RESEARCH

Lymph/angiogenesis contributes to sex differences in lung cancer through oestrogen receptor alpha signalling

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Abstract

Oestrogen signalling pathways are emerging targets for lung cancer therapy. Unravelling the contribution of oestrogens in lung cancer development is a pre-requisite to support the development of sex-based treatments and identify patients who could potentially benefit from anti-oestrogen treatments. In this study, we highlight the contribution of lymphatic and blood endothelia in the sex-dependent modulation of lung cancer. The orthotopic graft of syngeneic lung cancer cells into immunocompetent mice showed that lung tumours grow faster in female mice than in males. Moreover, oestradiol (E2) promoted tumour development, increased lymph/angiogenesis and VEGFA and bFGF levels in lung tumours of females through an oestrogen receptor (ER) alpha-dependent pathway. Furthermore, while treatment with ERb antagonist was inefficient, ERa antagonist (MPP) and tamoxifen decreased lung tumour volumes, altered blood and lymphatic vasculature and reduced VEGFA and bFGF levels in females, but not in males. Finally, the quantification of lymphatic and blood vasculature of lung adenocarcinoma biopsies from patients aged between 35 and 55 years revealed more extensive lymphangiogenesis and angiogenesis in tumour samples issued from women than from men. In conclusion, our findings highlight an E2/ERa-dependent modulation of lymphatic and blood vascular components of lung tumour microenvironment. Our study has potential clinical implication in a personalised medicine perspective by pointing to the importance of oestrogen status or supplementation on lung cancer development that should be considered to adapt therapeutic strategies.

Key Words

- ▶ lymphangiogenesis
- ▶ angiogenesis
- ▶ lung cancer
- ▶ oestrogen receptor
- ▶ sex
- ▶ gender
- ▶ tamoxifen
- ▶ microenvironment

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Introduction

Development of personalised medicine in cancer care is the challenge of the 21st century (Schleidgen *et al.* 2013). To ensure that patients benefit from the most appropriate therapies, it is mandatory to identify specific mechanisms underlying individual responses to therapies. There is now

increasing clinical evidence linking sex differences to lung diseases such as asthma, chronic obstructive pulmonary disease, as well as lung cancer (Townsend *et al.* 2012).

Historically, incidence rates of lung cancer were higher among men than women. This pattern has now

reversed in young population, since lung cancer incidence rates are currently higher among young women than men (Lewis *et al.* 2014, Jemal *et al.* 2018). These observations cannot be fully explained by sex differences in smoking behaviours. Population-based studies and clinical trials have also identified disparities in age, smoking practices and histological subtypes between men and women (Wakelee *et al.* 2006, Katcoff *et al.* 2014). Among non-smokers, women are 2.5-fold more susceptible than men to develop lung cancer at a younger age, and they display a higher prevalence for adenocarcinoma (Siegfried 2001, Wakelee *et al.* 2007, Townsend *et al.* 2012). Two major pathways could contribute to these differences: the epidermal growth factor (EGF)/EGFR and sex steroids (Cadranet *et al.* 2011, Siegfried & Stabile 2014).

Clinical and experimental data strongly support a contribution of oestrogens to lung cancer development (Siegfried & Stabile 2014, Rodriguez-Lara *et al.* 2018). Indeed, elevated 17 β -oestradiol (E2) levels and higher expression of aromatase predict lower overall survival in lung cancer patients (Mah *et al.* 2007). Moreover, observational series show that breast cancer patients receiving anti-oestrogen therapy exhibit a reduced risk of developing subsequent lung cancer and display lower mortality rates from lung cancer (Bouchardy *et al.* 2011, Chu *et al.* 2017). Exogenous E2 administration was linked to an increased lung tumour growth of human tumour xenografts in female immunodeficient mice (Stabile *et al.* 2002), as well as boosted lung tumour development in a transgenic animal model (Hammoud *et al.* 2008). However, the potential influence of menopausal hormone therapy on lung cancer incidence and survival remains unclear (Schabath *et al.* 2004, Greiser *et al.* 2010, Chlebowski *et al.* 2016). Overall, the contribution of oestrogens in lung cancer is largely studied and debated, especially regarding the complexity of the mechanisms sustaining their action. Therefore, the use of patient-adapted anti-oestrogen therapy still remains poorly considered for lung cancer.

Tumour microenvironment, especially lymphatic and blood vasculatures, strongly contributes to tumour development and dissemination (Paupert *et al.* 2011, Dieterich & Detmar 2016, De Palma *et al.* 2017). Although E2 has been shown to regulate angiogenesis, there is still a paucity of data regarding its effect on lymphatic endothelium, especially during tumour lymphangiogenesis. Nevertheless, lymphedema, a lymphatic disorder associated to accumulation of fat and fibrosis in limbs due to impaired lymphatic function, is related to hormonal status and is sex linked (Alitalo *et al.* 2005).

Recent reviews highlighted the organ specificity of both lymphatic (Petrova & Koh 2018, Wong *et al.* 2018) and vascular beds (Nowak-Sliwinska *et al.* 2018). Despite an abundant literature showing a direct pro-tumour impact of E2 on lung cancer cells expressing oestrogen receptors (ERs), little is known about its effects on lung tumour microenvironment, especially lymphangiogenesis or angiogenesis associated to lung cancer.

E2 binds two major receptors, ER alpha (ERa) and ER beta (ERb), belonging to the nuclear receptor family (Hamilton *et al.* 2017) and the G protein-coupled oestrogen receptor (GPER) (Barton *et al.* 2017), a seven transmembrane domain protein. Several studies evidenced that human lung cancer cells predominantly express ERb (reviewed in Baik & Eaton 2012, Rodriguez-Lara *et al.* 2018) and that ERb sustains lung tumour growth in murine models (Pietras *et al.* 2005, Hershberger *et al.* 2009, Zhao *et al.* 2011, Tang *et al.* 2014). On the other hand, it has been reported ERa-dependent growth-promoting genes are upregulated in lung cancer (Pietras *et al.* 2005, Pietras & Marquez-Garban 2007) and ERa expression is increased in lung tumours from women (Raso *et al.* 2009, Rouquette *et al.* 2012). In addition, GPER is also enhanced in human lung cancer (Jala *et al.* 2012). Altogether, these data indicate that the molecular pathways sustaining the impact of sex and oestrogen pathways on lung cancer cell growth and more specifically on lung cancer lymphangiogenesis and angiogenesis are still insufficiently understood.

