Platelet-Derived Growth Factor and Spatiotemporal Cues Induce Development of Vascularized Bone Tissue by Adipose-Derived Stem Cells

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Vasculature is essential to the functional integration of a tissue-engineered bone graft to enable sufficient nutrient delivery and viability after implantation. Native bone and vasculature develop through intimately coupled, tightly regulated spatiotemporal cell–cell signaling. The complexity of these developmental processes has been a challenge for tissue engineers to recapitulate, resulting in poor codevelopment of both bone and vasculature within a unified graft. To address this, we cultured adipose-derived stromal/stem cells (ASCs), a clinically relevant, single cell source that has been previously investigated for its ability to give rise to vascularized bone grafts, and studied the effects of initial spatial organization of cells, the temporal addition of growth factors, and the presence of exogenous platelet-derived growth factor-BB (PDGF-BB) on the codevelopment of bone and vascular tissue structures. Human ASCs were aggregated into multicellular spheroids via the hanging drop method before encapsulation and subsequent outgrowth in fibrin gels. Cellular aggregation substantially increased vascular network density, interconnectivity, and pericyte coverage compared to monodispersed cultures. To form robust vessel networks, it was essential to culture ASCs in a purely vasculogenic medium for at least 8 days before the addition of osteogenic cues. Physiologically relevant concentrations of exogenous PDGF-BB (20 ng/mL) substantially enhanced both vascular network stability and osteogenic differentiation. Comparisons with the bone morphogenetic protein-2, another pro-osteogenic and proangiogenic growth factor, indicated that this potential to couple the formation of both lineages might be unique to PDGF-BB. Furthermore, the resulting tissue structure demonstrated the close association of mineral deposits with pre-existing vascular structures that have been described for developing tissues. This combination of a single cell source with a potent induction factor used at physiological concentrations can provide a clinically relevant approach to engineering highly vascularized bone grafts.

Introduction

TISSUE ENGINEERING APPROACHES to generate vascularized bone grafts could potentially revolutionize treatment of massive bone loss due to traumatic injuries, cancer, and congenital defects. Vasculature is essential to the long-term functional outcomes of a large bone graft to ensure sufficient nutrient delivery and postimplantation viability. While vasculature plays a necessary and intimate role in bone development, inducing the formation of vascularized bone tissues *in vitro* remains a challenge because the factors that promote each lineage may be detrimental to the other.^{1–3} This incompatibility has led researchers to develop various methods to encourage vasculature to form concurrently or in a sequential manner with osteogenic differentiation, including precise dosing regimens of induction factors,^{4–6} various

cocktail media,^{7,8} and pretreating multiple cell types separately followed by a recombination in a unified graft.^{5,6,9–11} Yet, each of these approaches relies on mitigating competing factors, resulting in an imbalance with suboptimal results for one or both of the tissue components. Therefore, there is still an unmet need for a clinically translatable approach to induce robust formation of both vasculature and bone within a unified graft and using a single autologous cell source.

Adipose-derived stromal/stem cells (ASCs) are a promising, clinically relevant cell source to supply both the osteogenic and vascular components of a vascularized bone graft. In addition to their well-studied osteogenic capacity,^{12,13} ASC cultures have also been shown to give rise to endothelial cells^{14,15} and lumen-containing vessels.^{16–18} However, the ability of ASCs to undergo direct differentiation to endothelial cells remains contentious.¹⁹ Recent

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findings suggest that these vessels may arise due to a minute subpopulation of residual committed endothelial cell progenitors that undergoes extensive proliferation and self-assembly to form vascular networks.^{16,20} Recently, a few groups have shown promise in the potential of using ASCs^{4,21} or fresh adipose stromal vascular fraction (SVF)^{22,23} to form vascularized bone. However, robust vascular network development coupled with dense mineral deposition has yet to be demonstrated, which may limit the extent of integration and functional outcome of the tissue. This problem is a fundamental challenge for all approaches toward engineering a vascularized bone graft, including those that involve the combination of osteoblasts or mesenchymal stem cells (MSCs) with a separate endothelial cell source.²⁴

