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Optimal Scaffold Design and Effective Progenitor Cell Identification for the Regeneration of Vascularized Bone

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Abstract— **Bone tissue engineering offers perhaps the most attractive treatment option for bone repair/regeneration as it eliminates complications of other bone grafting options (i.e., availability and immunogenicity issues of autografts and allografts, respectively). However, scaffold-based bone tissue engineering is largely limited by inadequate vascaularization, and as a result, bone formation is often restricted to the construct's periphery. In this study, we offer a two-pronged approach to overcome periphery-limited bone and vascular formation. We have developed optimally designed, mechanically strong, biodegradable scaffolds with increased porosity and interconnectivity. We have also identified and isolated superior, clinically-relevant cell populations (peripheral blood-derived endothelial progenitor cells (EPCs), and bone marrow-derived mesenchymal stem cells (MSCs)). In combination, we have developed a synthetic graft system suitable for the regeneration of vascularized bone.**

I. Introduction

SUCCESSFUL bone repair and regeneration using a scaffold-based tissue engineering approach critically requires (i) an effective mechanically stable and biodegradable scaffold with sufficient porosity and interconnectivity to allow for efficient mass transport of oxygen and nutrients, and (ii) clinically feasible cell populations that can induce vascularization and bone formation [1], [2]. Otherwise, bone formation and vascularization is largely limited to the periphery of the scaffold constructs *in vitro* and *in vivo*, due to decreased mass transport-dependent cell survival, proliferation and differentiation in the construct's interior regions [3] - [9].

In this study, we offer a two-pronged approach to overcome periphery-limited bone and vascular formation. Firstly, we will design biodegradable scaffolds with increased porosity and interconnectivity, and mechanical compatibility to human cancellous bone. We will then investigate superior and clinically-relevant cell sources for enhanced bone formation and vascular invasion (i.e., endothelial progenitor cells and mesenchymal stem cells), and study their performance on our newly and optimally designed biodegradable scaffolds. The objective of this work is to design optimal scaffolds and identify superior osteo- and endothelial- progenitor cell combination for effective vascular invasion and enhanced bone regeneration.

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I. Materials & Methods

A. Macro-porous Scaffold Fabrication. Poly(85lactide-*co*-15glycolide) (PLGA) microsphere scaffolds with increased pore size and pore volume were developed via a "thermal sintering and porogen leaching" method previously described [10]. Briefly, PLGA microspheres (diameter 425-600 μm) and a porogen, NaCl (diameter 200-300 μm), were mixed at specific ratios upon packing into a steel mold containing disc shapes (8 mm diameter, 2 mm height), and thermally sintered at 100°C. Specific dry weight PLGA:NaCl ratios included 90:10, 80:20, 70:30, 60:40 and 50:50. The porogen was subsequently leached out by soaking the composite PLGA/NaCl scaffolds in distilled water for 2 hours, resulting in scaffolds with increased pore size compared to control scaffolds (PLGA scaffolds fabricated without NaCl). Scanning electron microscopy (SEM) was used to image the morphology of the macro-porous and control scaffolds.

B. Scaffold Characterization. Scaffold specimens were imaged using conebeam micro-focus X-ray computed tomography to render three-dimensional models for direct quantitation of porosity connectivity (μCT40, Scanco Medical AG). Serial tomographic images were acquired, and three-dimensional images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering. Segmentation of solid scaffold from open porosity was performed in conjunction with a constrained Gaussian filter to reduce noise. Direct measurements of internal porosity included volume fraction, size, connectivity, accessible internal pore volume, and accessible solid surface area of scaffold (as a function of pore dimension). The accessible volume and surface parameters provide direct measurements of the pore volume and surface available to cell infiltration as a function of minimum pore dimension, using a distance transformation algorithm similar to Moore *et al*. [11]

