both MSC source and culture conditions [128–130].

In general, EVs from healthy cells have a beneficial effect on recipient cells; on the contrary, EVs from cancer cells, have a detrimental influence also on MSCs [131]. MSCs exposed to tumor EVs acquire a series of functions such as migration to the tumor site [54, 132], production of proinflammatory cytokines [133], induction of prometastatic niches [134, 135], promotion of tumor growth *in vivo* [130], epithelial-to-mesenchymal transition induction [136, 137], recruitment of neoplastic cells in the BM [138], improvement of angiogenesis [139, 140], and modulation of the immune system [141–143].

Intriguingly, the crosstalk between tumor cells and MSCs seems to occur with a certain sequence: tumor cells, through EVs, communicate and modify MSCs; these reprogrammed MSCs, in response, produce EVs that can return on cancer cells or other cells creating a favorable environment for tumor [144, 145].

In HMs, less is known about the effect of neoplastic EVs on MSCs.

In CLL, Ghosh et al. found that MVs play an important role in the activation of the microenvironment in favor of disease progression [146]. CLL-MVs can activate the AKT signaling pathway in BM-MSCs by inducing the production of vascular endothelial growth factor, an important element for CLL cell survival [147]. In addition, Paggetti et al. demonstrated that CLL-derived Exo induce an inflammatory phenotype in endothelial cells and MSCs resembling the phenotype of cancer-associated fibroblasts [148]. In this way, leukemic Exo create a favorable environment for promoting CLL progression.

Exo derived from adult T-cell leukemia/lymphoma cells induce changes in cellular morphology and promote proliferation in MSCs by transferring epigenetic regulators, like *miRNA21* and *miRNA155* [149].

Horiguchi et al. found that EV *miRNA7977* derived from AML/MDS $CD34^+$ cells, is transferred into BM-MSCs where it reduces the poly binding protein 1 levels by compromising their ability to support $CD34^+$ cells [150]. Huan et al. studied the role of Exo in developing the BM AML niche [151]. They

reported that leukemic Exo are taken up by BM stroma. These Exo deliver important AML pathogenesis mRNA such as FLT3, NPM1, IGF-IR, and CXCR4. In addition, they carry *miRNA150* which binds the receptor for SDF-1 and CXCR4 mRNA. Consequently, these Exo reduce the expression of CXCR4 and thus cell migration versus SDF-1 of target cells. The CXCR4/SDF-1 axis is fundamental for HSPC retention in BM and their differentiation. The last AML-Exo effects are an altered proliferation and migration of BM-MSCs and hematopoietic progenitor cell lines, by reprogramming the BM microenvironment [151].

Recently, Kumar et al. showed that, in *in vitro* and *in vivo* models, AML-Exo are internalized by BM cells, increase long-term HSC population, and alter stromal compartment [152]. They induce the osteoblast inhibitor DKK1 expression in MSC progenitors decreasing their osteoblast differentiation potential. AML-EVs reduce the expression of factors that support normal hematopoiesis such as CXCL12, KITL, and IL-7 in MSCs. These modified stromal cells enhance leukemia growth at the expense of normal hematopoiesis [152]. In another context, Exo released by CML cells stimulate BM-MSCs to produce IL-8, which, in turn, promote both *in vitro* and *in vivo* leukemic cell survival [153]. MVs containing "leukemic" transcripts from CML cells transfer these mRNAs in healthy MSCs, increasing their proliferation [108]. Finally, *miRNA146a* in EVs from MM cells is transferred in MSCs inducing the secretion of elevated levels of cytokines which improved both MM cell viability and migration [154].

Collectively, the reported data demonstrate that EVs derived from HM cells are efficiently transferred into MSCs to transform the BM microenvironment into a niche that supports malignancy at the expense of HSCs, although the mode of transformation is still uncertain.

6. Conclusions

In conclusion, EVs constitute a new bidirectional communication system between BM microenvironment and SCs. In fact, SCs, including HSCs and MSCs, are not only EV donors but also targets of EVs derived from BM cells. Specifically, immune cells communicate with MSCs via EVs modulating their phenotype. In addition, EVs represent a tool in mediating the MSC capacity to support HSPCs, improving their survival and clonogenic potential in physiological conditions.

In different HMs, EVs are significantly induced compared to healthy controls. Neoplastic EVs exert oncogenic functions that (1) boost malignancy through autocrine signaling, (2) induce a suppression of HSPC functions and SC malignant transformation, (3) modify the BM environment in favor of cancer/leukemic cells acting also on MSCs. These last cells exposed to tumor EVs acquire a series of functions such as migration to the tumor site, production of proinflammatory cytokines, induction of prometastatic niches, promotion of tumor growth *in vivo*, recruitment of neoplastic cells in the BM, improvement of angiogenesis, and modulation of the immune system. Overall, "tumor modified" MSCs release EVs that play an active role in supporting a favorable environment for malignant cells at the expense of normal hematopoiesis.

In order to render more transparent the field of EVs, an EV-TRACK platform is created to collect biological and technical information of EVs [155]. Further studies are needed to clarify not only the mechanism of action of EVs in disease and health, but also to define EV population-specific identity and cell origin, and the standardization of protocols for their isolation and characterization.

Conflicts of Interest

The authors declare no conflicts of interest.

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Hindawi
Stem Cells International
Volume 2018, Article ID 9468085, 13 pages
https://doi.org/10.1155/2018/9468085

Research Article

Chemical Activation of the Hypoxia-Inducible Factor Reversibly Reduces Tendon Stem Cell Proliferation, Inhibits Their Differentiation, and Maintains Cell Undifferentiation

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Received 4 September 2017; Accepted 6 December 2017; Published 11 March 2018

Academic Editor: Margherita Maioli

Adult stem cell-based therapeutic approaches for tissue regeneration have been proposed for several years. However, adult stem cells are usually limited in number and difficult to be expanded in vitro, and they usually tend to quickly lose their potency with passages, as they differentiate and become senescent. Culturing stem cells under reduced oxygen tensions (below 21%) has been proposed as a tool to increase cell proliferation, but many studies reported opposite effects. In particular, cell response to hypoxia seems to be very stem cell type specific. Nonetheless, it is clear that a major role in this process is played by the hypoxia inducible factor (HIF), the master regulator of cell response to oxygen deprivation, which affects cell metabolism and differentiation. Herein, we report that a chemical activation of HIF in human tendon stem cells reduces their proliferation and inhibits their differentiation in a reversible and dose-dependent manner. These results support the notion that hypoxia, by activating HIF, plays a crucial role in preserving stem cells in an undifferentiated state in the "hypoxic niches" present in the tissue in which they reside before migrating in more oxygenated areas to heal a damaged tissue.

1. Introduction

Stem cell-based therapies have promised to be an attractive approach in tissue regeneration, especially with the discovery that adult tissues possess a reservoir of progenitor cells that can be isolated and cultured in vitro [1]. However, one of the main obstacles that have been encountered is the relatively small number of adult stem cells present in tissues and their very limited proliferative capacity. Moreover, while stem cells can be easily isolated from patients, they usually tend to quickly lose their potency in vitro with passages, as they differentiate and become senescent. A possible approach to overcome these limitations is to culture cells under low oxygen levels (below 21%) [2–5]. However, maintaining the correct in vitro culturing conditions under reduced oxygen tensions is quite complex. In fact, it requires special hypoxic chambers to avoid subjecting cells to continuous hypoxia/reoxygenation cycles during medium changes. Moreover, results are often conflicting, as many authors reported both the inhibition and promotion of cell proliferation even under moderate hypoxia (5–10%) [6–9]. Clearly, one confounding element is that adult stem cells are a very heterogeneous class of different cell subtypes, which are tissue specific. In addition, isolation techniques and culturing conditions in vitro heavily affect their phenotype. Therefore, we can speculate that these inconsistencies in the literature are likely due to a distinct response of each stem cell subtype to hypoxia. Nonetheless, under reduced oxygen tensions, it is

well established that the alpha subunit of the hypoxia inducible factor (HIF-1α) is stabilized [10], and this causes a signaling cascade greatly affecting stem cell metabolism and differentiation [11–14] (Figure 1(a)). One interesting feature of HIF mechanism of activation is that its stabilization relies on the inhibition of a family of prolyl hydroxylases (PHDs) that require oxygen to work; thus, their activity is reduced under oxygen starvation (Figure 1(b)). Interestingly, it has been shown that a chemical inhibition of PHD activity, using for instance 2-oxoglutarate analogous like dimethyloxalylglycine (DMOG), can mimic the effects of hypoxia in activating HIF even under normal oxygen tension (Figure 1(c)). Therefore, it could be envisioned to add these inhibitors in the culture medium to finely modulate stem cell proliferation and differentiation, avoiding the complex control of the oxygen tension.

On these bases, in this work, we studied the effects of HIF-1α stabilization, obtained by chemically inhibiting the PHDs, on human tendon stem cells, that we recently isolated for the first time from the human rotator cuff tendons [15, 16].

2. Methods

2.1. Cell Isolation and Culture. Human tendon stem cells (hTSCs) were isolated from supraspinatus tendon specimens collected during arthroscopic rotator cuff repair, according to a previously reported procedure [15]. The protocol study was approved by the Hospital Ethical Committee with authorization number 2642 (Sept. 19, 2011). Samples from supraspinatus tendons (4–8 mm wide) were collected from 6 patients after signed informed consent, kept in HypoThermosol (BioLife Solutions) at 4°C, and processed separately within 24 h, according to the procedure described below. Samples were washed with phosphate-buffered saline (PBS) (Euroclone), cut into small pieces, and digested for 90 min with collagenase type I (3 mg/mL; Worthington) and dispase (4 mg/mL; Gibco, Life Technologies) in PBS at 37°C. After centrifugation, cell pellets were resuspended in the following culture medium: α-Minimal Essential Medium (α-MEM) (Sigma-Aldrich) supplemented with 2 mM glutamine (Euroclone), 1% antibiotic-antimycotic mixture (Euroclone), and 20% (*v*/*v*) fetal bovine serum (FBS) (HyClone, Thermo Fisher Scientific). Cells were then filtered with a cell strainer (70 mM; BD Falcon) and plated in 150 cm^2 dishes. Adherent cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every 2-3 days. All experiments were carried out with cells at passage four to six after isolation [17].

2.2. Flow Cytometric Analysis. hTSCs were detached and collected in PBS at a concentration of 2×10^6 cells/mL. Specific binding sites were blocked with a blocking solution (50% 1X PSB, 50% FBS) for 30 minutes at room temperature (RT). Cells were stained with antibodies against human: PE-conjugated CD105, PE-conjugated CD90, FITC-conjugated CD73, conjugated CD45, PerCP-eFluor 710-conjugated CD34, and FITC-conjugated HLA-DR, for 10 minutes at 4°C. The respective isotype antibodies were used as controls. Cell samples were analyzed with a Navios flow cytometer (Beckman Coulter) equipped with Kaluza software (Beckman Coulter).

2.3. Chemical Activation of HIF-1α with DMOG. DMOG was prepared by solubilization in H_2O, as suggested by the manufacturer's protocol (Sigma Aldrich). Twenty-four hours after seeding, cells were either cultured for 96 h in normal growth medium with different concentrations of DMOG (0.01 mM, 0.1 mM, and 1 mM) or in normal growth medium without DMOG (controls). The medium was changed every 48 h in all experiments unless specified. To study the reversibility of the effects of DMOG on cell viability and proliferation capacity, multilineage differentiation potential of hTSCs was tested upon DMOG removal from the culture medium. To this purpose, cells were first cultured in the presence of different concentrations of DMOG for 96 h, as described above, washed twice with PBS, detached, and then reseeded and grown in normal medium without DMOG.

2.4. Western Blot Analysis. Control hTSCs or treated with different concentrations of DMOG for 96 h were rinsed twice with cold PBS, harvested, and lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were measured by the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), following the manufacturer's protocol. Proteins (30 μg) were denatured by boiling for 5 min in sample buffer (6.5 mM Tris-HCl, pH 6.8, 2% SDS (*w*/*v*), 10% glycerol (*v*/*v*), 5% 2-mercaptoethanol (*w*/*v*), and 0.01% bromophenol blue (*w*/*v*)) and separated on a 10% polyacrylamide gel in denaturing conditions. Proteins were subsequently transferred into nitrocellulose membranes by electroblotting using 100 volts for 2 hours. Then, the membranes were incubated overnight in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl), 0.1% (*v*/*v*) Tween 20 (TBS-Tween) containing 5% (*w*/*v*) dried milk or 5% (*w*/*v*) bovine serum albumin (BSA; Sigma-Aldrich) for the blocking buffer. Blots were incubated with a primary antibody specific for HIF-1α (Cell Signaling Technology) diluted 1 : 1000 in the appropriate blocking solution for three hours at room temperature. Membranes were then washed four times for 10 min with TBS-Tween and then incubated with the appropriate secondary antibody horseradish peroxidase (HRP) conjugated for 1 hour at room temperature. After four washes in TBS-Tween, the protein bands were detected using an ECL detection kit (Thermo Fisher Scientific) as described by the manufacturer. Relative protein expression levels were calculated normalizing data on Lamin A/C, which was used as internal control.

