Published in final edited form as:

Exp Lung Res. 2011 April ; 37(3): 144–154. doi:10.3109/01902148.2010.490288.

MECHANISM FOR NICOTINE-INDUCED UP-REGULATION OF WNT SIGNALING IN HUMAN ALVEOLAR INTERSTITIAL FIBROBLASTS

Reiko Sakurai, Laura M. Cerny, John S. Torday, and Virender K. Rehan

Departments of Pediatrics, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, David Geffen School of Medicine at UCLA, 1124 West Carson St., RB1, Torrance, CA 90502, USA

Abstract

Nicotine exposure alters normal homeostatic pulmonary epithelial-mesenchymal paracrine signaling pathways, resulting in alveolar interstitial fibroblast (AIF)-to-myofibroblast (MYF) transdifferentiation. Since the AIF vs MYF phenotype is determined by the expression of Peroxisome Proliferator-Activated Receptor (PPAR) γ and Wingless/Int (Wnt) signaling, respectively, we hypothesized that nicotine-induced AIF-to-MYF transdifferentiation is characterized by the down-regulation of PPAR γ , and the up-regulation of the Wnt signaling pathway. As nicotine is known to activate PKC signaling, we also hypothesized that in AIFs, nicotine-induced up-regulation of Wnt signaling might be due to PKC activation. Embryonic human lung fibroblasts (WI38 cells) were treated with nicotine (1×10^{-6} M) for either 30 minutes or 24 hours, with or without 30 minute pretreatment with calphostin C (1×10^{-7}), a pan-PKC inhibitor. Then we examined the activation of PKC (p-PKC) and Wnt signaling (p-GSK-3 β , β -catenin, LEF-1, and fibronectin). Furthermore, activation of nicotinic acetylcholine receptors (nAChR)- $\alpha 3$ and $-\alpha 7$, and whether a PPAR γ agonist, Rosiglitazone, blocks nicotine-mediated Wnt activation were examined. Following nicotine stimulation, there was clear evidence for nAChR- $\alpha 3$ and $-\alpha 7$ up-regulation, accompanied by the activation of PKC and Wnt signaling, which was further accompanied by significant changes in the expression of the down-stream targets of Wnt signaling at 24h. Nicotine-mediated Wnt activation was almost completely blocked by pretreatment with either calphostin C or RGZ, indicating the central involvement of PKC activation and Wnt/PPAR γ interaction in nicotine-induced up-regulation of Wnt signaling, and hence AIF-to-MYF transdifferentiation, providing novel preventive/therapeutic targets for nicotine-induced lung injury.

Keywords

Chronic lung disease; Lipofibroblast; Myofibroblast; Nicotine; Peroxisome Proliferator-Activated Receptor; Wnt Signaling

Address for Reprint Requests and Correspondence: Virender K. Rehan, MD, Department of Pediatrics, Los Angeles Biomedical Research Institute at Harbor UCLA Medical Center, David Geffen School of Medicine at UCLA, 1124 West Carson Street, Torrance, CA 90502, vrehan@labiomed.org.

Presented, in part, at the Pediatric Academic Societies' Meeting, Honolulu, Hawaii, May 2008

Introduction

There is strong epidemiologic and experimental evidence that fetal exposure to maternal smoking during gestation results in detrimental long-term effects on lung growth and function (1–10). Significant suppression of alveolarization, functional residual capacity, airway patency, and forced expiratory flow volumes have been demonstrated in the offspring of smoke-exposed pregnancies (9,10). Moreover, the effect of prenatal smoke exposure on lung growth and function seems to be greater than that of postnatal and childhood smoke exposure (11). However, the molecular mechanisms underlying the effects of *in utero* smoke exposure on lung structure and function are incompletely understood. Although there are many agents in smoke that may be detrimental to the developing lung, there is compelling evidence to support nicotine as the main agent affecting lung development in the fetus of the pregnant smoker (12–15).

Since alveolar interstitial fibroblasts play a key role in both normal lung development and injury/repair, we have focused on nicotine's effect on lung fibroblast differentiation (16,17). Using embryonic WI38 human fetal lung fibroblasts as a model, we have recently shown that *in vitro* nicotine exposure induces pulmonary AIF-to-MYF transdifferentiation, *i.e.*, to a phenotype that is not conducive to normal alveolar homeostasis, and in fact is the hallmark of all chronic lung diseases (18). This nicotine-induced AIF-to-MYF transdifferentiation is characterized by significant decreases in AIF's lipogenic markers such as PPAR γ , and increases in key myogenic markers such as fibronectin and α SMA. Since the PPAR γ and Wnt signaling pathways are central in determining the lipofibroblastic phenotype versus the myofibroblastic phenotype, in the present studies, we tested whether nicotine-induced down-regulation of PPAR γ signaling is accompanied by the concomitant up-regulation of Wingless/Int (Wnt) signaling. Further, we determined if Protein Kinase C (PKC), a known intracellular effector of nicotine's effects is centrally involved in nicotine-induced Wnt activation (19,20). We hypothesized that nicotine exposure of the developing lung fibroblast down-regulates PPAR γ expression and up-regulates the Wnt signaling pathway, and nicotine-induced activation of PKC signaling is centrally involved in nicotine-induced Wnt activation. Further, we have reasoned that understanding of the specific molecular mechanism(s) underlying AIF-to-MYF transdifferentiation will allow targeting of specific molecular intermediates to prevent nicotine-induced LIF-to-MYF transdifferentiation, and hence nicotine's detrimental effects on lung development and function.

