Growth Factor-Mediated Migration of Bone Marrow Progenitor Cells for Accelerated Scaffold Recruitment

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Detailed Terms
Title:
Growth factor-mediated migration of bone marrow progenitor cells for accelerated scaffold recruitment

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PDGF-BB; TGF-\(\beta\)1; HB-IGF-1; scaffolds; migration; progenitor cells.
ABSTRACT

Tissue engineering approaches using growth-factor functionalized, acellular scaffolds to support and guide repair driven by endogenous cells is thought to require a careful balance between cell recruitment and growth-factor release kinetics. The objective of this study was to identify a growth factor combination that accelerates progenitor cell migration into self-assembling peptide hydrogels in the context of cartilage defect repair. A novel 3D gel-to-gel migration assay enabled quantification of the chemotactic impact of platelet-derived growth factor-BB (PDGF-BB), heparin-binding insulin-like growth factor-1 (HB-IGF-1), and transforming growth factor-β1 (TGF-β1) on progenitor cells derived from subchondral bovine trabecular bone (BM-PCs) encapsulated in the peptide hydrogel [KLDL]3. Only the combination of PDGF-BB and TGF-β1 stimulated significant migration of BM-PCs over a 4-day period, measured by confocal microscopy. Both PDGF-BB and TGF-β1 were slowly released from the gel, as measured using their 125I-labeled forms, and they remained significantly present in the gel at 4 days. In the context of augmenting microfracture surgery for cartilage repair, our strategy of delivering chemotactic and pro-anabolic growth factors in KLD may provide the necessary local stimulus to help increase defect cellularity, providing more cells to generate repair tissue.
I. INTRODUCTION

Acellular scaffolds functionalized with cell-stimulatory molecules are widely used in tissue-engineering\textsuperscript{(1-4)}. This approach relies on endogenous cells to populate the scaffold following surgical implantation. Matching the timescale of the release of the cell-stimulatory molecules from the scaffold with the invasion kinetics of endogenous cells into the scaffold would presumably be important.

For cartilage repair, microfracture surgery is used in part to access the endogenous progenitor cell population, but the resulting repair tissue is often mechanically inferior fibrocartilage, and lateral integration with native cartilage can be incomplete\textsuperscript{(5,6)}. \textit{In vitro} and \textit{in vivo} studies have shown that the addition of cell-free scaffolds functionalized with growth factors(GFs) may help induce chondrogenesis of endogenous progenitor cells, neotissue production by these cells, and subsequent integration of neotissue with host tissue\textsuperscript{(1,7-14)} (Fig. 1A).

One candidate class of scaffolds for augmenting microfracture is self-assembling peptide hydrogels\textsuperscript{(15,16)}. These injectable hydrogels assemble upon exposure to physiological pH and ionic strength at concentrations less than 0.5%, allowing ample space for cells to deposit neotissue. The self-assembling sequences KLD and RAD have been shown to foster chondrogenesis of bone marrow progenitor cells and matrix production by primary chondrocytes\textsuperscript{(13,16,17)}. Once assembled, acellular KLD has an equilibrium modulus of \(~1\text{kPa}\), while chondrocyte-seeded KLD supports the assembly of functional neo-tissue with a modulus of \(~95\text{kPa}\) after 4 weeks of culture\textsuperscript{(16,18)}. Initial \textit{in vivo} studies demonstrated KLD to both be biocompatible and show promise towards stimulating repair\textsuperscript{(1,19)}. 
Many pro-anabolic GFs have been investigated in tissue engineering approaches to stimulate growth, such as insulin-like growth factor-1 (IGF-1) in the case of cartilage tissue (20,21), vascular endothelial growth factor (VEGF) for cardiac tissue (4,22,23), fibroblastic growth factor-2 (FGF-2) and nerve growth factor (NGF) for neural tissue (24), and epidermal growth factor (EGF) for hepatic tissue (24). Growth factors are also frequently used to stimulate progenitor cell differentiation in vitro, such as transforming growth factor beta-1 (TGF-β1) to stimulate chondrogenesis of mesenchymal progenitor cells (25). When pharmacologically delivered to the body globally, however, some GFs, such as IGF-1, can trigger systemic adverse events (26-28). Local delivery of such GFs by functionalization to scaffolds or particles (24,29), encapsulation in microspheres (20,30), or the development of fusion proteins (31) have extended the viability of these GFs as useful tissue engineering tools. The fusion protein heparin-binding insulin-like growth factor-1 (HB-IGF-1), for example, stimulates matrix production by chondrocytes through a single dose delivered via a self-assembling peptide hydrogel scaffold (13,31). Such local delivery allows for lower doses than injection methods, reducing toxicity and costs.

