Localized BMP-4 release improves the enthesis of engineered bone-to-bone ligaments

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1 INTRODUCTION

Over 100,000 ACL reconstructions are performed in the United States annually with an associated cost of over $1.5 billion. A particular area of importance for successful reconstruction is the regeneration of the enthesis, the interface between the bone and ligament or tendon graft. The ACL enthesis is a fibrocartilaginous transition, composed of 4 distinct but continuous zones: the ligament proper, unmineralized fibrocartilage, mineralized fibrocartilage, and bone. As the tissue approaches bone, the composition of the tissue shifts to accommodate changes in mechanical loading. The ligament proper consists of a highly aligned extracellular matrix (ECM) containing primarily type I collagen, and to a lesser extent type III collagen and proteoglycans, that is suited to resisting tensile load. The tissue transitions gradually to unmineralized fibrocartilage, which contains type I, II, and III collagen and an enrichment of proteoglycans such as aggrecan as well as the glycoprotein tenascin C which assists in resisting the compressive loads found at the enthesis. A tidemark demarcates the boundary between unmineralized and mineralized fibrocartilage; the latter is distinguished by the expression of type X collagen and progressively greater mineral content and stiffness as it transitions toward bone. Lastly, the bone to which the ligament is attached is mainly composed of a

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KEYWORDS
ligament injury, musculo-skeletal system, tendon injury

Successful anterior cruciate ligament (ACL) reconstruction requires a functional enthesis, the interface between a bone and ligament or tendon. The enthesis normally exhibits a gradient of tissue phenotypes to smooth the transition from the compliant ligament to stiff bone and decrease stress concentrations between the 2 tissues. However, this structure does not fully regenerate after surgical repair leading to increased rupture rates and incidence of osteoarthritis. We have previously engineered ligaments between 2 cylindrical brushite cement anchors using human ACL fibroblasts entrapped in a fibrin gel. Using this model, we hypothesized that the local application and release of growth factors from the brushite cement anchors will promote fibrocartilage formation and improve interface failure load. Of 5 chondrogenic growth factors (BMP-2, BMP-4, and BMP-7 and TGF-β1 and TGF-β3) tested, we identified TGF-β1 as having a significant negative effect (P = .0001), while the local release of BMP-4 at the enthesis of engineered ligaments improved the interface failure load by up to 38% compared to control conditions. BMP-4 treatment increased the expression of the enthesis-related genes Sox9, aggrecan, and tenascin C and the inhibitor of mineralization osteopontin. These data suggest that BMP-4 drives a shift toward a fibrocartilaginous phenotype resulting in a stronger engineered enthesis.
highly mineralized type I collagen matrix in addition to other non-collagenous proteins such as alkaline phosphatase, osteopontin, SPARC (secreted protein acidic and rich in cysteine), and osteocalcin. The highly aligned collagen matrix originating in the ligament proper continues through the fibrocartilage region becoming less aligned as the surface area increases at the attachment to bone. This gradient of tissue phenotypes and their mechanical properties help to smooth the transition from the compliant ligament to the stiff bone preventing damaging stress concentrations between the 2 tissues.

In vivo, the fibrocartilage interface develops after birth, originating from a small, hedgehog-responsive population of cells at the interface of the nascent tendon/ligament and bone. These cells develop into the characteristic longitudinal rows of rounded fibrocartilage cells. As the fibrocartilaginous enthesis matures into its characteristic 4-zone morphology, these zones also are distinguished by the expression of key regulators of the respective tissue types, namely the transcription factor scleraxis in tendon and ligament, the transcription factor Sry-related HMG box 9 (Sox9) in cartilage, and runt-related transcription factor 2 (Runx2) in bone. The developmental signals required for this process have not been fully determined, but both chemical signals—from the developing tendon and bone—and mechanical signals—from loading the tissue—are required for development of the mature fibrocartilaginous enthesis.

