The FASEB Journal article fj.15-279984. Published online October 6, 2015.

Hydrogel biophysical properties instruct coculture-mediated osteogenic potential

Kaitlin C. Murphy,* Roberta S. Stilhano,*,† Debika Mitra,* Dejie Zhou,* Samir Batarni,* Eduardo A. Silva,* and J. Kent Leach*,‡,1

*Department of Biomedical Engineering and ‡Department of Orthopaedic Surgery, School of Medicine, University of California, Davis, Davis, California, USA; †Department of Biophysics, Federal University of Sao Paulo, Sao Paulo, Brazil

ABSTRACT **Cell-based approaches for bone formation require instructional cues from the surrounding environment. As an alternative to pharmacological strategies or transplanting single cell populations, one approach is to coimplant populations that can establish a new vasculature and differentiate to bone-forming osteoblasts. Mesenchymal stem/stromal cells (MSCs) possess osteogenic potential and produce numerous angiogenic growth factors. Endothelial colony-forming cells (ECFCs) are a subpopulation of endothelial progenitor cells capable of vasculogenesis *in vivo* and may provide endogenous cues to support MSC function. We investigated the contribution of the carrier biophysical properties to instruct entrapped human MSCs and ECFCs to simultaneously promote their osteogenic and proangiogenic potential. Compared with gels containing MSCs alone, fibrin gels engineered with increased compressive stiffness simultaneously increased the osteogenic and proangiogenic potential of entrapped cocultured cells. ECFCs produced bone morphogenetic protein-2 (BMP-2), a potent osteoinductive molecule, and increases in BMP-2 secretion correlated with gel stiffness. Coculture of MSCs with ECFCs transduced to knockdown BMP-2 production abrogated the osteogenic response to levels observed with MSCs alone. These results demonstrate that physical properties of engineered hydrogels modulate the function of cocultured cells in the absence of inductive cues, thus increasing the translational potential of coimplantation to speed bone formation and repair.—Murphy, K. C., Stilhano, R. S., Mitra, D., Zhou, D., Batarni, S., Silva, E. A., Leach, J. K. Hydrogel biophysical properties instruct coculture-mediated osteogenic potential. *FASEB J.* 30, 000–000 (2016). www.fasebj.org**

Key Words: fibrin · mesenchymal stem cell · ECFC · BMP-2 · osteogenesis

Of the more than 6.2 million fractures that occur each year, approximately 5–10% will suffer from impaired healing, resulting in nonunions (1). Current treatments include autograft and allograft bone, but their limitations have prompted the search for alternative treatment strategies including synthetic biomaterials and the use of recombinant osteoinductive proteins such as bone morphogenetic protein-2 (BMP-2). However, these approaches suffer from inherent delays in tissue repair that are dependent upon the presence of the patient's own responsive cells to achieve bone healing.

Cell-based therapies for bone regeneration aim to overcome limited availability of locally responsive endogenous cells by implanting autologous or allogenic cell populations to directly or indirectly contribute to bone formation. Mesenchymal stem/stromal cells (MSCs) are under investigation for restoring lost bone volume; they have the potential to undergo differentiation toward the osteoblastic lineage upon osteogenic induction (2, 3) and secrete trophic factors that stimulate angiogenesis (4, 5). Endothelial cells have been implanted into bone defects to promote vascularization *in situ* and participate in the formation of new blood vessels, allowing the native repair processes to unfold (6). When MSCs and endothelial cells are codelivered, synergistic paracrine signaling enhances neovascularization and mineralization (7–9). However, the collection of autologous endothelial cells requires painful dermal biopsies that fail to provide clinically relevant numbers of cells, thereby requiring prolonged cell culture and delaying treatment. Alternatively, endothelial colony-forming cells (ECFCs) are a subpopulation of endothelial progenitor cells that can be obtained from peripheral blood in high numbers and are easily expanded (10, 11), exhibit robust proliferative and vasculogenic potential (12), and represent a promising, clinically relevant cell population. Under hypoxic conditions *in vitro*, ECFCs proliferate and migrate better than human microvascular endothelial cells, a commonly used endothelial cell type (12). Despite their seemingly superior therapeutic potential, the capacity of ECFCs to promote bone formation when deployed with MSCs is poorly understood.

The injection of cells to the target site seeks to localize cells at the defect and eliminate delays or off-target effects associated with cell homing strategies. Local deployment of cells using biomaterials further enhances efficacy of cell

Abbreviations: α-MEM, minimum essential medium α; ALP, alkaline phosphatase; BMP-2, bone morphogenetic protein-2; ECFC, endothelial colony-forming cell; EGM-2, endothelial cell growth medium-2; GF-Def, growth factor-deficient; MMP, matrix metalloproteinase; MSCs, mesenchymal stem/stromal cells; TBST, Tris-buffered saline supplemented with Tween 20

[1] Correspondence: Department of Biomedical Engineering, University of California, Davis, 451 Health Sciences Dri., Davis, CA 95616, USA. E-mail: jkleach@ucdavis.edu

doi: 10.1096/fj.15-279984

therapies by reducing the number of cells that migrate from the defect while enhancing cell persistence and providing instructional cues to direct cell function. In view of deploying coculture populations for tissue repair, it is particularly important to consider the needs of each cell type. Endothelial cell populations remodel their microenvironment to promote vasculogenesis, and therefore benefit from more compliant materials (13, 14). Conversely, MSCs differentiate into cells of the osteoblastic lineage more robustly in stiffer biomaterials (15–17). Therefore, many biomaterials used for cell-based therapies of bone formation are ill suited for endothelial cell delivery (18). Fibrin hydrogels are a promising platform for cell delivery as fibrin naturally occurs in the body, serving as a scaffold for leukocytes and endothelial cells during tissue regeneration (19). We previously reported that supplementation of the pregel fibrinogen solution with 2.3% (w/v) NaCl modifies the fibrin fiber structure and increases compressive stiffness, thereby promoting the osteogenic and proangiogenic potential of entrapped MSCs while avoiding osmolarity-related effects (16, 20). Entrapped cells are briefly exposed to salinity levels higher than physiologic values, but the NaCl diffuses out of the gels within the first hour (20). Importantly, these gels are formed from relatively low fibrinogen concentrations and undergo gelation in a time course that enables survival of entrapped cells. Thus, the manipulation of the physical properties of fibrin gels may provide a viable platform to simultaneously instruct endothelial and MSC function for bone formation.

We hypothesized that tailoring the biophysical properties of the cell carrier would instruct heterotypic cell function for enhancing bone formation. To explore this hypothesis, we entrapped human MSCs and ECFCs within fibrin gels engineered *via* supplementation with NaCl to modulate biophysical properties while keeping composition constant. We assessed gel material properties and the osteogenic and proangiogenic potential of entrapped cells. Furthermore, we explored the mechanism of how ECFCs modulate osteogenic potential of the system. The results of these studies offer enhanced translational relevance for using cell-based therapies in tissue repair.

MATERIALS AND METHODS

Cell culture

Human bone marrow-derived MSCs (Lonza, Walkersville, MD, USA) were used without additional characterization. MSCs were expanded in standard culture conditions (37°C, 21% O_2, 5% CO_2) in α-MEM supplemented with 10% fetal bovine serum (JR Scientific, Woodland, CA, USA) and 1% penicillin/streptomycin (Mediatech, Herndon, VA, USA) until use at passages 4–5. Human umbilical cord blood ECFCs were generously provided by Dr. Mervin Yoder (Indiana University, Indianapolis, IN, USA) and isolated using a protocol approved by the Institutional Review Board of the Indiana University School of Medicine as previously described (21). Adherent ECFCs were cultured on tissue culture plastic coated with 5 $\mu g/cm^2$ rat tail collagen I (BD Biosciences, San Jose, CA, USA) in endothelial cell growth medium-2 (EGM-2) medium with Lonza's SingleQuot supplements (hydrocortisone, gentamicin, human VEGF, human basic fibroblast growth factor, human epidermal growth factor, human insulin-like growth factor [IGF], and heparin) and further supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Growth factor-deficient (GF-Def) EGM-2 was prepared with serum-containing EGM-2 but lacking VEGF, fibroblast growth factor, and IGF. Culture-expanded ECFCs (passages 12–13) were used for all studies.