2.5. Cell Morphology and Proliferation. Cells were plated at a concentration of 2.6×10^3 cells/cm^2 and cultured in normal growth medium in the presence of DMOG (0 mM, 0.01 mM, 0.1 mM, and 1 mM), as described above. Cell morphology was examined with a phase-contrast microscope (Axiovert 40 CFL, Zeiss, equipped with a Moticam 2300 camera, Motic) at each time point to assess the effects of DMOG treatment on hTSC phenotype. Cell growth curves were prepared after harvesting with Trypsin-EDTA solution

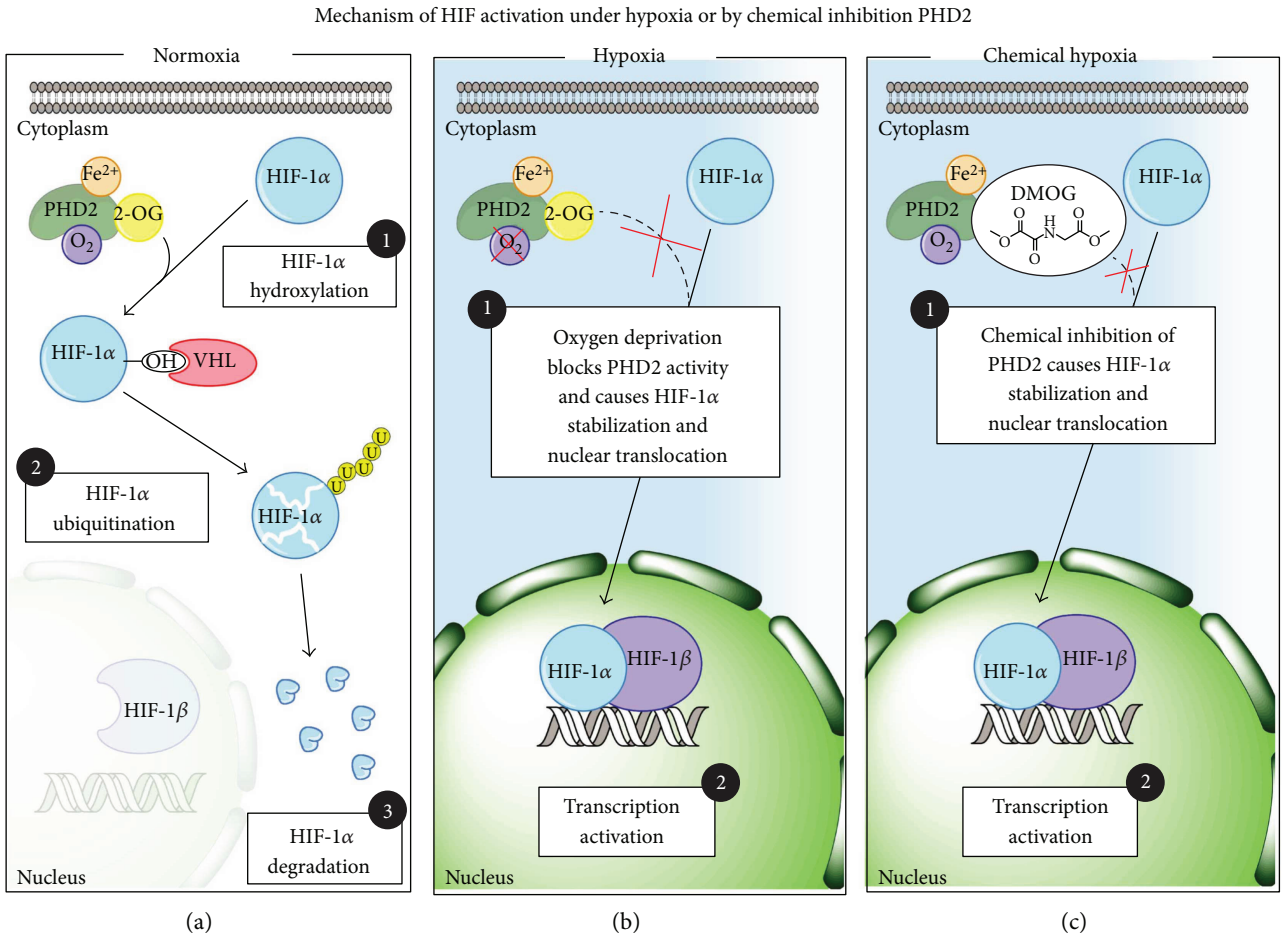

Figure 1: Schematic representation of different mechanisms of HIF-1α activation. The picture shows a schematic representation of (a) HIF-1α degradation by proteasome under normoxic conditions, (b) HIF-1α stabilization under hypoxic conditions mediated by the oxygen deprivation which causes the inhibition of PHD2 activity, and (c) chemical-induced HIF-1α stabilization under normoxic conditions by the inhibition of PHD2 activity through DMOG treatment.

(Sigma-Aldrich) by counting cells with a Countess Cell Counter (Invitrogen, Life Technologies), according to the manufacturer's procedure. Cell viability was determined by trypan blue dye exclusion assay. All assays were carried out in triplicates for each sample.

2.6. Cell Viability by MTT Assay. hTSCs were plated in 12-well plates (1×10^4 cells/well) and treated with different concentrations of DMOG or cultured in the growth medium alone. At each time point (0, 24, 48, and 72 h), two hours before collection, the reconstituted 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/mL in PBS; Sigma-Aldrich) was added to the medium (10% of the final volume). Following a two-hour incubation at 37°C, cells were lysed by adding an amount of MTT solubilization solution equal to the original culture medium volume, gently pipetting to completely dissolve the MTT formazan crystals. The MTT reduction was spectrophotometrically measured at a wavelength of 570 nm.

2.7. Cell Viability Assay by RealTime-Glo™. Cell viability was assessed using RealTime-Glo MT Cell Viability Assay (Promega) after 48 and 96 h of DMOG treatment (0 mM, 0.01 mM, 0.1 mM, and 1 mM) according to the manufacturer's protocol. The luminescent signal was measured at 48 and 96 h using VICTOR X3™ Multilabel Plate Reader. Briefly, MT cell viability substrate and NanoLuc® enzyme were both diluted 1 : 1000 in culture medium at a final volume of 100 μL per well. Cells were seeded at a density of 500 cells/well in a 96-well plate. Data were normalized to the untreated control.

2.8. Cytotoxicity Assay by CellTox Method. CellTox™ cytotoxicity assay (Promega) was used to investigate the cytotoxic effect of DMOG treatment, as the assay measures changes in membrane integrity that occur as a result of cell death. Briefly, cells were seeded in a 96-well plate at a concentration of 500 cells/well and incubated for 48 and 96 h with DMOG (0 mM, 0.01 mM, 0.1 mM, and 1 mM) and tested according to manufacturer's protocol. After each incubation, an equal volume of 100 μL of CellTox buffer containing 1 : 500 dilution CellTox Green Dye was added to each well and incubated at room temperature for 15 minutes. The signal was measured using the VICTOR™ X3 Multilabel Plate Reader with an excitation wavelength of 485–500 nm and emission

Table 1

Gene	Primer sequence
S14	Fw: 5′-GTGTGACTGGTGGGATGAAGG-3′
	Rev: 5′-TTGATGTGTAGGGCGGTGATAC-3′
VEGF	Fw: 5′-CAACATCACCATGCAGATTATGC-3′
	Rev: 5′-TCGGCTTGTCACATTTTTCTTGT-3′
NANOG	Fw: 5′-GGTCCCAGTCAAGAAACAGA-3′
	Rev: 5′-GAGGTTCAGGATGTTGGAGA-3′
OCT4	Fw: 5′-AGGAGAAGCTGGAGCAAAA-3′
	Rev: 5′-GGTCGAATACCTTCCCAAA-3′
KLF4	Fw: 5′-GACTTCCCCCAGTGCTTC-3′
	Rev: 5′-CGTTGAACTCCTCGGTCTC-3′
Tenascin C	Fw: 5′-CGGGGCTATAGAACACCAGT-3′
	Rev: 5′-AACATTTAAGTTTCCAATTTCAGGTT-3′
COL1A1	Fw: 5′-GGGATTCCCTGGACCTAAAG-3′
	Rev: 5′-GGAACACCTCGCTCTCCA-3′
PPAR-γ	Fw: 5′-TTCCTTCACTGATACACTGTCTGC-3′
	Rev: 5′-GGAGTGGGAGTGGTCTTCCATTAC-3′
LPL	Fw: 5′-AGAGAGAACCAGACTCCAATG-3′
	Rev: 5′-GGCTCCAAGGCTGTATCC-3′
ALP	Fw: 5′-CGCACGGAACTCCTGACC-3′
	Rev: 5′-GCCACCACCACCATCTCG-3′
SOX9	Fw: 5′-GTACCCGCACTTGCACAAC-3′
	Rev: 5′-TCGCTCTCGTTCAGAAGTCTC-3′

filter of 520–530 nm. Data were normalized to the untreated control.

2.9. Cell Viability by Annexin V/PI Flow Cytometric Assay. Cell viability was measured by flow cytometry on cells cultured for 96 h in normal growth medium with different concentrations of DMOG (0, 0.01, 0.1, and 1 mM) using Annexin V-FITC Apoptosis Detection Kit (eBioscience), according to the manufacturer's protocol.

Briefly, adherent cells were trypsinized, washed in PBS by gentle shaking, and resuspended at the concentration of $5 * 10^5$ cells/mL with 200 μL of a specific binding buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl, and 2.5 mM $CaCl_2$) containing 5 μL of Annexin V-FITC. After incubation for 10 min in the dark at room temperature, cells were washed in PBS, resuspended in 190 μL of binding buffer, and then stained with 10 μL propidium iodide (20 μg/mL). Samples were immediately acquired with a Navios Flow Cytometer (Beckman Coulter) and analyzed using Kaluza 1.2 software (Beckman Coulter).

2.10. RNA Extraction and Real-Time PCR. Total RNA was isolated using TRIzol® Reagent (Ambion, Life Technologies), and 1 μg of extracted RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (BioRad), according to the manufacturer's instructions. Real-time PCR was performed in a 96-well plate with 10 ng of cDNA as a template, 0.2 μM primers, and 1x Power SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies) in a 20 μL final volume per well using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The mRNA levels of vascular endothelial growth factor (VEGF), Nanog homeobox (NANOG), octamer-binding transcription factor 4 (OCT4), Kruppel-like factor 4 (KLF4), Tenascin C, collagen type I alpha-1 (COL1A1), peroxisome proliferator-activated receptor-γ (PPAR-γ), lipoprotein lipase (LPL), human alkaline phosphatase (ALP), and SRY-box9 (SOX9) were assessed. Ribosomal protein S14 (S14) was used as the housekeeping gene in all quantitative analyses (Table 1).

Amplification protocol: initial denaturation at 95°C for 3 minutes followed by 40 cycles of 5 seconds each at 95°C and 30 seconds at 57°C. Relative quantification of target genes was performed in triplicates, analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to the corresponding S14 values.

2.11. Adipogenic Differentiation. Cells were plated at a concentration of 3×10^4 cells/cm^2 and cultured in DMEM-low glucose (Sigma-Aldrich), 10% FBS (HyClone, Thermo Fisher Scientific), 4 mM L-glutamine (Euroclone), and 1% antibiotic-antimycotic mixture (Euroclone), with the addition of the mesenchymal stem cell adipogenesis kit (Millipore) for 21 days, according to the manufacturer's instructions. The adipogenic medium was changed every other day. At day 21, Oil Red O solution (Millipore) was used to stain lipid droplets of derived adipocytes, according to the manufacturer's procedures. All photomicrographs were acquired with an Axiovert 40 microscope (Zeiss) equipped with a Moticam 2300 camera (Motic). The mRNA expression of adipogenic markers including PPAR-γ and LPL were also assessed on days 7 and 21 by real-time PCR, as described above [17].

2.12. Osteogenic Differentiation. Cells were plated at the concentration of 3×10^4 cells/cm^2 and cultured in the osteogenesis induction medium, which was constituted of DMEM-low glucose (Sigma-Aldrich), 10% FBS (HyClone, Thermo Fisher Scientific), 4 mM L-glutamine (Euroclone), and 1% antibiotic-antimycotic mixture (Euroclone), supplemented with 0.1 μM dexamethasone, 50 μg/mL L-ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate (all reagents from Sigma-Aldrich) for 17 days. The osteogenic medium was changed every other day. At day 17, Alizarin Red solution (Millipore) was used to detect calcium deposition in derived osteoblasts, according to the manufacturer's instruction. All photomicrographs were acquired with an Axiovert 40 microscope (Zeiss) equipped with a Moticam 2300 camera (Motic). The expression of osteogenic marker ALP was determined by real-time PCR on days 5 and 17, as described above [17].

2.13. Chondrogenic Differentiation. Chondrogenic differentiation was performed using StemPro Chondrogenesis Differentiation Kit (Life Technologies), according to the manufacturer's instruction. Briefly, cells were resuspended

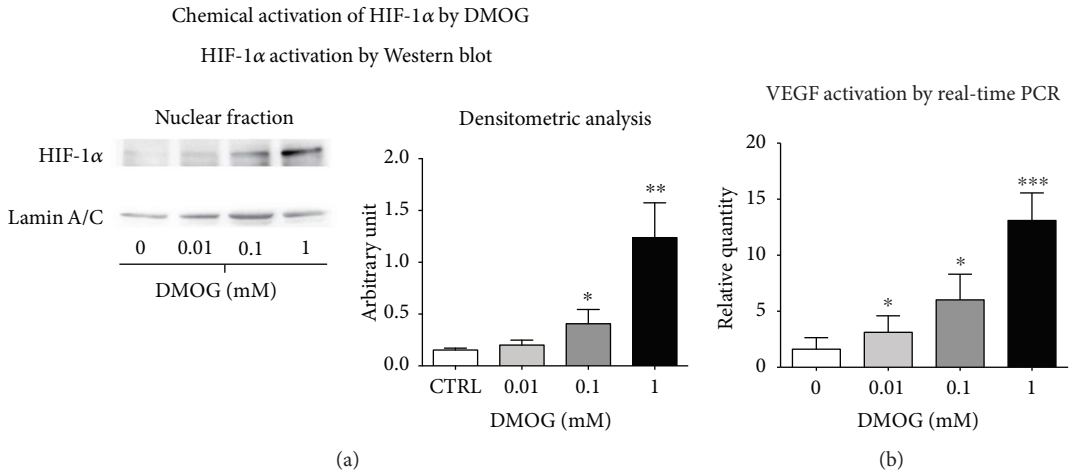

Figure 2: HIF-1α chemical activation with DMOG. HIF-1α expression was analyzed by Western blot (a). The activation of HIF-1α was also analyzed measuring the expression of VEGF by real-time PCR (b). Western blots were quantified by densitometry and results expressed with arbitrary units. Real-time PCR data are expressed as fold change as compared to control cells without DMOG treatment. p values were calculated using Student's t-test. Only p values < 0.05 are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

in growth medium at the concentration of 1.6×10^7, and 5 μL droplets were seeded in the center of a multiwell plate wells. After two hours, the chondrogenesis induction medium provided by the kit was added. The chondrogenic medium was changed every 2-3 days for 14 days. Alcian blue solution (Bio-Optica) was used to stain acid mucopolysaccharides formed during the differentiation process. The expression of chondrogenic marker SOX9 was determined by real-time PCR on days 5 and 14, as described above [17].

2.14. Statistical Analysis. Statistical analysis was performed using GraphPad Prism v 6.0 software (GraphPad Software Inc.). Data were typical results from three replicate experiments for each of the six patient-derived cell lines and were expressed as the mean ± standard deviation (SD). The Shapiro-Wilk normality test was used to evaluate the normal distribution of the sample.

One-way analysis of variance (ANOVA), followed by two-tailed, paired Student's t-test or Mann–Whitney test according to the characteristics of the data distribution, wherever applicable, was used for multiple comparisons. Linear contrasts were used to assess for changes in the mean values with increasing doses of DMOG. The significance level was set at p value lower than 0.05.