Because of the importance of the enthesis in load transfer, ACL ruptures are commonly repaired using bone-patel lar tendon-bone autografts, taken from the middle third of the healthy patellar tendon. These grafts contain intact entheses, allowing rapid bone-to-bone healing. However, significant donor site morbidity, including rupture of the patellar tendon, has resulted in a shift toward hamstring tendon autografts despite their impaired integration with bone. Poor regeneration of the enthesis after repair with a hamstring autograft contributes to higher re-rupture rates and osteoarthritis. Tissue-engineered grafts present a potentially limitless source of tissue for reconstruction. We have previously engineered bone-to-bone ligaments formed between 2 cylindrical brushite cement anchors using human ACL fibroblasts entrapped in a fibrin gel. These can be engineered from autologous cells isolated from the patient, reducing the possibility of rejection, disease transmission, and donor site morbidity while promoting faster rehabilitation. However, engineered ligaments are currently limited by the structural and mechanical immaturity of the tissue and the fact that a functional enthesis has yet to be recreated in vitro. Le Nihouannen and colleagues have previously demonstrated that growth factors can be adsorbed onto brushite for sustained, localized release. Using our engineered bone-to-bone ligament model, we hypothesized that local application and release of growth factors from the brushite anchors at the interface between the soft tissue and mineral will promote fibrocartilage formation, as evaluated by quantifying genes expressed in the different zones of the native enthesis, and thus improve interface failure load. Five growth factors that have been associated with fibrocartilage formation were used in this study: bone morphogenetic protein (BMP)-2, BMP-4, and BMP-7 and transforming growth factor (TGF)-β1 and TGF-β3. The objectives of this work were to (a) identify 1 or more growth factors important for interface failure load; (b) characterize their adsorption and local release; and (c) evaluate the potential of the growth factor(s) to produce fibrocartilage in an engineered ligament model.

2 | MATERIALS AND METHODS

2.1 | Materials

All materials were obtained from Sigma (St. Louis, MO) unless otherwise indicated below.

2.2 | Tissue collection and cell isolation

The University of California Davis Institutional Review Board approved all procedures and protocols. Remnants of human anterior cruciate ligaments (ACL) were collected during standard ACL reconstruction surgeries with informed consent. The ligament remnants were digested as previously described. Briefly, ligaments were washed 5 times in sterile phosphate-buffered saline (PBS) and then placed in a 5% antibiotic/antimycotic (ABAM) solution for 2 hours. The tissues were digested overnight at 37°C in 0.1% type II collagenase (Lot No. 749475, Cat. No. 17101015; Thermo Fisher Scientific Inc., Waltham, MA) dissolved in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum (FBS) (Lot No. FB22024N, Cat. No. FB52000; Biosera, Kansas City, MO) and 1% penicillin. The resulting fibroblasts were collected by centrifugation (1500 × g for 5 minutes), washed 3 times with growth media (DMEM containing 10% FBS and 1% penicillin), and then plated on 15-cm tissue culture plates. All experiments were performed on cells prior to passage 6.

2.3 | Formation of functionalized brushite cement anchors

Cylindrical brushite cement anchors were produced as described previously. Briefly, 1 g of β-tricalcium phosphate
was combined with 1 mL of a solution of 3.5 mol/L orthophosphoric acid and 100 mmol/L citric acid, and the resulting cement was transferred to a cylindrical mold containing single minutien pins and centrifuged for 1 minute at 3700 × g, before being allowed to set overnight at room temperature. Prior to use, the anchors were sterilized by soaking in 70% ethanol for 30 minutes and air-dried in a biological safety cabinet. Functionalized brushite cement anchors were produced by soaking the anchors in 40 µL growth factor solutions (or their respective reconstitution buffers) for 12 hours in a 96-well plate; specific growth factors used and their concentrations were experiment-dependent as described in subsequent sections. Following adsorption, the anchors were removed from the solutions and were dried for 1 hour in a biological safety cabinet (Figure 1A). Control anchors did not undergo an additional soaking step.

### 2.4 Formation of engineered ligaments

Ligament constructs were engineered as previously described. Briefly, 2.5 × 10⁵ ACL fibroblasts were suspended in growth media containing 5.8 units of thrombin (Lot No. 070M7351V, Cat. No. T4648-1KU), 20 µg aprotinin (Cat. No. A3428), and 2 µg 6-aminohexanoic acid (Cat. No. 07260-100 g). 714 µL of this cell/thrombin solution was dispersed onto each plate containing 2 cylindrical brushite cement anchors pinned 12 mm apart. The fibrin gel was formed by adding 286 µL of a 20 mg/mL fibrinogen solution (Lot No. 029K7636V, Cat. No. F8630-5G) and agitating the dish to thoroughly combine the mixture. Fibrin gelation was apparent within 1 minute of the agitation step. The plates were then incubated at 37°C with 5% CO₂ for 15 minutes to allow for complete gelation. Following gel formation, 2 mL of feed media consisting of growth media supplemented with 5 ng/mL TGF-β1 (Lot No. 0611209, Cat. No. 100-21; Peprotech, Rocky Hill, NJ), 200 µmol/L ascorbic acid (Cat. No. A8960-5G), and 50 µmol/L L-proline (Cat. No. P5607-25G) was added to each plate. The constructs were cultured for 14 days with media changes every other day. The cells contracted the fibrin resulting in the formation of a linear tissue between the 2 brushite cement anchors (Figure 1B).

