ORIGINAL ARTICLE

Preformed Vascular Networks Survive and Enhance Vascularization in Critical Sized Cranial Defects

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Vascular networks provide nutrients, oxygen, and progenitor cells that are essential for bone function. It has been proposed that a preformed vascular network may enhance the performance of engineered bone. In this study vascular networks were generated from human umbilical vein endothelial cell and mesenchymal stem cell spheroids encapsulated in fibrin scaffolds, and the stability of preformed vascular networks and their effect on bone regeneration were assessed in an *in vivo* bone model. Under optimized culture conditions, extensive vessel-like networks formed throughout the scaffolds *in vitro*. After vascular network formation, the vascularized scaffolds were implanted in a critical sized calvarial defect in nude rats. Immunohistochemical staining for CD31 showed that the preformed vascular networks survived and anastomosed with host tissue within 1 week of implantation. The prevascularized scaffolds enhanced overall vascularization after 1 and 4 weeks. Early bone formation around the perimeter of the defect area was visible in X-ray images of samples after 4 weeks. Prevascularized scaffolds may be a promising strategy for engineering vascularized bone.

Keywords: prevascularization, coculture, bone tissue engineering, angiogenesis, mesenchymal stem cells

Introduction

DESPITE ADVANCES IN RECONSTRUCTIVE SURGERY and regenerative medicine, large volume bone defects remain a significant clinical challenge.¹ Current treatment options have a number of drawbacks, including limited donor tissue availability, donor site morbidity, infection risk, and immune rejection.² In addition, grafts may fail due to a lack of integration with host tissue and core necrosis. Tissue-engineered bone grafts have the potential to overcome many of these limitations.³

Vascularization is critical for the success of tissue-engineered bone grafts, as blood vessels supply oxygen, nutrients, and osteoprogenitor cells necessary for bone healing or remodeling after injury. Vascularization can be induced in bone grafts through the delivery of proangiogenic growth factors,^{4–6} though vascular ingrowth is often too slow to overcome core ischemia and graft necrosis in larger volume applications. Various surgical approaches can enhance construct vascularization using the patient's own healing capacity,^{7,8} but these procedures often require

multiple surgeries and their success is still limited by the rate of tissue formation. Designing an implant with an established vascular network that quickly connects and establishes flow with host vessels may enhance implant viability and improve outcomes.

Scaffolds seeded with endothelial cells (ECs) and bone progenitor cells have been examined for bone tissue engineering after a brief *in vitro* culture time before implantation. In these conditions, the cells are unlikely to have sufficient time to form extensive network structures *in vitro*.^{9–11} Failure of these implanted cells to organize and establish flow may result in hypoxia and low cell survival rates, which is hypothesized to contribute to poor bone formation in these co-seeded scaffolds.^{12,13} Various studies demonstrate vascular tube formation *in vitro* but have not evaluated the performance of these vascularized systems *in vivo*.^{14,15} The survival and anastomosis of prevascularized scaffolds have been successfully demonstrated in subcutaneous implantation.^{16–19} However, the survival, anastomosis, and function of preformed vascular networks may vary significantly when implanted in the bone niche. For the success of tissue-engineered bone

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grafts, preformed vascular networks must be studied in bone healing environments.

In this study, we demonstrate the formation of extensive, interconnected vessel-like networks using ECs and mesenchymal stem cells (MSCs) cocultured within fibrin scaffolds. In this system, MSCs were essential for EC assembly into networks. The prevascularized scaffolds were then implanted in a bone critical sized defect model to examine their effect on bone regeneration and vascularization *in vivo*.

Materials and Methods

Cell culture and spheroid formation

Human bone-marrow-derived mesenchymal stem cells (hMSCs; Lonza, Walkersville, MD) were cultured in T75 flasks in complete MSC growth medium (MSCGM; Lonza) and used at passages 4–6. Human umbilical vein endothelial cells (HUVECs; Lonza) were cultured in T75 flasks in complete endothelial growth medium-2 (EGM-2; Lonza) and used at passages 3–5. Upon reaching confluence, cells were washed with phosphate-buffered saline (PBS) and trypsinized. To create spheroids, varying ratios of HUVECs and MSCs (0%, 10%, 30%, 50%, 70%, 90%, or 100% MSCs, 5000 cells per spheroid) were incubated overnight in 150 μ L EGM-2 with 0.25% wt/vol. methylcellulose in a nonadherent round-bottomed 96-well plate.²⁰

Fibrin scaffold formation

Fibrin gel scaffolds were formed by combining 80 μ L each of human fibrinogen (4 mg/mL; Sigma-Aldrich, St. Louis, MO) and bovine thrombin (4 U/mL; Sigma-Aldrich) in a 48-well plate for a final concentration of 2 mg/mL. Spheroids were placed on top of the base fibrin layer (one spheroid per scaffold for the *in vitro* studies, five per scaffold for the animal study) and encapsulated in a top layer of 80 μ L each fibrinogen and thrombin, for a final total volume of 320 μ L. Samples ($n=5$ per group per time point) were incubated in EGM-2 at 37°C and 5% CO₂ for 7, 14, or 21 days. After incubation, samples were fixed in 10% phosphate-buffered formalin (Fisher Scientific, Pittsburgh, PA) for 20 min for subsequent analysis.