C. Cell Isolation.

Mesenchymal Stem Cells (MSCs). Bone marrow aspirates (5 mL) were obtained from the iliac crests of New Zealand White rabbits. Bone marrow aspirates were layered over a Percoll gradient and centrifuged at 2000 rpm for 20 min at room temperature. Mononuclear cells at the interface were recovered and washed three times with phosphate buffer saline (PBS). The cell suspension was seeded in 150 mm tissue culture plates containing Dulbecco's-modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 mg/mL streptomycin, and 100 U/mL penicillin. MSC cultures grew at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed after 4 days with washes of PBS. The medium was subsequently replaced every 3 days. When the culture reached 90% confluency, the MSCs were recovered with 0.25% trypsin containing 0.01%

EDTA, and passaging was performed.

Endothelial Progenitor Cells (EPCs). Fifty milliliters of peripheral blood was collected via terminal exsanguation from one healthy New Zealand White rabbit in heparinized tubes. Bone marrow aspirates (5 mL) were also obtained from the iliac crests of the same New Zealand White rabbit. The mononuclear fractions from both sources were separated using Lymphoprep and suspended in endothelial growth media (i.e, endothelial basal medium-2 (EBM-2, Lonza), supplemented with 10% fetal bovine serum, 100 mg/mL streptomycin, 100 U/mL penicillin, and EGM-2 SingleQuots and plated on collagen (Sigma) -coated 150 mm plates. Peripheral blood-derived and bone marrow-derived EPCs were grown at 37°C with humidified 95% air/5% CO_2. After 4 days of culture, non-adherent cells were discarded by gentle washing with PBS, and fresh medium was applied. The medium was subsequently replaced every 3 days. When cells reached 90% confluency, cells were digested with 0.25% trypsin containing 0.01% EDTA, then passaged accordingly. Cells were cultured for 3-4 weeks, or until they began to display a cobblestone morphology.

D. Alkaline Phosphatase Activity. MSCs and EPCs cultured alone, and in co-culture on tissue culture plate (initial seeding density of 50,000 cells per well in 24-well plate) was maintained in a 1:1 mix of endothelial growth media and osteogenic media (i.e., α-MEM supplemented with 10% FBS, 100 mg/mL streptomycin, 100 U/mL penicillin, 10 nM dexamethasone, 3mM β-glycerophosphate and 50 µg/ml ascorbic acid). After 14 days in culture, samples were harvested and the cell lysates were collected. Alkaline phosphatase activity, an early osteogenic marker, of MSCs and EPCs alone and in co-culture was measured using an alkaline phosphatase (ALP) substrate kit (Bio-Rad) as previously described by Nukavarapu *et al.* [12]. ALP activity was normalized to the total protein from each individual sample (BCA Protein Kit, Thermo Scientific).

E. *In Vitro* Angiogenesis Assay. To investigate the angiogenic potential of PB-EPCs and BM-EPCs, the cells were encapsulated in an angiogenic matrix (10^6 cells/mL Matrigel) for 7 days. Two groups (n=3) were formed: Matrigel with only PB-EPCs, and only BM-EPCs. The groups were embedded in paraffin for sectioning. The number of branch points was counted under a phase contrast microscope in ten random fields of ten random sections, stained with Mayer's hematoxylin and eosin [13]. Results are expressed as mean ± SD.

G. Cell Seeding and Co-culture on Scaffolds. PLGA microsphere scaffolds were sterilized by immersing the scaffolds in 70% ethanol for twenty minutes. Scaffolds were then washed three times in sterile PBS before exposing them to UV radiation for one hour. After cell trypsinization and re-suspension, a 40 µl cell suspension containing a total of 250,000 cells was seeded onto each scaffold. Three experimental groups were evaluated: (1) PLGA scaffolds seeded with EPCs, (2) PLGA scaffolds seeded with MSCs, and (3) PLGA scaffolds seeded with EPCs and MSCs at a 1:1 ratio. The cell-seeded scaffolds were incubated for 30 minutes at room temperature to allow for cell adhesion onto the scaffolds. The cell-scaffold constructs were cultured in a 1:1 mix of osteogenic and endothelial growth media, and maintained for 2 days in an incubator at 37°C, 5% CO_2, and 95% humidified air.

