Stem Cells International

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Editorial Board

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Hindawi
Stem Cells International
Volume 2018, Article ID 4879379, 3 pages
https://doi.org/10.1155/2018/4879379

Editorial

The Stem Cell Niche: Interactions between Stem Cells and Their Environment

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Received 23 August 2018; Accepted 26 August 2018; Published 14 October 2018

Stem cells constitute a source of self-renewing cells with a potential to differentiate into distinct tissues. In the embryo, these cells supply the multiple different cell lineages necessary to generate functional organs. Adult tissues retain somatic stem cells with capabilities for specific tissue turnover and repair. Embryonic and adult stem cell research has shown that stem cell fates are controlled by their specialized microenvironment, referred to as the stem cell niche, via direct cell-cell interactions and the molecular signals emitting from the niche. The niche is formed by the ensemble of stromal cells and the factors they produce, including adhesive signals, soluble factors, and matrix proteins (Figure 1). While we have some understanding of the interactions between adult stem cells and their environment, the requisite components of the stem cell niche are still unclear. Furthermore, tissue-specific stem cells are likely to reside in specialized niches that require further characterisation in each tissue. Progress towards understanding and building a stem cell niche will be necessary to advance *in situ* applications of *in vitro* reprogrammed pluripotent stem cells, differentiated stem cells, and targeted tissue-specific stem cell expansion in tissue regeneration. This may additionally lead to better understanding of how abnormal microenvironments, such as the leukaemic stem cell niche, can contribute to cancer initiation and progression.

Schofield first postulated the hypothesis of a specialized stem cell niche for haematopoietic cells [1]. Since then, a range of stem cell niches regulating tissue turnover and maintenance has been identified and characterised. Even adult tissues previously regarded as postmitotic are now known to be maintained by low levels of steady-state cell replacement during the life course; however, this may not be sufficient under pathological conditions of injury or degenerative diseases. Research on the mechanisms underlying stem cell niche regulation and the strategies to replicate such natural microenvironments *in vitro* could be used to expand stem cells *ex vivo* without losing their native properties.

The stem cell niche typically has a spatial organisation that provides anatomical and functional interactions contributing to stem cell fate specification as well as maintenance of existing clones. These interactions are mutual and dynamic. Stem cells, particularly transformed cancer stem cells, can determine or reprogramme their niche. Stem cell plasticity in response to injury is contained within this environment instructive of stem cell fates [2]. On the other hand, many stem cells show a decline in function over the lifetime, which may underlie the ageing process in organisms [3]. The contribution of the microenvironment to stem cell fate bias is still unclear and requires further investigation. Considerable challenges remain in deducing commonalities, as well as stem cell niche-specific mechanisms, amongst the variety of stem cell supportive microenvironments.

As it is becoming clearer that the niche contributes to the maintenance of stem cell identity, the study of both is needed for understanding and recreating stem cell properties. What

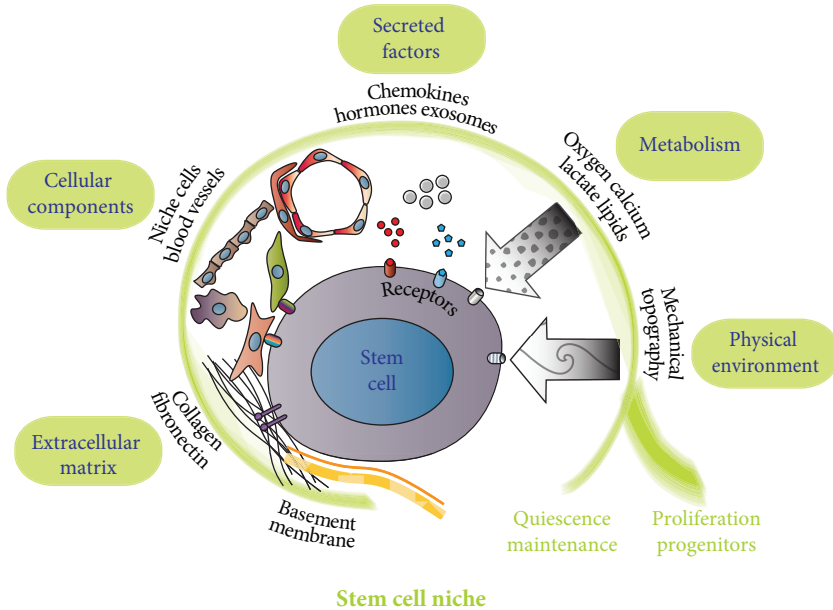

Figure 1: Schematic diagram of the stem cell niche. Shown here is a stem cell with the interacting factors and components of the microenvironment known to regulate resident stem cells, which can be maintained as proliferating or quiescent cells. The actual niche architecture and components may vary for different types of embryonic, adult stem cells and progenitor cells. Not drawn to scale are examples of various cellular niche elements (fibroblasts, MSCs, pericytes, endothelial cells, macrophages, tissue-specific niche architecture cells, and the interacting receptors); extracellular matrix (collagen, fibronectin, basement membrane, and the interacting integrins); secreted factors (exosomes, chemokines, hormones, and the signalling receptors); metabolism producing nutrients and redox state and their channels and transporters; and physical forces within the niche architecture and the corresponding mechanoreceptors.

links these various stem cell niches? During the development of an organism, morphogenetic cell movements and proliferation lead to the formation of the organs in the body. For the main populations of progenitor cells, this process involves a gradual specification induced by the developmental signals they encounter in their changing cellular environments, as they divide and expand into tissues. After completion of mammalian development, some multipotent progenitors and stem cells remain dedicated to tissue turnover in organs. Nested in various tissue-specific locations, they can undergo a range of cell fate changes essential for tissue homeostasis. Niches are typically remnants not of the early organ fields but of later stem cell locations in organogenesis, e.g., bone marrow, sutures in a bone [4, 5].

Cancer stem cells can determine their own *de novo* niche formation during cancer progression, showing that the niche mechanisms are at risk of misappropriation and alterations [6, 7]. The well-studied niches for haematopoietic stem cells, intestinal stem cells, and skin stem cells, as well as the examples of the hair follicle, mammary gland, and neural stem cell niches, have shown that tissue embedded adult stem cell states can include actively dividing cells as well as cells in a state of quiescence [8]. Such states are controlled by signalling in the niche. Embryonic stem cells can also adopt different stem cell states, depending on culture conditions mimicking the signalling conditions of embryonic environments at either blastocyst or epiblast stages; yet, cultured cells show epigenetic changes compared to their embryonic counterparts [9]. The responsiveness of adult stem cells and embryonic stem cells to their environment offers the prospect of bioengineered niches recapitulating developmental potential for biomedical applications [10].

This special issue presents novel research and concepts that link *in vivo* stem cell function to the niche, including research on *in vitro* stem cell niche modelling. An introduction to the adult stem cell niche is provided by S. Bardelli and M. Moccetti, who review recent advances in translational medicine approaches aiming to mimic the natural adult stem cell niche for regenerative medicine. Advances in intestinal stem cell (ISC) niche research are reviewed by L. Meran et al. from the Li group, focusing on the extracellular and cellular niche components; N. Gjorevski and P. Ordóñez-Morán summarizing recent studies on *in vivo* and *in vitro* models of the ISC niche; and B. C. E. Peck et al. from the Sethupathy group presenting the mutual interactions between the ISC niche and gut microbiota and reviewing the available tools to study these interactions. There is increasing recognition of the importance of the bone marrow vascular niche in stem cell regulation in the bone. S. K. Ramasamy reviews recent advances in understanding the heterogeneity and structure of the blood vessels in the bone and their functions in regulating mesenchymal and haematopoietic stem cells. A. Mauretti et al. from the Bouten group and C. Aguilar-Sanchez et al. from the Pennings group provide updates on the debate regarding the function, occurrence, and microenvironment of cardiac progenitor cells.

The cancer stem cell niche may contribute to cancer progression and resistance against chemotherapy, presumably through niche protection of cancer stem cells that are considered a root cause of the cancer relapse. Strategies targeting

interactions between cancer stem cells and their niche are discussed with respect to pancreatic cancer by J. Zhao et al. and ovarian cancer by M. Varas-Gody et al. Mesenchymal stem cell (MSC) research reports by D. Aboalola and A. Youssef in the Han laboratory show their regulation by insulin growth factor and its binding protein, as well as at the onset of osteogenic differentiation and myogenic differentiation, respectively. The role of MSC microRNAs in extracellular vesicles promoting skin repair is investigated by A. da Fonseca Ferreira et al. in the Assis Gomes laboratory. A circadian dynamics study by E. H. Rogers et al. in the Hunt lab shows interesting differences between MSCs originating from different tissues, which may be relevant to tissue engineering and stem cell therapies. Isolation techniques for corneal stroma-derived cells are functionally compared by R. Nagymihály et al. from the Petrovsky laboratory indicating changes in the expression profile of markers compared to the *in situ* state of these stem cells. H. Albalushi et al. from the Stukenborg laboratory report on the stabilising effects on human ES cells when grown on new laminin 521 substrates providing a more controllable culture microenvironment.

Conflicts of Interest

Sari Pennings, Karen Liu, and Hong Qian declare that there is no conflict of interest regarding the publication of the papers or the manuscripts that they edited for this Special Issue.

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Karen J. Liu
Hong Qian

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Hindawi
Stem Cells International
Volume 2018, Article ID 1247857, 13 pages
https://doi.org/10.1155/2018/1247857

Review Article

Cardiac Stem Cells in the Postnatal Heart: Lessons from Development

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Received 4 March 2018; Accepted 23 May 2018; Published 24 June 2018

Academic Editor: Andrea Ballini

Heart development in mammals is followed by a postnatal decline in cell proliferation and cell renewal from stem cell populations. A better understanding of the developmental changes in cardiac microenvironments occurring during heart maturation will be informative regarding the loss of adult regenerative potential. We reevaluate the adult heart's mitotic potential and the reported adult cardiac stem cell populations, as these are two topics of ongoing debate. The heart's early capacity for cell proliferation driven by progenitors and reciprocal signalling is demonstrated throughout development. The mature heart architecture and environment may be more restrictive on niches that can host progenitor cells. The engraftment issues observed in cardiac stem cell therapy trials using exogenous stem cells may indicate a lack of supporting stem cell niches, while tissue injury adds to a hostile microenvironment for transplanted cells. Engraftment may be improved by preconditioning the cultured stem cells and modulating the microenvironment to host these cells. These prospective areas of further research would benefit from a better understanding of cardiac progenitor interactions with their microenvironment throughout development and may lead to enhanced cardiac niche support for stem cell therapy engraftment.

1. Cell Turnover in the Heart: A Loss of Mitotic Potential

The heart has been a focus since the earliest medical research, yet some of the basic knowledge of heart cell biology has remained uncertain for almost a century. Before the concept of stem cells was known, a question was how the heart could maintain its essential function as a hard working organ throughout a human lifespan. A comparative lack of dividing cells had been observed in the adult heart by early histological detection of mitotic cells. Analyses of DNA synthesis in rodent heart tissues over subsequent decades indicated that the rate of DNA synthesis was extremely low in normal heart muscle and slightly increased in injured adult heart, whereas it was much higher during development and until adolescence [1]. Cardiomyocytes were found to stop dividing in the postnatal period when a switch occurs from hyperplasia to hypertrophy during terminal differentiation, and further heart growth is achieved through cell enlargement [2]. In rodents, this was detected by an increase in binucleated cells produced by cardiomyocytes synthesising DNA without completing cell division [3]. Human cardiomyocytes, which are less frequently arrested in a binucleated state (26–60%) than rodent cells (up to 90%), instead show increasing mononuclear polyploidy in the first decades of life [2–4]. Binucleated cells were speculated to provide metabolic benefit through increased transcription of mRNA [5], at the expense of cell renewal.

For many decades, it was taught that the heart was essentially restricted in cell number after birth, unable to regenerate after injury, and adapting to increased workload through cell enlargement. Studies using labelling and other techniques had nevertheless suggested some cardiomyocyte renewal; this was proposed to balance a rate of cell loss through apoptosis and called for a reevaluation of the terminally differentiated state of ventricular myocytes in the adult

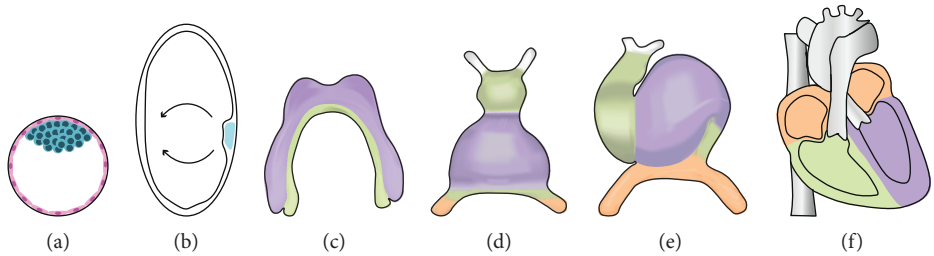

Figure 1: Summary of mouse heart development. (a) E3.5 preimplantation blastocyst stage showing pluripotent inner cell mass (ICM); (b) E6.5-gastrulating embryo showing mesoderm formation (arrows); (c) at E7.5, myocardial progenitor cells migrate to form the cardiac crescent; (d) at E8, the cardiac crescent fuses at the midline to form the early cardiac tube; (e) at E8.5, the cardiac tube forms a loop; (f) at E12.5–E15.5: the chambers undergo septation.

mammalian heart [6, 7]. The highest reported heart cell renewal rates raised the prospect of several tissue replacements per lifetime, as well as new cardiomyocyte generation after injury [8]. This led to a widening range of experimental data [9] and a useful revision of the dogma, but it was not easily understood in view of the clinical prevalence of heart failure, a chronic condition highlighting the lack of cardiac regenerative capacities. However, it was noted that organ damage including fibrosis is irreversible even in organs with high cell turnover, suggesting these are separate issues [6]. The field was more reconciled with studies using a method based on ^{14}C isotope decay measurement in humans. This estimated the rate of cardiomyocyte DNA synthesis in adulthood as less than 1% per year, following a gradual decrease from childhood [4, 10]. It was calculated that less than half of cardiomyocytes may be replaced during a normal lifespan [10]. Interestingly, in adult heart, the cell renewal rates of endothelial cells (>15% per year) and mesenchymal cells (<4% per year) were much higher than those of cardiomyocytes [4]. The overall arrest in cell division of cardiomyocytes after birth in mammals is not as yet explained but is associated with downregulation of positive cell cycle regulators, as well as centrosome disassembly [3, 11]. The potential for cell division is thought more likely to be retained in mononucleated cells or in smaller cells [5]. In lower vertebrates, however, the mitotic apparatus seems preserved [11]. Zebrafish displays a higher regenerative potential of organs including the heart, where the response to injury was found to reactivate cardiomyocyte proliferation of a subset of cells undergoing limited dedifferentiation [12–14]. In mammals, a low rate of cardiovascular replacement was confirmed and traced back to existing dividing cardiomyocytes [15].

Following revision and debate, it was proposed that cell turnover in the mammalian heart muscle occurs at a very low rate [16], which may contribute to its structural maintenance. It is normally insufficient to heal the heart after injury and in disease, but conditions or drugs may be identified that can stimulate the cells retaining mitotic potential [10]. Such cells remain abundant in lower vertebrates, but in mammals, these cells are predicated on rare mitotic cardiomyocytes or on the existence of progenitor and stem cells in an adult cardiac niche. The key to understanding the fate of proliferating cells in the adult heart may be found during its development, when active cell division is supported in dynamic cardiac microenvironments.

2. Heart Development: Assembling Progenitor Cells from Different Sources

Heart development is marked by growth transitions producing a cardiac tube and then causing its looping and partitioning until it reaches its final full-sized chambered heart structure (Figure 1). The process starts soon after embryo gastrulation at embryonic day 6.5 (E6.5) of mouse development, when the mesoderm is formed between the ectoderm and the endoderm germ layer during ingression through the primitive streak. The earliest cardiac progenitors are bilateral groups of cells that originate in the anterior mesoderm and migrate and extend across the ventral midline into a cardiac crescent at E7.5, which is referred as the first or primary heart field [17]. They are joined by a second group of progenitor cells from the underlying pharyngeal mesoderm forming the second heart field [18]. By E8, cardiac crescent cells migrate to the midline, merging the abutments of this arch to form a primitive heart tube. This is composed of beating cardiomyocytes lined with endothelial endocardial cells, separated by an extracellular matrix (ECM) named cardiac jelly. Endocardial cell commitment is thought to occur prior to their migration into the heart field [19]. The slightly later differentiating cells from the second heart field add to the ends of the heart tube to become the arterial and venous poles [18]. The initial heartbeat is found at the inflow region of this heart tube, but subsequent pacemaker cells are thought to arise from the right lateral plate mesoderm [20, 21]. At this stage, the heart tube already functions as a valveless pump with a compound mechanism [22]. Looping and bulging of this rapidly growing tube creates the left ventricle from primary heart field cells, and most of the right ventricle and the outflow tract from secondary heart field cells. By E10.5, the venous poles have pushed up anteriorly and dorsally to form the future atria composed of cells from both fields. Development is completed with the septation of the chambers and valve formation from endocardial cushions by E15.5 [23]. During this time, cells of neural crest origin migrate from the dorsal neural tube and complete the separation of the outflow tracts. Progenitor cells from the extracardiac mesoderm, termed the third heart field, migrate to an anterior location on the heart where they form a transient structure, the proepicardium. Proepicardial cells generate the epicardium by gradually covering the heart towards its apex [24, 25]. Lineage tracing showed that proepicardial cells

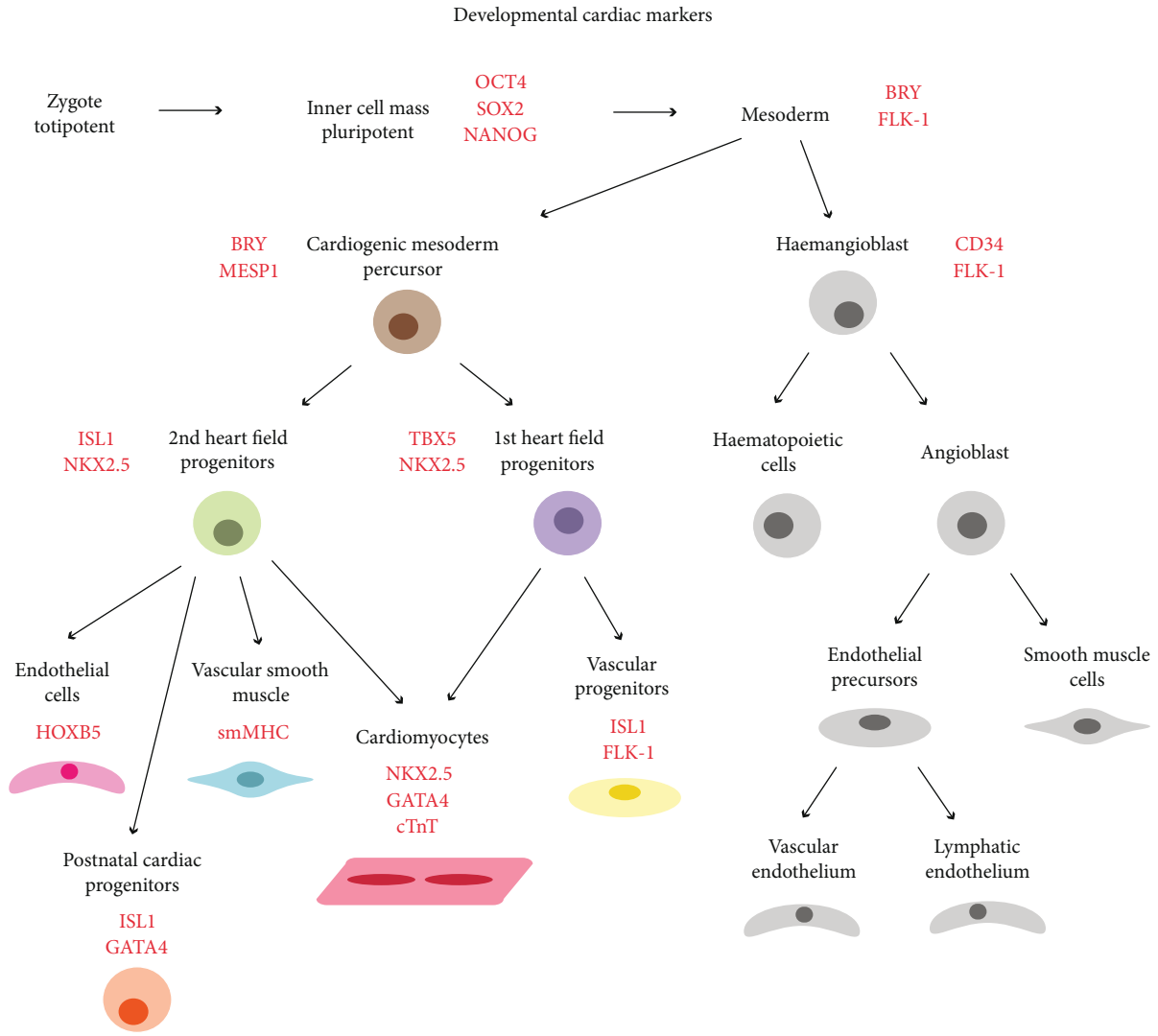

Figure 2: Summary of some of the cell differentiation stages with characteristic transcription factor markers (red) during embryonic development from zygote to cardiac and endothelial tissue.

are also the source of coronary vasculature cells [19]. The niche created at the interface between epicardium, myocardium, and nascent vasculature recruits migrating fetal macrophages of yolk sac origin [26]. Cardiac fibroblasts are also thought to originate from cells migrating out of the proepicardial organ or from epithelial to mesenchymal transition (EMT) during valve formation [27]. Importantly, this suggests that the role of the early mesoderm progenitors in organogenesis is taken over by a proepicardial niche in later cardiac development [24].

3. Developmental Signalling Environments: Inducing Cell Proliferation and Differentiation

Signalling from the surrounding microenvironment directs the transcription regulation of the developmental programme of the heart, necessary for differentiation (Figure 2) as well as proliferation. Specification of the cardiac progenitors is induced by endoderm-produced bone morphogenic protein (BMP) and suppressed by neural Wnt signals [28]. *Gata4* and NKX2.5 are the central transcription factors common to cardiac progenitors, whereas *Tbx5* and ISL1 are specific to cardiac progenitors of the first and second heart fields, respectively [29, 30]. These progenitors differentiate primarily into cardiomyocytes forming heart muscle but also endocardial cells forming the endothelial lining, as well as endothelial cells and vascular smooth muscle cells forming the blood vessels. Cardiomyocytes can further specialise into pacemaker cells generating the electrical impulses and the Purkinje cells conducting these [20, 21]. Other progenitors lead to the cardiac fibroblasts in connective tissues, the epicardial cells forming the outer layer of the heart, pericytes, and resident immune cells [23]. Signalling between these cells further determines morphogenesis in the developing heart [31]. For instance, during development, embryonic cardiac fibroblasts promote cardiomyocyte proliferation through ECM/β1 integrin signalling. In addition, endocardial release of neuregulin 1 (NRG-1) regulates cardiomyocyte differentiation and proliferation

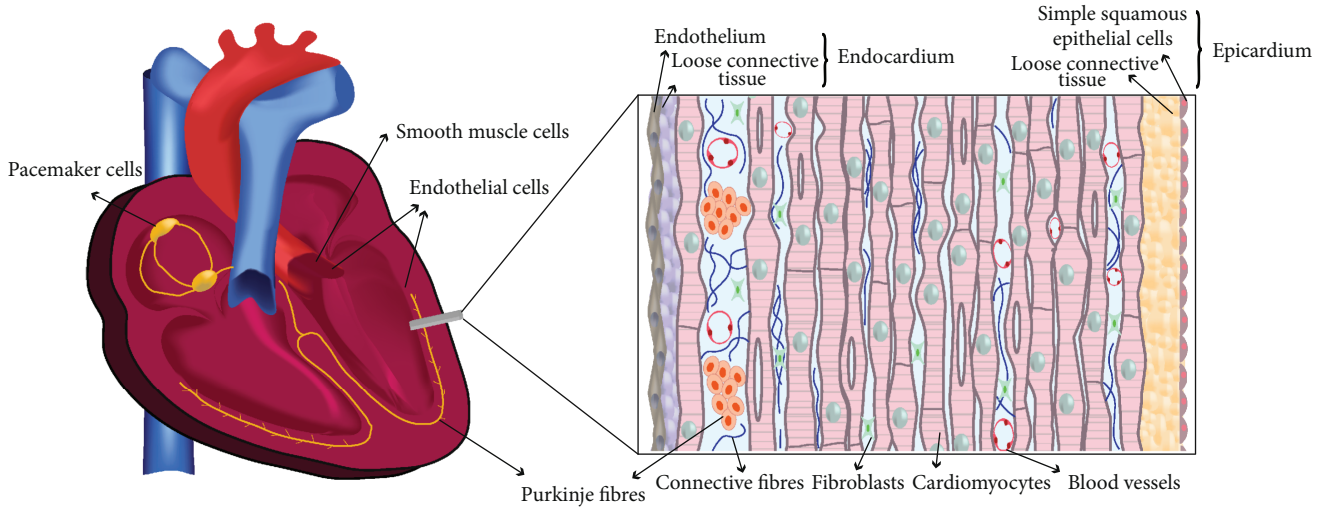

Figure 3: Adult heart architecture with left ventricle wall cross section showing the myocardium organisation with the endocardium lining and epicardium outer layers. Cell types drawn are mature cardiomyocytes, cardiac fibroblasts in their collagen matrix, endothelial cells of the endocardium and capillaries, Purkinje fibres, and epithelial and connective tissue cells of the epicardium.

necessary for trabecular growth within the ventricles, along with NOTCH1, VEGFR-2, and FGF signalling [32]. In turn, the myocardium releases ANG-1 required for differentiation and proliferation of the endocardium [33]. Myocardium also releases TGF-β, BMP, Wnt, and Notch signals regulating the EMT of cells in the endocardium during valve development [31]. Conduction cells differentiate from a subset of contractile cardiomyocytes in response to paracrine signals including endothelin-1 [34]. Epicardial retinoic acid (RA) activates FGF signalling important for proliferation in compact myocardium and for inducing downstream Wnt signalling promoting EMT for growth of the coronary vasculature. In turn, signalling from the myocardium regulates epicardial development [31, 35].

The dependence on signalling pathways in heart development [28] shows that these provide proliferation and differentiation cues from the earliest specification of progenitors in the cardiac crescent to the final heart chamber formation. Niche interactions occur through soluble paracrine signals or physical contacts through integrins and cadherins, which are coupled with cytoplasmic receptors that transduce these signals to the nucleus, where they regulate transcription [36]. Additionally, heart morphogenesis is directed by mechanoregulation from the nascent circulation, pressure load, and myocardium contractility [37, 38]. These signals are transduced via various cell sensors that respond to flow, pressure, stretching, and rhythmicity [39]. The resulting differential gene expression patterns are supported and stably propagated through new cell lineages by epigenetic mechanisms [40]. Heart developmental gene regulation was shown to be determined by chromatin remodelling, histone acetylation and methylation, and DNA methylation [41–45]. The heart has not only provided an early example of the contributions of epigenetic modifiers of gene expression to organogenesis; interestingly, it showed a partial reactivation of developmental histone deacetylases in adult disease [46–49]. In addition, the chromatin-remodelling complex BRG1 was reported to reactivate in response to cardiac stress [50, 51]. However, outside a developmental environment, adult cardiomyocyte reactivation results in cell growth rather than proliferation [49]. Similarly, the fate of progenitors seems to be affected by the transition from developing tissues to the mature configuration of the adult heart.

4. The Adult Cardiac Microenvironment: Confining Space and Signals for Function

Adult mammalian heart tissue has a specialised architecture that serves its essential contractile function (Figure 3). Cardiomyocytes are characterised by the ability of a subset of sinoatrial and atrioventricular nodal or Purkinje cells to generate action potentials and beat spontaneously; the automaticity of these cardiac pacemaker cells involves hyperpolarisation-activated and cyclic nucleotide-gated (HCN) channels [52, 53]. Contraction of cardiac muscle is produced by myofibrils formed by chains of sarcomeres, in which actin filaments interact with myosin filaments, the structural integrity of which is essential [54]. The left ventricular wall consists of lamellar units of myocardial cells in a helical arrangement, which gradually shifts in angle from a left-handed myocyte spiral in the outer zone, through a circumferential zone in the middle part, to a right-handed spiral in the inner zone of the wall [55]. In sections taken across the dense wall, these cardiomyocytes also show connections in radially twisted transmural sheets, which are less tightly coupled towards the inside wall of the left ventriculum [56].

A three-dimensional network of connective tissue surrounds and connects these myocardial sheets, lamellae, and cells, through an extracellular collagen matrix termed the perimysial weave [56]. This interstitial collagen is produced by cardiac fibroblasts, which are present in similar numbers as the cardiomyocytes in the adult heart [57, 58]. Cell-sorting measurements have shown that the proportion of fibroblasts is species specific, and that it is low in embryonic

heart but increases during late foetal and neonatal growth [59], reflecting its longer period of proliferation. Nevertheless, the fibroblasts also stop dividing after heart maturation, although this is thought to be due to a quiescent state from which a subset can reenter the cell cycle [59]. Cardiac fibroblast markers such as discoidin domain receptor 2 (DDR2) and vimentin can distinguish these cells from cardiomyocytes expressing α-myosin heavy chain (α-MHC), cardiac troponin T (cTnT), HCN4, and NKX2.5 [60]. However, some cell markers are not found present in the whole population of cardiac fibroblasts or are not specific to this cell type alone [61]. This unusual fibroblast cell type can conduct electrical signals via connexins through gap-junctional coupling with each other as well as with cardiomyocytes [61, 62], showing it contributes structurally as well as functionally to heart function.

Other abundant occupants of the heart are the endothelial cells, which can be endocardial (the lining of the heart) or vascular (coronary arterial, venous, capillary, and lymphatic cells) [19]. Recent methodology suggests that endothelial cells are more numerous than the other main cell types, but they only make up a small volume [58]. A high density of capillaries in the myocardial interstitial space ensures the supply of oxygen and nutrients to other cells, as well as communication via paracrine factors released by endothelial cells including nitric oxide, reactive oxygen species, endothelin-1, natriuretic peptides, and cytokines [35]. The epicardium forms the outer layer of the adult heart composed of connective tissue, adipose tissue, and surrounding mesothelium, a single layer of epithelial cells in contact with the pericardial fluid [25]. In addition to coronary vessels and nerves, the subepicardium niche environment remains host to macrophages and several other cell types identified by electron microscopy, including immature cardiomyocytes [26, 63]. Several of these cell types have mesenchymal stem cell (MSC) characteristics or other markers of potential progenitor cells in the heart [64].

Overall, the dense construction of mature myocardium embedded in a fibroblast matrix with the endothelial capillary network, with signalling integral to cardiac physiology and its contractile function, leaves few potential sites for adult cardiac stem cell-supporting niches. The loose connective subepicardial tissue surrounding the heart remains a separate niche environment featuring mixed cell types including potential progenitors. These are known to differentiate into several cell types, including cardiomyocytes [65]. Interstitial spaces around blood vessels in the myocardium are further high nutrient environments for interactions between resident and itinerant cells. Microscopic evidence for adult stem cell niches was reported at such locations in the atria and apex [6, 66].

5. The Cardiac Microenvironment in Disease: Stress Signals and Responses

Cardiac fibroblasts can proliferate in response to pathological stimuli [57, 59]. The source of these activated cardiac fibroblasts was initially thought to include resident cells and circulating progenitors [57], rapidly infiltrating a site of injury. More recent lineage tracing studies suggest that the response involves mainly resident cardiac fibroblasts, although contributions from perivascular cells and epicardial cells are possible [61, 67, 68]. Abnormal ECM changes during injury cause activated cardiac fibroblasts to undergo a TGF-β-induced and mechanoregulated differentiation to myofibroblasts, which express αSMA, fibronectins, stress fibres, and contractile activity. This initial response to heart injury can eventually lead to cardiac remodelling and chronic heart failure [69]. Further unexpected roles of myofibroblasts have been suggested in regulating apoptotic engulfment [70].

Cell division of preexisting cardiomyocytes is low but is increased adjacent to areas of myocardial injury, whereas it is reduced by aerobic respiration-mediated oxidative DNA damage [15, 71]. Stress signalling in the myocardium furthermore switches on genes encoding fetal isoforms of proteins [72]. Normally quiescent epicardial cells also proliferate to form epicardium-derived cells (EPDCs) that differentiate into mesenchymal cells; whether these can subsequently populate the adult myocardium is under debate [27]. An important extrinsic factor altering the cardiac niche is the inflammatory response occurring after myocardial infarction. In the ischemic phase, the infarcted tissue suffers necrosis and release of cytokines triggered by tumor necrosis factor TNF-α. Reperfusion brings on a further damaging inflammation response with recruitment of neutrophils, monocytes, and further cytokines, which trigger fibroblast proliferation and neovascularization [73]. The gross changes following cardiac injury and inflammation lead to an increase in myofibroblasts that will initially repair, then remodel the heart with a more rigid ECM [74]. This maladaptive response overshadows the reactivation of other progenitors or proliferating cells in this overall nonregenerative environment.

6. Resident Cardiac Progenitors: Uncovering Residual Heart Developmental Capacity

The existence of progenitor populations in the adult heart has been the focus of many studies [75]. The criteria in the search for cardiac progenitor cells are that they should reside in the heart as a self-renewing pool of multipotent cells able to differentiate into the main cardiac lineages. Table 1 summarises reported examples of cardiac resident side population cells, ISL1+ progenitors, c-Kit+ cells, Sca1+ cells, epicardial progenitors, and mesenchymal cells. Here, we will focus on the cells that have been investigated in more detail.

6.1. Cardiac c-Kit+ Cells. Multipotent, clonogenic, and self-renewing c-Kit+ cells isolated from bone marrow were first claimed to be able to substantially regenerate injured myocardial tissue [82]. Resident c-Kit+ cells in the heart were also reported to have this regenerative capacity [83, 84], suggesting a role in cardiac repair. In the heart, these cells were found together with supporting cells in niches that controlled the migration and differentiation of residing c-Kit+ cells [66]. However, other research groups reported that c-Kit+ cells in adults did not become cardiomyocytes [85, 86], or that they

Table 1: Cardiac progenitor cells and their activity in the heart.

Cardiac resident progenitor type	Characteristics	Cardiac cell fate contribution	References
Side population cells	Perivascular cells of undetermined origin; can grow as cardiospheres	Embryonic heart: cardiomyocytes, endothelial cells Adult: endothelial cells	[76–78]
ISL1+ cardiac progenitors	Major population of undifferentiated cardiac progenitors during development	Embryonic heart: cardiomyocytes Adult: cardiomyocytes (rare)	[79–81]
c-Kit+ cells	Cardiovascular progenitors during development, may be confined to endothelial fate or localised to niches in adult	Embryonic heart: endothelial cells, cardiomyocytes Adult: endothelial cells, cardiomyocytes (rare)	[66, 82–94]
Sca-1+ cells	Heart resident endothelial cells sharing characteristics with mesenchymal cells and side population cells	Embryonic heart: mesenchymal, endothelial, other Adult: cardiomyocytes (low-level replacement)	[81, 95, 96]
Epicardial progenitors	Capable of epithelial-to-mesenchymal transition, multipotent progenitor potential during development, may be reinducible in adult	Embryonic heart: cardiomyocytes, cardiac fibroblasts, coronary smooth muscle cells Adult: myofibroblasts and smooth muscle cells, cardiomyocytes upon induction	[27, 29, 65, 97–101]
Mesenchymal/stromal cells	Expressing MSC/stromal cell markers, restricted multipotency compared to other MSC	Adult heart: mainly cardiac stromal/fibroblast cells, limited cardiomyocyte potential	[4, 64, 102–105]

became cardiomyocytes through cell fusion with very low frequency [87], or that c-Kit+ cells could indeed generate new cardiomyocytes during ageing and after injury, but in extremely small quantities [88]. While this generated a debate regarding the reliability and sensitivity of the reporter mouse models [89, 90], it was demonstrated that an endothelial population of cells in mouse hearts expressed *c-Kit*, inconsistent with its role as a marker of uncommitted cells [91]. This result supported the finding that c-Kit+ cells are endothelial cells that are very infrequently capable of dedifferentiating to cardiac stem cells [88, 91]. More recent lineage tracing experiments confirmed a small subset of cardiomyocytes (~0.03%) expressing *c-Kit* in the adult heart, in addition to more abundant c-Kit+ cardiac endothelial fates [92]. This study and others cautioned that lineage tracing is based on a binary readout potentially overestimating contributions from transient or low expression, as actual cardiac *c-Kit* expression levels in cardiac resident cell populations isolated from adult heart may be low. On the other hand, underestimating factors in c-Kit+ cell genetic fate-mapping studies may include inefficient Cre recombinase activity in cardiac stem cells and deleterious consequences of *c-Kit* haploinsufficiency resulting from genetic manipulation of the endogenous *c-Kit* gene locus. These technical limitations have contributed to the ongoing debate on rare adult stem cell populations, which confirmed the low abundance of cardiac stem cells among the c-Kit+ cardiac cells and their very low levels of *c-Kit* expression [93]. Nevertheless, c-Kit+ identifies cardiovascular progenitors during development capable of differentiating into the major cardiac lineages until at least the neonatal stage in mammals, so an important question is how their cardiac myogenic capacity is largely lost in the adult [85, 94].

6.2. Epicardial Progenitors. During development, epicardium-derived cells are known for their capability of undergoing epithelial-to-mesenchymal transition (EMT), invading the heart and differentiating into other cells, such as the cardiac fibroblasts [27]. A subset of Wilm's tumour 1 Wt1(+) mouse epicardial cells was reported to differentiate into cardiomyocytes and integrate into the myocardium [97]. Furthermore, a population of multipotent *Tbx18*-expressing proepicardial progenitors was reported to differentiate into cardiomyocytes, cardiac fibroblasts, and coronary smooth muscle cells [29]. These genetic lineage tracing studies relied on the epicardial specificity of these markers during development, and their findings were complicated by reports already detecting these markers in the myocardium [98, 99]. In adult mice, epicardium-derived progenitors residing in the epicardium and subepicardium were proposed to be resident adult cardiac stem cells. Stimulating the reexpression of the embryonic epicardial marker *Wt1* in these cells by priming with thymosin β4 peptide was reported to enhance their response to subsequent injury and promote differentiation to cardiomyocytes [65]. However, this reprogramming of epicardial to cardiomyocytes was not observed when thymosin β4 treatment was administered after myocardial injury, when this was tested as a treatment model [100]. The adult epicardium remains a niche for progenitors that undergo EMT upon myocardial infarction and migrate to the subepicardium, where they differentiate into myofibroblasts and smooth muscle cells [101].

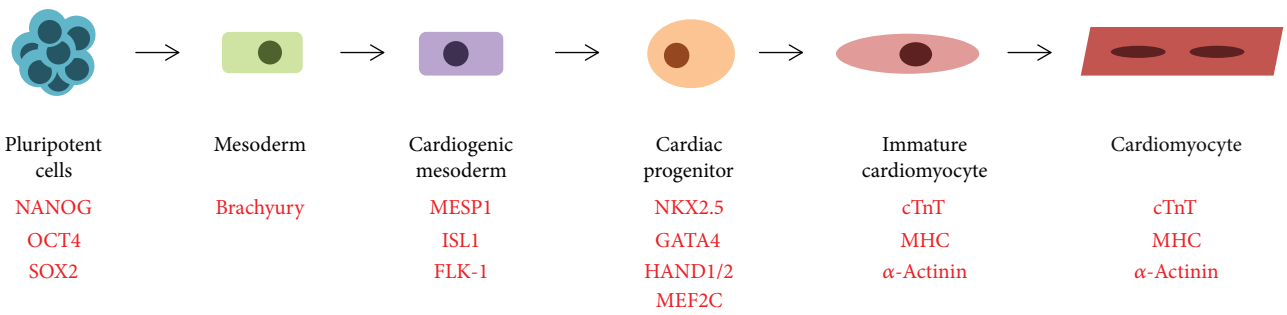

Figure 4: Diagram illustrating differentiation of pluripotent cells to cardiomyocytes. Markers for identification are shown for each step (red). ES or iPS cells differentiate towards mesoderm and cardiac mesoderm through cardiac progenitors and become mature, spontaneously contracting cardiomyocytes.

6.3. Cardiac Mesenchymal Cells. MSCs are adult stem cells that can be isolated from many tissues and on this basis may be resident in the heart [102]. However, cardiac MSCs are not unambiguously distinguishable by specific markers or morphology, so they have been defined by their differences from other cells or grouped with related cell types based on shared markers [4, 64]. Furthermore, MSCs are defined by self-renewal and multipotency criteria following in vitro tissue culture, while their primary in situ properties in many organs are still under debate [103]. Adult human heart pericytes purified from myocardium express MSC/stromal cell markers, but their multipotency seems restricted [104]. Cardiomyocyte differentiation potential was reported to be limited in cardiac mesenchymal cells, whether these had been derived from myocardium or from subpericardium origins [101, 104]. In a myocardial infarction model, these cells contributed paracrine benefits but differentiated into mesenchymal cells, not cardiomyocyte or endothelial fates [101]. Adult cardiac-resident MSC-like stem cells with a proepicardial origin were described as colony-forming units-fibroblasts (CFU-Fs), which expressed platelet-derived growth factor receptor alpha *(Pdgfrα)* and *Sca1* [105]. These can give rise to many cell fates but mainly cardiac stromal/fibroblast cells.

The adult cardiac niche contains quiescent stem cells and progenitors; some of which can reactivate in response to injury, but current evidence suggests that these cells generate primarily noncardiomyocyte cell fates or contribute to the low rate of cardiomyocyte turnover. Adult heart progenitors that can differentiate into other cell types such as endothelial cells are regarded as beneficial for tissue maintenance and regeneration through their prosurvival and angiogenic functions [79]. In strategies aiming to repopulate the heart, exogenous stem cells such as MSC and reprogrammed cells have received considerable attention as an accessible and more abundant source of stem cells.

7. Reprogrammed Cardiomyocytes: Recreating Heart Developmental Potential

7.1. Cardiomyocytes from Pluripotent Stem Cells. Among the cell replacement approaches towards cardiac regeneration is the use of cardiomyocytes obtained by in vitro derivation from embryonic stem (ES) or induced pluripotent stem (iPS) cells [106]. Similar to development, a pluripotent stem cell in a dish differentiates through the stages of mesoderm, cardiac mesoderm, and then cardiac progenitor before finally giving rise to a cardiomyocyte (Figure 4), with markers allowing identification of each differentiation stage [107]. ES and iPS cells begin to differentiate in culture by forming embryoid bodies [108, 109] when LIF is removed from the culture medium [110, 111]. The differentiation process is directed by the expression of transcription factors, recapitulating in vivo developmental stages of differentiation [110] (Figure 2). A cardiomyocyte-like fate also occurs directly in differentiation medium containing fetal calf serum, nonessential amino acids, and beta-mercaptoethanol [108, 112].

One of the issues of producing cardiomyocytes from fibroblast-derived iPS cells for cell therapeutic use is the heterogeneity of lineages, in which the cells will differentiate, resulting in a variety of cells aside from cardiomyocytes and raising tumourigenicity concerns. Inhibition or activation of specific signalling pathways, such as treatment with glycogen synthase kinase 3 inhibitors and then Wnt signalling inhibitors [113] or optimising the levels of the signalling molecule BMP4, key players in the specification of cardiac mesoderm, improved the efficiency of cardiomyocyte induction from iPS cells [28, 114]. Another issue is that cardiac lineage cells mature during the differentiation process in vivo while their beating frequency and sarcomere organisation increases. However, cardiomyocyte cultures obtained from ES or iPS cells consist mostly of immature cells with varying levels of sarcomeric organisation and inconsistent spontaneous contraction [115, 116]. Coculturing of cardiomyocytes differentiated from ES cells with endothelial cells was reported to improve their maturity and upregulate several microRNAs, which when transfected could replicate the effect [117]. This shows that developmental microenvironments can assist in directing pluripotent stem cells to form cardiac progenitors and cardiomyocytes in vivo, as well as in vitro.

7.2. Cardiomyocytes Transdifferentiated from Somatic Cells. Adult cardiomyocytes can also be obtained by derivation of other somatic cells, such as fibroblasts [75, 106]. Cell reprogramming combines a dedifferentiation of fibroblasts to induced pluripotent stem (iPS) cells with directed differentiation to cardiomyocytes. Transdifferentiation is a technique in which differentiated cells are reprogrammed to different

cell lineages by direct conversion, without going through a pluripotent stage. This has permitted the production of, for example, neurons [118], cardiomyocytes [119], or endothelial cells [120]. A clinical advantage of transdifferentiation of somatic cells is that they could be taken from the patient, thus reducing the danger of rejection, although such treatment may not be cost-effective and there is some debate regarding the immunogenicity of reprogrammed cells [121, 122].

It has been shown that transdifferentiation reprogramming occurs without passing through an ISL1+ cardiac progenitor cell stage [119]. These cells show activation of genes that are specific for cardiomyocytes, such as ryanodine receptor 2 *(Ryr2)*, connexin43 *(Gja1)*, cTnT, and α-MHC [119]. Three cardiac development transcription factors, *Gata4*, *Mef2c*, and *Tbx5*, are sufficient for cardiomyocyte induction in vitro and were also used to reprogramme cardiac fibroblasts to cardiomyocytes in mice in vivo after myocardial infarction, reducing scar tissue [123]. Fibroblast conversion to cardiomyocytes could be increased several-fold by adding *Hand2* to the transcription factor cocktail [124] or by enhancing *Mef2c* expression relative to *Gata4* and *Tbx5* [125]. Through further refinement, cardiomyocyte reprogramming was achieved to specific cardiac cells such as Purkinje [126] or pacemaker cells [127]. An alternative transdifferentiation protocol used three iPS factors, *Oct4*, *Klf4*, and *Sox2*, to initiate reprogramming and then direct cardiomyogenesis by adding BMP4 and inhibiting Janus kinase (JAK1) [128, 129].

8. Stem Cell Engraftment in the Cardiac Niche: Stem Cell Therapies Needing Microenvironments

The use of exogenous allogeneic stem cell injection strategies has focused on various populations: bone marrow-derived cells including MSCs, progenitor cell populations, pluripotent cells, and in vitro differentiated cells. Early reports suggested that injected stem cells were not detectably retained inside cardiac niches, but that positive effects were nevertheless observed as a result of paracrine signalling by these cells. A meta-analysis of stem cell therapy in the mouse model demonstrated a significant improvement in left ventricular ejection fraction [130]. The first reported animal studies detected engrafted cells [131, 132]; however, very low engraftment rates following intramyocardial cell injection are common. Myocardial engraftment in the rat model was improved using a prosurvival cocktail with Matrigel [133], suggesting that the allogeneic stem cell microenvironment can be modulated.

A large number of clinical trials have since demonstrated a good safety record for injecting stem cells into patients after myocardial infarction. Comprehensive surveys of these trials concluded an overall modest efficacy in improving cardiac function, indicating considerable variance and discrepancy with animal studies, while the long-term benefits remained uncertain [134–138]. For instance, of a dozen studies using injected bone marrow mononuclear cells, three noted an improved left ventricular ejection fraction, whereas other studies did not record differences in the patients' cardiac parameters, and the optimal timing of these injections after cardiac injury also remained unclear [135]. The much larger BAMI randomised controlled trial may be more conclusive on bone marrow mononuclear cells [134]. On the other hand, MSCs are immune-privileged, permitting the use of allogeneic transplants, and they are also a better characterised homogeneous population [106]. Smaller studies evaluating allogeneic bone marrow MSC injections were generally encouraging [139–141]. Overall, these and other studies indicate that the benefits of cardiac cell therapy may depend on more purified cell populations or better reprogrammed cells, while there is no consensus regarding the best cell type materials, which are still evolving [134].

In addition, cardiac stem cell therapies have much to gain from improved cell engraftment. Cell retention, long-term engraftment, and cell survival have been ongoing issues, with an estimated 1% of donor cells surviving after 4 weeks. Low engraftment is due to initial washout and thereafter the hostile inflammatory environment of the injured tissue, immune rejection, or the lack of mechanical resistance of the donor cells [134]. It is also possible that these stem cells undergo epigenetic changes in culture [142, 143]. Strategies towards enhancing cell survival by preconditioning the cells for the microenvironment of the transplantation site have been encouraging, as well as modulation of the microenvironment at the injured cardiac site [133, 134]. This shows that further investigation of potential adult cardiac niches and a better understanding of the processes by which developmental progenitors are supported by their cardiac microenvironment could lead to more effective cardiac stem cell therapies.

Conflicts of Interest

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

Acknowledgments

This work was supported by the British Heart Foundation, the Biotechnology and Biological Sciences Research Council, and The Genetics Society. The authors thank their colleagues for the helpful comments on the manuscript.

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Hindawi
Stem Cells International
Volume 2018, Article ID 7127042, 9 pages
https://doi.org/10.1155/2018/7127042

Research Article

Laminin 521 Stabilizes the Pluripotency Expression Pattern of Human Embryonic Stem Cells Initially Derived on Feeder Cells

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Received 17 March 2017; Revised 4 November 2017; Accepted 7 December 2017; Published 18 February 2018

Academic Editor: Sari Pennings

Human embryonic stem (hES) cells represent an important tool to study early cell development. The previously described use of human recombinant laminin (LN) 521 represented a step forward in generating clinically safe culture conditions. To test the short-term effect of LN521 on cultured hES cells, five male hES cell lines were cultured on human foreskin fibroblasts (hFFs), Matrigel, LN521, and LN121 and characterized by qPCR, immunofluorescence analysis, as well as their potential for three-germ layer differentiation. Variations in gene expression related to pluripotency, stemness, and testicular cells at different passages and culture conditions were evaluated by qPCR. All cell lines expressed pluripotency markers at protein and RNA level and were able to differentiate into cell types of the three germ layers after being cultured on LN521 for nine passages. Reduction in variation of pluripotency marker expression could be observed after culturing the cells on LN521 for nine passages. hES cells cultured on LN521 exhibited less differentiation, faster cell growth, and attachment when compared to hES cells cultured on LN121 or Matrigel. Our results indicate a positive effect of LN521 in stabilizing pluripotency gene expression and might be the first step towards more controllable and robust culture conditions for hES cells.

1. Introduction

Human embryonic stem (hES) cells, together with induced pluripotent stem cells, provide a unique platform to study molecular and cellular mechanisms in humans. Although hES cells are isolated at a very early stage of development, between five to eight days after fertilization [1, 2] and have the potential to give rise to the three germ layers, different cell lines seem to vary in their capacity to proliferate and to differentiate. They exhibit diverse expression profiles and seem to prefer various differentiation pathways [3, 4]. In addition to these cell line-specific profiles, the differentiation potential has been shown to be method- and even laboratory-dependent [5, 6]. Thus, new strategies involving the employment of well-defined and controlled culture conditions are needed to establish robust hES cell differentiation protocols.

Conventionally, hES cells are cocultured on feeder cells [7], but the use of hES cells in future personalized medicine

requires xeno-free and ideally even feeder-free culture conditions [8–10]. Such xeno- and feeder-free culture conditions are needed to avoid immunogenicity, microbial or viral contamination, and batch-to-batch variability of the culture matrices used [11]. In the first attempts to create a feeder-free culture system, Matrigel which is a protein mixture derived from mouse sarcoma cells, containing laminin (LN) 111, type IV collagen, perlecan, and nidogen, as well as several unknown components and growth factors, was used [12]. To a large degree, these unknown components and the batch-to-batch variability of Matrigel complicate comparability between hES cell experiments [13].

In order to avoid variability, well-defined culture conditions, involving, for example, purified matrix proteins such as LN521, combined with xeno-free media, have been designed to further increase the reliability and reproducibility of various differentiation protocols used [8, 14–16]. Recently used for directive differentiation of human pluripotent stem cells into several cell types, for example, hepatic cells, retinal pigment epithelial cells, and dopaminergic neurons [17–19], these culture conditions can be seen as a major step towards the application of pluripotent stem cell lines in personalized medicine.

In addition to the already mentioned advantages of using LN521, a reduction of DNA damage in hES cells cultured on LN521, compared with cultures on mouse feeder cells, has been reported as soon as after a single passage [20]. However, evaluation of gene expression profiles involving several hES cell lines generated on feeder cells and transferred onto LN521 with special focus on the differences during the first passages and the effects on pluripotency gene expression has not been performed.

Hence, in the present study, we aimed to investigate the short-term effects of LN521 on the expression of different genes, including the expression of common pluripotency genes in feeder cell-derived hES cell lines after being transferred to and cultured on LN521 for up to nine passages.

2. Material and Methods

2.1. Ethics. Ethics approval for the derivation and differentiation of the hES cell lines used in this study was obtained from the Regional Ethics Committee in Stockholm (Dnr. 454/02).

2.2. Cell Lines. Human ES cell lines HS360, HS364, HS380, HS401, and HS420, all karyotype 46,XY, were derived by mechanical isolation of the inner cell masses of six-day-old embryos. Karyotypes were confirmed to be normal 46,XY in all five cell lines after 17–40 passages on human foreskin fibroblasts (hFFs). Before culturing the hES cells on LN521- (LN521-02, human recombinant laminin 521, #80104, Biolamina, Sweden), LN121- (LN121-02, human recombinant laminin 121, test samples from Biolamina, Sweden), and Matrigel- (Corning Matrigel hESC-qualified matrix, 354277, Corning, Bedford, MA, US) coated plates, hES cells were cultured on mitotically inactivated hFFs (CRL-2429, ATCC, Manassas, VA, USA) in knock-out (KO) DMEM medium (KO-DMEM, 10829018, Invitrogen, Life Technologies, Paisley, Scotland) containing 20% KO serum replacement (KO-SR, 10828028, Invitrogen), 0.5% penicillin-streptomycin (15140122, Invitrogen), 2 mM L-GlutaMAX (35050038, Invitrogen), 1% nonessential amino acids (11140035, Invitrogen), 0.5 mM 2-mercaptoethanol (31350010, Invitrogen), and recombinant human fibroblast growth factor 2 at 8 ng/ml (FGF2, 234-FSE/CF, R&D Systems, Minneapolis, USA). In addition to hES cell cultures on hFFs in DMEM, as described above, hES cells were cultured on inactivated hFFs (CRL-2429, ATCC, Manassas, VA, USA) in NutriStem medium (NutriStem hESC XF, 05-100-1A, Biological Industries Israel Beit Haemek Ltd., Israel) at 37°C in 5% CO_2, with daily medium change. The cell colonies were passaged mechanically at four- to six-day intervals.

To enable hES cell culture on LN521- and LN121-coated plates, LN521 and LN121 were slowly thawed at 4°C for one to two hours and diluted at a 1 : 10 ratio with DPBS containing $CaCl_2$ and $MgCl_2$ ($DPBS^{++}$) to a final concentration of 10 μg/ml. The plates were then coated with LN521 or LN121 at 5 $\mu g/cm^2$ overnight at 4°C followed by one hour at 37°C or for two hours at 37°C according to the manufacturer's instructions. The hES cells were cultured in NutriStem medium (NutriStem hESC XF, 05-100-1A, Biological Industries Israel Beit Haemek Ltd., Israel) at 37°C and with 5% CO_2, with daily medium change.

To perform hES cell culture on Matrigel-coated plates, Matrigel was thawed on ice at 4°C overnight. Plates were incubated with Matrigel diluted 1 : 84 in NutriStem at 4°C overnight or at room temperature for 1 hour.

The cells were passaged enzymatically using TrypLE select (12563011, Invitrogen) every five to seven days. After three to four passages, the cells were expanded to collect material for analysis.

2.3. Spontaneous Differentiation: Embryoid Body Formation. At passage nine, after reaching confluence on LN521-coated plates, hES cells were allowed to form embryoid bodies (EBs) in 24-well ultralow attachment plates (#3473; Corning) for 14 days in NutriStem without growth factors (#06-5100-01-1A; Biological Industries). The EBs were then either plated on LN521-coated culture plates or chamber slides (#354104, Corning) for an additional 14 days. After a total of 28 days, differentiated cells were either used for gene expression analysis or fixed with 4% paraformaldehyde for immunocytofluorescence analysis.

2.4. RNA Isolation and cDNA Amplification. Human ES cells were harvested using enzymatic digestion with TrypLE select. RNA isolation was performed using an RNeasy kit (#74104, Qiagen, Germany) according to the manufacturer's protocol and treated with DNase (RNase-free DNase Set, 79254, Qiagen). cDNA was synthesized using an iScript cDNA synthesis kit (#170-8891, Bio-Rad), following the manufacturer's protocol.

2.5. Gene Expression Analysis

2.5.1. Taqman Low-Density Assay. To compare the five hES cell lines, the expression of 96 genes was analysed using Taqman low-density (TLD) array cards (4385344, Applied

Biosystems, Carlsbad, CA 92008, USA), designed by the International Stem Cell Initiative, according to the manufacturer's protocol. Briefly, the TLD array cards were preloaded with 96 TaqMan probes which were run at the same time. Included in the 96 genes were markers of undifferentiated stem cells, pluripotency maintenance, stemness and differentiation, and six internal controls. The data was analysed using RQ-Manager 1.2 software. All the hES cell lines were run in three biological replicates.

2.5.2. Quantitative Real-Time PCR. Quantitative PCR was performed using TaqMan Universal PCR Master Mix (#4369016, Applied Biosystem) with TaqMan probes (Applied Biosystem) according to the manufacturer's protocol (Supplementary Table 1). GAPDH was used as an internal control.

2.6. DNA Isolation and Karyotyping. After harvesting the hES cells with TrypLE, DNA was isolated using a DNeasy Blood and Tissue kit (69504, Qiagen) according to the manufacture's manual. Photospectrometry was used to control DNA concentration and purity. Karyotyping was performed at the Finnish Functional Genomics Centre, Turku, Finland, on the basis of BACs-on-Beads technology, using bead-bound bacterial artificial chromosome probes, which allows the analysis to be performed with only a low amount of DNA isolated from the whole cell pool, as described previously [21].

2.7. Immunocytofluorescence Analysis. For immunocytofluorescence and immunofluorescence analysis, the cells were fixed with 4% paraformaldehyde for 15 minutes, washed three times with 1× phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton X-100 (#108643, Merck, Germany) in PBS for ten minutes. After blocking for one hour with 5% donkey serum (125558, Jackson ImmunoResearch), 1% bovine serum albumin (BSA; 001-000-162, Jackson ImmunoResearch) and 0.1% Tween 20 (#655205, Merck) in 1× PBS at room temperature, the cells were incubated with primary antibodies in antibody solution (1% donkey serum, 0.1% BSA, and 0.1% Tween20 in PBS) at 4°C overnight (Supplementary Table 2). On the next day, the cells were washed three times with 1× PBS and incubated with secondary antibodies (donkey anti-mouse Alexa Fluor 488 conjugated (715-546-150, Jackson ImmunoResearch) or donkey anti-rabbit Cy3 conjugated (711-166-152, Jackson ImmunoResearch)) in an antibody solution for one hour at room temperature in the dark and afterwards counterstained with DAPI at 1 μg/ml (PureBlu DAPI, 135-1303, BioRad) for 15 minutes. The slides were visualized and images were taken using a confocal microscope (LSM700, Zeiss, Germany). A detailed description of the primary antibodies used in the study can be found in Supplementary Table 2.

2.8. Statistical Analysis. Gene expression (qPCR) was calculated as the mean ± standard deviation (SD) of triplicates run for each cell line. Expression was normalized against the expression of *GAPDH* as a housekeeping gene. One-way ANOVA was applied to compare the differences between the five cell lines (SigmaPlot 11.0, Sysat Software Inc., California, USA). Following the Shapiro–Wilk test for normality, pair-wise multiple comparisons were performed by using the Holm–Sidak procedure or Tukey's test (SigmaPlot 11.0, Sysat Software Inc.). A difference was considered to be statistically significant if the p value was <0.05.

TLD array data was normalized using *GAPDH* as a housekeeping gene control, and undetected genes were removed. To separate and compare gene expression profiles of cell lines, the dCT means of replicates were plotted in a heat map, separating cell lines using hierarchical clustering. Moreover, common patterns of expression were investigated via intersection of genes with high expression, arbitrarily defined as exhibiting $dCT < 5$, as well as low expression ($dCT > 15$).

2.9. Coefficient of Variation Calculation. The coefficient of variation (CV) of each gene was obtained by calculating the ratio between the SD and the mean value of all the cell lines together. Based on the CV value, variation was classified into four groups: (I) up to 10%, (II) 11–25%, (III) 26–50%, and (IV) more than 50%.

3. Results

3.1. Heterogeneous Gene Expression Levels of hES Cell Lines Transferred onto LN521. To examine the behaviour of hES cells derived on hFFs during the first passages in feeder-free culture conditions, we grew five male hES cell lines (HS360, HS364, HS380, HS401, and HS420) for four passages (p4) on LN521-coated plates. The colonies formed monolayers and continued to grow without signs of accelerated differentiation (Figure 1(a)).

To confirm the pluripotent nature of the five hES cell lines, gene expression analysis of 96 genes using TLD arrays revealed similar but not identical expression profiles (Figure 1(b) and Supplementary Table 3). At p4, all five cell lines exhibited enhanced expression of nine genes (*ZFP42*, *TDGF1*, *SFRP2*, *PODXL*, *NR6A1*, *CD9*, *DNMT3B*, *IFITM1*, and *LIN28*), which are all related to stemness, and reduced expression of four genes (*TAT*, *PAX4*, *IAPP*, and *DDX4*), which are related to differentiation (Figure 1(c)). Karyotypes were confirmed to be 46,XY in all five cell lines after p4 on LN521 (data not shown).

3.2. Stable Pluripotency of hES Cell Lines Derived on hFFs and Transferred onto LN521. The pluripotency status and potential influence of the culture method per se on the five hES cell lines cultured on LN521 were tested after prolonged culture (nine passages). The morphology of cultured cells was not changed compared with cells cultured up to p4 (Figures 1(a) and 2(a)). All five cell lines expressed a set of five commonly known pluripotency genes at the protein level. Immunofluorescence staining revealed nuclear expression of NANOG, SOX2, and POU5F1 and cytoplasmic expression of SSEA4 and TRA-1-60 (Figures 2(b)–2(f) and Supplementary Figure 1). Quantitative analysis of the pluripotency genes expressed in the nuclei, in all five cell lines, revealed no significant differences between the cell lines in

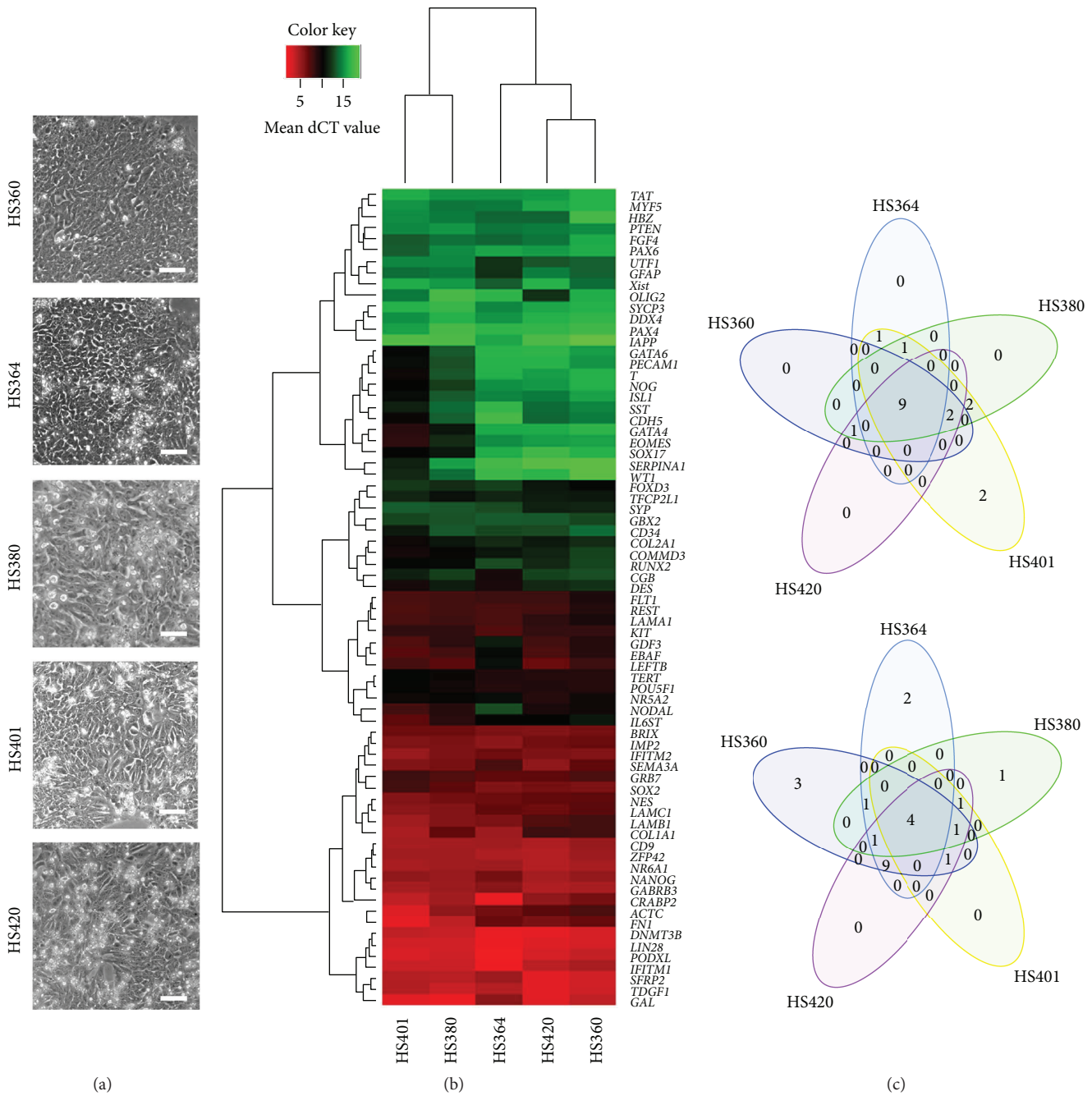


Figure 1: hES cells derived on hFFs and cultured to p4 on LN521. All cell lines (HS360, HS364, HS380, HS401, and HS420) cultured on LN521 exhibited typical hES cell morphology at p4 (a). The hierarchical clusters of gene expression levels for all the cell lines analysed by TLD arrays show a heterogeneous expression profile specific for every cell line. Red colour represents high gene expression (low dCT value) and green colour represents low gene expression (high dCT value) (b). As shown by the Venn diagram, all five cell lines exhibited higher expression of nine stemness-related genes and lower expression of four genes related to differentiation (c).

the expression of NANOG (98% ± 2%), POU5F1 (98% ± 1%), and SOX2 (99% ± 1%) (Supplementary Table 4).

After p9 on LN521, the ability of the cells to differentiate spontaneously into the three germ layers (ecto-, meso-, and endoderm) *in vitro* by the formation of EBs was tested. All hES cell lines expressed markers of ectoderm, mesoderm, and endoderm after 28 days of spontaneous differentiation (Figures 2(g)–2(i) and Supplementary Figure 2). Cells differentiating to endoderm revealed AFP secretion. Cells exhibiting expression of TUJ1 in their cytoskeletons demonstrated cells of ectodermal origin. Cells positive for alpha SMA protein expression were identified as mesodermal cells. qPCR analysis confirmed the RNA expression of endoderm-, mesoderm-, and ectoderm- as well as trophoblast-related genes. *GATA6*, *AFP*, and *DDX4* were used as endodermal, *KDR* and *ACTC1* as mesodermal, *NEUROD1* and *PAX6* as ectodermal markers, and *KRT7* as a trophoblast marker (Supplementary Figure 3).

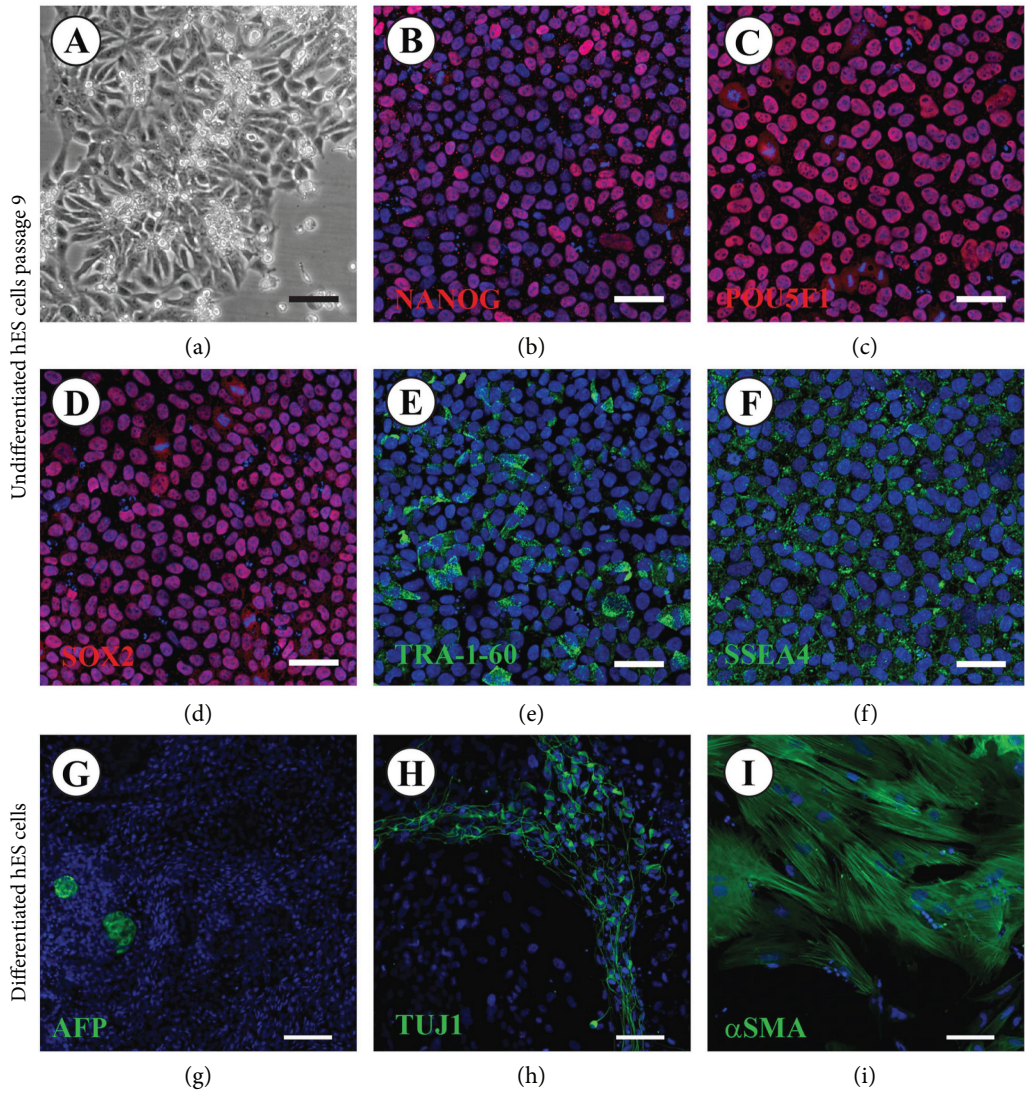


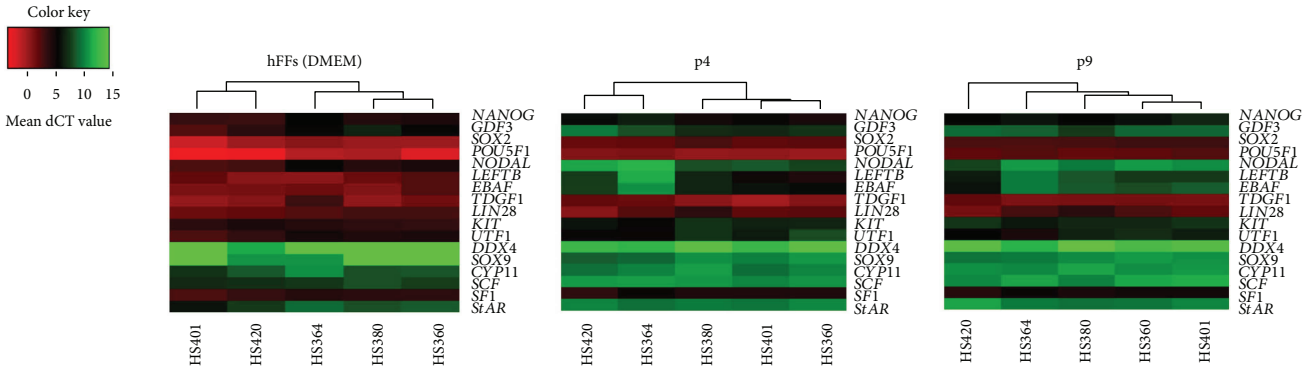
Figure 2: hES cells derived on hFFs and cultured to p9 on LN521. All five cell lines transferred from hFFs onto LN521 exhibited normal hES cell morphology (a). Immunofluorescence analysis of NANOG (b), POU5F1 (c), SOX2 (d), SSEA4 (e), and TRA-1-60 (f) expression, shown for HS380 as a representative cell line, revealed the presence of pluripotency markers after p9 on LN521 (red staining: NANOG, POU5F1, and SOX2; green staining: SSEA4 and TRA-1-60; and blue staining: DAPI). Scale bars: 50 μm. Immunofluorescence analysis of the expression of differentiation markers for endoderm (AFP) (g), ectoderm (TUJ1) (h), and mesoderm (alpha SMA) (i), shown in HS380 as a representative cell line, demonstrates the potential of hES cells cultured on LN521 to differentiate spontaneously into the three germ layers (green staining: AFP, alpha SMA and TUJ1; blue staining: DAPI). Scale bars: 50 μm.

3.3. Similarity in Expression Levels of Genes Related to Pluripotency. In order to investigate the effect of utilizing LN521 as a matrix for hES cell culture, we analysed the expression of 17 genes by qPCR. Since LN521 has been shown to be present in decellularized matrix of the human adult testis [22], we included markers of male gonadal cells in the analysis (Figure 3 and Supplementary Table 5). When comparing the gene expression profile of cells cultured on hFFs in DMEM with cells cultured on hFFs in NutriStem, a similar expression pattern was observed (Supplementary Table 6).

However, when comparing the gene expression profiles of hES cultured on hFFs and LN521 at p4, among the 17 genes investigated, 14 genes showed more than 50% gene expression variation on hFFs compared to 11 genes showing more than 50% gene expression variation on LN521. While at p9 of LN521 culture, only seven genes showed more than 50% variation (Figure 3).

Homogenization of pluripotency gene expression in all five cell lines was observed when comparing expression variation between p4 and p9. At p9, two pluripotency genes (*NANOG* and *GDF3*) showed a variation of less than 50% (34% and 28%, resp.) from more than 50% at p4 (54% and 66%, resp.). *POU5F1* and *SOX2* showed a reduction in variation from less than 50% (32% and 26%, resp.) at p4 to less than 25% (21% and 20%, resp.) at p9.

To explore the impact of culturing hES cells using LN521 matrix on other genes important for early cell development, we analysed RNA expression of genes related to stemness and germ and somatic cell markers at p4 and p9. *NODAL*,



GENE	hFFs (DMEM)		LN521, p4		LN521, p9	
	Rel. Exp. (mean)	SD	Rel. Exp. (mean)	SD	Rel. Exp. (mean)	SD
GDF3	0.071	0.083	0.005	0.003	0.002	0.001
NANOG	0.069	0.041	0.027	0.015	0.016	0.006
POU5F1	8.425	7.394	0.860	0.279	0.251	0.053
SOX2	1.872	1.996	0.261	0.068	0.150	0.030
EBAF	0.641	0.948	0.010	0.008	0.006	0.009
LEFTB	0.420	0.400	0.021	0.027	0.006	0.007
NODAL	0.096	0.066	0.002	0.002	0.001	0.001
TDGF1	0.656	0.384	0.742	0.505	0.561	0.534
UTF1	0.084	0.065	0.015	0.012	0.018	0.014
CYP11	0.003	0.002	0.001	0.0004	0.001	0.0001
DDX4	0.000	0.000	0.0001	0.0001	0.0001	0.0001
KIT	0.074	0.033	0.012	0.007	0.008	0.003
LIN28	0.406	0.354	0.317	0.248	0.254	0.179
SCF	0.005	0.002	0.0005	0.0001	0.0004	0.0002
SF1	0.099	0.046	0.054	0.022	0.043	0.016
SOX9	0.000	0.000	0.001	0.001	0.001	0.0003
StAR	0.005	0.006	0.001	0.0003	0.001	0.0003

Figure 3: Homogenization of the expression of pluripotency genes and genes related to male germ cells and somatic gonadal cells after prolonged culture of hES cells on LN521 presented as heat maps for dCT values on hFFs at p4 and LN521 at p4 and p9. Red colour represents high gene expression (low dCT value) and green colour represents low gene expression (high dCT value); undetected (low/no expression) samples was given a dCT value of 15. (Below) a table of the mean and SD values of the five cell lines showing the relative expression values of pluripotency genes and genes related to stemness and male gonadal cells to *GAPDH*. The coefficient of variation is calculated as SD/mean and the variations among the lines are classified into four groups: less than 10% (green), 11% to 25% (yellow), 26%–50% (orange), and more than 50% (red).

LEFTB, *EBAF*, *TDGF1*, *UTF1*, and *LIN28* showed a variation of more than 50% for both p4 and p9. However, variation in the expression of *LEFTB*, *UTF1*, and *LIN28* was reduced at p9 compared to p4 (Figure 3).

Genes related to gonadal cells showed a different pattern of variation in their expression between p4 and p9. *DDX4* and *StAR* showed no variation in their expression, while *KIT*, *CYP11*, *SF1*, and *SOX9* showed less variation at p9. In contrast, SCF showed more variation in its expression at p9 (Figure 3).

To investigate the difference between LN521 and other matrices used for hES cell culture, we cultured two hES cell lines (HS380 and HS360) on LN521, LN121, and Matrigel for four passages (Supplementary Figures 4 and 5) and one hES cell line (HS380) for nine passages on LN521 and LN121 and seven passages for Matrigel (Supplementary Figure 4). We observed a higher incidence of differentiation in cells cultured on Matrigel and LN121 compared to those cultured on LN521. In addition, hES cells were attaching and growing at a lower rate on LN121 compared to LN521. The differences between LN521, LN121, and Matrigel became more pronounced with prolonged culture (p7 to p9) compared to p4 (Supplementary Figure 4).

4. Discussion

Since the first isolation of hES cells, in 1998, the derivation and establishment of clinically safe hES cell lines have proven to be challenging [23, 24]. Incompletely defined culture conditions and the use of xenogenic materials for hES cell culture and expansion have limited interpretation and understanding of the mechanisms controlling self-renewal, pluripotency, and differentiation characteristics. Thus, the possibility to use human pluripotent stem cells in personalized medicine is limited [13]. The establishment of more defined culture conditions, for example, through the use of synthetic matrices would result in better-controlled conditions.

It was suggested that hES cells express four different isoforms of laminin, LN521, LN121, LN511, and LN111, since they express alpha 1, alpha 5, beta 1, beta 2, and gamma 1 laminin chains [9]. In addition, alpha 5 chain laminins are found in the inner cell mass (ICM) of the mouse blastocyst

which indicates that alpha 5 containing laminin isoforms are part of stem cell niches [25]. Therefore, we thought that LN521 might be an optimal matrix not only for cultures of newly derived pluripotent stem cells but also for hES cells previously derived and cultured on hFFs. A successful and robust adaptation of hES cells derived on hFFs and cultured in feeder- and xeno-free conditions using LN521 would allow that more and well-characterized hES cell lines can be used to establish defined differentiation protocols.

In the present study, the expression profiles of five XY hES cell lines, which were derived on hFFs, were analysed in regard to their pluripotency after short-term culture on LN521. We cultured hES cell lines (HS360, HS364, HS380, HS401, and HS420) on LN521 to p4 and p9. All five hES cell lines grew in a monolayer on LN521-coated wells, as previously described [9]. A confluent layer was observed within five to seven days without showing accelerated signs of differentiation. After being transferred from hFFs to LN521 and cultured to p4, all five cell lines showed a normal 46,XY karyotype. These results are in line with those of previous studies showing the feasibility of LN521 as a matrix for hES cell cultures in xeno-free and chemically defined culture systems [8]. However, the long-term impact on their genomic stability needs to be examined further to confirm the results of our short-term cultures.

Despite the overall pluripotent nature of the five cell lines, variation in expression of genes for pluripotency and differentiation was observed, indicating cell line-specific expression patterns. However, when comparing the expression of pluripotency genes (*SOX2*, *NANOG*, *POU5F1*, and *GDF3*) between the five cell lines cultured on hFFs and LN521 (p4), a homogenized expression profile was observed for hES cells cultured on LN521. Moreover, with prolonged culture (p9) on LN521, the gene expression variation was reduced further when compared to p4 on LN521. This homogenization between different cell lines cultured on LN521 indicates more predictable and robust culture conditions for hES cells, which are useful requirements for the establishment of directed and cell line-independent differentiation protocols.

To assess if this homogenization effect occurs due to the culture on LN521 or the change to NutriStem, cell culture medium, we cultured four cell lines (HS360, HS364, HS380, and HS401) for four passages on hFFs in two different media, in DMEM with supplements and in NutriStem. Our results showed a similar picture of variation in the expression of pluripotency genes and genes related to stemness in both cell culture media. This indicates that change to NutriStem medium when culturing the cells on LN521 had no obvious effect.

The expression of LN521 in the decellularized matrix obtained from human testis has been described earlier [22]. Therefore, we examined potential effects of LN521 on gene expression levels in early germ and somatic cells, by including markers usually expressed in human foetal male gonads. We investigated the expression of genes related to the specification and appearance of early stages of primordial germ cells in humans (*KIT, UTF1, LIN28,* and *DDX4*) and genes related to gonadal somatic cells (*CYP11, SOX9, SCF, StAR*, and *SF1*) as well as genes expressed in undifferentiated hES cells and related to the NODAL pathway (*EBAF, LEFTB, NODAL*, and *TDGF1*), which plays a major role in early cell fate, including germ-cell specification and endoderm specification as well as maintenance of the pluripotent status of stem cells [26]. Our results showed that LN521 had no effect on the expression levels of genes related to early gonadal cells, for example, for germ-cell specification and differentiation.

In addition, we observed that LN521 provides a more suitable matrix for hES cell attachment, growth, and self-renewal compared to Matrigel and LN121. A detailed study of the effect of long-term culture of hES on LN521 and LN121 compared with the short-term culture described in this study will be needed in the future to confirm the stability of gene expression profiles of hES cells cultured on LN521. The difference in the alpha chain between LN521 and LN121 as culture matrices may explain the diverse effects on cell attachment and gene expression levels on the hES cells. However, as mentioned above, the effect of this difference and its impact needs a thorough study for better understanding.

5. Conclusions

Our results demonstrate that LN521 supports hES cell growth and has a positive effect on maintaining the cells in a normal balanced pluripotent state. Culture on LN521 leads to homogenization of the gene-expression profiles of the different hES cell lines derived on hFFs, used in this study. Similar expression profiles of different hES cell lines might result in more predictable and therefore controllable behaviour of these cells in various differentiation protocols, which are required for the possible use of these cell lines in the field of future regenerative medicine.

Additional Points

Highlights. Laminin 521 provides suitable culture conditions for adaptation to feeder- and xeno-free conditions of hES cells derived and cultured on feeder cells. Laminin 521 has a positive effect in homogenizing pluripotent gene expression profiles between hES cell lines cultured for up to nine passages. Laminin 521 has no obvious effect on the expression of genes related to male germ cells and somatic gonadal cells, when used as a matrix for hES cell cultures.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Halima Albalushi, Jan-Bernd Stukenborg, and Outi Hovatta conceived and designed the experiments. Halima Albalushi, Magdalena Kurek, Luise Landreh, and Kristín Rós Kjartansdóttir performed the experiments. Kristín Rós Kjartansdóttir and Leif Karlsson performed the TLD assay analysis. Halima Albalushi, Magdalena Kurek, Jan-Bernd Stukenborg, and Olle Söder assisted in the immunofluorescence analysis and interpretation. Halima Albalushi and Jan-Bernd Stukenborg wrote and all authors edited the manuscript.

Acknowledgments

The authors are grateful to Dr. L. Antonsson for her assistance with the cell cultures and to Biolamina for sending test vials of LN121 for their experiment. Karyotyping was performed at Finnish Functional Genomics Centre, University of Turku, Turku, Finland. The authors would also like to gratefully acknowledge the aid of the core facility at Novum (BEA (Bioinformatics and Expression Analysis)), which is supported by the Board of Research of the Karolinska Institutet and the Research Committee of the Karolinska University Hospital. Financial support was received from Sällskåpet Barnavård in Stockholm, the Sigurd and Elsa Goljes Minne Foundation, the Swedish Research Council/Finnish Academy of Science, Kronprinsessan Lovisas Förening för Barnasjukvård/Stiftelsen Axel Tielmans Minnesfond, Samariten Foundation, and the Emil och Wera Cornells Foundation. Magdalena Kurek was financially supported by EU-FP7-PEOPLE-2013-ITN 603568 "Growsperm". Halima Albalushi was financially supported by a grant from the Sultan Qaboos University in Oman.

