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Estrogen regulates pulmonary alveolar formation, loss, and regeneration in mice

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Massaro, Donald, and Gloria DeCarlo Massaro. Estrogen regulates pulmonary alveolar formation, loss, and regeneration in mice. *Am J Physiol Lung Cell Mol Physiol* 287: L1154–L1159, 2004. First published August 6, 2004; doi:10.1152/ajplung.00228.2004.—Lung tissue elastic recoil and the dimension and number of pulmonary gas-exchange units (alveoli) are major determinants of gas-exchange function. Loss of gas-exchange function accelerates after menopause in the healthy aged and is progressively lost in individuals with chronic obstructive pulmonary disease (COPD). The latter, a disease of midlife and later, though more common in men than in women, is a disease to which women smokers and never smokers may be more susceptible than men; it is characterized by diminished lung tissue elastic recoil and presently irremediable alveolar loss. Ovariectomy in sexually immature rats diminishes the formation of alveoli, and estrogen prevents the diminution. In the present work, we found that estrogen receptor-α and estrogen receptor-β, the only recognized mammalian estrogen receptors, are required for the formation of a full complement of alveoli in female mice. However, only the absence of estrogen receptor-β diminishes lung elastic tissue recoil. Furthermore, ovariectomy in adult mice results, within 3 wk, in loss of alveoli and of alveolar surface area without a change of lung volume. Estrogen replacement, after alveolar loss, induces alveolar regeneration, reversing the architectural effects of ovariectomy. These studies *1*) reveal estrogen receptors regulate alveolar size and number in a nonredundant manner, *2*) show estrogen is required for maintenance of already formed alveoli and induces alveolar regeneration after their loss in adult ovariectomized mice, and *3*) offer the possibility estrogen can slow alveolar loss and induce alveolar regeneration in women with COPD.

chronic obstructive pulmonary disease; elastic recoil; estrogen receptor mutants; ovariectomy

ACROSS THE FULL BREADTH OF mammalian body mass, the area of the lung's gas-exchange structures (alveoli) is directly proportional to the organism's resting O_2 consumption (30). However, there is sexual dimorphism in alveolar architecture. Virgin female mice and rats have smaller alveoli and, per body mass, more alveoli and more alveolar surface area than same-age virgin males of their respective species (16). These differences become apparent about the time of sexual maturity and are present even though body mass-specific O_2 consumption is the same in males and nonpregnant females of the same age and species (16). During pregnancy and lactation, O_2 consumption can double (16). Thus female animals, and probably women during premodern times, evolved spending most of their reproductive life pregnant or lactating and, therefore, with a higher "steady-state" body mass-specific O_2 consumption than males.

The onset of architectural alveolar sexual dimorphism at about the time of sexual maturity suggested a role for ovarian hormones in alveolar development; this was confirmed by demonstrating ovariectomy in 21-day-old rats results in larger and fewer alveoli at 2 mo of age and that estrogen replacement prevents the impaired formation of alveoli (17). The demonstration of a requirement for estrogen for the formation of a full complement of alveoli in female rats (17) led to the present work in which we tested the hypothesis that estrogen receptors are required for the formation of a full complement of alveoli. Because this hypothesis was not falsified, we tested the hypothesis that ovariectomy in adult mice causes alveolar loss and that estrogen replacement, after alveoli were lost, results in alveolar regeneration. This hypothesis was also not falsified.

MATERIALS AND METHODS

Animals and experimental manipulations. Adult C57BL/6J mice underwent bilateral sham or bilateral ovariectomy at Jackson Laboratory. We received them a week later and began studies 2 wk later, i.e., 3 wk after surgery. Some mice were injected subcutaneously with sesame seed oil (the vehicle for estradiol) or equivolume estradiol in sesame seed oil (10 μg/kg body mass) daily for 3 wk; we injected 0.5 μl/g body mass. This dose of estrogen results in concentrations of estrogen that are lower than a much higher dose that provides physiological blood levels during estrus in mice but are supraphysiological during diestrus (12). Therefore, the dose we used probably provides physiological blood concentrations of estradiol during estrus and diestrus. At the time the mice were killed, examination of the peritoneal cavity verified the absence of ovaries in mice purported to have undergone bilateral ovariectomy and the presence of ovaries in sham-ovariectomized mice. We purchased female estrogen receptor (ER)-$\alpha^{-/-}$, ER-$\beta^{-/-}$, and wild-type (wt; C57BL/6) female mice from Taconic Farms. These mice did not receive treatments. All mice were allowed Rodent Lab Chow 5001 (Ralston Purina) and tap water ad libitum and were housed in the Department of Comparative Medicine on a 12:12-h light-dark cycle at 21°C. Mice were killed by cutting large vessels in the abdomen after establishing a surgical level of anesthesia with xylazine (~10 mg/kg) plus ketamine (~75 mg/kg). All procedures were approved by the Georgetown University Animal Care and Use Committee and comply with the National Institutes of Health guidelines.

