Bone Tissue Engineering with Perivascular Stem Cells

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular & Integrative Physiology

by

Aaron Watkins James

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Molecular, Cellular & Integrative Physiology
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Historically, mesenchymal stem cells (MSC) have been isolated in laboratory culture after several weeks, being selected from heterogeneous cell suspensions based on their ability to adhere and proliferate. Since 2001, human subcutaneous adipose tissue has been recognized as a rich source of tissue resident MSC. The isolation of perivascular progenitor cells from human adipose tissue by a cell sorting strategy was first published in 2008. Since this time, the interest in using pericytes and related perivascular stem cell (PSC) populations for tissue engineering has significantly increased. Herein, we describe a set of experiments in mouse and rat orthopaedic injury models, which examine the utility and advantages of PSC for bone tissue regeneration. In each model, PSC are compared to an unpurified stromal population taken from the same patient’s sample (termed SVF, or stromal vascular fraction). Next, we examine the effects of several growth and differentiation factors (GDFs) on PSC mediated bone repair, including BMP2 (Bone morphogenetic protein 2), NELL-1, and Wnt signaling ligands. Results showed that adipose-derived PSC are readily obtainable from human lipoaspirate without the need for culture, are able to form bone in vivo without the need for pre-differentiation, and induce superior vascularized bone formation in comparison to unpurified stromal cell populations taken from the same patient
sample. PSC have striking functional overlap with other MSC types, including their prominent paracrine function as a rich source of GDFs, but also their ability to directly participate in tissue regeneration. Unlike other MSC types, PSC frequency, viability, and osteogenic differentiation appear unphased in the context of osteoporotic conditions. These observed similarities and differences beg compelling questions regarding the phenotypic overlap between PSC and other MSC types. Finally, several candidate molecules enhance the osteogenic differentiation of PSC. Among these, the novel Wnt signaling regulator NELL-1 stimulates PSC to induce greater vascularized bone than either treatment alone. In aggregate, PSC represent an abundant and uncultured MSC source with significant translational potential for bone tissue repair.
The dissertation of Aaron Watkins James is approved.

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DEDICATION PAGE

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Ch. 1. Identification and isolation of perivascular stem cells from human adipose tissue

1.1 Pericytes as a mesenchymal stem cell (MSC) population

Rouget first described the mural cells of capillaries closely associated with the endothelium. In 1923, Zimmermann renamed these cells as pericytes and described their contractile function around microvessels. Prior to 2008, scattered observations had described the possible multipotentiality of pericytes, including an ability to undergo osteogenic, chondrogenic, fibrogenic, and adipogenic differentiation and with high expression of growth and differentiation factors. Covas et al. had identified MSC-like cells within human veins, but did not show in situ localization. A perivascular niche for MSC-like cells had also been hypothesized by Sarugaser et al., primarily based on in vitro differentiation studies down a single lineage and ability for self-renewal. However, it was not until Crisan et al. utilized a combination of immunohistochemical and flow cytometry analysis that the mesenchymal stem cell (MSC) identity of pericytes was fully appreciated. Using a combination of immunohistochemical markers the localization of pericytes was performed, including NG2, CD146, PDGFRB (Platelet derived growth factor receptor beta), and αSMA (Smooth muscle actin). As expected and across diverse human organs, pericytes were ubiquitously identified around arterioles, capillaries and venules. Next, Crisan et al. found that isolated pericytes express MSC markers, including CD73, CD90 and CD105. They further identified the multilineage differentiation potential of FACS purified pericytes, including an ability to differentiate down muscle, bone, fat and cartilage lineages. This study was the first of its kind to identify and confirm the MSC attributes of pericytes. Since this time, multiple independent investigators have confirmed the MSC attributes of pericytic / perivascular cells (see for a review). In addition, several research groups have identified pericytes as native progenitor cells that are involved in endogenous tissue development and repair.
1.2 Adventitial Cells: A Second Perivascular Population

Beyond pericytes, other progenitor cells types have been identified within the walls of blood vessels. Mesoangioblasts, so named as they are able to differentiate into endothelial cells and other mesodermal lineages, had been already demonstrated in the embryonic dorsal aorta\textsuperscript{1, 15}. Moreover, hematopoietic stem cells are generated from a hemogenic endothelium in the embryonic aortic wall\textsuperscript{16}. As well, prior studies have clearly shown that cells within the adventitia of larger vessels have MSC characteristics. Hu \textit{et al.} identified Sca-1 (Stem cell antigen-1) expression and other MSC markers within the adventitia of the mouse aorta\textsuperscript{17}. Under appropriate conditions, these Sca-1+ cells differentiated into smooth muscle cells\textsuperscript{17}. In agreement with these results, Passman \textit{et al.} described a population of Sca1+CD34+ckit-CD140b+ progenitors localized in the \textit{tunica adventitia} which were highly active for Sonic hedgehog (Shh) signaling\textsuperscript{18}. These Sca1+ adventitial progenitors were described to retain the potential to differentiate not only into smooth muscle cells but also into osteoblasts and endothelial-like cells\textsuperscript{18}. Since then, investigators have isolated CD34+ multipotent progenitors from human arteries and veins\textsuperscript{19, 20}. For example, Campagnolo \textit{et al.} had previously characterized a similar CD34+ cell population from the saphenous vein (termed “saphenous vein-derived progenitor cells”)\textsuperscript{19}. Likewise, Pasquinelli \textit{et al.} described a similar CD34+ cell with MSC properties from the thoracic aorta\textsuperscript{20}. After our group started the large scale isolation of adipose tissue-derived pericytes, it became clear that within the blood vessels of adipose tissue there was likewise a non-pericyte fraction with MSC characteristics. This led to the identification of a second (non-pericyte) perivascular cell with MSC characteristics within human white adipose tissue\textsuperscript{21}. These cells (identified by expression of CD34 and negativity for CD146 and CD31) were identified in the tunica adventitia of larger arteries and veins – and were thus named \textit{adventitial cells}. Similar to murine Sca1+ cells, human adventitial cells do not express any of the smooth muscle cell markers αSMA, NG2,
CD146 and PDGFRβ that are expressed by human pericytes\textsuperscript{21}. A schematic summarizing the location, FACS purification, and terminology for perivascular progenitor cells is shown in Fig. 1-1. A brief summary of FACS derivation and \textit{in situ} markers for pericytes and adventitial cells is shown in \textbf{Table 1-1}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Schematic representation of perivascular progenitor cells, their location, terminology used, and FACS derivation. From left to right, small vessel such as capillaries are composed of pericytes (green) which are intimately associated with the underlying endothelium (orange). The vascular lumen appears red. In larger vessels, the intima contains endothelial cells (orange), media is composed primarily of smooth muscle (grey) and adventitia contains a second MSC progenitor cell type, termed adventitial cells (blue). Enzymatic digestion of human tissues followed by FACS based purification allows for removal of non-MSC cell types such as tissue resident and migratory hematopoietic cells (CD45\textsuperscript{+} cells), endothelial cells (CD146\textsuperscript{+}CD34\textsuperscript{+}), and adipose tissue (by mechanical removal). Pericytes (green) and adventitial cells (blue) are further segregated based on their differential expression of CD146 and CD34. When combined, pericytes and adventitial cells are termed perivascular stem cells (or PSC).}
\end{figure}

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Table 1-1: Brief comparison of pericytes and adventitial cells} & \\
\hline
& Pericytes & Adventitial cells & \\
\hline
\textbf{FACS profile} & CD146\textsuperscript{+}CD34\textsuperscript{-}CD45\textsuperscript{-} & CD146\textsuperscript{-}CD34\textsuperscript{+}CD45\textsuperscript{-} & \\
\hline
\textbf{In situ markers} & aSMA, CD146, NG2, PDGFRβ, Nestin, CD44, CD73, CD90, CD105 & CD34, CD44, CD73, CD90, CD105 & \\
\hline
\end{tabular}
\end{table}

1.3 \textit{Human subcutaneous adipose tissue as a source for MSC}

Since the original description of adipose tissue as a source of multilineage stem cells in 2001\textsuperscript{22}, the preclinical study of adipose-derived cells for repair and regeneration of tissues has increased dramatically (reviewed in \textsuperscript{23}). The theoretical advantages of adipose-derived cells over other cell types for biologic bone regeneration strategies are numerous. For example, while autograft bone
remains the gold standard for bone repair, its disadvantages are significant, including donor site morbidity, extended operating time complications, and limited autogenous supply\textsuperscript{24-26}. On the other hand, bone marrow mesenchymal stem cells (BMSC) have long been studied for their application to bone tissue engineering. However, BMSC have distinct disadvantages over adipose-derived cells, including limitations in autogenous supply of bone marrow aspirate as compared to lipoaspirate, and relatively lower cell yield of BMSC in comparison to adipose-derived stem cells (ASC) (estimated at $\sim 10^4$ stem cells vs. $\sim 10^6$ stem cells/40 ml)\textsuperscript{27-29}. Finally, human adipose-derived stem cells (ASC) have been used successfully to heal small animal\textsuperscript{30, 31} and large animal skeletal defects\textsuperscript{32}.

Despite the advantages of adipose tissue-derived cells for bone regeneration, there exist clear hurdles that must be overcome for their successful clinical use. For example, ASC are defined by their adherence to cell culture plates and \textit{ex vivo} expansion. This \textit{ex vivo} expansion is time-consuming, costly, and increases the risk of immunogenicity, infection and genetic instability\textsuperscript{33, 34}. As an alternative to ASC, the cells of non-cultured total stromal vascular fraction (SVF) from adipose tissues have been used. SVF of adipose tissue is a highly heterogeneous cell population, including non-mesenchymal stem cell types, such as inflammatory cells, platelets, hematopoietic stem cells, and endothelial cells, among others\textsuperscript{35}. Variability in cell composition presents clear disadvantages for FDA (Food and Drug Administration) approval of a future stem cell-based therapeutic, potentially including reduced safety, purity, identity, potency and efficacy. Notably these are the criteria upon which the CBER (Center for Biologics Evaluation and Research) evaluates stem cell-based product applications\textsuperscript{36}. For example, given the heterogeneity of SVF, precise product characterization is not feasible leading to lack of SVF product identity. Batch-to-batch variability and non-uniformity in effect (again attributable to variable stem cell content) reduces the efficacy and potency of SVF based therapies, and potentially reduces product safety.

With these regulatory hurdles in mind, our approach was to reduce the inherent heterogeneity
within SVF to obtain a safer and more efficacious stem cell-based therapeutic.

To address this issue of SVF heterogeneity, previous investigators have used FACS to purify fat derived cell populations, with some success. For example, cell sorting of adipose tissue has allowed for enrichment of 'osteoprogenitor,'\textsuperscript{37} or 'chondroprogenitor'\textsuperscript{38} cell types – but not the isolation of multipotent mesenchymal stem cells (MSC). Importantly, these prior approaches utilized a specific cell signaling receptor or co-receptor (e.g. CD105) to partition for an ASC subset population. In contrast, partitioning of a SVF based on cell identity for the purposes of tissue engineering had not yet been performed. Our alternative solution has been to identify the native 'perivascular' phenotype of MSC and prospectively isolate perivascular MSC using multicolor FACS.

1.4 Purification of perivascular stem cells (PSC) from human subcutaneous adipose tissue

Leveraging prior work by Crisan, Corselli and others (see Sections 1.1 and 1.2 above), we first confirmed that adipose tissue is a potential tissue source of both subpopulations of PSC, including pericytes as adventitial cells\textsuperscript{10, 39-42}. With the use of three antibodies recognizing CD45, CD34, and CD146, we were able to successfully partition microvascular pericytes (CD34-CD146+CD45-) surrounding microvessels and capillaries\textsuperscript{10, 43}, and ‘adventitial cells’ (CD34+CD146-CD45-)\textsuperscript{44} associated with larger blood vessels (Fig. 1-2). Representative scatter plots are shown in Figure 1-2A-C. Here, DAPI staining was performed to remove DAPI+ non-viable cells (Fig. 1-2A), followed by exclusion of CD45+ hematopoietic cells (Fig. 1-2B). This was followed by further partitioning based on CD146 and CD34 to derive distinct pericyte (CD146+CD34-CD45-) and adventitial cell (CD146-CD34+CD45-) populations. When combined, these two perivascular progenitor cell populations are termed PSC (perivascular stem cell).
Next, we examined the frequency of PSC within human adipose tissue. With N=70 unique samples of lipoaspirate, we observed the mean prevalence of pericytes and adventitial cells to be 17.1% and 22.5% of the total viable cell yield of SVF (Fig. 1-2D). Collectively, PSC comprise on average 39.6% of total viable SVF. Importantly, those culture derived cells within adipose tissue that are non-PSC do not express typical MSC markers nor undergo mesodermal lineage differentiation\textsuperscript{44}.