Fibrin gel preparation

Fibrin gels were formed as we previously described (16, 20, 22). This fabrication process resulted in fibrin gels with a final fibrinogen concentration of 20 mg/mL (Calbiochem, Gibbstown, NJ, USA), 2.5 U/mL thrombin (Calbiochem), 20 mM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO, USA), and 250 KIU/mL aprotinin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), all in PBS. Gels made in PBS without additional NaCl supplementation contained 0.8% (w/v) NaCl (Sigma-Aldrich), whereas gels supplemented with NaCl had a final concentration of 2.3% (w/v) NaCl. A total volume of 80 μL was added to each cylindrical polydimethylsiloxane mold (5 mm in diameter), and the contents were allowed to gel for 1 h in standard culture conditions. The polydimethylsiloxane sheet was then carefully lifted from the culture dish, leaving behind the undisturbed fibrin gels, and the gels were transferred to 24-well tissue culture plates containing medium. The medium was refreshed after 1 h, ensuring that cellular responses were due to the material properties of the hydrogels and not NaCl content.

To add clinical relevance, all cells were used directly from the cryovial. The cryopreservation solution was removed *via* centrifugation, and cells were resuspended in the fibrinogen pregel solution. Fibrin gels contained 4×10^5 MSCs, 4×10^5 ECFCs, or a combination of 2×10^5 MSCs and 2×10^5 ECFCs for a final concentration of 5×10^6 cells/mL in each gel. All gels were cultured in a 1:1 mixture of α-MEM and GF-Def EGM-2 without osteogenic supplements or growth factors to promote the survival of both cell populations. The day of gel fabrication was denoted as d −1. Gels were maintained in standard cell culture conditions with medium changes every 3 d.

Assessment of gel mechanical and morphologic properties

Rheological properties of fibrin gels, with or without cells, were measured on a Discovery HR-2 hybrid stress-controlled rheometer (Thermal Analysis Instruments, New Castle, DE, USA) equipped with an 8 mm parallel plate geometry on a stage heated to 37°C. Gels were tested at a frequency of 0.5 Hz and a logarithmic sweep from 0.1 to 10 μN·m with 10 points per decade. The shear storage modulus was determined by averaging at least 10 points in the linear viscoelastic region.

The compressive moduli of fibrin gels were measured using an Instron 3345 Compressive Testing System (Norwood, MA, USA). Gels were allowed to swell for 1 h in PBS, blotted, and then loaded between 2 flat platens and compressed at 1 mm/min. Compressive moduli were calculated from the linear portions of the force–displacement graph for strain ranging from 0 to 5% (16).

The contraction of fibrin gels due to activity of entrapped cells was measured at 0 and 21 d by visually following morphologic changes in gel volume. Gels were imaged using a Nikon Eclipse TE2000U microscope (Melville, NY, USA) and Andor Zyla digital camera (Oxford Instruments, Abingdon, United Kingdom) and gel area was calculated in NIS Elements (Nikon).

Cellular response to engineered fibrin gels

The osteogenic response of MSCs entrapped within engineered fibrin gels was assessed from gels collected at 0, 7, 14, and 21 d. Gels were rinsed in PBS and sonicated in 400 μL passive lysis

buffer (Promega, Madison, WI, USA). Samples were centrifuged at 5000 rpm for 10 min to pellet the cell debris, and the supernatant was collected. The supernatant was analyzed for intracellular alkaline phosphatase (ALP) activity using a *p*-nitrophenyl phosphate colorimetric assay, and cell-secreted mineral within fibrin hydrogels was measured using *o*-cresolphthalein complexone as previously described (16, 20, 23). DNA content was quantified from the supernatant using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Rochester, NY, USA). ECFC secretion of BMP-2 was quantitatively determined from the conditioned medium after 7 d in culture. The medium was refreshed 24 h before collection, and the concentration of BMP-2 was determined using a human-specific BMP-2 ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

MSC secretion of VEGF was quantitatively determined from the conditioned medium of coculture-containing fibrin gels at designated time points. The medium was refreshed 24 h before collection, and the concentration of VEGF was determined using a human-specific VEGF ELISA kit (R&D Systems) according to the manufacturer's instructions.

To investigate the proangiogenic potential of entrapped cells, conditioned medium was collected from fibrin gels at 7 d and used to stimulate ECFC proliferation and tubule formation as we previously described (24). The mitogenic response of ECFCs to conditioned medium was determined by seeding ECFCs at 7500 cells/cm^2 in EGM-2 in a 12 well plate and allowing cells to attach overnight. The medium was then refreshed with a 1:4 volume ratio of coculture-conditioned medium to GF-Def EGM-2 and cultured for 72 h. Each well was then rinsed with PBS and cells were lysed with passive lysis buffer. DNA content was quantified as described above. For tubule formation, 100 μL of Growth Factor Reduced Matrigel (BD Biosciences) was pipetted into 48 well plates and allowed to gel at 37°C for 1 h. ECFCs were seeded on Matrigel at 30,000 cells/cm^2 in GF-Def EGM-2, and a mixture of conditioned medium and GF-Def EGM-2 was added in a 1:4 volume ratio. Cells were cultured for 16 h, after which they were stained with calcein acetoxymethyl ester (3 μg/mL in PBS; Invitrogen, Carlsbad, CA, USA) for 30 min. Average number of branch points per field of view and average tubule length were quantified using NIS Elements (Nikon). For both assays, EGM-2 served as the positive control, while GF-Def EGM-2 served as the negative control.

Detection of BMP-2 production in ECFCs by Western blot

To quantify BMP-2 expression by ECFCs entrapped in fibrin gels, gels were first collected in sample buffer [0.1% Triton-X 100, 0.01% Tris-EDTA, 0.1% protease inhibitor cocktail (Calbiochem)]. Samples were sonicated and spun down, and total protein was quantified using the BCA assay (Thermo Fisher Scientific). Protein (20 μg per sample) was added to 10 μL 4× XT sample buffer (Bio-Rad, Hercules, CA, USA), boiled for 5 min to reduce protein, and loaded onto 12% Tris-HCl polyacrylamide gels (Bio-Rad). Gels were electrophoresed for 60 min at 180 V. Protein was then transferred to nitrocellulose membranes (Invitrogen) for blotting, and protein was electrophoretically transferred *via* the iBlot system (Invitrogen). After transfer, blots were incubated in Tris-buffered saline supplemented with Tween 20 (TBST; 10 mM Tris, 100 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% nonfat milk for 1 h to block nonspecific protein binding. After blocking, blots were incubated overnight at 4°C with a primary polyclonal anti-human BMP-2 antibody (ab82511, Abcam, Cambridge, MA, USA; 1:100 in TBST) or anti-human β-actin (Cell Signaling Technology, Danvers, MA, USA; 1:200 in TBST). After rinsing with TBST, blots were incubated for 1 h with a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (1:1000; Cell Signaling Technology). Signal detection was achieved with the ECL Western Blotting Substrate (Thermo Fisher Scientific) and signals were recorded using the ChemiDoc MP System (Bio-Rad). Between antibodies, the membrane was stripped at 55°C for 60 min in stripping buffer (0.1 M glycine, pH 2.8). Protein bands were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA) and normalized to the levels of β-actin as the loading control.

Investigating cellular crosstalk when entrapped in fibrin gels

To probe the interaction between cells in coculture, we blocked common participants in matrix remodeling, angiogenesis, and bone formation. Matrix metalloproteinases (MMPs) were blocked with GM6001 (Calbiochem, 10 μM), a broad-spectrum MMP inhibitor, by addition directly to the fibrin gels during fabrication (25). The bioactivity of secreted VEGF and BMP-2 was blocked by adding antibodies to $VEGF_{165/121}$ (500 ng/mL) or BMP-2 (2 μg/mL; R&D Systems) to the medium as described (24, 26). Medium was refreshed every 2 d, and gels were collected at 7 and 21 d to assess mineral deposition.

