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Title

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Permalink

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Journal

Stem Cells, 40(7)

ISSN

1066-5099

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Publication Date

2022-07-27

DOI

10.1093/stmcls/sxac031

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Stem Cells, 2022, **40**, 691–703
https://doi.org/10.1093/stmcls/sxac031
Advance access publication 16 April 2022
Original Research

Wnt5a Promotes AT1 and Represses AT2 Lineage-Specific Gene Expression in a Cell-Context-Dependent Manner

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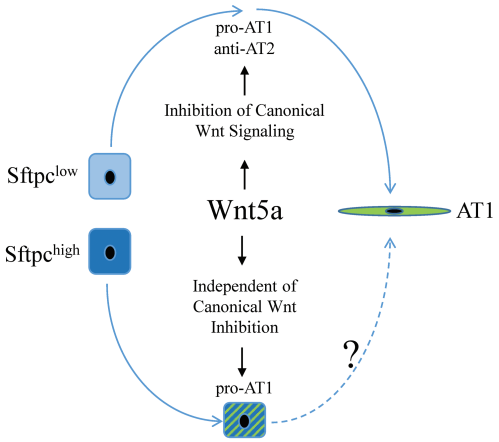
Abstract

Lung maturation is not limited to proper structural development but also includes differentiation and functionality of various highly specialized alveolar cell types. Alveolar type 1 (AT1s) cells occupy nearly 95% of the alveolar surface and are critical for establishing efficient gas exchange in the mature lung. AT1 cells arise from progenitors specified during the embryonic stage as well as alveolar epithelial progenitors expressing surfactant protein C (Sftpc^{pos} cells) during postnatal and adult stages. Previously, we found that Wnt5a, a non-canonical Wnt ligand, is required for differentiation of AT1 cells during the saccular phase of lung development. To further investigate the role of Wnt5a in AT1 cell differentiation, we generated and characterized a conditional Wnt5a gain-of-function mouse model. Neonatal Wnt5a gain-of-function disrupted alveologenesis through inhibition of cell proliferation. In this setting Wnt5a downregulated β-catenin-dependent canonical Wnt signaling, repressed AT2 (anti-AT2) and promoted AT1 (pro-AT1) lineage-specific gene expression. In addition, we identified 2 subpopulations of Sftpc^{high} and Sftpc^{low} alveolar epithelial cells. In Sftpc^{low} cells, Wnt5a exhibits pro-AT1 and anti-AT2 effects, concurrent with inhibition of canonical Wnt signaling. Interestingly, in the Sftpc^{high} subpopulation, although increasing AT1 lineage-specific gene expression, Wnt5a gain-of-function did not change AT2 gene expression, nor inhibit canonical Wnt signaling. Using primary epithelial cells isolated from human fetal lungs, we demonstrate that this property of Wnt5a is evolutionarily conserved. Wnt5a therefore serves as a selective regulator that ensures proper AT1/AT2 balance in the developing lung.

Key words: Wnt5a; Wnt signaling; lung development; AT1; AT2; lung epithelial progenitor; organoid.

Graphical Abstract



Two subpopulations of Sftpc^{low} and Sftpc^{high} AT2 cells exist in mouse neonatal lung during alveologenesis. In Sftpc^{low} subpopulation, Wnt5a inhibits canonical Wnt signaling, represses AT2 (anti-AT2) and promotes AT1 (pro-AT1) lineage-specific gene expression. In Sftpc^{high} subpopulation, Wnt5a exhibits pro-AT1 effects through a mechanism independent of inhibition of canonical Wnt signaling.

Received: 13 December 2021; Accepted: 5 April 2022.
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Significance Statement

Alveolar type 1 cells (AT1s) are critical for efficient gas exchange in the lung. They can also reprogram into AT2 cells during acute lung injury. Reduced or lack of AT1s, for example in the lungs of preterm neonates threatens extrauterine survival. Similarly, many adult lung diseases involve injuries to AT1s. Thus, studies directed at understanding the mechanisms of AT1 regeneration are highly significant. This study revealed a previously unknown Wnt5a function in regulating AT1 cell lineage-specific gene expression. The results, therefore, may have significant implications for future development of preventive or therapeutic strategies for lung diseases involving AT1 injury.

Introduction

Alveologenesis and Alveolar Type 1 Cell Differentiation

Lung development in the mouse commences around embryonic day 9.5 (E9.5) and lasts until postnatal day 30 (P30). In humans, it spans from 4 weeks of gestation to young adulthood.[1] The early phase of lung development which includes embryonic and pseudoglandular stages involves extensive branching morphogenesis and cell fate specification to establish the conducting airway structure.[1,2] Subsequently, during lung maturation that includes the canalicular, saccular, and alveolar stages of lung development the distal gas-exchange units, or alveoli, are formed. A key step in lung maturation is differentiation of mature and functional alveolar type 1 and alveolar type 2 cells (AT1s and AT2s). AT1s are large, flat, and thin epithelial cells through which oxygen and carbon dioxide are exchanged across the alveolar-capillary barrier. Approximately 95% of the alveolar surface is covered by AT1s. The remainder is occupied by AT2s which produce surfactant to reduce alveolar surface tension. A subpopulation of AT2s serves as facultative stem cells in lung maintenance and repair. Previously, AT1s were considered terminally differentiated. However, studies have shown that AT1s exhibit plasticity and are capable of reprogramming into AT2s during acute neonatal lung injury.[3,4] In the mouse, AT1 progenitors are specified between E13 and E15. They play an important role in directing differentiation of progenitors of secondary crest myofibroblasts (SCMFs), also named alveolar myofibroblast, which are essential for alveologenesis.[5] After birth, AT1s are derived from Sftpc^{pos} AT2s both during normal development and in repair of injury.[6,7] AT2 to AT1 transition is associated with downregulation of canonical Wnt signaling.[8,9] Because of its functional importance in gas exchange, AT1 differentiation is a vital process in lung maturation. Reduced or lack of mature AT1s as in the lungs of extremely preterm neonates, severely threatens extrauterine survival. In addition, because AT1s are directly exposed to the outside environment and frequently damaged in various lung injuries, regeneration of AT1s is key to restoring lung function. Thus, understanding the mechanisms that govern AT1 cell differentiation and regeneration is of great importance for lung development and injury repair.

Wnt5a in the Lung

Wnt ligands are a family of lipid modified, cysteine rich signaling molecules. In mammals, 19 *Wnt* genes have been identified whose encoded proteins signal through several intracellular pathways. These include both β-catenin (Ctnnb1)-dependent canonical Wnt pathways and Ctnnb1 independent non-canonical Wnt pathways.[10,11] Wnt5a mainly activates Ctnnb1 independent, non-canonical Wnt signaling.[12] It can also activate canonical Wnt signaling in the presence of certain receptors such as Frizzled 4.[12] Several studies from our group and others have demonstrated that Wnt5a signaling plays essential roles in normal lung development,[13-16] and homeostasis as well as stem cell maintenance.[17] Accumulating evidence also implicates dysregulated *WNT5A* expression in pathogenesis of several lung diseases such as chronic obstructive pulmonary disease (COPD),[18] pulmonary arterial hypertension (PAH),[19] idiopathic pulmonary fibrosis (IPF)[20-22], and bronchopulmonary dysplasia (BPD).[15,23] Importantly, both aberrant up- and downregulation of *WNT5A* expression have been detected in different disease conditions indicating complex roles of Wnt5a signaling in the lung.

Recently, we found that Wnt5a signaling is essential for lung maturation.[15] In the saccular stage, inactivation of Wnt5a disrupted AT1 differentiation. Wnt5a inactivation in both saccular and alveolar stages inhibited myofibroblast differentiation and migration. To further determine the roles of Wnt5a signaling in vivo, we generated a Wnt5a gain-of-function model, *Rosa26-rtTA;tetO-Wnt5a (Wnt5a^{Rosa})*, which expresses *Wnt5a* upon administration of doxycycline. We found that Wnt5a gain-of-function during postnatal days 4 and 5 (P4 and P5) disrupted alveologenesis, inhibited cell proliferation, decreased myofibroblast markers, and increased expression of AT1 lineage-specific markers in *Wnt5a^{Rosa}* lungs. Further analysis using *Sftpc-GFP*, a separate transgenic model, identified 2 distinct, Sftpc^{high} (GFP^{high}) and Sftpc^{low} (GFP^{low}) subpopulations in neonatal lungs. Interestingly, while Wnt5a promotes expression of AT1 lineage-specific genes in both subpopulations, it inhibits AT2 lineage-specific genes only in Sftpc^{low} cells. The ability of Wnt5a to promote AT1 and repress AT2 lineage-specific gene expression was also found during mouse fetal lung development when epithelial progenitor cell fate is specified. Importantly, using human fetal lung epithelial progenitors, we demonstrate that the novel role of Wnt5a described in this study is evolutionarily conserved between mouse and humans.

Materials and Methods

Mouse Breeding and Genotyping

All animals were maintained and housed in the animal facility of the University of Southern California in pathogen-free conditions according to a protocol approved by the USC Institutional Animal Care and Use Committee (Los Angeles, CA, USA).

The *Rosa26-rtTA;tetO-Wnt5a (Wnt5a^{Rosa})* mice were generated by breeding *Rosa26-rtTA*[24] and *tetO-Wnt5a* mice (Jackson Laboratory, stock No: 022938).[25] The *Wnt5a^{Rosa}* mice were mated with *Sftpc-GFP* mice[26] to generate triple transgenic *Wnt5a^{Rosa};Sftpc-GFP mice*. *Sftpc-Wnt5a;TOPGAL* mice were generated by breeding *Sftpc-Wnt5a*[14] and *TOPGAL* mice[27] (the Jackson Laboratory, stock No: 004623). Genotype of the transgenic mice was determined by PCR with DNA isolated from mouse tail tips. The primer sequences for genotyping each transgenic mouse are as follows:

Rosa26-rtTA: (Rosa-5) 5′-GAGTTCTCTGCTGCCTC CTG-3, (Rosa-3) 5′-CGAGGCGGATACAAGCAATA-3′, and (rtTA-3) 5′-AAGACCGCGAAGAGTTTGTC-3′.

tetO-Wnt5a: (17815) 5′-ACAAAGACGATGACGAC AAGC-3′, (17816) 5′-CACACCTTCTTCAATGTACTG-3′, (OIMR7338) 5′-CTAGGCCACAGAATTGAAAGATCT-3′, and (OIMR7339) 5′-GTAGGTGGAAATTCTAGCATCATC C-3′.

