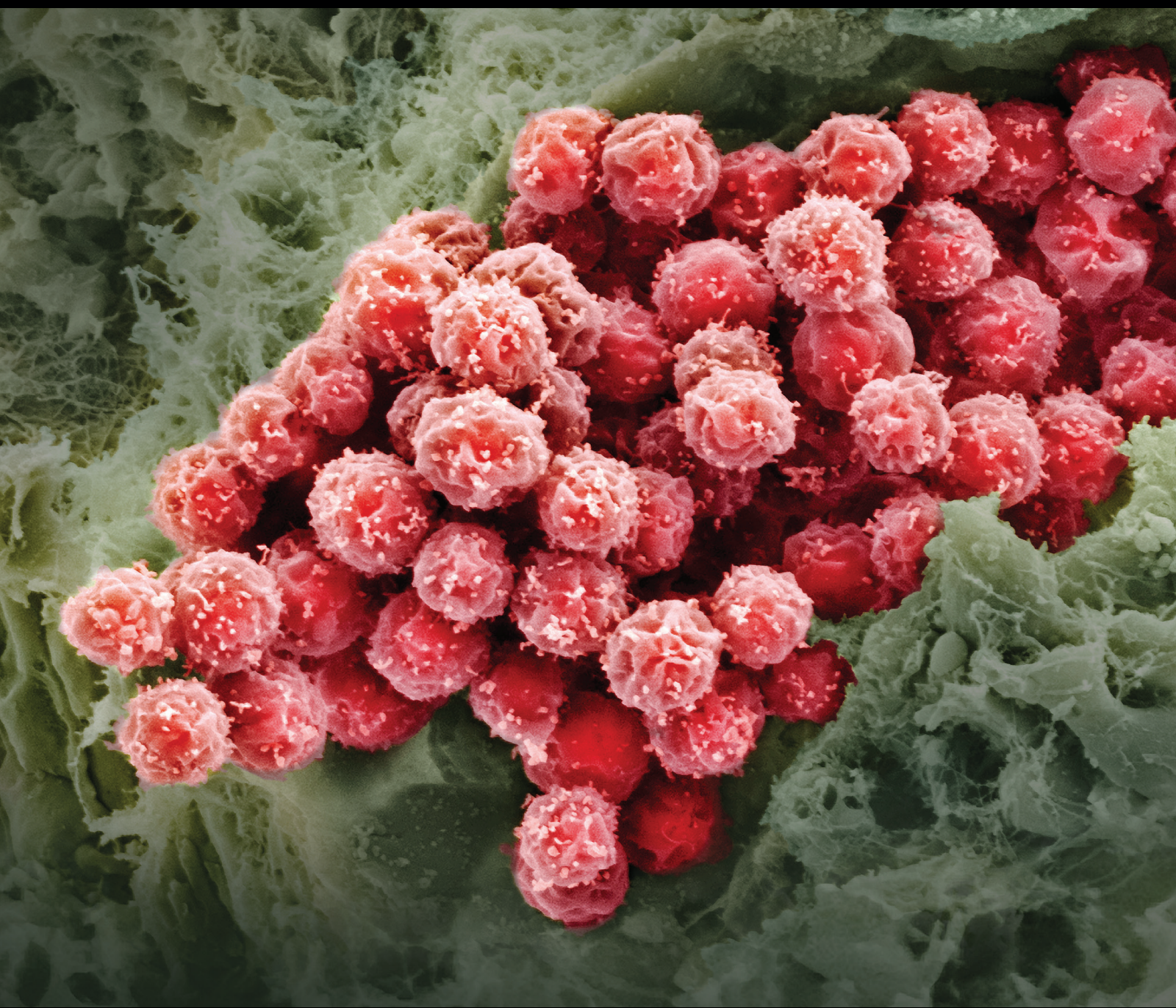
Stem Cells International

Targeted Strategies to Modulate Stem-Cell-Relevant Pathways

Guest Editors: Jonathan W. Lowery, James A. Ankrum, Shoichiro Kokabu, and Renjing Liu



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Editorial Board

Contents

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Editorial

Targeted Strategies to Modulate Stem-Cell-Relevant Pathways

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Modulation of stem cell behavior is of significant interest to the biomedical community and could lead to novel therapeutic advances in treating disease. Achieving this goal requires specific strategies that manipulate the pathways regulating stem cell plasticity and behavior. The accumulating evidence indicates that just a few main signaling pathways regulate most types of stem cells, which suggests that strategies that modulate one type of stem cell might hold broad usefulness. However, as stem cell research becomes more and more specialized, investigators studying a particular pathway or behavior in one specialty can miss a breakthrough advancement made in another specialty.

In this special issue we have collected reports and reviews of pathways that are critical to regulating the function and fate of mesenchymal stem cells (MSCs), induced pluripotent stem cells (IPSCs), and endothelial progenitor cells (EPCs). While each report is focused on the fate and function of a particular type of progenitor cell or a particular pathway, mechanisms at play in one cell type may be directly relevant to other cell types as well.

The multipotent nature of MSCs makes them an attractive cellular source for regenerative medicine. While many reports exist describing the potential of MSC to repair damaged tissues following trauma, our understanding of the role of MSC in repair of polytrauma, that is, in tissues suffering more than two injuries, is still in its infancy. In this special issue, M. Huber-Lang and colleagues provided a summary of studies that shed light on the potential of MSC as a therapeutic target for treatment of polytrauma. Moreover, the authors present examples that add to both sides of the debate on whether MSC are "actors" that drive tissue regeneration or are "targets" for attacks by the immune system following polytrauma.

S. Kokabu et al. also focus on MSCs, examining the reciprocal relationship between differentiation of this cell type into osteogenic versus adipogenic lineages. Particular attention is paid to the function of the transcriptional regulator Transducin-Like Enhancer of Split 3 (TLE3), which has recently been implicated in regulating the commitment between these two lineages. Additionally, S. Kokabu and colleagues propose future areas of research which may lead to the ability to control adipogenic versus osteogenic differentiation in the bone marrow microenvironment.

Related to this, J. W. Lowery et al. survey the strategies that are available to modulate the Bone Morphogenetic Protein (BMP) signaling pathway, which potently induces both osteogenic and adipogenic differentiation of MSCs. The authors detail the currently available natural and engineered ligands, extracellular antagonists, ligand traps, and kinase inhibitors. Numerous examples of each strategy in specific

settings and applications are presented. J. W. Lowery and colleagues also propose future areas for study in order to advance the ability to control behavior of MSCs, other stem cell populations, and somatic cells alike.

J. Zhao et al. examine the ability of late-outgrowth EPCs (LO-EPCs) to home to sites of injury after intravenous infusion via a series of *in vitro* experiments. LO-EPCs are capable of differentiating into endothelial cells, but are a rare cell type in circulation, making their *ex vivo* expansion necessary prior to therapy. In contrast to leukocytes and MSC which exhibit enhanced adhesion to inflamed endothelium, J. Zhao and colleagues reported no enhancement in LO-EPC adhesion in inflamed *in vitro* conditions. However, attachment was enhanced when the subcellular extracellular matrix was exposed. Disruption of endothelial barrier integrity by subconfluent seeding or incubation with anti-VE cadherin blocking antibodies resulted in increased LO-EPC adhesion, which the authors go on to show that it appears to be dominated by adhesion to fibronectin and vitronectin in the ECM. Thus, in contrast to MSC and leukocytes, disruption of endothelial integrity appears to be critical to facilitate LO-EPC homing.

Finally, P. Nagaria et al. examine how the method of conferring pluripotency affects the DNA damage response in cord blood myeloid progenitors and fibroblasts. The authors find that, in contrast to standard methods, a high-fidelity stromal-activated method results in IPSCs that closely resemble embryonic stem cells in their ability to repair double-stand DNA damage via non-homologous end joining and in their expression of c-MYC-mediated transcriptional signature. These findings are highly relevant to investigators working in the IPSC field and are potentially applicable to the safe clinical translation of IPSC-based therapies in patients.

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Research Article

High-Fidelity Reprogrammed Human IPSCs Have a High Efficacy of DNA Repair and Resemble hESCs in Their MYC Transcriptional Signature

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Human induced pluripotent stem cells (hiPSCs) are reprogrammed from adult or progenitor somatic cells and must make substantial adaptations to ensure genomic stability in order to become "embryonic stem cell- (ESC-) like." The DNA damage response (DDR) is critical for maintenance of such genomic integrity. Herein, we determined whether cell of origin and reprogramming method influence the DDR of hiPSCs. We demonstrate that hiPSCs derived from cord blood (CB) myeloid progenitors (i.e., CB-iPSC) via an efficient high-fidelity stromal-activated (sa) method closely resembled hESCs in DNA repair gene expression signature and irradiation-induced DDR, relative to hiPSCs generated from CB or fibroblasts via standard methods. Furthermore, sa-CB-iPSCs also more closely resembled hESCs in accuracy of nonhomologous end joining (NHEJ), DNA double-strand break (DSB) repair, and C-MYC transcriptional signatures, relative to standard hiPSCs. Our data suggests that hiPSCs derived via more efficient reprogramming methods possess more hESC-like activated MYC signatures and DDR signaling. Thus, an authentic MYC molecular signature may serve as an important biomarker in characterizing the genomic integrity in hiPSCs.

1. Introduction

Although human induced pluripotent stem cells (hiPSCs) resemble hESCs in many respects [1, 2], the therapeutic utility of hiPSCs is limited by low reprogramming efficiency [3–6] and poor genomic integrity [7–10]. A deeper understanding of the mechanisms that control these roadblocks will be vital for the use of hiPSCs in regenerative medicine. Reprogramming efficiency is controlled by intrinsic and extrinsic microenvironmental factors that are determined by the method employed [5]. Standard protocols often utilize inefficient and potentially mutagenic retroviral mediated transgene factor expression (e.g., OSKM: *OCT4*, *SOX2, KLF4,* and *C-MYC*, or OSNL, i.e., *OCT4, SOX2, NANOG,* and *LIN28*) [11]. More clinically useful nonviral, nonintegrating methods have also been widely employed (e.g., plasmids, microRNA), albeit with a significantly reduced reprogramming efficiency [11]. The choice of somatic donor and *in vitro* microenvironmental conditions also significantly influences reprogramming efficiency. For example, we previously demonstrated that bone marrow stromal cell (MSC) activation robustly activated MYC complex-regulated genes of pluripotency that subsequently facilitated high-quality reprogramming of human myeloid progenitors (MP) differentiated from $CD34^+$ hematopoietic stem-progenitor cells [12]. Activation of MYC-regulated factors potentially enhanced the rate and efficiency of reprogramming [13]. MYC may also play a key role in regulating promoters and microRNAs associated with core pluripotency-associated genes [14, 15]. These findings implicate targets of the MYC network not only in playing a

key role in controlling the efficiency of reprogramming, but also in maintaining stem cell pluripotency.

Efficacious DNA double-strand break (DSB) repair is a key element in maintenance of high genomic integrity [16, 17]. In mammalian cells, homologous recombination repair (HR) provides precise, error-free DSB repair by using a homologous sister chromatid as a template for repair [18]. In contrast, repair by nonhomologous end joining (NHEJ) joins DNA ends directly and is thus prone to errors [19]. In hESCs, repair of DSBs occurs mainly by HR [17]. We and others have reported a form of DSB end-joining repair in hESCs that is relatively error-free [17, 20, 21]. However, overall DNA repair properties in reprogrammed cells are more heterogeneous than hESCs [22, 23]. For example, we previously demonstrated that hiPSCs derived from mesenchymal stem cells (MSCs) or fibroblasts were more deficient than hESCs in DSB end-joining capacity despite similarities in the precision of repair between them [20]. These studies suggest that efficient DSB repair properties confer an advantage in achieving completion of faithful reprogramming to an authentic hESC-like state [24]. However, the mechanisms that control efficient DSB repair during reprogramming are unclear.

MYC, which can associate with the E-box elements in the promoters of several DSB repair genes and can amplify the cell's transcriptional program by binding to promoter and enhancer elements, represents a strong candidate for regulation of DSB repair in pluripotent cells [25, 26]. Determining these mechanisms not only is critical in finding the most efficient way to derive iPSCs, but also can be applied to measures ensuring the safe clinical use of iPSCs with high genomic integrity. To address these questions, we evaluated previously reported human CB-derived sa-CB-iPSCs generated with high efficiencies (1–4% input cells) and compared them to CB- and fibroblast-derived hiPSCs derived via standard methods (<0.001–0.5% input cells) [27]. Our data reveal that in response to radiation-induced DNA damage, sa-CB-iPSCs possessed a DDR signature that more closely resembles that of hESCs. These sa-CB-iPSCs also possess lower baseline levels of endogenous DNA DSBs and a greater accuracy of DSB end-joining, compared to standard CB-iPSCs and fibroblast-iPSCs. Moreover, we show that C-MYC may play an important role in facilitating a stringent and high-fidelity DSB response in hESCs and hiPSCs. Collectively, our data suggest that more efficient activation of MYC-associated DDR signaling during reprogramming or DSB damage may enhance the genomic integrity of hiPSCs and increase their ultimate clinical utility.

2. Materials and Methods

Ethics Statement (Human Embryonic Stem Cell Lines). All hESC lines used in this study were obtained commercially from the WiCell Research Institute (Wisconsin International Stem Cell Bank, WISCB). The use of all WISCB-donated hESC lines in these studies was approved by the Johns Hopkins Embryonic Stem Cell Research Oversight (JHU-ESCRO) Committee and the University of Maryland School of Medicine Embryonic Stem Cell Research Oversight Committee (UMSOM-ESCRO) and conforms strictly to standards of both institutions, including written informed consent. All experiments conducted in these studies also conformed to guidelines outlined for hESC and pluripotent stem cell research by the National Academy of Sciences and the National Institutes of Health (NIH).

2.1. Cell Culture. Pluripotent stem cells were routinely cultured on irradiated primary murine embryonic fibroblasts (MEF), derived from embryos of CF1 and DR4 F1 mice at embryonic days of 12.5 or 13.5 (P2/P3), or purchased from GlobalStem (Rockville, MD). Human pluripotent stem cell cultures were maintained in DMEM/F12 (Invitrogen) medium supplemented with 20% Knockout Serum Replacement (KOSR; Gibco), 0.1 mM MEM nonessential amino acids (Gibco), 1 mM L-glutamine (Gibco), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 4 ng/mL FGF2 (R&D Systems, Minneapolis, MN) at 37°C, 5% CO_2, and 85% relative humidity. The medium was changed daily on hESCs and hiPSC cultures. For experiments, human pluripotent stem cells were first transitioned from MEF feeder layers onto a BD-Matrigel™ (BD Biosciences) matrix precoated plate and cultured in mTESR1™ medium (Stem Cell Technologies, Vancouver, Canada). The mTESR1 growth media were replenished daily. Purified (>95%) human $CD34^+$ CB progenitors (also referred to as "starting CB progenitors") from pooled donors were purchased from AllCells (Emeryville, CA) and cultured in the hematopoietic growth medium (HPGM).

2.2. Generation of Episomal hiPSCs. Detailed methods for generation and characterization of hiPSC lines were previously described [12, 28]. Details of hiPSC lines are provided in Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3826249. In brief, sa-CB-iPSC lines (CB6.2, 6.13, 19.11, and E12C1) were derived via nucleofection of stromal-activated $CD34^+$ MP with 7 or 4 episomal factors (7F, SOKMNLT; SOX2, OCT4 (POU5F1), KLF4, c-MYC, NANOG, LIN28, and SV40L T antigen; 4F, SOKM) using the AMAXA II Nucleofector device (Lonza). Standard episomal CB-iPSC lines were derived without stromal activation with either four (4F; SOKM) or seven episomal factors (7F) from either CB-derived $CD34^+$ MP (4F: E17C1, E20C2, and E24C1) or CB-derived unsorted mononuclear cells (7F: iCB9, iCB8, and iCB2.5) [29], kindly provided by Dr. Igor Slukvin (University of Wisconsin-Madison). Skin fibroblast-derived hiPSC line iHUF3, derived with four retroviral factors (SOKM), was previously described (Byrne et al.) and kindly provided by Dr. Renee Reijo-Pera (Stanford University) [27]. Requests for hiPSC lines should be addressed to Elias T. Zambidis (ezambid1@jhmi.edu).

2.3. Gene Expression Microarrays. Details of the microarray analysis were described before [12]. Human HT-12 Expression BeadChip arrays (Illumina, San Diego, CA) were used for microarray hybridization to examine the global gene expression of hESC, hiPSC, and starting populations ($CD34^+$ progenitors and fibroblasts). The NIH Gene Expression Omnibus has issued the accession numbers GSE44425 (Figure 1,

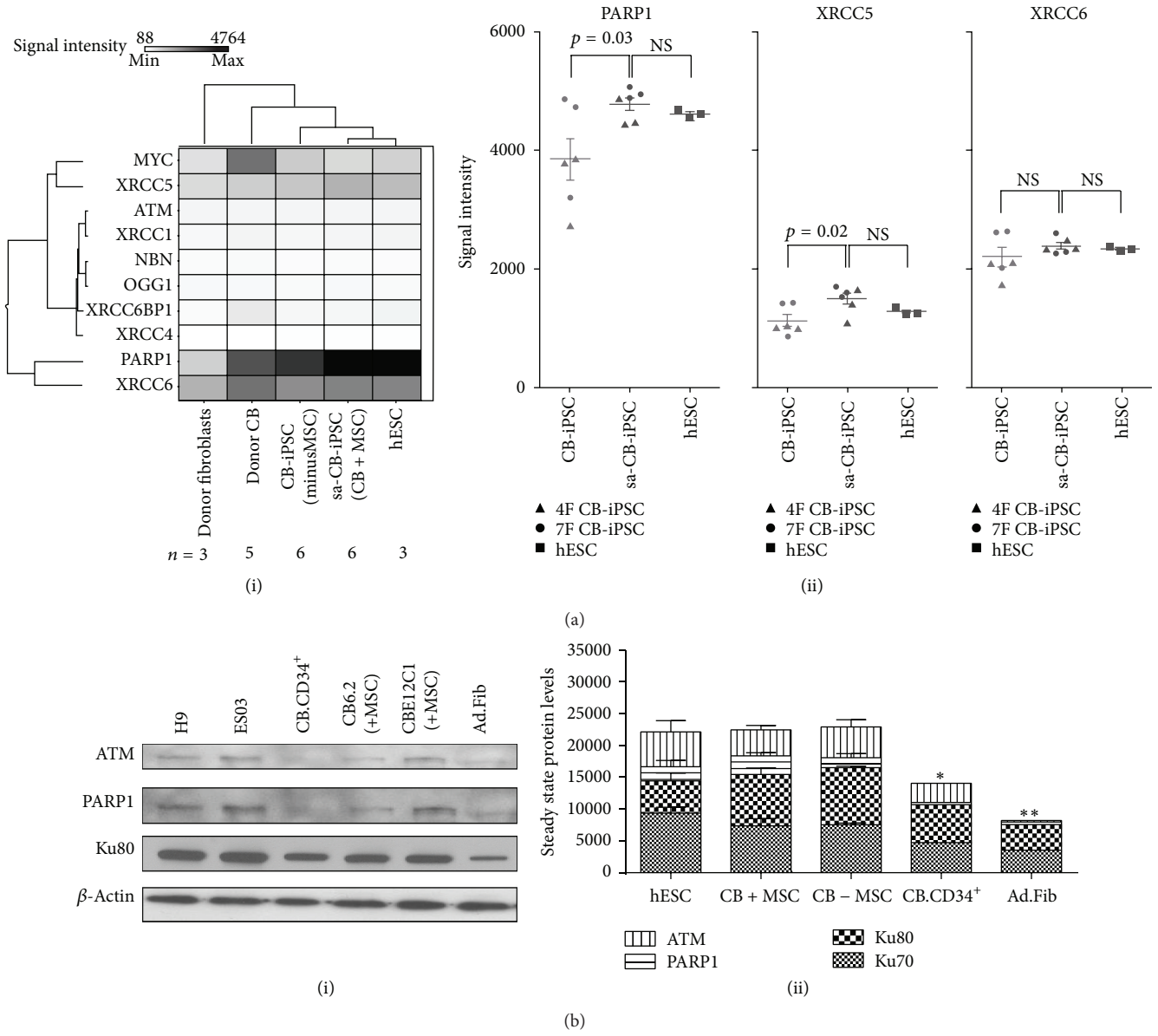

Figure 1: CB progenitors and CB-derived iPSCs closely resemble hESCs DNA repair gene expression signature. Microarray gene expression of selected DNA repair genes. (a)(i) Shown are hierarchical clustering heatmaps of mRNA from donor fibroblasts, donor $CD34^+$ CB, and CB.iPSC derived with (+MSC) and without (−MSC) bone marrow stromal cell activation. hiPSC lines included sa-CBiPSC derived from stromal-activated $CD34^+$ MP ($n = 6$; E5C3, E12C5, and E17C6: 6.2, 6.13, and 19.11), standard CB-iPSC, lines derived from $CD34^+$ MP without stromal activation ($n = 3$, E17C1, E20C2, and E24C1), and standard CB-iPSC lines derived from CB unsorted mononuclear cells ($n = 3$, iCB9, iCB8, and iCB2.5). hESC lines included ($n = 3$) H9, H7, and ES03. Signal intensities are from averaged independent biological replicate microarray samples (n as indicated). Expression array data depicts normalized values of the mean transcript levels for a subset of DDR genes in each group of the indicated cell lines. (a)(ii) Dot plots represent the normalized values of the signal intensities for PARP1/XRCC5/XRCC6 with corresponding p values between categories indicated in the array data in (a)(i) (n as indicated in (a)(i)). Paired tests with significance $p < 0.05$ ($*$) or without significance (NS; $p > 0.05$) with values of control hESC are indicated (▲ = 4F CB.iPSC; ● = 7F CB.iPSC). (b)(i) Representative Western blot from the whole cell lysates of hESCs (H9 and ES03), CB ($CD34^+$), two independent sa-CB-iPSCs (6.2 and E12C1), and adult fibroblasts (Ad.Fib) showing the steady state levels of PARP1 and Ku80 and ATM. β-Actin was used as the loading control. (b)(ii) Graphical representation of Western blots by ImageJ quantified-densitometry analysis normalized to β-actin ($n = 3$) in hESC (H9, ES03, and H7), sa-CB-iPSC (CB6.2, CB6.13, and CB19.11), standard CB mononuclear CB-iPSC (iCB9, iCB8, and iCB2.5), CB ($CD34^+$), and adult fibroblasts (Ad.Fib). Results are representative of the mean of two independent experiments of each set ± SEM, $^*p < 0.05$ and $^{**}p < 0.01$, based on 2-way ANOVA (multiple comparisons test) on combined expression of genes.

Figure 5(c)) for the deposited microarray data related to the above manuscript.

2.4. DNA Damage, Apoptosis, and MYC Inhibition Studies. For irradiation (IR) studies, pluripotent stem cells were exposed to X-ray radiation using a Pantak HF320 X-Ray machine (250 kV peak, 13 mA; half-value layer, 1.65 mm copper) at a dose rate of 2.4 Gy/min. For experiments involving MYC inhibitor (10058-F4, Sigma-Aldrich, St. Louis, MO), the cells were treated with either the control solvent (DMSO) or the drug at dose of 50 μM for 24 h before X-ray IR. Following 24 h treatment, the medium was replaced before exposure to IR. For knockdown studies, siMYC (ON-TARGETplus™, Dharmacon, Thermo Fisher Scientific) was utilized. The cells were transfected with siMYC (2 μg) using Lipofectamine™ 2000 (Life Technologies), 48 h before exposure to IR.

2.5. Whole Cell Extracts and Nuclear Extracts. Whole cell extracts were prepared with lysis buffer (25 mM Tris-HCl (pH 7.5), 333 mM KCl, 1.3 mM EDTA, and 4 mM DTT) with protease inhibitor cocktail (Roche, Branchburg, NJ) and phosphatase inhibitors cocktail (Sigma-Aldrich). Nuclear extracts were prepared using the CelLytic Nuclear Extraction Kit (NEXTRACT™, Sigma-Aldrich) without the use of any detergents. The nuclear extracts used for the DNA repair assay were dialyzed against the E-buffer (20 mM Tris-HCl (pH 8.0), 20% glycerol, 0.1 M K(OAc), 0.5 mM EDTA, and 1 mM DTT).

2.6. Immunoblotting Analysis. 20 μg of proteins was separated by electrophoresis through either 4–10% or 4–15% polyacrylamide gradient gels (Mini-PROTEAN TGX) (Bio-Rad Laboratories, Hercules, CA) and then transferred to PVDF membranes (Thermo Fisher Scientific, Waltham, MA). After blocking, membranes were probed with primary antibodies mouse Ku70 (1 : 500, E-5, SC17789, Santa Cruz Biotech (SCB), Dallas, TX), Ku80 (Calbiochem, EMD Millipore NA54), PARP1 (1 : 2000, CS # 9532, Cell Signaling, Beverly, MA), p53 (1 : 1000, CS # 9282), pATM (1 : 1000, Millipore, Billerica, MA), γH2AX (Millipore, Clone JBW301, 05-636), β-actin (1 : 5000, Sigma-Aldrich), and β-tubulin (CS # 2128) as loading controls. After probing with adequate secondary antibodies (anti-mouse IgG-CS and anti-rabbit, BioLegend, San Diego, CA), proteins expression was detected using enhanced chemiluminescence (ECL; 100 mM Tris-HCl (pH 8.5), luminol, coumaric acid, and hydrogen peroxide).

2.7. In Vitro NHEJ Assays (Plasmid Reactivation: PUC18 and EJ5-ISce1). We used the DNA repair fidelity assay (PUC18-based) as described before [30]. For the assay, 2 μg of EcoRI linearized PUC18 was incubated with 4 μg of nuclear extract. Reactions (in 20 μL volume) were carried out in ligation buffer (50 mM triethanolamine-HCl (pH 7.5), 60 mM KOAc, 50 μM deoxynucleotide triphosphates, 2 mM ATP, 1 mM DTT, and 100 μg/mL BSA). The mixture was incubated for 16 h at 18°C. Following the incubation, 10 ng of purified plasmid DNA was used to transfect *Escherichia coli* strain DH5α. Transformed cells were plated on LB agar plates, including 100 μg/mL carbenicillin, 20 mg/mL X-gal, and 200 mg/mL isopropyl-1-thio-β-d-galactopyranoside. To allow for spontaneous rejoining/incomplete EcoRI cutting, assay controls were conducted without nuclear extract. In addition, to correct for bacterial plating numbers and determine whether nuclease activity was affecting plasmid efficacy, cells were plated on Luria-Bertani agar without carbenicillin.