Morphologic and morphometric studies. In anesthetized mice, we intubated the trachea, punctured the diaphragm, and instilled 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, into the trachea at a transpulmonary pressure of 20 cmH_2O. The trachea was ligated, the lungs were removed from the chest, and fixation was

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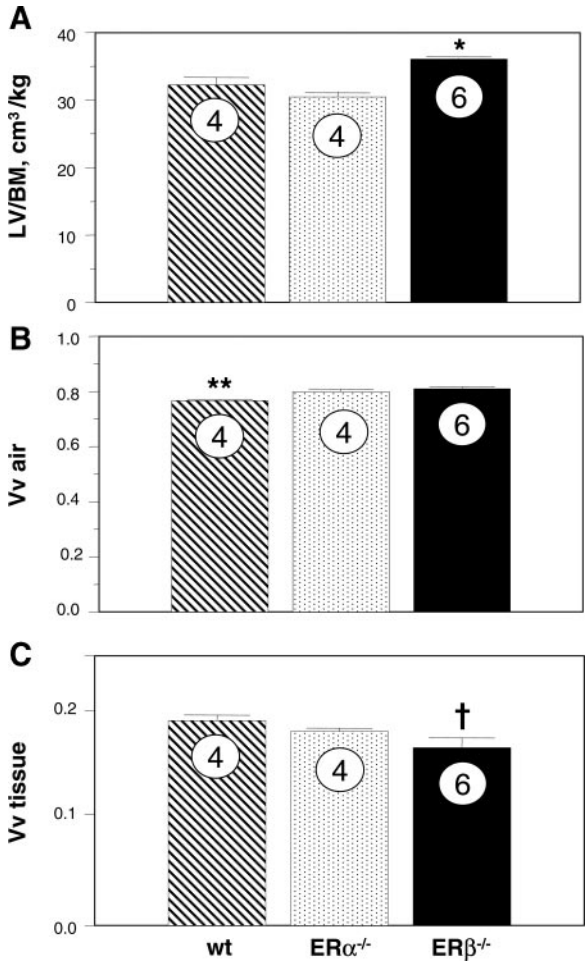

Fig. 1. Lung volume (LV) and volume density (Vv) of gas-exchange air and tissue of wild-type (wt), estrogen receptor (ER)-$\alpha^{-/-}$, and ER-$\beta^{-/-}$ mice. Lungs of adult female mice were fixed at a transpulmonary pressure of 20 cmH_2O, lung volume was measured by water displacement, and the lungs were then processed for morphometric analysis. *A*: lung volume/body mass (BM); *B*: volume density of gas-exchange region (Vv air); *C*: volume density of gas-exchange region tissue (Vv tissue). Means ± SE are provided. Nos. within bars indicate the no. of mice. *$P < 0.001$ and **$P \leq 0.009$ vs. each other mean; †$P = 0.015$ vs. wt.

continued for 2 h at 0–4°C. Lung volume was measured by volume displacement (26), lungs were cut into blocks, and blocks were selected for further processing using a systematic sampling technique (7). The selected blocks were washed in cacodylate buffer, postfixed 1 h at 4°C in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated, and embedded in epoxy resin (13–18).

Serial sections from three blocks per mouse were cut at ~0.8 μm thickness to a depth of 150–250 μm. To identify alveolar air spaces, gas-exchange structures were followed through a complete set of prints of serially sectioned lung (13–18). An alveolus was defined as a gas-exchange structure with a mouth that communicated with a common air space, which was designated an alveolar duct.

