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Intrauterine exposure to nicotine through maternal vaping disrupts embryonic lung and skeletal development via the *Kcnj2* potassium channel

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Abstract

Smoking cigarettes during pregnancy is associated with adverse effects on infants including low birth weight, defective lung development, and skeletal abnormalities. Pregnant women are increasingly turning to vaping [use of electronic (e)-cigarettes] as a perceived safer alternative to cigarettes. However, nicotine disrupts fetal development, suggesting that like cigarette smoking, nicotine vaping may be detrimental to the fetus. To test the impact of maternal vaping on fetal lung and skeletal development in mice, pregnant dams were exposed to e-cigarette vapor throughout gestation. At embryonic day (E)18.5, vape exposed litter sizes were reduced, and some embryos exhibited growth restriction compared to air exposed controls. Fetal lungs were collected for histology and whole transcriptome sequencing. Maternally nicotine vaped embryos exhibited histological and transcriptional changes consistent with impaired distal lung development. Embryonic lung gene expression changes mimicked transcriptional changes observed in adult mouse lungs exposed to cigarette smoke, suggesting that the developmental defects may be due to direct nicotine exposure. Fetal skeletons were analyzed for craniofacial and long bone

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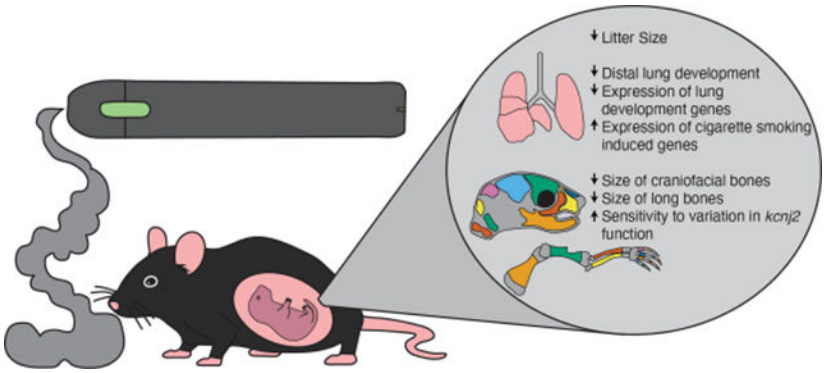
lengths. Nicotine directly binds and inhibits the Kcnj2 potassium channel which is important for bone development. The length of the maxilla, palatal shelves, humerus, and femur were reduced in vaped embryos, which was further exacerbated by loss of one copy of the *Kcnj2* gene. Nicotine vapor exposed $Kcnj2^{KO/+}$ embryos also had significantly lower birth weights than unexposed animals of either genotype. *Kcnj2* mutants had severely defective lungs with and without vape exposure, suggesting that potassium channels may be broadly involved in mediating the detrimental developmental effects of nicotine vaping. These data indicate that intrauterine nicotine exposure disrupts fetal lung and skeletal development likely through inhibition of Kcnj2.

Graphical Abstract



Nicotine vaping disrupts lung airway development, lung gene expression, craniofacial and long bone development, and increases sensitivity of skeletal consequences to variations in *Kcnj2* function.

Keywords

vaping; e-cigarette; nicotine; Kcnj2; Kir2.1; craniofacial; lung; skeletal; development

Introduction

Smoking cigarettes during pregnancy poses a substantial threat to the developing fetus including increased risk of premature birth, fetal or infant death and a range of birth defects [1,2], but the risks of vaping electronic-cigarettes (e-cigarettes, also termed “e-cigs”, “vapes”) are only beginning to be understood. E-cigs have recently increased in popularity, especially among young adults [3–6]. While about half of women cigarette smokers quit smoking during pregnancy [7,8] due to known risks [2,9–12], the prevalence of nicotine consumption via vaping does not decrease in pregnant populations suggesting that vaping is perceived to be safer during pregnancy [8,13]. The popularity of vaping among young people, the addictive nature of nicotine, and the lack of perceived risk suggest that vaping during pregnancy will likely increase over time. Identifying the effects of maternal e-cigarette exposure on fetal development is essential to inform public health messaging and protect offspring health.

E-cigarette liquid (e-liquid) contains propylene glycol, vegetable glycerin, flavorings, and nicotine [14]. Vaping devices deliver nicotine by heating the e-liquid to produce an inhalable

aerosol as opposed via tobacco combustion with traditional cigarettes. E-cigarettes often contain a higher dose of nicotine than traditional cigarettes [14]. Up to eight times higher nicotine concentrations were measured in rodents after e-cigarette exposure compared to cigarette exposure [15]. Nicotine passes through the placenta to fetal circulation where it can accumulate to reach higher levels than in the maternal plasma [16]. Nicotine can directly disrupt the development of multiple tissues and organs, including the fetal lung and skeletal system, which are especially vulnerable to gestational nicotine exposure [17].

The detrimental effects of maternal cigarette smoking on fetal lung development and adult lung disease are well established [18,19]. The risks of vaping came to attention in 2019 through a nation-wide outbreak of e-cigarette, or vaping, product use associated lung injury (EVALI), a rare, but severe lung disease linked only to certain products [20]. Chronic vaping is now known to be associated with lung disease [21]. The effect of maternal vaping on the developing lung is still under investigation. Mouse and human studies show disrupted alveolar and bronchial development, immune dysregulation, and increased incidence of lung disease in the offspring [22]. Studies have chiefly focused on long term functional outcomes or disruption of specific pathways. Whole transcriptome analysis in mouse embryos vaped during the entirety of gestation has not yet been reported. This is a critical gap in knowledge as transcriptional changes, potentially driven by epigenetic alterations acquired during maternal vaping, have been shown to lead to postnatal structural and functional lung defects [22].

Embryonic nicotine exposure has teratogenic effects on bone development [12,23]. Gestational delivery of nicotine in drinking water causes postnatal dysmorphic facial shape in mice, affecting the lower jaw (mandible) and cranial sutures [24,25]. Additionally, intraperitoneal or subcutaneous delivery of nicotine reduces the size of the palatal shelves and increases incidence of cleft palate[26,27]. Nicotine inhibits *Kcnj2* potassium channels [28], which are important for craniofacial and axial skeletal development suggesting a potential mechanism by which fetal nicotine exposure could impair skeletal development [29–33]. If maternally vaped nicotine reaches the developing fetus, it may inhibit *Kcnj2* channels to disrupt skeletal development. While the role of *Kcnj2* has not been established in lung development, this ion channel and other Kcnj family members are expressed in the lung [34,35], and thus could potentially mediate the effects of nicotine on lungs as well.

Here, we used a mouse model of maternal vaping to test the hypothesis that moderate daily exposure to nicotine vapor during the entirety of gestation disrupts fetal lung and skeletal development assessed at E18.5, the last day of *in utero* mouse development. We show that maternal vaping during pregnancy results in decreased litter size with some vape exposed embryos experiencing severe growth restriction. Vaped E18.5 lungs displayed defective distal lung development with decreased airspaces. We found *Kcnj2* knockout ($Kcnj2^{KO/KO}$) lungs to have defective lung development with decreased airspaces, but this phenotype is not enhanced by exposure to maternal vaping. Wildtype maternally vaped lungs have transcriptomic changes consistent with broadly disrupted developmental signaling programs. Upregulation of pathways induced by cigarette smoke in the adult mouse lung in maternally vaped embryonic lungs suggests maternal nicotine vaping directly disrupts embryonic lung development. Embryos exposed to nicotine vapor throughout gestation had shorter femurs

and humerus bones and craniofacial differences that were enhanced by loss of one copy of *Kcnj2.* Loss of both copies of Kcnj2 removes the exacerbation of phenotypes upon vaping exposure. These results suggest that maternal nicotine vaping may directly impact embryonic development through inhibition of *Kcnj2* channels.

Methods

Mouse models and maternal vaping in mice

Wildtype (C57BL/6) and *Kcnj2*$^{KO/+}$ (FVB.129-Kcnj2$^{tm1Swz/J}$ Stock No. 005057/Kcnj2, Jackson Labs) dams were bred to wildtype and *Kcnj2*$^{KO/+}$ males. Pregnant dams were exposed to nicotine vapor in a Teague-TE2 smoking machine adapted for use with a commercially available e-cigarette containing 2.4% freebase nicotine. Mice used as controls were exposed to room air only in the same chamber. Dams were treated for 4 hours daily during pregnancy starting on the day the vaginal plug was observed (considered as embryonic day 0). In the four-hour exposure window, mice were exposed to four e-cigarette cartridges resulting in 96 mg (4.0 mL × 24 mg/mL) of total nicotine exposure. To measure biologically relevant nicotine exposure, a Cotinine ELISA (Calbiotech # CO096D) was performed on maternal blood plasma collected from tail bleeds on day 18.0 of gestation immediately following final e-cigarette exposure. ELISA assays were performed in duplicate. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Colorado School of Medicine in accordance with established guidelines for animal care.

