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Cellular interactions regulate stem cell differentiation in tri-culture

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ABSTRACT

Currently, the mechanism governing the regeneration of the soft tissue-to-bone interface, such as the transition between the anterior cruciate ligament (ACL) and bone, is not known. Focusing on the ACL-to-bone insertion, this study tests the novel hypothesis that interactions between cells from the ligament (fibroblasts) and bone (osteoblasts) initiate interface regeneration. Specifically, these heterotypic cell interactions direct the fibrochondrogenic differentiation of interface-relevant cell populations, defined here as ligament fibroblasts and bone marrow stromal cells (BMSC). The objective of this study is to examine the effects of heterotypic cellular interactions on BMSC or fibroblast growth and biosynthesis, as well as expression of fibrocartilage-relevant markers in tri-culture. The effects of cell–cell physical contact and paracrine interactions between fibroblasts and osteoblasts were also determined. It was found that, in tri-culture with fibroblasts and osteoblasts, BMSC exhibited greater fibrochondrogenic potential than ligament fibroblasts. The growth of BMSC decreased while proteoglycan production and TGF-β3 expression increased. Moreover, tri-culture regulated BMSC response via paracrine factors, and interestingly, fibroblast–osteoblast contact further promoted proteoglycan and TGF-β1 synthesis as well as induced SOX9 expression in BMSC. Collectively, the findings of this study suggest that fibroblast–osteoblast interactions play an important role in regulating the stem cell niche for fibrocartilage regeneration, and the mechanisms of these interactions are directed by paracrine factors and augmented with direct cell–cell contact.

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BMSC; fibroblast; fibrocartilage; interface; osteoblast; tri-culture

Introduction

Primary load-bearing soft tissues such as the anterior cruciate ligament (ACL) connect to bone through a characteristic fibrocartilaginous interface, which exhibits a controlled spatial distribution of cell populations, matrix composition, and structure (1–7). These direct insertions are common in the musculoskeletal system, in which the fibrocartilaginous interface consists of contiguous non-calcified and calcified zones. In the non-calcified region, ovoid fibrochondrocytes are surrounded by a matrix containing both types I and II collagen, as well as glycosaminoglycans (GAG), while hypertrophic fibrochondrocytes are found in a matrix containing type X collagen and high alkaline phosphatase (ALP) activity at the calcified interface zone (3,6,8). This controlled matrix heterogeneity is important for minimizing stress concentrations and facilitating the transfer of complex loads between soft and hard tissues (2,9,10). Unfortunately, this critical interface is not regenerated following ACL reconstruction, which mechanically anchors the autologous tendon-based reconstruction graft to the bone. Due to a lack of biological fixation, functional integration of tendon grafts with the surrounding bone following reconstruction has been challenging, compromising long-term clinical outcome and functionality.

Currently, the mechanism governing the regeneration of the soft tissue-to-bone interface is not well understood. It has been observed that while ACL reconstruction procedures do not result in the anatomical regeneration of the soft tissue-to-bone enthesis, a fibrovascular matrix that later matures and reorganizes into a fibrocartilage-like tissue is found between the autologous graft and bone within the tibial or femoral tunnel (11,12). Interestingly, this interface-like tissue is observed to form only at regions where the ACL graft is in direct contact with bone. Based on these observations, it is hypothesized that fibroblast–osteoblast interactions are key modulators of the cell phenotype found at the soft tissue-to-bone junction, and these heterotypic cellular interactions are able to initiate events leading to fibrocartilage regeneration. This hypothesis was tested previously using a biomimetic co-culture model, allowing for both cell–cell contact and soluble factor interactions between the cell types native to the bone and ligament (13). It was observed that fibroblast–osteoblast

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interactions modulated their respective phenotypes, and co-culture resulted in the upregulation of fibrochondrocyte-related markers, such as type II collagen and cartilage oligomeric protein (13).

However, fibroblast–osteoblast co-culture was not sufficient to lead to fibrocartilage formation, suggesting that other cell types must be involved in this healing process. The initial exposure of osteoblasts and fibroblasts to one another post-injury can modulate the response of either resident or recruited interface-relevant cell populations at the graft-bone junction. In this study, interface-relevant cell populations are defined as tendon or ligament fibroblasts (Fb) and bone marrow stromal cells (BMSC), which are present following ACL reconstruction, and have been shown to have the potential to differentiate into insertion fibrochondrocytes based on published studies. For example, BMSC pre-embedded in a fibrin gel and coated on tendon grafts have been reported to promote the formation of a fibrocartilaginous tissue between the graft and bone, suggesting a potential role for stem cells in fibrocartilage regeneration (14). In addition, during development, the interface is shown to be derived from the ligament (5,15–17), and cells residing in the ligament or tendon graft have been reported to exhibit fibrochondrocytic or chondrocytic phenotypes under controlled conditions (18–21). It is clear that both fibroblasts and BMSC have fibrochondrogenic potential and are therefore the focus of this study. It is hypothesized that fibroblast–osteoblast interactions can direct the fibrochondrogenic differentiation of these cells. To test this hypothesis, the first objective of this study is to determine the effects of tri-culture with fibroblasts and osteoblasts on the growth and phenotypic responses of either ACL fibroblasts or BMSC. Specifically, a tri-culture model has been established (Figure 1), within which interface-relevant cells (fibroblasts or BMSC) are embedded in hydrogel, while osteoblast and fibroblast monolayers are established on each side of the gel, mimicking the spatial transition across the native insertion from ligament to the interface and then to the bone region. This biomimetic cell interaction model provides a physiologically relevant culturing environment (2D and 3D), while exerting spatial control over cell distribution, establishing distinct fibroblast, interface, and osteoblast regions. Using this model, the