Fig 1-2. Human white adipose tissue houses distinct pericyte and adventitial cell populations which together represent perivascular stem cells (PSC). (A) After exclusion of DAPI+ dead cells and (B) CD45+ hematopoietic cells, two perivascular stem cell populations were isolated with differential expression of CD34 and CD146. (C) Distinct CD34+146- adventitial cells, and CD34-CD146+ pericytes were obtained, and combined for later study. (D) The relative number of pericytes and adventitial cells are depicted as a fraction of the total DAPI- SVF cells. Pericytes and adventitial comprise approximately 17.1% and 22.5%, respectively. ‘Perivascular stem cells’ or PSC then represent on average 39.6% of total viable cells. Further methodologic details: Human SVF derived from a collagenous digestion was incubated with a mixture of the following directly conjugated antibodies: anti-CD34-PE (DAKO; 1:100), anti-CD45-APC (Santa Cruz Biotechnologies; 1:100), and anti-CD146-FITC (Abd Serotec; 1:100). All incubations were performed at 4°C for 15 min in the dark. Before sorting, DAPI (Invitrogen; 1:1000) was added for dead cell exclusion, the solution was then passed through a 70 μm cell filter and then run on a FACS Aria cell sorter (BD Biosciences). In this manner, distinct microvessel pericytes (CD34-CD146+CD45-) and adventitial cells (CD34+CD146-CD45-) were isolated, and combined to constitute the PSC (perivascular stem cell) population. Reproduced with permission from James AW, Zara JN, Zhang X, Askarinam A, Goyal R, Chiang M, Yuan W, Chang L, Corselli M, Shen J, Pang S, Stoker D, Ting K, Peault B, Soo C. Perivascular stem cells: a prospectively purified mesenchymal stem cell population for bone tissue engineering. Stem Cells Transl Med. Jun 2012; 1(6):510-9. PMCID: PMC3659717.
Having shown that PSC can be derived from human adipose tissue by FACS in significant numbers, we next set upon a detailed analysis of how variation between lipoaspirate samples influences PSC derivation. Of note, since our original report\textsuperscript{45}, a ‘twin center’ follow-up study has been published with primarily confirmatory observations\textsuperscript{46}. First, mean cell yield, viability and frequency were examined across all samples (Fig. 1-3). In our experience with 60 samples, we observed a somewhat variable total cell yield, as expressed in total human SVF cells per 100 mL tissue lipoaspirate. Most commonly, total SVF cell yield fell within a range between 10 – 70x10\textsuperscript{6} cells per 100 mL (mean: 39.4x10\textsuperscript{6}, median: 34.3x10\textsuperscript{6}) (Fig. 1-3A). Cell viability among SVF, as determined by DAPI- cells by flow cytometry, was above 70% in nearly all cases (mean: 81.2%, median 89.8%) (Fig. 1-3B). Next, the prevalence of CD45+ cells was analyzed, performed so as to exclude hematopoietic cells from PSC isolates. CD45+ hematopoietic cells most commonly represented less than 40% of SVF in most cases (mean: 24.4%, median 24.5%) (Fig. 1-3C). The two known perivascular stem cell populations were next analyzed: pericytes and adventitial cells. Pericytes most frequently represented 30% or less of total SVF (mean: 19.5%, median 16.7%) (Fig. 1-3D). Adventitial cells represented 40% or less of total SVF (mean: 23.8%, median 22.6%) (Fig. 1-3E). When added in combination, the total PSC content most commonly fell between 30-60% of total viable SVF (mean: 43.2%, median 41.7%) (Fig. 1-3F). Conversely, non-PSC cells among the CD45- cell fraction represented approximately 32.3% of total SVF. Of note, PSC isolation from human adipose tissue has been a process that has been continually refined over a period of years. In fact, the most recent cell isolates have demonstrated a significant reduction in batch-to-batch variability and improved cell purity\textsuperscript{46}. 


Fig 1-3. Yield, viability, and frequency of PSC in human white adipose tissue. (A) Total SVF cell yield, as represented by total SVF cells ($10^6$) per 100 mL tissue lipoaspirate. Blood and saline of lipoaspirate fluid is not included in these calculations. Each bar indicates the number of lipoaspirate samples falling within the stated range of values. (B) Percentage cell viability, as determined by % DAPI- cells by flow cytometry. (C) Percentage hematopoietic cells, as determined by % CD45+ cells among viable (DAPI-) cells by flow cytometry. (D) Percentage pericytes, as determined by % CD146+, CD34- cells among viable (DAPI-) cells. (E) Percentage adventitial cells, as determined by % CD146-, CD34+ cells among viable (DAPI-) cells. (F) Percentage PSC, as determined by % pericytes + % adventitial cells. Reproduced with permission from James AW, Zara JN, Corselli M, Askarinam A, Zhou AM, Hourfar A, Nguyen A, Megerdichian S, Asatryan G, Pang S, Stoker D, Zhang X, Wu B, Ting K, Peault B, Soo C. An abundant perivascular source of stem cells for bone tissue engineering. Stem Cells Transl Med. Sep 2012; 1(9):673-84. PMCID: PMC3659737.

1.5 Variation in PSC yield by time in cold storage
In a clinical setting it may be very difficult to coordinate the harvest of liposuction with its immediate processing for cell isolation. Theoretically, this would require the availability and a high degree of coordination between surgical and scientific/technical personnel. In our research experience, samples were processed in a range of timeframes, sometimes greater than 72 hours after liposuction was performed. In such circumstances, refrigerated storage of liposapirate was necessary. To determine the effects of cold-storage on PSC isolation, we stratified the data of cell yield, viability, and identity by hours of storage at 4°C (grouped per 24 hour period, Fig. 1-4). Total SVF cell yield showed a non-significant reduction after 72 hours (Fig. 1-4A). Remarkably, cell viability showed no difference even at 72 hours, as determined by DAPI staining (Fig. 1-4B). Next, the prevalence of CD45+ hematopoietic cells was analyzed. A non-significant reduction in percentage of CD45+ cells was observed across all timepoints greater than 24 hours cold-storage (Fig. 1-4C). The two known PSC populations were then analyzed, pericytes and adventitial cells (Fig. 1-4D, E). Interestingly, pericytes showed a gradual increase in percentage prevalence with increasing time cold storage, ranging from 14.2% for those samples processed immediately to 28.3% for those processed greater than 72 hours post-harvest (Fig. 1-4D). Conversely, adventitial cells showed a non-significant reduction in percentage prevalence with increasing time (Fig. 1-4E). Finally, the percentage prevalence of PSC was unchanged by hours cold-storage (Fig. 1-4F). Of note, our follow-up study examined even longer extended cold storage times, up to 185.5 hours. Interestingly, with prolonged cold storage a significant reduction in CD45+ inflammatory cells is noted along with increasing variation in PSC yield (especially noted after 92.5 hours). These results suggest that cold storage of PSC is feasible when necessary, however changes in cell composition of SVF are identifiable after prolonged storage times.
Fig 1-4. Effects of storage on PSC yield, viability, and frequency. Samples were shipped and stored as unprocessed lipoaspirate at 4°C prior to digestion, staining and FACS isolation. Characteristics of cell yield, viability and frequency were assessed, stratified by hours storage. Ranges of hours storage include 0-24, 25-48, 49-72, or >72 hours, calculated from shipment to processing. Maximum storage time was 168 hours. (A) Total SVF cell yield, as represented by total SVF cells (10^6) per 100 mL tissue lipoaspirate. (B) Percentage cell viability, as determined by % DAPI- cells. (C) Percentage hematopoietic cells, as determined by % CD45+ cells among DAPI- cells. (D) Percentage pericytes, as determined by % CD146+, CD34- cells among DAPI- cells. (E) Percentage adventitial cells, as determined by % CD146-, CD34+ cells among DAPI- cells. (F) Percentage PSC, as determined by % pericytes + % adventitial cells. Reproduced with permission from James AW, Zara JN, Corselli M, Askarinam A, Zhou AM, Hourfar A, Nguyen A, Megerdichian S, Asatryan G, Pang S, Stoker D, Zhang X, Wu B, Ting K, Peault B, Soo C. An abundant perivascular source of stem cells for bone tissue engineering. Stem Cells Transl Med. Sep 2012; 1(9):673-84. PMCID: PMC3659737.

1.6 Variation in PSC yield by patient demographics

The age of an organism has well described influences on MSC frequency and absolute number
within the bone marrow compartment. For example, older age generally reduces BMSC frequency and absolute numbers in mouse\textsuperscript{47} and human studies\textsuperscript{48, 49}, although there exists some debate within the literature on this subject in human BMSC\textsuperscript{50}. We next set out to examine if similar differences exist with patient age among human PSC (Fig. 1-5). For this purpose, variation in cell yield, viability and identity was analyzed by patient age, stratified by decade of life (Fig. 1-5). A non-significant reduction in cell viability was observed with patient age ≥51 (Fig. 1-5B). Percentage prevalence of pericytes showed a non-significant reduction with patient age >31 (Fig. 1-5D). In all respects, however, no significant change in yield, viability or identity was observed with patient age.
Fig 1-5. Effects of age on PSC yield, viability and frequency. Samples were derived from both genders and a wide range of ages (24-69 years). Characteristics of cell yield, viability and frequency were assessed, stratified by age. Parameters of yield, viability and frequency included: (A) Total SVF cell yield, as represented by total SVF cells (10^6) per 100 mL tissue lipoaspirate. (B) Percentage cell viability, as determined by % DAPI- cells. (C) Percentage hematopoietic cells, as determined by % CD45+ cells among DAPI- cells. (D) Percentage pericytes, as determined by % CD146+, CD34- cells among DAPI- cells. (E) Percentage adventitial cells, as determined by % CD146-, CD34+ cells among DAPI- cells. (F) Percentage PSC, as determined by % pericytes + % adventitial cells. Reproduced with permission from James AW, Zara JN, Corselli M, Askarinam A, Zhou AM, Hourfar A, Nguyen A, Megerdichian S, Asatryan G, Pang S, Stoker D, Zhang X, Wu B, Ting K, Peault B, Soo C. An abundant perivascular source of stem cells for bone tissue engineering. Stem Cells Transl Med. Sep 2012; 1(9):673-84. PMCID: PMC3659737.

Next, cell parameters of yield, viability and identity were stratified by patient gender (Fig. 1-6A-F). Mean SVF cell yield was significantly reduced in male as compared to female patients (Fig. 1-6A). No significant difference across genders was observed with cell viability, percentage of CD45+ cells, or percentage of pericytes (Fig. 1-6B-D). Conversely, the percentage prevalence of adventitial cells and PSC were slightly increased among male patients (Fig. 1-6E,F).

Next, the effects of age of menopause were assessed on cell parameters of yield, viability and identity (Fig. 1-6G-L). For the purposes of this stratification, a mean age of menopause (51 years) was used to determine presumed menopausal status among female samples. Cell viability and percentage prevalence of adventitial cells were both non-significantly reduced among post-menopausal women (Fig. 1-6H,K), while percentage prevalence of pericytes was non-significant increased after menopause (Fig. 1-6J). Thus, menopausal status had no statistically significant effects on any cell parameters of yield, viability or identity.
Fig 1-6. Effects of gender on human perivascular stem cell (PSC) yield, viability, and frequency. Samples were derived from both genders and a wide range of ages (24–69 years). Characteristics of cell yield, viability, and frequency were assessed, stratified by either gender (A–F) or menopausal status (G–L). Menopausal status was estimated on the basis of female age less than or greater than 51. Parameters of yield, viability, and frequency were as follows. (A, G): Total SVF cell yield, as represented by total SVF cells ($10^6$) per 100 ml of tissue liposapirate. (B, H): Percentage of cell viability, as determined by percentage of DAPI− cells. (C, I): Percentage of hematopoietic cells, as determined by percentage of CD45+ cells among DAPI− cells. (D, J): Percentage of pericytes, as determined by percentage of CD146+, CD34− cells among DAPI− cells. (E, K): Percentage of adventitial cells, as determined by percentage of CD146−, CD34+ cells among DAPI− cells. (F, L): Percentage of PSCs, as determined by percentage of pericytes + percentage of adventitial cells. Abbreviations: PSC, perivascular stem cell; SVF, stromal vascular fraction. Reproduced with permission from James AW, Zara JN, Corselli M, Askarinam A, Zhou AM, Hourfar A, Nguyen A, Megerdichian S, Asatryan G, Pang S, Stoker D, Zhang X, Wu B, Ting K, Peault B, Soo C. An abundant perivascular source of stem cells for bone tissue engineering. Stem Cells Transl Med. Sep 2012; 1(9):673-84. PMCID: PMC3659737.
Next, patient samples were analyzed by body mass index (BMI) \textbf{(Fig. 1-7)}. Patients were stratified by non-overweight (BMI < 25 kg/m$^2$), overweight (BMI 25-30 kg/m$^2$), and obese (BMI > 30 kg/m$^2$). With increasing BMI, a non-significant trend was observed toward reduced SVF cell yield \textbf{(Fig. 1-7A)}. The percentage prevalence of cells within SVF was observed to vary by BMI \textbf{(Fig. 1-7C-F)}. With increasing BMI, a trend toward increased frequency of hematopoietic cells, and reduced frequency of pericytes and PSC was observed. Thus, changes in BMI seemed to result in some effects on cell yield and identity, although none of the observed trends achieved statistical significance.

