Micromechanics and Mechanoresponsivity of the Developing Porcine Meniscus

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Introduction

The meniscus is critical for knee joint stability and load distribution. Unfortunately, its limited endogenous cell-mediated repair capacity means that meniscus injuries often fail to heal in adults.1 Given the superior repair capacity of juvenile meniscus tissues, understanding their initial formation and specialization mechanisms during embryonic development may provide insights that could be harnessed for tissue regeneration in the adult. Our group previously examined the embryonic formation and postnatal maturation of the murine meniscus, revealing that its region-specific matrix composition and cellular phenotypes are established prenatally.[2-3] Furthermore, we showed that mechanical forces that arise from muscle loading and cellular contraction are essential for meniscus formation and specialization, while reduced postnatal weightbearing has little effect on meniscus morphology and micromechanics.[4,5] While these studies provide critical insight to the mechanoregulation of meniscus morphogenesis, translatability of the murine model is limited by its small size and mechanical and morphological distinctions relative to humans. To overcome these limitations, the goal of the present study was to establish a more translationally relevant model of meniscus development using the Yorkshire pig. Here, we established the timeline for key events in knee joint formation in early gestation (28-45 days after fertilization, E28-45) and evaluated matrix micromechanics and emergent mechanoresponsivity of meniscus cells in pigs from early gestation (E45), mid-gestation (E84), and newborn (P1) stages.

Methods

Timed Pregnancies and Embryo Collection

Adult female Yorkshire pigs were artificially inseminated at the National Swine Research Center (NSRRC, Columbia, MO). Pregnancy was confirmed via ultrasound, and sows were euthanized on embryonic day 45 or 84, or on postnatal day 1.

Micromechanics

Freshly dissected left knee joints were embedded in OCT and cryo-sectioned into 10µm coronal sections. AFM nanoindentation was performed in 1X PBS using polystyrene microspherical tips (Ø25µm, k~0.6N/m) at ≥10 different locations within the inner and outer meniscus per specimen. The effective indentation modulus (Eind) was calculated from the finite thickness-corrected Hertz model.[3]

Histology

Left hindlimbs were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned to 5µm (sagittal and coronal orientations). Cellularity and proteoglycan distribution were assessed from Safranin O/Fast Green staining.

Cell Isolation

Menisci from the right hindlimbs were identified and isolated under a dissecting microscope. At E84 and P0, menisci were segmented into inner and outer regions. All tissues were minced into ~1mm3 pieces and cultured in basal medium (DMEM, 10% FBS, 1% anti-anti) to allow for cell egress from the tissue fragments.

Mechanoresponse Assay

Isolated cells were cultured on fibronectin-coated polyacrylamide (PA) hydrogels (5 or 55kPa) or glass for one day in basal medium. Cells were fixed and stained for YAP (AF-488), actin (phalloidin AF-555), and nuclei (Hoechst 33342, excitation 350nm). Confocal z-stack images were obtained at 10X magnification and processed in Cell Profiler to quantify cell area and YAP localization.

Results

Histological assessments of hindlimb development across early gestation timepoints (E28-42) were used to establish the timing of knee joint formation in the Yorkshire pig. Skeletal rudiments were apparent at E28, as indicated by cartilaginous condensations at the prospective femur and tibia locations (Figure 1a).

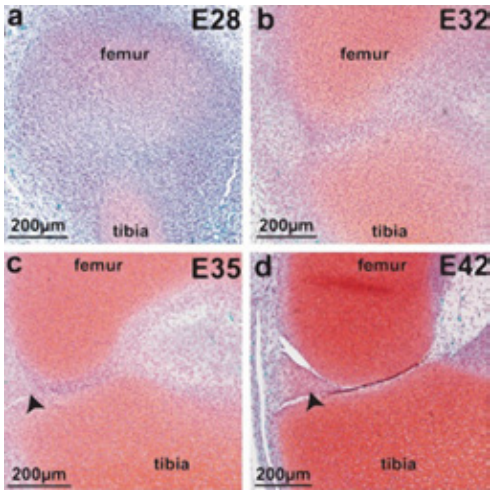

Figure 1. Knee joint formation during early embryonic development in the pig.