The selector method (6), which allows alveoli to be selected based on number, uninfluenced by size, shape, or, orientation, was used to select alveoli for analysis. The point-sample intercepts method (9) was used to determine the volume of an alveolus, and the number of alveoli was calculated (13–18).

Alveolar surface area was determined using sections ~0.8 μm thick that were cut from each of 10 tissue blocks. This provided 10 sections/mouse, which were stained with toluidine blue. Sections were photographed, and final prints were at a magnification of ×160. Alveolar surface area was determined by point and intersection counting (34).

Statistical analysis. Because we did not find differences of alveolar dimensions between uninjected ovariectomized mice and vehicle-injected ovariectomized mice, we placed these mice in a single group that we designated ovariectomized. For each parameter measured or calculated, the value for individual animals in each experimental group was averaged, and the SE was calculated. A one-way ANOVA and a post hoc Student-Newman-Keuls test were used to test if the numerical differences among mean values were statistically significant (29).

RESULTS

Lung volume and alveolar dimensions in wt and estrogen receptor mutant mice. Body mass-specific lung volume was higher in ER-$\beta^{-/-}$ mice than in wt or ER-$\alpha^{-/-}$ mice (Fig. 1*A*). Volume density of gas-exchange region air was greater in each mutant group than in wt mice (Fig. 1*B*). The volume density of gas-exchange region tissue was lower in ER-$\beta^{-/-}$ mice than in wt mice (Fig. 1*C*). The volume of an average alveolus was greater (Figs. 2*A* and 3), and body mass-specific number of alveoli was lower (Fig. 2*B*), in each mutant group compared

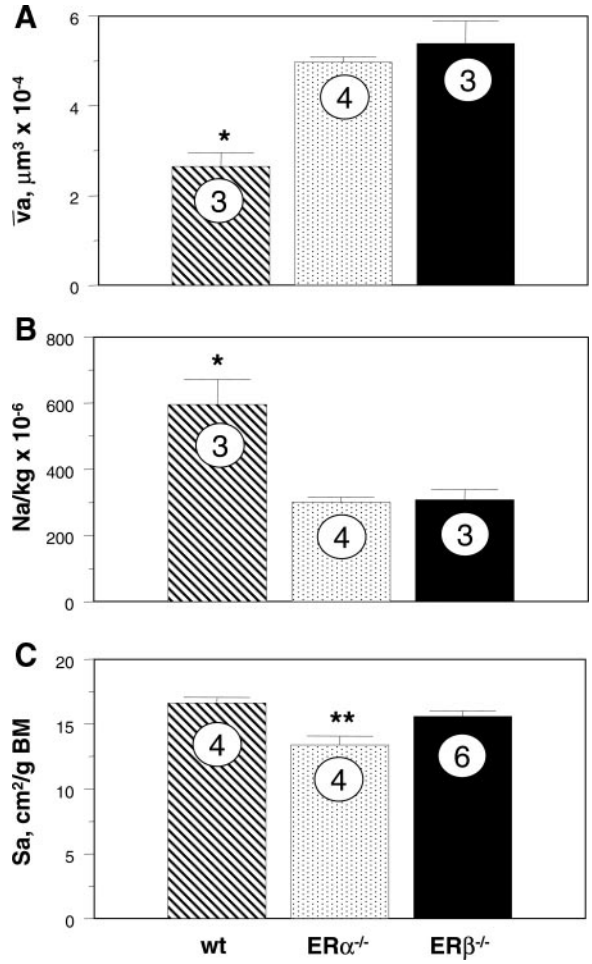

Fig. 2. Alveolar size, number, and surface area of wt, ER-$\alpha^{-/-}$, and ER-$\beta^{-/-}$ adult female mice. Lung tissue was fixed as described in the legend to Fig. 1. Means ± SE are given. Nos. within bars indicate the no. of mice. $\bar{v}a$, Volume of an average alveolus; Na/kg, no. of alveoli/body mass; *Sa*, alveolar surface area. *$P \leq 0.003$ and **$P \leq 0.020$ vs. each other mean.

