Stem Cells International

Environmental Stimulus on Stem Cell Behaviour

Lead Guest Editor: Josef Buttigieg
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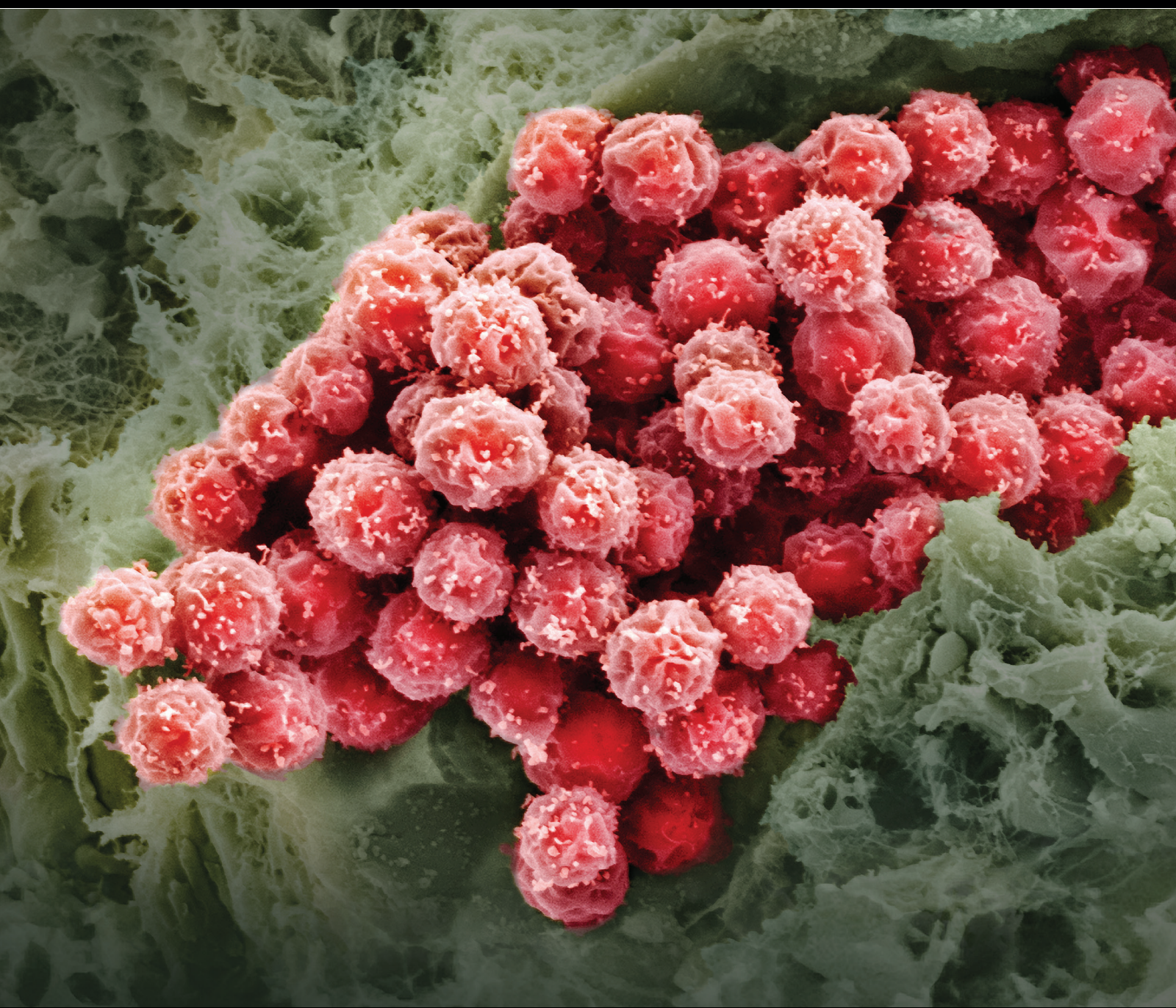

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Editorial Board

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Editorial

Environmental Stimulus on Stem Cell Behaviour

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Stem cells are unique in their ability of self-renewal as well as being able to differentiate into multiple cell types. Due to this unique physiology, stem cells are often studied in the context of regenerative medicine, as they can replace lost or damage cells, thus potentially reversing deficits encountered during disease or injury. Furthermore, stem cells play a unique ability during the development of different tissue systems. Pools of these cells serve to provide the progenitor cells required to differentiate into mature cells. Although there is much potential not only in regenerative medicine but also in terms of understanding, the development of various tissues, many key gaps exist in our understanding of stem cell physiology.

This special issue has the main goal of further advancing the field of stem cell biology by presenting research articles that examine not only stem cell biology but also the environment that they reside in. The environment surrounding stem cells is thought to play a key role in not only regulating their self-renewal process but also in influencing their differentiation fates. These environmental cues can arise from a variety of sources. Signalling molecules, both from autocrine and paracrine sources, can be derived from hormones, ATP, and even neurotransmitters.

In the living organism, stem cells reside in environments that experience significantly lower PO_2 values compared to the external atmospheric content. This fact, in addition to the special metabolic requirements of undifferentiated cells, likely plays a critical role in stem cell biology. Changes in PO_2 due to development or pathology can result in the activation of a variety of genes involved in both metabolism and development. Related to changes in PO_2 values is the production of reactive oxygen species (ROS). O_2 acts as the terminal electron acceptor in the mitochondrial electron transport chain. Due to changes in O_2 environment or inhibition of the ETC, there can be an increase or decrease in ROS production. Due to the extra electron, ROS can induce a variety of changes in the cell by altering gene transcription or even altering protein function.

It is our hope that these articles presented here will help not only answer current questions on stem cell biology but also guide future research.

Acknowledgments

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Eftekhar Eftekharpour
Josef Buttigieg

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Research Article

Molecular Genetic Analysis of Human Endometrial Mesenchymal Stem Cells That Survived Sublethal Heat Shock

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High temperature is a critical environmental and personal factor. Although heat shock is a well-studied biological phenomenon, hyperthermia response of stem cells is poorly understood. Previously, we demonstrated that sublethal heat shock induced premature senescence in human endometrial mesenchymal stem cells (eMSC). This study aimed to investigate the fate of eMSC-survived sublethal heat shock (SHS) with special emphasis on their genetic stability and possible malignant transformation using methods of classic and molecular karyotyping, next-generation sequencing, and transcriptome functional analysis. G-banding revealed random chromosome breakages and aneuploidy in the SHS-treated eMSC. Molecular karyotyping found no genomic imbalance in these cells. Gene module and protein interaction network analysis of mRNA sequencing data showed that compared to untreated cells, SHS-survived progeny revealed some difference in gene expression. However, no hallmarks of cancer were found. Our data identified downregulation of oncogenic signaling, upregulation of tumor-suppressing and prosenescence signaling, induction of mismatch, and excision DNA repair. The common feature of heated eMSC is the silence of MYC, AKT1/PKB oncogenes, and hTERT telomerase. Overall, our data indicate that despite genetic instability, SHS-survived eMSC do not undergo transformation. After long-term cultivation, these cells like their unheated counterparts enter replicative senescence and die.

1. Introduction

Mesenchymal stem cells (MSC) are self-renewing multipotent cells, which hold a great potential in regenerative medicine and tissue engineering reflected by more than 500 MSC-based clinical trials registered with the NIH [1, 2]. MSC were isolated from multiple sources, such as bone marrow, adipose tissue, blood vessel walls, peripheral and umbilical cord blood, Wharton's jelly, and Fallopian tubes.

Currently, the MSC of endometrium (eMSC) attract growing attention. Comparing with other MSC types, eMSC show a higher vasculogenic and anti-inflammatory potential [3]. These valuable features are associated with a special role of eMSC in every month endometrium growth [3]. Cultured eMSC are being applied in clinical trials, and encouraging results have been reported [4].

Typically, to accumulate clinically relevant cell mass, isolated stem cells should be expanded in vitro. An important point to consider is the genetic stability of stem cells during long-term cultivation. Genome stability ensures oncogenic safety and is a crucial risk factor in stem cell-based therapies. However, the literature data concerning the genome maintenance during prolonged cultivation of human MSC are ambiguous. Mounting evidence indicates that long-term MSC expansion may be accompanied with an occurrence of chromosomal abnormalities [5, 6]. It is well known that

chromosome abnormalities boost the tumor development. However, MSC even with chromosomal alterations showed progressive growth arrest and entered replicative senescence during prolonged cultivation. Currently, there are no studies reporting transformation of human MSC during long-term culturing in vitro even despite genome instability [5, 6]. Some papers on spontaneous malignant transformation [7, 8] were later retracted from publication. Using DNA fingerprinting and/or short tandem repeat analysis to compare "transformed" and normal MSC, it was found that in reality, "transformed" cells were crosscontaminated with cells of various permanent cell lines [9]. Nonetheless, some authors suggest that spontaneous malignant transformation of human MSC is not completely excluded [10]. Long-term cultivation of bone marrow- and liver-derived MSC produced transformed cells with tumorigenic potential. High-resolution genome-wide DNA array and short tandem repeat profiling confirmed a shared origin of the transformed cells and parental MSC [10]. The results of this publication have not yet been confirmed.

Stress response of stem cells is under active investigation [11]. However, the fate of stem cells cultivated after exposure to damaging factors is poorly monitored. Hyperthermia is an important ecological and therapeutic factor. Heat shock (HS) response has been studied for decades. The research was mainly focused on the expression of heat shock proteins (HSP) and heat shock factors (HSF) as well as their involvement in the regulation of various cellular functions. Traditionally, it was considered as a nonmutagen, that is, the agent not inducing DNA damage. Recently, it becomes clear that HS induces DNA damage and affects DNA integrity [12]. It was reported that HS-induced chromosomal instability in cancer cells [13]. HS response of human MSC is badly studied. Usually, HS is considered as an inductor of apoptosis or necrosis. The results of our studies demonstrated that eMSC unlike embryonic stem cells responded to sublethal temperature by stress-induced premature senescence (SIPS) [14, 15]. Treated cells exhibited gamma-H2AX-foci. It points to DNA damage as the appearance of gamma-H2AX-foci is a marker for DNA double-strand breaks.

The ability of HS to generate DNA damage makes it a convenient tool for the investigation of safety margins related to eMSC genome instability and transformation. To be appropriate for preclinical and clinical trials, a biological product (eMSC in our case) should have wide safety margins [16]. Since MSC transplantation is often accompanied by inflammatory processes triggering fever and hyperthermia in patients, we aimed to investigate genomic stability and possibility of malignant transformation in eMSC that stayed alive after SHS. SHS was induced at 45°C during 30 minutes. This particular design of treatment was chosen because in contrast to cells in vivo that can live maximum at 41-42°C [17], cultured cells may have maximal temperature threshold at 45.5°C [14]. It should be noticed that the minimum temperature producing a burn is 44°C but injured tissues are able to recover. In this study, we used methods of classic and molecular karyotyping, next-generation sequencing, and transcriptome functional analysis. Also, we compared our results with published data on gene expression changes in hTERT-transformed human bone marrow mesenchymal stem cells [18].

2. Materials and Methods

2.1. Cells and Treatment. The study was performed on eMSC derived from desquamated endometrium in the menstrual blood [19]. The cells were maintained in DMEM/F12 medium (Gibco, United States) with 10% bovine fetal serum (HyClone, United States), 1% antibiotic-antimycotic solution, and 1% GlutaMAXTM (Gibco, United States). The cells were subcultured 1 : 3 twice a week using 0.05% trypsin with EDTA (Invitrogen, United States).

2.2. HS Conditions. eMSC exposed to SHS were plated in 3 cm Petri dishes at density 10^5 cells/plate. The next day, dishes sealed with Parafilm were submerged into water bath at 45°C for 30 min and then returned to 37°C in CO_2 incubator. Survived cells (about 3×10^4 cells) were plated in a 3 cm Petri dish after 3 days. Upon reaching the monolayer, these cells were subcultured under the same conditions as intact cells. At the 6th passage after HS, the cells were analyzed using classic and molecular karyotyping, next-generation sequencing, and transcriptome and functional analysis.

2.3. G-Banded Karyotyping. The cells were seeded with a density of $14\text{-}15 \times 10^3$ cells/cm^2. Mitostatic agent colcemid (Sigma, United States) in dose 30 μg/mL was added after 24-25 h for 1 h. Then the medium was removed and the cells were harvested with 0.05% trypsin and centrifuged. The pellet was suspended and treated with 0.56% KCl hypotonic solution for about 1 h. The cell suspension was centrifuged (1300 rpm), resuspended, and fixed on ice by methanol mixed with acetic acid in the ratio 3 : 1. The fixative was changed three times, the total fixation time being 1.5 h. The fixed material was dropped onto cold and wet slides. The slides were air-dried for 1 week. Chromosomes were G-banded with Giemsa stain (Fluka, United States) after preliminary trypsinization. Metaphase plates with well-spread chromosomes were assayed under an Axio Scope microscope (Carl Zeiss, Germany). The chromosomes were identified according to the international nomenclature [20]. A total of 65 metaphases (31 of intact and 34 of SHS-survived eMSC) were analyzed.

2.4. Molecular Karyotyping. It was carried out in Genoanalitica (Moscow, Russia) using HumanCytoSNP-12 kit (Illumina®, United States) according to the manufacturer's recommendations. They were seeded with a density of 3×105 cells per 6 cm plate and lysed in 72 h for DNA isolation. DNA preparation, hybridization, washing, staining, and sample scanning were done according to the standard protocols of the company Illumina. The samples were hybridized on high-density oligonucleotide arrays with 3×105 isothermic probe-covered unique gene and intergenic areas of the human genome. The scanning was done with iScan (Illumina, United States). The results were analyzed with Genome Studio Genotyping Module and BlueFuse software (Illumina, United States).

2.5. SA-β-Gal Assay. Cell staining for β-galactosidase (β-gal) activity was performed using Senescence β-Galactosidase Staining Kit (Cell Signaling Technology) according to the manufacturer's protocol and quantified microscopically by counting X-gal-positive cells among not less 500 cells.

2.6. Next-Generation Sequencing. Sample preparation for NGS and sequencing on the Illumina platform were performed in Genotek company (Moscow, Russia). RNA was extracted using Pure Link RNA Mini Kit (AMBION, Life Technologies). After that, mRNA was extracted from total RNA using magnetic beads (Sileks). cDNA libraries were prepared using NEBNext® mRNA Library Prep Reagent Set for Illumina (New England Biolabs). In this approach, mRNA was fragmented, cDNA was synthesized, end repaired, and ligated to unique sequencing adaptors to form cDNA libraries. Dual indexing was performed by PCR with NEBNext Multiplex Oligos for Illumina (dual index primers set 1). Quality control of prepared libraries was made using Bioanalyzer 2100 (Agilent Technologies). Sequencing of cDNA libraries was done on an HiSeq2500 (Illumina) in rapid run mode with read length 100 nt.

Next-generation sequencing was done by parallel measurement of three biological samples both for control and SHS-treated eMSC. The NGS reads were trimmed using trimmomatic software specially developed for Illumina NGS data, with default parameters [21]. The trimmed reads were mapped to canonical nonredundant human transcriptome presented in RefSeq database [22] using Bowtie 2 software [23, 24]. This aligner became a de facto standard within mapping pipelines and shows a remarkable tolerance both to sequencing errors and indels [25]. Bowtie 2 was used with the "very sensitive" preset of parameters, which allows the most sensitive and accurate mapping (at the expense of speed). Only the nonambiguous mappings were counted.

The differential expression was determined as in the previous work [26]. The obtained counts were analyzed using the "limma" package (implemented in *R* environment) specially developed for whole-transcriptome analyses of differentially expressed genes [27]. Comparison of different software packages showed that limma is the method of choice for our goals [28]. Taking into account recommendation in limma manual, genes with counts below 10 in all probes were discarded. This procedure gave 10,880 genes for analysis of differential gene expression. The data normalization methods presented in limma (quantile, scale) were tested as well as the trimmed mean method from the edgeR package [29]. The results were similar. The results obtained with quantile normalization are shown.

2.7. Gene Module Analysis. The biological processes and molecular pathways enriched in differentially expressed genes were found similar to previous works [30, 31]. The biological processes were taken from GO database [32]. For each GO category (process), all its subcategories were collected using GO acyclic directed graphs, and a gene was regarded as belonging to a given category if it was mapped to any of its subcategories. As a source of molecular pathways, the NCBI BioSystem was used, which is the most complete compendium of molecular pathways from different databases [33]. The redundancy was removed by uniting entries with identical gene sets.

The hypergeometric distribution of probability (implemented in *R* environment) was used for determination of statistical significance of observed-to-expected ratios of gene numbers in different biological processes or molecular pathways in the gene samples (as compared to total gene set). The contrast test was used for analysis of gene expression folds. In this test, the mean expression fold of genes belonging to each process/pathway is compared with the mean fold of the total gene set. For evaluation of two-tailed statistical significance of an obtained contrast between these folds, 20,000 random samplings were taken from total gene set (of a size equal to the number of genes in a process/pathway). This method is preferable to parametric or nonparametric tests because normal distribution that is required for parametric tests is usually absent, whereas nonparametric tests can lose a considerable amount of information. The random-sampling test is distribution independent and retains all information. In both hypergeometric test and contrast test, the adjustment of obtained p values for multiple comparisons was done according to the method by Storey and Tibshirani [34]. This procedure gives q value, which can be considered as p value corrected for multiple tests.

2.8. Protein-Protein Interaction Network Construction and Analysis. The protein-protein interactions (PPI) were taken from the STRING database [35]. We choose this database because STRING places its focus on functional relationship between two proteins, contributing to a common biological purpose and contains interactions from multiple sources: experimental interactions imported from primary databases, pathways from manually curated databases, and statistical and semantic links between proteins, obtained from Medline abstracts and a large collection of articles [35].

The PPI networks were visualized also using the STRING server. We analyzed dense-connected components of protein-protein interaction networks for proteins encoded by genes demonstrating expression difference between SHS-survived and control eMSC by more than 8-folds (the top one-quarter of genes with the most differing expression). The induced and inhibited proteins were analyzed separately.

2.9. The Identification of Hub Proteins That Are Causal Regulators in Modular and Network Organization. Protein interaction network consists of nodes and edges, where each node stands for a protein and the edges represent interactions [36]. The number of edges per node characterizing the number of interacting proteins is termed a degree. Nodes with the highest degree are defined as hubs [37]. The degree is a fundamental parameter that is usually adopted to evaluate the nodes in a network for the identification of evolutionary conserved causal regulators in modular organization and networks [38, 39]. As recommended by Han et al. [40], nodes with degree greater than 5 were labeled as hubs.

2.10. PPI Network and Cluster Analysis. Biological networks are composed by subnetworks implicated in various

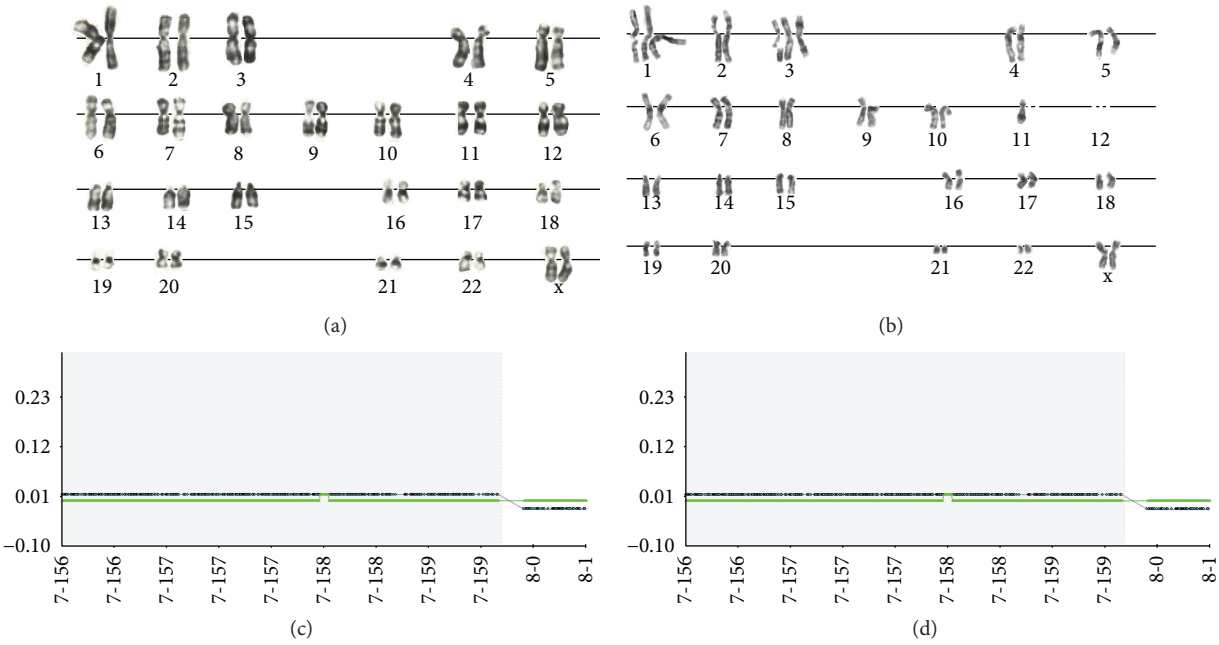


Figure 1: Karyotype analysis of eMSC. (a) G-banded karyotype of intact eMSC. (b) G-banded karyotype of SHS-survived eMSC. The picture illustrates trisomy and near-centromere breakage of chromosomes 1, 3, monosomy of chromosomes 11; absence of 2 homologs of chromosome 12 is seen in cells after SHS. (c) Molecular karyotyping of intact eMSC; (d) Molecular karyotyping of SHS-survived eMSC; *abscissa*: length of chromosome 7 in genome, rel. units; *ordinate*: logarithm of signal intensity, rel. units; *arrow* shows duplication in chromosomes 7, locus 7q36.3, and size 62 kB; *dots on black solid line*: deviations in each single base-pair difference in the DNA sequence according to the signal intensity; *green solid line*: deviations in single base-pair difference in the DNA sequence from the norm with signal intensity combined with genotype.

biological processes. To identify these subnetworks, we applied K-means clustering using the STRING sever. After the clustering, function annotation of clusters was made. The function annotation included GO (gene ontology) analysis and KEGG (Kyoto encyclopedia of genes and genomes) pathway analysis. The Benjamini method was used to control the false discovery rate (FDR) to correct the p value.

3. Results and Discussion

3.1. SHS Triggers Chromosome Instability in eMSC. In this study, we applied HS induction at 45°C during 30 minutes. This temperature is used in clinical practice for local and regional hyperthermia [41]. Clinical hyperthermia involves temperature elevation in the range of 39–45°C. Temperatures above 45°C sustained for more than several minutes usually lead to protein denaturation and cell death typically via necrosis. It is known that mesenchymal stem cells are present in many tissues of an adult organism and thus heating can lead to cell damage. This design of heating was used in a large number of studies since this treatment is sublethal and effectively induces a cellular response to heat stress [12, 14, 15]. Our previous studies performed with the same SHS induction design demonstrated that SHS triggered premature senescence of eMSC in culture. It was found that SHS-induced DNA damage response displayed in appearance of gamma-H2AX-foci. Nevertheless, 35% of cells were alive after SHS. Survived cells maintained key stem cell properties, including self-renewal features, CD marker expression pattern, and differentiation capacity [15]. Thus, SHS model "45°C during 30 minutes" is a very convenient one for the evaluation of tumorigenic potential with wide safety margins.

In this study, we assessed chromosome stability in eMSC that survived SHS using G-banding and molecular karyotyping. G-banding of these cells revealed random structural and functional chromosome abnormalities (aneuploidy, chromosome breakages) (Figure 1(b)). Control eMSC maintained the normal karyotype (Figure 1(a)). In contrast to G-banding, the results of molecular karyotyping performed with microarrays did not reveal the difference between intact and SHS-treated eMSC cells. The analysis identified in both intact and SHS-survived cell small duplicated fragment (62 kB) in the chromosome 7 (7q36.3) signifying that the defect is of the donor origin (Figures 1(c) and 1(d)). The comparison of the data obtained by G-banding and molecular karyotyping suggests that although hyperthermia disturbs karyotype structure, stress-related chromosome rearrangements are random. Karyotype destabilization was observed in immortalized Chinese hamster fibroblasts after long-term cultivation under mild hyperthermia [42] as well as in mouse embryonal carcinoma exposed to severe heat stress [13].

The inconsistency between G-banding and molecular karyotyping may originate from their different focuses. G-banding visualizes only large-scale genetic instability, including aneuploidy, chromosome breakages, and chromosome rearrangements (deletions and insertions) that involve more than 5 Mb [43]. The advantage of this method is that it works

with high accuracy even at the level of one cell [44]. Also, G-banding still is the only tool for the detection of balanced rearrangements (i.e., that do not change gene dosage balance) and low-level mosaicism, both of which are not detectable by molecular karyotyping [45]. Molecular karyotyping or "chromosomal microarray" evaluates both large- and small-scale rearrangements covering 0.5–1.0 Mb, including single-nucleotide polymorphism (SNP) and gene copy number variations. However, clinical sensitivity of molecular karyotyping depends on the proportion of potentially pathogenic rearrangements because it reveals pathogenic changes only if they occur in no less than 10% of all analyzed cells [44].

In our study, the abnormalities detected by molecular karyotyping were not detected by G-banding because of their small size. For example, the loss of heterozygosity, identified by molecular karyotyping, was not detected by G-banding. In turn, the failure of the identification of chromosome breakages by molecular karyotyping may originate from the occurrence of breakages at different chromosomal loci and from the preservation of the amount of genetic material. Also, molecular karyotyping did not confirm trisomy and monosomy because both abnormalities were observed in the number of cells less than the threshold sensitivity of the molecular karyotyping method. Thus, a comparison of the data of G-banding and molecular karyotyping provided a detailed description of eMSC karyotype at various levels of genome organization.

Molecular mechanisms of hyperthermia-related chromosome instability have been little investigated. Currently, we know that SHS response is rapid and dynamic and results in induction of several hundred and repression of several thousand genes [46]. The master regulators of SHS response are transcription regulator HSF and chaperones of HSP family. It was recently shown that stressful conditions impair the ability of these regulators to appropriately coordinate pathways of DNA repair, cytoskeleton maintenance, and chromosome segregation [47–49]. As a result, HSF1 and HSP90 function impairment may promote genetic instability [49].

Karyotypic changes are closely linked with the cancer development and tumor progression [50]. Recent research highlighted that cancer risk is heavily influenced by extrinsic factors [51]. Chromosomal rearrangements observed in SHS-treated cells pose the risk of their immortalization and malignancy. To investigate tumorigenic potential of these cells, we performed the entire transcriptome sequencing and bioinformatic analysis with particular attention to transformation-related traits.

3.2. SHS-Survived eMSC Transcriptomic Landscape. To obtain a genome-wide picture of gene activity difference in SHS-survived versus control eMSC, we performed NGS transcriptome sequencing and bioinformatic analysis of enriched molecular pathways for differentially expressed genes. A total of 8724 protein-coding genes were identified as differentially expressed at 2-fold threshold. Approximately, similar amounts of genes were induced or inhibited. 758 genes were upregulated and 729 genes were downregulated by more than 10-folds, whereas 4514 genes were upregulated and 4210 were downregulated by more than 2-folds. We did not reveal differential expression of the modules related to HS response coordinated by HSP proteins and HSF transcription factors.

Although fold change analysis of differentially expressed genes is the most commonly used approach for the identification of specific biological traits and potential biomarkers, this approach can overlook biologically meaningful molecules without large fold change such as transcription factors operating commonly at 20–150% expression difference [52]. To overcome this difficulty, we perform gene module analysis. For this propose, we selected GO categories and BioSystem pathways enriched for differentially expressed genes with high significance ($q < 0.10$). The selection yielded more than 100 gene modules (Figure 2). Surprisingly, about 85% of these modules were induced and only 15% were inhibited. The inconsistency between the numbers of activated and inhibited genes and the numbers of activated and inhibited gene modules (about 50% versus 50% and 85% versus 15%) suggests that the whole gene modules were activated in SHS-survived cells, whereas the inhibited genes were scattered among many modules achieving a level of significant enrichment only in a few of them.

The examination of functional distribution indicated that the induced modules related mostly to energy metabolism, transcription, mRNA splicing and processing, protein turnover, cell cycle, DNA repair, E-cadherin signaling, senescence, and tumor. The activation of modules related to energy metabolism, transcription, and protein turnover suggests that SHS-survived eMSC exhibited activated gene expression. The induction of modules implicated in the regulation of the first half of cell cycle may originate from unscheduled DNA synthesis that usually accompanies genome instability and manifestations of senescence [53]. The inhibited gene modules related lymphocyte mediated immunity involved in inflammation and cell migration (Figure 2) suggesting that SHS eMSC demonstrate better anti-inflammatory and stress-protecting properties compared to untreated cells.

The predominant induction of gene modules motivated us to investigate complex interplay between separate genes and functional modules using protein-protein interaction (PPI) network analysis. It is well established that compared to differential expressions of separate proteins and gene module analysis, PPI networks demonstrate better match between datasets and have an ability to provide more comprehensive insight to the data [54]. Also, in contrast to canonical pathways covering only a fraction of the true protein-protein interactions that occur within a cell, the networks can be constructed from extensive experimental data, the literature, and public databases of molecular interactions [55, 56].

To obtain detailed PPI data, we constructed separate networks for genes that were induced or inhibited in SHS-survived versus control eMSC by more than 8-folds. For this purpose, we took interactions with the highest confidence ($S > 0.9$, Figure 3(a)) and extracted the whole connected components. The network of induced genes contained larger clusters and more connections compared to the network of the inhibited genes (Figures 3(a) and 3(b)), confirming a modular character of transcriptome activation

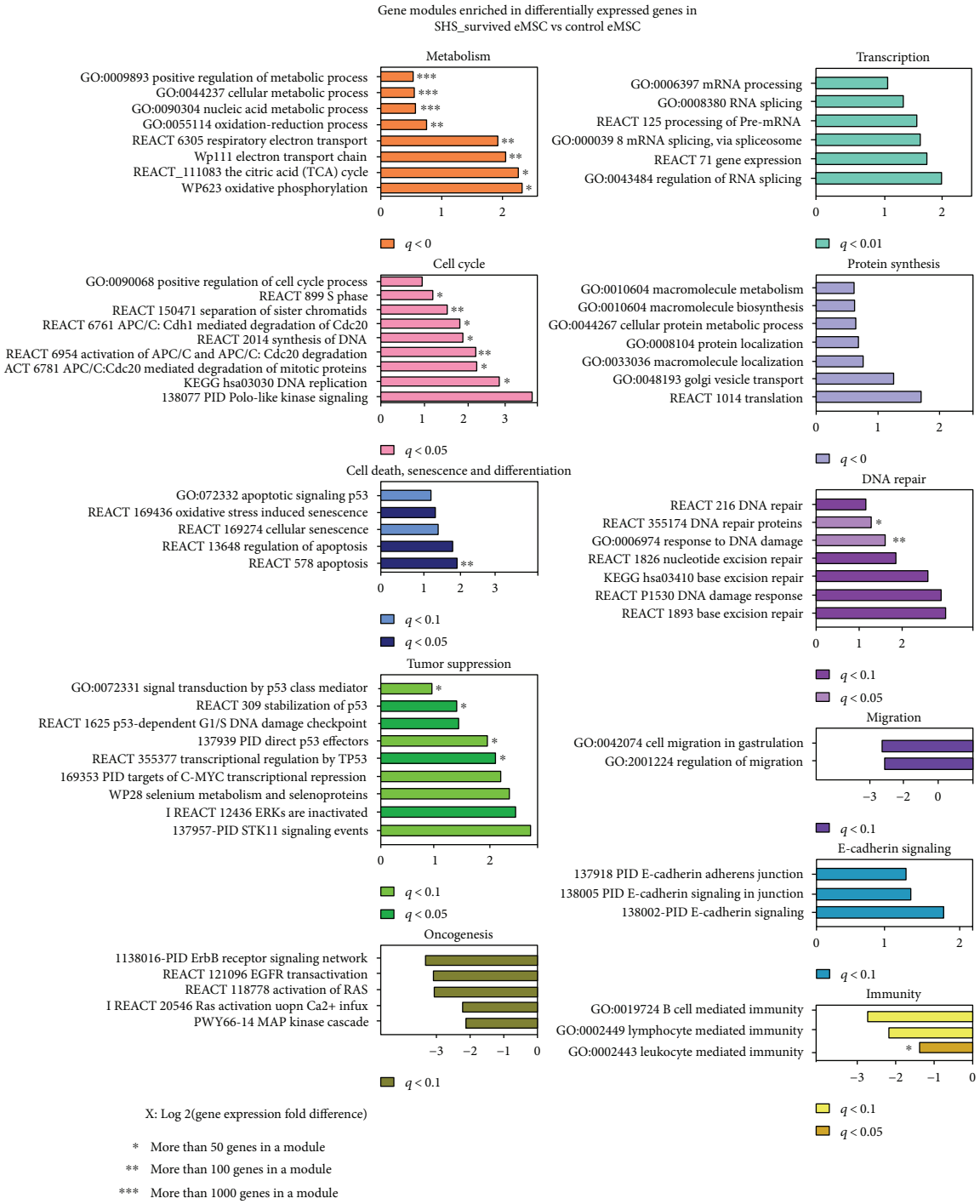


Figure 2: Gene modules that are induced or repressed in SHS-survived versus control eMSC with high significance ($q < 0.1$). This figure illustrates general metabolic activation in SHS-survived versus untreated eMSC seen from the upregulation of modules related to energy metabolism, cell cycle, protein turnover, DNA repair, and transcription. The figure shows increased tumor suppression that is evident from the upregulation of TP53 signaling, the induction of the module "targets of MYC transcriptional repression" uncovering the mechanism of Myc suppression in eMSC cells and from the decreased activity of oncogenic pathways.

in SHS-survived cells compared to control. Also, in accordance with the analysis of GO categories and BioSystem pathways, the clustering analysis with cluster functional characterization by gene module enrichment analysis using STRING database revealed many clusters. The main clusters were related to cell cycle (G1-S transition, S-phase, and metaphase), growth, protein synthesis, transcription, DNA repair, apoptosis, and tumor suppression in the induced network. The clusters related to signaling by oncogenes of RAS, MAPK, EGFR, JUN families, DNA damage checkpoint (ATM, ATR, BRCA1, and CHEK1), as well as olfactory and adrenal receptors, and negative metabolism regulation were

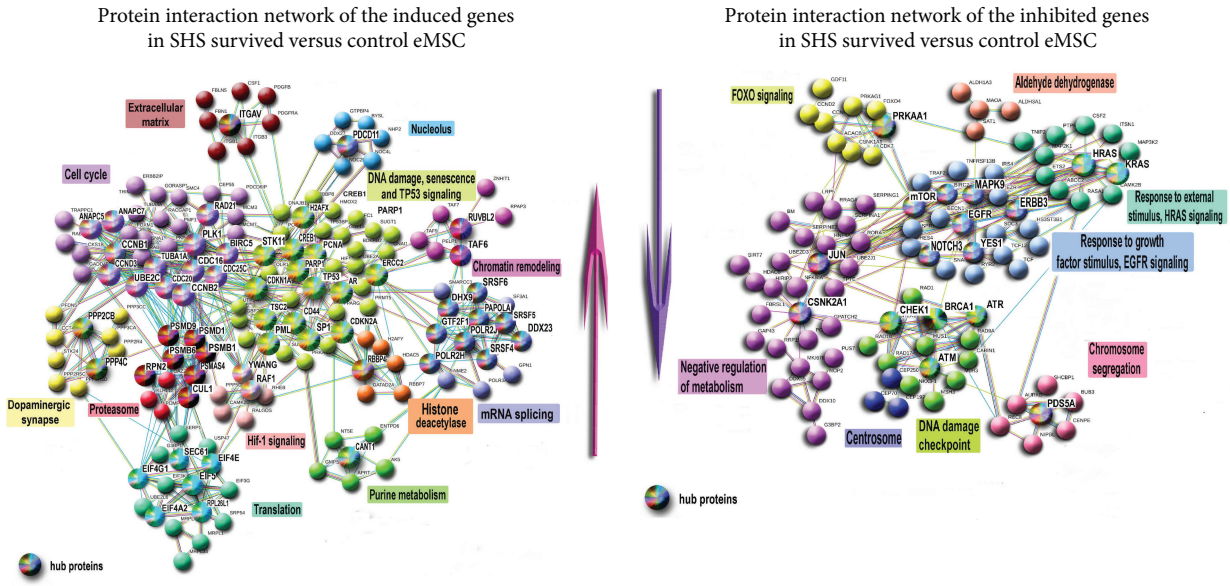


Figure 3: PPI networks for the most induced and inhibited genes in SHS-survived versus untreated eMSC. (a) PPI network for the induced genes. The network contains clusters related to cell cycle, proliferation, transcription, translation, and chromatin remodeling. It shows that SHS-survived eMSC have more active transcriptome than untreated cells. Large TP53-regulated gene cluster with many hub proteins related to tumor suppression (TP53, STK11, CDKN1a, CDKN2A, TSC2) and excision DNA repair (PARP1, SP1, PML and ERCC2) indicates that SHS-survived eMSC trigger stress protection. (b) PPI network for the inhibited genes-B containing gene cluster involved in DNA damage checkpoint coordinated by ATM, ATR, BRCA1, CHK1, and gene cluster related to chromosome segregation demonstrates DNA instability in SHS-survived eMSC. Two gene clusters related to HRAS and growth factor signaling regulated by HRAS and KRAS oncogenes and YES1, EGFR1, ERBB3, and mTOR growth factors point to the weakened prooncogenic signaling. Overall, these data are in good agreement with chromosome instability in SHS-survived cells. The network was constructed using STRING server at interaction confidence > 0.9. Clusterization was done with K-means clustering. Hub proteins, that is, the ones having more than 5 connections, are marked with multicolor large buttons; node proteins are indicated with plain small buttons.

found in the inhibited network (Figure 3(b)). Notably, the decreased activity of DNA damage checkpoint proteins may trigger unscheduled initiation of DNA replication [53]. Overall, the data of network analysis are in good agreement with the data of gene module examination and indicate that SHS-survived cells differ from control cells by gene expression and metabolic activation.

3.3. Transcriptome-Wide Analysis of Transformation-Related Features

3.3.1. CD Marker Expression Pattern. One of the most important questions of studies concerning stem cell research and implications in regenerative medicine is whether these cells can provide safety from oncogenic transformation. To investigate transformation-related features, we first examined whether HS-survived eMSC maintain appropriate CD marker expression pattern, that is, express mesenchymal stem cell-specific multipotent CD markers (CD13, CD29, CD44, CD73, CD90, and CD105) and do not express hematopoietic CD markers (CD34 and CD45) [18]. From the results of NGS data analysis, SHS-survived eMSC increased expression of CD13 (ANPEP) (2.3 folds), CD29 (ITGB1) (2.6 folds), CD44 (by 2.4 folds), and CD73 (NT5E) (2.8 folds) did not change the expression of CD90 (THY1) and CD105 (ENG) and decreased the expression of hematopoietic markers CD34 (SPN) (by 3.6 folds) and CD45 (PTPRC) (by 2.1 folds) compared to control (see Figures 4(a) and 4(b)) suggesting that these cells have normal phenotype inherent for nontransformed eMSC. This result is in good agreement with our previous data obtained by the method of immunophenotyping with eMSC after the same treatment [15]. Figure 4(a) illustrates protein interaction network for these CD markers indicating that all of them (with the exception of CD90 (THY1)) are interconnected by tight functional links. However, this encouraging result still does not guarantee safety. Recent studies indicated that human mesenchymal stem cells of bone marrow that were experimentally transformed with hTERT continued to express the normal CD pattern [18].

Therefore, to further examine manifestation of cancer-related features in SHS-survived eMSC compared to control, we checked our data according to the criteria of Hanahan-Weinberg describing the hallmarks of cancer [57]. These criteria include (1) increased and sustainable proliferation unbalanced by senescence and apoptosis, (2) growth suppressor inhibition, (3) cell death resistance, (4) replicative immortality, (5) angiogenesis, and (6) invasion and metastasis. It is worth noting that currently, Hanahan and Weinberg's work has more than 11,000 citations and thus can be considered as a classical blueprint for identification of transformation-related traits.

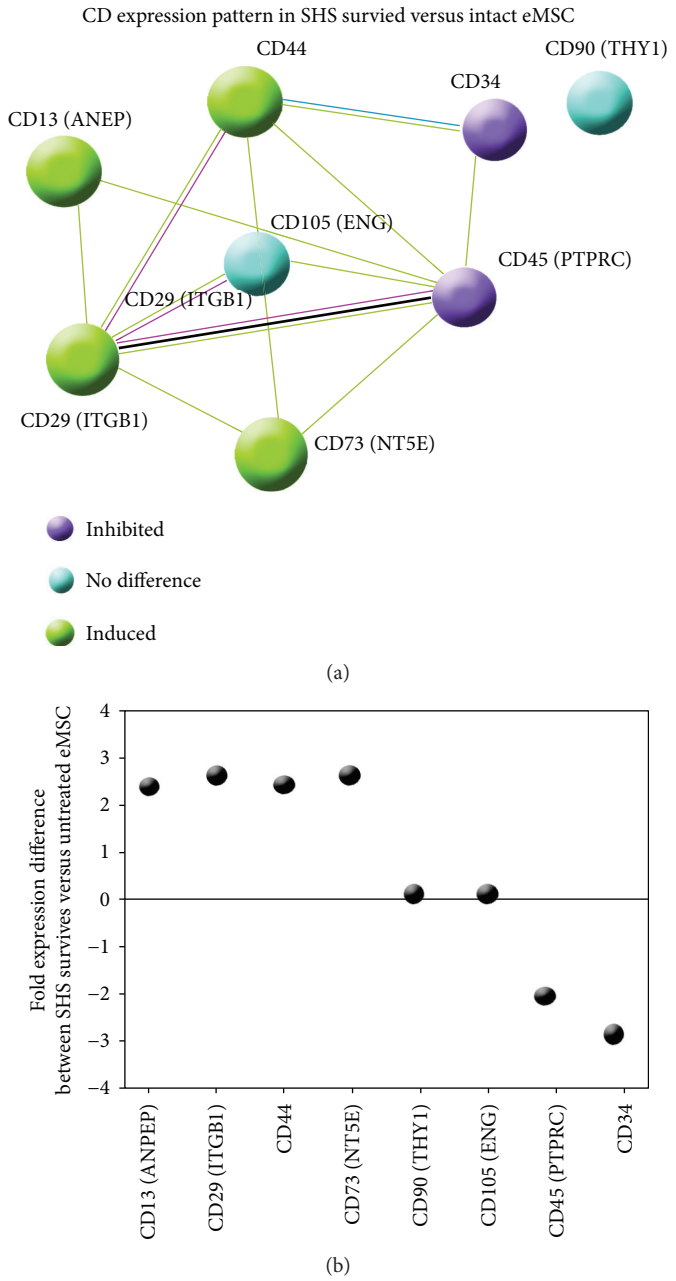


FIGURE 4: CD marker expression pattern in SHS-survived eMSC. (a) PPI network illustrating interactions between CD markers at interaction stringency $S = 0.7$ and the network constructed with STRING server. (b) Expression difference of CD markers between SHS-survived versus intact eMSC.

To characterize the data with regards to Hanahan-Weinberg criteria, we first revealed enriched GO categories and BioSystem pathways for 8724 genes (differentially expressed at 2.0-fold threshold) and searched among these modules the ones related to cell cycle, proliferation, growth, pluripotency, multipotency, cell senescence, apoptosis, oncogenes, tumor suppression, telomere extension and maintenance, angiogenesis, metastasis, and epithelial to mesenchymal transition (EMT). Significance levels were set at $p < 0.01$ and $q < 0.15$ (Supplementary Table 2). These thresholds were chosen on the ground of recommendations of GSEA group and other authors [58, 59]. Also, we performed a detailed functional analysis of genes unified by clusters in protein interaction networks (Figures 4(a) and 4(b)). Below, we provide a brief description of the obtained results.

3.4. Proliferation-Senescence Axis Characterizing the Hallmark "Ability to Maintain Sustainable Cell Proliferation Unbalanced by Senescence and Apoptosis." One of the most fundamental hallmarks of transformed cells is their ability to sustain proliferation by uncontrollable release of growth and proliferation-promoting signals, which is followed by the disruption of signaling attenuating proliferation [57].

To evaluate the activity of proliferation-related gene modules, we selected GO categories and BioSystem pathways enriched for differentially expressed genes containing in their title terms related to proliferation and cell cycle (Figure 2, Supplementary Table 2). Compared to control, SHS-survived eMSC showed increased activity of modules coordinating the first half of cell cycle including G1-S transition, S-phase, DNA replication, chromosome segregation, and cell cycle regulation (Figure 2, Supplementary Table 1). Also, the examination revealed several fold increase of APC/CDC20 and PLK1 signaling. Deregulation of these pathways may trigger aneuploidy by means of premature chromosome separation [60, 61].

The PPI network consisting of induced genes (Figure 3(a)) contained a large cell cycle-related cluster with hubs and nodes related to S-phase (CCNB1 and CCNB2, CCND3, CDC16, MCM3, MCM7, and CDC25) and chromosome separation (PLK1, ANAPC5, 7, CDC20, and TUBA1A). Consistently, the network of downregulated genes contained a cluster of regulators of sister chromatid cohesion containing PDS5A, CENPE, BUB3, and AURKB (Figure 3(b)). Thus, the gene module and protein interaction network analysis points to inappropriate chromosome segregation and confirm the results of G-banding revealed aneuploidy in SHS-survived cells.

To find out whether the induction of cell cycle-related modules was compensated by processes implicated in senescence and cell death, we selected the modules containing terms "senescence," "aging," and "cell death." The analysis identified several induced modules related to senescence and a substantial amount of modules implicated in TP53 signaling (Figure 2, Supplementary Table 1).

The PPI network of induced genes related to senescence contained a big cluster with TP53 protein as a hub and important regulators related to TP53 stabilization, and aging (CDKN2A (p16), CDKN2AIP (p16 interacting protein), and CDKN1A (p21)) as targets (Figure 3(a)). No modules and network clusters of senescence were found to be downregulated. Thus, our data indicate that manifestations of increased proliferative signaling and DNA instability are well balanced by prosenescence pathways in SHS-survived eMSC suggesting it does not meet the first Hanahan-Weinberg criterion entitled as "sustainable proliferation unbalanced by senescence and apoptosis" (Figure 3).