3. Results

3.1. Effects of DMOG on HIF-1α Stabilization on hTSCs. To chemically activate HIF-1α, hTSCs were cultured under normoxia in the presence of DMOG at different concentrations (0.01 mM, 0.1 mM, and 1 mM) for 96 h and compared to control cells that were cultured in normal growth medium without DMOG (Figure 2). Analysis of HIF-1α nuclear localization by Western blot revealed that the factor was activated by DMOG in a dose-dependent manner (Figure 2(a)). Similarly, analysis of VEGF, one of the main HIF-1α target genes, showed an expression increase by real-time PCR that was proportional to the concentration of DMOG in the culture medium (Figure 2(b)). Cell morphology analysis by phase-contrast microscopy revealed no noticeable differences between control and DMOG-treated hTSCs (Figure 3(a), 96 h of DMOG treatment). However, DMOG at 1 mM concentration induced cell suffering, as the formation of vacuoles and cell proliferation arrest could be observed throughout the culture plate (Figure 3(a)). The evaluation of the effects of DMOG on cell proliferation revealed a slight, although not statistically significant, increase when cells were treated with 0.01 mM DMOG (Figure 3(b)). On the other hand, a dose-dependent reduction could be initially observed at 0.1 mM after 72 h of treatment, reaching a significant decrease of 51%, 72.6%, and 77.7% in the presence of 1 mM DMOG at 48, 72, and 96 h, respectively, as compared to control cells (Figure 3(b)). These results were confirmed by MTT cell metabolic activity assay where a DMOG dose-dependent reduction could be observed at all time points (24, 48, and 72 h), reaching a 53.1% reduction at 1 mM, as compared to control cells (Figure 3(c)). Similarly, RealTime-Glo™ cell viability tests showed a DMOG dose-dependent signal reduction, with the exception of 0.01 mM DMOG that showed a significant increase at 96 h as compared to control cells (Figure 3(d)). Cell toxicity tests revealed no significant differences at all DMOG concentrations at 48 and 96 h, with the exception of 0.01 mM DMOG that showed a minor, yet statistically significant, decrease in cell toxicity at 96 h. Cell death analysis by Annexin V test revealed no significant cell apoptosis at all tested DMOG concentrations (Figure 3(f)).

3.2. Effects of HIF-1α Stabilization on hTSC Stemness. To evaluate the effects of DMOG on hTSC stemness, the expression of stem cell markers NANOG, OCT4, and KLF4 was determined by real-time PCR after a 96 h DMOG treatment at 0.01 mM, 0.1 mM, and 1 mM and compared to those of control cells (Figure 4(a)). The results showed a statistically significant trend increase of the mRNA levels of NANOG, OCT4, and KLF4, as compared to control cells, and that it was proportional to the concentration of DMOG used (red

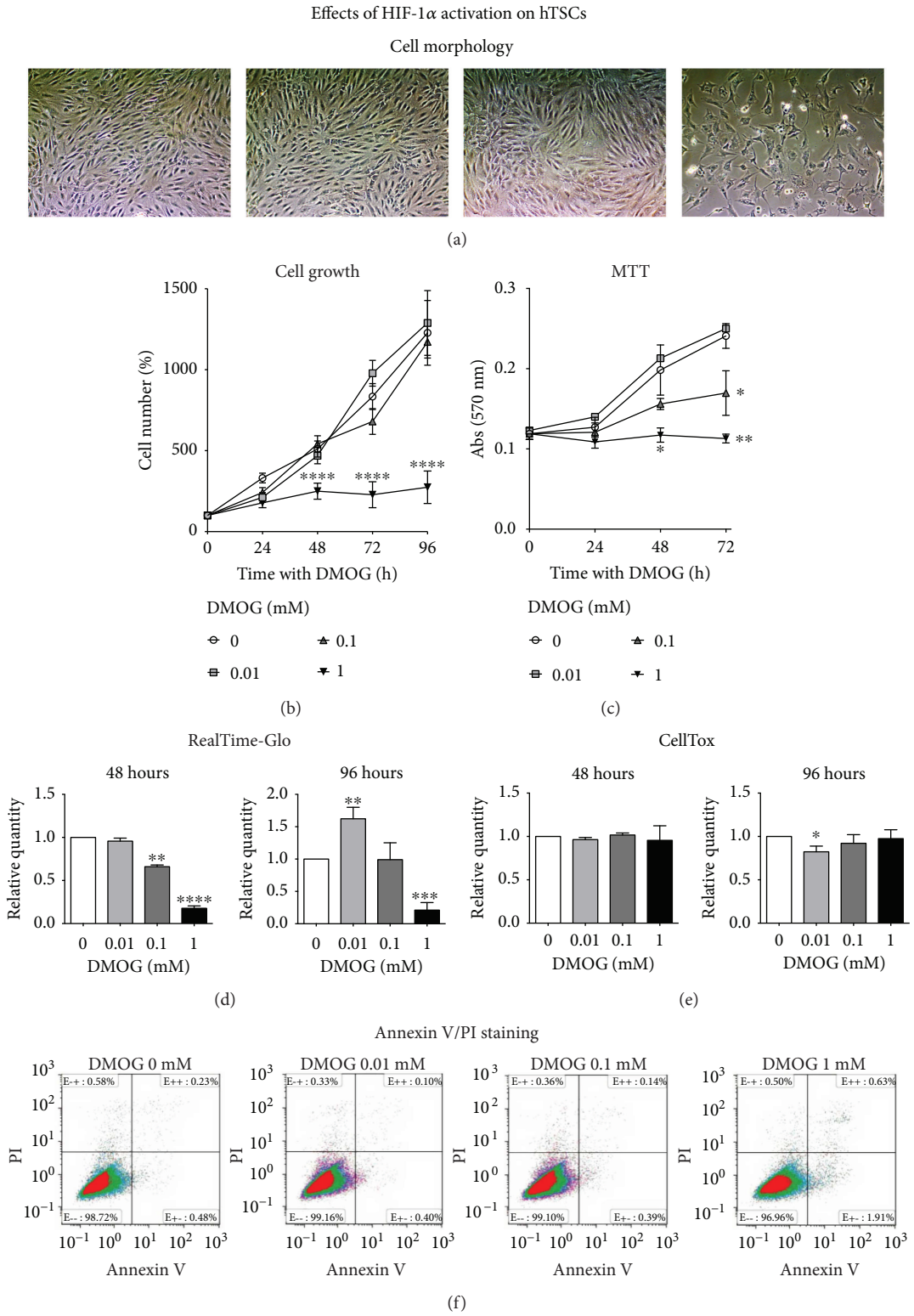

Figure 3: Effects of HIF-1α activation on hTSC morphology, proliferation, and viability. (a) Phase-contrast microphotographs (original magnification ×10), (b) cell growth curves, (c) MTT assay, (d) RealTime-Glo assay, (e) CellTox assay, and (f) Annexin V/PI staining of hTSCs during a 96 h treatment with different concentrations of DMOG (0.01 mM, 0.1 mM, and 1 mM) in normal growth medium and compared to control cells cultured without DMOG (0 mM). All experiments were performed in triplicates. Error bars show the mean ± SD of six and three different experiments in the case of cell growth curves and MTT assay, respectively. p values were calculated using Student's t-test. Only p values < 0.05 are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, as compared to control cells. Bar = 100 μm.

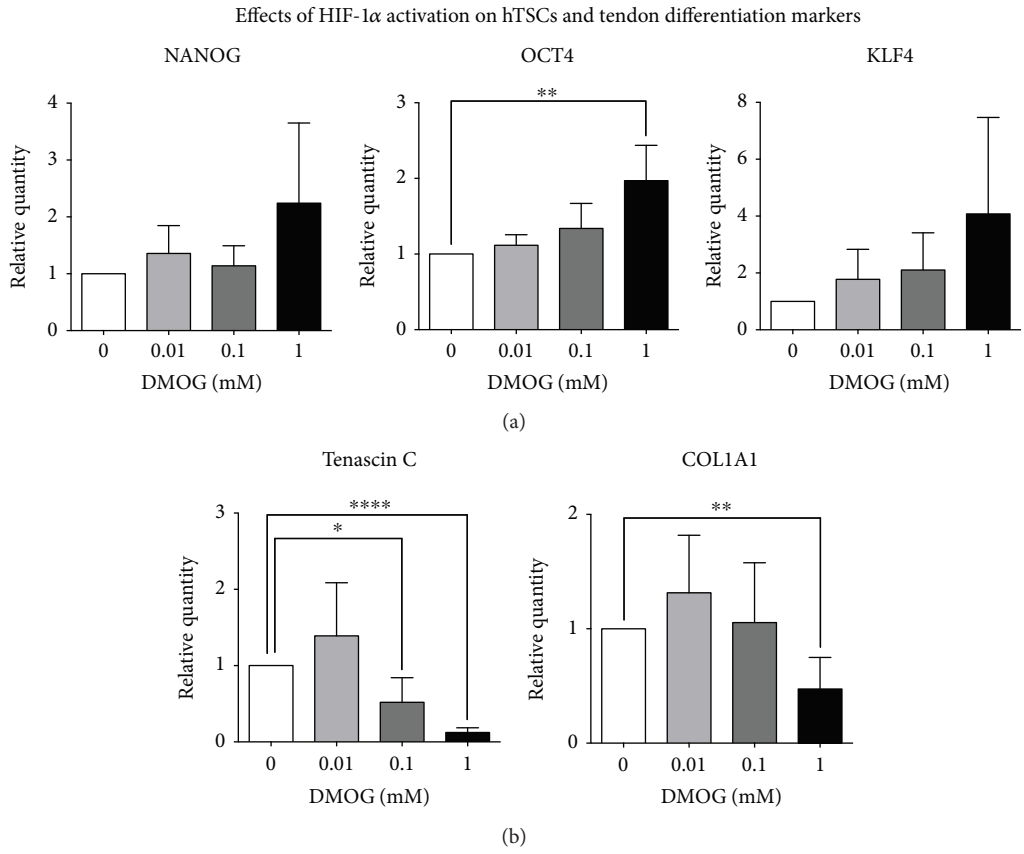

Figure 4: Effects of HIF-1α activation on stem cell and tendon marker expression. (a) Gene expression of NANOG, OCT4, and KLF4 by real-time PCR after a 96 h DMOG treatment, as compared to control-untreated cells. (b) Gene expression of Tenascin C and COL1A1 by real-time PCR after a 96 h DMOG treatment, as compared to control-untreated cells. Values are expressed as fold changes relative to control cells. Data are expressed as means ± SD of six different experiments. One-way ANOVA was used to test for differences among DMOG-treated cell groups. Bonferroni post hoc test was performed to compare individual DMOG-treated group cells against each other to see where the significant differences lie. Linear contrast analysis (analysis of variance) was used to assess for changes in the mean values with increasing doses of DMOG (red lines). Only p values < 0.05 are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$, as compared to control cells.

tendency line, Figure 4(a)). The mRNA levels of Tenascin C and COL1A1 tendon markers were measured by real-time PCR in hTSCs exposed to different concentrations (0.01 mM, 0.1 mM, and 1 mM) of DMOG for 96 h. The results showed a statistically significant trend decrease of the mRNA levels of both tendon markers, which was proportional to the concentration of DMOG used (red tendency line, Figure 4(b)). In particular, the expression of both Tenascin C and COL1A1 showed a slight, although not significant, increase when cells were treated with 0.01 mM DMOG, as compared to control cells. On the other hand, a significant reduction, which was concentration dependent, could be observed at 0.1 mM and 1 mM DMOG, reaching an 8.1- and 2.1-fold decrease for Tenascin C and COL1A1, respectively, at 1 mM DMOG concentration (Figure 4(b)).

3.3. Effects of HIF-1α Stabilization on hTSC Differentiation. To assess the effects of HIF-1α activation on the multipotency of hTSCs, cells were induced to differentiate toward osteoblasts, adipocytes, and chondroblasts by culturing them in the appropriate differentiation medium for 17, 21, and 28 days, respectively, in the presence of DMOG at different concentrations (0, 0.01, 0.1, and 1 mM), as described in the Methods (Figure 5). The adipogenic differentiation was qualitatively evaluated by Oil Red O staining revealing a reduction in the amount of lipid intracellular droplets, which was proportional to the amount of DMOG in the culture medium (Figure 5, left column). The osteogenic differentiation was assessed qualitatively by Alizarin Red S staining, which showed that the amount of calcium deposits was markedly reduced, in a dose-dependent manner, when cells were cultured in the presence of DMOG, as compared to control cells (Figure 5, central column). The chondrogenic differentiation was assessed qualitatively by Alcian blue staining, showing a deposition of extracellular matrix proteins when cells were cultured in the presence of DMOG, especially at 1 mM, as compared to control cells (Figure 5, right column). The effects of HIF-1α activation on adipogenesis and osteogenesis were also analyzed quantitatively by determining the mRNA levels of adipogenic markers PPAR-γ and LPL, osteogenic marker ALP, and chondrogenic marker SOX9 by real-time PCR (Figure 6). The results showed a significant decrease of all markers that was proportional to the concentration of DMOG used (Figure 6). In particular, PPAR-γ showed 2.8-,

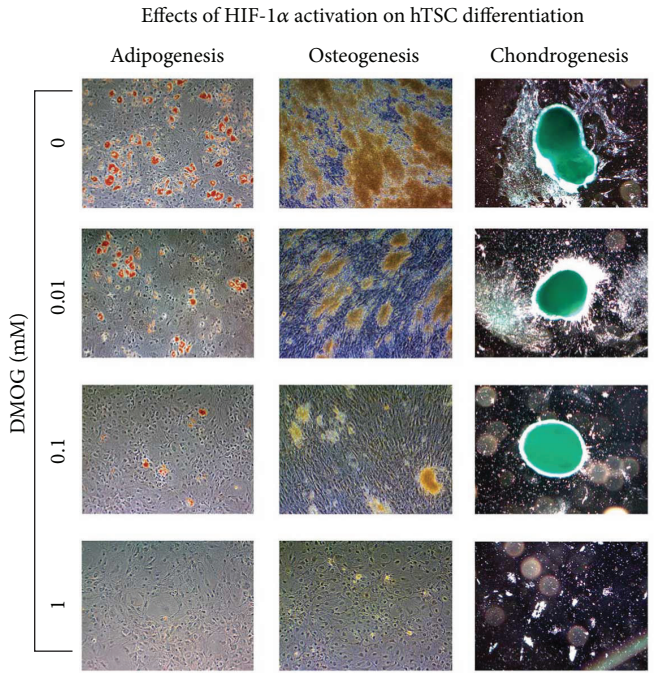

Figure 5: Effects of HIF-1α activation on in vitro differentiation of hTSCs toward the adipogenic, osteogenic, and chondrogenic phenotypes. Adipogenic, osteogenic, and chondrogenic differentiation ability of hTSCs in the presence of DMOG at different concentrations (0 mM, 0.01 mM, 0.1 mM, and 1 mM) in the appropriate differentiation medium was qualitatively evaluated by (a) Oil Red O, (b) Alizarin Red S staining, and (c) Alcian blue staining, respectively. (a) Lipid intracellular droplets (red) in the adipocytes were stained with Oil Red O solution. (b) Alizarin Red S staining revealed the presence of calcium deposits (yellowish-brown). (c) Alcian blue staining was used to stain acid mucopolysaccharides formed during the differentiation process. Typical results are shown. Original magnification ×10. Bar = 100 μm.

31.7-, and 59.7-fold expression decrease, while LPL showed 2.3-, 10.1-, and 109.2-fold decrease for 0.01 mM, 0.1 mM, and 1 mM DMOG treatment, as compared to control cells, respectively (Figure 6). Osteogenic differentiation in the presence of DMOG was inhibited, as revealed by a significant ALP decrease that was proportional to the concentration of DMOG used (Figure 6). In particular, ALP showed 3.7-, 15.1-, and 386-fold decrease when cells were incubated with 0.01 mM, 0.1 mM, and 1 mM DMOG, respectively, as compared to control cells (Figure 6).