Our prior studies showed that vascular assembly of ASCs is dependent on heterotypic cell–cell interactions, specifically through dense clustering of cells and endogenous platelet-derived growth factor (PDGF) signaling. This endogenous behavior is reminiscent of what occurs in native tissues, as proliferating endothelial cells in nascent vessels secrete PDGF-BB to recruit pericytes for vascular maturation and stabilization.^{25,26} PDGF-BB is also a major factor secreted by activated platelets to stimulate repair mechanisms in wounded tissues such as bone.^{27–29} A number of studies have demonstrated that *in vivo* administration of exogenous PDGF-BB significantly enhances bone formation.^{30–32} Yet, *in vitro* studies with MSCs have reported that while exogenous PDGF-BB induces greater proliferation, it has no effect on,^{33–35} or may even be inhibitory to,^{36,37} osteogenic differentiation. With regard to ASCs, it has been shown that during osteogenic differentiation, their expression of the PDGF receptor β (*PDGFR- β*) is upregulated.³⁸ However, the ability of PDGF-BB signaling to directly enhance the osteogenic capabilities of ASCs is not known.

This study focuses on recapitulating the heterotypic interactions needed to maximize the codevelopment of vasculature and bone. Specifically, we explore the potential of cellular aggregation and temporal presentation of factors to induce the cell–cell signaling events required to stimulate ASCs to self-organize into vascularized bone. In addition, we examine whether exogenous PDGF-BB can synergize complex tissue formation in ASC cultures by enhancing vascular stability and osteogenic differentiation. This overall approach provides us with a robust protocol to engineer vascularized bone with ASCs *in vitro*.

Materials and Methods

ASC isolation

Cellular isolation was performed at the Stem Cell Biology Laboratory, Pennington Biomedical Research Center, under an Institutional Review Board approved protocol (#PBRC 23040) according to published methods.³⁹ Briefly, fresh human subcutaneous adipose lipoaspirate was obtained under informed consent from Caucasian female donors ($n=2$) undergoing elective liposuction surgery, with an average age of 46 years and average body mass index of 29.1. The lipoaspirate tissue was processed to isolate the adherent population from the stromal vascular cell fraction, as previously described.¹⁶ The adherent population (passage 0) was trypsinized and cryopreserved⁴⁰ for shipment to the Johns Hopkins University.

ASC expansion and characterization

ASCs were thawed and expanded for one passage in a growth medium: high-glucose DMEM (Gibco Invitrogen) with 10% fetal bovine serum (FBS; Atlanta Biologicals), 1% penicillin/streptomycin (GIBCO Invitrogen), and 1 ng/mL FGF-2 (PeproTech). Cells were then trypsinized and used at passage two for all experiments. The phenotypic profile of the cells at this passage was examined via flow cytometry for mesenchymal (CD73, CD105) and vascular markers (CD31, CD34, VEGFR-2, and α -smooth muscle actin [α SMA]). Briefly, detached cells were suspended in phosphate-buffered saline (PBS) containing 2% FBS and incubated with monoclonal antibodies conjugated to fluorescein isothiocyanate or phycoerythrin for 30 min at 4°C. Cells were analyzed with a flow cytometer (BD Accuri C6). All antibodies were purchased from BD Biosciences.

Spheroid formation via hanging drop

Cells were trypsinized and resuspended at a concentration of 400,000 cells/mL (this varied in the aggregation study, where different cluster sizes were being compared) in a culture medium containing 0.24% (w/v) methylcellulose (Sigma). The cell suspension was pipetted as 10- μ L drops onto inverted Petri dish caps, which were then reverted and placed on dish bottoms containing sterile water to reduce evaporation. Dishes were incubated at 37°C overnight to allow cellular aggregation at the air–liquid interface. The dish caps were then flooded with PBS to allow bulk collection and transfer of the spheroids to conical tubes.

