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## Perinatal nicotine exposure-induced transgenerational asthma: Effects of reexposure in F1 gestation

Jie Liu<sup>1</sup>, Celia Yu<sup>2</sup>, Terence M. Doherty<sup>2</sup>, Omid Akbari<sup>3</sup>, Patrick Allard<sup>4,5</sup>, Virender K. Rehan<sup>1,4,5</sup>

<sup>1</sup>Department of Pediatrics/Lundquist Institute for Biomedical Innovation, Harbor-UCLA Medical Center, Torrance, CA, USA

<sup>2</sup>Lundquist Institute for Biomedical Innovation, Harbor-UCLA Medical Center, Torrance, CA, USA

<sup>3</sup>Department of Molecular Microbiology and Immunology, Keck School of Medicine, USC, Los Angeles, CA, USA

<sup>4</sup>David Geffen School of Medicine, UCLA, Los Angeles, CA, USA

<sup>5</sup>Institute for Society and Genetics, UCLA, Los Angeles, CA, USA

### Abstract

In a rat model, perinatal nicotine exposure results in an epigenetically driven multi-and trans-generationally transmitted asthmatic phenotype that tends to wane over successive generations. However, the effect of repeat nicotine exposure during the F1 (Filial 1) gestational period on the transmitted phenotype is unknown. Using a well-established rat model, we compared lung function, mesenchymal markers of airway reactivity, and global gonadal DNA methylation changes in F2 offspring in a sex-specific manner following perinatal exposure to nicotine in only the F0 gestation, in both F0 and F1 (F0/F1) gestations, and in neither (control group). Both F0 only and F0/F1 exposure groups showed an asthmatic phenotype, an effect that was more pronounced in the F0/F1 exposure group, especially in males. Testicular global DNA methylation increased, while ovarian global DNA methylation decreased in the F0/F1 exposed group. Since the offspring of smokers are more likely to smoke than the offspring of nonsmokers, this sets the stage for more severe asthma if both mother and grandmother had smoked during their pregnancies. Increased gonadal DNA methylation changes following nicotine reexposure in the F1 generation suggests that epigenetic mechanisms might well underlie the transgenerational inheritance of acquired phenotypic traits in general and nicotine-induced asthma in particular.

**Correspondence:** Virender K. Rehan, Department of Pediatrics, Lundquist Institute for Biomedical Innovation, Harbor-UCLA Medical Center, 1124 W. Carson Street, Torrance, CA 90502, USA. vrehan@lundquist.org.

#### AUTHORS CONTRIBUTIONS

V.K. Rehan conceived and designed research. J. Liu and C. Yu conducted experiments. V.K. Rehan, J. Liu, and P. Allard analyzed data. J. Liu, P. Allard, O. Akbari, T.M. Doherty, and V.K. Rehan wrote the manuscript.

#### ETHICS APPROVAL

All animal procedures were approved by the Institutional Animal Care and Use Committees at the Lundquist Institute at Harbor-UCLA Medical Center.

#### CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

## Keywords

cigarette smoke; epigenetics; pregnancy; methylation; multigenerational

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## 1 | INTRODUCTION

Strong epidemiological and experimental evidence links perinatal exposure to cigarette smoke and nicotine to offspring asthma.<sup>1–4</sup> Even exposure to great grandmother smoking during pregnancy, irrespective of maternal smoking status during pregnancy, is linked to offspring asthma.<sup>5–10</sup> This phenomenon, known as the transgenerational effect, indicates that the adverse health impact of smoking is heritable and persistent and has been observed in both laboratory animals and clinically. Though there are only a handful of studies, the evidence suggests that the perinatal smoke/nicotine-induced lung phenotype tends to wane with successive generations that are no longer exposed,<sup>5,10</sup> a feature that is also common to other stimuli whose effects are transgenerationally transmitted.<sup>11–13</sup> It is important to point out that the offspring pulmonary phenotype induced by perinatal nicotine exposure is similar to that induced following cigarette smoke exposure, and is characterized by increased total airway resistance (Rrs) and decreased total lung compliance (Cdyn). This phenotype is also accompanied by a lung molecular profile characteristic of an asthmatic airway, that is, predominant mesenchymal expression of myogenic genes.<sup>1,2,8–10</sup> This phenotype is transmitted to not just the first- and second-generation offspring, but also to naïve third generation offspring, indicating a true transgenerational effect.<sup>9,10</sup> Our first report of this phenomenon in rodents has now been corroborated by several other laboratories.<sup>8,14,15</sup>

The mechanism underlying transgenerational transmission of the nicotine-induced asthma phenotype remains incompletely understood. Since germ cells are the bridge between the generations, it is not surprising that epigenetic alterations in gonads of the nicotine-exposed offspring have been implicated.<sup>8,9</sup> However, the effect of repeat exposure to nicotine during a subsequent generation, for example, filial (F)1 gestation following exposure in F0 gestation on the asthmatic phenotype in F2 generation is unknown. This is clinically relevant, since the offspring of smokers are more likely to smoke than those of nonsmokers.<sup>16–18</sup> Furthermore, this question is important to investigate not only from asthma pathogenetic and epidemiological perspectives, but also from a fundamental biological perspective, since it can potentially provide insight into how acquired traits in general are transmitted to progeny in humans in ways outside those of classical Mendelian inheritance.

We reasoned that a repeat exposure to nicotine in sequential generations would solidify the pathologic effects of the stimulus on the asthma phenotype. We hypothesized that animals exposed to nicotine during development that are *re-exposed* during F1 gestation will display exacerbation of the asthma phenotype in F2 offspring, when compared to F2 animals receiving nicotine exposure only during F0 gestation. To test this hypothesis, we used an established rat model of perinatal nicotine exposure-induced multi- and trans-generational transmission of the asthma phenotype.<sup>9,10</sup> Since the nicotine-induced asthma phenotype in the model studied is sex-specific (more robust in males),<sup>9,10,19</sup> and it is possible that the mode of transgenerational transfer might be mediated via germline-specific mechanisms,<sup>20</sup>

all analyses were performed in a sex-specific manner. *We report that sequential nicotine exposure in a subsequent pregnancy causes a more robust asthma-like phenotype in succeeding generations, especially in males, compared to its exposure in only one pregnancy.* These findings have important consequences for offspring of smokers who are more prone to smoke than the general population is, since if they also smoke during pregnancy, their offspring may be at a significantly greater risk of suffering more severe pulmonary consequences.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

Nicotine hydrogen tartrate was acquired from Sigma-Aldrich (St. Louis, MO). All plasticware and other supplies were purchased from Corning (Corning, NY), Thermo Fisher Scientific (Waltham, MA), and Invitrogen (San Diego, CA).

### 2.2 | The animal model

The perinatal nicotine exposure rat model used herein has been described previously.<sup>9,10,19</sup> Briefly, time-mated, first-time pregnant, pair-fed Sprague-Dawley rat dams (F0, four separate dams for each group) (Charles River Laboratories, Inc) received either placebo (saline diluent) or nicotine (1 mg/kg subcutaneously) in 100  $\mu$ L volumes daily during gestation, from embryonic day 6 to postnatal day (PND) 21. F1 pups, delivered spontaneously at term, were breast fed *ad libitum*. F1 male and female littermates were weaned at PND 21 and maintained in separate cages as breeders to generate F2 offspring. At around PND 60, 4 F1 females and 4 F1 males from separate litters were mated to generate F2 pups for each group. Using the same nicotine administration strategy as utilized in F0 gestation, F1 pregnant dams were classified into three groups: (1) Not exposed to nicotine in either F0 or F1 gestation (control group, C group); (2) exposed to nicotine in only F0 gestation (nicotine exposed, N group); and (3) exposed to nicotine in both F0 and F1 gestations (nicotine reexposure group, NN group). The experiment was repeated at least four times using separate breeders. At PND 21, F2 pups were studied to assess pulmonary function, tracheal contractility, and expression of key mesenchymal airway contractility proteins and the corresponding signaling pathway markers in both lung and tracheal tissue by immunoblotting and immunostaining. In addition, global gonadal (testis and ovary) DNA methylation was determined.

All animal procedures were performed at the Lundquist Institute at Harbor-ULCA Medical Center following the National Institutes of Health guidelines for the care and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committees at the Lundquist Institute (Approval # 30352). In line with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association, animals were euthanized with an excess of pentobarbital (200 mg/kg) administered intraperitoneally followed by cervical dislocation for the pups, and severance of the major artery for the dams due to their bigger size as the secondary method of euthanasia.

### 2.3 | Pulmonary function testing

Pulmonary function studies were performed following previously described methods.<sup>9,10,21</sup> Briefly, the pups were deeply anesthetized with ketamine (70 mg/kg; Bioniche Teoranta, Inverin, Galway, Ireland) and xylazine (7 mg/kg; Akorn, Decatur, IL), tracheostomized, and ventilated using a small animal ventilator (MiniVent, Harvard Apparatus, Cambridge, MA). Next, pups were exposed to increasing concentrations of aerosolized methacholine (0, 1.25, 2.5, 5, 10, and 20 mg/mL) over a period of 3 minutes each, and Rrs and Cdyn were measured and plotted as a function of the methacholine concentration administered.