In this study, we report that the sex of lung microenvironment affects lung tumour development. Orthotopically grafting of syngeneic lung cancer cells into pulmonary parenchyma of immunocompetent mice revealed that lung tumours grow faster in female mice than in males. E2 increased tumour progression in female mice and enhanced lymphangiogenesis and angiogenesis through an ERa-dependent pathway. Furthermore, while treatment with ERb antagonist was inefficient, treatment by ERa antagonist and tamoxifen decreased lung tumour growth in females but not in males. Finally, lymphangiogenesis and angiogenesis were higher in lung adenocarcinoma samples issued from young women as compared to those obtained from young men.

Materials and methods

Human samples and ethical study approval

Human lung tumour samples and endometrium tissue were provided by the Biobank of the University Hospital of Liège (BHUL, University of Liège and CHU of Liège,

Belgium) to perform a retrospective study in accordance with the current legislation and recommendations of the Ethical Committee of the University Hospital of Liège.

Animals and ethical study approval

C57BL/6J mice were purchased from Charles River (France). Tie2-Cre⁺/ERa^{lox/lox} mice and their control littermates Tie2-Cre⁻/ERa^{lox/lox} in C56BL/6J background were generated as described previously (Billon-Gales *et al.* 2009). Mouse genotyping is provided in Supplementary Fig. 1 (see section on [supplementary data](#) given at the end of this article). All animals were maintained within the accredited Mouse Facility and Transgenics GIGA platform of the University of Liège (Belgium). All animal experiments were conducted in accordance with the Federation of European Laboratory Animal Science Associations and were approved by the Local Ethical Committee of the University of Liège.

Reagents

E2 was purchased from Sigma-Aldrich. MPP dihydrochloride (1,3-bis(4-hydroxyphenyl)-4-methyl-5-(4-(2-piperidinylethoxy)phenol)-1H-pyrazole dihydrochloride); PHTPPP (4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo(1,5-a)pyrimidin-3-yl)phenol) and G15 ((3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline) were purchased from Tocris Biosciences (R&D system).

Cell cultures

Male mouse Lewis Lung Carcinoma cells transfected with luciferase gene (LLC-Luc, LL/2-luc-M38) were purchased from American Type Culture Collection (ATCC) and Caliper Lifesciences (Hopkinton, MA, USA), respectively. LLC-Luc cells were authenticated by Leibniz-Institute DSMZ using STR DNA typing and Cytochrome Oxidase subunit 1 (COI) alignment respectively. All cells were used within ten passages after authentication. Cells were routinely cultured in DMEM (Gibco Invitrogen Corporation) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Lonza, Basel, Switzerland), 2mM glutamine and 100IU/mL penicillin/streptomycin (ThermoFisher Scientific).

Cell proliferation and viability assays

LLC-Luc cells were cultured for 24h in red phenol-free medium (Gibco Invitrogen Corporation) supplemented

with 10% of heat-inactivated and dextran-coated charcoal-treated foetal bovine serum (FBS-cs, Lonza). Cells were then cultured in medium with 2% FBS-cs supplemented with either 10% FBS (positive control) or E2 (10⁻¹⁰–10⁻⁷M) or MPP (10⁻⁸M) or PHTPP (10⁻⁸M) or G15 (10⁻⁷M) or vehicle (DMSO 0.001%) or cisplatin as positive control (100µM, #P4394, Sigma-Aldrich). To investigate cell proliferation, LLC-Luc cells were incubated during 24h with methyl-³[H]thymidine (Perkin Elmer Life Sciences) and radioactivity was measured with a b-counter (Beckman, LS-5000-CE); to measure cell viability, an MTT test (#11465007001, Roche) was performed in accordance with manufacturer's instructions.

Western blotting

Mouse testis and ovary tissues were collected as described below. Cells and tissues were lysed in RIPA buffer supplemented with a protease inhibitor (Complete, Roche). Primary antibodies used for immunostaining were anti-ERa (clone 60C, #04-820, Millipore; F10, #sc-8002, Santa Cruz), anti-ERb (PPZ0506, #417100, Invitrogen/ThermoFisher Scientific), anti-GPER (#sc-48525, Santa Cruz) and anti-HSC70 (B-6, #sc-7298, Santa Cruz). After incubation with appropriated HRP-conjugated secondary antibodies, immunoreactions were revealed using the enhanced chemiluminescence kit (ThermoFisher Scientific). Images were acquired by a LAS4000 digital camera (FujiFilm, Japan).

Mouse orthotopic model of lung cancer

When required by the experimental protocol, 5-week-old mice were gonadectomised. Ovaries and testis tissues were collected as control samples for Western blot assays. Two weeks after surgery, a group of females were treated with subcutaneous slow-releasing E2 pellets (OVX+E2) (75µg/kg/day, #ME2-60 days, Belma Technologies, Belgium) (Gerard *et al.* 2017). Ten days later, LLC-Luc cells (2×10⁶ cells/mice) were instilled into lungs as previously described (Rocks *et al.* 2012). For ER antagonist treatments, mice were either subcutaneously injected with MPP, PHTPP (1mg/kg in peanut oil) or DMSO (vehicle, 5% in peanut oil) 5 days a week or received a subcutaneous pellet of tamoxifen (5mg/60 days release, Innovative Research of America, FL, USA). All treatments were started 2 weeks before the LLC-Luc cell instillation and were conducted until euthanasia at day 21 after tumour cell instillation. To monitor lung tumour growth, luciferin (150mg/kg in PBS, #E160E, Promega) was injected intraperitoneally

and luciferase bioluminescence was measured using the bioluminescent IVIS imaging system (Xenogen-Caliper, Hopkinton, MA, USA).