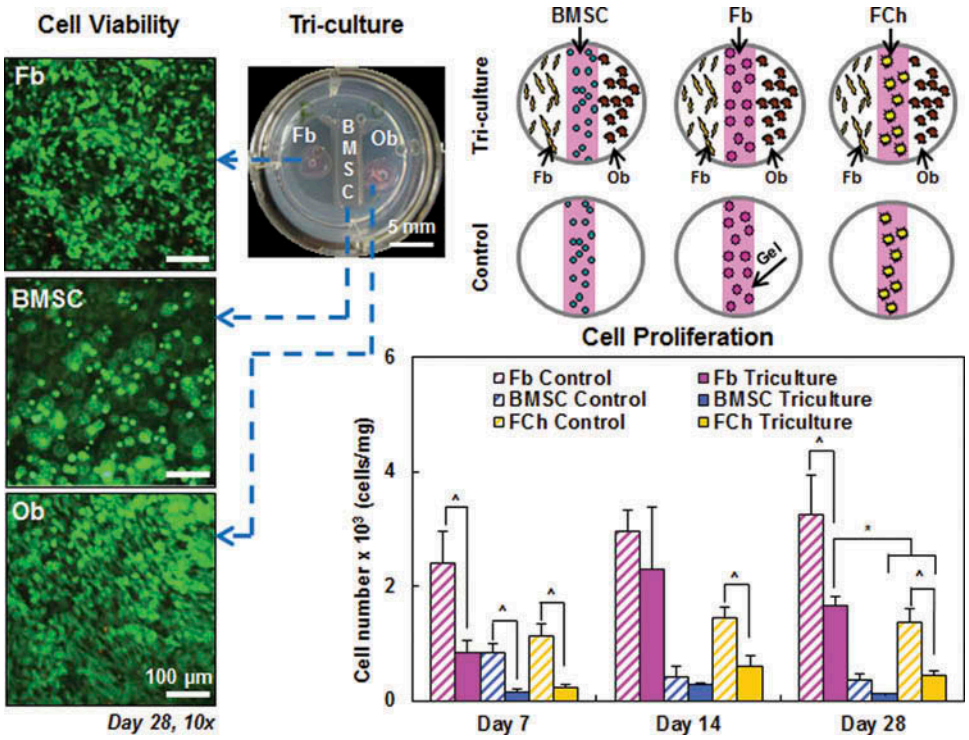

Figure 1. Effects of tri-culture on interface-relevant cell viability and proliferation. The tri-culture model established three cell regions representative of the ligament-to-bone junction: ligament fibroblast (Fb) only, interface relevant cell type [Bone Marrow Stromal Cells (BMSC), Insertion Fibrochondrocytes (FCh) or Fb] only, and osteoblast (Ob) only. All cell types were viable (n = 3/group) over time and BMSC remained spherical in tri-culture while both fibroblasts and osteoblasts exhibited a spindle-shaped morphology, Live/dead, Day 28, 10x. Cell proliferation is significantly lower in tri-culture for all interface-relevant cell populations (n = 6, $p < 0.05$), with the highest cell number found in Fb ($p < 0.05$). *$p < 0.05$ significantly different from other cell types in tri-culture; ^$p < 0.05$ significantly different from single-culture control.

effects of tri-culture on fibrochondrogenic differentiation of either fibroblasts or BMSC can be examined. Moreover, as a positive control, insertion fibrochondrocytes will be isolated from the ACL-to-bone enthesis, and the response of interface-relevant cell populations will be compared to those of fibrochondrocytes in tri-culture.

The second objective of the study is to distill the effects of physical cell–cell contact and paracrine communications between fibroblasts and osteoblasts on BMSC differentiation. Given that the ACL graft is juxtaposed against subchondral bone following ACL reconstruction, fibroblast and osteoblast interactions will likely be mediated by a combination of paracrine and/or autocrine signaling factors, as well as cell–cell physical contact (22–24). Specifically, the contributions of paracrine signaling and cell–cell contact will be examined using conditioned media and mixed tri-culture models, respectively. The expression of interface-related markers, such as proteoglycans, types I and II collagen, TGF-β1 and TGF-β3 will be determined. It is anticipated that insights into the mechanism governing regeneration of the fibrocartilage interface between soft tissue and bone will enable the design of bioactive fixation devices or synthetic ligament grafts which promote integrative and functional ACL reconstruction.

Materials and methods

Cells and cell culture

Primary fibroblasts and osteoblasts were isolated from explant cultures of anterior cruciate ligament (ACL) and trabecular bone fragments harvested from calves (<1 week old, Fresh Farm, Rutland, VT) following published protocols (13,25,26). Briefly, a midline longitudinal incision extending from the distal femur to the tibia was made in the knee under aseptic conditions. The patella was removed after retracting the skin and subcutaneous fascia, after which a deep incision was made into the joint capsule in order to expose the femoral condyle and the tibial plateau. The ACL was identified and excised from the joint after removing the synovial sheath, and the ACL-to-bone insertion was isolated by removing both ligament and bone tissues. Trabecular bone fragments were cored from subchondral bone of the same joint.

For explant cultures of fibroblasts and osteoblasts, the tissue was minced and then incubated in fully supplemented Dulbecco's Modified Essential Medium (DMEM) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 1% non-essential amino acids, 1% penicillin-streptomycin (Mediatech, Herdon, VA), 50 μg/ml gentamicin sulfate (Mediatech) and 0.025% amphotericin B (Mediatech). Cell outgrowth was observed after one week, and only cells from the second or third migration were used, as cell phenotypes have been shown to be maintained in these cultures (13,25,26). Primary ACL-to-bone insertion fibrochondrocytes were harvested following enzymatic digestion of bovine insertion fibrocartilage (27), whereby the tissue was minced and digested overnight with 0.1% w/v collagenase in DMEM supplemented with 1% FBS and 2% penicillin-streptomycin (27).