For the EJ5-*Isce1* assay, we used a protocol adapted from the one designed by Bennardo and colleagues but modified for *in vitro* plasmid reactivation analysis [31]. Briefly, the pimEJ5GFP reporter plasmid (Addgene Plasmid 44026) [31] was enzymatically linearized with *I-Sce1* (New England Biolabs (NEB), Ipswich, MA) at 37°C overnight. Linearized plasmid was dephosphorylated using Shrimp Alkaline phosphatase (SAP) (NEB), and column 500 ng DNA was incubated with dialyzed nuclear extracts (2 μg) of respective cell lines, and ligation reactions were performed in ligation buffer (10x T4 ligase buffer, 2 mM ATP, and 50 μM deoxynucleotide triphosphates). Following *in vitro* ligation, the plasmid DNA was column-purified and GFP genes were PCR-amplified using the primers p1 (Fwd) 5′-CTGCTAACCATGTTCATGCC-3′ and p2 (Rev) 5′-AAGTCGTGCTGCTTCATGTG-3′, as described by Bennardo et al. [31]. Following the PCR, we redigested plasmid with *I-Sce1* to differentiate between NHEJ repair that was completed with *I-Sce1* restoration (S+) and repair completed with loss of *I-Sce1* site (i.e., "S−" with deletions). Undigested and digested PCR products were fractionated on 2% agarose and visualized with the GelStar™ Nucleic Acid Stain (Lonza). S-fragment was excised from the gel and cloned into PCR2.1® TOPO (Life Technologies). Cloned products were transformed into OneShot® TOP10 chemically competent cells (Life Technologies) and plated on LB plates with kanamycin resistance. DNA from 5 colonies from each experiment was sequenced using the M13 primers. A total of 15 colonies were analyzed from three independent experiments, and TOPO plasmids were sequenced at the UMB TGL/Biopolymer core facility.

3. Results

3.1. CB Progenitors and CB-Derived iPSCs Closely Resemble hESCs in DNA Repair Gene Expression Signature. Previous studies indicated that progenitor donor cells were more amenable than differentiated cells in reprogramming to a pluripotent state [32, 33]. We performed microarray-based analysis to determine the DDR gene expression profile of hiPSCs (Table S1) derived via different methods (Figure 1(a)(i)). We found that donor $CD34^+$ CB progenitors cluster more closely with hESCs than adult fibroblasts (Ad.Fib) donors in baseline expression of DNA repair genes, including poly (ADP-ribose) polymerase 1-PARP1 (involved in single-strand break repair and DSB repair), XRCC5 (*a.k.a.* Ku80), and XRCC6 (*a.k.a.* Ku70) (involved in NHEJ DSB repair). Of note, expression of MYC and XRCC6 in CB progenitors was even higher than that for hESCs (Figure 1(a)(i)) [20]. Additionally, PARP1 and XRCC5 were expressed at higher baseline levels in sa-CB-iPSCs than in standard CB-iPSC lines (Figure 1(a)(ii)).

To determine whether the levels of expression of these repair gene transcripts translated into functional differences in protein levels, we performed immunoblot analyses on hiPSCs from these representative categories. Although steady state protein levels of ATM, Ku80, and PARP1 in sa-CB-iPSCs were similar to standard CB-iPSCs and hESCs, donor $CD34^+$ CB progenitor baseline expression of these DNA repair proteins more closely resembled hESCs ($^*p < 0.05$), compared to Ad.Fib ($^{**}p < 0.01$ difference) (Figures 1(b)(i) and 1(b)(ii)). These results suggested that $CD34^+$ CB progenitors may already possess hESC-like expression of DDR pathway components, even prior to initiation of reprogramming.

3.2. Sa-CB-iPSCs Resemble hESCs in Their DDR Response to Radiation. Irradiation (IR) elicits several posttranslational modifications of the components of DDR pathway. Irradiated hESCs and hiPSCs rapidly activate the ataxia telangiectasia and Rad3-related (ATR) and ataxia telangiectasia mutated (ATM) kinase-dependent DDR signaling [34], phosphorylating targets, such as p53 and H2AX [17, 35]. While ATR responds mainly to single strand breaks (SSBs) and stalled replication forks, ATM is activated in response to DSBs. Moreover, ATM deficiency confers hypersensitivity to IR [36].

To determine the efficacy of DDR, representative CB-derived hiPSCs (i.e., sa-CB-iPSC (CB6.2), standard CB-derived hiPSC (iCB9), and fibroblast -derived (iHUF3)) [34] were treated with IR (2 Gy) and compared with IR-treated hESCs (i.e., H9 and ESO3). To examine the DSB response in IR-treated hiPSCs, we performed immunoblotting for phosphorylation of H2AX on Ser139 (γH2AX), which functions to assemble DSB repair factors [35]. In hESCs (H9 and ES03) and sa-CB-iPSC (CB6.2), γH2AX expression was evident at 4 h after IR (Figures 2(a) and 2(c)), indicating activation of a DSB response. All tested hiPSCs exhibited kinetics of H2AX phosphorylation similar to hESCs (Figures 2(a)–2(c)). Interestingly, hESCs and hiPSCs did not differ significantly in the expression levels of total ATM protein (Figures 2(a) and 2(b)). Notably, hESCs and CB-derived hiPSCs, including sa-CB-iPSC (CB6.2) and standard CB-iPSC (iCB9), demonstrated activation of ATM via phosphorylation at Ser1981 up to 4 h following IR (Figures 2(a)(i), 2(a)(ii), 2(b), and 2(d)). Interestingly, in comparison to hESCs and CB-iPSCs, fibroblast-derived iHuF3 cells exhibited less robust phosphorylation of ATM following exposure to 2 Gy IR ($^*p < 0.05$) (Figures 2(a), 2(b), and 2(d)).

We next examined the activity of another ATM target, the tumor suppressor p53, whose expression is stabilized upon DNA damage, thus activating the DNA binding function of p53. Posttranslational modification of p53 via phosphorylation at Ser15 has been proposed to be an important mechanism by which p53 is stabilized and its functions are regulated [37]. However, phosphorylation is not an absolute necessity for DNA damage-induced stabilization of p53 [37]. Our results show that P53 activation, measured by monitoring total p53 protein and phosphorylation at Ser15, occurred with similar kinetics in all the hiPSCs and hESCs, with levels increasing between 0 and 4 h after IR (Figures 3(a)–3(c)). Moreover, standard hiPSC lines (e.g., iCB9 and iHuF3) consistently displayed higher baseline levels of total p53 protein in untreated controls, in comparison to hESC (H9), ESO3, and sa-CB-iPSC (CB6.2) (Figures 3(a)–3(d)). In our observation, activation of p53 in cells following IR is mostly contributed by the stabilization of total p53 protein, as the relative changes in levels of phosphorylated protein were insignificant when its expression is normalized to total p53 (except for CB6.2 (2 h), $p < 0.05$) (Figure S2).

We next investigated apoptotic responses of hiPSC cell lines to IR-induced damage. All pluripotent stem cells have been reported to exhibit hypersensitivity to radiation, with substantial cell death observed within 24 h after exposure to a low dose of ionizing radiation (1-2 Gy IR) [17, 20, 34]. We therefore reasoned that cells with higher levels of cytotoxic DSBs may induce apoptosis to avoid genotoxic stress. Using PARP1 cleavage as an apoptotic marker, IR-exposed cells were examined by immunoblotting. Notably, there were only subtle differences observed in the kinetics of PARP1 cleavage among hESCs and all hiPSCs. PARP1 was observed predominantly in the cleaved form 4 h after IR in all examined cell lines (Figures 3(a), 3(b), and 3(d)). These results indicated that despite the subtle differences in levels of DNA damage, reprogramming renders all hiPSCs equally hypersensitive to ionizing radiation-induced apoptosis.

3.3. Sa-CB-iPSCs More Closely Resemble hESCs in Nonhomologous End Joining (NHEJ) Repair. Differences in baseline levels of DNA damage markers between hiPSCs noted above may also be accounted for by differences in DSB repair [17]. For example, increased DSB formation could result from decreased efficiency of repair, which can lead to increased error-prone repair or misrepair. Thus, we next determined whether the CB.iPSCs derived with the same factors but using distinct episomal reprogramming methods demonstrated different DSB repair efficiencies. We employed an established end-joining plasmid-reactivation repair assay and observed that hESC H9 and sa-CB-iPSC CB6.2 displayed the lowest percentage of misrepair (approximately 3%). In contrast, standard hiPSCs iHuF3 and iCB9 possessed a significantly higher percentage of misrepair (approximately 8–12%; $^*p < 0.05$), when either compatible DSB ends or noncompatible DSB ends (which require additional processing steps in end joining) were used (Figures 4(a)(i) and 4(a)(ii)). To further confirm these results, we utilized an additional modified end-joining assay designed by Gunn and Stark [38] that measures DSB repair junctions representing repair of complementary or noncomplementary ends (Figure S1). We incubated *I-Sce1*-linearized pimEJ5GFP plasmid with nuclear extracts of pluripotent cell lines for measurement of *in vitro* plasmid reactivation (Figure 4(b)(i)), and the *I-Sce1* resistant fraction ("S–" products) was further analyzed for quantification and characterization of DNA deletions (Figure 4(b)(ii)). Sequencing of approximately 10–15 "S–" DNA clones recovered from end-joining experiments using H9 and CB6.2 extracts indicated that deletions in the DSB junctions were mainly in *I-Sce1* overhangs and were restricted to 1–5 nucleotides (nt) (33% and 54%, resp.). In contrast, only 1 out of 11 (9%)

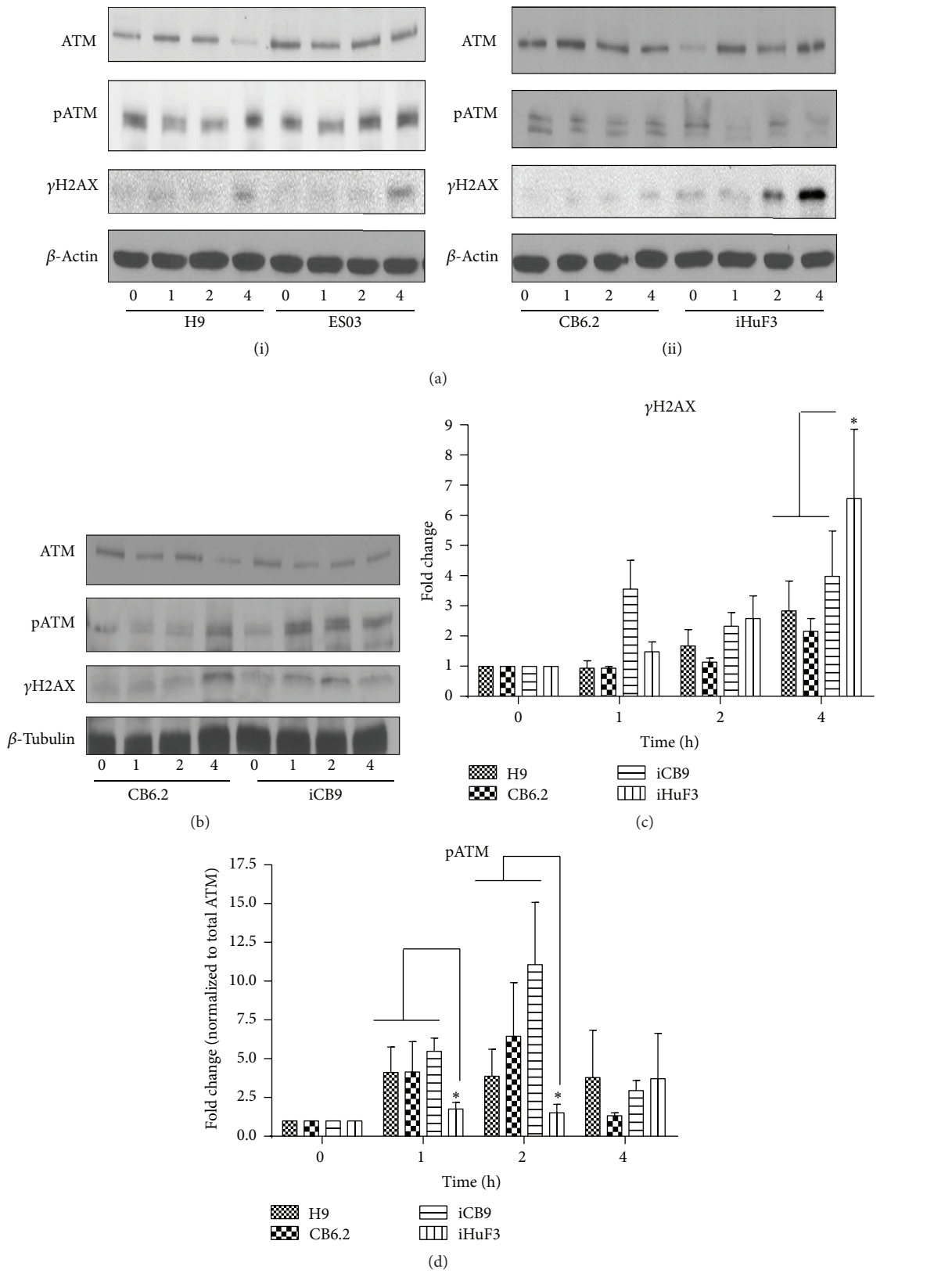

Figure 2: sa-CB-iPSCs closely resemble hESCs in DSB damage response to radiation. ((a)(i), (a)(ii), and (b)) Representative Western blot analysis depicting the expression of phosphorylated ATM (pATM) and H2AX (γH2AX) in cell lysates from H9, ES03, CB6.2, iCB9, and iHuF3 at time 0 and at 1 h, 2 h, and 4 h after IR. β-Actin and β-tubulin were used as loading controls. Cells were exposed to IR (X-ray; 2 Gy) recovered at the indicated time points and immunoblotting was performed to analyze the kinetics of DDR protein expression. ((c) and (d)) Densitometry analysis of the Western blots for (c) γH2AX and (d) pATM (normalized to total ATM), using ImageJ software. Statistical significance of the data was determined using 2-way ANOVA analysis with Bonferroni posttests to compare the replicates (three independent experiments). γH2AX expression in iHuF3 is significantly different at 4 h compared to the following (versus H9 and CB6.2, $^{*}p < 0.05$). pATM expression in iHuF3 is significantly different at 1 h and 2 h, compared to all other cell lines ($^{*}p < 0.05$).

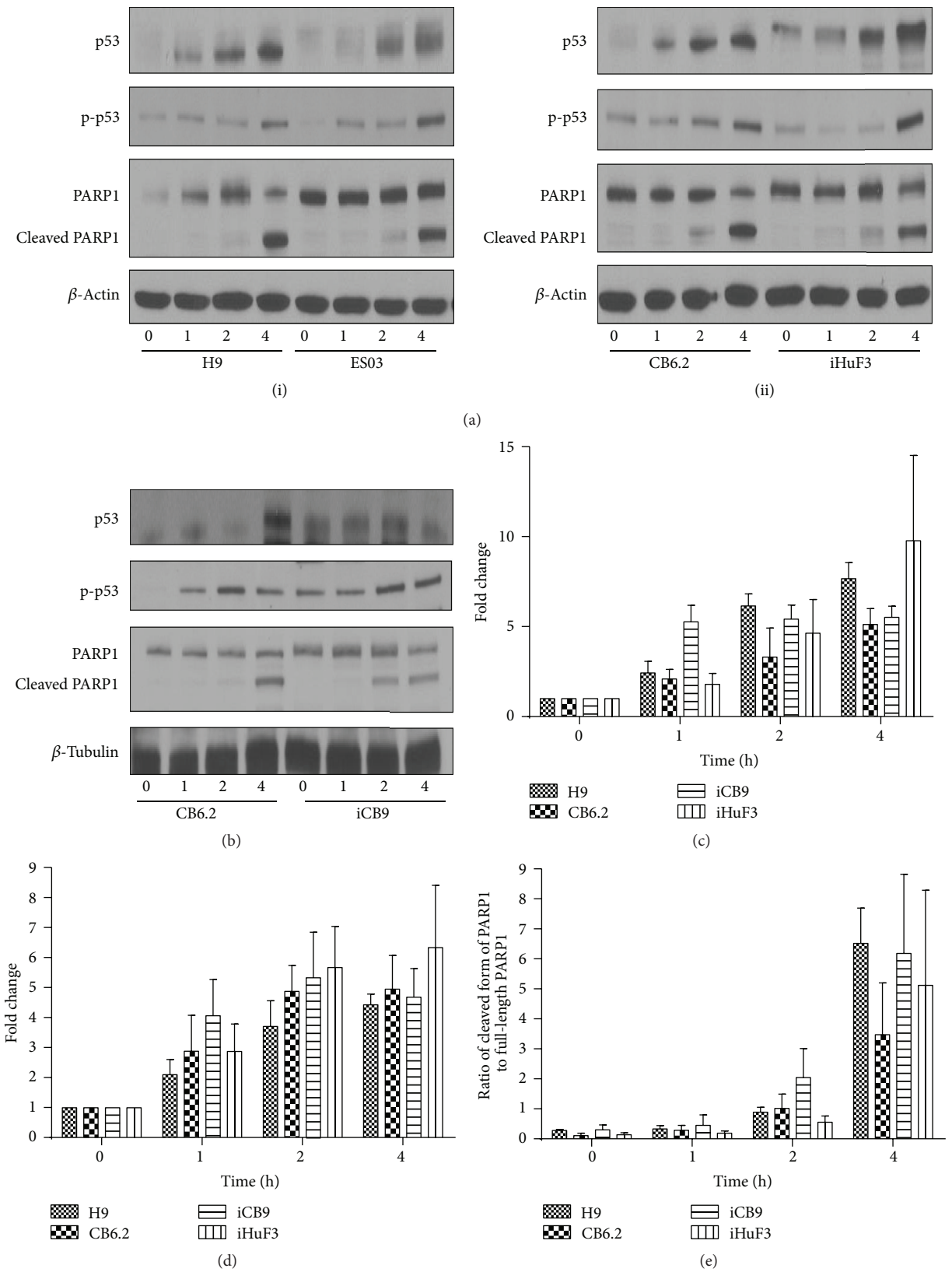

Figure 3: hESCs and hiPSCs have similar kinetics of apoptotic response to radiation exposure. ((a)(i), (a)(ii), and (b)) Representative Western blot analysis depicting the expression of p53 and PARP1 (full-length: 116 kDa; cleaved form: 89 kDa) in cell lysates from H9, ES03, CB6.2, iCB9, and iHuF3 at time 0 h and at 1 h, 2 h, and 4 h after IR. (c–e) Densitometry analysis of the western blots for measurement of (c) total p53, (d) phosphorylated $p53^{Ser15}$ (p-p53), and (e) PARP1 cleavage, using ImageJ software. Statistical significance of the data was determined using 2-way ANOVA with Bonferroni posttests to compare the replicates (three independent experiments).

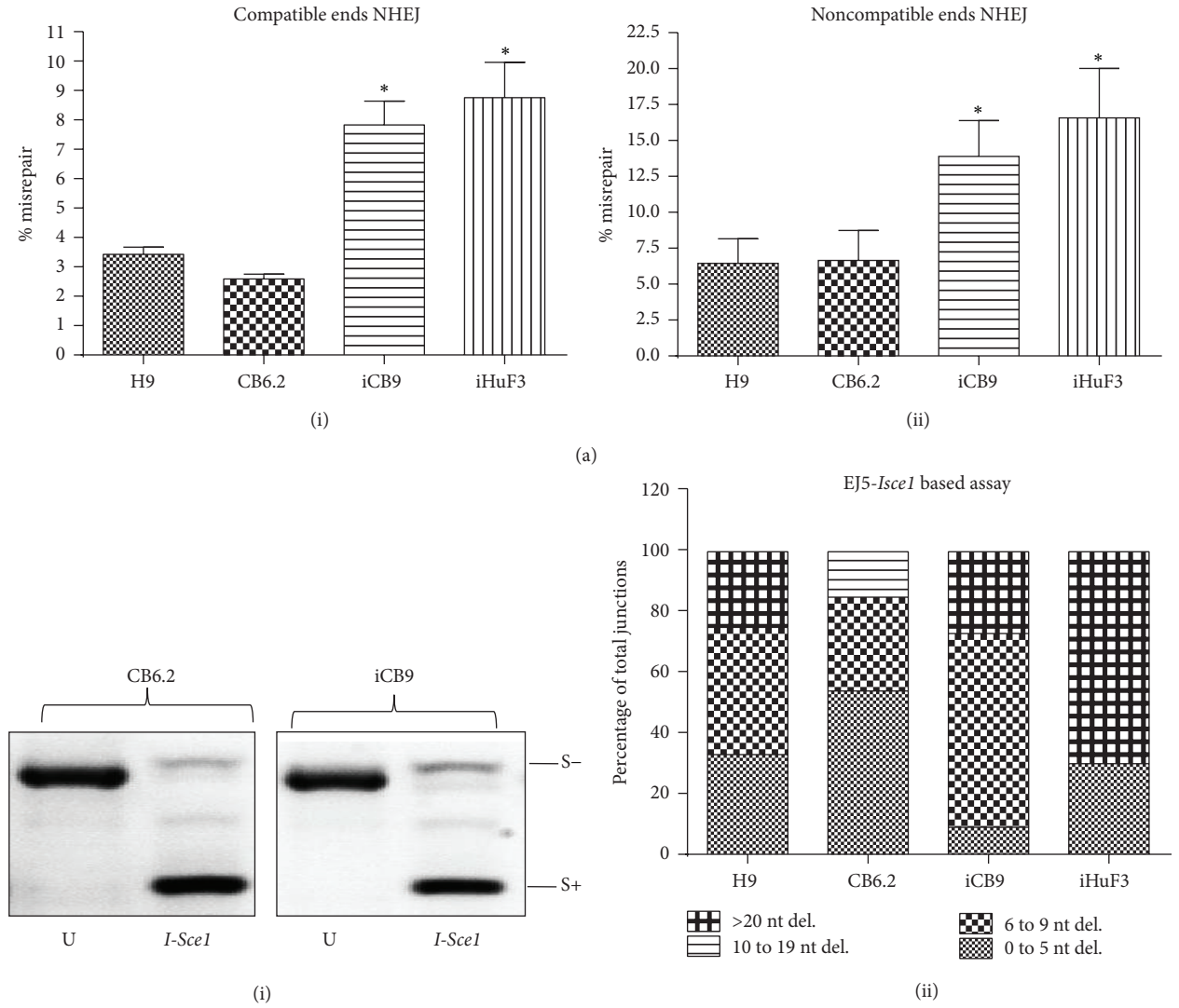

Figure 4: sa-CB-iPSC closely resembled hESC showing greater accuracy of nonhomologous end joining (NHEJ) repair. ((a)(i) and (a)(ii)) Analysis of repair products indicating percentage of misrepair in the *in vitro* PUC18-based end-joining assay. The misrepair % is calculated by dividing the total # of white colonies by total # of colonies, that is, blue + white, recovered from transformation of the repair products. (a)(i) demonstrates the % misrepair when the dialyzed nuclear lysates from respective cell lines are incubated with PUC18 linearized using *EcoR1*, giving compatible DNA ends; and (a)(ii) demonstrates the % misrepair when the dialyzed nuclear lysates from respective cell lines are incubated with PUC18 linearized using two restriction endonucleases (*Kpn1*/*Sac1*), giving noncompatible DNA ends. Statistical significance of the data was determined using one-way ANOVA with Bonferroni posttests to compare all pairs of columns (cell lines). The data is significantly different for H9 or CB6.2 versus iCB9 or iHuF3 ($^*p < 0.05$). (b)(i) Shown is a representative gel image of the PCR products from CB6.2 and iCB9 that are redigested with *I-Sce1* or left uncut (U). All the S+ products on the gel represent correct repair that restores the *I-Sce1* site in the plasmid. (S−) products represent the *I-Sce1* resistant repair products, which were cloned into TOP10 competent cells. (b)(ii) The clones, each representing different repair products, were analyzed by sequencing across *I-Sce1* junction. Data represents ~10–15 clones analyzed in H9, CB6.2, iCB9, and iHuF3. The data is significantly different for iCB9 versus H9 and CB6.2 (0–5 nt/6–9 nt deletions) or iHuF3 versus H9, CB6.2, and iCB9 (>20 nt deletions) ($^*p < 0.05$).

junctions that were recovered from iCB9 extracts showed 1–5 nt deletions ($^*p < 0.05$, compared to H9, CB6.2), 64% of the junctions had 6–9 nt deletions, and ~27% of the junctions had >20 nt deletions. Strikingly, we observed that 70% of junctions (7 out of 10) recovered from iHuF3 had deletions >20 nt ($^*p < 0.05$, compared to H9, CB6.2, and iCB9) (Figure 4(b)(ii)). This confirms that DNA end joining in sa-CB-iPSC CB6.2 more closely resembles that of hESCs and is less error-prone, compared to end-joining in the fibroblast-derived standard hiPSCs.

