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**Adipose-Derived Perivascular Stem Cells Heal Critical Size
Mouse Calvarial Defects**

A thesis submitted in partial satisfaction of the requirements for the
degree Master of Science
in Oral Biology

by

Silva Megerdichian

2013

ABSTRACT OF THE THESIS

Adipose-Derived Perivascular Stem Cells Heal Critical Size Mouse Calvarial Defects

By

Silva Megerdichian

Master of Science in Oral Biology

University of California, Los Angeles, 2013

Professor Xinli Zhang, Co-Chair

Professor Shen Hu, Co-Chair

Adipose tissue has attracted great interest as an alternative stem cell source with the same multipotent properties as mesenchymal stem cells (MSCs). However, the stromal vascular fraction (SVF) of adipose tissue contains a heterogeneous population of cells, which hamper its regenerative ability. We previously purified human adipose-derived

Perivascular Stem Cells (PSCs) from SVF and showed that PSCs were capable of making significantly more bone compared to the SVF when implanted in the mouse muscle.

Objectives: To evaluate the effectiveness of PSCs in the healing of mouse critical-size calvarial defects. **Methods:** Critical size (3mm) defects were created in the parietal bone of 50 adult SCID mice. Defects were either treated with an apatite coated PLGA scaffold alone, scaffold with human SVF or scaffold with human PSCs. Healing was monitored by live MicroCT scans at 0,2,4 and 6 weeks post injury. At 8 weeks postoperative, subjects were sacrificed and high-resolution microCT scanning was performed to observe total bone formation followed by histological analysis. Immunohistochemistry was performed to study expression of key osteogenic markers such as Osteopontin (OPN), Osteocalcin (OCN), Bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF).

Results: PSCs resulted in a significantly more healing of the defect sites (69%) as opposed to 25% healing in the SVF models at 6 weeks. H&E staining revealed that the PSC group showed more and higher quality new bone in comparison with the SVF samples.

Immunohistochemical staining of the defect sites showed that PSCs induced significant increases in osteogenic growth factor elaboration (BMP2, VEGF), significant evidence of OPN+, OCN+ osteogenesis, and ultimately significant calvarial defect re-ossification.

Conclusion: Our results indicate that adipose-derived PSCs are a new cell source for future efforts in skeletal regenerative medicine. Further research to investigate PSCs induced healing in larger animals is needed to further unfold the healing potential of PSCs and understand its mechanism of action.

The thesis of Silva Megerdichian is approved.

Hu, Shen, Committee Co-Chair

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2013

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DEDICATION

I dedicate this work to my husband Allen, for his unconditional love, support, and encouragement and to my daughter Nara, the light and the love of my life.

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INTRODUCTION:

Bone and cartilage loss caused by traumatic injury, congenital defects, periodontal disease, surgical resection, and craniofacial reconstruction that exceed the size at which complete healing occurs naturally are termed “critical” and require adjunctive therapies such as tissue grafts to heal properly.

Generating calvarial defects is an optimal model to test bone regeneration under non-load bearing conditions.¹ For the past 25 years, calvarial defects have been used as an in vivo model to test bone replacement materials and bone regenerative therapies.² A Critical-size defect is originally defined as “the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal”.³ In adults and children over two years of age, large cranial defects do not re-ossify successfully, posing a substantial biomedical burden.⁴

Current surgical strategies for the healing of skeletal tissue

Current surgical methods include application of autogenous (calvarial, rib or hip) graft, allogeneic (cadaveric) bone, and alloplastic materials.⁵ Traditionally, autografts have been used as the leading graft material; however, shortcomings in availability, additional surgical site for the patient and donor site morbidity have limited their clinical use.⁶ Therefore, searching for more efficient methods of

treating bony defects without using autogenous grafts is a continuous challenge for researchers and clinicians. Research in tissue engineering has enabled transferring cells and growth factors in biodegradable scaffolds to the injury site, which supply the needed factors to recreate an appropriate environment for cellular proliferation and differentiation necessary for tissue healing.

Growth factors such as bone morphogenetic proteins (BMPs) including BMP-2, BMP-4, and BMP-7 have demonstrated great osteogenic potential and have been used as a substitute for autografts in spinal fusion and long bone fractures in humans.⁷ In particular, it has been demonstrated that BMP2 is a necessary component of the signaling cascade that governs fracture repair.⁸ However, there are concerns regarding clinical applications of BMP2, since currently its high, superphysiologically required doses, can induce undesirable heterotrophic bone formation.^{7,9} This limitations warrant a clear need for an appropriate alternative to currently available techniques for bone tissue repair.

Use of stem cells in skeletal regenerative therapy

Since the discovery of bone marrow derived stromal cell osteogenesis in the 1960s, tissue engineering with adult multipotent stromal cells has evolved as a promising approach to restore structure and function of bone compromised by injury or disease.¹⁰ In 1961, Friedenstein reported a tightly coordinated bone formation with characteristic alkaline phosphatase activity within autologous transitional epithelium grafted into guinea pig abdominal wall.¹¹ In subsequent years,

Friedenstein explored osteogenic potential of other tissues, and, in 1970, reported *in vitro* osteogenesis of a subpopulation of bone marrow cells.¹² Ever since, the local application of multipotent osteogenic stem cells combined with a scaffold has gained significant popularity in efforts to improve bone defect healing. Mesenchymal stem cells (MSCs) are used as a primary source of self-renewing precursor cells for mesoderm-type tissue regeneration such as osteoblasts, adipocytes and chondrocytes.¹³ Traditionally, MSCs have been isolated mainly from the bone marrow (BMSCs), and have shown that in the presence of an osteoinductive medium, cultured BMSCs will successfully convert to osteoblasts.¹⁴ However, due to the following limitations, bone marrow is not an ideal MSC source for regenerative medicine: First, Harvesting bone marrow is a highly invasive procedure, second, the number of stem cells and their differentiation potential decrease by increasing age.¹⁵ These limitations have prompted researchers to look elsewhere to harvest MSCs more effectively. Thus far, MSCs have been isolated from numerous tissues including periosteum,¹⁶ adipose,¹⁷ synovial membrane,¹⁸ skeletal muscle,¹⁹ lung,²⁰ deciduous teeth,²¹ umbilical cord,²² blood,²³ skin,²⁴ and ear,²⁵ to mention some. Our lab and several others have looked into utilizing adipose tissue derived stem cell populations, as a readily available regenerative stem cell source for bone repair.^{4,26-28}

Adipose tissue as a stem cell source for regeneration of bony defects

Adipose tissue has attracted great interest as an alternative stem cell source to bone

marrow, primary due to the ease of obtaining clinical samples, and the higher number of stem cells yielded.²⁹ As opposed to bone marrow, adipose tissue is available in large quantities and it is more readily harvestable in the form of lipoaspirate, a disposable byproduct of liposuction.¹⁵ It has been shown that MSCs isolated from the adipose tissue are very similar to bone marrow MSCs, and can be grown easily under standard tissue culture conditions.³⁰ Previous studies confirm that MSCs derived from human adipose tissue and bone marrow share the same fibroblastoid morphology, have similar multipotential differentiation capacity and express the same typical set of surface marker proteins.¹⁵ It has been shown that bone marrow aspirate yields 10^4 stem cells per 40 ml, in comparison with adipose-derived stem cells estimated at 10^6 stem cells per 40 ml of human lipoaspirate.^{31,32} Thus far, human adipose-derived stromal cells (hASCs) have been shown to heal critical size skeletal defects in both small and large animals.^{26,33} Previously, hASCs have been first cultured in vitro and expanded. Culturing cells is a time consuming process which contradicts with their efficient clinical application, besides, it can introduce immunogenicity, infection and genetic instability.³⁴ Therefore, we believe that bypassing the culture stage and directly isolating and applying the hASCs to the injury site would make the clinical application of the adipose derived stem cells a more practical and safer procedure. Human ASCs are isolated from the stromal vascular fraction (SVF) of human lipoaspirate and expanded in culture dish. SVF is obtained by digestion of the lipoaspirate using the collagenase enzyme, then the suspension is centrifuged, adipocytes are separated, and the remaining cell pellet is

termed the stromal vascular fraction.³⁵ SVF was originally described as a mitotically active source of adipocyte precursors in 1968.³⁶ These cells morphologically resembled fibroblasts and were demonstrated to differentiate into pre-adipocytes and functional adipose tissue in vitro.³⁷ However, the idea of adipose-derived stem cells became more commonly acknowledged when Zuk et al demonstrated in 2001 that SVF contains large numbers of mesenchymal stem cells like cells that could be induced to differentiate into adipogenic, chondrogenic, myogenic, and osteogenic lineages.³⁰