Despite these promising in vitro results with locally delivered anabolic growth factors, it is believed that the benefits of these anabolic GFs will not be realized if the GFs diffuse out of the acellular scaffold prior to endogenous progenitor cell invasion in vivo. We therefore hypothesized that the addition of chemotactic GFs to the scaffold accelerates the migration of endogenous cells into the scaffold, providing potential enhanced benefit from the anabolic GFs.
Previous studies by Ozaki et al., Fiedler et al., and others, using modified Boyden chamber assays, have shown generally that mesenchymal stem cell chemotaxis can be stimulated by platelet derived growth factor-BB (PDGF-BB), and this effect is enhanced when combined with IGF-1 or transforming growth factor α (TGF-α) (32-37). Thus, we further hypothesized that the addition of PDGF-BB to our self-assembling peptide hydrogel scaffold provides a synergistic chemotactic effect with a pro-chondrogenic member of the TGF-family, TGF-β1, or the pro-anabolic GF HB-IGF-1.

Building upon the detailed literature characterizing KLD as a suitable environment for progenitor cell chondrogenesis and chondrocyte matrix production, this work focuses on assessing KLD as an environment for GF-induced migration (17,38-41). We hypothesized that the addition of pro-migratory GFs to KLD would result in increased cell migration into the gel. To test our hypothesis, we developed a novel 3D gel-to-gel migration system and used this system to evaluate the chemotactic impact of GFs on progenitor cells in KLD (Fig. 1B) (42). Specifically, using a cell population relevant to the endogenous cells accessed during microfracture, we studied the effects of the GFs PDGF-BB, TGF-β1, and HB-IGF-1 (individually and in combination) for their chemotactic potential in our 3D hydrogel scaffold. Further, we quantified the release kinetics of the candidate GFs from the scaffold in order to interpret their participation in cell migration.

II. MATERIALS & METHODS

**Cell harvest, expansion, and encapsulation:** Since the cells accessed in microfracture surgery reside below the microfracture punctures, we isolated progenitor
cells isolated from the stroma associated with subchondral trabecular bone. Mesenchymal progenitor cells associated with the marrow stroma found in immature trabecular bone (BM-PCs) were isolated from the subchondral trabecular bone of 1-2 week old bovine calf knee joints in a manner modified from Haynesworth et al. and Li et al. (43,44) (Fig. 2A) (Research 87, Marlborough, MA). Two 9mm osteochondral plugs were drilled from the condyle, and the cartilage layer was removed. The remaining trabecular bone explants were finely diced using a scalpel (Fig. 2B) and placed in primary expansion medium consisting of low glucose Dulbecco’s modified Eagle’s medium (DMEM) (Mediatech Inc., Manassas, VA) with 10% fetal bovine serum (FBS) (GE Healthcare Life Sciences, Hyclone Labs, Logan, UT), 100U/mL penicillin, 100µg/mL streptomycin, and 0.25µg/mL amphotericin (PSA), 10mM 4-(2-hydroxyethyl)-1-piperazaineethanesulfonic acid (HEPES) (Invitrogen, Carlsbad, CA), and 1ng/mL basic fibroblastic growth factor (FGF-2) (R&D Systems, Minneapolis, MN) (17). The bone fragments were rinsed vigorously with this medium and vortexed to mechanically drive out the cells. The resulting cell suspension was filtered through a 70µm-pore strainer and plated in flasks at 2.5x10^5 cells/cm² in expansion medium for 24 hours. The medium was removed and replenished, and cells were cultured until primary colonies reached 80% confluence (Fig. 2C). BM-PC colonies were removed from flasks with 0.05% trypsin in 0.53mM EDTA (Corning, Corning, NY), and frozen until further use. BM-PCs were passaged 1-2 times by plating with a seeding density of 1x10^3 cells/cm² in expansion medium containing 5ng/mL FGF-2 and expanding until 80-90% confluent. These expanded BM-PCs were encapsulated in 3.5mg/mL KLD self-assembling peptide hydrogels at 10x10^6 cells/mL, as described previously (17), for use in matrix production.
and migration studies. For matrix production studies, the cell-seeded hydrogels were cultured in chondrogenic culture medium consisting of high glucose DMEM, 1% insulin-transferrin-selenium + bovine serum albumin & linoleic acid(ITS+Premix)(BD Biosciences, Bedford, MA), 0.4mM proline(Sigma-Aldrich, St. Louis, MO), 37.5µg/mL ascorbate-2-phosphate(A2P)(Wake Chemicals, Richmond, VA), 10mM HEPES, PSA, 0.1mM non-essential amino acids(NEAA)(Sigma-Aldrich), 1mM sodium pyruvate(Invitrogen), 10ng/mL rhTGF-β1(Peprotech, Rocky Hill, NJ), and 0.1µM Dexamethasone(Invitrogen) at 37°C and 5% carbon dioxide, for 22 days(39,45).

