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RESEARCH ARTICLE | *Electronic Cigarettes: Not All Good News?*

In utero exposures to electronic-cigarette aerosols impair the *Wnt* signaling during mouse lung development

Alexandra Noël,[1] Shannon Hansen,[1] Anusha Zaman,[1] Zakia Perveen,[1] Rakeysha Pinkston,[1,2] Ekhtear Hossain,[1] Rui Xiao,[3] and Arthur Penn[1]

[1]*Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana;* [2]*Health Research Center, Department of Environmental Toxicology, Southern University and A&M College, Baton Rouge, Louisiana; and* [3]*Department of Anesthesiology, Columbia University Medical Center, New York, New York*

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Noël A, Hansen S, Zaman A, Perveen Z, Pinkston R, Hossain E, Xiao R, Penn A. In utero exposures to electronic-cigarette aerosols impair the *Wnt* signaling during mouse lung development. *Am J Physiol Lung Cell Mol Physiol* 318: L705–L722, 2020. First published February 21, 2020; doi:10.1152/ajplung.00408.2019.—Currently, more than 9 million American adults, including women of childbearing age, use electronic-cigarettes (e-cigs). Further, the prevalence of maternal vaping now approaching 10% is similar to that of maternal smoking. Little, however, is known about the effects of fetal exposures to nicotine-rich e-cig aerosols on lung development. In this study, we assessed whether in utero exposures to e-cig aerosols compromised lung development in mice. A third-generation e-cig device was used to expose pregnant BALB/c mice by inhalation to 36 mg/mL of nicotine cinnamon-flavored e-cig aerosols for 14–31 days. This included exposures for either 12 days before mating plus during gestation (preconception groups) or only during gestation (prenatal groups). Respective control mice were exposed to filtered air. Subgroups of offspring were euthanized at birth or at 4 wk of age. Compared with respective air-exposed controls, both preconception and prenatal exposures to e-cig aerosols significantly decreased the offspring birth weight and body length. In the preconception group, 7 inflammation-related genes were downregulated, including 4 genes common to both dams and fetuses, denoting an e-cig immunosuppressive effect. Lung morphometry assessments of preconception e-cig-exposed offspring showed a significantly increased tissue fraction at birth. This result was supported by the downregulation of 75 lung genes involved in the *Wnt* signaling, which is essential to lung organogenesis. Thus, our data indicate that maternal vaping impairs pregnancy outcomes, alters fetal lung structure, and dysregulates the *Wnt* signaling. This study provides experimental evidence for future regulations of e-cig products for pregnant women and developmentally vulnerable populations.

electronic-cigarettes; in utero exposures; lung development; maternal vaping; pregnancy

INTRODUCTION

Commercially available electronic-cigarettes (e-cigs) were first introduced in China in 2003 (105). Ever since, the e-cig market has been growing exponentially and is projected to exceed $60 billion globally by 2025, surpassing sales of conventional cigarettes (91). In addition to recreational use, e-cigs serve as a replacement vehicle for inhaled delivery of nicotine and are considered by some as a smoking cessation aid device, although this designation has not been approved by the US Food and Drug Administration (FDA). Battery-operated e-cig devices do not combust tobacco; rather, they use heat to produce an inhalable aerosol from a liquid mixture of nicotine, flavoring chemicals, and humectants, including propylene glycol (PG) and vegetable glycerin (VG) (11). Despite the paucity of supporting evidence, e-cigs are being advertised and are perceived by the general public, including pregnant women, as a safer alternative to combustion tobacco products. Notwithstanding the fact that e-cigs have been studied for barely a decade, direct support of their safety (or toxicity) relating to human health is still lacking (106). However, this is starting to change, as it was recently reported that there were over 350 cases of vaping-associated pulmonary illness (VAPI), with a few cases leading to death, in the US (14). This emphasizes the critical and urgent need for research in tobacco regulatory science.

Although cigarettes and e-cigs both deliver nicotine, qualitative and quantitative differences exist between aerosols produced from e-cig devices and those produced by conventional tobacco combustion cigarettes. The latter contains about 10–15 mg of nicotine per cigarette, while e-liquids are commercially available with nicotine concentrations ranging from 0 to 36 mg/mL (17). In addition, e-liquids, according to the FDA, are composed of a mixture of "generally regarded as safe" (GRAS) food additives, including flavoring chemicals, as well as PG and VG. Heating the e-liquid via an e-cig device produces an e-cig aerosol, which is a complex mixture of fine and ultrafine particles, as well as gases that contain at least 30 different reactive chemicals, including tobacco-specific nitrosamines, metals, and volatile organic compounds and carcinogenic polycyclic aromatic hydrocarbons and aldehydes (16, 96). While GRAS items can be considered safe when ingested, their safety following heating, aerosolization, and subsequent inhalation has not been established.

In the United States, more than 10% of pregnant women smoke during their pregnancy, and the rate of maternal vaping is estimated to be similar (105). While the effects of maternal smoking on fetal development are well documented, it is unclear whether maternal vaping will cause similar effects to the developing fetus. According to a recent published survey, the prevalence of e-cig use is 29% among pregnant tobacco users (53). To date, however, there are very few clinical or

Address for correspondence: A. Noël, Dept. of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Skip Bertman Dr., Baton Rouge, LA 70803.

epidemiological studies that have directly investigated the contribution of e-cig aerosol exposures to pregnancy outcomes and fetal lung development. We only found one cohort study that reported a relative risk of 3.1 for having a small-for-gestational-age newborn when pregnant women used only electronic nicotine delivery systems (ENDS) during pregnancy compared with unexposed pregnant women (13). Little is known about the effects of prenatal e-cig exposures on subsequent lung health. There has been only a very limited number of experimental studies in rodents (68, 85, 89). It was previously demonstrated that in utero or early-life exposures to e-cig aerosol alter central nervous system development (54), impair cognitive behaviors (75), cause vascular dysfunction (81), and lead to decreased lung and body weight (70) in the offspring. Specifically regarding the lungs, in utero exposures to nicotine in rats induced epigenetic alterations, evidenced by histone acetylation in the lung tissue, that resulted in an asthmatic phenotype in the offspring (89). Recently, another study demonstrated that in utero exposures to e-cig aerosols caused global DNA methylation in mouse offspring (15). These two studies suggest that in utero nicotine and e-cig aerosol exposures can induce epigenetic modifications, potentially leading to increased risk of developing pulmonary diseases. Another study showed that early-life postnatal exposure to e-cig aerosol for 10 days reduced body weight and decreased postnatal lung growth in mice (70). Taken together, these studies suggest that in utero exposures to nicotine or e-cig aerosols alter or delay normal fetal lung organogenesis and pose, possibly via epigenetic mechanisms, a risk for the development of chronic adult lung diseases, including asthma. Despite these studies, the mechanisms by which e-cig aerosols impair pregnancy outcomes and subsequently induce cellular, molecular, and epigenetic changes on the developing lung are poorly understood.

The Barker hypothesis (the developmental origins of health and disease) suggests that risk factors for the intrauterine environment can affect fetal development during sensitive critical periods, leading to an increase in the risk of developing specific diseases later in life (3). Lung organogenesis occurs during gestation, and this time represents a sensitive window of lung vulnerability to environmental stressors. Studies from our laboratory and others have shown that in utero secondhand smoke (SHS) exposure causes postnatal declines in lung function and predisposes to adult lung diseases, including emphysema, as well as causes exacerbated responses to ovalbumin-induced asthma (77, 112). Nicotine, plus the inflammation and oxidative stress induced by the chemicals present in cigarette smoke (CS) and SHS, appears to be responsible for these in utero effects (58, 66). In particular, nicotine is thought to play a crucial role in these altered lung development processes because it crosses the human placenta and accumulates in the fetal respiratory tract (42, 88). Nicotine is a well-established agonist for the nicotine acetylcholine receptor (nAChR) in humans, and nAChRs are present in the fetal lungs (107). Thus, fetal exposure to nicotine, activating nAChRs and its associated molecular and cellular responses early on in the underdeveloped lung, can lead to alterations or delays in development. Experimental studies involving in utero exposures to nicotine showed a reduction in the number of bronchial-alveolar attachment points, resulting in reduced alveolarization that in turn decreases the lung surface area and, consequently, affects lung function (116). Although e-cigs are devices for inhalable delivery of nicotine, e-cig aerosol composition is different from that of CS or SHS, as it includes heated and aerosolized flavorings and other chemicals in addition to nicotine. Thus, a clear need exists for direct evaluation of the effects induced by e-cig aerosol exposures on pregnancy outcomes and on neonatal lung development.

Lung developmental pathways include *Wnt*, *Shh*, *Fgf*, *Egf*, *Notch*, and *Tgf-β/Bmp* (22, 85). These pathways are active under specific and complex evolving spatiotemporal patterns that orchestrate lung organogenesis through four distinct anatomical stages: pseudoglandular, canalicular, saccular, and alveolar (22, 85). The Wingless/integrated (*Wnt*)/β-catenin signaling plays a key role in lung development because it is involved in branching morphogenesis, including distal branching of the lung, and differentiation of lung cell types (22, 85). For instance, *Wnt5a* gene knockout (KO) mice have smaller lungs compared with their wild-type counterparts, and alveolar development in those mice is delayed (57). Additionally, the Sonic hedgehog (*Shh*) signaling dose-dependently controls the morphogenesis of the lung and is critical for primary branching morphogenesis (22, 44). *Shh*-deficient mice exhibit lung hypoplasia (59, 73, 83). In mice, canonical *Wnt* signaling is active during the four stages of lung organogenesis, while starting at *embryonic day 10*, *Shh* is expressed in the lung epithelium (73, 80). These data clearly demonstrate that these pathways are critical for fetal lung development and that dysregulation of the *Wnt* and *Shh* pathways during lung organogenesis by an environmental stressor, including e-cig aerosol, may potentially cause physiological alterations in the respiratory tract (85).

While adverse effects on fetal lung development induced by nicotine, CS, and SHS are well documented, it is imperative to better identify and understand the specific toxicity of e-cig aerosols to provide scientific evidence to better inform healthcare providers caring for pregnant women. In this study, we assessed whether in utero exposures to e-cig aerosols compromised lung development in mice. Using our well-established in utero exposure mouse model (77, 113), pregnant mice were exposed by inhalation to cinnamon-flavored e-cig aerosols containing 36 mg/mL of nicotine for either 12 days before mating and during gestational *days 1–19* or only from gestational *days 6–19*. The aims of the present study were to determine pregnancy outcomes and assess fetal and neonatal lung development in the offspring at birth and at 4 wk of age, with a focus on genes that are essential for appropriate embryonic lung growth, including those related to *Wnt* and *Shh* pathways.

METHODS

E-Cig Aerosol Exposures and Animal Protocols

We used an e-liquid with a nicotine concentration of 36 mg/mL to replicate the nicotine intake of heavy smokers (>1 pack of cigarettes/day) and that of JUUL-type device users. JUUL appeared on the US market only in 2015 and is currently the most popular ENDS device on the US market, capturing 75% of ENDS sales (45). Each JUUL pod contains nicotine salt in concentration of 36 mg/mL or 59 mg/mL, the latter being equivalent to the nicotine content in one pack of cigarettes. Although the e-liquid nicotine concentration of 36 mg/mL used in this study appears to be high, *1*) it represents the highest concentration available on the US market; *2*) it reflects the nicotine intake of heavy smokers, and *3*) it is a lower concentration than that

contained in high-strength JUUL pods, which are currently the most popular ENDS in the US (45). We chose a cinnamon-flavored e-liquid because cinnamaldehyde has been identified as a high-priority flavoring chemical for evaluation of respiratory hazard (28). Cinnamaldehyde is highly cytotoxic, both in vitro and in vivo (2, 5, 7, 18, 33). In addition to its presence in cinnamon-flavored e-liquids, it was detected in 44% of 27 non-cinnamon-labeled e-liquids tested (7). Therefore, in this study we selected to use a cinnamon-flavored e-liquid to evaluate its lung developmental effects following in utero exposures since *1*) this flavor has been shown to be toxic to the respiratory tract, and *2*) there is a high presence of cinnamaldehyde in both cinnamon-labeled and non-cinnamon-labeled e-liquids.

