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ORIGINAL RESEARCH

Penetrance of Severe Pulmonary Arterial Hypertension in Response to Vascular Endothelial Growth Factor Receptor 2 Blockade in a Genetically Prone Rat Model Is Reduced by Female Sex

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BACKGROUND: We have previously reported important strain differences in response to SU5416 (SU, a vascular endothelial growth factor receptor 2 inhibitor) in rats and have identified a specific colony of Sprague-Dawley rats that are hyperresponsive (SD^{HR}) to SU alone and develop severe pulmonary arterial hypertension (PAH) with a single injection of SU, even in the absence of hypoxia. Interestingly, SD^{HR} rats exhibit incomplete penetrance of the severe PAH phenotype with an "all-or-none" response to SU alone, which provides a unique opportunity to assess the influence of female sex and sex hormones on susceptibility to PAH after endothelial injury in a genetically prone model.

METHODS AND RESULTS: SD^{HR} rats were injected with SU (20 mg/kg SC) and, in the absence of hypoxia, 72% of male but only 27% of female rats developed severe PAH at 7 weeks, which was associated with persistent endothelial cell apoptosis. This sex difference in susceptibility for severe PAH was abolished by ovariectomy. Estradiol replacement, beginning 2 days before SU (prevention), inhibited lung endothelial cell apoptosis and completely abrogated severe PAH phenotype in both male and ovariectomized female rats, while progesterone was only protective in ovariectomized female rats. In contrast, delayed treatment of SD^{HR} rats with established PAH with estradiol or progesterone (initiated at 4 weeks post-SU) failed to reduce lung endothelial cell apoptosis or improve PAH phenotype.

CONCLUSIONS: Female sex hormones markedly reduced susceptibility for the severe PAH phenotype in response to SU alone in a hyperresponsive rat strain by abolishing SU-induced endothelial cell apoptosis, but did not reverse severe PAH in established disease.

Key Words: disease penetrance ■ endothelial cell apoptosis ■ estrogen paradox ■ pulmonary arterial hypertension ■ sex hormones

Pulmonary arterial hypertension (PAH) is a progressive disease that is characterized by sustained elevation in pulmonary arterial pressure, complex pulmonary arterial remodeling, and microvascular rarefaction. Ultimately, right ventricular (RV) pressure overload leads to right-sided heart failure and death.[1] PAH is predominantly a disease of women,[2] and PAH registries from various regions of the world have reported female-to-male ratios ranging from 1.7:1 to 4:1.[2–4] Nonetheless, once PAH has developed, data from these registries demonstrate that women exhibit better survival than men,[2,3,5] consistent with a protective effect of female sex hormones on the progression of PAH, a phenomenon termed the "estrogen paradox".[2]

Sex differences have been studied in experimental models and female sex is often associated with more

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CLINICAL PERSPECTIVE

What Is New?

- This is the first report of the effects of female sex hormones (and withdrawal of female sex hormones) on penetrance of pulmonary arterial hypertension (PAH) phenotype in response to SU5416 in a genetically susceptible animal model, which is relevant for the understanding of sex differences in prevalence of human PAH.
- This study also showed that the protective effects of female sex hormones on lung vasculature is time dependent and is lost once PAH is established.
- This is the first study reporting the female sex–specific protective effects of progesterone in experimental pulmonary hypertension, and also the first report to demonstrate the link between female sex hormones and endothelial cell apoptosis, which is believed to be the central trigger for initiation of PAH development.

What Are the Clinical Implications?

- PAH is associated with several genetic mutations; however, not all patients with these mutations develop the disease; importantly, more females develop PAH but reasons still remain unclear.
- Data from this study demonstrate that steady-state high levels of either estrogen or progesterone prevent penetrance of disease while the periodic withdrawal of sex hormones at the end of the estrous cycle may increase susceptibility to PAH. These findings suggest a novel paradigm by which cyclical fluctuations in levels of female sex hormone may play a role in determining vulnerability to development of PAH.
- This study also highlights the importance of progesterone in the development of PAH that has largely been ignored.

Nonstandard Abbreviations and Acronyms

BMPR2	bone morphogenetic protein receptor-2
EC	endothelial cell
PAH	pulmonary arterial hypertension
PH	pulmonary hypertension
SU	SU5416, a vascular growth factor receptor inhibitor
SD^{HR}	hyperresponsive Sprague-Dawley
RVH	right ventricular hypertrophy
RVSP	right ventricular systolic pressure

variable and less severe pulmonary hypertension (PH); eg, in the monocrotaline and chronic hypoxia models.[6–8] In contrast, in some genetic models, female sex is associated with greater susceptibility to PH particularly when penetrance of the PH phenotype is low.[9] Currently, the model that best recapitulates clinical PAH is induced by a single-injection SU5416 (SU), an inhibitor of vascular endothelial growth factor receptor 2, followed by a 3-week exposure to hypoxia (10% O_2).[10–12] This model is characterized by elevation of pulmonary arterial pressures in the systemic range and development of complex arterial remodeling, including occlusive and plexiform-like lesions.[10–12] The SUHx model is widely used to study pathobiology of PAH, as well as for the assessment of novel therapeutic agents for treatment of PAH.[13] In the SUHx model, both male and intact female rats developed PH of similar severity; however, removal of female sex hormones by ovariectomy has been reported to exacerbate RV remodeling in the SUHx model in female rats.[14] Furthermore, estradiol supplementation was reported to block the development of PAH in ovariectomized female rats in the SUHx model.[14]

Our laboratory has previously reported strain differences in the SUHx model of PAH.[11] We also identified a hypersensitive substrain of Sprague-Dawley rats, obtained from a Canadian supplier (Charles River Laboratories), that developed severe and progressive PAH in response to single SU injection (20 mg/kg subcutaneous) in the absence of hypoxia. This susceptibility to SU alone is associated with several genetic mutations, which are unique to this specific colony of Sprague-Dawley rats and not present in other colonies (unpublished observations). Interestingly, the development of PAH was bimodal, with male rats either developing the full severe PAH phenotype (ie, responders) or no PAH (ie, nonresponders), suggestive of incomplete penetrance in this genetically susceptible strain. In the present study, we now report a marked sex difference in susceptibility for severe PAH in SD^{HR} rats after a single dose of SU alone, with female sex being strongly protective, attributable to the beneficial effects of female sex hormones in preventing endothelial cell (EC) injury and apoptosis induced by vascular endothelial growth factor receptor blockade. We also show that administration of female sex hormones can block the development of PH; whereas estradiol is equally beneficial in males and females, progesterone is only effective in preventing PH in female rats, and treatment with neither hormone reversed established disease.

METHODS

Data S1 shows detailed descriptions of the methods. All animal care and study protocols were approved

by the University of Ottawa Animal Care Committee and conducted according to the guidelines from the Canadian Council for Animal Care. Methods used in the analysis and materials used to conduct the research will be made available upon request to any researcher for purposes of reproducing the results or replicating the procedure.

SU5416 Hyperresponsive Phenotype Characterization

Male and female Sprague-Dawley rats (Charles River Laboratories, QC, Canada) were subjected to a single injection of SU5416 (20 mg/kg SC).[11] Following SU treatment rats were housed under normoxic conditions for 7 weeks and RV systolic pressure (RVSP), RV hypertrophy (RVH), and lung vascular remodelling were measured as previously described.[15] To study the effects of female sex hormones on SU-induced PH, rats were treated with 17β-estradiol or progesterone at 2 days before (pretreatment), 2 days post- (early posttreatment), and 4 weeks post-SU injection (delayed posttreatment) (Figure S1 and S2).

Lung Histological Measurements

Hematoxylin and eosin staining of paraformaldehyde-fixed lung sections and histological measurements were performed as previously described.[15]

Western Blotting

Western blot analysis of whole lung protein extract (50 μg) was performed as previously described[15] using primary antibodies to cleaved caspase-3, progesterone receptor, or β-actin.

Caspase 3/7 Activity Assay

Caspase 3/7 activity in lung lysates was assessed using Apo-ONE Homogeneous Caspase-3/7 Assay as previously described.[15]

Cleaved Caspase 3 and von Willebrand Factor Immunohistochemistry

Immunohistochemistry of paraformaldehyde-fixed lung sections was performed using Rabbit-specific HRP/DAB (ABC) Detection Immunohistochemistry Kit (Abcam plc) as described earlier.[16]

RESULTS

Protective Effect of Female Sex Against Penetrance of PAH in SD^{HR} Rats

SD^{HR} rats exhibited a bimodal response to SU alone (Figure 1A) and a cutoff was derived mathematically to separate responder from nonresponder animals using the data for RVSP (43.3 mm Hg) and RVH (0.43) (Figure S3).[17] Interestingly, 68% of male SD^{HR} rats (23 of 34) exhibited the severe PAH phenotype in response to SU, whereas only 27% of the female rats (7 of 26) were responsive to SU alone (Figure 1A), highlighting important sex differences in penetrance of PAH in this unique model. Nonetheless, the severity of PAH was similar in the responder male and female rats (RVSP 104±17 mm Hg versus 101±14 mm Hg, respectively), whereas RV pressures where equally low in male and female nonresponder rats (RVSP 35±5 mm Hg versus 31±4 mm Hg, respectively). To determine whether the penetrance of PAH was modified by female sex hormones, we tested SU response in ovariectomized versus nonoperated female SD^{HR} rats (intact) with cyclical endogenous estradiol production. A significantly greater proportion of ovariectomized rats responded to SU alone compared with intact female SD^{HR} rats (Figure 1B and 1C); however, there were no differences in the severity of PH in the responder animals, assessed by increases in RVSP and RVH. Furthermore, nonresponder rats showed normal lung vascular structure, whereas a similar degree of occlusive vascular remodeling was observed in both intact and ovariectomized responder rats (Figure 1D and 1E), with the possible exception of medial wall thickness in midsize (50–100 μm) vessels, which was significantly greater in the ovariectomized animals (Figure 1F through 1I and Figure S4 and S5).

Exogenous Estradiol Abrogates Penetrance of PAH in Both Ovariectomized Female and Male SD^{HR} Rats But Progesterone Treatment Is Protective Only in Ovariectomized Female SD^{HR} Rats

To confirm the role of the major female sex hormones in modifying the penetrance of PAH, we supplemented the ovariectomized female and male SD^{HR} rats with exogenous estradiol or progesterone using continuous-release subcutaneous pellets implanted 2 days before SU injection. The exogenous delivery of female sex hormones resulted in a sustained elevation in plasma levels of estradiol and progesterone (Figure S6). Interestingly, estradiol, but not progesterone, treatment resulted in lower bodyweights in both male and ovariectomized female rats compared with placebo-treated rats (Figure S7). Continuous estradiol treatment, starting 2 days before SU injection, completely blocked the development of severe PAH in response to SU alone in ovariectomized female (Figure 2A) and male (Figure 2B) rats. Similarly,

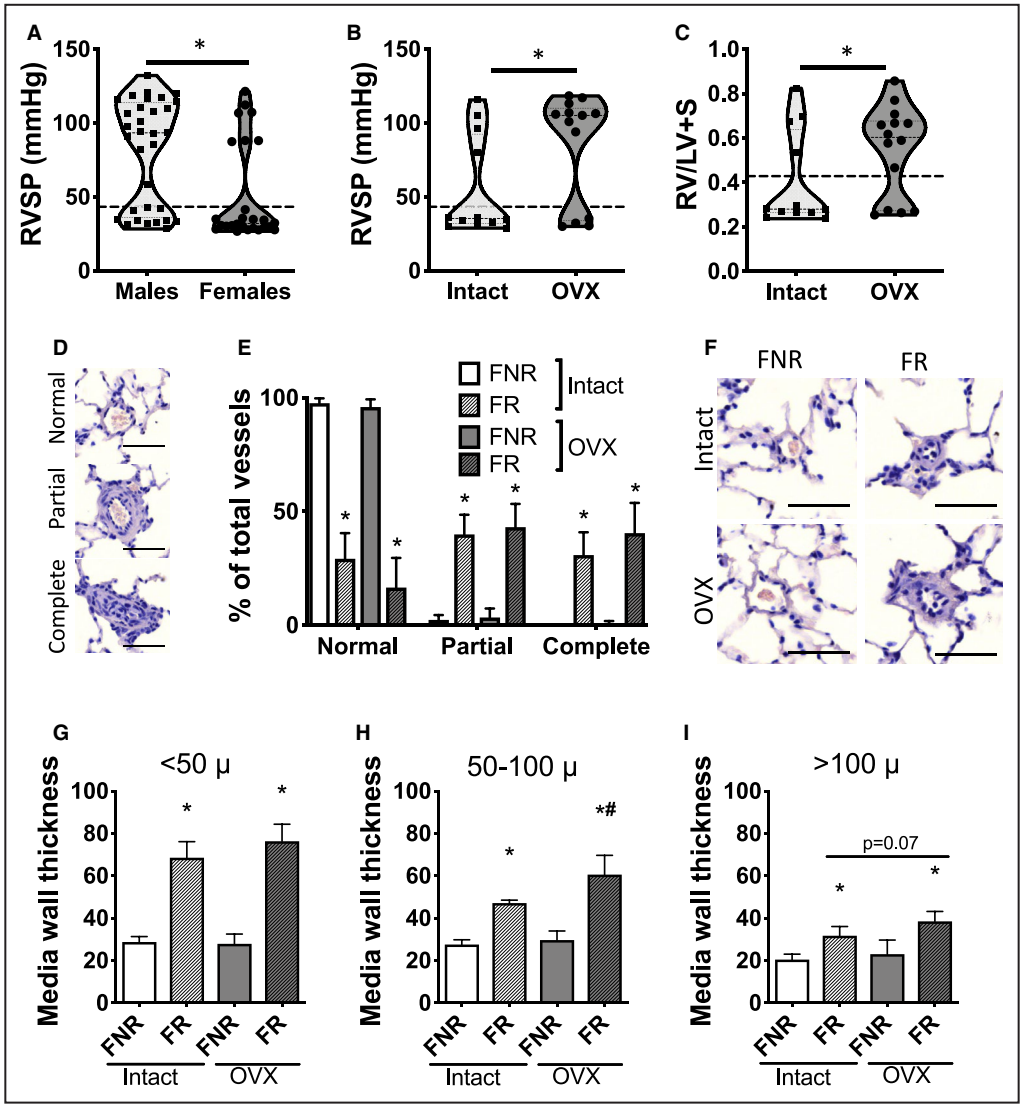

Figure 1. Sex differences in SU5416 (SU, a vascular endothelial growth factor receptor 2 inhibitor)-induced severe pulmonary arterial hypertension.
A, Right ventricular systolic pressure (RVSP) of male and female rats at 7 weeks post-SU injection. **P*<0.05 vs male rats (Fisher exact test). **B**, RVSP and **C**, RV hypertrophy of intact and ovariectomized (OVX) female hyperresponsive Sprague-Dawley (SD^{HR}) rats at 7 weeks post-SU injection. **P*<0.05 vs intact female rats (Fisher exact test). **D**, Representative micrograph images demonstrating normal and partial or completely occluded vessels in the lungs. Scale bar=50 μm. **E**, Vascular occlusion in responder female (FR) and female nonresponder rats (FNR) from intact or OVX female SD^{HR} rats (n=4–9 per group), **P*<0.05 vs nonresponder rats from the same group (Tukey multiple comparison test). **F**, Representative micrograph images demonstrating media wall thickness in FR or FNR rats from intact or OVX female SD^{HR} rats. Scale bar=50 μm. Media wall thickness of (**G**) <50 μm, (**H**) 50 to 100 μm, and (**I**) >100 μm diameter vessels of FR and FNR rats from intact and OVX female SD^{HR} rats (n=4–9 per group), **P*<0.05 vs FNR rats from the same group. $^{\#}P$<0.05 vs intact (Tukey multiple comparison test). RV/LV+S indicates right ventricular/left ventricular+septum.

muscularization of different sized pulmonary arteries and occlusive arterial remodeling was blocked by estradiol treatment in both ovariectomized female and male SD^{HR} rats (Figure 2E and 2F). In contrast, continuous treatment with progesterone abrogated the development of PAH in ovariectomized female rats (Figure 3A) but not in male rats (Figure 3B). Moreover, the protective effects of progesterone on medial wall thickening and occlusive arteriopathy induced by SU was seen only in ovariectomized female rats (Figure 3C and 3D) and not in male rats (Figure 3E and 3F).