**Analysis of matrix production and cell proliferation:** Thorough characterization of the chondrogenic capacity of bone marrow progenitor cells in KLD was previously reported by our lab, including both immunohistochemistry of collagen types I and II, as well as qPCR for the temporal evolution of gene expression for collagens types I & II, aggrecan, SOX9, osteocalcin and PPAR-γ(17). We also measured toluidine blue staining, sGAG production, and hydroxyproline content, and confirmed that these measures provided complementary surrogate markers of matrix production by chondrogenic cells(17,38). At day 22 in the present study, synthesis rates of sGAG and total protein were assessed by radiolabel incorporation using 5µCi of $^{35}$S-sulfate and 10µCi of $^3$H-proline(Perkin Elmer, Waltham, MA), respectively(46); total sGAG output was measured by DMMB dye binding, collagen content was measured by hydroxyproline reaction with p-dimethylaminobenzaldehyde, and DNA content was measured by Hoechst dye binding, as described previously(47-49). Histological staining for the spatial appearance of sGAG using toluidine blue was performed as described.
previously(16,17). N=6-8 gels per time point.

**Gel-to-gel migration assay:** We developed a novel gel-to-gel migration assay shown schematically in Fig. 1B. TGF-β1(100ng/mL), PDGF-BB(100 ng/mL)(Peprotech), or HB-IGF-1(500nM), along with all of their combinations, were tested by premixing them in 3.5mg/mL KLD self-assembling peptide hydrogel. The quantities of GFs added were not high enough to alter the assembly or mechanical properties of the KLD. Gels were cast into wells of a 48-well plate and assembled overnight in low glucose DMEM, PSA, 10mM HEPES, 1% FBS(migration medium). Based on the literature, this low concentration of FBS was chosen to avoid obscuring potential pro-migratory effects of the GFs being studied while maintaining cell viability during the course of the 4 day study(50). A thin layer of green fluorescent beads(polystyrene with 2% divinylbenzene, mean diameter 15.45µm; Bangs Laboratories, Inc., Fishers, IN) was added atop this gel, followed by the casting of a second KLD hydrogel encapsulating 150,000 BM-PCs (2x10^6cells/mL, chosen to optimize imaging and quantification)(Fig. 1B). These green fluorescent beads demarked the interface between the two gels to assist in confocal quantification of migrating cells. Hydrogels were cultured for 4 days at 37°C, then fixed with 4% PFA and stained with propidium iodide in a permeabilization buffer consisting of 0.5% Triton X-100(Sigma-Aldrich) and 0.5% BSA in PBS. Multichannel fluorescent confocal microscopy was used to image wells over the depth of the gels in 5µm steps, and migration was quantified using a modified MATLAB script developed previously(42,51). Briefly, 3D coordinates of cells and beads were calculated using a modified hybrid 2D/3D spot finding algorithm that determines fluorescent spot positions
for each 2D image slice and then reassembles them into the initial 3D shape. Variance in the fluorescent bead positions due to the curvature of the surface of the bottom gel was accounted for by comparing cell positions to the mean bead position ± 1-2 standard deviations. For fields of view only containing 1 bead, a standard deviation of 25µm was used.