H. Evaluation of Gene Expression. For the detection of gene expression of bone morphogenic protein-2 (BMP-2) and vascular endothelial growth factor-A (VEGF-A) of MSCs and EPCs, alone and co-cultured on macro-porous PLGA scaffolds were evaluated using the RT-PCR (Applied Biosystems, ABI Prism, 7900 HT Sequence Detector system). After 2 days in culture, the scaffolds were washed once with PBS, and the total RNA from cells on scaffolds were isolated using an RNeasy mini kit (Qiagen) following the manufacturer's protocol. After isolation, 3 µg of RNA was converted to cDNA and amplified. Accumulation of PCR products was monitored and quantified using SYBR Green and the comparative CT method in which the accumulated PCR products for each of the genes examined is normalized to the house keeping gene GAPDH. The primers for each gene were purchased from Applied Biosystems (ABI) and catalogue numbers are as follows: BMP-2 (Oc03824113_s1), VEGFA (Oc03395999_m1), GAPDH (Oc03823402_g1).

II. Results & Discussion

In this study, we developed biodegradable scaffolds with increased pore size and pore volume (i.e., macro-porous, Fig. 1A), yet mechanically stable. This was successfully achieved through mixing a porogen (i.e., NaCl crystals, 200-300 µm diameter) with PLGA microspheres (425-600 µm diameter), thermal sintering, followed by porogen leaching. SEM imaging demonstrated that after porogen leaching (Fig. 1A), there was visually an increase in pore size compared to scaffolds fabricated with PLGA microspheres alone (i.e., control PLGA scaffolds).

MicroCT imaging was used to reconstruct 3D models of the macro-porous and control scaffolds for nondestructive measurements of porosity. The porosity and mean pore size of the biodegradable scaffolds corresponded to the amount of porogen used. Specifically, the percent accessible volume for pore sizes with the diameter of 100 µm was 34.4%, and 48.1% for 0% NaCl/100% PLGA scaffolds, and 20% NaCl/80% PLGA scaffolds, respectively. Further, the percent accessible volume for pore sizes of 200 um is 9.1%, and 37.4% for 0% NaCl/100% PLGA scaffolds, and 20% NaCl/80% PLGA scaffolds, respectively. Data and images describing scaffold pore interconnectivity in Fig. 1B and 1C are presented as a function of pore size, providing direct measurements of externally accessible pore space through the full range of diametric pore dimension. Macro-porous scaffolds displayed significantly higher percentage of accessible pore volume in the 200-400 µm range in comparison to control scaffold. A pore size of approximately 300 micrometers has been established as the required pore size for neo-vascularization of bone constructs *in vivo* (3). Also, in control scaffolds, a sphere with a diameter of 300 µm can access only approximately 2% of the total pore volume, whereas the same sphere can access approximately 16% of the pore volume of the macro-porous PLGA scaffold (Fig. 1C). Thus, we effectively increased the accessible

volume available for cell infiltration throughout the scaffold. Furthermore, although the compressive modulus (CM) and strength (CS) for macro-porous scaffold are lower than the control scaffold (control CM: 362.4 ± 73.2 MPa, control CS: 11.2 ± 1.8 MPa; macro-porous CM: 248.6 + 63.3 MPa, macro-porous CS: 4.8 + 1.8 MPa), they are in the range of human trabecular bone mechanical properties (i.e., compressive modulus 50-800 MPa and compressive strength 1-10 MPa) [14].