![FIGURE 1](image-url) Formation of engineered ligaments. A, Functionalized brushite cement anchors were produced by soaking in a growth factor solution (GF) for 12 h and drying for 1 hr prior to use in the formation of engineered ligaments. B, Human ACL fibroblasts were seeded in a fibrin gel formed in a 35-mm dish around 2 functionalized brushite cement anchors. Over a 14-day culture period, the cells cause the contraction and digestion of the fibrin, forming a collagenous ligament-like tissue between the 2 anchors. C, After a 14-day culture, the engineered ligaments were tested using a custom-built uniaxial tensile tester using reverse molded grips. D, Failure occurred at the mineral-ligament interface allowing maximal tensile load (MTL) to be used as interface failure load.
2.5 | Uniaxial tensile testing

Mechanical testing was performed as described previously. Briefly, the dimensions of each construct were determined using a digital caliper, and the ligaments were then placed in a custom-built tensile tester (Figure 1C). The sample was kept submerged in phosphate-buffered saline and loaded to failure without preconditioning. The tensile test used a constant elongation rate of 0.4 mm/s. From the resulting deformation and load data, the interface failure load was calculated as the maximal tensile load (MTL) (newtons) achieved during the test because failure consistently occurred at the interface (Figure 1D). The load and deformation data were also normalized to the cross-sectional area (CSA) of the graft and initial length to generate stress and strain data. From the slope of the linear region of the stress-strain curve, we obtained the Young’s modulus, and the maximal stress value was used for ultimate tensile strength (UTS).

2.6 | Hydroxyproline determination of collagen content

Collagen content of the sinewes was determined using a hydroxyproline assay. After mechanical testing to failure, the constructs were removed from their brushite cement anchors and dried in an oven for 30 minutes at 120°C. Each sample was then weighed and hydrolyzed in 200 µL of 6 N HCl at 120°C for 2 hours and then dried for 1.5 hours at the same temperature. The resulting dehydrated pellet was resuspended in 200 µL of hydroxyproline buffer (containing 173 mmol/L citric acid, 140 mmol/L acetic acid, 588 mmol/L sodium acetate, 570 mmol/L sodium hydroxide) and then further diluted at 1:40 in that buffer. 150 µL of chloramine T solution was added to each sample, mixed, and incubated at room temperature for 20 minutes. 150 µL of aldehyde-perchloric acid containing 60% 1-propanol, 5.8% perchloric acid, and 1 mol/L 4-(dimethylamino)benzaldehyde was then added to each tube before being vortexed and incubated at 60°C for 15 minutes. The tubes were then cooled for 10 minutes and the samples read at 550 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments Limited, Winooski, VT). Hydroxyproline was converted to collagen mass assuming hydroxyproline contributes to 13.7% of the dry mass of collagen. The collagen fraction was determined by dividing the collagen content by the mass of the tissue.

2.7 | Box-Behnken design of experiments

To determine the contributions and interactions of chondrogenic growth factors to the interface failure load of engineered ligaments, we applied a Box-Behnken incomplete multifactorial design using Design-Expert® software (StatEase, Inc., Minneapolis, MN). The design resulted in 54 different combinations and concentrations of growth factors in which 3 levels of each factor were tested and the resulting response measures for each combination analyzed in a quadratic surface response plot. The factors that we selected for study were those with some implicit or explicit role in fibrocartilage development. Specifically, we used bone morphogenetic protein (BMP)-2 (Lot No. 0410255, Cat. No. 120-02), BMP-4 (Lot No. 07103463, Cat. No. 120-05), and BMP-7 (Lot No. 1210264, Cat. No. 120-03) and transforming growth factor (TGF)-β1 (Lot No. 0611209, Cat. No. 100-21) and TGF-β3 (Lot No. 0209P410, Cat. No. 100-36E) (all obtained from Peprotech, Rocky Hill, NJ). Brushite cement anchors were functionalized as described above with 1 of the 49 combinations of these 5 growth factors at concentrations from 50 to 1000 ng/mL as determined by the Design-Expert® software. An additional 5 grafts were formed with anchors functionalized with the 525 ng/mL of each growth factor to determine the biological variability of the system and provide a statistical control for the study. The collagen content and mechanics of the grafts were determined after 14 days in culture.