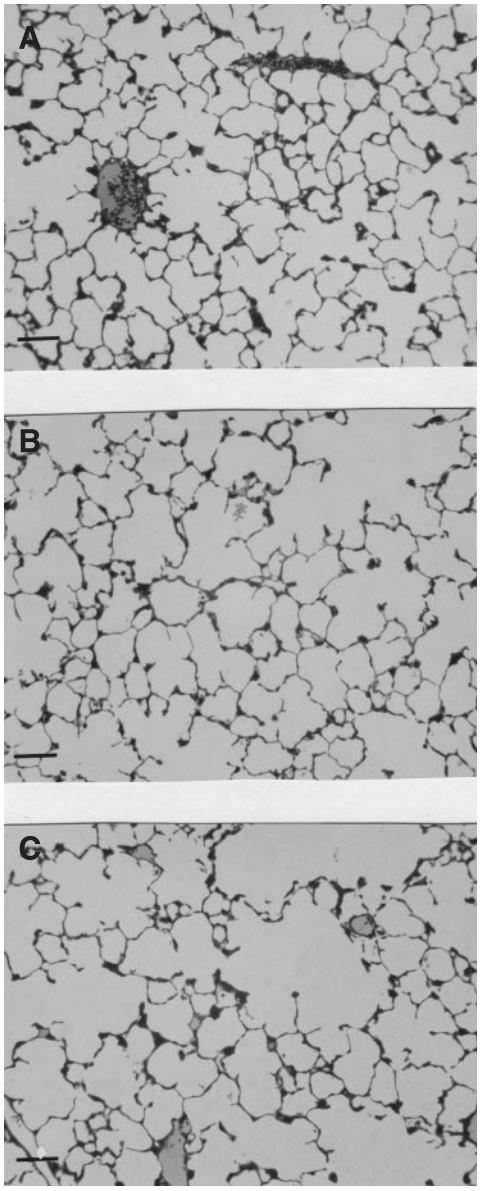

Fig. 3. Histological sections from lungs of adult female wt (*A*), ER-$\alpha^{-/-}$ (*B*), and ER-$\beta^{-/-}$ (*C*) mice. Lungs were fixed as described in Fig. 1. Scale bar = 50 μm.

with wt mice. Body mass-specific alveolar surface area was lower in ER-$\alpha^{-/-}$ mice than in wt or ER-$\beta^{-/-}$ mice (Fig. 2*C*).

Ovariectomy, estrogen replacement, lung, and alveolar dimensions. Total lung volume/body mass was not different among the groups (Fig. 4*A*). The volume density (fraction) of air in the gas-exchange region was lower, and the volume density of gas-exchange tissue was higher, in estrogen-treated ovariectomized mice than in either other group (Fig. 4, *B* and *C*). We do not have a ready explanation for the absence of an effect of ovariectomy plus oil treatment on the volume density of air and tissue in light of the lower volume density air and higher volume density tissue in ovariectomy plus estrogen mice (Fig. 4, *B* and *C*). Three weeks after surgery (not tested sooner), bilateral overiectomized mice had larger alveoli, a lower body mass-specific number of alveoli, and less alveolar surface area than sham-ovariectomized mice (Fig. 5). The loss of alveoli took place without morphological evidence of destructive emphysema-like changes (Fig. 6), consistent with the absence of an effect of ovariectomy on total lung volume. Three weeks of estrogen replacement (not tested sooner), begun 3 wk after ovariectomy, i.e., at a time alveolar loss had occurred, resulted in alveolar regeneration, as indicated by smaller alveoli, a higher body mass-specific number of alveoli, and a greater body mass-specific alveolar surface area in estrogen-treated mice compared with oil-treated mice (Figs. 5 and 6).

DISCUSSION

Sexual dimorphism of alveolar architecture. The lung's only recognized essential function is to provide sufficient gas-exchange surface to meet the organism's requirement for oxygen and elimination of CO_2. This functional need is reflected in the direct linear relationship, across the full range of mammalian body mass, between O_2 consumption and alveolar