Finally, it was tested whether the effects of HIF-1α activation could be reverted. To this purpose, hTSCs were grown in the presence of 0.01 mM, 0.1 mM, and 1 mM DMOG for 96 h, harvested, replated at the same cell concentration, and then cultured in normal growth medium (Figure 7(a)). Cells were then harvested and counted every 24 h for 4 days, starting 24 h after seeding. Cell growth analyses revealed that cells pretreated with 0.01 mM and 0.1 mM DMOG could regain the normal proliferation capacity, analogous to control cells, when switched back to the normal growth medium (Figure 7(b)). On the other hand, cells treated with 1 mM DMOG did not completely recover from DMOG treatment, at least within the experiment timeframe, as they showed a markedly proliferation reduction at 72 h, reaching 58.4% decrease at 96 h, as compared to control cells (Figure 7(b)). MTT assays confirmed cell growth experiments, revealing that DMOG treatment effects could be reverted within 72 h after switching cells in normal growth medium, with the exception of 1 mM DMOG, that still showed a significant reduction (about 36.2% at 72 h), as compared to control cells (Figure 7(c)). Then, the reversibility of the effects of HIF-1α activation on cell differentiation was determined. To this purpose, cells were first pretreated for 96 h with DMOG, followed by 96 h in normal growth medium to reestablish normal proliferation, and then differentiated in DMOG-free medium. Analysis of the adipogenic, osteogenic, and chondrogenic differentiation markers revealed no significant difference between DMOG pretreated and control cells at all concentrations ($p > 0.05$) (Figures 7(c) and 7(d)).

4. Discussion

Adult stem cells have been found to reside in hypoxic tissue compartments often referred to as "stem cell niches." Although the microenvironment and the molecular signals present in the niches have been only partially elucidated, it is now accepted that a low-oxygen tension helps to maintain the undifferentiated state of the progenitor cells [18, 19]. Moreover, it has been shown that culturing stem cells in vitro in low-oxygen incubators mimics the "niche," at least partially and cells seem to maintain their potency without differentiating [18, 20]. However, as already reported in the Introduction, the effects of low oxygen on adult stem cells appear to be very variable and cell subtype dependent, and it has been also reported that it can reduce stem cell proliferation and favor differentiation. Clearly, finding a way of

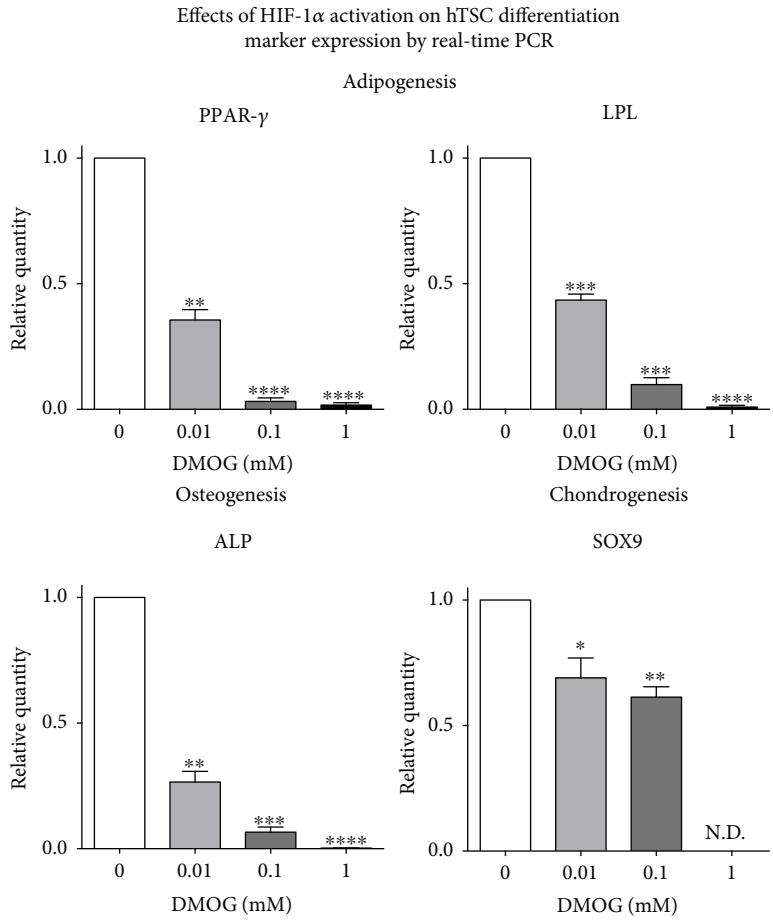

Figure 6: Quantitative evaluation of the effects of HIF-1α activation on in vitro differentiation of hTSCs toward the adipogenic, osteogenic, and chondrogenic phenotypes. Peroxisome proliferator-activated receptor-γ (PPAR-γ), lipoprotein lipase (LPL), and human alkaline phosphatase (ALP) expression by real-time PCR in hTSCs induced to differentiate toward adipocytes, osteoblasts, and chondroblasts in the presence of different concentrations of DMOG (0.01 mM, 0.1 mM, and 1 mM). Control cells were cultured under the same conditions but without DMOG. Values are expressed as fold changes relative to control cells. Data are expressed as the mean ± SD of three different experiments. p values were calculated using Student's t-test. Only p values < 0.05 are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, as compared to control cells.

amplifying stem cells without compromising their potency is vital for reaching the cell numbers needed for their therapeutic application. Along this line, the use of hypoxic incubators has been shown to be feasible, and the possibility of pharmacologically mimic oxygen deprivation represents a simpler and cost-effective alternative. Chemically mimicking the hypoxic niche has several advantages over culturing cells in low oxygen: (a) a drug can be easily synthesized on a large scale, (b) the methodology does not require especially designed and expensive incubators, (c) the drug dosage can be easily adjusted and controlled, and (d) the treatment can be suspended at any time. On the other hand, controlling the oxygen tension can be very cumbersome, especially when cells are cultured for a prolonged time and the media needs to be replaced without exposing cells to normal-oxygen tension. This can be achieved only with specially designed and expensive culturing chambers, which are not commonly used in most laboratories or research hospitals. Actually, most studies under hypoxic conditions are performed with normal incubators connected to a nitrogen tank; thus, cells are constantly exposed to cycles of low/high oxygen. This unfortunately mimics the ischemia/reperfusion conditions that might be detrimental for maintaining stem cells, as they could stimulate their differentiation, as it happens, for instance, after a cardiac infarct [21].

On these bases, with so many conflicting results, it is very hard to come up with a general picture. However, it is clear that under oxygen deprivation, any stem cell activates a common response mechanism, which is mainly regulated by the hypoxia-inducible factor. In particular, HIF-1α, which is activated under low-oxygen tension, has been shown to play a pivotal role in stem cell differentiation [11, 18]. In particular, the activation of HIF-1α has been shown to inhibit stem cell differentiation [3, 22]. Moreover, activation of HIF-1α is known to enhance the cell defense machinery against apoptosis [23–26]. This is another crucial issue in stem cell therapy, as most injected cells usually die because they have to survive in a very hostile environment while trying to reach the damaged tissue. Indeed, preconditioning stem cells by activating HIF-1α has been shown to increase their survival

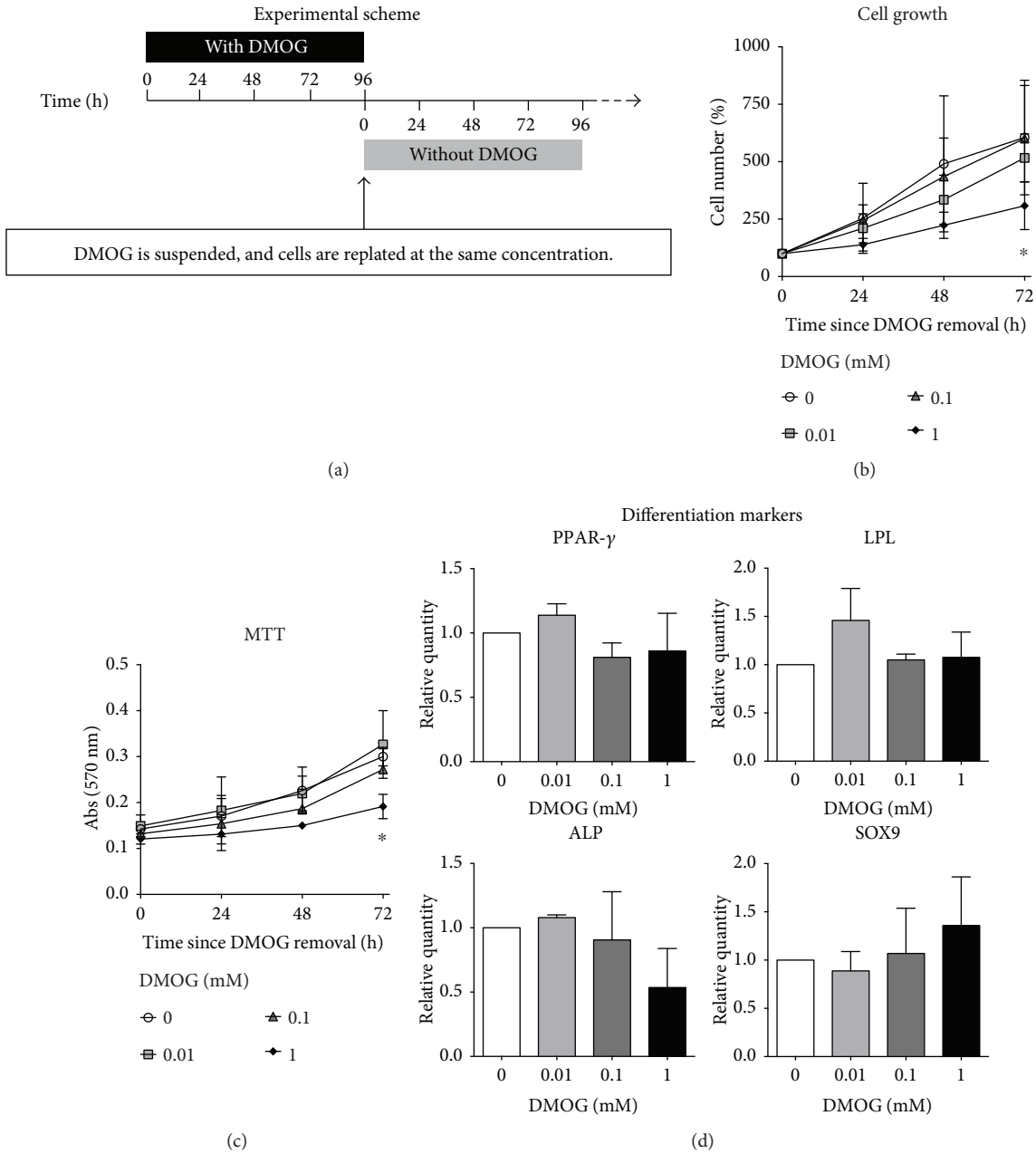

FIGURE 7: Reversibility of HIF-1α activation effects on hTSC proliferation, viability, and differentiation markers. (a) Schematic representation of the experimental setup. hTSCs were cultured in the presence of different concentrations of DMOG (0 mM, 0.01 mM, 0.1 mM, and 1 mM) in normal growth medium for 96 h, washed with PBS, detached, replaced at the same concentration in normal growth medium without DMOG, and then left overnight before successive analyses at 24, 48, 72, and 96 h after seeding. (b) Cell growth curves and (c) MTT assay of hTSCs after culturing DMOG-pretreated cells for 96 h in DMOG-free medium normal and compared to control cells (0 mM DMOG). (d) Peroxisome proliferator-activated receptor-γ (PPAR-γ), lipoprotein lipase (LPL), and human alkaline phosphatase (ALP) expression by real-time PCR in hTSCs induced to differentiate toward adipocytes, osteoblasts, and chondroblasts after a 96 h preconditioning with DMOG, followed by 96 h in normal DMOG-free growth medium, and then differentiation in DMOG-free medium. Control cells were cultured under the same conditions but without DMOG. Values are expressed as fold changes relative to control cells. All experiments were performed in triplicates. Data are expressed as the mean ± SD of six in the case of cell growth curves and three different experiments in case of MTT assay and differentiation marker expression. p values were calculated using Student's t-test. Only p values < 0.05 are indicated: $^*p < 0.05$, as compared to control cells.

after injection and ultimately obtain better regenerative results [27, 28]. Thus, in this study, we tested the effects of HIF-1α activation on human adult tendon stem cells, which we recently isolated from the rotator cuff supraspinatus tendon [15]. This tissue is quite hypoxic, as it is poorly vascularized in the adults, and this could explain, at least

partially, its limited healing capacity. To have a better control on HIF-1α activation, we decided to modulate it with a pharmacological approach using DMOG at different concentrations, which allowed to easily fine-tune HIF-1α expression. As we anticipated, in this study, we found that tendon stem cells cultured in the presence of DMOG reduce their proliferation capability and the effect is proportional to the concentration used. This is in agreement with other reports on adult stem cells cultured under hypoxia [29, 30]. Actually, a tendency, although not significant, toward a proliferation increase could be observed at very low DMOG concentration (0.01 mM), which is also in agreement with other literature reports, showing that moderate hypoxia can stimulate stem cell proliferation, while extreme hypoxia induces them to exit the cell cycle and enter a quiescent state [9]. Nonetheless, we found that DMOG effects on cell proliferation are reversible, as cells could regain their proliferation capability once the molecule is removed from the culture medium. We also found that stem cell markers NANOG, OCT4, and KLF4 are slightly upregulated upon DMOG supplementation and this increase is proportional to the DMOG concentration used. Moreover, tendon-specific markers decrease during DMOG treatment, supporting the notion that chemically induced hypoxia can keep stem cells into a more undifferentiated state. Furthermore, DMOG effects on tendon stem cell differentiation toward osteoblasts and adipocytes were also assessed. The results showed that differentiation is hindered when HIF-1α is activated in the presence of DMOG and that the inhibition is proportional to the concentration of DMOG used, supporting the notion that stem cells reside in the tissue in hypoxic niches, where HIF-1α is activated and the differentiation is inhibited, until cells migrate to more oxygenated areas. Actually, the literature on this issue is quite controversial. In fact, while some reports show a direct increase in the differentiation markers upon in vitro differentiation of hypoxia-activated mesenchymal stem cells [29, 31–34], other reports showed a marked reduction of differentiation [3, 18, 22, 30, 35–40]. Indeed, while the general notion is that a more primitive stem cell should give better regenerative results, it could be argued that a more committed stem cell is more prone to differentiate than an immature one, especially when different stem cell progenitors are compared in the same differentiation timeframe. In fact, at this stage, we cannot exclude that DMOG pretreated cells, as they show a higher expression of stem cell markers, could be more effective in regenerating damaged tissue in vivo.