**PDGF-BB loading efficiency in KLD hydrogels:**

A radiolabeled form of PDGF-BB\(^{125}\text{I}-\text{PDGF-BB}\) (Perkin Elmer) was used to assess its entrapment in KLD, following a method described previously for a similar self-assembling peptide hydrogel, RAD([RADA]\(_4\))(13,52). Just before use, free \(^{125}\text{I}\) label was first removed from 100µL of 19µCi/mL (1.9µg/mL) stock solution of \(^{125}\text{I}\)-PDGF-BB by Sephadex G-25 gel filtration chromatography (GE Healthcare Life Sciences, Marlborough, MA) (Supp. Fig 1). The resulting intact, labeled protein fractions were pooled and concentrated in 10,000MW cutoff Centricon centrifuge filters (EMD Millipore, Billerica, MA) spun at 3,000g at 4°C for 3 hours. The buffer was exchanged to a solution containing 10% sucrose (Sigma-Aldrich), 25mM HEPES, and 0.1% BSA through a subsequent 3,000g spin overnight at 4°C. The resulting retentate was combined with unlabeled PDGF-BB to a final total mixed species mass of 250ng, premixed into unassembled KLD to a final peptide concentration of 3.5mg/mL and a final PDGF-BB concentration of 335ng/mL, and 50µL gels were cast at the bottom of autoclaved low-retention tubes (n=10). Gel assembly was initiated by the addition of phosphate-buffered saline (PBS) (Sigma-Aldrich) + 1% PSA, and gels were incubated at 37°C at 5% CO\(_2\) on a rotary shaker. Three hours following assembly, the baths were removed from the gels,
and bath, gels, and tubes were counted via gamma counter for 2 minutes. Using similar methods, entrapment of TGF-β1 in KLD was previously characterized by Kopesky et al. using $^{125}$I-TGF-β1 (Perkin Elmer, Waltham, MA) (40), and entrapment of HB-IGF-1 in the similar self-assembling peptide hydrogel RAD was previously characterized by Florine et al. (13).

**GF release from KLD hydrogels:** Radiolabeled forms of PDGF-BB ($^{125}$I-PDGF-BB) and HB-IGF-1 ($^{14}$C-HB-IGF-1) were used to assess their release from KLD. (Release of TGF-β1 from KLD was previously reported by our lab (Kopesky et al.) using $^{125}$I-TGF-β1 (Perkin Elmer, Waltham, MA) (40).) $^{125}$I-PDGF-BB: The release of $^{125}$I-PDGF-BB from KLD was determined using a method similar to that described previously for $^{125}$I-TGF-β1 (40). Free $^{125}$I label was first removed from 40μL of 58μCi/mL (1.9μg/mL) stock solution of $^{125}$I-PDGF-BB by Sephadex G-25 gel filtration chromatography, as in the loading efficiency experiment above. The retentate following buffer exchange was premixed into unassembled KLD to a final peptide concentration of 3.5mg/mL, and 50μL gels were cast at the bottom of autoclaved low-retention tubes. Assembly was initiated with PBS + 1% PSA (n=14). Gels were incubated at 37°C at 5% CO$_2$ on a rotary shaker, and bath aliquots were removed and replenished every 1-2 days for 2 weeks. Aliquots, gels, and tubes were counted via gamma counter for 2 minutes following the two-week incubation. $^{14}$C-HB-IGF-1: The release profile of HB-IGF-1 from KLD was determined as described previously for the similar self-assembling peptide hydrogel RAD (13). Briefly, $^{14}$C-HB-IGF-1 was mixed with acellular KLD prior to gel assembly at a final GF concentration of 615nM, and PBS + 1% PSA was added to trigger self-assembly. Gels
were incubated at 37°C, and bath samples were removed and replenished every 24 hours for the first 8 days, and every 48 hours for days 8-16. On day 16, baths were removed, gels were mechanically disrupted, and radiolabeled protein was measured in bath and gel samples by liquid scintillation counting.

**Statistical analyses:** Values are mean ± SEM. Comparisons use general linear mixed effects models and Tukey’s HSD post hoc test (p<0.05), (JMP 11, SAS, Inc., Cary, NC).

### III. RESULTS

**BM-PC matrix production and cell proliferation:** We assessed the matrix production capacity of both passage-1 (P1) BM-PCs and passage-2 (P2) BM-PCs, to determine if four additional population doublings altered their ability to produce matrix. Histological assessment of P2 BM-PCs encapsulated in KLD hydrogels with toluidine blue for sGAG following 22 days of culture showed strong, disperse staining, indicative of high proteoglycan content (Fig. 3). Both P1 and P2 BM-PCs continued to proliferate following hydrogel encapsulation (Fig. 4A). Both populations also showed robust sGAG synthesis and deposition (Fig. 4B, C), along with increasing collagen deposition over the time course of the study (Fig. 4D). Previous studies using bovine BMSCs in KLD scaffolds have shown a similar evolution of sGAG synthesis and deposition, and the sGAG to be associated predominantly with aggrecan production (17). Together these data suggest a chondrogenic potential that is consistent with previous studies using BMSCs derived from cortical bone marrow (17,39).
**GF-stimulated gel-to-gel BM-PC migration:** To assess the ability of BM-PCs to invade a GF-loaded gel, BM-PCs were encapsulated in a second gel atop the first (Fig 1B). The mean position of the green fluorescent beads in the construct was used to demark the interface between the two gels. Cells that moved beyond one standard deviation past the mean bead position into the bottom gel were considered to have migrated, and the subset of cells that moved beyond two such standard deviations were considered to have migrated strongly (Fig. 5). All 8 combinations of TGF-β1, PDGF-BB, and HB-IGF-1 were tested for their impact on BM-PC migration in KLD hydrogels. The combination of TGF-β1 and PDGF-BB induced statistically significant migration compared to the GF-free control, whereas increases seen with all other GF combinations were not statistically significant (Fig. 6). The percent of cells that migrated strongly under the combination treatment of TGF-β1 and PDGF-BB was significantly higher than for TGF-β1 alone, HB-IGF-1 alone, or the combination of PDGF-BB and HB-IGF-1, while the percent of cells that migrated strongly under the PDGF-BB treatment was significantly higher than the combination of PDGF-BB and HB-IGF-1.