Immunofluorescent staining and analysis

Immunofluorescent staining was performed for the EC marker CD31 and α -SMA, expressed by hMSC. After fixation, samples were washed in PBS and blocked with 6% normal goat serum (MP Biomedicals, Solon, OH). Samples were incubated overnight with 1:200 mouse antihuman CD31 (clone JC70A; Dako, Carpinteria, CA) and 1:600 rabbit anti- α -SMA (Abcam, Cambridge, MA) primary antibodies with 2% normal goat serum in phosphate buffered saline (PBST) (0.5% Triton X-100 in PBS) at 4°C overnight. After incubation, samples were washed in PBST, and incubated with 1:200 tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antimouse IgG and 1:200 fluorescein isothiocyanate-conjugated donkey antirabbit IgG secondary antibodies (Jackson ImmunoResearch, West Grove, PA) in PBST for 4 h at room temperature. Samples were imaged with a Zeiss LSM 5 PASCAL confocal microscope. Images were taken in a z-stack through the full volume of EC outgrowth, and two-dimensional (2D) pro-

jections were created for all samples using Pascal software (Carl Zeiss, Germany). If necessary, mosaic images were stitched together using the MosaicJ plugin for ImageJ (NIH).^{21,22}

The projection images were used for analysis of EC network outgrowth and analyzed using Axiovision AC 4.5 (Carl Zeiss). The area of the CD31-positive cellular network was manually outlined and measured for all samples. For length and diameter measurements, eight individual vessel-like structures approximately evenly distributed around the circumference of the spheroid were randomly selected for each sample. Length was measured from the center of the spheroid core to the end of the vessel-like structure. Diameter was measured across the width of the structure.

Cranial defect surgeries

All animal experiments were performed at Edward Hines, Jr. VA Hospital in accordance with protocols approved by the Institutional Animal Care and Use Committee (protocol #H13-021). Cranial defect surgeries were performed on 30 male athymic RNU nude rats (Charles River Laboratories, Wilmington, MA) aged 13–15 weeks according to an established procedure.²³ In brief, rats were anesthetized using 3% isoflurane and placed in a small animal stereotaxic instrument (Kopf Instruments, Tujunga, CA) to stabilize the skull. Five hundred microliters lidocaine (1%) was injected subcutaneously as a local analgesic. A 3 cm incision was made longitudinally along the midline of the skull, and the periosteum was preserved and pulled back to expose the calvarium. An 8 mm diameter, full thickness defect was created using a trephine bur under saline irrigation. Two scaffolds were placed in each defect (total implanted scaffold volume is 640 μ L). The periosteum was sutured closed using 5-0 resorbable sutures, and skin was sutured using 4-0 nylon sutures. Drops of cefazolin were administered along the suture line to prevent infection. Animals were sacrificed at 1 week to assess the survival and anastomosis of the implanted vascular networks, and at 4 weeks to assess vascularization and initial bone growth ($n=5$ rats per group per time point).

At the time of sacrifice, animals were perfused with AlexaFluor 647-conjugated GSL I-isolectin B₄ (Invitrogen, Carlsbad, CA) to label perfused vasculature. Animals were anesthetized with 3% isoflurane maintained throughout the procedure. Isolectin (250 μ L of 400 μ g/mL isolectin in PBS) was injected through tail vein using a 27 gauge butterfly needle, flushed with heparinized saline (1% heparin in 0.9% sodium chloride), and circulated for 10 min. After lectin circulation, animals were euthanized through CO₂ overdose and fixed through perfusion fixation with 10% phosphate-buffered formalin. Harvested samples were formalin fixed and prepared for X-ray imaging. After X-ray imaging, samples were decalcified overnight in Cal-Ex decalcifying solution (Fisher Scientific), and paraffin embedded for histological staining.

