Shift from androgen to estrogen action causes abdominal muscle fibrosis, atrophy, and inguinal hernia in a transgenic male mouse model

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Inguinal hernia develops primarily in elderly men, and more than one in four men will undergo inguinal hernia repair during their lifetime. However, the underlying mechanisms behind hernia formation remain unknown. It is known that testosterone and estradiol can regulate skeletal muscle mass. We herein demonstrate that the conversion of testosterone to estradiol by the aromatase enzyme in lower abdominal muscle (LAM) tissue causes intense fibrosis, leading to muscle atrophy and inguinal hernia; an aromatase inhibitor entirely prevents this phenotype. LAM tissue is uniquely sensitive to estradiol because it expresses very high levels of estrogen receptor-α. Estradiol acts via estrogen receptor-α in LAM fibroblasts to activate pathways for proliferation and fibrosis that replaces atrophied myocytes, resulting in hernia formation. This is accompanied by decreased serum testosterone and decreased expression of the androgen receptor target genes in LAM tissue. These findings provide a mechanism for LAM tissue fibrosis and atrophy and suggest potential roles of future nonsurgical and preventive approaches in a subset of elderly men with a predisposition for hernia development.

Significance

Inguinal hernia is one of the most common disorders that affect elderly men. A major pathology underlying inguinal hernia is the fibrosis and other degenerative changes that affect the lower abdominal muscle strength adjacent to the inguinal canal. Here we describe a critical role of estrogen and its nuclear receptor that enhance fibroblast proliferation and muscle atrophy, leading to inguinal hernia. Further research may reveal a potential role of estrogen ablation to prevent muscle fibrosis or hernia in a subset of elderly men.

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Sex steroid hormones change as men age. Age-related changes in serum estradiol (E$_2$) levels in men are conflicting, as some studies have reported increases but others have noted unchanged or even decreased E$_2$ levels with advancing age (17–24). Conversion of circulating testosterone (T) to E$_2$ via aromatase expression in bulky tissues—namely, skeletal muscle and adipose tissue—produces the majority of estrogen in men (25, 26). Human muscle and adipose tissue aromatase expression or activity has been found to increase with advancing age, which coincides with the incidence of inguinal hernia (25, 27–29). In the 1930s, two separate laboratories reported that 40% of male mice that received postnatal estrogen injections or ovarian grafts developed scrotal hernias (8, 30). Estrogen injections initiated as early as postnatal day 28 or as late as 30 wk of age led to the development of scrotal hernia within a few weeks (8). These data were suggestive of a direct link between local tissue estrogen and acquired inguinal hernias and do not support the possible contribution of a congenital defect. The underlying cellular and molecular mechanisms, however, remain unknown. Conversely, serum T levels decrease by 20% by age 50 and by 50% by age 80 y in association with decreased skeletal muscle mass in men (31–33). A higher ratio of E$_2$ to T was observed in elderly with compared with younger men (31). It remains unclear whether the age-related shift in the estrogen to T ratio in the LAM tissue causes muscle fibrosis and atrophy and predisposes a subset of older men to develop inguinal hernia.

Aromatase is the only enzyme that catalyzes the conversion of T to E$_2$. The tissue distribution patterns of aromatase expression in humans and mice are markedly distinct. In male mice, aromatase is expressed only in the testes, gonadal fat, and brain via 3 promoters, whereas humans use 10 distinct promoters to express aromatase in many peripheral tissues, including skeletal muscle. We recently generated mice expressing aromatase from the human promoter (Arom$^{hum}$) to mimic human physiology with respect to aromatase expression and estrogen production (34). Arom$^{hum}$ mice physiologically express the human aromatase gene in many peripheral tissues, including skeletal muscle (25, 35, 36). Intriguingly, male Arom$^{hum}$ mice exhibit increased estrogen levels in peripheral LAM tissues, low serum T levels, and scrotal hernia formation mimicking what has been observed in a subset of older men. Thus, we used this mouse model to test the hypothesis that alterations in E$_2$, T, and their nuclear receptors, estrogen receptor-α (ERα) and androgen receptor (AR), in LAM tissues lead to fibrosis, skeletal muscle atrophy, and the development of scrotal hernias. We also investigated some of the underlying cellular and molecular mechanisms behind hernia formation.

