Faslodex Inhibits Estradiol-Induced Extracellular Matrix Dynamics and Lung Metastasis in a Model of Lymphangioleiomyomatosis

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Lymphangioleiomyomatosis (LAM) is a destructive lung disease primarily affecting women. Genetic studies indicate that LAM cells carry inactivating tuberous sclerosis complex (TSC)–2 mutations, and metastasize to the lung. We previously discovered that estradiol increases the metastasis of TSC2-deficient cells in mice carrying xenograft tumors. Here, we investigate the molecular basis underlying the estradiol-induced lung metastasis of TSC2-deficient cells, and test the efficacy of Faslodex (an estrogen receptor antagonist) in a preclinical model of LAM. We used a xenograft tumor model in which estradiol induces the lung metastasis of TSC2-deficient cells. We analyzed the impact of Faslodex on tumor size, the extracellular matrix organization, the expression of matrix metalloproteinase (MMP)–2, and lung metastasis. We also examined the effects of estradiol and Faslodex on MMP2 expression and activity in tuberin-deficient cells *in vitro*. Estradiol resulted in a marked reduction of Type IV collagen deposition in xenograft tumors, associated with 2-fold greater MMP2 concentrations compared with placebo-treated mice. Faslodex normalized the Type IV collagen changes in xenograft tumors, enhanced the survival of the mice, and completely blocked lung metastases. *In vitro*, estradiol enhanced MMP2 transcripts, protein accumulation, and activity. These estradiol-induced changes in MMP2 were blocked by Faslodex. In TSC2-deficient cells, estradiol increased MMP2 concentrations *in vitro* and *in vivo*, and induced extracellular matrix remodeling. Faslodex inhibits the estradiol-induced lung metastasis of TSC2-deficient cells. Targeting estrogen receptors with Faslodex may be of efficacy in the treatment of LAM.

Keywords: tuberin; estrogen receptor antagonist; matrix metalloproteinase; extracellular matrix

Lymphangioleiomyomatosis (LAM) is a progressive and destructive pulmonary disease with a striking gender predisposition, affecting women almost exclusively. Pathologically, LAM is

CLINICAL RELEVANCE

This study demonstrates that the estradiol receptor antagonist Faslodex completely blocked estradiol-promoted lung metastasis and enhanced the survival of mice carrying xenograft tumors. Faslodex offers potential therapeutic advantages for women with lymphangioleiomyomatosis.

characterized by the diffuse proliferation of abnormal smooth muscle cells and by the cystic destruction of the lung parenchyma (1–4). LAM occurs in 30 to 40% of women with tuberous sclerosis complex (TSC) (5, 6). In a series of patients at the Mayo Clinic, LAM was the third most frequent cause of TSC-related death, after renal disease and brain tumors (7). LAM can also occur in women who do not have germline mutations in the genes *TSC1* or *TSC2* (“sporadic LAM”). Inactivating mutations of both alleles in the *TSC1* or *TSC2* gene have been found in LAM cells from patients with both TSC LAM and sporadic LAM (8, 9).

About 60% of women with the sporadic form of LAM also demonstrate renal angiomyolipomas. The presence of *TSC2* mutations in LAM cells and renal angiomyolipoma cells from women with sporadic LAM, but not in normal tissue, has led to the hypothesis that LAM cells spread to the lungs via a metastatic mechanism, although LAM cells possess a histologically benign appearance (8, 10). The only proven treatment for end-stage LAM is lung transplantation, which carries significant 1-year mortality, and after which LAM can recur in the transplanted lungs (10, 11). Genetic and fluorescent *in situ* hybridization analyses of recurrent LAM after lung transplantation support this “benign metastasis” model (10).

The protein products of *TSC1* and *TSC2*, hamartin and tuberin, respectively, form heterodimers (12, 13) that inhibit the small GTPase Rheb (Ras homologue enriched in brain) via tuberin's highly conserved GTPase-activating domain. The loss of tuberin or hamartin leads to hyperactivation of the mammalian target of rapamycin complex–1 (mTORC1), which has been observed in LAM cells. The female predominance of LAM, coupled with genetic data indicating that LAM cells are metastatic, suggests that estrogen may promote the metastasis of cells with mTORC1 activation. Both LAM cells and angiomyolipoma cells express estrogen receptor- α (ER α) (14). We previously discovered that estradiol increases concentrations of circulating tumor cells and pulmonary metastases of tuberin-null cells in a xenograft model of LAM (15). Tumor metastasis is a multistep event involving the dissemination of tumor cells from the primary tumor to seed at remote tissue and form metastatic lesions. One of the initial steps of metastasis involves the degradation of the basement membrane. Matrix metalloproteinases (MMPs) are involved in the degradation of extracellular matrix (ECM), thereby facilitating tumor-cell invasion, metastasis, and angiogenesis (16). Elevated concentrations of MMP1, MMP2, MMP9, MMP14 (17–20), and