To knock down BMP-2 expression, ECFCs were transduced (multiplicity of infection 10) using Mission shRNA lentivirus transduction particles (TRCN0000058193) and pLKO-puro Non-Target shRNA Control Transduction particles (Sigma-Aldrich) in growth medium containing 8 μg/mL polybrene (Sigma-Aldrich). Cells were placed under selective pressure 48 h after infection using 2 μg/mL puromycin (Sigma-Aldrich). For determination of transduction efficiency, all cell types were transduced using a lentiviral-driven GFP construct (pLVTHM) (Addgene plasmid #12247). Knocked-down expression was confirmed by Western blot for BMP-2.

Statistical analysis

Data are presented as means ± SD. Statistical analysis was performed using 2-way ANOVA with Bonferroni correction for multiple comparisons or paired *t* tests when appropriate. All statistical analysis was performed in Prism 6 software (GraphPad, La Jolla, CA, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Gel contraction by entrapped cells is modulated by hydrogel physical properties

Changes in construct morphology were assessed by examining gel area over time (**Fig. 1*A*, *B***). At d 0 (24 h after synthesis), constructs formed without additional salt had contracted to half their original area and continued to contract over the 21 d study. Conversely, fibrin gels formed with 2.3% (w/v) NaCl did not contract significantly over time.

The initial rheological properties of fibrin hydrogels were characterized with and without cells to elucidate the contributions of NaCl and cells to the material properties. In the absence of cells, fibrin gels formed with 2.3% (w/v) NaCl were comparable in storage modulus to those formed without NaCl supplementation (Fig. 1*C*). When cells were present, the storage modulus decreased significantly in gels formed with 2.3% (w/v) NaCl; however, there were no significant differences in gels formed without NaCl supplementation. In contrast to the storage moduli, acellular

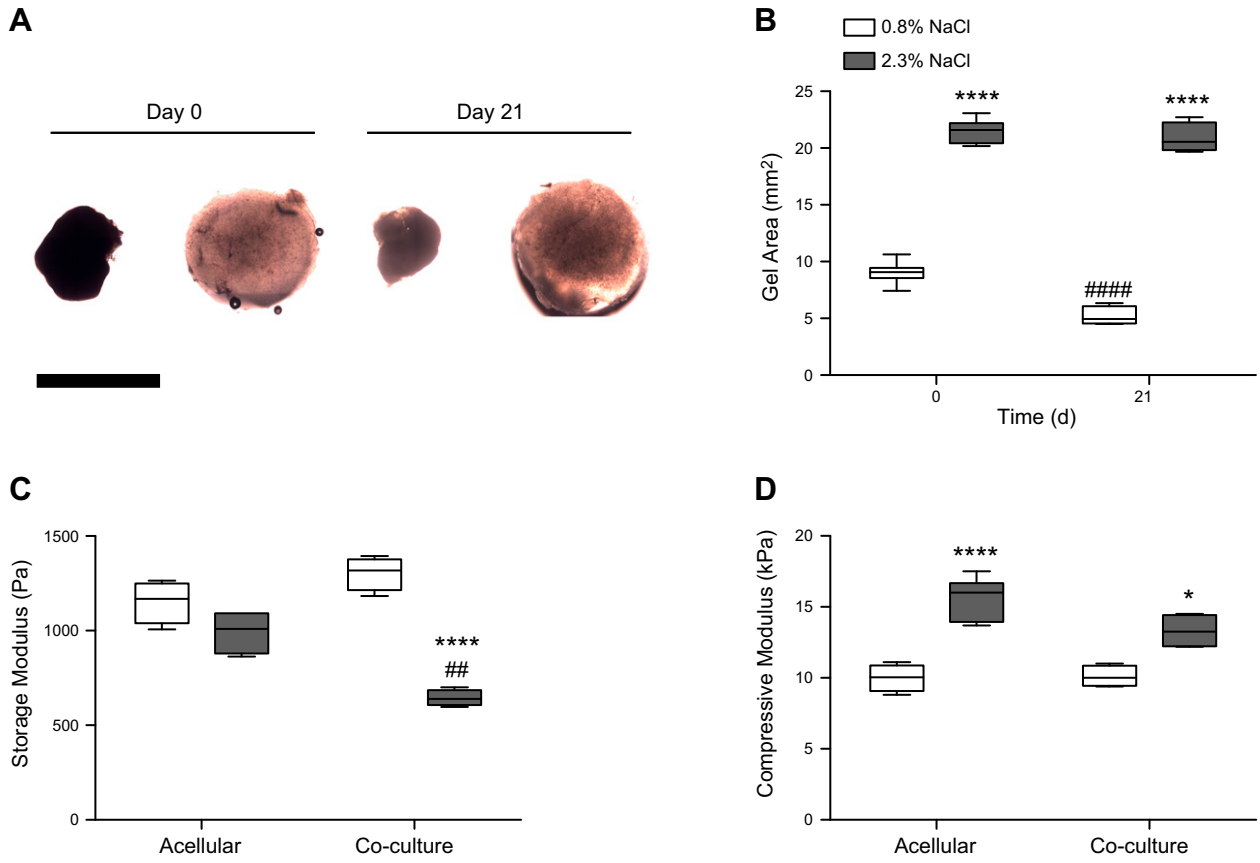

Figure 1. Hydrogel material properties are dependent on NaCl content. *A*) Representative images of fibrin gels over time. Scale bar, 5 mm. *B*) Quantification of gel contraction over time ($n = 6$). ****$P < 0.0001$ *vs.* 0.8% NaCl, $^{\#\#\#\#}P < 0.0001$ *vs.* d 0. *C*) Shear storage moduli of acellular and cellular fibrin gels ($n = 4$). ****$P < 0.0001$ *vs.* 0.8% NaCl, $^{\#\#}P < 0.01$ *vs.* 2.3% NaCl. *D*) Compressive moduli of acellular and cellular fibrin gels ($n = 4$). ****$P < 0.0001$ *vs.* 0.8%, *$P < 0.05$ *vs.* 0.8%. All data are represented as boxes marking median, 25th and 75th percentiles, and whiskers mark minimum and maximum values.

gels supplemented with 2.3% (w/v) NaCl exhibited significantly higher compressive moduli than those formed without NaCl supplementation. The compressive moduli were unchanged when cells were present (Fig. 1*D*).

Osteogenic potential of coculture is enhanced with addition of NaCl to fibrin gels

ALP activity, an early marker of osteogenic differentiation, correlated with NaCl concentration (**Fig. 2*A***). After 7 d, the coculture fibrin gels formed with 2.3% (w/v) NaCl contained significantly greater ALP compared with cells entrapped in gels formed with 0.8% (w/v) NaCl, and these significant increases were sustained for the 21 d study. There were no statistically significant differences in DNA except for at d 14 (Fig. 2*B*).

Cell-secreted mineral was quantified as a late-stage marker of osteogenesis. Gels fabricated with 2.3% (w/v) NaCl induced the most MSC-secreted mineral. Calcium levels increased over time for all gel formulations, demonstrating the ability of these fibrin gels to nucleate calcium and enable mineral growth (Fig. 2*C*). We then quantified the ability of endothelial cells to promote the osteogenic potential of the MSCs by measuring the endogenous secretion of BMP-2 in the medium as a function of salt content and associated gel properties. BMP-2 expression followed the same trend as the expression of osteoblastic markers in entrapped cells. Although BMP-2 expression was nearly identical at d 0, ECFCs entrapped in gels fabricated with 2.3% (w/v) NaCl secreted significantly more BMP-2 by d 7 (Fig. 2*D*).

