Electrical stimulation enhances cell migration and integrative repair in the meniscus

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Electrical signals have been applied towards the repair of articular tissues in the laboratory and clinical settings for over seventy years. We focus on healing of the meniscus, a tissue essential to knee function with limited innate repair potential, which has been largely unexplored in the context of electrical stimulation. Here we demonstrate for the first time that electrical stimulation enhances meniscus cell migration and integrative tissue repair. We optimize pulsatile direct current electrical stimulation parameters on cells at the micro-scale, and apply these to healing of full-thickness defects in explants at the macro-scale. We report increased expression of the adenosine A2b receptor in meniscus cells after stimulation at the micro- and macro-scale, and propose a role for A2bR in meniscus electrotransduction. Taken together, these findings advance our understanding of the effects of electrical signals and their mechanisms of action, and contribute to developing electrotherapeutic strategies for meniscus repair.

Electric fields are known to guide the development and regeneration of many tissues, including cartilage1-3. However, the exact roles of electrical signals in regulating the biosynthetic activity and homeostasis of articular tissues remain elusive, although preclinical and clinical studies have demonstrated superior healing following their application4-6. The meniscus is of particular interest, as knee arthroscopy for meniscus intervention is the most performed procedure by orthopaedic surgeons7. In the past, the entire meniscus was routinely removed by total meniscectomy, but long-term outcomes have since demonstrated that the incidence and severity of osteoarthritis is proportional to the amount of tissue removed8. Moreover, the extent of intrinsic repair after surgery is largely determined by the location of the injury: while meniscus tears in the vascularized, outer tissue region can undergo repair, those in the avascular inner region, similar to cartilage, do not heal, and the damaged tissue must instead be removed9. Therefore, general wisdom in orthopaedics has been that vascularity is necessary for healing, and the regional variation that exists within the meniscus has led to novel approaches to overcome the limited treatment options for injuries in the inner region.

The biochemical composition of the meniscus also varies by region, with predominantly type I collagen in the more fibrous outer region, and a mixture of types I and II collagen in the more cartilaginous inner region10. The bulk of the remaining extracellular matrix (ECM) is composed of negatively charged glycosaminoglycans (GAGs)11, which hydrate the tissue, contribute to its compressive properties, and also allow for electrical activity12. After meniscus injury, increases in GAG levels in the synovial fluid peak early, and persist out to four years after injury13. The synovial environment after injury also has elevated levels of IL-1 and TNF-α14-16, which act in concert to increase the production of nitric oxide (NO), prostaglandin E2 (PGE2), and matrix metalloproteinases (MMPs), increase the release of GAGs, and decrease the synthesis of collagen in the meniscus17-19. The full-thickness defect model in explants has been employed extensively in the study of meniscus repair in the presence of IL-1 and TNF-α, demonstrating dose-dependent decreases in integration strength and tissue repair over sustained supplementation20, and long-term potentiation of effects even after acute exposure21. The application of dynamic loading on meniscus explants in the presence of IL-1 was found in turn to combat the cytokine’s inflammatory effects on integrative repair22.

The endogenous electrical potentials during physiological loading of articular cartilage have been studied using theoretical23-25 and experimental26-28 models, and these native electrical signals have been implicated in transducing mechanical signals to cells within tissues26,29-30. A variety of electrical stimulation modalities investigated in 2-D and 3-D models of cartilage in vitro and cartilage repair in vivo4-6 were shown to significantly increase cell proliferation31,32 and GAG synthesis33,34, upregulate the expression of ECM genes35, and reduce the production of...
inflammatory mediators such as IL-1 and MMPs. However, questions remain as to how electrical signals influence cells and propagate their effects. The adenosine receptors have been implicated in the electrotransduction of pulsed electromagnetic fields in cartilage and. Stimulation of the high-affinity A2a and low-affinity A2b adenosine receptors resulted in elevated cyclic AMP and subsequent activation of anti-inflammatory pathways via protein kinase A and EPAC, which in turn lead to the suppression of NO and PGE2 and downstream feedback inhibition of TNF-α and IL-1β. We therefore hypothesized that electrical stimulation will enhance repair of the meniscus and control the inflammatory events underlying tissue degradation.