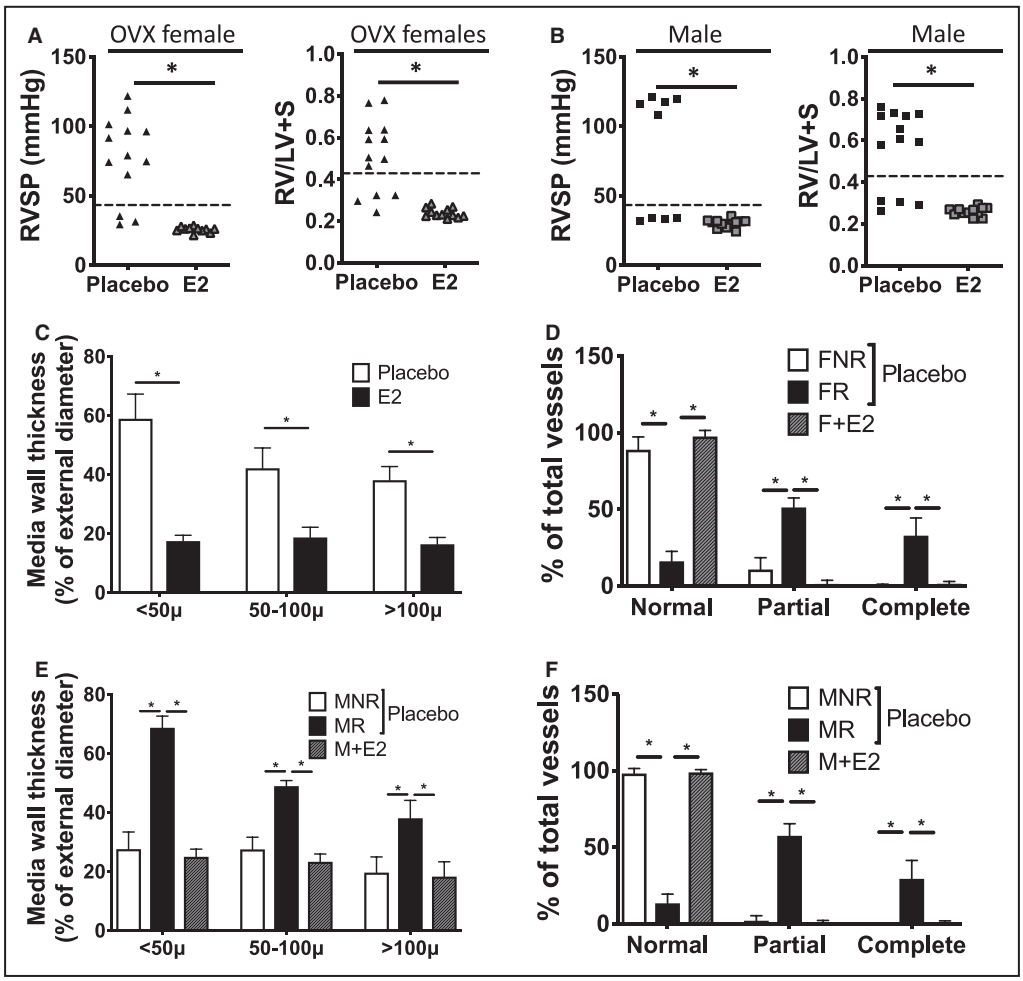

Figure 2. Effect of continuous estradiol treatment, beginning 2 days before SU (SU5416, a vascular growth factor receptor inhibitor) injection, on SU-induced pulmonary arterial hypertension.
A, Right ventricular (RV) systolic pressure (RVSP) and RV hypertrophy of 17β estradiol (E2)– or placebo-treated ovariectomized (OVX) female hyperresponsive Sprague-Dawley (SD^{HR}) rats at 7 weeks post-SU injection. **P*<0.05 vs placebo (Fisher exact test). **B**, RVSP and RV hypertrophy of E2- or placebo-treated male SD^{HR} rats at 7 weeks' post-SU injection. **P*<0.05 vs placebo (Fisher exact test), (**C**) media wall thickness, and (**D**) vascular occlusion of placebo-treated FR and nonresponder female (FNR), and F+E2-treated OVX female SD^{HR} rats (n=4–6 per group), **P*<0.05 vs FR (Student *t* test and Tukey multiple comparison test, respectively). **E**, Media wall thickness and (**F**) vascular occlusion of placebo-treated responder (MR) and nonresponder male (MNR) and M+E2-treated male SD^{HR} rats (n=4–6 per group), **P*<0.05 vs MR (Tukey multiple comparison test). RV/LV+S indicates right ventricular/left ventricular+septum.

Effect of Treatment With Female Sex Hormones on Persistent EC Apoptosis in SD^{HR} Rats

Previous studies have demonstrated that SU induces apoptosis of pulmonary vascular EC and inhibition of pulmonary vascular EC apoptosis abrogates the development of PAH in the SUHx model.[10,11] We have previously demonstrated that the severe PAH phenotype in SD^{HR} rats was associated with exaggerated EC apoptosis in the lung microvasculature.[11] Since estradiol is known to exert prosurvival effects on ECs, we studied its effects on pulmonary vascular EC apoptosis in the SU^{HR} rat strain. A significant increase in cleaved caspase-3 expression and caspase-3/7 activity was observed post-SU alone in the lung tissue from ovariectomized female responder rats compared with nonresponder rats (Figure 4A), and this increase in cleaved caspase-3 expression and activity was blocked by estradiol treatment (Figure 4A). Similarly, responder male rats had a greater increase in cleaved caspase-3 expression and activity in the lung homogenate compared with nonresponder rats and again the increased cleaved caspase-3 expression and activity were abolished by estradiol treatment (Figure 4B). Moreover,

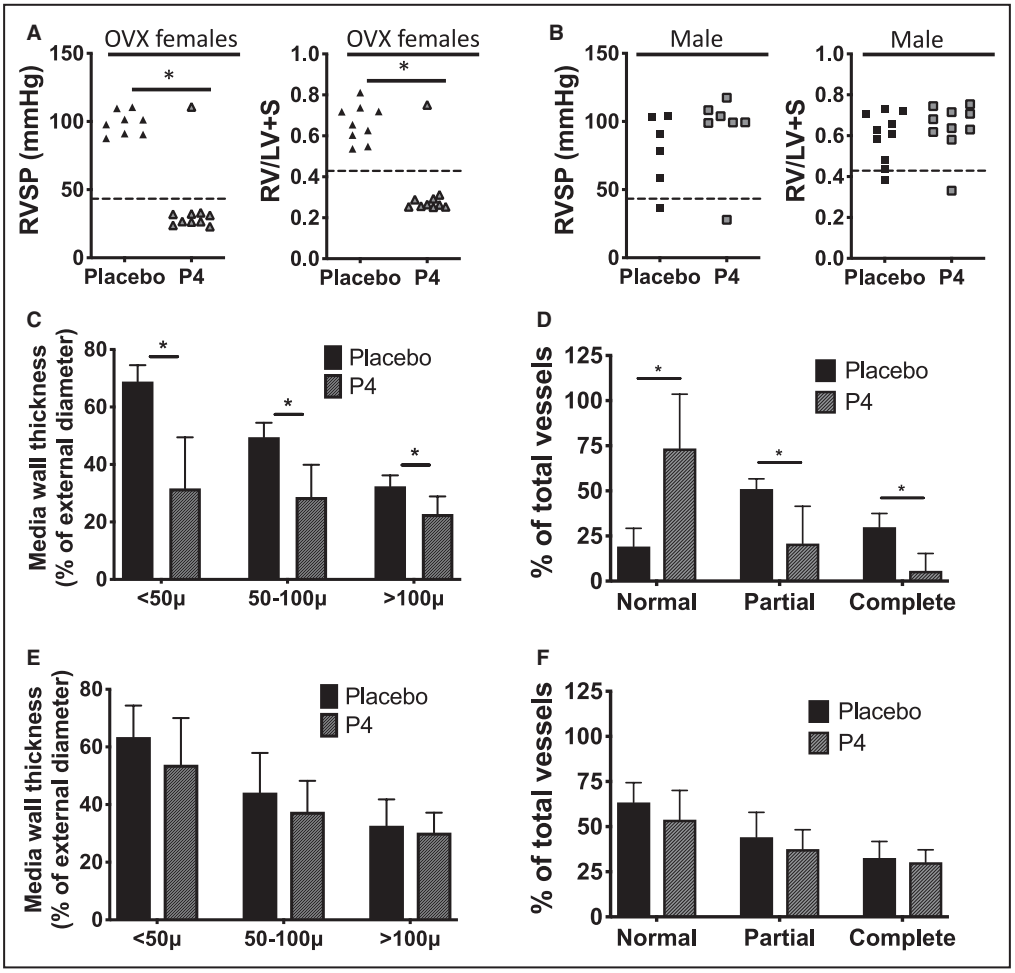

Figure 3. Effect of continuous progesterone treatment, beginning 2 days before SU (SU5416, a vascular growth factor receptor inhibitor) injection, on SU-induced pulmonary arterial hypertension.

A, Right ventricular systolic pressure (RVSP) and right ventricular (RV) hypertrophy of progesterone (P4) or placebo-treated ovariectomized (OVX) female hyperresponsive Sprague-Dawley (SD^{HR}) rats at 7 weeks post-SU injection. *P<0.05 vs placebo (Fisher exact test). **B**, RVSP and RV hypertrophy of P4- or placebo-treated male SD^{HR} rats at 7 weeks' post-SU injection. **C**, Media wall thickness and (**D**) vascular occlusion of placebo- and progesterone-treated OVX female SD^{HR} rats (n=3–6 per group), *P<0.05 vs placebo (Student t test). **E**, Media wall thickness and (**F**) vascular occlusion of placebo- and P4-treated male SD^{HR} rats (n=5–6 per group) (Student t test). RV/LV+S indicates right ventricular/left ventricular+septum.

cleaved caspase-3 immunohistochemistry confirmed the presence of pulmonary vascular EC apoptosis in the responder rats compared with nonresponder rats, which was blocked by estradiol treatment (Figure 4C and 4D; Figures S8–S10). As expected, continuous progesterone treatment inhibited increased cleaved caspase-3 expression and caspase-3/7 activity in the lung homogenate of ovariectomized female (Figure 4E) but not male hyperresponsive rats (Figure 4F). We also explored changes in bone morphogenetic protein receptor-2 (BMPR2) and phosphorylated SMAD (homologues of the Drosophila protein, mothers against decapentaplegic [Mad] and the Caenorhabditis elegans protein Sma) 1/5/9; however, no significant difference in BMPR2 or phosphorylated SMAD 1/5/9 was observed among responder, nonresponder, or female sex hormone–treated (2 days before SU injection) male or ovariectomized female rats (Figure S11 and S12).

To exclude a confounding effect of pretreatment with female sex hormones on the absorption or metabolism of SU, estradiol or placebo pellets were implanted in the ovariectomized female SD^{HR} rats 2 days after SU injection. As before, the increase in RVSP and RVH observed after SU in placebo-treated ovariectomized female SD^{HR} rat was completely blocked by estradiol treatment (Figure S13A and S13B). As well, early post-SU treatment with estradiol also inhibited increased cleaved

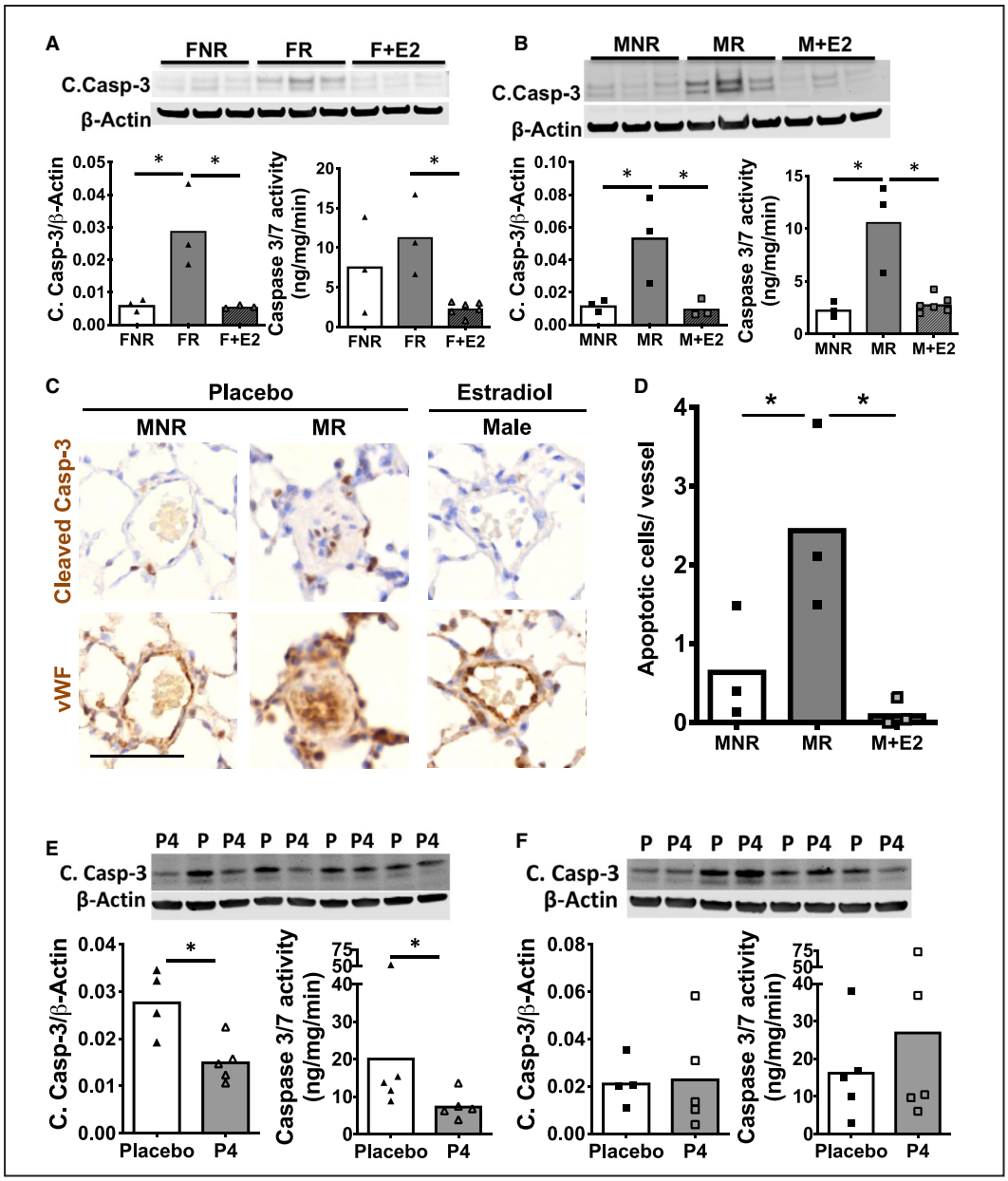

Figure 4. Protective effects of female sex hormones against prolonged endothelial cell apoptosis in SU (SU5416, a vascular growth factor receptor inhibitor)-induced pulmonary arterial hypertension.
A, Cleaved caspase-3 (Casp-3) expression and Casp-3/7 activity in lung homogenates of placebo-treated responder female (FR) and nonresponder female (FNR) rats, and 17β-estradiol (E2) (F+E2)-treated ovariectomized (OVX) female hyperresponsive Sprague-Dawley (SD^{HR}) rats (n=3–6 per group), **P*<0.05 vs FR (Tukey multiple comparison test). **B**, Cleaved Casp-3 expression and Casp-3/7 activity in lung homogenates of placebo-treated responder male (MR) and nonresponder male (MNR), and 17B estradiol-treated (M+E2) male SD^{HR} rats. (n=3–6 per group), **P*<0.05 vs MR (Tukey multiple comparison test). **C**, Representative images and (**D**) quantification of cleaved Casp-3–positive pulmonary vascular endothelial cells in placebo-treated MR and MNR, and M+E2-treated male SD^{HR} rats (n=3 per group), **P*<0.05 vs MR (Tukey multiple comparison test). Scale bar=50 μm. **E**, Cleaved Casp-3 expression and Casp-3/7 activity in lung homogenates of placebo (P) or progesterone (P4)-treated OVX female SD^{HR} rats (n=3–6 per group), **P*<0.05 vs placebo (Student *t* test). **F**, Cleaved Casp-3 expression and Casp-3/7 activity in lung homogenates of placebo (P) or progesterone (P4)-treated male SD^{HR} rats (n=3–6 per group) (Student *t* test). vWF indicates von Willebrand factor.

caspase-3 expression (Figure S13C) and caspase-3/7 (Figure S13D) activity in lung homogenates compared with placebo treatment.

Female Sex Hormones Fail to Reverse Established PAH in Ovariectomized Female SU^{HR} Rats

We next investigated the effects of implanting the estradiol, progesterone, or placebo pellets at 4 weeks post-SU, at which time PAH was established in this model. Animals demonstrating hyperresponsive phenotype at 4 weeks were randomized to receive placebo or estradiol treatment and there was no significant difference in baseline RVSP between placebo- and estradiol-treated rats (Figure 5A). After 4 weeks of treatment, (8 weeks post-SU), estradiol had no significant effect on RVSP or RVH (Figure 5A). Similarly, delayed treatment with progesterone resulted in no significant changes in RVSP or RVH compared with placebo (Figure 5B). These data suggest a loss of protective effects of female sex hormones once the severe PAH phenotype was established in this model. Moreover, when the baseline and post-treatment data were plotted for each individual animal, no improvement in hemodynamics was seen with estradiol (Figure 5C) or progesterone (Figure 5D) treatments; rather, progression of PAH was observed in nearly all animals.