### 2.4 | Tracheal tension studies

The whole trachea was excised immediately at animal sacrifice and dissected free of connective tissue in ice-cold modified Krebs-Ringer bicarbonate buffer (expressed as mM concentrations: 118.3 NaCl, 4.7 KCl, CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25.0 NaHCO<sub>3</sub>, and 11.1 glucose). An approximately 6 mm tracheal ring was resected from the midsection of each trachea and suspended in an organ chamber containing 10 mL of modified Krebs-Ringer bicarbonate buffer solution maintained at 37 ± 0.5°C and aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4). Each ring was suspended with two stirrups, each passed through the lumen. One stirrup was anchored to the bottom of the organ chamber, and the other stirrup was connected to a strain gauge (model FT03C; Grass Instrument, Quincy, MA) for the measurement of isometric force, as described previously.<sup>9,10,20</sup> For tracheal tension measurements, each tracheal ring was initially stretched to its optimal resting tension, which was achieved by stepwise stretching in 0.1 g increments, until the contractile response to 100 mM KCl reached a plateau. Tracheal rings were allowed to equilibrate for 1 hour, after which the effect of acetylcholine was determined at least 30 minutes after the administration of nitro-L- arginine.

### 2.5 | Western analysis

The isolated lungs and tracheas were flash frozen in liquid nitrogen, then homogenized and sonicated in four volumes of ice cold lysis buffer containing 50 mM of β-glycerophosphate (pH 7.4), 150 mM of NaCl, 1.5 mM of EGTA, 1 mM of EDTA, 1% of Triton X-100, 100 mM of NaF, 2 mM of Na<sub>3</sub>VO<sub>4</sub>, 1 mM of dithiothreitol, 1 mM of phenylmethylsulfonyl fluoride, 1 mM of benzamidine, 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, and 2 μg/mL of pepstatin A. After centrifugation at 13200 *g* for 15 minutes at 4°C, the supernatant was used for Western blot analysis to determine protein levels of fibronectin, α-smooth muscle actin (αSMA), calponin, collagen I & III, nicotinic acetylcholine receptor (nAChR) α3 and α7, β-catenin, lymphoid enhancer-binding factor-1 (LEF-1), and peroxisome proliferator-activated receptor gamma (PPARγ). The total protein concentration of the supernatant was measured by the bicinchoninic acid method, using bovine serum albumin as the protein standard. Aliquots of the supernatant, each containing 30 μg of protein, were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked with Tris-buffered saline (TBS) containing 5% of nonfat dry powdered milk (wt/vol) for 1 hour at room temperature. After a brief rinse with TBS containing 0.1% of Tween 20 (TBST), the protein blots were incubated in 1:250 diluted anti-fibronectin monoclonal antibody (catalog no.

610078, BD Biosciences), 1:10 000 diluted anti- $\alpha$ -SMA monoclonal antibody (catalog no. A2547, Sigma), 1:6000 diluted anti-calponin monoclonal antibody (catalog no. C-2687, Sigma), 1:500 diluted anti-collagen I polyclonal antibody (catalog no. RDIMCOIII1abr, Fitzgerald Industries), 1:1000 diluted anti-collagen III monoclonal antibody (catalog no. C7805, Sigma), 1:400 diluted anti-nicotinic AChR $\alpha$ 3 (catalog no. sc-5590, Santa Cruz Biotechnology), 1:20 000 diluted anti-nicotinic AChR $\alpha$ 7 (catalog no. N8158, Sigma), 1:1000 diluted anti- $\beta$ -catenin (catalog no. sc-7963, Santa Cruz Biotechnology), 1:500 diluted anti-LEF-1 (catalog no. sc-28687, Santa Cruz Biotechnology), 1:1000 diluted anti-PPAR $\gamma$  (catalog no. sc-7196, Santa Cruz Biotechnology), and 1:4000 diluted anti-Glyceraldehyde 3 Phosphate Dehydrogenase (GAPDH) monoclonal antibody (catalog no. MAB374, Millipore) overnight at 4°C. After washing three times with TBST, the blots were incubated in 1:1000 (fibronectin), 1:10 000 ( $\alpha$ -SMA), 1:6000 (calponin), 1:2500 (collagen I), 1:2000 (collagen III), 1:3000 (AChR $\alpha$ 3), 1:20 000 (AChR $\alpha$ 7), 1:2500 ( $\beta$ -catenin, LEF-1, and PPAR $\gamma$ ), 1:4000 (GAPDH) diluted horseradish peroxidase-conjugated anti-mouse, rat or rabbit secondary antibody for 1 hour at room temperature. After three more washes in TBST, the blots were exposed to X-ray film using HyGLO Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, INC., Metuchen, NJ) and developed. The relative densities of the protein bands were determined with ImageJ software (National Institutes of Health, Bethesda, MD. <https://imagej.nih.gov/ij/download.html>) and normalized to the density of GAPDH.

## 2.6 | Immunofluorescence staining

Immunofluorescence staining of  $\alpha$ -SMA, calponin, PPAR- $\gamma$ ,  $\beta$ -catenin, and LEF-1 was performed as previously described<sup>9,10,20</sup> and was merged using ImageJ software (National Institutes of Health, Bethesda, MD). Briefly, trachea and lungs were inflated *in situ* with 4% paraformaldehyde in phosphate buffer at a standard inflation pressure of 5 cm H<sub>2</sub>O and fixed following previously described methods. Five 5  $\mu$ m sections were incubated with mouse monoclonal antibodies against  $\alpha$ -SMA (1:1000 dilution; catalog no. A2547, Sigma), calponin (1:250 dilution; catalog no. C2687, Sigma),  $\beta$ -Catenin (1:50 dilution; catalog no. sc-7963, Santa Cruz Biotechnology), and rabbit polyclonal antibodies against LEF-1 (1:50 dilution; catalog no. sc-28687, Santa Cruz Biotechnology), and PPAR $\gamma$  (1:50 dilution, catalog no. SC-7196, Santa Cruz Biotechnology) at 4°C overnight and then Alexa Fluor 488 goat anti-mouse IgG (1:250 dilution for  $\alpha$ -SMA and Calponin, catalog no. A32723, Invitrogen), Alexa Fluor 594 goat anti-mouse IgG (1:50 dilution for  $\beta$ -Catenin, catalog no. A-11005, Invitrogen), and Alexa Fluor 488 goat anti-rabbit IgG (1:50 dilution for LEF-1, catalog no. A32731, Invitrogen), and Alexa Fluor 594 goat anti-rabbit IgG (1:50 dilution for PPAR $\gamma$ , catalog no. A-11037, Invitrogen) was applied to the sections for 1 hour at room temperature. The sections were washed with phosphate-buffered saline, then mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) for visualization under a fluorescence microscope by a single blinded investigator.

## 2.7 | Global DNA methylation

Genomic DNA from testes and ovaries was isolated using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA, Cat. No.: 51304) according to the manufacturer's instructions and the DNA concentration was determined using a NanoDrop 2000. Global DNA methylation was

quantified using a MethylFlash Methylated DNA Quantification Kit (Epigentek, Cat. No.: P-1034) according to the manufacturer's instructions. The amount and percentage of methylated DNA (5-mC) in the total DNA extract was calculated based on a standard curve generated using a methylated DNA positive control.

## 2.8 | Statistics

Using Excel (Office 365, Excel 2016), data were analyzed from at least four independent sets of experiments ( $n = 4-7$  per group). Levene's test was used to test for homogeneity. This test uses an  $F$  test to test the null hypothesis that the variance is equal across groups. A  $P$  value less than .05 indicates a violation of the assumption, which was not the case for our data set. Analysis of variance for multiple comparisons with Bonferroni *post hoc* correction was used, with the exposure group and sex as independent variables. A  $P$  value  $< .05$  was considered to indicate statistically significant differences among the experimental groups.

## 3 | RESULTS

### 3.1 | Effect of F1 and F0 perinatal nicotine exposures on F2 offspring pulmonary function

On comparing the effect of perinatal nicotine exposure in only F0 gestation and both F0 and F1 gestations *vs.* the control group on overall pulmonary function (N and NN groups *vs.* C group), we found that the Rrs was significantly increased and the Cdyn significantly decreased in both groups ( $P < .05$  *vs.* control group, for both Rrs and Cdyn). However, the effect was more pronounced in the NN group ( $P < .05$  *vs.* N group, Figure 1A,B,D,E). This was true for both males and females. However, on comparing the effect between the sexes, males exhibited significantly more pronounced effects ( $P < .05$  *vs.* females) (Figure 1C,F). The acetylcholine-induced increase in tracheal constriction was also significantly greater in the NN group ( $P < .05$  *vs.* C and N groups), but this effect was evident only in males (Figure 2), which is consistent with our previously published observations.<sup>9,10,19</sup>

### 3.2 | Effect of F1 and F0 perinatal nicotine exposures on F2 offspring markers of airway structure and contractility

We next compared lung mesenchymal tissue protein levels of airway structure and contractility, which are known to be affected by perinatal nicotine exposure. Consistent with the pulmonary functional data, Western analysis for fibronectin,  $\alpha$ SMA, calponin, and collagens 1 and 3 showed increases in the N and NN groups in both males and females, but the effect was significantly higher in NN *vs.* N group ( $P < .01$ ) (Figure 3A,B). nAChRs  $\alpha$ 3 and  $\alpha$ 7, known mediators of nicotine's effects on the developing lung, also showed a similar trend, that is a significant increase in nicotine exposure groups *vs.* the control group. Again, the increase was significantly higher in the NN group ( $P < .01$  *vs.* N group) (Figure 3A,B). The increases in levels of mesenchymal proteins in nicotine-exposed groups by Western analysis were corroborated by immunostaining. For example,  $\alpha$ SMA and calponin immunofluorescence staining of lung tissue from both males and females showed exposure-dependent increases, with a greater increase seen in the NN group (Figure 4). Next, the expression of mesenchymal markers of airway structure and contractility (fibronectin,  $\alpha$ SMA, calponin, collagen 1), and nAChRs  $\alpha$ 3 and  $\alpha$ 7 in trachea were assessed. In line with the tracheal contractility data (Figure 2), the increased levels of these proteins were seen

only in male tracheas of N and NN groups, with a significantly higher effect in the NN group (*vs.* N group,  $P < .01$ ) (Figure 5A). Notably, there was no effect on the levels of these proteins in female tracheas in both N and NN groups (Figure 5B). These Western analyses were again corroborated by immunostaining for  $\alpha$ SMA and calponin proteins in the tracheal tissue (Figure 6).