Lung tumour immunohistochemical analysis

To evaluate tumour density, paraffin-embedded lung tumour sections (5 µm) were stained with hematoxylin and eosin (H/E). For each mouse, we collected eight slides separated by 50 µm. Numeric images were obtained with NanoZoomer 2.0-digital slide scanner (Hamamatsu Photonics, Japan). On each slide, the lung tumour area and the total lung area were measured by computer assisted image analysis with Matlab software (MathWorks, Inc., MA, USA). The ratio of these two measures (lung tumour area/total lung area) corresponds to the lung tumour density. To obtain the lung tumour density for one mouse, we calculated the mean of the eight densities measured from the eight slides of the same lung.

Immunolabelings were carried out using anti-CD31 (#ab28364, Abcam), anti-podoplanin (D2-40, #MA1-83884, ThermoFisher Scientific), anti-LYVE1 (#AF2125, R&D System) or anti-ERa (1D5, #M7047, Dako) antibodies. Slides were then incubated with appropriate biotin-coupled secondary antibodies (Dako) and with streptavidin/Alexa 555 or streptavidin/Alexa 488 (#S21381, #A11055, Invitrogen Corporation). Slides were mounted with DAPI fluoromount G (SouthernBiotech, AL, USA). Numeric images were obtained with NanoZoomer 2.0-digital slide scanner (Hamamatsu Photonics, Japan) or recorded with fluorescence microscope (VANOX AHBT3, Olympus). For each mouse tumour sample, a minimum of five optical fields that cover the entire tumour section were recorded and a mean density was calculated. Lymphangiogenesis and angiogenesis densities were quantified as a ratio between the area occupied by LYVE1, PDPN or CD31 staining in the tumour and the area of the tumour. Image analysis was performed with Matlab software (MathWorks, Inc, MA, USA) as previously described (Pequeux *et al.* 2012).

EdU incorporation assay

Two hours before euthanasia, mice received a peritoneal injection of 5-ethynyl-2'-deoxyuridine (EdU, 2.5 mg/mouse, ThermoFisher Scientific). EdU incorporation in proliferating cells was evidenced using Click-it EdU cocktail kit (Molecular Probes) in accordance with manufacturer's instructions. Slides were mounted with aquapolymount (Polysciences, Hirschberg an der Bergstrasse, Germany).

Corneal assay

The ophthalmic cauterisation of cornea was performed and analysed, as previously described (Detry *et al.* 2013). Briefly, mice were anaesthetised with ketamine hydrochloride (100 mg/kg body weight) and xylazine (10 mg/kg body weight) by peritoneal injection; their eyes were locally anaesthetised with Unicaïne 0.4% drops (Thea Pharma, Wetteren, Belgium). After anaesthesia, an ophthalmic cauterisation (Optemp II V; Alcon Surgical, Fort Worth, TX, USA) was performed in the central part of the cornea. Corneas were recovered and they were dissected at day 7 post injury. Tissue was fixed during 1 h in 70% ethanol at room temperature, washed in PBS and blocked during 1 h in milk 3%/BSA 3% (Nestlé, Brussels, Belgium; Acros Organics, NJ, USA). To highlight lymphatic and blood vessels, tissues were first incubated overnight with polyclonal goat anti-mouse LYVE1 (1:200, #AF2125, R&D system) and monoclonal rat anti-mouse CD31 (1:200, #01951D, BD Biosciences Pharmingen, San Jose, CA, USA), then with rabbit anti-goat/Alexa Fluor 488 (1:200, #A21222, Molecular Probes) or goat anti-rat/Alexa Fluor 546 (1:200, #A11035, Molecular Probes) antibodies, respectively. Corneas were whole-mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and pictures were acquired with FSX100 microscope (Olympus). These experiments were performed on males, females, gonadectomised-mice and ovariectomised female mice treated with subcutaneous slow-releasing E2 pellets (75 µg/kg/day, #ME2-60 days, Belma Technologies, Liège, Belgium). This cornea assay was also carried out on female mice treated with subcutaneous injections of MPP, PHTPP or with G15 (1 mg/kg/day, Tocris Biosciences, R&D system) for 3 weeks (5 times/week). These treatments started 2 weeks before thermal cauterisation until euthanasia.

Protein quantification by Milliplex assay

Proteins from LLC-Luc lung tumours were extracted and analysed by Milliplex assay of mouse angiogenesis/growth factor, accordingly to manufacturer's instructions (Mouse Angiogenesis/Growth Factor Magnetic Bead Panel – Cancer Mutliplex Assay #MAGPMAG-24K, Merck).

RNA *in situ* hybridisation (RNAscope)

The mRNA *in situ* hybridisation of ERa (ESR1), CD31 (PECAM) and podoplanin (PDPN) was measured on human lung tumour (men and women) and endometrium tissue sections with the RNAscope assay (Advanced Cell

Diagnostics, Bioké, Leiden, The Netherlands) according to manufacturer's instructions. Briefly, paraffin-embedded tissue sections (5 µm) were deparaffinised and hybridised in duplex, either with Hs-ESR1 (#310301, Bioké) and Hs-PECAM1-O1-C2 (#487381-C2, Bioké) probes or with Hs-ESR1 (#310301, Bioké) and Hs-PDPN-C3 (#539751-C3, Bioké) probes or with RNAscope 3-plex negative control probe (#320871, Bioké). Hybridisation signal was amplified with RNAscope Multiplex Fluorescent reagent kit V2 (#323100, Bioké) and TSA Plus Fluorescent kits (#NEL745001KT, #NEL744001KT, PerkinElmer). Images were recorded with a confocal Olympus Fluoview 1000 microscope (Olympus America) at 40× of magnification.