Given the wealth of background information on electrical stimulation, inflammation, and adenosine receptor signaling in cartilage repair, it may come as a surprise that very little published information exists in the context of the meniscus. Only recently, meniscus cells from the outer region were found to migrate more quickly in 2-D culture than inner cells, in the presence of static direct current (DC) fields. However, electrical stimulation studies have demonstrated a disparity in migration behavior between 2-D and more physiologically relevant 3-D environments. Moreover, pulsatile electric fields (EFs) are already used in the clinical setting for related conditions. At this time, the effects of applied EFs on meniscus cells and subsequent development of novel repair strategies are only beginning to be understood. We investigated the effects of pulsatile direct current electric field stimulation on meniscus cell migration in a micropatterned 3-D hydrogel system. Our micro-scale system also enabled study of the paracrine signaling between meniscus and vascular endothelial cells, in concert with electrical stimulation. Electric fields are known to induce VEGF receptor signaling in endothelial cells, so we expected that patterns of meniscus cell migration that result from the differences in intrinsic vascularity within the tissue will be further enhanced by interactions between electric fields and endothelial cells. Moreover, little is known about the signal transduction pathways that respond to electrical stimuli in meniscus cells. Screening and optimization of stimulation parameters at the micro-scale enabled us to identify potential pathways involved in electrotransduction. By translating from the micro- to the macro-scale, we established stimulation regimes that enhanced integrative repair of full-thickness defects in meniscus, and demonstrated promising therapeutic effects of electrical stimulation on meniscus healing.

Results
Electrical stimulation has differential effects on meniscus cell migration. Electrical stimulation of the meniscus was investigated using three distinctly different yet related experimental model systems in which inner and outer meniscus cells or explants were subjected to pulsatile direct current electrical stimulation: (a) micropatterned three-dimensional hydrogels with encapsulated inner or outer meniscus cells, (b) micropatterned 3-D hydrogels with spatially distributed meniscus and endothelial cells, and (c) a macroscopic in vitro model of meniscus healing (Fig. 1). When cultured in the micropatterned 3-D hydrogel system, meniscus cells migrated over six days of culture, with the stimulated cells demonstrating enhanced migration relative to non-stimulated control cells (Fig. 2a). Notably, both inner and outer meniscus cells exhibited similar increases in migration with applied electrical signals at 3 V/cm, 1 Hz, 2 ms pulse duration (Fig. 2b), despite the variation in repair response between their respective tissue regions. When injected charge, or the total amount of charge delivered during one stimulus pulse, was maintained at a constant field strength of 3 V/cm, further increases in cell migration were gained as the frequency of stimulation increased to 10 Hz and the pulse duration decreased to 0.2 ms (Fig. 2c). The combinations of 3 V/cm, 0.1 Hz, 20 ms pulse duration, and 3 V/cm, 100 Hz, 0.02 ms pulse duration were also tested, but the longer pulse duration associated with 0.1 Hz led to a more rounded, quiescent cell appearance rather than the spread-out, migrating cell phenotype seen at the channel edge. The increase in frequency to 100 Hz did not markedly improve the migration behavior of inner or outer meniscus cells, likely a result of too brief of a refractory period for cells to fully respond to subsequent stimulation pulses.

No apparent difference in positive BrdU staining, indicative of cellular proliferation, was apparent between the inner and outer cells, with and without stimulation (Fig. 2d), suggesting that differences in cell motility over six days of culture were not the result of proliferation only. Finally, type I collagen, a key ECM component throughout the meniscus, exhibited trends of elevated gene expression in both inner and outer cells when the frequency of electrical stimulation was increased while maintaining constant injected charge (Fig. 2e).

Cooperative action of electrical stimulation and endothelial cells on meniscus cell migration. Co-culture with human umbilical vein endothelial cells (HUVECs) was investigated in the context of regional variation in healing between the inner and outer meniscus. Notably, HUVECs further potentiated the effects of electrical stimulation on meniscus cell migration (Fig. S1). Stimulating HUVECs alone in the hydrogel system led to increased expression of EDN1, PDGFA, and PDGFB genes (Fig. 3a), which encode key angiogenic factors (endothelin-1, PDGF-A, B) that modulate the behaviors of chondrocytes and meniscus cells, suggesting a dual role of electrical stimulation in upregulating angiogenic factors that specifically enhance meniscus cell migration, in addition to promoting cell migration in general. Although meniscus cells alone exhibited greater migration in response to the 3 V/cm, 10 Hz, 0.2 ms pulse duration regime, meniscus cell migration in co-culture with HUVECs at 10 Hz stimulation was not significantly greater than without HUVECs at 10 Hz, but was significantly greater than in non-stimulated meniscus cells with HUVECs (Fig. 3b). The cooperative action of stimulation and co-culture was observed in the 3 V/cm, 1 Hz, 2 ms pulse duration regime (Fig. 3b), suggesting that frequency-dependent interactions between chemical and electrical stimuli require optimization for collaborative effects. In this system, the expression of angiogenic factors by HUVECs was upregulated dramatically with stimulation at 1 Hz as compared to non-stimulated controls, whereas the effect of increasing frequency from 1 to 10 Hz was much smaller (Fig. 3a). These findings may account for the positive trends seen in meniscus cell migration with co-culture at 1 Hz stimulation that were not apparent at 10 Hz.