Results

Humanized Aromatase Expression and Physiological Estrogens Production in LAM Tissues Are Associated with Scrotal Hernia Formation in Arom$^{hum}$ transgenic mice. Arom$^{hum}$ mice were generated and characterized as previously described (34). Briefly, we isolated a human BAC clone containing the human aromatase coding sequence flanked by its full-length 93-kb 5′-regulatory region and the 3′-polyadenylation site, and injected the BAC clone DNA into pronuclear mouse embryos to generate transgenic mice with a single copy of the transgene in the germline. We obtained six male transgene-positive founders in two independent male transgenic lines, F1771 and F1772. The Arom$^{hum}$ F1771 line included a >78-kb 5′-flanking region encompassing the distal promoters I.4, I.7, I.f, as well as the proximal cluster of promoters I.6 and I.3/PII (Fig. L4), whereas the Arom$^{hum}$ F1772 line had a 4.3-kb 5′-flanking region containing only the proximal promoters I.6 and I.3/PII (34). Bilateral scrotal hernias were observed in 90–100% of male Arom$^{hum}$ mice by 12 wk from all two lines established from the founders, whereas none of the WT animals developed any hernias. Because all founder lines showed a similar phenotype, we primarily reported the results obtained from a single founder from the Arom$^{hum}$ F1771 line (referred to as Arom$^{hum}$ in this report). The scrotal hernia sacs contained abdominal viscera, including gonads, gonadal fat, urinary bladder, and bowel (Fig. 1B). In Arom$^{hum}$ mice, LAM tissues became progressively fibrotic and distended, comprising the major part of the hernia wall, contiguous with the scrotum (Figs. 1C and 2C and D). Human aromatase expression driven by its native promoters in Arom$^{hum}$ mice resembled the human pattern of aromatase expression (SI Appendix, Table S1) (25, 35–37).

Because inguinal hernia is associated with fibrosis of the LAM tissue, we analyzed the expression and promoter usage of aromatase and estrogen formation in the abdominal muscle tissue of Arom$^{hum}$ mice. We demonstrated that human aromatase mRNA but not mouse aromatase mRNA was readily detectable in LAM tissue, upper abdominal muscle (UAM) tissue, and quadriceps muscle (QM) tissue of Arom$^{hum}$ mice, whereas both human and mouse aromatase mRNA was absent in these muscle tissues in WT mice (Fig. 1D). 5′-RACE showed that aromatase mRNA was transcribed primarily from promoter I.4 of the human aromatase gene in abdominal muscle and QM tissues of Arom$^{hum}$ mice (SI Appendix, Table S1). Additionally, exon-specific RT-PCR showed that human aromatase expression was driven primarily...
by promoters I.4 and to a lesser extent PII in abdominal muscle tissues of *Arom*<sup>hum</sup> mice (Fig. 1E). The aromatase mRNA expression profile and promoter usage in other tissues of male *Arom*<sup>hum</sup> mice are summarized in SI Appendix, Table S1. Thus, human aromatase expression is driven by its native promoters in a wide variety of male *Arom*<sup>hum</sup> mouse tissues, including skeletal muscle (25, 35–37).

To determine whether humanized aromatase expression in the *Arom*<sup>hum</sup> mouse extragonadal tissue, including abdominal muscle, is accompanied by a change in tissue estrogen levels and circulating hormone levels, we measured tissue concentrations of estrogens [estrone (E<sub>1</sub>), E<sub>2</sub>, and estriol (E<sub>3</sub>)] using liquid chromatography-tandem mass spectrometry (LC-MS<sup>2</sup>) (Fig. 1F), and also compared peripheral serum levels of E<sub>2</sub> and the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) using LC-MS<sup>2</sup> and radioimmunoassay (SI Appendix, Fig. S1). Compared with WT mice, total estrogen levels in LAM tissues were significantly higher by 2.6-fold in *Arom*<sup>hum</sup> mice. Most importantly, LAM tissue levels of the biologically potent estrogen, E<sub>2</sub>, were 3.1-fold higher in *Arom*<sup>hum</sup> mice compared with WT littermates (Fig. 1F). However, serum E<sub>2</sub> levels measured by LC-MS<sup>2</sup> were not significantly different between WT and *Arom*<sup>hum</sup> males (Fig. 3C and SI Appendix, Fig. S1A). Serum FSH concentrations were significantly lower in *Arom*<sup>hum</sup> mice (64%) compared with WT mice. Decreased FSH levels in the *Arom*<sup>hum</sup> mice may be due to increased E<sub>2</sub> production via human aromatase expression in hypothalamic tissue (SI Appendix, Table S1). Serum LH concentrations were lower but did not reach significance in *Arom*<sup>hum</sup> mice compared with WT littermates. These results show that local LAM tissue E<sub>2</sub> levels, but not circulating E<sub>2</sub> levels, led to hernia formation in male *Arom*<sup>hum</sup> mice.

