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Ovary-dependent emphysema augmentation and osteopontin induction in adult female mice

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ABSTRACT

Biological differences between the sexes greatly impact the development and severity of pulmonary disorders such as emphysema. Recent studies have demonstrated crucial roles for osteopontin (OPN, also known as SPP1) in lung inflammation and alveolar destruction in human and experimental emphysema, but the impact of gender on OPN action remains unknown. Here, we report ovary-dependent induction of *Opn* mRNA with augmentation of experimental emphysema in adult female mice. Both male and female mice developed emphysematous lungs following intra-tracheal administration of porcine pancreatic elastase; however, compared with male mice, female mice developed more severe injury-related inflammation and pathologic alterations of the lungs. Notably, we observed female-specific induction of the *Opn* gene upon lung injury. Ovariectomy blocked this induction, with attenuation of lung inflammation and alveolar destruction, demonstrating the essential role of ovaries in injury-related *Opn* induction and augmentation of emphysema in adult female mice. Lastly, pre-treatment of adult female mice with pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, which blocks ATP-mediated wound response, suppressed *Opn* mRNA induction upon lung injury, resulting in attenuation of enhanced lung inflammation. Together, our findings define a novel, ovary-dependent mechanism underlying gender-specific augmentation of emphysema through transcriptional control of the *Opn* gene.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is an incurable, progressive respiratory illness characterized by limited airflow due to lung inflammation and structural matrix destruction [1]. Emphysema, a major component of COPD, is defined as the enlargement of alveolar air spaces, accompanied by bronchiolar fibrosis in the proximal airways. These pathological features result from low-grade inflammation elicited by chronic exposure to cigarette smoke (CS). Because smoking habits strongly influence the prevalence rate of COPD, gender effect was initially believed to have only a minor effect on COPD development and severity. However, studies comparing female and male smokers demonstrate that women tend to exhibit more severe symptoms including larger reductions in FEV_1 (the forced expiratory volume in one second) and higher mortality, after adjustment for smoking intensity [2–4]. In experimental emphysema, CS-exposed female mice exhibit more severe phenotypes in terms of inflammation, alveolar destruction, and respiratory dysfunction of the lung when compared with male counterparts [5]. Therefore, a female-specific mechanism underlying augmentation of COPD might exist.

Osteopontin (OPN) is a pleiotropic cytokine involved in a wide range of biological functions including bone metabolism, immune response, and cancer metastasis [6]. Elevated OPN levels have been reported in the lungs of patients with asthma, COPD, or pulmonary fibrosis, or a combination of the above [7–11] and are linked to pathological features of these diseases, including eosinophil and neutrophil infiltration [12,13], goblet cell hyperplasia [14,15], airway hyper-responsiveness [13,14], and fibrosis [11,15,16]. Shan et al. have recently demonstrated in mice that chronic exposure to CS induces *Opn* mRNA expression in lung dendritic cells, which in turn stimulates differentiation of Th17 cells and thus interleukin 17A (IL-17A)-driven inflammation, eventually leading to emphysema [17,18]. In contrast, *Opn* gene deficiency in mice attenuates

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CS-induced immune cell infiltration, IL-17A production, and alveolar destruction [17], demonstrating that OPN actively contributes to emphysema pathogenesis by modulating both innate and adaptive immunity.

Extracellular ATP is a danger-signaling molecule receiving extensive attention in the field of pulmonary medicine. Lung injury caused by chemicals and mechanical ventilation triggers ATP release, which recruits immune cells and fibroblasts through the activation of purinergic receptors, leading to inflammation and fibrosis of the lungs, respectively [19,20]. Like elevated OPN levels, elevated extracellular ATP concentrations have been reported in the lungs of smokers and former smokers, especially those suffering from COPD. Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), a potent purinergic receptor antagonist, ameliorates lung inflammation and alveolar destruction in CS-exposed mice [21], indicating that the purinergic receptor–mediated wound response actively contributes to emphysema pathogenesis. Despite the functional importance of OPN and purinergic receptors in emphysema pathogenesis, their roles in disease severity and gender difference have not yet been defined.

