Microstructural heterogeneity directs micromechanics and mechanobiology in native and engineered fibrocartilage

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Treatment strategies to address pathologies of fibrocartilaginous tissue are in part limited by an incomplete understanding of structure–function relationships in these load-bearing tissues. There is therefore a pressing need to develop micro-engineered tissue platforms that can recreate the highly inhomogeneous tissue microstructures that are known to influence mechanotransductive processes in normal and diseased tissue. Here, we report the quantification of proteoglycan-rich microdomains in developing, ageing and diseased fibrocartilaginous tissues, and the impact of these microdomains on endogenous cell responses to physiologic deformation within a native-tissue context. We also developed a method to generate heterogeneous tissue-engineered constructs (hetTECs) with non-fibrous proteoglycan-rich microdomains engineered into the fibrous structure, and show that these hetTECs match the microstructural, micromechanical and mechanobiological benchmarks of native tissue. Our tissue-engineered platform should facilitate the study of the mechanobiology of developing, homeostatic, degenerating and regenerating fibrous tissues.

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Injury and degeneration of fibrocartilaginous tissues, such as the knee meniscus and the intervertebral disc annulus fibrosus, have significant consequences in terms of socioeconomic cost and quality of life1,2. Despite the importance of these tissues in the activities of daily living, their structure–function relationships across multiple length scales is poorly understood in developing, healthy and diseased states. Without this information, discovery and development of effective treatment strategies to address pathology has been hindered. Moreover, while there exist tissue-engineered platforms that can recapitulate various aspects of healthy native-tissue structure and function3–7, these do not generally address emergent tissue pathology or its consequences on tissue structure, mechanical properties and biology. To that end, we set out to probe native-tissue multi-scale structure–function relationships and to develop micro-engineered platforms to advance our understanding of tissue development, homeostasis, degeneration and regeneration in a more controlled manner.

Micro-engineered platforms that include pathological features would enable the precise control of the biochemical, structural and mechanical properties of the cellular microenvironment. However, a limiting factor in designing such platforms is our incomplete understanding of the multi-scale structure–function relationships of native fibrocartilages. For example, mechanical strain transfer from the tissue to cellular level is highly non-uniform in these tissues8–11, yet the mechanisms responsible for this inhomogeneity have not been identified. Indeed, while numerous biomechanical investigations have addressed tissue-level structure–function relationships using idealized schematic representations of highly ordered collagen structure12–14, recent evidence suggests that the microstructure of many types of fibrocartilage is inhomogeneous. This inhomogeneity is characterized by aligned fibrous microdomains (FmDs) containing fibre interruptions and junctions with non-fibrous proteoglycan-rich microdomains (PGmDs) that are interspersed throughout the FmDs (refs 8,9,15). While it is generally thought that such microstructural features regulate tissue-to-cell mechanical signal transfer, and thereby alter the in situ mechanobiologic response (that is, early calcium signalling and eventual gene expression)9,13,15–17, this has not been experimentally evaluated. Given this emerging appreciation of the importance of multi-scale structure and its contribution to mechanics and biology in native fibrocartilage, it is imperative to further develop this area and inform the design of tunable micro-engineered platforms for studying context-dependent mechanotransduction of tissue physiology and pathology.

In the present work, we quantified the prevalence of PGmDs in developing and ageing fibrocartilaginous tissues and evaluated how cells within distinct microdomains of the tissue respond to physiologic deformation. In doing so, we demonstrated that the immediate intracellular calcium response to external mechanical perturbation in PGmDs and FmDs is distinct and context dependent. Next, we developed an approach to generate heterogeneous tissue-engineered constructs (hetTECs) containing ‘engineered-in’ PGmDs within an otherwise FmD structure and demonstrated that these tissue analogues could match the microstructural, micromechanical and mechanobiological benchmarks established by the native tissues. Collectively, these findings establish the emergent multi-scale structure of native fibrocartilage and its impact on micromechanics and mechanobiology, and provide a highly controlled micro-engineered platform in which to study the mechanobiology of developing, homeostatic, regenerating and degenerating fibrocartilaginous tissues and to develop therapeutics to restore function.

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Prevalence of PGmDs in the fibrocartilage microstructure

To quantify how the number and size of PGmDs change with development and ageing, we examined Picrosirius red- and Alcian blue-stained outer meniscus tissue sections obtained from fetal (late trimester), juvenile (3–5 months) and adult (1–3 years) bovine animals, as well as menisci obtained from human donors (21–65 years) of varying body mass index (BMI; 12.4–64.4 kg m$^{-2}$).

PGmDs, defined by localized Alcian blue-stained inclusion $>10,000\,\mu$m$^3$, were present in the bovine tissues of all age groups (Fig. 1a–c) and in most of the human tissues (Fig. 1d). Second-harmonic generation (SHG) imaging illustrated a near lack of organized collagen fibres within the PGmDs, with strong alignment and organization in the surrounding FmD regions (Fig. 1e). The number of PGmDs (per mm$^2$) significantly increased from fetal to juvenile bovine groups, with no further change observed between juvenile and adult (Fig. 1f). The area of PGmDs progressively increased with development and ageing in these bovine tissues, with the PGmD area in the adult menisci reaching levels that were significantly larger than all other groups (Fig. 1g).