E-cig aerosol and high-efficiency particulate air (HEPA)-filtered air exposures were conducted as described by Noël et al. (76), in 5-L whole body exposure chambers. An e-liquid composed of 36 mg/mL of nicotine, cinnamon flavor, and a 50:50 PG-to-VG ratio (EC Blend) was aerosolized by a SCIREQ third-generation e-cig generator with the atomizer's resistance and battery voltage set at 1.5 Ω and 4.2 V, respectively. Vaping was conducted under a topography profile of 3-s puff duration and a 55-mL puff volume every 30 s. This vaping topography profile is representative of e-cig users' behaviors and produces total particulate matter (TPM) levels similar to what users inhale (6, 26, 27, 34, 43). Indeed, it was reported in a vaping topography study that 235 was the maximum number of puffs per day taken by e-cig users (20, 60). This is very similar to our exposure profile producing a total of 240 puffs per day. Test atmosphere samples were collected for TPM gravimetric analysis, at a flow rate of 1 L/min throughout the experiment, in a cassette holding a 25-mm hydrophilic glass fiber filter with a 0.7-μm pore size (Millipore, AP4002500). The TPM concentration was monitored continuously in real time via a MicroDustPro (Casella). The average TPM level in the e-cig aerosol exposure chamber was 0.23 mg/puff ± 0.05 (standard error of the mean).

The experimental study design is presented in Fig. 1, *A* and *B*. The preconception exposure groups were composed of adult BALB/c male and female mice (Harlan, Indianapolis, IN; 12 and 10 wk of age, respectively) that were exposed to either e-cig aerosol or HEPA-filtered air for 2 h/day for 12 days before mating (2:1 female-to-male ratio) in addition to 2 h/day for the female mice alone from gestational *days 1–19*. The preconception group females were mated with exposure-matched males. The prenatal exposure groups were composed of pregnant BALB/c mice (10 wk of age) exposed from gestational *days 6–19* for 2 h/day to either e-cig or HEPA-filtered air. Prenatal females were mated with nonexposed males (2:1 female-to-male ratio). We chose to expose groups of pregnant female mice from *days 6–19* of gestation to ensure exposure during the critical and sensitive window of lung organogenesis, which starts at embryonic day 8.5 with the initiation of the pseudoglandular stage (74, 108). This in utero exposure model is used routinely in our laboratory (77, 112, 113). In utero exposures beginning 2 days before the onset of lung development allow for evaluating how e-cig aerosol exposures affect lung organogenesis. At the start of each set of exposures (preconception and prenatal), there were 11–12 female mice per group. The pregnancy rates, number of live offspring per group, and number of offspring per dam are shown in Fig. 1, *C* and *D*.

All the offspring from the preconception groups were euthanized at birth (*postnatal day 0*, PND0), whereas the offspring from the prenatal groups were euthanized either at birth (PND0) or at *postnatal day 28* (PND28) (4 wk of age). At birth, offspring were euthanized by an intraperitoneal injection of Beuthanasia-D (Schering-Plough, NJ) followed by cervical dislocation. The offspring were weighed and measured crown to rump. The number of live and stillborn offspring were counted and recorded. Afterward, blood was collected, and the lungs were excised. Lungs were either placed in RNA*later* for RNA extraction or on dry ice for protein expression or fixed with 10% formalin for histopathological evaluation. Dams were euthanized

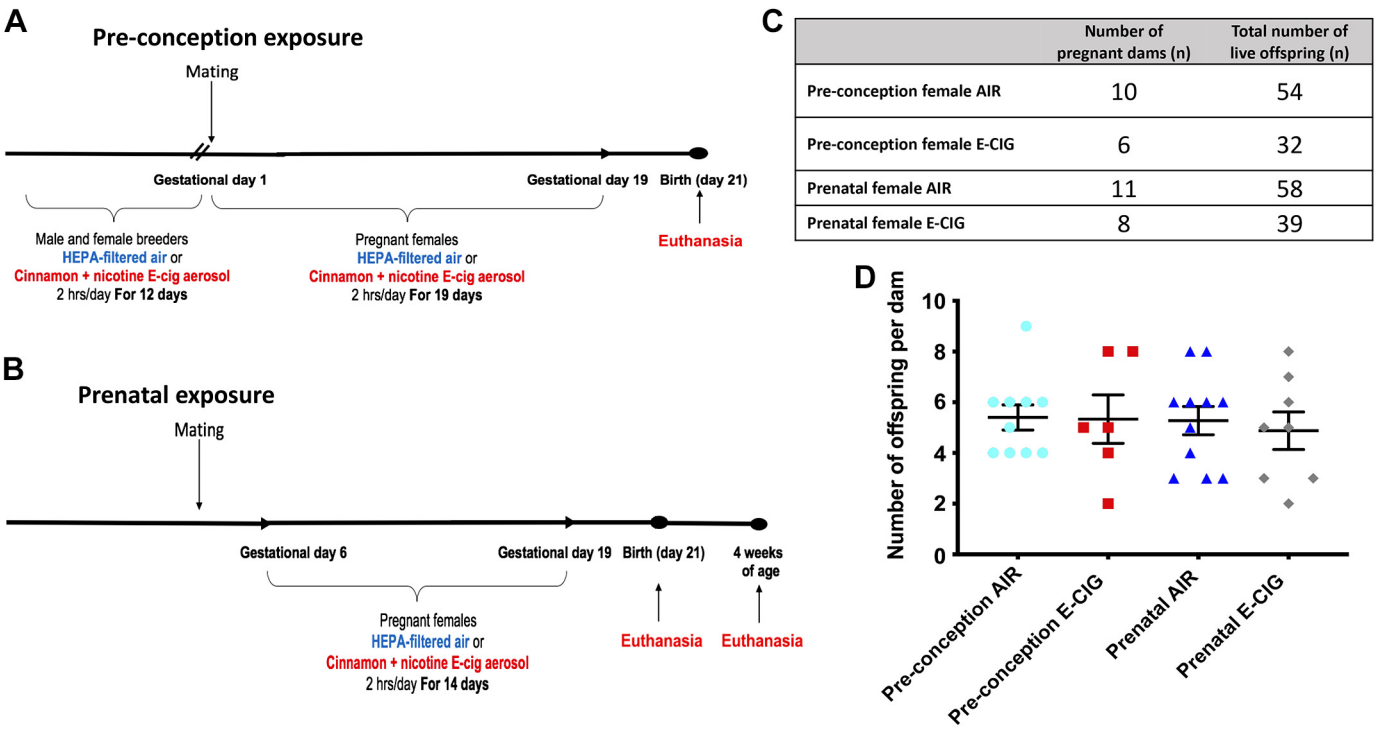

Fig. 1. Experimental design and pregnancy outcomes. *A*: preconception exposure groups were composed of adult BALB/c male and female mice exposed to either electronic-cigarette (e-cig) aerosol or high-efficiency particulate air (HEPA)-filtered air for 2 h/day for 12 days before mating, in addition to 2 h/day for the female mice alone from gestational *days 1–19*. *B*: prenatal exposure groups were composed of female BALB/c mice exposed from gestational *days 6–19* for 2 h/day to either e-cig or HEPA-filtered-air. *C* and *D*: at the start of each set of exposures (preconception and prenatal) there were 11–12 female mice per group, with various outcomes in terms of pregnant dams and number of live offspring.

immediately after euthanization of their offspring via an intraperitoneal injection of Beuthanasia-D. All mice were housed in an AAALAC-approved animal care facility at the School of Veterinary Medicine of the Louisiana State University under a 12:12-h light-dark cycle (from 6:00 AM to 6:00 PM). The mice had access to water and food ad libitum, except during the 2-h exposure periods. Mice were housed and handled in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. All procedures and protocols were approved by the Louisiana State University Institutional Animal Care and Use Committee.

Pulmonary Function Testing of Dams: flexiVent

Pulmonary function of preconception dams ($n = 4–5$ per group) was measured via the flexiVent system (SCIREQ, Montreal, Canada). As previously described (77), mice were anesthetized by a ketamine/xylazine cocktail, tracheostomized, and placed in the forced oscillation measurements flexiVent system. Measurements were accepted only if the coefficient of determination was > 0.95, assuring the fit of the single compartment model. For each mouse, five measurements per parameter were averaged. Lung resistance (R), compliance (C), and elastance (E) were calculated using the single compartment model. Following the procedure, mice were euthanized by an intraperitoneal injection of Beuthanasia-D (Schering-Plough, NJ).

Serum Collection and ELISAs: Cotinine, Placental Growth Factor, and 17β-Estradiol

Following euthanasia, blood samples of preconception adult male mice, dams, and offspring were collected from the heart and allowed to clot at room temperature for at least 30 min. Samples were then centrifuged at 4,800 rpm (Eppendorf model 5418) for 5 min at room temperature. The resulting supernatant, i.e., serum samples, were stored at −80°C for subsequent analysis. Preconception adult male mice breeders' serum cotinine levels were determined using an enzyme-linked immunosorbent assay (ELISA) (Mouse/Rat Cotinine ELISA, cat. no. CO096D-100, Calbiotech, CA) according to the manufacturer's instructions. Preconception and prenatal dams' serum were used to quantify placental growth factor (PlGF) and 17β-estradiol concentrations via ELISAs. The mouse PlGF ELISA kit (cat. no. ab197748, Abcam, Cambridge, MA) and the estradiol ELISA kit from Eagle Biosciences, Inc. (cat. no. DCM003, Nashua, NH) were used according to the manufacturers' instructions.

Lung Morphometric Analysis

The lungs of the PND28 offspring were inflated and pressure fixed (25 cm) with buffered formalin (10%) administered by intratracheal instillation. The lungs of the PND0 offspring were also fixed with buffered 10% formalin. We followed previously published procedures (82, 112, 113) for sectioning and staining 5-mm thick lung sections. We performed hematoxylin and eosin (H&E) staining on 5-mm thick paraffin-embedded tissue sections. Lung morphometric analysis was based on previously published procedures (30). For the 4-wk-old offspring, we calculated the mean linear intercept (Lm) and surface area per unit volume (SApUV), as previously described (30). For the PND0 offspring, we calculated the air/tissue fraction with parenchymal airspace profiling (111). In total, 15 sections were analyzed per sample, with $n = 5–6$ samples per group. Images were obtained with a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan).

Immunohistochemistry

All paraffin-embedded sections were cut at 4 microns and air dried overnight at room temperature (~23°C). Slides were incubated in a 60°C oven for 30 min before deparaffinization and antigen retrieval. Enzymatic retrieval occurred in the autostainer (Dako Autostainer Link 48, Dako Colorado Inc., Ft. Collins, CO). After heat-induced epitope retrieval, slides were rinsed 3 times in distilled water, place in Tween-buffered saline (TwBS) until ready for immunohistochemistry (IHC) staining. For the IHC, the endogenous enzyme block was performed with 3% hydrogen peroxide (incubation 10 min), followed by a TwBS rinse (5 min). We used normal goat serum (1:50; cat. no. S-1000, Vector Laboratories, Burlingame, CA) diluted in TwBS (30 min) to perform the protein block. Then, we incubated the primary antibody: Uteroglobin (1:2,000; rabbit polyclonal, cat. no. ab40873, Abcam, Cambridge, MA) for 30 min. This was followed by the incubation (30 min) with the secondary antibody-biotinylated anti-rabbit IgG (1:200; cat. no. PK-2200, Vector Laboratories, Burlingame, CA). Then, the avidin-biotin complex (cat. no. BA-1000, Vector Laboratories, Burlingame, CA) was incubated for 30 min. We used the ImmPACT Nova Red, Peroxidase substrate kit (cat. no. SK-4805, Vector Laboratories, Burlingame, CA) as the substrate-chromogen (8 min). This was followed by TwBS and distilled water rinse for 5 min. Finally, we counterstained the samples with Hematoxylin (1:10; Ref 812, ANATECH LTD, Battle Creek, MI) in distilled water for 5 min.

Lung Harvest and mRNA Extraction

The lungs of the dams, PND0 and PND28 offspring were harvested for mRNA extraction. Following previously described procedures (82, 112, 113), we used a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE) to assess the quantity and purity of RNA samples.