Because chronic exposure to CS activates innate immunity mediated by neutrophils and macrophages, both of which secrete proteinases, including elastase, which disrupts the lung extracellular matrix, the process leading to emphysema can be mimicked by a single intra-tracheal administration of porcine pancreatic elastase (PPE) in experimental animals [22–24]. In the current study, we demonstrated *Opn* mRNA induction, and enhanced inflammation and alveolar destruction, following PPE-induced lung injury in adult female mice. Both ovariectomy and PPADS pretreatment successfully repressed the injury-related *Opn* mRNA induction in adult female lungs, resulting in reduced lung inflammation and reduced structural alterations. These findings suggest that ovary-dependent transcriptional control of the *Opn* gene plays a role in the female-specific augmentation of experimental emphysema in mice and that it may have clinical implications for emphysema in female patients.

2. Materials and methods

2.1. Animals

Wild-type C57BL/6N mice (6- to 8-wk old) were obtained from the Sankyo Lab Service Corporation, Inc. (Tokyo, Japan). Mice were housed in a specific pathogen-free environment and allowed access to food and water ad libitum. Bilateral ovariectomy was performed on 6-wk-old mice, which were then housed for 3 wk to eliminate endogenous ovarian hormones, confirmed by uterine regression, before experiments were conducted. Emphysema mice were generated by intra-tracheally administering 5 units of porcine pancreatic elastase (PPE; Elastin Products, Co. Inc., Owensville, MO, USA) in a total volume of 25 μL saline by using a microspray (Penn-Century, Inc., Wyndmoor, PA, USA) under anesthesia. As the control, mice were given saline using the same procedure described above. To block purinergic receptor signaling, mice were administered 4.8 μg of pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid dissolved in 20 μL of saline (400 μM solution) intra-nasally 30 min before PPE administration. Bronchoalveolar lavage fluid (BALF) and lungs were collected at 24 h or indicated time points after PPE administration for further analysis.

2.1.1. BALF cell analysis

BALF cell analysis was performed as previously reported [24]. Briefly, BALF (2 mL) was centrifuged at 540 × *g* for 10 min at 4 °C. The supernatant was collected and stored at −80 °C until further use, and the cell pellet was suspended in 1 mL of PBS. The total cell number in BALF was determined by using a hemacytometer. To obtain cell counts of particular types of inflammatory cells, less than 50,000 cells from each BALF were spun at 640 rpm for 2 min at room temperature onto glass microscope slides by using a Shandon Cytospin 4 (Thermo Electron, Waltham, MA, USA), and the cells were stained with Diff Quik (International Reagents Corporation, Osaka, Japan). At least 200 cells per mouse were counted under bright-field microscopy for this differential cell analysis.

2.1.2. Lung histology

Four weeks following the elastase administration, lungs were collected and fixed with 4%(w/v) buffered paraformaldehyde phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24 h at 4 °C, and then embedded in paraffin. Coronal sections, 6-μm thick and encompassing the left lobes, were cut with a microtome from the paraffin-embedded lungs. To determine the extent of alveolar destruction, the sections were de-paraffinized, rehydrated, and then stained with H&E. The mean linear intercept, as a measure of intralveolar wall distance, was calculated as described previously [24].

2.1.3. Real-time PCR

Right lung lobes were frozen in liquid nitrogen immediately after isolation and were then fractured with a Multi Bead Shocker (Yasui Kikai Co., Osaka, Japan). Total RNA was extracted from the fractured lung powder by using TRI reagent (Invitrogen, Carlsbad, CA, USA), followed by DNase treatment (TURBO DNase; Ambion, CA, USA) to eliminate contaminated genomic DNA. For cDNA synthesis, 1.5 μg of total RNA was reverse-transcribed by using PrimeScript (Takara, Tokyo, Japan). Real-time PCR was performed for genes encoding osteopontin by using SYBR Premix Ex Taq II (Takara) in a total volume of 20 μL β-actin was used as the internal control. For calculating absolute copy numbers of target genes, serially diluted plasmids containing target gene cDNAs were used to generate a standard curve of each target gene. Melting temperature was used to confirm the specificity of the reactions. Forward and reverse primers used were as follows:

β-actin (5′-CTC CTA GCA CCA TGA AGA TCA-3′, 5′-CCT GCT TGC TGA TCC ACA TC-3′)
Osteopontin (5′-AGA ATC TCC TTG CGC CAC AG-3′, 5′-ATC GTC ATC ATC GTC GTC CAT-3′)

2.1.4. Statistics

Data from experimental replicates were pooled and are presented as means ± SEM. All data were statistically analyzed by 2-tailed Mann–Whitney *U* tests (for comparisons of two groups) or Kruskal–Wallis tests with Dunn's post-tests (for comparisons of multiple groups). Statistical significance was set at $P < 0.05$.