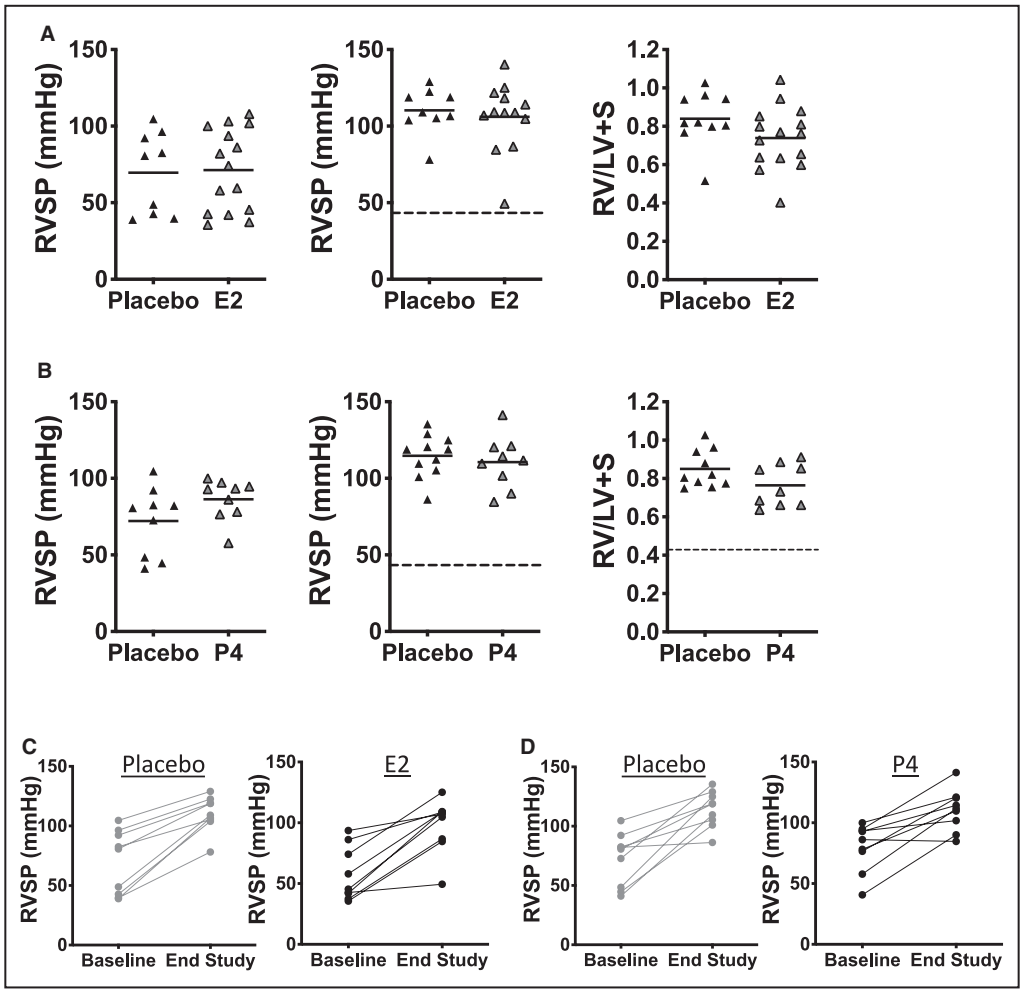

Figure 5. Effect of continuous estradiol treatment, beginning 4 weeks after SU (SU5416, a vascular growth factor receptor inhibitor) injection, on SU-induced pulmonary arterial hypertension.
A, Baseline right ventricular systolic pressure (RVSP) (4 weeks), end-study RVSP (8 weeks), and right ventricular (RV) hypertrophy for 17β estradiol (E2) or placebo-treated ovariectomized female hyperresponsive Sprague-Dawley (SD^{HR}) rats (Fisher exact test). **B**, Baseline RVSP (4 weeks), end-study RVSP (8 weeks), and RV hypertrophy for progesterone (P4)- or placebo-treated ovariectomized female SD^{HR} rats (Fisher exact test). Change in RVSP of individual rats from baseline to end study in (**C**) E2- or placebo- and (**D**) P4- or placebo-treated ovariectomized female SD^{HR} rats (paired *t* test). RV/LV+S indicates right ventricular/left ventricular+septum.

Lack of Inhibition of Lung Microvascular EC Apoptosis by Estradiol or Progesterone in Established PAH in SD^{HR} Rats

In contrast to early treatment with estradiol, no significant reduction in cleaved caspase-3 expression was seen in the lung homogenates when estradiol treatment was administered at 4 weeks post-SU (Figure 6A and 6B). Nonetheless, estradiol treatment resulted in reduced weight gain and increased expression of progesterone receptors (A and B), both downstream target genes of estrogen receptor activation, in lung homogenate of estradiol-treated rats compared with placebo-treated rats (Figure S14), confirming the biological activity of exogenous estradiol. Loss of inhibitory effects of progesterone on cleaved caspase-3 expression was also observed in female SD^{HR} rats (Figure 6C).

DISCUSSION

PAH is characterized by profound sex differences with respect to incidence and severity of disease.[18] While sex differences in PAH severity has been a focus of several studies,[14,19–21] it has been more challenging to study the effect of biological sex on disease penetrance because of the lack of an appropriate animal model of PAH exhibiting the incomplete penetrance. In most commonly used PH models, including the chronic hypoxia, monocrotaline, and SUHx models, the response to the stimulus is normally distributed with all animals developing PH to a greater or lesser extent.[11,13] Thus, the "penetrance" of PH in these models is always close to 100%, and one can only evaluate the effects of interventions, such as estrogen infusion or ovariectomized, on the severity of disease.[14,19–21] In this study, we used a unique substrain of Sprague-Dawley rats that develops severe PAH in response to a single injection of SU alone[11], with an "all-or-none" bimodal distribution mirroring the incomplete penetrance of hereditary PAH. Moreover, the SD^{HR} substrain demonstrates a profound sex difference in the observed penetrance of the severe PAH phenotype in response to SU alone, with 70% of male and only 30% of female rats developing disease. Thus, this model provides ideal opportunity to explore the influence of female sex and sex hormones in determining susceptibility to PAH in a genetically prone background.

In the present study, there was a clear sex dependence in penetrance of the severe PAH phenotype in this substrain that was eliminated by ovariectomized. As well, continuous delivery of exogenous estradiol completely prevented the development of severe PAH

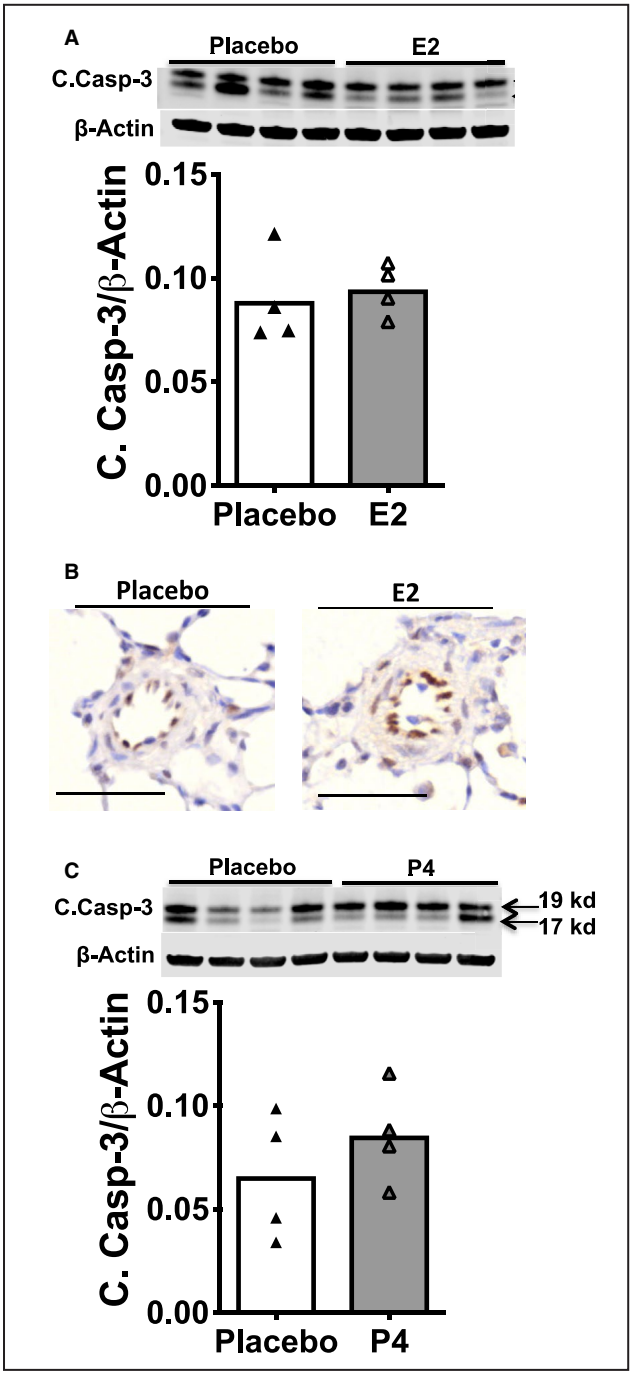

Figure 6. Loss of protective effects of female sex hormones on endothelial cell apoptosis with delayed treatment.
A, Cleaved caspase-3 (Casp-3) expression in lung homogenates of placebo- or 17β estradiol (E2)–treated ovariectomized female hyperresponsive Sprague-Dawley (SD^{HR}) rats (Student *t* test). **B**, Representative images of cleaved Casp-3 positive pulmonary vascular endothelial cells in placebo- or F+E2-treated ovariectomized female SD^{HR} rats. Scale bar=50 μm. **C**, Cleaved Casp-3 expression in lung homogenates of placebo or progesterone (P4)-treated ovariectomized female SD^{HR} rats (Student *t* test).

in SD^{HR} rats treated with SU alone in both male and ovariectomized female SD^{HR} rats. The abrogation of the severe PAH phenotype by exogenous estrogen suggests that a high constant level of estradiol may be more protective than the fluctuating endogenous levels seen during the normal estrus cycle of female rats. This was true regardless of whether estrogen delivery was initiated before or 2 days after SU injection, ruling out a confounding effect of estrogen on the pharmacodynamics of SU. Therefore, our results not only confirm previous reports showing that circulating estradiol is protective in various preclinical models[14,20,21] but also show for the first time a major effect of female sex and sex hormones on penetrance of the severe PAH phenotype in a genetically susceptible model.

The role of progesterone has received much less attention in studies of the effects of sex hormones in PAH. Progesterone has been shown to increase nitric oxide synthesis, decrease secretion of endothelin-1 from vascular ECs, and protect against endothelial barrier disruption, which could be beneficial in PAH.[22,23] Indeed, the continuous delivery of progesterone using osmotic minipumps has been shown to reduce the severity of PH phenotype in female rats subjected to monocrotaline.[19] However, the effects of progesterone on penetrance PAH have not been previously investigated. In the present study, our results provide the first evidence of the protective effects of progesterone on pulmonary vascular EC apoptosis and demonstrate that steady-state higher levels of progesterone protect against the penetrance of PH phenotype. This observation is consistent with the inverse relationship observed between plasma progesterone levels and the risk of PAH in humans.[24] Interestingly, the protective effect of progesterone was apparent only in female rats. This could not be explained by differences in progesterone receptor expression, which was similar both in male and female rats, and further investigation into the mechanisms of sex-specific differences on the vascular protective effects of progesterone is warranted.

While a strong influence of female sex on the penetrance of BMPR2 mutations has been well documented in humans, this is in the opposite direction to that seen in the rat model, with ≈40% of females harboring a disease-causing mutation ultimately developing PAH compared with only ≈15% of males.[25] This apparent paradox might be explained by the artificial nature of most animal models of PH in which the disease is initiated by a 1-time administration of an injurious stimulus (ie, monocrotaline or SU). As injury is induced at random relative to the hormonal cycle, the likelihood is that exposure to an endothelial toxin corresponds to a phase of the cycle when female sex hormone levels are relatively high, as this represents the most prolonged phase of the estrus cycle. Indeed, the well-known variability in response of female versus male rats to PH-inducing agents may be a reflection of the inherently random nature of the timing of injury. In contrast, PAH in humans is thought to be the result of a cumulative exposure to injurious environmental factors, often in the context of inherent genetic susceptibility, occurring over a period of many years.[26] Furthermore, a more subtle toxic stimulus may only induce disease when exposure corresponds to a period of greater susceptibility; for example, as might occur during abrupt withdrawal of the protective influence of female sex hormones at the end of the estrus cycle, thereby creating a window of vulnerability for endothelial injury and apoptosis. The "window of vulnerability" paradigm would mainly influence susceptibility to developing disease, rather than its severity; therefore, it may also provide an explanation for the so-called estrogen paradox. Specifically, the higher average levels of female sex hormones in women compared with men may have a beneficial overall effect on disease progression, as has been previously suggested,[14,20,27] and possibly on RV function and adaptation as well.[14] The window of vulnerability paradigm could also explain female predisposition in certain transgenic mouse models in which PH develops spontaneously.[9,28]

The mechanisms underlying the beneficial effects of circulating female sex hormones in experimental PH remain unclear. Like estradiol, progesterone is a potent vasodilator and has important vascular protective effects.[22,23] Estradiol and progesterone have both been demonstrated to prevent endothelial dysfunction[29] and exert potent vasodilation in the pulmonary circulation.[30,31] Estradiol has also been shown to inhibit smooth muscle cell proliferation and have antiapoptotic effects on ECs, both of which could be beneficial in PAH. Indeed, EC apoptosis is recognized as an essential trigger for the development of PH in the SUHx model of PAH[10,11], as well as in patients with hereditary PAH with disease-causing BMPR2 mutations.[32,33] In addition, inhibition of EC apoptosis using the nonspecific caspase inhibitor, Z-Asp-CH2-DCB, abrogated the development of the PAH phenotype in the SUHx model.[10] In the present study, we show that early treatment with estradiol or progesterone in female rats abolished the lung microvascular EC apoptosis in SD^{HR} rats after exposure to SU, and this completely prevented the development of PH in male and ovariectomized female animals. These findings strongly suggest that the beneficial effects of circulating female sex hormones in reducing the susceptibility for developing severe PAH in this genetically prone model were related to their important effects on EC survival and the inhibition of SU-induced EC apoptosis. In contrast, treatment with sex hormones had no marked effect on EC apoptosis or the PAH phenotype when given to animals with established disease, 4 weeks after exposure to SU. While it remains unclear why the beneficial effects of estradiol

were lost in the treatment model, this is consistent with the idea that different mechanisms may contribute to PAH initiation and progression; the former may be dependent on EC injury and apoptosis, whereas the latter may involve emergence of hyperproliferative and apoptosis-resistant vascular cells, which contribute to occlusive arterial remodeling, a characteristic feature of later disease in the SUHx model and human patients with PAH.[34] Such a cancer-like dysregulation of cell growth may not be beneficially affected by sex hormones.

Previous studies have suggested that local synthesis and metabolism of estrogens in the lung vasculature can play an important role in the pathogenesis of PAH. In ECs, SU has been shown to increase CYP1A1, an estrogen-metabolizing enzyme, and aromatase, an enzyme involved in estrogen synthesis, by mechanisms involving the aryl hydrocarbon receptor.[35] The resulting increases in local production of estradiol in response to SU were shown to mediate increased EC and pulmonary arterial smooth muscle cell proliferation.[35] As well, inhibitors of the aryl hydrocarbon receptor normalized aromatase and estrogen levels in the chronic hypoxia (10% O_2) and SUHx models, and reduced the severity of PH.[36,37] Moreover, alterations in estrogen metabolism with accumulation of deleterious estrogen metabolites have been reported in the plasma of patients with PAH,[38,39] including 16-hydroxyestradiol, which support a contribution of local estrogen metabolism to the progression of this disease.[40] While we did not assess the role of local estrogen or its metabolites in modulating the penetrance of the PAH phenotype in SD^{HR} rats, our data would suggest that any deleterious influence by this mechanism was clearly outweighed by the profound beneficial effects of endogenous and exogenous circulating female sex hormones in this model. However, an increase in the production of estrogen metabolites in later stages of disease may contribute to vascular cell proliferation, and this could provide another explanation for the lack of any therapeutic effects of delayed administration of estrogen in our model.

Together, the present report highlights a protective role of female sex in a unique model of severe PAH that mimics the variable penetrance of disease-causing mutations in humans. We now show that estrogen, and progesterone in female rats, act as EC survival factors and reduce lung endothelial apoptosis in response to injury, thereby decreasing the likelihood of developing disease. Moreover, these findings suggest a novel paradigm by which cyclical fluctuations in levels of female sex hormone may play a role in determining vulnerability to development of PAH, which may explain the divergent influence of female sex in determining susceptibility to disease between rodent models and the human patient.

