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SHORT REPORT

Molecular profile of clonal strains of human skeletal stem/progenitor cells with different potencies☆

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Abstract
Bone marrow stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) are fibroblastic reticular cells, a subset of which is composed of multipotent skeletal stem cells (SSCs). SSCs/BMSCs are able to recreate a bone/marrow organ in vivo. To determine differences between clonogenic multipotent SSCs and similarly clonogenic but non-multipotent BMSCs, we established single colony-derived strains (SCDSs, initiated by individual Colony Forming Unit-Fibroblasts) and determined their differentiation capacity by vivo transplantation. In this series of human SCDSs (N = 24), 20.8% formed fibrous tissue (F), 66.7% formed bone (B), and 12.5% formed a bone/marrow organ, and thus were multipotent (M). RNA isolated from 12 SCDSs just prior to transplantation was analyzed by microarray. Although highly similar, there was variability from one SCDS to another, and SCDSs did not strictly segregate into the three functional groups (F, B or M) by unsupervised hierarchical clustering. We then compared 3 F-SCDSs to 3 M-SCDSs that did segregate. Genes associated with skeletogenesis, osteoblastogeneis, hematopoiesis, and extracellular matrix were over-represented in M-SCDSs compared with F-SCDSs. These results highlight the heterogeneity of SSCs/BMSCs, even between functionally similar SCDSs, but also indicate that differences can be detected that may shed light on the character of the SSC.

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Introduction

Bone marrow (BM) stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) were first identified and characterized by Friedenstein and Owen as a rapidly adherent, fibroblastic population of cells that contain a subset of multipotent stem cells (reviewed in Owen and Friedenstein, 1988). These cells are capable of recreating the hematopoietic microenvironment when transplanted in vivo (Friedenstein et al., 1974) by generating a bone/marrow organ. These ectopic ossicles have been consistently found to be composed of bone, hematopoiesis-supporting stroma, marrow adipocytes of donor origin, and hematopoiesis of recipient origin (Balakumaran et al., 2010; Dieudonne et al., 1998; Krebsbach et al., 1997; Kuznetsov et al., 1997; Sacchetti et al., 2007). Subsequent studies have shown that these skeletal stem cells (SSCs, Bianco and Robey, 2004) are self-renewing, sub-endothelial cells that line BM sinusoids (pericytes) and send out processes that intercalate into areas of hematopoiesis (Sacchetti et al., 2007). Consequently, SSCs are hypothesized to be important participants in the hematopoietic stem cell (HSC) niche (Mendez-Ferrer et al., 2010; Sacchetti et al., 2007).

Much work has been done on studying the biological activities of BMSCs in vitro. While in vitro assays are valuable tools to address specific questions, they are not well suited for studying the biological activities of SSCs directly, due to the fact that the latter represent only a subset of cells within the BMSC population. Furthermore, there is no single marker or set of markers that can efficiently separate SSCs from non-multipotent BMSCs (Bianco et al., 2008), and even if there were, ex vivo expansion would result again in a mixture of stem cells and more committed cells due to the kinetics of cell division (reviewed in Neumuller and Knoblich, 2009). If one assumes that stem cell division is strictly asymmetrical (one cell remaining a stem cell, the other a more committed cell), the stem cell subset would rapidly be diluted by transiently amplifying cells that are not stem cells (Kuznetsov et al., 2004). In addition, while SSCs are clearly a component of the HSC niche, current culture conditions required for support of human HSCs in vitro are not optimal (Lymperi et al., 2010). For these reasons, in vivo transplantation is the gold standard by which to characterize the differentiation capacity of a clonal BMSC population, in particular with regard to the formation of hematopoiesis-supportive stroma, a defining feature of SSCs (Bianco, 2011). Furthermore, only a subset of freshly isolated BMSCs are capable of density-independent growth [Colony Forming Unit-Fibroblasts (CFU-Fs)], and the resulting clones are heterogeneous in their in vitro differentiation potential (Muraglia et al., 2000; Pittenger et al., 1999; Russell et al., 2010 as examples), and their ability to recreate a bone/marrow organ in vivo (Friedenstein, 1980; Gronthos et al., 2003; Kuznetsov et al., 1997; Sacchetti et al., 2007). In these studies, 10–20% of the single colony-derived strains (SCDSs, initiated by individual CFU-Fs) formed a bone/marrow organ, while the remainder formed only bone (35–45%) or fibrous tissue (35–55%).

Currently, the molecular profile of subsets of SSCs/BMSCs with varying differentiation potentials is largely undefined. Larsen et al. previously established transcription profiles that distinguish between immortalized clones with and without the ability to form bone in vivo (Larsen et al., 2010). Clones that formed bone had increased expression of extracellular matrix genes, and those that did not form bone expressed immune response-related genes. Here we present data from primary unmodified SCDSs. We first established the functionality of human SCDSs by in vivo transplantation, and then compared the molecular signature of SCDSs that regenerated a complete bone/marrow organ with those that formed only fibrous tissue.