2.8 | Determination of BMP-4 adsorption and release by ELISA

To indirectly determine the amount of BMP-4 adsorbed to brushite anchors, the remaining BMP-4 solutions (0.01, 0.1, 1, 10, 100 µg/mL) after removing the functionalized anchors were diluted in buffer (100-, 5000-, 10 000-, or 50 000-fold, respectively) and the BMP-4 remaining in the solution was quantified using the human BMP-4 Quantikine ELISA Kit (Lot No. 316035, Cat. No. DBP400; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

In a separate experiment, the amount of BMP-4 released from functionalized anchors into the culture media was determined. Anchors functionalized with 10 µg/mL BMP-4 were used to form engineered ligaments, and over a period of 30 days, 100 µL samples of the culture media were collected every other day. The media samples were diluted 100-fold and BMP-4 was quantified by ELISA as described above.

2.9 | BMP-4 dose response

Brushite cement anchors were functionalized with BMP-4 soaking solutions consisting of a range of concentrations. A dose response was initially performed with 10-fold increasing concentrations of BMP-4 up to 100 µg/mL (data not shown), but as 10 µg/mL BMP-4 showed the greatest
Effect, we focused on this or greater levels of BMP-4 peaking with a maximum BMP-4 solution concentration of 50 μg/mL. Control groups consisted of ligaments engineered with dry, untreated anchors (CON) and ligaments engineered with anchors soaked in the buffer solution (0 μg/mL) used to reconstitute the BMP-4 as per the manufacturer’s instructions. Overall, dose response experimental groups consisted of the following: CON, 0, 10, 25, and 50 μg/mL BMP-4.

2.10 Effect of BMP-4 on gene expression at the engineered enthesis

To determine the effect of BMP-4 on gene expression, constructs were formed around single anchors centrally placed in silicone-coated 12-well plate wells using 0.5 mL fibrin gel per construct with the same proportions of components as previously used. Over the course of several days, a ring of engineered ligament tissue formed around each of the anchors, approximating the enthesis region of whole engineered ligaments spanning 2 brushite cement anchors. These so-called enthesis constructs were cultured in feed media supplemented with 0 (buffer), 10, or 100 ng/mL BMP-4. In supplementing the culture media with BMP-4, the cells within the enthesis construct would be exposed to a uniform amount of BMP-4 to minimize variability and more precisely control BMP-4 dosage and treatment duration. After 10 days of culture, constructs were placed in RNAzol RT reagent (Molecular Research Center, Inc., Cincinnati, OH) for subsequent analysis of gene expression. RNA was quantified and 1 μL of RNAzol and a metal bead. The samples were homogenized by shaking at 50 Hz for 3 minutes followed by a 2-minute rest and another 2 minutes at 50 Hz in a TissueLyser LT (Qiagen, Frederick, MD). The beads were removed and RNA isolation was performed as per the manufacturer’s instructions. Total RNA was extracted from the engineered enthesis using RNAzol RT reagent (Molecular Research Center, Inc., Cincinnati, OH). For homogenization, tissues were transferred to a 2-mL Eppendorf tube with 250 μL of RNAzol and a metal bead. The samples were homogenized by shaking at 50 Hz for 3 minutes followed by a 2-minute rest and another 2 minutes at 50 Hz in a TissueLyser LT (Qiagen, Frederick, MD). The beads were removed and RNA isolation was performed as per the manufacturer’s instructions. Total RNA was quantified and 1 μg of RNA from each sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) for subsequent analysis of gene expression. Quantitative real-time PCR (qPCR) was performed using Eppendorf Mastercycler ep realplex4 thermal cycler (Eppendorf, Hauppauge, NY) with primers to scleraxis (fwd-5′-CTGGCCCTCAGCTACATCTC, rev-5′-GCAGAA GGTGCAAGATCGTGGG), collagen 1a1 (fwd-5′-AGAGGCTGGATGGTGGG, rev-5′-GTTTTGGGGTGTCTTGG), Sox9 (fwd-5′-AGGCCTCCTGATCGGAGTT, rev-5′-GGATCCATGGCTCAGGACT), aggrecan (fwd-5′-CT GCAGAAGCTGCTTCCG, rev-5′-CTGCAGGCTCAGGACT), TAT, rev-5′-TGAGGGAGGATTGTAAGAC), osteopontin (fwd-5′-GCCGAGGGTATGAGGTT, rev-5′-TGAG GTGAGTCCTGCTG), SPARC (fwd-5′-TGCCCTGA TGAGACAGGAGTTG, rev-5′-GGACAGATTAGCTCC ACA), and tenascin C (fwd-5′-ATTCTGGGAAGCTGC TGAC; rev-5′-GCTTGGGTTTGATGCGGATACC). Gene expression analysis was based on the 2−ΔΔCT method, using GAPDH (fwd-5′-ACAGCCTCAAGATCATCAGC; rev-5′-ATGAGTCCCTCACCAGATACC) as a reference gene. The absolute CT values of GAPDH were not different in any of the experimental groups.

