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Lymph/angiogenesis contribute to sex differences in lung cancer through ERalpha signalling

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Abbreviations:

bFGF basic Fibroblast Growth Factor

CD31 Cluster of Differentiation 31

COPD Chronic Obstructive Pulmonary Disease

Cx Castrated

DAPI 4',6-diamidino-2-phénylindole

DMEM Dulbecco's Modified Eagle Medium

DMSO dimethylsulfoxyde

E2 17b-oestradiol

EdU 5-ethynyl-2'-deoxyuridine

EGF Epidermal Growth Factor

EGFR Epidermal Growth Factor Receptor

ER Estrogen Receptor

FBS Foetal Bovine Serum

FBS cs dextran-coated charcoal treated Foetal Bovine Serum

GPER G Protein-coupled Estrogen Receptor 1

HRT Hormone Replacement Therapy

LLC Lewis Lung Carcinoma

Luc Luciferase-transfected

LYVE1 Lymphatic vessel endothelial hyaluronan receptor 1

OVX Ovariectomised

PDPN Podoplanin

Tmx Tamoxifen

VEGF Vascular Endothelial Growth Factor

VEGFR2 Vascular Endothelial Growth Factor Receptor 2

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Abstract

Estrogen signalling pathways are emerging targets for lung cancer therapy. Unravelling the contribution of estrogens in lung cancer development is a pre-requisite to support the development of sex-based treatments and to identify patients who could potentially benefit from anti-estrogen 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 grew faster in female mice than in males. Moreover, estradiol (E2) promoted tumour development in female mice and increased lymph/angiogenesis and levels of VEGFA and bFGF in lung tumours of females through an estrogen receptor (ER) alpha-dependent pathway. Furthermore, while treatment with ERbeta antagonist was inefficient, ERalpha 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 to 55 years old revealed more extensive lymphangiogenesis and angiogenesis in tumour samples issued from women than from men. In conclusion, our findings highlight an E2/ERalpha-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 estrogen status or supplementation on lung cancer development that should be considered to adapt therapeutic strategies.

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 (COPD), 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 of lung cancer are currently higher among young women than men (Jemal, et al. 2018; Lewis, et al. 2014). 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 (Katcoff, et al. 2014; Wakelee, et al. 2006). 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; Townsend et al. 2012; Wakelee, et al. 2007). Two major pathways could contribute to these differences: the epidermal growth factor (EGF)/EGFR and sex steroids (Cadranel, et al. 2011; Siegfried and Stabile 2014).

Clinical and experimental data strongly support a contribution of estrogens to lung cancer development (Rodriguez-Lara, et al. 2018; Siegfried and Stabile 2014). Indeed, elevated 17b-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-estrogen therapy exhibit a reduced risk of developing subsequent lung cancer and display lower mortality rates from lung cancer (Bouchardy, et al.

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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 (MHT) on lung cancer incidence and survival remains unclear (Chlebowski, et al. 2016; Greiser, et al. 2010; Schabath, et al. 2004). Overall, the contribution of estrogens 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-estrogen therapy still remains poorly considered for lung cancer.

Tumour microenvironment, especially lymphatic and blood vasculatures, strongly contributes to tumour development and dissemination (De Palma, et al. 2017; Dieterich and Detmar 2016; Paupert, et al. 2011). 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 and 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 estrogen 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 coupled-protein estrogen 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 and

Eaton 2012; Rodriguez-Lara et al. 2018) and that ERb sustains lung tumour growth in murine models (Hershberger, et al. 2009; Pietras, et al. 2005; Tang, et al. 2014; Zhao, et al. 2011). Otherwise, ERa-dependent growth-promoting genes are up-regulated in lung cancer (Pietras et al. 2005; Pietras and 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 of estrogen pathway 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 syngeneic lung cancer cells into pulmonary parenchyma of immunocompetent mice revealed that lung tumours grew 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 & Methods

Human samples and ethical study approval

Human lung tumour samples, endometrium and testis tissues 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.

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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. 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 (FELASA) 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, Abingdon, UK).

Cell cultures

MCF-7 cells (HTB-22™) and male mouse Lewis Lung Carcinoma cells transfected with luciferase gene (LLC-Luc, LL/2-luc-M38) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and Caliper Lifesciences (Hopkinton, MA, USA), respectively. MCF-7 and 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 10 passages after authentication. Cells were routinely cultured in DMEM (Gibco Invitrogen Corporation, Paisley, United Kingdom) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Lonza, Basel, Switzerland), 2 mM glutamine and 100 UI/ml penicillin/streptomycin (ThermoFisher Scientific, MA, USA).

Cell proliferation and viability assays

LLC-Luc cells were cultured for 24h in red phenol-free medium (Gibco Invitrogen Corporation, Paisley, UK) supplemented with 10% of heat-inactivated and dextran-coated charcoal treated foetal bovine serum (FBS-cs, Lonza, Basel, Switzerland). Cells were then cultured in medium with 2% FBS-cs supplemented with either 10% FBS (positive control) or E2 (10^{-10}M to 10^{-7}M) or MPP (10^{-8}M) or PHTPP (10^{-8}M) or G15 (10^{-7}M) or vehicle (DMSO 0.001%) or cisplatin as positive control (100µM, #P4394, Sigma-Aldrich, St-Louis, MO, USA). To investigate cell proliferation, LLC-Luc cells were incubated during 24h with methyl-^{3}[H]thymidine (Perkin Elmer Life Sciences, Boston, MA, USA) and radioactivity was measured with a b-counter (Beckman, LS-5000-CE); to measure cell viability, an MTT test (#11465007001, Roche, Basel, Switzerland) 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, Basel, Switzerland). Primary antibodies used for immunostaining were anti-ERa (clone 60C, #04-820, Millipore; F10, #sc-8002, Santa Cruz, CA, USA), anti-ERb (PPZ0506, #417100, Invitrogen/ThermoFisher Scientific, MA, USA), anti-GPER (#sc-48525, Santa Cruz, CA, USA) and anti-HSC70 (B-6, #sc-7298, Santa Cruz, CA, USA). After incubation with appropriated HRP-conjugated secondary antibodies, immunoreactions were revealed using the enhanced chemoluminescence kit (Thermofisher Scientific, MA, USA). Images were acquired by a LAS4000 digital camera (FujiFilm, Japan).

Mouse orthotopic model of lung cancer

When required by the experimental protocol, five weeks old mice were gonadectomised. Ovaries and testis tissues were collected as control samples for western blot assays. Two

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weeks after surgery, some 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 ($2x10^6$ 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/60days release, Innovative Research of America, FL, USA). All treatments were started 2 weeks before the LLC-Luc cell instillation and were conducted until sacrifice at day 21 after tumour cell instillation. To monitor lung tumour growth, luciferin (150mg/kg in PBS, #E160E, Promega, WI, USA) 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 (5µm) were stained with hematoxylin and eosin (H/E). For each mouse, we collected 8 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) correspond to the lung tumour density. To obtain the lung tumour density for one mouse, we calculated the mean of the 8 densities measured from the 8 slides of the same lung.

Immunolabelings were carried out using anti-CD31 (#ab28364, Abcam, Cambridge, United Kingdom), anti-podoplanin (D2-40, #MA1-83884, Thermofisher Scientific, MA, USA), anti-LYVE1 (#AF2125, R&D System, Abingdon, UK) or anti-ERa (1D5, #M7047, Dako, Glostrup, Denmark) antibodies. Slides were then incubated with appropriate biotin-coupled

secondary antibodies (Dako, Glostrup, Denmark) and with streptavidin/alexa 555 or streptavidin/Alexa 488 (#S21381, #A11055, Invitrogen Corporation, Paisley, UK). 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, Belgium). For each mouse tumour sample, a minimum of 5 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 sacrifice, mice received a peritoneal injection of 5-ethynyl-2'-deoxyuridine (EdU, 2.5mg/mouse, ThermoFisher Scientific, MA, USA). EdU incorporation in proliferating cells was evidenced using Click-it EdU cocktail kit (Molecular Probes, Merelbeke, Belgium) in accordance with manufacturer's instructions. Slides were mounted with aquapolymount (Polysciences, Hirschberg an der Bergstrasse, Germany).