Materials and methods

Generation of BM single cell suspensions

A suspension of BM nucleated cells (BMNCs) was prepared from human trabecular bone from surgical waste of a single donor (female, 43 years-old) according to NIH guidelines as previously described (reviewed in Robey et al., 2014). Briefly, BM was gently scraped from bone fragments into growth medium [α-MEM, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Invitrogen), and 20% lot-selected, non-heat inactivated fetal bovine serum (HyClone)], and the fragments were washed extensively to remove marrow. After pelleting by centrifugation, bone marrow nucleated cells (BMNCs) were resuspended in growth medium, and passed through a 16 gauge needle, and subsequently through a 70 μm cell strainer (Becton Dickinson) to remove aggregates.

Generation of SCDSs and non-clonal BMSCs

SCDSs were prepared as previously described (Robey et al., 2014). BMNCs were plated at low density (2×10^3 nucleated cells/cm^2) into 150 mm^2 tissue culture dishes (Becton Dickinson), and cultured in growth medium at 37 °C for 14 d without any media replacements. After 14 d, single colonies with >50 cells were randomly selected and individually isolated. Only colonies with a round morphology and obvious separation from surrounding colonies were chosen. The colonies were isolated by attaching a cloning cylinder (Bel-Art Products) to the dish using sterile vacuum grease (Baxter Healthcare Corp.). Cells were detached with 0.05% Trypsin/EDTA (Invitrogen), and transferred to 1 well of a 6-well dish (Becton Dickinson) with growth medium. Once the cells approached confluency, they were passaged consecutively into one 75 cm^2 flask (Becton Dickinson), and then into two 75 cm^2 flasks. Once these flasks became confluent, the cells were detached and used for RNA isolation and in vivo transplantation. In some cases, SCDSs generated previously and cryopreserved from another donor, were used in experiments described below. Non-clonal BMSC cultures were established by plating BMNCs at a density of 5×10^6–5×10^7 cells/75 cm^2 tissue culture flask in growth medium as previously described (Robey et al., 2014).

RNA isolation

RNA was isolated from at least 5×10^5 cells from each SCDS or non-clonal BMSC cultures with TRIzol (Invitrogen), and further purified by using a combination of chloroform phase

separation and RNeasy Mini Kits (Qiagen) according to the manufacturer's protocol.

In vivo transplantation of SCDSs

2×10^6 cells from each SCDS were suspended in growth medium and incubated at 37 °C on a rotator with 40 mg of sterile hydroxyapatite/tricalcium phosphate particles (HA/TCP, Zimmer). After 90 min, the particles and cells were collected by brief centrifugation and transplanted subcutaneously into the flank of an immunodeficient mouse (NIH-*Lyst*$^{bg\text{-}J}$*Foxn1*^{nu}*Btk*^{xid}, Charles River). After 8 wks, the mice were euthanized and the transplants harvested (Krebsbach et al., 1997).

Histological scoring of in vivo transplants

Each transplant was fixed in 4% paraformaldehyde for 3 d, and decalcified in 0.25 M EDTA. Decalcification was confirmed by X-ray (Faxitron, 30 V for 40 s using Kodak PPL film). After decalcification, the transplants were embedded in paraffin and sectioned (5 μm). Each transplant was sectioned such that a section was taken every 100 μm to generate at least five sections, in order to ensure procurement of a representative sample through the entire thickness of each transplant. Sections were stained with H&E and examined microscopically. Each section was given a separate semi-quantitative score ranging from 0 to 4 for both bone and hematopoiesis formation by two independent, trained observers (see Mankani et al., 2004). Bone and hematopoiesis were scored independently of each other; for example, a section with exuberant bone formation but less abundant hematopoiesis could receive a score of 4 for bone formation, but only 1 for hematopoiesis.

Gene expression microarray analysis

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. RNA was quantified and 5 μg was processed for microarray analysis (LMT, NCI, Frederick, MD). RNA was reverse transcribed to form cDNA, and hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays, composed of more than 54,000 probe sets and 1,300,000 distinct oligonucleotide features that analyze the expression level of over 47,000 transcripts and variants, including 38,500 well-characterized human genes. Three independent replicates for each of the experimental conditions were carried out and analyzed to control for intra-sample variation. Comparative analyses of expressed genes that were over/under-represented by >2-fold were carried out using the GeneSpring software. Signal intensity values were normalized using RMA summarization and baseline transformation to median of all samples was performed. Entities were filtered based on their signal intensity values. A total of 45,371 out of 54,675 entities passed the test where 1 out of 6 samples have signal intensity values between 20 and 100 percentiles. Hierarchical clustering was performed on filtered signal intensity (>20.0), non-averaged, fold change > 2. Gene ontology analysis was done using fold change > 2, and a p-value cutoff of 0.1, as a p-value cutoff 0.05 resulted in no significant GO groups. A fold change analysis (>10 fold) was performed to generate a list of top genes over/under-represented between groups. Statistical analysis was performed using the Student's *t*-test (fold change ≤ 2, corrected p-value ≤ 0.05).