3.5. Proapoptotic and Prooncogenic Signaling Balance Characterizing the Hallmarks "Evading Growth Suppressors" and "Cell Death Resistance." In contrast to normal cells, cancer cells evade programs of negative regulation of proliferation executed mostly by tumor-suppressing genes and pathways [57]. The most important of them are presented by TP53 gene and their signaling. Compared to control, SHS-treated eMSC show the induction of pathways coordinated by TP53 and other important pathways promoting tumor suppression (Figure 2, Supplementary Table 1). Also, the important finding was the strong activation of the BioSystem pathway "validated targets of C-MYC transcriptional repression." The upregulation of this pathway including genes that are usually silent when MYC is active indicating

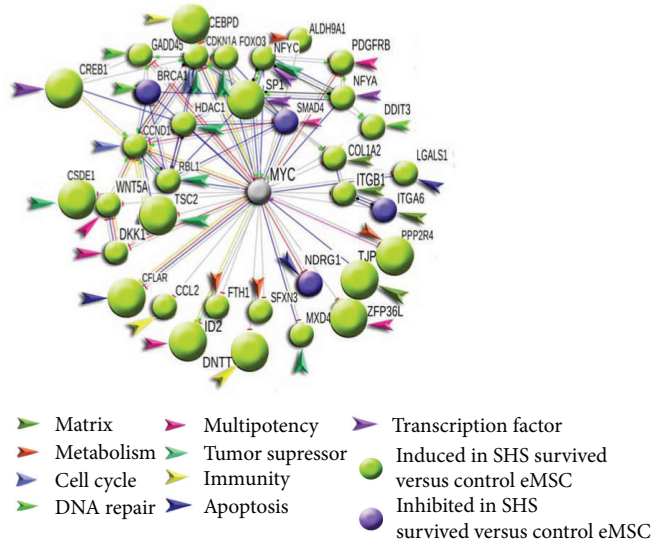


Figure 5: Gene composition of BioSystem pathway "validated targets of C-MYC transcriptional repression." The figure illustrates gene functional distribution and expression gradient through the pathway and indicates that all genes (31 induced and 4 inhibited) are interconnected with MYC as genes that are directly repressed by MYC. The repressing type of interactions is indicated with small red solid lines (bricks) near buttons. MYC silence is indicated with a *grey button*. The network is constructed using STRING server at interaction stringency $S = 0.9$.

that eMSC possess particular system protecting against MYC induction even under severe stress caused by DNA instability and confirms our NGS results pointing to the absence of MYC expression in SHS-untreated and SHS-treated cells. This result is surprising because DNA instability associated with aneuploidy, polyploidy, or chromosome breakages usually enhances cancer phenotype and activates C-MYC [26, 62–65].

To examine gene functional distribution and expression gradient through the pathway "validated targets of C-MYC transcriptional repression," we constructed protein interaction network containing all 35 proteins this pathway. Figure 5 indicates that all genes (31 induced and 4 inhibited) are interconnected with MYC as genes that are directly repressed by MYC. Most of the induced genes are related to DNA repair, tumor suppression, and multipotency. Thus, protein interaction network indicates that "validated targets of C-MYC transcriptional repression" pathway contains mainly activated genes, which should be repressed if MYC was activated. This confirms that HS-survived cells may have specific barrier against MYC activation. Thus, our data indicate that HS-survived eMSC do not have death resistance.

It is well established that cell death resistance may be triggered by impaired proapoptotic and cell death-related signaling accompanied by elevated signaling of oncogenesis [57]. From our results, SHS-survived eMSC demonstrated the upregulation of modules involved in apoptosis and cell death (Figure 2, Supplementary Table 1).

To investigate signaling implicated in oncogenesis, we selected gene modules with terms related to cancer, transformation, tumors of various origins, and well-known

oncogenes from different families of growth factors, serine/threonine, and tyrosine kinases (ABL, EGFR, ERBB, JAK, MAPK, and AKT), GTPases (RAS), and transcription factors (MYC, JUN, and FOS). The selection yielded no induced pathways. In contrast, the inhibited prooncogenic modules were quite numerous evidencing in favor of nontransformed state of SHS-survived eMSC (Figure 2, Supplementary Table 1). Thus, compared to untreated eMSC, SHS-survived eMSC demonstrated the downregulation of pathways implicated in signaling coordinated by RAS, ERBB, and MAPK.

Notably, our data revealed no expression of a master regulator of PI3K signaling PKB alpha (AKT1) and MYC oncogene in untreated eMSC and in SHS-survived ones. The important role of RAS, EGFR, ERBB, MAPK, AKT1, and MYC in transformation is currently well established and was reviewed in [57]. Also, the activity of tumor marker carcinoembryonic antigen-related cell adhesion molecule (CEACAM5) was decreased by more than 16-folds as well as the other proteins of CEACAM family. The implication of CEACAM family proteins in cancer progression and metastasis has been recently underlined by Bajenova and coauthors [66, 67]. The authors revealed the tight interaction between CEACAM proteins and MAPK, TGF-beta, and EMT pathways.

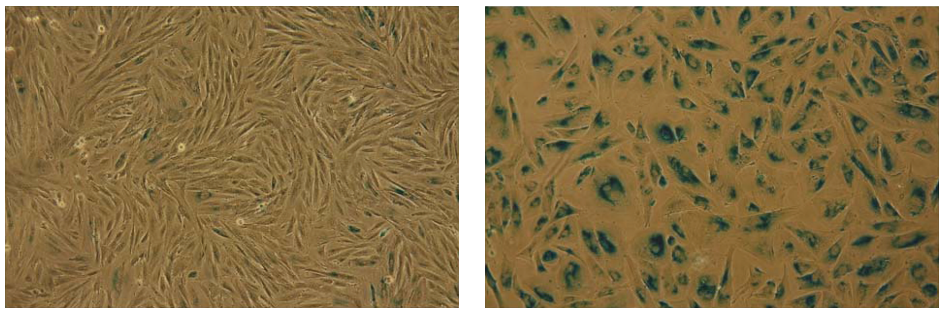
In accordance with the results of gene module analysis pointing to the nontransformed state of SHS-survived eMSC, the data of the examination of general PPI network constructed for the induced and inhibited genes revealed no oncogene containing clusters among the network of the induced genes and revealed three tight transformation-related clusters implicated in FOXO, HRAS, and EGFR signaling among the network of the inhibited genes (Figure 2, Supplementary Table 1).

3.6. Activity of Telomere- and Telomerase-Related Gene Modules as Indicators of the Hallmark "Replicative Immortality." Gene modules implicated in replicative immortality were searched by the titles containing terms related to telomere and telomerase. The search gave no results. To additionally verify the absence of features related to "replicative immortality," we analyzed genes participating in telomere extension, protection, and maintenance. The information about these genes were found in the TeloPIN database describing telomeric proteins and interaction network in mammalian cells [68]. The examination of genes hTERT, TRF1, TRF2, TIN2, RAP1, POT1, and TTP1 indicated that SHS-treated eMSC express only two genes (POT1 and TTP1) providing telomere protection. These genes were induced by 15.6- and 12.4-folds in the treated versus untreated eMSC. Other genes were not expressed. The absence of telomerase activity was found in other types of human mesenchymal stem cells including hematopoietic and nonhematopoietic stem cells such as neuronal, skin, adipose tissue, intestinal crypt, mammary epithelial, pancreas, adrenal cortex, and kidney [69].

3.7. Gene Modules Characterizing the Hallmarks "Angiogenesis" and "Invasion and Metastasis." The examination of gene modules with terms related to angiogenesis in their titles gave no results. Cell program related to invasion and metastasis was inhibited. It is evident from the dramatically decreased activity of transcription factors of SNAI and ZEB families (Supplementary Table 1) that are known to regulate prometastatic program of EMT [70]. Also, there are no manifestations of N-cadherin to E-cadherin switching, which is also an important EMT feature [70]. Moreover, the pathways of "stabilization and expansion of the E-cadherin adherens junction" and "E-cadherin signaling in keratinocytes" were induced (Supplementary Table 1). Accordingly, the two processes related to migration, including "positive regulation of neuron migration" and "cell migration involved in gastrulation," were downregulated confirming that the metastatic potential was not activated in SHS-survived eMSC (Figure 2, Supplementary Table 1).

3.8. Characteristic-Enabling Transformation. Besides the hallmarks of cancer [57], Hanahan and Weinberg identified characteristic-enabling transformation, including genome instability Warburg effect, inflammation, and avoided immune destruction. Of these characteristics, SHS-survived eMSC demonstrate only chromosome instability (Figure 1). In agreement with this observation, the transcriptome analysis identified features of chromosome missegregation and premature separation described above (Figures 2 and 3, Supplementary Table 1). Also, our data revealed features of DNA damage response modification, specifically, the increased activity of single-strand DNA repair and the impairment of double-strand DNA repair (DDR). The induction of modules related to DNA excision repair, TP53-dependent G1/S DNA damage checkpoint and network clusters containing TP53, TP53BP1, DDIT3, ERCC2, PARP1, and RFC1 DNA repair regulators was accompanied by the coordinated downregulation of the protein interaction network cluster containing ATM, CHEK1, BRCA1, and ATR (Figures 2 and 3, Supplementary Table 1). All these genes are well-known regulators of double-strand DNA damage sensing and repair and for the coordination of DNA replication checkpoint and appropriate S-phase entry (ATR and CHEK1) [71, 72]. Fortunately, the weakening of DDR is associated with compensatory induction of pathways involved in DNA-based excision repair, apoptosis, and senescence. The activation of these pathways (particularly the ones of single-strand DNA repair) may guard SHS-survived eMSC from transformation even despite prominent genomic instability [73]. Thus, our analysis revealed both flaws and strengths of eMSK SHS resistance and explained why these cells escape transformation despite extensive genomic instability.

The other cancer-enabling characteristic related to metabolic switches towards Warburg effect was not identified. Energy metabolism-related gene modules were activated and were implicated mainly to aerobic respiration and mitochondrial electron transport chain (Figure 2, Supplementary Table 1). Our data did not identify features of Warburg-like metabolic modifications, including the induction of modules of glycolysis and glutaminolysis and the inhibition of modules related to aerobic respiration. This result indicates that SHS-survived eMSC do not show transformation-related metabolic modification.



(a) (b)

Figure 6: SA-beta-Gal staining of SHS-survived cells at the 6th (a) and 19th (b) passages.

Gene modules implicated in transformation enabling characteristics related to inflammation and immune destruction were coordinately downregulated and were presented by modules of immunity mediated by B- and T-lymphocytes, immunoglobulines, interleukins, and adrenergetic receptor signaling (Figure 2, Supplementary Table 1), confirming the absence of these transformation-related characteristics in SHS-survived eMSC and their anti-inflammatory eMSC properties.

3.9. Outcome of Long-Term Cultured eMSC-Survived SHS. SHS-survived eMSC analyzed at the 6th passage after HS were then expanded further. They were subcultured for another 19 passages and slowly died. During the passaging, these cells demonstrated gradually reduced proliferation and morphological abnormalities manifested in cell enlargement. In other words, these cells slowly entered into the replicative senescence state. These observations were supported by the results of SA-beta-Gal activity in cells at various passages. The enzyme activity is a marker of cellular senescence. Figure 6 shows SHS-survived cells at the 6th (A) and 19th (B) passages after SHS exposure. It is seen that at the moment of karyotyping, next-generation sequencing and transcriptome functional analysis (passage 6) cells had typical fibroblast-like morphology and did not exhibit SA-beta-Gal activity (Figure 6(a)). At the 19th passage, Figure 6(b) exhibits cell enlargement and bright staining of SA-beta-Gal-positive cells that are hallmarks of the replicative senescence. These cells ceased proliferation and then died. So, in spite of genetic instability, SHS-survived cells remain mortal and nontransformed.

3.10. Comparison of Our Results with the Data on Transformed Bone Marrow Mesenchymal Stem Cells (bmMSC). The detailed investigation of Hanahan-Weinberg hallmarks of cancer indicates that although SHS-survived eMSC show genomic instability, these cells can be considered as nontransformed cells. It is reasonable to compare our data with the data obtained with experimentally transformed mesenchymal stem cells. For this propose, we used the data on gene expression changes in human bone marrow mesenchymal stem cells after hTERT transformation [18]. We made gene-by-gene comparison of gene sets containing the most severely deregulated genes during hTERT-induced transformation of bone marrow MSC with our results. Of

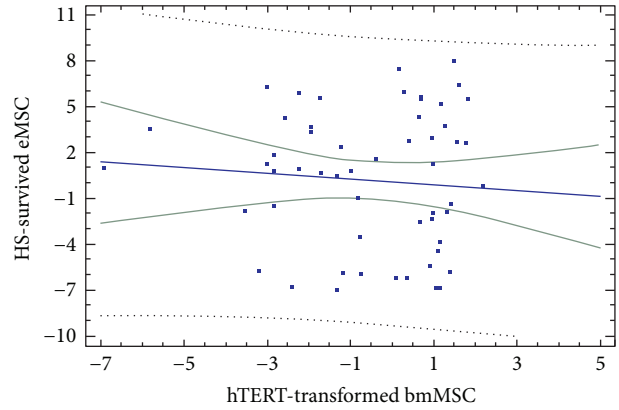


Figure 7: The absence of correlation between gene expression changes in hTERT-transformed bmMSC and SHS-survived eMSC ($r = -0.10$, $p > 0.5$).

123 genes, only 21 genes changed expression in the same direction in hTERT-transformed and SHS-survived eMSC cells, whereas 42 genes changed expression in opposite direction. The remaining 60 genes were expressed in control and hTERT-transformed cells and were not expressed in intact and SHS-survived eMSC (Supplementary Table 2).

The regression analysis revealed the absence of correlation between gene expression changes in hTERT-transformed bmMSC and SHS-survived eMSC ($r = -0.10$, $p > 0.5$, Figure 7). In accordance, the binomial test revealed the significant prevalence of the fraction of gene changing expression in bmMSC and eMSC in the opposite directions over the fraction of gene changing expression in the same direction ($p < 10^{-5}$).

To further verify the absence of transformation features in SHS-survived eMSC, we compared the activity of gene modules enriching for above-said sets containing 22, 45, and 44 genes (Supplementary Table 3). The examination of the 45-gene set indicated that in contrast to hTERT-transformed bmMSC, SHS-treated eMSC show increased ability to differentiation and cell adhesion and decreased PI3K-AKT signaling. The analysis of the 22-gene set shows that both hTERT-transformed and SHS-survived eMSC increase proliferative potential after treatment. The investigation of the 44-gene set revealed the silence of cancer-related modules in SHS-survived cells compared to hTERT-transformed cells

(Supplementary Table 3). Thus, the obtained results indicate that SHS-survived eMSC do not show features of transformation revealed in bmMSC after hTERT treatment.

4. Conclusion

Generally, our data indicated that compared to untreated cells, SHS-survived eMSC show higher genome instability, which exert global effect on gene expression and activity of gene modules related to essential biological pathways. The induction of energy metabolism, protein turnover, and transcription give SHS-survived cells beneficial properties helping them to overcome high-energy needs that are necessary to counteract aneuploidy and chromosome breakages. The evaluation of Hanahan-Weinberg criteria of transformation entitled as "hallmarks of cancer" and "cancer-enabling characteristics" did not reveal features of malignancy even despite genomic instability in eMSC. This result indicates that SHS-survived eMSC possess strong defense system protecting them against transformation. Indeed, we identified several lines of defense. The first line is provided by the ability of eMSC to decrease prooncogenic pathway activity (specifically, pathways regulated by oncogenes of RAS, Pi3k, MAPK, and ERBB families) in response to DNA damage and aneuploidy. The second line originates from the coordinated induction of tumor-suppressing pathways, including the pathways of TP53, p21 (CDKN1A), and p16 (CDKN2A) signaling, and pathways implicated in DNA repair. The third line is the increased protection against of single-strand breakages of DNA seen from the activation of pathways related to DNA excision and mismatch repair. Also, we found the silence of oncogenes MYC, AKT1/PKB, and hTERT telomerase that may further increase protection against transformation. Overall, our data suggest that despite genetic instability, SHS-survived eMSC do not undergo transformation and entered replicative senescence after prolonged expansion in culture, confirming their mortality and oncological safety.

Conflicts of Interest

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

Authors' Contributions

Vinogradov A. E., Shilina M. A., and Anatskaya O. V. contributed equally to this work. Fridlyanskaya I. I., Grinchuk T. M., and Nikolsky N. N. are senior authors.

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Supplementary Materials

Supplementary Table 1. Gene modules enriching for genes that are significantly induced or repressed in SHS-survived vs control eMSC. Supplementary Table 2. Comparion of gene expression changes in SHS survuved vs untreated eMSC (present study) and in hTERT transformed vs intact bone marrow MSC (*the data from Takeuchi et al, 2015). the data from Takeuchi et al, 2015). Supplementary Table 3. Comparison of gene module activity enriching for 22, 45 and 44 gene sets in hTERT and SHS treated bmMSC and eMSC. (*Supplementary materials*)

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Review Article

The Immunomodulatory Effects of Mesenchymal Stem Cell Polarization within the Tumor Microenvironment Niche

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Mesenchymal stem cells (MSCs) represent a promising tool for cell therapy, particularly for their antitumor effects. This cell population can be isolated from multiple tissue sources and also display an innate ability to home to areas of inflammation, such as tumors. Upon entry into the tumor microenvironment niche, MSCs promote or inhibit tumor progression by various mechanisms, largely through the release of soluble factors. These factors can be immunomodulatory by activating or inhibiting both the adaptive and innate immune responses. The mechanisms by which MSCs modulate the immune response are not well understood. Because of this, the relationship between MSCs and immune cells within the tumor microenvironment niche continues to be an active area of research in order to help explain the apparent contradictory findings currently available in the literature. The ongoing research aims to enhance the potential of MSCs in future therapeutic applications.

1. Introduction

The tumor microenvironment is composed of extracellular matrix and nontumor stromal cells (fibroblasts, endothelial, and immune cells). Communication between the tumor and stromal cells plays a pivotal role in the progression of cancer [1]. Mesenchymal stem/stromal cells (MSCs) [2] represent one population of cells that are found within the tumor stroma and have shown potential for either promoting or inhibiting tumor growth [3]. MSCs are often utilized for their therapeutic potential since they have the capacity to differentiate into nonhematopoietic cell lineages, promote tissue repair and regeneration, and modulate immune responses [4, 5]. Although MSCs isolated from the bone marrow (BM-MSCs) are the most commonly studied, MSC populations also can be obtained from many other tissue sources, including the placenta, skin, adipose tissue, and Wharton's jelly [6]. While the characteristics used to define these cells vary by laboratory, generally, MSCs share an ability to adhere to plastic *in vitro* and possess an immunophenotype that includes detectable expression of cluster of differentiation (CD) 105, CD73, and CD90 and negative expression of CD45, CD34, CD14, CD19, CD3, and human leukocyte antigen- (HLA-) DR surface markers [5–7]. In addition, MSCs are characterized by their capacity to differentiate at a minimum into adipogenic, chondrogenic, and osteogenic lineages *in vitro* [5, 8]. MSCs have emerged over the last decade as a promising modality for cell therapy, for applications ranging from regenerative medicine to tumor therapy.

Some of the general advantages of using MSCs for cell therapy include the ease of expansion and storage ex vivo and their ability to avoid immune clearance [9]. In addition, MSCs home to sites of injury, where they secrete extracellular matrix components, chemokines, and cytokines [10]. The secretome of these cells has functions associated with chemoattraction and modulation of immune cells, angiogenesis, and support of cellular growth and proliferation [11]. Because MSCs home towards tumors in a similar fashion as they home to injury sites, they can be useful for delivering cell-based therapeutics to tumor sites. The mechanisms utilized by MSCs to home towards tumors include several signaling axes, including stromal cell-derived factor (SDF-1 or CXCL12), its receptors, C-X-C-chemokine receptor type 4 (CXCR4), and related chemokine signals (CXCL10, CXCR3),

as well as the immune regulatory cytokine transforming growth factor beta (TGFβ) [10]. However, despite progress in understanding the nature and function of MSCs within the tumor microenvironment, many questions remain unanswered regarding their safety and efficacy for clinical use. This is due to the duality associated with MSC signaling once they reach the tumor microenvironment. For example, depending on the context and tumor type, MSCs have been found to either support or inhibit tumor progression [12].

MSCs elicit many of these effects on other cells through the release of paracrine factors, which can cause primary tumor resensitization and cancer cell apoptosis (Figure 1(a)). MSCs that infiltrate tumors come from local or distant sources [13] and may differentiate and/or transdifferentiate into normal resident cells in an attempt to contribute to tissue repair (Figure 1(a)). Within the tumor microenvironment, MSCs are able to induce cancer cell survival, stemness, and chemoresistance following their differentiation into cancer-associated fibroblasts (CAF) and by their release of soluble factors favoring angiogenesis and immune suppression (Figure 1(b)). Once MSCs have infiltrated the tumor microenvironment, the presence of tumor necrosis factor (TNF), interleukin- (IL-) 1, and interferon γ (IFNγ) or hypoxic conditions all stimulate MSCs to release proangiogenic and immunosuppressive factors including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and IL-6 and IL-8 [14]. Some of these paracrine factors released by MSCs such as IL-10 and TGFβ attract immune cells locally, where MSCs can inhibit their activation and proliferation [15]. The combination of angiogenic and immunosuppressive factors allows for tumors to escape the immune surveillance, proliferate, and metastasize [16]. However, contradictory evidence has been shown also regarding the ability of MSCs to inhibit tumor growth.

The discrepancies surrounding the ability of MSCs to either promote or inhibit tumor progression include factors such as tissue source, individual secretomes, nature of interactions with cancer cells and immune cells, type of cancer or cancer cell lines, and experimental conditions [10, 12]. Additionally, several studies are designed with MSCs from healthy donors which are functionally different from MSCs from cancer patients which likely undergo cellular and molecular changes in direct or indirect (secretome) interactions with cells in the tumor stroma [12, 17]. Therefore, there needs to be a much better understanding of the mechanism(s) regarding the communication between MSCs and immune cells within the tumor microenvironment and how that might impact tumor progression. Gaining a better understanding of these factors might allow clinicians to harness the MSC secretory phenotype in order to optimize their therapeutic potential against cancer.

2. Mesenchymal Stem/Stromal Cells and Their Microenvironment

In recent years, as cellular therapy using MSCs has become a therapeutic option to treat numerous diseases, several studies have examined the role of the microenvironment on MSC biology. When MSCs are introduced into a pathological milieu, then, they can exhibit increased or reduced survival and can alter their differentiation or immunomodulatory characteristics based on the physical and biochemical features of the microenvironment encountered. The response of MSCs to environmental cues might alter their phenotype towards proinflammatory or anti-inflammatory activities, depending on the context, and this duality of function has been compared to the polarization observed in macrophages. It is well established that macrophages may become either proinflammatory or anti-inflammatory depending on the cytokine environment to which they are exposed [18]. IFNγ plus LPS promotes predominantly M1 or proinflammatory macrophages with a relative increase in TNFα production and a reduction in IL-10 secretion. IL-4, on the contrary (alone or with IL-10 and TGFβ), promotes M2 macrophages, with a prominent anti-inflammatory phenotype with marked IL-10 release. This has led investigators to pursue the concept that MSCs, similar to macrophages, can be rendered either proinflammatory or anti-inflammatory depending on the cues they receive from their microenvironment. And this plasticity may help to explain their ability to be both pro- and antitumorigenic. Several environmental stimuli can impact how MSCs alter their apoptotic, proliferative, migratory, differentiation, and secretory profiles (Figure 2), and these will be detailed in the following section.

2.1. Hypoxia. Oxygen tension within a tumor is highly heterogeneous and can be found present at levels almost as low as anoxic conditions (close to no oxygen). Increasing hypoxia (low oxygen) within a tumor could be an indicator of tumor progression and can lead to the selection of highly invasive cancer cells with greater resistance to therapies. Differences in the oxygen tension levels in the tumor microenvironment have been associated with the modulation of properties of tumor stromal components [19]. Often, MSCs are usually *in vitro* in normoxic conditions, a higher oxygen tension level than what would be found *in vivo* [20]. Studies suggest that differences in oxygen tensions can lead to changes in the commonly studied properties of MSCs such as stemness [21], differentiation [16, 22–24], and secretome [25].

For example, hypoxia can promote MSC proliferation, enhance their migration, and maintain their stemness [21–23, 26–31]. The yield of differentiation into nonhematopoietic lineages is also altered in these cells as a result of variable oxygen tensions. The osteogenic differentiation potential has been reported to be increased under hypoxic conditions when compared to that of normoxic-cultured MSCs [23, 29, 30]. Chondrogenic differentiation has been inhibited by culture under hypoxic conditions [25]. The adipogenic differentiation potential has been reported to be either enhanced [32] or inhibited [25, 29], and the difference in findings could be attributed to differences in MSC sources, as well as to differences in experimental conditions.

In laboratory settings, a wide variety of experimental protocols is used to study the effect of low oxygen tensions on MSC behavior, leading to difficulties in the comparison of

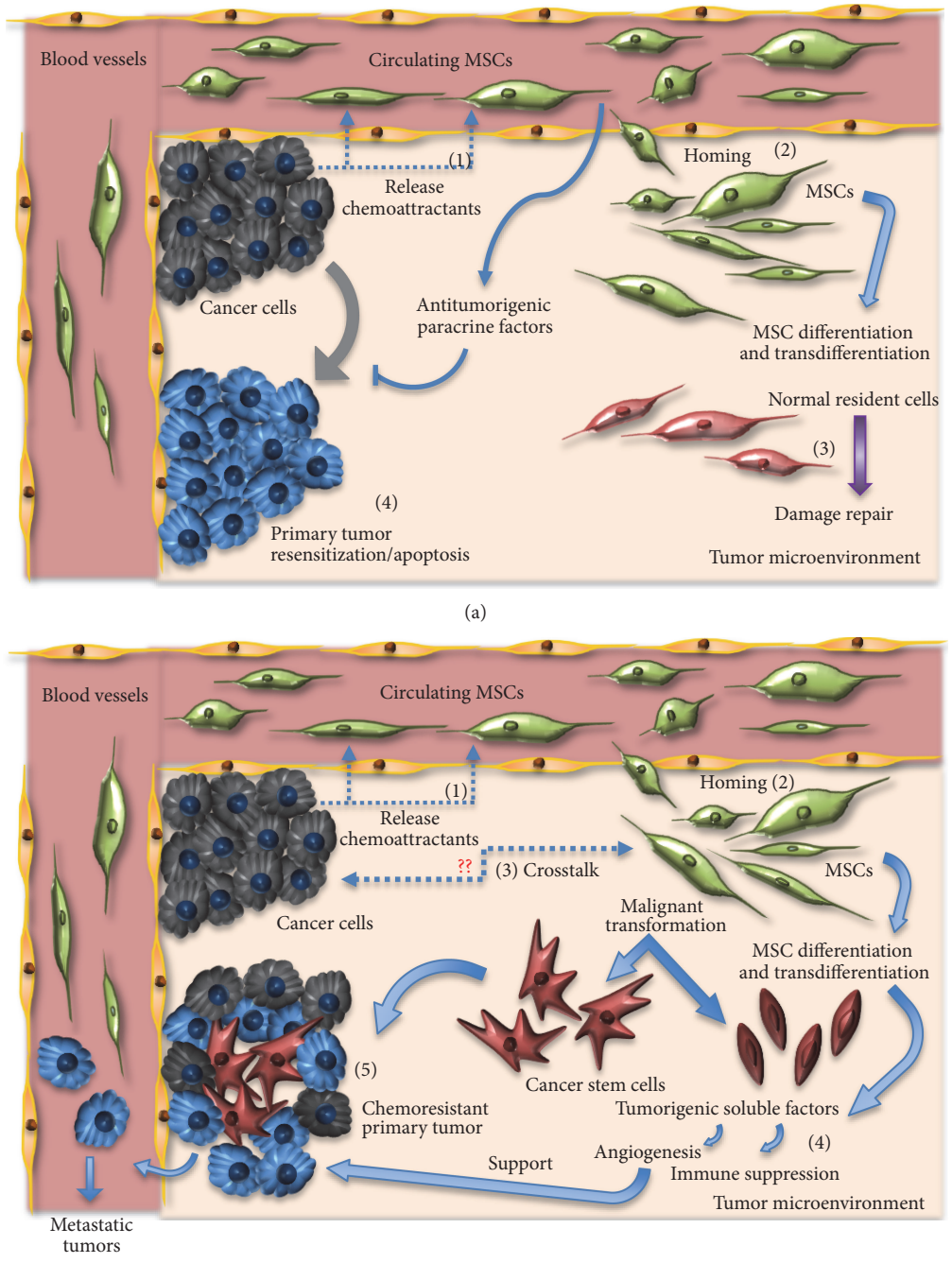

Figure 1: The role of MSCs in the tumor microenvironment. (a) The antitumor effects of MSC. Circulating MSC may release antitumor paracrine factors causing primary tumor resensitization and cancer cell apoptosis, while infiltrating MSCs may differentiate to contribute to tissue repair. MSCs arrive at tumors following chemoattraction (1), home towards tumors (2), with the goal of performing damage repair (3), and induce primary tumor resensitization and apoptosis (4). (b) The protumorigenic effects of MSCs. Infiltrating MSCs are attracted to tumors via chemoattractants (1), home to tumors (2), participate in secretory crosstalk with tumor cells (3), release proangiogenic and immune-suppressive soluble factors (4), and may support the growth of chemoresistant tumors (5).

the data. However, as indicated by Buravkova et al., the available data can be divided into two main groups utilizing duration of exposure as a common ground: MSCs grown under normoxic conditions and later exposed to acute short-term hypoxia (up to 72 hrs) and MSCs cultured permanently under hypoxic conditions [26]. From this perspective, the effects of hypoxia on MSC properties seem to occur in two phases; a short-term acute treatment under hypoxic conditions causes cell damage involving apoptosis, followed by adaptation mechanisms including a switch to an anaerobic glycolysis metabolism [26, 27] and maintenance of an undifferentiated multipotent state [26].

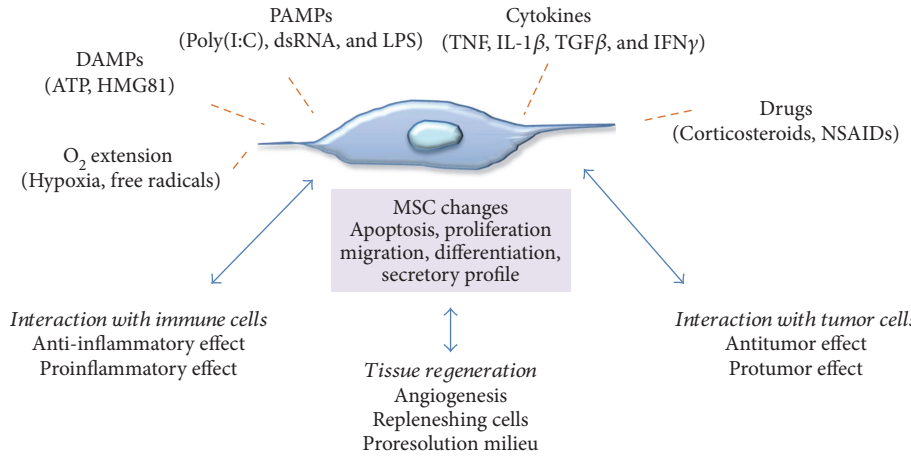

Figure 2: Interactions of MSCs with the microenvironment. Summary of environmental factors that can influence cellular responses of MSCs in the tumor microenvironment and/or tissue regeneration settings.

Paradoxically, pointing to a dual role for MSCs, some studies suggest that hypoxia pretreatment can promote more efficient cartilage repair [24], likely through induction of hypoxia-inducible factor 1 alpha. Likewise, umbilical cord-derived MSCs preconditioned with hypoxia can be more efficient in treating mouse hind limb ischemia [33]. Hypoxia may induce a significant increase in triglycerides, fatty acids, and diacylglycerols in MSCs, whereas blocking hypoxia might enhance production of angiogenic factors like VEGF and angiopoietin-2 [34]. Hypoxic conditions might also enhance the supportive role of MSCs on endothelial progenitors, for example, in diabetic rats with hind limb ischemia [35], which might be detrimental in the context of tumors, for example. However, the consequence of changes in oxygen tensions within the tumor microenvironment in the context of MSC interactions is not yet well understood. It is known that several soluble molecules are upregulated by MSCs in response to hypoxia, including cell cycle-regulating proteins such as VEGF and IGF-II [23, 28] and hypoxia-inducible factors (HIFs) with roles in the promotion of macrophage recruitment, primary tumor growth [32], and metastasis of breast cancer [32, 36] and induction of proangiogenic and chemotactic secretion factors such as MCP-1, IL-8, and RANTES [25]. The altered MSC secretome would likely impact promotion of tumor growth and alter infiltration of immune cells.

2.2. MSCs and Cytokines. When exposed to TNFα, a major proinflammatory cytokine, MSCs display reduced proliferation and caspase-dependent apoptotic pathways are activated through p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal protein kinase (JNK) pathways [21]. Conversely, TNFα can stimulate MSC migration and ICAM-1 expression, a molecule important in transendothelial migration of MSC [20]. TNFα pretreatment also can induce secretion of proangiogenic growth factors including fibroblast growth factor (FGF) 2, VEGF, or IL-8, promoting microvessel formation [36], but reduces the ability of MSCs to block neutrophil influx and improve perfusion of the jejunum in another model [37]. Interestingly, TNFα also can modulate anti-inflammatory effects on MSCs, enhancing prostaglandin E2 (PGE2) release and promoting IL-10-expressing anti-inflammatory macrophages [38]. Moreover, TNFα can induce the release of a potent anti-inflammatory protein called TNFα-stimulated gene 6 or TSG-6 [39].

Another example of a cytokine with effects on MSCs is interferon gamma or IFNγ. MSCs get "licensed" with T cell inhibitory properties when this cytokine is produced by $CD4^+$ helper T cells and cytotoxic CD8 T lymphocytes. There seems also to be some species-specific changes in MSCs in response to IFNγ, leading to upregulation of IDO in human MSCs and upregulation of iNOS in murine MSCs. The net result is that IDO ultimately depletes tryptophan in the local microenvironment, rendering lymphocytes inactive and unable to proliferate, while iNOS increases local NO concentration, leading to inactivation of several proinflammatory genes in lymphocytes [40]. IFNγ also upregulates ICAM1 and VCAM1 in MSCs [41], assisting these cells in contacting lymphocytes and other immune cells for a maximized effect. MSCs treated with IFNγ also can express inhibitory costimulatory molecules such as B7 family coregulatory molecules B7-H1 [42], which can interact with $CD4^+$ lymphocytes and block cell proliferation, promoting T cell anergy. Interestingly, there appears to be a critical threshold of an IFNγ concentration that can activate the immunosuppressive MSC effects [43], a threshold that might help explain differences among studies, even though differences in donor sources or culture conditions also may play a role in modifying the impact of IFNγ on MSCs. IFNγ can induce MHC classes I and II on MSCs, rendering them immunogenic [44]. Finally, another cytokine shown to block the anti-inflammatory properties of MSCs by reducing iNOS expression is TGFβ [45], pointing to a feedback mechanism in the microenvironment whereby TGFβ induction might promote the resolution of inflammation and/or tissue regeneration.

2.3. MSCs and Damage-Associated Molecular Pattern Molecules (DAMPs) or Pathogen-Associated Molecular Patterns (PAMPs). DAMPs include nuclear high-mobility group box 1 protein (HMGB1), a chromatin-associated

protein; cytosolic proteins such as S100 and purine metabolites (adenosine triphosphate, uric acid); and extracellular matrix (ECM) elements such as hyaluronic acid fragments. These proteins and ECM components are typically released into the microenvironment following tissue damage due to inflammation or other physical, chemical, or biological insults [46]. These molecules are sensed through binding to either Toll-like receptors TLR2 and 4 or receptors for advanced glycosylation products by MSCs, augmenting in many cases the trafficking and proliferation of MSCs. HMGB1 also can modulate the expression of IDO by MSCs, an immunomodulatory enzyme. Additionally, uric acid and S100A4 can act as chemoattractants of MSCs and enhance their immunomodulation effects by stimulating the expression of IL-10 and IDO in immune-suppressive lymphocytes [47]. For PAMPs, molecules produced by invading microorganisms, the detection by MSCs can occur via cytosolic membrane-bound pattern recognition receptors (PRR) [46]. TLRs are a subset of these PRR and are abundant in several immune and epithelial cells. TLRs will be discussed in more detail in Section 3 of this review.

2.4. Mesenchymal Stem/Stromal Cells and Drugs. It is important to also understand the effect that drugs can have on MSCs, since cells infused during regenerative therapies might be coadministered with agents such as corticosteroids or nonsteroidal anti-inflammatory drugs. The immunomodulatory properties of MSCs depend on their production of sufficient amounts of prostaglandins (PGE2) [48]. Coadministration with NSAIDs, for example, might reduce the effects of PGE2 and modify the effects of MSCs at sites of inflammation. On the other hand, tumor therapy applications might use gene-modified MSCs in coadministration with chemotherapeutics or immunotherapies to enhance their effects. MSCs loaded with chemotherapeutics can be used as delivery vehicles to tumors, as cultured MSCs are capable of selectively homing to and surviving in a variety of preestablished solid tumors (breast, colon, melanoma, and others) while being excluded from normal tissues [49]. The safe use of MSCs to treat cancer or noncancer diseases in patients that have undiagnosed, early-stage cancer requires understanding the fate and functions of MSCs and their interactions with tumors. Interestingly, instead of gene-modified MSCs, incorporation of drug-laden nano/microparticles inside the cell or on the cell surface also can be done [49].

The next sections of this review will focus on the changes MSCs impart onto the tumor microenvironment that relate to immune cell changes and also on the mechanisms of MSC polarization, which may help explain the dual antitumorigenic and protumorigenic nature of these cells as they interact and modulate various effects on tumor cells.

3. The Interaction of MSCs and the Immune System in the Tumor Microenvironment

The primary role of the immune system is to defend the body against the external environment and pathogens. The induction of specific immune responses, such as the production of antibodies to a particular pathogen, is known as an adaptive or acquired immune response and is typically acquired during the lifetime of an individual as an adaptive response to a specific pathogen. This distinguishes such responses from innate immunity, which is a type of inborn defense in that its action does not depend upon prior exposure to a particular pathogen. Innate immunity and adaptive immunity are orchestrated by sets of interacting but distinct cell types, and both responses are found to be important in tumor elimination or relapse, depending on the context. MSCs can impact both innate and adaptive immune responses within tumor of various types.

3.1. Innate Immunity Changes Mediated by MSCs in the Tumor Microenvironment. Without a properly functioning innate immune system, aberrant cell populations run the risk of going unchecked within an immunosuppressive environment conducive for the progression of cancer. Cell types of the innate immune system, including macrophages, natural killer (NK) cells, and dendritic cells (DC), possess mechanisms optimized for the detection and removal of tumor cells. The coordinated activation and response of the innate immune system are quite complex, involving the recruitment and maturation of a wide range of cell types. The inflammatory tumor microenvironment plays a key role in the recruitment of many of these innate immune cells through the release of proinflammatory cytokines [50]. Upon recruitment, these cells are then further differentiated in order to properly carry out their designated functions to restore a homeostatic microenvironment. The initiation of the proinflammatory signal may depend on local expression of IFNβ by DC to promote initial innate recognition of tumors [51]. Infectious disease models have indicated at least three pathways of innate immune sensing that can drive IFNβ upregulation at the transcription level. These are Toll-like receptor (TLR) signaling, retinoic acid-inducible gene 1 (RIG-I) sensing of cytosolic RNA, and the stimulator of interferon genes (STING) pathway sensing of cytosolic DNA from dying tumor cells [26]. In the case of tumors, the same mechanisms can be utilized to activate the innate system by "sterile immunity" with the participation of these innate immune sensing pathways, which involve stress-associated or damage-associated molecular patterns triggering innate immune activation.

Emerging mechanisms associated with infiltrations of innate immune cells into tumors have shown an important functional role for another component of the tumor microenvironment—stromal cells. The supportive stromal cell populations contribute their own cytokines that impact the innate immune response. MSCs are one of the stromal cell populations being actively studied in order to elucidate the effects they have on innate immune cell recruitment and functionality, due to their ability to influence multiple types of innate immune cells (Figure 3) [52]. Here, we highlight a few of the mechanisms in which MSCs influence the specific cell populations of the innate immunity.

3.1.1. Immune-Activating Effects of MSCs on Innate Immunity Cells within the Tumor Microenvironment. The

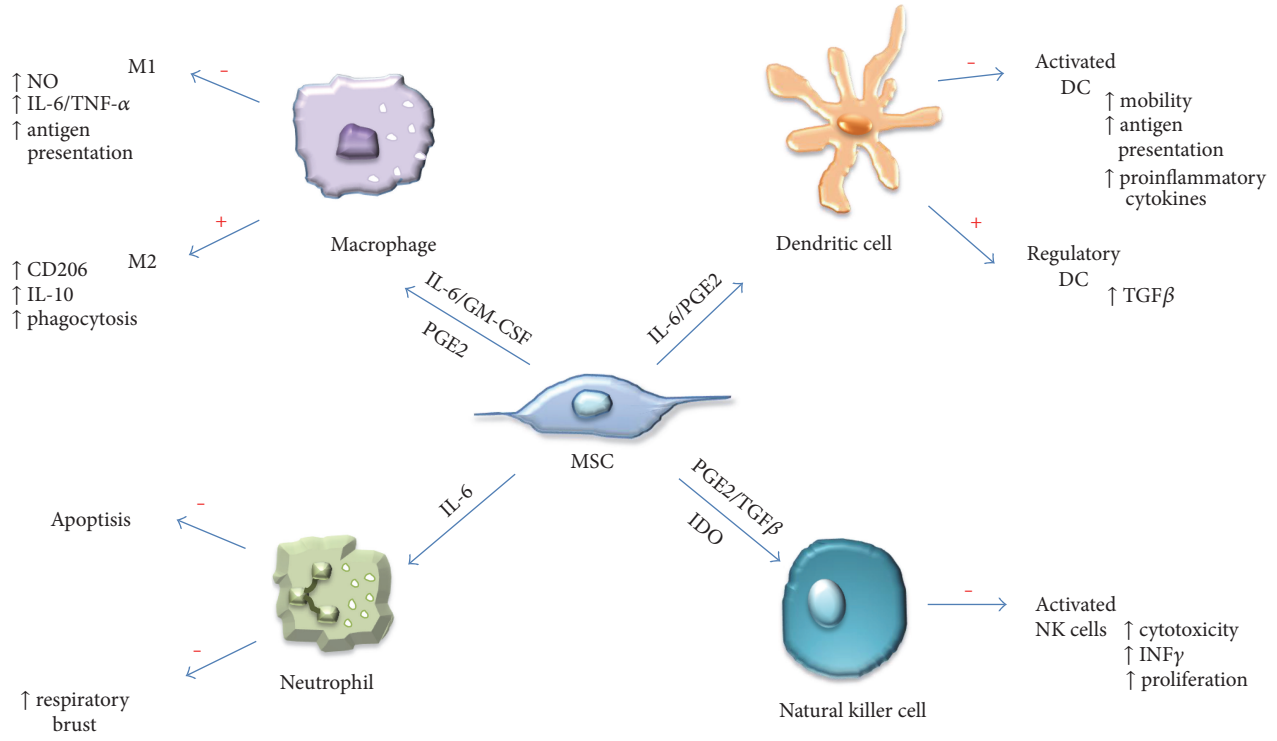

Figure 3: Interactions of MSCs with innate immune cells. MSCs utilize diverse molecular mechanisms to suppress or promote innate immune cells. MSCs suppress macrophage polarization to M1, although they favor M2 polarization. MSCs inhibit NK cell and DC activation, differentiation, and effector functions. MSC-derived PGE2 contributes to all of these effects. MSC-produced IL-6 suppresses neutrophil apoptosis and respiratory burst and also contributes to inhibiting DC function. In the presence of IL-6 and GM-CSF, MSCs also can affect macrophage function, while TGFβ and IDO suppress NK cell function. In addition, MSCs also favor the generation of regulatory DCs. Effects of MSC: + indicates function promotion and − indicates function suppression.

literature has few reports on immune-activating effects of MSCs on the innate immunity within tumors. Some immune-activating effects might include the effects of MSCs in increasing the phagocytic ability of cocultured macrophages. One study showed that macrophages cocultured with MSCs expressed high levels of CD206 and IL-10 and low levels of IL-12, suggesting development of alternatively activated macrophages [53]. Although the cocultured macrophages also expressed high levels of IL-6 and low levels of TNFα compared to controls, functionally, they displayed a higher level of phagocytic activity. These MSC-educated macrophages might represent a unique type of alternatively activated macrophage with a potentially significant role in tissue repair. Whether this macrophage type can be promoted within the tumor microenvironment following interaction with MSCs is not known.

Although far less is known about the role of neutrophils in interaction with MSCs, some studies suggest that polarization of MSCs via TLR3 (but not so much TLR4) activation preserves viable and functional neutrophils by amplifying the antiapoptotic effects that resting BM-MSCs would normally exert on these cells [54]. In addition, TLR3- and TLR4-activated BM-MSCs enhance the function of neutrophils, and the mechanism appears to be via the MSC secretome, as there was an absence of cell contact during incubation. Neutralizing experiments with MSC from various tissue sources revealed that the biological effects exerted on neutrophils by TLR3-activated MSC were mediated by the combined action of IL-6, IFNβ, and granulocyte macrophage colony-stimulating factor (GM-CSF), while those exerted by TLR4-activated MSC mostly depended on GM-CSF. MSC thus can sustain and amplify the functions of neutrophils in response to TLR3 and TLR4 triggering and may consequently contribute to inflammatory disorders. Another study determined that BM-MSCs can enhance the ability of neutrophils to phagocytize bacteria and to promote bacterial clearance in the peritoneum and blood [55]. The beneficial effects of MSCs could be reversed upon neutrophil depletion, demonstrating the importance of neutrophils for this MSC response in a model of sepsis. The role of any interactions between neutrophils and MSCs in the tumor microenvironment is unknown, but these studies would suggest that MSCs might be able to enhance the respiratory burst and other functions of neutrophils, which could act in a tumor-inhibitory manner.