Fibrin gel properties modulate the proangiogenic response of entrapped cells

The concentration of VEGF in the conditioned medium was measured *via* ELISA to determine the effect of NaCl content on the proangiogenic potential of entrapped cells. At d 1, cells entrapped in gels formed with 2.3% (w/v) NaCl exhibited significantly reduced VEGF secretion compared with those formed without NaCl supplementation. However, this response was reversed by d 7, and no significant differences were apparent between the 2 gel formulations at d 14 and 21 (**Fig. 3*A***).

We characterized the functional bioactivity of secreted endogenous growth factors by testing the ability of conditioned medium to stimulate ECFC proliferation and tubule formation. Conditioned medium from gels formed with 0.8 and 2.3% (w/v) NaCl supported ECFC proliferation as well as the positive control of EGM-2 and

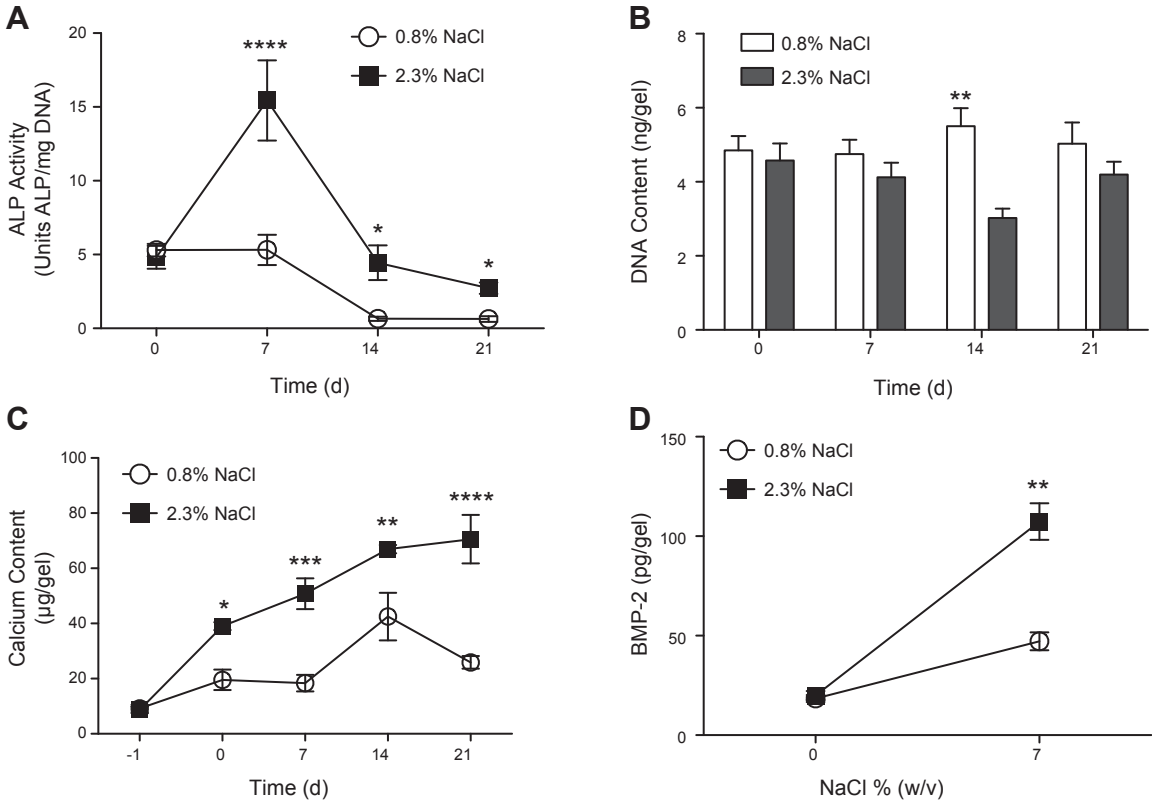

Figure 2. Osteogenic response of coculture population entrapped in fibrin gels is increased by NaCl supplementation. *A*) ALP activity ($n = 4$). ****$P < 0.0001$ *vs.* 0.8%. *B*) DNA content ($n = 4$). **$P < 0.01$ *vs.* 0.8% NaCl at d 7. *C*) Cell-secreted calcium ($n = 4$). *$P < 0.05$ *vs.* 0.8% NaCl, **$P < 0.01$ *vs.* 0.8% NaCl, ***$P < 0.001$ *vs.* 0.8% NaCl, ****$P < 0.0001$ *vs.* 0.8% NaCl. *D*) BMP-2 concentration in conditioned medium collected at 0 and 7 d ($n = 4$). **$P < 0.01$ *vs.* 0.8% NaCl. Data are represented as means ± SD.

significantly better than GF-Def EGM-2 (Fig. 3*B*). We observed increases in the number of branch points and tubule length when ECFCs were treated with conditioned medium from 2.3% (w/v) NaCl gels compared with 0.8% (w/v) NaCl gels (Fig. 3*C*, *D*). There were no statistically significant differences between these values and our positive control, EGM-2. These trends are qualitatively illustrated in Fig. 3*E*, *F*.

Investigating the crosstalk mechanism between MSCs and ECFCs in fibrin gels

MSCs alone or MSCs and ECFCs were suspended in fibrin gels containing 2.3% (w/v) NaCl, and the activity of endogenous BMP-2, VEGF, and MMPs was blocked with known inhibitors to each molecule. At both 7 and 21 d, gels containing a coculture of MSCs and ECFCs contained more calcium than gels loaded with MSCs alone. After 7 d, groups in which VEGF and BMP-2 were blocked contained significantly less calcium than the coculture control group (**Fig. 4*A***). However, by d 21 only the cocultured gels treated with BMP-2 antibody exhibited impaired mineralization to levels below MSCs alone. At both 7 and 21 d there was no statistically significant difference between the MSC control group and the coculture group cultured with BMP-2 antibody. Although we detected a slight increase in mineralization from gels containing MSCs alone when MMPs were blocked at d 7, this observation was not sustained after 21 d (Fig. 4*B*). DNA values were not statistically significant between groups (data not shown).

Increased osteogenic potential of cocultures is dependent upon BMP-2 secretion by ECFCs

To further investigate the effect of BMP-2 on the crosstalk of MSCs and ECFCs, we examined the expression of BMP-2 by MSCs, ECFCs, or a coculture population when entrapped in fibrin gels. MSCs did not secrete detectable BMP-2, and there were no statistically significant differences in BMP-2 expression between the ECFC and coculture samples (**Fig. 5*A, B***). The effective knockdown of BMP-2 was confirmed by Western immunoblotting (Fig. 5*C*, *D*).

BMP-2 knockdown ECFCs were then cocultured with MSCs in a 2.3% (w/v) NaCl fibrin gel to validate the role of BMP-2 secretion on the osteogenic potential of MSCs. At d 7, MSCs cultured with BMP-2 knockdown ECFCs exhibited significantly less ALP activity than both the MSC and coculture control group, yet this trend was reversed at d 21 (**Fig. 6*A***). Quantitative analysis of calcium content revealed a statistically significant reduction in osteogenic potential of MSCs cultured with BMP-2 knockdown ECFCs compared with the coculture control, a finding evident at both d 7 and 21 (Fig. 6*B*). DNA content increased in all groups over 21 d (Fig. 6*C*).