qRT-PCR analysis

RNA was extracted from cells that remained after generation of in vivo transplants and microarray analysis (SCDS-61, SCDS-11, SCDS-99, SCDS-107), from SCDSs isolated from another donor, and from non-clonal BMSCs. RNA (1.0 μg) was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, #170-8891). Quantitative RT-PCR (qPCR) was performed using a CFX-96 Real Time System paired with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA). qPCR reactions were set up using iQ SYBR Green Supermix (Bio-Rad #170-8882) according to the kit's instructions. Primers were designed using Beacon Designer 6 software (Premier Biosoft International, Palo Alto, CA): hSFRP2 (NM_003013) — F: AGGACAA CGACCTTTGCATC, R: CAGGCTTCACATACCTTTGGA; hCNN1 (NM_001299.4) — F: ACTTCATGGACGGCCTCA, R: TGGGTTG ACTCATTGATCTTCTT; RPL13a (NM_012423) — F: GGAGAA GAGGAAAGAGAAAGC, R: GGCAACAATGGAGGAAGG; GAPDH (NM_002046.3) — F: TGCACCACCAACTGCTTAGC, R: GGCAT GGACTGTGGTCATGAG. qPCR results, expressed as critical threshold (C_T) values, were normalized to the levels of RPL13a or GAPDH, generating ΔCT values; levels of relative expression were calculated as $2^{-\Delta CT}$.

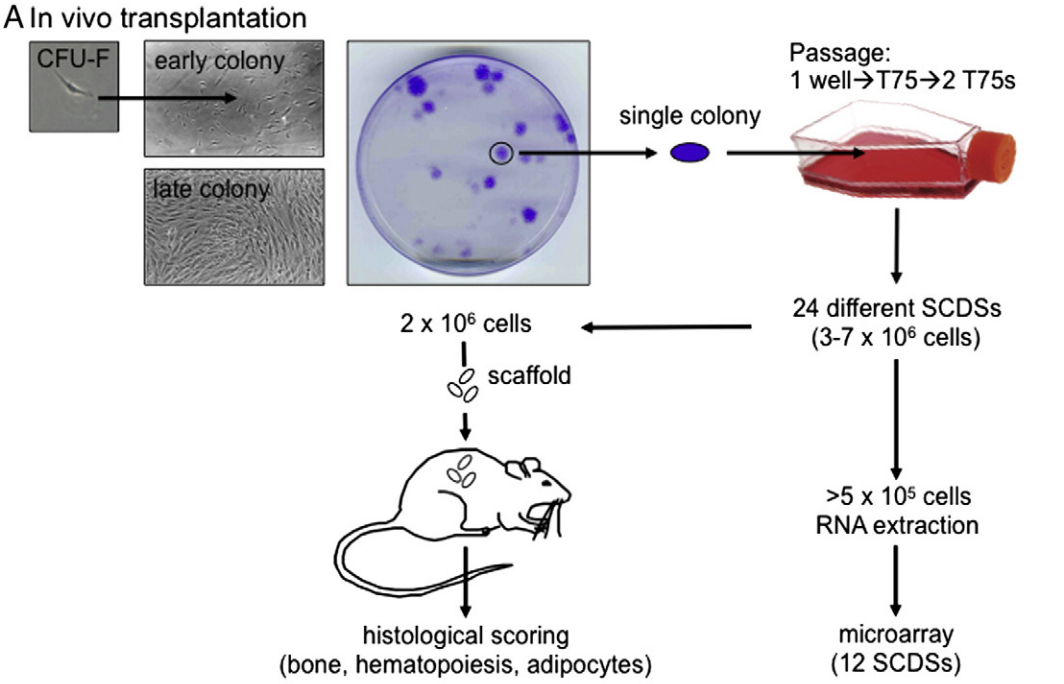
Results

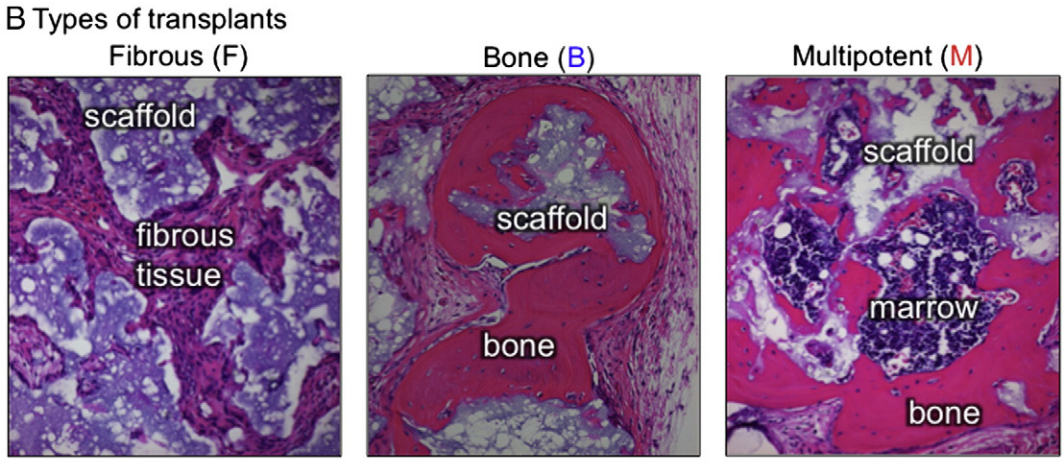
Generation and transplantation of SCDSs

SCDSs of hBMSCs were expanded under basal conditions, and their in vivo differentiation potential was assayed according to the scheme represented in Fig. 1A. Of the 114 hBMSC colonies originally selected, ~50% ceased to proliferate. Of those that continued to proliferate, 24 were randomly selected, transplanted, and harvested after 8 wks. Of note, no adipocytic differentiation was noted in any of the SCDSs. Between ~3 and 7×10^6 cells were available at the time of transplant.

