Correlating the Effects of Bone Morphogenic Protein to Secreted Soluble Factors from Fibroblasts and Mesenchymal Stem Cells in Regulating Regenerative Processes *In Vitro*

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The capacity to regenerate complex tissue structures after amputation in humans is limited to the digit tip. In a comparable mouse digit model, which includes both distal regeneration-competent and proximal regeneration-incompetent regions, successful regeneration involves precise orchestration of complex microenvironmental cues, including paracrine signaling via heterogeneous cell–cell interactions. Initial cellular processes, such as proliferation and migration, are critical in the formation of an initial stable cell mass and the ultimate regenerative outcome. Hence, the objective of these *in vitro* studies was to investigate the effect of soluble factors secreted by fibroblasts and mesenchymal stem cells (MSCs) on the proliferation and migration of cells from the regeneration-competent (P3) and -incompetent (P2) regions of the mouse digit tip. We found that P2 and P3 cells were more responsive to fibroblasts than MSCs and that the effects were mediated by bi-directional communication. To initiate understanding of the specific soluble factors that may be involved in the fibroblast-mediated changes in migration of P2 and P3 cells, bone morphogenic protein 2 (BMP2) was exogenously added to the medium. We found that changes in migration of P3 cells were similar when exposed to BMP2 or co-cultured with fibroblasts, indicating that BMP signaling may be responsible for the migratory response of P3 cells to the presence of fibroblasts. Furthermore, BMP2 expression in fibroblasts was shown to be responsive to tensile strain, as is present during wound closure. Therefore, these *in vitro* studies indicate that regenerative processes may be regulated by fibroblast-secreted soluble factors, which, in turn, are modulated by both cross-talk between heterogeneous phenotypes and the physical microenvironment of the healing site.

Introduction

LIMB REGENERATION IS of growing interest in the field of regenerative medicine due to the increased number of amputations occurring every year, with ~200,000 annually in the United States alone.¹ Amphibians are capable of regenerating complex structures after injury,^{2,3} while the regenerative capacity of mammals is more limited.^{4,5} However, the potential to regenerate in humans is not completely absent, as illustrated by multiple clinically documented cases of digit regeneration.^{6,7} Recent tissue engineering strategies have started exploring treatment modalities to promote regeneration,^{8,9} but a further understanding of the regenerative processes is needed to successfully regenerate all the tissues of whole digits and limbs.

Regeneration in mouse digits is level specific in that amputation at the distal end of the phalangeal element 3 leads to regeneration, while amputation at the more proximal phalangeal element 2 leads to only wound healing.¹⁰ Stromal

cells have been isolated from the connective tissue (excluding such tissues as the nail, skin, fat, and muscle) of the regeneration-competent and regeneration-incompetent regions to generate P3 and P2 cells, respectively.¹¹ Transplantation of these cells in amputated digits results in distinct patterns of localization, with P3 cells at the regenerating bone and P2 cells in the connective tissue.¹¹ Differences in cell proliferation and migration were also evident in *in vitro* studies, however, where P3 cells were significantly more proliferative than P2 cells for a number of two- and three-dimensional microenvironments.¹² The mechanisms that regulate these differences in proliferative and migratory capacity, processes necessary for the formation of a stable cell mass during the initial stages of regeneration, are still unknown.

The initial response to injury is critical in determining whether only wound healing or else a regenerative response will occur.⁴ Immediately after amputation, multiple cell phenotypes from neighboring tissues are all present and potentially interacting. In addition, complete repair of the

digit tip, ultimately, involves multiple specialized phenotypes, including endothelial cells, mesenchymal stem cells (MSCs), fibroblasts, and skeletal cells,^{13,14} in close proximity. Thus, understanding the interactions of these cell types during tissue redevelopment is critical for promoting successful regeneration.