Collectively, changes in patient age, gender and BMI led to infrequent and small changes in cell parameters of PSC isolation. In aggregate, these data collected from $n=60$ patients show evidence of reproducibility of PSC isolation.
Fig 1-7. Effects of body mass index (BMI) on PSC yield, viability and frequency. Samples were derived from non-overweight (BMI <25 kg/m²), overweight (BMI 25-30 kg/m²) and obese patients (BMI >30 kg/m²). Characteristics of cell yield, viability and frequency were stratified by patient BMI. (A) Total SVF cell yield. (B) Percentage DAPI- cells. (C) Percentage CD45+ hematopoietic cells. (D) Percentage pericytes, (CD146+, CD34- cells). (E) Percentage adventitial cells, (CD146-, CD34+ cells). (F) Percentage PSC, pericytes + adventitial cells. Reproduced with permission from James AW, Zara JN, Corselli M, Askarinam A, Zhou AM, Hourfar A, Nguyen A, Megerdichian S, Asatrian G, Pang
1.7 Variation in PSC characteristics by osteoporotic status

Osteoporosis is a complex metabolic disease process that results in a significant reduction of BMSC number, proliferation, osteogenic differentiation, and bone-forming activity\(^{47, 51, 52}\). In contrast, the effects of osteoporosis on adipose tissue derived MSC (termed ASC) are somewhat controversial. For example, Veronesi et al. found no significant differences among ASC derived from ovariectomy (OVX)- and sham-treated rats\(^5^3\). In contrast, Pei et al. reported marked decreases in ALP activity and Ocn expression among rat ASC after OVX\(^5^4\). Others groups have observed that age and osteoporotic fracture history have less appreciable effects on human ASC proliferation and osteogenic differentiation when compared to BMSC\(^5^5\). We hypothesized that this variation in the literature may reflect the heterogeneity of culture-derived ASC, and that a purified cell population may demonstrate more consistent resistance to osteoporotic conditions.

To investigate, purified PSC were isolated from human patients with or without a clinical history of osteoporosis (Fig. 1-8). Results demonstrated a remarkable resilience to the detrimental effects of osteoporosis among purified human PSC. PSC derived from osteoporotic donors demonstrated no significant difference in cell frequency, cell viability, or total cell yield (Fig. 1-8A). No significant difference was observed in bone nodule formation \textit{in vitro} between osteoporotic and non-osteoporotic PSC samples (Fig. 1-8B). In line with this observation, minimal significant changes in gene expression were observed among key transcription factors, growth and differentiation factors (GDFs), and osteogenic inhibitors after stratification by osteoporosis status (Fig. 1-8C). Of these genes, only the adipogenic transcription factor \textit{PPARG} showed a significant upregulation among osteoporotic PSC. In agreement with this observation, osteoporotic PSC showed an increase in lipid accumulation under adipogenic conditions (Fig. 1-
Although this was the sole functional difference observed between osteoporotic and non-osteoporotic PSC.

**Fig 1-8. Effects of osteoporotic status on PSC yield and cell characteristics.** Human PSC were isolated from patients with or without a clinical history of osteoporosis. (A) No significant difference in PSC frequency (% of stromal vascular fraction), cell viability, or total PSC yield (10^6 cells / 100 mL liposapirate) was identified between osteoporotic and non-osteoporotic patient samples. (B) Under osteogenic conditions, no significant difference in bone nodule formation was observed (assessed by Alizarin red staining and photometric quantification). N=3 patient samples per group. Osteogenic conditions include DMEM, 15% FBS, 100 µg/mL ascorbic acid and 10 mM β-glycerophosphate. (C) Gene expression among human PSC samples with or without a clinical history of osteoporosis, as assessed by qRT-PCR in growth medium conditions (DMEM, 10% FBS). Among key transcription factors (RUNX2, PPARG) and secreted proteins (see above), no significance differences were observed based on osteoporotic status. The exception was increased transcript abundance of PPARG among osteoporotic PSC. (D) Under adipogenic conditions, osteoporotic PSC showed increased adipogenic differentiation, by Oil red O staining and photometric quantification. Partially reproduced with permission from Lee S, Zhang X, Shen J, James AW, Chung CG, Hardy R, Li C, Girgius C, Zhang Y, Stoker D, Wang H, Wu BM, Peault B, Ting K, Soo C. Brief Report: Human Perivascular Stem Cells and Nel-Like Protein 1 Synergistically Enhance Spinal Fusion in Osteoporotic Rats. Stem Cells 2015 Oct; 33(10): 3158-63.

In summary, results showed that adipose-derived PSC were isolated by FACS in sufficient numbers for clinical use while bypassing the need for cell culture. Moreover, the isolation and in some cases cell characteristics of PSC are remarkably unaffected by variation in patient
demographics, such as age, gender, BMI, and clinical history of osteoporosis. In sum, these characteristics are major justification for the further study of PSC in tissue engineering applications. From here, we examined PSC in vivo transplantation in three preclinical models of bone formation / regeneration, to be discussed in the subsequent three chapters. In vivo models are summarized in Table 1-2.

<table>
<thead>
<tr>
<th>Model</th>
<th>Species/strain</th>
<th>Cells used</th>
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<td>scid mouse</td>
<td>Human PSC</td>
<td>NELL-1, BMP2</td>
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<tr>
<td>Calvarial bone defect</td>
<td>scid mouse</td>
<td>Human PSC</td>
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<td>Lumbar spinal fusion</td>
<td>Athymic rat</td>
<td>Human PSC</td>
<td>NELL-1</td>
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Ch. 2. Intramuscular implantation of PSC

2.1 Transplanted PSC undergo bone formation in vivo

FACS derived pericytes and adventitial cells had previously been known to have the ability to deposit bone nodules in vitro\textsuperscript{10, 21}. Moreover, perivascular cells have long been supposed to be the cell type culpable for vascular ossification\textsuperscript{56, 57}. Nevertheless, the ability of PSC to induce bone formation in vivo once isolated and transplanted had to date not been evaluated. First, we confirmed that in standard osteogenic medium, PSC showed robust alkaline phosphatase staining and bone nodule formation in osteogenic differentiation medium (shown after 5 and 10 days in culture, respectively, Fig. 2-1A,B). Freshly derived PSC were next placed on a collagen sponge without pre-differentiation or growth factor stimulation, and inserted intramuscularly. A collagen sponge was used as a relatively inert carrier to examine the spontaneous formation of bone in vivo. Over the course of six weeks, spontaneous ectopic ossification was observed by radiography (Fig. 2-1C), 3-dimensional computed tomography (CT) (Fig. 2-1D), and bone formation was observed by histologic analysis (Fig. 2-1E,F). In contrast, intramuscular
implantation of absorbable collagen sponge alone is known to have no bone forming effects\(^5\). Collectively, these studies showed that PSC (like their individual ‘pericyte’ and ‘adventitial’ components) undergo osteogenic differentiation \textit{in vitro}, and that transplanted PSC have bone forming potential \textit{in vivo}.

Fig 2-1. Human perivascular stem cells (PSC) are an ‘osteocompetent’ cell population. (A,B) PSC were cultured under osteogenic conditions (10% FBS, 100 µg/ml ascorbic acid, 10 mM \(\beta\)-glycerophosphate). (A) Representative image of alkaline phosphatase staining at 5 d differentiation. (B) Representative image of Alizarin red staining, 10 d differentiation. (C-F) PSC were implanted in the thigh complex of a scid mouse using a collagen sponge carrier (2.5\(\times\)10\(^5\) cells, sponge size 2.0x1.0x0.5cm). (C) High resolution radiography at 2 and 4 weeks post-implantation. (D) 3-Dimensional MicroCT reconstructions at Th90. (E,F) Pentachrome staining of histological sections. Eb: ectopic bone, cb: cortical bone, m: muscle. N=5 implants, from N=1 patient specimens. Reproduced with permission from James AW, Zara JN, Zhang X, Askarinam A, Goyal R, Chiang M, Yuan W, Chang L, Corselli M, Shen J, Pang S, Stoker D, Ting K, Peault B, Soo C. Perivascular stem cells: a prospectively purified mesenchymal stem cell population for bone tissue engineering. \textit{Stem Cells Transl Med}. Jun 2012; 1(6):510-9. PMCID: PMC3659717.

2.2 PSC form greater ectopic bone than unpurified SVF

Having demonstrated that PSC are an ‘osteocompetent’ cell source, we next sought to directly compare bone formation in patient-matched, purified PSC to unsorted SVF (Fig. 2-2). For this head-to-head determination, the same numbers of viable SVF cells or PSC were implanted (2.5\(\times\)10\(^5\)) intramuscularly in the thigh complex of a scid mouse. SVF and PSC samples were
derived from the same patient’s sample of adipose tissue. For these studies, an osteoinductive carrier was used: demineralized bone matrix (DBX Putty®) – which consists of morselized cortical-cancellous demineralized bone chips with sodium hyaluronate (Musculoskeletal Transplant Foundation, Edison, NJ). At four weeks post-implantation, 2- and 3-dimensional CT images showed significantly increased bone formation in PSC in comparison to SVF samples (Fig. 2-2A,B). CT quantification demonstrated a statistically significant increase in Bone Volume (BV) and Bone Mineral Density (BMD) in PSC-treated samples (Fig. 2-2C,D). Histologic analysis was performed by H&E staining (Fig. 2-2E). Histomorphometric quantification of bone area per random high-powered field showed increased bone among PSC-treated samples (Fig. 2-2F). Immunohistochemical staining for bone specific markers Bone sialoprotein (BSP) and Osteocalcin (OCN) was performed (Fig. 2-2G,I). In each instance, photographic quantitation of positive staining intensity showed a significant increase in both bone markers in PSC treated specimens (Fig. 2-2H,J). Importantly, we confirmed that those cells surrounding and interspersed between bone chips were of predominantly human origin (Fig. 2-3), using immunohistochemical staining for human-specific MHC (Major Histocompatibility Complex) Class I molecule expression (Fig. 2-3A) or via fluorescent labeling of SVF cells or PSC prior to implantation (Fig. 2-3B). Thus, equivalent numbers of purified PSC result in increased ectopic bone formation in comparison to patient-matched SVF cells.
Fig 2-2. PSC undergo more robust differentiation in comparison to human SVF. Equal numbers of viable SVF cells or PSC (2.5x10^5) from the same patient samples were implanted intramuscularly in the thigh of a scid mouse. An osteoinductive demineralized bone matrix (DBX®) putty was used as scaffold. Assessments were performed at 4 weeks post-implantation. (A,B) MicroCT images of SVF and PSC-treated samples. Representative 3-dimensional microCT reconstructions at Th90 (left) and corresponding 2-dimensional axial slices (right). (C,D) Analysis of bone volume (BV) and bone mineral density (BMD) among SVF- and PSC-treated samples. Th50-120. (E) Representative H&E staining. (F) Representative histomorphometric quantification of bone area per 100x field. (G) Representative bone sialoprotein (BSP) immunohistochemistry and (H) quantification of relative staining per 400x field. (I) Representative osteocalcin (OCN) immunohistochemistry and (J) quantification of relative staining per 400x field. Histomorphometric quantification calculated from N=6 random microscopical fields. N=12 implants per cell type, N=3 patient specimens. *P<0.05 in comparison to control as assessed by a Student’s t-test. eb: ectopic bone; f: femur. Reproduced with permission from James AW, Zara JN, Zhang X, Askarinam A, Goyal R, Chiang M, Yuan W, Chang L, Corselli M, Shen J, Pang S, Stoker D, Ting K, Peault B, Soo C. Perivascular stem cells: a prospectively purified mesenchymal stem cell population for bone tissue engineering. Stem Cells Transl Med. Jun 2012; 1(6):510-9. PMCID: PMC3659717.