3.4. C-MYC Maintains the DDR and NHEJ in hESCs and Is Required for Less Error-Prone Repair in sa-CB-iPSCs. MYC modules, along with Core and Polycomb group genes, represent key gene circuits that contribute to the ES cell expression signature [39]. C-MYC depletion from the reprogramming

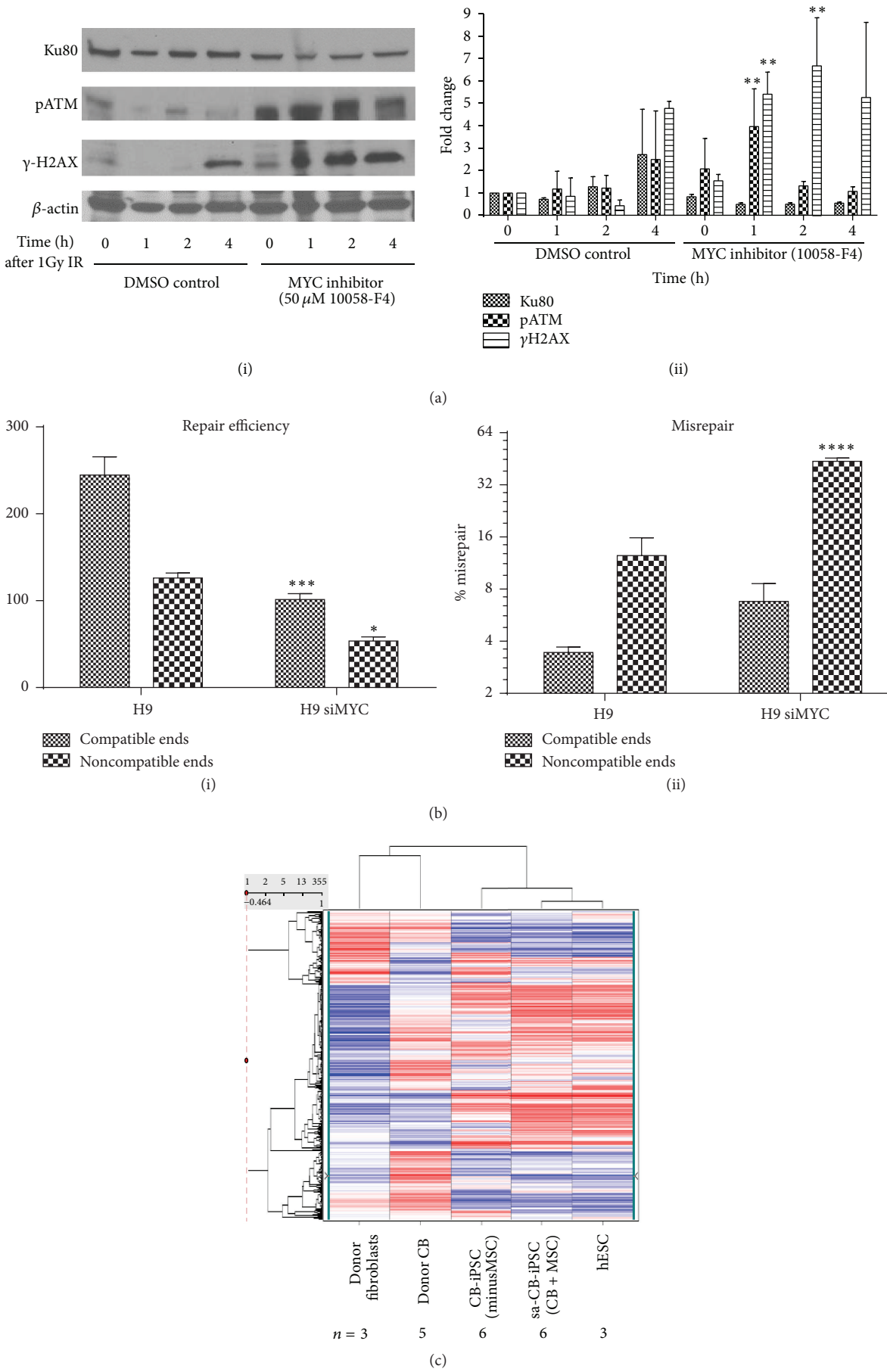

Figure 5: Continued.

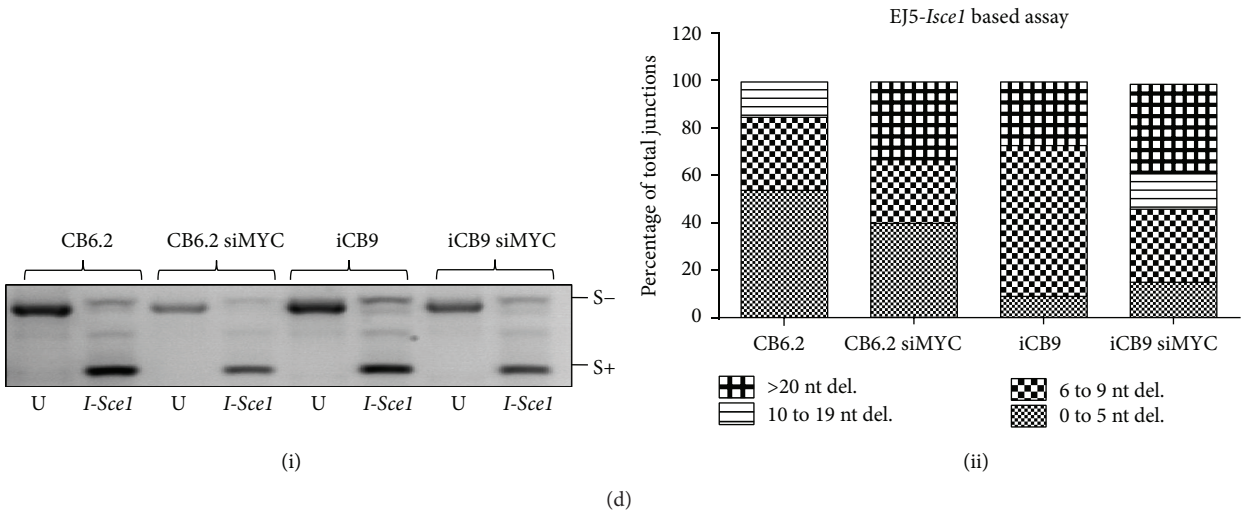

Figure 5: C-MYC maintained high-quality and high-efficiency NHEJ and is required for less error-prone DSB repair. (a)(i) Western blot analysis from whole cell extracts of H9 treated with either solvent control (DMSO) or MYC inhibitor (10058-F4) for 24 h at 50 μM, exposed to IR (1 Gy), and collected at indicated time points. (a)(ii) Densitometry analysis comparing the means from three independent western blots as in ((a)(i)). Statistical significance of the data was determined using 2-way ANOVA with Bonferroni posttests (γH2AX is significantly different between DMSO and MYC inhibition at 1 h, 2 h, and 4 h, $p < 0.05$; pATM is significantly different at 1 h, $p < 0.05$; Ku80 is significantly different at 0 h and 2 h, $p < 0.05$). ((b)(i) and (b)(ii)) The graph represents (i) efficiency of end-joining repair and (ii) percentage of misrepair in linearized PUC18 (with compatible ends) following incubation with extracts from H9 cells ± MYC siRNA. Repair efficiency is calculated by counting the total number of colonies (correctly repaired (blue) + incorrectly repaired (white)) from *in vitro* assays. Statistical significance was determined using paired t-test analysis ($p < 0.01$ between data sets H9 versus H9 siMYC). (c) Shown is the heatmap of $\log_2$ mean-subtracted normalized values of signal intensities from averaged independent biological replicate microarray samples ($n = 3$–6 per condition) representing the expression of genes in MYC module in mRNA from donor fibroblasts, donor CB ($CD34^+$ population), and CB-iPSC lines (*i.e.,* CB.iPS + MSC and CB.iPS (minusMSC)). ((d)(i) and (d)(ii)) (i) Shown is a representative gel image of the PCR products recovered from CB6.2 and iCB9 with or without treatments with siMYC. The PCR products are either redigested with *I-Sce1* or left uncut (U). (S–) products represent the *I-Sce1* resistant repair products. These (S–) fragments are cloned into TOP10 competent cells. (ii) The clones, each representing different repair products, were analyzed by sequencing near *I-Sce1* junction. Data represents ~10–15 clones analyzed in H9, CB6.2, iCB9, and iHuF3. The data is significantly different ($^*p < 0.05$) for CB6.2 versus CB6.2 siMYC (>20 nt deletion) and iCB9 versus iCB9 siMYC (>20 nt deletion). Results are representative of the mean of two independent experiments of each set ± SEM; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, based on t-test analysis.

cocktail significantly reduces the efficiency of reprogramming [40]. Interestingly, sa-CB-iPSCs were characterized by hESC-like MYC-regulated expression module and robustly expressed MYC complex genes [12]. In a different context, C-MYC has also been shown to regulate the transcription of several key DSB repair genes including Ku70 and BRCA1 in somatic cells [26]. Therefore, we questioned whether C-MYC contributes to enhanced efficacy and efficiency of repair in hESCs. As a proof of principle, C-MYC was depleted in hESC H9, using chemical inhibitor 10058-F4, which prevents MYC/MAX association and downstream signaling [41]. Following C-MYC inhibition (50 μM, 24 h), the control and drug-treated cells were exposed to IR (1 Gy) and cells were examined at 0, 1, 2, and 4 hours after IR for expression of DDR proteins by immunoblotting. Notably, compared with untreated cells, C-MYC inhibition resulted in increased levels of γH2AX 1 h after IR and persists until 4 h after IR (Figures 5(a)(i) and 5(a)(ii)). Whereas pATM expression changes after IR in untreated cells are more subtle, C-MYC inhibition results in persistence of pATM that decreases by 4 h. While Ku80 expression decreases with C-MYC inhibition, it is not significant compared with controls. These data suggest that C-MYC is involved in the radiation-induced DSB repair response in hESCs, facilitating repair.

We therefore next determined whether MYC inhibition reduced quality and efficiency of DNA end-joining in H9 cells. Remarkably, siRNA-mediated MYC knockdown (KD) in H9 resulted in a significant decrease in total NHEJ efficiency of these cells, as measured by counting total number of colonies (blue plus white) in an *in vitro* PUC18 assay (Figure 5(b)(i)). Moreover, we also observed a significant increase in the proportion of misrepaired colonies from hESC H9 cells treated with MYC siRNA (Figure 5(b)(ii)).

Since MSC activation of CB donors during reprogramming robustly activated MYC complex genes of pluripotency and facilitated high-capacity reprogramming of human MP differentiated from $CD34^+$ cells [12], we sought to evaluate the MYC module expression networks in sa-CB-iPSC versus other hiPSC lines. Interestingly, microarray expression of MYC-regulated circuit genes in sa-CB-iPSC was more hESC-like relative to standard CB-iPSC (Figure 5(c)). We next determined whether inhibition of C-MYC affected the quality of end-joining in these categories of hiPSCs. For these experiments, we utilized *I-Sce1*-based assays (Figure S1) and

measured DSB repair in these cells *in vitro* (see Section 2). As shown in Figure 5(d)(i), the majority of the GFP genes amplified from the PCR reaction were S+ (*I-Sce1* sensitive), indicating that these extracts mostly produced distal-end joining products that are error-free. However, to determine the character of the errors from plasmid reactivation, PCR products resistant to *ISce1* restriction digestion (S−) were cloned into PCR2.1. Remarkably, similar to C-MYC depletion end-joining results in H9 (Figure 5(d)(ii)), analysis of DSB repair junctions indicated that the efficacy of DNA end-joining significantly deteriorated and became more error-prone when C-MYC was depleted in sa-CB-iPSC (CB6.2) ($^{*}p < 0.05$) (Figure 5(d)(ii)). Specifically, while none of the 13 clones from WT CB6.2 had deletions of >20 nt, ~ 33% of clones (5 out of 15) showed deletions of >20 nt when C-MYC was depleted ($^{*}p < 0.05$). Interestingly, in WT iCB9, 20% of clones had deletions of >20 nt nucleotides that further increased following C-MYC KD (38% versus 27% in WT) (Figure 5(d)(ii)). These results imply that C-MYC gene expression signature is linked to efficacious NHEJ DSB repair in pluripotent cells. Moreover, these data indicate that expression of C-MYC gene expression circuits in hiPSCs could be an important indicator of not only overall efficiency of reprogramming, but also overall DDR signaling and, in particular, repair of DSBs.

4. Discussion

Generating hiPSCs from adult cells represents one of the most exciting developments in regenerative medicine. However, potential clinical applications of hiPSCs are severely hampered by low efficiency of production and suboptimal genomic integrity. One study estimated that ~13% of hESC and hiPSC cultures demonstrated aberrant aneuploid karyotypes [42]. Comparative genomic analyses have revealed a high frequency of DNA copy-number variations (CNVs) in hiPSCs when compared to either hESCs or somatic cells of origin [9, 43]. DNA damage and inaccurate "follow-up" repair mechanisms likely present a significant source of genomic aberrations [44]. For example, reprogramming methods may introduce DNA lesions in the form of lethal DSBs [44]. DSB lesions are introduced by ectopic expression of reprogramming factors and appear to develop irrespective of the reprogramming methodology (i.e., integrative or nonintegrative) [44, 45]. DSB repair components also play an important role in controlling the efficiency of reprogramming [44, 46–50]. Cells that are impaired in HR genes, such as BRCA1/BRCA2 or NHEJ factor DNA ligase IV (LIG4), show significantly decreased capacity for reprogramming [44, 49]. However, it is not well understood whether the features that promote reprogramming further translate into hiPSCs with more robust and efficacious DSB repair properties.

Our study demonstrates that CB-iPSCs generated with high efficiency (sa-CB-iPSC) possess an hESC-like C-MYC transcript signature and have a DDR that more closely resembles hESCs, relative to hiPSCs derived via standard methods. Moreover, sa-CB-iPSCs also performed end-joining DSB repair with less errors, compared with standard CB.iPSCs. Notably, depletion of C-MYC led to increased end-joining errors, suggesting for the first time that MYC-regulated circuits may be required for maintaining genomic integrity in hiPSCs.

Cell differentiation leads to a decline in DNA repair capacity, which can further lead to accumulation of DNA damage and mutations [32, 33]. In contrast, stem progenitors possess greater overall capacity for efficient DNA repair. Stem-progenitor cells may also be more amenable to cellular reprogramming, compared with differentiated somatic cells [12, 51, 52]. However, sa-CB-iPSCs derived from human myeloid progenitors through MSC activation signals are generated even more efficiently (1–4%) and possess minimal interline variability when differentiated to vascular progenitors, compared with hiPSCs derived from CB mononuclear cells generated without MSC activation (0.2–0.3%) [52]. While no significant differences in baseline expression of mRNA transcripts and translated proteins for DDR genes were observed between CB-iPSCs derived via different methods, most significant differences emerged when these cells were analyzed for their DSB repair activities. sa-CB-iPSCs exhibited end-joining repair which was less error-prone and more closely resembles DSB repair properties in hESCs.

Repair of nonligatable ends by NHEJ requires an end-processing step for ligation and thus is prone to errors resulting in deletions of a few nucleotides at DSB repair junctions. IR damage induces NHEJ-mediated DNA misrepair events in late G2 cell cycle stage [53]. Interestingly, ATM suppresses genomic aberrations and incorrect end utilization during NHEJ, known as "distal-end joining," formed as a consequence of multiple DSBs due to genotoxic stress [54, 55]. Although hESCs can uniquely employ high-fidelity NHEJ that can operate independently of ATM [56], hiPSCs perform error-prone DSB repair in particular when exposed to genotoxic stress [53]. Our studies indicate that despite similarities in levels of total ATM and ATM phosphorylation kinetics after IR, sa-CB-iPSCs and standard CB-iPSC have differences in NHEJ responses. In particular, standard fibroblast and CB-iPSCs demonstrated a higher percentage of large deletions (≥20 nt) in DSB junctions, compared to sa-CB-iPSCs and hESCs. Remarkably, "error-proneness" of NHEJ significantly escalates when pluripotent cells are subjected to IR stress under conditions of MYC inhibition.

MYC is an important regulator of transcription in hESCs and is one of the key factors employed in the generation of hiPSCs. Indeed, ectopic MYC is necessary for efficiently generating iPSCs [57, 58]. MYC interacts with the NuA4 complex, a regulator of ESC identity. and is the master regulator of a key ESC transcription program [14, 59, 60]. MYC also activates high telomerase activity during reprogramming via regulation of TERT [61]. Hematopoietic growth factor (GF) stimulation of myeloid progenitors differentiated from $CD34^{+}$ CB cells activates C-MYC-regulated modules to hESC-like levels and facilitates their pluripotency induction [12]. These GF-activated progenitors robustly overexpress MYC complex genes, which have been found to be vital for pluripotency and facilitation of somatic reprogramming [12]. Interestingly, the C-MYC module signature in ESCs highly resembles the C-MYC module that is found in cancer cells [39]. Our data reveals that hESCs and sa-CB-iPSCs

have a similar C-MYC module signature. Moreover, MYC inhibition results in more repair errors in hESC and hiPSCs. Thus, while NHEJ in somatic cells is considered error-prone [19], in normal pluripotent cells, C-MYC appears to be required for maintaining a more error-free NHEJ repair. Notably, putative C-MYC binding sites have been identified in the regulatory regions of several NHEJ genes, suggesting a potential mechanism through which C-MYC may maintain error-free NHEJ in hESCs and hiPSCs [26, 62].

In conclusion, our studies show that the various methods for generating hiPSCs may affect the pathways that regulate genomic integrity. Further characterization is required to determine how these pathways are interconnected and will enable improvement of the genomic integrity of hiPSCs. Knowing that C-MYC is also a master regulator of chromatin modifications [13, 60], its role in facilitating repair might be not only transcriptionally regulated but also epigenetically controlled. Thus, further elucidation of the role of C-MYC in maintenance of genomic integrity, regulating the balance between "good repair" and "bad repair" in pluripotent cells, is required.

Competing Interests

Under a licensing agreement between Life Technologies and the JHU, Elias T. Zambidis is entitled to a share of royalty received by the university for licensing of stem cells. The terms of this arrangement are managed by JHU in accordance with its conflict of interests policies. This does not alter the authors' adherence to journal policies on sharing data and materials.

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Review Article

A Survey of Strategies to Modulate the Bone Morphogenetic Protein Signaling Pathway: Current and Future Perspectives

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Bone morphogenetic proteins (BMPs) constitute the largest subdivision of the TGF-β family of ligands and are unequivocally involved in regulating stem cell behavior. Appropriate regulation of canonical BMP signaling is critical for the development and homeostasis of numerous human organ systems, as aberrations in the BMP pathway or its regulation are increasingly associated with diverse human pathologies. In this review, we provide a wide-perspective on strategies that increase or decrease BMP signaling. We briefly outline the current FDA-approved approaches, highlight emerging next-generation technologies, and postulate prospective avenues for future investigation. We also detail how activating other pathways may indirectly modulate BMP signaling, with a particular emphasis on the relationship between the BMP and Activin/TGF-β pathways.

1. Introduction

Bone morphogenetic proteins (BMPs) constitute the largest subdivision of the TGF-β family of ligands. To date, approximately thirty distinct human proteins are named BMPs and some have additionally been assigned as Growth/Differentiation Factors (GDFs). However, important differences exist among these molecules with regard to pathway mechanics and effects on cellular behavior. This imprecise nomenclature can cause confusion when discussing BMP ligands and their role in human physiology or disease. Clarification may come, however, by focusing on the downstream pathway activated by each ligand rather than name alone. The intracellular effectors SMAD1/5/8 actuate the "bone morphogenetic protein" activity (i.e., autoinduction of bone at extraskeletal sites) originally described by Urist [1, 2]. Proteins that participate in the activation of SMAD1/5/8, then, are *bona fide* components of the canonical BMP signaling cascade. On this basis, it is possible to identify approximately thirteen *bone fide* BMP ligands in humans. *Bona fide* human bone morphogenetic proteins (BMPs) (less common alternative names are in parentheses) are as follows:

BMP2 (BMP2A, BDA2A).

BMP4 (BMP2B, BMP2B1, MCOPS6, OFC11, and ZYME).

BMP5.

BMP6 (VGR, VGR1).

BMP7 (OP-1).

BMP8A.

BMP8B (OP-2).

BMP9 (GDF2, HHT5).

BMP10.

BMP15 (GDF9B, ODG2, and POF4).

GDF5 (BMP14, OS5, LAP4, BDA1C, CDMP1, SYM1B, and SYNS2).

GDF6 (BMP13, KFM, KFS, KFS1, KFSL, SGM1, CDMP2, LCA17, MCOP4, SCDO4, and MCOPCB6).

GDF7 (BMP12).

It is this narrow definition of BMP signaling that we utilize in this review article.

Bone morphogenetic proteins (BMPs) are unequivocally involved in the modulation of several stem cell populations

including embryonic stem cells (ESCs), induced pluripotent stem cells, intestinal stem cells, and mesenchymal stem cells (reviewed in [3–6]). For instance, in embryonic primordial germ cell differentiation, BMP signaling activates a transcriptional network and reexpression of the pluripotency markers *Nanog* and *Sox2* [7]. Mouse ESCs also require dose dependent BMP pathway activation to maintain pluripotency [7]. Genetic inactivation studies demonstrate that *Bmp7* is essential for the maintenance of nephron progenitor cells and its absence promotes premature arrest of nephrogenesis [8]. Additionally, complete removal of BMP signaling sends inactive hair follicle (HF) stem cells into premature proliferation while ectopic expression of BMP4 reduces HF induction and leads to baldness [9]. These findings support the idea that BMP signaling acts as a gatekeeper in stem cells preventing execution of differentiation programs; however other studies demonstrate that BMPs may also elicit the opposite effect. This is often accomplished in collaboration with other signaling pathways. For example, in human ESCs BMPs work in concert with FGF2 to drive mesendoderm differentiation into cardiac, hematopoietic, pancreatic, and liver lineages [10]. The same study suggests that cells derived from mouse ESCs further differentiate into hematopoietic mesoderm cells driven by cooperation between BMP, TGF-β, and Wnt signals [10]. And, BMP pathway activation is a potent activator of osteochondral differentiation in mesenchymal stem cells [11]. Thus, depending on the stem cell population in question, BMP signaling may act in a context-specific manner to either stimulate differentiation or promote maintenance of pluripotency.

This widespread yet context-dependent role of BMP signaling in modulating stem cell behavior requires appropriate regulation of BMP signaling for the development and homeostasis of numerous human organ systems [12]. Aberrations in the BMP pathway or its regulation are increasingly associated with diverse human pathologies (reviewed in [13–16]). Concomitant with this increased clinical significance, there is a growing need to develop effective strategies that modulate BMP signaling as a means of regulating stem cell populations. Tremendous gains have been made in recent years, but these exciting advances have often occurred within areas that may have been overlooked by nonspecialists. Thus, in this review we wish to provide a wide-perspective on the modulation of BMP signaling by paying particular attention to strategy rather than specific application *per se*, though numerous reported applications are noted in the main text and supplemental tables. We briefly outline the current FDA-approved approaches, highlight emerging technologies, and postulate prospective avenues for future investigation. We also detail how activating other pathways may indirectly modulate BMP signaling, with a particular emphasis on the relationship between the BMP and Activin/TGF-β pathways.

2. Strategies to Activate the BMP Pathway

In this section, we highlight several strategies to activate the BMP pathway. These different approaches are schematized in Figure 1.

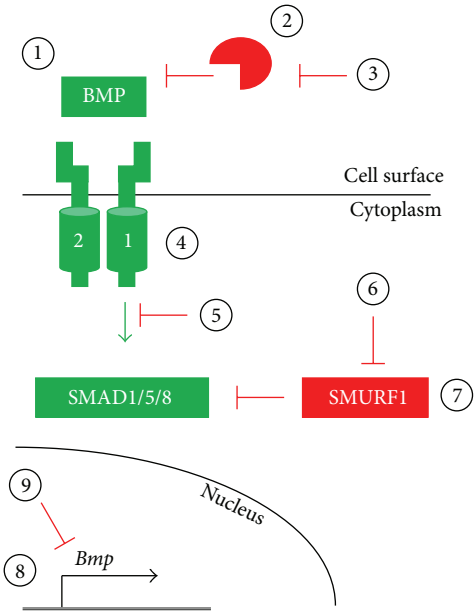

Figure 1: Potential strategies for modulating the BMP pathway. (1–3) The BMP pathway may be activated by exogenous natural or engineered BMP ligands or by expression of such ligands via gene transfer techniques (1). Ligand-induced BMP pathway activation may be inhibited by extracellular ligand traps, such as naturally-occurring antagonists or neutralizing antibodies, via delivery of recombinant protein or expression via gene transfer techniques (2). Endogenous extracellular BMP antagonists, such as Noggin or Chordin, may be inhibited via neutralizing antibodies or small molecules, resulting in increased BMP signaling (3). (4-5) The endogenous BMP pathway inhibitors FKBP12 and Casein Kinase 2 may be inactivated by delivery of FK506 and CK2.3, respectively, thereby increasing signal transduction (4). Alternatively, BMP receptor-mediated activation of the SMAD effectors may be blocked by kinase inhibitors (5). (6-7) Persistence of BMP signaling may be modulated by regulating the SMURF1-mediated ubiquitination of SMAD effector proteins by disrupting SMURF1 interaction with SMADs by small molecule inhibitors (6) or by increasing SMURF1 protein levels (7). (8-9) BMP pathway component expression may be elevated by increasing transcription or alleviating microRNA-mediated translational silencing (8). Alternatively, BMP pathway component levels may be reduced by reducing transcription and/or translation rates (9).

2.1. Natural and Engineered Ligands. The potential for clinical application of the BMP pathway was discovered decades prior to the identification of the BMP ligands [1, 2]. In these original reports, BMP activity liberated from the bone matrix was shown to promote ectopic bone formation. Several osteogenic proteins were then cloned, expressed as recombinant human proteins, and demonstrated to induce bone formation [17], heralding the potential for clinical applicability in orthopedics, which came to actualization in 2001 when recombinant human (rh) BMP7 (OP-1, Stryker) received a humanitarian device exemption (HDE) from the US FDA "for use as an alternative to autograft in recalcitrant long bone nonunions where use of autograft is unfeasible and alternative treatments have failed" (FDA). This was followed

in 2002 when rhBMP2 (InFuse Bone Graft, Medtronic) received FDA medical device approval for use in anterior lumbar interbody fusion. The FDA subsequently approved rhBMP2 for use in several additional spine fusion approaches. rhBMP7 received a second HDE in 2004 for use in posterolateral lumbar fusion, and rhBMP2 received additional FDA approval for use in open tibial fractures in 2004 and oral-maxillofacial applications including sinus augmentation and localized alveolar ridge augmentation in 2007 (FDA). Several ongoing or upcoming clinical trials evaluate the usefulness of rhBMP2 and rhBMP7 in additional orthopedic/dental applications (https://clinicaltrials.gov/).

Recombinant BMPs have a high production cost for clinical use, which raises concern about their cost-effectiveness [18, 19]. As detailed in Table 1, this has prompted several groups to produce relatively short biomimetic peptides and/or to optimize BMP sequences for synthesis in *E. coli* [20–40]. Additionally, numerous studies have demonstrated the feasibility of a gene transfer approach for production of natural or engineered BMP ligands *in vivo* (Tables S1–S7 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/7290686). Several of these studies accomplished cell type specific and/or regulated BMP synthesis. One very interesting idea put forth involves ingesting bacteria that express BMPs for localized production in the gastrointestinal tract [41], which might be advantageous for treating conditions like inflammatory bowel disease (Table S7).