MATERIALS AND METHODS

Reagents

Nicotine bitartrate was acquired from Sigma Biochemicals (St. Louis, MO). Rosiglitazone maleate (RGZ) was obtained from SmithKline Beecham Pharmaceuticals (Philadelphia, PA). Calphostin was purchased from Calbiochem (San Diego, CA). D-tubocurarine, bungarotoxin, and mecamylamine were purchased from Sigma Biochemicals (St. Louis, MO). Calyculin A was purchased from Upstate (Temecula, CA). Other antibodies were obtained from specific vendors described in Western blot analysis.

Cell culture

The human embryonic cell line, WI38, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Minimum Essential Medium (MEM) +10% Fetal Bovine Serum at 37°C in 6-well plates, 4-well slides, 60 mm, and 100 mm culture dishes, as needed. At 70–80% confluence, the cells were treated with nicotine (1×10^{-9} or 1×10^{-5} M) with or without other specific interventions as described below.

Isolation of total cellular RNA

Total RNA was isolated by lysing the cells in 4M guanidinium thiocyanate, followed by extraction with 2M sodium acetate (pH 4.0), phenol, and chloroform/isoamyl alcohol. RNA was precipitated with isopropanol, collected by centrifugation, vacuum dried, and then dissolved in diethylpyrocarbonate-treated water (4). Integrity of RNA was assessed from the visual appearance of the ethidium bromide-stained ribosomal RNA bands following fractionation on a 1.2% (wt/vol) agarose-formaldehyde gel and quantitated by absorbance at 260 nm.

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT PCR probes used included;- GSK-3 β , sense 5'-CAGCAGCCTTCAGCTTTTGG-3', antisense 5'-CCGGAACATAGTCCAGCACCAG-3'; LEF-1, sense 5'-GGGATGTTCGCCGAGATCAGTCATCC-3', antisense 5'-CGGTACCTGATGTAGGCAGCTGTCATTC-3'; TCF7, sense 5'-TCAGGGAAGCAGGAGCTG-3', antisense 5'-TTCTTGATGGTTGGCTTCTTG-3'. Complementary DNA (cDNA) was synthesized from 2 μ g of total RNA by RT using 100 U of Superscript reverse transcriptase II (Invitrogen, Inc., Carlsbad, CA) and random primers (Invitrogen, Inc.) in a 20 μ l reaction containing 1 $\times$ Superscript buffer (Invitrogen, Inc.), 1mM deoxy-NTP mix, 10mM dithiothreitol, and 40 U ribonuclease inhibitor. Total RNA and random primers were incubated at 65°C for 5 min, followed by incubation at 42°C for 50 minutes. Adding a denaturing enzyme at 70°C for 15 min terminated the reaction. For PCR amplification, 1 μ l of cDNA was added to 24 μ l of a reaction mixture containing 0.2 μ M of each primer, 0.2 mM deoxy-NTP mix, 0.5 U AccuPrime Taq DNA Polymerase (Invitrogen, Inc.), and 1 $\times$ reaction buffer. PCR was performed in a RoboCycler (Stratagene, Inc., La Jolla, CA). Initially, we obtained standard curves for the cycle number and the absorbance optical density for each of the markers examined by RT-PCR. The cycle number (30 to 38) for each PCR reaction was chosen so that the absorbance of the amplified product was in the linear range. The PCR products were visualized on 2% agarose gels by ethidium bromide staining, and the gels were photographed under UV light. Band densities were quantified using the Eagle Eye II System (Stratagene, Inc.). The expression of different mRNAs was normalized to 18s mRNA.

