Stem Cells International

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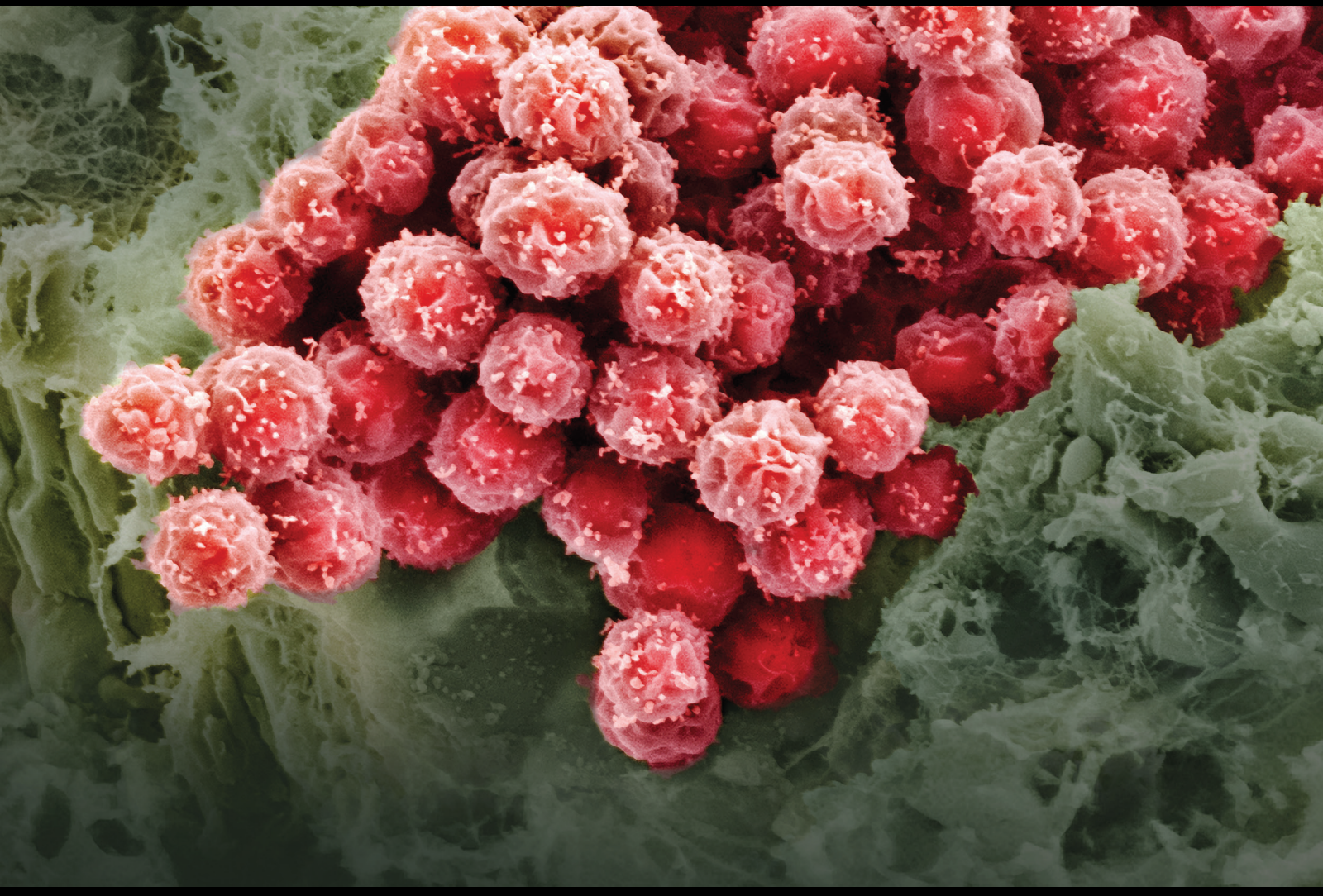

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Review Article

Dental Pulp Stem Cell Heterogeneity: Finding Superior Quality "Needles" in a Dental Pulpal "Haystack" for Regenerative Medicine-Based Applications

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Human dental pulp stem/stromal cells (hDPSCs) derived from the permanent secondary dentition are recognised to possess certain advantageous traits, which support their potential use as a viable source of mesenchymal stem/stromal cells (MSCs) for regenerative medicine-based applications. However, the well-established heterogeneous nature of hDPSC subpopulations, coupled with their limited numbers within dental pulp tissues, has impeded our understanding of hDPSC biology and the translation of sufficient quantities of these cells from laboratory research, through successful therapy development and clinical applications. This article reviews our current understanding of hDPSC biology and the evidence underpinning the molecular basis of their heterogeneity, which may be exploited to distinguish individual subpopulations with specific or superior characteristics for regenerative medicine applications. Pertinent unanswered questions which still remain, regarding the developmental origins, hierarchical organisation, and stem cell niche locations of hDPSC subpopulations and their roles in hDPSC heterogeneity and functions, will further be explored. Ultimately, a greater understanding of how key features, such as specific cell surface, senescence and other relevant genes, and protein and metabolic markers, delineate between hDPSC subpopulations with contrasting stemness, proliferative, multipotency, immunomodulatory, anti-inflammatory, and other relevant properties is required. Such knowledge advancements will undoubtedly lead to the development of novel screening, isolation, and purification strategies, permitting the routine and effective identification, enrichment, and expansion of more desirable hDPSC subpopulations for regenerative medicine-based applications. Furthermore, such innovative measures could lead to improved cell expansion, manufacture, and banking procedures, thereby supporting the translational development of hDPSC-based therapies in the future.

1. Introduction

The dental pulp tissues of postnatal human teeth are now well-established to harbour a unique and varied source of mesenchymal stem/stromal cells (MSCs). Due to the essential roles that MSCs play during tissue development and in mediating reparative responses within the dentine-pulp complex [1], there is a strongly held belief that the dental pulp offers a potentially viable source of MSCs for regenerative medicine-based applications. Such principles are based on their availability and ease of isolation using minimally invasive techniques from the pulpal tissues of extracted

teeth, whilst mitigating many of the ethical issues associated with the collection of MSC populations from other tissue sources, coupled with their similarities to human bone marrow-derived MSCs (hBMMSCs), the current "gold standard" MSC source [2–4].

MSCs within both human exfoliated deciduous teeth (stem cells from human exfoliated deciduous teeth (SHEDs) [5]) and the permanent secondary dentition (human dental pulp stem/stromal cells (hDPSCs) [6, 7]) have been widely isolated and characterised, in terms of their distinct cell surface marker expression profiles, self-renewal and clonogenic characteristics, proliferative capacities, multipotent differentiation capabilities (e.g., dentinogenic, osteogenic, chondrogenic, adipogenic, myogenic, neurogenic, hepatogenic, and angiogenic lineages), and other desirable genotypic/phenotypic properties [8–12]. Therefore, hDPSCs, particularly those isolated from extracted third molar teeth due to orthodontic reasons, have received considerable attention for the development of more effective stem/stromal cell-based regenerative therapies. Indeed, despite much hDPSC-based research being dedicated toward demonstrating their beneficial effects as a regenerative dental pulpal therapy during endodontics [13, 14], hDPSCs have also been shown to promote tissue repair following transplantation into various animal model defects *in vivo*, related to pathologies associated with other clinical disciplines, such as orthopaedics, neurology, ophthalmology, hepatology, and cardiology [8, 11, 12, 15].

Despite such advances in our understanding of hDPSC biology and the continued development and evaluation of hDPSC-based therapies for clinical use, challenges remain which impact on the potential and exploitation of this MSC source in regenerative medicine. Although methodologies exist enabling the routine isolation of hDPSCs from dental pulp tissues, as with MSCs sourced from other tissues [16–19], an unequivocal issue which merits significant consideration is the established heterogeneous nature of MSC populations within the dental pulp, as isolated hDPSCs are invariably comprised of many individual subpopulations with contrasting biological and regenerative characteristics. Such traits have presented a major obstacle to the translational development of hDPSC-based therapies for clinical application, especially if hDPSC subpopulations possess divergent proliferation and differentiation properties to permit predictable and reproducible regenerative outcomes. Ever since the pioneering work of Gronthos et al. [6, 7], who originally described the characterisation of a unique population of postnatal hDPSCs from the dental pulp of human third molar teeth, the heterogeneity between hDPSC subpopulations within dental pulp tissues has been indisputable. Specifically, hDPSCs shared a similar immunophenotype to hBMMSCs and exhibited a high degree of clonogenicity, self-renewal, rapid proliferative rates, and multipotency capabilities, including differentiation into odontoblast-like cells and the production of sporadic, but densely calcified, nodules. Subcutaneous hDPSC transplantation into immunocompromised mice also resulted in the formation of a functional mineralised dentine-like tissue and associated dental pulp-like tissue *in vivo*, distinct to that formed by hBMMSCs [6, 7, 20]. However, further analysis of individual hDPSC subpopulations derived from single-cell colonies revealed significant differences in their proliferative and odontogenic potentials. Despite heterogeneous, multicolony hDPSC population expansion being capable of achieving >120 population doublings (PDs) *ex vivo*, only 20% of purified clonogenic hDPSC subpopulations were capable of proliferating >20PDs. Furthermore, only two-thirds of these hDPSC subpopulations were capable of forming abundant ectopic dentine *in vivo* [6–8].

Although Gronthos et al. concluded that isolated hDPSCs only represent a minor fraction of the total cell number within dental pulp tissues (approximately 400 fibroblastic colony-forming unit (CFU-F) colonies per 10^5 cells plated) [6, 7], additional confirmation of the limited proportion of isolated hDPSC subpopulations capable of undergoing extensive *ex vivo* expansion and odontogenesis further highlighted the considerable heterogeneity surrounding the proliferative, lineage differentiation and other biological characteristics of hDPSC subpopulations. Thus, such circumstances have since confounded efforts to purify and profile large numbers of particular hDPSC subpopulations with the desired genotypic and phenotypic qualities required for the development of MSC-based therapies. As it has been estimated that undifferentiated MSC populations only comprise around 0.001-0.01% of the total number of cells within tissues such as adult human bone marrow [21], the procedures used for the harvesting of sufficient quantities of hDPSC subpopulations for evaluation and development for clinical use become a significant consideration. As a result of such low MSC yields from native tissues, extensive *ex vivo* expansion is often necessary to obtain sufficient cell numbers for successful therapy development, especially where allogenic MSC-based therapies are concerned [22, 23], with typically reported MSC therapeutic doses in the range of 10^8 cells for routine cell transplantations [24, 25].

Consequently, a remaining challenge to the routine use of hDPSCs for regenerative medicine-based applications is the identification of particular molecular markers capable of discriminating hDPSCs with superior regenerative properties, versus lesser quality subpopulations. This review provides a comprehensive overview of our current understanding of hDPSC biology and the molecular basis behind hDPSC heterogeneity. Based on this knowledge, we further outline some of the key advances which have led to particular cellular markers being harnessed to distinguish between hDPSC subpopulations, in terms of their proliferative, multipotency, immunomodulatory, and other regenerative properties overall. Such characteristics may subsequently be exploited for the development of strategies that allow for the selective screening, improved isolation, and enrichment of superior quality hDPSC subpopulations from dental pulp tissues, leading to improved cell expansion, manufacture, and banking, thereby supporting the translational development of hDPSC-based therapy development.

2. Current Understanding of hDPSC Biology

2.1. Development and Stem Cell Niche Locations. Despite dental pulp being recognised as a highly vascularised and

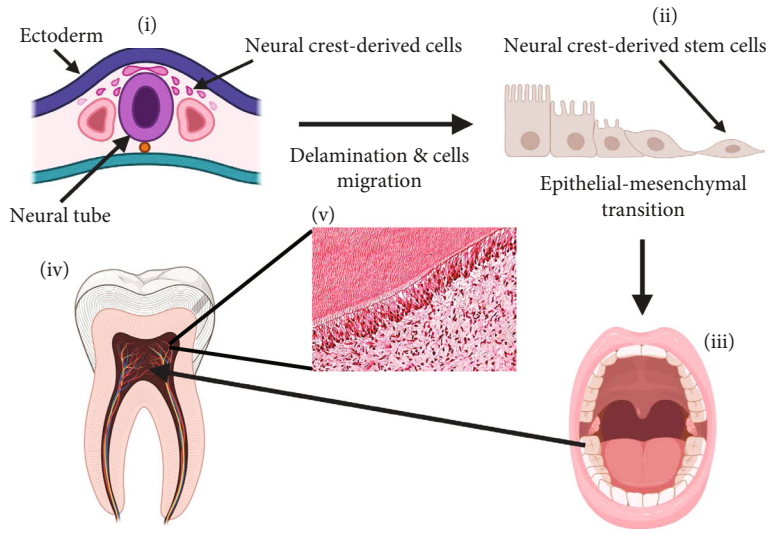

Figure 1: Summary of the events involved in hDPSC formation from migrating neural crest-derived cells during embryonic tooth development. (i) During development, neural crest-derived cells delaminate from the periphery of the neural tube and migrate to the oral region. (ii) Neural crest-derived cells undergo epithelial-mesenchymal transition, differentiating into neural crest stem cells and (iii) subsequently into several other cell types and tissues within the craniofacial region. (iv–v) These include the various cell types which comprise the dentine-pulp complex, including hDPSCs.

innervated tissue comprising of a multi-heterogeneous population of cells [1], hDPSCs are established to be ectomesenchymal-derived stem cells, originating during embryonic tooth development from migrating cranial neural crest cells and possessing MSC-related properties [26–29]. During development, neural crest-derived cells delaminate from the periphery of the neural tube, migrate to the oral region, and undergo epithelial-mesenchymal transition, differentiating into neural crest stem cells and subsequently into several other cell types and tissues within the craniofacial region (Figure 1). As the self-renewal and multipotency of premigratory and postmigratory neural crest cells are thought to be maintained by neural crest-derived MSCs within developing tissues [30, 31], neural crest cells confer the advantageous regenerative properties of MSCs within the craniofacial region, including hDPSCs [11, 12, 27–33].

In postnatal tissues, hDPSCs remain quiescent within their stem cell niche microenvironments of the healthy dentine-pulp complex [34, 35], for instance, through their differentiation into newly formed odontoblast-like cells or restoration of pulpal fibroblast composition during tertiary (reparative) dentinogenesis [1, 32, 36–38]. Although the ontogeny, anatomical locations, and identities of hDPSCs within the dental pulp remain to be fully established, initial studies proposed that hDPSCs originate from within the cell-rich subodontoblast layer residing adjacent to the postmitotic primary odontoblasts, from fibroblast-like cell populations within the dental pulp stroma, and from perivascular regions associated with the pulpal vasculature [32, 37–41]. However, as hDPSCs adjacent to the primary odontoblasts are unlikely to contribute significantly to the regeneration of odontoblast-like cells during tertiary dentinogenesis [42], pericyte-derived subpopulations within the perivascular niche have since been established to possess particularly prominent roles in responding to tissue injury within the dentine-pulp complex, although contributions from nonpericyte-derived MSCs also occur [37–44]. Consequently, it is now believed that hDPSCs exist within several different niches throughout the dental pulp, albeit with distinct intrinsic characteristics and regenerative properties based on their respective locations within the tissue [3, 45, 46]. Thus, it has been speculated that the hDPSC subpopulations with contrasting developmental lineages within the dental pulp respond differently during tissue repair, which may account for the diverse proliferative and odontogenic responses originally observed within the dentine-pulp complex, following transplantation of individual single colony-derived hDPSC strains [1, 3, 6, 7].

2.2. Immunophenotypic Features. In accordance with the minimal criteria stipulated for the classification of human MSCs by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cell and Gene Therapy (ISCT) [47], hDPSCs exhibit adherence to tissue culture plastic under standard culture conditions and specific cell surface antigen expression (positive CD73, CD90, and CD105 expression and negative CD11b, CD14, CD19, CD34, CD45, CD79α, and human leukocyte antigen-[HLA]-DR expression) and exhibit multipotent differentiation capabilities of osteogenic, chondrogenic, and adipogenic lineages. However, although some controversy still surrounds the appropriateness and use of these specific criteria, particularly with contradictory reports around the expression of certain hematopoietic stem cell markers [48], it has been widely confirmed that hDPSCs demonstrate positive expression of the ISCT-recommended MSC markers, CD73 (5′-ectonucleotidase), CD90 (Thy-1), and CD105 (endoglin), and negative expression of the hematopoietic stem cell markers, CD3, CD8, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD71, CD79α, CD117, and HLA-DR [6, 8,

9, 11, 12, 49–52]. However, there is currently no specific marker for hDPSCs, and although expression of a wide range of other mesenchymal, embryonic, neural crest, and other cell surface markers has been extensively examined, the heterogeneous nature of hDPSC subpopulations within dental pulp tissues and their distinct immunophenotypic characteristics have led to considerable inconsistencies and diversity being displayed in their marker expression profiles [9, 11, 48]. Nonetheless, in addition to CD73, CD90, and CD105, hDPSCs have been reported to express numerous other MSC surface markers, such as CD13 (aminopeptidase N), CD29 (β_1-integrin), CD44, CD166 (activated-leucocyte cell adhesion molecule), and CD271 (low-affinity nerve growth factor receptor, LANGFR/p75) [9, 11, 49–56]. Consistent with their proposed location within the perivascular niche [37–44], hDPSCs have also been demonstrated to positively express perivascular cell (STRO-1 (stromal precursor antigen 1), STRO-3, and PDGFR-β (platelet-derived growth factor receptor-β)), endothelial cell (CD106, vascular cell adhesion molecule-1; CD146, melanoma cell adhesion molecule), smooth muscle cell (α-smooth muscle actin (αSMA)), and pericyte (3G5, ribosomal protein S14; NG2, neuron-glial antigen 2) markers, with hDPSCs predominantly presenting a pericyte-associated phenotype [6, 9, 12, 43, 44, 50–53, 56, 57].

Analysis of embryonic stem cell markers has revealed varying levels of OCT4 (octamer-binding transcription factor-4), NANOG (homeobox transcription factor), SOX2 (SRY- (sex determining region Y-) box 2), SSEA4 (stage-specific embryonic antigen-4), and Slug expression in hDPSCs, which regulate stem cell properties such as self-renewal, multi/pluripotency, and mesenchymal lineage commitment [9, 11, 49, 50, 53, 58–60]. Furthermore, hDPSCs exhibit positive gene expression for self-renewal and multipotency marker, BMI-1 [53, 61, 62]. Based on their neural crest origins, expression of various neural lineage markers has also been identified in hDPSCs, including CD117 (c-Kit), CD271, Nestin, glial fibrillary acidic protein (GFAP), β-III tubulin, S100, Notch 1, musashi-1, synaptophysin, microtubule-associated protein 2 (MAP-2), and oligodendrocyte-associated CNPase [11, 12, 49, 50, 52, 53, 63–65].

2.3. Self-Renewal and Multilineage Differentiation Characteristics. High self-renewal capabilities are one of the defining features of hDPSCs [2, 6–8]. Although hDPSCs and hBMMSCs both share similar spindle-shaped morphologies, gene expression profiles, and differentiation pathways overall, hDPSCs have been shown to maintain higher colony-forming efficiencies and proliferation rates than hBMMSCs, associated with the elevated expression of cell cycle-related genes, such as cyclin-dependent kinase 6 and insulin-like growth factor 2 (IGF-2), by hDPSCs [6–8, 66]. Indeed, heterogeneous hDPSC populations have been proven to possess considerable expansion potentials achieving >120PDs *ex vivo*, although considerable variations in the proliferative capacities of individual hDPSC subpopulations have been highlighted, as most are only capable of achieving <40PDs in culture [6–8, 52, 53, 67].

Under basal conditions, hDPSCs express osteogenic marker genes, including runt-related transcription factor 2 (RUNX2), type I collagen, dentine sialophosphoprotein (DSPP), osteocalcin, osteopontin, osteonectin, alkaline phosphatase, and bone morphogenetic proteins (BMP-2, BMP-4); adipogenic marker genes, such as peroxisome proliferator-activated receptor γ (PPARγ), lipoprotein lipase (LPL), leptin, and adipophilin; chondrogenic markers, such as type II collagen and SOX9; and myogenic markers, such as αSMA, myosin, myogenin, and desmin [2, 6–8, 12, 49]. Such genotypic qualities support the extensive plasticity displayed by hDPSCs, a hallmark feature which makes these populations such attractive propositions in regenerative medicine, in terms of their potential to mature into more specialised cells for the potential repair of dental and nondental tissues throughout the body [11, 15, 27–29]. Under appropriate inductive conditions *in vitro*, hDPSCs can be induced to undergo differentiation into numerous cell types associated with both mesodermal and nonmesodermal (ectodermal and endodermal) lineages, including odontoblasts, osteoblasts, chondrocytes, adipocytes, glia cells, neuronal cells, oligodendrocytes, Schwann cells, retinal ganglion-like cells, endothelial cells, pancreatic cells, cardiomyocytes, hepatocytes, melanocytes, skeletal muscle cells, and bladder smooth muscle cells [8–12, 15, 50], well beyond the minimum multilineage differentiation criteria stipulated for hBMMSCs by the ISCT [47]. Such findings have subsequently led to further evaluation of hDPSC differentiation potency and regenerative potentials *in vivo*, most notably following transplantation into various animal disease and trauma models [8–12, 15].

2.4. Immunomodulatory and Anti-Inflammatory Properties. In addition to their proliferative and differentiation characteristics, hDPSCs have further been demonstrated to possess potent immunomodulatory and anti-inflammatory properties. hDPSCs do not express the major HLA class II surface antigen and are capable of inhibiting $CD4^+$ and $CD8^+$ T-cell proliferation and proinflammatory cytokine production, in addition to inducing their apoptosis. Such responses are induced by the secretion of soluble factors, such as human leukocyte antigen G5 (HLA-G5), interleukins (IL-6, IL-10), transforming growth factor-β_1 (TGF-β_1), hepatocyte growth factor (HGF), and Fas ligand (FasL), via hDPSC-derived exosome release and through the induction of endoplasmic reticulum (ER) stress in T-cells [68–75]. hDPSCs can also prevent T-helper 17 (Th17) cell activation, whilst stimulating regulatory T cell (Treg) differentiation, and suppress B cell proliferation and differentiation, influencing immunoglobulin production [69, 71–73, 76]. hDPSCs further attenuate activated peripheral blood mononuclear cell (PBMC) proliferation via TGF-β_1, indoleamine 2,3-dioxygenase (IDO), and HGF secretion [77, 78] and regulate the differentiation and functions of macrophage subtypes through IDO-mediated inhibition of tumour necrosis factor-α (TNF-α) secretion and development of the proinflammatory M1 macrophage phenotype, in addition to stimulation of anti-inflammatory M2 macrophage polarisation by inhibiting Toll-like receptor (TLR) and nuclear factor κB (NFκB) signalling [78, 79].

3. Markers Implicated in Distinguishing hDPSC Subpopulations with Distinct Characteristics

In light of the established heterogeneous nature of hDPSC subpopulations, a comprehensive understanding of the biological characteristics of hDPSCs has been crucial to efforts aimed at developing strategies for their exploitation as novel tissue regeneration therapies for clinical applications. Consequently, numerous studies have now reported the isolation and characterisation of single colony-derived clonal populations of hDPSCs utilising strategies and specific biological characteristics as markers, to selectively obtain more refined subpopulations for regenerative medicine purposes. A summary of these proposed heterogenic markers, their subcellular locations, and the purported hDPSC characteristics which these markers identify is presented in Figure 2 and Table 1.

3.1. Cell Surface Markers. To date, the most widely analysed and exploited biological characteristics described for the detection and purification of particular hDPSC subpopulations have been based on their relative expression of specific mesenchymal, embryonic, and neural crest cell surface markers [11, 50, 51]. Notably, early characterisation studies utilised a combination of perivascular markers (STRO-1, CD146, and 3G5), to colocalise STRO-1 and CD146 to the microvasculature and confirm that most hDPSCs reside within the perivascular niche of dental pulp tissues [43].

Numerous studies have since employed these and a variety of additional cell surface markers for the isolation and discrimination of distinct hDPSC subpopulations. STRO-1^+/$CD146^+$ subpopulations have been identified as highly proliferative, multipotent hDPSCs and are often coexpressed with other embryonic stem cell markers, such as OCT4 and NANOG, which aid the maintenance of MSC characteristics [59, 80]. These subpopulations have been shown to possess superior colony-forming efficiencies, compared to their STRO-1^-/$CD146^-$ counterparts, and able to proliferate >40PDs. STRO-1^+/$CD146^+$ hDPSCs are also capable of forming dentine/pulp-like structures [12], although certain STRO-1^+/$CD146^+$ clones were reported to exhibit restricted differentiation potential [80]. Thus, further attention has focussed on the characterisation of STRO-1^+ hDPSC subpopulations also positive for hematopoietic stem cell markers, c-kit^+ (CD117) and $CD34^+$. These multipotent subpopulations can undergo osteogenic differentiation *in vitro* [81], whilst c-kit^+/$CD34^+$/STRO-1^+ hDPSCs also coexpressing flk-1 (vascular endothelial growth factor receptor 2 (VEGFR2)) not only have strong osteogenic capacities but are also capable of angiogenic differentiation *in vitro*, codifferentiating into osteoprogenitor and endothelial progenitor cells [50, 82]. Furthermore, STRO-1^+/c-Kit^+/$CD34^-$ and STRO-1^+/c-Kit^+/$CD34^+$ hDPSCs have been proposed to represent distinct subpopulations, with contrasting cell proliferation, stemness, and differentiation properties, especially in terms of their ectodermal lineage capabilities, with STRO-1^+/c-Kit^+/$CD34^+$ hDPSCs possessing a greater propensity towards neurogenic commitment [83]. It has since been shown that STRO-1^+/c-Kit^+/$CD34^+$ hDPSCs, expressing CD271, Nestin, and SOX10, are capable of differentiating into Schwann cell-like cells *in vitro* and promoting axonal regeneration *in vivo* [84]. Thus, these studies suggest that a larger pool of hDPSCs exist within dental pulp tissues with enhanced multipotency towards mesodermal and ectodermal lineages, represented within a highly proliferative STRO-1^+ population comprising several interrelated subpopulations [85].

Further studies into $CD146^+$ hDPSCs have determined that these subpopulations are capable of promoting mineralisation and regenerating the dentine-pulp complex *in vivo*, identical to that formed by multicolony-derived hDPSCs [42, 86]. *In vivo* regenerated dentine and dentine-pulp complex were also significantly thicker and displayed uniform expression of dentine matrix protein-1 (DMP-1) and DSPP. Alternatively, $CD146^-$ subfractions have been reported to exhibit potent neurogenic potential, with the additional elevated expression of neurotrophic factors [87].

As $CD271^+$ cells are regarded as being of neural crest origin [63, 64], these have been identified as subpopulations with enhanced neurogenic potential, exhibiting high expression for neural markers, such as Nestin, SOX1, and SOX2, and the ability to differentiate into the neuronal cell lineage, compared to $CD271^-$ hDPSCs [88]. Although positive CD271 expression is relatively low across all hDPSCs, highly proliferative, multipotent hDPSC subpopulations exhibit no CD271 expression ($CD271^-$), unlike their low proliferative/unipotent $CD271^+$ counterparts [52, 53]. In accordance with CD271 expression being proposed to significantly influence multipotent differentiation capabilities in hDPSCs, $CD271^-$ subpopulations have been demonstrated to possess superior colony-forming efficiencies, prolonged proliferation, and multilineage potential *in vitro*, in addition to enhanced bone formation capabilities *in vivo* [64, 65]. Furthermore, CD271 has recently been identified to be highly expressed within STRO-1^+/c-Kit^+/$CD34^+$ hDPSC subpopulations, possessing slow proliferation rates, reduced stemness, and early-onset senescence, compared to their STRO-1^+/c-Kit^+/$CD34^-$ counterparts [83]. However, despite differences in CD271 expression, both hDPSC subpopulations exhibited similar osteogenic, myogenic, and adipogenic differentiation, although STRO-1^+/c-Kit^+/$CD34^+$ hDPSCs expressing CD271 demonstrated greater neurogenic lineage commitment. That said, not all studies have demonstrated complete inhibition of multipotent differentiation in $CD271^-$ expressing hDPSCs [63, 83].

Other MSC markers demonstrated to distinguish particular traits between hDPSC subpopulations include CD105, as $CD105^+$ hDPSC subpopulations exhibit high proliferative, migratory, and multipotent differentiation potentials, especially towards the angiogenic lineages, exhibiting high expression of vascular endothelial growth factor (VEGF) and other proangiogenic factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) [89]. Upon transplantation into a mouse hind limb ischaemia model, $CD105^+$ hDPSCs were able to regenerate high densities of capillaries within sites of injury. Such findings may be related to their ontogeny, as CD105 is a membrane glycoprotein expressed in vascular endothelium, as $CD105^+$ cells

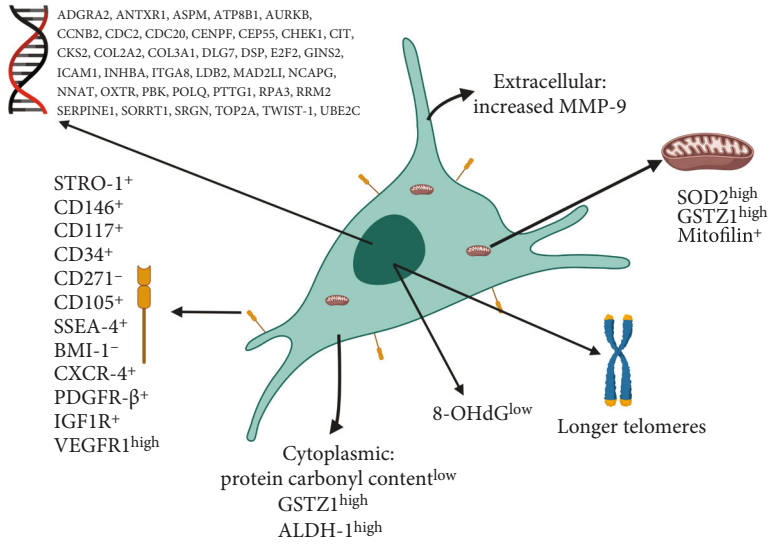

Figure 2: Summary of the subcellular locations of the proposed heterogenic gene, protein, and other biochemical markers implicated in distinguishing high stemness, proliferative, multipotency, and other regenerative characteristics between hDPSC subpopulations.