Supplementary Materials

Supplementary 1. Figure 1: Immunofluorescence analysis of NANOG, POU5F1, SOX2, SSEA4 and TRA-1-60 expression, shown for HS360, HS364, HS401 and HS420 cells, revealed the presence of pluripotency markers after p9 on LN521 (red staining: NANOG, POU5F1 and SOX2; green staining: SSEA4 and TRA-1-60; blue staining: DAPI). Scale bars: 50 μm.

Supplementary 2. Figure 2: Immunofluorescence analysis of protein expression in the three germ layers (endoderm (AFP), ectoderm (TUJ1) and mesoderm (alpha SMA)) was positive for all markers in HS360, HS364, HS401 and HS420 cells cultured on LN521 after spontaneous differentiation. Green staining: AFP, alpha SMA and TUJ1; blue staining: DAPI. Scale bars: 50 μm.

Supplementary 3. Figure 3: QPCR analysis of genes related to differentiation for all cell lines on LN521: mesodermal markers KDR and ACTC1; ectodermal markers PAX6 and NEUROD1; endodermal markers GATA6, AFP and DDX4; trophoblast marker KRT7. Gene expression relative to that of *GAPDH* is shown before (grey bars) and after spontaneous differentiation (red bars). Abbreviations: ND: not detected.

Supplementary 4. Figure 4: Comparison of RNA expression of pluripotency genes, genes related to stemness and genes expressed in the male gonadal cells, for hES cells cultured on three different matrices (LN121, LN521 and Matrigel). The differences in the gene expression for HS380 at p4 on LN121, LN521 and Matrigel, and at p9 on LN121 and LN521, and at p7 on Matrigel are presented as heatmap for dCT value normalized to *GAPDH*. Red colour represents high gene expression (low dCT value) and green colour represents low gene expression (high dCT value). Undetected (low/no expression) samples was given a dCT value of 13. Abbreviation: SD: standard deviation; dCT: delta cycle threshold.

Supplementary 5. Figure 5: Comparison of RNA expression of pluripotency genes, genes related to stemness and genes expressed in the male gonadal cells, for hES cells cultured on three different matrices (LN121, LN521 and Matrigel). The differences in the genes expression for HS360 at p4 on LN121, LN521 and matrigel are presented as heatmap for dCT value, normalized to *GAPDH*. Red colour represents high gene expression (low dCT value) and green colour represents low gene expression (high dCT value). Undetected (low/no expression) samples was given a dCT value of 13. Abbreviations: dCT: delta cycle threshold.

Supplementary 6. Table 1: Detailed information for the TaqMan primers (Applied Biosystems, ThermoFisher Scientific) used to analyse the RNA expression of genes related to pluripotency, stemness and differentiation, and genes expressed in male gonadal cells. Table 2: Detailed information for the primary antibodies used to analyse the protein expression of pluripotent, mesodermal, endodermal and ectodermal cells as well as for IgGs used as negative controls.

Supplementary 7. Table 3: Data set of the mean dCT value (±SD) of gene expression for cell lines cultured on LN521 (four passages), normalized to *GAPDH*. Mean dCT values (±SD) for all genes included in the TLD array. Abbreviations: dCT: delta cycle threshold; SD: standard deviation; TLDA: taqman low density array.

Supplementary 8. Table 4: Quantitative analysis of protein expression of pluripotency genes (NANOG, POU5F1 and SOX2) in five hES lines cultured on LN521 for 9 passages. No significant difference between the lines in the expression of all three genes. Counting is presented as mean (±SD) of the percentage of the number of positive cells compared to total number of cells. Abbreviation: SD: standard deviation.

Supplementary 9. Table 5: Data set of the mean dCT values (±SD) of gene expression of cell lines cultured on hFFs, LN521 p4 and LN521 P9, normalized to *GAPDH*. Mean dCT values (±SD) for all genes was analysed with qPCR. Abbreviations: dCT: delta cycle threshold; SD: standard deviation; hFFs: human foreskin fibroblasts.

Supplementary 10. Table 6: hES cells cultured on feeder cells in DMEM at p3 and p4 with supplements and NutriStem at p4 show no difference in expression variation of pluripotency genes and genes related to stemness. The mean values (±SD) for the five cell lines showing the relative expression to *GAPDH* of pluripotency genes and genes related to stemness and genes expressed in male gonadal cells. The coefficient of variation is calculated as SD/mean and the variations among the lines are classified into four groups: less than 10% (green), 11% to 25% (yellow), 26%–50% (orange) and more than 50% (red). Abbreviation: SD: standard deviation; Rel. Exp.: relative expression.

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Hindawi
Stem Cells International
Volume 2017, Article ID 9841035, 14 pages
https://doi.org/10.1155/2017/9841035

Research Article

Extracellular Vesicles from Adipose-Derived Mesenchymal Stem/Stromal Cells Accelerate Migration and Activate AKT Pathway in Human Keratinocytes and Fibroblasts Independently of miR-205 Activity

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Received 3 March 2017; Revised 27 July 2017; Accepted 23 August 2017; Published 5 November 2017

Academic Editor: Sari Pennings

Mesenchymal stem/stromal cells (MSCs) are promising tools in cell therapy. They secrete extracellular vesicles (EVs) that carry different classes of molecules that can promote skin repair, but the mechanisms are poorly understood. Skin wound healing is a complex process that requires the activity of several signaling pathways and cell types, including keratinocytes and fibroblasts. In this study, we explored whether adipose tissue MSC-derived EVs could accelerate migration and proliferation of keratinocytes and fibroblasts, activate the AKT pathway, and promote wound healing *in vivo*. Furthermore, we evaluated if EV effects are miR-205 dependent. We found that MSC EVs had an average diameter of 135 nm. Keratinocytes and fibroblasts exposed to EVs exhibited higher levels of proliferation, migration, and AKT activation. Topical administration of EVs accelerated skin wound closure. Knockdown of miR-205 decreased AKT phosphorylation in fibroblasts and keratinocytes, whereas migration was decreased only in keratinocytes. Moreover, knockdown of miR-205 failed to inhibit AKT phosphorylation in fibroblasts and keratinocytes exposed to EVs. About the mechanism of EV effects, we found that incubation with EVs prevented inhibition of AKT activation by miR-205 knockdown, suggesting that EVs activate AKT independently of miR-205. In conclusion, we demonstrated that EVs are a promising tool for wound healing.

1. Introduction

Mesenchymal stem/stromal cells (MSCs) can be isolated from many adult tissues [1]. Human adipose tissue was chosen as the source of stem cells in this study, due to its easy access and high mesenchymal stem cell population. The extraordinary plasticity of these cells was originally thought to contribute to their well-established therapeutic efficacy in a wide variety of disease models, as well as in human clinical trials. However, recent findings suggested that MSCs have a

remarkable influence on the microenvironment by secreting a wide range of bioactive factors. This ability may contribute more to tissue repair than to their capacity for transdifferentiation [2].

MSC secretome comprises a wide variety of biomolecules, including cytokines, growth factors, and RNAs. These molecules could modulate the microenvironment and, thus, be responsible for the majority of beneficial effects of stem cells in tissue repair [3]. Recent studies indicated that MSCs also secrete extracellular vesicles (EVs) that contribute to cell-cell communication. After internalization, EVs may alter recipient cell fate by delivering transcriptional factors able to modulate cellular properties [4, 5].

On the other hand, wound repair is a complex process controlled by many secreted factors, including soluble proteins and RNAs [6]. Many preclinical and clinical studies have demonstrated that administration of MSCs accelerates wound closure, reduces scarring, and promotes collagen synthesis and angiogenesis [7, 8]. However, the mechanisms and factors responsible for the improvement of wound healing process during stem cell therapy are still poorly understood.

Several biochemical pathways coordinate skin integrity restoration [9]. The pathway regulated by miR-205 is involved in cell migration and proliferation. Lack of miR-205 results in epidermal defects caused by impaired proliferation, whereas genetic deletion of miR-205 leads to neonatal mortality in mice [10]. This microRNA modulates AKT activation and, therefore, increases migration in epithelial cells and promotes wound healing [11].

In this study, we evaluated the ability of adipose-derived MSC EVs to increase cell proliferation, migration, and AKT activation in human keratinocytes and fibroblasts and to promote wound healing *in vivo*. Furthermore, we examined whether EV effects on migration and activation of AKT were dependent on miR-205. Here, we demonstrated that EVs and its molecules are promising tools for wound healing.

2. Materials and Methods

2.1. MSC Isolation and Culture. MSCs were isolated from subcutaneous adipose tissue donated freely by lipoplasty surgery patients according to the regulations approved by the Universidade Federal de Minas Gerais's Research Ethics Committee (348/2016). Cell extraction was carried out as described by Zuk et al. [12]. Briefly, tissue samples were washed twice with phosphate-buffered saline (PBS, pH 7.4; Thermo Fisher Scientific, Waltham, MA, USA) to remove blood cells and incubated with 0.1% collagenase solution (Thermo Fisher Scientific) for 45 min at 37°C. After incubation, samples were centrifuged at 300 ×g for 7 min, the supernatant was discarded, and the remaining pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; cat. number: 12800-017, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin/streptomycin (PS; Sigma-Aldrich, St. Louis, MO, USA). Cells were transferred to culture flasks and kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Culture medium was changed every 3 days.

2.2. Characterization of MSCs by Cytometry. MSCs (10^6 cells per sample) were incubated with 0.4 μg of primary antibodies against CD73 (Thermo Fisher Scientific, cat. number: 41-0200), CD90 (Thermo Fisher Scientific, cat. number: 14-0909-82), CD105 (Thermo Fisher Scientific, cat. number: 12-105742) (traditional membrane stem cell markers), CD19 (Thermo Fisher Scientific, cat. number: 11-0199-42), and CD45 (Thermo Fisher Scientific, cat. number: 11-9459-42) (hematopoietic cell markers) for 30 min at 4°C [13]. After washing, cells were incubated with a secondary antibody for 30 min at 4°C, washed again, and suspended in PBS. Alexa Fluor 488 goat anti-mouse (Thermo Fisher Scientific) was used for unconjugated primary antibodies. In control experiments, cells were incubated only with the secondary antibody to exclude nonspecific binding. Cytometry was performed using a Guava® EasyCyte™ 6–2 L (Millipore, Temecula, CA, USA). For each sample, at least 5000 events were acquired. Results were analyzed with FlowJo 7.6.5 software (LLC, Ashland, OR, USA).

2.3. Differentiation of Chondrocytes, Adipocytes, and Osteocytes. MSCs were incubated for 21 days in specific differentiation media, as previously described [12]. Following incubation, cells were fixed and stained by Von Kossa, oil red O, and alcian blue stains to visualize osteogenic, adipogenic, and chondrogenic differentiation, respectively. Although other strategies could be used to verify osteogenic and chondrogenic differentiation, Von Kossa and alcian blue stains are still regarded as good methods for that purpose. Next, cells were imaged using a QIClick camera (QImaging, Surrey, BC, Canada) coupled to an IX70 Olympus microscope controlled by Image-Pro Plus 7.0.1 (Media Cybernetics, Rockville, MD, USA).

2.4. EV Isolation. EVs were isolated as previously described [14]. Briefly, stem cells were incubated with serum-free DMEM for 48 h at 37°C, in the atmosphere of 95% air and 5% CO_2. This step was necessary to assure that the medium collected had only EVs from stem cells, as serum-free DMEM does not contain EVs. After the incubation period, the medium underwent successive centrifugation steps. The first centrifugation was at 300 ×g for 7 min; then, the supernatant was centrifuged for 15 min at 1000 ×g, followed by another supernatant centrifugation for 40 min at 10000 ×g. Then, the supernatant was ultracentrifuged (100000 ×g for 70 min). Finally, the supernatant underwent the last ultracentrifugation (100000 ×g for 70 min), and the resulting pellet was composed of EVs. All centrifugation steps were carried out at 4°C. Cells used for EV extraction were in the fourth or fifth passage. For every complete experiment, EVs from stem cells from three different patients were isolated and used, totalizing 45 samples from 48 different patients in this study.

2.5. EV Characterization and Concentration. EVs isolated from serum-free DMEM as described above were resuspended in PBS and analyzed using NanoSight LM 10 system and Nanoparticle Tracking Analysis software (Malvern Instruments Ltd., Malvern, UK). We used 1.9×10^8 vesicles in each experiment.

2.6. Isolation and Culture of Human Keratinocytes and Fibroblasts. Human keratinocytes were extracted as described by Zonari et al. [8]. Human skin samples were donated with consent from the patients that underwent abdominoplasty surgery according to regulations specified by the Universidade Federal de Minas Gerais Research Ethics Committee (55698116.2.0000.5149). The skin was separated from adipose subcutaneous tissue with scissors, and the fatty tissue was discarded. Then, skin samples were fragmented into 5 mm^2 pieces and incubated in dispase solution (2.5 UI) for 16 h at 4°C. After this, the dermis and epidermis were separated with tweezers (fibroblasts were extracted from the dermis and keratinocytes—from the epidermis). The dermis was incubated in a 0.1% collagenase solution (Thermo Fisher Scientific) for 3 h at 37°C, and the epidermal fragments were incubated with 0.05% trypsin solution for 5 min at 37°C. After the incubation period, a cell scraper was used to remove the fragments of the epidermis to release keratinocytes. The resulting material was then passed through a 100 μm filter to only collect the cells that were then placed in culture flasks with keratinocyte serum-free medium (KSFM) and cultured at 37°C in the atmosphere of 95% air and 5% CO_2. After a 3 h incubation in collagenase solution, dermal fragments that remained undigested were discarded, and the supernatant underwent centrifugation for 7 min at 300 ×g. The resulting pellet was resuspended in DMEM containing 10% FBS and 1% PS and transferred to a culture flask. Cells were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, and the medium was changed every three days.

2.7. Growth Curves. The growth curves were performed in keratinocytes and fibroblasts seeded in 24-well plates (5×10^4 cells per well) and exposed to the medium with or without MSC EVs. The concentration of vesicles used was 3.16×10^7 per mL and each well was exposed to one mL of enriched medium. Cells were counted in a Neubauer chamber daily for eight consecutive days. Cell counts were used to plot a growth curve.

2.8. Western Blot. For experiments with EVs, cells were exposed to enriched medium and the concentration of vesicles used were 3.16×10^7 per mL. Initially, total protein samples were isolated from cells exposed to EVs using a sterile cell scraper and a lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 8.0, 0.5% Nonidet P-40). Total protein concentration was quantified by the Bradford method (Bradford, 1973), and then, samples underwent electrophoresis. Proteins were then transferred to polyvinylidene fluoride membranes using a Trans-Blot® SD semidry transfer cell (BioRad, Hercules, CA, USA). Immunoblotting was performed by standard methods, as previously described [15]. Primary antibodies against total AKT (Cell Signaling Technology, Danvers, MA, USA; cat. number 9272, 1 : 1000), phospho-AKT (Cell Signaling Technology; cat. number 4060, 1 : 500), phospho-histone 3 (Sigma-Aldrich; cat. number MFCD01633984, 1 : 500), total histone H3 (Sigma-Aldrich; cat number MFCD01322157, 1 : 2000), Alix (Cell Signaling Technologies, cat. number: 2171), and CD9 (cat. number: MA1 80-307) were used. Band density analysis was performed using ImageJ software (https://imagej.nih.gov/ij/).

2.9. Scratch Wound Healing Assay. Human keratinocytes and dermal fibroblasts were seeded in 6-well plates at 80% confluence. After exposure to KSFM or DMEM (depending on the cell type) for 48 h, a scratch was made on the cell monolayer with a sterile 200 μL pipette tip. Wells were washed with PBS and then exposed to MSC EVs. The concentration of vesicles used were 3.16×10^7 per mL in each well. Scratch closure was monitored by imaging at 0, 12, and 24 h. Scratch areas were measured by Image-Pro Plus Software.

2.10. Excisional Wound-Splinting Model. All animal experiments were performed after obtaining an approval from the University Research Ethics Committee (168/2013). The excisional wound-splinting model was used as described by Wang and colleagues [16]. This model facilitates the approximation between rat and human skin wound healing by reducing skin traction. Briefly, 24 male Wistar rats (220 g) were divided into two groups: test group and negative control group. Animal groups were further divided into three subgroups of four animals, for assessments at 7, 14, and 21 days after exposure to gel preparations. Rats were anesthetized using an intraperitoneal injection of ketamine and xylazine. Excision wounds (5 mm in diameter) were made on rat dorsal skin with a mini punch. Sterile silicone rings were glued around the wounds with a high-performance glue. Animals were placed in individual cages and received aspirin for pain relief. Throughout the experiment, they had free access to food and water.

2.11. Gel with EVs. Hydroxyethyl cellulose aqueous gel (2%) was made in sterile conditions and admixed to EV suspension (1.9×10^8 vesicles) in a 1 : 1 ratio. The resulting 1% hydroxyethyl cellulose gel comprising MSC-derived EVs was aseptically applied daily on wounds of animals in the test group, whereas animals from the control group were treated with plain 1% hydroxyethyl cellulose gel without EVs. Wound parameters were assessed at 7, 14, and 21 days after the wound was made. Images were taken every day.

2.12. Real-Time PCR. Human keratinocytes and dermal fibroblasts were seeded in 6-well plates (5×10^5 cells per well). RNA was extracted with a mirVana® RNA extraction kit (Thermo Fisher Scientific) and used for reverse transcriptase reaction. We used a TaqMan probe kit (Thermo Fisher Scientific, cat. number 4427975); amounts of RNA and cDNA were as recommended by the manufacturer. The reactions were read using a 7900HT Fast Real-Time PCR System and analyzed with SDS 2.4 software (Thermo Fisher Scientific). Relative gene expression was calculated by the $2^{-(\Delta\Delta Ct)}$ method [17].

2.13. Transfection of siRNA. Transfection was conducted as recommended by the manufacturer. Human dermal fibroblasts and keratinocytes were seeded at 60–70% confluence and then exposed to a suspension of Lipofectamine 2000 (Thermo Fisher Scientific) with 25 nM siRNA in Opti-MEM (Thermo Fisher Scientific) for 5 h. The siRNAs used

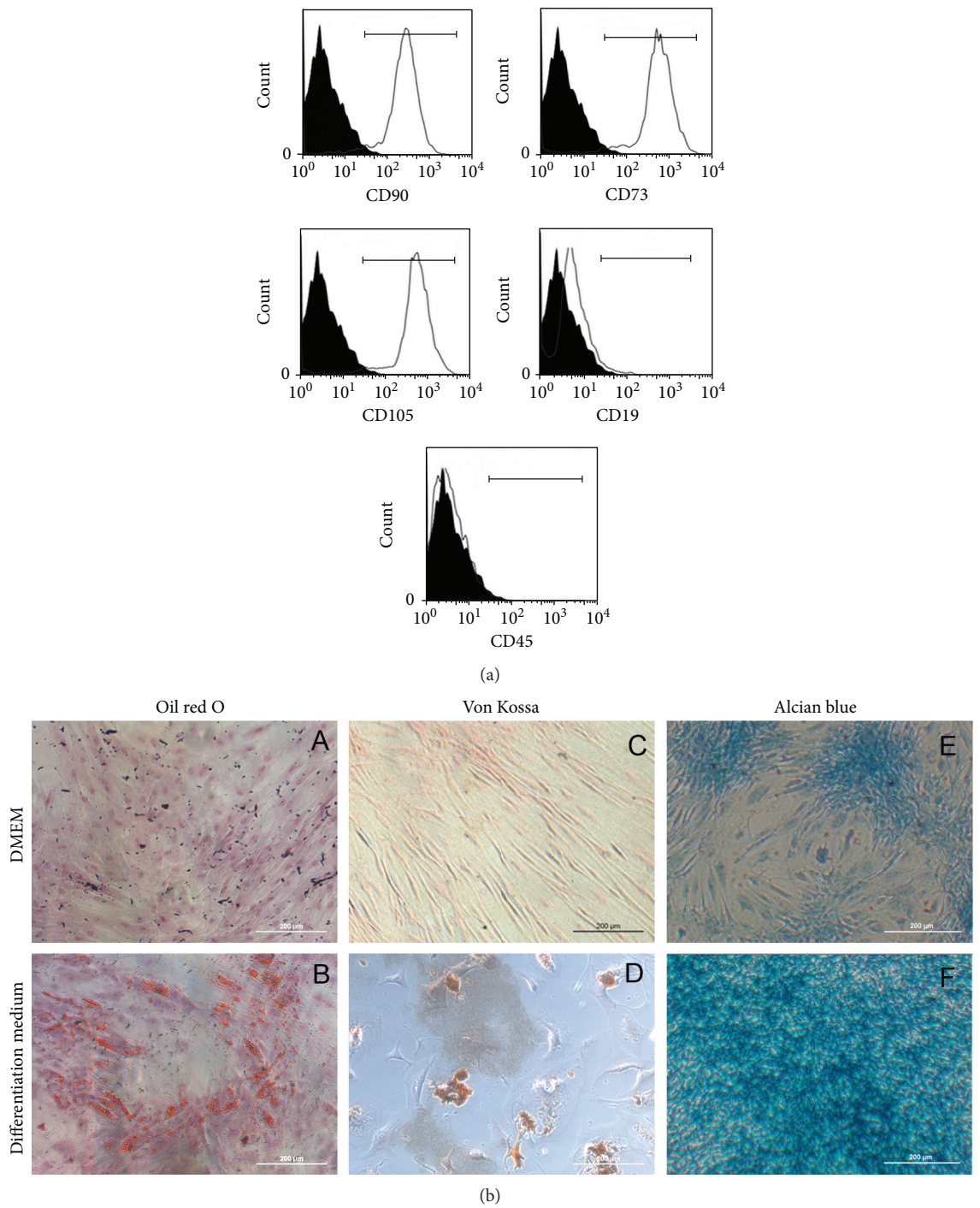

Figure 1: MSC characterization. (a) Cytometry graphs in solid black curves refer to the negative controls (cells incubated with secondary antibody) and nonfilled curves indicate the marker tested. The cell population expressed CD90, 73, and 105 and did not express CD19 and 45. (b) Brightfield images are showing that MSCs are able to differentiate in adipocytes, osteocytes, and chondrocytes. Undifferentiated cells were grown only in DMEM (A, C, and E) and show only background staining for oil red O on (A), Von Kossa on (C), and alcian blue on (E). Once incubated in differentiation-inducing culture mediums, adipose-derived MSCs showed positive staining for oil red O on (B), Von Kossa on (D), and for alcian blue on (F). Scale Bar = 200 μm. Representative images of three biological replicates that were conducted independently.

were all purchased from Thermo Fisher Scientific: mirVana miRNA inhibitor, hsa-miR-205-5p (assay ID: MH11015; catalog number: 4464084); mirVana miRNA mimic, hsa-miR-205-5p (assay ID: MC11015, catalog number: 4464066); and mirVana miRNA inhibitor negative control number 1 (catalog number: 4464076). After the incubation, the

transfection medium was discarded and replaced by DMEM or KSFM, depending on the cell type. Tests were performed after 48 h.

2.14. Statistical Analysis. The data shown represent at least three independent experiments and are expressed as the mean ± standard deviation. The statistical analyses were performed with GraphPad software. The experiments with more than two data groups were compared by using one-way ANOVA and the Bonferroni posttest. P values of at least <0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. Characterization of MSCs. Initially, MSCs were characterized according to the three criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [18]. First, cell-surface antigen profile was ascertained by staining the cells with specific monoclonal antibodies, followed by cytometry analyses (Figure 1). We investigated the expression levels of cell markers CD90 (91.4% ± 0.1%), CD73 (92.9% ± 0.1%), CD105 (92% ± 0.1%), CD19 (4.9% ± 0.1%), and CD45 (1.3% ± 0.1%). These percentages of labeled cells indicated acceptable culture purity according to the criteria proposed by Bourin et al. [18]. MSCs adhered to the plastic material of the plates and showed fibroblast-like morphology (polygon-like or spindle-like with processes, Figure 1(b)). Finally, MSCs were capable of differentiating into adipocytes, osteoblasts, and chondrocytes (Figure 1(b)). After 21 days of exposure to differentiation medium followed by oil red O, Von Kossa, or alcian blue staining, it was possible to observe differentiation of stem cells into different types of cells, which displayed fat droplets in their cytoplasm, mineral deposits, and proteoglycan inclusions, respectively. These results suggested that the cell population isolated from subcutaneous adipose tissue in this study indeed comprised true MSCs, classified according to the criteria of the Tissue Stem Cell Committee of the International Society for Cellular Therapy [18].

3.2. Characterization of EVs Isolated from MSCs. Next, EVs were analyzed by a NanoSight LM 10 system, using Nanoparticle Tracking Analysis (NTA) software. Results of these experiments are shown in Table 1 and in Figure 2. The average diameter of the vesicles observed in our preparations was 135 nm. We also determined their density, spreading speed, and sample viscosity. As shown in Figure 2, the curve base is broad, indicating that there is variation in size within the population. Accumulating data support the notion that the content, size, and membrane composition of EVs are highly heterogeneous and dynamic, depending on the cellular source, state, and environmental conditions [19, 20]. Also, we performed Western blot for Alix and CD9, two proteins present in EVs. Many efforts have been made by the scientific community to isolate EV subtypes, but the results were inconclusive. Hence, in this work, we assumed that we dealt with a mixture of different EV populations [19].

Table 1: Extracellular vesicles isolated from MSCs conditioned by serum-free DMEM.

Characteristic	Sample data
Average size	135 nm
Concentration	1.89×10^8 particles/mL
Spreading speed	1.201 nm·s^{-1}
Sample viscosity	0.90 cP

Data were generated by the Nanoparticle Tracking Analysis software.

3.3. EVs Increased Proliferation of Fibroblasts and Keratinocytes. To assess whether MSC EVs could induce proliferation of fibroblasts and keratinocytes *in vitro*, cells of both types were exposed to MSC EVs and two kinds of experiments were conducted. As shown in Figure 3(a), growth curves of fibroblasts and keratinocytes indicated that MSC EVs increased cell proliferation, which would be a beneficial factor for wound healing [9, 21]. Western blot analysis showed greater phosphorylation of histone H3, a mitosis marker [22], in cells of both types when exposed to MSC EVs (Figures 3(b) and 3(c)). Those results indicated that EVs modified epithelial cell proliferation. Previously, it has been reported that EVs from stem cells induced cell proliferation by modulating microenvironments and promoting mitosis [23, 24]. As cell proliferation is a key step in skin wound healing, and its impairment is directly associated with the formation of chronic wounds, the capacity of stem cell EVs to accelerate this process may be a useful therapeutic feature.

3.4. EVs Induced Migration of Fibroblasts and Keratinocytes. Epidermal keratinocytes and dermal fibroblasts were used in scratch wound healing assays, as described in Materials and Methods. As shown in Figure 4, the presence of EVs in the culture medium was associated with a more rapid decrease in the area affected by the initial wound, indicating increased migration after 24 h of exposure to MSC EVs in cells of both types. Cell migration in the wound bed is essential for skin wound healing, and it is known to be affected in some chronic conditions, for example, in diabetic chronic wounds [25]. It has been suggested that one of the reasons of skin cell migration impairment in chronic wounds is the depletion of stem cells in those areas, which creates imbalance in the microenvironment and decelerates wound healing process. It will be interesting to substantiate in more direct experiments whether MSC EVs and their signals can indeed modulate cell migration, as suggested by our results (Figure 4) and by data from other groups [23, 24].

3.5. EVs Accelerated Wound Healing. Male Wistar rats were subjected to the excisional wound-splinting procedure and then exposed to aqueous hydroxyethyl cellulose gel with or without MSC EVs for 7, 14, and 21 days. We found that treated animal wounds closed more rapidly than control wounds (Figure 5(a)). Furthermore, we observed that in rats treated with the gel enriched with MSC EVs, wound closure occurred at a faster rate than in animals treated with EV-free gel (Figure 5(b)). Acceleration of wound

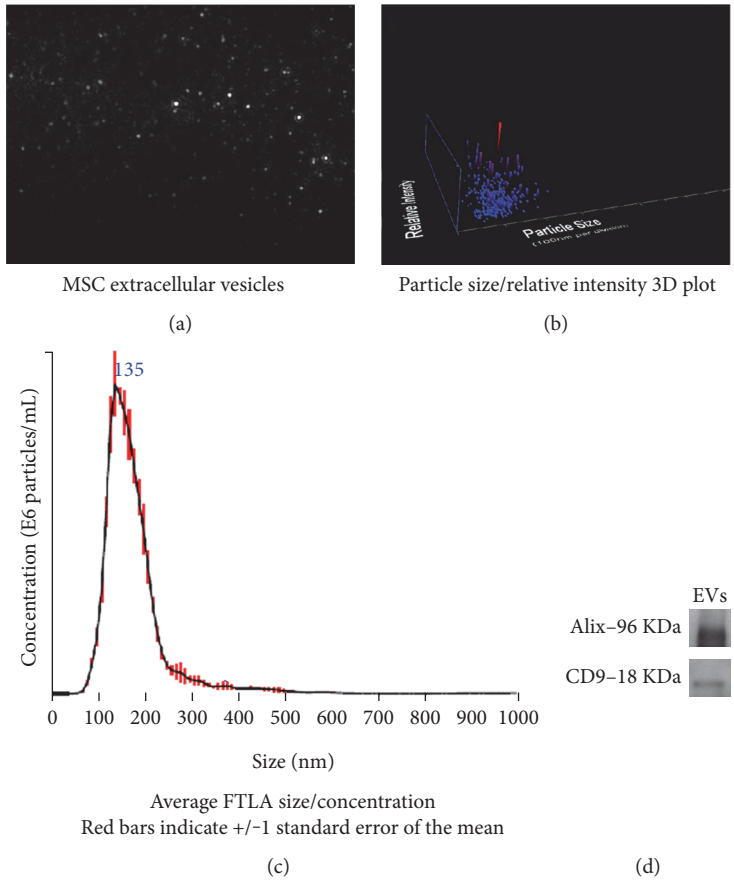

Figure 2: Physical characteristics of extracellular vesicles derived from mesenchymal stem/stromal cells. (a) Snapshot of extracellular vesicles derived from mesenchymal stem/stromal cells in solution shown as gray dots on a black background. (b) 3D plot of extracellular vesicle size distribution. The red dot represents vesicles with the predominant diameter. (c) 2D plot of extracellular vesicle size distribution. Average diameter size is 135 nm. (d) Western blot for two proteins present in EVs, Alix, and CD9. Representative images of at least three biological replicates that were conducted independently.

healing by stem cell-derived EVs has been reported previously. However, in those experiments, more invasive treatments, namely, intradermal injection of stem cell EV preparations were used [26]. In this work, we demonstrated comparable acceleration of wound healing by topical application of MSC EVs, which is a more advantageous setting that causes less therapeutic discomfort. Our results seem to be in accordance with suggestions of other authors about a promising potential of MSC EVs in skin wound healing [26, 27].

3.6. EVs Promoted AKT Phosphorylation in Fibroblasts and Keratinocytes. The AKT pathway is considered one of the major biochemical pathways regulating migration of epithelial cells. To assess whether MSC EVs induced AKT activation, Western blot assays were performed in fibroblasts and keratinocytes. As shown in Figure 6, exposure of keratinocytes and fibroblasts to MSC EVs was associated with higher levels of phosphorylated AKT, which would increase the overall activity of the AKT pathway. Sometimes, there are some double bands in the Western blots for total AKT. The heaviest band was used for quantifications that was the band always present in the phosphorylated form. Recently, other authors found that AKT phosphorylation was higher in skin cells exposed to MSC EVs [10, 11]. Those results are interesting from the therapeutic point of view, as modulation of the AKT pathway activity may be a promising avenue of research into novel stimulators of wound healing process.

3.7. Exposure of Fibroblasts and Keratinocytes to EVs Increased miR-205 Expression. It has been shown that one of the most abundant microRNAs in the skin, miR-205, is an important modulator of AKT pathway activity [11]. Moreover, we found the presence of miR-205 in EV samples from MSCs, through the Next Generation Sequencing experiments (data not shown). To evaluate whether MSC EVs modulated miR-205 expression, qPCR experiments were conducted in fibroblasts and keratinocytes after exposure to EVs for 24 h. We found that treatment with MSC EVs led to a ~3-fold increase in miR-205 expression level (Figure 7(a)). Although miR-205 is present inside the EVs from stem cells, we could not assume at this point that the increased expression observed in qPCR experiments is strictly due to the transfer from

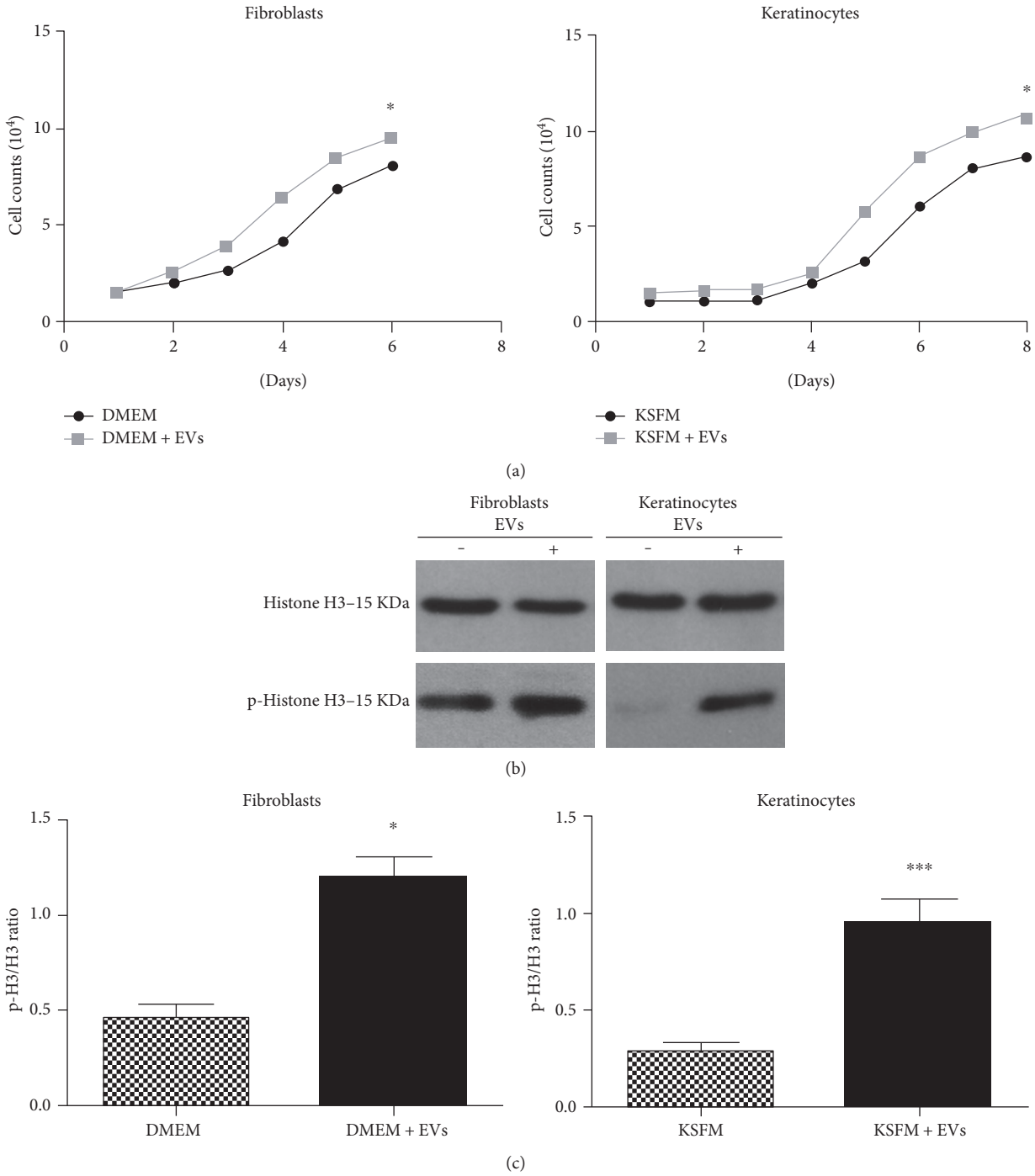

Figure 3: Extracellular vesicles derived from mesenchymal stem/stromal cells induce proliferation of fibroblasts and keratinocytes. (a) Growth curves comparing proliferation of fibroblasts (left graph) and keratinocytes (right graph) cultured in medium (DMEM or KSFM depending on cell type) in the absence (medium) or presence of extracellular vesicles (medium + EVs) derived from mesenchymal stem/stromal cells (MSCs). (b) A representative Western blot gel illustrating bands for total and phosphorylated histone H3 (p-histone H3). (c) Densitometry analysis of the p-H3/total H3 ratio. Incubation with MSC EVs was associated with higher relative levels of phosphorylated (activated) histone H3 in both fibroblasts and keratinocytes. Data are presented as the mean ± standard deviation of three biological independent experiments. Statistical significance of differences is indicated as follows: $^{*}P < 0.05$; $^{***}P < 0.001$.

EVs to cells with the experiments described here. As widely known, microRNA biology is complex and could be modulated by several factors, including other microRNAs [10, 11].

Next, we carried out qPCR experiments to verify expected changes in cellular miR-205 levels after its overexpression or knockdown. Transfection of 25 nM of miR-205 sequence increased the expression level of this microRNA ~ 6-fold in

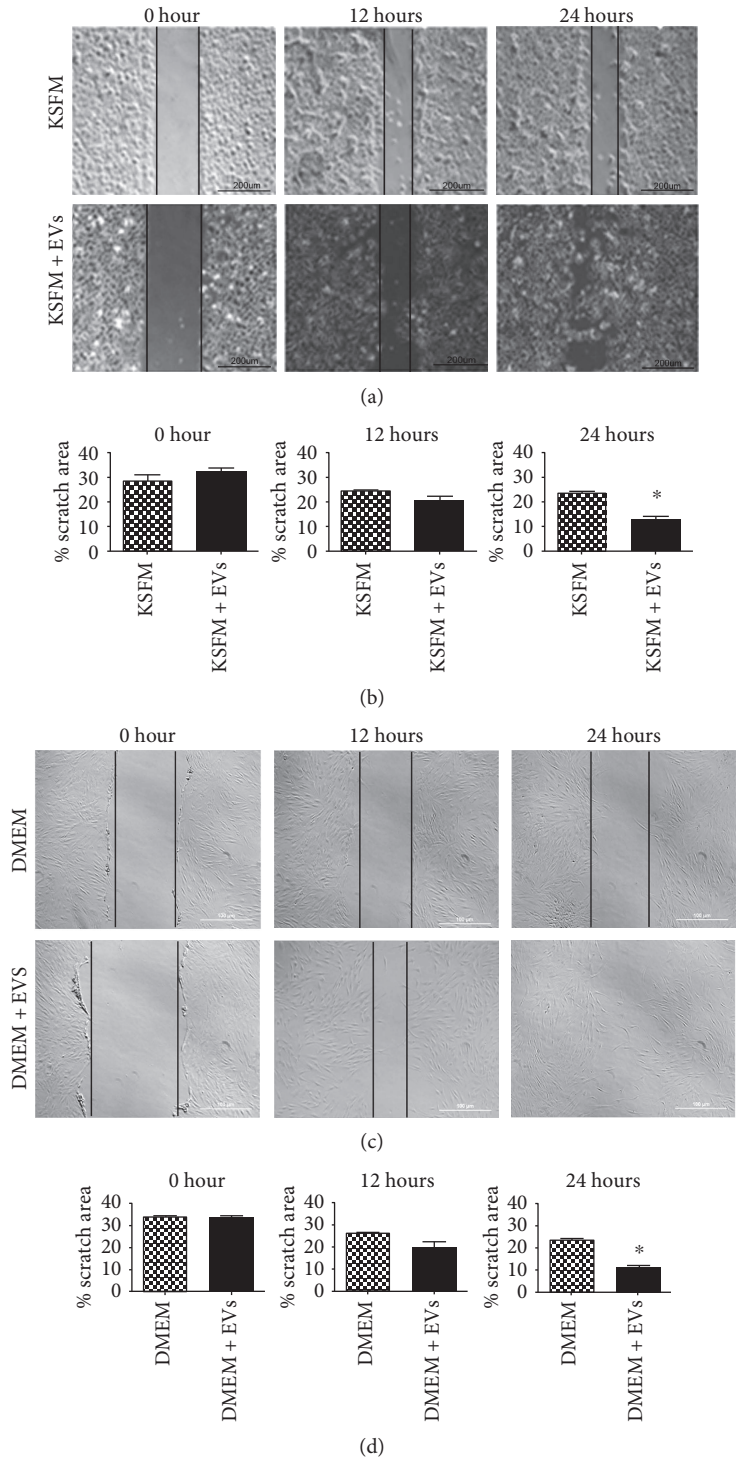

Figure 4: Extracellular vesicles derived from mesenchymal stem/stromal cells induce keratinocyte and fibroblast migration. (a) Phase contrast images of human keratinocytes cultured in DMEM in the absence (KSFM) or presence of extracellular vesicles (KSFM + EVs) derived from mesenchymal stem/stromal cells (MSCs) in 0, 12, and 24 h after scratch. Scale bar = 200 μm. (b) Relative areas affected by the scratch wound at the three time points of the experiments. Significantly smaller scratch wound area was observed in 24 h after the scratch following the exposure to MSC EVs, indicating faster keratinocyte migration. Data are presented as the mean ± standard deviation of three independent experiments. Statistical significance of differences is indicated as follows: $^*P < 0.05$. (c) Phase contrast images of human fibroblasts cultured in DMEM in the absence (DMEM) or presence of extracellular vesicles (DMEM + EVs) derived from mesenchymal stem/stromal cells (MSCs) in 0, 12, and 24 h after scratch. Scale bar = 200 μm. (d) Relative areas affected by the scratch wound at the three time points of the experiments. Significantly smaller scratch wound area was observed in 24 h after the scratch following the exposure to MSC EVs, indicating faster fibroblast migration. Data are presented as the mean ± standard deviation of three biological independent experiments.

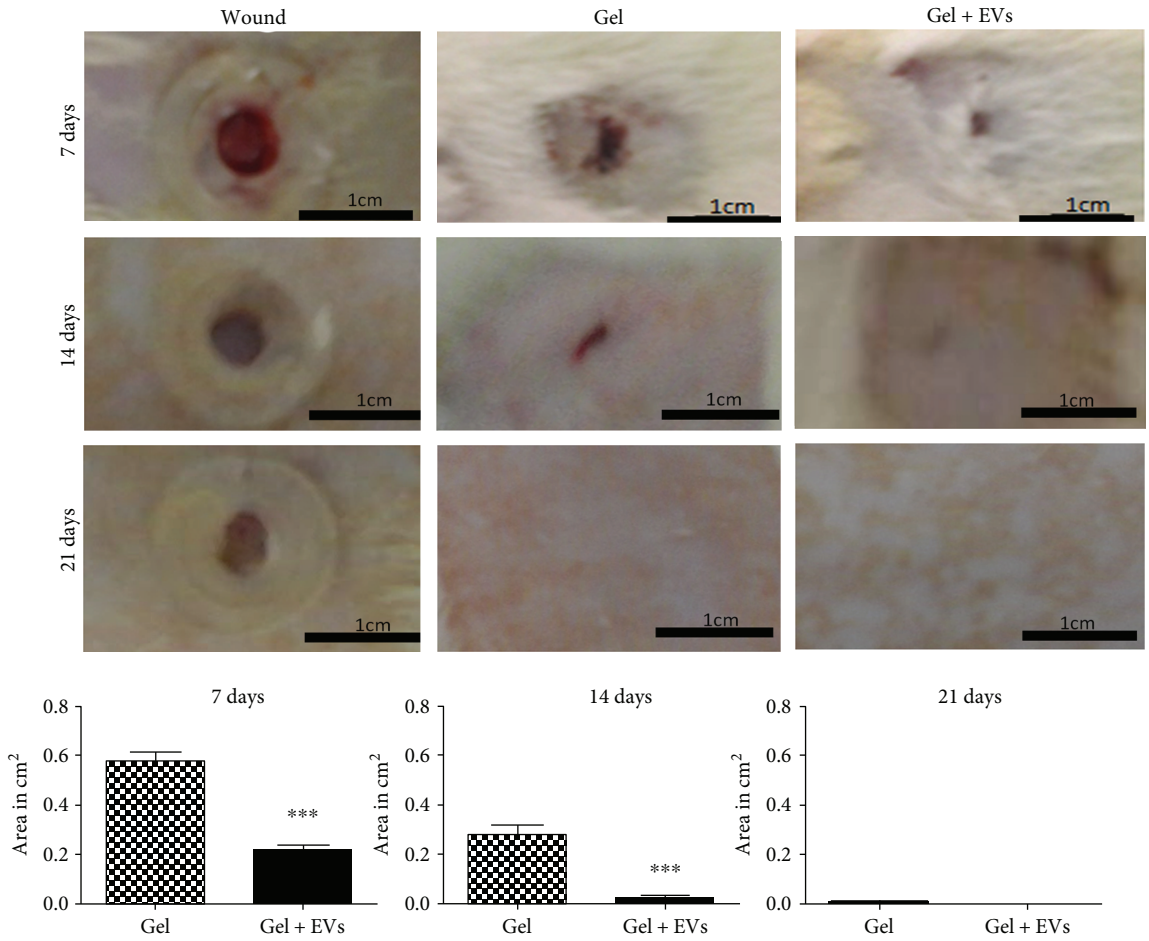

Figure 5: Gel enriched with extracellular vesicles derived from mesenchymal stem/stromal cells accelerated wound healing in rat excisional wound-splinting model. (a) Representative photographs of rat skin wounds that were either untreated (left column), exposed to hydroxyethyl cellulose gel (middle column), or exposed to hydroxyethyl cellulose gel enriched with extracellular vesicles (EVs) derived from mesenchymal stem/stromal cells at different time points of the experiment (7, 14, and 21 days after the wound was created). (b) Comparison of relative wound areas between negative control (gel) and test groups (gel + EVs). Data are presented as the mean ± standard deviation of three biological independent experiments. Statistical significance of differences is indicated as follows: $^{***}P < 0.001$.

both fibroblasts and keratinocytes (miR-205 mimic). We also examined the consequences of miR-205 knockdown in cells before (siRNA group) and after exposure to EVs (siRNA + EVs). We achieved 100% knockdown in both cell groups transfected with siRNA. Expression levels of miR-205 in cells exposed only to the transfection reagent lipofectamine (lipofectamine group) or lipofectamine plus a scramble sequence (scramble group) did not change, as expected (Figure 7(b)).

We also examined the effects of miR-205 overexpression and knockdown on AKT activation by performing Western blot analysis of the levels of phosphorylated and total AKT (Figure 7(c)). In human fibroblasts and keratinocytes, miR-205 overexpression did not induce significant AKT phosphorylation compared to the levels detected in cells treated with lipofectamine or scramble RNA. This result contrasted with the data reported by Yu et al. [11] who showed that miR-205 could induce AKT activation in keratinocytes. This could be attributed to the miR-205 concentration used. We decided not to increase the concentration of siRNA because we already had a 6-fold change in cells transfected with miR-205 mimic sequence that was around twice the amount of miR-205 found in the cells exposed to EVs. However, miR-205 knockdown led to lower levels of phosphorylated AKT in cells of both types, as previously demonstrated.

Properties of fibroblasts and keratinocytes exposed to MSC EVs were similar when miR-205 was silenced. They exhibited higher levels of AKT phosphorylation than cells treated with lipofectamine only. These results indicated that AKT activation upon exposure to MSC EVs was likely independent of miR-205 expression. Considering the complexity of EV cargo, it is known that other molecules present in the vesicles could work collectively, modulating the microenvironment, favoring migration in skin cells. We are currently analyzing others RNAs present to the EVs to further investigate other potential targets for wound healing process. Also, several growth factors could be present in the EVs. Perhaps many molecules could participate in the effects described in this paper.

Finally, we investigate the migration effects of miR-205 overexpression and knockdown in fibroblasts and

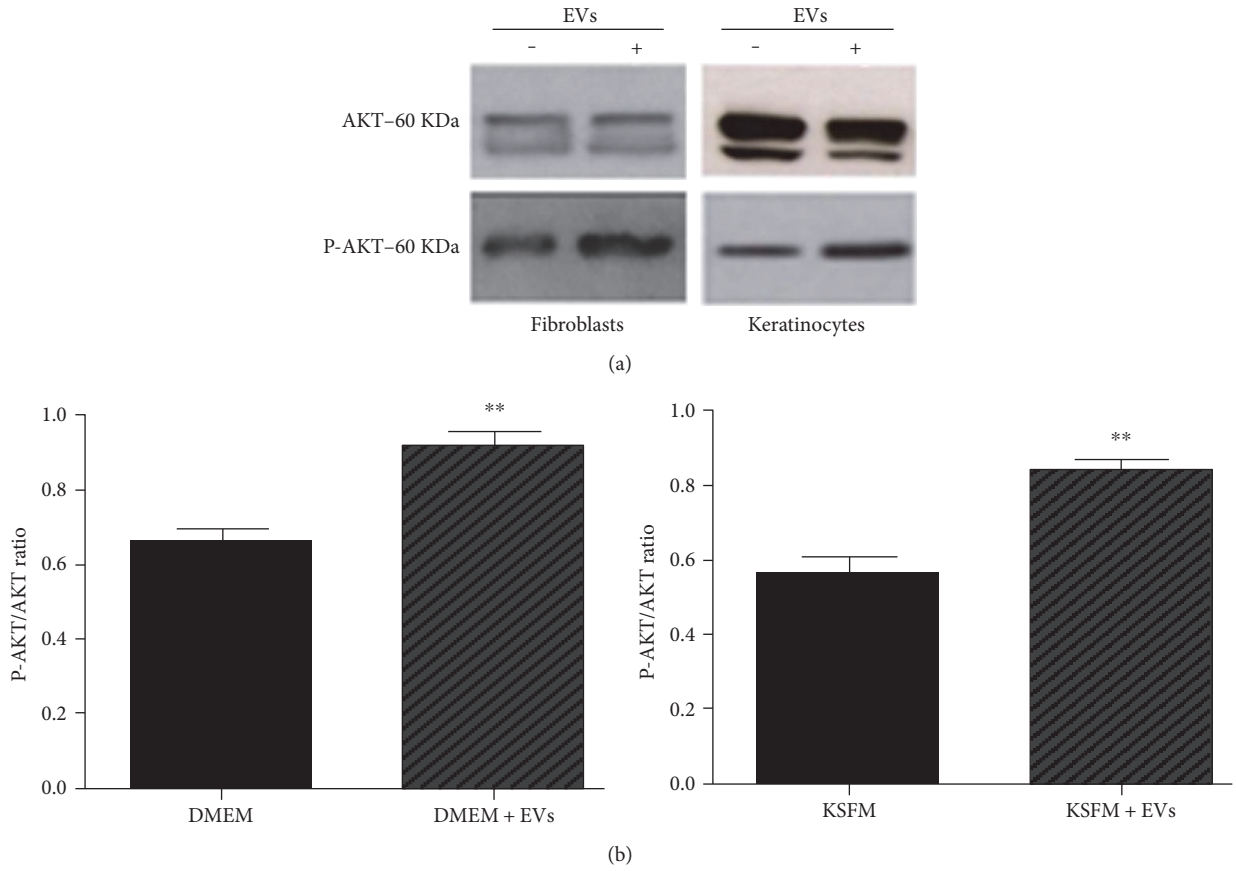

Figure 6: Exposure to extracellular vesicles derived from mesenchymal stem/stromal cells induced AKT phosphorylation in human keratinocytes and fibroblasts. (a) A representative Western blot gel illustrating bands for total and phosphorylated AKT (p-AKT) in cells before and after exposure to extracellular vesicles (EVs) derived from mesenchymal stem/stromal cells (MSCs). (b) Densitometry analysis of the p-AKT/total AKT ratio. Incubation with MSC EVs was associated with higher relative levels of phosphorylated (activated) AKT in both fibroblasts (left graph) and keratinocytes (right graph). Data are presented as the mean ± standard deviation of three biological independent experiments. Statistical significance of differences is indicated as follows: $^{**}P < 0.01$.

keratinocytes (Figure 8). The migration of fibroblasts was not affected by miR-205 overexpression or knockdown, but knockdown cells exposed to EVs showed faster migration. This result suggests that the migration of fibroblasts was independent of miR-205 and that EVs could have other molecules responsible for increasing fibroblast migration. On the other hand, migration of keratinocytes was increased by miR-205 overexpression. Accordingly, the knockdown of miR-205 made migration of keratinocytes similar to the levels of control groups, but the knockdown cells exposed to EVs had an increased migration. Taken together, these results suggest that miR-205 is important for keratinocyte migration, but, for the migration effect induced by EVs, its expression seems not to be essential.

Many studies are showing a complex role of miR-205 in many models [28]. For example, In HaCaT keratinocytes, miR-205 knockdown could promote migration in scratch wound healing assay. This could be due to the different cells used or because of the complex network of miR-205 [29]. In cancer, it acts either as an oncogene through facilitating tumor proliferation and initiation or as a tumor suppressor by inhibiting invasion and proliferation [28].

Considering the complexity of EV cargo, it is known that other molecules are present such as several growth factors and mRNAs. Perhaps many molecules could participate in the effects described in this paper. It is important, then, to fully elucidate the content of those EVs in terms of mRNAs, microRNAs, proteins, and other classes of molecules as to further explain the effects observed in this work.

4. Conclusion

In summary, our data suggest that cell migration and proliferation, key processes in skin wound healing, can be enhanced by exposure to MSC-derived EVs, which activate the AKT pathway in a miR-205-independent manner. Topical administration of a gel containing MSC EVs accelerated wound healing in an animal model, and this is an interesting finding as it opens perspectives for noninvasive model studies. For further development of this approach, it will be interesting to determine EV cargo and the molecules and mechanisms that mediate beneficial effects of MSC-derived EVs observed in this study.

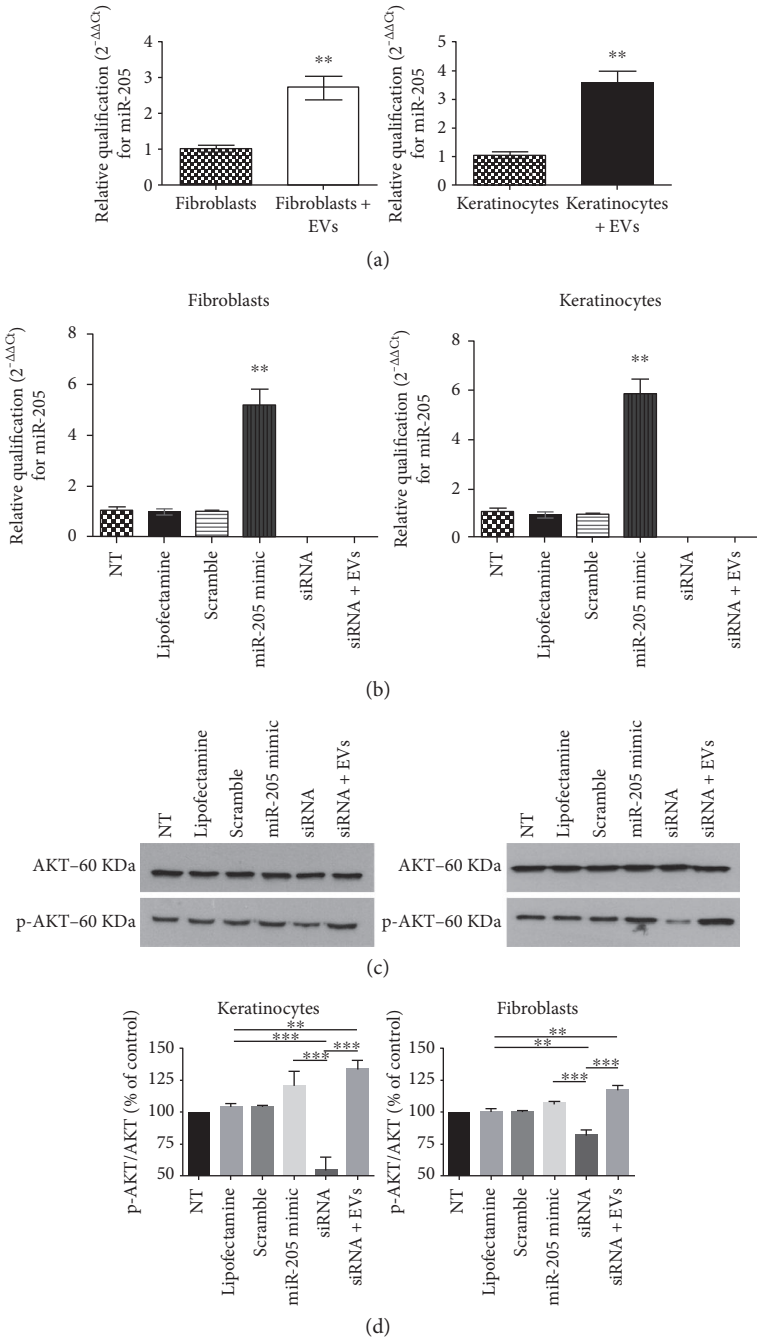

Figure 7: Effects of extracellular vesicles derived from mesenchymal stem/stromal cells on AKT phosphorylation are independent of miR-205. (a) Graphs illustrate relative levels of miR-205 in human fibroblasts and keratinocytes cultured in standard culture medium or in the medium containing EVs derived from mesenchymal stem/stromal cells (MSCs). Relative expression of miR-205 was higher in cells exposed to MSC EVs. qPCR data were normalized using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the mean ± standard deviation of three independent experiments. Statistical significance of differences is indicated as follows: $^{**}P < 0.01$. (b) Quantification of miR-205 relative expression levels by qPCR in fibroblasts (left graph) and keratinocytes (right graph) from the following experimental groups: nontreated (NT), exposed only to lipofectamine only (lipofectamine), transfected with scramble miR (scramble), transfected with synthetic miR-205 (miR-205 mimic), transfected with siRNA for miR-205 (siRNA), and, finally, transiently silenced for miR-205 and exposed to extracellular vesicles from mesenchymal stem/stromal cells (siRNA + EVs). Data are presented as the mean ± standard deviation. Statistical significance of differences is indicated as follows: $^{**}P < 0.01$. (c) Representative Western blot gels illustrating bands for total and phosphorylated AKT (p-AKT) from experimental groups described previously. (d) Densitometry analysis of the p-AKT/total AKT ratio in lysates of cells from different experimental groups. Data are presented as the mean ± standard deviation of three biological independent experiments. Statistical significance of differences is indicated as follows: $^{**}P < 0.01$; $^{***}P < 0.001$.

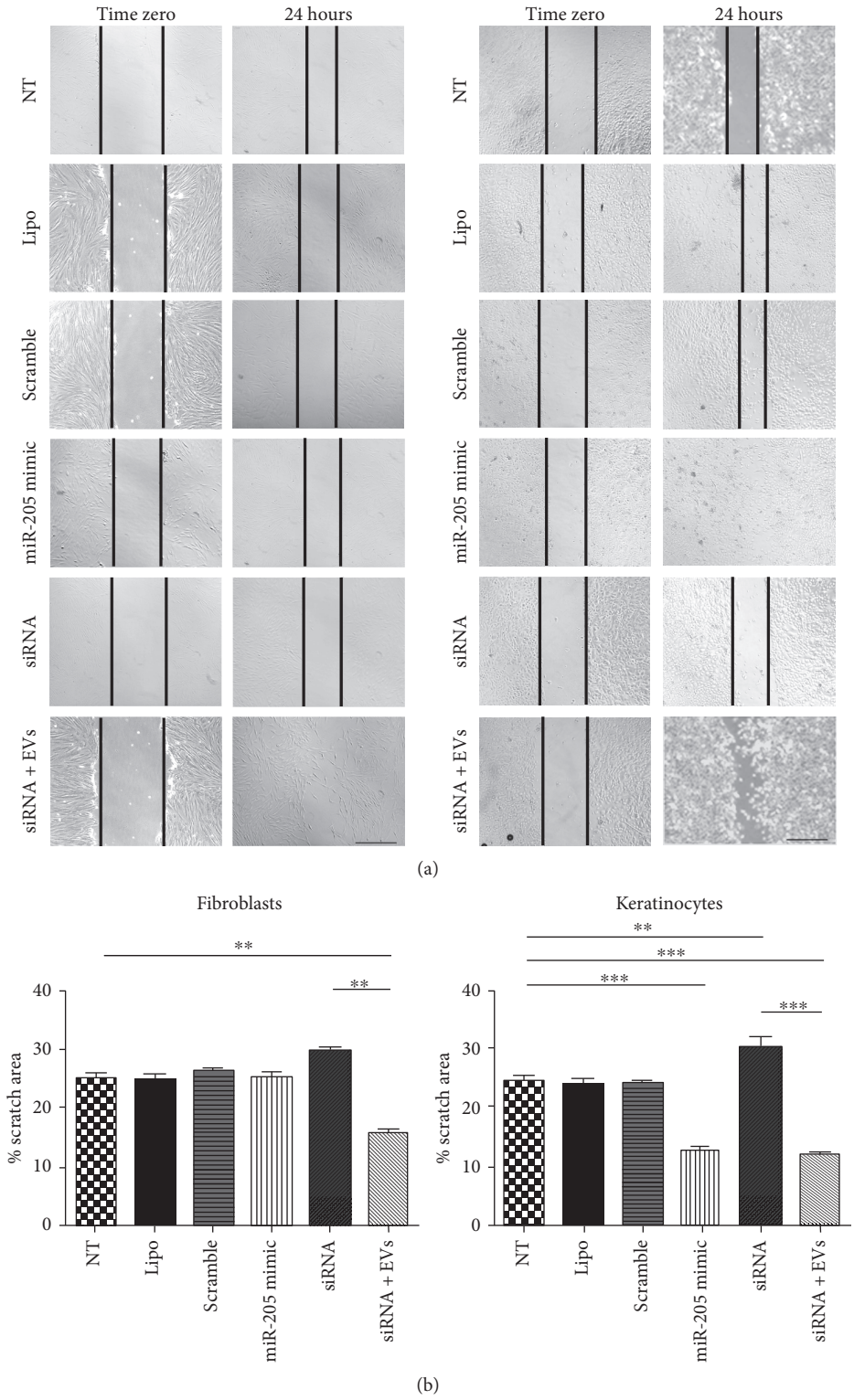

Figure 8: Effects of extracellular vesicles on the migration of fibroblasts and keratinocytes do not require miR-205. (a) Phase contrast images of scratch wound straight after the scratch was made (zero) and in 24 h after that in fibroblasts (left graph) and keratinocytes (right graph) from the following experimental groups: nontreated (NT), exposed only to lipofectamine only (lipofectamine), transfected with scramble miR (scramble), transfected with synthetic miR-205 (miR-205 mimic), transfected with siRNA for miR-205 (siRNA), and, finally, transiently silenced for miR-205 and exposed to extracellular vesicles from mesenchymal stem/stromal cells (siRNA + EVs). Scale bar = 200 μm. (b) Relative areas affected by the scratch wound in 24 h in all experimental groups. Data are presented as the mean ± standard deviation of three biological independent experiments. Statistical significance of differences is indicated as follows: $^{**}P < 0.01$; $^{***}P < 0.001$.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the NIH grant 1R03TW008709 and by grants from the "INCT-Regenera," "Rede Mineira de Engenharia de Tecidos e Terapia Celular (REMETTEC, RED-00570-16)," FAPEMIG, CAPES, and CNPq. The authors also thank "Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais" for its financial sponsorship. The authors would like to thank Editage (http://www.editage.com) for English language editing and Carolina de Andrade for Western blot assistance.

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Hindawi
Stem Cells International
Volume 2017, Article ID 9275248, 12 pages
https://doi.org/10.1155/2017/9275248

Research Article

Effect of Isolation Technique and Location on the Phenotype of Human Corneal Stroma-Derived Cells

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Received 29 March 2017; Revised 11 July 2017; Accepted 27 August 2017; Published 29 October 2017

Academic Editor: Karen Liu

Purpose. To determine the effect of the isolation technique and location upon the phenotype of human corneal stroma-derived cells (CSCs). *Methods*. CSCs were isolated from the corneal stroma center and periphery using the explant or enzymatic digestion technique. The native tissue was stained for functional markers, while cultured cells were analysed by FACS. PCR was used to determine gene expression in the cultured versus native cells. *Results*. The native stroma was positive for α-actinin, ALDH1A1, CD31, CD34, Collagen I, and Vimentin. Cultured cells expressed CD73, CD90, CD105, CD51, Nestin, CD49a, CD49d, ABCG2, and CD47. PCR demonstrated a significant upregulation of ALDH1A1, AQP1, ITGB4, KLF4, CD31, CD34, and CXCR4 in the native tissue, while the expression of ABCG2, ITGAV, Nestin, CD73, CD90, CD105, and Vimentin were significantly higher in the cultured cells. GPC did not change. *Conclusion*. The study finds no significant difference between the phenotype of CSCs generated by the explant or enzymatic digestion technique from the center or periphery of the stroma. Isolation of the cells can be performed without regard to the location and isolation technique used for research. Cultivated CSCs undergo a complete surface marker and genotype profile change compared to the state in situ.

1. Introduction

The human corneal stroma is responsible for two-thirds of the refractive power of the eye and occupies 90% of the corneal thickness. When affected by disease or trauma, the homeostasis and thus transparency of the tissue is compromised. This is partially due to presence of local edema and activation of resident corneal cells—keratocytes. These quiescent cells assume a dendritic cell morphology *in vivo* and synthesize collagens and proteoglycans forming the backbone of the tissue [1]; when activated, keratocytes can transform into myofibroblasts, associated with scar formation and ultimately loss of corneal transparency [2, 3]. Treatment for such cases usually involves lamellar or penetrating keratoplasty, but due to donor scarcity worldwide, alternative sources are seeked upon, such as bioengineered or decellularised corneas or prostheses [4, 5].

Cells derived from the human corneal stroma have been shown to possess trilineage differentiation potential and presence or absence of specific markers (e.g., $CD73^+$, $CD90^+$, $CD105^+$, $CD34^-$, $CD45^-$, $CD14^-$, $CD11b^-$, $CD79\alpha^-$, $CD19^-$, and HLA-DR^-), while adherence to plastic and exposure to serum render these cells equivalent to mesenchymal stem cells (MSCs) [6, 7]. Moreover, the immunosuppressive potential of CSCs has been demonstrated previously [8, 9], as well as their cell therapy potential in animal studies [10].

Establishment of CSC cultures is relatively easy. Nevertheless, many countries have access only to peripheral tissue remaining in unused corneal rings after keratoplasty. Use of enzymatic digestion to isolate CSCs out of the tightly packed collagen layers appears straightforward as well [11, 12], while the explant culture method of isolating pieces of corneal tissue to produce cells ex vivo has already been established. Many speculations yet remain about the true origin of the

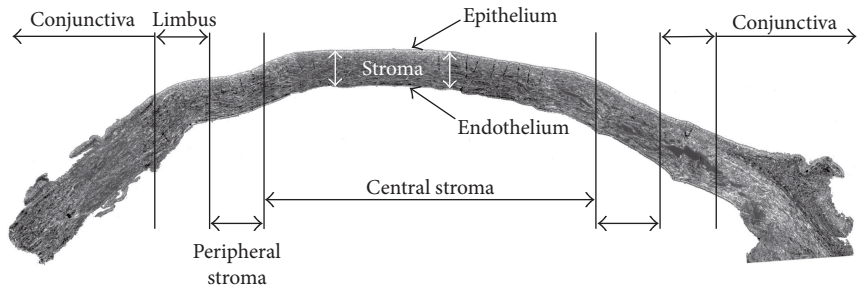


Figure 1: Anatomical features of the human cornea and sites of stromal cell isolation. Pieces of tissue were punched out from the indicated central and peripheral corneal regions by a surgical trephine (3 mm) for consequent digestion and/or culturing. The picture was taken by a phase contrast microscope and put together as an overlay (EVOS® FL microscope, Thermo Fisher Scientific).

outgrowing cells from the corneal stroma ex vivo [9, 12]. Most likely, it is the resident cells of the corneal stroma that get activated after isolation and possibly different side populations become dominant, eventually generating a culture of cells displaying a fibroblastoid morphology [9, 13, 14].

Various types of culture media have also been assessed to elucidate the best possible conditions for induction of *in vitro* stem cell phenotype in these cells [15]; however, no comparison to date has been carried out to determine whether cells isolated from different locations (cornea center versus periphery) display any difference. The present study aims to establish cornea stromal cultures by explant and enzymatic digestion methods from central and peripheral parts of the cornea and compare their phenotypical and genotypical properties for future application of these cells in corneal research or cell therapy purposes.

2. Materials and Methods

2.1. Isolation Procedure and Cultivation. Cadaveric tissue collection complied with the directive of the Helsinki Declaration and was approved by the National Medical Research Council (14387/2013/EKU-182/2013). Samples were obtained within 24 hours from death. Following disinfection by povidone iodine (Egis, Hungary) and rinsing with PBS of human bulbi, corneal buttons were dissected using scissors. The corneal epithelium, Descemet's membrane, and corneal endothelium were peeled off. To obtain equal-sized stromal explants, pieces of tissue measuring 3 mm in diameter were punched out from the corneal buttons with the help of a trephine from regions defined as the peripheral versus the central stroma, as shown in Figure 1. An equal number of punches were generated from the central versus peripheral regions in the same way described above and treated with 3 mg/mL (>125 CDU/mg) mixed collagenase solution (Sigma-Aldrich, St. Louis, MO, USA) for 3 hours at 37°C, with agitation.

Four types of CSC cultures were defined as central explant (CE) and peripheral explant (PE) versus central digested (CD) and peripheral digested (PD) from the same donors, referring to the location and presence or absence of enzymatic digestion, respectively. 24-well plates (Corning Costar, Sigma-Aldrich) were used to expand the cells. Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% Fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin-streptomycin (PS) (Sigma-Aldrich) was applied to the cells. Culture media was changed every alternate day. Cells up to passage 4 were used for the experiments.

2.2. Immunofluorescent Staining for Proliferation Marker Ki-67 in Cultured CSCs. CD, PD, CE, and PE were expanded in 24-well culture plates (Corning Costar). Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) and permeabilised using 0.1% Triton X-100 (Sigma-Aldrich). Bovine serum albumin (BSA) 1% (Sigma-Aldrich) diluted in phosphate-buffered saline (PBS) was applied as a blocking solution for 1 hour at room temperature. Samples were incubated with the primary Ki-67 (Sigma-Aldrich) antibody for 1 hour at room temperature. A phycoerythrin-conjugated secondary antibody was used to visualize the protein and finally, 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) counterstaining to stain the cell nuclei. Pictures were taken by an EVOS FL microscope (Thermo Fisher Scientific).

2.3. Immunofluorescent Staining of Native Corneal Tissue. For studying the protein expression in situ, corneal sections were prepared from paraffin-embedded tissues and stained for markers expressed by progenitor and/or stem cells (ABCG2, CXCR4, and Nestin). Proliferation- (Ki-67), function-related (ALDH1A1, Collagen I, and CD34), and MSC markers (CD73, CD90, CD105, and Vimentin), extracellular matrix and cell-adhesion components (Fibronectin, Collagen IV, and VE-Cadherin), and other molecules (α-actinin, ABCG5, and antifibroblast marker) were stained. More information about the antibodies used is shown in Table S1 available online at https://doi.org/10.1155/2017/9275248.

In brief, sections were deparaffinised and the nonspecific sites were blocked by 1% BSA (Sigma-Aldrich) for 1 hour at room temperature. Primary antibodies were applied overnight at 4°C. Following three times 5-minute wash by PBS containing 1% Tween-20 (PBST), Alexa Fluor 488 conjugated secondary antibodies were incubated on the sections for 1 hour at room temperature. DAPI counterstaining was performed to visualize the nuclei. Pictures were taken by a Zeiss Axio Observer Z1 (Carl Zeiss) microscope.

2.4. Fluorescence-Activated Cell Sorting (FACS). CSCs were subcultured in 150 cm^2 flasks (TPP, Sigma Aldrich) for FACS

analyses. Cells were collected by trypsinisation (Hyclone, GE Healthcare Life Sciences, Logan, Utah, USA) for surface protein expression analyses. After centrifugation at 1000 RPM, for 10 minutes, the cells were resuspended in FACS buffer (0.05% Na-azide and 0.5% BSA in DPBS). Three-color staining—fluorescein-isothiocyanate, phycoerythrin, and allophycocyanin-conjugated primary antibodies against ABCG2, CD31, CD34, CD44, CD47, CD49a, CD49d, CD51, CD73, CD90, CD105, and Nestin—were applied for 30 minutes at 4°C. FACS Calibur cytometer (BD Biosciences, Immunocytometry Systems) was used to measure the samples. Finally, data were analysed by Flowing Software 2.5 (Perttu Terho, Turku Centre for Biotechnology, University of Turku, Finland) and FCS Express 6 (De Novo Software, California, USA). More information about the antibodies is provided in Table S2.

2.5. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis. Native corneal stroma tissue free of epithelium and endothelium was collected from 3 donors. The tissue from the donors was homogenized and pooled. Total RNA was isolated by Qiazol reagent (Qiagen) and RNeasy mini kit (Qiagen) following the manufacturer's protocol. Similarly, total RNA was isolated from cultured cells separately for the 4 defined conditions by the RNeasy mini kit and pooled from 3 donors.

Nucleic acid concentrations were determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific). Random hexamers and Superscript III reverse transcriptase (Life Technologies, Waltham, MA, USA) were used to transcribe 1 μg RNA into cDNA. StepOnePlus RT-PCR system (Applied Biosystems) and Taqman Gene Expression assays were used to determine relative gene expression levels. Genes *ALDH1A1* (Hs00605167_g1), *ABCG2* (Hs01053790_m1), *AQP1* (Hs01028916_m1), *CD31* (Hs01065279_m1), *CD34* (Hs00990732_m1), *CD73* (Hs01573922_m1), *CD90*/*THY1* (Hs00174816_m1), *CXCR4* (Hs00607978_s1), *ENG* (*CD105)* (Hs00923996_m1), *GPC4* (Hs00155059_m1), *ITGAV* (Hs00233808_m1), *ITGB4* (Hs00236216_m1), *KLF4* (Hs00358836_m1), *Nestin* (Hs00707120_s1), and *Vimentin* (Hs 00185584_m1) were used for the analyses. 10 minutes at 95°C, then 40 cycles at 95°C (15 s) and 60°C for 1 minute was set for the measurements. Analysis of the data was done by the $2^{-\Delta\Delta Ct}$ method. Fold change (relative quantity) was determined relative to the expression level of the native stromal tissue. *18S RNA* (Hs03003630_g1) was used as a housekeeping gene. All samples were run in triplicates.

2.6. Statistical Analysis. One-way ANOVA and Student's *t*-test were applied to reveal statistical differences between different groups. Significance level was set to 0.95. p values less than 0.05 ($^{*}p < 0.05$; $^{**}p < 0.01$) were considered significant.

3. Results

3.1. Cell Morphology and Proliferative Activity. Cultures established by the enzymatic method (CD, PD) yielded CSCs immediately, and these cells proliferated fast (Figure 2(a)), reaching confluence within 10–12 days (Figure 2(b)). The explant cultures from the central and peripheral regions (CE, PE) showed no microscopically observable proliferative activity up until days 12–14, when the CSCs started actually migrating and proliferating around the edges of the explants (Figure 2(a)). The explant cultures reached confluency by days 20–25 after isolation (Figure 2(b)). No apparent morphological differences were observed between the CSCs produced from different locations (center versus periphery) and isolation technique (explant versus enzymatic digestion).

Ki-67 staining revealed a strong proliferative capacity of the cells in both the explant and the digested cultures isolated from the central and peripheral regions of the corneal stroma (Figure 3(a)). From all stained cells, $4.21 \pm 1.53\%$, $7.87 \pm 4.73\%$, $8.60 \pm 4.58\%$, and $10.95 \pm 4.42\%$ were positive for Ki-67 (Figure 3(b)) for the four different conditions: CD, CE, PD, and PE, respectively ($p = 0.43$).

3.2. Immunofluorescent Staining of the Native Corneal Stroma. In order to first demonstrate the differential expression patterns of cultured versus resident cells of the cornea, the expression of markers for stemness, mesenchymal, epithelial, endothelial cell origin, and extracellular matrix components and adhesion proteins was carried out in the native cornea (Figure 4 and Table 1, for a summary of the findings). The anterior stroma is defined as the first 1/3 of the thickness of the cornea proximal to the corneal epithelium, while posterior stroma is defined as the 2/3 thickness proximal to the corneal endothelium (Figure S1).

Expression of ABCG2 and ABCG5 could not be detected in situ in any of the corneal layers or regions. Strong and similar staining for ALDH1A1 and α-actinin was observed in the central and peripheral regions of the stroma. The presence of CD34 was confirmed by a strong signal coming from all parts of the stroma in situ and CD31 was detected as well. (Figure 4).

The expression of the major stromal component, Collagen I, showed strong positivity, while absence of Collagen IV could be detected throughout the stroma. The triad of MSC markers: CD73, CD90, and CD105 was negative throughout the native cornea, and markers like Ki-67, CXCR4, and Nestin could not be detected in the tissue either. The presence of Vimentin could be confirmed in the stroma, while Fibronectin appeared to be negative in the native corneal stroma. The expression of an antireticulocyte, fibroblast marker, and VE-cadherin was also found negative in this tissue (Figure 4).

3.3. Surface Protein Expression Profile of Cultured CSCs. FACS analyses revealed a high expression of MSC markers: CD73, CD90, and CD105, with no significant difference among the culture conditions (Figure 5 and Table S3). Adhesion molecules CD51, CD49a, CD49d, and integrin-related CD47 showed an increased expression, with no significant difference being detected between the conditions. Putative stem cell markers ABCG2 and Nestin were positive, too, while CD34 and CD31 were negative. Statistical analyses revealed no significant difference in the expression of the latter proteins when comparing the various isolation conditions used.

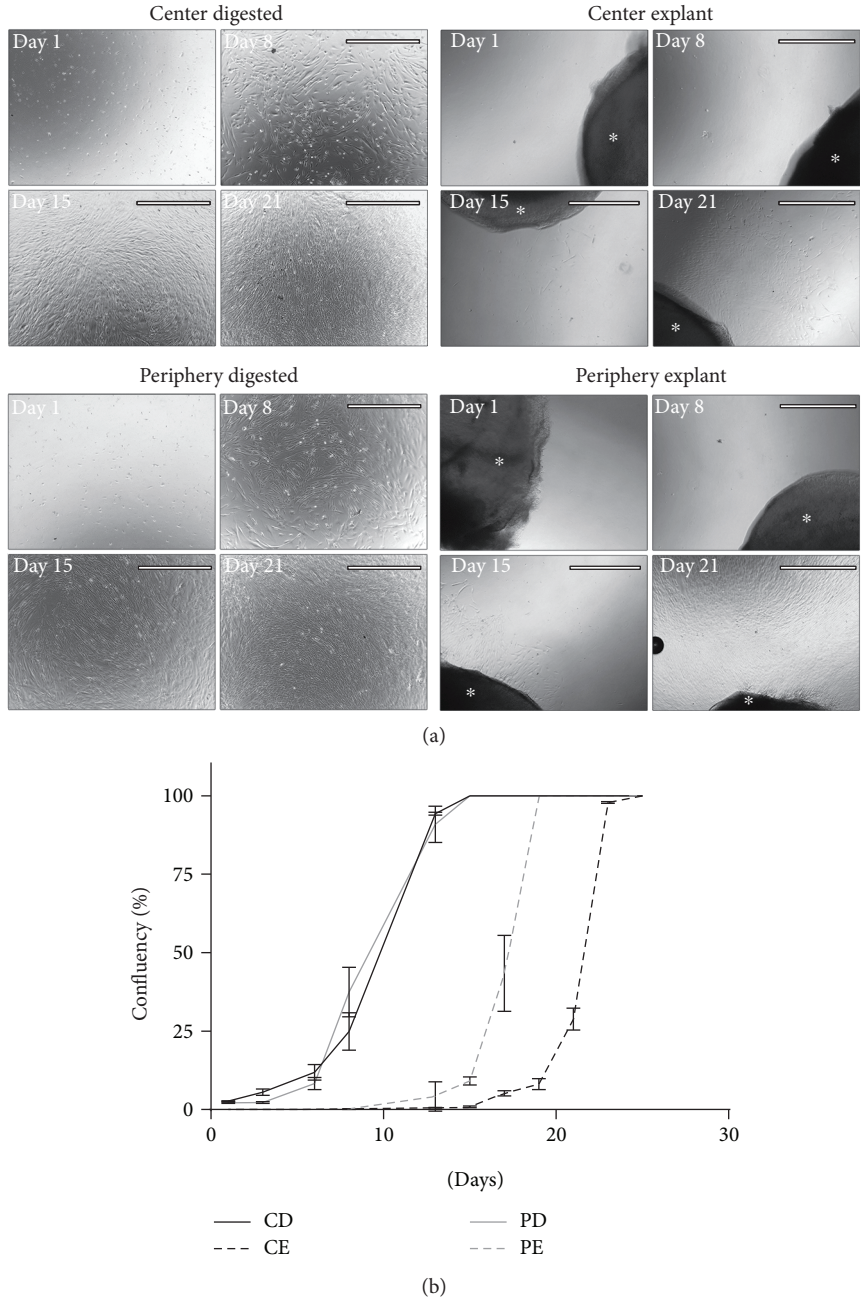


Figure 2: Phase contrast images of corneal stroma cell cultures and respective growth rates. Pictures show days 1, 8, 15, and 21 of cultivation for cells obtained from the central and peripheral regions of the stroma by enzymatic digestion and explant techniques (a). The scale bars represent 1000 μm. The explanted tissue is marked by an asterisk (*). Confluency of the cultured cells % ± SD was determined by ImageJ measurements for each isolation technique ($n = 3$), and the points were plotted accordingly for the given values on the respective days (b).

3.4. Gene Expression Pattern in Cultured CSCs and Native Corneal Stroma. CD73, CD90, and CD105/Endoglin were significantly expressed higher in the cultured CSCs compared to the native stroma (14-, 95-, and 25-fold; $p = 0.01$,$p < 0.01$, and $p < 0.01$, resp.). Furthermore, no difference could be detected in the expression of the latter genes in the different regions isolated by the two methods. Expression of Vimentin appeared to be 18-fold higher in the cultured CSCs compared to the native stroma ($p < 0.01$) with no difference between the various culture conditions. The expression of CD34 was significantly downregulated in cultured cells, as much as 5-fold, when compared to the expression found in the native tissue ($p < 0.01$). CD31 was significantly downregulated in cultured CSCs versus native cells ($p < 0.01$). Significantly lower expression of ALDH1A1 could be detected in the culture conditions compared to the native corneal stroma as well ($p < 0.01$) (Figure 6).

ABCG2 was expressed 60-fold more in primary CSCs compared to the native stromal cells ($p < 0.01$). Significantly lower expressions of AQP1, CXCR4, ITGB4, and KLF4 were

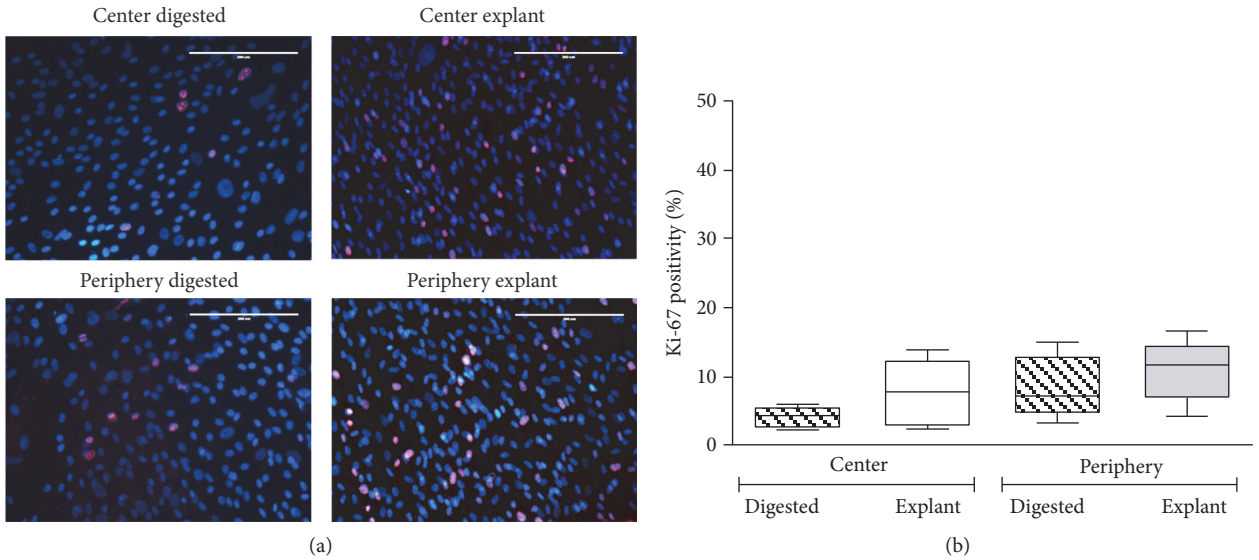


Figure 3: Immunofluorescent staining of nuclear Ki-67 in cultured cells. CSCs obtained from either the central or peripheral corneal stroma by digestion and explant techniques have been cultured for 21–30 days, respectively, and stained for proliferation marker Ki-67. The proliferation marker is shown in red with DAPI counterstaining (a). Relative quantity of Ki-67-positive cells (%) ± SD is shown (b) ($p = 0.43$). The scale bars represent 200 μm.

detected in the cultured CSCs compared to that of the native tissue ($p < 0.01$, $p < 0.01$, $p < 0.01$, and $p < 0.01$, resp.), while ITGAV and Nestin were significantly upregulated in the cultures ($p < 0.01$, $p < 0.01$). GPC4 expression was found to be unaffected by culturing ($p = 0.36$).