Sftpc-GFP: (forward) 5′-TTCACTGGTGTTGTCCCAAT-3′ and (reverse) 5′-GACTTCAGCTCTGGTCTTGTAG-3′

Sftpc-Wnt5a: (forward) 5′-CAGGAACAAACAGGCTTC AAAG-3′ and (reverse) 5′-TTCTATAACAACCTGGGCG AAG-3′.

TOPGAL: (forward) 5′-ATCCTCTGCATGGTCAGGTC-3′ and (reverse) 5′-CGTGGCCTGATTCATTCC-3′

Doxycycline Administration

Doxycycline (Fisher Scientific, cat#ICN19895501) was prepared in water at 2 mg/mL and administered (80 µg per mouse, 1 dose per day) to neonatal mice at P4 and P5 orally with a plastic feeding needle (Instech Laboratories, PA). Neonatal lungs were collected at P7 for morphological, immunofluorescent, and molecular biological analyses.

Neonatal Lung Fibroblast Isolation and Culture

Fibroblasts from neonatal lungs were isolated as previously described.[15] In brief, P5 neonatal lungs of control and *Wnt5a*^{Rosa} mice were dissected in HBSS solution (GIBCO24020-117), digested with dispase and dissociated with a gentle MACS dissociator (Miltenyi Biotec. Inc., San Diego, CA). The dissociated cells were filtered through 40 µm cell strainers, collected by centrifugation, and resuspended in DMEM containing 10% fetal bovine serum (FBS). The cells were then plated in cell culture plates and incubated at 37 °C with 5% CO_2 for 1 h. After removing floating cells, the attached fibroblasts were washed with PBS and cultured in fresh medium. Loosely attached cells were removed by repeated washes within 20 h of culture. When the cells grew to near confluence, they were trypsinized and stored in 10% DMSO (DMEM + 10%FBS + 10%DMSO) in liquid nitrogen for future use. Cells less than passage 5 were plated in 12-well plates in DMEM with 10%FBS and treated with doxycycline as specified in figure legends before collection for qRT-PCR and Western blot analyses.

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from mouse lungs and human organoids with Trizol reagent (ThermoFisher Scientific, MA) and Zymo Direct-zol RNA microprep kit (Zymo Research, CA). cDNA was synthesized using the EasyScript Plus cDNA synthesis kit following the manufacturer's protocol (Lamda Biotech, Inc. MO).

Quantification of selected genes by qRT-PCR was conducted using a LightCycler (Roche Applied Sciences, IN) and the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Sciences, IN) as previously described.[14] Each reaction contained 3 µl of FastStart SYBR green reaction mix plus enzyme, 2.5 µM primers and 0.5 µl of cDNA in a total volume of 20 µL. The running protocol consisted of 4 steps, which includes (1) pre-denaturation at 95 °C for 6 minutes, (2) amplification and quantification at 95 °C for 10 s, 62 °C for 15 s and 72 °C for 20 s for 45 cycles, followed by (3) melting curve analysis, and ending with (4) cooling to 4 °C. The reaction conditions were optimized so that each reaction showed a single peak melting curve. Relative ratios of a target gene mRNA in transgenic lungs compared with littermate control lungs were analyzed by the ΔΔCt method[28] using TBP (TATA-box binding protein) as the reference gene. Primers for qRT-PCR were designed using IDT PrimerQuest (https://www.idtdna.com/pages/tools/primerquest). Sequences of the primers are listed in Supplementary Table 1.

Immunofluorescence Staining

Immunofluorescence staining was conducted as previously described.[15] In brief, 5 µm paraffin lung sections were processed through xylene deparaffinization, rehydration with decreasing ethanol and antigen retrieval with citrate buffer, followed by blocking with normal serum and probing with primary antibodies at 4 °C overnight. The sections were then incubated with combinations of fluorescent conjugated anti-mouse, anti-rabbit, anti-sheep, or anti-goat IgG secondary antibodies (Jackson ImmunoResearch Laboratories, ING). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). Primary antibodies used are listed in Supplementary Table 2.

Human Fetal Lung Organoid Culture

All human fetal lung tissue samples were collected under Institutional Review Board Approval at both the University of Southern California and Children's Hospital Los Angeles. Consent for tissue donation was obtained after the patient had already made the decision for pregnancy termination by Dilation and Curettage or Dilation and Evacuation and was obtained by a different clinical staff member than the physician performing the procedure. All tissues were de-identified, and the only clinical information collected was gestational age and the presence of any maternal or fetal diagnoses. Lung samples ranging in age from 11.0 to 11.6 weeks of gestation were received immediately after elective terminations and transported on ice in high glucose DMEM. Distal epithelial tips were isolated from human fetal lungs as previously described[29] with minor modifications. Lung lobes were incubated in dispase (Corning, Cat#354235) on ice for 20 min and dissociated through 1 mL pipette tips. The distal epithelial airways were isolated from the mesenchyme and then cut with tungsten needles to separate the tips from conducting airways. Six to 8 pieces of epithelial tips were subsequently embedded in 100% Geltrex (Fisher Scientifics) in NUNC cell culture plates (Thermo Fisher Scientific, CA) with growth medium described previously[30] which contains N2 (0.5×), B27 (0.5×), GlutaMax (1×), ascorbic acid (50 µg/mL), MTG (0.45 µM) CHIR99021 (3 µM), FGF10 (10 ng/mL), KGF (10 ng/mL), BMP4 (10 ng/mL), and retinoic acid (0.05 µM), in IMDM/Ham's F12 medium (3/1). After 2 weeks, the organoids were removed from old matrix and re-embedded in fresh Geltrex with 2-3 pieces per well and further cultured under the same condition for 2 more weeks. Subsequently, the organoids were cultured in differentiation media containing N2 (0.5×), B27(0.5×), GlutaMax (1×), ascorbic acid (50 µg/mL), MTG (0.45 µM), FGF10 (10 ng/mL), KGF (10 ng/mL), dexamethasone (127 nM), cAMP (100 µM), and IBMX (100 µM) in IMDM/Ham's F12 medium (3/1) in the presence of 200 ng/mL recombinant WNT5a protein (R&D systems, MN) or vehicle before harvesting for qRT-PCR analysis.

Western Blot

Protein extracts were prepared from cultured cells with RIPA buffer (Sigma, MO), separated on 4-12% Tris-Glycine gels and blotted onto nitrocellulose membranes (Bio-Rad, CA). The membranes were probed with antibodies and analyzed with the SuperSignal West PICO chemiluminescent substrate as described by the manufacturer (Thermo Fisher Scientific, CA). Primary antibodies used for Western blot are listed in Supplementary Table S2.

Statistical Analysis

At least 3 independent control and mutant lungs were used for each morphometric and molecular biological analysis. Five images (10× magnification) from each lung were used to manually calculate mean linear intercept (MLI) as described.[31] For the ratios of Mki67 and Acta2 positive cells, 700 to 1300 cells were counted from each control or mutant lung. Quantitative data are presented as mean values ± the standard error of the mean (sem). *P*-values were calculated by 2-tailed Student's *t* tests and $P < .05$ was considered statistically significant.

Results

Arrested Alveologenesis in *Wnt5a*^{Rosa} Lungs

To determine the function of Wnt5a during alveologenesis, we generated a conditional Wnt5a gain-of-function model by breeding *Rosa26-rtTA*[24] and *tetO-Wnt5a*[25] mice to derive *Rosa26-rtTA;tetO-Wnt5a* (*Wnt5a*^{Rosa}) progeny in which doxycycline can activate expression of a mouse Flag-tagged Wnt5a protein.[25] Over 60% of the *Wnt5a*^{Rosa} mice that were administered doxycycline at P4 and P5 died between P8 and P10 with reduced body weight (*Wnt5a*^{Rosa} vs control at P7: 57.4 ± 9.5%) and signs of respiratory distress (gasping for air). The *tetO-Wnt5a* and *Rosa26-rtTA*, control littermates, were normal. Histological analysis of P7 mouse lungs revealed active alveologenesis in control lungs, represented by the presence of small distal alveolar clusters (arrows, Fig. 1H). In the *Wnt5a*^{Rosa} lungs, these structures were either missing (Fig. 1I) or collapsed (arrows, Fig. 1K), suggesting that Wnt5a gain-of-function disrupted alveologenesis. These findings were validated by increased MLI and reduced number of secondary crests in the *Wnt5a*^{Rosa} lungs (Fig. 1L and M).

Wnt5a Reduces Proliferation and Alters Differentiation of Multiple Alveolar Cell-Types

To determine the potential cause of the observed *Wnt5a*^{Rosa} lung phenotype, and because Wnt5a is known to regulate cell proliferation,[21,32] we examined cell proliferation in *Wnt5a*^{Rosa} lungs. Fig. 2A shows significantly decreased transcripts for 2 proliferation related genes, *Mki67* (0.22 ± 0.02, $P < .05$) and *Pcna* (0.53 ± 0.04, $P < .05$) in the *Wnt5a*^{Rosa} lungs. No changes were found in *Myc* mRNA. Correspondingly, manual scoring of Mki67-positive cells using multiple histological preparations also revealed a significant decrease in proliferating cells in *Wnt5a*^{Rosa} lungs (Ctrl: 14.9 ± 0.7%, *Wnt5a*^{Rosa}: 5.5 ± 0.2%, Fig. 2B and C).

Mesodermal control of alveologenesis is well supported by the results of multiple studies.[33,34] We therefore examined expression of myofibroblast markers and found that levels of *Acta2*, *Tagln*, and *Des* were drastically reduced in *Wnt5a*^{Rosa} lungs (Fig. 2D). Consistent with the latter, immunofluorescence staining with Acta2 antibody revealed decreased number of Acta2^{pos} cells in the *Wnt5a*^{Rosa} lungs (Fig. 2E and F).