**Entrapment of 125I-PDGF-BB in acellular KLD:**

To determine the initial entrapment efficiency of PDGF-BB in unassembled KLD, a mixture of 125I-PDGF-BB and PDGF-BB at a final concentration of 335ng/mL was added to KLD at a final concentration of 3.5mg/mL of peptide (see Methods). Three hours after initiation of gel assembly, 80% of the PDGF-BB initially loaded was entrapped in the gel.
**Release of $^{14}$C-HB-IGF-1 and $^{125}$I-PDGF-BB from acellular KLD:** To assess the release kinetics of HB-IGF-1 and PDGF-BB from KLD, we loaded $^{14}$C-HB-IGF-1 and $^{125}$I-PDGF-BB into unassembled KLD. We measured the release of the radioactive proteins into the bath over 2 weeks, as well as their final presence in the gel at 2 weeks, via scintillation counting for $^{14}$C and gamma counting for $^{125}$I (Fig. 7). The release profile of TGF-β1 from KLD, published by Kopesky *et al.* (40), is included in Figure 7 for comparison. A first-order exponential of the form $f = a(1 - e^{-bt})$ was fit to the release data to obtain a characteristic release time $\tau = \frac{1}{b}$ for each growth factor (Table 1). These data show that HB-IGF-1 is largely released from KLD within four days, while PDGF-BB is released more slowly and 15% still resides in the gel at two weeks. Separate experiments confirmed that PDGF-BB had little or no effect on cell viability, sGAG content or loss, or the biosynthesis of sGAG or total protein in adjacent cartilage tissue (see supplemental figures 2 & 3).

**IV. DISCUSSION**

Stimulus of tissue regeneration, mediated by acellular scaffolds functionalized with GFs, is thought to hinge on matched kinetics between endogenous cell recruitment into the scaffold and GF retention time in the scaffold. Motivated by an augmented microfracture strategy for cartilage repair, we developed a 3D gel-to-gel migration assay to test the chemotactic effect of GFs on BM-PCs. We demonstrated that the combination of PDGF-BB and TGF-β1 stimulates migration of BM-PCs into and through a 3D self-assembling peptide hydrogel scaffold when delivered as a single dose
premixed into the scaffold (Fig 6). A single premixed dose allows for easy translation to an *in vivo* surgical setting, but it raises the question of GF retention and release kinetics from the scaffold in order to bind to cell receptors once cells have entered the scaffold. Entrapment efficiency of PDGF was ~80%, suggesting that a significant amount of PDGF remained in the gel, consistent with previous results for TGF-β1(40). We measured GF release kinetics and showed that the characteristic exponential release time of PDGF-BB from KLD (in the geometry of Fig. 1B) to be 3.5 days, with 58% of the loaded PDGF-BB remaining in the scaffold at the 4-day time point of our 3D gel-to-gel migration studies (Table 1). Previous studies have shown even stronger retention of TGF-β1 in KLD (40). The substantial release of HB-IGF-1 from KLD indicates that a strong gradient of HB-IGF-1 was no longer present in our *in vitro* system after 4 days. Thus, chemotaxis induced by conditions containing HB-IGF-1 should not be expected in the geometry of Fig. 1B, despite the chemotactic properties previously reported for the IGF family of proteins in modified Boyden chamber assays using BMSCs (32,35). The range of release profiles observed between the three GFs reflects how they each interact with the scaffold in a unique manner, though the precise mechanistic differences in scaffold-GF interactions have not been elucidated. There are no known specific binding motifs between any of these three GFs and KLD, although factors that may impact these differences include size, amino acid content and sequence, conformation, net charge, and non-specific binding. Taken together, these data demonstrate that KLD scaffolds possess GF release kinetics that may effectively stimulate migration of BM-PCs in the scaffold while retaining a portion of GFs that may subsequently promote cartilage repair by localized BM-PCs *in vivo*.
Both synergistic and antagonistic interactions between PDGF-BB and TGF-β1 have been shown previously in a variety of cell/tissue systems, including murine embryos, human pulmonary smooth muscle cells, and murine wound healing. In our system, neither PDGF-BB nor TGF-β1 was able to induce statistically significant migration alone. These data are consistent with the murine embryo study by Wang et al., which demonstrated that sclerotome and surrounding mesenchyme cells require PDGF signaling for TGF-β1 mediated migration. The precise mechanism of action that describes how these GFs together induce migration of BM-PCs is not yet known, and would require quantification of migratory-associated cell signaling pathways; while beyond the scope of this study, such experiments are underway.