Protein determination and Western blot analysis

Protein determination was made using the Bradford dye-binding method (21). For Western blotting, briefly, cells were lysed using an extraction buffer [10 mM Tris (hydroxymethyl) aminomethane (Tris, pH 7.5), 0.25 M sucrose, 1 mM EDTA, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml each of pepstatin A, aprotinin, and leupeptin], and centrifuged at 140 $\times$ g for 10 min at 4°C. Equal amounts of protein (25 – 50 μ g) from the supernatants were dissolved in electrophoresis sample buffer and were subjected to sodium dodecyl sulfate-polyacrylamide (4–12% gradient) gel electrophoresis, followed by electrophoretic transfer to a nitrocellulose membrane. Nonspecific binding of antibody was blocked by washing with Tris-buffered saline (TBS) containing 5% milk for 1 h. The blot was then subjected to two brief washes with TBS plus 0.5% Tween 20, incubated in TBS plus 0.1% Tween 20, and the specific primary antibodies Nicotine acetylcholine (nACh) receptor- α 3 and - α 7 1:500, LEF-1 1:500, Santa Cruz Biotechnology, Santa Cruz, CA; P-PKC 1:1000, T β -catenin 1:2000, and P- β -catenin 1:1000, Cell Signaling, Boston, MA; Fibronectin 1:1000, Sigma, St. Louis, MO; PPAR γ 1:600 Cayman, Ann Arbor, MI, overnight at 4°C. Blots were then washed in TBS plus 0.1% Tween 20, and then incubated for 1 h in secondary antibody, washed, and developed with a chemiluminescent substrate [enhanced chemiluminescence (ECL); Amersham, Arlington Heights, IL] following the manufacturer's protocol. The densities of the specific protein bands were quantified using a scanning densitometer (Eagle Eye II still video system, Stratagene, La Jolla, CA). The blots

were subsequently stripped and reprobed with anti-GAPDH (1:5000, Chemicon, Inc., Temecula, CA) antibody to confirm equal loading of the samples.

β-catenin Staining

In brief, cells were cultured on Lab-Tek® 4-chamber slides under control and experimental conditions. At the end of the experimental period the slides were washed twice with ice cold PBS and then fixed in freshly prepared 4% paraformaldehyde. Fixed slides were washed in PBS, incubated for 10 minutes in 0.1% TritonX-100 (Sigma, St. Louis, MO). Then blocked with 5% normal goat serum (Jackson ImmunoResearch Lab, West Grove, PA) in PBS for 1 hour at room temperature to block non-specific binding, and then incubated with primary β-catenin antibody (1:200) overnight at 4°C. Secondary donkey anti-rabbit Alexa Fluor 568 (Invitrogen, Carlsbad, CA) was used at a 1:200 dilution for 1 hour. The slides were washed 3 times with PBS, and then mounted and cover-slipped with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Nuclear staining was performed with TO-PRO-3 Iodide (Invitrogen, Carlsbad, CA) at a 1:1500 dilution for 15 minutes and then visualized under confocal laser microscope (Leica microscope with TCS SP 2 confocal unit using x63/1.32 numerical aperture HCX PI Apo objective).

Statistical Analysis

The data for analysis were obtained from at least 3 independent set of experiments and Analysis of Variance for multiple comparisons with Newman-Keuls post-hoc test and Student's t-test, as indicated, were used to analyze the experimental data. $p < 0.05$ was considered to indicate significant differences in the expression of lipogenic and myogenic markers among the control, nicotine, and nicotine plus treatment groups.

Results

Evidence for nicotine-induced activation of Wnt signaling during AIF-to-MYF transdifferentiation

Using cultured WI38 cells, we have previously demonstrated that *in vitro* nicotine exposure resulted in AIF-to-MYF transdifferentiation, characterized by a decrease in the expression of adipogenic markers, and an increase in the expression of myogenic markers (18,22). Since canonical Wnt signaling is a key determinant of MYF differentiation (23,24), we have now examined if these changes were accompanied by the activation of Wnt signaling. A 24 hour exposure of WI38 cells to nicotine ($1 \times 10^{-9}M - 1 \times 10^{-5}M$) resulted in a dose-dependent increase in LEF-1 (Fig 1A), a key intermediate in the Wnt signaling pathway, and fibronectin (Fig 1B), a key down-stream target of Wnt signaling activation. As hypothesized, this was accompanied by a dose-dependent decrease in PPAR γ levels (Fig 1C). Nicotine-induced down-regulation of PPAR γ signaling and the activation of canonical Wnt signaling were further corroborated by a significant decreases in PPAR γ and GSK-3 β , and significant increases in LEF-1 and TCF mRNA expression (Fig. 2), and by the nuclear translocation of β-catenin, based on immunohistochemistry (Fig. 3).