Previous investigators have examined direct application of the SVF from adipose tissue for regeneration of skeletal defects. However, these experiments using unpurified SVF show poor and unreliable bone formation,³⁸ or lower bone repair efficacy relative to the cultured ASCs.³⁹ As mentioned, SVF is the total cellular yield after collagenase digestion and centrifugation minus adipose cells, but it also contains a heterogeneous population of cells including nonviable cells, hematopoietic cells, endothelial cells, inflammatory cells and etc. This highly heterogeneous nature of SVF is believed to negatively interfere with its ability to generate and repair bone.^{26,40} Therefore, in an attempt to improve the current techniques available for bone tissue repair, the SVF mixture has been purified down to only the cells with MSC property previously in our lab.^{40,41} Moreover, this purification of freshly isolated SVF cells, has been culture free and based on cell surface markers⁴⁰ in order to make the process safer and more clinically applicable.

Adipose- derived Perivascular Stem Cells (PSCs) for bone tissue engineering

Previously it has been shown that cells that are closely associated with small vasculature, named perivascular stem cells (PSCs) have MSC like characteristics.^{41,42} PSCs are found in multiple vascularized human organs as well as the adipose tissue, commonly associated with blood vessels.⁴³ Two distinct populations of human perivascular cells, with multipotent MSC-like qualities have been identified and purified: pericytes, which encircle small capillaries (diameter <10) and microvessels; and adventitial cells, which surround all larger blood vessels. ⁴¹ These PSC populations are distinguishable with their distinct cell surface markers, with Pericytes expressing CD45-, CD146+, CD34-,^{42,43} and adventitial cells expressing CD45-, CD146-, CD34+ (Hematopoietic cells: CD45+).⁴⁴ Due to these cell surface markers, these cells can be isolated and sorted by using multicolor fluorescence-activated cell sorting (FACS).⁴²

Recently, using this technique our lab has purified the SVF isolated from fresh lipoaspirate for multipotent PCSs from 60 patient samples and documented their mean cell yield, frequency and viability. We learned that within 60 samples, the total cell yield, as expressed in total SVF cells per 100 ml of tissue lipoaspirate was variable. However, generally the total SVF cell yield ranged between 10×10^6 and 70×10^6 cells.⁴⁰ Cell viability among SVF, as determined by 4',6-diamidino-2-phenylindole (DAPI) negative cells by flow cytometry, was above 70% in nearly all cases.⁴⁰ Lastly, in calculating prevalence, pericytes most frequently represented

17% of the total SVF and adventitial cells represented 22% of the total SVF.⁵ When the two populations were added to determine the percentage of total PSCs in the SVF, PSCs on average makeup approximately 40% of the total viable SVF.⁴⁰

Previously, it has been shown that both these populations of PSCs are able to undergo osteogenic differentiation.^{41,43} However, bone remodeling capacity of PSCs in direct clinical application without first culturing them has not been examined yet. As a preliminary effort by our lab to apply the freshly harvested and purified PSCs to an experimental model, PSCs were isolated and incorporated in a mouse intramuscular implantation model combined with Demineralized Bone Matrix (DBM) scaffold to compare their bone forming capacity to that of the traditionally derived SVF.⁴⁵ The results indicated that PSCs made significantly more bone in the muscle pouch model and showed greater osteogenic differentiation compared with the SVF.

In this study, we extend the application of PSCs to a mouse calvarial bone injury model. We hypothesize that purified PSCs are a more effective regenerative medium to use in bone repair, and will lead to a significantly greater healing of mouse critical-size calvarial defects compared to SVF.

MATERIAL AND METHODS:

PSCs and Animals subjects:

Adipose-derived SVF and PCs were purified from human lipoaspirate ($n = 60$ donor patient samples) obtained from patients undergoing cosmetic liposuction previously.⁴⁰ Briefly as described previously,^{40,44} in order to obtain the SVF, lipoaspirate was digested with collagenase type II and then adipocytes were separated and removed by centrifugation. The pellet was re-suspended in red-cell lysis buffer and the resulting SVF was either further processed for cell sorting (to isolate PCs) or loaded immediately onto scaffolds for in vivo application. PCs were purified by FACS by incubating the SVF with a mixture of anti-CD34, CD45 and CD146 antibodies. Before sorting, 4',6-diamidino-2-phenylindole (DAPI) was added for dead cell exclusion.⁴⁰ Total of 50, 6 weeks old male SCID mice (Charles River Laboratories, Wilmington, MA, <http://www.criver.com>) were used. Animals were divided into three different treatment groups: First, a scaffold only group serving as control group, in which a PLGA scaffold without cells was placed in the defect site ($n = 18$ mice); second a scaffold + SVF group ($n = 16$ mice split equally from $n = 4$ separate patient lipoaspirate samples) and lastly scaffold + PCs group ($n = 16$ mice split equally from $n = 4$ separate patient lipoaspirate samples).

Scaffold Fabrication:

Hydroxyapatite-coated poly lactic coglycolic acid (PLGA) scaffolds were fabricated

from 85/15 PLGA (inherent viscosity = 0.61 dl/g; Birmingham Polymers, Pelham, AL, <http://www.durect.com>) by solvent casting and a particulate leaching process. PLGA/chloroform solutions were mixed with 200-300 μm diameter sucrose to obtain 92% porosity (volume fraction), and compressed into thin sheets in a teflon mold. After freeze-drying overnight, scaffolds were immersed in three changes of double distilled (dd) H_2O to dissolve the sucrose, and gently removed from the teflon plate with a fine-tip spatula. After particulate leaching, all scaffolds were disinfected by immersion in 50%, 60% and 70% ethanol for 30 min each, followed by three rinses in sterile dd H_2O . All scaffolds were then dried under a laminar flow hood. Scaffolds were then implanted with SVF or PSCs to be prepared for implantation into the calvarial defect areas. Two hundred fifty thousand cells from each individual patient sample were re-suspended in 25 μl of growth medium (DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin) and placed directly onto the scaffold for 30 minutes (for empty scaffold controls, 25 μl of medium without cells was used). The scaffolds were subsequently submerged in 100 μl of growth medium for 8 hours of incubation. Before implantation, cell-seeded scaffolds were rinsed in sterile PBS to prevent transfer of medium derived growth factors or immunogens.

Surgical Procedures:

Critical size (3mm) calvarial defects were created in the right parietal bone of the animals using a high-speed dental drill. A 3mm defect in the cranium of mouse is

considered a non-healing defect that would need adjunctive therapy to re-ossify.⁵ Surgical Procedures were performed under isoflurane sedation. The surgical site was cleaned with alternating scrubs of Betadine and alcohol. An incision was made along the sagittal midline, slightly off to the right to open the scalp and expose the right parietal bone. The pericranium was removed with a sterile cotton swab. Using diamond-coated trephine bur, and under saline irrigation, unilateral full-thickness calvarial defects were created in the non-suture-associated parietal bone. Importantly the underlying dura matter was left undisturbed, since this tissue has been demonstrated to be critical for stem cell-mediated calvarial regeneration.⁴⁶ Finally, the skin was sutured with 6-0 vicryl, and Animals were postoperatively treated with buprenorphine for 48 h and trimethoprim/sulfamethoxazole for 10 days for pain management and infection prevention, respectively. Animals were housed and experiments were performed in accordance with guidelines of the Chancellor's Animal Research Committee of the Office for Protection of Research Subjects at the University of California, Los Angeles.