To determine the impact of Wnt5a gain-of-function on other alveolar cells, we further characterized markers of AT1, AT2 and endothelial cells in control and *Wnt5a*^{Rosa} lungs. We found increased expression of multiple AT1 lineage-specific markers (*Hopx*, *T1a*, and *Cav1*), while AT2 markers remained mostly unchanged in *Wnt5a*^{Rosa} lungs (Fig. 2G and I). Pdpn (also named T1α) staining in the *Wnt5a*^{Rosa} lungs was also clearly brighter than that of control lungs (Fig. 2H). In the endothelial lineage, *Flk1* expression was decreased (Fig. 2K), suggesting that endothelial progenitors (which express *Flk1*) may have been reduced by Wnt5a gain-of-function.[35]

Wnt5a Activates Ca^{2+}-Dependent Non-Canonical and Represses Canonical Wnt Signaling in Mesenchymal Cells

To further verify the function of Wnt5a in mesenchymal cells, we conducted in vitro analyses with primary fibroblasts isolated from P5 *Wnt5a*^{Rosa} and control lungs (Fig. 3). In support of the in vivo findings, in vitro activation of *Wnt5a* by doxycycline repressed cell proliferation as shown by reduced expression of *Mki67* (0.63 ± 0.09, $P < .05$) and *Pcna* (0.78 ± 0.04, $P < .05$) (Fig. 3A). Transcripts of canonical myofibroblast markers (*Acta2*, *Tagln*, and *Des*) were also decreased, although not significantly, when compared with the control cells. No significant changes were observed in markers of pericytes (*Pdgfrb* and *Ng2*) and lipofibroblasts (*Adrp*, *Tcf21*, *Fgf10*, and *Pparg*) (Fig. 3B).

To further understand the potential mechanism that mediates the effects of Wnt5a, we examined the activity of downstream signaling pathways. Wnt5a signaling mainly activates the β-catenin independent non-canonical Wnt pathways including the Ca^{2+} dependent and PCP pathways.[36] Western blot analysis revealed significantly increased phospho-CaMKII (p-CaMKII) in the fibroblasts with Wnt5a gain-of-function, indicating activation of the Ca^{2+}-dependent pathway by Wnt5a. No significant change was observed in levels of p-PKC and p-JNK (Fig. 3C to F).

Ca^{2+}-dependent non-canonical Wnt signaling has been shown to inhibit canonical Wnt signaling which regulates cell proliferation. Therefore, we determined the expression of canonical Wnt targets by qRT-PCR. Our results indicate that expression of canonical Wnt signaling target genes *Axin2* and *Tcf7* was significantly reduced in Wnt5a gain-of-function fibroblasts (Fig. 3G). In support of this finding, canonical Wnt signaling targets *Tcf7* and *Ccnd1* were also decreased in *Wnt5a*^{Rosa} lungs when compared to control lungs (Fig. 3H). Therefore, Wnt5a activates Ca^{2+}-dependent non-canonical Wnt signaling and represses canonical Wnt signaling in downregulating mesenchymal cell proliferation.

Wnt5a Differentially Promotes AT1, Represses AT2 Lineage-Specific Gene Expression, and Inhibits Canonical Wnt Signaling in Subpopulations of Sftpc^{pos} Cells.

AT1s arise from Hopx^{pos} progenitors specified in the embryonic period as well as Sftpc^{pos} AT2s after birth.[6,7] Since Wnt5a gain-of-function promoted AT1 differentiation during alveologenesis as evidenced by increased expression of several AT1 lineage-specific markers (Fig. 2), we characterized the impact of Wnt5a in the Sftpc^{pos} cells in neonatal lungs. To isolate Sftpc^{pos} cells, we used *Sftpc-GFP* mice in which

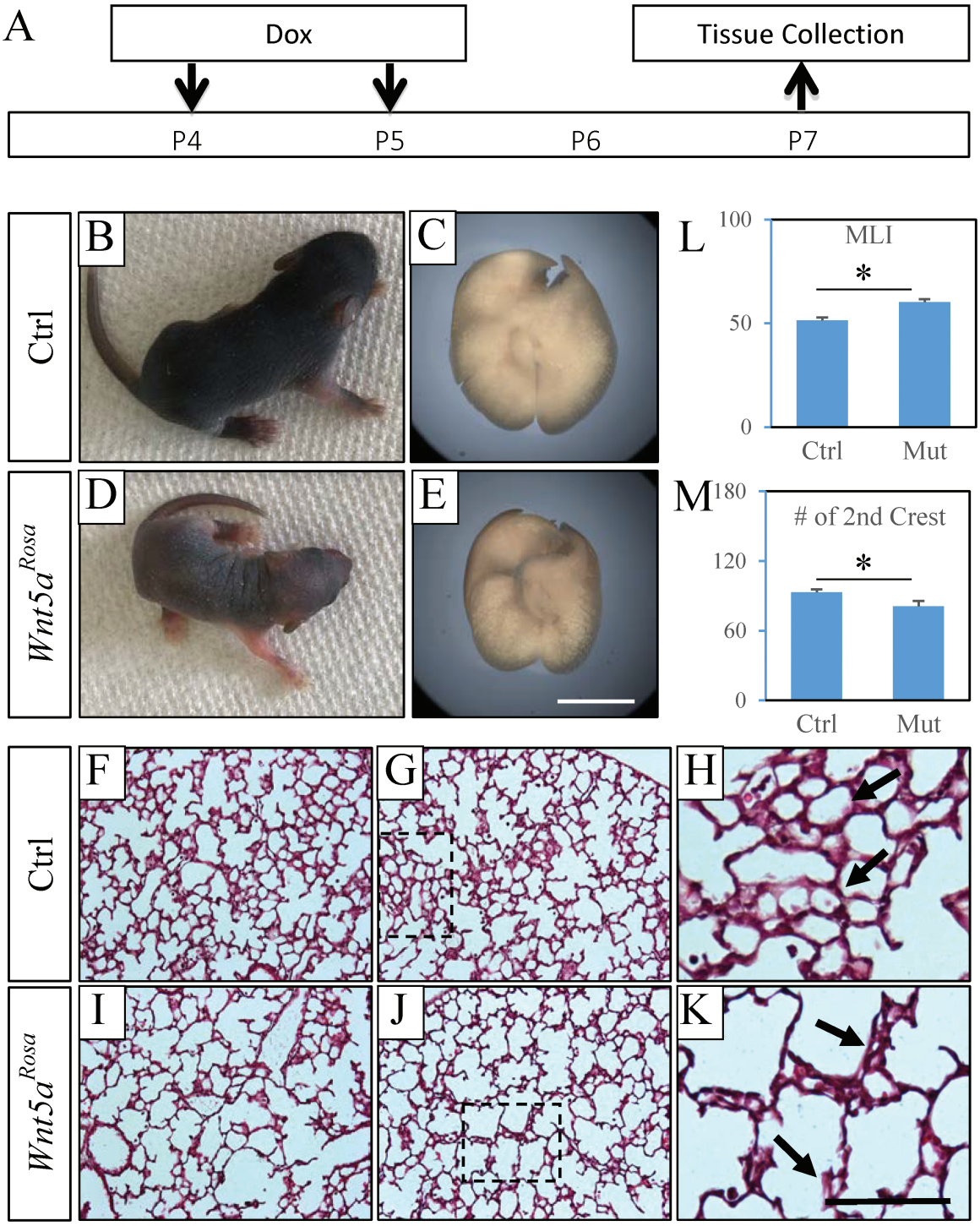

Figure 1. Wnt5a gain-of-function disrupts alveologenesis. (**A**) Schematic of the experimental plan. (**B-E**) Gross morphology of P7 control (**B-C**) and *Wnt5a*^{Rosa} (**D-E**) neonatal mice (**B, D**) and lungs (**C, E,** post-fixation). (**F-K**) H&E staining of P7 control (Ctrl, **F-H**) and *Wnt5a*^{Rosa} (**I-K**) lungs. Panels H and K show higher magnification of boxed areas in G and J, respectively. Arrows in H indicate clusters of small distal alveoli in control lungs. Arrows in K show collapsed distal alveoli in *Wnt5a*^{Rosa} lungs. (**L**) Mean linear intercept (MLI) of control and *Wnt5a*^{Rosa} lungs. (**M**) Number of secondary crests per unit area (0.39 mm^2) of control and *Wnt5a*^{Rosa} lungs. Five areas from each of 4 independent lungs were measured for MLI and number of secondary crests. Data represent mean ± SEM. * indicates $P < .05$. Scale bar: 5 mm for C and E, 300 μm for F, G, I, and J, 100 μm for H and K.

GFP is expressed exclusively in Sftpc^{pos} cells (Fig. 4A). FACS isolation of total GFP^{pos} cells from P7 *Sftpc-GFP* lungs revealed 2 distinct populations distinguished by different GFP expression levels (Fig. 4B and C). qRT-PCR analysis showed that the GFP^{low} subpopulation (Sftpc^{low}) expresses lower levels of AT2 and higher levels of AT1 lineage-specific markers when compared with GFP^{high} (Sftpc^{high}) population, which was distinguished by higher levels of AT2 and lower levels of AT1 lineage-specific markers (Fig. 4D and E). In addition, canonical Wnt targets such as *Axin 2*, *Ccnd1*, and *Jag1* were reduced, while *Tcf7* and *Lef1* were increased in the GFP^{low} subpopulation (Fig. 4F). To determine whether Wnt5a