Table 1: Summary of the cell surface, senescence and other relevant gene, protein and metabolic markers implicated in distinguishing high stemness, proliferative, multi-potency and other regenerative characteristics between hDPSC sub-populations.

Marker	Associated properties of hDPSC sub-populations	References
STRO-1$^+$	High colony forming efficiency, high proliferation, multi-potent	[59, 80, 83, 85]
CD146$^+$	High colony forming efficiency, high proliferation, multi-potent, high odontogenic differentiation	[43, 59, 80, 86]
CD146$^-$	High neurogenic differentiation	[87]
CD117$^+$ (c-kit$^+$)	High odontogenic, osteogenic, neurogenic, adipogenic, myogenic and angiogenic differentiation	[50, 81–84, 94]
CD34$^+$	Low stemness, low proliferation, high osteogenic, neurogenic and angiogenic differentiation	[50, 81–84]
CD271$^+$	Low proliferation, bi-/uni-potent, high neurogenic differentiation	[52, 53, 88]
CD271$^-$	High colony forming efficiency, high proliferation, multi-potent	[52, 53, 64, 65, 83]
CD105$^+$	High proliferation, high migration, multi-potent, high angiogenic differentiation	[89]
CD51$^+$ (CD140α^+)	High odontogenic, osteogenic and chondrogenic differentiation	[64]
SSEA-4$^+$	High proliferation, multi-potent, high osteogenic, chondrogenic and neurogenic differentiation, low adipogenic differentiation	[58]
BMI-1$^-$	High stemness, high proliferation, low multi-potency	[61, 62]
CXCR-4$^+$ (CD186)	High colony forming efficiency, high proliferation, multi-potent	[57, 91, 92]
PDGFR-β^+	High proliferation, high odontogenic differentiation	[93]
IGF1R$^+$	High stemness, high proliferation, multi-potent, immunomodulatory, anti-inflammatory	[94]
VEGFR1^{high}	High angiogenic differentiation	[95]
Long Telomeres	High proliferation, resistance to senescence, high stemness, multi-potent	[53, 103]
Low Oxidative DNA & Protein Biomarkers	High proliferation, resistance to senescence, high stemness, multi-potent	[53, 104]
SOD2^{high}	High proliferation, resistance to senescence, high stemness, multi-potent	[52, 53]
GSTZ1^{high}	High proliferation, resistance to senescence, high stemness, multi-potent	[52, 53]
Mitofilin$^+$	High stemness, multi-potent	[112]
ALDH-1^{high}	High stemness, multi-potent	[113]
MMP-9^{high}	High stemness, high proliferation, multi-potent, increased matrix remodelling	[116]

are also found within the perivascular niche. Similar results were evident in a cerebral ischaemic model, whereby their transplantation resulted in new vessel formation in the ischaemic zone and subsequently promoted neuronal regeneration by endogenous neuronal cells [89].

CD51$^+$/CD140α^+ hDPSCs have also been identified as a subpopulation capable of odontogenesis, osteogenesis, and chondrogenesis. Their odontogenic and osteogenic differentiation capacity was demonstrated to be greater than that of the STRO-1$^+$/CD146$^+$ hDPSCs, producing greater quantified alkaline phosphatase activity and mineralised tissue formation [64]. However, despite the expression of other MSC markers, such as CD29$^+$, CD44$^+$, and CD73$^+$, having been suggested to correlate with hDPSC stemness, these have been precluded as having potential benefits as markers for the isolation of hDPSC subpopulations [28].

Although the majority of isolated hDPSCs exhibit negative hematopoietic stem cell marker expression, a small percentage (≤2%) has been found to be positive for markers, such as CD34$^+$ and CD117$^+$ [8–12, 50, 51]. CD34$^+$ hDPSC subpopulations have reduced proliferative capacities, but with an enhanced neurogenic potential, compared to their CD34$^-$ counterparts [83, 84]. CD34$^+$ clones also express lower levels of MSC markers, such as CD133 and CD44. CD34$^+$ clones have the ability to undergo osteogenic, adipogenic, myogenic, and neurogenic differentiation. Most significantly, CD34$^+$ hDPSCs show superior neurogenic potential and are capable of differentiating into Schwann cells, which upon *in vivo* transplantation were capable of sciatic nerve regeneration in an animal model [83, 84]. CD34$^+$ hDPSC subpopulations express greater Nestin and CD271 than CD34$^-$ clones and express GFAP. Subpopulations coexpressing STRO-1$^+$/c-kit$^+$/CD34$^+$ were also able to undergo osteogenic, adipogenic, and myogenic differentiation *in vitro*, but no significant differences were evident in the differentiation capacities between CD34$^-$ and CD34$^+$ subpopulations [81, 83, 85]. CD117$^+$ hDPSCs have been found to be capable of osteogenic, adipogenic, myogenic, and neurogenic differentiation, coexpressing with STRO-1$^+$/CD34$^+$ as described above [81, 83, 85]. CD117 (c-Kit) is a tyrosine kinase III receptor that acts in concert with stem cell factor (SCF) as its ligand, with proposed roles in maintaining the self-renewal properties of hDPSCs [90]. However, CD117 expression was gradually lost during differentiation [82].

In terms of embryonic stem cell and self-renewal markers, SSEA-4$^+$ hDPSCs have been characterised as being highly proliferative subpopulations, with multipotent capacities towards osteogenic, chondrogenic, and neurogenic lineages, but impaired adipogenesis [58]. Furthermore, reduced BMI-1 expression has been correlated with the maintenance of stemness and extended proliferative properties in hDPSCs, albeit resulting in potential impairments in differentiation potential [61, 62]. Expression of the stromal cell-derived factor (SDF)-1α receptor and C-X-C chemokine receptor type 4 (CXCR-4, CD186) has further been identified to distinguish hDPSCs with greater colony formation efficiencies and proliferative and multilineage differentiation capacities than their CXCR4$^-$ counterparts [57, 91, 92]. Similarly, hDPSCs sorted by their expression of PDGFR-β demonstrated that PDGFR-β^+/c-kit$^+$ subpopulations exhibited enhanced proliferation and prominent odontogenic differentiation *in vitro*, coupled with enhanced mineralisation and dentine/pulp-like tissue formation *in vivo* [93]. IGF1 receptor (IGF1R), regarded as a pluripotent marker of embryonic stem cells, was also found to be expressed in hDPSCs, with IGF1R$^+$ subpopulations displaying both self-renewal and multipotency potentials, especially towards neurogenic and angiogenic lineages [94]. Similarly, enriched populations of VEGFR1^{high} hDPSCs have a strong ability to undergo angiogenic differentiation *in vitro*, producing increased blood vessel sprouting and neovascularisation than VEGFR1^{low} subpopulations [95].

3.2. Markers Related to Self-Renewal, Proliferation, and Resistance to Cellular Senescence. In light of the integral importance of self-renewal, clonogenicity, colony-forming efficiency, and *ex vivo* expansion potential to the development of hDPSCs for regenerative medicine, numerous studies have established a number of relevant cellular markers capable of distinguishing hDPSC subpopulations with superior self-renewal and proliferative capabilities. As detailed above, STRO-1$^+$/CD146$^+$, CD271$^-$, BMI-1$^-$, and CXCR-4$^+$ hDPSC subpopulations have been shown to possess superior colony-forming efficiencies and stemness properties than their STRO-1$^-$/CD146$^-$, CD271$^+$, BMI-1$^+$, and CXCR4$^-$ counterparts [57, 59, 62, 64, 65, 80, 83, 91, 92].

Another significant drawback of hDPSC heterogeneity stems from the original findings that only 20% of purified clonogenic hDPSC subpopulations are capable of undergoing >20PDs [6–8], prior to proliferative decline and the onset of replicative (telomere-dependent) senescence. Such events significantly impede the *ex vivo* expansion capabilities of hDPSCs necessary to produce sufficient cell numbers for clinical use, characterised by progressive telomere shortening, inhibition of G_1-S phase transition, and permanent growth arrest. This is associated with the loss of telomeric TTAGGG repeats, positive senescence-associated β-galactosidase staining, and increased tumour suppressor (p53 and retinoblastoma protein (pRb)) and cyclin-dependent kinase inhibitor (p21^{waf1} and p16^{INK4a}) gene expression [96, 97]. These events are recognised to significantly alter the MSC genotype and phenotype, ultimately leading to impaired cellular regenerative properties and disrupted local tissue microenvironment signalling mechanisms, through the secretome associated with the senescence-associated secretory phenotype (SASP) [96–98].

Although hDPSC susceptibility to replicative (telomere-dependent) and oxidative stress-induced (telomere-independent) premature senescence has previously been recognised [52, 53, 61, 62, 99], the relative expression levels of many cell surface markers have been implicated as being indicative of elevated rates of hDPSC proliferation and/or expansion potential, including STRO-1$^+$, CD34$^-$, CD90$^+$, CD105$^+$, CD117$^+$, CD146$^+$, CD271$^-$, CXCR4$^+$, PDGFR-β^+/c-kit$^+$, and IGF1R$^+$ [50, 57, 59, 64, 65, 80–83, 85, 86, 89, 91–94]. Reduced BMI-1 expression in hDPSCs has been demonstrated to delay replicative senescence and limit senescence marker (positive senescence-associated β-galactosidase staining and elevated p16^{INK4a} expression) detection [62].

Additional studies have focussed on understanding the telomere dynamics underlying hDPSC heterogeneity and the intrinsic mechanisms responsible for protecting highly proliferative hDPSC subpopulations from accelerated telomere erosion. SSEA-4$^+$ hDPSCs have been found to possess longer telomeres and higher proliferation rates, compared to SSEA-4$^-$ subpopulations [58]. More recently, significant variations in the *ex vivo* expansion capabilities of individual hDPSC subpopulations have been demonstrated, with highly proliferative hDPSCs capable of achieving >80PDs, whereas low proliferating hDPSCs only complete <40PDs before senescence, correlating with hDPSCs with high proliferative capacities possessing longer telomeres. This leads to the delayed detection of senescence-related markers, such as positive senescence-associated β-galactosidase staining and elevated p53, p16^{INK4a}, and p21^{waf1} expression [52]. Thus, it is likely that such highly proliferative hDPSCs are responsible for the extensive expansion potential of heterogeneous hDPSC populations (>120PDs) *in vitro*, as previously described [6–8]. Low proliferative hDPSC senescence was also associated with the loss of stem cell marker characteristics, positive CD271 expression, and impaired osteogenic/chondrogenic differentiation, in favour of adipogenesis. In contrast, highly proliferative hDPSCs exhibited no CD271 expression but retained stemness and multipotency capabilities, only demonstrating impaired differentiation following prolonged *in vitro* expansion (>60PDs). As most studies have only reported negligible reverse transcriptase human telomerase catalytic subunit (hTERT) expression in hDPSCs [52, 53, 61, 100, 101], hTERT is unlikely to be responsible for maintaining telomere integrity and the proliferative/multipotency capabilities of highly proliferative hDPSCs. Thus, the absence of hTERT implies that other intrinsic mechanisms may account for differences in telomere lengths, proliferation rates, and differentiation capabilities between high and low proliferative hDPSC subpopulations.

Oxidative stress is another prominent mediator of cellular senescence in MSCs, associated with the excessive generation of reactive oxygen species (ROS) at the expense of endogenous enzymic and nonenzymic antioxidant defence mechanisms, leading to indiscriminate oxidative damage to biomolecules, such as DNA, proteins, and lipids and accelerating premature senescence [53, 96, 97, 102]. As with previous studies confirming differences in replicative senescence susceptibilities between high and low proliferative hDPSCs [52], similar variations in the relative susceptibilities of hDPSC subpopulations to premature senescence have also been confirmed, following continual exposure to oxidative stress [53]. Although all hDPSC subpopulations exhibit accelerated susceptibilities to premature senescence, highly proliferative hDPSCs (CD271$^-$) showed most resistance to premature senescence, achieving 50-76PDs similar to untreated controls (>80PDs). In contrast, low proliferative subpopulations (CD271$^+$) collectively displayed accelerated premature senescence (4-32PDs), even in untreated controls. Whilst telomere lengths were largely unaffected by oxidative stress exposure, elevated premature senescence susceptibilities in low proliferative hDPSCs (2-10PDs) were accompanied by the loss of certain stem cell markers and increased oxidative DNA (8-hydroxy-deoxy-guanosine (8-OHdG)) and protein (protein carbonyl content) damage, absent in highly proliferative hDPSCs until 45-60PDs [53]. Such findings of enhanced low proliferative hDPSC subpopulation susceptibilities to oxidative damage are supported by recent single-cell Raman spectroscopy studies, which demonstrated distinctive decreases in nucleic acid and protein spectral intensities in low proliferative hDPSCs, as a consequence of accumulative exposure to ROS-induced biomolecular damage [103].

Further studies led to the discovery that increased superoxide dismutase 2 (SOD2) and glutathione S-transferase ζ1 (GSTZ1) expression and SOD activities were present in highly proliferative hDPSCs (10-25PDs), which declined during culture expansion [53]. However, low proliferative hDPSCs (2-10PDs) exhibited inferior SOD-, catalase-, and glutathione-related antioxidant expression and activities overall. As mitochondria are the principle cellular source of ROS during senescence, mitochondrial SOD2 and mitochondrial/cytosolic GSTZ1 are likely candidates as the principle protective enzymic antioxidant defence mechanisms against oxidative stress in highly proliferative hDPSC subpopulations, preventing mitochondrial damage and hDPSC senescence and leading to the extended maintenance of proliferative, stemness, multipotency, and other cellular characteristics [52, 53, 62, 96, 104–107]. Thus, such telomere length, senescence, oxidative stress, and antioxidant characteristics may be utilised as predictors of hDPSC proliferative and multipotency qualities for future regenerative medicine exploitation [108]. Evidence is increasingly emerging to highlight mitochondrial dynamics, metabolism, oxidative stress, and function as having a major impact on the phenotypic responses of hDPSCs and other MSC populations, such as differentiation [109–111]. Indeed, cell proliferation-inducing protein 52 (mitofilin) is an antagonist of mitochondrial activation during differentiation located within the inner mitochondrial membranes of hDPSCs, which becomes depleted during normal differentiation [112]. Consequently, the selective isolation of mitofilin$^+$ hDPSCs has been shown to result in the isolation of more primitive cells with greater differentiation efficiencies. Therefore, a better understanding of the molecular profiles, mitochondrial-related stem cell markers, and morphological characteristics of hDPSC mitochondria may further prove effective in the selection of superior quality hDPSC for clinical applications.

3.3. Multipotency or Specialised Differentiation Markers. Another essential facet of hDPSC stemness, which makes them appealing options for regenerative medicine, is their potential multipotency capabilities [11, 15, 27–29]. Consequently, a wide range of studies have implicated individual or collections of cell surface markers as being indicative of multipotent differentiation characteristics in hDPSC subpopulations, including STRO-1$^+$/CD146$^+$[59, 80], c-kit$^+$/CD34$^+$/STRO-1$^+$ [50, 81–83], CD51$^+$/CD140α^+ [64], CD105$^+$ [89], CD271$^-$ [52, 53, 64, 65, 83], CXCR4$^+$[57, 91, 92], and IGF1R$^+$ [94]. Similarly, SSEA-4$^+$ hDPSCs possess multipotent capacities towards osteogenic, chondrogenic,

Table 2: Summary of the marker genes identified to exhibit upregulated expression by microarray analysis, which distinguishes between high proliferative/multipotent hDPSCs and low proliferative hDPSC subpopulations. Adapted from Menicanin et al. [117].

Gene functions	Gene symbol
Cell cycle, mitosis, and cell division	ASPM, AURKB, CCNB2, CDC2, CDC20, CENPF, CEP55, CIT, CKS2, DLG7, MAD2L1, NCAPG, PBK, PTTG1, UBE2C
DNA repair and replication	CHEK1, E2F2, GINS2, POLQ, PTTG1, RPA3, RRM2, TOP2A
Transcription regulation	CENPF, E2F2, LDB2, PTTG1, TWIST-1
Cell proliferation and differentiation	CENPF, CHEK1, CIT, CKS2, TWIST-1

Table 3: Summary of the marker genes relating to multipotency, stemness, or differentiation, identified to be differentially expressed by microarray analysis, which distinguishes between high proliferative/multipotent hDPSCs and low proliferative/lineage-restricted hDPSC subpopulations. Adapted from Kobayashi et al. [80].

	Gene symbol
Genes positively correlated with multipotency	ATP8B1, DSP, ICAM1, INHBA, NNAT, OXTR, SERPINE1, SORT1, SRGN
Genes negatively correlated with multipotency	ADGRA2, ANTXR1, COL1A2, COL3A1, ITGA8

and neurogenic lineages but impaired adipogenesis [58]. However, reduced BMI-1 expression is associated with limited hDPSC differentiation potential [61, 62]. Furthermore, despite not distinguishing multipotent properties, certain cell surface markers have been attributed to the identification of hDPSC subpopulations with specific mesodermal or ectodermal differentiation lineage capabilities. For instance, STRO-1$^+$/c-Kit$^+$/CD34$^+$, CD146$^-$, and CD271$^+$ hDPSCs have a greater propensity towards neurogenic commitment [83, 84, 87, 88], whilst CD105$^+$, VEGFR1^{high}, and PDGFR-β^+/c-kit$^+$ subpopulations have strong tendencies for angiogenic [89, 95] and odontogenic [93] lineage differentiation, respectively. Thus, such limited lineage differentiation tendencies may point towards more refined indications for such subpopulations in future for more specific regenerative applications, such as nerve injury and cardiovascular or tooth repair, thereby utilising these hDPSCs for optimised clinical scenarios for which they appear best suited.

Multipotency has further been revealed to be influenced by hDPSC telomere lengths and relative susceptibilities to replicative senescence, with hDPSC subpopulations with longer telomere lengths and higher proliferation rates displaying tripotent osteogenic, chondrogenic, and adipogenic lineage differentiation, in contrast to low telomere length, unipotent hDPSCs which only exhibit adipogenesis [52, 58]. As high and low telomere length hDPSCs express CD271$^-$ and CD271$^+$ levels, respectively [52, 53], positive CD271 expression in low telomere length hDPSC subpopulations may explain their more lineage-restricted capabilities, considering the established inhibitory effects of CD271 on multipotent differentiation in hDPSCs [64, 65].

3.4. Immunomodulatory Markers. Of the various hDPSC characteristics which may be exploited for regenerative medicine purposes, the abilities of specific markers to selectively distinguish hDPSC subpopulations with exceptional immunomodulatory and anti-inflammatory potencies are the area which is currently most limited [11, 68–79]. However, IGF1R$^+$ hDPSCs have been reported to possess immunomodulatory and anti-inflammatory properties, following *in vivo* transplantation into a rodent hypoxia-ischemia model [94].

3.5. Other Markers Associated with Other Regenerative Characteristics. hDPSC subpopulations expressing the intracellular enzyme, aldehyde dehydrogenase-1 (ALDH-1), associated with improved stemness have been found to be capable of osteogenic, chondrogenic, and adipogenic differentiation. These subpopulations have also been immunolocalised to the perivascular niche and the perineurium of nerve bundles [113]. The relative abilities of hDPSC subpopulations have further been exploited through their contrasting migratory responses to GM-CSF in culture, with highly migratory hDPSCs also exhibiting improved proliferative responses and other regenerative properties overall [114, 115]. Although most studies have established hDPSC heterogeneity using two-dimensional (2D) culture approaches, a recent study demonstrated that highly proliferative/multipotent and low proliferative/unipotent hDPSCs in three-dimensional (3D) type I collagen gels exhibit comparable gel contraction capabilities and matrix metalloproteinase-2 (MMP-2) expression/activities, although highly proliferative/multipotent hDPSC subpopulations possess higher MMP-9 expression/activities, which may impact on the abilities of these subpopulations to regulate cellular functions within the stem cell niche and remodel/degrade 3D biomaterial scaffolds; and their regenerative properties overall [116].

Microarray studies by Menicanin et al. [117] compared the global gene expression profiles of highly proliferative/multipotent hDPSC clones with low proliferative potential cell clones with restricted differentiation potential, in order to identify potential biomarkers of highly proliferative subpopulations with multipotent capabilities. In total, 24 genes were identified to be upregulated in highly proliferative/multipotent hDPSCs associated with cell cycle progression, mitosis, and cell division; DNA repair and replication; gene transcription; and cell proliferation and differentiation (Table 2). A more recent study by Kobayashi et al. [80] reported similar findings, revealing 1227 genes that were related to multipotency, 90 of which were also associated

with stemness or differentiation. Based on their relative levels of expression, 14 of these 90 genes were selected as candidate hDPSC markers, particularly in relation to their multipotency, stemness, or differentiation characteristics, between high proliferative/multipotent hDPSCs and low proliferative/lineage-restricted hDPSC subpopulations (Table 3). Thus, the characterisation of such hDPSC subpopulations provided an array of novel candidate marker genes of increased stemness, proliferation, and multipotency, facilitating their improved isolation and the enrichment of superior quality hDPSCs for regenerative medicine applications.

4. Future Perspectives and Considerations

Although individual hDPSC subpopulations do share certain similarities, hDPSC heterogeneity is now a well-established concept. Furthermore, despite a greater insight into the complex molecular factors and processes that underpin hDPSC heterogeneity being achieved since their original characterisation by Gronthos et al. [6, 7] and advances in the identification of a plethora of purported markers that discriminate between distinct hDPSC subpopulations, many important questions remain to be addressed. Firstly, why do diverse niches of hDPSC subpopulations exist within the dentine-pulp complex with contrasting immunophenotypes and proliferative/differentiation potentials, despite these tissues primarily consisting of odontoblasts and pulpal fibroblasts [1, 3, 6, 7, 32, 39, 40, 118]. Certain hDPSC subpopulations would undoubtedly be responsible for replenishing odontoblasts and pulpal fibroblasts lost due to disease and trauma during tertiary dentinogenesis [1, 32, 36–44, 119]. However, considering the neural crest origins of ectomesenchymal-derived hDPSCs, together with the highly vascularised and innervated nature of dental pulp tissues [1, 26–32], it is reasonable to assume that other hDPSC subpopulations are responsible for vascular and neural cell replacement and the identification of neural and perivascular cell markers within the dental pulp [6, 9, 11, 12, 37–44, 49–53, 56, 57, 63–65]. That said, as the identification of stem cell markers is an essential prerequisite to enable the selective screening, isolation, and purification of hDPSC subpopulations for particular therapeutic applications, it remains plausible that additional minor hDPSC subpopulations exist within dental pulp tissues which are yet to be isolated and explored, due to a lack of understanding regarding their intrinsic stem cell marker properties, niche locations, and roles within the dentine-pulp complex.

Although the impact of underlying factors associated with the individual patient donors of the permanent dentition used as sources of hDPSCs, such as their genomic composition, age, gender, health, diet, and environmental and other unknown factors, on the genotypic and phenotypic characteristics of hDPSC subpopulations is still open to speculation and remains to be fully established, these are highly likely to contribute to the issue of hDPSC heterogeneity [19]. However, from a wider perspective, more pertinent questions which certainly provide significant contributions to the complex heterogeneous nature of hDPSCs surround the developmental origins, hierarchical organisation, and precise niche locations of individual subpopulations within dental pulp tissues, in addition to the extent to which the entire heterogeneous populations within the dental pulp are comprised of true multipotent hDPSCs or a collection of unique committed progenitor cells with specialised lineage-restricted differentiation capabilities [9, 80]. Furthermore, despite standard methods being established for the isolation and characterisation of hDPSCs [120], the methods of derivation, cell culture conditions, the stage of the cell cycle, and proliferation or commitment upon isolation again could be influential factors on the regenerative properties of hDPSCs and warrant additional consideration [19, 120].

4.1. Developmental Origins. Gronthos et al. [6, 7] originally proposed that the heterogeneous nature of hDPSCs may reflect differences in their developmental stages or may even represent different pulpal cell lineages. Since then, studies into the developmental potentials of different hDPSC clones have suggested that a number of subpopulations exist within the dental pulp, derived from either the mesoderm or ectoderm of migratory cranial neural crest cell origins [26–29, 64, 83]. Several studies indicate that intrinsic positional information can dictate the neural crest stem cell phenotype within tissues and that environmental signals can regulate neural crest cell developmental fate and differentiation. As postmigratory neural crest cells only comprise a small proportion of the larger DPSC population overall and their multipotency is believed to persist within tissues [27–33], it is plausible that such differential developmental origins within the DPSC population contributes to their heterogenic nature. However, pericyte-derived subpopulations within the perivascular niche have been ascribed principal roles in mediating tissue repair responses within the dentine-pulp [37–44]. It has also been proposed that the relative contribution of pericyte-derived and nonpericyte-derived MSCs to cell differentiation in tissues depends on the extent of the vascularity and its kinetics of growth and/or repair. Thus, in tissues with high vascularity, such as dental pulp, the pericyte contribution to MSCs would be expected to be considerable [1, 121].

Although details on the nature and developmental origins of individual hDPSC subpopulations within human dental pulpal tissues have largely remained elusive, much progress has been made through the study of a mouse incisor stem cell model, regarded as an attractive system for the study of adult dental stem cell biology [122]. This model has permitted investigations into the properties, distinct locations, and contributions of active MSC subpopulations to the constant growth and repair of dentine and pulp tissues within continuously erupting incisor teeth, to compensate for tissue loss during occlusion. These constantly active MSCs can be subsequently distinguished from lesser active MSCs resident within the molar teeth, which do not undergo continual growth in adult mice [32, 118]. It has been established that incisor MSCs are a heterogeneous population, consisting of cells from different neural crest-derived tissues, with the ectomesenchymal cells giving rise to dental pulp and odontoblast cells, as evident in humans. Furthermore, through exploitation of genetic-based lineage tracing, it

was revealed that differentiated odontoblasts originate from perivascular NG2$^+$ pericytes during mouse incisor growth [44, 123]. It has further been shown that all NG2$^+$ perivascular cells are derived from GLI family zinc finger 1 (Gli1$^+$) cells, which are preferentially localised surrounding the vasculature. Although the majority of Gli1$^+$ cells in the mouse incisor do not express classic MSC markers, such as CD44, CD73, CD105, CD146, or Nestin, Gli1$^+$ cells are activated in response to incisor injury. Thus, Gli1$^+$ cells are the major source for odontoblasts and pulp cells during incisor growth and repair, although in contrast to incisors, mouse molars do not contain Gli1$^+$ cells around the dental pulp vasculature, whereas NG2$^+$ pericytes are present [124]. However, as lineage tracing quantification indicated that only 15-16% of newly differentiated odontoblasts were derived from NG2$^+$ perivascular cells, other MSC-like cells of nonpericyte origin were also shown to be present in the dental pulp and contribute to the majority of odontoblasts. Indeed, as certain MSCs have been shown to differentiate from peripheral nerve-associated glial cells [125], lineage tracing of Schwann cells as the predominant glial cell type confirmed that odontoblast-like cells originate from neural crest-derived Schwann cells and Schwann cell precursors, thereby initiating reparative dentinogenesis and supporting dental pulp cells through the formation of Schwann cell-derived odontoblasts. Therefore, as Schwann cells do not express perivascular markers, pericytes and Schwann cells are regarded as distinct dental stem cell populations within the neurovascular bundle regions of mouse incisor dental pulp, with diverse contributions to homeostasis and repair.

Lineage tracing experiments have since shown that αSMA-expressing, perivascular niche-derived MSCs generate a small number of newly formed odontoblasts during primary dentinogenesis, although their contribution to the formation of new odontoblast-like cells during reparative dentinogenesis is far more significant [126]. Further studies have also identified that 30% of MSCs in continually growing mouse incisors exhibit positive CD90 expression during postnatal development, although CD90$^+$ MSCs decrease in number during adulthood [127]. However, following adult incisor injury, CD90$^+$ MSCs reappear and contribute to reparative processes, replenished by mitotic cells within the mouse incisor pulp, positive for hematopoietic stem cell marker, Celsr1$^+$. Such lineage tracing analysis has also discovered Axin2$^+$ cells in the odontoblast layer and the dental pulp in the proximal region of the mouse incisor, whose progeny contributes to dental pulp cell and odontoblast numbers, implying that Axin2$^+$ cells are transit amplifying cells (TACs) [127]. Similarly, Axin2$^+$ cells in mouse molars differentiate into new odontoblast-like cells that secrete the reparative dentine via Wnt/β-catenin signalling in response to injury [128]. Additionally, PDGFRβ^+ cells are recognised as identifying MSCs within the cervical loop region and TACs of the mouse incisor model [129], which are distinct MSC populations to those found in the neurovascular niches [123–125]. Therefore, markers, such as NG2$^+$, Gli1$^+$, CD90$^+$, αSMA$^+$, Celsr1$^+$, Axin2$^+$, and PDGFRβ^+, amongst others yet to be identified, may shed light on similar hDPSC subpopulations within the perivascular niches of human dental pulp tissues and their roles in repair and regeneration within the dentine-pulp complex.

4.2. Stem Cell Niches within the Dental Pulp. It is widely accepted that MSCs reside in quiescent states within various specialised niches and uniquely organised local microenvironments that enable the maintenance and regulation of MSC self-renewal, proliferation, migration, and differentiation in response to injury, via direct cell-cell/cell-matrix interactions and communications mediated through secreted factors [6, 130, 131]. From original suggestions that hDPSCs originate from niches within the cell-rich subodontoblast layer, the dental pulp stroma, and especially from perivascular regions surrounding the pulpal vasculature [32, 37–44], it is now believed that several stem cell niches exist within the dental pulp containing distinct multipotent hDPSCs and other regenerative characteristics, supported by the identification of hDPSC subpopulations of pericyte and nonpericyte origins during previous human and mouse localisation and lineage tracing studies, as described above. However, further research is still necessary to explore the precise locations of as yet unexplored stem cell niches within the dental pulp and the extent to which local niche microenvironments influence hDPSC heterogeneity. Not only would such undertakings help in understanding whether isolated multipotent hDPSCs are derived from one highly proliferative multipotent population or from many committed hDPSC progenitor subpopulations with distinct lineages, but also assist in endeavours to develop more novel 3D scaffold materials which recapitulate the physiochemical properties of the native stem cell niche microenvironment, leading to improved regulation of transplanted hDPSC regenerative responses *in situ* [132–134].