Gene Expression Analysis

Quantitative RT-PCR. As previously described (77), we performed quantitative RT-PCR (qRT-PCR) on cDNA samples of lung homogenates to determine the expression levels of a selection of genes. We used inventoried TaqMan Gene Expression Assays primer-probe sets (Applied Biosystems). cDNA samples from 4–6 mice in each group were assayed by qRT-PCR. Reaction volumes were 25 μL, and 40 reaction cycles were run for each gene in an Applied Biosystems 7300 Real-Time PCR System. We used the comparative cycle threshold ($\Delta\Delta C_T$) method to determine relative gene expression, with each gene normalized to hypoxanthine guanine phosphoribosyltransferase (Hprt1) expression. Results are reported as fold change over control ($2^{-\Delta\Delta CT}$).

RT² profiler PCR array. In the lungs of the PND0 and PND28 offspring, the expression of 84 *Wnt* Signaling genes was analyzed using a RT² PCR array (Qiagen) according to the manufacturer's instructions. After DNAse treatment, total RNA (0.5 μg) was reverse-transcribed utilizing the RT² First Strand Kit (Qiagen 330401), and cDNA was diluted with RNase-free water to a volume of 111 μL. Converted cDNA sample (100 μL) was mixed with RT² SYBR Green qPCR Master mix (Qiagen 330503). Equal aliquots (25 μL) were added onto the corresponding wells of the PCR Array plate, which contains the predispensed gene-specific primer sets, and the PCR was performed according to the cycling conditions of Applied Biosystems model 7300 real-time cyclers. Gene expression and fold change were calculated using the ΔΔCt method. ΔCt data were calculated using β2-microglobulin (*B2M*) as a housekeeping gene, and the fold change was calculated using the $2^{-\Delta\Delta Ct}$ method with the web-based PCR Array data analysis software ($n = 4$ per group).

Extraction of Proteins

After offspring were euthanized, as described by Noël et al. (77), lungs were removed, quickly frozen in dry ice, and then stored at −80°C until assayed. Frozen lung tissue samples were pulverized into fine powder using a mortar and pestle precooled in liquid nitrogen and quickly placed into 2-mL round bottom microcentrifuge tubes containing 300 μL of RIPA lysis buffer (cat. no. sc-24948, Santa Cruz Biotechnology, Dallas, TX) and four 2.3-mm zirconia/silica beads (cat. no. 11079125z, Biospec Products Inc.). Tissues were then lysed

completely with the aid of Tissue Lyser II (Qiagen, Germantown, MD) at 25 MHz for 2 min. The lysed tissue was centrifuged at 13,000 *g* for 5 min at 4°C. Proteins in the supernatants were aliquoted and stored at −80°C. We used bicinchoninic acid (BCA) protein assay kits (Pierce BCA Protein Assay Kit, cat. no. 23227, Thermo Scientific, Waltham, MA) to determine protein concentrations in the tissue supernatants.

Western Blotting

For the PND0 offspring, the levels of lung protein expression and phosphorylation were determined by Western blot analysis as described previously (77). Briefly, we incubated 15 μg of protein extracts from each of 20 different lung tissue samples (5 samples per group) in Laemmli buffer (cat. no. 161-0747, Bio-Rad Laboratories Inc., Hercules, CA) with 2-mercaptoethanol at 95°C for 5 min in a heat block (VWR Digital Heat Block) and resolved protein bands by SDS-PAGE on Any kD Criterion TGX Stain-Free Precast Gels (cat. no. 5678125, Bio-Rad) at 200 V. We used a 10–250 kDa prestained protein ladder (Precision Plus Protein All Blue Standards, cat. no. 161-0373, Bio-Rad) as a standard to monitor migration of proteins on the gel. Resolved protein bands on the gels were transferred onto nitrocellulose membranes (Trans-Blot Turbo Midi Nitrocellulose Transfer Packs, cat. no. 1704159, Bio-Rad) by the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% dry skim milk and incubated overnight at 4°C with specific primary antibodies against different proteins in phosphate buffer solution (PBS) containing 0.1% Tween-20 (PBS-T). All primary and secondary antibodies were obtained from Cell Signaling Technology Inc., Danvers, MA (WNT signaling antibodies sampler kit, cat. no. 2915). The primary antibody-antigen complexes were detected by incubating the membranes with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The blots were then washed three times with PBS-T and were detected with the aid of enhanced chemiluminescence (ECL) detection reagent (Clarity Western ECL Substrate, cat. no. 1705061, Bio-Rad). Western blot images were captured by the ChemiDoc Touch imaging system (Bio-Rad). The captured images were analyzed with Image Laboratory 5.2 (Bio-Rad). The relative protein expression levels were calculated after normalization to an internal control, β-ACTIN.

Uncropped blots used to determine protein expression are shown in Supplemental Fig. S1 (see https://doi.org/10.17632/cg4zg75mgs.2).

Ingenuity Pathway Analysis

As previously described (78), gene expression data (including results from the RT^2 Profiler) were analyzed with Ingenuity Pathway Analysis (Qiagen, Ingenuity Systems, Redwood City, CA). As described previously (112), we examined the Ingenuity Analysis Knowledge Database to identify the most significantly enriched gene networks and canonical pathways ($P < 0.05$). Subsequently, we created custom gene interaction networks to examine the links between specific genes and significantly related functional networks and pathways.

Statistical Procedures

All biological outcomes were analyzed by the Student's *t* test for pairwise comparisons or by ANOVA followed by the Tukey's test for multiple comparisons. Results are presented as means ± SE. Gene expression results are presented as fold change > ± 1.5 compared with respective air control group. Results were considered statistically significant at $P < 0.05$. We carried out statistical analyses with the GraphPad Prism 8 Software (GraphPad Software, San Diego, CA).

RESULTS

Cotinine Levels and Lung Nicotinic Receptor Gene Expression Confirm Exposure to Nicotine-Rich E-Cig Aerosols

Because cotinine has a half-life of ~1 h in mice (115) and the dams and PND0 offspring were euthanized at least 2 days after the last e-cig exposure (last exposure on gestational *day 19* and birth on *day 21*), we determined serum cotinine levels only in the preconception male breeders immediately following the last e-cig aerosol exposure. E-cig aerosol-exposed male mice had high levels of serum cotinine (150.4 ± 22 ng/mL) (Fig. 2*A*), confirming the nicotine intake through our e-cig aerosol whole body exposure system (76). Additionally, because nicotine is an agonist for the nAChR, we determined the lung gene expression of the nicotinic acetylcholine receptor α7 subunit (α7nAChR) in both dams and PND0 offspring. We found that dams exposed to e-cig aerosols had significantly increased gene expression of this nicotinic receptor with a 1.8-fold increase in the preconception dams and a 2.7-fold increase in the prenatal dams compared with their respective air control groups (Fig. 2*B*). As nicotinic receptors are activated by nicotine (109), this confirms the nicotine exposure through e-cig aerosols in the exposed dams. In the offspring, only the preconception e-cig group showed a significant downregula-

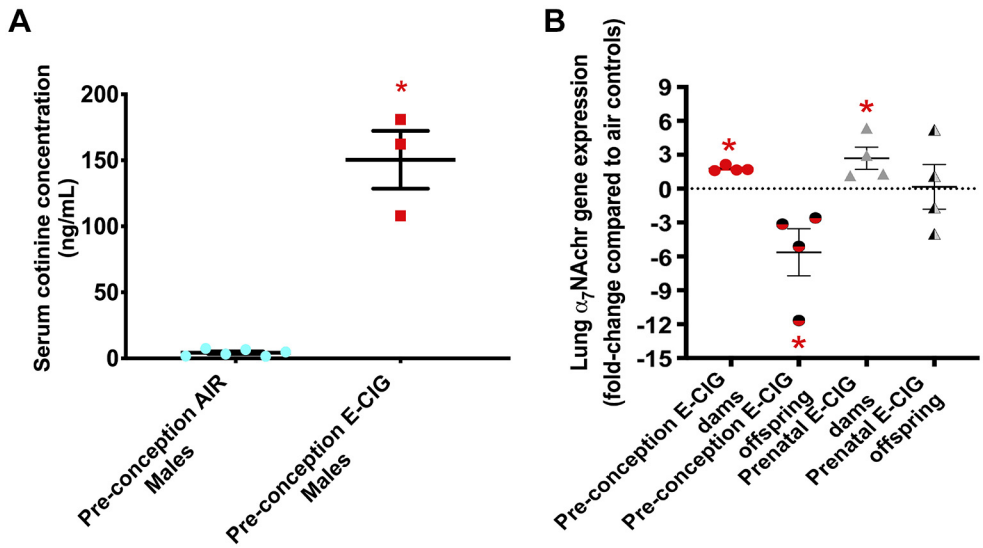

Fig. 2. Cotinine levels and lung nicotinic receptor gene expression confirm exposure to nicotine-rich electronic-cigarette (e-cig) aerosols. *A*: 12 days of cinnamon-flavored e-cig aerosol exposures containing 36 mg/mL of nicotine significantly increased the serum contine concentration in exposed male mice. Data represent the mean ± SE for $n = 3$–6 mice per group. Student's *t* test, $^*P < 0.05$: statistically different from the air group. *B*: lung gene expression of the nicotine acetylcholine receptor (α7nAChR) in both dams and offspring. For each exposure window in dams and *postnatal day 0* (PND0) offspring, data are expressed in fold changes compared with respective air control group for $n = 4$ mice per group. $^*P < 0.05$: statistically different from respective air control group.

tion of −5.6-fold of α7nAChR in the lungs compared with respective air controls (Fig. 2*B*). The paradox of nicotinic acetylcholine receptor regulation by nicotine proposes that chronic exposure or overactivation of the nicotinic receptors can lead to its downregulation (109). Our results suggest that undeveloped fetal lungs exposed to nicotine in utero for 19 days can lead to downregulation of the α7nAChR lung gene and support nicotine exposure in the offspring.

In Utero E-Cig Exposures Decrease Body Length and Birth Weight Through 4 Wk of Age in Offspring

To investigate whether in utero e-cig exposures adversely affect pregnancy outcomes, we exposed both male and female mice to either e-cig aerosol or HEPA-filtered air for 12 days before mating, added to gestational *days 1–19* for the female mice. Another group of female mice were exposed to these same aerosols solely from gestational *days 6–19*. All the live offspring (n = 32–58 per group, Fig. 1*C*) were included for the measurements of the body length and weight at birth. The e-cig aerosol exposure did not affect the litter size per dam, which ranged from 2 to 9 offspring per dam (Fig. 1*D*).

Compared with respective air-exposed controls, both preconception and prenatal exposures to e-cig aerosol significantly decreased the offspring birth length (Fig. 3*A*) (preconception e-cig: 2.84 cm vs. air: 3.01 cm; prenatal e-cig: 2.79 cm vs. air: 2.97 cm, Fig. 3*B*) and weight (preconception e-cig: 1.35 g vs. air: 1.55 g; prenatal e-cig: 1.33 g vs. air: 1.51 g, Fig. 3*C*). Additionally, the decreased body weight was sustained through 4 wk of age in the offspring exposed prenatally to e-cig aerosol (Fig. 3*D*). Overall, these results strongly suggest that in utero e-cig exposures have a negative effect on the physical growth of the newborns.

Maternal Vaping Impairs Lung Function and Disrupts Hormonal Levels in the Dams

After giving birth, the dams of the preconception groups were assessed for lung function measurements via the flexiVent system. As shown in Fig. 4*A*, the maximum respiratory elastance (Ers) of the preconception e-cig dams was significantly higher (38.1 ± 7.1 vs. 17.8 ± 2.8 cmH_2O/mL) than that of their respective air controls. The elastance represents the elastic stiffness or the resistance of the respiratory system to expand (69). Therefore, increased elastance translates into increased work of breathing (69).