2.11 Statistical analysis

The Box-Behnken data were analyzed as described by Box and Behnken 28 using the Design-Expert® software. A 2FI model was used for analysis as suggested for the data set. Data are presented as mean ± standard error of the mean (SEM). For all other experiments, GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) was used to conduct statistical analysis. Normality was assessed with the Kolmogorov-Smirnov test, and homogeneity of variance was assessed with the Brown-Forsythe test. A 1-way ANOVA was used to assess the presence of statistical significance between experimental conditions. Tukey’s HSD was used for post-hoc analysis for groups with equal variances and the Kruskal-Wallis test used for groups with non-equal variances. Statistical analyses and the type I error were maintained at α < .05 for all comparisons.

3 RESULTS

3.1 Effect of chondrogenic growth factors on interface failure load and collagen content

Engineered ligaments were produced using brushite cement anchors functionalized with varying combinations and concentrations of growth factors according to a Box-Behnken design. Five chondrogenic growth factors were tested: BMP-2, BMP-4, BMP-7, TGF-β1, and TGF-β3, at concentrations ranging from 50 to 1000 ng/mL. After a 2-week culture period, the interface failure load was determined. A 2-factor interaction (2FI) model was fitted to the experimental data using Design-Expert® software. ANOVA was used to assess the statistical significance of the model (P < .05) as well as the effects of the individual factors. The resulting response surface analysis of the Box-Behnken study showed that TGF-β1 has a significant negative effect (P = .0001), and while it was the only factor that reached significance, we also found that BMP-4 had the strongest positive (although not significant, P = .1598) effect on interface failure load (Figure 2A). BMP-2 and BMP-7 also showed small positive
effects individually but exhibited synergistic improvement in interface failure load together (data not shown). At the peak concentrations of both of these factors, the interface failure load is modeled to approach that of the effect of BMP-4 alone. TGF-β3 was observed to have a small positive effect on interface failure load, but when combined with increasing concentrations of BMP-4, the addition of TGF-β3 had a negative effect (Figure 2B). BMP-2 alone tended to have a small positive effect on interface failure load, but as MTL did not increase in combination with BMP-4 and in fact BMP-2 tended to decrease the maximum effect of BMP-4, this suggests that there may be competition between the 2 growth factors (Figure 2C). Even though TGF-β1 was the only factor that reached significance ($P < .0001$), the observed strong trend ($P = .16$) of BMP-4 on interface failure load, together with the fact that BMP-4 is known to be important for the formation of the enthesis in vivo, led us to further investigate the effect of BMP-4 on the interface.

3.2 | BMP-4 is adsorbed to and released from brushite anchors

To identify whether BMP-4 modulates the mechanical properties through a bulk or local effect, we quantified BMP-4 adsorption and release from the brushite anchors. To determine how much BMP-4 could be adsorbed to the brushite cement anchors, anchors were soaked in 10-fold increasing concentrations of BMP-4 from 0.01 to 100 μg/mL. Increasing the amount of BMP-4 resulted in a proportional increase in the absolute amount of BMP-4 adsorbed to the anchor (Figure 3A). To determine the amount of BMP-4 released into the media over time, we engineered ligaments using anchors functionalized with 10 μg/mL BMP-4 and measured the amount of BMP-4 released in the culture media over a period of 30 days (Figure 3B). The cumulative release for each anchor was 58.0 ± 3.6 ng of BMP-4, with the majority being released within the first 7 days of culture. Considering the amount of BMP-4 adsorbed to the 2 anchors (approximately 338.7 ± 69.4 ng), over two-thirds of the original...
BMP-4 was still available for a local effect during the typical 2-week culture duration.