Part of the high cost of rhBMPs is related to the fact that large amounts of protein have been required for clinical use, leading multiple groups to engineer versions that have higher activity than the naturally-occurring ligand (Table 1). For instance, BMP2 chimerae containing segments from Activin A have been shown to be resistant to sequestration by the antagonist Noggin [35, 42–47], leading to greater signaling activity. Noggin-resistant versions of BMP7 and GDF5 bearing enhanced activity have also been described [48–50]. Other studies have utilized nonsignaling ligand decoys to neutralize Noggin [51–53] or potentiate receptor complex assembly [54–59]. In addition, heterodimeric ligands, such as BMP2/6, BMP2/7, and BMP4/7, have been designed to optimize receptor:ligand interactions and each of these display greater activity than the respective homodimer [60–70]. To the best of our knowledge, there are no ongoing clinical trials in humans with these second-generation ligands. One can envision combining the best features of these intelligently engineered molecules and/or production methods into an optimized BMP pathway activator best-suited for specific clinical uses.

2.2. Neutralizing Antibody and Small Molecule Approaches. BMP pathway activation is regulated by a large number of soluble antagonists [71]. Because these proteins operate in the extracellular space, they are attractive targets for strategies aimed at blocking their interaction with BMPs. The feasibility of this approach has been demonstrated by studies using neutralizing antibodies against Noggin or Gremlin in the contexts of pulmonary arterial hypertension (PAH) and spinal cord injury [72–74]. Additionally, the peptide CK2.3 reportedly disrupts the inhibitory interaction between Casein Kinase 2 and the BMP type 1 receptor BMPR1A [75]. Similarly, an *in silico* screen has identified several compounds that could bind to Noggin to disrupt its interaction with BMP ligands [76] and lead candidates have emerged from a screen for small molecules that potentially inhibit the E3 ubiquitin ligase SMURF1 by preventing its interaction with the BMP effectors SMAD1/5 and targeting them for degradation [77–79]. We are not aware of clinical trials of these antibodies or small molecules for increasing BMP signaling *in vivo* at present. The FDA-approved immunosuppressant tacrolimus (Astellas Pharma), which is also known as FK506, activates BMP signaling by inhibiting FKBP12 and is being tested in a clinical trial for the treatment of PAH (NCT01647945).

2.3. Regulation of Expression and/or Potentiating Activity. Enhancing the expression of BMP pathway components could serve as a means to increase signaling. Numerous stimuli have been reported to increase expression levels of BMP ligands or receptors (Table S8). Notably, several kinds of clinically relevant physical stimuli, such as pulsed electromagnetic fields, ultrasound, and mechanical loading, can positively modulate the BMP pathway at multiple levels [80–89]. Additionally, several FDA-approved drugs have been shown to regulate expression of BMP pathway components and/or potentiate BMP signaling. For instance, the statin drugs lovastatin and simvastatin increase BMP2 expression and signaling in several cell types and *in vivo* [90–95]. BMP2 expression and signaling are also increased by the Rho-kinase inhibitor fasudil [96, 97]. Pan-phosphodiesterase inhibition with pentoxifylline or selective inhibition with rolipram or sildenafil has been reported to potentiate BMP signaling as well [98–104].

Recent years have brought considerable attention to the role that microRNAs (miRNAs) play in gene expression, and several miRNAs have been implicated in negatively regulating the expression of BMP pathway components (Table 2 and Section 3). This opens the door, then, to an RNA interference strategy called "anti-miR" or "antagomiR" that targets miRNA and thereby alleviates translation repression. To date, a handful of studies have demonstrated the feasibility of anti-miRs to augment BMP pathway activity *in vitro* and in animal models (Table 2). This technology could prove useful as a means to increase expression of BMP pathway members, especially in scenarios where abnormal miRNA expression is involved in disease pathogenesis [105].

3. Strategies to Inhibit the BMP Pathway

In this section, we will highlight several strategies to inhibit the BMP pathway. These different approaches are schematized in Figure 1.

3.1. Natural and Engineered Antagonists and Small Molecule Inhibitors. The fact that BMP ligands are present in the extracellular environment makes them vulnerable to sequestration upstream of receptor binding on target cells, and

Table 1: Examples of engineered BMP pathway activators.

Category	Engineered version	Modification(s)	Reference(s)
BMP2-based	B2A (B2A2-K-NS)	BMP2-based peptide with heparin-binding domain that augments activity of BMP2 but has no signaling ability alone	[54–59, 151]
	BMP2-L51P	BMP2 mutant that augments activity of BMP2 but has no signaling ability alone	[51–53]
	BMP2_108	BMP2-based peptide; mimics activity of BMP2	[20]
	mBMP	BMP2-based peptide with mineral-binding domain; mimics activity of BMP2	[21]
	OPD	BMP2-based peptide; mimics or presumed to mimic activity of BMP2	[22]
	P1	BMP2-based peptide; mimics or presumed to mimic activity of BMP2	[23]
	P2	BMP2-based peptide; mimics or presumed to mimic activity of BMP2	
	P24	BMP2-based peptide; mimics or presumed to mimic activity of BMP2	[24, 25]
	PEP7	BMP2-based peptide; mimics or presumed to mimic activity of BMP2	[26]
	Unnamed	BMP2-based peptide; mimics or presumed to mimic activity of BMP2	[27–34]
BMP2/Activin A chimerae	AB204	Segmental-chimera of BMP2 and Activin A with enhanced activity over BMP2; Noggin resistant	[35, 42–46]
	AB204-I103Y	Variant of AB204; enhanced activity over BMP2 and AB204	[42]
	AB211	Segmental-chimera of BMP2 and Activin A with enhanced activity over BMP2; Noggin resistant	[35]
	AB215	Segmental-chimera of BMP2 and Activin A with enhanced activity over BMP2; Noggin resistant	[35, 47]
BMP2/BMP9 chimera	BB29	Segmental-chimera of BMP2 and BMP9 with enhanced folding when produced in *E. coli*	[35]
BMP6/BMP7 chimera	80-1	Segmental-chimera of BMP6 and BMP7 with reduced Noggin binding when compared to BMP7	[48]
BMP7-based	BMP7-E60K	BMP6-informed mutant with reduced Noggin binding	[48]
	THR-123	BMP7-based peptide	[36]
	Unnamed	BMP7-based peptide; mimics activity of BMP7	[27]
BMP9-based	MB109	BMP9-based peptide optimized for production in *E. coli*	[37]
	pBMP9	BMP9-based peptide with enhanced activity over BMP9	[38–40]
	SpBMP9	BMP9-based peptide with enhanced activity over BMP9	[40]
	Unnamed	BMP9-based peptide; mimics activity of BMP9	[27]
GDF5-based	GDF5-S94N	Naturally-occurring mutant with enhanced activity due to decreased inhibition by Noggin	[48]
	GDF5-N445K	Naturally-occurring mutant with enhanced activity due to decreased inhibition by Noggin	[49]
	GDF5-N445T	Naturally-occurring mutant with enhanced activity due to decreased inhibition by Noggin	[49, 50]
	GDF5-V453/V456	BMP2-informed variant of GDF5; enhanced activity over GDF5 and BMP2	[152, 153]
Heterodimers	BMP2/6	Heterodimer with enhanced activity over BMP2 and BMP6	[60, 61]
	BMP2/7	Heterodimer with enhanced activity over BMP2 and BMP7	[62–67]
	BMP4/7	Heterodimer with enhanced activity over BMP4 and BMP7	[68–70]

Table 2: Examples of microRNAs targeting BMP pathway components and their inhibition via anti-miR RNA interference.

miRNA	Target(s)/notes	Reference(s)	Anti-miR
miR-17-5p	*Bmpr2, Smad7*	[154, 155]	NR
miR-20a	*Bmpr2, Bambi, Crim1*	[154, 156]	[157]
miR-23b	*Smad4, Smad5*; also *Smad3*	[158]	NR
miR-26a	*Smad1, Smad4, Tob1*	[159–161]	[159, 160]
miR-27	*Acvr2a*; also *Tgfβr1* and *Smad2*	[162]	NR
miR-30a/b/c/d	*Bmp7, Smad1*	[163, 164]	[164]
miR-100	*Bmpr2*	[165]	NR
miR-122	*Hemojuvelin*	[166]	[166]
miR-125	*Bmpr2*	[167]	[167]
miR-130a	*Alk2*	[168]	NR
miR-135b	*Bmpr2, Smad5*; also *Alk4* and *Tgfβr2*	[169, 170]	NR
miR-140	*Bmp2*	[171]	NR
miR-145	Undetermined (possibly *Bmp4* indirectly)	[172]	NR
miR-148a	*ALK2*	[173]	NR
miR-153	*Bmpr2*	[174]	NR
miR-155	*Smad1, Smad5*	[175, 176]	NR
miR-199a*	*Smad1*	[177]	[177]
miR-200	*Bmp4,* indirectly	[178]	NR
miR-205	*Smad1*, *Smad4*	[179]	NR
miR-302	*Bmpr2*	[180]	NR
miR542-3p	*Bmp7*	[181]	NR

NR: not reported.

the extracellular antagonists Noggin, Gremlin, and Chordin might be used to regulate BMP signaling in this manner [71]. Numerous studies have exploited this relationship by administering recombinant BMP antagonists or delivering them via gene transfer (Tables S2, S4, and S6–S8). Once delivered, these antagonists typically sequester multiple BMP isoforms, which, depending on the specific application, may be advantageous or not. An alternative approach to enhance BMP:BMP antagonist interactions would be to employ soluble decoy receptors that comprise only the ligand binding domain of individual BMP receptors and, therefore, interact with ligands according to particular affinities (Table 3). An example of this kind of specificity can be observed with the soluble ALK1 (ALK1-ECD, Dalantercept, Acceleron Pharma), which is currently in clinical trials as a cancer therapy (NCT01458392, NCT01642082, NCT01720173, NCT01727336, and NCT02024087); ALK1-ECD preferentially sequesters BMP9 and BMP10 [106–111]. Greater specificity in ligand sequestration may also be achieved by using neutralizing antibodies raised against individual BMP ligands (Table 3). Investigators should be aware, however, that a high degree of homology exists between certain BMP ligands, such as BMP2 and BMP4 which are 92% identical, and this could make it challenging to specifically neutralize only one isoform when others are present. It is possible, also, that a specific BMP ligand could be inactivated via interaction with its prodomain [112] or via bespoke DNA aptamers [113].

BMP receptors are serine/threonine kinases, which makes them attractive targets for small molecules that block the kinase pocket and inhibit their activity. Considerable attention has been focused upon type 1 BMP receptors (ALK1/2/3/6) and the first kinase inhibitor reported was Dorsomorphin [114]. Though significant off-target effects are now noted for Dorsomorphin (Table 4), this molecule represents a key advancement in the field and has served as a guide for subsequent generations of analogues with greater specificity (Table 4). Some type 1 receptor selectivity

Table 3: Examples of BMP pathway modulation by receptor ECDs or neutralizing antibodies.

Molecule	Reference(s)
ACVR2A-ECD	[182]
ACVR2B-ECD	[182, 183]
Anti-ALK1 Ab	[184]
ALK1-ECD	[106–110]
ALK3-ECD	[185–188]
Anti-BMP2 Ab	[189, 190]
Anti-BMP4 Ab	[190–192]
Anti-BMP6 Ab	[193–195]
Anti-BMP7 Ab	[196, 197]
Anti-BMP10 Ab	[111]
BMPR2-ECD	[198]
Dragon-ECD	[194]
Anti-gremlin Ab	[72]
Hemojuvelin-ECD	[193, 199, 200]
Anti-noggin Ab	[73, 74]

Ab: antibody; ECD: extracellular domain.

Table 4: Small molecule inhibitors of BMP Type 1 receptors and examples of their use.

Molecule	Comment(s)	Reference(s)
1LWY	Dramatically enhanced selectivity for ALK2 versus other type 1 BMP receptors (approximate order of selectivity: ALK2 > ALK3 > ALK6); greatly reduced off-target effects compared to DM and LDN	[120]
DMH1	Pan-type 1 BMP receptor inhibitor (approximate order of selectivity: ALK3 > ALK1 > ALK6 > ALK2); reduced off-target effects compared to DM and LDN	[121, 122, 201–205]
DMH2	Pan-type 1 BMP receptor inhibitor (approximate order selectivity: ALK6 > ALK3 > ALK2); notable off-target effects, including BMPR2, TGFBR2, ALK4, ALK5, AMPK, and VEGFR2	[120, 201, 206]
DMH3	Presumed pan-type 1 BMP receptor inhibitor; reduced off-target effects compared to DM and LDN	[201]
Dorsomorphin (DM)	Pan-type 1 BMP receptor inhibitor (approximate order of selectivity: ALK2 > ALK3 > ALK1 > ALK6); notable off-target effects, including BMPR2, ACVR2A, ACVR2B, TGFBR2, ALK5, AMPK, VEGFR2, and PDGFRβ	[114, 121, 122, 124, 201, 202, 207–215]
K02288	Modestly enhanced selectivity for ALK1 and ALK2 versus other type 1 BMP receptors (approximate order of selectivity: ALK2 > ALK1 > ALK6 > ALK3); reduced off-target effects compared to DM and LDN	[121, 216, 217]
LDN-193189 (LDN)	Pan-type 1 BMP receptor inhibitor (approximate order of selectivity: ALK1~ALK2 > ALK3 > ALK6); notable off-target effects, including BMPR2, ACVR2A, ACVR2B, TGFBR2, ALK5, AMPK, VEGFR2, and PDGFRβ	[120–122, 124, 185, 191, 207–209, 216, 218–227]
LDN-212854	Significantly enhanced selectivity for ALK1 and ALK2 versus other type 1 BMP receptors (approximate order of selectivity: ALK2 > ALK1 > ALK3); reduced off-target effects compared to DM and LDN	[121]
LDN-214117	Dramatically enhanced selectivity for ALK2 versus other type 1 BMP receptors (approximate order of selectivity: ALK1, ALK2 > ALK3); greatly reduced off-target effects compared to DM and LDN	[123]
ML-347	Dramatically enhanced selectivity for ALK1 and ALK2 versus other type 1 BMP receptors (approximate order of selectivity: ALK2 > ALK1 $\gg$ ALK3); reduced off-target effects compared to DM and LDN	[122, 228]
VU5350	Pan-type 1 BMP receptor inhibitor (approximate order selectivity: ALK3 > ALK2 > ALK6); notable off-target effects, including BMPR2, TGFBR2, AMPK, and VEGFR2	[120]

has been reported among each of these compounds and it is conceivable that, in the near future, an investigator may be able to choose the most appropriate small molecule for a given application. For instance, activating mutations in ALK2 cause both fibrodysplasia ossificans progressiva (FOP) and pediatric intrinsic diffuse glioma (PIDG) [115–119]. Four candidate molecules, LDN-212854, LDN-214117, ML-347, and 1LWY, have recently been described as having dramatically enhanced selectivity for ALK2 (and the closely related ALK1) over the other type 1 receptors [120–123]; we are unaware of data directly comparing the *in vivo* efficacy of these four molecules head-to-head. Similarly, Tsugawa et al. concluded that differential type 1 receptor targeting underlies the finding that LDN-193189, DMH2, and VU5350 are effective in promoting liver regeneration in a rodent model while 1LWY is not [120].

It should be noted that some of these small molecules also target type 2 BMP receptors BMPR2, ACVR2A, and ACVR2B (Table 4), which might be advantageous in some experimental designs but could be problematic in others. And, given that ACVR2A and ACVR2B are also utilized by Activin and Activin-like ligands such as Myostatin, one must also keep in mind that Dorsomorphin and LDN-193189 can effectively block SMAD2/3 activation by these ligands [124].

3.2. Regulation of Expression. As mentioned in Section 2, several miRNAs have been shown to negatively regulate the expression of BMP pathway components (Table 2). In particular, translation of the BMP effector SMAD1 is repressed by at least four distinct miRNAs. And, some miRNAs, such as miR-155, target both SMAD1 and SMAD5. This raises the possibility that gene transfer of certain miRNA sequences singly or in combination could be useful as a means to impair effectors of the canonical BMP response. Proof of principle for this approach is found in several studies that utilized viral transduction or naked DNA delivery of miRNA to impact BMP signaling (Table 2). Similarly, knockdown of BMP pathway components as a means of reducing signaling *in vivo* has been accomplished by gene transfer in multiple scenarios and by various methods (Tables S2, S4, and S6). Notably, one emerging gene therapy strategy uses allele-specific RNA interference (ASP-RNAi) to selectively silence a single protein isoform, such as a constitutively active (ca) mutant [125]. Two separate groups have applied ASP-RNAi to the BMP pathway *in vitro* to knock down disease-causing caALK2 expression [126, 127]. This strategy is particularly amenable to FOP because the same point mutation underlies the vast majority of cases, thus enabling a single set of validated siRNAs to treat most patients [128]. ASP-RNAi could potentially be applied to disease-causing dominant negative mutations as well, such as those in *BMPR2* that are found in some heritable PAH patients and are associated with earlier onset and more severe disease than nonexpressed mutants [129].

In comparison to stimuli that positively modulate the BMP pathway, relatively few agents have been described to reduce expression and/or pathway activity (Table S9). Notably, the FDA-approved antianginal drug perhexiline reduces BMP signaling *in vitro* and decreases ossification in an ectopic assay [130]. BMP inhibition is also observed with a retinoic acid receptor-gamma agonist and a clinical trial is currently underway to examine this approach in reducing heterotopic ossification among patients with classic FOP (https://clinicaltrials.gov/).

4. Indirect Modulation of BMP Pathway Activity via Activating Other Pathways

A large body of literature describes effects on the BMP pathway when other signaling pathways are targeted. Many of these studies were designed to augment BMP signaling, especially in orthopedic and dental applications (Table S1) though other scenarios have also been evaluated (Tables S2–S7) and several ways that the cellular or tissue microenvironment can be altered to be more permissive to BMP signaling have come to light. One example of this is the synergy observed when intermittent parathyroid hormone therapy is combined with BMP2 or BMP7 in bone healing [131, 132].

Relatively little is known about how activating a different pathway can antagonize the effects of BMP signaling *in vivo*. One significant exception to this is the wide range of contexts in which the Activin/TGFβ and BMP pathways elicit distinctly opposing effects on the same cell type. Some examples of this includes early body patterning [133], angiogenesis [134], cell fate of type 2 alveolar epithelial cells [135], maintenance of epithelial cell polarity [136], and regulation of skeletal muscle mass [137, 138]. Also, imbalances in the ratio of TGFβ superfamily cytokines are increasingly associated with human diseases, including pulmonary and kidney fibrosis [139, 140], glaucoma [141, 142], asthma [143], and pulmonary arterial hypertension [144, 145]. This raises the intriguing possibility that the effects of Activin/TGFβ pathway inhibition, for example, on skeletal muscle mass or bone volume, could in part be due to reducing antagonism of the BMP pathway. Support for this idea comes from the fact that increasing the BMP pathway can have similar effects to inhibiting TGFβ signaling (e.g., [146–148]). While the Activin/TGFβ receptor kinase inhibitor SB431542 has been reported to increase BMP signaling in preosteoblasts [149] and BMP target gene expression in chondrocytes [150], most studies have not evaluated how modulating the BMP pathway alters transduction of the Activin/TGFβ pathway, or vice versa, so the extent to which this bidirectional antagonism impacts development and disease is not presently known. That said, in general, all cell types examined to date have the capacity to respond to BMPs, Activins, and TGFβs and these molecules are often present in the extracellular environment at the same time. Thus, how cells integrate BMP versus Activin/TGFβ information and make specific decisions is an important area for future research.

5. Methods

Studies germane to this topic were identified in http://pubmed.com/ by combining the following search terms: antagonism; antagonist; bmp; bone morphogenetic protein; gene therapy; inhibition; inhibitor; siRNA. Articles

retrieved were indexed to MEDLINE prior to January 6, 2016. Clinical trials were identified on https://clinicaltrials.gov/ and https://www.clinicaltrialsregister.eu/ prior to January 21, 2016. Specific applications highlighted are meant to be representative rather than exhaustive of the field and no endorsement by the authors of any particular application should be inferred.

Competing Interests

The authors declare no competing interests.

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Research Article

Disrupted Endothelial Cell Layer and Exposed Extracellular Matrix Proteins Promote Capture of Late Outgrowth Endothelial Progenitor Cells

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Late outgrowth endothelial progenitor cells (LO-EPC) possess a high proliferative potential, differentiate into vascular endothelial cells (EC), and form networks, suggesting they play a role in vascular repair. However, due to their scarcity in the circulation there is a requirement for *ex vivo* expansion before they could provide a practical cell therapy and it is currently unclear if they would home and engraft to an injury site. Using an *in vitro* flow system we studied LO-EPC under simulated injury conditions including EC activation, ischaemia, disrupted EC integrity, and exposed basement membrane. Perfused LO-EPC adhered to discontinuous EC paracellularly at junctional regions between adjacent cells under shear stress 0.7 dyn/cm^2. The interaction was not adhesion molecule-dependent and not enhanced by EC activation. LO-EPC expressed high levels of the VE-Cadherin which may explain these findings. Ischaemia reperfusion injury decreased the interaction with LO-EPC due to cell retraction. LO-EPC interacted with exposed extracellular matrix (ECM) proteins, fibronectin and vitronectin. The interaction was mediated by integrins $\alpha5\beta3$, $\alpha v\beta1$, and $\alpha v\beta3$. This study has demonstrated that an injured local environment presents sufficient adhesive signals to capture flow perfused LO-EPC *in vitro* and that LO-EPC have properties consistent with their potential role in vascular repair.

1. Introduction

Endothelial cells (EC) play an important role in regulating vascular homeostasis, modulating permeability, maintaining vascular tone, and responding to various stimuli by the production of bioactive substances [1]. Loss of endothelial integrity may cause a variety of deleterious consequences including acute events such as thrombus formation and predisposing to chronic pathology including transplant vasculopathy and atherosclerosis leading to complications such as coronary heart disease, stroke, and diabetes [2–5]. Endothelial integrity depends on a balance between the extent of endothelial cell injury and the capacity for endogenous repair. In healthy individuals, neighbouring mature endothelial cells can replicate locally and replace damaged cells [3]. However if injurious stimuli are prolonged and/or repeated or there is a large area of damage, endogenous repair may be inadequate [6] and require additional repair mechanisms.

Endothelial progenitor cells (EPC) could provide an alternative mechanism for maintenance and repair of damaged endothelium *in vivo*. Two types of EPC with distinct properties have been identified, early outgrowth EPC (EO-EPC) and late outgrowth EPC (LO-EPC) [7–11]. Early outgrowth EPC are short-lived cells (<2 weeks) and do not differentiate into EC *in vivo* but can restore endothelial function and enhance angiogenesis after tissue ischaemia via a paracrine effect [8, 12, 13]. However, they are a heterogeneous population of hematopoietic cells including monocyte-derived immune cells [12, 14, 15]; delivering large numbers of *ex vivo* expanded autologous EO-EPC might risk exacerbating immune response. LO-EPC, by contrast, are a homogeneous endothelial-like progenitor cell population that possess a high proliferative potential, differentiate into vascular endothelial cells, and form networks *in vitro* and *in vivo* [10, 16, 17]. We and others have shown that LO-EPC morphology and angiogenic function is preserved in patients with cardiovascular

risk factors and patients with end stage renal failure [16, 18]. Their proliferation, differentiation, and tube forming ability are increased by laminar shear stress [19–22] suggesting that they may contribute to autologous vascular repair. However LO-EPC are not abundant in the circulation [7, 23]. To use them therapeutically LO-EPC would need to be expanded *ex vivo* to high concentrations before being delivered back into the circulation. The fate of LO-EPC after delivery including their ability to home to and engraft at a site of injury is not known.

Vascular damage is characterised by endothelial cell activation and dysfunction that may progress to detachment leading to loss of endothelial integrity [3, 24]. Endothelial cell damage markers including endothelial microparticles derived from activated or apoptotic cells and whole endothelial cells can be detected in the circulation [25]. Once the endothelial monolayer is disrupted, the basement membrane is exposed to blood flow. This layer provides the primary physical support for endothelial cells and is composed of collagen type IV, collage type I, fibronectin, vitronectin, laminin, and several proteoglycans (including heparin sulphate proteoglycan) [26]. These local changes may influence LO-EPC homing and engraftment. In this study, we investigated the dynamic interaction of LO-EPC with normal endothelial cells, activated endothelial cells or those undergoing simulated ischaemia reperfusion injury, and different extracellular matrix (ECM) proteins. Understanding the interaction of LO-EPC under simulated injury conditions *in vitro* and the mechanism of LO-EPC capture from flow will provide us with a critical view on the practicality of using LO-EPC for endogenous repair.

2. Materials and Methods

2.1. Cell Culture. This study had full ethical approval from the institutional review board of the Clinical School, University of Cambridge, and written informed consent was obtained from all volunteers. Late outgrowth EPC were isolated as previously described [16]. Briefly, mononuclear cells (MNC) were isolated from 40 mls venous peripheral blood by density-gradient centrifugation with Ficoll-paque-1.077 (GE Healthcare, UK). The mononuclear cells were plated in a culture flask coated with type I collagen (BD, UK) and cultured at 37°C under 5% CO_2 atmosphere in endothelial basal medium (EBM) supplemented with SingleQuots (Lonza) and 20% Hyclone fetal calf serum (Fisher Scientific, UK). Nonadherent cells were removed after 3 days in culture and the medium was changed on alternate days. Colonies of LO-EPC appeared after 2 to 3 weeks in culture and exhibited typical cobblestone morphology. Once individual colony cell number reached 500–1000, the cells were passaged into a new collagen-coated flask. Subsequently cells were passaged at a 1 : 3 ratio into noncoated flasks. The medium was changed every other day. LO-EPC from passages 4–6 were used.

Human abdominal aorta endothelial cells (HAEC) were purchased from PromoCell, Germany. The cells were cultured in complete endothelial growth medium with 5% fetal calf serum (PromoCell). The medium was changed every other day. Cells from passages 3–6 were used.