X-ray imaging

Imaging was performed using Kevex PXS10-65 W micro-focus tungsten X-ray tube as a radiation source. The tube voltage and power were 50 kV and 25 W, respectively. The anode spot diameter was ~ 25 μ m. In this experimental setup

we used a pixelated (imaging) X-ray camera, specifically Shad-o-Box 2048 from Teledyne Rad-icon Imaging Corp. The collected images were 2048×1024 pixels, where each pixel size was $48 \mu\text{m}$. Camera used a $\sim 65 \mu\text{m}$ thick Gadox scintillation material ($\text{Gd}_2\text{O}_3:\text{Tb}$; Kodak Min-R 2190). By controlling source-to-object-to-detector distances (magnification effect) we obtained $\sim 50 \mu\text{m}$ full width at half maximum resolution at the object plane. The camera sensor (RadEyeEV) was chilled down to -15°C to decrease the dark current. This was achieved using a specially designed helium-flushed enclosure cooled by using TeTechnology, Inc. Peltier cooling assembly. This method allowed us to achieve camera's temperature stability of $\sim 0.01^\circ\text{C}$.

X-ray image analysis

The mean intensity within the defect area was quantified using a custom MATLAB script. Blinded full-resolution images were loaded, and the defect region was defined manually and confirmed with histological images.

Histology

Hematoxylin and eosin (H&E) and Masson's trichrome staining was performed on paraffin-embedded sections ($10 \mu\text{m}$ thickness) to visualize tissue structure. Stained sections were imaged using an Axiovert 200 inverted microscope ($5 \times$ objective, $1.10 \mu\text{m}/\text{pixel}$), and area of new bone formation within the defect was quantified as a percentage of total tissue area within the defect. Three nonconsecutive sections were analyzed per sample using Axiovision software.

Immunohistochemical staining

Immunohistochemical staining for CD31, an EC marker, was used to visualize vascularization within the defect area. Immunohistochemical staining for the osteogenic markers osterix and osteopontin was performed to evaluate bone regeneration. Paraffin-embedded sections ($10 \mu\text{m}$ thickness) were deparaffinized, rehydrated, and underwent antigen retrieval by incubating in target retrieval solution (Dako) for 30 min in a steamer. Sections were blocked with 5% normal goat serum and incubated with one of the three following primary antibodies at 4°C overnight: 1:700 rabbit polyclonal CD31 (Santa Cruz Biotechnologies, Santa Cruz, CA), 1:300 rabbit polyclonal osterix (Abcam), or 1:300 rabbit polyclonal osteopontin (Abcam). Secondary antibody staining was performed using an antirabbit Vectastain Elite ABC kit (Vector Labs, Burlingame, CA) according to the manufacturer's protocol, and stains were visualized with DAB (Invitrogen). Positive CD31 staining was confirmed based on the pattern of vessel staining and quantified as total number of vessels per tissue area.

Human-specific CD31 staining was used to identify the implanted human ECs. The same procedure was used as detailed above, replacing the primary antibody with 1:50 mouse antihuman CD31 (clone JC70A; Dako) and using an antimouse Vectastain Elite ABC kit (Vector Labs).

Immunofluorescent staining of frozen sections

Immunofluorescent staining for human-specific CD31 was performed on frozen tissue sections to compare with the perfused lectin and identify perfused human vessels. Sections

were thawed, washed in PBS, and blocked with 6% normal goat serum (MP Biomedicals). Sections were incubated at room temperature overnight with 1:100 mouse antihuman CD31 (clone JC70A; Dako) primary antibody with 2% normal goat serum in PBST (0.5% Triton X-100 in PBS). After incubation, samples were washed in PBST and incubated with 1:200 TRITC-conjugated goat antimouse IgG secondary antibody (Jackson ImmunoResearch) in PBST for 2 h at room temperature. Samples were imaged with a Zeiss LSM 5 PASCAL confocal microscope to identify TRITC-labeled human ECs and AlexaFluor 647-labeled perfused vessels. Human perfused vessel density (vessel structures positive for both perfused lectin and human CD31 per tissue area) was quantified using AxioVision software. Mean vessel density was calculated from four images of each of three nonconsecutive tissue sections per sample (total of 12 images per sample).

Statistical analysis

All data are shown as mean $\pm$ standard deviation. Statistical significance between groups was determined with *t*-test (human-specific vessel quantification), one-way (X-ray image data) or two-way (all other data) analysis of variance (ANOVA) using SigmaStat (San Jose, CA). Tukey's post hoc test was used to compare differences between groups. In all comparisons, $p < 0.05$ was considered statistically significant.

Results

Optimization of prevascularized scaffolds in vitro

Cellular outgrowth. Coculture spheroids of HUVECs and human MSCs were suspended in fibrin scaffolds and cultured in endothelial growth medium. Spheroids cultured in MSC growth medium did not form vessel-like structures (data not shown). The HUVEC/MSC ratio in the spheroids was varied, and in EC media cellular outgrowth from the spheroid was observed throughout the culture period through brightfield microscopy in all conditions (Fig. 1). Immunofluorescent staining was performed for the EC marker CD31 and α -SMA, which is expressed by MSCs,²⁴ to identify the cell types and structures present. Staining showed that MSC migration preceded HUVECs at all time points. The total MSC outgrowth area was quantified at 1, 2, and 3 weeks through brightfield microscopy (Fig. 1D). Outgrowth area varied with the amount of MSCs in the spheroid and increased with time. The 50% MSC group had the highest amount of outgrowth area of $37.70 \pm 2.08 \text{ mm}^2$ at 3 weeks. Statistical analysis of the cellular outgrowth after 3 weeks is available in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/tea).