Statistical analysis

Results were analysed with GraphPad Prism 5.0. Statistical analyses were assessed with Student *t*-test or one-way ANOVA followed by Bonferroni post-test for Gaussian distribution, with Mann–Whitney or Kruskal–Wallis for non-Gaussian distribution and with two-way ANOVA for grouped analysis. Mann–Whitney was also used to compare independent experimental groups. The *P* value was expressed as followed: **P*<0.05; ***P*<0.01; ****P*<0.001; NS, not significant.

Results

Female mice develop larger lung tumours than males, through an E2-dependent pathway

To understand the impact of sex on lymphangiogenic and angiogenic processes associated to lung adenocarcinoma development, we used an orthotopic syngeneic lung cancer model, which was developed in immunocompetent mice in order to preserve the integrity of the lung microenvironment.

Twenty-one days after intratracheal LLC-Luc administration, bioluminescent signals produced by lung tumours were higher in females as compared to males (Fig. 1A). Quantification of tumour area on histological lung sections confirmed that females developed approximately two-fold larger lung tumours than males (Fig. 1B). To evaluate the effects of endogenous oestrogen in sex-related differences regarding lung tumour development, females were ovariectomised (OVX) and LLC-Luc tumour implantation in lung parenchyma was measured. Interestingly, OVX female mice displayed a decreased lung tumour growth as compared to naive females and to OVX females supplemented with

exogenous E2 (OVX+E2) (Fig. 1C and D). By contrast, lung tumour growth was not affected by gonadectomy in males, even after E2 supplementation (Fig. 1E and F). This suggests a specific effect of endogenous and exogenous oestrogens on tumour growth in female lungs but not in males.

E2 increases LLC-Luc cell proliferation *in vivo* but not *in vitro*

LLC-Luc cells used in the orthotopic lung cancer model expressed GPER, but not ERα receptor (Fig. 2A). ERβ expression was assessed using the anti-ERβ antibody PPZ0506, the only specific and commercially available antibody validated by Andersson *et al.* (2017). No expression of ERβ was detected in LLC-Luc protein extracts.

Treatment of LLC-Luc cells with increasing E2 concentrations, ranging from 10^{−10} M to 10^{−7} M, for 24, 48 or 72 h did neither affected LLC-Luc proliferation (Fig. 2B and C) nor cell viability (Fig. 2D). FBS and cisplatin were used as positive and negative controls in the proliferation or viability assays, respectively. In addition, the combined treatment of cells with E2 and ER antagonists, MPP (ERα antagonist), PHTPP (ERβ antagonist) or G15 (GPER antagonist) did not modulate cell proliferation (Fig. 2E). These results highlight that E2 does not directly increase lung cancer cell proliferation, despite GPER expression by those cells.

To assess LLC-Luc cell proliferation *in vivo*, tumour-bearing mice were intraperitoneally injected with EdU (Fig. 2F). Interestingly, EdU density was higher in LLC-Luc lung tumours when OVX mice were treated with E2 (OVX+E2) (Fig. 2G). In these tumours, the expression of ERα by cancer cells was not induced *in vivo* (Fig. 2H and I). However, some positivity was associated to lymphatic and blood vessels, as shown by co-immunostainings (yellow staining) of ERα and LYVE1 (Fig. 2H) or CD31 (Fig. 2I).

Altogether, these results highlight that when lung cancer cells do not express ERs, E2 can still promote lung tumour growth *in vivo*.

E2 increases lymphangiogenesis and angiogenesis in females

A significant higher lymphatic vessel density was detected in tumours grown in female lungs as compared to male counterparts (Fig. 3A and B). While ovariectomy (OVX) decreased tumour lymphatic vessel density,

Figure 1

Orthotopic graft of LLC-Luc cells into lung parenchyma grows faster in females than in males. (A) *In vivo* bioluminescent signals derived from LLC-Luc lung tumours imaged either in whole mice (upper panel) or in dissected pulmonary lobes (lower panel) from male and female mice. The right panel shows bioluminescent intensity quantification over time ($n = 8$), $**P < 0.01$, 2-way ANOVA. (B) Hematoxylin/eosin staining of LLC-Luc lung tumours from male ($n = 24$) and female ($n = 23$) mice (scale bar = 1 mm) and quantification of lung tumour density (tumour area/total lung area) on the right panel, eight slides spaced with 50 μm were analysed per sample, $*P < 0.05$, Mann-Whitney. (C) *In vivo* bioluminescent signals derived from LLC-Luc lung tumours imaged in female, OVX female and OVX + E2 female mice. The right panel shows bioluminescent intensity quantification over time ($n = 8$), $*P < 0.05$, 2-way ANOVA. (D) Hematoxylin/eosin staining and quantification of lung tumour density (tumour area/total lung area) of LLC-Luc lung tumours from females and OVX female mice treated or not with E2 (scale bar = 1 mm) ($n = 8$), 8 slides spaced with 50 μm were analysed per sample, $*P < 0.05$, 1-way ANOVA. (E) *In vivo* bioluminescent signals derived from LLC-Luc lung tumours imaged either in whole mice of male ($n = 8$), gonadectomised (Cx) male ($n = 8$) and Cx + E2 male ($n = 7$) animals. The right panel shows bioluminescent intensity quantification over time, 2-way ANOVA. (F) Hematoxylin/eosin staining and quantification of the lung tumour density (tumour area/total lung area) of LLC-Luc lung tumours from males ($n = 8$) and gonadectomised male mice treated (Cx + E2, $n = 7$) or not with E2 (Cx, $n = 8$) (scale bar = 1 mm), 8 slides spaced with 50 μm were analysed per sample, 1-way ANOVA.