Fibrin encapsulation

Settled spheroid pellets were resuspended in fibrinogen (8 mg/mL final; Sigma), followed by the addition of thrombin (2 U/mL final; Sigma) to initiate gelation. Fibrin gels (35 μ L) containing 40 spheroids each (in the aggregation study, the number of spheroids per gel was varied to keep the total cell number constant) were pipetted into 6-mm-diameter wells and incubated at 37°C for 30 min to allow complete gelation before the addition of the culture medium. Samples were then cultured for 2 to 3 weeks, depending on the experiment, with media changed every other day.

Media preparation

The vascular medium (VM) consisted of the endothelial basal medium-2 (Lonza), 6% FBS, 1% penicillin/streptomycin, 10 ng/mL VEGF₁₆₅, 1 ng/mL FGF-2, and 1 μ g/mL L-ascorbic acid-2-phosphate (Sigma). For monolayer osteogenic differentiation experiments, the control medium consisted of the low-glucose DMEM (Gibco Invitrogen), 6% FBS, and 1% penicillin/streptomycin; the osteogenic medium (OM) consisted of the control medium plus 10 mM β -glycerophosphate (Sigma) and 50 μ M L-ascorbic acid-2-phosphate. For osteogenic differentiation in fibrin gels, OM also included 10 ng/mL VEGF₁₆₅ and 1 ng/mL FGF-2 to support vascular viability. The composition of FGF-2 and dexamethasone in the VM and OM were optimized to support both vascular and osteogenic development (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). Media were supplemented with additional growth factors (i.e., PDGF-BB or bone morphogenetic protein-2

[BMP-2]) depending on the experiment. All growth factors were purchased from PeproTech.

Osteogenic differentiation assays

For monolayer experiments, ASCs were seeded at 5000 cells/cm² and cultured in either the control medium or OM for 21 days, with the addition of either PDGF-BB or PDGFR inhibitor tyrphostin AG1295 (Santa Cruz Biotechnology). Mineralization was assessed via Alizarin Red S staining and quantification of the calcium content. For fibrin gel experiments, samples were cultured in the VM for up to 8 days followed by up to 12 days of OM supplemented with VEGF and FGF-2. Mineralization was assessed via quantification of the calcium content normalized to the DNA content.

For Alizarin Red S staining, monolayers were washed twice with PBS, fixed with 3.7% formaldehyde for 20 min, and then washed again three times. Samples were subsequently incubated for 10 min with 40 mM Alizarin Red S (Sigma) and washed extensively before imaging. To quantify the calcium content in each sample, monolayers or whole fibrin gels were washed twice with PBS, and then incubated with 0.5 N HCl overnight at 4°C with agitation. The calcium content in the sample supernatants was quantified using a colorimetric Calcium LiquiColor Test (Stanbio). The DNA content was quantified using a PicoGreen dsDNA quantitation kit (Molecular Probes) as previously described.¹⁶

Whole-mount immunostaining

All incubation and washing steps were carried out at 4°C with gentle agitation. Samples were fixed with 3.7% formaldehyde for 3 h, and then washed with PBS three times for 30 min each. Fixed gels were carefully removed from their wells and transferred to microcentrifuge tubes for subsequent staining procedures. Gels were permeabilized and blocked for 4 h with 0.2% Triton X-100 and 5% normal goat serum (Sigma) in PBS with 0.1% Tween (PTw). Samples were then incubated overnight with primary antibodies diluted in a blocking solution, followed by three 1-h washes in PTw. Primary antibodies included mouse anti-CD31 (4 µg/mL; Santa Cruz Biotechnology) and either rabbit anti-laminin (7 µg/mL; Sigma) or rabbit anti-osteocalcin (10 µg/mL; Santa Cruz Biotechnology). Samples were then incubated overnight with DyLight 488-conjugated goat anti-mouse and DyLight 649-conjugated goat anti-rabbit (both 1:400; Jackson ImmunoResearch) diluted in the blocking solution, followed by three 1-h washes in PTw. Lastly, samples were blocked in 5% normal mouse serum for 4 h, incubated overnight with Cy3-conjugated mouse anti- α SMA (7 µg/mL;

Sigma), and then washed three times for 1 h each. Gels were mounted in 70% glycerol in chamber slides and imaged using a Zeiss LSM 510 confocal microscope with a 5 $\times$ objective.