The maternal serum placental growth factor (PlGF) concentration was slightly but nonsignificantly decreased in both e-cig-exposed groups compared with their respective controls (Fig. 4*B*); however, we found a strong negative Pearson cor-

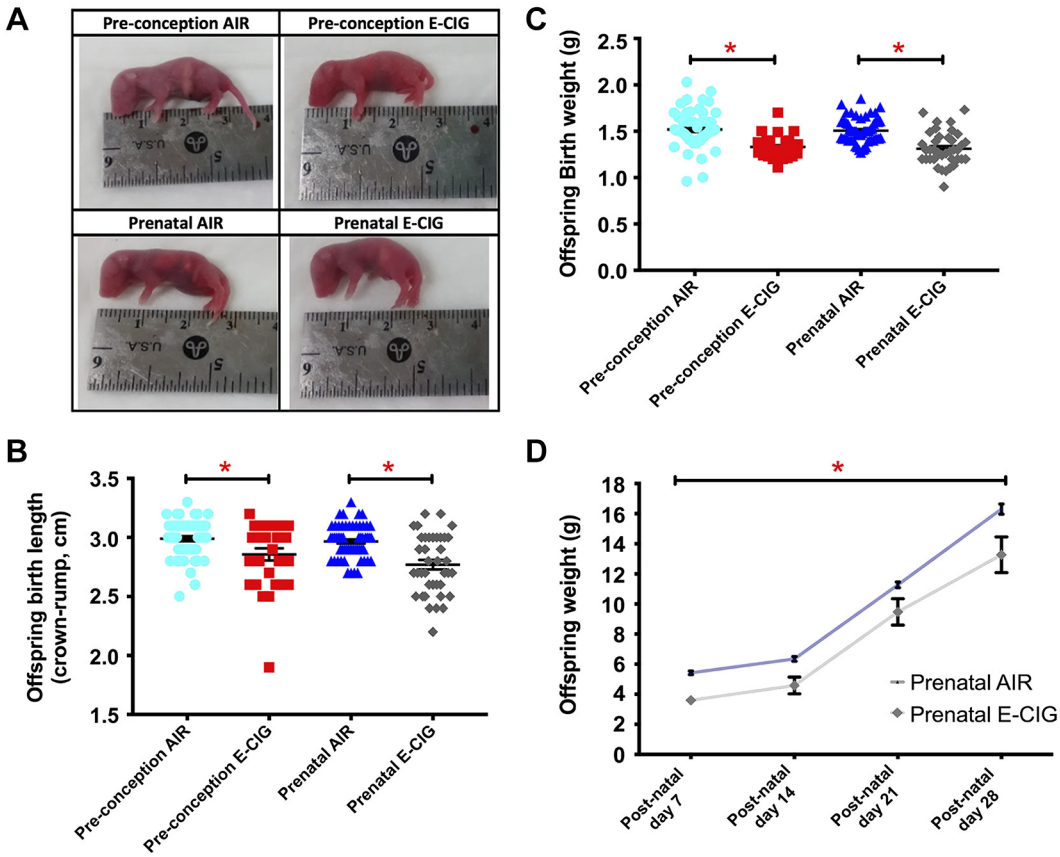

Fig. 3. In utero electronic-cigarette (e-cig) exposures decrease body length and birth weight though 4 wk of age in offspring. *A*: representative images of offspring at birth from each exposure group. *B* and *C*: in utero e-cig exposures significantly decrease birth length and weight compared with air controls. Data represent the mean ± SE for n = 32–58 mice per group. Student's t test, $^*P < 0.05$: statistically different from the respective air group. *D*: decreased body weight was maintained through *postnatal day 28* (PND28) in prenatal e-cig-exposed offspring compared with air controls. Data represent the mean ± SE for n = 7 prenatal e-cig mice per group (2 males and 5 females) and n = 19 prenatal air mice per group (6 males and 13 females). Student's t test, $^*P < 0.05$: statistically different from the air group at all time points, i.e., PND7, 14, 21, and 28.

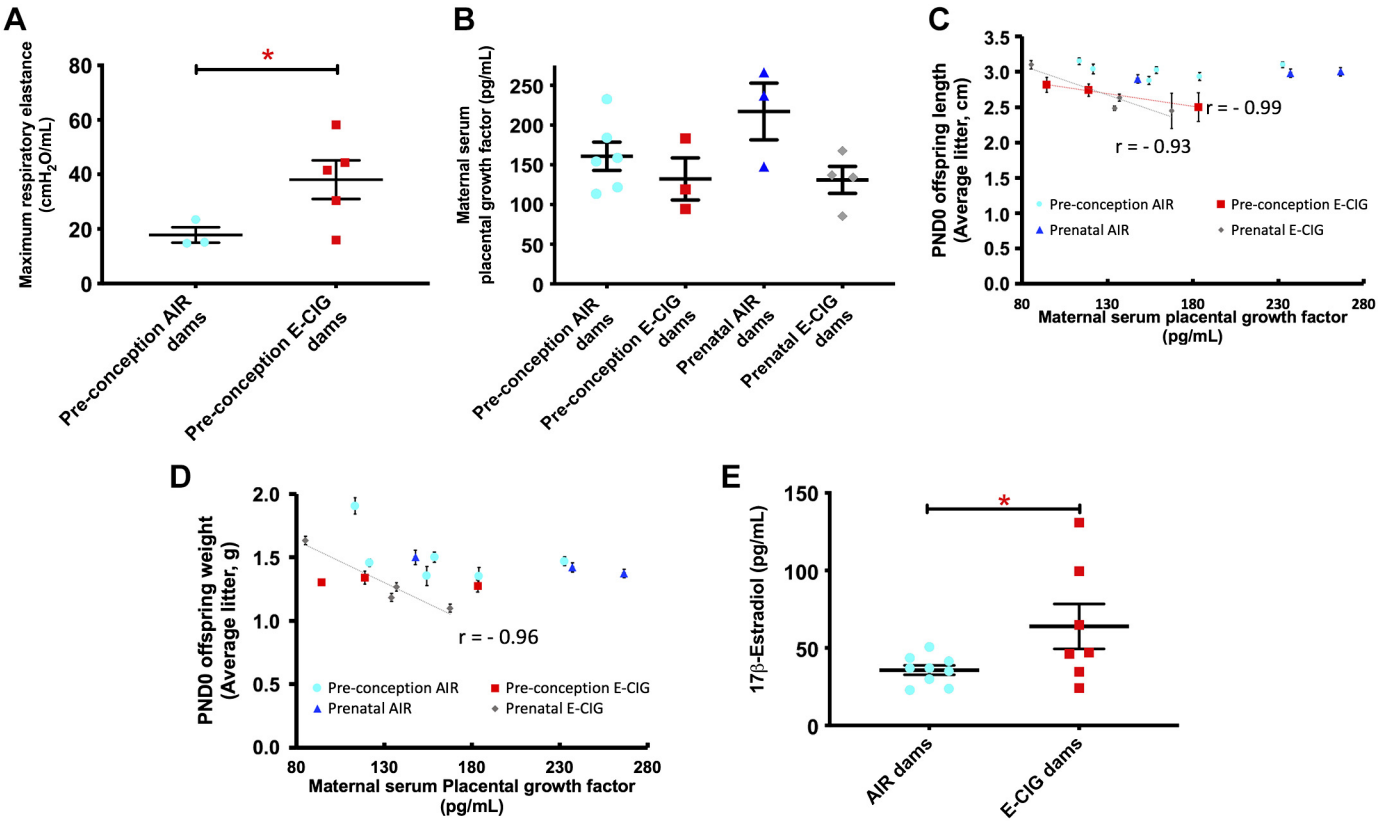

Fig. 4. Maternal vaping impairs lung function and disrupts hormonal levels in dams. *A*: lung function data measured by the flexiVent system revealed that dams exposed preconceptually to electronic-cigarette (e-cig) aerosols had increased maximum respiratory elastance. Data represent the mean ± SE for $n = 3$–5 mice per group. Student's *t* test, $^*P < 0.05$: statistically different from the air group. *B–D*: maternal serum placental growth factor (PlGF) levels were lower in e-cig-exposed dams, with inverse correlations between each dam's PlGF concentration and its respective litter length and weight at birth. Data represent the mean ± SE for $n = 3$–6 mice per group. PND0, *postnatal day 0*. *E*: maternal serum 17β-estradiol concentration was significantly elevated in e-cig-exposed dams (preconception + prenatal) when compared with air control-exposed dams. Data represent the mean ± SE for $n = 7$–9 mice per group.

relation ($r = -0.99$) between individual preconception e-cig-exposed dam serum PlGF concentration and each dam's respective average litter length (Fig. 4*C*). The serum PlGF concentrations ranged from 94 to 183 pg/mL for the preconception e-cig group and that of the respective control group ranged from 113 to 232 pg/mL. A strong negative Pearson correlation ($r = -0.96$) also was observed for individual prenatal e-cig-exposed dam serum PlGF concentration and each dam's respective average litter weight (Fig. 4*D*). The serum PlGF concentrations ranged from 85 to 167 pg/mL for the prenatal e-cig group and that of the respective control group ranged from 147 to 266 pg/mL. Overall, the results suggest that e-cig exposures decreased maternal serum PlGF concentration, which correlates with decreased offspring measurements at birth.

The maternal serum 17β-estradiol concentration was increased, although nonsignificantly, in both e-cig exposed groups compared with their respective controls (data not shown). Yet after combining both e-cig groups and comparing results to those of the combined air control groups, we observed a significant increase in the maternal serum 17β-estradiol concentration in the dams exposed to e-cig aerosols (Fig. 4*E*). Overall, these results in the dams (Fig. 4, *A* to *E*) show that e-cig exposures during pregnancy can affect lung function and disrupt the endocrine environment, which may potentially disturb fetal development.

E-Cig Exposures Modulate Maternal-Fetal Lung Immuno-Inflammatory Responses

To better understand the maternal-fetal interactions related to the lungs, we evaluated the expression of selected inflammation-related genes in both dams and offspring. In the preconception group, we found that 7 inflammation-related genes were downregulated in the lungs, including 4 genes (*Stat6*, *Gata3*, *Stat5a*, and *Il-1β*) common to both dams and fetuses (Fig. 5*A*). In contrast, prenatal e-cig exposures dysregulated 7 lung genes in the offspring, including *Il-5*, *Il-13*, *Stat5a*, and *Hmox1*, all of which were upregulated, whereas only 2 genes (*Il-4* and *Il-6*) were downregulated in the dams (Fig. 5*B*). Of note, those 2 genes were also downregulated in the preconception dams (Fig. 5*A*). Overall, independent of the maternal exposure window, maternal–fetal lung immuno-inflammatory responses were modulated by e-cig exposures.

In Utero E-Cig Exposures Alter the Lung Structure of the Offspring at PND0 and PND28

To investigate whether in utero e-cig exposures affect lung structural development, we carried out a morphometric analysis of the offspring lung tissue at PND0 and PND28. Morphometry assessments of preconception e-cig-exposed offspring' lungs versus air-exposed lungs at PND0 showed significantly increased tissue fraction compared with airspace