Radiographic Imaging

Live microcomputed tomography (microCT) was used to longitudinally assess in vivo bone formation in the calvarial defects and monitor healing from 0 to 6 weeks, at two weeks intervals. Using an isolated imaging chamber that provided continuous delivery of 1%–3% isoflurane anesthesia and temperature control at 36°C, all animals under-went a non-contrast enhanced microCT scans at the

appropriate time points. Subjects were then scanned using a microCAT II (Siemens Preclinical Solutions, Malvern, PA, <http://www.medical.siemens.com>) imaging system accomplished in 20 minutes time. MicroCT images were created using Feldkamp reconstruction at a 200 μm resolution. Data were analyzed and quantified by AMIDE (A Medical Image Data Examiner; <http://amide.sourceforge.net>) software. At 8 weeks postoperative, subjects were sacrificed and high-resolution microCT scanning was performed. Samples were fixed with formalin and imaged using high-resolution microCT (Skyscan 1172F; Skyscan, Kontich, Belgium, <http://www.skyscan.be>) at an image resolution of 19.73 μm and analyzed using the DataViewer, Recon, CTAn, and CTVol software provided by the manufacturer.

Histology and Immunohistochemistry

Following microCT analysis, samples were decalcified in 19% EDTA and embedded in paraffin. Hematoxylin/eosin (H&E) staining was performed to confirm osteogenesis in the defect sites and study the quality of bone formation. For immunohistochemistry, samples were deparaffinized and incubated with 3% H₂O₂ for 20 minutes to block endogenous peroxidase activity. Then blocked with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for one hour to block non-specific sites. Primary antibodies against osteopontin (OPN) and osteocalcin (OCN) to detect osteogenesis in the defect areas (Santa Cruz

Biotechnology Inc., Santa Cruz, CA), were used. Samples were also stained against osteogenic growth factor BMP2 (Santa Cruz Biotechnology) and VEGF (Santa Cruz Biotechnology). In order to confirm actual presence of human cells in the defect areas where SVF and PSC were used, major histocompatibility complex I (MHC class I) (Santa Cruz Biotechnology), and human proliferating cell nuclear antigen (PCNA) (Dako, Glostrup, Denmark) were used. Primary antibodies were first added commonly at a dilution of 1/100 to each section and incubated at 37°C for 1 h and at 4°C overnight. Biotinylated secondary antibody (Dako Corporation, Carpinteria, CA) was added to samples the next day, followed by an hour of bench top incubation. Next, (Avidin Biotin Complex) ABC (Vector Laboratories, Burlingame, CA) was applied to the sections, AEC plus substrate in red color (Dako, Carpinteria, CA) was used as a chromagen, and the sections were counterstained with light Hematoxylin. Photomicrographs were acquired using an Olympus BX51 microscope and MicroFire digital microscope camera with Picture Frame software (Optronics, Goleta, CA) connected to a light microscope with an original magnification of 20X and 40x. Data for immunohistochemistry was quantified and presented in the form of graphs expressed as relative positive staining of different treatment groups using Photoshop (Adobe Systems Inc., San Jose, CA, <http://www.adobe.com>).

Statistical analysis

For statistical analysis, appropriate analysis of variance (ANOVA) was used first to compare all these groups together. Next, a post hoc Tukey's test was performed to compare between each two specific groups. (* $P < 0.05$, ** $p < 0.01$)

RESULTS:

Radiographic evaluation

Serial live microCT scans of subjects between 0 to 6 weeks post operative taken at 2 weeks intervals indicated that defects with PSC impregnated scaffolds had robust re-ossification of the defect site, significantly greater than either SVF-treated or scaffolds only defects. The scan results were quantified as relative defect size, calculated as the unfilled defect area over the original defect area, using Photoshop (Adobe Systems Inc., San Jose, CA, <http://www.adobe.com>)

The quantified results show that defects with PSCs had up to 61.5% and 69% healing at 4 and 6 weeks respectively, whereas defects with SVF showed only 20 to 25% healing at 4 and 6 weeks (Fig. 1) At the 8th week post surgery, samples were harvested and high-resolution microCT scanning with 3D reconstructions was performed. The least amount of bone formation was seen in the control defects with no cells. Defects with SVF-treated scaffolds led to minimal scattered bone formation with no significant difference with the control group. In contrast, defects with PSC-treated scaffolds led to significant defect re-ossification over the 8 weeks period (Fig.2)

Histological analyses

Next, histologic analysis of the defect sites was performed by H&E staining. Images were taken so that the lateral defect edge would be visible to be able to differentiate

the old from the new bone. In agreement with the radiographic observation, control and SVF treated samples showed insignificant scattered new osteoid formation. Instead, defects with PSC-loaded scaffolds showed considerable newly formed bone (Fig. 3). Also, the newly formed bone in the PSC samples appeared to have a normal, mature bone structure with osteocytes in lacunae and healthy marginal osteoblasts. In summary, histological and radiographic analysis together confirmed that PSCs led to significant calvarial defect re-ossification, whereas patient-matched SVF cells did not.

Immunohistochemical analyses

Protein expression of various key osteogenic markers was studied by performing immunohistochemistry. First, bone matrix protein expression, including OPN and OCN, was examined (Fig. 4,5). Osteopontin has been implicated as an important factor in bone remodeling, by serving to initiate the process by which osteoclasts develop their ruffled borders to begin bone resorption.⁴⁷ OPN is expressed early in mesenchymal cell differentiation and is related to cell migration as well as osteogenesis.⁴⁸ OPN staining revealed that all treatment groups had numerous OPN+ cells; this is reasonable, considering that OPN is an intermediate marker of osteogenesis.⁴⁸ Moreover, in addition to the osteoblasts, OPN is also expressed by fibroblasts and preosteoblasts,⁴⁹ that may be contributing to the more profuse positive staining of OPN. However, PSC-treated scaffolds were showed strong staining of OPN+ bone-lining osteoblasts and OPN+ osteocytes within the defect site.

The quantified data of OPN staining in Fig. 4B also confirmed that on average, samples with PSC, expressed significantly higher (three times more) OPN relative to the samples with SVF and 12 times more OPN relative to samples with scaffold only ($P<0.01$). Samples treated with SVF however, showed no significant quantitative difference relative to the control subjects.

Similarly, OCN staining was most predominant within osteoids of PSC-treated scaffolds (Fig 5A), and less frequent among hyper-cellular areas of scaffold and SVF-treated scaffold groups. OCN staining quantification also confirmed that PSC treated samples expressed 2.5 times more OCN compared to the control and SVF treated samples ($P<0.01$). However, data obtained from the SVF samples did not show a difference from the control subjects in OCN staining (Fig 5B).

Next, expression of BMP2 and VEGF was examined (Fig. 6,7). As expected, we found that the cellular areas within the PSC treated scaffolds had a more intense BMP2 and VEGF staining, as opposed to the control and SVF groups which showed mostly BMP2 and VEGF negative defect sites. Quantitative analysis of the BMP2 staining also confirmed that defects with PSCs had a 20-fold increase compared with the control and 8 fold increase compared with the SVF samples (Fig 6B). Samples treated with SVF showed no significant difference in their staining level compared with the control subjects. Quantitative analysis of the VEGF staining presented a 2.5 fold increase in the PSCs samples over the control group ($P<0.05$), and 1.5 times more staining relative to the SVF group (non-significant).