(Received in original form November 21, 2012 and in final form January 27, 2013)

This work was supported by the LAM Foundation, the Adler Foundation, the LAM Treatment Alliance, grant DK51052 from the National Institute of Diabetes and Digestive and Kidney Diseases, and National Heart Lung and Blood Institute grants HL31147 (E.P.H.) and HL098216 (J.J.Y.).

Author Contributions: C.L. performed immunoblotting analyses of xenograft tumors. X.Z., J.D.M., and E.K.S. performed real-time RT-PCR analyses. E.Z. performed real-time RT-PCR analyses, performed and analyzed trichrome staining data, and quantified Ki67, estrogen receptor- α , and Type IV collagen in xenograft tumors. A.P. analyzed xenograft tumors for drug effects. Y.S. measured matrix metalloproteinase–2 activity and examined the effects of Faslodex on mitogen-activated protein kinase activation in xenograft tumors. T.A.M. performed immunohistochemical staining. J.J.Y. and E.P.H. contributed to the design, implementation, and supervision of the study, and wrote the manuscript. All authors retained full access to the data, and approved the final version of the manuscript.

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Am J Respir Cell Mol Biol Vol 49, Iss. 1, pp 135–142, Jul 2013

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Originally Published in Press as DOI: 10.1165/rcmb.2012-0476OC on March 22, 2013

Internet address: www.atsjournals.org

cathepsin K (21) have been observed in LAM lung nodules. MMP9 was implicated as a biomarker for monitoring therapeutic responses to doxycycline in one LAM patient (22). MMP2 is one of the MMPs that play a critical role in the breakdown of ECM components, including Type IV collagen. Glassberg and colleagues reported that estrogen induced MMP2 expression and activity in LAM-derived smooth muscle cells, which were blocked by the nonspecific MMP inhibitor doxycycline *in vitro* (23). Moir and colleagues found that LAM-derived smooth muscle cells produced a higher concentration of MMP2, which was suppressed by doxycycline treatment *in vitro* (24). High concentrations of MMP2 transcript have been observed in cultured tuberin-null LAM patient-derived cells (25). Despite these findings, the molecular and cellular mechanisms that underlie the female predominance of LAM remain incompletely understood.

We report here that estradiol increases MMP2 expression and activity in TSC2-deficient cells, which are blocked by the estrogen receptor antagonist Faslodex, a drug approved by the United States Food and Drug Administration for treating breast cancer (26–28). *In vivo*, estradiol alters the extracellular matrix organization, increases the expression MMP2, and reduces the accumulation of extracellular matrix protein Type IV collagen in TSC2-deficient xenograft tumors in mice. Faslodex normalizes extracellular matrix organization, inhibits estradiol-promoted lung metastases, and enhances the survival of estradiol-treated mice bearing xenograft tumors of TSC2-deficient cells. Based on these preclinical studies, we propose that the estrogen receptor antagonist Faslodex may be of therapeutic benefit in LAM.

MATERIALS AND METHODS

Cell Culture and Reagents

Eker rat uterine leiomyoma-derived (ELT3) cells (29) were cultured in IIA complete medium. We used 17- β -estradiol (Sigma Chemical Co., St. Louis, MO), PD98059 (Cell Signaling, Boston, MA), ICI182780 (Tocris, Minneapolis, MN), or GM6001 (ENZO Life Science International, Farmingdale, NY) as indicated.

Animal Studies

All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Brigham and Women's Hospital. Female ovariectomized and male CB17-*SCID* mice were used as described previously (15). Faslodex (1 mg/day, intramuscular injection) treatment was initiated 1 day after cell inoculation.

Immunoblotting and Antibodies

The antibodies used included anti-MMP2 (Chemicon, Billerica, MA), anti-Type IV collagen (Abnova, Walnut, CA or Abcam, Cambridge, MA), anti-Ki67 (BioGenex), anti- β -actin (Sigma Chemical Co.), anti-ER α (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-S6 (Ser235/236), anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204), and anti-p44/42 MAPK (Cell Signaling).