Evidence for Protein Kinase C activation, mediating nicotine-induced up-regulation of Wnt signaling

To study the mechanism of nicotine-induced AIF-to-MYF transdifferentiation, using RT-PCR, we have previously observed up-regulation of the nicotinic acetylcholine receptor (nAChR)s- $\alpha 3$ and $\alpha 7$ by AIFs following stimulation with nicotine (18). Since in other systems nicotine-induced up-regulation of nAChR has been linked to Protein Kinase C (PKC) activation (19,20), we hypothesized that in AIFs, nicotine-induced stimulation of Wnt signaling would also be mediated by PKC activation. Nicotine treatment of AIFs

resulted in PKC activation within 5 minutes, which was also evident at all other time-points examined (Fig 4A). When WI38 cells were stimulated with nicotine (1×10^{-6} M) for 30 minutes following pretreatment with calphostin C (1×10^{-7} M), a specific and potent pan-PKC inhibitor, it completely blocked the nicotine-induced activation of PKC, and the down-regulation of GSK-3 β (Fig 4B). Similarly, 24h treatment of WI38 cells with nicotine following 1h pretreatment with calphostin completely blocked the nicotine-induced up-regulation of LEF1 and fibronectin (Fig. 5), indicating the central involvement of PKC activation in nicotine-induced up-regulation of Wnt signaling.

Evidence that cross-talk between the PPAR γ and Wnt signaling pathways modulates nicotine-induced activation of Wnt signaling

Since we have previously demonstrated that nicotine-induced AIF-to-MYF transdifferentiation can be blocked by the PPAR γ agonist RGZ, we next examined whether RGZ would block nicotine-induced activation of Wnt signaling. Indeed, pretreatment of AIF with RGZ blocked the decrease in phospho- β -catenin at 30 minutes (Fig. 6A), and the nicotine-induced increases in LEF-1 and fibronectin levels (Fig. 6) at 24h, clearly suggesting cross-talk between the PPAR γ and Wnt signaling pathways. This is further supported by the fact that nicotine-induced nuclear translocation of β -catenin (Fig. 3) was also blocked by pretreatment with RGZ.

Evidence that nicotine-induced activation of Wnt signaling is mediated by nicotinic acetylcholine receptor $\alpha 7$

We have previously documented the expression of the specific nAChRs $\alpha 3$ and $\alpha 7$ by WI38 cells, which, at least in part, have been implicated in mediating nicotine-induced effects on developing lung structure and function (18,25). To examine the relationship between nicotine-induced up-regulation of nAChRs and Wnt activation, we first examined the specificity of the nAChR inhibitors d-tubocurarine against nAChR $\alpha 3$ and $\alpha 7$, bungarotoxin against nAChR $\alpha 7$, and mecamylamine against nAChR $\alpha 3$ in blocking nicotine-induced up-regulation of these specific receptor subtypes in WI38 cells. Pretreatment of WI38 cells with d-tubocurarine, bungarotoxin, or mecamylamine blocked nicotine-induced up-regulation of nAChR $\alpha 3$ and nAChR $\alpha 7$, nAChR $\alpha 7$, and nAChR $\alpha 3$, respectively (Fig. 7A). However, pretreatment with only d-tubocurarine and bungarotoxin blocked the nicotine-induced increase in LEF-1 and fibronectin, whereas pretreatment with mecamylamine had no effect (Fig. 7B).

Discussion

Though nicotine-induced activation of PKC (19) and the involvement of PKC in mediating Wnt signaling (26,27) has been demonstrated previously, to our knowledge this is the first report that unequivocally implicates the central involvement of PKC activation in mediating nicotine-induced up-regulation of Wnt signaling. Based on the data included in this report, we propose that nicotine, acting specifically through nAChR $\alpha 7$, activates PKC, which in turn down-regulates GSK-3 β , inhibiting β -catenin phosphorylation. As a result, there is cytoplasmic accumulation and nuclear translocation of β -catenin, where it binds to LEF-1/TCF, which in turn results in fibronectin induction. Furthermore, the PPAR γ ligand RGZ clearly blocked both nicotine-induced stabilization of cytoplasmic β -catenin, and its nuclear translocation, and up-regulation of LEF-1 and fibronectin. Of note, though in this series of experiments, we did not determine the specific PKC isoform activated in WI38 cells on treatment with nicotine, depending on the cell-type and stimulus involved, activation of specific isoforms has previously been suggested during myofibroblast differentiation (19,28).

Depending upon the cell-type and system involved, both positive (29,30) and negative (31,32) interactions between PPAR γ and Wnt signaling have been reported, and β -catenin seems to be the key Wnt signaling intermediate that mediates these interactions. For example, similar to our observations, following PPAR activation in mesenchymal cells, Liu and Farmer have reported decreased s-catenin levels, and thereby repressed canonical Wnt signaling (33). This functional interaction between s-catenin and PPAR is likely to be facilitated by the catenin binding domain within PPAR that is highly homologous to the catenin binding domain in TCF/LEF (32).