Regardless of whether these emergent microdomains represent a normal part of ageing or a pathologic state in fibrocartilages, we postulate that an optimal PGmD number and size may exist to support mechanical compression in the normal fibrocartilages, and that these inclusions further accumulate and grow in size with the onset of pathology. Indeed, PGmDs are present in the healthy fibrocartilage-rich, wrap-around tendons (that support both tensile and compressive loading), but when these tissues are translocated to bear only tension in vivo, PGmD area decreases significantly$^{44}$. Furthermore, PGmDs accumulate to an excessive degree in tendinopathic tissue and compromise tendon mechanical function$^{19-22}$. This suggests that PGmDs may form as a consequence of development and mechanical adaptation in normal tissues, but also can undergo maladaptive remodelling and further accumulate and grow in size with the onset of pathology.

| No. of PGmDs (per mm$^2$) | PGmD area ($\times 10^4\,\mu$m$^2$) | Human | Juvenile | Fetal | Adult |
|---------------------------|-------------------------------|-------|---------|-------|-------|
| 0.13                      | $p^* < 0.0006$                |       |         |       |       |
| $< 0.0006$                |                               |       |         |       |       |
| $< 0.0006$                |                               |       |         |       |       |
| $< 0.0006$                |                               |       |         |       |       |

**Strain transfer in native fibrocartilage micromechanics**

Regardless of whether these emergent microdomains represent a homeostatic or diseased state, they most certainly create abrupt changes in structure that influence macro-to-micro scale strain transfer in the tissue. To investigate this, we performed uniaxial tensile tests on fresh fetal, juvenile and adult bovine outer meniscus specimens using a custom confocal-mounted microtensile device$^{46-48}$ that enabled simultaneous tracking of endogenous cells (Fig. 2a,b). The presence of PGmDs within analysed regions was confirmed after testing via histological indexing to the region of interest (Fig. 2c).

Strain analysis showed that while the strain fields within the extracellular matrix (ECM) were heterogeneous (Supplementary Fig. 2a–d), average strain transfer from the tissue to ECM level was linearly correlated in all age groups (Fig. 2d–g and Supplementary Fig. 2e). In fetal tissues, where smaller PGmDs were
Figure 2 | PGmDs attenuate local strain transmission and cell deformation in fibrocartilage. a, Schema of the mechanical test. Tissue samples were stretched uniaxially while simultaneously monitoring the position and size of cells and nuclei in situ via confocal microscopy. b, Lagrangian strain at the tissue and local ECM length scale was computed by tracking centroids of surface markers and cell nuclei, respectively. Cell strain was calculated by measuring the change in the long axis of individual cells. c, Histology (Alcian blue and Picrosirius red) showing the presence of PGmDs within an imaged region and a micrograph of cell nuclei in both PGmD and FmD. Scale bar, 250 μm. d-g, Strain transfer from the tissue to ECM (FmD and PGmD) in bovine fetal (d), juvenile (e), adult (f), and non-ovine human (g) outer meniscus; PGmD regions showed attenuated strain transfer. *, p < 0.05 versus FmD via extra-sum-of-squares F-test; m, slope of linear regression (average strain transfer ratio). Dashed line represents 1:1 relationship. n = 10–20 triads for each FmD and PGmD. h, Finite-element-simulated longitudinal strain map of untreated and ChABC-treated specimens at 10% applied strain. Arrow indicates PGmD. i, PGmD/FmD strain ratio from experimental data and finite-element (FE) simulations of untreated and ChABC-treated specimens. j, Digestion of proteoglycans (via ChABC treatment) from PGmDs resulted in a partial recovery of strain transfer in juvenile meniscus specimens. *, p < 0.05 versus FmD and #, p < 0.05 versus PGmD via extra-sum-of-squares F-test. n = 21 triads for ChABC treated. All data are presented as mean ± s.e.m.
Figure 3 | PGmDs alter local cell mechnano-response to applied tissue-level mechanical deformation. a, Representative time-series confocal snapshots at 0, 60 and 324 s, and corresponding histology (Alcian blue and Picrosirius red) for a juvenile meniscus tissue. Cells in FmDs respond to 3% strain with an increase in the number of calcium oscillations over time, as indicated by the yellow arrowheads. Conversely, cells in PGmDs do not respond to stretch, but have an elevated baseline calcium signal. White outline indicates PGmD area. Scale bar, 250 μm. b, Representative fluorescence intensity traces for cells in FmDs and PGmDs after stretch. c, Percentage of responding cells at baseline in the FmD and PGmD regions. #, p < 0.0001 versus FmD via Student’s t-test (two-tailed and unpaired). n = 21 and 23 for FmD and PGmD, respectively. d, Change in the percentage of responding cells with applied stretch relative to baseline levels. n = 5–7 per strain group. One-way ANOVA (p < 0.05) with Tukey’s post hoc. e, Percentage of responding cells (from Fig. 3d) plotted against measured ECM strain, showing relative strain dependence. All data in a–e are presented for juvenile meniscus. All data are presented as mean ± s.e.m.