4.3. Hierarchical Organisation. The ability to self-renew is one of the defining features of hDPSCs, recognised as involving either the slow cell division of an original mother stem cell to generate daughter cells with identical developmental potential to the original mother stem cell during symmetric division; or mother stem cell division into an identical copy of the mother cell and a highly proliferative TAC, possessing multipotent differentiation capabilities during asymmetric division. However, as TACs further divide to form larger colonies, they achieve a more mature progeny with reduced proliferative capabilities and the induction of replicative senescence, becoming more lineage-restricted. Consequently, stem cells expanded during development are maintained in quiescent states within the homeostatic stem cell niche microenvironments and participate in tissue repair as required, upon exposure to tissue perturbations or stressors [135, 136]. TACs have been suggested to arise within the postnatal dental pulp in response to cavity-induced injury and first to differentiate into new odontoblast-like cells [37].

As with MSCs derived from other tissues, the presence of a hierarchy amongst hDPSCs in adult dental pulp, with a small subpopulation of self-renewing, highly proliferative multipotent stem cells resident within a larger compartment of predominantly less proliferative and more committed, bipotent or unipotent subpopulations, has been proposed

for some time [6, 7, 137]. Nonetheless, the findings of more recent reports on hDPSC heterogeneity would certainly support the hierarchical model, in that small minority subpopulations within the dental pulp are highly proliferative, multipotent hDPSCs, whilst the majority are low proliferative, more lineage-restricted bipotent or unipotent hDPSCs [52, 53]. These highly proliferative, multipotent hDPSCs are believed to be responsible for maintaining the stem cell pool through their self-renewal ability and differentiation into cells of different lineages [1, 6]. As highly proliferative, multipotent hDPSCs with longer telomeres are expanded *ex vivo* and become senescent, they lose their proliferative capabilities and also become more lineage-restricted with bipotent or unipotent properties [52, 53]. As asymmetric stem cell division involves true mother cells giving rise to a multitude of differentiated daughter cells without themselves going through a high number of cell divisions, only a limited amount of telomere shortening occurs in such cells, thereby maintaining telomere length integrity [138]. Therefore, the characterisation of individual highly proliferative/multipotent and low proliferative/unipotent hDPSC subpopulations with contrasting telomere length profiles conceivably lends credence to the existence of a hierarchical arrangement amongst hDPSCs residing within the dental pulp.

Differential gene expression profiles between highly proliferative/multipotent hDPSC subpopulations and their low proliferative, bipotent or unipotent counterparts may also reflect these being different entities within the hierarchical organisation, as these genes associated with key responses, such as cell cycle progression, mitosis, and cell division; DNA repair and replication; cell proliferation; stemness; and differentiation, were commonly identified to be upregulated in highly proliferative/multipotent hDPSCs, which would expectedly contribute to the superior retention of stemness, proliferative, and differentiation characteristics versus their low proliferative, bipotent/unipotent counterparts (Tables 2 and 3) [80, 117]. Thus, hDPSC heterogeneity can be determined via distinct gene expression and the functionality and frequency of cell cycle transitions.

Nucleoside labelling and lineage tracing studies using the mouse incisor stem cell model [32, 118, 122] have exploited the differences between quiescent/slow-cycling (label-retaining) cells and fast-cycling cells (TACs), to investigate the tissue locations of rapidly cycling and slow-cycling cells. Using this approach, TACs expressing genes associated with polycomb repressive complex 1 (Prc1), such as Ring1a (Ring1) and Ring1b (Rnf2), have been confirmed as the most rapidly cycling cells in the mouse incisor dental pulp, located immediately distal to slow-cycling MSCs and crucial for mediating the TAC phenotype via Wnt/β-catenin signalling [139]. Furthermore, the Gli1$^+$ cells located within neurovascular bundles which provide the vast majority of odontoblasts and pulp cells during incisor growth and repair have been reported to colocalise with slow-cycling cells within the dental pulp [124]. CD90$^+$ slow-cycling cells have been elucidated to be DPSCs which contribute to odontoblasts and pulp cells throughout the life of the mouse incisor and responsible for incisor growth [125]. However, CD90$^+$ DPSCs only contribute to a small proportion of odontoblasts and pulp cells, correlating with the proportion of CD90$^+$ slow-cycling cells. Additional studies have found that around half of pulp cells and odontoblasts in the mouse incisor model were glial-derived, located within the population of slow-cycling cells with nonglial-derived, pericyte populations possibly contributing to the remainder [125]. Therefore, current evidence suggests that fast-cycling cells account for cell replenishment to maintain tissue homeostasis, whereas quiescent/slow-cycling cells act as a "reservoir" to be initiated to supply TACs, upon tissue injury [127, 140]. However, in contrast to hDPSC biology as a whole, much less emphasis has been directed towards understanding their TACs, despite their integral roles in repair and regenerative responses within the dentine-pulp complex.

4.4. Isolation, Purification, Characterisation, and Culture. Protocols for the routine isolation, purification, characterisation, and culture of hDPSCs are well-documented [9, 11, 19, 120, 141–143]. However, although the diverse range of methodologies reported for the harvesting of hDPSCs from dental pulp tissue achieve such aims overall, these do not overcome some of the current challenges which remain, in terms of addressing issues surrounding the consistent isolation and enrichment of hDPSCs with enhanced stemness, proliferative, and multipotent differentiation characteristics; in particular, as such hDPSCs are regarded as minor populations in the dental pulpal milieu [1, 6, 7, 52, 53, 137]. Thus, the development of novel strategies to permit the standard screening, collection, and expansion of particularly high-potency hDPSC subpopulations from dental pulp tissues would certainly be the key to meeting existing inadequacies relating to hDPSC heterogeneity.

Technical advances are being made, with most exploiting the molecular characteristics associated with hDPSCs with enhanced stemness, proliferative, and multipotent differentiation properties, as described above. Of these, the differential mesenchymal, embryonic, and neural crest cell surface marker profiles reported between hDPSC subpopulations have been most widely exploited to date, particularly using antibody- and molecular biology-based techniques, such as fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS), or real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) [11, 51, 52, 55, 56]. However, despite the multitude of cell surface markers identified, uncertainty remains whether such markers are specifically associated with the undifferentiated progenitor state of hDPSCs or even if these markers are actually MSC specific, or whether particular markers are more useful in identifying distinct hDPSC subpopulations individually or with other coexpressed markers. That said, even studies involving multiple stem cell markers have proven challenging, as it is difficult to distinguish whether hDPSC subpopulations are mutually exclusive of each other and if the characteristics of subpopulations that express a certain cell surface marker differ to other subpopulations that coexpress the same marker with additional surface markers. Furthermore, as most studies share a common limitation of having a small sample size of donors from which the hDPSCs were harvested, it remains to be established

whether hDPSC molecular profiles are representative across a wider population or a consequence of donor-donor variation. Alternative initiatives to address some of the concerns regarding the use of cell surface markers have included the identification of new cell surface proteome markers in hDPSCs isolated from single donors, using label-free mass spectrometry [56]. Of the 101 CD markers and 286 non-CD cell surface markers, these included TNF receptor superfamily proteins (CD40, CD120a, CD261, CD262, CD264, and CD266), integrins (α-4, α-6, and α-10), and IL receptors (CD121a, CD130, CD213a1, CD217, and CDw210b), which could be utilised for the more precise identification and isolation of hDPSCs. Similarly, multiparametric flow cytometry has recently been reported to permit the detection of several cell surface molecules, with which to characterise and identify the phenotypes of heterogeneous hDPSC subpopulations, both *in vitro* and *in vivo* [144]. The cell surface plasma membrane of hDPSCs has further been the focus of innovative studies aimed at enriching, separating, and identifying putative membrane protein markers by mass spectrometry, such as CD9, CD10 (neprilysin), and CD63, a novel approach which may be utilised for the characterisation and profiling of hDPSC subpopulations in future [145].

Gene expression profile comparisons of highly proliferative/multipotent hDPSC versus low proliferative/lineage-restricted hDPSCs have led to the discovery of potential genotypic marker genes for the selective isolation and purification of highly multipotent hDPSCs for regenerative medicine applications (Tables 2 and 3) [80, 117]. In contrast to these ground-breaking microarray studies, proteomic profiling comparisons of highly proliferative/multipotent hDPSC versus low proliferative/lineage-restricted hDPSCs have been much less in comparison [146, 147]. However, in studies involving low numbers of clones obtained from multiple donors, gene expression and proteomic differences amongst clones isolated from multiple donors may not truly reflect genotypic and phenotypic differences between highly proliferative/multipotent and low proliferative/lineage-restricted hDPSCs, but the genetic backgrounds of the donors instead [80]. Consequently, gene expression and proteomic analyses should be performed with patient-matched, highly proliferative/multipotent hDPSCs and low proliferative/lineage-restricted hDPSCs isolated from individual donors, thereby eliminating the influences of donor variation.

An alternative and greater understanding of hDPSC subpopulations within the dental pulp may be obtained utilising single-cell RNA sequencing (scRNA-seq), which allows the transcriptomic profiling of thousands of individual cells and is widely applied in stem cell biology for the analysis of MSC heterogeneity and the provision of specific markers by cell clustering, predicting cell fate by making trajectories, understanding the difference or dysregulation between different cell types, stage, or status, and providing indications for lineage tracing studies [148, 149]. Such sophisticated approaches can be expanded further through the combination of DNA, RNA, protein, and/or the epigenomic analyses, to permit the high-dimensional dissection of single cells, which offers great potential for understanding the regulation of molecular pathways. scRNA-seq, coupled with lineage tracing studies, has now been performed using mouse models, which have further highlighted the diverse nature of DPSCs within the developing tooth [150]. Additional reports utilising scRNA-seq have confirmed the high level of MSC heterogeneity within the dental pulp complex, with the existence of an active pool of DPSCs responsible for the formation of the principle mesenchymal-derived cell types, odontoblasts and distal/apical dental pulp cells, which produce a continuum of transient cell states [151]. The apical region of the dental pulp also contains active progenitor-like cell and precursor stromal-like cell subpopulations. Such findings were further validated in human wisdom teeth, which continue to grow until later in life. In comparison of molar hDPSC subpopulations to DPSCs within the mouse incisor dental pulp, the hDPSCs within the nongrowing human tooth preferentially exhibited a transcriptional state of more mature cells associated with the distal pulp, whilst those in the growing human tooth possessed a more apical-like transcriptomic profile [151]. Thus, it appears that nongrowing teeth are particularly characterised by a default distal pulp-like state, whilst an apical-like state is a signature of growing dental tissues. The presence of DPSC subpopulations and quiescent/active cell populations within the apical tooth region are supported by analogous scRNA-seq studies involving the mouse incisor model, which have identified a subpopulation of Runx2$^+$/Gli1$^+$ cells within the heterogeneous Gli1$^+$ population [152]. These Runx2$^+$/Gli1$^+$ cells are not MSCs in nature but are located in close proximity to MSCs and TACs, where they maintain the MSC niche and regulate TAC functions, via IGF signalling. These studies have further confirmed the occurrence of a novel Foxd1$^+$ DPSC subpopulation in the apical region near the epithelial labial cervical loop, where these are capable of differentiating towards odontogenic or pulpal lineages [150, 151].

As highly proliferative/multipotent hDPSCs are minority subpopulations within dental pulp tissues, the development of noninvasive strategies capable of successfully discriminating between hDPSC subpopulations with contrasting proliferative and differentiation capabilities *in situ* would be immensely beneficial for the selective screening and isolation of more desirable hDPSCs for *in vitro* assessment and therapy development. Consequently, single-cell Raman spectroscopy signatures obtained for highly proliferative/multipotent and low proliferative/lineage-restricted hDPSCs have been proven to be a viable noninvasive tool for the rapid screening and isolation of superior quality hDPSCs from dental pulp tissues *in situ*, thereby overcoming issues surrounding hDPSC heterogeneity [103].

5. Implications of Advances in Our Understanding of hDPSC Heterogeneity

It is undeniable that our understanding of the molecular basis underlying hDPSC heterogeneity has improved significantly in recent years. Although hDPSC heterogeneity has hindered their development for clinical application, the need for a greater understanding of their molecular characteristics has led to the identification of a wide variety of novel cell surface, gene, protein, and metabolic markers, purported to

reliably discriminate between hDPSC subpopulations with contrasting stemness, proliferative, multipotency, immunomodulatory, anti-inflammatory, and other relevant regenerative properties. Importantly, considering that hDPSCs only constitute a minor fraction of the total cell content within dental pulp overall, the identification and subsequent isolation and enrichment of highly proliferative, multipotent hDPSCs as even smaller minority subpopulations within the dental pulp become a much greater challenge. In light of the extensive *ex vivo* expansion required to obtain sufficient cell numbers for successful MSC-based therapy development [22, 23], highly proliferative hDPSC subpopulations with extended proliferative lifespans that retain stemness and multipotency capabilities could be regarded as ideal candidates to progress towards *ex vivo* laboratory evaluation and translational development as regenerative medicine-based therapies for broad clinical applications. That said, it has been suggested that lesser proliferative hDPSC subpopulations may be better suited in the development of more specialised tissues, in line with their restricted differentiation potentials down certain lineages, thereby expediting their possible tailoring towards more specific regenerative purposes.

Through the identification of new potential markers which distinguish hDPSC subpopulations with specific or superior characteristics, these will undoubtedly lead to advancements in the development of novel screening, isolation, and purification strategies, permitting the routine and effective identification, enrichment, and expansion of specific hDPSC subpopulations from whole dental pulp tissues for regenerative medicine applications. However, despite these recent advancements, many important aspects of hDPSC biology remain unanswered, which significantly impacts on their development as cellular therapeutics. For instance, despite cell surface proteins being extensively regarded as viable markers to distinguish hDPSC subpopulations, limitations in their specificity highlight the requirement for the identification of further markers, especially those capable of differentiating between highly proliferative/multipotent and low proliferative/lineage-restricted hDPSCs. As numerous markers were identified during gene expression profiling studies [80, 117], more extensive studies into the profiles of highly proliferative/multipotent and low proliferative/lineage-restricted hDPSC subpopulations using various genomic and proteomic technologies warrant further investigation. However, such studies would benefit from the inclusion of larger cohorts of patient donors, to confirm the most reliable gene, protein, or metabolic markers identified and discount the influences of donor genetic variations.

The reasons behind hDPSC heterogeneity remain to be fully established, although the developmental origins, hierarchical organisation, and stem cell niche locations of hDPSC subpopulations are strong contributory factors to the ultimate question as to what extent heterogeneous hDPSC populations within the dental pulp are derived from true multipotent hDPSCs or many different committed cell subpopulations exhibiting more specialised lineage-restricted differentiation capabilities [9, 80]. Nonetheless, existence of the hierarchical model is supported by recent reports that small minority subpopulations within the human dental pulp are highly proliferative, multipotent hDPSCs, whilst the majority are low proliferative, more lineage-restricted bipotent or unipotent hDPSCs [6, 7, 52, 53, 137, 138], further supported by the discovery of rapidly cycling TACs and slow-cycling cells relevant to the hierarchical structure, within the mouse incisor stem cell model [32, 44, 118, 122–129, 139, 140]. Such studies have further confirmed the prominent presence of DPSC subpopulations within both perivascular and neural niche locations associated with the neurovascular bundles of mouse incisor dental pulp, with contrasting roles in tissue homeostasis and repair [32, 44, 118, 122–129, 139, 140], helping to corroborate the presence of pericyte-derived hDPSC subpopulations within the perivascular niche of human dental pulp [37–44].

It is unquestionable that the development of transgenic mouse models to study stem cell incisor repair, coupled with technological advances in lineage tracing and scRNA-seq [32, 44, 118, 122–129, 139, 140, 148–152], has aided insights into DPSC subpopulation heterogeneity within the mouse incisor model, through the identification of specific cell types, status, and functions. An existing drawback of these techniques is the broad expression levels of currently used gene markers between neural crest cell and DPSC subpopulations. Additionally, as most lineage tracing experiments of dental pulp cells have solely been performed using the mouse incisor repair model, a key question remains over the relative applicability of such studies to nongrowing molar teeth in mouse models, in addition to whether comparative findings would be evident within human incisors and molars. Indeed, species variations in the cell subtypes and transcriptional profiles involved in tooth self-renewal have recently been highlighted between continuously erupting mouse incisor and nonerupting molars, in addition to growing and mature teeth in humans [151]. However, other recent reports have utilised scRNA-seq technologies to map the transcriptional landscape of the various cell populations that comprise human teeth, including the hDPSCs and other cell types within the dental pulp and their niche microenvironments [153]. By utilising such approaches, hDPSCs were characterised by their higher expression of Frizzled-related protein (FRZB), Notch receptor 3 (NOTCH3), CD90 (THY1), and smooth muscle myosin heavy chain 11 (MYH11), in line with their MSC and perivascular nature. Additionally, despite previous ethical concerns surrounding the possible use of lineage tracing experimentation in humans, recent developments have also demonstrated that it is now possible to trace human cell lineages using natural variations in nuclear/mitochondrial DNA and in DNA methylation status [154, 155]. Thus, although further characterisation studies into the diversity of neural crest cell and mouse DPSC subpopulations *in vivo* are warranted, such combined lineage tracing and scRNA-seq analyses of hDPSCs in human dental pulp tissues could allow us to finally address the remaining issues restricting the translational development of hDPSCs for future clinical use, through a better understanding of the cellular and molecular mechanisms regulating tooth development, homeostasis, and tissue repair, relevant to improved regenerative therapies in the future.

6. Final Conclusions

It is inevitable that hDPSC heterogeneity has posed major hurdles to their translational development and evaluations in clinical trials. Indeed, it is recognised that only a limited number of hDPSC-based clinical trials have occurred to date, due to concerns regarding the optimisation of isolation and culture expansion protocols, safety, mechanisms of action, good manufacturing practice (GMP), and quality control procedures and regulations [16, 19, 120, 156, 157]. Therefore, by addressing these remaining issues and harnessing their specific properties overall, the utilisation of specific markers for the discrimination of more desirable highly proliferative/multipotent hDPSC subpopulations could become a routine strategy for their selective isolation and purification in the future. Such innovative measures would ultimately aid their overall expansion, assessment and efficient hDPSC manufacture, cryopreservation, and banking [9, 12, 16, 18, 156, 158], thereby supporting the successful translational development of more effective hDPSC-based regenerative therapies for a wide range of potential dental and nondental clinical applications.

Abbreviations

2D: Two-dimensional
3D: Three-dimensional
8-OHdG: 8-Hydroxy-deoxy-guanosine
α-SMA: α-Smooth muscle actin
ALDH-1: Aldehyde dehydrogenase-1
BMP: Bone morphogenetic protein
CFU-F: Fibroblastic colony-forming unit
CXCR-4: C-X-C chemokine receptor type 4
DMP-1: Dentine matrix protein-1
DSPP: Dentin sialophosphoprotein
ER: Endoplasmic reticulum
FACS: Fluorescence-activated cell sorting
FasL: Fas ligand
FRZB: Frizzled-related protein
GFAP: Glial fibrillary acidic protein
Gli1: GLI family zinc finger 1
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GMP: Good manufacturing practice
GSTZ1: Glutathione S-transferase ζ1
hDPSC: Human dental pulp stem/stromal cell
HGF: Hepatocyte growth factor
HLA: Human leukocyte antigen
hTERT: Human telomerase catalytic subunit
IDO: Indoleamine 2,3-dioxygenase
IGF1R: IGF1 receptor
IGF-2: Insulin-like growth factor 2
IL: Interleukin
ISCT: International Society for Cell and Gene Therapy
LANGFR: Low-affinity nerve growth factor receptor
LPL: Lipoprotein lipase
MAbs: Monoclonal antibodies
MACS: Magnetic-activated cell sorting
MAP-2: Microtubule-associated protein 2
MHC: Major histocompatibility complex
MMP: Matrix metalloproteinase
MSC: Mesenchymal stem/stromal cell
MYH11: Smooth muscle myosin heavy chain 11
NFκB: Nuclear factor κB
NG2: Neuron-glial antigen 2
NOTCH3: Notch receptor 3
OCT4: Octamer-binding transcription factor-4
PDGFR-β: Platelet-derived growth factor receptor-β
PDs: Population doublings
PPARγ: Peroxisome proliferator-activated receptor γ
pRb: Retinoblastoma protein
Prc1: Polycomb repressive complex 1
qRT-PCR: Quantitative reverse transcription polymerase chain reaction
ROS: Reactive oxygen species
RUNX2: Runt-related transcription factor 2
SASP: Senescence-associated secretory phenotype
SCF: Stem cell factor
scRNA-seq: Single-cell RNA sequencing
SDF-1α: Stromal cell-derived factor-1α
SOX: SRY- (sex-determining region Y-) box
SOD2: Superoxide dismutase 2
SSEA-4: Stage-specific embryonic antigen-4
STRO: Stromal precursor antigen
TAC: Transit-amplifying cell
TGF-β_1: Transforming growth factor-β_1
Th17: T-helper 17
TLR: Toll-like receptor
TNF-α: Tumour necrosis factor-α
VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor.

Data Availability

No additional data were available to support this review article.

Disclosure

The presented manuscript is largely based upon the PhD thesis of coauthor Dr. Nadia Alaidaroos and the BDS Project Dissertation of coauthor Zi Kok.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Zi Y. Kok and Nadia Y.A. Alaidaroos are joint first authors.

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Review Article

Key Markers and Epigenetic Modifications of Dental-Derived Mesenchymal Stromal Cells

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As a novel research hotspot in tissue regeneration, dental-derived mesenchymal stromal cells (MSCs) are famous for their accessibility, multipotent differentiation ability, and high proliferation. However, cellular heterogeneity is a major obstacle to the clinical application of dental-derived MSCs. Here, we reviewed the heterogeneity of dental-derived MSCs firstly and then discussed the key markers and epigenetic modifications related to the proliferation, differentiation, immunomodulation, and aging of dental-derived MSCs. These messages help to control the composition and function of dental-derived MSCs and thus accelerate the translation of cell therapy into clinical practice.

1. Introduction

Mesenchymal stromal cells (MSCs) are plastic-adherent fibroblast-like cells [1] that have self-renewal and multidifferentiation potential and strong proliferative ability [2–4]. Bone marrow mesenchymal stromal cells (BMMSCs) are widely recognized in tissue regeneration. However, there are several challenges to obtaining BMMSCs, including pain, morbidity, and low harvested cell number. Thus, alternative sources for BMMSCs must be identified [5, 6]. Dental-derived MSCs have unique clinical advantages, such as easy access and remarkable tissue reparative/regenerative potential, and they have been proposed as ideal candidates for MSCs-based tissue regeneration [7–9].

Tissue regeneration and maintenance are dependent on MSCs [10]. However, a barrier to realize the therapeutic potential of MSCs is their intrinsic heterogeneity, which is also observed for dental-derived MSCs [11–13]. Cell subpopulations within heterogeneous MSCs cultures vary in their regenerative potential, including proliferation potential [14], differentiation [15], and immunomodulatory ability [16]. In MSCs therapy, cells are the active substances in medicines. Although variation is inevitable, our limited ability to detect and control heterogeneity poses challenges for the production of MSCs therapies. Heterogeneity has been cited as a possible factor contributing to the variability in treatment outcomes of MSCs therapies in clinical trials [17–19]. Variation in the regenerative potential among cell subsets in MSCs cultures may confound trial results and slow or arrest the translation of MSCs therapy into clinical practice [20].

Cell-to-cell variation in MSCs function is initiated in vivo in the MSCs niche [21], is evident within single-cell-derived MSCs colonies, and is exacerbated by replicative stress during in vitro cultivation [22]. A focus of ongoing research on dental-derived MSCs heterogeneity is to elucidate key markers. Cellular key markers can be used to noninvasively and nondestructively isolate specific cell subpopulations from MSCs cultures for clinical applications and research [23]. It may also help to regulate the regenerative potential of dental-derived MSCs. Besides, recent studies suggest that epigenetic alterations in cells occur in response to intrinsic cellular inheritance and external environmental stimuli to maintain the homeostasis of cells and niche cells [24, 25]. Epigenetic modification stimulates potentially reversible changes to gene expression, thus presenting exciting opportunities for clinical dental-derived MSCs interventions. Here,

Table 1: Characteristics of dental-derived MSCs.

Cell type	Origin	Multipotentiality (*in vitro*)	Application	References
DPSCs	Dental pulp tissue	Osteo/odontogenic Adipogenic Chondrogenic Vascular Neurogenic	Immunoregulation Angiogenesis Nerve injure treatment Dentin/pulp complex formation	[213–217]
SHEDs	Human exfoliated deciduous teeth	Osteo/odontogenic Adipogenic Chondrogenic Neurogenic	Angiogenesis Dental/pulp complex formation	[46, 218–220]
SCAPs	Apical papilla	Osteo/odontogenic Adipogenic Neurogenic	Dentin/pulp complex formation, spinal injure treatment Angiogenesis	[221, 222]
PDLSCs	Periodontal ligament	Osteo/cementogenic Adipogenic Chondrogenic Neurogenic	Immunosuppressive effects Periodontal disease	[8, 223–225]
DFCs	Dental follicle	Osteo/cementogenic Odontogenic Adipogenic Chondrogenic Neurogenic	Periodontal tissue Angiogenesis Pulp tissue formation	[54, 226, 227]
TGSCs	Apical papilla in the developing tooth germ	Adipogenic Osteogenic Neurogenic Adipogenic Chondrogenic Endothelial	Liver disease Dental defects	[32, 228, 229]
GMSCs	Gingiva	Osteogenic Chondrogenic Neurogenic Adipogenic	Nerve regeneration, mandibular defects Immunomodulatory properties	[31, 43, 230]

we first reviewed the heterogeneity of dental-derived MSCs. Then, we discussed the key markers and epigenetic modifications that support regeneration potential.

2. Dental-Derived MSCs and Their Heterogeneity

The oral region contains a variety of distinct MSCs populations, including mesenchymal stromal cells of dental pulp (DPSCs) [26], mesenchymal stromal cells of apical papilla (SCAPs) [27], mesenchymal stromal cells of human exfoliated deciduous teeth (SHEDs) [28], mesenchymal stromal cells of periodontal ligament (PDLSCs) [29], dental follicle cells (DFCs) [30], mesenchymal stromal cells of gingiva (GMSCs) [31], and mesenchymal stromal cells of human tooth germ (TGSCs) [32]. These dental-derived MSCs have less cytoplasm and are spindle-shaped, and they are positive for CD13, CD29, CD44, CD73, CD90, CD105, CD106, CD146, CD166, and CD271 but negative for CD3, CD8, CD11b (or CD14), CD15, CD19 (or CD79α), CD33, CD34, CD45, CD71, CD117, and HLA-DR [26, 27, 29, 33–35]. Since oral tissues develop from migrating cranial cells, dental-derived MSCs display ready availability and high proliferation ability [36, 37]. Moreover, they possess multidifferentiation potential and can differentiate into adipocytes [32, 38], chondrocytes [39, 40], osteoblasts [41, 42], neuronal cells [43–45], and endothelial cells [46, 47] (Table 1).

Issues related to cell heterogeneity are getting more and more attention in the field of MSCs research. A more comprehensive understanding of the variability of transplantable populations will help maximize the potential of any MSCs therapy [48]. According to the International Society for

Cellular Therapy, dental-derived MSCs meet all the minimum criteria that define MSCs [49]. Even so, this definition does not necessarily identify a homogeneous population of MSCs. Instead, it describes a group of heterogeneous cells that exhibit variability at the level among tissues of origin, individual donors, clonal subpopulations, and single cells [12, 50, 51].

2.1. Tissue and Donor Heterogeneity and Functional Variation. Every tissue or organ has its evolutionary origin. The composition of a tissue, in terms of cell types, stands behind the overall functionality. The heterogeneity of cell types increases throughout the evolution in every functional entity [52]. A large amount of evidence shows that MSCs from different tissues have differences in marker profiles, gene expression patterns, and tendency to differentiate into specific cell types [38, 53–55]. GMSCs were isolated and characterized as 90% derived from neural crest cells (cranial neural crest cell-derived GMSCs, N-GMSCs) and 10% derived from the mesoderm (mesoderm GMSCs, M-GMSCs). N-GMSCs express a high level of Fas-ligand (FasL), which induces T cell apoptosis and immune tolerance [56]. In comparison with M-GMSCs, N-GMSCs are more likely to differentiate into chondrocytes and neural cells in vitro and induce T-cell apoptosis [57]. Moreover, N-GMSCs can be induced into neural crest stem-like cells via the activation of RhoA-ROCK/Yes-associated protein 1 (YAP1) signaling [58]. These results indicate that N-GMSCs possess a superior capacity for immune regulation and differentiation than M-GMSCs. Indeed, there is mounting evidence that cultured cells retain a "memory" of their previous environments [59–61]. The highly heterogeneous nature of DPSCs is believed to be due to multiple progenitor cell populations existing in different locations of the dental pulp that may have different proliferation and differentiation abilities [62]. Different niches have been found in situ associated with the vasculature, within the pulpal stroma, in the subodontoblast layer, and among peripheral nerve-associated glial cells [63].

In addition, even from the same tissue source, dental-derived MSCs show tremendous variability between donors [13, 64, 65]. Similarly, as donor age increases, MSCs availability, self-renewal capacity, and differentiation potential have been reported to decline. Many animal and human studies have established the effects of increasing donor chronological age on the impairment of dental-derived MSCs regenerative capabilities [66–69]. In studies that involved small numbers of clones obtained from multiple donors, differences in gene expression among clones obtained from multiple donors might have reflected the different genetic backgrounds of the donors, rather than phenotypic differences between multipotent MSCs and committed progenitor cells [70]. Analysis of gene expression profiles among clones obtained from a single donor may allow researchers to eliminate the differences in genetic backgrounds that are associated with the use of multiple donors [71].