Lung histology and measurement

The fetal left lung was isolated, fixed overnight in formalin and embedded in paraffin. Airspace areas were quantitated in H&E-stained tissue sections using the Trainable Weka Segmentation plugin in ImageJ (NIH) by a blinded examiner. High resolution images of lung sections were overlayed by a mask to remove cellularized areas of the tissue from analysis. Airway and blood vessel lumens were manually excluded from the analysis. Area measurements were obtained using the Measurement plugin in ImageJ.

Lung whole transcriptomic analysis

The fetal right lungs were isolated, preserved in RNAlater solution (Thermo Fisher), and immediately frozen at −80C. Total RNA was isolated at the same time from all samples using the RNeasy Mini Kit (Qiagen). Only RNA with RIN score > 8 was used. Library prep and sequencing of 40 million paired end reads was carried out by the University of Colorado Genomics Shared Resource Facility. Differential gene expression and pathway enrichment analysis was carried out using standard bioinformatics pipelines by the University of Colorado Cancer Center Biostatistics and Bioinformatics Shared Resource Facility.

Skeletal Staining and measurement

Embryos for skeletal staining were collected at E18.5. Alizarin red and alcian blue skeletal staining was performed as previously described[29]. Quantification of skeletal stain measurements was performed in ImageJ. *Fontanelle area*: Fontanelle area was defined as the total area in millimeters (mm^2) between the frontal bones from a dorsal view of the

skull. *Premaxilla Area:* Premaxilla area was defined as the total area of the premaxilla in mm^2 when seen from a lateral view. *Premaxilla length*: Premaxilla length was determined by drawing a line straight from the anterior superior tip of the premaxilla to the maxilla. *Palate shelf length:* Palate shelf length was determined by measuring the length between the posterior-medial point of the horizontal plate of the palatine bone and the anterior-medial point of the horizontal plate of the palatine bone. *Mandible height*: Mandibular height was defined as the distance between the molar alveolus of dentary and the inferior point of the mandibular body. *Mandible length*: Mandibular length was determined by measuring the length between the most anterior point of the mandible and the posterior point of the condylar process. *Femur and humerus lengths*: Femur and humerus bone lengths were measured as the total length between the anterior and posterior most points of ossification. *Femur* width: Femur width was measured as the width at the medial point of the ossified shaft of bone. *Humerus width*: Humerus width was determined by measuring the humerus at the widest point of the deltoid tuberosity. Example measurements for each metric can be seen outlined in red or yellow on the WT images of the respective structures (Fig. 7–8)

Statistical Analysis

T-tests were used to determine statistical significance across genotype and treatments. On bar graphs, error bars represent standard deviation. Box and whisker and violin plots are plotted as minimum value to maximum value. A p value of <0.05 was considered statistically significant in this study.

Results

Maternal nicotine vaping in mice leads to disrupted fetal development

We modified a Tegue-TE2 smoking machine for use with e-cigarettes (Fig. 1A). Two e-cigarettes are placed in the top of the machine, aerosol is drawn into the mixing chamber, and a vacuum draws the aerosol over the mice in the exposure chamber. We exposed pregnant wildtype (C57BL/6) and *Kcnj2*$^{KO/KO}$ dams to room air or e-cigarette vapor containing 2.4% nicotine throughout gestation with 4 second puffs and 20 second interpuffs for 4 hours per day. Exposure began upon observation of the vaginal plug (E0.5) and terminated on E18.0. At E18.5, embryos were collected, lungs were harvested for histology and whole transcriptome sequencing and skeletons were isolated and stained with alcian blue and alizarin red. Exposure of dams to e-cigarette vapor was confirmed through maternal plasma cotinine levels. Exposed dams had plasma cotinine levels of 35.12±6.59 ng/mL, n=3 litters (Fig. 1B). While we did not collect enough plasma from pups to measure their cotinine levels, others have shown that nicotine crosses the mouse placenta efficiently to the extent that cotinine levels are approximately equal in pup plasma as in their nicotine-dosed dams [26,36]. Active smokers are considered to have cotinine levels higher than 10 ng/mL, but heavy smokers can have levels up to 500 ng/mL [37]. Similar to mice, nicotine crosses the human placenta, but in humans, fetal nicotine levels are up to 15% higher than maternal concentrations [16]. As a result, our study represents a low to moderate exposure of nicotine.

We measured litter size and embryo weight to determine how e-cigarettes effect fetal outcomes in wildtype mice. Some embryos from nicotine vaped litters were severely

underdeveloped compared to littermates (Fig. 2A–B). There was no relationship between underdeveloped embryos and location in the uterine horns. We found that litters from nicotine vaped embryos were significantly reduced in size (7.8±1.6 pups, n=6 vs 6±1 pup, n=3, p=0.03 by t-test) (Fig. 2C). We did not observe a significant overall difference in birthweights of nicotine vaped embryos (1.27±0.11 g, n=29 vs 1.23±0.22 g, n=12 p=0.27, Fig. 2D). The number of embryos and dams analyzed are represented in Table 1.

Maternally nicotine-vaped embryos have defective distal lung development

To examine the impact of maternal vaping on respiratory development, lungs were isolated from room air-exposed (termed to as "control") and vaped wildtype and *Kcnj2*$^{KO/KO}$ E18.5 embryos. The solitary left lung was formalin-fixed and paraffin embedded to evaluate tissue structure in H&E stained tissue sections (Fig. 3A–3E). At E18.5 the embryonic lung is undergoing the saccular stage of development, during which the alveolar sacs start to form in preparation for air breathing and gas exchange [38]. This requires the progressive thinning of the interstitium and enlargement of airspaces (known as saccules), which are readily apparent on histology during normal mouse development. We quantitated airspace area in lung tissue sections and found that vaped wildtype lungs had reduced airspace area compared to controls (1588 +/− 43.58 AU vs 1330 +/− 34.87, p<0.0001) (Fig. 3F). Lack of saccular enlargement due to maternal vaping may represent either a general delay in lung development or a specific disruption of saccular refinement.

To test the possibility that maternal nicotine vaping effects lung development through inhibition of *Kcnj2*, we assessed overall lung histology and quantitated airspaces in control and vaped *Kcnj2*$^{KO/KO}$ lungs. We found that control *Kcnj2*$^{KO/KO}$ mice had severely abnormal lungs with significantly smaller airspaces than wildtype control lungs (1332 +/− 41.33 AU vs 1588 +/− 43.58 AU, p=0.0033) (Fig. 3F) indicating a novel role for *Kcnj2* in lung development. Interestingly, airspace area of *Kcnj2*$^{KO/KO}$ lungs was not significantly different between control and vaped embryos (1332 +/− 41.33 AU vs 1419 +/1 31.29 AU, p=0.086), suggesting that *Kcnj2* loss of function is at least in part responsible for the lung phenotypes observed (Fig. 3F).

To characterize the lung development defect, the right lungs from vaped and control embryos were used for whole transcriptome RNA sequencing (RNAseq). Differential gene expression analysis demonstrated that vaping lead to 1,483 upregulated and 1,080 downregulated genes (log2 fold change>1; FDR > 0.05). Pathway analysis revealed that downregulated genes were highly enriched for pathways controlling lung development, consistent with a general developmental delay phenotype [38] (Table 1). We used a published list of 391 mouse lung developmental genes [39] to show that their differential expression clearly distinguishes vaped versus control samples, with most genes being downregulated (a subset is presented in Fig. 4A). These included genes regulating epithelial structure and the actin cytoskeleton, and developmental signaling pathways like Notch, BMP/TGF-beta, Wnt, and Hedgehog signaling (Fig. 4A–F). This is consistent with the defective or delayed lung development demonstrated by histology (Fig. 3A–B). Wnt signaling is known to be dysregulated in association with e-cigarette use and cigarette smoking [40]. Our data indicate a broad disruption of developmental signaling pathways that control lung morphogenesis

by maternal vaping during pregnancy. Additionally, Kcnj channels, including *Kcnj2* were downregulated in the lungs of WT vaped embryos (Supp. Fig. 1).

We also observed a marked reduction in genes that control ciliogenesis and ciliated cell formation in the vaped wildtype lungs (Fig. 5A). Ciliated airway epithelial cells are fundamental to host defense through the mucociliary clearance of inhaled contaminants [41]. Ciliated cells first appear at E16.5 and continue to form through postnatal development [42]. Consistent with our transcriptomic data, we observed overall fewer ciliated cells and more immature ciliated cells with short cilia in the vaped compared to control embryos (Fig. 5B–C). These data indicate that airway epithelial differentiation is disrupted by maternal vaping.