Histological analysis and scoring of SCDS transplants

Sections of transplants were stained with H&E, examined histologically, and given an independent score ranging from 0 to 4 for both bone and hematopoiesis (Mankani et al., 2004). Those clonal strains that formed only fibrous tissue were considered to be devoid of SSCs, and were termed fibrous (F), those that formed bone without supporting hematopoiesis were considered to be unipotent (B), and those that formed bone and supported formation of marrow (stroma and adipocytes of donor origin, hematopoiesis of recipient origin) were considered to be multipotent (M) (Fig. 1B). Of the 24 SCDSs transplanted, 5 (20.8%) formed fibrous tissue (F), 16 (66.7%) formed bone (B), with scores between 1 and 3, and 3 (12.5%) were multipotent (M) (Fig. 1C). These results are consistent with what has been reported previously. Notably, only M transplants were found to have adipocytes; adipocytes were not found in F or B transplants.





C Scoring

	Fibrous	Bone Only				Multipotent		
Transplant Score - Scale of 0-4 (Bone Score/HP Score)	0/0	1/0	2/0	3/0	4/0	3/1	3/2	4/2
Number of Clones	5	5	7	3	1	1	1	1
	20.8%	66.7%				12.5%		

Figure 1 A) Experimental design for generation of single colony-derived strains (SCDSs), assessment of functionality by in vivo transplantation and profiling by microarray analysis. B) Types of transplant. Twenty-four SCDSs were transplanted in vivo with hydroxyapatite/tricalcium phosphate ceramic particles as a scaffold, and then scored on a scale of 0–4 for the presence of bone and hematopoiesis. The SCDSs were categorized as generating fibrous tissue (no bone, no support of hematopoiesis) (F), bone-forming only (B), and as multipotent (M) based on the formation of bone and support of hematopoiesis. C) The scores of individual transplants were categorized, and the percentages of each type (F, B or M) are indicated.

Analysis of SCDS gene expression using microarray

RNA from a total of 12 SCDSs (5 F, 4 B, and 3 M) was analyzed by microarray. All 12 SCDSs were allowed to undergo unsupervised hierarchical clustering based on total gene expression (Fig. 2A). Based on the heat map, the SCDSs were highly similar to one another, but there also was a fair amount of variability. In addition, the SCDSs did not strictly segregate into the three distinct functional groups (F, B and M). The 3 M-SCDSs clustered tightly together, but F-SCDSs clustered into 2 different groups.