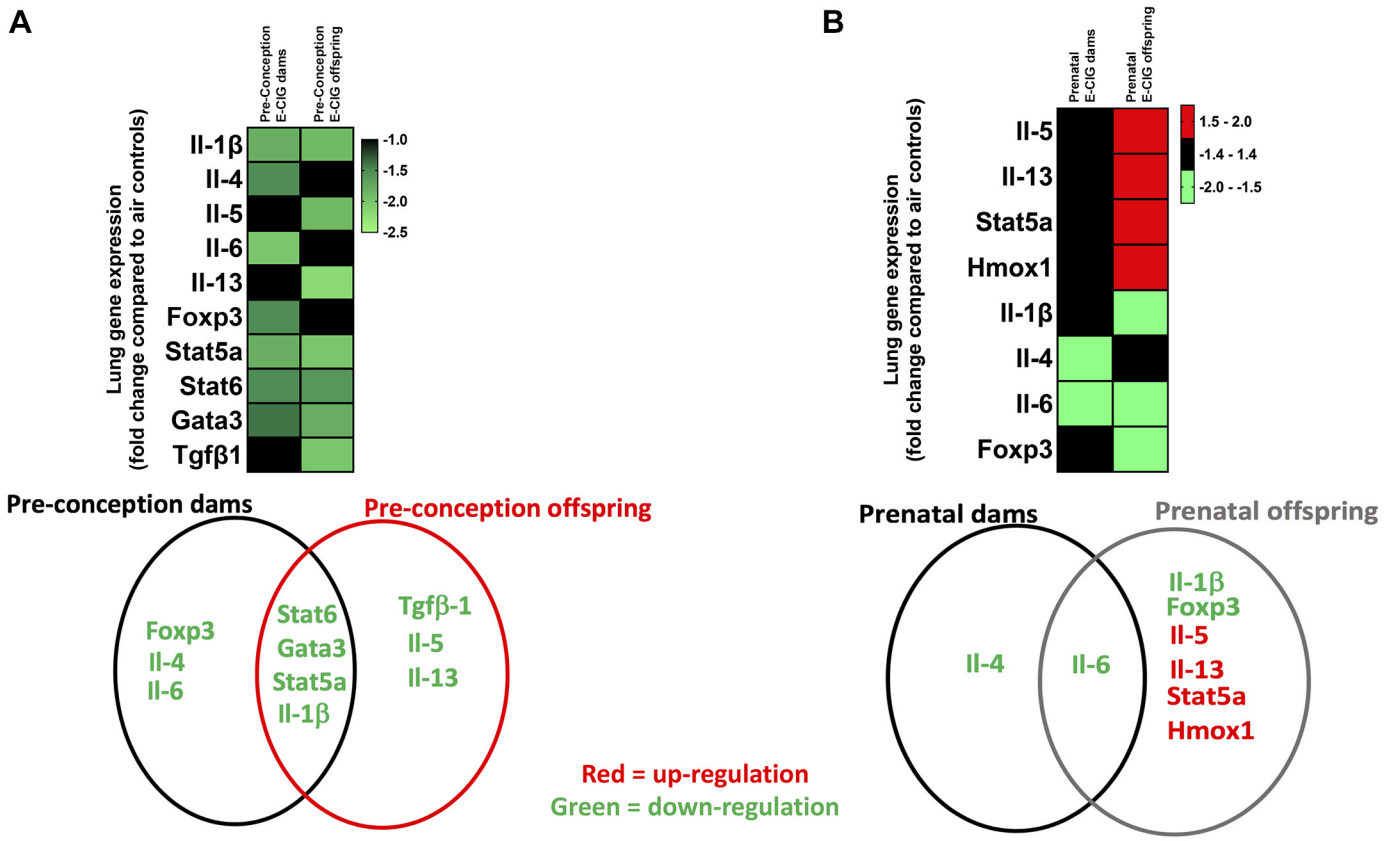

Fig. 5. Electronic-cigarette (e-cig) exposures modulate maternal-fetal lung immune-inflammatory responses. *A*: expression of lung inflammation-related genes in both preconception dams and *postnatal day 0* (PND0) offspring. Data are expressed in fold changes compared with respective air control group for $n = 3–6$ mice per group. *B*: expression of lung inflammation-related genes in both prenatal dams and PND0 offspring. Data are expressed in fold changes compared with respective air control group for $n = 3–6$ mice per group. Red denotes upregulation and green denotes downregulation.

(Fig. 6, *A* and *B*). Furthermore, in the embryonic lung, *Notch* regulates the balance of ciliated, secretory and neuroendocrine cells in the airway epithelium. Postnatally, *Notch* signaling prevents airway club cells from differentiating into goblet cells. Our data show that preconception exposures to e-cig aerosols decreased *Notch2* lung gene expression at PND0 (Fig. 6*C*), and, as evidenced by immunohistochemistry for anti-uteroglobin antibody, increased the percentage of club cells in the lungs (Fig. 6, *D* and *E*). Additionally, at PND28, the prenatal e-cig-exposed group had significantly higher Lm values compared with the respective air control group (Fig. 6, *F* and *G*). Overall, these results indicate that in utero e-cig exposures alter lung structure at birth. This is associated with decreased *Notch2* gene expression, which is involved in airway epithelial cell differentiation. Additionally, lung structural damage is sustained through 4 wk of age.

In Utero E-Cig Exposures Dysregulate Lung Gene and Protein Expression, with the Preconception Exposure Downregulating Genes of the Wnt *Signaling*

Next, to uncover the molecular mechanisms associated with altered lung structure following in utero exposures to e-cig aerosols, we assessed the expression of *Tgf-β1*, *Igf1*, *Igf2*, *Shh*, *Fos*, *Fosl1*, *Muc5ac*, and *Mmp12*, in addition to 84 *Wnt* signaling genes in the lungs of the offspring. The alterations in lung structure of the preconception e-cig group were supported by the downregulation of *Shh* in addition to 75 lung genes involved in the *Wnt* signaling, which are essential to lung organogenesis. These genes included *Wnt1*, *Wnt3a*, *Lef1*, and *Sfrp1*, whose dysregulation has been associated with lung hypoplasia (41) (Fig. 7*A*). Conversely, the prenatal e-cig group showed at PND0 dysregulation of 3 genes (> twofold change), which were upregulated and included *Mmp7*, *Wnt10a*, and *Frzb* (Fig. 7*A*). At PND28, the lung molecular changes in the prenatal e-cig-exposed group returned to baseline levels, with only 1 dysregulated gene (upregulation of *Fosl1*) (Fig. 7*A*). At the protein level, we found significantly increased expression levels of WNT5A/B, DVL2, DVL3, and pLRP6 proteins in the prenatal e-cig-exposed group at PND0 compared with their respective controls (Fig. 7, *B* and *C*), thus confirming the overall upregulation direction for the molecular changes in this prenatally e-cig aerosol-exposed group.

The gene expression results for the preconception e-cig exposure group were further reinforced by the Ingenuity Pathway Analysis (IPA). The analysis of the genes dysregulated by preconception e-cig aerosol exposures reveal that the interconnections between the 75 downregulated genes (Fig. 7) were related to decreased growth and proliferation of lung cells (Fig. 8).

Overall, our data indicate that maternal vaping of nicotine-rich aerosols impairs pregnancy outcomes and alters fetal lung structure via molecular mechanisms associated with *Shh*, *Wnt*, and *Notch2* pathways.

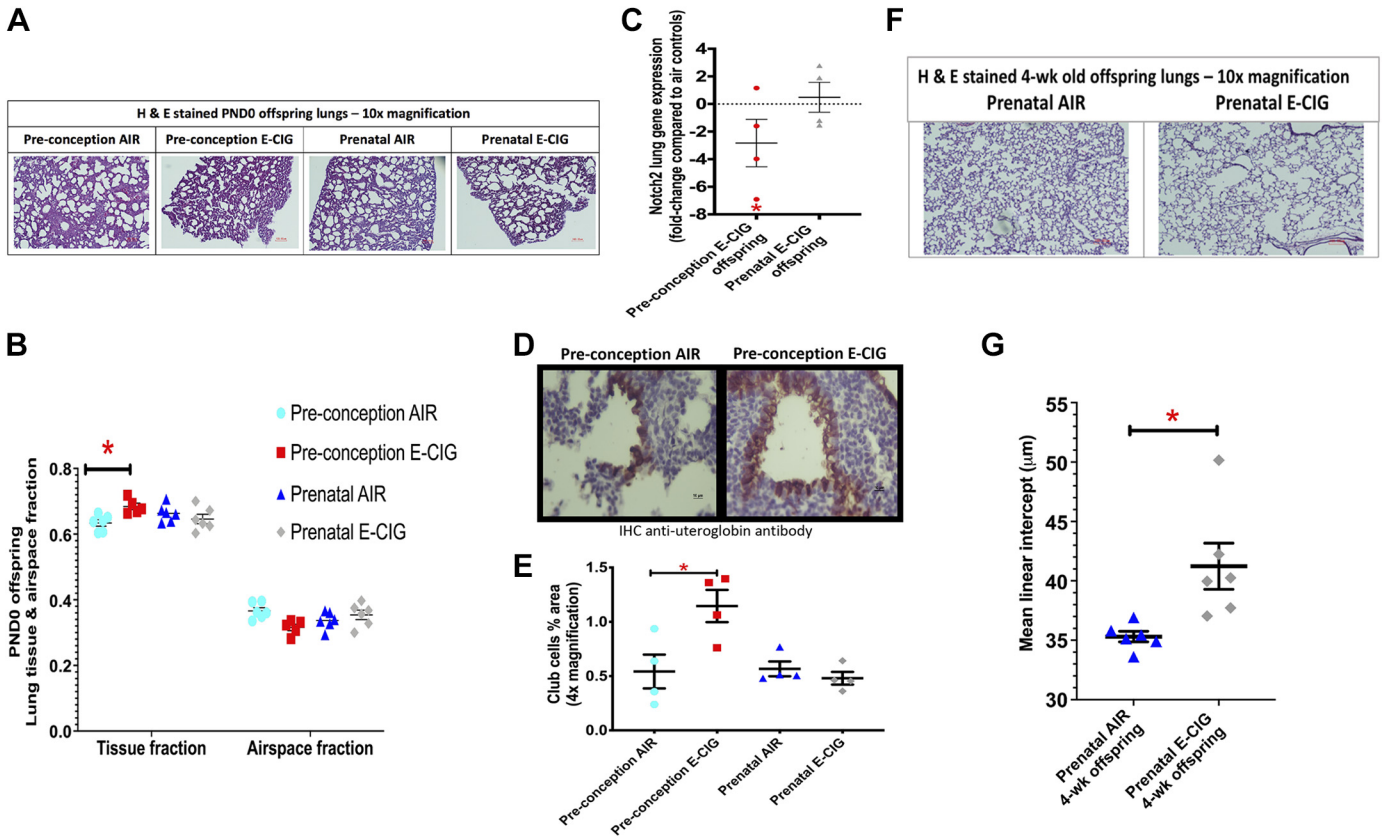

Fig. 6. In utero electronic-cigarette (e-cig) exposures alter the lung structure of the offspring at *postnatal day 0* (PND0) and *28* (PND28). *A*: representative images of hematoxylin-eosin (H&E)-stained lung tissue slides from offspring at birth from each exposure group. Scale bar = 100 μm. *B*: calculated air/tissue fraction with parenchymal airspace profiling from PND0 offspring of each exposure group. Data represent the mean ± SE for $n = 5–6$ mice per group. Student's *t* test, $*P < 0.05$: statistically different from respective air group. *C*: lung gene expression of *Notch2* in PND0 offspring. Data are expressed in fold changes compared with respective air control group for $n = 4$ mice per group. $*P < 0.05$: statistically different from respective air group. *D*: representative images of immunohistochemistry-stained lung tissue for uteroglobin in preconception PND0 offspring. Scale bar = 10 μm. *E*: quantification in percentage area of stained cells (club cells) at ×4 magnification by ImageJ. Data represent the mean ± SE for $n = 4$ mice per group. Student's *t* test, $*P < 0.05$: statistically different from respective air group. *F*: representative images of H&E-stained lung tissue slides from 4-wk-old offspring in the prenatal exposure groups. Scale bar = 100 μm. *G*: calculated mean linear intercept (Lm) values of lung tissue from 4-wk-old prenatally exposed offspring. Data represent the mean ± SE for $n = 5–6$ mice per group. Student's *t* test, $*P < 0.05$: statistically different from the air group.