2.2. Interaction of LO-EPC and HAEC under In Vitro Shear Flow. 3×10^4 HAEC were plated directly on Ibidi μ-Slide VI 0.4 Luer slides (Thistle Scientific LTD, UK) 48 hours before the experiments. HAEC were either left untreated, or treated with 0.05 ng/mL TNFα for 4 hours, or subjected to ischaemia for 4 hours followed by reperfusion overnight, before being connected to the flow system. The flow system was set up as previously described [27, 28]. Briefly, to perfuse the cells in the flow system, one end of the Ibidi slide was attached by silicon rubber tubing to an electronic valve, which allowed smooth switching between the LO-EPC suspension and wash buffer (1% BSA in DPBS, Sigma, UK) held in vertical syringe barrels, and the other end of the Ibidi slide was attached by silicon rubber tubing to a Harvard syringe pump (Harvard Apparatus, UK). The flow rate of 0.4 mL/min was pump-controlled. The equivalent shear stress (τ) exerted on Ibidi μ-Slide VI 0.4 Luer slide surface at a flow rate of 0.4 mL/min (Φ) was 0.7 dyn/cm^2, which was calculated from the equation "$\tau = \eta 176.1\Phi$". η (dynamical viscosity) was 0.01 dyn·s/cm^2. LO-EPC were labelled with Dil-Ac-LDL to distinguish them from HAEC after adhesion. After insertion of the Ibidi slide into the flow system, the slide was washed for 2 min with 1% BSA in DPBS (perfusion buffer). A total of 4×10^5 labelled LO-EPC in 1.5 mL perfusion buffer were then perfused at a shear stress of 0.7 dyn/cm^2 for 4 min. An additional 2 min wash was applied to remove nonadherent cells. The interaction of LO-EPC with endothelial cells was observed and recorded during the LO-EPC perfusion, and the images were retained. The number of adherent LO-EPC was counted and expressed as adherent cells per square millimetre. Videomicroscopic recordings were made and analyzed offline using computerized image analysis software (Image ProPlus and Image J). The interactions of LO-EPC with HAEC were easily observed on the video and individual cell motion including rolling, tethering, transient adhesion, and firm arrest was recorded. The flow experiments were conducted at 37°C within a Perspex chamber.

2.3. Interaction of LO-EPC and ECM under In Vitro Shear Flow. 100 μg/μL of collagen IV (BD, UK), collagen I (BD, UK), fibronectin (Sigma, UK), vitronectin (Invitrogen, UK), or laminin (Sigma, UK) was preplated to Ibidi μ-Slide VI 0.4 Luer slides for 1 hour at 37°C. The Ibidi slide was then connected to the flow system as described above. The slide was washed for 2 min with 1% BSA in DPBS. A total of 4×10^5 LO-EPC in 1.5 mL were perfused at a shear stress of 0.7 dyn/cm^2 for 4 min. An additional 2 min wash was applied to remove the nonadherent cells. Cells were subsequently fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with PBS, and stained with Hoechst dye (1 μg/mL) for 30 min. The number of adherent LO-EPC was imaged, counted, and expressed as adherent cells per square millimetre.

2.4. Static Interaction of LO-EPC with ECM. 100 μg/μL of collagen IV, collagen I, fibronectin, vitronectin, or laminin was preplated to Ibidi μ-Slide VI 0.4 Luer slides for 1 hour at 37°C before rinsing twice with DPBS. 2×10^4 LO-EPC were

seeded on each ECM-treated Ibidi slide and incubated at 37°C for 45 min. After washing twice with DPBS, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and stained with Hoechst for 30 min. The total number of adherent cells was imaged, counted, and expressed as adherent cells per square millimetre.

2.5. Flow Cytometric Analysis of Cell Surface Markers. To study TNFα mediated HAEC activation, control HAEC and HAEC treated with 0.05 ng/μL TNFα (R&D System, UK), for 4 hours at 37°C, were collected in 100 μL 1% bovine serum albumin (BSA, Sigma) in PBS and incubated with fluorescein conjugated anti-human E-selectin antibodies (R&D System), APC conjugated anti-human ICAM-1 (BD), and PE/Cy5 conjugated anti-human VCAM-1 (Bio Legend, UK), together with the respective isotype control antibodies. Forward-side scatter plots were used to exclude dead cells. Data analysis was performed using CELL Quest software (BD) and FlowJo.

The expression of VE-Cadherin in LO-EPC and HAEC was quantified by flow cytometry using a FITC conjugated antibody against human VE-Cadherin (Abcam, UK), together with its corresponding isotype.

2.6. Immunofluorescence Staining. VE-Cadherin expression was visualized by immunofluorescence staining. Control HAEC and HAEC treated with ischaemia reperfusion injury were fixed for 15 min in 4% paraformaldehyde. Cells were then blocked for 30 min in 3% (w/v) BSA in TBS at pH 7.4 (blocking buffer). FITC conjugated VE-Cadherin antibodies (Abcam, UK) were diluted 1 : 50 in blocking buffer and incubated with cells at 4°C overnight.

2.7. Cell Staining of Dil-Acetylated-Low Density Lipoprotein (Dil-Ac-LDL). Both endothelial cells and LO-EPC can take up Dil-Ac-LDL. To label LO-EPC with Dil-Ac-LDL, LO-EPC were incubated with 10 μg/mL of Dil-Ac-LDL (Molecular Probes, Invitrogen) for 1 hour at 37°C and then washed twice with PBS. Labelled LO-EPC were distinguished from HAEC after adhesion.

2.8. VE-Cadherin Blocking Studies. Purified mouse antibody against human VE-Cadherin was used to block the surface expression of VE-Cadherin in HAEC (clone: BV9, Biolegend, UK). 3×10^4 HAEC were incubated with 50 μg/mL of antibody for 1 hour at 37°C before being connected to the flow system as described above. The cells were washed for 2 min with 1% BSA in DPBS prior to perfusion, with a total of 4×10^5 LO-EPC in 1.5 mL of 1% BSA in DPBS.

2.9. Ischaemia Reperfusion Injury. Ischaemia reperfusion injury was simulated by anoxic (O_2 < 1% and CO_2 > 5%) and acidotic conditions with glucose and pyruvate deprivation as described previously [29]. LO-EPC were incubated with a minimal volume of ischaemia solution (118 mM NaCl, 24 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot H_2O$, 2.5 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgCl_2$, 0.5 mM sodium·EDTA·$2H_2O$, 20 mM sodium lactate, and 16 mM KCl, pH 6.2) under hypoxia in an anaerobic bag (BDH), at 37°C for 4 h. Cells were then transferred to a 37°C incubator with 5% CO_2 with additional complete culture medium for reperfusion overnight.

2.10. Integrin Blocking Studies. 1.5 mL of 4×10^5 LO-EPC was incubated with 10 μg/mL anti-ITG $\alpha 5\beta 1$ (Millipore, UK), anti-ITG $\alpha V\beta 3$ (Millipore, UK), and anti-ITG $\alpha v\beta 1$ (Bioss, Antibodies-online.com) or no antibodies (control) for 30 minutes at 37°C with gentle rotation. LO-EPC were then perfused onto fibronectin pretreated Ibidi slides under a shear stress of 0.7 dyn/cm^2. After perfusion, the slides were washed for an additional 2 min to remove nonadherent cells. Adherent cells were then fixed with 4% paraformaldehyde and stained with Hoechst. The cells were imaged, counted, and expressed as adherent cells per square millimetre.

2.11. Live Cell Image Acquisition and Analysis. Live cell imaging of the interaction of LO-EPC with HAEC or ECM was performed using a digital imaging system coupled to an inverted microscope under flow conditions. The camera was set up to observe the top view of the rolling of LO-EPC on endothelial cells or ECM. The images were acquired though Image Pro software. The images were taken from a representative field of view every 30 seconds for 5 min from the start of LO-EPC perfusion. The sequence of events including LO-EPC rolling, tethering, and binding was recorded. The total number of adherent LO-EPC was stained with Hoechst or Dil-Ac-LDL and determined by counting the total adherent cells in 3–6 fields of view. The rolling velocity was observed but no specific measurements were recorded in this study.

2.12. Statistical Analysis. All values are expressed as mean ± SE from at least three separate experiments. Within each independent experiment, at least duplicate measurements were performed. One way ANOVA with Newman Keuls *post hoc* test was used to determine significance for all experiments. A probability value of $p < 0.05$ was considered statistically significant and is indicated by $*$, and $p < 0.01$ is indicated by $**$.

3. Results

3.1. Interaction of LO-EPC with Human Abdominal Aorta Endothelial Cells under Flow. The interaction of LO-EPC with a human abdominal aortic endothelial cell (HAEC) monolayer was assessed under 0.7 dyn/cm^2 shear stress at 37°C. Figure 1(a) shows LO-EPC adhere to a subconfluent HAEC monolayer (3×10^4) at gaps between adjacent HAEC paracellularly rather than adhering to superficial (luminal) surface of the HAEC. Interaction did not occur on a completely confluent HAEC monolayer (6×10^4) (Figure 1(b)). The number of cells adhering to complete confluent and subconfluent HAEC was 3.25±0.38 and 21.46 ± 1.81/mm^2, respectively. Dilution of the seeded HAEC to 1×10^4 to increase the intercellular spacing between HAEC decreased the adhesion of LO-EPC to HAEC (data not shown), suggesting that LO-EPC preferentially form adjacent contacts with HAEC. Similar rolling velocity was observed regardless of HAEC confluence. The adherent LO-EPC appeared as round cells

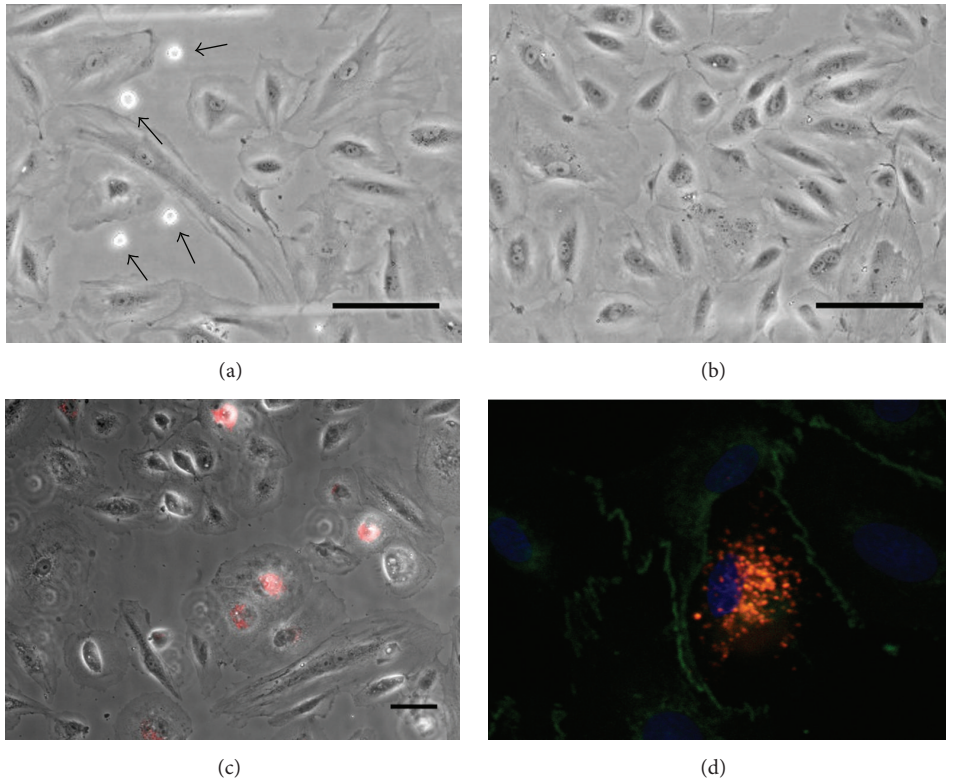

Figure 1: Representative phase-contrast live cell images of interaction of LO-EPC with 3×10^4 subconfluent (a) and 6×10^4 confluent (b) HAEC monolayers under shear stress 0.7 dyn/cm^2. 4×10^5 LO-EPC were perfused for 4 min. Arrows indicated adherent LO-EPC which only adhered paracellularly at junctional regions of discontinuity between two cells in subconfluent HAEC monolayer. Scale bar 30 μm. After adhesion LO-EPC spread and establish cell-cell interaction (c). LO-EPC were labelled with DiI-Ac-LDL shown red. Scale bar 60 μm. VE-Cadherin staining revealed the formation of lateral junction between LO-EPC and HAEC (d). LO-EPC were labelled with DiI-Ac-LDL shown red and VE-Cadherin expression shown green.

initially, but they could withstand the shear forces, spread rapidly after firm adhesion, and start to establish cell-cell connections under 0.7 dyn/cm^2 shear stress (Figure 1(c)). Immunofluorescence staining of VE-Cadherin in adherent LO-EPC revealed adhesion junction formation between LO-EPC and HAEC (Figure 1(d)), while Claudin-5 (tight junction protein) and PECAM showed more diffuse staining in adherent LO-EPC (data not shown), suggesting VE-Cadherin promotes a homotypic type of recognition between LO-EPC and HAEC. Adhesion of LO-EPC did not disrupt the HAEC morphology and monolayer structure and no transmigration of LO-EPC was observed. This was in contrast to monocytes, which interacted only by binding superficially to a confluent HAEC layer and then transmigrating (data not shown).

3.2. The Interaction of LO-EPC with HAEC Was Not Adhesion Molecule-Dependent under Flow. TNFα (0.05 ng/mL) was used to induce HAEC activation to investigate whether this enhanced the interaction with LO-EPC. HAEC activation was characterised by increased expression of cell surface adhesion molecules E-selectin, ICAM-1, and VCAM-1 in HAEC (Figure 2(a)). Activation did not increase the interaction with LO-EPC under 0.7 dyn/cm^2 shear stress (Figure 2(b)) and there was no difference seen in rolling velocity, suggesting that adhesion molecules did not mediate LO-EPC rolling or adhesion to HAEC under flow conditions. This contrasted with monocytes in which the interaction increased when HAEC were activated. When 10^6 monocytes were perfused the number of adherent monocytes increased from 6.01 ± 0.67 cells per millimetre square in untreated HAEC to 45.74 ± 4.03 cells per millimetre square in activated HAEC ($p < 0.01$) under 0.7 dyn/cm^2 shear stress.

3.3. Vascular Endothelial- (VE-) Cadherin Mediates the Interaction of LO-EPC with HAEC under Flow. VE-Cadherin is an endothelium specific adhesion protein prominently located at junctions between endothelial cells suggesting it may play a role in initiating the interaction of LO-EPC with HAEC. We showed that LO-EPC had higher expression levels of VE-Cadherin compared to HAEC (Figure 3(d)). Higher expression of VE-Cadherin could contribute to LO-EPC adherence to HAEC paracellularly.

To investigate the involvement of VE-Cadherin in the interaction of LO-EPC with HAEC under dynamic flow, an antibody against VE-Cadherin was used to block the surface expression of VE-Cadherin (Figures 3(a) and 3(b)). Blocking VE-Cadherin in HAEC reduced the interaction of LO-EPC with HAEC significantly (Figure 3(c)), without significantly

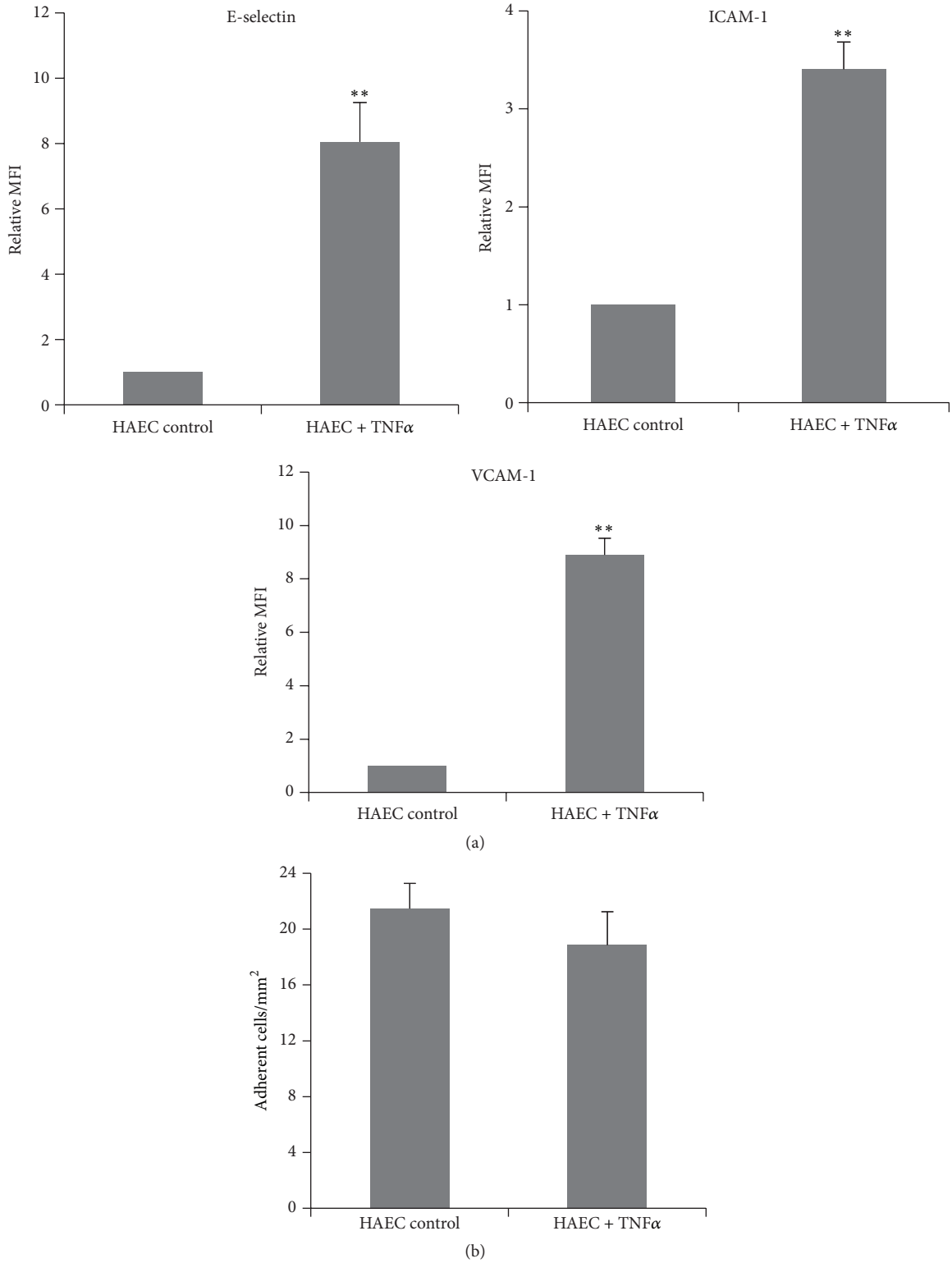

Figure 2: Interaction of LO-EPC with activated HAEC. Surface expression of adhesion molecules in HAEC after stimulation with 0.05 ng/mL TNFα (a). Surface expressions of E-selectin, ICAM-1, and VCAM-1 were quantified using flow cytometry. The graph shows relative Mean Fluorescence Intensity (MFI) normalised to untreated cells and represents the mean ± SE of three experiments. $^{**}p < 0.01$. Activation of HAEC did not increase the interaction with LO-EPC (b). 4×10^5 LO-EPC were perfused to 3×10^4 HAEC for 4 min and the data represented as mean ± SE of three experiments.

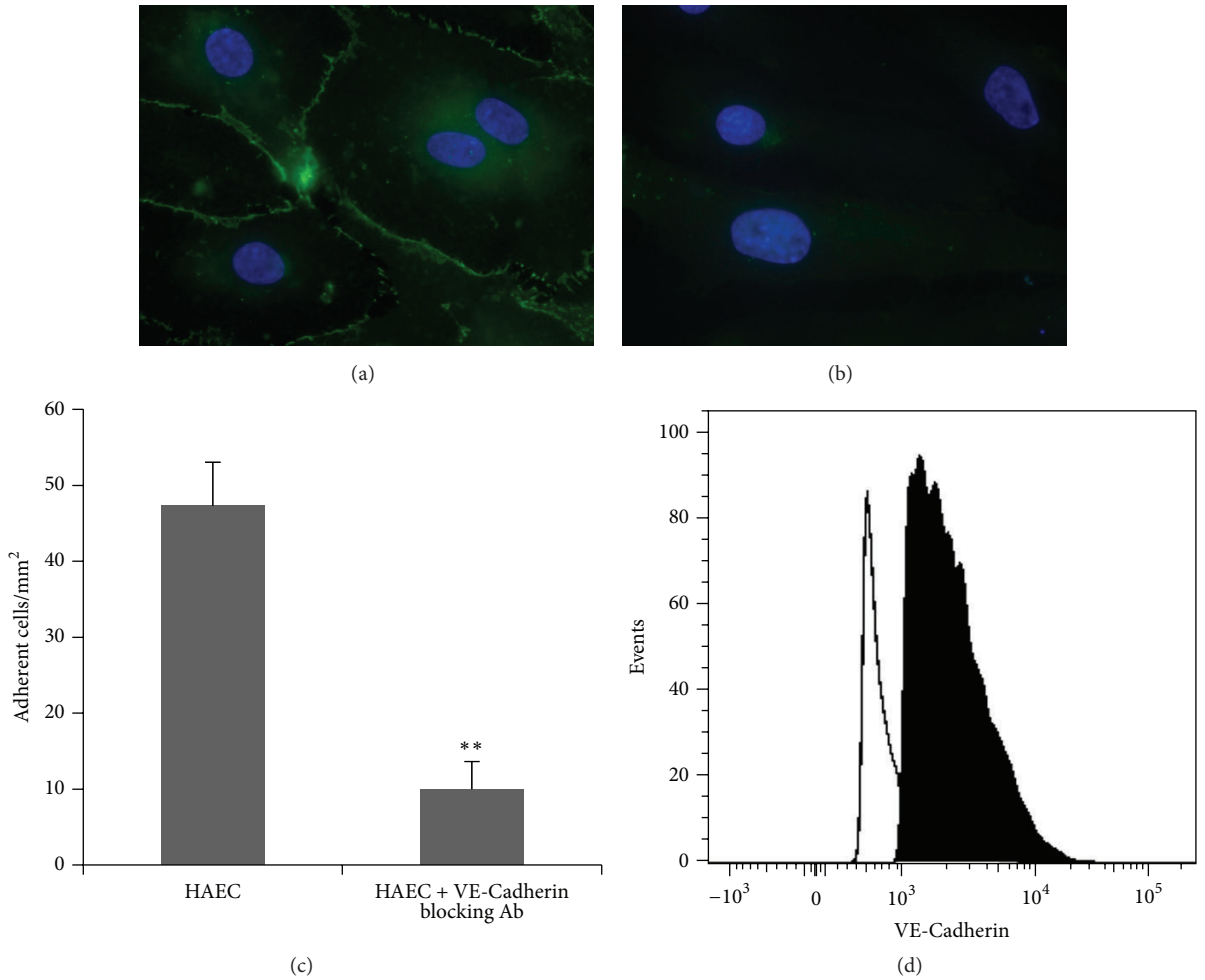

Figure 3: Representative microscopic images of VE-Cadherin expression in control HAEC (a) and HAEC incubated with anti-VE-Cadherin antibody for 1 hour at 37°C (b). Scale bar 20 μm. Effect of VE-Cadherin on dynamic interaction of LO-EPC with HAEC. 4×10^5 LO-EPC were perfused (c). The data was represented as mean ± SE of three experiments. $^{**}P < 0.01$. Representative flow cytometric histograms illustrating VE-Cadherin expression in LO-EPC and HAEC (d). The lined histogram represents VE-Cadherin expression in HAEC and the filled histogram represents VE-Cadherin expression in LO-EPC.

changing the rolling velocity (observation only), confirming the role of VE-Cadherin in LO-EPC adhesion to HAEC under 0.7 dyn/cm^2 shear stress.

3.4. Decreased Interaction of LO-EPC with HAEC after Ischaemia Reperfusion Injury under Flow. HAEC treated with simulated ischaemia reperfusion *in vitro* under flow showed decreased interaction with LO-EPC compared to normal HAEC (Figure 4(a)). Four hours of ischaemia followed by reperfusion caused HAEC retraction and detachment, also demonstrated by significantly more floating cells in the supernatant compared to untreated control HAEC. There was no significant difference in VE-Cadherin expression between control HAEC and HAEC with ischaemia reperfusion injury (Figures 4(b)–4(d)). This suggests that the decreased interaction between LO-EPC and HAEC after ischaemia reperfusion injury may be due to cell retraction and increased intercellular space between HAEC rather than being VE-Cadherin related.

3.5. Interaction of LO-EPC with Extracellular Matrix Proteins. When injured endothelial cells retract and/or detach, interstitial basal membrane is exposed. Endothelial basal membranes are comprised of several extracellular matrix (ECM) proteins, including collagen IV, collagen I, fibronectin, vitronectin, and laminin. We compared the adhesion of LO-EPC to different ECM. There was no significant difference in LO-EPC binding to collagen IV, collagen I, fibronectin, and vitronectin under static conditions (Figure 5(a)). Under 0.7 dyn/cm^2 shear stress, however, LO-EPC bind to different ECM with different strengths, with a higher adhesive strength for fibronectin and vitronectin (Figure 5(b)). There were no signs of toxicity of these substrates on LO-EPC. In addition LO-EPC adhere more avidly to fibronectin and vitronectin than they do to HAEC (Figure 4(a)).