2.2. Clonal Heterogeneity and Functional Variation. Further study revealed that the clonal variation of dental-derived MSCs superimposes the difference among tissue- and donor-dependent differences. For example, DPSCs from multiple colonies can reach more than 120 population doublings (PDs), while single-colony-derived DPSCs can proliferate only up to 10-20 PDs [13]. Approximately two-thirds of DPSCs derived from single colonies can form the same amount of dentin as multicolony DPSCs. The other one-third generated only a limited amount of dentin. Studies have demonstrated that each colony is originally derived from the clonal expansion of a single progenitor cell [72]. However, there is growing appreciation that cellular phenotype can be highly variable, even within a clone [22]. Within the same colony, DPSCs of different cell shapes and sizes may be observed. If seeded on dentin, some DPSCs will be transformed into odontoblast-like cells with polarized cell bodies and a cell process extending into the existing dentinal tubules [73, 74]. When SHEDs clones derived from a single colony are transplanted into immunodeficient mice, only a quarter of the clones have the same potential to generate ectopic dentin-like tissue equivalent to that produced by multicolony-derived SHEDs clones [28]. Thus, cell-to-cell variation exists at every level, where the heterogeneity between clones has been noticed, and this must be taken into account when using this cell type in any basic scientific research or clinical application [22].

Functional variation at multiple levels extends to the molecular and epigenetic status of MSCs [71, 75]. Young et al. reported the ability of murine DPSCs clones derived from single cells to differentiate into immature neuron-like cells and oligodendrocyte-like cells in vitro. Significantly, only DPSCs clones with high nestin mRNA expression levels successfully differentiated into microtubule-associated protein 2 (Map2) and neurofilament- (NF-) positive neuron-like cells [50]. Alraies et al. identified differences between high (A3) and low (A1 and A2) proliferative capacity DPSCs populations, even from the same pulpal tissue sample [76]. They found that high proliferative capacity DPSCs exhibited longer telomeres but lacked CD271. It suggested that proliferative and regenerative heterogeneity is related to contrasting telomere lengths and CD271 expression between DPSCs populations. A highly dynamic histone modification response was evident in mineralizing DFCs, but not in DPSCs, and the latter cell type expressed higher levels of the pluripotency-associated genes octamer-binding transcription factor- (OCT-) 4 and NANOG [77]. The authors concluded that the two neural crest-derived MSCs populations were distinguished by epigenetic repression of dentinogenic genes and the dynamic histone enrichment in DFCs during mineralization. It highlighted the essential role of epigenetic mechanisms in the terminal differentiation of dental-derived MSCs and lineage commitment.

Such functional variability may provide an opportunity to identify MSCs subpopulations that are most suitable to drive a series of tissue restoration [75]. Moreover, it motivates ongoing work to reveal molecular or epigenetic markers of MSCs differentiation potential, as discussed later in this review.

3. Key Markers of Dental-Derived MSCs

For the application of dental-derived MSCs in tissue engineering and regenerative medicine, it is important to

optimize their isolation and preserve their phenotypic properties. The presence of key markers in the MSCs niche will help to break down the heterogeneous barrier of dental-derived MSCs [20, 78]. This section summarizes the key molecules that regulate dental-derived MSCs proliferation, differentiation, immunomodulation, and aging in the MSCs niche. This study will provide key targets and a certain theoretical basis for maintaining MSCs characteristics and promoting MSCs-mediated tissue regeneration. Table 2 lists some key markers in the field of dental-derived MSCs.

3.1. Key Markers of Proliferation Potential. Dental-derived MSCs are a reliable cell resource for tissue regeneration, and they need to be expanded largely in vitro, which requires cells to have superior proliferation and self-renewal potential [79]. It is necessary to explore the key markers related to proliferation to provide useful information for obtaining high-quality MSCs.

STRO-1 is a marker that recognizes a trypsin-insensitive epitope on perivascular cells, and it has been used to isolate MSCs populations from human and rat dental pulp and has shown enhanced proliferation potential [80]. Ranga Rao et al. found a gradual decrease in STRO-1 and transcription factor expression (OCT4, NANOG, and nestin) with an increase in the passage number of GMSCs [14]. A previous study showed that the STRO-1^+/$CD146^+$ SCAPs demonstrated higher colony-forming unit (CFU) efficiency and much higher expression of several embryonic and neural markers (stage-specific embryonic antigen-3 (SSEA-3); Nanog; OCT3/4; and nestin) than nonsorted SCAPs and the STRO-1^-/$CD146^+$ subpopulation [81]. Moreover, STRO-1^+ selected DPSCs show effective hard tissue formation when seeded into a calcium phosphate ceramic scaffold [82, 83]. These results suggested that STRO-1^+ cells may represent a very promising adult MSCs source with enhanced multipotent MSCs properties.

Alkaline phosphatase (ALP) is abundantly expressed in undifferentiated cells, such as induced pluripotent stromal (iPS) cells/embryonic stromal (ES) cells, preimplantation embryos (2-cell embryos to blastocysts (inner cell mass)) and embryonic ectoderm at the egg-cylinder stage, primordial germ cells (PGCs), and immature spermatogenic cells [84]. ALP is also a marker of neuronal progenitor cells, human myogenic progenitor cells (also called "pericytes"), and BMMSCs [85–87]. Inada et al. found that among the five primarily isolated SHEDs, two exhibited higher degrees of ALP activity and higher OCT-3/4 expression. Furthermore, these two lines proliferated faster than the other three lines and were easier to program into iPS cells [88]. Moreover, Yu et al. found that the ALP^+ subpopulation of PDLSCs had higher levels of STRO-1 and CD146 than ALP^- cells, even after a high number of passages. ALP^+ cells expressed significantly higher levels of stemness-associated genes, NANOG, OCT-4, and sex-determining region Y-box- (SOX-) 2 than ALP^- cells [89]. In summary, ALP^+ cells may represent a population with a higher proliferation rate than ALP^- cells. Further studies are needed to understand the roles of ALP in stemness in other dental subpopulations.

Nuclear factor I-C (NFIC) is regarded as a key regulator of tooth development. NFIC deficiency causes aberrant odontoblasts and abnormal dentin and periodontium formation, and it ultimately leads to short molar roots [90]. Zhang et al. found that overexpression of NFIC increases cell proliferation in SCAPs [91]. NFIC silencing could prolong the G1 phase of the cell cycle in SCAPs [92]. Moreover, Zhang et al. demonstrated that NFIC can markedly promote the proliferation of rat DFCs [93].

3.2. Key Markers of Differentiation Potential. The multipotent properties of dental-derived MSCs make them a valuable cell source for regeneration [6]. Osteogenic differentiation, chondrogenic differentiation, and adipogenic differentiation are the minimum requirements for the differentiation ability of MSCs. In addition, vascularity and innervation are two properties that cannot be sacrificed when considering tissue regeneration. In particular, a limitation of the apical foramen is that it requires the ingrowth of nerve fibers and blood vessels from apical tissues when regenerating parts of the tooth [94].

CD146 is a cell adhesion molecule and an integral membrane glycoprotein at the intercellular junction. It was originally identified as a tumor marker for melanoma [95]. Additionally, CD146 is a MSCs marker that is associated with angiogenic, neurogenic, and mineralization abilities [96]. $CD146^+$ BMMSCs possess high migration ability and are stromal cells that support hematopoiesis [97]. Matsui et al. found that $CD146^+$ DPSCs have higher mineralization ability than nonseparated cells, $CD146^-$ cells, and $CD146^{+/-}$ cells. Moreover, transplanted $CD146^+$ cells generated clear dentin/pulp-like structures in immunocompromised beige mice. Immunohistochemical studies detected dentin matrix protein-1 (DMP1), dentin sialophosphoprotein (DSPP), and human mitochondria in transplanted DPSCs [98]. This result suggests that $CD146^+$ cells may exhibit a high osteoblastic potential, which is consistent with previous studies [99].

CD271 or p75 neurotrophin receptor (NTR) is a well-conserved transmembrane pro-neurotrophin/neurotrophin receptor that plays critical roles in the maintenance of nerve cell viability [100]. CD271 has been proposed to be a neural MSCs marker that defines a cell population with neurogenic potential in the adult brain subventricular zone (SVZ) [101] and subgranular zone (SGZ) [102]. CD271 is expressed at low levels (<10%) in DPSCs. $CD271^+$ DPSCs have higher expression levels of SOX1 (neural precursor cell marker), SOX2 (cell pluripotency marker), and nestin (neural stem cell marker) than $CD271^-$ DPSCs. This result suggests that $CD271^+$ DPSCs may denote a subpopulation with greater neurogenic potential [103]. In addition, Alvarez et al. used a combination of the three surface markers CD51/CD140α, CD271, and STRO-1/CD146 to isolate homogenous populations of PDLSCs. $CD271^+$ cells had a higher dental/osteogenic potential and led to the greatest upregulation of osteogenic marker genes, like distal-less homeobox 5 (DLX5), runt-related transcription factor 2 (RUNX2), and bone gamma-carboxyglutamate protein (BGLAP) during the induction process [15].

Table 2: Key markers of dental-derived MSCs.

Study mode	Characterization	Key markers	Mechanism	Function (partly)	References
Proliferation potential					
Human SCAPs					
(i) STRO-1^{+}/CD146^{+} (ii) STRO-1^{-}/CD146^{+}	(i) Plastic adherent (ii) Osteo/odontogenic, adipogenic, neurogenic differentiation	STRO-1	(i) (+) DSPP, BSP, ALP, BGLAP, BMP2, Runx2, NFL-L, nestin, NCAM, and β-tub-III	In vitro (i) As a marker for subpopulation (ii) With higher CFU efficiency	[81]
Human PDLSCs					
(i) ALP^{+} (ii) ALP^{-}	(i) Plastic adherent (ii) Osteogenic, adipogenic, and neurogenic differentiation (iii) Positive for STRO-1, CD73, CD90, CD106, and CD146	ALP	(i) (+) STRO-1, CD146 NANOG, OCT4, SOX	In vitro (i) As a marker for subpopulation (ii) Express higher level of stemness genes	[89]
DFCs (rat)					
(i) Overexpress NFIC	(i) Plastic adherent (ii) Colony formation (iii) Osteogenic and adipogenic differentiation (iv) Positive for CD29, CD44, CD90, and negative for CD34 and CD45	NFIC	(i) (+) ALP, Col I, Runx2	In vitro (i) As a marker for regulating DFCs (ii) Promote the proliferation and osteogenic/cementogenic differentiation	[91]
Differentiation potential					
Human DPSCs		CD146			
(i) CD146+ (ii) CD146- (iii) CD146+/-	(i) Plastic adherent (ii) Osteogenic and adipogenic differentiation		(i) (+) ALP, osteocalcin, DMP1, DSPP	In vitro (i) As a marker for subpopulation (ii) With higher osteo/dentinogenic and adipogenic differentiation In vivo (i) Generated clear dentin/pulp-like structures	[98]
Human DPSCs		CD271/ p75 NTR,			
(i) CD271^{+} (ii) CD271^{-}	(i) Plastic adherent		(i) (+) nestin, SOX1, SOX2 and SOX9	In vitro (i) As a marker for subpopulation (ii) With greater neural differentiation potential	[102]
Human DPSCs (i) STRO-1^{+}/c-Kit^{+}/CD34^{+} (ii) STRO-1^{+}/c-Kit^{+}/CD34^{-}	(i) Plastic adherent (ii) Osteogenic and adipogenic differentiation	CD34	(i) (+) CD271 and nestin, β-galactosidase, MAP-2, Neu-N, synapsin	In vitro (i) As a marker for subpopulation (ii) With higher efficiency of neurogenic commitment	[106]
Human SCAPs		WIF1			
	(i) Plastic adherent				[108]

Table 2: Continued.

Study mode	Characterization	Key markers	Mechanism	Function (partly)	References
(i) Overexpress WIF1			(i) By activating OSX (ii) (+) ALP, DSPP, and DMP1	In vitro (i) As a marker for regulating SCAPs (ii) Enhance dentinogenic differentiation potential In vivo (i) Generated greater bone/dentin-like tissues	
Human SCAPs					
(i) Knockdown GNAI3	(i) Plastic adherent (ii) Odonto/osteogenic differentiation (iii) Positive for CD44 and CD90 but negative for CD14 and CD45	GNAI3	(i) By suppressing JNK/ERK signaling (ii) (+) DSPP, Runx2, OSX, OPN, OCN, and BMP4	In vitro (i) As a marker for regulating SCAPs (ii) Promoting proliferation, migration and odonto/osteogenic differentiation	[117]
Immunomodulatory					
Human PDLSCs					
(i) STRO-1^+/$CD146^+$ (97.1%)	(i) Plastic adherent	STRO-1/CD146	(i) (-) CD1b	In vitro (i) As a combination of markers for subpopulation (ii) Regulating DC-mediated T-cell proliferation	[124]
Cellular aging					
Human DPSCs					
(i) Young DPSCs/MDPSCs (ii) Aged DPSCs/MDPSCs	(i) Plastic adherent (ii) Angiogenic, neurogenic, odontogenic/osteogenic, and adipogenic differentiation (iii) Positive for CD29, CD44, CD73, and CD90, and negative for CD31	Migratory response to G-CSF	(i) A small age-dependent increase: SA-β-gal, p16, p21, IL-1β, IL-6, IL-8, Groα	In vitro (i) As a marker for subpopulation (ii) With high proliferation, migration, and regeneration potential is independent of age	[130]
Human PDLSCs					
(i) Activate YAP	(i) Plastic adherent (ii) Osteogenic, chondrogenic, and adipogenic differentiation (iii) Positive for CD44, CD73, CD90, and CD105), and negative for CD11b, CD19, CD34, CD45, HLA-DR	YAP	(i) (+) P-MEK, P-ERK, P-P90RSK and P-Msk (ii) (-) Bcl-2 family members (Bak, Bid, and Bik)	In vitro (i) As a marker for regulating PDLSCs (ii) Promote proliferation, accelerating the cell cycle, inhibiting apoptosis, and delaying senescence	[135, 136]
Human DPSCs					
(i) Overexpress TGF-β1	(i) Plastic adherent (ii) Osteogenic, chondrogenic and adipogenic differentiation (iii) Positive for CD90, CD44, CD105, and CD73, and negative for CD34, CD45, CD11b, and HLA-DR	TGF-β1		In vitro (i) As a marker for regulating DPSCs (ii) Have positive effect on proliferation, cell cycle, and prevents cellular senescence and apoptosis	[40]

CD34 is a transmembrane phosphoglycoprotein that was discovered for the first time in hematopoietic stromal cells (SCs). Clinically, it is related to the selection and enrichment of hematopoietic SCs during bone marrow transplantation [104, 105]. In addition, CD34 is assumed to act as a negative marker for MSCs [49]. Pisciotta et al. found that STRO-1^+/c-Kit^+/$CD34^+$ DPSCs showed a much higher efficiency of commitment compared to STRO-1^+/c-Kit^+/$CD34^-$ DPSCs, which was demonstrated by the expression of β-III tubulin and the shift to a neuron-like shape following the induction [106]. Moreover, Carnevale et al. demonstrated that STRO-1^+/c-Kit^+/$CD34^+$ DPSCs expressed Schwann cell markers, such as p75NTR, glial fibrillary acidic protein (GFAP), and S100 calcium binding protein B (S100B), after incubation in appropriate induction media. The integration of the graft of the DPSCs-collagen scaffold complex into a sciatic nerve defect in rats contributed directly to nerve fiber regeneration and myelination in vivo [107].

Wnt inhibitory factor 1 (WIF1) belongs to a family of secreted modulators of Wnt proteins. A recent study suggested that WIF1 may enhance the dentinogenic differentiation potential in SCAPs via its regulation of OSX. Moreover, in vivo transplantation experiments revealed that dentinogenesis in SCAPs was enhanced by WIF1 overexpression [108]. Other members of the Wnt modulator family, including secreted frizzled-related proteins (sFRPs), play different roles in Wnt signaling depending on the cell subtype and model used [109–112]. Guanine nucleotide binding proteins (GNAIs) are a family of regulatory proteins responsible for molecular signal transduction of extracellular signals to the intracellular environment [113]. GNAI3 has been demonstrated to play a role in regulating various cellular processes, including proliferation, cytokinesis, apoptosis, migration, and invasion [114–116]. GNAI3 is primarily expressed in Hertwig's epithelial root sheath (HERS) and the surrounding mesenchyme in mice. Moreover, knockdown of GNAI3 could inhibit the proliferation, migration, and odonto/osteogenic differentiation of $CD90^+$/$CD44^+$/$CD45^-$/$CD14^-$ SCAPs by inactivating c-Jun N-terminal kinase (JNK) and extracellular-signal regulated kinase (ERK) signaling pathways [117].

3.3. Immunomodulatory Key Markers. MSCs-based immunomodulation may play an essential role in the regeneration of different tissues. The immunomodulatory and tropic capacity of transplanted MSCs contributes to the creation of a microenvironment that promotes the activation of endogenous tissue repair mechanisms, and it is now considered to be the major mechanism underlying the therapeutic effects of these cells in vivo [118]. Similar to MSCs from other tissues, dental-derived MSCs possess a strong immunomodulatory ability [6, 119, 120]. Potential mechanisms underlying the immunomodulatory effects of MSCs include enzyme expression, soluble factor production, and cell-to-cell contact [121].

STRO-1^+ cells in MSCs have significantly enhanced inhibitory effects on lymphocyte proliferation compared with STRO-1^- cells; thus, STRO-1^+ cells impart stronger immunoregulatory effects than STRO-1^- cells [122, 123]. A previous study showed that the STRO-1^+ $CD146^+$ subpopulation of PDLSCs inhibit T cell proliferation by suppressing the expression of the nonclassical major histocompatibility complex-like glycoprotein CD1b on dendritic cells [124]. The priming of dental-derived MSCs with interferon-gamma (IFN-γ), tumor necrosis factor- (TNF-) α, and interleukin- (IL-) 1β usually enhances their immunosuppressive ability and could be considered a feedback mechanism that dampens exacerbated immune responses [121]. A recent study of human DPSCs showed that their ability to inhibit peripheral blood mononuclear cell (PBMC) proliferation and B cell immunoglobulin production was significantly enhanced by IFN-γ and inhibited by anti-IFN-γ antibodies [125].

3.4. Key Markers of Cellular Aging. MSCs aging is a negative process from the perspective of cell-based therapies because all advantageous functions may become limited with age. Dental-derived MSCs show clear losses in proliferation capacity with increasing donor age, and they also show donor age-related decreases in maximal life span and proliferation rate [126]. Under standard cell culture conditions, DFCs exhibit cellular senescence after being expanded by more than 14 cell passages [127]. With aging, the proliferation and osteogenic/adipogenic/chondrogenic differentiation potential of PDLSCs decreased while the apoptosis of PDLSCs increased. Moreover, the immunosuppressive ability of PDLSCs decreased with aging [128].

Signs of senescent cells include cell growth arrest, DNA damage foci, and senescence-associated β-galactosidase expression, and identifying these markers represents a reliable method for detecting senescent cells [129]. Horibe et al. isolated DPSCs subsets based on their migratory response to granulocyte colony stimulating factor (G-CSF) (MDPSCs) from young and aged donors. In long-term culture, MDPSCs showed a small age-dependent increase in senescence-associated β-galactosidase (SA-β-gal) production and senescence markers, including p16, p21, IL-1β, IL-6, IL-8, and Groα. The regenerative potential of aged MDPSCs was similar to that of young MDPSCs in an ischemic hindlimb model and an ectopic tooth root model [130]. Autologous transplantation of MDPSCs with G-CSF in pulp-ectomized teeth in dogs augmented the regeneration of pulp tissue. Furthermore, MDPSCs from aged donors were as potent as those from young donors [131]. Notably, MDPSCs showed no significant age-related changes in biological properties, such as stability, regenerative potential, and senescence marker expression.

The Hippo pathway is a newly discovered signaling network that is evolutionarily and functionally conserved and has been shown to play a critical role in controlling organ size by regulating both cell proliferation and apoptosis [132, 133]. As a Hippo signaling transcriptional coactivator, YAP plays pivotal roles in MSCs fate and organ size control [134]. Jia et al. discovered that activated YAP promotes proliferation, accelerates the cell cycle, inhibits apoptosis, and delays senescence in human PDLSCs [135]. Knockdown of YAP inhibits the proliferation activity and induces apoptosis of human PDLSCs with the involvement of the Hippo pathway and shows crosstalk with the Erk and Bcl-2 signaling pathways

[136]. Transforming growth factor- (TGF-) β1 is a potent stimulator of tissue regeneration and is abundant in the bone matrix [137]. Salkin et al. found that TGF-β1 transfection has a positive effect on proliferation and the cell cycle and prevents cellular senescence and apoptosis. They suggested that TGF-β1 overexpression with gene transfer may improve the biological potential of DPSCs and could represent an option instead of transmission of recombinant protein into cells from the outside [40]. However, the effects of TGF-β1 associated with cell senescence are controversial. A previous study demonstrated that treatment with TGF-β1 induced PDLSCs senescence, which is characterized by increasing in senescence-associated β-galactosidase activity and both p16 and p21 expression. Furthermore, TGF-β1 treatment demonstrated the capacity to induce the production of reactive oxygen species (ROS). Of note, the addition of a ROS scavenger successfully rescued TGF-β1-induced PDLSCs senescence [138]. These results indicated that the regulatory mechanism of TGF-β1 in cell senescence is quite different in various cells.

4. Epigenetic Modifications in Dental-Derived MSCs

Epigenetic modifications regulate gene expression without changing the DNA sequence and affect cell development and differentiation. DNA methylation, histone posttranslational modification, and noncoding RNA play primary roles in epigenetic mechanisms [139]. The rescuing potential of MSCs is under the control of different kinds of signals, including the environment, which epigenetically regulate their differentiation processes [140]. Recently, the epigenetic modifications that regulate dental-derived MSCs were revealed; thus, the use of epigenetics to improve the therapeutic potential of dental-derived MSCs has been highlighted. Therefore, summarizing these multiple epigenetic modifications associated with the differentiation process and determining how these modifications could be reversed are of paramount importance. Table 3 lists some epigenetic biological targets in the field of dental-derived MSCs.

4.1. DNA Methylation. DNA methylation is an essential epigenetic mechanism that plays a vital role in the development and differentiation of early embryos by regulating gene expression patterns. The global DNA methylation landscapes of early-life human tissues, such as oocytes, blastocysts, or placenta, are characterized by specific genome-wide hypomethylation compared to differentiated tissue postimplantation [141]. A previous study found that SHEDs have partially methylated domains (PMDs) that are close to the inner cell mass (ICM) and placental methylome. The methylation status of related genes changes under inflammation. For example, 44% of normal dental pulp tissues show complete methylation, while 93% of inflamed dental pulp tissue samples contain IFN-γ genes that are only partially methylated or unmethylated. In addition, IFN-γ transcription does not occur in the pulp tissue that shows per-methylation [142].

DNA methylation refers to the process in which methyl groups are transferred to cytosine bases of DNA and converted into 5-methylcytosine [143]. This process is catalyzed by DNA methyltransferases (DNMTs). The DNA methyltransferase family includes DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L. Several studies suggest that DNA demethylation levels are correlated with the osteogenesis capacity of MSCs and that DNMT inhibitors could downregulate DNA methylation to improve osteogenesis [144, 145]. 5-Azacytidine (5-aza), a DNMT inhibitor, works by integrating into the DNA structure to prevent DNA from interacting with DNMTs, and it also stimulates DNMT degradation [146]. Liu et al. found that high glucose conditions increased the DNA methylation levels of PDLSCs and blocked osteogenic differentiation ability. 5-Aza-2′-deoxycytidine (5-aza-dC) could rescue the osteogenic differentiation capacity of PDLSCs through activation of the canonical Wnt signaling pathway and the upregulation of osteogenesis-related genes (ALP, OCN, osteopontin (OPN), and OSX) [147]. Upon treatment with 5-aza-2′-deoxycytidine (5-aza-CdR), the odontogenic differentiation capacity of DPSCs is enhanced. 5-Aza-CdR upregulates odontogenic markers (DSPP and DMP1) and transcription factors (RUNX2, DLX5, and OSX), increases ALP activity, and accelerates calcified nodule formation [148]. In addition, myogenic differentiation is also improved after treatment with 5-aza. Nakatsuka et al. used 5-aza to investigate the myogenic differentiation potential of mouse DPSCs. DNA demethylation induced by 5-aza and forced expression of myogenic differentiation 1 (Myod1) upregulated muscle-specific transcription factors, such as myogenin and paired box 7 (Pax7) [149].

The ten-eleven translocation (Tet) family is a group of recently identified demethylases capable of modifying DNA by hydroxylating 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) [150]. Three Tet family members (Tet1, Tet2, and Tet3) show distinct expression patterns depending on the cell or tissue type and developmental stage [151, 152]. This discovery revealed a new mechanism by which the Tet enzyme regulates DNA demethylation. Yu et al. found that downregulation of Tet1 and Tet2 led to the hypermethylation of the Dickkopf WNT signaling pathway inhibitor 1 (DKK-1) promoter, activated the WNT signaling pathway, and increased the expression of FasL, and it also improved the immune regulation ability of PDLSCs. Importantly, Tet1/Tet2-downregulated PDLSCs showed a significantly increased therapeutic effect on DSS-induced colitis mice [153]. This result indicated that the Tet/DKK-1/FasL cascade may serve as a promising target for enhancing PDLSCs-based immune therapy.

4.2. Histone Posttranslational Modifications. The posttranslational modification of histones mainly occurs at the N-end of the tail protruding from the nucleosome core, and this modification plays an essential role in chromatin remodeling and gene expression regulation. In detail, distinct histone amino-terminal modifications can generate synergistic or antagonistic interaction affinity for chromatin-associated proteins, which in turn dictate dynamic transitions between transcriptionally active or transcriptionally silent chromatin states

Table 3: Epigenetic modifications of dental-derived MSCs.

Study mode	Characterization	Epigenetic molecules	Mechanism	Function (partly)	References
Human DPSCs/peri-implantitis DPSCs					
(i) Upregulate LINC00968	(i) Plastic adherent (ii) Osteogenic differentiation	LINC00968	(i) LINC00968 regulates RUNX2 expression by sponging miR-3658 (ii) (+) Runx2, Osx, and ALP	In vitro (i) As a therapeutic target of DPSCs (ii) Promote osteogenic differentiation and bone formation In vivo (i) Generate new bone and nodes in graft	[178]
Human DPSCs					
(i) Overexpress lncRNA-CCAT1	(i) Plastic adherent	lncRNA-CCAT1	(i) (+) collagen I, OPN, and OCN (ii) (-) miR-218	In vitro (i) As a therapeutic target of DPSCs (ii) Promote cell proliferation and differentiation	[231]
Human DPSCs					
(i) Overexpress lncRNA H19	(i) Plastic adherent (ii) Osteogenic differentiation (iii) Positive for CD73, CD90, CD105, and CD146, and negative for CD34 and CD45	lncRNA H19	(i) By inhibiting the DNMT3B-mediated methylation of DLX3 (ii) (-) SAHH	In vitro (i) As a therapeutic target of DPSCs (ii) Promote the odontogenic differentiation	[204]
Human DPSCs					
(i) Overexpress lncRNA MEG3	(i) Plastic adherent (ii) Osteogenic differentiation	lncRNA MEG3	(i) (+) SMURF1 (ii) (-) miRNA-543, OSX, OPN, OCN, and RUNX2	In vitro (i) As a therapeutic target of DPSCs (ii) Inhibit osteogenic differentiation	[232]
Human DPSCs					
(i) Silence SNHG7	(i) Positive for CD29, CD44, and CD90 (ii) Osteogenic differentiation	lncRNA SNHG7	(i) (+) miR-1226-3p and miR-210-5p (ii) (-) OCN, ALP, DMP1, BSP, BMP2, and DSPP	In vitro (i) As a therapeutic target of DPSCs (ii) Inhibits osteogenic differentiation	[170]
Human PDLSCs					
(i) Inhibit HDAC6	(i) Plastic adherent (ii) Osteogenic differentiation (iii) Positive for CD73, CD90, CD146, and CD29 and negative for CD34 and CD45	HDAC6	(i) (-) Acetylation of p27Kip1	In vitro (i) As a therapeutic target of PDLSCs (ii) Accelerate senescence, reduced osteogenic differentiation, diminished migration capacities	[167]

Table 3: Continued.

Study mode	Characterization	Epigenetic molecules	Mechanism	Function (partly)	References
Human PDLSCs					
(i) Knockdown circRNA CDR1as	(i) Plastic adherent (ii) Osteogenic differentiation	circRNA CDR1as	(i) (+) miR-7 (ii) (-) GDF5(BMP14), Smad1/5/8, and p38 MAPK phosphorylation	In vitro (i) As a therapeutic target of PDLSCs (ii) Inhibit osteogenic differentiation (iii) In vivo (iv) Less bone formation and a larger defect area	[184]
Human PDLSCs					
(i) Overexpress miRNA-132	(i) Plastic adherent (ii) Osteogenic and adipogenic differentiation (iii) Positive for STRO-1, CD73, CD90, CD105, and CD146 and negative for CD14, CD19, CD34, CD45, and HLA-DR	miR-132	(i) (-) GDF5, Runx2, OCN, and ALP	In vitro (i) As a therapeutic target of PDLSCs (ii) Inhibits osteogenic differentiation	[233]
Human PDLSCs					
(i) Downregulate lncRNA MEG3	(i) Plastic adherent (ii) Osteogenic differentiation (iii) Positive for CD90, CD105, and CD146 and negative for CD45, CD34, CD11b, CD19, and STRO-1	lncRNA MEG3	(i) (+) IGF1 (ii) (-) miRNA-27a-3p	In vitro (i) As a therapeutic target of PDLSCs (ii) Promote osteogenic differentiation	[234]
Human PDLSCs					
(i) Overexpress lncRNA SNHG1	(i) Plastic adherent (ii) Osteogenic, chondrogenic, and adipogenic differentiation (iii) Positive for CD73 and CD90 and negative for CD31 and CD34	lncRNA SNHG1	(i) By EZH2-mediated H3K27me3 methylation of KLF2 promotor (ii) (-) OCN, OSX, and ALP	In vitro (i) As a therapeutic target of PDLSCs (ii) Inhibit osteogenic differentiation	[235]
PDLSCs from periodontitis patients					
(i) Overexpress lncRNA -POIR	(i) Plastic adherent (ii) Osteogenic differentiation	lncRNA-POIR	(i) (+) FoxO1 (ii) (-) miR-182, cyclin D1, c-myc, and Axin	In vitro (i) As a therapeutic target of PDLSCs (ii) Promote osteogenic differentiation (iii) In vivo (iv) Promote osteogenesis	[236]

Table 3: Continued.