Bone marrow stromal cells (BMSC) were isolated following previously developed protocols (33). Briefly, bone marrow was extracted from the femoral cavity with a spatula, diluted with fully supplemented DMEM (1:20), and gently vortexed to achieve a homogenous mixture. The BMSC were isolated using the adherence exclusion method (28), and hematopoietic cells and other non-adherent cells were removed with successive media changes. All cultures were maintained in fully supplemented DMEM with 10 μg/ml L-ascorbic acid and 3 mM β-glycerophosphate at 37 °C and 5% CO_2. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. The chondrogenic and osteogenic potential of the isolated BMSC were evaluated and confirmed prior to this study.

Tri-culture model

To evaluate heterotypic cellular interactions, a biomimetic tri-culture model was designed to represent the multi-cellular regions at the ACL-to-bone insertion. The tri-culture model was established by pre-coating the surface of a tissue culture well (surface area ~ 400 mm^2) with 2% agarose (type VII) in order to control cell interactions, as well as prevent unwanted cell migration and adhesion. As shown in Figure 1, fibroblasts and osteoblasts were seeded separately on Thermanox™ tissue culture coverslips (5×10^4 cells/coverslip) and placed on opposing sides of the same tissue culture well. Interface-relevant cells (fibroblasts or BMSC) were embedded in agarose gel (1×10^7 cells/ml). Briefly, 4% wt/vol agarose was combined 1:1 with cell suspension to attain a final agarose concentration of 2% wt/vol. Following gelation, cell-seeded agarose was then cut into strips averaging 0.12 cm^3 in volume, and placed in the center of wells, between the segregated cultures of fibroblasts and osteoblasts, to establish tri-culture of three distinct and separate cell populations: fibroblast-only, interface-relevant cell-only, and osteoblast-only (Figure 1, from left to right). In addition to being biomimetic, another advantage of the tri-culture model is that the response of each cell

population can be analyzed independently. Moreover, the tri-culture model is designed to permit paracrine signaling between at least three types of cells, as well as allow for analysis of the effects of direct cell–cell physical contact between select populations.

Effects of tri-culture on cell response

The first objective of this study focuses on the effects of fibroblast and osteoblast interactions on the responses of two interface-relevant cell populations—ligament fibroblasts and BMSC. For the positive control, insertion fibrochondrocytes were similarly cultured in tri-culture, while individual cultures of fibroblasts, BMSC or fibrochondrocytes in agarose hydrogel served as single-culture controls (Figure 1).

Effects of mode of cellular interactions in tri-culture – paracrine effects

The second objective of this study was to examine the effects of the type of cellular interaction (paracrine factors vs. cell–cell contact) on the differentiation of interface-relevant cell types into fibrochondrocytes. Specifically, the effects of paracrine factors from fibroblast–osteoblast co-culture, as well as fibroblast–osteoblast physical contact, on the responses of BMSC were evaluated. To isolate the effects of soluble factors, a conditioned media model was used in this study (29). Briefly, conditioned media was collected from the parallel cultures of fibroblasts-only, osteoblasts-only, and fibroblast–osteoblast co-culture, each seeded at a density of 5×10^4 cells/well. The conditioned media was first centrifuged at 1000 rpm in order to remove any cells or debris, and was subsequently mixed with fresh fully supplemented DMEM (1:1) and added to monolayer cultures of BMSC (5×10^4 cells/well) every other day. BMSC monolayers in fresh fully supplemented DMEM served as a control, and experimental groups included BMSC receiving either fibroblast-conditioned media (Fb→BMSC), osteoblast-conditioned media (Ob→BMSC) or fibroblast–osteoblast co-culture conditioned media (Co→BMSC). At 1, 3, 7, 14 and 21 days, the effects of conditioned media on BMSC growth ($n = 6$), ALP activity ($n = 6$), and GAG deposition ($n = 3$) were evaluated.

Effects of mode of cellular interactions in tri-culture – physical contact

Upon examining the contribution of paracrine factors on BMSC differentiation, the second mode of cellular interactions evaluated here was the role of fibroblast–osteoblast physical contact on BMSC differentiation in tri-culture. Briefly, BMSC were tri-cultured with fibroblasts and osteoblasts using the aforementioned tri-culture model (Figure 1). However, instead of seeding fibroblasts or osteoblasts separately on each side of the BMSC-seeded agarose gel, a 1:1 mixture of fibroblasts and osteoblasts were seeded on both sides of the BMSC (5×10^4 cells/section). This resulted in three regions in the well: Fb/Ob, BMSC, Fb/Ob, with defined heterotypic cell–cell contact compared to the segregated tri-culture model. The response of BMSC in mixed tri-culture was compared to that of BMSC in segregated tri-culture. Single cultures of BMSC, fibroblasts, and osteoblasts were used as controls. The effects of fibroblast–osteoblast physical contact on BMSC growth ($n = 6$), ALP activity ($n = 6$), GAG production ($n = 6$), and gene expression for SOX9 and TGF-β3 ($n = 4$) were determined after 1, 7, 14 and 21 days. Additionally, TGF-β1 and TGF-β3 secretion ($n = 6$) during tri-culture were measured after 14 days.

Cell growth and proliferation

For all groups, total DNA per sample ($n = 6$) was measured using the PicoGreen® dsDNA assay (Molecular Probes, Eugene, OR) following the manufacturer's suggested protocol. Briefly, at each time point, the samples were collected and weighed, and subsequently homogenized mechanically. Following homogenization, the cells were lysed with 0.1% Triton-X solution. The amount of DNA per sample was correlated to fluorescence intensity measured with a microplate reader (SPECTRAFluor Plus, Tecan, Research Triangle Park, NC) using a standard curve, at the excitation and emission wavelengths of 485 nm and 535 nm, respectively. The total number of cells per sample was obtained using the conversion factor of 8 pg of DNA per cell (30,31). Cell number in the hydrogel region was normalized by the corresponding wet weight of each sample and shown as a fold increase compared to day 1 samples.