DISCUSSION

Wnt and *Shh* pathways are critical throughout lung development by being involved in the cross-regulation of lung organogenesis (108). Therefore, *Wnt* and *Shh* may well play important roles in the context of impaired lung development due to in utero exposures to environmental stressors (51, 56, 85). In the particular setting of in utero e-cig aerosol exposures, however, the cellular and molecular mechanisms affecting lung development are currently unknown. It was previously reported that in utero exposures to cigarette smoke decreased the expression of *Wnt*-related genes in the lungs of BALB/c mice offspring at birth (23). In addition, nicotine has been shown to dysregulate *Wnt* signaling (50, 93). Furthermore, the *Wnt* signaling is involved in nicotine-induced lung adverse effects in embryonic human lung fibroblasts (93). In utero exposures to aerosols represent a distinctive route of administration, as there is no direct contact between the fetal lungs and the e-cig aerosols but rather passive systemic exposures to chemicals, including nicotine and aldehydes, through the placenta and therefore via the maternal circulation (25). Here, we showed that preconception and prenatal exposures to e-cig aerosols decrease birth weight and body length and impair lung development in mice. In addition to lung structural changes, we found that in utero e-cig aerosol exposures in PND0 offspring downregulated the expression of *Shh* and 75 *Wnt*-related lung genes. In our model, this implies that *Wnt* signaling may play a key role in the molecular mechanisms associated with impaired lung development following in utero exposure to e-cig aerosols. We have identified two key pathways, *Wnt* and *Shh*, which are involved in lung organogenesis and are dysregulated following exposures to e-cig aerosols. Our findings, for the first time, suggest that in utero e-cig aerosol exposures alter lung structure and delay lung maturation. While these results may be associated with downregulation of the *Wnt* signaling, more research is needed to determine whether in utero e-cig aerosol exposure's effects on *Wnt* signaling have a causal relationship with altered lung development. Our data also suggest distinct embryotoxic changes based on the in utero exposure window, which are important new findings for this type of mixture. Taken as a whole, this suggests that immediate and possibly even lasting alterations in the structure and function of the lung may arise from in utero e-cig

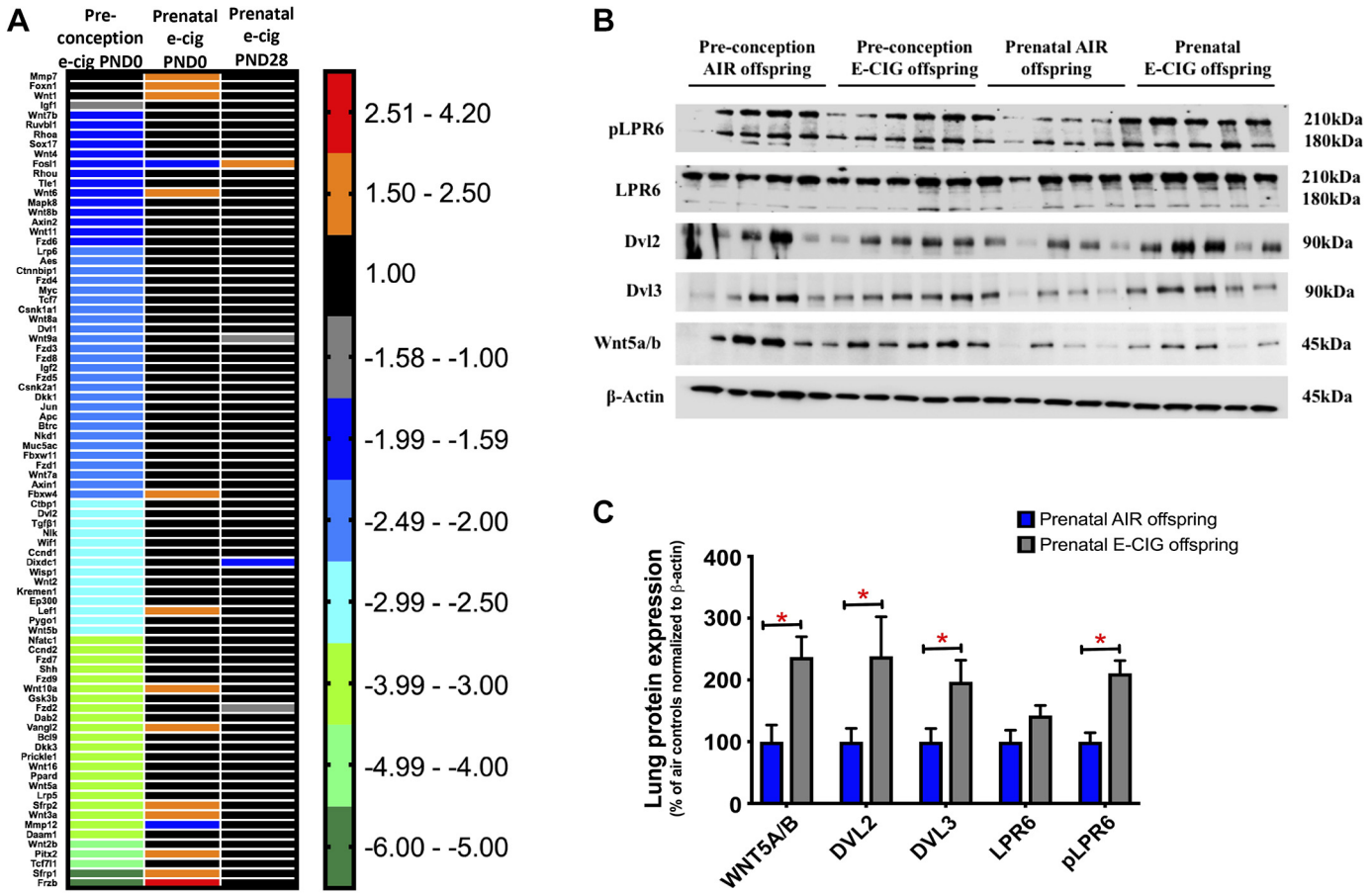

Fig. 7. In utero electronic-cigarette (e-cig) exposures dysregulate lung gene and protein expression, with the preconception exposure downregulating genes of the *Wnt* signaling. *A*: expression of 84 *Wnt* signaling genes in the lungs of *postnatal day 0* (PND0) and *28* (PND28) offspring analyzed using a RT^2 PCR array. Data are expressed in fold changes compared with respective air control group for $n = 4$ mice per group. *B*: representative image of gel used for Western blot analysis of *Wnt* signaling-related proteins in lungs of PND0 offspring of each exposure group; $n = 5$ mice per group. *C*: quantification of Western blot results for prenatal exposure groups expressed as a percentage of respective air control group and normalized to β-actin. Data represent the mean ± SE for $n = 5$ mice per group. Student's *t* test, $^*P < 0.05$: statistically different from the air group.

aerosol-induced dysregulation of key pathways orchestrating lung development.

First, it is important to recognize that physico-chemical characterization of an e-cig aerosol is influenced by the device settings and the e-liquid composition. 'Mod-tank-style' e-cig devices have customizable features for the atomizer's resistance, the battery voltage, and the device wattage, which customers use to obtain specific heating conditions. During e-cig use, the e-liquid is heated at 200°C or greater (11) and the thermal degradation of VG and PG produce emissions of harmful aldehydes, including formaldehyde and acetaldehyde, known to be potent threats to human health, with formaldehyde being a proven human carcinogen (32, 96). When voltages > 5 V are applied to third- or fourth-generation e-cig devices, they produce e-cig aerosols containing formaldehyde at similar or higher levels (1.8 μg/puff) than those found in CS (32, 35, 36, 48, 99). The flavoring agent incorporated into the e-liquid can also have intrinsic toxic properties. The concentration of flavoring chemicals in e-liquids range from 10 to 50 mg/mL, and this includes cinnamaldehyde (7). Cinnamaldehyde is the flavoring aldehyde that is mainly responsible for producing the flavor of cinnamon. We used a cinnamon flavored e-liquid. It was previously shown that out of 24 e-liquid flavors tested, cinnamon e-liquid was the most potent in inhibiting the survival of human embryonic stem cells and pulmonary fibroblasts in addition to mouse neural stem cells (2). By using human embryonic stem cells simulating the initial periods of prenatal development, it was thus demonstrated that cinnamon-flavored e-liquid is embryotoxic in vitro (2). This suggests that cinnamaldehyde flavoring can adversely affect prenatal development. Subsequently, several in vitro studies also showed that cinnamon-flavored e-liquids and cinnamaldehyde are highly cytotoxic (IC_{50} below 1%) and genotoxic to macrophages, neutrophils, and bronchial epithelial cells (5, 7, 18, 33). Most importantly, it was proven that cinnamon-flavored e-liquids are cytotoxic, independent of nicotine concentration (5). Taken together, scientific evidence has established that cinnamon-flavored e-liquids and cinnamaldehyde are cytotoxic to numerous cell types, as well as being embryotoxic (2, 5, 7, 18, 33). Therefore, the in utero effects of cinnamon-flavored e-cig aerosol on developing mouse lungs reported here cannot be generalized to all e-cig aerosols. Although the e-cig aerosol we used in our study also contained nicotine, the effects that we observed may be mostly driven by cinnamaldehyde toxicity; thus, this suggests that the in utero effects of e-cig aerosol on the developing lungs may be flavor specific.

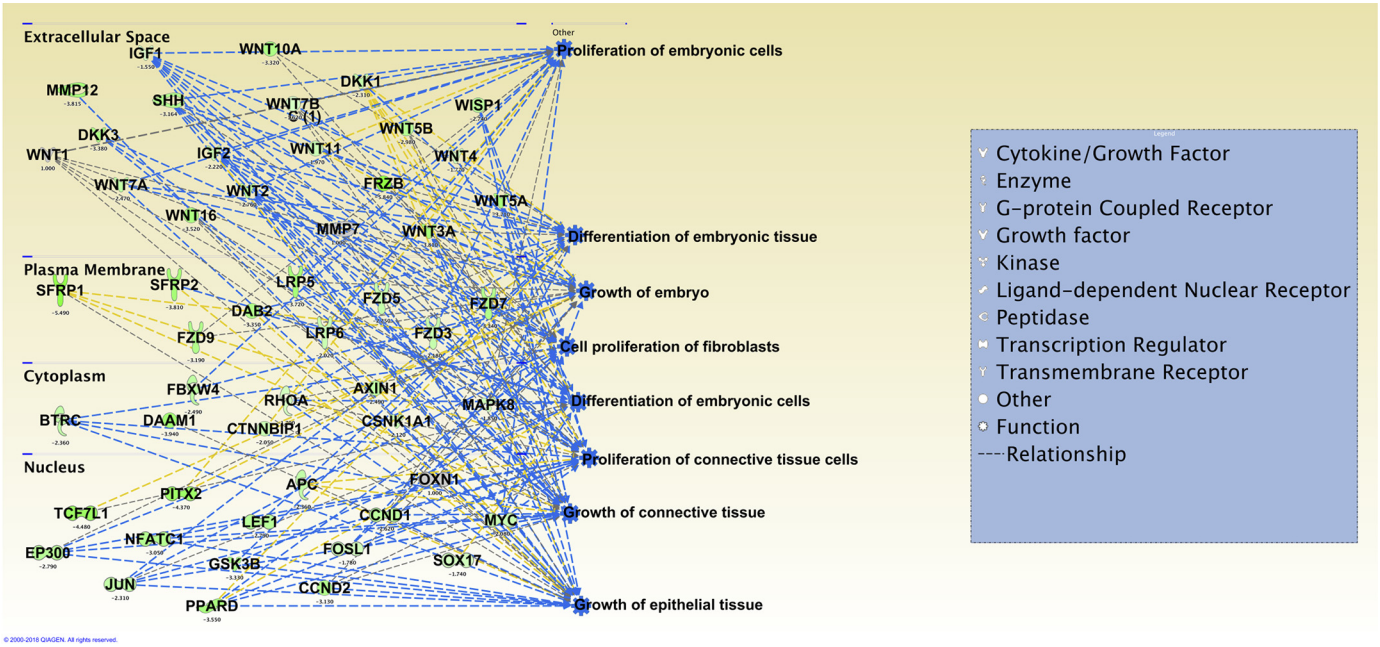

Fig. 8. The gene interaction networks impacted by preconception electronic-cigarette (e-cig) aerosol exposures are associated with decreased growth and proliferation of lung cells. Ingenuity Pathway Analysis (IPA) of genes dysregulated by preconception exposures to e-cig aerosols in *postnatal day 0* (PND0) offspring. [Reprinted with permission from QIAGEN Silicon Valley.]

Thus, various factors can influence e-cig aerosol production and composition. These factors are associated with the ratios and constituents of the e-liquid formulation, which impact the chemical component of the aerosol, as are the atomizer's resistance and voltage applied to the e-cig device, which influence the heating conditions used to aerosolize the e-liquid and therefore the physical characteristics of the aerosol. Because it is in the aerosol form that the e-liquid constituents interact with biological matrices, the device settings and e-liquid are factors that can significantly impact the toxicity of the inhaled e-cig aerosol. Consequently, these variables need to be taken into account when comparing experimental in vitro, in vivo, and human e-cig studies. We used a third-generation e-cig device, which operated with an atomizer's resistance and battery voltage set at 1.5 Ω and 4.2 V, respectively. These conditions are representative of e-cig users' operational settings (20, 60) and were not intended to simulate extreme conditions of sub-ohm or high-wattage vaping.