3.1.2. Immune-Suppressive Effects of MSC on Innate Immunity Cells within the Tumor Microenvironment. The majority of the effects reported in the literature for the interaction of MSCs with innate immunity cells are of an immune-suppressive nature and involve several cell types, including macrophages, natural killer cells, and dendritic cells. Macrophages represent a major cell population involved in the innate immune response. The importance

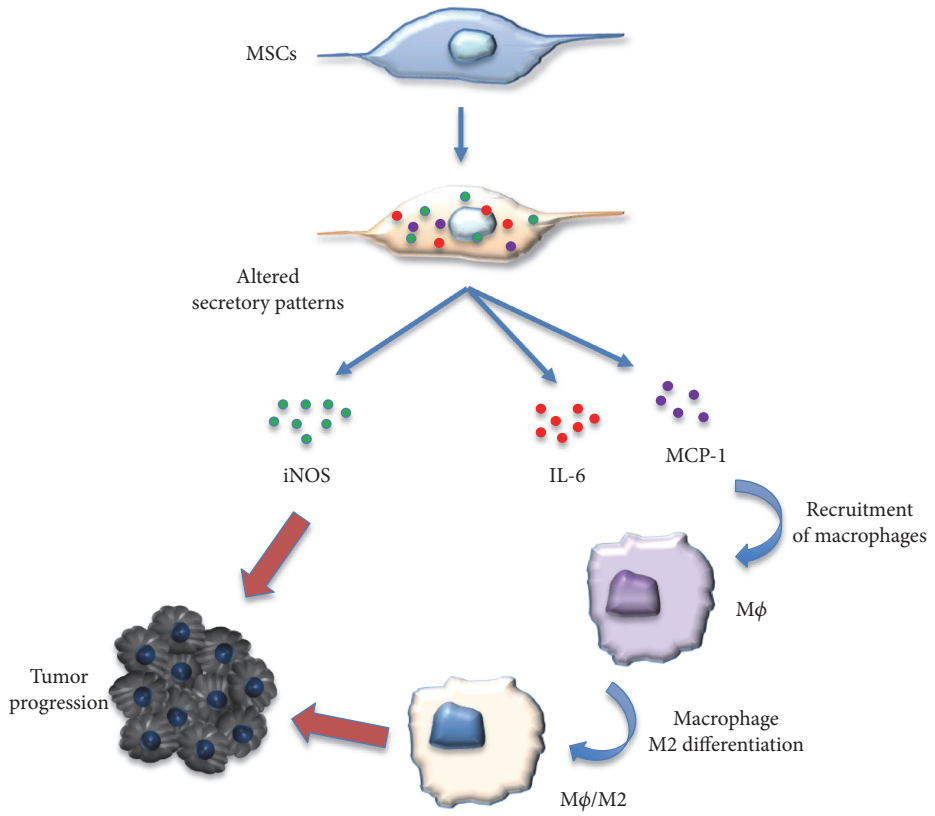

Figure 4: Interaction between macrophages and the MSCs in promoting tumor growth. M1 macrophages could activate the MSCs to adopt a regulatory phenotype, and the MSCs with an altered secretory profile promoted tumor growth by iNOS and MCP1 and induced macrophages toward M2-like macrophages.

of macrophages and immunosurveillance of tumors has been well established [56]. However, these cells can have drastically different functions depending on their polarization state. In fact, macrophages can elicit anti-inflammatory (M1) or inflammatory (M2) properties following exposure to different polarization signals [18]. Many of these polarization signals come from cytokines released within the niche of the tumor microenvironment. Their recruitment and maturation are tightly regulated to ensure proper control and remove aberrant cell populations.

MSCs actively influence the function of macrophages by influencing their polarization status (Figure 4) [57]. For example, MSCs shift the polarization of macrophages from a TNFα-secreting M1 signature to an immunosuppressive IL-10-expressing phenotype which may be mediated through a prostaglandin- (PGE-) 2-based mechanism [58]. Wolfe et al. also demonstrated that conditioned media collected from MSCs were able to induce an M2 phenotype in macrophages indicative of an upregulation of arginase 1 and CD206 [59]. This shift was also accompanied by an increase in IL-6 production by MSCs following coculture with M2 macrophages. While the duality of IL-6 expression is still under investigation, its expression has been associated with a range of protumorigenic functions including increased proliferation, angiogenesis, and immunosuppression [60]. Furthermore, MSCs isolated from neoplastic pancreatic tissue polarized macrophages into an M2 phenotype to a greater extent than "normal" or naïve MSCs [61]. This suggests that MSCs isolated from tumors change in response to the tumor microenvironment. Collectively, these studies suggest that the presence of MSCs can influence the fate of macrophages, which may alter their ability to detect and eliminate cancer cells, and this would create a more immunosuppressive environment.

Natural killer (NK) cells are a lymphatic cell population involved with the innate immune response. They play an essential role in the detection of cancer by discriminating healthy from unhealthy cell populations in order to properly mitigate a cytotoxic immune response [62]. The differential expression of CD56 has suggested that NK cells are present as a heterogeneous cell population within tumors, with distinct cytokine profiles and cytotoxic potential [63]. And similar to macrophages, MSCs can influence NK cell function through a range of processes. For instance, MSCs from umbilical cord, bone marrow, and adipose tissues have shown immunomodulatory effects by inhibiting activation of the $CD56^{low}$ subset of NK cells, which was accompanied by a decrease in TNFα expression [64]. MSCs also were able to decrease the proliferation and NK cell function by increasing the expression of the suppressor of cytokine signaling 3 in a recent model of sepsis [65]. MSCs isolated from acute myeloid leukemia and lung cancer tissues or cultured in the presence of conditioned media from HeLa cells showed an increase in TLR4 expression compared to naive MSCs. This

resulted in decreased cytotoxic function of NK cells potentially through the decreased release of the proinflammatory cytokines IL-6 and IL-8 by these MSCs compared to naïve BM-MSCs [66].

Interestingly, MSCs isolated from cancer patients do not always attenuate the function of NK cells. In fact, MSCs isolated from acute lymphoblastic leukemia patients increased the cytotoxic functionality of NK cells to a greater extent than healthy MSCs [67]. The influence of MSCs on the recruitment of NK cells has also been considered a potential therapeutic strategy. Genetically manipulated MSCs overexpressing sirtuin 1 have been shown to decrease tumor size in a subcutaneous tumor mouse model partly through the recruitment of NK cells [68].

Dendritic cells (DCs) play an important role in maintaining the activation of both the innate and adaptive immune response partially by being an important antigen-presenting cell (APC) type [69]. MSCs have shown the ability to alter the function of DCs. MSCs are able to suppress the maturation of DCs through the secretion of IL-10 and through activation of the signal transducer and activator of transcription (STAT)3 signaling, promoting decreased IL-12 production by DCs [70]. MSCs isolated from placental tissue were shown to attenuate the maturation process of human DCs as well as to alter the DC secretome by decreasing the secretion of IL-12 and IL-23 [71]. Within the tumor microenvironment, MSCs suppressed the ability of DC-mediated T cell mechanisms including IFNγ secretion and tumor cytotoxicity by reducing the amount of available cysteine excretion through a STAT3 mechanism [72]. MSCs isolated from chronic myeloid leukemia patients induced secretion of higher levels of TGFβ from DCs which in turn increased the differentiation of regulatory T (Treg) cell populations [73]. While still an active area of research, Tregs have been shown to be important in immunosuppression and have been linked to cancer progression [74].

The maturation and recruitment of cells associated with the innate immune response are tightly regulated by cell-to-cell and paracrine communication within the tumor microenvironment. MSCs represent a biologically active stromal population that has been shown to influence these processes in a range of innate cell populations, highlighting the importance MSCs have in the regulation of the innate immune response even when not directly involved in the immune response.

3.2. Changes in the Adaptive Immunity Response Mediated by MSC in the Tumor Microenvironment. Tumors are antigenic, and some display hundreds of mutations in coding exons, representing a large repertoire of antigens as potential targets for immune system recognition. But despite expression of abundant antigens, most cancers progress and evade destruction by the immune system. Analysis of the tumor microenvironment in patients with a variety of solid tumors has revealed that a major subset of tumors shows evidence of a CD8 T cell-infiltrated phenotype, but these become functionally inhibited by several mechanisms. These include programmed death ligand 1, expressed on tumor cells to limit activated T cell development and response; indoleamine-2,3-dioxygenase (IDO); and FoxP3$^+$ Treg cells. The development of this phenotype appears, in part, to be promoted by type I interferon signaling and DCs. Another interaction that appears to have a role in driving adaptive immune cell infiltration and persistence is the presence of stromal cells, such as MSCs, in the tumor microenvironment. MSCs impact adaptive immune cell recruitment and phenotypes in different ways, by promoting either immune activation or immune suppression within the tumor microenvironment (Figure 5).

3.2.1. Immune-Activating Effects of MSCs on Adaptive Immunity Cells within the Tumor Microenvironment. Immune activation by MSCs stems from the ability of these cells to activate allogeneic T cells in mixed leukocyte reactions (MLR) [4], an assay that assesses how T cell populations react to external stimuli by activation and proliferation. In a similar coculture model, MSCs have the ability to stimulate resting T cells to become activated and to proliferate [75]. Also, MSCs can behave as conditional APC in syngeneic immune responses [76], whereby TLR-activated MSCs recruit and activate immune inflammatory cells, likely through the secretion of proinflammatory cytokines by MSCs [77]. The clinical implications of this immune-activating phenotype are unknown, and whether these observations can be extended to MSC derived from other tissues is unclear at this time.

A very interesting report showed that human adipose-derived MSCs (ASCs) could induce what the authors called an "explosive" T cell proliferation, effectively activating resting immune cells [75]. When cocultured with peripheral blood mononuclear cells (PBMC), ASCs upregulated IL-6, IL-8, TNF, FGF, and VEGF, as well as IDO, suggesting strong crosstalk between cell populations. Following removal of ASC from the coculture, PBMC showed a large increase in proliferation, with a 25-fold increase after 7 days. The proliferating fraction of PBMC consisted of CD4 T cells with high CD25 expression, with FoxP3 cells increasing from 5 to 8.5%. These results suggest that ASCs can stimulate the activation and proliferation of Treg-type cells. Treg could be associated with tumor promotion or tumor inhibition, depending on the context. For example, another study reported the increase in Treg cells when ASCs isolated from breast cancer tissue were used in coculture with PBMC lymphocytes [78], and the effect was promotion of what appeared to indicate an anti-inflammatory reaction within breast tumors based on cytokine expression (IL-4, IL-10, TGFβ, CD25, and CCR4). It remains to be seen whether the net effect would have been tumor suppression or promotion, but the authors suggested that the likely effect would be tumor promotion. Other anti-inflammatory effects were observed in a clinical study where inflammatory nasal polyps (sometimes precancerous) were treated with ASCs. In ASC-treated patients, the proportions of CD4 and CD8 T cells decreased, with reductions in levels of Th2-type cytokines (IL-4 and IL-5) and significant increases in levels of Th1 cytokines (IFNγ and IL-2), as well as of regulatory cytokines (TGFβ and IL-10) [79]. Also, ASCs appeared to have immune regulatory effects by reducing the eosinophilic inflammation of nasal polyps. Downregulation

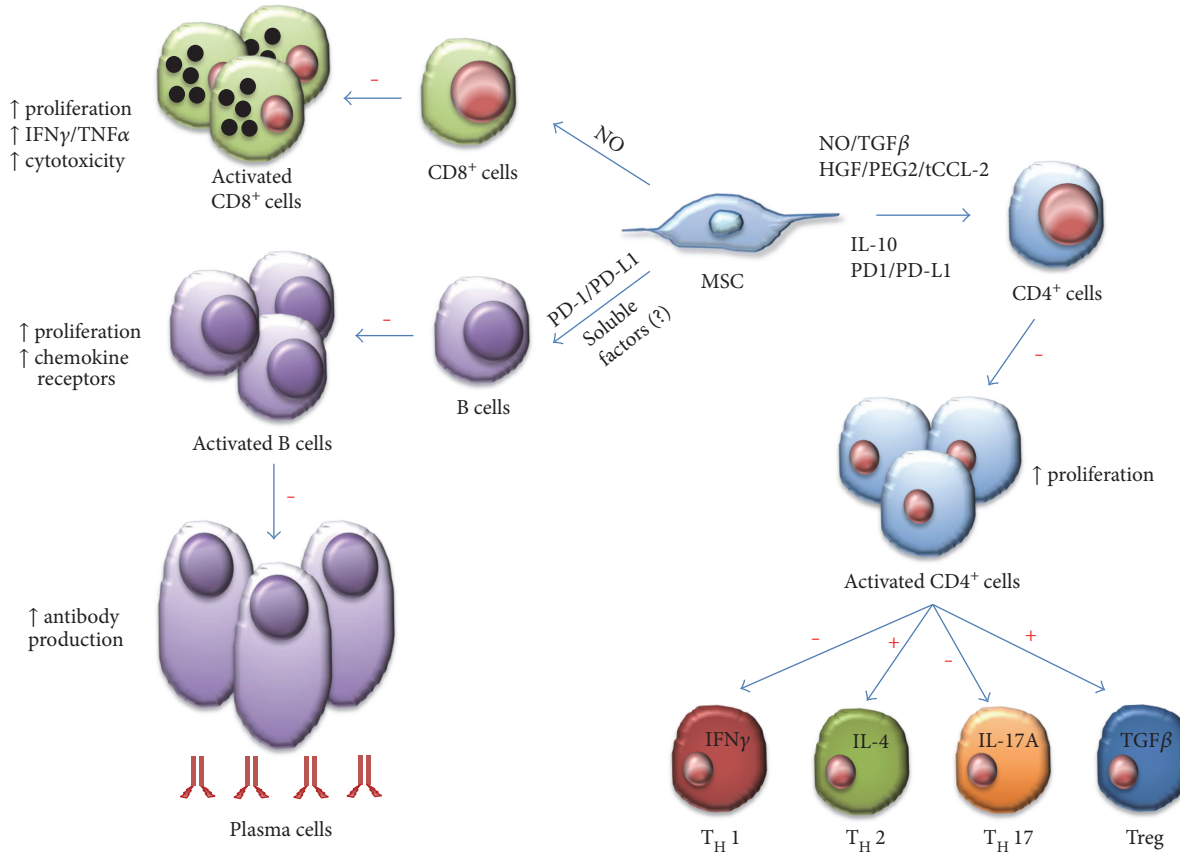

FIGURE 5: MSC immune suppression or promotion of adaptive immune cells. MSCs inhibit several aspects of B cell activity, including activation, proliferation, chemokine receptor expression, and differentiation to becoming antibody-secreting plasma cells. Unknown soluble factors and PD-1/PD-L1 ligation mediate these effects of MSCs on B cells. MSC can induce NO in response to inflammatory cytokine detection to suppress $CD8^+$ T cell proliferation, cytokine production, and cytotoxicity. In response to activation by certain cytokines, $CD4^+$ T cells can differentiate into numerous effector populations. MSCs produce soluble factors (NO, TGFβ, HGF, PGE2, truncated CCL-2, and IL-10) and membrane-bound molecules (PD-1 ligation) to achieve suppression of $CD4^+$ T cell proliferation and the polarization of $CD4^+$ T cells towards TH1 and TH17 cells. MSCs also favor the development of TH2 and anti-inflammatory Treg populations. Effects of MSC: + indicates promotion and − indicates suppression.

of activated T lymphocytes and a Th2 immune response and upregulation of a Th1 and eosinophilic inflammation could prevent progression of those nasal polyps predisposed to becoming tumors.

A provocative new idea to explain the dichotomy between the pro- and antitumor effects of MSCs may depend not only on how they recruit components of the immune system but on their localization within the body when the tumor first starts to develop. A recent study showed opposite effects on breast tumor growth when MSCs were coinjected or injected distantly [80]. Interestingly, in a 4T1 model of breast tumor development, the only variation was the site of injection of MSCs, demonstrating opposite effects on tumor growth for the first time in the same animal model (Figure 6). When injected locally with 4T1 tumor cells (coinjection), MSCs could initially promote the migration and invasion abilities of tumor cells but no significant difference was observed in late-stage lung metastasis. Coinjection of MSCs promoted angiogenesis by participating in the establishment of the tumor stroma. The distant injection of MSCs resulted in tumor-specific migration, presumably to provide structural and functional support to the tumor via differentiation into fibroblastic-like cells and pericytes. However, distant injection inhibited tumor progression and appeared to be directly related to promoting altered immune cell populations within the tumor. Treg and myeloid-derived suppressor cells (MDSC) were decreased significantly; CD8 T cells and APC were increased as a trend (although not significantly). Gene expression profiles of immune cells in the spleen and cytokine analyses of serum suggested that upregulation of TNF, IFNγ, TLR3, and IL-12 might explain the antitumor activity of distantly injected MSCs [80]. These interesting findings suggest that naïve MSCs are a double-edged sword in the modulation of tumor growth. In order to harness the potential of MSCs, several groups have genetically modified these cells with the goal of assessing their potential therapeutic effectiveness for a variety of cancers.

Tumor microenvironments are very similar to active sites of chronic inflammation. Since MSCs are able to home to inflammatory sites, researchers explored the possibility of using genetically engineered MSCs as delivery vehicles for

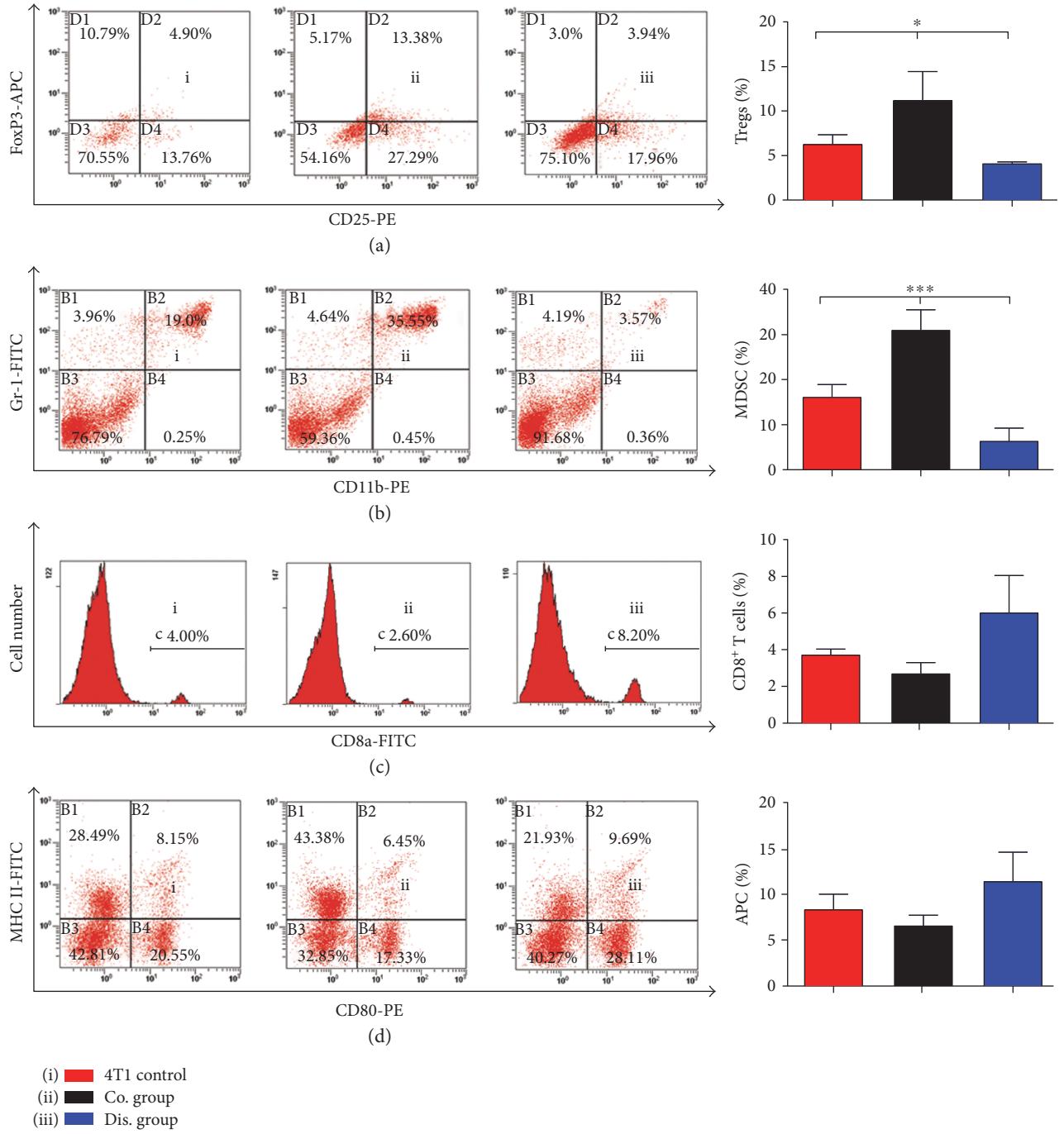

FIGURE 6: Flow cytometry shows changes in the proportion of immune cells in the spleen of mice on day 30 postinoculation. (a) Change in the proportion of Tregs ($CD4^+CD25^+Foxp3^+$) of $CD4^+$ T cells when gated for positive CD4-FITC cells ($n = 3$; one-way analysis of variance [ANOVA]). Proportions of MDSCs ($CD11b^+Gr\text{-}1^+$; $n = 3$; one-way ANOVA) (b), $CD8^+$ T cells ($n = 3$; one-way ANOVA) (c), and APCs ($CD80^+MHC\ II^+$; $n = 3$; one-way ANOVA) (d) were also altered. $^*p < .05$ and $^{***}p < .001$.

antitumor therapies. The first studies utilized MSC engineered to express anticancer genes such as IFNβ, showing that MSC can engraft and release their therapeutic products within the tumor microenvironment. MSCs that were gene-modified to deliver the immune-stimulating factor (LIGHT), a member of the TNF superfamily, also could induce breast tumor regression *in vivo* [9]. MSC-LIGHT retained tropism towards tumors and stimulated a potent antitumor response that promoted an influx of T cells into tumors and inhibited tumor growth. CD4 T cells were found to play a role in the induction phase of the immune response, and CD8 T cells were essential for the effector phase.

Gene-modified ASCs also can display antitumor activity *in vivo*, and this ability has been shown for a variety of gene products. For example, ASCs stably modified to

express IFNγ promote significant antimelanoma effects as compared to recombinant IFNγ treatment alone [81], suggesting that the mode of delivery of an antitumor cytokine in the tumor microenvironment is critical to its effectiveness. Also, this study showed that ASCs have immune modulatory properties that enhance the effects of IFNγ in the tumor microenvironment. ASC-IFNγ engrafted into the tumor stroma inhibited tumor growth and angiogenesis, prevented a systemic increase of Treg, increased $CD8^+$ T cell infiltration (but not IL-2^+ cells), and prolonged the survival of mice. A study by our group with ASC stably expressing the glycoprotein and antiangiogenic protein pigment epithelial-derived factor (PEDF) also has shown strong antitumor effects *in vivo* [82]. Like the IFNγ study, our group also observed that delivery of molecules secreted by gene-modified ASCs to tumors appears to be more potent than delivering recombinant proteins, as in the case of PEDF. These are interesting observations, but it remains unknown why molecules secreted from ASC are more potent in their antitumor activity—perhaps this can be attributed to the combination of cytokine production with a high production of extravesicles (exosomes) by ASCs. Exosomes bud from the cell to carry mediators which include proteins, miRNA, and mRNA [51], conveying regenerative signals during normal homeostasis, as well as relaying immune modulatory and therapeutic signals during tissue damage between tumor and stromal cells. The role of MSC-derived exosomes is being heavily investigated for therapeutic applications and holds promise for cancer therapy.

3.2.2. Immune-Suppressive Effects of MSCs on Adaptive Immune Cells within the Tumor Microenvironment. The effect of MSC on immune suppression is also well defined in the literature. These effects emerge from cell-cell interactions between MSCs, including ASCs, and both innate and adaptive immune cells [83–85] and are partly mediated through TLR pathways typically through inhibition of T cell proliferation [86]. TLR4 activation has immunesuppressive effects involving vascular cell adhesion molecule 1- and intercellular adhesion molecule 1-mediated binding of immune cells and TLR3 activation via hyaluronic acid interactions [54, 87]. MSC immune-suppressive abilities also can be mediated by the release of soluble factors with anti-inflammatory effects, including TGFβ, IDO, inducible nitric oxide synthase (iNOS), PGE2, and G-CSF [10]. MSCs also can prevent autoimmunity, as seen in a CCL2-dependent recruitment of myeloid-derived suppressor cells (MDSC), in a mouse model of experimental autoimmune uveitis [88]. Several reports point to a role for ASCs in inducing Treg cells, including those under lower oxygen (5% O_2) conditions, and other physiologically relevant mechanisms thought to involve cell-cell contact [89].

An alternative source of MSCs is perirenal ASCs, and these have been shown to enhance the percentage of induced Treg cells (iTreg) from effector cells through methylation of a region of the FoxP3 gene promoter [90]. iTreg had immunosuppressive capacities comparable to those of natural Treg (nTreg), and their induction was IL-2 receptor-dependent. The mechanisms employed by MSCs to inhibit effector T cell proliferation seem to overlap with the mechanisms involved in Treg induction, yet they do not interfere with this cell type's function. The inflammatory state also influences which types of chemokines ASCs express, potentially influencing the types of immune cells recruited to the tumor microenvironment. A study examined a coculture of ASCs with alloactivated PBMC in MLR, with proinflammatory cytokines IFNγ, TNFα, and IL-6 or under control conditions. In the presence of proinflammatory cytokines, ASC upregulated (by >200-fold) the expression of T lymphocyte attractants C-X-C chemokine ligand motif- (CXCL) 9, CXCL-10, and CXCL-11 and also upregulated the neutrophil, monocyte, and eosinophil attractants CXCL1 and CXCL6 (by >7-fold). The pattern of chemokine induction by ASC appeared to depend on the inflammatory stimulus. In ASC cultured with MLR, the expression of CCL-2, CCL-5, CCL-13, CCL-20, and CCL-28 was increased significantly compared to that in control ASC. Culture of ASC with proinflammatory cytokines strongly increased the expression of CCL-2, CCL-5, CCL-7, CCL-8, and CCL-13 but had no effect on the lymphocyte attractants CCL-20 and CCL-28 [91]. Thus, ASCs can be altered by different inflammatory conditions and, importantly, can be preconditioned *in vitro* for potential clinical immune therapy use.

Interestingly, although the research on MSCs has mainly focused on their effects on T cells [17] with data regarding the modulatory effects of MSCs on alloantigen-specific humoral response in humans being scarce, it has been demonstrated recently that MSCs significantly affect B cell functioning [92]. ASCs support the survival of quiescent B cells and target B cell differentiation towards B regulatory cells (Breg, $CD19^+CD24^{hi}$ $CD38^{hi}$). Such an effect could impact B cell responses in immune diseases such as rheumatoid arthritis, but the effect in the solid tumor microenvironment is currently unknown. The effect on blood tumors such as chronic lymphocytic leukemia (CLL) appears to be bidirectional activation between bone marrow MSC and tumorigenic B cells. Coculture of MSCs protected CLL B cells from both spontaneous and drug-induced apoptosis [1]. The CD38 expression was upregulated in CLL B cells with MSC coculture. In MSC, ERK phosphorylation and AKT phosphorylation were detected when CLL B cells and MSC were separated by a transwell, indicating soluble factor activation of MSC. This study adds to the evidence that in human tumors, including hematological malignancies, stromal cell interaction with tumor cells significantly impacts the critical features of both cell types.

4. Polarization of MSC Can Help Explain Their Dual Effect on Tumorigenesis and Immune Modulation

There is increasing evidence that the activity of human MSCs is greatly modulated by the stimulation of TLRs. TLRs are a

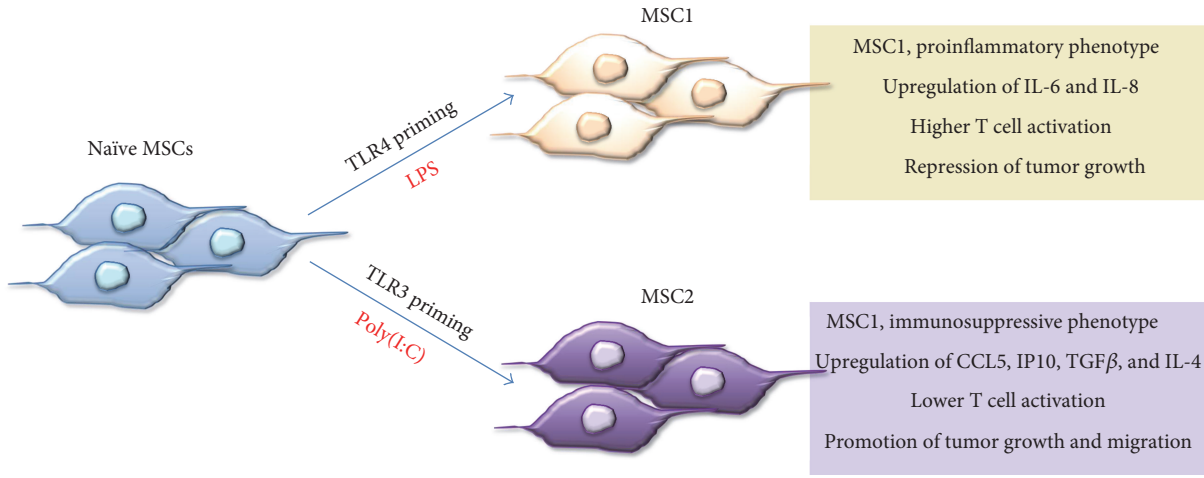

Figure 7: Interactions of MSC with the microenvironment leading to polarization. MSC polarization by different stimuli such as LPS or poly(I:C) through stimulation of either TLR4 or TLR3 receptors, respectively. Two polarized MSC phenotypes emerge from TLR stimulation, which represent a proinflammatory phenotype (MSC1, antitumorigenic) and immune-suppressive phenotype (MSC2, protumorigenic).

family of pattern recognition receptors that act upon recognition of pathogen-associated molecular patterns. These triggers act to promote intracellular signaling mechanisms leading to the synthesis and secretion of cytokines by leukocyte subsets and nonimmune cells. Eleven TLRs (TLR1–11) have been identified so far in human cells [93]. Several of these TLRs have been reported to be expressed by MSCs, at different levels depending on the tissue of origin. BM-MSCs have been reported to express TLR1-2 [94–98], TLR3 [86, 94–99], TLR4 [66, 86, 94–99], TLR5-6 [95–99], TLR7 [94–96], TLR9 [94–97], and TLR10 [97]. Wharton jelly-derived MSCs have been reported to express a similar TLR profile than BM-MSCs with the notable absence of TLR4 [95]. And ASCs have been reported consistently to express TLR1–6 and TLR9 [95, 97, 100] and less consistently to express TLR7 [100] and TLR10 [97, 100]. These receptors have been associated with the modulation of multiple MSC properties, including differentiation capability [86], migration [86, 94], extracellular matrix deposition [86], secretome [3, 66, 86, 94–101], immunomodulation [3, 86, 94, 102], and modulation of tumor progression [66, 101]. However, inconsistent reports of the modulation of these properties are found in the literature. As we have discussed in previous sections, the role of MSCs in immunomodulation is primarily achieved by the secretion of cytokines that affect the activity of immune cells. Thus, multiple groups have examined the role of changes in the secretome of MSCs upon TLR ligation (primarily TLR3 and TLR4) in order to understand the role of TLR2 in MSC-mediated immunomodulation.

Multiple studies report similar responses from the stimulation of TLR3 or TLR4 on BM-MSCs and ASCs with their respective agonists LPS and polyinosinic:polycytidylic acid (poly(I:C)). Stimulation with these ligands has been reported to promote the expression of cytokines and chemokines with roles in immunomodulation and inflammation such as CXCL10 [94], IL-6 [86, 94–97, 100, 101], IL-8 [86, 94–96, 100], CCL5 [95, 96], IL-12 [94, 95, 97], IL-27 [95], IL-23 [95], IL-1β [96], MIP3α [97], TNFα [94, 97], and CCL2 [97]. Similarly, TLR ligation with poly(I:C) and LPS has been associated with activation of NFκB signaling [100]. Although many of the upregulated cytokines have roles in the modulation of immune cells, including neutrophils, lymphocytes, DCs, macrophages, and NK cells, there is little to no consensus on the effect of TLR ligation on immunomodulation. In 2009, Lombardo et al. concluded that TLR ligation did not have a significant effect on the immunogenic properties of hASCs when they evaluated the immune-modulating activity on peripheral blood lymphocyte proliferation or activation [100]. In contrast, Cassatella et al. and Liotta et al. reported a reduction in the immunosuppressive activity of MSCs that promoted the T cell and neutrophil survival, activation, and response upon TLR ligation [54, 102].

Although most studies report similar results following stimulation with poly(I:C) or LPS, contrasting phenotypes have been described for the stimulation of these TLRs in BM-MSCs. These phenotypes were described as the proinflammatory MSC1 and the immunosuppressive MSC2 [86] (Figure 7). Characterization of these phenotypes led to the understanding that the low-level exposure of MSCs to the TLR4 ligand generates the MSC1 phenotype, whereas the ligation of TLR3 to double-stranded RNA or poly(I:C) generates the MSC2 phenotype. MSC1 shows an increased synthesis and secretion of proinflammatory cytokines and chemokines, such as IL-6 and IL-8, whereas MSC2 has increased production of immunosuppressive mediators such as IP-10 and CCL5. Other phenotypes are polarized as well. For instance, MSC1 has been reported to permit T lymphocyte activation and attenuate the tumor progression relative to naïve MSCs. On the contrary, MSC2 has been associated with the suppression of T lymphocytes and the promotion of tumor growth and metastasis [3, 86]. Multiple factors account for the variability of responses seen upon the stimulation of TLRs in MSCs, including tissue of origin [95], species of origin, and environmental conditions [97, 98].

Exposure to inflammatory cytokines has been reported to alter the TLR profile of MSCs [96, 98], causing an upregulation of TLR2-3 [95, 96, 98], TLR4 [95, 98], and TLR7 [96] and a downregulation of TLR6 [98] which causes a change in the responsiveness of the cells to TLR stimulation [98]. Additionally, Romieu-Mourez et al. reported that the combination of TLR3 and TLR4 stimulation with inflammatory cytokines increases the response of the MSCs and can synergistically upregulate the secretion of certain cytokines and enzymes [96]. IFNα and poly(I:C) synergistically upregulated IL-12, TNFα, CCL5, IFNβ, and iNOS. Similarly, the two ligands acted in synergy with IFNγ for the upregulation of CCL5, TRAIL, TNFα, IL-12, and iNOS. Additionally, increased neutrophil chemotaxis was observed when attracting the cells with conditioned media from hMSCs treated with IFNγ and LPS. This increased chemotaxis was associated with the increased secretion of IL-6 and IL-8 resulting from this combined treatment.

In 2015, tumor-derived MSCs from acute myeloid leukemia and lung cancer tissues and cells cultured in conditioned media from HeLa cells were reported to present a higher expression of TLR4 compared to unsorted MSCs [66]. These MSCs were also found to have a lower secretion of IL-6 and IL-8 than other MSCs. However, the secretion of these cytokines experiences a more significant expression enhancement upon LPS stimulation. Additionally, NK cell proliferation was suppressed by these $TLR4^+$ MSC, and the suppression was enhanced by the activation of the cells with LPS.

The study of TLR ligation effects on MSCs could provide key information relevant to the development of treatments for the targeting of different types of cancer [103] and inflammatory diseases [104–106]. However, the current understanding of these phenomena is challenging due to the variability in stimulation conditions (i.e., ligand concentration, base media, time of exposure), sources (i.e., donor, tissue of origin, species), and culture conditions (i.e., media passaging) of cells among the multiple studies, resulting in the inconsistent phenotypes currently found in the literature.

5. Harnessing the Power of MSC for Immune Therapy

Understanding and harnessing the polarization of MSCs in the tumor might provide us with tools to augment effectiveness of current and future immune therapies. Eight current or recent clinical studies point to the promise of using MSCs (naïve or gene-modified) for cancer therapy. These studies include several phase I trials including MSC expressing IFNγ or MSC bearing the sodium symporter gene for ovarian cancer therapy, as well as allogeneic BM-MSC for localized prostate cancer, and MSC bearing GX-051 for advanced head and neck cancer. Phase 1-2 trials are being held for MSC coinfused with hematopoietic stem cells for treating leukemia and other myeloproliferative disorders and MSC bearing CRAd (oncolytic adenoviruses) for children and adults with multiple types of metastatic and refractory solid tumors.

Additionally, since MSCs (and ASC) are found in stromal cell niches of various tumor types, it is possible that MSC can exploit the properties related to tissue repair to promote tumorigenesis and/or protect epithelial cells from the effects of chemotherapy. By restoring the MSC ability to modulate anticancer immunity, perhaps one can hijack the tumor to favor infiltration of immune cells and reduce tumor bulk or reduce metastasis.

6. Conclusions

MSC biology exhibits high plasticity and thus should be studied in a relevant environmental context. The several studies we have reviewed here only begin to shed light on the effects of each variable in the microenvironment on tumor progression, as each variable is usually tested and reported in isolation. In reality, MSCs either are in their natural environment or are homing to new environments to encounter various signals at the same time and in various sequences. The complexity of tumor microenvironments, for example, makes the interpretation of studies that focus on a few molecules difficult, and it is important to remember to draw limited conclusions about each study. The same principle goes for tumor-derived MSCs or stromal cells being used to deliver anticancer therapy or modulate cancer immunity. Once MSCs arrive at the cancer site, they meet epithelial tumor cells and stromal cell-derived factors in high concentrations, including cytokines, chemokines, other immunomodulatory small molecules, and various DAMPs coming from dying malignant cells. Ultimately, the result of all these environmental factors will determine how MSCs will actually behave *in vivo*. Different cells contribute to tumor development, including the classically described tumor stromal cell components and also MSC in the local tumor stroma, interconnected in a network of crosstalk and mutual modulation. The network includes cell-cell contact and a specific secretory profile acquired by MSCs during this interaction, enabling them to perform as proinflammatory cells or as anti-inflammatory cells and to alter the tumor microenvironment. The application of MSC for cell therapy purposes will have several benefits over that of other stem cells including induced pluripotent stem cells including the possibility of easy availability, low in vitro manipulation requirements, potential autologous application, and a lower risk of tumorigenicity. MSCs have high plasticity in adapting to different tumor microenvironments, raising the possibility of experimental modulation or priming of their "phenotype." The therapeutic potential of MSCs offers enormous hope for treating tissue defects and numerous diseases, including cancer.

7. Future Directions

MSCs can self-renew, differentiate into multiple lineages, and exhibit proangiogenic and immunomodulatory effects. Along with these intrinsic properties, MSCs exhibit natural tropism toward inflamed tissue, which has led to the clinical application of these cells in different therapies. However, a significant barrier is the inability to localize the cells to the tissue of interest due to low homing efficiency, poor engraftment, and low cell retention. To circumvent these challenges, it is critical to develop engineering strategies that can

improve tissue engraftment as well as enhance the therapeutic potential of these cells. The therapeutic application of MSC (or ASC) will require a relatively long-term culturing method, which can result in senescence of cells and a potential reduction in the therapeutic activity of transplanted cells. If the immune-suppressive or immune-stimulatory capacity of MSC can be restored via careful and purposeful "polarization," their application might be harnessed to its full therapeutic potential.

Conflicts of Interest

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

Acknowledgments

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Review Article

Regulatory Role of Redox Balance in Determination of Neural Precursor Cell Fate

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In 1990s, reports of discovery of a small group of cells capable of proliferation and contribution to formation of new neurons in the central nervous system (CNS) reversed a century-old concept on lack of neurogenesis in the adult mammalian brain. These cells are found in all stages of human life and contribute to normal cellular turnover of the CNS. Therefore, the identity of regulating factors that affect their proliferation and differentiation is a highly noteworthy issue for basic scientists and their clinician counterparts for therapeutic purposes. The cues for such control are embedded in developmental and environmental signaling through a highly regulated tempo-spatial expression of specific transcription factors. Novel findings indicate the importance of reactive oxygen species (ROS) in the regulation of this signaling system. The elusive nature of ROS signaling in many vital processes from cell proliferation to cell death creates a complex literature in this field. Here, we discuss the emerging thoughts on the importance of redox regulation of proliferation and maintenance in mammalian neural stem and progenitor cells under physiological and pathological conditions. The current knowledge on ROS-mediated changes in redox-sensitive proteins that govern the molecular mechanisms in proliferation and differentiation of these cells is reviewed.

1. Introduction

The central nervous system (CNS) consists of the brain and spinal cord, which are comprised mainly of neurons, astrocytes, oligodendrocytes, and microglial cells. The earliest evidence that proliferating cells contribute to postnatal neurogenesis was proposed in mid-1960s [1, 2]; however, it took three more decades to find evidence of proliferating multipotential neural stem and progenitor cells in cell cultures of the embryonic and adult mammalian brain [3, 4] and spinal cord [5]. Shortly after these reports, other elegant studies showed the detailed anatomical location of these cells that are collectively known as neural precursor cells (NPCs) [6]. The NPCs play an important role in the generation of glial and neuronal cells in development and also function as a reservoir for normal tissue turnover [7]. The involvement of NPCs in memory formation and their capacity to proliferate and differentiate into different nervous tissue cells have inspired extensive research in the pursuit of an ultimate cure for the treatment of many diseases that are often associated with neural cell death, including neurodegenerative diseases, stroke, and spinal cord injury. Despite more than two decades of research, the details on factors that regulate NPC proliferation and differentiation are not quite clear. Neurotrauma and stroke have been shown to affect NPC population through a mixture of promoting cell proliferation and inhibitory factors for migration and appropriate differentiation [8, 9]. However, despite some increase in NPC proliferation in response to injury, the extent of their contribution towards efficient cell replacement and tissue repair remains very limited. Although the negative effects of trauma on NPCs have been attributed to the hostile posttrauma extracellular milieu [10, 11], the underlying mechanism involved in the postinjury rise in NPC proliferation remains

unexplored. The identification of these factors that increase the capacity of NPCs for proliferation may lead to the identification of novel therapies.

Reactive oxygen species (ROS) are naturally generated in the mitochondria as an inevitable part of the oxidative phosphorylation respiration. The overall level of ROS is increased after any stress conditions including neurotrauma, creating a dual-edge sword that induce the removal of damaged tissue and initiate the repair process. ROS and their contribution to biological systems can be compared to the need for table salt in our diet; while a moderate amount of salt is needed, excessive quantity will undermine our health. In this manuscript, we aim to review the current literature on potential involvement of ROS in the regulation of NPC proliferation and differentiation. We will discuss some of the underlying signaling systems and antioxidant systems that have been shown to play a role in these processes.

2. Neural Stem and Progenitor Cells or Neural Precursor Cells (NPCs)

The NPCs are responsible for the normal turnover of the neural cell tissue. The common cardinal properties of the NPCs are their ability to self-renew and their capacity to differentiate to different neural cells. The formation of new neurons in adults is specifically localized to two regions: the subventricular zone (SVZ) and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus in the CNS. The SVZ niche has been well studied in detail and is responsible for the generation of new neurons for the olfactory bulb. There are four cell types found in this region: the ependymal cells (Ep) line the ventricle and separate the cerebrospinal fluid from the SVZ. A highly proliferating cell type, known as neuroblasts or type A, is arranged in a chain [12]. These cells are migratory in nature and move in the rostral migratory stream to reach the olfactory bulb. A second type identified by their slow proliferation rate is known as type B cells. These cells are large quiescent cells with long processes and are located in close proximity next to the ependymal cells. These cells display a glial phenotype and contain GFAP intermediate filaments. Type B cells generate type C cells that are scattered in SVZ, representing transiently amplifying cells. Type C can divide once or twice and give rise to type A neuroblasts [13]. Thus, the SVZ is a region with a heterogeneous cell population, each with a different proliferative capacity. A recent study elegantly shows that a distinct subtype of NPCs in SVZ directly responds to signals originated from the hypothalamus ventromedial nucleus (satiety centre) [12]. Direct innervation of SVZ was shown to induce or impede cell proliferation in specific subtypes of NPCs mediated by the availability or the scarcity of food. The authors postulate that this may specifically affect the neuronal population of the olfactory bulb neurons for the animal [12]. Understanding the factors that regulate NSC proliferation can be used for the identification of novel therapies.

3. ROS and Oxidative Stress

Reactive oxygen species (ROS) are produced as a by-product of cellular metabolism. However, ROS production in aerobic organisms is majorly integrated as a signaling system rather than a negative regulator [14]. ROS levels are tightly regulated by a variety of means resulting in a balance between ROS production and their consumption known as redox balance. This balance is cell specific and reflects the cellular metabolism, specific function, and even the stage of cell cycle. For instance, young/proliferating cells have the highest levels of reducing antioxidants and therefore have a more reduced redox balance. As the cells proceed to differentiation, redox balance becomes more oxidized in aged/dying cells [15]. The redox balance also determines the cells' function, for example, immune cells that are involved in the phagocytosis of foreign pathogens generate high levels of ROS [16], while hepatocytes are equipped with reducing antioxidants to neutralize the many xenobiotics entering our body through the gastrointestinal system [17].

There are three major sources of ROS in the cells: mitochondria, cell membrane, and endoplasmic reticulum (ER). One of the most common forms of ROS, the highly reactive superoxide anion $[O_2^{\bullet}]^-$, is formed in mitochondrial complex III (cytochrome C oxidase) and complex I, located in the inner membrane of mitochondria as the components of mitochondrial electron transport chain [18, 19]. Additionally, cytosolic superoxide is produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) located in the cellular plasma membrane and the endoplasmic reticulum (ER) [18]. Superoxide anions can react with nitric oxide (NO) to generate the highly damaging peroxynitrite ($ONOO^-$) [20]. Superoxide is enzymatically converted to hydrogen peroxide (H_2O_2) by superoxide dismutase located in the cytosol (SOD1) and mitochondria (SOD2). Despite lower reactivity of H_2O_2, this ROS has a longer half-life and can diffuse across biological membranes [21]. These properties make H_2O_2 an important player in cellular signaling. The damaging effects of H_2O_2 resulted from its conversion to hydroxyl radical ($OH^{\bullet}$) in the Fenton reaction catalyzed by Ferrous ion (Fe_2^+) [22]. The hydroxyl radical ($OH^{\bullet}$) is the most reactive and damaging free radical in biological systems and can extract electrons from other molecules, including membrane phospholipids. This results in the formation of lipid radicals that generate a chain reaction of lipid radical and lipid peroxide formation. Although vitamin E has been credited for scavenging lipid free radicals, the vitamin E itself is converted to α-tocopherol radical that must be quenched by vitamin C and thiol antioxidants [23]. Lipid peroxides can also go under the Fenton reaction and produce more hydroxyl radicals that exacerbate the cellular damage [24]. Therefore, the regulation of the peroxide (H_2O_2 and ROOH) level is a critically important task in maintaining cellular health. Thiols containing peptides and proteins are the prime antioxidant pool involved in the management of peroxides. In the following sections, we will examine the known effect of ROS in cell signaling, especially in the context of NPC cell proliferation, and will

review some of the antioxidants that have been shown to affect NPC proliferation and differentiation.