Lastly, immunohistochemistry was performed to confirm the presence of human

cells within the defect sites by human MHC class I antigen, and human (Proliferative Cell Nuclear Antibody) PCNA markers (Fig. 7, 8). As expected, we obtained positive staining both in PSC and SVF treated samples, indicating existence of human cells in the defect areas. It is noteworthy that the PSC samples had more intense staining of the PCNA compared with the SVF samples, which may be suggestive of a more superior regenerative ability of the PSC cells.

In summary, PSCs appeared to be a more effective target cells for bone regeneration, compared to SVF, as they led to a significantly higher calvarial defect ossification with adequate radiographic and histologic evidence. PSCs stimulated a more improved osteogenesis and bone remodeling, revealed by the OPN and OCN staining and induced more pro-osteogenic growth factors such as BMP2 and VEGF in the defect sites. Moreover, PSCs are able to form bone in vivo without the need for in vitro-expansion. Avoidance of the culturing stage is highly advantageous and can significantly increase safety and practicality in the clinical use of PSCs.

DISCUSSION:

In this study, we demonstrated that adipose derived human perivascular stem cells are a purified and homogeneous MSC like population, and are superior to unsorted SVF of the adipose tissue for skeletal defect healing. We used a mouse calvarial defect model to examine the efficacy of PSCs in skeletal defect re-ossification. When defect healing through live MicroCT images was monitored, a much more robust and efficient repair of the defect areas was observed with the PSC impregnated scaffolds. Indeed, at the end of the 8-week period when subjects were sacrificed, we did observe a significant increase in the amount of new bone formation in the PSC loaded defects. Performing H&E staining on the samples confirmed the presence of a significantly more newly formed bone in the defect areas loaded with human PSCs, as opposed to a minimal new bone formation in the control and SVF samples. In addition, the H&E staining revealed that in the PSC subjects, the newly formed bone was made of mature bone structure. In most cases, in the PSC samples it was difficult to detect the defect edge since the new bone had beautifully meshed into the old bone, while the control and SVF samples exhibited an obvious cut off at the defect edge and only some scattered unorganized bone formation was noted. The radiographic and histologic findings confirmed that PSCs showed an improved bone regenerative ability compared to a patient-matched SVF, both in terms of the quantity and quality of bone regeneration. These results make adipose-derived PSCs a new cell source for future clinical efforts in bone

regeneration and healing. Moreover, PSCs are plentiful within adipose tissue, constituting an average 40% of total viable SVF fraction.⁴⁵ Therefore, it has been estimated that even patients with minimal excess body fat could donate autologous fat tissue for PSC harvest.⁴⁵ For example, 200 ml of lipoaspirate would theoretically yield 31 million cells, which is estimated to be adequate for healing of a 2-cm mid-diaphyseal femoral defect.⁴⁰ Considering that on average, 2000-5000 ml of lipoaspirate is extracted during one session of liposuction,⁵⁰ 200ml of lipoaspirate is a clinically relevant stem cell numbers extractable from any patient who will need autologous adipose cells for regenerative purpose. However, these numbers are an estimation based on the number of the PSC cells used so far to heal mouse calvarial defects. Future studies of PSC application in the healing of larger defects (perhaps in larger animals) or spinal fusion models can provide us with a better understanding of the amount of lipoaspirate needed for the healing of a certain size defect and its clinical applicability. Also, practically speaking, it may be very challenging to immediately isolate PSC cells from a fresh lipoaspirate for clinical use. In such setting, most likely refrigerated storage of lipoaspirate would be necessary. While doing this study, we found that samples were being processed even more than 72 hours after liposuction. In order to determine the effects of cold storage of lipoaspirate on cell yield, viability and frequency, the data of cell yield, viability, and identity were grouped by hours of storage at 4°C per 24-hour period.⁴⁰ Excitingly, cell viability showed no difference after 72 hours of storage. Next total cell yield and frequency were calculated. There was a non significant decrease in total cell yield,

and the percentage prevalence of PSCs did not change by hours of cold storage. Moreover, it was investigated if changes in patient age, gender or BMI would make a difference in cell yield, viability, and frequency of PSCs and observed that these variations made small and non-significant changes in the data.⁴⁰

Next through immunohistochemistry, it was confirmed that cells of human origin truly existed in the defect sites by performing staining against human markers. Indeed both SVF and PSC groups responded positively to human MHC class I and PCNA markers with no positive staining obtained in the scaffold only group.

Also, it was interesting to see a consistent increase of the PCNA staining in the PSC samples compared with the SVF group, possibility indicative of the improved proliferation and/or survival of human PSCs within the mouse calvarial defect sites.

Next, through immunohistochemistry we were able to gain valuable insight on how PSC stimulated ontogenesis relates with expression of bone matrix proteins such as OPN and OCN. OPN and OCN are extracellular matrix proteins synthesized and secreted during the process of osteoblast differentiation and mineralization.⁵¹

While OPN is an early and marker of bone formation, OCN indicates the later phase of bone formation.⁵¹ In relation to OPN, all treatment groups showed some level of OPN expression, however the PSC group were the only subjects that constantly displayed OPN+ bone-lining osteoblasts and OPN+ osteocytes within the newly formed bone in the defect sites. OPN is expressed early in mesenchymal cell differentiation and is related to cell migration as well as osteogenesis.⁴⁸ Therefore, expression of osteopontin is an early indicator of osteogenic differentiation and is

commonly used to identify osteoprogenitor cells.⁵² This partially explains the numerous OPN+ cells in all treatment groups, since all groups have some level of mesenchymal stem cells, however, what we are interested in knowing is that which one of regenerative mediums (SVF or PSCs) will more successfully convert more of the osteoprogenitor cells into osteoblasts. Therefore, we progressed to perform osteocalcin staining, since OCN marks the later phase of bone formation and indicates the mineralization process implemented by the calcification of the osteocytes in the collagen layer.⁵³ In comparison to untreated and SVF treated bone, osteocalcin expressions in the PSC groups were elevated 2.5 fold after 8 weeks. The higher intensity of OCN positive cells in the PSC group can be suggestive of an earlier and more abundant calcification of the newly formed bone in this group. Moreover, OCN staining was observed to be in a more scattered distribution pattern, which is reasonable, considering that osteocalcin is a secreted extracellular matrix protein. Overall, the pattern of OPN and OCN expression and their quantitative values suggested that the PSC group had the most developed healing process. These results indicate that in vivo differentiation of undifferentiated adipose-derived PSCs is possible, and that the stem cells themselves contributed to osteogenesis in the mouse calvaria, in the absence of added growth factors such as bone morphogenetic proteins.