Corneal assay

The ophthalmic cauterization of cornea was performed and analysed, as previously described (Detry, et al. 2013). Briefly, mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and xylazine (10 mg/kg body weight) by peritoneal injection; their eyes were locally anesthetized with Unicaïne 0.4% drops (Thea Pharma, Wetteren, Belgium). After anaesthesia, an ophthalmic cauterization (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 hour in 70% ethanol at room temperature, washed in PBS and blocked during 1 hour in milk3%/BSA3% (Nestlé, Brussels,

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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, Abingdon, UK) 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, Merelbeke, Belgium) or goat anti-rat/Alexa Fluor 546 (1:200, #A11035, Molecular Probes, Merelbeke, Belgium) antibodies, respectively. Corneas were whole-mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and pictures were acquired with FSX100 microscope (Olympus, Japan). These experiments were performed on males, females, gonadectomised-mice and ovariectomised female mice treated with subcutaneous slow-releasing E2 pellets (75µg/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 (1mg/kg/day, Tocris Biosciences, R&D system, Abingdon, UK) for 3 weeks (5 times/week). These treatments started 2 weeks before thermal cauterization until sacrifice.

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, Darmstadt, Germany).

RNA in situ hybridization (RNAscope)

The mRNA in situ hybridization 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 (5µm) were deparaffinized and hybridized in duplex, either with Hs-ESR1 (#310301, Bioké, Leiden,

The Netherlands) and Hs-PECAM1-O1-C2 (#487381-C2, Bioké, Leiden, The Netherlands) probes or with Hs-ESR1 (#310301, Bioké, Leiden, The Netherlands) and Hs-PDPN-C3 (#539751-C3, Bioké, Leiden, The Netherlands) probes or with RNAscope 3-plex negative control probe (#320871, Bioké, Leiden, The Netherlands). Hybridization signal was amplified with RNAscope Multiplex Fluorescent reagent kit V2 (#323100, Bioké, Leiden, The Netherlands) and TSA Plus Fluorescent kits (#NEL745001KT, #NEL744001KT, PerkinElmer, MA, USA). Images were recorded with a confocal Olympus Fluoview 1000 microscope (Olympus America, Waltham, MA, USA) at 40X of magnification.

Statistical analysis

Results were were analysed with GraphPad Prism 5.0 (San Diego, CA, USA). 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.

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

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297 approximately 2-fold larger lung tumours than males (Fig. 1B). To evaluate the effects of
298 endogenous estrogen in sex-related differences regarding lung tumour development, females
299 were ovariectomised (OVX) and LLC-Luc tumour implantation in lung parenchyma was
300 measured. Interestingly, OVX female mice displayed a decreased lung tumour growth as
301 compared to naive females and to OVX females supplemented with exogenous E2 (OVX+E2)
302 (Fig. 1C-D). By contrast, lung tumour growth was not affected by gonadectomy in males,
303 even after E2 supplementation (Fig. 1E-F). This suggests a specific effect of endogenous and
304 exogenous estrogens on tumour growth in female lungs but not in males.

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

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

310 Treatment of LLC-Luc cells with increasing E2 concentrations, ranging from 10^{-10}M to 10^{-}
311 ^{7}M, for 24, 48 or 72 hours did neither affected LLC-Luc proliferation (Fig. 2B-C) nor cell
312 viability (Fig. 2D). FBS and cisplatin were used as positive and negative controls in the
313 proliferation or viability assays, respectively. In addition, the combined treatment of cells
314 with E2 and ER antagonists, MPP (ERa antagonist), PHTPP (ERb antagonist) or G15 (GPER
315 antagonist), did not modulate cell proliferation (Fig. 2E). These results highlight that E2 does
316 not directly increase lung cancer cell proliferation, despite GPER expression by those cells.

317 To assess LLC-Luc cell proliferation *in vivo*, tumour-bearing mice were intraperitoneally
318 injected with EdU (Fig. 2F). Interestingly, EdU density was higher in LLC-Luc lung tumours
319 when OVX mice were treated with E2 (OVX+E2) (Fig. 2G). In these tumours, the expression
320 of ERa by cancer cells was not induced *in vivo* (Fig. 2H-I). However, some positivity was

associated to lymphatic and blood vessels, as shown by co-immunostainings (yellow staining, white arrows) of ERa and LYVE1 (Fig. 2G) or CD31 (Fig. 2H).

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-B). While ovariectomy (OVX) decreased tumour lymphatic vessel density, 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. Similarly to lymphatic vessel, blood vessel density was increased in lung tumours grown in female mice (Fig. 3A-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-D). VEGFA and bFGF levels were increased in LLC-Luc lung tumours grown in females as compared to males and in OVX females treated with E2 as compared to OVX mice (Fig. 3E-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 neither lymphangiogenesis nor angiogenesis (Fig. 3H).

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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-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 estrogen receptors, we tested pharmacological inhibitors of ERa (MPP), ERb (PHTPP) and GPER (G15) on the cornea injury model in wild-type 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 unique estrogen 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, et al. 2011). In female animals, both MPP and tamoxifen decreased tumour growth, while PHTPP was not efficient (Fig. 5A-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-D). No modulation was observed in vessels when mice were treated with PHTPP (Fig. 5C-D). Levels of VEGFC and VEGFD were not modified in these experimental conditions (Fig 5E-F). In contrast, treatment with MPP or tamoxifen decreased VEGFA and bFGF levels measured in lung tumours from females (Fig. 5G-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-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 to 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%)

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and in woman (62.7%) samples as assessed by nuclear immunohistochemical staining (Fig. 6B).

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 endothelia 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, estrogens 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 estrogens and lung tumour growth, especially through a direct action on ER-positive cancer cells (Hammoud et al. 2008; Liu, et al. 2015; Mah et al. 2007; Tang et al. 2014). The originality of this study is to highlight that sex and estrogenic 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

the 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 (Dieterich and Detmar 2016; Paupert et al. 2011; Petrova and 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 (Billon-Gales et al. 2009; Kisanuki, et al. 2001; 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 (Billon-Gales et al. 2009; Brouchet, et al. 2001; Guivarc'h, et al. 2018; Kim, et al. 2014; Pequeux et al. 2012). 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 wild-type 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

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445 in regulation of lymphatic and blood endothelia functions under pathological conditions.

446 For the first time, we demonstrate the regulation of lymph/angiogenesis by E2/ERa signalling
447 in lung cancer. We delineate that endogenous or exogenous E2 contributes to increase
448 lymph/angiogenesis and levels of VEGFA and bFGF in lung tumours of females. This is in
449 line with previous reports showing an E2-dependent upregulation of VEGFA and bFGF in
450 several tissues (Garmy-Susini, et al. 2004; Pequeux et al. 2012) and with the ERa-dependent
451 upregulation of VEGFR2 observed in myometrial and retinal microvascular endothelial cells
452 treated with E2 (Gargett, et al. 2002; Suzuma, et al. 1999). While VEGFA overexpression is
453 correlated with a poor prognosis in lung cancer patients, VEGFC levels are not associated
454 with survival (Zhan, et al. 2009). In the same model, lymphangiogenesis was not increased
455 through VEGFC or VEGFD overexpression, but was correlated with an increase of VEGFA
456 and bFGF levels. Although the action of VEGFC and VEGFD on VEGFR3 is the major
457 pathway for lymphangiogenesis promotion (Alitalo and Detmar 2012; Zhang, et al. 2010),
458 VEGFA and bFGF were also described to stimulate this process (Cao, et al. 2012; Cursiefen,
459 et al. 2004; Detry et al. 2013). Especially, bFGF has been described to interact with VEGFC
460 and to collaboratively promote tumour growth, lymphangiogenesis and metastasis in an
461 experimental mouse model of fibrosarcoma (Cao et al. 2012). These data suggest that the
462 increase of bFGF observed in females and E2-treated mice could reinforce the action of
463 VEGFC to increase lymphangiogenesis even if VEGFC levels are not directly modulated.