EC network formation. HUVEC network formation was observed through confocal microscopy imaging of CD31-stained samples (Fig. 2). HUVEC networks appeared to form within the areas first invaded by MSCs. The total outgrowth area of HUVEC varied with HUVEC/MSC ratio within the spheroid (Fig. 2G). With HUVEC-only spheroids (0% MSC group), vessel-like structures were not observed at any time point (Fig. 2D). Minimal sprouting and cellular migration were visible with 0% MSCs. CD31-positive structures were not observed in the 100% MSC group at any time point. In all other groups containing HUVECs, cells sprouted outward

FIG. 1. MSC outgrowth area varies with cell ratio. (A–C) Brightfield images of HUVEC/MSC spheroids (50% MSC group) encapsulated in fibrin scaffolds in a 48-well plate over time. Scale bar in each image is 500 μm. (D) MSC outgrowth area was quantified from brightfield images, and varied with culture time and HUVEC/MSC ratio. The 50% MSC group had highest MSC outgrowth after 3 weeks of culture. Statistical significance bars shown for week 3 time point only. *indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$, and ****indicates $p < 0.0001$. $n = 5$ samples per group. HUVEC, human umbilical vein endothelial cell; MSC, mesenchymal stem cell. Color images available online at www.liebertpub.com/tea

throughout the fibrin to form interconnected networks of vessel-like structures (Fig. 2A–C) with lumens visible in some of the vessels (Fig. 2E). The EC network area increased with time in all groups (Fig. 2G). The 50% MSC group had the highest EC network area at all time points ($0.33 \pm 0.08 \text{ mm}^2$ at 1 week, $0.74 \pm 0.30 \text{ mm}^2$ at 2 weeks, and $1.80 \pm 0.27 \text{ mm}^2$ at 3 weeks). This higher area was statistically significant relative to the 10% ($p < 0.0001$), 70% ($p < 0.0001$), and 90% ($p < 0.0001$) MSC groups at week 3. Statistical analysis of the EC network area, length, and diameter after 3 weeks is available in Supplementary Table S1.

The length of individual vessel-like structures also increased with time (Fig. 2H). After 3 weeks of culture, the 50% MSC group had the highest vessel length ($736 \pm 88 \mu\text{m}$). This increase was statistically significant against 10% MSC ($p < 0.0001$), 70% MSC ($p = 0.0083$), and 90% MSC ($p < 0.0001$). The diameter of individual vessel-like structures tended to increase with time (Fig. 2I). After 3 weeks of culture, the 50% MSC group exhibited the largest vessel diameter of $39.84 \pm 2.24 \mu\text{m}$. This increase was statistically significant against 10% MSC ($p = 0.0024$).

The spatial relationship between HUVECs and MSCs was assessed through immunofluorescent staining for CD31 and α -SMA, and visualized with confocal microscopy (Fig. 2F). MSCs and HUVECs were often in proximity, with some MSCs appearing to orient in a pericyte-like fashion on the outer surface of the EC structures. However, this observation was not sufficient to determine the specific role of the MSCs in network development.

Multispheroid networks. For the cranial defect study, scaffolds were prepared with five encapsulated spheroids

distributed throughout a 320 μL fibrin scaffold. Two scaffolds were implanted in each effect (8 mm in diameter, ~1 mm in depth). Scaffolds contained either HUVEC/MSC spheroids (50% MSC, 2500 of each cell type per spheroid) or MSC-only spheroids (2500 cells per spheroid). Fibrin-only control scaffolds were also used for comparison. All samples were cultured for 3 weeks before implantation. Prevascularized samples were immunofluorescent stained for CD31 to assess network formation (Fig. 3). The general sprouting pattern was similar to that of scaffolds with one spheroid. Individual vessel-like structures from nearby spheroids appeared to sprout toward each other and connect (Fig. 3B). Each spheroid had an individual sprouting area of $1.65 \pm 0.32 \text{ mm}^2$, with a total spheroid network area of $7.01 \pm 1.28 \text{ mm}^2$ per scaffold, resulting in an ~10% vascular density (total vessel area/total scaffold area) within the scaffold. Individual vessel-like structures had an average length of $1.03 \pm 0.09 \text{ mm}$ and diameter of $17.85 \pm 0.95 \mu\text{m}$.