Studies by Steadman et al. and others have theorized that one cause for the formation of fibrocartilage rather than hyaline cartilage following microfracture surgery may be an insufficient quantity of progenitor cells populating the defect in the initial days post-surgery. Our strategy of growth factor-mediated acceleration of progenitor cell migration may have the potential to increase the number of cells recruited to a scaffold-filled defect in augmented microfracture surgery, and thereby increase the likelihood of hyaline cartilage production both through higher cellularity and growth factor-enhanced matrix synthesis.

The data presented here, combined with data from the literature, show that both PDGF-BB and TGF-β1 are retained within KLD at significant levels over the 4 day period during which our migration studies were performed. To induce directed cell migration, cells must be exposed to gradients in chemotactic factors rather than a
bolus release(60,61). Our results imply that the release kinetics of PDGF-BB and TGF-β1 from KLD are slow enough to generate a gradient and induce chemotaxis in our *in vitro* system. Thus, the slow local release of premixed chemotactic growth factors from KLD may have the potential to accelerate the population of a defect with progenitor cells *in vivo*. Previous studies by Koutsopoulos *et al.* demonstrated that protein release rates from the self-assembling peptide RAD can be tuned by adjusting the scaffold concentration(62), indicating the release rates of our chemotactic growth factors could be tuned to meet the required kinetics of cell migration *in vivo*. While the scope of the present study is focused on the context of microfracture surgery, the concept of using a self-assembling peptide in combination with chemotactic growth factors could be extended to other tissues, such as neural(63), myocardial(64), or hepatic(65).

While the complex cell population accessed in microfracture surgery presents a challenge for experimental models, bone marrow-derived mesenchymal progenitor cells are commonly used *in vitro* to simulate them(66-70). To isolate BM-PCs, bone marrow stroma is typically scooped from the diaphyseal marrow cavity of long bones, aspirated from the iliac crest, or separated from sponge-like network of trabecular bone(43,68). These techniques have also been successfully applied to an array of species, including human, canine, feline, porcine, lapine, and bovine(43,71-77). Regardless of the physiological location of marrow harvested, these techniques all yield progenitor cells capable of undergoing chondrogenesis, whether in pellet culture or 3D scaffolds, such as KLD(17,39,44,66,68,70,77-80). While a phenotypic characterization of the cell population accessed in microfracture or used to simulate them *in vitro* would be
interesting, a complex cell population is an unavoidable product of the surgical technique.

A limitation of this study regarding translation to an in vivo setting concerns the role of TGF-β1. While TGF-β1 has shown promise in combination with PDGF-BB in vitro, its use for in vivo application raises concerns, as active TGF-β1 has been shown to induce fibrosis when injected into animal joints or delivered locally via scaffold, microparticle, or gene vector(1,28,81-83). To avoid an adverse effect of TGF-β1, one possibility is that moderate local activation of latent TGF-β1 found in native synovial fluid by chemical or mechanical forces may provide the necessary cues to stimulate migration if combined with PDGF-BB(84,85). Alternatively, engineering approaches designed to locally deliver exogenous latent rather than active TGF-β1 have shown promising preliminary results in rats(86). A strong, local dose of a pro-chemotactic GF(e.g. PDGF-BB), however, may be sufficient to induce a chemotactic effect similar to that observed in vitro. An additional limitation of this study is the gel-to-gel experimental setup used in vitro as compared to the bone-to-gel geometry in vivo. This difference may result in altered cell migration kinetics, but may also be overcome by tuning the scaffold density or growth factor dose. Initial rabbit studies with KLD have demonstrated the capacity of the scaffold to support cell migration in vivo, and the work presented here suggests that the addition of chemotactic GFs may have the potential to increase cellularity in the defect(1).