4. Role of ROS in Cell Signaling: A Basic Understanding

Although the ROS-mediated damage has been well studied, developments of new tools and techniques during the last two decades have increased our knowledge of the ROS physiological importance in the cells. In prokaryotes, several transcription factors are directly regulated by ROS-mediated oxidation of their thiol groups located on cysteine residues [25]. The same principle applies in higher organisms where the distribution of cysteine is disproportionally high. The human proteome encodes about 214000 cysteine residues, reflecting a selective enriching of this amino acid during evolution [26]. The importance of cysteine in cell physiology has been related to the chemical property of sulfur that enables stable covalent bonds with major elements (C, N, O, H, and P) and transitional metal ions [27]. The thiol group in cysteine is readily oxidized by reactive ROS such as H_2O_2 and forms sulfenic acid (–SOH). This is highly unstable and is quickly converted to a disulfide bond. This step is reversible by thiol antioxidants; however, under excessive oxidative conditions, cysteine is further oxidized and forms the irreversible sulfinic (–SO2H) or sulfonic (–SO3H) acid [28]. Nitrosylation and glutathionylation are other forms of oxidative modification of cysteine residues that lead to changes in the structure and function of the cellular proteins [29]. Methionine is another thiol containing amino acid that responds to changes in the redox state of the cell by transient oxidation (methionine sulfoxide) and reduction by methionine oxide reductase [30]. These properties offer a unique position for cysteine/methionine as the interface between the extracellular environment (exposome) and the cellular proteome mediated through ROS. This is well exemplified in the original discovery of NPCs by their responsiveness to growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF2) [31]. The effect of these growth factors is mediated through activation of NOX and generation of superoxide radical. This is quickly converted to H_2O_2 that will then activate the downstream signaling by *activation* of tyrosine kinases and concurrent *inactivation* of phosphatases to optimize the effect of the extracellular factors [32]. Our lab has recently shown that the level of ROS can affect cell-signaling machinery between cell survival and cell death [33]; therefore, the levels of ROS must be tightly regulated. We further showed that oxidative modification of methionine residues in cathepsin L may be responsible for a balance between the protective autophagy and apoptosis [34]. The thiol group in glutathione, thioredoxin, and glutaredoxins has a low redox potential enabling them to act as an electron buffering pool and therefore a central player in the regulation of cell signaling. The redox sensitivity of signaling proteins in cell proliferation has not been adequately investigated. New advances in the field of redox biology will identify new players in this field.

5. ROS as a Mediator in Growth Factor Response and Cell Proliferation

Several extracellular signals such as growth factors trigger cell proliferation. Extracellular ligand binding activates receptor tyrosine kinases (TrK), and sequential phosphorylation of its components relays the stimulus intracellularly to initiate the cell cycle machinery. Phosphatases act on kinases to prevent continuous stimulation and provide temporal and spatial signal specificity. Although the profile of underlying proteins involved in this process are well identified, recent advances in redox biology indicate the key role of ROS in the feed-forward amplification of the signaling cascade [35]. The identification of cysteine residues in the active site of these key signaling proteins mediate the redox regulatory of ROS and thiol antioxidant systems in these processes. Growth-promoting external stimuli that can elevate intracellular ROS levels predominantly originate from plasma membrane-bound NADPH oxidase (NOX). These ROS signals result in the inhibition of protein tyrosine phosphatases (PTP) in their vicinity, through oxidation-driven structural changes. This inhibition is reversible with reducing agents or proteins. PTPs at these sites are required to modulate the receptors' sensitivity for growth factors. This phenomenon is involved in the regulation of cell proliferation and is affected by cellular seeding density in cell culture conditions. At low density, elevated ROS levels in growing cells inhibit PTP activity [36]. Thus, TrK function is promoted/prolonged in these conditions, resulting in increasing cell proliferation rate. Cell-cell contact is known to decrease cell proliferation rate through ROS signaling modulating receptor phosphorylation/dephosphorylation activity. Such "contact inhibition" is associated with decreased intracellular ROS levels which activate phosphatases to ameliorate growth factor signaling in the cell culture system [37, 38]. Growth-promoting external stimuli can elevate intracellular ROS levels, through NOX in the plasma membrane.

EGF receptor is one of the well-studied signaling pathways that is involved in the regulation of NPC proliferation in developing mouse brain [31]. The activation of EGF receptors and the enhancement of cell proliferation in this system is known to be mediated by transient increase in H_2O_2 levels via small GTPase Rac1-stimulated NOX1 [39]. H_2O_2 elevation is crucial to relay the changes in the phosphorylation status of tyrosine receptor proteins (EGF receptor and phospholipase C-γ1) for the activation of intracellular signaling pathways. Notably, the inhibition of H_2O_2 production by increased catalase activity decreased the proliferation of human epidermoid carcinoma cells [40]. Besides EGF, the presence of bFGF receptors on NPCs is associated with the "stemness" of these cells resulting in colonial expansion in vitro in response to these growth factors [31]. Platelet-derived growth factor (PDGF) receptor (PDGFR) also plays a role in the development of CNS and neuroprotection [41]. PDGFR is also known to regulate oligodendrocyte progenitor formation and neuronal specification [42, 43]. Naïve NPCs do not express PDGFR but the expression of this receptor is rapidly increased in a subset of NPCs that are fated towards immature neurons and oligodendrocytes.

PDGF also stimulates neuronal fate adaptation in dividing neural progenitor cells by promoting the expansion of immature neurons [44]. The mitogenic effect of PDGFR is similar to the activation of EGFR through transient elevation of intracellular H_2O_2 levels by NOX1. NOX1-mediated H_2O_2 elevation is also associated with induced proliferation in rat smooth muscle cells and human hepatic stellate cells [45, 46]. A direct relevance of nontoxic H_2O_2 addition (2–4 μM) and high proliferation of NPCs has been reported where inhibition of NOX decreases NPC proliferation and neurogenesis [47]. Conversely, a previous report claims that H_2O_2 treatment decreases NPC proliferation [48]; however, it is noteworthy that concentrations of H_2O_2 used in this study were beyond its physiological levels (up to 100 μM). Further confirmation of ROS inhibitory effect on NPC proliferation was shown after the upregulation of cellular antioxidant levels by N-acetylcysteine (NAC, a precursor of glutathione). Enhanced cellular reducing capacity increased O2A progenitor population (oligodendrocyte and type 2 astrocyte bipotent progenitors) and decreased oligodendrocyte differentiation [49], indicating that low ROS levels are important for cell proliferation and limit differentiation. Conversely, increase in mitochondrial-generated superoxide load or knockout of SOD2 was associated with decreased NPCs in developing mice brain [19]. Similarly, NPC-specific ataxia-telangiectasia mutated (ATM) knockout shows elevated intracellular ROS levels which negatively affected cell proliferation and neurogenesis [48]. This contradictory findings may indicate the delicate role of ROS balance in NPC expansion (proliferation) and differentiation. NPCs can be quiescent but will undergo activation with spiking ROS levels and proliferate to give rise to more NPCs. Similarly, differentiating cells have more ROS than its ancestor progenitors due to metabolic shift from aerobic glycolysis to oxidative phosphorylation [50, 51]. An in vitro report showed that post mitotic exit of neuroblasts to form immature neurons was associated with increased ROS levels and enhanced levels of mitochondrial electron transport proteins [52]. These information cumulatively indicate that ROS levels in a concentration-dependent manner modulate NPC proliferation and differentiation; however, the fine-tuning of signaling is regulated by many transcription factors.

6. ROS and NPC Proliferation

It is a well-accepted fact that all stem cells reside in areas with much lower oxygen than with the ambient oxygen levels, yet much of our knowledge on NPCs' biology comes from culturing these cells in normal tissue culture incubators containing the atmospheric 21% oxygen. The beneficial effect of lowered oxygen on NPCs was reported originally by Studer et al. [53], which showed increased proliferation and cell survival in these cells. Moreover, the positive effect of normal oxygen levels on the proliferation of NPCs has been extensively reviewed [54]. Additionally, cell differentiation was also affected by increased expression of neuronal markers when NPCs from developing midbrain were exposed to low oxygen pressure ($3 \pm 2\%$) [53]. The beneficial role of this physiological normoxia or "physoxia" has been partially credited to lower ROS levels in these conditions which improved cell survival of primitive neural stem cells [55]. The underlying mechanism in these conditions is well illustrated by positive regulatory domain-containing protein 16 (*Prdm16)*, which is preferentially expressed in primitive stem cell pools. A decrease in Prdm16 level increases ROS levels, causing depletion of hematopoietic and neural stem cells by increasing oxidative stress-mediated cell death. The regulatory role of Prdm16 is applied through its promoter control for hepatocyte growth factor (Hgf) gene with antioxidative properties. Thus, ROS elevation is correlated with decreased Prdm16 and downregulation of its downstream target Hgf [56]. Opposing observations of ROS-positive effects on NPC proliferation came from an elegant study by Le Belle's group [47]. In this report, the authors convincingly showed that highly proliferative neural stem cells contain increased levels of ROS, and experimental manipulation of their levels severely affected normal NPCs in vitro and in vivo [47]. However, these experiments were all conducted under normal atmospheric oxygen concentrations, posing the question whether these findings are relevant to physiological oxygen levels in the CNS.

The complexity of ROS involvement in proliferation and differentiation is further elevated by the type of ROS or the antioxidants used in these experimental conditions. An example of the different ROS-subtype effects on cell proliferation has been shown previously [57]. This group showed that in vascular smooth muscle cells, superoxide anions generated by xanthione/xanthione oxidase increased cell proliferation through the induction of Id3, a DNA-binding inhibitor protein. However, the cell proliferation was inhibited when cells are treated with H_2O_2, where the transcription factor gut-enriched Kruppel-like factor (GKLF) is induced. The group showed that Id3 : GKLF ratio regulates the cell proliferation, as overexpression of Id3 overcomes the inhibitory effect of GKLF. This study showed that specificity of ROS effects may be mediated through different redox-sensitive proteins.

ROS also are involved in NPC differentiation as was recently reviewed [51]. Overall, NPCs' proliferation has been inversely related to oxygen levels, and therefore in hypoxic zones, NPCs remain mostly proliferative. Upon the induction of mitochondrial activity and oxidative phosphorylation, the rise in ROS levels or exposure to stressful conditions inhibits proliferation and promotes cell differentiation [51]. Understanding ROS production and regulation in NPCs provide a window of opportunity to optimize cell proliferation and differentiation.

7. ROS Regulated Proteins and Transcription Factors

Redox signaling is a fast-paced and transient process in which electrons are transferred between the oxidizing ROS and redox-sensitive proteins, such as tyrosine kinases and phosphatases [58]. The interaction between ROS and these signaling molecules results in the transduction of external signals to NPC proliferation and differentiation. A sophisticated antioxidant system is required to maintain the balance

between ROS levels and available antioxidants. Several transcription factors are known to be activated by the redox status of the cell. These include ATM, FoxOs, Nrf2, HIF1α, and APE1 that are known to regulate redox-driven signals with regard to NPC fate determination.

7.1. ATM Regulates NPC Self-Renewal and Differentiation. Ataxia-telangiectasia mutated (ATM) is a serine/threonine protein kinase involved in redox balance, DNA repair, and cell proliferation [59]. ATM was reported to be essential for adult neurogenesis as high levels of ATM expression are seen in neuronal progenitors, which decreases during differentiation, suggesting a role for ATM in the maintenance of proliferating NPCs. Ataxia-telangiectasia patients exhibit an aberrant neuronal differentiation, which is possibly due to excessive yet abnormal proliferation of neuronal progenitors [60]. Under normal condition, elevated ROS levels activate ATM, which leads to the downstream activation of p53, causing senescence in proliferating cells [61]. ATM regulates ROS levels in self-renewing NPCs, as its knockout increases ROS levels leading to oxidative stress, resulting in decreased proliferation of NPCs which can be rescued by antioxidants or p38 MAPK inhibitor [48]. This study suggests that excessive upregulation of ROS levels is detrimental for cell proliferation.

7.2. FoxOs. FoxO proteins (forkhead transcription family O) are a group of transcription factors that have consensus binding site. FoxO is known to double life expectancy in *Caenorhabditis elegans.* Interestingly, there is a strong association with human longevity and the involvement of a genetic variant of FoxO3A. Animal studies suggest a correlation between FoxO3 and insulin and insulin-like growth factor signaling [62, 63]. FoxO1 is essential in the activation of redox-sensitive Oct4, a key modulator of pluripotency in stem cells, thus maintaining their embryonic pluripotency [64]. There is a cell-specific regulation of FoxO in NPCs where FoxO1 is exclusively expressed in slow-proliferating NPCs (type B) and is excluded in doublecortin positive (Dcx+) neuronal progenitors (type A); this indicates a cell stage-specific expression/regulation [65]. Thus, FoxO1 is associated with the repression of differentiation and the maintenance of stemness and regulates the stem cell reservoir. FoxO3 is also known to maintain NPC pool homeostasis by regulating proliferation and differentiation [66]. An evolutionarily conserved interaction of MST1-FOXO plays an important role in oxidative stress response in mammalian neurons. A similar interaction of orthologs CST1-DAF-16 is reported to increase life span in the nematode worm [67]. The oxidative stress response is mediated through FoxOs in many stem cell types, including hemopoietic stem cells [68]. A direct deletion of FoxOs in neural precursor cells results in megalocephaly or enlarged brain, suggesting increased proliferation of NPCs that can result in the depletion of NPC pool in the adult brain [69]. FoxO-null mice display an increased level of ROS in their NPC pool which results in reduced self-renewal of the stem cell supply [69]. Interestingly, FoxOs are induced in neurodegenerative diseases and spinal cord injury, which can potentially promote recovery [70, 71].

Cumulatively, the current literature indicates a regulatory role for FoxOs on NPC proliferation and differentiation. This includes the regulation of antioxidant systems [72], which control ROS levels that are ultimately involved in maintaining the NPCs' reservoir as well as their fate determination. Understanding such complexity can have a potential application in aging and neurotrauma.

7.3. HIF1α. Hypoxia is a condition when a tissue receives less oxygen than its surrounding region. The occurrence of hypoxia is physiological during development and pathological in neurotrauma. During embryonic development, most of stem cell niches experience hypoxia [73] that requires adaptive changes to survive these conditions. Hypoxia inducible factor-1 alpha (HIF1α) is an oxygen sensor that is induced by low oxygen levels but is degraded rapidly in normoxia [74]. Hypoxia can increase NPC proliferation and reduce apoptosis by HIF1α dependent and independent pathways [55]. HIF1α is essential in maintaining neural stem cells in mice adult SVZ and is required for maintaining the vasculature by inducing vascular endothelial growth factor (VEGF) [75]. The role of HIF1α in CNS development is well documented by its involvement in vasculogenesis as well as embryogenesis [76]. Under pathological conditions such as stroke, ROS are generated after the interruption of blood supply (hypoxia) and again after the restoration of blood supply (reperfusion). Although the damaging effects of ROS on cellular health has been well documented [77], interestingly, ROS generation has been also shown to be correlated with increased neurogenesis [78–80]. HIF1α is also known to maintain the NPCs in a quiescent state in the adult and embryonic brain [75, 81]. ROS induces transactivation of NFκB and in turn induces HIF1α promoter in pulmonary artery smooth muscles [82]. Redox regulation of HIF1α is well established through regulation of its stability by ROS levels [83, 84]. Therefore, oxidative modification of this master transcription factor can affect the downstream genes including VEGF, p21, p53, and Bcl-2. As a therapeutic approach, transplanting HIF1α overexpressing NPCs improved neurological function in rat after cerebral ischemia via increasing survival and providing microvascularization [85]. Thus, hypoxia and ROS levels can play a crucial role in defining stem cell properties and increasing cell proliferation, which can be utilized for novel therapeutic strategies.

7.4. PTEN-PINK1. Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene, which is frequently mutated in many human cancers [86, 87], but also has been associated with social deficits and autism spectrum disorder in mice [88]. The presence of PTEN results in NPCs exiting cell cycle at the G_0 phase and increased self-renewal by AKT1 downregulation [89]. PTEN deficiency causes hyperproliferation but does not cause stem cell depletion. The PTEN depletion causes cell proliferation irrespective of growth factor dependency [90]. PTEN is a redox-sensitive protein, which controls NPC proliferation. Growth factors such as EGF and PDGF stimuli can cause elevated ROS mediated by NOX which

reversibly oxidize PTEN to promote cell proliferation [91]. This reversible oxidative inhibition of PTEN modulates the proliferation of NPCs when required. This phenomenon has been taken advantage of by several cancer cells. As discussed before, Nox2-associated growth factor signaling for proliferation occurs via PTEN inhibition (transient oxidation). A study reported that adult hippocampal progenitors are maintained by oxidizing PTEN upon FGF signaling [92]. Interestingly, PTEN knockout neurospheres show increased proliferation under basal conditions and do not further increase proliferation when exposed to elevated ROS conditions. In contrast, neurospheres from wild-type and PTEN heterozygous mice responded to H_2O_2 by increased hyperproliferation [47]. Thus, PTEN is involved in the generation of cancer-initiating stem cells by adaptation of reduced growth factor responsiveness. PTEN-induced putative kinase 1 (PINK1) is a downstream of PTEN and is known to be involved in mitochondrial function. PINK1 knockout results in increased ROS generation and thus oxidative stress [93, 94]. In a recent study, PINK1 is shown to increase during normal mice development and its deletion results in decreased gliogenesis, without affecting NPC proliferation and neuronal or oligodendrocyte differentiation. Given the fact that astrocyte differentiation pathway is altered but NPC proliferation is unaffected upon PINK1 knockout, understanding the underlying mechanisms that regulate the ROS-PINK1protein control of gliogenesis remains to be explored. Mitochondrial ROS generation was not changed during in vitro NPC differentiation [95]. This is not the case in mouse embryonic fibroblasts, where mitochondrial ROS levels and oxidative stress are exacerbated upon PINK1 global knockout [93]. The difference in mechanisms for such phenotype is yet to be explored. Overall, understanding PINK1 may reveal the mechanisms behind reactive astrogliosis.

7.5. p53. p53 protein functions as a tumor suppressor, and a negative controller of cell proliferation is mediated by p21, a key negative regulator of cell cycle and apoptosis in adult NPCs [96]. Although the role of p53 in the differentiation of embryonic stem cells has been shown [97, 98], its involvement in NPC differentiation remains to be identified. Conversely, a paralog of p53 known as p73 is crucial in maintaining the neurogenic pool by regulation of the NPCs' self-renewal and proliferation in both embryonic stage and throughout the adulthood [99]. A direct correlation of p53 in ROS generation in mouse embryonic NPCs has been reported [100]. The study suggests that a fine-tuning of ROS generation is controlled by p53. The study further reveals that elevated ROS is correlated with early neurogenesis and propagation of DCX+ precursor cells. Their data suggests that knockout of p53 is associated with increased ROS that in turn activates AKT-PI3Kinase pathway which forces the NPCs to commit to neuronal fate [100]. Although these results contradict the theory which says increased endogenous ROS is required for multipotent stem cell proliferation [47], the difference could come from the additional stress levels after p53 knockout that can cause enhanced oxidative stress beyond the threshold for proliferation and therefore affects the NPCs' fate commitment. P53 also regulates apoptosis as well as ROS regulation and NPC metabolism which eventually regulate proliferation.

7.6. Nrf2. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a master transcription factor that induces a battery of genes with a common conserved sequence known as the antioxidant response element (ARE) in their promoters [101]. Nrf2 is dynamically regulated by ROS levels in neural stem cells [102]. Under normal conditions, Nrf-2 is quickly ubiquitinated and destined for degradation; however, when exposed to increased ROS levels during oxidative stress conditions, Nrf2 is translocated into the nucleus where it binds to ARE and increases a set of antioxidant proteins [103], including members of glutathione and thioredoxin systems. Quinone compounds such as tertiary butyl hydroquinone (tBHQ) can also increase Nrf2 protein stability and thus increase cellular defense [104]. The upregulation of Nrf2 is known to enhance NPCs' survival against any oxidative stress [104]. This has been shown in SVZ-derived NPCs in culture or after ischemia, which correlates with induced neurogenesis [103]. The effect of Nrf2 is partially mediated by Notch1 signaling, which is conserved in neurogenesis but not in gliogenesis [105]. A recent report suggests a putative role of Nrf2 in neuronal fate commitment through ROS signaling [106]. The group elegantly showed that physiological levels of ROS mediate Nrf2 concerted signaling that eventually affects NPCs' self-renewal and fate determination [106]. The exact role of Nrf2 on NPCs in neurodegenerative diseases for its potential therapeutic application remains to be investigated.

8. NPC Metabolism and Mitochondria

Cellular metabolism also affects NPC proliferation and differentiation. The early embryonic NPCs, also known as primitive NPCs, are mostly dependent on glycolysis [107]. As development progresses and nutritional requirements change, there is a shift from glycolysis to oxidative phosphorylation [108, 109]. Quiescent stem cells residing in the SVZ are similar to a primitive cell type and depend on glycolysis. Oxidative phosphorylation is the main form of metabolism in proliferating NPCs. However, a forced disruption of mitochondrial metabolism in NPCs will switch their metabolism back to glycolysis, resulting in their quiescence via p53 inactivation [110]. Increased oxidative damage in mitochondria that leads to mitochondrial DNA damage decreases self-renewal in NPCs, which can be rescued by the antioxidant N-acetyl cysteine (NAC) [111]. Another study by the same group in induced pluripotent stem cells provided similar observations including decreased proliferation with mitochondrial mutagenesis, an effect which could be rescued with mild concentration of NAC and mitochondria-targeted antioxidant mitoQ. Interestingly, higher levels of antioxidants resulted in decreased proliferation [112]. These studies suggest that high levels of ROS are detrimental for NPC proliferation and differentiation. This may also imply the potential application of antioxidants for diseases that are associated with abnormal changes in NPC proliferation

[112]. Mitochondria are one of the major sources of cellular ROS, and certain ROS can leak through the membrane affecting the overall cell health. Mitochondrial structural dynamics are also known to affect ROS generation that can influence the self-renewal and differentiation in NPCs [106]. Sox2-positive NPCs contain elongated mitochondria and show lower ROS levels compared to Sox2-negative progenitors with fragmented mitochondria and higher ROS levels. Moreover, low ROS in Sox2+ NPCs contain reduced Nrf2 which enhances Notch signaling, and thus, NPCs self-renewal is maintained; whereas in Sox2-negative progenitors, ROS elevation activates Nrf2 signaling which results in reduced self-renewal and induced differentiation. Antioxidant NAC reversed the condition and increased cell proliferation [106]. A more recent study reveals ultrastructural evidence for mitochondrial function alterations in the adult NPCs. Rounded mitochondria display with rudiment OxPhos and electron transport chain (ETC) machineries in NPCs while intermediate progenitors (IPCs) have elongated mitochondria with functional OxPhos and ETC components [113]. This shows the metabolic shift in quiescent NPCs to become actively dividing IPCs. We could appreciate the effect of metabolism in altering ROS levels and thus fate determination.

9. ROS, Autophagy, and NPCs

Autophagy is a dynamic process regulating energy utilization and recycling damaged proteins and organelles. It is said that autophagy is a response to starvation condition, but a basal level of autophagy occurs naturally in all the cells. There is a developing interest to understand autophagy mechanism in NPCs in self-renewal and differentiation. Basal autophagy regulates mitophagy to keep the mitochondrial ROS production at bay [114, 115]. This is an essential step to maintain stem cell properties. Mammalian target of rapamycin (mTOR) signaling and other autophagy inducers also are known to regulate NPC maintenance [116, 117]. Redox control of autophagy in stem cells in physiology and pathophysiology are an emerging topic [118, 119]. FIP200 is an essential protein that initiates autophagy in NPCs. Specific deletion of FIP200 in NPCs results in defected SVZ and dentate gyrus in adult mice. Such deletion of FIP200 also results in the accumulation of p62, increased mitochondria, and elevated ROS levels. The impairment in self-renewal was rescued by p53 ablation or NAC treatment; however, the defected neurogenesis was not rescued by p53 ablation but was amendable to NAC [120]. This suggests the importance of autophagy in regulating ROS levels by controlling mitochondria in postnatal cells but not in embryonic NPCs. This may represent the differential mechanisms in different developmental stages. In a follow-up study in the same direction, the group tested few other autophagy regulators and found that Atg5 and Atg16L1 conditional deletion impaired autophagy but not p62 accumulation in postnatal NPCs. The accumulation of p62 in FIP200 null mice causes aberrant NPC properties and increased $O_2^{\bullet-}$ levels by SOD1 inhibition. FIP200 and p62 double knockout resulted in the recovery of NPC properties and reduced ROS levels but no reduction in elevated mitochondria number [121]. This accumulating evidence shows how aberrant ROS production due to autophagy defect leads to decreased NPC health. This is relevant in any CNS diseases where autophagy is affected.

10. Regulation of ROS Levels: Involvement of Thioredoxin Family of Proteins

Protein oxidation-reduction is majorly governed by thiol-based protein systems such as thioredoxin and glutathione systems, and therefore, many potent antioxidants mimicking these thiols are used as potential therapeutic molecules. Thioredoxin family of proteins consists of reduced (active) thioredoxin (Trx), which physically interacts with peroxides or other substrate proteins and reduces their oxidized thiol groups. Two thiol groups in Trx active site (Cys32-Gly-Pro-Cys35) provide the reducing electrons for the reduction of oxidized proteins or the oxidizing ROS [122]. Trx is oxidized in these reactions but will be regenerated by Trx reductase using electrons from nicotinamide adenine dinucleotide phosphate (NADPH) [123]. Thioredoxin-interacting protein (Txnip) is a natural inhibitor, physically interacting with Trx1 and Trx2, preventing their physical interaction with the substrate [29]. Trx1-TrxR1 oxidoreductase pair is found in cytosol, but Trx2 and TrxR2 are specific to mitochondria with a similar function to their cytoplasmic counterparts [123]. Trx1 plays a crucial role in cell proliferation by reducing a key enzyme, ribonucleotide reductase, in DNA synthesis [124]; however, other reducing factors such as glutathione and dithiothreitol failed to replicate Trx effect on cell proliferation [125]. This indicates that Trx growth-promoting role may be mediated by other properties such as the regulation of cellular response to growth factors [126]. Trx is also involved in many cellular activities which is mediated by its reducing capacity for DNA binding in some transcription factors, as shown for Oct4 that controls cell proliferation [127]. Higher levels of Trx in some cancers have been linked to enhanced cell proliferation and resistance to oxidative stress, and therefore, Trx and TrxR are targeted in anticancer therapies [128, 129]. Trx is secreted from some cells through a leaderless pathway although the mechanism of action in extracellular space is not identified [130]. Supplementing the growth medium with Trx1 has been shown to increase cell proliferation in a series of solid tumors [126]. Yet there are currently no receptors identified for Trx; however, minimal uptake of Trx has been reported [126]. In the nervous system, Trx1 is secreted from astrocyte that has been linked to neuroprotection [131]. A recent study shows that Trx1 addition in vitro and in vivo increases NPC proliferation and promotes neurogenesis [132]. The study showed that the number of Dcx^+ cells was increased in this study in embryonic NPCs [132]. New emerging evidences indicate that Trx effect on hematopoietic stem cell proliferation might be mediated through the regulation of ROS. A growing list of substrates for Trx gives an insight and depth of Trx influence in cell proliferation. For instance, its interaction with senescence-associated proteins ASK1 and Txnip prevents apoptosis induction and cell cycle arrest, respectively [133, 134]. The fact that Trx is stimulated by the antioxidant and oxidant response elements in

its promotor region makes Trx a first response protein in stressful conditions [135]. The negative regulator of Trx, Txnip, has been correlated with the induction of oxidative stress through ROS production [136]. Oxidative stress or increased ROS can lead to Trx-Txnip dissociation, which can stabilize Txnip function in senescence as well as inflammasome activation [29]. Txnip has also been linked to reactive astrogliosis in diabetic retinopathy [137]. Thus, major antioxidant systems may contribute to therapeutic applications by modulating cell proliferation and differentiation.

11. Neural Stem Cells in Pathology

Acute neurotrauma, chronic neurodegenerative diseases, and aging are shown to affect NPC population [138] including increased proliferation in acute stroke and spinal cord injury [79, 139]. Conversely, depletion of NPCs or lack of their proliferation is a characteristic feature of chronic diseases such as Alzheimer's and Parkinson's diseases [140]. All of these conditions are associated with aberrant ROS signaling. Mild oxidative challenge by buthionine sulfoximine (BSO) and other oxidants has been shown to decrease self-renewal (proliferation) of NPCs and modulates multipotentiality by increasing astrocyte differentiation while decreasing neurogenesis. Increased Sirt1 is one of the factors for this effect at least in defected neurogenesis. Sirt1, a multifaceted nicotinamide adenine dinucleotide- (NAD-) dependent histone deacetylase, is involved in energy metabolism and transcriptional regulation [141]. Increased Sirt1 expression decreases Mash1 expression in an epigenetic manner [50]. In another study with similar concept, H_2O_2 treatment promoted cell death and inhibited the protective autophagy in Sirt1 knockout cells in mouse embryonic stem cells [142]. Such experimental conditions may be useful in mimicking the CNS pathological conditions with elevated oxidative stress. Aging results in reduced stem cell pool in brain and other tissues. This is caused by chronic decrease in mitochondrial functions and NAD+ levels; nicotinamide phosphoribosyltransferase (Nampt) is the enzyme required to synthesize NAD+. Ablation of Nampt, a rate-limiting enzyme, results in the depletion of neural stem cell pool [143]. Repletion of NAD+ rescues mitochondrial function and increases stem cell pool and thus regenerative capacity by NAD+-induced sirtuin pathways restoring normal mitochondrial functions in aging tissues [144]. In reviewing this body of literature, one must appreciate the differential response of proliferating and quiescent NPCs under oxidative stress conditions.

Overall, these studies indicate the involvement of ROS in the regulation of proliferation and differentiation of NPCs and that the ROS-antioxidant balance is an important factor in this process. As NPCs have the potential for the treatment of different disorders and neurotrauma in the nervous system, it is critical to use translationally related approaches to direct NPCs towards a specific phenotype.

12. Conclusion

Redox biology is a fast expanding field in modern biology. The advances in biochemistry have enabled new techniques to examine the changes in proteins that are mediated by a simple oxidation by oxidizing ROS. The conformational change is fast and can be quickly reversed by oxidoreductase proteins using protons from NADPH. This form of signaling is increasingly identified in many systems. The level of ROS level determines the outcome: low levels are acting locally in the vicinity of the ROS source such as the membrane NADPH and are involved in signaling, but high levels initiate a full-scale oxidative damage on cellular macromolecules and cause cell damage. Antioxidants are important players that can determine the cellular response. However, evidence of susceptibility of antioxidant proteins to oxidative damage has been shown in the literature, including our group. It is therefore important to carefully dissect the physiological and pathophysiological levels of ROS when addressing any particular effect of ROS in a biological system.

NPCs are located in a low oxygen niche in SVZ and SGZ, and therefore ROS signaling system can effectively regulate their proliferation and differentiation. Cellular antioxidant systems can potentially modulate the ROS levels and therefore must be examined cautiously when addressing the redox-mediated changes in NPCs. Additionally, in vitro examination of NPCs and their response to ROS manipulation is often performed in normal atmosphere (21%) oxygen pressure which is significantly higher than the normal physiological concentration of oxygen (3–5%) in the brain. Therefore, specific attention to details must be applied in these conditions. Introduction of complementary approaches including redox western blotting in these studies can potentially identify new redox-sensitive proteins or systems and expand our understanding in this field. The employment of powerful genetic modifications has enabled in vivo examination of NPCs; however, using knockout animal models is always associated with potential upregulation of compensatory systems that may obscure the results.

Age-dependent or disease-associated changes in the redox status of the brain suggest that antioxidant therapy may be a potential therapeutic application or intervention to induce NPCs healing capacity. It must be noted that although targeting ROS scavenging has constantly failed to produce any results, the quest for finding novel therapies that can modulate the cellular reducing capacity may be an effective approach to block ROS-mediated effects in CNS. Future therapy shall be formulated in fine-tuning the redox balance utilizing antioxidants and redox-sensitive proteins.

Conflicts of Interest

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

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Research Article

Protective Effects of Chronic Intermittent Hypobaric Hypoxia Pretreatment against Aplastic Anemia through Improving the Adhesiveness and Stress of Mesenchymal Stem Cells in Rats

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Aplastic anemia (AA) is a common malignant blood disease, and chronic intermittent hypobaric hypoxia (CIHH) has a beneficial effect against different diseases. The aim of the present study was to investigate the protective effect of CIHH against AA and underlying mechanisms. 5-Fluorouracil and busulfan treatment induced AA model in rats with reduction of hematological parameters and bone marrow tissue injury and decrease of the colony numbers of progenitor cells. CIHH pretreatment significantly reduced the incidence rate of AA and alleviated above symptoms in AA model. The adhesive molecules of bone marrow mesenchymal stem cells (BMMSCs) in AA model, VLA-4, VCAM-1, and ICAM-1 were upregulated, and those of CD162 and CD164 were downregulated by CIHH pretreatment. The expressions of HIF-1α and NF-κB in BMMSCs were also decreased through CIHH pretreatment. Overall, the results demonstrated for the first time that CIHH has an anti-AA effect through improving the adhesiveness and stress of mesenchymal stem cells in rats. CIHH could be a promising and effective therapy for AA.

1. Introduction

Aplastic anemia (AA), an acquired bone marrow failure syndrome [1], manifests itself not only in excessive reduction of hematopoietic stem cells (HSCs) [2] and immune disorders [3, 4] but also in a deficiency of hematopoietic microenvironment (HIM) [5, 6]. Currently, there is growing interest in the adhesiveness of stem cells as a pivot for regeneration therapy against aplastic anemia.

The normal hematopoietic function of bone marrow needs both normal HSCs and normal hematopoietic microenvironment (HIM). The advanced research showed that improving HIM of bone marrow in AA patients will promote the effect of therapies for AA with a doubled result. Bone marrow stromal tissue is an important part of HIM, and bone marrow mesenchymal stem cells (BMMSCs) are the most important component cells of bone marrow stromal tissue, known as bone marrow stromal cells in the early literature. BMMSCs can secrete various hematopoietic factors supporting the proliferation and multidifferentiation in host HSCs [6]. So BMMSCs play an important nutritional support role on bone marrow HSCs and form the structural basis for the survival and executive function of bone marrow HSCs. In the process of bone marrow hematopoiesis, cell adhesion

molecules (CAMs) mediate the combination between hematopoietic stem cells and bone marrow stromal cells or matrix molecules, which also can mediate the homing of hematopoietic stem cell [7]. Therefore, the therapy via enhancing the adhesiveness and nutritional support between the bone marrow hematopoietic stem cells and mesenchymal stem cells might be valuable for AA treatment.

Hypoxia is well known to promote the production of red blood cells [8]. Researches in vitro showed that hypoxia could promote the proliferation of HSCs and bone marrow mesenchymal stem cells (BMMSCs) and could inhibit their differentiation [9]. Hypoxia has been proved to mobilize multipotential mesenchymal stem cells into peripheral blood [10, 11]. Chronic intermittent hypobaric hypoxia (CIHH), a special kind of hypoxia simulating high-altitude hypoxia, has been demonstrated to have beneficial effects on the body. For example, CIHH protects the heart, the central nervous system, and the liver against ischemia/reperfusion or hypoxia/reoxygenation injuries [12–14]. Our previous studies showed that CIHH had no effects on basic cardiac function but protected the heart against ischemia/reperfusion injury [15–17]. Recently, our research showed that CIHH treatment had preventive and therapeutic effects on some diseases, such as collagen-induced arthritis [18, 19], renal vascular hypertension [20], and fructose-fed metabolic syndrome in rats [21], and on diabetes through improving liver insulin resistance in diabetic rats [22, 23]. However, the effects of CIHH on AA and their underlying mechanisms have yet to be elucidated.

Our present work is focused on the preventive effect of CIHH pretreatment on AA induced by the combination of 5-FU and BU in rats. The experimental hematology, the primary progenitor cell culture, the long-term bone marrow explant culture (LTBMC-Ex) methods, flow cytometric (FCM) analysis, and Western blotting method were used to investigate the protective effect of CIHH against AA and underlying mechanisms.

2. Materials and Methods

2.1. Preparation and Evaluation on AA Rat Model. Briefly, adult male Sprague-Dawley rats were given a 5-fluorouracil (5-FU) (J&K Scientific LLC, USA) injection (150 mg/kg) intraperitoneally in the first day. Then, busulfan (BU) (20 mg/kg) (Sigma-Aldrich, USA) was given intraperitoneally in the seventh day and was injected weekly. Fifty milligrams pure BU was dissolved in 5 mL acetone and was further diluted with 20 mL bacteriostatic water to make final concentration (2 mg/mL). The whole AA induction lasts 28 days. The values of red blood cell (RBC), white blood cell (WBC), and platelet (PLA) in peripheral blood should be decreased more than half at least. The evaluation of AA was mainly according to the examination of bone marrow, especially bone marrow biopsy [24–26].

2.2. Animal Grouping, CIHH Treatment, and Protocols. Adult male Sprague-Dawley rats were provided by the Hebei Key Laboratory of Laboratory Animal Science (Shijiazhuang, China). All animals were treated in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication number 85-23, revised 1996). All experimental procedures were reviewed and approved by the Ethics Committee for the Use of Experimental Animals at Hebei Medical University.

The adult male Sprague-Dawley rats (6 weeks, 180~220 g body wt, clean grade) were randomly divided into four groups: control (Con), CIHH pretreatment (CIHH), aplastic anemia induction (AA), and CIHH plus AA (CIHH + AA) groups. In AA rats ($n = 20$), aplastic anemia was induced by an injection of 5-FU and BU. CIHH rats ($n = 20$) were only treated with CIHH (simulated 3000 m altitude, 5 hours per day for 28 days, $PO_2 = 108.8$ mmHg, from 8 : 00 to 14 : 00) [16]. The CIHH + AA rats ($n = 20$) were treated with CIHH before AA induction. Control rats ($n = 20$) received neither AA induction nor CIHH. During the experiment, the health condition and physical activity of the rats were monitored regularly. All rats were sacrificed with an overdose of pentobarbital sodium (100 mg/kg, i.v.) at 56d.

The whole duration for animal treatment lasts 56 days. For AA rats, coinjection of 5-FU and BU was given at 29d, and the samples of blood, bone marrow, and femora tissue were collected at 56d. For CIHH + AA rats, CIHH treatment was given from the 1st day to 28d, a 5-FU and BU coinjection was given at 29d, and the samples were collected to assess the outcomes at 56d. For CIHH rats, CIHH treatment was only given from the 1st day to 28d and then lived in normoxic condition until sacrificed at 56d. For control rats, a physiological saline injection was given at 29d and sacrificed at 56d in normoxic condition. During the experiment, the health condition and physical activity of the rats were monitored regularly. All rats were sacrificed with an overdose of pentobarbital sodium (100 mg/kg, i.v.) at 56d.

2.3. Analysis of Peripheral Hemogram in General. The blood sample was collected once a week until being sacrificed. Approximately 500 μL of blood were collected by postocular venous plexus from each group using 500 μL ethylene diamine tetraacetie acid (EDTA) anticoagulated. The values of RBC, WBC, PLA, HGB, and HCT were determined by using standard laboratory techniques.

2.4. Bone Marrow Histomorphology and Pathology. After adherent soft tissue and epiphyses were removed from the long bones (femur and tibia) of rats, the bone tissues from femur were fixed in 4% paraformaldehyde solution for 48 hours. Then, the samples of bone were immersed in 20% EDTA solution for 4 weeks for decalcification. After routine dehydration and paraffin embedding, the tissue was cut into 5 μm thick sections that pasted on a polyuridylic acid-treated microscopic slide for 48 hours baking at 60°C and H-E staining. After H-E staining, hyperplasia of bone marrow hematopoietic tissue was observed under an upright microscope.

2.5. Culturation of Hematopoietic Progenitor Cells (HPCs). BMMNCs were assayed for colony-forming unit-granulocyte-macrophage (CFU-GM), colony-forming unit-

erythroid (CFU-E), burst-forming unit-erythroid (BFU-E), and colony-forming unit-mixed lineage (CFU-Mix) in semisolid culture medium. Briefly, BMMNCs were cultured in Iscove's modified Dulbecco's medium (Invitrogen, USA) containing 1% (wt/vol) methylcellulose (Sigma-Aldrich, USA), 1% (wt/vol) bovine serum albumin (BSA) (Sigma-Aldrich, USA), 1×10^{-4} M β-mercaptoethanol (β-ME) (Sigma-Aldrich, USA), 20% (vol/vol) screened fetal bovine serum (FBS) (Sigma-Aldrich, USA), and 30% (vol/vol) horse serum (HS) (Sigma-Aldrich, USA). The culture system was mixed and seeded into 24-well culture plates. Each sample was seeded into 3 wells. Each well contained 2×10^5 BMMNCs with 0.1 μg/mL rat granulocyte macrophage colony-stimulating factor (GM-CSF) (Sigma-Aldrich, USA), 5 IU/mL rat stem cell factor (SCF) (Sigma-Aldrich, USA), 1 IU/mL rat interleukin-3 (IL-3) (Sigma-Aldrich, USA), and 10 U/mL rat erythropoietin (EPO) (Sigma-Aldrich, USA). The total volume of culture system was 0.3 mL. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere, and colonies were scored on day 3 for CFU-E (aggregates $\geq$ 50 hemoglobinized cells) [27], on day 7 for BFU-E (aggregates $\geq$ 200 hemoglobinized cells) [28], on day 10 for CFU-GM (aggregates $\geq$ 50 nonhemo-globinized cells) [29], and on day 12 for CFU-Mix (aggregates $\geq$ 50 hemoglobinized and nonhemoglobinized cells) [30].

2.6. Long-Term Bone Marrow Explant Cultures (LTBMC-Ex) In Vitro for Mesenchymal Progenitor Cells (MPCs). Under sterile condition, small 0.2mm^3 fragments of bone marrow were explanted to 75 mm culture dish containing 4 mL of RPMI-1640 supplemented with 30% FBS. The culture medium also contains 1% bovine serum albumin (BSA), 10^{-4} M β-mercaptoethanol (β-ME), 100 ng/mL rat stem cell factor (SCF), 50 ng/mL rat interleukin-3 (IL-3), and 50 ng/mL rat granulocyte macrophage colony-stimulating factor (GM-CSF) [31]. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 air for 4 weeks. After days 14, 19, and 25 of culture, the numbers of colonies deriving from CFU-Fs (aggregates $\geq$ 8 to 20 spindle-shaped nonhemoglobinized cells) [10, 31] were scored using an inverted microscope.

2.7. Generation of BMMSCs. BMMSCs were obtained from rat femoral BM as previously described [10]. Briefly, bones were cleaned of adherent soft tissue, epiphyses were removed, and marrow was harvested by inserting an 18-gauge syringe needle into one end of the bone shaft and seeded at a density of 2×10^6 cells per mL in 10 mL Dulbecco's modification of Eagle's medium (DMEM) (Invitrogen, USA) culture medium 20% (vol/vol) screened fetal bovine serum (FBS). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. All nonadherent cells were removed by changing the medium during 48 hours; thereafter, medium was changed twice a week. The monolayer of adherent cells was trypsinized by 0.25% trypsin-EDTA when it was half confluent, resuspended in culture medium, and seeded at 1×10^4 cells per mL in 10 mL DMEM culture medium at 37°C in a humidified 5% CO_2 atmosphere again (passage 1 [P1]).

2.8. Flow Cytometric Analysis of the Surface Markers on BMMSCs. Flow cytometric analysis was performed to evaluate the surface markers of BMMSCs with a FACSCalibur flow cytometer (FACS Canto™ II, BD Biosciences, USA) using a 488 nm argon laser. Cells from single-cell suspension were incubated for 60 minutes at 4°C with monoclonal antibodies (Abs) against rat antigens, including CD90, CD44, CD73, CD34, and CD45. Irrelevant isotype-identical Abs (clone F8–11-13; Serotec) served as negative control. Specific and unspecific Ab binding was detected with a secondary phycoerythrin-labeled anti-mouse Ab (Serotec). Samples were analyzed by collecting 10,000 events using Cell-Quest software (Becton, Dickinson and Company).

2.9. Measurement of the Expression of Very Late Antigen-4 (VLA-4), Vascular Cell Adhesion Molecule-1 (VCAM-1), Intercellular Cell Adhesion Molecule-1 (ICAM-1), CD162, and CD164 in BMMSCs. The protein expression of VLA-4, VCAM-1, ICAM-1, CD162, and CD164 on passage 2 (P2) BMMSCs was analyzed by FCM and Western blotting. Firstly, the changes in expression of adhesion molecules were determined by fluorescent intensity using FCM. The BMMSC suspensions are divided into 6 tubes and the corresponding fluorescence-labeled monoclonal antibodies (5 μL): FITC anti-rat VLA-4 monoclonal antibody (eBioscience, USA), FITC anti-rat VCAM-1 monoclonal antibody (eBioscience, USA), FITC anti-rat ICAM-1 monoclonal antibody (eBioscience, USA), FITC anti-rat CD162 monoclonal antibody (eBioscience, USA), FITC anti-rat C164 monoclonal antibody (eBioscience, USA), and IgG (eBioscience, USA) were added into the tubes containing 50 μL BMMSC suspensions, respectively. There were about 5×10^5 cells in each tube. The contents in the tubes were mixed vertically and kept for 30 minutes at room temperature in the dark. Then, the tube was centrifuged in 1200g centrifugal force for 10 minutes at 4°C. The supernatant was decanted. The cells were washed for 3 times and diluted into 400~600 μL with PBS. At least 10,000 cells for each sample were obtained and analyzed with Cell Quest 3.0 software. Negative control was set up for all experimental groups to eliminate nonspecific fluorescence. The expression of adhesive molecules (AM) is determined by the fluorescence intensity. Secondly, the changes in expression of adhesion molecules were confirmed by Western blotting. The Western blot analysis was performed according to previously established methods [22].

2.10. Measurement of the Expression of HIF-1α and NF-κB in BMMSCs. The total protein expression of HIF-1α and the total and nuclear level of protein expression of NF-κB in BMMSCs were measured by Western blotting. The Western blot analysis was performed according to previously established methods [22]. Briefly, BMMSCs were collected and lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). All samples were normalized according to the protein concentrations and separated in 10% SDS-PAGE gels and then transferred to nitrocellulose filter membranes (Pall Corporation, Washington, NY) using the wet transfer blotting system (Bio-Rad, Hercules, CA). The following antibodies were used for Western blotting: anti-

HIF-1α (Santa Cruz Biotechnology), anti-NF-κB (Abcam), and anti-GAPDH (Abcam). Goat anti-rabbit secondary antibody was obtained from Santa Cruz Biotechnology. The gray levels of blots were analyzed using ImageJ software.