Image analysis

Confocal z-stacks of immunostained gels were z-projected and thresholded for subsequent quantification of vessel network parameters ($n=6$ images per group). Thresholded images were analyzed with AngioQuant software⁴¹ to quantify the total vessel length, area, and interconnectivity. ImageJ software (NIH) was used to quantify pericyte coverage of vessels, which was defined as α SMA+ area within at least 5 µm of the abluminal face of vessel networks. Briefly, vessel networks were selected in the CD31 channel of thresholded image composites, and selections were enlarged by 5 µm at all edges. These enlarged selections were applied to the α SMA channel, and percent α SMA+ area within the selection was quantified (denoted as % vessel pericyte coverage). The mean intensity of the α SMA+ population was quantified by measuring the mean pixel intensity within the nonthresholded α SMA channel viewfield. Lastly, to quantify the amount of basement membrane protein deposited along the vessels, the enlarged vessel selection was applied to the nonthresholded laminin channel, and the mean pixel intensity within the selection area was quantified (denoted as laminin mean intensity).

Statistical analysis

Quantitative data are expressed as mean $\pm$ standard error. Multigroup comparisons were determined by one-way ANOVA with the Tukey's test for *post hoc* analysis. Significance levels are denoted as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

Cell characterization

ASCs at passage 2 were primarily positive for mesenchymal markers CD73 (98.4%) and CD105 (80.1%) and predominantly negative for markers of the endothelial lineage (5.2% CD34+; 0.3% VEGFR+; and 0.8% CD31+) or the pericyte marker α SMA (1.1%) (Fig. 1).

Effects of cellular aggregation on vascular morphogenesis

To study the effects of cellular aggregation on vascular morphogenesis of ASCs in a 3D fibrin gel, cells were encapsulated either as a monodispersed cell suspension or

FIG. 1. Phenotypic distribution of initial cell population. Adipose-derived stromal/stem cells (ASCs) at passage 2 were primarily positive for mesenchymal markers CD73 and CD105 and predominantly negative for markers of the endothelial lineage and the pericyte marker, alpha-smooth muscle actin (α SMA).

spheroids containing various cell numbers (1000, 4000, or 10,000 cells per spheroid) (Fig. 2A). The total cell number remained constant among groups (1.6×10^5 cells/sample). After 14 days of culture in the VM, spheroid aggregates yielded significantly larger (up to 2.1-fold) and more interconnected (up to 5.5-fold) vessel networks than monodispersed cells (Fig. 2B–D). Vessel coverage with pericytes and laminin also increased with increased initial aggregation, up to 4000 cells per spheroid (Fig. 2E, F). With the largest spheroid size (10,000 cells per spheroid), the spatial distribution of the networks was less uniform with larger distances between aggregates. Based on these results, spheroid aggregates containing 4000 cells each were used for the remaining studies.

Sequential addition of factors

To determine the effects of osteogenic factors on vascular morphogenesis, fibrin-encapsulated spheroids were cultured for 0, 4, 8, or 12 days in the VM before being transitioned to the OM for the remaining culture period (Fig. 3A). All groups

were cultured for a total of 12 days. ASCs cultured in the VM for 8 days before addition of osteogenic factors yielded 3.2-fold longer vascular networks than those cultured in the OM for the entire period (Fig. 3B). Interestingly, the mean α SMA+ intensity increased significantly with increasing length of time in the OM (Fig. 3C), with an apparent increase in the number of α SMA+ and a reduction in their association with the nearby vessels. Extended culture in the OM yielded 2.4-fold less laminin deposition around vessels than in the VM (Fig. 3D). Thus, while vascular factors were still present in the OM, delaying the addition of osteogenic factors by at least 8 days provided more favorable conditions to induce vascular network growth. This sequential protocol resulted in early mineral deposits located along vessel tracks (Fig. 3E).