Using the 1 Hz stimulation regime, gene expression profiles of the meniscus cells were further investigated to elucidate how stimulation and co-culture cooperate to enhance migration. As seen in cultures of meniscus cells with electrical stimulation alone, both inner and outer cells showed increases in COL1A2 expression in response to combined stimulation at 1 Hz and co-culture with HUVECs (Fig. 3c). Although inner and outer meniscus cells demonstrated similar migration behavior in co-culture with HUVECs, they do so in response to different angiogenic factors secreted by endothelial cells, as seen at the gene expression level. Both types of cells responded to stimulation in co-culture with HUVECs by increased expression of EDNRA, encoding endothelin receptor type A (Fig. 3c). However, outer cells responded to the secretion of PDGF isoforms by HUVECs with increased expression of PDGFRα and PDGFRβ, encoding PDGF receptors α and β, while no such changes were detected for inner cells. These results demonstrate that electrical stimulation and endothelial cells collaborate to activate meniscus cell receptors at the gene expression level, suggesting potential synergy between the biophysical and biochemical stimuli.

Electrical stimulation enhanced integrative repair of meniscus explants. Using a macroscopic in vitro model of meniscus healing, translation of the micro-scale findings to the macro-scale revealed a significant increase in the integration strength of full-thickness
defects in explants stimulated at 3 V/cm, 10 Hz, 0.2 ms pulse duration, after six weeks of culture (Fig. 4a). Stimulation at 1 Hz, 2 ms pulse duration initially corresponded to decreases in integration strength over the first four weeks of culture, but surpassed control conditions without stimulation by day 42, albeit to a lesser extent than stimulation at 10 Hz. The underlying basis for the enhanced integration strength of defects in explants stimulated at 10 Hz was further explored by assaying for biochemical content and visualizing the distribution of cells and ECM within the tissue. The overall biochemical content of explants without stimulation decreased throughout six weeks of culture, consistent with previous studies of explant stability in long-term culture, in the absence of growth factor supplementation.49,50 (Fig. 4b). However, the GAG and OHP content of explants stimulated at 10 Hz was not significantly different than at day 0, in comparison to explants without stimulation, which were significantly lower than initial values (Fig. 4b). In general, stimulation led to significant upward trends in DNA and OHP content of explants after six weeks of culture, as compared to explants without stimulation (Fig. 4b).

Histological evaluation revealed that the defect interface appeared more closely apposed in explants stimulated at 10 Hz than in control explants (Fig. 4c). Specifically, newly synthesized matrix was observed at the interface, containing sulfated GAGs and collagens, as evidenced by Alcian blue and Picrosirius red staining, respectively. BrdU labeling was performed to assess the effects of electrical stimulation on cell proliferation, yielding more BrdU-positive cells at the interface of stimulated explants, and indicating a moderate role of electrical stimulation in triggering cell proliferation in explants over long-term culture. Taken together, these data suggest that in addition to tissue repair, electrical stimulation acts to maintain cells and overall ECM composition, and prevent explant degradation in vitro.

Antii-inflammatory effects of electrical stimulation on meniscus explants. Evaluation of the components from media collected...
throughout six weeks of culture revealed increases in endogenous TNF-\(\alpha\) and IL-1\(\beta\) production by explants without stimulation, compared to those receiving stimulation at 3 V/cm, 10 Hz, 0.2 ms pulse duration, particularly within the first four weeks (Fig. S2a, b). The elevated cytokine levels in explants without stimulation corresponded with changes in NO production (Fig. S2c), MMP activity (Fig. S2d), and GAG release (Fig. S2e), which were typical of catabolic degradation: in comparison to stimulated explants, greater NO production, MMP activity, and GAG release were detected in explants without stimulation, suggestive of an anti-inflammatory, anti-catabolic, and stabilizing effect of electrical stimulation in long-term culture in