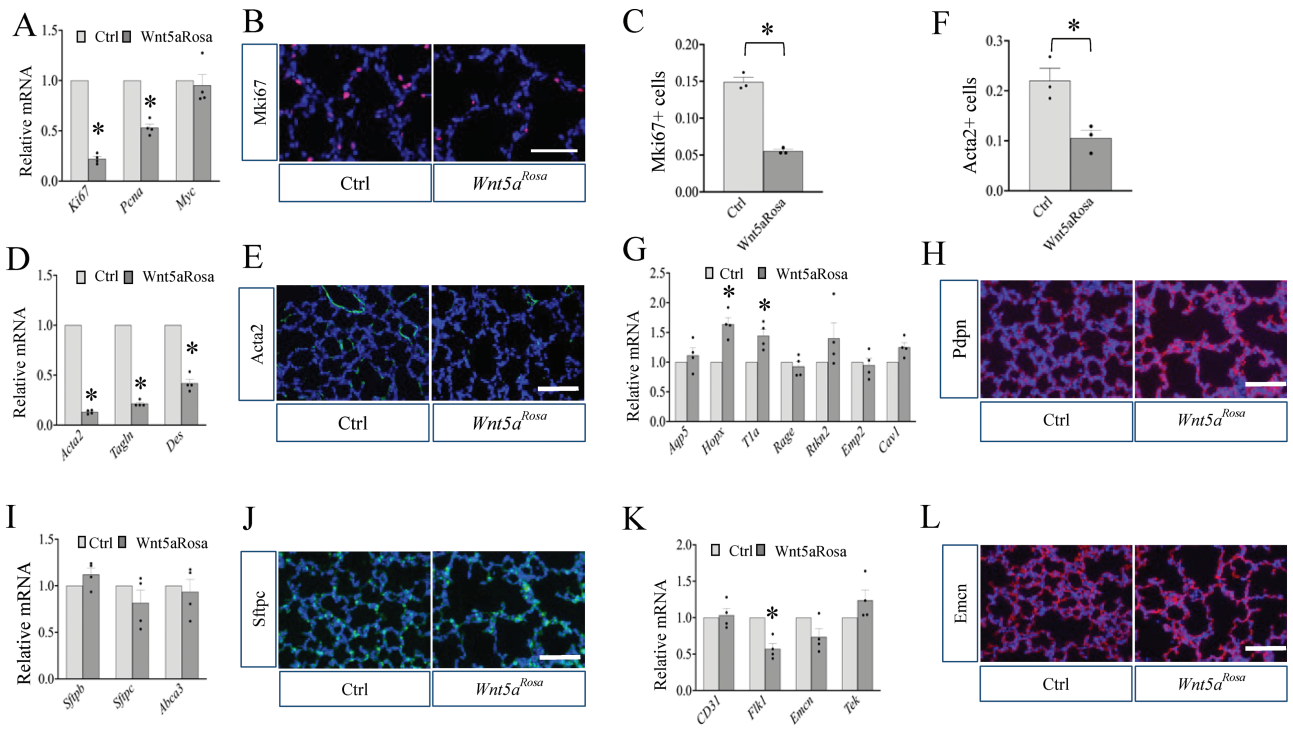

Figure 2. Wnt5a reduces proliferation and alters differentiation of multiple alveolar cell-types during alveologenesis. (**A**) Relative mRNA levels of proliferation markers by qRT-PCR analyses. (**B**) Immunostaining of Mki67 (red) in control and *Wnt5a*^{Rosa} lungs. (**C**) Percentage of Mki67-positive cells (Mki67+) in total Dapi-positive cells in control and *Wnt5a*^{Rosa} lungs. (**D**) Relative mRNA levels of myofibroblast markers by qRT-PCR analyses. (**E**) Immunostaining of Acta2 in control and *Wnt5a*^{Rosa} lungs. (**F**) Percentage of Acta2 positive cells (Acta2+) in total Dapi-positive cells in control and *Wnt5a*^{Rosa} lungs. (**G**) Relative mRNA levels of AT1 genes by qRT-PCR analyses. (**H**) Immunostaining of Pdpn (T1a) in control and *Wnt5a*^{Rosa} lungs. (**I**) Relative mRNA levels of AT2 genes by qRT-PCR analyses. (**J**) Immunostaining of Sftpc in control and *Wnt5a*^{Rosa} lungs. (**K**) Relative mRNA levels of endothelial markers by qRT-PCR analyses. (**L**) Immunostaining of Emcn in control and *Wnt5a*^{Rosa} lungs. Data represent mean ± SEM. * indicates $P < .05$. $n = 4$ (qPCR) and 3 (cell ratio). Scale bar: 50 μm in B; 100 μm in E, H, J, and L.

differentially impacted the 2 subpopulations, we generated triple transgenic, *Sftpc-GFP*; *Wnt5a*^{Rosa} model and examined the expression of lineage-specific AT1 and AT2 markers in each of the 2 subpopulations in control and transgenic lungs. As shown in Fig. 5A, gain of Wnt5a function increased AT1 lineage-specific markers but inhibited those of AT2 in the GFP^{low} population. In addition, both cell proliferation and canonical Wnt targets were decreased by Wnt5a in the GFP^{low} population (Fig. 5B and C). In the GFP^{high} population, Wnt5a gain-of-function also increased AT1 lineage-specific markers (Fig. 5D). Importantly however, no significant alteration was observed in cell proliferation, expression of AT2 lineage-specific genes, or canonical Wnt targets (Fig. 5D to F). Of note however, certain canonical Wnt targets such as *Ccnd1* were in fact increased. These data demonstrate that the activity of Wnt5a in regulating canonical Wnt signaling and AT2 vs AT1 lineage-specific genes in epithelial cell subpopulations is highly selective and dependent on developmental status.

Wnt5a Promotes AT1, Represses AT2 Lineage-Specific Gene Expression and Inhibits Canonical Wnt Signaling in Mouse Fetal Lungs

AT1 cell fate is specified around embryonic days E13 to E15, when AT1 progenitors are identifiable as Hopx^{pos} cells in the stalk region of distal epithelial airways.[37] To determine whether Wnt5a affects AT1 cell fate specification, we characterized the lungs of E15 *Sftpc-Wnt5a* transgenic mice, in which *Wnt5a* is over-expressed in Sftpc^{pos} epithelial cells.[14] As shown in Fig. 6A, Wnt5a gain-of-function moderately, but significantly, increased AT1 lineage-specific gene signature and decreased the AT2 gene *Sftpc* (*Aqp5*: 1.28 ± 0.05, $P < .05$; *Hopx*: 1.15 ± 0.03, $P < .05$; *Sftpc*: 0.48 ± 0.06, $P < .05$). Immunofluorescence staining revealed that spatial distribution of Hopx^{pos} AT1 progenitors and Sftpc^{pos} epithelial progenitors remains similar between the control and transgenic lungs (Fig. 6B).

To determine whether Wnt5a affects canonical Wnt signaling in mouse fetal lung epithelial cells, we mated the *Sftpc-Wnt5a* mice with canonical Wnt reporter mouse line, *TOPGAL*. The *TOPGAL* transgenic mice express β-galactosidase (*LacZ*) in response to activation of canonical Wnt signaling.[27] β-gal staining revealed decreased *LacZ* activity by Wnt5a in the *Sftpc-Wnt5a;TOPGAL* lungs as compared to the littermate controls (Fig. 6C). In support of the latter finding, qRT-PCR showed that expression of *LacZ* and canonical Wnt target *Tcf7* were reduced in *Sftpc-Wnt5a;TOPGAL* and *Sftpc-Wnt5a* lungs, respectively (Fig. 6D to F).

Wnt5a Promotes AT1 Lineage-Specific Gene Expression in Human Fetal Lung Epithelial Progenitors

In sum, the studies described above demonstrate that Wnt5a regulates not only postnatal epithelial cells during the process of alveologenesis but also drives fetal epithelial progenitors

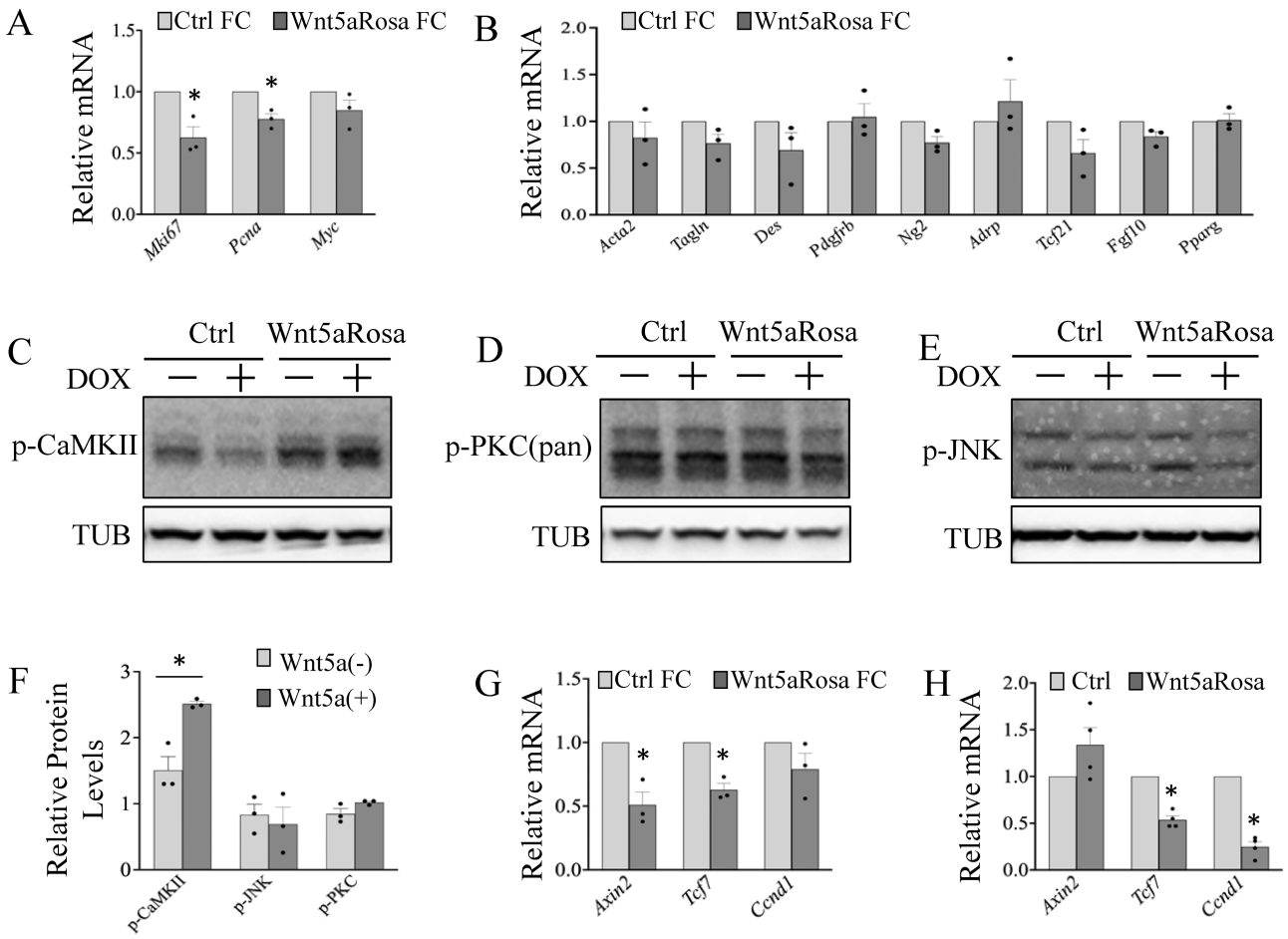

Figure 3. Wnt5a activates CaMKII, inhibits canonical Wnt signaling, and represses proliferation in fibroblast cells. Primary fibroblasts were isolated from P5 control and *Wnt5a*^{Rosa} lungs, cultured in 12-well plates and treated with doxycycline (0.1 μg/mL) for 24 h and then analyzed by qRT-PCR or Western blot. (**A**) Relative mRNA levels of proliferation markers by qRT-PCR analyses. (**B**) Relative mRNA levels of myofibroblast (*Acta2, Tagln, Des*), lipofibroblasts (*Adrp, Tcf21, Fgf10, Pparg*) and pericytes (*Pdgfrb, Ng2*) markers by qRT-PCR analyses. (**C-E**) Western blot analyses of p-CaMKII, p-PKC, and p-JNK in control and *Wnt5a*^{Rosa} fibroblast cells with or without doxycycline (DOX). α-Tubulin (TUB) was used as loading control. (**F**) Quantification of Western blot results from 3 independent experiments. Value of Wnt5a (–) represents relative protein levels (normalized to α-tubulin) between *Wnt5a*^{Rosa} and control cells without DOX. Value of Wnt5a (+) represents relative protein levels (normalized with α-tubulin) between *Wnt5a*^{Rose} and control cells with DOX. (**G, H**) Relative mRNA levels of canonical Wnt signaling target genes in primary fibroblasts **(G)** and P7 lungs **(H)** by qRT-PCR analyses. Data represent mean ± SEM. * indicates $P < .05$. $n = 3$.

