Estrogen Receptor Expression Changes After Puberty in the Skeletally Immature Porcine Anterior Cruciate Ligament

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INTRODUCTION: Anterior cruciate ligament (ACL) injuries are increasing in the pediatric population [1,2]. In certain sports, female athletes are 3-4x more likely to incur an ACL injury [3,4]. Several papers have drawn associations between menstrual cycle phase and injury rate [5,6], although sample sizes are often small and hormone levels are not tracked [7]. Hormone receptors for estrogen and progesterone are present in adult ACL tissue, yet these findings are not well-characterized spatially or during skeletal growth [8]. Thus, there is a need for additional studies to assess the impact of hormones on the ACL, particularly during growth. Pigs serve as a model to study the ACL during growth [9] and experience increases and cyclic variation in serum sex hormone levels, similar to humans [10]. The objective of this work is to compare gene expression of matrix molecules and both gene and protein expression of hormone receptors in the ACL and its anteromedial (AM) and posterolateral (PL) bundles before and after puberty.
METHODS: The AM and PL bundles of the ACL were collected from female Yorkshire crossbreed pigs prior to puberty (approximately 2 months old) or post-puberty (8-12 months old) (n=6 animals/age; Figure 1A). Given the focus on female sex hormones, only female animals were used. All animals were collected from a range of studies performed at North Carolina State University and approved by IACUC. The bundles were immediately snap-frozen in liquid nitrogen for gene expression and western blot analysis or placed in optimal cutting temperature for immunofluorescence and histological staining. Uterine tissue was also harvested from post-pubescent animals as a positive control for hormone receptors. RNA was extracted, concentration was assessed, and transcriptional analysis was performed to analyze extracellular matrix protein-related genes and hormone receptor genes (nCounter MAX Analysis System). SDS-PAGE gels were used to separate proteins by size. Protein bands were blotted with anti-estrogen receptor alpha (ERα) or anti-G protein-coupled receptor 30 (GPR30) followed by secondary antibodies. Blots were imaged, and relative protein levels were quantified (ImageJ). Immunofluorescence was done on tissue sections to examine spatial presence of ERα and GPR30. Multiple unpaired t-tests were conducted with Holm-Sidak adjustment for multiple comparisons for gene expression differences, and unpaired t-tests were performed for relative differences in ERα protein expression. Analyses were performed at alpha=0.05 or adjusted for multiple comparisons.
RESULTS: In terms of gene expression (Figure 1B), bone-morphogenic protein 12 (GDF7/BMP12), lumican (LUM), and collagen 3α1 (COL3A1) all had higher expression in post-pubescent AM bundles compared to pre-pubescent AM bundles. In the pre-pubescent AM bundle, there was greater expression of several collagens, elastin (ELN), and tenomodulin (TNMD). When comparing PL bundles across age, cartilage oligomeric protein (COMP) had greater expression in post-pubescent PL bundles, and TNMD had greater expression in pre-pubescent PL bundles. In terms of hormone receptors, expression of ERα (ESR1) was higher and expression of GRP30 (GPER1) was lower in post-pubescent tissues (Figure 2A). Similar differences across age were found in the PL bundle, though not statistically significant. There were no differences in expression of genes encoding estrogen receptor-beta, or progesterone receptor between pre- and post-pubescent ACLs (Figure 2A). For protein expression, via western blotting, ERα was detectable in the ACL of 1 of 6 pre-pubsecent and 6 of 6 post-pubescent animals in both bundles (blots for AM bundle shown in Figure 2B). Quantitatively, ERα expression was significantly higher in the post-pubescent ACLs (both bundles), while GPR30 expression lower (though not statistically significant) (Figure 2B). Via immunofluorescence, positive signal for both ERα and GPR30 was observed across both bundles for all tissues, except for one post-pubescent AM bundle tissue. Both estrogen receptors were seen in cells in collagen-rich regions and within the interfascicular matrix area. Across age, there was decreased staining intensity for GPR30 in the post-pubescent group, similar to the age-related differences in gene and protein concentration.
DISCUSSION: When characterizing the ACL across development (pre- and post-pubescent), the post-pubescent ACLs had greater ERα gene and protein expression than pre-pubescent ACLs. While previous literature has shown hormone receptors in the ACL [8], this study shows that receptor expression in the ACL changes during skeletal growth. Given the catabolic effects of estrogen on the ACL in in-vitro culture [11], the changes in estrogen receptors may alter the sensitivity of the ACL to changing hormone levels in-vivo. In the western blots for ERα, we did notice bands at different molecular weights at both ages, which suggest reactivity with other protein complexes in the ACL and would explain the apparent differences between western blot and immunofluorescence results. Beyond hormone receptors, the increased expression of collagen and tenomodulin in pre-pubescent animals indicate a matrix-synthesis focused environment and the increased expression of lumican and matrix metalloproteinase III indicate matrix homeostasis in post-pubescent animals. To better understand potential mechanisms of action, additional studies should also focus on tracking hormone receptor changes in the ACL relative to estrus cycle phase and serum hormone levels and controlled in-vitro tissue culture experiments where the hormones and the receptors are monitored.
SIGNIFICANCE/CLINICAL RELEVANCE: This study describes changes in estrogen receptors within cells in the ACL before and after puberty onset. Such changes could be related to increased injury risk, though further mechanistic studies are needed.
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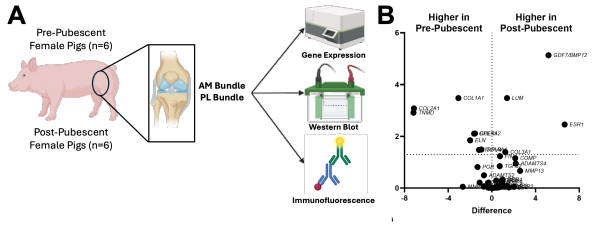

Figure 1. (A) Schematic of study design to assess changes in gene and protein expression between pre- and post-pubescent ACLs. (B) Varying gene expression across growth for the AM bundle.

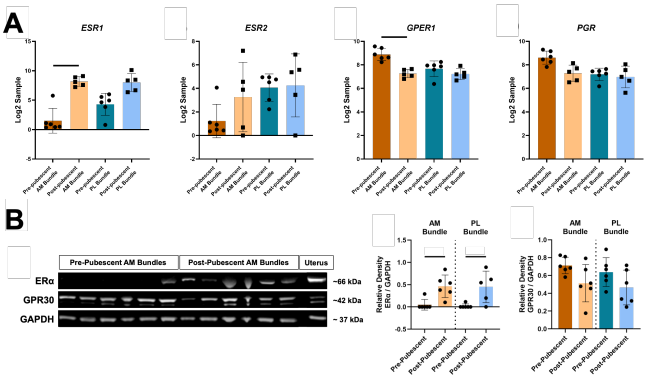

Figure 2. Changes in sex hormone (A) gene expression and (B) protein expression across growth in both ACL bundles. Bars indicate statistical significance ($p<0.05$).