Differences in rolling velocity were observed when examining the motion of LO-EPC interaction with ECM under the microscope. The rolling of LO-EPC on fibronectin and vitronectin coated surfaces was slower (data not shown),

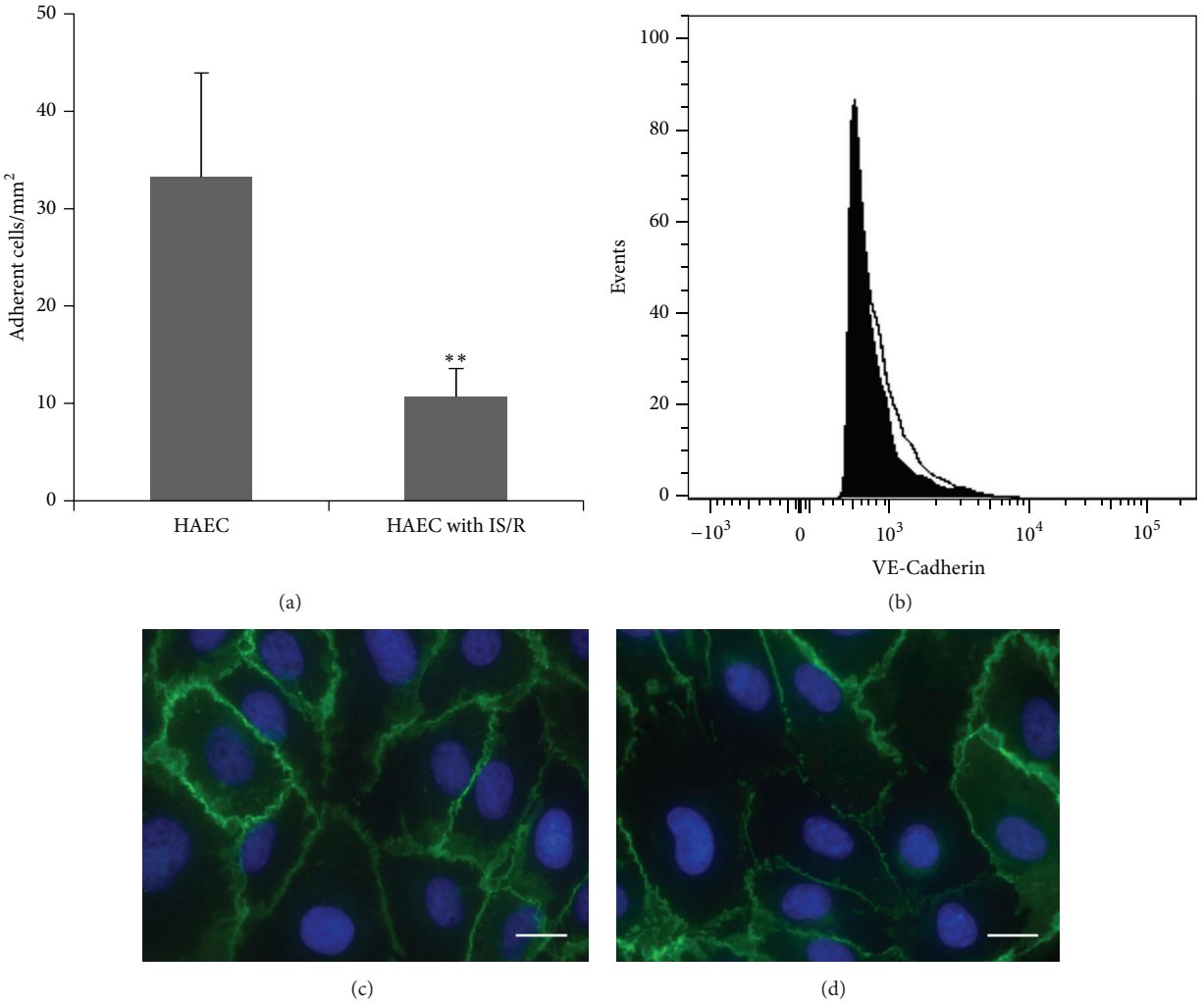

Figure 4: Dynamic interaction of LO-EPC with control HAEC and HAEC with ischaemia reperfusion injury (a). The data represented as mean ± SE of three experiments. $^{**}p < 0.01$. Representative flow cytometric histograms of VE-Cadherin expression in control HAEC and HAEC with ischaemia reperfusion injury (b). The filled histogram represents VE-Cadherin expression in control HAEC and the unfilled histogram represents VE-Cadherin in HAEC with ischaemia reperfusion injury. Representative microscopic images of VE-Cadherin expression in control HAEC (c) and HAEC with ischaemia reperfusion injury (d). Scale bar 20 μm.

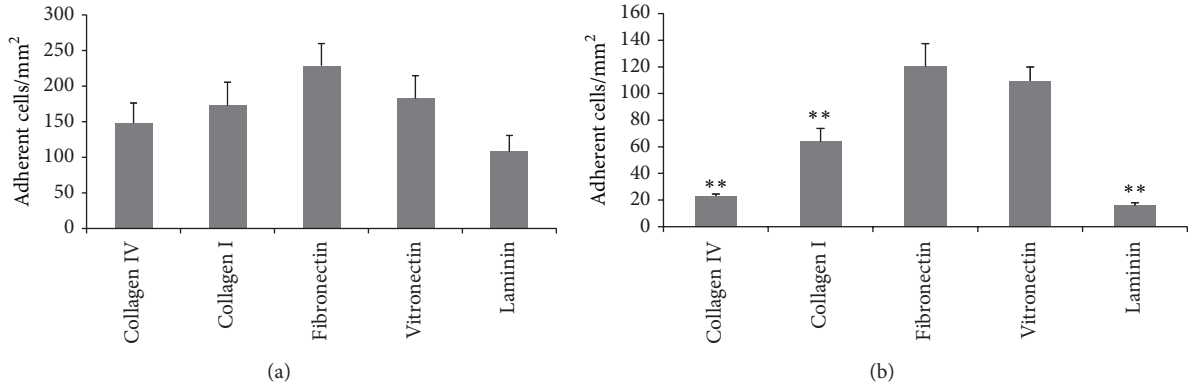

Figure 5: LO-EPC adhesion to various extracellular matrix proteins under static and flow conditions. 2×10^4 of LO-EPC were plated to Ibidi μ-Slide VI 0.4 Luer slides for 45 min to study the static adhesion of LO-EPC to various ECM (a). Dynamic adhesion of LO-EPC to various extracellular matrix proteins under 0.7 dyn/cm^2 shear stress (b). 4×10^5 EPC were perfused into Ibidi μ-Slide VI 0.4 Luer slides coated with 100 μg/mL collagen IV, collagen I, fibronectin, vitronectin, and laminin, respectively. The data was represented as mean ± SE of three experiments. $^{**}p < 0.01$ compared to fibronectin.

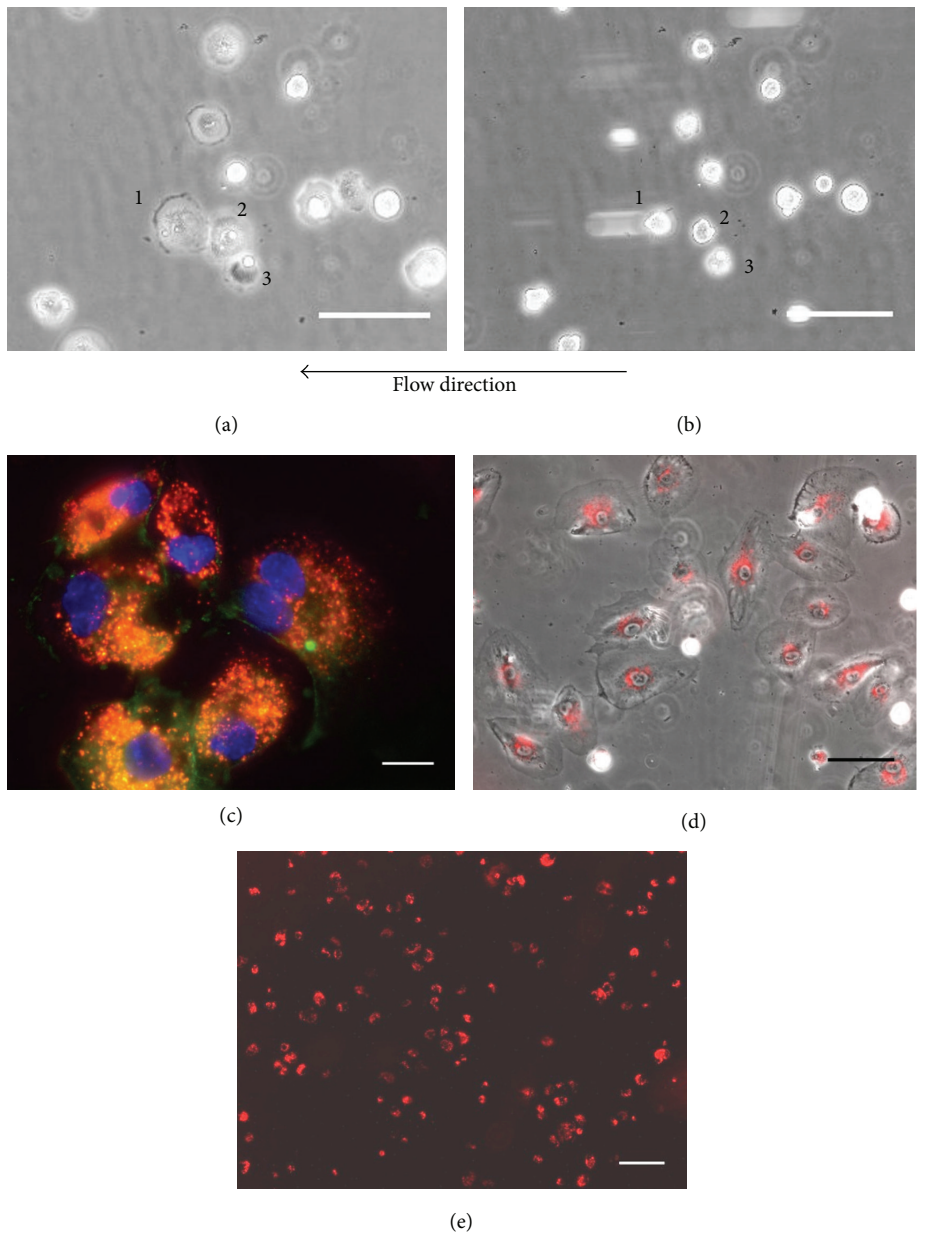

FIGURE 6: LO-EPC (4×10^5) were perfused over fibronectin-coated Ibidi slides and time lapse imaging was used to visualise and record LO-EPC adhesion. Representative still images show three adherent cells after initial capture (a, b) after 4 min of perfusion (0.7 dyn/cm^2). Scale bar represents 30 μm. Adherent LO-EPC form lateral adhesion junction (c). LO-EPC labelled with DiI-Ac-LDL shown red and VE-Cadherin expression shown green. Scale bar 20 μm. 24 hours after adhesion to fibronectin, LO-EPC spread and proliferate (d). Scale bar represents 60 μm. The adherent LO-EPC effectively cover a fibronectin-coated surface (e). Scale bar represents 110 μm.

suggesting that fibronectin and vitronectin influenced both rolling and adhesion phases of interaction with LO-EPC.

Figure 6(a) shows that, under a shear stress of 0.7 dyn/cm^2, LO-EPC readily attach and spread on a fibronectin coated surface. Rolling LO-EPC appeared as round cells initially, but rapidly spread, formed cell-cell connections upon firm adhesion, and withstood a total of 10 min perfusion under flow (Figures 6(a) and 6(b)). LO-EPC displayed coordinated adhesion behaviour under flow with sequential events of rolling along the surface for short distances and episodes of transient tethering prior to firm adherence. Immunofluorescence staining of VE-Cadherin in adherent LO-EPC confirmed the formation of lateral junctions (Figure 6(c)). 24 hours after adhesion, LO-EPC had proliferated (Figure 6(d)) and a large surface area was covered by adherent LO-EPC (Figure 6(e)).

3.6. Exposed Fibronectin Enhanced the Cell-Cell Interaction between LO-EPC and HAEC. Interaction of LO-EPC with HAEC that had undergone ischaemia reperfusion was increased when HAEC had been seeded on to fibronectin (Figures 7(a) and 4(a)). There was no significant difference

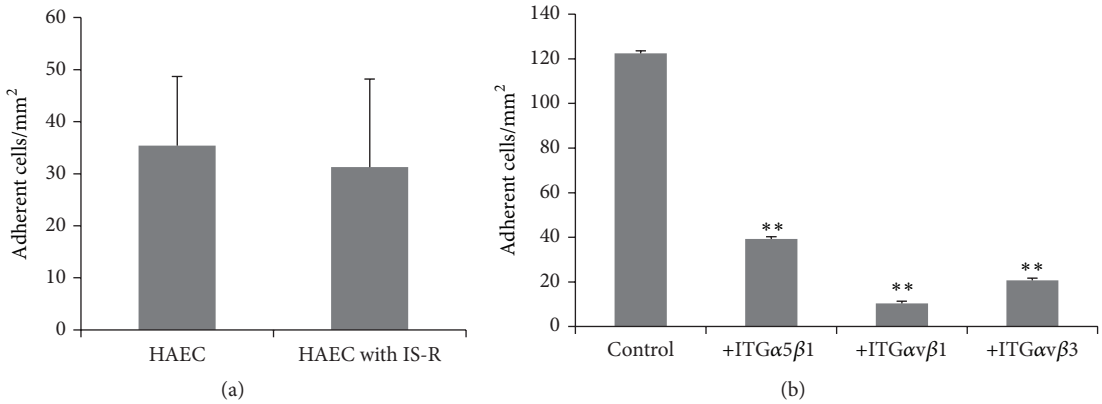

Figure 7: The influence of fibronectin on the dynamic interaction of LO-EPC with control HAEC and HAEC with ischaemia reperfusion injury under 0.7 dyn/cm^2 shear stress. 3×10^4 HAEC were plated into Ibidi μ-Slide VI 0.4 Luer slides which had been precoated with 100 μg/mL fibronectin. The data represent a mean ± SE of three experiments. LO-EPC adhesion to fibronectin was integrin dependent (b). LO-EPC binding to fibronectin was blocked by antibodies against integrin $\alpha 5\beta 1$, integrin $\alpha v\beta 1$, and integrin $\alpha v\beta 3$. The data represent the mean ± SE of three experiments. $^{**}p < 0.01$ compared to the control (no blocking antibodies).

between the dynamic interactions of LO-EPC with control HAEC and HAEC with ischaemia reperfusion injury (Figure 7(a)), suggesting fibronectin could promote the cell-cell interaction of LO-EPC with a retracted or discontinuous HAEC in order to aid in the reformation of the endothelial cell monolayer.

3.7. Integrins Mediate Adhesion of LO-EPC on Extracellular Matrix Proteins under Flow. We have shown previously that there is differential integrin gene expression in LO-EPC, with higher expression of integrin monomers αv, $\alpha 5$, $\beta 1$, and $\beta 3$ and higher cell surface expression of integrin heterodimers $\alpha 5\beta 3$, $\alpha v\beta 1$, and $\alpha v\beta 3$ [16]. Using blocking antibodies against integrins $\alpha 5\beta 3$, $\alpha v\beta 1$, and $\alpha v\beta 3$, the interaction of LO-EPC with fibronectin was significantly decreased. The data suggested that the interaction between LO-EPC and ECM was mediated largely by these three integrins $\alpha 5\beta 3$, $\alpha v\beta 1$, and $\alpha v\beta 3$ (Figure 7(b)).

4. Discussion

Adhesion of LO-EPC to injury sites involves both cell-cell and cell-matrix interactions. Enabling direct interaction between endothelial cells and ECM is likely critical for LO-EPC homing and performing vascular repair at the injury site. The most extensively studied cell-cell interaction under dynamic flow is the interaction of leukocytes with endothelial cells [27, 30, 31]. Studies on the dynamic interaction of early outgrowth EPC with endothelial cells showed a strong resemblance to that of leukocyte interactions with activated endothelial cells; they share some common features of a coordinated sequence of multistep adhesive events including an initial phase of rolling and final firm adhesion [32, 33]. The initial phase of leukocyte rolling *in vivo* is mediated by P-selectin and firm adhesion is mediated by E-selectin, ICAM-1, and VCAM-1 [32, 33]. Until now there has been no information on the dynamic interaction of LO-EPC with endothelial cells. Using an *in vitro* flow system to simulate physical conditions of blood circulation *in vivo*, we showed that LO-EPC did not interact with confluent EC under flow but readily adhered and spread where there were discontinuities in the EC monolayer. The interaction occurred paracellularly at gaps in the intercellular junctions between EC and was not critically adhesion molecule-dependent since upregulating the cell surface adhesion molecules E-selectin, ICAM-1, and VCAM-1 in HAEC did not alter the interaction of LO-EPC with EC under flow. The adhesion mechanism is distinct and in contrast to the interaction of early outgrowth EPC [33], monocytes [31], and mesenchymal stem cells (MSC) [34] in which the interactions with endothelial cell were all adhesion molecule-dependent. One possible explanation for the apparent different adhesion mechanism used by LO-EPC may be the integrin expression profile of LO-EPC. LO-EPC show low expression of integrins $\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha X\beta 2$, and $\alpha D\beta 2$ [16], which are responsible for binding to vascular ligands such as ICAM-1, ICAM-2, and VCAM-1 [35, 36]. LO-EPC also have lower expression of integrin $\alpha 4\beta 1$, whereas MSC engage VLA-4 (integrin $\alpha 4\beta 1$)/VCAM-1 to mediate firm adhesion on EC [34]. Different cells may use different adhesion mechanisms depending on the respective adherent properties as demonstrated previously in the interaction of tumour cells and endothelial cells. Cells from different tumour types interact with the endothelial surface using different mechanisms depending on adhesion molecules expressed on the tumour and endothelial cell surface [37].

The interaction of LO-EPC with HAEC occurred paracellularly suggesting that cell-cell contact on lateral surfaces may play a role in initiating this interaction. Vascular endothelial-(VE-) Cadherin is a strictly endothelial specific adhesion junction protein, prominently localised at endothelial cell lateral borders and mediates homotypic cell-cell adhesion [38–40]. "Homophilic interactions" between LO-EPC and EC suggest that VE-Cadherin may mediate initial adhesion of LO-EPC to endothelial cells. VE-Cadherin, CD31, and CD146 are typically associated with a more mature endothelial phenotype; however we showed that LO-EPC had a higher

level of VE-Cadherin compared to mature endothelial cells (HAEC), which may be significant in ensuring that LO-EPC resist tractive flow forces. The adhesion junction formation observed in the adherent LO-EPC with HAEC confirmed that the adhesion of LO-EPC with HAEC was at least partly mediated by VE-Cadherin. Interactions between VE-Cadherin activate the cellular cascade signalling pathways further strengthening the cadherin interaction [41]. Indeed, blocking VE-Cadherin in the endothelial cells reduced their interaction with LO-EPC interaction under dynamic flow. In addition, VE-Cadherin regulates various cellular processes such as cell proliferation and modulates vascular endothelial growth factor receptor functions as well as being involved in VE-Cadherin-mediated contact inhibition of cell growth [42, 43]; therefore LO-EPC would fulfil nearly every function required by reparative cells. A similar interaction pattern was also observed previously in the interaction of blood-borne tumour cells with endothelial cells. The preferred tumour cells interactions prior to tumour cell extravasation occur at sites near to endothelial intercellular junctions [44]. Although VE-Cadherin is a strictly EC specific adhesion molecule it is also expressed by aggressive melanoma tumours [45]. Development of long-term firm adhesions depends on the collaborative interactions of several adhesion proteins including tight junction protein and PECAM. Ayalon et al. showed that there were spatial and temporal relationships between VE-Cadherin and PECAM-1 in regulating endothelial cell-cell interaction [46]. Cadherins became organized on the cell surface much earlier than PECAM-1 and served as the nucleation sites for subsequent and adjacent assembly of PECAM-1 adhesions [46]. The reciprocal role of these junctional proteins in regulating stable junction organization and biological activity in the adherent LO-EPC remains to be clarified.

When endothelial cells are subjected to ischaemia reperfusion injury, the dynamic interaction of LO-EPC with EC was decreased. Koto et al. reported that hypoxia could disrupt the barrier function of neural blood vessels through changes in the expression of adhesion junction protein claudin-5 in endothelial cells [47]. However, our data showed that ischaemia reperfusion injury did not significantly influence VE-Cadherin expression in EC, suggesting that decreased interaction was unlikely to be due to disrupted VE-Cadherin function. This was in agreement with Chen et al. who showed that 4 hours of ischaemia did not cause significant changes in mRNA expression of VE-cadherin and claudin-5 in endothelial cells in the lung [48]. The decreased interaction we observed was likely due to an increase in the size and number of intercellular spaces caused by cellular retraction under these conditions. This is consistent with our observation that increasing the cell spacing of a monolayer also decreased LO-EPC interaction.

The extracellular matrix (ECM) beneath the endothelium is a highly organized complex network of collagens, fibronectin, vitronectin, laminin as well as proteoglycans, glycoproteins, and bound growth factors. They form a thin sheet-like matrix to create varying degrees of tissue tensile strength to preserve the function and integrity of blood vessels [22, 49–51]. When endothelial cells are damaged, ECM components will be exposed on the luminal surface. So far there has been little study of adhesion of LO-EPC to ECM under dynamic flow. Angelos et al. showed that LO-EPC could interact with fibronectin and the number of LO-EPC adhering to a fibronectin coated surface was influenced by the perfused cell density and shear stress [52]. In this study, we compared LO-EPC adhesion strength to different ECM and demonstrated that LO-EPC are highly adhesive to fibronectin and vitronectin but less so on collagen IV, collagen I, and laminin under a shear stress of 0.7 dyn/cm^2. Different ECM interact with cells via different cell surface integrin receptors [53, 54]. We showed that the interaction of LO-EPC with fibronectin was strongly dependent on integrins $\alpha5\beta1$, $\alpha V\beta1$, and $\alpha V\beta3$. The involvement of integrin $\alpha5\beta1$ in LO-EPC with matrix protein is in agreement with other published works [22, 52] in which increased adhesion of LO-EPC to fibronectin was generated by LO-EPC producing multiple contacts of $\alpha5\beta1$ with a fibronectin-coated surface and the contact area growing during the first 20 minutes of attachment [52]. Previously we showed that there was higher gene expression of integrin subunits $\alpha5$, αv, $\beta1$, and $\beta3$, moderate expression of $\alpha6$ and αE, and low level expression of other integrin subunits, and that integrins $\alpha5\beta1$, $\alpha V\beta3$, and $\alpha V\beta3$ have higher cell surface expression in LO-EPC [16], the receptors for fibronectin (integrins $\alpha5\beta1$, $\alpha V\beta1$, and $\alpha V\beta3$) [55] and vitronectin (integrin $\alpha V\beta3$) [56]. The receptor for collagen IV and collagen I (integrins $\alpha1\beta1$ and $\alpha2\beta1$) [57, 58] and laminin (integrins $\alpha3\beta1$, $\alpha6\beta1$, $\alpha7\beta1$, and $\alpha6\beta4$) [58] were expressed at lower levels in LO-EPC. The constitution of endothelial basal membranes varies between different vascular beds and fibronectin and vitronectin are not normally involved in maintaining tissue structure and are found at lower levels in quiescent vessels [59, 60]. Both fibronectin and vitronectin are present in the bloodstream [61] with serum concentrations of 300 μg/mL and 200–300 μg/mL, respectively [62, 63]. When an endothelial cell monolayer is damaged, the cells leak into the injured area and are rapidly deposited in injured tissue, becoming a prominent constituent of the endothelial basement membrane, and provide an adhesive scaffold for the recruitment of cells [49, 62, 63]. The higher local concentrations of fibronectin and vitronectin after injury and the higher binding strength with LO-EPC make the injury site a strong target for capturing LO-EPC homing to an injury site.

Although in this study we examined the interaction of LO-EPC with HAEC and ECM separately, these two processes are closely linked and occur concomitantly, especially in the initial phase of vascular injury. Endothelial cells and the supporting matrix exist in a state of "dynamic reciprocity" to serve and regulate each other. ECM not only provides a substrate for cell attachment and spreading, contact guidance for cell migration, and a scaffold for building tissues but also serves as a reservoir for growth factors [64]. EC are primarily responsible for the synthesis and deposition of these ECM [64]. We found that fibronectin and vitronectin provided superior adhesion for LO-EPC compared to HAEC. Adhesion to ECM helped LO-EPC to establish junctional adhesion with HAEC as shown by EC with ischaemia reperfusion

injury causing decreased dynamic interaction with LO-EPC which was restored when EC were seeded on fibronectin.

It was reported that mature endothelial cells increase deposition of collagen IV, fibronectin, and laminin under hypoxic condition which may contribute to the complex interplay between endothelial cells and ECM [50]. EPC deposited collagen IV, fibronectin, and laminin to a greater extent than mature EC [65]. Therefore, using autologous LO-EPC therapeutically could amplify these benefits and enhance endogenous repair.

Limitations of This Study. (1) In this study 0.7 dyn/cm^2 shear stress was used to investigate the dynamic interaction of LO-EPC with endothelial cells and ECM. Angelos et al. showed that the number of adherent LO-EPC/cm^2 exhibited a biphasic response with the optimal shear stress for late outgrowth EPC binding to fibronectin at 1 dyn/cm^2 [52], a biphasic response similar to both neutrophils and monocytes binding to the endothelium under flow [66, 67]. The adhesive strength under flow not only depends on adhesive signals, but also depends on shear stress. Higher shear stress could interfere with the binding strength by increased rolling velocity or might help with binding if modelling of microvillus deformation is accurate [68]. Future studies will investigate the influence of different shear stresses on interaction of LO-EPC with endothelial cells and ECM.

(2) The model used in this study was a simplified one. *In vivo*, exposed endothelial basal membrane is not only a target for LO-EPC; but it will also attract platelets and other immune cells. Platelets aggregate immediately after endothelial denudation and adhere to ECM by platelet-specific integrin αIIbβ3 [60]. Activated platelets play a role not only in thrombosis but also in inflammation, immune responses, and atherosclerotic disease [69]. Recently it was reported that activated platelets could also support adhesion and migration of circulating progenitor cells [70]. Platelet-coated ECM may represent an attractive adhesive surface promoting arrest of circulating CD34+ progenitor cells *in vitro* as well as *in vivo* [71]. Whether platelets and other inflammatory cytokines encourage or prevent LO-EPC interaction with endothelial cells and ECM under flow perfusion will be investigated in future experiments.

5. Conclusion

In conclusion, we have demonstrated that discontinuous endothelial monolayer and exposed ECM were sufficient adhesive signals to capture LO-EPC from flow perfusion *in vitro* and that LO-EPC demonstrate appropriate properties to effect vascular repair. Further studies are needed to examine whether these adhesive signals are effective under different shear stresses and strong enough to capture LO-EPC from blood circulation *in vivo*.