Intercellular signaling is critical for the induction and progression of regeneration, which is regulated by a complex signaling network involving numerous growth factors. Fibroblasts play a major role in the secretion of growth factors that exert chemotactic effects on neighboring cells during amphibian regeneration,³ as well as wound healing^{15,16} where they are subject to tensile forces during closure.¹⁷ Similarly, during tissue repair, MSCs are known to participate through the secretion of soluble factors.^{18,19} In the digit tip regeneration model, skin fibroblasts are present in the wound environment immediately after amputation, while MSCs are exposed to the wound site after the degradation at the bone stump has occurred.²⁰ In addition, it has been found that both WNT signaling for blastema growth²¹ and bone morphogenic protein 2 (BMP2) secretion for the formation of bone^{22,23} are critical for proper tissue regrowth. Thus, the delicate balance and complex integration of paracrine signaling in the wound environment, which consists of both heterotypic phenotypes and mechanical cues, contributes a great extent to the successful regenerative outcome of the digit tip. Use of *in vitro* culture models can help assess the potential paracrine signaling between these regeneration-relevant phenotypes, especially within the context of defined mechanical cues. Thus, the objective of these studies was to investigate the effect of soluble factors secreted by fibroblasts and MSCs on the *in vitro* proliferation and migration of regeneration-competent (P3) and -incompetent (P2) cells.

Materials and Methods

Cell culture

P2 and P3 culture. Cells isolated from the connective tissue (excluding such tissues as the nail, skin, fat, and muscle) of CD1 mouse phalangeal elements (digits II–IV) were a generous gift from Dr. Ken Muneoka (Tulane University). P2 (from phalangeal element 2) and P3 (from phalangeal element 3) cells are from amputation planes of the mouse considered regenerative-incompetent and -competent, respectively.²⁴ These mesenchymal cell populations lack expression of markers for hematopoietic stem cells and keratinocyte progenitors. As previously described,^{11,12} these cells were expanded at 37°C/5% CO₂/21% O₂ on fibronectin (BD Biosciences, San Jose, CA)-coated tissue culture plastic in culture medium consisting of Dulbecco's Modification of Eagles Medium/MCDB supplemented with ITS + 1 (Sigma, St. Louis, MO), 2% ES-qualified fetal bovine serum (Invitrogen, Grand Island, NY), 0.5 × 10^{−9} M Dexamethasone (Sigma), 10^{−4} Ascorbic Acid 2-phosphate (Sigma), 20 ng/ml PDGFββ, 20 ng/ml EGF (R&D Systems, Minneapolis, MN), and antibiotics. Before reaching confluency, cells were passaged using 0.05% Trypsin (Invitrogen) and replated at a density of 8000 cells/cm². At passage 5, expanded cells were frozen and stored in LN₂ until just before use in experiments.

Fibroblast and MSC culture. Human neonatal (NEO) and adult (AD) skin fibroblasts were purchased from ATCC

(Manassas, VA), and C57BL/6 mouse MSCs were purchased from Invitrogen. Basal medium for NEO, AD, and MSCs was Eagle's Minimum Essential Medium (ATCC), Iscove's Modified Dulbecco's Medium (ATCC), and Alpha Minimum Essential Medium (CellGro, Manassas, VA), respectively, supplemented with 10% fetal bovine serum (Fisher Scientific) and antibiotics. Cells were used at passage 4 for experiments.

Co-culture and conditioned medium studies

P2 and P3 cells were co-cultured with fibroblasts or MSCs using transwell membranes that enable soluble communication between cell types. To determine the role of these cells in the proliferation of P2 and P3 cells, fibroblasts (neonatal: NEO and adult: AD) and MSCs were seeded at 15,000 cells/cm² onto cell culture transwell inserts with a 1 μm pore size (Falcon, Franklin Lanes, NJ) and grown for 1.5 days in P2/P3 culture medium. Concomitantly, P2 and P3 cells were seeded at 5000 cells/cm² on fibronectin-coated six well plates, grown for 24 h in culture medium, and then serum starved overnight. The cell culture inserts with fibroblasts or MSCs were then transferred into the wells to enable soluble factor communication with either P2 or P3 cells. Parallel experiments with P2 and P3 cells cultured alone served as controls. After 24 h, proliferation was evaluated by cell number in fixed samples of the six-well plates. Samples were stained using Hoescht 33528 (AnaSpec, Fremont, CA), and 10× magnification fluorescent images were acquired and digitized to contrast labeled nuclei. ImageJ software then quantified the number of nuclei per field. Five fields were analyzed per sample.