Independent effects of exogenous PDGF-BB on each lineage

The effects of exogenous PDGF-BB on osteogenic differentiation and vascular morphogenesis of ASCs were studied

FIG. 2. Effects of cellular aggregation on vascular morphogenesis. (A) ASCs were encapsulated in the fibrin gel as monodispersed cells or as spheroid aggregates (sph) of different sizes. (B) Vascular networks developed after 14 days of culture in the vascular medium (VM), staining positively for CD31 (green), α SMA (red), and laminin (blue). Compared to monodispersed cultures, vascular networks derived from spheroids were longer (C), more interconnected (D), and displayed greater coverage by pericytes (E) and laminin (F). Scale bars=500 μ m. Significance indicated as * p <0.05, ** p <0.01, or *** p <0.001 versus monodispersed. Color images available online at www.liebertpub.com/tea

FIG. 3. Sequential addition of factors. (A) Fibrin-encapsulated spheroids were cultured for 0, 4, 8, or 12 days in the VM before switching to the osteogenic medium (OM). All samples were cultured for 12 days. (Scale bar = 500 μ m). Total vascular network length (B) and laminin coverage (D) were significantly higher when the transition to the OM was delayed by several days, whereas α SMA staining intensity increased substantially with longer culture periods in the OM (C). (B–D) Significance indicated as * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ versus 0 days. (E) The stepwise protocol resulted in early mineral deposits being deposited in the vicinity of established vessels (arrows). Scale bar = 50 μ m. Color images available online at www.liebertpub.com/tea

independently. ASCs were cultured in the OM for 21 days with the addition of either the PDGF-BB (0, 2, or 20 ng/mL) or PDGFR inhibitor AG1295 (0, 1, or 10 μ M). In the presence of exogenous PDGF-BB, ASC calcium deposition was significantly increased in a dose-dependent manner throughout the culture (Fig. 4A) as well as on a per cell basis (Fig. 4B). In the presence of AG1295, total calcium deposition was decreased in a dose-dependent manner (Fig. 4A), whereas calcium deposition per cell was unchanged relative to the OM-only group in the presence of the inhibitor (Fig. 4B).

In a separate experiment, fibrin-encapsulated spheroids were cultured in the VM for 14 days with the addition of PDGF-BB or AG1295. Addition of low-concentration (2 ng/mL) PDGF-BB resulted in a slight, 1.4-fold increase in the total vessel length (Fig. 4C, D). Overall, however, PDGF stimulation did not have statistically significant effects on the total vessel length (Fig. 4D). Vessels were noticeably wider with the addition of 20 ng/mL PDGF-BB (Fig. 4C). This was reflected quantitatively with a statistically larger total vessel area divided by the total vessel length ($p < 0.001$, data not shown). Pericyte coverage was reduced substantially by 2.9-fold in the presence of exogenous PDGF-BB, and only slightly reduced with the addition of AG1295 (Fig. 4E).

Combined effects of exogenous PDGF-BB on vascularized bone

To determine the overall effects of PDGF-BB on the co-development of vasculature and bone within the same culture, encapsulated spheroids were induced in a sequential manner of the VM for 8 days followed by the OM for 12 days with either 0, 2, or 20 ng/mL PDGF-BB added throughout. Exogenous PDGF-BB resulted in significantly greater vascular density and osteogenic differentiation than cultures without PDGF-BB (Fig. 5D). Specifically, the vascular network length was 2.7-fold greater when 20 ng/mL PDGF-BB was added (Fig. 5A, E), and the calcium content per cell was 5.2-fold greater (Fig. 5H). Osteocalcin production was semi-quantitatively measured to be 1.4-fold greater as well (Fig. 5C, G). Conversely, α SMA intensity was 2.1-fold less in the presence of 20 ng/mL PDGF-BB (Fig. 5B, F).

