Sex-specific lung diseases: effect of oestrogen on cultured cells and in animal models

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ABSTRACT Sex prevalence in lung disease suggests that sex-specific hormones may contribute to the pathogenesis and/or progression of at least some lung diseases, such as lung adenocarcinoma, lymphangioleiomyomatosis (LAM) and benign metastasising leiomyoma (BML). Oestrogen is an important hormone in normal lung development and in the pathogenesis of female predominant pulmonary diseases. *In vivo* and *in vitro* studies have facilitated our understanding of disease pathogenesis and discovery of potential therapeutic targets. Oestrogen promoted disease progression in cell and animal models of lung adenocarcinoma, LAM and BML. Specifically, oestrogen enhanced tumour growth and metastasis in animal models of these diseases. Furthermore, 17β-estradiol (E_2), the most abundant form of oestrogen in humans, increased the size and proliferation of cultured cells of lung adenocarcinoma and LAM. Coupled with the known mechanisms of oestrogen metabolism and signalling, these model systems may provide insights into the diverse effects of oestrogen and other hormones on lung diseases. Anti-oestrogen treatments that target key events of oestrogen synthesis or signalling, such as aromatase activity, oestrogen receptors and signalling pathways, may offer additional opportunities for clinical trials.

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Introduction

Sex differences in lung disease populations suggest that sex-specific hormones, *e.g.* oestrogen, play a role in disease pathogenesis (table 1, fig. 1) [1–12]. Asthma, one of the most common lung diseases, is diagnosed more frequently in males than females before puberty. However, asthma is diagnosed more frequently in adult females [13–15]. Chronic obstructive pulmonary disease (COPD), once considered a disease of males, is increasingly prevalent among females; in contrast, the proportion of males diagnosed with COPD appears to have plateaued or decreased [16, 17]. Female prevalence of COPD has been associated with the increased use of tobacco [18, 19]. The increase of tobacco-related lung cancer appears to parallel the incidence of COPD in females [20].

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TABLE 1 Sex prevalence in lung diseases

Pulmonary disease	First author [ref.]	Subjects n	Male:female ratio	Females %
LAM	Ryu [1]	230	0:230	100
BML	Pitts [2]	100	0:100	100
Lung adenocarcinoma	Muscat [3]	337	69:126	65
PAH	Badesch [4]	1166	230:936	80
CT-ILD	Cottin [5]	34	10:24	70
IPF	Gribbin [6]	920	568:352	38
	Baumgartner [7]	248	149:99	40
ILD	Kornum [8]	21 765	12 639:9126	42
	Coultas [9]	258	136:122	47
PLCH	Vassallo [10]	102	40:62	61
NSIP	Travis [11]	67	22:45	67
	Travis [11]	386	189:197	51
RB-ILD	Portnoy [12]	32	15:17	53

LAM: lymphangioleiomyomatosis; BML: benign metastasising leiomyoma; PAH: pulmonary arterial hypertension; CT-ILD: connective tissue-associated interstitial lung disease; ILD: interstitial lung disease; PLCH: pulmonary Langerhans' cell histiocytosis; NSIP: nonspecific interstitial pneumonia; RB-ILD: respiratory bronchiolitis-associated interstitial lung disease.

Although sex specificity of some lung diseases is apparent, the lack of comprehensive studies and epidemiological data poses a major challenge in determining sex differences in disease populations. Both lymphangioleiomyomatosis (LAM) and benign metastasising leiomyoma (BML) have been primarily diagnosed in females and, thus, female hormones seem likely to contribute to disease progression [1, 2]. In agreement, there are only a few isolated case reports that document the occurrence of clinically significant LAM in males [21, 22]. Female predominance was also apparent in lung adenocarcinoma [3], pulmonary arterial hypertension [4] and connective tissue-associated interstitial lung disease (ILD) [5], whereas male predominance was observed in idiopathic pulmonary fibrosis [6, 7]. Evidence of sex predominance is inconclusive for ILD [8, 9], pulmonary Langerhans' cell histiocytosis [10], nonspecific interstitial pneumonia [11], and respiratory bronchiolitis-associated ILD [12]. Potential contributions of oestrogen to disease pathogenesis may be better assessed through additional research using model systems.