In studies that assess the migratory potential of P2 and P3 cells, co-culture and conditioned medium experiments were configured in a similar manner as those described earlier. Neonatal fibroblasts, adult fibroblasts, and MSCs were seeded onto transwell membranes as in the proliferation assay, except that P2 and P3 cells were seeded on fibronectin-coated six well plates at 10,000 cells/cm². After 2 days of independent culture, the inserts were transferred into the wells to enable soluble factor communication for 12 h and then, a 200 μL pipet tip was used to create a scratch (~240 μm wide). After 6 h, the migratory potential of cells was determined by fluorescently labeling the nuclei with Hoechst and acquiring 10× magnification images. The original scratch region was defined by measurements on phase images of 10 independent samples. Based on the average of these measurements, a rectangular area (212 × 1032 pixels) was applied to each fluorescent image. The number of nuclei contained within this region of interest was then quantified for five fields per sample.

In order to determine the role of bi-directional versus uni-directional soluble signaling, conditioned medium from fibroblasts and MSCs was used. For these studies, the conditions were as described earlier except that after culture of the neonatal fibroblasts, adult fibroblasts, and MSCs, the medium was collected and then transferred to the P2 and P3 cells. The assessments of proliferation and migration were as described earlier.

BMP treatment

The responsiveness of P2 and P3 cells to BMP signaling was determined by supplementing the medium with BMP2.

To determine whether BMP2 can activate SMAD 1, P2 and P3 cells were initially seeded at 10,000 cells/cm² and grown for 1 day with subsequent serum starvation overnight. BMP2 (R&D Systems) was then added to the medium for a range of concentrations (0–300 ng/mL) for 30 min. Samples were prepared for flow cytometry analysis of protein expression. Cells were fixed in 4% formaldehyde for 10 min at 37°C, permeabilized with 90% methanol for 30 min, and then labeled with primary and secondary antibodies. Antibodies used were for BMPR1A and BMPR2 (Abgent, San Diego, CA), as well as phospho-SMAD1/5 and SMAD1 (Cell Signaling, Danvers, MA). Fluorescence was detected using a BD FACSCanto II. For each sample, the positive expressing cells were defined as being above the 98% level of the 2° antibody-only controls.

In studies that evaluated the effect of BMP signaling on cell proliferation and migration, P2 and P3 cells were treated with fresh medium supplemented with either BMP2 (100 ng/mL) or Noggin (100 ng/mL) (both from R&D Systems) and analyzed as described for the co-culture studies.

Strain applied to fibroblasts

Mechanical loading was applied to fibroblasts using a Flexcell International system (Hillsborough, NC) to determine the effect of the physical microenvironment on BMP expression. Neonatal fibroblasts were seeded at 10,000 cells/cm² on collagen type I-coated silicone membranes of custom plates (Flexcell International) and allowed to attach for 2 days. A cyclic tensile strain of 10% at 1 Hz (in a sinusoidal pattern) was then applied for 48 h and analyzed for gene expression.

Gene expression of BMP markers was evaluated using real-time polymerase chain reaction. Cells from static and strain samples were lysed, homogenized using QIAshredders (Qiagen, Germantown, MD), and stored at –80°C. RNA was isolated using the RNeasy kit (Qiagen) and quantified with a Nanodrop[®] spectrophotometer. From each sample, 1 µg of RNA was converted into cDNA (Superscript[®] III First-strand Synthesis Kit; Invitrogen) and analyzed using SYBR[®] Green on a StepOnePlus[™] PCR System (Applied Biosystems, Foster City, CA). Primers were custom designed (Primer Express[®] Software v3) for *BMP2*, *BMP4*, and *GAPDH* (primer sequences are listed in Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/tea). Gene expression levels were quantified using standard curves and are reported as normalized to *GAPDH* expression.

Statistical significance

Data are presented as mean ± SEM for *n* = 3. Treatment effects were analyzed using a one way analysis of variance or a Student's *t*-test. Significance is based on *p*-values of <0.05.