Figure 2

E2 increases LLC-Luc cell proliferation *in vivo*, but not *in vitro*. (A) Western blot of oestrogen receptors (ER) on LLC-Luc cell lysates: ERα (66 kDa), ERb (56 kDa), GPER (42 kDa). HSC70 (70 kDa) was used as loading control. Proteins isolated from mouse ovary and testis were used as positive controls. (B) *In vitro* proliferation of LLC-Luc cells treated with vehicle, E2 (10^{-9} M) or FBS 10% (positive control) for 24, 48 and 72 h, $***P < 0.001$ vs vehicle, $n = 6$, 2-way ANOVA. (C) *In vitro* proliferation of LLC-Luc cells treated with vehicle, E2 (10^{-10} M to 10^{-7} M) or FBS 10% (positive control) for 48 h, $***P < 0.001$ vs vehicle, $n = 6$, 1-way ANOVA. (D) *In vitro* viability of LLC-Luc cells treated with vehicle, E2 (10^{-10} M to 10^{-7} M) or cisplatin (10^{-4} M, positive control) for 48 h, $***P < 0.001$ vs vehicle, $n = 6$, 1-way ANOVA. (E) *In vitro* proliferation of LLC-Luc cells treated with ERα, ERb or GPER selective antagonists (MPP 10^{-8} M, PHTPP 10^{-8} M, G15 10^{-7} M) in combination or not with E2 (10^{-9} M), $n = 6$, 1-way ANOVA. (F) *In vivo* LLC-Luc cell proliferation analysed by EdU immunofluorescent staining (scale bar = 500 μm in upper panel, scale bar = 100 μm in lower panel) in OVX female mice treated or not with E2. (G) Quantification of EdU density (EdU area/lung tumour area) on LLC-Luc tumours from OVX females ($n = 26$) and OVX + E2 females ($n = 23$), $*P < 0.05$, t -test. (H) Double-immunofluorescent staining and their zoom of LYVE1 (red) and ERα (green), colocalisation of stainings (yellow) and DAPI (blue) in LLC-Luc lung tumours, scale bar = 50 μm. Uterus tissue was used as positive control, scale bar = 50 μm. (I) Double-immunofluorescent staining and their zoom of CD31 (red) and ERα (green), colocalisation of stainings (yellow) and DAPI (blue) in LLC-Luc lung tumours, scale bar = 50 μm.

E2 supplementation of OVX mice (OVX+E2) was able to rescue it. In male mice, there was no effect of castration and E2 treatment on lung tumour lymphangiogenesis. Similar to lymphatic vessel, blood vessel density was increased in lung tumours grown in female mice (Fig. 3A and B). Furthermore, when OVX females were used, E2 treatment increased tumour-related blood vessel network,

while no modulation was observed in males. VEGFC, VEGFD, VEGFA and bFGF, the main prolymphangiogenic and proangiogenic factors, were measured by Milliplex assay in lung tumour samples. VEGFC and VEGFD levels were not modulated between experimental groups (Fig. 3C and D). VEGFA and bFGF levels were increased in LLC-Luc lung tumours grown in females as compared to

Figure 3

E2 increases lymph/angiogenesis through ER α . (A) Immunofluorescent staining (scale bar = 50 μ m) of LYVE1 (green) or CD31 (red) in LLC-Luc lung tumours from female ($n = 11$), OVX ($n = 15$) or OVX + E2-treated ($n = 18$) female mice, and from male ($n = 12$), Cx ($n = 5$) or Cx + E2-treated ($n = 5$) male mice. (B) Quantification of LYVE1 density (LYVE1 area/lung tumour area) and CD31 density (CD31 area/lung tumour area) in lung tumours of these mice, $*P < 0.05$; $**P < 0.01$, Mann-Whitney or 1-way ANOVA. (C) VEGFC, (D) VEGFD, (E) VEGFA and (F) bFGF levels measured by Milliplex and reported to the total amount of protein (mg) in lung tumour lysates issued from male, female, OVX female and OVX + E2 female mice, $*P < 0.05$, 1-way ANOVA. (G) CD31/LYVE1 staining (for each condition: left panel, scale bar = 1 mm; right panel, scale bar = 500 μ m) and quantification (CD31 or LYVE1 area/total cornea area) in cornea of female mice ($n = 10$), OVX ($n = 10$) or OVX + E2 ($n = 10$) mice, $*P < 0.05$; $***P < 0.001$, 1-way ANOVA, or (H) in cornea of male ($n = 18$) or castrated male mice (Cx, $n = 8$), Mann-Whitney.

males and in OVX females treated with E2 as compared to OVX mice (Fig. 3E and F).

In order to evaluate if vascular modulation by E2 was only related to lung tumour-associated processes or if it could be extended to other pathological lymphangiogenic and angiogenic processes, a model of cornea injury was used to concomitantly study lymphangiogenic and angiogenic response in an inflammatory context (Detry *et al.* 2013). In this model, OVX females displayed decreased lymphatic and blood vessel densities, which were restored upon E2 supplementation (OVX+E2) (Fig. 3G). Castration (Cx) of males did not modulate either lymphangiogenesis or angiogenesis (Fig. 3H).

Altogether, these results show that E2 promotes pathological lymphangiogenesis and angiogenesis only in females and increases VEGFA and bFGF in lung tumours.

Prolymphangiogenic and proangiogenic effects of E2 are mediated by ERa

To define whether ERa signalling contributes to the sex-dependent control of lymphangiogenesis and angiogenesis in lung cancer, the impact of ERa deletion in lymphatic and blood endothelial cells was evaluated by performing experiments in Tie2-Cre/ERa^{lox/lox} mice.

When compared to males, the increase of lung tumour growth observed in female control Tie2-Cre⁻/ERa^{lox/lox} mice was inhibited in Tie2-Cre⁺/ERa^{lox/lox} mice (Fig. 4A). In addition, the increase of lung tumour growth induced by E2 in OVX female control Tie2-Cre⁻/ERa^{lox/lox} mice was abrogated in Tie2-Cre⁺/ERa^{lox/lox} mice (Fig. 4B).

Quantification of the lymphatic and blood vessel networks in tumours that grew in Tie2-Cre⁺/ERa^{lox/lox} mice (ERa-deficiency in vessels) did not show any statistical variation between the four aforementioned experimental groups (Fig. 4C and D). Moreover, by applying the model of cornea injury to these Tie2-Cre⁺/ERa^{lox/lox} mice, we did not observe any difference of lymphangiogenesis and angiogenesis responses between the experimental groups (Fig. 4E). To evaluate the potential contributions of the other oestrogen receptors, we tested pharmacological inhibitors of ERa (MPP), ERb (PHTPP) and GPER (G15) on the cornea injury model in WT female mice. MPP decreased both corneal lymphangiogenesis and angiogenesis confirming ERa signalling involvement (Fig. 4F). However, PHTPP and G15 did not exert any effect on lymphatic and blood vessel networks.