464 Since the female microenvironment of lung tumours appeared to be more sensitive to
465 estrogens through ERa signalling, female mice were treated with an ERa antagonist or with
466 tamoxifen, an anti-estrogen therapy largely used in breast cancer patients. Interestingly, ERa
467 antagonist or tamoxifen decreased lung tumour growth and lymph/angiogenesis.
468 Concomitantly, these treatments also reduced VEGFA and bFGF, but not VEGFC and
469 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 sex specificities evidenced in lung cancer patients, where young women (30-39 years old) display higher lung cancer incidence than young men (Jemal et al. 2018).

In summary, this study emphasizes that female microenvironment sustains more efficiently lung tumour development than the male one. Especially, estrogens 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 estrogen status or supplementation on lung cancer development that should be considered to adapt therapeutic strategies.

Conflict of interest statement: The authors have nothing to disclose.

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Author Contribution

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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Figure Legends

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=1mm) and quantification of lung tumour density (tumour area/total lung area) on the right panel, 8 slides spaced with 50µm were analysed per sample, *p<0.05, Mann-Whitney. (C) *In vivo* bioluminescent signals derived from LLC-Luc lung tumours imaged either in whole mice of 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=1mm) (n=8), 8 slides spaced with 50µm 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), gonadectomized (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 gonadectomized male mice treated (Cx+E2, n=7) or not with E2 (Cx, n=8) (scale bar=1mm), 8 slides spaced with 50µm 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 estrogen receptors (ER) on LLC-Luc cell lysates: ERa (66kDa), ERb (56kDa), GPER

(42kDa). HSC70 (70kDa) 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 72h, ***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 48h, ***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 48h, ***p<0.001 vs vehicle, n=6, 1-way ANOVA. (E) *In vitro* proliferation of LLC-Luc cells treated with ERa, 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=500µm in upper panel, scale bar=100µm 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 ERa (green), colocalisation of stainings (yellow) and DAPI (blue) in LLC-Luc lung tumours, scale bar=50µm. Uterus tissue was used as positive control, scale bar=50µm. (I) Double-immunofluorescent staining and their zoom of CD31 (red) and ERa (green), colocalisation of stainings (yellow) and DAPI (blue) in LLC-Luc lung tumours, scale bar=50µm.

Figure 3. E2 increases lymph/angiogenesis through ERa. (A) Immunofluorescent staining (scale bar=50µm) 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

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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=1mm; right panel, scale bar=500µm) 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.

Figure 4. Prolymphangiogenic and proangiogenic effects of E2 are mediated by ERa. (A) Hematoxylin/Eosin (H/E) staining of LLC-Luc lung tumours issued from Tie2-Cre^{-}/ERa$^{lox/lox}$ male (n=7) or female (n=7) mice and from Tie2-Cre^{+}/ERa$^{lox/lox}$ male (n=8) or female (n=5) mice (scale bar=1mm) and quantification of the tumour density (tumour area/total lung area), 8 slides spaced by 50µm were analysed per sample, *p<0.05, Mann-Whitney. (B) Hematoxylin/Eosin (H/E) staining of LLC-Luc lung tumours from Tie2-Cre^{-}/ERa$^{lox/lox}$ OVX (n=6), OVX+E2 (n=6) female mice and from Tie2-Cre^{+}/ERa$^{lox/lox}$ OVX (n=7), OVX+E2 (n=5) female mice (scale bar=1mm) and quantification of the tumour density (tumour area/total lung area), 8 slides spaced by 50µm were analysed per sample, *p<0.05, Mann-Whitney. (C) LYVE1 immunofluorescent staining (scale bar=50µm) and quantification (LYVE1 area/lung tumour area) in LLC-Luc lung tumours in Tie2-Cre^{+}/ERa$^{lox/lox}$ 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=50µm) and quantification (CD31 area/lung tumour area) in LLC-Luc lung tumours in Tie2-Cre^{+}/ERa$^{lox/lox}$ male (n=8), female (n=5), female OVX (n=7) and female OVX+E2 (n=5) Kruskal-Wallis. (E) LYVE1/CD31 staining (scale bar=500µm) and quantification (LYVE1 or CD31 area/total cornea area) of blood and

lymphatic vessels in cornea of Tie2-Cre^{+}/ERa$^{lox/lox}$ 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=500µm) 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), ERa antagonist (MPP 10^{-8}M, n=13), ERb 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 or ERb antagonist 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, 1mg/kg), ERb antagonist (PHTPP, 1mg/kg), n=8; *p<0.05, 2-way ANOVA. (B) Hematoxylin/Eosin staining of LLC-Luc lung tumours (scale bar=1mm) 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 of CD31 positive blood vessels in LLC-Luc lung tumours (scale bar=50µm) 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<0.01, ***p<0.001, Kruskal-Wallis. (D) Immunofluorescent staining (scale bar=50µm) 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. 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, 1mg/kg), ERb antagonist (PHTPP, 1mg/kg) (n=8), 2-way ANOVA. (J) Hematoxylin/Eosin staining of LLC-

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Luc lung tumours (scale bar=1mm) 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 of CD31 positive blood vessels in LLC-Luc lung tumours (scale bar=50µm) 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. (L) Immunofluorescent staining (scale bar=50µm) 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.

Figure 6. Lymph/angiogenesic vasculature and ERa status in human lung adenocarcinoma biopsies. (A) Characteristics of human lung tumours (histology, age, smoker status). (B) Representative immunohistochemical staining of ERa (blue) in human lung tumour biopsies according to sex (men: n=23, women: n=51) and separated as ERa-positive (ERa+) and ERa-negative (ER-) tumours, scale bar=50µm. (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 ERa expression (ERa+ : men=14, women n=30; ERa- : 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 50µm 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 ERa expression (ERa+ : men=14, women n=30; ERa- : men n=9, women n=19), *p<0.05, t-test, scale bar=250 µm on upper panel and 50µm on lower panel. (E) Representative ERa (green), PDPN (red) and CD31 (red) mRNA detection by RNAscope on human lung tumour

sections (scale bar=10μm). Endometrium is used as positive control for ERa expression. Cell nuclei are stained with DAPI.

Supplementary figure legends

Suppl. Fig.1: Genotyping of Tie2-Cre^{+}/ERa$^{lox/lox}$ (Cre+) and Tie2-Cre^{-}/ERa$^{lox/lox}$ (Cre-) mice by PCR on DNA extracts from the tail. A. Expression of Cre-recombinase and of FGF2 used as internal positive control of PCR. B. Expression of ERa$^{lox/lox}$ and of ERa$^{-/-}$ corresponding to esr1 without exon 2.