2.11. Statistical Analysis. Data were expressed as means ± SEM, and enumeration data were expressed as percentage. The unpaired Student *t*-test was used to determine the differences between two groups, and the comparison of enumeration data between the two groups was analyzed with χ^2 test. One-way ANOVA (Dunnett's multiple comparison test) was used to determine the differences among the multiple groups. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. CIHH Treatment Reduced the Incidence Rate of AA. In the CIHH + AA group, 4 out of 20 (20%) rats developed AA. However, 14 out of 20 (70%) rats in the AA group developed AA. So the incidence rate of AA in the CIHH + AA group was significantly lower than that in the AA group ($P < 0.05$).

3.2. CIHH Treatment Improved the Reduction of Hematological Parameters in Peripheral Blood of AA Rats. AA rats displayed anemia, hemorrhage tendency, and infection symptoms. The body weight of AA rats was decreased significantly. Blood examination results showed that the peripheral blood cells in AA rats were decreased to 1/2 of baseline in 21d AA induction. WBCs were reduced at first; then RBC, PLA, HGB, and HCT were reduced (Figure 1), which matched the characters of AA rats [1]. During following 28d after stopping BU injection, blood hemogram in AA rats was reduced continuously. But the hematological parameters of peripheral blood in CIHH + AA rats could effectively antagonize those deviations of blood compared with those of the AA group ($P < 0.01$). There were no significant differences of hematological parameters of peripheral blood between the control and CIHH group.

3.3. CIHH Treatment Improved the Destroyed Bone Marrow Tissue in AA Rats. There were no significant differences of pathologic morphology in bone marrow tissue between control (Figures 2(a) and 2(b)) and CIHH (Figures 2(c) and 2(d)) rats. While in AA rats, the hematopoietic cells such as megakaryocytes in bone marrow were significantly reduced, the bone marrow hematopoietic scaffold structure was loosened, and the bone marrow reticular fibers were decreased. The number and structure of bone marrow capillary were abnormal, and mesenchymal blood sinus of bone marrow was dilated (Figures 2(e) and 2(f)), while the suppression of bone marrow in CIHH + AA rats were significantly improved compared with AA rats (Figures 2(g) and 2(h)).

3.4. CIHH Treatment Increased Both the Hematopoietic Progenitor Cells (HPCs) and Mesenchymal Progenitor Cells (MPCs) in AA Rats. Progenitor cell assays were performed on the BM samples. As shown in Figures 3(a)–3(d), numbers of HPCs were 174.53 ± 5.75, 77.27 ± 1.55, 42.37 ± 1.43, and 219.33 ± 8.05 in CFU-GM, BFU-E, CFU-E, and CFU-Mix per 10^6 cells in control rats ($n = 30$), respectively. The numbers of HPCs were 52.77 ± 2.78, 27.20 ± 1.76, 14.10 ± 1.31, and 60.90 ± 2.71 in CFU-GM, BFU-E, CFU-E, and CFU-Mix per 10^6 cells in AA rats ($n = 30$), respectively (approximately 1/3 of baseline). While in CIHH + AA rats, numbers of HPCs were significantly increased compared with AA rats ($P < 0.01$), which were 154.93 ± 7.56, 70.37 ± 2.35, 37.10 ± 1.14, and 205.63 ± 6.85 in CFU-GM, BFUE, CFU-E, and CFU-Mix per 10^6 cells, respectively ($n = 30$).

We next examined the colony numbers of MPCs at 14d, 19d, and 25d. A dramatic decrease in MPCs frequency was observed in AA rats (approximately 1/4–1/6 of baseline) (Figure 3(e)). Numbers of MPCs were 3.09 ± 0.68, 9.45 ± 0.90, and 7.09 ± 0.64 at 14d, 19d, and 25d CFU-Fs per 10^6 cells in AA rats ($n = 30$), respectively. While in CIHH + AA rats, numbers of MPCs were 16.82 ± 0.94, 29.55 ± 1.07, and 42.36 ± 1.57 at 14d, 19d, and 25d CFU-Fs per 10^6 cells ($n = 30$), respectively, which significantly increased compared with AA rats ($P < 0.01$).

3.5. Surface Markers of BMMSCs. Flow cytometric analysis was performed to assay the surface markers of BMMSCs. The BMMSCs were closely resembled in a homogeneous layer in each group (Figure 4(a)). These cells expressed CD90 ($97.3 \pm 3.5\%$), CD44 ($98.5 \pm 0.6\%$), and CD73 ($96.5 \pm 2.5\%$), while there was an absence of CD34 ($2.8 \pm 1.7\%$) and CD45 ($1.8 \pm 0.5\%$) (Figure 4(b)).

3.6. CIHH Treatment Increased the Expression of VLA-4, VCAM-1, and ICAM-1 and Decreased the Expression of CD162 and CD164 in BMMSCs of AA Rats. Compared with the control group, the protein expression of VLA-4, VCAM-1, and ICAM-1 in BMMSCs of AA rats was significantly decreased ($P < 0.01$), but the expression of CD162 and CD164 was significantly increased in AA rats ($P < 0.01$) while CIHH treatment increased the expression of VLA-4, VCAM-1, and ICAM-1 and decreased the expression of CD162 and CD164 ($P < 0.01$, Figure 5).

3.7. CIHH Treatment Decreased the Expression of HIF-1α and NF-κB in BMMSCs. Compared with the control group, the protein expression of HIF-1α and NF-κB in BMMSCs in AA rats was significantly increased ($P < 0.01$). However, after CIHH treatment, the protein expression of HIF-1α and NF-κB was significantly decreased in BMMSCs in CIHH + AA rats ($P < 0.01$, Figure 6).

4. Discussion

In the present study, CIHH treatment reduced the incidence rate of AA, improved the abnormality of hematological parameters in peripheral blood of AA rats, and restored the destroyed bone marrow tissue in AA rats. CIHH treatment also increased the colony numbers of both hematopoietic progenitor cells (HPCs) and mesenchymal progenitor cells (MPCs) in AA rats. CD90, CD44, and CD73 were expressed on BMMSCs, while there was an absence of CD34 and CD45. CIHH treatment increased the expression of VLA-4, VCAM-1, and ICAM-1 in BMMSCs of AA rats but decreased the

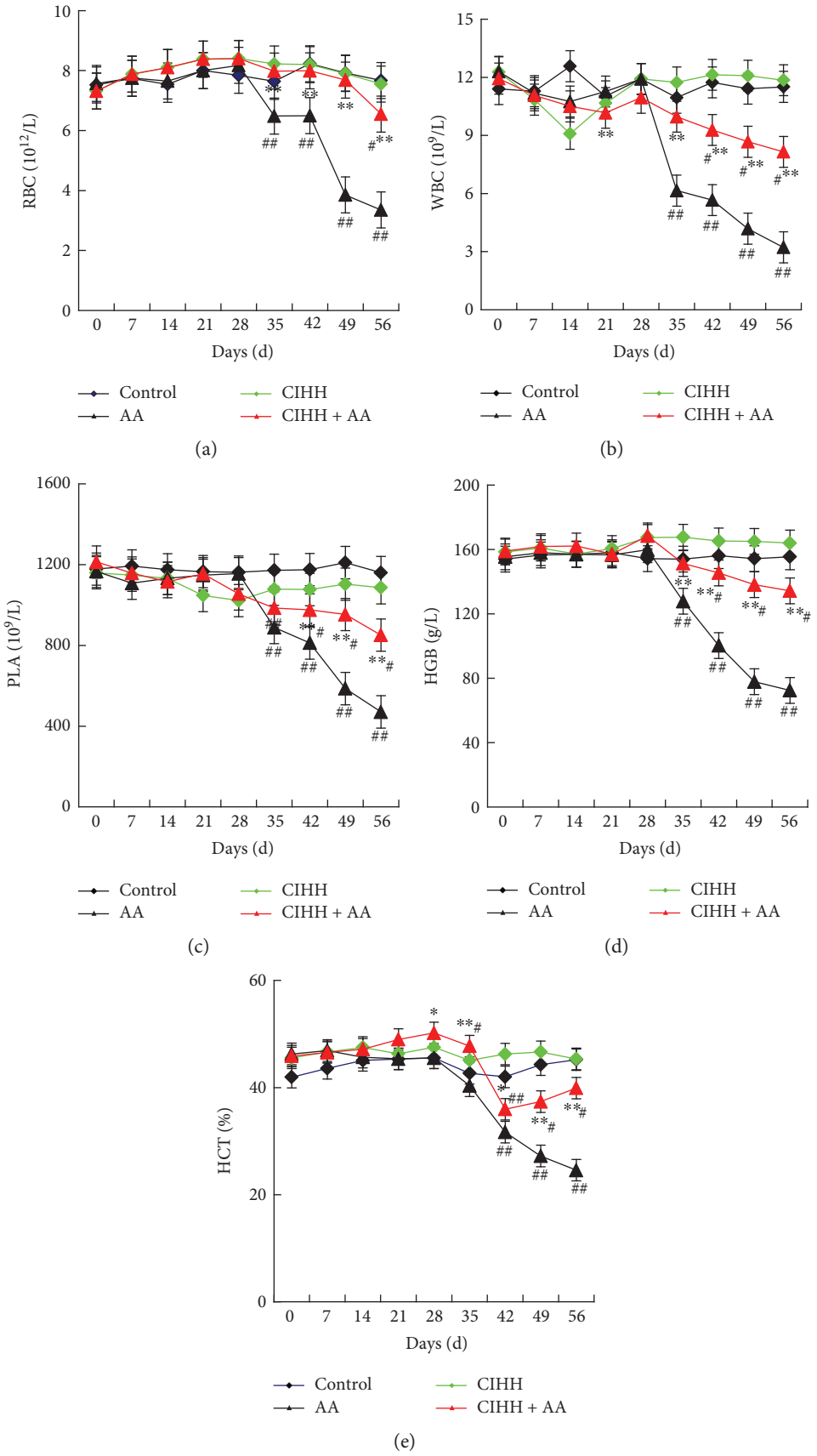

Figure 1: The hematological parameters of peripheral blood in each group. (a–e) The values of RBC, WBC, PLA, HGB, and HCT. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the AA group.

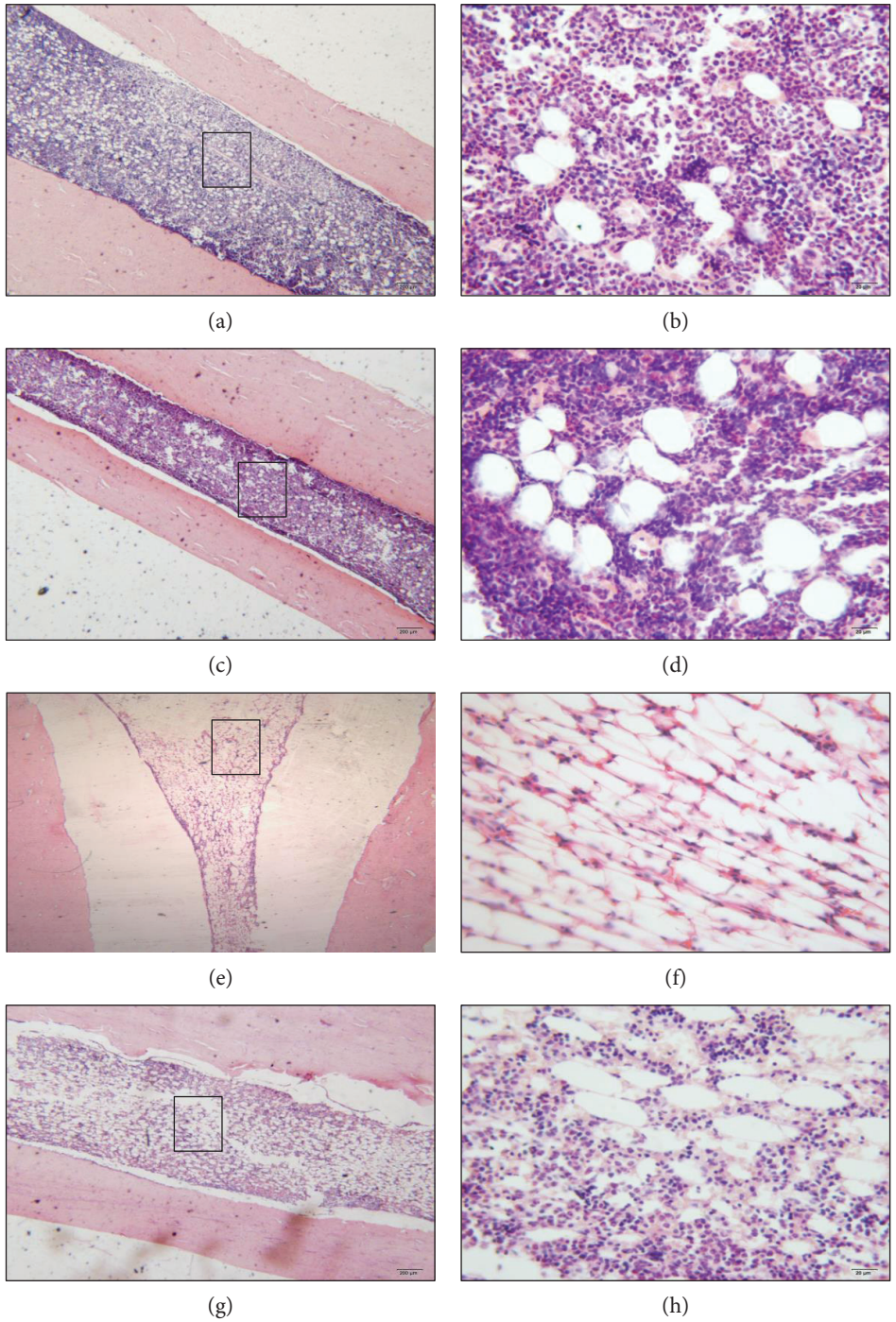

Figure 2: The pathologic morphology of bone marrow tissue in each group. (a, b) Normal bone marrow in the control group; (c, d) normal bone marrow in the CIHH group; (e, f) lessened and damaged bone marrow in the AA group; and (g, h) improved bone marrow in the CHH + AA group. (H-E staining: a, c, e, and g × 40; b, d, f, and h × 400).

expression of CD162 and CD164. CIHH treatment decreased the expression of HIF-1α and NF-κB in BMMSCs.

Although several studies suggested that the HSC damage, immunity disorder, and defects on hematopoietic microenvironment might play roles in the development of bone marrow failure in AA, the exact mechanism for AA is still not clear. Traditional treatment of AA focuses on the repair of damaged HSCs and regulation of immune function. The advanced research found that improving HIM of bone marrow in AA patients will promote the curative effect for AA with a doubled result. Research has shown that there is the damage of HSCs and HIM [32] in AA rat model induced by BU.

The result in our study showed that CIHH could effectively antagonize the decreasing of bone marrow CFU-F, recover the mature fibroblast-like stromal cell layer that

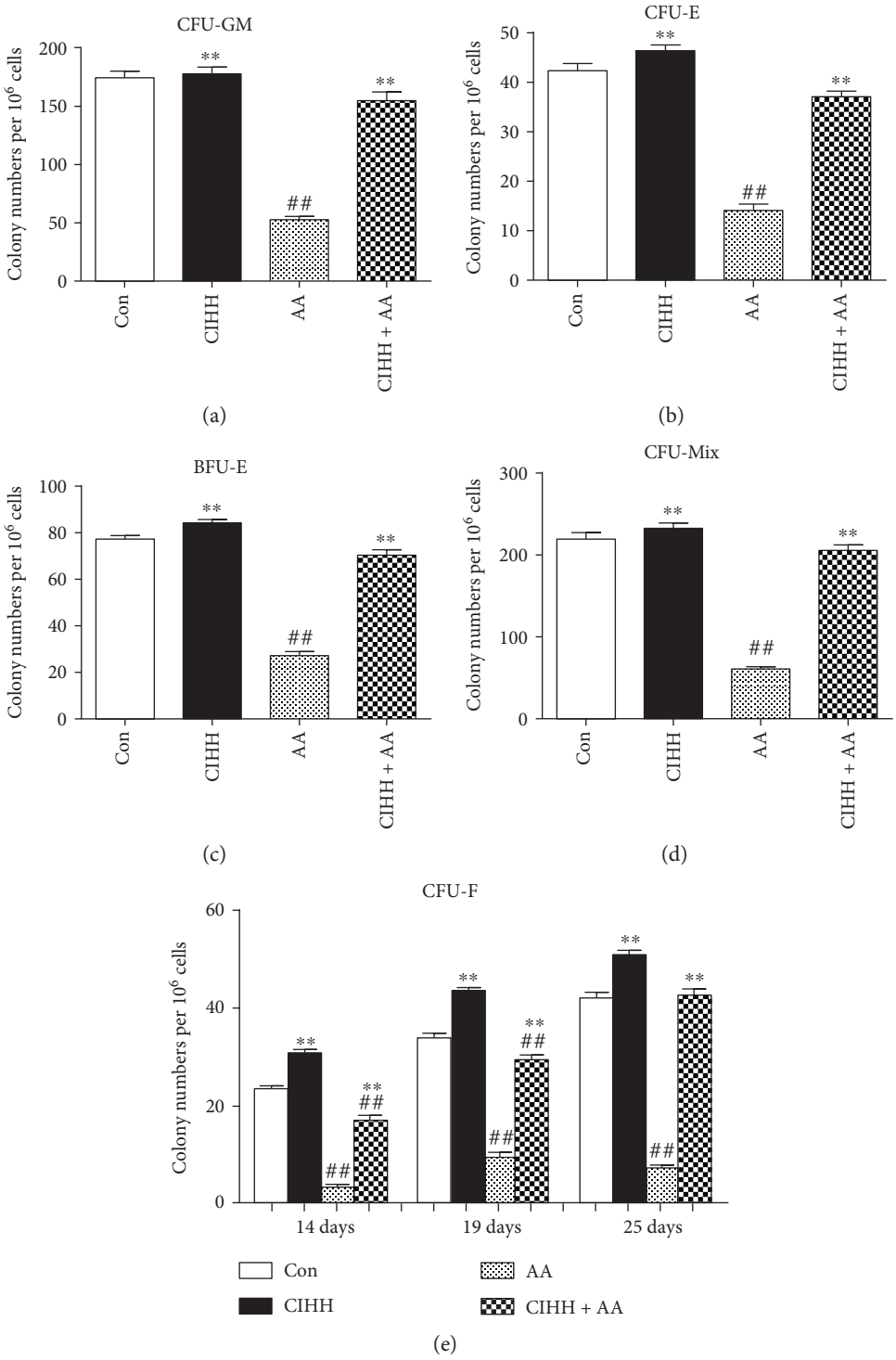

Figure 3: The colony numbers of hematopoietic and mesenchymal progenitor cells in bone marrow of each group. (a–d) Hematopoietic progenitor cells: CFU-GM, CFU-E, BFU-E, and CFU-Mix; (e) mesenchymal progenitor cells: CFU-F. $^{\#\#}P < 0.01$ versus the control group. $^{**}P < 0.01$ versus the AA group.

formed in bone marrow, and support the hematopoiesis of bone marrow in AA rats. The in vitro study revealed that persistent hypoxia stimulates CFU-F proliferation [33], but we do not know the effect of CIHH on CFU-F. Combining the studies of ours and others, we guess that the enhancement of CIHH on CFU-F (1) stimulates T lymphocyte of bone marrow to produce positive hematopoietic regulator, such as IL-3 and stem cell factor and to promote hematopoietic cells growth and proliferation [34] and (2) improves autocrine and paracrine of BMMSCs. The fibroblasts in the

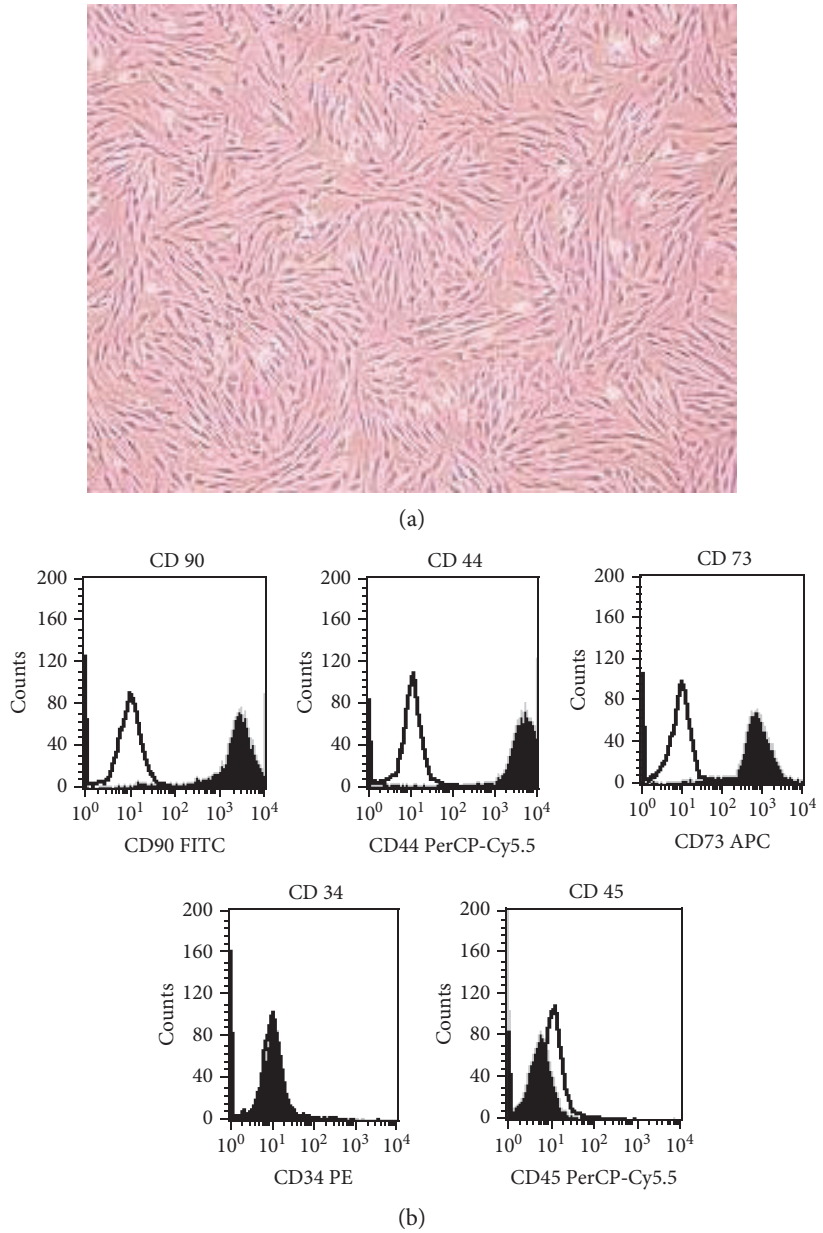

Figure 4: Morphology and surface markers of BMMSCs. (a) Morphology of BMMSCs, observed by phase contrast microscopy. (b) Flow cytometric analysis of the surface markers (CD90, CD44, CD73, CD34, and CD45). Fluorescence intensity histograms with specific antibodies for membrane antigens (black line) and irrelevant isotypic-matched Ab as negative control (black area).

colonies are the main component of stromal cells in bone marrow, which can reflect the function of BMMSCs to a certain degree [35].

There is a great number of adhesion ligands on the surface of BMMSCs, which can combine to adhesion receptors on the surface of hematopoietic stem/progenitor cells. So BMMSCs are often described as the soil of HSCs and play a key role in hematopoiesis through stimulating the proliferation and differentiation of hematopoietic stem/progenitor cells [36] or through the extracellular medium [7]. Therefore, the abnormality of adhesion ligand expression in BMMSCs will affect the growth of hematopoietic stem/progenitor cells in the bone marrow microenvironment. It is reported that the expression of BMMSC adhesion molecules in AA patients was low [37], the level of SCF was decreased, and the BMMSCs grew slowly and were easy to differentiate into adipocytes. These adhesion molecules constitute a complex supporting hematopoiesis network, which is the molecular basis of the support of hematopoiesis by stromal cells [38]. On the other hand, hypoxia can regulate the bone marrow hematopoietic microenvironment [5], improve the capacity of bone marrow, and increase the expression of certain adhesion molecules in bone marrow cells [39].

In this study, we focus on the adhesion molecules VLA-4, VCAM-1, ICAM-1, CD162, and CD164. VCAM-l and VLA-4 are involved in the adhesion process between bone marrow hematopoietic stem cells and mesenchymal stem cells. VLA-4 plays a role mainly in adhesion between cell and

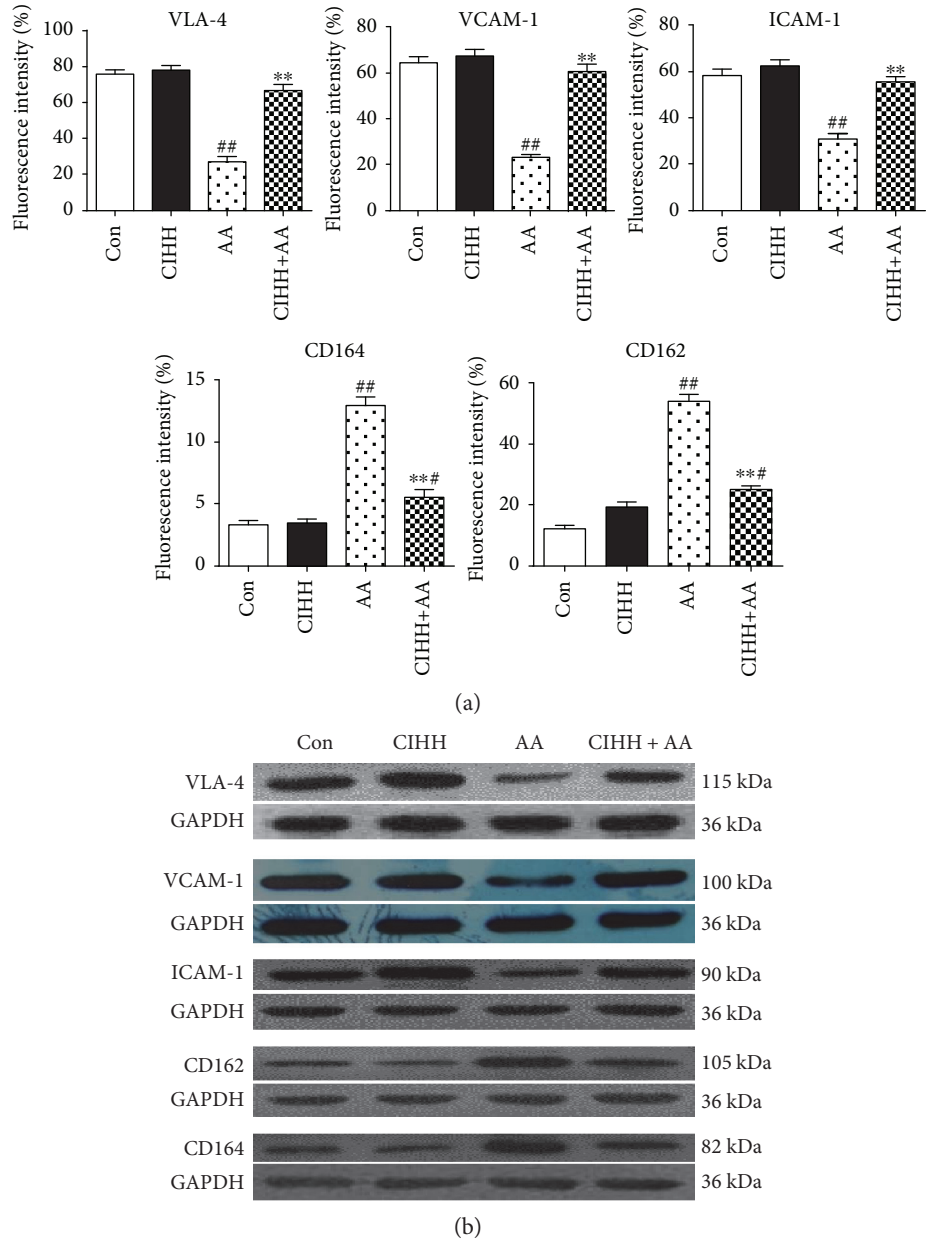

Figure 5: The protein expression of VLA-4, VCAM-1, ICAM-1, CD162, and CD164 in BMMSCs of each group. (a) Fluorescent intensity of VLA-4, VCAM-1, ICAM-1, CD162, and CD164 in BMMSCs by flow cytometry. (b) Representative total protein expression of VLA-4, VCAM-1, ICAM-1, CD162, and CD164 in BMMSCs by Western blotting. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the control group. $^{**}P < 0.01$ versus the AA group.

extracellular matrix and is related to cell migration and differentiation [40]. Studies have shown that the adhesion of bone marrow hematopoietic cells and bone marrow stromal cells/extracellular matrix mediated by VLA-4 is the basis of the bone marrow cell proliferation. ICAM-1 is widely distributed in hematopoietic and nonhematopoietic cells and can regulate the adhesion between hematopoietic progenitor cells and bone marrow stromal cell/extracellular matrix layer. The expression of VCAM-1 can be increased by the stimulation of IL-l β, IL-4, or TNF-α and is a key factor in hematopoietic cell proliferation and differentiation. Studies showed that the direct expression of ICAM-1 is closely correlated with the clinical efficacy on AA treatment. CD162 is expressed in hematopoietic progenitor cells, lymphocytes, and granulocytes and is the only receptor of CD62P (P-selectin) [41]. CD162 mRNA can be expressed in mature $CD34^+$ cells, and the capacity of CD162 in combination with P-selectin is gradually decreased along with $CD34^+$ cell differentiation and matures [42]. There are two possible mechanisms for the inhibition of CD162 on the proliferation of $CD34^+$ cells. One is that the adhesion between CD162 and $CD34^+$ cells mediates the apoptosis of $CD34^+$ hematopoietic stem/progenitor cells. The other is that the differentiation of $CD34^+$ hematopoietic stem/progenitor cells into cell proliferation

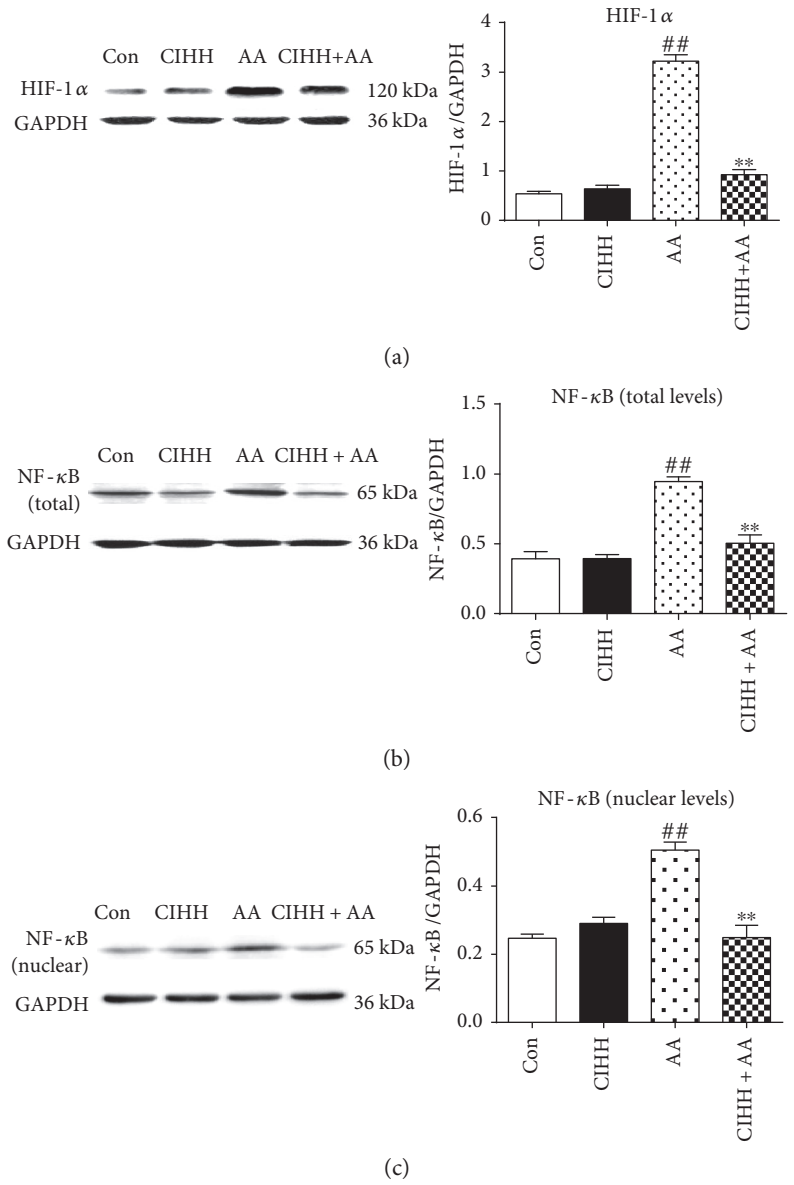

Figure 6: The protein expression of HIF-1α and NF-κB in BMMSCs. (a) Representative and quantitative analysis of expression of HIF-1α in total levels. (b) Representative and quantitative analysis of expression of NF-κB in total levels. (c) Representative and quantitative analysis of expression of NF-κB in nuclear levels. $^{\#\#}P < 0.01$ versus the control group. $^{**}P < 0.01$ versus the AA group.

cycle is inhibited [43]. But the exact mechanism for CD162 action is unclear yet. CD164, expressed in hematopoietic stem/progenitor cells, lymphocytes, macrophages/monocytes, and epithelial cells, plays a role in the adhesion and proliferation in hematopoietic stem/progenitor cells and bone marrow stromal cells [44]. CD164 promotes the adhesion between $CD34^+$ cells and bone marrow stromal cells and inhibits the proliferation of the hematopoietic stem/progenitor cells at the same time [45]. So CD164 is a negative regulatory factor on the proliferation of hematopoietic progenitor cells [46].

The adhesion dysfunction may reduce the adhesion of hematopoietic cells and mobilize excessive pluripotent stem cells from the adhesion area into the blood circulation which may be destroyed, resulting in bone marrow hematopoietic failure eventually. However, CIHH treatment can effectively antagonize the adhesion dysfunction in AA rats and recover the hematopoietic microenvironment. There are two possible mechanisms for CIHH anti-AA through adhesion molecule action: (1) CIHH can improve the adhesiveness by regulating the expression of BMMSC adhesion molecules in AA rats and promoting BMMSCs to secrete more hematopoietic factors. And CIHH also can enhance the sensitivity of BMMSCs to hematopoietic factors and improve the homing of hematopoietic cells back to the marrow, which promotes the homing of different stages of hematopoietic cell into the hematopoietic specific area in bone marrow. (2) CIHH treatment can make the pluripotent stem cells continuously into niche to be saved up through regulating the BMMSC adhesion function.

Finally, we investigated the effect of CIHH on protein expression of HIF-1α and NF-κB in BMMSCs of AA rats. NF-κB and HIF-1α are two important signal transduction molecules involved in hypoxia, inflammation reactions, and stress under physiological and pathological conditions. But it is not clear if the role of NF-κB and HIF-1α in AA. HIF-1α is a nuclear transcription factor that plays a role in hypoxic condition. HIF-1α regulates the expression of angiogenesis-related genes, apoptosis-related gene, and EPO [47]. It is known that the normal bone marrow cavity itself is in hypobaric hypoxia condition. Qian et al. [48] found that HIF-1α could activate caspase-3 and promote apoptosis of bone marrow stromal cells in hypoxia and reoxygenation condition. If the hypoxia microenvironment in bone marrow is aggravated in AA, caspase-3 can be activated and apoptosis of bone marrow cells can be induced. NF-κB is a kind of eukaryotic transcription factor and participates in the expression of genes regulating immune, inflammation, apoptosis, and cell proliferation process [49]. NF-κB signal pathway can be activated by various stress stimulators such as cytokines, bacteria, virus, ultraviolet radiation, and free radical. The activated NF-κB protein can enhance not only the transcription level of cytokines but also acute stress reactive protein gene [50]. Walmsley et al. found that the proximal HIF-1α gene promoter site contained the activated NF-κB-binding site [51]. So the hypoxia could raise the transcription of HIF-1α through NF-κB pathway [52]. The overexpression of HIF-1α can promote NF-κB activity and increase the reaction of stress [53]. The latest research showed that NF-κB protein plays a key role in the development of hematopoietic cells [54]. Both signaling pathways of NF-κB and HIF-1α in AA rats could promote the expression for each other through crosstalking, resulting in excess stress of bone marrow cells in AA rats. CIHH has an effective effect against the increase of stress reaction, which will inhibit the overexpression of NF-κB and HIF-1α ultimately.

In conclusion, this study demonstrated for the first time that CIHH has an antiaplastic anemia effect, which might be related to the improvement on adhesiveness and stress of mesenchymal stem cells.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Jing Yang and Li Zhang contributed equally to this study.

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Research Article

The Leukemic Stem Cell Niche: Adaptation to "Hypoxia" versus Oncogene Addiction

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Previous studies based on low oxygen concentrations in the incubation atmosphere revealed that metabolic factors govern the maintenance of normal hematopoietic or leukemic stem cells (HSC and LSC). The physiological oxygen concentration in tissues ranges between 0.1 and 5.0%. Stem cell niches (SCN) are placed in tissue areas at the lower end of this range ("hypoxic" SCN), to which stem cells are metabolically adapted and where they are selectively hosted. The data reported here indicated that driver oncogenic proteins of several leukemias are suppressed following cell incubation at oxygen concentration compatible with SCN physiology. This suppression is likely to represent a key positive regulator of LSC survival and maintenance (self-renewal) within the SCN. On the other hand, LSC committed to differentiation, unable to stand suppression because of addiction to oncogenic signalling, would be unfit to home in SCN. The loss of oncogene addiction in SCN-adapted LSC has a consequence of crucial practical relevance: the refractoriness to inhibitors of the biological activity of oncogenic protein due to the lack of their molecular target. Thus, LSC hosted in SCN are suited to sustain the long-term maintenance of therapy-resistant minimal residual disease.

1. Introduction

Most stem cell studies, and definitely all of the earliest ones, were carried out using hematopoiesis as a model of tissue regeneration. Likewise, hypotheses to describe the tissue environment where normal stem cells are maintained were developed within a hematological context. Schofield organized this issue conceptually in 1978, putting forward the "stem cell niche" (SCN) model [1] to define bone marrow (BM) sites dedicated to the maintenance of hematopoietic stem cells (HSC). The model predicted that this maintenance is achieved by preventing HSC commitment to differentiation. In other words, if HSC proliferate within the SCN environment, they would do without losing stem cell potential, that is, undergoing the so-called "self-renewal," which is a defining feature of stem cells. The SCN model was supported by experimental findings indicating that HSC and less immature hematopoietic progenitor cells (HPC) are compartmentalized rather than randomly distributed in BM, HSC being located preferentially close to the bone surface and HSC/HPC instead in proximity of the central sinus [2–5]. Hematopoietic cells homing in BM after exogenous transplantation follow a similar compartmentalization pattern [6]. The definition of the relationship between "endosteal SCN," where HSC are maintained, and "vascular SCN," where HSC commitment to differentiation and HPC clonal expansion are driven, was completed much later [7, 8]. Hereafter, the acronym SCN is used to indicate SCN where HSC are maintained.

That metabolic factors take part in the regulation of hematopoiesis emerged from studies indicating that low oxygen concentrations in the incubation atmosphere enhance the yield of hematopoietic cultures [9–15]. The issue was deepened in the early 1990s in our laboratory using 1% oxygen to culture murine BM cells. In 1% oxygen, HSC maintenance was enhanced while the overall hematopoietic output was reduced. HPC were indeed suppressed, the more severely the lower their hierarchical level. These studies provided the first mechanistic implementation of the Schofield's SCN model and led to putting forward the hypothesis of a "hypoxic SCN" dedicated to hosting and preserving HSC selectively from HPC [16]. The effects of low oxygen were later confirmed, by us and others, for human hematopoiesis and long-term-repopulating HSC [17–21]. When we addressed directly the role of low oxygen in modulating self-renewal, we found that one replication cycle in cultures incubated in 1% oxygen boosts stem cell potential. Such an effect is lost when cycling is sustained for more than one cycle and does not occur in air or in the presence of interleukin-3 (IL-3). This indicates that HSC self-renewal occurs immediately after HSC rescue from quiescence to cycling, provided this happens in low oxygen, which therefore appears as a crucial factor to spare stem cell potential. Thus, low oxygen maintains HSC in a state where proliferation is allowed, but not commitment to differentiation. The latter is instead typically driven when proliferation is extensively stimulated, such as in the presence of IL-3 [22].

Although tissue areas where stem cell potential is maintained are commonly referred to as "hypoxic SCN," low-oxygen tensions represent a physiological feature of SCN. Indeed, while the nonphysiological sea-level atmospheric oxygen concentration (20-21%) is considered a physiological standard ("normoxia") for cell culture incubation, oxygen tensions corresponding to 0.1–5% concentration actually characterize the microenvironment of a number of different tissues. Thus, close to the lower end of this range, oxygen tensions are normoxic for HSC but hypoxic for HPC and the bulk of hematopoietic cell population. This issue is reviewed elsewhere [23–25].

To take advantage of their selective homing in tissue areas at the lowest oxygen tensions, HSC need a complex pattern of metabolic adaptation which is not shared by HPC. It is worth noting here that, in keeping with what summarized above, the term "adaptation" is commonly used with a reverse meaning. The term refers in fact to the capacity of cells to home at the lower oxygen tensions actually found in tissues ("adaptation to hypoxia"), instead of defining the conditions enabling cells to stand the nonphysiologically elevated oxygen concentrations used for cell culture ("adaptation to hyperoxia") [25]. When it comes to defining the metabolic peculiarity of HSC with respect to HPC, one must specify that this peculiarity does not simply consist of the compatibility with tissue "hypoxia," as either HSC or HPC exhibit a "hypoxic" metabolic profile [26]. Rather, as mentioned above, HSC, different from HPC, are capable to stand the lowest physiological oxygen tensions (0.1–1%, referred to as very low oxygen) and for extended times. The regulative role of hypoxia-inducible factor-α (HIFα) signalling on hematopoiesis needs to be considered within this context. HIF1α upregulation via transcriptional activation and/or protein stabilization is known as the key driver of cell "adaptation to hypoxia." However, as its stabilization threshold is 2% oxygen or even higher [27], HIF1α alone cannot select HSC from HPC or confer upon HSC all the features enabling their exclusive homing in the very low-oxygen SCN. In other words, HIF1α stabilization is necessary but not sufficient condition for HSC maintenance. HIF1α activity is indeed required for the maintenance of HSC as well as leukemia stem cells (LSC) of chronic myeloid leukemia (CML) [28, 29]. Thus, while the overall "adaptation to hypoxia" requires HIF1α upregulation, enhanced glycolysis and reduced mitochondrial/respiratory activity, only a small minority of hypoxia-adapted cells is capable to stand the very low-oxygen tensions typical of SCN [30]. The characterization of this cell subset is of course of great interest, especially in oncological settings.

The use of 0.1% oxygen in the incubation atmosphere of leukemia cell populations led us to detecting a crucial feature of the SCN-adapted LSC subset [31, 32]. In an environment where all cells are subjected to HIF1α-driven metabolic adaptations (see above), we found that only a minority of CML or murine erythroleukemia (MEL) cells persists throughout extended incubation times. In these cells, while stem cell potential is maintained, the oncogenic protein responsible for disease is lost ("oncogene suppression") [33, 34]. In the case of CML, this loss was found to occur when the shortage of glucose complicates that of oxygen [35]. This points to the existence of a leukemia cell subset combining the adaptation to energy shortage with the suppression of oncogenic signals. Such a combination is likely to be a key factor enabling this cell subset to survive within the selective SCN environment. A crucial by-product of oncogene suppression in CML is that SCN-adapted cells are refractory to the tyrosine kinase inhibitors (TKi) which target the constitutive enzymatic activity of the BCR/Abl oncogenic protein responsible for disease [33, 35], in keeping with findings obtained in other laboratories [36–38]. Refractoriness to therapy due to the fact that its molecular target is suppressed in some surviving cells, LSC in particular, possibly represents the most straightforward explanation of the long-term persistence of therapy-resistant minimal residual disease (MRD) of CML [31, 32]. The data reported in this paper indicated that oncogenic proteins and signals are suppressed in several different leukemias incubated at very low-oxygen concentration. The relevance of this phenomenon as a general aspect of leukemia cell adaptation to severe energy restriction is discussed.

2. Materials and Methods

2.1. Cells and Culture Conditions. K562 and KCL22 (human CML), NB4 (human acute promyelocytic leukemia), Kasumi-1 (human acute myeloid leukemia), and MEL cell lines were cultured in RPMI 1640 (containing 2 g/l of D-glucose) supplemented with 10% foetal bovine serum, 50 units/ml penicillin, 50 mg/ml streptomycin, and 2 mM/l-glutamine (all from EuroClone, Paignton, UK). Exponentially

growing cells were plated at 3×10^5 or 3×10^4 cells/ml and incubated at 37°C in water-saturated atmosphere containing 0.1% O_2, 94.9% N_2, and 5% CO_2 in an anaerobic workstation (Concept 400, Baker Ruskinn, York Road, or DG250, Don Whitley Scientific, Shipley, Bridgend, UK) or in normoxia (21% O_2 and 5% CO_2) in a conventional cell culture incubator. Cell viability was measured by trypan blue (#F-7378, Sigma-Aldrich, St. Louis, MO, USA) exclusion test.

2.2. Protein Extraction and Western Blotting. Cells were lysed in Laemmli buffer and protein concentration was determined by the BCA method (Pierce™ BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA). Extracted proteins were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Merck-Millipore, Billerica, MA, USA) by electroblotting. Membranes were blocked in a 1 : 1 dilution of Odyssey blocking buffer (OBB; LI-COR® Biosciences, Lincoln, NE, USA) with PBS and then incubated at 4°C overnight with primary antibody in a 1 : 1 dilution of OBB with PBS-0.1% Tween 20 (T-PBS). Primary antibodies used were rabbit polyclonal anti-pCRKL (#3181) from Cell Signaling Technology (Danvers, MA, USA); rabbit polyclonal anti-c-Abl (sc-131), anti-erythropoietin-receptor (EPo-R; sc-697) and anti-ERK1 (sc-93), mouse monoclonal anti-RARα (sc-515796) and anti-tubulin (sc-32393), and goat polyclonal anti-ETO (sc-9737) and anti-GAPDH (sc-20357) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit polyclonal anti-histone 4 (H4; #07-108) from Merck-Millipore; goat anti-R-MuLV gp70 antiserum, recognizing gp55 in MEL cells, kindly provided by Dr. Sandra Ruscetti (Laboratory of Cancer Prevention, National Cancer Institute, Frederick, MD, U.S.A.); and rabbit anti-ARD1, produced in Dr. Nathalie Mazure's laboratory. After washing with T-PBS, membranes were incubated for 1 h at room temperature in OBB 1 : 1 with PBS containing IRDye800CW (1 : 20000)- or IRDye680 (1 : 30000)-conjugated secondary antibody (LI-COR). Antibody-coated protein bands were visualized by Odyssey Infrared Imaging System Densitometry (LI-COR Biosciences, Lincoln, NE, USA), as previously reported [39].