Alkaline phosphatase (ALP) activity

Sample ALP activity ($n = 6$) was quantified using an enzymatic assay based on the hydrolysis of p-nitrophenyl phosphate (pNP-PO_4) to p-nitrophenol (pNP) (32). Briefly, at each time point, the samples were collected, homogenized, and lysed with 0.1% Triton-X solution. To analyze ALP activity, the samples were first incubated at 37 °C for 2 hours in 0.1 M Na_2CO_3 buffer containing 2 mM $MgCl_2$ with disodium p-nitrophenyl

phosphate (pNP-PO_4) as the substrate. Standards were prepared by serial dilutions of 0.5 mM p-nitrophenol (pNP) in Na_2CO_3 buffer. Absorbance was measured at 405 nm, and enzymatic activity was expressed as total nM of pNP/hour/cell.

Proteoglycan production and deposition

Proteoglycan production (n = 6) was measured using the Blyscan™ assay (Biocolor Ltd., Newtownabbey, UK) following the manufacturer's suggested protocol (33). Briefly, sample homogenates were lyophilized, and digested with papain for 16 hours at 65 °C. The amount of GAG/sample was correlated using a standard curve to absorbance intensity measured at 620 nm. Proteoglycan deposition was also evaluated histologically by Alcian Blue staining (7). Briefly, the samples (n = 3) were fixed with 10% neutral buffered formalin for 10 minutes, rinsed with phosphate buffered saline, and stained with 1.0% Alcian Blue overnight.

Gene expression

Expression of fibrocartilage markers (types I and II collagen, aggrecan, TGF-β3, and SOX9) was measured by reverse transcription polymerase chain reaction (RT-PCR) (25,34,35) at day 14. Briefly, total RNA was isolated (n = 4) using the Trizol® extraction method (Invitrogen, Carlsbad, CA), and reverse transcribed into cDNA using the SuperScript™ First-Strand Synthesis System (Invitrogen), followed by amplification of the cDNA product using recombinant Taq DNA polymerase (Invitrogen). All genes were amplified for 30 cycles in a thermal cycler (Brinkmann, Westbury, NY). Gene expression levels were normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

TGF-β1 and TGF-β3 levels in conditioned media

The temporal concentrations of TGF-β1 and TGF-β3 in the collected conditioned media from all groups were measured using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA, n = 6, Quantikine®, R&D Systems, Minneapolis, MN). Briefly, samples were incubated in wells pre-coated with TGF-β1 or TGF-β3 soluble receptor Type II (R&D Systems) and sandwiched with an enzyme-linked polyclonal antibody specific for TGF-β1 or TGF-β3 (R&D Systems). The wells were then coated with a 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution supplied by the manufacturer, incubated for 20 minutes at room temperature, and mixed with a sulfuric acid stop solution. Sample absorbance at 450 nm was measured using a spectrometer (Tecan, Research Triangle Park, NC).

Statistical analysis

Data are presented as the mean ± standard deviation. For statistical analysis, a multi-way analysis of variance (ANOVA) was performed to determine the effects of tri-culture, cell type, time and modes of cellular interactions (fibroblast- vs. osteoblast- vs. co-culture-conditioned media; and mixed tri-culture vs. segregated tri-culture) on cell number, GAG content, ALP activity, interface-relevant gene expression and TGF-β1 and TGF-β3 secretion levels. The Tukey-Kramer post-hoc test was used for all pair-wise comparisons and statistical significance was attained at $p < 0.05$. Statistical analyses were performed using JMP (SAS Institute, Cary, NC).

Results

Effects of tri-culture on cell viability and growth

In tri-culture (Figure 1), three distinct regions were established, with fibroblasts and osteoblasts on each side of the well and interface-relevant cells (Fb, BMSC, or FCh) seeded in the middle region. As shown in Figure 1, all cell types in tri-culture remained viable over time. Fibroblasts and osteoblasts formed confluent monolayers populating their respective coverslips, while interface-relevant cells adopted a spherical morphology (Figure 1) within the hydrogel. During the first week of tri-culture, cell number for all groups remained relatively constant in the hydrogel (Figure 1). Subsequently, for single-culture controls, fibroblast proliferation was significantly greater than that of either BMSC or fibrochondrocytes, while no significant difference was observed over time between BMSC and fibrochondrocyte control groups. Overall, compared to the single-culture controls, tri-culture significantly decreased cell growth for all cell types (Figure 1). As such, fibroblasts in tri-culture measured a significantly higher cell number when compared to tri-cultured BMSC or fibrochondrocytes.

Effects of tri-culture on glycosaminoglycan production

In both single-culture and tri-culture, fibroblasts produced a basal level of GAG, which did not change over time, while GAG production increased over time for tri-cultured BMSC and fibrochondrocyte groups (Figure 2, $p < 0.05$). As expected, insertion fibrochondrocytes