**The Development of Scrotal Hernias Is Associated with Fibrosis and Myocyte Atrophy in LAM Tissue of *Arom*<sup>hum</sup> Mice.** We studied WT and *Arom*<sup>hum</sup> mice from 1 to 26 wk of age for the development of hernias. Minimal lower abdominal bulging was first observed in 75% of *Arom*<sup>hum</sup> mice at 4 wk, with noticeable bulging by 8 wk in all *Arom*<sup>hum</sup> mice, followed by frank scrotal herniation by 12 wk in all *Arom*<sup>hum</sup> mice (Fig. 2A). Hernia size continued to increase during the whole observation period (up to 26 wk of age) (Fig. 2B).

We also studied the effects of muscle aromatase expression and estrogen production on skeletal muscle histology. UAM and QM did not show any major structural differences between WT and the *Arom*<sup>hum</sup> mice (Fig. 2C and SI Appendix, Fig. S4), while the morphology of extended LAM tissue that comprises the major part of hernia wall was markedly altered in *Arom*<sup>hum</sup> mice (Fig. 1B and C). At 24 wk, LAM tissue of *Arom*<sup>hum</sup> mice showed a marked decrease in myocyte size and centralized nuclei, indicating myocyte atrophy (Fig. 2C, yellow arrow, *Inset*). Masson’s trichrome staining of LAM sections showed a marked increase in collagen deposition in the context of myocyte atrophy in *Arom*<sup>hum</sup> mice compared with WT mice (Fig. 2D–F). Together, these results clearly indicate that hernia formation induced by LAM E<sub>2</sub>...
administration of an aromatase inhibitor prevents scrotal hernia development in Arom<sup>hum</sup> mice. Aromatase inhibitors represent the most effective endocrine treatment for postmenopausal breast cancer (34, 38). The role of aromatase inhibitors in prevention of scrotal hernias, however, is unknown. Here we investigated whether blockade of aromatase activity using a slow-release aromatase inhibitor pellet (letrazole, 10 μg/d per mouse) can prevent scrotal hernia development in Arom<sup>hum</sup> mice. Intriguingly, none of the Arom<sup>hum</sup> mice administered subcutaneous letrazole at 3 wk of age developed scrotal hernia until the end of treatment (15 wk of age) (Fig. 3A and B). Serum E<sub>2</sub> levels were not significantly different between WT and Arom<sup>hum</sup> mice, but they significantly decreased in Arom<sup>hum</sup> mice after letrazole treatment (Fig. 3C). T levels in vehicle-treated Arom<sup>hum</sup> mice were lower compared with WT mice and were increased by levels comparable to those in WT mice after letrazole treatment (Fig. 3D). Similarly, FSH levels in vehicle-treated Arom<sup>hum</sup> mice were significantly lower compared with WT mice and increased to normal (WT) levels with letrazole treatment (Fig. 3E). However, letrazole treatment did not change serum LH levels in either WT or Arom<sup>hum</sup> mice, nor did it change serum E<sub>2</sub>, T, or FSH levels in WT mice (Fig. 3C–F). Most importantly, LAM tissue E<sub>2</sub> levels were 6.3-fold higher in Arom<sup>hum</sup> mice compared with WT littermates and letrazole treatment completely restored LAM E<sub>2</sub> levels to normal (WT) levels (Fig. 3G). Development of fibrosis in the LAM tissue of Arom<sup>hum</sup> mice was completely prevented with letrazole treatment (Fig. 3H). The myofiber cross-sectional area in untreated Arom<sup>hum</sup> mice was significantly smaller than in WT mice, indicating muscle atrophy. Letrozole treatment completely prevented myofiber atrophy in Arom<sup>hum</sup> mice (Fig. 3I and J). These data demonstrate that aromatase inhibition via letrazole treatment can inhibit LAM aromatase activity, restore LAM tissue E<sub>2</sub> levels and serum T and FSH levels to normal levels, and prevent LAM tissue fibrosis, muscle atrophy, and hernia formation.