4. Discussion

Cells derived from the corneal stroma can be a good source for corneal research, drug testing, and future cell therapy purposes in the eye or other organs [9, 16]. CSCs derived from explants from the central part of the human corneal stroma have been extensively characterized by us recently [6]. These cells display MSC-like properties *in vitro*, including trilineage differentiation potential and immunosuppressive characteristics. Such features, however, appear not to be characteristic of the resident cells *in vivo*. It is still debated whether cells obtained from the stroma stem-like cells are actually stromal keratocytes that undergo morphological and functional changes or a different small progenitor population existent *in vivo*, which gets activated ex vivo.

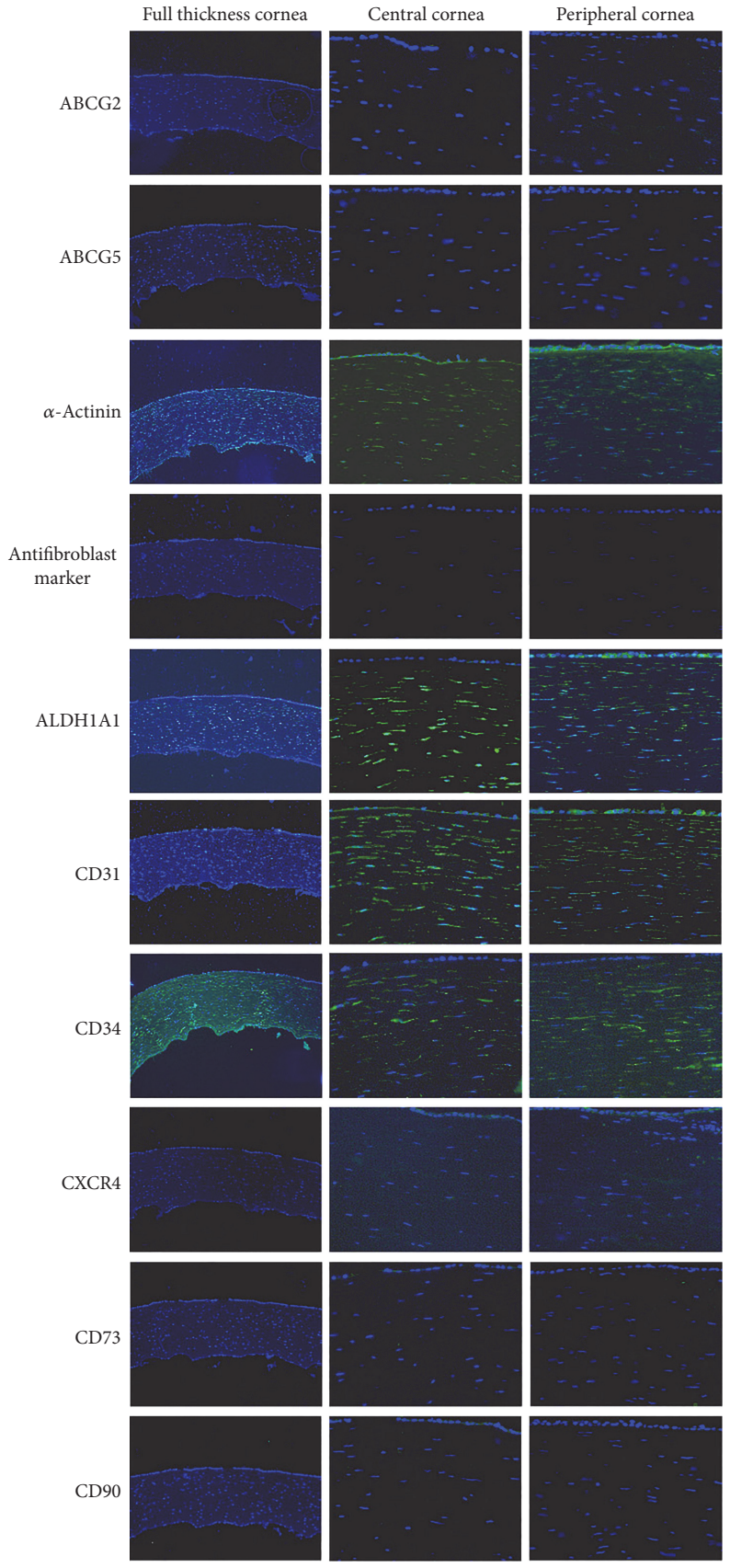
The shortage of donor corneas worldwide [17] and the availability of corneal rings remaining after keratoplasty in many research groups worldwide justify a comparison and clarification which of the different sources or techniques for isolating corneal stromal cells can be used. Most of the available tissue following surgery contains stroma from the periphery, such as that remaining after penetrating keratoplasty, DMEK, or DSAEK procedures.

Immunostaining of the native corneal tissue revealed no significant difference in the expression of previously described markers in the central versus peripheral parts of the cornea. The native corneal stroma showed no expression of the putative stem cell marker—the efflux protein ABCG2—while a strong staining was observed in the cultures from both the central and peripheral regions produced by both techniques of isolation (explant versus enzymatic). This difference in the expression found in the cultured CSCs versus the native cells could also be confirmed at the gene expression level. Upregulation of ABCG2 may result in a stronger resistance of cultured cells to externally applied therapy (e.g., chemotherapy), while cancerous cells have been known to exploit use of such molecules to survive harsh conditions [18, 19]. A population of murine adult stem cells stained by Hoechst 33342 has been shown to discharge the dye through ABCG2 and could be inhibited by verapamil [20]. Similarly, a side population has been identified in the human limbal epithelium [21]. No expression of another member of the ATP-binding cassette transporter superfamily, such as ABCG5, could be found in the native cornea.

Expression of ALDH1A1, a corneal crystalline, is essential for the maintenance of transparency, downregulation of which is associated with corneal haze [22]. Strong staining was observed in the corneal stroma, which was equally distributed along the central and peripheral regions of the cornea. Interestingly, its expression was significantly downregulated in the culture conditions compared to the native tissue.

All layers of the cornea expressed α-actinin, including the stroma. Since keratocytes express this protein, the marker should not be used alone for excluding presence of fibroblastic cells *in vivo* or ex vivo [23].

CD31 is usually expressed by vascular endothelial cells and is likely to be involved in leukocyte migration. The role of CD31 in the attraction and adhesion of polymorphonuclear cells in corneal wound healing has been demonstrated before [24]; however, its role in an undamaged and purely avascular tissue such as the native cornea remains unclear.



(a)

Figure 4: Continued.

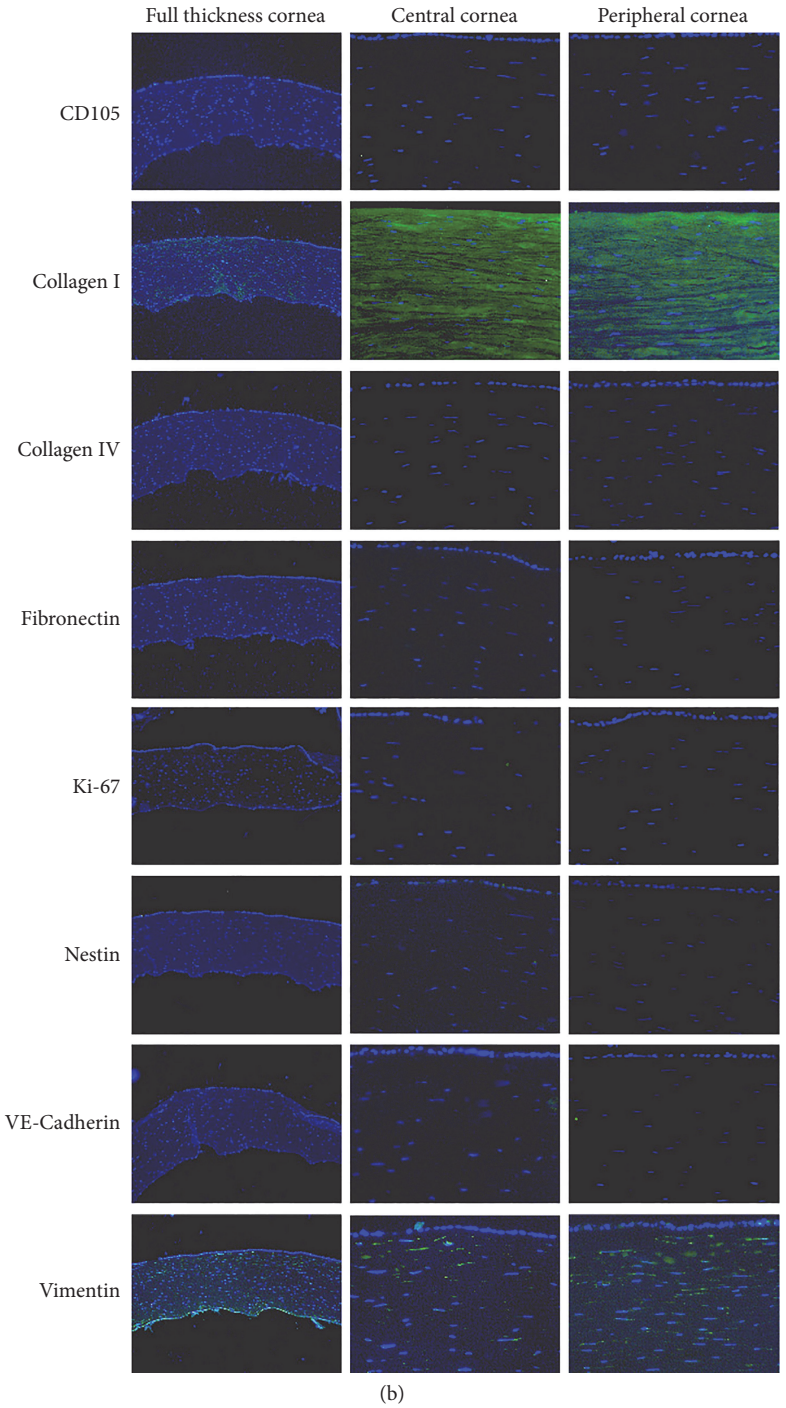


Figure 4: Immunofluorescent staining of normal human full thickness corneal sections. Images were acquired at 10x (left column) and 40x (middle and right columns) magnifications for each marker. Proteins and markers were stained by Alexa Fluor 488 conjugated secondary antibodies (green). DAPI (blue) counterstaining was applied to visualize the cell nuclei.

Interestingly, the ex vivo cultured CSCs elicited no CD31 positivity by the means of isolation applied here. The RT-qPCR analysis confirmed a statistically significant down-regulation of CD31 in the cultured versus the native cells.

Little is known about the pleiotropic functions of CD34, which is often referred to as the marker of hematopoietic progenitors, while evidence suggests this marker to likely function in immunological processes, such as regulating migration and mobility of eosinophil granulocytes and dendritic cells, as demonstrated in animal knockout experiments [25]. Several studies have demonstrated the presence of CD34 in keratocytes and its loss over the cultivation time ex vivo [16, 26]. Strong staining of the corneal stroma could indeed be detected in the native corneas, which was not the

Table 1: Distribution of corneal stroma markers in the various corneal regions.

Marker	Peripheral stroma	Central stroma	Anterior stroma	Posterior stroma
ABCG2	−	−	−	−
ABCG5	−	−	−	−
ALDH1A1	++	++	++	+
α-Actinin	++	++	++	+
CD31	+	+	+	+
CD34	++	++	++	++
CD73	−	−	−	−
CD90	−	−	−	−
CD105	−	−	−	−
Collagen I	++	++	++	++
Collagen IV	−	−	−	−
CXCR4	−	−	−	−
Fibroblast marker	−	−	−	−
Fibronectin	−	−	−	−
Ki-67	−	−	−	−
Nestin	−	−	−	−
VE-Cadherin	−	−	−	−
Vimentin	++	++	++	++

"–" stands for no staining, "+" for a medium intensity signal, and "++" for a strong staining.

case in the cultured cells. Gene expression analysis further supported this finding, and a 5-fold decrease in the expression of CD34 was observed in the cultured CSCs compared to the native stroma. This is also in line with the findings of others, which still leaves the function of this protein to be further elucidated in the cornea [16]. Aquaporin-1 is an important factor in keratocyte migration during wound healing, *in vivo*, which is downregulated, yet still expressed *in vitro* in an animal model [27].

The triad of MSC markers—CD73, CD90, and CD105—was found to be negative in situ, in contrast to the strong positivity observed in all cultured cells ex vivo. The same results were found when comparing the gene expression of the cultured versus native cells. These findings are supported by recent findings in another independent study [28], which shows the strong potential corneal stroma cells have, as well as their ability to get activated upon cultivation ex vivo. Gaining expression of the latter three proteins, together with a loss of CD34 during ex vivo cultivation, is what renders CSCs to have MSC-like phenotype, according to the International Society for Cellular Therapy (ISCT). Other types of MSCs of different origin demonstrate similar surface phenotype characteristics (high expression of CD90 and CD105) [29–32]. CD90 is believed to be involved in the cellular adhesion to the matrix and other cells, inflammation, fibrosis, migration, and tumour growth, [33] while CD73 is likely responsible for the immunosuppressive role of MSCs [34]. This feature has been demonstrated by us recently, as well as [6]. These molecules are retained over long-term and multiple passages on the cells, as demonstrated by us and others equally [35].

Characterizing the extracellular matrix which makes the backbone of the cornea is also very important to elucidate the difference between the corneal stroma cells *in vivo*, while in their niche, and ex vivo. Abundance of the major component of the corneal stroma, Collagen I, was indeed found throughout the native stroma, while Collagen IV was absent. Integrin αV (CD51) was found to be expressed on cultured cells and an upregulation was observed at a gene level, while Integrin β4 was significantly downregulated ex vivo compared to in situ.

These findings further strengthen the cultured cells respond to a change in the environmental niche surrounding them, which is likely compensated by deposition of de novo synthesized collagen ex vivo (data not shown).

Fibronectin was also not present in the native, intact corneas, which confirms the corneal wound healing properties of this extracellular matrix component. Deposits of Fibronectin have been shown to appear in the epithelium and stroma soon after penetrating trauma, although it disappears over the course of two weeks [36].

The presence of Ki-67 could not be detected in the native corneal sections either. This further confirms there was no trauma affecting the control, native epithelium, or the other layers of the cornea, thus indicating presence of induced cell proliferation, although both explant and enzymatic technique generated cultures from the different corneal regions displayed actively proliferating Ki-67-positive cells.

The mesenchymal marker Vimentin was found to be expressed in the stroma. This is in line with the findings of others. Vimentin has also been shown in knockdown studies to cause development of corneal haze [37]. The neural stemness marker Nestin could not be detected in the native corneal stroma. This protein is known to be expressed in spherical cultures generated from murine corneas, while putative precursor Nestin-positive population has been shown previously in the peripheral cornea of rabbits [38, 39]. Nestin was found to be upregulated in the cultured cells when compared to the native tissue. The protein is expressed in proliferating cells and is believed to have a role in the reorganization of intermediate filaments.

KLF4, an important stemness marker, has been associated with tissues exposed to the outside world [40]. The cornea is the first and most important mechanical barrier of the eye. We hereby show a decreased gene expression of KLF4 in cultured CSCs, compared to the native stroma. The putative stem cell migratory/homing marker CXCR4 [41, 42] was not detected in situ by immunostaining, while low levels were detected by PCR. Others reported similar, low amounts of the functional CXCR4 in cultured MSCs, but blocking the molecule has led to a decreased homing response to bone marrow [43]. CXCR4 has also been implicated in the invasion of malignant cells and epithelial-mesenchymal transition (EMT) [44].

Altogether, a general upregulation of stemness and mesenchymal cell markers was observed in the ex vivo cultivated CSCs, with a downregulation of function-related molecules, which should all be considered when treating such cells as

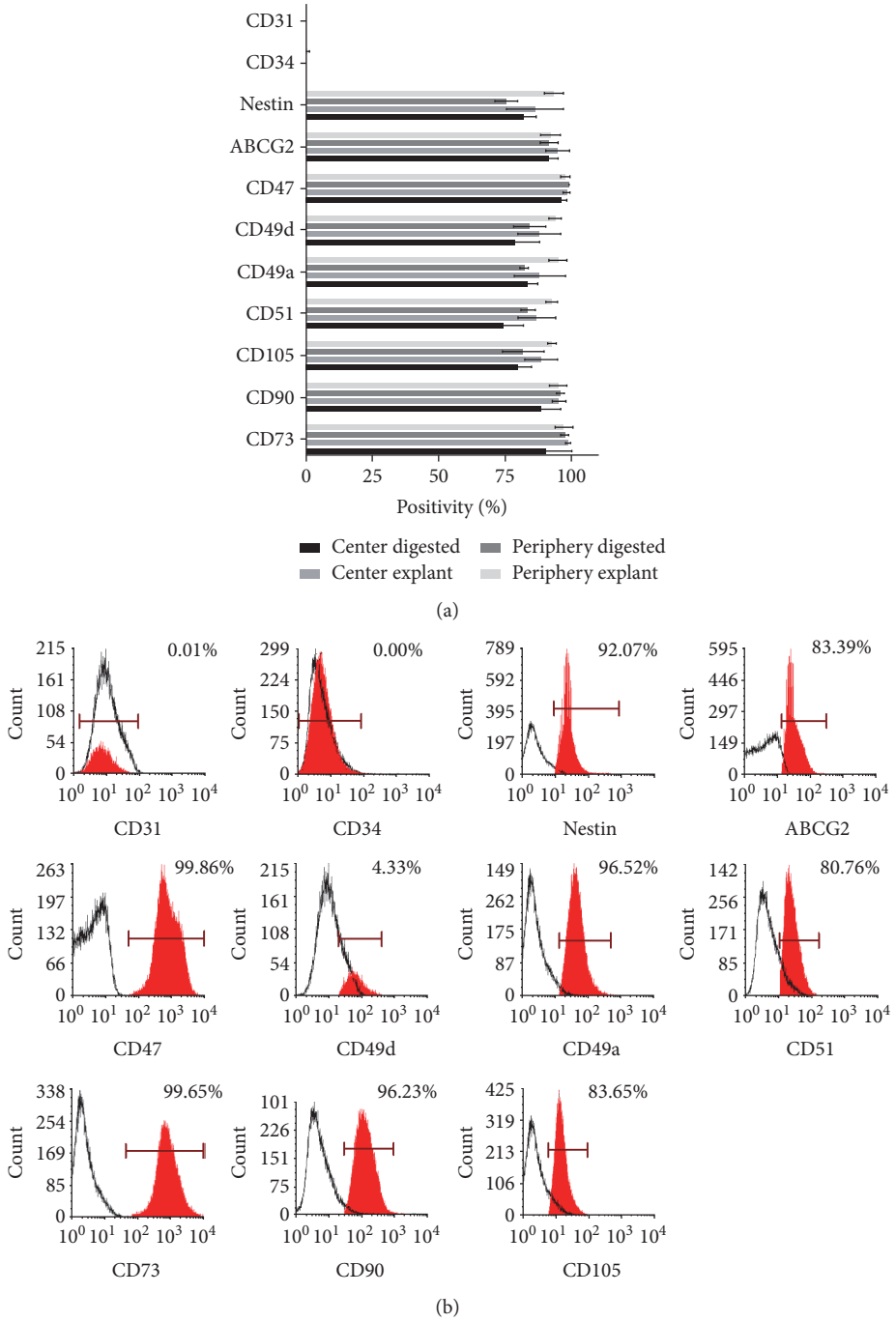


Figure 5: Percent of positive cells for given surface markers in the four different conditions ± SD is shown (a) ($n = 3$). Representative histograms of the FACS analysis, showing a PE donor, with the respective isotype controls, depicted in white color, with the stated antibodies in red, after Overton histogram subtraction (% of positive cells is shown) (b).

MSCs. The potential of corneal stromal cells has been demonstrated numerous times in animal studies before; however, it seems like certain compromises should be made when using such cells as part of *in vitro* study models, due to the striking differences *in vivo* and *in vitro*.

The present study shows no phenotypic or genotypic difference between CSCs produced by the digestion or explant methods from the central or peripheral regions of the cornea. However, the gene expression and protein profile of native corneal stroma cells compared to ex vivo expanded CSCs shows that the latter likely adapt from an *in vivo* extracellular matrix niche to an adopted environment and presence of serum in the culture medium. Such a change in the expression profile shows how dynamic corneal stroma cells can be to the environmental niche and likely to wounds or inflammation on the surface of the eye and intracorneally.

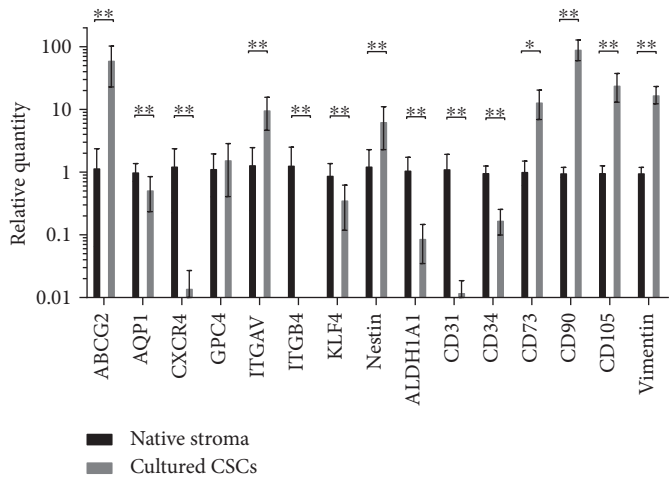


Figure 6: Gene expression profile of the native corneal stroma compared to cultured CSCs. Relative quantities are shown on a logarithmic scale, in the native and pooled cultured cells from the different isolation/cultivation methods; mean relative quantity (RQ) ± standard deviation (SD) is represented. Significance values are depicted as $^{*}p < 0.05$ and $^{**}p < 0.01$. 18S RNA was used as a housekeeping gene, with the expression levels of the cultured CSCs compared to that of the native tissue ($n = 3$). The RNA from the native tissue was prepared by mincing the stromal tissue from 3 donors into pieces, followed by extraction of total RNA.

It remains to be further examined whether such changes are reversible, or if it is beneficial for the cells to change their gene and protein expression if used for treatment of corneal, eye, or other conditions in human cell therapy. Most likely, a future biopsy taken from any part of a healthy live donor, despite the procedure being invasive, could yield viable, expanding populations of stromal cells exhibiting mesenchymal stem cell-like properties.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Richárd Nagymihály and Zoltán Veréb contributed equally to this work.

Acknowledgments

Richárd Nagymihály has been supported by an EU COST Action BM1302 travel grant to perform parts of their experiments at the host site at the Norwegian Center for Stem Cell Research/Center for Eye Research, University of Oslo, Norway. Goran Petrovski and the Stem Cells and Eye Research Laboratory, Department of Ophthalmology, Faculty of Medicine, University of Szeged, Hungary, have been supported by the National Brain Research Program (KTIA_NAP_13-A_III/9 and GINOP-2.3.2-15-2016-00006) (Hungary), cofinanced by the EU and the European Regional Development Fund. Furthermore, the project was also funded through the Center for Eye Research (CER), Department of Ophthalmology, Oslo University Hospital and University of Oslo and the Norwegian Financial Mechanism 2009–2014 under Project Contract no. MSMT-28477/2014, Project 7F14156.

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Hindawi
Stem Cells International
Volume 2017, Article ID 2057168, 13 pages
https://doi.org/10.1155/2017/2057168

Research Article

Comparing Circadian Dynamics in Primary Derived Stem Cells from Different Sources of Human Adult Tissue

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Received 12 May 2017; Revised 8 August 2017; Accepted 23 August 2017; Published 23 October 2017

Academic Editor: Sari Pennings

Optimising cell/tissue constructs so that they can be successfully accepted and integrated within a host body is essential in modern tissue engineering. To do this, adult stem cells are frequently utilised, but there are many aspects of their environment *in vivo* that are not completely understood. There is evidence to suggest that circadian rhythms and daily circadian temporal cues have substantial effects on stem cell activation, cell cycle, and differentiation. It was hypothesised that the circadian rhythm in human adult stem cells differs depending on the source of tissue and that different entraining signals exert differential effects depending on the anatomical source. Dexamethasone and rhythmic mechanical stretch were used to synchronise stem cells derived from the bone marrow, tooth dental pulp, and abdominal subcutaneous adipose tissue, and it was experimentally evidenced that these different stem cells differed in their circadian clock properties in response to different synchronisation mechanisms. The more primitive dental pulp-derived stem cells did not respond as well to the chemical synchronisation but showed temporal clock gene oscillations following rhythmic mechanical stretch, suggesting that incorporating temporal circadian information of different human adult stem cells will have profound implications in optimising tissue engineering approaches and stem cell therapies.

1. Introduction

Without adult stem cells (ASCs), mammalian tissues would not be able to maintain their natural homeostasis, as these cells play a vital part in the replenishment and repair processes, whereby old or damaged cells become replaced. Stem cells are characterized by their extraordinary abilities to self-renew through cell division over a long period of time and to be able to differentiate and give rise to organ- or tissue-specific cells in response to internal or external stimuli. It is these homeostatic and regenerative abilities that allow them to regularly repair and replace damaged or injured tissues. *In vivo*, a combination of chemical, biological, and physical cues present in the stem cell niche contribute to the direction of mesenchymal stem cell (MSC) fate, which allows them to have such a broad multilineage differentiation potential.

Recently, there has been a drive to investigate the use of Oct-4 expressing dental pulp-derived mesenchymal-like stem cells (DPSCs) in tissue engineering, as the differentiation capacity of MSCs may be restricted by their tissue of origin. These newly discovered cells offer an alternative to bone marrow-derived MSCs (BMSCs), as they have been shown to have a more potent differentiation potential than BMSCs. DPSCs have been shown to have a faster proliferation rate as well as the potential to differentiate into not only several mesenchymal cell types but also neurogenic cells, as well as being highly advantageous in terms of accessibility [1]. DPSCs are able to differentiate into neural cells as they originate from the migrating neural crest cells during embryogenesis. Dental pulp therefore consists of ectomesenchymal elements, containing neural crest-derived cells that exhibit both plasticity and multipotency [2]. The stem cells that reside within the dental pulp are extremely protected from external stimuli in their "sealed niche." The embryonic tissues found there remain undifferentiated within the jaws until the only organogenesis event which occurs after birth.

Another, more accessible source of adult stem cells that is currently being investigated is from the adipose tissue (ADSCs); adipose tissue offers an abundant source of MSCs which can be obtained in large quantities with minimal patient discomfort. Both DPSCs and ADSCs are more favourable to BMSCs as they also result in much lower site morbidity. All three have been investigated in terms of their differentiation capacities; for example, Davies et al. [3] found that DPSCs exhibited the highest potential to produce a mineralised matrix, but ADSCs and BMSCs showed enhanced dentinogenic and mineral volume. Stanko et al. [4] found that these three cell types showed no differences in terms of cell morphology or MSC surface marker expression. However, they did find significant differences regarding the expression of several pluripotency genes; BMSCs and ADSCs produced similar protein levels of several pluripotency markers but the DPSCs showed significant differences in the amount of protein products observed, including a lower expression of Oct3/4. These differences were hypothesised to reflect the mixed embryonic stem cell origin of DPSCs.

One temporal cue recently discovered to regulate mesenchymal stem cell potential and differentiation capacity is governed by the circadian rhythm [5–7]. The mammalian circadian rhythms are orchestrated by a hierarchy of self-sustained tissue oscillators. The suprachiasmatic nucleus (SCN) in the anterior hypothalamus of the brain coordinates a number of peripheral tissue oscillators to regulate a coherent rhythm of a multitude of outputs regulating metabolism, physiology, and behaviour [8]. The molecular mechanisms that regulate the circadian clockwork are evolutionarily conserved and cell-autonomous, whereby a network of autoregulatory transcriptional-translational feedback loops drive circadian expression patterns of the core clock components [9]. The primary transcriptional-translational feedback loop (TTFL) is controlled by the basic helix-loop-helix transcription factors CLOCK and BMAL1 (i.e., ARNTL). When these two proteins heterodimerize, they are able to bind to *cis*-regulatory enhancer elements within target core clock genes as well as many clock-controlled genes (CCGs) [10, 11]. Core clock genes include *period (Per1, Per2*, and *Per3)* and *cryptochrome* (*Cry1* and *Cry2*); these two proteins also heterodimerize and repress their own transcription by negatively regulating the CLOCK:BMAL1 complex [12, 13]. The CLOCK:-BMAL1 heterodimers also regulate the transcription of retinoic acid-related orphan nuclear receptors, REV-ERBs (i.e., NR1D1 and NR1D2) and RORs, which form part of the stabilising loop. These are known to bind to retinoic acid-related orphan receptor response elements (ROREs), which are present in the *Bmal1* (i.e., *ARNTL*) promoter. REV-ERBs repress transcription of *Bmal1*, whereas RORs activate the transcription [14]. Both positive and negative autoregulatory loops constitute a circadian molecular clock and take approximately 24 hours to complete.

A recent field of investigation has shown that the clock genes can directly influence ASC and progenitor cell activation and differentiation, within their tissue-specific niches. For example, disrupting the clock gene *Bmal1* leads to increased adipogenesis, and thus the attenuation of Bmal1 expression *in vitro* in preadipocytes leads to a downregulation of the Wnt signalling pathway and increased adipogenesis [15]. However, in mature adipocytes, it has been found that BMAL1 is highly expressed in differentiated cells; when BMAL1 was knocked-down by RNA interference, the mature 3T3-L1 cells were only able to accumulate minimum amounts of lipid droplets in the cells. Furthermore, adenovirus-mediated BMAL1 expression resulted in the induction of several factors involved in lipogenesis, many of which showed clear circadian rhythm in mice adipose tissue [16]. This conflicting research shows that the circadian rhythm has a clear role in cell differentiation and may have differential roles in the different stages of differentiation, but this still requires extensive further research.

The involvement of circadian clocks in the regulation of adult stem cell activation is not only niche-specific but can also act at the cell population level. Janich and colleagues showed that the circadian clock has a differential role in regulating the activation of coexisting epidermal stem cell populations. The authors found that the genes regulating stem cell niche dormancy, activation, and differentiation contained several putative BMAL1/CLOCK-binding sites. Deleting *Bmal1* leads to circadian arrhythmia, decreased expression of Wnt-related genes, and TGF-β inhibitors and causes progressive accumulation of dormant stem cells. Deleting *Per1/2*, conversely, results in progressive depletion of dormant stem cells [5]. A subsequent study showed that the stem cells responded differently to differentiation cues at certain times of the day. Interestingly, different proliferation-related or differentiation-related genes were expressed at different times of the day; for example, DNA replication and cell division related pathways were highly expressed in the dark phase, as opposed to in the light phase, when differentiation pathways were more highly expressed [6].

The endogenous mammalian circadian clock has a period of approximately 24 hours, which is reset daily by external cues, known as zeitgebers. The most potent of these is a daily light cue, which entrains the clock in the SCN in mammals through reticulo-hypothalamic signalling mechanisms. The SCN relays this information to peripheral tissue clocks via diffusible neuro-endocrine signals [17]. Another important timing signal which has the capacity to entrain daily rhythms is via systemic factors such as growth factors and hormones. Glucocorticoids, which are a class of steroid hormones that bind to the glucocorticoid receptor (GR) present in almost every vertebrate cell, have been implicated in synchronising peripheral circadian rhythms. A recently discovered entrainment mechanism for the clock that requires fundamental research is by mechanical stimulation, whereby cells in different tissues in the body are subjected to very different levels of mechanical strain. Mechanical vibrations have the capability of resetting the clock in *Drosophila melanogaster*; it has been demonstrated that rhythmic mechanical stimulation of the chordotonal organs can synchronise the *Drosophila* circadian clock. *Drosophila* with loss of function mutations in their period gene did not exhibit this ability of synchronization through vibrations, highlighting

the importance of functional clock systems for mechanical entrainment [18].

Previous extensive work in MSCs has demonstrated significant effects that mechanical forces *in vitro* can exert on their proliferation and differentiation properties. For example, Tirkkonen et al. [19] used vibrational loading to cause the differentiation of human ADSCs towards bone-forming cells and inhibition of adipogenesis. The authors found that the hADSCs cultured in osteogenic conditions were sensitive to vibrational loading, and their osteogenic differentiation was enhanced with high-frequency vibration. It has also been recently published that the mechanical environment of the epithelial stem cell niche within the mammalian mammary tissue controls the amplitude of the molecular clock oscillations, which is altered upon environmental (e.g., ageing) and genetic clock disruption [7].

Interestingly, unlike adult stem cells such as BMSCs and ADSCs, which are capable of circadian synchronization by hormonal and growth factor signals such as dexamethasone [20] and serum shock [21]. Embryonic stem (ES) cells lack a ~24 h circadian rhythm and do not display the core TTFL required for circadian clock gene expression. However, upon differentiation, ES cells in culture can gain a molecular circadian rhythm, which can also be reversed when the cells are reprogrammed with the addition of *Oct3/4*, *Sox2, Klf4*, and *c-Myc* [22]. However, investigating the circadian rhythm in embryonic cells presents many challenges and the way by which we undertake our experiments may result in a disruption of endogenous oscillation(s) [23]; therefore, this lack of circadian rhythm in embryonic stem cells may not be as strict as it previously appeared.

It was hypothesised here that adult mesenchymal stem cells from different human tissue sources at different anatomical locations within the body may exhibit different circadian dynamics and respond to temporal cues differently and that the more primitive adult stem cells such as DPSCs would be less responsive to circadian synchronisation cues compared to BMSCs or ADSCs. In addition to well-established chemical synchronisation using a synthetic glucocorticoid, dexamethasone [24], mechanical stimulation was also used in a circadian paradigm to investigate whether different human adult stem cells could be entrained using mechanical cues. The applied method investigating mechanical stimulation, using a uniaxial stretch rig composed of flexible silicone substrates at 6.66% radial distension and frequency of 1 Hz, was selected as these parameters have previously found to be within physiological range. Published data has previously shown that a uniaxial strain between 5–15% with a frequency of 1 Hz is preferable for MSCs and shows positive effects on proliferation and collagen synthesis [25]. Furthermore, O'Cearbhaill et al. [26] found that radial distensions of 5% and frequencies of 1 Hz caused mechanosensitive effects including cell reorientation parallel with the direction of flow and adapted morphologies, highlighting that there is a significant cytoskeletal restructuring in these mechanically stimulated MSCs compared to static controls.

If viable, this mechanical stretch paradigm could offer a synchronisation method that excludes the need to use chemical or thermal approaches to synchronise the circadian clock, which would be hugely advantageous in tissue engineering and regenerative medicine. In this research, the circadian differences in adult stem cells derived from different human adult tissues with respect to their expression of several core clock genes, stabilising clock genes and stem cell markers in both dynamic and static conditions have been determined and compared. The results reveal differential circadian gene expression patterns in human adult stem cells derived from different tissue sources upon glucocorticoid synchronisation. It is also shown that rhythmic mechanical stimulation has the ability to entrain some human stem cells, which provides a novel clock synchronisation approach independent of chemical or temperature cues. Such a clock synchronisation protocol may prove more advantageous in future tissue engineering applications, leading to significant developments in both age-related diseases and tissue engineering and synchronising stem cell therapies.

2. Materials and Methods

2.1. Isolation of Adipose-Derived Stromal Cells. Human lipoaspirate was harvested during a lipofilling procedure after breast tumour removal. Lipoaspirate (5 g) was washed by addition of serum-free DMEM medium (Gibco, UK) and centrifuged for 3 min at 500*g*. The washed fat layer was moved to a new tube to which a digestion solution containing 10 mL of DMEM medium (Gibco, UK) and 20 μL of collagenase type 2 (Sigma, UK) was added. This was placed on a roller mixer for 30 min at 37°C. 10 mL of DMEM medium containing 10% FBS and penicillin/streptomycin (Sigma, UK) was added and the tube was spun for 5 min at 1000*g*. The oil layer was removed and discarded, and undigested fat fraction was placed into a T25 falcon flask, with the addition of complete growth DMEM medium as above. The remaining supernatant was discarded and the pellet was resuspended and placed in a separate T25 flask, with the addition of complete growth DMEM medium. Both of these fractions were cultured until confluency and used at passage 2.

2.2. Cell Culture of BMSCs and DPSCs. Primary human BMSCs and DPSCs were obtained commercially (Lonza and BioEden Limited, resp.) and expanded in Dulbecco's Modified Eagle Medium (GlutaMAX; Gibco, UK) supplemented with 1% penicillin/streptomycin and 10% FBS and incubated at 37°C in 5% CO2. Cells were trypsinised upon reaching 70–80% confluency and used before reaching passage 7.

2.3. Clock Synchronisation with Dexamethasone. Cells were seeded into 6-well plates in complete growth medium as above until confluency. They were synchronised using 100 nM dexamethasone and total RNA collected every four hours over a circadian cycle at the following time points after synchronisation (h): 16, 20, 24, 28, 32, 36, 40, 44, 48, and 52. The cells were exposed to the dexamethasone for 1 h and then incubated in complete growth medium until sample

Table 1: qPCR primer sequences (human).

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
Bmal1	TGCCTCGTCGCAATTGG	ACCCTGATTTCCCCGTTCA
Per2	GTCCAGCCCCCACCTTTC	GGGAAGGAATAACTGGGTAGCA
Per1	CTCAGTGGCTGTCTCCTTCC	GAGCCAGGAGCTCAGAGAAG
Rev-ErbAα	CTTCAATGCCAACCATGCAT	CCTGATTTTCCCAGCGATGT
Sox2	GAGAGAAAGAAAGGGAGAGAAG	GAGAGAGGCAAACTGGAATC

collection. Negative controls received no dexamethasone but were exposed to the same media changes and conditions.

2.4. Mechanical Stimulation. Flexible silicone chambers were coated in fibronectin for 1 h before cells were seeded into the chambers and allowed at least 24 h to settle and attach. Once confluent, the chambers were loaded into a unique uniaxial stretch rig and stretched for 3 consecutive days in a rhythmic manner (frequency 1 Hz, 6.66% stretch, 12 h ON/12 h OFF). After day 3 of rhythmic stimulation, cells were allowed to rest and were collected at the following times over 1.5 circadian days following mechanical stimulation (h): 16, 20, 24, 28, 32, 36, 40, 44, 48, and 52.

2.5. Quantitative RT-qPCR. Total RNA was extracted using TRI-reagent (Sigma) as per the manufacturer's specifications. Total RNA was determined using nanodrop spectroscopy before cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen), with Oligo dT at 50°C for 40 min in a 20 μl reaction. Real-time RT-PCR was carried out on cDNA samples with SYBR Green PCR Supermix (Bio-Rad) using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad) under the following cycling conditions: 95°C for 3 min; 40 cycles of 95°C for 10 s; and 60°C for 30 s. Results were normalised relative to a housekeeping gene *GAPDH* expression. Primers were designed against the following genes: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), aryl hydrocarbon receptor nuclear translocator-like (*ARNTL* or *Bmal1*), period 2 (*Per2*), period 1 (*Per1*), nuclear receptor subfamily 1 group D member 1 (*NR1D1* or *Rev-ErbAα*), and SRY (sex determining region Y)-Box 2 (*Sox2*) (primer sequences are listed in Table 1).

2.6. Statistical Analyses. Data were expressed as the mean ± standard error. Statistical analyses were performed, following the determination of normal distribution, using one-way analysis of variance (ANOVA), with Tukey HSD post hoc, or the independent *t*-test at a confidence level of 95% (SPSS 24 Software). In order to determine circadian gene rhythmicity and its significance, cosinor periodogram analysis was used, made available online by the Refinetti circadian biology group at Boise State University (http://www.circadian.org/softwar.html). p values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Human Stem Cells Derived from Different Sources of Adult Tissue Show Differential Circadian Clock Gene Expression Profiles. Asynchronous cultures of BMSCs, ADSCs, and DPSCs were allowed to grow to confluency in complete growth medium, before their RNA was extracted and a PCR "clock panel" of genes was used to analyse their relative mRNA expression. As expected, different human adult stem cells did exhibit significantly varying amounts of clock gene expression, with the BMSCs and DPSCs appearing to show contrasting expressions of *Bmal1*, a component of the positive arm of the molecular clock, and *Per2*, a component of the negative arm (Figure 1). Interestingly, *Bmal1* was significantly higher in DPSCs compared to BMSCs and ADSCs, whilst *Per2* was significantly higher in BMSCs compared to DPSCs and ADSCs. *Per1* and *Rev-ErbA* were also both significantly higher in DPSCs than ADSCs and BMSCs. In contrast, *Bmal1*, *Per1*, and *Rev-ErbA* were not significantly different in expression levels between BMSCs and ADSCs.

3.2. Glucocorticoid Stimulation Using Dexamethasone Leads to Circadian Synchronisation in BMSCs and ADSCs but Not DPSCs. Next, the three human adult stem cell types were grown to confluency and clock synchronised using synthetic glucocorticoid, 100 nM dexamethasone for a period of 1 h, after which, their media was replaced with complete growth medium. RNA samples were initially taken at two opposite circadian phases at either 20 h or 32 h post synchronisation to examine any initial differences at opposite phases. The antiphasic relationship of *Bmal1* and *Per2* could be clearly seen with the two genes peaking and troughing at opposite circadian times, respectively. For example, in ADSCs and DPSCs, *Bmal1* expression was much higher at 32 h than at 20 h whilst *Per2* was much higher at 20 h compared to 32 h (Figure 2). Interestingly, the opposite appeared to be observed for the BMSCs, which showed higher *Bmal1* and lower *Per2* expression at 20 h versus 32 h, respectively. The mRNA expression of a component of the stabilising loop in circadian machinery was also examined. It was found that *Rev-ErbAα* expression showed temporally different expressions at the two circadian time points in different human adult stem cell types and interestingly appeared to peak at 32 h similarly to *Per2* in BMSCs, whilst at the same circadian

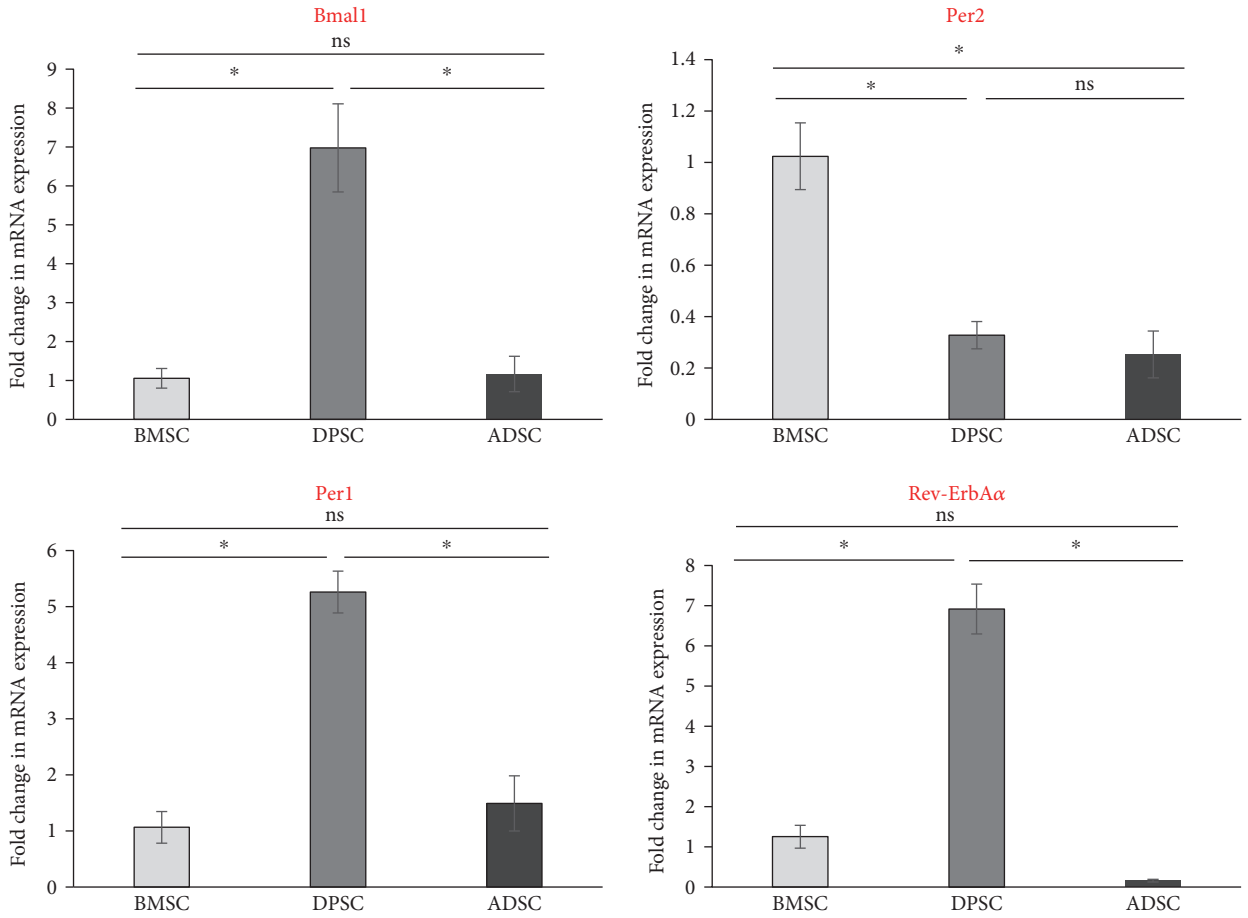

FIGURE 1: Clock gene expression in unsynchronised adult stem cells. Quantitative RT-PCR analyses comparing the relative mRNA expression levels of core clock genes in mesenchymal-like adult stem cells derived from human bone marrow, dental pulp, and adipose tissue. Data is expressed as the mean of ΔCt ± SEM normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent samples, $^{*}p < 0.05$ (one-way ANOVA).

time point, it peaked similar to *Bmal1* in the ADSCs and DPSCs.

As significant differences were observed between the two initial time points, temporal clock gene expression profiles over 1.5 circadian days were then compared in order to get a clearer understanding of the clock gene expression, omitting the first 0.5 circadian day (0–15 h) in order to exclude the transient effects of dexamethasone, as published previously [7]. Samples were collected every four hours starting with 16 h postdexamethasone synchronisation and ending at 52 h, in order to more closely investigate circadian gene dynamics between the three different human adult stem cell types (Figure 3). Both the ADSCs and BMSCs showed robust oscillations of clock gene expressions which is a characteristic antiphasic temporal pattern of synchronised cells reported previously. For example, in the BMSCs, *Bmal1* troughed whilst *Per2* peaked at 32 h postdexamethasone synchronisation. In contrast, in ADSCs, *Bmal1* peaked and *Per2* troughed around 16–20 h, showing a clear antiphasic relationship. On the other hand, the more primitive DPSCs, however, did not exhibit oscillating expressions of the circadian clock genes, and no clear temporal patterns of clock gene expression could be observed following dexamethasone synchronisation.

When analysed by cosinor periodogram (Table 2), *Bmal1* expression was shown to exhibit significant circadian rhythmicity in BMSCs ($p = 0.007$), whilst *Per2* was just short of significance ($p = 0.076$), and all three circadian clock genes (*Bmal1, Per2*, and *Rev-ErbAα*) showed circadian rhythmicity in ADSCs ($p = 0.006$, $p = 0.021$, and $p = 0.001$, resp.). However, no core clock genes were found to exhibit significant circadian rhythmicity in DPSCs, except for *Rev-ErbAα* which nearly reached significance ($p = 0.055$).

In order to confirm cell synchronisation by dexamethasone and exclude the possibility that changing the cell culture growth medium itself following dexamethasone synchronisation had any synchronising effects on cells, unsynchronised BMSCs were used as a negative control and collected temporally having received no stimulation other than the same media changes as the above time-course experiment. As expected, no circadian rhythm of core clock genes *Bmal1* or *Per2* was observed in unsynchronised BMSCs (Supplementary Fig. 1 available online at https://doi.org/10.1155/2017/2057168).

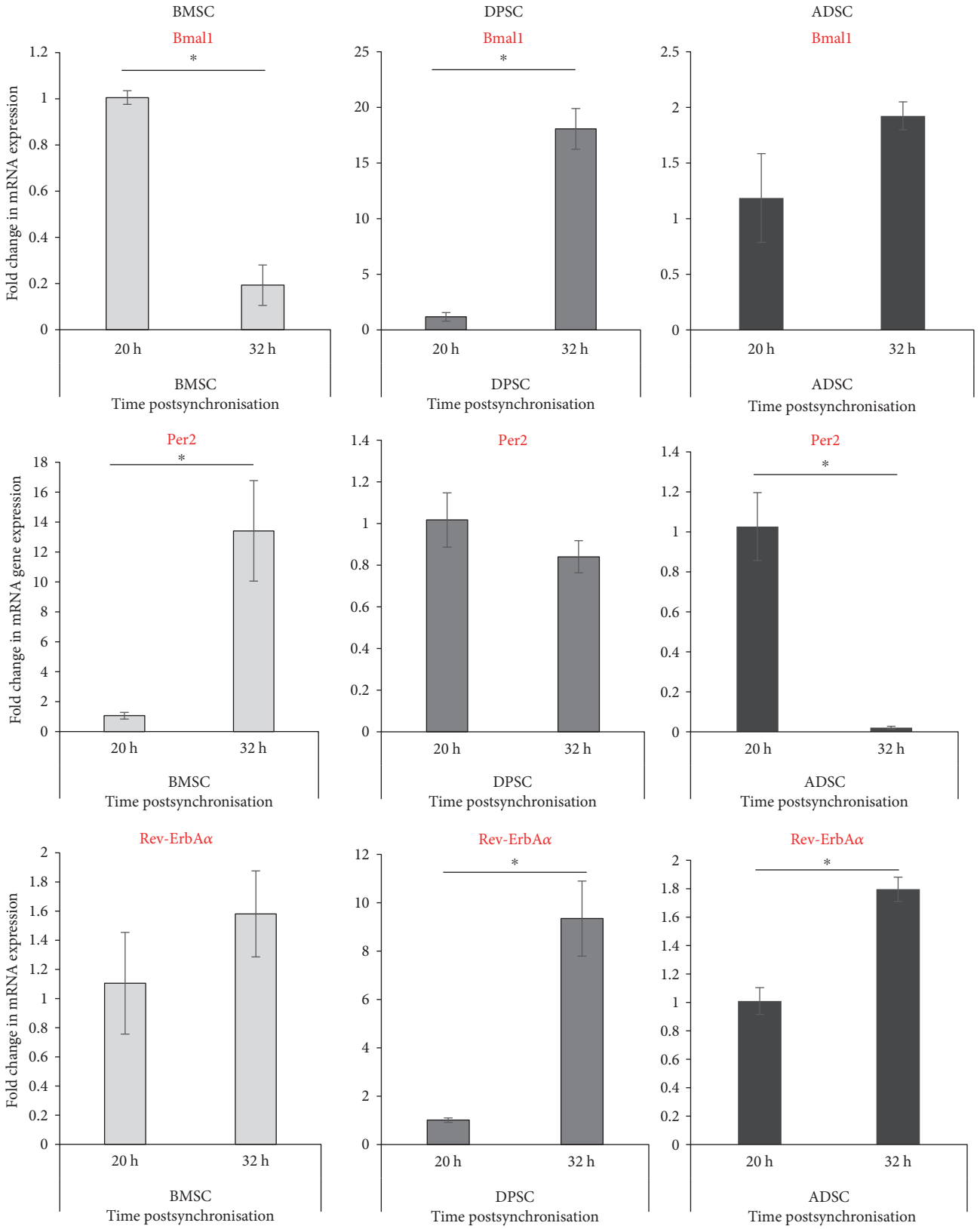

Figure 2: Clock gene expression in synchronised human adult stem cells at two opposite circadian phases. Quantitative RT-PCR analyses showing the expression levels of clock genes after synchronisation with dexamethasone at two opposite circadian phases 12 h apart (20 h versus 32 h). Data are expressed as the mean of ΔCt ± SEM relative to the 20 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments, $^{*}p < 0.05$ (independent *t*-test).

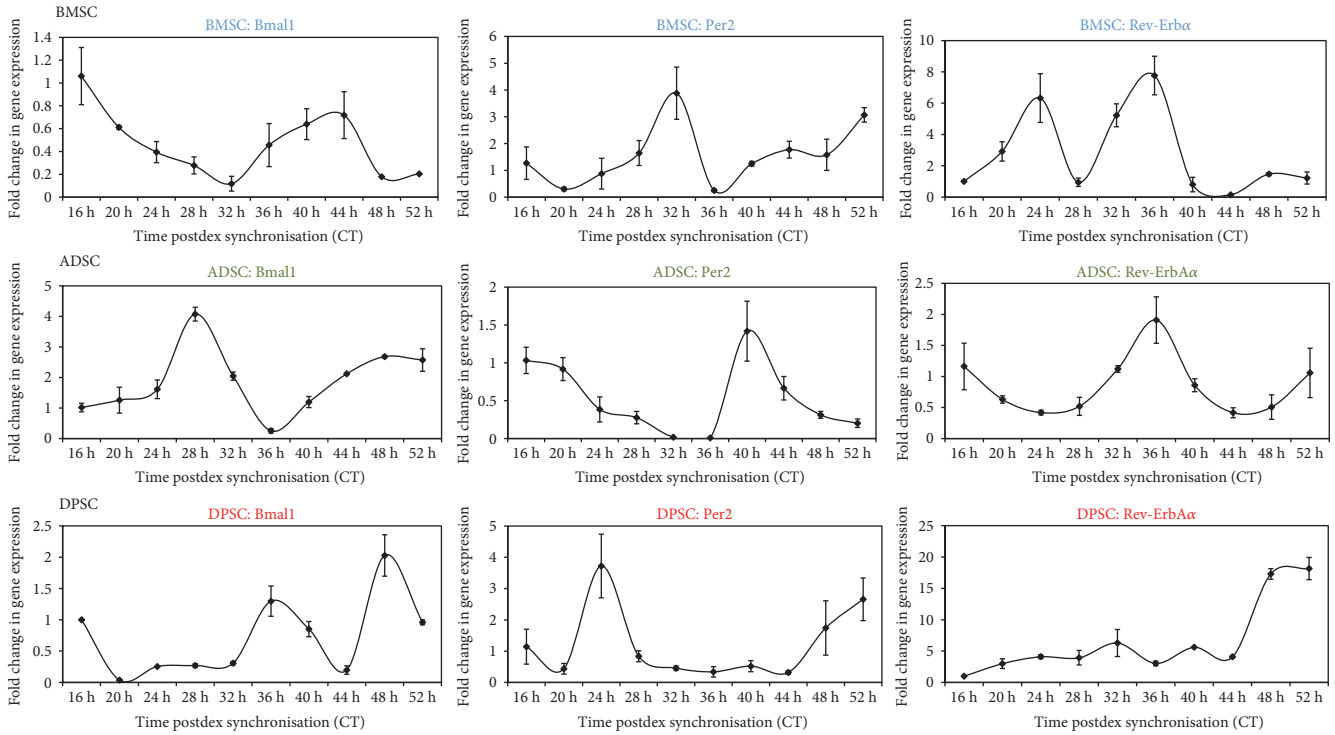

Figure 3: Circadian rhythm dynamics in human adult stem cells following synchronisation with dexamethasone. Quantitative RT-PCR analyses showing temporal expression profiles of clock genes collected every four hours between 16 h–52 h following synchronisation with dexamethasone. Data are expressed as the mean of ΔCt ± SEM relative to 16 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments.

Table 2: Cosinor analysis of circadian clock rhythmicity in human BMSCs, ADSCs, and DPSCs following dexamethasone synchronisation.

Cell	Gene	Period (h)	*p* value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
BMSC	Bmal1*	23.9	0.007	−76	0.331	0.451	76.2
	Per2	20.2	0.076	−261	1.099	1.597	52.1
	Rev-erbA	26.0	0.391	−287	1.853	3.158	23.6
ADSC	Bmal1*	20.0	0.006	−289	1.264	1.885	77.4
	Per2*	23.4	0.021	−98	0.502	0.486	66.6
	Rev-erbA*	21.8	0.001	−314	0.611	0.914	85.1
DPSC	Bmal1	20.0	0.571	−9	0.325	0.720	14.9
	Per2	26.0	0.101	−176	1.089	1.071	47.9
	Rev-erbA	26.0	0.055	−266	4.952	4.938	56.2

*Significance according to the cosinor analysis software.

3.3. DPSCs Can Be Entrained by Rhythmic Mechanical Stretch Synchronisation. In order to find a novel method of synchronising the DPSCs, it was hypothesised that as these stem cells are encapsulated in a tight niche in the tooth but are still subjected to a substantial amount of mechanical stimulation, they could potentially be entrained using mechanical means, if not chemical. Therefore, both BMSCs, used as a positive control here as these cells were previously reported to respond to mechanical stretch, and DPSCs were seeded onto fibronectin-coated, flexible silicone chambers (Figure 4(a)) and subjected to 3 days of rhythmic cyclical mechanical stretch, undergoing 12 h of cyclical stretching followed by 12 h of relaxation. After this regime was completed, samples were collected in the absence of mechanical stimulation at two opposite temporal phases at either 20 h or 32 h following their last exposure to the stretch (omitting the first 12 h to exclude any transient effects) and the clock gene expressions were analysed (Figure 4(b)), in a similar manner to the experimental design following dexamethasone exposure. Here, in striking contrast to synchronisation with dexamethasone, the clock gene levels in DPSCs appeared to peak and trough in a characteristic antiphasic manner for *Bmal1* and *Per2,* respectively; in both BMSCs and DPSCs *Bmal1* peaked in expression at 20 h, in contrast to *Per2* which

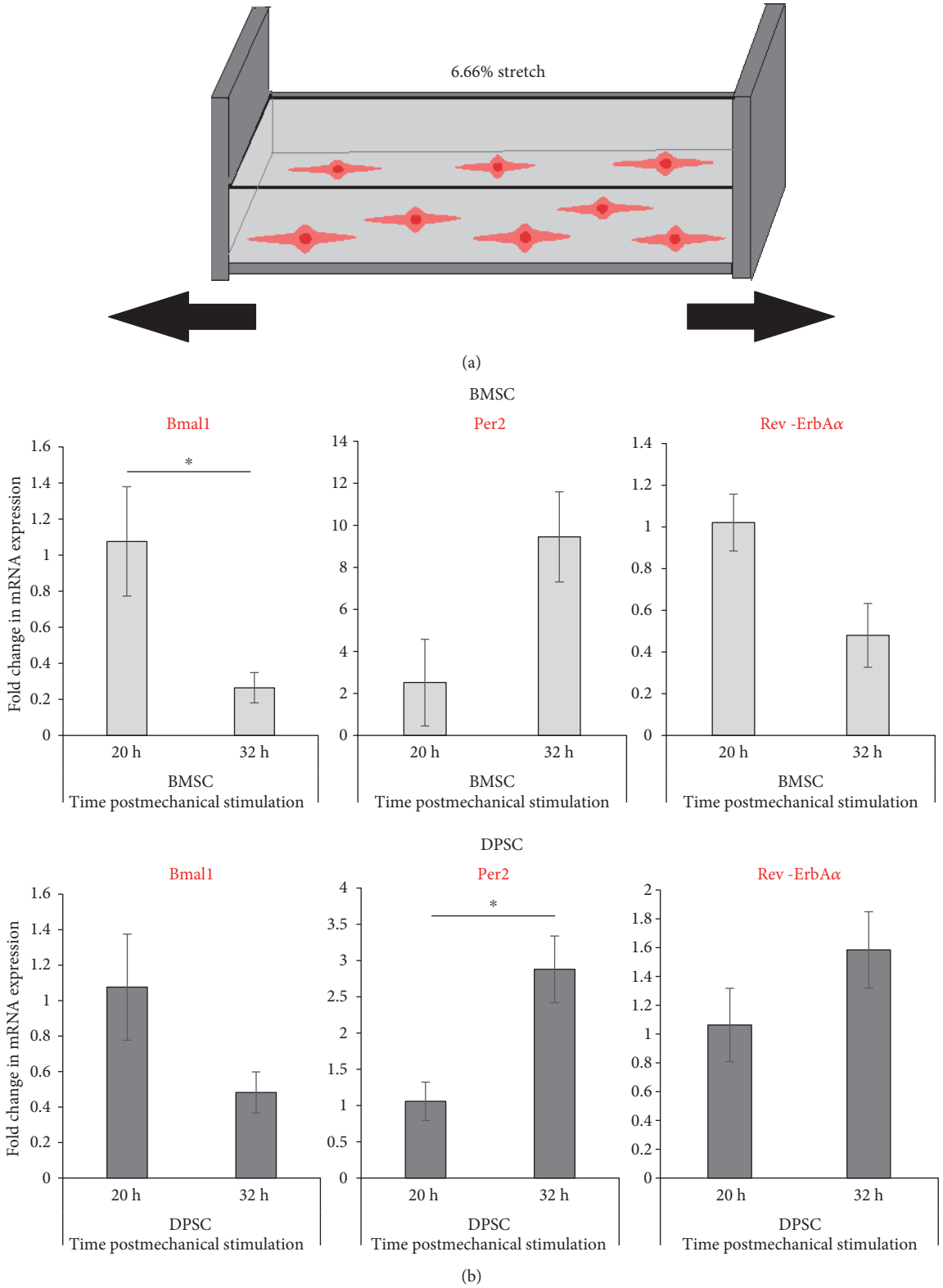

Figure 4: Clock gene expression in human adult stem cells following mechanical rhythmic stimulation paradigm. (a) Schematic diagram showing the setup of the mechanical stretch apparatus; cells were seeded into silicone chambers and stretched for 3 days using a unique uniaxial stretch rig with offset cams (1 Hz, 6.66% stretch, 12 h ON (stretch), 12 h OFF (rest)). (b) Quantitative RT-PCR analyses showing the expression levels of clock genes at two opposite circadian phases (20 h versus 32 h) following rhythmic mechanical stimulation protocol. Data are expressed as the mean of ΔCt ± SEM relative to the 20 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments, $^{*}p < 0.05$ (independent *t*-test).

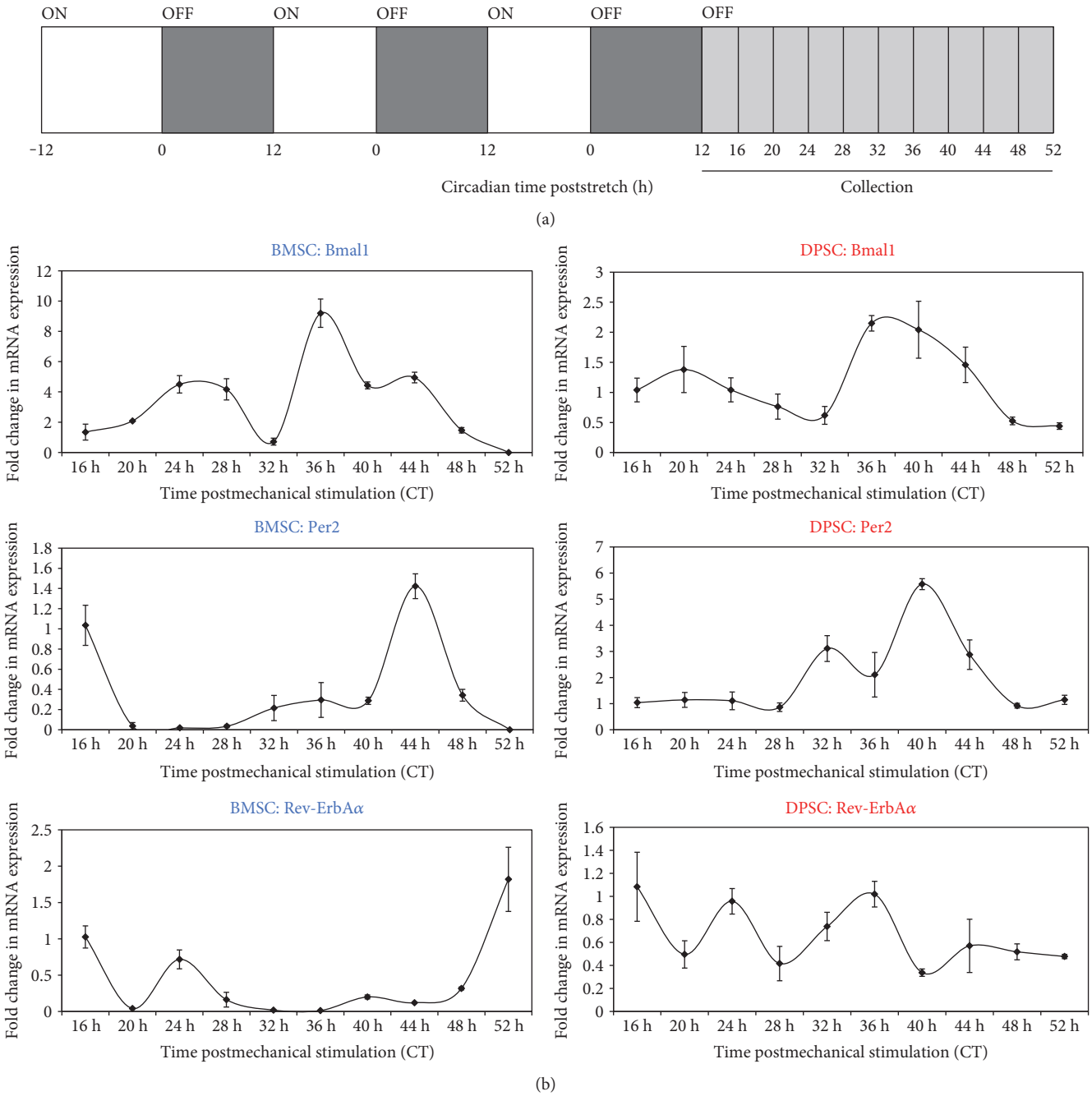

Figure 5: Circadian gene dynamics in human adult stem cells after rhythmic mechanical stimulation protocol. (a) Schematic diagram outlining the schedule of the 12 h ON : 12 h OFF (stretch : rest) regime followed by sample collection every 4 h between 16 h–52 h. (b) Quantitative RT-PCR analyses showing temporal expression profiles of clock genes following 3 days of rhythmic mechanical stimulation. Data are expressed as the mean +/− of ΔCt ± SEM relative to 16 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments.

peaked at the opposite circadian time point, being much higher in expression at 32 h, compared to 20 h.

Again, samples were then collected over a longer time course to further investigate these significant differences in gene expression and gain a finer temporal resolution over a 1.5 circadian cycle, the clock gene expressions were analysed every four hours 16 h to 52 h postrhythmic mechanical stimulation (Figure 5(a)). From these longer circadian mechanical time courses, it was evident that both DPSCs and BMSCs could produce oscillating expression of core clock genes using rhythmic mechanical stimulation. In the DPSCs, *Bmal1* expression was clearly at its trough at 32 h, which was the circadian time point at which *Per2* appeared to initially peak (Figure 5(b)). When analysed by cosinor periodogram (Table 3), *Bmal1* expression was shown to exhibit significant circadian rhythmicity in DPSCs ($p = 0.02$).

Table 3: Cosinor analysis of circadian clock rhythmicity in human BMSCs and DPSCs following synchronisation by rhythmic mechanical stretch.

Cell	Gene	Period (h)	p value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
BMSC	Bmal1	26.0	0.571	−346	1.754	3.568	21.6
	Per2	26.0	0.123	−51	0.428	0.343	45.0
	Rev-erbA	26.0	0.559	−171	0.316	0.397	15.4
DPSC	Bmal1*	20.0	0.020	−135	0.664	1.146	67.0
	Per2	26.0	0.132	−354	1.362	2.179	43.9
	Rev-erbA	21.2	0.505	−49	0.155	0.669	17.8

*Significance according to the cosinor analysis software.

Surprisingly, no circadian clock genes were found to show significant circadian rhythmicity in BMSCs.

3.4. The Pluripotency Marker Sox2 Shows Initial Cyclical Gene Expression following Glucocorticoid and Mechanical Synchronisation. Using both synchronisation mechanisms, it was next determined if any pluripotency stem cell markers displayed an initial circadian gene expression pattern following either glucocorticoid or rhythmic mechanical stimulation. To this end, *Sox2* was analysed and its temporal expression determined every four hours over one circadian day under the same synchronisation conditions as described above in the three stem cell types (Figure 6). *Sox2* showed initial cyclical expression in both BMSCs and ADSCs but not DPSCs following dexamethasone synchronisation. Strikingly, it was found that *Sox2* showed a very similar pattern of gene expression to that of *Rev-ErbAα* (Supplementary Fig. 2).

When analysed by cosinor periodogram (Table 4), *Sox2* expression was shown to exhibit significant initial circadian rhythmicity in ADSCs ($p = 0.007$) when exposed to dexamethasone synchronisation, whilst it was just short of significance in DPSCs following dexamethasone ($p = 0.074$) and cyclical mechanical stretch ($p = 0.087$). However, no significant circadian rhythmicity in *Sox2* could be observed in BMSCs, despite it showing a similar pattern.

4. Discussion

In this research, it has been shown that human stem cells derived from different human adult tissues did exhibit different levels and temporal expression patterns of core clock genes, stabilising loop genes and stem cell markers. It was also observed that the adult stem cells from different sources did indeed respond to circadian synchronising signals very differently as predicted; for instance, the BMSCs appeared to synchronise more readily in response to chemical stimulation than mechanical stimulation, but the DPSCs were much more responsive to entrainment by mechanical means. It has been experimentally evidenced that the more primitive human stem cells such as DPSCs have a different profile of the molecular circadian rhythm, in terms of both the relative levels of clock gene expression and the oscillating temporal patterns of gene expression after synchronisation by chemical means (i.e., dexamethasone exposure). It appears that the DPSCs are much less responsive to the dexamethasone synchronisation, which may be due to less developed circadian components which relay these signals. Indeed, their relative unresponsiveness to dexamethasone may be due to their early developmental origin from the migrating neural crest cells and their resulting ectomesenchymal composition. Therefore, upon DPSC differentiation, it is possible that they will gain responsiveness to circadian synchronisation and/or undergo maturation of the apparatus necessary for circadian gene oscillations. This is consistent with the research by Yagita et al. showed that embryonic stem cells do not have the capacity for circadian synchronisation by chemical means using forskolin; but upon differentiation and maturation, this ability can be gained [22]. In contrast, our results confirm previous findings that the more mature MSCs derived from the bone marrow and adipose tissue did exhibit robust clock rhythms that were responsive to glucocorticoid synchronisation and showed clear antiphasic relationships of the positive and negative arms of core TTFLs. For example, Wu et al. showed similarly oscillating expressions of clock genes in cultures of murine and human BMSCs in response to dexamethasone [19] whilst Huang et al. demonstrated that human BMSCs and ADSCs have circadian oscillations induced by serum shock [20].

As the DPSCs could not be synchronised by chemical means, a novel synchronising mechanism to which they may respond was sought. Circadian mechanosensory entrainment has been previously investigated by Simoni et al., who found that 12 h : 12 h cycles of vibration and silence, respectively, were sufficient to synchronise the daily locomotor activity of *Drosophila melanogaster* [18]. Moreover, it has recently been published that a mechanical environment of the epithelial stem cell niche within the mammalian mammary tissue controls the amplitude of the molecular clock oscillations [7]. Therefore, rhythmic mechanical stretch was utilised as an entraining factor for adult human stem cells using a uniaxial mechanical stretch apparatus. Following the stretch entrainment and subsequent analysis by cosinor periodogram, differences were observed in the phasing and period of the clock genes in the same stem cell types, highlighting how the entrainment mechanisms lead to different effects. It was observed that the DPSCs can be entrained by rhythmic mechanical stretch and appeared more responsive than the BMSCs; the *Bmal1* gene oscillations induced by rhythmic mechanical stimulation in the DPSCs resembled the temporal clock gene

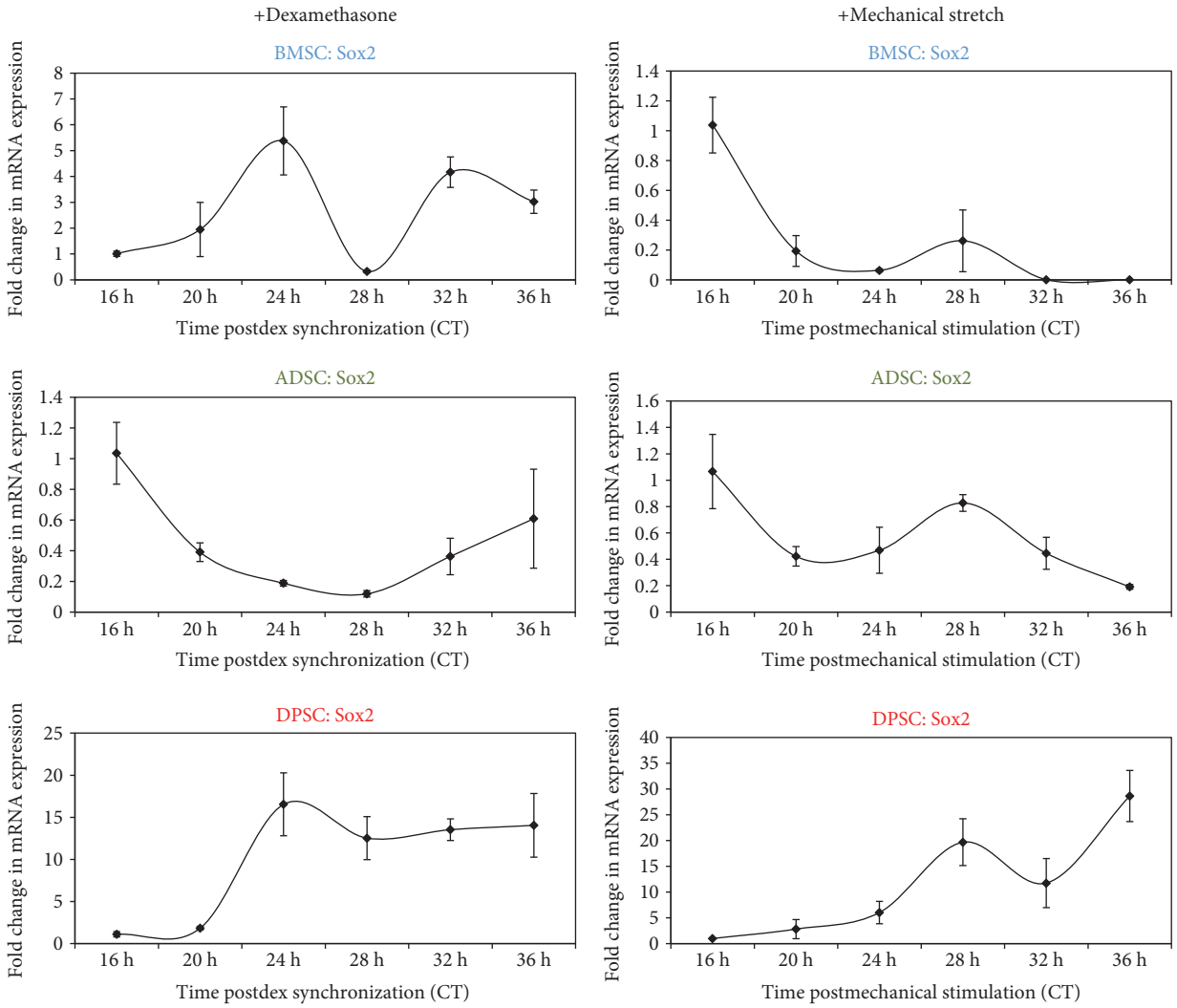

Figure 6: Human pluripotency marker SOX2 shows cyclical temporal gene expression in adult stem cells initially following dexamethasone or rhythmic mechanical stimulation. Quantitative RT-PCR analyses showing temporal expression profile of the pluripotency marker *Sox2* synchronisation with dexamethasone or rhythmic mechanical stimulation and collected every four hours over 1 circadian cycle (16 h–36 h). Data are expressed as the mean of ΔCt ± SEM relative to 16 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments.

Table 4: Cosinor analysis of pluripotency marker *Sox2* rhythmicity in human BMSCs, ADSCs, and DPSCs following synchronisation by dexamethasone and rhythmic mechanical stretch.

	Cell	Period (h)	*p* value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
Dex	BMSC	26.0	0.658	−218	1.114	2.348	19.0
	ADSC*	21.8	0.009	−348	0.371	0.354	74.3
	DPSC	26.0	0.074	−261	6.463	7.800	52.5
Stretch	BMSC	26.0	0.541	−61	0.328	0.279	41.2
	ADSC	26.0	0.863	−107	0.119	0.569	9.3
	DPSC	26.0	0.087	−311	9.926	11.858	55.7

*Significance according to the cosinor analysis software.

expression patterns seen in BMSCs in response to dexamethasone. This may be due to the fact that *in vivo*, DPSCs experience significant mechanical stimulation in the form of jaw movement, occlusion forces, and hydrostatic pressures and are one of the few stem cell niches to also experience thermal shock and extreme temperature fluctuations and so are much more likely to respond to this form of stimulation. Moreover, when the initial expression profile of the

pluripotency marker *Sox2* was compared to the profile of the stabilising loop gene *Rev-ErbAα*, a strikingly similar pattern was observed following synchronisation with dexamethasone in BMSCs and ADSCs. In contrast, the rhythmic mechanical stimulation in DPSCs was able to induce the cyclical expression of the core clock genes as well as a pluripotency marker *Sox2*. These results therefore suggest a novel regulation of the *Sox2* gene which may be under both circadian and mechanical controls in different stem cell types.

The circadian clock in mammals has been extensively shown to have a key regulatory role on various tissue systems, including musculoskeletal tissues. It is therefore vital that such temporal regulation be taken into account when optimising and integrating any cellular/tissue constructs into the body, if one hopes for more successful tissue engineering strategies. For instance, it has previously been shown that the circadian regulation is significantly involved in the establishment of osseointegration under vitamin D regulation. Here, KEGG pathway analysis showed the potential association of the circadian rhythm with the success of implant osseointegration [27]. The circadian rhythm has also been shown to regulate coexisting populations of epidermal stem cells at opposite phases of the clock, which are differentially prone to activation by external stimulation [5]. Furthermore, research indicates that epidermal stem cells differ in their responsiveness to proliferation- and differentiation-like cues over a 24 h cycle [6], which may have vast implications in tissue engineering, where one cellular state may be favoured to encourage successful implant integration.

Overall, these findings suggest that the mechanism of entraining stem cell clocks by using each unique stem cell optimal synchronisation method offers an insightful way in which stem cells can be 'primed' to respond to the desired tissue engineering applications. With this in mind, mechanical entrainment of human adult stem cells allows for a noninvasive means by which the circadian clock in human adult stem cells can be directed and controlled whilst maintaining their appropriate clock timing, thus avoiding the need for additional exogenous chemical or thermal stimuli.

Conflicts of Interest

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

Acknowledgments

The authors would like to acknowledge support from the Analytical Biosciences Group of the Royal Society of Chemistry, MRC-ARUK Centre for Integrated Research on Musculoskeletal Ageing (Vanja Pekovic-Vaughan and John A. Hunt), and Welcome Trust Institutional Strategic Fund Fellowship (Vanja Pekovic-Vaughan). Furthermore, the authors thank BioEden Limited for the generous donation of the dental pulp stem cells used in this study. These stem cells were generated using the same technology used to process their customer's samples.

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Hindawi
Stem Cells International
Volume 2017, Article ID 6406025, 10 pages
https://doi.org/10.1155/2017/6406025

Review Article

Remodeling the Human Adult Stem Cell Niche for Regenerative Medicine Applications

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Received 21 March 2017; Accepted 17 August 2017; Published 27 September 2017

Academic Editor: Karen Liu

The interactions between stem cells and their surrounding microenvironment are pivotal to determine tissue homeostasis and stem cell renewal or differentiation and regeneration *in vivo*. Ever since they were postulated in 1978, stem cell niches have been identified and characterized in many germline and adult tissues. Comprehensive studies over the last decades helped to clarify the critical components of stem cell niches that include cellular, extracellular, biochemical, molecular, and physical regulators. This knowledge has direct impact on their inherent regenerative potential. Clinical applications demand readily available cell sources that, under controlled conditions, provide a specific therapeutic function. Thus, translational medicine aims at optimizing *in vitro* or *in vivo* the various components and complex architecture of the niche to exploit its therapeutic potential. Accordingly, the objective is to recreate the natural niche microenvironment during cell therapy process development and closely comply with the requests of regulatory authorities. In this paper, we review the most recent advances of translational medicine approaches that target the adult stem cell natural niche microenvironment for regenerative medicine applications.

1. Introduction: Highlights for the Translation of the Adult Stem Cell Niche Concept into Therapeutic Applications

Multipotent stem cells are critical biotherapeutics for regenerative medicine because of their innate ability to restore the structure and function of adult damaged tissues or organs. As a matter of fact, self-renewal, clonogenicity, and multipotentiality are the main common features of adult stem cells. In the transition from preclinical studies to clinical application, however, we should consider a number of hurdles in manipulating stem cells and implement clinically oriented approaches to control stem cell fate and function.

The niche is a highly dynamic microenvironment that can adapt to physiological or diseased conditions [1, 2]. The interest in targeting the stem cell niche grows and the opportunity of its remodeling represents a potential valuable therapeutic target for regenerative medicine [3–5]. Within the endogenous niche, multipotent stem cells are thoroughly connected with their surroundings and receive constant input which directs their fate. Ex vivo, culture conditions can thus modify the characteristics of cells towards their fates and further enhance their regenerative potential. Well-characterized adult niches vary in size and complexity: human adult stem cells can reside as individual cells within niches distributed throughout tissues. In other cases, multiple stem cell clusters are identified, as in the bulge of hair follicles or in the forebrain subventricular zone. Temporally speaking, adult stem cells can occupy a single invariant niche throughout postnatal life, for example, in the central nervous system; on the contrary, hematopoietic stem cells constantly recirculate from one bone marrow compartment to another and further activate hematopoiesis in extramedullary niches, such as in the liver and in the spleen in stress conditions, for instance during hematopoietic malignancies [6, 7]. These

strategies well comply with the concept of the dynamic innate regenerative capacity of the human body.

To target the stem cell niche, it might be necessary to regulate its various components such as cell-to-cell contact, cell to extracellular matrix interactions, and mechanical and electrical stimuli in a temporally and spatially regulated manner [8, 9]. Controlling all the niche components is an unattainable goal; however, this biological complexity translates into compelling manufacturing processes for reliable, quality-assured, and cost-effective products for stem cell-based therapies [10].

Manufacturing of cell therapy products (CTPs) for clinical application typically requires challenging steps such as the specific definition of identity, potency, and purity of each CTP. These definitions are largely therapy dependent. Towards this purpose, the US Food and Drug Administration (FDA) releases the current Good Manufacturing Practice (cGMP) guidelines and the International Conference on Harmonisation (ICH) introduces a systematic approach to process manufacturing and product management based on scientific knowledge and risk assessment [2, 11]. Overall, while developing a robust manufacturing process, it is essential to identify the critical characteristics to ensure product quality that are directly linked to its safety and efficacy. Stem cell expansion may be a critical step to determine CTP quality. Variability of stem cell identity, potency, and purity is particularly relevant to CTP manufacturing, and every attempt is made to mitigate the sources of this variability. For this very reason, the reagents used in CTP manufacturing are constantly improved. Many CTPs, formerly cultured in animal serum or feeder layers, are now cultured in chemically defined, xenofree or serum-free, cGMP conditions, with the specific purpose of reducing product variability [12, 13]. It is a critical challenge in current clinical translation to maintain ex vivo the precise characteristics of an identified stem cell and its surrounding microenvironment [14, 15].

In the following sections, we discuss the major challenges to limit adult stem cell product variability, and we describe, to the best of our knowledge, the most recent advances for their clinical translation. In general, we highlight the fact that "Clearly, fundamental scientific and medical questions reside within the niche" [16] to develop efficacious stem cell therapy products.

2. Mimicking the Natural Physical Microenvironment: Composition of the Extracellular Matrix for Clinical Applications

Contact with the extracellular matrix (ECM) and with other cells represents an important mechanism by which adult stem cells sense the microenvironment and make decisions about their fate [17]. The precise design of cellular biophysical microenvironment is a promising approach with the purpose of controlling stem cell behavior [18, 19]. Furthermore, the modulation of stem cell fate *in vitro* through an artificial microenvironment may efficiently avoid the need for direct genetic manipulation, which is more problematic for clinical application. Employing an artificial ECM aims at recreating the *in vivo* three-dimensional (3D) microenvironment.