2.3 PSC induce greater vascularization of ectopic bone than unpurified SVF

We next turned our attention to the effects of PSC transplantation on blood vessel formation. For this comparison, an equal number of patient-matched unsorted SVF cells or purified PSC were again implanted intramuscularly in an immunocompromised mouse model. Four weeks after cell transplantation, H&E staining was first used to visualize and quantify the size and number of blood vessels. Representative H&E stained sections of SVF-treated and PSC-treated samples showed a notable difference in the size and number of blood vessels (Fig. 2-4A,B, top). Semiquantitative histomorphometric analysis of H&E-stained images showed a significant increase in both mean blood vessel number (Fig. 2-4C) and blood vessel area (Fig. 2-4D) among PSC-treated samples. To further confirm these differences, immunohistochemistry was performed for VEGF (Vascular endothelial growth factor). Samples treated with PSC showed greater VEGF staining in comparison to those treated with SVF (Fig. 2-4A,B, middle). This was quantified, showing a
significant increase in VEGF expression in the PSC-treated samples in comparison to SVF (Fig. 2-4E). Next, SVF-treated and PSC-treated implants were examined for expression of vWF (von Willebrand Factor) by immunohistochemistry. Representative images showed greater vWF immunostaining in the PSC-treated samples than in the SVF-treated samples (Fig. 2-4A,B, bottom). Finally, CD31 immunofluorescent staining was performed in samples implanted with red fluorescent-tagged MSC (Fig. 2-4F). This allowed for co-visualization of both implanted MSC and CD31+ endothelium. We found that red-labeled PSC frequently were found in a close association with CD31+ endothelial cells within the vasculature. In contrast, the red-labeled SVF cells showed are more sporadic and less close association with blood vessels. These studies collectively confirmed the significantly larger effect that PSC have on vascularization in comparison to unsorted SVF. Interestingly, we also found that transplanted PSC have a qualitative tendency to ‘return’ back to their perivascular locale once implanted. A similar phenomenon of ‘perivascular return’ has been noted in pericyte transplantation studies in the myocardium.69.
PSC-treated implants show increased vascularization as compared to SVF. Equal numbers (2.5×10^5) of either PSC or SVF cells loaded onto DBX putty were implanted intramuscularly in a scid mouse thigh complex. Samples were harvested 4 weeks post implantation. Specimens were stained with H&E and for VEGF and von Willebrand Factor (vWF). Histomorphometric semiquantitative analysis of respective 400× images followed using the Adobe Photoshop magic wand tool. (A) H&E, VEGF, and vWF staining among SVF-treated implants. (B) H&E, VEGF, and vWF staining among PSC-treated implants. (C) Blood vessel number, based on n=10 H&E images per group. (D) Mean blood vessel area among implants. (E) VEGF quantification among implants.
area, based on \( n=10 \) H&E images per group at a tolerance of 30. (E) VEGF quantification, based on \( n=5 \) images per group and tolerance of 30. (F) CD31 immunofluorescent staining, appearing green with red fluorescent-labeled cells. DAPI is used as a nuclear counterstain, appearing blue. White arrow indicates close association of PSC with a blood vessel. \( \ast p<0.05 \). Reproduced with permission from Askarinam A, James AW, Zara JN, Corselli M, Pan A, Chang L, Rackohn T, Stoker D, Zhang X, Ting K, Peault B, Soo C. Human Perivascular Stem Cells Show Enhanced Osteogenesis and Vasculogenesis with NELL-1 Protein. *Tissue Eng Part A.* Jun 2013; 19(11-12): 1386-97. PMCID: PMC3638559.

2.4 Increasing SVF cell numbers does not rescue its bone forming potential

Having demonstrated that equal numbers of PSC form increased bone as compared to SVF, we next asked as to whether this difference could be overcome by simply adding more SVF cells. For this purpose, two concentrations of cells were used in the same intramuscular model: either \( 2.5\times10^4 \) or \( 2.5\times10^5 \) cells per implant. All SVF or PSC samples were patient-matched. If reduced MSC numbers were the only difference between SVF and PSC, then increasing the SVF cell number 10-fold would surely mitigate any observed differences. However, we found this not to be the case (Fig. 2-5). Namely, PSC formed greater bone in comparison to SVF irrespective of cell concentration. This was observed by microCT reconstructions of representative samples (Fig. 2-5A), microCT quantifications of BV and BMD (Fig. 2-5B,C), and representative histological fields (Fig. 2-5D,E). Thus, a simple reduction in stem cell number likely does not explain the difference in bone formation between SVF and purified PSC.
Fig 2-5. Comparison of variation in cell number on PSC or SVF-mediated ectopic bone formation. SVF or PSC were implanted intramuscularly in a scid mouse on a DBX® Putty scaffold, at two different cell dosages (2.5*10^4 and 2.5*10^5 viable cells). Bone formation was assessed at 4 weeks post-implantation. (A) Representative 3-dimensional microCT reconstructions. Th40 used. (B) Relative bone volume (BV), Th40 used for quantifications. (C) Relative bone mineral density (BMD). (D,E) Representative H&E staining of (D) SVF and (E) PSC laden implants at each cell concentration. N=4-10 implants per treatment group, N=2 patient specimens. *P<0.05 in comparison to SVF 2.5*10^4, **P<0.05 in comparison to SVF 2.5*10^5. Reproduced with permission from James AW, Zara JN, Zhang X, Askarinam A, Goyal R, Chiang M, Yuan W, Chang L, Corselli M, Shen J, Pang S, Stoker D, Ting K, Peault B, Soo C. Perivascular stem cells: a prospectively purified mesenchymal stem cell population for bone tissue engineering. Stem Cells Transl Med. Jun 2012; 1(6):510-9. PMCID: PMC3659717.

In aggregate, these studies found for the first time that adipose-derived PSC induce greater amounts of vascularized bone formation than their unpurified stromal counterpart, even when derived from the same patient sample. Moreover, this was the first time that an adipose-derived purified stem cell fraction was used successfully to engineer bone tissue while bypassing a culture-based expansion period. As well, our studies suggested that the significant differences in PSC mediated bone formation could not be attributed solely to an enrichment process for MSC / osteoprogenitor cells.
3.1 Human PSC heal critical sized calvarial defects

Having demonstrated that transplanted PSC induce ectopic bone formation exceeding that of an unpurified stromal population, we next sought to define PSC efficacy in bone healing. A critical-sized (non-healing) calvarial defect model was used (3mm parietal bone, full-thickness defect). For these experiments, scaffold seeded with either PSC or unsorted SVF, from the same patient sample and at identical cell numbers, were implanted into defects ($n=4$ separate patient samples used). A scaffold without cells served as a further control. Osteoinductive scaffolds were composed of hydroxyapatite coated-poly(lactic-co-glycolic acid) (PLGA) as previously described $^{30, 31}$. Advantages of apatite coated PLGA scaffolds include: (1) osteoinductive, osteoconductive properties to support bone healing, (2) previously optimized porosity for tissue ingrowth and neovascularization, and (3) highly reproducible scaffold composition and dimensions.

Results showed that PSC-laden scaffolds led to significant defect re-ossification, while SVF-laden scaffolds did not. This was observed by high-resolution micro computed tomography (microCT) scanning and 3D reconstructions (Fig. 3-1A). Very little ossification was observed among scaffolds without cells (left, Fig. 3-1A). SVF-seeded scaffolds led to scattered ossification (middle, Fig. 3-1A), while PSC-treated scaffolds showed much more robust ossification by 8 weeks postoperative (right, Fig. 3-1A). Serial live microCT scans were performed every two weeks postoperative, and were next quantified as percentage healing of the original defect size, using Adobe Photoshop (Fig. 3-1B). SVF-seeded scaffolds led to minimal healing with no significant difference to scaffolds without cells. In contrast, PSC-seeded scaffolds led to progressive re-ossification of the defect site (up to 61.5 and 69% healing at 4 and 6 weeks, respectively), significantly higher than either SVF-seeded scaffolds or scaffolds without cells.
Notably, defects without any treatment (without a scaffold) showed no defect re-ossification \((n=3\) animals, *data not shown*).

Next, histologic examination of the calvarial defect site was performed after H&E and Masson's trichrome staining (*Fig. 3-1C*). Photographs were taken at the lateral defect edge. Upon treatment of either control scaffolds or SVF-seeded scaffolds, an abrupt cutoff was clear where the surgically created defect was performed (left and middle panels, *Fig. 3-1C*). In these samples, minimal scattered new osteoid formation was observed in serial sections (*data not shown*). In stark contrast, defects treated with PSC-seeded scaffolds showed considerable new-formed bone (right panels, *Fig. 3-1C*). Histomorphometric analysis of the defect site confirmed these qualitative findings, showing an increase in significant increase in Bone Area (B.Ar) with PSC application (*Fig. 3-1D*). In summary, PSC led to significant calvarial ossification while patient-matched SVF cells did not.
Fig 3-1. Calvarial healing by microCT and histology. SVF or PSC were used to treat a 3mm diameter parietal bone defect in a SCID mouse. A custom-made hydroxyapatite coated PLGA scaffold was used as a carrier. Defects were either treated with an empty scaffold, or scaffold seeded with cells (scaffold + SVF, or scaffold + PSC). (A) 3 dimensional reconstructions of control, SVF, or PSC treated calvarial defects shown at 8 weeks postoperative. CT Threshold of 40 was used. (B) Relative defect healing as assessed by 0, 2, 4 and 6 weeks post-operative by serial live microCT scans. Relative defect area was calculated using a top-down view of the calvaria using AMIDE software images, followed by Adobe Photoshop quantification of relative defect size. (C) Representative H&E and Masson’s Trichrome images for the defect site. Images are taken from the lateral defect edge to delineate old from new bone. N=16-18 mice per treatment group split equally between N=4 separate patient samples. Black scale bar: 50 μm. Yellow scale bar: 25 μm. (D) Histomorphometric analysis, including Bone Area (B.Ar) and % B.Ar (B.Ar/T.Ar). Reproduced with permission from James AW, Zara JN, Corselli M, Askarinam A, Zhou AM, Hourfar A, Nguyen A, Megerdichian S, Asatian G, Pang S, Stoker D, Zhang X, Wu B, Ting K, Peault B, Soo C. An abundant perivascular source of stem cells for bone tissue engineering. Stem Cells Transl Med. Sep 2012; 1(9):673-84. PMCID: PMC3659737.
Finally, we next examined protein expression of various key osteogenic molecules by immunohistochemistry within PSC- or SVF-treated calvarial defects (Fig. 3-2). First, bone associated protein expression, including Osteopontin (OPN) and Osteocalcin (OCN), was examined (Fig. 3-2A,B). Consistent with its role as an intermediate marker of osteogenesis\(^6^0\), all three treatment groups showed numerous OPN+ cells. PSC-treated scaffolds were the only treatment group, however, to show OPN+ bone-lining osteoblasts and OPN+ osteocytes within the defect site (far right, Fig. 3-2A). For OCN, consistent with its known role as a late marker of osteogenesis\(^6^1\), a more scattered distribution of positive staining was observed. This was most predominant within bone matrix present in PSC-seeded scaffolds (far right, Fig. 3-2B), and less frequent among scaffold and SVF-seeded scaffold groups (left and middle panels, Fig. 3-2B).

Next, two GDFs of central importance in bone formation were examined: Bone morphogenetic protein-2 (BMP-2) and Vascular endothelial growth factor (VEGF) (Fig. 3-2C,D). Within scaffold only and SVF-seeded scaffold groups, the cellular elements within the defect site were predominantly BMP2 negative, with only sporadic staining for VEGF (left and middle panels, Fig. 3-2C,D). In stark contrast, cellular areas within the PSC-treated scaffold group were intensely BMP2+, VEGF+ (right panels, Fig. 3-2C,D). Finally, we sought to confirm the persistence of human cells within the defect site, either by human MHC Class I, or anti-human proliferating cell nuclear antigen (PCNA) immunohistochemistry (Fig. 3-2E,F). Indeed, we found persistence of human SVF or PSC at 8 weeks post-operative (Fig. 3-2E,F). Interestingly, we observed greater staining for human specific markers in PSC-seeded specimens, suggesting improved engraftment, proliferation, or cell survival of PSC within the calvarial defect site.
Fig 3-2. Calvarial healing by immunohistochemistry. SVF or PSC were used to treat a 3mm diameter parietal bone defect in a scid mouse. A custom-made hydroxyapatite coated PLGA scaffold was used as a carrier. Defects were either treated with an empty scaffold, or scaffold seeded with SVF or PSC. Immunostaining was performed for: (A) Osteopontin (OPN), (B) Osteocalcin (OCN), (C) Bone Morphogenetic Protein-2 (BMP2), (D) Vascular Endothelial Growth Factor (VEGF), (E) MHC Class I, and (F) Proliferating Cell Nuclear Antigen (PCNA). N=16-18 mice per treatment group split equally between N=4 separate patient samples. Black scale bar: 50 μm. Blue scale bar: 25 μm. Reproduced with permission from James AW, Zara JN, Corselli M, Askarinam A, Zhou AM, Hourfar A, Nguyen A, Megerdichian S, Asatryan G, Pang S, Stoker D, Zhang X, Wu B, Ting K, Peault B, Soo C. An abundant perivascular source of stem cells for bone tissue engineering. Stem Cells Transl Med. Sep 2012; 1(9):673-84. PMCID: PMC3659737.