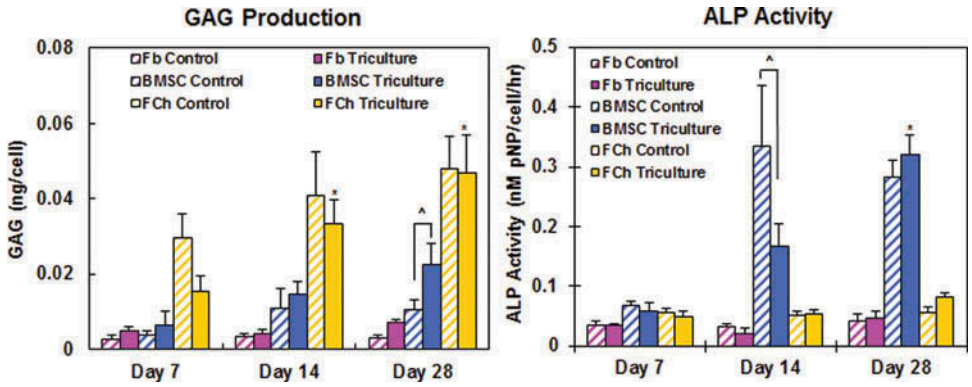

Figure 2. Effects of tri-culture on proteoglycan production and alkaline phosphatase (ALP) activity. In tri-culture, the insertion fibrochondrocytes (FCh) produced the most glycosaminoglycans (GAG), followed by bone marrow stromal cells (BMSC), while fibroblasts (Fb) measured the least amount of GAG ($n = 6$, $p < 0.05$). The BMSC tri-cultured group also produced more GAG than BMSC single-culture controls ($p < 0.05$). Ligament fibroblasts (Fb) and fibrochondrocytes (FCh) measured a similar level of alkaline phosphatase (ALP) activity regardless of culturing condition (tri-culture vs. single-culture control). In contrast, BMSC measured significantly higher ALP levels than all other cell types ($p < 0.05$), and interestingly, ALP activity was reduced in tri-culture at day 14 ($p < 0.05$), resulting in a delayed peak in BMSC ALP activity at day 28 compared to day 14 in the BMSC single-culture control. *$p <$ 0.05 significantly different from other cell types in tri-culture; ^$p < 0.05$ significantly different from single-culture control.

produced significantly more GAG when compared to either fibroblasts or BMSC in tri-culture at all time points (Figure 2), while tri-culture had no apparent effect on fibrochondrocyte GAG production. Interestingly, by day 28, a significantly higher GAG content was measured in the BMSC group in tri-culture when compared to single-culture control (Figure 2). Histological staining confirmed positive proteoglycan deposition for fibrochondrocyte and BMSC groups in tri-culture, while only background staining was observed for fibroblasts in tri-culture.

Effects of tri-culture on alkaline phosphatase activity

Basal levels of ALP activity were detected in all fibroblast and fibrochondrocyte groups over time (Figure 2). In contrast, ALP activity increased from day 7 to day 14 for the BMSC control ($p < 0.05$), with the highest activity level detected at day 14. In tri-culture, while the ALP activity of BMSC was significantly lower than that of the single-culture control at day 14 (Figure 2), this activity was comparable to that of the control by day 28 (Figure 2). Compared to the fibroblast and fibrochondrocyte groups, BMSC consistently exhibited a higher ALP activity after day 14 in both single-culture and tri-culture ($p < 0.05$).

Effects of tri-culture on the development of interface-related markers

It was observed that type I collagen expression by both fibroblasts and BMSC was significantly higher in tri-culture compared to single-culture controls (Figure 3). However, type I collagen expression by the fibrochondrocyte groups was lower compared to fibroblasts and BMSC at day 28 in tri-culture (Figure 3, $p < 0.05$). Type II collagen expression was evident in both BMSC and fibrochondrocyte control groups, as well as their respective tri-cultured groups, while it was not detectable in fibroblast groups. No significant difference was found between single-culture control and tri-culture in both BMSC and fibrochondrocyte groups. The collagen II:I expression ratios for the BMSC and fibrochondrocyte groups were comparable in both tri-culture and control (Figure 3). Histology staining for type II collagen deposition shows that, while fibrochondrocytes produced type II collagen in both tri-culture and single-culture controls, BMSC type II collagen deposition was evident only in tri-culture (Figure 3), and not in single-culture controls (data not shown). Analysis of the expression of TGF-β3 showed that, in single-culture controls, all groups expressed comparable levels of TGF-β3 (Figure 3). Interestingly, in tri-culture, TGF-β3 expression was significantly higher for BMSC when compared to either the fibroblast or fibrochondrocyte group (Figure 3).

Effects of fibroblast-osteoblast interactions: Paracrine factors

In the conditioned media studies, it was observed that, while media collected from fibroblasts exerted no significant effect on BMSC growth, osteoblast-conditioned media and conditioned media collected from a 1:1 co-culture of osteoblasts and fibroblasts significantly