Immunohistochemistry

Sections were deparaffinized, incubated with primary antibodies and biotinylated secondary antibodies, and counterstained with Gill's hematoxylin. Trichrome staining was performed using reagents from Polysciences (Warrington, PA).

Gelatin Zymography

Conditioned media from cultured cells were collected and subjected for 10% SDS-PAGE containing 0.1% gelatin (Invitrogen, Carlsbad, CA).

Whole lysates were extracted from xenograft tumors, using m-PER buffer (Pierce) without protease inhibitors. The MMP2 standard was acquired from Chemicon.

Real-Time RT-PCR

RNA from cultured cells and xenograft tumors was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA). Gene expression was quantified by using One-Step qRT-PCR Kits (Invitrogen) in a Real-Time PCR System (Applied Biosystems, Grand Island, NY) and normalized to β -actin control, or by using SYBR green (SG) quantitative PCR on an ABI 7500 HT machine (Applied Biosystems) and normalized to glyceraldehyde 3-phosphate dehydrogenase.

Matrigel Invasion Assay

ELT3 cells were preincubated with estradiol or vehicle for 24 hours, and then seeded into a six-well BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ) in the presence of GM6001 or control. Twenty-four hours later, invading cells were stained with crystal violet and quantitated.

Statistical Analyses

Statistical analyses were performed using the Student *t* test when comparing two groups. Results are presented as the means $\pm$ SDs of experiments performed in triplicate. The log-rank test was performed for the Kaplan-Meier survival plot.

RESULTS

Estradiol Increases MMP2 Expression and Activity in TSC2-Deficient ELT3 Cells *In Vitro*

To determine whether estradiol regulates MMP2 expression in TSC2-deficient cells, we treated cultured ELT3 cells with estradiol, and analyzed the concentrations of MMP2 by immunoblotting. Estradiol increased MMP2 accumulations by 5- to 7-fold, with the highest concentrations at 2 hours after treatment (Figure 1A). Furthermore, we found that preincubation with the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 for 30 minutes, or the estradiol receptor antagonist ICI182780 (Faslodex or Fulvestrant) for 4 hours, strongly blocked estradiol's enhancement of MMP2 accumulation after 2 hours of treatment (Figures 1B and 1C), consistent with our previous finding that estradiol stimulates p42/44 MAPK in ELT3 cells (15). Furthermore, we found that ICI182780 (Faslodex) inhibited the estradiol-stimulated phosphorylation of p44/42 MAPK (Figure 1D).

To determine whether MMP2 activity and expression are regulated by estradiol, we used gelatin zymography. Within 5 minutes of estradiol stimulation, the active MMP2 concentration was increased by 3-fold. This estradiol-stimulated MMP2 activity was sustained through 120 minutes of stimulation (Figures 1E and 1H). At 2 hours, MMP2 activity was increased by 2.5-fold, and was blocked by PD98059 (Figures 1F and 1H). To determine whether estradiol-stimulated MMP2 activity is mediated by the estradiol receptor, we treated ELT3 cells with ICI182780 for 4 hours, followed by estradiol stimulation. At 24 hours, MMP2 activity was increased by 8-fold (Figure 1F). ICI182780 almost completely blocked estradiol-stimulated MMP2 activity ($P < 0.01$) (Figures 1G and 1H).

Estradiol Reduces Extracellular Matrix Organization

MMPs are zinc-containing endopeptidases that degrade components of the ECM. To determine whether estradiol regulates ECM integrity in the tumor stroma, we examined the morphology of the ECM in primary tumors from ovariectomized female