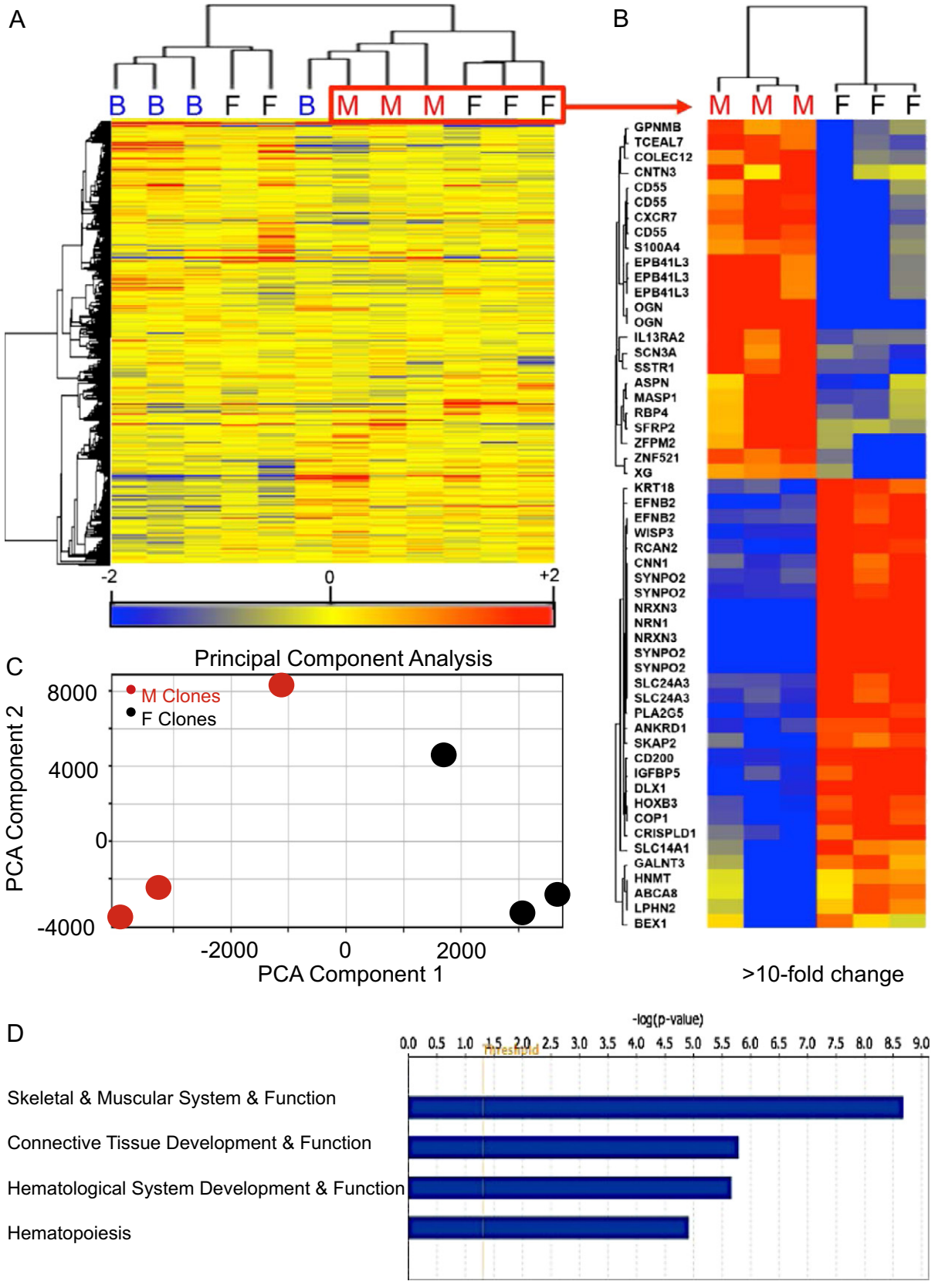


Figure 2 Microarray analysis of 12 SCDSs with known functionality based on the results of in vivo transplantation (5 F, 4 B, and 3 M). A) Unsupervised hierarchical clustering and the heat map generated by microarray. B) Unsupervised hierarchical clustering and heat map of 3 M-SCDSs (M-61, M-11, M-2), and the three F-SCDSs (F-99, F-017, F-109) that were used for further analysis of the patterns of gene expression. C) Principle component analysis of the 3 M-SCDSs (red circles) and the 3 F-SCDSs (black circles). D) GO groups highly represented in M-SCDSs compared to F-SCDSs.

Table 1 Genes differentially expressed by M-SCDSs relative to F-SCDSs.

Gene name	Symbol	Fold change	Direction
A. Genes involved in skeletogenesis			
Mesenchyme homeobox 2	*MEOX2*	4.77	Up
GNAS complex locus	*GNAS*	2.17	Up
Eyes absent homolog 1	*EYA1*	2.74	Up
Paired related homeobox 1	*PRRX1*	2.03	Up
Calponin 1, basic, smooth muscle	*CNN1*	11.27	Down
B. Genes involved in osteoblast differentiation			
Bone morphogenetic protein 2	*BMP2*	2.65	Up
Bone morphogenetic protein 4	*BMP4*	2.34	Up
Secreted frizzled-related protein 2	*SFRP2*	33.14	Up
Msh homeobox 2	*MSX2*	2.41	Up
Insulin-like growth factor 1	*IGF1*	6.30	Up
Meningioma 1	*MN1*	2.90	Up
Wnt1-inducible-signaling pathway protein 1	*WISP1*	2.83	Up
C. Genes involved in hematopoiesis			
Bone morphogenetic protein 4	*BMP4*	2.34	Up
Intercellular adhesion molecule 1 (CD54)	*ICAM1*	2.01	Up
Interleukin 8	*IL8*	2.86	Up
Chemokine (C-X-C motif) ligand 1	*CXCL1*	4.27	Up
D. Small leucine rich repeat proteoglycans			
Decorin	*DCN*	2.33	Up
Osteoglycin	*OGN*	35.30	Up
Osteomodulin	*OMD*	9.93	Up
Asporin	*ASPN*	26.43	Up

2 F-SCDSs clustered with 3 B-SCDS, while 3 others clustered with each other, and along with M-SCDSs. One B-SCDS clustered separately with the M-SCDSs.

Because of the heterogeneity noted, the 3 F-SCDSs that clustered together and the 3 M-SCDSs were chosen for further analysis, as it would allow us to determine the differences in gene expression between two extremes of the differentiation spectrum (multipotent vs. fibrous). Heat maps showing all genes (Fig. 2A), and only the genes that were at least 10-fold differentially expressed between the M-SCDSs and F-SCDSs (Fig. 2B), revealed that there were considerable differences between SCDSs with the same in vivo differentiation potential (see also Supplementary Table 1 for genes over-and under-represented at >10-fold and >5-fold, GEO accession number: GSE647890). This is further reflected in principle component analysis of these SCDSs. While the M and F groups separate, those within each group are not tightly clustered (Fig. 2C).

Ingenuity pathway analysis (IPA) of higher-level functions

IPA was used to determine which biological and molecular functions were significantly associated with the genes that were different between the M-SCDSs and F-SCDSs by at least two-fold (Fig. 2D). Among the functional categories identified were those associated with Skeletal and Muscular System Development and Function, Connective Tissue Development and Function, (both pointing to the inherent osteogenic nature of M-SCDSs), Hematological System Development and Function, and Hematopoiesis, again highlighting the participation of M-SCDSs in the HSC niche.