Noncellular niches represent the first attempt for the development of defined physical culture conditions. The most recent advances towards therapeutic application include the development of synthetic bioinformative substrates designed at the micro- and nanoscale level [20]. Microtopography and nanotopography modulate cell behavior including adhesion, self-renewal, proliferation, and differentiation and represent emerging powerful tools. Physical constraints of their microenvironment, including micro- and even nanoscale geometric information, are detected by cells: rigidity, stiffness, and geometry of the substrate influence stem cell behavior [21–23]. These technologies have been adapted from the microelectronics industry and employ techniques such as surface micropatterning, chemical etching, and soft lithography to obtain organized pattern and regular geometries, microfluidics, and nanoscale-engineered three-dimensional (3D) biomimetic scaffolds for high-throughput studies. Lutolf et al. showed that the 3D topography of the substrate, in synergy with its defined matrix composition, can facilitate stem cell differentiation and alignment, if clinically needed [24, 25]. Nanoscale, micropatterned, and highly flexible membranes can be used to develop retinal pigment epithelium layers for minimally invasive implantation within the eye [26]. As of today, nanotopography is equally important as a defined culture medium formulation in the optimization of stem cell culture conditions [27–29].

The mechanisms by which topographic information of the biomimetic niche influence stem cell behavior are not completely understood; they appear to involve changes in cytoskeletal organization and structure, mainly at the level of integrins in the cellular membrane as a response to the geometry and size of the ECM. This interaction activates concomitant intracellular signaling cascades and guides stem cell behavior [30, 31]. Additionally, defined surfaces such as synthetic peptides containing the Arg-Gly-Asp (RGD) motif for cell attachment are still fairly new and represent a successful option for cell expansion [32–34].

In general, synthetic peptides and surfaces offer the advantage of being animal component free (ACF) and are potentially scalable. Matrigel, a poorly defined complex ECM isolated from the murine Engelbreth–Holm–Swarm tumor, would not be the ideal choice for clinical applications [35, 36]. Recombinant versions of single-ECM proteins, such as fibronectin and laminin, exist and offer the opportunity of designing a whole ACF cell environment. However, at present, recombinant proteins are still cost prohibitive for large-scale cell therapy product manufacturing.

Biocompatible hydrogel-based ECMs are employed for the culture of stem cells. Hydrogels are 3D macromolecule platforms obtained by the crosslinking of hydrophilic polymers. Collagen, fibrin, hyaluronic acid, alginate, dextran, chitosan, and agarose are used as components for hydrogels [37, 38]. However, fine modulation of their mechanical properties, degradation rate, and reproducibility is a challenge. Consequently, hydrogels polymerized with synthetic (chemically defined) peptides such as polylactic-glycolic acid

(PLGA) or polyethyleneglycol (PEG) are developed [39, 40]. Many biodegradable synthetic hydrogel-based products are approved for clinical use by the FDA and they are specifically designed for each clinical application. These defined biomimetic ECMs are effective in creating an adequate microenvironment for adult stem cells; however, it does not seem that they are sufficient to guarantee long-term maintenance of stem cells *in vitro*. Thus, we further analyze the additional important components of the stem cell niche to proceed to clinical application.

3. Moving towards Standardization of Cell Therapy Products: A Chemically Defined Microenvironment

In vitro, cultured cells are subjected to an environment whose main components are, together with the substrate, the culture medium, the atmosphere, and cell-to-cell interactions. Each of these components participate to the complex network of signaling pathways that eventually determine stem cell fate [22, 23]. Stem cell culture is widely employed in basic research and its optimization produces expanded cells in clinically relevant numbers [28, 32]. Culture media and their supplements provide the most fundamental nutrients to cultured cells: essential amino acids, a carbon source (typically glucose and galactose), basic salts, lipids, metal ions, a buffer system to maintain pH, an iron carrier (e.g., transferrin), growth factors, or hormones. Media supplements provide adhesion factors and they favor protection from shear forces (e.g., through surfactants or albumin). Overall, the medium and its components mimic as much as possible the situation *in vivo*.

A universally optimal culture condition does not exist because stem cells are all different. Stem cell culture parameters are defined for each stem cell type and designed on their intended therapeutic use [41, 42]. Feeder layers supply growth factors, cytokines, and other extracellular matrix components such as leukemia inhibitory factor (LIF), activin, Wnt, bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF), laminin, and vitronectin to maintain an undifferentiated state. These cell culture conditions are ill defined: Mallon et al. reported that feeder cells show batch-to-batch variability to maintain human embryonic stem cell (hESC) self-renewal and limited culture scale-up [43]. Negative results related to xenotransmission are also detected in long-term culture [44]. This demonstrates the unsuitability of cellular feeder layers as a culture component. Thus, studies on the development of feeder-free, possibly serum-free, and physicochemically defined culture systems are strongly encouraged.

Good Cell Culture Practice (GCCP) and Good Manufacturing Practice (GMP) represent the leading guidelines to establish standardized protocols for cell-based therapy and regenerative medicine [45]. As a matter of fact, the design of fully defined media able to maintain stemness, or alternatively to induce differentiation towards well-defined phenotypes, is a point of major interest for stem cell culture today. Chemically defined media used for the growth of Chinese Hamster Ovary (CHO) represent an instructive lesson from the past.

The advantage of defined media, aside from the desirable ethical reduction or complete absence of fetal bovine serum (FBS), is the precise chemical composition which thus facilitates a controlled culture environment for the selective growth of cells. Defined culture conditions allow the establishment and the maintenance of phenotypically well-defined and karyotipycally stable cells.

Cell culture conditions are further optimized by the implementation of specific stem cell supplements, that is, recombinant growth factors or cytokines. The selection of the medium additives and their concentrations, especially the growth factors, is critical since it could variably affect the cultured cells. Adult stem cells require ex vivo-specific growth factors that mimic their native microenvironment.

Growth factors act as mitogens that stimulate cell proliferation and in some cases are crucial to maintain cell characteristics. The most commonly used growth factors in ACF or xenofree (XF) media for human adult stem cells include basic fibroblast growth factor, epidermal growth factor, transforming growth factor-β, vascular endothelial growth factor, and platelet-derived growth factor [46, 47]. Most of these growth factors are available as recombinant proteins and are widely used for cell therapy applications.

The specificity of growth factors, their concentration, and synergistic effect play a crucial role in achieving an optimized, cell-specific, defined culture medium. Notably, growth factor requirements can be not only cell-type specific but also species specific: LIF supports the expansion of a mouse but not human ESCs. Secreted molecules, such as colony-stimulating factor and stem cell factor (Kit ligand), play important roles in cell survival.

Cell-to-cell interactions involving other classes of molecules are also important: interactions between Eph tyrosine kinase receptors and their Ephrin transmembrane ligands regulate adult stem cell proliferation and migration [48].

Efficient stem cell manufacturing *in vitro* is crucial to guarantee a long-term therapeutic effect *in vivo*. This critical issue increases our knowledge on the fine regulation of stem cell microenvironment and moves translational research into effective and more reproducible clinical trials.

4. Bioreactors: 3D Mechanical Force Mimicking the Controlled Oxygen Perfusion in Stem Cell Niches

For decades, cells are cultured under an atmospheric oxygen pressure that is much higher than the one experienced in their niches *in vivo*. Cell culture incubators normally preserve atmospheric partial oxygen pressure (pO2) which is around 150 mmHg (21% O_2). *In vivo*, physiological pO2 ranges between 50 and 5 mmHg (7–0.7%). Thus, the term "normoxia" referred to standard cell culture systems does not refer to physiological conditions. Lowering the pO2 is beneficial for various adult stem cell types [49]: Wion et al. reported that bone marrow mesenchymal stem cell

expansion was more efficient at 2% pO2 [50]. Additionally, the pO2 found in adult stem cell niches is variable.

The stem cell culture medium is dynamic and changes rapidly due to the release and/or consumption of numerous metabolites. For this reason, continuous perfusion of cell cultures with fresh medium through controlled bioreactors is considered a valuable option to standardize cell-manufacturing processes. Bioreactors utilize mechanical forces to influence biological processes under closely controlled conditions. They provide spatially homogeneous cell distribution; deliver physiological relevant concentrations of oxygen, carbon dioxide, and nutrients in the culture medium; and provide physical stimuli to regulate stem cell differentiation and proliferation. In bioreactors, stem cells are expanded in stirred vessels or on perfused scaffolds, and their culture pH and oxygen values are monitored. This controlled process is beneficial in terms of stem cell expansion and differentiation compared to conventional static culture conditions, although autocrine and paracrine loops might be disturbed [51]. The implementation of sensitive monitoring systems and control algorithms is required to increase cell product reproducibility. Various types of bioreactors exist and are employed in the manufacturing of stem cell therapy products.

5. Reduction of Animal-Derived Components: Serum-Free Culture and Its Impact on the Niche Microenvironment

Serum is a mixture of a large number of components, and its composition is partly uncharacterized. Slight variations in its composition influence key properties of cells because they are highly sensitive to culture conditions. Thus, serum introduces an unknown variable into the culture system, and this represents a challenge to generate consistent and quality-assured cells in clinical-scale production [52, 53].

In cell culture, the use of fetal bovine serum (FBS) as a medium supplement is most widespread. The major function of serum in stem cell culture media is to provide multiple elements that correspond to the *in vivo* condition: hormonal factors for cell growth and proliferation transport proteins that carry hormones, minerals, trace elements (e.g., transferrin), and lipids (e.g., lipoproteins). Additionally, it stabilizes pH with factors inhibiting proteases (such as α-antitrypsin or α2-macroglobulin), supplies adhesion molecules of the extracellular matrix, and contains factors that protect against shear forces [54, 55].

The critical problems related to the presence of FBS in stem cell culture are batch-to-batch variability, fluctuating availability, unexpected cell characteristics, and potential cytotoxicity of uncharacterized factors [56–58]. Gstrauntha-ler et al. raised several ethical issues concerning the use and collection of FBS [59, 60]. Most importantly, the immunogenicity of cells cultured in FBS has proven to be challenging for their use in therapeutic strategies.

Most regulatory agencies tolerate the use of xenogenic components in culture media in phase I clinical trials. However, later phase trials are required to employ serum-free or at least xenofree media. Mendicino et al. reported recently that FBS is employed during manufacturing in over 80% of the investigational new drug (IND) applications for mesenchymal stem cell (MSC) products submitted to the FDA [61]. The concentration of FBS ranges from 2 to 20%, with 10% FBS being the most common concentration. Serum consumption increases on the average of 10%–15% annually, which suggests that the demand for serum will soon exceed the actual availability. Safety concerns represent sound reasons to search for serum substitutes or serum-free media [62–64].

The major benefits of establishing serum-free cell culture systems are in the direction of standardization, that is, limitation of the cell therapy product variability, and elimination of a potential source of contamination [65, 66]. Of note, serum-free media are generally more cell specific.

Adult stem cells cannot survive in the absence of serum-specific growth factors as well as other unidentified factors in the serum. In serum-free culture, a separate attachment substrate is required. Human plasma fibronectin is a common adhesion substrate used in serum-free systems [67]. Human platelet lysates (HPLs) are considered a valuable FBS alternative for adult stem cell expansion [68]. Platelet granules contain various growth factors and cytokines that can be released by freeze/thaw-mediated lysis, sonication, or chemical treatment. Due to the wound healing property of platelets *in vivo*, growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1), platelet-derived epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), together with attachment factors (fibronectin and vitronectin), and protease inhibitors are exploited for their use [69]. However, hPL preparations are subjected to donor-to-donor variations.

Pooled human AB serum (HABS) is an additional alternative: it supports proliferation of human mesenchymal stromal cells (hMSCs) and maintains their characteristics throughout ex vivo expansion [70]. Furthermore, human umbilical cord blood serum (hUCBS) is a rich source of soluble growth factors. hUCBS supports the growth, proliferation, and differentiation of the resident stem cell population in the fetal blood. Cord blood defines distinct characteristics in cord blood-derived stem cells, and this supplement may constitute a unique microenvironment to support ex vivo culture of adult stem cells [71]. However, the drawbacks of hUCBS are various, likewise any other blood-derived alternative to FBS. In general, the possibility of contamination from adventitious agents, lot-to-lot variability, and limitation of collection volumes remain a challenge. Contamination issues are kept controlled by strict adherence to blood bank quality standards.

To overcome the issue of limited collection availability, recombinant forms of human serum albumin are commercially available. Recombinant human serum albumin (r-HSA) is used instead of purified human serum albumin (HSA) [72]. r-HSA is structurally identical to HSA but it is free from viral and prion contamination, and it guarantees high batch-to-batch consistency. Recombinant human albumin is more likely to be compliant with regulatory requirements and may serve as an ACF ancillary product for cell therapy and regenerative medicine applications

[56, 61]. The major disadvantage is the price, which is several times higher for r-HSA than for purified HSA.

A few serum-free media are also commercially available. Unfortunately, the composition of commercially available proprietary serum-free media is generally unknown. Manufacturers usually do not disclose this information that is often requested by regulatory authorities in clinical settings.

The process of developing serum-free media or adapting stem cells to serum-free culture media is complex and time consuming. However, the development of these defined media should be encouraged in view of their intended clinical application. As stem cell therapy industry advances and clinical trials reach their later phases, culture process validation, scale-up, and quality assurance of critical raw material are highly requested.

Addressing this need results in significant changes to current culturing technologies for a beneficial shift towards more qualified and compelling therapies. Table 1 shows an overview of the current alternatives for clinical applications.

6. The Cardiac Stem Cell Niche in Regenerative Medicine

Extracellular matrix (ECM) composition is precisely regulated during normal heart development and its dysregulation results in structural and functional heart diseases.

The heart is a biomechanical organ in which the mechanical stress on cardiac cells mainly arises from the hemodynamic load. Dysregulation of either preload in diastole or afterload in systole contributes to the pathogenesis of congenital or adult heart disease. The microenvironment of stem cells in the adult myocardium includes cardiomyocytes, vasculature, interstitial cells, and extracellular matrix, each of them representing a potential target to enhance the regenerative potential of the heart after injury.

Cardiovascular diseases represent a major public health priority. Specifically, patients who suffer from myocardial infarction may encounter adverse remodeling that can ultimately lead to heart failure. Prognosis of patients affected by heart failure is very poor with 5-year mortality close to 50%. Despite the impressive progress in the clinical treatment of heart failure in recent years, heart transplantation is still required to avoid death as the result of the inexorable decline in cardiac function. Nonetheless, the morbidities associated with heart transplantation and the limited organ supply demand the development of new stem cell-based approaches for regenerative medicine [82–84].

The human heart is one of the organs which regenerates less in the body, or at least, its regenerative potential is clearly lower than the intestine, liver, bone, or skin [85]. However, some degree of cardiomyocyte renewal has to be recognized [86, 87]. Despite the fact that proliferative rates are clearly small and quite difficult to detect, they raise the question whether such innate processes could be increased and employed therapeutically. Given these observations, the main objective of cardiac regenerative medicine is to replace damaged heart cells and, therefore, to restore the physiological structure and function of the organ [88, 89]. Various clinical trials employ adult stem cells to regenerate the heart. The past decade highlighted the most instructive stem cell-based studies for cardiac diseases. These first-generation adult stem cell therapies for myocardial regeneration were promising in small animal models but beneficial effects in humans were far more moderate [90]. Consequently, the objective of second-generation therapeutic approaches is the enhancement of cellular properties and survival to restore the normal function of the myocardium.

Current investigation deals with combinatory approaches that employ multiple stem cell types. Preconditioning stem cells *in vitro* with growth factors, hypoxic treatment, or antiaging reagents enhances cellular engraftment, survival, and differentiation before administration. An example of this valuable approach involves the "cardiopoietic" guidance of multipotent adult stem cells: Behfar et al. employed a specific cardiogenic cocktail for human mesenchymal stem cells through manipulation of their culture environment [91]. The authors assessed this approach in the C-CURE trial (ClinicalTrials.gov Indentifier: NCT00810238) and in the larger CHART-1 (ClinicalTrials.gov Indentifier: NCT01768702) clinical trial to treat ischemic heart failure. So far, preliminary results indicate a positive although not statistically significant trend in the treated group.

Engineered scaffolds represent 3D myocardial tissue for adult stem cell culture; this approach includes synthetic porous scaffolds or scaffold-free cell sheets to increase cardiac contractility and output. In an effort to use physicochemically defined components, recombinant human laminin and recombinant human fibronectin in our hands (Figure 1, unpublished results) are used [92].

Complex 3D ECM, including ECM obtained from decellularized hearts, provides a superior microenvironment over single 2D ECM components with regard to cardiac stem cell structural organization and function [93–95]. Hydrogels are an effective alternative to scaffolds: they create a synthetic microenvironment for cells *in vitro* and are subsequently administered into the myocardium as a patch or injected into the damaged region of the heart. 3D bioprinting recently emerged as an exciting technological advancement for the construction of 3D myocardial tissue: it is now possible to print native cardiac tissue or custom-made patient-specific devices for cardiovascular diseases.

Exosomes carrying noncoding RNAs are important players for intercellular communication in the heart. MicroRNAs (miRNAs) and long noncoding RNAs (lncRNAs) act as critical regulators of cardiac development and disease: they necessitate the implementation into future efforts at mimicking the cardiac microenvironment *in vitro*. miR-15, miR-17, mrR-133a, miR-199a, miR-210, miR-451, and miR-499 improve myocardial structure and function after ischemia or infarction.

Future models may expand into gene therapies: the analysis of mononuclear polyploid cells naturally occurring in regenerative tissues represents a more recent approach [96].

7. Conclusions

Several studies performed in the last decades highlight the importance of the microenvironment in which human

Table 1: A comprehensive overview of the current available alternatives to recreate the stem cell microenvironment *in vitro* for clinical applications.

Component of the native stem cell niche microenvironment	Function *in vivo*	Corresponding component *in vitro*	Most recent alternatives
Extracellular matrix (ECM)	Physical adhesion; Cell orientation; Stem cell fate	Scaffolds or matrices (2D or 3D) Coating substrates	Hydrogels [39, 40]; Synthetic peptides (RGD) [73]; Micro- and nanotopographic biomimetic scaffolds [20, 74]
Chemical microenvironment	Provides fundamental nutrients (salts, ions, lipids, etc.); Buffering system	Cell culture medium	Cell-type-specific chemically defined (serum-free) culture medium [75, 76]
	Adhesion factors; Protection from shear forces; Cell proliferation	Fetal bovine serum (FBS)	Human platelet lysates [77]; Human pooled AB serum [78]; Human umbilical cord blood serum [71]; Recombinant human serum albumin [72]; Serum-free (or reduced FBS) culture systems [79]
	Cell proliferation	Feeder cells; Growth factors	Feeder-free systems [44]; Recombinant human growth factors [80]
	Cell metabolism and survival	Oxygen	Bioreactor-controlled oxygen perfusion [51, 81]

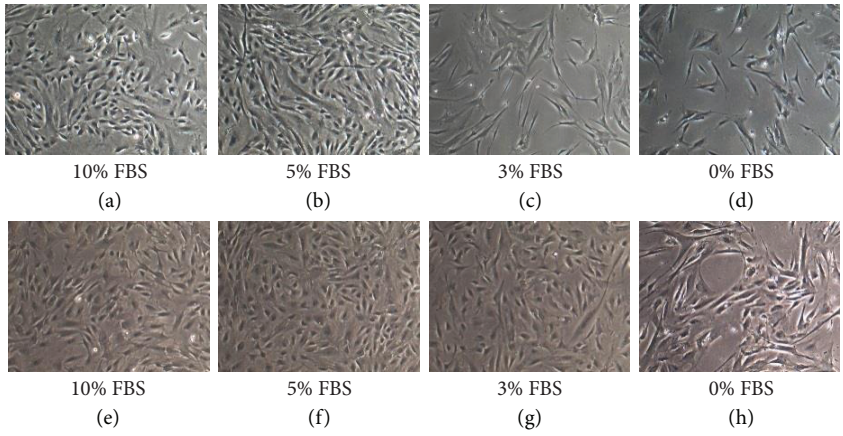

Figure 1: (a, b, c, d) Human cardiac biopsy-derived stem cells cultured in sequentially optimized serum-free culture medium on recombinant human fibronectin-coated surface. (e, f, g, h) Control culture of human cardiac biopsy-derived stem cells in commercially available serum-free proprietary medium (Essential 6™, Gibco) on fibronectin-coated surface. Authors' unpublished results.

stem cells grow and maintain their peculiar characteristics. Various components of the human stem cell niche are clarified, and the objective of recreating an appropriate native microenvironment is the current objective of regenerative medicine.

Manufacturing human adult stem cells as therapeutics should preferably be performed in animal component-free or reduced animal component systems to avoid the risk of zoonoses. Ideally, the cell culture systems that are engineered for this purpose will minimize exposure to animal cells and proteins by using primarily human or recombinant human components. Furthermore, it is highly desirable to employ physicochemically defined culture media, possibly devoiding complex mixtures such as animal or human serum.

We are moving closer to producing stem cell therapy products that have very limited contact with animal products and thus are better candidates for use in regenerative therapies. Although many challenges lie ahead in the industrialization of CTP manufacturing, we find much reason for optimism. Decades of experience with industrial cell line culture processes lay the foundation of engineering CTPs such as bioreactor scale-up, analysis of cellular metabolism, medium design, optimization of expansion strategies, and process control. Meanwhile, our understanding of how cells interact with their environment is improving, and controlled systems that mimic the cellular microenvironment are generating important data sets which are increasingly focused on molecular and cellular information. In parallel, our general understanding of the molecular basis of stem cell states, including adhesion properties, metabolic needs, clonogenicity, and proliferation control, is progressing. Such findings emphasize the importance of a multidisciplinary approach

for the development of engineered products, involving the connection of many disciplines such as cell and molecular biology, materials science, biomedical engineering, and medicine. The global perspective is the implementation of a comprehensive cell therapy product including a defined serum-free culture medium, a perfusion system, biosensors, and micro- or nanoscale-designed scaffolds mimicking as far as possible the niche microenvironment that is known to modulate stem cell function. The future lies probably in the development of 3D modular biomimetic systems assembled according to the final purpose of the stem cell culture, for example, stemness maintenance or control of cell differentiation towards clinically relevant cell phenotypes.

This massive development requires time and resources and may also involve remarkable changes to be implemented into the original manufacturing process. Additionally, full characterization of the final stem cell product after process development changes is crucial to verify comparability to the original product. It is also critical to carefully examine the quality, safety, and availability of the specific components implemented into the system to ensure that the selection meets the needs for further scale-up of the process and resulting therapeutic product.

The future of CTPs relies on the development of cost-effective technologies for cell manufacturing. Given the inherent complexity of CTPs and their production processes, appropriately designed approaches will be essential in transforming today's experimental CTPs into available therapeutics.

The advancement of the knowledge and optimization of the integral components of the human stem cell niche are instrumental in this ambitious goal.

Conflicts of Interest

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

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Hindawi
Stem Cells International
Volume 2017, Article ID 5046953, 10 pages
https://doi.org/10.1155/2017/5046953

Review Article

Structure and Functions of Blood Vessels and Vascular Niches in Bone

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Received 5 May 2017; Revised 26 July 2017; Accepted 23 August 2017; Published 17 September 2017

Academic Editor: Hong Qian

Bone provides nurturing microenvironments for an array of cell types that coordinate important physiological functions of the skeleton, such as energy metabolism, mineral homeostasis, osteogenesis, and haematopoiesis. Endothelial cells form an intricate network of blood vessels that organises and sustains various microenvironments in bone. The recent identification of heterogeneity in the bone vasculature supports the existence of multiple vascular niches within the bone marrow compartment. A unique combination of cells and factors defining a particular microenvironment, supply regulatory signals to mediate a specific function. This review discusses recent developments in our understanding of vascular niches in bone that play a critical role in regulating the behaviour of multipotent haematopoietic and mesenchymal stem cells during development and homeostasis.

1. Introduction

Recent advancements in vascular biology have increased our understanding and knowledge of blood vessels and their characteristics during various physiological and pathological conditions. Blood vessels not only act as a transport conduit system but also play important roles in organ development, tissue morphogenesis, inflammation, barrier formation, and wound healing [1–4]. In addition, active involvement of blood vessels in the pathogenesis of a number of diseases suggests a fundamental need to understand these versatile transport networks in the body [5]. Blood vessels form an integral part of the skeletal system playing multiple roles in the maintenance of bone homeostasis. The importance of blood vessels in bone was initially recognised by surgeons during repair and healing of bone fractures [6, 7]. The essential role played by the bone vasculature during skeletal development [8–10] and fracture repair [6, 9, 11] has been an intense field of research. Further, the cell-specific contributions in pleiotropic functions of bone such as regulating whole body metabolism [12–14], brain functions [15–17], and mineral homeostasis [18–20] still need to be understood.

Blood vessels in bone are reported to provide nurturing microenvironments to haematopoietic stem cells (HSCs) [21, 22] and mesenchymal stem cells (MSCs) [23, 24]. Various microenvironments in bone still need to be characterised well to understand their function during development, growth, and disease. Recent technical advances in bone imaging have substantially improved our fundamental knowledge of skeletal blood vessels. This review aims to provide an overview of recent developments and contemporary understandings of the bone vasculature and its microenvironments.

2. Structure and Characterisation of Blood Vessels in the Skeletal System

2.1. Skeletal Blood Circulation. Bone has an extensive network of blood vessels (Figure 1) consuming almost 10–15% of resting cardiac output [25, 26]. The spatial arrangement of blood vessels enables efficient and optimal delivery of oxygen and nutrients to various locations within the bone marrow compartment. Irrespective of the bone type, the main blood supply of bones is derived from arteries entering the cortical region, which connect with medullary sinusoids

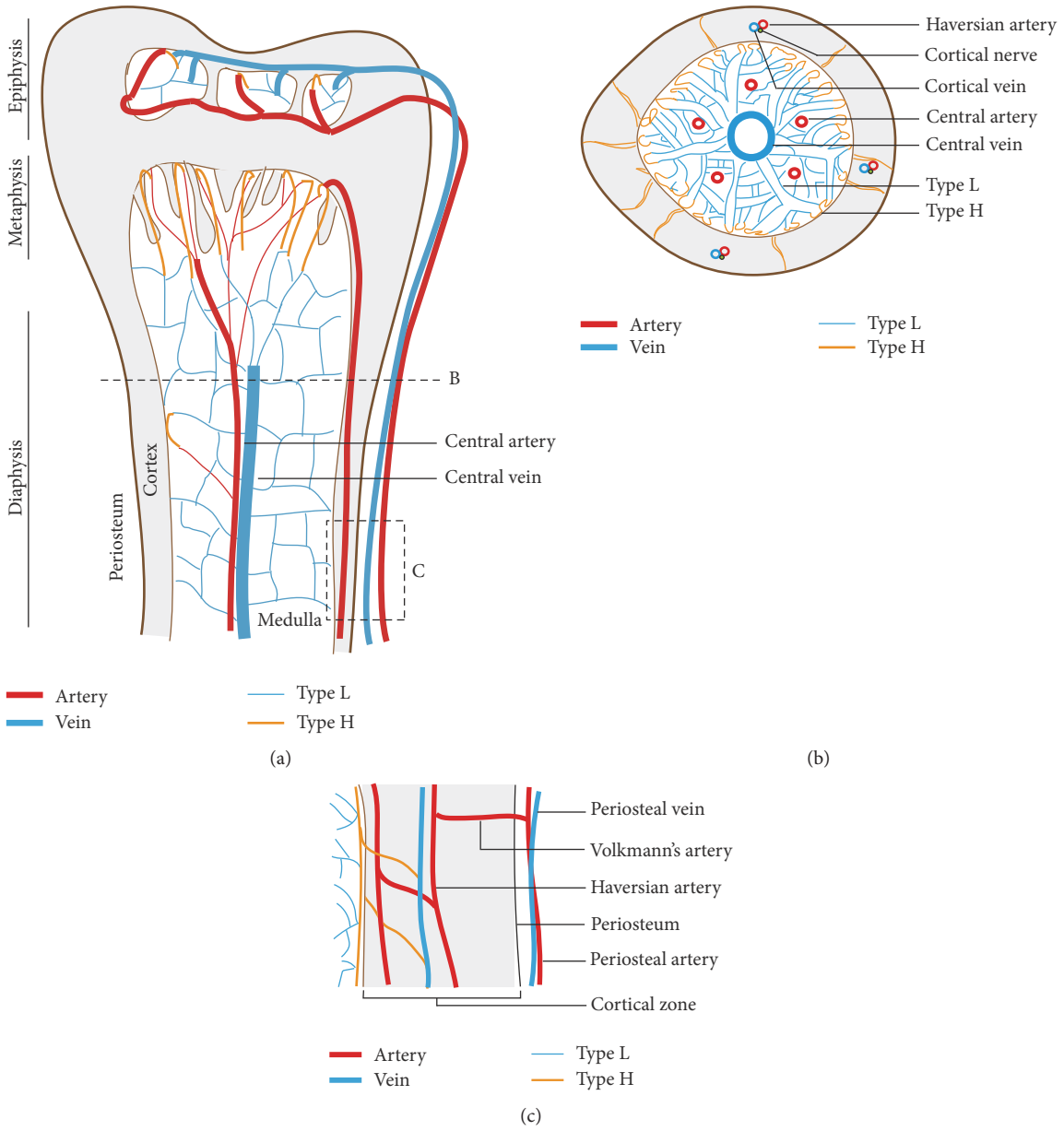

Figure 1: Blood vessel arrangement in long bone. (a) Longitudinal view demonstrates arrangement of arteries, veins, and capillaries in the epiphysis, metaphysis, and diaphysis regions of long bone. Arteries branch into smaller arterioles and terminate in type H capillaries. Type H capillaries are localised near osteoprogenitors in the metaphysis and endosteum regions. Type L capillaries are sinusoidal vessels terminating in the central vein. (b) Transverse view shows bone vascular pattern in cortical and medullary regions of long bone. A large central vein and a few nutrient arteries are prominent in the medullary region. (c) Arrangement of blood vessels showing the connection between cortical and medullary blood flow. Periosteal blood vessels are connected intermittently with cortical blood vessels.

to finally exit the bone through veins [27, 28]. However, shape and type of skeleton can possibly affect the arrangement of capillary network existing between arteries and veins. Typical long bones, such as the femur and tibia, are supplied by several arteries and arterioles, which are classified based on their region of blood supply. The central artery also called as nutrient artery enters bone through a foramen and branches into a number of smaller arteries and arterioles to supply maximum regions of adult bone. It sustains high blood pressure to reach distant locations, usually terminating into capillaries present in the metaphysis and endosteum. There is a central large vein that receives blood from capillaries present in various regions and drains deoxygenated blood and nutrient waste from bone [29]. Periosteal arteries supply the outer surface of bone and are connected to Haversian arteries present in the cortical region through Volkmann's arteries. Haversian arteries run parallel to the longitudinal axis of the long bone in the cortex while shorter Volkmann's arteries run perpendicular to the long bone axis [30, 31]. Haversian arteries eventually converge into metaphyseal capillaries to deliver blood into the medullary region. In contrast, the blood supply from epiphyseal arteries does

not have a route to enter the medullary region of long bones, thus maintaining a separate blood circulation in the epiphysis region. Epiphyseal arteries enter the bone from a heavy network of periarticular vascular plexus present near the ends of long bones. The veins draining the epiphyseal blood are relatively smaller compared to the vein present in the medullary region (Figure 1).

2.2. Heterogeneity in Blood Vessels. Divergence in arterial blood supply envisages the existence of multiple veins and capillary subtypes in bone. However, diversity within these blood vessels has not been well appreciated until recently. Fenestrated or sinusoidal capillaries form the majority of blood vessels in the skeletal vasculature. These are highly branched networks of blood vessels present in the marrow cavity of bones. Sinusoidal endothelial cells express vascular endothelial growth factor receptor-3 (VEGFR3) while bone arterial endothelium is negative for Vegfr3 [32]. Vascular structures in bone can be demarcated as laminin$^{+/low}$Sca-1$^{-/low}$ sinusoids, Sca-1^{+}laminin^{+} endosteal vessels, and Sca-1^{+}laminin^{+} central arteries [33]. Investigating blood vessels during postnatal development led to the identification of a new blood vessel subtype called type H present in actively growing regions of bone. They are named type H as they express high levels of blood vessel markers, endomucin (Emcn) and CD31 (Pecam1) compared to sinusoidal vessels, which express low levels of these markers thereby termed as type L [8, 10].

In an actively growing bone, type H vessels are present in the metaphysis and endosteum regions, while type L vessels predominate the whole medullary region. Type H capillaries are linearly structured, columnarly arranged blood vessels in comparison to a branched network of type L capillaries. The leading fronts of type H vessels, which mediate angiogenesis in bone, contain bulge-shaped lumenised structures [10, 29]. However, the functional significance of these unique structures in the vascular front remains unknown. Arteries and arterioles express ephrin B2 (Efnb2) and are negative for Emcn expression. A subpopulation of endothelial cells within type H endothelium, expressing both Efnb2 and Emcn, is proposed to generate arteriolar blood vessels (Efnb2+, Emcn−). This subfraction of type H blood vessels displays expression of other arterial markers such as Sox17 and neuropilin-1 [34]. Arteries are tightly enwrapped by α-smooth muscle actin+ (αSMA+) mesenchymal cells, while smaller arterioles have αSMA− and platelet-derived growth factor receptor beta+ (PDGFRβ+) perivascular cells. Multiple types of bone mesenchymal cells and their association with blood vessel subtypes are discussed later in this review. Thus, the bone vasculature is heterogeneous, unique, and needs profound investigation to understand tissue-specific vascular modifications and specialised functions.

3. Blood Flow and Oxygenation in Bone

The spatial arrangement of blood vessels is intricate and unique in every tissue to provide proper oxygen and nutrient supply to the whole tissue or organ. The organisation of distinct blood vessel subtypes in long bones indicates a peculiar blood flow pattern. Blood velocity is higher in type H vessels compared to type L vessels. When blood flows down from type H capillaries, blood velocity drops with each vascular branch in the metaphysis to attain a characteristic low velocity for type L capillaries in the diaphysis. Frequent branching and joining of vascular networks in the diaphysis maintain low blood velocity in the diaphyseal capillaries [29, 35].

3.1. Oxygen Status in Bone Vascular Microenvironments. The peculiar blood flow pattern in bone coincides with oxygen status of the bone microenvironment. Measurement of local oxygen tension (pO_2) in live mice indicated that pO_2 is higher in the endosteal bone region than in the deeper sinusoidal regions. Endosteal regions are vascularised by type H capillaries and arterioles compared to type L vessels in sinusoidal regions [36]. It has also been illustrated that low vascular permeability in arterial and type H vessels maintain low reactive oxygen species (ROS) in the microenvironment compared to fenestrated, highly permeable sinusoids [35]. Analysing HSCs in Hoechst-perfused mice showed that the localisation of long-term HSCs (LT-HSCs) is limited to the least perfused regions in the BM [37, 38]. The low oxygen or hypoxic microenvironment supports maintenance of HSCs and protects them from damage caused by oxygen stress [39]. Hypoxia-dependent stabilisation of hypoxia inducible factor (HIF) is essential for the canonical HIF-mediated signalling pathway that plays divergent roles in blood vessels [8, 34], mesenchymal cells [40], and haematopoietic cells [39, 41, 42] in the BM microenvironment.

Hypoxia and HIF-1α-mediated regulation of chondrocyte growth and survival is essential for chondrogenesis and growth plate development [43, 44]. An important downstream target of HIF-1α is VEGF, a fundamental factor required for blood vessel formation in physiological and pathological conditions [45]. VEGF plays a pleiotropic role in regulating several processes during bone development, growth, and repair [46]. Genetic studies in chondrocytes illustrated essential functions of their VEGF in angiogenesis and bone formation in addition to regulating chondrogenesis [47, 48]. Thus, hypoxia-mediated regulation of HIF controls VEGF levels to couple blood vessel growth and osteogenesis in bone [49, 50]. Stabilisation of HIF in osteoprogenitors results in the expansion of the HSC niche and promotion of erythropoietin production in bone [51]. HSCs also exploit HIF signalling to precisely regulate their cell cycle and quiescence status in the BM [42].

Genetic and pharmacological manipulations of blood flow in developing zebra fish affected nitric oxide synthase signalling in primitive HSCs resulting in defective HSC development [52]. Blood flow is also an important player in mobilising haematopoietic cells from bones to various organs and tissues. In mice, sinusoidal vessels having high permeability promote migration and differentiation of HSPCs [35]. A declining number of type H vessels and arterioles with age in bone leads to reduced skeletal blood perfusion and HSC function [29, 34]. In addition, manipulating blood flow in bone leads to defective angiogenesis and bone formation [29], suggesting blood flow as a potential cause of age-related bone loss. These compelling evidences argue the importance of blood flow in

maintaining skeletal homeostasis by regulating bone formation and haematopoiesis.

3.2. Clinical Importance of Blood Flow in the Skeleton. Despite differences in bone structures, studies from rodent models have been beneficial for the development of therapeutic strategies to target human bone diseases. Basic understanding of blood vessels and blood flow in the skeletal system is mainly derived from findings in rodent models. Rodents show age-related bone loss similar to humans. Remarkably, identification of decrease in bone arterial capillaries with age in mice [34] corresponds with age-associated decline in femoral arterial blood flow in humans [53]. Recent demonstration of decreased type H vessels in aged and osteoporotic human subjects [54] highlights the significance of investigating skeletal blood vessels in rodents.

Increasing number of clinical evidences indicate the importance of blood flow in maintaining homeostasis of the skeletal system. Reduced blood supply was measured in bones of elderly women with osteoporotic conditions [55]. Impairment of blood supply to bone causes death of bone cells leading to the development of osteonecrosis condition [56]. A comparative study in patients with unilateral arterial occlusive disease showed deleterious effect of defective blood flow on bone mineralisation [57]. Defects in blood flow in the subchondral region has been identified as a potential mechanism in generating osteoarthritis [58]. Systemic disorders such as diabetes [59], chronic obstructive pulmonary disorders [60], and hypertension [61] that impair vascular perfusion are associated with bone defects. Moreover, blood supply is critical for initiation of callus formation during fracture healing and repair [6]. Defective blood vessel formation is observed at fracture sites showing delayed healing and regeneration processes [50]. Disuse-induced osteopenia conditions such as bed rest and hindlimb unloading have also been associated with changes in blood supply to bone [62, 63]. In spite of clinical data supporting experimental findings, further research is required to understand molecular mechanisms involved in the generation of these clinical conditions.

4. The Vascular Microenvironment for Mesenchymal Cells

4.1. Types of Mesenchymal Stem and Progenitor Cells Forming Bone Marrow Stroma. Mesenchymal lineage cells comprising a majority of bone marrow stromal cell population form an important component of the bone marrow microenvironment. Multipotent mesenchymal stem and progenitor cells (MSPCs) can generate various types of bone marrow mesenchymal stromal cells including osteoblasts, chondrocytes, adipocytes, and reticular cells. Understanding the hierarchical relationship of BM stromal cells is still an intensive area of research. Although perivascular origin of MSPCs in different organs has been suggested [23], distinct waves of stromal cells have been identified in the developing bone marrow [64, 65]. Genetic lineage tracing techniques has provided significant knowledge in understanding the heterogeneity associated with BM mesenchymal cells. Nestin-GFP+ cells wrap endothelial cells (ECs) that form arteries and type H capillaries. Perivascular Nestin-GFP+ cells were identified to mark early MSPCs, which can generate bone marrow stroma and bone-lineage cells [66]. Similarly, osterix+ neonatal mesenchymal cells possess the potential to generate bone lineage cells, chondrocytes, adipocytes, and BM stroma. In contrast, osterix+ embryonic and adult mesenchymal cells show limited potential [64]. Remarkably, both osterix+ and Nestin-GFP+ cells are present near type H capillaries and absent around perisinusoidal type L capillaries [8, 35]. Perisinusoidal stromal cells expressing leptin receptor (LepR) were suggested to contribute to bone-lineage cells when marked early during development [67]. Remarkably, LepR expression in adult mesenchymal cells promotes their adipogenic potential inhibiting osteogenic cell fate [68]. LepR+ cells contribute to C-X-C motif chemokine ligand 12 (Cxcl12) expressing cells in the bone marrow [69]. Cxcl12 expressing Nestin-negative mesenchymal cells provide the HSC supporting microenvironment [70]. Therefore, it will be interesting to use an inducible (*–CreER*) system to understand stage-specific contribution of LepR+ cells in BM stroma.

In contrary to perivascular MSPCs, cells of nonperivascular origin have also been identified to contribute to bone lineage cells and BM stroma. Lineage tracing cells of chondrogenic origin using *Col2-CreER* system demonstrated their potential to form bone lineage cells and Cxcl12-abundant reticular stromal cells [65]. Similarly, lineage tracing using other chondrogenic systems such as Sox9- and Aggrecan-*CreER* also confirmed the cells' potential to generate multiple mesenchymal lineage cells. Identification of Gremlin1 as a marker for cells with osteochondroreticular potential indicates the possible existence of distinct progenitor subtypes within the pool of MSPCs. Clonally expanding Gremlin1+ cells were identified in growth plate and metaphysis region and they lack adipocyte differentiation potential [71]. These studies demonstrate the existence of heterogeneity in MSPCs and need to understand subtypes within the population to identify their hierarchical relationship.

4.2. Localisation of Mesenchymal Stromal Cells in the Vascular Niche. Localisation of MSPCs suggests that multiple regions within the bone marrow microenvironment can support and provide niches for MSPCs. Col2+, Sox9+, and Aggrecan+ cells are located on the growth plate, which is an avascular region [65]. Gremlin1+ cells are present in both growth plate and metaphysis regions [71]. Nestin-GFP+ cells are located around arteries and in the metaphysis [35, 66]. PDGFRβ+ mesenchymal cells show a distribution pattern similar to Nestin-GFP+ cells [34]. Majority of osterix+ cells are located around type H vessels in the metaphysis [8, 64]. LepR+ and Cxcl12+ cells are largely localised around type L (perisinusoidal) vessels [67, 68]. Chondrocytes are present in the avascular zone, typically in the growth plate or epiphysis region of bones [65]. Osteogenic progenitors are specifically localised around type H vessels in the metaphysis and endosteum regions [8]. Fat cells or adipocytes preferentially present in perisinusoidal space of the diaphysis

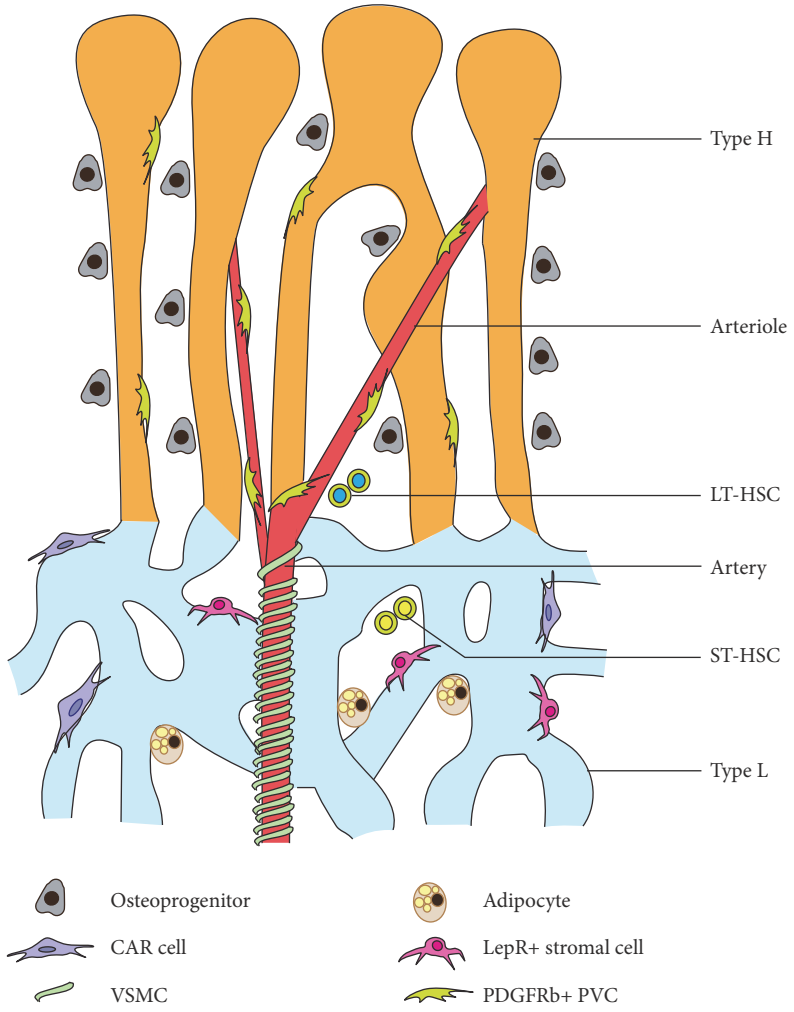

Figure 2: Vascular microenvironments in bone. Multiple types of perivascular mesenchymal stromal cells are supported by distinct subtypes of vascular structures in the bone marrow microenvironment. Arteriolar niche supports long-term HSCs (LT-HSC) while sinusoidal niche maintains short-term and cycling HSCs (ST-HSCs).

[68]. Reticular cells are also localised around type L vessels in the perisinusoidal region [67, 69]. Vascular smooth muscle cells are αSMA+ periarterial cells, tightly wrapping arteries in the bone marrow microenvironment [8, 34]. Thus, subpopulations of heterogenic BM mesenchymal stromal cells preferentially localise around specific blood vessel subtypes, suggesting the existence of specialised vascular microenvironments (Figure 2).

Evidences suggest the central role played by blood vessels in supporting the local microenvironment. High expression of pro-osteogenic factors in type H vessels generates the microenvironment required for osteoprogenitors. Promoting type H capillaries in bone results in improved osteoprogenitor numbers [8, 10]. Similarly, platelet-derived growth factor B (PDGF-B) released by endothelium binds to PDGF receptor present on mesenchymal cells to activate growth mediated signalling pathways [72]. Overexpression of PDGF-B in bone endothelium results in increased PDGFRβ+ perivascular mesenchymal cells in the bone marrow [34]. Mesenchymal cells also release angiogenic factors such as VEGF, angiopoietin, FGF, and BMP [3, 4] to maintain a mutual relationship in regulating a specific bone marrow microenvironment.

5. Blood Vessels in the Haematopoietic Stem Cell (HSC) Microenvironment

5.1. Bone Endothelial Cells in HSC Maintenance. A strong interdependence of ECs and HSCs has been illustrated during both primitive and definite haematopoiesis [22, 73, 74]. The importance of the BM vasculature was initially appreciated only in thrombopoiesis, stem cell mobilization, and homing [21]. Identification of long-term (LT) HSCs' occurrence near blood vessels generated an immense interest in the field to understand the bone marrow vascular niche [75]. Cultured ECs from nonhaematopoietic organs such as heart and liver were identified to maintain HSCs *in vitro*, while ECs from kidney lacked this potential [76]. Later, identification of tissue-specific molecular signals in ECs [77] suggested unique potential of bone marrow endothelium in

Table 1: Genetic studies illustrating functions of endothelial factors in bone are summarised below.

Factors	Modification	Functions	Reference(s)
Cxcr4	EC-specific deletion (induced)	Increased vascular permeability HSPC egress	[35]
Cxcl12	EC-specific deletion (constitutive)	Decreased HSC frequency	[69, 70]
Dll1	EC-specific deletion (induced)	Monocyte development	[90]
Dll4	EC-specific deletion (induced)	Regulates type H vessels Coupling of angiogenesis and osteogenesis haematopoiesis	[10, 34]
Fbw7	EC-specific deletion (induced)	Reactivating type H vessels in aged bones induce arterioles formation increase PDGFRb+, alpha-SMA+ mesenchymal cells increase HSC frequency	[10, 29, 34]
Fgfr1/2	EC-specific deletion (induced)	Impaired vascular integrity reduced HSPCs and MSPCs	[35]
Gp130	EC-specific deletion (constitutive)	Hypocellular marrow, marrow dysfunction, and splenomegaly	[78]
Hif1a Vhl	EC-specific deletion (induced)	Regulates type H vessels Coupling of angiogenesis and osteogenesis	[8]
Pdgfb	EC-specific overexpression (induced)	Increased PDGFRb+, alpha-SMA+ mesenchymal cells	[34]
Pecam1	Global deletion	No substantial change in blood vessels	[29]
Scf	EC-specific deletion (constitutive)	Decreased HSC frequency	[81]
Sele	Global deletion	Promotes HSC quiescence and resistant to irradiation	[80]

profoundly supporting HSCs and haematopoiesis compared to ECs from other organs.

Endothelial specific deletion of glycoprotein 130 (gp130), a subunit of receptors that bind IL-6 chemokine family, resulted in hypocellular marrow and reduced HSC numbers [78]. Regeneration of sinusoidal ECs after irradiation was severely affected upon inhibiting VEGFR2 signalling with a blocking antibody [79]. E-selectin was suggested to be exclusively expressed in the bone marrow endothelium, and deletion of this gene enhances HSC quiescence and resistance to irradiation [80]. In addition to direct cell contact, ECs were illustrated to release soluble factors called angiocrine factors to regulate the HSC microenvironment [2]. Cxcl12 and stem cell factor (Scf) are important and widely investigated angiocrine factors of BM ECs involved in regulating HSC homeostasis. Endothelial cell-specific deletion of Scf resulted in decreased HSC numbers with reduced repopulation potential upon BM transplantation [81]. In a similar study, deletion of Cxcl12 in ECs resulted in depletion of HSCs and their long-term repopulating activity [69]. In a recent study, activation of Notch signalling in ECs led to the expansion of both cellular and angiocrine components of the HSC microenvironment. Endothelial Notch signalling promoted formation of new type H capillaries, small arterioles, PDGFRβ+ perivascular mesenchymal cells, and cellular Scf levels [34].

5.2. Arteriolar Microenvironments for Long-Term HSCs. Arteriolar microenvironments consisting of arterial ECs and surrounding NG2+ mesenchymal cells were demonstrated to maintain HSC in a quiescent state [82]. Similarly, sinusoidal blood vessels and surrounding LepR+ mesenchymal cells were also shown to provide microenvironments to maintain HSC population [83]. In another study, α-catulin GFP+ c-Kit+ HSCs were shown to localise in the central marrow region consisting of a sinusoidal microenvironment formed by sinusoidal blood vessels and LepR+ mesenchymal cells. It also proposes a single perisinusoidal microenvironment for both quiescent and dividing HSCs [84]. Arterial blood vessels having less permeability were shown to maintain HSC in a low ROS compared to highly permeable sinusoids involved in cell trafficking and homing [34, 35]. Endothelial Notch signalling-mediated amplification of arteriolar blood vessels leads to the expansion of HSC niches that result in increased HSC numbers and their function in young mice [34]. Recently, Hoxb5 expression in BM was identified to demarcate LT-HSCs population. Spatial localisation of Hoxb5+ HSCs shows that they are directly attached to VE-cadherin+ ECs, indicating their close association with blood vessels in the BM microenvironment [85]. The study does not provide further details regarding the vascular microenvironment near Hoxb5+ HSCs. It will be interesting to understand the localisation of Hoxb5+ HSCs in the context of multiple vascular compartments present in the BM microenvironment.

6. Concluding Remarks

Despite emerging interest in the bone vasculature and that manipulating blood vessels might regulate the BM microenvironment, our knowledge of heterogeneous vascular niches and endothelial regulatory factors is limited, to gain insight into the vessel-mediated organisation of the BM microenvironment. Table 1 summarises important factors studied in bone endothelial cells and their specific functions. It has become increasingly evident that the bone vasculature is highly complex, heterogeneously composed of distinct blood vessel types, and endowed with specialized functions that control bone formation, haematopoiesis, and bone regeneration. ECs forming these heterogeneous blood vessels along with their released angiocrine factors and supporting surrounding cell types contribute to the formation of multiple microenvironments in the bone marrow. In addition, local oxygen status generated by the organisation of capillaries

and microcirculation regulates the behaviour and functions of microenvironments. Involvement of multiple factors and cell types suggests the existence of disciplined regulation mechanisms to control the integrity of local niches. Dissecting cellular and molecular components of these local microenvironments will enhance our understanding of clinically significant HSCs and MSCs in bone.

Bone mass is severely affected in physiological changes such as ageing and in systemic diseases such as diabetes [86] and hypothyroidism [87]. The BM microenvironment is modified in accordance with these physiological and pathological conditions in the body. These changes perhaps involve amplification or reduction of a specific microenvironment within the bone marrow compartment to compensate changes in the whole body physiology. For example, age-related physiological changes lead to loss of bone mass and are associated with the loss of type H vessels which provide the supportive microenvironment for osteoprogenitors [8]. Similarly, changes in the BM microenvironment were observed during cancer and metastasis [88, 89]. These evidences strongly argue that the dynamic nature of the bone marrow microenvironment undergoes modifications based on the local and systemic demands and functions.

ECs playing a central role in constructing and orchestrating various microenvironments in the BM could potentially serve as an excellent target to manipulate specific niches in bone. Reactivation of type H vessels in aged mice could promote neo-osteogenesis, leading to new bone formation and increase in bone mass [8, 29]. Despite its potential therapeutic applications, limited knowledge of the bone vasculature severely affects our understanding of the organisation and localisation of microenvironments in bone. Characterising heterogeneous blood vessels and their endothelial-derived factors and further insights on the cellular and molecular components of microenvironments are critical to unravel the interaction and role of blood vessels in regulating the bone marrow architecture in various physiological and pathological conditions.

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

Funding was provided by the Wellcome Trust, the Royal Society, and the Medical Research Council, UK.

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Hindawi
Stem Cells International
Volume 2017, Article ID 7471582, 20 pages
https://doi.org/10.1155/2017/7471582

Review Article

Cardiac Progenitor Cells and the Interplay with Their Microenvironment

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Received 31 March 2017; Accepted 26 July 2017; Published 17 September 2017

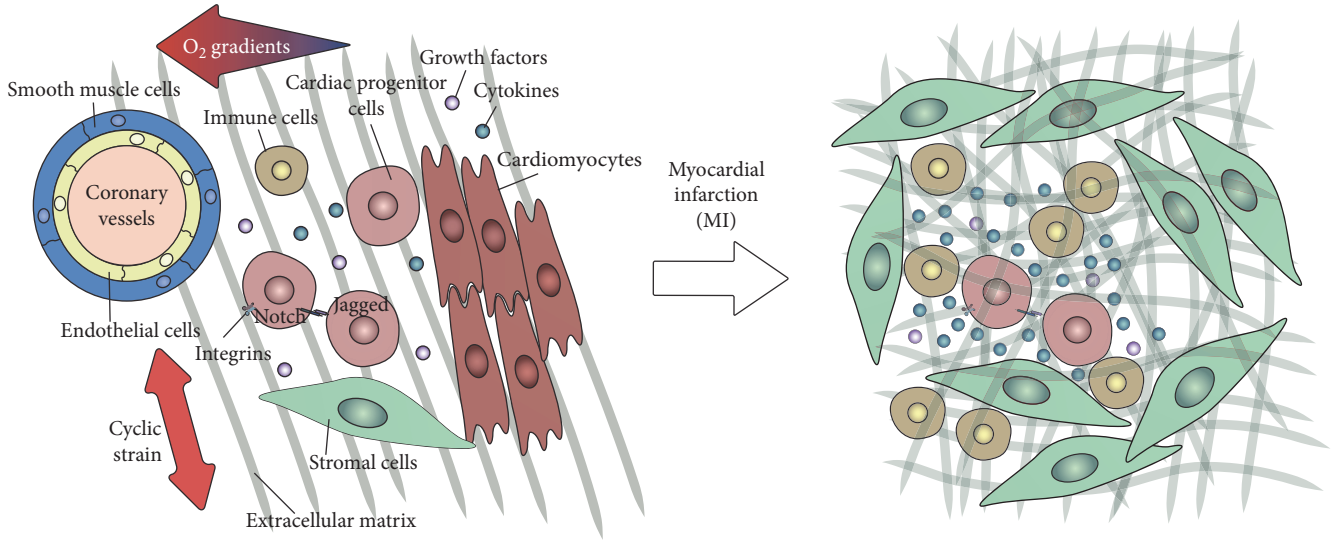
Academic Editor: Sari Pennings

The microenvironment plays a crucial role in the behavior of stem and progenitor cells. In the heart, cardiac progenitor cells (CPCs) reside in specific niches, characterized by key components that are altered in response to a myocardial infarction. To date, there is a lack of knowledge on these niches and on the CPC interplay with the niche components. Insight into these complex interactions and into the influence of microenvironmental factors on CPCs can be used to promote the regenerative potential of these cells. In this review, we discuss cardiac resident progenitor cells and their regenerative potential and provide an overview of the interactions of CPCs with the key elements of their niche. We focus on the interaction between CPCs and supporting cells, extracellular matrix, mechanical stimuli, and soluble factors. Finally, we describe novel approaches to modulate the CPC niche that can represent the next step in recreating an optimal CPC microenvironment and thereby improve their regeneration capacity.

1. Introduction

Cardiac tissue is a composite material consisting of contractile and supportive cells surrounded by extracellular matrix (ECM) and is intertwined with nervous and vascular networks. An ischemic event, such as a myocardial infarction (MI), not only induces cell death but also affects the tissue structure and composition. This can eventually lead to loss of cardiac function due to changes in the key players of the cardiac microenvironment: (1) stem/progenitor cells and supporting cells, (2) extracellular matrix (ECM) proteins, (3) the mechanical environment of the cells and the matrix, such as the cyclic strain provided by the beating heart, and (4) soluble factors, such as oxygen and cytokines (Figure 1(a)). In this review, we omit to describe vascular components, innervation, and electrical conduction, as these are extensively reviewed elsewhere [1–3], although their derivatives, such as oxygen gradients and cyclic strain, are included.

The myocardium shows very limited self-renewal; nevertheless, the notion of the heart as a terminally differentiated organ, incapable of regenerating after injury, has been challenged by abundant evidence in the last decade [4, 5]. There is ongoing debate over whether cardiac regeneration is to be attributed to dedifferentiation and proliferation of cardiomyocytes [6, 7] or to differentiation of cardiac stem or progenitor cells [8–10], which makes it difficult to identify the ideal therapeutic target. Nevertheless, the existence of resident cardiac progenitor cells (CPCs) in the heart and their relevance for cardiac regeneration have been demonstrated by several studies [10–13]. CPCs have emerged as a promising candidate for cardiac regeneration, due to their differentiation potential [10, 14] and the ability to produce and remodel ECM proteins [15]. Moreover, after acute MI,



(a) Healthy cardiac microenvironment

(b) Diseased cardiac microenvironment

Figure 1: The cardiac progenitor cell resident microenvironment. (a) The simplified representation shows some of the key players of the healthy CPC niche: (1) cellular elements (CPCs and supporting cells: cardiomyocytes, endothelial cells, smooth muscle cells, stromal cells, and immune cells) and cell-cell interactions such as signaling via Notch; (2) extracellular matrix (ECM); (3) mechanical stimuli, such as the cyclic strain provided by the beating heart; and (4) soluble factors, such as cytokines, oxygen gradients, and growth factors. (b) Simplified representation of the infarcted heart, where the microenvironment is altered and the niche components modified: (1) cardiomyocyte death and infiltration of myofibroblasts and immune cells; (2) excessive and disordered formation of ECM; (3) increased ECM stiffness and thus altered mechanical behavior; and (4) increased secretion of growth factors and cytokines.

the number of CPCs in the adult increases and differentiation into the cardiac lineages takes place [6]. However, the post-MI microenvironment can affect CPC behavior: in chronic infarcts, CPCs are characterized by decreased telomerase activity, leading to impaired cell division and cellular senescence, as well as increased CPC apoptosis [6].

The traditional cell therapy approach to treat a MI entails isolation of CPCs, their expansion *in vitro*, and transplantation into the infarcted area [16]. Despite the immediate benefits on cardiac function, this treatment has shown very limited improvement on the long term [17–19], mainly due to low cell survival and engraftment in the host tissue [20]. In fact, a MI creates a hostile environment for the injected progenitor cells, due to the inflammatory response and tissue alterations, such as scar tissue formation, triggered by the cardiac injury, as extensively described elsewhere [20–22] (Figure 1(b)).

In the adult, stem or progenitor cells reside in specific microenvironments, referred to as "niches," that protect stem cells and regulate their fate and functions [23–25]. Stem cell niches are stored in specific anatomical compartments, located in tissue areas that are shielded from external damaging stimuli [23, 26].

In the adult mouse heart, putative progenitor cell niches have been identified in the atria, base-mid region, and apex [27]. To date, however, the cardiac progenitor cell niche is still largely uncharacterized and most studies have been performed on mouse models [27, 28].

For cell therapy, cells are isolated from their "resident niche" and expanded in an "*in vitro* niche," prior to transplantation into the "diseased niche" of the infarcted heart tissue (Figure 2(a)). An alternative to cell therapy is to promote the regeneration provided by endogenous CPCs, for instance by promoting the migration of CPCs to the damaged cardiac area (and to the "diseased niche"), as well as their proliferation and differentiation (Figure 2(b)). Another potential approach is to generate new, or engineered, microenvironments for the cells, in order to recreate optimal conditions to enhance their regenerative potential. Currently, there is a lack of knowledge on the composition and similarities of these three niches and on the interplay between CPCs and the niche components.

Therefore, in this review, we highlight the key elements of all potential CPC niches and discuss the interplay between CPCs and the niche components. Improved knowledge on the CPC niches and the CPC-niche interactions will enhance our insight into CPC behavior and the influence of the niche on CPC regenerative capacity, which can ultimately help modulate the microenvironment to promote the regenerative potential of CPCs. In the last part of this review, we therefore provide an overview on recent advances in the field of engineered cardiac microenvironments, which can represent the next step in exploring and modulating the CPC niche and CPC behavior for cardiac repair.

2. The Cardiac Resident Progenitor Cell

The presence of CPCs in the fetal and adult heart in mammals (including humans) has been extensively described (reviewed by [11]). Yet, CPCs are not conclusively defined

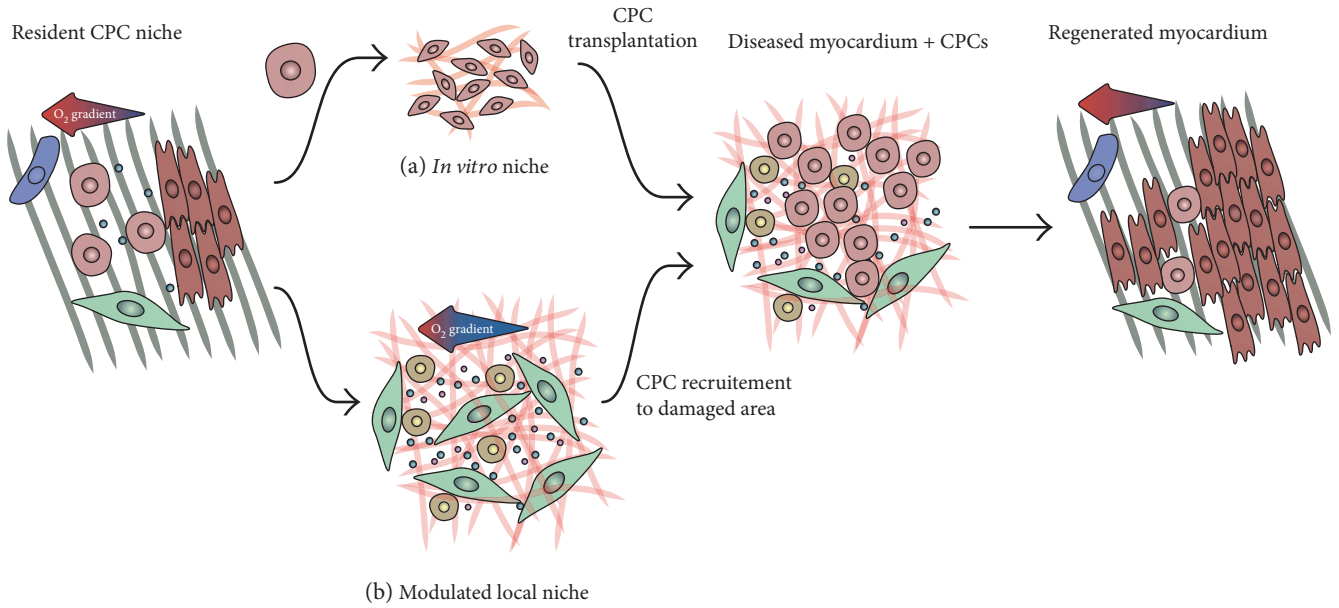


Figure 2: The CPC microenvironments. For therapeutic application, CPCs can be isolated from their resident niche and (a) cultured in an *in vitro* niche, prior to transplantation into the infarcted heart, or (b) the local microenvironment can be modulated in order to recruit CPCs to the injured area. The aim of both approaches is to regenerate the myocardium thanks to CPC proliferation and differentiation into cardiomyocytes.

Table 1: Summary of the cardiac cell populations described in this review.

Cell type	Cardiac resident		Self-renewal	Multipotent	Activation after injury	Improvement of cardiac function	Defined CPC	[Refs]
	Embryonic	Adult						
Side population	+	+	+	+	+	+	Yes	[6, 9, 11, 14, 29–45]
c-kit$^+$ cells	+	+	+	+	+	+	Yes	[6, 8–12, 35, 46–74]
Sca-1 cells	+	+	+	+	+	+	Yes	[6, 9–13]
Isl1 progenitors	+	?	+	+	?	?	No	[9, 11, 47, 62, 88–99]
Cardiospheres	+	+	+	+	?	?	No	[9, 11, 47, 62, 87, 88]

and the nomenclature stem/progenitor cell is often used in a generic sense. However, whereas "stem cells" replicate indefinitely and are pluripotent, "progenitor cells" can only divide a limited amount of times and are multipotent. To prevent misunderstanding, in this review, we propose a definition for CPC, based on key characteristics and functions of these cells. To be identified as a *cardiac progenitor*, a cell should (1) reside throughout the heart in both embryonic and adult stage and be (2) self-renewing and (3) multipotent, that is, able to differentiate in minimally three of the four cardiac cell types (cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts). Furthermore, the cell should (4) be activated during cardiac injury and have regenerative potential proven by the fact that (5) transplantation of these cells into the diseased heart has favorable effects on cardiac function.

In this section, we will describe the populations of cells that we include among CPCs, as well as others that are often classified as CPCs but that we exclude from the CPC definition (Table 1), with a focus on their regenerative potential.

2.1. Cardiac Side Population Cells. The identification of cardiac progenitor cell population in the heart goes back to the beginning of the twenty-first century. Since 2002, several studies described the presence in the adult mouse heart of cardiac side population cells (cSPCs) [29–32], which were later identified also in fetal and adult hearts of rats and humans [33–35].

In vitro studies have proven the ability of these cells to self-renew with retained side population phenotype [14, 30, 32, 36], as well as their multipotency. Differentiation potential into cardiomyocytes [14, 33, 37–40], endothelial cells [36, 39, 41], and smooth muscle cells [39] was confirmed by transplantation studies into the injured heart [31, 33, 41]. Differentiation of side population cells into fibroblasts has so far only been shown after transplantation into the cryoinjured rat heart [33].

cSPCs are activated in murine injured hearts [42, 43], and more clinically relevant, they were also activated in human hearts in response to injury [6, 38, 44]. The regenerative potential of the cardiac side population was tested in

three transplantation studies in animal models of cardiac injury. Only one of the performed transplantation studies [30, 31, 33] assessed the functional recovery, reporting increase of injection fraction [30].

2.2. c-kit$^+$ and Sca-1$^+$ Cardiac Cells. While no single marker exclusively identifies CPCs, there is a strong agreement that specific surface markers, like type III tyrosine kinase receptor c-kit (CD117) and Sca-1, identify cardiac progenitor populations. During early development, both markers are primarily haematopoietic stem cell markers present in the bone marrow. In 2003, both cells types were identified in the myocardium of adult rodents [10, 45]. These cell populations are heterogeneous and thereby share similarities but are also distinct, although it is suggested that they both originate from the same resident precursor cell [46].

2.2.1. c-kit$^+$ Cardiac Cells. c-kit$^+$ cardiac resident progenitor cells are probably the most studied CPC population. Following the discovery in 2003, the presence of c-kit$^+$ cells was confirmed not only in human [47] and mouse [48–50] from the developing to adult heart [51–54] but also in other mammals, including dog [55], pig [56], and sheep [57].

Self-renewal of c-kit$^+$ CPCs has been assessed *in vitro* [45, 47, 55], and c-kit$^+$ cells appear to be the most undifferentiated progenitor population [58]. Despite controversy about the multipotency of c-kit$^+$ CPCs [46], several *in vitro* approaches revealed differentiation potential towards all four cardiac cell types, although the extent of differentiation is species- and developmental stage-dependent [10, 35, 47, 50, 55, 59, 60]. In fact, transplantation studies showed that c-kit$^+$ CPCs are more prone to differentiate toward endothelial and smooth muscle cells rather than cardiomyocytes and fibroblasts [8, 45, 50, 61]. This suggests that fetal and neonatal-derived c-kit$^+$ CPCs only have potential to differentiate into cardiomyocytes, while adult-derived cells are more predisposed to differentiate into vascular cells only [48, 62].

A number of studies have verified the presence of c-kit$^+$ cells in hypertrophic adult human hearts [12], after myocardial infarction [6, 50, 53, 63] and after ischemia/reperfusion injury [64, 65]. In chronic heart failure, both increases and decreases in cell numbers have been described [6, 66, 67].

Transplantation of adult c-kit$^+$ cells in preclinical studies in rodents revealed that attenuation of scar formation and left ventricular function [47, 68–70] was mainly induced by a paracrine mechanism. Although more preclinical research is needed to fully understand the contribution of c-kit$^+$ cells to cardiac regeneration, the first clinical trial of Cardiac Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO, NCT00474461) was conducted [71, 72]. The data of this randomized phase 1 trial reported the induction of myocardial regeneration by c-kit$^+$ cells [72–74]. Despite the limited number of patients and the lack of placebo controls in this randomized phase 1 trial, as well as the expression of concern by *The Lancet*, both preclinical and clinical outcomes suggest a contribution of c-kit$^+$ cells to cardiac repair.

2.2.2. Sca-1$^+$ Cardiac Cells. Resident Sca-1$^+$ cells are found in fetal and adult mouse and human hearts, in the atria, the intra-atrial septum, the myocardium, and the epicardium [10, 12, 54, 73, 74].

Human Sca1$^+$ cells harbor telomerase activity, which characterizes their proliferative potential and their ability to self-renew [73, 75, 76]. They display a mesenchymal profile and have gene expression comparable with c-kit$^+$ cells, although murine Sca-1$^+$ CPCs have shown the highest correlation with cardiomyocytes and thereby seem to be the most committed to cardiomyogenic differentiation [58]. Culture-expanded Sca-1$^+$ cells can be differentiated into cardiomyocytes in the presence of oxytocin or 5-azacytidine treatment [13, 75, 76], and the cardiac differentiation potential is enhanced by addition of transforming growth factor-beta (TGF-β) [75, 76]. Next to cardiomyocytes, Sca-1$^+$ cells can differentiate into endothelial cells and smooth muscle cells, as observed both *in vitro* [10, 74, 75, 77] and *in vivo*. Transplantation of isolated adult murine Sca-1$^+$ induced revascularization and revealed differentiation into cardiomyocytes and endothelial cells in infarcted mouse heart [13, 74, 76]. Similarly to c-kit$^+$ cells, versatility of differentiation of Sca1$^+$ cells is developmental stage- and subpopulation-dependent. Whereas fetal cells are very suitable for cardiomyogenic and angiogenic development, adult cells prefer smooth muscle cell differentiation [78].

As for cSPCs and c-kit$^+$ cells, Sca-1$^+$ cells are present in the hypertrophic human heart [12] and the number of resident cells is expanding after myocardial infarction [74]. Transplantation of both fetal and adult Sca-1$^+$ cells, of both murine or human origin, into the mouse injured heart limits structural and functional deterioration and thereby attenuates impairment of contractility. This regenerative potential is mediated by differentiation of Sca-1$^+$ cells and via paracrine mechanisms [74, 76, 79, 80].

Despite these functional benefits of Sca-1$^+$ cells *in vivo*, no clinical trials are conducted. The fact that there is no Sca-1 homologue present in the human genome raises questions about the epitope on human Sca-1$^+$ CPCs that is recognized, which hampers their clinical application [81]. Interestingly, a panel of antibodies has recently been published that were specifically raised against resident human Sca-1$^+$ CPCs. These antibodies, such as mAb C19, recognize CPCs in human heart tissue, and isolated C19$^+$ cells have CPC characteristics and differentiate into the same lineages as Sca-1$^+$ CPCs. This discovery might be a step forward for the application of these human CPCs in clinical trials [82].

2.3. Isl1$^+$ Cell and Cardiospheres. Other cardiac cell types are often included among putative CPCs, such as Isl1$^+$ cells and cardiospheres [46, 60, 83–86]. These cells reside in the heart; however, they do not (yet) respond to our definition of CPCs. Hereafter, we report what is known about Isl1$^+$ cells and cardiospheres and explain why we excluded them from the CPC classification.

2.3.1. Isl1$^+$ Progenitors. LIM-homeodomain transcription factor Isl1 positive cardiac cells share many of the characteristics of the CPCs described so far. Isl1$^+$ cells are present in

the developing heart [87], and the number of Isl1$^+$ cells residing in the heart is substantially decreasing from fetal to neonatal and adult stages [62, 87–91]. Their distribution is comparable with Sca-1$^+$ cells; the location is conserved between rodents and human [90]. Postnatal Isl1$^+$ cells can proliferate on cardiac mesenchymal feeders [85, 92, 93], and they have been shown to differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells [85, 90, 94]. Recently, local upregulation of Isl1$^+$ after ischemia/reperfusion in the adult mouse heart has been observed [95]. However, at the moment, there is no evidence of the regenerative potential of these cells, due to a lack of data regarding the beneficial effects on cardiac function after transplantation into the diseased heart. Nevertheless, there is interest in their therapeutic value, as shown by a clinical trial designed by Assistance Publique—Hôpitaux de Paris (NCT02057900).

2.3.2. Cardiospheres and Cardiosphere-Derived Cells. Often included also among CPCs are cardiospheres and cardiosphere-derived cells, first described by Messina et al. in 2004 [96]. Cardiospheres can be obtained from human atrial or ventricular biopsies from embryonic, fetal, and postnatal mouse hearts [96–98]. Cells migrating from the tissue explants spontaneously form cardiac multicellular spheroids when cultured on poly-D-lysine-coated culture plates. The cell monolayer growing after seeding cardiospheres on fibronectin-coated culture plates is known as cardiosphere-derived cells [99]. Although they unquestionably reside in the heart, due to the methods used to obtain cardiospheres and cardiosphere-derived cells, the origin of these cells is difficult to determine. Although most cardiosphere-derived cells in culture are known to express the endoglin marker CD105 [98], this mesenchymal and hematopoietic marker is not exclusive and thus cannot be used to specifically define these cells *in vivo* as CPCs. Moreover, activation after injury cannot be proven due to the lack of a specific marker for these cells. Nevertheless, cardiospheres and cardiosphere-derived cells are self-renewing and can form cardiomyocytes, endothelial cells, and smooth muscle cells [96, 98–100].

Cardiospheres contain a mixed cell population, including c-kit$^+$ cells as well as endothelial precursors and mesenchymal cells [99]. Moreover, the expression of ECM proteins and integrins, as well as the gradients of oxygen and nutrients that are thought to occur between the periphery and the core of the spheroid, makes cardiospheres an *in vitro* model mimicking the CPC niche [99, 100]. As such, cardiospheres can be used to study the CPC-niche interactions *in vitro*, beside their potential therapeutic application.

Adult cardiospheres and cardiosphere-derived cells have proven to have beneficial effects on cardiac function in murine and porcine animal models [101–107], and these broadly positive findings have led to two clinical trials, CADUCEUS and ALLSTAR. The Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction (CADUCEUS, NCT00893360) trial is a phase I randomized study in which patients received cells three months after myocardial infarction [108]. Although the study was not powered, transplantation of cells was safe and led to reduction in infarct size and increase in area of viable myocardium, unfortunately without change in ejection fraction of the left ventricle [108, 109]. The positive outcomes initiated the start of a follow-up phase II clinical trial—Allogenic Heart Stem Cells to Achieve Myocardial Regeneration (ALLSTAR) [110].

Above, we described the various populations of resident CPCs that have been identified in the heart. All these cells have a heterogeneous nature and although they express different markers, they might be more similar than they are different. It is even suggested that all these described CPCs represent the same population and that the difference lies in the method of determination or their differentiation stage [47]. It is therefore important to precisely characterize and purify the CPC population, and further research is required. Nonetheless, although CPCs might not be rigorously defined, these cells have potential in cardiac regeneration.

3. The CPC Microenvironment

The regenerative potential of the heart is determined not only by the characteristics of CPCs but also by the influence of the microenvironment on their functions. In this section, the key components of the CPC niche will be described. These are (1) cellular components, represented by supporting cells; (2) cyclic strain, as provided by the cardiac beating; (3) extracellular matrix, which provides both mechanical and biochemical stimuli; and (4) soluble factors (such as cytokines) and oxygen tension, which can play a pivotal role in determining stem cell behavior (schematically represented in Figure 1(a)).

3.1. Supporting Cells. In both healthy and diseased hearts, cells interact with each other directly via cell-cell contact or indirectly by the expression of paracrine factors (Figure 3). Interactions can be isotypic (same cell types) or heterotypic (cells of different phenotypes) and the crosstalk between different populations will affect not only the cardiac function but also the regenerative potential. These interaction processes are complex and mostly unknown in the CPC niche. The role of the direct contact between CPCs and supporting cells is difficult to unravel. Most knowledge is derived from *in vitro* experiments, and crosstalk outcomes are mainly based on paracrine effects. In this section, we will focus on the interactions of CPCs with supporting cells and their importance for cardiac repair.

3.1.1. Cardiomyocytes. In the first *in vivo* study about the cardiac niche and putative supporting cells, connexins and cadherins were detected in cellular contacts between CPCs and cardiomyocytes as well as between CPCs and fibroblasts [27, 47, 111]. However, these connections were not observed between CPCs and endothelial cells and between CPCs and smooth muscle cells [27]. Cardiomyocytes are able to transfer information to CPCs (and vice versa) through gap junctions. Coculture of CPCs with cardiomyocytes promotes their expansion and results in beating CPCs, together with expression of cardiomyocyte-specific proteins and well-organized sarcomeres [14, 37, 90, 96, 112], a process probably regulated by TGF-β [113, 114] and indirectly via the Wnt/beta-catenin

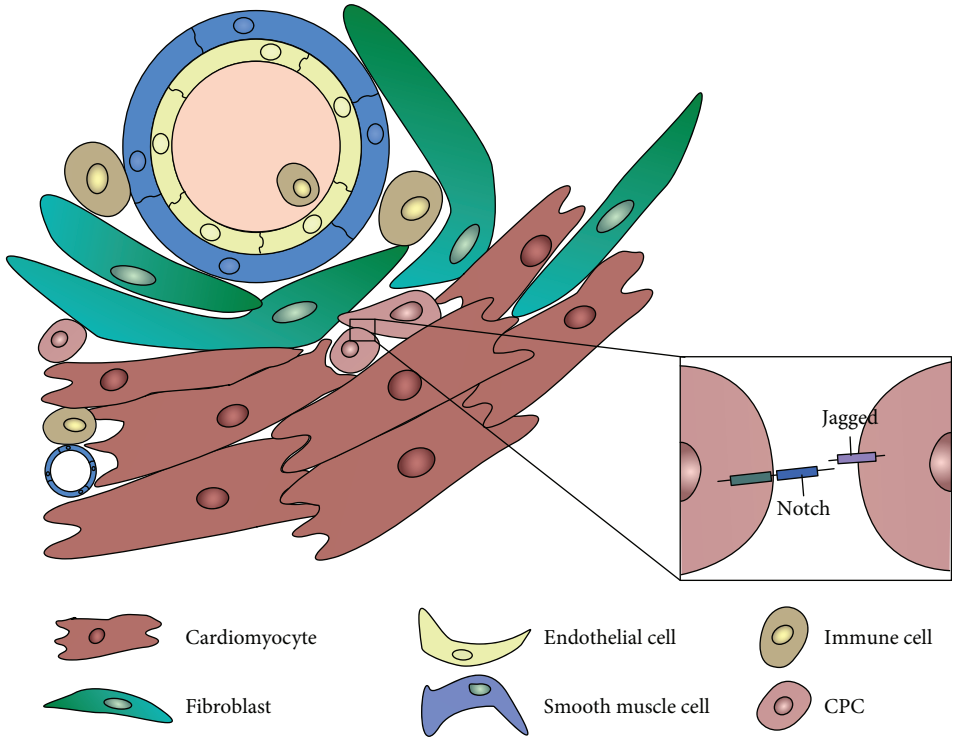


Figure 3: Cell-cell interactions in the CPC niche. CPCs interact with each other and with supporting cells (cardiomyocytes, fibroblasts, endothelial cells, smooth muscle cells, and immune cells), both via direct cell-cell signaling (such as the Notch pathway) and paracrine signaling.

signaling system [115]. Therefore, coupling of CPCs with cardiomyocytes is critical to control the cardiac fate, and lack of appropriate interaction may hamper CPC differentiation [116]. However, cardiomyocytes might not solely stimulate differentiation toward the cardiomyogenic lineage. In fact, under hypoxia, cardiomyocytes produce vascular endothelial growth factors (VEGF), which might induce endothelial differentiation of the CPCs [115]. At the same time, CPCs can express growth factors and cytokines, which besides being necessary for their proliferation and senescence [117] are also important for cardiomyocyte proliferation, cell survival, and prevention of hypertrophy [118].

3.1.2. Endothelial Cells. Since CPCs are often found in the perivascular area, interaction with endothelial cells and smooth muscle cells is plausible, although cell-cell interactions were not observed [27]. It can be hypothesized that endothelial cell-CPC interaction is regulated via Notch, since Notch receptors are predominantly expressed by the vascular endothelium [119]. Notch signaling is crucial for cell fate decisions that underlie cardiomyogenic and vessel formation [119, 120]. Since Notch signaling is a highly conserved pathway that acts via cell-cell contact, it will be discussed in more detail later on. Indirect interactions between endothelial cells and CPCs, via the production of VEGF, might not only promote CPC migration [121] but also regulate CPC differentiation towards endothelial or smooth muscle cells [39, 41].

3.1.3. Immune Cells. Myocardial injury causes inflammation by activation of immune cells, which are involved in cardiac repair as well as scar tissue formation. Hence, crosstalk between CPCs and immune cells is likely to take place, although there are no proven interactions. Transplantation studies revealed that CPCs are able to dampen the immune response and thereby influence cardiac repair [122]. However, the mechanisms underlying CPC modulation of the immune system are not completely unrevealed, as it is the case for mesenchymal stem cells [123–126].

(1) Macrophages. Macrophages are a heterogeneous population of both protective and cytotoxic cells. They play a cardioprotective role by maintaining cardiac homeostasis via interactions with other cardiac cells [127]. Macrophages are able to produce growth factors (e.g., IGF-1, VEGF, and TGF-β), which stimulates CPC proliferation and induces differentiation towards both cardiomyocytes and endothelial cells [128, 129]. On the other hand, CPCs are able to polarize macrophages away from their proinflammatory phenotype, although the exact mechanism behind it is unclear. It is not known toward which cell type the polarization acts, although it was proven not to be toward the anti-inflammatory phenotype [130]. We therefore assume that a cardioprotective effect arises from the interaction between macrophages and CPCs.

(2) Natural Killer Cells. Natural killer cells, a subset of the innate lymphoid cell compartment, are effectors of the innate immune system, which are essential in allogeneic transplantation. Their cytotoxic effects are mediated by exocytosis of granules that perforate the target cell to trigger

apoptosis [131]. Little is known about these cells and CPCs, but a recent study by Boukouaci et al. revealed that CPCs are protected from killer cell cytotoxicity within an inflammatory context [132]. On the other hand, CPCs are able to downregulate the toxicity of natural killer cells and bias cytokine secretion towards an anti-inflammatory state. Retention of CPCs is improved by this crosstalk with natural killer cells and contributes to cardiac regeneration [132].

(3) Mast Cells. Mast cells are bone marrow-derived precursors, and although their number increases in the failing heart, their exact role in cardiac disease and regeneration is understudied. Moreover, mast cells express similar markers as CPCs [133]. It is known that CPCs share distinct characteristics with mast cells, but not all CPCs are mast cells [133]. Both mast cells and CPCs are located in the perivascular area, although cell contact is not reported. Paracrine effects can be assumed since mast cells produce several cytokines, growth factors, and angiogenic factors that are all involved in cardiac repair [134]. During mast cell degranulation, TGF-β is released, which as described earlier is important for CPC differentiation [135].

3.1.4. Stromal Cells

(1) Fibroblasts. Together with cardiomyocytes, fibroblasts were the first supporting cells of CPCs to be discovered [27, 47]. Like cardiomyocytes, fibroblasts are connected to CPCs via gap and adherens junctions. Not only do fibroblasts maintain the supporting matrix of the CPC niche [115] (the importance of the ECM-cell interaction will be discussed later on in a dedicated paragraph), but they also might influence the differentiation potential of CPCs. It has recently been shown that fibroblast-conditioned medium can induce differentiation via the Wnt signaling pathway [136] and that fibroblasts produce angiogenic and antiangiogenic factors [115]. Fibroblasts originate mainly from the epicardium [137]; therefore, interactions between epicardium-derived cells (EPDCs) and CPCs need to be described.