Cranial defect study

Vascularization within defect. Samples were harvested at 1 and 4 weeks after implantation. At the time of harvest, all defects were filled with tissue. The fibrin scaffolds were observed within the samples harvested at 1 week but not at 4 weeks. Tissue within the defects appeared more dense in week 4 samples. There were no observable differences in gross appearance between treatment groups.

After processing and sectioning, vascularization within the defect area was assessed through immunohistochemical staining for CD31 (Fig. 4) using a general antibody (rat and human), and the mean blood vessel density (number of CD31⁺

FIG. 2. EC network formation is dependent on MSCs. (A–C) Confocal microscopy images of immunofluorescent staining for CD31 (HUVEC, red) show formation of EC network in 50% MSC spheroids after 1 week (A), 2 weeks (B), and 3 weeks (C) of culture. Scale bar is 200 μ m. (D) CD31 staining of HUVEC-only spheroid after 2 weeks of culture. No sprouting or outgrowth is observed. Scale bar is 100 μ m. (E) CD31 staining of 50% MSC group after 3 weeks shows appearance of lumen and branching of tubular structures. Scale bar is 50 μ m. (F) CD31 and α -SMA staining (MSC, green) shows proximity of MSCs to ECs in 50% MSC group after 3 weeks of culture. Scale bar is 50 μ m. (G) Area of EC network was quantified from two-dimensional projections of three-dimensional z-stacks of confocal images. The 50% MSC group exhibited the highest network area after 3 weeks. In all, 0% and 90% MSC groups were significantly different from all other groups at 3 weeks. The length (H) and diameter (I) of individual vessels were quantified, with the highest of both in the 50% MSC group after 3 weeks of culture. Statistical significance bars shown for week 3 time point only. ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$. $n = 5$ samples per group. EC, endothelial cell. Color images available online at www.liebertpub.com/tea

vessels per square millimeter) was quantified (Fig. 4G). Vessels were observed throughout the entire defect area. Prevascularized scaffolds had the highest mean blood vessel density at both 1 week (205 ± 26 vessels/mm²) and 4 week (173 ± 30 vessels/mm²) time points. At 1 week, this density was significantly greater than both fibrin controls (104 ± 17 vessels/mm², $p < 0.0001$) and MSC-only scaffolds (114 ± 15 vessels/mm², $p = 0.0004$), and significant relative to fibrin scaffolds at 4 weeks (104 ± 27 vessels/mm², $p = 0.0114$).

Human CD31 analysis. Immunohistochemical staining was performed using a human-specific CD31 antibody to identify the implanted human cells (Fig. 5). After 1 week of implantation, human CD31-positive vessels were observed within the defect area, with erythrocytes visible within the lumens (Fig. 5B). The human vessels were primarily

localized to the fibrin scaffold and invaded tissue, and were not observed within the surrounding bone tissue at 1 week. Even after 4 weeks of implantation, perfused human vessels were present and visible within the tissues (Fig. 5C).

Human vessel density was calculated using human-specific CD31 staining and perfused lectin. Human CD31-positive structures were not visible in fibrin-only or MSC-only groups (Fig. 5E). Samples with prevascularized scaffolds at 1 week had a human vessel density of 11.3 ± 5.6 vessels/mm² and a density of 15.9 ± 9.1 vessels/mm² at 4 weeks (Fig. 5D). There was no statistically significant difference between 1 and 4 weeks.

Histological analysis of bone formation. H&E and Masson's trichrome staining was performed to assess tissue structure and bone regeneration (Fig. 6). After 1 week of

FIG. 3. Vascular network formation was extensive in multi-spheroid scaffolds used for *in vivo* studies. **(A)** Confocal microscopy mosaic image of immunofluorescent CD31 staining of MSC/HU-VEC (1:1 ratio) spheroids encapsulated in fibrin scaffolds after 3 weeks of culture. Scale bar is 1 mm. **(B)** Vessel-like structures sprouting from two nearby spheroids appear to connect with each other, forming interconnected spheroid networks. Scale bar is 200 μ m. Color images available online at www.liebertpub.com/tea

FIG. 4. Prevascularized fibrin scaffolds increase overall vascularization after cranial defect implantation. Nonspecific (human and rat) CD31 staining (*red arrows*) shows presence of blood vessels within the defect space in fibrin-only (**A, D**), MSC-only (**B, E**), or prevascularized (**C, F**) scaffolds after 1 week (**A–C**) and 4 weeks (**D–F**) of implantation. At week 1, some of the implanted fibrin is visible (*blue asterisks*). **(G)** Mean blood vessel density as measured from images of CD31 staining. Prevascularized scaffolds resulted in the highest blood vessel density at both 1 and 4 weeks. *indicates $p < 0.05$, ***indicates $p < 0.001$, and ****indicates $p < 0.0001$. $n = 5$ samples per group. Scale bar in all images is 50 μ m. Color images available online at www.liebertpub.com/tea