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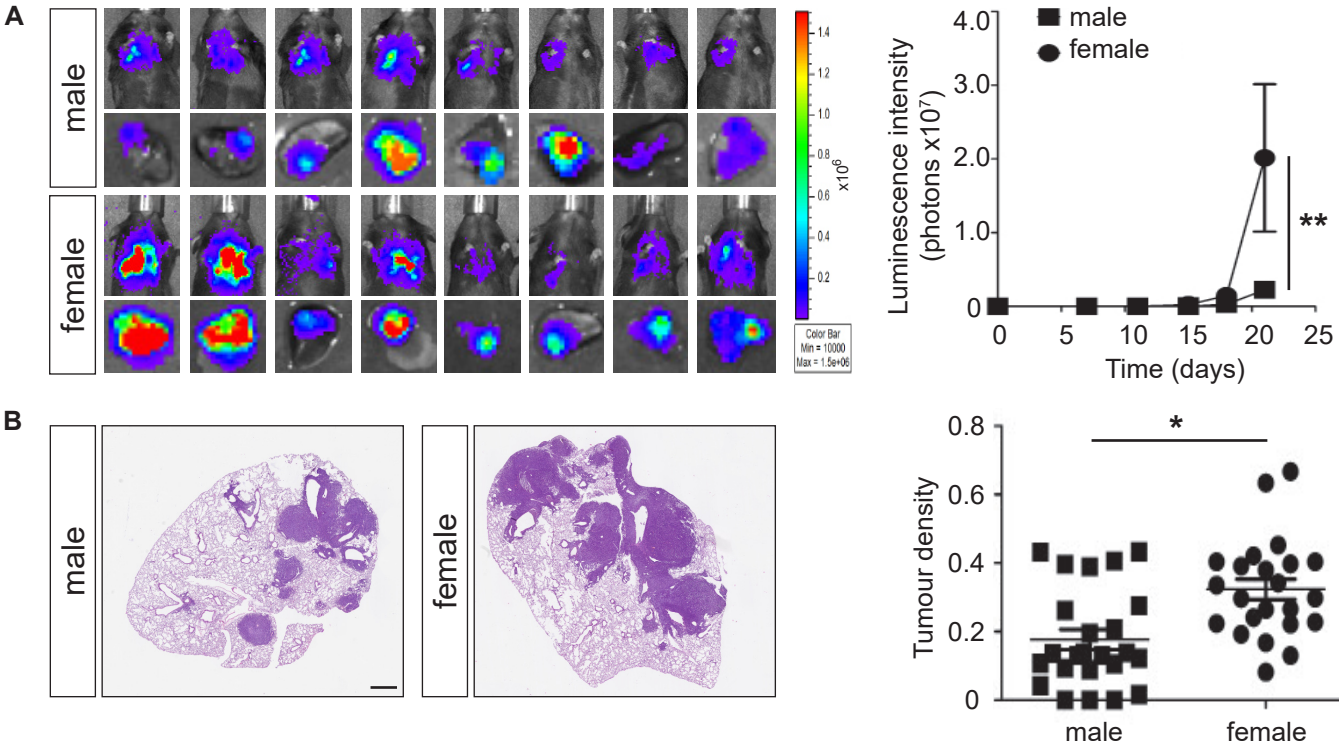