towards an AT1 fate as well as modulating canonical Wnt activities in mice. To determine whether this novel function of Wnt5a in mice is evolutionarily conserved in human lungs, we isolated distal tip epithelial progenitors from 11.0- to 11.6-week gestational age human fetal lung tissues and cultured them in organoid growth medium.[30] After 4 weeks of culture, the organoids were treated with recombinant WNT5a protein for 3 days and then analyzed for gene expression by qRT-PCR. As shown in Fig. 7B, WNT5a treatment increased expression of the AT1 lineage-specific signature genes (*PDPN*: 2.24 ± 0.46, $P = .07$; *HOPX*: 1.89 ± 0.23, $P < .05$; *AGER*:1.90 ± 0.47, $P = .15$). No significant inhibition of AT2 lineage-specific genes or proliferation markers was observed. In fact, even though statistically insignificant, *SFTPC* was indeed increased. For canonical WNT signaling targets, *LEF1* was significantly increased while *AXIN2* and *TCF7* remained unchanged (*LEF1*: 1.52 ± 0.11, $P < .05$) (Fig. 7C). Therefore, the pro-AT1 effects of WNT5a are indeed evolutionarily conserved, while the impact on AT2 genes and canonical WNT signaling is variable across mouse and human species.

Discussion

In the current study, we describe previously unappreciated, novel and cell-type selective functions of Wnt5a in modulating AT1 and AT2 lineage-specific gene expression. During mouse alveologenesis and in mouse fetal lungs Wnt5a promotes AT1 and inhibits AT2 lineage-specific gene expression, concurrent with inhibition of canonical Wnt signaling. Importantly, during alveologenesis and only in a select subpopulation of mouse AT2 cells, those distinguished by high levels of Sftpc expression, Wnt5a acts to promote AT1 lineage-specific genes without inhibiting canonical Wnt signaling. The latter function of Wnt5a is evolutionarily conserved in fetal human epithelial progenitors. Thus, Wnt5a regulates alveolar epithelial lineages in a highly temporal and cell-type selective manner

Wnt5a Exhibits Pro-AT1 and Anti-AT2 Functions, the Latter Associated With Inhibition of Canonical Wnt Signaling

AT1 cell differentiation is essential for both normal lung development and injury repair. In the current study, we found

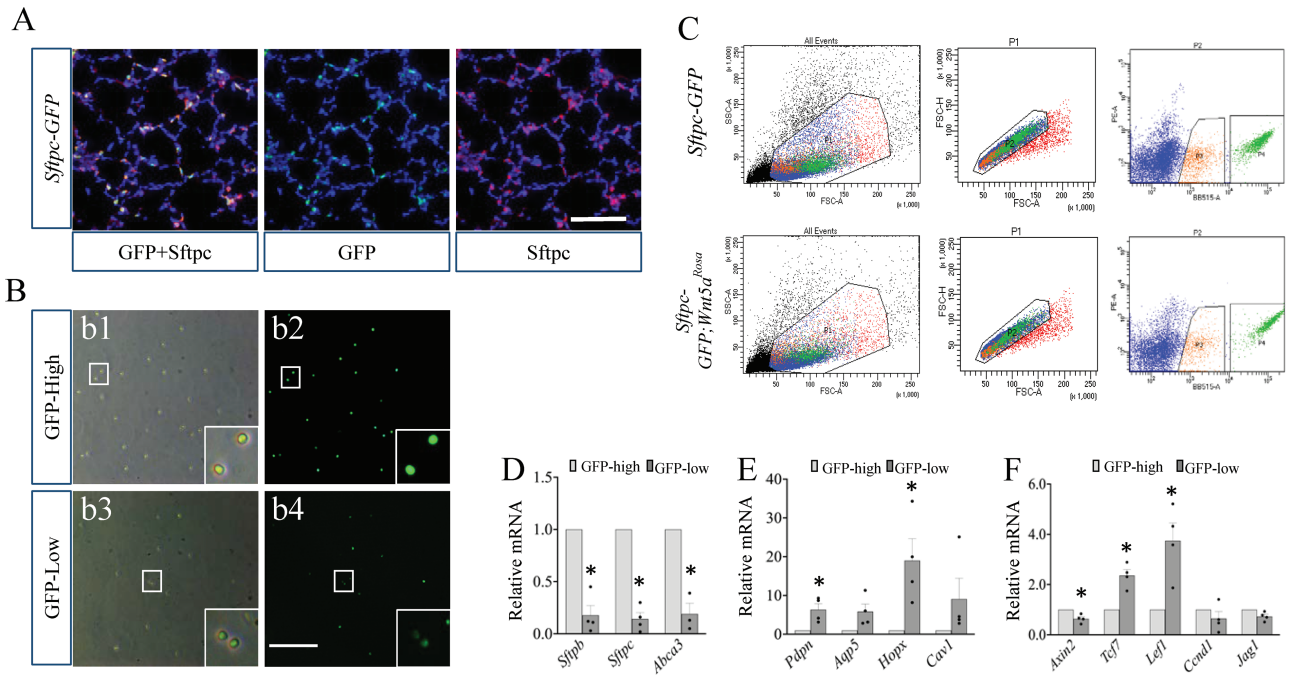

Figure 4. Two populations of Sftpc^{pos} alveolar epithelial cells are present in neonatal lungs. (**A**) Immunostaining of GFP (green) and Sftpc (red) in P7 *Sftpc-GFP* lungs. Almost all GFP^{pos} cells are Sftpc^{pos}. (**B**) Bright field (**b1, b3**) and fluorescent (**b2, b4**) images of FACS sorted GFP^{high} (**b1, b2**) and GFP^{low} (**b3, b4**) cells. Insets show higher magnification of boxed areas. (**C**) Gating strategy of FACS sorting of GFP^{pos} cells from *Sftpc-GFP* (control) and *Sftpc-GFP; Wnt5a^{Rosa}* lungs. (**D**) Relative mRNA levels of AT2 markers (GFP^{low} vs GFP^{high}). (**E**) Relative mRNA levels of AT1 markers (GFP^{low} vs GFP^{high}). (**F**) Relative mRNA levels of canonical Wnt targets (GFP^{low} vs GFP^{high}). Data represent mean ± SEM. * $P < .05$. $n = 4$. Scale bar: 100 μm in A, 200 μm in B.

that Wnt5a promotes AT1 (pro-AT1) and represses AT2 (anti-AT2) lineage-specific gene expression in mouse fetal lungs and in alveolar stage Sftpc^{low} cells. Our data demonstrate that the anti-AT2 function of Wnt5a occurs concurrently with downregulation of canonical Wnt signaling (Supplementary Table S3). Consistent with these findings, canonical Wnt signaling has been shown to play an important role in maintaining AT2 progenitors while inhibition of canonical Wnt signaling has been thought to promote AT1 differentiation.[8] A recent study by Kanagaki et al further demonstrated that inhibiting canonical Wnt signaling in pluripotent stem cells induces AT1 differentiation.[9] Interestingly, we found that in Sftpc^{high} cells during mouse lung alveologenesis and in human fetal lung epithelial progenitors, Wnt5a promotes AT1 without inhibiting AT2 lineage-specific gene expression (Figs. 5 and 7). Therefore, Wnt5a is able to execute the pro-AT1 function independent of the anti-AT2 function in certain cell populations. Furthermore, the pro-AT1 function is independent of inhibiting canonical Wnt activity (Supplementary Table S3), suggesting that the pro-AT1 and anti-AT2 functions of Wnt5a are mediated through different mechanisms distinct from the pathway that involves downregulation of canonical Wnt signaling.

Wnt5a Regulates Canonical Wnt Signaling Depending on Discrete Physiological and Pathological Conditions

Wnt5a has been shown to repress canonical Wnt signaling.[38] It can also activate canonical Wnt signaling in the presence of certain receptors. In neonatal lungs, we found that Wnt5a gain-of-function represses canonical Wnt signaling in both fibroblasts (Fig. 3G and H) and a particular subpopulation of Sftpc^{pos} cells (ie Sftpc^{low} cells, Fig. 5C). In contrast, this inhibitory effect of Wnt5a was not observed in the Sftpc^{high} cells (Fig. 5F). Furthermore, expression of *Ccnd1* was increased by Wnt5a in the Sftpc^{high} cells. Therefore, in neonatal epithelial cells Wnt5a's impact on canonical Wnt signaling is dependent on the cellular context. This cellular contextual dependence of Wnt5a may be critical in understanding the physiological and pathological mechanisms underlying lung development and disease. For example, in models of COPD, Wnt5a inhibited canonical Wnt signaling and exacerbated airspace enlargement.[18] Consistent with the latter, Wnt5a and Wnt5b repressed both canonical Wnt signaling and the growth of alveolar epithelial progenitors in lung organoid culture.[39] In comparison, in a hyperoxia-induced BPD mouse model, increased mesenchymal Wnt5a was associated with active canonical Wnt signaling.[23] In adult lung homeostasis, Wnt5a is enriched in the niche mesenchymal cells surrounding and maintaining the AT2 stem cells which exhibit higher canonical Wnt activity represented by expression of Axin2 (Axin2^{pos}).[17] These data collectively demonstrate the complexity of the Wnt5a signaling pathway in executing its multi-functional roles in different aspects of lung biology.

