Impaired Chondrogenesis Within Female Rat Osteochondral Defects Is Associated with Elevated Wnt, Pro-inflammatory Signaling

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INTRODUCTION: Articular cartilage has limited repair capacity following injury. Surgical techniques that activate endogenous chondroprogenitor cells, such as marrow stimulation, yield fibrocartilage that does not reproduce native articular cartilage and degenerates with time. The patient specific factors contributing to poor repair outcome require further study. Prior reports suggest that females have worse repair outcomes [1,2], but any sex disparity in repair tissue formation has not been established. Preclinical studies have not sufficiently addressed sex differences in cartilage repair: most used male subjects exclusively. When examining cartilage repair in both male and female Lewis rat littermates, we noted pronounced variability in early fibrocartilage deposition. A preliminary comparison suggested sex differences in repair quality. The goal of the present study was to rigorously compare early repair tissue composition and repair cell transcriptomes between young adult male and female rats receiving an osteochondral defect (OCD) that models marrow stimulation.

METHODS: Study design: Animal studies were conducted as pre-approved by the local Institutional Animal Care and Use Committee. Osteochondral defects (1 mm diameter, 1 mm deep) were made in each patellar groove of young adult (3 month old) male and female Lewis and Brown Norway rats (at least 8 per strain-sex combination). At 4 weeks post-injury (wpi) – when mesenchymal cell recruitment, expansion, and chondrogenic differentiation have taken place – repair tissue samples were collected for histology and gene expression by RT-qPCR or for transcriptomic analysis using single cell RNA sequencing (scRNA-seq). Outcome measures: Paraffin-embedded femora were sectioned in the transverse plane and stained with Safranin O/Fast Green or immunostained for collagen types I and II, SOX9, and Ki67. The area of Safranin O (red) staining and overall defect filling were quantified using ImageJ. Total RNA was isolated from bulk repair tissue collected with a 1.0 mm biopsy punch for RT-qPCR. Additional male and female Lewis rats (4 knees per sex) were included for scRNA-seq: 1.5 mm repair tissue cores (including adjacent articular cartilage) were collagenase digested and depleted of erythroid lineage cells using magnetic beads. The resulting pooled cell suspensions were used for library preparation using a 10X Genomics workflow and sequenced at a depth of >20,000 reads per cell. Differential gene expression with gene ontology (GO) enrichment analysis was performed between individual male and female clusters [3]. Statistical analysis: For histological and qPCR measures, male and female mean values were compared by Welch's t-test. For scRNA-seq, differential gene expression between sexes for each cell cluster was calculated using a standard negative binomial statistic.

RESULTS: Repair characterization: In males, 4 wpi repair consisted of fibrocartilage in the chondral phase that stained positive for Safranin O and was immunopositive for both SOX9 and type I collagen; hyaline cartilage undergoing active endochondral ossification was typically found in the subchondral phase. In stark contrast, female defects were filled with fibrotic tissue in the chondral phase that lacked proteoglycan staining, with reduced ossification in the subchondral phase (**Fig. 1B**). Defect filling was inferior in females (**Fig. 1A-B**). Consistent with histology, RT-qPCR demonstrated reduced expression of chondrogenic markers including Acan in females (**Fig 1C**). To determine whether trends were strain specific, we replicated the comparison in Brown Norway rats: similar deficiencies in filling and chondrogenic marker expression were observed in this distinct strain. scRNA-seq analysis: Nearly 75K high quality cells (38K male; 36K female) were sequenced from Lewis rat repair tissues. Seurat cluster analysis revealed an expected mixture of mesenchymal and hematopoietic lineages (**Fig. 2A**). Focusing on the mesenchymal cells, 9 distinct clusters were identified: vascular pericytes and endothelial cells, two fibroblastic clusters (one Prg4+, one Prg4-), two fibrochondrocyte clusters (Sox9+/Col1a1+), one osteoprogenitor cluster (Runx2+/Bglap+), and articular chondrocytes (Cytl1+/Cilp+) (**Fig. 2B**). The proportion of total mesenchymal cells was higher in the pooled male isolate, consistent with repair tissue histology (**Fig. 1B**). The proportion of fibrochondrocytes was reduced in the pooled female sample. Differential gene expression analysis revealed reduced chondrogenic (Sox9, Acan, Col2a1) but not osteogenic (Runx2) transcripts in the female fibrochondrocytes. These differences explain the downregulation of multiple chondrogenesis-related GO terms within female repair cells (**Fig. 2C**), which also displayed downregulation in gene sets related to energy metabolism, ECM synthesis, and proteostasis compared to the corresponding male clusters. In contrast, multiple female clusters were enriched for gene sets related to inflammation and canonical Wnt signaling. Elevated expression of Wnt response genes and cell proliferation markers were confirmed in female repair tissues.