Figure 1. Estradiol increases matrix metalloproteinase (MMP)-2 expression and activity in tuberous sclerosis complex (TSC)-2-deficient Eker rat uterine leiomyoma-derived (ELT3) cells *in vitro*. (A) ELT3 cells were grown in phenol red-free and serum-free media for 24 hours, and then stimulated with 1 μ M estradiol (E_2) for 0, 0.5, 2, 4, or 12 hours. Concentrations of MMP2 were measured by immunoblot. A β -actin immunoblot was included as a loading control (ctrl). (B) ELT3 cells were incubated with the mitogen-activated protein kinase kinase (MEK1/2) inhibitor PD98059 for 30 minutes, or (C) the estrogen receptor antagonist ICI182780 (ICI) for 4 hours, and then treated with 10 nM E_2 for 0, 2, or 4 hours. (D) MMP2 and phospho-44/42 mitogen-activated protein kinase (P-MAPK) were measured by immunoblotting. (E) Conditioned media from ELT3 cells after 10 nM E_2 stimulation for 0, 5, 15, or 120 minutes were collected, and protein concentrations were measured. MMP2 activity was examined using gelatin zymogram gels. Std, standard. (F) ELT3 cells were preincubated with PD98059 (PD) for 30 minutes, followed by 10 nM E_2 stimulation for 0, 0.25, 2, or 4 hours. (G) ELT3 cells were preincubated with ICI182780 (ICI) for 4 hours, followed by 10 nM E_2 stimulation for 0 and 24 hours. The conditioned media were collected. MMP2 activity was examined using gelatin zymogram gels. Densitometry analyses show the fold of change in MMP2 activity. * P < 0.05 and ** P < 0.01, Student *t* test. (H) MMP2 activity in conditioned media collected from PD98059-treated and ICI182780-treated cells was examined using a gelatin zymogram gel.

and intact male mice treated with placebo or estradiol. Compared with placebo-treated animals, the xenograft tumors from estradiol-treated animals exhibited a disruption of the ECM network (Figures 2Aa, 2Ab, 2Ba, and 2Bb), associated with a 60% reduction of Type IV collagen in both female and male mice (Figures 2Ac, 2Ad, 2Bc, and 2Bd).

Estradiol Increases MMP2 Accumulation in Tumor Cells *In Vivo*

Type IV collagen can be degraded by MMPs, and particularly by MMP2. To determine whether MMP2 is associated with the estradiol-dependent behavior of TSC2-deficient cells, we measured the expression of MMP2 in tumor lysates with immunoblotting. Estradiol increased the concentrations of MMP2 by 1.8-fold and 2.5-fold in tumors from female and male mice, respectively (P < 0.05; Figures 3A and 3B).

Faslodex Blocks Estradiol-Promoted Lung Metastases *In Vivo*

To investigate the *in vivo* effects of inhibiting the estradiol receptor pathway in estradiol-induced alterations of the ECM, we used the estradiol receptor antagonist Faslodex (Fulvestrant). Faslodex is approved by the United States Food and Drug Administration for the treatment of ER-positive metastatic breast cancer. Mice were implanted with either placebo or estradiol pellets 1 week before cell inoculation, and were then treated with Fulvestrant (1 mg/day, intramuscular). Eight weeks after inoculation, estradiol-treated mice demonstrated

a mean tumor area of 251 ± 65 mm², whereas placebo-treated mice demonstrated a mean tumor area of 107 ± 47 mm² (P < 0.05) (Figure 4A), consistent with our previous work (15). Interestingly, Faslodex exerted no effect on estradiol's promotion of the growth of primary tumors of TSC2-deficient cells. Mice treated with Faslodex plus estradiol (E_2) demonstrated a mean tumor area of 259 ± 83 mm² (P = 0.03, Faslodex plus E_2 to Faslodex plus placebo; P = 0.86, Faslodex plus E_2 to E_2) (Figure 4A). To confirm the effects of Faslodex *in vivo*, we performed Western blot analyses of ER α in xenograft tumors. Faslodex decreased the concentration of ER α protein by 40% (Figure 4B). We also quantified ER α positivity in immunohistochemistry (IHC)-stained sections, and found that estradiol enhanced nuclear ER α by nearly 2-fold, and this enhancement was blocked by Faslodex treatment (Figures 4C and 4D), further confirming that Faslodex was effective *in vivo*. Faslodex treatment did not affect phosphorylated ribosomal protein S6 (Figures 4B and 4C). To examine the effects of Faslodex on the growth of xenograft tumors, we performed Ki67 staining. Estradiol increased Ki67 nuclear positivity by 50% (Figures 4C and 4D), whereas Faslodex did not alter Ki67 positivity (Figures 4C and 4D). Furthermore, Faslodex treatment decreased the estradiol-stimulated phosphorylation of p44/42 MAPK by Western blotting (Figure 4E) and by immunohistochemical staining (Figure 4F). These results demonstrate that Faslodex was effective in inhibiting the estrogen receptor, but did not alter cell growth in the primary tumors.