Difference Between Subpopulations of Sftpc^{pos} Cells, and Between Mouse and Human Lung Epithelial Progenitors

Sftpc^{pos} cells are heterogenous in the neonatal lungs. They contain differentiated AT2s and transient AT1/AT2 cells which are differentiating toward AT1 or AT2 cell fate. Using the *Sftpc-GFP* model, the alveolar stage Sftpc^{pos} cells can be distinguished and isolated as GFP^{low} (Sftpc^{low}) and GFP^{high} (Sftpc^{high}) subpopulations. The GFP^{low} cells express lower levels of *Sftpc*

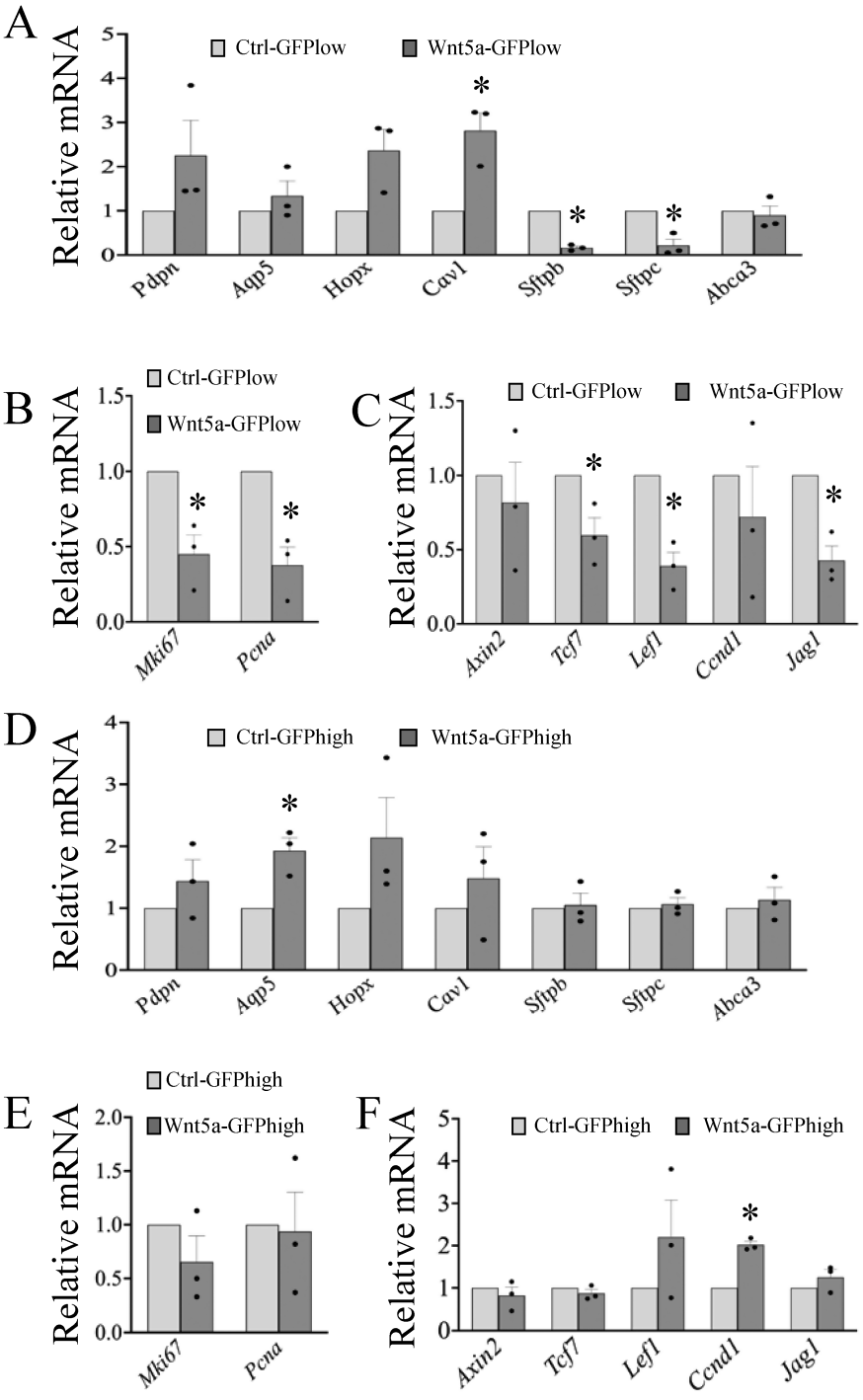

Figure 5. Wnt5a promotes AT1 differentiation in both GFP^{low} and GFP^{high} cells but represses AT2 genes and canonical Wnt signaling only in the GFP^{low} subpopulation. (**A**) Relative mRNA levels of AT1 and AT2 genes in GFP^{low} cells (*Wnt5a^{Rosa}* vs control). (**B**) Relative mRNA levels of proliferation markers in GFP^{low} cells (*Wnt5a^{Rosa}* vs control). (**C**) Relative mRNA levels of canonical Wnt targets in GFP^{low} cells (*Wnt5a^{Rosa}* vs control). (**D**) Relative mRNA levels of AT1 and AT2 genes in GFP^{high} cells (*Wnt5a^{Rosa}* vs control). (**E**) Relative mRNA levels of proliferation markers in GFP^{high} cells (*Wnt5a^{Rosa}* vs control). (**F**) Relative mRNA levels of canonical Wnt targets in GFP^{high} cells (*Wnt5a^{Rosa}* vs control). Data represent mean ± SEM. * indicates $P < .05$. $n = 3$.

and higher levels of AT1 lineage-specific genes, suggesting that they may represent the AT1/AT2 transitional cell population. Interestingly, a recent study reported that 2 distinct AT2 subpopulations were detected in adult mouse lungs by using a Sftpc^{creERT2};tdTomato$^{flox/flox}$ model.[40] The authors found that the Tom^{high} (Sftpc^{high}) cells represent more mature AT2 cells while the Tom^{low} (Sftpc^{low}) cells function as epithelial progenitors and respond to pneumonectomy. In the present study we compared alveolar stage GFP^{low} and GFP^{high} cells with the mouse fetal epithelial progenitors and found that the GFP^{low} cells are similar to mouse fetal epithelial progenitors in terms of AT1 and AT2 lineage-specific gene expression as well as canonical Wnt responsiveness to Wnt5a. For example, Wnt5a represses AT2 lineage-specific genes and canonical Wnt signaling in both fetal epithelial progenitors and GFP^{low} cells, but, importantly, not in GFP^{high} cells. Therefore,