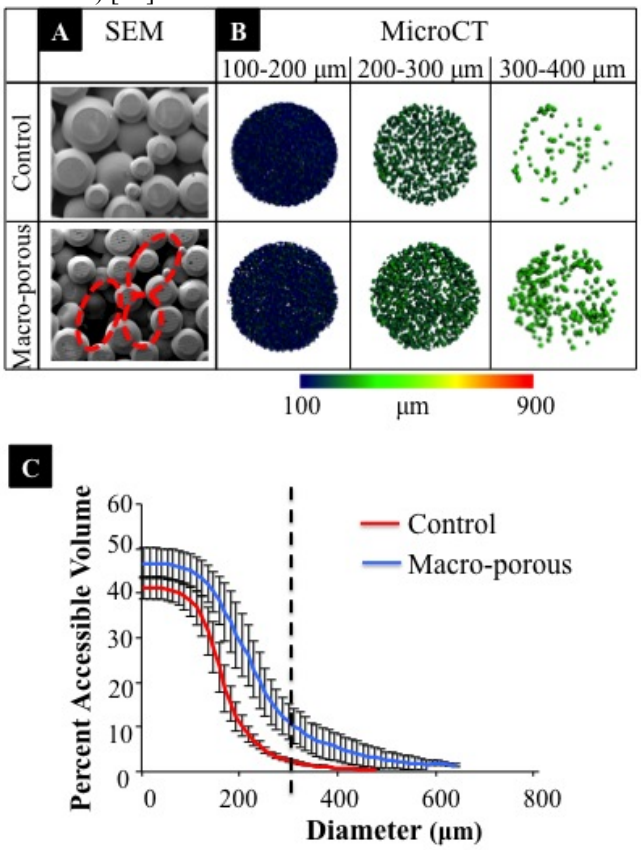

Figure 1. (A) SEM images of control and macro-porous PLGA microsphere scaffolds. Macro-porous scaffolds, developed via a combined thermal sintering and porogen leaching technique, displayed increased pore sizes (highlighted by red dashed circles). (B) Interconnected volume accessible to spherical objects with specific diameter range (i.e., 100-200 µm, 200-300 µm, 300-400 µm) in control and macro-porous scaffolds. (C) Macro-porous scaffolds display significantly higher percent accessible volumes with higher pore diameters as compared to control scaffold. Dashed line illustrates percent accessible volume of control and macro-porous scaffolds for an object with a diameter of 300 µm.

We next isolated clinically-relevant cell populations established to enhance vascularization and bone formation *in vitro* and *in vivo* [15], [16], specifically peripheral blood derived - endothelial progenitor cells (EPCs) and bone marrow derived – EPCs and mesenchymal stem cells (MSCs) [13], [17]-[24]. Previous studies have demonstrated EPCs and MSCs promote enhanced bone regeneration via the stimulation of neo-vascularization [23], [25]-[27]. We isolated EPCs from two very clinically relevant sources, peripheral blood and bone marrow (i.e., PB-EPCs and BM-EPCs), and assessed their angiogenesis capabilities, as well as their ability to enhance the expression of an early bone marker when co-cultured with mesenchymal stem cells (MSCs). PB-EPCs formed significantly higher numbers of networks and branch points than BM-EPCs after culturing in Matrigel for one week (Fig. A-C). Furthermore, we systematically examined the combination of these two cell

