Characterization of Skeletal Stem Cells via Novel Biomarkers

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Abstract

Mesenchymal stem cells (MSCs), also called skeletal stem cells (SSCs), have been traditionally categorized by their ability to differentiate into bone, cartilage, and fat, combined with their expression of key cell surface biomarkers. One key problem that exists, however, is that these biomarkers are not cell-type specific and are not indicative of the stemness potential of these cells, and therefore are poorly reliable. To fill in the gap in literature, we attempted to identify alternative cell surface biomarkers and transcription factors present in skeletal stem cells that were extracted from the bone marrow and those derived from pluripotent stem cells. Our results confirmed a significant decline in the ability of skeletal stem cells derived from pluripotent stem cells to proliferate and differentiate into adipocytes and osteocytes after passage 3. Further, analysis of cell surface biomarkers and transcription factors showed that biomarkers commonly used to identify skeletal stem cells remain highly expressed in skeletal stem cells that had lost their ability to differentiate into the three lineages. However, it was found that integrin alpha-6 (CD49f) and the transcription factors GATA6, PRDM16, SIM2 and SOX11 were significantly upregulated in skeletal stem cells isolated from the bone marrow and cells derived from pluripotent stem cells when compared to fibroblasts. These transcription factors and CD49f also changed their expression in skeletal stem cells in later passages, which had lost their ability to proliferate and differentiate. Our results suggest that CD49f and the expression of transcription factors GATA6, PRMD16, SIM2 and SOX11 can be used to define skeletal stem cells derived from pluripotent stem cells.

Introduction

Skeletal stem cells derived from the bone marrow (BM-SCCs), previously termed mesenchymal stem cells are known for their self-renewal abilities and their ability to give rise to adipocytes, osteoblasts, chondrocytes and stroma which supports hematopoiesis (Bianco, 2014). The following characteristics have been devised to define SSCs in vitro: 1) adherence to plastic, 2) positively express cell-surface markers CD73, CD90 and CD105, 3) negatively express hematopoietic and endothelial markers, and finally, 4) capacity to differentiate into bone, cartilage and fat *in vitro* (Dominici et al., 2006). Using these minimal criteria, the isolation of potential SSCs has been reported from a variety of sources, including adipose tissue, (Shi et al., 2003) dental pulp, (Schwab & Gargett, 2007) endometrial stroma, (Ryu et al., 2012) palatine tonsil (Romanov et al., 2003) and umbilical cord blood, (Brown et al., 2009) suggesting their widespread presence in the body. However, even given their wide-distribution in the body, some researchers argue that when compared to BM-SSCs, SSCs from other sources are inherently different and do not actually contribute to skeletal development (Bianco et al., 2014). A novel alternative source for SSCs comes from differentiation of human pluripotent stem cells (hPSC-SSCs). These include embryonic stem cells (ESCs) (Villa-Diaz, Brown & Liu, 2012) and induced pluripotent stem cells (iPSCs), (Takahashi, Tanabe & Ohnuki, 2007) the latter are obtained by reprogramming somatic cells into fully pluripotent cells (Lorenz, 2008). Further adding to this debate is the observation that several key biomarkers commonly used to identify SSCs are also expressed in fibroblasts and remain expressed in SSCs that are no longer able to differentiate (Sacchetti, Funari & Michienzi, 2007), as can be seen in Figures 1 and 2. Therefore, this suggests that these biomarkers are not cell-type-specific, nor are they truly indicative of undifferentiated SSCs.

Novel biomarkers have been recently proposed to characterize SSCs. One such marker is melanoma-associated cell adhesion molecule (MCAM), also known as CD146. CD146 has been found to be expressed in self-renewing osteoprogenitor subendothelial cells of the BM stroma (Tormin, Li & Brune, 2011) and in perivascular regions (Cattoretti et al., 1993). For example, when transplanted to heterotopic sites, CD146-positive cells isolated from the BM support a hematopoietic microenvironment (Tormin, Li & Brune, 2011).