Figure 2 | Electrical stimulation enhanced meniscus cell migration. (a) Representative images of inner meniscus cell migration in a micropatterned hydrogel system with (10 Hz) and without (0 V) electrical stimulation, before (day 0) and after 3 days of pre-culture and 3 days (day 6) of stimulation (3 V/cm, 10 Hz, 0.2 ms pulse duration) or no stimulation (0 V). Scale bar: 200 \(\mu\)m. (b) Optimization of stimulation frequency. Parameters were held constant at 3 V/cm, 2 ms pulse duration, with varying frequency (0.1, 1, 10 Hz). Migration distances at day 6 demonstrated increases for inner and outer meniscus cells at 1 Hz versus cells without stimulation (0 V). *\(p < 0.05\) vs. 0 V; \(n = 38–194\). (c) Maintenance of charge injection. Field strength was held constant at 3 V/cm, with varying frequency and pulse duration. A higher frequency (10 Hz) and shorter pulse duration (0.2 ms) further enhanced inner and outer meniscus cell migration at day 6. *\(p < 0.05\) vs. 0 V; **\(p < 0.05\) vs. 0 V, 1 Hz; \(n = 68–252\). (d) Immunofluorescence staining of migrating inner and outer cells at day 6 with (3 V/cm, 10 Hz, 0.2 ms pulse duration) or without (0 V) stimulation, against BrdU for proliferating cells and DAPI for nuclei. No overt differences in cell proliferation were apparent between the cell types with and without stimulation. Scale bar: 200 \(\mu\)m. (e) Fold change \((2^{\Delta\Delta C_T})\) in gene expression of COL1A2 in meniscus cells at day 6 relative to cells at day 0, respectively, both normalized to GAPDH. Increasing frequency of stimulation with maintenance of injected charge (3 V/cm, 1 Hz, 2 ms or 3 V/cm, 10 Hz, 0.2 ms pulse duration) led to upward trends in gene expression. *\(p < 0.05\) for linear trend; \(n = 5–10\).
Figure 3 | Cooperative action of electrical stimulation and endothelial cells on meniscus cell migration. (a) Fold change \( \left( 2^{-\Delta \Delta C_{T}} \right) \) in gene expression of \( EDN1, PDGFA, \) and \( PDGFB \) in HUVECs at day 6 relative to cells at day 0, respectively, both normalized to GAPDH. Increasing frequency of stimulation with maintenance of injected charge (3 V/cm, 1 Hz, 2 ms or 3 V/cm, 10 Hz, 0.2 ms pulse duration) led to upward trends in gene expression. * \( p < 0.05 \) for linear trend; \( n = 3–4 \). (b) Optimization of electrical stimulation parameters for co-culture of HUVECs and meniscus cells. Meniscus cell migration with HUVECs and stimulation at 3 V/cm, 10 Hz, 0.2 ms pulse duration (10 Hz) was greater than with HUVECs alone at day 6 (left). * \( p < 0.05 \) vs. + HUVEC; \( n = 37–154 \). Meniscus cell migration with HUVECs and stimulation at 3 V/cm, 1 Hz, 2 ms pulse duration (1 Hz) demonstrated cooperative, upward trends with the addition of each stimulus at day 6 (right). * \( p < 0.05 \) for linear trend (analysis of groups indicated by the same letter); \( n = 24–194 \). (c) Fold change \( \left( 2^{-\Delta \Delta C_{T}} \right) \) in gene expression of \( COL1A2, EDNRA, PDGFRA, \) and \( PDGFRB \) in meniscus cells at day 6 relative to cells at day 0, respectively, both normalized to GAPDH. Increasing frequency of stimulation with maintenance of injected charge (3 V/cm, 1 Hz, 2 ms or 3 V/cm, 10 Hz, 0.2 ms pulse duration) led to upward trends in gene expression. * \( p < 0.05 \) for linear trend (analysis of groups indicated by the same letter); \( n = 3–10 \).
These changes in explants without stimulation occurred early in culture, within the first three weeks, but their biochemical composition continued to decrease for the remainder of the culture period.

Adenosine A2b receptor plays a role in meniscus electrotransduction. Adenosine receptors in meniscus were identified by gene expression analysis of migrating cells, with and without stimulation at 3 V/cm, 10 Hz, 0.2 ms pulse duration. In hydrogel-encapsulated...
cells, the expression of ADORA1, ADORA2A, and ADORA3, encoding the adenosine A1, A2a, and A3 receptors, respectively, was minimal at day 0 ($2^{-\Delta \Delta C_T} \ll 10^{-4}; n = 4$) and at day 6 after culture, with and without stimulation ($2^{-\Delta \Delta C_T} \ll 10^{-1}; n = 4$), but ADORA2B encoding A2bR was upregulated in meniscus cells with electrical stimulation at 3 V/cm, 10 Hz, 0.2 ms pulse duration (Fig. 5a). Translation to the protein level was evident through immunofluorescence staining of migrating meniscus cells at day 6, against A2bR and DAPI for nuclei. Stimulated cells exhibited positive staining for A2bR, but not cells without stimulation. Arrows indicate the left edge of channels. Scale bar: 200 μm. (c) Immunofluorescence staining in meniscus explants at day 42 with 3 V/cm, 10 Hz, 0.2 ms pulse duration and without electrical stimulation (0 V), of A2bR and nuclei. Cells in stimulated explants only exhibited positive staining for A2bR. Boxes (top) indicate the areas pertaining to high magnification images (bottom). Scale bar: 50 μm.