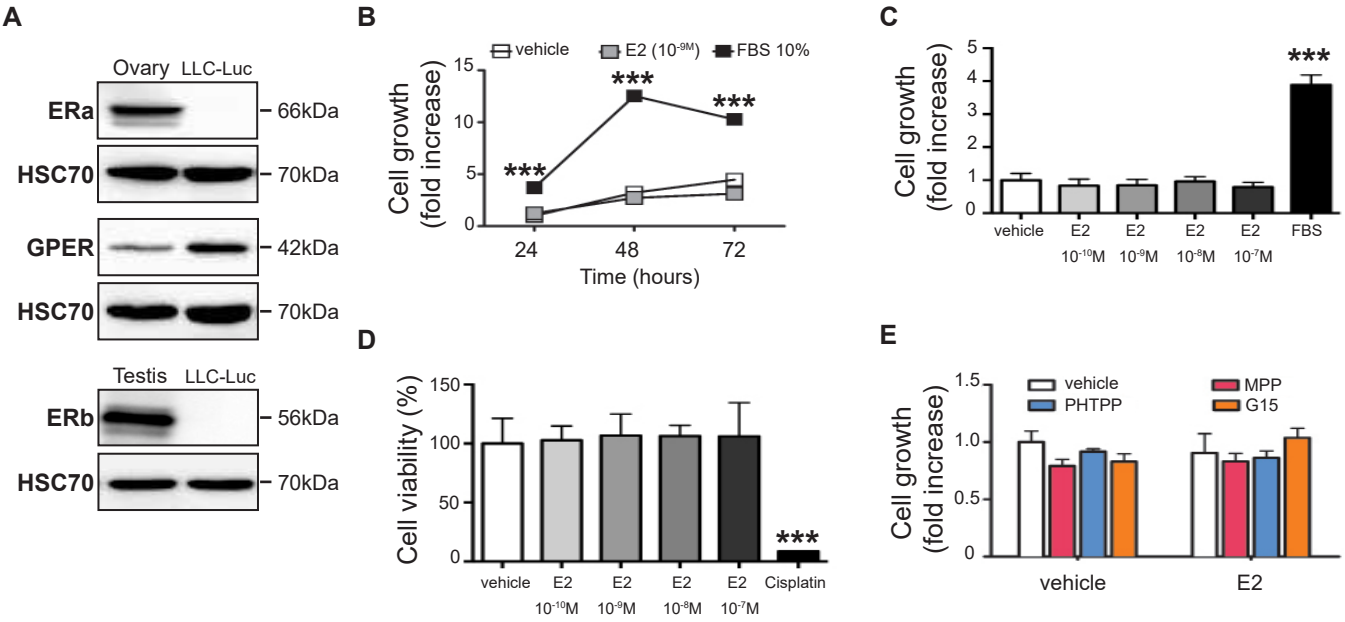

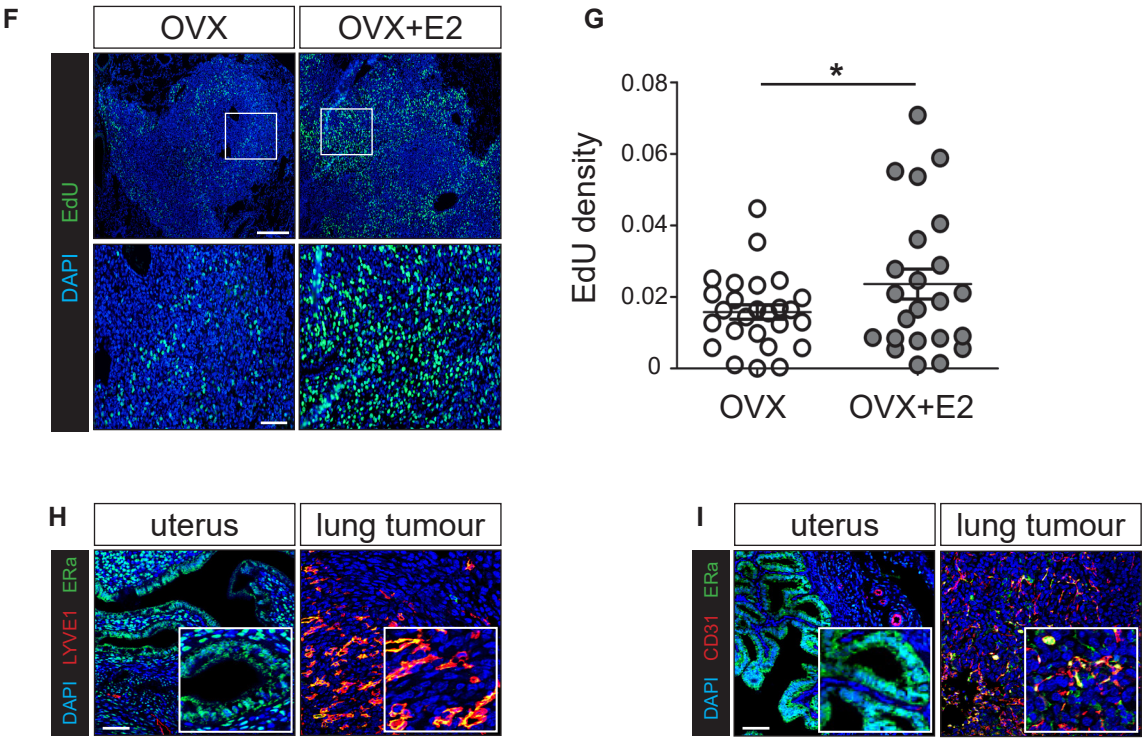

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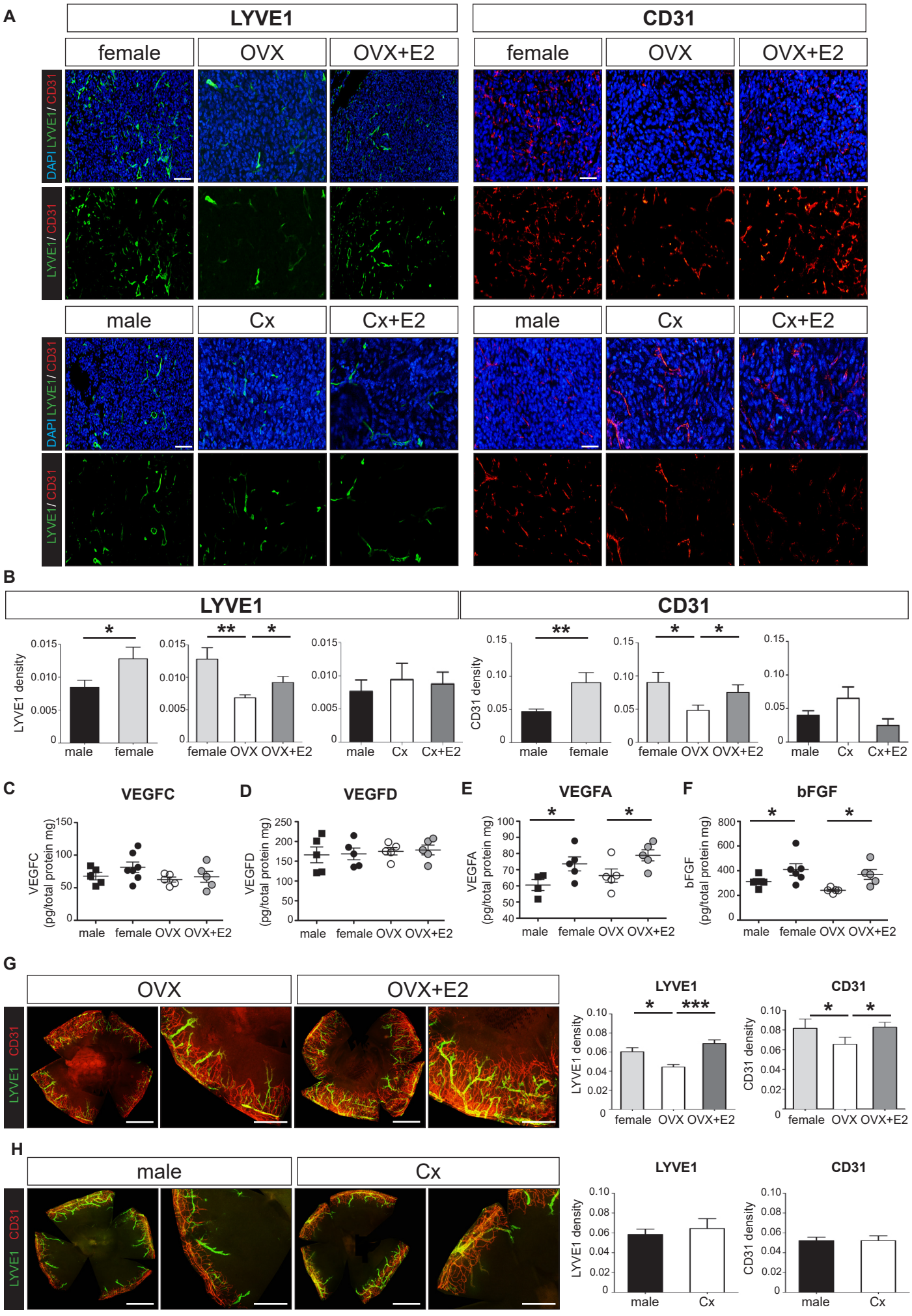