Further immunohistochemical assays were performed to study the effects of PSC mediated bone healing on pro-osteogenic growth factors such BMP2 and VEGF. Some of the most important mediators of bone repair have been osteogenic bone

morphogenetic proteins.⁵⁴ Expression of bone morphogenetic proteins including, BMP-2, play an essential role in initiating the early cascade of bone healing, ectopic bone formation, and adult ossification.⁷ Therefore we compared BMP2 expression for all three-treatment groups and observed a significant increase in the level of BMP2 expression associated with PSC induced osteogenesis (8 times higher than the SVF group). BMP-2 stimulates proliferation of both chondrocytes and osteoblasts and increases bony matrix production.⁵¹ BMPs have also been shown to induce mesenchymal stem cells differentiation into osteoblasts.⁵⁵ Increased levels in expression of BMP-2 in the PSC treated subjects underline the superior osteoinductive properties of the PSCs within a bony environment. In addition, immunohistochemical staining against VEGF also showed an increased angiogenic activity during the healing of the PCS impregnated calvarianl defects. Angiogenesis has been identified as another key factor determining the outcome of bone repair.⁵⁶ Accordingly, inhibition of angiogenic growth factors leads to negative alteration of bone repair, while administration of angiogenic growth factors is capable of stimulating bone formation.⁵⁷ The increased levels of VEGF in the PSC loaded samples therefore, could suggest that PSCs may also stimulate bone healing by inducing vasculogenesis. This stimulatory and protective effect of PSCs over the endothelial cells has been explored previously by several investigators and it has been demonstrated that in fact PSCs secrete VEGF, which promote the survival of the endothelial cells.⁵⁸ This combined positive effects of PSCs on VEGF and PBP2 expression, bring us to suggest the possibility that one of the ways that PSCs may

induce bone formation could be through paracrine induction of pro-vasculogenic and pro-osteogenic growth factors. Another factor that improved PSC induced bone formation may have been the elimination of non-MSC type cells from the SVF suspension and obtaining pure PSCs as a regenerative medium. As mentioned earlier, both subpopulations of PSCs have been shown by clonal analysis to be purified ancestors of MSCs. Therefore, sorting for human PSCs markers ensured a highly purified MSC population for implantation, eliminating endothelial cells, hematopoietic cells, inflammatory cells and etc.³⁰ Some existing theories suggest that endothelial cells may actually inhibit differentiation of MSCs and therefore inhibit osteogenesis by negatively regulating the differentiation of MSCs.^{59,60} These investigations show that human endothelial cells inhibit bone marrow MSC differentiation into mature osteoblasts by interfering with Osterix expression.⁵⁹ With this evidence in mind, the enhancement of bone formation in the PSC samples in part may be due to the elimination of the endothelial cell component of the SVF mix.

In conclusion, this study suggests that Adipose-derived human PSCs are a new cell source for future clinical efforts in bone regeneration and healing. PSCs showed an improved bone regenerative ability compared to a patient-matched SVF, both in terms of the quantity and quality of bone regeneration. Furthermore, this study importantly demonstrates that PSCs are able to form bone in vivo without the need for in vitro-expansion. Avoidance of the culturing stage is highly advantageous and can significantly increase safety and practicality in the clinical use of PSCs.

FIGURES:

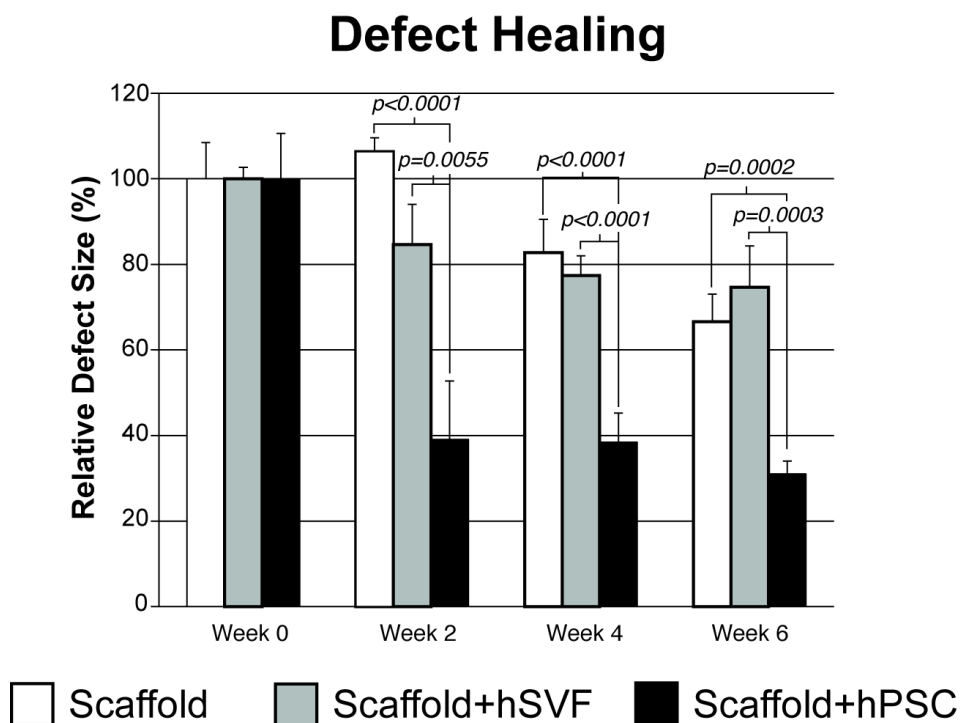


Figure 1. Mean Relative defect healing quantification of live MicroCT

Relative defect healing at 0, 2, 4, and 6 weeks postoperative by serial live microCT scans. $n = 16 - 18$ mice per treatment group split equally among $n = 4$ separate patient samples. 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. Defect healing progress is shown by amount of bone formation over the entire defect area, expressed as mean percentage healing. For hPSC samples between 0 to 4 week, there is 60% healing, compared to 18% healing in the scaffold only and only 20% healing in the SVF group. By week 6, 69% of the defect size is shown to have re-ossified in the PSC group, whereas only 25% healing is shown in the SVF group.

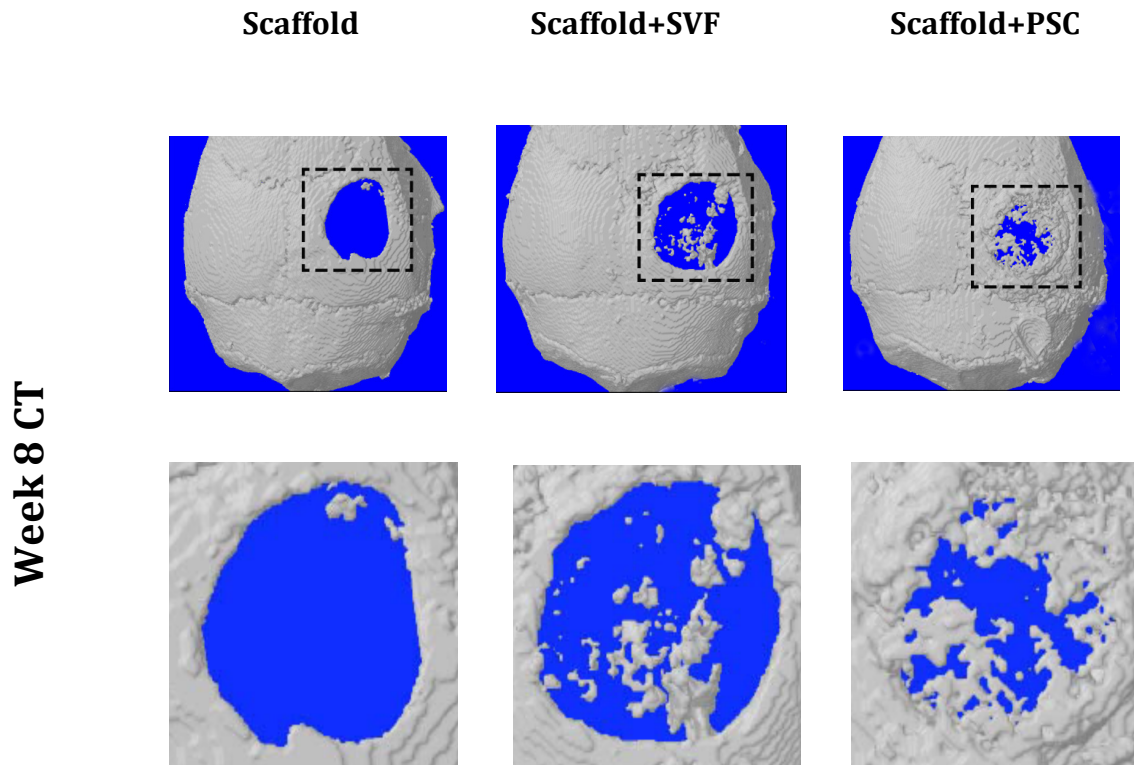


Figure 2: High resolution MicroCT scanning 8 weeks post mortem

Three-dimensional reconstructions of control, SVF, or PSC treated calvarial defects at 8 weeks postoperative. Significant re-ossification of the defect area is observed in the PSC group, as opposed to minimal osteogenesis in the SVF group and negligible healing in the scaffold only group.