Higher Estrogen Sensitivity in LAM Tissue Is Accounted for by Higher ERα Levels in Stromal Fibroblasts. E<sub>2</sub> exerts its biological effects by binding and signaling through at least three distinct receptors, ERα, ERβ, and Gpr30 (39–41). The levels of ERα and ERβ differ in different muscle groups are markedly different (42). In Arom<sup>hum</sup> mice, ERβ mRNA levels were significantly lower in LAM tissue compared with UAM tissue (SI Appendix, Fig. S2A). Moreover, ERα mRNA levels were 350- and 597-fold higher than ERβ mRNA levels in UAM tissue and 610- and 1,786-fold higher than ERβ mRNA levels in LAM tissue of WT and Arom<sup>hum</sup> mice, respectively (SI Appendix, Fig. S2B). Additionally, although Gpr30 mRNA levels were significantly higher in LAM tissues than UAM tissues, ERα mRNA levels were 510- and 617-fold higher than Gpr30 mRNA levels in LAM tissue and 263- and 244-fold higher than Gpr30 mRNA levels in LAM tissue of WT and Arom<sup>hum</sup> mice, respectively (SI Appendix, Fig. S2C and D). Most importantly, Gpr30 mRNA levels were significantly lower in LAM fibroblasts compared with UAM fibroblasts (SI Appendix, Fig. S2E). ERα mRNA levels were 47- to 54-fold higher than Gpr30 mRNA levels in UAM fibroblasts and 277- to 215-fold higher than Gpr30 mRNA levels in LAM fibroblasts of WT and Arom<sup>hum</sup> mice, respectively (SI Appendix, Fig. S2F). Thus, ERα seems to be the predominant receptor that plays a key role in estrogen sensitivity and hernia formation in LAM tissue of Arom<sup>hum</sup> mice. Furthermore, in muscle tissue, ERα mRNA levels were highest in LAM, modest in UAM, and lowest in QM in both WT and Arom<sup>hum</sup> mice (Fig. 4E). ERα mRNA levels in LAM of all mice were significantly higher than those in UAM. QM ERα mRNA levels were significantly lower than UAM and LAM in all mice. ERα protein levels tended to be higher but did not reach significance in LAM homogenates compared with UAM homogenates in either WT or Arom<sup>hum</sup> mice (SI Appendix, Fig. S3).

Because muscle is a heterogeneous tissue consisting of myocytes, stromal cells (fibroblasts), vascular endothelial cells, and other cell types, it is possible that specific expression of ERα in a particular cell type may not be accurately assessed by analyzing whole-tissue homogenates. Thus, we isolated fibroblasts from
UAM and LAM tissues of WT mice and found that mRNA and protein levels of ERs were markedly higher in LAM fibroblasts than UAM fibroblasts (Fig. 4 B and C). Primary fibroblast specificity was confirmed by the presence of a fibroblast marker (α-SMA) and the absence of a myocyte marker (MyoD). We used immunohistochemistry (IHC) to further confirm ERα protein levels in LAM fibroblasts of WT and Arom<sup>hum</sup> mice. ERα immunoactivity was almost exclusively observed in the stromal fibroblasts, but rarely present in myocytes (Fig. 4D and SI Appendix, Fig. S4). The H-score of ERα<sup>+</sup> stromal cells was significantly higher in LAM tissue than in UAM tissue in both WT and Arom<sup>hum</sup> mice, and furthermore, it was markedly higher in Arom<sup>hum</sup> LAM tissue than in WT LAM tissue (Fig. 4E). The stromal component in QM had negligible ERα expression (SI Appendix, Fig. S5). Therefore, locally produced E2 in LAM tissues seems to be mediated via high LAM fibroblastic ERα expression, resulting in hernia formation in Arom<sup>hum</sup> mice.

**ERα-Target Gene Expression Induced by Local E2 Excess in LAM Tissue Is Increased in Arom<sup>hum</sup> Mice.** To identify the early molecular events responsible for estrogen-induced muscle tissue fibrosis, myocyte atrophy, and hernia formation, we performed an RNA expression microarray analysis of LAM in WT and Arom<sup>hum</sup> mice at the age of 3 wk (before the start of morphologic changes in the muscle or hernia development). Multidimensional scaling analysis of the datasets from Arom<sup>hum</sup> and WT mice showed statistically significant differences; 92 genes were expressed preferentially in LAM of Arom<sup>hum</sup> mice and 33 genes were preferentially expressed in WT mice (Table 1 and Dataset S1). Pathway analysis listed the hepatic fibrosis pathway as the first up-regulated canonical pathway, and E2 was listed as the third upstream regulator in Arom<sup>hum</sup> mice (Tables 2 and 3). The expression of the previously established estrogen-target gene Greb1 was significantly higher (1.89-fold) in Arom<sup>hum</sup> mice than in WT mice. We verified the mRNA levels of Greb1 and several other estrogen-target genes (Pgr, Cnd1, and Adora1) by real-time PCR in tissue lysates and primary fibroblasts of UAM and LAM from 3-wk-old WT and Arom<sup>hum</sup> mice (43–46). In general, mRNA levels of these estrogen-responsive genes tend to be higher in abdominal muscle tissues or fibroblasts with higher E2 levels (Arom<sup>hum</sup> vs. WT mice) and with higher ERα expression (LAM vs. UAM) (Fig. 4 F–M). Furthermore, to investigate whether ERs mediates the effects of E2 in LAM fibroblasts, we treated LAM primary fibroblasts from WT or Arom<sup>hum</sup> mice with E2 in the presence or absence of the E2/ERα antagonist ICI 182780 or the ERα-selective E2 antagonist methyl-piperidino-pyrazole (MPP). E2 increased mRNA levels of Pgr and Greb1 after 48-h of treatment. ICI 182780 or MPP inhibited the stimulatory effect of E2 on gene expression of Pgr and Greb1 (Fig. 4 N and O and SI Appendix, Fig. S7 A, B, E, and F). ERα knockdown significantly down-regulated mRNA levels of Pgr and Greb1, which could not be restored with E2 treatment (Fig. 4 P and Q and SI Appendix, Figs. S6 and S7 I and J). These results strongly suggest that estrogen action in LAM fibroblasts of Arom<sup>hum</sup> mice was drastically enhanced via locally produced estrogen and high ERα expression, leading to hernia formation.