### 3.3 | Effect of F1 and F0 perinatal nicotine exposures on key signaling pathways that determine the nicotine-induced lung phenotype in F2 lungs

Since Wnt and PPAR $\gamma$  signaling pathways are centrally involved in the development of the perinatal nicotine-induced asthmatic lung phenotype, these were examined next. Lung  $\beta$ -catenin and LEF-1 protein levels increased in exposure-dependent fashion, indicating Wnt activation and an accompanying decrease in PPAR $\gamma$  protein levels in whole lung lysates of both males and females (Figure 7). On probing these markers in tracheas,  $\beta$ -catenin and LEF-1 protein levels increased and PPAR $\gamma$  protein levels decreased in male tracheas, consistent with tracheal contractility data. No changes were seen in female tracheas of nicotine exposed (both N and NN groups) animals (*vs.* controls) (Figure 8). The lung and tracheal tissue Wnt and PPAR $\gamma$  signaling data were also verified with immunostaining, with increases in  $\beta$ -catenin and LEF-1 protein levels and a decrease in PPAR $\gamma$  protein levels occurring only in male tracheas (Figure 9).

### 3.4 | Effects of F1 and F0 perinatal nicotine exposures on global DNA methylation in F2 offspring gonads

Gonadal DNA methylation has been suggested to be involved in the transgenerational transmission of nicotine-induced asthma. Therefore, we next determined the effect of nicotine reexposure during F1 gestation following exposure during F0 gestation on global DNA methylation in the testes and ovaries of F2 males and females. We observed an exposure-dependent effect on global DNA methylation which was increased in testes, but decreased in the ovaries in nicotine exposed gonads. We also found that the effects on the NN *vs.* N groups were more pronounced (Figure 10).

## 4 | DISCUSSION

Nicotine exposure during F0 gestation only as well as during both F0 and F1 gestations resulted in a clearly asthmatic phenotype in the F2 offspring, compared to the control group. *However, the F0 and F1 exposure group demonstrated a significantly more robust asthmatic phenotype, as indicated by the pulmonary function and the lung and tracheal structural and contractility protein data. These changes were more pronounced in males.* Accompanying these changes, the global DNA methylation was significantly greater in testes, but lower in ovaries in the nicotine exposure during both F0 and F1 gestation group *vs.* the nicotine exposure in only F0 gestation group. These data suggest that reexposure during the second pregnancy exacerbated the phenotype acquired from exposure to nicotine in the first pregnancy.

Despite well-publicized risks and preventive efforts, more than 10% of the US women still smoke during pregnancy, resulting in at least 400 000 smoke-exposed infants/year.<sup>22</sup>

Moreover, an even greater number of women and children are exposed to secondhand and thirdhand smoke.<sup>23</sup> This is highly relevant, since exposure to cigarette smoke is the most important preventable determinant of childhood respiratory illnesses. An estimated 20% of healthcare costs (~\$1 billion annually) for childhood respiratory illnesses are directly attributable to maternal smoking.<sup>24</sup> Multiple lines of evidence suggest that most effects of maternal smoking during pregnancy are attributable to nicotine exposure.<sup>2,3,25,26</sup> Despite warnings from the Food and Drug Administration and elsewhere, there has been a recent surge in the use of e-cigarettes (*i.e.*, inhaled nicotine), and due to their perceived safety, the use of e-cigarettes during pregnancy has increased sharply among women of reproductive age.<sup>27,28</sup> Since fetal exposure to nicotine occurs via transplacental transfer, it can be argued that nicotine in maternal circulation might be equally harmful, irrespective of the maternal mode of exposure, that is inhalation, ingestion, transdermal, etc. Our findings therefore might be relevant to both prevention and public policy recommendations regarding nicotine exposure including that via e-cigarettes.

It is generally believed that most DNA methylation changes induced by various environmental exposures are erased at every generation, but different organisms show different degrees of germline reprogramming,<sup>13,29</sup> and there are incontrovertible examples of retention of environmentally induced epigenetic marks across generations.<sup>11,12,30–32</sup> This has been convincingly demonstrated in worms and flies,<sup>12,13</sup> though the evidence in mammals is somewhat sparse. The finding of progressively increased gonadal DNA methylation alterations (NN group > N group > control group) with a progressively enhanced F2 offspring asthma phenotype (*vs.* N and C control groups) not only suggests a causative association, but also suggests that the nicotine-induced germline DNA methylation changes escape germline reprogramming during development, at least at the global level. However, since the DNA methylation studies were performed on whole testes and ovaries and not on isolated germ cells, it is possible that the reported DNA methylation changes also reflect contributions from non-germ cells in gonads. It is also possible that germline DNA methylation alterations are not the underlying mechanism driving nicotine-induced lung phenotypes across generations. For example, it is possible that other epigenetic mechanisms (*e.g.*, histone modifications, small RNAs, prions, hormones, etc.) might lead to similar DNA methylation patterns and pulmonary phenotypes across generations.<sup>33</sup> To attribute environment-induced germline epigenetic change to transgenerational transmission of an acquired phenotype decisively, in addition to proving the retention of the specifically altered epigenetic change (*e.g.*, a locus-specific methylation change), it is also necessary to rule out the transmission of the offending stimulus, that is, nicotine-induced alteration in DNA sequence.

To our knowledge, our data are the first to provide experimental evidence for the enhanced transmission of an acquired characteristic following reexposure to the inciting stimulus in a subsequent generation in a mammalian model. Whether the inherited phenotype will remain stable in subsequent generations remains to be seen. In *Drosophila*, exposure to heat shock or osmotic stress induces a new epigenetic state and specific gene derepression that is transmitted to the subsequent generation. In fact, exposure to heat stress over multiple generations causes the inheritance of defective heterochromatin over multiple successive generations, but it gradually returns to the normal state, suggesting that the new epigenetic



state was unstable.<sup>11</sup> Whether the same phenomenon applies to our model requires testing in the F3 generation and beyond.

In our model, nicotine exposure in both the F0 and F1 pregnancies encompassed most of the pregnancy and lactation periods, hence it is difficult to untangle the precise developmental timing of nicotine exposure to account for the resultant more severe F2 asthmatic phenotype. Though unlikely, it is theoretically possible that the more severe F2 asthmatic phenotype in the NN group would have resulted from nicotine exposure of germ cells *via* breast milk. Studies limiting nicotine exposure exclusively to the pre- or postnatal period are required to help decipher this effect.

Overall, our data clearly show that an additional exposure to the inciting stimulus during development in a subsequent pregnancy provides further strength to the phenotypic alteration induced by the original stimulus during a prior pregnancy, though it remains unclear whether this transmission is accomplished *via* the male or female germline (or both). Further studies are needed to unravel the exact manner and the germline involved in transmission of the nicotine-induced asthmatic phenotype. Such data might also provide a hitherto unrecognized additional explanation for the ever-increasing asthma epidemic. Moreover, the consequences of sex-specificity of the transgenerational inheritance of perinatal nicotine-induced remains unclear; however, we have previously suggested that nicotine's male-specific effect on the developing upper airway provides a selection advantage, since it counters the detrimental effects of male hormones on the developing lung parenchyma.<sup>19</sup> The translational relevance of our study is highlighted by the fact that the dose of nicotine (1 mg/kg body weight) used in our model is approximately equivalent to that used by a moderately heavy (5–9 cigarettes/day) human smoker, that is, the range in habitual smokers being 0.16 to 1.8 mg/kg body weight.<sup>34–37</sup> At this dose, the pulmonary morphologic, structural, molecular, and functional changes seen in our model are similar to those demonstrated in other perinatal nicotine exposure models.<sup>1–3,38,39</sup>

## 5 | CONCLUSIONS

Nicotine reexposure in the F1 generation following its exposure in F0 gestation exacerbates the asthma phenotype seen in the F2 generation offspring. Since the offspring of smokers are more likely to smoke than the offspring of nonsmokers, it likely sets the stage of a more severe asthma phenotype if both mother and grandmother had smoked during their pregnancies. Increased global DNA methylation in the testes, but decreased global DNA methylation in the ovaries following nicotine reexposure in the F1 generation corroborates the interpretation that epigenetic mechanisms might well underlie the transgenerational inheritance of acquired phenotypic traits in general, and nicotine-induced asthma in particular.