Figure 5 | Putative role of adenosine A2b receptor in mediating electrical stimulation of meniscus. (a) Fold change ($2^{-\Delta \Delta C_T}$) in gene expression of ADORA2B in meniscus cells at day 6 relative to cells at day 0, respectively, both normalized to GAPDH. The addition of electrical stimulation (3 V/cm, 1 Hz, 2 ms or 3 V/cm, 10 Hz, 0.2 ms pulse duration) upregulated expression of the gene encoding the adenosine A2b receptor (A2bR). * $p < 0.05$ vs. 0 V; $n = 3–4$. (b) Immunofluorescence staining of migrating meniscus cells at day 6 with (3 V/cm, 10 Hz, 0.2 ms pulse duration) and without (0 V) stimulation, against A2bR and DAPI for nuclei. Stimulated cells exhibited positive staining for A2bR, but not cells without stimulation. Arrows indicate the left edge of channels. Scale bar: 200 μm. (c) Immunofluorescence staining in meniscus explants at day 42 with (3 V/cm, 10 Hz, 0.2 ms pulse duration) and without electrical stimulation (0 V), of A2bR and nuclei. Cells in stimulated explants only exhibited positive staining for A2bR. Boxes (top) indicate the areas pertaining to high magnification images (bottom). Scale bar: 50 μm.

Discussion

Electrical stimulation is a versatile treatment modality that has yet to be fully explored in the context of injuries to the meniscus, in which negatively charged glycosaminoglycans form the basis for endogenous electrical activity. We demonstrate that pulsatile direct current electric fields enhance meniscus cell migration in a micro-patterned hydrogel system, and the integrative repair of meniscus defects in an in vitro explant model of meniscus healing. Notably, the responses of meniscus cells from the inner and outer regions were comparable, suggesting that the meniscus cells from both regions can be induced to migrate and promote healing by external electrical signals.

In our 3-D systems, electrical stimulation alone induced similar migration behavior in cells isolated from both the inner and outer regions, whereas previous studies have shown that outer cells migrate more quickly than inner cells during 2-D galvanotaxis. This
The galvanotaxis studies42. The inner and outer meniscus cells used in the present study, but are relevant due to the differences in relative potency of IL-1α and β in porcine meniscus explants57. Moreover, the effects of TNF-α and IL-1 were not independent, and interplay between the two cytokines in activating pro-inflammatory pathways has been documented14–19. The lower levels of TNF-α and IL-1β in explants receiving the optimal stimulation regime suggest inhibitory effects of electrical stimulation on their production. In contrast, increases in these cytokines were observed in explants without stimulation, with corresponding increases in NO production, MMP activity, and subsequent GAG loss in media. These two observations correlate with previous findings demonstrating that inhibition of MMPs can significantly reduce GAG loss in culture50. The loss of explant stability in the absence of electrical stimulation, particularly in the first half of the culture period, corresponds with the observation that meniscus injury disrupts tissue homeostasis and initiates a series of events furthering degradation53–57.

The migration and anti-inflammatory responses elicited by electrical stimulation occur via the activation of membrane receptors on meniscus cells. It has been previously postulated that electromagnetic fields can serve as first messengers in signaling towards tissue repair59. Based upon the findings in this study, a new model of electrotransduction in meniscus could be proposed: after meniscus injury, TNF-α and IL-1 levels are elevated, progressing toward further tissue degradation (Fig. 6a). Within this environment, the application of pulsatile DC electrical stimulation selectively activates the adenosine A2bR receptor, producing the second messenger cAMP, which triggers anti-inflammatory pathways that reduce NO production, MMP activity, and GAG release, and inhibit the initial pro-inflammatory cytokines (Fig. 6b).