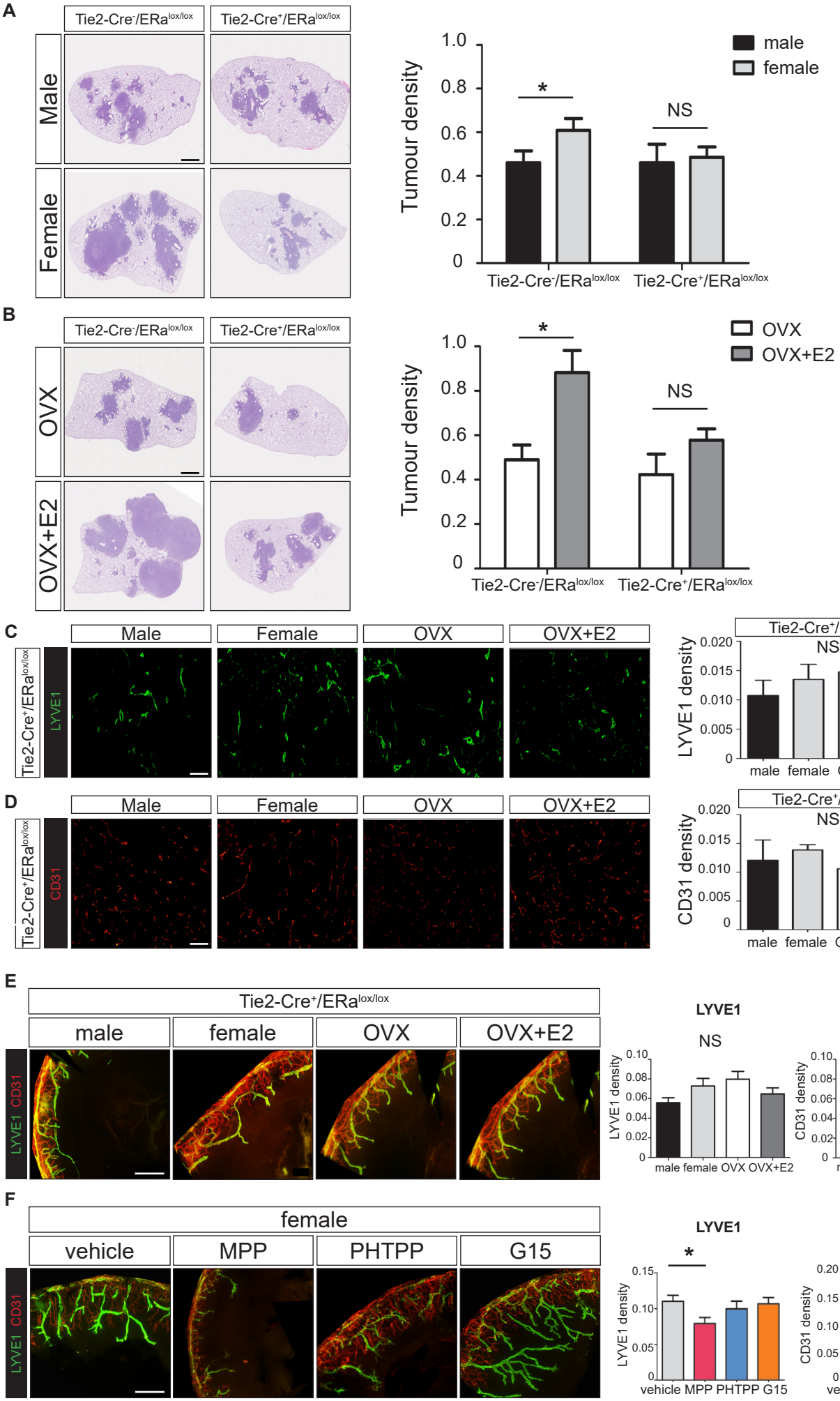

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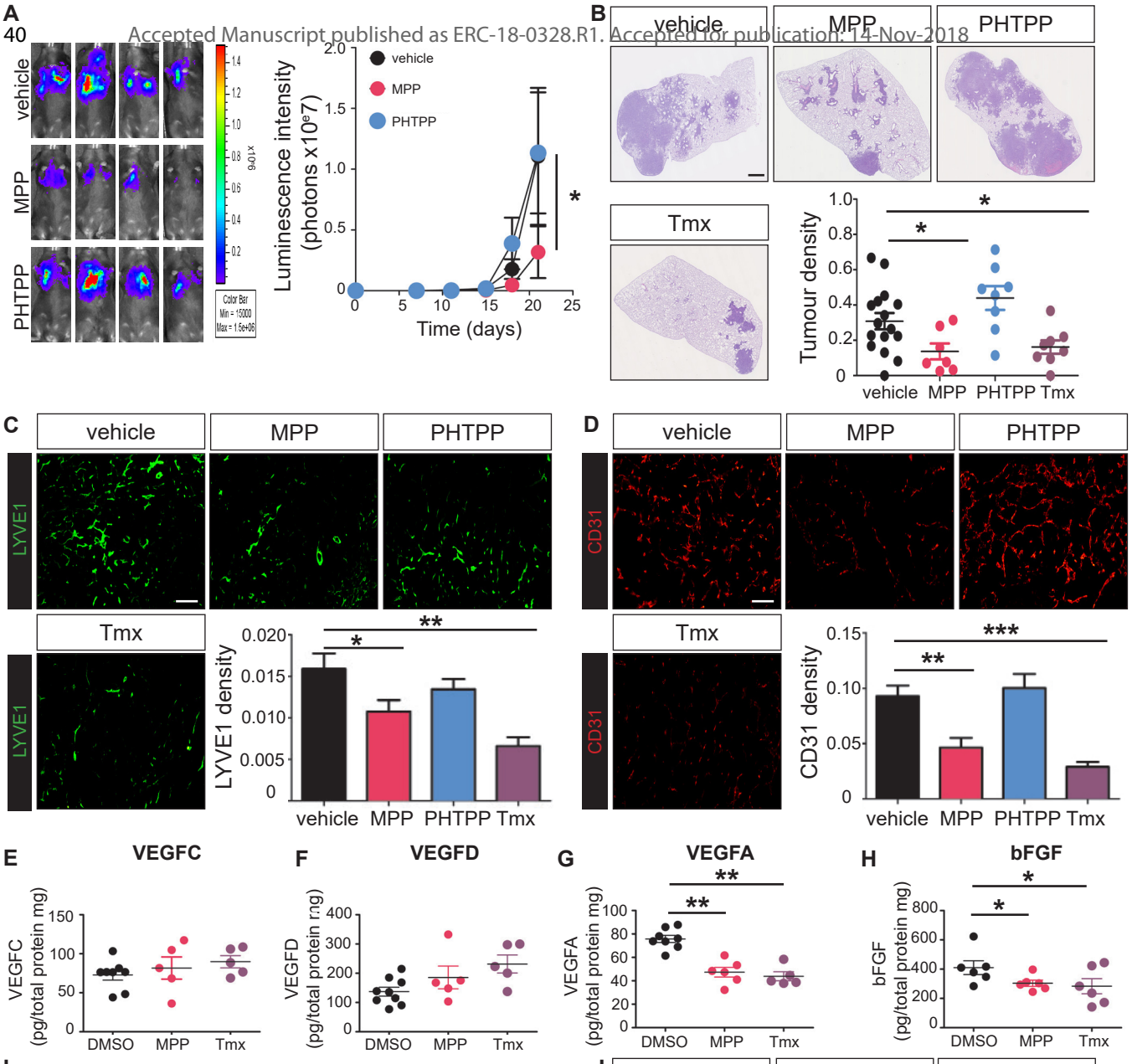
FEMALE