Competing Interests

The authors indicate no potential conflict of interests.

Acknowledgments

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Review Article

Mesenchymal Stem Cells after Polytrauma: Actor and Target

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Mesenchymal stem cells (MSCs) are multipotent cells that are considered indispensable in regeneration processes after tissue trauma. MSCs are recruited to damaged areas via several chemoattractant pathways where they function as "actors" in the healing process by the secretion of manifold pro- and anti-inflammatory, antimicrobial, pro- and anticoagulatory, and trophic/angiogenic factors, but also by proliferation and differentiation into the required cells. On the other hand, MSCs represent "targets" during the pathophysiological conditions after severe trauma, when excessively generated inflammatory mediators, complement activation factors, and damage- and pathogen-associated molecular patterns challenge MSCs and alter their functionality. This in turn leads to complement opsonization, lysis, clearance by macrophages, and reduced migratory and regenerative abilities which culminate in impaired tissue repair. We summarize relevant cellular and signaling mechanisms and provide an up-to-date overview about promising future therapeutic MSC strategies in the context of severe tissue trauma.

1. Mesenchymal Stem Cells: A Multifaceted Adult Stem Cell Population

Mesenchymal stem cells, also referred to as multipotent mesenchymal stromal cells (MSCs), have first been isolated from bone marrow and characterized as a nonhematopoietic stem cell population with multilineage mesenchymal differentiation potential [1, 2]. Subsequently, cells with a MSC-like phenotype have been described in various neonatal (e.g., umbilical cord, placenta, and cord blood) and adult tissues (e.g., adipose tissue, synovial membrane, cartilage, bone, skin, muscle, liver, and lung) [3–6]. Functional characteristics of those cell populations seem to depend to a certain extent on the tissue source [5]. Moreover, MSCs have been attributed to a mixed developmental origin [6]. Since MSCs have received rapidly growing interest as a therapeutic tool or target in regenerative medicine the International Society for Cellular Therapy proposed the following minimal criteria for defining MSC: (1) adherence to plastic, (2) expression pattern of several surface markers (positive: CD73, CD90, and CD105; negative: CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLADR surface molecules), and (3) osteogenic, adipogenic, and chondrogenic differentiation potential [7]. These minimal criteria clearly define heterogenous cell populations with widespread distribution in the body [5]. However, they have been used in most studies so far. In a more stringent sense, CD146-positive subendothelial cells from bone marrow have been proposed as clonogenic, self-renewing multipotent skeletal stem cells which also support hematopoiesis [8]. Besides high proliferation capacity [1] and migratory activity in response to chemoattractive factors [9] the differentiation potential into various mesenchymal lineages such as osteoblasts, chondrocytes, adipocytes, tenocytes, and muscle cells [10] or a certain transdifferentiation capacity [11], for example, into neural cell types [12] or hepatocytes [13], attracted much interest in the context of regenerative medicine. The original concept was that MSCs could regenerate tissues by engraftment and differentiation into the respective tissue-specific cell types. Later it was recognized that MSC could additionally support regenerative processes

by secretion of trophic factors and by immunomodulatory activity [14–16]. The relative contribution of these synergistic functionalities is not clearly defined so far and may depend on the origin of the involved MSC population, the respective target tissue, the severity and kind of tissue damage, and the extent of local and systemic inflammatory reaction.

2. Polytrauma: A Multifaceted Challenge

Polytrauma has been defined as two or more injuries (multiple injuries) with at least one injury or the sum of all injuries being life-threatening [17]. The pathophysiological consequences of polytrauma are extremely complex and do not reflect the sum of all separate injuries but rather a unique global amplified challenge of all organs [18]. Even remote tissues which were primarily not injured become affected by the systemic danger response to various pathogen- and danger-associated molecular patterns often resulting in systemic inflammatory response syndrome (SIRS), sepsis, and finally multiple-organ dysfunction syndrome (MODS) endangering life a second time. Thus, polytrauma may in principal transform any organ and single cell into "actors" driving the danger response *after trauma* and thereby adding to tissue damage proposed as "second hit." Subsequently, all cells may theoretically also transmogrify to a "target" of the general danger response, in particular *per* the inflammatory reaction, coagulatory response, complement attack, oxidative burst reaction, bacterial invasion, and so forth [19]. The multifaceted cellular response to polytrauma also includes cells with a physiologically high regenerative potential such as MSCs. After severe trauma MSCs may be challenged by the balancing act between cellular recruitment and immunomodulation to promote healing versus inactivation and death with resulting impairment or absence of sufficient healing. Although clinical data are rare, there is growing experimental evidence that the relative contributions of these MSC functions are critical for understanding the role of MSCs in mediating recovery (or the lack thereof) in the context of polytrauma.

3. Recruitment of MSCs after Polytrauma

MSCs are crucial for the initiation of regenerative processes. Inconsistent numbers of circulating cells have been detected in experimental and clinical trauma settings [20–23], and their homing behaviour to bone marrow or migration to damaged tissue remains elusive. Furthermore, bone marrow-derived MSCs revealed enhanced proliferative capacity which was somehow dependent on the severity of trauma [24]. The trauma-triggered mobilization of MSCs from the bone marrow can be caused by hypoxia [25], various danger-associated molecular patterns (DAMPs, e.g., histones and mitochondrial debris), and chemoattractants (e.g., [26]), all of which are generated after severe injury. When synchronically exposed to key mediators of the trauma response, such as IL-1β, IL-6, IL-8, C3a, and C5a (in concentrations corresponding to those measured in the blood of polytrauma patients), MSCs exhibited an increased chemotactic activity. Particularly the central complement activation product C3a was able to remarkably enhance their migratory activity [27]. Similarly, the anaphylatoxin C5a has been found to be a chemoattractant for MSCs in higher concentrations [28], implying that complement activation at the injury site may result in a strong chemotactic signal for MSC recruitment. However, other established factors also enable MSCs to migrate towards the place of injury: they have been shown to relocate to fracture sites target-specifically in response to soluble mediators including the chemokine stromal cell-derived factor-1 (SDF-1) [29]. Granulocyte colony stimulating factor (G-CSF) represents another potent MSC mobilization factor. In patients with severe trauma, G-CSF has recently been demonstrated to be upregulated more than 50-fold and even higher in case of an additional hemorrhagic shock [30]. In turn, G-CSF may not only mobilize MSCs but also induce a bone regenerative response, for example, by an increased expression of bone morphogenetic protein-2, growth differentiation factor-9, IL-10, IL-8, and nodal growth differentiation factor, as recently shown *in vitro* [31]. During neurotrauma, lysophosphatidic acid (LPA), a bioactive phospholipid, has been demonstrated to play a causative pathophysiological role [32]. Interestingly, LPA is also known to be an effective mobilizer of MSC [33]. Further inflammatory mediators generated after polytrauma [19], such as tumor necrosis factor (TNF), macrophage migration inhibitory factor (MIF), and extracellular HMGB-1 (high mobility group box 1) as a key DAMP, are potent recruiters for MSCs to the site of injury [33].

It is noteworthy, however, that almost all tissues are home to residential MSC-like cells which after infliction of injury may initiate tissue regeneration independently of or even despite additionally recruited MSCs. In this regard, a recent study was unable to detect MSCs in the human blood circulation under conditions such as end-stage renal or liver disease or during heart transplant rejection and thus proposed that bone marrow disruption caused by multiple fractures rather than solid organ injury may be the reason for MSCs to appear in the circulation [21].

It is crucial that MSCs are not only mobilized to injured tissue, but also able to adequately differentiate upon arrival. However, MSC differentiation mechanisms after polytrauma are rarely investigated. We and others have proposed C5a-C5a receptor (C5aR) interactions to be involved in osteogenic differentiation since C5aR was increasingly expressed as human MSCs differentiated to osteoblasts [34, 35]. Furthermore, the altered C5aR expression profile upon differentiation was strongly dependent on the urokinase receptor (uPAR) and NF-κB pathway, indicating that the uPAR-C5aR-NF-κB signaling cascade controls osteogenic differentiation in MSCs [35]. Apart from MSCs, CD34-positive progenitor cells are also considered competent in osteogenic and endothelial differentiation, and their numbers in circulation have also been reported to be increased up to 7 days after severe trauma [36].

4. MSCs as Actors after Trauma

Regardless of their origin, migrated and resident MSCs are thought to sustainably modulate the local and systemic inflammatory response after trauma and to induce and control the regenerative processes in damaged tissue (Figure 1).

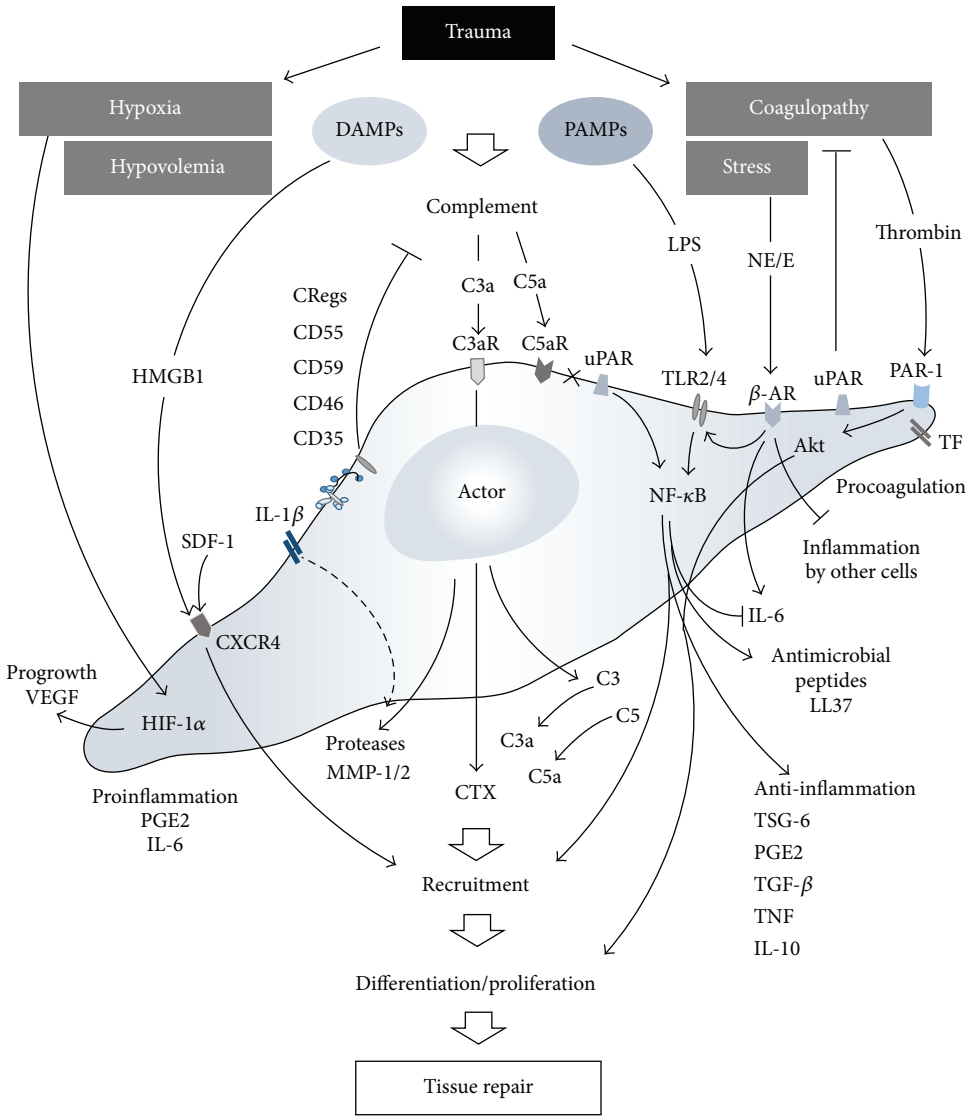

Figure 1: After trauma, MSCs are challenged with local and systemic hypoxia, hypovolemia, disturbances in coagulation, and released danger molecules, inducing them to act as mediators in vast numbers of processes and ideally contributing to successful tissue repair. C3aR: complement C3a receptor; C5aR: complement C5a receptor; CRegs: complement regulatory proteins; CTX: chemotaxis; CXCR4: C-X-C chemokine receptor type 4; DAMPs: damage-associated molecular patterns; HIF-1α: hypoxia inducible factor-1 alpha; HMGB-1: high mobility group box 1; IL: interleukin; LPS: lipopolysaccharides; MMP: matrix metalloproteinase; NE/E: norepinephrine/epinephrine; β-AR: beta-adrenergic receptor; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; PAMPs: pathogen-associated molecular patterns; PAR-1: protease-activated receptor 1; PGE2: prostaglandin E2; SDF-1: stromal cell-derived factor-1; TF: tissue factor; TGF-β: transforming growth factor beta; TLR: toll-like receptor; TNF: tumor necrosis factors; TSG-6: tumor necrosis factor-inducible gene 6 protein; uPAR: urokinase receptor; VEGF: vascular endothelial growth factor.

The main character of MSCs after trauma appears multifaceted and may include growth-enhancing, antiapoptotic, anti-inflammatory, antioxidative, antimicrobial, and other features as recently comprehensively reviewed for single acute organ injuries [37]. However, in the context of combined trauma (e.g., tissue trauma plus hemorrhagic shock) and polytrauma, there is still uncertainty of how MSCs act.

It is established that the MSCs are potent anti-inflammatory actors. In experimental polytrauma, bone marrow MSC application inhibited LPS-associated acute lung injury (ALI) and underlying TLR2/4 upregulation within the lungs and remarkably shifted the proinflammatory cytokines towards an anti-inflammatory cytokine profile [38].

Exposure of MSCs to IL-1β concentrations found in serum early after polytrauma resulted in generation and release of metalloproteinase-1 (MMP-1), tumor necrosis factor-inducible gene 6 (TSG-6), cyclooxygenase-2, and prostaglandin E synthase, all of which act as key immunomodulators of the posttraumatic response [27]. Furthermore, IL-1β-triggered TSG-6 generation by MSCs may switch the

proinflammatory M1 macrophage phenotype towards the rather anti-inflammatory M2 macrophage phenotype and thereby improve wound healing [39].

Polytrauma-induced massive activation and subsequent dysfunction of the coagulation and complement system [40] may also determine MSC behaviour. Thrombin as a central coagulation molecule in the activated clotting cascade after polytrauma results in expansion of MSCs via protease-activated receptor- (PAR-1-) mediated Akt signaling and subsequent robust upregulation of c-MYC [41]. When exposed to the key activation product of the related complement system, C3a, in concentrations measured early after multiple injuries, MSCs significantly upregulated angiogenic factors such as vascular endothelial growth factor (VEGF), CXCL8/IL-8, but also IL-6. In turn, these factors induced *in vitro* minimal tube formation of endothelial cells indicative of angioneogenesis [42].

Bone marrow-derived MSCs also exhibit innate procoagulatory activity most likely based on the expression of tissue factor (TF) on MSCs, resulting in increased clotting, decreased fibrinolysis, and microvascular obstructions [43] which may reflect conditions found in advanced stages of acute trauma-induced coagulopathy. Concerning platelets within the clotting process, platelet-derived growth factors (PDGF) and other platelet-originated products are able to induce MSCs expansion *ex vivo*. In the setting of severe trauma, serum PDGF-AA and PDGF-BB levels were associated with the number of MSCs obtained from the bone marrow of the injured patients [23]. Contrary to other reports, that study failed to show a significant increase in bone marrow homing of MSC, nor could a significant recruitment of MSCs into the peripheral blood be observed after severe injury, irrespective of the trauma severity. Nevertheless, serum from polytrauma patients induced MSC proliferation in a PDGF-associated manner [23].

Concerning complement generation, MSCs do in fact express various complement receptors, such as C3aR and C5aR, [44] by which they are able to sense chemotactically active anaphylatoxins. Furthermore, MSCs are also capable of generating key complement components, such as C3 and C5 [34], and thus after cleavage by various activated coagulation factors may generate the potent anaphylatoxins C3a and C5a, both of which can induce all classical signs of local and systemic inflammation found after severe tissue injury. Indeed, MSCs were found as a complement activator upon exposure to ABO-matched human blood resulting in production of C3a which in turn governs the immunomodulatory features of MSCs and the interactions with other immune cells [45].

As further action mechanisms of MSCs *after injury*, hypoxia during trauma-hemorrhagic shock not only may support preservation of undifferentiated MSCs but also may increase their regenerative potential and moreover may activate hypoxia inducible factor-1 (HIF-1) in MSCs which in turn results in an increased expression of VEGF for neovascularization [25].

Whether all these effects of MSCs are due to the direct cellular actions, the secretion of cytokines, or (in part) microvesicles shed from MSCs is unknown. Extracellular MSC vesicles have been shown to protect against hypoxia-induced acute kidney injury. Interestingly, when the MSC-derived vesicles were generated in a simulated inflammatory micromilieu, the microvesicles containing tetraspanins failed to reverse the kidney injury. In contrast, effective microvesicles originated from otherwise untreated MSCs contained the complement factors C3, C4A, and C5 [46] which may assist in further cell recruitment and induction of regeneration processes [47, 48].

Paracrine and endocrine functions of MSCs have recently been more and more in the focus of research [49]. Besides the inflammation-modulatory functions, MSCs seem also to influence endothelial and epithelial permeability resulting in an enhanced clearance of alveolar fluid [50]. This may be of particular importance for polytrauma-induced blood-organ barrier dysfunction and associated multiple-organ dysfunction syndrome. In this context, in both murine polytrauma model and polytrauma patients, we have recently shown evidence of the tight junction molecule, junctional adhesion molecule-1 (JAM-1), circulating in the blood [51]. In a rodent ischemia-reperfusion (I/R) injury model of the superior mesenteric artery, bone marrow-derived MSCs acted as inhibitors of zonula-occludens-1 (ZO-1) downregulation and tight junction disruption via a TNF-controlled mechanism [52]. These observations support the idea of MSCs improving crucial cellular barrier functions after severe tissue trauma.

5. MSCs as Targets after Trauma

Besides their function as "activators" and "suppressors" of the systemic inflammatory response *after trauma*, MSCs are equipped with a broad arsenal of defense mechanisms against immunological attacks. Thus, they seem to present an important "target" cell for the immune system after multiple injuries (Figure 2).

The procoagulatory surface mainly formed by tissue factor (TF) expression on MSCs [43] renders these cells as potential focal points of fibrin generation and subsequent effective cellular immobilization. This process might also be supported by expression of the plasminogen activator inhibitor 1(PAI-1) on MSCs [53]. As a potential defense mechanism against this fibrin "cladding," fibrinolytic factors (e.g., uPAR) are expressed on MSCs [53] which in concert with various released proteases may dissolute any fibrin thrombi.

The MSCs represent a major target for complement attacks. Abundant deposition of the C3 fragments iC3b and C3dg on MSCs and thus opsonization of the MSCs exposed to ABO-matched allogenic human blood have been found [45]. To counteract a harmful complement attack and opsonization MSCs express a remarkable variety of membrane bound complement regulatory proteins (CRegs), such as protectin (CD59), decay accelerating factor (CD55), and membrane cofactor protein (CD46) [34]. Furthermore, MSCs also release factor H which results in direct inhibition of C3 cleavage and opsonization [54]. However, despite these potent complement inhibitory strategies, contact of MSCs with serum (e.g., provided by massive transfusions after polytrauma) may overwhelm these defense mechanisms and

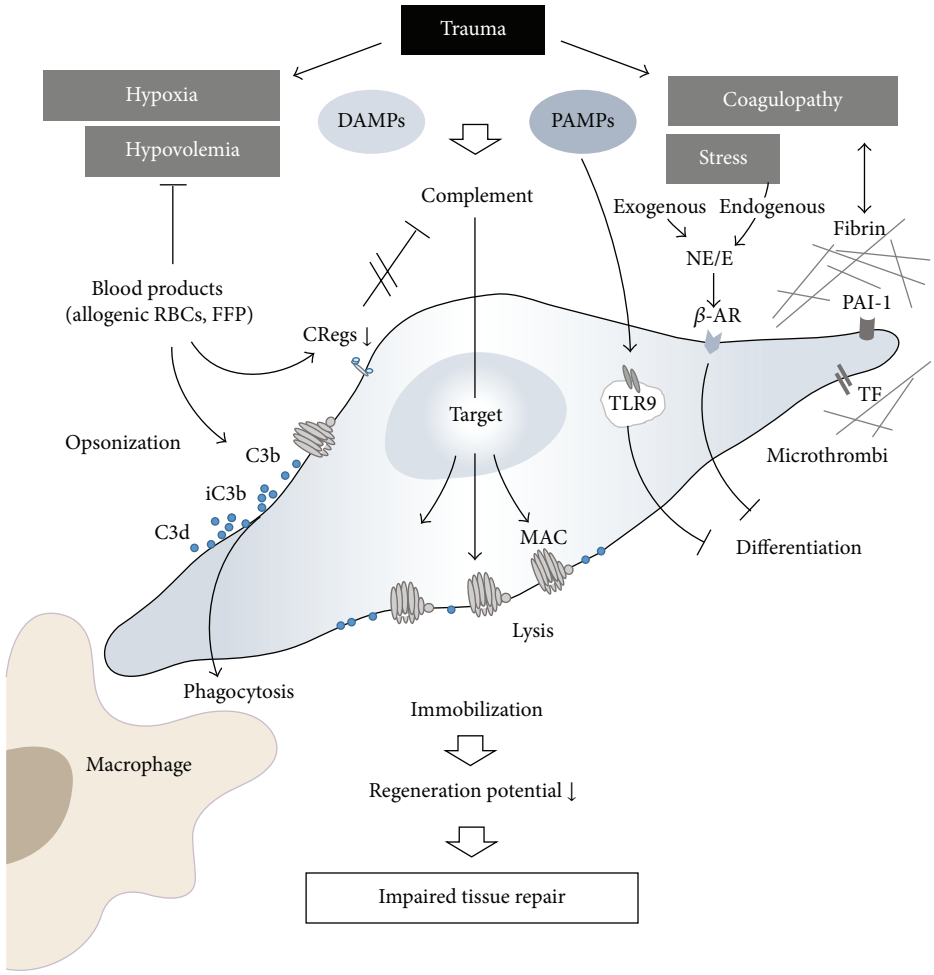

Figure 2: MSCs also function as targets of pathophysiological processes after trauma, leading to complement opsonization and macrophage phagocytosis and reduction in differentiation potential or ability to migrate to the site of injury and finally resulting in the impairment of regenerative potential and tissue repair. See text for detailed information. CRegs: complement regulatory proteins; DAMPs: damage-associated molecular patterns; FFP: fresh frozen plasma; MAC: membrane attack complex; NE/E: norepinephrine/epinephrine; β-AR: beta-adrenergic receptor; PAI-1: plasminogen activator inhibitor 1; PAMPs: pathogen-associated molecular patterns; RBC: red blood cells; TF: tissue factor; TLR9: toll-like receptor 9.

result in serum-induced cytotoxicity [55]. Experimentally, adoptively transferred MSCs in mice deficient in C3 or in mice after C3 depletion (by cobra venom factor) exhibited significantly reduced MSC injury *in vivo* compared to MSCs in wildtype mice [55]. These findings indicate that complement inhibitory strategies in MSCs are crucial for survival and regenerative potential of these cells after trauma. All of the abovementioned CReg proteins on leukocytes are somehow dysregulated early after polytrauma in humans [56]. Possibly, also on MSCs, the CReg shield might be disturbed after multiple injuries and therefore may turn MSCs into targets for a fatal complement attack.

Circulating histones and mitochondria have been identified as DAMPs in patients after severe tissue injury [57–59], inducing a robust inflammatory response. Furthermore, MSC fate determination including differentiation seems to be crucially dependent on histone-modifying enzymes and various transcription factors [60]. Thus, it is tempting to speculate that polytrauma conditions may manipulate histone signatures and thereby disturb regenerative potential of MSCs. However, further research has to elucidate underlying mechanisms.

Exposure to trauma-released mitochondria, mitochondrial DNA, and debris [59] leads to toll-like receptor (TLR) activation in MSCs which in turn may result in an antagonization of MSC differentiation into a specific tissue [61]. Thus, mitochondrial DAMPs may significantly alter MSC proliferation and differentiation and may affect MSC multipotency [61], finally leading to an impaired or altered regeneration after severe tissue trauma.

It is important to consider that, directly after polytrauma, there is a strong stress reaction resulting in an extensive release of endogenous catecholamines, including epinephrine and norepinephrine. Interestingly, activation of the corresponding β-adrenoreceptor on MSCs leads to inhibition of their differentiation potential [62]. To what

extent additionally applied exogenous catecholamines (e.g., norepinephrine), given to stabilize hemodynamic function, will compromise tissue regeneration by suppressing MSC function or differentiation is of great clinical interest and needs to be clarified in future translational studies.

6. Therapeutic Potential of MSCs in Polytrauma

6.1. Current Challenges. A major challenge is the transfer of the numerous *in vitro* findings of multifaceted MSC functions to relevant and reliable preclinical studies and finally the translation to the clinical setting. The optimal MSC source (e.g., bone marrow, adipose tissue, and umbilical cord), the timing after trauma, the administration route, and number of applied cells remain to be defined for the polytrauma situation. In addition, possible immunosuppressive functions of MSC in a polytrauma-induced compromised immunological situation may increase the risk of life-threatening infections. Noteworthily, the acute trauma situation does not allow time and tissue consuming procedures for cell isolation, characterization, and expansion rendering an autologous MSC transplantation strategy questionable. Furthermore, socioeconomic considerations with high logistic demands (inclusive GCP/GLP-conform MSC preparation), high costs, and high variability of the individual injury pattern currently prevent a broad therapeutic platform for MSC in polytrauma patients.