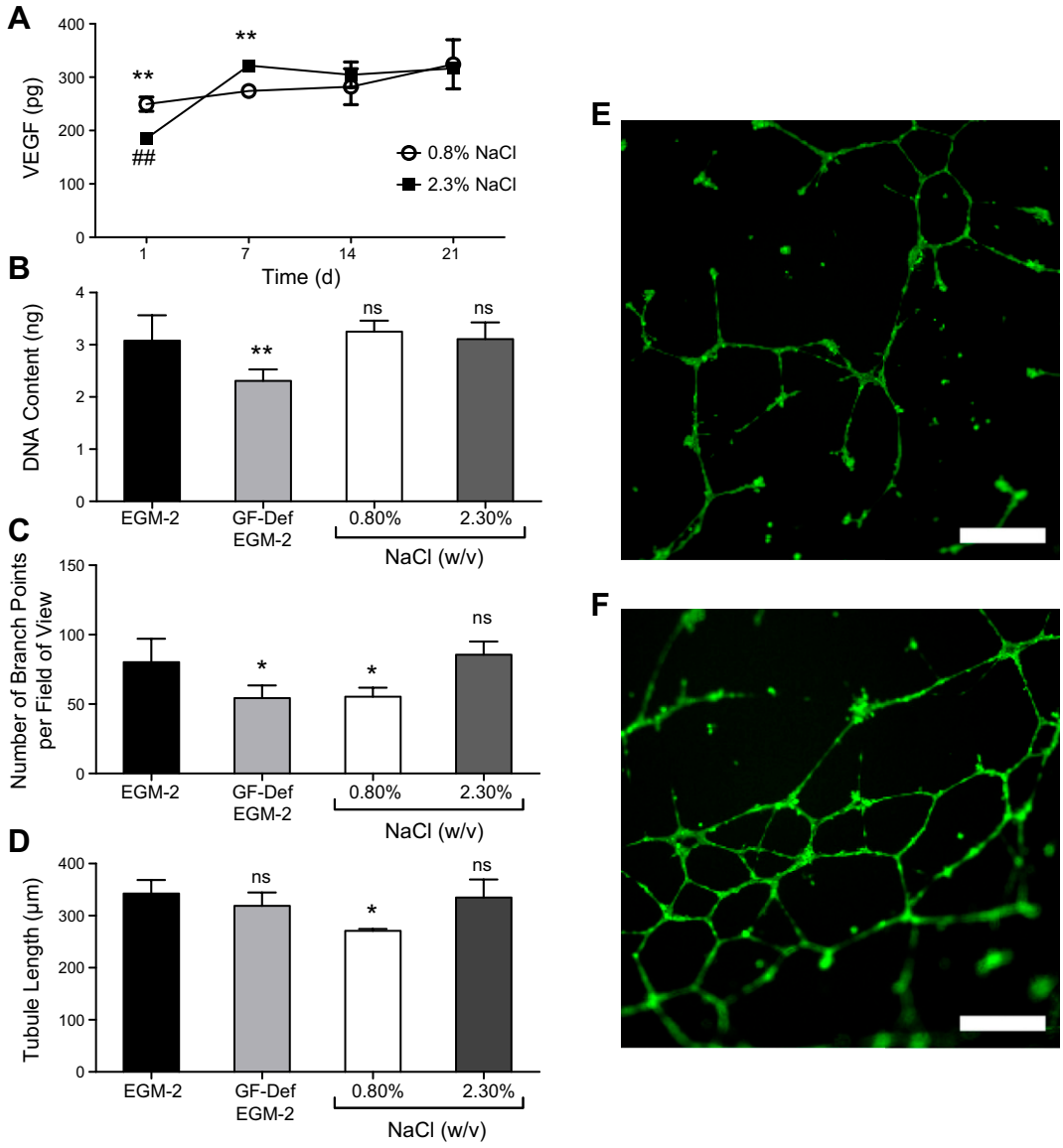

Figure 3. Proangiogenic response of entrapped coculture population within fibrin gels is maintained with NaCl supplementation. *A*) VEGF concentration in conditioned medium ($n = 4$). $^{**}P < 0.01$ *vs.* 2.3% NaCl at day 0 and *vs.* 0.8% NaCl at d 7, $^{\#\#}P < 0.01$ *vs.* 2.3% NaCl at d 7, 14 and 21. *B*) Endothelial cell proliferation measured through DNA content in response to MSC-conditioned medium ($n = 4$). ns, no significant differences *vs.* EGM-2. $^{**}P < 0.01$ *vs.* EGM-2. *C*) Endothelial cell tubulogenesis on Matrigel in the presence of MSC-conditioned medium measured by the number of branch points per field of view ($n = 4$). ns, no significant differences *vs.* EGM-2. $^{*}P < 0.05$ *vs.* EGM-2. *D*) Endothelial cell tubulogenesis on Matrigel in the presence of MSC-conditioned medium as measured by tubule length ($n = 4$). ns, no significant differences *vs.* EGM-2. $^{*}P < 0.05$ *vs.* EGM-2. All data are represented as means ± SD. *E*) Representative image of endothelial cell tubulogenesis on Matrigel in the presence of MSC-conditioned medium from fibrin gels formed with 0.8% NaCl and (*F*) 2.3% NaCl. Scale bars, 500 μm.

DISCUSSION

Bone is a highly vascularized tissue and depends upon the establishment of a robust vasculature to repair itself (27). The requirement of a vascular supply motivates various strategies to accelerate vasculogenesis prior to osteogenesis. Cell-based approaches offer one method to provide cells at the defect site that can immediately establish this new vasculature and directly contribute to bone formation. The coimplantation of bone- and vessel-forming cell populations results in a more robust vasculature than either cell population transplanted alone (28, 29). In addition to participating in vasculogenesis, endothelial cells may promote osteogenesis through cell–cell communication *via* gap junction and connexin 43 activity or expression of BMP-2 (7, 30). This interaction motivates the delivery of coculture populations versus presenting a single cell type. However, for a coculture population to thrive, it is imperative to consider the needs of both cell types. Kaigler *et al.* reported that in order for human microvascular endothelial cells to enhance MSC osteogenic potential, the 2 cell types must be in direct contact with each other. Although their work was carried out using poly (lactic-co-glycolic acid) scaffolds, hydrogels allow for more intimate colocalization and interaction between the heterogeneous cell populations (7). These data demonstrate

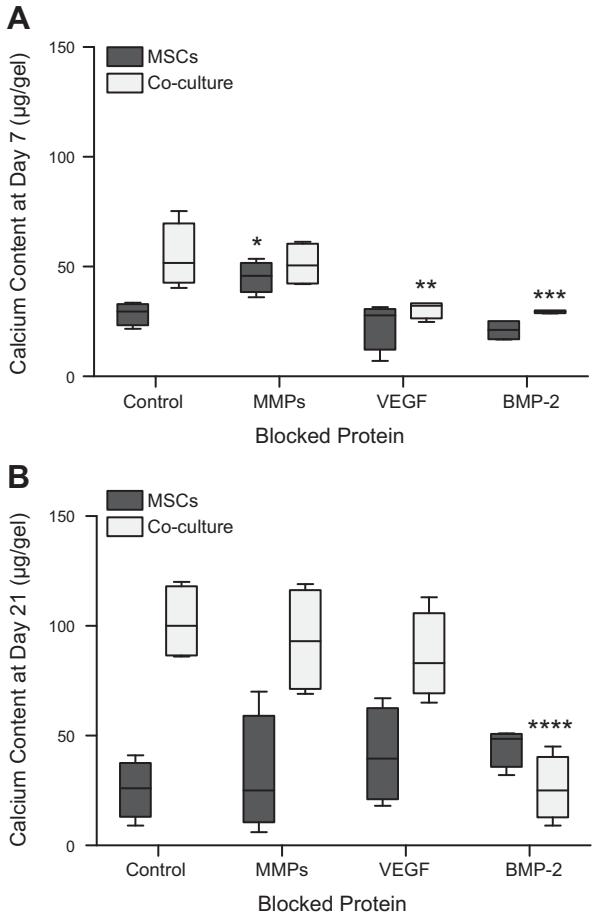

Figure 4. Osteogenic potential of entrapped cells in the presence of various antagonists. *A*) Cell-secreted calcium at d 7 (n = 4). *$P < 0.05$ *vs.* MSC control, **$P < 0.01$ *vs.* coculture control, ***$P < 0.001$ *vs.* coculture control. *B*) Cell-secreted calcium at d 21. ****$P < 0.0001$ *vs.* coculture control at d 21. All data are represented as boxes marking median, 25th and 75th percentiles, and whiskers mark minimum and maximum values.

that fibrin gels possessing enhanced material properties can dually stimulate the proangiogenic and osteogenic potential of entrapped ECFCs and MSCs, thereby enhancing the quantity of resulting mineralized tissue.