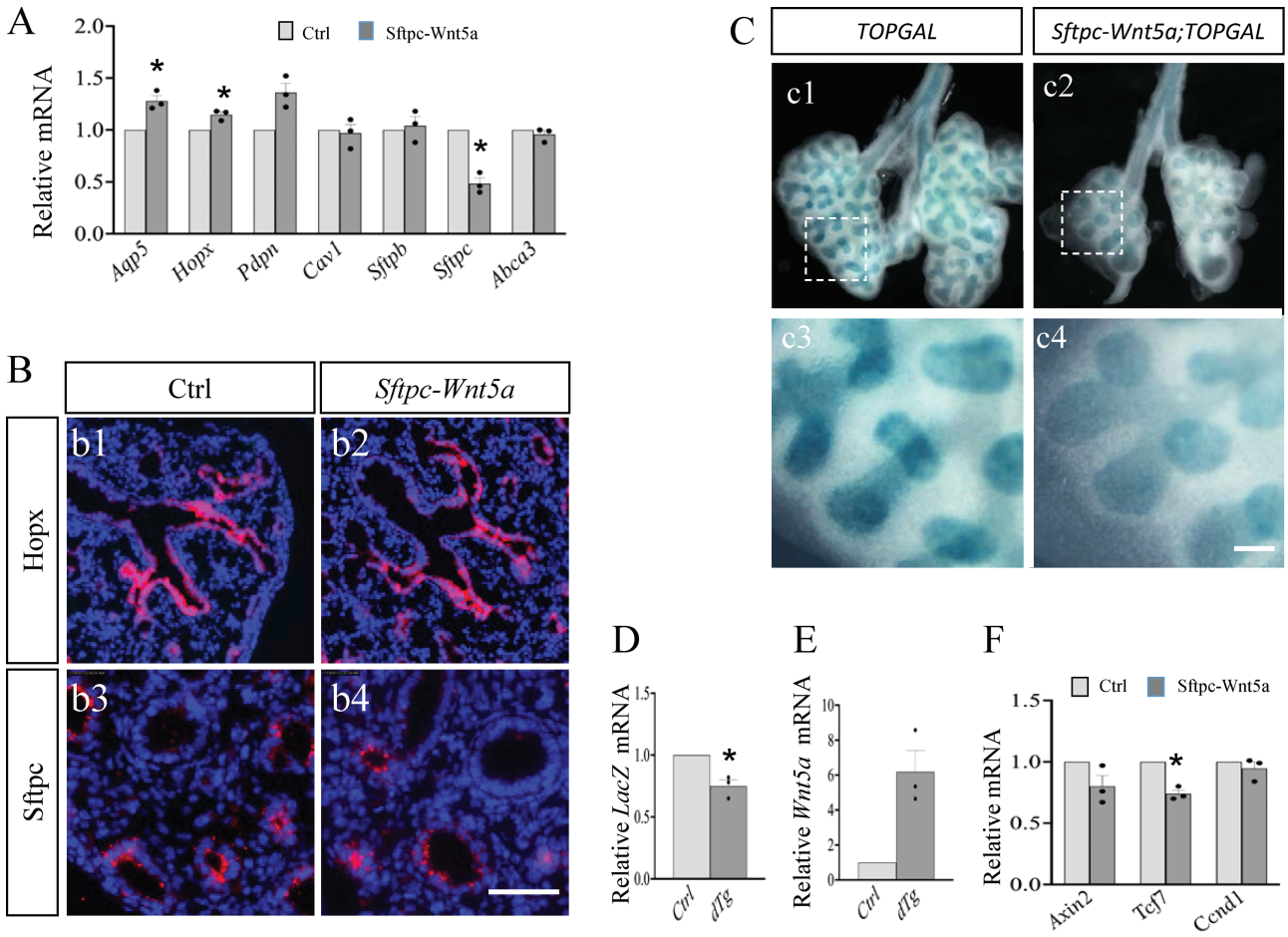

Figure 6. Wnt5a promotes AT1 gene expression but represses *Sftpc* and canonical Wnt signaling during mouse fetal lung development. (**A**) Relative mRNA levels of AT1 and AT2 genes in E15 *Sftpc-Wnt5a* lungs when compared with that of littermate control lungs. (**B**) Immunostaining of Hopx (**b1, b2**) and Sftpc (**b3, b4**) in control (**b1, b3**) and *Sftpc-Wnt5a* (**b2, b4**) lungs. (**C**) β-Gal staining of E13 *TOPGAL* (control, **c1, c3**) and *Sftpc-Wnt5a;TOPGAL* (**c2, c4**) lungs. Panels **c3** and **c4** show higher magnification of boxed areas in **c1** and **c2**, respectively. (**D, E**) qRT-PCR analyses of *LacZ* (**D**) and *Wnt5a* (**E**) in *TOPGAL* (Ctrl) and *Sftpc-Wnt5a;TOPGAL* (dTg) lungs. (**F**) Relative mRNA levels of canonical Wnt signaling targets in E15 *Sftpc-Wnt5a* lungs when compared with that of littermate control lungs. Data represent mean ± SEM. *$P < .05$. $n = 3$. Scale bar: 100 μm in b1 and b2, 50 μm in b3 and b4, 400 μm in c1 and c2, 100 μm in c3 and c4.

the GFP^{low} cells may retain certain progenitor properties and plasticity and are more sensitive to the stimulus (Wnt5a) for AT2 to AT1 transition, when compared with the mature AT2s (GFP^{high}). The origin of the GFP^{low} population remains to be further determined. It is likely that they represent transient AT1/AT2 cells originating from AT2 progenitors or from AT1 progenitors undergoing AT1 to AT2 differentiation.[37] In addition, it is also possible that these cells are derived from the bipotent AT1/AT2 progenitors that are present during the saccular stage of lung development.[41]

Our data also demonstrate important common features as well as differences between mouse and human fetal lung epithelial progenitors in response to Wnt5a. As summarized in Supplementary Table S3, Wnt5a activates AT1 lineage-specific gene expression in both cells. However, Wnt5a negatively regulates AT2 lineage-specific gene and canonical Wnt activity in mouse but not in human fetal lung epithelial progenitors. Further transcriptomic and proteomic analyses of Wnt receptors and mediators, as well as elucidating the nature of the crosstalk signaling will help to understand the underlying mechanisms.

Multi-Functional Roles of Wnt5a Signaling in Lung Maturation

Previously, we found that inactivation of Wnt5a disrupted alveologenesis by inhibiting myofibroblast differentiation and migration. In the current study, Wnt5a gain-of-function also disrupted alveologenesis by inhibiting proliferation of fibroblasts, which occurs via activation of p-CaMKII-mediated non-canonical and inhibition of canonical Wnt signaling. Therefore, the functional repertoire of Wnt5a appears to be multimodal dependent on different cellular activities. What is certain is that dysregulated *Wnt5a* adversely affects alveologenesis, indicating that finely regulated Wnt5a signaling is required for normal lung development. This feature is true for many of the key growth factors or signaling molecules that play essential roles in morphogenesis, tissue maintenance, cellular differentiation, and regeneration. Therefore, a nuanced understanding of the multifunctional properties of Wnt5 signaling is essential for exploring its potential application in translational medicine.

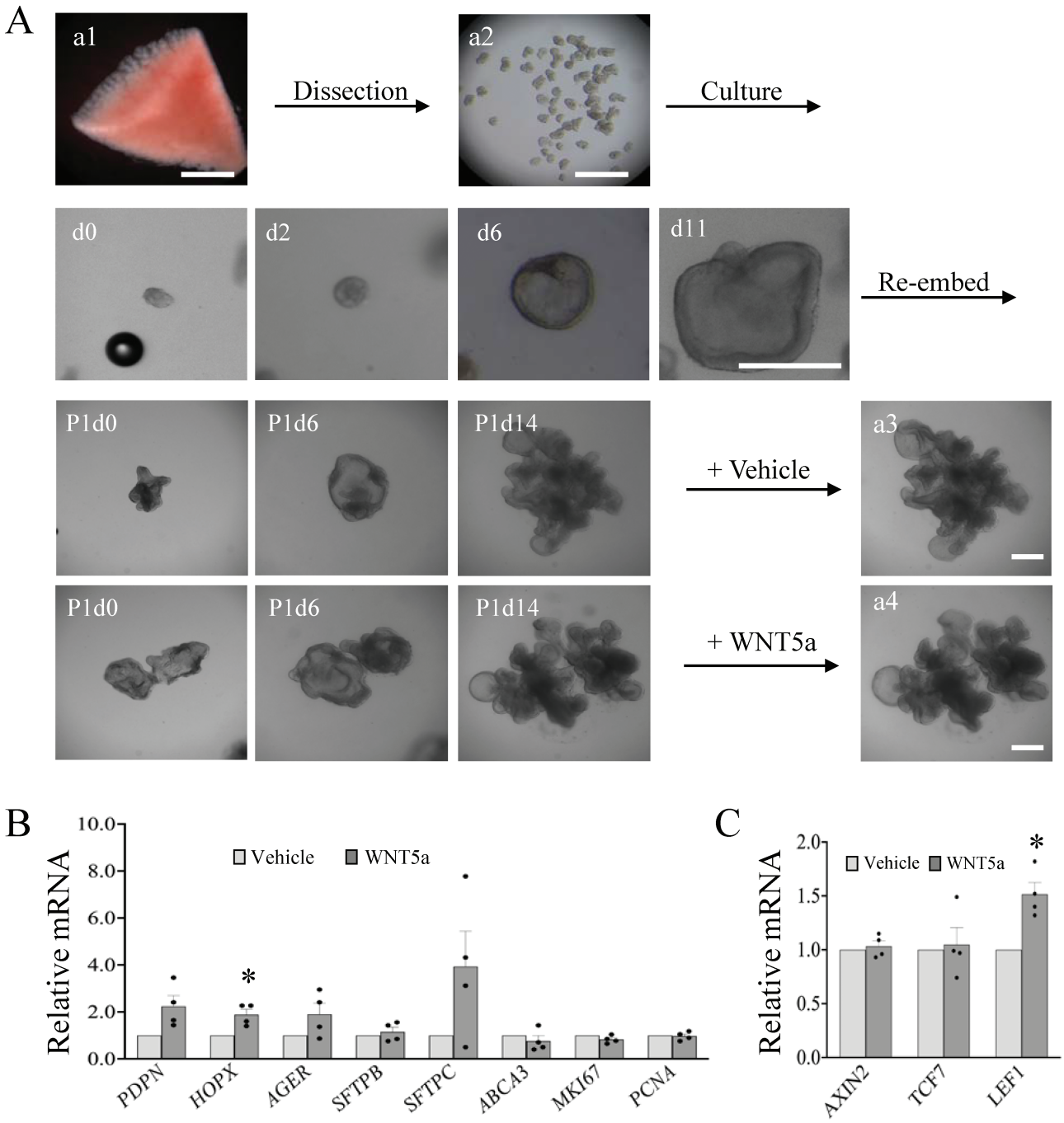

Figure 7. Wnt5a promotes AT1 gene expression in human fetal lung epithelial progenitors. (**A)** Illustration of the organoid culture procedures. **a1:** Lung tissue of 11.5 weeks gestational age. **a2:** Distal epithelial tips isolated from the fetal lungs. **d0, d2, d6, d11:** representative organoids derived from distal epithelial tips cultured in Geltrex for 0, 2, 6 and 11 days, respectively. Organoids were re-embedded to fresh Geltrex after 2 weeks of culture. **P1d0, P1d6, P1d14:** Re-embedded organoids were continue cultured for 0, 6 and 14 days as passage 1 (P1). By 2 weeks, the organoids were treated with either vehicle (**a3**) or recombinant WNT5a (200 ng/mL, **a4**) for 3 days and collected for qRT-PCR analyses. (**B**) Relative mRNA levels of AT1, AT2, and proliferation markers in organoids treated with recombinant WNT5a for 3 days when compared with that cultured in vehicle. (**C**) Relative mRNA levels of canonical WNT targets (WNT5a vs vehicle). Data represent mean ± SEM. **P* < .05. *n* = 4. Scale bar: 2 mm in a1, 500 μm in all panels except a1.

Conclusions

The results of the current study demonstrate a previously unappreciated and novel function of Wnt5a in regulating proliferation and differentiation of AT1s, AT2s and myofibroblasts during lung maturation. The data show that Wnt5a promotes AT1 cell differentiation during fetal and neonatal mouse lung development. In addition, Wnt5a represses AT2 lineage-specific genes in a subpopulation of Sftpc^{pos} cells in concert with inhibition of canonical Wnt signaling. Therefore, Wnt5a is essential in regulating canonical Wnt signaling for proper lung development. Interaction of Wnt5a and canonical Wnt signaling plays a critical role in maintaining a balance between AT1 and AT2 cell differentiation during lung maturation. Finally, our finding that the pro-AT1 function of Wnt5a is evolutionarily conserved between mice and humans

speaks to the crucial role of Wnt5a as a principal regulator of lung epithelial progenitors.

Acknowledgments

We thank Hongyan Chen for excellent technical support. We thank Dr. Stijn De Langhe (University of Alabama at Birmingham, AL) for providing the Rosa26-rtTA mice. We thank Melissa L. Wilson (Department of Preventive Medicine, University of Southern California) and Family Planning Associates for coordinating fetal tissue collection.

Funding

This work was supported by the National Heart, Lung and Blood Institute, National Institutes of Health [HL144932, HL122764 (C.L. and P.M.), HL143059 (P.M.), R35 HL135747 (Z.B. and P.M.), HL114959 (B.Z.)], the Hastings Foundation (Y.C., P.M., Z.B.), the Francis Foundation (Y.C.), and the Baxter Foundation (Y.C.).

Conflict of Interest

The authors declared no potential conflicts of interest.