ARTICLE INFORMATION

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Supplementary Material

Data S1
Figures S1–S14

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Supplemental Material

Data S1.

Supplemental Methods

SU5416 hyper-responsive phenotype characterization

Male and female Sprague Dawley (SD, Charles River laboratories, QC, Canada) rats weighing 150-200 g were used for this study. A total of 241 rats were utilized for the study (79 males, 38 females and 124 ovariectomized (OVX) females). PH was induced by a single subcutaneous injection of SU5416 (SU: 3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1,3-dihydroindol-2-one) (Tocris, Bristol, United Kingdom) in 0.5% carboxymethyl cellulose.[10, 11] Following SU treatment the rats were housed under normoxic condition for 7 weeks. PH phenotype was characterized by measuring RVSP, RV hypertrophy and lung vascular remodelling as described below (Fig S1 and S2). To study sex differences in penetrance of PH phenotype in male (n=34) and female (n=26) SD^{HR} rats were injected with SU (Fig S1A) and 2 male rats died before the endpoint. To explore role of female sex hormones in penetrance of PH, OVX rats (n=14) and sham female SD^{HR} rats (n=12) were treated with SU 2 weeks after OVX surgery (Fig S1B). One OVX rat died before endpoint.

Sex hormone treatment

Sex hormone replacement was performed using continuous slow-release pellets (17β-estradiol: 0.5mg pellet, s.c., 60-day release; progesterone: 150mg pellet, sc, 60-day release). At 2 days before, 2 days post or 4 weeks post-SU injection, rats were randomized to receive estradiol, progesterone or placebo pellets (Figures S1C, 1D, 2A and 2B). Rats were anaesthetized by isoflourane inhalation and slow-release pellets were implanted sub-cutaneously on the dorsal side at the base of the neck. Topical bupivacaine was applied immediately after wound closure and twice daily for one day post-surgery. Buprenorphine (s.c., 0.03mg/kg) was administered 1 hour prior to surgery and once daily for two days post-surgery. For estradiol pre-treatment (Fig S1C and 1D) experiments, 25 male SD^{HR} rats

(Placebo: 13 rats; estradiol: 12 rats) and 26 OVX female SD^{HR} rats (Placebo: 13 rats; estradiol: 13 rats) were included and 4 male and 2 female placebo treated rats died before endpoint. For progesterone pre-treatment (Fig S1C and 1D) study, 20 male SD^{HR} rats (Placebo: 10 rats; progesterone: 10 rats) and 20 OVX female SD^{HR} rats (Placebo: 10 rats; progesterone: 10 rats) were included. Four male and 2 female placebo treated rats and 3 progesterone treated male rats died before endpoint. For early post-treatment (Fig S2A), 20 OVX female SD^{HR} rats (Placebo: 10 rats; estradiol: 10 rats) were included and 1 placebo treated rat died before endpoint. For 4-week treatment experiments, RVSP measurement was performed to confirm development of PH and rats with RVSP >35mmHg were included in the study and randomized to receive placebo or sex hormone containing pellets. Rats with RVSP <35mmHg at 4 weeks do not develop severe PH (based on extensive experience with this model) and; therefore, these rats were excluded from the experiments designed to investigate the effects of female sex hormones on established PH. Twenty five OVX female SD^{HR} rats (Placebo: 10 rats; estradiol: 15 rats) were included in the study of estradiol reversal treatment (Fig S1D), and 19 OVX female SD^{HR} rats (Placebo: 10 rats; progesterone: 9 rats) were included to study progesterone reversal treatment (Fig S1D).

Measurement of RVSP and RV hypertrophy

RVSP was measured using high-fidelity pressure catheters (Transonic-Scisense Inc., ON, Canada) at 4 weeks post-SU (Baseline) and at 7- or 8-weeks post-SU (end study). For RV catheterization, rats were anaesthetized by an intraperitoneal injection of xylazine (7 mg/kg) and ketamine (35 mg/kg). The pressure catheter was inserted into the right jugular vein and advanced through the superior vena cava and right atrium into the RV. Hemodynamic parameters were recorded and analyzed using the LabScribe3 software (iWorx, Dover, NH, USA). At 7 weeks, after data acquisition, animals were euthanized by exsanguination under anaesthesia. The heart was excised, and the ventricles were dissected from the atria, the aorta and the pulmonary trunk. The RV and left ventricle (LV) and septum (S) were separated, and RV hypertrophy was calculated by measuring the ratio of RV weight to LV plus septum weight (RV/LV+S, Fulton index). The operators acquiring the RVSP and RV

hypertrophy data were blinded to the treatment allocation. For 4-week RVSP measurement, rats were anaesthetized by isoflourane inhalation and topical bupivacaine was applied immediately after wound closure and twice daily for one day post-surgery. Buprenorphine (s.c., 0.03mg/kg) was administered 1 hour prior to surgery and once daily for two days post-surgery.

Lung histological measurements

The left lobe of the lung was inflated via the trachea with 50:50 OCT/saline solution (Tissue-Tek OCT; Qiagen, Mississauga, ON, Canada) and then removed. The left lobe was then cut into thick cross sections and fixed in 4% paraformaldehyde (PFA) for 24 h, rinsed and washed in PBS for 8 hr and stored in 70% ethanol until the day of paraffin embedding. Tissue blocks were sectioned (5μm thickness) with a microtome (Leica Microsystems, Concord, ON, Canada), placed onto poly-L-lysine-coated slides, dried at 37°C for 16 hours and then dewaxed and rehydrated through graded alcohols. For microscopy and quantitative morphometry of the lung, hematoxylin and eosin (H&E) staining was performed with standard protocols. Images were acquired by Panoramic DESK (3DHISTECH, Hungary) scanscope using Panoramic Scanner and analyzed using Panoramic Viewer (3DHISTECH, Hungary). Ten random high-power fields (100X magnification) for each rat were analyzed for media wall thickness, total vessel count and vascular occlusion. Media wall thickness as percent of external diameter was estimated as described previously.[15] Percent Medial wall thickness = ((distance between the internal and external lamina × 2)/external diameter) × 100. For total vessel count, all the vessels were counted from the 10 random fields. The numbers of normal and completely or partially occluded distal arterioles (<100 μm) were quantified from the random fields.

Western blotting

Right lung was collected at the end of study, flash-frozen in liquid nitrogen and stored at -80 °C until further processing. Lung lysates were prepared in CelLytic™ MT Cell Lysis Reagent (Sigma, ON, Canada) containing cOmplete™ protease inhibitor cocktail (Sigma, ON, Canada) and PhosSTOP™ (Sigma, ON, Canada) and using the TissueLyser (Qiagen, ON, Canada) two cycles of 25hz for 3 min.

The tissue lysate was then centrifuged at 12000xg for 10 min and the supernatant was collected. Protein concentration of the protein extract was determined colorimetrically by the DC Protein Assay kit (Bio-Rad, ON, Canada), using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis of lung protein extract (50 µg) was performed with NuPAGE® Novex® 4-12% Bis-Tris Protein Gels (ThermoFisher Scientific, ON, Canada). Following transfer of the separated proteins to nitrocellulose membranes (NOVEX iBLOT Gel transfer Stacks, ThermoFisher Scientific, ON, Canada), blots were blocked with 2% BSA in PBS-T (PBS containing 0.1% Tween 20, pH 7.4). After blocking, blots were incubated with primary antibodies to cleaved caspase-3 (Cell Signalling Technologies, Cat# 9661S), progesterone receptor (Abcam, Cat# ab16661), BMPR2 (BD Biosciences, Car# 612292), phospho-SMAD1/5/9 (Cell Signalling Technologies, Cat# 9511S) or β-actin (ThermoFisher Scientific, Cat# A5441) for overnight at 4 °C. Then the blots were washed for three times for 15 min with PBS-T and incubated with appropriate IRDye® anti-rabbit or anti-mouse secondary antibodies (LI-COR Biotechnology, NE, USA) in 2% BSA/PBS-T. Further, the blots were washed for three times for 15 min with PBS-T and imaged with Odyssey® imaging system (LI-COR Biotechnology, NE, USA). The blots were quantified using the Image Studio™ Software (LI-COR Biotechnology, NE, USA) and expressed as a percentage of control to reduce the variation between blots.

Caspase 3/7 activity assay

Caspase 3/7 activity in the lung lysates was assessed using Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega Cop, WI, USA) according to manufacturer's protocol with slight modifications. Briefly, lung lysates were diluted to 1 µg/µL with CelLytic™ MT Cell Lysis Reagent. Then, 50 µL of diluted reagent (substrate and buffer combined) was added directly to 50 µL samples and incubated at 25 °C for 2.5 hr. Fluorescence was measured every 30 min using excitation wavelength of 480 nm and emission wavelength of 520 nm. Caspase activity was calculated using gain of fluorescence between 30 min intervals. Amount of metabolized substrate was determined from standard curve of Rhodamin 110.

Cleaved caspase 3 and von Willebrand Factor (vWF) immunohistochemistry

PFA fixed and paraffin embedded tissue were sectioned (5μm thickness) with a microtome (Leica Microsystems, Concord, ON, Canada), placed onto poly-L-lysine-coated slides, dried at 37°C for 16 hours and then dewaxed and rehydrated through graded alcohols. Antigen retrieval was performed using Citric Acid Based Antigen Unmasking Solution (Vector Labs, Cat# H3300) according to manufacturer's protocol. Immunohistochemistry was performed using Rabbit specific HRP/DAB (ABC) Detection IHC Kit (Abcam, Cat# ab64261) according to manufacturer's protocol. Sequential sections were used for cleaved caspase-3 (Cell Signalling Technologies, Cat# 9661S) and Von Willebrand Factor (vWF, Abcam, Cat# ab6994) immunohistochemistry. The primary antibodies (cleaved caspase-3 at 1:40 and vWF at 1:400) were diluted in 1% BSA in PBS and each section was incubated overnight at 4 °C with 80 μL diluted antibody. Images were acquired by Panoramic DESK (3DHISTECH, Hungary) scanscope using Panoramic Scanner and analyzed using Panoramic Viewer (3DHISTECH, Hungary). Ten random high-power fields (100X magnification) for each rat were analyzed for cleaved caspase-3 positive endothelial cells.

Cleaved caspase 3 and smooth muscle actin (SMA) immunofluorescence staining

PFA fixed and paraffin embedded tissue were sectioned (5μm thickness) with a microtome (Leica Microsystems, Concord, ON, Canada), placed onto poly-L-lysine-coated slides, dried at 37°C for 16 hours and then dewaxed and rehydrated through graded alcohols. Antigen retrieval was performed using Citric Acid Based Antigen Unmasking Solution (Vector Labs, Cat# H3300) according to manufacturer's protocol. Slides were washed in PBS than permeabilized with 0.25% triton X-100 (Sigma Aldrich, Cat# T8787) in PBS for 15 min at room temperature. Slides were washed in PBS-T, prior to blocking with 5% goat serum (Rockland immunochemicals Inc. Cat# B304) – 2% bovine serum albumin (Wisent Inc. Cat# 800-095-EG) for 1h at room temperature. Slides were incubated overnight at 4°C with primary antibodies: rabbit anti-cleaved caspase 3 (Cell Signalling

Technologies, Cat# 9661S) 1:50 dilution, and mouse anti-actin (alpha smooth muscle; Sigma-Aldrich, Cat# A5228) 1:200 dilution. The slides were then washed 3x in PBS-T followed by incubation with secondary antibodies, goat anti mouse Alexa Fluor 488 (Thermofisher, Cat# A32723) and goat anti-rabbit Alexa Fluor 594 (Thermofisher, Cat# A-11037), at 1:400 dilution for 1h at room temperature. Samples were washed 3x in PBS-T, 1x in PBS than auto-fluorescence was further quenched using Vector TrueView Autofluorescence quenching kit (Vector Labs, Cat# VECTSP8400) for 4min immediately followed by 3x PBS wash. Slides were counterstained with DAPI (Sigma, Cat# D9542) for 10min at 5μg/ml. Cells were washed 3x in PBS-T and mounted using VECTASHIELD vibrance antifade mounting media (Vector Labs, Cat# VECTSP8400) and allowed to dry at room temperature for 1h. Slides were stored at 4C and imaged using the Zeiss Imager M2.

Statistical analysis

The study evaluated the effect of biological sex on the penetrance of the PAH phenotype by comparing response to SU alone between male and female SD rats. To study the role of female sex hormones in modifying penetrance of PAH, the response to SU alone in male or OVX female rats was compared between animals receiving placebo or female sex hormones (estradiol or progesterone). For statistical comparisons, Student's t-test or One-Way ANOVA (>2 groups) were performed followed by Tukey multiple comparison test with significance level of $p<0.05$. For comparison of proportion change, odds ratio was calculated and Fisher's exact test was performed to calculate statistical significance. Data are represented as mean ± standard error of mean unless otherwise stated. Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software Inc. CA, USA).

Figure Legends:

Figure S1. Schematic diagrams demonstrating experimental procedures for study of A) penetrance of pulmonary arterial hypertension (PAH) in male vs female SD^{HR} rats in response to SU, B) effect of ovariectomized (OVX) on penetrance of PAH in response to SU; and effects of female sex hormones (17β-estradiol/ progesterone) pre-treatment on PAH penetrance in response to SU in C) male and D) OVX female SD^{HR} rats.

Figure S2. Schematic diagrams demonstrating experimental procedures for study of the effects of female sex hormones (17β-estradiol/ progesterone) A) early post-treatment and B) delayed post-treatment on penetrance of pulmonary arterial hypertension (PAH) in response to SU5416 in ovariectomized (OVX) female SD^{HR} rats.

Figure S3. Mathematical derivation of cutoff values for separation of bimodal distribution of A) right ventricular systolic pressure (RVSP) and B) RV hypertrophy in the SD^{HR} rats subjected to SU5416 in absence of hypoxia.

Figure S4. Representative low magnification images demonstrating vascular remodelling in the lungs of intact female non-responder (FNR) and responder (FR) SD^{HR} rats in response to SU.

Figure S5. Representative low magnification images demonstrating vascular remodelling in the lungs of ovariectomized (OVX) female non-responder (FNR) and responder (FR) SD^{HR} rats in response to SU5416.

Figure S6. Bar graph showing plasma A) estradiol and B) progesterone concentration in placebo or estradiol/progesterone treated SD^{HR} rats. Values represent mean±SD, *p<0.05 vs placebo.

Figure S7. Effect of continuous estradiol treatment, beginning 2 days before SU5416 injection, on body weights of SD^{HR} rats. Bar graph demonstrating body weights of male or female SD^{HR} rats treated with A) estradiol or B) progesterone at baseline and end study. Values represent mean±SD, n=12-14 per group, *p<0.05 vs placebo.

Figure S8. Representative low magnification images demonstrating cleaved caspase-3 positive vascular endothelial cells in the lungs of responder male SD^{HR} rats at 7 weeks post SU5416 injection.

Figure S9. Representative low magnification images demonstrating cleaved caspase-3 positive vascular endothelial cells in the lungs of responder female SD^{HR} rats at 7 weeks post SU5416 injection.

Figure S10. Representative immunofluorescence images demonstrating cleaved caspase-3 and smooth muscle acting staining in the lungs of vehicle or estradiol treated ovariectomized (OVX) female SD^{HR} rats at 7 weeks post SU5416 injection. Arrows demonstrate cleaved caspase-3 positive cells in the intima.

Figure S11. Effect of continuous estradiol treatment, beginning 2-days before SU injection, on lung bone morphogenic protein receptor 2 (BMPR2) and phospo-SMAD1/5/9 expression. A) Images and bar graph demonstrating BMPR2 and phospho-SMAD1/5/9 expression in lung homogenates of male SD^{HR} rats treated with placebo or estradiol. **B)** Images and bar graph

demonstrating BMPR2 and phospho-SMAD1/5/9 expression in lung homogenates of ovariectomized (OVX) female SD^{HR} rats treated with placebo or estradiol.

Figure S12. Effect of continuous progesterone treatment, beginning 2-days before SU injection, on lung bone morphogenic protein receptor 2 (BMPR2) and phospo-SMAD1/5/9 expression. A) Images and bar graph demonstrating BMPR2 and phospho-SMAD1/5/9 expression in lung homogenates of male SD^{HR} rats treated with placebo or progesterone. **B)** Images and bar graph demonstrating BMPR2 and phospho-SMAD1/5/9 expression in lung homogenates of ovariectomized (OVX) female SD^{HR} rats treated with placebo or progesterone.

Figure S13. Effect of continuous estradiol treatment, beginning 2 days after SU injection, on SU5416 induced pulmonary arterial hypertension (PAH). A) Right ventricular systolic pressure (RVSP) and **B)** RV hypertrophy of estradiol (E2) or placebo treated ovariectomized (OVX) female SD^{HR} rats at 7 weeks post SU5416 injection. **C)** Cleaved caspase-3 expression and **D)** caspase-3/7 activity in lung homogenates of placebo or estradiol (E2) treated OVX female SD^{HR} rats, n= 3-6 per group, *$p<0.05$ vs placebo.