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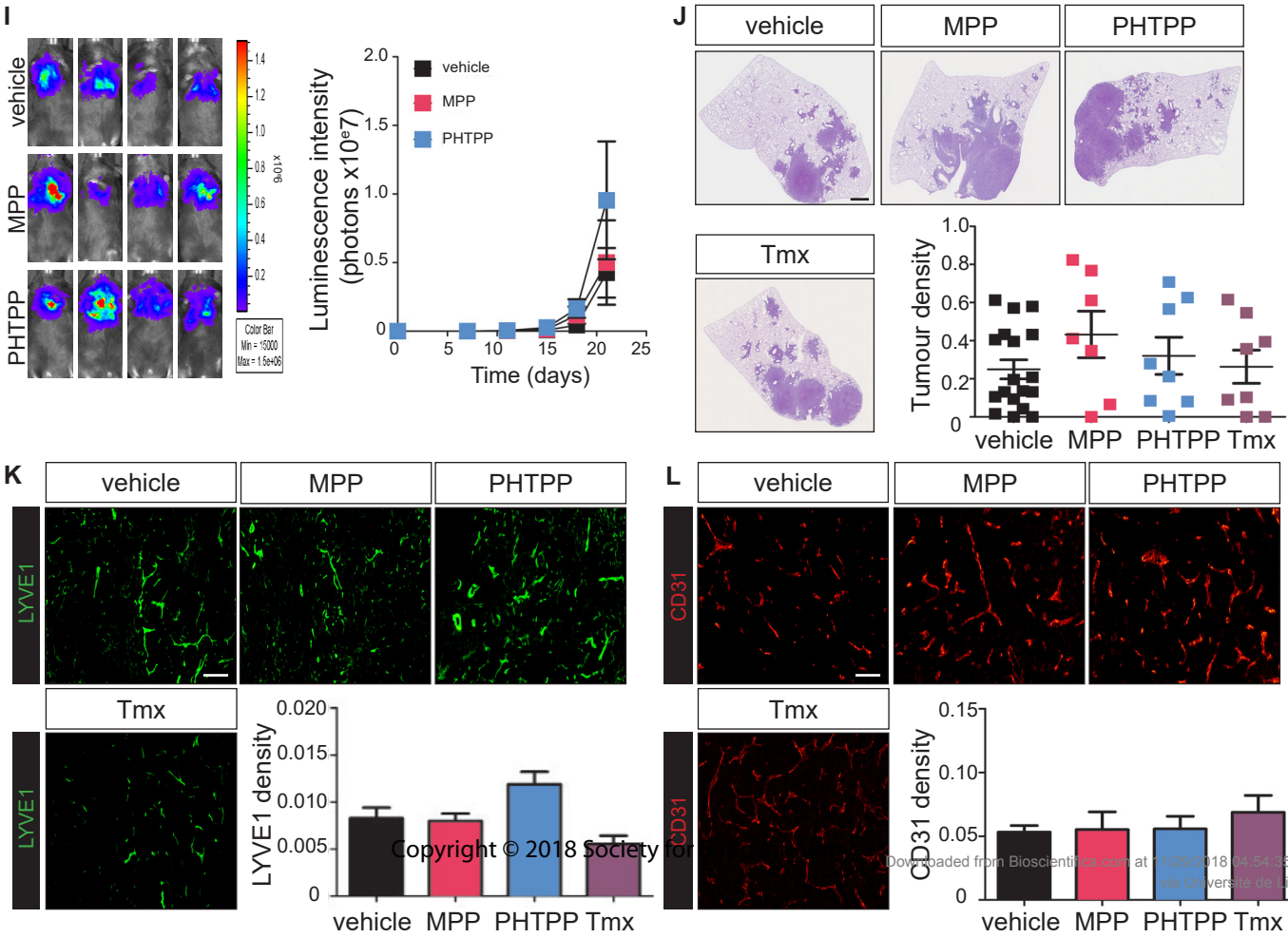
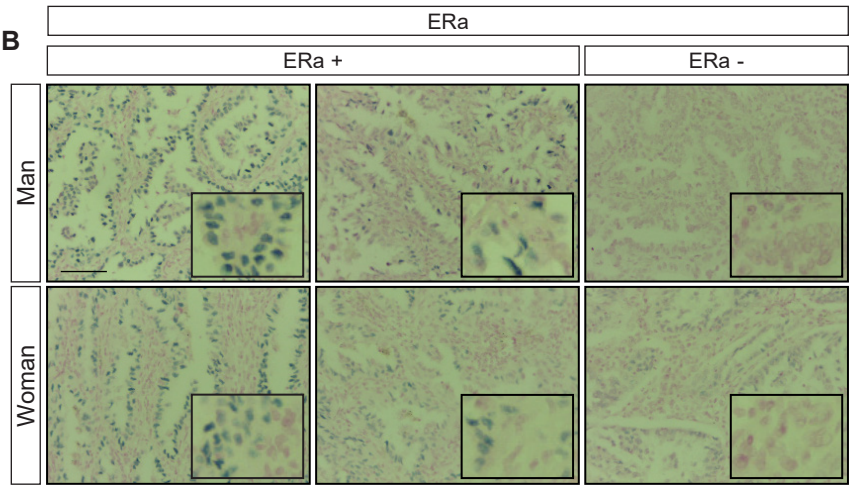
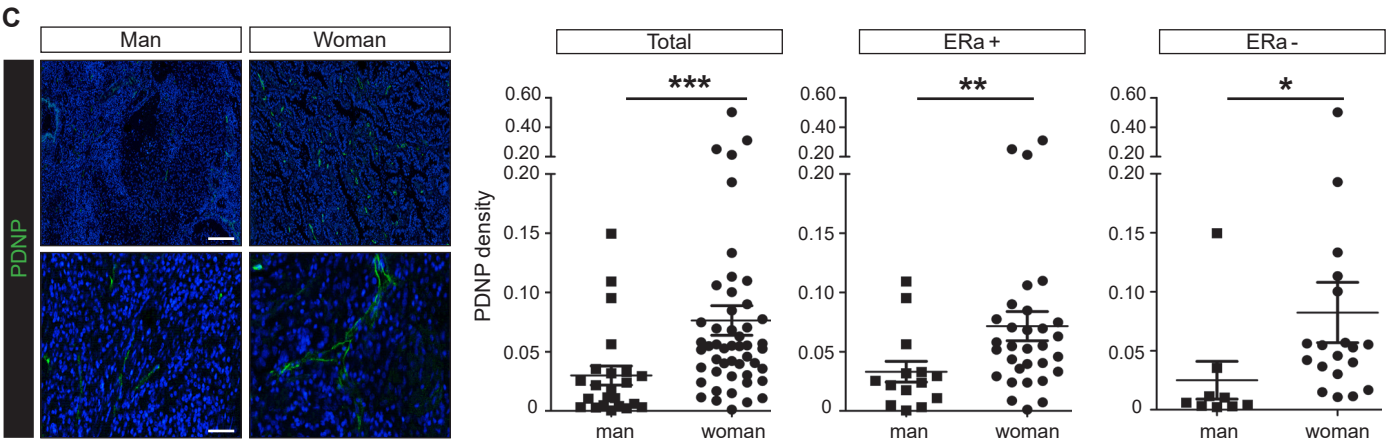
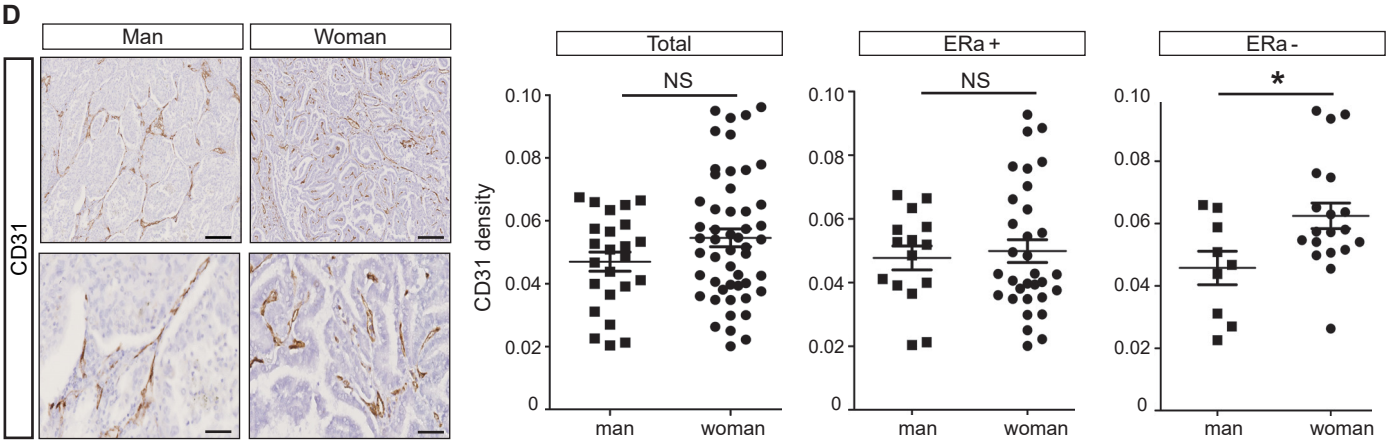


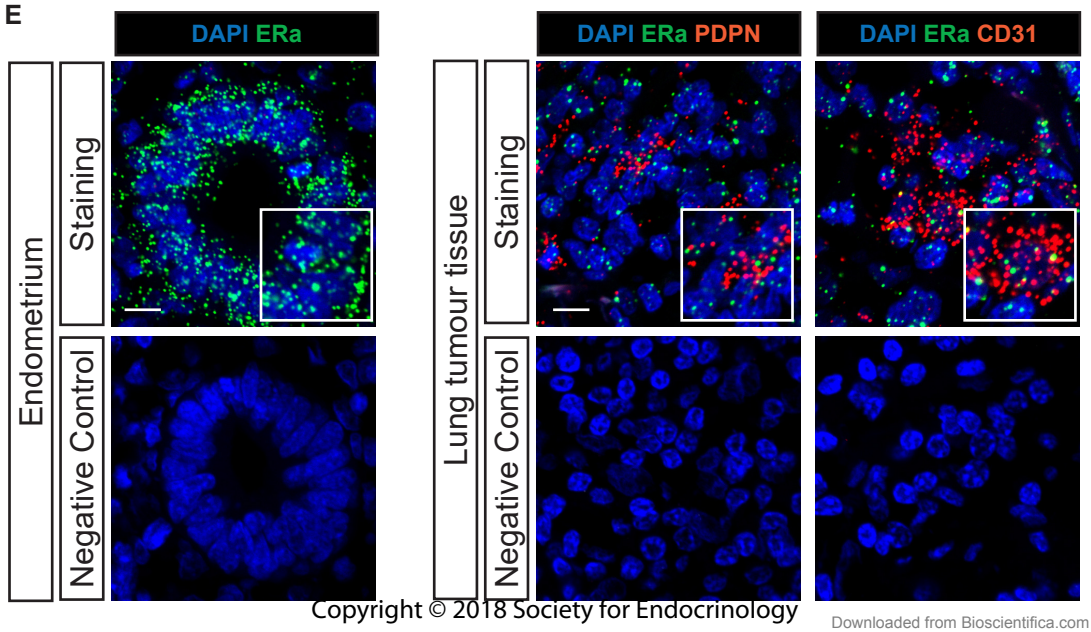
A

Gender/Description	Man	Woman
Adenocarcinoma	100% (23/23)	100% (51/51)
Age	40-55 (Mean = 50)	37-51 (Mean = 51)
Smoker	100% (23/23)	98% (50/51)