2.1.5. Study approval

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the Musashino University, Japan, and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

3. Results and discussion

3.1. Enhanced acute neutrophil infiltration to lungs upon elastase treatment in adult female mice

To gain insight into the mechanisms underlying gender differences in the pathogenesis and disease severity of emphysema, we generated elastase-induced experimental emphysema in male,

female, and ovariectomized mice and estimated lung inflammation by measuring the number of various inflammatory cell types in the bronchioalveolar lavage fluid. Through a time-course study of lung inflammation we found that acute inflammation occurred at 24 h after PPE treatment in adult female mice (Fig. S1). As shown in Fig. 1, a significant increase in neutrophil infiltration to lungs was observed in both male and female mice compared with saline-treated controls. We also observed an enhanced inflammatory response in PPE-treated female mice compared with PPE-treated male mice (Fig. 1). Interestingly, the augmentation phenotype in PPE-treated female mice was not observed in PPE-treated ovariectomized mice (Fig. 1), demonstrating that ovaries are required for the augmentation of acute inflammation upon lung injury in adult female mice.

3.2. Augmentation of emphysema in adult female mice

Next we addressed if ovaries influence emphysematous lung formation in PPE-treated adult female mice. Four weeks after PPE treatment, we found chronic macrophage accumulation, a characteristic feature of emphysema, in the lungs of adult female mice but not male mice (Fig. 2A). Furthermore we observed a slight but significant increase in neutrophil number in the lungs of PPE-treated female mice compared with their male counterparts (Fig. 2A). Importantly, the chronic accumulation of macrophages in PPE-treated female lungs was blocked by ovariectomy (Fig. 2A), and the slight increase in neutrophil number was also blocked in PPE-treated ovariectomized mice (Fig. 2A). There is a report that elastin fragment, acting as a chemotactic molecule, recruits blood monocytes and thus causes macrophage accumulation in CS-exposed mice [25]. However, considering the elapsed time from PPE administration together with the accumulation of macrophages in female mice but not ovariectomized mice (Fig. 2A), it very unlikely that exogenous elastase directly contributed to the macrophage accumulation observed in female lungs in our study. This suggests that there is an alternative, ovary-dependent mechanism through which monocytes migrate and reside in emphysematous lung tissue in the absence of elastin fragment.

We then examined structural alterations of the alveolar walls in PPE-treated mice. At 4 wk post-treatment, air space enlargement due to alveolar destruction was detected in male, female, and ovariectomized mice (Fig. 2B). Although CS exposure causes moderate and irregular, multifocal air space enlargement throughout the parenchyma in mice [5], our administration of PPE via a microspray led to strong and relatively homogenous structural alterations. Quantitative morphometric assessment by measuring the mean linear intercept (Lm) revealed significantly larger alveolar spaces in PPE-treated female mice (μm) than in PPE-treated male mice (Fig. 2C), which is consistent with the CS-induced experimental emphysema model [5]. Most importantly, ovariectomy significantly reduced the Lm values of adult females to a level that was similar to that in males (Fig. 2C). Collectively, these results indicate that ovary-dependent augmentation of emphysema pathogenesis emerges within 24 h after lung injury, which consequently influences the severity of alveolar destruction (Figs. 1 and 2).