Given this lack of effect on primary tumor growth, the impact of Faslodex on lung metastases in this model is striking and unexpected. Faslodex completely blocked lung metastases in the

Figure 2. Estradiol disrupts extracellular matrix organization in TSC2-deficient xenograft tumors. Primary tumor sections from placebo-treated and estradiol-treated female mice (A) and male mice (B) were stained with hematoxylin and eosin (H&E) (Aa and Ab, and Ba and Bb) and Type IV collagen (Ac and Ad, and Bc and Bd). The percentage of positive Type IV collagen (Col IV) staining in tumor stroma was quantified from five random fields per section, using Metamorph (MDS Analytical Technologies, Inc., Center Valley, PA). * $P < 0.05$, Student's t test.

presence of estradiol ($P < 0.05$; Figure 4G), and increased the survival of estradiol-treated mice ($P < 0.05$; Figure 4H). These results point toward a fundamental role of the estrogen receptor in the dissemination and invasion of TSC2-deficient cells.

Faslodex Normalizes Estradiol-Induced ECM Disruption and Inhibits MMP2 Expression *In Vivo*

Histologically, xenograft tumors from Faslodex plus placebo-treated animals exhibited dense bundles of collagen fibers (Figure 5A). The xenograft tumors from animals treated with Faslodex plus estradiol exhibited a well-maintained ECM network (Figure 5A), similar to that of placebo-treated animals (Figures 2 and 5A). We further examined the abundance of collagen fibers using trichrome staining, and found that estradiol reduced trichrome staining in tumors, whereas Faslodex treatment restored trichrome staining to placebo-treatment concentrations (Figure 5B). We next measured the concentrations of MMP2 in xenograft tumors, using Western blot analysis. Estradiol elevated

Figure 3. Estradiol increases MMP2 expression and activity in TSC2-deficient ELT3 cells *in vivo*. (A) Concentrations of MMP2 protein were measured by immunoblotting in primary tumors from placebo-treated ($n = 6$) and estradiol-treated ($n = 7$) female SCID mice, and (B) from placebo-treated ($n = 4$) and estradiol-treated ($n = 4$) male SCID mice. A β -actin immunoblot was included as a loading control. Scatterplots show the relative concentrations of MMP2 normalized to β -actin. * $P < 0.05$, Student t test.

MMP2 protein concentrations relative to placebo, and Faslodex plus estradiol treatment reduced the MMP2 protein concentration compared with estradiol treatment (Figure 5C). The activity of MMP2 was elevated by estradiol treatment, and decreased by Faslodex plus estradiol treatment (Figures 5D and 5E). These data indicate that Faslodex reverses estradiol-induced MMP2 expression and activity *in vivo*.

Faslodex Inhibits the Estradiol-Enhanced Expression of ECM Genes

To determine how Faslodex normalized ECM organization *in vivo*, we examined the expression of seven ECM genes known to be regulated by estradiol, including collagen Type VIII α 1 (*COL8A1*), laminin γ 2 (*LAMC2*), semaphorin 3A (*SEMA3A*), spondin 2 (*SPON2*), fibrillin 1 (*FBNI*), microfibrillar-associated protein 4 (*MFAP4*), and extracellular superoxide dismutase (*SOD3*). Xenograft tumors from estradiol-treated mice expressed higher concentrations of *COL8A1* (18-fold), *LAMC2* (1.8-fold), *SEMA3A* (2.5-fold), *SPON2* (4-fold), and *MFAP4* (2.5-fold) compared with tumors from placebo-treated mice ($P < 0.05$; Figure 6A). These increases were inhibited by Faslodex treatment. Estradiol decreased the expression of the Type IV collagen gene *Col4A5* by 3-fold, which was reversed by Faslodex treatment (Figure 6B). To determine whether the action of estradiol on ECM integrity was mediated by MMPs, we performed a Matrigel invasion assay. We found that estradiol stimulated the invasion of TSC2-deficient ELT3 cells. The pretreatment of the cells with GM6001, a matrix metalloproteinase inhibitor, blocked estradiol-induced invasion by 60% (Figure