3.3 | BMP-4 treatment alters the mechanical properties of engineered ligaments

Engineered ligaments formed using untreated brushite cement anchors (CON) or anchors functionalized with a soaking solution of 0 (equal volume of buffer), 10, 25, or 50 μg/mL BMP-4 were cultured for 14 days before uniaxial tensile testing. These concentrations were selected as preliminary experiments found that the interface failure load tended to increase with greater concentrations of BMP-4 up to 10 μg/mL (data not shown). The 10 μg/mL BMP-4 condition consistently produced the greatest interface failure load of all concentrations tested, with a 38% increase over control condition \((P = .025; \text{Figure 4A})\) and a 21.6% increase over the buffer condition \((P > .05; \text{Figure 4A})\). While the 10 μg/mL BMP-4 treatment improved interface failure load, this was not due to improvement in material properties of the engineered ligaments (Figure 4B and 4C), nor was it due to improvement in the collagen fraction (Figure 4E). The 10 and 25 μg/mL groups showed significantly increased mass (Figure 4F), on the order of 1 milligram, which cannot be fully accounted for with a concurrent increase in CSA (Figure 4D) or an increase in collagen content. Anchors treated with concentrations of BMP-4 greater than 10 μg/mL produced constructs with significantly poorer interface failure load and material properties, especially at 50 μg/mL BMP-4 at which level the interface failure load dropped to 72.1% that of the control group \((P < .0001; \text{Figure 4A})\) and 75.4% that of the buffer-treated (0 μg/mL) group \((P < .0001; \text{Figure 4A})\). In summary, BMP-4 was found to have a concentration-dependent effect on the mechanical properties of the interface and altered ECM content.

3.4 | BMP-4 treatment increases the expression of enthesis-associated genes

After establishing a BMP-4-mediated increase in the interface failure load of engineered ligaments that was not due

![Figure 4](image-url)
to increased collagen content in the graft, we sought to better characterize the changes in the phenotype of the cells near the interface. To model this area, we produced a 3D engineered enthesis by creating enthesis-only constructs around single brushite cement anchors in 12-well plates. These constructs were treated with 0, 10, or 100 ng/mL BMP-4 supplemented in the feed media (Figure 5A). The amount of BMP-4 was selected following calculation of the localized BMP-4 levels in the BMP-4 release experiment. These concentrations of BMP-4 approximate the range of BMP-4 that the cells would experience from released BMP-4 in the culture media (up to ~50 ng; Figure 3B) or at the tissue near the anchor (up to ~100 ng per anchor; Figure 3A). After 10 days of treatment, the constructs were processed for qPCR (Figure 5B-I). The tendon/ligament-specific marker scleraxis decreased approximately 20-fold with the high BMP-4 level, whereas the enthesis-related markers Sox9 (7.4-fold), aggrecan (12.1-fold), and tenasin C (2.2-fold) all increased. The bone-specific markers Runx2 and SPARC were unchanged, whereas the expression of osteopontin, an inhibitor of mineralization, was found to significantly increase with BMP-4 treatment ($P < .0001$) (up to 155.5-fold). Together, these data suggest that BMP-4 is inducing a change in the transcription profile that is consistent with a shift from ligament toward fibrocartilage and specifically non-mineralized fibrocartilage phenotype.

### 4 DISCUSSION

Using a fibrin cast ligament surrounding brushite cement anchor model, we have demonstrated that growth factors have a significant effect on the mechanical integrity of the engineered enthesis. Brushite cement anchors functionalized with TGF-$\beta$1 decreased, whereas BMP-4 increased the strength of the interface between an engineered ligament and brushite cement. We found that the local release of growth factors from anchors soaked in 10 $\mu$g/mL BMP-4 resulted in a 38% increase in the strength of the engineered enthesis compared to control conditions. Further, gene expression analysis suggested that this improvement in function occurred concomitant with a shift in transcriptional profile from a tendon/ligament toward that of fibrocartilage.