Another marker, nerve growth factor receptor, also known as CD271, selectively identifies SSCs from the BM (Lee et al., 2009). Furthermore, CD49f, also known as integrin α6, is highly conveyed in fetal BM-SSCs (Yang et al., 2015), while its expression decreases in adult cells (Zhang, Wang & Liu, 2018). During *in vitro* expansion of SSCs, it has also been previously shown that CD49f expression gradually decreases following each passage, while its knockdown reduces the potential for differentiation (Wagner et al., 2005).

Given this information, an updated phenotypic analysis of SSCs should include not only biomarkers, but also include the expression of transcription factors due to their heavy involvement in controlling transcription of thousands of genes that give rise to cell identity. Toward this end, the transcriptome of SSCs isolated from the BM and other sources have been reported (Wagner et al., 2015). Nevertheless, due to the heterogenous nature of cell origins, a consensus of transcription factors present in SSCs has not been reached. Therefore, we aimed to define the phenotypic characteristics of SSCs combining not only expression of cell surface markers, but also to include transcription factors for a more holistic viewpoint. We began by comparing BM-SSCs to ESC-SSCs and iPSC-SSCs. Inclusion of fibroblasts was necessary in our analysis to identify non-specific SSC biomarkers. Additionally, SSCs induced into adipocytes and osteoblasts were included to identify biomarkers specific for undifferentiated SSCs.