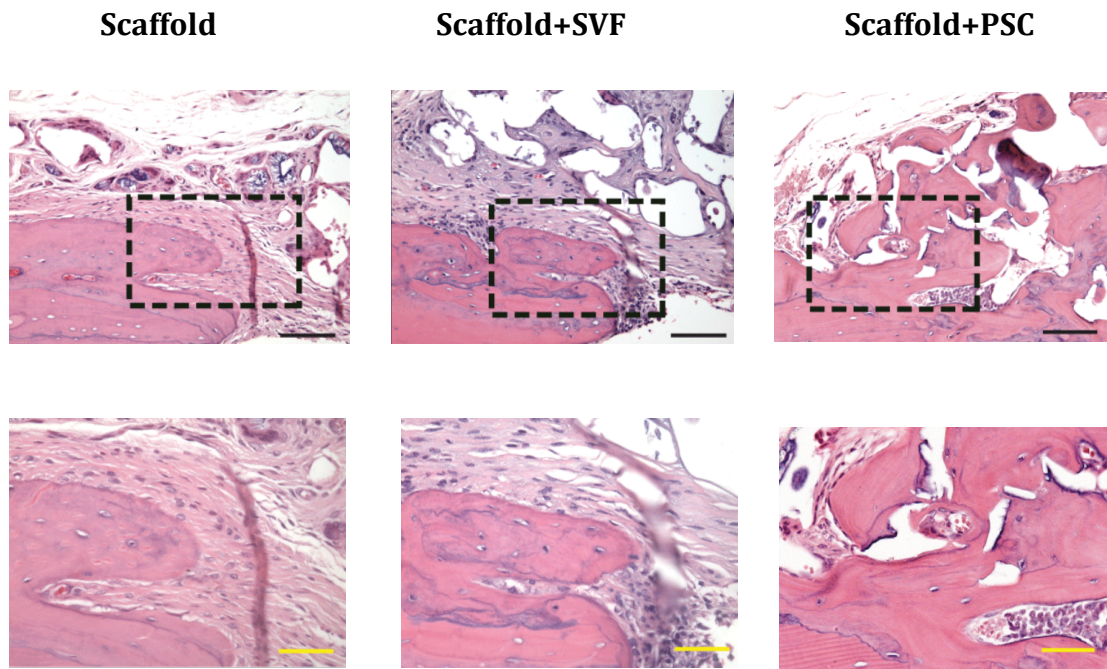


Figure 3. Representative hematoxylin/eosin images for the defect site

Images are taken from the defect edge to define old bone from newly formed bone. With treatment of either control scaffolds or SVF-treated scaffolds, an abrupt cutoff was clear where the surgically created defect was performed. PSC treated samples however, showed a notable continuity from the old into the new bone area with osteocytes in the lacunae of the newly formed bone. Black scale bars =50 μ m. Yellow scale bars = 25 μ m.

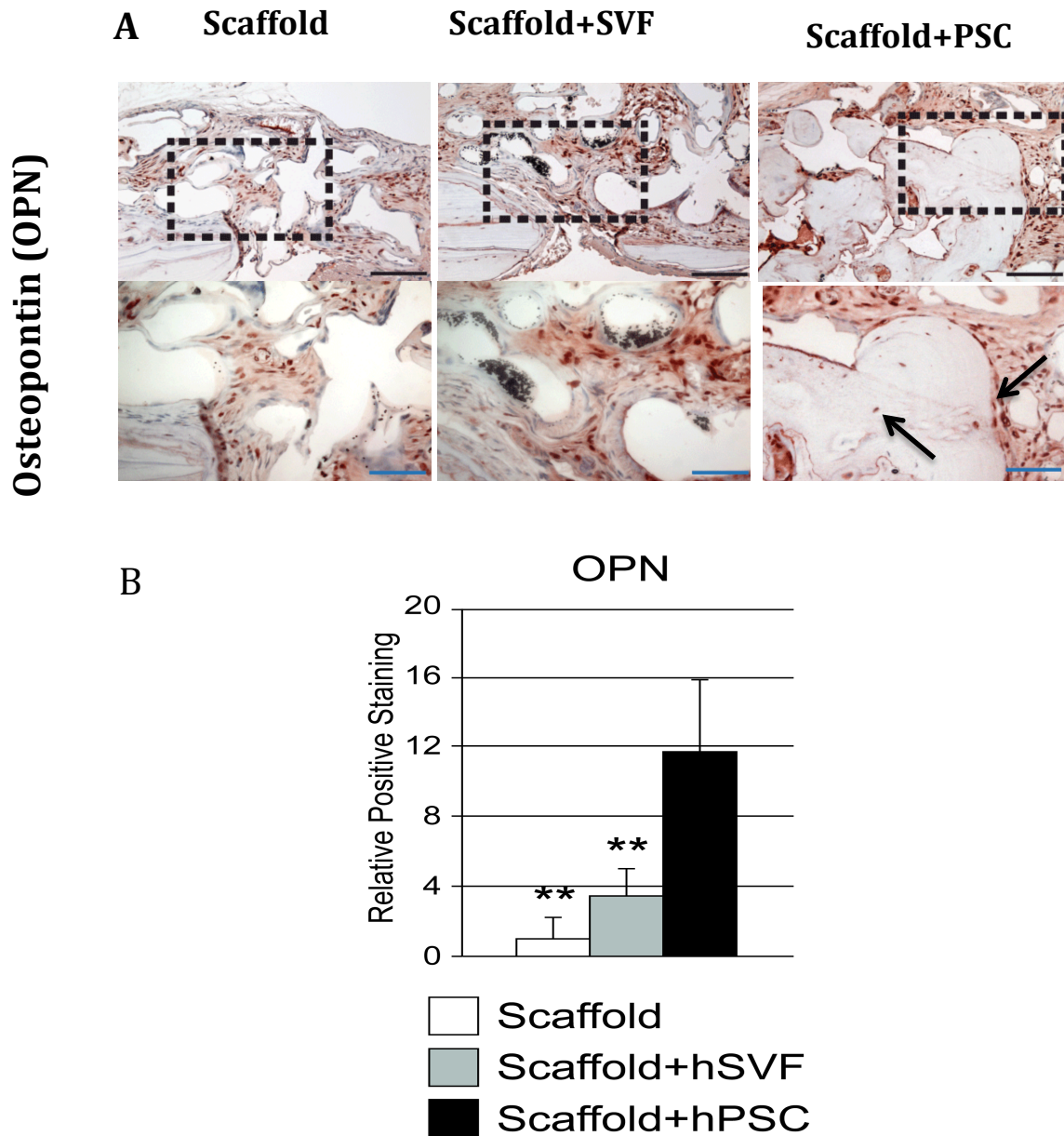


Figure 4: Immunohistochemistry showing Osteopontin (OPN) expression

(A) Representative OPN staining showing the PSC treated scaffolds to be the only group with OPN+ bone-lining osteoblasts and OPN+ osteocytes within the defect site.(shown by arrows) (B) Quantification of OPN staining, $n = 16 - 18$ mice per treatment group. * $p < 0.05$, ** $p < 0.01$ for paired t-test between PSC/SVF and PSC/Scaffold groups. Scale bars = $50 \mu\text{m}$ (top rows), $25 \mu\text{m}$ (bottom rows)