Cultured cells and animal models can be valuable for exploration of disease pathogenesis, progression and underlying mechanisms. Cultured cells are often used to elucidate the biochemical mechanisms that dictate physiological phenomena in lung diseases, as well as to evaluate effects of endogenous or exogenous oestrogen on cellular biology. Experimental small animals, particularly rats and mice, are frequently used as models to investigate human lung physiology and pathophysiology. Methodologies for experimentally altering oestrogen levels either surgically (*e.g.* ovariectomy) or chemically (*e.g.* gonadotropin-releasing hormone agonists or oestrogen inhibitors) can provide important insights into sex effects on lung diseases.

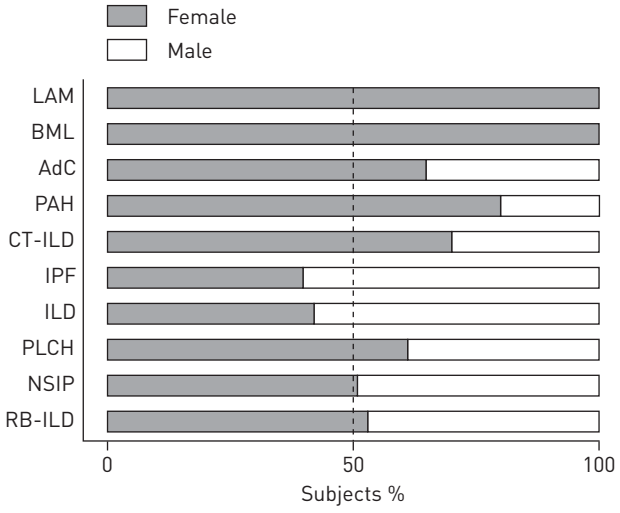

FIGURE 1 Sex prevalence among adults with lung disease. LAM: lymphangioleiomyomatosis; BML: benign metastasising leiomyoma; AdC: lung adenocarcinoma; PAH: pulmonary arterial hypertension; CT-ILD: connective tissue-associated interstitial lung disease; IPF: idiopathic pulmonary fibrosis; ILD: interstitial lung disease; PLCH: pulmonary Langerhans' cell histiocytosis; NSIP: nonspecific interstitial pneumonia; RB-ILD: respiratory bronchiolitis-associated interstitial lung disease. - - - -: 50:50 female:male ratio.

Oestrogen metabolism and signalling in lung diseases

Oestrogen, a female hormone that is important in normal lung development, enhances growth of female-specific tumours, such as breast cancer and endometrial adenocarcinoma [23, 24]. 17β-estradiol (E_2), the most abundant form of oestrogen in females, and other steroid hormones are synthesised from cholesterol [25]. Aromatase (CYP19) is a cytochrome P-450 enzyme that catalyses the rate-limiting conversion of testosterone to E_2 [26–28]. E_2 can be metabolised by the CYP1A1 and CYP1B1 enzymes to catechol oestrogens, 2-hydroxyestradiol (2-HE) and 4-hydroxyestradiol (4-HE), which can be further metabolised to methoxyestrogens (*e.g.* 2-methoxyestradiol (2-ME)) *via* catechol-*O*-methyltransferase [29]. E_2, acting through oestrogen receptors ERα and ERβ, has both genomic and nongenomic effects [30, 31]. Genomic actions result from alteration of gene expression, such as transcription of the vascular endothelial growth factor (VEGF)-A gene [32, 33], which is mediated by binding of E_2-bound nuclear receptors to the oestrogen-response element. Nongenomic activities reflect the more rapid activation of signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) pathways [30, 31, 34].

Lung adenocarcinoma

Lung cancer is now the leading cause of cancer-related death in females [20]. Although smoking remains the primary risk factor for lung cancer, female predominance in lung adenocarcinoma, the most common histological subtype of nonsmall cell lung cancer (NSCLC), in both smokers and nonsmokers suggests that female hormones also have a significant role in its pathogenesis [35–38]. The decrease in risk for this lung cancer subtype after menopause also supports the hypothesis that endogenous oestrogen predisposes females to the disease [39].

Oestrogen synthesis and signalling have been shown to contribute to the growth and proliferation of lung adenocarcinoma *in vitro*. Aromatase activity was detected in human NSCLC cell lines, primary tumour tissues and metastases [40]. The presence of aromatase enabled lung carcinoma cells to produce their own oestrogen and, thus, is linked to the poor survival rates in females [41–43]. Numerous studies have confirmed the presence of functional ERα and ERβ at nuclear and cytoplasmic sites in lung adenocarcinoma and other NSCLC cells [39, 44–48]. Interaction of ERs, progesterone receptors and growth factor signalling was suggested to contribute to clinical outcomes of lung adenocarcinoma [49]. Interaction of both genomic and nongenomic actions was proposed to promote cell growth [48, 50]. Although the functions of the receptors remain incompletely understood, the presence of ERs, particularly ERβ, in NSCLC cell lines and primary cells was associated with poor survival outcomes for lung adenocarcinoma in both sexes [42, 51–54].