There is a growing body of evidence suggesting the critical involvement of Wnt signaling in lung development (34–37). Wnts, a family of 19 secreted glycoproteins, which bind to cell surface receptors called Frizzleds (Fz), trigger the intracellular signaling cascades involving either the canonical β -catenin-dependent, or the non-canonical pathways. For Wnt/Fz interactions to occur, the presence of the co-receptors, low-density lipoprotein (LRP) 5 and 6, is required. The expression of various Wnts, Fzs, Dvls, and other related proteins such as LEF-1, and TCFs has been demonstrated to be spatio-temporally specific. In the mouse embryo, Wnt signaling has been shown to be active throughout lung development (36). β -catenin is expressed in the alveolar epithelium and adjoining mesenchyme, and has been shown to be central to the formation of the peripheral airways of the lung, responsible for gas exchange, but not for the formation of the proximal airways (34,37). Using fetal rat lung explants in culture as a model of spontaneous lung development, we have previously shown the spontaneous down-regulation of Wnt, and the spontaneous up-regulation of the PTHrP/PPAR γ signaling pathways during late fetal lung development (35). In addition to its critical role during normal lung development, Wnt signaling has recently been suggested to be important in the pathogenesis of pulmonary fibrosis (38–40). The observations presented here further complement these data, and suggest that nicotine's effect on the AIF Wnt signaling pathway might play a critical role in *in utero* nicotine-induced lung injury.

Because of the failure to eliminate maternal smoking during pregnancy, combined with the lack of understanding of the molecular mechanisms involved, up until now there has been no specific intervention to prevent the pulmonary consequences of *in utero* smoke exposure (4,41). We have recently proposed that specific disruption of pulmonary alveolar epithelial-mesenchymal interactions results in interstitial AIF-to-MYF transdifferentiation, which may be the final common pathway through which various non-inflammatory and inflammatory triggers lead to chronic lung damage in the premature infant (17). Alveolar interstitial fibroblast-to-MYF transdifferentiation results in failed alveolarization in the developing lung, which leads to an arrest of pulmonary growth and development, the hallmarks of *in utero* smoke-induced lung damage (4,42). The data presented here suggest that in addition to the previously described molecular mechanisms for *in utero* nicotine-induced lung damage, there is also an up-regulation of AIF Wnt signaling. We have previously demonstrated the down-regulation of the PTHrP-stimulated cAMP-dependent PKA pathway in this process, which normally induces the AIF lipogenic phenotype, characterized by expression of such lipogenic features as triglyceride accumulation and the expression of PPAR and ADRP (18,22). Importantly, lipogenic AIFs sustain alveolar type II cell growth and differentiation, whereas MYFs do not (43). Therefore, nicotine-induced AIF-to-MYF transdifferentiation may provide a specific mechanism for failed alveolarization observed in association with maternal smoke exposure.

In summary, in addition to previously proposed mechanisms for the detrimental effects following *in utero* nicotine exposure, our data, for the first time, provide evidence for an integrated mechanism for the up-regulation of Wnt signaling, and the down-regulation of PPAR γ through direct effects of nicotine on the developing mesenchyme that could permanently alter the structure and function of the developing lung by disrupting critically

important epithelial-mesenchymal interactions. More importantly, specific interventions that augment the pulmonary mesenchymal lipogenic pathway could ameliorate this very complex, nicotine-induced *in utero* lung injury.

Acknowledgments

We are grateful to P. Guo, PhD, for technical assistance with this work.

GRANT SUPPORT

Supported by grants from the TRDRP (14RT-0073, 15IT-0250, and 17RT-0170), the NIH (HL75405, HD51857, HD58948 and HL55268).