In this study, fibrin gels exhibited compressive moduli of 10–15 kPa and elastic moduli ranging from 600–1200 Pa, representing physical properties on par with other materials utilized in non-weight-bearing bone repair and regeneration in the head and neck region (31, 32). These values are congruous with our previously published data (16), and the addition of cells did not significantly alter the compressive moduli of either gel formulation. However, we observed reductions in storage moduli with increasing NaCl content that became more apparent upon the addition of cells. These differences may arise because the compressive modulus represents the resistance to compression of both the hydrogel fibers and entrapped fluid, and rheological analysis assesses only the ability of the gel fibers to resist shear stress without consequence to water content. These trends are consistent with other reports of hydrogel material properties (33, 34).

The results of this study confirm that fibrin hydrogel biophysical properties can be engineered to simultaneously support the therapeutic potential of both MSCs and ECFCs. The supplementation of the pregel solution with NaCl provides a tool to enhance hydrogel material properties, has no bearing on the osmolality of the cell microenvironment, and does not impair cell viability (20). These engineered hydrogels promoted the osteogenic and proangiogenic potential of the entrapped cell populations. This duality is especially significant, as we previously reported that osteogenically induced MSCs on tissue culture plastic exhibit reduced proangiogenic potential (24, 35). In agreement with our monolayer studies, we further showed that as the osteogenic potential of entrapped MSCs increased *via* culture in fibrin gels with higher NaCl content and associated stiffness, the proangiogenic potential of entrapped cells decreased (16). Upon the addition of ECFCs to the system, we now show that stiffer fibrin gels support both the osteogenic and proangiogenic potential of the entrapped cells. These studies were carried out in the absence of soluble cues to observe the effect of the material alone on cell function. In addition, we noted that the total calcium content was significantly greater in the coculture gels compared with our previous findings with only MSCs entrapped in fibrin gels, demonstrating the synergistic effect of coculture and hydrogel stiffness on the osteogenic potential of MSCs.

The increased mineralization of coculture populations compared with MSCs alone prompted an investigation into the crosstalk mechanism between ECFCs and MSCs in fibrin gels. These studies were performed in the stiffer 2.3% (w/v) NaCl gel due to the robust osteogenic response of entrapped cells. MMPs, VEGF, and BMP-2 were chosen as target molecules due to their roles in the angiogenic and osteogenic functions of ECFCs and MSCs, respectively. Our results confirm that ECFCs produce BMP-2, and BMP-2 is the predominant signaling molecule responsible for the increased mineralization of these coculture populations. Although MSCs do not produce BMP-2, ECFCs secrete BMP-2 as a paracrine signaling molecule (36). Numerous studies have evaluated and identified the role of BMP-2 on the enhanced osteogenic potential of MSCs when cocultured with endothelial cells (7, 37), yet this is the first study to evaluate the role of crosstalk between ECFCs and MSCs in a fibrin hydrogel. BMP-2 may affect multiple signaling pathways and contribute to osteogenic differentiation through as yet unidentified synergies. Additionally, this study provides a comprehensive comparison of different signaling molecules and their direct effect on MSC deposition of calcium. We investigated VEGF due to its potency in promoting angiogenesis and bone formation *in vivo* (38, 39). ECFCs do not secrete VEGF (40), and MSCs do not have VEGF receptors (35, 41). Therefore, the addition of a pan-VEGF antibody served to abrogate the paracrine signaling from the MSCs to the ECFCs. However, the use of the VEGF antagonist did not reduce the total calcium content in coculture fibrin gels. As the ECFCs continued to produce BMP-2, this potent osteoinductive molecule likely impaired VEGF-secretion by MSCs. In addition, ECFCs can use BMP-2 in an autocrine fashion to enhance angiogenesis through the Smad1 and ERK

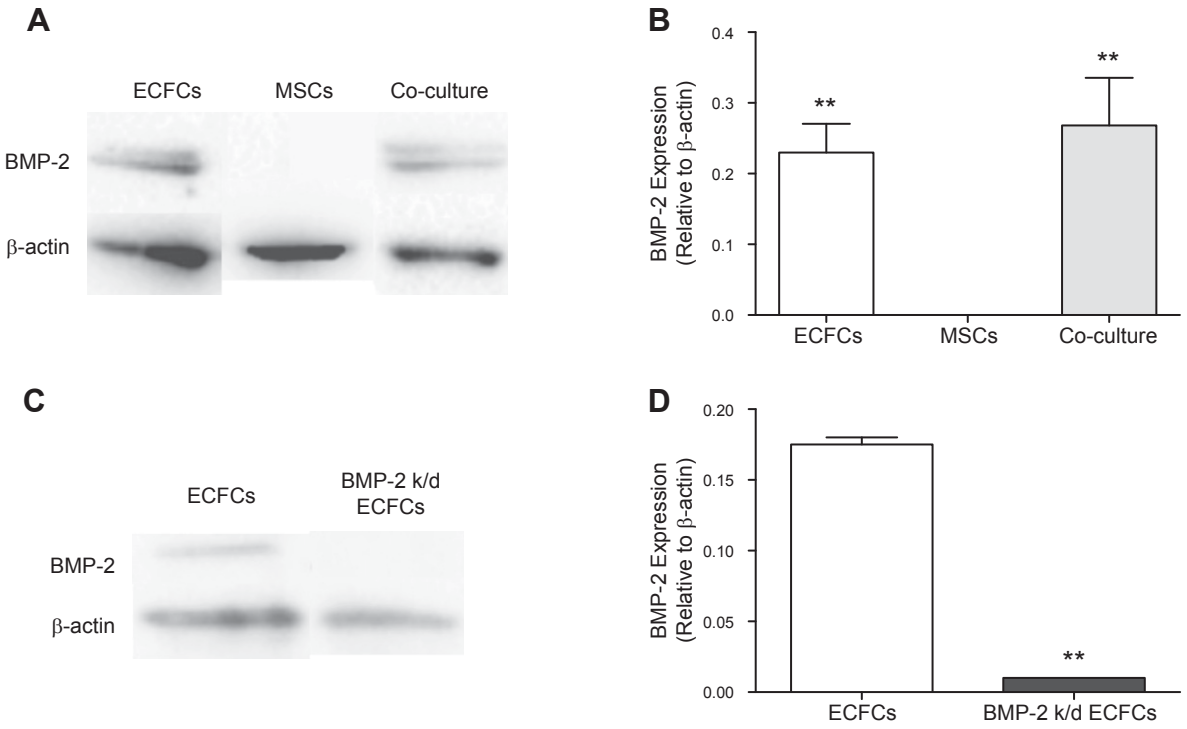

Figure 5. BMP-2 expression in ECFCs is modulated by lentiviral knockdown. *A*) Western blotting was performed to detect production of BMP-2 in ECFCs, MSCs, and coculture populations. *B*) Densitometry analysis of BMP-2 Western blot ($n = 3$). $^{**}P < 0.01$ *vs.* MSC. *C*) Western blot of ECFCs and ECFCs with BMP-2 expression knocked down. *D*) Densitometry analysis of BMP-2 Western blot ($n = 2$). $^{**}P < 0.01$ *vs.* ECFCs. All data are represented as means ± SD.

pathways (42). Therefore, in this coculture system, the ECFCs are not dependent on VEGF for their continued function. Finally, MMPs were interrogated due to their capacity to remodel fibrin gels and facilitate tubulogenesis. The addition of MSCs to endothelial cells in a dense fibrin matrix enhanced capillary morphogenesis by upregulating MMPs (43). However, the addition of a broad-spectrum MMP antagonist did not diminish the coculture's capacity to produce mineral. Overall, these findings suggest that although VEGF and MMPs are key mediators in ECFC function, they are not directly linked to ECFC secretion of BMP-2, which promotes the osteogenic potential of MSCs within a fibrin gel.