Figure S14. Effect of continuous estradiol or progesterone treatment, beginning 4 weeks after SU injection, on lung progesterone receptor (PR) expression and body weights. A) Images and bar graph demonstrating increased PRa and PRb expression in lung homogenates of ovariectomized (OVX) female SD^{HR} rats treated with estradiol. Values represent mean±SD, n=4 per group, *$p<0.05$ vs placebo. **B)** Bar graph showing lower body weights of OVX female SD^{HR} rats treated with estradiol compared to placebo treated rats. Values represent mean±SD, n=9-14 per group, *$p<0.05$ vs placebo. **C)** Images and bar graph showing PRa and PRb expression in lung homogenates of OVX female SD^{HR} rats treated with progesterone. Values represent mean±SD, n=4 per group. **D)**

Bar graph showing no effect of progesterone treatment on body weights of OVX female SD^{HR} rats.

Values represent mean±SD, n=9 per group.

Figure S1

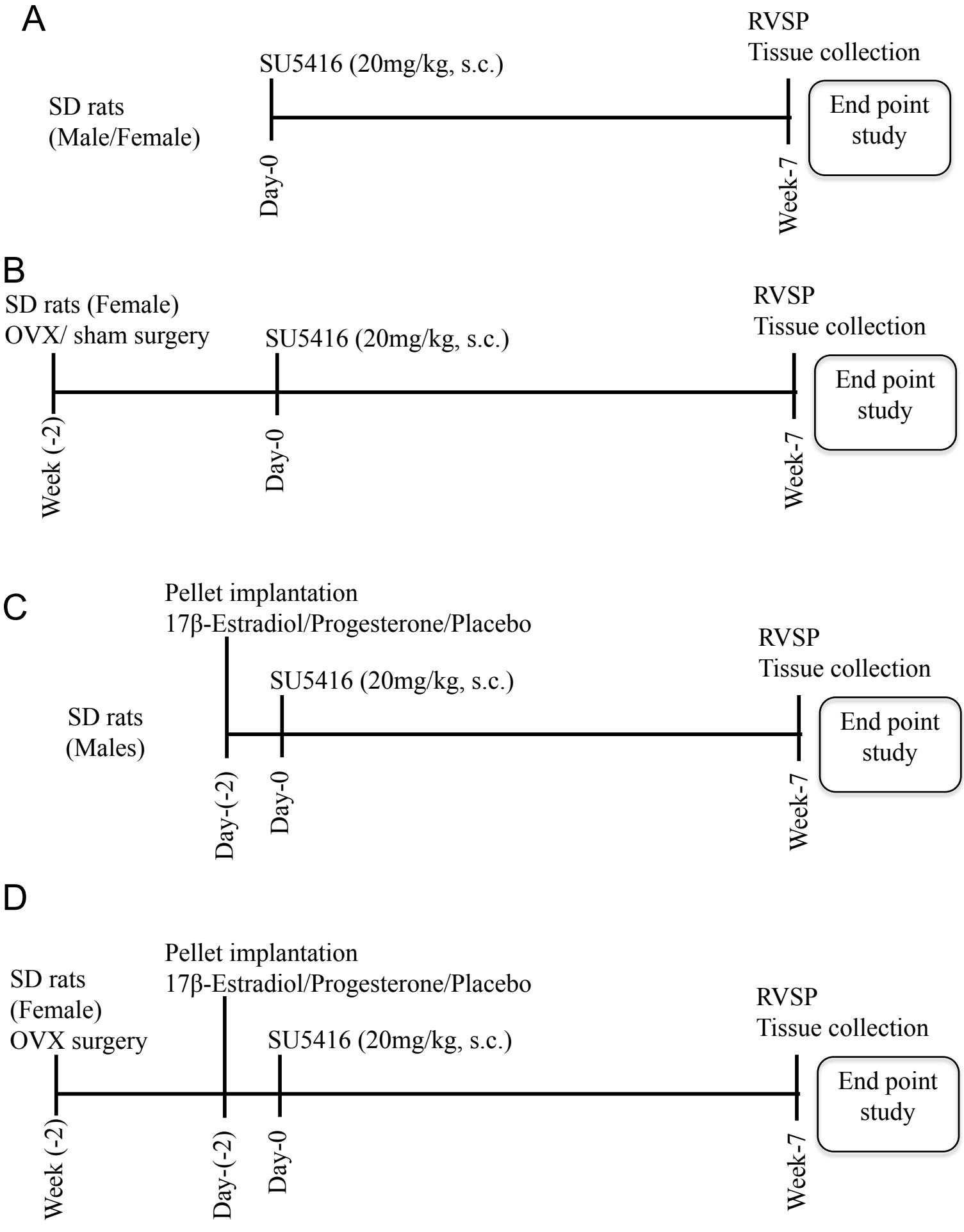

A
SD rats
(Male/Female)
SU5416 (20mg/kg, s.c.)
RVSP
Tissue collection
End point
study
Day-0
Week-7
B
SD rats (Female)
OVX/ sham surgery
SU5416 (20mg/kg, s.c.)
RVSP
Tissue collection
End point
study
Week (-2)
Day-0
Week-7
C
Pellet implantation
17β-Estradiol/Progesterone/Placebo
SU5416 (20mg/kg, s.c.)
RVSP
Tissue collection
SD rats
(Males)
End point
study
Day-(-2)
Day-0
Week-7
D
SD rats
(Female)
OVX surgery
Pellet implantation
17β-Estradiol/Progesterone/Placebo
SU5416 (20mg/kg, s.c.)
RVSP
Tissue collection
End point
study
Week (-2)
Day-(-2)
Day-0
Week-7

Figure S2.

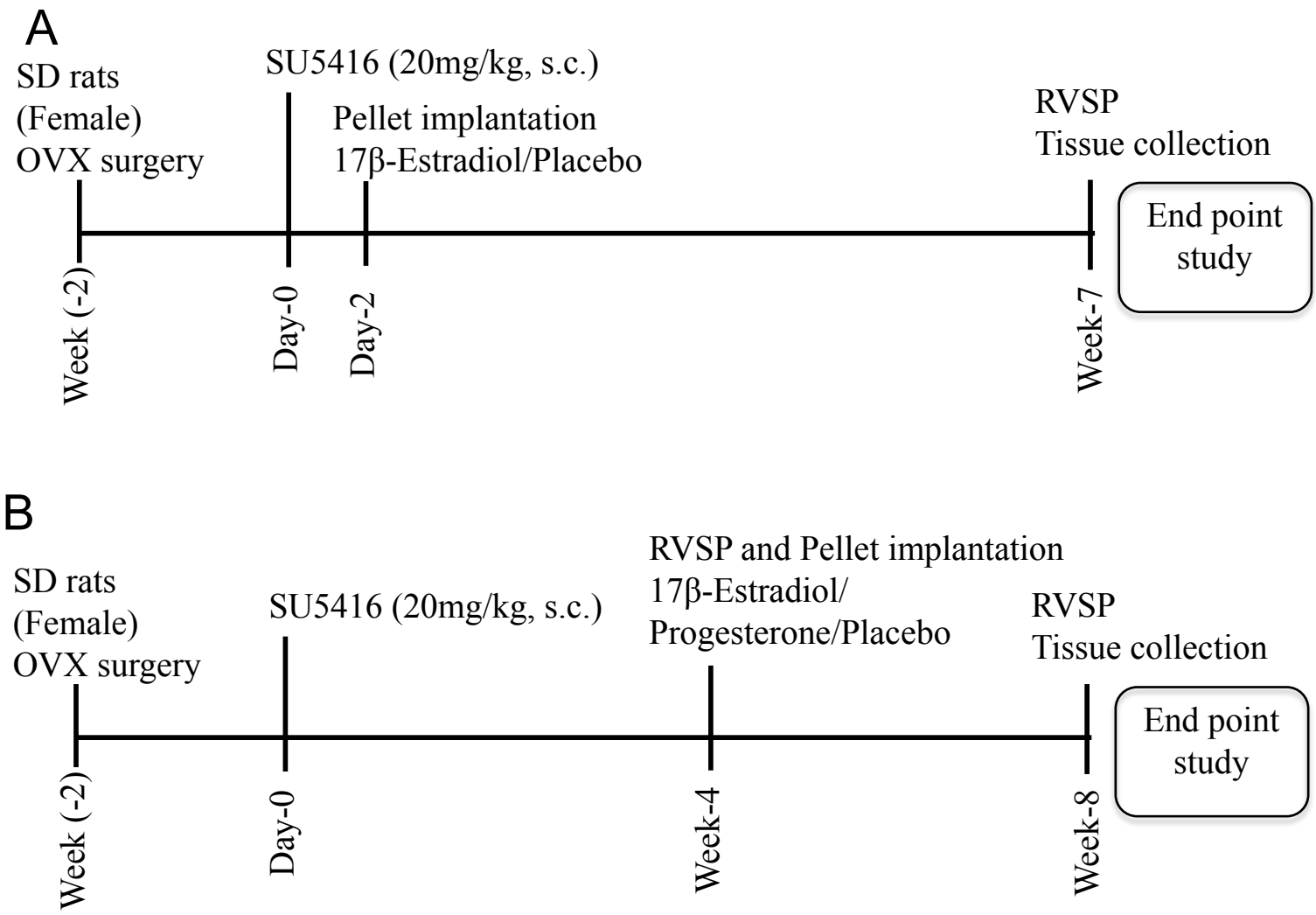
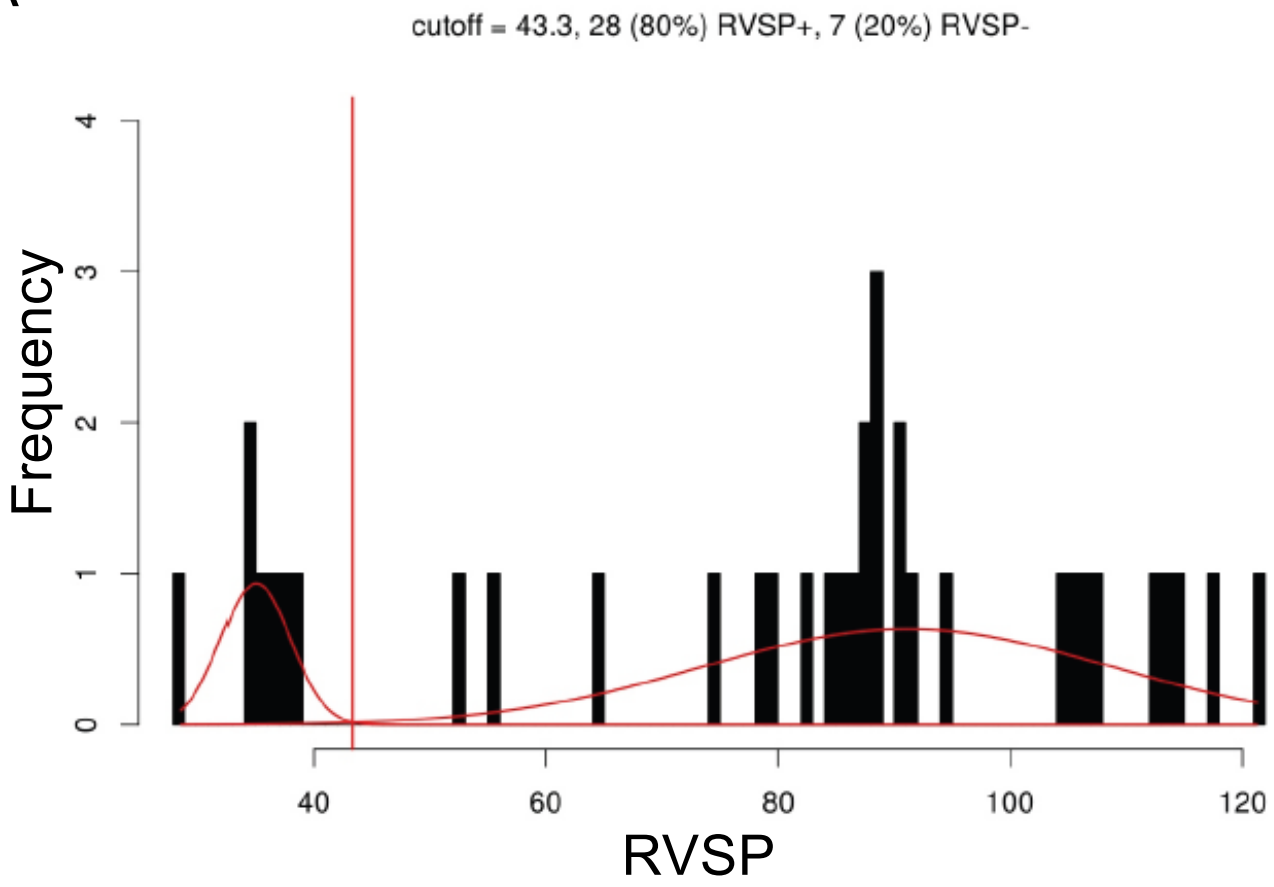

A
SD rats
(Female)
OVX surgery
SU5416 (20mg/kg, s.c.)
Pellet implantation
17β-Estradiol/Placebo
RVSP
Tissue collection
End point
study
Week (-2)
Day-0
Day-2
Week-7
B
SD rats
(Female)
OVX surgery
SU5416 (20mg/kg, s.c.)
RVSP and Pellet implantation
17β-Estradiol/
Progesterone/Placebo
RVSP
Tissue collection
End point
study
Week (-2)
Day-0
Week-4
Week-8

Figure S3

A


cutoff = 43.3, 28 (80%) RVSP+, 7 (20%) RVSP-
Frequency
0
1
2
3
4
40
60
80
100
120
RVSP