Transcriptomic response in maternally vaped embryonic lungs is similar to cigarette smoke exposed adult mouse lungs

Maternal nicotine vaping upregulated multiple pathways in the embryonic lung including those related to mitochondrial dysfunction, protein expression and degradation, metabolism, and DNA damage (Table 2). This suggested that the embryonic lung tissue may be directly damaged by exposure to vape components, which may lead to the developmental defects observed by histology and downregulated developmental gene expression (Fig. 4A–F). We found an overlap in the genes altered by maternal nicotine vaping in embryonic lungs and those altered by directly inhaling cigarette smoke in the adult lungs (Fig. 6A, Supplemental Table 1) [43,44]. Cigarette smoking gene signature list is shown in Supplemental Table 1. We showed that similar to cigarette smoking, the glutathione metabolism, pyrimidine metabolism, and phosphotidylinostitol signaling pathways were also upregulated by maternal vaping (Fig. 6B–D, Supplemental Table 1). This conserved response to exposures indicates that vape products likely reach and directly impact the developing lung. The developmental phenotypes are not simply due to maternal effects (ex. placental restriction) [40,45–47]. Further, it suggests that maternal vaping and adult smoking may disrupt lung structure and function through at least partially overlapping mechanisms.

Nicotine-vaped embryos have craniofacial defects that are exacerbated by reduced Kcnj2 function

We measured the length of several craniofacial structures to determine how embryonic exposure to e-cigarettes impacts craniofacial outcomes in wildtype and *Kcnj2* heterozygous ($Kcnj2^{KO/+}$) and homozygous knockout ($Kcnj2^{KO/KO}$) animals. WT vaped embryos had a reduction in premaxilla area and length compared to WT control embryos (2.28±0.10 mm^2, n=4 vs 2.049±0.18 mm^2, n=5 and 1.11±0.022 mm, n=4 vs 1.027±0.069 mm, n=5, Fig. 7A'–B', 7H–I). Fontanelle area, palate shelf length, and mandibular ramus height were not significantly different in WT vaped embryos (Fig. 7A–A", 7B–B", 7G, 7J–L). Interestingly, *Kcnj2* heterozygous animals were sensitized to the effects of nicotine vapes while *Kcnj2* homozygous knockout littermates were not affected. Premaxilla area and length phenotypes were exacerbated in vaped $Kcnj2^{KO/+}$ mice compared to control $Kcnj2^{KO/+}$ mice (2.13±0.17 mm^2, n=5 vs 1.75±0.18 mm^2, n=5 and 1.03±0.053 mm, n=5 vs 0.92±0.058 mm, n=5, Fig. 7C'–D', 7H–I). Gestational vaping caused the emergence of additional phenotypes in $Kcnj2^{KO/+}$ offspring. Fontanelle area was significantly larger in nicotine

vaped *Kcnj2*$^{KO/+}$ mice (1.27±0.15 mm^2, n=5, vs 2.15±0.34 mm^2, n=5, Fig. 7C–D, 7G). Both mandible length and mandibular ramus height were significantly reduced in nicotine vaped *Kcnj2*$^{KO/+}$ mice specifically (5.34 ±0.22 mm, n=5, vs 5.12±0.16 mm, n=5 and 1.39±0.084 mm, n=5, vs 1.28±0.053 mm, n=5,Fig. 7C'–D', 7K–L). Additionally, *Kcnj2*$^{KO/+}$ embryos had significantly smaller palatal shelves when exposed to nicotine vapes (1.27 ±0.072 mm, n=5, vs 1.075±0.069 mm, n=5, Fig. 7C"–D", 7J). These craniofacial phenotypes are shared with *Kcnj2*$^{KO/KO}$ mice. However, craniofacial phenotypes of *Kcnj2*$^{KO/KO}$ mice were not exacerbated by maternal nicotine vaping. Fontanelle area, mandible length, and mandibular ramus height were not significantly different between treatments in *Kcnj2*$^{KO/KO}$ mice (Fig. 7E–E", 7F–F", 7G, 7K–L). Premaxilla and palate measurements were not taken from *Kcnj2*$^{KO/KO}$ mice because these structures are absent in embryos of this genotype (Fig. 7E'–F' and 7E"–F"). Importantly, these craniofacial measures were significantly decreased between vaped wildtype and vaped *Kcnj2*$^{KO/+}$ offspring (Fig. 7), while they are largely unchanged when animals are not exposed to nicotine (Fig. 7). Together, these data suggest that inhibition of Kcnj2 is responsible for nicotine-induced craniofacial defects in offspring.

Nicotine-vaped embryos have shorter femurs and humerus bones that are exacerbated by reduced Kcnj2 function

We measured the length of the humerus and femur to determine how fetal exposure to nicotine vapor impacted long bone growth and development in wildtype and *Kcnj2* heterozygous and homozygous knockout animals. We found that humerus length was significantly reduced in nicotine vaped wildtype mice compared to control mice (2.61±0.021 mm, n=29, vs 2.49±0.052mm, n=12, Fig. 8A–B, 8G). The humerus length was significantly more reduced when animals lacked one copy of *Kcnj2* (2.63±0.03 mm, n=14, vs. 2.37±0.01, n=5, Fig. 8C–D, 8G). However, the reduction in humerus length between vaping treatments was not significant in *Kcnj2*$^{KO/KO}$ embryos (2.59±0.047 mm, n=4, vs 2.43±0.071 mm, n=4, Fig. 8E–F, 8G). Femur lengths were also significantly reduced by exposure to fetal exposure to nicotine vapor in wildtype (2.17±0.02 mm, n=29, vs 2.04±0.05 mm, n=12, Fig. 8A'–B', 8H). While *Kcnj2*$^{KO/+}$ mice do not have significantly shorter femurs from wildtype, pups that were exposed to 2.4% nicotine vapor throughout gestation had a more significant reduction in femur length than *Kcnj2*$^{KO/+}$ room air controls (2.16±0.034mm, n=14 vs. 1.98±0.024 mm, n=5, Fig. 8C'–D', 8H'). Like the humerus, there was no significant difference in femur length in *Kcnj2*$^{KO/KO}$ embryos (Fig. 8E'–F', 8H). Long bone lengths in vaped *Kcnj2*$^{KO/+}$ offspring were significantly reduced from un-vaped wildtype controls, but not significantly decreased from vaped wildtype. Together, these data suggest that nicotine-induced inhibition of Kcnj2 reduces long-bone length in offspring.

Discussion

Here, we provide evidence that maternal nicotine vaping can cause fetal lung and skeletal defects. We show that embryos from nicotine vape exposed dams are occasionally growth restricted consistent with developmental delay and growth restriction data from human maternal cigarette smoking [6,48]. One in five babies born to mothers who smoke cigarettes during pregnancy present with low birth weight [6,48]. The phenotypes seen in the severely delayed embryos resemble those of embryos that have been exposed to high levels of

nicotine during gestation [26]. We did not see a significant decrease in weight in the embryos exposed to nicotine vapor. Gestational e-cigarette exposure at cotinine levels four times higher than our model reduced birth weight [47,49]. Cigarette smoking during pregnancy decreases fetal birth weight in a dose dependent manner [50]. Perhaps higher doses of nicotine would significantly affect fetal birth weight. Our data are valuable in parsing the effects of vaping nicotine during pregnancy in low to moderate smoking populations. Our study is differentiated from other rodent maternal vaping paradigms by daily 4h nicotine exposure during the entirety of gestation, in contrast to other studies which only exposed the embryos during smaller developmental windows or used intermittent dosing [47,49,51,52]. Further, we specifically focus on the mouse E18.5 timepoint, considered as the last day of embryonic development, to assess developmental outcomes without confounding effects from fetal loss or postnatal development or adaptation.

We show that at E18.5, maternally vaped wildtype mouse lungs have smaller airspaces compared to room air exposed mice. This is consistent with disruption of the saccular stage of lung development (E17.5 to P5), during which alveolar spaces or “sacs” form, then expand through the thinning of the inter-airspace septa [38]. A separate study, which exposed embryos to e-cigarette vapor only from E6 to E19 daily for 2h also noted thickened septa at P0 [49]. Due to long term exposure during the entirety of lung development, we cannot conclude that vaping specifically disrupts sacculation. The observed phenotypes are also consistent with a general lung developmental delay that may have multiple drivers. Interestingly, data suggest that these defects may be overcome in the postnatal period, as another study using the 2h daily gestational dosing model reported no histological differences at P5 and P11 [47]. Vaped mice that occasionally display a growth restriction phenotype (see above) had even smaller airspaces. We speculate that such animals are likely to have difficulties with or may be unable to transition to air breathing upon birth.