Calcium signalling in native fibrocartilage mechanobiology

To assess the effect of tissue micro-scale heterogeneity on mechanobiology, we next examined how strain at the ECM level regulates intracellular calcium signalling in cells within FmDs and PGmDs in response to tissue-level tensile strain. Cells within PGmDs had an elevated baseline signal intensity compared with those within FmDs (Fig. 3a,b). Furthermore, the PGmD resident cells had a lower frequency of spontaneous calcium oscillations (<5%) compared with FmDs (~20%) before strain and were much less responsive to applied strain compared with cells in FmDs (Fig. 3c,d and Supplementary Fig. 5). Application of tissue-level strain (6%) increased the peak amplitude of calcium transients in FmD cells, but did not change the duration or frequency of their oscillations, consistent with our previous findings18 (Supplementary Fig. 6a–d). Moreover, the percentage of FmD cells showing calcium oscillations progressively increased in response to the level of applied strain, with significantly more cells responding at 6% and 9% strain compared with 0 and 3% (Fig. 3d). In contrast, application of tissue-level strain had no effect on cells in the PGmDs in terms of the peak of cells responding (Fig. 3d). This is likely due to the strain shielding that occurs within the PGmDs, where the ECM...
Fabrication of heterogeneous tissue-engineered constructs

The above findings indicate that fibrous tissues contain microdomains whose composition, mechanical properties and mechanosensitivity differ from the bulk ECM of these fibrous tissues. While it is not clear whether these microdomains represent a homeostatic response to mechanical demands or a pathologic consequence of ageing and/or disease, they certainly play a major role...

strain was less than 3% when 9% strain was applied to the tissue (Figs 2e and 3e). Indeed, the change in the percentage of responding cells was less than 5% from baseline for both FmD and PGmD cells when the local matrix strain was under 3% (Fig. 3e). These results suggest that attenuation of externally applied strain within PGmDs has a profound effect on the calcium responsivity of cells located within these microdomains.

Fabrication of heterogeneous tissue-engineered constructs

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in tissue physiology. To address this in a more tractable format, we developed hetTECs to mimic the microstructural, micromechanical and mechanobiological factors observed in native fibrocartilage.

To fabricate hetTECs, menenchymal stem cell (MSC) micro-pellets and meniscus fibrochondrocytes (MFCs) were seeded onto aligned electrospun nonfibrous poly-e-caprolactone (PCL) scaffolds.\(^2, 28\) (Fig. 4a). On day 3, an additional sheet of scaffold was layered on top of the construct (Fig. 4a), and constructs were maintained in a chemically defined medium containing TGF-β3 for up to 8 weeks. Visualization of the cells on day 3 confirmed that MSC micro-pellets (red/orange) were well dispersed within the proliferating MFC scaffolds (green) attached to the scaffold (Fig. 4b). Histological analyses after 1 week revealed that MSC micro-pellets deposited proteoglycans, forming PGmDs, while MFCs deposited collagen in the vicinity of the PGmD (Fig. 4c). By 4 weeks, MFCs had continued to lay down a collagen-rich matrix (Fig. 4c). By 8 weeks, the size of PGmDs significantly increased, to the level of the juvenile meniscus, and these domains had become more fully ensheathed within the surrounding collagenous matrix (Fig. 4c,d). HetTECs containing only FmD and both FmDs and PGmDs accumulated increasing proteoglycan content with time (Supplementary Fig. 7a). Similar trends were observed for total collagen content (Supplementary Fig. 7b), with constructs containing PGmDs alone producing the least amount of collagen. Immunostaining showed that type II collagen was strongly localized within the PGmDs by week 8 (Fig. 4e), while type I collagen was uniformly dispersed within the surrounding FmD (Fig. 4f). Occasionally, PGmDs showed cellular outgrowths rich in type II collagen (red arrow on Fig. 4e) that connected neighbouring PGmDs over time (Supplementary Fig. 8). Despite the density of type II collagen within the engineered PGmDs, this collagenous architecture was less dense and less ordered than was observed in the surrounding FmDs at week 4 and 8 (Supplementary Fig. 9), similar to that observed in native tissue (Fig. 1e). These differences in collagen type and structure had profound effects on the cell nucleus morphology, with nuclei in FmDs being highly aligned and elongated in the fibre direction, while the nuclei of cells in PGmDs were smaller, rounder and randomly oriented (Supplementary Fig. 1a–c).

To determine the molecular identity of cells located in the FmD and PGmD, quantitative RNA fluorescent in situ hybridization (FISH) was used to identify individual aggrecan (AGG) and GAPDH messenger RNA at the single-cell level.\(^2, 29, 30\) The AGG/GAPDH mRNA ratio was significantly greater in the cells in the PGmD compared with cells in the FmDs (Fig. 4g,h). Here, it is important to note that while PGmDs exhibit an increased AGG/GAPDH ratio, the overall proteoglycan content does not increase over time (Supplementary Fig. 7) because the engineered PGmDs constitute only a small fraction of the entire hetTECs.

Bulk construct mechanics were measured in tension at week 8. HetTECs with PGmDs alone did not alter the construct Young’s modulus in comparison to unseeded PCL scaffolds (Fig. 4i). Conversely, seeding of MFCs to produce the FmD significantly increased the Young’s modulus (Fig. 4i), suggesting that the type I collagen-rich matrix deposited by MFCs is essential for establishing the macro-scale tensile function of the hetTECs. The tensile modulus of hetTECs containing only FmD was similar to those including both FmDs and PGmDs, suggesting that the PGmDs did not compromise the bulk tissue function, consistent with native tissue.

### HetTEC micromechanics and mechanobiology

To determine how tissue to ECM-level strain transfer in hetTECs is compared with the native tissue, we performed uniaxial tension testing on hetTECs while simultaneously tracking DAPI-stained nuclei. With strain, hetTECs containing PGmDs showed highly heterogeneous strain fields, where the strain magnitude within the PGmD was considerably lower than that of the surrounding FmD (Fig. 5a). Consistent with our measures of native tissue, quantification of strain transfer in the FmD was direct (80–90% strain transfer ratio), while in the engineered PGmDs strain transfer was significantly attenuated (0–15% strain transfer ratio) at both 4 and 8 weeks (Fig. 5b and Supplementary Fig. 11). Similar trends were observed in hetTECs containing only PGmD or FmD regions (Fig. 5b and Supplementary Fig. 11). These results demonstrate that hetTECs with engineered-in PGmDs recapitulate the heterogeneous strain transfer to the micro-scale level that was observed in both human and bovine juvenile and adult tissues.