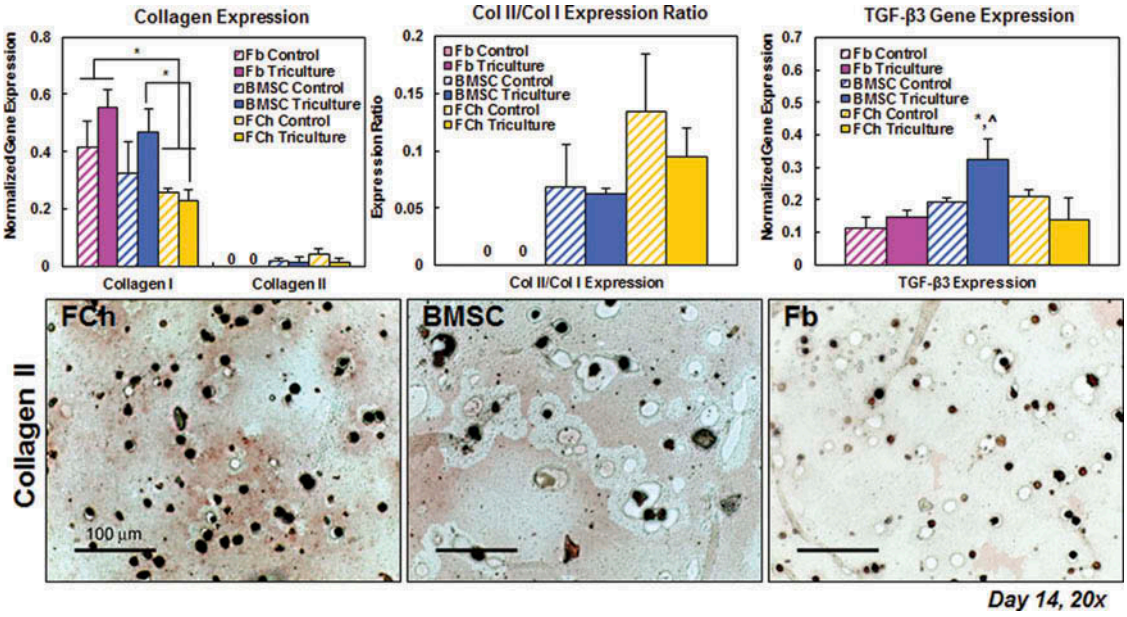

Figure 3. Effects of tri-culture on the expression of interface relevant markers. All cell types continue to express type I collagen through Day 14 ($n = 6$), while the highest collagen II/collagen I expression ratio ($n = 6$) was seen in insertion fibrochondrocytes (FCh), followed by bone marrow stromal cells (BMSC) at Day 14. Deposition of type II collagen ($n = 3$) was only evident for the tri-cultured BMSC and FCh groups, with only background staining seen in the fibroblast (Fb) tri-cultured group, Day 14, 20x. An upregulation of TGF- β3 expression was only observed in the BMSC tri-cultured group at Day 14 ($n = 6$, $p < 0.05$). *$p < 0.05$ significantly different from other cell types in tri-culture; ^$p < 0.05$ significantly different from single-culture control.

suppressed BMSC proliferation compared to controls fed with fully supplemented DMEM alone (Figure 4). The ALP activity of BMSC increased over time in the control group, while conditioned media from all groups significantly suppressed BMSC ALP activity at day 28 (Figure 4). Compared to the fibroblast-conditioned media, conditioned media collected from osteoblasts and co-culture groups further suppressed BMSC ALP activity (Figure 4). No significant difference was found between the osteoblast- and co-culture-conditioned media groups in terms of ALP activity. When BMSC were exposed to fibroblast- and co-culture-conditioned

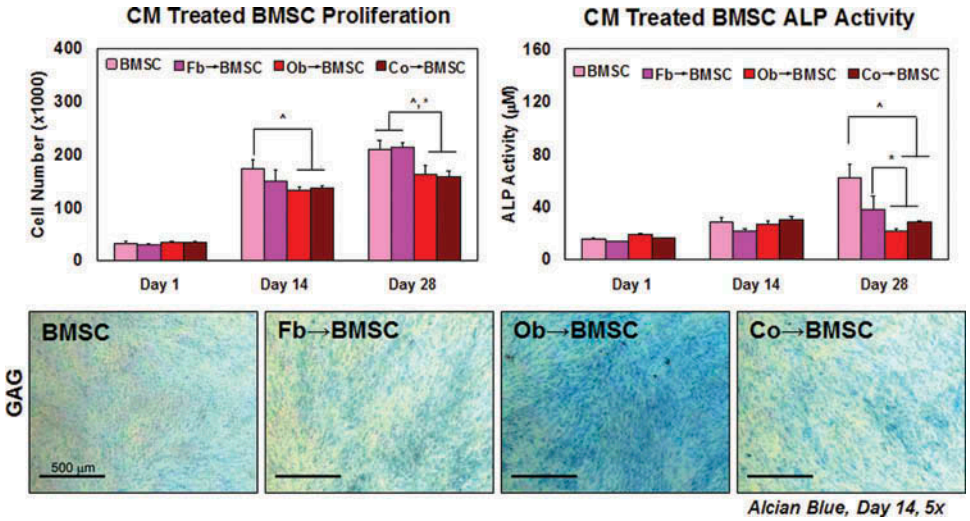

Figure 4. Paracrine effects on the response of interface relevant cells. When treated with conditioned media (CM) harvested from osteoblasts only or osteoblast-fibroblast co-culture, both bone marrow stromal cell (BMSC) proliferation and alkaline phosphatase (ALP) activity were significantly reduced ($n = 6$, $p < 0.05$). Osteoblast CM also increased GAG deposition by BMSC, as evident in positive Alcian blue staining seen in the BMSC group (Day 14, 5x, $n = 3$). *$p < 0.05$ significantly different from other cell types in tri-culture; ^$p < 0.05$ significantly different from single-culture control.

media, minimal GAG staining was observed (Figure 4). Interestingly, BMSC exposed to osteoblast-conditioned media exhibited positive staining for GAG deposition by day 14 (Figure 4).

Effects of fibroblast-osteoblast interactions: physical contact

In terms of BMSC response in mixed tri-culture vs. segregated tri-culture, it was observed that cell number remained constant over time in all groups, with no significant difference detected between groups at any time point. Interestingly, GAG production by BMSC increased over time for all groups, and was significantly higher in tri-culture when compared to the single-culture control (Figure 5). The highest GAG content was observed in mixed tri-culture when compared to both single-culture and segregated tri-culture (Figure 5). The ALP activity of BMSC in single-culture peaked at day 14. In contrast, tri-culture delayed this peak in ALP activity and significantly decreased its intensity at day 14 (Figure 5).