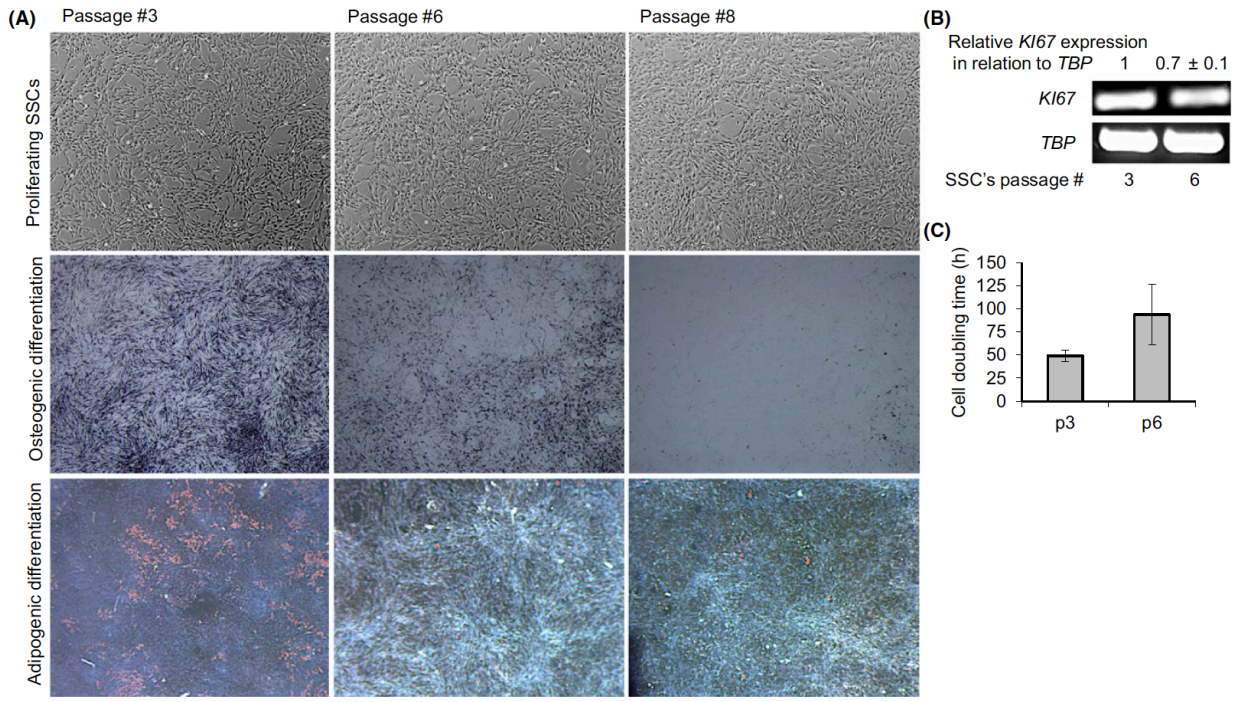

Figure 1: Skeletal stem cells lost potential for differentiation and proliferation over time (1A). hPSCs were differentiated into SSCs and cultured for eight consecutive passages. The top panel in the figure demonstrates micrographs of SSCs at passages 3, 6 and 8 to showcase that the spindle-like appearance of these cells remains consistent through all passages. The middle and bottom panel show micrographs of osteoblasts and adipocytes from hPSC-SSCs at passages 3, 6 and 8 after staining for alkaline phosphatase and Oil Red O, respectively. RT-PCR gel showing reduction of KI67 expression in hPSC-SSCs at passage 6 compared to cells at passage 3 can be seen in 1B. TBP was used as loading control and to normalize the expression of KI67 for the densitometry analysis of bands. 1C indicates cell doubling time in hours (h) of hPSC-SSCs at passages 3 and 6.

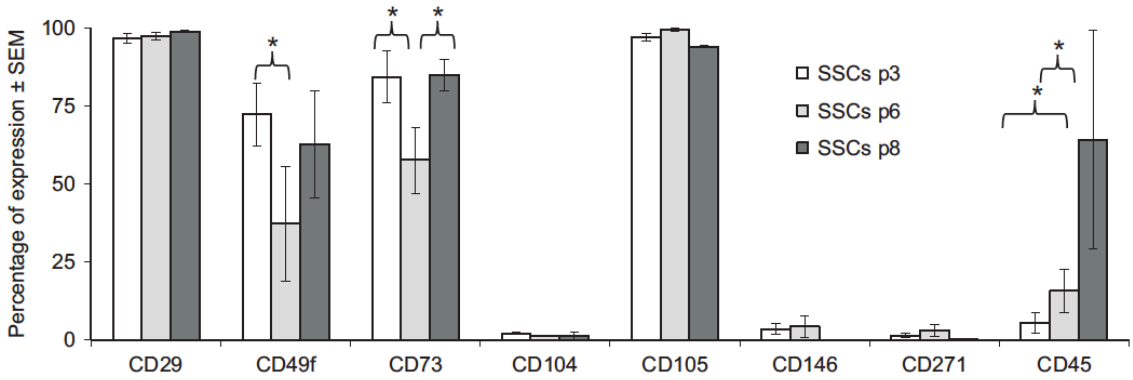

Figure 2: Expression of cell surface biomarkers in hPSC-SSCs cultured for eight consecutive passages. Flow cytometry analysis was performed at passages 3, 6 and 8 to quantify the percentage of cells with positive expression of CD29, CD49f, CD73, CD104, CD105, CD146, CD271 and CD45. Asterisks (*) denote statistically significant differences ($P < 0.050$) detected by T-test.

Materials and Methods

Replicates

All experiments were repeated in triplicates and with multiple human ESC and iPSC lines.

Culture and differentiation of human pluripotent stem cells

NIH-approved human ESC lines H1, H7 and H9 (WiCell Research Institute) and CHB8 and CHB10 (Children's Hospital Corporation, Boston, MA) and three iPSCs derived in our laboratory (hGF2-iPSCs, hGF4-iPSCs and hFF iPSCs) were cultured on Matrigel® hESC-Qualified Matrix (Corning, Corning, NY, USA) with StemFlex Medium (Gibco® Life Technologies, Waltham, MA, USA), in incubators with high humidity and 5% CO2 at 37°C.

Undifferentiated colonies were passaged using TrypLE Express (Gibco® Life Technologies). Pluripotent stem cells differentiation into SSCs was induced as previously described (Brown et al., 2009). In subsequent culture, SSCs were seeded at a density of ~7 x10^{13}cells/cm^2. To induce osteogenic differentiation, ~3 x 10^3cells/well were seeded in six-well plates and incubated in StemMACS™ OsteoDiff Media (Miltenyi Biotec, Auburn, CA, USA) for 10 days. Media was changed every 3rd day. Cells were then fixed with 10% formalin for 20 minutes at RT and stained for alkaline phosphatase (SIGMAFAST BCIP/NBT, Sigma-Aldrich, St. Louis, MO, USA) to verify osteoblast differentiation. For adipogenic differentiation, ~5 x 10^3 cells/well were seeded in six-well plates and incubated in Stem MACS™ AdipoDiff Media (Miltenyi Biotec) for 21 days. Media was changed every 3rd day. To verify adipocyte differentiation, cells were stained with Oil red O according to the pre-established protocol.