The developing lung expresses a variety of Kcnj channels, including *Kcnj2*, which is broadly expressed by epithelial, endothelial, and mesenchymal cell types [34,35]. We find that room air control $Kcnj2^{KO/KO}$ embryos have disrupted distal lung development compared to wildtype embryos. This is a novel finding and adds to the growing field of ion channel control of morphological development. It has been previously shown that homozygous ablation of another Kcnj channel, *Kcnj13* (Kir7.1), which is strongly expressed by cells of the developing lung buds, causes moderate retardation of lung development similar to what we see in our vaping model [35,53,54]. Ion homeostasis via Kcnj channels is necessary for actin driven cell shape changes during lung morphogenesis [53,55]. It is likely that additional Kcnj channels have a role in lung development.

To determine if nicotine vapor impairs lung development through *Kcnj2*, a nicotine-suppressed receptor, we quantified airspace areas from control and vaped $Kcnj2^{KO/KO}$ embryos. Interestingly, vaped $Kcnj2^{KO/KO}$ mice do not have a significant reduction in airspace area when compared to control $Kcnj2^{KO/KO}$ embryos. Although the room air control $Kcnj2^{KO/KO}$ lungs are moderately disrupted, the lack of significantly different phenotype in vaped $Kcnj2^{KO/KO}$ lungs raises the possibility that *Kcnj2* is a target of nicotine to delay lung development.

Underlying the aberrant lung architecture, we show large scale disruption of gene expression in maternally vaped wildtype embryos. Gene expressions changes were dominated by markedly downregulated developmental signaling pathways such as Notch, Wnt, TGFbeta/BMP, and Hedgehog, which are well known regulators of lung development [35,38,39,56]. These complex programs cooperatively drive the formation, elongation and branching of the airways followed by the creation and subsequent enlargement of the airsacs. We show downregulated canonical Wnt signaling (reduced target genes *Axin2*, *Myc*, *Ccnd1*). Wnt signaling plays a critical role in sacculation, including the differentiation of alveolar epithelial cells that mediate gas exchange. Consistent with our data, candidate-based gene expression studies in other maternal vaping models also showed disrupted Wnt signaling [47,49]. BMP signal transduction components, including several BMP ligands and Smad genes, were significantly downregulated in vaped embryos. Additionally, *Kcnj* genes were downregulated within the lung as well. Loss of *Kcnj2* decreases BMP signaling in the palate [29]. We show that loss of Kcnj2 severely disrupts lung development. Future studies are needed to determine if Kcnj2 contributes to BMP signaling in the lung. RNAseq data also showed a broad disruption of the Notch pathway in vaped lungs. Notch is most well-known as the regulator of the ciliated vs. secretory cell fate decision in the developing airways [56]. Consistently, we see a marked downregulation of ciliated cell related transcripts by vaping. Ciliated cell loss and aberrant Notch gene expression has also been observed in smokers and in patients with chronic obstructive pulmonary disease [57]. Consistent with our transcriptional data, we observed fewer ciliated cells in maternally vaped fetal lung tissue.

Our gene expression data show broadly upregulated DNA damage and repair pathways. Recent studies demonstrated that nicotine and other e-cigarette additives can cause DNA damage in lung cell lines [58]. We also show that gene expression changes strongly overlap between maternally vaped embryonic mouse lungs and adult mouse lungs exposed to cigarette smoke [43]. Both models show dysregulation of pyrimidine and glutathione metabolism and phosphatidylinositol signaling pathways. This supports our conclusion that disruption of mouse lung developmental programs by maternal vaping results from direct exposure of embryonic lung tissues to e-cigarette vape components introduced from the maternal circulation via the placenta. While mechanisms are not yet clear and likely to be complex, we suggest that disruption of lung development may occur through the deleterious effect of nicotine, the shared component in vapes and traditional cigarettes and which readily crosses the placenta [16].

Maternal nicotine use has teratogenic effects on embryonic bone development [12,23]. Our data adds to a body of work strengthening the evidence that nicotine exposure, either through cigarette smoking or vaping, during embryonic morphogenesis has adverse effects on craniofacial and skeletal development [59,60 26 61]. Our data suggest that nicotine induced skeletal defects occur at least in part through *Kcnj2* inhibition. First, nicotine inhibits *Kcnj2* currents at plasma concentrations that are found in people who smoke or vape nicotine [28]. Second, fetal nicotine exposure causes craniofacial defects in mice that phenocopy homozygous loss of *Kcnj2* including reduced size of the pre-maxilla, maxilla, mandibles, palate, and increased the size of the fontanelle in mice [29,30,33]. Third, clinical studies have shown that fetal nicotine exposure through maternal cigarette smoking is associated with smaller stature and increased incidence of cleft palate and other craniofacial abnormalities

[48,62]. Fourth, *Kcnj2* disruption in humans causes similar phenotypes to the defects that are caused by nicotine exposure by cigarette smoking in humans including palatal and dental defects, smaller mandible, limb abnormalities, and shorter stature [31,32,63,64]. Given that *Kcnj2* regulates BMP signaling for craniofacial patterning and bone development, we hypothesize that nicotine inhibits Kcnj2 to disrupt BMP-dependent skeletal development [29,30,65]. However, further studies testing whether nicotine-induced inhibition of Kcnj2 disrupts BMP signaling are needed to draw this conclusion. In summary, our data suggest that nicotine is a likely causative agent common to cigarettes and vapes to cause skeletal abnormalities.

Our work suggests nicotine exposure through maternal vaping crosses the placenta and directly hinders fetal lung development through down regulation of several developmental signaling pathways. Together with previously published research, our work supports a model in which nicotine inhibits *Kcnj2* channels to disrupt craniofacial and axial skeletal development (Fig. 9).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Maternal e-cigarette use disrupts lung development
- Maternal nicotine vaping decreases expression of lung development genes and increases expression of cigarette smoking associated genes
- Embryonic exposure to e-cigarettes decreases the size of craniofacial and long bones
- Loss of one copy of *Kcnj2* sensitizes embryos to the skeletal consequences of nicotine vaping

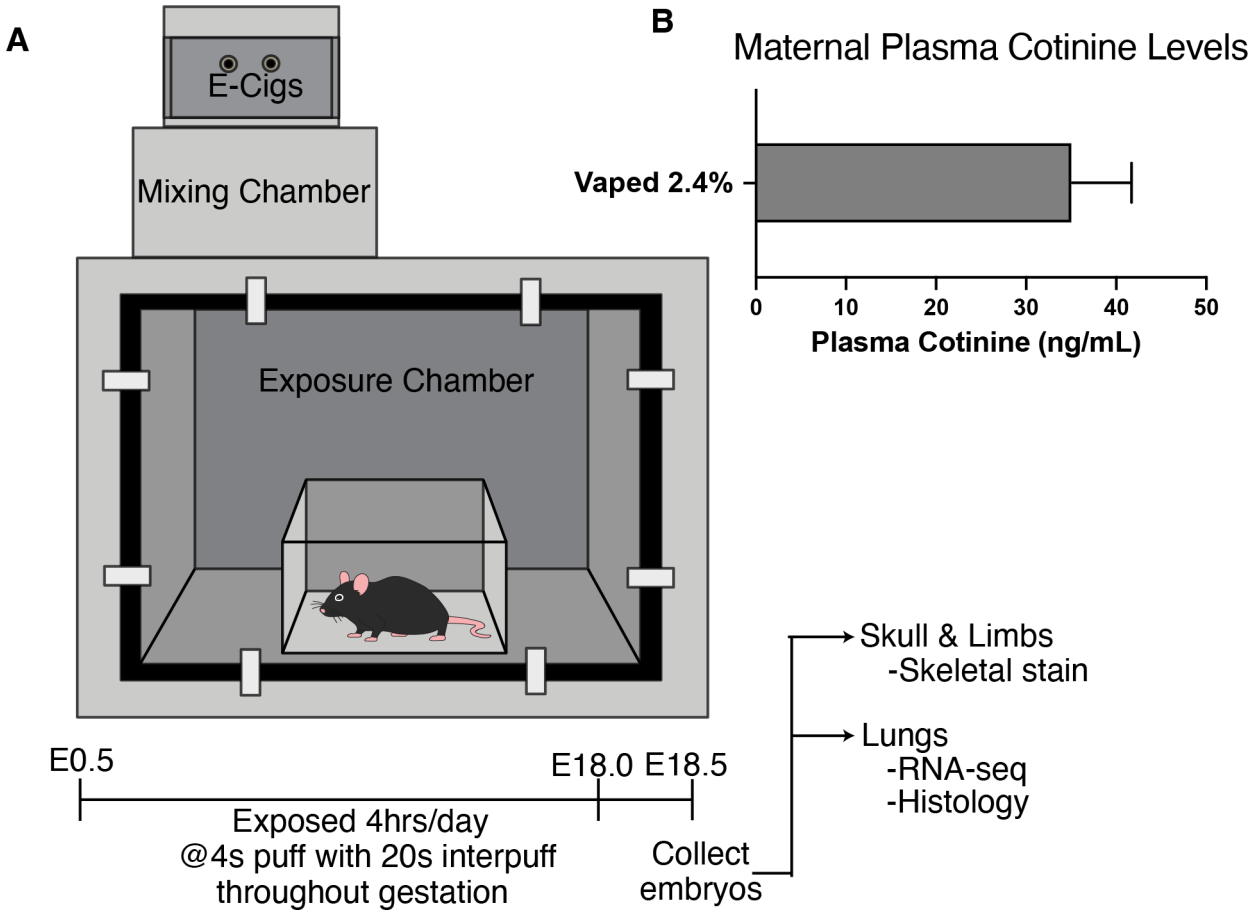

Figure 1: Exposure paradigm and plasma cotinine levels

A) Experimental design showing e-cigarette exposure chamber, exposure paradigm, and sample processing. B) Plasma cotinine levels from dams exposed to 2.4% nicotine vapes. Error bar represents SEM.