To further probe the recapitulation of native-tissue functionality in engineered hetTECs, we next monitored intracellular calcium signalling in response to applied strain. At 0% strain, cells within FmDs showed spontaneous calcium oscillations and cells within the PGmDs showed elevated baseline intensity (Fig. 5c and Supplementary Fig. 12a,b). Application of 6% strain caused a greater number of FmD cells to respond compared with cells within PGmDs, which were largely unresponsive to applied strain (Fig. 5c,d). Furthermore, the relative baseline calcium signalling intensities measured in hetTECs also mimicked the calcium signalling intensities measured in native tissues, where cells within PGmD exhibited higher baseline intensities than the cells within FmDs (Figs 3b and 5c). These findings demonstrate that the mechanobiologic response (intracellular calcium signalling) of cells in hetTECs mirrors (and is perhaps even more sensitive than; Supplementary Fig. 12c–f) that seen in cells within the juvenile native meniscus microdomains.

### Outlook

While this work quantified the prevalence of PGmDs in fibrocartilage during maturation and ageing and established their micromechanical and mechanobiologic roles in native tissue, one central question that remains unanswered is whether the PGmDs are beneficial or detrimental to tissue health and function. During development of fibrocartilaginous tissues, PGmDs do not play an important and beneficial role. Indeed, there may be an optimal number and size of PGmDs in healthy tissue, but PGmDs may also accumulate to a pathologic degree with disease progression or in response to injury. For instance, PGmDs are present in healthy wrap-around tendons\(^19\) (where they help the tissue resist lateral compression) but also over-accumulate in the tendinopathic tissues\(^15, 23, 31\) (where they compromise tensile load-bearing capacity). Interestingly, within injured tendons, tendon-derived progenitor cells participate in regeneration of the FmD regions, but can also aberrantly undergo chondrogenesis\(^32\), resulting in a pathologic level of PGmDs in the repairing tissue. This may suggest that the presence and accumulation of PGmDs during ageing could be a consequence of aberrant differentiation of endogenous stem and progenitor cells, perhaps as a result of micro-scale damage and altered local microenvironments within the otherwise ordered FmDs. Answering such questions may ultimately aid in the development of effective therapeutic strategies to modulate and maintain the optimal level of the PGmDs in ageing fibrocartilaginous tissues and restore such optimal levels in disease or following injury.

Answering such questions in native tissues is difficult, however, given their complexity and the scarcity of tissues with controllable levels of pathology. To develop a more tractable and defined framework in which to study the impact of PGmDs, we engineered hetTECs inclusive of PGmDs, producing constructs that matched the microstructural, micromechanical and mechanobiologic benchmarks of native tissue (Fig. 6). These hetTECs may serve as an in vitro platform in which to study the mechanobiology of developing and pathologic tissues in a more controllable fashion, and to develop therapeutic strategies to halt or reverse the...
Figure 5 | HetTECs reproduce native-tissue domain-dependent strain transfer characteristics and mechano-response. a, Colour maps of local ECM strain (\(E_{xx}\); loading and fibre direction) for hetTECs matured for 8 weeks with application of 0, 3, 9 and 15% strain. Red dashed outline indicates the location of a PGmD within the hetTEC. b, Quantification of local ECM strain within FmD and PGmD regions of hetTEC at week 8. PGmD only: construct containing only PGmD/MSC-micro-pellets; FmD only: construct containing only FmD/seeded MFCs; PGmD/FmD: construct containing both PGmD and FmD components. 

\[ p < 0.0001 \] versus FmD via extra-sum-of-squares \(F\)-test; 
\[ p < 0.0001 \] versus PGmD/FmD (FmD) via extra-sum-of-squares \(F\)-test; 
\( m \), slope of linear regression (average strain transfer ratio). Dashed line represents 1:1 relationship. 

\( n = 35–50 \) triads per group.

c, Representative calcium response for hetTEC cells in FmDs and PGmDs over 600 s before or after the application of 6% strain. As in native tissue, cells in PGmDs do not respond to strain and have a higher baseline calcium level.

d, Percentage increase in responding cells in hetTEC FmD and PGmD regions with application of 6% strain. 

\[ p = 0.04 \] versus FmD via Student’s \(t\)-test (two-tailed and unpaired). 

\( n = 3 \) per group. All data are presented as mean ± s.e.m.

Figure 6 | a–c, HetTECs match the microstructural (a), micromechanical (b) and mechanobiological (c) benchmarks established by heterogeneous native fibrocartilages. Rectangle in a indicates range of PGmD area in native tissues. Data are presented as mean ± s.d. Red and blue shaded areas in b indicate 99% confidence interval of linear regression of FmD and PGmD ECM native-tissue strain. Red and blue symbols show response in hetTEC microdomains after 8 weeks of culture. Data are presented as mean ± s.e.m. Blue and red traces in c show calcium signalling in PGmD and FmD regions of native and engineered hetTECs in response to applied strain. All native tissue shown are from juvenile bovine meniscus.