3.3. Female-specific induction of Opn gene in injured lungs

A strong correlation between OPN accumulation in plasma, sputum, and lung biopsies of patients with COPD and their disease severity has been reported [9,10,18]. Given such clinical observations, and the crucial roles of OPN in the pathogenesis of human and experimental emphysema [17,18], we predicted that Opn would be implicated in the enhanced severity of emphysema pathogenesis in adult female mice. Therefore, we performed real-time PCR to determine whether lung injury by PPE alters the expression level of the *Opn* gene in adult female mice, and if so, whether this alteration is controlled through ovaries. At 24 h after PPE treatment, we found a significant increase in *Opn* expression in the lungs of adult female mice (Fig. 3A) but not in those of male mice. Interestingly, this induction was completely suppressed by ovariectomy (Fig. 3A), revealing an ovary-dependent transcriptional control of OPN in the early pathogenesis of emphysema of adult females. Our finding gives rise to an apparent inconsistency with human emphysema in which OPN accumulation is observed in the lungs of both male and female patients with COPD [9,10,18]. We cannot exclude the possibility that a slight but continuous tissue damage by chronic CS exposure activates gene expression of *OPN* independent of ovaries; however, considering that the prevalence rate of COPD increases with age [26], biological aging may also exert a non-negligible impact on OPN expression. Supporting this, age-related Opn elevation and tissue rejuvenation by Opn neutralization have been reported in skeletal muscle of male mice [27].

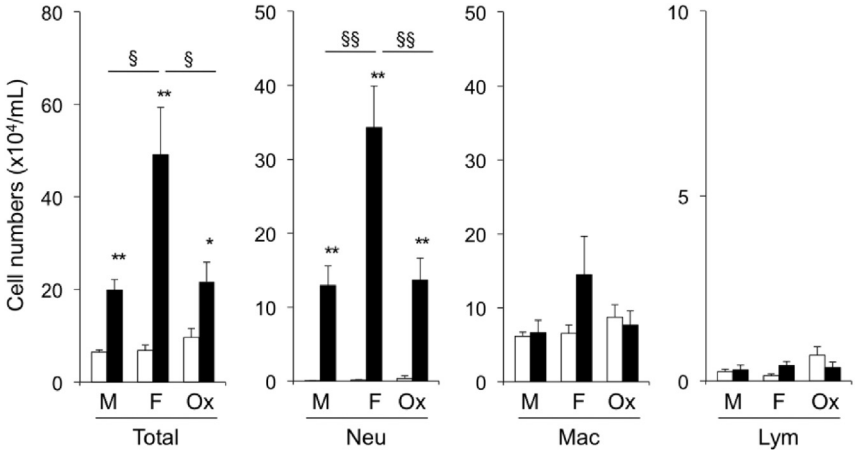

Fig. 1. Lack of augmentation of acute lung inflammation in ovariectomized female mice at 24 h after elastase treatment. Mice were treated with porcine pancreatic elastase (PPE) (closed column, n = 9 to 17 per group) or saline (open column, n = 3 to 13 per group). The cell numbers of total cells, neutrophils (Neu), macrophages (Mac), and lymphocytes (Lym) in the bronchioalveolar lavage fluid (BALF) of male (M), female (F), and ovariectomized (Ox) mice at 24 h after treatment are shown. Data are presented as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ versus saline-treated (control) mice, Kruskal–Wallis test. $^{\S}P < 0.05$; $^{\S\S}P < 0.01$ versus PPE-treated female mice, Kruskal–Wallis test.