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## Abbreviations:

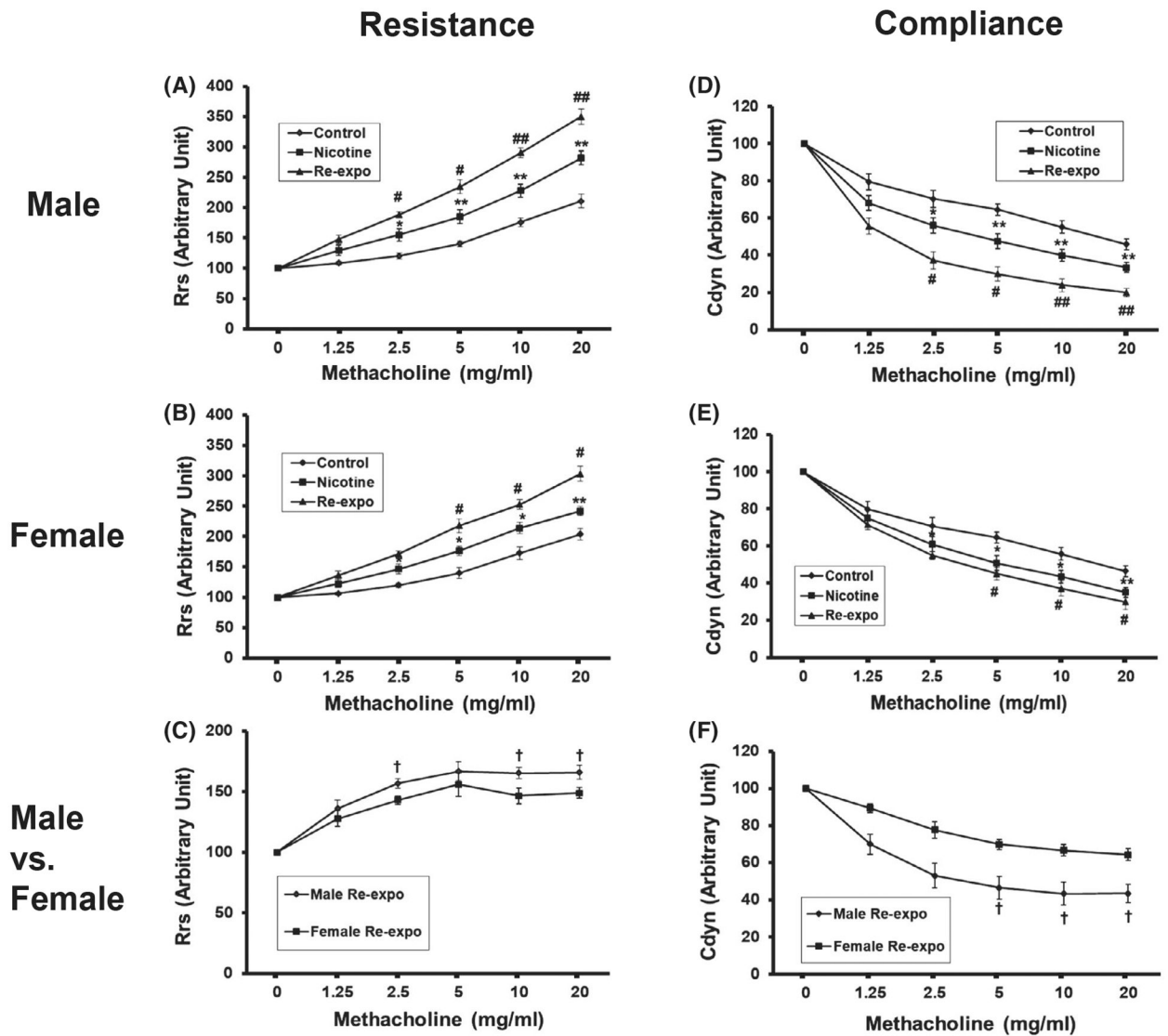
<b>Cdyn</b>	total lung compliance
<b>F0, F1, and F2</b>	filial 0, 1, and 2
<b>GAPDH</b>	glyceraldehyde 3-Phosphate Dehydrogenase
<b>LEF-1</b>	lymphoid enhancer-binding factor-1
<b>nAChR</b>	nicotinic acetylcholine receptor
<b>PAGE</b>	polyacrylamide gel electrophoresis
<b>PND</b>	postnatal day
<b>PPAR<math>\gamma</math></b>	peroxisome proliferator-activated receptor gamma
<b>Rrs</b>	total airway resistance
<b>SDS</b>	sodium dodecyl sulfate
<b>TBS</b>	tris-buffered saline
<b>TBST</b>	tris-buffered saline containing Tween 20
<b><math>\alpha</math>SMA</b>	$\alpha$ -smooth muscle actin

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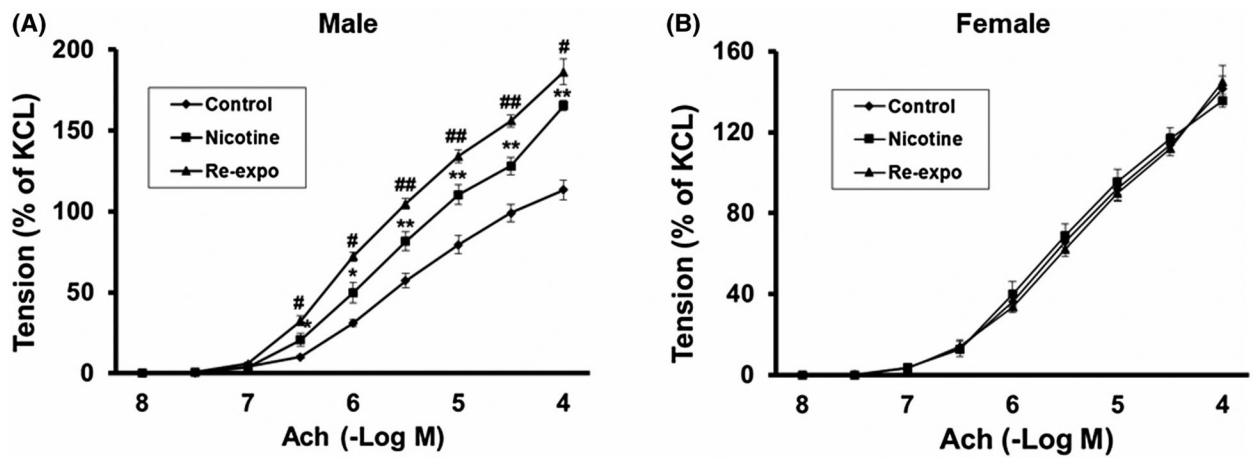
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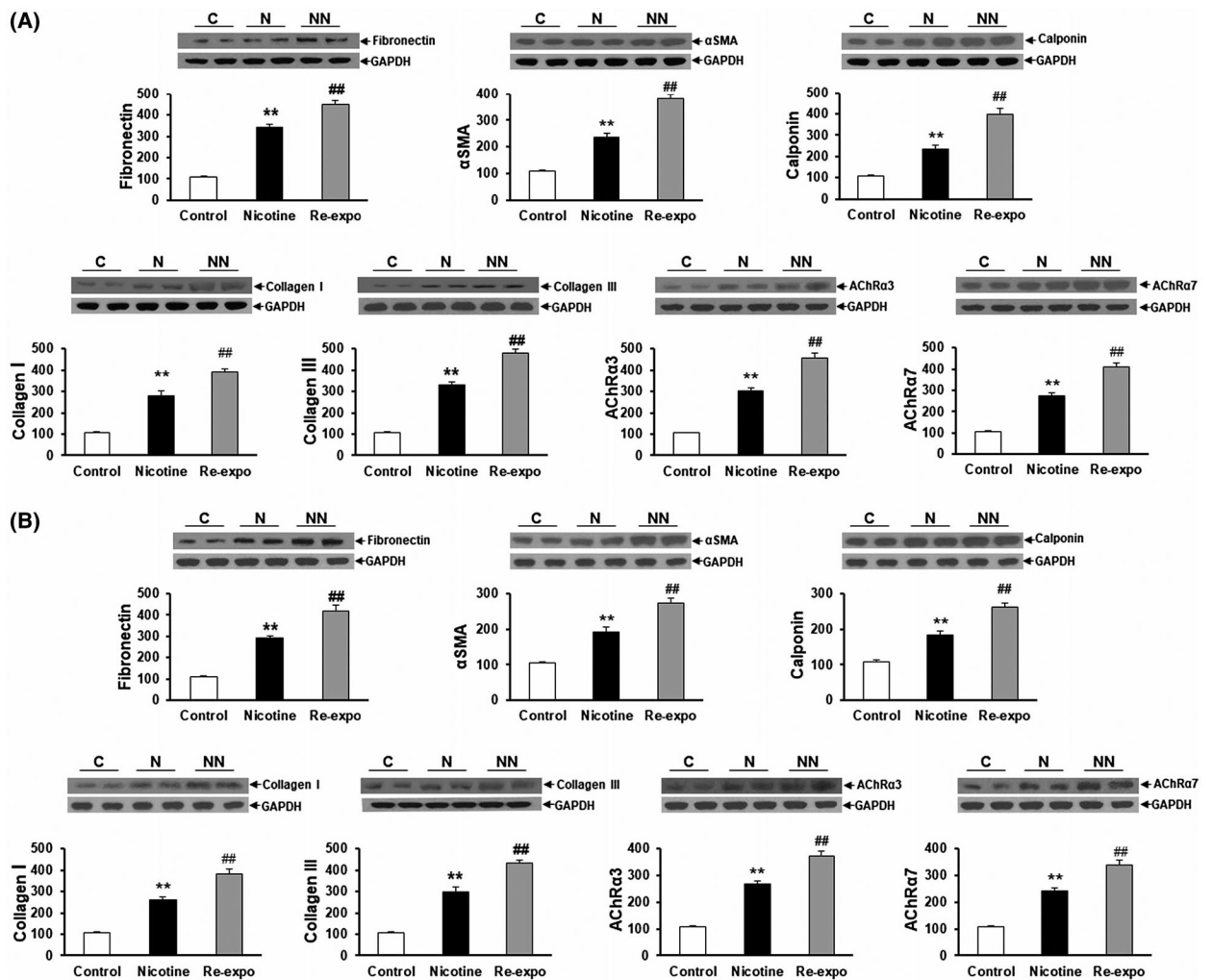
**FIGURE 1.**