**FIGURE 5** BMP-4 increases the expression of enthesis-related genes in 3D single anchor constructs. A, Methodological setup for the gene expression study. Gene expression of (B) scleraxis, (C) collagen 1a1, (D) Sox9, (E) aggrecan, (F) tenasin C, (G) Runx2, (H) SPARC, and (I) osteopontin. Data are presented as mean ± SD with $n = 4$ for all groups. * indicates significant difference from control ($P < .0001$), and § indicates significant difference from all other groups ($P < .05$). Data are representative of 3 independent experiments.
Adapting the technique first described by Le Nihouannen and coworkers to functionalize brushite cement by adsorbing protein, we have directly determined the effect of growth factors on the strength of an engineered enthesis. To achieve this goal, we used a Box-Behnken partial factorial design of experiments approach. In this way, we were rapidly able to determine the effects of different combinations and concentrations of growth factors on the engineered enthesis. To our surprise, TGF-β1 had a significant negative effect on interface failure load (Figure 2). This was unexpected because TGF-β signaling plays an important role in fibrosis and in the development of ligaments and cartilage in vivo. However, due to the widely varying role of TGF-β signaling which can affect hundreds of different target genes, it is likely that the stimulatory effects of TGF-β are cell type–dependent and it has a stronger ligamentogenic rather than chondrogenic effect in ligament cells, which could potentially decrease the strength of the interface.

Even though none of the growth factors in the DOE had a significant positive effect on interface failure load, BMP-4 showed a clear trend to improve the strength of the interface. Furthermore, the interaction between the closely related BMP-2 and BMP-7, where high levels of BMP-2 or BMP-7 decreased the positive effects of BMP-4, suggested that the BMPs can increase interface failure load but that BMP-4 had the strongest effect of the BMPs tested. BMP signaling has an established role in regulating chondrogenesis and therefore, various BMPs including BMP-4 have been used to promote chondrogenic differentiation in human embryonic stem cells, human mesenchymal stem cells, and human chondrocytes. In tendon or ligament cells, a few groups have investigated the chondrogenic effects of BMP-2, BMP-6, and BMP-7. However, the potential of BMP-4 to promote chondrogenesis in ligament cells has not been studied previously despite the fact that BMP-4 is known to be important for the formation of the bone ridge at the interface between tendon and bone. Our DOE results suggested a trend toward improved enthesis strength using BMP-4. This, together with the prior use of BMP-4 in promoting in vitro chondrogenesis and its role in the developing tendon-bone interface, led us to investigate the effects of BMP-4 on the development of our engineered enthesis. Anchors treated with 10 μg/mL BMP-4 showed a small but significant improvement in interface failure load (Figure 4A) without a change in collagen (Figure 4E). BMP-4 had varying effects depending on the concentration, with concentration-dependent differences on the interface vs the ligament material properties. At 10 μg/mL BMP-4, the interface failure load was improved with little change to material properties, whereas at 25 μg/mL BMP-4 decreased both interface failure load and material properties (Figure 4). In both groups, the collagen fraction (Figure 4E) and mass (Figure 4F) were not different, implying that there is a change in the non-collagenous ECM content responsible for the differences in mechanical properties. Gene expression data confirmed that BMP-4 is able to change the expression of cartilage-associated ECM molecules (Figure 5D-F). The concentration-dependent effect, that is, that high BMP-4 decreased bulk material properties, may stem from the amount of BMP-4 released into the media, which at high concentrations decreased SCX expression and therefore would be expected to decrease the bulk mechanical properties of the engineered ligament.

Although there was only a 22% increase in the strength of the engineered enthesis treated with 10 μg/mL BMP-4 compared to the buffer treatment, there was a dramatic shift in gene expression in cells exposed to BMP-4 within an engineered enthesis. To our knowledge, this is the first report of a localized shift in cellular phenotype induced by BMP-4. This shift in phenotype likely underlies the improved mechanical properties of the interface of tissues engineered with the adsorption of 10 μg/mL BMP-4. Cells within engineered enthesis constructs treated with the level of BMP-4 experienced near the mineral interface of engineered ligaments showed a 20-fold decrease in the expression of the tendon/ligament-specific marker scleraxis. Concomitant with the decrease in scleraxis was an increase in the expression of genes enriched in fibrocartilage: Sox9 (7.4-fold), aggrecan (12.1-fold), and tenascin C (2.2-fold) (Figure 5D-F). The expression of the bone-specific transcription factor and master regulator of osteogenesis (Runx2) remained unchanged (Figure 5G). Expression of osteopontin and SPARC was also evaluated, both of which are involved in regulating mineralization and cell-matrix interactions. While SPARC expression remained unchanged, the expression of osteopontin, an acidic glycoprotein that inhibits mineralization, was significantly increased (up to 155.5-fold) with BMP-4 (Figure 5I). Osteopontin has also recently been shown to be localized in the mouse supraspinatus and quadriceps tendon enthesis in addition to being present at mineralized tissue interfaces of bone and cartilage and regions of ectopic calcification in tendons. Consistent with our gene expression analysis, Xu et al have shown that aggrecan and osteopontin are upregulated in response to BMP-4 in periodontal ligament cells. The increase in expression of chondrogenic genes and osteopontin, together with the decrease in scleraxis expression, suggests that in the presence of high BMP-4, the cells at the engineered enthesis shift from a ligament to a fibrocartilage phenotype. However, this has yet to be demonstrated at the protein level. Even though we showed a positive effect of 10 μg/mL BMP-4-adsorbed brushite cement anchors on interface failure load after 14 days in culture and a positive effect of BMP-4 on
fibrocartilage-related gene expression after 10 days in culture, greater concentrations of adsorbed BMP-4 were shown to have a negative effect on interface failure load. Given that the release profile of BMP-4 indicates that most of the adsorbed BMP-4 remains at the enthesis for local release, studies at additional time points would shed light on the longer term effects of BMP-4.