FIG. 5. Human EC networks are perfused after cranial defect implantation. (A, B) Staining for human-specific CD31 shows presence of implanted human blood vessels after 1 week of implantation (red arrows). (B) Erythrocytes are visible surrounded by human CD31-positive structures, suggesting perfusion of implanted vascular networks. (B inset) Vessel-like structures outside the defect area (black arrows) do not exhibit positive human-CD31 staining, indicating antibody specificity. (C) Confocal microscopy images of prevascularized scaffold at 4 weeks. Colocalization of human-CD31 stain and perfused isolectin indicates survival and perfusion of implanted human vessels. (D) Quantification of perfused human vessels in prevascularized scaffolds after 1 and 4 weeks of implantation as measured from confocal images. Human CD31-positive vessels were not present in fibrin or MSC-only groups. (E) Confocal microscopy images of MSC-only scaffold at 4 weeks. A perfused blood vessel is visible but not positive for hCD31. Scale bar in all images is 50 μ m. Color images available online at www.liebertpub.com/tea

implantation, soft tissue filled the defect space in all samples, but bone regeneration was not observed. At 4 weeks, neobone formation was observed in all samples at the perimeter of the defect. Highly organized collagenous tissue and immature bone were observed throughout the span of the defect, particularly in prevascularized and MSC-only scaffolds (Fig. 6B, C). Formation of mineralized tissue within the defect area was quantified from histological images, and classified as mature or immature. Prevascularized scaffolds resulted in the highest percent tissue area of both mature ($8.63\% \pm 2.96\%$) and immature ($36.34\% \pm 8.63\%$) bone (Fig. 6G), though the level was not statistically significant relative to MSC-only scaffolds ($6.53\% \pm 5.18\%$ mature, $31.28\% \pm 14.8\%$ immature) or fibrin controls ($4.32\% \pm 1.63\%$ mature, $28.26\% \pm 6.89\%$ immature).

Immunohistochemical staining for osteopontin, an extracellular matrix component of bone,²⁵ and osterix, a transcription factor involved in bone formation,²⁶ was performed to assess bone regeneration after 4 weeks of implantation (Fig. 7). Positive staining for osteopontin was observed in all groups (Fig. 7A, C, E), particularly in regions of dense tissue. Positive stain for osterix was observed within cell nuclei in all groups (Fig. 7B, D, F).

Bone formation through X-ray imaging analysis. X-ray imaging was used to evaluate calcification within the cranial defect area (Fig. 6D–F). Higher intensity was observed in the edges of the explants due to the bone thickness and skull curvature. No calcified tissue was observed in the defect region at 1 week in any experimental group. At 4 weeks,

FIG. 6. Bone formation after cranial defect implantation of prevascularized fibrin scaffolds after 4 weeks. (A–C) Histological images of H&E staining of (A) fibrin-only, (B) MSC-only, and (C) prevascularized scaffolds. The defect area is denoted by dashed black lines. New mature bone formation is indicated by blue asterisks, and new immature bone formation is indicated by blue arrows. Scale bar in each image is 500 μ m. (D–F) X-ray images of (D) fibrin, (E) MSC-only, and (F) prevascularized scaffolds. (G) Bone formation as percentage of total tissue area quantified from histological images. Prevascularized scaffolds resulted in the most bone formation though this increase was not statistically significant. (H) Bone formation as measured by mean intensity value within defect area quantified from X-ray radiographs. No statistically significant differences were found between groups. $n=5$ samples per group. H&E, hematoxylin and eosin. Color images available online at www.liebertpub.com/tea

FIG. 7. Immunohistochemical staining for osteogenic markers in samples harvested after 4 weeks of cranial defect implantation. Positive staining (appearing as red-brown) for osteopontin (A, C, E) and osterix (B, D, F) was present in samples receiving (A, B) fibrin-only scaffolds, (C, D) MSC-only scaffolds, and (E, F) prevascularized scaffolds. (G, H) shows images of negative controls. Scale bar in panels (A–F) is 300 μ m, and scale bar in panels (G–H) is 500 μ m. Color images available online at www.liebertpub.com/tea

mineralization was observed around the defect perimeter in the prevascularized samples and the MSC-only scaffolds (Fig. 6E, F). MATLAB was used to quantify mean intensity values within the defect space in the 4 week time point samples (Fig. 6H). No statistically significant differences were observed between the groups.