Cell doubling time calculations

Doubling time was calculated with the following formula: ((Duration of cell culture (hours)) * (log(2)))/(log(Final concentration) − log(Initial Concentration)), using Roth V. 2006 Doubling Time Computing (http://www.doubling-time.com/compute.php).

Flow cytometry analysis

Cells were harvested and prepared for flow cytometry analysis according to pre-established protocol (Villa-Diaz et al., 2012). The following PE-conjugated antibodies were used: CD29, CD49f, CD73, CD104, CD271, CD146, CD105 and CD45. A FACSCalibur instrument (Becton Dickinson) to record 10,000 events per sample was used. Data was analyzed using CELLQUEST software (Becton Dickinson, Franklin Lakes, NJ, USA). The percentage value of positive cells for each antibody was calculated by subtracting the isotype control value from the detected value of each antibody.

RNA isolation, preparation, quantitative real-time PCR and reverse transcription PCR

Total RNA extraction, purification, reverse transcription into cDNA and qRT-PCR using TaqMan probes were done as previously described (Villa-Diaz, et al., 2012). We determined gene expression by quantitative real-time PCR on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative RNA expression levels of target genes were analyzed by the comparative ΔΔCT method using the housekeeping gene TBP as an internal control. Expression levels of genes were normalized to expression levels of control samples and reported as fold changes and changes greater than 2-fold in relative mRNA expression were considered significant. For reverse transcription PCR, 1 μL of total RNA was reverse transcribed using SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen,

Carlsbad, CA, USA). The primer sequences for KI67 forward: TTGTGCCTTCACTTCCACAT and reverse: CTGGTAATGCACACTCCACCT. The TBP forward: CTCCCACCCAAAGTCTGATGA and reverse: GCCATAAACCAAGCAGGACG. The cDNA synthesis and pre-denaturation were carried out at 95°C for 2 minutes. Amplification for PCR was performed for 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The final extension cycle was run at 72°C for 10 minutes. Finally, 14 µL of PCR product was loaded onto a 2.0% agarose gel. Band densitometry analysis was performed using ImageLab 6.0 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Experiments were performed in triplicate, and data is expressed as mean value ± SEM and analyzed by an unpaired T-test. Levels of statistical significance were set at $p < 0.05$.

Results

We found that hPSC-SSCs maintained their spindle-like morphology over eight passages. However, their ability to differentiate into osteoblasts and adipocytes was significantly compromised. We saw a reduction from passage 3 to passage 6. By passage 8, differentiation status was almost non-existent (Figure 1A). Calculating cell doubling time revealed a significant decrease in proliferation rates between passage 3 and 8 that was confirmed by a 30% decrease in *KI67* RNA levels (Figure 1B,1C). This indicates that differentiation and proliferation were reduced in SSCs during *in vitro* propagation. Additionally, flow cytometry analysis of hPSC-SSCs at passages 3, 6 and 8 demonstrated that CD105 and CD29 biomarkers remained highly expressed in during all cell passages, while CD146 and CD271 were minimally expressed (Figure 2). Markers CD49f and CD73 were highly expressed at passage 3, decreased significantly ($p < 0.05$) by passage 6 and returned to high levels at passage 8. Expression of CD45 was low at passage 3 and increased with subsequent passages. Due to the fact that CD49f heterodimerizes with CD29 and CD104 we investigated the expression of both CD29 and CD104. The latter of the 2, however, was found to be have consistently low expression in these cells.