Growth and proliferation *in vitro* of human NSCLC cell lines and primary lung adenocarcinoma cells were enhanced by oestrogen-stimulated gene expression in a receptor-dependent manner [48, 55–58]. *In vitro*, ERα or ERβ levels were correlated with increased NSCLC proliferation [56, 59]. Proliferation of normal lung fibroblasts and lung tumour cells *in vitro* was similarly induced by oestrogen in a dose-dependent manner [60]. Oestrogen accelerated growth of lung adenocarcinoma xenografts in mice through increased VEGF secretion and angiogenesis [61]. Oestrogen-promoted tumour progression in a mouse model of lung adenocarcinoma induced with *K-ras* oncogene activation and *p53* tumour suppressor gene deletion has been reported [62]. In that study, pharmacological administration of oestrogen had increased the number of tumours and tumour volume, whereas tumour development patterns were similar in ovariectomised female and male mice, consistent with the regulation of tumour progression by endogenous oestrogen [62].

A greater incidence of lung cancer-related death in female than male double-mutant *K-ras* and *p53* mice strongly suggests that oestrogen may also contribute to lung metastasis [63]. Oestrogen facilitates lung metastasis by affecting tumour cells in the circulation, as well as by modulating host factors, *i.e.* angiogenesis, vascular permeability and stromal effects [64]. The ER antagonist tamoxifen was shown to decrease the expression of matrix metalloproteinase (MMP)-9 and thus the metastatic capability of ER-positive NSCLC cell lines [65]. Further details of anti-oestrogen treatment studies are discussed below. Although the effects of oestrogen on metastasis of lung adenocarcinoma are not completely understood, the findings mentioned above are consistent with stimulatory roles of oestrogen on the pathogenesis of lung adenocarcinoma.

KATO *et al.* [66] reported a murine model of lung adenocarcinoma that resulted from post-translational protein modification by ADP-ribosylation, a reaction in which the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD^+) is transferred to an acceptor (fig. 2). Bacterial toxins, including *Pseudomonas aeruginosa* cytotoxins and pertussis toxins [67], employ ADP-ribosylation to disrupt normal cellular metabolic or biosynthetic pathways. Human and other mammalian tissues use ADP-ribosylation to control metabolic, biosynthetic and regulatory pathways [67, 68]. ADP-ribosyltransferases catalyse the modification of acceptors [69–71], and hydrolases cleave the ADP-ribose–acceptor bond [72–76]. ADP-ribosylarginine

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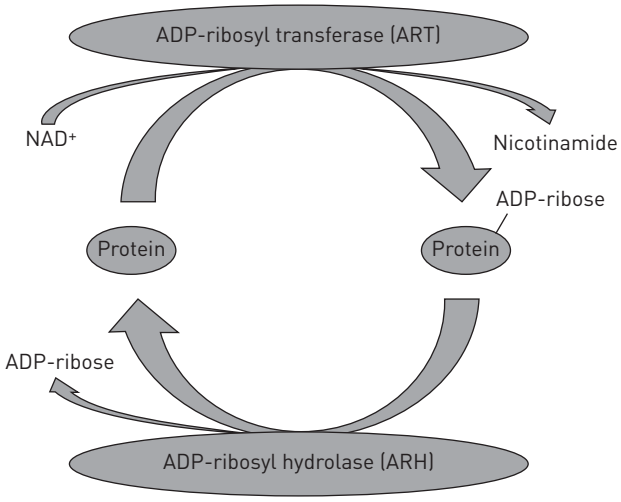

FIGURE 2 The mono-ADP-ribosylation cycle, and the enzyme activities of ADP-ribosyltransferase (ART) [69, 70] and ADP-ribosyl hydrolases (ARH) [66, 72, 73]. ARTs are responsible for transferring the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD^+) to an acceptor amino acid. ARH catalyses the hydrolysis of the bound ADP-ribose protein bond resulting in the regeneration of free protein.

hydrolase (ARH1) reverses the ADP-ribosylation of arginine by cleaving the ADP-ribose-(arginine) protein bond to restore the native protein [75, 76]. *ARH1*$^{+/-}$ and *ARH1*$^{-/-}$ mice developed greater numbers of lung adenocarcinomas than *ARH1*$^{+/+}$ wild-types. Moreover, ARH1-deficient cells in culture exhibited higher proliferation rates and larger numbers of colonies than their wild-type counterparts. Tumorigenesis appears due to loss of the functioning ARH1 allele. The occurrence of adenocarcinoma in heterozygous mice with loss of the functioning allele leads to the conclusion that ARH1 is a tumour suppressor gene and, thus, plays a critical role in progression of lung adenocarcinoma.