(2) Epicardium-Derived Cells (EPDCs). CPCs are often found in the subepicardial region, which mostly consists of EPDCs. EPDCs have a crucial modulatory role during cardiac development, and their activation after injury [138–141] also suggests the same role in the adult heart [142]. Due to these characteristics, some groups even suggest that the epicardium is a source of progenitor cells [143, 144]. The presence of CPCs near the epicardium and EPDCs suggests that important interactions occur between CPCs and EPDCs. Previous research showed that EPDCs stimulate the migration and proliferation of CPCs [141, 145, 146]. Coculture of CPCs with EPDCs revealed induction of metalloproteinases and their inhibitors, which affected infarct size [146]. In fact, matrix remodeling is not only important to prevent cardiac dilation after injury, but it also plays a role in maintaining the supporting network of the CPC niche. Moreover, coculturing stimulated angiogenesis and thereby improved cardiac function. The interaction between CPCs and EPDCs is reciprocal and results in synergistic action, leading to improved cardiac function. This beneficial effect is at least partly explained by paracrine stimulation [146].

(3) Telocytes. Another stromal cell type which is present in the subepicardial region is the telocyte, formally known as interstitial Cajal-like cells [147]. Telocytes are in close vicinity with CPCs, and stromal synapses and adherens junctions are formed between the cells both *in vitro* and *in vivo* [148, 149]. These adherens junctions not only control the retention of CPCs but also might be important for division and migration of CPCs [149]. Therefore, it is assumed that telocytes provide guidance and nursing for CPCs to stimulate their activation, proliferation, and differentiation leading to cardiac repair [148, 150, 151]. Furthermore, telocytes produce growth factors (e.g., VEGF) [152] and macromolecular signals, such as microRNAs [153], which might influence the differentiation potential of CPCs [151].

3.1.5. Cell-Cell Signaling via Notch in CPCs. Cell-cell contact-dependent signaling is an essential component of the niche and has an important influence on cellular behavior. Notch signaling is a fundamental and highly conserved pathway that acts via direct cell-cell communication and has a key role in the heart. Notch regulates a number of cell functions, such as survival, proliferation, and differentiation, as well as tissue development and homeostasis. In mammals, four Notch proteins have been identified (Notch1–4), which can bind to ligands of the Delta or Serrate/Jagged families expressed by neighboring cells, as extensively reviewed elsewhere [154, 155] (Figure 3). Following cleavage by ɣ-secretase, Notch intracellular domain (NICD) is released and translocates to the nucleus, where it regulates the expression of target genes, such as members of the Hes and Hey families [154–156], as well as Nkx2.5 in cardiac cells [157].

Notch signaling represents an essential element of the cardiac microenvironment. Firstly, Notch plays a crucial role in cardiomyogenesis, and Notch mutations have been linked to several congenital heart and heart valve defects [154–156]. Active Notch signaling is needed for CPC differentiation [158], whereas in cardiomyocytes, Notch is activated during embryonic development [155, 159] and inactivated during maturation [160] and after birth (reviewed by [159]). Secondly, several studies demonstrated the reactivation of Notch in adult cardiomyocytes after MI, in small animal models [157] as well as in humans [161, 162]. This shows the key role of Notch in cardiomyocyte survival [163, 164] and cardiac repair after injury [159, 165].

In the adult mouse, about 60% of c-kit$^+$ CPCs expresses the Notch1 receptor, and signaling with surrounding cells, either CPCs or myocytes, is mediated by Jagged1 [157]. Notch signaling strongly depends on timing and dosage [166, 167]; it is needed for proliferation and expansion of the CPC pool [160] and is essential for CPC cardiomyogenic differentiation [157, 158]. The activation of Notch1 by Jagged1 in mouse c-kit$^+$ CPCs promotes the nuclear translocation of N1ICD and enhances its colocalization with the cardiac transcription factor and Notch target gene Nkx2.5 [157]. However, Notch becomes undetectable when differentiating CPCs lose their proliferative capacity, showing

that its downregulation is needed for terminal differentiation [160]. Moreover, overexpression of Notch1 in mouse c-kit$^+$ CPCs leads to improved resistance to oxidative stress, and injection of these cells in mouse infarcted hearts resulted in an enhanced cardioprotective effect, as shown by smaller infarct length and area, functional improvement, and larger capillary density as compared to control cells (where endogenous Notch was activated via Jagged1) [120].

In view of these findings, Notch has been proposed as a potential therapeutic target for treating myocardial disease. Hydrogels functionalized with a peptide mimic of Jagged1 were shown to activate Notch signaling in rat c-kit$^+$ CPCs, and injection of CPCs embedded in these hydrogels led to improved cardiac repair in a mouse MI model [168].

Yet, very little is known about Notch in human CPCs. Recent studies, by our group and by others, have studied Notch signaling in cardiac progenitors cultured as multicellular spheroids, or cardiospheres (if isolated from adult myocardial tissue). The spheroid model better mimics the *in vivo* cell-cell interactions as compared to cell monolayers and therefore represents an interesting and promising model to study cell-cell signaling (see paragraph 4, "Approaches to modulating the CPC microenvironment"; [169]). The formation of cardiospheres from adult explant-derived cells increased the expression of Notch1 and Notch3 receptors [170]. Moreover, multicellular spheroid formation was shown to activate Notch signaling in both fetal and adult CPCs and more evidently so in combination with hypoxic culture, indicating the pivotal role of niche-like environmental conditions on a fundamental cellular pathway such as Notch signaling.

Understanding the role and activity of this key regulatory pathway in human endogenous CPCs will be crucial for the improvement of CPC-based cardiac regeneration therapies.

3.2. Extracellular Matrix. Cardiac cells are surrounded by a highly organized and dynamic network, known as the ECM that forms the cardiac tissue [171]. The cardiac ECM is composed of different proteins, proteoglycans, and glycosaminoglycans that form a fibrillar mechanical support in which cells are embedded. These structural components include collagen types I, III, and V, as well as elastin that provides resilience to the cardiac tissue [172–174]. Furthermore, proteoglycans such as tenascin-C and decorin contribute to the cardiac tissue and are crucial for the stability and integrity of the ECM [175, 176]. Next to structural components, the ECM is composed of nonstructural elements that regulate important cellular functions, such as adhesion, proliferation, and differentiation. These are primarily type IV collagen, laminins, and fibronectin [172, 177]. Moreover, within the ECM network, different cell types secrete soluble macromolecules in the extracellular space, such as VEGF, TGF-β, and stromal cell-derived factor 1-α (SDF1-α), which regulate and stimulate important cellular processes [178–180].

Cardiac fibroblasts are primarily responsible for the production and remodeling of ECM [181], both under healthy and under pathological conditions. During and following a myocardial infarction, fibroblasts become activated and secrete an abundance of ECM components to compensate for the loss of cardiomyocytes. Eventually, this leads to excessive ECM formation and scarring, with adverse effects on the contractility of the cardiac tissue. Altogether, this fibrotic environment might not be ideal for the injected progenitor cells. For this reason, the effect of the ECM environment on progenitor cell survival and function should be investigated to improve the CPC contribution to cardiac regeneration. Below, recent literature on the interaction of CPCs with native and/or synthetic ECM *in vitro* and *in vivo* is summarized and discussed, with a focus on ECM properties such as stiffness, architecture, and composition.

3.2.1. Integrins. CPC adhesion to its environment is essential for the connection between intracellular components and the ECM. Via focal adhesions (FAs), CPCs can sense their environment and respond accordingly. In general, FAs are transmembrane protein complexes that directly link ECM components or other cells to intracellular actin junctions, intermediate filaments, and sarcomeres [182]. Important components of these transmembrane proteins are integrins, which are heterodimers consisting of a combination of α and β subunits. In mammals, 24 types of receptors can be formed and each combination has a specific binding affinity to a different ECM component. For CMs, the most occurring integrins are $\alpha1\beta1$, $\alpha5\beta1$, and $\alpha7\beta1$, which bind specifically to collagen type I (COL), fibronectin (FN), and laminin (LN), respectively, with $\beta1$ being the prevalent β subunit [182]. Furthermore, the protein expression of different types of integrin also changes from neonatal CMs, where the dominant subunit is $\alpha5$, to adult CMs, where the $\alpha5$ is replaced by $\alpha7$ subunit [182, 183]. Similar changes in integrins expression can also be observed in response to pathological conditions [182]. Human fetal CPCs subjected to cardiomyogenic differentiation protocol *in vitro* showed unvaried expression but increased clustering of integrin $\beta1$, indicating FA maturation and improved mechanosensing with early cardiac differentiation [184]. These studies underline the importance of integrins in the heart and suggest that the FA expression of CPCs and interactions with specific ECM components should also be studied to be able to guide CPCs towards specific lineages.

3.2.2. CPC-ECM Interactions

(1) ECM Composition. The versatility of interactions between CPCs and their direct surroundings generates the possibility to obtain highly regulated and regenerative cellular responses via intracellular signaling. It is currently known that the stiffness, composition, and/or structure of the natural ECM has an effect on progenitor commitment *in vitro* [185]. More importantly, the response that the ECM evokes on cells are different depending on the cell type. This has been tested by culturing cardiac progenitor cells on different substrates *in vitro* and studying the cellular behavior and functions associated with cardiac regeneration. The first studies were initiated by French et al., who studied c-kit$^+$ Sprague-Dawley rat CPC behavior, that is, cardiomyogenic gene expression, cell survival, and proliferation, cultured on decellularized porcine ventricular ECM (cECM) or standard

collagen type I (COL), which more closely resembles the biochemical composition of a scar following a MI [186]. Interestingly, early cardiac genes for GATA-binding protein-4 (GATA-4), Nkx2.5, α-myosin heavy chain, and troponin C and T were increased when CPCs were cultured for 2 days on cECM compared to COL. Moreover, fibroblast and endothelial/smooth muscle cell-specific genes decreased and remained constant, respectively, for CPCs cultured on cECM compared to COL. In a recent study, human Sca-1^+ cells originating from either fetal heart (fCPCs) or adult hearts (aCPCs) were encapsulated and cultured in three-dimensional (3D) hydrogels consisting of either cECM or COL [187]. Similarly, gene expression of early cardiac markers, that is, GATA-4, Nkx2.5, myocyte enhancer factor 2c (Mef2c), and myosin light chain 2v (MLC2v), increased when fCPCs and aCPCs were cultured in cECM compared to COL after 4 days. Furthermore, after 7 days, these markers increased when fCPCs were cultured in cECM and remained constant for aCPCs. One explanation for the minimal increase in cardiac markers after a longer period of time could be the development of an endogenous microenvironment by CPCs that decreases the early biochemical effect of cECM. Additionally, improved proliferation and survival was observed for both cECM-coated surfaces and for CPCs encapsulated in cECM hydrogels.

These findings indicate the importance of the biochemical composition for the early maturation of fCPCs towards cardiac specific lineages. Nevertheless, more information is needed on the specific ECM composition to be able to understand which of the components generates the beneficial response of CPCs towards cardiac-derived ECM, considering the fact that CPCs reside in a specific, yet complex, niche that strongly determines their behavior [27]. In a follow-up study by French et al., CPCs were cultured on cECM and were COL-, FN-, and LN-functionalized. In a follow-up study by French et al., CPCs were cultured and subjected to different cyclic strains on Bioflex plates functionalized with cECM, COL, FN, or LN [188]. Proliferating cell nuclear antigen (PCNA) was used as a measure to determine the proliferation response of CPCs cultured for 30 hours on the different substrates. The highest number of PCNA positive cells was observed on substrates functionalized with fibronectin, demonstrating the benefit of a single ECM component compared to the whole complex cardiac ECM. Fibronectin has been shown to be crucial for the expansion of human CPCs during development and after a MI [189]. Additionally, endogenous fibronectin production by human CPCs has been observed after 7 days in static culture [15]. Interestingly, the beneficial effect of fibronectin on the proliferation of CPCs seems to diminish at strain magnitudes of 10–15%. These findings suggest the importance of fibronectin on the initial proliferation response of CPCs; however, the effect can be overruled by other microenvironmental components such as cyclic strain and/or stiffness.

(2) ECM Stiffness. The data described so far suggest that an ideal biochemical environment is not sufficient to completely obtain the desired regenerative response, but that mechanical and/or structural stimuli also contribute to a favorable response. To illustrate this, c-kit$^+$ human pediatric CPCs were cultured in neonatal or adult ECM derived from Sprague-Dawley rats and combined with fibrin to create 3D hybrid hydrogels with a range of Young's moduli, that is, 2, 8, 14, and 32 kPa [190]. By increasing Young's modulus of the neonatal and adult ECM-fibrin hybrid hydrogel from 2 to 8 kPa, the gene expression of cardiac titin decreased, whereas it increased at higher moduli. These findings suggest that ECM stiffness has an effect on the genetic behavior of CPCs in terms of cardiac titin expression. Encapsulating CPCs in an environment with a stiffness that resembles the native mechanical properties would provide better conditions to study the development of CPCs into mature cardiomyocytes. However, this is complicated by the fact that stiffness values may differ between neonatal, fetal, and adult heart, between healthy and diseased conditions, and also between species [191].

To study the effect of ECM stiffness on cardiac stem/progenitor cell maturation, hydrogels with time-dependent and development-mimicking stiffnesses were developed based on thiolated-hyaluronic acid (HA) and crosslinked with poly(ethylene glycol) (PEG) diacrylate. By growing precardiac embryonic stem cells on HA-hydrogels with elastic moduli ranging from 1 to 10 kPa, a 60% increase in myofibril orientation and a 3-fold increase in Troponin T expression was observed compared to cells grown on mechanically static polyacrylamide hydrogels [192]. A recent example of modulating the ECM stiffness is shown by Choi et al., where a sol-to-gel transitional gelatin-PEG-tyramine (GPT) hydrogel with tunable mechanical properties was developed [193]. Hydrogels with elastic moduli of 1.8, 2.8, 5.8, and 8.1 kPa were created by varying the H_2O_2 concentration. Interestingly, CPCs isolated from 9-week-old Sprague-Dawley rats showed inhibited f-Actin organization and decreased proliferation in stiffer GPT hydrogels (elastic moduli of 5.8 and 8.1 kPa) compared to lower stiffnesses (1.8 and 2.8 kPa) [197]. However, an enhanced expression level of early cardiac differentiation markers was observed in GPT hydrogels with higher elastic moduli. These results strongly suggest an inhibition of proliferation and enhancement and differentiation in cardiac stem/progenitor cells as a result of increasing the ECM stiffness.

3.3. Cyclic Strain. The adult human heart beats 60–100 times per minute every day. Cells that reside in the myocardium are constantly subjected to this mechanical loading, which thus represents a significant component of the cardiac microenvironment that can influence the regenerative response of resident CPCs. However, while many studies have focused on the mechanoresponse of contractile cardiomyocytes, the effect of cyclic strain on CPCs has only been investigated by a few research groups. We recently elucidated the mechanoresponse of human Sca-1^+ CPCs. Cells were cultured on 2D substrates coated with collagen IV, which together with laminin represents the main component of the cardiomyocyte basement membrane. Whereas undifferentiated CPCs did not show a preferential orientation upon application of uniaxial cyclic strain, CPCs in the early stage of cardiomyogenic differentiation (predifferentiated) oriented perpendicularly to the main direction of the stretch (strain avoidance behavior) after 48 hours [184]. The different responses appear to be

due to the development of the mechanosensing structures, such as focal adhesions (FAs) and actin stress fibers (the *mechanosome*), that we demonstrated to occur during the early phase of cardiac differentiation [184]. In the study of French et al. [188] mentioned above, cyclically strained rat c-kit$^+$ CPCs displayed a different orientation response when cultured on different ECM coatings. After 24 hours, rat CPCs displayed a strong strain avoidance response on fibronectin and collagen I. On the other hand, the strain avoidance response on cECM was much weaker as compared to collagen I and fibronectin, whereas almost no strain avoidance was observed on laminin [188]. Taken together, the reported studies suggest that CPCs on the natural cardiac ECM are less responsive to cyclic strain as compared to single ECM components (fibronectin, collagen I). Furthermore, the mechanoresponse of CPCs is weakened on certain ECM proteins (laminin, collagen IV). It is tempting to speculate that this behavior might be related to the affinity of different integrins for the ECM proteins. It would be interesting to investigate which integrins are expressed by the CPCs on the different substrates, and especially on the naturally derived cardiac ECM, and relate this expression pattern to the CPC mechanoresponse (as previously done in other cell types by [194, 195]).

It should be noted that the above studies are limited by their 2D setup, which does not resemble the 3D physiologic environment. In a study by van Marion et al. [196], the effects of human Sca-1$^+$ CPC engraftment in collagen I/Matrigel hydrogels were investigated. Whereas CPCs showed a random orientation in stress-free hydrogels, in statically constrained hydrogels, they aligned along the direction of the strain after 24 hours. This effect was even more pronounced at day 9 of culture, showing that CPCs become readily mechanosensitive in 3D. Furthermore, already after 24 hours of culturing in the 3D hydrogels, the cardiac differentiation markers were upregulated as compared to the 2D culture, indicating an increased differentiation capacity of the CPCs towards the cardiomyocyte phenotype in 3D.

Detailed investigation of the mechanosensing of (human) CPCs in 3D environments is needed in order to provide a closer clue of the response of these cells to the mechanical stimuli provided by the cardiac microenvironment.

3.4. Soluble Factors and Oxygen Tension. After a myocardial infarction, cardiac cells are immediately exposed to hypoxia, due to the temporary lack of oxygen. Hypoxia has been shown to regulate the behavior of several stem and progenitor cells by dramatically influencing fundamental signaling pathways, such as Notch and Oct4, that determine self-renewal and multipotency [197–199]. In response to low oxygen tension, cells express hypoxia-inducible factors (HIFs), with HIF-1α being the key mediator of the cellular adaptive response to hypoxia [200]. For example, HIF-1α is induced in the ischemic myocardium after MI [201]. HIF-1 directly regulates the transcription of the chemokine stromal cell-derived factor 1 (SDF-1) [202] and its receptors CXCR4 [203], which play an important role in the mobilization of progenitor cells [204–206]. Moreover, the upregulation of SDF-1 in ischemic tissues is directly proportional to the reduction of oxygen tension [202]. The interaction between SDF-1 and CXCR4 plays a crucial role in the mobilization and migration of circulating progenitor cells in ischemic tissues [202, 207, 208].

For cardiac regeneration, the response of resident progenitor cells to low oxygen tension is of great interest, due to the potential contribution of these cells to the cardiac regenerative mechanisms [38]. In this respect, a number of studies on murine CPCs have been conducted.

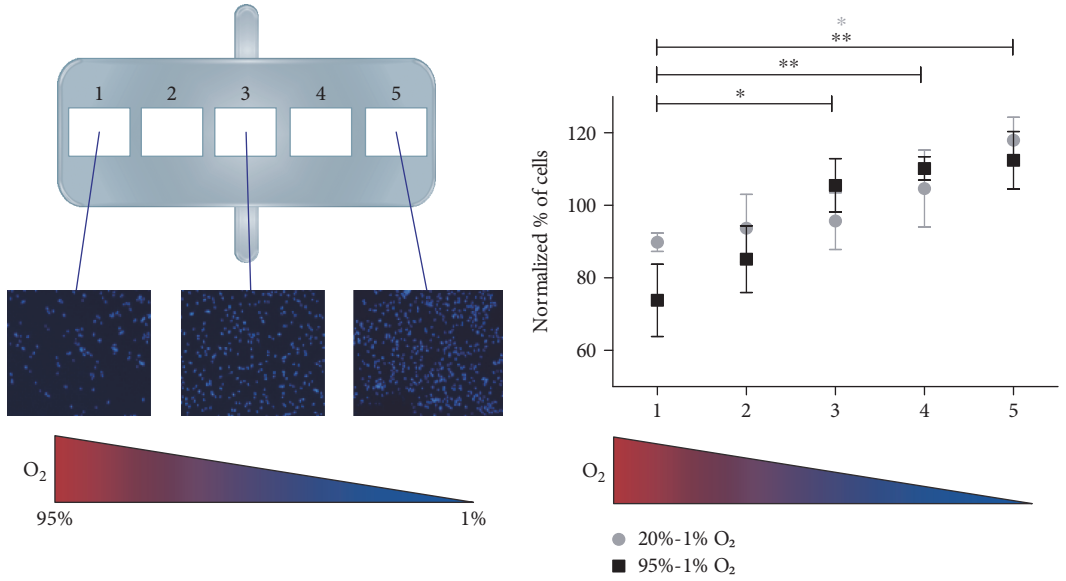
These cells express the SDF-1 receptors CXCR4 and CXCR7 [209, 210]. In room air conditions (20% O_2), CPCs show very limited expression of CXCR4; however, under (harsh) hypoxia (0.1% O_2), expression of both the CXCR4 receptor and the chemokine SDF-1 is greatly enhanced [209]. SDF-1 induces CPC migration in a time- and dose-dependent manner [209, 210]; this SDF-1-induced migration is however abolished by knockdown of CXCR4 or CXCR7 [210], demonstrating the crucial role of the SDF-1/CXCR4 and SDF-1/CXCR7 axis for CPC motility. Pretreatment of murine CPCs with hypoxia results in increased migration toward SDF-1 *in vitro*, suppressed by cell transfection with CXCR4 shRNA [209], once again indicating the key role of the SDF-1/CXCR4 interaction for CPC motility. Additionally, hypoxic pretreatment results in improved recruitment of the CPCs to the ischemic myocardium in a mouse MI model [209], suggesting a potential therapeutic benefit was offered by this procedure.

The response to hypoxia of human CPCs is less known. In a study by van Oorschot et al. [211], human Sca1$^+$ CPCs displayed increased proliferation and motility when cultured under low oxygen tension (1% O_2). The motility and migration of these cells were also enhanced by culture in 1% O_2-conditioned media [211]. Moreover, human Sca1$^+$ CPCs displayed an increase in cell motility directly proportional to the reduction of oxygen tension, similarly to the SDF-1 induction observed in ischemic tissues by Ceradini and Gurtner [212]. In a hypoxia-gradient microfluidics chip, where high O_2 tension was applied on one end (20% or 95%) and 1% O_2 at the other end, an increasing number of human Sca1$^+$ CPCs was detected after 24 hours towards the condition of the lowest oxygen tension (Figure 4). CPC displayed similar proliferation in all the areas of the chip, thereby suggesting that the higher amount of cells in the hypoxic area is indeed due to CPC migration.

This suggests that, under hypoxic conditions, not only do human CPCs show improved motility but they also release chemoattractants and their receptors. However, the induction of SDF1 and CXCR4/CXCR7 in human CPCs has not been investigated yet. Given the data here reported on murine CPCs and on other human progenitor cells, a mechanism similar to the SDF-1/CXCR4 axis might take place.

4. Approaches to Modulate the CPC Microenvironment

The CPC niche is complex and its importance for cardiac differentiation, maturation, and contribution to repair is largely unknown. For a better understanding, engineering approaches to recapitulate the native cardiac



(a) Oxygen gradient device

(b) Local cell amount

Figure 4: CPCs migrate toward lower oxygen concentration in an oxygen gradient device. (a) In a PDMS device (showed in the schematic representation) where 20% or 95% O_2 was applied at one end, and 1% O_2 at the other end, an increasing number of CPCs were observed at the lower oxygen side after 24 hours. Representative images show the increased amount of cells (nuclei stained with Hoechst 33342, blue). (b) The quantification of cell number (normalized to the initial value after seeding) is reported as mean ± SD ($n = 4$; $^*P < 0.05$; $^{**}P < 0.01$).

microenvironment are required. For recreating the CPC niche *in vitro*, several key components are of importance. In this part of the review, we will focus on current *in vitro* engineering approaches to mimic the cell natural environment (Figure 5).

As previously stated, the CPC microenvironment or niche should display key characteristics, such as optimal biochemical, physical, and mechanical properties, to enhance the regenerative response of CPCs. This ideal microenvironment should therefore stimulate either proliferation or differentiation, or elicit a beneficial effect on the paracrine signaling of CPCs. To date, little is known of the exact characteristics of this ideal niche and what is necessary to obtain optimal CPC contribution to cardiac regeneration.

Multicellular spheroids are scaffold-free spherical cell aggregates that mimic in the most simplistic way the conditions of the niche [99, 100, 213]. As compared to 2D cell culture, spheroids provide improved cell-cell and cell-ECM interactions, as well as gradients of soluble factors, such as oxygen and nutrients [213–216]. Therefore, cell spheroids are used as a model to study cell behavior in a 3D environment that better resembles the *in vivo* conditions. At the same time, they could entail major advantages for clinical use over the injection of cells grown as a monolayer, especially in the treatment of cardiac disease (as extensively described by [169, 216]).

However, an engineered microenvironment could provide more specific signals to CPCs, and its characteristics might be tunable to elicit a distinct response. Currently hydrogels, decellularized ECM, and synthetic matrices are used to create 3D cardiac environments that take into account cell-matrix interactions, as described above. However, though these matrices mimic ECM-like features, they not always resemble the mechanical strength of the native tissue [217]. Unfortunately, only a small amount of studies are performed with CPCs in matrices to create an engineered CPC-niche, although recently these are being increasingly explored [97, 188, 196, 218, 219]. Other cell types, which do not match the definition of CPCs of this review, are more prominently used in hydrogels, and the knowledge gained from these studies might be interesting to engineer the CPC niche. Cardiosphere-derived cells in both alginate [220] or biodegradable poly-(N-isopropylacrylamide) hydrogels showed cardiomyogenic differentiation and proliferation [221] and provided functional benefits [222].

However, these approaches lack to take into account some key aspects of the cardiac microenvironment. By making more use of biomaterials that can form well-defined and "smart" microenvironments, more knowledge can be extracted to finally be able to define the ideal CPC niche. Recently, scalable engineered and force-generating human myocardium was produced under well-defined conditions using embryonic stem cells, induced pluripotent stem cell-derived cardiomyocytes, and fibroblasts [223]. Interestingly, extensive evidence for cardiac molecular maturation and functional tissue formation was obtained using RNA sequencing techniques. According to Tiburcy et al., the most important responses that determine the degree of *in vitro* maturation of human cardiomyocytes are artificial electrical pacing [224], mechanical stimulation (uniaxial and cyclic load) [225], and cocultures with fibroblast-like cells [226]. Finally, they were able to develop a model that can be used to screen drugs, study heart repair or model heart

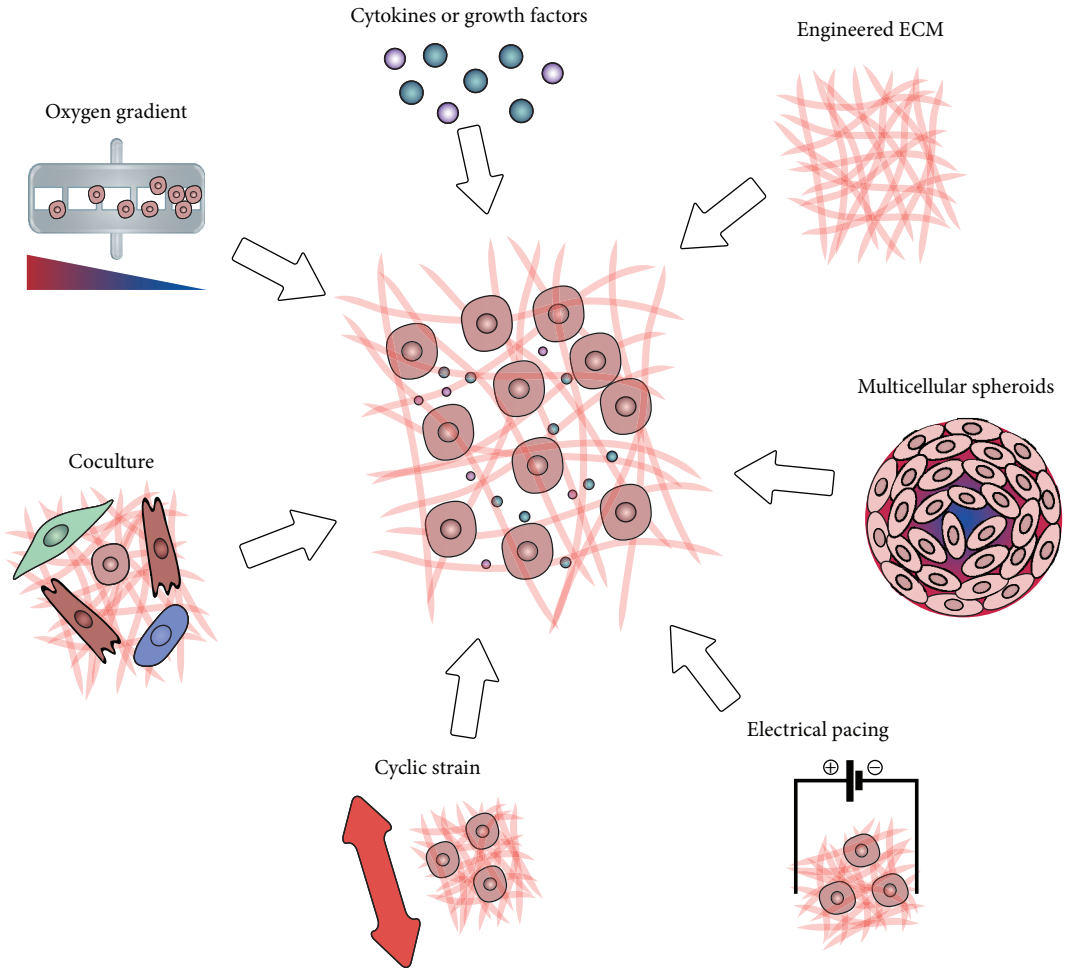


Figure 5: *In vitro* approaches to modulate the CPC niche. Strategies to optimize the regenerative potential of CPCs by modulating their microenvironment include (clockwise) the following: engineering the ECM with synthetic or naturally derived polymers with the right composition and physical properties; recreating a niche-like environment by growing cells as multicellular spheroids; applying electrical pacing and/or cyclic strain; co- and multiculture of different cell types to optimize cell-cell interactions, with or without surrounding ECM; and modulating the cell recruiting potential held by gradients of oxygen and cytokines and growth factors.

disease, and to study the endogenous repair of CPCs. Another method to create well-defined microenvironments is by making use of micro- and nanoscale engineered biological systems on a chip [227]. With these techniques, different niche components, such as mechanical [228–230], electrical [231], or topographical cues [232], can be carefully modulated and stem cell responses can then be studied in more detail. For instance, Morez et al. have shown improved cardiomyocyte differentiation from CPCs using silicone parallel microgrooves (10 μm wide and 3 μm deep) *in vitro* [233].

Further *in vitro* research is needed on the influence of different niche components on the behavior and regenerative potential of CPCs, in order to make the final next step towards the successful endogenous cardiac repair by CPCs.

5. Conclusive Remark

In this review, we have defined cardiac resident progenitor cells according to their behavior and characteristics. Although there is ongoing debate and controversy about the presence of CPCs in the heart and their regenerative potential, a considerable amount of evidence shows that these cells exist and reside in the fetal and adult (human) heart in specific niches. We have highlighted the key components of CPC niches and the interplay of CPCs with niche elements.

As reported, the CPC niche is very complex in structure and composition and the relative and combined effects of individual niche elements on CPC function and regenerative potential is, to date, far from clear. Better understanding of the effect of the niche on cell behavior could lead to strategies to optimize their contribution to cardiac repair. Therefore, we concluded this review by describing how engineering *in vitro* approaches, that take into account the key factors and attempt to mimic the native niche, can enhance the regenerative response of CPCs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Arianna Mauretti, Sergio Spaans, and Noortje A. M. Bax contributed equally to this work.

Acknowledgments

This research was funded by the People Program (Marie Curie Actions) of the European Union's Seventh Framework Program FP7-People-2012-ITN "TECAS" under Grant Agreement no. 317512 and FP7-People-2013-CIG under Grant Agreement no. 618623 and a grant of the Dutch Heart Foundation (DHF-2014T013).

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Hindawi
Stem Cells International
Volume 2017, Article ID 2348485, 17 pages
https://doi.org/10.1155/2017/2348485

Research Article

Insulin-Like Growth Factor Binding Protein-6 Alters Skeletal Muscle Differentiation of Human Mesenchymal Stem Cells

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Received 27 February 2017; Revised 7 July 2017; Accepted 19 July 2017; Published 14 September 2017

Academic Editor: Sari Pennings

Insulin-like growth factor binding protein-6 (IGFBP-6), the main regulator of insulin-like growth factor-2 (IGF-2), is a component of the stem cell niche in developing muscle cells. However, its role in muscle development has not been clearly defined. In this study, we investigated the role of IGFBP-6 in muscle commitment and differentiation of human mesenchymal stem cells derived from the placenta. We showed that placental mesenchymal stem cells (PMSCs) have the ability to differentiate into muscle cells when exposed to a specific culture medium by expressing muscle markers Pax3/7, MyoD, myogenin, and myosin heavy chain in a stage-dependent manner with the ultimate formation of multinucleated fibers and losing pluripotency-associated markers, OCT4 and SOX2. The addition of IGFBP-6 significantly increased pluripotency-associated markers as well as muscle differentiation markers at earlier time points, but the latter decreased with time. On the other hand, silencing IGFBP-6 decreased both pluripotent and differentiation markers at early time points. The levels of these markers increased as IGFBP-6 levels were restored. These findings indicate that IGFBP-6 influences MSC pluripotency and myogenic differentiation, with more prominent effects observed at the beginning of the differentiation process before muscle commitment.

1. Introduction

Unlike embryonic stem cells which are derived from the early embryo, placental mesenchymal stem cells (PMSCs) are derived from human placentae that are usually discarded following delivery, and therefore a readily available and noncontroversial source of adult stem cells for possible use in tissue regenerative therapies in human patients [1–3]. Placental mesenchymal stem cells are available in large numbers and capable of differentiating into cells of all three germ layers depending on the type and concentration of niche factors to which the cells are exposed to *in vitro*. The pathways activated by these cells during differentiation into specific mesodermal cell types illustrate the mechanisms by which these cells differentiate *in vitro* and *in vivo* and may provide important information on the developmental processes of tissues and organs during embryogenesis and in the adult.

Skeletal muscle development is a highly coordinated stepwise process utilizing a series of transcriptional factors, and structural and enzymatic proteins expressed to mark the different stages of skeletal muscle development. During myogenesis, committed progenitors differentiate into muscle lineage by upregulating the myogenic regulatory factors (MRFs) as well as muscle commitment transcription factors (Pax3 and Pax7), followed by the expression of early muscle cell markers (MyoD and myogenin) [4]. After commitment, the cells start to fuse together to form multinucleated fibers and express muscle-specific proteins, such as myosin heavy chain (MHC) [4]. It is believed that in recovery and regeneration after muscle injury in the adult, this process is recapitulated.

Mesenchymal stem cells isolated from bone marrow have the ability to differentiate into myocytes [5, 6]. However, these cells have limited availability and do not have the ability to form fused skeletal muscle *in vitro* [7, 8]. Adipose-derived stem cells are another source of stem cells that can differentiate into skeletal muscle [9]. Although these cells are readily available, they have limited muscle recovery [10]. Therefore, the need to find a stem cell population that will eliminate the problems related to other stem cells was our main priority.

The insulin-like growth factor (IGF) family of peptides regulates cell growth, differentiation, and the maintenance of cell survival through several signal transduction pathways [11]. This family includes two IGF peptides, IGF-1 and IGF-2, three cell surface receptors, type-1 and type-2 IGF receptors, insulin and hybrid receptors, and six IGF binding proteins (IGFBPs) [4]. IGF-1 and IGF-2 are circulating and intercellular peptides that function as potent mitogens for many different cell types, which are mediated by binding to IGF-1R, a membrane receptor tyrosine kinase [12]. IGFBPs are carriers for IGFs in the circulation and in the extracellular fluid compartment [13], protecting them from degradation [12, 14], delivering them to specific tissues, and modulating the biological actions of IGFs. IGFBP-6 is a 30 kDa secreted protein, and unlike other IGFBPs, has a significantly higher affinity (~70–100-fold) for IGF-2 than IGF-1 [15, 16]. IGFBP-6 has been demonstrated to modulate IGF-2 activity via inhibiting IGF-2 binding to the IGF-1R or directly independent of IGF-2 binding to the receptor [17, 18]. IGF binding proteins, including IGFBP-6, are secreted into the extracellular environment where they interact with IGFs. They are also localized intracellularly suggesting that IGFBPs may have biological actions independent of IGFs [19].

The IGF family has been shown previously to play a major role in muscle development. IGF-1R knockout mice die soon after birth due to breathing difficulties due to lack of functional respiratory muscles [20, 21]. IGF-2 is expressed abundantly in the developing skeletal muscle and is a major factor for muscle growth, differentiation, and regeneration [22]. When IGF-2 is knocked down, myogenesis does not occur [22]. During development, IGFBP-6 is expressed abundantly in developing muscle cells and is also required for myogenesis [22]. Previous studies in our laboratory have described IGF-independent functions of IGFBP-6 by interacting with Ku proteins in regulating cell fate in a skeletal muscle cell line [23]. Also, another study showed that IGFBP-6 inhibits angiogenesis but promotes migration in an IGF-independent manner [17].

To our knowledge, the biological roles of IGFBP-6 in the differentiation of stem cells into the muscle lineage have not been reported. In this study, we determined if PMSCs can differentiate into skeletal muscle when exposed to muscle differentiation promoting conditions and then characterized the effects of IGFBP-6 on the differentiation of PMSCs into skeletal muscle.

2. Materials and Methods

2.1. Isolation of PMSCs. PMSC isolation and experiments were conducted in accordance with the approval from the Health Sciences Research Ethics Board of Western University. Informed consent was obtained from healthy women undergoing therapeutic termination of pregnancy, and the PMSCs used in this study were isolated from 15 weeks preterm placental tissues. After surgery, chorionic villi were dissected, washed, minced with surgical scissors and forceps, and then subjected to enzymatic digestion with collagenase IV (369 IU/mg), hyaluronidase (999 IU/mg) (Sigma-Aldrich, Oakville, ON), and DNase I (2000 IU/mg) (Hoffmann-LaRoche, Mississauga, ON) for 10 minutes at room temperature, followed by 0.05% trypsin (Gibco/Invitrogen, Mississauga, ON) for 5 minutes at room temperature. The sample was then washed for 10 minutes with 10% FBS in DMEM/F12 medium, and the resulting single cell suspension was separated by density centrifugation over a Percoll (Sigma-Aldrich, Oakville, ON) discontinuous gradient using a modified protocol by Worton et al. [24].

2.2. Cell Culture. Cells from Percoll gradient fractions number 3 and number 4 were plated on to T75 flasks, cultured, and maintained using DMEM/F12 media supplemented with 15% FBS serum (Gibco/Invitrogen, Mississauga, ON) and FGF-2 (50 ng/mL) (Sigma-Aldrich, Oakville, ON) containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin-B. The nonadherent cells were discarded at the time of media change, which was performed every 72 hours. The adherent cells were cultured until they reach 90% confluence. Cells were then passaged 1 : 2 approximately once per week using 0.05% trypsin for 10 min at 37°C for 3 passages. Fourth passaged cells were stored at −80°C in 1 mL of freezing media (30% FBS and 10% DMSO in DMEM/F12 media). When needed, vials were thawed and cells were resuspended in normal culture media (25 ng/mL FGF-2 and 15% FBS in DMEM/F12). Only PMSCs of passage 3 or 4 were used in the experiments.

2.3. Flow Cytometry Analysis. Cells were trypsinized for 10 minutes using recombinant trypsin (TrypLE EXpress, Gibco/Invitrogen, Mississauga, ON) diluted 1 : 1 in PBS, at 37°C. After the cells were detached from the flask, trypsin was neutralized with 10% FBS in DMEMF/12 medium, cells were washed and incubated for one hour with fluorochrome-labeled primary antibody against MSC markers. CD73 (number 550256) (BD Pharmingen, San Jose, CA), PE-conjugated CD105 (number 12-1057-73) (eBioscience, San Diego, CA), and CD-117/c-Kit (sc-13508) (Santa Cruz Biotechnology, Dallas, TX) were used (Supplementary Figure 1).

2.4. Muscle Differentiation. Cells were plated in the presence of the muscle growth media (fetal bovine serum 0.05 mL/mL, fetuin 50 μg/mL, epidermal growth factor 10 ng/mL, basic fibroblast growth factor 1 ng/mL, insulin 10 μg/mL, and dexamethasone 0.4 μg/mL) for 48 hours before changing to the skeletal muscle differentiation media, which is serum-free medium containing 10 μg/mL insulin (PromoCell, Heidelberg, Germany). Cells were grown in six-well plates in a standard tissue culture incubator at 37°C in 5% CO_2.

2.5. IGFBP-6. Recombinant Human IGFBP-6 (ProSpec, East Brunswick, NJ) was resuspended in sterile MilliQ-H_2O and added to the media at a concentration of 375 ng/mL. IGFBP-6 was added every 3 days at the time of media change. IGFBP-6 concentration was determined by a dose-response experiment using PMSCs in muscle differentiation media (Supplementary Figure 2A). IGFBP-6 was added every 3 days at the time of media change because that was the time it took for IGFBP-6 secreted levels to be lower than the control (Supplementary Figure 2B).

2.6. Downregulation of IGFBP-6 Expression by siRNA. To silence the endogenous IGFBP-6 expression, IGFBP6 siRNA (h) with a pool of 3 target-specific 19–25 nt siRNAs was used (Santa Cruz Biotechnology, Dallas, TX). 8 μL of Lipofectamine (Invitrogen, Mississauga, ON) with either 8 μL of scrambled or IGFBP-6 siRNA was added to 100 μL of DMEM/F12 media (Invitrogen, Mississauga, ON) for 40 minutes at room temperature; the concentration of siRNA was 80 nM. The siRNA solution was then added to the 60% confluent cells and incubated for 5 hours at 37°C. Muscle growth media (1.5 mL) was added to the cells for 48 hours, and then it was replaced with 2 mL of muscle differentiation media. New siRNA was added every 3 days during the change of media, and the experiment was performed for 7 days.

2.7. Immunocytochemistry. PMSCs were grown and differentiated on glass cover slips, stained with primary antibodies, and incubated at 4°C overnight. The primary antibodies were washed using 0.1% Tween-20 in PBS (3 times for 5 minutes), and cells were then incubated in the dark with the secondary antibody. The secondary antibody was washed 0.1% Tween-20 in PBS, and the nuclear stain was added for 7 minutes and then rinsed. The cover slips were mounted for 2 hours, and images were taken using a Zeiss confocal microscope. Each antibody was performed in triplicate.

2.8. Immunoblotting. Following experiment completion, each cell lysate containing 20 μg of protein was added to 6x SDS gel loading buffer (1% β-mercaptoethanol, 1% SDS, 30% glycerol, 0.0012% bromophenol blue, Tris-HCl 0.28 M, and pH 6.8). Samples were boiled for 5 minutes at 95°C, then placed on ice for 3 minutes, and centrifuged at 3000 rpm for 20 seconds before loading. Samples were resolved by molecular weight using 10% SDS polyacrylamide gels and then transferred onto polyvinyldenefluoride (PVDF) membranes (Bio-Rad, Hercules, California) using a Trans-Blot Turbo (Bio-Rad, Hercules, California) with an optimized protocol depending on the protein size. Membranes were blocked with 5% nonfat dry milk, gently shaked for 1 hour at room temperature in Tris-HCl buffer saline pH 8.0 with 0.1% Tween-20 (TBS-T). Blots were then washed with TBS-T (3x for 10 min) followed by incubation at 4°C overnight with specific primary antibodies in 5% BSA or 5% nonfat dry milk in TBS-T following the manufacturer's protocol. Then membranes were washed and incubated for 1 hour at room temperature with the corresponding secondary HRP antibody. Resolved protein bands were detected using chemiluminescence, and images were taken using the VersaDoc Imager (Bio-Rad, Hercules, California). Western blots were performed in triplicate.

2.9. Quantification of the IGFBP-6 and IGF-2 Secretion by Enzyme-Linked Immunosorbent Assay (ELISA). Human IGFBP-6 (RayBiotech®, Burlington, ON) and IGF-2 (ALPCO, Salem, NH) ELISA kits were used to measure the amount of IGFBP-6 and IGF-2 secreted into the media of different treatment conditions. Standards and samples were loaded into the wells and the immobilized antibody bound IGFBP-6 or IGF-2 present in the sample. The wells were washed, and biotinylated anti-human antibody was added. After washing, HRP-conjugated streptavidin was added; then a TMB substrate solution was used to develop a blue color in proportion to the amount of IGFBP-6 or IGF-2 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color was measured at 450 nm using Multiskan Ascent analysis software.

2.10. Aldehyde Dehydrogenase (ALDH) Activity. PMSC ALDH activity was assessed by flow cytometry. An Aldefluor™ kit (Stem Cell Technologies, Vancouver, BC) was used as per the manufacturer's protocol. 5 μL of the activated Aldefluor reagent/mL was added, and the cells were incubated for 45 minutes. Cells were centrifuged for 5 minutes and resuspended in 500 μL of ice-cold Aldefluor assay buffer. ALDH activity was measured using flow cytometry. Samples were run in triplicate.

2.11. Antibodies. For pluripotency markers, we used OCT4 antibody (N-19: sc-8628) (Santa Cruz Biotechnology, Dallas, TX), and SOX2 (2683-1) (Epitomics, Burlington, ON, CAN). For muscle differentiation markers, Pax3/7 (E-10:sc365613), MyoD (M-318: sc-760), myogenin (F5D: sc-12732), and myosin heavy chain (H-300: sc-20641) (Santa Cruz Biotechnology, Dallas, TX) were used. For loading control, pan-actin Ab-5 (Thermo Fisher Scientific, Fremont, CA) was used. For IGFBP-6, antibody (H-70: sc-13094) (Santa Cruz Biotechnology, Dallas, TX) was used. The secondary antibodies used for immunoblotting were goat anti-rabbit (number 170-6515) or anti-mouse (number 170-6516) HRP-conjugated antibodies (Bio-Rad, Hercules, CA), or donkey anti-goat antibody (Santa Cruz Biotechnology, Dallas, TX). The secondary antibodies used for immunocytochemistry were green-Alexa 488 or red-Alexa 568 (Invitrogen, Mississauga, ON).

2.12. Statistical Analysis. All experiments were run in triplicates, and the specific protein levels were quantified and normalized for loading with the level of pan-actin in each lane. GraphPad Prism Software 5.0 was used to generate all graphs and analyses. A two-way ANOVA followed by a Bonferroni's multiple comparison test or a one-way ANOVA followed by a Student t-test was used, and significant difference was considered when $P < 0.05$. Graphic representation values are presented as mean ± SEM (shown as variance bars).

3. Results

3.1. PMSCs Can Differentiate into Skeletal Muscle. To determine if PMSCs can differentiate into skeletal muscle, PMSCs

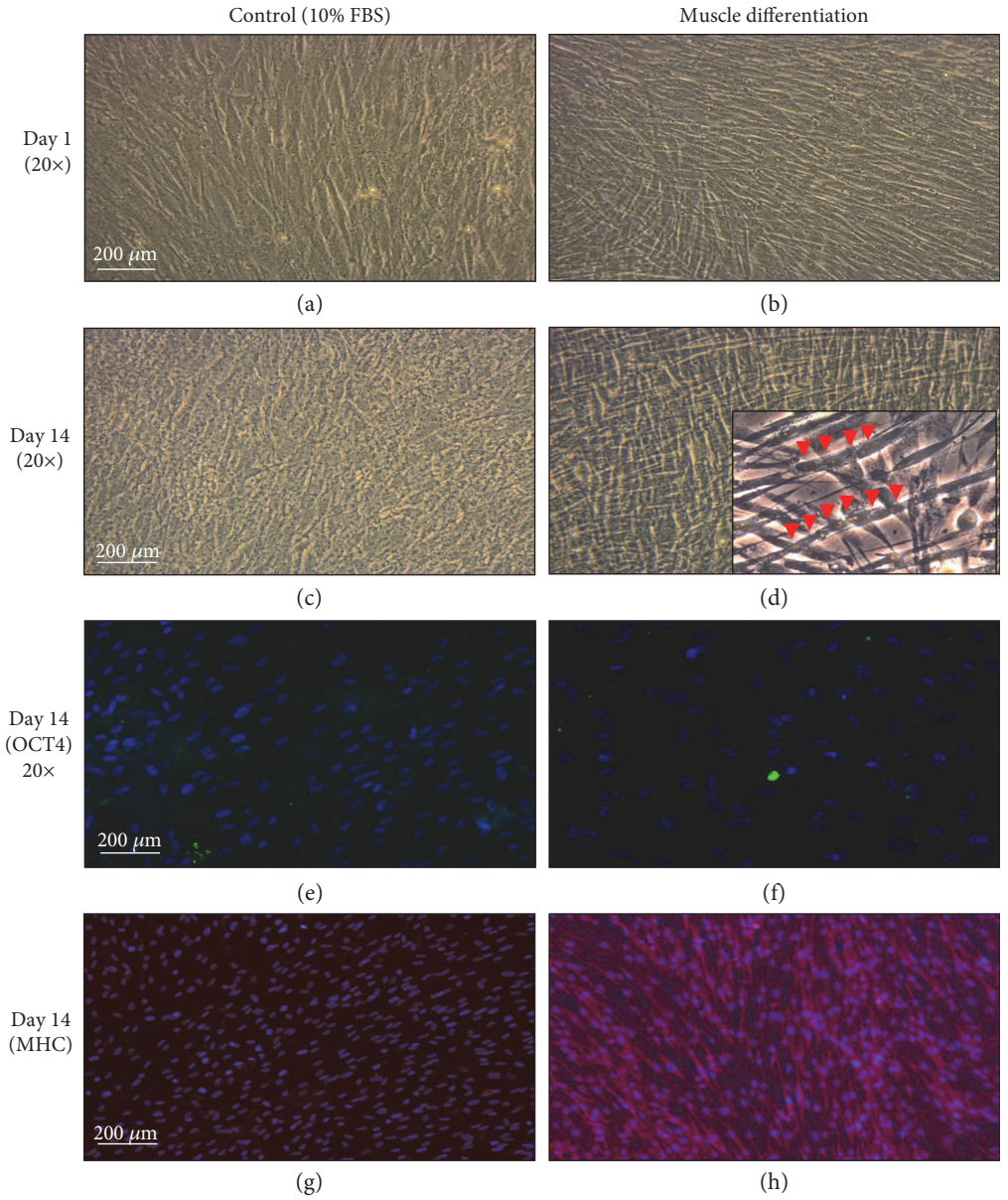


Figure 1: PMSCs cultured under muscle differentiation conditions showed muscle morphology with lower OCT4 and higher MHC at 14 days postdifferentiation. (a, b) Compared to cells cultured under standard conditions in 10% FBS, PMSCs grown in muscle differentiation media showed skeletal muscle morphology as early as day 1 postdifferentiation (20x). (c, d) At 14 days postdifferentiation, PMSCs grown in muscle differentiation media showed increased skeletal muscle fiber compaction and the formation of multinucleated fibers (20x). 40x magnification is shown in the bottom right corner. Red arrows indicate multinucleated muscle cells. (e–h) Cells grown in muscle differentiation media showed less OCT4 (green-Alexa 488, λ-488 nm) (20x) and more MHC immunoreactivity (red-Alexa 568, λ-568 nm), when compared to PMSCs in 10% FBS at 14 days postdifferentiation (10x). Nuclei were stained with Hoechst dye (blue, $\lambda = 340$ nm). Experiment was performed in triplicate.

were grown under muscle differentiation conditions for up to 14 days. Compared to PMSCs grown in nondifferentiating conditions (10% FBS), differentiated PMSCs showed muscle morphology as early as day 1 postdifferentiation (compaction and elongated appearance) (Figures 1(a) and 1(b)), and cells continued to differentiate forming multinucleated fibers at day 14 (Figures 1(c) and 1(d) and Supplementary Figure 3A and B). Associated with these morphological changes, pluripotency-associated marker (OCT4) immunoreactivity appeared low (Figures 1(e) and 1(f)), and muscle differentiation marker (MHC) immunoreactivity was high (Figures 1(g) and 1(h)) when compared to control cells (10% FBS). In addition, PMSCs under muscle differentiation conditions showed lower cell counts per field compared to undifferentiated controls (Supplementary Figure 3C).

Under muscle differentiation conditions, PMSCs decreased pluripotency-associated protein levels of OCT4 and SOX2. OCT4 levels were reduced at day 1 compared to control with further decrease at 14 days postdifferentiation (Figure 2(a)). In addition, SOX2 levels were lowered and

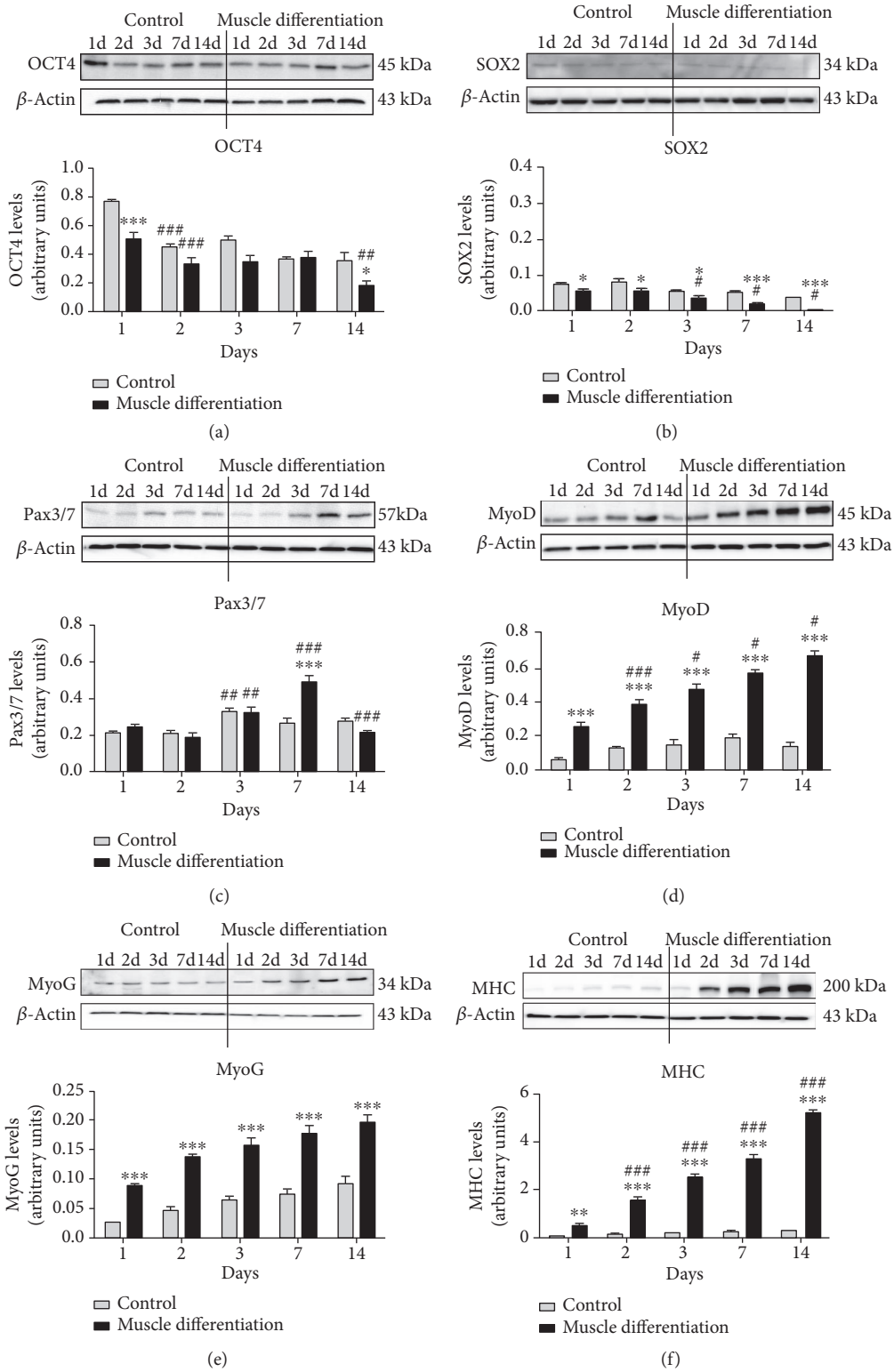


Figure 2: PMSCs cultured under muscle differentiation conditions increased levels of muscle markers (Pax3/7, MyoD, MyoG, and MHC) and decreased OCT4 and SOX2 levels using Western blots. Compared to PMSCs cultured under control conditions, (a) OCT4 was decreased at 1 and 14 days under muscle differentiation conditions. (b) SOX2 levels were decreased at each time point under muscle differentiation conditions. (c) Pax3/7 was increased at day 7 and decreased by day 14 under muscle differentiation conditions. (d) MyoD, (e) MyoG, and (f) MHC were increased at each time point under muscle differentiation conditions. Protein levels were quantified by densitometry and normalized to β-actin. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ comparing control to muscle differentiation conditions, or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ comparing the same treatment over time.

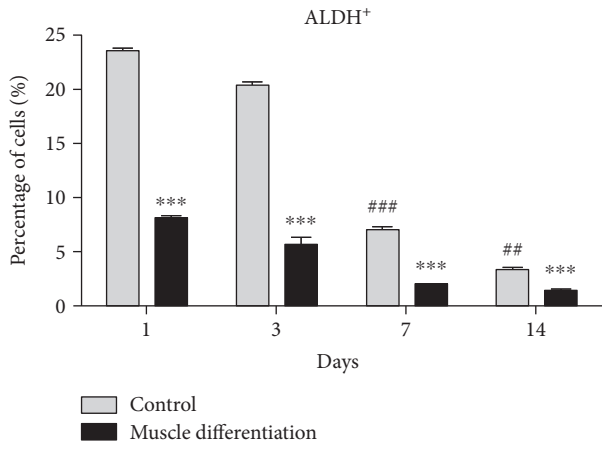


Figure 3: PMSCs cultured under skeletal muscle differentiation conditions showed a decreased frequency of cells with high ALDH activity. Compared to PMSCs cultured under control conditions, PMSCs cultured under differentiated conditions showed significantly decreased frequency of cells with high ALDH activity. Even under control culture conditions, PMSCs showed diminished ALDH-activity over time. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{***}P < 0.001$ comparing control to muscle differentiation conditions, or $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ comparing the same treatment over time.

nearly diminished by day 14 in cells under muscle differentiation conditions compared to control (Figure 2(b)). Muscle commitment marker Pax3/7 was increased at day 7, followed by a decrease at day 14 in PMSCs under muscle differentiation conditions compared to control (Figure 2(c)), suggesting that PMSCs under muscle differentiation conditions are committed to the muscle lineage and are proceeding to muscle differentiation. This was confirmed by the protein levels of muscle markers (MyoD, MyoG, and MHC) that increased significantly over time under muscle differentiation conditions (Figures 2(d), 2(e), and 2(f)). Collectively, these findings indicate that PMSCs differentiate into skeletal muscle under appropriate culture conditions, and this cell differentiation model could be consistently used to study muscle development *in vitro*.

We used the Aldefluor assay to determine the frequency of primitive progenitor cells with high ALDH activity. In this context, high ALDH activity is a conserved characteristic of proliferative progenitor cells of multiple lineages [25, 26]. As differentiation occurs towards a more mature cellular phenotype, ALDH activity is reduced. Compared to PMSCs grown under nondifferentiation conditions, there was a decrease in the frequency of cells with high ALDH activity ($ALDH^+$ cells) under muscle differentiation conditions at days 1 to 14 (Figure 3 and Supplementary Figure 4). Moreover, ALDH activity was also decreased over time when cultured under control conditions (10% FBS) (Figure 3). These findings suggested that PMSCs comprised of a heterogeneous population that slowly differentiated during maintenance in standard culture conditions and PMSCs stimulated to differentiate into skeletal muscle immediately decreased ALDH activity at earlier time points.

3.2. PMSCs Express IGFBP-6 during Myogenic Differentiation. PMSCs under skeletal muscle differentiation conditions were investigated if they expressed IGFBP-6. Using immunocytochemistry, PMSCs cultured under differentiation conditions showed high intracellular IGFBP-6 immunoreactivity compared to PMSCs cultured under control conditions (Figures 4(a) and 4(b)). Using immunoblotting at multiple time points, following day 2 of differentiation, IGFBP-6 levels gradually decreased in PMSCs cultured under differentiation conditions but remained higher than time-matched controls (Figure 4(c)). Using ELISA detection in PMSC-conditioned media, there was an increase in the levels of IGFBP-6 secreted into the media, confirming that developing muscle cells express IGFBP-6 which is actively secreted into the extracellular space (Figure 4(d)). Therefore, the synthesis of IGFBP-6 increased as the cells became more differentiated towards the muscle lineage.

3.3. IGFBP-6 Affects Multipotency of the Developing Muscle Cells from PMSCs before Muscle Commitment. To test the effects of extracellular IGFBP-6 on developing muscle cells, recombinant human IGFBP-6 was added to the muscle differentiation media. Addition of extracellular IGFBP-6 into the culture media increased intracellular IGFBP-6 detection by Western blots, suggesting that recombinant human IGFBP-6 induced a positive feedback effect or was taken up by the differentiating cells (Figure 5(a)). Furthermore, stimulation in IGFBP-6 increased OCT4 and SOX2 levels concomitant to the increased IGFBP-6 levels (Figures 5(b) and 5(c)). Interestingly, IGFBP-6 supplementation also increased Pax3/7 levels suggesting enhanced PMSC commitment towards the skeletal muscle lineage (Figure 5(d)). The fact that these two events occurred simultaneously suggests that IGFBP-6 possibly had these effects on different population of cells in culture.

Finally, IGFBP-6 treatment increased the levels of muscle-specific markers, MyoD, MyoG, and MHC, at the earlier time points with a decline over time in the prolonged presence of increased extracellular IGFBP-6 compared to unsupplemented muscle differentiation conditions (Figures 5(e), 5(f), and 5(g)). Collectively, these data suggested that IGFBP-6 promoted PMSC commitment to the muscle lineage as an immediate effect but maintained pluripotency-associated markers and delayed muscle differentiation at later time points, as seen with the decreased protein level of muscle differentiation markers.

Due to the fact that both pluripotency-associated and differentiation markers increased by IGFBP-6 treatment in a time-dependent manner, we tested the cells for ALDH activity to determine the frequency of PMSCs that maintained high ALDH progenitor phenotype. PMSCs under muscle differentiation in the presence of IGFBP-6 increased ALDH activity compared to PMSCs under muscle differentiation alone at days 1 to 14 (Figure 6 and Supplementary Figure 5), suggesting that IGFBP-6 addition prolonged primitive progenitor phenotype in PMSCs cultured under

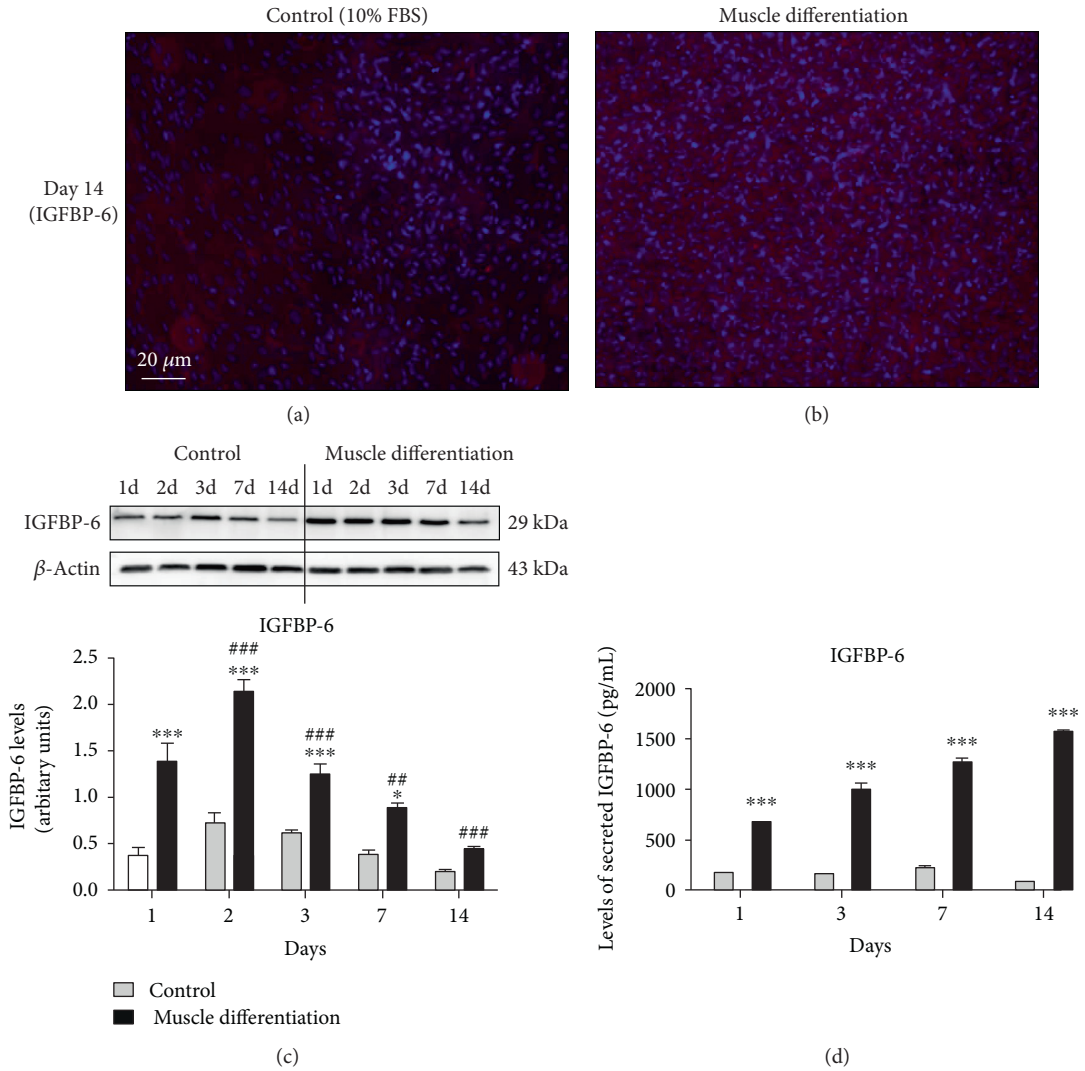
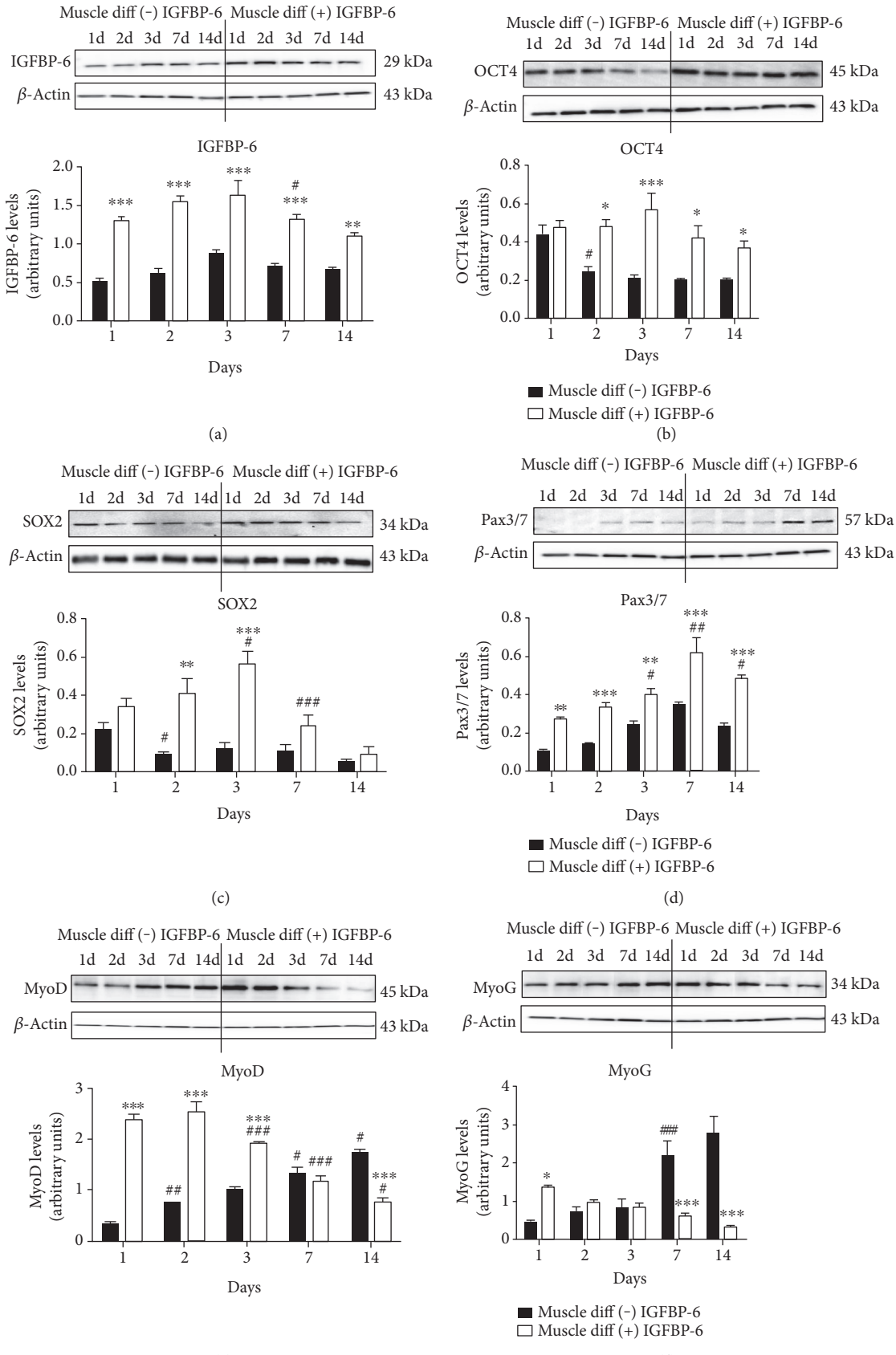


Figure 4: PMSCs cultured under skeletal muscle differentiation conditions showed increased IGFBP-6 expression and secretion. (a, b) PMSCs cultured under muscle differentiation conditions showed higher IGFBP-6 staining (red-Alexa, λ-568 nm) when compared to PMSCs under control conditions (10% FBS) at 14 days postdifferentiation. Nuclei were stained with Hoechst dye (blue, $\lambda = 340$ nm). (c) Using Western blots, IGFBP-6 protein levels in PMSCs cultured under differentiation conditions were increased at each time point compared to control conditions. Under muscle differentiation conditions, IGFBP-6 levels peaked at 2 days postdifferentiation and gradually decreased from days 3 to 14. Protein levels were quantified by densitometry and normalized to β-actin. (d) Using ELISA, IGFBP-6 and (e) IGF-2 secretion into the media was increased under muscle differentiation conditions compared to control conditions. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{*}P < 0.05$, and $^{***}P < 0.001$ comparing control to muscle differentiation conditions, or $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ comparing the same treatment over time.

muscle differentiation conditions. Further immunocytochemistry analyses at day 14 revealed that compared to unsupplemented conditions, PMSCs treated with IGFBP-6 showed more muscle compaction (Figures 7(a), 7(b), 7(c), and 7(d)). Moreover, MHC immunoreactivity appeared equivalent with or without IGFBP-6 supplementation (Figures 7(e) and 7(f)) with less number of cells (Figure 7(g)). These findings suggest that the increase in both pluripotency-associated and differentiation markers resulted from the impact of changing culture conditions (cellular environment) on a heterogeneous population of undifferentiated and differentiated cells.

3.4. Endogenous IGFBP-6 Is Required for the Differentiation of PMSCs to Skeletal Muscle. To evaluate the effects of IGFBP-6 silencing on pluripotency-associated and muscle differentiation markers in PMSCs, IGFBP-6 knockdown by siRNA was used during muscle differentiation over 7 days. As predicted, PMSC expression of IGFBP-6 was decreased for 1-2 days after IGFBP-6 knockdown compared to scrambled siRNA control. However, IGFBP-6 levels were equivalent to scrambled controls by day 3. Readministration of IGFBP-6 siRNA at day 3 prolonged IGFBP-6 reduction, but IGFBP-6 returned to control levels by day 6 (Figure 8(a)). These findings suggest that differentiating PMSCs have a



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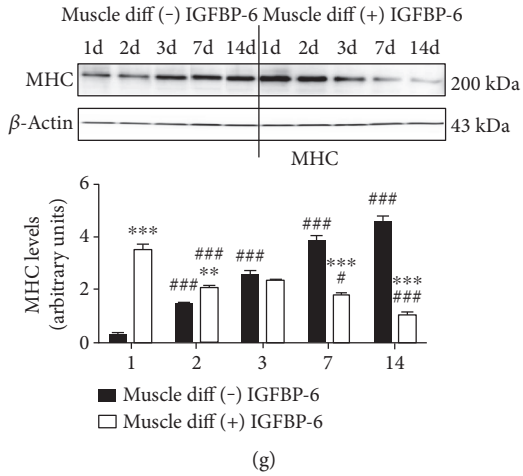


(g)

Figure 5: PMSCs treated with IGFBP-6 increased pluripotency-associated and muscle differentiation markers. IGFBP-6, OCT4, SOX2, Pax3/7, MyoD, MyoG, and MHC protein levels were quantified within PMSCs grown in muscle differentiation media with or without IGFBP-6 (375 ng/mL) supplementation using Western blots. (a) IGFBP-6 treatment increased IGFBP-6 levels as compared to PMSCs grown in muscle differentiation media only. IGFBP-6 treatment also increased pluripotency-associated markers (b) OCT4 and (c) SOX2 levels. (d) IGFBP-6 treatment increased muscle lineage commitment marker Pax3/7 at each time point. Muscle differentiation markers (e) MyoD, (f) MyoG, and (g) MHC levels increased with IGFBP-6 treatment at early time points (1–3 days) but showed reduced levels at later time points (7–14 days). Protein levels were quantified by densitometry and normalized to β-actin. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ comparing treatments, or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ comparing the same treatment over time.

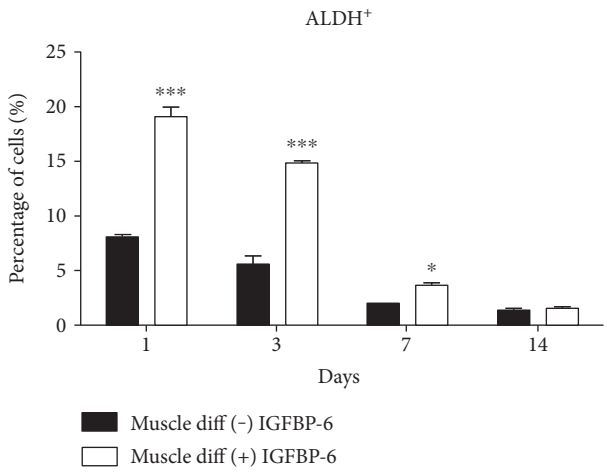


Figure 6: IGFBP-6 treatment increased the frequency of PMSCs with high ALDH activity. At days 1, 3, and 7, PMSCs treated with IGFBP-6 showed increased frequency of cells with high ALDH-activity. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{*}P < 0.05$ and $^{***}P < 0.001$.

high capacity to express IGFBP-6 and overcame siRNA knockdown within 3 days in culture. Alongside IGFBP-6 knockdown, we observed a reduction in pluripotency-associated markers for OCT4 (Figure 8(b)) and SOX2 (Figure 8(c)) concomitant with reduced IGFBP-6 levels, suggesting that IGFBP-6 may be important for maintaining potency which needs to be further investigated. Concomitantly, there was an increase in muscle commitment marker Pax3/7 that was reduced by day 3 (Figure 8(d)). Similarly, levels of the muscle lineage differentiation markers MyoD, MyoG, and MHC were all decreased at early time points after IGFBP-6 knockdown (Figures 8(e), 8(f), and 8(g)). Increased protein levels of muscle commitment marker and reduced levels of muscle differentiation markers suggest that endogenous IGFBP-6 knockdown initiated PMSCs commitment to the muscle lineage but delayed muscle differentiation.

As both pluripotency-associated and differentiation markers were decreased with IGFBP-6 silencing, ALDH activity was determined. Silencing of endogenous IGFBP-6 expression in PMSCs, decreased ALDH activity compared to control (scrambled siRNA) in a time-dependent manner (Figure 9 and Supplementary Figure 6). However, there was no change in cell morphology with IGFBP-6 silencing (Figure 10(a)) when compared to the control (Figure 10(b)) at day 7 postdifferentiation. On the other hand, IGFBP-6 knockdown decreased IGFBP-6 production and secretion as IGFBP-6 levels were reduced in PMSC-conditioned media at all time points as measured by ELISA (Figure 10(c)).

4. Discussion

Stem cell research has progressed in recent years, and the promise of using stem cells in tissue regeneration and cellular therapies are closer to becoming a reality in the clinics [27, 28]. However, before they can be used reliably and safely in regenerative medicine, it is essential to understand how factors within the stem cell microenvironment influence lineage commitment and differentiation as stem cell fate is altered by the culture conditions *in vitro* [29]. In addition,

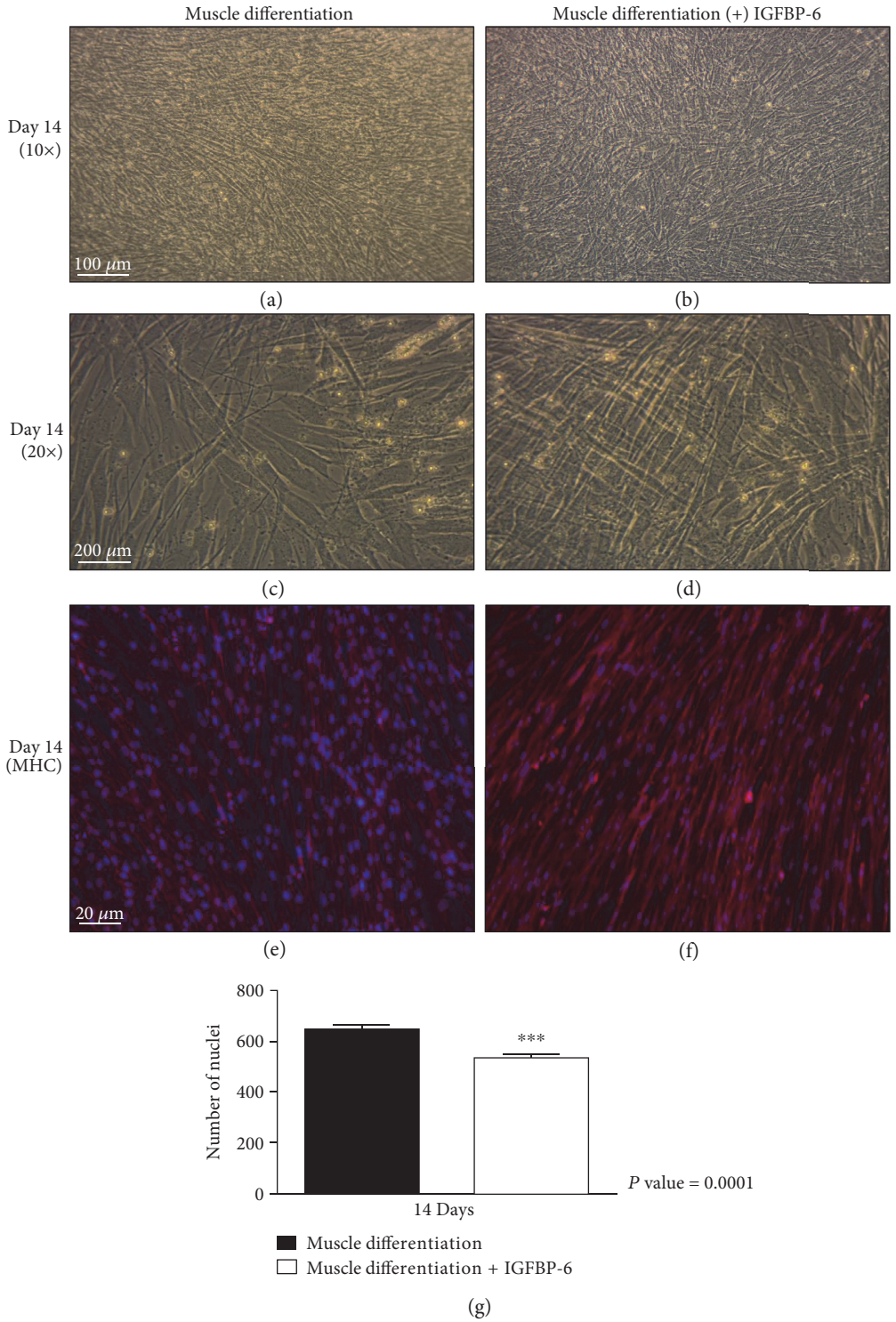
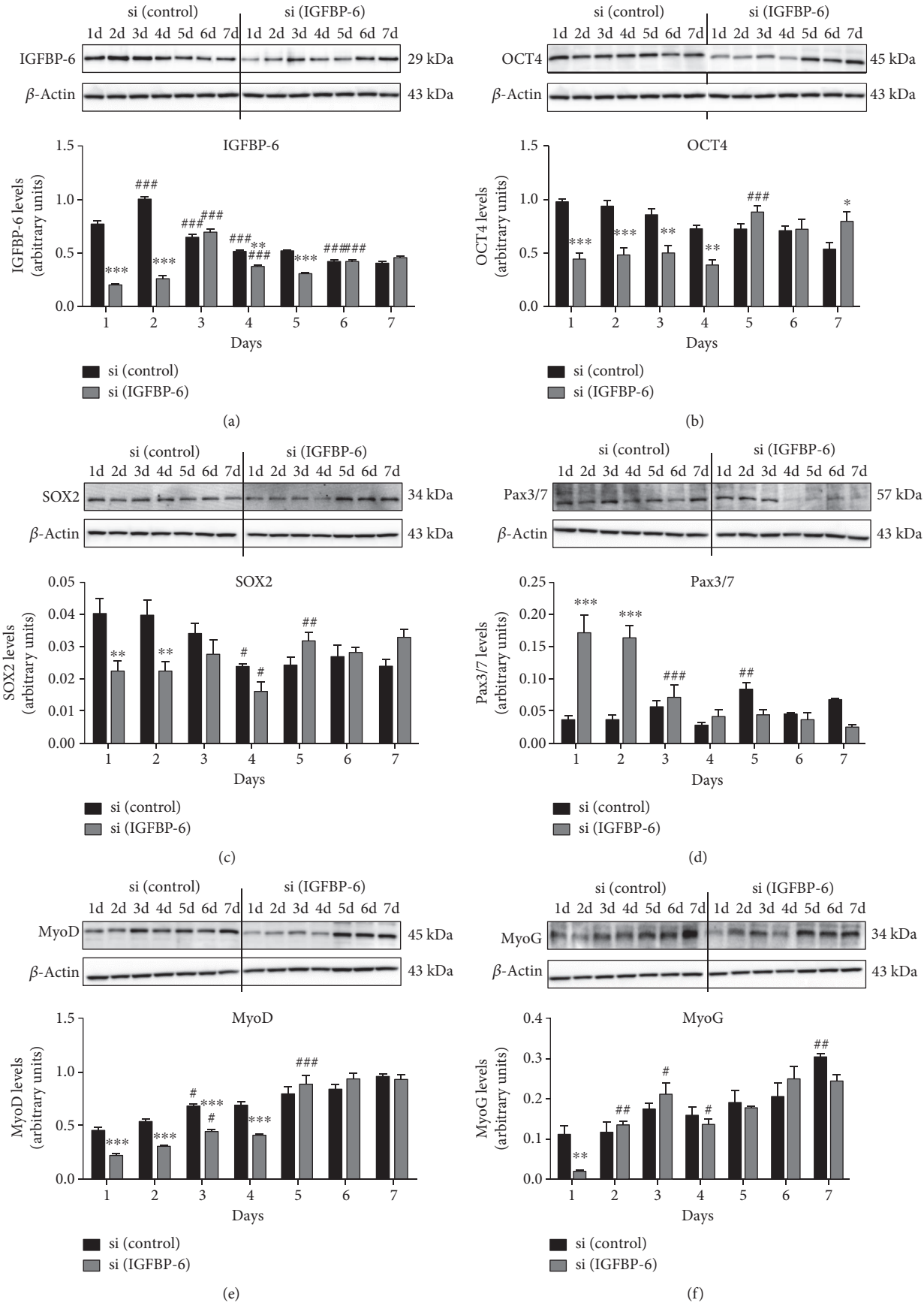


Figure 7: PMSCs under muscle differentiation conditions treated with IGFBP-6 showed more muscle compaction with lower cell count and similar immunofluorescence for MHC compared to cells under muscle differentiation alone at 14 days. Compared to PMSCs cultured under muscle differentiation alone, PMSCs treated with IGFBP-6 showed high cell compaction at day 14 (10x) (a, b) and (c, d) 20x. (e, f) Using immunocytochemistry, PMSCs were immunoreactive for MHC (red-Alexa, λ-568 nm), with no change in immunoreactivity with IGFBP-6 treatment. Nuclei were stained with Hoechst dye (blue, $\lambda = 340$ nm). Images are representative of 3 technical replicates. (g) PMSCs treated with IGFBP-6 had lower cell count at 14 days postdifferentiation compared to control PMSCs. Data is presented as the mean ± SEM of 15 different fields from 3 independent experiments. One-way ANOVA followed by Student's t-test, $^{***}P < 0.0001$.