Results

Fibroblast soluble factors

These studies focused on the effects of soluble communication between fibroblasts and the P2 and P3 cells. The effects on P2 or P3 proliferation of fibroblasts in indirect co-culture were evaluated by cell number after 24 h. Representative phase images of P2 cells cultured alone as a

control, or co-cultured with neonatal or adult fibroblasts, qualitatively show differences in cell number (Fig. 1A). When nuclei-labeled fluorescent images were quantified, it was found that the presence of neonatal and adult fibroblasts significantly (*p* < 0.001) increased the proliferation of P2 cells (Fig. 1B left). To determine whether these observed changes were due to unilateral communication from the fibroblasts to the stromal cells, the P2 cells were exposed instead to only the conditioned medium from the fibroblasts. For the same medium volume, treatment duration, and sample size, no changes in the proliferation of P2 cells were found to be statistically significant (Fig. 1B right). Together, these results indicate that communication of soluble factors between fibroblasts and P2 cells can induce increased P2 proliferation, but that these effects are not solely mediated by the fibroblasts.

FIG. 1. Fibroblast effects on P2 and P3 cell proliferation. P2 and P3 cells were cultured alone (CON), exposed to co-culture with neonatal (NEO) or adult (AD) fibroblasts, or exposed to conditioned medium from neonatal (CM_{NEO}) or adult (CM_{AD}) fibroblasts for 24 h. Representative phase (A: top) or nuclei-labeled fluorescent (A: bottom) images of P2 cells are shown. Proliferation was assessed by quantifying cell number in the fluorescent images for P2 (B) or P3 (C) cells exposed to co-culture (B, C: left) or conditioned medium (B, C: right). Values presented are mean ± SEM (*n* = 3), with asterisks indicating significance (***p* < 0.01, ****p* < 0.001).

The effects of fibroblasts on P3 cells were similarly evaluated as for P2 cells. Consistent with previous publications^{11,12} the inherent proliferative rate of P3 cells is markedly higher than that of P2 cells (3–6× greater in cell number for these studies). Nonetheless, proliferation of P3 cells, unlike that of P2 cells, was not significantly ($p=0.6$) affected by the presence of neonatal or adult fibroblasts in indirect co-culture (Fig. 1C left). To ascertain whether the P3 cells were inhibiting fibroblast-mediated secretion that might affect proliferation, fibroblast conditioned medium was applied to P3 cell populations. Under these conditions as well, P3 cell number was not significantly changed ($p=0.7$; Fig. 1C right). Thus, fibroblast-secreted soluble factors were not found to affect P3 proliferation.

The effect of fibroblast co-culture on the migratory potential of P2 and P3 cells was evaluated using a scratch test. For confluent P2 or P3 cells, a scratch was created and the region was defined using phase images (Fig. 2A top). After 6 h, nuclei-labeled fluorescent images of fixed samples (Fig. 2A bottom) were assessed to determine the number of cells in the scratch region (Fig. 2B). Both neonatal and adult fibroblasts significantly ($p<0.01$) decreased the migration of P2 cells (Fig. 2C left). This response was not reproduced, however, when the cells were only exposed to the conditioned medium of fibroblasts (Fig. 2C right). Thus, P2 cell migration is affected by the presence of fibroblasts, but not when exposed only to fibroblast-secreted factors.

The different fibroblast sources differentially regulated P3 cell migration in a co-culture configuration. P3 migration was significantly ($p<0.5$) increased by soluble factor communication with neonatal fibroblasts, but was unaffected by adult fibroblasts (Fig. 2D left). Neonatal fibroblasts had a greater effect through conditioned medium than co-culture, with a 1.7-fold versus 1.4-fold increase (Fig. 2D right). In addition, conditioned medium from adult fibroblasts induced a significant ($p<0.001$) effect on P3 cell migration (Fig. 2D right), where co-culture had no detectable effect. These results indicate that fibroblast effects on P3 migration are donor dependent and that P3 cells interactively modulate the fibroblast-mediated effects.

MSC soluble factors

The effects of MSCs, a cell type known to secrete trophic factors during tissue repair,²⁵ on P2 and P3 cells were evaluated as described earlier. MSC co-culture had no detectable effect on P2 cell number (Fig. 3A), but induced a significant ($p<0.01$) decrease in the number of P3 cells (Fig. 3B). MSC-conditioned medium alone, however did not affect P3 proliferation (Fig. 3C right), indicating that interactive cross-talk of P3 cells and MSCs was necessary for the observed effects on P3 proliferation. Migration of both cell types, however, was nonresponsive to MSC co-culture or conditioned medium (Fig. 3B, C). These results indicate that MSCs had a limited effect on regeneration-relevant processes through secretion of trophic factors.