While the release of HB-IGF-1 from KLD may be too rapid in the geometry of Fig. 1B to fully test its chemotactic capacity on BM-PC migration, delivery of HB-IGF-1 from the hydrogel scaffold may still have an important function in cartilage regeneration. HB-
IGF-1 has been shown to induce sustained production of aggrecan and collagen in chondrocyte-seeded RAD scaffolds, and is thus a promising candidate stimulant for generating repair tissue. Additionally, delivery of HB-IGF-1 from the scaffold to adjacent cartilage explant tissue in vitro has already been shown to stimulate chondrocyte biosynthesis within these explants(13). Thus, HB-IGF-1 has the potential to stimulate repair and integration at the cartilage-scaffold interface. In this context, the KLD scaffold could provide local delivery of GFs such as HB-IGF-1 to the damaged cartilage interface, allowing HB-IGF-1 to serve its intended role as a pro-anabolic GF acting at the site of damage. Together, the ability of a scaffold to retain certain growth factors(e.g., PDGF-BB and TGF-β1) while locally delivering others(e.g., HB-IGF-1) demonstrates the dual role of such a hydrogel scaffold, and its capacity to be tailored to a particular tissue repair application.

V. CONCLUSIONS

This study demonstrates that the combination of PDGF-BB + TGF-β1 has the capacity to accelerate progenitor cell migration into a 3D self-assembling peptide scaffold. Delivery of pro-anabolic GFs such as HB-IGF-1 may also stimulate cell biosynthesis within the scaffold and in adjacent cartilage tissue. We found that multiple growth factors with varying release kinetics can be delivered simultaneously in a single dose via the self-assembling peptide KLD. This multi-growth factor approach could be used to address multiple tissue regeneration challenges at once, including cell recruitment, tissue production, and integration. In the context of augmenting microfracture surgery for cartilage repair, our strategy of delivering chemotactic and pro-anabolic growth factors in KLD may provide the necessary local stimulus to help
regenerate hyaline-like tissue. An extensive rabbit model is currently being used to test these results in vivo, though it is beyond the scope of this paper and will be presented in a detailed follow-up study.

VI. ACKNOWLEDGEMENTS

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AUTHOR DISCLOSURE STATEMENT

James Pancoast and Dr. Richard Lee are founders of ProteoThera, Inc.; Brigham and Women’s Hospital has filed for intellectual property on HB-IGF-1 listing them as inventors. James Pancoast is an employee and Richard Lee is a board member and consultant for ProteoThera, Inc. Dr. Grodzinsky has equity in 3-D Matrix, Ltd., Japan.

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Figure captions
Table 1: Quantification of the release kinetics of $^{14}$C-HB-IGF-1, $^{125}$I-PDGF-BB, and $^{125}$I-TGF-β1 from KLD hydrogels: The characteristic release time, $\tau$, for each growth factor was determined by fitting a first-order exponential of the form $f=a(1-e^{-bt})$ to the data shown in Fig 7, where $\tau=1/b$.

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<th>Growth Factor</th>
<th>$\tau$ (days)</th>
<th>Release at 4 days</th>
<th>Release at 2 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-HB-IGF-1</td>
<td>1.62</td>
<td>86%</td>
<td>95%</td>
</tr>
<tr>
<td>$^{125}$I-PDGF-BB</td>
<td>3.50</td>
<td>58%</td>
<td>83%</td>
</tr>
<tr>
<td>$^{125}$I-TGF-β1</td>
<td>6.20</td>
<td>16%</td>
<td>30%</td>
</tr>
</tbody>
</table>

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Fig. 1: Concept and experimental design: (A) The addition of a hydrogel scaffold to augment the standard microfracture approach; (B) 3D gel-to-gel migration assay setup: A KLD hydrogel premixed with GFs was cast at the bottom of a 48-well plate well. A second KLD hydrogel encapsulating 150,000 BM-PCs was cast on top of the first gel, with a layer of green fluorescent beads used to demark the interface between the two gels. Constructs were incubated in migration media for 4 days (see Methods), fixed, and BM-PC migration was assessed with confocal microscopy.
Growth factor-mediated migration of bone marrow progenitor cells for accelerated scaffold recruitment (doi: 10.1089/ten.TEA.2015.0524)