Effect of perinatal nicotine reexposure on alterations in total airway resistance (Rrs) and total lung compliance (Cdyn) after methacholine (Mch) challenge in male and female rats. Compared with the nicotine exposure group, in the nicotine reexposure there was a significant increase Rrs and a decrease in Cdyn after Mch challenge in both the males (A and D) and females (B and E). Please note that compared with the female group, with nicotine reexposure there was a significant increase in Rrs (C), and a significant decrease in Cdyn (F) after Mch challenge in male rats. Values are means ( $\pm$  SE) ( $n = 6-7$  for each group). \* $P < .05$ , \*\* $P < .01$  vs. control; # $P < .05$ , ## $P < .01$  vs. nicotine exposure group; † $P < .05$  vs. female group

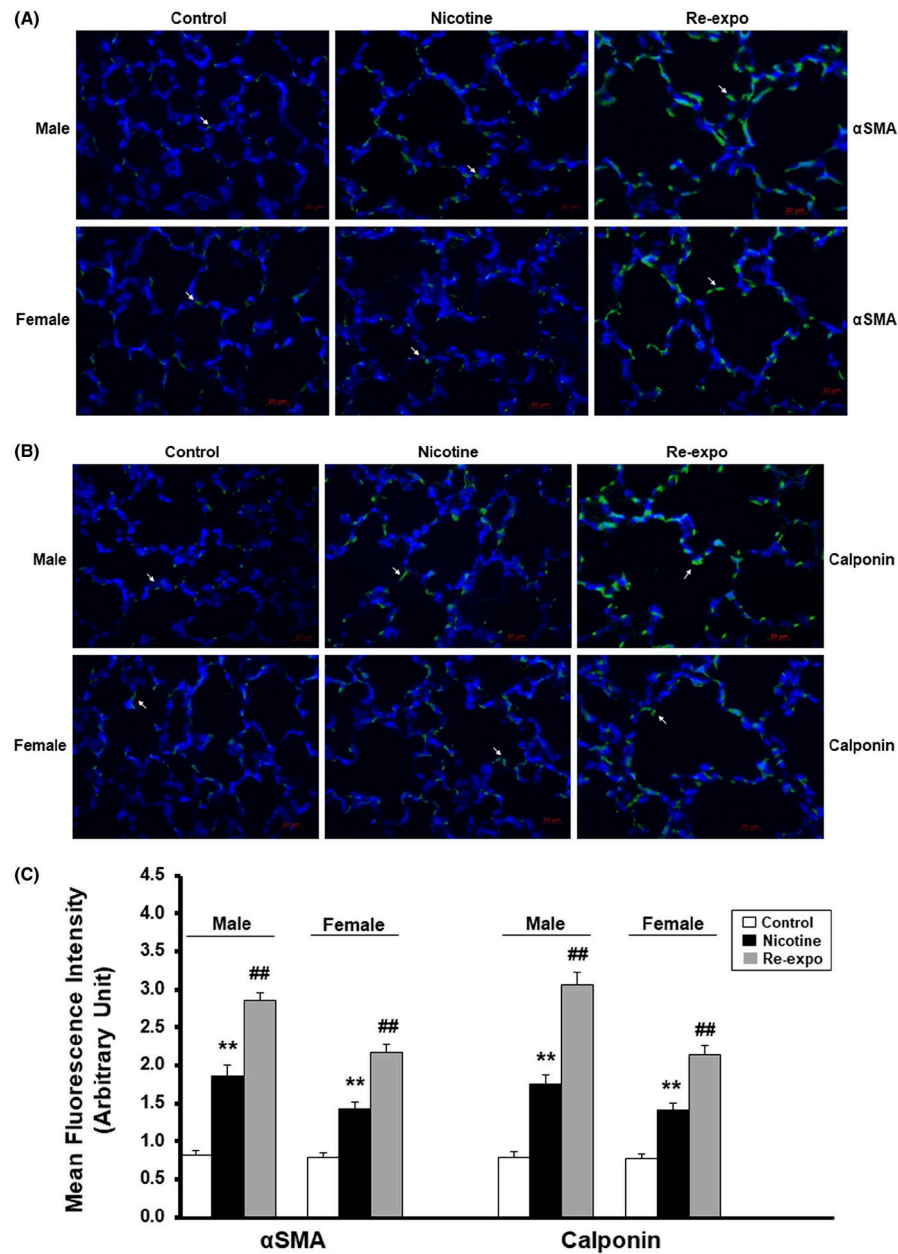


**FIGURE 2.**

Effect of perinatal nicotine reexposure on tracheal constriction response to acetylcholine (Ach) in male and female rats. Compared with the nicotine group, with nicotine reexposure there was a significant increase in the tracheal constriction response to Ach in male rats (A). In contrast, in female rats, there was no increase in the tracheal constriction in response to Ach in the nicotine exposure and reexposure groups when compared to the control group (B). Values are means ( $\pm$  SE) ( $n = 6-7$  for each group).  $*P < .05$ ,  $**P < .01$  vs. control group;  $\#P < .05$ ,  $\#\#P < .01$  vs nicotine exposure group

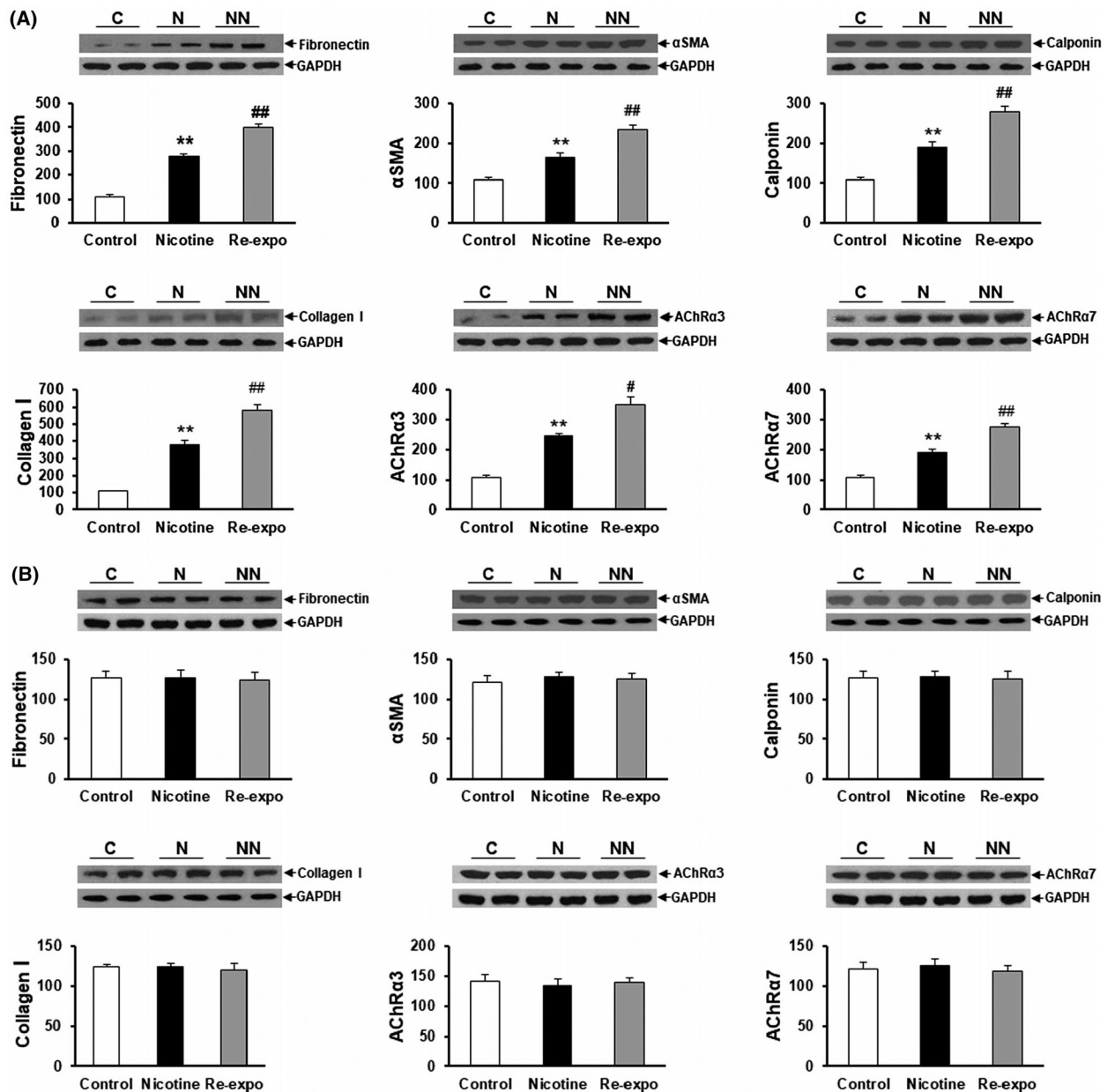
**FIGURE 3.**

Effect of perinatal nicotine reexposure on protein levels of mesenchymal markers of airway structure and contractility in the male (A) and female (B) rat lung. Compared with the nicotine exposure group, in the nicotine reexposure group the protein levels of fibronectin, αSMA, calponin, collagens I and III, and Ach receptors (AChRs) α3 and α7 were increased significantly. Upper panels show representative Western blots for these markers and for GAPDH with C, N, and NN standing for control, nicotine exposure, and nicotine reexposure, respectively. In males, collagen I, αSMA, and calponin share one GAPDH; AChRα3 and AChRα7 share another; and fibronectin and collagen III, yet another (A); the same pattern applies to females (B). Lower panels show the densitometry values of the markers normalized to GAPDH. Values are means ( $\pm$  SE) ( $n = 4$  for each group). \*\* $P < .01$  vs. control group; ## $P < .01$  vs. nicotine exposure group