**The Role of Fibroblast Proliferation and Excessive ECM Formation in Estrogen-Induced Hernia Formation.** Fibroblasts constitute the key cell type of the stroma and are important for wound healing and collagen formation in fibrotic tissues (47). To further determine the underlying mechanisms of locally produced estrogen in fibroblast proliferation, function, and hernia formation in LAM, K67 immunostaining (indicating cell proliferation) was performed in UAM, LAM, and QM of WT and Arom<sup>hum</sup> mice. K67 immunoreactivity was predominantly observed in the LAM stromal component and scarcely present in myocytes of WT and Arom<sup>hum</sup> mice vs. WT mice.

### Table 1. Microarray analysis of LAM tissue in WT and Arom<sup>hum</sup> mice

| Gene symbol | Definition | Fold-change | Adjusted P value |
|-------------|------------|-------------|-----------------|
| **Top 13 up-regulated fibrotic genes in LAM of Arom<sup>hum</sup> mouse** | | |
| Kiss1 | KISS-1 metastasis-suppressor | 31.13 | 0.009 |
| Ren1 | Renin 1 structural | 7.87 | 0.026 |
| Emb | Embigin | 5.20 | 0.020 |
| Krt8 | Keratin 8 | 4.38 | 0.005 |
| Timp1 | Tissue inhibitor of metalloproteinase 1, transcript variant 2 | 4.19 | 0.009 |
| Krt7 | Keratin 7 | 3.05 | 0.007 |
| Spon2 | Spondin 2, extracellular matrix protein | 2.83 | 0.058 |
| Krt18 | Keratin 18 | 2.71 | 0.013 |
| Spon1 | Spondin 1, (f-spondin) extracellular matrix protein | 2.54 | 0.009 |
| Tnc | Tenascin C | 2.52 | 0.079 |
| Plod2 | Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2 | 2.42 | 0.018 |
| Eln | Elastin | 2.38 | 0.020 |
| Col8a1 | Collagen, type VIII, alpha 1 | 2.21 | 0.040 |

Fibrotic genes were up-regulated and androgen-responsive genes were down-regulated in Arom<sup>hum</sup> mice vs. WT mice.

### Table 2. Ingenuity pathway analysis revealed top three up-regulated canonical pathways in Arom<sup>hum</sup> mice

| Top three up-regulated canonical pathways | P value |
|----------------------------------------|--------|
| Hepatic fibrosis/hepatic stellate cell activation | 1.18E-04 |
| Agranulocyte adhesion and diapedesis | 1.18E-03 |
| Parkinson’s signaling | 1.99E-03 |
Arom<sup>hum</sup> mice. K67 immunostaining, although low, could be detected in UAM stromal tissue and was negligible in the QM stromal component of both mice. The highest percentage of K67<sup>+</sup> nuclei was observed in LAM stromal cells of Arom<sup>hum</sup> mice (Fig. 5 A and B). Letrozole treatment strikingly decreased the number of K67<sup>+</sup> fibroblasts in LAM tissue of Arom<sup>hum</sup> mice (Fig. 5C). These results show that estrogen-induced-fibroblast proliferation was increased in vivo in LAM tissue of Arom<sup>hum</sup> mice.