2.3. Measurement of Glucose Concentration in Culture Medium. Medium samples were harvested at the indicated times and stored frozen at −20°C until analysis was performed by the glucose hexokinase method using the ADVIA 2400 Chemistry System (Siemens, Camberley, Surrey, UK).

2.4. Statistical Analysis. Statistical analyses were performed using Student's *t*-test and GraphPad Prism software. A p value less than 0.05 was considered statistically significant.

3. Results

The incubation of CML cell lines in atmosphere at 0.1% O_2 was paralleled by a time-dependent suppression of BCR/Abl protein, which did not occur in cells incubated at 21% O_2 (Figures 1(a) and 1(b)), in keeping with what was previously shown [33, 35, 40]. These findings are confirmed and extended here to primary CML cells explanted from BM of a patient in blast crisis (Figure 1(c)). Figures 1(a) and 1(b) also show that incubation in low oxygen led to a decrease in the phosphorylated form of CRLK, a major BCR/Abl downstream substrate used as read-out of BCR/Abl activity. Thus, the suppression of BCR/Abl protein abolished, as expected, its tyrosine kinase activity. The kinetics of BCR/Abl suppression varied depending on the cell line analysed. Indeed, in K562 cells, BCR/Abl protein level was undetectable starting from day 3 of incubation in low oxygen (Figure 1(a), blot on the left), while in KCL22 cells, BCR/Abl protein suppression occurred at day 4 (Figure 1(b)).

Previous studies carried out in our laboratory revealed a close relationship between suppression of BCR/Abl protein in CML cells and glucose exhaustion from culture medium [35]. These findings are confirmed and extended here by measuring the time course of glucose concentration in cultures of KCL22 cells and in K562 cells plated at different concentrations (Figures 1(a) and 1(b), graphs). In the experiments carried out with KCL22 cells, glucose got exhausted between day 3 and day 4, when BCR/Abl protein expression became undetectable (Figure 1(b)). In K562 cell cultures where 3×10^5 cells/ml were plated at time zero, glucose was already exhausted on day 3, when BCR/Abl protein expression became undetectable (Figure 1(a), blot on the left). In K562 cell cultures established with 3×10^4 cells/ml, glucose was still relatively high on day 7 and exhausted on day 10, findings perfectly in keeping with those relative to BCR/Abl protein expression (Figure 1(a), blot on the right). These data confirmed the relationship between glucose consumption and BCR/Abl protein suppression.

The effects of incubation at 0.1% O_2 of a number of non-CML leukemia cell lines are shown in Figure 2. MEL and Kasumi-1 cells underwent a time-dependent suppression of the oncogenic protein(s) driving the disease (Figures 2(a) and 2(b)), in keeping with previous observations [34, 41]. These findings are extended here to NB4 cells (Figure 2(c)). It is to note in particular that in MEL cells, either the gp55 protein of Friend's virus or the EPo-R, both contributing to oncogenic signalling in these cells, was suppressed. In Kasumi-1 cells, the oncogenic driver AML1/ETO protein behaved likewise. In NB4 cells, incubation in low oxygen led to the suppression of the oncogenic driver PML/RARα, but not the normal RARα protein; a lysate of K562 CML cells was added to the electrophoretic run as negative control.

4. Discussion

This paper shows that incubation at very low-oxygen tensions determines the suppression of driver oncogenic proteins and signals in a number of leukemia cell populations, leading to hypothesizing that this suppression is a widespread phenomenon occurring in different types of cancers, including solid neoplasias. That the triggering of suppression mechanism is related to the onset of severe energy restriction rather than simply to "adaptation to hypoxia" is supported by the fact that, in all types of leukemia tested, oncogene suppression occurred only after 3-4 days of incubation in very low oxygen. Consequently, the

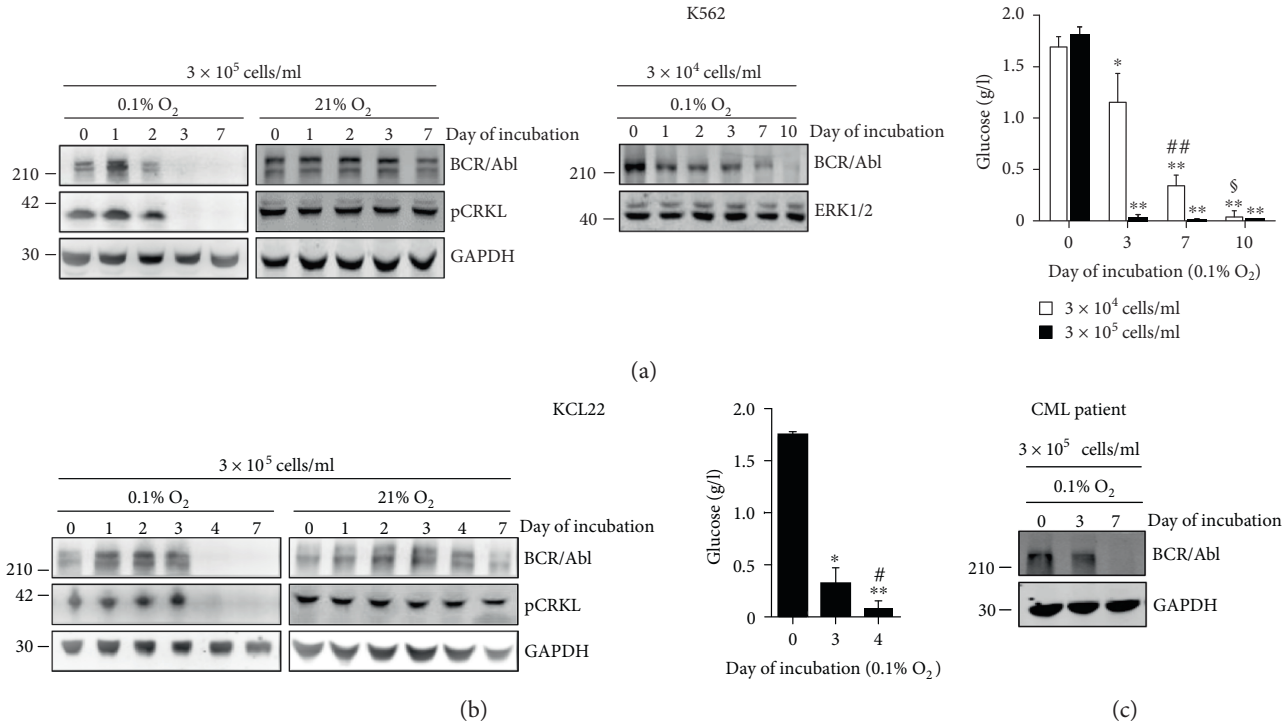

Figure 1: Suppression of BCR/Abl protein in CML cells undergoing "adaptation to hypoxia." K562 cells (a), KCL22 cells (b), or blast-crisis primary cells (c) were plated at the indicated time-zero cell concentrations and incubated in atmosphere at 0.1% or 21% O_2. Cell lysates obtained at the indicated incubation times were immunoblotted using anti-c-Abl (detecting BCR/Abl) or anti-phospho-CRKL antibodies or, as loading equalization control, anti-GAPDH or anti-ERK1/2 antibodies; migration of molecular weight markers is indicated on the left (kDa). One out of three independent experiments with similar outcome is shown. Glucose concentration in the medium of cultures incubated at 0.1% O_2 was measured at the indicated incubation times as described in Materials and Methods. Values are means ± SD of data from 3 independent experiments; $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ versus time 0; $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ versus day 3; $^{\S}p \leq 0.01$ versus day 7.

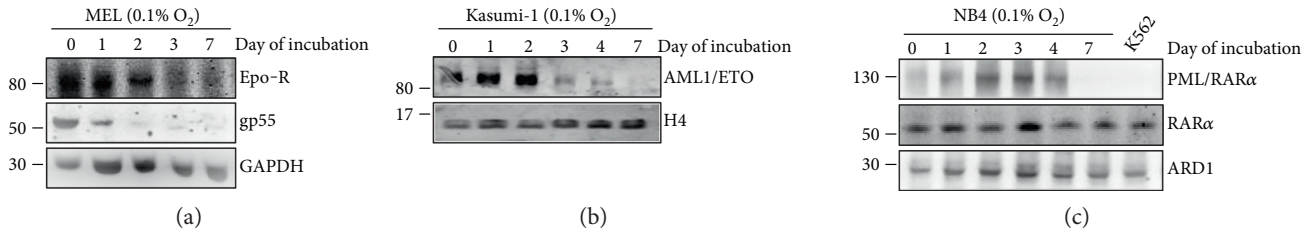

Figure 2: Suppression of oncogenic proteins driving non-CML blood neoplasias in the course of cell "adaptation to hypoxia." MEL (a), Kasumi-1 (b), or NB4 (c) cells were incubated in atmosphere at 0.1% O_2 and lysed at the indicated times, and total cell lysates were subjected to immunoblotting with the indicated antibodies. GAPDH, H4, or ARD1 were detected to verify loading equalization. Migration of molecular weight markers is indicated on the left (kDa). For each cell population, one out of three independent experiments with similar outcome is shown.

phenomenon cannot be simply (or directly) ascribed to HIF1α activation, which is driven within minutes of cell challenged with oxygen shortage. Based on the previous finding that BCR/Abl suppression in low oxygen parallels the onset of glucose shortage [35] and on the data reported here, what we call "severe energy restriction" seems actually to consist of glucose exhaustion from culture medium.

The time-dependent suppression of oncogenic proteins we found as a widespread response of leukemia cells to incubation in low oxygen needs to be discussed within the issue of the general effects of "hypoxia" on protein expression [42]. Indeed, the ATP demand for protein synthesis under "hypoxia" has been estimated to drop to about 7% of that in "normoxia" [43]. This drop occurs initially at the level of translation and later extends to transcription level [44]. Despite such a general decrease in protein synthesis, mRNA translation continues of course for factors important for adaptation to "hypoxia," such as HIF-α and HIF-β, vascular endothelial growth factor, and platelet-derived growth factor [45]. On the other hand, we found that, in CML cells, a number of not directly hypoxia-related proteins, such as tubulin for example, are not affected by energy shortage following

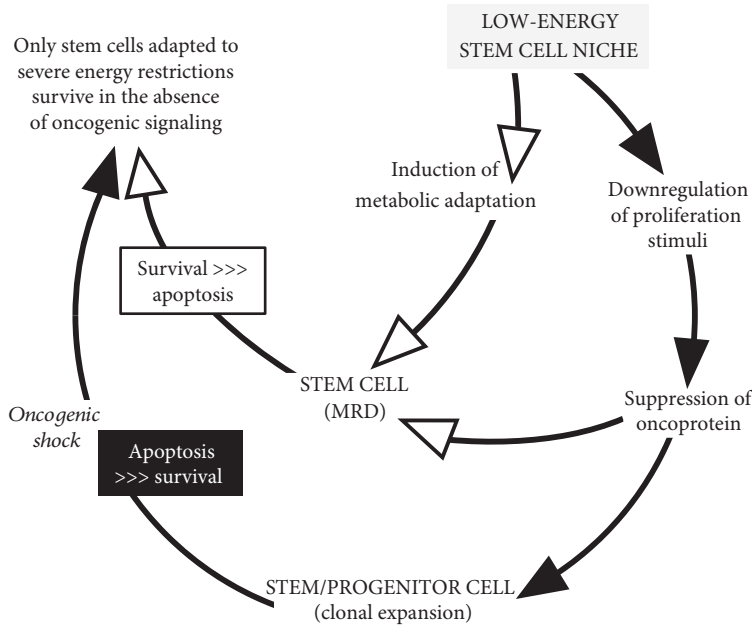

Figure 3: Metabolic adaptation lets stem cell escape oncogene addiction and oncogenic shock. Suppression of oncogenic signalling is necessary to prevent stimuli driving commitment to clonal expansion and differentiation from antagonizing the long-term maintenance of stem cell properties in the SCN. Oncogene suppression puts under stress (black arrowheads/box) stem/progenitor cells committed to clonal expansion and differentiation, which are oncogene-addicted. Thus, in the SCN, these cells would be subjected to prevalent proapoptotic stimuli and undergo the "oncogenic shock." On the contrary, stem cells which metabolically adapt to SCN environment become independent of oncogene signalling (lose oncogene addiction) and escape oncogenic shock (white arrowheads/box), ensuring MRD maintenance.

either oxygen or glucose deprivation [40]. However, the relationship of downregulation of protein expression to oxygen and glucose shortage remains to be deepened.

As briefly mentioned in the Introduction, oncogene suppression under severe energy restriction most likely mirrors the fact that this condition is incompatible with sustained oncogenic signalling which drives commitment to clonal expansion and differentiation. The experiments reported in this paper did not address directly the regulation of LSC maintenance in the function of the expression of driver oncogenes. However, previous data obtained for some of the cell lines used here indicate that, under conditions driving oncogene suppression, cell growth is markedly reduced while stem cell potential is not suppressed [33–35]. In this respect, it is worth pointing out that stabilized leukemia cell lines are highly heterogeneous populations which comprise a full spectrum of different functional phenotypes and include cycling or quiescent cell subsets endowed with stem cell potential [34]. Based on these premises, one can propose that LSC are capable in principle to survive in the absence of oncogenic signalling and that metabolic pressure in the low-energy SCN exploits this capacity and actually selects LSC which have lost oncogene addiction and are capable to stand oncogene suppression (Figure 3). On the contrary, in LSC which do not undergo metabolic adaptation, oncogene addiction is maintained, so that oncogene suppression would result in the induction of apoptosis, the so-called "oncogenic shock." In other words, maintained oncogenic stimulation (enforcing commitment to differentiation and clonal expansion) makes LSC unfit to home in SCN, where they would suffer of the prevalence of proapoptosis over prosurvival stimuli upon withdrawal of oncogenic signalling [46–48].

The loss of oncogene addiction within the SCN implies a de facto reversion of LSC to the normal HSC phenotype, as long as they remain under conditions where the balance within the regulation of LSC compartment is in favour of the maintenance of stem cell potential rather than of commitment to differentiation [48]. Revertant LSC are likely to rely on physiological extra-/intra-cellular signals for support of their survival and self-renewal. In other words, the loss of oncogenic signalling in LSC should be considered functionally equivalent to HSC deprivation of cytokine signalling which drives commitment to differentiation and extensive proliferation, such as that of IL3 [22]. However, LSC adapted to the SCN environment maintain their leukemic genetic signature, so that they are capable, when transferred to growth-permissive conditions outside the SCN, to regenerate oncogenic protein-expressing/protein-dependent cells and thereby to rescue drive to clonal expansion and relapse of disease [33, 35, 40]. Thus, oncogene suppression is not a genetically blocked event but a fully reversible phenotypical adaptation, according to the "chiaroscuro stem cell" model proposed by Quesenberry et al. in 2002 to define the relationship between the HSC and HPC phenotypes [49]. This model may be integrated with the model, proposed by Reya and coworkers, defining two alternative scenarios for the generation of cancer stem cells [50], that is, the oncogenic transformation of a normal (self-renewing) stem cell or the staminalization (acquisition of self-renewal) of a

normal progenitor cell. Our data seem to indicate that these two scenarios are to be considered complementary rather than alternative and to correspond, respectively, to LSC capable or incapable to adapt to and home in SCN.

A crucial, and of high practical relevance, consequence of oncogene suppression is that SCN-adapted LSC which reverted to a normal HSC phenotype exhibit complete refractoriness to inhibitors of the biological activity of the oncogenic protein, due to the lack of their molecular target [31, 32]. The combination of refractoriness to therapy with long-term persistence in tissues makes SCN-adapted LSC the best candidates to sustain MRD in vivo. As far as CML is concerned, the presence of these LSC is probably the main reason of the failure of BCR/Abl-targeting TKi to eradicate LSC, suppress MRD, and prevent relapse of disease [51]. In this scenario, it is predictable that even next-generation TKi will be equally ineffective, as we already observed in vitro (manuscript submitted). All above urges to investigate on the mechanisms of LSC adaptation to severe oxygen and nutrient shortage in view of the design of therapeutic strategies directed to eradicate, rather than to control, leukemia. However, a drawback preventing a useful application of such strategies is represented by the revertant nature of metabolically adapted LSC, which implies the risk of seriously damaging the HSC pool while trying to eradicate LSC. In this respect, current therapeutic approaches directed to suppress oncogene-addicted leukemia/cancer cells committed to clonal expansion and differentiation while tolerating the maintenance of LSC self-renewal at the subclinical level may turn out to represent a safer scenario.

Conflicts of Interest

The authors declare no conflict of interest.

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Review Article

Environmental Factors That Influence Stem Cell Migration: An "Electric Field"

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Environmental Stimulus of Electric Fields on Stem Cell Migration. The movement of cells in response to electric potential gradients is called galvanotaxis. In vivo galvanotaxis, powered by endogenous electric fields (EFs), plays a critical role during development and wound healing. This review aims to provide a perspective on how stem cells transduce EFs into directed migration and an understanding of the current literature relating to the mechanisms by which cells sense and transduce EFs. We will comment on potential EF-based regenerative medicine therapeutics.

1. Introduction

Stem cells reside in restricted microenvironments where their development and behaviour are controlled by a variety of cues. Signalling from the niche and microenvironment controls many aspects of stem cell behaviour including regulation of quiescence versus proliferation, as well as regulating modes of division and migration. The environmental cues that activate signalling pathways are often subtle and can vary in homeostatic versus pathological conditions.

Electric fields (EFs) are a physical cue inherent in physiological environments. They are typically created when charged particles (ions) are separated across cell membranes thereby creating transmembrane electric potential differences or across sheets of coupled cells in epithelium thereby creating transepithelial potential differences [1]. When an injury occurs, transepithelial potentials are disrupted. For instance, a skin wound can disrupt the insulating cell barrier thus creating a potential of zero volts at the injury site. A new electric potential difference is then created between the injury site and the regions surrounding the wound. This EF is significantly greater near the wound edge, dropping off with distance from the wound [2]. Depending on species, location of injury (skin, cornea), distance from the wound edge, and time after injury, the strength of the EF can vary extensively from around 0.6 to 200 mV/mm [3–5]. These injury-generated potentials are critical for directing the migration of cells at the wound margin towards the injury site because disrupting the EF prevents wound closure [6–7]. Similarly, EFs are critical for tissue development. These EFs can range from 10 to 20 mV/mm as is found beneath the neural plate ectoderm, to larger EFs of 1000 mV/mm across the neural tube [8, 9]. The removal or reversal of these in vivo EFs causes developmental defects such as tail abnormalities and malformed limbs [9–11]. Hence, EFs provide an important environmental cue that regulates cell behaviour during development and following injury.

For many years, it has been recognized that EFs are dominant cues that guide the persistent migration of many different cell types. Many cell populations from mammalian, amphibian, and fish species have been identified to undergo galvanotaxis: the directional migration of cells in an EF. These cells include neural crest cells [12], somatic epithelial cells of the cornea [13–15], lens [16], and retina [17], vascular endothelial cells [18], Schwann cells [19], leukocytes [20],

macrophages [21], keratinocytes [22, 23], osteoblasts and osteoclasts [24], chondrocytes [25], and fibroblasts [26]. In addition, stem cells and their progeny from the central nervous system (forebrain and spinal cord-derived) [27, 28] and human mesenchymal stem cells [29, 30] as well as human embryonic and induced pluripotent stem cells [31] undergo galvanotaxis. The direct current EF intensities that are used to induce in vitro migration of different cell types and ages of organisms (i.e., embryonic to adult) vary considerably ranging from as low as 3 mV/mm to greater than 1000 mV/mm [19, 21]. This range of EFs is comparable to those found in vivo during development and wound healing [3, 8].

2. Neural Stem Cells and Endogenous Electric Fields

While a variety of cells are responsive to EFs during development and in adulthood, stem cells are of particular interest due to their regenerative potential. Regulating the behaviour of tissue-specific stem cells has garnered much attention. Tissue-specific stem cells are self-renewing and multipotent with their progeny restricted to generating cells specific to their tissue of origin. Understanding the cues that regulate the behaviour of tissue-specific stem cells and their progeny is a priority for regenerative medicine.

Neural stem cells reside in the germinal zone of the developing central nervous system and are found in the periventricular regions of the adult nervous system [32, 33]. The forebrain subependyma lining the lateral ventricles is a neurogenic region that contains the largest population of neural stem cells in adulthood. In vitro, individual neural stem cells isolated from the subependyma can proliferate to form clonally derived clusters of cells termed "neurospheres" in the presence of mitogens (epidermal growth factor and fibroblast growth factor). A neurosphere consists of a pure population of stem cells and their progeny (together termed neural precursor cells) (NPCs) [32, 33]. There is great interest in harnessing the regenerative potential of these cells to promote neural repair. One method to control NPC migration is through EFs.

EFs in neural tissue are normally present due to neural oscillations and display complex patterns. The effects of these endogenous EFs on cell migration are mostly unknown. Interestingly, under physiological conditions in the adult brain, the rostral migratory stream is a well-defined pathway where ongoing neurogenesis and NPC migration occurs towards the olfactory bulb. Along this pathway, a small 3 mV/mm EF is present and it is speculated that this endogenous EF may play a role in the in vivo directed migration of NPCs [34]. Cao et al. demonstrated that reversing the field in ex vivo tissue slices resulted in reversed direction of migration [34]. However, this has not been explicitly tested by disrupting the EF in vivo. Endogenous EFs are also generated as a result of injury. Following an ischemic insult, there is prolonged cellular depolarization which causes a drop in extracellular direct current potential [35–36]. This change in potential could contribute to directing NPC migration to the site of injury as is observed following cortical stroke [37–39]. These findings are of particular interest when considering whether one could use EFs to enhance endogenous NPC cell migration to injury sites as a means to enhance neural repair.

Notably, EFs have also been shown to promote axon outgrowth, increasing the length and directing the growth of axons from embryonic chick explants and *Xenopus* embryonic neurons [40–42]. Furthermore, a number of neural cell types have been demonstrated to undergo galvanotaxis including oligodendrocyte precursors [43], astrocytes [44], and neuronal cells [45]. The reader is directed to reviews done by Yao et al. where nervous system cell migration in direct current EFs has been discussed in more detail [46, 47]. In this review, we will focus on the role of EFs in regulating cell migration of neural stem and progenitor cells.

3. Cell Mechanisms for Sensing and Transducing Electric Fields

Cells undergoing galvanotaxis have speed, velocity, and directionality. Speed is a measure of how fast the cells are migrating. Velocity is a measure of how fast the cells are migrating towards the cathode or anode. Finally, directionality is a measure of the cells' straight line path towards the cathode or the anode. These components of galvanotaxis can be decoupled, that is, change in directedness without changing velocity or speed, highlighting the complexity of the mechanisms involved in galvanotaxis [27, 48]. Although there are many unknowns related to the cellular mechanisms that underlie galvanotaxis, it is well established that ion channels are critical for sensing and transducing EFs. In response to EFs, both intracellular molecules polarize and channels themselves polarize. In turn, this affects the normal ion flow through ion channels causing polarized intracellular response [48, 49]. The polarized response can then trigger cytoskeletal changes directing the cell's migration.

3.1. Neural Stem Cells. Adult-derived NPCs respond to EFs by migrating towards the cathode. This EF-induced migration is specific to undifferentiated NPCs, and they respond within 15 minutes of application of a 250 mV/mm EF. Differentiated progeny of NPCs (neurons, astrocytes, and oligodendrocytes derived from neurospheres in the presence of fetal bovine serum) do not migrate in the presence of an EF [27]. Interestingly, the directed migration of undifferentiated NPCs is regulated, in part, by epidermal growth factor (EGF). In the absence of EGF, undifferentiated NPCs have reduced directedness and velocity. Indeed, in the presence of the EGFR antagonist erlotinib, a significant loss in NPC migration velocity was observed [27]. Meng et al. suggested that EGFR is redistributed in the cell membrane in response to EF application [28]. Further, EGFR downstream effector phosphoinositide 3-kinase (PI3K) has key roles in actin polymerization. Phosphatidylinositol-3,4,5-trisphosphate (PIP3) and actin were increased at the leading cathodal edge. Thus, EGFR senses the EF while the activation of the PI3K pathway, which could include Rho GTPases as reviewed by Hanna and

El-Sibai, enables actin polymerization at the leading edge thereby contributing to propelling the cell forward during galvanotaxis [50]. Notably, calcium is also required for actin polymerization and the extension of processes at the leading edge of migrating cells [51], and perhaps not surprisingly, galvanotaxis of NPCs is in part regulated by calcium. Buffering extracellular calcium in an EF of 115 mV/mm for 1.5 hours prevents NPC migration [52]. Thus, the polarization of actin and the influx of calcium contribute to persistent cell migration towards the cathode.

Further highlighting the importance of receptors, Li et al. showed that *N*-methyl-*D*-aspartate receptors (NMDARs) are important in explant cultures of embryonic germinal zone from the developing brain [53]. When placed in the presence of an EF, a subpopulation of cells in the explant cultures undergoes cathodal migration. In addition, NMDAR downstream pathways of Rac1 activator Tiam1, phosphorylated p21-activated kinase 1, and actin were upregulated in response to the EF. Moreover, application of an NMDAR antagonist significantly decreased cell migration to the cathode and inhibited the increased associations between NMDAR and its downstream pathways. Since the explant cultures contained a mixed population of stem cells, progenitor cells and mature neural cells, it is not possible to determine which receptor and/or intracellular signalling pathway was specific (or not) to the EF-induced migration of stem versus somatic cells [53]. Despite this caveat, the study reveals that multiple receptors and intracellular signalling pathways are involved in transducing EFs to NPC migration.

Another cue that plays a role in the EF-induced migration of NPCs is cell-matrix/cell-cell interactions. Cao et al. examined pure populations of adult-derived NPCs and cells from a human neuroblastoma cell line and found increased expression of the cell adhesion proteins N-cadherin and β-catenin in the presence of EFs [54]. There was a concomitant upregulation of the P2Y purinoreceptor in the presence of the EF, and interestingly, blocking the receptor using drugs or siRNA resulted in a loss of directedness in NPCS [54]. To note, cell-cell contact has been demonstrated to have effects in altering the sensitivity of epithelial cells to EFs. Clustered epithelial cells respond to lower EFs than single cells though they required more time in the presence of the EF before aligning [55]. Furthermore, fibroblasts have shown different galvanotactic behaviour depending on extracellular matrix molecules as their migration changes with different collagen I substrate concentrations [26]. Hence, cell-matrix and cell-cell interactions appear to play a role in galvanotaxis as well.

3.2. Nonneural Stem Cells. The galvanotactic response of a variety of nonneural stem cell populations has been explored. In vitro studies support the idea that the galvanotactic response of cells varies depending on the passage number of the cells. For instance, passage 1 mesenchymal stem cells migrated towards the anode while more extensively passaged cells (passage 3-4) that were in culture for longer periods migrated towards the cathode. One possible explanation for this finding is that the mesenchymal stem cells were differentiating into a more mature chondrogenic phenotype with continued passaging, and chondrocytes have been shown to migrate towards the cathode [56]. Zhao et al. demonstrated that human bone marrow-derived mesenchymal stem cells will migrate towards the anode but the migration speed and direction is reduced with higher passage number [30]. These findings reinforce the fact that cultured cells, outside of their in vivo niche, can change their behaviour depending on environmental cues.

Most interestingly, the galvanotactic response of human-derived mesenchymal stem cells was shown to be donor dependent [29]. Bone marrow-derived mesenchymal stem cells from three independent donors showed cathodal migration. However, their migration speeds, displacement, and time delay between application of EF and onset of migration were all different. This work highlights the fact that optimization of EF application is critical for potential application in vivo.

3.3. Somatic Cells. Much of the work analyzing how cells sense and transduce EFs into migratory behaviours has been done on somatic cells. It is likely that the galvanotactic response of stem cells and somatic cells share some similarities; however, this is yet to be established.

Nakajima et al. performed a large-scale screening for ion transporter expression in human cells during galvanotaxis [48]. The importance of inwardly rectifying potassium channel Kir4.2 was established in a number of cell lines including human corneal epithelial cells (cathode-migrating cells), immortalized human keratinocytes (anode-migrating cells), and a human breast adenocarcinoma line (anode-migrating cells). They demonstrated that Kir4.2 knockdown via siRNA interfered with galvanotaxis in each of these cell types in EFs up to 500 mV/mm whereby the breast adenocarcinoma cells displayed reduced migration speed whereas the corneal epithelial cells and keratinocyte migration speed was not changed. This highlighted the differences in mechanisms depending on cell type and the fact that it was independent of whether galvanotaxis was anodally versus cathodally directed. The proposed mechanisms through which this channel senses the EF is through the polarization of polyamines in the cell. There was no obvious polarization of Kir4.2 channels in the presence of EFs; however; the intracellular polyamines which bind to the Kir4.2 channel were polarized to the cathode in both cathode- and anode-migrating cells [48]. This asymmetric distribution affects how the polyamines bind to the Kir4.2 channel which can cause local changes in membrane potential, osmolality, and ionic environment which in turn is important for galvanotaxis. Similar to NPCs, PIP3 is involved in galvanotaxis of human corneal epithelial cells, immortalized human keratinocytes, and a human breast adenocarcinoma line. Knocking down the EF-sensing Kir4.2 inhibited PIP3 from polarizing to the leading edge of the process during galvanotaxis [48]. As previously discussed, this protein is involved in actin polymerization whereby the polymerization of actin could push the cell forward at the leading edge.

A number of other channels have been shown to play a role in galvanotaxis. Yang et al. showed that the epithelial

sodium channel (ENaC), a heterotrimeric channel which mediates transepithelial sodium transport and water balance in polarized epithelia, plays a role in the directionality of galvanotaxis in keratinocytes [49]. Epithelial keratinocytes from an αENaC (the major pore-forming subunit of the channel) knockout mouse and human keratinocytes with an ENaC siRNA-mediated knockdown migrated with similar or increased speed compared to wild-type controls. However, their migration was undirected in the presence of an EF. Of interest, they found that a human lung epithelial cell line that did not undergo galvanotaxis underwent directed migration in the presence of an EF when ENaC was overexpressed. Furthermore, unlike the Kir4.2 channels, ENaC polarized to the cathode side of keratinocytes in the presence of an EF. The knockout of ENaC prevented the formation of stable lammellipodial protrusions on the leading edge of keratinocytes in the presence of an EF [49]. Thus, ENaC senses EFs and can contribute to galvanotaxis through lamellipodia. This highlights two different pathways by which channels can sense EFs: through the polarization of small molecules that bind to the channel and/or through the polarization of the channels themselves.

Similar to its importance in NPC migration, Trollinger et al. found that EF application in conditions that decreased calcium influx (using strontium, a calcium substitute) resulted in a significant decrease in the directionality (94% decrease), and to a lesser extent, a reduction in speed (33% decrease) for human keratinocytes [57]. Hence, calcium is important in the galvanotactic migratory response of both stem and somatic cells.

A review by Funk discussed how mammalian cells sense and transduce EFs through calcium influx and Na(+)/H(+) exchangers (NHE3) [58]. The galvanotaxis of cathode-directed osteoblasts, anode-directed osteosarcoma cells, and cathode-directed HEK293 cells were compared and contrasted. Both osteoblast cells and osteosarcoma cells had a detectable increase in intracellular calcium levels initiated in the trailing edge in the presence of an EF [59]. This increase in calcium level could activate the protein kinase C (PKC) pathway either directly or through phosphatidylinositol 4,5-bisphosphate (PIP2), whose expression was also modified during EF exposure, and second messenger signalling lipid diacylglycerol [60–62]. PKC was shown to contribute to the patchy localization of NHE3 which colocalized with β-actin at the leading edge membrane protrusions [61, 62]. Furthermore, γ-tubulin complexes interacted with phosphorylated NHE3 in patches in the leading edge of the cell [61]. Indeed, the γ-tubulin was thought to aid in establishing the microtubule polarity within the cell as they were observed at the microtubule-organizing centres which could help orient the cell in the presence of persistent cues such as EFs [63].

Although there are known mechanisms through which cells sense and transduce EFs into migration, many questions persist. The different signalling pathways that regulate cell migration to the cathode or anode still remain unsolved. While both osteoblasts (cathode-directed) and osteosarcoma (anode-directed) cells express similar channels such as NHE3, NHE1, and Na, K-ATPase (NaKA), they are differentially activated in the presence of EFs and their inhibition has different effects on their migratory behaviour [60]. Further, intracellular and extracellular pH has also been shown to be important. The presence of H+ clouds at the leading edges of cathodally directed osteoblast cell migration has been observed; however, this was not seen in anodally directed osteosarcoma cell migration. These findings are consistent with the idea that intracellular changes in pH may dictate the direction of migration [60]. Reducing extracellular pH can lead to a complete reversal of the direction of migration, causing human keratinocytes to migrate to the anode instead of undergoing cathodal migration as is seen under physiological pH conditions [64]. Additionally, cells respond to EFs in a dose-dependent manner. Bovine epithelial cells migrate in opposite directions depending on the value of the EF. They migrate towards the anode between 150 and 200 mV/mm and towards the cathode at 50 mV/mm [16]. Thus considering the environment and the strength of the EF cues will be critically important for promoting directed cell migration.

4. Clinical Application of Electrical Stimulation for Therapeutic Interventions

Electrical stimulation therapies are currently used in the clinic and have been implemented to promote wound healing of chronic ulcers, albeit with limited success [65]. Transcranial direct current electrical stimulation (tDCS) and deep brain stimulation (DBS) are in clinical practice for neural stimulation of a variety of disorders including mood disorders such as depression, epilepsy, hypokinetic movement disorders, and psychiatric diseases [66, 67]. The cellular mechanisms underlying the success of these strategies are not well established. For tDCS, it is suggested that the success of the treatment relates to the orientation of the neurons being activated, NMDA sensitivity for neuroplasticity, intracortical neurotransmitter concentrations, changes in transmembrane proteins, or changes in cortical connectivity and/or spinal connectivity as recently reviewed by Roche et al. [68]. The optimal stimulation parameters in humans have not yet been clearly established. Although for tDCS, <2.5 mA is considered the "standard," higher stimulation has been employed in some studies [69].

For DBS, a range of frequencies, pulse widths, and amplitude values as well as the differences between constant-current and constant-voltage have been reported [70, 71]. The location of the implanted electrodes and the disorder being treated play a large role in determining the best stimulation parameters for successful treatment [70, 71]. It is proposed that DBS mimics a lesion thereby leading to inhibition of local neuronal networks, disruption of abnormal firing in the brain, and/or it could also involve stimulation-induced release of neurotransmitters locally and through larger networks as reviewed by Chiken et al. and Herrington et al. [72, 73]. DBS also promotes synaptic plasticity and network reorganization which could explain the slower recovery responses observed following DBS [73]. In terms of cell-specific effects, it has been proposed that astrocytes play a key role in DBS therapies by regulating neuronal

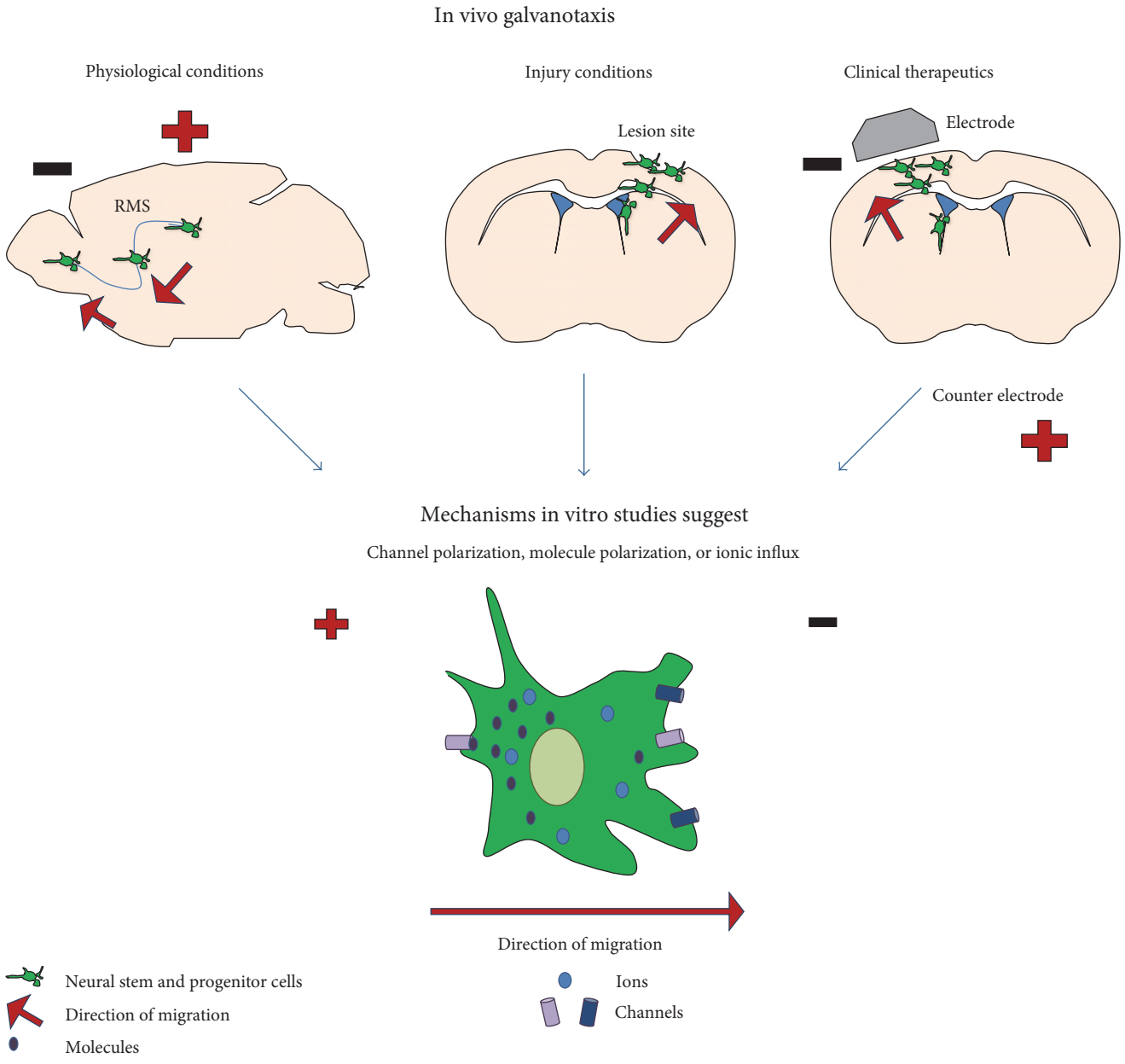

Figure 1: Overview of how electric fields affect stem cells in vivo and how the electric fields can be sensed. Sagittal brain section featured in the top left, coronal brain sections featured in the top middle and right. RMS: rostral migratory stream.

activity and contributing to long-term potentiation and depression [74].

There is significantly less research on the effects of these clinical stimulation parameters on NPCs; however, the evidence that exists supports the hypothesis that NPCs are affected. In the case of tDCS, Rueger et al. performed 10 days of transcranial stimulation and examined cell migration 3 days after the last stimulation [75]. They reported that cathodal tDCS (but not anodal) increased the number of endogenous NPCs within the cortex (outside their normal in vivo subependyma niche) [75]. One interpretation is that the NPCs migrated towards the cathode, similar to what is seen in vitro. In a more recent separate study, Keuters et al. transplanted fluorescently labelled and iron oxide-labelled NPCs into the striatum and the corpus callosum of rats and used the same stimulation parameters as Rueger et al. (15 minutes per day at 500 μA for 10 days) [75, 76]. They demonstrated that anodal tDCS increased the migration of transplanted NPCs within the striatum and on the corpus callosum in the rat brain. The stimulation did not lead to directed migration, but instead, the cells were dispersed more widely in the stimulated brain [76]. These findings suggest that EF induced a more rapid migration of cells within the brain parenchyma while the lack of optimization of the stimulus parameters could account for the undirected migration. Electrical stimulation can also affect the

differentiation profile of NPCs. In vitro, electrical stimulation of NPCs enhances differentiation towards neurons, although, immediately after stimulation of NPCs remain undifferentiated [27, 52, 77]. In vivo, there has been reported enhanced neurogenesis with significant increases in doublecortin-positive neuroblasts in the subependyma of the adult rodent forebrain at 2 days post-tDCS [78]. Further, a separate study supported the activation of NPCs with DBS, reporting increased proliferation in the neurogenic dentate gyrus of the rodent brain [79]. Finally, increased cell proliferation has been observed in the human brains of Parkinson's patients that received DBS, compared to normal and untreated Parkinson's brains [80]. Taken together, these studies suggest that electrical stimulation used in current clinical therapies can activate NPCs.

Based on the ability of electric stimulation to activate NPCs, we propose that electrical stimulation paradigms could be developed to stimulate NPC migration and attract more stem cells to injury sites. Accordingly, to translate the in vitro galvanotaxis studies that direct stem cell migration to the clinic, it is important to consider the use of clinically relevant waveforms for EF delivery. To this end, Babona-Pilipos et al. demonstrated, for the first time, that a balanced biphasic monopolar EF at 400 Hz was able to direct NPC migration in vitro [77]. This is an exciting step forward as the use of charge-balanced stimulation would reduce electrochemical reactions that could create toxic by-products through degradation of the implanted electrode, which would necessarily occur if direct current EFs were applied [81]. Other negative effects of overstimulation of neural tissue include enhanced inflammatory reactions including the activation of endogenous microglia [81]. The careful use of EF stimulation for neural repair could harness the ability of EFs to direct cell migration to injury sites and contribute to the goal of wound healing in the nervous system.

5. Conclusions

Much work has been done to examine the galvanotactic response of somatic cells and stem cell populations; however, a thorough understanding of how EFs direct cell behaviour is not well established. Work to date has revealed that EFs activate a number of channels and that variations in the extracellular and intracellular environment as well as the distribution of channels on the membrane contribute to the galvanotactic response (Figure 1). Cell-specific factors such as cell type and differentiation state, along with time in culture, cell-matrix, and cell–cell contact can influence the galvanotactic response of cells. Further insight into the underlying factors and mechanisms involved in cell migration in response to EFs will enhance the application of EFs in regenerative strategies.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Vitamin C in Stem Cell Biology: Impact on Extracellular Matrix Homeostasis and Epigenetics

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Transcription factors and signaling molecules are well-known regulators of stem cell identity and behavior; however, increasing evidence indicates that environmental cues contribute to this complex network of stimuli, acting as crucial determinants of stem cell fate. L-Ascorbic acid (vitamin C (VitC)) has gained growing interest for its multiple functions and mechanisms of action, contributing to the homeostasis of normal tissues and organs as well as to tissue regeneration. Here, we review the main functions of VitC and its effects on stem cells, focusing on its activity as cofactor of Fe^{+2}/αKG dioxygenases, which regulate the epigenetic signatures, the redox status, and the extracellular matrix (ECM) composition, depending on the enzymes' subcellular localization. Acting as cofactor of collagen prolyl hydroxylases in the endoplasmic reticulum, VitC regulates ECM/collagen homeostasis and plays a key role in the differentiation of mesenchymal stem cells towards osteoblasts, chondrocytes, and tendons. In the nucleus, VitC enhances the activity of DNA and histone demethylases, improving somatic cell reprogramming and pushing embryonic stem cell towards the naive pluripotent state. The broad spectrum of actions of VitC highlights its relevance for stem cell biology in both physiology and disease.

1. Introduction

L-Ascorbic acid (vitamin C (VitC)) was extensively studied over the last century because it plays an essential role for proper folding and deposition of collagen proteins, which are the most abundant proteins in the human body and have a strong impact on the composition/structure/biomechanical features of the extracellular matrix (ECM). Human cells are unable to synthesize VitC, and therefore, it must constantly be restored through the diet. Indeed, under VitC deprivation, human cells are unable to generate and maintain healthy tissues, in particular those rich in collagens such as the skin, bones, and cartilage, and VitC deficiency in humans causes scurvy, a complex syndrome characterized by generalized ECM dissolution and tissue disintegration. It is only until recently that ECM homeostasis was considered the unique molecular mechanism influenced by VitC availability. In the last years, the use of cutting-edge technologies (next-generation sequencing and advanced microscopy) to study stem cell biology have broadened enormously our knowledge of VitC activities. Specifically, VitC has emerged as a key regulator of stem cell identity/behavior, influencing pluripotency, self-renewal, and differentiation. VitC enhances somatic cell reprogramming, that is, the generation of induced pluripotent stem cells (iPSCs), and pushes embryonic stem cells toward a naive state of pluripotency by modulating the cellular epigenetic profile [1–3]. The strong biological, biotechnological, and medical significance of VitC-dependent molecular mechanisms become even more relevant taking into account another key VitC-dependent cellular modification, that is, collagen hydroxylation, which is the most abundant posttranslation modification found in the human proteoma. In this review, we will focus on the recent progress made on the influence of VitC on stem cell biology and its implications for regenerative medicine.

2. Vitamin C Metabolism and Functions

VitC is a naturally occurring small carbohydrate (3-keto-L-gulofuranlactone) synthesized by a two-step reaction mainly

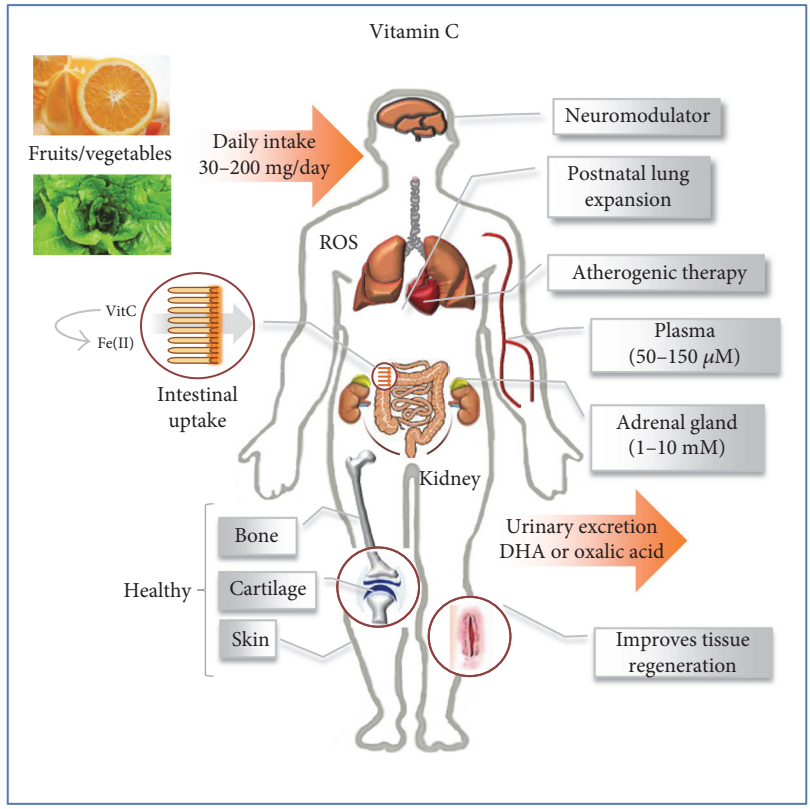

Figure 1: Vitamin C metabolism and activities. Vitamin C, in humans, must be introduced by daily intake through diet. It plays crucial roles both for the proper function of healthy organs and tissues and for tissue repair and regeneration. VitC may act as a scavenger against reactive oxygen species (ROS) and as a chelator, for example, iron metabolism. Both VitC and its catabolic product, dehydroascorbate (DHA), are excreted through urine.

from L-galactose or D-galacturonic acid in green plants [4]. Humans are unable to synthesize VitC due to the lack of the L-gulonolactone oxidase (GLO) enzyme and therefore are strictly dependent on an exogenous source of VitC. Its level is maintained in a range between micromolar in the blood plasma (~50 μM) and millimolar (~1–10 mM) inside the cells (Figure 1), with the highest levels found in pituitary and adrenal gland cells where it is accumulated through the activity of highly specific transport systems encoded by the *SLC23A1* and *SLC23A2* genes, also known as *SVCT1* and *SVCT2* [5–7]. VitC is continuously catabolized by oxidation to dehydroascorbate (DHA), which in turn is converted into oxalic acid [8]. The main route of elimination of VitC and DHA is urinary excretion (Figure 1). Oxalate is one of the major end products of VitC breakdown in humans, and this may cause accumulation of calcium oxalate stones and nephrocalcinosis; thus, susceptible people should avoid systematic ingestion of vitamin C supplements [9].