Thus and in summary, despite persistence of engrafted cells, SVF was observed to be ineffective in inducing calvarial defect ossification with minimal evidence of radiographic or histologic differences in comparison to scaffold without cells. In stark contrast, PSC induced significant increases in osteogenic growth factor elaboration (BMP2, VEGF), significant evidence of OPN+ and OCN+ osteoblasts, and ultimately significant calvarial defect re-ossification.
Ch. 4. PSC mediated spinal fusion

Although the previously examined calvarial defect model was of a critical size (non healing without intervention), it is widely regarded as a permissive model for bone healing. This perspective can be attributed to several factors: (1) the outer layer of the meninges is a rich source of osteoprogenitor cells and osteoinductive GDFs, (2) very little mechanical stress is placed on the defect itself, and (3) only a thin expanse of bone is needed for complete defect reossification. In contrast, lumbar spinal fusion is a far more challenging and functionally demanding ‘litmus test’ for the bone forming ability of PSC. Toward this end, the bone forming potential of PSC was examined in a posterolateral lumbar spinal fusion procedure model, performed in 8-week-old athymic rats. Briefly, the transverse processes of lumbar (L)4 and L5 were exposed, decorticated, and PSC-based implant material was placed between the transverse processes bilaterally to induce spinal fusion. The surgical procedure is fully described in prior publications.

In these experiments, a DBX putty was again used as in our previously described intramuscular implantation model. DBX is a moldable putty that allows for easy packing into a defect site of variable anatomic dimensions. Potential known disadvantages of demineralized bone matrix products include variable demineralization resulting in variable osteoinductivity and variation in cytokine content between production lots and across vendors. Importantly, all experiments were performed using a single vendor and on a single lot of DBX, thereby excluding ‘interbatch variability’ as a potential confounding factor in bone formation outcomes.

At 4 weeks post implantation, flexion/extension forces were used to evaluate gross intervertebral motion. Fusion was graded using a 5-point scale, as previously described, with a score of 4
or greater considered fused. PSC treatment resulted in significantly increased spinal fusion rates in comparison with the acellular control. All three dosages of PSC (0.15×10^6, 0.5×10^6, and 1.5×10^6) resulted in fusion rates of 100%, 80%, and 100%, respectively, compared with 20% fusion in the acellular control group (Fig. 4-1). Our unpublished observations found an approximate 20% fusion rate with SVF application. These results suggested that PSC application led to functional significant bone formation and mechanical stability, while SVF application showed no difference in comparison to the control / acellular carrier material.

![Graph](image)

**Fig 4-1. Spine fusion rates with PSC treatment.** Acellular control was compared with three concentrations of PSC-based bone graft substitutes in an athymic rat posterolateral spinal fusion model. Spines were harvested 4 weeks postoperative and analyzed by manually applying flexion and extension forces against the L4/L5 vertebrae. (A) An average score of ≥ 4 was considered fused. (B) Fusion was apparent in 20% (1 of 5) of control-treated samples and 100% (6 of 6), 80% (4 of 5), and 100% (6 of 6) in 0.15 × 10^6, 0.50 × 10^6, and 1.50 × 10^6 PSC-treated animals, respectively. ***, p ≤ .01 compared with control. No significant difference in fusion scores or rates was observed between PSC-treated groups. Reproduced with permission from Chung CG, James AW, Asatrian G, Chang L, Nguyen A, Le K, Bayani G, Lee R, Stoker D, Pang S, Zhang X, Ting K, Peault B, Soo C. Human perivascular stem cell-based bone graft substitute induces rat spinal fusion. *Stem Cells Transl Med.* May 2015; 4(5): 538. PMCID: PMC4414212.

Having demonstrated by manual palpation that PSC significantly enhance mechanical stability, we next performed quantitative high-resolution micro-CT analyses. Three-dimensional reconstructions of the spines displayed large bilateral bone masses at level L4:L5 in PSC-treated groups with bone bridging between adjacent transverse processes suggesting complete bony fusion (Fig. 4-2A). In contrast, the acellular control group demonstrated significantly reduced
bone formation, as well as clear clefts between the two transverse processes, indicating incomplete fusion (Fig. 4-2A).

Next, micro-CT data analysis was performed to quantify the newly formed bone. Region of interest selection was performed so as to exclude all native bone from analyses. PSC-treated groups demonstrated a dose-dependent trend of increased bone mineral density (Fig. 4-2B). In PSC-treated samples, the bone volume density (or bone volume/tissue volume) exhibited a statistically significant increase as compared with the acellular control group (Fig. 4-2C). In addition, PSC-treated groups displayed a trend toward increased trabecular thickness (Fig. 4-2D) and a statistically significant, dose-dependent increase in trabecular bone number (Fig. 4-2E).
**Fig 4-2. Spine fusion radiography with PSC treatment.** Micro-computed tomography (micro-CT) analysis. (A) Reconstructions of high-resolution micro-CT scans, shown in frontal and axial planes. (B–F) Micro-CT quantifications were next performed for bone mineral density (B), fractional bone volume (C), trabecular thickness (D), trabecular number (E), and trabecular spacing (F). ***, p ** .01 compared with control. No significant difference in micro-CT parameters was observed between PSC-treated groups. Abbreviations: BMD, bone mineral density; BV/TV, fractional bone volume; PSC, human perivascular stem cell; Tb. N, trabecular number; Tb. Sp, trabecular spacing; Tb. Th, trabecular thickness. Reproduced with permission from Chung CG, James AW, Asatrian G, Chang L, Nguyen A, Le K, Bayani G, Lee R, Stoker D, Pang S, Zhang X, Ting K, Peault B, Soo C. Human perivascular stem cell-based bone graft substitute induces rat spinal fusion. *Stem Cells Transl Med.* May 2015; 4(5): 538. PMCID: PMC4414212.
To further explore the effects of PSC in promoting bone formation, histological analyses were performed by H&E and Masson’s trichrome staining (Fig. 4-3A,B). The acellular control group showed decellularized cortical/cancellous bone chips (DBX material) in a predominantly hypocellular fibrous background, with minimal new-formed woven bone (Fig. 4-3A,B). In marked contrast, PSC-treated groups showed DBX material connected and contiguous with significant new-formed woven bone. Moreover, with escalating PSC dosing, an increase in hypertrophic chondrocytes was observed indicative of endochondral ossification (Fig. 4-3A, yellow arrows). Increased osteoid was also observed in a slight dose-dependent manner in PSC-treated samples (Fig. 4-3B, green arrows). Further, histomorphometric quantification of bone area/total implant tissue area revealed significantly increased bone in PSC-implanted samples as compared with the acellular control group, with a slight dose-dependent effect seen with escalating PSC dosages (Fig. 4-3C).

To confirm that PSC-treatment induced increased endochondral bone formation, we next performed immunohistochemical staining for OCN. We observed that PSC-treated samples exhibited both significant and dose-dependent increase in OCN staining compared with acellular control (Fig. 4-3D). Additionally, we examined the efficiency of PSC in promoting osteoblast and bone formation through BSP (Bone Sialoprotein)-positive staining. Once again, PSC-treated groups showed significantly increased relative staining in a dose dependent manner (Fig. 4-3E).
MSC populations are known to induce in vivo bone formation by both direct ossification as well as indirect stimulation of neighboring osteoprogenitor cells. To test the extent to which PSC induce bone formation via direct and indirect effects, species specific immunohistochemical stains were next performed (in the above studies all PSC are of human origin and were implanted in to
rats, allowing for detection of species specific antigens). Immunohistochemical staining for human-specific MHC Class I molecules (hMHC) confirmed that with increasing doses of PSC application, increasing human antigen staining was observed (Fig. 4-4A). This was quantified as the number of hMHC-positive bone-lining osteoblasts and bone-resident osteocytes (Fig. 4-4C,D). These results showed that PSC have a direct effect in new bone formation, and that increasing PSC dosages resulted in increasing direct ossification. Next, parallel staining was performed using rat-specific MHC (rMHC) (Fig. 4-4B). Similar to the pattern of hMHC expression, with increasing dosages of PSC an increasing amount of rat-specific MHC staining was observed. When quantified, an increase in both rMHC positive, cuboidal, bone-lining osteoblasts and bone-resident osteocytes was observed with increasing dosages of PSC (Fig. 4-4C,D). Notably, rat-specific osteoblasts outnumbered human-specific osteoblasts by a ratio of 3.9–10:1 (Fig. 4-4C). Likewise, rat-specific osteocytes outnumbered human-specific osteocytes by a ratio of 7.8–30.8:1 (Fig. 4-4D). Interestingly, a significant number of human-derived osteoblast and osteocytes persisted, indicating direct differentiation and incorporation into the bone matrix. Moreover, a significant dose-dependent effect was observed between PSC treatment groups. Thus and in summary, immunohistochemistry for species-specific antigens verified that not only do PSC play a direct role in bone formation, but also PSC plays a role in paracrine support of host osteoprogenitor cell recruitment and/or differentiation.
Fig 4-4. Species specific cell tracking among spine fusion samples treated with PSC. (A, B): Immunohistochemical staining of human- (A) or rat-specific (B) MHC. (C, D): Quantifications for osteoblasts (C) and osteocytes per higher power field (D). Blue scale bar = 100 μm. *, p ≤ .05 compared with rat-specific control values; **, p ≤ .01 compared with rat-specific control values; ##, p ≤ .01 compared with human-specific control values. A significant dose-dependent effect was observed between PSC-treated groups. Abbreviations: hMHC, human-specific major histocompatibility complex; HPF, higher power field; PSC, human perivascular stem cells; MHC, major histocompatibility complex; Ob, osteoblasts; Ot, osteocytes; rMHC, rat-specific major histocompatibility complex. Reproduced with permission from Chung CG, James AW, Asatrian G, Chang L, Nguyen A, Le K, Bayani G, Lee R, Stoker D, Pang S, Zhang X, Ting K, Peault B, Soo C. Human perivascular stem cell-based bone graft substitute induces rat spinal fusion. Stem Cells Transl Med. May 2015; 4(5): 538. PMCID: PMC4414212.

In summary, our data demonstrate that adipose-derived FACS-purified PSC can successfully induce functional bone tissue formation and reliable rat lumbar spinal fusion. This represents the first instance which PSC were shown to induce clinically relevant bone formation able to withstand the application of biomechanical force.

Ch. 5. Combining growth and differentiation factors in PSC for bone repair

Despite accumulating data to suggest the preclinical efficacy of PSC-mediated bone tissue
engineering – multiple lines of evidence suggest that the bone forming advantage of PSC may be squarely attributed to paracrine effects. For example, in our previously discussed rat spinal fusion model, immunohistochemical detection of species-specific antigens shows that osteoblasts and osteocytes within new bone are far more commonly of host rather than donor origin (see again Fig. 4-4C,D). Thus, the majority of new-formed bone is not of PSC derivation, and an underlying paracrine mechanism was hypothesized. Supporting this hypothesis, our original descriptions showed that pericytes and PSC are robust cytokine sources. For example, human pericytes secrete 5-20 times more Fibroblast growth factor-2 (FGF-2), VEGF, and Keratinocyte growth factor than classically derived adipose tissue or cord blood MSC.

In an effort to shed further light on this, we performed whole transcriptome sequencing (RNA Seq) comparing PSC to unpurified SVF from the same patient sample. As expected, a large number of genes encoding for growth and differentiation factors (GDFs) associated with bone formation were upregulated among PSC, including members of the FGF (Fibroblast growth factor), BMP (Bone Morphogenetic protein), VEGF, and PDGF (Platelet-derived growth factor) families, as well as Wnt associated signaling elements (unpublished observations). For example, BMP2 showed an over 320-fold increase in transcript abundance in PSC as compared to patient-matched SVF. Likewise, FGF2 showed a 135-fold increase in transcripts among PSC. Conversely, a number of well described inhibitory factors in bone formation showed a relative reduction among purified PSC. These included, for example, DKK1 (Dickkopf-1) which showed an up to 33-fold reduction in transcript abundance. These data bolster the hypothesis that PSC, like other MSC types, are efficient producers and elaborators of GDFs for bone repair. Moreover, established factors that can act as ‘brakes’ on bone formation, such as DKK1, appear to be reduced in expression among purified PSC. However, the extent to these specific differences in transcriptional profiles underlie the bone-forming advantage of PSC was entirely unknown. We reasoned that supplementation of GDFs enriched among PSC may either speed PSC mediated bone formation, or ‘rescue’ the
poor bone forming efficacy of SVF.

