Effect Of Hormonal Changes on Anterior Cruciate Ligament Fibroblast Gene Expression in a Porcine *in vitro* Model of Pre-Pubescent Female Development

Madison E. Thompson[1], Jacob D. Thompson[2,3], Brenna R. Pugliese[3,5], Lauren V. Schnabel[3,4], Matthew B. Fisher[2,3]

[1]Department of Computer Science, North Carolina State University, [2]Joint Department of Biomedical Engineering, North Carolina State University and the University of North Carolina – Chapel Hill, [3]Comparative Medicine Institute, North Carolina State University, [4]College of Veterinary Medicine, North Carolina State University, [5]Department of Clinical Sciences, NC State University College of Veterinary Medicine, Raleigh, NC

CMI COMPARATIVE MEDICINE INSTITUTE

THE SCHNABEL LAB
PIONEERING EQUINE HEALTH

Introduction

- Skeletally immature adolescent female athletes experience ACL injuries 3-8 times more often than males[1]
- Several studies have shown that in addition to differential skeletal development, high estrogen and progesterone levels may predispose adolescent females to ACL injury[2,3]
- It is hypothesized that estrogen and progesterone increase the inflammatory response of ligament fibroblasts

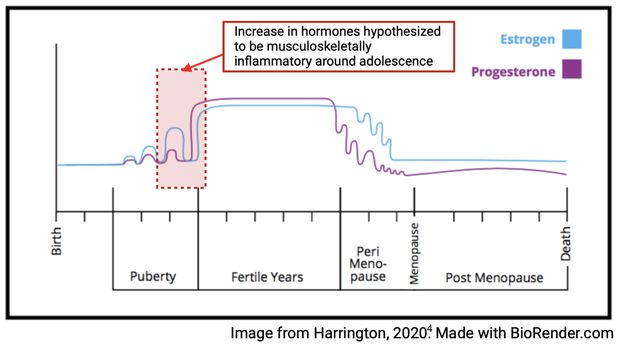
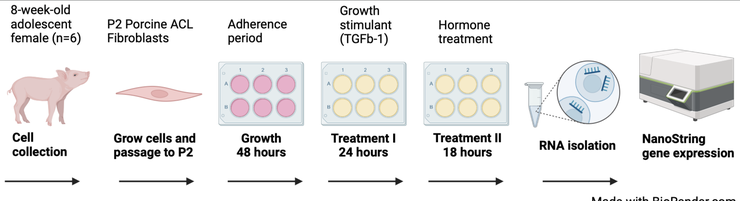


Image from Harrington, 2020[4]. Made with BioRender.com

Objective: utilize an *in vitro* porcine ACL model to determine whether estrogen and progesterone play a critical role in musculoskeletal gene expression

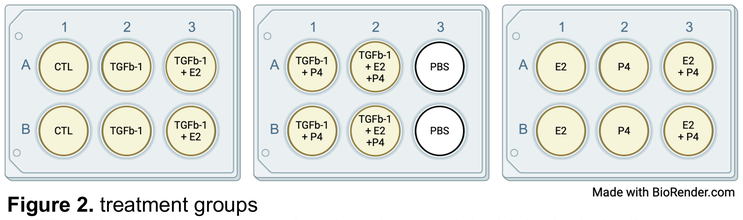
Methodology



Made with BioRender.com

Figure 1. Experimental design

- Porcine ACL Fibroblasts harvested from 8-week-old females were passaged to P2 and allowed to grow for 48 hours
- Select groups were stimulated for 24 hours with TGFb-1 to simulate pubescent spikes in estradiol and progesterone
- Hormone treatments were applied with stimulation for 18 hours
- RNA was analyzed for gene expression



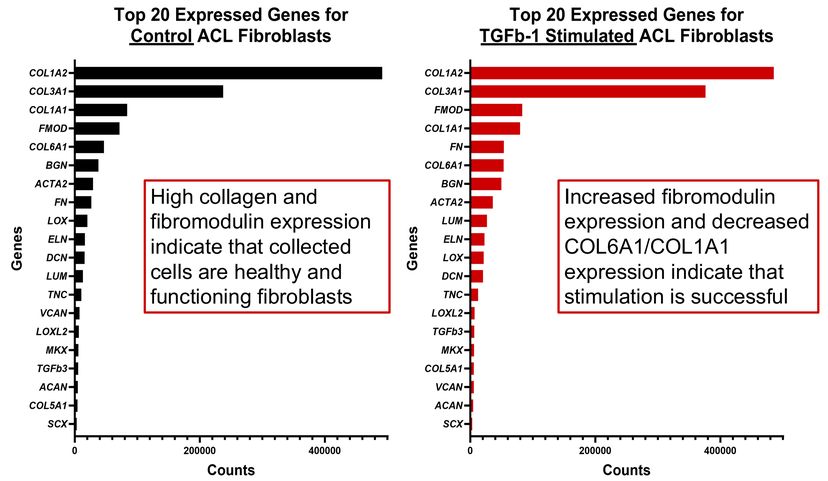
Made with BioRender.com

Figure 2. treatment groups

- P2 ACL fibroblasts were plated and treated in biological duplicates at a concentration of 85,000 cells per well
- 4 of the 8 treatment groups were stimulated with 10 ng/mL TGFb-1
- Groups receiving estrogen were treated with 1000 pg/mL E2
- Groups receiving progesterone were treated with 100 ng/mL P4