The future joint line was established by E32 (Figure 1b), and primitive menisci were visible, but not yet separated from the adjacent cartilaginous structures at E35 (Fig. 1c, arrowhead). The menisci were fully formed and separated from the articular cartilage at E42 (Figure 1d, arrowhead). Subsequent tissue and cellular analyses were performed from E45 onwards, at which point the menisci were readily isolated (Figure 2). Safranin O/fast green staining of menisci from these later timepoints showed that regional matrix specification, namely, proteoglycan enrichment within the inner meniscus, was evident in these tissues (Figure 3).

AFM nanoindentation revealed rapid stiffening of the meniscus primitive matrix throughout gestation (Figure 4) The microscale modulus of the outer zone was significantly greater than the inner zone at all three timepoints (#$p < 0.03$). In the inner meniscus, Eind increased from 3.5 ± 1.4 kPa (mean ± SD) at E42 to 18.2 ± 4.3 kPa at P1, while Eind of the outer meniscus increased from 6.5 ± 2.5 kPa to 26.1 ± 8.0 kPa. Together with our histological observations, these data indicate that the inner and outer meniscus develop distinct compositional and mechanical

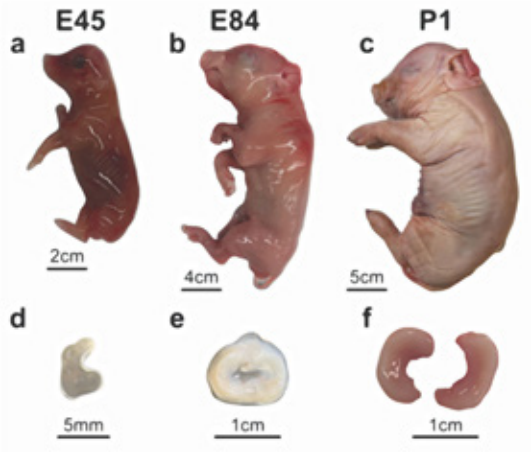

Figure 2. Gross anatomy **(A-C)** and isolated menisci **(D-F)** of embryonic newborn pigs.

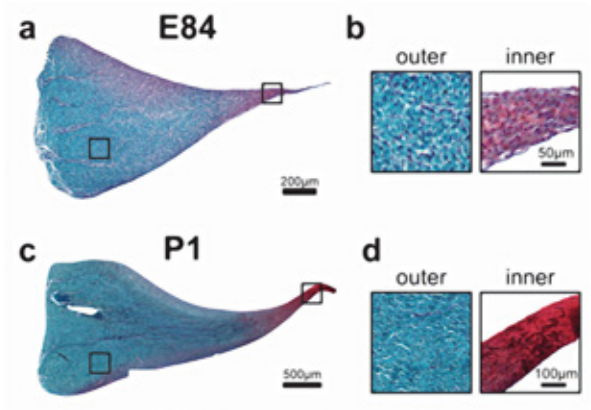

Figure 3. Regional specification is apparent in the developing pig meniscus.

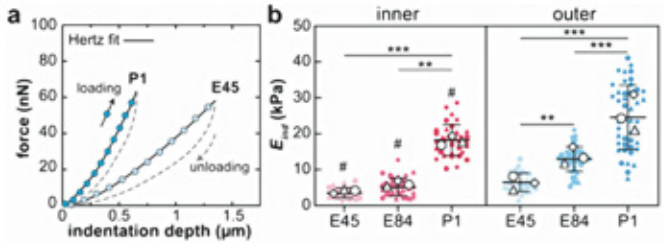

Figure 4. (A) Representative indentation force vs. depth curves for early embryonic and newborn outer meniscus; **(Bb)** Nanoindentation moduli (Eind) of inner (left) and outer (right) meniscus increase throughout gestation (**$p<0.001$, ***$p<0.0001$), and Eind of the inner region was less than that of the outer region (#$p<0.05$). For each animal ($n=3$), ≥10 indentation locations were tested in each region. White points represent the average Eind from one animal, and colored points of the same shape correspond to multiple indentations from the same animal.

microenvironments early in prenatal development that undergo continued specialization through the end of gestation, and these changes appear to initiate earlier in the outer zone.