The expression of all biomarkers was comparable to levels acknowledged in foreskin and gingival fibroblasts with the exception of CD49f, which was low (<10%) in fibroblasts (data not shown). Difference in expression of CD49f between fibroblasts and SSCs was validated at RNA levels, with an 88.9 ± 37.9 (mean ± SEM)-fold increase in SSCs in relation to fibroblasts.

We investigated the mRNA levels of transcription factors previously identified in BM-SSCs and from synovial tissue (Kubo et al., 2009) and compared the expression levels to fibroblasts, hESCs, iPSCs derived from the same pool of fibroblasts, hPSC-SSCs and BM-SSCs (Figure 3A). We found that transcription factor *ETV1* was similarly expressed in fibroblasts,

iPSCs and SSCs group, while it was significantly lower in BM-SSCs. The remaining four transcription factors (*ETV5, FOXP1, HMGA* and *KLF12*) were equally expressed between fibroblasts and SSCs. In contrast, relative mRNA expression of *GATA6, PRDM16, SIM2* and *SOX11* was significantly upregulated in SSCs compared to fibroblasts.

We further looked into the mRNA expression of *ETV5, FOXP1, HMGA, KLF12* and *ITGA6* in hPSC-SSCs at an early passage (passage 3), which proved to have an increased capability for osteogenic and adipogenic differentiation compared to a later passage (passage 8) (Figure 3B). When comparing the early vs. later passages, the mRNA expression of *GATA6* decreased, *PRDM16* and *SIM2* remained the same, and *SOX*11 presented a non-significant increase in later passage cells. In contrast, *ITGA6* mRNA levels had an 8-fold increase in late passage cells. We hypothesize that this is reflective of the increase in CD49f expression observed by flow cytometry at passage 8 (Figure 2).

Furthermore, investigation of the mRNA expression of *ETV5, FOXP1, HMGA, KLF12* and *ITGA6* in hPSC-SSCs at passage 3 and in equivalent cells in early process of differentiation into osteoblasts and adipocytes revealed interesting results. We found that there was decreased expression of *ITGA6, GATA6* and *SIM2* in cells undergoing adipogenic and osteogenic differentiation, while there was no difference in *PRDM16* levels. Finally, *SOX11* mRNA levels increased during adipogenic differentiation.