Altogether, these results support that ERa is the only oestrogen receptor that mediates E2 effects on lymphangiogenesis and angiogenesis.

Treatment with ERa antagonists inhibits lung tumour growth, lymphangiogenesis and angiogenesis in females, but not in males

LLC-Luc tumour growth was evaluated in females and males treated with MPP (ERa antagonist), PHTPP (ERb antagonist) or tamoxifen (Tmx), an ER antagonist widely used in female patients suffering from hormone-dependent breast cancer (Early Breast Cancer Trialists' Collaborative Group 2011). In female animals, both MPP and tamoxifen decreased tumour growth, while PHTPP was not efficient (Fig. 5A and B). In addition, females treated with MPP or tamoxifen presented reduced tumour lymphatic or blood vessel networks when compared to control mice treated with vehicle (Fig. 5C and D). No modulation was observed in vessels when mice were treated with PHTPP (Fig. 5C and D). Levels of VEGFC and VEGFD were not modified in these experimental conditions (Fig. 5E and F). In contrast, treatment with MPP or tamoxifen decreased VEGFA and bFGF levels measured in lung tumours from females (Fig. 5G and H), while PHTPP had no effect (Supplementary Fig. 2).

In males, none of the used antagonists (MPP, PHTPP, tamoxifen) was able to modulate LLC-Luc tumour development (Fig. 5I and J), lymphangiogenesis (Fig. 5K) or angiogenesis (Fig. 5L).

These results show sex specific reactivity towards treatments targeting ERa signalling.

Lymphangiogenesis and angiogenesis rates are higher in lung adenocarcinoma samples of women than men

To validate the clinical relevance of our findings, we measured lymphatic and blood vessel densities on human lung cancer biopsies and evaluated if lymphangiogenesis and angiogenesis were differentially regulated in lung cancer developing in women or in men. For this retrospective study, a cohort of patients with lung cancer ($n=74$) and aged between 35 and 55 years old has been selected to ensure that lung carcinogenesis had occurred before menopause (Fig. 6A). Although the mean age was similar for both experimental groups and all subjects included in this study were smokers, except one woman, one noteworthy data is that almost twice more biopsies were available from women ($n=51$) than from men ($n=23$) (Fig. 6A). Similar percentage of cancer cells positive for ERa was detected in man (60.9%) and in woman (62.7%) samples as assessed by nuclear immunohistochemical staining (Fig. 6B).

Figure 4

Prolymphangiogenic and proangiogenic effects of E2 are mediated by ERα. (A) Hematoxylin/eosin (H/E) staining of LLC-Luc lung tumours issued from Tie2-Cre⁺/ERα^{lox/lox} male ($n = 7$) or female ($n = 7$) mice and from Tie2-Cre⁺/ERα^{lox/lox} male ($n = 8$) or female ($n = 5$) mice (scale bar = 1 mm) and quantification of the tumour density (tumour area/total lung area), 8 slides spaced by 50 μ m were analysed per sample, $*P < 0.05$, Mann-Whitney. (B) Hematoxylin/eosin (H/E) staining of LLC-Luc lung tumours from Tie2-Cre⁺/ERα^{lox/lox} OVX ($n = 6$), OVX + E2 ($n = 6$) female mice and from Tie2-Cre⁺/ERα^{lox/lox} OVX ($n = 7$), OVX + E2 ($n = 5$) female mice (scale bar = 1 mm) and quantification of the tumour density (tumour area/total lung area), 8 slides spaced by 50 μ m were analysed per sample, $*P < 0.05$, Mann-Whitney. (C) LYVE1 immunofluorescent staining (scale bar = 50 μ m) and quantification (LYVE1 area/lung tumour area) in LLC-Luc lung tumours in Tie2-Cre⁺/ERα^{lox/lox} male ($n = 8$), female ($n = 5$), female OVX ($n = 7$) and female OVX + E2 ($n = 5$) mice, Kruskal-Wallis. (D) CD31 immunofluorescent staining (scale bar = 50 μ m) and quantification (CD31 area/lung tumour area) in LLC-Luc lung tumours in Tie2-Cre⁺/ERα^{lox/lox} male ($n = 8$), female ($n = 5$), female OVX ($n = 7$) and female OVX + E2 ($n = 5$) Kruskal-Wallis. (E) LYVE1/CD31 staining (scale bar = 500 μ m) and quantification (LYVE1 or CD31 area/total cornea area) of blood and lymphatic vessels in cornea of Tie2-Cre⁺/ERα^{lox/lox} male ($n = 8$), female ($n = 5$), OVX female ($n = 7$) and OVX + E2 female ($n = 5$) mice, Kruskal-Wallis. (F) LYVE1/CD31 staining (scale bar = 500 μ m) and quantification (LYVE1 or CD31 area/total cornea area) of blood and lymphatic vessels in cornea of wild-type female mice treated with vehicle ($n = 11$), ERα antagonist (MPP 10^{-8} M, $n = 13$), ERβ antagonist (PHTPP 10^{-8} M, $n = 12$) or GPER antagonist (G15 10^{-7} M, $n = 9$), $*P < 0.05$, Kruskal-Wallis.