Through our DOE, we have shown that BMP-2 and BMP-7 had no significant effects on the interface failure load of engineered ligaments. This was surprising because both BMP-2 and BMP-7 have been shown to upregulate chondrogenic markers in bovine ligament fibroblasts and spinal ligament tissue, respectively. In addition, BMP-2 has been extensively used in studies testing its efficacy in promoting ligament/tendon-bone integration. BMP-7 has also been used in this context. However, the effectiveness of either of these BMPs to promote bone integration has been equivocal. In contrast, very few studies have probed the effect of BMP-4 on tendon/ligament-bone healing. Coen et al showed that in a rat model of biceps tenodesis, the administration of a lentiviral vector for BMP-4 in the bone tunnel and graft end improved graft pullout strength but showed no histological improvement. Anderson et al utilized bone-derived extract containing BMP-4 in addition to BMP-2, BMP-3, BMP-5, BMP-6, and BMP-7 to improve rabbit ACL reconstruction, but this study lacked information on the specific contribution of the different types of BMPs. In light of our DOE data, BMP-4 may be a better candidate for future studies looking to improve tendon-bone healing than BMP-2 or BMP-7.

A few groups have attempted to engineer a functional tendon- or ligament-bone unit in vitro. A key challenge in this process lies in creating and maintaining different cell phenotypes that are found in the native enthesis. Strategies have included (a) seeding different cell types representative of the different regions found in the enthesis; (b) modifying the regional properties, such as mechanical, structural, and bound/soluble factors, of a scaffold to support a heterogeneous cell population; and (c) modifying regional mechanical properties of a scaffold to promote cell differentiation. Each of these processes has been successful to a point; however, a functional enthesis spanning tendon/ligament to bone has been very difficult to reproduce in vitro.

In the current work, our approach was to modify a 2-phase construct consisting of a uniform cell population within an engineered ligament tissue flanked by bone-like brushite cement, a type of calcium phosphate known to be osteoconductive and bioresorbable. By locally applying bioactive factors at the interface between the 2 phases to promote the formation of a fibrocartilaginous phase, we hoped to reproduce the biological signals involved in the development of the fibrocartilaginous enthesis. An advantage of our model is that it is functional, as both the mechanical and phenotypic improvements to the interface are measurable. While the current size of the constructs limits its clinical relevance, they can be scaled up by manufacturing larger brushite cement anchors and engineering ligaments with an increased construct volume. In addition, due to this study’s focus on soluble factors, a further limitation of the current work is the absence of mechanical stimulation. Cells within tendons and ligaments have the ability to transition to fibrocartilage as an adaptation to compressive load both at the enthesis and in regions where they wrap around bony pulleys. This in vitro model is well suited for the application of mechanical stimulation, and combined with the method to locally apply growth factors at the enthesis demonstrated here, future avenues of study could include the contributions and potential interactions of mechanical and chemical signaling.

5 | CONCLUSIONS

We have used a method for the local release of growth factors to alter the mechanics of an engineered enthesis. Using a fibrin cast ligament model, we have shown that functionalizing the brushite cement anchors at either end of the ligament with TGF-β1 has a significant negative effect on enthesis strength, whereas 10 μg/mL BMP-4 significantly improves the failure load of the interface. The improved interface failure load was not the result of increased collagen production, but may result from a shift in the phenotype of the cells at the interface from tendon/ligament to fibrocartilage. These results suggest that functionalizing grafts with BMP-4 could improve clinical outcomes during ACL reconstruction surgeries.

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