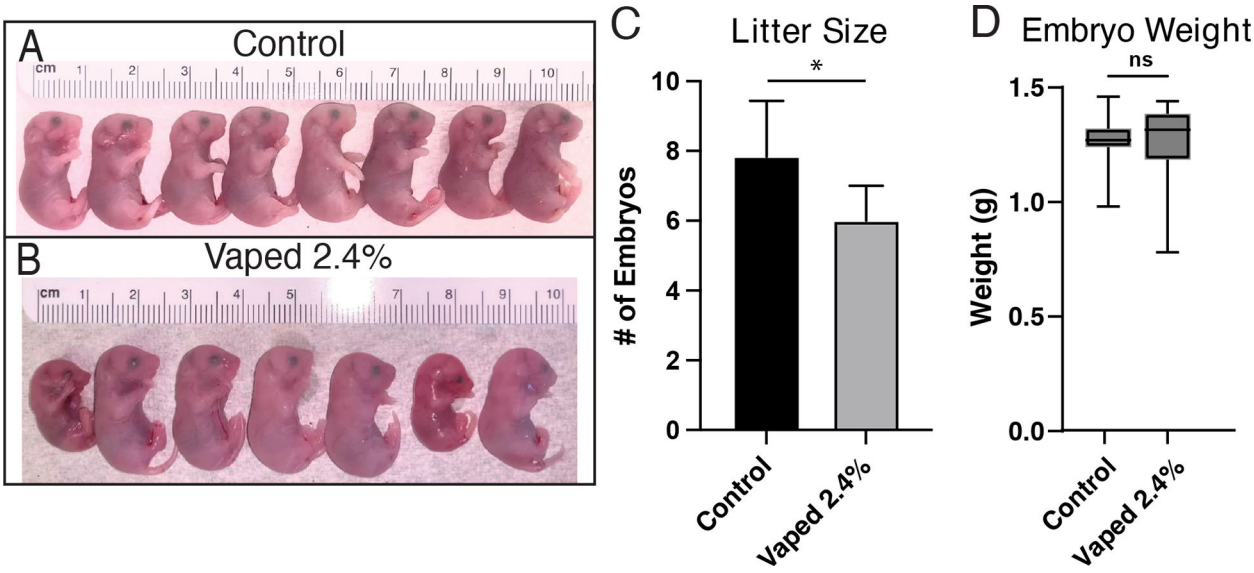

Figure 2: Nicotine vaping effects on litter size and embryo weight

A) Representative litter of E18.5 embryos room from air control dam. B) Representative litter of E18.5 embryos from 2.4% nicotine vaped dam. C) Representative litter weights from pictured litters in A and B. D) Number of embryos per litter from either room air control or 2.4% nicotine vaped dams. E) Weights of embryos from room air control or 2.4% nicotine vaped dams. (*$p<0.05$ by t-test, ns=not significant)

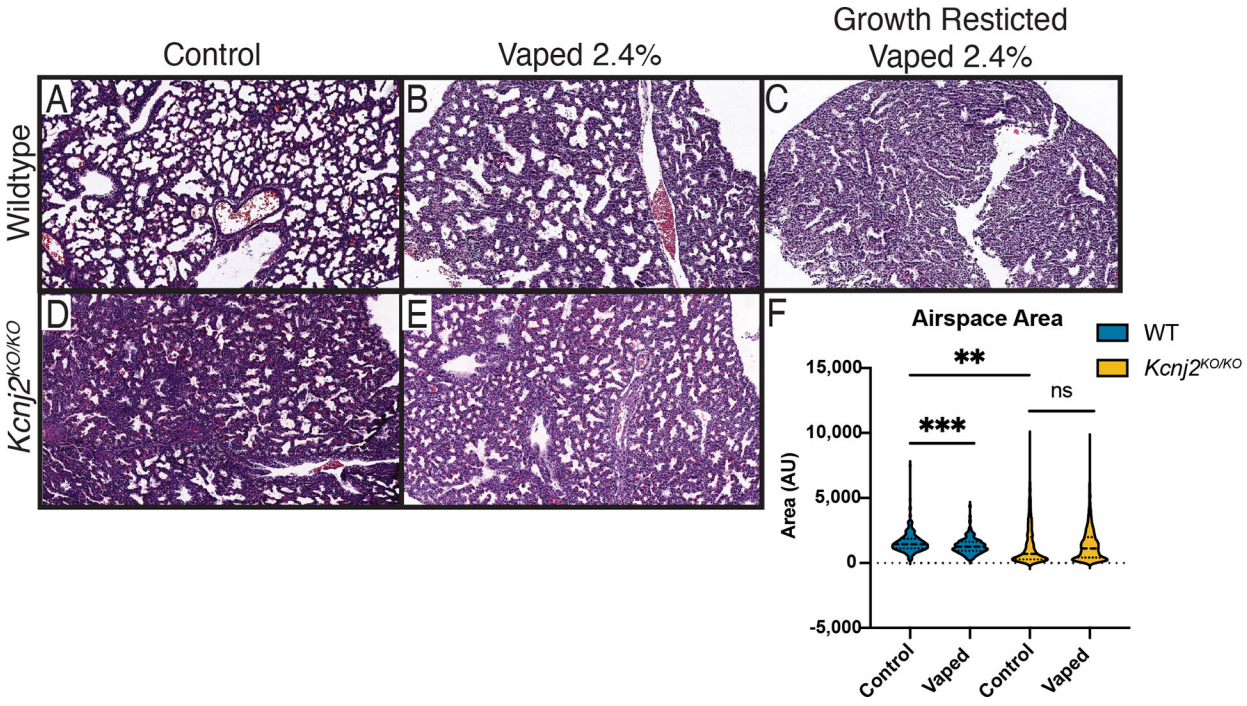

Figure 3: Maternal nicotine vaping disrupts fetal lung development

Representative H&E stained E18.5 lungs from a room air control wildtype embryo (A), normal sized 2.4% nicotine vaped wildtype embryo (B), growth restricted 2.4% nicotine vaped wildtype embryo (C), room air control *Kcnj2*$^{KO/KO}$ embryo (D), and 2.4% nicotine vaped *Kcnj2*$^{KO/KO}$ embryo (E). F) Quantification of airspace area of room air control vs 2.4% nicotine vaped wildtype and *Kcnj2*$^{KO/KO}$ embryos. (**p<0.005, ***p<0.0005 by t-test, ns=not significant)