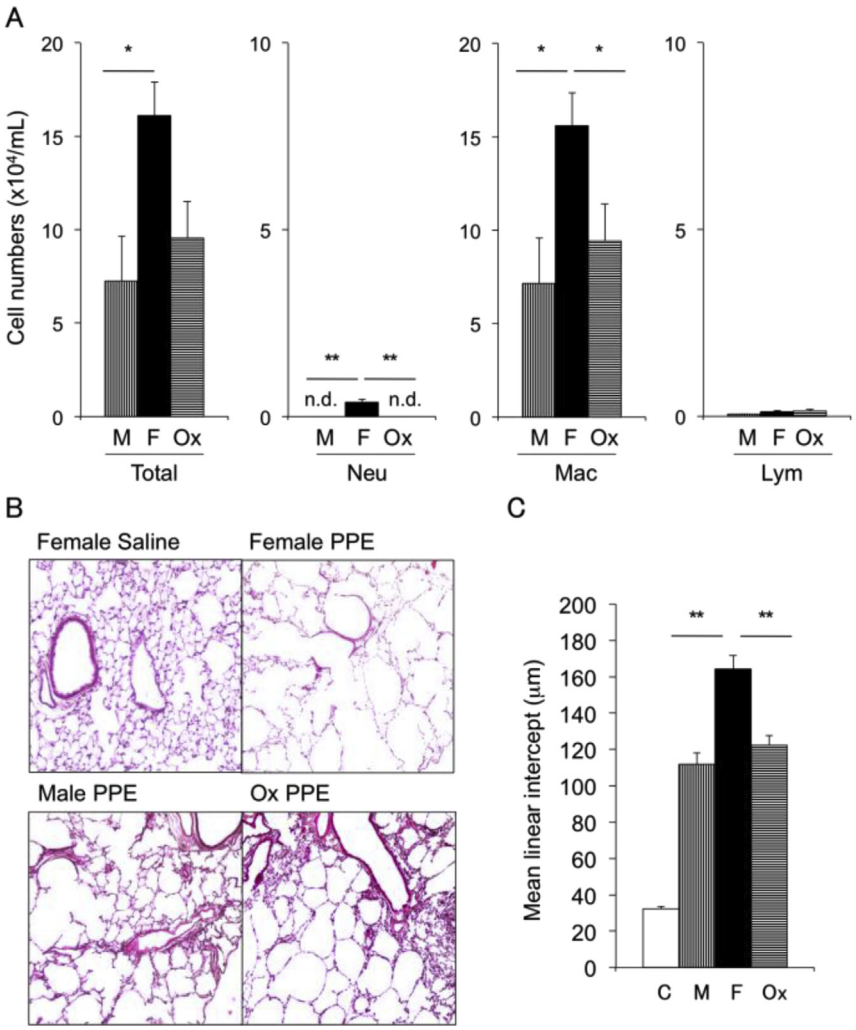

Fig. 2. Reduced severity of emphysema in ovariectomized female mice compared with other female mice at 4 wk after elastase treatment. (A) Cell numbers in the BALF of male (M; n = 4), female (F; n = 19), and ovariectomized (Ox; n = 4) mice at 4 wk after PPE treatment. Data are presented as means ± SEM. n.d. not detected; $^{*}P < 0.05$; $^{**}P < 0.01$ versus PPE-treated female mice, Kruskal–Wallis test. (B) Representative H&E staining of lungs from indicated mice. (C) Size of air spaces from indicated mice. Data are presented as means ± SEM (n = 3 per group). $^{**}P < 0.01$ versus PPE-treated female mice, Kruskal–Wallis test.

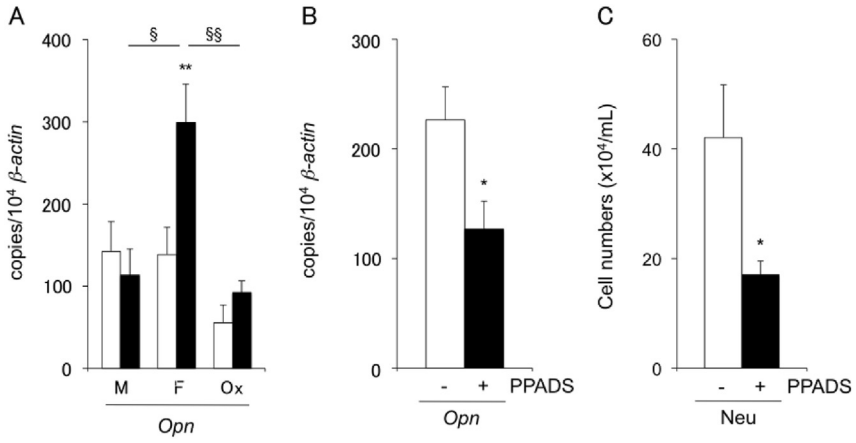

Fig. 3. Loss of *Opn* gene induction in lungs of elastase-treated, ovariectomized female mice. (A) *Opn* mRNA expression in lungs from male (M), female mice (F) and ovariectomized mice (Ox) at 24 h after PPE treatment. Open column shows saline-treated control group (n = 6 to 10 per group) and closed column shows PPE-treated group (n = 7 to 19 per group). Data are presented as means ± SEM; $^{**}P < 0.01$ versus control mice, Kruskal–Wallis test. $^{§}P < 0.05$; $^{§§}P < 0.01$ versus PPE-treated female mice, Kruskal–Wallis test. (B) *Opn* mRNA expression in lungs from saline- and PPADS-pretreated female mice at 24 h after PPE treatment (n = 4 per group). Data are presented as means ± SEM. $^{*}P < 0.05$ versus saline-pretreated PPE females, Mann–Whitney *U* test. (C) Neutrophil numbers in BALF (n = 4 per group). Data are presented as means ± SEM. $^{*}P < 0.05$ versus saline-pretreated PPE females, Mann–Whitney *U* test.