Figure 5

Lung tumour treatment with ERA/ERb antagonists or with tamoxifen. (A) *In vivo* bioluminescent signals and quantification of LLC-Luc lung tumours in female mice treated with vehicle (control group), Era antagonist (MPP, 1 mg/kg), ERb antagonist (PHTPP, 1 mg/kg), $n = 8$; $*P < 0.05$, 2-way ANOVA. (B) Hematoxylin/eosin staining of LLC-Luc lung tumours (scale bar = 1 mm) and quantification of tumour density (lung tumour area/total lung area) in females treated with vehicle; MPP; PHTPP and Tamoxifen (Tmx), $*P < 0.05$, Mann-Whitney. (C) Immunofluorescent staining (scale bar = 50 μ m) and quantification (LYVE1 area/lung tumour area) of LYVE1 positive vessels in LLC-Luc lung tumours of female mice treated with vehicle ($n = 7$), MPP ($n = 8$); PHTPP ($n = 7$) or Tmx ($n = 8$), $*P < 0.05$, $**P < 0.01$, 1-way ANOVA. (D) Immunofluorescent staining of CD31 positive blood vessels in LLC-Luc lung tumours (scale bar = 50 μ m) and quantification of the CD31 density (CD31 area/lung tumour area) in tumours of female mice treated with vehicle ($n = 16$), MPP ($n = 8$); PHTPP ($n = 8$) or Tmx ($n = 8$), $***P < 0.001$, Kruskal-Wallis. Milliplex analysis of (E) VEGFC, (F) VEGFD, (G) VEGFA, (H) bFGF concentrations in LLC-Luc lung tumour lysates from vehicle-, MPP- and Tmx-treated females; $*P < 0.05$, $**P < 0.01$, 1-way ANOVA. (I) *In vivo* bioluminescent signals and quantification of LLC-Luc-derived bioluminescence in lungs of male mice treated with vehicle (control group), Era antagonist (MPP, 1 mg/kg), ERb antagonist (PHTPP, 1 mg/kg) ($n = 8$), 2-way ANOVA. (J) Hematoxylin/eosin staining of LLC-Luc lung tumours (scale bar = 1 mm) and quantification of tumour density (lung tumour area/total lung area) from males treated with vehicle, MPP, PHTPP and Tamoxifen (Tmx), Kruskal-Wallis. (K) Immunofluorescent staining (scale bar = 50 μ m) and quantification (LYVE1 area/lung tumour area) of LYVE1 positive vessels in LLC-Luc lung tumours of male mice, treated with vehicle ($n = 8$), MPP ($n = 6$); PHTPP ($n = 7$) or Tmx ($n = 7$), 1-way ANOVA. (L) Immunofluorescent staining of CD31 positive blood vessels in LLC-Luc lung tumours (scale bar = 50 μ m) and quantification of the CD31 density (CD31 area/lung tumour area) in lung tumours of male mice ($n = 17$), treated with vehicle ($n = 8$), MPP ($n = 7$); PHTPP ($n = 7$) or Tmx ($n = 7$), Kruskal-Wallis.

Podoplanin (PDPN) staining on tissue samples of lung adenocarcinoma showed that lymphangiogenesis was higher in women than men, independently of ERa status in cancer cells (Fig. 6C). CD31 staining showed that angiogenesis was higher in ER-negative lung adenocarcinoma from women, when patient cohorts were selected by ERa status (Fig. 6D). In addition, we evidenced by RNAscope methodology that ERa mRNA was expressed by CD31- and PDPN-positive cells (Fig. 6E) of these lung adenocarcinoma sections.

Discussion

This study highlights the contribution of lymphatic and blood endothelium in the sex-dependent modulation of lung cancer progression. Based on histological analysis of human lung adenocarcinoma samples and on experimental *in vivo* models, our study shows that the 'female microenvironment' offers a better soil for lung tumour development. Notably, oestrogens in females promote lymphangiogenesis and angiogenesis in an ERa-dependent manner. This new original concept is supported by drastic reduction of lymph/angiogenesis and tumour growth upon treatment with pharmacological ERa antagonist and tamoxifen, which showed efficacy only in females but not in males.

Several preclinical and clinical studies reported a positive correlation between oestrogens and lung tumour growth, especially through a direct action on ER-positive cancer cells (Mah *et al.* 2007, Hammoud *et al.* 2008, Tang *et al.* 2014, Liu *et al.* 2015). The originality of this study is to highlight that sex and oestrogenic status of the host lung parenchyma regulates the development of lung adenocarcinoma cells by modulating lymphangiogenesis and angiogenesis. Our results are in accordance with reviews highlighting underappreciated sex differences in vascular physiology and pathophysiology (Boese *et al.* 2017, Stanhewicz *et al.* 2018). Angiogenesis is one of the leading processes that support cancer development allowing tumours to be fuelled with oxygen and nutrients (De Palma *et al.* 2017). Tumour-associated lymphangiogenesis more recently emerged as an active player and a novel potential therapeutic target, due to its contribution (1) in antigen, fluid and metastatic cell transport, (2) in the regulation of cancer stemness and (3) in immunomodulation (Paupert *et al.* 2011, Dieterich & Detmar 2016, Petrova & Koh 2018).

A key finding is that lymph/angiogenesis was markedly higher in lung adenocarcinoma of woman patients than men, especially when cancer cells did not

express ERa, although ERa is expressed by lymphatic and blood endothelial cells. We applied an orthotopic syngeneic model of lung cancer in immunocompetent mice lacking ERa in Tie2-positive cells (Tie2-Cre⁺/ERa^{lox/lox}) and therefore in lymphatic and blood endothelium (Kisanuki *et al.* 2001, Billon-Gales *et al.* 2009, Morfoisse *et al.* 2018). Through this genetic approach, we delineated that ERa signalling mediates an E2-dependent lung tumour lymph/angiogenesis in females, while ERb and GPER are not involved. Our results are in line with studies reporting that major E2-related blood endothelial functions are mediated through ERa (Brouchet *et al.* 2001, Billon-Gales *et al.* 2009, Pequeux *et al.* 2012, Kim *et al.* 2014, Guivarc'h *et al.* 2018). Although ERa signalling has been recently reported to mediate E2 protective effects on secondary lymphedema (Morfoisse *et al.* 2018), the contribution of E2/ERa signalling to tumour lymphangiogenesis had not been reported yet. In line with our data, lymphangiogenesis was also increased in the heart of mouse after myocardial infarction when cardiomyocytes overexpressed ERa (Mahmoodzadeh *et al.* 2014). Interestingly, the prolymphangiogenic effect of the E2/ERa pathway that we observed on lung tumour lymphangiogenesis in females could also be extended to inflammatory-related lymphangiogenesis. Indeed, the use of Tie2-Cre⁺/ERa^{lox/lox} mice or treatment of WT mice with a pharmacological inhibitor of ERa prevented lymphangiogenesis in the cornea injury assay. Altogether, these results support a significant contribution of E2/ERa signalling in regulation of lymphatic and blood endothelia functions under pathological conditions.