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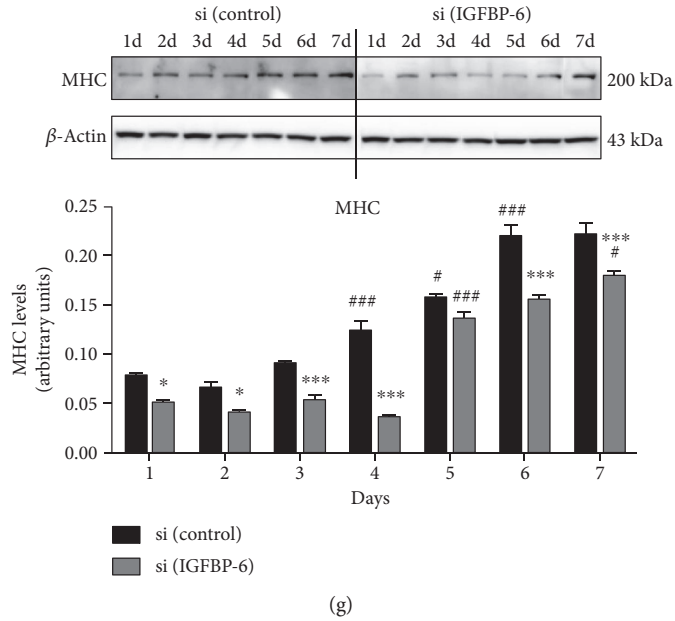

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Figure 8: IGFBP-6 knockdown in PMSCs under muscle differentiation conditions reduced muscle markers protein levels. PMSCs were treated with siRNAs against IGFBP-6 or scrambled siRNA control every 3 days in muscle differentiation media. Using Western blots, (a) IGFBP-6 levels were significantly decreased at 1 and 2 days after siRNA treatment but recovered to control levels at day 3. When IGFBP-6 siRNA was reintroduced at day 3, there was a significant decrease for up to 5 days which returned to control levels at day 6. IGFBP-6 siRNA treatment decreased (b) OCT4 and (c) SOX2 levels at the early time points then returned to control levels at day 5. In contrast, muscle cell commitment marker (d) Pax3/7 was increased at days 1 and 2 when IGFBP-6 was knocked down. Muscle differentiation markers: (e) MyoD, (f) MyoG, and (g) MHC levels were decreased at early time points but recovered to control levels by day 5. Protein levels were quantified by densitometry and normalized to β-actin. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ comparing siRNA treatments, or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ comparing the same treatment over time.

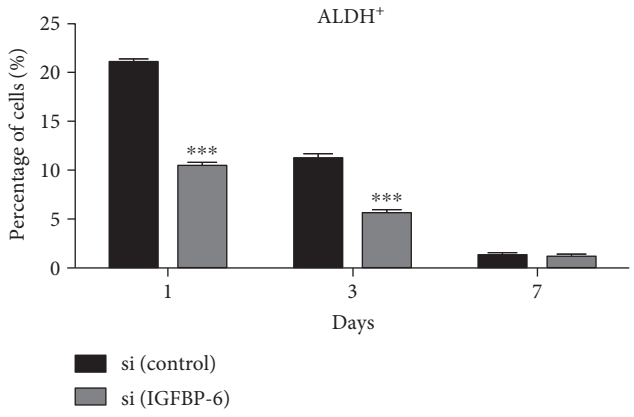

Figure 9: IGFBP-6 siRNA in PMSCs cultured under muscle differentiation conditions decreased the frequency of cells with high ALDH activity. PMSCs treated with IGFBP-6 siRNA showed significantly reduced frequency of cells with high ALDH activity. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{***}P < 0.001$.

most current cellular therapies are expected to utilize pluripotent or multipotent stem cells that are already poised to generate into a desired lineage of committed progenitor cells by culturing them under specific culture conditions prior to therapy. Congenital muscular dystrophies represent a potential genetic disorder that may be amenable to cellular therapies due to accessibility and possible incorporation of new functional skeletal muscle cells into diseased tissues after transplantation [30, 31]. The results from this study are the first to provide insight on how IGFBP-6 can be used to modulate muscle lineage commitment and differentiation from readily available PMSCs *in vitro*.

To be able to use stem cells to treat Duchenne muscular dystrophy and to be approved for clinical trials, cells need to be from a readily available source, maintain the muscle differentiated state, avoid immune rejection by the host, avoid tumorigenesis, and can be easily injected. Human placental mesenchymal stem cells achieve these criteria.

The human placenta is usually discarded tissue after birth and represents a rich source of adult mesenchymal stem cells for the development of tissue regeneration therapies [2, 3, 32]. PMSCs have greater cell expansion and passage number *in vitro* than mesenchymal stem cells isolated from bone marrow [1]. They also demonstrate lower tumorigenicity [33] and higher immunotolerance capacity to reduce the possibility of triggering an immune response [34]. Thus, placental stem cells could provide an ethical and readily available source of stem cells for future experimental and clinical applications.

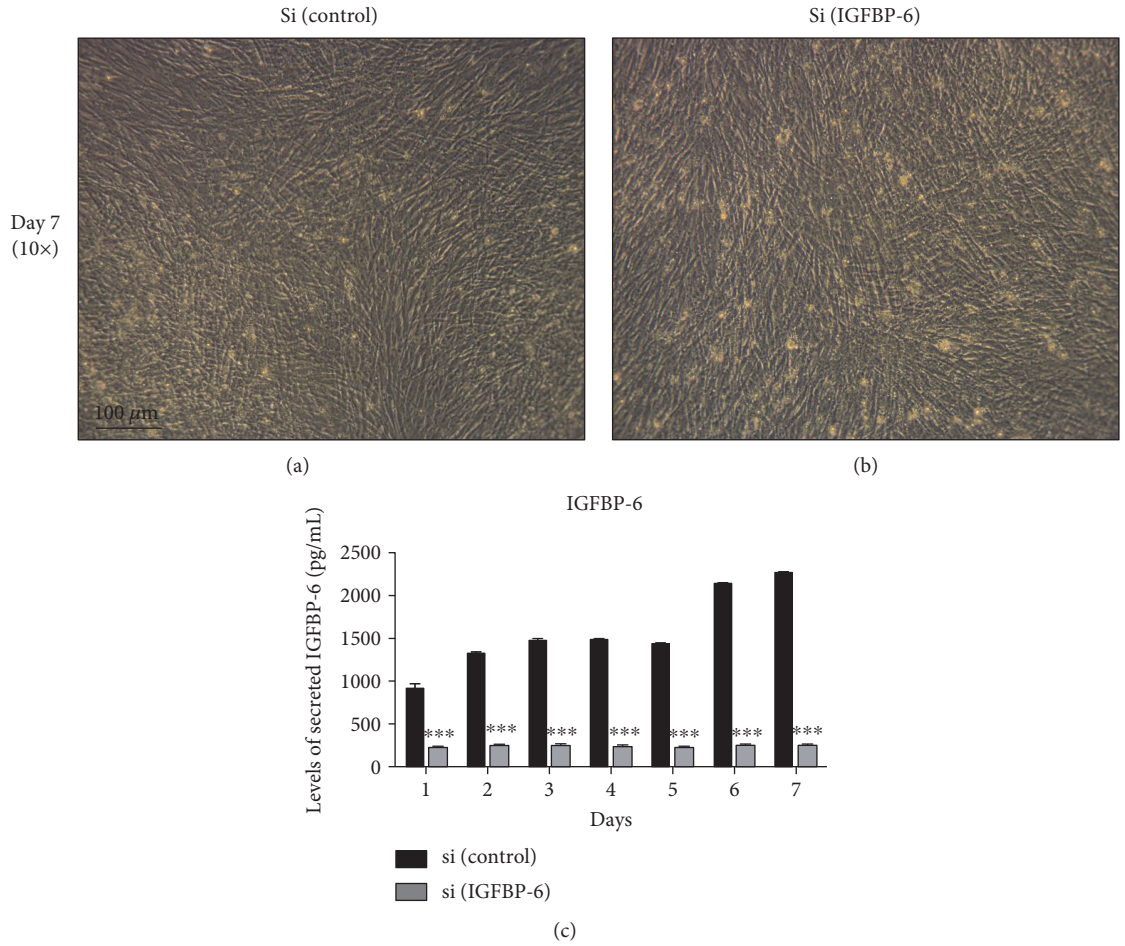

Figure 10: IGFBP-6 siRNA treatment maintained PMSCs cell morphology and inhibited IGFBP-6 secretion. PMSC skeletal muscle morphology was maintained for 7 days under muscle lineage differentiation conditions with (a) scrambled siRNA or (b) IGFBP-6 siRNA treatment. (c) Using ELISA, IGFBP-6 secretion was decreased at each time point with IGFBP-6 siRNA treatment that was applied every 3 days. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{***}P < 0.001$. ELISA sensitivity: highest amount detectable 60,000 pg/mL; lowest amount detectable 82.3 pg/mL. Standard curve $R^2 = 0.97$.

The IGF family plays a central role in muscle development, differentiation, growth, and regeneration [20–22, 35, 36]. In Duchenne muscular dystrophy, IGF-1 activates muscle growth and hypertrophy and appears to improve the loss of muscle mass [37]. IGFBPs are the carriers for IGFs in the circulation [7], protecting them from degradation [12, 38] and delivering them to specific tissues and thus modulate the biological actions of IGFs. Also, IGFBPs increase the half-life of IGF peptides in the circulation and control their access to the IGF-1R, thus playing an important role in IGF-regulated cell metabolism, development, and growth. In recent years, it has become apparent that the IGFBPs can be expressed and maintained within the cellular environment and have functions independent of IGFs [14]. Several IGF binding proteins have been shown to be important in myogenesis and are expressed in developing muscle cells. Ren et al. reported that in C2C12 myoblast cells and in primary skeletal muscle cells, IGFBP-5 acts in an IGF-dependent manner to promote myogenesis by binding to IGF-2 and promoting its interaction with the IGF-1R [39]. Knockdown of IGFBP-5 impaired myogenic differentiation by reducing myogenin, myosin heavy chain, and IGF-2 expression [39]. In L6E9 skeletal myoblasts, IGFBP-4 and IGFBP-6 were accumulated during myogenesis, with IGFBP-4, and not IGFBP6, inhibiting IGF-1 induced muscle differentiation [40]. These findings suggested the important role of IGFBPs in the muscle differentiation of both primary and cell lines of skeletal muscle lineage. Our study is the first to demonstrate the role of IGFBP-6, which is specific for the embryonic IGF, IGF-2, in muscle development using PMSCs.

The aim of this study was to characterize the effects of IGFBP-6 on the early differentiation stage before PMSCs commit to the muscle lineage. As shown, when PMSCs were cultured under muscle differentiation-specific conditions, they showed the capacity to differentiate into multinucleated muscle fibers and commit to the muscle lineage. The biological effects of IGFBP-6 on this differentiation process, as determined by pluripotency-associated markers (OCT4 and SOX2), muscle commitment (Pax3/7), and differentiation (MyoD, MyoG, and MHC), were significantly changed at

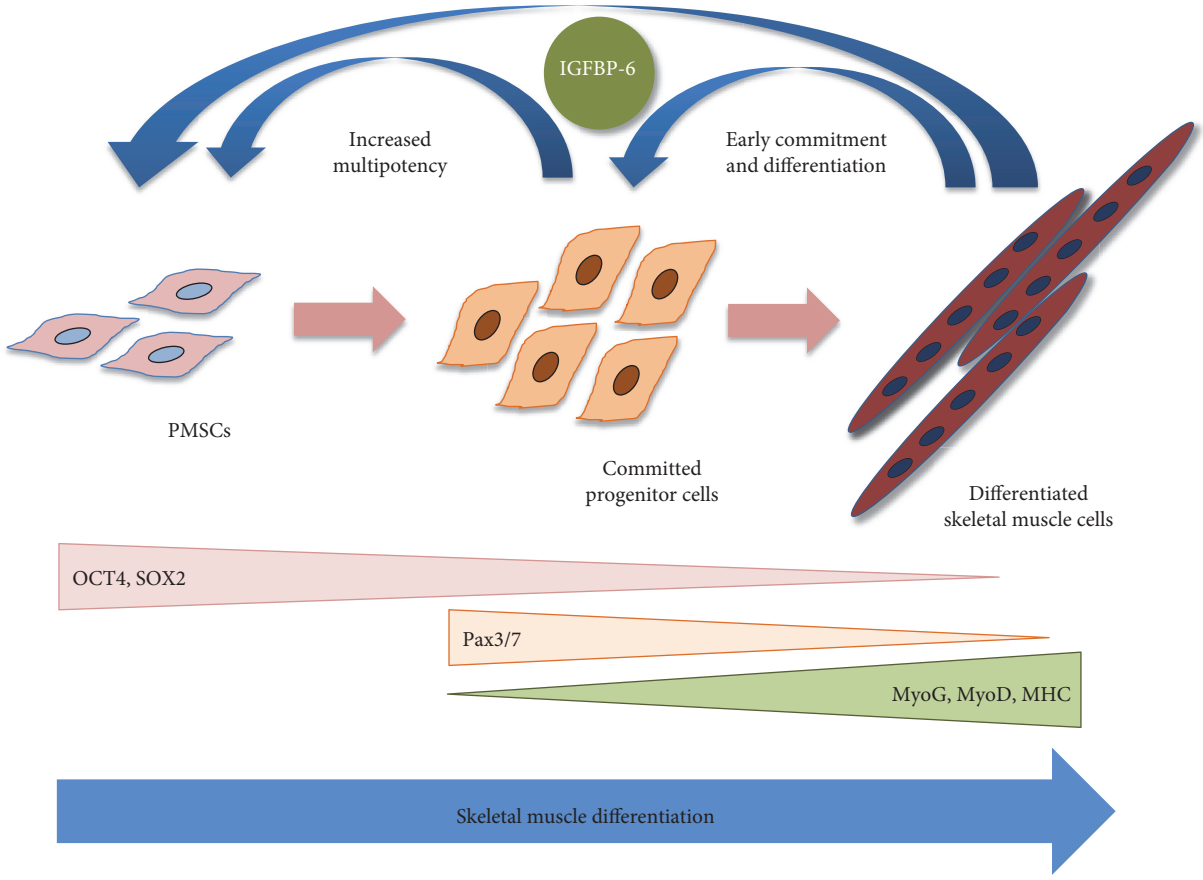

Figure 11: Model of IGFBP-6 functions on PMSC differentiation into skeletal muscle. PMSCs under normal growth conditions (10% FBS) express high levels of pluripotency-associated markers OCT4 and SOX2. As these cells commit towards the skeletal muscle lineage, increased IGFBP-6 correlated with increased Pax3/7 that decreased as differentiation markers (MyoG, MyoD, and MHC) were increased. Both committed and differentiated muscle cells continued to express and secrete IGFBP-6. As IGFBP-6 increased, there was an increase in multipotency markers, as well as, an earlier commitment and differentiation towards the muscle lineage. Thus, IGFBP-6 was required for maintaining multipotency and enhancing muscle commitment and differentiation.

the earlier time points. Thus, IGFBP-6 induced muscle differentiation and could potentially be used to guide skeletal muscle regeneration using stem cell therapy.

IGFBP-6 was highly expressed in developing muscle cells [41, 42]; however, its role in muscle development is unclear. Previous studies from our laboratory using human fetal tissues have demonstrated that IGFBP-6 mRNA was expressed abundantly in the skeletal muscle, heart, and skin and prevalent in the regions of active cellular division and differentiation, suggesting that the protein is synthesized in these tissues and has autocrine/paracrine actions in the developing cells [43]. In another study from our laboratory, we reported that IGFBP-6 mRNA was expressed in low abundance in the chorionic villi of placenta during the second and third trimesters [44], suggesting that this IGFBP-6 is expressed in specific population of cells in this tissue (e.g., mesenchymal stem cells) and/or that the expression is increased only when PMSCs are induced to differentiate into a specific lineage such as skeletal muscle.

The findings in this current study using PMSCs suggest that stem cells in the developing myotome or MSCs in a developed muscle tissue express IGFBP-6 in significant levels during differentiation, indicating IGFBP-6 as an integral protein during muscle development. In fact, as muscle differentiation progressed *in vitro*, the intracellular IGFBP-6 decreased gradually due to the increased capacity to secrete IGFBP-6 into the culture medium, indicating multiple roles for IGFBP-6, both intracellular and extracellular in muscle development. Thus, IGFBP-6 activities may switch from intracellular IGF-independent actions to more paracrine IGF-dependent or IGF-independent actions as muscle differentiation occurs. Interestingly, the increase in extracellular IGFBP-6 by the addition of IGFBP-6 to the culture medium significantly increased cellular IGFBP-6 (intracellular or cell associated) with a concurrent increase in pluripotency-associated markers OCT4 and SOX2. The increase in intracellular IGFBP-6 suggests that IGFBP-6 was likely internalized or associated with the cell surface. A previous report from our laboratory demonstrated the intracellular actions of IGFBP-6 in the cytoplasm and nucleus of skeletal muscle cell line RD cells which is likely an IGF-independent actions of IGFBP-6 [19].

When extracellular IGFBP-6 was supplemented into PMSC cultured under muscle differentiation conditions, the muscle commitment marker Pax3/7 was increased at all time

points of study, while other muscle differentiation markers increased only at the earlier time points. As the differentiation progressed, IGFBP-6 treatment inhibited complete myogenic differentiation as demonstrated by decreased muscle differentiation markers MyoD, MyoG, and MHC. These findings together with the higher OCT4 and SOX2 levels indicate that IGFBP-6 promotes the commitment of PMSCs towards the muscle lineage, while the prolonged presence delays the differentiation process. Moreover, increased IGFBP-6 in the MSC microenvironment is expected to reduce the bioavailability of IGF-2 due to its high affinity for the peptide, confirmed by IGF-2 ELISA (Supplementary Figure 7A). Thus, it is likely that the increased IGF-2 secretion by the differentiating muscle cells will have a biologic impact on muscle development which will be further investigated.

Knockdown of IGFBP-6 using siRNA decreased both intracellular and secreted IGFBP-6. This knockdown-mediated decrease in OCT4 and SOX2 supports the evidence that IGFBP-6 enhances pluripotency of PMSCs. In contrast, the significant early increase in Pax3/7 with IGFBP-6 silencing supports an earlier commitment towards the myogenic lineage. The increase in Pax3/7 could be due to the presence of a greater availability of extracellular IGF-2 (Supplementary Figure 7B), which is being recruited to the commitment process or could be due to actions independent of IGFBP-6. In contrast, the muscle differentiation markers (MyoD, MyoG, and MHC) were all reduced after IGFBP-6 knockdown, suggesting that IGFBP-6 is required for the muscle differentiation process. Therefore, IGFBP-6 supports PMSC multipotency and its loss leads to an early commitment towards the myogenic lineage but delayed differentiation.

Studies in various cell lines have shown mostly an inhibitory action of IGFBP-6 mainly via IGF-2-dependent actions. In L6A1 myoblast, IGFBP-6 inhibited muscle differentiation induced by IGF-2 but not IGF-1 [45]. Previous reports on the effects of IGFs on muscle differentiation were using mouse cell lines [46–49]; thus, our study is one of the first to show the effects of IGFBP-6 on human mesenchymal stem cell differentiation into skeletal muscle *in vitro*.

Overall, we have demonstrated in this study that IGFBP-6 has both endogenous and exogenous actions that can promote or inhibit PMSC multipotency or differentiation. Exogenous IGFBP-6 exposure facilitates muscle lineage commitment while a prolonged exposure can inhibit late stage differentiation. Therefore, endogenous IGFBP-6 is required for maintaining multipotency and delaying commitment and enhancing late stage differentiation.

In conclusion, PMSCs are able to differentiate into skeletal muscle cells under appropriate environment or niche conditions and that this process is enhanced by the increase in extracellular IGFBP-6 and delayed by silencing the endogenous expression as evident by alterations in both pluripotent and muscle differentiation markers (Figure 11). A balance between endogenous and exogenous levels of IGFBP-6 is required for the complete muscle differentiation process, and since IGFBP-6 has intracellular as well as extracellular effects, whether the response occur dependent or independent of IGFs (particularly IGF-2) will be further delineated.

Disclosure

Some data in this paper has been presented as a poster at the Endocrine Society's 96th Annual Meeting and Expo. Data was also submitted as part of a PhD thesis [50].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by CIHR Grant 111024.

Supplementary Materials

Supplementary Figure 1: PMSC Isolation from 15 weeks preterm placenta. (A) The dissected villous tissue was digested enzymatically and cells were separated using a discontinuous Percoll gradient. Five cell fractions were typically obtained corresponding to five different densities and cells were isolated from layers 3, 4, and 5. (B) Phase contrast images of the isolated PMSCs, from all three layers, grown in culture after 4 weeks. (C) PMSCs from passage 4 of all three layers were positive for CD73 and CD105 (>98%), and were negative for CD117 (<1%) (measured by flow cytometry). Flow cytometry histograms are representative of all 3 layers from 3 placental tissue as they showed the same results. Supplementary Figure 2: IGFBP-6 levels in response to IGFBP-6 supplementation in PMSCs under skeletal muscle differentiation conditions. (A) PMSCs cultured under muscle differentiation conditions showed increased IGFBP-6 protein levels, using western blots, in response to different doses of recombinant human IGFBP-6 protein supplementation with 375 ng/mL and 450 ng/mL having the highest band intensity. (B) IGFBP-6 secretion into the media was increased with the supplementation of recombinant human IGFBP-6 protein (375 ng/mL) that reduced by time and was lower compared to control at day 3. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{***}P < 0.001$. Supplementary Figure 3: PMSCs cultured under muscle differentiation conditions showed the formation of multi-nucleated fibers and lower cell count compared to control. (A) At 14 days post-differentiation, PMSCs are immunoreactive for MHC (Red-Alexa 568, λ-568 nm) with cell alignment and multi-nucleated fiber formation (5X). Nuclei, were stained with Hoechst dye (blue, $\lambda = 340$ nm). (B) PMSCs grown in muscle differentiation media showed multi-nucleated skeletal muscle fiber formation (40X). Black arrows indicate the multi-nucleated muscle. (C) PMSCs under muscle differentiation conditions showed lower cell count per field compared to control. Data is presented as the mean ± SEM of 15 different fields from 3 independent experiments. One-way ANOVA followed by a Student's t-test, $^{**}P < 0.01$. Supplementary Figure 4: PMSCs cultured under skeletal muscle differentiation conditions showed a decreased frequency of cells with high ALDH-activity. Representative flow cytometry dot plots showing the frequency of

PMSC with high ALDH-activity with Aldefluor and an inhibitor of ALDH (DEAB) or with ALDH alone when cultured under control (10% FBS) or muscle differentiation conditions at (A) day 1, (B) day 3, (C) day 7, (D) and day 14. Supplementary Figure 5: IGFBP-6 treatment increased the frequency of PMSCs with high ALDH-activity. Representative flow cytometry dot plots with Aldefluor and an inhibitor (DEAB) or with ALDH alone in PMSCs cultured under muscle differentiation conditions with or without IGFBP-6 addition at (A) day 1, (B) day 3, (C) day 7, (D) and day 14. Supplementary Figure 6: IGFBP-6 siRNA in PMSCs cultured under muscle differentiation conditions decreased the frequency of cells with high ALDH-activity. Representative flow cytometry dot plots with Aldefluor and an inhibitor of ALDH (DEAB) or with ALDH alone of PMSCs treated with IGFBP-6 siRNA at (A) day 1, (B) day 3, and (C) day 7 under muscle differentiation conditions. Supplementary Figure 7: IGF-2 secretion in PMSCs treated with IGFBP-6 or IGFBP-6 siRNA under muscle differentiation conditions. (A) IGF-2 levels secreted into the media were significantly decreased at each time point after IGFBP-6 addition compared the control. (B) After treatment with siRNA against IGFBP-6 compared to controls (scrambled siRNA), IGF-2 levels increased at the first 48 hours with siRNA treatment applied every 3 days. Data is presented as the mean ± SEM of 3 independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test was performed to determine $^{*}P < 0.05$, $^{**}P < 0.001$. (*Supplementary materials*)

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Hindawi
Stem Cells International
Volume 2017, Article ID 4576327, 17 pages
https://doi.org/10.1155/2017/4576327

Research Article

Regulation of Osteogenic Differentiation of Placental-Derived Mesenchymal Stem Cells by Insulin-Like Growth Factors and Low Oxygen Tension

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Received 27 February 2017; Accepted 20 July 2017; Published 12 September 2017

Academic Editor: Sari Pennings

Placental mesenchymal stem cells (PMSCs) are multipotent cells that can differentiate *in vitro* to multiple lineages, including bone. Insulin-like growth factors (IGFs, IGF-1 and IGF-2) participate in maintaining growth, survival, and differentiation of many stem cells, including osteoprogenitors. Low oxygen tension (PO_2) can maintain stem cell multipotency and impede osteogenic differentiation. In this study, we investigated whether PMSC osteogenic differentiation is influenced by low PO_2 and by IGFs. Our results indicated that low PO_2 decreased osteogenic markers RUNX2 and OPN; however, re-exposure to higher oxygen tension (room air) restored differentiation. IGFs, especially IGF-1, triggered an earlier expression of RUNX2 and enhanced OPN and mineralization. RUNX2 was phosphorylated in room air and augmented by IGFs. IGF-1 receptor (IGF-1R) was increased in low PO_2 and reduced by IGFs, while insulin receptor (IR) was increased in differentiating PMSCs and enhanced by IGF-1. Low PO_2 and IGFs maintained higher IR-A which was switched to IR-B in room air. PI3K/AKT was required for osteogenic differentiation, while MEK/ERK was required to repress an RUNX2 and OPN increase in low PO_2. Therefore, IGFs, specifically IGF-1, trigger the earlier onset of osteogenic differentiation in room air, whereas, reversibly, low PO_2 impedes complete differentiation by maintaining higher multipotency and lower differentiation markers.

1. Introduction

Mesenchymal stem cells (MSCs), found in many adult tissues, are responsible for tissue repair and regeneration after injury or disease [1]. Unlike embryonic stem cells (ESCs), MSCs are less tumorigenic and have a more restricted mesendodermal lineage-specific differentiation towards myocytes, osteoblasts, chondrocytes, adipocytes, stromal fibroblasts, and endothelial cells [1–4]. Also, MSCs can modulate the immune response and have been used successfully in graft-versus-host disease-resistant patients [5]. Therefore, MSCs are promising candidates for stem cell-based therapies to treat many adult and paediatric diseases, such as sickle cell disease [6], rheumatic diseases [7], lymphoma [8], and heart failure [9]. In bone, MSC transplantation has been used to correct bone malformation and injury. In children, osteogenesis imperfecta (OI) is a severe genetic disorder of mesenchymal cells with a deficit for type I collagen which is important for matrix deposition and mineralization [10]. There is no treatment for OI; however, an allogeneic bone marrow transplantation has been shown to successfully accelerate linear growth and increase total body bone mineral density in OI children [10].

Although bone marrow MSCs were used for stem cell therapy [11, 12], placental MSCs (PMSCs) are abundant and readily available and do not require invasive techniques

for isolation [13]. PMSCs are isolated from different compartments of the placenta (the amnion, the chorion, and the fetal membranes) and have a higher proliferation capacity than bone marrow MSCs [13]. MSCs are dependent on their surrounding microenvironment for maintaining stem cell identity [14, 15], and they differentiate by specific promoting factors via a tight transcriptional network signaling loss of multipotency and initiating linage-specific progenitor differentiation. In osteoblast differentiation, runt-related transcription factor 2 (RUNX2) is the initiation transcription factor that upregulates transcription of genes required for bone matrix deposition and mineralization including osteopontin (OPN), osteocalcin (OCN), type I collagen, and alkaline phosphatase [16–19]. RUNX2 is phosphorylated by the mitogen-activated protein kinase (MAPK) at the C-terminal proline-serine-threonine (PST) region, which is required for its transcriptional activity and DNA binding to promoter regions of osteogenic genes, such as OPN and OCN [20, 21].

Stem cell differentiation condition is controlled by soluble factors, small molecules, hormones, and growth factors [15]. The insulin-like growth factors (IGFs, IGF-1 and IGF-2) can promote and stimulate stem cell differentiation towards several lineages from all three germ layers [22–26], including osteoblast differentiation [27] and *in vivo* bone development [28, 29]. IGF-1 and IGF-2 and their receptor IGF-1R have a strong association with osteogenesis and are abundantly expressed specifically in mature osteoblasts and osteoclasts in autocrine/endocrine mechanism [27]. IGF-1- and IGF-1R-null mice show underdeveloped short bone, low bone mineral density, and delayed calcification, whereas IGF-2-null mice show no major skeletal defects [28]. Therefore, IGF-1 and IGF-2 may have distinct functions in osteogenic differentiation.

In vivo, hypoxia-inducible fator-1α- (HIF1α-) knockout mice have decreased trabecular bone volume, reduced bone formation rate, and reduced proliferation of osteoblasts during long bone development [30]. In contrast, an overexpression of HIF1α in osteoblasts leads to the development of extremely dense and heavily vascularized long bones [31]. Therefore, low oxygen tension, which stabilizes HIF1α, is required for gene regulation required for healthy bone development. *In vitro*, low oxygen tension promotes stem cell proliferation, self-renewal, and multipotency [32] but inhibits osteoblast differentiation [33]. However, reexposure to room air restores complete differentiation and may potentiate osteogenic differentiation [34, 35]. Hence, long-term exposure to low oxygen tension is inhibitory to stem cell differentiation, whereas a short-term exposure can play a role in directing stem cell fate towards a more robust osteogenic differentiation [34].

IGFs and low oxygen tension are natural niche components of osteogenic microenvironment, which are shown to affect later stages of osteogenic differentiation during the mineralization period of MSC differentiation [36, 37]. However, the combined effect of these two microenvironmental factors on the commitment and early differentiation stages is not clear. In this study, we used preterm PMSCs to study the role of IGF-1 and IGF-2 signaling in combination with low oxygen tension in osteogenic differentiation. We show that low oxygen tension inhibits PMSC osteogenic differentiation, and IGF-1 more than IGF-2 enhances differentiation via specific signaling pathways mediated via IGF-1R/IR, PI3K, MEK1/2, and RUNX2 phosphorylation.

2. Material and Methods

2.1. PMSC Isolation. PMSCs were isolated from early gestation (10–13 weeks) human placentae. After informed consent was taken, placentae were collected from patients who underwent therapeutic pregnancy termination. Immediately after surgery, placentae were dissected under sterile conditions and small pieces of chorionic villi were collected. Tissue samples were minced mechanically and subjected to a process consisting of two steps of enzymatic digestion with (1) collagenase IV/hyaluronidase and (2) DNase I followed by (3) trypsin/EDTA. Each enzymatic step was performed for 20 min at 37°C, followed by 10 min wash at 4°C in a solution of PBS supplemented with 10% fetal bovine serum (FBS) (Gibco, Mississauga, ON). Cells released during digestion were passed through a tissue mesh (45 μm) to obtain a single cell suspension. Next, cells were separated on a Percoll (Sigma) discontinuous gradient according to a modified protocol by Worton et al. for hematopoietic stem cell isolation [38] and then seeded in DMEM/F12 media (Gibco, Mississauga, ON) supplemented with 10% FBS and antibiotic-antimycotic solution. After 4 days, media was changed and nonadherent cells were washed with media to leave behind adherent PMSCs forming colonies. PMSC colonies were characterized by flow cytometry for presence/absence of cell surface markers CD90, CD73, CD105, and CD117/c-kit as published previously [39].

2.2. Osteogenic Differentiation and Incubation in Low Oxygen Tension. Cells were cultured and maintained using DMEM/F12 media supplemented with 10% ES-FBS and FGF-2 (100 ng/mL) (Gibco, Mississauga, ON). Before treatments, cells were cultured in DMEM/F12 supplemented with 10% FBS only. Upon treatment, PMSCs were plated at 70% confluency in nondifferentiation conditions (15% FBS/DMEMF12) or in the presence of osteogenic stimulatory conditions (15% osteogenic differentiation FBS, 10^{-8} M dexamethasone, 50 μg/mL ascorbic acid, and 3.5 mM β-glycerophosphate) (STEMCELL Technologies, Vancouver, BC). For IGF-1 or IGF-2 treatments, 100 ng/mL of either IGF was added to a reduced FBS level of basic 2% osteogenic differentiation media; IGFs were added fresh at every media change. The relative effect of different IGF and oxygen tension treatments on calcium deposits was compared using alizarin red and alkaline phosphatase staining. The signaling of the MEK/ERK pathway or the PI3K/AKT pathways was inhibited by the continuous presence of U0126 (5 μM) or LY294002 (10 μM), respectively. Cell cultures were then placed in either a 5% CO_2 incubator or a hypoxia chamber, which was filled with a mix of 1% O_2, 5% CO_2, and balanced N_2 (BOC Canada Ltd., Toronto, ON) for 15 min to ensure saturation using a Hudson 5590 Oxygen Monitor (Hudson, Ventronics Division, Temecula, CA).

Thereafter, the chamber was placed in a tissue culture incubator at 37°C.

2.3. Alizarin Red Staining and Quantification. To assess these morphological changes in differentiated versus nondifferentiated cells, cells were cultured for 14 days and then fixed with 4% formaldehyde for 30 min at RT. Then, cells were stained either with 1% alizarin red solution for 10 min at RT or with NBT/BCIP reagent (Vector Labs, Burlington, ON) as per the manufacturer's protocol. Both stainings were then solubilized with 10% cetylpyridinium chloride in 10 mM sodium phosphate buffer (pH 7.0) as previously described [27]. The absorbance of 200 μL solubilized staining was read at $\lambda = 570$ nm using a plate reader. The absorbance was then normalized to total protein content per well in micrograms.

2.4. RT-PCR for Insulin Receptor Isoforms in PMSCs. Total RNA was extracted from differentiated and nondifferentiated PMSCs using the PureLink RNA Mini Kit (Ambion, Burlington, ON) as per the manufacturer's protocol. 1–3 μg total RNA was reverse-transcribed using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Burlington, ON) and oligo $(dT)_{20}$ primers. End-point PCR reactions were run in 25 μL using the Eppendorf 96-well plate thermocycler. Human *IR* isoforms in the same cDNA sample were detected using primers amplifying exon 11 including *IR-B* (250 bp) or excluding *IR-A* (214 bp) and amplified as published previously [40]. Human *RPL13a* levels were used as the reference endogenous control for normalization of the target mRNAs. Amplification conditions were run at 92°C for 5 min followed by 30 cycles of 92°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec.

2.5. Immunoblotting. To detect protein level changes, 10–20 μg each of cell lysate samples was resolved by SDS-PAGE and then transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 5% bovine serum albumin or 5% nonfat dry milk in 1x TBS (Tris-buffered saline) for 1 hr at room temperature. Blots were then washed in 1x TBS 0.1% Tween 20 (TBS-T) (3× for 5 min) followed by incubation at 4°C overnight with primary antibodies as per the manufacturer's protocols. Blots then were washed using TBS-T (3× for 10 min) and were incubated with the corresponding secondary HRP-labelled antibody for 1 hr at RT. Immunocomplexes were detected by ECL and documented using VersaDOC™ Imaging System (Bio-Rad).

2.6. Antibodies. In this study, the following antibodies were used to detect the IGF system: phospho-p44/42 MAPK (#4377), p44/42 MAPK (#9102), phospho-AKT (Ser473, #4051), and AKT (#9272) (Cell Signaling Technologies, Burlington, ON) and IGF-1Rα (N-20, sc-712) and IR-α (N-20, sc-710) (Santa Cruz Biotech., Santa Cruz, CA). For multipotency markers, we used OCT3/4 antibody (N-19, sc-8628) (Santa Cruz Biotech., Santa Cruz, CA) and SOX-2 (2683-1) (Epitomics, Burlington, ON). For the osteogenic differentiation markers, we used RUNX2 (#8486) (Cell Signaling Technologies, Burlington, ON), phospho-RUNX2 (PA5-12988) (Thermo Fisher Scientific, Burlington, ON), and OPN (K-20, sc-1059) (Santa Cruz Biotech., Santa Cruz, CA). For the loading control, we used pan-Actin Ab-5 (#MS-1295) (Thermo Fisher Scientific, Fremont, CA). The secondary antibodies used for immunoblotting were goat anti-rabbit (#170-6515), anti-mouse (#170-6516) HRP-conjugated antibodies (Bio-Rad Laboratories, Hercules, CA), or donkey anti-goat antibody (sc-2020) (Santa Cruz Biotech., Santa Cruz, CA).

2.7. Statistical Analysis. All experiments were run in triplicates from three independent experiments each; whenever possible, three or more PMSC primary lines were used from preterm placentae. All graphs and analyses were generated using GraphPad Prism Software 5.0 (GraphPad Software, San Diego, CA). A two-way ANOVA with Bonferroni post hoc test was used for the PMSC WST1 proliferation assay and densitometry quantifications. Data are expressed as mean ± standard error of the mean (SEM); values were considered significant when $P < 0.05$.

3. Results

3.1. Effect of Low Oxygen Tension on PMSC Osteogenic Differentiation. In differentiation conditions, PMSCs had greater morphological changes over 14 days in room air by alizarin red staining (Figures 1(a) and 1(b)). Compared to room air, low oxygen tension stabilized HIF1α and enhanced cell proliferation at day 14 (Figure S1 available online at https://doi.org/10.1155/2017/4576327) but inhibited osteogenic differentiation over the same time period (Figures 1(a) and 1(b)). Based on quantification of staining, PMSCs showed spontaneous differentiation into osteogenic-like cells in room air at day 3 that was inhibited by low oxygen tension (Figure 1(b)). Therefore, low oxygen tension prevented spontaneous and osteogenic medium-derived differentiation of PMSCs.

To monitor PMSC multipotency and differentiation, levels of pluripotency-associated proteins OCT4 and SOX2 and early osteogenic commitment transcription factor RUNX2 and the later marker OPN were measured at day 3, 7, and 14 (Figure 1(c)). Although PMSCs were under differentiation conditions, OCT4 and SOX2 levels were consistently higher in low oxygen tension compared to room air (Figure 1(c)). Upon differentiation at day 14, OCT4 levels were slightly increased in room air and decreased in low oxygen tension (Figure 1(d)), whereas SOX2 levels were slightly decreased in room air and low oxygen tension in comparison to nondifferentiation conditions (Figure 1(e)). RUNX2 levels were increased upon differentiation in room air which was lowered by low oxygen tension (Figure 1(f)). While RUNX2 was robustly increased at day 3 in room air, osteogenic differentiation occurred gradually as demonstrated by OPN expression, a late marker of differentiation and matrix formation (Figure 1(g)). Again, low oxygen tension inhibited an increase in OPN levels. Therefore, low oxygen tension reduced osteogenic differentiation by maintaining higher multipotency (higher OCT4 and SOX2) and lowering early commitment (lower RUNX2) and later differentiation (lower OPN) towards the osteogenic lineage.

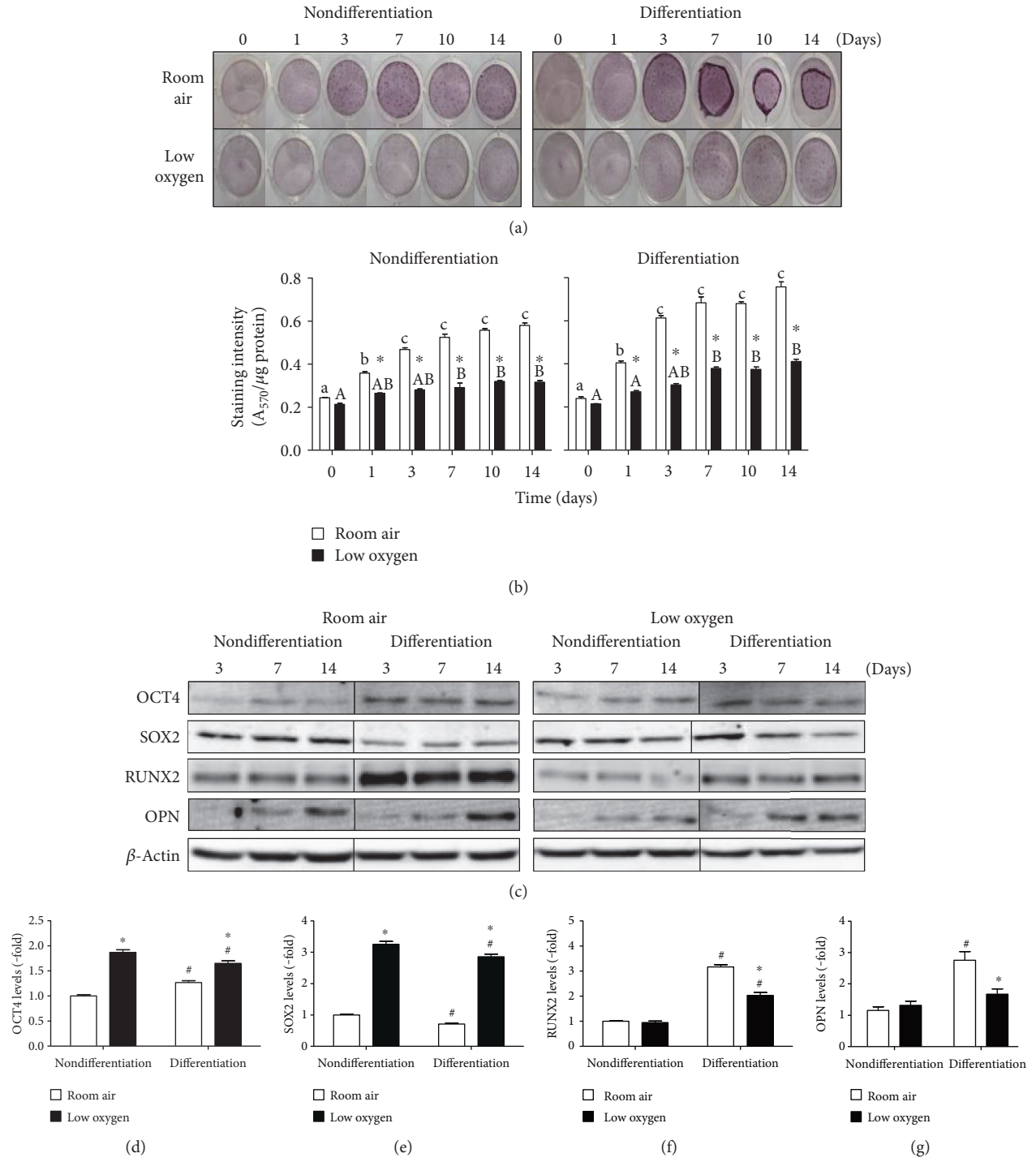


Figure 1: PMSC differentiation under low oxygen tension and its effect on osteogenic differentiation and multipotency. PMSCs were cultured for 14 days in nondifferentiation or osteogenic differentiation conditions containing 15% FBS in room air (20% O_2) or low oxygen levels (1% O_2). Treatments were stopped after 1, 3, 7, 10, and 14 days for alizarin red staining to confirm (a) PMSC differentiation morphology and quantified in (b) (two-way ANOVA, $P < 0.05$, $N = 4$). $*$ indicates significance between room air and low oxygen tension at each time point; lowercase letter (a, b, and c) indicates significance between time points within room air condition, uppercase letter (A, B) indicates within low oxygen tension. (c) Immunoblots showing protein levels of pluripotency-associated and differentiation markers from cell lysates isolated at 3, 7, and 14 days. Quantifications of day 14 samples show protein levels for (d) OCT4, (e) SOX2, (f) RUNX2, and (g) OPN in nondifferentiation and differentiation conditions. Quantification levels shown were normalized to β-actin, a protein loading control (two-way ANOVA, $P < 0.05$, $N = 3$). $*$ indicates significance between room air and low oxygen tension; # indicates significance between nondifferentiation and differentiation within the same oxygen tension.

3.2. Reversibility of the Inhibitory Effect by Low Oxygen Tension on Osteogenic Differentiation. We evaluated whether low oxygen tension irreversibly blocks PMSC differentiation by re-exposure to room air following exposure to low oxygen tension. By introducing PMSCs into room air for the last 7 days of differentiation, alizarin red staining increased to similar levels as in room air (Figures 2(a) and 2(b), left). This effect was also confirmed by alkaline phosphatase staining, which was even increased to higher levels than room air (Figures 2(a) and 2(b), right). Moreover, re-exposure to room air decreased OCT4 and SOX2 levels in comparison to low oxygen tension (Figures 2(c) and 2(d) and S2). RUNX2 levels were also robustly increased upon re-exposure to room air (Figures 2(e) and S2). These data demonstrate that low oxygen tension can decrease but does not block osteogenic differentiation of PMSCs.

3.3. Effect of Insulin-Like Growth Factors on PMSC Osteogenic Differentiation in Low Oxygen Tension. The role of IGFs in mediating the differentiation process was investigated by adding a fresh dose of IGF-1 or IGF-2 (100 ng/mL) with every medium change for 14 days under room air or low oxygen tension conditions (Figures 3(a) and 3(b)). In non-differentiation conditions, there was an increase in alizarin red staining indicating an increase in spontaneous differentiation in room air (enhanced at day 7 with IGF-1 and day 14 with IGF-2) (Figure 3(c)) and less in low oxygen tension (only at day 3 with both IGFs with no further increase) (Figure 3(e)). In differentiation conditions, IGFs (IGF-1 more than IGF-2) enhanced osteogenic differentiation morphologically, in both room air and low oxygen tension, as indicated by increased numbers of calcification centers and higher order of cell organization—as shown by intense alizarin red staining (Figures 3(b) and 3(g)). Interestingly, IGF-1 and IGF-2 in room air enhanced staining intensity starting at day 3 and reached maximum by day 7, whereas control (IGF-free conditions) required 14 days to reach the same level (Figure 3(d)). Low oxygen tension reduced PMSC differentiation and inhibited the effects of IGF-1 or IGF-2 (Figure 3(f)).

The effect of IGFs on PMSC multipotency and differentiation markers was also determined using immunoblotting (Figure S3). In room air, IGFs (IGF-2 more than IGF-1) maintained high OCT4 levels at days 3 and 7, which disappeared at day 14 (Figure 4(a), left). In low oxygen tension, IGFs maintained higher levels of OCT4 throughout differentiation (Figure 4(a), right). SOX2 was increased only by IGF-1 at day 3 in room air (Figure 4(b), left). In low oxygen tension, IGF-1 and IGF-2 maintained lower levels of SOX2 during differentiation (Figure 4(b), right). In contrast, RUNX2 was increased by IGF-1 and IGF-2 in the early stages of differentiation (day 3) and was maintained at higher levels only by IGF-1 at day 14 (Figure 4(c), left). Low oxygen tension abolished this IGF-1 effect on RUNX2 (Figure 4(c), right). However, it seems that the IGF-1 effect on increasing RUNX2 levels is delayed by low oxygen tension, in the preconditioning study after the exposure to room air (Figure S2). OPN increased by day 14 in room air in the absence of IGFs (Figure 4(d), left), whereas IGF-1 increased OPN levels at day 7 and further increased at day 14 only in room air (Figure 4(d), left). On the other hand, IGF-2 was opposite to IGF-1 and caused a reduction in OPN levels. Low oxygen tension inhibited any increase in OPN levels and also prevented the IGF-1-mediated increase shown in room air (Figure 4(d), right). These data support that IGF-1 has an important role in osteogenic differentiation, and higher oxygen tension is needed to promote osteogenic differentiation.

3.4. Role of Insulin-Like Growth Factor Receptors in PMSC Osteogenic Differentiation under Low Oxygen Tension. IGF-1 and IGF-2 can signal via the insulin-like growth factor-1 receptor (IGF-1R) or the insulin receptor (IR) to promote proliferation and differentiation. In room air, IGF-1R did not change upon differentiation and was not affected by IGFs, whereas IR was increased in differentiating PMSCs and upregulated by IGF-1 (Figures 6(a), 6(b), and 6(c)). In low oxygen tension, the IGF-1R was increased in differentiation conditions but reduced by both IGF-1 and IGF-2 (Figure 5(b), right), whereas no change in IR was observed in differentiation conditions (Figure 5(c)). IR exists in two isoforms, IR-A and IR-B, which can determine the differentiation status of stem cells. A higher ratio of IR-B : IR-A possibly suggests a more differentiated state towards the osteogenic lineage. In PMSCs, IR-B : IR-A ratio was low in non-differentiated PMSCs (Figure 6(d)). Upon differentiation, the level of IR-B : IR-A increased gradually in room air but not in low oxygen tension (Figure 6(e)). This was caused by the elevated expression of IR-A than of IR-B in low oxygen tension (Figure S4, right). IGF-1 or IGF-2 increased the IR-B : IR-A ratio earlier at day 3 that was reduced at days 7 and 14 in room air, unlike the consistent lower ratio in low oxygen tension (Figures 6(f) and 6(g)).

3.5. Downstream Insulin-Like Growth Factor Signaling Mediates Osteogenic Differentiation. Downstream kinases of IGF receptor signaling, p-ERK1/2 and p-AKT, are major signaling kinases to mediate an IGF effect. In PMSCs, p-ERK1/2 decreased gradually over the differentiation process in room air, while it was maintained higher in low oxygen tension at days 3 and 7 (Figure 5(a)). A significant decrease in ERK1/2 levels was detected at day 14 regardless of oxygen tension. The addition of IGF-1 or IGF-2 caused a further reduction in p-ERK1/2 levels in room air and low oxygen tension (Figure 5(a)). p-AKT was not significantly changed in room air and even in the presence of IGFs (Figure 5(b), left). On the other hand, IGF-1 increased the levels of p-AKT in low oxygen tension during differentiation (Figure 5(b), right). During osteogenic differentiation, RUNX2 is phosphorylated by MAPK (MEK1/2), which can affect its DNA binding and protein-protein interactions. Only in room air, p-RUNX2 levels were elevated at day 14 in the absence of IGFs, whereas the addition of IGF-1 or IGF-2 increased p-RUNX2 levels to ~8 and ~4 folds, respectively (Figure 5(c), left). In low oxygen tension, RUNX2 phosphorylation was not upregulated throughout the differentiation (Figure 5(c), right).

To specify the role of signaling kinases in mediating PMSC differentiation, U0126 and LY294002 were used to inhibit MEK1/2 and PI3K, the upstream kinases of ERK1/2

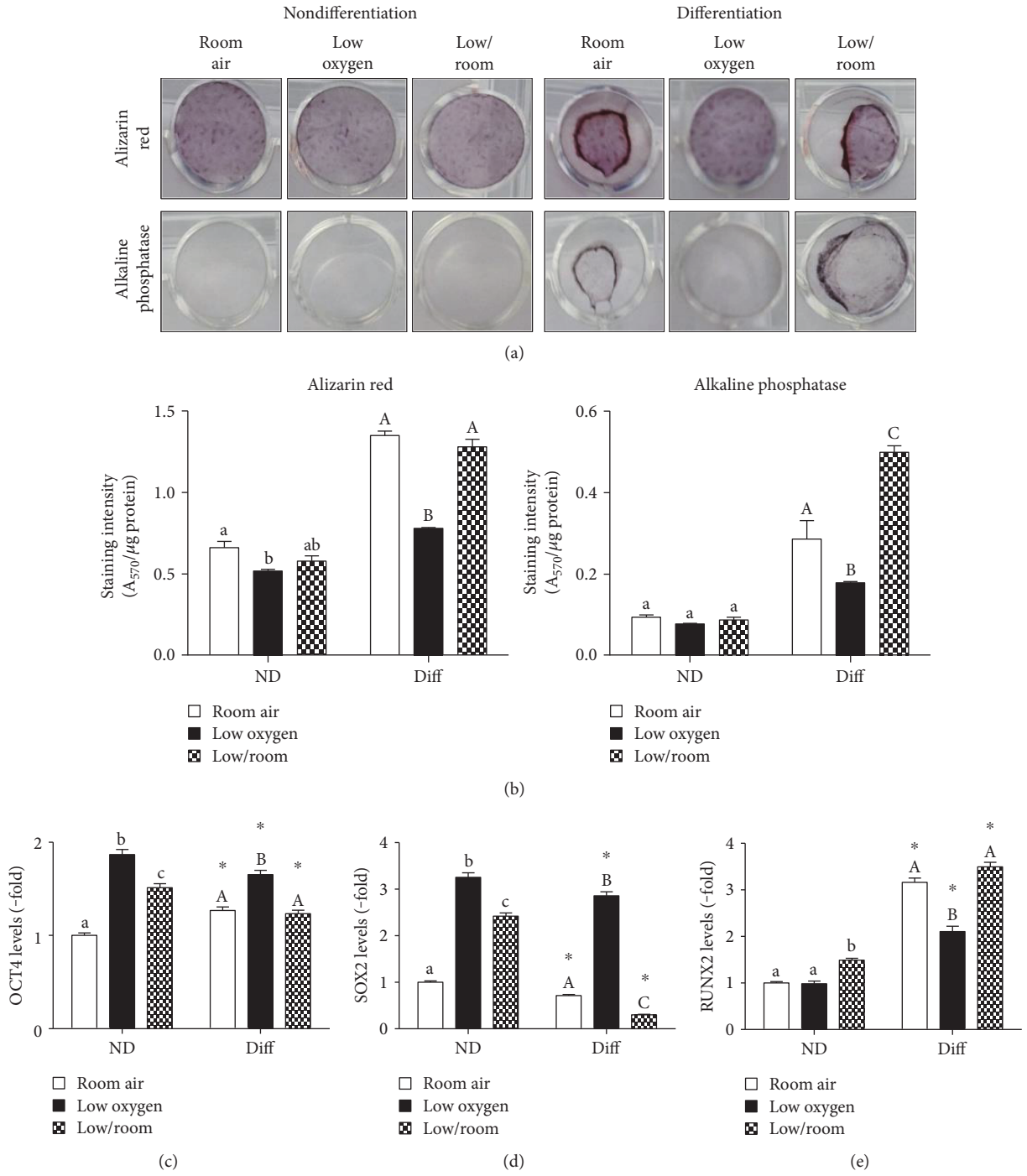


Figure 2: Effect of low oxygen tension preconditioning on PMSC osteogenic differentiation and multipotency. PMSCs were cultured in nondifferentiation or osteogenic differentiation conditions containing 15% FBS in room air (20% O_2) or low oxygen levels (1% O_2) for 14 days as in Figure 1. For low/room treatment, PMSCs were cultured for 7 days in low oxygen and followed by 7 days in room air (shown in the third panel). Alizarin red or alkaline phosphatase staining was used to detect calcium deposition and enzyme expression changes as shown morphologically in (a) and quantified in (b) (two-way ANOVA, $P < 0.05$, $N = 4$); lowercase letter (a, b, and c) indicates significance between oxygen tension effects within nondifferentiation condition, uppercase letter (A, B, C) indicates within differentiation conditions. From immunoblots shown in Figure S2, protein levels were quantified in (c) OCT4, (d) SOX2, and (e) RUNX2 levels. Quantification levels shown were normalized to β-actin, a protein loading control (two-way ANOVA, $P < 0.05$, $N = 3$). $*$ indicates significance between room air and low oxygen tension; lowercase letter (a, b, and c) indicates significance between oxygen tension effects within nondifferentiation condition, uppercase letter (A, B, and C) indicates within differentiation conditions.

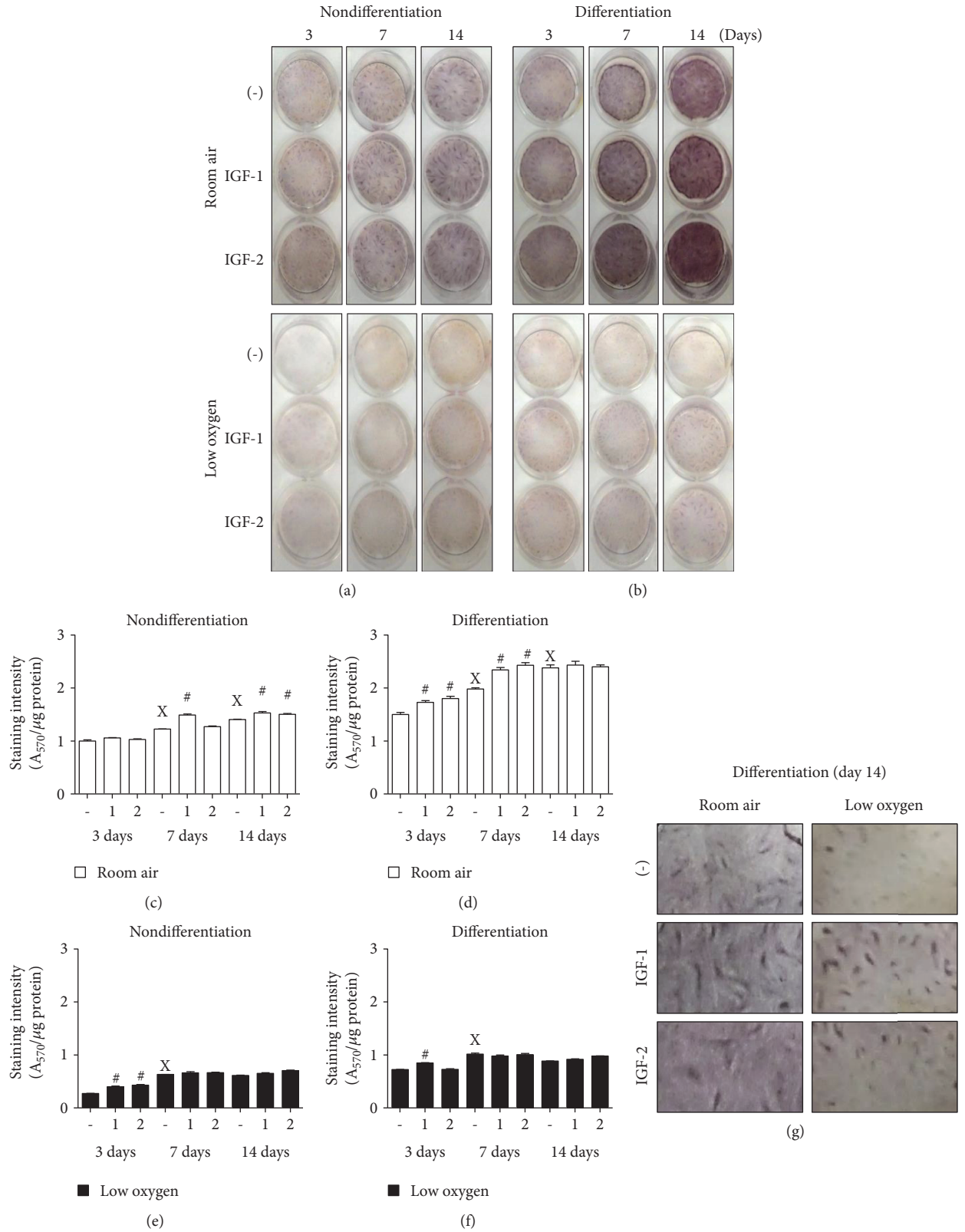


Figure 3: PMSC differentiation under low oxygen tension is regulated by IGFs. PMSCs were cultured in nondifferentiation or differentiation conditions containing 2% FBS in the presence or absence of 100 ng/mL of IGF-1 or IGF-2, in room air (20% O_2) or low oxygen levels (1% O_2). Treatments were stopped after 3, 7, and 14 days for (a-b) alizarin red staining and quantified in (c-d) (two-way ANOVA, $P < 0.05$, $N = 4$). X indicates significance between different days without IGFs; # indicates significance of IGF addition compared with no IGFs in the same day. (g) Calcification centers and cell organization of differentiated PMSCs stained with alizarin red from day 14 are shown in higher magnification.

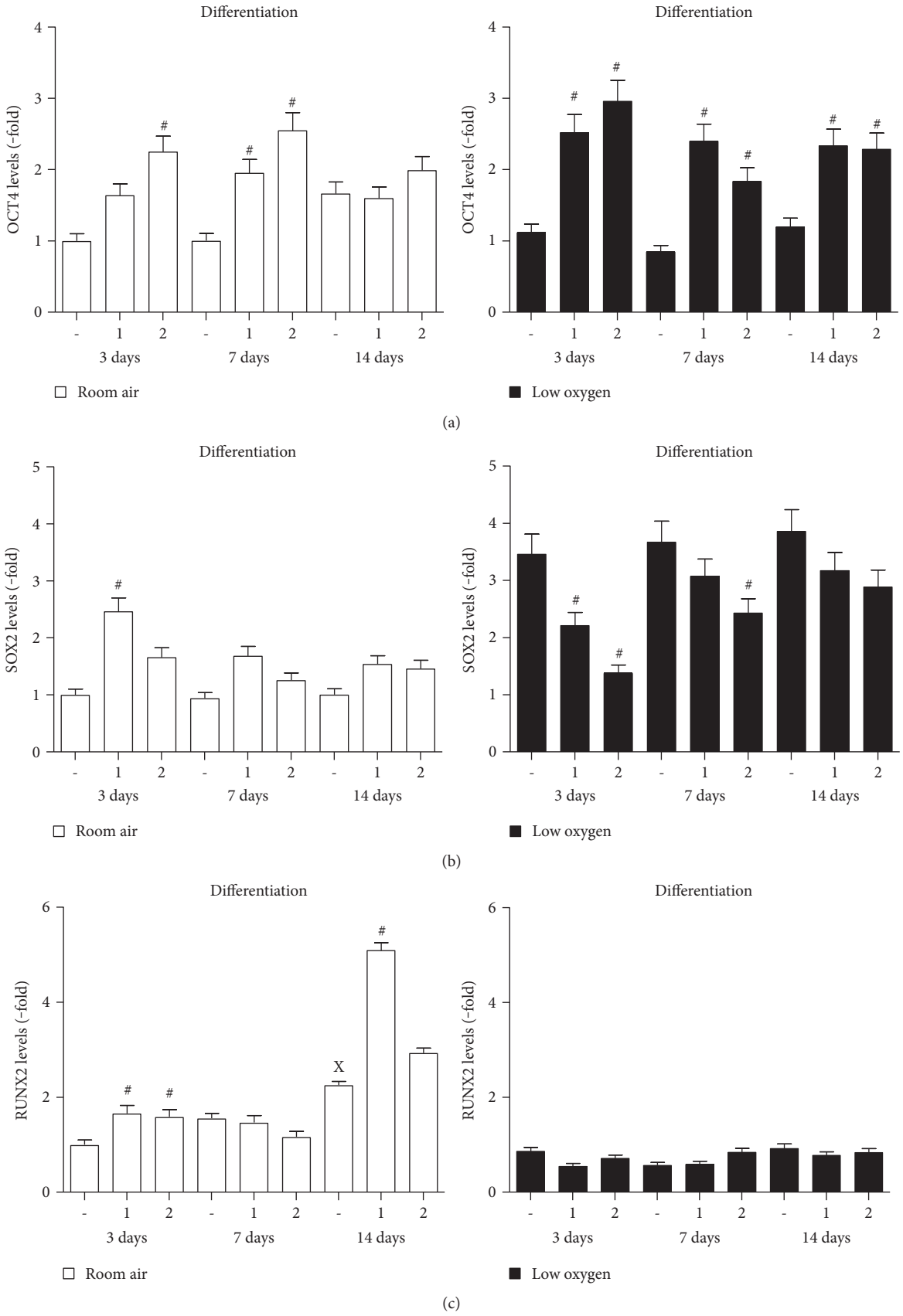
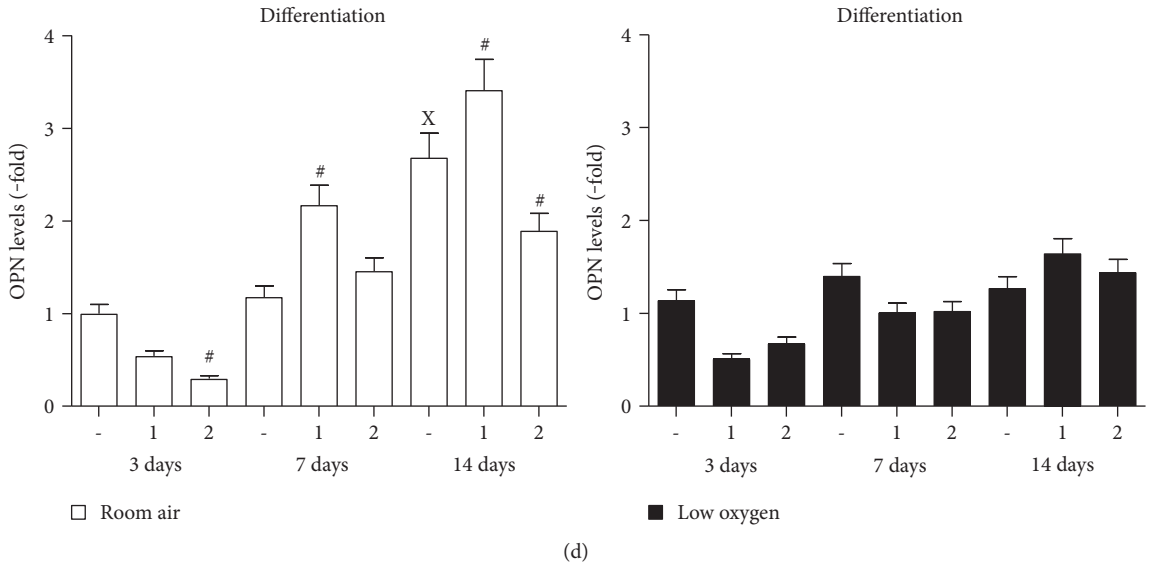


Figure 4: Continued.



(d)

Figure 4: PMSC multipotency and differentiation are regulated under low oxygen tension by IGFs. PMSCs were cultured for 14 days in osteogenic differentiation conditions containing 2% FBS in the presence or absence of 100 ng/mL of IGF-1 or IGF-2 in room air (20% O_2) or low oxygen levels (1% O_2). Treatments were stopped after 3, 7, and 14 days. Immunoblots, shown in Figure S3, were used to quantify the changes in protein levels of (a) OCT4, (b) SOX2, (c) RUNX2, and (d) OPN induced by IGFs over time. Quantification levels were normalized to β-actin, a protein loading control (two-way ANOVA, $P < 0.05$, $N = 3$). X indicates significance between different days without IGFs; # indicates significance of IGF addition compared with no IGFs in the same day.

and AKT, respectively. After 14 days, the alizarin red staining was reduced more by LY294002 than by U0126 (Figures 7(a) and 7(b)) without affecting PMSC viability (Figure S5). U0126 did not change the levels of OCT4 and SOX2 in differentiating PMSCs (Figures 7(c) and 7(d)); however, RUNX2 and OPN were increased in low oxygen tension (Figures 7(e) and 7(f)). This demonstrates that the repression of osteogenic differentiation by low oxygen tension may be mediated by MEK1/2 signaling. LY294002 reduced OCT4, SOX2, RUNX2, and OPN in differentiation conditions but more so in low oxygen tension (Figures 7(c), 7(d), 7(e), and 7(f)). Therefore, PI3K signaling has a dual role in PMSCs—to maintain multipotency and to promote osteogenic differentiation.

The effects of the inhibitors were assessed by phosphorylation of ERK1/2 and AKT. The addition of U0126 and LY294002 every 48 hours reduced phosphorylation of ERK1/2 and AKT (Figure S6), respectively. At day 14 of differentiation, U0126 decreased p-ERK1/2 levels in room air and not in low oxygen tension (Figure 7(g)). LY294002 inhibited AKT phosphorylation in room air and even lower in low oxygen tension conditions (Figure 7(h)), yet LY294002 increased p-ERK1/2 levels in low oxygen tension (Figure 7(g)). RUNX2 phosphorylation was unaffected with U0126 in room air but was decreased with LY294002 (Figure 7(i)). In low oxygen tension, p-RUNX2 was increased only with U0126 during differentiation (Figure 7(i)). Therefore, the balance of MEK1/2 and PI3K signaling is required to mediate osteogenic differentiation, especially in low oxygen tension, as their misregulation can cause PMSC differentiation.

4. Discussion

The successful use of stem cells for cell-based therapies requires an optimization of stem cell survival and potency *in vitro*, preventing cell death *in vivo* postinjection [41]. In this study, we used low oxygen tension and IGFs to determine their combined effect on the commitment and differentiation of PMSCs towards the osteogenic lineage (Figure 8). We found that low oxygen tension increased PMSC proliferation, induced higher OCT4 and SOX2 levels, blocked differentiation and mineralization, and reduced the IGF-mediated early onset of osteogenic differentiation. Low oxygen tension also increased the levels of IGF-1R in differentiated PMSCs. In comparison, insulin receptor expression was increased in room air with an elevated IR-B, opposite to IR-A which was enhanced by low oxygen tension. Upon differentiation, RUNX2 levels were increased with loss of pluripotency-associated proteins. Only in room air, RUNX2 was phosphorylated and enhanced by IGF-1 and IGF-2 which may explain the more robust osteogenic differentiation level.

In vitro, osteogenic differentiation follows a three-phase process: a differentiation phase (days 0–5), a matrix formation phase (days 5–12), and a mineralization phase (days 12–19) [37]. In this study, we investigated the role of IGFs in combination of low oxygen tension in the commitment and matrix formation phases of PMSC differentiation (days 0–14). In this process, RUNX2 is strongly detected in preosteoblasts, immature osteoblasts, and early osteoblasts [18, 42]. We demonstrated that RUNX2 is expressed in early differentiation in room air and inhibited by low

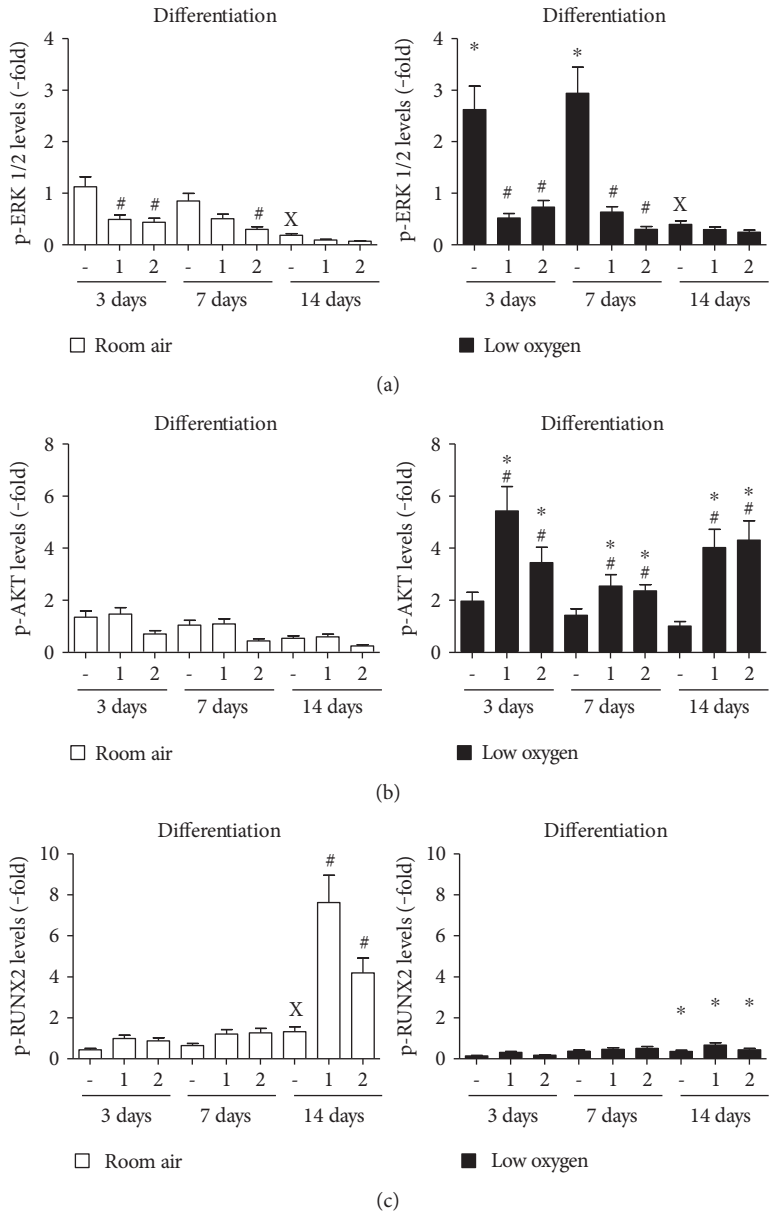


Figure 5: Effect of oxygen tension and IGF-1 or IGF-2 on signaling downstream kinases of cell surface receptors during osteogenic differentiation. Similar to Figures 4 and 6, PMSCs were cultured for 14 days in osteogenic differentiation conditions containing 2% FBS in the presence or absence of 100 ng/mL of IGF-1 or IGF-2 in room air (20% O_2) or low oxygen levels (1% O_2). Treatments were stopped after 3, 7, and 14 days. Immunoblots, in Figure S3, were used to quantify protein levels of (a) p-ERK1/2, (b) p-AKT, and (c) p-RUNX2 over the three days. These kinases/phosphoproteins were normalized to their total kinase/phosphoprotein level and β-actin (two-way ANOVA, $P < 0.05$, $N = 3$). X indicates significance between different days without IGFs; # indicates significance of IGF addition compared with no IGFs in the same day; $*$ indicates significance between room air and low oxygen tension.

oxygen tension. OPN, as a late osteogenic differentiation marker, was also repressed by low oxygen tension. IGFs enhanced PMSC differentiation only in room air (IGF-1 has a greater effect than IGF-2) where low oxygen tension abolished their effect. Previously, IGF-1 was shown to increase the levels of RUNX2 [26], and similarly in PMSCs, IGF-1 and IGF-2 increased RUNX2 levels as early as day 3 and maintained elevated levels at day 14. Also, OPN was elevated as early as day 7 with IGF-1, which increased further at day 14.

IGFs enhance the differentiation function by promoting growth, inhibiting apoptosis, and upregulating matrix maturation (increased type I collagen) and mineralization [36]. *In vivo*, the use of MSCs that overexpress IGF-1 improves fracture healing by accelerating bone cell differentiation [43]. Signaling through IGF-1R in MSCs during differentiation is regulated by PI3K/AKT and not by MAPK signaling (in a positive feedback loop) which also inhibits apoptosis in osteoblasts [29, 43]. In this study, we showed that the inhibition of PI3K caused a significant reduction in the

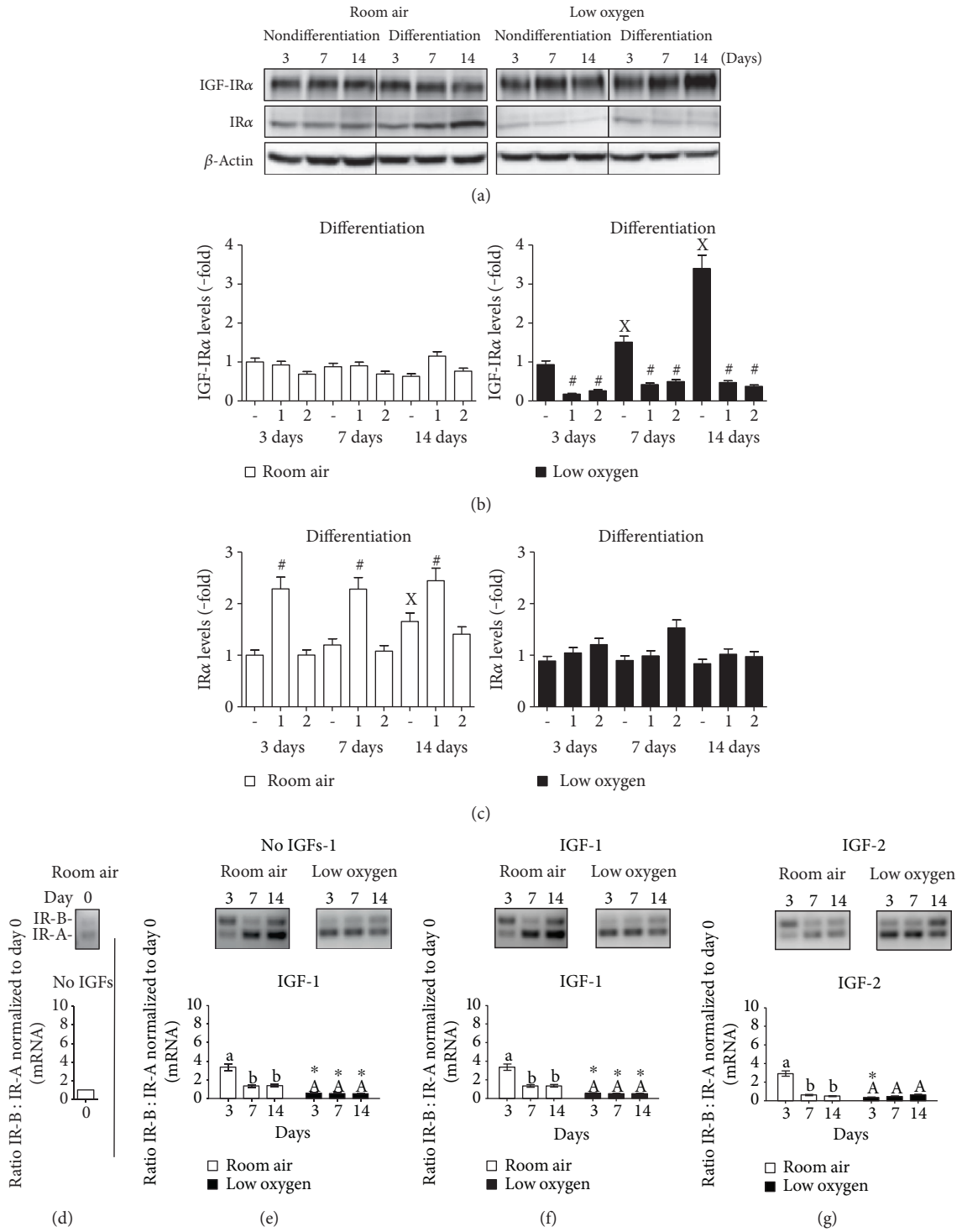


Figure 6: IGF-1R and IR levels and its isoforms (IR-A and IR-B) in differentiating PMSCS are regulated by oxygen tension and IGFs. PMSCs were cultured for 14 days in nondifferentiation or osteogenic differentiation conditions containing 2% FBS in the presence or absence of 100 ng/mL of IGF-1 or IGF-2 in room air (20% O_2) or low oxygen levels (1% O_2). Treatments were stopped after 3, 7, and 14 days. Immunoblots were used to detect levels of (a) IGF-1R and IR in the absence of IGFs over time. Quantification of immunoblots, shown in Figure S3, shows the IGF-1 or IGF-2 effect on (b) IGF-1R and (c) IR over the three days. Levels were normalized to β-actin, a protein loading control (two-way ANOVA, $P < 0.05$, $N = 3$). X indicates significance between different days without IGFs; # indicates significance of IGF addition compared with no IGFs in the same day. By end-point PCR, mRNA levels of IR-A versus IR-B were measured and a ratio was calculated and normalized to total IR in (d) undifferentiated day 0 PMSCs, (e) differentiation without IGFs, (f) differentiation with IGF-1, and (g) differentiation with IGF-2 (two-way ANOVA, $P < 0.05$, $N = 3$). $*$ indicates significance between room air and low oxygen tension; lowercase letter (a, b) indicates significance between time points within room air condition, uppercase letter (A) indicates within low oxygen tension.

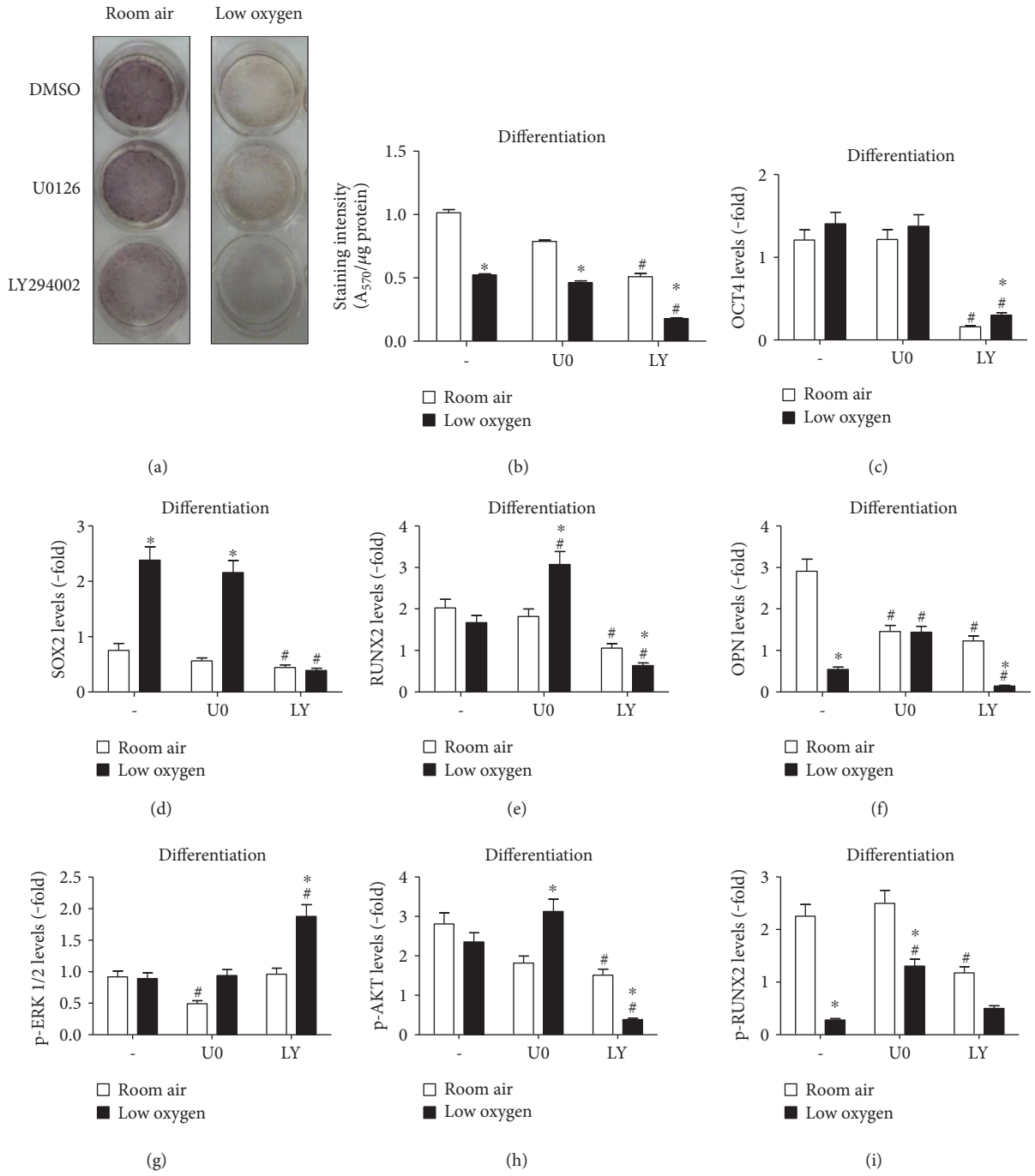


Figure 7: PMSC differentiation is mediated via MEK1/2 and PI3K signaling and their inhibition effect on ERK1/2, AKT, and RUNX2 phosphorylation under low oxygen tension. PMSCs were cultured for 14 days in osteogenic differentiation conditions containing 2% FBS in room air (20% O_2) or low oxygen levels (1% O_2). During the 14 days, cells were continuously exposed to (5 μM) U0126 or (10 μM) LY294002 in differentiation media. Treatments were stopped at 14 days and stained with alizarin red to confirm (a) PMSC differentiation morphology changes with the inhibitors and quantified in (b) (two-way ANOVA, $P < 0.05$, $N = 4$). Immunoblots, shown in Figure S7, were used to quantify protein levels of (c) OCT4, (d) SOX2, (e) RUNX2, (f) OPN, (g) p-ERK1/2, (h) p-AKT, and (i) p-RUNX2 induced by signaling inhibition. Quantification levels were normalized to β-actin, a protein loading control; additionally, each phosphoprotein was normalized to its total protein (two-way ANOVA, $P < 0.05$, $N = 3$). $*$ indicates significance between room air and low oxygen tension; # indicates significance between DMSO control and inhibitor.

multipotency markers (OCT4 and SOX2) and the osteogenic markers (RUNX2 and OPN) supporting that PI3K is required not only to maintain multipotency but also to regulate PMSC differentiation.