The physiological role of low-affinity A2bR has been less characterized than the high-affinity A2aR, which has been studied extensively for anti-inflammatory activity in the context of osteoarthritis and rheumatoid arthritis50–52. However, activation of the A3dR leading to elevated cAMP, has been shown to inhibit MMP-1 production in fibroblast-like synoviocytes from RA patients62, and in this study, we report for the first time in meniscus, among the class of adenosine receptors, the increased expression of A2bR following electrical stimulation at both the micro- and macro-scale. In order to fully elucidate the role of A2bR in meniscus electrotransduction, further experiments using receptor agonists and antagonists must be tested in meniscus defect models, with the addition of exogenous sources of pro-inflammatory cytokines to the system, and measurement of changes in adenyl cyclase and cAMP levels. To this end, our micro-scale system for meniscus cell migration will allow for blocking or knock-down of the adenosine A2b receptor by antagonists or gene silencing, in order to verify the integral role of A2bR via short-term experiments, and subsequent application at the macro-scale in long-term culture of explants, for confirmation at the tissue level. Moreover, cells and explants in this study were derived from juvenile bovine meniscus, requiring future studies using adult or even osteoarthritic tissue to confirm and extend findings. While growth factors were not supplemented to medium in this system, the addition of TGF-β3 alone63 or TGF-β1 in the presence of IL-1β has been shown to significantly enhance meniscus repair, meriting the further study of any potential synergy between electrical and chemical stimuli. However, it should be noted that the reparative and protective effects of electrical stimulation alone, as shown in this study, are more immediately translatable to the clinic, without the need for in vivo delivery of growth factors. Moreover, the integration strength of explants stimulated at the optimal regime is among the highest reported in literature for this model of meniscal repair, even in the absence of exogenous growth factors64–66.

The contribution of growth factors was explored at the micro-scale through the co-culture of endothelial cells, as a model of clinical methods that increase the vascular response to the inner region67,68, which does not naturally undergo repair. Although up to 30% of
the meniscus is vascularized in adults\(^\text{67}\), and endothelial cells from previously shown to act on chondrocytes\(^\text{47}\) and meniscus cells\(^\text{48}\). Meniscus cell membrane, which stimulate adenylyl cyclase via G\(_{s}\) and HUVEC behavior\(^\text{46}\), and in our system, upregulated the gene expression of electrical signals on HUVECs, which have been shown to regulate compared to either stimulus alone. The cooperative effects of electrical signals on cells from the inner and outer regions are validated in co-culture with HUVECs cooperatively enhanced the migration of meniscus cells in a 3-D environment. Despite the variation in vascularity of their respective tissue regions, inner and outer cells demonstrated trends of increased migration in the presence of both stimuli as their respective tissue regions, inner and outer cells demonstrated a disparity that was further amplified by electrical signals. However, the upregulation of ET\(_{x}\)R gene expression in cells of both regions with co-culture suggests that ET-1 signaling is involved in the vascular response of the outer region for healing. Moreover, the limited healing potential of the inner region could be enhanced if ET-1 is supplemented to the avascular tissue, which does not receive these signals natively in the absence of endothelial cells. If these differential paracrine mechanisms of HUVECs on cells from the inner and outer regions are validated in future studies, by applying conditioned medium or specific angiogenic factors, we not only gain a further understanding of the variation in repair response between the two regions, but we also identify the angiogenic factors that are natively absent in the inner region, but can be supplemented in order to promote healing.

In addition, we found that the coordinated action of EFs and HUVECs on meniscus cell migration was more apparent using a different set of stimulation parameters than for EFs alone. This observation, as well as the variation in migration response seen during initial optimization of parameters for meniscus cells only, is in line with previous theories that a window exists in which productive electrical stimulation occurs\(^\text{33}\), and parameters must be optimized for all relevant cell types within the system. Moreover, although the co-culture studies were performed at one fixed channel-to-channel distance, the system can be easily modified to vary this distance by simply changing the design of the micropattern, in order to identify a length scale over which paracrine signaling occurs. Although it was not tested in this study, the interaction of electrical stimulation and endothelial cells is pertinent at the tissue level as well, with implications for clinical and adjuvant therapies. However, in vitro models of meniscus repair cannot fully replicate the regional variation in vascular environment within the tissue and its influence on healing\(^\text{44}\).

In summary, the effects of pulsatile electric field stimulation on meniscus repair were demonstrated for the first time. Optimization of electrical stimulation regimes for cell migration was achieved at the micro-scale with short-term experiments, and then applied at the macro-scale towards significant integrative repair of full-thickness defects in meniscus explants. In situ stimulation of explants revealed another role of electric fields in modulating inflammation and reducing tissue degradation. The dual functions of electrical stimulation in meniscus support its use in the clinical setting, after surgical intervention, to recruit cells for tissue repair and to prevent the matrix degradation that leads to osteoarthritis. Future establishment of therapeutic strategies, by testing stimulation regimes and translating to macro-scale tissue repair, has the potential to overcome current limitations in meniscus healing.