2.1. ROS Neutralizer and Iron Chelator. VitC is considered the most relevant naturally occurring reducing substance [10]. Inside the cells, VitC cooperates to maintain the intracellular redox balance. VitC reduces reactive oxygen species (ROS), including superoxide anion (O_2^{-1}), hydroxyl radical (OH^-), singlet oxygen (O_2^*), and hypochlorous acid (HClO), which are generated during mitochondrial oxidative phosphorylation (aerobic ATP generation). ROS regulate several signaling pathways involved in pluripotency, including MAPKs, ERKs, p38MAPKs, JNKs, and MAPK phosphatases. Interestingly, VitC inhibits NFkB activation in human cell lines (U937, HL-60, and MCF-7) and in primary cells (HUVEC) in a dose-dependent manner [11]. ROS inactivation results in VitC oxidation to dehydroascorbic acid (DHA), which in turn alters cellular homeostasis. DHA can be reduced to VitC (DHA → VitC) by enzymatic and nonenzymatic activities involving glutathione and homocysteine, which regenerate/recycle VitC [12, 13]. Besides its role as antioxidant, VitC exerts a chelator activity; indeed, by reducing ferric to ferrous ($Fe^{+3} \rightarrow Fe^{+2}$) iron and by generating soluble iron complexes, VitC efficiently enhances the absorption of nonheme iron at the intestine level [14–17]. The chromaffin granule cytochrome b_{561} (CGCyt b_{561}) and the duodenal Cyt b_{561} (DCyt b_{561}) are transmembrane oxidoreductases [18, 19], which contribute to recycle VitC from DHA and enhance iron absorption. Indeed, while CGCyt b_{561} catalyzes the transfer of electrons from cytoplasmic VitC to intravesicular DHA (DHA → VitC), DCyt b_{561} transfers electrons from cytoplasmic VitC to Fe^{+3} ions in the intestinal lumen, thus generating soluble Fe^{+2} ions which are eventually taken up by the cells through a Fe^{2+} transporter [20, 21]. As recently reviewed [22], VitC impacts on iron metabolism also stimulate ferritin synthesis, inhibit lysosomal ferritin degradation and cellular iron efflux, and induce iron uptake from low-molecular weight iron-citrate complexes.

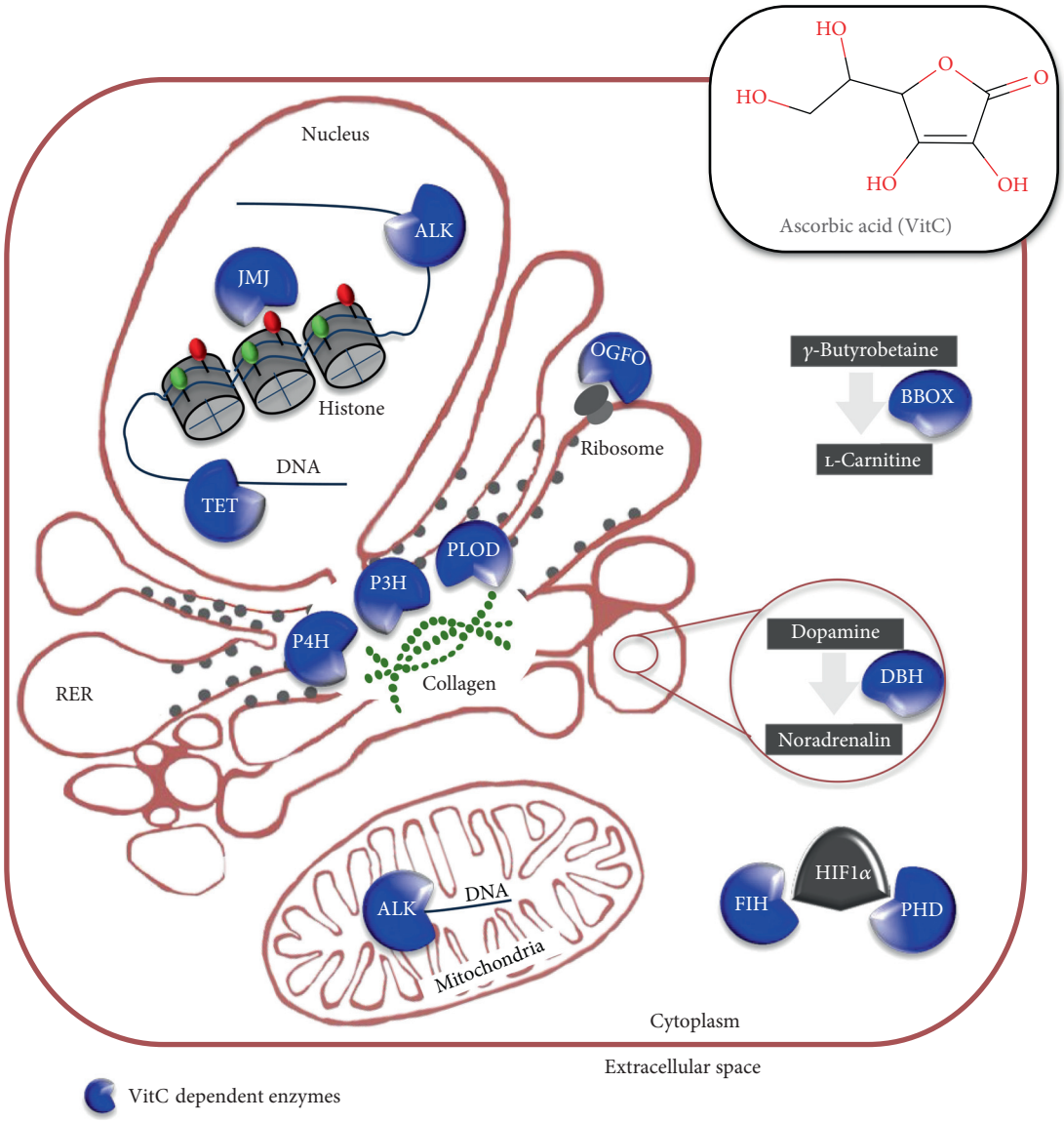

Figure 2: Localization and functions of VitC-dependent mono- and dioxygenase enzymes. ALK: RNA and DNA demethylase family; JMJ: jumonji histone demethylases; TET: ten-eleven translocation DNA demethylases; OGFO: 2-oxoglutarate- and Fe^{2+}-dependent oxygenase; PLOD: procollagen-lysine_α-KG_5-dioxygenases; P3H: collagen prolyl 3-hydroxylase; P4H: collagen prolyl 4-hydroxylases; BBOX1: γ-butyrobetaine dioxygenases; DBH: dopamine beta-monooxygenase; PHD: HIF-prolyl hydroxylase; FIH: factor inhibiting HIF.

2.2. Enzymatic Cofactor/Enhancer. Besides its role as antioxidant, VitC is essential for the activity of a family of mono- and dioxygenases enzymes (EC 1.14.11) by providing the electrons required to keep the prosthetic metal ions in the reduced/active form, specifically Cu^{+1} (cuprous) for the monoxygenases and Fe^{+2} (ferrous) for the dioxygenases [23, 24]. In mammals, VitC-dependent oxygenases catalyze the hydroxylation of DNA, peptides/proteins, and lipids as well as a wide variety of small molecules. For instance, VitC is the cofactor of the γ-butyrobetaine dioxygenase (BBOX1), which catalyzes the final step of L-carnitine biosynthesis (Figure 2). This enzyme is involved in the transport of fatty acids inside the mitochondria for β-oxidation and modulates osteogenesis and chondrogenesis in adipose- and bone marrow-derived stem cells [25]. Similarly, VitC is required for the conversion of the neurotransmitter dopamine to noradrenalin (dopamine beta-monooxygenase (DBH)), the metabolism of tyrosine and the amidation of peptide hormones. VitC-dependent enzymes also include the prolyl hydroxylases that regulate the hydroxylation and thus the degradation of the hypoxia-inducible factor (HIF). Conversely, 5-(hydroxymethyl)-2-furfural (5-HMF) stabilizes HIF protein by reducing VitC level [26]. Of note, VitC enhances the activity of the asparaginyl hydroxylase factor inhibiting HIF-1 (FIH-1), which is an important suppressor of hypoxia-inducible factor (HIF) activity [27]. Interestingly, HIF regulates stem cell pluripotency and self-renewal controlling specific signaling pathways and transcription factors [28]. Most remarkably, VitC enhances the activity of a specific class of RNA and/or DNA demethylases, that is, the human AlkB homologue enzymes (VitC/Fe^{+2}/αKG-dependent dioxygenases) [29, 30], including ABH1 (or

ALKBH1), which catalyzes the demethylation of 3-meC in DNA and RNA [31]; ABH2 (ALKBH2), which catalyzes the oxidative demethylation of 1-methyladenine [32]; ABH3 (ALKBH3), which repairs methylated RNA [33]; and FTO, which demethylates 3-methylthimidine (3-meT) and 3-methyluracil [34], whose variants were found associated with obesity in both children and adults [35]. Another class of VitC/Fe^{+2}/αKG-dependent demethylases has recently gained great attention, due to their key role in somatic cell reprogramming, specifically, the jumonji (JHDM, KDM) family, which are engaged in histone demethylation (chromatin-modifying oxygenases) and the DNA demethylases of the ten-eleven translocation (TET) family [36–40]. Finally, the P4HA and PLOD enzymes belong to the same family of VitC/Fe^{+2}/αKG-dependent dioxygenases and catalyze the hydroxylation of collagen proline and lysine residues. Thus, VitC activity is essential for the activity of key epigenetic enzymes as well as for the conversion of procollagen to collagen. Moreover, it has recently been reported that VitC stimulates the iron-mediated nonenzymatic conversion of the oncometabolite α-hydroxyglutarate (2-HG) into α-ketoglutarate [41]. 2HG is a competitive inhibitor of α-ketoglutarate dioxygenases [42]. Therefore, VitC influences the epigenetic signature, the metabolism (fatty acid catabolism), and the microenvironment (collagen/ECM composition) of the cells, thus pointing to a key role of VitC availability in shaping cell identity/behavior.

2.3. VitC Localization. By acting as antioxidant, VitC is required in all the subcellular compartments but particularly inside the oxidative organelles (mitochondria, endoplasmic reticulum, and peroxisomes). Indeed, by acting as an enzymatic cofactor, its requirement depends on the enzymes' subcellular localization (Figure 2). For instance, histone and DNA demethylases are all located in the nucleoplasm. Specifically, AlkB human homologues (ALBH or ALKBH enzymes) are located in the nucleoplasm and inside the mitochondria where they catalyze the demethylation of 3-meC residues on DNA and RNA, respectively [31]. Collagen prolyl/lysyl hydroxylases are localized in the ER lumen [43], HIF prolyl hydroxylases (PHDs) in the cytosol, and dopamine β-monooxygenase and peptidylglycine α-hydroxylating monooxygenase (PHM) in the synaptic and secretory vesicles. Since VitC is a water-soluble molecule, specific transport systems should be activated to keep optimal VitC concentrations in each subcellular compartment. Two families of transporters are associated with VitC transport in human cells, the sodium- (Na^{+1}-) coupled ascorbic acid transporters (SVCTs; SLC23), which are highly specific for reduced VitC, and the members of the glucose transporter (GLUT; SLC2) family, some of which also transport DHA. SVCT2 transporter colocalizes with the protein disulfide isomerase (PDI) and marks both ER and mitochondrial membranes. Interestingly, the embryonic brain cortex of SVCT2 KO mouse mutants produces significant lower levels of several neurotransmitters, including dopamine and norepinephrine [44] and, furthermore, SVCT2 knockdown mitochondria inefficiently transport VitC [45]. Finally, homozygous Slc23a1$^{-/-}$ mice die at birth with respiratory failure and intraparenchymal brain hemorrhage [46]. SLC23A1 is essential for renal reabsorption and hepatic accumulation of VitC but not for its intestinal transport [47]. Interestingly, specific polymorphisms in the sodium-dependent VitC transporter 2 gene increase the risk of incident of acute coronary syndrome in women, but not in man [48]. So far, the transporters that facilitate VitC accumulation in the intraluminal ER have not been characterized at a molecular level. However, defects in the subcellular distribution of VitC may cause diseases and aging. For instance, impaired mitochondrial uptake of VitC/DHA and thus a VitC shortage in the mitochondrial matrix should provoke a defective removal of ROS. Indeed, it has recently been hypothesized that VitC may be channelled from the nucleoplasm to the ER lumen through the ER subdomain nuclear envelope [43], which may eventually reduce the level of VitC in the nucleus and thus its availability for the epigenetic enzymes. A similar subcellular redistribution of VitC may occur as a consequence of a rapid and massive accumulation of collagen synthesis, for instance upon stimulation with transforming growth factor-β (TGFβ). Thus, it is interesting to hypothesize that changes in VitC levels in the different subcellular compartments could impact on the redox status, the epigenetic signature, and the ECM composition and eventually modify cell behavior. In this respect, it is important to take into account that VitC subcellular distribution likely depends on the expression levels and binding affinities of the different VitC-dependent enzymes and carriers; however, our knowledge is still limited and this issue needs to be further studied [43].

3. VitC-Dependent Regulation of ECM/Collagen Homeostasis

The extracellular matrix (ECM) is a complex mix of fibrillar proteins and polysaccharides synthesized and secreted by the cells in the extracellular space. Indeed, with the exception of the neural extracellular matrix [49], the ECM has a fibrillar structure in most tissues and provides a structural scaffold to the surrounding cells that is essential for tissue/organ morphogenesis, as well as for their regeneration after injury. Collagens are the most abundant proteins in the ECM and thus the most abundant proteins in mammals (~30% of total protein mass) [50] making up 90% of the bone tissue [51]. Collagens are crucial for the development and maintenance of the skin, cartilage, tendons, ligaments, and of the blood vessels and are deposited in the ECM where they generate supramolecular assemblies/complexes contributing to the shape and mechanical properties of tissues, such as the tensile strength in the skin and the ligament resistance to traction [52]. VitC thus impacts on ECM homeostasis by regulating collagen synthesis and maturation.

3.1. Collagen Synthesis. VitC promotes the transcription of collagen genes and/or increases the stability of collagen mRNA in many different cell lines, including human skin fibroblasts [53, 54], PAT cells [55], and murine 3T3-L1 preadipocytes [56]. Also, profibrotic cytokines of the TGFβ family stimulate collagen synthesis, especially in wound

healing and fibrotic diseases [57]. Interestingly, activation of the TGFβ pathway enhances collagen synthesis and reduces collagen degradation in different cell lines, including human mesenchymal stem cells [58], human marrow stromal cell [59], human dermal fibroblasts [60–62], glomerular mesangial cells [63], lung alveolar epithelial cells [64], and vascular smooth muscle cells (VSMCs) [65], thus resulting in fibrosis/ECM accumulation. In line with these findings, in human dermal fibroblasts, several collagen-coding genes, including *COL1A2*, *COL3A1*, *COL6A1*, and *COL6A3*, have been identified as TGFβ/SMAD3 targets in human dermal fibroblasts [66]. Moreover, vitamin D-induced reduction of intestinal fibrosis has been associated with the inhibition of the canonical TGFβ/SMAD3 pathway [67]. In renal epithelial cells, TGFβ regulates collagen deposition by recruiting mTOR kinase (through noncanonical TGFβ pathway) [47, 68]. Interestingly, mTOR regulates HIF-1α, which in turn is controlled by VitC and regulates the transcription of *COL1A2* (collagen I α2) gene. Indeed, knockdown of DEPTOR, an mTOR signaling inhibitor, induces collagen expression; conversely knockdown of RAPTOR, which conversely is a positive regulator of mTOR, inhibits collagen expression. TGFβ can increase collagen synthesis also by inducing the cleavage of the cAMP response element-binding protein 3-like 1 (CREB3L1) transcription factor [69]. Of note, collagen synthesis may be induced also independently of the TGFβ signaling as described during hypoxia-dependent mesenchymalization of human lung epithelial A549 cell line [70].

3.2. Collagen Prolyl and Lysyl Hydroxylases. Collagens are synthesized as procollagen molecules, which are subjected to numerous posttranslational modifications, that is, hydroxylation of L-pro and L-lys residues, glycosylation of L-lys and hydroxylysine residues, and sulfation of tyrosine (Tyr) residues (see [71]). Collagen synthesis also requires the activity of specific posttranslational enzymes that are inactivated by the formation of the collagen triple helix. First, collagen hydroxylation is required for the correct folding of procollagen polypeptide chains into stable triple helical molecules. Collagen lysyl hydroxylases, also known as procollagen-lysine_α-KG_5-dioxygenases, encoded by *PLOD1*, *PLOD2*, and *PLOD3* genes, are VitC-dependent enzymes that catalyze the lysine hydroxylation [72, 73]. Collagen prolyl 4-hydroxylases (P4Hs) are VitC-dependent enzymes that catalyze the proline hydroxylation in collagens. Collagen prolyl hydroxylation involves three isoforms of the P4HA subunit (P4HA1, P4HA2, and P4HA3) that form A_2B_2 tetramers with P4HB and eventually P4H1, P4H2, and P4H3 holoenzymes, respectively. Collagen prolyl hydroxylation is the major posttranslational modification in the human proteoma [74]. In the absence of P4H activity, the procollagen molecules are unable to exit the ER [75, 76]. Interestingly, it has been previously reported that PH activity is induced early during wound healing and that its induction is associated with the onset of collagen biosynthesis and deposition [77]. Increasing evidence suggest that VitC-dependent collagen hydroxylation positively correlates with tumor aggressiveness. Recently, it has been shown that silencing of P4HA2 (collagen prolyl 4-hydroxylase α subunit 2) expression inhibits proliferation and suppresses the most aggressive phenotype of breast cancer cells in vivo [78]. Of note, treatment with the P4H inhibitor ethyl 3,4-dihydroxybenzoate induces a similar phenotype [79]. Interestingly, P4HA1 is highly expressed in aggressive prostate cancer and it is essential for in vivo cancer progression [80].

3.3. Collagen Signaling. Collagen precursors (procollagen/~300 nanometers in length) are synthesized in the endoplasmic reticulum (ER), packaged into transport vesicles, and delivered to Golgi cisternae where fibrillogenesis occurs. The resulting collagen fibrils are the longest (up to millimeters in length) and the largest protein polymers in vertebrates [51, 81]. Collagens are degraded in the extracellular microenvironment through the activity of zinc-dependent endopeptidases, that is, the matrix metalloproteinases (MMPs), which are the key enzymes involved in physiological (development and tissue repair) and pathological (tumorigenesis and metastasis) processes [82]. Collagens participate in cell-matrix interactions acting as functional ligands of several receptor families including glycoprotein VI (GPVI), inhibitory leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1), Endo180 (urokinase-type plasminogen activator-associated protein), integrins, and dimeric discoidin receptors (DDR1 and DDR2) [83–85]. Of note, DDR receptors are powerful inhibitors of collagen deposition (fibrillogenesis) [86]. These collagen-receptor interactions modulate cell growth, differentiation, and migration. Of note, β(1) integrins are required for correct embryoid body formation and cardiac fate specification and differentiation of induced pluripotent stem cells [87].

3.4. Influence of ECM in Tissue Generation, Regeneration, and Cancer. VitC influences ECM composition/structure, and it is now evident that the mechanical features of the ECM influence normal and cancer stem cell behavior [88–93]. For instance, while soft substrates (0.6 kPa polyacrylamide gels coated with of type-1 collagen) sustain embryonic stem cell (ESC) self-renewal [94], rigid/stiff substrates induce differentiation/lineage specification of mesenchymal stem cells (MSCs) [95]. ECM structure also impacts on tumor cells' behavior. Indeed, breast cancer malignancy is associated with ECM stiffening [88], and increased collagen deposition and collagen fiber diameter, through ROCK activation, drive epidermal hyperplasia [90]. Interestingly, VitC-dependent collagen prolyl and lysyl hydroxylases are key regulators of breast cancer metastasis [79, 96, 97]. In particular, HIF-1-mediated induction of VitC-dependent PLOD2 enzyme is required for deposition of fibrillar collagen and increase tumor stiffness [96]. Recently, the use of soft fibrin gels allowed the isolation of a subpopulation of highly tumorigenic melanoma cancer cells, named tumor-repopulating cells (TRCs) [98]. Of note, TRC self-renewal relies on a specific epigenetic modification, that is, VitC-dependent demethylation of the histone 3 lysine 9 (H3K9) [99]. Indeed, silencing of H3K9 demethylases inhibits TRC self-renewal [99].

Besides its involvement in tumor cell progression, it is well known that ECM is a key determinant within the stem

cell niche, influencing also normal stem cell behavior and identity. Indeed, several signals and factors produced by the ECM have been reported to integrate with other signaling pathways and transcription factors, thus finely modulating stem cell proliferation, self-renewal, and cell fate decisions both in vitro and in vivo. In particular, integrin receptors respond to ECM signals, regulating stem cell differentiation in early embryogenesis. Indeed, integrin $\beta 1$ is essential for inner-cell mass development [100], and laminin-deficient embryos are unable to undergo epiblast differentiation and cavitation [101]. In line with these findings, type-1 collagen (collagen-1) facilitates mESC self-renewal in vitro [102]. Moreover, VitC-dependent collagen synthesis is essential for the induction of ESC cardiac differentiation, which in turn is impaired by two inhibitors of collagen synthesis (l-2-azetidine carboxylic acid and cis-4-hydroxy-d-proline) [103]. Of note, ECM signaling also influences self-renewal of various somatic stem cells, safeguarding epidermal stem cell compartment [104], neural stem cell maintenance and behavior [105], and hematopoietic stem cell self-renewal and differentiation [106]. Furthermore, ECM is crucial for the skeletal system development, function, and repair, impacting on different stem cell types such as osteoblasts/osteoclasts involved in bone remodeling [107] and mesenchymal stem cells [108], chondrocytes and tenocytes [109], and muscle stem cells (for a review, see [110]). Based on these observations, VitC availability might influence stem cell phenotype/behavior by regulating ECM stiffness and homeostasis and thus playing a crucial role for tissue function.

4. VitC as Epigenetic Modifier

Epigenetic changes are important regulators of gene expression both in development and in diseases. Among them, addition of a methyl group to the C5 position of cytosine on DNA and to the lysine at different positions (K4, 9, 27, and 36) of histone 3 (H3) represents the major and best characterized epigenetic modification. VitC impacts the epigenetic signature of the cells by promoting the activity of the Fe^{2+} and αKG-dependent dioxygenases involved in DNA and histone demethylation.

4.1. VitC and DNA Demethylation. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and plays a pivotal role in modulating transcription activity and cellular identity. In mammals, it mostly involves the cytosine residues of CpG dinucleotides. Global DNA demethylation occurs early during embryo development, at the preimplantation stages, that is, before the inner cell mass (ICM) specification [111, 112]. Conversely, a widespread DNA remethylation occurs during gastrulation and leads to lineage restriction and loss of pluripotency. Aberrant DNA methylation is a hallmark of cancer [113, 114], and global DNA hypomethylation is associated with poor prognosis in tumor patients [115]. Global changes in DNA methylation patterns have been associated with cardiovascular diseases, essential hypertension, inflammation, autoimmune diseases, and infections [116–120]. DNA demethylation depends on the catalytic activity of the VitC/Fe^{+2}/αKG-dependent TET (ten-eleven translocation) enzymes, which convert 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC). A further TET-mediated oxidation of 5-hmC to 5-formylcytosine (5-fC) and to 5-carboxylcytosine (5-caC), together with the activation of the base excision repair mechanism, lead to a complete demethylation process [38]. Of note, blocking VitC entry into the cells by phloretin and/or preventing (knocking down) the expression of Tet genes (*Tet1*, *Tet2*, and *Tet3*) by short interference RNAs (siRNA) significantly reduces VitC-dependent 5-hmC induction [121]. Moreover, treatment of cells with glutathione, an antioxidant agent, does not alter the level of 5-hmC; thus, indicating that VitC-dependent induction of 5-hmC is not due to its activity as an antioxidant, thus supporting a key role of VitC as a cofactor for TET DNA dioxygenases. Interestingly, in hepatocellular carcinoma cells, VitC enhances the demethylating effect of 5-Azacytidine (a DNA methyl transferase inhibitor), inducing the expression/activity of Tet enzymes and increasing the levels of 5-hmC [122].

4.2. VitC and Histone Demethylation. The transfer of a methyl group to lysine and arginine residues of histone proteins is the principal epigenetic modification that occurs on histones and is part of the epigenetic mechanisms that controls stem cell homeostasis. This reaction is catalyzed by histone methyltransferases, which, as DNMTs, use S-adenosylmethionine as donor of methyl groups. While for DNA methylation, only one methyl group is added to cytosine, for histones, up to three or two methyl groups can be added to lysine and arginine, respectively. The resulting mono-, di-, and trimethylated residues can either promote or silence chromatin, depending on the methylated residue. As for DNA methylation, also histone methylation is a reversible process, which depends on the activity of histone demethylases, such as the VitC/Fe^{+2}/α-KG dependent demethylases, that is, the JmjC domain-containing histone demethylases. Thus, VitC enhances the activity of several JmjC domain-containing histone demethylases, inducing histone demethylation and contributing to establish the epigenetic signature of the cells. For instance, VitC counteracts H3K9 and H3K36 and DNA methylation induced by exogenously provided L-Proline in ESCs [123].

4.3. VitC-Dependent Dioxygenases in Stemness, Fibrosis, and Cancer. VitC availability has been reported to be crucial for stem cell identity and plasticity. Increased VitC availability (100 μg/ml) in mouse ESCs promotes widespread DNA demethylation through TET activity [3] and pushes ESCs towards a naive state of pluripotency, which can be placed between the naive/2i and FBS/LIF cultures [1]. VitC induces extensive DNA hypomethylation also in human embryonic stem cells (hESCs) [124]. VitC maintains the methylated pattern and regulates the expression of the imprinted Dlk1-Dio3 cluster in ESCs and acquisition of full pluripotency [125]. Indeed, VitC promotes TET activity (DNA demethylation) and enhances the generation of mouse and human-induced pluripotent stem cells (iPSCs) leading to the transcriptional activation of pluripotency gene network [121, 126, 127]. Similarly, VitC promotes histone

demethylation, reducing the level of H3K9me3 and enhancing the pre-iPSC to iPSC transition [2, 128, 129]. Accordingly, mouse embryonic fibroblasts (MEFs) upon KD of the Tet enzymes fail to undergo the mesenchymal to epithelial transition necessary for the reprogramming process [128, 130, 131]. In line with these findings, VitC activity exerts a key role in the early stages of embryo development, as suggested by several developmental defects induced by VitC deficiency. Indeed, although the knowledge of the mechanism is still limited, several evidence indicate a key role of VitC-dependent activity of DNA and histone demethylases during early embryonic development. Indeed, VitC-induced Tet3 activity is required for epigenetic reprogramming of the zygotic paternal DNA and for the subsequent demethylation of the maternal DNA [132]. Moreover, VitC promotes a second round of demethylation in primordial germ cells (PGC) [133].

Epigenetic changes induced by VitC are important modulators of cell identity and are also considered hallmarks of several pathological conditions. In particular, different types of cancers, including leukemia, melanoma, colorectal adenoma, and gastric cancers, show reduced levels of 5-hmC, which may alter the normal regulation of gene expression and lead to malignant transformation. Interestingly, mutations that result in decreased expression and/or altered function of TET enzymes, as well as mutations in *SVCT* genes that reduce the normal uptake of VitC and thus the level of 5-hmC, have been described in human cancers [134, 135]. Interestingly, restoring the levels of 5-hmC could at least in part decrease malignancies [134]. While the role of epigenetic alterations, that is, reduced 5-hmC levels, has been mostly implicated and studied in cancers, their relevance in other diseases remains still poorly understood. A growing interest is emerging in the context of fibrotic diseases. Although different studies support the idea that the acquisition of the profibrotic characteristics in different pathologies is associated with epigenetic modifications that control changes in gene expression profiles, the impact of DNA methylation on the acquisition of the profibrotic features is still under debate [136].

5. VitC in Stem Cells

Scurvy is characterized by a generalized tissue disintegration, dissolution of intercellular ECM, which induces an excessive proliferation of undifferentiated cells and a reversion to a primitive form of the tissue [137]. This suggests a putative role of the VitC-collagen/ECM integrity on the control of precursor cells' proliferation. Interestingly, stromal changes (ECM depolymerization) also arise at the invasive front or in the proximity of invading neoplastic cells of aggressive tumors, where VitC mostly accumulates [138]. Furthermore, several evidence indicate that VitC stimulates the proliferation of different mesenchyme-derived cell types including osteoblasts, adipocytes, chondrocytes, and odontoblasts [103, 108, 139–146], as well as the proliferation of immunologically relevant T cells [147] and hyalocytes (eye vitreous cells) [148]. Depending on the concentration used, the incubation time, and the cell type analyzed, VitC can inhibit and/or induce stem cell proliferation and/or the differentiation (Table 1). For instance, VitC safeguards the differentiation potency of bone marrow-derived multipotent *mesenchymal stem cells* (MSCs) stimulating their in vitro proliferation [139]. In these cells, VitC stimulates ECM secretion (collagen and glycosaminoglycan). *Adipocyte stem cells* (ASCs) are a heterogeneous population of MSCs that can differentiate into adipocytes, osteoblast, and chondrocytes. L-Ascorbate-2-phosphate (A2-P or Asc-2-P, 250 μM) enhances proliferation of human ASCs and induces the formation of an ASC sheet displaying abundant extracellular matrix (ECM) deposition [149]. Of note, A2-P-treated ASCs maintain both high levels of expression of pluripotency-associated transcription factors *Sox2*, *Oct4*, and *Nanog* and their differentiation capabilities along adipogenic and osteogenic (mesoderm) lineages. This is highly relevant since ASCs cultivated in the absence of VitC tend to lose their stemness and pluripotency. Moreover, A2-P-treated ASCs display enhanced *hepatogenic* (endoderm) and *neurogenic* (ectoderm) transdifferentiation capabilities under specific conditions [149]. Most relevantly, collagen synthesis appears to be required for VitC activity [149]. VitC covalently coupled to a methyl methacrylate polymer enhances the proliferation of bone marrow-derived human MSCs [143]. VitC also promotes proliferation of *cardiac progenitor cells* [150]. A mix of micronutrients, including VitC, transferrin, glutathione, selenite, and ethanolamine, sustains the in vitro expansion/self-renewal of mouse *intestinal stem cells* (ISCs) [151]. ISCs are located at the base of the intestinal crypts and, under appropriate stimuli, are able to divide and differentiate into mature epithelial cells. VitC (40 μg/ml) promotes the proliferation of *spermatogonial stem cells* (SSCs) and reduces the generation of ROS while it induces the expression of the *Bcl-2* (antiapoptotic) gene [152]. Of note, this activity is highly specific of VitC.

5.1. VitC Positively Regulates the Pluripotency Genes. VitC, like retinoic acid (vitamin A) and calciferol (vitamin D), modulates gene expression [153]. VitC induces *Nanog* expression in ESCs and safeguards pluripotency [154]. Similarly, VitC enhances the expression of *Nanog* in mouse teratocarcinoma-derived EC cells (F9) and inhibits the retinoic acid-induced differentiation of EC cells through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway [155]. At mechanistic level, VitC induces STAT2 phosphorylation, which in turn activates *Nanog* transcription [155]. VitC enhances telomerase activity in *periodontal ligament stem cells* (PDLSCs) and upregulates the expression of extracellular matrix type-I collagen, fibronectin, integrin β1, and the stem cell markers *Oct4*, *Sox2*, and *Nanog* as well as osteogenic markers *Runx2*, *Alp*, and *Ocn* [156]. In hESCs, VitC induces the expression of CD30, which is a biomarker for malignant cells in Hodgkin's disease and embryonal carcinoma cells, through a dramatic loss of DNA methylation of a CpG island in the CD30 promoter [157]. Furthermore, VitC is a competitive inhibitor of adenylate cyclase [158] and thus could repress the expression of the genes controlled by the cAMP-dependent pathway [159]. The molecular mechanism(s) involved in

Table 1

Cell type	Effect of exogenously supplied vitamin C	Concentration	Ref.
ASCs (adipocyte stem cells)	↑ Proliferation	250 μg/ml	[149]
hMSCs (human mesenchymal stem cells)	↑ Proliferation	50 μg/ml	[143]
CPC (cardiac progenitor cells)	↑ Proliferation	10–250 μg/ml	[150]
ISCs (intestinal stem cells)	↑ Proliferation/self-renewal	10–250 μM	[151]
Caprine SSCs (spermatogonial stem cells)	↑ Proliferation	40 μM	[152]
Cord blood-derived MSCs (mesenchymal stem cells)	↓ Proliferation	500 μM	[163]
TBV2 ESCs	↑ Naive state of plurypotency	50–150 μg/ml	[1]
ESCs	↑ Naive state of plurypotency	10–500 mM	[3]
Ovarian follicles	↑ Survival	50 μg/ml	[182]
Porcine oocytes	↑ Maturation	50 μg/ml	[181]
J1 ESCs	↑ Pluripotency marker expression	50 μg/ml	[154]
Periodontal ligament stem cells (PDLSCs)	↑ Telomerase activity	20–50 μg/ml	[156]
Bone marrow-derived MSCs (mesenchymal stem cells)	↑ Osteoblastic differentiation	50 μg/ml	[162]
Cord blood-derived MSCs (mesenchymal stem cells)	↑ Osteogenesis	250 μM	[163]
ESCs	↑ Osteoclastogenesis	50 μg/ml	[145]
hMSCs (human mesenchymal stem cells)	↑ Tenogenesis	50 μg/ml	[93]
E14 ESCs	↑ Adipocyte differentiation	25 μg/ml	[165]
C6R8 mESCs	↑ Cardiogenesis	10–100 μM	[170]
Fgfr1$^{-/-}$ R1 mESCs	↑ Rescue of cardiomyocyte differentiation	10 μM	[171]
iPSCs	↑ Cardiogenesis	10–250 μg/ml	[150]
HaCaT cells	↑ Epidermal keratinocyte differentiation	1 mM	[141]
Bone marrow-derived MSCs (mesenchymal stem cells)	↑ Smooth muscle cell differentiation (SMCs)	30 μM	[169]; [168]
Mesencephalic precursor cells	↑ Dopaminergic neuron differentiation	100 μM	[167]; [166]
Pre-iPSCs from MEF	↑ Reprogramming	25–50 μg/ml	[2]
B-cells	↑ Reprogramming	50 ng/ml	[125]
Porcine somatic cells	↑ Reprogramming	50 μg/ml	[179]
MEFs	↑ Reprogramming	25–50 μg/ml	[131]

VitC-dependent induction of pluripotency markers are not well understood and deserve further investigation.

5.2. Impact of VitC on Stem Cell Differentiation. A vast body of evidence supports the notion that supplemental VitC improves the differentiation and maintenance of mesenchyme-derived connective tissues, including adipose tissue, cartilage, bone, and blood (Figure 3). For instance, a combination of VitC and beta-glycerophosphate promotes differentiation of mouse ESCs toward the osteoblast lineage [160]. VitC also enhances osteoblastic differentiation of adipocyte-derived progenitor cells [161]. Moreover, a combination of staphylococcal enterotoxin C and VitC (50 μg/ml) promotes osteoblastic differentiation in bone marrow-derived MSCs [162]. Furthermore, supplemental VitC (250 μM) enhances osteogenesis from umbilical cord blood-derived MSCs, whereas higher VitC concentrations (500 μM) reduces MSC proliferation [163]. Low levels of VitC induce osteogenic differentiation of MG-63 osteosarcoma cell line, whereas at higher doses induce apoptosis [164]. These findings are relevant considering that impaired MSC bone differentiation potential induces osteosarcoma [164]. VitC also enhances osteoclastogenesis in ESCs [145], whereas a cocktail including VitC (25 μg/ml), rosiglitazone, insulin, T3, dexamethasone, and indomethacin, significantly increases ESC adipocyte differentiation [165]. VitC enhances differentiation of mesencephalic precursor cells into dopaminergic neurons [166, 167]. It has been shown that VitC promotes epidermal keratinocyte differentiation and that downregulation of protein kinase C activity has abolished the prodifferentiating effect of VitC [141]. A mix of TGFβ and VitC (30 μM) promotes differentiation of bone marrow-derived MSCs into *smooth muscle cells* (SMCs) [168, 169]. VitC improves cardiac differentiation of mouse ESCs [170]. Of note, VitC promotes cardiac differentiation only when supplemented to the culture medium in a specific time window (day 2–6 differentiation) [150]. Remarkably, VitC addition at the early phases of the differentiation process overcomes the loss of cardiomyocyte differentiation ability in fibroblast growth factor receptor 1 (Fgfr1) knockout (Fgfr1$^{-/-}$) mESCs [171]. The molecular mechanisms underlying VitC-dependent regulation of stem cell differentiation process are largely unknown. The antioxidant activity of VitC is unlikely to be the primary underlying mechanism given that other antioxidants do not induce the same phenotypic transition/differentiation process. Most likely, VitC

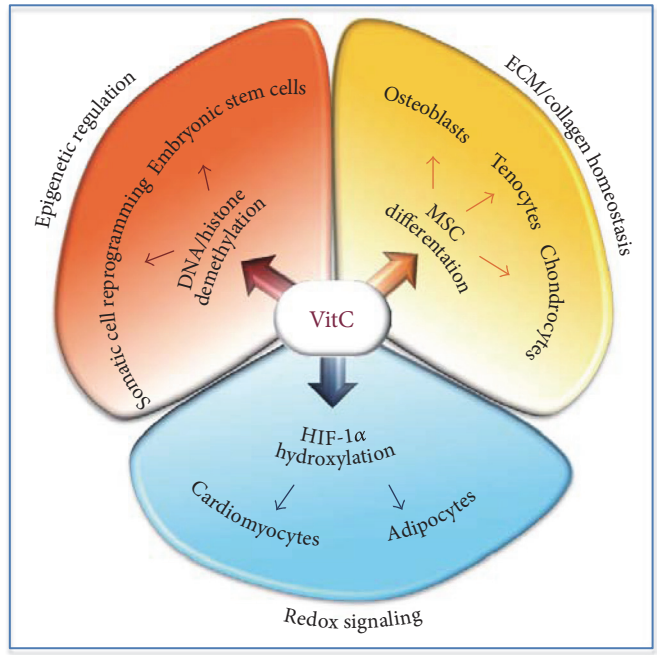

Figure 3: Molecular mechanisms underlying VitC activity on stem cells. VitC-dependent regulation of epigenetic modifications, ECM remodelling and redox balance, control embryonic stem cell self-renewal/proliferation, somatic cell reprogramming, and stem cell differentiation.

may influence stem cell differentiation by modulating DNA and histone demethylation. Accordingly, Tet1 and Tet2 double-knockout mESCs display developmental defects when injected in the mouse blastocyst [172]. Unlike wild-type mESCs, *Tet1* knockdown mESCs generate teratomas containing predominately immature glandular tissues (endoderm) surrounded by stromal cells (mesoderm) and trophoblastic giant cells, thus indicating that *Tet1* is essential for cell lineage specification [173]. Moreover, Tet1/2/3 triple knockout results in impaired differentiation potential of mESCs [174]. Furthermore, *Tet2* silencing in hematopoietic stem cell impairs differentiation and alters hematopoiesis [175], whereas it induces neuroectoderm differentiation in ESCs [176]. Conversely, Tet2 overexpression sustains mESC self-renewal while it impairs differentiation [177].

5.3. VitC in Somatic Cell Reprogramming. It is well known that the chemical composition of the medium strongly influences the epigenetic and biological properties of iPSCs [178]. Indeed, only under specific growth conditions, iPSCs acquire an ESC-like gene expression profile and epigenetic signature. In line with its ability to maintain pluripotency, supplemental VitC improves mouse and human somatic cell reprogramming [2] (Figure 3). Interestingly, addition of VitC (50 ng/ml) prevents aberrant DNA hypermethylation of the imprinted *Dlk1-Dio3* gene cluster and improves the generation of fully pluripotent iPSCs [125]. VitC treatment enhances the expression of pluripotency markers (*Oct4*, *Sox2*, and *Klf4*) during reprogramming of porcine somatic cells through nuclear transfer [179]. Of note, other antioxidants, such as N-acetylcysteine (NAC) and vitamin E, are unable to similarly improve cell reprograming, thus suggesting that this activity does not rely, at least primarily, on the role of VitC as antioxidant [170]. Several reports have led to the conclusion that VitC promotes the generation of induced pluripotent stem cells (iPSCs) through the activation of both histone-demethylating dioxygenases (JMJ) and TET DNA-demethylating enzymes [131]. A putative role of VitC as enhancer of the collagen synthesis/maturation has not been analyzed. Here, we speculate that somatic cells such as fibroblast may suffer VitC starvation in their nucleus, due to an excessive utilization at the level of ER (collagen synthesis). Exogenously, added VitC may thus compensate this nuclear VitC starvation, thus enhancing DNA and histone demethylation and the expression of pluripotency genes. This intriguing hypothesis may explain at least in part VitC effects on reprograming and deserves further investigation.

6. Biotechnological and Medical Applications

VitC is used for the in vitro production of mammalian embryos [180]. Indeed, in a narrow window of concentrations, VitC improves oocyte maturation and the subsequent development of preimplantation embryos. For instance, added at 50 μg/ml, but not at 100 μg/ml, VitC improves porcine oocyte maturation by increasing the cleavage rates and the total cell numbers per blastocyst and by reducing apoptotic cell death [181]. Of note, it has been established that treatment with VitC increases the pregnancy rate in pigs [179]. Furthermore, in vitro culture of ovarian follicles is an emerging tool for fertility preservation. It has been reported that VitC (50 μg/ml) supplementation significantly enhances the survival of early stage (primary) ovarian follicles (<80 μm) cultured in alginate hydrogels, avoiding the breakdown of the follicular basement membrane [182]. In correlation, at the cellular level, VitC upregulates the expression of

extracellular matrix (ECM) and cell adhesion molecules [182]. In adult humans, VitC accumulates in the brain, acting as antioxidant and as neuromodulator for acetylcholine and noradrenaline release [183]. In correlation, recent studies have suggested that supplemental VitC could be beneficial for the treatment of neurodegenerative disorders [184]. As a reducing agent, VitC is used for the treatment of methemoglobinemia [185], an autosomal recessive disorder provoked by the deficiency of methemoglobin reductase (OMIM 250800). VitC is extensively used to attenuate the symptoms of common cold [186–190]. In correlation, it has been reported that DHA, the oxidized form of VitC, inhibits the multiplication of viruses of three different families: herpes simplex virus type 1 (HSV-1), influenza virus type A, and poliovirus type 1, perhaps at the step of nucleocapsid formation occurring inside the Golgi cisternae of infected cells [191]. It has been proposed that VitC may contribute to maintain a healthy skin by altering the gene expression profile of dermal fibroblasts [192]. Indeed, VitC has modified significantly the expression of more than 250 genes in vitro cultured human dermal fibroblasts [192]. The transcriptome modifications involve mainly genes related with regulation of the cell cycle and/or mitosis, DNA replication and/or repair, lipid and glucose metabolism, cytoskeleton and ECM remodeling, and collagen biosynthesis [192]. It has also been observed that an increased intake of VitC improves tissue regeneration after surgical trauma, myocardial infarction, and thermal burns. The effect of VitC on cancer prevention and/or regression has been extensively studied, but the results are controversial and beyond the scope of this review. It has recently been shown that high concentrations of dehydroascorbate (DHA), that is, the oxidized form of VitC, induce oxidative stress and cell death in cancer cells [193], thus refurbishing the interest in the use of supranutritional doses of VitC as anticancer. Nonetheless, a high-dose VitC regimen as anticancer therapy has some contraindications [194] and raises some significant questions [195]. Finally, the effects of VitC on the behavior/identity of cancer stem cells have not been studied but would deserve attention.

7. Conclusions

An increasing number of reports reveal that VitC impacts on stem cell plasticity/identity and that this largely depends on its ability to sustain the activity of several Fe^{+2}/αKG dioxygenase enzymes, which catalyze the hydroxylation (oxidation) of different biological substrates located in specific cellular compartments. Specifically, in the nucleus, VitC modulates the activity of several DNA and histone hydroxylases, whereas in the endoplasmic reticulum, VitC acts as cofactor of collagen hydroxylases. Therefore, VitC is able to modify simultaneously both the epigenetic/gene expression profile and the extracellular matrix (microenvironment) of stem cells. In some cases, the effect of VitC activity appears to be dose dependent within a physiological concentration range. However, whether or not VitC-dependent reactions may rely on specific VitC concentrations requires further investigation, due to the current limited knowledge of the key molecular and biochemical features (i.e., expression profiles, molecular interactions, and kinetic parameters) of the enzymes and/or transporters in the different cellular compartments.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Wnt5a Signaling in Normal and Cancer Stem Cells

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Wnt5a is involved in activating several noncanonical Wnt signaling pathways, which can inhibit or activate canonical Wnt/β-catenin signaling pathway in a receptor context-dependent manner. Wnt5a signaling is critical for regulating normal developmental processes, including stem cell self-renewal, proliferation, differentiation, migration, adhesion, and polarity. Moreover, the aberrant activation or inhibition of Wnt5a signaling is emerging as an important event in cancer progression, exerting both oncogenic and tumor suppressive effects. Recent studies show the involvement of Wnt5a signaling in regulating normal and cancer stem cell self-renewal, cancer cell proliferation, migration, and invasion. In this article, we review recent findings regarding the molecular mechanisms and roles of Wnt5a signaling in stem cells in embryogenesis and in the normal or neoplastic breast or ovary, highlighting that Wnt5a may have different effects on target cells depending on the surface receptors expressed by the target cell.