5. Conclusions

In conclusion, in this study, we found that a chemical activation of HIF-1α reduces human tendon stem cell proliferation, increases the expression of stem cell markers, and reduces their commitment toward the tendon phenotype. These effects are reversible upon DMOG removal from the culture medium. Moreover, DMOG present in the differentiation medium reversibly inhibits stem cell differentiation. Further studies to test whether DMOG pretreatment has any beneficial effects in vivo in tendon stem cell regeneration capacity are currently undergoing in our laboratories.

Abbreviations

ALP: Alkaline phosphatase
COL1A1: Collagen type I alpha-1
DMOG: Dimethyloxalylglycine
HIF-1α: Hypoxia inducible factor-1α
KLF4: Kruppel-like factor 4
LPL: Lipoprotein lipase
NANOG: Nanog homeobox
OCT4: Octamer-binding transcription factor 4
PHDs: HIF prolyl hydroxylases
PPAR-γ: Peroxisome proliferator-activated receptor-γ
S14: Ribosomal protein S14
SOX9: SRY-box9
VEGF: Vascular endothelial growth factor.

Disclosure

The authors alone are responsible for the content and writing of the paper.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

All authors have read and approved the final submitted manuscript.

Acknowledgments

This work was partially supported by a grant (Fondi Linea 2, Tipologia B) from the Department of Biomedical Sciences for Health, University of Milan, Milan, Italy.

Supplementary Materials

Supplementary Figure 1: characterization of hTSCs by flow cytometry. Flow cytometric analysis of antigens CD34, CD45, HLA-DR, CD73, CD90, and CD105. Peaks of specific antigens are shown in green while peaks of respective isotype controls are shown in red. *(Supplementary Materials)*

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Hindawi
Stem Cells International
Volume 2017, Article ID 9497325, 16 pages
https://doi.org/10.1155/2017/9497325

Review Article

The Effects of Different Factors on the Behavior of Neural Stem Cells

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Received 8 August 2017; Accepted 18 October 2017; Published 20 November 2017

Academic Editor: Margherita Maioli

The repair of central nervous system (CNS) injury has been a worldwide problem in the biomedical field. How to reduce the damage to the CNS and promote the reconstruction of the damaged nervous system structure and function recovery has always been the concern of nerve tissue engineering. Multiple differentiation potentials of neural stem cell (NSC) determine the application value for the repair of the CNS injury. Thus, how to regulate the behavior of NSCs becomes the key to treating the CNS injury. So far, a large number of researchers have devoted themselves to searching for a better way to regulate the behavior of NSCs. This paper summarizes the effects of different factors on the behavior of NSCs in the past 10 years, especially on the proliferation and differentiation of NSCs. The final purpose of this review is to provide a more detailed theoretical basis for the clinical repair of the CNS injury by nerve tissue engineering.

1. Introduction

The central nervous system (CNS), consisting of the brain and the spinal cord, is the most important part of the nervous system. In the CNS, a large number of nerve cells get together to constitute different circuits or networks so that the CNS can transmit, store, and process information, generate all kinds of mental activities, and control animal behaviors. However, once the CNS is damaged, it is extremely difficult to be cured, which involves both internal and external causes. The internal cause is that the regenerative ability of nerve cells in the brain and the spinal cord is weak and the loss of cells cannot be supplemented by the proliferation of mature nerve cells. The external cause is the formation of the microenvironment that inhibits nerve regeneration in injured sites, including the production of inhibitory factors that inhibit the regeneration of axons, the lack of neurotrophic factors that promote cell regeneration, and the formation of glial scars that impede axonal passage. For the past few years, the continuous development and improvement of cell transplantation technique have provided the possibility for the repair of the CNS injury.

Neural stem cells (NSCs) that can self-renew and proliferate and have the potential to differentiate into neurons, astrocytes, and oligodendrocytes in the nervous system are not only mainly distributed in the ventricular zone (VZ) and subventricular zone (SVZ) but are also distributed in the hippocampus dentate gyrus of adult mammals, olfactory bulb, spinal cord, compartment, striatum essence, cerebellum, cerebral cortex, and other parts [1]. Based on the characteristics of self-renewal, proliferation, multilineage differentiation, low immunogenicity, and migration of transplanted NSCs *in vivo*, NSC has become a very promising cell type for the treatment of the CNS injury. One of the purposes of NSC transplantation is to replace or complement the missing or dysfunctional neurons in the CNS. In addition, NSCs can also promote neuranagenesis by continuous secretion of neurotrophic factors.

Scientific studies have demonstrated that it is not ideal to simply transplant NSCs into the injured area because the NSCs implanted are primarily transformed into astrocytes

[2]. Consequently, to clarify the mechanism of the factors that regulate the behavior of NSCs is the key for us to repair the CNS injury successfully. This paper summarizes the effects of different factors (including physical factors, chemical factors, biological factors, and material factors) on the behavior of NSCs in the past decade and provides a more detailed theoretical basis for the clinical repair of the CNS injury by nerve tissue engineering.

2. Physical Factors

Under normal circumstances, most of the NSCs in the CNS are in a resting state, but all of them have the potential to differentiate. When subjected to endogenous and exogenous stimulations, these NSCs will be activated, showing different growth and differentiation behavior [3]. Here, we summarize the physical factors that regulate the fate of NSCs from the perspectives of physical stimulation and oxygen treatment.

2.1. Physical Stimulation. In recent years, plenty of studies have shown that external stimuli such as sound, light, electricity, magnetism, and acupuncture can induce NSCs to respond. The effects of low-intensity pulsed ultrasound (LIPUS) on the cell viability, proliferation, and neural differentiation of induced pluripotent stem cell-derived neural crest stem cells (iPSCs–NCSCs) have been reported. Under the different output intensities of LIPUS: 0, 100, 300, 500, 700, 900, 1100, 1300, and 1500 mW/cm^2, the viability and proliferation of iPSCs–NCSCs were obviously enhanced after 2 days, and the genes of neurofilament (NF-M), β-tubulin III (Tuj1), S100β, and glial fibrillary acidic protein (GFAP) were upregulated after 4 days by LIPUS of 500 mW/cm^2 [4].

Humans have been living together with radiation, such as cosmic radiation and ground radiation and man-made radiation. Computed tomography (CT) and X-ray are typically used in hospitals to examine patients, and we use mobile phones and computers every day. Thus, whether radiation affects the human body or cells has caused widespread global concern. Different doses of X-rays were used to irradiate the NSCs, and researchers found that NSCs could restore the abilities of proliferation and differentiation after a short stop of proliferation temporarily when irradiated at low doses, such as 1 Gy; conversely, NSCs exposed to relatively high doses (>5 Gy) lost these capabilities [5]. Over the past years, NSCs have been exposed to the radiation of radio-frequency electromagnetic field (RF-EMF) 900 MHz with specific absorption rate (SAR) = 2 W/kg [6], RF-EMF 1710 MHz with SAR = 1.5 W/kg [7], RF-EMF 1800-900 MHz with SAR = 1.6 W/kg [8] that confirmed the effects of RF-EMF on the proliferation and apoptosis of the NSCs. In general, these studies indicate that proliferation and differentiation of the NSCs depend on the source, intensity of radiation, and the duration of exposure.

The use of electrotherapy has a long history, and the past application of electrotherapy treatment of diseases was mostly empirical. With the development of the research on human bioelectricity, it has been found that bioelectric phenomena are widespread in organisms, which involve embryonic development and tissue repair after injury. A culture system with the functions of supporting stem cell growth and regulating the levels of electric current has been invented. The biphasic current stimulator chip with indium tin oxide (ITO) electrodes generated both positive and negative currents. The results showed that biphasic electrical currents (BECs) obviously promoted the proliferation and neuronal differentiation of NSCs [9]. Another experiment was conducted to study the effect of electric field on cell migration and differentiation. The 115 V/m direct current (DC) electric field increased the migration of neural precursor cells (NPCs) by approximately four times compared with the control group through the calcium-dependent mechanism and caused NPC membrane depolarization without breaking and also facilitated the differentiation of NPCs into neurons rather than glial cells [10].

The effects of magnetic field on the nervous system have been studied a lot so far. Results showed that moderate magnetic field could change the function of the CNS, the extremely low-frequency magnetic field could affect the survival and apoptosis of nerve cells, and the pulsed magnetic field could relieve pain in rats. Repeated transcranial magnetic stimulation (rTMS) is a new method for the treatment of many neurological disorders. NSCs and neural progenitor cells were subjected to the rTMS with 1 Hz and 30 Hz. After 2 weeks, compared with the blank control group, the formation of neurospheres was significantly increased with the rTMS; furthermore, the number of neurospheres in the 30 Hz stimulation group was the largest. There was no significant difference in the neuronal differentiation between the two stimulation groups, but both of them were increased by 1.5 times compared with the control group [11]. Although there are numerous studies in this area, the mechanism of the magnetic field on the specific regulation of NSCs is still not clear.

With the electromagnetic pulse being widely used in all walks of life, the impact of pulsed electromagnetic fields (PEMFs) on NSCs has attracted more and more attention. High-intensity PEMFs were applied to neonatal rats with 0.1 Hz and 0.5–10 tesla (T) [12]. The results showed that the survival rates of NSCs in the 3.0 T and 4.0 T groups were higher than those in other groups, indicating that the growth of NSCs was promoted. On the contrary, the 6.0 T, 8.0 T, and 10.0 T groups significantly inhibited the growth of NSCs. Thus, 4.0 T was the most favorable PEMF intensity for the growth of NSCs.

Acupuncture is a form of alternative medicine in which thin needles are inserted into the body. It is a common method of treatment in traditional Chinese medicine, which has been used to induce or activate the proliferation and differentiation of endogenous NSCs for the treatment of CNS injury with the advantages of safe source, no immune response, and no ethical problems. It has been reported that Sprague-Dawley (SD) rats were divided into the sham-operated group, model group, and acupuncture group, and acupuncture was performed daily at the specific acupuncture points of the brain traumatic rats. After 7 days, the number of nestin, neurofilament protein- (NF-) 200 and GFAP-positive cells was most in the acupuncture group [13]. Another similar experiment was conducted at different times to detect the marker proteins expressed by the three groups of NSCs [14].

The results showed that acupuncture was an effective method to promote nerve regeneration and functional recovery.

2.2. Oxygen Treatment. Oxygen is one of the indispensable conditions for cell survival, which participates in the tricarboxylic acid cycle to produce energy for cell growth, proliferation, and synthesis of various desired components. It is well known that oxygen concentration mediates many ischemic behaviors and cerebral ischemia can cause the activation of NSCs. The effects of oxygen on the proliferation and differentiation of NSCs have been studied [15]. Researchers studied the effects of 0, 1, 2, 3, 4, 10, and 20% oxygen concentrations on NSCs. They found that the proliferation rate and the proportion of neuronal differentiation of NSCs in 2% oxygen were the greatest and the number of dead cells gradually increased with the decrease of oxygen concentration. However, the duration of low oxygen pretreatment is still controversial [16]. To solve this problem, another team attempted to culture NSCs in 5%, 10%, and 20% oxygen for 72 hours and 120 hours. Finally, they reported that the optimum concentration and time of low oxygen pretreatment were 5% oxygen for 72 hours, which could maximize the proliferation and neuronal differentiation of NSCs [17].

NSCs were cultured in three mediums: hypoxic conditioned medium (HCM) with 1% and 4% oxygen and normoxic conditioned medium (NCM). The experimental results showed that 4% HCM could facilitate the proliferation of NSCs and the differentiation into neurons as much as possible. However, this result was inhibited after the addition of PI3K/AKT and JNK inhibitors. Ultimately, researchers summarized that 4% HCM promoted the proliferation and differentiation of NSCs might be mainly through the PI3K/AKT pathway [18]. These findings raise the possibility of curing CNS injury with the NSCs.

So far, researchers have investigated the effects of physical factors on the behavior of NSCs in a macroscopic manner without any specific regulatory mechanisms. Thus, using physical methods for the treatment of CNS injury still has a long way to go.

3. Chemical Factors

3.1. Drugs. Drug treatment is the most traditional method, and the history of which is almost as long as the history of mankind itself. Although great progress has been made in medicine, we still cannot use drugs to cure the CNS injury. At present, numerous researchers have attempted to transplant NSCs which are stimulated by drugs into the sites of CNS injury in order to make NSCs proliferate and differentiate into the desired direction.

3.1.1. Chinese Herbal Medicine. After screening a number of Chinese herbal medicines and their active ingredients *in vitro* and *in vivo*, Chinese herbal medicine has been demonstrated to have various effects on NSCs in many aspects [19]. Salvianolic acid B is the most abundant and bioactive content of the salvianolic acids in danshen [20]. It was found that 20 μg/mL of salvianolic acid B was favorable for the proliferation and neuronal differentiation of NSCs and the growth-promoting activity was associated with the number of NSCs in the G2/S phase [21]. Tetramethylpyrazine (TMP), an active element derived from traditional Chinese medicine Ligusticum Chuanxiong, is widely used for the treatment of ischemic stroke [22]. The proliferation and differentiation of NSCs treated with TMP under the hypoxia condition have been studied. The phosphorylation of extracellular signal-related protein kinase (ERK) and p38 in the mitogen-activated protein (MAP) kinase family were involved in these experiments [23]. In another study, NSCs were isolated from the hippocampus of neonatal 1 day rats; then, NSCs were cultured in different concentrations of epimedium flavonoids (EF) without growth factors. Researchers concluded that EF had neurotrophic factor-like function to promote NSC proliferation directly. On the other hand, compared with the 10% fetal bovine serum (FBS) control, EF significantly improved the level of neuron differentiation and migration of NSCs [24]. Studies have shown that panax notoginseng saponins (PNS) have functions of protecting brain tissue and antiaging. The result that 17.5 μg/mL PNS was most conducive to the proliferation of NSCs has been confirmed [25]. Area density, optical density, and the numbers of nestin/BrdU, nestin/vimentin, and nestin/Tuj1 positive cells were significantly increased after oxygen glucose deprivation (OGD) and PNS were given, suggesting that PNS benefited the nerve regeneration in the brain ischemic injury. Polygala tenuifolia Willd is used as the treatment and prevention of dementia, and its main active ingredient is named tenuigenin. Under the influence of tenuigenin, more neurospheres were formed and the number of Tuj1-positive cells and GFAP-positive cells was significantly higher than that of the same volume of the phosphate-buffered solution (PBS) control group [26], which might be the underlying reason of the medicinal value of tenuigenin (seen in Table 1).

Gastrodiae rhizome [27], *Astragalus mongholicus* [28], Angelica [29], and other herbs are well-known, and many researchers have studied their effects on the proliferation and differentiation of NSCs. In conclusion, Chinese herbal medicine has shown great potential in inducing the proliferation and differentiation of NSCs. With the deepening of research, Chinese medicine scholars are expected to explore a new regulatory system for NSCs so that Chinese medicine can take a further step in stem cell research.