**Methods**

Cell isolation. Juvenile bovine meniscus cells were isolated from the inner and outer regions as previously described\(^\text{1}\). Briefly, the menisci of calves were obtained from a commercial source (Green Village Packing Company), dissected within 36 h of slaughter, and sectioned into inner (2/3) and outer (1/3) regions. The tissue was then minced into 1–2 mm\(^3\) pieces, and plated on tissue culture plates in basal medium consisting of high glucose DMEM, 1\% antibiotic-antimycotic, 10\% FBS, and 50 \(\mu\)g/\(\mu\)L ascorbate 2-phosphate. Over 2–3 weeks, cells migrated out of the tissue pieces, and were expanded to passage 2.

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords of term delivery, collected according to an active IRB at Columbia University (IRB-AAAC48399). According to the protocol, tissue samples were fully deidentified and there was no patient information available to the investigators. Umbilical cords were rinsed with PBS, and the umbilical vein lumen was infused with 2.5\% trypsin solution and clamped at both ends. Enzymatic digestion occurred at 37° C for 15 min, and the resulting cell digest was collected by rinsing the vein with PBS and centrifugation at 300 \(\times\) g for 5 min. The cell pellet was resuspended on 25 cm\(^2\) tissue culture flasks in endothelial growth medium (EGM-2, Lonza), and expanded to passage 5.
Explant culture. Juvenile bovine meniscus explants were harvested from the central tissue region using sterile 4 mm Ø biopsy punches, and cut to 1.5 mm height using a custom microtome device. A 1.5 mm Ø central core was punched and immediately replaced into the explant ring to simulate a full-thickness defect. Explants were cultured for 3 days in basal medium prior to the start of each experiment.

Cell migration assay. The micropatterned three-dimensional hydrogel system used in cell migration studies was previously established for the study of human mesenchymal stem cells and HUVECs in co-culture. Briefly, the poly(dimethylsiloxane) (PDMS; 9:1 elastomer:curing agent; Syngard 184 silicone elastomer kit, Dow Corning) micropattern consisted of two parallel channels (1 cm length, 1000 µm width, 200 µm height, channel-to-channel distance: 2000 µm; Fig. 1b). Prior to cell encapsulation, the PDMS surface was blocked using a 5% solution of FBS in sterile distilled water.

In single channel studies (Fig. 1a), inner or outer meniscus cells were encapsulated at 3.5 x 10^5 cells/mL each in 1.8% fibrin (bovine fibrinogen, MP Biomedicals; thromboplastin from bovine liver; Sigma-Aldrich) and printed into single channels on plastic slides. The cell-fibrin suspension was allowed to polymerize for 15 min, before removal of the PDMS mold and encapsulation in an additional layer of 1.8% fibrin to permit cell migration. In co-culture studies (Fig. 1b), inner or outer meniscus cells and HUVECs were encapsulated at 3.5 x 10^5 cells/mL each in 1.8% fibrin, printed into two parallel channels on plastic slides, and covered by another layer of 1.8% fibrin.

Single channels of inner or outer meniscus cells served as controls. Cultures were maintained in EGM-2 over 6 days after encapsulation, and migration was monitored by bright field imaging at days 0 and 6, using a Olympus IX81 microscope with an IX2-UCB digital camera and Metamorph software.

Electrical stimulation of cells. After 3 days of pre-culture without stimulation, the cells were transferred into custom bioreactors with carbon electrodes (Ladd Research Industries) spaced 2.5 cm apart (Fig. 1a). Bioreactors were connected by platinum wire to an electrical stimulator (Grass Technologies) generating continuous pulses of 3 V, corresponding to a field strength of 3 V/cm, and either 1 Hz and 0.2 ms pulse duration or 10 Hz and 0.2 ms pulse duration. Each row was stimulated in a custom bioreactor (Fig. 1c), consisting of a 5 cm x 2 cm x 2 mm long-melting-temperature agarose. All samples were dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned to 8 µm thickness. Sections were stained with hematoxylin and eosin (H&E) for nuclei and cytoplasmic elements, respectively. Acantha blue (pH 1.0) for sulfated GAGs, and Picrosiris red for collagen fibers. For immunofluorescence staining of A2bR in paraffin-embedded samples, antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). Staining and histological fluorescence staining at bidwells' time points.