To assess the effects of oestrogen on this murine model of lung adenocarcinoma, five sex groups were compared. Female-equivalent mice comprised female, female ovariectomised treated with oestrogen and male plus oestrogen mice, whereas male-equivalent mice were male and female ovariectomised mice. Mouse embryonic fibroblasts grown from wild-type, heterozygous (+/-), and homozygous (-/-) ARH1 mice were injected intravenously or subcutaneously into all groups. Tumour growth in female-equivalent mice was more rapid than in male-equivalent counterparts, clearly consistent with a role of endogenous oestrogen in tumorigenesis. Moreover, tumour growth rate in female ovariectomised mice was similar to that of male mice. Oestrogen administration further enhanced tumour growth rates, suggesting that oestrogen levels might be directly correlated with the pathogenesis of lung adenocarcinoma.

LAM and BML

LAM is a rare multisystem disease characterised by cystic lung destruction, pleural and/or ascitic chylous effusions, and renal angiomyolipomas (AMLs) [1, 77–81]. Cystic lung destruction results from the growth and proliferation of abnormal smooth muscle-like LAM cells. LAM occurs almost exclusively in females either sporadically or in association with tuberous sclerosis complex (TSC) [81], an autosomal-dominant genetic disorder caused by mutations in either the *TSC1* or *TSC2* tumour suppressor genes [82, 83]. In response to extracellular factors (*e.g.* nutrients, hormones, hypoxia), the protein products of two *TSC* genes form a TSC1-TSC2 heterodimer, which modulates activation of the mTOR pathway [84]. mTOR is the master regulator of cell-cycle progression, growth and proliferation *via* phosphorylation of ribosomal protein S6 and 4E-BP1, making it a therapeutic target in many cancers and tumour-related diseases [85–88].

BML is a rare disease that appears to result from the benign metastasis of smooth muscle cells to the lung [89]. Despite the inherent aetiology in its name, BML is poorly understood with several hypotheses proposed to describe its multifocal proliferative properties and metastatic potential [2, 89, 90]. Behaviour of LAM cells is similar to that of lung cancer in terms of growth, proliferation, cell survival in the circulation, and metastasis [91–93]. Striking patho-physiological similarities between LAM and BML have enabled researchers to correlate findings in models of either disease and clinical observations in patients with LAM or BML [94, 95]. The evident female predominance among patients, together with correlation of clinical lesion grades with hormonal levels at menarche, pregnancy, menstruation and menopause, are all consistent with the hypothesis that oestrogen contributes to progression of LAM as well as BML [2, 80, 96].

The observations that both ERs were found in the affected lungs, LAM cells and uterine leiomyoma of BML are consistent with a receptor-dependent model of progression of these diseases [2, 97–99]. Aromatase

presence and activity in either disease has not been described, although there are clinical reports of the use of the aromatase inhibitor letrozole as a treatment for BML [96, 100]. Among other reports of effects of female hormones on cell growth and proliferation in LAM models, *in vitro* studies of TSC2-null Eker rat embryonic fibroblasts revealed that prolactin, regulated by oestrogen [101], an important reproductive hormone, increased proliferation *via* activation of MAPK and other protein kinase pathways [102]. Observations on the proliferative effects of oestrogen using Eker rat uterine leiomyoma-derived smooth muscle cells (ELT3) *in vitro* were similar [103], as were findings with primary cultures of human AML cells on genomic and nongenomic effects of oestrogen [104].

To explore the hypothesis that oestrogen induces changes in cell size as well as proliferation in cells grown from skin of TSC-LAM patients, we measured rat and human cells with different methodologies (fig. 3). In preliminary experiments with rat and human fibroblasts lacking TSC2, sirolimus decreased cell size and E_2 treatment increased it. E_2 also increased proliferation of both human TSC2-heterozygous and TSC2-null LAM cells. Moreover, E_2 effects on cell size and number were prevented by MAPK kinase (MEK) 1/2 inhibitors. These observations may offer clues to the elucidation of biochemical mechanisms that regulate cell size and proliferation, including effects of oestrogen on mTOR and other signalling pathways, and perhaps an insight into potential therapeutic targets for anti-oestrogen treatments.