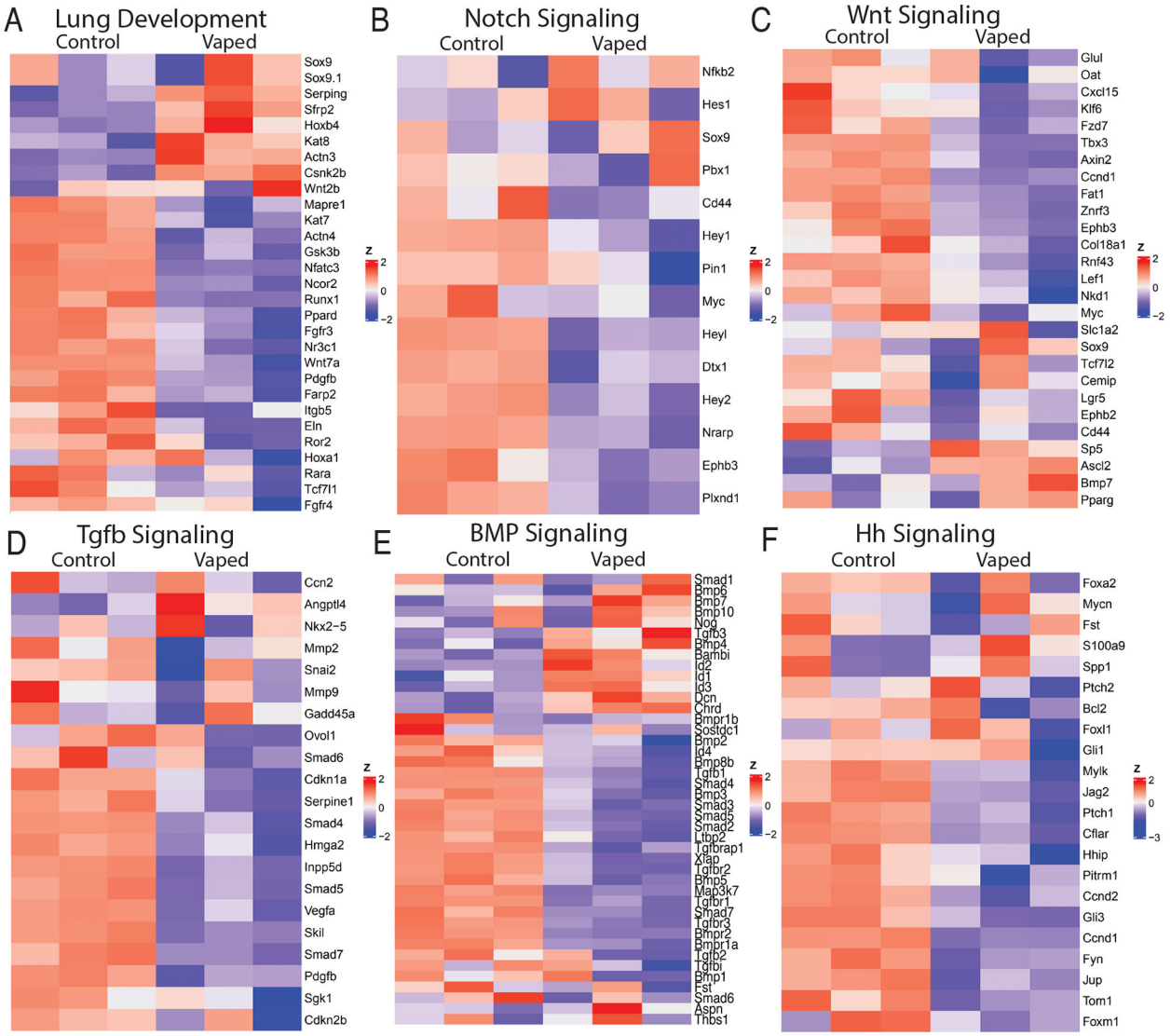

Figure 4: E-cigarette consequences on fetal airway developmental signaling Maternal nicotine vaping disrupts developmental gene expression in the lung.

A) Heatmaps of differentially expressed genes in control and 2.4% nicotine vaped wildtype embryos show disruption of lung development related genes (A) and genes of the Notch (B), Wnt (C), Tgfb (D), BMP (E), and Hh (F) signaling pathways.

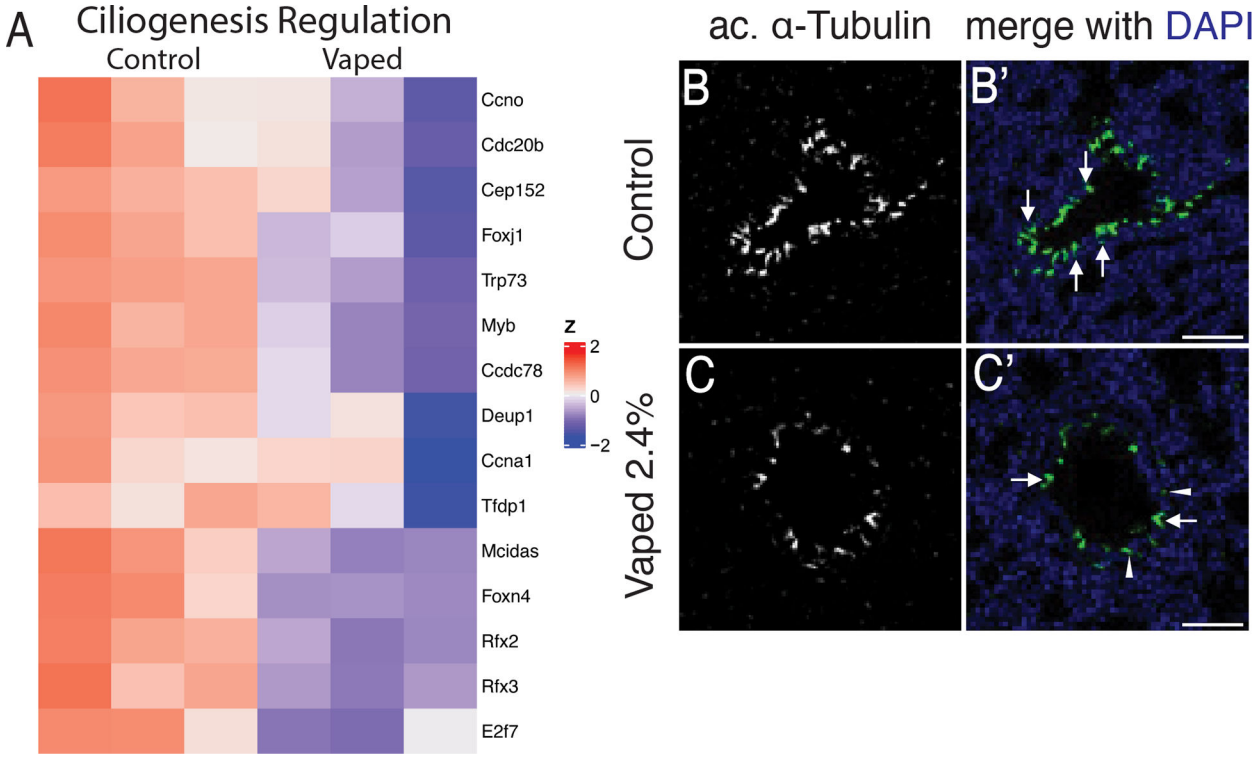

Figure 5: Maternal nicotine vaping leads to reduced ciliated cell formation in embryonic airways

A) Heatmap of differentially expressed ciliogenesis regulation RNAs in control and 2.4% nicotine vaped wildtype embryos. B) Acetylated alpha-Tubulin (green) labeling shows that vaped lungs contain overall fewer ciliated cells and more immature (arrowhead) ciliated cells compared to control lungs that contain mostly mature (arrow) ciliated cells. DAPI (blue) labels nuclei.

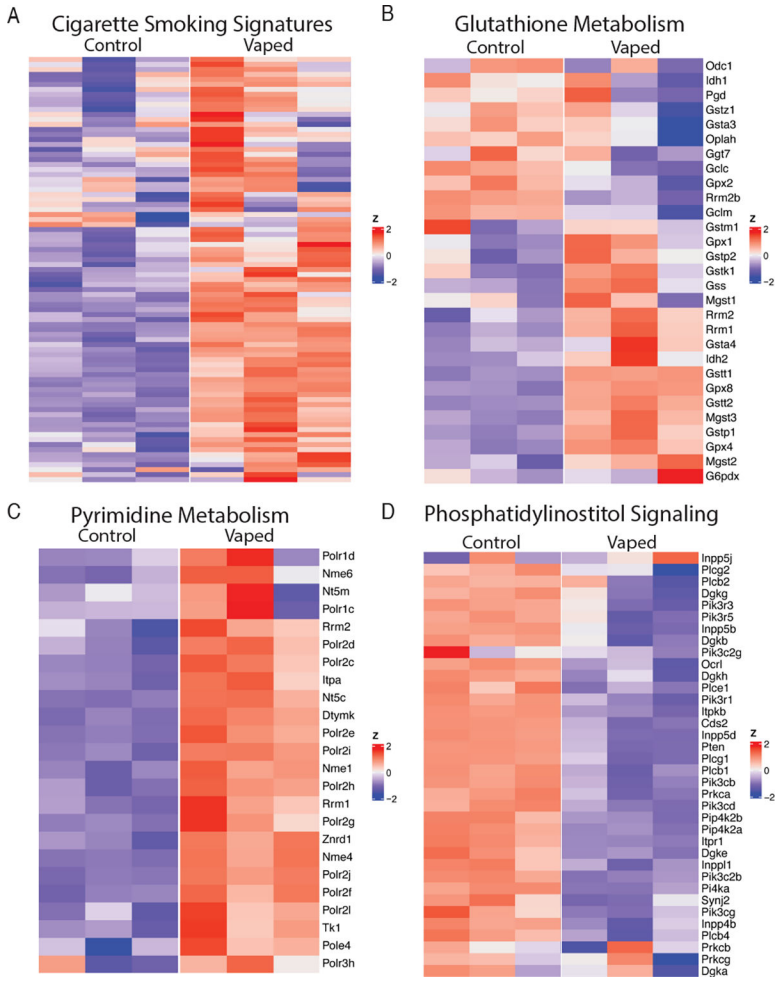

Figure 6: Adult lung cigarette smoking associated genes are also disrupted in maternally nicotine vaped embryonic lungs

A) Heatmap showing differential expression of gene lists identified in adult mouse lungs exposed to cigarette smoke (Martin et al., 2016) between control and 2.4% nicotine vaped wildtype lung tissue. Heatmap showing differential expression of cigarette smoking induced Glutathione (B), Pyrimidine (C), and Phosphatidylinositol (D) pathway genes (Miller et al., 2017).