To date, we only found one cohort study on pregnancy and neonatal outcomes following gestational exposure to e-cig aerosols (13). This study showed that the risk ratio (RR) of having a newborn that is small-for-gestational-age at birth is RR = 3.1 in pregnant ENDS-only users compared with unexposed pregnant women (13). Furthermore, experimental studies in rodents reporting birth length and/or weight changes following in utero exposures to e-cig aerosols are very limited (15, 54, 70, 75, 81, 97). These studies report variable results. Whereas three studies showed no significant differences for birth weight and neonatal weight gain in mice exposed in utero to e-cig aerosol with or without nicotine (e-liquid nicotine concentration ranging from 13 to 18 mg/mL) (15, 54, 75), three other studies demonstrated decreased birth weight and reduced weight gain in neonatal mice or rats exposed to e-cig aerosols, with and without nicotine (e-liquid nicotine concentration of 18, 24, and 50 mg/mL) during gestation and at the end of the gestation plus lactation period (70, 81, 97). In the present study, we found that exposures to e-cig aerosols containing cinnamon flavor and 36 mg/mL of nicotine decreased body length and birth weight through 4 wk of age in offspring (Fig. 3). Low birth weight can be caused by several maternal, placental, or fetal conditions, including maternal smoking (46). We showed that in utero exposures to cinnamon-flavored e-cig aerosol containing 36 mg/mL of nicotine significantly elevated serum cotinine levels in male breeders and upregulated the lung gene expression of α7nAChR in dams (Fig. 2), thereby confirming nicotine exposure in exposed breeders. Because nicotine induces vasoconstrictive effects, including reduction of the blood flow to the placenta, maternal smoking has been associated with intrauterine growth restriction (IUGR) (46, 55). P1GF is a placental biomarker measured in the maternal circulation (8), and low maternal plasma levels of PlGF have been associated with fetal growth restriction (8). We found a strong negative correlation between individual preconception e-cig-exposed dam serum PlGF concentration and each dam's average litter length; and likewise, a negative correlation between individual prenatal e-cig-exposed dam serum PlGF and each dam's average litter weight (Fig. 4, *C* and *D*). Thus, our data suggest that lower maternal serum PlGF concentrations (range: 85–183 pg/mL) from e-cig-exposed dams may be correlated with lower birth length (range: 2.45–3.10 cm) and weight (range: 1.10–1.63 g) of PND0 offspring compared with air-treated dams (serum PlGF range: 113–266 pg/mL) and respective offspring length (range: 2.88–3.15 cm) and weight (range: 1.28–1.91 g) (Fig. 4, *C* and *D*). Moreover, we found that e-cig exposures increased maternal serum 17β-estradiol concentration (Fig. 4*E*). It has previously been reported that freebase nicotine dose-dependently increases estradiol secretion in vitro in human granulosa cells (9). Estradiol is a

hormone that plays key roles during gestation, particularly regarding the placenta and normal fetal development (40, 62, 72, 84). In vivo studies in nonhuman primates revealed that increased levels of serum estradiol early in pregnancy can affect placental blood flow, potentially resulting in fetal growth restriction (10). In humans, elevated maternal estradiol levels in the first trimester are correlated with increased risk of low birth weight in the offspring (40, 84). In the present study, although maternal estradiol levels were measured shortly after birth, the significant increase for this crucial hormone in dams exposed to e-cig aerosols compared with the air-exposed dams could affect the intrauterine environment. Taken together, these results suggest that use of e-cigs containing nicotine may impact maternal hormonal balance (i.e., levels of PlGF and 17β-estradiol), which may be a possible mechanism by which the intrauterine environment is altered via nicotine's known vasoconstrictive effect decreasing placental blood flow, leading to IUGR, with resulting reductions in fetal growth and birth weight. Although we cannot identify nicotine or cinnamaldehyde flavoring chemical as being responsible for the low birth weights, our data are in line with previous reports in rodents using e-liquids with higher levels of nicotine (> 18 mg/mL) (70, 81, 97). Overall, our data and other research support that rodent gestational exposures to e-cig aerosols with nicotine can decrease the measurements of the offspring at birth. This is in line with results regarding newborns' small-for-gestational-age at birth in a recent human cohort study that evaluated the effects of ENDS exposure during pregnancy (13).

The effects of CS on the immune system during pregnancy are still largely unknown (86); however, in a prospective cohort study, it was shown that CS can stimulate the maternal immune system for both inflammatory and anti-inflammatory responses early during pregnancy (61). CS is known to play dual roles on innate and adaptive immunity by either enhancing immune responses or weakening defense mechanisms to pathogens (87). Thus, CS can be an immunosuppressant for cells of the respiratory tract (71), and a growing body of evidence is showing that exposure to e-cig aerosols leads to the suppression of immune-inflammatory responses in nasal and respiratory cells (18, 63, 68, 90, 110). In the present study, we found that preconception exposures to e-cig aerosols downregulated the expression of lung genes (*Foxp3*, *Gata3*, *Il-4*, and *Il-6*) involved in adaptive as well as innate (*Stat5a*, *Stat6*, *Il-1β*) immunity in the dams (Fig. 5*A*) (87). Also, two adaptive immunity-related cytokines (*Il-4* and *Il-6*) were downregulated in prenatal e-cig-exposed dams (Fig. 5*B*). While the importance of T regulatory cells for the maternal-fetal tolerance during pregnancy has previously been shown (39), in our study, preconception e-cig-exposed dams had a 1.5-fold decreased in the gene expression of *Foxp3*, a key transcription factor for the suppressive function of T regulatory cells. This suggests that in our model, preconception and prenatal e-cig aerosol exposures have an immunosuppressive effect on the lungs of pregnant mice and may impact immunological tolerance. In contrast, in a study conducted by Chen et al. (15), exposures to e-cig aerosols (containing tobacco flavor with or without 18 mg/mL of nicotine) for 6 wk before pregnancy, plus during gestation, in addition to 20 days during lactation, led to increased protein expression of TNF-α and IL-1β in the lungs of e-cig-exposed dams, while IL-6 was only increased in the group exposed to e-cig without nicotine compared with air controls (15). It is unclear why these data relating to dams' lung inflammatory responses to e-cig aerosols differ from our results; however, as mentioned previously, in vivo e-cig studies may not be directly comparable. The exposure regimen (31 vs. 83 days), e-cig device settings used (12 vs. 30 W), and e-liquid composition (cinnamon vs. tobacco flavor; 36 vs. 0 or 18 mg/mL of nicotine) are factors that were strikingly different in these two studies and can significantly impact the toxicity of the inhaled e-cig aerosols. This suggests that each e-cig aerosol-related toxicity may be unique to the particular exposure system, thereby complicating the regulatory component of new alternative tobacco products.

In our study, in addition to the downregulation of *Stat6*, *Gata3*, *Stat5a*, and *Il-1β* genes found in the preconception e-cig-exposed dams, *Tgfß-1* and two Th2-related cytokines (*Il-5* and *Il-13*) were also downregulated in the respective offspring (Fig. 5*A*). Given its immune suppressive properties, *Tgfß-1* regulates the homeostasis of immune cells proliferation (39, 52). Also, during lung morphogenesis *Tgfß-1* is crucially involved in epithelial-mesenchymal interactions, particularly relating to epithelial branching points (92). It has previously been reported that a decrease in *Tgfß-1* signaling resulted in alveolar immaturity in mice (1). In our prenatal e-cig-exposed offspring, among the 7 dysregulated genes, *Il-5*, *Il-13*, *Stat5a*, and *Hmox1* were upregulated (Fig. 5*B*). This suggests that in the lungs of offspring, at the molecular level, prenatal e-cig exposures can activate Th2 innate immune responses (*Stat5a*, *Il-5*, and *Il-13*), which are associated with eosinophilia, a hallmark feature of asthma (49). These results are similar to those obtained by Chen et al. (15), who found increased gene expression of *Il-5*, *Il-13*, and *TNF*-α in the lungs of 1-day-old offspring exposed to nicotine-free tobacco-flavored e-cig aerosols in utero (15). Overall, the data suggest that in our model, preconception and prenatal e-cig aerosol exposures can modulate maternal-fetal immune interactions, resulting in two types of responses: with preconception exposures leading to immunosuppression that may potentially affect fetal lung organogenesis (further discussed below) and prenatal exposures promoting Th2 inflammation and oxidative stress responses in the developing lungs that may potentially predispose to early-onset asthma.

Further, to evaluate the degree to which in utero exposures to e-cig aerosol impaired lung structural development in the offspring, we assessed lung morphometric measurements. As mentioned previously, lung organogenesis is divided into four distinct molecular and histological stages, and at PND0, the lungs of mice are in the saccular stage (95, 108). Anatomically, this stage is characterized by the thinning of the interstitial tissue, which by reducing the thickness of the interstitium and mesenchyme, enables the airways and future alveolar walls to lengthen and enlarge, subsequently leading to the formation of the airspaces that are vitally required for gas exchange (47, 95, 108). As shown in Figs. 6, *A* and *B*, we found that in the preconception e-cig-exposed PND0 offspring, the lung tissue fraction was significantly increased and the airspace fraction significantly decreased compared with their respective air control group. These anatomical changes evidenced by increased lung tissue fraction suggest that the interstitium of the preconception e-cig-exposed offspring's lungs had not yet fully progressed into the thinning phase; thus, this exposure delayed the normal developmental processes associated with the lungs'

saccular stage. Moreover, molecular markers of lung morphogenesis, including lung gene expression of *Notch2* and the immunohistochemical staining of Club cells, were altered by preconception e-cig exposures (Fig. 6, *C–E*). In epithelial differentiation during embryonic lung development, *Notch* signaling regulates the fate of ciliated and secretory cell populations (103). The major role of this pathway is to prevent cell differentiation and maintain stem cell populations (101). Activation of this pathway leads to the suppression of differentiation, while deactivation leads to targeted cellular differentiation (101). In our in utero e-cig exposure model, we found that the PND0 offspring from the preconception e-cig exposure group exhibited a 2.8-fold downregulation of the *Notch2* gene (Fig. 6*C*). In human adult lungs, *Notch* signaling-related genes are downregulated in smokers and, to a greater extent, in smokers with chronic obstructive pulmonary disease (COPD), whose lungs are usually characterized by atypical epithelial differentiation (101). It is thought that this downregulation allows for epithelium repair and cellular differentiation (101). Our results suggest that the preconception e-cig exposure may make the lungs prone to cell differentiation. In addition, the predominant cell type in the bronchial epithelium is the Club cell (117). Club cells are nonciliated secretory cells that play key functions in *1*) regeneration of lung cells after injury, via *Notch* signaling-regulated differentiation into ciliated, goblet, and type II alveolar cells; *2*) immunomodulation; and *3*) detoxification (31, 102). Using immunochemistry techniques, we found that uteroglobin expression, a protein secreted by bronchial Club cells, was significantly increased in the lung tissue of the preconception e-cig-exposed offspring (Fig. 6, *D* and *E*). It was previously shown in mice genetically predisposed to epithelial cell dysfunction that hyperoxia-induced oxidative stress accelerated the proliferation of Club cells in the bronchial epithelium (31). In our model, the in utero e-cig exposure may have increased the proliferation of Club cells when compared with the air control group. Overall, our data indicate that preconception exposures to e-cig aerosol affect the lung structure of the offspring at PND0, by *1*) delaying thinning of the lung epithelium, characteristic of the saccular stage, and *2*) downregulating *Notch* signaling, resulting in epithelial cell differentiation, with increased expression of Club cell-secreted protein.