Growth of TSC2-null human AML cells *in vitro* and pulmonary metastasis of TSC2-null Eker rat lymphoma cells (ELT3) in mice were stimulated by oestrogen *via* both genomic transcription-dependent and nongenomic MAPK-dependent pathways [105]. These findings were consistent with other *in vitro* and *in vivo* observations using rat LAM-TSC models [106, 107]. Moreover, inhibiting MEK1/2 with CI-1040 also interfered with the survival and growth of TSC2-null cells in mice [105]. A recent study describes a novel TSC-LAM murine model, which was generated by intratracheal instillation of TSC2-deficient, patient-derived AML cells, for monitoring systemic tumorigenesis, dissemination and drug responses in real-time using single photon emission computed tomography/computed tomography, providing potential preclinical drug-testing options [108]. Oestrogen accelerated the growth of immortalised AML cells *via* the activation of Akt and downstream signalling pathways in a mouse xenograft model [109].

We also studied the effects of oestrogen on murine models of LAM and BML. We established the five sex groups, *i.e.* three female equivalent and two male equivalent, as described for the ARH1 mouse models. We injected patient-derived LAM or BML cells either subcutaneously or intravenously and measured, respectively, local tumour growth or growth of metastases in lung and other organs. Preliminary data showed that tumour growth was more rapid in female-equivalent than male-equivalent mice. Furthermore, pharmacological oestrogen treatment resulted in rates of growth that exceeded those observed in female mice. These data are clearly consistent with contributions of endogenous oestrogen to tumorigenesis. Additional studies are required to clarify other effects of oestrogen on mechanisms of disease pathogenesis.

Despite their apparently benign morphology, LAM cells exhibit metastatic behaviours that can be attributed to the modified actin cytoskeleton that results from impaired *TSC1* and/or *TSC2* function and downstream activation of mTOR [84]. Chemotactic migration of *TSC2*-null cells was enhanced, whereas cell adhesion and Rho activity were decreased [110]. Overexpression of TSC1 (hamartin) increased Rho activity, focal adhesion and stress fibre formation [111]. MMP-2 activity, an indicator of tumour cell invasiveness or metastatic capability, was increased by ER activation in spindle-shaped LAM cells, consistent with clinical observations of MMP activation in patients with LAM [112, 113]. Lymphatic involvement in tumour cell dissemination and metastasis has also been documented [114, 115]. Oestrogen was shown to promote

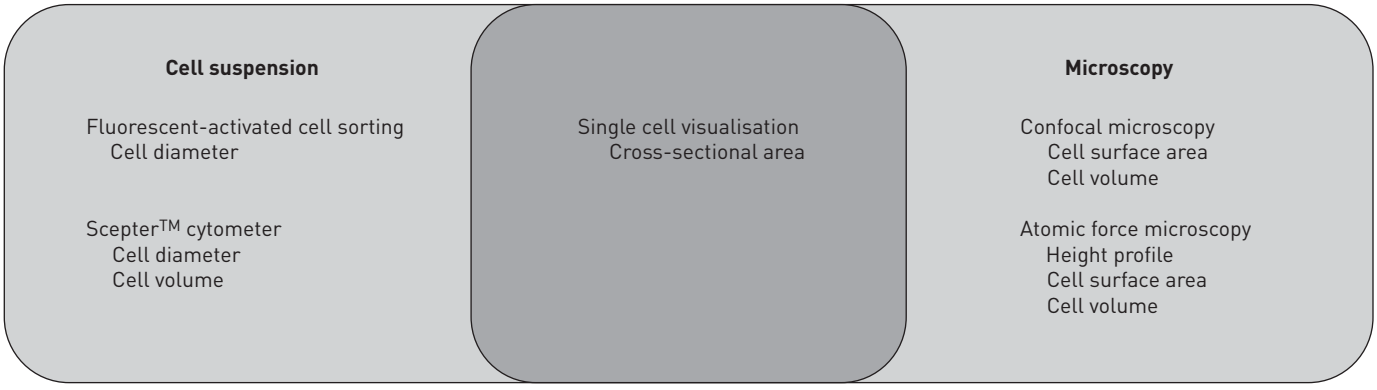

FIGURE 3 Venn diagram summarising the various laboratory methods for measuring cell size and the size parameters acquired by each method.