Figure 4. Faslodex blocks estradiol-promoted lung metastases of TSC2-deficient cells. ELT3 cells were subcutaneously injected into female ovariectomized mice implanted with estradiol or placebo pellets. Animals were treated with Faslodex (1 mg/kg/day by intramuscular injection) starting 1 day after cell inoculation. (A) The tumor area was calculated at 8 weeks after cell inoculation. (B) Immunoblot analyses of estrogen receptor- α (ER α) and phospho-S6 (P-S6) (serine 235/236) were performed in tumors from E₂ or E₂ plus Faslodex (E₂ + F)-treated mice. A densitometry analysis of ER α was performed. (C) Tumor sections were stained with ER α , phospho-S6 (P-S6) (Ser235/236), and Ki67. (D) Percentages of cells with nuclear immunoreactivity for ER α and Ki67 were scored from five random fields per section. (E) Immunoblot analysis of phospho-p44/42 MAPK (threonine 202/tyrosine 204) was performed in tumors from all treatment groups. (F) Tumor sections of E₂ or E₂ plus Faslodex (E₂ + F)-treated mice were stained with phospho-p44/42 MAPK (threonine 202/tyrosine 204). (G) The numbers of lung metastases in female mice were scored: placebo (P) ($n = 10$), E₂ ($n = 9$), placebo plus Faslodex (P + F; $n = 5$), and E₂ plus Faslodex (E₂ + F; $n = 4$) mice. * $P < 0.05$, Student t test. (H) Kaplan-Meier analysis of overall survival in mice bearing xenograft tumors. The log-rank test was performed for the Kaplan-Meier survival plot. Differences were considered significant at $P < 0.05$. NS, no significance.

6C), consistent with our hypothesis that MMPs mediate the metastatic effects of TSC2-deficient cells.

DISCUSSION

We and others used ELT3 cells as a model of LAM because they are smooth muscle-derived, express ER α and the progesterone receptor, and respond to estradiol stimulation *in vitro* and *in vivo* (29, 30). We previously found that estradiol promotes

the metastasis of TSC2-deficient ELT3 tumors (15). We report here that estradiol alters the architecture of ELT3 subcutaneous xenograft tumors. This alteration was associated with decreased Type IV collagen and increased cellular MMP2. We also found a marked increase of MMP2 transcript levels in xenograft tumors from estradiol-treated mice. *In vitro*, estradiol enhanced the expression, accumulation, and activity of MMP2 in a MEK1/2-dependent manner. In mice bearing xenograft tumors, the administration of the estrogen receptor antagonist Faslodex

Figure 5. Faslodex normalizes estradiol-induced extracellular matrix (ECM) disruption and MMP2 expression *in vivo*. ELT3 cells were subcutaneously injected into female ovariectomized mice implanted with estradiol or placebo pellets. Animals were treated with Faslodex (1 mg/kg/day by intramuscular injection), starting 1 day after cell inoculation. (A) Primary tumor sections from female mice were stained with hematoxylin and eosin. (B) Collagen fibers in primary tumors were examined using trichrome staining. (C) In xenograft tumors from placebo (P), estradiol (E₂), and estradiol plus Faslodex (E₂ + F)-treated mice ($n = 3$), MMP2 protein concentrations were examined using immunoblot analyses. (D) MMP2 activity was examined using gelatin zymogram gels. A densitometry analysis was performed, and showed the fold of change in MMP2 activity. * $P < 0.05$ and ** $P < 0.01$, Student t test. (E) MMP2 activity in xenograft tumors from estradiol-treated mice was examined by gelatin zymogram gel.

normalized extracellular matrix organization, inhibited estradiol-promoted lung metastases, and enhanced the survival of estradiol-treated mice bearing xenograft tumors.

Metastasis is a multistep process (31), and steroid hormones may promote the metastasis or dissemination of LAM cells to the lung through several distinct mechanisms. Our data support a model in which estradiol induces disruption of the ECM of LAM nodules and reduces concentrations of Type IV collagen, thereby promoting the dissemination of LAM cells (Figure 6D). This is in agreement with our previous finding that estradiol increased the concentration of circulating disseminated tumor cells (15), and with the identification by Crooks and colleagues of circulating LAM cells in the blood, urine, and chylous fluid of patients with LAM (32). The degradation of elastic fibers has been observed in regions of smooth muscle cells within LAM nodules (17), and Type IV collagen has been found to colocalize with MMP2 in LAM nodules (18). Collectively, these findings reveal a possible connection between ECM alterations and MMP accumulation during the pathogenesis of LAM. Furthermore, tuberin-null, LAM-associated,

angiomyolipoma-derived cells were previously observed to express higher MMP2 transcript levels than TSC2-reexpressing cells (25). Our data indicate not only that MMP2 concentrations are increased in TSC2-deficient cells, but also that estradiol further enhances MMP2 activity. This is consistent with studies by Glassberg and colleagues in LAM-derived smooth muscle cells (23), and by Goncharova and colleagues in TSC2-null lung lesions in a preclinical model of LAM (2). Collectively, these findings and our *in vivo* data support the notion that estradiol synergizes with the loss of TSC2 to enhance MMP2 expression.