Primary fibroblasts isolated from LAM tissue of WT mice were treated with physiological doses of E2 (0.1 nM to 10 nM) for 24 h; cell proliferation, indicated by proliferating cell nuclear antigen levels, and fibrosis, indicated by ColIα1, were increased in proportion to the dose of estrogen (Fig. 5D). ERα protein levels were decreased after E2 treatment, suggesting the effectiveness of the E2 treatment. Interestingly, RNA microarray analysis of LAM of Arom<sup>hum</sup> mice demonstrated that of the top 33 up-regulated genes, 13 are known to be involved in the fibrotic response (Table 1). The higher expression of the fibrosis-related genes Kiss1, Ren1, Emb, Timp1, Spon2, and Elh in LAM of Arom<sup>hum</sup> mice compared with WT mice was verified by real-time PCR (Fig. 5 E–J). In Arom<sup>hum</sup> mice, mRNA levels of Kiss1, Ren1, Emb, Timp1, and Spon2 in LAM were also significantly higher compared with UAM (Fig. 5 E–J). Microarray analysis showed that the fibrotic pathways were activated in LAM tissue of Arom<sup>hum</sup> mice (Table 2). Furthermore, we treated LAM primary fibroblasts from WT or Arom<sup>hum</sup> mice with E2 in the presence or absence of the E2/ER antagonist ICI 182780 or the ERα-selective E2 antagonist MPP. E2 increased mRNA levels of Kiss1 and Spon2 after a 48-h treatment. ICI 182780 or MPP inhibited the stimulatory effect of E2 on these two fibrotic gene expressions (Fig. 5 K and L and SI Appendix, Fig. S7 C, D, G, and H). ERα knockdown significantly down-regulated mRNA levels of Kiss1 and Spon2 in the presence of E2 treatment (Fig. 5 M and N and SI Appendix, Figs. S6 and S7 K and L). Together, these results demonstrated that E2 excess and higher ERα expression induced not only fibroblast proliferation but also a sharp increase in ECM formation in LAM fibroblasts of Arom<sup>hum</sup> mice.

Arom<sup>hum</sup> Mice also Display Decreased Circulating T Levels and Androgen-Responsive Gene Expression. Intriguingly, we found that circulating T levels were significantly lower in Arom<sup>hum</sup> mice than in WT controls (Fig. 6A) and were restored to levels comparable to those in WT mice after letrozole treatment (Fig. 6B). These data suggest that elevated brain aromatase and estrogen formation in Arom<sup>hum</sup> mice may suppress gonadotropins, leading to decreased testicular T secretion. An alternative interpretation could be that increased aromatase activity leads to T depletion via converting it into E2. This, however, is unlikely because the roughly estimated rate of conversion of serum T to E2 is about 0.3% in Arom<sup>hum</sup> mice (48). T exerts its biological action via binding to the AR. Besides the full-length AR (AR-FL), mRNA levels of AR45, one of the AR variants (49), were also reported to be present in skeletal muscle tissue (50, 51). Using primers that amplify total AR (both AR-FL and AR45) in UAM and LAM tissues, we found similar levels of total AR mRNA in WT and Arom<sup>hum</sup> mice (Fig. 6B). Using an antibody that specifically recognizes only AR-FL, AR-FL immunostaining was observed in both myocytes and stromal cells (Fig. 6C). Interestingly, using a second antibody recognizing both AR-FL and AR45 in mouse and human for immunoblotting, both AR-FL and AR45 proteins were identified in abdominal skeletal muscle tissues (Fig. 6D). Human prostate cancer cell line LNCaP expressed both AR-FL and AR45 and served as positive controls.
Mice (Fig. 6 and 7) encode androgen-response expression pattern, human ERα expression significantly higher in the stromal cells of LAM tissue from hernia patients (H-score: 29.06 ± 5.86) compared with tissues from hernia-free patients (H-score: 7.64 ± 1.59). ERα expression was negligible in myocytes (Fig. 7 B and G). AR-FL expression in LAM myocytes was significantly lower in hernia patients (H-score: 20.88 ± 2.17) than in hernia-free patients (H-score: 59.16 ± 17.39) (Fig. 7 C and H). Stromal cell proliferation indicated by Ki67 staining was significantly increased in hernia patients (1.2%) compared with hernia-free controls (0.3%) (Fig. 7 D and I). Thus, cell-specific ERα expression pattern, cell proliferation, and fibrosis in human indirect inguinal hernia tissue are similar to those observed in the Aromh mouse hernia model.

Discussion

Aromh mice represent a unique and pathologically relevant experimental model to study the relationship between aromatization of androgen to estrogen, the downstream estrogenic and androgenic effects in muscle tissue, and LAM fibrosis and atrophy, leading to hernia development. Human aromatase expression driven by its alternatively used cognate promoters (L4 and to a lesser extent PIID) in Aromh mice resembled the human pattern of age-related increase in aromatase expression and estrogen formation in peripheral tissues, including LAM tissue, and an accompanying decrease in circulating T levels (25). Our data strongly indicate that locally produced E2 acting on highly estrogen-sensitive and ERα-rich LAM fibroblasts led to stromal fibrosis, myocyte atrophy, and eventually inguinal hernia formation. The role of E2 in this phenomenon is clear because the inhibition of E2 production by an aromatase inhibitor prevented hernia formation. The possibly contributing roles of decreased T levels that are observed in Aromh mice and restored to normal with an aromatase inhibitor, however, are less clear. Decreased T levels are possibly due to increased brain E2 levels via the brain expression of human aromatase, leading to decreased gonadotropin and then testicular T secretion. As the incidence of inguinal hernia and peripheral aromatase expression increase with age in men, our findings have a particular clinical significance not only for understanding the mechanisms underlying maintenance of skeletal muscle mass in various body sites, but also for assessing hernia risk and developing strategies for hernia prevention in a subset of elderly men (27–29).