5.1. Bone morphogenetic protein2 (BMP2) promotes PSC osteogenic and adipogenic differentiation

BMP2, one of the GDFs highly expressed among PSC, is the most commonly used protein for local bone regeneration (INFUSE® Bone Graft)\textsuperscript{71}. We next sought to determine: (1) if BMP2 would be an appropriate stimulus for increased PSC mediated bone formation, and (2) if BMP2 could 'rescue' the poor-bone healing phenotype of SVF. Toward this end, BMP2 was combined with either cellular constituent (SVF or PSC) and placed in our previously described scid mouse intramuscular implantation model. After 4 weeks, 2D Axial CT scans showed a clear increase in bone formation among both SVF and PSC treated samples in response to BMP2 (Fig. 5-1A,B). In addition, a striking trend was observed in which a thin cortical shell (cs, indicated by white arrows) was observed in BMP2 treated specimens that extended well beyond the usual ectopic bone margins (Fig. 5-1A,B). Quantification of BV and BMD were next performed with BMP2 treated specimens. First, we again found that BV and BMD were increased in PSC as compared to SVF-treated samples (far left, Fig. 5-1C,D). However, the effects of BMP2 were to some extent unexpected. While BMP2 produced a significant increase in BV (Fig. 5-1C), it had a negative and opposing effect on BMD as compared with SVF or PSC alone (Fig. 5-1D). Histological evaluation was more revealing (Fig. 5-1E,F). In SVF and PSC samples without BMP2, bone chips were closer together (Fig. 5-1E) and were interspersed by cells with fibroblastic morphology (left, Fig. 5-1F). In BMP2-treated SVF or PSC samples, however, bone chips were farther apart, and predominantly interspersed by lipid droplets (Fig. 5-1F). In order to quantify the adipogenic response produced by BMP2, relative lipid droplet number per 100x field was determined. Within both SVF- and PSC-treated samples, BMP2 induced a statistically significant increase in lipid droplet number (Fig. 5-1G). Thus, BMP2 had both pro-osteogenic, but also pro-adipogenic effects within both SVF- and PSC-treated implants and may not be the most appropriate cytokine
stimulus for induction of PSC-mediated high quality bone formation.

Fig 5-1. Effects of BMP2 on SVF or PSC-mediated ectopic bone formation. SVF or PSC implanted intramuscularly in a scid mouse with or without BMP2 on a DBX® Putty scaffold and assessed 4 weeks post-implantation. (A,B) Colorized, representative, 2-dimensional, axial microCT images. A scale bar below indicates colorization at various
thresholds. (C) Relative bone volume (BV). Values are normalized to SVF+DBX only. Th100 used. (D) Relative bone mineral density (BMD). Values are normalized to SVF+DBX only. (E,F) Representative H&E images at low and high magnification. N=8 implants per cell type, N=1 patient specimen. (G) Relative lipid droplet number per 100x field. Means calculated from 10 random microscopic fields. *P<0.05 in comparison to SVF control, **P<0.05 in comparison to PSC control. Cs: cortical shell, eb: ectopic bone, f: femur. Reproduced with permission from James AW, Zara JN, Zhang X, Askarinam A, Goyal R, Chiang M, Yuan W, Chang L, Corselli M, Shen J, Pang S, Stoker D, Ting K, Peault B, Soo C. Perivascular stem cells: a prospectively purified mesenchymal stem cell population for bone tissue engineering. Stem Cells Transl Med. Jun 2012; 1(6):510-9. PMCID: PMC3659717.

5.2 NELL-1 promotes PSC osteogenic differentiation and vascularization

In search of alternative GDFs rather than BMP2, in a candidate fashion we next examined how the novel factor NELL-1 may enhance bone formation among SVF or PSC. NELL-1 is a GDF in preclinical development that has potent pro-osteogenic effects in small and large mammalian models (reviewed in \(^72\)). NELL-1 is a unique secreted protein of 810 amino acids first studied in the context of human craniofacial skeletal development, where NELL-1 was noted to be osteoinductive and its overexpression associated with human craniosynostosis (CS)\(^73\). Since that time, transgenic Nell-1-overexpressing mice have been observed to recapitulate a CS-like phenotype\(^74\). Conversely, Nell-1-deficient mice (as developed by N-ethyl-N-nitrosourea (ENU)-induced mutagenesis) exhibit cranial and vertebral bone defects with undermineralization\(^75\). Mechanistically, NELL-1 binds to the cell surface receptor integrin \(\beta_1\)\(^76\) and regulates activity of the master osteogenic transcription factor, Runx-related transcription factor-2 (Runx2)\(^77\). Unlike BMP2, NELL-1 had been recently described to have anti-adipogenic effects\(^78\).

In order to test the extent to which NELL-1 could effectively be paired with our cellular constituents for improved bone formation, NELL-1 recombinant protein was next added to SVF- or PSC-laden intramuscular implants. At 4 weeks post implantation, PSC-treated implants again showed increased bone formation in comparison with SVF, as shown by CT reconstructions (Fig. 5-2A,B) and quantification (Fig. 5-2C,D). The addition of NELL-1 led to a significant increase in BV and BMD among PSC-treated implants (Fig. 5-2C,D, far right column). Histologic and histomorphometric analysis revealed similar findings between treatment groups (Fig. 5-2E-H).
Bone chips were noted to be closer together among PSC-treated or PSC + NELL-1-treated implants (Fig. 5-2E,F). Histomorphometric calculations of bone area and bone area/tissue area confirmed these observations (Fig. 5-2G,H). A significant increase in both parameters was observed between PSC-treated and SVF-treated implants. Moreover, PSC + NELL-1 led to a significant increase over PSC treatment alone. Unlike BMP2, minimal lipid was observed among NELL-1-treated samples.
Fig 5-2. Effects of NELL-1 on PSC- or SVF-mediated ectopic bone formation. SVF or PSC in a scid mouse muscle pouch with DBX Putty, with or without NELL-1, assessed at 4 weeks post implantation. (A) Representative three-dimensional microcomputed tomography (microCT) reconstructions. Threshold (Th) 90 was used. (B) Colorized representative two-dimensional microCT images. A scale bar below indicates colorization at various thresholds. (C) Relative BV; Th50–120 used. (D) Relative BMD. (E, F) Representative hematoxylin and eosin staining at low (E) and high (F) magnifications. (G, H) Histomorphometric analysis of mean bone area (G) and mean bone area/tissue area (H) as calculated from 30 random high-powered-fields. n = 9 implants per treatment group, n = 1 patient specimen. *, p < .05 in comparison with SVF control; #, p < .05 in comparison with PSC control; **, p < .05 in comparison with SVF with same dose NELL-1. See supplemental online Table 5 for treatment groups. Abbreviations: BMD, bone mineral density; BV, bone volume; PSC, human perivascular stem cell; SVF, human stromal vascular fraction; NELL-1, Nel-like molecule 1. Reproduced with permission from James AW, Zara JN, Zhang X, Askarinam A, Goyal R, Chiang M, Yuan W, Chang L, Corselli M, Shen J, Pang S, Stoker D, Ting K, Peault B, Soo C. Perivascular stem cells: a prospectively purified mesenchymal stem cell population for bone tissue engineering. Stem Cells Transl Med. Jun 2012; 1(6):510-9. PMCID: PMC3659717.

We next sought to confirm our observations of additive bone formation with NELL-1 + PSC, via immunohistochemical analysis of our intramuscular implantation samples (Fig. 5-3). Specific stains included the bone matrix protein, OCN (Fig. 5-3A), and the GDFs important in bone formation BMP2 (Fig. 5-3B) and BMP7 (Fig. 5-3C). OCN staining across all groups was located primarily within DBX chips and the newly formed bone. The PSC-treated groups showed greater staining relative to the NELL-1-treated groups, while the combination PSC and NELL-1-treatment showed the highest staining intensity (Fig. 5-3A). This observation was confirmed by a semi-quantitative assessment of immunohistochemical staining distribution (Fig. 5-3D). Next, BMP2 immunohistochemistry and semi-quantification were performed (Fig. 5-3B, E). Across all samples, BMP2 staining was primarily observed among the cells surrounding bone particles. As with OCN, the combined treatment of PSC and NELL-1 resulted in significantly higher staining of BMP2 than either cells or cytokine alone. Finally, BMP7 immunohistochemistry and quantification were performed (Fig. 5-3C, F). A similar overall distribution of BMP7 was noted in comparison to BMP2. Again, the PSC and NELL-1 combination treatment induced significantly higher expression of BMP7 when compared to either cell or cytokine treatment alone. Thus, immunohistochemical staining for the bone matrix protein OCN and differentiation factors BMP2 and BMP, revealed an additive effect of PSC when combined with NELL-1.
Fig 5-3. Immunohistochemical analysis of NELL-1 + PSC mediated ectopic bone formation. (A–C) Immunohistochemistry was performed on the four previously mentioned experimental groups (DBX, DBX+NELL-1, DBX+PSC, and DBX+PSC+NELL-1). Semiquantitative analysis of the relative stain intensity was determined, using the magic wand tool of Adobe Photoshop on random 400× fields. (A) Representative images of Osteocalcin (OCN) immunohistochemistry and corresponding relative stain quantification (D), based on n=5 images per group and tolerance of 30. (B) Representative images of BMP-2 immunohistochemistry and corresponding relative stain quantification (E), based on n=8 images per group and tolerance of 30. (C) Representative images of bone morphogenetic protein-7 (BMP-7) immunohistochemistry and corresponding relative stain quantification (F), based on n=8 images per group and tolerance of 30. **p<0.01. Reproduced with permission from Askarinam A, James AW, Zara JN, Corselli M, Pan A, Chang L, Rackohm T, Stoker D, Zhang X, Ting K, Peault B, Soo C. Human Perivascular Stem Cells Show Enhanced Osteogenesis and Vasculogenesis with NELL-1 Protein. Tissue Eng Part A. Jun 2013; 19(11-12): 1386-97. PMCID: PMC3638559.
Our prior observations suggested that implanted PSC induce greater vascularization of new bone formation, including greater vessel numbers, size and density (see again Fig. 2-4). From prior studies, NELL-1 protein has been shown to have significant effects on blood vessel formation. Immunohistochemical studies have shown that NELL-1 is expressed in a perivascular location in both mouse and man (unpublished observations). Since then, NELL-1’s importance in blood vessel formation was shown with the observation that Nell-1 deficient embryos show reduced vasculogenesis during mid-gestation. Previous in vitro studies also demonstrated an increase in VEGF expression among human pericytes grown in NELL-1 supplemented osteogenic medium. We next formally examined whether the combination of NELL-1 with PSC resulted in increased bone vascularization in comparison to either treatment alone. Toward this end, we examined intramuscular implantation samples treated with either NELL-1 protein, purified PSC, or both in combination (Fig. 5-4).

Samples were first stained with H&E (Fig. 5-4A). H&E-stained images and corresponding semiquantitative histomorphometric analysis revealed an increase in the blood vessel number (Fig. 5-4B) and size (Fig. 5-4C) in the NELL-1-treated and PSC-treated samples relative to the DBX control. The PSC+NELL-1-treated samples showed the highest blood vessel number and greatest blood vessel size relative to either cell or GDF treatment alone. This finding is reflected in VEGF staining and quantification (Fig. 5-4D,E). Semiquantification of staining images showed a significant increase in VEGF staining in groups treated with both PSC and NELL-1 as compared to the groups with each treatment alone. These studies demonstrated the ability of NELL-1 to enhance PSC-mediated vascularization concomitant with bone formation.
**Fig 5-4. NELL-1 enhances PSC-mediated vascularization in vivo.** H&E staining and VEGF immunohistochemistry were performed on the four previously mentioned experimental groups (DBX, DBX+NELL-1, DBX+PSC, and DBX+PSC+NELL-1). Histomorphometric semiquantitative analysis of respective 200× images followed using the Adobe Photoshop magic wand tool. (A) Representative H&E images. (B) Blood vessel number, based on n=10 H&E images per group. (C) Mean blood vessel area, based on n=10 H&E images per group. (D) VEGF stain quantification, based on n=7 images per group at a tolerance of 30. (E) Representative image of VEGF immunohistochemistry. *p<0.05. Reproduced with permission from Askarinam A, James AW, Zara JN, Corselli M, Pan A, Chang L, Rackohn.
5.3 NELL-1 and PSC synergistically induce spinal fusion in osteoporotic rats

Until this point, all studies examined the effects of PSC mediated bone formation in healthy animals. However, and as we previously mentioned, the cell characteristics of PSC appear to be predominantly unchanged when isolated from osteoporotic patients (see again Fig. 1-8). The development of a bone graft substitute that is efficacious in the osteoporotic patient is a major health need. Most bone graft substitute options (including those using demineralized bone matrix, BMSC or BMP2) are either reduced in efficacy\cite{47, 51, 52} or contraindicated\cite{80} in the osteoporotic patient. Thus, development of a bone graft substitute with efficacy in osteoporotic bone would constitute a major advance in the field.

Osteoporosis in the context of MSC transplantation is complex, and requires consideration of both the donor status from which the cells are derived, and the host bone in which they are delivered. Studies to date have only considered osteoporotic status of either ‘host bone’ or ‘donor MSC’ separately\cite{54, 81-83}, and a relative analysis of ‘host’ versus ‘donor’ osteoporotic status in MSC transplantation has not yet been performed. As mentioned, osteoporosis is well known to reduce BMSC number and bone-forming activity\cite{47, 51, 52, 84}. Unlike BMSC, PSC demonstrate a remarkable resilience to the detrimental effects of osteoporosis, especially when derived from osteoporotic donors (see again Fig. 1-8). Next, we sought to examine how osteoporotic status of the host animal affects PSC mediated bone healing outcomes.