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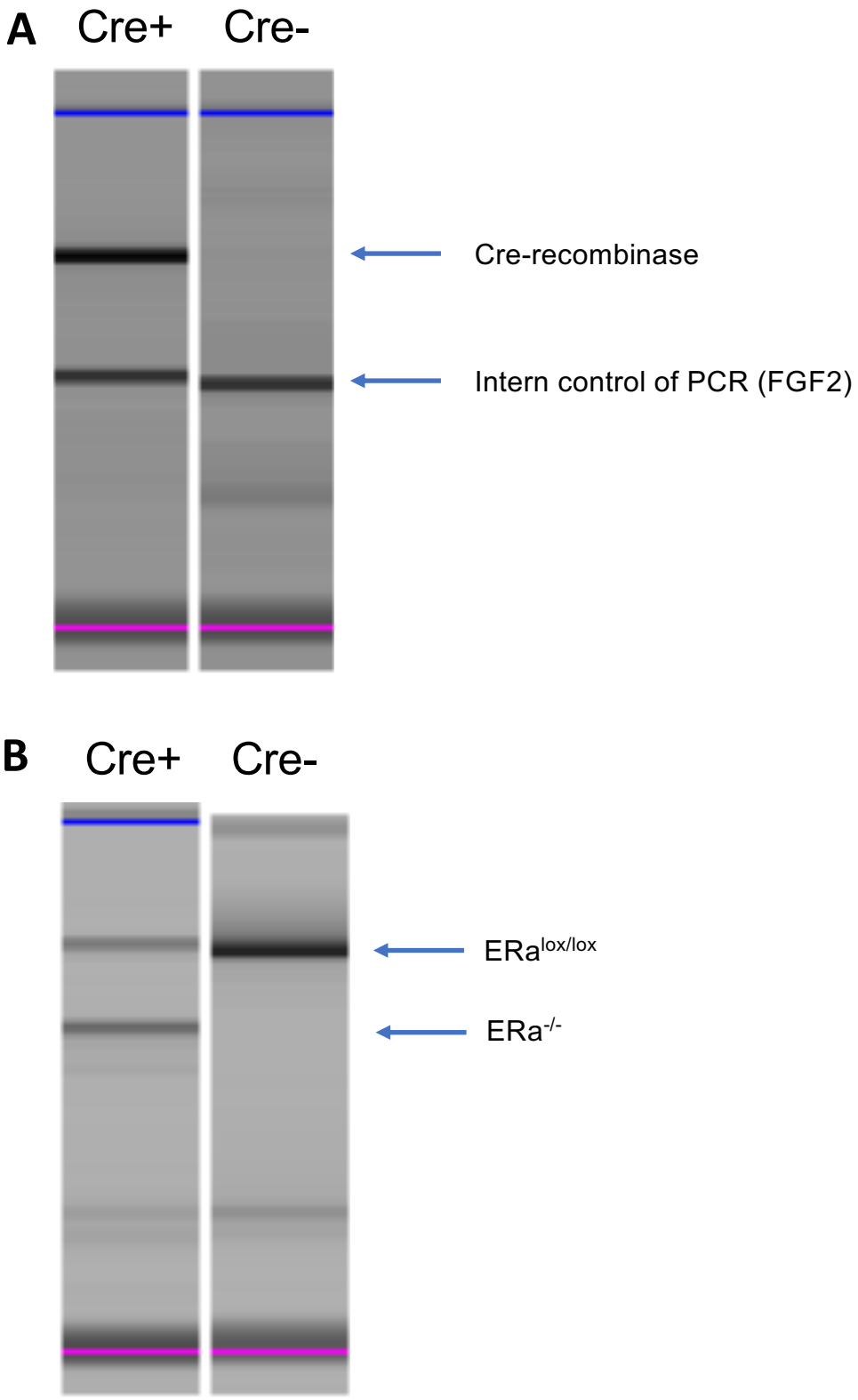

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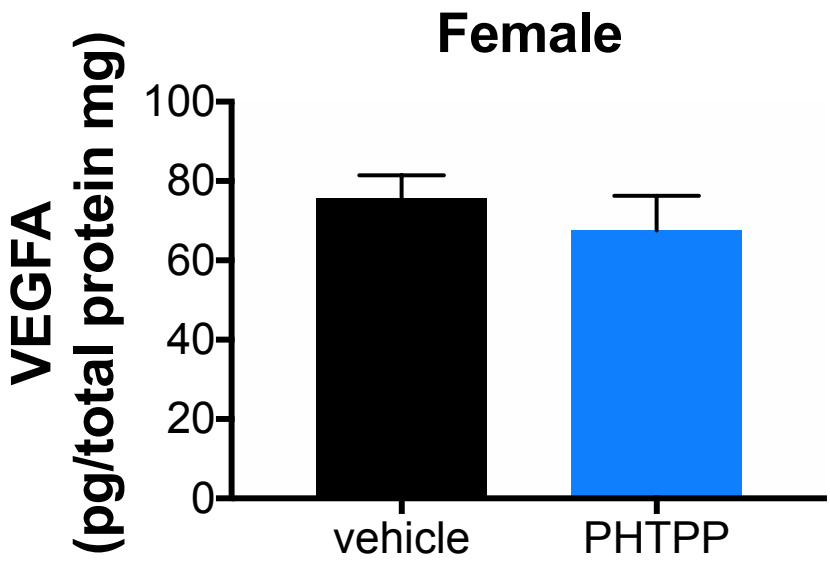
A



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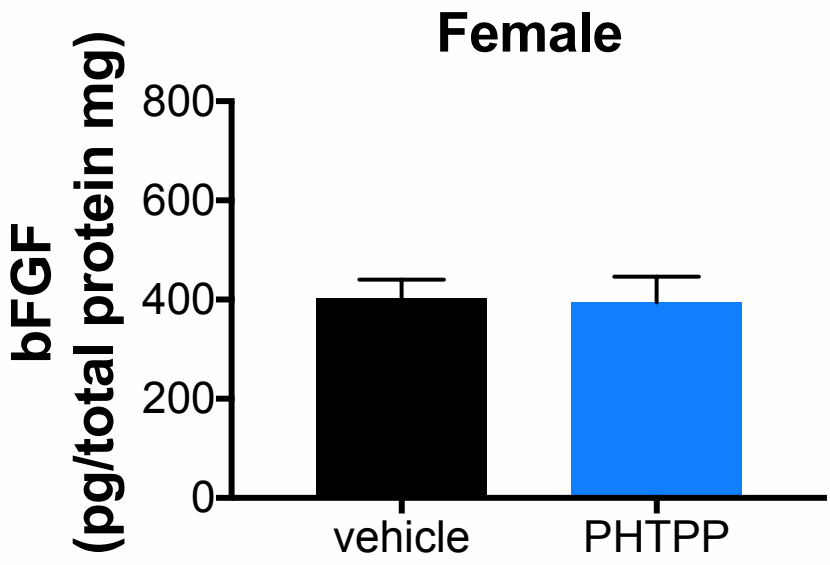

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