Previous studies showed that postnatal systemic administration of exogenous estrogen or ubiquitous overexpression of a full-length aromatase cDNA with strikingly high circulating estrogen levels led to the formation of scrotal hernias in mice; in utero estrogen exposure was not necessary for this phenotype (9, 54). Our humanized aromatase mouse model is unique in that the estrogenic effect on LAM tissue and hernia formation are primarily mediated via local estrogen production by aromatase activity from the human CYP19A1 (aromatase) gene expressed in the skeletal muscle tissue, with normal circulating E2 levels. This model of muscle atrophy and hernia development is therefore more physiologically relevant to these common pathologies observed in a subset of elderly men.

Estrogen exerts its physiological functions by binding to its receptors ERα, ERβ, and GPR30 (39–41). Studies of ER knockout mice show that ERα is primarily involved in the classic actions of estrogens (i.e., sexual differentiation, fertility, uterine...
function, and lactation) (39, 40). ERβ has been shown to play biological roles in the central nervous system, the immune system, the ovary, and the prostate (55, 56). GPR30, on the other hand, has been linked to certain physiological and pathological effects regulated by estrogen on the central nervous, immune, renal, reproductive, and cardiovascular systems (41, 57–59). Because ERβ and Gpr30 mRNA levels in LAM or UAM tissues are either barely detectable or extremely lower than ERα in our hands, the estrogenic effects on LAM fibrosis and hernia are most likely mediated primarily by ERα signaling. Indeed, we found that ERα mRNA and protein were predominantly present in the prominent perimuscular stromal fibroblast compartment of LAM tissue in both WT and Arom<sup>hum</sup> mice. Additionally, ERα levels in LAM tissue fibroblasts were higher than those in UAM or QM tissues. Moreover, ICI 182780 (which opposes E<sub>2</sub> action via degradation of the ER), MPP (which is an ERα-selective E<sub>2</sub> antagonist), or ERα knockdown diminished estrogenic gene expression in LAM fibroblasts. Thus, we conclude that locally produced estrogen in Arom<sup>hum</sup> LAM tissue is mediated via fibroblastic ERα, giving rise to increased stromal cell proliferation, fibrosis, muscular atrophy, and hernia development. This explains, at least in part, why atrophy and fibrosis develop in LAM tissue of Arom<sup>hum</sup> mice, but not in other muscle groups (i.e., UAM and QM).

Gene-expression profiling allowed us to identify a number of molecular pathways and target genes activated early by E<sub>2</sub>/ERα in LAM tissues before the hernias become manifest. These pathways include ERα-driven fibroblast activation and fibrosis pathways (44–46, 60, 61). Consistent with these findings, E<sub>2</sub> is found to induce fibrosis in pathologic tissues, including gynecomastia, uterine fibroid tumors, and the skin of patients with systemic sclerosis (62–64). Several known E<sub>2</sub>/ERα target genes were highly expressed in LAM tissue of Arom<sup>hum</sup> mice. Moreover, some of these genes, (e.g., Greb1 and Pgr) were selectively induced in primary fibroblasts of LAM tissue, suggesting that these effects took place primarily in the fibroblast compartment of LAM tissue. Greb1 is a chromatin-bound ER coactivator and is essential for ER-mediated transcription (61). Greb1 is also one of the most highly estrogen-inducible genes and correlates well with changes in ER activity following breast cancer treatment (43, 65). Thus, Greb1 may also contribute to fibroblast proliferation in LAM. Moreover, the estrogen-responsive fibrotic genes, including Kiss1, Krt8, Krt7, Spon2, Krt18, Tnc, Plod2, and Eln, were also increased in LAM of Arom<sup>hum</sup> mice (Table 1), as has been reported for several other tissues (66–72). On the other hand, several other well-known estrogen response genes (e.g., Adora1, Tff1, and Susd3) were not induced by aromatase expression in LAM tissue (44, 45, 73), suggesting epigenetic differences between tissues or cell types may account for E<sub>2</sub>/ERα induction of a select group of genes.