References

1. Maritz GS. Effect of maternal nicotine exposure on growth in vivo of lung tissue of neonatal rats. *Biol Neonate*. 1988; 53:163–70. [PubMed: 3370261]
2. Moshhammer H, Hoek G, Luttmann-Gibson H, Neuburger MA, Antova T, Gehring U, Hrubá F, Pattenden S, Rudnai P, Slachtova H, Zlotkowska R, Fletcher T. Parental smoking and lung function in children: an international study. *Am J Respir Crit Care Med*. 2006; 173:1255–63. [PubMed: 16484675]
3. Chen MF, Kimizuka G, Wang NS. Human fetal lung changes associated with maternal smoking during pregnancy. *Pediatr Pulmonol*. 1987; 3:51–8. [PubMed: 3588051]
4. Collins MH, Moessinger AC, Kleinerman J, Bassi J, Rosso P, Collins AM, James LS, Blanc WA. Fetal lung hypoplasia associated with maternal smoking: A morphometric analysis. *Pediatr Res*. 1985; 19:408–12. [PubMed: 4000765]
5. Cunningham J, Dockery DW, Speizer FE. Maternal smoking during pregnancy as a predictor of lung function in children. *Am J Epidemiol*. 1994; 139:1139–52. [PubMed: 8209873]
6. Hanrahan JP, Tager IB, Segal MR, Tosteson TD, Castile RG, Van Vunakis H, Weiss ST, Speizer FE. The effect of maternal smoking during pregnancy on early infant lung function. *Am Rev Respir Dis*. 1992; 145:1129–35. [PubMed: 1586058]
7. Sekhon HS, Keller JA, Proskocil BJ, Martin EL, Spindel ER. Maternal nicotine exposure upregulates collagen gene expression in fetal monkey lung: association with $\alpha 7$ nicotinic acetylcholine receptors. *Am J Respir Cell Mol Biol*. 2002; 26:31–41. [PubMed: 11751201]
8. Pierce RA, Nguyen NM. Prenatal nicotine exposure and abnormal lung function. *Am J Respir Cell Mol Biol*. 2002; 26:1013.
9. Early developmental origins of impaired lung structure and function. *Early Human Development*. 2005; 81:763–771. [PubMed: 16081227]
10. Wang L, Pinkerton KE. Detrimental effects of tobacco smoke exposure during development on postnatal lung function and asthma. *Birth Defects Research Part C: Embryo Today*. 2008; 84:54–60.
11. Tager IB, Ngo L, Hanrahan JP. Maternal smoking during pregnancy. Effects on lung function during the first 18 months of life. *Am J Respir Crit Care Med*. 1995; 152:977–83. [PubMed: 7663813]
12. Pastrakuljic A, Schwartz R, Simone C, Derewlany LO, Knie B, Koren G. Transplacental transfer and biotransformation studies of nicotine in the human placental cotyledon perfused in vitro. *Life Sci*. 1998; 63:2333–42. [PubMed: 9877223]
13. Dempsey D, Jacob P 3rd, Benowitz NL. Accelerated metabolism of nicotine and cotinine in pregnant smokers. *J Pharmacol Exp Ther*. 2002; 301:594–8. [PubMed: 11961061]
14. Luck W, Nau H, Hansen R, Steldinger R. Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers. *Dev Pharmacol Ther*. 1985; 8:384–95. [PubMed: 4075937]
15. Szuts T, Olsson S, Lindquist NG, Ullberg S, Pilotti A, Enzell C. Long-term fate of [^{14}C]nicotine in the mouse: retention in the bronchi, melanin-containing tissues and urinary bladder wall. *Toxicology*. 1978; 10:207–20. [PubMed: 705796]