In these experiments, we sought to maximize the clinical relevance of using coculture populations for bone formation. Compared with fully mature somatic cells, MSCs and ECFCs possess increased clinical relevance due to differences in cell proliferation and ease of isolation. ECFCs can be noninvasively harvested from peripheral blood, and endothelial cells from human umbilical cords or dermal biopsies are limited in number and restricted to autologous use (44, 45). In these studies, all cells were used directly from cryovials and once entrapped in fibrin cells were cultured in the absence of potent osteogenic and proangiogenic supplements that may mask the effect of material properties on

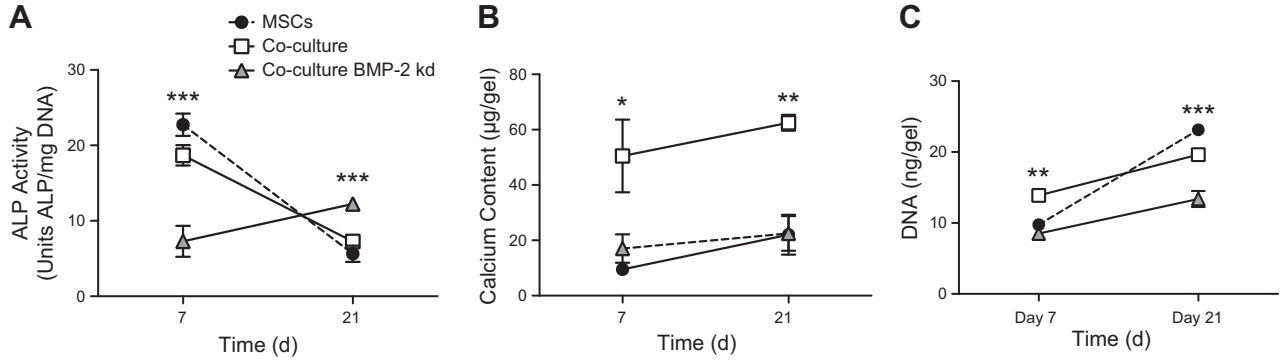

Figure 6. Interrogation of the role of BMP-2 secretion by ECFCs on the osteogenic potential of entrapped MSCs. *A*) ALP activity ($n = 4$). $^{***}P < 0.001$ *vs.* MSCs and coculture at d 7 and 21). *B*) Cell-secreted calcium ($n = 4$). $^{*}P < 0.05$ *vs.* MSCs and coculture BMP-2 knockdown ECFCs at d 7; $^{**}P < 0.01$ *vs.* MSCs and coculture BMP-2 knockdown ECFCs at day 21. *C*) DNA content ($n = 4$). $^{**}P < 0.01$ *vs.* MSCs and coculture BMP-2 knockdown ECFCs at d 7, $^{***}P < 0.001$ *vs.* MSCs and coculture at d 21. All data are represented as means ± SD.

instructing cell function. Finally, fibrin is approved by the U.S. Food and Drug Administration as a biomaterial. The physiological concentrations of fibrinogen and thrombin necessary to fabricate these materials could feasibly be harvested from patients to use autologous proteins (46).

The results of this study confirm that NaCl can be used to tailor fibrin gels to support both the proangiogenic and osteogenic potential of MSCs when cocultured with ECFCs *in vitro*. Furthermore, this coculture population exhibits increased osteogenic potential compared with a monoculture of MSCs, and this enhanced function is due to the ECFC secretion of BMP-2. FJ

This work was supported by a U.S. National Institutes of Health, National Institute of Dental and Craniofacial Research Grant R03-DE021704, and the AO Foundation (Davos, Switzerland) (C10-39L to J.K.L.). K.M. was supported by the American Heart Association Western States Affiliate Predoctoral Fellowship. The authors declare no conflicts of interest.