B

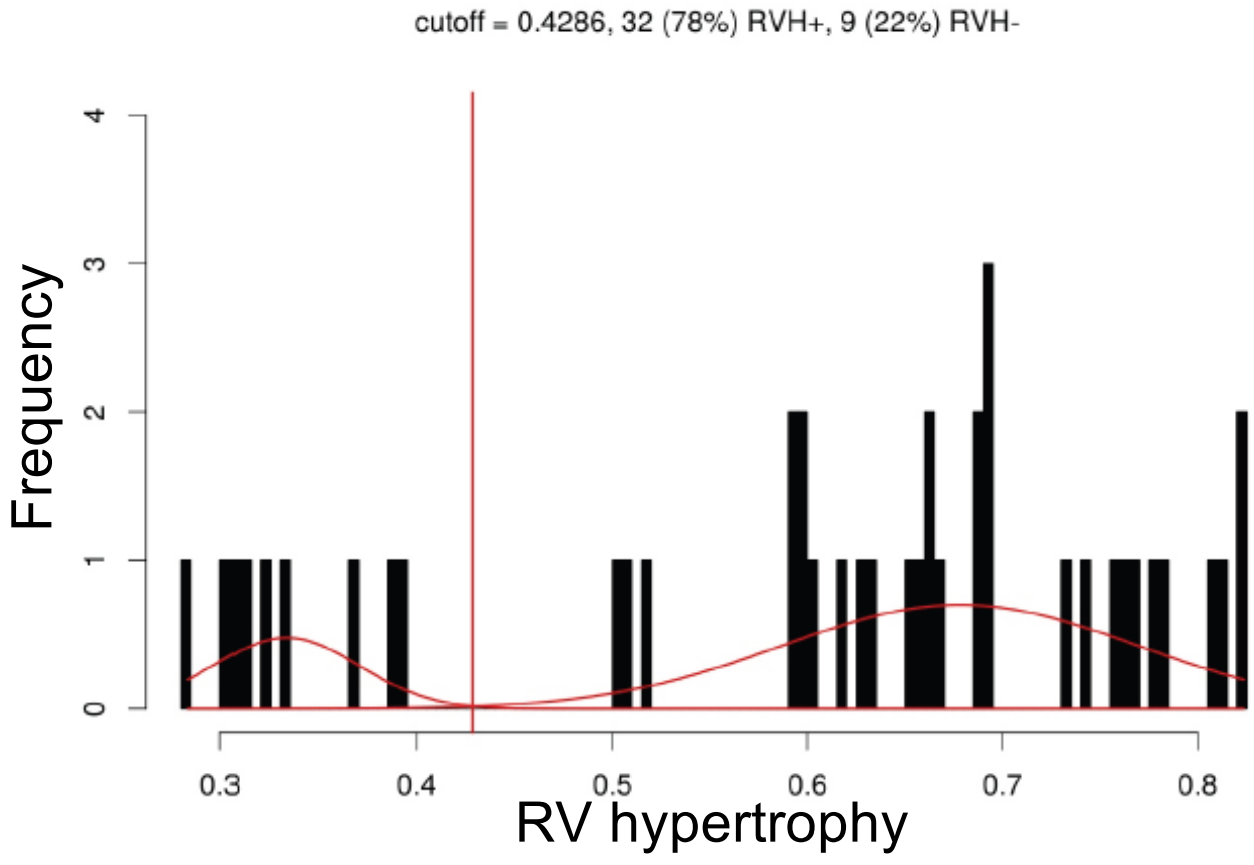
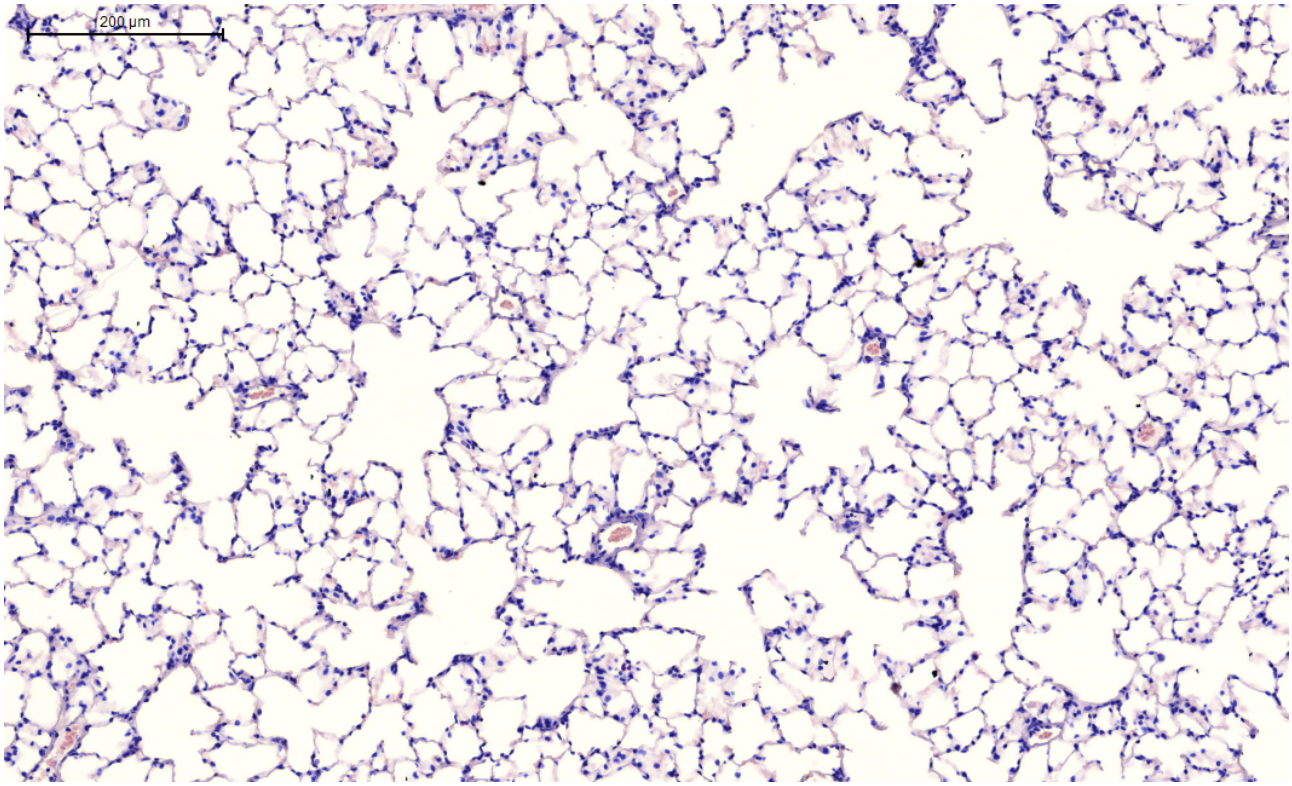

cutoff = 0.4286, 32 (78%) RVH+, 9 (22%) RVH-
Frequency
0
1
2
3
4
0.3
0.4
0.5
0.6
0.7
0.8
RV hypertrophy

Figure S4

Intact FNR



Intact FR

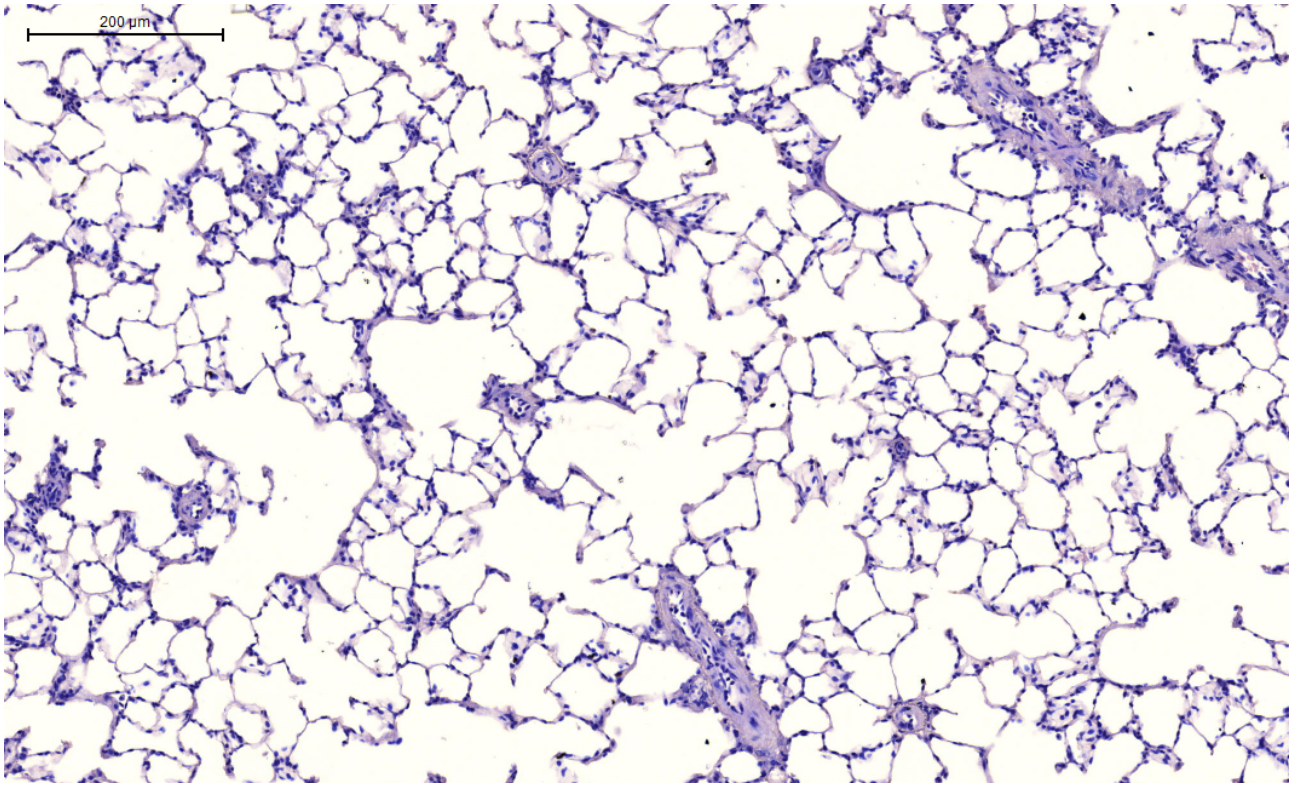
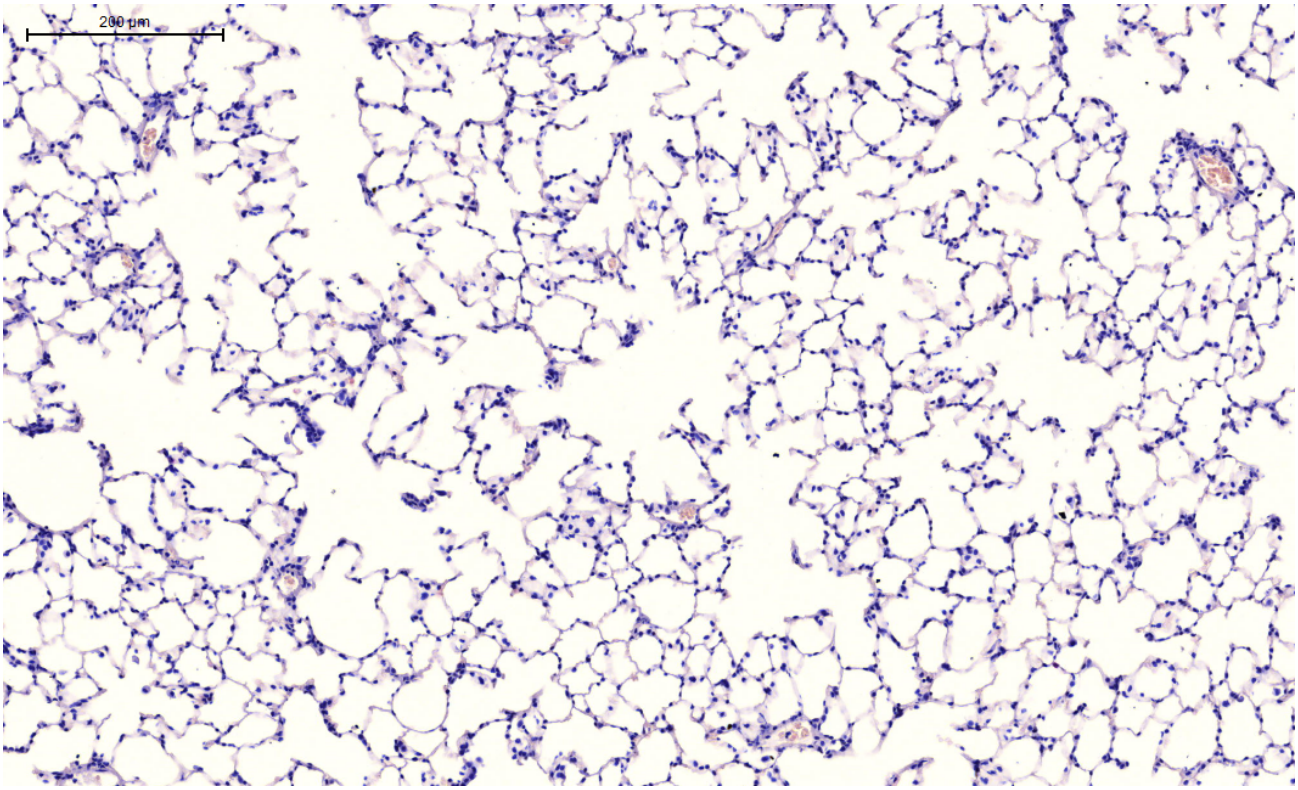


Figure S5

OVX FNR



OVX FR

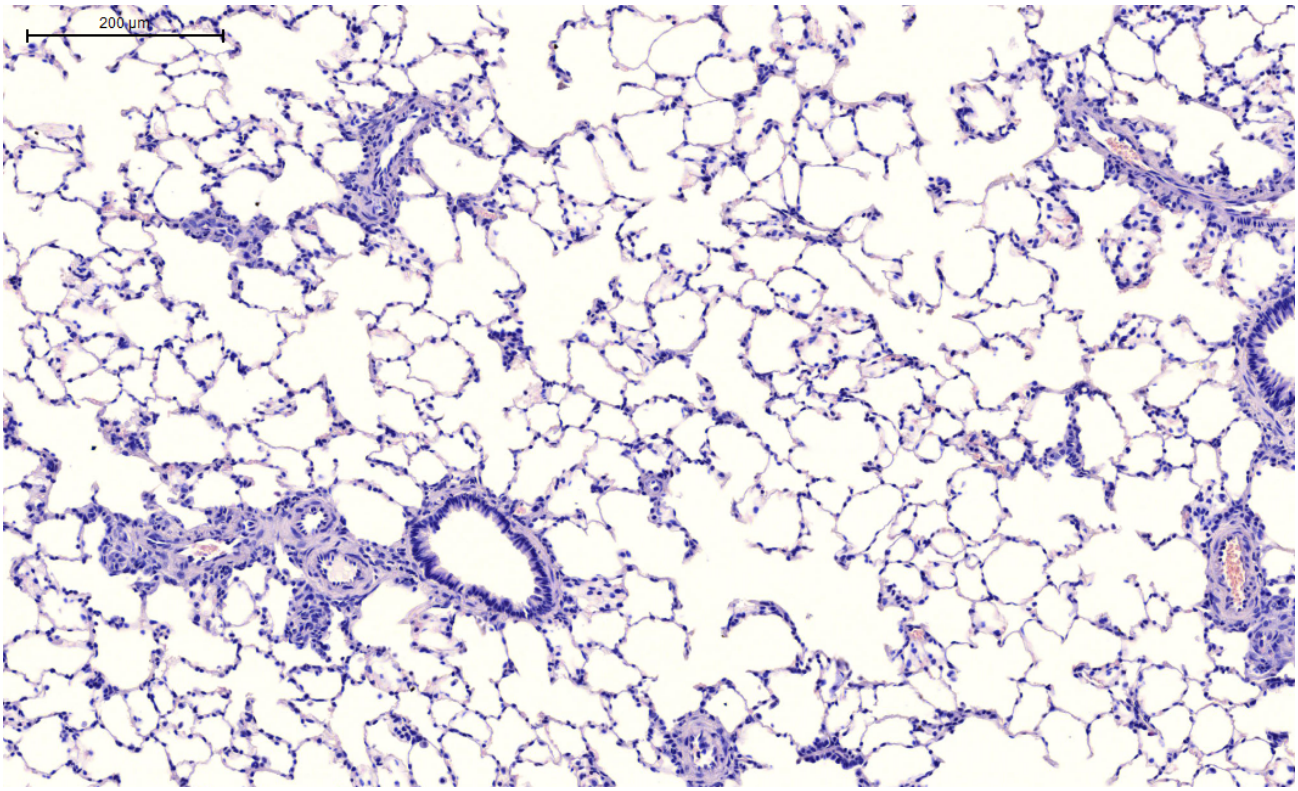
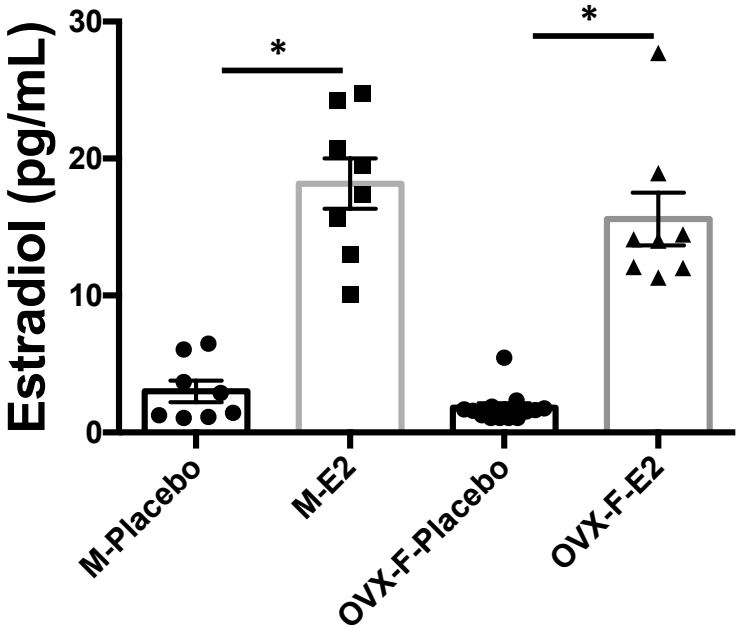