Discussion

Prevascularized scaffolds have shown promise for rapid vascularization of engineered tissue constructs,^{18,19,27} and have been proposed as a potential advantage for bone tissue engineering. However, the examination of prevascularized biomaterials in the treatment of bone defects *in vivo* has been limited. In this study, we examined the ability of fibrin scaffolds containing extensive vascular networks formed from

HUVEC/MSC coculture spheroids to enhance vascularization and bone regeneration in a rodent cranial defect model.

The vasculature serves a crucial role in bone homeostasis and function, and is active in bone healing and remodeling processes. Angiogenesis is one of the first steps of bone fracture healing, and adequate vasculature is required for successful regeneration.²⁸ It has been suggested that the development of a vascular network is the limiting factor in tissue regeneration.²⁹ The bone endothelium actively recruits circulating bone progenitor cells for delivery into remodeling sites,²⁸ and secretes a number of factors that stimulate osteogenesis.³⁰ Therefore, a tissue-engineered bone graft that accelerates graft vascularization and perfusion may improve outcomes.

In this study, fibrin was chosen as a scaffold for its proangiogenic properties, its role in the body's own bone

healing process, and its demonstrated ability to foster cellular sprouting.²⁰ Fibrin is a major component of the fibrous callus formed after bone fracture, serves as the provisional matrix for tissue regeneration, and provides many cellular cues to aid wound healing, making it an excellent biomaterial for tissue engineering strategies.^{31,32} Fibrin is frequently used as a supplement to increase vascularization within biomaterial systems both *in vitro* and *in vivo*.^{6,33,34} We have found that fibrin can support robust vascular network formation *in vitro*²⁰ at a level and complexity that we have not been able to accomplish in collagen or other scaffolds.³⁵ Platelet-rich fibrin has shown to increase bone regeneration when combined with MSC sheets³⁶ or autogenous bone.³⁷ In addition, previous reports suggest that fibrin scaffolds may support greater levels of bone regeneration in comparison with collagen scaffolds.^{38,39} For these reasons, this study sought to investigate if culturing preformed vascular networks within fibrin scaffolds further enhanced tissue vascularization and bone healing.

This study uses HUVEC/MS C spheroids to create vascular networks within fibrin scaffolds *in vitro*. The ability of HUVECs to create three-dimensional (3D) vessel-like networks within biomaterial scaffolds is well established.^{18,40,41} HUVECs were used in this study to evaluate the ability of prevascularized scaffolds to improve tissue vascularization and regeneration in a bone defect model, but they are not a clinically viable EC source. For this work to transition to the clinic, the prevascularization technique could be modified to incorporate autologous ECs in place of HUVEC, such as endothelial progenitor cells isolated from peripheral blood, ECs differentiated from an autologous stem cell source (such as induced pluripotent stem cells), or other clinical EC sources.⁴² In addition, future work may require multiple EC types to prevascularize a biomaterial, as distinct bone-specific EC subtypes have been shown to improve osteogenesis and angiogenesis.⁴³

EC networks can be generated in biomaterial scaffolds by the self-assembly of cells suspended in the matrix or by sprouting from cellular aggregates, or spheroids.⁴⁴ The use of cell spheroids allows for increased cell–cell and cell–matrix interactions, a closer approximation of the *in vivo* environment relative to self-assembly vasculogenesis models.^{45,46} The ECs sprout from the spheroids and form an interconnected network that inosculates with networks from other spheroids. In vasculogenesis models in which ECs are suspended within a biomaterial, the cells typically form many short vessel-like structures that may not persist and often do form a network connected throughout the biomaterial volume.

The results from the *in vitro* optimization of HUVEC/MS C coculture spheroids demonstrate the importance of MSCs in network formation *in vitro*. EC sprouting was not observed in the absence of MSCs, which could indicate that HUVECs may rely on MSCs for sprouting and network formation to occur. This is consistent with our observations of MSCs in proximity to HUVEC vessel-like structures (Fig. 2F) and with findings from previous studies.^{34,47–49} MSCs have extensive intercellular interactions with ECs that enhance angiogenesis,^{50–52} which may contribute to the formation of HUVEC networks observed in this study. In all coculture ratios studied, the MSC sprouted out much farther than the HUVEC, covering nearly the entire area of the fibrin scaffold after 2–3 weeks, while the EC network sprouted a shorter distance and lagged behind the MSCs. The MSC outgrowth area increased rapidly between

weeks 1 and 2 (Fig. 1D), whereas the HUVEC network area increased highest from weeks 2 to 3 (Fig. 2G). MSCs are highly migratory, which could contribute to the increased sprouting distance. We found that spheroids containing 50% MSCs resulted in the highest cellular outgrowth and EC network formation, as well as the highest length and diameter of vessel-like structures (Fig. 2G–I). Other studies have investigated EC/MS C cocultures in different systems and found similar trends, with higher amounts of MSCs to be more vasculogenic than a higher proportion of ECs.^{40,49} Cell type, source, matrix composition, and stiffness all likely play a role in determining the optimal conditions for a given system.