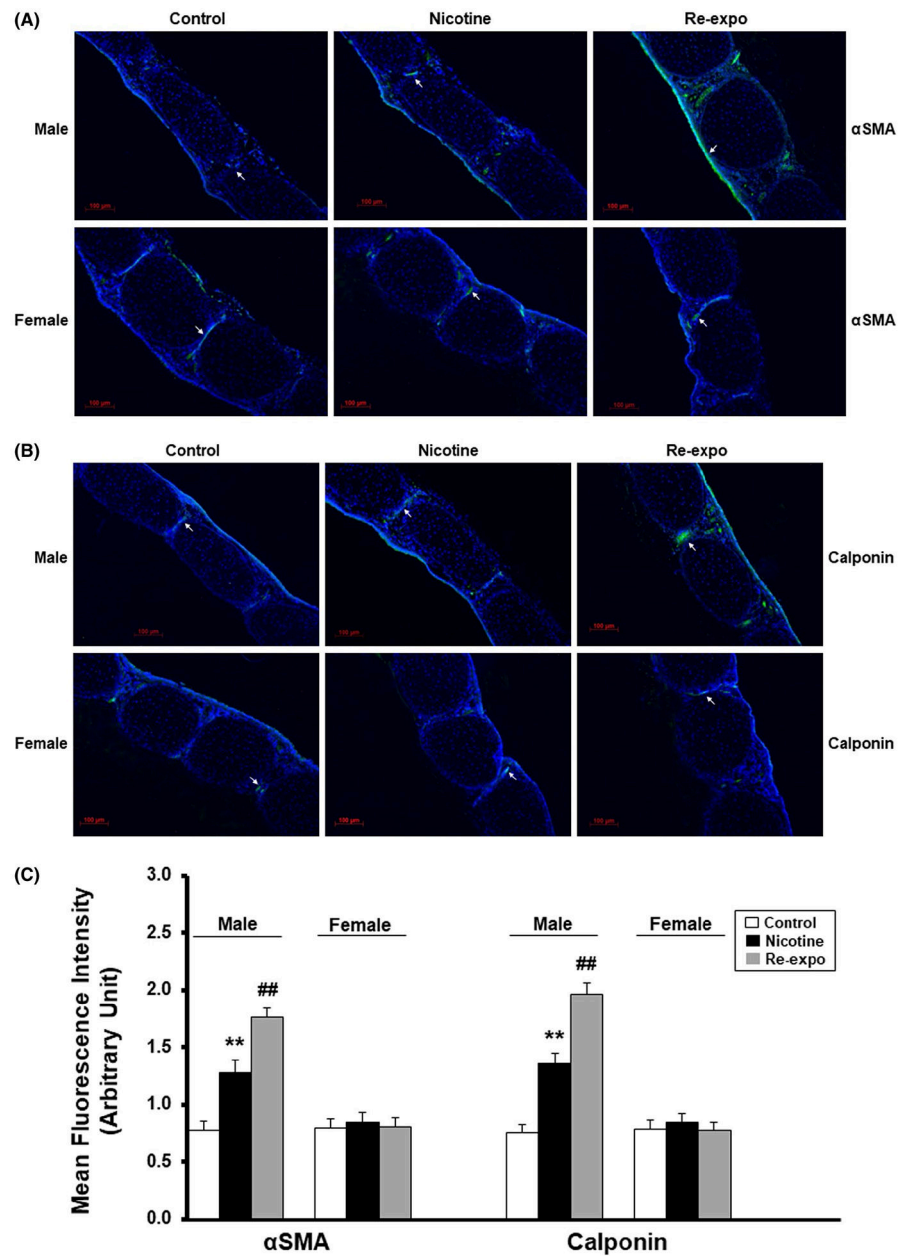


**FIGURE 4.** Effects of perinatal nicotine reexposure on protein levels of mesenchymal markers of airway structure and contractility in paraformaldehyde-fixed, paraffin-embedded male and female rat lung sections from at least four animals in each group. Compared with the nicotine exposure group, in the nicotine reexposure group, the staining for  $\alpha$ SMA (green stain) (A) and calponin (green stain) (B) increased significantly in both male and female lungs. But in the nicotine reexposure group there was more intense staining of these markers in male lungs vs. female lungs. The mean fluorescence intensity measurements of 8–10 images using ImageJ also showed a similar trend (C).

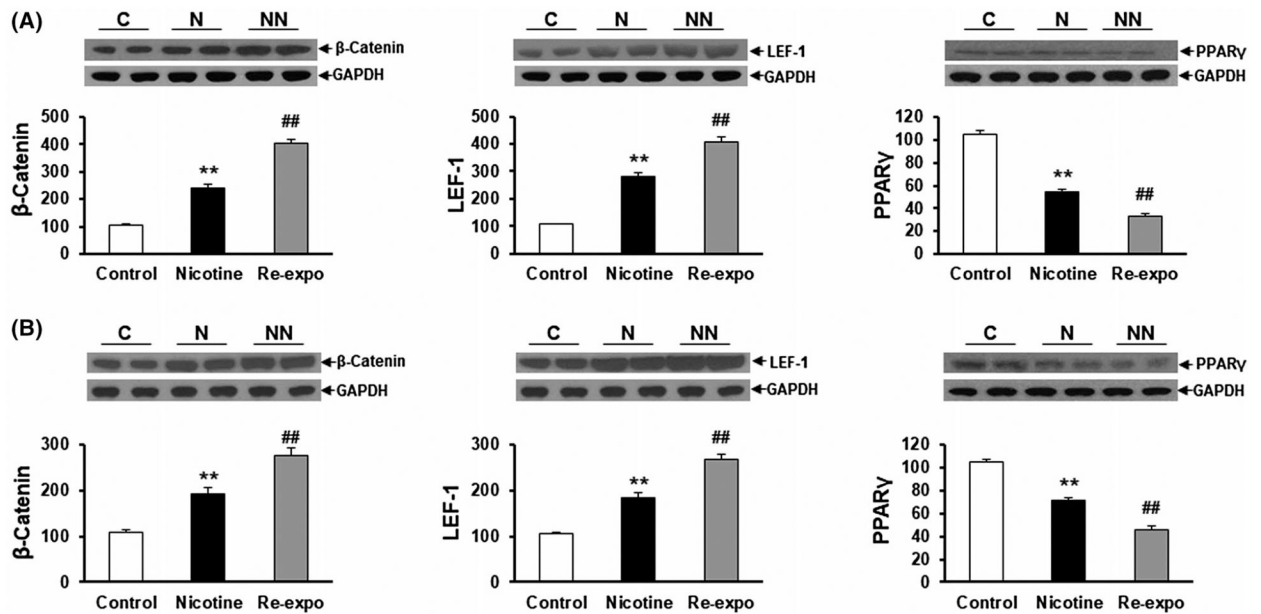


**FIGURE 5.**

Effects of perinatal nicotine reexposure on alterations in mesenchymal markers of airway structure and contractility in the male and female rat trachea. Compared with the nicotine exposure group, in the nicotine reexposure group the protein levels of fibronectin,  $\alpha$ SMA, calponin, collagens I, and Ach receptors (AChRs)  $\alpha$ 3 and  $\alpha$ 7 increased significantly in males (A), but did not change in females (B). Upper panels show representative immunoblots for the specific markers and GAPDH. In males, fibronectin, AChR $\alpha$ 3 and AChR $\alpha$ 7 share one GAPDH (A); the same pattern applies to females (B). Lower panels show the densitometry values of the markers normalized to GAPDH. Values are means ( $\pm$  SE) ( $n = 4$  for each group). \*\* $P < .01$  vs. control group; ## $P < .01$  vs. nicotine exposure group