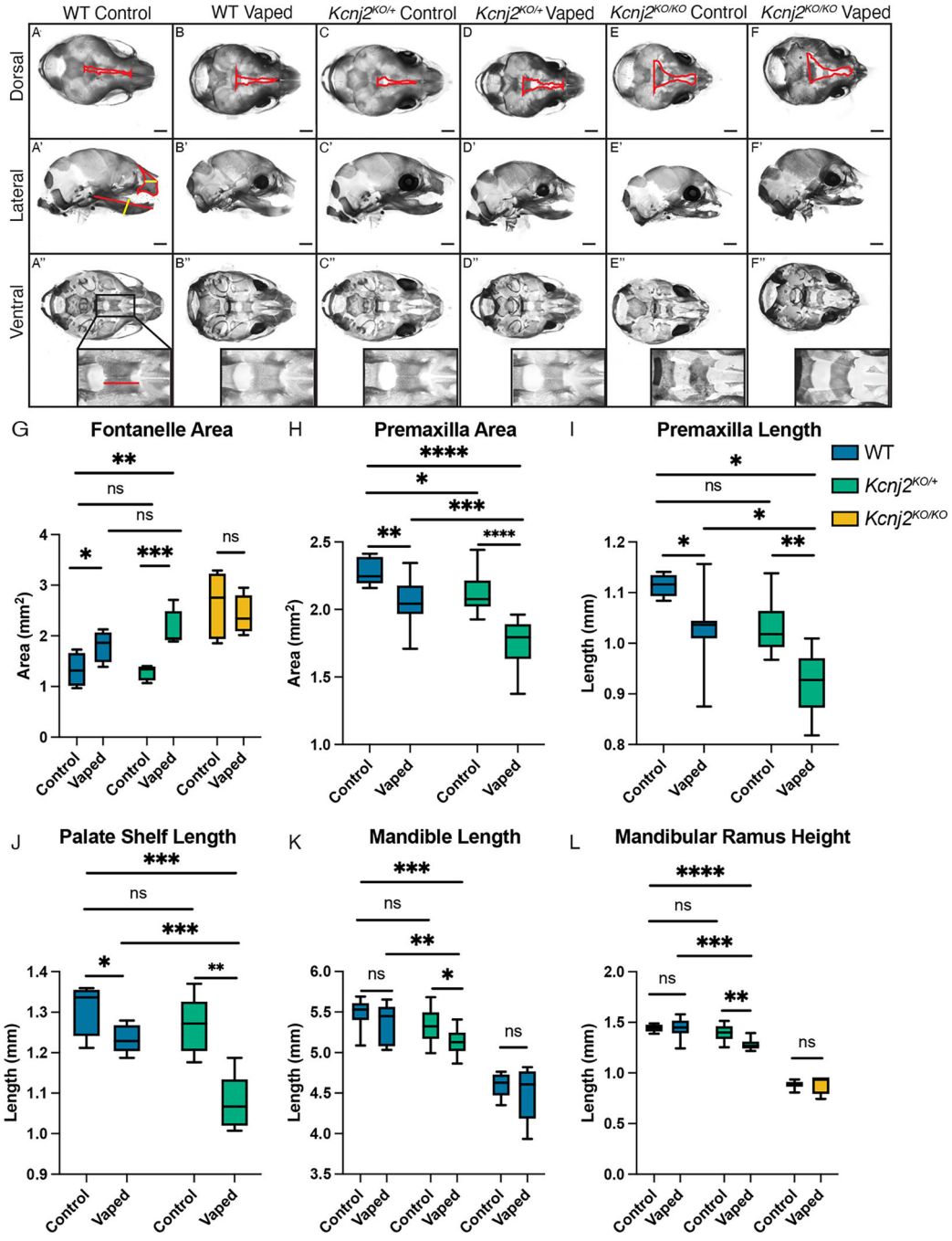

Figure 7: Craniofacial outcomes of maternal nicotine vape exposed embryos

A, A', A") Dorsal, lateral, and ventral (mandible removed) views of E18.5 WT room air control skeletal stained skull. Red outline in dorsal view denotes boundaries used to quantitate fontanelle area. Red outline in lateral view denotes boundaries of premaxilla area quantification. Horizontal yellow line in lateral view shows premaxilla length measurement. Vertical yellow line in lateral view shows mandibular ramus height measurement. Horizontal red line in lateral view shows mandible length measurement. Inset in ventral view show magnification of palatal shelves. Red line in inset denotes measurement of palate shelf

length. B-B") Skeletal stain of WT 2.4% nicotine vaped skull. C-C") Skeletal stain of *Kcnj2*$^{KO/+}$ room air control skull. D-D") Skeletal stain of *Kcnj2*$^{KO/+}$ 2.4% nicotine vaped skull. E-E") Skeletal stain of *Kcnj2*$^{KO/KO}$ room air control skull. F-F") Skeletal stain of *Kcnj2*$^{KO/KO}$ 2.4% nicotine vaped skull. G) Fontanelle area measurements. H) Premaxilla area measurements. I) Premaxilla length measurements. J) Palate shelf length measurements. K) Mandible length measurements. L) Mandibular ramus height measurements. Scale bars represent 1mm. (*p<0.05, **p<0.005, ***p<0.0005 by t-test, ns=not significant). Comparisons between *Kcnj2*$^{KO/KO}$ and WT or un-vaped *Kcnj2*$^{KO/+}$ are significantly different as previously published, but statistical significance of these measurements is not shown to simplify interpretation.