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pulmonary metastases using the ELT3 ($TSC2^{-/-}$) uterine leiomyoma cells, although whether or not effects of oestrogen and the hamartin-tuberin complex are related remains unknown.

Pulmonary fibrosis

Idiopathic pulmonary fibrosis has been implicated as a male-predominant disease. However, the role of oestrogen in this disease has not been clearly elucidated. The finding that pulmonary fibrosis is prevalent in males leads to the proposal that oestrogen could have protective effects. The results of some of the animal models are controversial but it has been shown that oestrogens could have protective effects for the development of pulmonary fibrosis [116–118]. Interestingly, the severity of pulmonary fibrosis also appears to depend on sex [119–122]. While the majority of these findings support a protective role of oestrogen in experimental pulmonary fibrosis, further studies are warranted.

Therapeutic studies of oestrogen in lung diseases

Numerous, albeit diverse, effects of oestrogen on progression of lung diseases led to speculation about the potential efficacy of anti-oestrogen therapies that target key regulators of oestrogen synthesis and signalling, *e.g.* aromatase and ERs, respectively (table 2). Aromatase inhibitors such as letrozole inhibit enzymatic production and thereby lower oestrogen levels [123]. Oestrogen-receptor downregulators and selective oestrogen-receptor modulators bind specifically to ERs to alter their activity and signalling [124].

Effects of anti-oestrogens on progression of the lung diseases discussed here have been studied using cell culture and animal models. Sizes of lung adenocarcinoma xenografts were significantly smaller after cells were administered with, rather than without, the aromatase inhibitor anastrozole [40]. ER antagonist tamoxifen decreased the extent of metastatic spread of cultured ER-positive NSCLC cells [65]. Combined treatment with tamoxifen and gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, decreased proliferation and increased apoptosis of lung adenocarcinoma cell lines [125].

Sirolimus, the presently favoured treatment for LAM, directly inhibits mTOR activity, thus, slowing disease progression. Clinical trials showing that sizes of AML tumour were partially restored after withdrawal of sirolimus [126] prompted investigation of other therapeutic options, *e.g.* anti-oestrogen agents, together with or without sirolimus [126, 127]. Tamoxifen reduced VEGF levels in $Tsc1^{+/-}$ mice and reduced the development of female $Tsc1^{+/-}$ mouse liver haemangiomas, tumours that are related to renal AMLs and LAM cells [128]. Tamoxifen was also shown to accelerate the growth of AML cells, which differs from its effect on the $Tsc1^{+/-}$ mouse liver haemangiomas [104]. In a recent paper, Li *et al.* [129] reported that ER antagonist faslodex decreased tumour size by inhibiting oestrogen-induced ECM remodelling, expression of MMP-2 and metastasis to the lung. Such effects of anti-oestrogen agents on lung diseases in model systems are certainly cause for optimism, albeit still incomplete. Further investigation should help us to better understand their potential therapeutic value.

Conclusion

Based on our findings, as well as those from literature summarised in this review, oestrogen plays a significant role in the pathogenesis of predominantly female lung diseases. There is also evidence that oestrogen may have variable effects on other lung diseases, including those that are predominantly found in males. Oestrogen is probably not the only sex hormone that influences lung disease incidence, severity and/or pathogenesis, nor does it necessarily promote disease progression. Rather, the complex interactions between multiple hormones, receptors and/or signalling pathways probably influence sex predominance and the defining characteristics of lung diseases. Additional investigation of sex differences using cultured cells and animal models may provide valuable information on the association between lung

TABLE 2 Types of anti-oestrogen therapy

Therapy	Mechanism	Examples
Aromatase inhibitor	Blocks the enzymatic activity of aromatase and inhibits 17β-oestradiol (E_2) synthesis	Letrozole, anastrozole, exemestane
Oestrogen receptor downregulator	Binds directly to oestrogen receptors and thus inhibits oestrogen binding Reduces number of oestrogen receptors Alters the shape or conformation of oestrogen receptors	Fulvestrant
Selective oestrogen receptor modulator	Selectively binds to oestrogen receptors and thus inhibits oestrogen binding	Tamoxifen, raloxifene, toremifene

pathophysiology and endocrine factors. Studies of anti-oestrogen and other anti-hormonal treatments may ultimately improve therapeutic options for patients with lung diseases.

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