In a similar experiment, we directly compared PDGF-BB with BMP-2 to determine whether these results are unique to PDGF-BB signaling, as BMP-2 has also been shown to be both pro-osteogenic and proangiogenic. Encapsulated spheroids were cultured in the VM for 8 days followed by the OM for 12 days with the addition of either 20 ng/mL

FIG. 4. Independent effects of exogenous platelet-derived growth factor-BB (PDGF-BB) on each lineage. Exogenous PDGF-BB increased mineral deposition in osteogenic cultures, as indicated by Alizarin Red S staining (**A**) and calcium quantification (**B**). Conversely, blocking endogenous PDGF receptor signaling with AG1295 greatly reduced the overall mineral deposition (**A**), but not on a per-cell basis (**B**). In vascular cultures, vessel network length was minimally affected by PDGF signaling (**C**, **D**), whereas pericyte coverage was substantially reduced in the presence of exogenous PDGF-BB (**C**, **E**). Scale bar = 500 μ m. Significance indicated by brackets in (**B**, **D**, **E**) as * p < 0.05, ** p < 0.01, or *** p < 0.001. Significance indicated in (**B**) as #### p < 0.001 versus PDGF groups, and all OM groups were significantly greater (p < 0.001) than control. Color images available online at www.liebertpub.com/tea

PDGF-BB or 20 ng/mL BMP-2. Cultures with PDGF-BB resulted in 4.2-fold greater calcium deposition and 5.6-fold greater calcium per cell than those with BMP-2 (Fig. 6H). The total vascular network length was also 2.3-fold higher in the presence of PDGF-BB than BMP-2 (Fig. 6A, E).

Discussion

Prevascularizing tissue-engineered grafts before implantation has been shown to accelerate anastomosis with host vasculature and increase the survival of the tissue construct.^{42–44} However, engineering vascularized bone has been a challenge in that, the factors that are traditionally used in the differentiation medium to induce either osteogenic differentiation or angiogenic behaviors can be mutually inhibitory when combined in mixed cultures.⁵ The mutual inhibition is particularly devastating for the vascular component, as vessels have proven to be delicate and unstable in the presence of osteogenic cues *in vitro*. This study has demonstrated that exogenous PDGF-BB helps circumvent these issues by enhancing vascular growth and stability, as well as promoting robust osteogenic differentiation and

mineralization. This is the first demonstration of cooperative growth yielding robust vascular networks within the dense mineralized matrix.

Our previous studies demonstrated that cultured ASCs contain minute vascular subpopulations that are fully capable of forming stable vascular structures on 2D substrates when seeded at high densities.¹⁶ An interesting observation in that earlier study was that the cells spontaneously self-assembled into dense clusters of cells before extensive vascular growth, suggesting that heterotypic cell-cell interactions were important to the process. Here we confirm that cellular aggregation is essential to the formation of dense, stable vascular networks by ASCs. The aggregation of ASCs into spheroids before encapsulation into 3D fibrin gels significantly enhanced their resulting vascular network density and interconnectivity, pericyte coverage, and deposition of vascular basement membrane. This novel finding complements previous work demonstrating that aggregation of pure endothelial cells into spheroids or on the surface of microbeads reduces apoptosis and greatly enhances vascular sprouting.^{45,46} ASCs specifically have been shown to reside in perivascular sites in native adipose tissues^{47,48} and

FIG. 5. Combined effects of exogenous PDGF-BB on vascularized bone. Encapsulated spheroids were cultured for 8 days in the VM, followed by 12 days of the OM with 0, 2, or 20 ng/mL of PDGF-BB added throughout the entire culture period. Total vessel length (A, E), osteocalcin production (C, G), and calcium deposition (H) increased, while α SMA staining intensity (B, F) decreased with the increasing PDGF-BB concentration (D) overlay of all channels. Scale bar = 500 μ m. Significance indicated as * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ versus 20 ng/mL PDGF-BB. Color images available online at www.liebertpub.com/tea

stabilize vascular networks *in vitro*.^{48,49} Therefore, aggregation may also serve to bring the pericyte-like cells closer to the endothelial subpopulation to help support and stabilize them.