Meniscus progenitor cells migrated from isolated meniscus segments onto tissue culture polystyrene over 5-7 days Figure 5). These cells were allowed to proliferate for an additional 7-10 days, then were passaged and seeded onto PA hydrogels or glass slides. Meniscus progenitors isolated at all three gestational timepoints exhibited increased mechanoactivation (i.e., greater cell area and increased YAP nuclear localization) with increasing substrate stiffness (Figure 5). Interestingly, P1 outer zone cells exhibited higher YAP nuclear localization than P1 inner zone or E42 cells, even on soft substrates.

Discussion

In the present work, we begin to establish a detailed, multiscale timeline of the coordinated morphological and mechanobiological changes that govern knee joint development in a translationally relevant porcine model. Consistent with prior investigations in the mouse, we show that meniscus formation and knee joint cavitation proceed rapidly following skeletal rudiment condensation and establishment of the interzone at the prospective joint

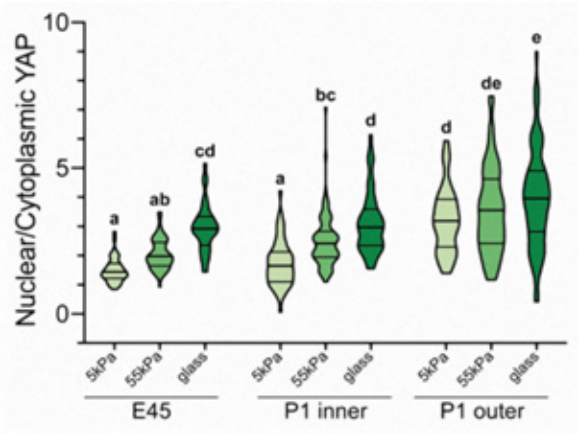

Figure 5. YAP nuclear: cytoplasmic ratio on substrates of different stiffnesses. Groups not sharing a common letter are significantly different ($p<0.05$).

line.[4,5] Histological and micromechanical assessments revealed that microenvironmental distinctions between the proteoglycan-rich inner meniscus and type I collagen-rich outer meniscus are apparent as early as E45 in the pig. At this point, embryos and hindlimbs are sufficiently large to allow meniscal tissue isolation with the aid of a dissection microscope. The differing regional matrix composition and micromechanics at this timepoint suggests resident cell identity and matrix synthesis are determined early in embryonic development. Furthermore, cells from E45 exhibited a characteristic mechanosensitive response (e.g., increased cell spreading as a function of substrate stiffness, indicating that resident meniscal cells are able to sense and respond to their mechanical microenvironment as soon as cavitation is complete. Interestingly, P1 outer zone cells showed a distinct mechanoresponse from P1 inner zone and E45 cells, wherein nuclear YAP localization was greater on all substrate stiffnesses, suggesting exposure to a stiffer microenvironment in vivo may enhance mechanosensitivity.

Future work will continue probing the phenotypic and mechanosensitive attributes of the developing meniscus, for example, by examining the heterogeneity of these cells and the matrix they synthesize via single cell RNAseq and immunohistochemistry, to establish the spatiotemporal characteristics of meniscus specialization throughout development. By uncovering the mechanisms by which emergent meniscus cells respond to biophysical cues to establish a mature, functional meniscus in the pig, we aim to shed light on mechanobiologic mechanisms that could be harnessed for injury repair and regeneration in adult fibrous tissues.

Acknowledgements

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