The lung structural changes induced by the preconception e-cig aerosol exposures were further supported at the molecular levels with the downregulation of key genes associated with lung morphogenesis, including *Shh* signaling (*Btrc*, *Ccnd1*, *Ccnd2*, *Gsk3b*, *Csnk1a1*, *Fbxw11*), *TGF-β* signaling (*Rhoa*, *Myc*, *Ep300*), *Notch* signaling (*Notch2*, *Ctbp1*, *Dvl1*, *Dvl2*), and *Il-17* signaling (*Fosl1*, *Jun*, *Gsk3b*), in addition to the majority of the genes relating to the *Wnt* signaling (Fig. 7). In line with these results, it was previously demonstrated that maternal cigarette smoke exposures suppressed *Wnt* signaling-related genes (*Foxa2*, *Fzd-7*, *Egf*, *Ctnnb1*, *Fn1*, and *Pdgfra*) in fetal lungs of exposed BALB/c mice at birth (23). Two of those six *Wnt*-related genes, *Fzd-7* and *Ctnnb1*, were similarly downregulated by in utero e-cig aerosol exposures, by negative three- and negative twofold, respectively, in our preconception group (Fig. 7*A*). Moreover, *Wnt/Notch* signaling-related genes (*Dll1*, *Notch2*, *Notch3*, *Hes5*, *Hey1*, and *Hey2*) are downregulated in the lungs of smokers as well as smokers with COPD (101). Taken together, these data suggest that cigarette smoke and e-cig aerosol can inhibit the expression of fetal and adult lungs' genes involved in the *Notch* and *Wnt*/β-catenin pathways. The *Wnt* signaling is essential for lung organogenesis, as it is active throughout the development of the fetal lung. In the embryonic lung, it regulates cellular fate, proliferation and differentiation, stem cell maintenance, and lung branching morphogenesis (41, 104). Numerous in vitro and in vivo studies using gene knockdown approaches as well as knockout and transgenic mouse models enabled isolation and identification of the roles and functions of specific *Wnt*-related genes in lung morphogenesis (41). For instance, using *Wnt2* and *Wnt2b* mutant mice, Goss et al. (37) demonstrated that loss of *Wnt2* resulted in lung hypoplasia (incomplete development of the lungs), while the double knockout (*Wnt2/2b*) mice exhibited lung agenesis (absence of bronchus, parenchyma and vessels) (37). In our study, the preconception e-cig exposure downregulated the gene expression of *Wnt2* and *Wnt2b* (−2.8 and −4.15-fold change, respectively), in the lungs of the PND0 offspring compared with their respective air control group (Fig. 7). Another study using $Wnt4^{-/-}$ mice revealed that fetal lungs had reduced cell proliferation in lung buds and exhibited significant pulmonary hypoplasia (12). We found that the preconception e-cig exposure downregulated *Wnt4* by −1.8-fold compared with air controls (Fig. 7). We cannot confirm that lung hypoplasia occurred in our study because we did not measure lung weight at birth; however, the downregulation of *Wnt2*, *Wnt2b*, and *Wnt4* (fold change range: −1.8 to −4.15; Fig. 7), which are all related to lung hypoplasia in mice, suggests that in our model in utero e-cig aerosol exposures may delay lung maturation processes and further supports our lung morphometry data (Fig. 6). Additionally, $sFrp1^{-/-}$ and $Lrp5^{-/-}$ mice display altered alveolar growth (29, 65), and in our in utero model, preconception exposures to e-cig aerosols decreased the expression of those 2 genes by −5.5- and −3.7-fold, respectively (Fig. 7). Also, *Wif1*-deficient mice exhibit impaired lung epithelial cells maturation (114). We had a −2.6-fold downregulation of *Wif1* in the lungs of preconception e-cig-exposed PND0 offspring (Fig. 7). Along with the downregulation of *sFrp1*, *Lrp5*, and *Wif1* (fold change range: −2.6 to −5.5; Fig. 7), this suggests that preconception e-cig exposures may impact alveolorization processes in addition to lung epithelial cell differentiation. Moreover, it was previously reported that *Wnt5a* knockout mice have atypical distal lung morphology, evidenced by increased enlargement of the distal airways, in addition to delayed lung maturation processes demonstrated by wider intersaccular intertitium (57). This further supports our preconception e-cig exposure lung morphometry results (Fig. 6, *A* and *B*), where, in the saccular stage, the lung tissue fraction was significantly increased in the e-cig-exposed PND0 offspring compared with their respective air control group, and this was accompanied by a −3.7-fold downregulation of the *Wnt5a* gene (Fig. 7). Thus, our data suggest that at both the anatomical (Fig. 6) and molecular (Fig. 7) levels, preconception exposures to e-cig aerosols affected lung developmental processes. Overall, the lung gene expression results for the preconception e-cig exposed group were further reinforced by the Ingenuity Pathway Analysis, which revealed that the interconnections between the 75 downregulated genes (Fig. 7) were related to decreased growth and proliferation of lung cells (Fig. 8). Taken together, our results suggest that in utero exposures to e-cig aerosols lead to altered

growth of the offspring's lung epithelium due to downregulation of *Shh* and *Wnt* pathways.

In addition, prenatal exposure to e-cig aerosols dysregulated the expression of only three lung genes by a fold change greater than two in PND0 offspring. These genes: *Mmp7*, *Wnt10a*, and *Frzb*, had an increased expression ranging from 2.0- to 4.1-fold (Fig. 7). Of note, in human samples, MMP7 protein expression in the lungs was increased in idiopathic pulmonary fibrosis (IPF) patients compared with controls (4). Additionally, *Wnt10a* and *Frzb* gene expression are increased in mice treated with bleomycin to induce IPF pathogenesis (21, 79). Expression of these 3 genes had returned to baseline levels by PND28, and only *Fosl1* was upregulated by 2.2-fold (Fig. 7). *Fosl1* lung gene expression is increased in mice treated with bleomycin and gefitinib, an epidermal growth factor receptor (EGFR) inhibitor (98). Furthermore, in our PND0 offspring prenatally exposed to e-cig aerosols, while no significant differences were observed at the transcription level for *Wnt5a* and *b*, *Dvl2* and *3*, or *Lrp6*, we found significant upregulation of those genes at the protein level (Fig. 7, *B* and *C*). Although the transcriptome and translatome are biochronologically related entities, mRNA and protein expression have been reported to be moderately-to-weakly correlated (38, 64, 94). Tian et al. (100) analyzed 425 pairs of mRNA-protein data sets from mouse cell lines and found a Pearson correlation coefficient (r_p) of only 0.59. More recently, with a data set of 6,672 mRNA-protein pairs, Schiller et al. (94) reported a Pearson correlation coefficient as low as 0.39 for significant transcriptome and proteome results in lungs of mice treated with bleomycin to induce IPF (94). They found that WNT3A was significantly regulated at the proteomic level, while not showing any significance at the mRNA level (94). This is similar to the results we obtained for WNT5A/B (Fig. 7). The biological reasons for the quantitative discrepancies between mRNA and protein expression levels are still not well defined; however, they include posttranscriptional and posttranslational mechanisms (e.g., changes in rates of mRNA and protein synthesis), as well as protein turnover involving protein half-life, resulting from synthesis and degradation processes (38, 64, 94). It is thought that the quantitative transcriptome–proteome relationship is better correlated for genes that are highly expressed rather than for genes that have a low expression level (38). Nonetheless, while transcriptomic results can suggest associations, it is the protein abundance within a cell that drives the molecular signaling and interactions (38). Overall in the present study, with the prenatal exposures to e-cig aerosols, the gene and protein expression results point in the same direction and suggest that this exposure may increase the expression of genes (and subsequently proteins) involved in lung development and diseases, including IPF.

Moreover, epidemiological and experimental studies showed that in utero exposures to CS can impact lung alveolorization processes (19, 24, 25, 67). In human infant lungs, it was previously shown that in utero exposures to CS resulted in increased distance of attachment points (25). Similarly, another study in guinea pigs demonstrated that in utero exposures to CS increased the distance between alveolar attachments at 27 days of age (24). In rats, in utero plus postnatal exposures to nicotine altered the offspring's lungs architecture by impairing alveolar development, evidenced by decreased radial alveolar counts (number of alveoli in the lungs) in the offspring exposed to nicotine, which resulted in emphysema-like injury (67). Another study in rats highlighted that maternal exposures to CS reduced the number of saccules and increased the size of these sacs, leading to lung hypoplasia in exposed offspring (19). In mice, the alveolorization period starts at PND4 and ends around PND28 (74, 108). In the study reported here, prenatal exposures to e-cig aerosols at PND28 increased mean linear intercept values (Fig. 6, *F* and *G*). Reduced alveolorization has been associated with decreased overall growth of newborns (25), and we found that offspring prenatally exposed to e-cig aerosols had a lower body weight at 28 days of age (Fig. 3*D*). It is possible that prenatal exposures to e-cig aerosols have a distinct and separate effect on alveolar formation, occurring during the alveolorization stage. A similar effect with in utero exposures to nicotine was observed in rats (67). Overall, we showed that prenatal exposures to e-cig aerosols led to a sustained lower body weight at PND28 and increased airspace size in the offspring's lungs. This indicates that prenatal e-cig exposures disturbed alveolar development and suggests that alterations in lung structure are sustained in early postnatal life, which may be a factor negatively affecting lung function later in life.

Limitations

This study was not designed to address the individual contribution of in utero exposures to cinnamon flavoring or nicotine-containing aerosols on the mouse lung development. We investigated the effects produced by an e-liquid that contained cinnamon flavoring chemicals and nicotine to recreate a 'real-world' exposure scenario where e-cig users are exposed to all of these harmful chemicals at once. The effects of in utero exposures to nicotine alone on the developing lungs have previously been reported elsewhere (50, 67, 89). However, the pulmonary effects following exposures to heated nicotine from a third-generation e-cig device, which are currently poorly documented, may differ. As mentioned above, cinnamaldehyde, the cinnamon flavoring chemical, is embryotoxic and cytotoxic to several lung cell types (2, 5, 7, 18, 33). Therefore, with its proven potent intrinsic toxicity, cinnamaldehyde may be primarily responsible for the effects on the developing lung reported in this study. Taken together, generalization of the results presented here must be made with caution, as they are most probably not representative of all e-cig aerosol exposures, whose toxicological effects may be dependent on e-cig device settings, PG/VG ratios, nicotine concentration, and the presence of flavoring chemicals (2, 5, 7, 18, 32, 33, 35, 36, 48, 96, 99).

Summary and Proposed Mechanisms

Preconception exposures to 36 mg/mL of nicotine cinnamon-flavored e-cig aerosol for 12 days before mating plus during gestational *days 1–19* in BALB/c dams significantly increased the lung gene expression of the nicotine receptor (α7nAChR), confirming the nicotine exposure (Fig. 2*B*), and increased the respiratory elastance of the lungs (Fig. 4*A*). Also, there was a strong negative correlation between each dam's serum PlGF concentration and respective litter birth length (Fig. 4*C*), in addition to increased levels of 17β-estradiol in the serum of the dams (Fig. 4*E*). It is known that nicotine can disrupt the endocrine environment during pregnancy (10, 46,

55), as evidenced here, and that this can decrease placental blood flow, leading to IUGR and resulting in the decreased birth weight and length in exposed offspring that we observed (Fig. 3, *B* and *C*). In the lungs of the dams and the offspring, preconception exposures to e-cig aerosols modulated the maternal-fetal immune interactions by suppressing the expression of lung genes related to immunity and inflammation (Fig. 5*A*). This is in line with recent studies showing that e-cig aerosols can have an immunosuppressive effect (18, 68, 110). In terms of fetal lung structure-associated anatomical and molecular changes induced by in utero e-cig exposures, we found that 75 lung genes essential to lung organogenesis and mostly related to the *Wnt* signaling were downregulated (Fig. 7). *Wnt5a* knockout mice have delayed lung maturation processes, as evidenced by wider intersaccular intertitium (57). The lungs of the PND0 offspring exposed preconceptually to e-cig aerosols were in the saccular stage and had a −3.7-fold downregulation of the *Wnt5a* gene, and their lung tissue fraction was significantly increased (Figs. 6 and 7). This confirms at both the anatomical (Fig. 6) and molecular (Fig. 7) levels that preconception exposures to e-cig aerosols impair fetal lung development. As per the Ingenuity Pathway Analysis, the relationships between those dysregulated genes (Fig. 7) were related to decreased growth and proliferation of lung cells (Fig. 8). Overall, our results suggest that preconception exposures to e-cig aerosols impair *Wnt* signaling during mouse lung development and alter fetal lung structure.

Conclusions

The results presented here strongly suggest that e-cig use during pregnancy may not be safe for the respiratory systems of either the mothers or the fetuses. Altered fetal lung development results in pulmonary immaturity and may predispose infants, children and even adults to lung diseases. Thus, this study helps provide scientific evidence for future regulations and public health policies on e-cigs, a new alternative tobacco product, with a particular focus on vulnerable populations, including pregnant women and their offspring. More research is needed to determine the long-term pulmonary effects associated with the molecular and epigenetic changes that are induced by in utero e-cig exposures.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.N. and A.P. conceived and designed research; A.N., S.H., A.Z., Z.P., and E.H. performed experiments; A.N., S.H., A.Z., Z.P., R.P., and R.X. analyzed data; A.N. interpreted results of experiments; A.N. prepared figures; A.N. drafted manuscript; A.N. and A.P. edited and revised manuscript; A.N., S.H., A.Z., Z.P., R.P., E.H., R.X., and A.P. approved final version of manuscript.

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