Study mode	Characterization	Epigenetic molecules	Mechanism	Function (partly)	References
Human PDLSCs					
(i) Upregulate lncRNA TUG1	(i) Plastic adherent (ii) Osteogenic differentiation (iii) Positive for STRO-1 and CD146 and negative for CD45 and CD31	lncRNA TUG1	(i) (+) Lin28A, Runx2, OCN, and ALP	In vitro (i) As a therapeutic target of PDLSCs (ii) Promote osteogenic differentiation	[237]
Human PDLSCs					
(i) Overexpress lncRNA TUG1	(i) Plastic adherent (ii) Osteogenic differentiation (iii) Positive for STRO-1 and CD146 and negative for CD34 and CD45	lncRNA TUG1	(i) (+) Smad2/7, Runx2, OCN, and ALP (ii) (-) miRNA-222-3p	In vitro (i) As a therapeutic target of PDLSCs (ii) Promote osteogenic differentiation	[238]
Human PDLSCs					
(i) Silence lncRNA XIST	(i) Plastic adherent (ii) Osteogenic differentiation	lncRNA XIST	(i) (+) Runx2, OCN, and ALP (ii) (-) miR-214-3p	In vitro (i) As a therapeutic target of PDLSCs (ii) Promote osteogenic differentiation	[239]
Human PDLSCs					
(i) Overexpress lncRNA MORT	(i) Plastic adherent	lncRNA MORT	—	In vitro (i) As a therapeutic target of PDLSCs (ii) Inhibit proliferation, affect the recurrence of periodontitis	[240]
Human SCAPs					
(i) Depletion of KDM2A or BCOR	(i) Plastic adherent (ii) Osteogenic differentiation	KDM2A/BCOR (Complex)	(i) By promoting methylation of the SFRP2 (ii) (+) OSX	In vitro (i) As a therapeutic target of SCAPs (ii) Promote osteo-/dentinogenic differentiation In vivo (i) Enhance bone/dentin-like tissue formation	[112]
Human SCAPs					
(i) Silence KDM2A	(i) Plastic adherent (ii) Chondrogenic and adipogenic differentiation	KDM2A	(i) (+) SOX2 and NANOG	In vitro (i) As a therapeutic target of SCAPs (ii) Enhance adipogenic and chondrogenic differentiation	[39]

Table 3: Continued.

Study mode	Characterization	Epigenetic molecules	Mechanism	Function (partly)	References
Human SCAPs					
(i) Overexpress DLX5	(i) Plastic adherent (ii) Osteogenic differentiation	DLX5	(i) (+) KDM4B, DSPP, DMP1, OPN, and OSX	In vitro (i) As a therapeutic target of SCAPs (ii) Promote osteo-/dentinogenic differentiation In vivo (i) Promote osteo-/dentinogenesis	[241]
Human SCAPs					
(i) Overexpress KDM2B	(i) Plastic adherent (ii) Chondrogenic differentiation	KDM2B	(i) (-) COL1, COL2, and SOX9	In vitro (i) As a therapeutic target of SCAPs (ii) Inhibit the chondrogenic differentiation	[242]
Human SCAPs					
(i) Knock down KDM3B	(i) Plastic adherent (ii) Osteogenic differentiation	KDM3B	(i) (-) ALP, RUNX2, OSX, DSPP, and OCN	In vitro (i) As a therapeutic target of SCAPs (ii) Inhibit the osteo-/odontogenic differentiation, proliferation, and migration	[243]
Human SCAPs					
(i) Overexpress miR-34a	(i) Plastic adherent (ii) Osteogenic differentiation	miR-34a	(i) (-) NOTCH2 (ii) (+) DSPP, RUNX2, OSX, and OCN	In vitro (i) As a therapeutic target of SCAPs (ii) Promote osteo-/odontogenic differentiation	[244]
Human SCAPs					
(i) Overexpress miR-141-3p	(i) Plastic adherent (ii) Positive for CD29, CD73, CD90, and CD105 and negative for CD34 and CD45	miR-141-3p	(i) (-) YAP	In vitro (i) As a therapeutic target of SCAPs (ii) Imped proliferative ability, promote senescence	[245]
Human SCAPs					
(i) Overexpress lncRNA-H19	(i) Plastic adherent (ii) Osteogenic differentiation	lncRNA-H19	(i) (+) miR-141, SPAG9 (activate p38 and JNK pathways), Runx2, DSP, and ALP	In vitro (i) As a therapeutic target of SCAPs (ii) Promote osteo/odontogenic In vivo (i) Enhance the osteo/dentinogenesis	[246]

Table 3: Continued.

Study mode	Characterization	Epigenetic molecules	Mechanism	Function (partly)	References
Human DFCs					
(i) Downregulate lncRNA MEG3	(i) Plastic adherent (ii) Osteogenic differentiation (iii) Positive for CD29, CD44, and CD90 and negative for CD31, CD34, and CD45	lncRNA MEG3	(i) Activate Wnt/β-catenin signaling pathway by decreasing H3K27me3 occupation at the Wnt gene promoters (ii) (+) β-catenin, ALP, RUNX2, and OCN (iii) (-) EZH2	In vitro (i) As a therapeutic target of DFCs (ii) Promote osteogenic differentiation	[247]
Human DFCs					
(i) Overexpress lncRNA HOTAIRM1	(i) Plastic adherent (ii) Osteogenic differentiation	lncRNA HOTAIRM1	(i) By maintaining the hypomethylated state of the HOXA2 promoter (ii) (+) HOXA2, ALP, and RUNX2 (iii) (-) DNMT1	In vitro (i) As a therapeutic target of DFCs (ii) Inhibit proliferation and promote osteogenesis	[248]
Human DFCs					
(i) Overexpress miR-101	(i) Plastic adherent (ii) Osteogenic differentiation	miR-101	(i) (+) SP7 (osterix)	In vitro (i) As a therapeutic target of DFCs (ii) Promote osteogenic differentiation	[249]
Human GMSCs					
(i) Overexpress miR-3940-5p	(i) Plastic adherent (ii) Osteogenic differentiation	miR-3940-5p	(i) (+) p15INK4b, p18INK4c, p19INK4d, Cyclin A, DSPP, DMP1, and DLX 5 (ii) (-) Cyclin E	In vitro (i) As a therapeutic target of GMSCs (ii) Inhibit cell proliferation, enhanced the osteo/dentino genic differentiation	[250]

[154]. The more common histone modifications include methylation, acetylation, phosphorylation, and ubiquitination. Among them, histone acetylation has been widely studied in the field of dental-derived MSCs [155]. Acetylation is the only modification that directly causes a structural relaxation of chromatin by neutralizing the charge of histones [156]. The balance of the acetylation process depends on the role of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs cause histidine acetyltransferases to add negatively charged acetyl groups, which weaken the interaction between DNA and histone residues, while HDACs remove acetyl groups [157].

The vital role of the acetylation process in maintaining the balance between osteoblastic bone formation and osteoclastic bone resorption is crucial for bone tissue homeostasis [158]. The acetylation of H3K14 (histone H3, lysine 14) and H3K9 (histone H3, lysine 9) can promote osteogenic differentiation of dental-derived MSCs [159]. HDACs are downregulated during the osteogenic differentiation of dental-derived MSCs [160, 161]. The use of HDAC inhibitors effectively increases the acetylation of H3K9K14 (histone H3, lysines 9 and 14) and promotes the expression of bone-related genes [162, 163]. Valproic acid (VPA), a short-chain fatty acid, can inhibit class II HDACs. Paino et al. demonstrated that HDAC2 silencing in DPSCs leads to increased expression of OPN and bone sialoprotein (BSP) and downregulates the mRNA levels of osteocalcin (OCN), which resembles the effect of VPA [164]. This result suggests that the specific inhibition of an individual HDAC by RNA interference could only enhance a single aspect of osteoblast differentiation, resulting in selective effects. It has been reported that the glucocorticoid receptor (GR) plays a key role in this regulation. HDAC2 binds to GR and inhibits its translocation into the nucleus; however, when HDAC2 is inhibited by VPA, GR can enter the nucleus, thereby affecting the expression of OCN [165].

Histone deacetylase 6 (HDAC6) is a class IIb HDAC with a unique duplicated deacetylase domain and ubiquitin-binding domain [166]. Interestingly, HDAC6, as a critical regulator of PDLSCs aging, can deacetylate p27Kip1. Loss-of-function experiments suggested that pharmacologic inhibition of the deacetylase activity of HDAC6 accelerated PDLSCs senescence and impaired MSCs activities, which showed reduced osteogenic differentiation and diminished migration capacities; thus, HDAC6 may be a new target for intervention in the aging process of PDLSCs [167].

4.3. Noncoding RNAs. Noncoding RNAs (ncRNAs) play an essential role in histone modification, gene silencing, and targeting DNA methylation. They are divided into short ncRNAs, with lengths of less than 30 nucleotides, and long ncRNAs, with more than 200 nucleotides [168]. ncRNAs regulate gene expression through transcription and posttranscriptional control. The overall activity and functional balance of gene networks are maintained by lncRNA/miRNA/mRNA regulatory interactions [169]. For example, a total of 89 lncRNAs, 1,636 mRNAs, and 113 miRNAs were differentially expressed after DPSCs differentiation. Simultaneously, an array of signaling pathways, including phosphoinositide-3-kinase–protein kinase B, TGF-β, and Wnt, were also affected. The lncRNA SNHG7 was shown to inhibit the odonto/osteogenic differentiation of DPSCs when silenced [170].

MicroRNAs (miRNAs) are vital regulators that promote the intrinsic properties of MSCs, such as their self-renewal, pluripotency, and differentiation capacities, and miRNAs have a length of approximately 20-22 nucleotides. MiRNAs extensively regulate cell functions by affecting the abundance and translation efficiency of homologous mRNAs. Single miRNAs can target numerous gene sites on mRNA transcripts. In contrast, targeting multiple miRNAs can jointly target a single mRNA [171–173]. MiRNAs are believed to be novel regulators in the differentiation of dental MSCs by targeting related genes. During osteogenic differentiation, the expression of 116 miRNAs was altered significantly in PDLSCs. The upregulated miRNAs were miR-654-3p and miR-4288 and the downregulated miRNAs were miR-34c-5p, miR-218-5p, miR-663a, and miR-874-3p. The prediction of target genes suggested that these significantly altered miRNAs may impact the osteogenic differentiation of PDLSCs by targeting osteogenesis-related genes [174].

Long noncoding RNAs (lncRNAs) (>200 nucleotides) are the largest ncRNA transcript family in the human genome and participate in transcription and posttranscriptional and epigenetic regulation of genes [175]. lncRNAs can fold into complex secondary or higher-order structures, and they show greater potential and versatility for gene regulation than miRNAs [176]. lncRNAs can act as RNA decoys and miRNA target site decoys. They bind to specific combinations of regulatory proteins and play essential roles in chromatin modification and processing of mRNA targets. A recent study showed that lncRNAs can cross-talk with mRNAs through competition for shared miRNA-response elements. In this circumstance, lncRNAs function as competitive endogenous RNAs (ceRNAs), which correspond to miRNA sponges or antagomirs, to affect the expression levels and activities of miRNAs, thereby repressing miRNA targets and causing an additional level of posttranscriptional regulation [177]. Previous studies have shown that lncRNAs regulate gene expression and function by competing with miRNAs for binding to target mRNAs [178]. Two thousand and one hundred sixty-two lncRNAs were differentially expressed between PDLSCs and GMSCs. These lncRNAs could be potential regulators, especially those with higher fold change (FC), such as lncRNA-n336841, lncRNA-n341766, and lncRNA-n333720 [179].

Circular RNAs (circRNAs) are widely distributed in organisms and represent a type of ncRNA with a cyclic covalent structure that has a high degree of evolutionary conservation and tissue cell expression specificity [180, 181]. circRNAs are more stable than linear RNAs due to their resistance to ribonuclease digestion [182]. Chen et al. revealed the circRNA expression profile in DPSCs during odontogenic differentiation. 43 upregulated circRNAs and 144 downregulated circRNAs were found in the process of dental differentiation. These differentially expressed genes are rich in signaling pathways that regulate the pluripotency of MSCs, such as the Wnt signaling pathway and the TGF

signaling pathway [183]. Recently, circRNAs were also shown to function as ceRNAs to regulate the effect of miRNAs on their target genes during cell differentiation. Previous studies have found that circRNA cerebellar degeneration-related protein 1 (CDR1) competitively inhibits miR-7 and stimulates the expression of growth differentiation factor-(GDF-) 5, thereby promoting the osteogenic differentiation of PDLSCs. This process activates the Smad1/5/8 and p38 MAPK differentiation pathways [184]. In addition, CDR1as acted as a miR-7 sponge to activate the ERK signaling pathway and thus mediated the inhibitory effect of lipopolysaccharide (LPS) on cell proliferation. Knockdown of CDR1as promotes the inhibition of PDLSCs proliferation induced by LPS [185].

5. Conclusions

Among the regenerative strategies, dental-derived MSCs-based techniques have demonstrated particular promise [186, 187]. Several preclinical studies and clinical trials have been performed using dental-derived MSCs for the treatment of dental and nondental diseases, such as neurodegenerative diseases and autoimmune and orthopedic disorders [188–191]. Moreover, no adverse events that may be related to cell transplantation have been reported [192, 193]. These suggest the efficacy and safety of dental-derived MSCs-based therapy. However, previous studies have illustrated the difficulty in generating a consistent population of cells for therapeutic use. Even with tissue from a single donor, controlled culture conditions, and the expansion of single cells, each clone produces a distinct population with widely different morphology, growth kinetics, gene expression profile, and epigenetic status [194–196]. Based on this, we could consider that it may be necessary in the future to establish MSCs banks based on the heterogeneity of dental-derived MSCs, in case of a need to screen for cells prior to clinical use [12].

In addition, clonal cultures serve as an extremely useful research tool to identify desirable properties of cells within mixed populations. In future studies, screening of single cell-derived clones on a larger scale to that described in this report will serve to further understand cell heterogeneity and its impact on the development of MSCs-based therapies [50]. We recommend that, whenever possible, studies performed at the population level should be validated in terms of the principal findings using clonally expanded populations. This would clarify whether the response is common to all MSCs, or only to selected subpopulations [22]. Moreover, functional diversity within a MSCs colony must be considered in the design of experiments and trials for even nonclonal MSCs populations and can be mitigated or even exploited when the mechanisms of onset are better understood.

Cell therapy entails the administration of living cells that have been purified, propagated, or differentiated to create a cell product for a specific therapeutic need [197]. Identifying key markers that support cell functions is a significant aspect of the development of dental-derived MSCs therapies. It allows the optimization of population selection by selectively screening and isolating better quality dental-derived MSCs for in vitro expansion and assessment, aiding the translational development of more effective MSCs-based therapies for clinical evaluation and application [76]. Strategies to isolate, purify, and propagate subpopulations of adult MSCs may, therefore, contribute to the development of cell therapy products with enhanced clinical benefit in the future. Moreover, cell reprogramming and the induction of pluripotency depend critically on the control of the epigenetic tags linked to cell differentiation [198]. Therefore, the study of these multiple epigenetic modifications associated with the differentiation process, and how these could be reversed, is of paramount importance [25, 140, 199]. The ideal situation is when key markers of dental-derived MSCs could be analyzed and used to identify different cell types or subpopulations in the complex tissue [200]. The epigenome information from the same set of single cells could be used subsequently to investigate how different epigenetic layers regulate transcription [201]. Finally, to build a causal relationship between genotype and phenotype, it will be ideal to knock out key component genes for MSCs in vivo using gene-editing technologies [202, 203]. This control over dental-derived MSCs composition and function will accelerate the translation of cell therapy into clinical practice.

Although the results of the present research on dental-derived MSCs are promising, many of the key markers and epigenetic modifications discussed here have yet to be validated in an animal model [81, 89, 204]. There are currently less clinical research reports on dental-derived MSCs. A key challenge in therapeutic application of MSCs appears to be that the surface markers commonly related to in vitro functionality are not necessarily associated with the corresponding activity in vivo [205]. Based on this, we encourage verifying first in animal models and then in clinical trials all the promising surface markers and epigenetic modifications that have been identified based on the in vitro function of MSCs. Key molecules that are predictive of clinical outcome are candidates to use as quality attributes for robust and reproducible manufacturing of MSCs therapies [20, 206, 207]. Moreover, clinicians need to be encouraged to pay more attention to the research progress of dental-derived MSCs and develop new methods for clinical application. Small advances in the clinical application of dental-derived MSCs will bring great encouragement to researchers [208–210]. Similarly, the development of basic research will accelerate the clinical application of dental-derived MSCs [211, 212].

Abbreviations

MSCs:	Mesenchymal stromal cells
SCs:	Stromal cells
BMMSCs:	Bone marrow mesenchymal stromal cells
DPSCs:	Mesenchymal stromal cells of dental pulp
SCAPs:	Mesenchymal stromal cells of apical papilla
SHEDs:	Mesenchymal stromal cells of human exfoliated deciduous teeth
PDLSCs:	Mesenchymal stromal cells of periodontal ligament
DFCs:	Dental follicle cells
GMSCs:	Mesenchymal stromal cells of gingiva

TGSCs: Mesenchymal stromal cells of human tooth germ
FASL: Fas-ligand
N-GMSCs: Cranial neural crest cell-derived GMSCs
M-GMSCs: Mesoderm GMSCs
YAP1: Yes-associated protein 1
PD: Population doublings
Map2: Microtubule-associated protein 2
NF: Neurofilament
OCT-4: Octamer-binding transcription factor-4
SSEA-3: Stage-specific embryonic antigen-3
iPS: Pluripotent stromal
ES: Embryonic stromal
PGCs: Primordial germ cells
ALP: Alkaline phosphatase
SOX2: Sex-determining region Y-box-2
NFIC: Nuclear factor I-C
DMP1: Dentin matrix protein-1
DSPP: Dentin sialophosphoprotein
NTR: Neurotrophin receptor
SVZ: Subventricular zone
SGZ: Subgranular zone
DLX5: Distal-less homeobox 5
BGLAP: Bone gamma-carboxyglutamate protein
GFAP: Glial fibrillary acidic protein
S100B: S100 calcium binding protein B
WIF1: Wnt inhibitory factor 1
SFRPs: Secreted frizzled-related proteins
GNAIs: Guanine nucleotide binding proteins
HERS: Hertwig's epithelial root sheath
JNK: c-Jun N-terminal kinase
ERK: Extracellular-signal regulated kinase
IFN-γ: Interferon-gamma
PBMC: Peripheral blood mononuclear cell
G-CSF: Granulocyte colony stimulating factor
MDPSCs: DPSC subsets based on their migratory response to G-CSF
SA-β-gal: Senescence-associated β-galactosidase
CFU: Colony-forming unit
OCT-4: Octamer-binding transcription factor-4
IL-1β: Interleukin-1β
TGF-β1: Transforming growth factor-β1
ROS: Reactive oxygen species
PMDs: Partially methylated domains
ICM: Inner cell mass
DNMTs: DNA methyltransferases
5-Aza: 5-Azacytidine
5-Aza-dC: 5-Aza-2′-deoxycytidine
5-aza-CdR: 5-Aza-2′-deoxycytidine
OPN: Osteopontin
Pax7: Paired box 7
Myod1: Myogenic differentiation 1
Tet: Ten-eleven translocation
5-Mc: 5-Methylcytosine
5-hmC: 5-Hydroxymethylcytosine
DKK-1: Dickkopf WNT signaling pathway inhibitor 1
HATs: Histone acetyltransferases
HDACs: Histone deacetylases
H3K14: Histone H3, lysine 14
H3K9: Histone H3, lysine 9
H3K9K14: Histone H3, lysines 9 and 14
VPA: Valproic acid
BSP: Bone sialoprotein
OCN: Osteocalcin
GR: Glucocorticoid receptor
HDAC6: Histone deacetylase 6
ncRNAs: Noncoding RNAs
miRNAs: MicroRNAs
lncRNAs: Long noncoding RNAs
ceRNAs: Competitive endogenous RNAs
circRNAs: Circular RNAs
FC: Fold change
CDR1: Cerebellar degeneration-related protein 1
GDF: Growth differentiation factor
SFRPs: Secreted frizzled-related proteins.

Conflicts of Interest

The authors declare that they have no competing interests.

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Research Article

Single-Cell Transcriptome Analysis of Human Adipose-Derived Stromal Cells Identifies a Contractile Cell Subpopulation

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The potential for human adipose-derived stromal cells (hASCs) to be used as a therapeutic product is being assessed in multiple clinical trials. However, much is still to be learned about these cells before they can be used with confidence in the clinical setting. An inherent characteristic of hASCs that is not well understood is their heterogeneity. The aim of this exploratory study was to characterize the heterogeneity of freshly isolated hASCs after two population doublings (P2) using single-cell transcriptome analysis. A minimum of two subpopulations were identified at P2. A major subpopulation was identified as contractile cells which, based on gene expression patterns, are likely to be pericytes and/or vascular smooth muscle cells (vSMCs).

1. Introduction

Human adipose-derived stromal cells (hASCs) have gained increasing attention as a potential cell therapy product. hASCs are classified as multipotent, fibroblast-like, plastic adherent cells that can easily be expanded in vitro into adipocytes, osteoblasts, and chondrocytes [1, 2]. A distinct advantage of hASCs is that a relatively large number of cells can be extracted from the adipose tissue with minor donor site morbidity [2, 3]. This has sparked worldwide growth of a new research field and industry.

It is well known that the cellular fraction isolated from the adipose tissue (stromal vascular fraction; SVF) and the resultant hASCs grown in culture are heterogeneous [1, 2, 4]. It is also well established that the expansion of heterogeneous cell populations leads to clonal selection and a loss of diversity [5–7]. It is unclear however whether the heterogeneity of the SVF, or the loss thereof during expansion, contributes to or impedes the potential therapeutic use of hASCs. A great deal of effort is thus being made to better understand the heterogeneous nature of the isolated cell populations.

Transcriptome analysis enables a comprehensive understanding of the molecular mechanisms that lead to functional variation and heterogeneity of cell populations. The characterization of cells based on protein-coding ribonucleic acid (RNA) that will be translated into functional proteins allows researchers to look at the transcriptome as a precursor of the proteome. Until recently, most transcriptome work has been conducted on pooled populations of cells providing an average of gene expression levels across the cell types present [8]. This masks the uniqueness and heterogeneity of gene expression patterns in individual cells, resulting in cells that are abundant being studied predominantly, while rare cell populations remain poorly characterized [9]. Single-cell RNA sequencing (scRNA-Seq) technologies constitute a powerful tool to quantify intrapopulation heterogeneity by studying gene expression patterns in individual cells [10, 11]. To date, scRNA-Seq has been used to dissect various heterogeneous cell populations and complex tissues, such as bone marrow-

derived mesenchymal stromal cells (BM-MSCs) [12] and cells of the hematopoietic lineage [13].

Various studies have previously explored the heterogeneity of human and mouse ASCs at a single-cell level; however, most of these studies focused on the SVF [14–16]. For certain clinical applications, the heterogeneous hASC population may have to be expanded ex vivo to obtain clinically relevant cell numbers. We have thus undertaken an exploratory study to characterize cellular heterogeneity in freshly isolated, cultured hASCs at P2, using scRNA-Seq analysis.

2. Materials and Methods

2.1. Tissue Collection. Approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Reference number: 241/2015). Adipose tissue was obtained from healthy donors undergoing liposuction procedures, who had given written informed consent. Each donor was assigned a unique reference number to ensure anonymity. All donor information was kept confidential.

2.2. Isolation and Maintenance of Adipose-Derived Stromal Cells. Adipose tissue was processed using an adaptation of the Coleman method as published elsewhere [17]. The resulting SVF was resuspended in complete growth medium (CGM) consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin/-streptomycin (p/s). Cells were counted and seeded in CGM at a density of 5000 cells per cm^2 in 25 or 75 cm^2 culture flasks. Three hASC cultures (A20, A28 and A10) were isolated and maintained at 37°C, 5% CO_2 in CGM. Cultures were expanded ex vivo for two rounds (P2). Cells were dissociated [using 3 mL 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) for 7 min at 37°C] when they reached confluence and reseeded at a density of 5000 cells/cm^2.

2.3. Immunophenotyping of Human Adipose-Derived Stromal Cells. Flow cytometric analysis was used to determine immunophenotype before the start of each experiment. A six-color antibody panel was designed in-house based on the published criteria for ASCs [1, 18]. The following antibodies were included in the panel: CD34-PE-Cy7 (Clone 581; Beckman Coulter, Miami, USA), CD36-APC (Clone 5-271; Biolegend, California, USA), CD44-APC-Cy7 (Clone IM7; Biolegend, California, USA), CD45-Brilliant Violet™ (BV)510 (Clone HI30; BD Biosciences, New Jersey, USA), CD90-BV421 (Clone 5E10; BD Biosciences, New Jersey, USA), and CD105-PE (Clone 43A3; Beckman Coulter, Miami, USA).

Antibodies were added to 1×10^5 cells from each cell suspension (5 μL of each antibody), placed in a flow cytometry tube (FCT) and incubated at room temperature (RT) in the dark for 20 min. A second FCT containing 1×10^5 unstained cells was prepared as a negative control and was used to set the negative/positive boundaries. After the incubation period, 1 mL phosphate buffered saline (PBS) containing 2% p/s was added to the aliquot. The sample was centrifuged at 300 g for 5 min, and the resulting supernatant was removed. The samples were phenotypically characterized using a Gallios flow cytometer (Figure S1 and Table S1; Beckman Coulter, Miami, USA). A total of 18 000 to 18 500 events were acquired for each sample and analyzed using Kaluza flow cytometry analysis software (version 2.1; Beckman Coulter, Miami, USA). Single-color FCTs (i.e., cells stained with only one monoclonal antibody per tube) were used to calculate the color compensation matrix in order to correct for spectral spill over into other detector channels.

2.4. Sorting of Human Adipose-Derived Stromal Cells. Sorting was done to ensure that only single, viable hASCs < 25 μM in diameter, i.e., that were not too big for the microfluidic channels of the C1™ Single-Cell Auto Prep Array Integrated Fluidics Circuit (IFC) plates (Fluidigm, California, USA) were selected for analysis. hASC markers, CD90, and CD44 were included in the sort criteria to ensure inclusion of most hASC subpopulations at P2. CD45, a common leukocyte marker, was included to ensure that hematopoietic cells were excluded from the sort.

The sample was centrifuged at 300 g for 5 min at RT, the supernatant was removed, and 5 μL of each antibody together with a viability marker (Propidium iodide; PI) was added to the sample. Viability ranged between 96 and 100%. The sample was incubated for 20 min at RT, protected from light. After the incubation period, the sample was washed with 1 mL PBS containing 2% p/s. The sample was centrifuged at 300 g for 5 min at RT, and the supernatant was removed. The sample was resuspended in 1 mL CGM for sorting using the FACSAria™ Fusion cell sorter (BD Biosciences, New Jersey, USA).

A sequential gating strategy was used to sort hASCs (see Figure S2). A side scatter (SS) vs. forward scatter (FS) two-parameter dot plot was used to exclude debris. Larger cells with stronger FS signals were also excluded due to the risk of potentially blocking the microfluidic channels of the IFC plate (Figure S2(a)). The SS area vs. SS width dot plot was used to exclude aggregates (Figure S2(b)), ensuring sorting of single cells. Viable cells were included based on negative PI staining (Figure S2(c)). Cells negative for CD45 and positive for CD44 and CD90 were included in the sort criteria (Figure S2(d)–(e)). The objective behind the gating strategy was thus to ensure that only single, viable, $CD90^+$, $CD44^+$, and $CD45^-$ cells were selected and sorted.

The sort was performed using a neutral density filter of 2 and a nozzle size of 130 μm. Sort purity was set on "Purity." Cells (300 000) were sorted into preprepared sort tubes containing 2 mL CGM. After the sort, the samples were centrifuged, and the supernatant was removed. The sorted cells were resuspended in 500 μL PBS (resulting concentration = 600 cells/μL).

2.5. Single-Cell Capture, Lysis, Reverse Transcription, and Amplification. The Fluidigm C1™ Single-Cell Auto Prep System (Fluidigm, California, USA) together with IFC plates (17 to 25 μm in size) were used to capture single cells. The Clontech® SMARTer® technology (Takara Bio, USA, Inc.) was used for the lysis, reverse transcription, and amplification of the resulting complementary DNA (cDNA), as per the manufacturer's instructions.

2.6. Library Preparation and Sequencing. Library preparation and sequencing were done by the Beijing Genomics Institute (BGI), Tai Po, Hong Kong, China.

2.7. Bioinformatic Pipeline. Analysis of the scRNA-Seq data was done using the RNA-Seq protocol of BCBIO, a bioinformatic-specific pipeline designed for automated, high throughput sequencing analysis (https://github.com/chapmanb/bcbio-nextgen). Data quality was determined using the FASTQC program, and the samples were aligned to the human reference genome (GRCh38) using HISAT2 (hierarchical indexing for spliced alignment of transcripts) [19]. Gene quantification was performed using featureCounts [20].