For the first time, we demonstrate the regulation of lymph/angiogenesis by E2/ERa signalling in lung cancer. We delineate that endogenous or exogenous E2 contributes to increase lymph/angiogenesis and levels of VEGFA and bFGF in lung tumours of females. This is in line with previous reports showing an E2-dependent upregulation of VEGFA and bFGF in several tissues (Garmy-Susini *et al.* 2004, Pequeux *et al.* 2012) and with the ERa-dependent upregulation of VEGFR2 observed in myometrial and retinal microvascular endothelial cells treated with E2 (Suzuma *et al.* 1999, Gargett *et al.* 2002). While VEGFA overexpression is correlated with a poor prognosis in lung cancer patients, VEGFC levels are not associated with survival (Zhan *et al.* 2009). In the same model, lymphangiogenesis was not increased through VEGFC or VEGFD overexpression, but was correlated with an increase of VEGFA and bFGF levels. Although the action of VEGFC and VEGFD on VEGFR3 is the major pathway for lymphangiogenesis promotion

Figure 6

Lymph/angiogenic vasculature and ERα status in human lung adenocarcinoma biopsies. (A) Characteristics of human lung tumours (histology, age, smoker status). (B) Representative immunohistochemical staining of ERα (blue) in human lung tumour biopsies according to sex (men: $n = 23$, women: $n = 51$) and separated as ERα-positive (ERα+) and ERα-negative (ERα-) tumours, scale bar = 50 μm . (C) Immunofluorescent staining of PDPN and quantification of PDPN density (PDPN stained tumour area/lung tumour area) in human lung tumours according to sex (men $n = 23$, women $n = 49$) and ERα expression (ERα+: men $n = 14$, women $n = 30$; ERα-: men $n = 9$, women $n = 19$), $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Mann-Whitney, scale bar = 250 μm on upper panel and 50 μm on lower panel. (D) Immunohistochemical staining of CD31 and quantification (CD31 stained tumour area/lung tumour area) in human lung tumours according to sex (men $n = 23$, women $n = 51$) and ERα expression (ERα+: men $n = 14$, women $n = 30$; ERα-: men $n = 9$, women $n = 19$), $*P < 0.05$, t -test, scale bar = 250 μm on upper panel and 50 μm on lower panel. (E) Representative ERα (green), PDPN (red) and CD31 (red) mRNA detection by RNAscope on human lung tumour sections (scale bar = 10 μm). Endometrium is used as positive control for ERα expression. Cell nuclei are stained with DAPI.

(Zhang *et al.* 2010, Alitalo & Detmar 2012), VEGFA and bFGF were also described to stimulate this process (Cursiefen *et al.* 2004, Cao *et al.* 2012, Detry *et al.* 2013). Especially, bFGF has been described to interact with VEGFC and to collaboratively promote tumour growth, lymphangiogenesis and metastasis in an experimental mouse model of fibrosarcoma (Cao *et al.* 2012). These data suggest that the increase of bFGF observed in females and E2-treated mice could reinforce the action of VEGFC to increase lymphangiogenesis even if VEGFC levels are not directly modulated.

Since the female microenvironment of lung tumours appeared to be more sensitive to oestrogens through ERa signalling, female mice were treated with an ERa antagonist or with tamoxifen, an anti-oestrogen therapy largely used in breast cancer patients. Interestingly, ERa antagonist or tamoxifen decreased lung tumour growth and lymph/angiogenesis. Concomitantly, these treatments also reduced VEGFA and bFGF, but not VEGFC and VEGFD levels. These observations corroborate clinical data showing that tamoxifen decreases lung cancer probability in patients (Bouchardy *et al.* 2011, Chu *et al.* 2017). Tamoxifen also prevents the protective effect of E2 on secondary lymphedema (Morfoisse *et al.* 2018). In addition, tamoxifen can also reduce capillary tube formation and endothelial cell migration by decreasing platelet-related VEGFA discharge (Johnson *et al.* 2017). More interestingly, these treatments failed to impact lung tumour growth and lymph/angiogenesis in males. Our results thus provide an explanation for the increased incidence of lung cancer in young women (30–39 years old) compared to men (Jemal *et al.* 2018).

In summary, this study emphasises that female microenvironment sustains more efficiently lung tumour development than the male one. Especially, oestrogens increase lymph/angiogenesis through an ERa-dependent pathway. In accordance, treatment by ERa antagonist or tamoxifen decreases lung tumour growth and lymph/angiogenesis in females but not in males.

In addition to shedding light on sex issues in lung cancer in young patients, our study has potential clinical implication by pointing to the importance of oestrogen status or supplementation on lung cancer development that should be considered to adapt therapeutic strategies.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0328>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C D performed the experiments and prepared the figures and the manuscript. N R developed the *in vivo* model of lung cancer, gave scientific advices along the study and critically reviewed the manuscript. S B developed and performed all computer-assisted quantification analysis. I P, A G and M G-C contributed to *in vitro* and *in vivo* experiments and critically reviewed the manuscript. L B supported the study with critical clinical advices. A G supported the study with scientific advices all along the study and critically discussed study design and manuscript. F L contributed to the genesis of the project, supported the study with scientific advices along the study and critically reviewed the manuscript. D C supervised the project, was responsible for finding funding and critically reviewed the manuscript. C P initiated and supervised the project, was responsible for finding funding, designed the experiments, analysed data and wrote the manuscript.

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