16. McGowan SE, Torday JS. The pulmonary lipofibroblast (lipid interstitial cell) and its contributions to alveolar development. *Annu Rev Physiol.* 1997; 59:43–62. [PubMed: 9074756]
17. Torday JS, Rehan VK. A paracrine model for lung development, disease, and treatment-perspective. *Pediatr Res.* 2007; 62(1):2–7. [PubMed: 17515838]
18. Rehan VK, Wang Y, Sugano S, Romero S, Chen X, Santos J, Khazanchi A, Torday JS. Mechanism of nicotine-induced pulmonary fibroblast transdifferentiation. *Am J Physiol Lung Cell Mol Physiol.* 2005; 289:L667–76. [PubMed: 15951329]
19. Roman J, Ritzenthaler JD, Gil-Acosta A, Rivera HN, Roser-Page S. Nicotine and fibronectin expression in lung fibroblasts: implications for tobacco-related lung tissue remodeling. *FASEB J.* 2004 Sep; 18(12):1436–8. [PubMed: 15247149]
20. Jaimes EA, Tian RX, Raij L. Nicotine: the link between cigarette smoking and the progression of renal injury? *Am J Physiol Heart Circ Physiol.* 2007 Jan; 292(1):H76–82. [PubMed: 16920799]
21. Bradford M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
22. Rehan VK, Sakurai R, Wang Y, Huynh K, Torday JS. Reversal of nicotine-induced alveolar lipofibroblasts-to-myofibroblast transdifferentiation by stimulants of parathyroid hormone-related protein signaling. *Lung.* 2007; 185(3):151–9. [PubMed: 17401602]
23. Cossu G, Tajbakhsh S, Buckingham M. How is myogenesis initiated in the embryo? *Trends Genet.* 1996; 12:218–23. [PubMed: 8928226]
24. Torday JS, Rehan VK. The evolutionary continuum from lung development to homeostasis and repair. *Am J Physiol Lung Cell Mol Physiol.* 2007 Mar; 292(3):L608–11. [PubMed: 17085519]
25. Sekhon HS, Keller JA, Proskocil BJ, Martin EL, Spindel ER. Maternal nicotine exposure upregulates collagen gene expression in fetal monkey lung: association with $\alpha 7$ nicotinic acetylcholine receptors. *Am J Respir Cell Mol Biol.* 2002; 26:31–41. [PubMed: 11751201]
26. Chen RH, Ding WV, McCormick F. Wnt signaling to beta-catenin involves two interactive components. Glycogen synthase kinase-3 β inhibition and activation of protein kinase C. *J Biol Chem.* 2000; 275:17894–9. [PubMed: 10749878]
27. Kinoshita N, Iioka H, Miyakoshi A, Ueno N. PKC delta is essential for Dishevelled function in a noncanonical Wnt pathway that regulates *Xenopus* convergent extension movements. *Genes Dev.* 2003; 17:1663–76. [PubMed: 12842914]
28. Bogatkevich GS, Tourkina E, Silver RM, Ludwicka-Bradley A. Thrombin Differentiates Normal Lung Fibroblasts to a Myofibroblast Phenotype via the Proteolytically Activated Receptor-1 and a Protein Kinase C-dependent Pathway. *Am J Physiol Lung Cell Mol Physiol.* 2003; 285:334–343.
29. Handeli S, Simon JA. A small-molecule inhibitor of Tcf/ β -catenin signaling down-regulates PPAR γ and PPAR δ activities. *Molecular Cancer Therapeutics.* March 1.2008 7:521–529. [PubMed: 18347139]
30. Jansson EA, Are A, Greicius G, Kuo IC, Kelly D, Arulampalam V, Pettersson S. The Wnt/ β -catenin signaling pathway targets PPAR γ activity in colon cancer cells. *PNAS.* 2005; 102(5): 1460–1465. [PubMed: 15665104]
31. Girnun GD, Smith WM, Drori S, Sarraf P, Mueller E, Eng C, Nambiar P, Rosenberg DW, Bronson RT, Edelmann W, Kucherlapati R, Gonzalez FJ, Spiegelman BM. APC-dependent suppression of colon carcinogenesis by PPAR γ . *Proc Natl Acad Sci USA.* 2002; 99:13771–13776. [PubMed: 12370429]
32. Liu J, Wang H, Ying Zuo Y, Farmer SR. Functional Interaction between Peroxisome Proliferator-Activated Receptor γ and β -catenin. *Molecular and Cellular Biology.* August; 2006 26(15):5827–5837. [PubMed: 16847334]
33. Liu J, Farmer SR. Regulating the balance between peroxisome proliferator-activated receptor γ and β -catenin signaling during adipogenesis. A glycogen synthase kinase 3 β phosphorylation-defective mutant of β -catenin inhibits expression of a subset of adipogenic genes. *J Biol Chem.* 2004; 279:45020–45027. [PubMed: 15308623]
34. Mucenski ML, Wert SE, Nation JM, Loudy DE, Huelsken J, Birchmeier W, Morrissey EE, Whitsett JA. β -catenin is required for specification of proximal/distal cell fate during lung morphogenesis. *J Biol Chem.* 2003; 278:40231–8. [PubMed: 12885771]

35. Torday JS, Rehan VK. Up-regulation of fetal rat lung parathyroid hormone-related protein gene regulatory network down-regulates the Sonic Hedgehog/Wnt/betacatenin gene regulatory network. *Pediatr Res*. 2006; 60:382–388. [PubMed: 16940239]
36. Okubo T, Hogan BLM. Hyperactive Wnt signaling changes the development potential of embryonic lung endoderm. *J Biol*. 2004; 3:11. [PubMed: 15186480]
37. Tebar M, Destree O, de Vree WJ, Ten Have-Opbroek AA. Expression of the Tcf/Lef and sFrp and localization of beta-catenin in the developing mouse lung. *Mech Dev*. 2001; 109:437–440. [PubMed: 11731265]
38. Chiloi M, Poletti V, Zamo A, Lestani M, et al. Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am J Pathol*. 2003; 162:1495–502. [PubMed: 12707032]
39. Pongracz JE, Stockley RA. Wnt signaling in lung development and diseases. *Respir Res*. 2006; 7:15. [PubMed: 16438732]
40. Alejandre-Alcazar MA, Kwapiszewska G, Reiss I, Amarie OV, Marsh LM, Sevilla-Perez J, Wygrecka M, Eul B, Kobrich S, Hesse M, Schermuly RT, Seeger W, Eickelberg O, Morty RE. Hyperoxia modulates TGF-beta/BMP signaling in a mouse model of bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol*. 2007 Feb; 292(2):L537–49. [PubMed: 17071723]
41. Federal Trade Commission Cigarette Report for 2002. Federal Trade Commission; Washington DC: 2004. <http://www.ftc.gov/reports/cigarette/041022cigaretterpt.pdf>
42. Sekhon HS, Jia Y, Raab R, Kuryatov A, Pankow JF, Whitsett JA, Lindstrom J, Spindel ER. Prenatal nicotine increases pulmonary alpha7 nicotinic receptor expression and alters fetal lung development in monkeys. *J Clin Invest*. 1999 Mar; 103(5):637–47. [PubMed: 10074480]
43. Torday JS, Torres E, Rehan VK. The role of fibroblast transdifferentiation in lung epithelial cell proliferation, differentiation, and repair in vitro. *Pediatr Pathol Mol Med*. 2003 May-Jun; 22(3): 189–207. [PubMed: 12746170]