While ASC spheroids were able to sprout dense vascular networks in the VM, vascular growth was severely limited in the OM, which consisted of VM plus β -glycerophosphate and higher ascorbic acid. β -glycerophosphate is an essential initiator of mineralization, as cells cannot synthesize their own phosphate. However, it may be a possible source of vascular inhibition after it is broken down into inorganic phosphate by an alkaline phosphatase activity.⁵⁰ High inorganic phosphate levels (>2.5 mM) have been shown to induce endothelial cell apoptosis via increased generation of reactive oxygen species.⁵¹ To compensate for this, delaying the addition of osteogenic factors by 1 week allowed the vessels to grow and establish stable networks before inducing osteogenic differentiation of the nonendothelial subpopulation. This sequential process is similar to what occurs in bone development and fracture repair, in which, an invading vasculature is a prerequisite for the entry of osteogenic progenitors and subsequent mineralization.^{52,53} This established vascular network serves to bring nutrients and progenitor cells to the highly active site of regeneration,^{27,54} as well as serving as a template around which mineral will

be deposited.⁵³ Establishment of vascular networks before osteogenesis helped to stabilize the networks with pericytes and increased laminin deposition, possibly shielding them from the inhibitory factors in the second phase.

This study has demonstrated that exogenous PDGF-BB at 20 ng/mL further helps to circumvent issues of mutual inhibition by enhancing both the osteogenic and vascular development of ASCs. In osteogenic cultures, PDGF-BB markedly increased mineral deposition overall and on a per cell basis. Conversely, addition of the inhibitor AG1295 reduced overall mineral deposition, while the relative amount per cell remained unchanged. These results suggest that PDGF signaling might not be required for osteogenic differentiation of ASCs, but it may significantly amplify their responsiveness to osteogenic cues, leading to the production of greater amounts of mineral. This response may be somewhat unique to ASCs, as previous studies have shown that PDGF signaling either has no effect or even an inhibitory effect on the osteogenic differentiation of bone marrow-derived MSCs.³³⁻³⁷

In vascular cultures, PDGF signaling had minimal effects on vascular network properties, with only low levels of exogenous PDGF-BB contributing to a slight increase in network density. PDGF signaling did, however, significantly affect pericyte coverage of the vessels. Exogenous PDGF-BB

FIG. 6. Direct comparison of PDGF-BB versus bone morphogenetic protein-2 (BMP-2) on vascularized bone. Encapsulated spheroids were cultured for 8 days in the VM, followed by 12 days of the OM with the addition of 20 ng/mL of either PDGF-BB or BMP-2. Exogenous BMP-2 induced no significant changes in either the vascular network length (A, E) or calcium content (H) versus the control group (VM-OM), whereas PDGF-BB was substantially greater than both. (B, F) α SMA staining intensity was much greater with BMP-2 versus PDGF-BB, but only slightly greater than control. (C, G) Osteocalcin staining intensity was similar between PDGF-BB and BMP-2 groups (D) overlay of all channels. Scale bar = 500 μ m. Significance indicated as * p < 0.05, ** p < 0.01, or *** p < 0.001 versus 20 ng/mL PDGF-BB. Color images available online at www.liebertpub.com/tea

caused a significant decrease in pericyte coverage, likely by masking endogenous gradients and stimulating the pericytes to migrate randomly. Interestingly, the inhibitor AG1295 did not significantly reduce pericyte coverage. This is possibly a result of the cellular aggregation before encapsulation, in which, the growing vessels are already in direct contact with pericyte-like cells, thereby reducing the necessity to recruit pericytes via a PDGF gradient.