BMP treatment of P2 and P3 cells

Since BMP signaling is necessary for the successful formation of bone during digit tip regeneration *in vivo*,^{22,24} we investigated the potential effects that BMP signaling may

FIG. 2. Fibroblast effects on P2 and P3 cell migration. P2 and P3 cells were cultured alone (CON), exposed to co-culture with neonatal (NEO) or adult (AD) fibroblasts, or exposed to conditioned medium from neonatal (CM_{NEO}) or adult (CM_{AD}) fibroblasts. A representative phase image (A: top) is shown of P3 cells co-cultured with neonatal fibroblasts taken immediately after a scratch was made and the scratch region was defined (red dotted line). Also shown is a representative nuclei-labeled fluorescent image of a P3 sample fixed at 6 h after the scratch was made (A: bottom). Migration was assessed by quantifying cell number within the scratch regions (B) for samples exposed to indirect co-culture (C, D: left) or conditioned media (C, D: right). Values presented are mean $\pm$ SEM ($n=3$), with asterisks indicating significance (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Color images available online at www.liebertpub.com/tea

have on regenerative-relevant cells *in vitro*. Initially, P2 and P3 cells were evaluated for protein expression of BMP receptors and were found to have high levels in both cell types (~85% positive for BMPRI1A and ~99% positive for BMPRI2; Fig. 4A). Both P2 and P3 cells also had similar endogenous expression levels of SMAD1 (Fig. 4B left). When evaluating for BMP signaling, both P2 and P3 cells again had comparably high levels of phosphorylated SMAD 1/5 when treated with 300 ng/mL of BMP2 (Fig. 4B right; double arrow head) compared with nontreated controls (Fig. 4B right; single arrow head). This sensitivity to BMP2 activation was robust in that there was a consistent activation for each cell type when treated with a range of concentrations

FIG. 3. Mesenchymal stem cells (MSC) effects on P2 and P3 cell proliferation and migration. P2 and P3 cells were cultured alone (CON), exposed to indirect co-culture with MSC, or MSC- conditioned medium (CM_{MSC}). Both P2 (A, B) and P3 (C, D) cells were analyzed for changes in proliferation (A, C) and migration (B, D) due to co-culture (A–D: left) or conditioned media (A–D: right). Values presented are mean $\pm$ SEM ($n=3$), with asterisks indicating significance (* $p<0.05$).

FIG. 4. BMP receptor expression and response in P2 and P3 cells. A representative histogram of BMPR1A and BMPR2 protein expression is shown for P2 (yellow) and P3 (blue) cells (A). To assess activation with BMP2, protein expression of SMAD 1 (B: left) and phosphorylated SMAD 1/5 (B: right) for untreated (black, single arrow head) and BMP2-treated (300 ng/mL; red, double arrow head) P2 and P3 cells are shown. Protein expression for 2° antibody-only staining controls is also shown (filled gray). BMP, bone morphogenic protein. Color images available online at www.liebertpub.com/tea

(25–300 ng/mL; Supplementary Fig. S1). Thus, these data indicate that both P2 and P3 cells have the receptors which are necessary to bind BMP and are equally activated along the SMAD pathway in response to BMP2 treatment.

The ability of P2 and P3 cells to respond to BMP signaling motivated investigation into BMP-induced changes in cellular processes. P2 and P3 cells were treated with either BMP2 or Noggin (an antagonist of BMP2) and assessed for proliferation and migration. Noggin significantly ($p<0.01$) increased the migration of P2 cells (Fig. 5A right), and BMP2 significantly ($p<0.05$) decreased cell proliferation of P3 cells. P3 cell migration, in turn, was altered by both BMP and Noggin (Fig. 5B right). BMP2 significantly increased ($p<0.001$) while Noggin significantly ($p<0.01$) decreased the migration of P3 cells (Fig. 5B right), which shows that key BMP signaling molecules may regulate the migration of P3 cells. Since P3 migration was similarly up-regulated when exposed to neonatal fibroblasts, conditioned medium from neonatal fibroblasts, and BMP2, this suggests that BMP2 may be one of the key fibroblast-secreted soluble factors which modulates P3 cell behavior.

