Morphogenesis and Mechano-Response of the Developing Porcine Meniscus

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INTRODUCTION: The meniscus plays a pivotal role in maintaining joint stability and distributing loads in the knee. However, injuries to the meniscus in adults pose a challenge due to a lack of endogenous cell-mediated repair. Although advances have been made in recent decades, restoration of native tissue structure and biomechanical function after injury is still not yet fully realized. Notably, tissue repair is superior in younger tissues and as such, consideration of how the meniscus initially forms and matures may offer insight into regenerative strategies that could be harnessed for adult repair. To address this, our group has detailed the embryonic formation and postnatal maturation of the murine meniscus. These studies revealed that the defined cellular phenotypes and matrix characteristics of the meniscus, including region-specific cartilaginous inner and fibrous outer zones, are already established before birth. This regional specialization is further refined during postnatal growth and maturation, ultimately leading to mature functional tissue [1,2]. These studies also showed that mechanical forces (generated by muscle contraction and cellular force-generating machinery) are essential for embryonic tissue formation [3,4], but that postnatal weight-bearing had a lesser effect [5]. While these studies highlight the mechanically regulated alterations taking place at the cellular and matrix levels during murine meniscus morphogenesis, the mouse model has several limitations that impact its translatability. These include its small size, different loading conditions, and other morphological features that are distinct from humans (e.g., mice develop ossicles in the meniscus horn). To further this line of inquiry in a human-relevant species, the purpose of this study was to determine the timing of knee joint formation in the pig, to establish methods for meniscus cell isolation, and to assay the emergent mechano-responsivity of early meniscus progenitors in this model.

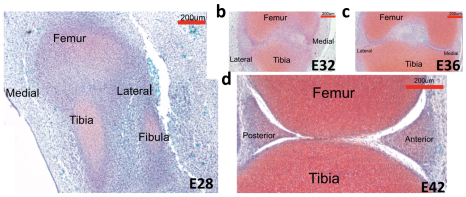

Figure 1: Safranin-O/Fast Green staining of porcine embryonic hindlimbs. a) Skeletal rudiments are apparent at at E28, b) the future joint line is established by E32, and c) by E36, the meniscus has formed but has not yet separated from surrounding tissues. d) By E42, the meniscus has fully formed and separated from the adjacent articular cartilage. Scale: 200 microns.

METHODS: Timed Pregnancies & Embryo Collection: Adult female Yorkshire pigs were artificially inseminated at the National Swine Research and Resource Center (NSRRC). Pregnancy was confirmed by ultrasound, and sows were euthanized on embryonic (E) days 28, 32, 36, or 42, or on postnatal (P) day 1. Embryos or piglets were shipped on ice, and the hindlimbs were harvested. Histological Analysis: Hindlimbs from each stage were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into serial 5µm-thick frontal/sagittal sections. Cellularity and proteoglycan distribution were assessed by Safranin-O/Fast Green staining. Cell Isolation: E42 and P1 menisci from the right hindlimb were identified and isolated using a dissecting microscope. P1 menisci were segmented into an inner and outer region and all tissue was chopped into smaller fragments and cultured on tissue culture plates in basal medium (DMEM, 10% FBS, anti/anti) to allow for cell egress from the tissue fragments. Cells were cryo-preserved at passage 0 and 1 for subsequent studies. Mechano-Response Assay: Isolated embryonic/postnatal cells were cultured on fibronectin-coated polyacrylamide (PA) hydrogels (Soft: 5kPa or Stiff: 55kPa) or glass. Following 1-day in basal media, cells were fixed and immuno-stained for YAP (labeled with Alexa Fluor (AF)-488), actin (phalloidin-AF555), and nuclei (Hoechst 33342, excitation: 350). Imaging: Confocal microscopy was used to obtain fluorescent z-stack images at 10X magnification. Images were processed in Cell Profiler to quantify cell area and YAP nuclear to cytoplasmic ratio. Statistics: 2-way ANOVA using Graphpad Prism was used to make comparisons between groups, with significance set at $p<0.05$.

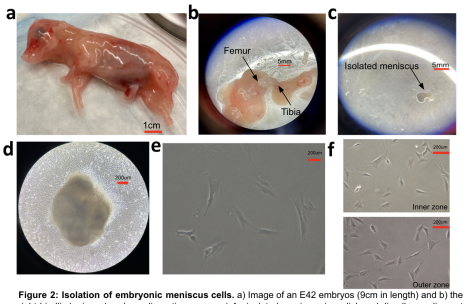

Figure 2: Isolation of embryonic meniscus cells. a) Image of an E42 embryos (9cm in length) and b) the right hindlimb viewed under a dissecting scope. c) An isolated meniscus in a dish and d) cells crawling out of a meniscus segment after 5 days of culture. E42 (e) and P1 (f, inner and outer) meniscus cells in culture at passage 1.