PDGF-BB appeared to play a different role in maintaining vascular stability in the presence of osteogenic factors. Using a sequential protocol of a vascular phase followed by an osteogenic phase, the addition of 20 ng/mL PDGF-BB was able to support vascular growth and stability as well as amplify mineral deposition. There are some hypotheses in the literature about how exactly this occurs. A recent review discusses the possible roles of PDGF-BB signaling in terms of bone fracture repair.²⁷ When bone is injured, high concentrations of PDGF-BB may cause vascular pericytes and other osteoprogenitors to populate the injury site and increase production of mineral. These cells may also respond to PDGF-BB by secreting higher levels of VEGF to recruit angiogenesis toward the regenerating tissue.^{27,55} In the case of our own study, this increased endogenous production of VEGF may be furthering the stability of established vessels in the osteogenic environment. It may also be possible that the vessels in these cultures are responding directly to the exogenous PDGF-BB via PDGFRs.^{56,57} However, further stud-

ies are needed to identify the specific mechanisms underlying these morphogenic events within the ASC cultures.

An interesting observation in our study was that the expression of α SMA changed dramatically throughout the induction period. α SMA aids cellular contractility and is primarily expressed by vascular smooth muscle cells and pericytes.⁵⁸ In our study, initial ASC cultures exhibited only 1% α SMA-positive population. Before the application of osteogenic supplements, α SMA+ cells were primarily located in perivascular locations. However, following exposure to osteogenic supplements, there was a dramatic increase in the intensity and number of cells expressing this marker. This change in expression profile reflects what is observed in native mineralizing bone: osteoblasts upregulate expression of α SMA during active mineralization, whereas resting osteoblasts and osteocytes do not express α SMA.⁵⁹ However, the current study also showed that ASCs treated with 20 ng/mL PDGF-BB in the presence of osteogenic cues exhibited substantially lower α SMA expression at day 20 than those cultured without PDGF-BB even though the mineral content in PDGF-BB cultures was significantly higher suggesting that a α SMA expression alone does not directly correlate with increased osteogenesis.

Lastly, we examined whether this mutually beneficial response exhibited by cells in the presence of PDGF-BB was unique by comparison to BMP-2, the most widely studied and clinically applied pro-osteogenic growth factor. BMP-2

has been shown to significantly enhance osteogenic differentiation and *in vivo* bone growth,⁶⁰ as well as indirectly enhance vascular growth via promotion of VEGF production.⁶¹ The current study shows through direct comparison that PDGF-BB is considerably more effective than BMP-2 at both amplifying osteogenic differentiation of ASCs and maintaining vascular stability when applied at 20 ng/mL. Importantly, this concentration of PDGF (20 ng/mL) is only 5 to 10 times the concentration in normal human serum⁶² and is within the physiological range measured during bone injury.⁶³ Clinical safety is imperative, as supraphysiological concentrations may lead to adverse side effects such as bone overgrowth and inflammation.⁶⁴ Together, these results indicate that, in combination with ASCs, PDGF-BB may be better suited than BMP-2 for the enhancement of both bone and vasculature at physiological concentrations.

In summary, we have demonstrated that ASCs from a single source can be driven to form robust vascularized bone with the addition of exogenous PDGF-BB. Cellular aggregation was key in encouraging heterotypic cell-cell interactions and inducing dense, stable vascular networks. These factors, together with a delayed addition of osteogenic cues were essential to recapitulate intrinsic developmental profiles that instructed the cells to grow into complex tissue grafts with minimal manipulation compared to existing models. This induction protocol using physiological levels of PDGF-BB can be utilized to engineer vascularized bone grafts with greater efficiency and potential for subsequent integration and functionality.

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Disclosure Statement

The authors have no conflicts of interest or disclosures to report.

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