T is the major substrate of the aromatase enzyme. T is not only converted by aromatase to E<sub>2</sub> in target tissues for estrogenic action, but is also converted by 5α-reductase-1 or -2 into a potent and nonaromatizable androgen, DHT (74). mRNA levels of 5α-reductase-1 or -2 were undetectable or barely detectable in LAM tissue. Thus, in LAM, androgen action must be provided primarily via an interaction of T with AR. In fact, in double

![Fig. 7](image_url)  
**Fig. 7.** (A) Masson’s trichrome staining (MTS), (B) immunostaining for ERα, (C) AR-FL, and (D) Ki67 in LAM tissue from hernia-free and hernia patients. Yellow arrows indicate atrophic myocytes. Red arrows indicate brown positive staining. (Scale bar, 50 μm; Magnification: Insets in A–D, 40×.) Quantification of the percentage of connective tissue or fibrotic area (E) and myofiber area (F), the average H-score (range 0–300) for ERα in fibroblasts (G) and AR-FL in myocytes (H), and the percentage of Ki67<sup>+</sup> nuclei (I) in fibroblasts from hernia-free and hernia patients. Two-tailed Student’s t test, *P < 0.05, **P < 0.01, n = 6 per group.

![Fig. 8](image_url)  
**Fig. 8.** Schematic demonstrating the effect of a shift from androgen to estrogen action induced by human aromatase gene expression in LAM tissue on fibrosis, myocyte atrophy, and hernia formation in mice.
5α-reductase knockout mice (for both types 1 and 2), T was shown to exert androgenic action via AR (74). T and AR exert anabolic effects on skeletal muscle, resulting in muscle protein synthesis and increased muscle mass (75, 76). Overall, AR-FL seems to be the predominant AR type in both LAM and UAM tissue, suggesting a more important role of AR-FL in both WT and Arom<sup>−/−</sup> LAM tissues. The precise role of AR-FL in abdominal muscle tissue, however, needs further investigation. We also found that serum T was significantly lower in Arom<sup>−/−</sup> mice, which may contribute to decreased androgen action. These results indicate that low circulating T levels, together with local estrogen excess, shift the steroid balance from androgen to estrogen in Arom<sup>−/−</sup> mice or in a subset of elderly men, leading to muscle atrophy and hernia, possibly via decreased muscle mass. In summary, the lower portion of the LAM tissue adjacent to the scrotal opening is particularly enriched with ERα-expressing fibroblasts and thus sensitive to E2. Expression of aromatase in mice, which directly converts circulating T to E2 locally in the muscle or in the brain, leads to stromal proliferation, muscle atrophy, and hernia development in LAM tissue via up-regulation of estrogenic action and down-regulation of androgenic action (Fig. 8). All Arom<sup>−/−</sup> mice uniformly developed fibrosis, myocyte atrophy, and hernia, which was entirely blocked and prevented by an aromatase inhibitor. There are currently no established or novel medical therapies for primary prevention of inguinal hernias associated with skeletal muscle fibrosis and atrophy in a subset of men. In particular, the surgical repair of a recurrent hernia is quite problematic and carries a high risk of treatment failure or recurrence. Intriguingly, our findings uncover a previously unrecognized mechanism for LM fibrosis and atrophy and inguinal hernia formation and open new horizons for drug development to prevent hernia, especially recurrent hernia after surgery in vulnerable populations, such as elderly men. Currently available aromatase inhibitors or analogs of androgen may provide alternative or complementary management modalities in addition to surgical repair.

**Materials and Methods**

Arom<sup>−/−</sup> Mouse Maintenance, Hernia Assessment, and Letrozole Treatment. The Arom<sup>−/−</sup> mouse (FVB/N background) was generated and genotyped in our laboratory, as previously described (34). Arom<sup>−/−</sup> transgenic mice contain the complete human aromatase coding region, a >75-kb promoter region including promoters I.4, I.7, I.1f, I.6, I.3 and PII, and the 3′-polyadenylation site. Mice were maintained on a 14-h light:10-h dark cycle with standard chow (7921: Harlan Teklad) and water available ad libitum. All animal procedures were approved by and conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee at Northwestern University. Animals were randomly used for all experiments in a blinded manner. Hernia development in 32 Arom<sup>−/−</sup> male mice was monitored by weekly visual inspection and palpation from 3 to 26 wk of age. Age-matched WT male littermates were used as controls. Hernia dimensions were measured using a digital caliper, and hernia area was calculated by the formula, area (mm<sup>2</sup>) = length (mm) × width (mm). At the designated end-points, UAM, LAM, and QM from each individual mouse were resected, with one-half of the tissue snap-frozen in liquid nitrogen, and the other half fixed in 4% phosphate-buffered paraformaldehyde for histological and IHC analyses. All tissue and serum samples were collected from mice between 10:00 AM and 12:00 PM (noon) to avoid possible variability in daily hormone fluctuations. In some experiments, male mice were randomly treated with letrozole (10 μg per mouse) using 90-d continuous-release pellets (Innovative Research of America) or control pellets starting at 3 wk of age (77).

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