Furthermore menopausal women who have been losing ovarian function show elevated serum levels of OPN [28]. Nonetheless, our findings clearly demonstrate that injury-related *Opn* induction in lungs is tightly controlled by ovaries, and further suggest that Opn acts as a possible molecular switch defining the severity of pathogenesis of emphysema.

3.4. Purinergic receptor–driven Opn induction upon elastase treatment in adult female mice

Purinergic receptors serve as stress sensors for tissue injury, so antagonizing their action prevents lung inflammation and alveolar alterations in CS-exposed mice [21]. Therefore, we addressed whether blockade of purinergic receptor signaling impairs *Opn* mRNA induction in adult female mice and thereby ameliorates the enhanced disease pathogenesis despite the presence of ovaries. Pre-treatment of adult female mice with a potent purinergic receptor antagonist, PPADS, suppressed injury-related *Opn* mRNA induction (Fig. 3B). Most importantly, consistent with reduced lung inflammation in CS-exposed *Opn*-deficient mice [17], the enhanced inflammatory response of adult female mice was blocked with PPADS (Fig. 3C), demonstrating that ovary-dependent *Opn* induction triggered by purinergic receptor signaling defines the severity of emphysema pathogenesis of adult female mice. OPN plays an important role in Th17-mediated adaptive immunity in the pathogenesis of human and experimental emphysema [17,18]. Because we failed to detect continuous Opn induction at 4 wk after PPE treatment in adult female mice (data not shown), we haven't addressed whether Opn influences the severity of the adaptive immunity in the chronic phase of emphysema. However, within 48 h of PPE treatment, the Th17 cell population and concomitant IL-17A secretion increases in the lungs of adult female mice [29]. Most importantly, IL-17A gene knockout reduces the severity of acute lung inflammation, alveolar enlargement, and further static lung compliance of PPE-treated adult female mice [29]. Therefore, ovary-dependent Opn induction may modulate not only neutrophil-mediated innate immunity but also Th17-mediated adaptive immunity, thus giving rise to gender differences in the disease severity of experimental emphysema.

Ovariectomy and estrogen receptor knockout experiments in mice demonstrate the physiological importance of estrogen in alveolar development and maintenance, and its protective effects against acute lung injury, and age-associated lung dysfunction [30–32]. In contrast, our data show that ovariectomy exerts augmentative effects against inflammation and alveolar destruction in the process of emphysema formation. Therefore, the conclusions of these studies and our study differ with respect to the pathological roles for ovaries and their products in pulmonary disorders of females, but we reach the same conclusion that adult female mice under estrogen deficiency tend to exhibit the same degrees of injury-related inflammation of the lungs, and respiratory functions as adult male mice. It is currently unclear whether ovaries differentially modulate lung pathogenesis depending upon physiological stresses or pathological stimuli in adult females; however, our results raise the important question of whether clinical pulmonary conditions in women may be affected by menstrual cycle, pregnancy, lactation, menopause, contraceptive pills, and chemotherapies, all of which significantly influence ovarian functions.

In summary, we found that ovaries control *Opn* mRNA induction upon lung injury, and the severity of inflammatory response and concomitant alveolar destruction. Moreover, we showed that prevention of injury-related *Opn* induction by PPADS ameliorates the lung inflammation necessary for emphysematous lung formation in adult female mice. Understanding the transcriptional control of the *Opn* gene by ovaries under pathological conditions in mouse could provide a foundation for establishing gender-specific therapeutic strategies to prevent and reverse emphysema and potentially other female-predominant pulmonary disorders such as severe asthma in humans.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.081.

Transparency document

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