The multispheroid network fibrin scaffolds demonstrated the ability to form robust, interconnected vascular networks *in vitro*. The length of individual vessel-like structures was higher than that in scaffolds with only one spheroid, possibly due to cellular signaling between spheroids. The total EC network area of the multispheroid scaffolds before implantation is ~10% of the total scaffold area. Previous studies investigating vascularization in a cranial defect model have reported ~1%¹¹ to 4%⁵³ vascular area, and ~4% vascular volume.^{4,54} While the vessel density is slightly higher than mature bone, vascular density is increased during healing, and the networks in the fibrin scaffolds are primarily capillary sized, which is distinct from the more complex structures in mature bone tissue.

Prevascularized scaffolds increased overall defect vascularization after 1 and 4 weeks of implantation relative to MS C-only and fibrin-only scaffolds (Fig. 4G). This may be a result of the implanted vessels or the secretion of soluble factors to induce vascularization. However, analysis of human-specific CD31 staining showed that prevascularized scaffolds anastomosed with host tissue within 1 week of implantation (Fig. 5B), and functional human vessels were observed at 4 weeks postimplantation (Fig. 5C), suggesting that this increase results, at least in part, from the survival of the vessels themselves. The density of human vessels remained similar between 1 and 4 weeks (Fig. 5D), indicating that the implanted vessels are maintained over time. In addition, the overall vascular density is greater in prevascularized samples than with MSCs alone, which are thought to produce more angiogenic factors than ECs. Previous work has shown HUVEC/MS C spheroid network anastomosis within 9 days after subcutaneous implantation.¹⁸ In a cranial window model, implanted ECs anastomosed with host vessels within 2 weeks of implantation, and were retained *in vivo* for 1 year.⁵⁵ However, the cranial window model is substantially distinct from common implantation models used to evaluate tissue-engineered constructs. To the authors' knowledge, there have not been any previous studies to determine short-term anastomosis of vascular structures implanted in a bone defect model. EC-laden scaffolds implanted in bone defects have been assessed at time points over 4 weeks due to the kinetics of bone formation.⁵⁶ In other work, functional implanted ECs were identified in cranial defects 6^{12,34,57} and 12 weeks⁵⁷ postimplantation.

While the prevascularized structures inosculated with host vasculature and increased overall vascular density, histology and X-ray imaging showed limited bone formation at 4 weeks in all groups. Minimal calcification is expected at these relatively early time points. Longer implantation may be needed to provide insight into any advantages in tissue maturation and calcification. HUVECs have been shown to promote

osteogenesis, induce bone formation, and stimulate bone repair.^{40,58–60} We observed nonsignificant increases in bone formation with prevascularized scaffolds after 4 weeks. Continuous spans of highly organized collagenous tissue across the length of the defect were observed in samples with prevascularized scaffolds after 4 weeks, in addition to the presence of early osteogenic markers (Fig. 7). The presence of osteopontin and osteonin indicates osteoblast activity and active bone matrix deposition and formation. These findings suggest that mature bone formation may occur at a later time point. The reduced bone formation could be related to the low stiffness of the fibrin scaffolds. Longer time points, inclusion of osteoinductive factors, or predifferentiation of the MSCs may aid in the formation of mature bone.

We found no statistical difference in bone formation between MSC-only and HUVEC/MSC prevascularized scaffolds, similar to other studies investigating coseeded scaffolds.^{9,11–13,34} Other studies have suggested that acellular scaffolds regenerate higher amounts of vasculature and bone than cellularized multilayered scaffolds.⁵⁴ However, this finding may not hold when applied to human bone defects found clinically, which are orders of magnitude larger than rat critical sized cranial defects. The clinical success of a tissue-engineered bone graft depends on a number of factors, but vascularization is a key component of tissue formation.⁶¹ The prevascularized scaffolds developed in this study create rapidly vascularized tissue that may prove to be essential for graft survival.

Conclusions

This study has demonstrated the formation of vascular networks from HUVEC/MSC coculture spheroids encapsulated in fibrin scaffolds. The extent of network formation was dependent on the ratio of the two cell types, and MSCs were required. A spheroid composition of 50% MSCs resulted in the most expansive vascular network, with vessel-like structures >700 μ m long after 3 weeks of culture. Multiple spheroids were encapsulated in a single scaffold to form expansive interconnected networks within a scaffold for study in a critical sized defect model. When implanted, the preformed vascular networks survived and perfused with host vasculature within 1 week of implantation. Prevascularized fibrin scaffolds increased overall vascularization. This study demonstrates that preformed vascular networks rapidly perfuse and enhance vascularization in a bone defect model.

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Disclosure Statement

No competing financial interests exist.

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