3.1.2. Western Medicine. Under the background of the continuous emergence of new drugs and the substantial increase of medical level, the drug market for the treatment of CNS diseases has been growing rapidly. It has been demonstrated that ketamine could affect the proliferation and differentiation of NSCs *in vitro* [30]. Thereout, ketamine was injected into the subventricular zone (SVZ) of neonatal rats. The results demonstrated that ketamine could alter neurogenesis by inhibiting the proliferation of NSCs, preventing the differentiation into astrocytes and promoting the differentiation into neurons [31]. Sphingosine-1-phosphate (S1P) is a potent lipid mediator transducing intracellular signals, which regulates cells' biological behavior in the CNS [32, 33]. Fingolimod (FTY720), a structural analog of S1P, has been used to affect the brain-derived NSCs [34]. The research showed that

Table 1: Effects of drugs or active ingredients on the proliferation and differentiation of NSCs.

Drugs or active ingredients	Cells	The role of proliferation	The role of differentiation	Mechanism
Salvianolic acid B [21]	NSCs from cortex of E13.5 mice	Nestin-positive cells ↑	The expression of tau ↑; the expression of GFAP ↓	Cells in G2/S phase
Tetramethylpyrazine [23]	NSCs from cortex of E15 SD fetal rats	BrdU-positive cells ↑	β-Tubulin III-positive cells ↑; β-tubulin III/nestin-positive cells ↑; GFAP-positive cells ↓; GFAP/nestin-positive cells ↓	The expression of cyclin D1 ↑; the expression of P-ERK ↑; the expression of P-JNK with little change; the phosphorylation of P38 ↓
Epimedium flavonoids [24]	NSCs from hippocampus of 1d neonatal rats	BrdU-positive cells ↑	Tuj1-positive cells ↑; NF-200-positive cells ↑; GFAP-positive cells ↑	
Panax notoginseng saponins [25]	NSCs from hippocampus of 1d neonatal rats	Nestin-positive cells ↑; BrdU-positive cells ↑	Nestin/BrdU-positive cells ↑; nestin/vimentin-positive cells ↑; nestin/Tuj1-positive cells ↑	
Tenuigenin [26]	NSCs from hippocampus of E16 rats	BrdU-positive cells ↑	β-Tubulin III-positive cells ↑; GFAP-positive cells ↑	
Gastrodiae rhizome [27]	NSCs from human embryos	Viability ↑	The expression of nestin ↓; the expression of Tuj1 ↑; the expression of MAP2 ↑; dendrites ↑	The expression of Sox2 ↓
Astragaloside IV [28]	NSCs from hippocampus of adult SD rats	BrdU-positive cells ↑	BrdU/MAP2-positive cells ↑; BrdU/GFAP-positive cells ↑	The expression of NGF ↑
Angelica [29]	NSCs from embryonic rats	Nestin ↓ compared with the hypoxia group; nestin ↑ compared with the control group		
Ketamine [31]	NSCs from the SVZ of 7d neonatal male SD rats	Nestin/BrdU-positive cells ↓	The expression of β-tubulin III ↑; the expression of GFAP ↓; the expression of nestin ↓	
Fingolimod [34]	NSCs from E13.5 SD rats	CCK-8 ↑	The expression of GFAP ↑	
Simvastatin [36]	NSCs from embryonic rats	Nestin-positive cells ↑	GFAP-positive cells ↑; MAP2-positive cells ↑	The expression of Notch1 ↑

the proliferation and migration of NSCs were promoted and the formation of astrocytes was increased instead of significant neuronal differentiation. Simvastatin is an essential antihypertensive drug for basic medical systems. The proliferation and neuronal differentiation of NSCs induced by simvastatin showed a long-term neurorestoration effect on the injured brain, which was mediated by the activation of the Notch signaling pathway [35, 36]. For this reason, simvastatin has become one potential treatment for patients with traumatic brain injuries (seen in Table 1).

Compared with traditional Chinese herbal medicine, western medicine is adopted for the purpose of symptomatic treatment and fast acting. However, it needs further exploration for radical cure and less side effects on chronic diseases and incurable diseases such as CNS injury.

3.2. Other Chemical Molecules. Recently, chemical molecules have been widely used to guide the biological function of NSCs and their different structures and properties have exhibited different effects on NSCs. An increase of evidence has suggested that hydrogen sulfide (H_2S) could act as a novel neuromodulator to intervene in the advances in treating brain diseases. Sodium hydrosulfide (NaHS), the H_2S donor, induced the proliferation of NSCs associated with extracellular signal-regulated kinase ERK1/2 and the neuronal differentiation associated with the expression of differentiation-related genes [37]. Epigallocatechin-3-gallate (EGCG), the major component of green tea polyphenols with antibacterial and antioxidant properties, is not toxic when it is less than 10 μM. At this point, EGCG stimulated the proliferation of NSCs and the formation of neurospheres, and most importantly, neuronal differentiation was promoted by EGCG via the activation of the PI3K/AKT signaling pathway [38].

Although many chemical molecules had a role in promoting the proliferation and differentiation of NSCs, researchers also analyzed the effects of the chemical molecules that are

harmful to humans in life on NSCs. Bisphenol-A (BPA), an endocrine disrupter commonly used as a surface coating for canned food, was fed daily to pregnant rats. Studies revealed that BPA significantly altered the expression of neurogenic genes and the Wnt signaling pathway genes. In other words, BPA impaired the proliferation and differentiation of NSCs via the Wnt/β-catenin signaling pathway [39]. As we all know, many unhealthy food contain saturated fatty acids (SFAs). Excessive intake of SFAs is the main cause of elevated cholesterol, secondary to atherosclerosis increasing the risk of coronary heart disease. Palmitic acid (PA) is one of the SFAs. The greater the dose of PA, the stronger the inhibition of NSC proliferation. PA was even cytotoxic at high concentrations. Besides, results also showed that PA promoted NSC differentiation into astrocytes by activating Stat3 and had little effect on neuronal differentiation [40]. Recently, 6-OH-PBDE-47, the metabolite of polybrominated diphenylether-47 (PBDE-47) used as a flame retardant [41], has been studied. Researchers found that 6-OH-PBDE-47 was more cytotoxic for adult NSCs than its parent compound and its inhibition of neurogenesis was associated with the inhibition of the ERK5 signaling pathway [42].

3.3. Chemical Functional Groups. Previous studies have demonstrated that surface chemistry was able to modulate cell-matrix adhesions [43] and chemical functional groups were capable of regulating the growth and differentiation of cells [44–46]. For these reasons, glass coverslips were modified by the hydroxyl (–OH), sulfonic ($-SO_3H$), amino ($-NH_2$), carboxyl (–COOH), mercapto (–SH), and methyl ($-CH_3$) groups for culturing NSCs to study the effects of different functional groups on the adhesion, migration, and differentiation of NSCs [47]. On the $-NH_2$ surface, the number of cells migrated from the neurospheres was the largest; conversely, the number on the –OH surface was the least. NSCs cultured on the $-NH_2$ surface exhibited an increase on neuronal differentiation, while the $-SO_3H$ surface was more favorable for the differentiation of NSCs into oligodendrocytes. On the –COOH surface and the –SH surface, NSCs showed similar effects on migration and viability and tended to differentiate into glial cells. In addition, a great deal of astrocytes was observed on the –OH surface and the $-CH_3$ surface. Hence, the chemical functional group-modified surface provides a reliable chemical method for the design of biomaterials for nerve tissue engineering.

From the years of research, it is clear that chemical factors such as drugs, other chemical molecules, and chemical functional groups can affect the growth of NSCs through some proliferation and differentiation-related genes or signal pathways and provide a theoretical and experimental basis for cell therapy of treating CNS diseases. However, there are still many problems that need to be overcome, such as the cytotoxicity, clinical application, and drug response.

4. Biological Factors

Up to now, modern neuroscience has been developed to study the structure and function of the nervous system at the biomolecular level to clarify the mechanism of neural activities. Previous studies have shown that both the physical factors and the chemical factors have a significant effect on the proliferation and differentiation of NSCs. Similarly, the use of growth factors, proteins, cells, and other biological factors can also regulate the behavior of NSCs.

4.1. Proteins. Proteins are the material basis of all life and are important parts of the body cells. Based on the functions of proteins, researchers have been trying to use proteins to regulate the growth and differentiation of NSCs to achieve the purpose of repairing the CNS injury.

4.1.1. Neurotrophins and Growth Factors. The method based on various growth factors is still the most important way to regulate the proliferation and differentiation of NSCs. Neurotrophins and growth factors are typical biologically active molecules, as well as the essential substances in cell growth, which promote the growth, development, and integrity of neurons and glial cells. So far, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), nerve growth factor (NGF), and epidermal growth factor (EGF) [48] have been the most commonly used factors.

BDNF stimulated the proliferation of NSCs and significantly increased the differentiation of NSCs into neurons and oligodendrocytes; in addition, BDNF upregulated the expression of Wnt/β-catenin signaling molecules (Wnt1, β-catenin). Nevertheless, these promoting effects were blocked when the specific inhibitor of the Wnt signaling pathway IWR1 was added, indicating that BDNF acted on NSCs by triggering the Wnt/β-catenin signaling pathway [49]. Recently, the research about using BDNF for the treatment of Alzheimer's disease [50] has also been carried out. NT-3 transfecting bone marrow-derived NSCs (BM-NSCs) has been reported [51]. It was found that NT-3 promoted the proliferation and differentiation of BM-NSCs into cholinergic neurons and increased the level of acetylcholine (ACh) in the supernatant. Compared to this method, another team transduced NT-3 into the rat embryonic cortical NSCs [52]. The result showed that NT-3 was beneficial to the proliferation and neuronal differentiation of NSCs and greatly improved the survival rate of NSCs. NGF, the earliest discovered factor in the neurotrophic factors, has been studied the most thoroughly so far, with the dual biological function of providing neuronal nutrition and promoting synaptic growth. When NGF was added to the medium containing basic fibroblast growth factor (bFGF), the number of NSC proliferation was 17 times higher than that of the serum-free medium control group and 2.5 times higher than that of the treatment group with bFGF alone in the medium [53]. Now, researchers are increasingly concerned about the effects of multiple neurotrophic factors on NSCs. They explored the combination of NGF, BDNF, and bFGF to induce NSCs. After one week, the experimental results reflected that the proportion of differentiated neurons in the multifactor groups (bFGF + NGF, bFGF + BDNF, NGF + BDNF, and NGF + BDNF + bFGF) was significantly higher than that of the single-factor groups (NGF, BDNF, and bFGF) and the proportion of neurons was the highest in

the NGF + BDNF + bFGF group. Besides, NSCs continued to proliferate over time in all groups [54].

The effects of neurotrophins and growth factors on the regulation of NSCs can accelerate the recovery of neurological function, promote the growth of neurons and dendrites, and provide the feasibility for the treatment of senile dementia, neurasthenia, and spinal cord injury.

4.1.2. Other Proteins. The cell cycle which is closely connected with the development, proliferation, and differentiation of NSCs is controlled by the activation and inactivation of cell cycle-related proteins. Cyclin D1 causes cells to enter the S phase by forming a complex that inactivates pRb through the interaction with cyclin-dependent kinase 4 or 6 [55]. The knockdown of cyclin D1 resulted in the apoptosis of NSCs and inhibited the differentiation of NSCs into astrocytes with no effect on the neuronal differentiation [56]. Cell cycle-dependent kinases (Cdk) also play a key role in regulating cell cycle. After double knockout of Cdk2 and Cdk4 in mice, a phenomenon of ablation was observed between the intermediate zone and the cortical plate [57]. In addition, researchers found that the compensation of Cdk2 was the root cause of NSC proliferation and the double knockout NSCs tended to differentiate into neurons. Leucine-rich repeat and Ig domain-containing Nogo receptor interacting protein-1 (LINGO-1) is a nervous system-specific transmembrane protein. After 6 days of differentiation in LINGO-1 neutralized cultures, the number of neurons differentiated by NSCs increased three times, and the number of astrocytes had a slight increase. However, the neutralization of LINGO-1 did not significantly influence the total number of cells compared to the untreated control group [58]. The mental retardation-associated protein srGAP3 has been demonstrated that it could affect the morphology, behavior, and function of SHSY-5Y cell line [59] and was associated with mental retardation [60], long-term memory [61], and neurogenesis. The results showed that the viability and proliferation of NSCs decreased significantly when srGAP3 was knocked out (LV3-srGAP3 infection). After culturing in a differentiation medium 7 days, the number of nestin and β-tubulin III-positive cells in the srGAP3 knockdown group was more than that of the control group (LV3-NC infection) and the number of GFAP-positive cells decreased [62].

4.2. Cocultivation of Cells. In order to establish a culture system which is more similar to the environment *in vivo* so that cells can communicate with each other and support the growth mutually, cell coculture technique has been developed. Coculture system contains the following functions: inducing cells to differentiate into other types of cells; maintaining cell function and viability; and regulating cell proliferation. At present, cell cocultures have been extensively used in cell research.

Endothelial cells (ECs) are one of the most common cells cocultured with NSCs. It has been found that ECs stimulated the proliferation and differentiation of NSCs with vascular endothelial growth factor (VEGF), possibly by activating the Notch, Wnt, and Pten signaling pathways. The expression of the Notch signaling pathway-related genes (*notch2*, *numb*, *Hes1*, and *Psen1*), the Pten signaling pathway-related genes (*Pten*, *Akt1*, and *PIP3*), and the Wnt signaling pathway-related genes (*Wnt3a* and *β-catenin*) increased significantly in *in vitro* coculture conditions [63–65].

Recent data suggest that microglia are associated with neurogenesis. When activated by endotoxins, microglias inhibited neurogenesis *in vivo*. In contrast, microglia activated by cytokines interferon- (IFN-) g and interleukin- (IL-) 4 enhanced the neuronal differentiation [66]. Here, NSCs were cocultured with microglia collected from ischemic or excitotoxic injured brain. Microglia released mitogenic factors that promoted the proliferation of NSCs and NSCs differentiated into neurons and oligodendrocytes as soon as possible [67]. It is a known fact that astrocytes are one of the differentiation products of NSCs. Studying the effects of astrocytes on the behavior of NSCs has attracted the interest of researchers. An *in vivo* experiment was designed in which both astrocytes and NSCs were transplanted into the ischemic striatum of the transient middle cerebral artery occlusion (MCAO) rat model [68]. Definitively, cotransplantation resulted in a higher survival, proliferation, and neuronal differentiation of transplanted NSCs than that of NSCs transplanted alone.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) have been widely used in tissue engineering. Consequently, researchers tried to coculture BM-MSCs with NSCs. The proliferation and neuronal differentiation of NSCs and high expression of various growth factors were induced by BM-MSCs, but the glial differentiation was inhibited. In addition, protecting NSCs against the neurotoxin 6-hydroxydopamine was another beneficial effect of BM-MSCs on NSCs [69]. In light of the above findings, the mechanism of neurogenesis induced by BM-MSCs was worth exploring. The result reflected that the enhancement of the proliferation and differentiation of NSCs was concerned with the upregulation of chemokine (C-C motif) ligand 2 (CCL2) released from BM-MSCs [70].