Evaluation of meniscus cell migration. Bright field images were processed using a custom MATLAB program to track cell migration. Briefly, the program applied the Sobel edge detection algorithm and eliminated small regions of isolated, single meniscus cells. Each image was subdivided into regions corresponding to ~1580 µm of the channel length, for which average channel-to-channel distances were calculated.

Real-time PCR. The total RNA of meniscus cells or HUVECs after six days of culture was extracted by TRIzol reagent (Invitrogen) and reverse transcribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Meniscus cells and HUVECs were also collected at day 0 prior to encapsulation and processed to facilitate follow-up cell analysis (2-3d). Real-time PCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR System using Fast SYBR Green Master Mix (Invitrogen), custom bovine primers for COLIA2, EDNRA, PDGFRα, PDGFRβ, ADORA1, ADORA2B, ADORAS, and GAPDH, and custom human primers for EDN1, PDGFRA, PDGFRβ, and GAPDH.

Mechanical integration testing of explants. The integration of full-thickness defects in explants was tested with a custom device consisting of a 1.33 mm Ø indenter in series with a 50 g load cell, placed above a cup with a 2 mm Ø hole. Prior to testing, the height of each sample was measured using a digital caliper. The indenter was placed at a ramp rate of 0.3% of the sample height per second, until the entire core was pushed fully through the outer ring. Integration strength was calculated as the ratio of the peak force to the surface area of contact between the central core and outer ring. After testing, samples were collected for further biochemical analysis.

Biochemical analysis of explants and media. The biochemical composition of explants was evaluated for DNA, sulfated GAG, and OHP content. Briefly, samples were lyophilized for 24 h and digested at 60°C for 16 h in a papain solution containing 125 µg/mL papain (Sigma-Aldrich), 50 mmol phosphate buffer (pH 6.5), and 2 mmol acetylated casein (Sigma-Aldrich). DNA content of the sample digests was obtained using the PicoGreen dsDNA quantitation kit (Invitrogen), according to the manufacturer's protocol. Sulfated GAG content was measured using the 9,14-dimethylpentynyl blue dye-binding (DMBB) assay. Hydroxyproline (OHP) content was quantified by a modified acid hydrolysis assay. Media collected at each change was assayed for TNF-α, IL-1β, and NO production, MMP activity, and GAG release using the DMBB assay. Briefly, TNF-α and IL-1β production were quantified directly on media samples using the bovine TNF-α (GenWay Biotech) and IL-1β (Thermo Fisher Scientific) ELISA kits, respectively. Prior to NO analysis, media samples were filtered through Vivipur 500 units (10,000 MWCO PES filters, Sigma-Aldrich), and total NO production in media samples was determined by quantification of nitrate and nitrite, according to manufacturer's protocol (nitrate/nitrite colorimetric assay kit, Cayman Chemical Company). The activity of MMP-1, -2, -3, -9, -13, and -14 was assessed via cleavage of a fluorescent peptide substrate (PEPDBA808, BioZyme), adapted from a previously published protocol. Media samples were incubated in 2.5 µM p-aminoxybenzyl fluorescein (APMA; pH 7.0–7.5) in assay buffer (200 mM NaCl, 50 mM Tris, 5 µM CaCl2, 10 µM ZnSO4, 0.01% Brij 35, pH 7.5) or in assay buffer alone at 37°C for 5 h. Samples were then diluted twofold with assay buffer containing 20 µM substrate at 37°C for 2 h, and measured at 485 nm excitation and 530 nm emission. Total MMP activity was calculated as the difference in fluorescence readings between samples incubated with and without APMA.

Histological and immunofluorescence staining of cells and explants. Cell migration studies were incubated with 5-bromo-2-deoxyuridine (BrdU, Invitrogen) to assess cellular proliferation. Single channel studies of meniscus cells were incubated with BrdU labeling reagent (1:50) at 37°C for 4 h, and explants (1:50) at 37°C overnight. Samples were then fixed for further histological processing.

Statistical analysis. For cell migration studies, 1-way ANOVA with Tukey post tests or post tests for linear trend were performed with Prism (α = 0.05). For integration studies, 2-way ANOVA with Bonferroni post tests and 1-way ANOVA with post tests for linear trend were also performed with Prism. All data are presented as mean ± SEM.
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Author contributions
X.Y., P.G.C. and G.V.N. designed research; X.Y. and D.E.A. performed research; X.Y., D.E.A., P.G.C. and G.V.N. analyzed data; X.Y. and G.V.N. wrote the paper. All authors discussed the results and commented on the paper.

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