RUNX2 exerts its transcriptional activity via binding to its cognate DNA site in promoter regions of osteogenic differentiation genes (such as, OCN and OPN) [44]. RUNX2 is phosphorylated by the MEK/ERK pathway *in vitro* [20]

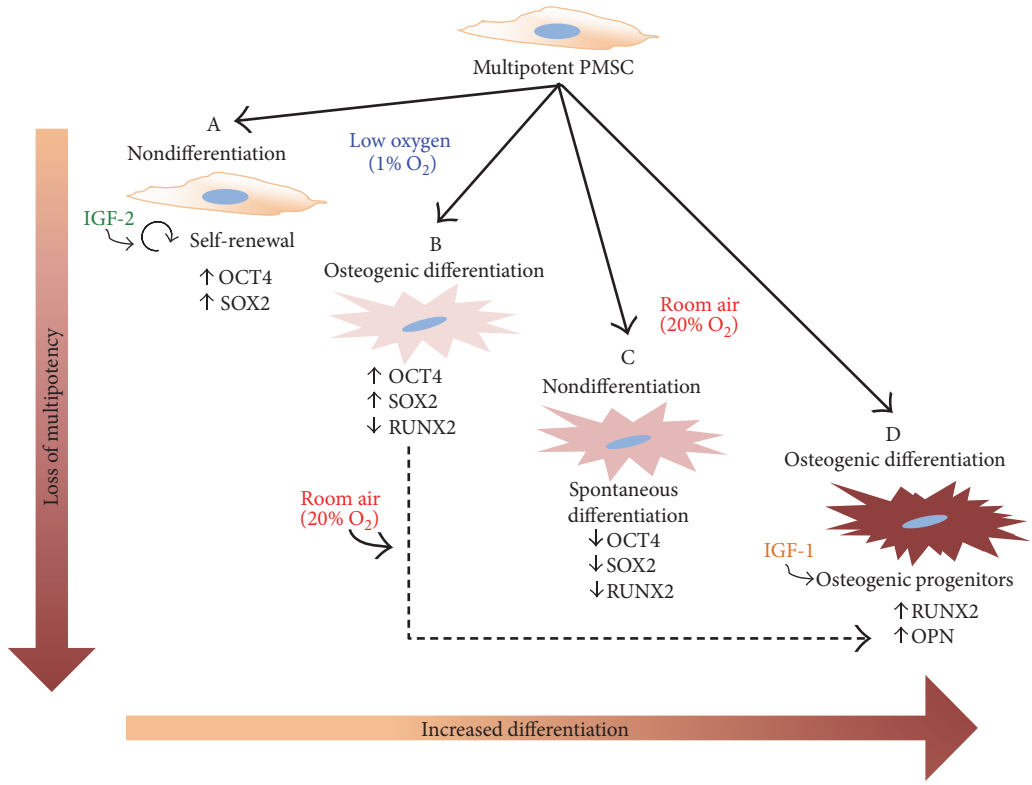


Figure 8: PMSC osteogenic differentiation in the presence of IGFs and low oxygen tension. Summary diagram showing stem cell differentiation model in room air versus low oxygen. PMSCs can differentiate into osteogenic lineage only in room air, while maintaining PMSCs in low oxygen tension impedes this differentiation process. (A) In low oxygen tension, nondifferentiation conditions maintain self-renewal (higher proliferation) and IGF-2 can maintain higher expression of OCT4 and SOX2. (B) In differentiation media, PMSCs show an impeded differentiation with higher OCT4 and SOX2 and lowered RUNX2, which recovers following room air exposure. (C) In room air, spontaneous differentiation occurs with lower OCT4 and SOX2 and lower RUNX2. (D) In differentiation media, PMSCs fully differentiate towards osteoblastic progenitors with higher RUNX2 and OPN, which enhanced by IGF-1. Cell morphology and alizarin red staining increase as PMSCs lose multipotency and commit to the osteogenic lineage.

and *in vivo* [45] which triggers its transcriptional activity. In PMSCs, IGF-1 and IGF-2 increased the phosphorylation of RUNX2 at day 14 of PMSC differentiation, thereby enhancing differentiation and osteogenic gene transcription. These data demonstrate that IGF-1, more than IGF-2, is a potent growth factor required by PMSCs to complete the differentiation process. In bone marrow MSCs, FGF2 signaling via MEK/ERK was shown to induce RUNX2 phosphorylation that activates an OCN promoter [21]. Also, in HBME cells, IGF-1 induced the RUNX2 DNA binding activity by the MEK/ERK pathways in a sequential activation process [46]. Therefore, the MAPK pathway connects between the cell surface receptor signaling (e.g., IGF-1R) and RUNX2 phosphorylation to advance osteogenic differentiation. On the contrary, one report showed that MEK1/2 signaling is suppressive for osteoprogenitor differentiation in a neurofibromatosis type I mouse model [47], indicating that inhibition of MEK1/2 signaling is required for osteogenic differentiation. Interestingly, MEK1/2 inhibition elevated total and phosphorylated levels of RUNX2 and OPN in PMSCs in low oxygen tension; however, it could not rescue mineralization. Therefore, MEK1/2 signaling can be oxygen tension dependent that may repress osteogenic differentiation in low oxygen tension by lowering RUNX2 and OPN levels.

The transcriptional network of OCT4 and SOX2 in maintaining pluri-/multipotency and repressing differentiation has been shown in mouse and human embryonic stem cells. In mESCs, mesodermal lineage specification is determined by OCT4 and SOX2: a balanced expression maintains pluripotency, while the upregulation of OCT4 relative to SOX2 induces mesendodermal lineage specification and downregulation of OCT4 relative to SOX2 induces neural ectodermal lineage specification [48, 49]. In human ESCs, OCT4 maintains an embryonic stem cell state and represses extraembryonic differentiation, while SOX2 is required to suppress the differentiation towards the mesendodermal [50]. In PMSCs, osteogenic differentiation increased the expression of OCT4 and decreased the expression of SOX2, as expected in a mesodermal differentiating lineage, only when placed in room air. Low oxygen tension upregulated both OCT4 and SOX2 maintaining a more multipotent state even under differentiation conditions, similar to a previous study [51]. Also, we observed that differentiating PMSCs upregulated OCT4 prior to differentiation, which could be important for facilitating differentiation. In fact, in ESCs, OCT4 was required for

in vivo and *in vitro* differentiation processes and OCT4-deficient cells were unable to differentiate [52].

Oxygen tension is increasingly recognized as an important factor of the stem cell niche in proliferation, migration, metabolism, and differentiation. In bone fractures, oxygen level can go as low as 0.1% (~0.76 mmHg) [37, 53]. Also, oxygen tension at the fracture site of the femoral head can be low at 17.3–19.9 mmHg and even lower at 1 cm away from fracture site (12.5–12.8 mmHg) [54]. The hypoxia-inducible factor (HIF) system is responsible for downregulating the expression of osteoblast commitment genes, such as RUNX2 [37], and a complete differentiation is obscured due to inhibiting the expression of downstream genes responsible for mineralization and matrix formation (OPN and OCN) [37]. However, few contradicting reports have shown that low oxygen tension may favour osteogenic differentiation and mineralization of MSCs which was referred to be dependent on the oxygen tension used during stem cell expansion following initial isolation [55]. Another report also argues that mineralization occurs in low oxygen tension; however, RUNX2 and alkaline phosphatase levels were reduced, compared with those in room air [56]. In this study, low oxygen tension impeded the complete differentiation and calcification of PMSCs, but RUNX2 levels were elevated compared with those in nondifferentiation conditions. This inhibition was reversed upon exposure to higher oxygen tension as was demonstrated by the elevated alkaline phosphatase staining levels compared with room air (Figure 2). Indeed, stem cell preconditioning was shown to improve healing and survival of fractured bone following transplantation [34]. The inhibition of differentiation by low oxygen tension (with stabilized HIF1α) inhibits a metabolic switch required for osteogenic differentiation [57], which relies on the upregulation of mitochondrial function and aerobic respiration (with downregulation of HIF1α) [58]. Therefore, glycolysis in undifferentiated MSCs is not sufficient for ATP production, as differentiating MSCs switch to oxidative phosphorylation to meet the high energy demand of differentiation processes, matrix deposition, and mineralization [57].

Low oxygen tension inhibited the enhanced IGF-mediated osteogenic differentiation in PMSCs, which can be due to ligand/receptor signaling downregulation. In a previous report, we noticed that low oxygen tension upregulates the levels of IGF-1R and IR [59]; therefore, it is possible that IGF ligands are obscured from the receptors. IGF-binding proteins (IGFBPs), such as IGFBP-1 and IGFBP-3, are upregulated by low oxygen tension, and their binding affinity to IGFs can block the interaction between IGFs and their receptors [60, 61]. A recent report demonstrated by siRNA studies that the upregulation of hypoxia-responsive IGFBP-3 in adipose-derived and bone marrow stem cells reduced their osteogenic differentiation and mineralization in low oxygen tension [62]. Also in PMSCs, IGFBP-3 is upregulated by low oxygen tension (data not shown), and therefore, it may bind to IGF-1 and inhibit its actions via the IGF-1R activation, abolishing the IGF-1-induced osteogenic differentiation presented in room air. This mechanism needs to be investigated.

IGF-1R and IR signaling pathways are indispensable for postnatal bone growth and turnover. In patients with osteoporosis, primary osteoblasts have an impaired IGF-1R signaling decoupled from IGF-1 stimulation [63], which causes lower proliferation rate and differentiation and therefore bone loss. In insulin-dependent diabetic patients, the low insulin levels can lead to osteopenia, increased risk of fragility fracture, and poor bone healing [64, 65]. In mice, the knockout of IGF-1R in osteoblasts causes a reduced trabecular bone volume with defective mineralization [66]. On the other hand, mice lacking the IR in osteoblasts have reduced trabecular bone; however, unlike IGF-1R knockouts, bone was normally mineralized [67]. Hence, IGF-1R signaling is essential for coupling matrix biosynthesis to sustain mineralization. Indeed, IGF-1R or IR presence affects osteoblast number/abundance in bone, in which the absence of IGF-1R does not change the number of osteoblasts but the knockdown of IR severely reduces the number of osteoblasts in bone [66, 67]. Interestingly, conditional knockdown of IGF-1R from osteoblasts greatly increases their insulin responsiveness via the IR which can partially compensate for the IGF-1R by promoting proliferation and mineralization [68]. Even though the IR can compensate for IGF-1R loss, the IGF-1R is essential for augmenting these signaling interactions for normal bone growth and turnover. Between the two IR isoforms, IR-B is abundantly expressed in differentiating MSCs and mature osteoblasts while IR-A was abundantly expressed in proliferating cells [69]. Therefore, the IR increases with a higher ratio of IR-B to IR-A in differentiating osteoblasts [69]. In our PMSCs, we showed that the IR-A is elevated by low oxygen tension in differentiation conditions; however, IR-B was the dominant isoform in room air. Unlike IR-A, which mediates mitogenic actions involved in increased cell proliferation, atherosclerosis, and cancer, IR-B is responsible for metabolic action to facilitate metabolism, cell differentiation, and increased longevity [70]. This suggests that differentiating osteoblasts express higher IR-B levels to utilize more glucose for metabolism and possibly to accommodate the higher energy demand in differentiating cells.

In summary, we have shown in this study that PMSCs can successfully differentiate towards the osteogenic lineage; thus, they may be an alternative source to bone marrow for adult MSCs. Preconditioning in low oxygen tension and the use of IGFs (mainly IGF-1) to stimulate PMSCs are promising strategies to generate osteogenic progenitor cells for tissue regeneration therapy in bone diseases and repair. Further, *in vivo* studies in animal models using these strategies will be required to determine the successful engraftment of PMSCs for the regenerative therapy in OI or bone fractures.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by the CIHR Grant 111024.

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Hindawi
Stem Cells International
Volume 2017, Article ID 8387297, 10 pages
https://doi.org/10.1155/2017/8387297

Review Article

Intestinal Stem Cell Niche Insights Gathered from Both *In Vivo* and Novel *In Vitro* Models

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Received 31 March 2017; Accepted 3 July 2017; Published 7 September 2017

Academic Editor: Karen Liu

Intestinal stem cells are located at the base of the crypts and are surrounded by a complex structure called niche. This environment is composed mainly of epithelial cells and stroma which provides signals that govern cell maintenance, proliferation, and differentiation. Understanding how the niche regulates stem cell fate by controlling developmental signaling pathways will help us to define how stem cells choose between self-renewal and differentiation and how they maintain their undifferentiated state. Tractable *in vitro* assay systems, which reflect the complexity of the *in vivo* situation but provide higher level of control, would likely be crucial in identifying new players and mechanisms controlling stem cell function. Knowledge of the intestinal stem cell niche gathered from both *in vivo* and novel *in vitro* models may help us improve therapies for tumorigenesis and intestinal damage and make autologous intestinal transplants a feasible clinical practice.

1. Introduction

The intestine represents the most vigorously renewing adult tissue, which undergoes rapid turnover in order to prevent damage from stress factors; its tissue-specific stem cells are essential for tissue homeostasis in the adult organism [1]. These undifferentiated cells residing at the bottom of the crypts of Lieberkühn are able to produce a large number of differentiated progeny as well as to self-renewal. Due to their relevant function, many efforts have been done in the last years to define the exact localization of the intestinal stem cells and its properties. There is now evidence that at least two types of stem cells coexist in the small intestine. Best characterized are the leucine-rich-repeat-containing G-protein-coupled receptor 5-expressing ($Lgr5^+$) stem cells which divide approximately every 24 hours, and they are interspersed between the terminally differentiated Paneth cells [2]. The *Lgr5* gene was selected from a panel of intestinal Wnt targets for its restricted crypt expression (columnar base cells, CBC) and was identified as a marker gene of stem cells in the small intestine and colon [2]. Very recent findings have found that $Lgr5^+$ stem cell population is not homogenous. The expression of the RNA-binding protein Mex3a labels a slowly cycling subpopulation of $Lgr5^+$ ISCs that contribute to all intestinal lineages. Thus, Mex3a defines a reserve-like ISC population within the $Lgr5^+$ compartment [3]. The second type of stem cells are located at the +4 position of the intestinal crypt and are called label-retaining cells (LRCs) as they show long-term label retention upon irradiation damage and pulse labeling with BrdU. These cells remain quiescent and act as a reserve population that can give rise to all intestinal cell lineages after tissue damage [4–8]. Some reports point out that there is an apparent dichotomy between quiescent versus cycling stem cells that in fact reflect a continuum of phenotypes dictated by different thresholds of expression of key regulators (e.g., signals and/or transcription factors) that modulate stem-like functions [7, 9–13]. Future experiments for a better identification of these mechanisms and the features of the +4 LRC stem

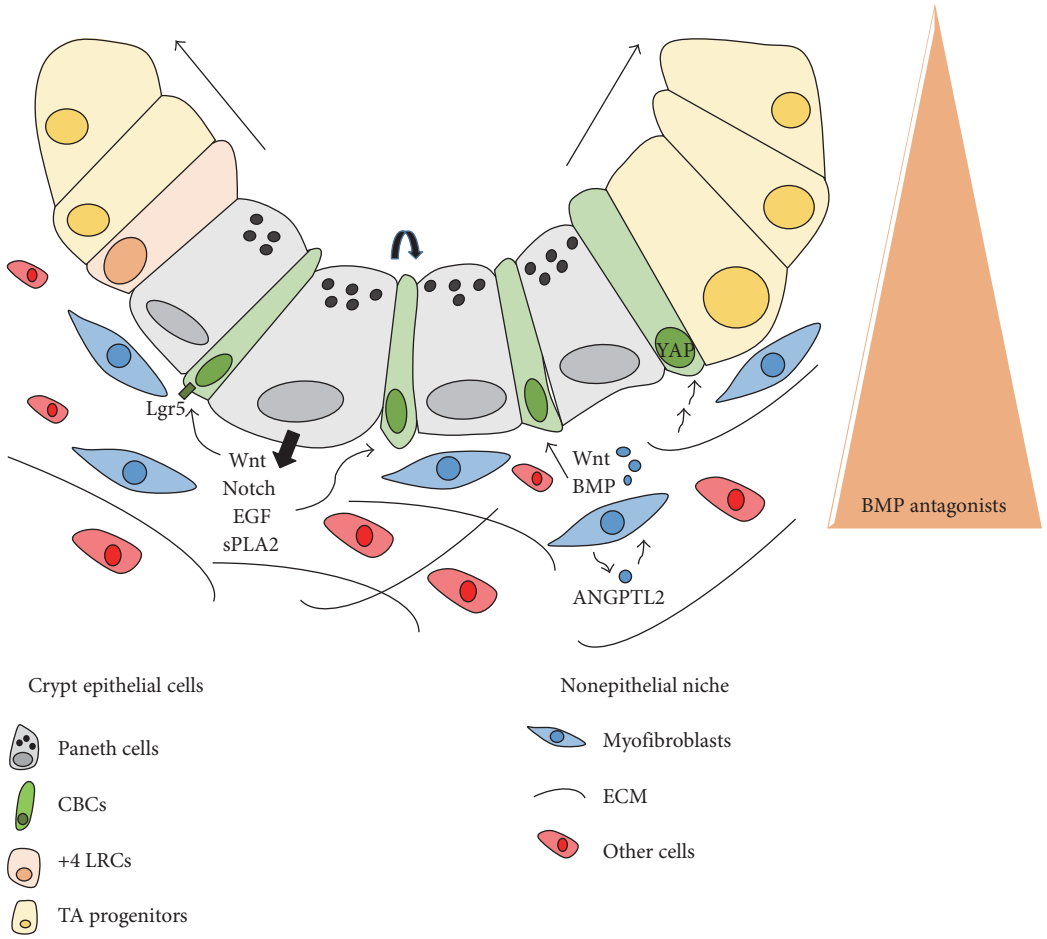

Figure 1: The stem cell niche of the small intestine. Epithelial and nonepithelial environments support the signals required for stem cell maintenance. Among them, Wnt and Notch signaling have been defined as major determinants for stem cell self-renewal, for proliferation/differentiation of stem cells in the crypt. Stromal BMP antagonists regulate the crypt-villus axis, and the extracellular cell matrix (ECM) support signals that control stem cell fate. Other cells: neural, immune, and endothelial cells. TA: transit-amplifying progenitors; sPLA2: secreted phospholipases A2.

cell populations are still needed in order to understand the capacity of the intestinal tissue to induce a regenerative response under (radiation induced) tissue injury. In this review, we will mostly focus on the *in vivo* and *in vitro* models for intestinal CBC stem cell niche.

Control of proliferation, self-renewal, and lineage specification of the stem cells in the crypt are believed to be directed by an actively regulated process based on cell-cell and cell-stroma interactions [14]. The ISC niche or microenvironment is composed of epithelial and underlying nonepithelial cells within the lamina propia populated by stromal, immune, endothelial, and neural cells that support paracrine and/or autocrine signaling (Figure 1). The ISC niche also comprises the extracellular matrix (ECM), a highly dynamic structure that continuously undergoes controlled remodelling, mediated by metalloproteinases that are responsible for ECM degradation [15]. The ECM interacts with the different cells in the niche to regulate stem cell fate [16] (Figure 1). Overall, the components of the niche tightly modulate Wnt, Notch, epidermal growth factor (EGF), bone morphogenic protein (BMP)/transforming growth factor (TGF) β, and Hedgehog signaling pathways to maintain proliferation/differentiation balance [17–19].

Functional analysis of stem cells and their environment has been hampered by a lack of suitable *in vitro* systems allowing long-term culture and until some years ago, the only possible strategy to analyse such interactions for a potential role in intestinal development, homeostasis, damage or tumorigenesis was the time-consuming tissue-specific mouse models. For example, *Achaete-scute complex homolog 2* (Ascl2) was reported to be responsible for controlling intestinal stem cell fate by using transgenic mice [20]. In 2009, two groups developed a three-dimensional (3D) culture model of freshly isolated crypt cells from murine small intestine and colon [21–23], and later this method was set up for human samples [24, 25]. These assays maintain basic crypt-villus physiology and permit long-term intestinal epithelial expansion as sphere-like organoids. The stem cells are embedded in Matrigel, a gelatinous protein mixture secreted by mouse sarcoma cells containing structural proteins such as laminin, entactin, and collagen in combination with several growth stimuli essential for crypt proliferation (the Wnt agonist R-

spondin1, EGF, and the BMP inhibitor Noggin). Single-sorted Lgr5$^+$ stem cells are sufficient to give rise to organoids in culture which contain all differentiated lineages: Paneth cells at the base of the crypt and enteroendocrine, goblet cells, and enterocytes that migrate upwards the villus. Importantly, these cultures allow ex vivo monitoring intestinal stem cell function with respect to self-renewal and production of rapidly dividing crypt progenitor cells and differentiated lineages and are therefore comparable to the *in vivo* situation [21]. In this review, we will compare *in vivo* models to the most novel *in vitro* technology which will improve in the next years our understanding of stem cell behavior.

2. *In Vivo* Models of the Stem Cell Niche

2.1. CBC Epithelial Niche. Stem cells require the support of neighbouring epithelial cells to maintain their function. The epithelial niche modulates several signaling cascades, the Wnt pathway being one of the main regulators of stem cell self-renewal. Genetic deletion of the Wnt pathway's main players (β-catenin, Tcf4 knockout models) or ectopic expression of the secreted Wnt antagonist Dickkopf-1 (Dkk1) disrupts intestinal epithelial homeostasis, leading to crypt loss, reduced proliferation, and altered differentiation [26–28]. Similarly, overactivation of the Wnt pathway in mice, by overexpression of the Wnt agonists roof plate-specific spondin 1 (R-spondin1) and R-spondin3 or by deleting *Adenomatous polyposis coli (Apc)*, drives hyperplasia and increases the expansion of intestinal stem cell niche [29–31]. The Wnt target genes, *EphB2*, *EphB3*, and their ligands ephrins, are key coordinators of migration and proliferation in the stem cell niche. EphB knockouts show that these proteins determine cell positioning along the crypt-villus axis in the intestinal epithelium [32]. Furthermore, EphB signaling promotes cell-cycle reentry of progenitor cells and contributes to the mitogenic activity in the adult mouse small intestine and colon [33].

"What is the main source of epithelial Wnt signals within the intestine?" The secretory Paneth cells adjacent to CBCs secrete Wnt3, and they constitute an important part of the small intestinal stem cell niche. They are known to produce bactericidal products such as lysozyme and cryptdins/defensins, and in addition, they can also produce TGFα, Notch (Dll4), and EGF factors that regulate stem cell maintenance [34, 35]. Reduction of number of Paneth cells in Gfi1$^{-/-}$ mouse model, transgenic expression of diphtheria toxin A under the Paneth cell-specific cryptdin 2 promoter (*CR2-tox176*), and conditional deletion of *Sox9* showed that the stem cells were coincidently decreased in number [34]. Some studies indicate that these cells are dispensable for small intestinal homeostasis; however, it should be determined in additional mouse models able to also achieve a total disruption of Paneth cells [36]. Although, this exact type of cells are not present in the colon, Clarke's group found goblet cells interdigitated with Lgr5$^+$ stem cells that contained a distinct cKit/CD117$^+$ crypt base subpopulation which expressed the Notch ligands Delta-like (Dll) 1, Dll4, and EGF. *In vivo*, this colonic cKit population was regulated by Notch signaling [37]. Later on, Clevers lab described equivalent cells called regenerating islet-derived family member (Reg) 4$^+$-expressing deep crypt secretory (DCS) cells (called Paneth/goblet-like cells) that are intermingled with the Lgr5$^+$ colonic stem cells at the base of the crypt. These cells also produce Wnt and Notch factors to support essential growth and maintenance signals. In these mouse experiments, the ablation of these types of cells resulted in loss of stem cell function and disruption of colon homeostasis [38]. When Paneth or DCS cells were sorted together with Lgr5$^+$ cells, the signals provided by them markedly increased differentiation and organoid growth from a single stem cell in the *in vitro* culture [34, 38]. Novel data have revealed that Paneth and DCS cells are also secreting the phospholipases A2 (sPLA2s) which inhibit Wnt pathway through intracellular activation of Yap1. Importantly, this cascade affects stem cell niche during homeostasis [39].

Several Notch mouse models evidence the impact of this pathway in epithelial stem cell niche. Indeed, Lgr5$^+$ stem cells are critically dependent on Notch, which depend on direct cell-cell contact as the Paneth cells are the main sources of Notch signals [40–46]. This is the case of simultaneous inactivation of Dll1 and Dll4 which resulted in the complete conversion of proliferating progenitors into postmitotic goblet cells, concomitant with the loss of Lgr5$^+$ SCs [41]. A negative regulatory mechanism of Wnt and Notch influencing intestinal stem cells in the gut was nicely shown by Tian et al. When Notch pathway was blocked, it perturbed intestinal stem cell function by causing a derepression of the Wnt pathway, leading to misexpression of prosecretory genes. Then, attenuation of Wnt rescued the phenotype associated with Notch blockade [43].

Other studies show *Leucine-rich repeats and immunoglobulin-like domains (Lrig) 1*, a direct Myc target gene as part of a negative feedback loop that modulates the proliferation of intestinal progenitor cells. *Lrig* knockout mouse induces upregulation of EGFR, ErbB2, and ErbB3 promoting downstream activation of c-Myc within intestinal stem and progenitor cells [17, 47]. The EGF pathway affects stem cell function by regulating the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and/or protein kinase C (PKC) pathways among other cascades [48]. These and further studies on this direction may lead to next-generation stem cell-based therapies.

2.2. CBC Nonepithelial Niche. Vigorous crosstalk between the epithelium and the underlying nonepithelial niche is required to define the crypt-villus axis. It is well established that mesenchymal cells secrete BMP antagonists such as Gremlin 1 and Gremlin 2 at the bottom of the crypts which supports compartmentalization [49]. Thus, BMP signaling is inhibited for a right intestinal epithelial renewal. Indeed, mouse transgenic overexpression of the BMP antagonist Noggin affects crypt expansion and increased stem cell numbers [50, 51]. Moreover, genetic models carrying BMPR1A inactivation or deficiency of its downstream effector PTEN show an inhibition of BMP signaling that enhances AKT activation and an increase in Wnt signaling [1, 50]. Hedgehog signaling is also involved in this crosstalk by modulating stromal BMPs [52]. The gradient of Wnt and BMP pathway

by diffusion of ligands along the crypt acts as a balance of cell differentiation/proliferation. Wnt is higher at the crypt base, whereas BMP pathway, which inhibits proliferation, has an opposite pattern of expression [1] (Figure 1).

Mesenchymal cells also secrete Wnt proteins, and R-spondins have been detected in the intestinal stroma [19, 53–55]. Experiments by using inducible mouse deletion (only epithelial cells) of Porcupine (an endoplasmic reticulum resident O-acyltransferase essential for the secretion and activity of all Wnts) showed that the cells had normal proliferation and differentiation, indicating that epithelial Wnt is dispensable for stem cell maintenance. Then, it was observed that intestinal stromal cells endogenously expressing Wnts and R-spondin3 support the growth of Porcupine-deficient organoids ex vivo, pointing out that stromal production of Wnts can fully support murine intestinal homeostasis [56].

All these data suggest that Wnt signals from Paneth cells can be replaced by stromal ones, so nonepithelial activation of Wnt pathway may support intestinal stem cell maintenance. On this direction, recent studies show that a subpopulation of mesenchymal cells marked by the winged-helix transcription factor Foxl1 is critical in maintaining stem cells. These cells produce Wnt factors, and their ablation reduces crypt growth. However, there is a need to better identify this subset of mesenchymal cells [57]. In addition, the autocrine secretion of ANGPTL2 by subepithelial myofibroblasts affects BMP production which then modulates intestinal organoid growth and size. Moreover, intestinal damage of *Angptl2* knockout mice reduces CBCs and influences Wnt pathway; however, ANGPTL2 is dispensable for intestinal homeostasis [58] (Figure 1).

Remarkably, some other cell populations in the niche (immune, endothelial, and neural cells) also influence the stem cell behaviour by modulating the different signaling cascades. They secrete growth factors, cytokines, and ligands that alter the stem cell fate [19, 59]. Upcoming work is required to reveal a more detailed comprehension of the interplay and components of this complex cellular network.

3. *In Vitro* Models of the ISC Niche

3.1. Intestinal Organoids. Animal models have provided invaluable insight into the nature and hallmarks of the intestinal stem cell, as well as the set of microenvironmental inputs that govern its behavior and constitute the intestinal stem cell (ISC) niche. However, in addition to ethical and practical considerations, animal-based studies suffer multiple limitations in the scope of scientific questions they can address. In particular, mouse models generally do not afford the dynamic and multifactorial observation and control that are required for securing comprehensive understanding of ISCs and their niche. Further, whereas the mouse intestine is an adequate approximation of its human counterpart, several crucial developmental and histological differences exist [60], and mouse-based studies may fall short in providing insight that is also relevant for humans. *In vitro* models of the ISC niche circumvent these problems by offering a level of accessibility and tractability that is difficult or impossible to achieve *in vivo*.

Driven by both basic research and therapeutic objectives, researchers have cultured stem cells *in vitro* for several decades. A decade ago, Sasai demonstrated that, aside from directing pluripotent stem cells to commit toward a certain lineage, thus obtaining populations of differentiated cells, stem cells and their progeny can follow their innate developmental programs and self-organize into structures that mimic multiple histological and functional aspects of real organs [61, 62]. These organ mimetics generated *in vitro* were termed organoids.

Intestinal organoids, or "miniguts", generated in the laboratory of Hans Clevers, were among the first types of stem cell-derived organoids reported [21, 24]. Sato et al. showed that intestinal crypts or single-dissociated Lgr5-expressing ISCs embedded in Matrigel and provided with niche signals, including R-spondin1, Noggin, and EGF, not only survive and proliferate but also undergo morphogenesis and differentiation to produce structures that approximate the adult intestine: crypt-like projections radiate outward from a spherical epithelial structure that surrounds a central lumen. In addition to cycling ISCs, housed at the proximal ends of the crypt-like buds, intestinal organoids contain all differentiated intestinal cell types, which are represented at the ratios found in the native intestine [63] and in spatial arrangements that closely mimic the patterning of the crypt-villus axis. Importantly, these structures reconstitute the principal geometric, architectural, and cellular hallmarks of the ISC niche—Lgr5-expressing ISCs are attached to a basement membrane-like hydrogel. ISCs and Paneth cells are represented in numbers and ratios reflecting those *in vivo*, thus forming a bud structure of similar in shape and size to those of the intestinal crypt. Small modifications of the culture protocol—notably, the addition of Wnt3a—allow for the culture of adult human ISC-derived intestinal organoids [24]. It should be emphasized, however, that these structures feature a round, cystic architecture, thus missing the crypt-like domains of mouse intestinal organoids.

In addition to adult ISCs, intestinal organoids have been generated from induced pluripotent stem cells (PSCs), using a protocol inspired by human embryonic development [64]. PSCs were first treated with activin A to induce the formation of definitive endoderm, which was then steered toward mid/hindgut fates by treatment with FGF4 and Wnt3a. Culturing of the resulting mid/hindgut spheroids in Matrigel, under conditions used for the culture of adult ISCs and crypts [21], gave rise to intestinal organoids. Notably, PSC-derived human organoids are organized into crypt- and villus-like domains, contain the major differentiated epithelial cell types, and, interestingly, are enveloped by a sheath of mesenchyme, comprised of myofibroblasts and smooth muscle cells, thus recapitulating an additional aspect of the ISC niche.

Aside from promising to revolutionize basic and clinical research, by serving as models of development and disease, platforms for drug discovery and toxicity screens and sources of tissue for cell-based therapies [23, 65], intestinal organoids complement *in vivo* studies in our quest to dissect the ISC

niche and define the mechanisms whereby it exerts its influence on stem cells. Indeed, to demonstrate that Paneth cells constitute the ISC niche, as discussed above, mouse models were used in conjunction with intestinal organoids [34, 66]. Likewise, organoids have been instrumental in elucidating the roles of various genes, including R-spondin and Lgr4/5 [67, 68] and YAP [69, 70] in the regulation of ISC fate.

The format of common organoid culture models allows for relatively easy and routine manipulations of the soluble microenvironment of ISCs. Beyond a set of soluble cues, the stem cell niche also comprises adhesion and mechanical signals from the surrounding ECM [71], which are likely to be as important as morphogens and growth factors in regulating ISC fate [72–75]. Typical intestinal organoid models, however, employ Matrigel—an ECM protein-rich hydrogel derived from the Engelbreth-Holm-Swarm sarcoma—as the 3D matrix. Matrigel, while clearly providing essential adhesive and mechanical cues, without which organoid formation would not be possible, remains a black box in regard to its contribution to the ISC niche. That is, Matrigel is a complex multicomponent mixture with ill-fined and variable biochemical and biophysical properties [76, 77] and the specific components and mechanisms whereby this material influences ISC fate are unclear. In the following sections, we will discuss recent advances in using biomaterials and bioengineering approaches to overcome the limitations of Matrigel, secure a more holistic understanding, and introduce additional levels of control over the ISC niche.

3.2. Toward a Synthetic ISC Niche: Using Synthetic Matrices to Deconstruct the Native Intestinal ECM. Synthetic hydrogels, comprising a water-swelled polymer network, can be rendered biocompatible and biofunctional through the incorporation of essential biological signals and used as well-defined alternatives to animal-derived ECM gels, such as collagen and Matrigel [78–80]. Moreover, these materials provide a biologically "blank" 3D environment into which biochemical and biophysical factors found in native tissues can be introduced and varied in a systematic and controlled manner, thus interrogating their cellular effects and evaluating them as potential stem cell niche components.

We recently took advantage of poly(ethylene glycol) (PEG) hydrogels to identify ECM components that control ISC fate and used this knowledge to construct well-defined and tunable matrices for the culture of ISCs and intestinal organoids [81] (Figure 2). Inspired by their localization to the basement membrane of mouse and human intestinal crypts *in vivo* [60, 74, 82–85], we assessed the effect of laminin-111, collagen-IV, fibronectin, hyaluronic acid, and perlecan on ISC self-renewal, differentiation, and organoid formation in the context of a 3D PEG hydrogel. We found that all components enhanced ISC survival and colony formation; laminin-111, collagen-IV, and fibronectin displayed the strongest positive effects. Notably, the fibronectin-derived RGD (Arg-Gly-Asp) peptide was sufficient in supporting ISC expansion in synthetic matrices. On the other hand, laminin-111 was of crucial importance for the concerted cycles of ISC self-renewal, differentiation, and morphogenesis that drive organoid formation; none of the other ECM components tested were even minimally effective. Although informative, our study examined the effects of only a handful of ECM components found *in vivo*. Future studies not only could take a system-level approach and expanded the number of ECM factors tested but also investigate potential interactive effects of multiple components. Sophisticated high-throughput approaches to generate and analyze multifactorial environments, which have already been used to study other stem cell systems [86, 87], seem ideally suited for further deconstructing the complexity of the ISC niche.

In addition to soluble and tethered molecular factors, ISCs *in vivo* experience physical signals from the microenvironment, including the mechanical properties of their surrounding ECM. The mechanical environment is now recognized as a major extrinsic regulator of multiple stem cell systems [88]. Our understanding of potential physical regulators of ISC fate is minimal, owing to the difficulty of performing controlled mechanical perturbations in both mouse models and Matrigel-based organoid culture. Nevertheless, recent *in vivo* studies provide clues that mechanical forces may directly control ISC proliferation in the colon [89]. We used the mechanically tunable PEG matrices to examine the effect of matrix mechanical properties on ISC expansion and organoid formation [81] and observed profound effects. In particular, we found that relatively stiff (shear modulus of ~1 kPa) matrices were optimal for ISC expansion, whereas ISC survival and colony formation in soft matrices was exceedingly low. In contrast, ISC differentiation and organoid formation were impaired by stiff matrices and only occurred in soft ones. We again took advantage of the versatility of the PEG hydrogel system to shed light on the molecular mechanisms underlying the mechanical effects on ISC expansion and organoid formation, which, surprisingly also accounted for the seemingly contradictory influence of stiffness on these two processes. In particular, we found that the matrix mechanics regulates ISC behavior by controlling the activity of Yes-associated protein 1 (YAP), which is a known mechanotransducer in other cellular systems [90, 91], and is also required for ISC expansion and organoid formation [69, 70, 81]. We showed that stiff matrices enhance ISC colony formation by inducing nuclear translocation of YAP in single-embedded ISCs. However, continued ISC proliferation within a stiff environment led to cell confinement and compression, which in turn resulted in gradual YAP inactivation. Creating matrices that are initially stiff enough to induce YAP activation but soften in a controlled manner to prevent cell compression rescued YAP inactivation and supported both ISC expansion and organoid formation. Thus, we used modular PEG-based matrices to unveil matrix stiffness as another important component of the ISC niche [81].

3.3. Future Perspective: Using Microengineering to Establish a Homeostatic ISC Niche In Vitro. A key difference between the intestinal niche *in vivo* and current organoid models is not only the types of niche components available but also the mode in which they are presented. Current organoid cultures contain essential niche signals, including Wnt and Notch

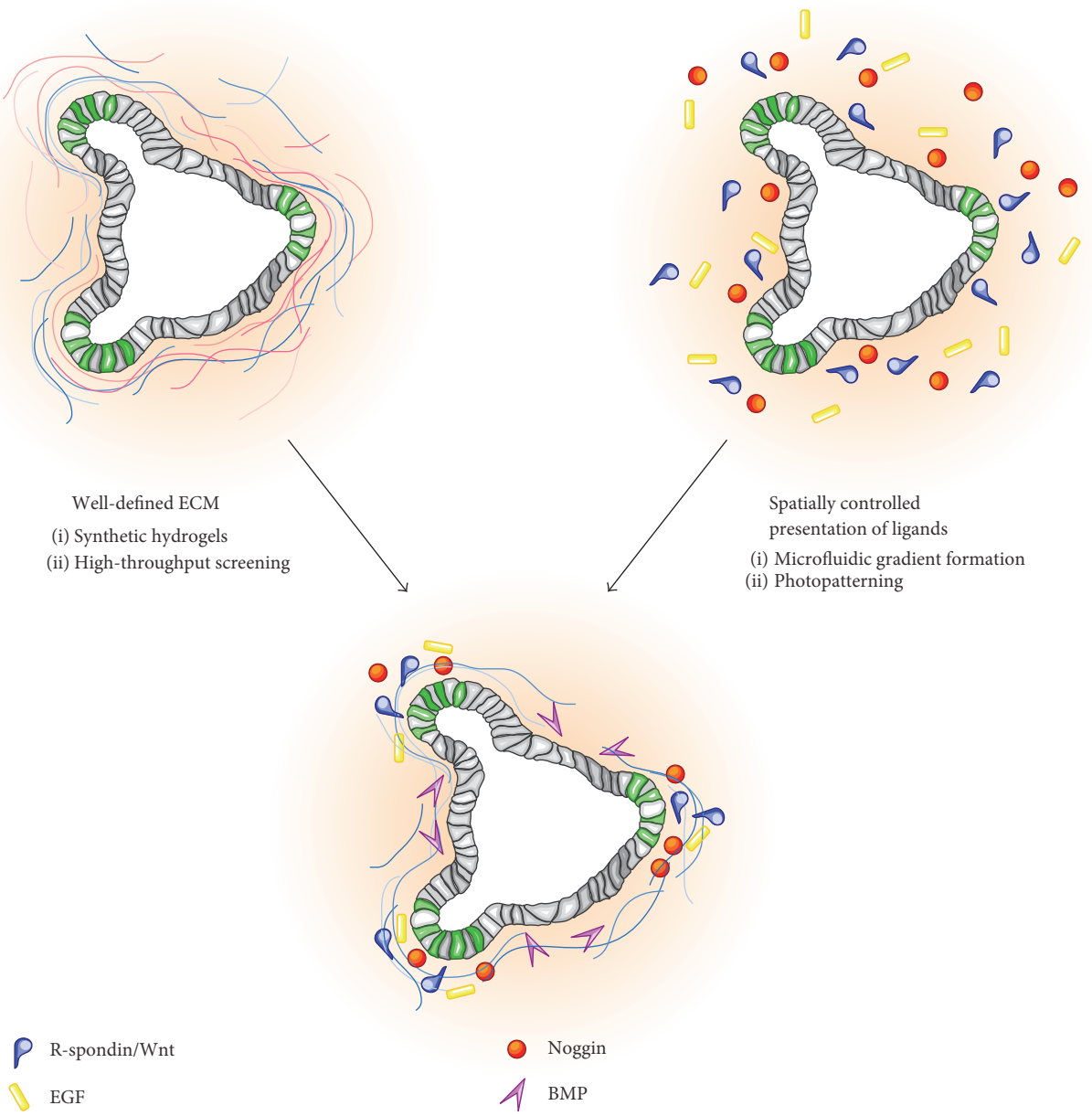

Figure 2: Engineering the ISC niche *in vitro*. Bioengineering approaches could further increase the tractability of organoid models and their fidelity to the real intestine. Synthetic matrices simplify the complexity of Matrigel and offer a powerful new toolkit with which to examine the effects of individual or combinations of ECM and mechanical niche signals. Microengineering approaches can be used to introduce spatial and temporal control over the biochemical and biophysical environment of ISCs, thereby mimicking the native niche more closely.

pathway agonists, and BMP inhibitors. However, these soluble components are included in the cell culture medium, wherein they eventually reach a uniform concentration. In contrast, master regulators of intestinal biology *in vivo* are presented in distinct spatiotemporal patterns, which are crucial for the regionalization of the intestine and the establishment of the ISC niche. For example, Wnt signals *in vivo* are produced by the subepithelial mesenchyme and Paneth cell and thus restricted to the bottom of the crypts where they are crucial for maintaining ISCs in a self-renewing state [92]. Bone morphogenetic protein (BMP) and Sonic Hedgehog (Shh) signals, are, on the other hand, enriched in the villus region, where they suppress proliferation and ensure differentiation into functional enterocytes [92]. This difference in the presentation of soluble cues may account for the fact that intestinal organoids are continuously expanding structures, with new crypt-like buds forming perpetually. Thus, intestinal organoids currently mimic a developmental or regenerative, rather than a homeostatic state, wherein ISC self-renewal is balanced by differentiation and apoptosis to establish a stable niche (the intestinal crypt).

Bioengineers have developed a number of strategies for controlling the spatiotemporal patterns of soluble and tethered cues in soft 3D media, similar to the matrices required

for organoid formation. Microfluidically generated morphogen gradients are perhaps the most widespread approach. Here, 3D hydrogels are microstructured using lithographic [93–95] or ablative [96] techniques. The resulting channels are loaded with a molecular of interest, which forms a gradient through the surrounding permeable gel. The shape of the gradient and, thus, the spatiotemporal mode of biomolecule delivery to encapsulated cells can be controlled by varying the concentration of the molecule at the source (the channel), the flow rate, and the diffusive properties of the permeable medium. Photopatterning approaches provide even finer spatial and temporal control over the distribution of mechanical and tethered molecular cues in 3D gels. These strategies use controlled illumination to locally change the properties of hydrogels that have been engineered to contain photosensitive building blocks [97–99]. The molecular changes induced can then be used to add or remove molecules or alter the mechanical stiffness of a desired region, at a desired time. We believe that these and other approaches for controlling the spatial and temporal presentation of diffusible or immobilized cues may be useful for the generation of the separate molecular zones seen in the native intestine, which could in turn contribute toward creating a more realistic *in vitro* model of a stable, homeostatic ISC niche (Figure 2).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Hindawi
Stem Cells International
Volume 2017, Article ID 5604727, 17 pages
https://doi.org/10.1155/2017/5604727

Review Article

Gut Microbial Influences on the Mammalian Intestinal Stem Cell Niche

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Received 4 May 2017; Accepted 2 August 2017; Published 22 August 2017

Academic Editor: Karen Liu

The mammalian intestinal epithelial stem cell (IESC) niche is comprised of diverse epithelial, immune, and stromal cells, which together respond to environmental changes within the lumen and exert coordinated regulation of IESC behavior. There is growing appreciation for the role of the gut microbiota in modulating intestinal proliferation and differentiation, as well as other aspects of intestinal physiology. In this review, we evaluate the diverse roles of known niche cells in responding to gut microbiota and supporting IESCs. Furthermore, we discuss the potential mechanisms by which microbiota may exert their influence on niche cells and possibly on IESCs directly. Finally, we present an overview of the benefits and limitations of available tools to study niche-microbe interactions and provide our recommendations regarding their use and standardization. The study of host-microbe interactions in the gut is a rapidly growing field, and the IESC niche is at the forefront of host-microbe activity to control nutrient absorption, endocrine signaling, energy homeostasis, immune response, and systemic health.

1. Introduction

The gastrointestinal (GI) tract is the primary site of nutrient absorption and digestion, a barrier to harmful toxins and pathogens, and the largest endocrine organ of the body involved in the maintenance of metabolic homeostasis. The intestinal epithelium comprises the innermost monolayer of cells in the GI tract that directly interfaces with the gut lumen and is replaced every 2-3 days in mice and 3–5 days in humans [1–3]. The monolayer is organized by units of villi (projections into the lumen) and crypts (invaginations into the lamina propria—connective tissue and immune cells that reside beneath the epithelial layer; see Figure 1). The villi contain specialized, differentiated cell types including cells of the absorptive lineage (e.g., enterocytes) and of the secretory lineage (e.g., enteroendocrine cells and goblet cells) [4]. The rapid renewal of these cells is driven by actively proliferating intestinal epithelial stem cells (IESCs) that reside at the base of the crypt in a functionally defined niche that includes epithelial Paneth cells as well as nearby nonepithelial cell types including immune cells of the lamina propria and stromal cells. The delicate balance in IESCs between self-renewal and differentiation controls intestinal epithelial homeostasis and regeneration, particularly in response to injury, inflammation, or altered microenvironment. The niche in which IESCs are embedded helps maintain this balance. In addition to the cell types mentioned above, microbiota residing in the intestinal lumen are key members of the IESC niche.

The intestine is a suitable environment for the habitation of a high density of microbes (>100 trillion bacteria, viruses, fungi, archaea, and protists) [5–9]. These resident microbes take part in a complex triangular ecological niche involving nutrients and host cells [5–7]. It is important to note, however, that the niche, much like the overall cellular composition, is nonuniform across different anatomical and functionally-distinct regions of the intestine, including the duodenum, jejunum, ileum, caecum, and colon. These different intestinal segments exhibit varying microbial density and composition and are subject to different nutritional and environmental exposures [8, 9]. Together with neighboring

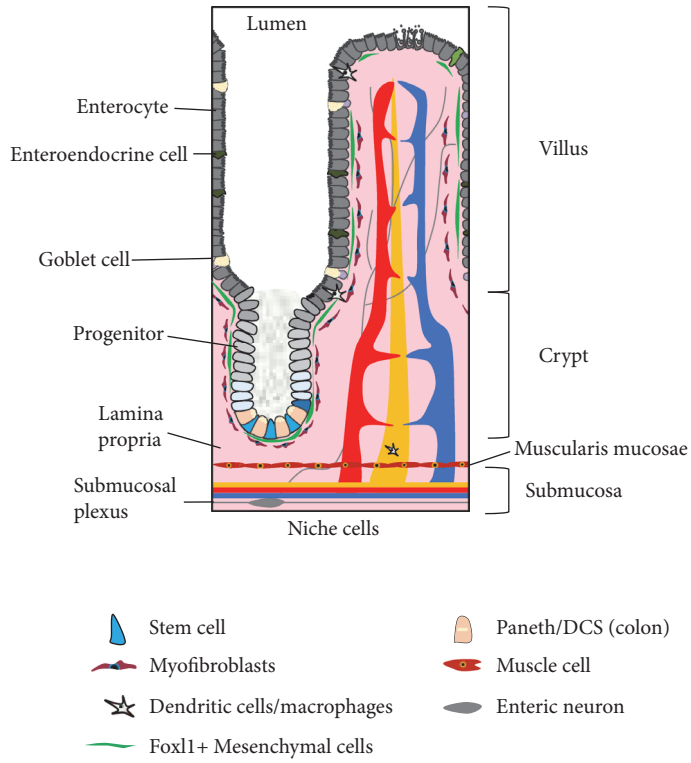

Figure 1: The intestinal stem cell niche. Intestinal stem cells have the capacity to generate, via a population of progenitor cells, all differentiated cell types of the intestinal epithelium including enterocytes, goblet cells, Paneth cells, and enteroendocrine cells. Those cell types that are known or suspected to comprise the intestinal stem cell niche include the adjoining Paneth cells of the small bowel, or the deep crypt secretory cells of the colon, as well as myofibroblasts, dendritic cells, macrophages, muscle cells, and enteric glia and neurons found in the subepithelial lamina propria and submucosal compartments of both small and large intestine.

host cells, the microbiota influence niche functions, and thereby modulate IESC behavior differently across the length of the intestine [10]. As such, it is important to consider regional differences in microbial composition that may contribute to different functions when studying the IESC niche. In what follows, we will provide an overview of the major cell types in the IESC niche and then a more detailed description of the known contributions of resident microbiota.

2. The Cell Types of the Intestinal Epithelial Stem Cell Niche

2.1. Intestinal Epithelial Stem Cells. The intestinal crypt in which IESCs reside harbors some IESCs-derived cell populations, including transit-amplifying progenitor cells, enteroendocrine cells (EECs), and Paneth cells [3, 11]. Under normal conditions, IESCs predominantly divide symmetrically [12, 13]. Certain stress contexts can trigger asymmetric division in order to prevent the hyperabundance of IESCs [14]. IESCs produce transit-amplifying progenitor cells that divide very rapidly (approximately every 12 hours) and comprise two-thirds of the base of the crypt. They progressively differentiate into various specialized intestinal epithelial cells (e.g., enterocytes) that generally migrate up the crypt-villus axis [12]. Once these differentiated cells reach the apex of the villus, they undergo anoikis (a form of programmed cell death, where cells detach from the extracellular matrix) and are released into the lumen of the intestine [15, 16]. Paneth cells and a subset of EECs represent exceptions to this pattern, as these cells can migrate downward toward the base of the crypt where IESCs reside, forming a part of the IESC niche. Paneth cells also have an increased lifespan relative to other differentiated cell lineages, estimated to be greater than 3-4 weeks before undergoing anoikis [17, 18]. And, while there are conflicting reports, some types of enteroendocrine cells may also survive longer than absorptive enterocytes [19, 20].

Crypt size, proliferative index, and the distribution of proliferative cells within the crypt are variable across the intestinal tract (see Figure 2, [8]). This type of regional variability is not uncommon in other organ systems with adult multipotent stem cells [21–23]. The actively cycling IESCs of the small intestine are located in the crypt base and are marked by high expression of several genes including *Lgr5*, *Olfm4*, and *Ascl2*, as well as by low expression of *Sox9* [24]. Slower cycling or reserve IESCs are marked by high expression of *Bmi1*, *Tert*, *Hopx*, *Lrig1*, and *Sox9*. However, these markers are not specific, as several of them are also found in actively cycling IESCs (e.g., *Lrig1*) or EECs (e.g., *Sox9*, [25]). Particularly fascinating is the observation that some secretory and absorptive progenitors exhibit plasticity; that is, the potential to revert back to IESCs in response to injury [26–29], suggesting that the reserve stem cell population is broader and less defined than certain differentiated

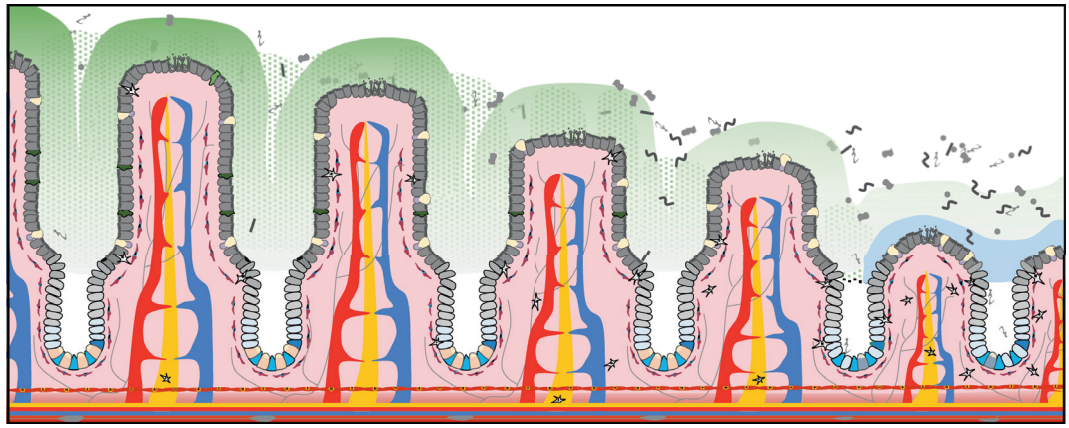

Figure 2: Regional differences along the small intestinal tract. The proximal-distal axis of the small intestine displays a gradient of various properties. Not only are mirobial loads progressively loads increased toward the ileal end of the intestine, but villus length gradually decreases in this same direction as well. The mechanisms by which luminal microbes could affect such changes in intestinal architecture may involve TLR activation, extracellular vesicles (EVs), metabolic byproducts, and/or other heretofore unspecified direct and indirect on intestinal epithelial stem cells.

IEC populations (also see reviews [30, 31]). Recent single-cell transcriptomic work has shown that there is heterogeneity even among Lgr5+ actively cycling IESCs [32]. This molecular heterogeneity was also seen in earlier studies comparing populations of $CD24^{lo}$ and side-population IESCs [33], as well as in a very recent RNA-seq-based comparison of IESC populations isolated by diverse methods [34].

The chromatin state, and many transcription factors and signaling cascades, regulate IESC stemness. The position of the IESC within the crypt is a major determining factor of its self-renewal capacity, driven in part by Wnt and Delta-Notch signaling [12, 35]. Transcription factors such as Klf5, Gata4, Gata6, Ascl2, and Yy1 have been shown to control intestinal stem cell fate, and their deficiency causes disruption of intestinal architecture [14, 36]. More recently, microRNAs too have emerged as key regulators of the niche and responders to environmental stimuli in IESCs [37, 38]. For example, miR-375 in murine IESCs is highly sensitive to the presence of microbes, and loss-of-function studies in ex vivo mouse enteroid cultures suggest that it may be a prominent regulator of intestinal epithelial proliferation [38]. For further detailed review of IESCs, we refer the reader to recent review article [39].

2.2. Paneth Cells. Paneth cells are epithelial cells of the small intestine that are located between and around IESCs and take part in shaping the crypt microenvironment and regulating microbial interactions within the crypt by secreting antimicrobial peptides [40]. They are present throughout the entire small intestinal tract, and they increase in number along the proximal-distal axis. Unlike villus epithelial cells that get replaced every 3–5 days, the life span of Paneth cells in the crypt is about 30 days [40]. As part of their role in the niche, they also release growth factors that directly influence the neighboring IESCs [40], cementing their role in the niche. Under environmental stress, Paneth cells act to protect and stimulate IESCs. For example, under conditions of caloric restriction, luminal cyclic adenosine diphosphate (cADP) derived from Paneth cells induces IESC. Interestingly, however, ablation of Paneth cells *in vivo* does not appear to impact IESC proliferation and differentiation [41] or the distribution of microbes within the gut [17], possibly due to compensatory responses by other niche cells [41]. Loss of Paneth cells has been shown to compromise the barrier integrity of the intestinal epithelium [42]. Recent work has suggested that the large intestine may also harbor Paneth cell-like deep crypt secretory (DCS) cells [43]. More work is needed however to evaluate these cells further and determine the extent to which they contribute to colon IESC niche functions [43]. For further detailed descriptions of Paneth cells, we refer the reader to the following reviews [44, 45].

2.3. Enteroendocrine Cells. Enteroendocrine cells (EECs) are occasionally located within the crypt and play a vital role in gut physiology and may contribute to the IESC niche microenvironment [46, 47]. Though EECs make up less than 1% of all intestinal epithelial cells, they have an important function in sensing the luminal environment (nutrients, bile acids, microbes, etc.) and secreting hormones, including Glp-1, Cck, Pyy, Gip, ghrelin, and neurotensin, in order to coordinate systemic energy regulation [48, 49]. There are many different subtypes of EECs based on the hormones that they most readily express and secrete. For example, both I cells and K cells are EEC subtypes that reside predominantly in the proximal small intestine, but secrete the hormones CCK and GIP, respectively, which have different endocrine effects [50, 51]. The abundance and types of EECs vary throughout the gastrointestinal tract; some EEC subtypes are found throughout the small and large intestine (e.g., N cells: neurotensin-secreting EECs), whereas others are found primarily in the small intestine (e.g., K cells, I cells, and S cells: gastric inhibitory peptide-, cholecystokinin-, and serotonin-secreting EECs, resp.) [50]. EECs are also abundant in the rectum, where they are found at the highest frequency in the GI tract other than the proximal small bowel [52–54]. It has been suggested that crypt EECs, or possibly

secretory progenitor cells in general, may comprise a reserve pool of IESCs that actively proliferate in response to intestinal injury [25, 49, 55, 56]. Their contributions to the maintenance of IESC function remain poorly characterized; however, it is known that certain EEC-secreted peptides, such as Glp-2, can serve as paracrine signaling molecules to promote intestinal epithelial proliferation [57]. EECs have also been shown to respond to microbe-derived peptides and therefore may act as a conduit signaling mechanism for the IESC niche [58]. For example, recently it was shown that colonic exposure to proteins from *Escherichia coli* stimulate Pyy and Glp-1 release from EECs in rats [59]. Much more work remains to be done in order to define more rigorously the functional importance of crypt EECs to the IESC niche.

2.4. Stromal Cells. In the adult intestine, the epithelium is surrounded by stromal cells of the mesenchymal lineage. These cells facilitate intercellular crosstalk through several factors that regulate IESC proliferation and differentiation and therefore are considered an integral aspect of the IESC niche. Subepithelial mesenchymal stromal cells produce bone morphogenetic proteins (BMPs), which are members of the TGF-β superfamily that antagonize Wnt signaling along the crypt-villus axis, thereby inhibiting IESC expansion and promoting epithelial cell differentiation [60]. Other mesenchymal cells including myofibroblasts secrete BMP inhibitors that promote Wnt-mediated IESC self-renewal [61]. Recently, a seminal study by Aoki et al. and Kaestner and colleagues described a small population of elongated *Foxl1*-expressing mesenchymal cells that envelop both the crypts and villi of the intestinal epithelium and produce a number of growth factors including those of the Wnt and Bmp family to support IESCs [62]. Ablation of these cells, but not other niche cells like Paneth cells, results in severely compromised crypt proliferation. These data suggest that the *Foxl1*+ mesenchymal cell population constitutes an essential component of the IESC niche [35]. In sum, the entire collection of subepithelial stromal cells mediates an intricate signaling network that maintains balance between IESC self-renewal and differentiation along the crypt-villus axis. Comprehensive characterization of the functional diversity of mesenchymal cells and their roles in the niche remains an active and important area of research.

Macrophages are crucial sentinels in the healthy intestinal lamina propria that are required for maintenance of intestinal homeostasis in the face of microbiota and food antigens [63]. Epithelial tuft cells and goblet cells mediate immune response to microbes and microbial-derived peptides by secreting chemokines to which these intestinal macrophages readily respond [64–68]. In both rodents and humans, intestinal macrophages are more numerous in the small intestine than in the large bowel. It is increasingly being recognized that macrophages, in addition to serving an innate immune function, can regulate intestinal stem cell function. Recently, Saha et al. found that radiation-induced intestinal injury is ameliorated by enhanced stem-cell proliferative function stimulated in part by macrophage-secreted Wnt factors [69].

2.5. Enteric Nervous System. The enteric nervous system (ENS) plays a vital role in many aspects of GI tract function, including orchestrating peristalsis and fluid secretion required for food digestion and nutrient absorption and sustaining a healthy luminal microbiome. Also, it has been found that the ENS can influence IESC function. For example, Lundgren et al. have shown that modification of mucosal afferent nerve function modulate IESC proliferation [70]. Given that enteric nerve cells act synchronously with clonally related neurons, the effect may be broadly translated across multiple crypts [71]. Moreover, in addition to their neural support roles, glial cells of the ENS also contribute to intestinal epithelial proliferation and repair after injury through the secretion of proepidermal growth factor (pro-EGF) [72, 73].

For detailed reviews of the diverse cells types within the IESC niche, see [11, 74, 75].

3. Role of Gut Microbiota in the Stem Cell Niche

To maintain gut homeostasis and proper function, IESCs must respond either directly or indirectly to apical luminal and basolateral abluminal factors, most notably gut microbiota and dietary components. Cells of the IESC niche have evolved a number of mechanisms to manage a constantly changing luminal microenvironment. Constituents of the intestinal microbiota and their products are potentially highly potent regulators of IESC activity due to their proximity to the intestinal epithelia, as well as their profound effects on host nutrition, metabolism, and mucosal barrier integrity.

3.1. Region-Specific Roles for Gut Microbiota in the Control of Intestinal Epithelial Renewal. Decades of research on murine models has revealed that luminal bacteria can shape a variety of morphological and functional features of different intestinal regions and cellular subpopulations. One of the oldest observations was made in the 1960s through studies of germ-free (GF) and antibiotic-treated mice and rats. It was noted that these rodents exhibited decreased villus height and crypt depth in the jejunum and ileum, increased villus height and decreased crypt depth in the duodenum, reduced mucosal surface area, lowered mitotic indices, reduced lamina propria volume, and slower transepithelial migration rates compared to conventionally raised (CR) animals [76–84]. These findings were suggestive of one or more of several possibilities. For example, shorter crypts could be indicative of decreased proliferation, and/or increased/premature differentiation, and/or progenitor apoptosis. Subsequent follow-up studies have evaluated these possibilities and are shedding light on the context-specific effects of colonization on intestinal physiology. Current state-of-the-art follow-up studies include whole transcriptome profiling (both aggregate and single-cell) and fluorescent immunohistochemistry for markers of active proliferation and apoptosis. For example, we recently demonstrated that genes in pathways associated with mitotic cell cycle are transcriptionally upregulated in jejunal cell populations enriched for stem cells of conventionalized animals relative to GF animals [38]. Yu et al. also demonstrated increased ileal crypt proliferation in ex-germfree mice in

response to colonization with microbiota from healthy infants relative to colonization with microbiota from infants with low weight gain [85]. In studies in which GF rodents were exposed to commensal microbes, increased colonic epithelial proliferation and deepened large bowel crypts were observed [86, 87]. Although this effect was reported in other small intestinal regions as well, it was evident that the magnitude of the effect of microbes on epithelial morphology is region-specific [88, 89]. Duodenal and jejunal intestinal epithelia from CR mice display slightly increased proliferation relative to ileum, despite the fact that duodenal and jejunal luminal bacterial loads are substantially less than what is found in the ileal lumen [90]. The potential primacy of microbial composition over total bacterial number on the control of intestinal epithelial proliferation was demonstrated by the observation that exposure to specific bacterial species such as the breast milk-derived probiotic strain *Lactobacillus reuteri* DSM 17938 induces intestinal epithelial proliferation while other strains like *L. reuteri* PTA 6475 do not [91]. Viruses may also contribute to overall intestinal epithelial morphology and physiology. For example, certain strains of murine norovirus can modulate innate immunity and mediate some negative effects on the intestinal epithelium of dextran sodium sulfate and certain antibiotic treatments [92].

3.2. Mechanisms of Microbial Influence on IESCs. Although it is clear that the presence of luminal microbes is correlated with structural and functional changes in IECs, it is often difficult to determine whether microbes or the experimental treatments that induce microbial changes are responsible for these effects. Modifications of diet and antibiotic treatments have been employed historically to alter the intestinal microbiota in order to study host effects. However, identifying the precise, and likely multiple, mechanisms by which microbiota influence the IESCs has proven challenging especially given the regional specificity and diversity of microbes and their derived metabolites. Regulation of IESCs by microbiota may occur either through direct and or indirect means, and understanding mechanisms of niche-microbe interactions has therapeutic relevance. Secreted factors that stimulate the Wnt/β-catenin signaling pathway are the primary means by which the niche offers support for IESCs. For example, following injury from radiation, mesenchymal stem cells activate the Wnt/β-catenin signaling pathway and support $Lgr5^+$ stem cell growth to promote regeneration [93]. Similarly, as mentioned above, Saha et al. demonstrated that macrophages secrete Wnt factors in exosomes to support the intestinal stem cell niche during regeneration and protect it from radiation-induced injury [69]. Yet, the extent to which these and other niche cells act in response to changes in the gut microbiota during homeostasis or following injury has not been fully elucidated.

3.2.1. Potential Mechanisms of Direct Influence. The intestinal stem cell niche has been described as being maintained under completely sterile conditions in the absence of injury [94–96]. However, microbes residing within intestinal mucosa, and indeed within healthy intestinal crypts, are well documented, which raises the possibility of direct regulation of intestinal stem cell physiology by gut microbiota. The earliest visualization of microbiota in direct contact with the intestinal epithelium was in the 1970s using scanning electron microscopy on mouse intestine. These studies showed microbes attached to the openings of the crypts of Lieberkühn via long webbing filaments [97–100], and not fully separated from the epithelium by the mucus layer. However, it was not until recently that microbes were visualized deep within crypts [101, 102]. One main challenge in identifying these crypt-based microbes stems from the use of common washing and fixation methods that dissolve or disturb microbial biofilms and host-mucins [98, 103]. Using a fixation method that preserves the biofilms, such as anhydrous Carnoy's fixative, together with extremely cautious sectioning techniques, has further improved visualization of microbes within intestinal crypts [5, 95, 99, 101, 102]. Current research suggests that crypt-based microbes are found primarily in the colon and caecum, which is consistent with the overall microbial density gradient within the gut [5, 101, 102]. Bacterial species found within the crypt, as identified by 16S sequencing, and fluorescent in situ hybridization (FISH) of murine colonic crypts, indicate the predominance of bacteria capable of aerobic metabolism, including species of *Acinetobacter* and *Proteobacteria* [5]. This finding is interesting given that the flora of the small intestine is also enriched for aerobes [104, 105]. Following GI infection, certain pathogenic microbes have been found to more frequently occupy the crypt niche, even in the upper GI tract, and it has been suggested that colonization of the crypts might promote pathogenic longevity leading to chronic infections, as is seen with *Helicobacter pylori* in the stomach [106]. On the other hand, the presence of residing *H. pylori* in gastric crypts also prevents secondary infections, a form of "colonization resistance," which may be beneficial to the host's health.

Less well studied is the possibility that microbiota may stimulate IESCs directly through the release of outer membrane vesicles (OMVs). Given that IESCs take up macrophage-derived exosomes [69], much like what has been observed in enterocytes, it is possible that IESCs also take up outer membrane vesicles (OMVs) produced by gram-negative bacteria localizing at the base of villi or within the crypts [107–109]. OMVs are similar in size to exosomes and are taken up via similar pathways, such as through caveolin or lipid raft-mediated endocytosis [110]. OMVs may carry bacterially derived and molecularly active peptides, virulence factors, small RNAs, and DNA, all of which could act to modify IESC gene expression patterns. Uptake of OMVs by IESCs has not been formally evaluated, though uptake of OMVs by other intestinal epithelial cells has been demonstrated [111]. This may suggest multiple possibilities by which microbes directly regulate IESC gene expression and cellular behavior.

3.2.2. Potential Mechanisms of Indirect Influence. Resident microbiota, as part of the symbiotic relationship with humans, metabolize and ferment foods in the intestinal lumen. Byproducts and metabolites from these processes

can be absorbed or act as receptor ligands by both the host as well as by other microbes within the gut. Some of the most widely studied microbial metabolites include short-chain fatty acids (SCFAs), which are produced primarily in the colon through the fermentation of dietary fibers. Kaiko et al. found that SCFA butyrate suppressed colonic stem cell proliferation [112], perhaps through receptors encoded by *Ffar3*, *Ffar2*, and *Niacr1* [113]. *Ffar2* is robustly expressed in mouse jejunal IESCs and is downregulated upon conventionalization [38]. Importantly, enterocyte metabolism of butyrate at the entrance of the colonic crypt was an important modulator of the SCFA dosage received by IESCs, suggesting that certain enterocytes may support the niche [114]. However, given the trace amounts of SCFAs in the small intestine, this may not be a prominent pathway regulating the small bowel IESC niche. In this same screen by Kaiko et al., nicotinic acid (or niacin) was found to have pro-proliferative effects on colonic stem cells [112]. Niacin is ingested or biosynthesized by the gut microbiota [115] and may therefore be a strong candidate for regulation of small intestine IESCs. Further research into small intestinal metabolites that regulate IESCs is warranted.

Microbial stimulation of non-IESC niche cells may result in the secretion of signaling peptides that in turn influence IESC physiology. For example, Paneth cells of the small intestine (and possibly DCS cells of the colon) form a major component of the IESC niche. They secrete a number of antimicrobial peptides and growth factors including lysozyme, α-defensins, WNT, EGF, and Notch to their neighboring stem cells, and when dysregulated leave the host more susceptible to infection and other physiological abnormalities (see reviews [40, 116–118]). TLR activation in Paneth cells is associated with the degranulation and secretion of defensins into the crypt [119–122], which would modulate the niche microenvironment. However, it is not yet clear what the precise effect of Paneth cell degranulation is on IESC physiology.

Other niche cells may provide more insight, though in some cases their actions on IESCs may be interdependent. Niche cells respond to various microbial signals (e.g., via TLR receptors) and metabolites (e.g., SCFA), resulting in a number of downstream stimuli that could alter IESC physiology. Some EECs, such as L-cells located along the crypt-villus axis, release Pyy and Glp-1 in response to microbial stimuli [119]. Pyy in turn stimulates intestinal epithelial proliferation and differentiation both *in vivo* and *in vitro* [123, 124]. EECs that reside outside the niche may also contribute toward the control of IESC behavior by serving as intermediates in multicellular signaling pathways initiated by resident microbes. Tuft cells have recently been shown to respond in part to parasites and helminthes by secreting IL-25 [125]. IL-25 induces innate lymphoid cells to secrete the IESC stimulating factor IL-13 [67], resulting in increased goblet and tuft cell differentiation [65]. IL-33 expression in intestinal stromal cells provides another possible mechanism by which microbes may regulate IESCs, as some microbes, including helminths and other parasites, induce IL-33 release from lymphocytes [126]. For example, it was recently shown that TNF-α- and IL-1β-stimulated IL-33 release from pericryptal fibroblasts in response to *Salmonella typhimurium* infection promotes secretory cell differentiation of IESCs [127]. Sources of IL-33 are not limited to these fibroblasts; epithelial cells may also express IL-33 thereby further regulating IESC differentiation.

Finally, microbiota-derived neurostimulatory peptides, including glutamate, serotonin, and GABA, as well as macronutrients like glucose and fatty acids, can act as neurotransmitters to stimulate the enteric nervous system, which in turn can regulate IESC function (see reviews Mazzoli and Pessione [58] and Neunlist and Schemann [128]).

4. Tools to Study Microbiota Interactions in the IESC Niche

A number of questions remain with regard to how the microbiota may influence the IESC niche. Over the past several decades, experimental models have been developed, which span *in vitro*, ex vivo, and *in vivo* methodologies (Table 1). Here, we touch on the most recently developed as well as the most widely used tools for studying IESC-microbe interactions.

4.1. In Vitro Models to Study Intestinal Host-Microbe Interaction. One of the most straight-forward and widely used *in vitro* cell culture models to study host-microbe interactions are coculture systems. Typically, an intestinal epithelial cell line (e.g., Caco-2, HIECs, T84, IEC6, and HT29s) will be seeded as a monolayer, on transwells, or on a scaffold device. Bacteria, or bacterial supernatant, or other microbes, may be added to the culture chamber either directly to the cells or separated by some type of membrane or barrier [129–131]. Metabolic, molecular, and physiological assays can then be conducted in the hours or days following. These coculture experiments are scalable, highly reproducible, and straightforward to conduct in most labs with standard cell culture equipment. Additional cell types, such as primary-derived macrophages or PBMCs [132–134], can be included in the coculture. Despite the ease of performing these coculture experiments, they harbor limitations with regard to mimicking *in vivo* physiological conditions. To address this limitation, researchers have recently developed interesting *in vitro* coculture microfluidic, scaffold, and three-dimensional (3D) systems [135–138]. For example, Chen et al. developed a tube culture system to coculture enterocyte-like Caco-2 cells, Goblet-like HT29-MTX cells, and H-InMyoFibs myofibroblast cell lines. The tube structure allows researchers to pass media and bacteria across cells, while also mimicking the oxygen and nutrient gradients present *in vivo* within the intestinal tract [137]. Nonetheless, many of the cell lines used are transformed and therefore may not always faithfully represent primary cells. Moreover, there exist no known cell lines for certain intestinal cell types such as Paneth cells [45].

4.2. Ex Vivo Models to Study Intestinal Host-Microbe Interaction. More recently, researchers have moved to the use of ex vivo three-dimensional (3D) primary enteroid and intestinal organoid models to evaluate epithelial-microbe interactions [38, 93, 139–141]. Intestinal tissue is isolated

Table 1

Coculture type	Description	Pros	Cons	Reference
Monolayer	An intestinal cell line (or ex vivo enteroids) is grown in monolayers on standard cell culture plate or transwell. Bacteria are added to the media and cocultured for hours or days.	(i) Assay effects of single bacterial strain or pathogen on IECs (ii) Quick growth (iii) Good reproducibility (iv) Bacteria exposure remains apical (v) Easily multiplexed (vi) Coculture IECs with other intestinal niche cell types available (vii) Easy genetic manipulated in culture via transfections or infection (viii) Certain assays are more easily applied to monolayers	(i) Cell lines are somewhat homogenous and poorly reflect niche cell behavior (ii) Poorly reflect the regional specificity of the intestine (iii) Bacteria can quickly outgrow epithelial cells (iv) Monolayers poorly reflect IE conditions or mucus layer physiology (v) Certain niche cells lack representative cell lines	[129–134, 148, 155]
3D-scaffold	Intestinal cell lines (or ex vivo enteroids) are seeded onto a fabricated 3D-scaffold. Bacteria are added to the media and cocultured for hours, days, or months.	(i) Assay effects of single bacterial strain or pathogen on IECs (ii) Coculture IECs with other intestinal niche cell types (iii) Quick growth (iv) Depending on model, can better replicate movement, morphology, rigidity, oxygen, and nutrient gradients relative to monolayer models	(i) Difficult setup and/or specialized materials or parts (ii) May be difficult to multiplex	[135–138, 156–159]
Mucosal explant	Intestinal tissue biopsies or slices are taken, and mucosa/submucosa can be isolated and plated on cell culture plates or transwell inserts. Selected bacterial strains are added to the media and cocultured for hours or days.	(i) Can easily assay effects of single bacterial strain or community on primary tissue (ii) Better replicates *in vivo* environment than monolayer, cell line cultures (iii) Good viability in presence of commensal microbes (iv) Produce the wide range of metabolites and cytokines found *in vivo*	(i) Cannot be passaged or replicated (ii) May require specialized media and expensive growth factors (iii) Difficult to identify cell-type-specific effects/responses to microbiota	[149, 150, 160, 161]

Table 1: Continued.

Coculture type	Description	Pros	Cons	Reference
Enteroids/ organoids	IESCs or crypts are isolated fresh or derived from induced pluripotent or embryonic stem cells and suspended in a collagen-rich matrix (Matrigel). Growth factors are added to the media to support their growth. Bacteria should be injected into the lumen or added to the media, as enteroids/organoids form with the villi on the inside and crypts projecting outward.	(i) Assay effects of single bacterial strain or community on primary tissue (ii) Better replicates *in vivo* environment than monolayer, cell line cultures (iii) Produce the wide range of metabolites and cytokines found *in vivo* (iv) Can be passaged indefinitely and cryopreserved (v) Can easily be genetically manipulated in culture via transfections, gymnosis, or infection (vi) Can generate from patient-derived tissue or any available genetic model	(i) Injection of bacteria requires specialized equipment and expert technical skill (ii) May require specialized media and expensive growth factors	[38, 61, 139, 152, 162–165]
Introduction model	Animals are derived or maintained in a GF (gnotobiotic) facility. Selected bacterial strains or mixed microbiota (such as reconstituted fecal matter) are introduced to the animals.	(i) Can assay effects of mono- or polycolonization (ii) Can colonize with patient-derived microbiota (iii) Variables can be tightly controlled (iv) GF animals can be maintained under GF conditions indefinitely (v) Highly reproducible	(i) GF mice have altered development and physiology (ii) Limited number of genetic models readily available at most gnotobiotic facilities (iii) Expensive to generate and house	[85, 166–171]
Depletion model	CR or specific-pathogen free animals are given broad spectrum antibiotics, typically in drinking water, to remove measurable traces of microbiota. Microbiota may be reintroduced to the animals passively, or through forced colonization.	(i) Assay effects of single bacterial strain or community on primary tissue (ii) No need for gnotobiotic facility or equipment (iii) Very affordable to conduct (iv) More realistic in terms of human disease and physiology (v) Any available genetic model can be used	(i) Antibiotic treatment alters host gene expression independent of microbiota [172] (ii) Does not fully eliminate all microbes	[42, 91, 172–174]

and single cells, crypts, or whole mucosa is extracted and grown in a collagen-rich matrix, such as Matrigel. Enteroids and organoids will grow into large 3D masses containing all mature cell types of the isolated tissue, which more accurately mimics *in vivo* physiology compared to *in vitro* models [142]. Enteroids refers to cultures consisting solely of intestinal epithelial tissue, whereas organoids are derived to contain multiple tissue types, such as epithelia, enteric nerves, myofibroblasts, and smooth muscle cells [143]. Enteroid cultures can be passaged indefinitely making them a viable alternative to immortalized cell lines. Of note, these structures can also be derived using induced pluripotent stem cells (iPSC cells) [144, 145]. Because enteroids will form sealed "lumens," with villi forming on the inside and crypts projecting outward, microbes should be microinjected into the lumens to evaluate host-microbe interactions (see [146, 147] for review). Microinjections of enteroids and organoids can be challenging. Moreover, the tendency of these structures to occasionally burst and then reseal can be prohibitive to long-term studies of injected microbes. Nevertheless, we recently demonstrated that IESCs grown in enteroid culture can be genetically manipulated using gymnosis to knockdown gene and microRNA expression [38]. Recently, monolayer versions of ex vivo enteroid culture systems have emerged, which expand the number of assays that can be performed, including patch clamps and live imaging studies [134, 148]. Less widely used are ex vivo mucosal explants and slice models, which, like organoids, contain a full complement of intestinal cell types [149, 150] and like coculture systems can be manipulated by adding microbes to the culture media (see review [151]). However, even with high oxygenation, small bowel explants have not been cultured successfully beyond 48 hours, and are not easily multiplexed like some enteroid systems [152], which severely limits their usefulness [149, 153]. Despite the advantages of using these culture systems, results of experiments intended to evaluate the effects on IESCs could be confounded by the presence of mature, differentiated intestinal cell types. Certain small molecules may assist in enriching for IESCs, for example, valproic acid and CHIR99021 [154], which could help clarify direct effects of microbes on IESCs.

4.3. In Vivo Models to Study Intestinal Host-Microbe Interaction. Finally, there are a number of *in vivo* methods to study the effect of microbiota on the intestinal stem cell niche. These models typically fall into one of two classes: introduction-based or depletion-based. In introduction models, a GF animal is exposed to microbes in a process termed "colonization." Depletion models on the other hand aim to remove microbiota from a CR animal through the exposure to broad-spectrum antibiotics. Sometimes, researchers may combine approaches and reintroduce microbiota following depletion [175–177]. There are benefits and limitations to both approaches.

While the systemic and intestinal physiology of GF mice is atypical, these animals provide a "blank slate" for researchers to evaluate the effects of single strains, defined sets of microbes, or undefined microbiota on the stem cell niche. However, as humans are never reared in GF conditions, the clinical utility of GF models is often questioned [178]. Nevertheless, GF animals provide a valuable resource. Attempts at generating GF animals began before the beginning of the 20th century using chickens and guinea pigs [179, 180]. However, multigenerational GF animals were not described until much later in the 20th century (see [180, 181] for review). Currently, GF animals are acquired surgically through aseptic caesarian section or embryo transfer, and then maintained under sterile conditions in specialized isolation chambers. Food, water, and bedding must be sterilized prior to being introduced to animals, and fecal matter as well as cage environments are regularly checked to verify that no microbes have unintentionally been introduced. While GF animals survive, and in fact may live longer than CR animals [182], they develop abnormally and have altered behavior, metabolism, digestion, and immune system function [180]. Colonization of GF animals with microbes elicits a robust immune response, which takes several weeks to normalize to a state more similar to that of CR animals [166–168, 183]. The dynamic process of conventionalization is an important consideration as animal age, length of colonization, and animal diet contribute to microbial community structure and immune response. Moreover, colonization dynamics demonstrate substantial regional specificity. Temporal and regional dynamics of GF mouse conventionalization have been examined, most notably in a series of papers by El Aidy and colleagues [166–168, 184]. From these studies and others, we know certain developmental processes have a limited timeframe during which microbial colonization of GF animals may restore phenotypic similarity, especially within the immune system, with CR animals (see review [185]). Temporal and regional changes are also quite robust, with genes involved in innate immunity being most different in the first couple of days following colonization and stabilizing between 2 and 3 weeks postcolonization [167, 186, 187]. Regionally, immune cell recruitment occurs more rapidly in the small intestine compared to the colon in the days postcolonization [167], which has the potential to affect niche response. Many studies have performed colonization at different ages and for different lengths of time, making cross-study comparisons challenging. Moreover, differences in housing conditions, bedding material, and nonsterilized foods can introduce variables that further confound cross-study comparisons. The evaluation of the *in vivo* effect of specific microbes can be achieved using GF animals. However, because early microbe exposure significantly affects immune development and other physiological functions, the results of some gnotobiotic experiments may not reflect what occurs in animals that have been exposed to microbes since birth [188]. Despite these limitations, GF models have been used successfully to evaluate the effect of microbiota on IESCs, including many studies employing laser capture microdissection (LCM) [42, 85, 172, 189, 190] to isolate and test the effect of microbiota on the niche. For example, using LCM, Yu et al. [85] assessed the effects of microbiota on crypt cell gene expression following colonization of GF animals with microbiota collected from neonatal patient samples. Others have shown specific effects of antibiotics

and colonization on gene expression in the intestinal crypts [172]. LCM of intestinal epithelial crypts includes several cell lineages, though it is possible to enrich for IESCs by genetically depleting Paneth cells [190]. This method, however, is labor intensive and does not result in high yields of RNA. As an alternative to LCM methods, we derived GF Sox9-EGFP reporter mice, which allow for the isolation of IESCs and progenitor cells using fluorescent-activated cell sorting (FACS), allowing for more precise assaying of cell-type-specific effects of microbiota [38].

Depletion of bacteria in CR animals using broad spectrum antibiotics is another approach for investigating the effect of microbiota on the stem cell niche. The major advantages are that such depletion-based approaches are substantially less expensive and quicker to conduct. However, there are several limitations. Notably, it has been shown that antibiotic treatment alone, irrespective of microbial depletion, can modify host gene expression and cause alterations to the intestinal epithelium, especially within the crypt compartment [172]. Moreover, complete elimination of microbiota using antibiotics is unlikely [169, 191], especially since most broad spectrum antibiotics specifically target bacteria, leaving enteric fungi and viruses to flourish. Nevertheless, antibiotic treatment continues to be a widely used model to investigate the effect of microbes on the host. It is likely that a combination of both introduction and depletion models could be helpful to evaluate fully the effect of microbial factors on the niche [192].

Another strategy that circumvents both gnotobiotic and antibiotic models is surgery to create isolated intestinal segments, such as Thiry-Vella fistulas, to determine the effect of autonomous microbial changes on intestinal function without experimental modification of the lumen [193]. However, this *in vivo* model eliminates normal luminal flow which of course does not properly reflect normal physiology. Despite the inherent limitations of all of the investigative methods, much has been learned concerning the mechanisms mediating microbial influences on host intestinal epithelial structure and function.

5. Conclusion and Discussion

The IESC niche constitutes a complex network of cell types expanding well beyond the epithelial layer to help govern the balance between IESC self-renewal and differentiation. The mammalian IESC is comprised of epithelial cells including IESCs, Paneth cells, and EECs, as well as nonepithelial components including stromal, neural, and immune cell types. It is also evident that gut microbiota have a prominent influence on intestinal epithelial physiology and stem cell function. However, the underlying mechanisms remain poorly understood and are still under active investigation. A major challenge is the isolation of functionally distinct cellular subpopulations and niche cells from the intestine as well as the difficulty in ascertaining the specific effect of individual microbes, metabolites, and other microbe-derived products. Several *in vitro*, ex vivo, and *in vivo* tools are available to investigate the relationship between host and microbe within the gut, and the research community has made substantial strides in the last decade. Nevertheless, several key questions remain, most notably the following: (1) Do IESCs respond to direct signals from gut microbiota? (2) Which niche cells are essential for proper microbial control of IESCs? (3) Do IESCs provide feedback to intestinal microbiota? (4) Does the niche contribute to the selection of microbes which reside in crypts, and what if any are the unique functions of the crypt-based microbes in regulating IESC behavior? (5) How are host-microbe interactions altered by diet, age, disease, or anatomic position along the GI tract? The answers to these questions will significantly advance our understanding of the role of host-microbe communication in normal intestinal physiology and in driving gastrointestinal diseases.

As we continue to address these and related important questions, moving forward, it is our opinion that special care must be taken to standardize relevant *in vitro*, ex vivo, and *in vivo* experiments in order to facilitate cross-study comparisons. For example, in terms of *in vivo* studies, given what we know of regional specificity and variability, we believe it is important whenever possible to report measurements from all three major small intestinal segments as well as the colon. Also, as rodents ingest bedding material, a considerable source of fiber, studies using animal models should include specifics as to bedding material, the diets used throughout the study course, the housing conditions (single versus cohoused, open versus closed ventilation, and light/dark cycles), the age at (and duration of) colonization, and the source, composition, and handling of the microbiota used for colonization, all of which have previously been shown to affect microbial composition.

The development of probiotics or engineered bacteria, as well as molecular strategies such as those based on microRNAs, represent exciting possibilities for modulating the gut microbiome and the IESC stem cell niche and thereby modifying intestinal physiology. Such efforts could in the long-term provide benefit to patients with a wide range of gastrointestinal diseases. With many recent advances in tools and technologies for exploring direct and indirect interactions between microbes and host IESCs, we anticipate significant progress in this area over the next decade.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Funding was generously provided by 1-16-ACE-47 (Praveen Sethupathy via American Diabetes Association). The authors would like to thank Dr. Jason Spence of the University of Michigan, Dr. Ilana Brito of Cornell University, and Dr. John Rawls of Duke University for their invaluable comments and suggestions on this manuscript. Additionally, the authors thank Dr. Susan Henning of the University of North Carolina for the informal discussions regarding the mammalian intestinal epithelial stem cell niche.