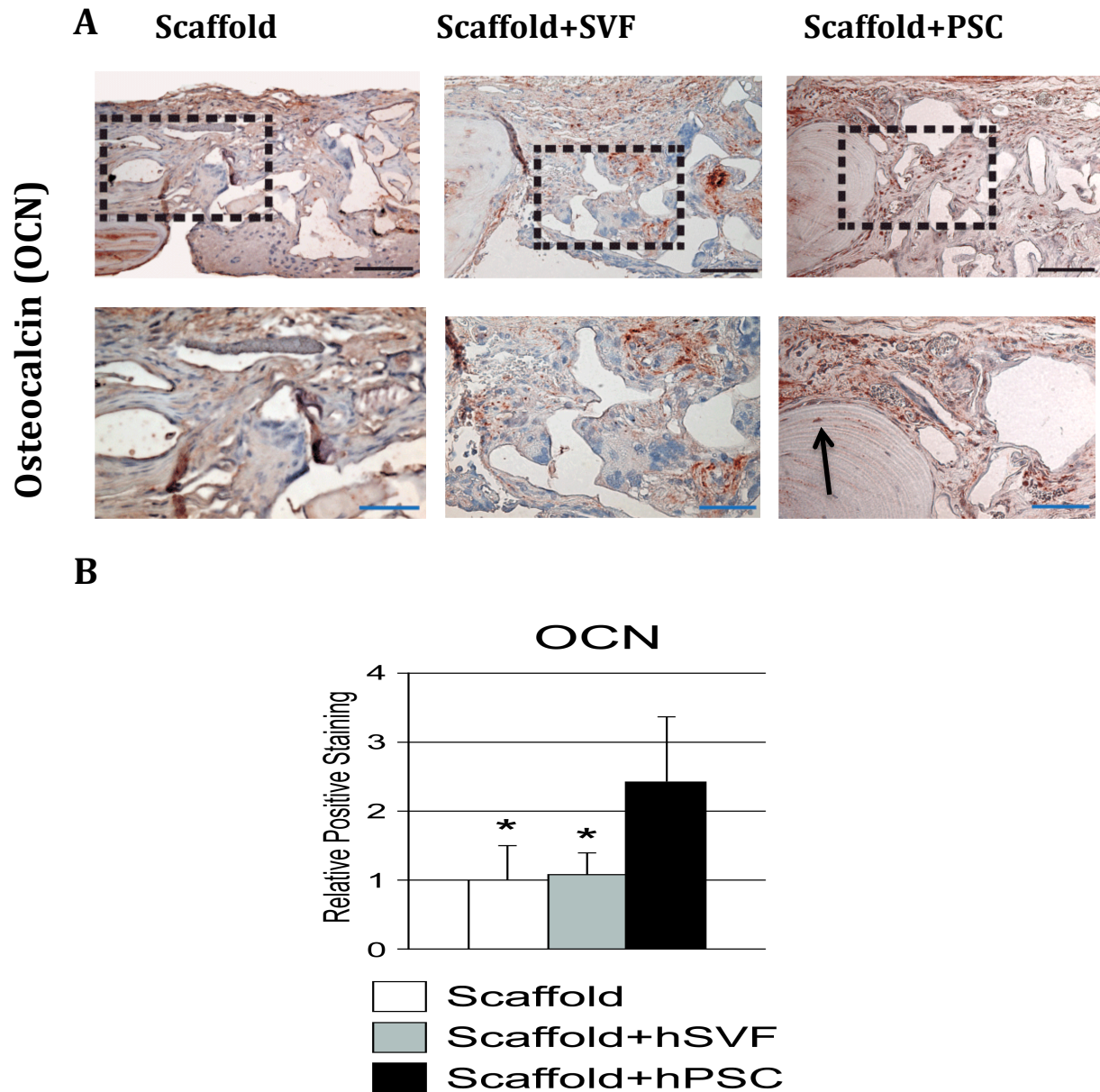


Figure 5: Immunohistochemistry showing Osteocalcin (OCN) expression
 (A) Representative OPN staining showing PSC treatment group being the only group to show OCN positive staining within the newly formed osteoid. (B) Quantification of OPN staining, $n = 16 - 18$ mice per treatment group. * $p < 0.05$, ** $p < 0.01$ for paired t-test between PSC/SVF and PSC/Scaffold groups.

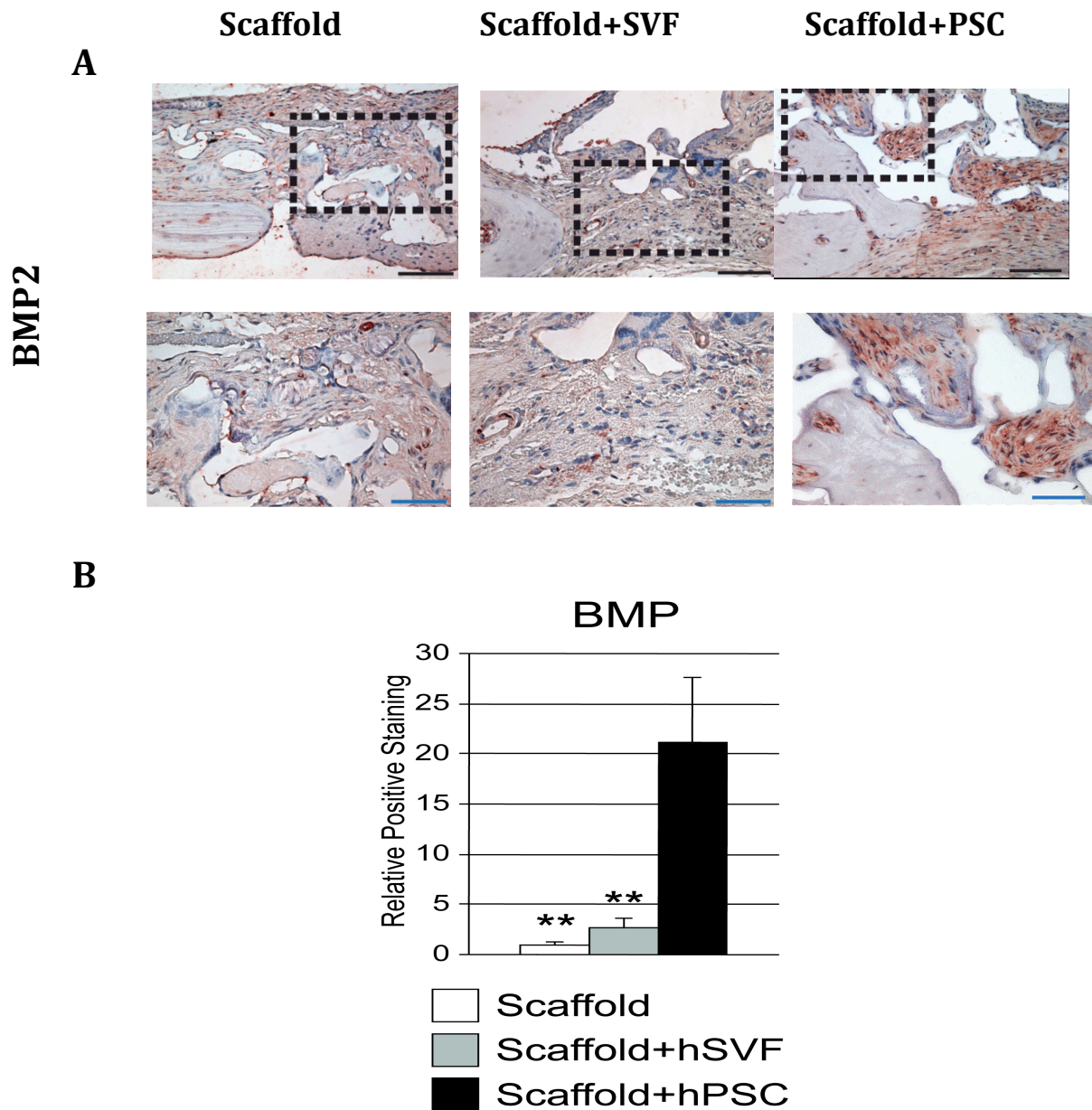


Figure 6: Immunohistochemistry showing BMP2 expression

(A) Representative staining showing significantly more positive BMP2 expression in hPCS samples compared with other treatment groups.