Toward this end, human PSC were applied either alone or in combination with NELL-1 protein in our previously described rat lumbar spinal fusion model (Fig. 5-4). Four weeks prior to spinal instrumentation, all animals had been rendered osteoporotic by ovariectomy (OVX). Post-OVX, the average BMD of the lumber vertebrae should a 10.2% reduction in comparison to sham
operated to control rats. Pilot studies showed that prior cell seeding densities of PSC were insufficient to induce significant bone formation in osteoporotic animals. Likewise, pilot studies with NELL-1 showed that concentrations shown to be efficacious in a non-osteoporotic animals were insufficient in osteoporotic rats. With these results in mind, higher seeding densities of PSC and higher concentrations of NELL-1 were now used in our osteoporotic rat spine fusion model, designated as ‘High P’ and ‘High N,’ respectively. Three dimensional micro-CT images showed that High P+N treatment resulted in significant new bone formation between the transverse processes, resulting in the radiographic impression of solid fusion (Fig. 5-4A). In contrast, the control group demonstrated clear clefts between the two transverse processes with minimal bone formation. Manual assessment of fusion (using the previously described 5 point scale) showed a mean fusion score of 4.7 among high P+N. In contrast, PSC or NELL-1 when applied alone resulted in a mean fusion score of 3.5 (in this scoring metric, a score of less than 4 is considered unfused). Quantitatively, the High P+N group exhibited a significant increase in BV of 82.6±1.97 mm3 compared to any other groups (p<0.01) (Fig. 5-4B). Similar findings were observed with measurements of implant BMD (Fig. 5-4B).
Fig 5-4. NELL-1 combined with PSC enhances spinal fusion among osteoporotic rats. (A) Representative images of microCT scanning of fusion mass with three-dimensional reconstruction from High P+N, High P, High N, and control groups four weeks after implantation. The High P+N group had marked bone formation around the transverse processes of L4 and L5 (arrow). In contrast, the control group demonstrated radiolucent spaces (arrow head). (B) Histomorphometric analyses of the fusion mass showed a significant increase in BV in rats treated with High P+N. The region of interest was defined as starting from the lower border of transverse process of L5 to the upper border of the transverse process of L4. Only graft material and new bone formation between the transverse processes of L4 and L5 were analyzed and quantified. P: human perivascular stem cells, N: Nell-like protein-1, microCT: micro-computed tomography, BV: bone volume, * p<0.05 and ** p<0.01. Bars, ±SD. Reproduced with permission from Lee S, Zhang X, Shen J, James AW, Chung CG, Hardy R, Li C, Girgisus G, Zhang Y, Stoker D, Wang H, Wu BM, Peault B, Ting K, Soo C. Brief Report: Human Perivascular Stem Cells and Nell-Like Protein 1 Synergistically Enhance Spinal Fusion in Osteoporotic Rats. Stem Cells 2015 Oct; 33(10): 3158-63.

Histologic analysis of each treatment group was now performed (Fig. 5-5). All samples showed at least some foci of endochondral ossification / active bone formation. However, in control-treated samples fibrous tissue was most common among bone interstices. In contrast, High N, High P, and High P+N samples showed significantly greater areas of chondroid matrix, endochondral ossification, woven bone formation and implant neovascularization. Additionally,
BSP immunohistochemistry demonstrated increased staining in new bone and cartilaginous tissue in High P and High P+N samples compared to the control samples (Fig. 5-5A).

Next, bone labeling studies were performed in which Calcein and Alizarin were injected and subsequently incorporated into mineralizing matrix. Overall, we observed a wider band of Calcein/Alizarin labeling in High P+N than other groups, suggesting more robust active ossification along the edges of the pre-existing demineralized bone matrix (DBX) scaffold (Fig. 5-5B). Next, images of Calcein/Alizarin labeling were compared with the distribution of human specific antigens (hMHC Class I). Results showed evidence of co-localization of human specific markers with bone labeling markers, confirming the direct involvement of PSC in ossification after transplantation (Fig. 5-5C).
Fig 5-5. Histology of NELL-1 + PSC in osteoporotic rat spinal fusion model. (A) Top panel: HE staining in low magnification images showing a dash line divides the bone mass formed over the transverse processes of vertebral bones (vB). The asterisk indicates the capsule of bone mass over the transverse processes. Middle panel: H&E staining in high magnification of black box area of corresponding top panel image reveals more active and mature bone formation areas (arrows) and blood vessels (V) in High P+N group over either High P or High N. Bottom panel: Immunohistochemical staining demonstrated positive brown staining for the BSP. In control and High N groups, the BSP positive chondrocytes were shown without bone formation. New bone and cartilage tissues were stained BSP positive in High P and High P+N samples. Overall, more positive cells were revealed in High P+N samples. (B) The active ossifications were observed along the edge of DBX particles (dark gray) using Calcein (green) and Alizarin red complexone (red) dynamic labeling in the superimposed images of bright light and fluorescent fields. Samples from High P+N revealed more robust activity of new bone formation in cryosection of undecalcified tissue. (C) The hMHC class I positive PSC were co-localized/embedded in mineralized matrix in cryosection of undecalcified tissue. Higher numbers of PSC positive of MHC class I (blue) were observed in High P+N group compared to High P. When the images were merged, bone formation was specifically correlated with the area of PSC (pink). H&E: hematoxylin and eosin, P: human perivascular stem cells (PSC), N: Nel-like protein-1 (NELL-1), BSP: bone sialoprotein, DBX: demineralized bone matrix, hMHC: human major histocompatibility complex, PSC: blue by Aminomethylcoumarin streptavidin or pink by merging with green and red. Images were acquired at 40× magnification for the top panel of (A) and 200× magnification for middle and bottom panel of (A) and (B, C) respectively, and the relevant scale bars were provided. Reproduced with permission from Lee S, Zhang X, Shen J, James AW, Chung CG, Hardy R, Li C, Girgius C, Zhang Y, Stoker D, Wang H, Wu BM, Peault B, Ting K, Soo C. Brief Report: Human Perivascular Stem Cells and Nel-Like Protein 1 Synergistically Enhance Spinal Fusion in Osteoporotic Rats. Stem Cells 2015 Oct; 33(10): 3158-63.
In aggregate, transplantation studies in osteoporotic animals suggest state that (1) host osteoporotic status to some extent negatively impacts the ability of either PSC or NELL-1 to exert in vivo bone forming effects, and (2) these negative effects induced by OVX can counteracted by increasing the dose of both cell and GDF components. Comparing this aggregate data with our studies among osteoporotic donors (see again Fig. 1-8), it appears that host but not donor osteoporotic status dictates PSC mediated bone healing outcomes.

5.4 Wnt signaling modulation of PSC osteogenic differentiation

Although NELL-1 has been shown to induce osteoprogenitor cell differentiation and subsequent bone formation across models, the intracellular mechanisms whereby this occurs had been previously unknown. At the same time that our studies regarding PSC + NELL-1 combined bone formation were performed, several lines of evidence pointed to the fact that NELL-1 positively regulated canonical Wnt signaling to induce bone formation. For example, we found that Nell-1 overexpression in a Wnt signaling reporter TOPgal mouse induced heightened endosteal Wnt signaling activity. Conversely, Nell-1 haploinsufficient mice showed reduced canonical Wnt signaling activity in the bone marrow and long bone cortical compartments. These observational data led to us to test if NELL-1 exerted a positive regulatory effect on Wnt/β-catenin signaling in MSC (Fig. 5-6).

Here, we confirmed the positive association between NELL-1 and Wnt/β-catenin signaling in MSC (Fig. 5-6). First, the M2-10B4 mouse bone marrow stromal cell (BMSC) line was treated with NELL-1 or WNT3A as a positive control. By three independent methods, NELL-1 induced a significant increase in Wnt/β-catenin (Fig. 5-6A-C). First, immunocytochemistry for active β-catenin showed increased staining (Fig. 5-6A). Western blot analysis for cytoplasmic and nuclear fractions demonstrated increased nuclear β-catenin (Fig. 5-6B,C). Finally, M2-10B4 cells expressing the TOPFLASH reporter system exhibited an increase in TCF/LEF1 activity (Fig. 5-
Next, we determined whether interference with Wnt/\(\beta\)-catenin signaling would impede NELL-1-induced osteogenic differentiation. To answer this, two antagonists of Wnt signaling were used: DKK-1 (Fig. 5-6E) or XAV939 (Fig. 5-6F). Results showed that both Wnt antagonists inhibited NELL-1’s induction of Runx2. The findings of NELL-1’s positive regulation of Wnt/\(\beta\)-catenin signaling was subsequently confirmed in both osteoclasts (Fig. 5-6G-J) and human BMSC cell types\(^{86}\). In summary, NELL-1 protein activates Wnt/\(\beta\)-catenin signaling in several MSC and osteoclastic cell types in vitro. Moreover, NELL-1’s induction of osteogenic differentiation is dependent on intact Wnt/\(\beta\)-catenin signaling.

Fig 5-6. NELL-1 activates Wnt/\(\beta\)-catenin signaling. (A–D) RhNELL-1 increases Wnt signaling in the M2-10B4 BMSC line. (A) Active \(\beta\)-catenin immunocytochemistry in M2-10B4 cells, treated with rhNELL-1, WNT3A or control (PBS). (B,C) Western blot analysis and quantification of cytoplasmic and nuclear \(\beta\)-catenin (\(N=3\) wells per treatment). (D) M2-10B4 cells were transfected with TOPFLASH reporter (\(N=4\) wells per treatment). (E,F) RhNELL-1 requires intact Wnt/\(\beta\)-catenin signaling for induction of OB differentiation (\(N=4\) wells per treatment). (E) M2-10B4 cells were treated with PBS or rhNELL-1 with or without DKK-1 for 3 days. Runx2 expression measured using qRT–PCR. (F) M2-10B4 cells were transduced with Runx2-EGFP reporter lentivirus and treated with PBS or rhNELL-1 with or without XAV939. Runx2 reporter assay was performed after 3 days. (G–I) RhNELL-1 increases Wnt signaling in the RAW264.7 osteoclast cell line (\(N=3\) wells per treatment). (G) Gene expression after 2 days with or without rhNELL-1. (H) Active \(\beta\)-catenin immunocytochemistry in RAW264.7 cells, treated with rhNELL-1, WNT3A or control (PBS). (I,J) Western blot and quantification with or without rhNELL-1. Black scale bars, 100 µm. Data points indicate the means, while error bars represent one s.e.m. In vitro experiments were performed in biological triplicate, unless otherwise described. Parametric
data were analyzed using an appropriate Student's t-test or a one-way ANOVA, followed by a post hoc Tukey's test. Nonparametric data were analyzed with a Mann–Whitney U-test or a Kruskal–Wallis one-way analysis. *P<0.05, **P<0.01.

Having revealed a new function of NELL-1 as a positive regulator of canonical Wnt signaling activity, we returned to our prior RNA-Seq data to examine how Wnt signaling activity compared between unpurified SVF and purified PSC. Interestingly, results showed that several agonists or positive regulators of canonical Wnt signaling were upregulated among PSC (*unpublished observations*). For example, WISP-1 showed a 72-fold upregulation among PSC, while DIXDC1 showed a 69.3-fold upregulation, and WNT16 showed a 21-fold upregulation. In contrast, a number of Wnt signaling antagonists and negative regulators of canonical Wnt signaling were observed to be upregulated among unpurified SVF. For example, CTNNBIP1 showed a 50-fold upregulation among SVF, while DKK1 (Dickkopf-1) showed a 33.3-fold upregulation among SVF.

Having shown differences in baseline Wnt signaling activity between SVF and PSC, we next examined the effects of Wnt signaling modulation in PSC osteogenic differentiation *in vitro* (**Fig. 5-7**). Exogenous treatment with two Wnt ligands were tested: WNT3A and WNT16. WNT3A was used as the most commonly studied canonical Wnt ligand. WNT16 was used as a mixed canonical / non-canonical Wnt ligand*87*, that showed enrichment among PSC by RNA-Seq. Studies have shown that WNT16 exerts relatively weak canonical signaling activity*88*, and in some cases can actually reduce overall canonical signaling activity via competitive binding with other stronger canonical Wnt ligands*88-90*. Results showed that sustained WNT3A treatment increased Wnt signaling activity (**Fig. 5-7A**), but resulted in inhibition of PSC *in vitro* osteogenic differentiation (**Fig. 5-7B,C**). This was observed across both gene markers of osteogenic differentiation (*RUNX2, ALP, OCN, Fig. 5-7B*) and bone nodule formation, as assessed by Alizarin red staining (**Fig. 5-7C**). In contrast, sustained WNT16 protein treatment resulted in a
decrease in canonical Wnt signaling activity (Fig. 5-7D), increase in gene markers of osteogenic differentiation (Fig. 5-7E) and increase in Alizarin red staining (Fig. 5-7F). In contrast to WNT16 mediated inhibition of canonical Wnt signaling, a significant increase in phospho-JNK was observed by western blot, including p46 and p54 isoforms (not shown). From these data several conclusions can be drawn: (1) continuous treatment with a canonical Wnt ligand represses PSC osteogenic differentiation (as has been shown in other human MSC types\textsuperscript{91-93}), whereas (2) continuous treatment with the mixed canonical-non-canonical Wnt ligand WNT16 resulted in a converse increase in PSC osteogenic differentiation, associated with JNK signaling activation. In addition, these data clearly suggest that NELL-1, although a positive regulator of canonical Wnt signaling, most likely exerts alternative bioactive effects beyond the nuclear localization of $\beta$-catenin to support its observed pro-osteogenic effects in PSC.