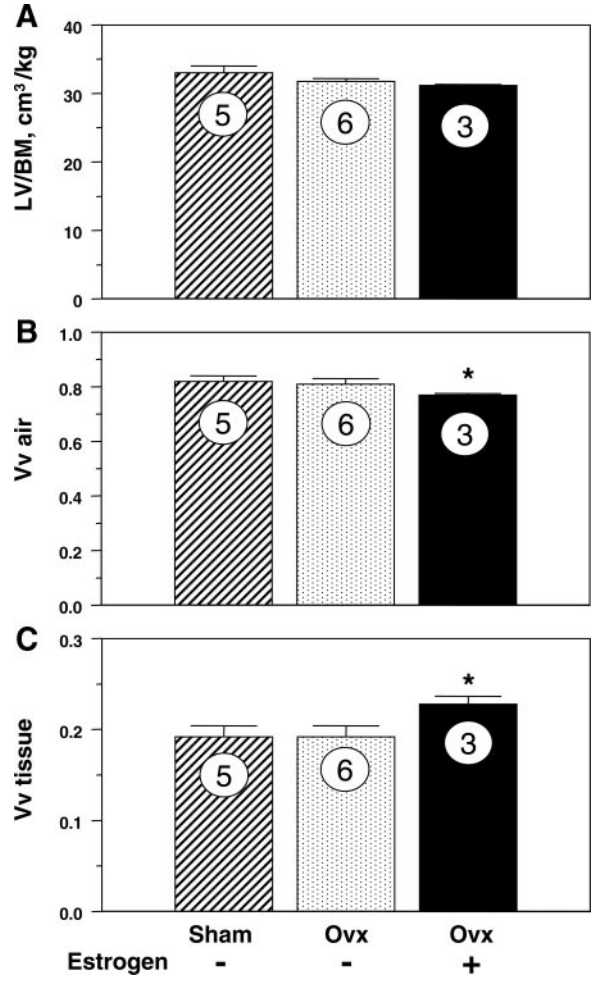

Fig. 4. Lung volume and gas-exchange region volume densities of sham-ovariectomy and ovariectomy mice. Three weeks after bilateral sham, or bilateral ovariectomy, adult mice were injected sc with vehicle or equivolume estradiol (10 μg/kg) daily for 3 wk. Lungs were fixed at a transpulmonary pressure of 20 cmH_2O, and lung volume was measured by volume displacement. Lungs were then processed for morphometric analysis. Means ± SE are given. Nos. within the bars indicate the no. of mice. *A*: lung volume/body mass; *B*, Vv air; *C*, Vv tissue; Ovx, ovariectomy. *$P \leq 0.05$ vs. each other mean.

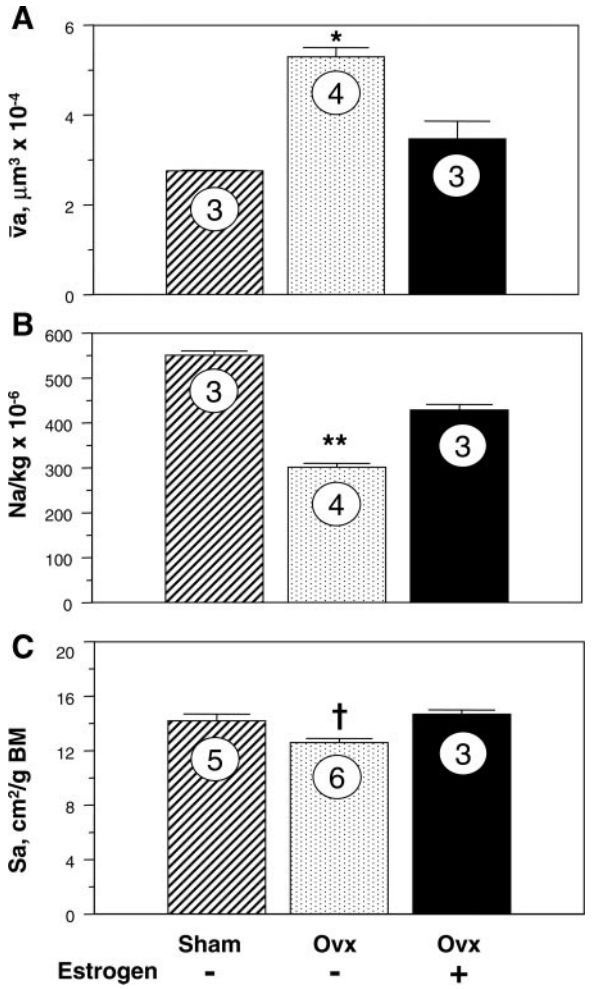

Fig. 5. Alveolar size, number, and surface area. Experiments were performed, and lung tissue was fixed and processed as described in the legend to Fig. 4. Means ± SE are given. Nos. within bars indicate the no. of mice. *A*: $\bar{v}a$; *B*, Na/kg; *C*, *Sa*/g. $^*P \leq 0.004$, $^{**}P \leq 0.0004$, and $\dagger P \leq 0.005$ vs. each other mean.

surface area (30). The match between O_2 need and alveolar surface is met, in spite of the presence of vastly different body mass and hence of different body mass-specific O_2 consumption (higher in small than in large organisms), by greater subdivision of the alveolar surface in small than in large organisms (30). Within species, alveolar body mass-specific surface area increases in response to prolonged increased O_2 need (8).