Analysis of the effects of fibroblast-osteoblast physical contact on SOX9 expression indicated that, while positive SOX9 expression was evident for all BMSC groups, BMSC in mixed tri-culture exhibited a significant up-regulation of SOX9 expression at day 14 compared to BMSC in segregated tri-culture or single-culture (Figure 5). In addition, the concentration of secreted TGF-β1 in the media was significantly higher in segregated tri-culture compared to the BMSC single-culture. Interestingly, the level of TGF-β1 was further enhanced in mixed tri-culture (Figure 5), and cells in mixed tri-culture continued to secrete TGF-β1 until day 14, with secretion levels dropping thereafter. By day 28, the TGF-β1 levels measured in both segregated and mixed tri-culture were found to be statistically comparable (Figure 5). No significant difference in secreted TGF-β3 levels was observed in the media collected from any group (data not shown).

Discussion

The long-term goal of this study is to elucidate the mechanisms directing the regeneration of the ligament-to-bone interface, focusing on the role of cellular interactions between resident cell populations at this critical junction. To this end, a biomimetic tri-culture model is used to evaluate the interactions between fibroblasts, osteoblasts, and interface-relevant cell populations found at the soft tissue-to-bone junction post-injury, as well as to determine the relevance of

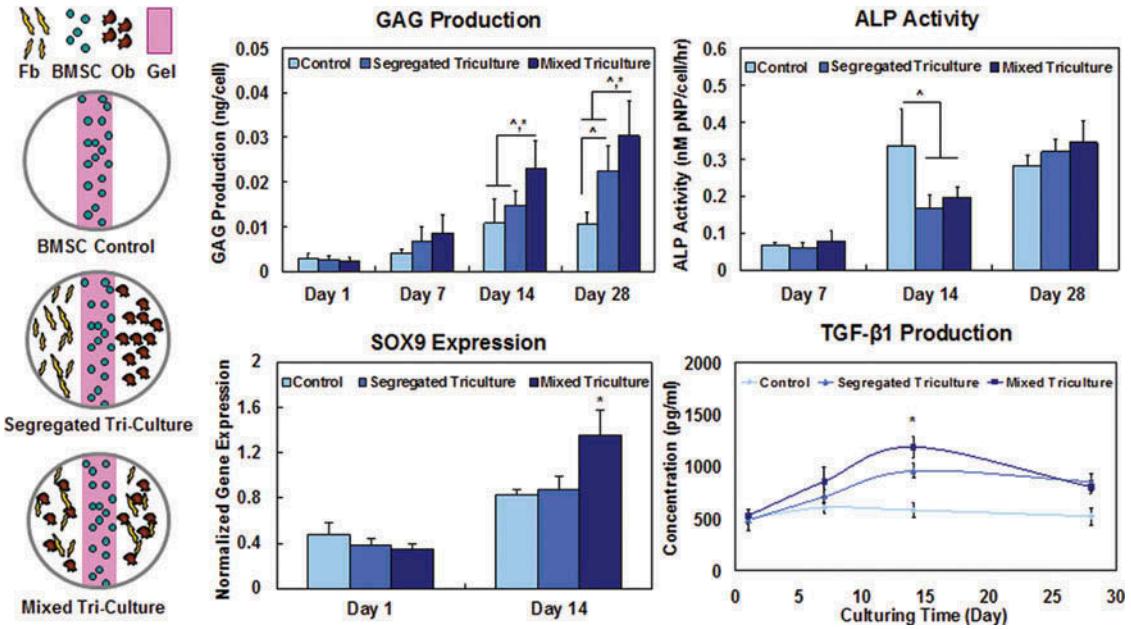

Figure 5. Effects of mode of osteoblast-fibroblast interactions on bone marrow stromal cells (BMSC) in tri-culture. Schematic of segregated and mixed tri-culture conditions is shown on the left. Regardless of the mode of cell interactions, tri-culture increased proteoglycan production ($p < 0.05$, $n = 6$) by bone marrow stromal cells (BMSC) and decreased their alkaline phosphatase (ALP) activity ($p < 0.05$, $n = 6$). Interestingly, BMSC proteoglycan production was significantly higher in the mixed tri-culture model where the fibroblasts and osteoblasts are in direct physical contact. Both elevated SOX9 expression ($n = 6$, $^*p < 0.05$) and higher TGF-β1 production were found in mixed tri-culture. $^*p < 0.05$ significantly different from other tri-culture model; $^\wedge p < 0.05$ significantly different from single-culture control.

Table 1. Summary of the effects of tri-culture on the behavior of interface-relevant cell types, relative to single-culture controls.

Cell type in tri-culture	Cell growth (Day 28)	Glycosaminoglycan (GAG) production (Day 28)	Alkaline phosphatase (ALP) activity (Day 14)	Gene expression (Day 14)		TGF-β1 production (Day 14)
				TGF-β3	SOX9	
Fibroblasts (Fb)	↓	–	–	–	N/A	N/A
Bone marrow stromal cells (BMSC) – Segregated	–	↑	↓	↑	–	↑
BMSC – Mixed	–	↑↑	↓	↑	↑	↑↑

these interactions in interface regeneration. The tri-culture model allows for the concurrent culture of three distinct and viable cell populations: fibroblasts, interface-relevant cells, and osteoblasts, as well as the investigation of both paracrine signaling and cell-cell contact effects. As summarized in Table 1, tri-culture induced fibrochondrogenic differentiation of BMSC, and this effect was mediated by soluble factors and enhanced by physical contact between osteoblasts and fibroblasts.