DISCUSSION: In support of the current findings, Fitzgerald et al. reported that, while MRL/MpJ 'superhealer' mice displayed overall improved cartilage regeneration compared to the C57BL/6 strain, males displayed improved long term healing compared to females; in BL6 mice, however, both males and females showed poor healing [4]. In the present study, scRNA-seq not only supported histological observations of reduced chondrogenesis by female repair cells but also revealed elevated inflammatory and Wnt signaling for multiple repair populations within the lesion microenvironment. Both signaling pathways can inhibit chondrogenic differentiation [5] and represent possible therapeutic targets for overcoming sex-specific deficits in cartilage repair.

SIGNIFICANCE/CLINICAL RELEVANCE: Current articular cartilage repair strategies that activate endogenous chondroprogenitor cells fail to restore tissue function, prevent long term degeneration and the onset of osteoarthritis. The current findings demonstrate a sex-specific chondrogenic deficiency within early cartilage repair tissue as well as mechanistic targets for improving structural repair and overall outcomes in female patients.

REFERENCES: Faber et al. *Cartilage* (2021) 13: 837S.; [2] Pachowsky et al. *J Orthop Res* (2014) 32:1341.; [3] Childress et al. *Osteoarthritis and Cartilage Open* (2025) 7: 100620. [4] Fitzgerald et al. *Osteoarthritis and Cartilage* (2008) 16: 1319. [5] Cleary et al. *J Tissue Eng Regen Med* (2015) 9: 332.

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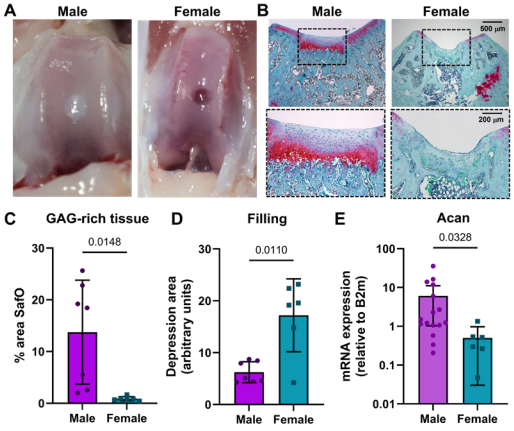

Figure 1. Female rats display inferior osteochondral defect repair characterized by poor chondrogenesis. (A) Representative images of repair tissues 4 weeks after generating a 1 mm osteochondral defect in the trochlear groove of age-matched male and female Lewis rats (3 mo at injury). (**B**) Safranin O / Fast Green staining for transverse sections at 4 weeks following injury. (**C-E**) Quantification of Safranin O-positive tissue area fraction (C) and defect filling (D) from histology as well as Acan transcript levels (plotted on log scale) in repair tissue (E). p values calculated using Welch's t-test.

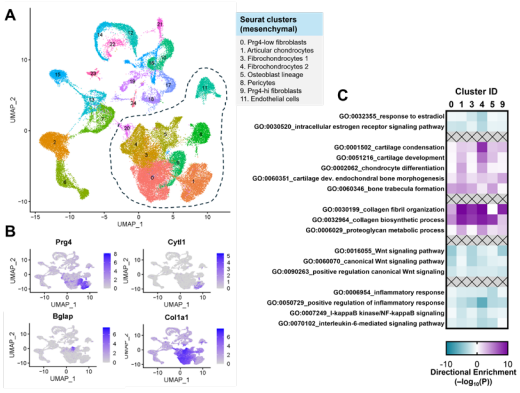

Figure 2. Differential gene expression analysis between chondrocytes in repair tissue versus intact articular cartilage. (**A**) Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) of cells isolated from male and female osteochondral repair tissue (plus surrounding articular cartilage) collected 4 weeks post injury. The total isolate (~75K) cells represents 4 pooled repair tissues per sex. The mesenchymal clusters are encircled with a dotted line, and annotated in the adjacent box. (**B**) Feature plots of select distinguishing mesenchymal marker genes. (**C**) Gene Ontology (GO) enrichment analysis for the major mesenchymal repair cells, with a focus on genes related to estrogen signaling, chondrogenesis, ECM production, Wnt signaling, and pro-inflammatory signaling. Gene sets relatively enriched in males are highlighted in violet, whereas those relatively enriched in females are highlighted in teal.