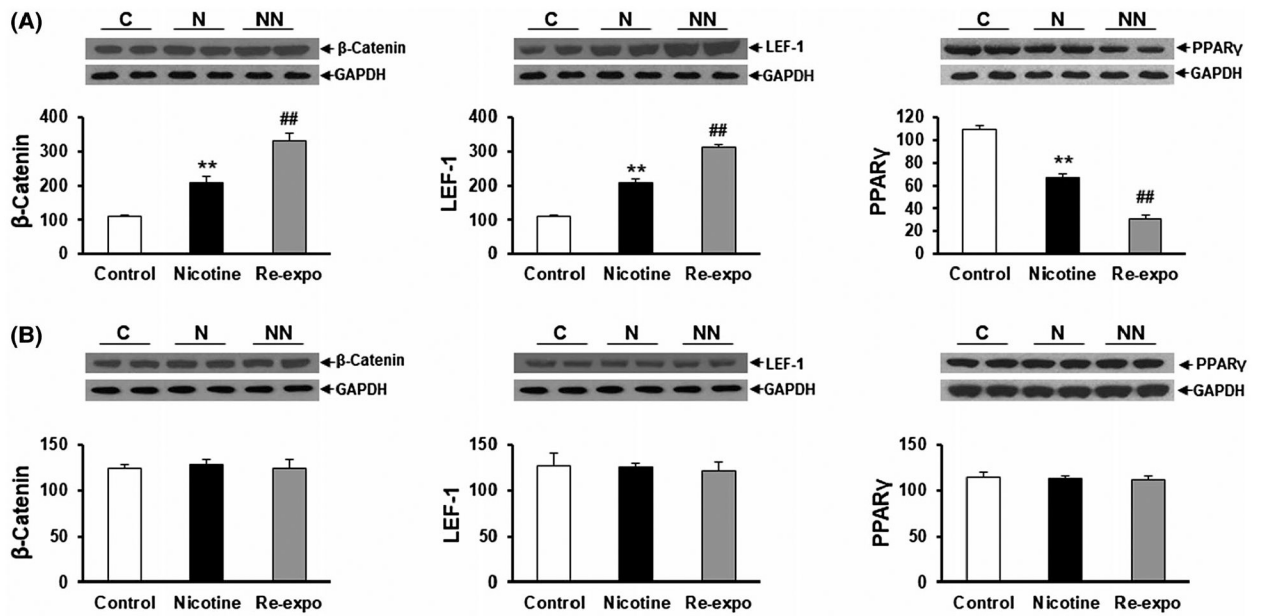


**FIGURE 6.** Effects of perinatal nicotine reexposure on alterations in mesenchymal markers of airway structure and contractility in paraformaldehyde-fixed, paraffin-embedded male and female rat tracheal sections from at least four animals in each group. Compared with the nicotine exposure group, in the nicotine reexposure group, the staining for  $\alpha$ SMA (green stain) (A) and calponin (green stain) (B) increased significantly in males, but did not change in females. The mean fluorescence intensity measurement of 8–10 images using ImageJ also showed a similar trend (C).

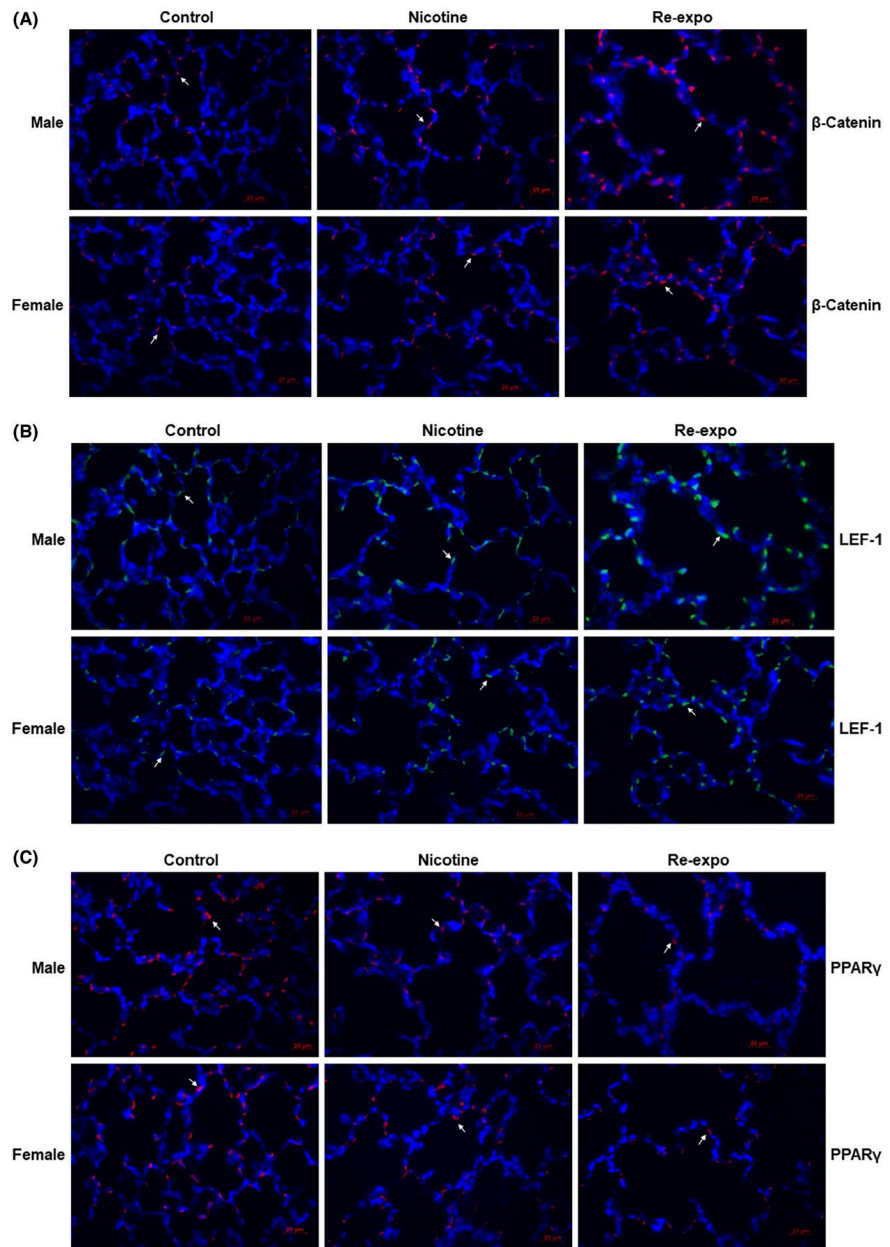


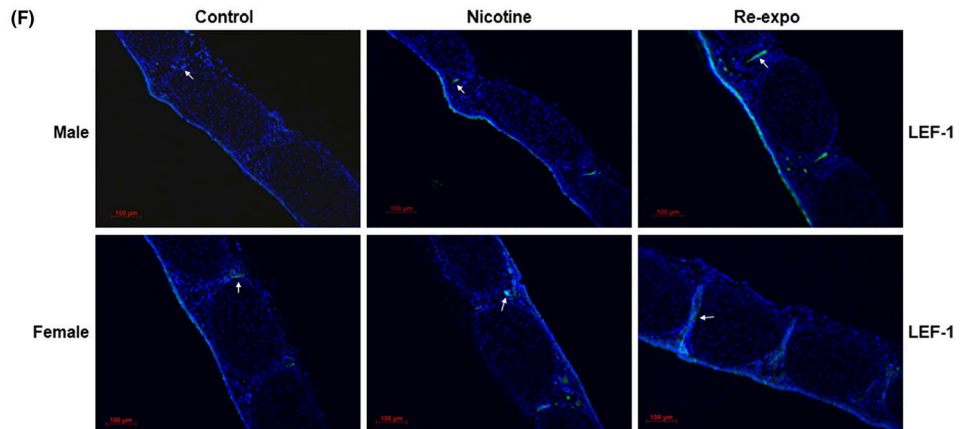
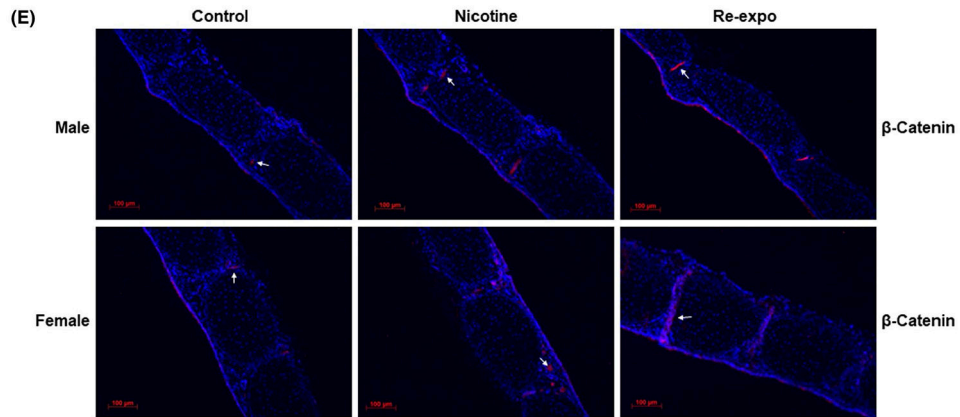
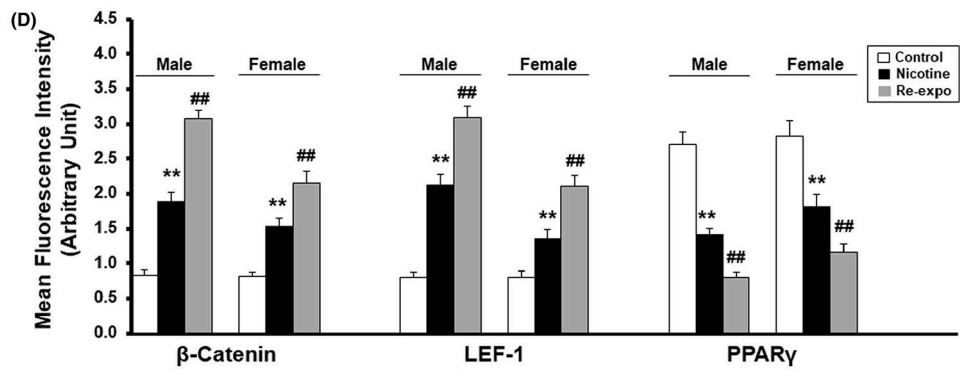
**FIGURE 7.**