6.2. Progress Made. Nevertheless, various preclinical studies have already addressed the therapeutic potential of MSC in single injury models of different tissues and organs [37, 63]. These experimental approaches include physical trauma of the skin [39, 64], muscle [65], skeletal tissues [66, 67], lung [68, 69], brain [70–72], and spinal cord [73], all of which are frequently affected in polytrauma patients (Annual Report 2013, TraumaRegister DGU®). Moreover, their therapeutic effect in specific pathophysiological situations frequently developing in polytrauma patients, for example, sepsis [74–76], has been studied. In most cases, the therapeutic strategies were based on the concept of MSCs as "actors" delivered by local or systemic cell transplantation. The majority of these studies on monotrauma models indicated therapeutic benefits, although the absolute number of transplanted cells systemically recruited to the site of the injury or surviving in injured regions after local injection was rather low. Therefore, reported therapeutic effects were mainly attributed to the release of trophic factors and immunomodulation [14]. In mice, systemic application of allogeneic MSCs leads to limited local recruitment and stimulation of bone formation assessed by μCT analysis in a fracture model while it had no additive effects on bone formation induced by repetitive mechanical stimulation [67]. This indicates that the trauma situation, most probably the posttraumatic inflammatory reaction, triggers this functionality. Since the respective environment is greatly dependent on the extent and combination of different traumatic injuries, the situation in a polytraumatized patient may be quite different. So far, only few studies addressed this highly relevant clinical situation. Thorax trauma occurs frequently in combination with other injuries and is highly relevant for the polytrauma mortality. Interestingly, chest trauma also influences the course of other injuries like fracture healing in rats [77, 78]. On the other hand, in the same species, the resulting histologic lung alteration is aggravated by parallel hemorrhagic shock or chronic stress. Systemic infusion of allogenic MSC in male rats reduced the lung injury score after lung contusion with hemorrhage or chronic stress [79, 80] and restored the disturbed bone marrow function characterized by reduced clonal growth of bone marrow cells and persistent anemia [79, 81]. In these models, MSC application also increased the relative amount of regulatory T cells [79, 80]. Even in the most compromised situation combining lung contusion, hemorrhagic shock, and chronic stress, the MSC therapy proved to be effective [82]. Since this situation more closely resembles the polytrauma setting in human patients, a therapeutic benefit through future application of MSCs can be expected. In another study where multiple fractures were combined with hemorrhagic shock in rats, systemic MSC application improved weight gain, physical activity, muscle atrophy, and fracture callus histology [83]. In the polytrauma situation, due to vascular damage and hypotension, prolonged ischemia of various organs may be another critical factor. In this context it could be shown that MSC treatment attenuated lung I/R injury in rats [84]. Furthermore, in a mouse model, intravenously applied allogeneic MSCs protected lung transplants from cold I/R injury [85]. In this study, the cell-therapeutic effects were associated with reduced cellular apoptosis, decreased infiltration of macrophages, neutrophils, and CD8+ cells, and lower amounts of TNF, IL-6, and TLR4 but higher expression of TSG-6, in lung tissue [85]. Most of the previously mentioned *in vivo* studies concentrated on major clinically relevant outcome parameters and not on underlying molecular processes. Based on the current knowledge in this field, it could be speculated that a combination of different processes might be involved as illustrated in Figures 1 and 2. Only in some studies on monotrauma models the presence of transplanted cells is documented in the injured tissue. Whether local recruitment and simply survival of transplanted cells are determining factors in regeneration after multiple injuries is not known so far.

6.3. Current Limitations. Numerous clinical trials are currently under way but only a very limited number address acute physical trauma situations [86]. As recently reviewed by Squillaro et al., 493 MSC-based clinical trials are currently listed in the National Institute of Health database, addressing various areas such as graft-versus-host disease, hematological disease, diabetes, organ transplantation, and inflammatory diseases [86]. Only two studies address acute lung injury [37], and, to our knowledge, no study has focussed on the polytraumatized patient. As mentioned above, due to the clinical situation and critical timing including limited time for autologous MSC expansion, polytraumatized patients would require allogenic application of MSCs in future studies. This may theoretically be feasible since allogeneic applications have already been performed in refractory lupus erythematosus patients and in steroid-resistant graft-versus-host disease patients without serious adverse effects [87, 88].

Furthermore, only limited information is available about differential immunosuppressive functionality [19, 89] as well as spatial and temporal regenerative potential of MSCs originated from different tissues. Consequently, great caution is necessary in clinical translation of experimental findings defining MSCs as "actors" and "targets" since MSCs resident in different tissues, MSCs mobilized after trauma, and MSCs after *ex vivo* expansion and transplantation may not function identically and thus may not be interchangeable.

6.4. Future Directions. A promising approach to address the therapeutic potential of MSC would be the injection of factors that systemically mobilize or locally target endogenous stem cells. Such a strategy was reported by Hannoush et al. for acute physical lung injury in male rats [68]. Systemic G-CSF application for 5 days prior to lung contusion leads to an increase of hematopoietic progenitor cell colony growth in the traumatized lung [68]. However, the question of whether MSCs were also systemically mobilized remained open in this study. Nevertheless, the resulting lung injury score was improved by G-CSF pretreatment and by SDF-1 injection into the lung (or by the combination of both) similarly to the effects seen after systemic application of allogeneic MSCs [68]. Strategies addressing the local recruitment of MSCs to date mainly investigate CXCR4 activation by SDF-1 [90]. As a future therapeutic avenue, modulation of the activated complement system may also support endogenous MSC recruitment since the anaphylatoxins C3a and in higher concentrations also C5a stimulate directed MSC migration as mentioned earlier [27, 28, 44, 91]. Noteworthily, in severe trauma situations, catecholamines via induction of genes involved in migration may support mobilization of MSCs [19, 92]. On the other hand, catecholamines were reported to inhibit differentiation into adipogenic, osteogenic, and chondrogenic lineage which may reflect differential activity of MSCs depending on the functional demand [92]. In addition, MSCs are able to inhibit the inflammatory response of other cells such as macrophages [93].

Micro-environment-tailored strategies to improve engraftment at the lesion site may include preconditioning with cytokines or growth factors, platelet-enriched plasma, complement regulators, hypoxia, genetic modifications, or modification of MSC surface structures with antibodies or coating with homing ligands [94–96]. Also, improving the survival of transplanted cells in a compromised milieu, for example, by hypoxic preconditioning in I/R injury in rats [84, 97] may offer the chance to further increase the therapeutic potential and to reduce the rather high numbers of cells that are usually applied. Immunoselection based on expression of specific functional markers reflects a further important strategy to direct cells to the insulted region of interest. This has recently been shown for selected CXCR4-positive MSCs, revealing a significantly improved migratory and healing profile and remarkable synchronic suppression of the systemic inflammatory reaction [29]. Other treatment strategies with the MSC secretome or MSC microvesicles have not yet been tested in the setting of multiple trauma. Nevertheless, they may be promising based on observations on other disease models [98, 99].

7. Conclusion

Numerous *in vitro* and *in vivo* observations clearly indicate that MSCs are central players in the complex network of pathophysiologic events after major trauma. Many questions, however, still remain open in order to therapeutically address MSCs as either "actors" or "targets" in the polytrauma setting. These include the optimal cell source (e.g., bone marrow, adipose tissue, and umbilical cord), the timing and balancing in the posttraumatic scenario of pro- and anti-inflammatory reactions, the application route and dosage of cells, and possible immunosuppressive functions of MSC in a compromised situation carrying the danger of life-threatening infections. Future translational studies are needed to answer these questions and to individually and beneficially utilize the ambivalent and multifaceted behaviour of MSCs.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Cell Fate and Differentiation of Bone Marrow Mesenchymal Stem Cells

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Osteoblasts and bone marrow adipocytes originate from bone marrow mesenchymal stem cells (BMMSCs) and there appears to be a reciprocal relationship between adipogenesis and osteoblastogenesis. Alterations in the balance between adipogenesis and osteoblastogenesis in BMMSCs wherein adipogenesis is increased relative to osteoblastogenesis are associated with decreased bone quality and quantity. Several proteins have been reported to regulate this reciprocal relationship but the exact nature of the signals regulating the balance between osteoblast and adipocyte formation within the bone marrow space remains to be determined. In this review, we focus on the role of Transducin-Like Enhancer of Split 3 (TLE3), which was recently reported to regulate the balance between osteoblast and adipocyte formation from BMMSCs. We also discuss evidence implicating canonical Wnt signalling, which plays important roles in both adipogenesis and osteoblastogenesis, in regulating TLE3 expression. Currently, there is demand for new effective therapies that target the stimulation of osteoblast differentiation to enhance bone formation. We speculate that reducing TLE3 expression or activity in BMMSCs could be a useful approach towards increasing osteoblast numbers and reducing adipogenesis in the bone marrow environment.

1. Introduction

In 2010, more than 10 million Americans over the age of 50 had osteoporosis with another 43 million Americans at risk for the disease [1]. It is estimated that greater than 1.5 million fragility fractures occur each year, with an annual health care cost of at least 14 billion US dollars [2]. By 2025, the health care expenditures for osteoporotic fractures will approach 25.3 billion US dollars [3]. Bone is constantly remodeled through the processes of bone formation by osteoblasts and bone resorption by osteoclasts. Osteoclasts are derived from hematopoietic stem cell precursors of the monocyte/macrophage lineage located in the blood and bone marrow [4]; conversely, osteoblast-lineage cells (osteoblasts and osteocytes) originate from bone marrow mesenchymal stem cells (BMMSCs) [5]. BMMSCs are a multipotent cell type that can give rise not only to osteoblast-lineage cells but also to a range of other cell types, including adipocytes [6] (Figure 1). In some pathological conditions, including senile osteoporosis, the balance between adipocyte and osteoblast differentiation is disrupted in this cell population such that adipocyte differentiation is increased relative to osteoblast differentiation and this is associated with reduced bone mass, increased bone fragility, and increased susceptibility to fracture [7]. Therefore, understanding the molecular mechanism(s) responsible for controlling the balance between osteoblastogenesis and adipogenesis in the adult bone environment is of great significance.

Table 1: The proteins regulate adipogenesis and osteoblastogenesis.

Number	Protein(s)	Function	Assay	Reference(s)
1	Msx2	Adipogenesis↓; osteoblastogenesis↑	*In vitro*	[66]
2	Dlk1/Pref-1	Adipogenesis↓; osteoblastogenesis↑	*In vitro*	[67]
3	TAZ	Adipogenesis↓; osteoblastogenesis↑	Zebrafish; *in vitro*	[68]
4	Wnt10b	Adipogenesis↓; osteoblastogenesis↑	Knockout mice; transgenic mice	[69]
5	LIP	Adipogenesis↓; osteoblastogenesis↑	*In vitro*	[70]
6	Dec1	Adipogenesis↓; osteoblastogenesis↑	*In vitro*	[71]
7	Hemooxygenase-1	Adipogenesis↓; osteoblastogenesis↑	*In vitro*	[72]
8	ID4	Adipogenesis↓; osteoblastogenesis↑	Knockout mice	[73]
9	Maf	Adipogenesis↓; osteoblastogenesis↑	Knockout mice	[74]
10	Pkd1	Adipogenesis↓; osteoblastogenesis↑	Knockout mice	[75]
11	sFRP-1	Adipogenesis↑; osteoblastogenesis↓	*In vitro*	[76]
12	ZFP467	Adipogenesis↑; osteoblastogenesis↓	*In vivo injection*	[77]
13	GIT2	Adipogenesis↓; osteoblastogenesis↑	Knockout mice	[78]
14	Wnt6	Adipogenesis↓; osteoblastogenesis↑	*In vitro*	[79]
15	Wnt10a	Adipogenesis↓; osteoblastogenesis↑	*In vitro*	[79]
16	VEGF	Adipogenesis↓; osteoblastogenesis↑	Knockout mice	[80]
17	Semaphorin 3A	Adipogenesis↓; osteoblastogenesis↑	Knockout mice	[81]
18	TLE3	Adipogenesis↑; osteoblastogenesis↓	*In vitro*	[19]
19	S100a16	Adipogenesis↑; osteoblastogenesis↓	*In vitro*	[82]
20	mTORC2	Adipogenesis↓; osteoblastogenesis↑	*In vitro*	[83]
21	Adiponectin	Adipogenesis↓; osteoblastogenesis↑	Knockout mice	[84]
22	Cysteine dioxygenase type 1	Adipogenesis↑; osteoblastogenesis↓	*In vitro*	[85, 86]
23	MYSM1	Adipogenesis↓; osteoblastogenesis↑	Knockout mice	[87]

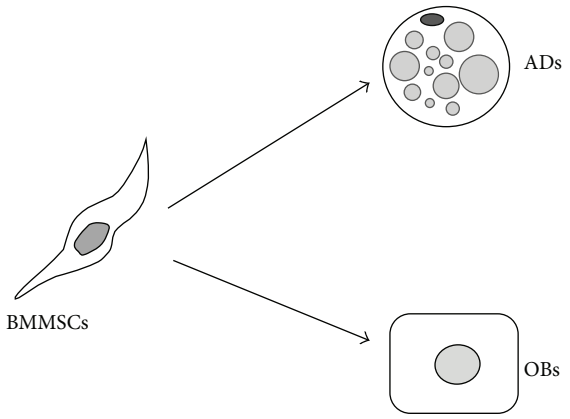

Figure 1: Bone marrow mesenchymal stem cells differentiate into both adipocytes and osteoblasts. Osteoblast and marrow adipocytes are derived from common progenitors, the bone marrow mesenchymal stem cells. BMMSCs: bone marrow mesenchymal stem cells; OBs: osteoblasts; ADs: adipocytes.

In this review, we will summarize the processes of osteoblast and adipocyte differentiation from BMMSCs, focusing on the role of Transducin-Like Enhancer of Split 3 (TLE3), which was recently reported to regulate osteoblastogenesis and adipogenesis. We also discuss the prospect of bone regenerative therapy by using stem cells.

2. Relationship between Adipogenesis and Osteoblastogenesis

Adipogenesis is driven by a complex and well-orchestrated signalling cascade composed of several key transcription factors, most notably proliferator-activated receptor- (PPAR-) γ and several members of the CCAAT/enhancer-binding family of proteins (C/EBPs) [8]. PPAR-γ is commonly referred to as the master regulator of adipogenesis because no factor has yet been identified that can induce normal adipogenesis in its absence [9].

BMP-SMAD signalling plays an important role in osteoblastogenesis by inducing expression of several critical transcription factors such as RUNX2, Osterix, DLX2, and DLX5 [10–12]. RUNX2 is essential for the commitment of mesenchymal stem cells to the osteoblast lineage and homozygous deletion of *Runx2* in mice results in a complete lack of osteoblasts [13, 14]. It appears that adequate RUNX2 is also dosage-dependent since haploinsufficiency of *Runx2* in mice or *RUNX2* in humans causes hypoplastic clavicles and delayed closure of the fontanelles, defects that are characteristic of cleidocranial dysplasia in humans [15, 16]. RUNX2 controls osteoblast-related genes such as *Osterix*, *collagen I*, and *osteocalcin* [17] and autoregulates the *Runx2* gene itself [18].

Several proteins have been reported to regulate both adipogenesis and osteoblastogenesis (Table 1) and, in general, adipogenesis is reciprocally related to osteoblastogenesis in

BMMSCs. However, the exact nature of the signals regulating the balance between osteoblast and adipocyte formation within the bone marrow space remains to be determined. In the sections below, we seek to bring attention to TLE3, which is a relatively understudied regulator of osteoblastogenesis and adipogenesis that is a member of the Groucho/TLE family of transcription factors [19].

3. Groucho/TLE Family Member

Groucho (Gro)/Transducin-Like Enhancer of Split (TLE) family members are transcriptional cofactors in metazoans that play critical roles during development and cell fate determination, including differentiation into fat and bone cells. The names "Gro" and "TLE" are used interchangeably in the literature and in sequence databases [20] and the *Drosophila* genome encodes a single Gro while the mouse and human genomes encode four members of each family [21].

Groucho/TLE proteins consist of a five-domain structure [22]: a highly conserved Q domain, which is a glutamine-rich region predicted to form two coiled-coil motifs that facilitates oligomerization of Gro/TLE molecules *in vitro* [23–25]; a glycine/proline rich (GP) domain, which is essential for interaction of Groucho/TLE proteins with histone deacetylases (HDACs) [23, 24, 26, 27]; a CcN domain, which contains a nuclear localization sequence and putative cdc2 and casein kinase II (protein kinase CK2) phosphorylation sites; a serine/proline rich (SP) domain, which is a region rich in serine/proline residues [22, 28–30]; and a highly conserved WD40 domain, which contains multiple tryptophan and aspartic acid tandem repeats, has been shown by X-ray crystallography to form a β-propeller, and binds many kinds of transcriptional factors [20, 31].

Groucho/TLE proteins do not bind DNA directly but are instead recruited by other transcription factors and are largely considered transcriptional corepressors since they often reduce the activity of a target transcriptional factor. However, the Groucho/TLE family member TLE3 was recently reported to induce the transcriptional activity of PPAR-γ, which is a master transcriptional regulator of adipogenesis [32], suggesting that the Groucho/TLE family may act as corepressors or coactivators in a context-dependent manner.

4. Distribution of TLE3 during Development

During development, TLE3 is expressed in the placenta [33] and homozygous null *Tle3* mutant mice are smaller than their heterozygous and wild type littermates. Most homozygous null *Tle3* mutant embryos demonstrate severe placental defects and die *in utero* [34]. TLE3 is also expressed in the developing nervous system where as the neural tube closes, its distribution shifts from the entire width of the neural plate to the dorsal region and ventricular zone; expression in the roof of the mesencephalon and metencephalon remains most pronounced at this stage. TLE3 is also expressed in the dorsal root ganglia and its expression in the newly formed somites becomes restricted to a dorsal, bracket-shaped group of cells corresponding to the dermamyotome [35].

In older mouse embryos expression of TLE3 in the central nervous system (CNS) is observed along the entire length of the brain and spinal cord in the ventricular zone, with the strongest expression in the layer of cells immediately lining the lumen. In the developing eye, TLE3 is located in the lens and the neural layer of the retina. Somatic expression of TLE3 continues in the dermamyotome and in the condensing sclerotome, forming the vertebrae and bones. Faint staining for TLE3 is also observed in the metanephros (embryonic kidney); tissues derived from the pharynx, including Rathke's pouch and the thymic primordial; the lining of the gut and tissues derived from the gut endoderm such as the epithelial walls of the bronchi of the lungs and the liver; and derivatives of the branchial arches such as the dorsum and intrinsic muscles of the tongue and the dental laminae of the tooth primordial [35].

In later stages of mouse development (16.5 days after conception), TLE3 expression is more restricted than at midgestation. For instance, *Tle3* mRNA is detected in the ventricular zone and the cortical plate of the cerebral cortex; the colliculus; the cerebellum; the olfactory lobe; nasal epithelia; whisker follicles primordia; epithelial cells of the salivary glands; basal layer of skin and hair follicles; and derivatives of the pharyngeal pouches including the lining of the cochlea, eustachian tube, esophagus, larynx, epiglottis, and the thymus [35]. TLE3 is also expressed by cells of the bone marrow [19] and brown and white adipose tissue [32], with the expression level of TLE3 increasing with adipocyte differentiation [19, 32].

5. TLE3 Enhances Adipocyte Differentiation and Suppresses Osteoblastogenesis

Adipocytes are classically classified into two kinds: white adipocytes and brown adipocytes. White adipocytes are optimized to store energy as triglycerides in large, unilocular lipid droplets. When metabolic needs arise, white adipocytes mobilize energy through hydrolysis of triglycerides and release of free fatty acids into the circulation [36]. White adipocytes express a battery of genes involved in lipid handing, triglyceride biosynthesis, triglyceride mobilization, and endocrine signalling [37–39].

Brown adipocytes derive their color from their high mitochondrial content. Unlike white adipocytes, brown adipocytes store energy primarily to provide an intracellular fuel source for thermogenesis [40]. During cold exposure, brown adipose tissue (BAT) executes a transcriptional program that promotes energy expenditure and thermogenesis. Induction of the gene encoding Mitochondrial Uncoupling Protein-1 (UCP1) is critical for brown fat thermogenesis [41, 42]. It has been thought that Ucp1 expression is restricted to BAT; however, recent studies have demonstrated that Ucp-1-positive cells can be detected even in white adipose tissue under certain circumstances. These cells are called "beige adipocytes" [43] and have characteristics of both white and brown adipose cells: during basal state, beige adipocytes

display unilocular morphology similar to white adipocytes, but upon cold stimulation, these cells acquire features of intermediate morphology ultimately resulting in expression of proteins typical for BAT and transformation of stored fat into the small lipid droplets typical for brown adipocytes [44–46].

While the transcriptional determinants of the white and brown adipocyte gene programs are incompletely understood, it is known that PPARγ is the master transcriptional regulator of both white and brown fat differentiation. In support of this, mice deficient in PPARγ lack both types of adipose tissue [9, 47–49]. Villanueva et al. [32] identified TLE3 as a cofactor for PPARγ and it was later confirmed that TLE3 enhances transcriptional activity of PPARγ, thereby inducing adipocyte differentiation of BMMSCs [19, 21]. Additionally, TLE3 disrupts the physical interaction between transcriptional cofactor PRDM16, which was identified as a key factor driving brown adipocyte linage development [43, 50], and PPARγ, thereby suppressing brown-fat-specific genes and inducing white-fat-specific genes; the net result of these effects is impaired fatty acid oxidation and thermogenesis [51]. We predict that TLE3 has some influence on beige adipocyte formation, but further studies are needed to examine this possibility.

Described above, osteoblast-lineage cells and marrow adipocytes are derived from a common progenitor, the BMMSCs. RUNX2 controls osteoblast-related genes and is essential for commitment to the osteoblast lineage [13, 14, 52]. RUNX2 interacts with Groucho/TLE family members, which act as corepressors of RUNX2 activity [53, 54]. For instance, TLE1 and TLE2 repress RUNX2-dependent activation of *osteocalcin* gene transcription [55]. And TLE3 suppresses BMP2-induced osteoblast differentiation of BMMSCs via recruiting HDAC and repressing RUNX2 transcriptional activity [19].

6. Expression of TLE3 Is Regulated by Canonical Wnt Signalling

The Wnt family of nineteen secreted glycoproteins has a critical role in regulating embryonic development, cell differentiation, and cell fate determination [56]. Wnts transduce two types of intracellular signalling referred to as canonical and noncanonical pathways. Canonical Wnt signalling, that is, signalling mediated by the effector β-catenin, has a key role in adult skeletal homeostasis and bone remodeling [57] by promoting differentiation and maturation of osteoblasts and, thereby, increasing bone formation [58]. In contrast, canonical Wnt signalling suppresses adipocyte differentiation [59].

Groucho/TLE family members, including TLE3, act as transcriptional corepressors of canonical Wnt signalling via binding to the downstream effectors TCF/LEF and inhibiting Wnt target gene transcription [20, 60–62]. According to Daniels and Weis [62], β-catenin that enters the nucleus upon activation of the Wnt pathway directly competes with Groucho/TLE proteins for TCF/LEF binding to accomplish gene regulation.

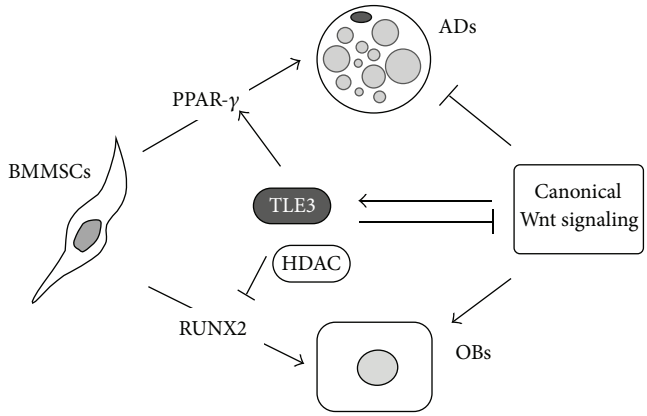

Figure 2: Model for the role of TLE3 in the bone marrow microenvironment. TLE3 directly induces adipogenesis and suppresses osteoblastogenesis of BMMSCs by acting on PPAR-γ and RUNX2, respectively. TLE3 also indirectly induces adipogenesis and suppresses osteoblastogenesis by repressing canonical Wnt signalling, which is capable of inducing osteoblastogenesis and inhibiting adipogenesis. In addition, canonical Wnt signalling induces TLE3 expression, suggesting that the induction of TLE3 by Wnt signalling may be part of a negative feedback loop during osteoblastogenesis and/or a positive feedback loop during adipogenesis in the adult bone marrow microenvironment. BMMSCs: bone marrow mesenchymal stem cells; OBs: osteoblasts; ADs: adipocytes.

Recently, Wnt responsive elements in the TLE3 promoter region were identified through comparative genomic analysis and functional analyses confirmed that expression of TLE3 is increased by Wnt signalling [21]. Given the opposing roles of TLE3 and Wnt signalling in BMMSCs differentiation, this finding suggests that induction of TLE3 by Wnt signalling is part of a negative feedback loop active during osteoblast differentiation and/or a part of a positive feedback loop during adipogenesis, suggesting that TLE3 regulates the cell fate of BMMSCs between osteoblasts and adipocytes (Figure 2).

7. Prospects for Therapy

Osteoporosis, which is one of the most abundant bone-related diseases, is characterized by low bone mass and microarchitectural deterioration of bone tissue that results in increased bone fragility and susceptibility to fracture [7]. The most commonly prescribed therapeutics are antiresorptives, such as calcitonin, estrogen, and bisphosphonates, that block osteoclast activity as a means to stabilize bone architecture. While efficacious in halting further bone loss, little or no new bone mass is added to the skeleton while on antiresorptive therapy. Recent data on the importance of continuous bone remodeling suggest that overuse of antiresorptives could lead to BRONJ (bisphosphonate-related osteonecrosis of the jaw) [63] and fracture in some patients [64]. Thus, development of new, effective therapies that target enhancing bone formation by stimulating osteoblast differentiation is required.

8. Conclusion

In this review we summarized the cell fate determination and the differentiation of BMMSCs and especially focus on the role of TLE3, which represses osteoblast differentiation and enhances adipocyte formation from BMMSCs. Therefore, we speculate that reducing TLE3 expression or activity in BMMSCs could be a useful approach towards increasing osteoblast numbers and reducing adipogenesis in the bone marrow environment. Recently, a delivery system involving dioleoyl trimethylammonium propane- (DOTAP-) based cationic liposomes attached to six repetitive sequences of aspartate, serine, and serine ($(AspSerSer)_6$) was utilized to deliver siRNAs specifically to bone formation surfaces [65]. Delivery of siRNAs against *Tle3* with this delivery system might be useful for reducing mRNA levels of TLE3 in bone without affecting other organs and/or tissues. Thus, developing effective methods of reducing TLE3 expression or activity in bone locally may shed light on novel bone formation therapies.

Competing Interests

The authors declare that they have no competing interests.

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