Author Contributions

C.L.: conceptualization, data curation, funding acquisition, investigation, methodology, resources, validation, project administration, supervision and writing—original draft, writing – review and editing; N.P.: data curation, investigation, methodology; S.M.S.: data curation, investigation, methodology, validation; J.Z., F.G., G.K.: investigation; B.Z.: funding acquisition, methodology, writing – review and editing; M.E.T., B.H.G., S.B.: resources, writing – review and editing; M.K.L.: methodology, writing – review and editing; Z.B.: conceptualization, funding acquisition, writing – review and editing; Y.-W.C.: funding acquisition, investigation, methodology, resources, writing – review and editing; P.M.: conceptualization, data curation, funding acquisition, methodology, resources, validation, project administration, supervision and writing—original draft, writing – review and editing.

Data Availability

Additional data that support the findings of this study are available on request from the corresponding author.

Supplementary Material

Supplementary material is available at *Stem Cells* online.

References

1. Volckaert T, De Langhe SP. Wnt and FGF mediated epithelial-mesenchymal crosstalk during lung development. *Dev Dyn*. 2015;244(3):342-366. https://doi.org/10.1002/dvdy.24234.
2. Morrisey EE, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev Cell*. 2010;18(1):8-23. https://doi.org/10.1016/j.devcel.2009.12.010.
3. Penkala IJ, Liberti DC, Pankin J, et al. Age-dependent alveolar epithelial plasticity orchestrates lung homeostasis and regeneration. *Cell Stem Cell*. 2021;28(10):1775-1789.e5. https://doi.org/10.1016/j.stem.2021.04.026.
4. Borok Z, Lubman RL, Danto SI, et al. Keratinocyte growth factor modulates alveolar epithelial cell phenotype in vitro: expression of aquaporin 5. *Am J Respir Cell Mol Biol*. 1998;18(4):554-561. https://doi.org/10.1165/ajrcmb.18.4.2838.
5. Zepp JA, Morley MP, Loebel C, et al. Genomic, epigenomic, and biophysical cues controlling the emergence of the lung alveolus. *Science*. 2021;371(6534):eabc3172.
6. Rock JR, Barkauskas CE, Cronce MJ, et al. Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. *Proc Natl Acad Sci USA*. 2011;108(52):E1475-E1483. https://doi.org/10.1073/pnas.1117988108.
7. Desai TJ, Brownfield DG, Krasnow MA. Alveolar progenitor and stem cells in lung development, renewal and cancer. *Nature*. 2014;507(7491):190-194. https://doi.org/10.1038/nature12930.
8. Frank DB, Peng T, Zepp JA, et al. Emergence of a wave of wnt signaling that regulates lung alveologenesis by controlling epithelial self-renewal and differentiation. *Cell Rep*. 2016;17(9):2312-2325. https://doi.org/10.1016/j.celrep.2016.11.001.
9. Kanagaki S, Ikeo S, Suezawa T, et al. Directed induction of alveolar type I cells derived from pluripotent stem cells via Wnt signaling inhibition. *Stem Cells*. 2021;39(2):156-169. https://doi.org/10.1002/stem.3302.
10. Wiese KE, Nusse R, van Amerongen R. Wnt signalling: conquering complexity. *Development*. 2018;145(12):dev.165902.
11. Katoh M. Canonical and non-canonical WNT signaling in cancer stem cells and their niches: cellular heterogeneity, omics reprogramming, targeted therapy and tumor plasticity. *Int J Oncol*. 2017;51(5):1357-1369. https://doi.org/10.3892/ijo.2017.4129.
12. Mikels AJ, Nusse R. Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biol*. 2006;4(4):e115. https://doi.org/10.1371/journal.pbio.0040115.
13. Li C, Xiao J, Hormi K, et al. Wnt5a participates in distal lung morphogenesis. *Dev Biol*. 2002;248(1):68-81. https://doi.org/10.1006/dbio.2002.0729.
14. Li C, Hu L, Xiao J, et al. Wnt5a regulates Shh and Fgf10 signaling during lung development. *Dev Biol*. 2005;287(1):86-97. https://doi.org/10.1016/j.ydbio.2005.08.035.
15. Li C, Smith SM, Peinado N, et al. WNT5a-ROR signaling is essential for alveologenesis. *Cells*. 2020;9(2):384. https://doi.org/10.3390/cells9020384.
16. Rieger ME, Zhou B, Solomon N, et al. p300/beta-Catenin interactions regulate adult progenitor cell differentiation downstream of WNT5a/protein kinase C (PKC). *J Biol Chem*. 2016;291(12):6569-6582. https://doi.org/10.1074/jbc.M115.706416.
17. Nabhan AN, Brownfield DG, Harbury PB, et al. Single-cell Wnt signaling niches maintain stemness of alveolar type 2 cells. *Science*. 2018;359(6380):1118-1123. https://doi.org/10.1126/science.aam6603.
18. Baarsma HA, Skronska-Wasek W, Mutze K, et al. Noncanonical WNT-5A signaling impairs endogenous lung repair in COPD. *J Exp Med*. 2017;214(1):143-163. https://doi.org/10.1084/jem.20160675.
19. Yuan K, Shamskhou EA, Orcholski ME, et al. Loss of endothelium-derived Wnt5a Is associated with reduced pericyte recruitment and small vessel loss in pulmonary arterial hypertension. *Circulation*. 2019;139(14):1710-1724. https://doi.org/10.1161/CIRCULATIONAHA.118.037642.
20. Newman DR, Sills WS, Hanrahan K, et al. Expression of WNT5A in idiopathic pulmonary fibrosis and its control by TGF-beta and WNT7B in human lung fibroblasts. *J Histochem Cytochem*. 2016;64(2):99-111. https://doi.org/10.1369/0022155415617988.
21. Vuga LJ, Ben-Yehudah A, Kovkarova-Naumovski E, et al. WNT5A is a regulator of fibroblast proliferation and resistance to apoptosis. *Am J Respir Cell Mol Biol*. 2009;41(5):583-589. https://doi.org/10.1165/rcmb.2008-0201OC.

22. Rydell-Tormanen K, Zhou XH, Hallgren O, et al. Aberrant nonfibrotic parenchyma in idiopathic pulmonary fibrosis is correlated with decreased beta-catenin inhibition and increased Wnt5a/b interaction. *Physiol Rep*. 2016;4(5):e12727. https://doi.org/10.14814/phy2.12727.
23. Sucre JMS, Vickers KC, Benjamin JT, et al. Hyperoxia injury in the developing lung is mediated by mesenchymal expression of Wnt5A. *Am J Respir Crit Care Med*. 2020;201(10):1249-1262. https://doi.org/10.1164/rccm.201908-1513OC.
24. Volckaert T, Campbell A, Dill E, et al. Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. *Development*. 2013;140(18):3731-3742. https://doi.org/10.1242/dev.096560.
25. van Amerongen R, Fuerer C, Mizutani M, et al. Wnt5a can both activate and repress Wnt/beta-catenin signaling during mouse embryonic development. *Dev Biol*. 2012;369(1):101-114. https://doi.org/10.1016/j.ydbio.2012.06.020.
26. Lo B, Hansen S, Evans K, et al. Alveolar epithelial type II cells induce T cell tolerance to specific antigen. *J Immunol*. 2008;180(2):881-888. https://doi.org/10.4049/jimmunol.180.2.881.
27. DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development*. 1999;126(20):4557-4568. https://doi.org/10.1242/dev.126.20.4557.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408. https://doi.org/10.1006/meth.2001.1262.
29. Nikolic MZ, Caritg O, Jeng Q, et al. Human embryonic lung epithelial tips are multipotent progenitors that can be expanded in vitro as long-term self-renewing organoids. *Elife*. 2017;6:e26575.
30. Chen YW, Huang SX, de Carvalho A, et al. A three-dimensional model of human lung development and disease from pluripotent stem cells. *Nat Cell Biol*. 2017;19(5):542-549.
31. Thurlbeck WM. Measurement of pulmonary emphysema. *Am Rev Respir Dis*. 1967;95(5):752-764. https://doi.org/10.1164/arrd.1967.95.5.752.
32. Yu XM, Wang L, Li JF, et al. Wnt5a inhibits hypoxia-induced pulmonary arterial smooth muscle cell proliferation by downregulation of beta-catenin. *Am J Physiol Lung Cell Mol Physiol*. 2013;304(2):L103-L111. https://doi.org/10.1152/ajplung.00070.2012.
33. Li C, Lee MK, Gao F, et al. Secondary crest myofibroblast PDGFRalpha controls the elastogenesis pathway via a secondary tier of signaling networks during alveologenesis. *Development*. 2019;146(15):dev.176354.
34. Li R, Bernau K, Sandbo N, et al. Pdgfra marks a cellular lineage with distinct contributions to myofibroblasts in lung maturation and injury response. *Elife*. 2018;7:e36865.
35. Tiozzo C, De Langhe S, Yu M, et al. Deletion of Pten expands lung epithelial progenitor pools and confers resistance to airway injury. *Am J Respir Crit Care Med*. 2009;180(8):701-712. https://doi.org/10.1164/rccm.200901-0100OC.
36. Kikuchi A, Yamamoto H, Sato A, et al. Wnt5a: its signalling, functions and implication in diseases. *Acta Physiol (Oxf)*. 2012;204(1):17-33. https://doi.org/10.1111/j.1748-1716.2011.02294.x.
37. Frank DB, Penkala IJ, Zepp JA, et al. Early lineage specification defines alveolar epithelial ontogeny in the murine lung. *Proc Natl Acad Sci USA*. 2019;116(10):4362-4371. https://doi.org/10.1073/pnas.1813952116.
38. Ishitani T, Kishida S, Hyodo-Miura J, et al. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. *Mol Cell Biol*. 2003;23(1):131-139. https://doi.org/10.1128/MCB.23.1.131-139.2003.
39. Wu X, van Dijk EM, Ng-Blichfeldt JP, et al. Mesenchymal WNT-5A/5B signaling represses lung alveolar epithelial progenitors. *Cells* 2019;8(10):1147.
40. Ahmadvand N, Khosravi F, Lingampally A, et al. Identification of a novel subset of alveolar type 2 cells enriched in PD-L1 and expanded following pneumonectomy. *Eur Respir J*. 2021;58(5):2004168. https://doi.org/10.1183/13993003.04168-2020.
41. Treutlein B, Brownfield DG, Wu AR, et al. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature*. 2014;509(7500):371-375. https://doi.org/10.1038/nature13173.