Figure S6

A


Estradiol (pg/mL)
30
20
10
0
*
*
M-Placebo
M-E2
OVX-F-Placebo
OVX-F-E2

B

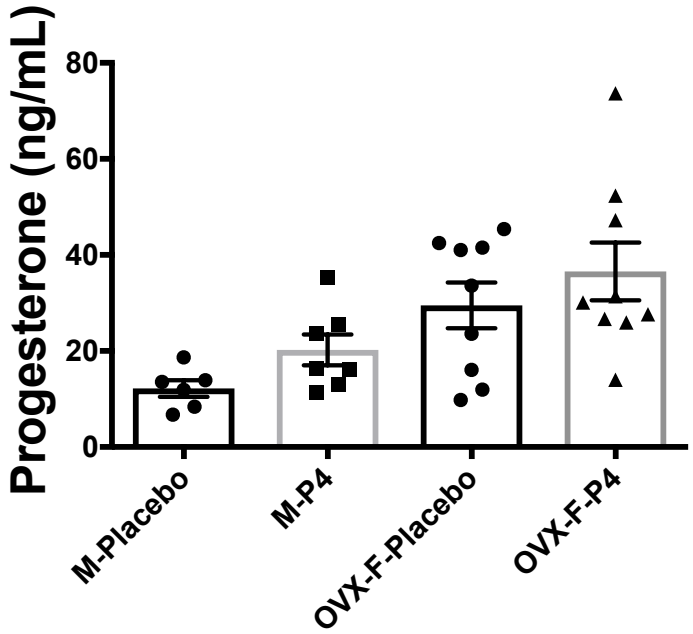
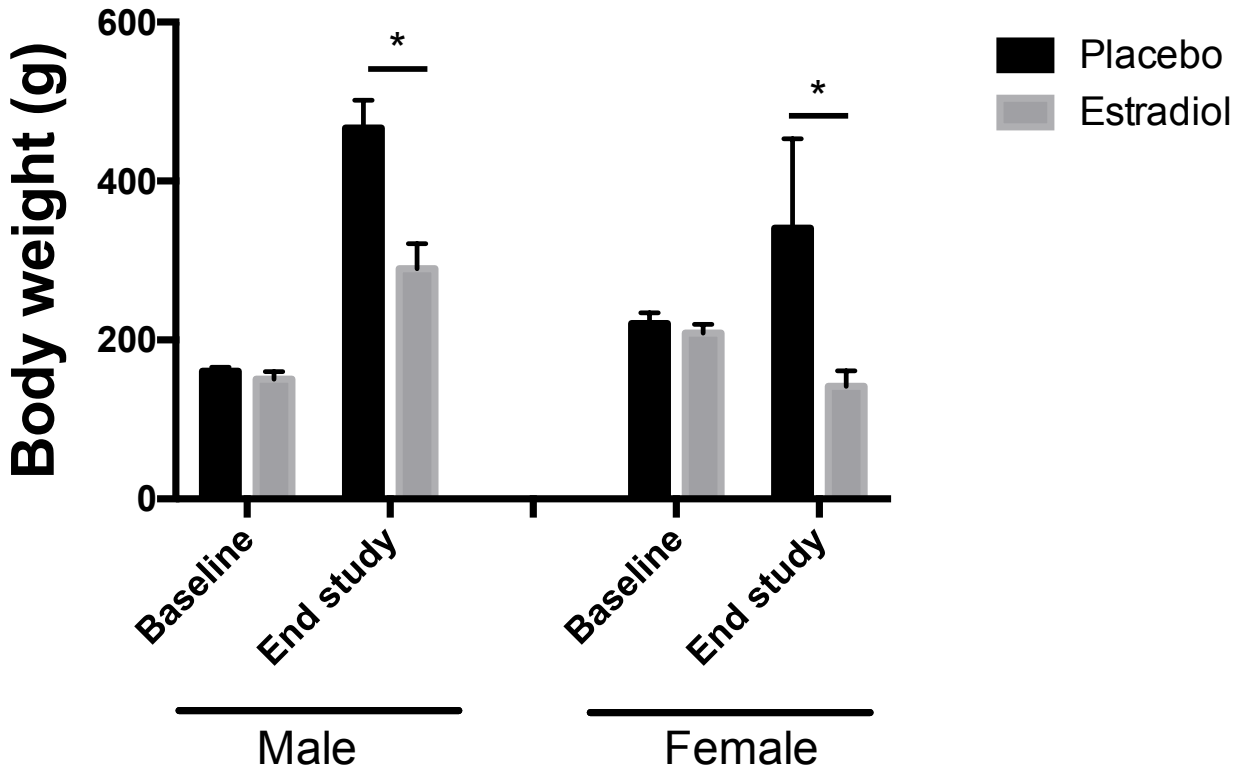

Progesterone (ng/mL)
80
60
40
20
0
M-Placebo
M-P4
OVX-F-Placebo
OVX-F-P4

Figure S7

A


Body weight (g)
600
400
200
0
*
*
Placebo
Estradiol
Baseline
End study
Baseline
End study
Male
Female

B

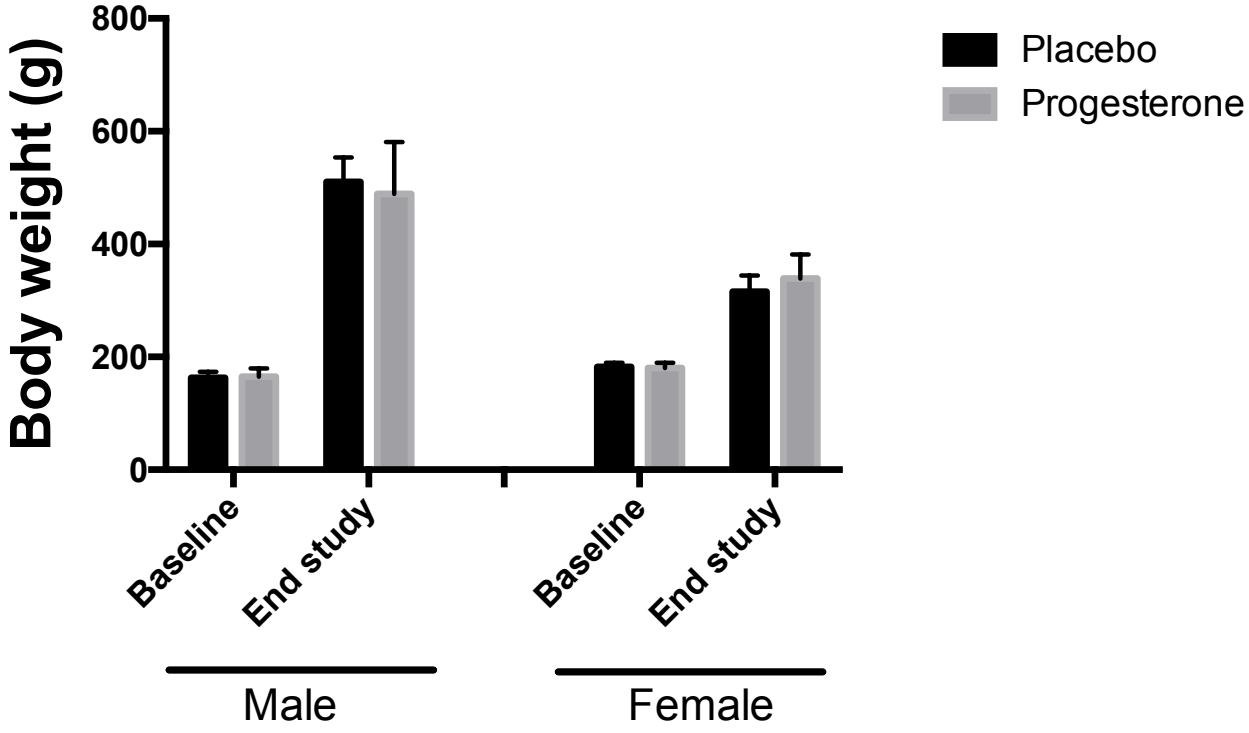

Body weight (g)
800
600
400
200
0
Placebo
Progesterone
Baseline
End study
Baseline
End study
Male
Female