Q.13 For example, hetTECs could be used to identify the PGmD area fraction in tissue at which tissue-level mechanical properties are compromised. Additionally, by probing the time course over which PGmDs grow in conditions of physiologic and altered loading (for example, partial transection, mechanical overload, fatigue), hetTECs may be used to determine progression of tissue degeneration.
whether the PGmDs develop as a consequence of mechanical adaptation or pathologic consequences of ageing, degeneration and/or injury.

While the current study focused solely on fibrocartilages, tissue microstructure and its influence on micromechanics and mechanobiology and the hetTEC construction algorithms that create micro-engineered heterogeneous features can be extended to other types of dense tissue that contain structural microdomains or develop them in response to damage or disease. Muscle, cardiovascular tissue, skin and neural tissue will benefit from these native tissue and hetTEC approaches applied to homeostasis and pathology. Together, these findings demonstrate the impact of emergent heterogeneous micro-scale tissue features on normal tissue function, and validate a method to micro-engineer heterogeneous tissue constructs containing these complex features to address and correct tissue pathology.

Methods

Methods and any associated references are available in the online version of the paper.

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Author contributions

W.M.H., S.J.H., T.P.D., J.F.D., C.M.M., L.J.S., R.L.D., R.L.M. and D.M.E. designed the final submission. W.M.H., S.-J.H., T.P.D., J.F.D., J.M.M., L.J.S., R.L.D., R.L.M. and D.M.E. performed the experiments. W.M.H., S.-J.H., T.P.D., J.F.D., C.M.M., L.J.S., R.L.D., R.L.M. and D.M.E. analysed and interpreted the data. W.M.H., S.J.H., R.L.M. and D.M.E. drafted the manuscript, and all authors edited the final submission.

Additional information

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Competing financial interests

The authors declare no competing financial interests.
Histological analysis of hetTECs. For histological analysis, constructs (at week 4 and 8) were fixed with 4% paraformaldehyde for 30 min at 37°C, and embedded in Cryoprep frozen section embedding medium (Optimal Cutting Temperature Compound, Fisher Scientific), frozen at −80°C, and cut into 6-µm-thick sections in the plane of the forming tissue. Sections were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. Primary antibody dilutions (anti-TGFB3 antibody; R302006, EMD Millipore) were incubated on sections for 1 h at room temperature. Signal was visualized using DAB chromagen reagent (DAB150 IHC Select, EMD Millipore) according to the manufacturer’s protocol. Stained sections were visualized and imaged using a bright-field microscope (Nikon Eclipse TS 100).

Quantitative RNA fluorescent in situ hybridization analysis. For RNA FISH, hetTECs were embedded in Cryoprep frozen section embedding medium (Optimal Cutting Temperature Compound, Fisher Scientific), frozen at −80°C, and cut into 6-µm-thick sections in the plane of the forming tissue. Sections were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. Primary antibody dilutions (anti-TGFB3 antibody; R302006, EMD Millipore) were incubated on sections for 1 h at room temperature. Signal was visualized using DAB chromagen reagent (DAB150 IHC Select, EMD Millipore) according to the manufacturer’s protocol. Stained sections were visualized and imaged using a bright-field microscope (Nikon Eclipse TS 100).

Biochemical composition of native tissues and hetTECs. Fresh (untreated), chondroitinas A/C (ChABC)-treated (1 M A and C; 4 h on a shaker at 37°C), and ChABC Tris buffer (50 mM Tris and 60 mM sodium acetate; 4 h on a shaker at 37°C)–treated bovine juvenile meniscal tissues were dehydrated in an oven at 60°C overnight and the dry weight was measured (n = 8 for each untreated and ChABC Tris buffer group). hetTECs (n = 5 for PGMD only, FmD only, and PGMD/FmD groups) were weighed and digested in proteinase K (0.1135:28:9001, Roche) at 60°C overnight. Digestate from hetTECs was hydrolysed overnight in 6 M HCl at 110°C. Total glycosaminoglycan (GAG) content was determined using the 1,9-dimethylhemoine blue assay76. GAG contents of native tissues and hetTECs were normalized to the sample dry and wet weights, respectively. Collagen content of hetTECs was determined using the ortho-hydroxyprobe assay, assuming a ratio of ortho-hydroxyprobe to collagen of 1:7.14 (ref. 37). Collagen content of hetTECs was normalized by the sample wet weight.

Mechanical evaluation of hetTECs. The Young’s modulus of intact hetTECs was evaluated at 8 weeks via uniaxial tensile testing (n = 4 for PGMD only, FmD only, and PGMD/FmD groups) using an Instron 5848 electromagnetic testing system equipped with a 100 N load cell (Instron). Cross-sectional area of each scaffold was measured using a custom non-contacting laser-based system4. Gauge length of the sample was determined using a digital calliper. Samples were pre-conditioned with 10 rotations of sinusoidal loading to 0.1 N at a frequency of 0.1 Hz. Samples were then ramped to failure at a strain rate of 0.1%/s. Tensile modulus was calculated by performing linear regression to the linear region of the stress–strain plot.

Sample preparation for micromechanical testing. Bovine meniscal tissues were obtained from fetal (n = 10), juvenile (n = 9) and adult (n = 8) knee joints within 12 h of euthanization using ImageJ (National Institutes of Health). For immunohistochemical detection of type I and II collagen, samples were incubated in proteinate K (S3020, Dako) for 4 min, followed by blocking with 10% normal goat serum (no. 00862Z, Life Technologies) for 30 min at room temperature. Primary antibody dilutions (Type I collagen: MAB3391, EMD Millipore; Type II collagen: 11-116B3, Developmental Studies Hybridoma Bank) were applied at a concentration of 10 µg ml⁻¹ overnight at +4°C, followed by washing and incubation with secondary antibody (DAB150 IHC Select; EMD Millipore) for 1 h at room temperature. Signal was visualized using DAB chromagen reagent (DAB150 IHC Select, EMD Millipore) according to the manufacturer’s protocol. Stained sections were visualized and imaged using a bright-field microscope (Nikon Eclipse TS 100).