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Hindawi
Stem Cells International
Volume 2017, Article ID 6012810, 9 pages
https://doi.org/10.1155/2017/6012810

Review Article

Targeting Cancer Stem Cells and Their Niche: Current Therapeutic Implications and Challenges in Pancreatic Cancer

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Received 31 March 2017; Revised 17 June 2017; Accepted 20 June 2017; Published 6 August 2017

Academic Editor: Hong Qian

Cancer stem cells (CSCs) have been identified as a subpopulation of stem-like cancer cells with the ability of self-renewal and differentiation in hematological malignancies and solid tumors. Pancreatic cancer is one of the most lethal cancers worldwide. CSCs are thought to be responsible for cancer initiation, progression, metastasis, chemoresistance, and recurrence in pancreatic cancer. In this review, we summarize the characteristics of pancreatic CSCs and discuss the mechanisms involved in resistance to chemotherapy, the interactions with the niche, and the potential role in cancer immunoediting. We propose that immunotherapy targeting pancreatic CSCs, in combination with targeting the niche components, may provide a novel treatment strategy to eradicate pancreatic CSCs and hence improve outcomes in pancreatic cancer.

1. Introduction

Pancreatic ductal adenocarcinoma, referred to in this review as pancreatic cancer, is one of the most lethal malignancies around the world. In 2012, an estimated 338,000 new cases were diagnosed and 330,000 deaths occurred worldwide [1]. Despite advances in the diagnosis and treatment of pancreatic cancer, there has been little improvement in the survival of the patients over the past two decades [2, 3]. The 5-year survival for all stages of pancreatic cancer diagnosed from 2007 to 2013 is 8.2% in the USA [4]. Pancreatic cancer continues to be a challenging disease. Radical resection remains the only potentially curative treatment. However, more than 50% of patients are diagnosed locally advanced or metastatic and only 15–20% of patients have resectable disease at the time of diagnosis [5]. Nevertheless, a significant proportion of patients who undergo surgical resection followed by adjuvant therapy will experience recurrence [6]. To date, chemotherapy is the main treatment option for patients with advanced pancreatic cancer [7, 8]. Several clinical trials have shown a modest survival benefit, such as FOLFIRINOX (oxaliplatin, irinotecan, leucovorin, and fluorouracil) and nab-paclitaxel plus gemcitabine [9, 10]. Improved understanding of the interactions between pancreatic cancer cells and the tumor microenvironment (TME) provides valuable therapeutic targets for pancreatic cancer [11]. For instance, targeting tumor-associated macrophages (TAMs) with CCR2 inhibition in combination with FOLFIRINOX in patients with borderline resectable and locally advanced pancreatic cancer has shown encouraging results with moderate toxicity in a phase Ib trial [12]. However, the clinical efficacy of systemic chemotherapy and molecular-targeted therapies, such as EGFR and VEGFR inhibition, in the management of pancreatic cancer is still considered unsatisfactory [13–15]. Therefore, exploring mechanisms involved in pancreatic cancer evolution is urgently required. Increasing evidence supports the idea that a subpopulation of pancreatic cancer cells, called pancreatic cancer stem cells (CSCs), plays a significant role in the process of tumor initiation, local invasion, distant

metastasis, chemoresistance, and relapse in pancreatic cancer [16, 17]. Therapeutic approaches to target CSCs are expected to have widespread clinical implications for pancreatic cancer treatment.

2. Overview of Pancreatic CSCs

The existence of CSCs and their role remained obscure largely due to technological challenges for a long time [18, 19]. During the past two decades, numerous studies have provided support for this concept. In 1997, Bonnet and Dick first identified $CD34^{++}CD38^{-}$ cells as CSCs in human acute myeloid leukemia [20]. Since then, CSCs have been identified in various solid tumors including breast cancer, brain tumor, pancreatic cancer, melanoma, head and neck cancer, and colorectal cancer [21–27]. All these findings reveal that CSCs, a subpopulation of cancer cells with the ability to self-renew and the capacity to proliferate and differentiate, are the driving force for cancer initiation, progression, metastasis, and chemoresistance [28–30].

Pancreatic CSCs were first identified in 2007. Li et al. established human pancreatic cancer xenografts in NOD/SCID mice. After 16 weeks, xenografts were digested and sorted for the markers of CD44, CD24, and epithelial-specific antigen (ESA)/epithelial cell adhesion molecule (EpCAM). Sorted cells were then injected into NOD/SCID mice. They identified a subpopulation of pancreatic cancer cells with the specific cell surface markers $CD44^{+}CD24^{+}ESA^{+}$ as pancreatic CSCs, which showed stem-cell-like properties of self-renewal, the ability to produce differentiated progeny, and upregulation of developmental signaling molecule sonic hedgehog [24]. Then, Hermann et al. demonstrated CD133 as a cell surface marker of pancreatic CSCs. $CD133^{+}$ pancreatic cancer cells were highly tumorigenic and resistant to gemcitabine. As few as 500 $CD133^{+}$ pancreatic cancer cells were capable of forming orthotopic tumors in athymic mice, but 10^{6} $CD133^{-}$ cells did not result in any tumor formation. Elimination of $CD133^{+}CXCR4^{+}$ pancreatic cancer cells significantly reduced the metastatic potential of pancreatic cancer [31]. In 2010, Rasheed et al. identified aldehyde dehydrogenase (ALDH) expression as a marker for pancreatic CSCs. ADLH-positive pancreatic cancer cells showed enhanced clonogenic growth and high migratory ability, which had a negative impact on the overall survival of patients with pancreatic cancer [32]. In 2011, Li et al. identified c-Met as a new marker for pancreatic CSCs. c-Met^{high} pancreatic cancer cells could form spheres and c-Met inhibitor or knockdown of c-Met significantly inhibited tumor sphere formation in vitro. c-Met^{high} cells had increased tumorigenic potential in mice. They established human pancreatic cancer xenografts in NOD/SCID mice and found that administration of c-Met inhibitors could inhibit tumor growth, reduce the population of pancreatic CSCs, and prevent metastases when given alone or in combination with gemcitabine [33]. In 2014, Bailey et al. described microtubule regulator, doublecortin and Ca^{2+}/calmodulin-dependent kinase-like 1 (DCLK1) as a morphologically and functionally distinct population of pancreatic CSCs. Pancreatic cancer cells expressing DCLK1 displayed high clonogenic potential. Inhibition of γ-secretase activity reduced the abundance of these cells in murine pancreatic intraepithelial neoplasia (PanIN) and prevented PanIN progression [34]. Fujiwara et al. identified CD166 expression as another important characteristic of tumorigenicity and invasive and migratory activities of pancreatic cancer cells. $CD166^{+}$ pancreatic cancer cells were more tumorigenic, while $CD166^{-}$ cells exhibited stronger invasive and migratory activities [35].

In addition to the identification of specific phenotypes, several studies aim to characterize of pancreatic CSCs based on gene expression analysis. Bao et al. reported that pancreatic CSCs ($CD44^{+}/CD133^{+}/EpCAM^{+}$) exhibited differential expression of more than 1600 mRNAs, including *BMP4*, *FoxQ1*, Sox4, and *Wnt3a*, compared with $CD44^{-}/CD133^{-}/EpCAM^{-}$ cells. The knockdown of FoxQ1 in pancreatic CSCs resulted in the inhibition of aggressive behaviour [36]. Skoda et al. identified 602 differentially expressed genes in pancreatic CSCs ($CD24^{+}/CD44^{+}/EpCAM^{+}/CD133^{+}$), including upregulated Wnt signaling (WNT2, WNT2B, FZD6, and FZD7), upregulation of LYN expression, and downregulation of FYN expression [37]. These differentially expressed genes are supposed to be essential for regulating functions and phenotypes of pancreatic CSCs. Recently, a study using a combined approach with high-sensitivity mutation detection and whole-transcriptome analysis of the same single cell to characterize CSCs in patients with chronic myeloid leukemia during treatment with tyrosine kinase inhibitors provides insights into disease evolution and points to new therapeutic targets [38]. This method which exemplifies how single-cell analysis can identify CSCs might be applied to other cancers, including pancreatic cancer.

According to the two most common models, intratumoral heterogeneity arises hierarchically and stochastically. These models explain CSCs from different perspectives and are not mutually exclusive [39]. Here, we mainly discuss the hierarchical model (Figure 1). According to this model, carcinogenesis occurs when stem cells, progenitor cells, or differentiated cells give rise to CSCs. Even though much effort has been made to identify and characterize pancreatic CSCs, the origin of pancreatic CSCs is still widely unknown [40]. One hypothesis is that pancreatic CSCs may originate from stem cells or progenitor cells that reside in normal tissues with accumulating mutations, which ultimately trigger a malignant transformation [41]. Pancreatic islets are formed by self-duplication of adult cells, and their formation does not rely on stem cells [42]. However, this does not preclude the existence of stem cells in the pancreas. On the other hand, it is also possible that mature cells may transform into CSCs. The pancreas is composed of endocrine cells (α-cells, β-cells, etc.), acinar cells, and ductal cells, which all derive from a common progenitor expressing Pdx1 [43]. Both ductal cells and acinar cells have been proposed as cellular origins for the development of pancreatic cancer [44, 45]. Under certain conditions, pancreatic ductal cells or acinar cells acquire genetic alterations and dedifferentiate into pancreatic CSCs. Finally, pancreatic CSCs and their differentiated progeny contribute to tumor heterogeneity.

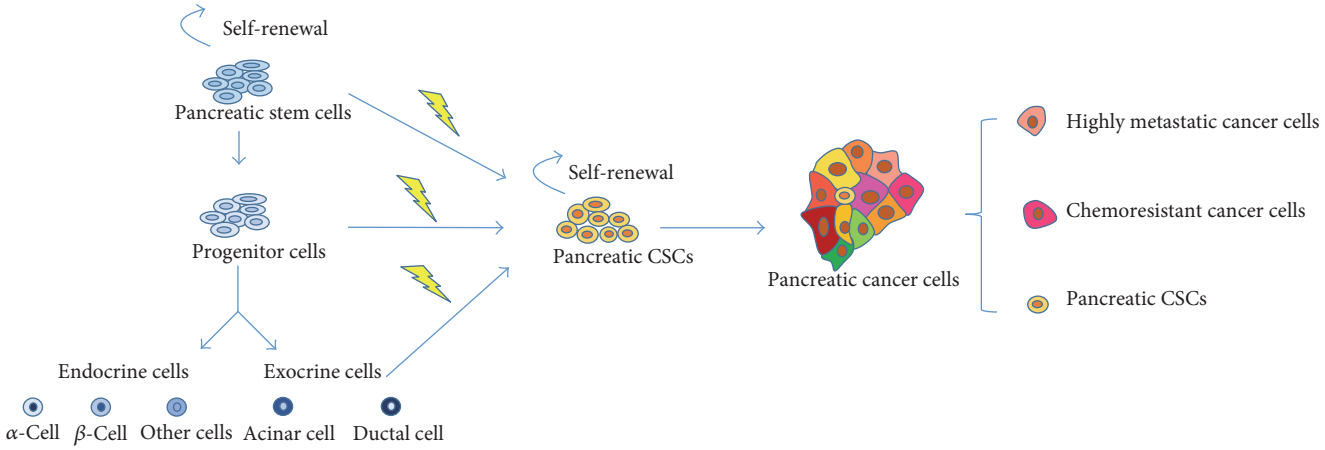

Figure 1: The origin of pancreatic CSC hypothesis. Normal stem cells give rise to progenitor cells that proliferate and differentiate into various types of mature cells, including α-cells, β-cells, acinar cells, and ductal cells. Pancreatic CSCs may originate from the transformation of normal stem cells or progenitor cells through the accumulation of mutations. On the other hand, under certain conditions, pancreatic ductal cells and acinar cells may acquire genetic alterations and dedifferentiate into pancreatic CSCs. Pancreatic CSCs have the ability of self-renewal and differentiation. Finally, pancreatic CSCs and their differentiated progeny contribute to tumor heterogeneity.

3. The Pancreatic CSC Niche

As is the case for normal stem cells, pancreatic CSCs require nutrients and signals from the surrounding microenvironment, also called pancreatic CSC niche, to achieve a dynamic balance between self-renewal and differentiation. As an anatomically distinct region within the TME, the pancreatic CSC niche is comprised of different types of cells and noncellular components, such as non-CSC cancer cells, cancer-associated fibroblasts (CAFs), pancreatic stellate cells (PSCs), immune cells, blood and lymphatic vessels, extracellular matrix (ECM), cytokines, chemokines, and growth factors [46].

Direct cell-cell interactions between pancreatic CSCs and stromal cells, as well as signaling pathways mediated through the expression and secretion of a range of growth factors and cytokines, play a key role in the regulation of pancreatic CSCs. PSCs can form a niche for CSCs to promote in vitro sphere formation and invasiveness by paracrine Nodal/Activin signaling [47]. TGF-β treatment significantly increases the proportion of pancreatic CSCs, which exhibit a high degree of epithelial-mesenchymal transition (EMT) and great invasion and migration activity in vitro [48]. Depletion of TAMs and inflammatory monocytes by inhibiting either the myeloid cell receptor colony-stimulating factor-1 receptor (CSF1R) or chemokine (C-C motif) receptor 2 (CCR2) decreases the number of pancreatic CSCs [49]. Another important contributor to the pancreatic CSC niche is CAFs. CAF-derived CXCL12 attracts CXCR4 expressing CSCs, and fibronectin secreted by fibroblasts promotes CSC attachment [50]. CAFs can stimulate stemness via activation of WNT and NOTCH pathways [51]. Pancreatic cancer is characterized by remarkable desmoplasia [52, 53]. CAF activation leads to the ECM remodelling [54, 55]. In normal tissues, the ECM has an effect on cell proliferation, differentiation, and migration [56]. Receptors expressed within the ECM allow stem cells to anchor to specific locations and communicate with surrounding cells within the niche. Loss of the ECM results in a decrease of stem cell numbers [57, 58]. The accumulation of the ECM in pancreatic cancer destroys the normal pancreatic architecture, promotes EMT, enhances CSC marker expression, and forms a barrier blocking therapeutics [59]. All these cellular and noncellular components establish a supportive niche to maintain the properties of CSCs and regulate their fate.

Targeting pancreatic cancer stroma is a promising new therapeutic option, but recent studies have spurred some controversy. Rhim et al. discovered that sonic hedgehog-deficient tumors had reduced fibroblast-rich desmoplastic stroma, aggressive behaviour, undifferentiated histology, increased vascularity, and heightened proliferation [60]. Ozdemir et al. found that depletion of CAFs and fibrosis led to enhanced numbers of pancreatic CSCs, immunosuppression, and reduced survival [61]. Saridegib is a small molecule targeting smoothened in the sonic hedgehog pathway. The inhibition of the hedgehog pathway depleted the tumor stroma, enhanced delivery of gemcitabine, and improved survival in a mouse model of pancreatic cancer [62]. However, a phase I/IIb trial of saridegib plus gemcitabine in patients with metastatic pancreatic cancer was stopped in 2012 because interim data showed that patients receiving the combination therapy had higher rates of progressive disease and lower overall survival than patients receiving placebo plus gemcitabine [63]. These findings suggest that some stromal elements might actually restrain tumor growth. Thus, the complex cross-talk between pancreatic cancer cells, including CSCs, and the stroma should be evaluated by further studies.

4. Resistance of Pancreatic CSCs to Chemotherapy

One key attribute of pancreatic CSCs is chemotherapy resistance, which may initially reduce the tumor bulk but fail to

eradicate CSCs, resulting in recurrence of pancreatic cancer. Notably, resistance of pancreatic CSCs to chemotherapy is mediated by both intrinsic factors of CSCs and extrinsic factors of the CSC niche.

Cioffi et al. found that miR-17-92, targeting NODAL/ACTIVIN/TGF-β1/p21 signaling, was suppressed in gemcitabine-resistant pancreatic CSCs. Overexpression of miR-17-92 cluster or knockdown of p21 could inhibit chemoresistance of pancreatic CSCs [64]. The ATP-binding cassette (ABC) transporter, ABCG2, is an important source of drug resistance in cancer [65]. However, Bhagwandin et al. found that in pancreatic cancer, ABCG2 did not efflux gemcitabine and inhibition of ABCG2 did not sensitize pancreatic CSCs to gemcitabine [66]. Family with sequence similarity 83 member A (FAM83A) could promote pancreatic CSC-like traits by activating the Wnt/β-catenin and TGF-β signaling pathways and chemoresistance in pancreatic cancer. Inhibition of FAM83A significantly enhanced the sensitivity of pancreatic cancer to gemcitabine [67]. Our previous study also defined a distinguished group called side population (SP) cells from a metastatic human pancreatic cancer cell line with highly tumorigenic and metastatic characteristics after orthotopic injection. In particular, these SP cells showed properties of pancreatic CSCs. Wnt, NOTCH, and EGFR signaling pathways associated with CSCs were altered in SP cells. The proportion of SP cells was significantly enriched when cultured with increasing concentrations of gemcitabine [68]. In addition, as a part of the TME, the pancreatic CSC niche also contributes to chemoresistance. Extensive fibrosis produced by PSCs results in significant hypoxia in the pancreatic CSC niche. In turn, hypoxia stimulates PSCs to induce fibrosis and angiogenesis [69]. This impairs drug delivery and stimulates EMT, promoting chemoresistance of pancreatic cancer cells [70]. In addition, aberrant accumulation of ECM in the pancreatic CSC niche can reduce the penetration of chemotherapeutic agents [71].

5. The Potential Role of Pancreatic CSCs in Cancer Immunoediting

Evading immune destruction is considered as a hallmark of cancer, but the mechanisms are not yet fully understood [72, 73]. The concept of cancer immunoediting describes the dynamic interaction between cancer and immune cells during cancer progression. Cancer immunoediting consists of three stages: elimination, equilibrium, and escape [74–76]. New mechanisms of immune escape are continuously discovered and translated to preclinical and clinical studies. Increasing studies have focused on the cross-talk between CSCs and immune cells, and recent findings raise the possibility that CSCs might get involved in the process of cancer immunoediting [75, 76]. Here, we speculate the potential role of pancreatic CSCs in different stages of cancer immunoediting (Figure 2).

In the elimination process, both innate and adaptive immune cells play a critical role in cancer immunosurveillance [77]. Several driver genes have been identified in pancreatic cancer, including tumor suppressor genes *CDKN2A*, *SMAD4*, and *TP53* and the oncogene *KRAS* [78–80]. Although immune response has been described to some of these antigens, the majority of T-cell antigens are located outside of classical driver mutations [81]. During pancreatic cancer initiation, malignant cells with these genetic mutations can upregulate activating NK cell receptor ligands and downregulate inhibitory ligands. For example, major histocompatibility complex class I-related chains A and B (MICA/B) are frequently expressed on the surface of pancreatic cancer cells. Such ligands bind to NKG2D on NK cells and other immune cells, activating NK cell cytotoxicity and leading to the release of proinflammatory cytokines, which facilitate the anticancer immune response [82]. Tumor-specific $CD8^+$ T-cells can recognize and eliminate pancreatic cancer cells expressing tumor-associated antigens [83]. However, pancreatic CSCs exhibit a quiescent behaviour and low immunogenicity, which probably makes them the right candidate to escape immune surveillance [84, 85].

In the equilibrium process, immune response and pancreatic cancer progression are balanced [86]. The quiescent behaviour and longevity of pancreatic CSCs makes it easy to accumulate genetic and epigenetic alterations and survive the equilibrium process [87]. Upon asymmetric division, a cancer stem cell generates a daughter stem cell for self-renewal and a daughter cell that undergoes further differentiation. The differentiated pancreatic cancer cells are subjected to immunosurveillance, and most of them could be detected and destroyed by the immune system as mentioned above. In contrast, poorly immunogenic cancer cells are more likely to escape from immunosurveillance. In breast cancer, the downregulation of MICA/MICB on CSCs promotes the resistance of breast CSCs to NK cell cytotoxicity and lung metastasis formation [88]. Whether pancreatic CSCs survive by this mechanism needs to be explored. In the meanwhile, the pancreatic CSC niche is not totally established yet. The dependence of pancreatic CSCs on their niche may restrain their rapid propagation [89]. The equilibrium process is functionally similar to the state of tumor dormancy [90]. The pancreatic CSCs may stay dormant for a long time before eventually becoming clinically apparent.

In the escape process, pancreatic cancer cells successfully evade immune destruction. Several factors can result in the weakening of the immune system, such as aging, immunosuppressive drugs, and systemic immunosuppression. On the other hand, the TME of pancreatic cancer is generally regarded as poorly immunogenic and could also contribute to immune escape of pancreatic CSCs [91]. Pancreatic cancer cells are able to reprogram the TME via secretion of immunosuppressive factors and recruitment of immunosuppressive cells, such as regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs), both of which can suppress the cytotoxicity of $CD8^+$ T-cells and NK cells [92–94]. Monocytic MDSCs increase the frequency of ALDH1 (Bright) pancreatic CSCs and promote mesenchymal features of pancreatic cancer cells through tumor-induced STAT3 activation [95]. Besides, as mentioned above, PSCs, CAFs, and TAMs can also support pancreatic CSCs growth and promote immunosuppression in the niche. The immunosuppressive niche allows pancreatic CSCs to rapidly produce specialized cancer cells with high metastatic potential

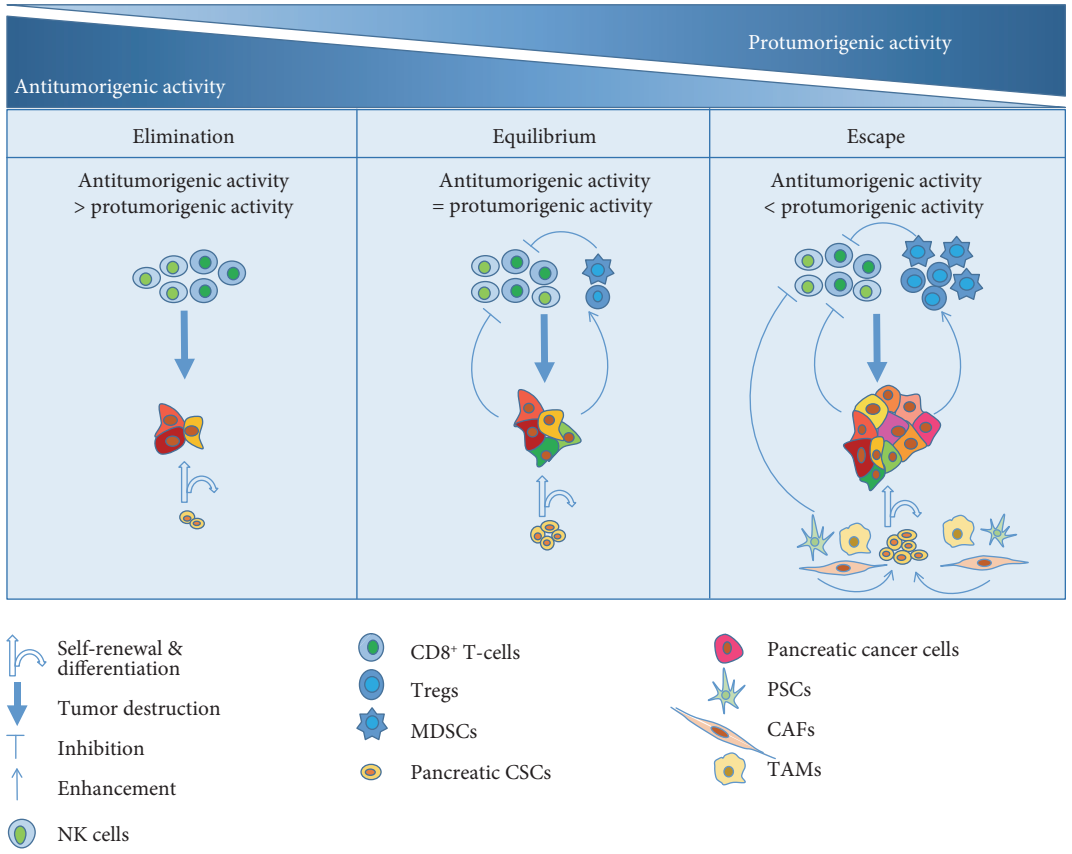

Figure 2: The potential role of pancreatic CSCs in cancer immunoediting. Elimination (left): in the elimination process, most of pancreatic cancer cells can be successfully detected and destroyed by the innate and adaptive system. However, pancreatic CSCs are believed to be immunologically privileged like normal stem cells. Low immunogenicity prevents pancreatic CSCs from recognition and elimination by the host immune system. Equilibrium (middle): in the equilibrium process, the immune system and pancreatic cancer cells that have survived the elimination process enter into a dynamic equilibrium. The function of the immune system can be negatively regulated by cancer cells and stromal cells. The majority of pancreatic cancer cells are destroyed, but some cancer cells acquire the ability to avoid immune destruction. The equilibrium process is functionally similar to the state of tumor dormancy. Escape (right): in the escape process, pancreatic cancer cells can inhibit host anticancer immunity by secretion of immunosuppressive factors and by recruitment of stromal cells, such as Tregs and MDSCs. Besides, PSCs, CAFs, and TAMs also support pancreatic CSC growth and promote immunosuppression. The immunosuppressive niche allows pancreatic CSCs to rapidly produce specialized cancer cells with high metastatic potential or chemoresistance. Finally, pancreatic CSCs and their differentiated progeny progressively grow into a visible tumor in the pancreas and even metastasize to distant sites.

or chemoresistance. Finally, pancreatic CSCs and their differentiated progeny progressively grow into a visible tumor in the pancreas and even metastasize to distant sites.

Although the biological properties of pancreatic CSCs may help to explain how pancreatic cancer avoid immune destruction, the underlying mechanisms of pancreatic CSCs in cancer immunoediting remain to be further investigated.

6. Conclusion

Remarkable research results have been made in identifying characteristics of CSCs in pancreatic cancer over the last decade. Pancreatic CSCs have been suggested to exhibit high resistance to current therapies. However, there has been limited progress in developing alternative therapeutic options to eradicate pancreatic CSCs. Recently, cancer immunotherapy has emerged as an attractive research field in cancer treatment. Immune checkpoint inhibitors targeting CTLA-4, PD-1, and PD-L1 have shown clinical benefit in patients with advanced melanoma, non-small-cell lung cancer, and several other cancers [96–98]. Several phase I/II clinical trials studying the safety and efficacy of immune checkpoint inhibitors are being conducted in pancreatic cancer. In spite of efficacy in mismatch repair-deficient patients, the response is very poor [99, 100]. Due to the potential role of pancreatic CSCs in cancer immunoediting, immunotherapy targeting pancreatic CSCs and the niche components may provide a novel treatment strategy for pancreatic cancer [101, 102].

Pancreatic CSCs express specific markers, including CD24, CD44, CD133, EpCAM, CXCR4, c-Met, and CD166, at levels substantially different from the bulk pancreatic cancer cells. These markers not only have proven useful for identification and isolation of pancreatic CSCs but also can be considered as potential targets for cancer immunotherapy [103]. In addition, targeting the niche components may also help to eliminate CSCs [104]. Schatton et al. reported that

CSCs inhibited T-cell activation by expression of PD-1 and B7.2 in melanoma [105, 106]. Lee et al. demonstrated preferential expression of PD-L1 on CSCs in head and neck cancer [107]. These findings raise the possibility that pancreatic CSCs might actively suppress anticancer immunity through CTLA-4 and PD-1 pathways. Assessment of the expression of immune checkpoint molecules on pancreatic CSCs and their niche will be necessary to verify whether this is the case in pancreatic cancer. In addition, Ames et al. found that NK cells preferentially killed pancreatic CSCs in vitro and intratumoral injection of activated NK cells in the human pancreatic cancer-bearing NSG mice significantly reduced the number of pancreatic CSCs and tumor burden [108].

Therefore, immunotherapy targeting pancreatic CSCs and their niche holds tremendous promise in pancreatic cancer treatment. Further research is urgently needed to improve our understanding of pancreatic CSCs and to develop more effective therapeutic strategies to eradicate pancreatic CSCs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Hindawi
Stem Cells International
Volume 2017, Article ID 7970385, 11 pages
https://doi.org/10.1155/2017/7970385

Review Article

Intestinal Stem Cell Niche: The Extracellular Matrix and Cellular Components

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Received 5 April 2017; Accepted 4 July 2017; Published 1 August 2017

Academic Editor: Karen Liu

The intestinal epithelium comprises a monolayer of polarised columnar cells organised along the crypt-villus axis. Intestinal stem cells reside at the base of crypts and are constantly nourished by their surrounding niche for maintenance, self-renewal, and differentiation. The cellular microenvironment including the adjacent Paneth cells, stromal cells, smooth muscle cells, and neural cells as well as the extracellular matrix together constitute the intestinal stem cell niche. A dynamic regulatory network exists among the epithelium, stromal cells, and the matrix via complex signal transduction to maintain tissue homeostasis. Dysregulation of these biological or mechanical signals could potentially lead to intestinal injury and disease. In this review, we discuss the role of different intestinal stem cell niche components and dissect the interaction between dynamic matrix factors and regulatory signalling during intestinal stem cell homeostasis.

1. Introduction

The intestinal epithelium is a monolayer of cells covering the entire lumen of the gut that constitutes an important barrier against the external environment. Both small and large intestine share similar glandular crypt structure where intestinal stem cells (ISCs) reside. Crypts are formed by epithelial invaginations into the extracellular matrix (ECM), cushioned by supportive stromal cells. The ISCs, marked by the leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), reside at the crypt base alongside their neighbouring Paneth cells [1]. The ISCs divide and give rise to daughter cells entering the transit-amplifying (TA) zone. The TA cells will then proliferate and migrate upwards towards the crypt-villus junction, where they terminally differentiate into all different cell types, including enterocytes, goblet cells, enteroendocrine cells, and tuft cells, before reaching the villus tip and being exfoliated into the lumen, with the exception of Paneth cells that will migrate downwards back to the stem cell zone. The whole ISC proliferation-differentiation journey from the base of the crypt to the villus tip lasts approximately 3–5 days [1–3].

ISCs in the crypt base are maintained by their surrounding niche for precise regulation of self-renewal and differentiation under homeostasis. The ISC niche can be categorised fundamentally into two major components: the "physical" niche and the "cellular" niche. The physical niche refers to the ECM, which comprises an intricate network of fibrous structural proteins (proteoglycans and glycoproteins) that act as scaffolding to maintain the three-dimensional architecture of the intestine. Examples of ECM components surrounding the intestinal crypts include fibronectins, laminin isoforms, collagens, and glycosaminoglycans (GAGs) [4–11]. The cellular niche refers to the stromal microenvironment that comprises all the resident cells embedded within the ECM. These include pericryptal myofibroblasts, fibroblasts, endothelial cells, pericytes, immune cells, neural cells, and smooth muscle cells, which secrete a wide range of matrix components and growth factors for the control of ISC proliferation and differentiation [12, 13]. In addition, Paneth cells constitute another important cellular niche intrinsically within the intestinal crypt. Paneth cells are direct neighbours of LGR5+ stem cells that provide crucial niche factors and signals to support ISC homeostasis [3].

Communication between the ISCs and their niche is regulated by multiple signalling pathways such as the Wnt/β-catenin cascade, Notch signalling, Transforming growth factor (TGF-β)/bone morphogenic protein (BMP) pathways, and Hedgehog pathways. Perturbations of these pathways or ECM homeostasis due to inflammation, toxins, chemotherapy, and nutritional deprivation can substantially affect the ISC niche, leading to increased susceptibility to intestinal diseases. The ECM is also suggested to act as a reservoir for growth factors via heparin sulfate proteoglycan binding, which may assist in establishing morphogen gradients [14]. These growth factors may also be released upon ECM degradation. In this review, we discuss the contributions of the ECM and cellular microenvironment to the ISC niche and highlight the signalling pathways involved in ISC regulation.

2. The Cellular Niche

The mesenchymal compartment of the intestinal lamina propria contains multiple stromal cell populations with distinct phenotypes and function. These include fibroblasts, myofibroblasts, endothelial cells, pericytes, neural cells, smooth muscle cells, and immune cells (Figure 1). The role of intestinal stromal cells in mucosal immunity and homeostasis has been extensively summarised and discussed in several comprehensive reviews, therefore will not be addressed in this review [13, 15, 16]. We will focus on the role of other mesenchymal cells as well as the Paneth cells in ISC homeostasis.

2.1. Mesenchymal Cells. Fibroblasts and myofibroblasts constitute the major cell components in the lamina propria. Fibroblasts drive wound healing but also have pathological implications in a range of diseases, including carcinogenesis, in various organs. Intestinal subepithelial myofibroblasts (ISEMFs), a member of the fibroblast family, are located in pericryptal regions throughout the lamina propria [17]. TGF-β is thought to be a key factor inducing myofibroblast differentiation [18]. ISEMFs play a pivotal role in the ISC niche by secreting crucial factors such as Wnt ligands and BMP antagonists for stem cell maintenance [13, 19, 20]. ISEMFs exhibit characteristics of both fibroblasts and smooth muscle cells with contractile ability. Upon wound healing, an inflammatory response triggers ISEMFs to secrete the matrix metalloproteinases (MMPs) for matrix remodelling [21]. Once the healing process resolves, ISEMFs will undergo apoptosis mediated in part by IL-1β [18, 22]. Excessive ECM deposition, on the other hand, is associated with a pathological persistence of activated ISEMFs such as in inflammatory bowel disease [22, 23].

Smooth muscle cells, present in close association with ISEMFs, form a thin layer of muscle (muscularis mucosa) to separate the lamina propria from the submucosa. The smooth muscle cells contract and relax to keep the muscularis mucosal layer under constant agitation [13]. This function serves to expel potentially toxic luminal contents out of the crypts and away from the ISC niche. Similar to ISEMFs, smooth muscle cells also express BMP antagonists to repress the differentiative BMP signal and maintain the Wnt activity in the crypt base [20].

Endothelial cells present in the lamina propria appear to be important in maintaining epithelial homeostasis. Previous data showed that radiation-induced injury triggered rapid endothelial apoptosis prior to epithelial death *in vivo* [24]. Importantly, loss of epithelial stem cells did not occur when endothelial apoptosis was blocked by basic fibroblast growth factor (bFGF) treatment or by genetic deletion of the acid sphingomyelinase (*ASMase*)—a gene that is required for radiation-induced endothelial apoptosis. Endothelial cells are also implicated in the induction of intramucosal immune responses [16, 25]. Further investigation is required to fully understand their niche role in ISC homeostasis.

Pericytes are periendothelial myofibroblast-like contractile cells wrapping around the capillaries, which regulate angiogenesis and capillary wall permeability via paracrine signalling [26]. However, the identity of the pericytes remains controversial regarding their ontogeny and progeny. Distinction between populations of pericytes and myofibroblasts is challenging since they express similar molecular markers [27]. Subsets of pericytes have been reported to be multipotent progenitors that may participate in tissue regeneration [28]. The specific role of pericytes in the ISC niche remains unclear. It is believed that pericytes may function similarly as ISEMFs based on their close developmental origin and identity [26, 27].

Neural cells are important for the intestinal epithelial growth. Bjerknes and Cheng showed that enteric neurons participate in the feedback loop that regulates epithelial growth and repair by expressing the glucagon-like peptide 2 (GLP-2) receptor [29]. The enteric nervous system consists of a large number of neurons and enteric glia cells (EGCs) that are interconnected to form the two ganglionated plexuses—the myenteric and the submucosal plexuses. EGCs are located both within the ganglia and in the extraganglionic regions, such as the lamina propria with close proximity to the intestinal crypts [30, 31]. In addition to their neuroprotective function, these mucosal EGCs are thought to play crucial roles in maintaining the intestinal epithelial barrier. Recent data show that EGC homeostasis postnatally is dependent on functional host-microbe interactions, indicating their role in regulating immune responses in the gut [32]. The EGCs also exert protective functions on the intestine by secreting factors such as epidermal growth factor (EGF) and TGF-β isoforms following inflammation or injury [33, 34].

2.2. Paneth Cells as ISC Niche. The sole importance of the stromal microenvironment as the ISC niche was challenged when ISC-derived epithelial culture was first established in 2009 in the absence of the mesenchymal niche [35]. The study showed that a single Lgr5-expressing ISC was able to grow three dimensionally into crypt-villus budding organoids with full proliferation and differentiation potential in a Matrigel-based culture. The specialised cells intermingled with ISCs at the crypt base—the Paneth cells, are later revealed to provide essential niche signals to their neighbouring stem cells [3]. Paneth cells are regarded as

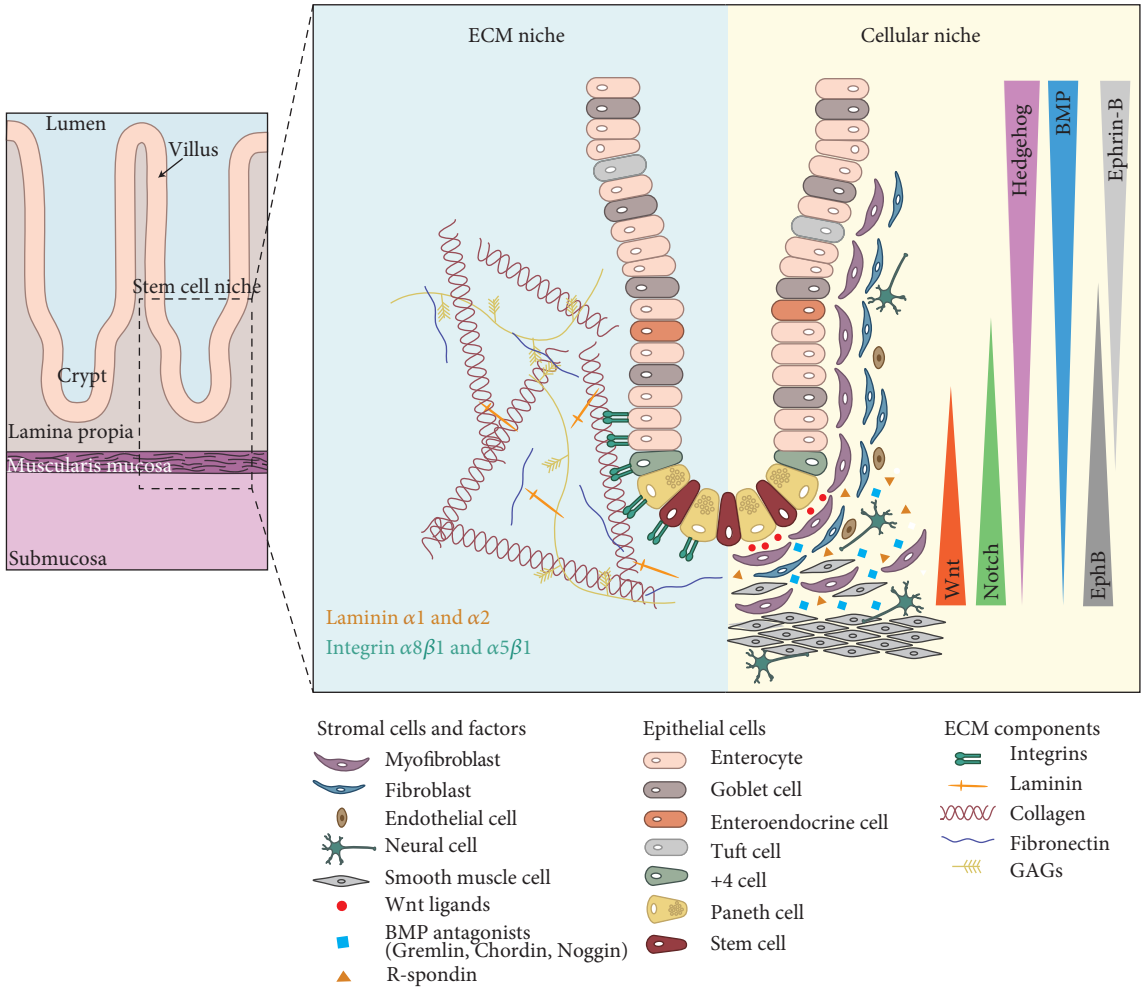

Figure 1: The intestinal stem cell niche. The intestinal epithelium comprises a monolayer of polarised columnar cells organised along the crypt-villus axis. Intestinal stem cells reside at the base of the crypts and continuously generate transit-amplifying (TA) daughter cells that differentiate into various mature cells in the villi (enterocytes, goblet cells, enteroendocrine cells, tuft cells, or Paneth cells). The crypt surrounding microenvironment is made up of both physical/structural and cellular niche to regulate ISC homeostasis. The physical niche includes collagen fibres, integrins, fibronectin filaments, laminins, and glycosaminoglycan, which form a highly structured network named as the extracellular matrix (ECM). The cellular niche includes pericryptal myofibroblasts, fibroblasts, endothelial cells, neural cells, and smooth muscle cells. The ECM and cellular niche interact and communicate with each other via different signalling pathways such as the Wnt, Notch, TGF-β/BMP, Eph/ephrin, and Hedgehog pathways for stem cell maintenance.

multifunctional guardians of the stem cell niche. They secrete antibacterial peptides such as lysozyme and defensins to sterilise the niche and are crucial for the mucosal defence mechanisms [36, 37]. In addition, they express signalling factors such as EGF, TGF-α, Wnt3, and the Notch ligand Dll4, which provide essential trophic support to ISCs [3]. Paneth cell depletion *in vivo* resulted in simultaneous loss of Lgr5+ stem cells, indicating its essential niche role in the gut.

3. The Physical Niche: Extracellular Matrix

Separating the mesenchymal compartment from the epithelial compartment is the basement membrane, which consists of two layers: the basal lamina positioned directly beneath epithelial cells and the underlying reticular sheet of matrix that anchors the epithelium to the lamina propria [38]. The basement membrane is a specialised ECM that is jointly produced by both epithelial and stromal cells and is mainly composed of laminins, collagen IV, and fibronectin. The presence of the basement membrane at the epithelial-mesenchymal interface is believed to play a crucial role in regulating epithelial cell homeostasis (comprehensively discussed in previous reviews [17, 39]). In the underlying connective tissue (lamina propria), several specific isoforms of the ECM components such as fibronectins, laminins, collagens, GAGs (e.g., heparan sulfate proteoglycans—also known as perlecan), and integrins are reported to be enriched at the intestinal crypt base, suggesting their potential role in ISC regulation [4–11, 38, 40–42]. A very recent study on matrix reconstitution of the matrix for intestinal organoid culture using minimal essential components provides direct and significant insight into the biochemical and biophysical roles of

the ECM in ISC homeostasis [43]. Here, we discuss the role of ECM in the ISC niche through various biological and mechanical parameters (Figure 1).

3.1. Biochemical ECM Roles in the ISC Niche. Collagen is the main structural protein in the ECM and is the most abundant protein in our body. The collagen superfamily contains at least 19 different subtypes, with types I, III, IV, and VI uniformly distributed in the healthy intestinal ECM [11, 44, 45]. However, increasing evidence suggests that type VI collagen (which interacts closely with type IV collagen of the basement membrane) is the key regulator for the mechanical microenvironment of the intestinal crypt cells via fibronectin and RGD (Arg-Gly-Asp)-dependent crypt cell interactions [4, 7]. Indeed, intestinal epithelial crypt cells have been demonstrated to secrete type VI collagen into the basal lamina of the intestinal basement membrane [7]. Increases in ECM collagen deposition augment tissue stiffness which alters integrin focal adhesions, growth factor receptor signalling, and acto-myosin and cytoskeletal-dependent cell contractility [46].

Laminin is one of the major glycoprotein constituents of the intestinal crypt basement membrane and is recognised to be particularly important in the establishment of epithelial cell polarity [10, 47]. Laminin subtypes are key components of small intestine and colon basement membranes. Laminin $\alpha 1$ and laminin $\alpha 2$ were shown to be enriched at the crypt regions, while laminin $\alpha 5$ was expressed strongly at the villus basement membrane [39, 47, 48]. Laminin $\alpha 5$ is believed to play a crucial role in establishing the mucosal pattern of the small intestine by maintaining the villus architecture [48, 49]. The recent study on the designer matrices for intestinal organoid culture has further demonstrated that laminin-111 ($\alpha_1\beta_1\gamma_1$) is important to enhance ISC survival and proliferation [43].

Fibronectin is a high molecular weight adhesive glycoprotein found in a wide range of tissues and plays important roles in cell adhesion, migration, growth, and differentiation. Fibronectin contains binding sites for many ECM proteins such as collagens, GAGs, and RGD peptides for cell surface receptors of the integrin superfamily, suggesting its multifunctional role in the ECM [5]. Intestinal fibronectin is secreted by fibroblasts as well as being expressed by epithelial cells and is located throughout the lamina propria [9, 40, 50]. Altered fibronectin deposition patterns are correlated with several intestinal disease states. For instance, upregulation of FN throughout epithelial cells is associated with intestinal fibrosis such as inflammatory bowel disease [5]. Strain forces exerted in the ECM *in vitro* have been shown to induce fibronectin-mediated epithelial cell migration by activating the extracellular signal-regulated kinase (ERK) and myosin light chain (MLC) signalling pathways, indicating the importance of fibronectin in wound closure and epithelial migration [51]. Fibronectin is also postulated to be an activator of the nuclear factor-κB (NF-κB) signalling pathway in the context of intestinal inflammation [5].

Integrins are heterodimeric receptors, consisting of α and β subunits that link the ECM with the intracellular cytoskeleton as part of the RGD-adhesion system, mediating cell anchorage, intracellular signalling, and mechanotransduction [4, 52]. Several integrin subunits and signalling components were previously shown to be expressed at high levels in the ISCs of the *Drosophila* midgut [53]. The study further demonstrated that integrin signalling is required for the maintenance and proliferation of intestinal stem cells but dispensable for multiple lineage differentiation. $\beta 1$ integrins have also been identified as key regulators for ISC proliferation and homeostasis by mediating Hedgehog signalling in a mouse genetic study [54]. The transmembrane $\alpha 5\beta 1$ integrin receptor has been shown to regulate many fibronectin-dependent biological effects in human tissues [55]. Integrin $\alpha 8\beta 1$ is another crucial mediator of intestinal crypt cell-matrix interaction via the focal adhesion kinase (FAK) signalling pathway [56–58]. Intestinal epithelial cells have also been shown to be regulated by integrin-linked kinase (ILK) through a fibronectin-dependent mechanism [59]. Overall, these studies suggest an essential role for integrins, in particular $\beta 1$ integrins in promoting ISC homeostasis.

Glycosaminoglycan molecules are thought to provide lubrication and structural integrity to cells in the intestinal ECM owing to their high viscosity and low compressibility, thereby providing a passageway between cells to facilitate cell migration [60, 61]. GAGs can function to organise collagen fibre deposition, stimulate angiogenesis, and inhibit coagulation [62]. The specific GAGs of physiological interest in the intestine are heparan sulfate, hyaluronic acid, heparin, and chondroitin sulfate [63]. Heparan sulfate proteoglycan (HSPG) is one of the most well-studied GAGs in the intestine. HSPGs are present in the ECM as linear polysaccharides, which are able to bind Wnt, Hedgehog, TGF-β, and FGF proteins in *Drosophila* and *Xenopus* studies [8, 64–66]. Intestine-specific HSPGs are found on the basolateral surface of intestinal epithelial cells and have been shown to promote intestinal regeneration by modulating Wnt/β-catenin signalling pathway, suggesting their role in ISC homeostasis [8, 67]. Hyaluronic acid is another chemically simple, high molecular weight, and nonbranching polymer of N-acetyl-glucosamine repeats that exists abundantly throughout the matrix. During disease processes such as in excessive inflammation, these polymers are cleaved to fragments of lower molecular weight that take on signalling roles [68, 69]. Hyaluronic acid binds to CD44, which is expressed on the plasma membrane of many cell types including ISCs [69]. It also binds to the Toll-like receptors TLR2 and TLR4, which are widely distributed in the gastrointestinal tract to mediate the host response to both commensal and pathogenic bacteria [70]. It has been shown that hyaluronic acid administration enhanced intestinal crypt survival of radiation-induced enteritis mediated through TLR4 and cyclooxygenase-2 (COX-2) [70, 71]. Together, the data suggest that GAGs constitute an important niche for ISC homeostasis.

3.2. Biomechanical ECM Roles in the ISC Niche. The biomechanical influence of the microenvironment is believed to play important roles in developmental processes, stem cell fate, and lineage determination [72]. Biophysical factors such as cell shape, ECM stiffness, and topography can all contribute to stem cell regulation. Cells perceive physical stimuli via

direct contact to the cell adhesion molecules, which allow the cytoskeleton to communicate with the adjacent ECM structures. This enables microenvironmental forces to be sensed and translated into intracellular messages, in a process termed mechanotransduction, to regulate a wide array of physiological processes [73]. The development of *in vitro* technology for the study of the matrix in the past decade has significantly advanced our understanding of the mechanical regulation of stem cell homeostasis. For example, a recent study using intestinal organoid cultures in customized matrices demonstrated that high matrix stiffness enhanced ISC expansion through yes-associated protein 1 (YAP)/Hippo pathway-dependent mechanism, whereas soft matrices promoted differentiation [43]. The Hippo signalling pathway is a key player of the ECM mechanotransduction that controls organ size by sensing the external mechanical forces (discussed in detail in the next section). The downstream key regulator YAP displays nuclear translocation and activation in response to mechanical tension, indicating its importance in cellular mechanosensing and mechanotransduction [74, 75].

In many organs, ECM topography undergoes constant dynamic remodelling whereby components are deposited, degraded, or modified by cues conveyed to the matrix by the surrounding cells [62]. The process of intestinal ECM remodelling is strongly associated with angiogenesis, cell migration, and differentiation as well as tumourigenesis, while ECM deposition and destruction occur via matrix metalloproteinases (MMPs) [76]. MMPs comprise a large family of at least 25 zinc-dependent endopeptidases capable of degrading all components of the ECM. They are classified according to substrate specificity and are associated with human diseases such as rheumatoid arthritis and cancer [77]. Intestinal organoids cultured in RGD-based hydrogels that were susceptible to MMP-mediated degradation demonstrated a proinflammatory phenotype with reduced stem cell maintenance [43]. The findings provide direct evidence that the ECM comprises an essential niche role for the regulation of ISCs.

4. Signalling Pathway Regulation in the ISC Niche

The cellular and mechanical niche components in the intestinal crypt communicate with each other via different signalling regulatory pathways to maintain the optimal microenvironment for ISC homeostasis. Here, we discuss the major signalling pathways that are essential for stem cell maintenance and repair (Figure 1).

4.1. Wnt. Wnt signalling is an evolutionary conserved pathway that plays a crucial role for the maintenance and proliferation of intestinal stem cells [78, 79]. Wnt ligands are secreted by various ISC niche cells, including the Paneth cells and the stromal cells surrounding the crypt [3, 80, 81]. Expression analysis in the intestine showed that Wnts 3, 6, and 9b are secreted predominantly by epithelial cells, whereas Wnts 2b, 4, 5a, and 5b are secreted by the mesenchyme [82]. Paneth cell-secreting Wnt3 constitutes the essential ISC niche factor for the stromal-free intestinal organoid culture *in vitro* [3, 83]. Interestingly, Wnt3 deletion or Paneth cell depletion *in vivo* in the gut did not affect intestinal homeostasis, suggesting a redundant role of Wnt ligands from the stromal microenvironment [80, 83].

R-spondin is a potent Wnt agonist that potentiates Wnt signalling in the presence of Wnt ligands via LGR-dependent mechanism [84]. A more recent study further demonstrates distinct, nonequivalent roles of Wnt and R-spondin ligands in ISC homeostasis using lineage tracing mouse models. While Wnt proteins confer a basal competency by maintaining R-spondin receptor expression (LGR4-6, RNF43, and ZNRF3 receptors), they are unable to induce ISC self-renewal and expansion alone *in vivo* without the presence of R-spondin ligands. The data suggest that R-spondin, rather than Wnt, plays the dominant role in controlling the size of the Lgr5+ ISC pool [85]. R-spondin proteins are secreted by the intestinal stromal niche to promote crypt proliferation and ISC maintenance [81, 84, 86]. Indeed, ex vivo stromal cell-free intestinal organoid culture is also dependent on the presence of R-spondin [35]. Depletion of Foxl1-expressing pericryptal mesenchymal cells *in vivo* led to suppression of Wnt activity and ISC proliferation due to the loss of Wnt ligands and R-spondin [87], supporting the important roles of Wnt and R-spondin in ISC maintenance. Similarly, another recent study shows that the CD34+ gp38+ pericryptal mesenchymal cells (also express Foxl1) are the major intestinal source for the ISC niche factors such as Wnt2b, R-spondin, and Gremlin1 [88]. These cells are in close proximity with Lgr5+ ISCs that constitute the key ISC microenvironment by promoting Wnt signalling and antagonising the BMP signalling (see below). On the other hand, several secretory Wnt antagonists such as SFRP-1 and Dkk-3 are also expressed in the stromal cells [82], suggesting the crucial role of ISC stromal niche in controlling the Wnt activity at the "just-right" level for stem cell homeostasis.

4.2. BMP. Mesenchymal-derived BMPs belong to the TGF-β family. TGF-β/BMP signalling inhibits intestinal epithelial stem cell expansion and promotes epithelial differentiation in the crypt [89, 90]. In contrast to Wnt signalling, BMP signals are activated in the villus and are suppressed toward the base of the crypt [89, 91]. Bmp4 is expressed throughout the lamina propria, while the BMP receptor (Bmpr1a) is expressed in the epithelial cells towards the villus [89]. BMP antagonists such as Gremlin1, Gremlin2, and Chordin are secreted by the ISEMFs and smooth muscle cells at the human colonic crypt bottom to repress BMP signalling, while BMP ligands are expressed in the upper colonic crypt to drive differentiation [20]. Similar to human colon, the BMP antagonist Noggin is also expressed at the stromal niche surrounding the crypt in the small intestine [89]. Transgenic expression of Noggin in the intestinal epithelia led to de novo crypt formation [92]. Another secreted protein, angiopoietin-like protein 2 (ANGPTL2), is also expressed by ISEMFs to inhibit *Bmp*2 and *Bmp7* expression via integrin $\alpha 5\beta 1$/NF-κB signalling and maintain ISC homeostasis [93].

Crosstalk between Wnt and BMP signalling is believed to play a key role in ISC homeostasis. Previous studies showed that deletion of Bmpr1a in mouse intestine caused rapid expansion of the stem cell compartment by enhancing Wnt activity [89]. Recent data further demonstrate that epithelial BMP signalling is crucial to restrict ISC expansion by direct Smad4-mediated repression of Wnt/stem cell signature genes [90]. Importantly, the stromal cells surrounding the intestinal crypt base secrete both Wnt (Wnt and R-spondin ligands) and BMP factors (Bmp antagonists such as Gremlin and Noggin) together to drive ISC proliferation [20, 87, 88]. R-spondin and Noggin also constitute the key growth factors for the stromal cell-free intestinal organoid culture, which can be replaced by coculturing with mesenchymal cells [35, 87, 88]. Together, these findings suggest that ISC stromal cells play an indispensable role for ISC homeostasis by modulating both Wnt and BMP signalling pathways.

4.3. Notch. Notch signalling is crucial for ISC maintenance and fate decision, where Notch inhibition resulted in reduced stem cell proliferation [94, 95]. The Notch signalling pathway is regulated through the presentation of the membrane-bound Notch ligand to an adjacent cell expressing the Notch receptor, suggesting the importance of close proximity between ISCs and their niche [96]. Notch receptor and ligand transcripts have been detected in both epithelial and mesenchymal cells of the developing and adult rodent intestine [97–99]. Paneth cells express the Notch ligands delta-like 1 and 4 (Dll1 and Dll4) and present these ligands to their adjacent Notch receptor-expressing ISCs for Notch activation [3]. Simultaneous deletion of Dll1 and Dll4 resulted in loss of ISCs and crypt proliferation, suggesting that Notch activation is required for ISC homeostasis [100]. Activation of Notch has also been shown to be crucial during intestinal epithelial regeneration [101].

Notch signalling is also important in lineage specification at the progenitor cells. Notch activation drives absorptive lineage differentiation, while Notch inactivation drives atonal homolog 1- (*Atoh1*-, also known as *Math1*) dependent secretory lineage differentiation [100, 102–108]. Atoh1 depletion in the intestine resulted in expansion of the crypt proliferative zone and promoted enterocyte over secretory cell differentiation [109]. On the other hand, disruption of Notch signalling caused rapid conversion of all proliferative crypt cells into goblet cells [105, 110, 111]. The ISC-specific marker Olfactomedin 4 (Olfm4) was shown to be a direct Notch target in the intestine [94]. Interestingly, murine Olfm4 has been described as a secreted ECM glycoprotein that promotes cell adhesion and binds to cell surface cadherins and lectins, suggesting a potential link between Notch signalling and the ECM niche [112].

4.4. Eph/ephrin. Cell positioning along the intestinal crypt-villus axis is controlled by the Eph/ephrin-mediated interaction and repulsion among the epithelial cells and is crucial for ISC homeostasis [113, 114]. Eph tyrosine kinase receptors and their ephrin ligands are expressed in most adult stem cell niches, often in counter gradients to regulate tissue boundary and stem cell proliferation [115]. *In vivo* studies and gene expression profiling experiments have shown that EhpB2 and EphB3 are both Wnt target genes and are expressed in the proliferative cells at the crypt bottom [113]. Deletion of both EphB2 and EphB3 in mouse intestine altered the positioning of proliferative and differentiated cells and caused mislocation of the Paneth cells scattering along the crypt-villus axis. In contrast to the EphB receptors, ephrin-B1 ligand is expressed in the differentiated cells in an opposite gradient [113]. Interaction of the receptor with its ligand prevents proliferating cells from migrating into the differentiated cell territory, thereby promoting compartmentalisation of the epithelial cells along the crypt-villus axis [113, 116]. In addition to the EphB family, multiple EphA receptors and their ligands are also expressed differentially in human colonic crypts. EphA1, EphA4, and EphA7 are expressed at the crypt bottom, while EphA2, EphA5, and the ephrin-A1 ligand are enriched at the upper colonic crypts [20]. The role of EphA-ephrin-A signalling in ISC homeostasis is yet to be determined. Together, Eph-ephrin signalling is believed to maintain ISC homeostasis by restricting the ISCs and Paneth cells at the crypt bottom for exposure to the key stem cell niche factors.

4.5. Hippo. Hippo signalling pathway is highly conserved and plays a central role in organ size control, stem cell renewal, and regeneration via extracellular mechanical forces [117]. The transcriptional coactivators YAP and TAZ have been shown to transduce mechanical cues to mediate biological effects in response to ECM elasticity and cell shape. YAP and TAZ are translocated to the nucleus for transcriptional activation in stiff matrix, while the two effector proteins are excluded from the nucleus in soft matrix [118]. Recent studies suggest an important role of Hippo signalling in regulating intestinal homeostasis and regeneration [119–121]. The effector protein YAP is mainly expressed throughout the intestinal crypt and promotes intestinal regeneration [119]. YAP and TAZ have been shown to induce both proliferation of crypt progenitor cells and differentiation of ISCs into goblet cells via TEADs- and Klf4-mediated transcription regulation, respectively [122]. YAP/TAZ-deletion was also found to impair intestinal organoid formation and prevent Apc loss-induced lethality by Wnt-mediated mechanism [123]. On the other hand, an inhibitory role for YAP in intestinal regeneration has been proposed. Overexpression of a constitutively active YAP-S127A mutant in mouse intestine led to the loss of proliferative crypts and Wnt signal suppression, whereas depletion of YAP in the gut caused hyperactive Wnt signalling and expansion of ISCs and niche cells during regeneration [120]. These paradoxical observations could possibly be explained by the complexity of the Hippo pathway such as cell-ECM interaction, nuclear-cytoplasmic shuttling of YAP/TAZ, and its crosstalk with Wnt signalling cascade. Further investigation on the effect of ECM dynamics to ISC maintenance in the context of Hippo signalling regulation will help understand the mechanical-cytoskeletal cues on stem cell homeostasis and regeneration.

4.6. Hedgehog. Hedgehog signalling is involved in stem cell maintenance, organogenesis, and tissue repair/regeneration

[124]. Paracrine Hedgehog signalling is crucial for intestinal crypt-villus axis formation during development. Expression of the two ligands Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) is limited to the intervillus pockets of the developing epithelium, while the expression of the receptors patched 1 (Ptch1) and patched 2 (Ptch2) and the effectors Gli1, Gli2, and Gli3 is restricted to the underlying mesenchyme [125]. Ihh is expressed in the differentiated epithelial cells in the villi of the adult small intestine and is crucial for epithelial integrity and wound healing [126]. Blockade of Hedgehog signalling inhibited villi formation and maintained intestinal crypt proliferation by enhancing Wnt/β-catenin activity [125]. Deletion of Shh or Ihh showed multiple gastrointestinal defect and reduced smooth muscle cells [127]. Intestinal-specific deletion of Ihh resulted in disruption of mesenchymal architecture and ECM deterioration via the loss of Bmp signalling and increased MMP synthesis [128]. In addition to regulating smooth muscle and myofibroblasts during development, Hedgehog signalling is also required to induce *Bmp4* expression in the stromal niche to regulate enteric neural cell differentiation [129]. Together, the data suggests that paracrine Hedgehog signalling from epithelial to mesenchymal cells promotes stromal niche formation, which in turn affects epithelial proliferation and differentiation. Hedgehog signalling in the gut represents one of the best examples of the close regulation between ISCs and their niche.

5. Conclusion and Future Perspectives

The cellular and ECM niches together constitute a dynamic microenvironment that is critical for intestinal tissue homeostasis. In this review, we provide an overview of the biochemical and mechanical cues originating from the matrix, as well as various vital signalling pathways derived from different cellular niche components that are important for the regulation of ISC maintenance and differentiation. Matrix proteins function in the ISC niche to provide the structural scaffold for maintaining the crypt-villus axis formation, transduce intracellular signalling via integrin binding, and act as a reservoir of growth factors that may be released upon proteolysis. Integrin-mediated stem cell anchoring has been recently shown to be crucial for the maintenance of the stem cell compartment in the epidermis, where human epidermal stem cells express high levels of β1 integrins [130]. It will be interesting to further explore the role of the integrin-mediated anchoring mechanism in compartmentalisation of the ISCs and Paneth cells in the intestine apart from the Eph/ephrin signalling.

ECM remodelling can influence the accessibility and the biological cues of the ISC niche. Given the growing evidence of the pivotal role of the microenvironment in inflammatory bowel disease and cancer, ECM components may represent appealing therapeutic targets. Recent studies suggest that epigenetic modification such as histone methylation and acetylation may regulate ISC proliferation and differentiation [131]. Further investigation on the potential link between the microenvironment and epigenetic mechanisms may provide an additional level of stem cell regulation. Recent advances on intestinal tissue engineering further highlight the significance between ISCs and their niche (both physical and biological). A greater understanding of the interplay between different cell populations in the ISC niche and their influence on ECM will shed light on both disease management and regenerative medicine.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Laween Meran and Anna Baulies contributed equally.

Acknowledgments

This review is a snapshot of ISC niche at the current status, and the authors apologize to many colleagues whose work could not be cited here due to space limitation. This work was supported by the Francis Crick Institute which receives its core funding from Cancer Research UK (FC001105), the UK Medical Research Council (FC001105), the Wellcome Trust (FC001105), and the European Union's Horizon 2020 research and innovation programme (668294). Laween Meran is funded by the NIHR Great Ormond Street Hospital Biomedical Research Centre.

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Hindawi
Stem Cells International
Volume 2017, Article ID 5263974, 8 pages
https://doi.org/10.1155/2017/5263974

Review Article

The Crosstalk between Ovarian Cancer Stem Cell Niche and the Tumor Microenvironment

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Received 10 April 2017; Accepted 3 July 2017; Published 27 July 2017

Academic Editor: Karen Liu

Ovarian cancer is one of the most important causes of cancer-related death among women in the world. Despite advances in ovarian cancer treatment, 70–80% of women who initially respond to therapy eventually relapse and die. There is evidence that a small population of cells within the tumors called cancer stem cells (CSCs) could be responsible for treatment failure due to their enhanced chemoresistance and tumorigenicity. These cells reside in a niche that maintains the principal properties of CSCs. These properties are associated with the capacity of CSCs to interact with different cells of the tumor microenvironment including mesenchymal stem cells, endothelial cells, immune cells, and fibroblasts, promoting cancer progression. This interaction can be mediated by cytokines, growth factors, lipids, and/or extracellular vesicles released in the CSC niche. In this review, we will discuss how the interaction between ovarian CSCs and the tumor microenvironment can contribute to the maintenance of the CSC niche and consequently to tumor progression in ovarian cancer.

1. Introduction

Among the different gynecological cancer, ovarian cancer (OVCA) is the most lethal one in women worldwide. Although OVCA accounts for only 3% of all cancer incidents, 6% of cancer-related deaths are caused by ovarian cancer, making it the fifth leading cause of cancer mortality in women [1]. The main contributing factor to the high mortality rate of OVCA is late diagnosis, and although the use of first-line chemotherapy (e.g., paclitaxel-platinum combination) is initially effective for most patients, around 70% of women with advanced OVCA (stages 3-4) relapse within a few years after treatment and die due to the development of drug resistance [2, 3]. A small population of cells termed cancer stem cells (CSCs) has been identified as important contributors to drug resistance in ovarian cancer because they possess molecular and cellular mechanisms identified as important contributors of chemoresistance [4–7].

CSCs constitute a subset of cells with self-renewal and differentiation properties that are distinguished from the bulk of tumor cells by their exclusive ability to perpetuate the growth of a malignant cell population indefinitely [8]. CSCs have different cellular characteristics involved in cancer pathogenesis, such as tumorigenesis, metastasis, and tumor resistance [8, 9]. The presence of CSCs, therefore, offers a plausible explanation for the high rate of relapse, even some months after the therapy, with an initial successful treatment [3, 10, 11]. The explanation for relapse has usually been explained by tumor cells acquiring a resistant phenotype; however, studies have shown that resistance can be associated with the capacity of CSCs to resist the initial treatment and then to interact with

different cell types of the tumor microenvironment to promote relapse and cancer progression [12, 13].

2. Tumor Microenvironment

The tumor microenvironment is the combination of noncancerous cells and the proteins produced by all the cells present in the tumor. The group of noncancer cells in the tumor is also defined as stroma and is composed of endothelial cells, cancer-associated fibroblasts (CAFs), adipocytes, mesenchymal cells, mesenchymal stem cells (MSCs; bone marrow derived (BM-MSCs) or carcinoma associated (CA-MSCs)), and cells from the immune and inflammatory systems (tumor-associated macrophages (TAM), regulatory T cells, etc.) [14, 15]. The participation of tumor stroma components in carcinogenesis and how the different cells of the tumor microenvironment contribute to induce tumor progression and metastasis has been extensively described [16, 17]. Stromal cells could be implicated in the acquisition of a specific phenotype by different processes, such as cell-cell and cell-matrix interaction, local release of soluble factors, generation of specific niches within the tumor, or conversion of cancer cells to CSCs [14, 15]. In the case of OVCA, the importance of the microenvironment in tumor progression can be explained by the bidirectional interaction between OVCA cells and their own stroma modulating the contents of the ascitic fluid promoting the protumoral phenotype of the stromal cells and regulating processes to favor tumor progression [18–22]. A good example of this interaction is tumor vascularization, which is essential for tumor growth and survival. The vascular endothelial growth factor (VEGF) is the most potent proangiogenic factor and is secreted by different types of cells including MSCs and endothelial and tumor cells [23]. In OVCA, VEGF induces the expression of CXCL12 receptor in vascular endothelial cells (VECs) and the hypoxic condition of the tumor induces the secretion of CXCL12 and VEGF acting together to induce angiogenesis [24]. MSCs have also been implicated in promoting angiogenesis by induction of VEGF and HIF1α expression in ovarian cancer [19]. In addition, OVCA cells secrete lysophosphatidic acid (LPA), a potent bioactive lysophospholipid that activates the expression and secretion of CXCL12 by MSCs, enhancing the resistance of OVCA cells to hyperthermia [25]. Several studies show the role of MSCs in tumor progression and how these cells interact with OVCA cells in response to different stimulus [19, 26, 27].

Cells acquiring tumorigenic traits (i.e., unregulated cell proliferation and resistance to cell death) are insufficient for tumor progression, and for that reason, multiple cell types are involved in this process, requiring effective cell-to-cell communication between cancer cells and local/distant microenvironments [28]. Cytokines, growth factors, and extracellular vesicles (EVs), including exosomes, could play an important role in this interaction and can influence proliferation, angiogenesis, chemoresistance, and metastasis [18]. In the next sections, we will discuss the importance of all these factors in the maintenance of the CSC niche and tumor progression.

3. Ovarian CSCs and Inflammatory Network

One of the hallmarks in cancer is the effect of inflammation in the tumor microenvironment and how the different components involved in the inflammatory process can contribute to tumor development [28, 29]. Several studies have shown the importance of different cytokines secreted by the tumor microenvironment in the regulation of CSCs [30]. In the case of ovarian cancer, it is known that an inflammatory state is considered a risk factor and can be associated with ovarian cancer development, drug resistance, and metastasis [31, 32]. Several cytokines have been described in circulation, ascites, and cyst fluid of patients with ovarian cancer [33–38] and also in the stroma and epithelium of tumors [39]. The presence of cytokines in the tumor stroma raises the possibility of activating signaling pathways related to the inflammatory network in all cell types of the stroma, including the ovarian CSCs. One of the cytokines present in the tumor microenvironment of ovarian cancer is IL-17 [40]. Xiang and collaborators demonstrated that IL-17 in ovarian cancer is produced by CD4+ T cells and CD68+ macrophages, tumor-associated macrophages (TAM) in the ovarian CSC niche, and the IL-17 receptor is expressed in a population of CD133+ CSCs [41]. The activation of this signaling pathway promotes self-renewal of the ovarian CSCs mediated by nuclear factor NFκB and p38 mitogen-activated protein kinase (MAPK) signaling pathway, contributing to the ovarian cancer progression [41].

Among the cell types associated with the tumor microenvironment, M2 macrophages, a type of TAM, have a significant effect on tumor progression in several types of cancer, including ovarian cancer. These cells can secrete different factors, including VEGF, TGF-β, PPAR-γ, IL-10, and IL-17 [42–45]. As we mentioned before, CD68+ macrophages can induce the self-renewal of ovarian CSCs, but evidence shows that ovarian CSCs can induce polarization of M2 macrophages. Transwell assays of ovarian CSCs with monocytes showed an increase in monocyte differentiation to macrophages with M2 phenotype, increase in the IL-10, decrease in TNF-α, and activation of PPAR-γ and NFκB [46, 47]. These results indicate that soluble factors, including cytokines, secreted by the ovarian CSCs, contribute to the M2 macrophage polarization to support the self-renewal of themselves.

In ovarian cancer, the proinflammatory state is not only induced by cells of the immune system. Other cells of the tumor microenvironment can contribute to generate different cytokines affecting the ovarian CSC niche. For example, in OVCA, metastasis occurs commonly in the omentum by the overexpression of ErbB3 in the tumor cells and the overexpression of neuregulin 1 in the omentum [48]. The omentum is an organ primarily composed of adipocytes, and these cells also can promote homing, migration, and invasion of ovarian cancer cells through release of cytokines such as IL-8 and IL-6 [49]. Both cytokines are also released by other sources in the tumor microenvironment and have been shown to regulate CSCs in other types of tumor [50–53]. Cytokines secreted by adipose tissue in ovarian cancer, therefore, may also regulate mechanisms related to CSCs. For

example, one mechanism used for ovarian CSCs to acquire chemoresistance is mediated by the high expression of Bcl_{xl}, and IL-6 secreted by the adipocyte increases the levels of Bcl_{xl} in chemosensitive ovarian cancer cells using the same mechanism used by ovarian CSC enhancing the proliferation, sphere formation, and tumorigenesis of ovarian cancer cells [54]. This data supports a role for cytokines released by adipocytes in ovarian cancer that can regulate CSCs.

As described before, in the tumor microenvironment, there are several types of interactions between different cell types. One of them is autocrine interaction that includes CSCs, which are able to secrete cytokines that will activate inflammatory signaling pathways in the same cell. For example, Wang and collaborators reported that CD133+ ovarian CSCs have the IL-23/IL-23 receptor axis activated, and the activation of this pathway promotes self-renewal and formation of ovarian CSCs by activation of the signaling pathways STAT3 and NFκB, thus contributing to tumor progression [55]. In the same way, CD133+ cells have CCL5 and its receptors upregulated, and their autocrine activation promotes invasion and migration via NFκB-mediated MMP-9 upregulation [56]. On the other hand, stem cells have the capacity to differentiate into other cell types by expressing different phenotypes, and CSCs are no exception. There is evidence that ovarian CSCs are able to differentiate into stromal cells supporting tumoral processes. One of these processes is angiogenesis, where new blood vessels are required for solid tumor maintenance, progression, and metastasis [57]. Alvero and colleagues demonstrated that CD44+ ovarian cancer cells, another subpopulation of ovarian cancer cells with stem-like properties different from CD133+ ovarian cancer cells, have the capability to be differentiated into a CD44+/VE-cadherin+/CD34+ cells phenotype and mimic the behavior of normal endothelial cells forming vessel-like structures in a VEGF-independent manner [58]. Supporting this discovery, Tang and colleagues showed that ovarian CSCs can activate NFκB and STAT3 signaling secreting CCL5 and activating this pathway in an autocrine manner to allow its own differentiation into endothelial cells to improve tumor angiogenesis [59]. These data highlights the importance of the inflammatory network in the tumor microenvironment, as well as the mechanisms by which cytokines can support the ovarian CSC niche.

4. Ovarian CSCs and Growth Factors

Growth factors play an important role in maintaining tissue homeostasis under physiological conditions, but in cancer, the same growth factors can be involved in tumor progression [60]. As with cytokines, cells from the tumor microenvironment are able to secrete growth factors and regulate processes that are important for tumor development such as angiogenesis and metastasis, including the function of CSC and tumor initiation [61, 62].

Cancer-associated fibroblasts (CAFs) are key components of the tumor stroma and could have an important role in ovarian cancer progression and metastasis [63]. Histological examination and gene expression analysis of ovarian tumor tissues have shown abundant fibrous stroma formation, overexpression of fibroblast growth factor 4 (FGF4), and stem cell-associated genes in samples enriched with CSCs in the presence of fibroblasts [64]. *In vivo* studies demonstrated that the capability to generate ovarian CSCs was enhanced in the presence of CAFs and the capacity of the fibroblast to enhance CSC properties was suppressed by knockdown of the FGF4 receptor (FGFR2), expressed preferentially in ovarian CSCs [64]. Supporting these data, there is evidence that the activation of FGF signaling can control the expansion and self-renewal of CSCs [65–67]. Moreover, FGF is able to induce angiogenesis through the autocrine induction of VEGF secretion [68]. VEGF is the master regulator of angiogenesis [69], but the function of VEGF in cancer is not limited to the generation of new blood vessels; it can also promote CSC properties in certain cancers [70]. In ovarian cancer, VEGF-A, a member of the VEGF family, stimulates ovarian CSCs through VEGFR2-dependent Src activation to upregulate the stem cell factor B cell-specific Moloney murine leukemia virus integration site 1 (Bmi1) [71].

CAFs can also participate in the generation and maintenance of the CSC niche via activation of the insulin growth factor receptor (IGF-IR), inducing Nanog expression and stem cell phenotype in cancer cells [72]. In ovarian cancer, the IGF signaling is involved in tumor progression and chemoresistance [73] and the activation of IGF-1R-AKT signaling by different chemotherapeutics agents increase the expression of genes involved in self-renewal (*Oct4*/*Sox2*/*Nanog*) and imparts functional heterogeneity in the ovarian CSCs during acquirement of chemoresistance [74].

Evidence also suggests that MSCs are recruited to the tumor microenvironment. A special type of MSCs has been identified associated to ovarian carcinoma called carcinoma-associated MSCs (CA-MSCs) and is present in the majority of human ovarian tumor samples [75]. One of the characteristic of the CA-MSCs is the upregulation of the TGF-β superfamily/BMP family members in comparison with control MSCs, and this activation in the BMP signaling pathways increases the population of ovarian CSCs promoting the CSC proliferation [75]. Other factors such as TNF-α and TGF-α, released by different types of cells in the tumor microenvironment, have also been identified with a potential role in the ovarian cancer progression [62]. The exact nature of their interactions with CSCs remains to be clearly established.

5. Ovarian CSCs, MicroRNA Regulation, and Extracellular Vesicles

Different cell types, including CSCs, can regulate the expression of small noncoding RNAs called microRNAs (miRNAs) to regulate several processes [76, 77]. In order to maintain the stemness of cancer cells, the tumor microenvironment can modulate the expression of miRNAs. Cui and collaborators showed that myeloid-derived suppressor cells (MDSCs), components of the tumor microenvironment, stimulate the expression of miRNA-101 in ovarian cancer cells and subsequently repress the corepressor gene C-terminal binding protein-2 (CtBP2), resulting in an increase in cancer cell stemness and metastatic and tumorigenic potential [78].

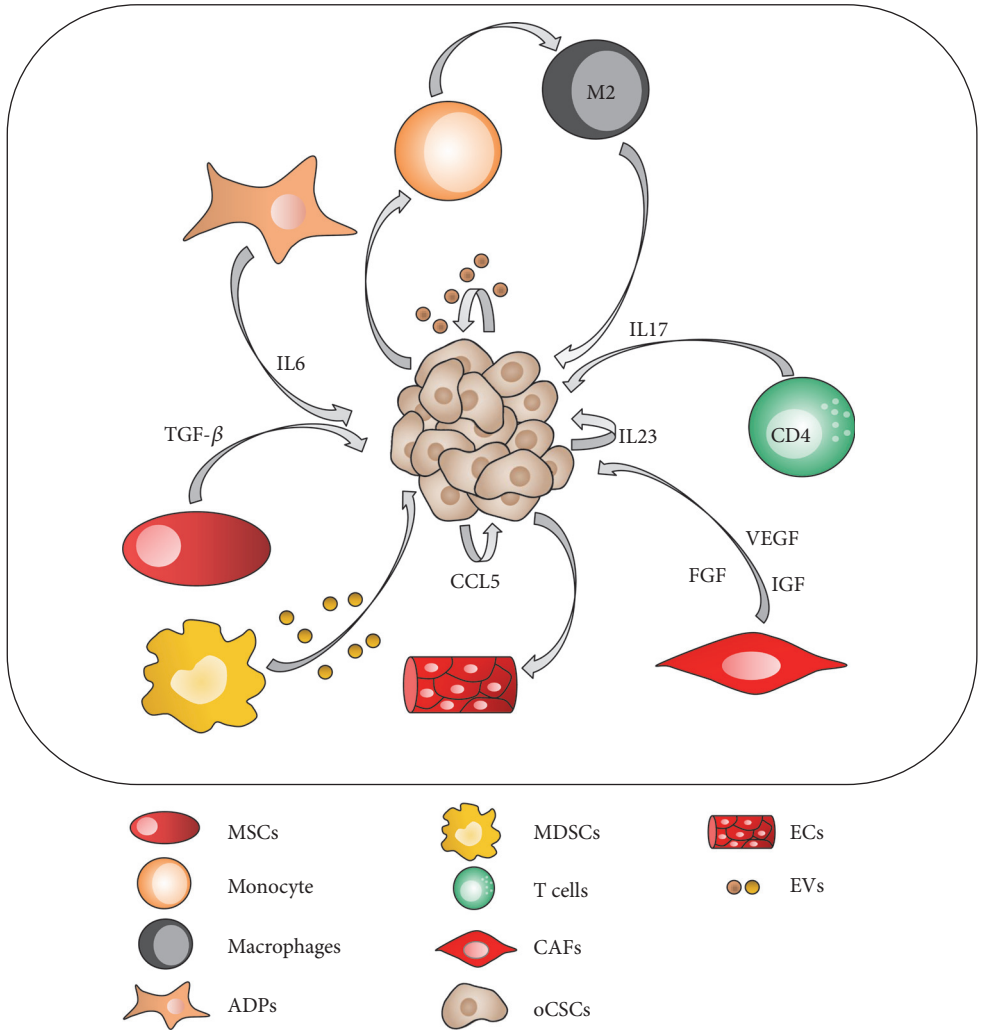

Figure 1: Schematic representation of the interaction between the ovarian cancer stem cell niche and the tumor microenvironment. T cells and M2 macrophages mediate self-renewal of oCSCs by secretion of IL-17. ADPs support tumorigenesis of oCSCs by secretion of IL-6. CAFs mediate self-renewal of oCSCs by secretion of FGF, VEGF, and IGF. MSCs mediate tumorigenesis of oCSCs by secretion of TGF-β. oCSCs induce differentiation of monocyte to M2 macrophages. oCSCs (CD133+) induce its own self-renewal by autocrine activation of IL-23 secretion. oCSCs induce tumorigenesis by CCL5 secretion (CD133+) and EV secretion. oCSCs (CD44+) induce its own differentiation to ECs by secretion of CCL5. MSCs: mesenchymal stem cells; ADPs: adipocytes; MDSCs: myeloid-derived suppressor cells, T cells; CAFs: cancer-associated fibroblasts; oCSCs: ovarian cancer stem cells; ECs: endothelial cells; M2: macrophages; EVs: extracellular vesicles.

How the MDSCs regulate the expression of this miRNA is still unclear, but one of the possibilities is that MDSCs could transfer these miRNAs by extracellular vesicles (EVs). EVs are small membrane vesicles capable of transferring contents between cells to function in cell-cell communication [79].

In the last decades, the communication and exchange of proteins, mRNA, and miRNAs mediated by EVs within the tumor microenvironment has acquired a big relevance in the regulation of tumor processes such as metastasis and chemoresistance [79–81], all processes where CSCs are involved. Some studies have described the role of EVs derived from CSCs in the tumor progression of renal, prostate, and breast cancer [82–84]. In ovarian cancer, although the release of EVs is very important to mediate tumor progression [85–87], the interaction between the tumor microenvironment and the ovarian CSCs mediated by EVs is still unclear.

6. Conclusion

The available evidence supports the hypothesis that the niche of ovarian CSCs plays an important role in the initiation of the tumor, but this role would not be possible without the interaction of the niche with the tumor microenvironment. This interaction, mediated by different types of factors, can be considered bidirectional; this communication allows the ovarian CSCs to maintain the stemness of the niche while differentiating the CSCs to other cell types of the tumor microenvironment in order to support tumor progression. Similarly, CSCs may modulate the function of different cells in the tumor microenvironment to support these tumorigenic properties.

Cellular communication among different cells in the tumor microenvironment is modulated by a variety of

messages such as cytokines, growth factors, EVs, and miRNAs (Figure 1), and how the microenvironment will interact is dependent on the needs of the CSC niche, and in what tumor process it will participate in. For example, a proinflammatory microenvironment, considered one of the hallmarks of cancer, is normally associated with tumor progression inducing proliferation, angiogenesis, and migration of cancer cells [28, 88]. IL-6 and CCL5 in the niche of the ovarian CSCs promote these processes, and CCL5 induces the differentiation of a subset of CSCs to generate ECs and support angiogenesis [54, 56, 59]. Other components of the proinflammatory network, such as IL-17 and IL-23, participate in the maintenance of the CSC niche promoting self-renewal, indicating their possible role in tumor initiation [41, 55].

Although IL-17 is secreted by CD4+ T cells and CD68+ macrophages in ovarian cancer, in other types of cancer, a population of FoxP3+ regulatory T cells (Treg), that under certain conditions express IL-17, plays a critical role in the regulation of CSCs [89]. Therefore, Treg could not only be modulating the tumor immunity by the inhibition of effector T cells but could also be regulating the tumor microenvironment and the release of different factors by the CSC niche.

Even though growth factors are considered one of the major regulators of the tumor progression process [60], they also participate in the self-renewal of CSCs and regulate their tumor initiation capacity [64, 71, 74]. This dual effect can be attributed to the heterogeneity of the ovarian tumor [90, 91]. Such a heterogeneity is also present in the ovarian CSC population [74, 92, 93] and could explain why the activation of the NFκB-STAT3 signaling in one subset of CSCs (CD133+) induces self-renewal while in another subset (CD44+) it induces differentiation to ECs [55, 59]. The presence of a different CSC population could also explain why different factors contribute to CSC self-renewal, though this could be attributed to the activation of the same signaling pathway by different factors as well.

The role of microRNAs and EVs in the interaction between ovarian CSC niche and the tumor stroma is still an area of ongoing investigation, but its importance in gene regulation and cell communication supports the idea that they must play an important role in the self-renewal of ovarian CSCs.

Finally, it is worth mentioning that the microenvironment of the fallopian tube epithelium (FTE) could be a contributing factor to the CSC niche, given that there are several hypotheses that this is the site where ovarian cancer originates [94]. The identification of a stem cell niche in the FTE and the presence of a cancer-prone stem cell niche in the mesothelium and tubal (oviductal) epithelium support the idea that the FTE could play a role in the maintenance of the CSC niche [95, 96].

Understanding these interactions and what is the contribution of the ovarian CSC niche and of the other components of the tumor in the development of ovarian cancer will allow us to gain the knowledge needed to generate therapies against tumor progression and relapse.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was funded by Fondecyt Iniciación (11150624) granted to Manuel Varas-Godoy.

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