Tensile strain effects on fibroblasts

Soluble factors secreted by fibroblasts are dependent on the cellular microenvironment, including the tensile forces that are present at the wound site during healing. Here, uniaxial cyclic tensile strain (10% at 1 Hz for 48 h) was applied (Fig. 6A) to determine effects on the gene expression of *BMP2* and *BMP4* in neonatal fibroblasts. Static

FIG. 5. BMP and Noggin effects on P2 and P3 cell proliferation and migration. P2 (**A**) and P3 (**B**) cells were analyzed for changes in proliferation (**A, B**: left) and migration (**A, B**: right) due to BMP2 (100 ng/mL) and Noggin (100 ng/mL) treatment. Values presented are mean $\pm$ SEM ($n=3$), with asterisks indicating significance (** $p < 0.01$, *** $p < 0.001$).

controls and strain samples looked morphologically similar (Fig. 6B). However, applied strain significantly ($p < 0.05$) decreased *BMP2* expression and significantly ($p < 0.05$) increased *BMP4* expression. These results indicate that the secretion of BMP-related soluble factors can be modulated by the physical microenvironment during healing.

Discussion

In these studies, we have shown that cells from the regeneration-competent (P3) and -incompetent (P2) regions of the mouse digit tip are responsive to paracrine signaling *in vitro*. In particular, we found that fibroblasts play a greater role than MSCs in regulating the proliferation and migration of P2 and P3 cells. The response of P2 cells to fibroblasts was seen in the indirect co-culture model but not through conditioned medium alone, illustrating that the cross-talk between these cell types induced the observed differences in proliferation and migration of P2 cells. P3 cells, on the other hand, were highly responsive to signaling from neonatal fibroblasts in both the indirect co-culture and conditioned media experiments with quantifiable changes in migration, which were recapitulated by exogenously adding BMP2 to the medium. Furthermore, we found that BMP secretion from neonatal fibroblasts can be modulated by applied tensile strain. Thus, these *in vitro* studies indicate that regenerative processes may be regulated by fibroblast-secreted soluble factors, which, in turn, are modulated by both the cross-talk between heterogeneous phenotypes and the physical microenvironment of the healing site.

One of the critical initial events in epimorphic regeneration is the assembly of cells to form a stable cell mass. Since there is a disproportionate representation of cell types after injury compared with the original heterogeneous composition^{14,26} cell accumulation should be a function of migration, differentiation, and/or proliferation. Previously, we had

FIG. 6. Strain effects on BMP expression for fibroblasts. Cyclic tensile strain was applied to neonatal fibroblasts. A schematic of the physical configuration (**A**) and representative phase images of static controls and strain samples (**B**) are shown. Gene expression of *BMP2* and *BMP4* expression was determined for both groups (**C**). Values presented are mean $\pm$ SEM ($n=3$), with asterisks indicating significance (* $p < 0.05$).

shown that P2 and P3 cells differ greatly with regard to proliferation and migration: P3 cells are more proliferative, while P2 cells are more migratory.¹² During regeneration *in vivo*, adjacent cell phenotypes may modulate these two processes by secretion of specific growth factors. In these *in vitro* studies, we showed that signaling from fibroblasts, in particular, can considerably adjust the proliferation and migration of P2 and P3 cells.

Co-culture of heterogeneous phenotypes can induce bi-directional signaling between the distinct cell types. Changes in migration and proliferation of P2 cells were seen during indirect co-culture but not from the conditioned medium alone. This indicates that P2 cells provide signals to the fibroblasts, which, in turn, then secrete factors that impact P2 cellular processes. P3 cells were susceptible to changes in migration from fibroblasts under both culture conditions, but effects were greater for conditioned medium alone. As a result, P3 cells likely dampen the fibroblast-induced changes by sending inhibitory signals to the fibroblasts. Thus, P2 and P3 cells are susceptible to changes due to soluble factors secreted by adjacent phenotypes at the wound site, but are active in modulating those effects.