Methods

Polymerization of aligned nanofibrous scaffolds. Aligned nanofibrous materials, poly-(ε-caprolactone) (PCL, mol. wt 80 kDa, Shenzhen Bright China Industrial) and a material of polylactic-co-glycolic acid (PLGA) 85:15, loaded into a 1:1 mixture of tetrahydrofuran and N,N-dimethylformamide (Fishier Scientific), were loaded into a syringe, and extruded at 2.5 ml h⁻¹ through an 18 G stainless-steel spinneret charged to create a voltage gradient of +1 kV cm⁻¹ over an air gap of 13 cm. Electrospinning jets were collected onto a cylindrical mandrel rotating at a surface velocity of 10 m s⁻¹ to direct alignment of collected fibres. PCL scaffolds (10 × 65 × 0.3 mm) were hydrated and sterilized in decreasing concentrations of ethanol (100, 70, 50, 30% ; 30 min per step). To enhance cell attachment, scaffolds were incubated in a solution of human fibronectin (20 µg ml⁻¹ in PBS, F4759, Sigma–Aldrich) for 12 h before cell seeding.

Fabrication of heterogeneous tissue-engineered constructs. To form engineered meniscal fibrochondrocytes (MFCs) were isolated from juvenile bovine outer menisci (3–6 months, Research 87; ref. 11) were sectioned into 1 mm³ cubes and MFCs were allowed to migrate out of the tissue over 1–2 weeks, after which they were expanded through passage 2 in a basal medium consisting of high-glucose DMEM containing penicillin/streptomycin/fungizone and 10% fetal bovine serum (FBS). MSCs and MFCs were isolated from the same donor. For each individual MFC population, MSCs were labelled with Cell Tracker red (Molecular Probes) to enable visualization with extended culture and were formed into micro-pellets (5,000 cells per pellet) by centrifugation at 300g for 5 min. Micro-pellets were cultured for one week in a chemically defined serum-free chondrogenic medium (high-glucose DMEM with 1× penicillin/streptomycin/fungizone, 0.1 µM dexamethasone, 50 µg ml⁻¹ ascorbate 2-phosphate, 40 µg ml⁻¹ L-proline, 100 µg ml⁻¹ sodium pyruvate, 100 µg ml⁻¹ L-glutamine, 5.25 µg ml⁻¹ insulin, 6.25 µg ml⁻¹ transferrin, 6.25 µg ml⁻¹ selenious acid, 1.25 mg ml⁻¹ bovine serum albumin and 5.35 µg ml⁻¹ linoleic acid) with 10 ng ml⁻¹ TGFβ3 to induce chondrogenesis (including production of proteoglycans)20. Meniscus fibrochondrocytes (MFCs) were isolated from juvenile bovine outer menisci (3–6 months, Research 87; ref. 35). Menisci were sectioned into 1 mm³ cubes and MFCs were allowed to migrate out of the tissue over 1–2 weeks, after which they were expanded through passage 2 in a basal medium consisting of high-glucose DMEM containing penicillin/streptomycin/fungizone and 10% fetal bovine serum (FBS). MSCs and MFCs were isolated from same donors. MFCs were labelled with Cell Tracker Green (CellTracker Green CMFDA, C7025, Molecular Probe) before seeding onto scaffolds.

Preparation of native tissues for PGMD quantification. Bovine menisci were obtained from fetal (late trimester; n = 12; Animal Technologies), juvenile (3–6 months; n = 11; Green Village Packing), and adult (3–5 years; n = 16; Green Village Packing) knee joints within 12 h of euthanization. Human menisci (n = 11; donor ages 21–65 years) were procured from a commercial and IRB-approved source (National Disease Research Interchange (NDRI)). Body mass index (BMI) for human donors was calculated on the basis of height and weight information provided by the vendor. The vendor provided medical history on each donor and indicated whether the donors had been previously diagnosed with knee osteoarthritis. Rectangular tissue samples were dissected from the outer central (body) region of each meniscus using a scalpel, in the axial plane. Dissected samples were fixed in 10% buffered formalin for 1 week at room temperature. Fixed samples were then paraffin-processed and stored in 70% ethanol (at 4°C overnight) using an epi-fluorescent microscope (Nikon Eclipse TE2000-U). For constructs intended for long-term culture, fresh scaffolds were layered on top of the original seeded scaffold, creating an internal space for organized tissue development. This entire construct was cultured in chemically defined media with TGF-β3, with care taken to not disturb the construct over the first three days. At this time point, to visualize micro-pellets and MFCs, a set of hetTECs were fixed with 4% paraformaldehyde for 20 min at 37°C, and imaged using an epi-fluorescent microscope (Nikon Eclipse TS2000-U). For constructs intended for long-term culture, fresh scaffolds were layered on top of the original seeded scaffold, creating an internal space for organized tissue development. This entire construct was cultured in chemically defined media with TGF-β3 for up to 8 weeks.