The effects of interactions between fibroblasts and osteoblasts on the fibrochondrogenic induction of ligament fibroblasts and BMSC were compared in this study in order to identify the most relevant cell type responsible for the formation of the fibrocartilage-like tissue often observed *in vivo*. To this end, cell organization in the tri-culture model is reminiscent of the scenario in which the autologous semitendinosus graft used in ACL reconstructions is in direct physical contact with subchondral bone inside the bone tunnel, and the interface-relevant cells, such as fibroblasts or BMSC, may be stimulated to form an interface-like matrix between these soft and hard tissues during tendon-bone healing. It was observed that in tri-culture, unlike fibroblasts, BMSC consistently exhibited fibrochondrogenic markers, such as proteoglycan and type II collagen deposition, and type I collagen expression, accompanied by a reduction in mineralization potential. The 3D hydrogel culture enables BMSC to cluster and form aggregates, which promotes a high degree of cell–cell contact, and this cell condensation has been reported to initiate chondrogenesis (13,36). Moreover, compared to the hydrogel control, tri-culture significantly enhanced proteoglycan production by BMSC, underscoring the role of cellular interactions in this induction process. Interestingly, while positive SOX9 expression is evident in both single-culture and segregated tri-culture, SOX9 is significantly upregulated in mixed tri-culture, which accelerates the interactions between osteoblasts and fibroblasts. Additionally, those in mixed tri-culture produced significantly more proteoglycans than those in segregated tri-culture, suggesting that fibroblast–osteoblast physical contact accelerated BMSC differentiation toward fibrochondrocytes.

While type I collagen was consistently expressed by BMSC, type II collagen was also up-regulated in tri-culture and in single-culture hydrogel controls. The expression ratio of types I and II collagen by BMSC was not statistically different from that of fibrochondrocytes, the positive control. More importantly, type II collagen deposition was only evident in BMSC exposed to tri-culture. These observations suggest that BMSC differentiation is facilitated by 3D culture in a hydrogel matrix, and further reinforced in tri-culture. Moreover, under the influence of fibroblast-osteoblast interactions, upregulated TGF-β3 expression and TGF-β1 production, key induction factors for BMSC chondrogenesis (36–38), are detected only in BMSC tri-culture. Interestingly, the production of TGF-β1 is also enhanced during mixed tri-culture, indicating the importance of direct osteoblast-fibroblast contact in this response. Stimulation with TGF-β1 has been reported to promote chondrogenesis, upregulating the expression of type II collagen and proteoglycan in equine MSC (39–41). It is therefore likely that the fibrochondrogenic induction observed here in BMSC tri-culture is mediated by TGF-β1 and its related signaling pathway.

In terms of mineralization potential, as expected, BMSC exhibit higher ALP activity than either fibrochondrocytes or fibroblasts. It is noted that the highest ALP activity for BMSC is measured in the single-culture control at day 14, while this peak is delayed for two weeks in tri-culture, reflecting a significant suppression in enzyme activity due to interaction with osteoblasts and fibroblasts. Conditioned media studies confirm this finding, whereby a significant decrease in ALP activity is seen when BMSC are exposed to osteoblast-conditioned media compared to fibroblast-conditioned media, indicating that cellular interactions between BMSC and osteoblasts play a more significant role in regulating BMSC mineralization potential in tri-culture. Interestingly, no significant difference in ALP activity between mixed and segregated tri-culture is observed, suggesting that paracrine signaling is largely responsible for this delay in enzyme activity.

This study also investigates the effects of soluble factors and cell–cell physical contact on BMSC

differentiation in tri-culture. In addition to decreased cell proliferation and ALP activity, higher proteoglycan deposition is observed when BMSC are exposed to osteoblast-conditioned media, while comparatively, fibroblast-conditioned media has less of an effect on BMSC behavior. It is emphasized here that some of the effects observed in tri-culture (reduced cell growth and lower ALP activity) are also seen in BMSC stimulated by either the co-culture- or osteoblast-conditioned media. Interestingly, while osteoblast-conditioned media promoted GAG deposition by BMSC, this effect is reduced when BMSC are exposed to conditioned media from co-culture. These differences highlight the inherent limitation of conditioned media studies, and the challenges of using these models to recapitulate the real-time cell–cell feedback that is activated by tri-culture.

In addition to paracrine interactions, fibroblast-osteoblast physical contact in mixed tri-culture is shown to expedite BMSC differentiation toward a fibrochondrocyte-like phenotype. For example, in addition to SOX9 upregulation, BMSC in mixed tri-culture produced significantly more proteoglycans than BMSC in segregated tri-culture, indicating that fibroblast-osteoblast physical contact is more potent than paracrine factors in BMSC induction. As such, given that a significantly higher secretion of TGF-β1 is found in the mixed-culture model at day 14, direct fibroblast-osteoblast contact may lead to the secretion of soluble factors that will further induce BMSC differentiation. It is likely that the regulation of BMSC differentiation under the influence of fibroblast-osteoblast interactions derive from a synergistic effect of both fibroblast-osteoblast physical contact and the paracrine factors secreted from such interactions.

Collectively, the results of this study demonstrate that fibroblast-osteoblast interactions significantly regulate BMSC differentiation and play a key role in the regeneration of a fibrocartilage-like interface. In addition to the biological regulation of interface regeneration, as the native ACL-to-bone interface is subjected to a complex mechanical loading environment (42), the mechanotransduction of the interface-relevant cells is also important in the fibrocartilage interface regeneration and maintenance. Future studies will focus on further optimizing the tri-culture model to study the combination of tri-culture and dynamic loading on the facilitation of interface regeneration, as well as the influence of substrate stiffness on cell induction. In addition to new understandings regarding the mechanism governing the regeneration of the interface between soft tissue and bone, findings from this study are critical for the design of a new generation of integrative fixation devices or synthetic ACL grafts that will enable integrative and functional ACL reconstruction.

Conclusions

In this study, a biomimetic tri-culture model was designed and optimized to recapitulate the soft tissue-to-bone junction, and was used to investigate the role of fibroblast-osteoblast interactions on BMSC differentiation toward an interface fibrochondrocytic phenotype. Tri-culture with fibroblasts and osteoblasts resulted in induced fibrochondrogenic differentiation of BMSC, and this effect was mediated by soluble factors and enhanced by physical contact between osteoblasts and fibroblasts.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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