Differential gene expression

Examination of genes in the IPA categories that were significantly over-represented in M-SCDSs by >2-fold revealed genes involved in skeletogenesis (Table 1A — *MEOX2*, *GNAS*, *EYA1*, *PRRX1*) and osteoblast differentiation (Table 1B — *BMP2*, *BMP4*, *SFRP2*, *MSX2*, *IGF1*, *MN1*, *WISP1*). Interestingly, *WISP3*, a close relative of *WISP1* was highly under-represented (Fig. 2B). Hematopoiesis-related genes were also over-expressed (Table 1C — *BMP2*, *ICAM1*, *IL8*, *CXCL1*), including CXCR7 (Fig. 2B), which binds to CXCL11 and CXCL12, although its role in hematopoiesis is not yet known. In addition, 4 members of the small leucine-rich repeat proteoglycan family (SLRP) were over-represented (Table 1D — *DCN*, *OGN*, *OMD*, *ASPN*). Based on our analyses, *SFRP2* was the most significantly over-represented gene, while *CNN1* was the most significantly under-represented (Table 1A). More information concerning the role of these genes in skeletogenesis, osteogenesis, hematopoiesis and extracellular matrix function can be found in the Supplementary Information.

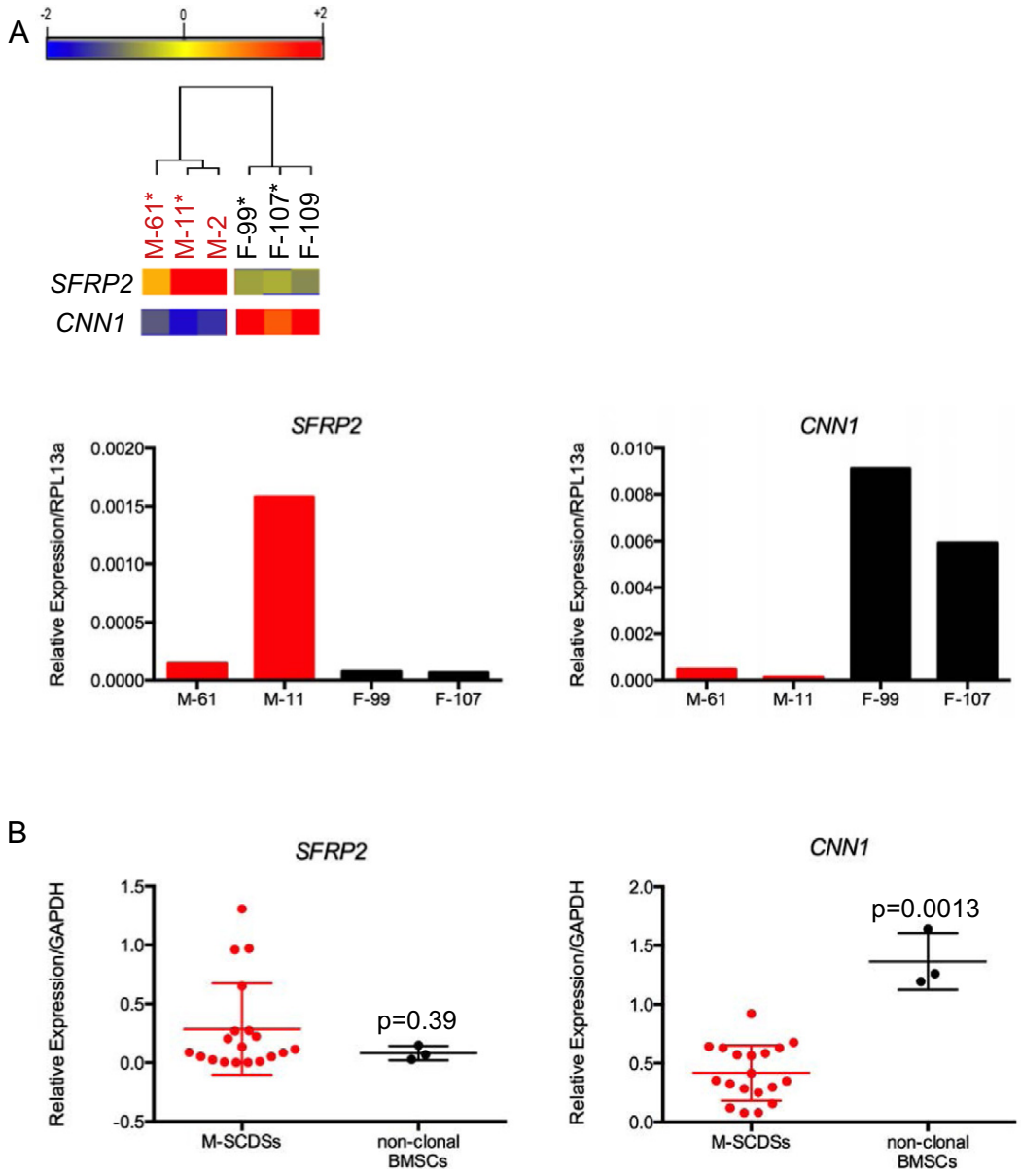


Figure 3 Evaluation of *SFRP2* and *CNN1* expression in M-SCDSs, F-SCDSs and non-clonal BMSCs by qRT-PCR. A) RNA was extracted from cells remaining after in vivo transplantation and microarray analysis (M-SCDS-61, M-SCDS-11, F-SCDS-99 and F-SCDS-107 indicated by asterisks); no more cells were available for any of the other SCDS strains shown in Fig. 2A). *SFRP2* (highly over-represented, Fig. 2B), was variably expressed in the two remaining M-SCDSs, coinciding with what was found in the heat map (Fig. 2B). On the other hand, expression of *CNN1* (highly under-represented, Fig. 2B), was under-expressed in both M-SCDSs and highly expressed in both F-SCDSs, consistent with what was found in the heat map (Fig. 2B). B) The ability of *SFRP2* and *CNN1* to distinguish between M-SCDSs from another donor compared to non-clonal BMSCs was examined. As with the original M-SCDSs (Fig. 3A), *SFRP2* was highly variable in the series of M-SCDSs from another donor, although several M-SCDSs expressed high levels. *CNN1* was clearly under-expressed in M-SCDSs compared with non-clonal BMSCs.

Determination of *SFRP2* and *CNN1* expression by qRT-PCR

RNA was extracted from all cells that were remaining (M-SCDS-61, M-SCDS-11, F-SCDS-99, F-SCDS-107, Fig. 3A), and analyzed for expression of *SFRP2* and *CNN1*, which were highly over-represented and under-represented in M-SCDSs, respectively. As shown in Fig. 3A, the expression of *SFRP2* was variable between the two M-SCDSs, with one having much higher expression than the other, reflective of what was observed on the heat map. On the other hand, *CNN1* was more consist, with a greatly reduced level of expression in M-SCDSs vs. F-SCDSs.

We next sought to determine whether the markers identified in the current series of SCDSs would be detected in the same pattern in SCDSs from a different donor. A number of M-SCDSs from another donor had been previously

cryopreserved; however, no F-SCDSs grown under the same conditions were available. For that reason, we compared the levels of *SFRP2* and *CNN1* expression in the M-SCDSs from the second donor to three different non-clonal populations of BMSCs, which are a mixture of cells at various stages of commitment. While there was a trend for higher expression of *SFRP2* in M-SCDSs (4/19 had much higher, and 4/19 had slightly higher expression), the difference compared with non-clonal BMSCs was not statistically different (Fig. 3B). This variability is similar to what was observed in the original series of M-SCDSs (Fig. 3A). Expression of *CNN1* by the M-SCDSs was significantly lower than in non-clonal BMSCs (Fig. 3B).