During regeneration *in vivo*, transplanted P3 cells respond to signals from the wound environment and migrate toward the site of injury at the bone stump.¹¹ This localization was not found for either a highly invasive cancer cell line or the P2 cells. Thus, positional honing for regeneration-competent P3 cells in this *in vivo* model is unique. Our studies which show that P3 migration can be mediated by soluble factors *in vitro* are consistent with an *in vivo* environment in which regenerative cells are recruited by factors present at the wound site. This suggests that complex interactions between cells involved in regeneration, as well as with the wound environment itself, play a key role in whether or not a regenerative response is elicited.

MSCs have the capacity to secrete growth factors and cytokines that are relevant for tissue repair and regeneration.^{27–29} In our studies, however, MSCs had no detectable effect on P2 cells and only affected P3 proliferation. In the mouse digit tip, endogenous regeneration occurs only for amputations distal to the bone marrow cavity. As regeneration progresses, the bone stump is degraded, which exposes the bone marrow and the MSCs within to the wound site.³⁰ Thus, instead of regulating proliferation and migration that dominate the early stages of regeneration, MSCs may play a greater role later in the process, perhaps by promoting cell differentiation. This would be consistent with MSC-directed differentiation toward osteocytes³¹ and chondrocytes³² observed in other systems.

During digit tip regeneration, BMP proteins are expressed (BMP2, BMP4, BMPRIA, and BMPRII) in the blastema and in the marrow region of the amputated stump.²² Knockdown studies in fetal and neonatal mice found that treatment with Noggin, a BMP antagonist, inhibited regeneration^{20,24} and that successful formation of bone during regeneration is dependent on BMP signaling.^{22,24} We found that both regeneration-competent and -incompetent cells have the receptors for BMP signaling and similarly phosphorylate SMAD 1/5 in response to BMP2. However, BMP2 differentially regulated the functional responses of these cells, in that P3 cells had a greater response than P2 cells with regard to proliferation and migration. Furthermore, changes in P3 migration were similar in response to coculture of neonatal fibroblasts, exposure to conditioned medium from neonatal fibroblasts, and treatment with BMP2. Together, these results suggest that BMP signaling to the stromal cells during epimorphic regeneration may be through the secretion of BMP2 by adjacent fibroblasts.

Previous *in vivo* studies³⁰ indicated that BMPs present at the wound site may be critical in modulating the regenerative response, and our own *in vitro* results showed that fibroblast-secreted BMP2 affected functional processes of the stromal P2 and P3 cells. It is already known that the tensile strains which develop during wound closure influence cell behavior.³³ Here, we determined that the applied tensile strain, in fact, altered the BMP expression in neonatal fibroblasts, specifically with a downregulation of *BMP2*. Thus, these results indicate that the physical microenvironment during the early stages of wound healing may mediate the initial processes critical for regeneration.

Successful epimorphic regeneration is dependent on well-controlled, overlapping, and interdependent events to re-establish properly arranged distinct tissues. Using regeneration-competent (P3) and -incompetent (P2) cells *in vitro*, we

have explored cellular and molecular interactions that may regulate regenerative processes *in vivo*. We have shown that fibroblast-mediated soluble factors can modulate proliferation and migration processes which are critical in the initial formation of a stable cell mass, while MSCs may be more involved in later stages of regeneration. Furthermore, we have shown that the BMPs, in particular BMP2, may be a key secretory factor and that the physical microenvironment can affect its availability at the wound site.

Complete regeneration of the mouse digit tip involves precise orchestration of complex microenvironmental cues. Recent research has begun to identify the types of cells and tissues that contribute to regeneration in the mouse digit tip.^{13,14} However, it is not fully understood how these cells and tissues, through chemical and/or physical signaling, respond to and modulate the microenvironment to subsequently reform the lost tissue. Here, we showed that fibroblast-secreted BMP signaling during wound healing may be one key factor in regulating the regenerative response. Further understanding of the cell–cell and cell–matrix interactions involved in endogenous repair, however, is still necessary to develop treatment modalities to promote regeneration *in vivo* after digit and limb amputation in humans.

Acknowledgments

The authors thank Tulane University, the Louisiana Board of Regents (KML), and the National Institutes of Health (#P20 GM103629) for supporting this work.

Disclosure Statement

The authors have no competing financial interests.

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Received: May 13, 2014

Accepted: May 20, 2014

Online Publication Date: July 3, 2014