In addition to the large number of neuronal deaths, CNS injury is difficult to repair due to the regenerated axons without the ability to pass through the glial scar. Olfactory ensheathing cell (OEC) is a unique type of glial cells derived from the olfactory placode and occurs along the olfactory nerve in both the peripheral and central nervous system [71]. OECs could help the axons of neurons pass through the glial scar to promote functional recovery. Furthermore, the proliferation, neuronal differentiation of NSCs, and the formation of axons were promoted when NSCs were cocultured with OECs [72].

Cell coculture technique is a relatively safe, new, and promising technology in nerve tissue engineering. The interaction of supporting growth and promoting differentiation between the cells can be observed through the coculture system. At present, this technology has good applications in stem cells, tumor biology, and other aspects.

4.3. MicroRNAs. MicroRNAs are one sort of endogenous and noncoding RNAs that downregulate gene expression

Table 2: Effects of microRNAs on the proliferation and differentiation of NSCs.

MicroRNA	The role of proliferation	The role of differentiation	Mechanism
miR-34a [75]	Postmitotic neurons ↑	Neurite elongation ↑	miR-34a ↑ → SIRT1 ↓ → P53 acetylation ↑
miR-125b [77]	BrdU-positive cells ↓	Nestin-positive cells ↓; Sox2-positive cells ↓; vimentin-positive cells ↓; MAP2-positive cells ↑; Tuj1-positive cells ↑; cell migration ↑	miR-125b ↑ → nestin ↓
miR-146 [81]	Neural sphere diameter ↓	β-Tubulin III positive cells ↓; GFAP-positive cells ↑	miR-146 ↑ → Notch1 ↓
miR-342-5p [82]	BrdU-positive cells ↓	PAX6-positive cells ↓; TBR2-positive cells ↑; GFAP-positive cells ↓	Notch → Hes5 ↓ → miR-342-5p ↑ → GFAP ↓
miR-184 [84]	BrdU-positive cells ↑	Tuj1-positive cells ↓; GFAP-positive cells ↓	MBD1 ↓ → miR-184 ↑ → Numbl ↑
miR-9 [85]	BrdU-positive cells ↓	Tuj1-positive cells ↑; GFAP-positive cells ↑	miR-9 ↑ → TLX ↓ → proliferation ↓, neurons ↑
miR-7 [86]	G1/S cell cycle arrest	Tuj1-positive cells ↑; MAP2-positive cells ↑; Nestin-positive cells ↓; vimentin-positive cells ↓	miR-7 ↑ → *Klf4* ↓ → proliferation ↓, neurons ↑
miR-124 [87, 88]	BrdU-positive cells ↓	Tuj1-positive cells ↑; NeuN-positive cells ↑; GFAP-positive cells ↓	miR-124 ↑ → TrkB ↑, Cdc42 ↑ → neurons ↑

through the translational inhibition or degradation of their target mRNA [73]. The role of microRNAs on NSCs has attracted extensive attention of researchers. In NSCs, miR-34a, miR-125b, miR-146, miR-342-5p, miR-184, miR-9, miR-7, and miR-124 are important regulators (seen in Table 2).

MiR-34a, a member of the miR-34 family, is encoded by its own transcripts [74]. It has been reported that the overexpression of miR-34a increased the neuronal differentiation and neurite outgrowth of NSCs, which involved the downregulation of the silent information regulator 1 (SIRT1) and the enhancement of p53-DNA-binding activity [75]. Another regulator of neuronal differentiation, miR-125b, is highly expressed in the CNS [76]. MiR-125b inhibited the proliferation of NSCs and promoted the neuronal differentiation and migration by inhibiting its downstream target nestin [77], which is a necessary cellular process (proliferation, differentiation, and migration) regulator of NSCs [78, 79]. Similarly, miR-146, which is mainly involved in the regulation of inflammation and innate immune [80], is one of the brain-specific miRNAs. It had the same inhibitory effect on the proliferation of NSCs as miR-125b. NSCs tended to differentiate into glial cells rather than neurons by inhibiting the expression of Notch 1 with the overexpression of miR-146 [81]. The Notch signaling pathway negatively regulated miR-342-5p by its transcriptional repressor Hes5. Studies have shown that transfection of miR-342-5p induced the apoptosis of NSCs, whereas the differentiation of NSCs into intermediate progenitor cells (INPs) was promoted. Notably, the suppression of the differentiation into astrocytes was regulated by miR-342-5p directly targeting GFAP [82]. Methyl-CpG-binding protein 1 (MBD1) has been demonstrated that it was capable of controlling cell growth [83]. Accordingly, the study was conducted to indicate that the high expression of miR-184 regulated by MBD1 directly promoted the proliferation of NSCs and inhibited their differentiation [84]. Numb-like protein (Numbl), which was the downstream target of miR-184, MBD1, and miR-184 together constituted a network to balance the proliferation and differentiation of NSCs.

The balance of self-renewal and differentiation of NSCs was closely related to the feedback loop formed by miR-9 and the nuclear receptor TLX [85]. The overexpression of miR-9 inhibited the proliferation of NSCs by suppressing the TLX expression, as well as accelerating neuronal differentiation. In contrast, TLX had the ability to inhibit the expression of miR-9 pri-miRNA, thereby avoiding the miR-9-induced proliferation and premature differentiation. It was found that miR-7 participated in the NSC self-renewal and differentiation by targeting Kruppel-like factor 4 (*Klf4*), a key gene for the NSC proliferation [86]. Data showed that the overexpression of miR-7 downregulated the *Klf4* gene, which in turn resulted in a decrease in the NSC proliferation and an increase in the neuronal differentiation.

MiR-124 also plays an important role in neuronal differentiation of NSCs. Six microRNAs (miR-124, miR-132, miR-134, miR-20a, miR-17-5p, and miR-30a-5p) were detected in the inner ear NSCs after 14 days of neuronal differentiation [87]. The expression of miR-124 was upregulated during neuronal differentiation, which made the tropomyosin receptor kinase B (TrkB) and the cell division control protein 42 homolog (Cdc42) upregulate, thus greatly promoting

Table 3: Effects of biomaterials on the proliferation and differentiation of NSCs.

Topography	Materials	Dimensions	Cells	Outcome
Film	Chitosan [90]		NSCs from cortex of E12 fetal rats	Proliferation ↑; astrocytes ↑
Film	Ultrananocrystalline diamond (UNCD) [91]		NSCs from cortex of E11.5 rat embryos	Proliferation most on hydrogen-terminated UNCD film; neuronal differentiation most on oxygen-terminated UNCD film
Film	PHA: PLA, PHBVHHx [92]		NSCs from neocortex of E13–15 rat embryos	Adsorption and proliferation more on PHBVHHx film; neuronal differentiation and neurite outgrowth more on PLA film
Hydrogel	Gelatin-hydroxyphenylpropionic acid (Gtn-HPA) [93]		NSCs from hippocampus of adult female Fischer 344 rats	Viability ↑; proliferation rate ↓; the expression of GFAP ↑; the expression of Tuj-1 ↑
Hydrogel	Hyaluronic acid (HA) [94]		NSCs from induced pluripotent stem cells	Glial, neuronal, or immature/ progenitor states ↑; proliferation ↑
Hydrogel	Polyethylene glycol [99]		NSCs from the BMSCs of 4 weeks SD rats	Viability ↑; proliferation ↑ slightly; neuron ↑; neurite outgrowth and extension
Nanofibers	Polyethersulfone (PES) [100]	283 nm, 749 nm, 1452 nm	NSCs from hippocampus of adult rats	40% ↑ in oligodendrocyte with 283 nm fibers; 20% ↑ in neuronal cells with 749 nm fibers; proliferation ↑; cell spreading ↑; cell aggregation ↓ with the decrease of fiber diameter
Nanofibers	Polyhydroxyalkanoates (PHA): PHB, P3HB4HB, PHBHHx [101]		NSCs from neocortex of E13–15 rat embryos	Proliferation and neuronal differentiation most in PHBHHx
Nanofibers	Poly-ε-caprolactone (PCL) [102]	550 ± 100 nm	NSCs from neocortex of E14.5 rat embryos	Proliferation ↑; neurons ↑; oligodendrocytes ↑

neuronal differentiation and neurite outgrowth, compared with other groups. Another team transplanted NSCs transfected with miR-124 into the spinal cord injury rats. They found that the overexpression of miR-124 increased the percentage of neurons, decreased the percentage of astrocytes, and reduced the lesion cavity volume of the spinal cord injury rats [88].

Taken together, miRNAs regulate gene expression by binding to 3′-untranslated regions (3′-UTR) of specific mRNAs [75, 89], thereby altering the proliferation and differentiation of NSCs and ultimately completing the regulation of nervous system development.

5. Material Factors

Material, one of the three elements of tissue engineering, plays an increasingly important role in transplanting NSCs to repair CNS injury. In recent years, with the development of tissue engineering, bio-scaffold materials which have good biocompatibility, biodegradability, three-dimensional structure, and good surface activity and are nontoxic, have effectively combined with NSCs to provide appropriate support and favorable microenvironment to enhance NSC survival, proliferation, and differentiation, so as to achieve the purpose of repairing trauma and rebuilding function. Generally, morphology, composition, and surface modification of materials are several important factors that affect cell behavior. Here, we summarized the effects of several major morphologies (film, hydrogel, and nanofiber) of different materials on the behavior of NSCs (seen in Table 3).

Film is the simplest 2D scaffold for cultured cells. Chitosan films (Chi-F), chitosan porous scaffolds (Chi-PS), and chitosan multimicrotubule conduits (Chi-MC) have been prepared to evaluate the effects on the proliferation and differentiation of NSCs. As a result, NSCs cultured on Chi-F exhibited the maximal proliferation but were more likely to differentiate into astrocytes. The proportion of neuronal differentiation of NSCs cultured on Chi-MC was the largest compared with that on Chi-F and Chi-PS [90]. By controlling the surface properties of ultrananocrystalline diamond (UNCD), the proliferation and differentiation of NSCs could be modulated [91]. The result indicated that the hydrogen-terminated UNCD film was most conducive to NSC proliferation; in addition, either the oxygen-terminated UNCD film or the hydrogen-terminated UNCD film could greatly increase the proportion of neuronal differentiation. Polyhydroxyalkanoate (PHA) is a common biopolymer material. The fusion protein PhaP-IKVAV was coated on the surfaces of two polyester materials of the same aliphatic family: poly (L-lactic acid) (PLA) and the

copolymer of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) (PHBVHHx). Ultimately, the levels of NSC adsorption and proliferation were stronger on the PHBVHHx film, whereas the neuronal differentiation and neurite outgrowth were more promoted on the PLA film [92].

Hydrogel is a network scaffold formed by the crosslinking of polymers with physical or chemical interactions. It is rich in water and has a porous structure and is conducive to material exchange, cell attachment, growth, and extension of protrusions. The gelatin-hydroxyphenylpropionic acid (Gtn-HPA) hydrogel was formed by enzyme-mediated oxidative crosslinking. The viability of NSCs encapsulated in the Gtn-HPA hydrogel was increased by approximately 8 times. Moreover, the Gtn-HPA hydrogel increased the proportion of neuronal differentiation to a greater extent compared with the blank control group [93]. Hyaluronic acid (HA) is a biocompatible and biodegradable biomaterial that is abundant in the connective tissue. The number of glial cells, neurons, or immature/progenitor cells was increased when NSCs were encapsulated into HA hydrogels and transplanted into the brain injury sites [94]. Studies have shown that polyethylene glycol (PEG) was a material with the abilities of suppressing the production of free radicals [95], inhibiting apoptotic cell death following traumatic spinal cord injury [96], supporting the repair of damaged neuronal membranes [97], and transferring oxygen and nutriments [98]. Arginyl glycyl aspartic acid (RGD) was bound to PEG to form a hydrogel that facilitated the survival, the neuronal differentiation of NSCs, and the growth and extension of neurites [99].

The electrospinning technique by which materials are made into three-dimensional nanofiber scaffolds has been used in tissue engineering. NSCs were cultured on laminin-coated electrospun polyethersulfone (PES) fiber meshes. With the decrease of fiber diameter, the level of NSC proliferation and migration increased, while the degree of cell aggregation decreased. Moreover, the differentiation of NSCs was significantly affected by the diameter of fibers [100]. Three PHA materials were selected as nanofiber scaffolds for cell culture, including poly (3-hydroxybutyrate) (PHB), copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate (P3HB4HB), and copolymer of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx). These three nanofiber scaffolds supported the growth and differentiation of NSCs, in which PHBHHx showed the strongest ability to promote the proliferation and neuronal differentiation of NSCs [101]. Researchers immobilized BDNF on poly-ε-caprolactone (PCL) nanofibers, which could support NSC proliferation and differentiation into neurons and oligodendrocytes [102] (seen in Table 3).

Artificial biomaterial scaffolds cannot only fill the tissue defects, which is conducive to the attachment, migration, and growth of endogenous and exogenous NSCs, but also regulate the microenvironment around the lesion by controlled releasing active factors so as to achieve nerve regeneration. Tissue engineering combines materials with NSCs to provide a promising approach for solving the barrier of nerve regeneration in the CNS.

6. Cell Signaling Pathways

The intrinsic regulatory mechanisms of the four factors discussed above affecting the behavior of NSCs may be related to the cell signaling pathways. Growing evidence has suggested that the self-renewal and differentiation of NSCs are due to the common integration of multiple cell signaling systems in the microenvironment of the cells. Consequently, elucidating the regulatory mechanisms of NSCs is critical for the study about the development of the nerve system, the repair of injured nerves, and cell transplantation for the treatment of CNS diseases. Here, we mainly focused on the mechanism of the regulation of NSCs through the Wnt and the Notch signaling pathways.

6.1. Wnt Signaling Pathway. The Wnt signaling pathway is a highly conserved signaling pathway in the evolution of species, which plays a vital role in the early development, organogenesis, tissue regeneration, and other physiological processes of animal embryos. The main components of the Wnt/β-catenin signaling pathway include the secreted protein Wnt family, the transmembrane receptor Frizzled family, low-density lipoprotein receptor-related protein (LRP), Dishevelled (Dsh), glycogen synthase kinase3β (GSK3β), Axin, β-catenin, adenomatous polyposis coli protein (APC), and the transcription factor T cell factor/lymphoid enhancer factor (TCF/LEF) family. When the Wnt is in a resting state, β-catenin, GSK3β, APC, and Axin constitute a degraded complex which causes the phosphorylation of β-catenin, the ubiquitination mediated by β-TrCP, and the degradation by protease eventually. When the Wnt ligand binds to the Frizzled and LRP5/6, the Wnt signaling pathway is activated to inhibit the formation of degraded complexes, reduce the activity of GSK3β, decrease the degradation, and increase the aggregation of β-catenin. Then β-catenin enters the nucleus and binds to the transcription factor of the TCF/LEF family to initiate the transcription of downstream target genes such as *Cyclin D1*, *neurogenin-1* (*Ngn-1*), and *Ngn-2* [103].