2.8. Quality Control Processing of the Single-Cell Data. Quality control metrics were applied to identify and remove poor quality samples from downstream analysis. A MultiQC report which can be viewed at http://wiki.bi.up.ac.za/scasc/multiqc was used to assess the quality of the data (Phred scores), the number of reads per sample, and the percentage alignment to the human reference genome (GRCh38). Cells with a Phred score < 30, reading depth of ≤ 100 000, or < 70% alignment to the human reference genome were removed from downstream analysis. Genes not expressed by three or more cells were removed from further analysis. Cells expressing < 200 genes were also removed. Cells expressing ≥ 10 000 genes were considered artifactual and were also removed. Further data analysis was done in *R* (version 3.5.2) [21] and RStudio (version 1.0.153) using the Seurat package (version 2.3.3) [22].

High levels of mitochondrial gene expression are usually indicators of cellular stress [23]; thus, samples containing > 10% mitochondrial genes were excluded from downstream analysis. Mitochondrial genes were also regressed out of the single-cell data due to possible interference in differential gene expression analysis. Various mitochondrial pseudogenes (*MTND1P23*, *MTND2P28*, *MTCO1P12*, *MTCO2P12*, *MTATP8P1*, *MTATP6P1*, *MTCO3P12*, and *MTCYBP45*) were highly expressed, which skewed the initial results and were therefore removed from downstream analysis.

Cell cycle genes were also regressed out of the dataset to prevent interference with downstream differential gene expression analysis [24].

Samples were normalized by employing global scale normalization (lognormalize). This function normalizes gene expression measurements for each cell to the total expression and multiplies this by a scale factor (10 000).

2.9. Batch Effect Correction Using Canonical Correlation Analysis. Initial clustering of the cells using the Seurat package resulted in clustering based on biological replicates (Figure S3), most likely due to biological and technical variability. A computational strategy introduced by Butler et al. (2018) referred to as canonical correlation analysis (CCA) was used to correct for this [22]. Heatmaps (Figure S4) and a biweight midcorrelation (bicor) saturation plot (Figure S5) were used to identify the most significant canonical correlation vectors (CCs) to align the three biological replicates for downstream analysis. Biological replicates (A20, A28, and A10) were aligned using 1000 genes with the highest dispersion in all three data sets, and six CCs were used for downstream clustering.

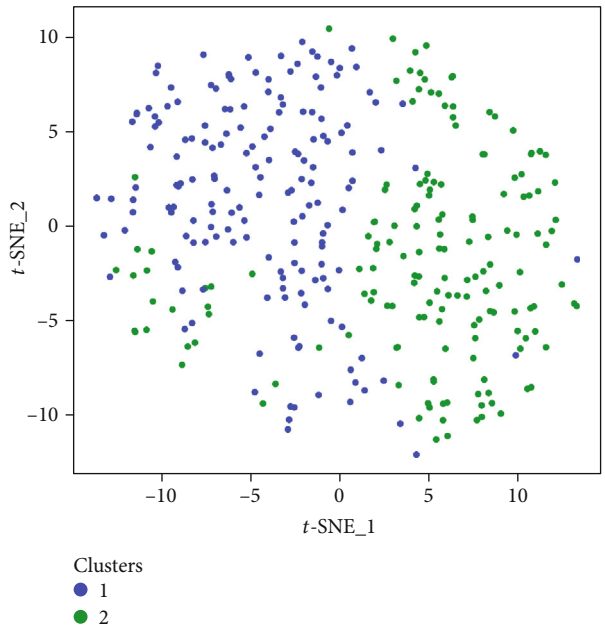
2.10. t-SNE Clustering Analysis. Clusters of transcriptomically similar cells were identified using a shared nearest neighbor (SNN) modularity optimization-based clustering algorithm [25], and nonlinear dimensional reduction (*t*-distributed stochastic neighbour embedding; *t*-SNE) was used to visualize possible clusters. A number of resolution parameters ranging from 0.5 to 1.2 were used. Differentially expressed genes (DEGs) specific to each cluster were identified using the FindAllMarkers function.

2.11. Statistical Analysis. DEGs for each cluster were identified based on a log fold change (logFC) ≥ 0.5. Statistical significance was tested using the nonparametric Wilcoxon rank sum test with Bonferroni correction. An adjusted *P* value of $\alpha \leq 0.01$ was considered significant.

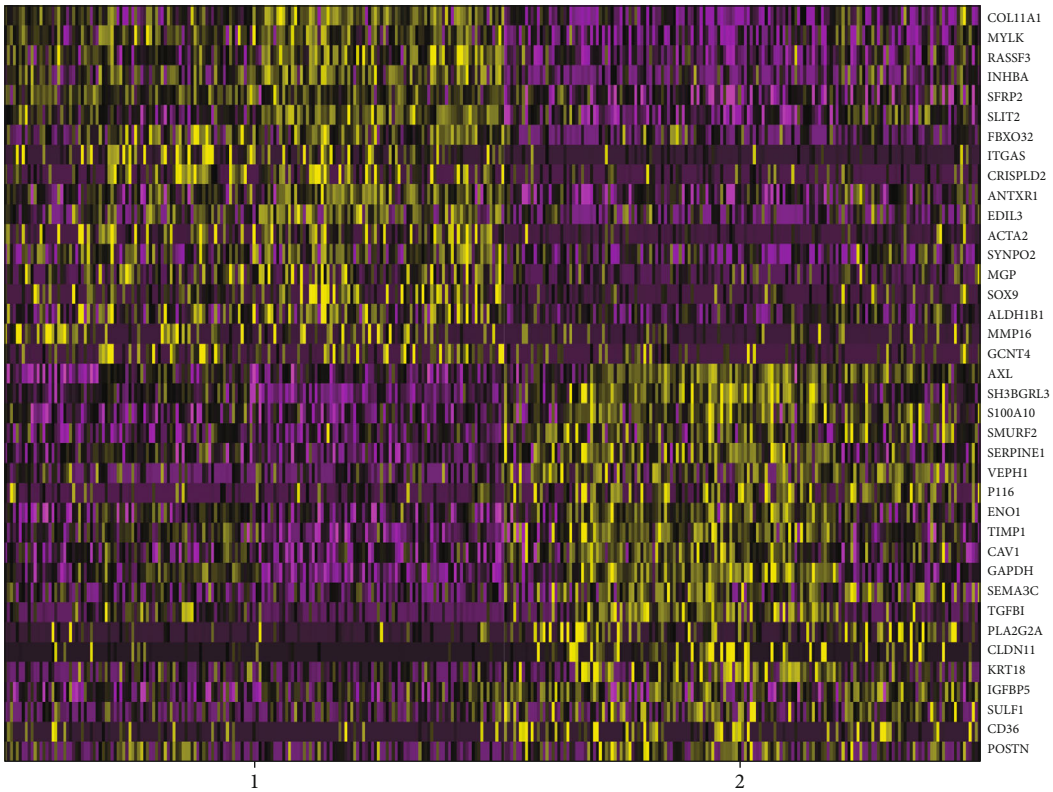
3. Results

A minimum of two and a maximum of five clusters were identified in the hASCs at P2. While identifying clusters, it is possible to change the clustering resolution. Increasing resolution usually results in an increased number of clusters identified. Two clusters were identified at resolution 0.5 (Figure 1(a)), while three clusters were identified at resolutions 0.6 and 0.7 (Figure 1(c)). Four clusters were identified at resolution 0.8 (Figure 1(e)) and resolutions 0.9 to 1.2 consistently identified five clusters (Figure 1(g)). DEGs were identified for each cluster number permutation (two to five clusters). The genes identified and reported were based on a positive logFC of ≥ 0.5, with a *P* value of ≤ 0.01. However, only genes with an adjusted *P* value of ≤ 0.01 were considered for further analysis. Figures 1(b), 1(d), 1(f), and 1(h) show heatmaps illustrating the DEGs for each cluster of the various cluster permutations identified (two, three, four and five clusters, respectively). The list of statistically significant DEG ($\alpha \leq 0.01$) can be viewed in Table S2.

Cluster 1 remained stable for resolutions 0.6 to 1.2. The list of statistically significant ($\alpha \leq 0.01$) DEGs also remained similar in this cluster across the different cluster number permutations (Table 1). Of these genes, two were consistently identified with a positive logFC of ≥ 1 for all the different cluster number permutations, namely, *ACTA2* and *COL11A1*. *COL11A1* codes for the proalpha1 (XI) chain of type XI collagen [26]. *ACTA2* codes for smooth muscle alpha-actin protein and is one of the best known molecular markers for contractile cells such as vascular smooth muscle cells (vSMCs) and pericytes [27–30]. The t-SNE plot in Figure 2 shows the expression pattern of both *ACTA2* and *COL11A1* in single hASCs and clearly shows that these genes are predominantly expressed in cells of cluster 1. Upon further investigation, other statistically significant DEGs were identified within cluster 1 that also encode for proteins involved in contractile cell structure and function. A list of

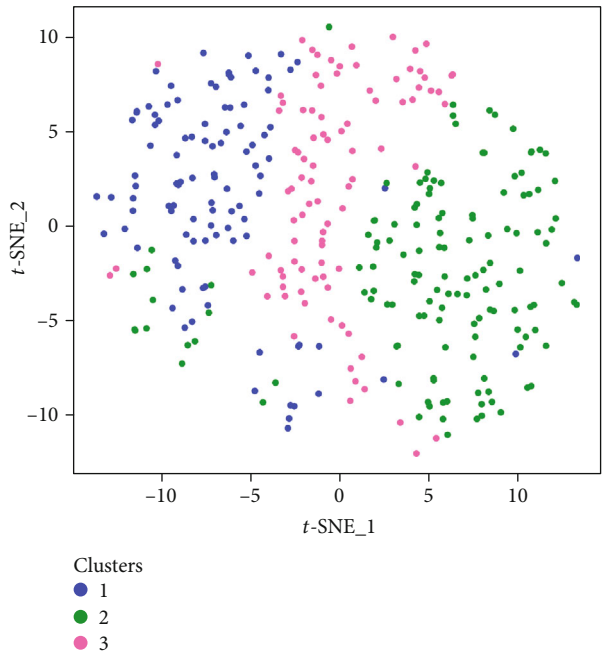


(a)

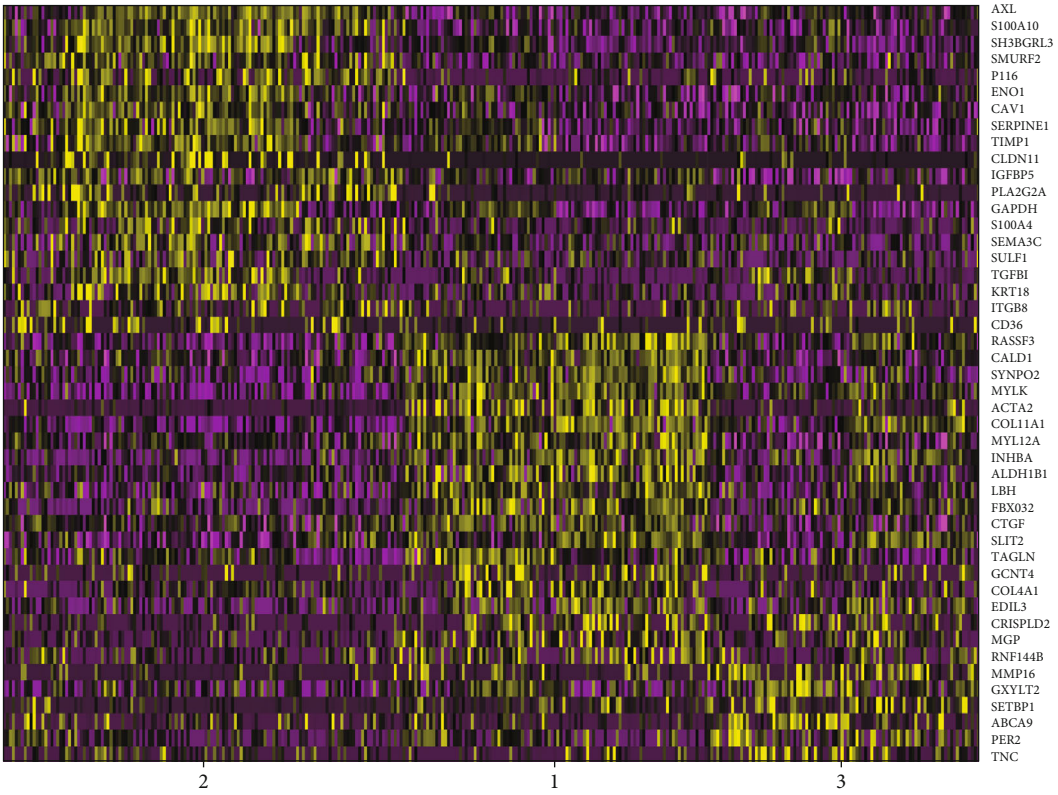


(b)

Figure 1: Continued.

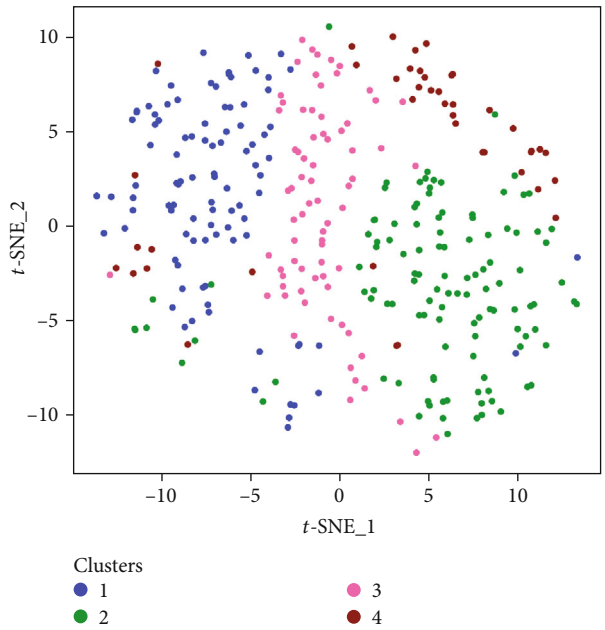


(c)

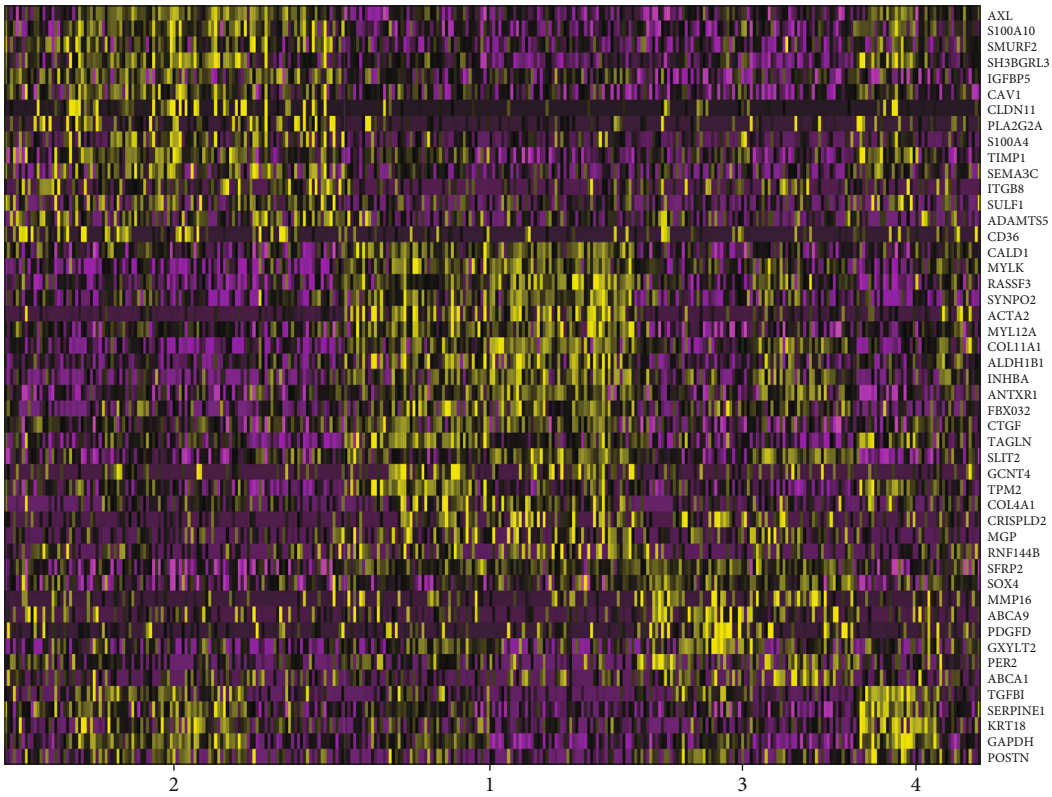


(d)

Figure 1: Continued.

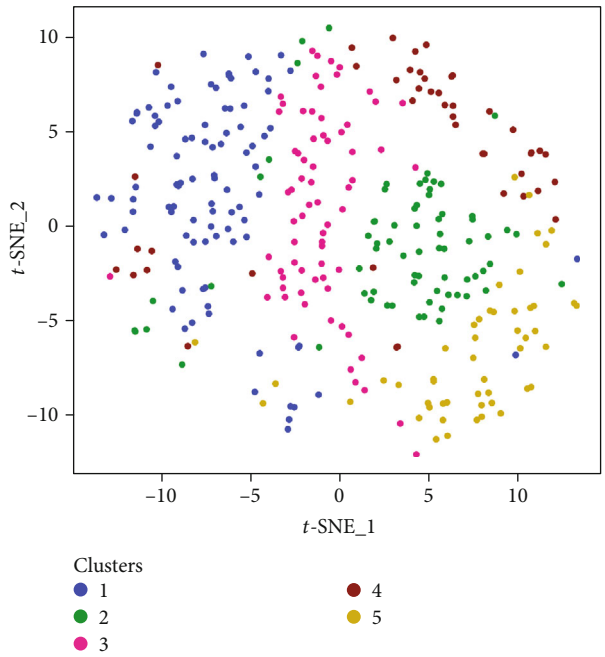


(e)

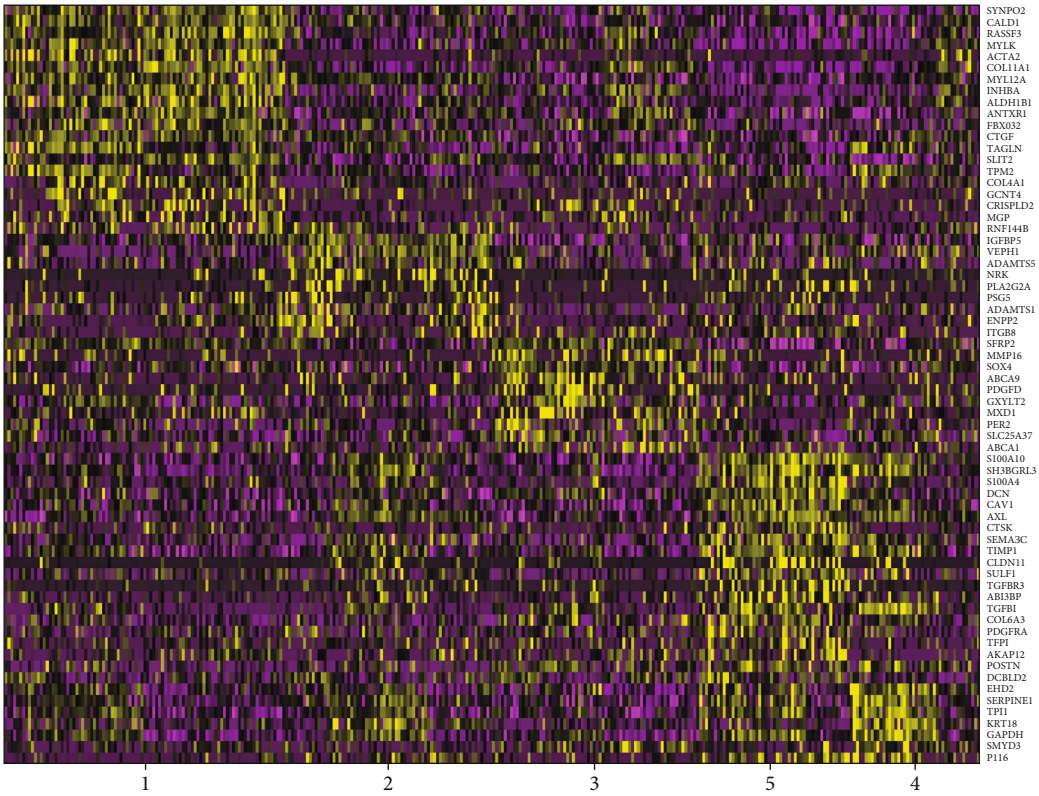


(f)

Figure 1: Continued.



(g)



(h)

Figure 1: *t*-SNE plots illustrating the different cluster number permutations and heatmaps illustrating the top DEGs for each cluster. The colored dot clouds on the tSNE plots represent individual clusters. (a) Two clusters were identified at resolution 0.5 while (c) three clusters were identified at resolutions 0.6 and 0.7. (e) Four were identified at resolution 0.8 and (g) resolution 0.9 to 1.2 consistently identified five clusters. For each cluster number permutation, there is a corresponding heatmap illustrating the top DEGs per cluster (two, three, four, and five clusters, respectively). Yellow represents highly expressed genes while purple represents poorly expressed genes.

Table 1: Statistically significant ($\alpha \leq 0.01$) DEGs identified in cluster 1 that encode for proteins that play a role in contractile cells.

Genes	Protein	Function	References
ACTA2	Alpha smooth muscle actin (α-SMA)	Involved in vascular contractibility and blood pressure homeostasis.	[15, 27–31]
COL11A1	Collagen alpha-1(XI) chain	One of three chains that make up type XI collagen.	[26, 28]
SYNPO2	Myopodin or synaptopodin-2	Nonmotor actin binding protein that interacts with α-SMA.	[28]
CALD1	Caldesmon (heavy and light chain)	Cytoskeletal protein that interacts with actin, tropomyosin, myosin, calmodulin, and phospholipids.	[28, 30–32]
TAGLN	Transgelin (SM22-α)	Nonmotor actin binding protein.	[27, 28, 31, 33]
MYLK	Myosin light chain kinase	Calcium/calmodulin dependent enzyme that phosphorylates myosin regulatory light chains.	[28, 34]
MYL12A	Myosin regulatory light chain 12A	Regulates smooth muscle contraction.	[28, 35]
TPM2	Beta-tropomyosin	Controls the binding of myosin and actin.	[28, 34]

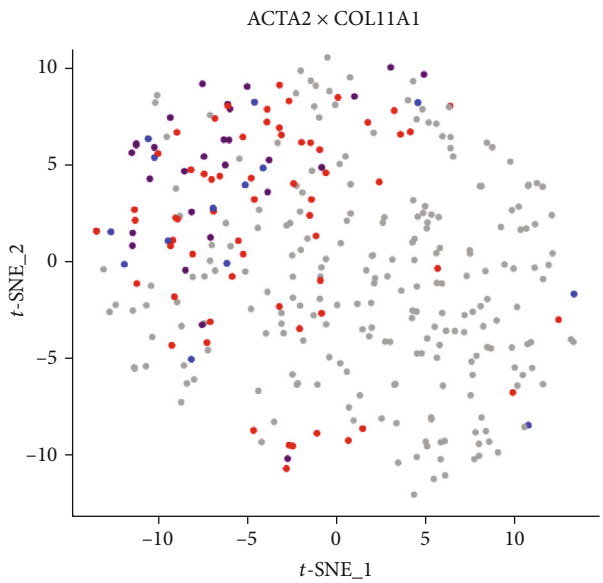


Figure 2: *t*-SNE plot illustrating the expression of *ACTA2* and *COL11A1* in individual hASCs. Each dot on the plot represents an individual cell. Cells expressing *ACTA2* are represented as blue dots while cells expressing *COL11A1* are represented as red dots. Cells expressing both are colored purple while the grey dots represent cells that express neither of the selected genes. The data shows the selected genes being predominantly expressed in cluster 1 cells.

these genes, the proteins they encode and their respective functions, are shown in Table 1.

Kumar et al. (2017) used bulk RNA-Seq techniques to study pericytes and vSMCs that were differentiated from mesenchymangioblasts derived from human-induced pluripotent stem cells (iPSCs) [28]. Their results showed that vSMCs and pericytes share expression of *ACTA2*, *TAGLN*, *TPM2*, and *MYLK*, while *MYL12A*, *CALD1*, *COL11A1*, and *SYNPO2* were only identified in vSMCs [28]. Based on the list of statistically significant DEGs (Table 1), it is hypothesized that cluster 1 is comprised of contractile cells including pericytes and/or vSMCs.

Cluster 2 remained stable at low resolutions (0.5 to 0.7) but became fragmented when the resolution was increased to 0.8. The additional clusters identified at the higher resolutions (clusters 4 and 5) showed very few DEGs and overlapping DEGs with cluster 2 at lower resolutions. Based on these observations, clusters 4 and 5 cannot conclusively be identified as separate subpopulations within this dataset.

Even though cluster 3 remained stable for most of the cluster number permutations (resolutions 0.6 to 1.2), only three DEGs were identified to be statistically significant ($\alpha \leq 0.01$) in this cluster: *MMP16*, *SFRP2*, and *SOX4*. The lack of significant DEGs in this cluster did not allow for clear identification of the cells and/or their function within this cluster.

4. Discussion

hASCs have shown great promise as a potential cellular therapeutic product for a number of diseases, and efforts are being made to better understand the heterogeneous nature of the isolated cell populations. Single-cell transcriptome technologies are powerful tools for studying cellular heterogeneity. Very few single-cell studies on hASCs have been published to date, and the majority have focused on the SVF. There is however little consensus on the number of subpopulations present in the SVF or the specific markers for each subpopulation. This is mainly due to different aims and methodologies employed by each research group. Rennert et al. (2016) studied 96 genes using single cells of human SVF and identified up to five subpopulations [14]. Hardy et al. (2017) also used single-cell technology to study two subpopulations in SVF, named adventitial stromal cells and pericytes, based on the following phenotypic markers: CD31, CD45, CD146, and CD34 [15]. Pericytes had a transcriptomic signature distinct from adventitial stromal cells. Each subpopulation could be further divided into aldehyde dehydrogenase (ALDH)-bright and ALDH-dim populations [15]. It was also reported that a minimum of six genes could be used to discriminate between pericytes and adventitial stromal cells [15].

Clonal expansion in primary cultures such as ASCs has been well documented [5–7, 36]. In an elegant study conducted by Selich et al. (2016) on MSCs derived from the umbilical cord, it was shown that the first severe loss of diversity in primary cell cultures is observed within the first three passages [5]. The second drop in clonal diversity could be

observed during further expansion as specific clones become dominant [5].

The cellular transcriptomic heterogeneity of hASCs cultured in vitro has not been comprehensively investigated at the single-cell level. In our study, the aim was to characterize heterogeneity of freshly isolated hASCs that had undergone two population doublings (P2; before severe loss of diversity). The objectives of this study were (a) to determine the number of subpopulations present in hASCs at P2 via clustering and (b) to identify statistically significant DEGs for each cluster.

hASCs were sourced from three female donors. The percentage of hASCs that expressed phenotypic markers was between 88 and 92% at P2. It is well documented that differentiation and proliferation potential of hASC decreases in older females; however, the hASC surface marker expression remains intact [37, 38].

Clustering was done at different clustering resolutions. hASCs were sourced from three different donors, and there was the possibility of clonal selection during expansion. However, scRNA-Seq analysis of 322 cells revealed limited heterogeneity with only two to five subpopulations present at P2. Two clusters remained consistent at resolutions 0.6 to 1.2, namely, clusters 1 and 3. Even though cluster 3 was consistently identified throughout, very few statistically significantly ($\alpha \leq 0.01$) DEGs could be identified for this cluster. In contrast, the list of statistically significant ($\alpha \leq 0.01$) DEGs (in particular, *ACTA2*, *COL11A1*, *SYNPO2*, *TAGLN*, *CALD1*, *MYLK*, *MYL12A*, and *TPM2*) identified for cluster 1 suggests that this subpopulation is comprised of contractile cells such as pericytes and/or vSMCs.

Pericytes and vSMCs are collectively known as perivascular cells. Their main function is to support and stabilize vascular networks and to regulate blood flow [4, 39]. vSMCs form the media of arteries and veins of varying complexity and are also found in the adventitia of large veins, while pericytes surround smaller blood vessels such as capillaries and postcapillary venules [27, 39, 40]. Both cell types are believed to be present in the SVF of the isolated adipose tissue [1, 2, 4]. A single-cell study by Hardy et al. (2017) determined that pericytes (identified based on sorting criteria) had a distinct transcriptomic signature that could be differentiated from the rest of the SVF using a minimum of six distinct molecular markers. One of these markers was *ACTA2* [15], which is one of the main molecular markers identified in cluster 1. In addition, Baryawno et al. (2019) did a comprehensive single-cell study on murine BM-MSCs. They identified a dominant subpopulation expressing *ACTA2* which they classified as pericytes [12]. *ACTA2* appears however to be expressed by both pericytes and vSMCs [30, 31]. In a recent study, Kumar et al. (2017) studied pericytes and vSMCs differentiated from mesenchymangioblasts [28]. Transcriptome data from that study revealed that vSMCs and pericytes share the expression of *ACTA2*, *TAGLN*, *TPM2*, *MYLK* and *MYL12A*, while *CALD1*, *COL11A1*, and *SYNPO2* that were only identified in vSMCs [28]. It is therefore hypothesized that cluster 1 cells are pericytes and/or vSMCs.