Histological analysis of hetTECs. For histological analysis, constructs (at week 1, 4, 8) were fixed with 4% paraformaldehyde for 30 min at 37°C, and embedded in Cryoprep frozen section embedding medium (Optimal Cutting Temperature Compound, Fisher Scientific), frozen at −80°C, and cut into 6-µm-thick sections in the plane of the forming tissue. Sections were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. Primary antibody dilutions (anti-TGFB3 antibody; R302006, EMD Millipore) were incubated on sections for 1 h at room temperature. Signal was visualized using DAB chromagen reagent (DAB150 IHC Select, EMD Millipore) according to the manufacturer’s protocol. Stained sections were visualized and imaged using a bright-field microscope (Nikon Eclipse TS 100).
12 h of euthanization. Isolated meniscus tissues were maintained in culture medium ( Dulbecco’s modified Eagle medium, DMEM; Mediatech) with 1% penicillin/streptomycin (Life Technologies) for no longer than 3 h. Immediately before fluorescent staining and mechanical testing, test specimens were trimmed to 12.0 ± 3.0 × 0.3–0.8 mm (length × width × thickness) using a scalpel.

Human meniscus tissues (age 45–50; n = 3; previously frozen; remaining tissues from the PGMd quantification study above) were obtained from NDRI (as above) and prepared in a similar manner. Double-layered hetTECs (at week 4 and 8) were sectioned from the top surface of the construct using a freezing stage microtome (Leica SM2400, Leica Biosystems) until the FmDs and PGmDs were prepared in a similar manner. Double-layered hetTECs (at week 4 and 8) were obtained from NDRI (as above) before fluorescent staining and mechanical testing, test specimens were trimmed to 12.0 ± 3.0 × 0.3–0.8 mm (length × width × thickness) using a scalpel.

To probe the influence of proteoglycans within native tissue, additional bovine juvenile meniscus tissues were dissected, trimmed and treated with 1 U ml−1 chondroitinase ABC (ChABC; n = 5; Sigma-Aldrich) or with ChABC Tris buffer (n = 6; 50 mM Tris and 60 mM sodium acetate) for 4 h on a shaker at 37 °C before fluorescent staining and mechanical testing.

Before micromechanical testing, cells and nuclei in native-tissue samples were stained with 0.01 μg ml−1 FM4-64 (Life Technologies) and NucBlue Hoechst 33342 (Life Technologies) in 1 × PBS, respectively. For human tissues and ChABC- and ChABC Tris buffer-treated samples, cell nuclei were stained using 10 μM DRAQ-5 (Cell Signaling Technology) for 30 min. Samples were washed in 1 × PBS to remove excess dye. Subsequently, tissue markers were applied to the surface of each block. Native-tissue samples were processed with a Sharpie permanent marker. Cell nuclei within hetTECs were stained with 4′,6-diamidino-2-phenylindole (DAPI, ProLong Gold anti-fade reagent with DAPI, P36955, Molecular Probes) before micromechanical testing.

Micromechanical testing to quantify strain transfer. To investigate how the tissue-level mechanical strain was transferred to the FmD and PGmD regions, and subsequently to the cells and nuclei within each of these regions in the native tissue, all samples were examined using micromechanical testing. The samples were placed in grips with tissue markers facing up and a 30 mN preload was applied to remove tissue slack. Incremental grip strains of 3, 6, 9, 12 and 15% were applied at a strain rate of 0.1% s−1. A 30 mN preload was applied to remove tissue slack. Incremental grip strains of 3, 6, 9, 12 and 15% were applied at a strain rate of 0.1% s−1. Multichannel z-stack confocal images were acquired at 0% strain and 30 s after each strain increment using a water-immersion lens (field of view: 360 × 360 μm; HCx50: 405 mm; 420–480 nm; FM4-64: 515 nm/LP 650 nm; DRAQ-5: 633 nm/LP 650 nm). The same group of cells and nuclei was tracked with each level of applied strain. The position of tissue surface markers was captured using a CCD (charge-coupled device) camera concurrently with confocal imaging. All testing was performed in a DMEM bath at room temperature.

During confocal imaging, PGMds were preliminarily identified using previously determined characteristics of nuclear shape within PGMds (ref. 8). That is, nuclei within PGMds were rounder, with a nuclear aspect ratio <0.25, while nuclei of cells located within FmDs were elongated with a nuclear aspect ratio >2.15 (ref. 8). Given that the average fetal NAR (1.95 ± 0.64) was smaller than average NAR in juvenile and adult tissues (2.31 ± 1.05), an NAR cutoff of 1.7 was used for fetal specimens to bin cells as being in an FmD or PGMd. Subsequent to mechanical testing, the presence of actual PGMds within the imaged regions was confirmed via histology (staining of PGMd with Alcian blue). At both 4 and 8 weeks, hetTECs were tested in a similar manner using a custom device mounted on an inverted epi-fluorescent microscope (Nikon T30, Nikon Instruments)19. For these samples, 0% to 15% grip-to-grip strain was applied in 3% increments19 at each strain increment, images of cell nuclei were acquired using a CCD camera and local strain was calculated as described below.

Multi-scale strain calculation. Two-dimensional (2D) tissue strains were calculated from the centroids of the surface tissue markers. Similarly, 2D extracellular matrix (ECM) Lagrangian strains were calculated from the position of the centroid of nuclear triads at each strain level (n = 20, 10 triads for FmD and PGMd of fetal; n = 20 for each FmD and PGMd of juvenile; n = 20, 15 for FmD and PGMd of adult; n = 21 for FmD and PGMd of ChABC Tris buffer; n = 10 for each FmD and PGMd of Tris buffer; n = 7 for each FmD and PGMd of human; n = 35–50 each for FmD and PGMd in hetTECs). Cellular (n = 10 for fetal PG; n = 20 all other groups) and nuclear (n = 25 for juvenile FmD; n = 20 all other groups) strains were calculated using ImageJ (National Institutes of Health) by measuring the change in length of the cell long axis in the native fetal, juvenile and adult tissues as a function of applied strain.