There is one example, of which we are aware, of alveolar architecture that seems to anticipate a future need for oxygen. Thus, although oxygen consumption almost doubles in rats during pregnancy and lactation, alveolar surface area is the same in age-matched virgin female, pregnant, and lactating rats (16). However, at the onset of sexual maturity, but not before, virgin female rats and mice have a higher body mass-specific number of alveoli and alveolar surface area than same-age virgin males. This sexual dimorphism is present in spite of an identical resting body mass-specific oxygen consumption within the same species in same-age males and females (16). We suggest the greater body mass-specific alveolar surface area in females, in spite of an identical resting body mass-specific oxygen consumption as males, was selected evolutionarily because it allowed females to meet the metabolic demands of reproduction, which in the wild would be almost continuous during most of female adult life, without adding to the energy demands of reproduction, a requirement to form additional lung.

Estrogen modulates the development of alveoli. Rats ovariectomized at age 21 days have, at age 2 mo, larger but fewer alveoli and a lower body mass-specific alveolar surface area than sham-ovariectomized rats (17). These differences are not because of differences in body mass-specific oxygen consumption and are prevented by estrogen replacement (17). Thus, among the hormones in which concentration is changed by ovariectomy, estrogen seems responsible for the impaired formation of alveoli after ovariectomy.

Estrogen receptors and alveolar architecture. The study just cited (17) provided strong evidence that estrogen receptors are present in the alveoli and that they play a role in the formation

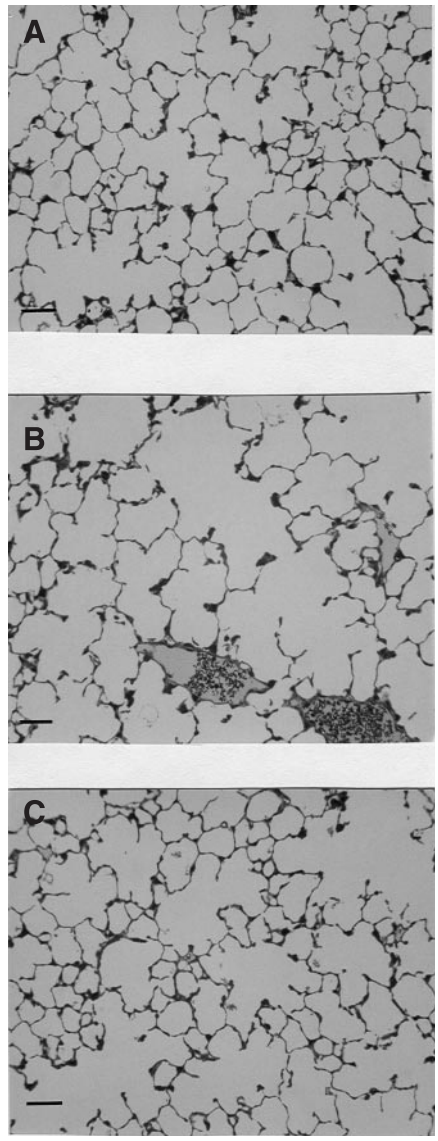

Fig. 6. Histological sections of lung from sham (*A*)-, bilateral ovariectomy (*B*)-, and bilateral ovariectomy plus estradiol (*C*)-treated mice. Scale bar = 50 μm.