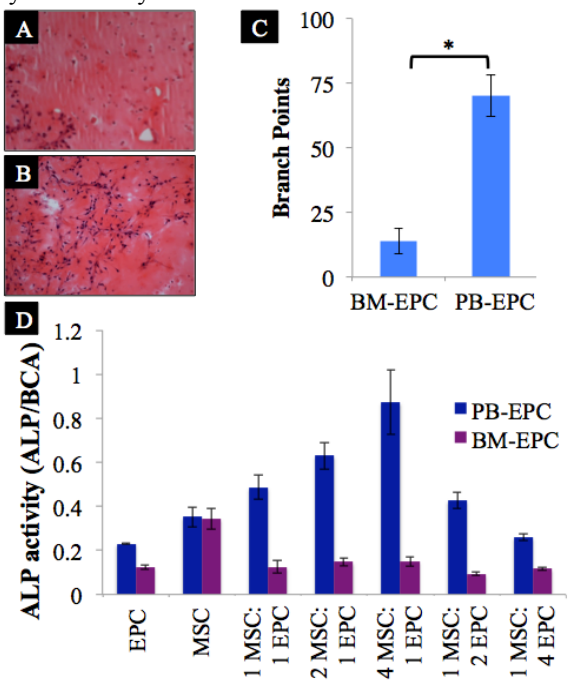

Figure 2. Angiogenesis assay showing network formation by (A) BM-EPCs and (B) PB-EPCs in Matrigel after 1 week *in vitro*. (C) BM-EPCs formed significantly less networks and branch points, as compared to PB-EPCs in Matrigel. (D) Comparison of ALP activity of PB-EPCs and BM-EPCs cultured alone, or in co-culture at various ratios with MSCs after 14 days in a mix of endothelial and osteogenic differentiation media.

types co-cultured with MSCs, by culturing MSCs with either PB-EPCs or BM-EPCs at various ratios, including 1:1, 1:2, 1:4, 2:1 and 4:1 (MSC:EPC). We observed significantly enhanced expression of alkaline phosphatase (ALP) when PB-EPCs were co-cultured with MSCs, on the contrary to BM-EPCs co-cultured with MSCs (Fig. 2D). From this, we selected EPCs isolated from peripheral blood to be a superior cell population for enhancement of angiogenesis and ostoegenesis when co-cultured with MSCs.

As a strategy to improve and enhance future implant survival and performance, we pre-vascularized our macro-porous scaffolds by culturing PB-EPCs and MSCs together. We co-cultured these two cell types for two days on our biodegradable scaffolds, which is the time we would pre-culture our scaffolds before implanting *in vivo*. At this time, both VEGF-A and BMP-2 expression, two critical markers for angiogenesis and bone formation, were increased when PB-EPCs and MSCs were co-cultured together on macro-

porous PLGA scaffolds (Fig. 3). Although this study focused on PLGA microsphere scaffold, the present strategy (i.e., pre-vascularized macro-porous scaffolds) can be applied to any biodegradable scaffold for successful bone regeneration.

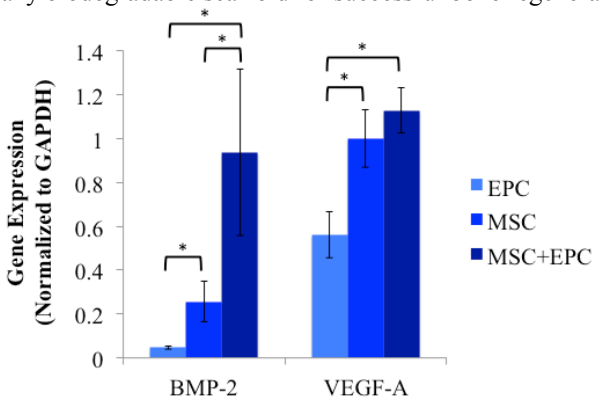

Figure 3. BMP-2 and VEGF-A gene expression in EPCs, MSCs, and co-cultured MSCs and EPCs on macro-porous PLGA scaffolds after 2 days *in vitro*.

III. Conclusions

In this study, we have developed novel macro-porous and mechanically stable, biodegradable scaffolds for bone tissue regeneration/repair. We have also identified peripheral-blood derived endothelial progenitor cells and mesenchymal stems cells to be superior cell populations to promote bone and vascular formation when co-cultured on our scaffolds. When implanted into a bone defect model *in vivo*, we hypothesize that the optimally designed scaffolds seeded with effective osteo- and endothelial- progenitor combination could promote angiogenesis, and significantly enhance bone formation. Ultimately, we believe our two-pronged approach will offer a practical and effective solution to address the lack of vascularization during bone regeneration.

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