Wnt7a is critical for self-renewal and neuronal differentiation of NSCs. In the hippocampal dentate gyrus of adult mice, a decrease in the expression of Wnt7a accelerated the withdrawal of NSCs from the cell cycle, and the proliferation of NSCs reduced significantly. Dramatically, the number of mature neurons differentiated by NSCs was also significantly reduced when the expression of Wnt7a was decreased. The study demonstrated that Wnt7a could regulate the expression of different downstream target genes in the transcriptional level to achieve the bidirectional regulation of NSC behavior. The activation of the Wnt7a/β-catenin-cyclinD1 pathway was able to stimulate NSC proliferation, and the activation of the Wnt7a/β-catenin-Ngn-2 pathway promoted neuronal differentiation [104] (seen in Figure 1(a)). At the same time, the overexpression of Wnt7a could increase the level of Ngn-1 mRNA and then induce the differentiation of NSCs into neurons in the cerebral cortex of mice [105] (seen in Figure 1(a)). Wnt3a has been reported to be involved in the survival, proliferation, and differentiation of NSCs through the Wnt/β-catenin pathway [106]. The feasibility

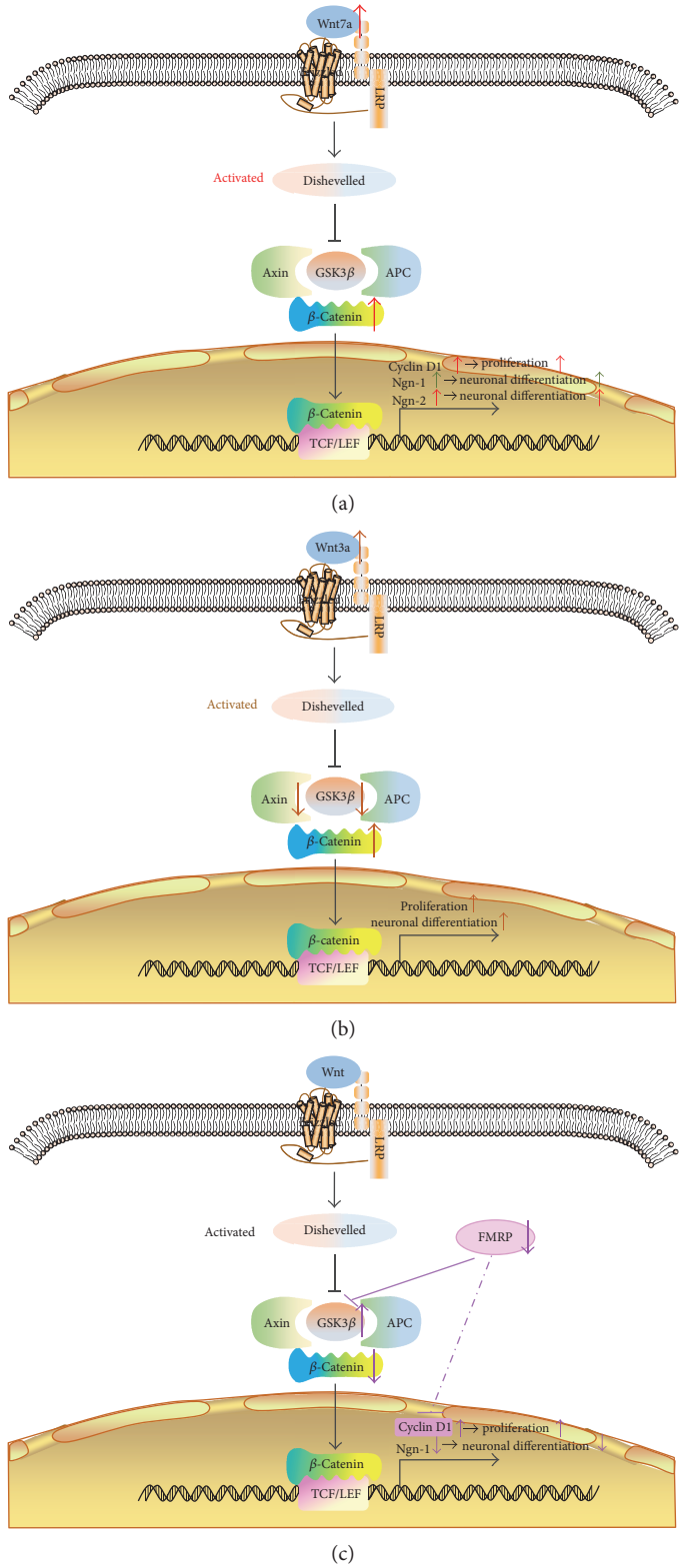

Figure 1: Effects of different proteins on the Wnt signaling pathway. (a) The increase in the expression of Wnt7a activated the Wnt7a/β-catenin-cyclinD1 pathway, which stimulated the proliferation of NSCs, and induced the Wnt7a/β-catenin-Ngn2 pathway, which promoted the neuronal differentiation (the red arrows). In the cerebral cortex of mice, the overexpression of Wnt7a could increase the level of Ngn-1 mRNA and then induce the differentiation of NSCs into neurons (the green arrows). (b) The overexpression of Wnt3a activated the Wnt signaling pathway and promoted the proliferation and neuronal differentiation of NSCs. (c) The loss of FMRP resulted in a decrease in the level of β-catenin, then downregulated the expression of *Ngn-1* and reduced neuronal differentiation.

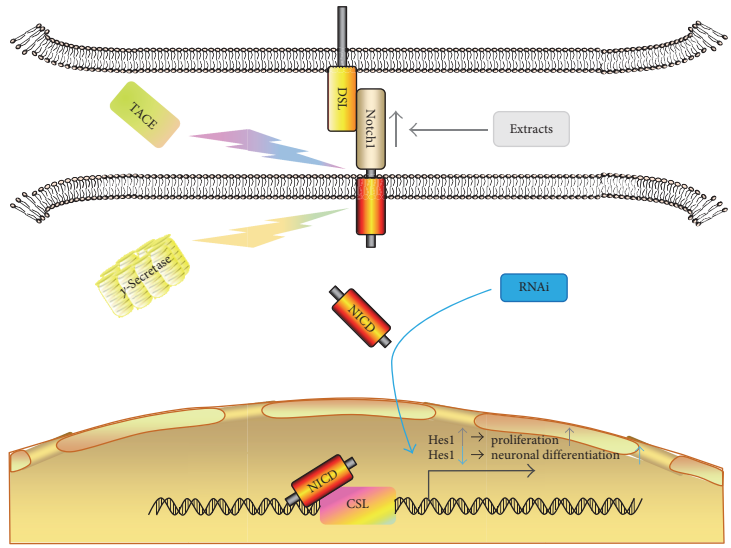

Figure 2: The upregulation and downregulation of *Hes1* in the Notch signaling pathway. After the TBI in mice, the RNAi downregulated the expression of *Hes1*, and the NSCs in the DG differentiated into neurons heavily (the blue arrows). The extracts from the injured spinal cord upregulated the expression of Notch1 and *Hes1*, activated the Notch signaling pathway, and promoted the proliferation of NSCs (the gray arrows).

of Wnt3a acting on NSCs to repair the retina has been investigated [107]. The overexpression of Wnt3a led to the instability of Axin and the inhibition of GSK3β activity, thereby activating the Wnt signaling pathway and ultimately promoting the proliferation and neuronal differentiation of NSCs (seen in Figure 1(b)). Fragile X mental retardation protein (FMRP) is a regulatory protein for hereditary mental retardation. The absence of FMRP caused a decrease in the level of β-catenin due to the disorder of GSK3β and then downregulated the expression of *Ngn-1* [108]. Ngn-1 was an initiator of early neuronal differentiation and an inhibitor of astrocyte differentiation [109, 110]. Eventually, the differentiation of neurons was reduced and the differentiation of glial cells was increased. In addition, the inhibition of NSC proliferation was weakened by the absence of FMRP (seen in Figure 1(c)).

6.2. Notch Signaling Pathway. The Notch signaling pathway is widespread and highly conserved in invertebrates and vertebrates. The core components of the classical Notch signaling pathway are mainly composed of Notch receptors (Notch1–4), Notch ligands (the Delta/Serrate/lag-2 protein (DSL), such as Jagged1, Jagged2, and Delta-like1–4), CSL (a class of DNA-binding proteins), and some regulatory molecules. When the Notch receptor binds to the ligand, the Notch intracellular domain (NICD) is released by the receptor after three times of shearing, then enters the nucleus to form a NICD/CSL transcriptional activator which activates the target genes of the basic-helix-loop-helix (bHLH) transcriptional repressor family [111]. Studies have shown that the Notch signaling pathway is one of the important signaling pathways that affect the self-renewal, differentiation, and internal stability of NSCs.

External factors mainly mediate the differentiation inhibitory signal through the "side inhibition" mechanism of the Notch signaling pathway, which prevents the differentiation of adjacent NSCs and promotes their proliferation. Thus, the Notch signaling pathway plays a key role in the self-renewal and maintenance of NSCs during the brain development. Some researchers reported that *Hes1* could promote the proliferation and inhibit the differentiation of NSCs [112, 113]. After the traumatic brain injury (TBI) in mice, the expression of *Hes1* was downregulated by RNA interference (RNAi). The NSCs in the dentate gyrus (DG) differentiated into neurons largely, which improved the spatial learning and memory ability of the mice and further restored the neurological function [114] (seen in Figure 2). The extracts from the injured spinal cord upregulated Notch1 mRNA, and the expression of *Hes1* subsequently activated the Notch signaling pathway to promote NSC proliferation [115] (seen in Figure 2). Nevertheless, silencing the expression of *Notch1* inhibited the division of NSCs and prevented NSCs from entering the cell cycle and maintaining self-renewal.

Several studies have demonstrated that the Wnt and Notch signaling pathways interact with each other during NSC differentiation. Wnt3a could upregulate the downstream target gene *Hes1* of the Notch signaling pathway and continue to inhibit the expression of *Hes5*, improve the level of *Mash1*, and then induce the proliferation of NSCs. The overexpression of *Hes5* inhibited the Wnt signaling pathway, downregulated *Mash1*, and induced neuronal differentiation [116] (seen in Figure 3(a)). Besides, when the *Ngn-2* expression dynamically oscillated, it was capable of inducing the expression of Delta-like1 (Dll1) in the adjacent cells and activating the Notch signaling pathway so that *Hes1* was upregulated to promote self-renewal of NSCs; when *Ngn-2* was expressed persistently, it inhibited the *Hes1* expression and promoted the differentiation of NSCs into neurons [117, 118] (seen in Figure 3(b)).

To sum up, the proliferation and differentiation of NSCs are involved in the coordination and integration of multiple

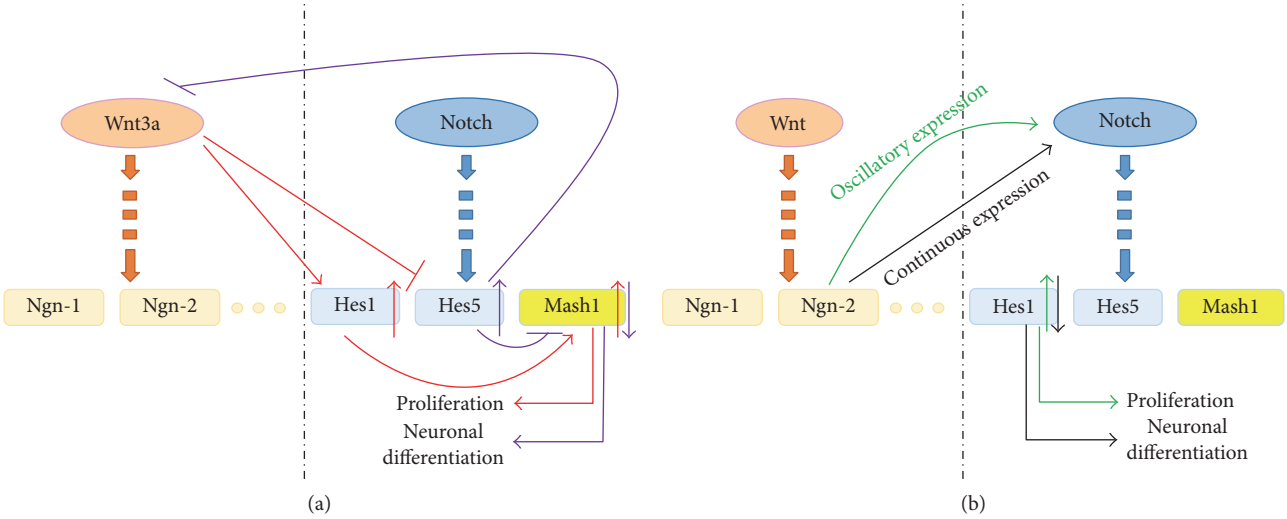

Figure 3: The interaction between the Wnt and the Notch signaling pathways. (a) Wnt3a could upregulate the expression of the downstream target gene *Hes1* of the Notch signaling pathway and inhibit the expression of *Hes5* and then induce the proliferation of NSCs (the red arrows). The overexpression of *Hes5* inhibited the Wnt signaling pathway, downregulated *Mash1*, and induced neuronal differentiation (the purple arrows). (b) The oscillatory expression of *Ngn-2* could activate the Notch signaling pathway so that *Hes1* was upregulated to promote the self-renewal of NSCs (the green arrows). The continuous expression of Ngn-2 inhibited the expression of *Hes1* and promoted the differentiation of NSCs into neurons (the black arrows).

cell signaling pathways. However, the precise regulation mechanism of NSCs is still not clear.

7. Conclusion

In this article, we mainly summarize the factors which have been widely studied in the last decade. These different factors have different effects on the behavior of NSCs which have become the candidate for repairing CNS injury due to their ability of self-renewal and multiple differentiation. Physical factors usually modulate the behavior of NSCs by controlling specific parameters and the sites of exertion. While chemical factors affect the behavior of NSCs according to the different molecular structures and properties of different chemical molecules, biological factors, such as neurotrophins, growth factors, and microRNAs, are involved in the endogenous regulation of the NSC proliferation and differentiation. Moreover, NSCs are also subject to exogenous regulation of the microenvironment which is constituted by adjacent cells and extracellular matrices (including various proteins). Materials provide the structural support for the injured sites, which forms a favorable microenvironment, thereby promoting the proliferation and differentiation of NSCs. It is interesting that the regulation of these four factors on the NSC behavior is closely related to the expression of related genes. Therefore, we believe that the cell signaling pathway is the underlying mechanism that regulates the NSC proliferation and differentiation by different factors.

Although significant progress has been made in the treatment of CNS diseases by using NSCs, there are still many problems that need to be addressed. For example, (1) the mechanism of precise regulation of NSCs after transplantation is still unclear; (2) there may be complications of transplantation; and (3) at present, most stem cell transplantation is only in the animal experimental stage and the application of the NSC transplantation in clinical still has a great risk. Researchers have gradually realized the existence of these problems. We believe that in the near future, these problems will be solved and NSCs will play a more and more important role in treating CNS diseases.

Conflicts of Interest

The authors declare no potential conflicts of interest.

Authors' Contributions

Lixiang Huang and Gan Wang contributed equally to this work.

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