(B) Quantification of the BMP2 staining, also confirming a significant increase in positive staining of BMP2, while SVF samples show no significance difference compared to the scaffold only subjects. * $p < 0.05$

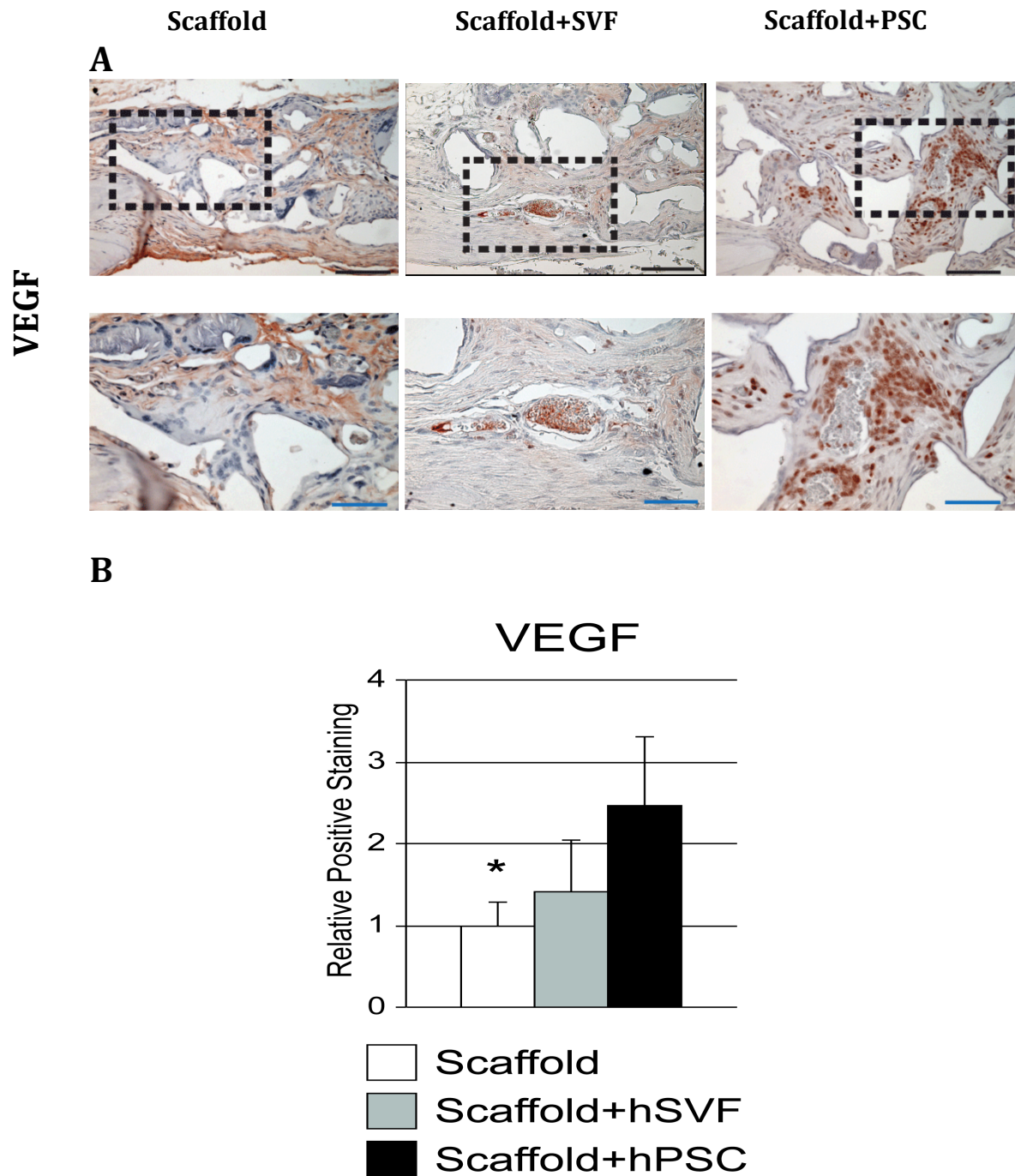


Figure 7: Immunohistochemistry showing VEGF expression

(A) Representative staining showing a much more intense staining in the PSC treated defects, compared to the control and SVF samples. (B) Quantitative analysis of VEGF staining showing a significant increase of 2.5 fold over the control subjects ($P < 0.05$) and a 1.5 fold mean increase (non-significant) over the SVF samples. SVF samples showed no significant difference over the control group.

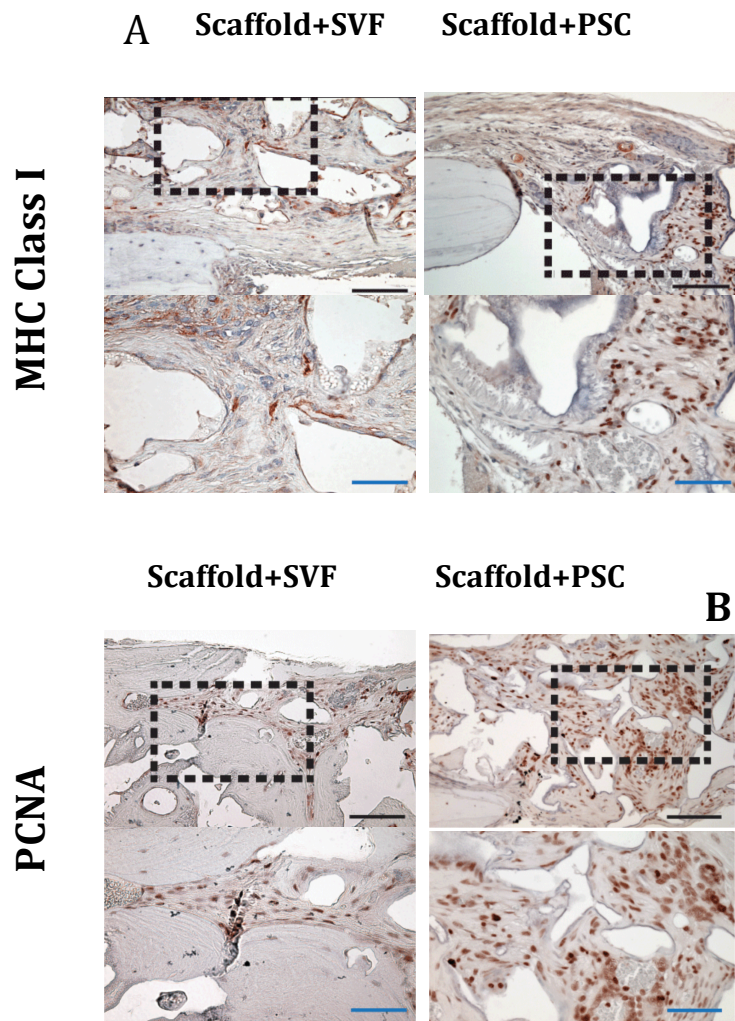


Figure 8: Human MHC class I, and PCNA immunohistochemistry

(A) A positive human MHC class I staining in both SVF and PSC samples confirms the presence of human cells within the defect areas. A more intense staining is observed in the PSC samples. **(B)** PCNA is used as another marker to confirm the presence of proliferating human stem cells in the defect areas. PCNA also shows a stronger staining in the PSC treated samples.

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