We acknowledge that the transcriptome is only a precursor to translated proteins and therefore, its analysis in isolation has limitations with regard to functional relevance. It would therefore be useful to confirm and distinguish between the different contractile cells; however, there is currently no single marker that can be used to distinguish between vSMCs and pericytes [39, 41], and an array of markers is therefore used. Many membrane-specific markers have been identified for pericytes which include CD146, neuron-glial antigen 2 (NG2), and ribosomal protein S14 (3G5) [27, 28]. vSMCs are usually identified via immunohistochemistry using proteins such as myocardin, transgelin, and myosin heavy chain 11 [28, 30]. Identifying membrane-specific proteins will allow for the isolation of specific clusters of cells by FACS. Of particular interest would be the presence or absence of pericytes within cluster 1. Pericytes have been reported by multiple research groups to be potential progenitor cells with the ability to differentiate into osteocytes, adipocytes, chondrocytes, and myocytes [4, 42–46], an ability shared by hASCs [1, 47]. If cluster 1 could be separated from the rest of the hASC population, it would be of interest to see whether the differentiation capabilities usually observed in hASCs could be ascribed to pericytes, an as yet undiscovered progenitor/stem cell population, or both. The hypothesized contractile subpopulation constituted 31% of the 322 hASCs studied at P2. It will be important to determine to what extent the differentiation capabilities of pericytes contributes to the overall differentiation potential of the broader heterogeneous hASC cell population. Collagen gel contraction assays could be useful to further confirm the functional capabilities of these cells.

MSC populations exhibit considerable heterogeneity between donors and within individual donor populations, and the mechanisms that regulate self-renewal and lineage specification remain largely unexplored. This creates significant obstacles in research and in efforts aimed at developing clinical manufacturing protocols to produce standardized MSC therapeutic products [48]. The functional variation and heterogeneity of MSCs from various sources are purported to be the main reasons for inconsistent and controversial results observed in clinical trials [48, 49]. Single-cell sequencing technologies are powerful tools that can be used to explore the heterogeneity within cell populations. Traditional sequencing approaches analyze pooled populations which masks heterogeneity as a result of poorly visible smaller subpopulations. Single-cell RNA-Seq allows identification and transcriptomic characterization of large and small subpopulations which may lead to the discovery of specific markers relevant to therapeutic applications. Understanding the mechanisms that regulate fate specifications could lead to tailored expansion of specific lineages with unique biological properties suited for specific therapeutic needs.

Standardization of isolation and expansion methods is a key element in quality control of MSC products. We recognize that the use of FBS in our experiments is a confounding factor. These results form part of an initial study in our group to explore the transcriptomic heterogeneity of hASCs cultured in vitro. We realize that these experiments would need to be repeated using good manufacturing practices (GMP) conditions for better clinical translation.

Another limitation of this study is that the C1™ IFC plates can only capture cells of a specific size range (17 to

25 μM). This leads to the noncapture of cells falling outside this size range. This creates a bias in the hASCs captured, which are usually quite heterogeneous in the cell size. Future experiments should therefore use a system that will allow capture of all cell sizes present.

5. Conclusions

In conclusion, this exploratory study has characterized the heterogeneity of hASCs at P2 using transcriptome analysis at the single-cell level. Our results reveal the presence of at least two subpopulations of cells. One of these subpopulations is believed to be contractile cells that include pericytes and/or vSMCs.

Data Availability

The single-cell RNA-Seq data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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Supplementary Materials

Is included as a separate file and includes the following figures and tables: Figure S1: representative dot plots illustrating the sequential gating strategy used to study the combined immunophenotypic profile of hASCs. The hASC population of interest was visualized on an FS vs. SS log two-parameter dot plot. (a) Single hASCs were included, and debris was excluded by gating around intact cells. The gated hASCs were then plotted on two-parameter dot plots, allowing for the simultaneous analysis of expression of two surface markers. (b) hASCs were identified that were positive for CD44 and CD90 (quadrant A++). (c) CD105 positive and CD45 negative (quadrant B-+). The final plot represents (d) $CD44^+$/-$CD90^+$/$CD105^+$/$CD45^-$ hASCs stained for CD36 and CD34. This figure is relevant to the Materials and Methods section. Also see Table S2. Figure S2: an example of the sequential gating strategy for the sorting of the hASCs. (a) SS vs. FS two-parameter dot plot was used to identify hASCs (region A) and exclude debris (region F). (b) SS area vs. SS width dot plot, gated on A, was used to exclude small clumps of cells, ensuring that only single cells (region B) are included in the downstream sorting region. (c) A PI vs. count histogram, gated on B, was used to select for viable cells (region C). Selection of the ASC population was further refined in the next two plots. First, a CD44 APC-Cy7/CD45 KO plot that was gated on viable cells was used. (d) Only cells positive for the CD44 and negative for CD45 were selected. The final selection for sorting was based on (e) positive staining of ASCs for CD44 and CD90, using a CD44 APC-Cy7 vs. CD90 PE-Cy5 plot. The logic behind the sequential gating strategy was to ensure that only single, viable, $CD90^+$, $CD44^+$, and $CD45^-$ cells were selected and sorted. This figure is relevant to the Materials and Methods section. Figure S3: *t*-SNE plot illustrating the batch effect observed during initial single-cell analysis using the Seurat package. The different colored dot clouds represent individual clusters of cells. During preliminary clustering, it was noticed that the cells were clustering according to their original identity, referring to the cell cultures from which they were originally isolated. This figure is relevant to the Materials and Methods section. Figure S4: heatmaps illustrating the top 20 DEGs for the first 12 CCs. The heatmaps show the top 20 DEGs (rows) identified for each cell (columns) in every CC. Yellow represents genes with positive scores (highly expressed genes) while purple represents negative scores (low level expressed genes). DEGs could be visualized (yellow and purple blocks) for up to 9 CCs. From 10 CCs onward, no distinct pattern could be distinguished. This indicates that CCs 1 to 9 could be used for downstream analysis. This figure is relevant to the Materials and Methods section. Figure S5: biweight midcorrelation (bicor) saturation plot illustrating the smoothed shared correlation strength versus CCs between the different datasets. The plot measured the correlation strength for 15 CCs of the different datasets. Each colored line on the graph represents the Seurat object of the individual datasets, named according to the cell culture from which the cells originate. The graph shows a saturation point (where the curve in the graph starts to flatten) at 6 CCs. The first 6 CCs were used for downstream analyses. This figure is relevant to the Materials and Methods section. Table S1. List of statistically significant ($\alpha \leq 0.01$) DEGs identified in the different cluster number permutations. This table is related to Figure 1, Figure 2, and Table 1. Table S2: percentage of hASCs (P2) from the three cultures used during the single-cell experiments which adhered to the phenotype: $CD44^+$/$CD90^+$/-$CD105^+$/$CD45^-$/$CD34^-$/$CD36^+$. All cultures adhered to the phenotypic criteria set out by the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). This table is relevant to the Material and Method section, and Figure S1. *(Supplementary Materials)*

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Research Article

Positively Correlated CD47 Activation and Autophagy in Umbilical Cord Blood-Derived Mesenchymal Stem Cells during Senescence

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Autophagy plays a critical role in stem cell maintenance and is related to cell growth and cellular senescence. It is important to find a quality-control marker for predicting senescent cells. This study verified that CD47 could be a candidate to select efficient mesenchymal stem cells (MSCs) to enhance the therapeutic effects of stem cell therapy by analyzing the antibody surface array. CD47 expression was significantly decreased during the expansion of MSCs in vitro ($p < 0.01$), with decreased CD47 expression correlated with accelerated senescence phenotype, which affected cell growth. UCB-MSCs transfected with CD47 siRNA significantly triggered the downregulation of pRB and upregulation of pp38, which are senescence-related markers. Additionally, autophagy-related markers, ATG5, ATG12, Beclin1, and LC3B, revealed significant downregulation with CD47 siRNA transfection. Furthermore, autophagy flux following treatment with an autophagy inducer, rapamycin, has shown that CD47 is a key player in autophagy and senescence to maintain and regulate the growth of MSCs, suggesting that CD47 may be a critical key marker for the selection of effective stem cells in cell therapy.

1. Introduction

Autophagy is a natural intracellular degradation mechanism that maintains cellular homeostasis by delivery to the lysosome [1]. The coordinated response to starvation and metabolic stress triggers autophagy to function in the recruitment and degradation of cytosolic proteins and organelles to remove and recycle any malfunctioning or unnecessary components [2]. In pathological conditions, including neurodegenerative diseases, cancer, and inflammatory diseases, autophagy is also induced and modulated. It is clear that autophagy plays a role in controlling inflammation and is closely implicated in human disease [1, 3, 4]. Autophagy, an intracellular self-degradation system, is responsible for the removal of damaged organelles or malformed proteins, in order to regenerate newer and healthier cells. The function of autophagy is related to cellular clearance, which is critical for development, differentiation, and tissue remodeling [5–8]. Therefore, understanding autophagy function is important to clarify the cause of diseases. Autophagy has a bifunctional role in cell survival and death, presumably by clearing potential toxic protein aggregates. Blocking autophagy suppresses cytochrome c release from mitochondria and caspase activation and induces cell viability [9].

Aging, affecting the decline in regenerative potential of stem cells, is coupled with a progressive reduction in the regulation of cellular and tissue interactions. It results in senescence, allowing irreversible arrest of cellular division. Insufficient clearance of accumulation in damaged components is mainly described in aging organisms. An alternating lysosomal degradation system and decreased autophagic activity were ensued by aging. Commonly, impaired and defective autophagy in aged cells has been identified [10, 11]. The decline in both the number and functionality of stem cells with aging may contribute to regenerative decline. It has been reported that autophagy is related to age-dependent changes in stem cells for maintenance of stemness and differentiation capacity [12]. Additionally, immunosuppressive properties of higher passage mesenchymal stem cells (MSCs)

are decreased, notwithstanding the stable phenotype of MSCs [13, 14]. There was a significant decline in self-renewal capacity with increased donor age and in vitro expansion [15–17]. Interestingly, higher passaged cells and cells from old donors showed less proliferative capacity. In addition, secretion of cytokines and growth factors from less proliferative cells was related to senescence-associated secretory phenotype, which is associated with senescence [18, 19]. Therefore, it is important to find key modulatory mechanisms of senescence markers to determine the quality of the in vitro cells for stem cell therapy.

CD47, known as integrin-associated protein (IAP), is a cell surface glycoprotein expressed in human cells and binds the ligands thrombospondin-1 (TSP-1) and signal-regulatory protein alpha (SIRPα) [20]. Clinically, the inhibition of CD47 is a potential therapeutic strategy for treatment of various cancers. The absence of CD47 results in phagocytosis of T-cell-mediated adaptive immunity by targeting CTLA and PD-1 [21]. Recent studies have focused on overexpressed CD47, which plays a key role in immune response in tumor cells [22–24].

In addition to immune responses, CD47 is involved in a range of cellular processes, including apoptosis, proliferation, adhesion, and migration. Activation of CD47 was correlated with enhanced proliferation of cancerous cells via the P13K/Akt pathway [25]. On the other hand, activation of CD47 with TSP-1 inhibited proliferation and suppressed expression of stem cell transcription factors, such as Sox2, Klf4, and Oct4 [26]. CD47 also leads to cell death in normal and tumor cells via apoptosis or autophagy. However, the relationship between CD47 and autophagy or senescence had a functional significance, which was unclear at this time. This study reported that CD47 is a key regulator of autophagy and senescence to maintain and orchestrate the aging of MSCs.

2. Materials and Methods

2.1. Cell Culture. The Institutional Review Board of MEDIPOST Co., Ltd. approved this study (MP-2014-07-1-1). To culture UCB-MSCs, UCB was collected in blood bags containing citrate phosphate dextrose adenine (CPDA-1), anticoagulant, within 24 h from mothers who gave informed consent. Human mononuclear cells (MNCs) were isolated from UCB with Ficoll-Hypaque solution (Sigma-Aldrich, St. Louis, MO, USA). The isolated MNCs were suspended in minimum essential medium α (Gibco, Carlsbad, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and gentamicin (Gibco) at 37°C in a 5% $05CO_2$ incubator. Medium was changed every three days. Nonadherent cells were washed out with medium changes. Adherent cells, MSCs, were subcultured at 70% confluence. Cell expansion was analyzed using the trypan blue exclusion method. Rapamycin (10 μM, Sigma-Aldrich), an inducer of autophagy, was also treated with UCB-MSCs (passage 6, P6) for 3 h.

2.2. Flow Cytometry and Cell Surface Antibody Screening with Lysoplates. To detect CD47 and stem cell markers of MSCs (P5), fluorescence-activated cell sorting was performed. Cells were detached and washed with DPBS and then incubated with fluorescein isothiocyanate- (FITC-) conjugated human CD47, CD14, CD45, CD146, and human leukocyte antigen- (HLA-) DR antibodies (BD Biosciences, San Diego, CA, USA) and phycoerythrin- (PE-) conjugated human CD49b, CD73, CD166, CD274, epidermal growth factor receptor (EGFR; BD Biosciences), CD90, and CD105 antibodies (Invitrogen, Carlsbad, CA, USA) for 20 min. An isotype control was also included [27]. Cells were washed with DPBS and fixed with 4% paraformaldehyde. MSC immunophenotypes were determined by flow cytometry on a MACS Quant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany), and expression of cell surface antigens was calculated for 10,000 gated-cell events. For screening cell surface markers, 5×10^5 cells isolated from two groups (Groups 1 and 2) were dispensed into 96-well round-bottom plates (BD Lysoplates, BD Biosciences), which were lyophilized with 242 human cell surface marker antibodies. Stained cells were washed and subsequently stained with an Alexa Fluor 647-conjugated goat-anti-mouse IgG secondary antibody (Thermo Fisher Scientific, Eugene, OR, USA). Surface markers were measured by flow cytometry on a FACSCalibur instrument (BD Biosciences) by calculating the percentage of cells per 10,000 cell events.

2.3. Human Autophagy Array. Autophagy-related protein levels were analyzed by Human Autophagy Array C1 (RayBiotech, Norcross, GA, USA) using 20 different antibodies. Briefly, 2 mg cell lysates were incubated with an antibody array membrane at 4°C. The day after, repeated washings with the provided buffer were performed. Each membrane was incubated with biotinylated antibody cocktail for 2 h, followed by treatment of HRP-Streptavidin for 2 h. After further washing, membranes were incubated with detection buffer and detected on a ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA). The intensity of the protein array was normalized against positive control spots.

2.4. SA β-Gal Staining. Cellular senescence was analyzed using a Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, MA, USA). UCB-MSCs at passages 4, 7, and 13 were 70% confluent. Cells were fixed with fixing solution for 5 min at RT. After washing twice with DPBS, cells were incubated with staining working solution for 48 h at 37°C in darkness. Cells were examined using an inverted microscope (Nikon, Japan). The average percentage of stained cells was calculated from four fields.

2.5. Western Blotting. Cells were lysed with radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA). A total of 15 μg of each protein extract was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane. Blocked membranes were incubated with primary antibodies (anti-human Beclin1, anti-human Atg5, anti-human Atg12, anti-human phospho-p38 MAPK (Thr190/Tyr182, pp38), and phospho-Rb (Ser780, pRB, Cell Signaling, Danvers, MA, USA); anti-human LC3B (Sigma-Aldrich); anti-human CD47 (Abcam, Cambridge, MA, USA); and anti-human

GAPDH (Novus bio, Centennial, CO, USA)) overnight at 4°C and then probed with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. A chemiluminescence immunoblotting system (GE Healthcare Life Sciences, Buckinghamshire, UK) was used to visualize and analyze the bands.

2.6. Small Interfering RNA (siRNA). Cells were transfected with CD47 small interfering RNAs (siRNAs) and scrambled siRNA (100 nM, Dharmacon, Lafayette, CO, USA) for 48 h using the DharmaFECT reagent according to the manufacturer's recommendations. Human CD47 siRNA with sequences 5′-GCAUGGCCCUCUUCUGAUU-3′, 5′-GUACAGCGAUUGGAUUAAC-3′, 5′-CAGAGAAGGUGAAACGAUC-3′, and 5′-UAACUGAAGUGAAGUGAUG-3′ was synthesized. Scrambled siRNA with sequences 5′-UGGUUUACAUGUCGACUAA-3′, 5′-UGGUUUACAUGUUGUGUGA-3′, 5′-UGGUUUACAUGUUUUCUGA, and UGGUUUACAUGUUUUCCUA-3′ was synthesized.

2.7. Immunocytochemistry. To detect CD47 and LC3B, cells were seeded on two-chamber slides. After washing with PBS, cells were fixed with 4% PFA and then blocked with 0.1% Triton X-100. Cells were incubated with CD47 primary antibodies (1 : 500, Abcam) and secondary antibody (anti-mouse Cy3-conjugated secondary antibody, 1 : 700, Jackson ImmunoResearch Europe Ltd., Newmarket, UK). LC3B was detected using a rabbit monoclonal primary antibody (1 : 1000, Abcam) followed by an anti-rabbit Alexa Fluor® 488 (1 : 700, ImmunoResearch Europe Ltd). Images were acquired using an LSM 800 confocal microscope (Zeiss, Oberkochen, Germany).

2.8. Statistical Analysis. All data is presented as the mean ± standard deviation. Statistical analysis was performed with a one-way analysis of variance (ANOVA) for experiments with more than two groups followed by Tukey's HSD test using GraphPad Prism (San Diego, CA, USA). All experiments were repeated at least three times. Data was considered statistically significant if $p < 0.05$.

3. Results

3.1. CD47 Affects Cellular Processes on UCB-MSCs. To confirm that cell surface protein on senescent MSCs controls the aging process, 242 human cell surface antibody screening was performed. Our previous study found that melanoma cell adhesion molecule (MCAM/CD146) among 242 human cell surface markers was downregulated with prolonged in vitro expansion, associated with cellular senescence [26]. In the screening results of senescent UCB-MSCs, cell surface markers, CD47, CD146, CD49b, CD274, and EGFR, which showed a significant difference between early and late passages, were selectively chosen. Proliferative rates on UCB-MSCs (P5) under the same conditions were measured in 10 different MSC lots to verify the effects of senescent states. The basic characteristics of the MSCs, such as stemness, are determined with stem cell surface markers (Table S1). MSCs were positive to CD90, CD73, CD166, and CD105 (≥85%). CD14, CD45, and HLA-DR MSC markers were negative (≤1%). The differentiation capacity is determined by ALPase staining and Von Kossa staining.

Depending on the proliferative rate, UCB-MSC lots were divided into two groups (Group 1 and Group 2). Group 2 had a higher cumulative population doubling (PD) at passage 5, compared to that of Group 1 (Figure 1(a)). Heat map analysis showed downregulated surface proteins (CD47, CD146, CD49b, CD274, and EGFR) in Group 1 (Figure 1(b)). Flow cytometric analysis revealed that only the CD146 and CD47 protein expressions were significantly suppressed in Group 1, with less proliferative capacity. There was a significant difference in CD47 levels between Group 1 and Group 2 (Figures 1(c) and 1(d)). To evaluate the cellular processes between senescence and autophagy, immunoblotting was performed with autophagy-related proteins, ATG5, ATG12, Beclin1, and LC3B in Groups 1 and 2 (Figure 1(e)). At the same time, Group 1 and Group 2 showed significant differences in autophagy-related proteins. The increased expression of ATG5, ATG12, Beclin1, and LC3B was analyzed, suggesting that senescent cells may regulate autophagic activity during passages.

3.2. The Relationship between CD47 and Cellular Senescence in the Late Passage UCB-MSCs. To evaluate the relationship between cellular senescence and cell surface marker CD47, CD47 expression was analyzed in Group 2 during passage from P4 to P13. Until P7, there was no significant change in CD47 expression in MSCs. However, CD47 expression was significantly decreased at a late passage, P13 (Figure 2(a)). Representative confocal microscopic images of CD47-positive cells at a late passage (P13) showed significantly decreased CD47 expression ($p < 0.01$, Figure 2(b)). Overall, passaging of MSCs downregulated cell surface CD47 expression. The fold changes in cell growth gradually decreased from P4 to P13 (Figure 2(c)). To clarify the general cellular senescence processes, SA β-gal activity was monitored during passaging from P4 or P7, and P13. SA β-gal positive cells gradually increased from P4 to P13. At P13, cellular senescent activity was significantly increased compared to early passages (P4 and P7) (Figure 2(d)). Moreover, western blotting showed decreased CD47 protein levels from P4 to P13. The level of pRB, a senescence-related protein, was significantly decreased at P13. Conversely, the level of pp38, a senescence-related protein, was significantly increased at P13 ($p < 0.01$, Figure 2(e)). Overall, the results demonstrated that UCB-MSC expansion increased senescent progression, consistent with downregulated CD47 expression.

3.3. The Relationship between CD47 and Autophagy in the Late Passage UCB-MSCs. The correlation between CD47 and autophagy phenotype at late passages of UCB-MSCs was confirmed by immunoblotting of autophagy-related markers and fluorescent staining of LC3B. To evaluate autophagy during passaging from P4 to P13 of UCB-MSCs, ATG5, ATG12, Beclin1, and LC3B expression levels on UCB-MSCs were markedly decreased at P13 (Figure 3(a)). Confocal microscopy images demonstrated markedly decreased density of LC3B-positive cells, an autophagosome

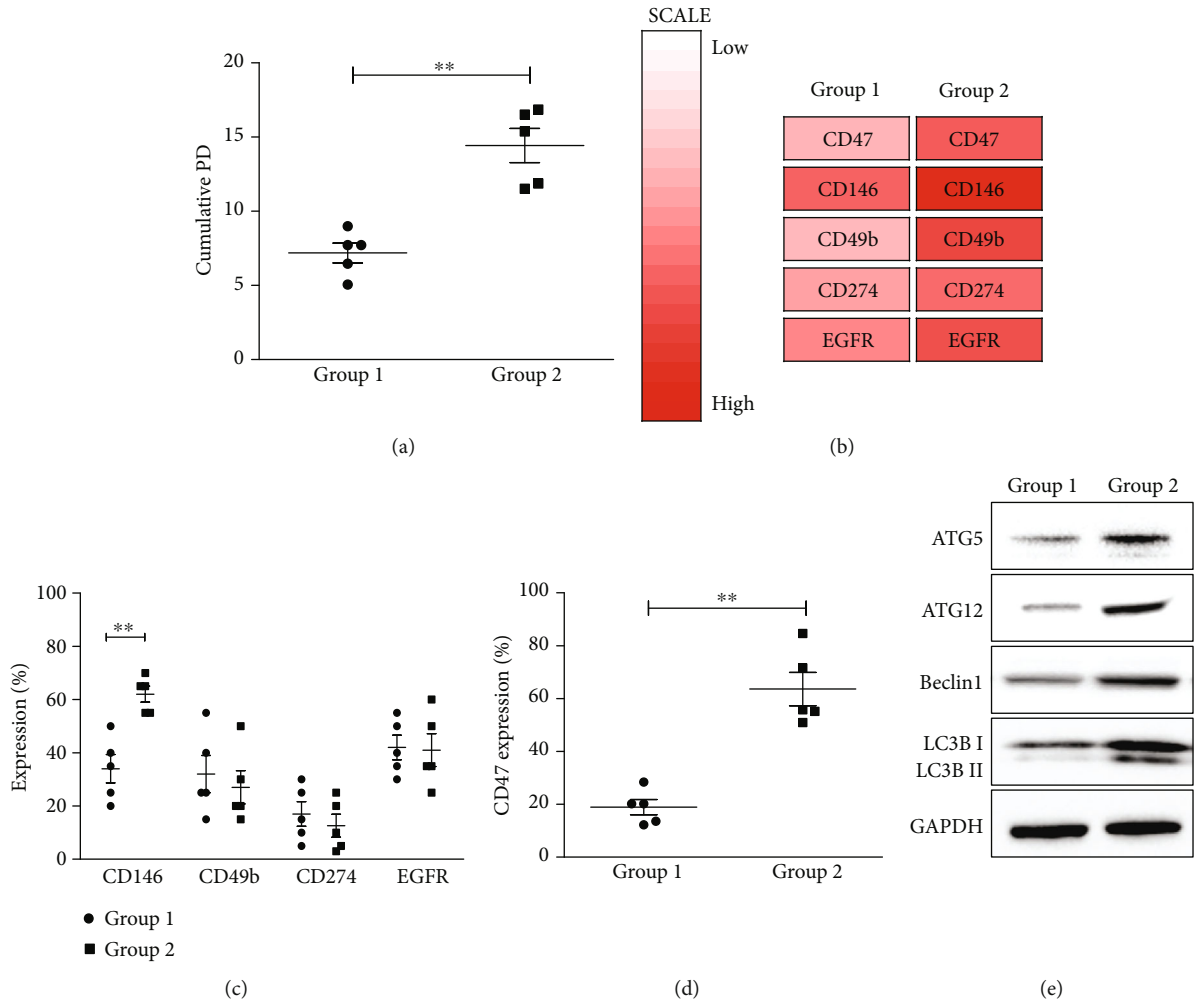


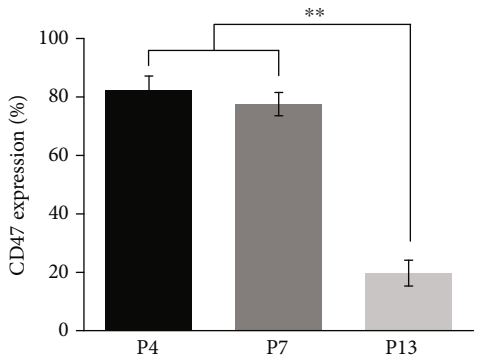
Figure 1: Screening results revealed different CD47 expressions on two groups, depending on cellular proliferation. (a) Comparative results of cumulative population doubling (CPD) at passage 5 showed higher proliferative capacity on Group 2. (b) Screening for cell surface proteins between two groups was shown with heat map analysis based on 242 cell surface antibody screening. The significant difference in cell surface antigen expression between two groups was revealed: CD47, CD146, CD49b, CD274, and EGFR. (c, d) Flow cytometry identified two CD markers (CD146 and CD47), showing significant difference expression. (e) Immunoblotting results demonstrated that the expression of autophagy-related proteins, ATG5, ATG12, Beclin1, and LC3B, downregulated on Group 1. Data is expressed as the mean ± SD; $n = 5$; $^{**}p < 0.01$, $^{*}p < 0.05$.

marker, in the late passage (Figure 3(b)). The result was consistent with downregulated protein levels of autophagy-related markers in UCB-MSCs. These results suggest that the senescent UCB-MSCs have lower autophagy, which can be correlated by the lower CD47 expression.

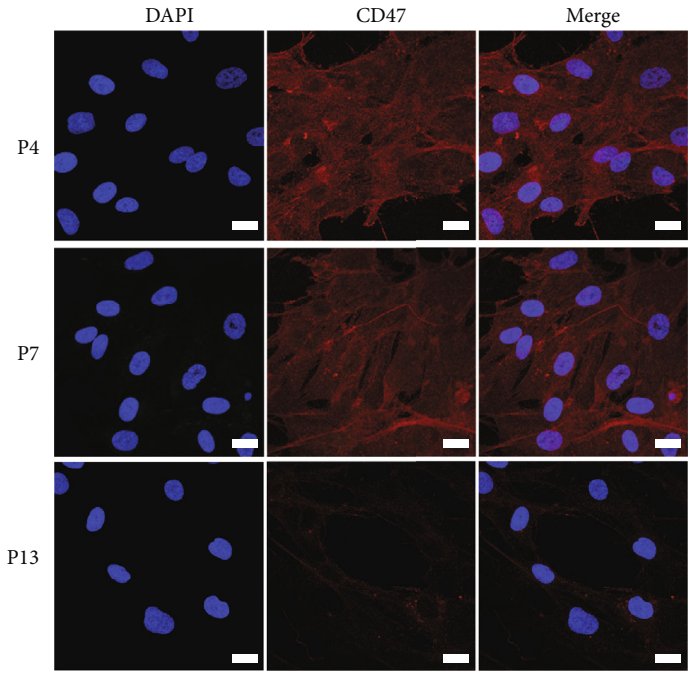
3.4. CD47 Knockdown Reduces Autophagy and Accelerates the Senescence of UCB-MSCs. To investigate the role of CD47 in autophagy and senescence, the cellular profile of CD47 knockdown UCB-MSCs and normal UCB-MSCs was assessed. CD47, a cell surface marker at passage 6, was silenced. MSCs were transfected with 100 nM CD47 siRNA or a scrambled control siRNA for 48 h. CD47 siRNA treatment significantly suppressed CD47 protein levels, as shown by flow cytometry results (Figure 4(a)).

Human autophagy array results showed significant differences in LC3A and LC3B protein levels between scrambled control siRNA-transfected and CD47 siRNA-transfected MSCs. The 60% protein levels of LC3A and LC3B, autophagy-related markers, were downregulated in CD47 siRNA-treated MSCs. In addition to LC3A and LC3B, ATG3, ATG5, ATG7, ATG13, Beclin, and LAMP1, autophagy-related proteins, revealed 20% suppression of protein levels with CD47 blockade. As depicted in Figures 4(b) and 4(c), the marked changes in LC3A and LC3B after CD47 siRNA treatment correlated with the alteration of CD47 protein levels. Overall, CD47 knockdown in UCB-MSCs reduced autophagy via LC3A and LC3B.

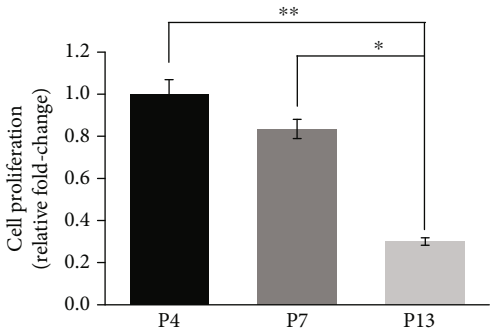
Interestingly, western blot results demonstrated that pRB and pp38 levels, senescence-related proteins, were also affected by CD47 knockdown. With CD47 siRNA transfection, pRB proteins were inhibited, whereas the level of pp38 proteins revealed a 14-fold increase in CD47 siRNA-transfected MSCs (Figure 4(c)). Relative fold changes of CD47, pRB, pp38, ATG5, ATG12, Beclin1, and LC3B were analyzed with significant differences between scrambled



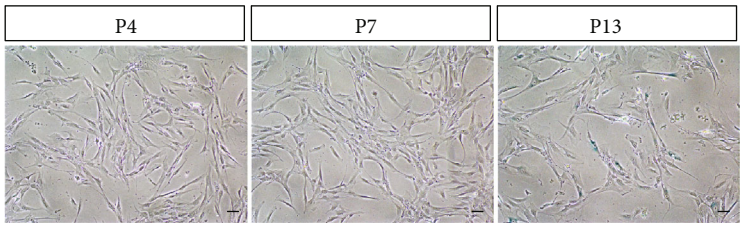
(a)

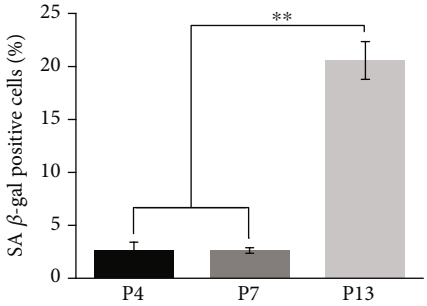


(b)



(c)





(d)

Figure 2: Continued.