Discussion

Here we establish a molecular signature for unmodified SCDSs that were initiated by individual multipotent SSCs in comparison with SCDSs that were initiated by cells that were not multipotent, based upon their differentiation capacity as determined by in vivo transplantation. Of note, all of our SCDSs were established by clonogenic cells (CFU-Fs), but only ~1 out of 5 was in fact multipotent, as has been reported previously by us, and others (Friedenstein, 1980; Gronthos et al., 2003; Kuznetsov et al., 1997; Sacchetti et al., 2007). This reinforces the notion that cultures of BMSCs should not be referred to as "stem cell" cultures (as is often the case), but as cultures in which of a subset composed of stem cells exist. Not even all CFU-Fs are stem cells, although their enumeration provides an approximation of the number of stem cells within a freshly isolated single cell suspension of BM (Bianco et al., 2008).

The results show that the molecular profiles of SDSCs were very similar to one another, but no two were alike, supporting the view that BM stromal CFU-Fs are heterogeneous. It has long been noted that upon plating of cells at clonal densities, there are differences in the size and growth habit (monolayer or multilayering) of colonies. Previous studies (e.g., Satomura et al., 1998) showed a positive correlation between rate of proliferation and multipotency of murine SCDS based on in vivo transplantation. In our current series, M-SCDSs appeared to proliferate slightly faster than F-SCDSs based on the number of days it took to reach the final harvest and the total number of cells generated, but this was not statistically significant (data not shown). Furthermore, colonies are composed of cells of different shapes and sizes, ranging from extended fibroblastic cells to large flat cells (Digirolamo et al., 1999; Owen and Friedenstein, 1988; Satomura et al., 1998). However, the morphological nature of the colony was not predictive of the outcome of in vivo transplantation assays (Satomura et al., 1998). When colonies are allowed to spontaneously differentiate upon prolonged culture, varying percentages of osteogenic, adipogenic or non-differentiated colonies arise (Owen and Friedenstein, 1988). This may be indicative of commitment of a particular CFU-F to one of the stromal cell phenotypes, as a reflection of the influences exerted on that CFU-F during embryonic growth, and post-natal development and homeostasis. With passage, the size and shape of the cells become more uniform; however, heterogeneity still persists, based on the fact that not all cells retain the ability to form colonies upon re-plating at clonal density (Friedenstein, 1976). This is most likely due to the kinetics of SSC self-renewal that are not yet well understood in mammalian systems (Neumuller and Knoblich, 2009). Furthermore, the rate of proliferation of cells within an established colony (as would be harvested at 14 d) is not synchronized, with cells in the periphery migrating and proliferating at a faster rate than those that are more central (Friedenstein, 1990). These differences result in cells within the colony being in different phases of the cell cycle, which can impact on gene expression. For example, alkaline phosphatase is shed from the cell during the G2 + M phase, and is slowly regained during G1 and S phases (Fedarko et al., 1990).