REFERENCES

1. American Academy of Orthopaedic Surgeons (2000) *Musculoskeletal Injuries Report: Incidence, Risk Factors and Prevention*, Rosemont, IL
2. Bajada, S., Mazakova, I., Richardson, J. B., and Ashammakhi, N. (2008) Updates on stem cells and their applications in regenerative medicine. *J. Tissue Eng. Regen. Med.* **2**, 169–183
3. Hoch, A. I., and Leach, J. K. (2014) Concise review: optimizing expansion of bone marrow mesenchymal stem/stromal cells for clinical applications. *Stem Cells Transl. Med.* **3**, 643–652
4. Leu, A., Stieger, S. M., Dayton, P., Ferrara, K. W., and Leach, J. K. (2009) Angiogenic response to bioactive glass promotes bone healing in an irradiated calvarial defect. *Tissue Eng. Part A* **15**, 877–885
5. He, J., Decaris, M. L., and Leach, J. K. (2012) Bioceramic-mediated trophic factor secretion by mesenchymal stem cells enhances in vitro endothelial cell persistence and in vivo angiogenesis. *Tissue Eng. Part A* **18**, 1520–1528
6. Kaigler, D., Krebsbach, P. H., Wang, Z., West, E. R., Horger, K., and Mooney, D. J. (2006) Transplanted endothelial cells enhance orthotopic bone regeneration. *J. Dent. Res.* **85**, 633–637
7. Kaigler, D., Krebsbach, P. H., West, E. R., Horger, K., Huang, Y. C., and Mooney, D. J. (2005) Endothelial cell modulation of bone marrow stromal cell osteogenic potential. FASEB J. **19**, 665–667
8. Grellier, M., Granja, P. L., Fricain, J. C., Bidarra, S. J., Renard, M., Bareille, R., Bourget, C., Amédée, J., and Barbosa, M. A. (2009) The effect of the co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres on mineralization in a bone defect. *Biomaterials* **30**, 3271–3278
9. Seebach, C., Henrich, D., Wilhelm, K., Barker, J. H., and Marzi, I. (2012) Endothelial progenitor cells improve directly and indirectly early vascularization of mesenchymal stem cell-driven bone regeneration in a critical bone defect in rats. *Cell Transplant.* **21**, 1667–1677
10. Melero-Martin, J. M., Khan, Z. A., Picard, A., Wu, X., Paruchuri, S., and Bischoff, J. (2007) In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood* **109**, 4761–4768
11. Ingram, D. A., Mead, L. E., Tanaka, H., Meade, V., Fenoglio, A., Mortell, K., Pollok, K., Ferkowicz, M. J., Gilley, D., and Yoder, M. C. (2004) Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* **104**, 2752–2760
12. Decaris, M. L., Lee, C. I., Yoder, M. C., Tarantal, A. F., and Leach, J. K. (2009) Influence of the oxygen microenvironment on the proangiogenic potential of human endothelial colony forming cells. *Angiogenesis* **12**, 303–311
13. Fioretta, E. S., Fledderus, J. O., Baaijens, F. P., and Bouten, C. V. (2012) Influence of substrate stiffness on circulating progenitor cell fate. *J. Biomech.* **45**, 736–744
14. Ghajar, C. M., Chen, X., Harris, J. W., Suresh, V., Hughes, C. C., Jeon, N. L., Putnam, A. J., and George, S. C. (2008) The effect of matrix density on the regulation of 3-D capillary morphogenesis. *Biophys. J.* **94**, 1930–1941
15. Engler, A. J., Sen, S., Sweeney, H. L., and Discher, D. E. (2006) Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677–689
16. Murphy, K. C., Hughbanks, M. L., Binder, B. Y., Vissers, C. B., and Leach, J. K. (2015) Engineered fibrin gels for parallel stimulation of mesenchymal stem cell proangiogenic and osteogenic potential. *Ann. Biomed. Eng.* **43**, 2010–2021
17. Kim, J., Park, Y., Tae, G., Lee, K. B., Hwang, C. M., Hwang, S. J., Kim, I. S., Noh, I., and Sun, K. (2009) Characterization of low-molecular-weight hyaluronic acid-based hydrogel and differential stem cell responses in the hydrogel microenvironments. *J. Biomed. Mater. Res. A* **88**, 967–975
18. Chen, Y. C., Lin, R. Z., Qi, H., Yang, Y., Bae, H., Melero-Martin, J. M., and Khademhosseini, A. (2012) Functional human vascular network generated in photocrosslinkable gelatin methacrylate hydrogels. *Adv. Funct. Mater.* **22**, 2027–2039
19. Janmey, P. A., Winer, J. P., and Weisel, J. W. (2009) Fibrin gels and their clinical and bioengineering applications. *J. R. Soc. Interface* **6**, 1-10
20. Davis, H. E., Miller, S. L., Case, E. M., and Leach, J. K. (2011) Supplementation of fibrin gels with sodium chloride enhances physical properties and ensuing osteogenic response. *Acta Biomater.* **7**, 691–699
21. Yoder, M. C., Mead, L. E., Prater, D., Krier, T. R., Mroueh, K. N., Li, F., Krasich, R., Temm, C. J., Prchal, J. T., and Ingram, D. A. (2007) Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* **109**, 1801–1809
22. Murphy, K. C., and Leach, J. K. (2012) A reproducible, high throughput method for fabricating fibrin gels. *BMC Res. Notes* **5**, 423
23. Decaris, M. L., and Leach, J. K. (2011) Design of experiments approach to engineer cell-secreted matrices for directing osteogenic differentiation. *Ann. Biomed. Eng.* **39**, 1174–1185
24. Hoch, A. I., Binder, B. Y., Genetos, D. C., and Leach, J. K. (2012) Differentiation-dependent secretion of proangiogenic factors by mesenchymal stem cells. *PLoS One* **7**, e35579
25. Ghajar, C. M., Kachgal, S., Kniazeva, E., Mori, H., Costes, S. V., George, S. C., and Putnam, A. J. (2010) Mesenchymal cells stimulate capillary morphogenesis via distinct proteolytic mechanisms. *Exp. Cell Res.* **316**, 813–825
26. Edgar, C. M., Chakravarthy, V., Barnes, G., Kakar, S., Gerstenfeld, L. C., and Einhorn, T. A. (2007) Autogenous regulation of a network of bone morphogenetic proteins (BMPs) mediates the osteogenic differentiation in murine marrow stromal cells. *Bone* **40**, 1389–1398
27. Das, A., and Botchwey, E. (2011) Evaluation of angiogenesis and osteogenesis. *Tissue Eng. Part B Rev.* **17**, 403–414
28. Yu, H., VandeVord, P. J., Mao, L., Matthew, H. W., Wooley, P. H., and Yang, S. Y. (2009) Improved tissue-engineered bone regeneration by endothelial cell mediated vascularization. *Biomaterials* **30**, 508–517
29. Koike, N., Fukumura, D., Gralla, O., Au, P., Schechner, J. S., and Jain, R. K. (2004) Tissue engineering: creation of long-lasting blood vessels. *Nature* **428**, 138–139
30. Herzog, D. P., Dohle, E., Bischoff, I., and Kirkpatrick, C. J. (2014) Cell communication in a coculture system consisting of outgrowth endothelial cells and primary osteoblasts. *Biomed Res. Int.* **2014**, 1–15
31. Kim, J., Kim, I. S., Cho, T. H., Lee, K. B., Hwang, S. J., Tae, G., Noh, I., Lee, S. H., Park, Y., and Sun, K. (2007) Bone regeneration using hyaluronic acid-based hydrogel with bone morphogenic protein-2 and human mesenchymal stem cells. *Biomaterials* **28**, 1830–1837
32. Chung, Y. I., Ahn, K. M., Jeon, S. H., Lee, S. Y., Lee, J. H., and Tae, G. (2007) Enhanced bone regeneration with BMP-2 loaded functional nanoparticle-hydrogel complex. *J. Control. Release* **121**, 91–99
33. Oelker, A. M., Berlin, J. A., Wathier, M., and Grinstaff, M. W. (2011) Synthesis and characterization of dendron cross-linked PEG hydrogels as corneal adhesives. *Biomacromolecules* **12**, 1658–1665
34. Wingate, K., Bonani, W., Tan, Y., Bryant, S. J., and Tan, W. (2012) Compressive elasticity of three-dimensional nanofiber matrix directs mesenchymal stem cell differentiation to vascular cells with endothelial or smooth muscle cell markers. *Acta Biomater.* **8**, 1440–1449
35. Binder, B. Y., Genetos, D. C., and Leach, J. K. (2014) Lysophosphatidic acid protects human mesenchymal stromal cells

from differentiation-dependent vulnerability to apoptosis. *Tissue Eng. Part A* **20**, 1156–1164
36. Smadja, D. M., Bièche, I., Silvestre, J. S., Germain, S., Cornet, A., Laurendeau, I., Duong-Van-Huyen, J. P., Emmerich, J., Vidaud, M., Aiach, M., and Gaussem, P. (2008) Bone morphogenetic proteins 2 and 4 are selectively expressed by late outgrowth endothelial progenitor cells and promote neoangiogenesis. *Arterioscler. Thromb. Vasc. Biol.* **28**, 2137–2143
37. Saleh, F. A., Whyte, M., Ashton, P., and Genever, P. G. (2011) Regulation of mesenchymal stem cell activity by endothelial cells. *Stem Cells Dev.* **20**, 391–403
38. Young, S., Patel, Z. S., Kretlow, J. D., Murphy, M. B., Mountziaris, P. M., Baggett, L. S., Ueda, H., Tabata, Y., Jansen, J. A., Wong, M., and Mikos, A. G. (2009) Dose effect of dual delivery of vascular endothelial growth factor and bone morphogenetic protein-2 on bone regeneration in a rat critical-size defect model. *Tissue Eng. Part A* **15**, 2347–2362
39. Leach, J. K., Kaigler, D., Wang, Z., Krebsbach, P. H., and Mooney, D. J. (2006) Coating of VEGF-releasing scaffolds with bioactive glass for angiogenesis and bone regeneration. *Biomaterials* **27**, 3249–3255
40. Joo, H. J., Song, S., Seo, H. R., Shin, J. H., Choi, S. C., Park, J. H., Yu, C. W., Hong, S. J., and Lim, D. S. (2015) Human endothelial colony forming cells from adult peripheral blood have enhanced sprouting angiogenic potential through up-regulating VEGFR2 signaling. *Int. J. Cardiol.* **197**, 33–43
41. Fierro, F. A., Kalomoiris, S., Sondergaard, C. S., and Nolta, J. A. (2011) Effects on proliferation and differentiation of multipotent bone marrow stromal cells engineered to express growth factors for combined cell and gene therapy. *Stem Cells* **29**, 1727–1737
42. Dyer, L. A., Pi, X., and Patterson, C. (2014) The role of BMPs in endothelial cell function and dysfunction. *Trends Endocrinol. Metab.* **25**, 472–480
43. Ghajar, C. M., Blevins, K. S., Hughes, C. C., George, S. C., and Putnam, A. J. (2006) Mesenchymal stem cells enhance angiogenesis in mechanically viable prevascularized tissues via early matrix metalloproteinase upregulation. *Tissue Eng.* **12**, 2875–2888
44. Lin, R. Z., Moreno-Luna, R., Li, D., Jaminet, S. C., Greene, A. K., and Melero-Martin, J. M. (2014) Human endothelial colony-forming cells serve as trophic mediators for mesenchymal stem cell engraftment via paracrine signaling. *Proc. Natl. Acad. Sci. USA* **111**, 10137–10142
45. Melero-Martin, J. M., De Obaldia, M. E., Kang, S. Y., Khan, Z. A., Yuan, L., Oettgen, P., and Bischoff, J. (2008) Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. *Circ. Res.* **103**, 194–202
46. Spotnitz, W. D. (2014) Fibrin sealant: the only approved hemostat, sealant, and adhesive-a laboratory and clinical perspective. *ISRN Surg.* **2014**, 203943

Received for publication July 31, 2015.
Accepted for publication September 21, 2015.