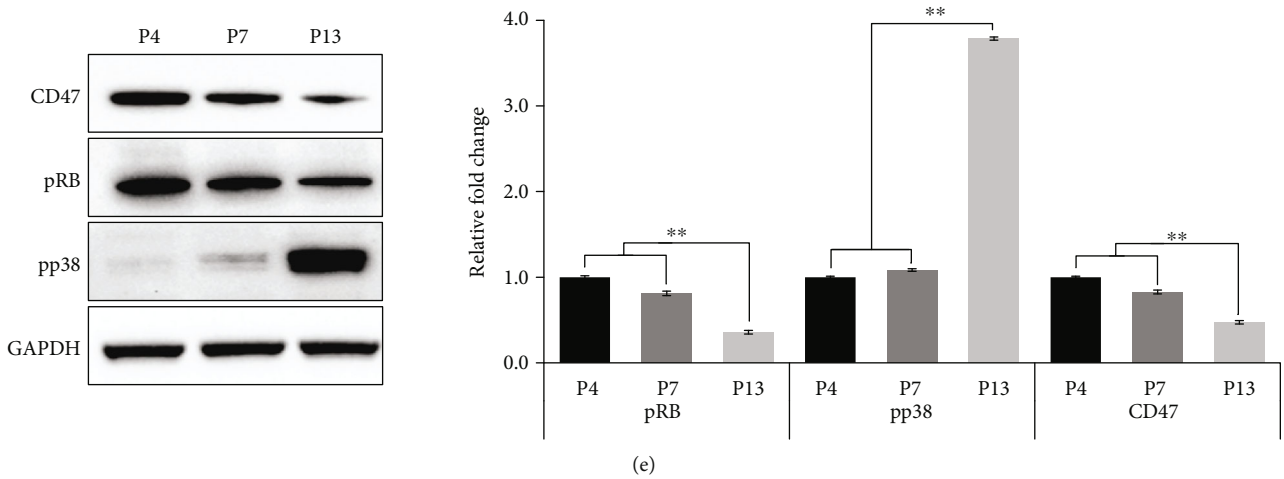


Figure 2: CD47 expressions and cellular senescence on late passages of UCB-MSCs were downregulated. (a) Flow cytometric analysis of CD47 expressions on UCB-MSCs during passaging was performed. (b) Representative immunofluorescence images of CD47-positive cells under passaging from passage 4 to 13. Scale bar 20 μm. (c) Cell proliferation was determined by measuring fold changes, with results normalized to the growth observed at passage 4. (d) SA β-gal staining was performed on UCB-MSCs at passages 4, 7, and 13. SA β-gal positive cells were quantified by determining the percentages of stained cells. Scale bar 50 μm. (e) Representative western blots of CD47, pRB, and pp38, cellular senescence-related markers, in UCB-MSCs subjected to passaging the cells. Semiquantification of protein expression levels of CD47, pRB, and pp38 was analyzed at passage 4 (P4), passage 7 (P7), and passage 13 (P13). Data is expressed as the mean ± SD; $n = 3$; $^{**}p < 0.01$, $^{*}p < 0.05$.

control siRNA and CD47 siRNA-transfected MSCs (Figure 4(d)). To verify the role of CD47 on proliferation, CD47-transfected MSC counts were demonstrated during day 5. Downregulated CD47 expression also inhibited UCB-MSC proliferation (Figure 4(e)). Representative immunofluorescent staining has been implicated in the relationship between LC3B and CD47. LC3B-positive cells were significantly reduced, correlated with CD47 protein levels, with CD47 siRNA transfection (Figure 4(f)). Thus, CD47 may affect LC3B expression that accumulates autophagy and triggers senescence in late passages of UCB-MSCs.

3.5. CD47 Influences Senescence in UCB-MSCs. Autophagic flux in CD47 siRNA-transfected UCB-MSCs is estimated by treatment of the inducer, rapamycin. Autophagy induction during passaging on MSCs converts LC3BI to LC3II and induces an increase in LC3B. Rapamycin, an inducer of autophagy, inhibits the mTOR signaling pathway, which downregulates autophagy indirectly by negatively regulating the transcription of genes required for lysosomal function. Rapamycin upregulated LC3BII expression by inducing autophagy in the scrambled control group (Figure 5(a)). However, immunoblotting results demonstrated that Beclin 1 and LC3B-II levels downregulated in CD47 siRNA transfected group were enhanced by rapamycin treatment. To measure autophagic flux, it is essential to determine the extent to which LC3BII is degraded in a lysosome-dependent manner and how much LC3 puncta are determined by fluorescent staining. The increase in the number of LC3 puncta of naïve cells in the scrambled control group in the presence of rapamycin represented the number of autophagosomes. LC3B was recruited to autophagosomes forming punctate structures, as indicated by the green color. However, CD47-transfected groups showed a decreased number of LC3B-positive puncta in the presence of rapamycin (Figure 5(b)). Together, these results establish that CD47 is critical for modulating autophagic flux via LC3B-related lysosome regulation.

4. Discussion

This study demonstrated CD47 as a critical mediator of proliferation and autophagy to maintain and control MSC senescence. Based on various beneficial functions, including stemness, differentiation potential, paracrine action, low immunogenicity, and tumorigenicity, MSCs have been widely applied in cell-based trials for broader-spectrum diseases. To obtain a sufficient number of cells for sufficient therapeutic effect, MSCs must be expanded during long-term in vitro culture, followed by premature senescence. Cellular senescence potentially induces poor clinical outcomes by producing growth arrest and reducing stem cell ability. Thus, the premature senescence of MSCs is a main concern that needs to be addressed to achieve better outcomes in cell therapy.

Cell surface markers were identified as quality-control markers to select functional MSCs, as suggested by previous reports. Our results indicated that the change in EGFR and CD49f protein expression was associated with the cell size of MSCs [28]. Additionally, surface markers, including CD264, CD142, and CD274, have been reported as new markers for isolating MSCs [29–31]. These markers were useful for quality control to characterize stem cell phenotypes destined for therapeutic treatment. Among them, CD264 is a surface marker to select aging MSCs unrelated to the chronological age of the donor; cells expressing this protein exhibit increased senescence-associated β-galactosidase (SA β-gal) activity and reduced differentiation potential and colony-

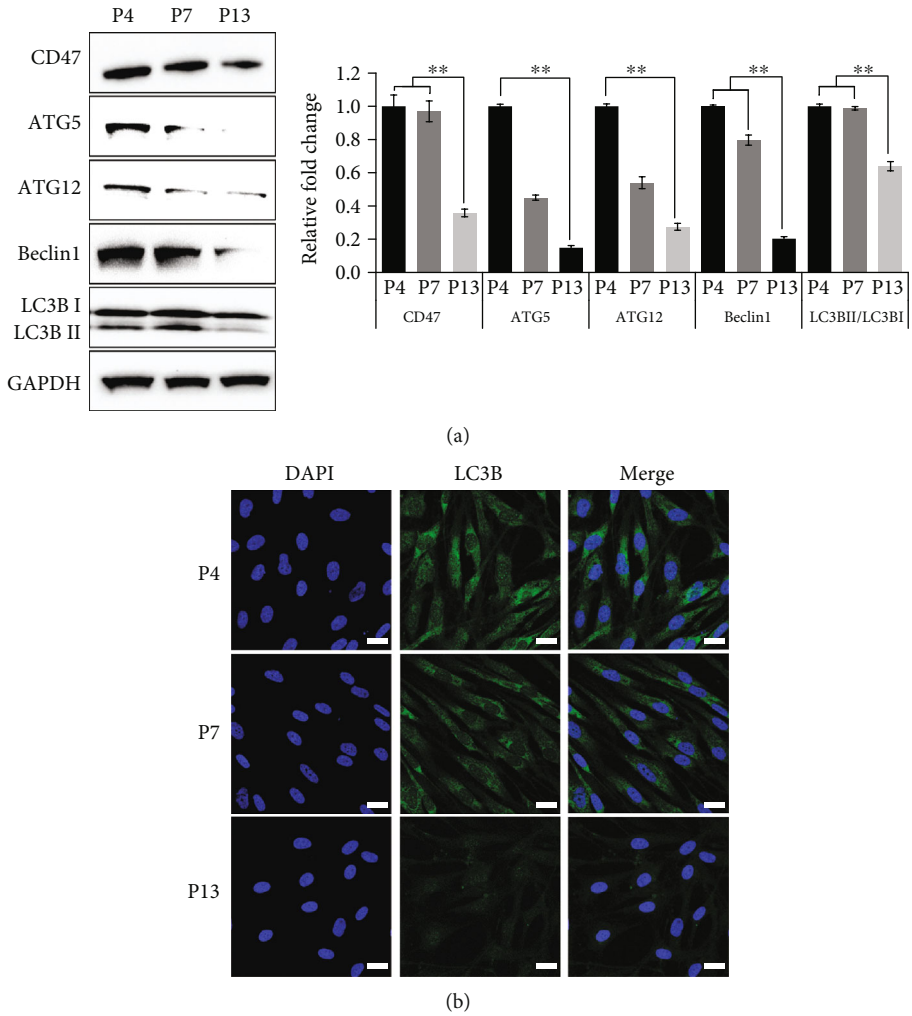
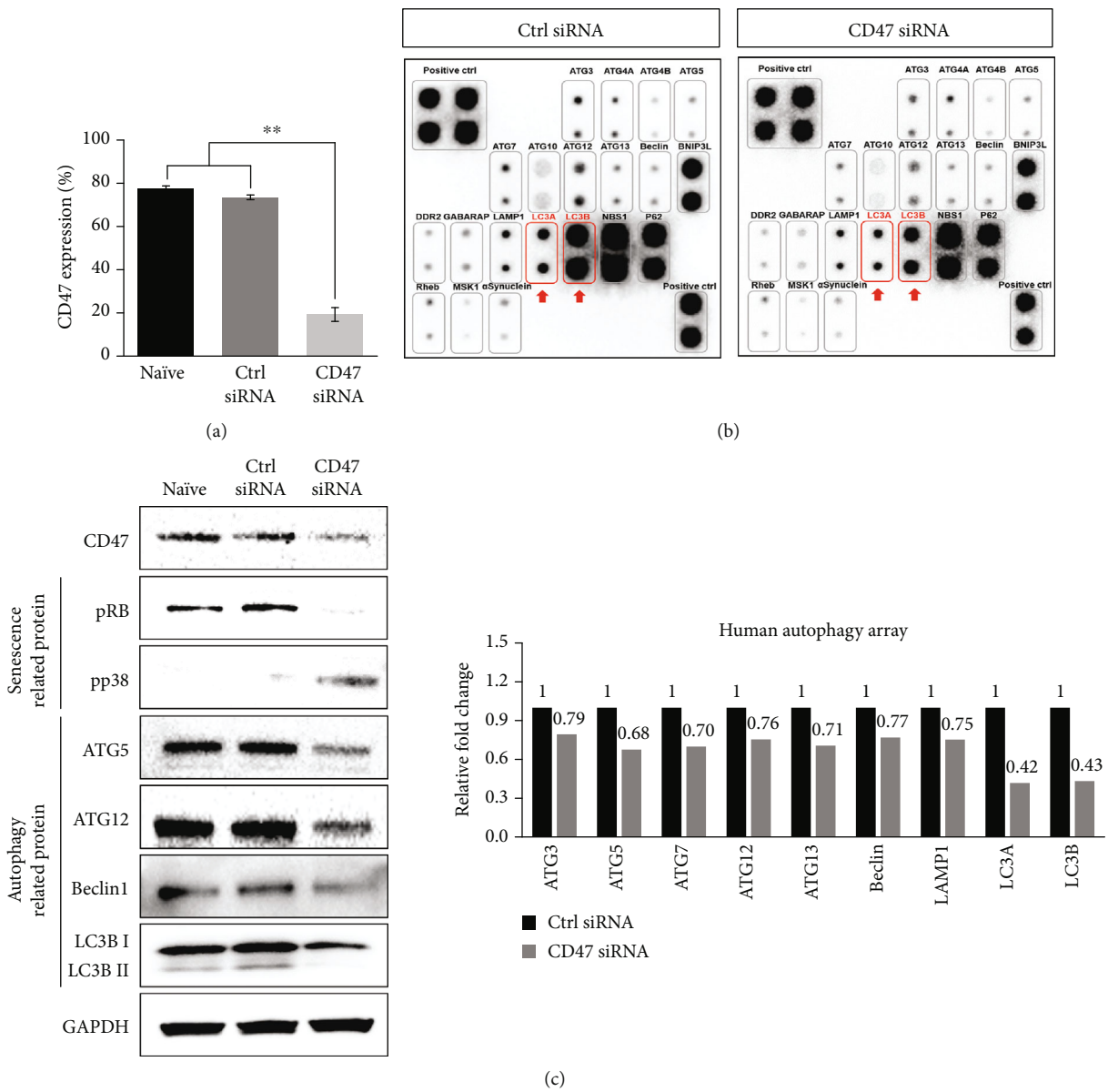


Figure 3: CD47 expressions and autophagy on UCB-MSCs were downregulated during passages. (a) Representative western blot results revealed autophagy-related protein expressions, CD47, ATG5, ATG12, Beclin1, and LC3B during passaging from P4 to P13. Relative fold changes of protein expressions were normalized to each protein level observed at P4. (b) LC3B-positive cells at different passages (P4, P7, and P13) were monitored by immunostaining. Scale bar 20 μm. Data is expressed as the mean ± SD; $n = 3$; $^{**}p < 0.01$.

forming efficiency compared to CD264$^-$ MSCs [29, 32]. To confirm that the cell surface proteins on senescent MSCs control the aging process, surface antibody screening was performed. It was previously found that melanoma cell adhesion molecule (MCAM/CD146) among 242 human cell surface markers was downregulated with prolonged in vitro expansion, associated with cellular senescence [27]. Moreover, the surface markers CD47, CD49b, CD274, and EGFR were selectively chosen, showing a significant difference between early and late passages. To validate the association between five surface markers and growth rate, protein levels of these surface markers in UCB-MSCs (early stage, P5) from 10 donors were measured under the same conditions, which could be classified into two groups by the CD47 protein level or growth potential (Group 1 vs. Group 2). Particularly, cells in Group 1 had stopped proliferation soon and observed faster senescence with a lower cumulative PD. Significantly lower levels of CD47 expression were also obtained in Group 1. Overall, the results provided evidence of CD47 as a candidate surface marker for selecting high-growth cells or late senescent cells.

Autophagy plays a key role in maintaining bioenergetic homeostasis through regulating molecular clearance or organelle turnover [33]. Unstable autophagy can lead to cell death or cellular senescence. Autophagy gradually declined cellular modulation with aging, resulting in loss of cellular efficiency [34]. Interestingly, autophagy is necessary for growth and differentiation of MSCs, suggesting that downregulation of autophagy can restrict the therapeutic effect of MSCs [12]. Thus, we hypothesized that autophagy might employ later senescence to control cell growth or senescence processing in MSCs. Senescent cells are identified and characterized by the senescence phenotype, triggering the stable repression of E2F-target genes and repressing some growth-enhancing genes through the recruitment of the retinoblastoma (Rb) tumor suppressor or p38 mitogen-activated [35, 36], as demonstrated by results on protein levels of p38 and Rb. Zhang et al. demonstrated that autophagy plays a



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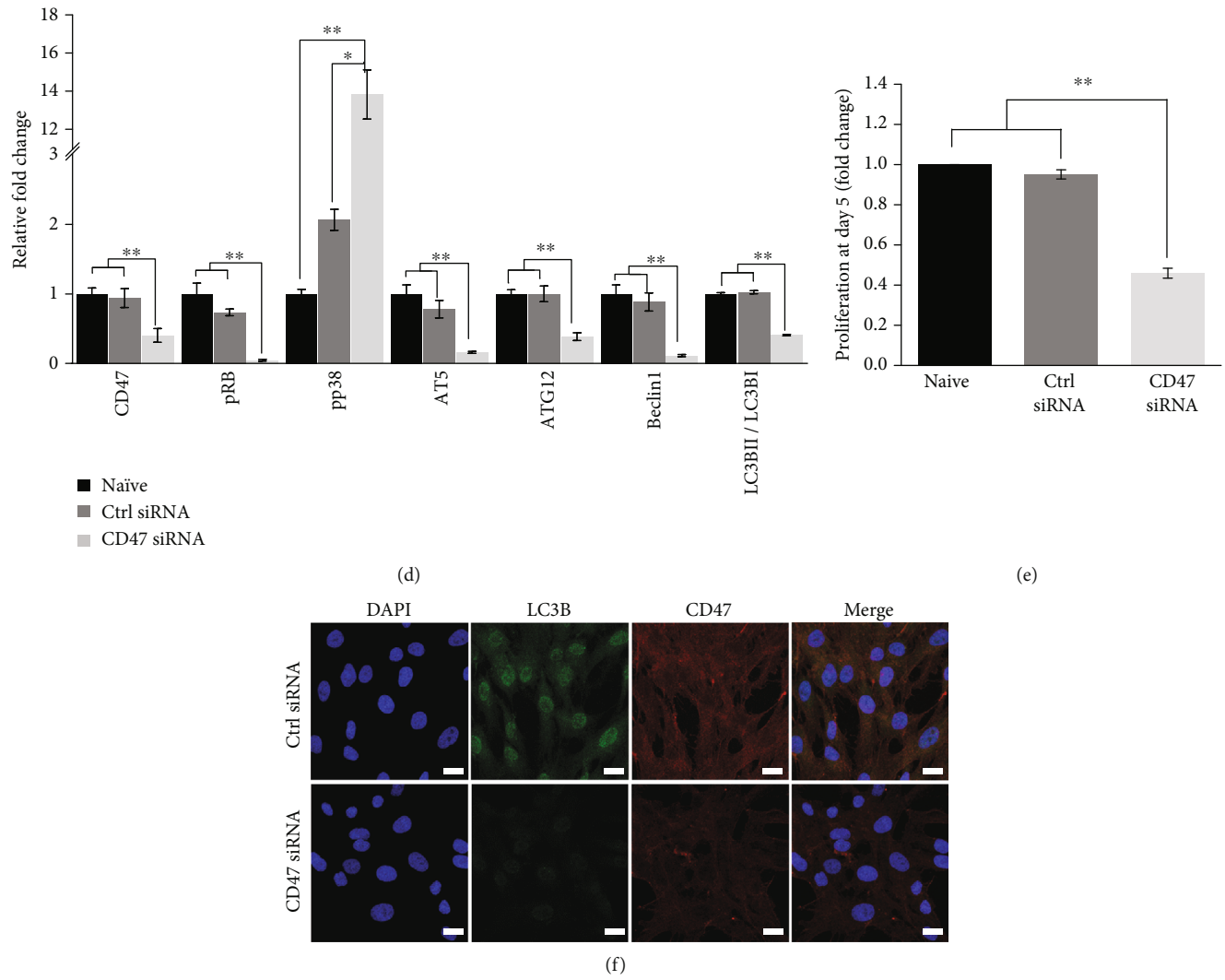

Figure 4: CD47 knockdown in UCB-MSCs reduces the autophagy and accelerates the senescence. (a) Flow cytometric analysis showed CD47-silenced UCB-MSCs downregulated CD47 cell surface markers at passage 6. (b) Human autophagy array was performed to reveal the significant autophagy-related protein level difference between control and CD47-silenced UCB-MSCs. Relative fold changes were normalized to control siRNA-transfected cells. Red box with arrow indicated LC3B proteins, showing significant difference. (c, d) Representative western blot analysis demonstrated relative changes of senescence-related proteins (pRB and pp38) and autophagy-related proteins (ATG5, ATG12. Beclin1, and LC3B) after CD47 siRNA transfection at passage 6. (e) Proliferation at day 5 after CD47 siRNA transfection was downregulated. (f) Immunostaining results with LC3B and CD47 antibodies on control siRNA and CD47 siRNA-transfected UCB-MSCs were determined. Scale bar 20 μm. Data is expressed as mean ± SD; $n = 3$; $^{**}p < 0.01$.

protective role in senescence, with autophagy activation before aging having the potential to delay MSC senescence. The ROS/JNK/p38 mechanism pathway plays a key mediating role in autophagy and delayed MSC senescence [37]. Autophagy uses a bulk protein and organelle degradation system and is the main homeostatic cellular recycling process. Three types of autophagy processes included the following: macroautophagy-, microautophagy-, and chaperone-mediated autophagy. Usually, macroautophagy is considered the major form of autophagy. Autophagy is controlled by double-membrane-bound structures called autophagosomes [1, 3, 38].

The autophagic pathway is classified into several phases: initiation, vesicle elongation, maturation, fusion, and degradation [39]. Beclin1, the mammalian ortholog of the yeast protein ATG6, has been known to play a crucial role in the autophagy initiation step [40]. Beclin1 plays a role in the phosphoinositide-3 kinase pathway to activate the formation of autophagic vacuoles [41]. Almost all Atg5 and Atg12 are covalently attached to each other and present as an Atg12-Atg5 conjugate within cells, forming a protein complex in the cytosol [42]. A small fraction of the Atg12-Atg5 complex targets the outer side of the phagophore, which is an intermediate structure during vesicle elongation [43]. LC3B is an autophagosomal ortholog of the yeast protein ATG8 and is a specific marker of autophagosome formation (maturation-fusion-degradation) [44]. LC3BI is localized in the cytoplasm, whereas LC3BII binds to autophagosomes. Autophagy stimulation leads to the conjugation of LC3II [45]. LC3BII binds to autophagosomes and is degraded by lysosomal hydrolases after the fusion of autophagosomes

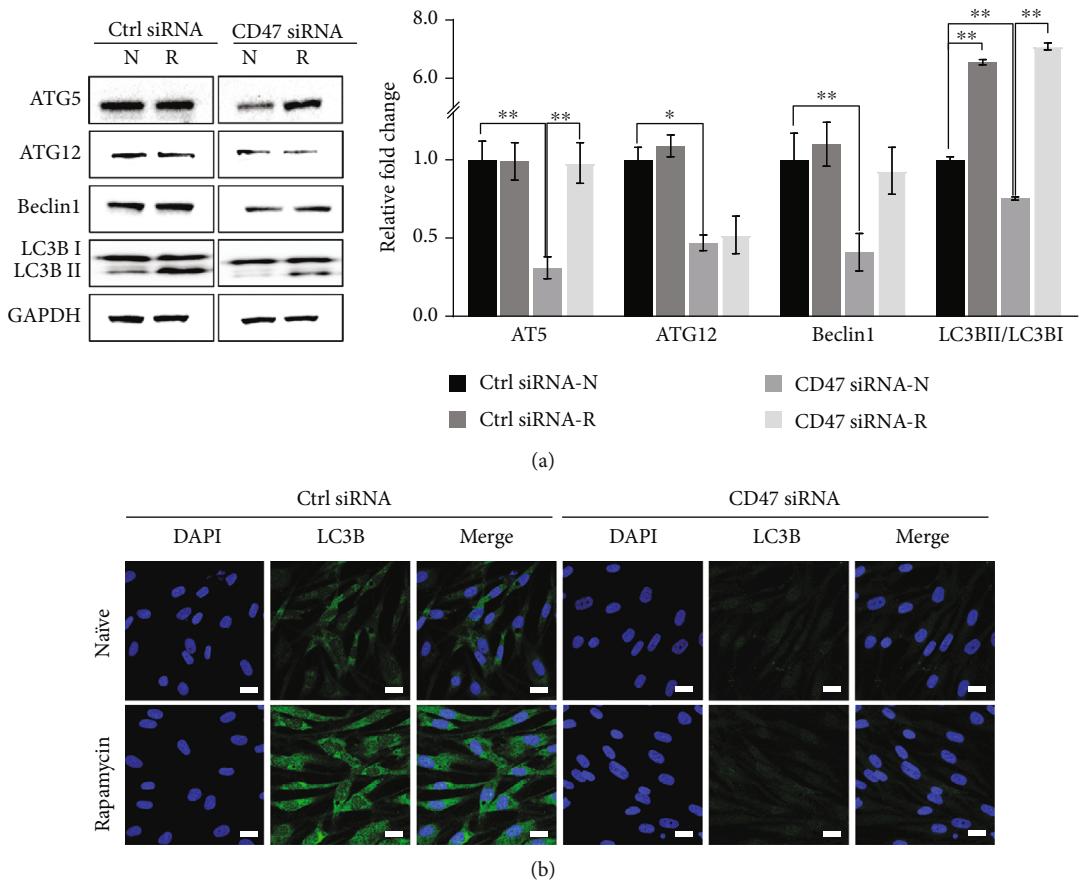

Figure 5: Autophagy flux. (a) Rapamycin (R, 10 μM), autophagy inducer, was treated in CD47 knockdown UCB-MSCs. Autophagy-related proteins were determined with immunoblotting analysis. Relative fold change of proteins, ATG5, ATG12, Beclin1, and LC3BII relative to LC3BI was quantified against protein levels of GAPDH. (b) Representative immunofluorescence images of LC3 with treatment of autophagy inducer or inhibitors showed significant different intensities on LC3 levels, contingent upon CD47 levels. Scale bar 20 μm. Data is expressed as the mean ± SD; $n = 3$; $^{**}p < 0.01$, $^{*}p < 0.05$.

with lysosomes [46]. Recent data has suggested that additional membranes are derived from the Golgi complex, mitochondria, and plasma membrane [47]; however, this process has not yet been admitted. In this study, various autophagy-specific markers (Beclin1, ATG5, ATG12, LC3BI, and LC3BII) were analyzed through the autophagy pathway. Moreover, to further examine the relationship of cellular mechanisms between CD47 expression and autophagy, autophagy-associated proteins were tested in two groups (Group 1 vs. Group 2). These groups showed significant differences in CD47 expression at an early stage (P5), suggesting that growth ability may control autophagic activity. Next, we provided evidence that CD47 expression is significantly inhibited during passaging (from P4 to P13) of MSCs through in vitro aging. Similarly, the autophagy phenotype was markedly reduced in passaged MSCs. To investigate the role of CD47 in autophagy and senescence, it was verified that CD47 siRNA-transfected MSCs exhibited an accelerated senescence phenotype. This data suggests that CD47 is a positive regulator of autophagy processing during cellular senescence.

Moreover, the direct role of CD47 in controlling autophagy activation was demonstrated using a protein array. The 60% of LC3B protein levels, a central protein in the autophagy pathway where it functions in autophagy substrate selection and autophagosome biogenesis, were blocked in CD47 siRNA-transfected MSCs. In addition to ATG3, ATG5, ATG7, ATG13, Beclin1, and LAMP1, autophagy-related proteins revealed 20% suppression of protein levels with CD47 inhibition. Rapamycin, an inducer of autophagy, inhibits the mTOR signaling pathway, which decreases autophagy indirectly by negatively regulating the transcription of genes required for lysosomal function [48]. As expected, rapamycin increased LC3BII expression by inducing autophagy in the scrambled control group. However, results from the CD47 siRNA-transfected group demonstrated that the levels of Beclin1 and LC3BII were activated by rapamycin treatment. To analyze autophagic flux, it is essential to determine the extent to which LC3BII is degraded in a lysosome-dependent manner and how much LC3 puncta are demonstrated with immune staining. The increase in the number of LC3 puncta in the control group in the presence of rapamycin represented the number of autophagosomes, whereas the CD47 siRNA groups revealed fewer LC3B-positive puncta in the presence of rapamycin. These results establish that CD47 is critical for modulating autophagic flux via LC3B-related lysosome regulation. Collectively, this data

suggests that CD47 can be a useful candidate marker for predicting the senescence status or good growth of MSCs.

Indeed, results demonstrated that CD47 plays a role in controlling MSC growth, and suppression of CD47 downregulated the senescence process in MSCs. Moreover, CD47 leads to cell death in normal and tumor cells via autophagy. For example, regulation of autophagy by CD47 was also reported in a model of transverse aortic constriction to expose left ventricular heart failure, which indicated that the presence of CD47 could enhance autophagy in injured heart muscle under conditions where TSP1 expression is also increased [49]. Importantly, this study demonstrated that CD47 downregulation resulted in decreased autophagy protein levels. Thus, this data provides new direct evidence that autophagy is a downstream target of CD47 during the development of cellular senescence in MSCs. Here, we observed a novel correlation between CD47 and autophagy in senescent MSCs. To obtain a sufficient number of functional cells, cellular senescence, inevitably induced by long-term culture, has to be evaluated with a therapeutic marker. CD47, as the cell surface marker, can be a useful marker for predicting cellular senescence and applied in potential quality-control assessments for MSC-based therapy. However, further research, which is in progress, is needed to clarify the downstream pathway to understand the positive regulation of autophagy by CD47 during cellular senescence.

5. Conclusion

CD47 expression markedly decreased during MSC expansion in vitro, with augmented CD47 downregulation accelerating the senescence phenotype, which affected cell growth. Collectively, this data indicated that CD47 is a key player in autophagy and senescence to maintain and orchestrate MSC growth. Collectively, these results suggest that CD47 is a novel quality-control marker for predicting senescence or selecting good growth in MSCs and could be valuable in quality-control evaluation and for enhancing the therapeutic effect of MSC-based therapy.

Data Availability

The datasets generated during the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest

The authors have no competing financial interests to declare.

Authors' Contributions

Gee-Hye Kim and Yun Kyung Bae contributed equally to this work.

Acknowledgments

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Supplementary Materials

Table S1: characteristics of MSCs. *(Supplementary Materials)*

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