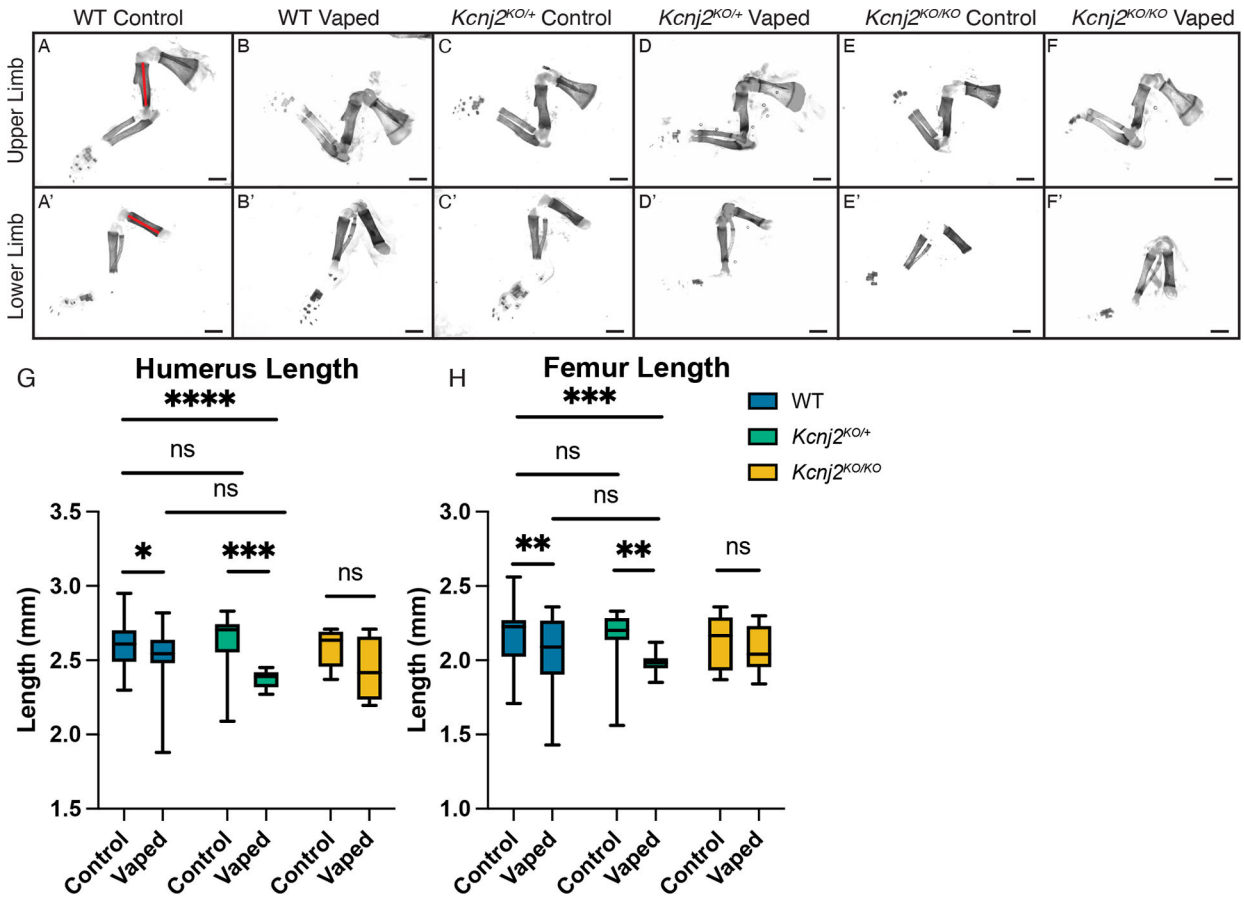

Figure 8: Long bone outcome of nicotine vaped embryos

A, A') Skeletal stained upper and lower limbs of E18.5 WT room air control embryo. Red line in upper limb panel indicates humerus boundaries. Red line in lower limb panel indicates femur measurement boundaries. B-B') Skeletal stain of WT 2.4% nicotine vaped limbs. C-C') Skeletal stain of *Kcnj2*$^{KO/+}$ room air control limbs. D-D') Skeletal stain of *Kcnj2*$^{KO/+}$ 2.4% nicotine vaped limbs. E-E') Skeletal stain of *Kcnj2*$^{KO/KO}$ room air control limbs. F-F') Skeletal stain of *Kcnj2*$^{KO/KO}$ 2.4% nicotine vaped limbs. G) Humerus length measurements. H) Femur length measurements. Scale bars represent 1mm. (*p<0.05, **p<0.005, ***p<0.0005 by t-test, ns=not significant).

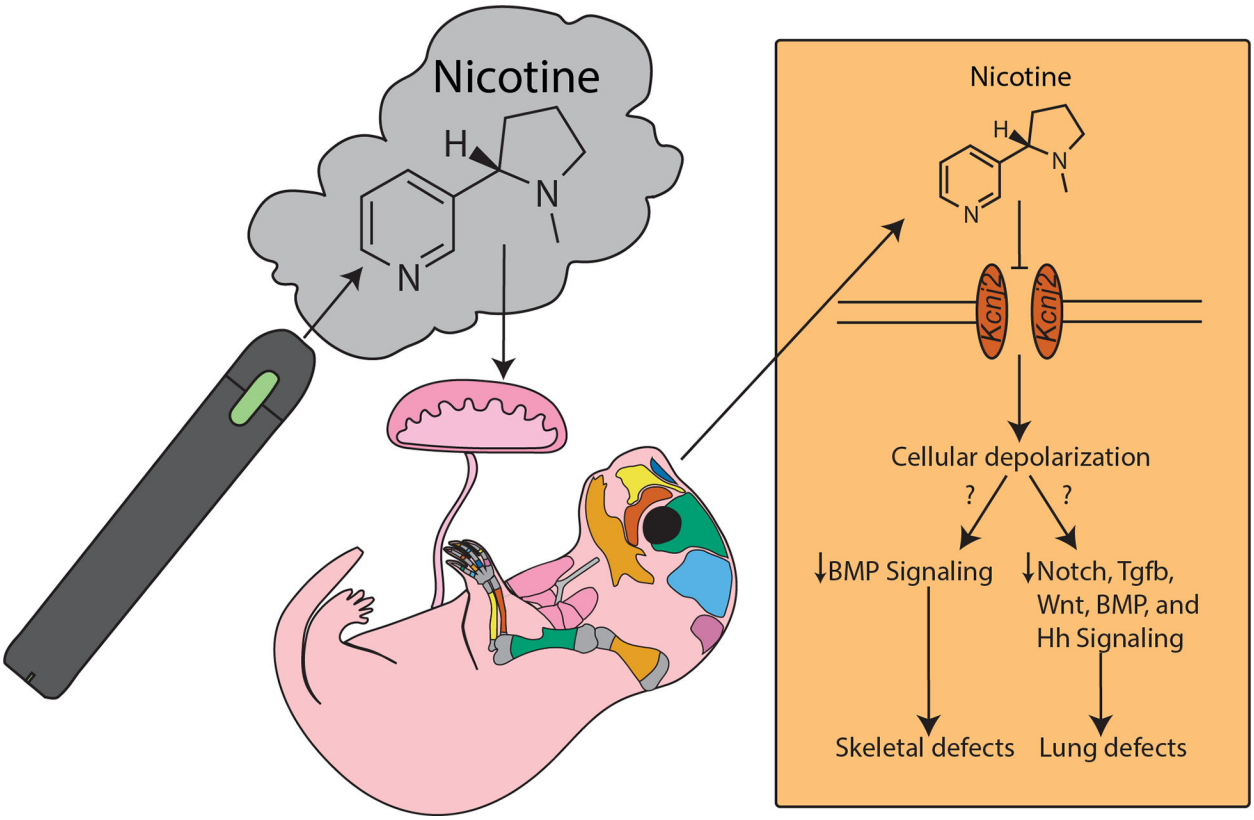

Figure 9: Nicotine vapes disrupt fetal lung and skeletal development through inhibition of *Kcnj2*

Model of proposed mechanism of nicotine vaping's effects on embryonic skeletal and lung development.

Table 1:

Population information for exposure groups. Breakdown of the dams and embryos by exposure group and genotype.

	Control	**Control**	**Control**	**Vaped**	**Vaped**	**Vaped**
Genotype	WT	*Kcnj2*$^{KO/+}$	*Kcnj2*$^{KO/KO}$	WT	*Kcnj2*$^{KO/KO}$	*Kcnj2*$^{KO/KO}$
Dams Exposed	2	4	N/A	1	2	N/A
Embryos (WT dam)	16	N/A	N/A	7	N/A	N/A
Embryos (*Kcnj2*$^{KO/+}$ *dam)*	12	14	4	5	5	4

Table 2:

Pathway analysis of gene expression changes in maternal nicotine vaped embryonic lungs Upregulated and downregulated KEGG pathway analysis of genes from 2.4% nicotine vaped wildtype lung tissue compared to room air controls.

UPREGULATED PATHWAYS	**p adj.**	**NES**
Mitochondrial dysfunction		
KEGG_OXIDATIVE_PHOSPHORYLATION	0.00371	2.98
Protein expression		
KEGG_PROTEASOME	0.00371	2.31
KEGG_RIBOSOME	0.00371	3.14
KEGG_SPLICEOSOME	0.00371	1.86
KEGG_PROTEIN_EXPORT	0.01922	1.77
Metabolism		
KEGG_GLUTATHIONE_METABOLISM	0.01060	1.83
KEGG_PYRIMIDINE_METABOLISM	0.01048	1.69
KEGG_ARACHIDONIC_ACID_METABOLISM	0.01048	1.87
DNA damage		
KEGG_DNA_REPLICATION	0.00450	2.06
KEGG_MISMATCH_REPAIR	0.01048	1.86
KEGG_BASE_EXCISION_REPAIR	0.01710	1.78
KEGG_NUCLEOTIDE_EXCISION_REPAIR	0.02766	1.69
KEGG_HOMOLOGOUS_RECOMBINATION	0.04183	1.67
DOWNREGULATED PATHWAYS	**p adj.**	**NES**
Epithelial structure		
KEGG_ADHERENS_JUNCTION	0.00450	−2.08
KEGG_REGULATION_OF_ACTIN_CYTOSKELETON	0.01220	−1.49
KEGG_FOCAL_ADHESION	0.01364	−1.46
Developmental signaling		
KEGG_NOTCH_SIGNALING_PATHWAY	0.00583	−1.82
KEGG_JAK_STAT_SIGNALING_PATHWAY	0.01035	−1.57
KEGG_MAPK_SIGNALING_PATHWAY	0.01048	−1.46
KEGG_ERBB_SIGNALING_PATHWAY	0.01108	−1.60
KEGG_WNT_SIGNALING_PATHWAY	0.01364	−1.51
Other signaling		

UPREGULATED PATHWAYS	**p adj.**	**NES**
KEGG_PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM	0.00583	−1.78
KEGG_INSULIN_SIGNALING_PATHWAY	0.00819	−1.56
KEGG_CHEMOKINE_SIGNALING_PATHWAY	0.00957	−1.56
KEGG_NEUROTROPHIN_SIGNALING_PATHWAY	0.00450	−1.75
KEGG ADIPOCYTOKINE SIGNALING PATHWAY	0.01734	−1.65
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	0.01922	−1.48
KEGG_RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY	0.02020	−1.65
Other		
KEGG_DORSO_VENTRAL_AXIS_FORMATION	0.00450	−2.01
KEGG_ENDOCYTOSIS	0.00466	−1.70
KEGG_FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS	0.00891	−1.65
KEGG_INOSITOL_PHOSPHATE_METABOLISM	0.01060	−1.73