In the most striking result of this study, the estradiol receptor antagonist Faslodex completely blocked estradiol-promoted lung metastasis and enhanced the survival of mice carrying xenograft tumors, while exerting no effect on the primary subcutaneous tumor. Faslodex, which is approved by the United States Food and Drug Administration for the treatment of breast cancer, is an estradiol receptor antagonist that disrupts ligand binding, receptor dimerization, and nuclear translocation, and accelerates the degradation of both ER α and ER β (27, 28).

Figure 6. Faslodex inhibits estradiol-enhanced expression of ECM genes. ELT3 cells were subcutaneously injected into female ovariectomized mice implanted with estradiol or placebo pellets. Animals were treated with Faslodex (1 mg/kg/day by intramuscular injection), starting 1 day after cell inoculation. RNA was isolated from primary tumors. (A and B) Transcript levels of collagen Type VIII α 1 (COL8a1), laminin γ 2 (LAMC2), semaphorin 3A (SEMA3A), spondin 2 (SPON2), fibrillin 1 (FBN1), microfibrillar-associated protein 4 (MFAP4), extracellular superoxide dismutase (SOD3), and COL4A5 were measured using real-time RT-PCR in primary tumors ($n = 3$). * $P < 0.05$, placebo versus estradiol. ** $P < 0.05$, E₂ versus E₂ + F, Student t test. (C) Matrigel invasion assay. ELT3 cells (20,000 cells) were seeded in a Matrigel-coated upper chamber and treated with E₂ (10 nM) or E₂ plus GM6001 (2 μ M). Cell invasion through the Matrigel after 24 hours of incubation was detected by crystal violet staining and quantitated. Data represent means $\pm$ SEMs ($n = 3$ in triplicate). * $P < 0.05$, E₂ versus E₂ + GM6001, Student t test. (D) Schematic illustration of the relationship between estradiol, Faslodex, and ECM remodeling. Cont, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The mechanisms through which Faslodex exerts this selective effect on metastasis will require further investigation. The interplay between ER α and ER β could be involved. LAM cells are believed to express both ER α and ER β (23). Although ER α plays a pro-proliferative role in most cell types, ER β can exert an antiproliferative action by controlling cell-cycle regulators (33) (34). Faslodex offers potential therapeutic advantages in LAM, compared with tamoxifen and other selective ER modulators (SERMs), because it does not involve estrogen agonist activity. We previously found that tamoxifen, a SERM, activated both genomic and nongenomic estradiol signaling pathways, and stimulated the growth of LAM patient-derived angiomyolipoma cells (35).

LAM is a disease of women in which the estrogen receptor is expressed in the lesional cells. Our data represent, to our knowledge, the first preclinical study of an estrogen-targeted therapy for LAM. The Multicenter International LAM Efficacy of Sirolimus Trial demonstrated that the mTORC1 inhibitor sirolimus stabilizes lung function and improves quality of life in women with LAM. However, upon drug withdrawal, the decline of lung function resumed (36), and women whose lung function declines

to the point of needing lung transplantation cannot remain on sirolimus while awaiting a donor lung because of the effects of sirolimus on wound healing. Therefore, despite tremendous progress in LAM, the urgent need remains for improved therapeutic strategies. Our promising preclinical results with Faslodex add to a growing list of agents approved by the United States Food and Drug Administration for their potential efficacy in LAM, including simvastatin (37), chloroquine (38), and doxycycline (39). Faslodex may be of particular benefit in premenopausal women, in whom higher estradiol concentrations may be associated with higher rates of lung function decline. Finally, we note that the mechanism of action and the toxicity profile of Faslodex are distinct from those of most other modalities under consideration, providing a novel opportunity for therapeutic benefit both alone and in combination with other agents.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Mr. Bonna Ith and Dr. Mark Perrella for specimen preparation. The authors also thank Damir Khabibullin, Chunrong Wang, and Faina Myachina for valuable technical assistance.

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