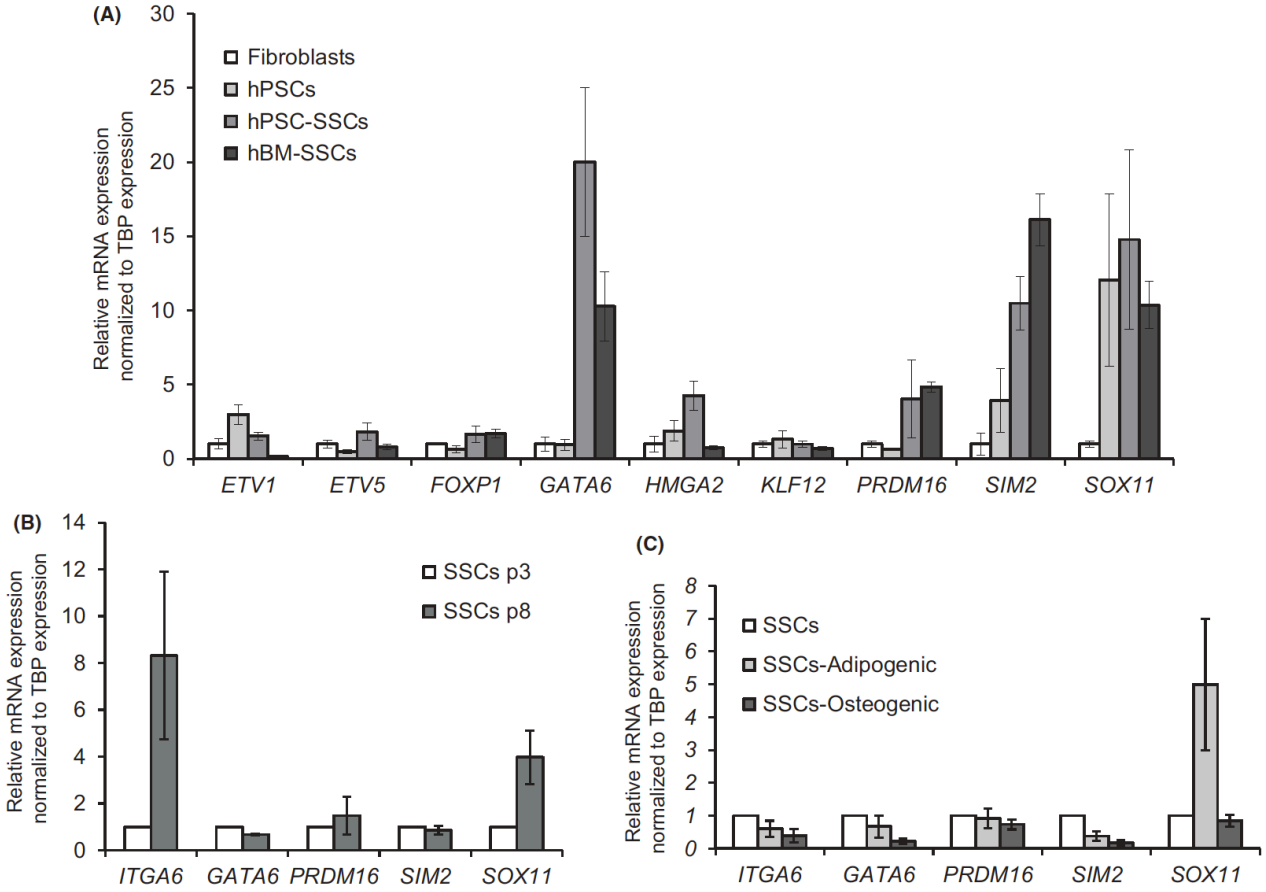

Figure 3: Identification of transcription factors expressed in skeletal stem cells. (A) Graph showing relative mRNA level expression of transcription factors (*ETV1, ETV5, FOXP1, GATA6, HMGA2, KLF12, PRDM16, SIM2* and *SOX11*) in human pluripotent stem cells (hPSCs), and skeletal stem cells derived from the pluripotent stem cells (hPSC-SSCs) and isolated from bone marrow extracts (hBM-SSCs). Graphs comparing the relative mRNA levels of *integrin α6 (ITGA6)* and transcription factors between PSC-SSCs at passage 3 to cells at passage 8 (B) and cells in early stage of adipogenic and osteogenic differentiation (C). The relative mRNA levels of each gene were normalized to reference gene *TBP*.

Discussion

Our data suggests CD49f and CD73 as biomarkers that more accurately define SSCs. This is due to the fact that their expression correlates with the differentiation potential at different passages. While biomarkers CD29, CD105, CD146 and CD271 are present in SSCs, they are also expressed at similar levels in fibroblasts and in SSCs that have lost differentiation abilities, further supporting our data. Our results indicated to not include ETV1, ETV5, FOXP1, HMGA2 and KLF12 as transcription factors to characterize SSCs as they are expressed at similar or lower levels to fibroblasts.

The increased expression of GATA6, PRDM16, SIM2 and SOX11 among BM-SSCs and hPSC-SSCs and the limited expression of GATA6 and SIM2 in undifferentiated cells reveal them as representative transcription factors that can be used in the characterization of SSCs. Previous studies in which knockout of GATA6, SIM2 and SOX11 in SSCs showed that there was inhibited self-renewal capacity, while knockout of PRDM16 was found to reduce adipogenic and osteogenic potential (Kubo et al., 2009). In the present study, we demonstrated that cells expressing low mRNA levels of these transcription factors have reduced proliferation levels and low capacity for differentiation into adipocytes and osteoblasts, indicating an important, functional value in functional SSCs.