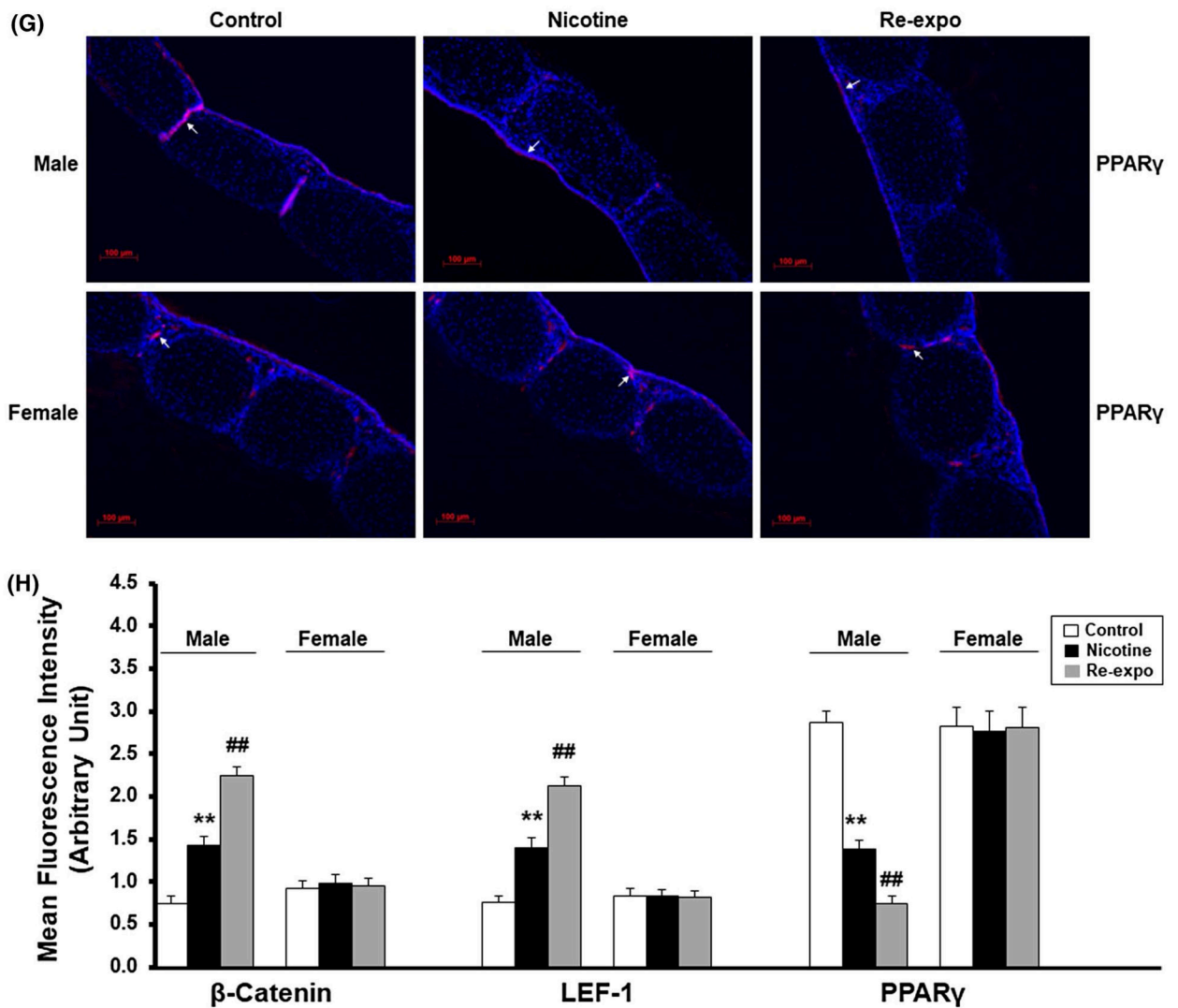
Effects of perinatal nicotine reexposure on alterations in Wnt and PPAR $\gamma$  signaling pathways in the male and female rat lung. Compared with the nicotine exposure group, with nicotine reexposure the protein levels of  $\beta$ -catenin and LEF-1 increased significantly, whereas PPAR $\gamma$  decreased significantly in both male (A) and female (B) lungs. Upper panels show representative Western blots for these markers and for GAPDH. In males,  $\beta$ -catenin and LEF-1 share a GAPDH (A); the same pattern applies to females (B). Lower panels show the densitometry values of the markers normalized to GAPDH. Values are means ( $\pm$  SE) ( $n = 4$  for each group). \*\* $P < 0.01$  vs. control group; ## $P < .01$  vs. nicotine group

**FIGURE 8.**

Effects of perinatal nicotine reexposure on alterations in Wnt and PPAR $\gamma$  signaling pathways in the male and female rat trachea. Compared with the nicotine exposure group, in the nicotine reexposure group, the protein levels of  $\beta$ -catenin and LEF-1 increased, whereas those of PPAR $\gamma$  decreased significantly in males (A), but did not change in females (B). Upper panels show representative immunoblots for the specific markers and GAPDH. Lower panels show the densitometry values of the markers normalized to GAPDH. Values are means ( $\pm$  SE) ( $n = 4$  for each group). \*\* $P < .01$  vs. control group; ## $P < .01$  vs. nicotine exposure group

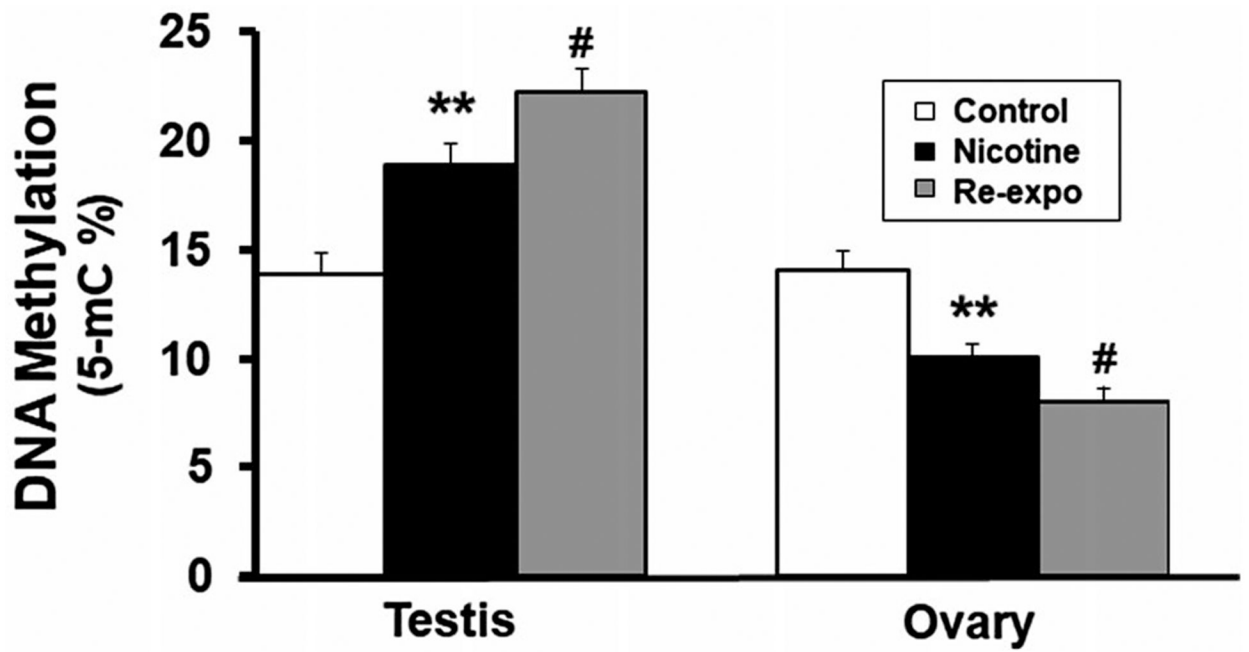






**FIGURE 9.**

Effects of perinatal nicotine reexposure on alterations in Wnt and PPAR $\gamma$  signaling pathways in paraformaldehyde-fixed, paraffin-embedded male and female rat lung, and tracheal sections from at least four animals in each group. Compared with the nicotine exposure group, in the nicotine reexposure group, the staining for  $\beta$ -catenin (A, red stain) and LEF-1 (B, green stain) increased significantly, whereas that for PPAR $\gamma$  (C, red stain) decreased significantly in both male and female lungs. Compared with the nicotine exposure group, with nicotine reexposure the staining for  $\beta$ -catenin (E, red stain) and LEF-1 (F, green stain) increased, whereas PPAR $\gamma$  (G, red stain) decreased significantly in male tracheas, but did not change in female tracheas. The mean fluorescence intensity measurement of 8–10 images using ImageJ also showed a similar trend (D and H).



**FIGURE 10.**

Effects of perinatal nicotine reexposure on global DNA methylation in rat offspring gonads. Compared to the nicotine exposure group, in the nicotine reexposure group, the level of global DNA methylation increased in testes, and decreased in ovaries. Values are means  $\pm$  SE (n = 6 for each group). \*\* $P < .01$  vs control; # $P < .05$  vs nicotine exposure group