Strain mapping. To confirm triad analysis, adult bovine mesenchymal tissues (n = 2) were prepared for mechanical testing as described above and analysed using texture correlation. Cell nuclei were stained using 10 μM DRAQ-5 (Cell Signaling Technology) for 30 min, and then washed in 1 × PBS. A 30 mN preload was applied after the sample was placed in the grips. Incremental strain rates of 1%, up to 4 or 9% were applied at a strain rate of 0.1% s−1. Z-stack confocal images were acquired at 0% strain and at each strain increment using a water-immersion 10× lens zoomed all the way out (field of view: 1,270.31 × 1,270.31 μm; DRAQ-5: 633 nm/LP 650 nm). At the end of testing, tissue auto-fluorescent images were acquired using a 405 nm laser at high power. Tested samples were fixed in 4% paraformaldehyde, cryo-sectioned, and stained with Alcian blue. Locations of the imaged regions were identified on histological slides by matching structural features between fluorescence auto-fluorescence and histology. To perform strain mapping, flattened the confocal z-stacks containing cell nuclei from each strain increment were analysed via texture correlation using VIC-2D (Correlated Solutions). For hetTECs, the same images used to calculate ECM strain from nuclear triads were also processed by strain mapping using VIC-2D.

Finite-element modelling. For finite-element analysis, a representative Alcian blue and Picrosiris red-stained histology section was used to generate a mesh of representative geometry consisting of a PGMd embedded in the FmD. The PGMd observed in the histology section was manually outlined and converted into a 2D finite-element mesh (FEbio; ref. 40) using a custom MATLAB program (Supplementary Fig. 13a). The FmD was modelled as a ‘Homes–Mow matrix reinforced with exponential power-law fibres using material properties taken from the previous work on fibrocartilage19. The PGMd was modelled as a ‘Homes–Mow matrix with a Donnan osmotic swelling parameter. Material properties for the PGMd were taken from the articular cartilage literature, where the mechanical behaviour of articular cartilage is dominated by the PG matrix and osmotic swelling. To simulate the physiologic interface between PGMd and FmD (as observed in the SHG image, Fig. 1e), a transition zone that separated the PGMd from the FmD was incorporated (Supplementary Fig. 13b). This zone was modelled as a ‘Homes–Mow matrix with a Poisson ratio equal to both PGMd and FmD. The transition zone modulus (E) and nonlinear stiffening term (β) were selected as the combination that best reproduced the experimental strain ratios (εFMd/εPGm) of the juvenile meniscus at 10% strain. To simulate the effect of GAG digestion via ChABC treatment, the PGMd fixed charge density was set to zero and the PGMd elastic modulus (E) was reduced by 98%, as described previously.22 The material properties for each region are summarized in Supplementary Table 2.

To monitor changes in intracellular calcium concentration in response to strain, tissue samples were gripped in the custom micromechanical test device mounted on a confocal microscope. A 30 mN preload was applied to remove tissue slack. Grip strains of 0 (n = 5 tissues), 3 (n = 6 tissues), 6 (n = 5 tissues) and 9% (n = 7 tissues) were applied at 0.1% s−1. For hetTECs, samples were stretched to grips strain of 6% at 0.1% s−1 (n = 3 per group). Before that (is baseline) and after strain application, time-series confocal images of the calcium signal was acquired every 4 s for 10 min using a 10× water-immersion lens (field of view: 900 × 900 μm2). The same groups of cells were tracked and imaged during and after strain. For hetTECs, samples were imaged in a plane located at 1/3 of the pellet height (determined from the z-stack), closest to the underlying scaffold surface (Supplementary Fig. 14). All samples were submerged in HBSS at room temperature throughout testing. For native tissues, the locations of putative PGMds were confirmed by histology as above.

Nuclear shape and calcium signalling analysis. ImageJ was used to determine the percentage of responding cells, nuclear area (n = 50 per group), and the nuclear aspect ratio (the ratio of the long axis to the short axis, n = 50 per group). A custom MATLAB program was used to quantify temporal characteristics (peak amplitudes, durations, number of peaks in 10 min, and time between peaks) of the calcium oscillations. For native tissues, the baseline percentage of responding cells was pooled from all groups at 0% strain in both FmD and PGMd regions.

Statistical analysis. For normally distributed data, Student’s t-test (two-tailed and unpaired), one-way ANOVA or two-way ANOVA was performed. For multiple comparisons, Tukey’s or Bonferroni post hoc tests were performed, as indicated in the figure captions. For data that did not fit a normal distribution, Mann–Whitney U or Kruskal–Wallis tests were performed. For non-parametric multiple comparison tests, a Bonferroni–Dunn’s post hoc test was performed. To compare strains across multiple length scales, linear regression was performed between Lagrangian tissue versus ECM strain, ECM strain versus cell strain, and cell strain versus nuclear strain for FmD and PGMd regions. The extra-sum-of-squares F-test was performed to compare the slopes of regression (that is, average strain transfer ratios). Significance was set at p < 0.05.

Code availability. Custom MATLAB programs used in this study are available on request.
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