Fig.1A

Fig.1C

Fig. 1. Effect of in vitro nicotine treatment on key protein levels of AIF differentiation markers
 A twenty four hour exposure of WI38 cells to nicotine (1×10^{-9} M – 1×10^{-5} M) resulted in dose-dependent increases in LEF-1 (Fig 1A) and fibronectin (Fig. 1B) levels, and a dose-dependent decrease in PPAR γ (Fig 1C) levels by Western analysis. Values are mean $\pm$ standard deviation; *, $p < 0.05$, control vs. nicotine, N=3.

Fig. 2. Effect of in vitro nicotine treatment on mRNA expression of key markers of AIF PPAR γ and Wnt Signaling

Nicotine treatment (1×10^{-6} M for 24h) of WI38 cells resulted in a significant decreases in the expressions of PPAR γ and GSK-3 β mRNA along with significant increases in the expressions of LEF-1 and α SMA mRNA. Values are mean $\pm$ standard deviation; *, $p < 0.05$, control vs. nicotine, N=3.

Fig. 3. Evidence that nicotine-induced activation of Wnt signaling can be blocked by PPAR γ agonist RGZ

WI38 cells were stimulated with nicotine (1×10^{-6} M) for 60 minutes with or without pretreatment with RGZ (1×10^{-5} M). As shown by confocal immunofluorescence imaging, RGZ pretreatment almost completely blocked the nicotine-induced nuclear translocation of β -catenin. Left hand column shows β -catenin (red fluorescence indicated with white arrows), middle column shows nuclear marker 4',6-diamidino-2-phenylindole (DAPI, blue staining), and the right hand column shows the merged images of these two stains (magnification x63).

Fig. 4A

Fig. 4. Evidence for Protein Kinase C activation on nicotine treatment of WI38 cells
 Nicotine treatment (1×10^{-6} M) of WI38 cells resulted in PKC activation within 5 minutes, which was also evident at all other time-points examined (Fig. 4A). When WI38 cells were stimulated with nicotine (1×10^{-6} M) for 30 minutes following 1h pretreatment with calphostin C (1×10^{-7} M), a specific and potent pan-PKC inhibitor, it completely blocked the nicotine-induced activation of PKC and the down-regulation of GSK-3 β (Fig. 4B). Values are mean $\pm$ standard deviation; *, $p < 0.05$, control vs. nicotine; #, $p < 0.05$, calphostin C vs. nicotine, N=3.

Fig. 5. Evidence for Protein Kinase C activation mediating nicotine-induced up-regulation of Wnt signaling

Twenty four treatment of WI38 cells with nicotine (1×10^{-6} M) following 1h pretreatment with calphostin (1×10^{-7} M) completely blocked the nicotine-induced up-regulation of LEF1 and fibronectin, indicating the central involvement of PKC activation in nicotine-induced Wnt up-regulation. Each bar represents the mean $\pm$ standard deviation. Values are mean $\pm$ standard deviation; *, $p < 0.05$, control vs. nicotine, $N=3$; #, $p < 0.05$, nicotine treated vs. nicotine + calphostin C, $N=3$).

Fig. 6. Evidence that cross-talk between the PPAR γ and Wnt signaling pathways modulates nicotine-induced activation of Wnt signaling

Pretreatment of WI38 cells with RGZ (1×10^{-5} M) blocked the decrease in phospho- β -catenin at 30 minutes (Fig. 6A), and the nicotine-induced increases in LEF-1 and fibronectin levels at 24h (Fig. 6B). Values are mean $\pm$ standard deviation; *, $p < 0.05$, control vs. nicotine, #= $p < 0.05$ RGZ vs. nicotine, N=3).

Fig. 7. Evidence that nicotine-induced activation of Wnt signaling is mediated by nicotinic acetylcholine receptor $\alpha 7$

Pretreatment of WI38 cells with d-tubocurarine, bungarotoxin, or mecaylamine blocked nicotine-induced up-regulation of nAChR $\alpha 3$ and nAChR $\alpha 7$, nAChR $\alpha 7$, and nAChR $\alpha 3$, respectively (Fig. 7A). However, pretreatment with only d-tubocurarine and bungarotoxin blocked the nicotine-induced increases in LEF-1 and fibronectin, whereas pretreatment with mecaylamine had no effect (Fig. 7B). (*, $p < 0.05$, control vs. nicotine, $N = 3$; #, $p < 0.05$, nicotine vs. nicotine + d-tubocurarine/bungarotoxin, $N = 3$).