![Fig 5-7. The effects of canonical and non-canonical Wnt signaling on osteogenesis of PSC. (A,B,C) WNT3A treatment during PSC osteogenic differentiation. (A) mRNA levels of AXIN2, CMYC, and DKK1 assessed by qRT-PCR in osteogenic differentiated PSC treated with WNT3A (50 ng/mL). (B) mRNA levels of RUNX2 (day 6), ALP (day 9), and OCN (day 6) in osteogenic differentiated PSC treated with WNT3A. (C) Bone nodule formation in PSC examined by AR staining after treatment with WNT3A for 12 days. (D,E,F) WNT16 treatment during PSC osteogenic differentiation. (D) mRNA levels of AXIN2, CMYC, and DKK1 assessed by qRT-PCR in osteogenic differentiated PSC treated with WNT16. (E) mRNA levels of RUNX2 (day 6), ALP (day 9), and OCN (day 6) in osteogenic differentiated PSC treated with WNT16. (F) Bone nodule formation in PSC examined by AR staining after treatment with WNT16 for 12 days. Scale bar= 200 µm. Quantification was performed using Adobe Photoshop CC 2015. Mean ± SD are shown. *$p< 0.05$, **$p< 0.01$ versus control. Unpublished data.]
Having demonstrated that PSC show heightened \textit{WNT16} expression and that WNT16 supplementation induces PSC osteogenic differentiation (see again Fig. 5-7), we next inquired as the effects of \textit{WNT16} knockdown on PSC osteogenesis (Fig. 5-8). Prior studies have shown that \textit{WNT16} knockdown inhibits BMP2 induced osteogenesis in human skeletal muscle derived MSC\textsuperscript{94}. Using siRNA targeting \textit{WNT16}, we observed a significant reduction in \textit{WNT16} gene expression in comparison to scramble siRNA (Fig. 5-8A). \textit{WNT16} knockdown was accompanied by a reduction in the expression of markers of osteogenic differentiation, including \textit{RUNX2} (\textit{Runt related transcription factor 2}), \textit{ALP} (\textit{Alkaline Phosphatase}), and \textit{OCN} (Fig. 5-8B). Likewise, \textit{WNT16} knockdown resulted in a significant reduction in biochemical staining associated with osteogenic differentiation, including ALP staining and quantification (Fig. 5-8C), and Alizarin red (AR) assessment of bone nodule deposition (Fig. 5-8D). In aggregate, these data point to alternative GDFs, such as WNT16, in the regulation of PSC osteogenic differentiation.
**Fig 5-8.** WNT16 knockdown inhibits osteogenic differentiation in PSC – (A-B) mRNA levels of WNT16 (A) and (B) osteogenic markers, RUNX2 (Run related transcription factor 2), ALP (Alkaline Phosphatase) and OCN (Osteocalcin) at 3 days after transfection of PSC with WNT16 or scramble siRNA. (C-D) (Left) Osteogenic differentiation in PSC after 12 days of osteogenic differentiation, as examined by alkaline phosphatase staining (ALP) (C) and Alizarin red (AR) staining (D). Scale bar= 200 µm. (Right) Quantification was performed using Adobe Photoshop CC 2015. Mean ± SD of PSC treated with WNT16 siRNA versus scrambled siRNA are shown. *p < 0.05, **p < 0.01. Unpublished data.

**Ch. 6 Discussion**

6.1. *Perivascular cells as a purified stem cell source*

Our studies demonstrate, from cell culture wares to osteoporotic animal models, that a stromal vascular population from adipose tissue can be purified based on cell identity for improved bone regeneration outcomes. Specifically, we have shown that human perivascular stem cells (or PSC) are superior to unsorted SVF in bone healing outcomes across model systems. Moreover, sufficient quantities of PSC can be harvested without the need for time-consuming and costly *in vitro* expansion protocols. Like other MSC types, PSC transcribe and secrete a wealth of GDFs that have osteogenic and vasculogenic properties. Like other MSC types, PSC exert their bone forming effects via direct ossification and indirect paracrine stimulation. Nevertheless, the end result of PSC transplantation across models is more expedient, stronger, and better vascularized bone tissue. Several other studies have attempted to partition adipose-derived SVF for tissue engineering purposes. However, existing studies segregate adipose cell therapies based on differential expression of cell surface signaling receptors, such as TGF-β co-receptors or BMP receptors. This approach is fundamentally distinct from that presented here, and is an attempt to retrospectively curate a mixed cell population rather than prospectively isolate a cell type of interest.

Several appealing but fallacious conclusions can be reached from our studies. The most often conceived are discussed briefly below. First, that *all MSC are perivascular in origin.* Vascular structures are virtually present in every adult organ, and the assumption that tissue resident MSC
are of a fundamental perivascular origin is provocative. However, much literature suggests the presence of MSC-like cells in numerous mesenchymal tissue compartments including periosteum\textsuperscript{97}, perichondrium\textsuperscript{98}, and dental associated tissues\textsuperscript{99}, to name just a few. Presuming a fundamental perivascular identity for MSC is no doubt overly reductive. Second, that PSC are a pure cell population. Based on their FACS derivation, PSC are highly homogenous in comparison to an unsorted stromal cell population. This statement is supported by our unpublished work looking at the transcriptome of PSC, as well as published observation showing minimal expression of endothelial and hematopoietic cell contaminants among PSC isolates\textsuperscript{46}. However, some degree of heterogeneity of course exists even within this FACS derived population. First, from a definitional standpoint, PSC represents a bipartite population of two related populations: pericytes and adventitial cells. Current theories suggest that adventitial cells are a more undifferentiated cell type, and that under appropriate conditions adventitial cells can adopt a pericyte-like phenotype\textsuperscript{21}. As well, other investigators have clearly shown that the pericyte population is able to be further segregated in vivo based on a several markers including SMA\textsuperscript{21}, NG2\textsuperscript{100-103}, and Nestin\textsuperscript{100-103}. Moreover, Sacchetti recently reported that anatomic site of origin seems to partially dictate the lineage differentiation potential of CD146+ pericytes\textsuperscript{104}. However, the further partitioning of pericytes ex vivo for the purposes of regenerative medicine is essentially unexplored territory. Third, that PSC are necessarily perivascular in origin. Immunohistochemical studies have shown overwhelming evidence that MSC reside in a perivascular locale, yet this is simply a snapshot in time. Accumulating evidence from the field of tumor biology suggests that tumor-derived cells can adopt a pericyte immunophenotype when adjacent to endothelium\textsuperscript{105-108}. Likewise, bone marrow derived cells can also adopt a functional perivascular phenotype when placed in appropriate in vitro models\textsuperscript{104}. These studies suggest a more fluid definition of pericytes, and the potential relationship between circulating MSC and tissue resident perivascular MSC should not be dismissed. Fourth and finally, that a single GDF is the principal factor to enhance PSC mediated bone formation. Notwithstanding our desire for a silver bullet, our studies make it
clear that a wide range of GDFs may be considered as osteoinductive molecules for PSC mediated bone formation (discussed in more detail in Section 6.4 below).

6.2 Mechanisms of PSC improved osteogenicity

While PSC showed improved bone formation in comparison to SVF cells across all experiments and all parameters, the underlying mechanism by which PSC produce higher bone formation is only partially elucidated. As mentioned, it is clear that PSC induce bone formation via both direct and paracrine effects. However, it is less clear whether PSC purification is efficacious primarily by the enrichment for desirable MSC-like cells, the removal of differentiation-inhibiting cells/factors, or some combination of the two. Our experiments refute the concept that PSC purification is a simple enrichment process (see again Fig. 2-5). For example, adding SVF cells at a 10:1 ratio in comparison to PSC still led to inferior bone formation. The alternative hypothesis is that within SVF there exists a differentiation inhibiting cell type. In this regard, endothelial cells have been described to negatively regulate the differentiation of MSC, such as ASC or BMSC\textsuperscript{109, 110}. For instance, human endothelial cells inhibit BMSC differentiation into mature osteoblasts by interfering with Osterix expression\textsuperscript{110}. With this evidence in mind, PSC may show enhanced bone formation in part by elimination of the endothelial cell component. From a molecular and cell signaling perspective, candidate brakes on the process of osteogenic differentiation have also been identified among SVF. For example, DKK1 transcripts are highly enriched among SVF, and this may be exerting negative effects. Most likely, the answer is a combination of these explanations, and that PSC purification is successful from a tissue engineering perspective both due to its positive selection and negative selection criterion.

6.3 Variation in perivascular stem cells across demographics

We analyzed the variation in PSC yield with differences in age, gender, BMI, and clinical history of osteoporosis. Remarkably, large variations were not observed with any of these demographics
suggesting that PSC could be isolated with success from virtually any patient. First, no statistically significant differences were observed with age. This was somewhat unexpected, as adipose and other tissue vascularity are reduced with patient age\textsuperscript{111, 112}. However, those undergoing liposuction were only from 24-69 years of age, and so with further extremes of patient age a significant impact in cell characterization may be appreciated. Second, stratification by gender showed only a minor increase in adventitial cells and PSC among male liposuction specimens. Other research groups have found increased osteogenic differentiation among male adipose-derived stromal cells (ASC)\textsuperscript{113, 114}, and it may be likely that this in fact represents an increased prevalence of PSC. Third, major differences were not observed when PSC were segregated by menopausal status or osteoporotic status. This is particularly interesting in that bone marrow MSC have been shown to be exquisitely sensitive to the systemic effects of osteoporotic bone loss. Finally, we observed that increasing BMI showed non-significant trends toward reduced cell yield, reduced prevalence of pericytes and increase prevalence of hematopoietic cells. The trend toward increased prevalence of CD45+ hematopoietic cells is consistent with other reports linking high BMI with inflammation\textsuperscript{115-117}. In addition, a trend toward a reduction in pericyte numbers was observed. This is also in agreement with previous reports showing reduced capillary numbers in adipose tissue of obese patients\textsuperscript{118}. In summary, accumulating data suggest that PSC can be isolated with success from virtually every patient. Particularly appealing is the notion that PSC represent a uniquely ‘osteoporotic resistant’ cell population.

6.4 Choice of osteoinductive GDF

The marriage of an osteocompetent cell source with osteoinductive growth factors is a logical union for skeletal tissue engineering. In the course of our study of PSC mediated bone formation, numerous candidate factors have emerged to augment, supplement or synergize with PSC. BMP2 was first studied, as the most commonly used, FDA approved osteoinductive GDF. Over
recent years, an increasing side effect profile of BMP2 has been observed (see \textsuperscript{119} for a review), including production of cystic bone voids\textsuperscript{120, 121} with inappropriate adipogenesis\textsuperscript{122}. In particular to PSC, we found that BMP2 induced a massive adipogenic response and showed poor bone quality. In contrast, NELL-1 showed additive pro-osteogenic / pro-vasculogenic effects when co-applied with PSC across model systems. NELL-1 appears to invoke a more ‘osteospecific’ response than BMP2, with no apparent induction of adipogenesis. However, our transcriptomics work has shown that numerous alternative signaling pathways are enriched among PSC, including FGF, PDGF, VEGF and Wnt signaling elements. The goal of pairing PSC with a differentiation factor for bone repair can be conceptualized as two fold. The first is to drive the osteogenic differentiation of PSC for direct bone formation. The second and less studied goal would be to shift the paracrine effects of PSC in a method to induce more robust bone formation among host osteoprogenitor cells. Preliminary data suggest that NELL-1 has positive regulatory effects on both aspects of PSC mediated bone formation.

\textit{6.5 Conclusions}

In summary, adipose-derived PSC are readily obtainable from human lipoaspirate without the need for culture, are able to form bone \textit{in vivo} without the need for pre-differentiation, and induce superior vascularized bone formation than unpurified stromal cell populations from the same patient sample. PSC have striking overlap with other MSC types, including their prominent paracrine function as a rich source of GDFs, but also their ability to directly participate in tissue regeneration. Unlike other MSC types, PSC frequency, viability, and osteogenic differentiation appear unphased in the context of osteoporosis. These observed similarities and differences beg compelling questions regarding the phenotypic overlap between PSC and other MSC types. Finally, several candidate molecules enhance the osteogenic differentiation of PSC. Among these, the novel Wnt signaling regulator NELL-1 stimulates PSC to induce greater vascularized bone than either treatment alone. In aggregate, PSC represent an abundant and uncultured
MSC source with significant translational potential for bone tissue repair.

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