1. Introduction

Stem cells, including embryonic stem cells and stem cells identified in adult tissues, have an ability to self-renew and to generate more differentiated progeny. The embryonic stem cell is derived from the inner cell mass of the blastocyst, is pluripotent, and can thus generate all the tissues of the body [1]. Stem cells in adult tissues reside in specialized niches, where they integrate various environmental and intrinsic signaling inputs that determine cell fate and maintain tissue homeostasis.

Wnt factors are a group of these signaling molecules that act on stem cells within the stem cell niche to help maintain their capacity for self-renewal. Wnt factors are known to bind to different receptors to transduce the canonical signaling pathway or noncanonical signaling pathway(s) that regulate diverse biological activities [2, 3]. The canonical Wnt signaling pathway is initiated when Wnt factors bind to Frizzled family receptors (Fzd) and low-density lipoprotein receptor-related protein 5/6 coreceptors (LRP5/6) to form complexes. This results in the recruitment of Axin and dishevelled (Dvl) to the plasma membrane and disruption of the β-catenin degradation complex, leading to accumulation of β-catenin in the cytoplasm, which then translocates to the nucleus, where it binds to T-cell factor (TCF)/lymphoid enhancer factor (LEF) family and activates the transcription of canonical Wnt target genes [4, 5]. In contrast, several Wnt factors activate β-catenin-independent pathways (noncanonical Wnt signaling pathways), known as the Wnt-$Ca2^{+}$ pathway or planar cell polarity (PCP) pathway, which act to direct cell migration during embryogenesis [6–8].

Nineteen secreted cysteine-rich Wnt family glycoproteins have been identified in mice and humans. During earlier mammalian embryogenesis or maintenance of adult tissue homeostasis, these Wnt factors and their receptors are dynamically expressed to activate appropriate signaling, to ensure the right balance between proliferation and differentiation. Perturbation of Wnt signaling with aberrant expression of Wnt factors, their receptors, or downstream signaling molecules may lead to the development of several human cancers [6, 9, 10]. Because the tumor initiation capacity seems to be restricted to a small population of tumor cells that are endowed with the capacity for self-renewal and differentiation, they often are referred to as cancer stem cells (CSCs, or tumor initiation cells) [11–14]. More importantly, the biology of embryonic stem cells, normal stem cells, and CSCs is highly interrelated. This is evident from the fact that molecular signals (e.g., Wnt

signaling) that define and maintain embryonic stem cell or adult normal stem cells are often aberrantly activated in tumor cells.

A major focus of this review is the role of Wnt5a signaling via different surface receptors in the regulation of stem cells in embryogenesis and in the normal or neoplastic breast or ovary, as there already are several excellent reviews that cover aspects of Wnt signaling in colon cancer [14, 15]. We summarized the latest advances on the signaling pathways that are activated by Wnt5a and that pose a conundrum for the rational design of drugs aimed at depleting the CSCs (or tumor initiation cells) within tumors while sparing the function of normal tissues.

2. Wnt5a Signaling Pathway

Wnt5a is a noncanonical Wnt ligand that is evolutionarily conserved and plays an important role in development. Homozygous Wnt5a knockout mice have perinatal lethality due to developmental defects [16]. Previous studies showed that Wnt5a can interact with Fzd2 and receptor tyrosine kinase-like orphan receptor 2 (ROR2) to activate Rac1 in a β-catenin-independent pathway. More recently, we demonstrated that Wnt5a induced heterooligermization of receptor tyrosine kinase-like orphan receptor 1/2 (ROR1/ROR2), which recruited and activated guanine exchange factors (GEFs), which in turn activated RhoA and Rac1, respectively, enhancing leukemia-cell chemotaxis and proliferation [17]. On the other hand, as another action of Wnt5a, it competed with Wnt3a for binding to Fzd2, thereby inhibiting Wnt3a-dependent LRP6 phosphorylation and β-catenin accumulation in vitro and in intact cells, inhibiting the capacity of Wnt3a to induce accumulation of β-catenin, and thereby inhibiting β-catenin-dependent Wnt signaling [18].

However, Wnt5a also could activate β-catenin-dependent pathway and induce secondary axis formation in Xenopus embryos that coexpressed the Fzd5 receptor [19, 20]. Subsequently, another study found that Wnt5a could inhibit canonical Wnt/β-catenin signaling in cells that expressed ROR2, but also could induce canonical Wnt/β-catenin signaling in cells that expressed Fzd4 and LRP5 [21]. Thus, a single Wnt5a ligand can have disparate effects on cells depending on receptor availability. Therefore, the cellular context dictates the effect of Wnt5a. This might account to the observation that the Wnt5a could exert either oncogenic or tumor suppressive effects in different cancers [18, 21–23].

3. Wnt5a Signaling in Embryonic Stem Cells

The Wnt pathway (triggered either by Wnt3a, Wnt5a, or Wnt6) also could be involved in the short-term maintenance of pluripotency of both mouse and human ES cells [24]. The first evidence for this was provided by a study of a pharmacological inhibitor of glycogen synthase kinase-3 (GSK3) that could modulate GSK3 activity, leading to activation of β-catenin canonical signaling and increased stability of c-Myc [25, 26]. Wnt5a and Wnt6 subsequently were found to be produced by embryonic fibroblast feeder cells, which could inhibit mouse ES cell differentiation in a serum-dependent manner. Direct activation of β-catenin, using constitutively active β-catenin (S33Y) without disturbing the upstream components of the Wnt/β-catenin, fully recapitulated the effect of Wnts on ES cells. Importantly, Wnt5a also is a potent inhibitor of β-catenin phosphorylation, thereby stabilizing β-catenin [5]. These data suggest that Wnt5a signals to stabilize β-catenin to further activate Wnt/β-catenin canonical signaling in mouse ES cells. Finally, the Wnt/β-catenin pathway can upregulate the mRNA for STAT3, a known regulator of ES cell self-renewal [27], suggesting a molecular mechanism whereby the Wnt/β-catenin pathway acts to prevent ES cell differentiation through convergence on the LIF/JAK-STAT pathway at the level of STAT3 [28]. However, what surface receptors are involved in the activation of Wnt5a/β-catenin signaling in ES cells and whether noncanonical Wnt signaling also could be activated by Wnt5a in ES cells remains unclear.

4. Wnt5a Signaling on Stem Cells in Normal Mammary Gland and Ovarian Tissues

A line of evidence supports the importance of Wnt in maintaining of mammary stem cells in mammary gland [29–31]. However, Wnt5a does not induce β-catenin/TCF transactivation activity in mammary gland tissue. The cells of the mammary gland are hyperproliferative in Wnt5a knockout mice, whereas in the presence of ectopic Wnt5a, ductal extension is inhibited [32]. These phenotypes are the inverse of the activation of canonical Wnt/β-catenin signaling. Therefore, Wnt5a may limit and control mammary gland growth by suppressing Wnt/β-catenin signaling. It is puzzling then that this inhibition of mammary gland growth was not observed in transgenic MMTV-Wnt5a strains [33].

In the *Drosophila* germarium, altered Wingless (Wg; the fly Wnt homologue) signaling occurs in ovarian germline stem cells (GSCs) through removal of its regulators such as dishevelled, armadillo, Axin, and shaggy; this causes GSC loss, influences follicle cell proliferation, and induces differentiation [34, 35]. Using conditional gene targeting for Wnt5a in ovarian granulosa cells (GC) results in the female subfertility associated with increased follicular atresia and decreased rates of ovulation. Further study found that Wnt5a regulates its target genes not by signaling via the Wnt/Ca^{2+} or planar cell polarity pathway, but rather by inhibiting the canonical pathway, causing both β-catenin and cAMP responsive element binding (CREB) protein levels to decrease via a glycogen synthase kinase-3β-dependent pathway in ovarian granulosa cells (GC). These data indicate that Wnt5a is required for normal ovarian follicle development and can antagonize gonadotropin responsiveness in granulosa cells by suppressing canonical Wnt/β-catenin signaling [36].

Collectively, Wnt5a is most likely to suppress β-catenin signaling in normal stem cells of mammary gland and ovarian tissues, although Wnt5a is able to activate β-catenin signaling in embryonic stem cells. Whether this difference is due to receptors that are differentially expressed in embryonic stem cells versus stem cells of normal mammary gland or the ovary needs to be further investigated.

5. Wnt5a Signaling in Breast Cancer Stem Cells

Wnt factors may affect various mammary epithelial cells and induce an expansion of stem cells during tumor progression [37–42]. Moreover, aberrant β-catenin expression was associated with basal, triple-negative breast cancers and poor clinical outcome [43, 44]. The occurrence of aberrant Wnt signaling in specific breast cancer subtypes is less likely dictated by activation caused by somatic mutations. Mutations have not been identified in *CTNNB1,* which encodes β-catenin, and mutations within *APC* have been identified in less than 20% of breast cancer patient samples [45]. Further investigation is needed to determine which Wnt ligands and receptors cause this aberrant Wnt/β-catenin signaling.

Recently, a study on MMTV-*Wnt1* mouse primary cells found that both recombinant Wnt3a and recombinant Wnt5a could enhance formation of mammospheres in vitro. Wnt5a-induced formation of mammospheres was not caused by an increase in canonical Wnt/β-catenin signaling, but was instead mediated by noncanonical Wnt signaling requiring the receptor tyrosine kinase ROR2 and the activity of the Jun N-terminal kinase, JNK, in mouse breast cancer cells [46]. This is consistent with the observation that patients with breast cancers that express ROR2 had a significantly shorter overall survival than those with tumors lacking expression of ROR2 [47]. However, silencing ROR2 in human breast cancer cell lines decreased both β-catenin-dependent and β-catenin-independent targets [47], suggesting that ROR2 may be involved in both β-catenin-dependent and β-catenin-independent Wnt signaling pathways. ROR1 predominately seems to be expressed by less well-differentiated tumors that have high potential for relapse and metastases and that also express markers associated with epithelial–mesenchymal transition (EMT) [48, 49]. Conversely, silencing ROR1 in human breast cancer cell lines could attenuate expression of genes associated with EMT and impair their migration/invasion capacity in vitro and their metastatic potential in vivo [49]. Very recently, Chien et al. reported that ROR1 expression might be an independent adverse prognostic factor in patients with triple-negative breast cancers [50]. Whether ROR1 can have a similar effect with ROR2 on breast cancer stem cell and what signaling pathway(s) are activated by ROR1 in breast cancer stem cells remains unknown.

A study on a mouse model of ErbB2-induced breast cancer found conflicting evidence on the effect of Wnt5a on human breast cancer stem cells. During ErbB2-induced mammary tumorigenesis, basal tumor-initiating cells (TIC), which exhibited enhanced tumorigenic capacity compared with the corresponding luminal progenitors, preferentially lost *Wnt5a* expression, as determined by transcript profiling analysis. Moreover, *Wnt5a* heterozygosity promoted tumor multiplicity and pulmonary metastasis. As a TGFβ substrate, luminal cell-produced Wnt5a induced a feed-forward loop that activated SMAD2 in a RYK- and TGFβR1-dependent manner to limit the expansion of basal TIC in a paracrine fashion, a potential explanation for the suppressive effect of Wnt5a on mammary tumorigenesis [51]. In this mouse model, it remains uncertain whether canonical Wnt/β-catenin signaling was activated or if Wnt5a also inhibited canonical β-catenin signaling to suppress basal TIC expansion.

The Weinberg laboratory studied immortalized human mammary epithelial cells (mMECs) and found that both the canonical and noncanonical Wnt pathways cooperated with TGFβ signaling in not only the maintenance, but intriguingly also the induction, of stem cell properties [52]. Moreover, only noncanonical Wnt ligands Wnt5a and Wnt16 were found upregulated in stem-like cells relative to non-stem-like cells [52]. Therefore, it is possible that Wnt5a or Wnt16 may activate either the canonical or noncanonical Wnt signaling pathway in a receptor context-dependent manner in human breast cancer stem cells.

6. Wnt5a Signaling in Ovarian Cancer Stem Cells

Similar to breast cancer, Wnt5a effect on ovarian cancer is also controversial. An early study of primary ovarian tumors ($n = 130$) that showed low levels of Wnt5a in ovarian cancer relative to normal ovary is predictive of a poor outcome [53]. Ectopic expression of Wnt5a inhibits the proliferation of human ovarian cancer cell line OVCAR5 both in vitro and in vivo orthotopic ovarian cancer mouse models. Mechanistically, ectopic expression of Wnt5a in OVCAR5 antagonizes canonical Wnt/β-catenin signaling and induces cellular senescence by activating the histone repressor A (HIRA)/promyelocytic leukemia (PML) senescence pathway [53].

In contrast, studies involving a large number of patients found that upregulation of Wnt5a was associated with a relatively poor prognosis [54–58]. Compared with the frequency of Wnt1 expression in ovarian cancer, Wnt5a was more frequently found in malignant epithelial ovarian cancer patients (80% out of a total 38) [54]. Of note, patients with ovarian cancers that express high levels of both Wnt1 and Wnt5a had a significantly lower probability of long-term survival than patients with ovarian cancers that did not express Wnt1 or Wnt5a. Furthermore, Wnt5a is prevalent in ascites fluid obtained from women with ovarian cancer [55], suggesting that it contributes to the ovarian tumor microenvironment. This is consistent with the observation that high levels of Wnt5a are associated with increased risk for metastasis [55].

Activating mutations in the canonical Wnt pathway is rare in ovarian cancer, with the exception of endometrioid ovarian cancers [59]. The contribution of canonical Wnt signaling to progression of ovarian cancer remains unknown. Conversely, studies on noncanonical Wnt/β-catenin signaling show that Wnt5a may regulate ovarian cancer EMT, migration, or metastasis via noncanonical signaling pathways [57, 60]. Consistent with this notion, recent studies have found that expression of ROR1, a receptor of Wnt5a, was highly expressed by high-grade and less-differentiated ovarian cancers and associated with a relatively short disease-free survival and overall survival compared to ovarian cancers that did not express ROR1 [61–63]. Ovarian cancers that express high levels of ROR1 had gene expression signatures associated with ovarian CSCs [62]. Furthermore, tumor-cell expression of ROR1 apparently correlates with the expression of ALDH1 and the capacity to form tumor

spheroids in vitro (both markers of CSC). Treating primary ovarian patient-derived xenograft (PDX) tumor cells, which express high levels of ROR1 with an anti-ROR1 mAb (UC-961 or cirmtuzumab), inhibited spheroid formation and migration in vitro and engraftment and re-engraftment in immune-deficient mice, indicating that ROR1 may influence ovarian cancer stem cell self-renewal. Further studies are needed to determine if Wnt5a is responsible for the influence that ROR1 apparently has on the biology of ovarian cancer stem cells.

7. Concluding Remarks

Wnt5a may play an important role in embryonic stem cell and organ development. However, the role of Wnt5a in cancer stem cells is varied and complex. It may suppress or promote tumor progression. To elucidate molecular mechanisms that drive altered cellular behavior, additional research on tissue-specific expression of specific receptors and coreceptors is needed. In particular, a more detailed understanding of the complex cross-talk between Wnt5a and specific receptors that are expressed in embryonic stem cells and that may be re-expressed or reactivated in cancer stem cells, but not in normal somatic tissues (e.g., ROR1 and ROR2), may enable development of specific inhibitors that block aberrant signaling and thereby favorably impact patient survival.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Effect of BMP-2 Delivery Mode on Osteogenic Differentiation of Stem Cells

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Differentiation of stem cells is an important strategy for regeneration of defective tissue in stem cell therapy. Bone morphogenetic protein-2 (BMP-2) is a well-known osteogenic differentiation factor that stimulates stem cell signaling pathways by activating transmembrane type I and type II receptors. However, BMPs have a very short half-life and may rapidly lose their bioactivity. Thus, a BMP delivery system is required to take advantage of an osteoinductive effect for osteogenic differentiation. Previously, BMP delivery has been designed and evaluated for osteogenic differentiation, focusing on carriers and sustained release system for delivery of BMPs. The effect of the delivery mode in cell culture plate on osteogenic differentiation potential was not evaluated. Herein, to investigate the effect of delivery mode on osteogenic differentiation of BM-MSCs in this study, we fabricated bottom-up release and top-down release systems for culture plate delivery of BMP-2. And also, we selected Arg-Gly-Asp- (RGD-) conjugated alginate hydrogel for BMP-2 delivery because alginate is able to release BMP-2 in a sustained manner and it is a biocompatible material. After 7 days of culture, the bottom-up release system in culture plate significantly stimulated alkaline phosphate activity of human bone marrow-mesenchymal stem cells. The present study highlights the potential value of the tool in stem cell therapy.

1. Introduction

An in vitro differentiation process to obtain specific cell type from stem cells is required for stem cell therapy. Stem cells can be manipulated in vitro under specific conditions that favor differentiation towards a designated somatic cell type [1]. Many studies have demonstrated manipulative techniques to direct stem cell differentiation through use of defined media, substrates and growth factors [2]. In particular, bone morphogenetic protein-2 (BMP-2) is a well-known inductive growth factor for osteogenic differentiation of various stem cells [3]. BMP-2 binds to microdomains on the cellular surface related to biological signal pathways, such as cognate receptors, to induce osteogenic differentiation [4]. Thus, the probability of BMP-2 binding to surface receptors should be maximized to enhance efficacy of osteogenic differentiation during BMP-2 treatment process in vitro.

Protein delivery system is a promising method for localized and sustained delivery of biologically active BMP-2 at the target sites [5]. Conventional methods involve the daily addition of BMP-2 to the culture medium and BMP-2 is assumed to be homogeneous as well as sufficient in the medium [6]. However, only a small amount reaches the cellular microdomains related to the biological signal pathways because of Brownian motion of BMP-2 in the culture medium. In contrast, BMP-2 released from a matrix utilizing a protein delivery system could efficiently bind to receptors on the cultured cells. However, such delivery

systems rarely focus on monolayer cultures subject to conventional techniques. Previous studies have not compared the effect of BMP-2 delivery modes on stem cells in a monolayer culture system that provides convenience and speed to obtain a large number of desired cells, such as osteocytes.

The purpose of this study was to investigate the effects of BMP-2 delivery mode on the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (BM-MSCs). To that end, BMP-2 was loaded to Arg-Gly-Asp (RGD) peptide-conjugated alginate hydrogel. We selected alginate as a base material for BMP-2 delivery in this study because alginate has valuable properties such as biocompatibility and gel-forming properties via ionic crosslinking using calcium in mild condition. In addition, this reaction is rapid and selective and produces high yields. Thus, this can be used as a carrier of BMP-2 and to create a suitable environment for cell culture. Human BM-MSCs were induced by using a bottom-up and top-down release system and the cells were characterized in terms of alkaline phosphatase (ALP) activity and differentiation. The results may provide a useful tool for expanding the potential applications of stem cell therapy.

2. Methods and Materials

2.1. Synthesis of Peptide-Modified Alginate. Sodium alginate (M_w = 200,000–300,000; FMC Biopolymer, Philadelphia, PA) was dissolved in a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at room temperature (pH = 6.5, 0.3 M NaCl). A peptide with the $(\text{glycine})_4$-arginine-glycine-aspartic acid-alanine-$(\text{serine})_2$-lysine (G4RGDASSK) sequence (Anygen, Seoul, Republic of Korea) was added to the alginate solution in the presence of *N*-hydroxysulfosuccinimide (sulfo-NHS; Pierce, Rockford, IL) and 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC, Sigma-Aldrich, St. Louis, MO). The peptide-modified alginate was purified by extensive dialysis with distilled water for 5 days (M_w cut-off = 3,500) and activated charcoal treatment and then sterilized with a 0.22 μm filter. The degree of substitution (DS) of the peptide was determined with the number of peptides per 100 uronic acid residues in the alginate chain. In this study, the DS was 0.15 [7].

2.2. Preparation of Hydrogels and Encapsulation of BMP-2. The purified and lyophilized RGD-modified alginate (60 mg) was dissolved in α-MEM (3 mL) and mixed with the Chinese Hamster Ovary (CHO) cell-derived recombinant human BMP-2 (R&D Systems, Minneapolis, MN). A calcium sulfate ($CaSO_4$) solution (20% w/v, 120 μL) was added to a second syringe. The two syringes were connected with a female connector, and the contents were quickly mixed. An alginate solution containing $CaSO_4$ formed a gel at 37°C for 20 min. The gel was used to punch out discs (8 mm diameter; 0.5, 1, and 2 mm thickness) used for release test of BMP-2 and for culture of cells.

2.3. Determination of the Kinetics of BMP-2 Release from RGD-Alginate Hydrogel Discs. To determine the degree of BMP-2 release from RGD-alginate hydrogel discs (2% w/v) with 0.5, 1, and 2 mm thickness, BMP-2-loaded scaffolds were sunk in 24-well culture plates containing 1 mL phosphate-buffered saline (PBS, pH 7.4; Sigma) and the culture plates were incubated at 37°C without agitation. At predetermined times, enzyme-linked immunosorbent assay (ELISA) was done to determine the kinetics of BMP-2 release from RGD-alginate hydrogel discs with various thicknesses. At each time point, supernatant was collected and the culture plates were replenished with fresh buffer. The amount of BMP-2 in the supernatants was measured using an ELISA kit (human beta BMP-2 Duoset; R&D Systems). Briefly, ELISA plates (NUNC, Polylabo, Strasbourg, France) were coated with the capture monoclonal antibody and then blocked with the bovine serum albumin (1 w/v%) and sucrose (5 w/v%) for 1 hour. After appropriately diluted supernatants were added, bound-BMP-2 was detected with biotin-conjugated anti-human BMP-2 polyclonal antibody. Streptavidin-conjugated horseradish peroxidase was then added to the plates. Enzyme substrate (tetramethylbenzidine and peroxide) was treated for 20 minutes, and the reaction was stopped by adding an acidic solution. Absorbance was measured at 450 nm range of PowerWave X340 plate reader (Bio-TEK Instrument, Inc., Winooski, VT). The amount of BMP-2 was calculated from a calibration curve based on known concentrations of BMP-2. Experiments were performed with five replicates of each supernatant.

2.4. Cell Culture and Differentiation. Human BM-MSCs were purchased (Lonza Ltd., Walkersville, MD) and cultured in accordance with a previously described method [8]. Briefly, cells were maintained in α-MEM (Gibco BRL, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 2 mM L-glutamine (Gibco BRL), 100 units/mL penicillin (Gibco BRL), and 0.1 mg/mL streptomycin (Gibco BRL). In this study, BM-MSCs were used after three to five passages. The stem cell markers (CD 44 (95.39%), CD 73 (93.13%), CD 90 (98.60%), and CD 105 (94.71%)) for BM-MSCs were analyzed by fluorescence activated cell sorting (FACS) analysis which characterized BM-MSCs at passage 5 by flow cytometry histograms (see Supplementary Figure 1 in Supplementary Material available online at https://doi.org/10.1155/2017/7859184).

To determine RGD modification of alginate, the BM-MSCs were seeded onto the surfaces of alginate hydrogel discs at density of 2×10^4 cells/cm^2. The discs were placed in 24-well culture plates and incubated at 37°C under 5% CO_2 atmosphere. After a 24 h culture period, discs were washed with PBS to remove nonadhered cells, and then photographs of the BM-MSCs adhering to the surface of the discs were taken using an optical microscope (Olympus, Tokyo, Japan). For the osteogenic differentiation of BM-MSCs, the BM-MSCs and RGD-modified alginate hydrogel discs containing BMP-2 (2 μg/disc) were applied as shown in Figures 2 and 3. Briefly, the BM-MSCs were seeded onto RGD-modified alginate hydrogel discs with BMP-2 and cultured with α-MEM for 7 days to obtain the bottom-up release system. To construct the top-down release system, BM-MSCs were seeded onto RGD-modified alginate hydrogel discs without BMP-2 and RGD-modified alginate hydrogel discs with BMP-2 were placed in Transwell inserts. Following the adhesion of the BM-MSCs,

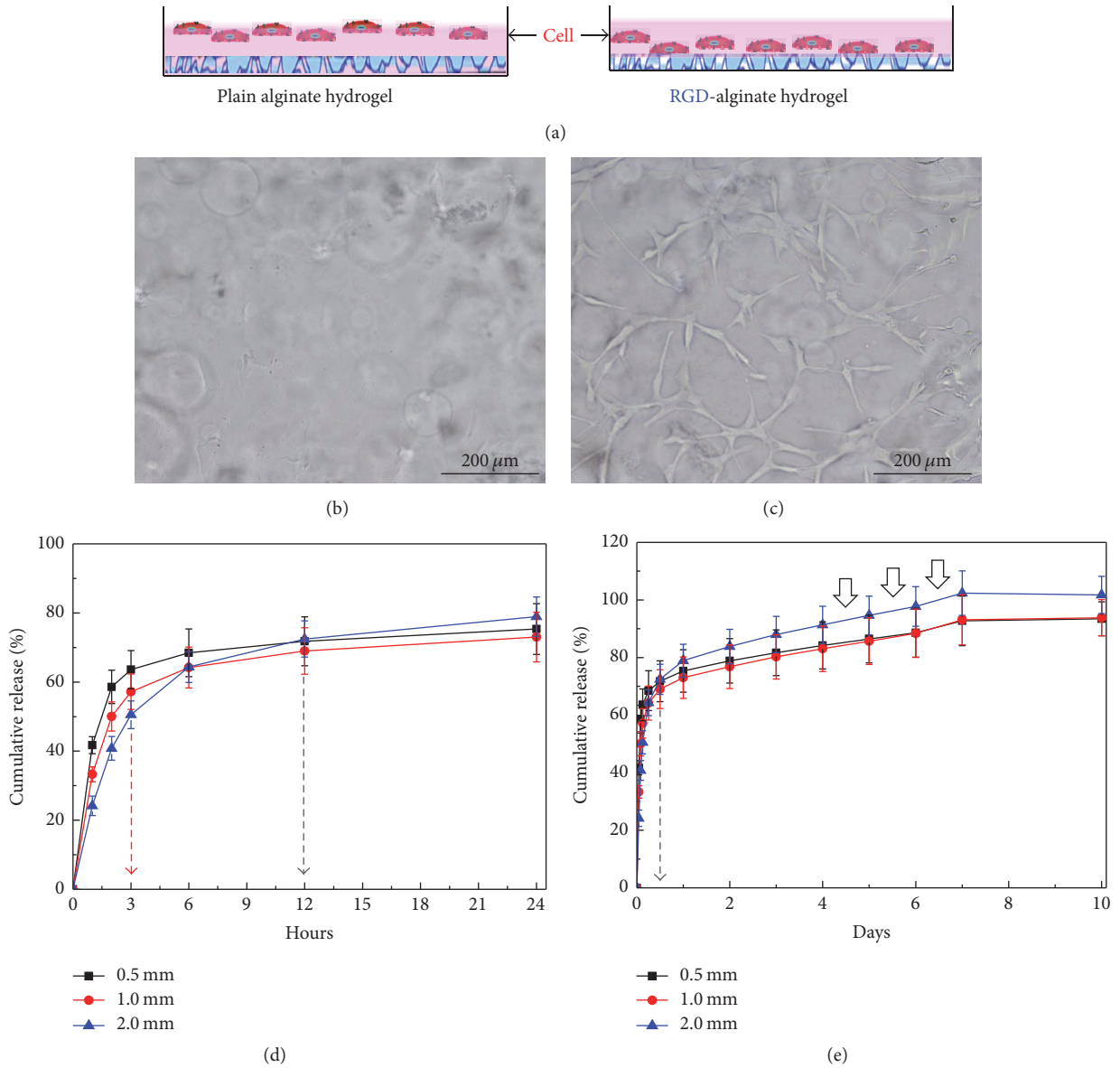

Figure 1: Role of RGD peptide in cell adhesion during culture of BM-MSCs. (a) Experimental design for plain and modified RGD-alginate hydrogel. Photographs of human BM-MSCs adhered on the surface of (b) unmodified alginate hydrogel disc and (c) RGD-modified alginate hydrogel disc 1 day after cell plating. The profiles of BMP-2 release from RGD-modified alginate hydrogel disc with various thicknesses (0.5, 1.0, and 2.0 mm) (d) for 24 hours and (e) 10 days. The amount of BMP-2 released from various hydrogel discs was determined by ELISA. The values represent the mean ± standard deviation ($n = 5$).

the cells were cultured with Transwell inserts containing RGD-modified alginate hydrogel discs with BMP-2 in α-MEM for 7 days, with the culture media changed every other day.

2.5. ALP Assay. To investigate the effects of BMP-2 delivery mode on BM-MSCs osteogenic differentiation, ALP activity as an early osteogenic differentiation marker was measured after 7 days, when cells on alginate hydrogel discs were stained using an ALP staining kit II (Stemgent, Lexington, MA) according to the manufacturer's instructions. The cells on each alginate gel disc were observed and photographed with an optical microscope (Nikon, Tokyo, Japan). In addition, the cells were lysed to quantify ALP activity as described previously [9]. ALP activity was normalized by the protein content, which was examined using the BCA protein assay reagent (Pierce Chemical, Rockford, IL). The BM-MSCs cultured on RGD-modified alginate without BMP-2 were used as negative control (Supplementary Figure 2).

2.6. Statistical Analysis. The quantitative data are expressed as means ± standard deviations (SD). Statistical analyses were performed by one-way analysis of variance (ANOVA) with Statistical Package for the Social Sciences (SPSS) software (SPSS Inc., Chicago, IL). A value of $p < 0.05$ was considered statistically significant.

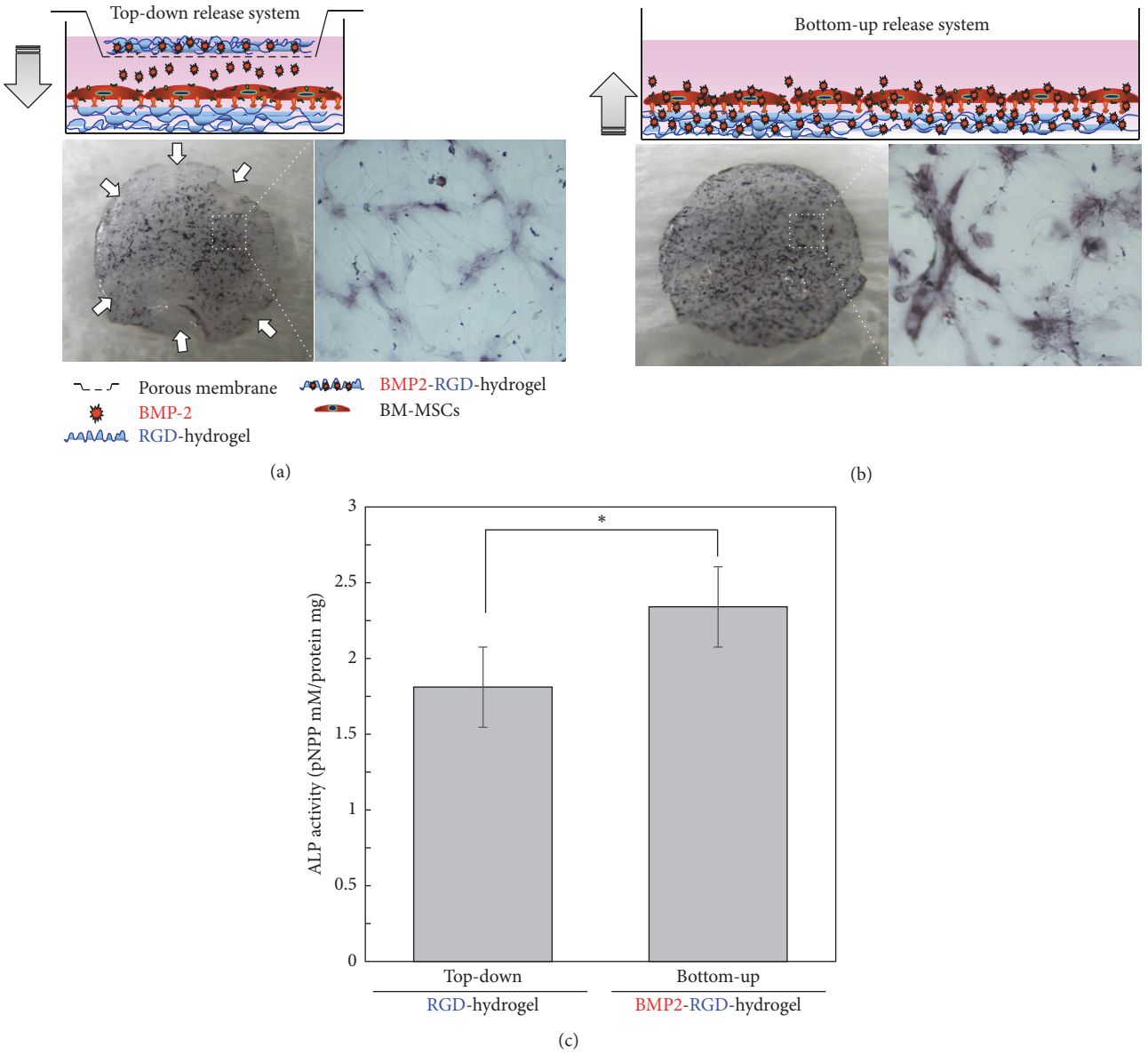

FIGURE 2: Schematic representation of culture system on alginate hydrogel disc used to assess the effect of BMP-2 delivery mode on osteogenic differentiation of human BM-MSCs and photographs of ALP stained human BM-MSCs. (a) Top-down release system, (b) bottom-up release system, and (c) quantification of ALP activity for BM-MSCs cultured under each mode of delivery. The values represent the mean ± standard deviation ($n = 3$). $^{*}p < 0.05$ compared with top-down release system at 7 days.

3. Results

3.1. Preparation of RGD-Modified Alginate Hydrogel for Human BM-MSC Culture. We first performed chemical conjugation of RGD sequence containing GGGGRGDASSK peptide into the alginate hydrogel. Because the attachment capacity of cells is low on alginate hydrogel, it is ideal for the investigation of RGD influence on adhesion of cells. Applicability was examined by plating cells (2×10^4 cells/cm^2) on an unmodified (control) and RGD-modified alginate hydrogel disc (Figure 1(a)). After 24 hours of culture, the unmodified group showed that the cells remained in suspension and were unable to adhere (Figure 1(b)). In contrast, cell adhesion in the RGD-modified group was favorable and the attached cells exhibited a fibroblastic morphology (Figure 1(c)). This result indicated that the peptide containing RGD was successfully conjugated to the alginate backbone, resulting in enhancements of cell adhesion on hydrogel disc that does not naturally possess adhesive properties.

3.2. Analysis of the Kinetics of BMP-2 Release from RGD-Alginate Hydrogel Discs with Various Thicknesses. Next, we loaded BMP-2 (2 μg/disc) into RGD-modified alginate hydrogel discs to determine the optimal thickness for releasing BMP-2 effectively into the cell culture plate. The RGD-modified alginate hydrogel discs with various thicknesses

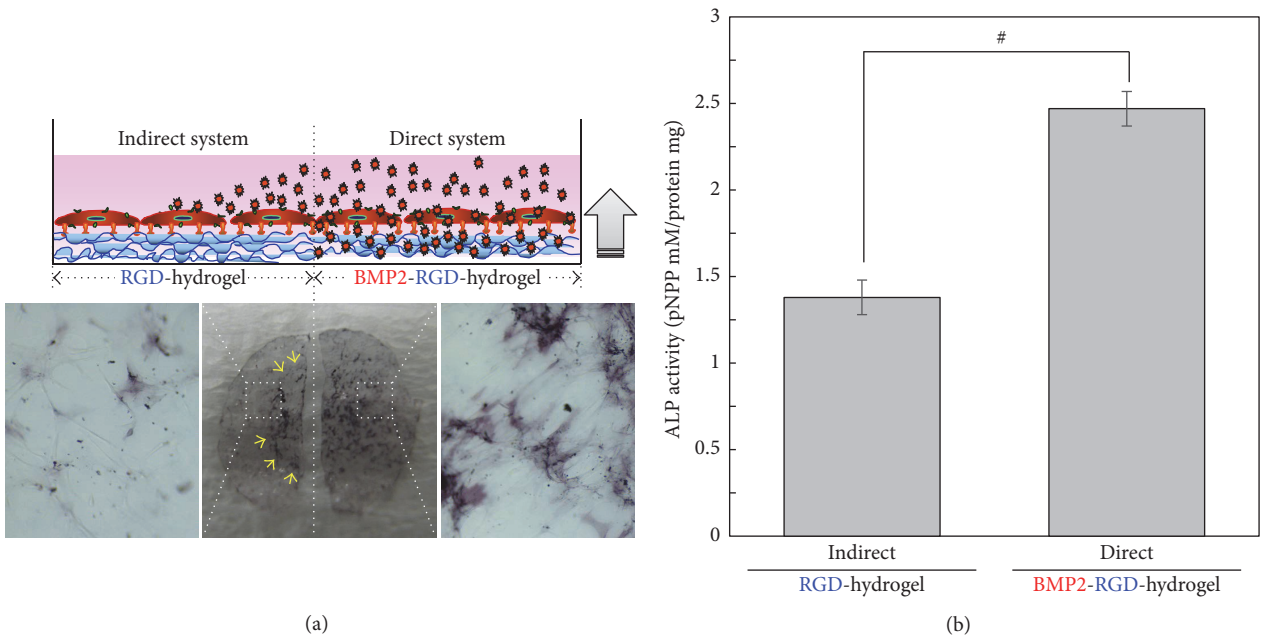

FIGURE 3: A top-down and bottom-up approach applied to a one culture dish. (a) ALP staining and (b) quantification of ALP activity for BM-MSCs cultured in their respective half. The values represent the mean ± standard deviation ($n = 3$). $^{\#}p < 0.05$ compared with direct bottom-up release system at 7 days.

(0.5 mm, 1.0 mm, and 2.0 mm) were fabricated and placed in cell culture plates to test the kinetics of BMP-2 release from alginate hydrogel discs. The release of BMP-2 from 0.5 mm thickness-alginate discs was more rapid than that from 2.0 mm thickness-alginate discs (Figure 1(d)). Almost all of the BMP-2 was released from the alginate hydrogel discs within the first 10 days (Figure 1(e)). Importantly, the release of BMP-2 from 2.0 mm thickness-alginate hydrogel discs was sustained for 12 hours. The BMP-2 release rate decreased as the thickness of alginate hydrogel discs increased. In addition, with the 0.5 mm discs, approximately 90% of the initially loaded BMP-2 was released over the first 10 days. In contrast, for the 2.0 mm discs, virtually all of the loaded BMP-2 was released over the first 10 days. The present findings indicate that the rate of BMP-2 release from alginate hydrogel disc can be controlled by the thickness of hydrogel disc. A disc thickness of 2.0 mm provides favorable kinetics of BMP-2 release for osteogenic differentiation of stem cells.

3.3. Osteogenic Differentiation of BM-MSCs in Top-Down and Bottom-Up Release Systems for BMP-2 Delivery. In order to investigate whether BM-MSCs differentiation was influenced by the mode of BMP-2 delivery, BM-MSCs were cultured in a top-down or bottom-up release system (Figure 2). Similar to standard protocols, the top-down approach was structured to release BMP-2 into the media through an 8 μm porous membrane. In contrast, the bottom-up system was set up in order to make the RGD-alginate hydrogel be in contact with BM-MSCs during BMP-2 release. This approach utilized adhesion and proximity in order to force the cells to be more interactive with the protein. The osteogenic differentiation of BM-MSCs was analyzed using ALP staining and ALP activity (mM/protein mg). Results of ALP staining showed that BM-MSCs cultured in the bottom-up release system were intensively stained compared to those in the top-down release system (Figures 2(a) and 2(b)), demonstrating that BM-MSC differentiation into osteogenesis occurred better in the bottom-up release system than the top-down release system. Additionally, we analyzed ALP activity (mM/protein mg) in both release systems. The bottom-up release system produced increased protein level in comparison to the top-down release system (Figure 2(c)), confirming that differentiation was more favorable in the bottom-up release system.

Based on these results, we confirmed that differentiation of stem cells was influenced by the method of release. To further investigate this, BM-MSCs in the same culture discs were divided into two sections consisting of an untreated RGD-hydrogel and modified BMP2-RGD-hydrogel. As expected, ALP stains appeared denser in the half of the discs receiving BMP-2 in a bottom-up manner (Figure 3(a)). However, it is very likely that BMP-2 released from the bottom-up system indirectly carried over to the untreated section, which may have influenced adjacent cells to exhibit signs of differentiation (Figure 3(a), yellow arrow). In addition, measured ALP activity remained higher in the direct-contact group regardless of disc division (Figure 3(b)).

4. Discussion

We investigated the effect of the delivery mode of BMP-2 on osteogenic differentiation of human BM-MSCs. The top-down release system and bottom-up release system were used to investigate the effects of release mode of BMP-2. The main difference of both systems is how the BMP-2 was transmitted to the cells.

Compared to conventional method for BMP-2 treatment in two-dimensional cell culture, the bottom-up release system presents several advantages. First, BMP-2 appeared to bind more rapidly and readily to receptors on cultured stem cells than BMP-2 released from top-down release system. In top-down release system (conventional method), BMP-2 released in the culture medium showed Brownian motion [10]. Thus, only a small amount of BMP-2 reaches the receptors related to osteogenic differentiation pathway on the cells. In contrast, BMP-2 released from bottom-up system may trigger cell signals more efficiently. Although the release profiles of BMP-2 in both systems are equal, the osteogenic differentiations of stem cells cultured on bottom-up release system were superior to those of cultures with top-down release system (Figure 2). This can be explained by the rapid binding of BMP-2 released from the alginate hydrogel to receptors on human BM-MSCs. Second, alginate gel as a BMP-2 reservoir may play an important role at the delivery site to ensure their proper biological activity. In this study, half of cell culture plate is coated with alginate hydrogel containing BMP-2 (direct system) and half of cell culture plate is coated with only alginate hydrogel without BMP-2 (indirect system) (Figure 3). The BMP-2 released from alginate hydrogel in the direct system rapidly triggered osteogenic differentiation of stem cells. The direct system induced ALP activity to a much greater extent than that of indirect system. In contrast, the osteogenic differentiation of stem cells was interrupted at indirect culture sites compared to that of direct system. This indicates that delivery mode of BMP-2 may also influence the efficacy of osteogenic differentiation.

In this study, daily addition of BMP-2 was not used as a control group. Several studies have reported successful osteogenic differentiation using various BMP-2 release systems [11, 12]. In these studies, cells were cultured in culture plates with BMP-2 added to the culture medium daily as control group for evaluation of BMP-2 release system. The daily addition of BMP-2 is based on the assumption that the total concentration of BMP-2 remains constant. However, actual amount of BMP-2 in culture media is different as indicated by BMP-2 release profile. Therefore, conventional control may be inefficient to test effect on osteogenic differentiation by delivery mode of BMP-2. Instead, we deliberately used control groups with equal release profile (top-down release system) to avoid complicating systemic factors for a more equal comparison of the two types of BMP-2 delivery mode.

Depending on the type of cell, BMP-2 is involved in the hedgehog pathway, transforming growth factor-beta signaling pathway, and cytokine-cytokine receptor interaction [13, 14]. Previous studies have reported its involvement in extra-embryonic endoderm derivation from human embryonic stem cells and chondrogenic commitment of MSCs as well as cardiomyocyte contractility [15–17]. While the action of BMP-2 is relatively extensive, in mesenchymal stromal cells, it serves as an important factor for osteogenic commitment in a ligand-dependent manner to activate downstream gene regulation via SMAD [18] (Figure 4). Thus, we believe that the enhancement of the osteogenic differentiation was caused by the increased osteogenic activity of BM-MSCs that was presumably triggered by the enhancement of BMP-2 binding

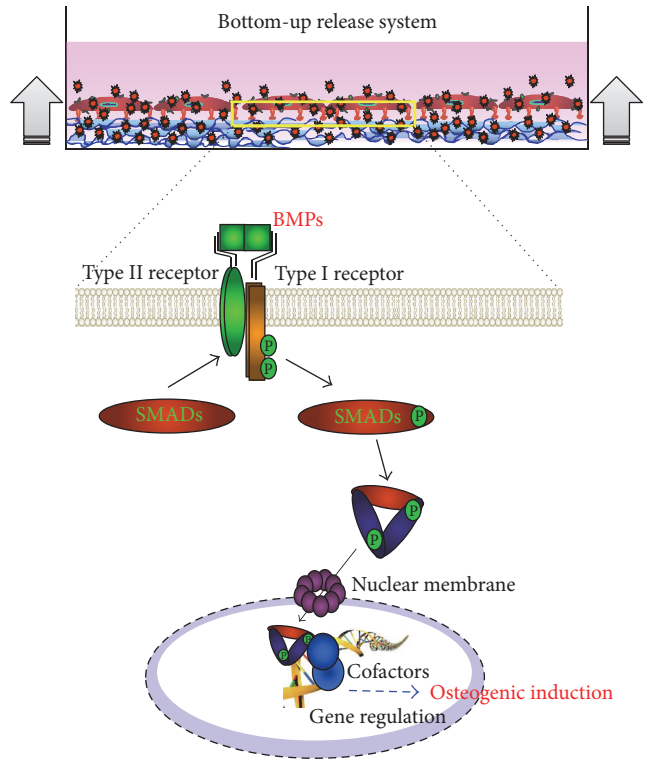

Figure 4: Schema of osteogenic differentiation through the BMP-2 signaling pathway. Type I and type II BMP receptors span the cell membrane and bind extracellular BMP ligand. Ligand binding to BMP receptor complexes activates signaling through type II-receptor-mediated phosphorylation of the type I receptor. In bottom-up release system, the probability of BMP-2 binding to receptors was maximized during BMP-2 treatment process.

in bottom-up release system. Future studies are needed to elucidate the molecular mechanisms of BMP-2 binding effects in the bottom-up release system.

In conclusion, osteogenic differentiation of BM-MSCs was significantly enhanced in the bottom-up release system compared to those of the top-down release system. These results show that the bottom-up release system could serve as a differentiation stimulator of stem cells. Thus, these findings could be useful for applications involving stem cell culture or differentiation studies that aim to advance cell utility in the field of stem cell therapy.

Disclosure

An earlier version of this work was presented as an abstract at Korea Polymer Society 2014 Fall Conference.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Taekhee Jung and John Hwan Lee contributed equally to this work as first authors.

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