Secondly, our study showed that SCDSs did not strictly segregate transcriptionally based on their differentiation potential as determined by in vivo transplantation. The basis for this is not clear, but may relate to a lack of knowledge concerning the stages of maturation of SSCs (pericytes) to more mature phenotypes (osteoblasts, adipocytes, stromal cells). Stages of osteogenic differentiation have been marked by use of mouse reporter lines that suggest that Runx2 is expressed in SSCs/BMSCs, and committed osteoprogenitors (Yoshida et al., 2002), Osterix is expressed in immature osteogenic cells (Maes et al., 2010), the Col1a1 2.3 kb promoter is active in more mature osteoblastic cells (Pavlin et al., 1992), and that Osteocalcin is expressed in very mature osteoblasts and osteocytes (Zhang et al., 2002). However, such staging for SSC/BMSC differentiation is not yet clear. Based on the hierarchical clustering (Fig. 2A), it can be speculated that a cell that initiated a B-SCDS that clustered with M-SCDSs represented a cell that was in transition from being multipotent to a committed osteogenic cell. Likewise, the individual cells that initiated the F-SCDSs that clustered with other B-SCDSs may have recently transitioned to a fibroblastic phenotype from an osteogenic phenotype. The fact that the F-SCDSs clustered into 2 distinct groups (one with B-SCDSs, the other with M-SCDS) suggests that while all of the F-SCDSs could not make bone in vivo, there may be at least two subsets of fibroblastic BMSCs. A plausible explanation for the fact that 3 F-SCDSs clustered with M-SCDSs may relate to the fact that committed osteogenic cells (B-SCDSs) have a quite different repertoire of expressed genes compared to those that do not exert an overt phenotype (M-SCDSs and F-SCDSs). Clearly, further investigation will be needed to establish the hierarchy of SSCs/BMSCs.

Despite the high degree of similarity between SCDSs and the lack of strict segregation by function, we did identify genes that were highly over-represented by comparing 3 M-SCDSs and 3 F-SCDSs (see Supplementary Information for the role of these differentially expressed genes in skeletogenesis, osteogenesis, hematopoiesis and extracellular matrix). While there are numerous reports on the molecular signature of "mesenchymal stem cells," most of these studies have focused on comparing profiles of "MSCs" from different tissues (e.g., Al-Nbaheen et al., 2013), or BMSCs after initiating differentiation (e.g., Delorme et al., 2009). One study (Papadimitropoulos et al., 2014) in which 3D culture conditions appeared to maintain stemness better that 2D conditions reported over-representation of IL8 and DNER, and under-representation of SYNPO2, NTN4 and LIMCH1 which were over- and under-represented by

>2-fold respectively (data not shown) in our M-SCDSs (with the exception of SYNPO2, which was >10-fold down regulated, see Fig. 2). Few studies have evaluated SCDSs for both their in vivo differentiation potential and their molecular signature. Larsen et al. (2010) reported that their immortalized bone-forming clone highly expressed extracellular matrix genes, including DCN, IL-8 and IFI27, all of which were expressed >2-fold higher in our M-SCDSs (Supplementary Table 1, and data not shown), but the status of hematopoiesis in transplants generated by this immortalized clone was not reported. In another study, clones isolated from different tissues, including BM, and profiled based on rate of proliferation and differentiation, identified *TWIST1* as highly over-represented (Menicanin et al., 2010), as it was in our M-SCDSs (>2-fold, data not shown).

The strengths of our studies are that we were able to detect differences in the molecular profiles of SCDSs derived from a single donor that were initiated by multipotent SSCs vs. BMSCs that were not. However, it is known that the growth rate, expression of markers and differentiation capacity are variable from donor to donor (Phinney et al., 1999). To address this issue, we used a series of M-SCDSs from another donor, and found that *SFRP2*, while highly expressed in some M-SCDSs, was not as robust in identifying M-SCDSs as decreased expression of *CNN1*. These data highlight the need to analyze a large series of SCDSs from more donors to identify reliable and predictive markers. Perhaps the more significant issue relates to the need to expand the cells ex vivo, which may exert proliferative stress that leads to shortened telomeres, DNA damage and changes in differentiation capacity, to obtain sufficient numbers of cells for establishment of their functionality by the in vivo transplantation assay and concomitant molecular profiling. As noted above, heterogeneity exists within colonies, thereby masking what may be profound differences between SSCs and cells that become more committed as the colony is established, and propagated. Analyzing freshly isolated single cells without ex vivo expansion, as recently performed on hematopoietic cells (Guo et al., 2013), would undoubtedly eliminate this issue. Nonetheless, our results begin to better describe the heterogeneous nature of SSCs/BMSCs that has been postulated, but not clearly defined at the molecular level. Future studies will attempt to adapt a single cell strategy, although it will not be possible to study what a given cell would do upon in vivo transplantation. The genes identified in our current study may help guide identification of a single cell profile representative of an SSC in this type of approach.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.02.005.

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