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Fig. 2: BM-PC isolation: (A) 9mm osteochondral plugs were harvested from the condyles of 1-2 week old bovine joints, and the cartilage was removed; (B) Remaining trabecular bone fragments were rinsed vigorously with media and mechanically disrupted to isolate cells; (C) Bright field microscopy of passage 1 BM-PCs following differential adhesion.
Fig. 3: Histology: Toluidine blue staining of P2 BM-PCs encapsulated in KLD following 22 days of culture showed strong disperse staining, indicative of high proteoglycan content.
Fig. 4: Assessment of BM-PC matrix production: P1 or P2 BM-PCs were encapsulated in KLD hydrogels and cultured for 22 days. (A) DNA content per hydrogel as measured by Hoechst dye binding; (B) sGAG content per hydrogel as measured by DMMB, normalized to wet weight; (C) Proteoglycan biosynthesis rate as measured by $^{35}$S-sulfate incorporation (D) Hydroxyproline content normalized to wet weight. n=6-8 gels per condition. Horizontal bars indicate statistically significant differences (p<0.05).
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**Fig. 5:** Example of confocal imaging and quantification of BM-PC migration and fluorescent bead position in the GF-free control (left) and PDGF-BB + TGF-β1 (right) conditions assessed using a spot finding algorithm(38): (A,B): 3D confocal image stack from the perspective of the objective below the well. (C,D): Profile perspective of the 3D confocal image stack. (E,F): Spot finding algorithm computations of confocal images in E and F. (G,H): Quantification of cell migration. Cells that migrated beyond one standard deviation (1SD) of the average bead position at the interface were considered to have migrated. The subset of these cells that migrated beyond two standard deviations (2 SD) were considered to have migrated strongly.
Fig. 6: 3D gel-to-gel migration of P1 BM-PCs over 4 days of stimulation by all 8 combinations and permutations of TGF-β1, PDGF-BB, and HB-IGF-1: Migration is expressed as the percent of cells migrated (blue bars) or the percent of cells strongly migrated (red bars) (see Fig 5). The combination of TGF-β1 and PDGF-BB induced statistically significant migration compared to the GF-free control. Horizontal lines over bars represent significant differences compared to GF-free control. * over bars represent significant differences compared to the combination of TGF-β1 and PDGF-BB. # over bars represent significant differences compared to the PDGF-BB alone.
Fig. 7: Release kinetics of PDGF-BB (n=14) and HB-IGF-1 (n=4) from KLD using their radiolabeled forms: The release of TGF-β1 (n=6), shown here for comparison, was previously published by Kopesky et al.(48). By 4 days, when migration studies were terminated (Fig 6), 42% of the PDGF-BB and 84% of the TGF-β1 remained in the scaffold, whereas only 14% of the HB-IGF-1 was retained.

Supp. Fig. 1: Experimental design for the release of GFs from KLD hydrogels to the medium: In the example of $^{125}$I-PDGF-BB, free $^{125}$I was removed by Sephadex G25 chromatography (as seen in fractions ~50-65). The remaining intact labeled $^{125}$I-PDGF-BB (fractions ~20-30) was encapsulated in KLD hydrogels, and release to the PBS bath was measured using a gamma counter.
**Supp. Fig. 2:** Impact of PDGF-BB on cartilage explant viability: 1-2 week old bovine cartilage explants were cultured in basal migration medium with and without soluble PDGF-BB (100ng/mL) for 4, 8, or 15 days and then fluorescently stained with FDA (green) for viable cells and propidium iodide (PI, red) for non-viable cells. (A) Example of explant cultured in basal migration medium for 15 days; (B) Explant cultured in basal migration medium + PDGF-BB (100ng/mL) for 15 days. Cartilage disks were stained immediately upon termination of culture, and images were taken with a 4x objective. Images show a minimal reduction in viability with the addition of PDGF-BB.

**Supp. Fig. 3:** Impact of PDGF-BB on cartilage explant matrix synthesis: 1-2 week old bovine cartilage explants were cultured in basal migration medium with and without soluble PDGF-BB (100ng/mL) for 4, 8, or 15 days. (A) sGAG content per hydrogel as measured by DMMB(43), normalized to wet weight; (B) Cumulative sGAG content lost to medium per day, as measured by DMMB; (C) Proteoglycan biosynthesis rate as measured by $^{35}$S-sulfate incorporation(41); (D) Total protein synthesis as measured by $^3$H-Proline incorporation(41). n=6-9 gels per condition.