Results: Gene Expression

Differential Gene Expression Indicates Effective Stimulation



Most Expressed Genes by Treatment and Functional Groups

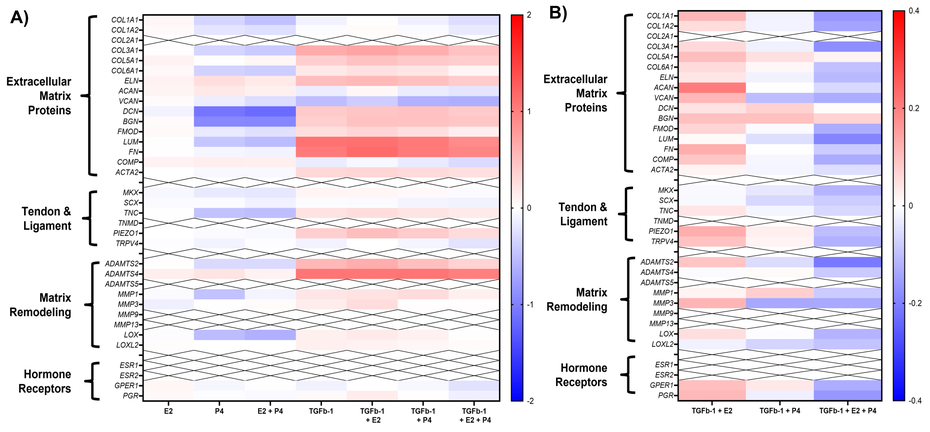


Figure 3. A) shows gene expression for stimulated and unstimulated treatment groups, normalized to the unstimulated control **B)** shows genes expression for stimulated treatment groups, normalized to the stimulated control.

Statistically Significant Expression of Critical Inflammatory Genes

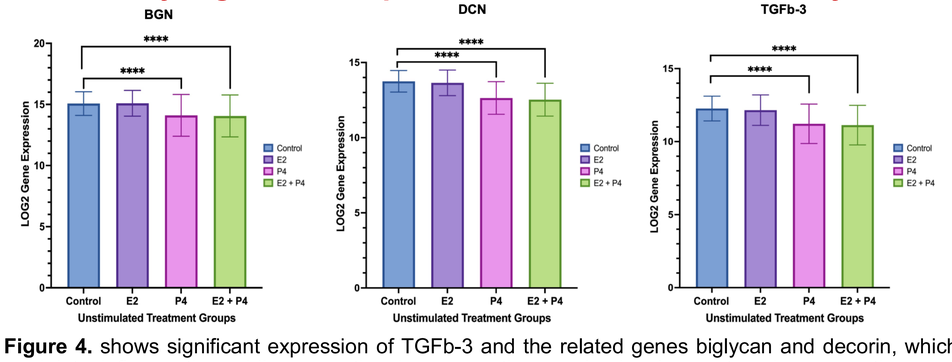


Figure 4. shows significant expression of TGFb-3 and the related genes biglycan and decorin, which modulate inflammation while supporting bone growth, muscle development and regeneration, and collagen fibril assembly. **A)** biglycan **B)** decorin, and **C)** TGFb-3 expression is significantly higher for unstimulated progesterone and estrogen and progesterone treatment groups.

Discussion

Progesterone has a greater effect on proteoglycan expression in ACL fibroblasts than estrogen

- TGFb-1 stimulates ECM production specifically for lumican, fibronectin, ADAMST2, and ADAMST4 in ACL fibroblasts
- Estrogen generally had a minimal impact on the gene expression of cultured ACL fibroblasts *in vitro*
- Both estrogen (E2) and progesterone (P4) have a minimal impact on TGFb-1 stimulation, but have a downregulation effect when combined

Limitations and Future Directions

- The effects of other hormones, such as testosterone and relaxin, should be studied
- Porcine estrogen receptors are less expressed *in vitro*
- Hormone concentrations and dosing timelines should be optimized for future experiments
- The optimized experiment will be studied in 3D models for various cell types, with preliminary chondrocyte data below

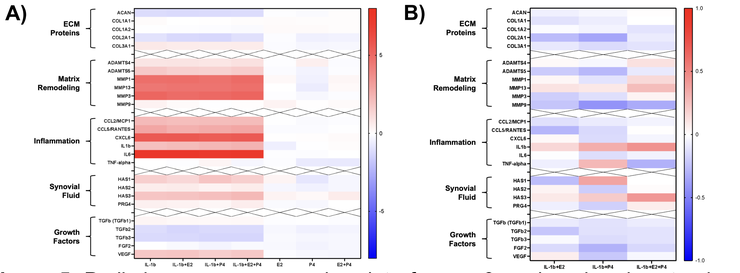


Figure 5. Preliminary gene expression data from n=3 equine chondrocytes in an OA model. **A)** shows all genes normalized to the unstimulated control and **B)** shows stimulated groups normalized to the stimulated control.

References

[1] Silvers-Granelli H. Why Female Athletes Injure Their ACL's More Frequently? What can we do to mitigate their risk? *International Journal of Sports Physical Therapy*. 2021;16(4). doi:https://doi.org/10.26603/001c.25467

[2] Stijak L, Kadija M, Djulejić V, et al. The influence of sex hormones on anterior cruciate ligament rupture: female study. *Knee Surgery, Sports Traumatology, Arthroscopy*. 2014;23(9):2742-2749. doi:https://doi.org/10.1007/s00167-014-3077-3

[3] Lin CY, Casey E, Herman DC, Katz N, Tenforde AS. Sex Differences in Common Sports Injuries. *PM&R*. 2018;10(10):1073-1082. doi:https://doi.org/10.1016/j.pmrj.2018.03.008

[4] Harrington J. Female Hormone Lifecycle. www.menopausenaturalsolutions.com. Published June 5, 2020. https://www.menopausenaturalsolutions.com/blog/female-hormone-lifecycle

Acknowledgements

I would like to thank the Comparative Medicine Institute for their financial and professional support that made this research possible. I would also like to thank the Schnabel Lab and the Fisher Lab for their mentorship and support throughout this project.