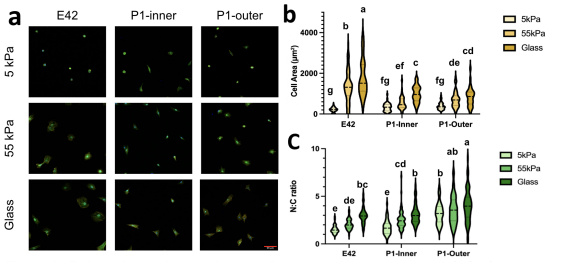

Figure 3: Embryonic porcine meniscus cells are mechano-responsive. a) Confocal images showing YAP(green), actin (yellow), and nuclei (blue) in E42, P1-inner, and P1-outer meniscus cells cultured on soft (5kPa) and stiff (55kPa) hydrogels and a glass control. Quantification of b) cell area size and c) YAP N:C ratio. Letters indicate differences between individual groups.

RESULTS: Histology across time points during early gestation identified key timepoints of porcine limb development. At E28 (Fig. 1a), skeletal rudiments were clearly evident, as indicated by cartilaginous condensations at the presumptive femur, tibia, and fibula. By E32 (Fig. 1b), the future joint line was well established, and by E36 (Fig. 1c), the menisci had condensed but had not yet separated from the adjacent structures. At E42 (Fig. 1d), the menisci were fully formed and completely separated from the neighboring articular cartilage. At this stage, the E42 embryo measures ~9cm in length (Fig. 2a) and microdissection reveals a fully formed joint (Fig. 2b), from which the meniscus can be readily isolated (Fig. 2c). When these menisci are removed, chopped into segments, and placed in tissue culture, meniscus progenitor cells exit the tissue within 5 days (Fig. 2d). These cells proliferate in culture, and appear similar to those isolated from P1 menisci (Fig. 2e-f). When placed on PA gels of increasing stiffness, meniscus progenitors from E42 (all zones) and P0 inner/outer zone were mechanosensitive (Fig. 3a). That is, E42, P0-inner, and P0-outer cells all increased in area with increasing stiffness (Fig. 3b). Similarly, for E42 and P1-inner cells, the YAP N:C ratio was higher on 55kPA and glass than on 5 kPa gels (Fig. 3c). Interestingly, outer zone cells (P1-outer) showed a higher YAP N:C ratio than either E42 or P1-inner cells, even on soft (5 kPa) substrates.

DISCUSSION: Consistent with other model systems, the porcine hindlimb undergoes a coordinated series of events during the formation of the knee joint, including condensation of the rudiments, establishment of a joint line, condensation of soft tissue, and eventual cavitation and separation. In the pig, this process appears complete by E42, approximately 1/3rd of the way through gestation. At this stage, embryos are sufficiently large that, with the aid of a dissection microscope, one can readily isolate the menisci and these tissues can be cultured to generate cells for in vitro experimentation. At P1, the meniscus can be further separated into an inner and an outer region, enabling assessment of regional variation. When plated on substrates of increasing stiffness, both E42 and P1 cells showed a characteristic mechanobiological response, increasing in spread area as a function of stiffness. This indicates that, as soon as the meniscus becomes a distinct entity (cavitation is complete), resident cells can respond to their mechanical microenvironment. Interestingly, the P1 inner meniscus and E42 cells showed the most similar response, while the P1-outer cells were distinct. Specifically, P1-outer cells had a higher level of N:C YAP at every stiffness. These findings may suggest that cells within the outer zone of the meniscus develop a different threshold for mechanical signaling, earlier in development. Future studies will continue to explore these mechanosensing attributes of embryonic meniscus cells, as well as query the heterogeneity in these populations using single-cell RNA seq and other assays to identify the spatiotemporal mechano-responsiveness of the meniscus throughout development.

SIGNIFICANCE: This study established the timing of embryonic knee joint formation in the pig and identified emergent mechanosensitivity in meniscus progenitors as a function of region and developmental state.

REFERENCES & ACKNOWLEDGEMENTS: [1] Tsinman+ FASEB J, 2021. [2] Kwok+ Acta Biomat, 2023. [3] Tsinman+ Trans ORS, 2022. [4] Tsinman+ JOR, 2023. [5] Fogarty+ JOR, 2023. This work was supported by the NIH and the VA.