The individual expression and function of these transcription factors in SSCs have been reported by previous groups. For instance, Sagi et al. in 2012 reported that GATA6 was highly expressed in PSC-SSCs and in SSCs isolated from BM, adipose tissue, spleen, thymus and aorta (Chuikov et al., 2010). Remarkably, others have found that unlike GATA6, which is expressed in all of the previously mentioned populations of SSCs, genes involved in mesoderm segmentation and somite development were differentially expressed in the same pool of SSC populations (Chuikov et al., 2010). This suggests that GATA6 could very well be a part of a vital signature of

SSCs. Further, PRMD16 is expressed in neural stem/progenitor cells and hematopoietic stem cells and has been found to promote maintenance of these populations by modulating oxidative stress (Shaw, Johnson & Kimber, 2010). In our study, we observed that PRDM16 is highly upregulated in SSCs when compared to fibroblasts; however, its expression remains unchanged during differentiation or in later passage cells, which have lost differentiation and most of their proliferation capacity.

Transcription factor SIM2 has been implicated during early fetal development and it is involved in development of the forebrain, ribs, vertebrae, limb skeletal muscles and kidneys (Goshu et al., 2002). In fact, one study found that SIM2-negative mice develop rib, vertebral and craniofacial abnormalities and die soon after birth due to breathing failure (Letourneau et al., 2015). This associates SIM2 in skeletal tissue development by SSCs and supports our finding of its expression in BM-SSCs and PSC-SSCs. Interestingly, SIM2 is associated with KLF4, NANOG, OCT4 (POU5F1) and SOX2 in mouse ESCs (Larson et al., 2012) and reports show significantly higher expression of SIM2 in human PSCs in comparison to fibroblasts (Figure 3A). This suggests a possible role for SIM2 in regulating hPSCs.

We found SOX11 to be significantly expressed in SSCs compared to fibroblasts. This expression remains constant even after differentiation and in cells from later passages. Previous publications have found that SOX11 expression decreases with consecutive expansions of SSCs, while its knockdown via siRNA reduces the proliferation and capacity for osteogenic differentiation (Kubo et al., 2009). Indeed, SOX11 has been identified as a key transcription factor in osteogenesis; SOX11-deficient mice develop multiple craniofacial and skeletal malformations, such as cleft palate or cleft lips, duplication in vertebrae L4,5 and kinked tails (Bhattaram et al., 2010).

Finally, we saw that integrin α6 (CD49f) expression decreases in cells during the expansion and differentiation processes, suggesting that CD49f is a more reliable biomarker of SSCs compared to other tested biomarkers. This is because CD49f is not expressed in fibroblasts and its levels become reduced in older cells that lost proliferation and differentiation abilities. These findings are corroborated by Yang et al. in 2015, who reported similar results in SSCs isolated from fetal and adult bone marrow. Yang and colleagues additionally found that knocking down CD49f reduces differentiation potential of SSCs. Furthermore, CD49f has been found to play an important role in the self-renewal of PSCs (Villa-Diaz et al., 2016), skeletal muscle stem cells, glioblastoma (Lathia et al., 2010) and breast cancer stem cells (Goel et al., 2014); and its expression has been identified in 35 populations of stem cells (Krebsbach & Villa-Diaz, 2017). Our flow cytometry data indicate that although close to 100% of cells were CD29 and CD105-positive during all 8 passages, only about 70% of PSC-SSCs were CD49f-positive at passage 3, and this expression decreased further in subsequent passages and in cells that lost differentiation and proliferation abilities. This suggests the presence of two sub-populations based on CD49f expression. We propose that the CD49f-positive subpopulation contains SSCs, while the CD49f-negative population may contain differentiated cells. This will be further verified in later experiments.

Conclusion

Our results distinguish GATA6, SIM2 and CD49f as reliable biomarkers that better indicate the functional status of SSCs. Such results have vital implications in regenerative medicine, including the SSC-based therapies proposed for a variety of diseases. There are currently ~900 clinical trials ongoing which involve SSCs or MSCs. The cells used in these trials are likely using traditional methods of characterizations, which we have demonstrated unreliable due to the fact that they are unable to differentiate between functional and non-functional cells. Thus, we propose inclusion of these new biomarkers which better identify functional SSCs in order to obtain more consistent outcomes that can be utilized in therapies.

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