of alveoli. Our present work indicates both known mammalian estrogen receptors, ER-α and ER-β, play a seemingly nonredundant role in alveolus formation. ER-$\alpha^{-/-}$ and ER-$\beta^{-/-}$ adult female mice have larger and fewer alveoli than wt female mice. However, ER-$\beta^{-/-}$ mice have diminished lung tissue elastic recoil, as evidenced by a higher body mass-specific lung volume at a pressure of 20 cmH_2O than either wt or ER-$\alpha^{-/-}$ mice. This greater lung volume accounts for the absence of a low alveolar surface area in ER-$\beta^{-/-}$ mice compared with wt mice and contributes to the larger size of alveoli in ER-$\beta^{-/-}$ than are present in wt mice. It also demonstrates the data recently reported by Patrone et al. (25), which claim to show ER-$\beta^{-/-}$ mice have fewer alveoli than wt mice, do not on their own support that claim. The lower lung recoil in ER-β mice with the resultant larger alveoli and the method they used to "count" alveoli would have indicated a lower number of alveoli whether present or not (25). By contrast, the lower body mass-specific number of alveoli in ER-$\beta^{-/-}$ mice in the present study demonstrates these mutants have fewer alveoli, as do ER-$\alpha^{-/-}$ mice, than wt mice. We suggest, based on the decreased elastic tissue recoil of lungs of ER-$\beta^{-/-}$ mice, the absence of ER-β results in a defect in the extracellular matrix that, in turn, leads to diminished formation of alveoli. The diminished formation of alveoli in ER-$\alpha^{-/-}$ mice, in the absence of altered lung elastic recoil, indicates ER-α regulates alveolus formation without an effect on the extracellular matrix that alters lung tissue recoil.

Ovariectomy, estrogen replacement, and alveolar turnover. Our present findings are consistent with the notion that molecules that induce, enhance, or are required for alveolus formation are required for the maintenance of alveoli after they are formed, i.e., ovariectomy in adult mice resulted in loss of alveoli, the absence of estrogen receptors diminished alveolus formation, and estrogen replacement in ovariectomized mice resulted in alveolar regeneration. In the same vein, signaling via retinoic acid receptor-γ is required for alveolus formation (19), and all-*trans* retinoic acid enhances (15) and induces alveolus formation (10, 14). By contrast, deficiency of vitamin A, which acts via its metabolites, in particular all-*trans* retinoic acid (22), results in loss of alveoli (1).

Potential clinical relevance. Chronic obstructive pulmonary disease (COPD) is more prevalent in men than in women. However, there is increasing evidence women are more susceptible than men to the harmful pulmonary effects of cigarette smoke and to the development of COPD, a disease of middle life and later, characterized by all-or-nothing progressive, presently irremediable, alveolar loss. Women age 45–60 yr, with a comparable degree of COPD as same-age men, have reached that state having smoked fewer cigarettes (20). About 85% of aged never smokers with COPD are women (3). Women may be more susceptible than men to the development of severe, early-onset COPD (27), and women die from COPD sooner than men with COPD despite having smoked less (21). After menopause, the age-related loss of gas-exchange function in women increases to rates of loss present in age-matched men (23). Women nonsmokers have more than a twofold higher prevalence of COPD than men (5). Estrogen replacement maintains (4) and improves (24) forced expiratory volume in 1 s in women after menopause. This may reflect an effect on conducting airways. Alternatively, or in addition, it could mean there are more alveolar attachments to conducting airways because of less loss of alveoli, alveolar regeneration, or a combination of these possibilities.

The evidence that estrogen is needed for the formation of alveoli and the presence of estrogen receptors in the lung in the perinatal period (2) raises the possibility insufficient estrogen may play a role in arrested alveologenesis in very prematurely born babies (11, 28). This possibility is strengthened by the present findings that ER-$\alpha^{-/-}$ and ER-$\beta^{-/-}$ mice, which had impaired estrogen signaling during development, have diminished formation of alveoli. The serum concentration of estrogen is 100-fold higher in pregnant than in nonpregnant women, and this high concentration of estrogen is present in the fetus (31). Therefore, the baby's serum concentration of estrogen plummets at birth, and, in prematurely born babies, this fall is developmentally premature, occurring at a time of rapid formation of alveoli. A very small trial provides suggestive evidence that estrogen replacement to very prematurely born baby girls (not reported for boys) results in less chronic lung disease (31–33).

In summary, estrogen signaling is required for alveolus formation for a full complement of alveoli during development (Fig. 2 and Ref. 17), for the maintenance of already formed alveoli (Fig. 5), and can induce alveolar regeneration in adult mice (Fig. 5). In postmenopausal women, estrogen maintains (4) and improves (24) lung function.

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