Figure S8

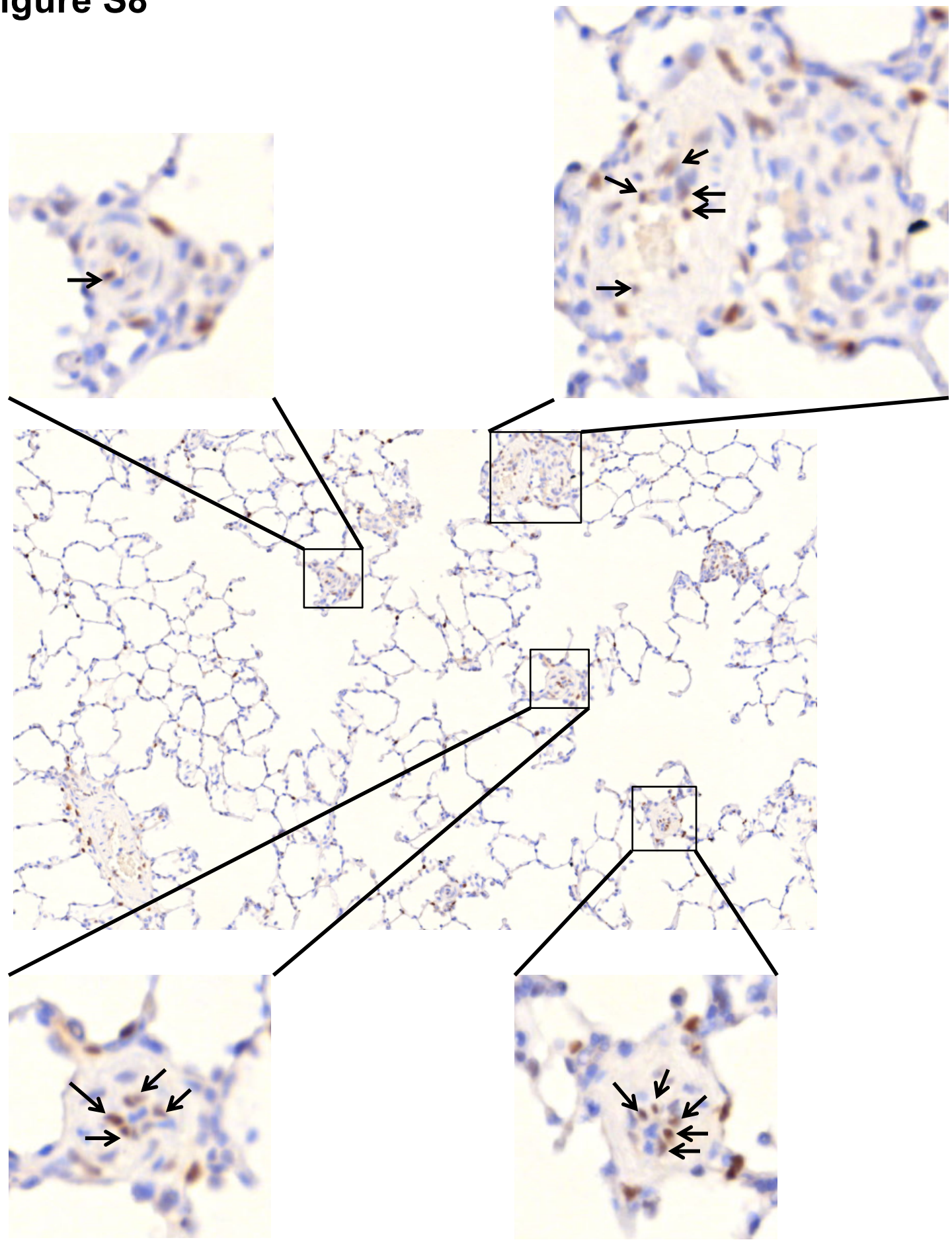


Figure S9

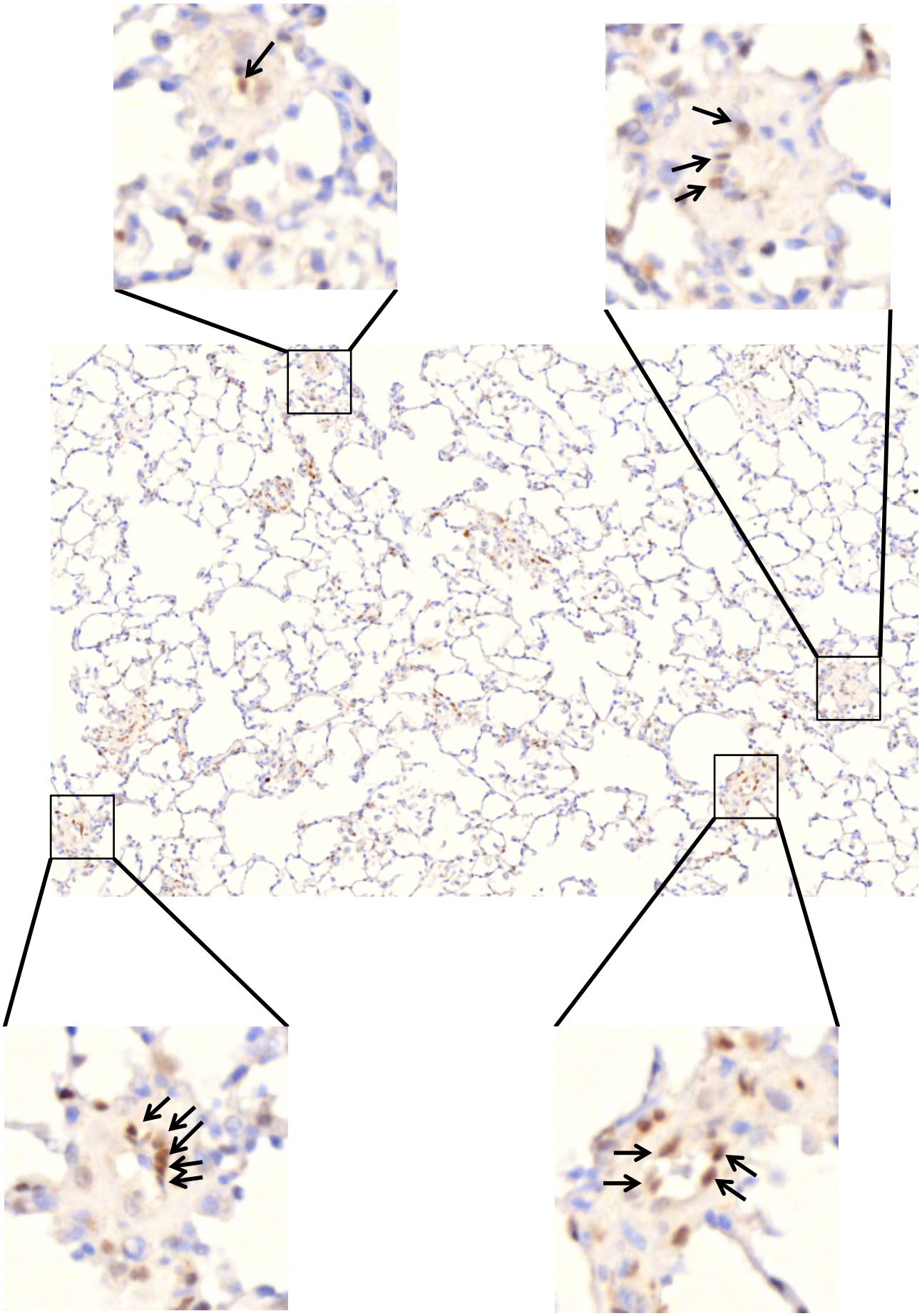


Figure S10

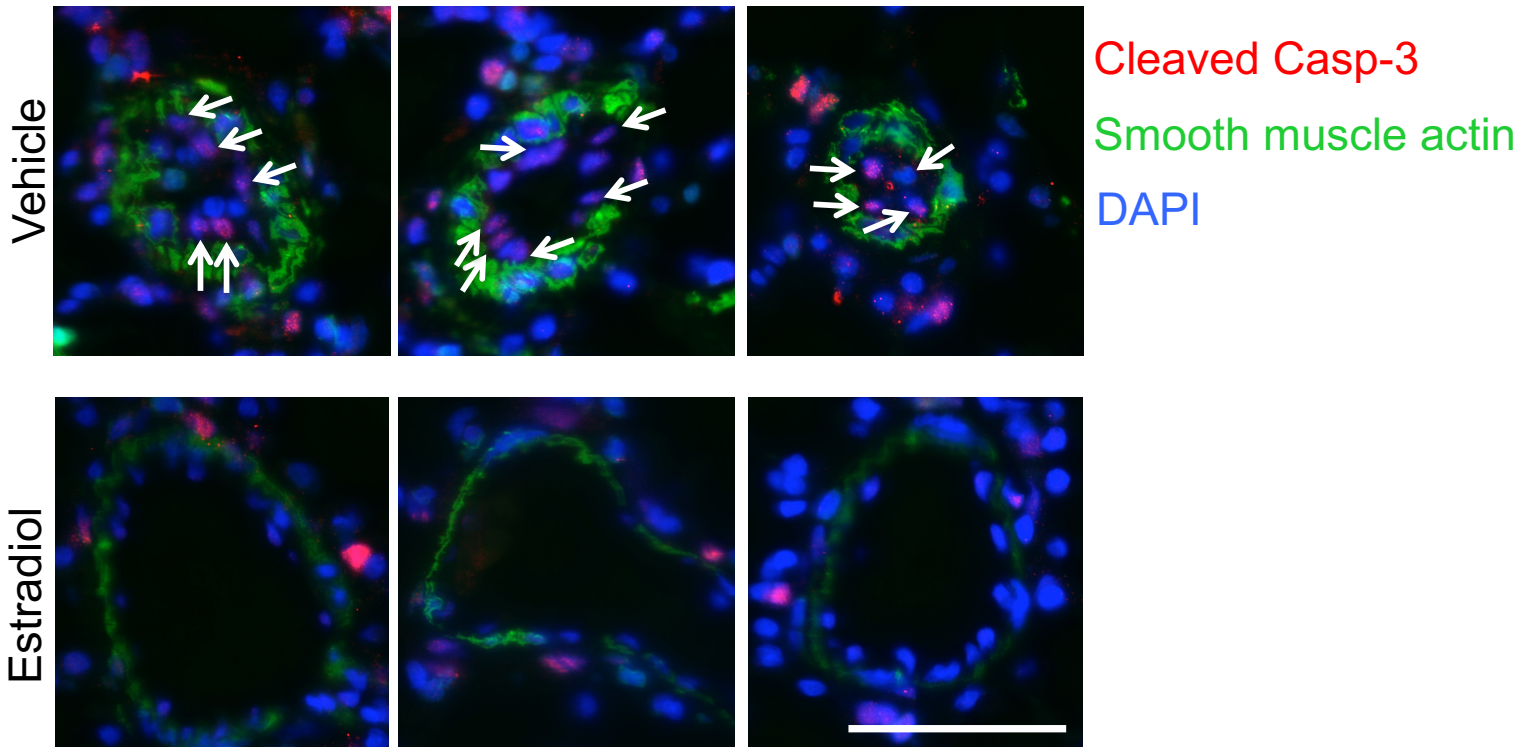


Figure S11

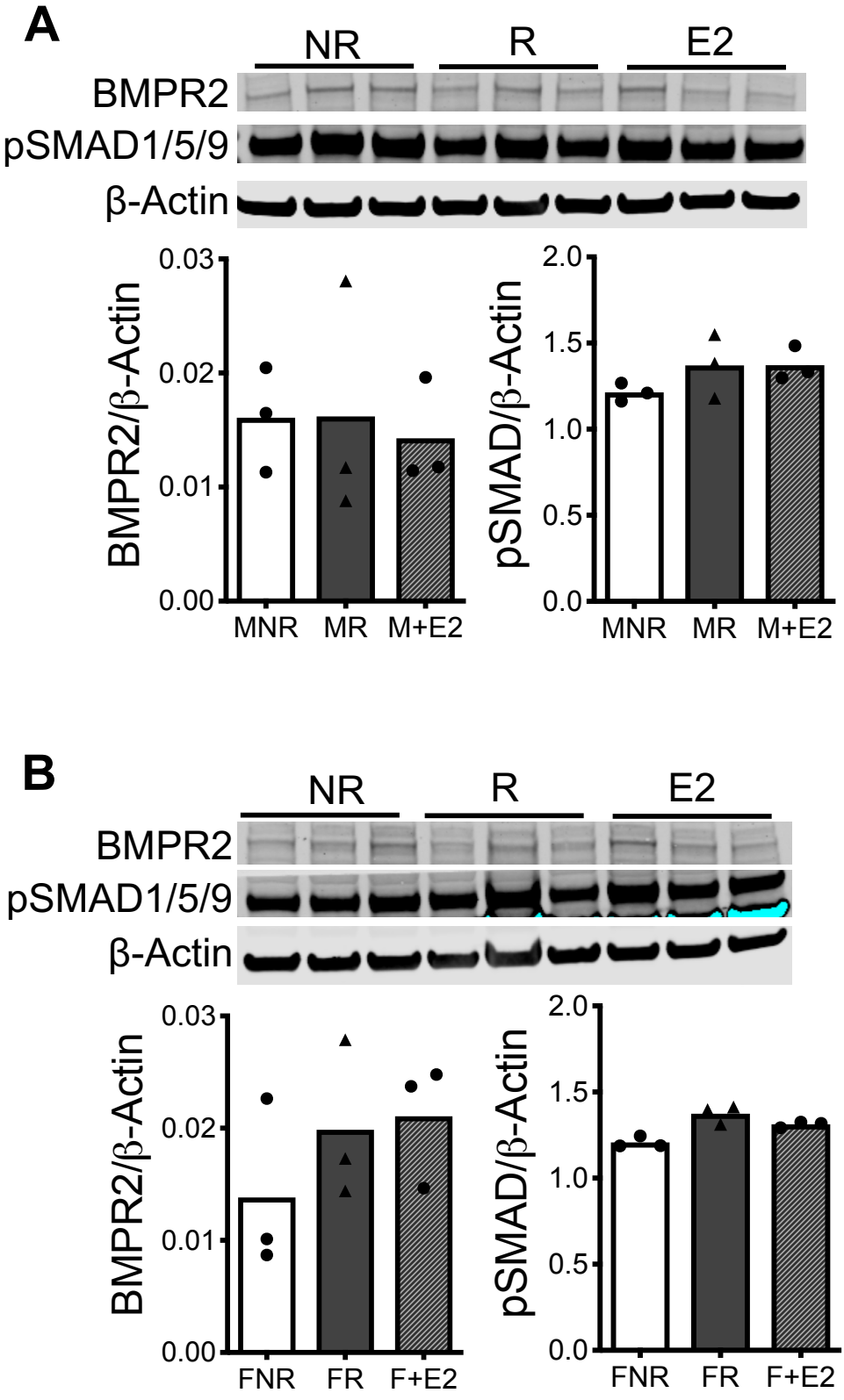

A
NR
R
E2
BMPR2
pSMAD1/5/9
β-Actin
BMPR2/β-Actin
0.03
0.02
0.01
0.00
MNR
MR
M+E2
pSMAD/β-Actin
2.0
1.5
1.0
0.5
0.0
MNR
MR
M+E2
B
NR
R
E2
BMPR2
pSMAD1/5/9
β-Actin
BMPR2/β-Actin
0.03
0.02
0.01
0.00
FNR
FR
F+E2
pSMAD/β-Actin
2.0
1.5
1.0
0.5
0.0
FNR
FR
F+E2

Figure S12

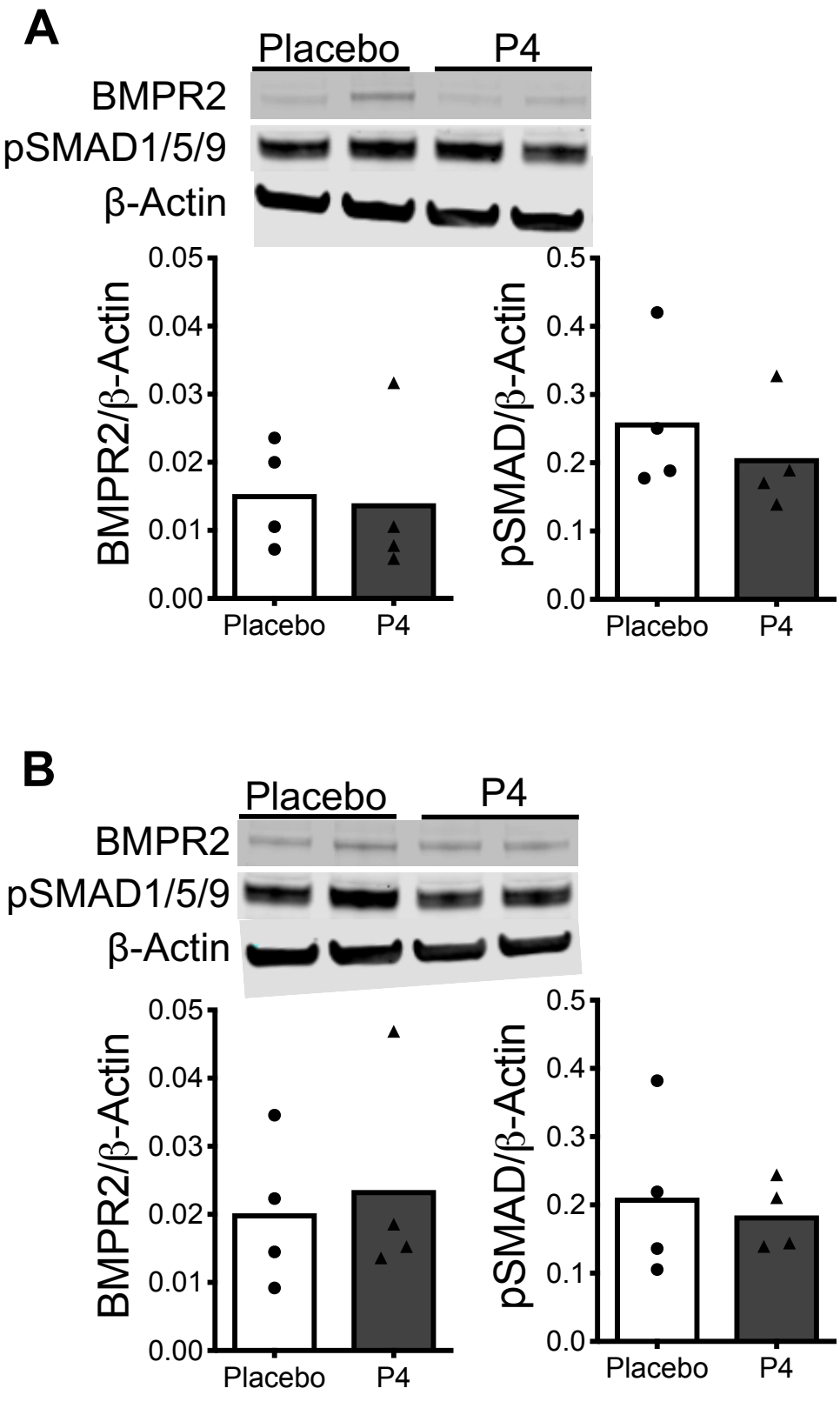

A
Placebo
P4
BMPR2
pSMAD1/5/9
β-Actin
BMPR2/β-Actin
0.05
0.04
0.03
0.02
0.01
0.00
Placebo
P4
pSMAD/β-Actin
0.5
0.4
0.3
0.2
0.1
0.0
Placebo
P4
B
Placebo
P4
BMPR2
pSMAD1/5/9
β-Actin
BMPR2/β-Actin
0.05
0.04
0.03
0.02
0.01
0.00
Placebo
P4
pSMAD/β-Actin
0.5
0.4
0.3
0.2
0.1
0.0
Placebo
P4

Figure S13

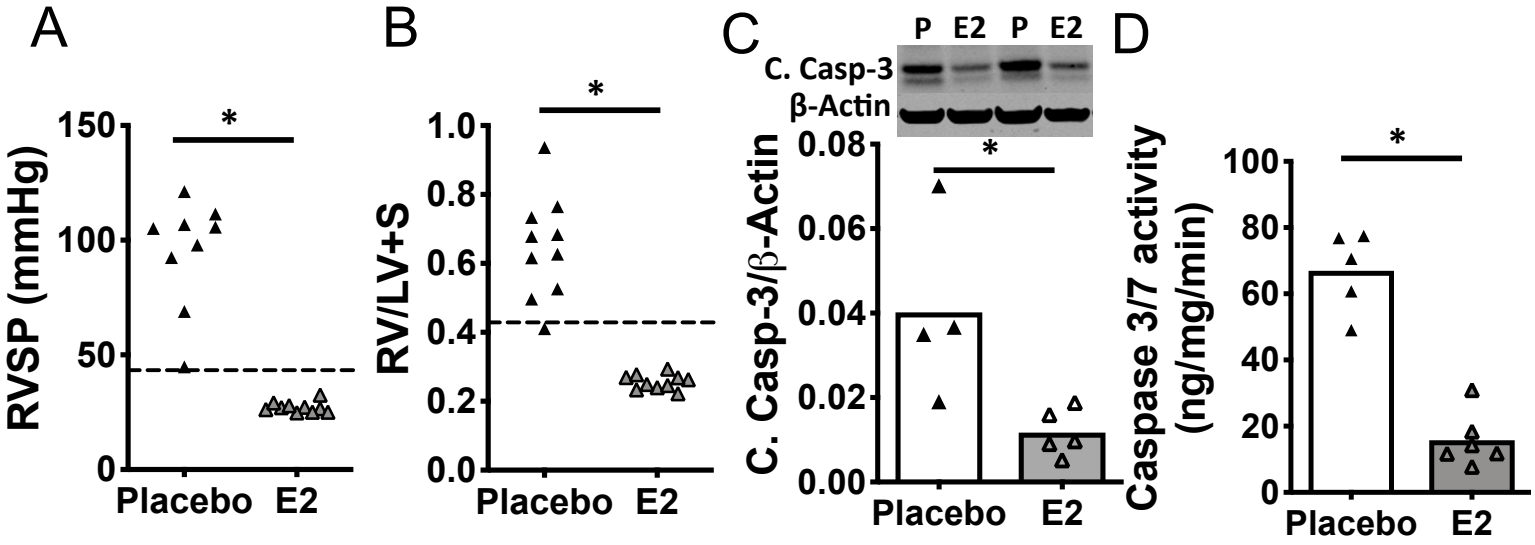
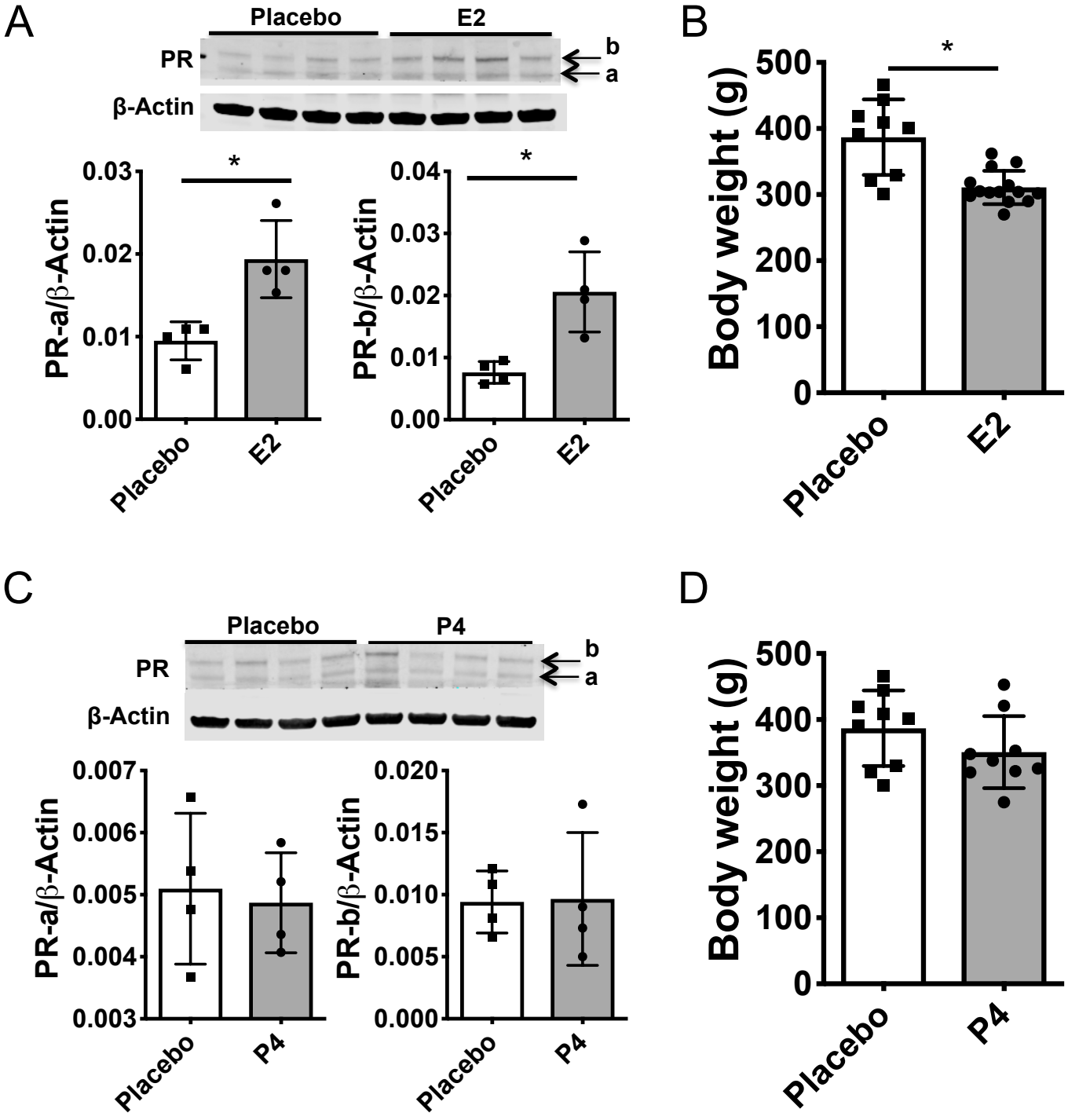

A
RVSP (mmHg)
150
100
50
0
*
Placebo
E2
B
RV/LV+S
1.0
0.8
0.6
0.4
0.2
0.0
*
Placebo
E2
C
P
E2
P
E2
C. Casp-3
β-Actin
C. Casp-3/β-Actin
0.08
0.06
0.04
0.02
0.00
*
Placebo
E2
D
Caspase 3/7 activity
(ng/mg/min)
100
80
60
40
20
0
*
Placebo
E2

Figure S14


A
Placebo
E2
PR
b
a
β-Actin
PR-a/β-Actin
0.03
0.02
0.01
0.00
*
Placebo
E2
PR-b/β-Actin
0.04
0.03
0.02
0.01
0.00
*
Placebo
E2
B
Body weight (g)
500
400
300
200
100
0
*
Placebo
E2
C
Placebo
P4
PR
b
a
β-Actin
PR-a/β-Actin
0.007
0.006
0.005
0.004
0.003
Placebo
P4
PR-b/β-Actin
0.020
0.015
0.010
0.005
0.000
Placebo
P4
D
Body weight (g)
500
400
300
200
100
0
Placebo
P4