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Regulation of lung endoderm progenitor cell behavior by miR302/367

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SUMMARY

The temporal and spatial control of organ-specific endoderm progenitor development is poorly understood. miRNAs affect cell function by regulating programmatic changes in protein expression levels. We show that the *miR302/367* cluster is a target of the transcription factor Gata6 in mouse lung endoderm and regulates multiple aspects of early lung endoderm progenitor development. *miR302/367* is expressed at early stages of lung development, but its levels decline rapidly as development proceeds. Gain- and loss-of-function studies show that altering *miR302/367* expression disrupts the balance of lung endoderm progenitor proliferation and differentiation, as well as apical-basal polarity. Increased *miR302/367* expression results in the formation of an undifferentiated multi-layered lung endoderm, whereas loss of miR302/367 activity results in decreased proliferation and enhanced lung endoderm differentiation. miR302/367 coordinates the balance between proliferation and differentiation, in part, through direct regulation of Rbl2 and Cdkn1a, whereas apical-basal polarity is controlled by regulation of Tiam1 and Lis1. Thus, miR302/367 directs lung endoderm development by coordinating multiple aspects of progenitor cell behavior, including proliferation, differentiation and apical-basal polarity.

KEY WORDS: Lung, MicroRNA, Progenitor, Mouse

INTRODUCTION

The proper balance between proliferation and differentiation of endodermal progenitors is crucial for development and function of endodermally derived tissues such as the lung. Characterization of the gene regulatory networks that regulates the expansion and differentiation of these progenitors has a profound impact on our understanding and treatment of lung disease. The entire epithelium of the lung is initially generated from a small pool of undifferentiated progenitor cells that is present in the anterior ventral foregut endoderm at E9.5 of mouse development (Morrisey and Hogan, 2010). By E11.5 in the mouse, distinct progenitors are found in the proximal versus distal regions of the branching airways (Morrisey and Hogan, 2010). These progenitors will differentiate into distinct epithelial lineages during lung development that are required for proper gas exchange in the adult lung. Disruption in these developmental processes can lead to severe neonatal lung disease.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate mRNA translation and stability at a post-transcriptional level. Many miRNAs are expressed in groups or clusters from a single primary transcript which is further processed by the Dicer-Drosha complex into mature 22 nucleotide fragments which bind to complementary regions within the 3′ untranslated (UTR) regions of target genes. An important role for the miRNA pathway in lung development has been demonstrated using a conditional deletion of Dicer in embryonic lung epithelium (Harris et al., 2006). The resulting mutant lungs exhibited severe branching defects and increased epithelial apoptosis. Despite this dramatic effect, only a few studies have shown an important role for specific miRNAs in endoderm development. In the lung, transgenic overexpression of *miR17-92* promotes proliferation and inhibits differentiation of lung endoderm progenitor cells, whereas deletion of *miRNA17-92* leads to a hypoplastic lung phenotype (Lu et al., 2007; Ventura et al., 2008).

We have previously shown that the transcription factor Gata6 plays an important role in regulating lung endoderm progenitor differentiation and proliferation (Zhang et al., 2008). In these studies, we have identified the *miR302/367* cluster as a target of Gata6 function in early lung endoderm. We have found that the *miR302/367* cluster is a direct target of Gata6 and is expressed at high levels up to E12.5 lung development, but declines rapidly as development proceeds. Increased or decreased *miR302/367* expression leads to an altered balance of lung endoderm progenitor proliferation and differentiation, as well as to disruption in apical-basal polarity. This balance between proliferation, differentiation and the disruption in progenitor cell polarity is controlled, in part, by expression of several key targets of miR302/367, including the tumor suppressor Rbl2, the cell cycle regulator Cdkn1a and the cell polarity factors Tiam1 and Lis1 (Pafah1b1 – Mouse Genome Informatics). Together, these studies demonstrate that miR302/367 controls multiple aspects of lung endoderm progenitor cell behavior that regulate their ability to form a single-layered epithelium required for functional gas exchange and postnatal respiration.

MATERIALS AND METHODS

RNA purification, RT-QPCR and miRNA microarray analysis

Total RNA was isolated from embryonic lungs at the indicated ages using Trizol reagent, reverse transcribed using SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), and used in quantitative real time

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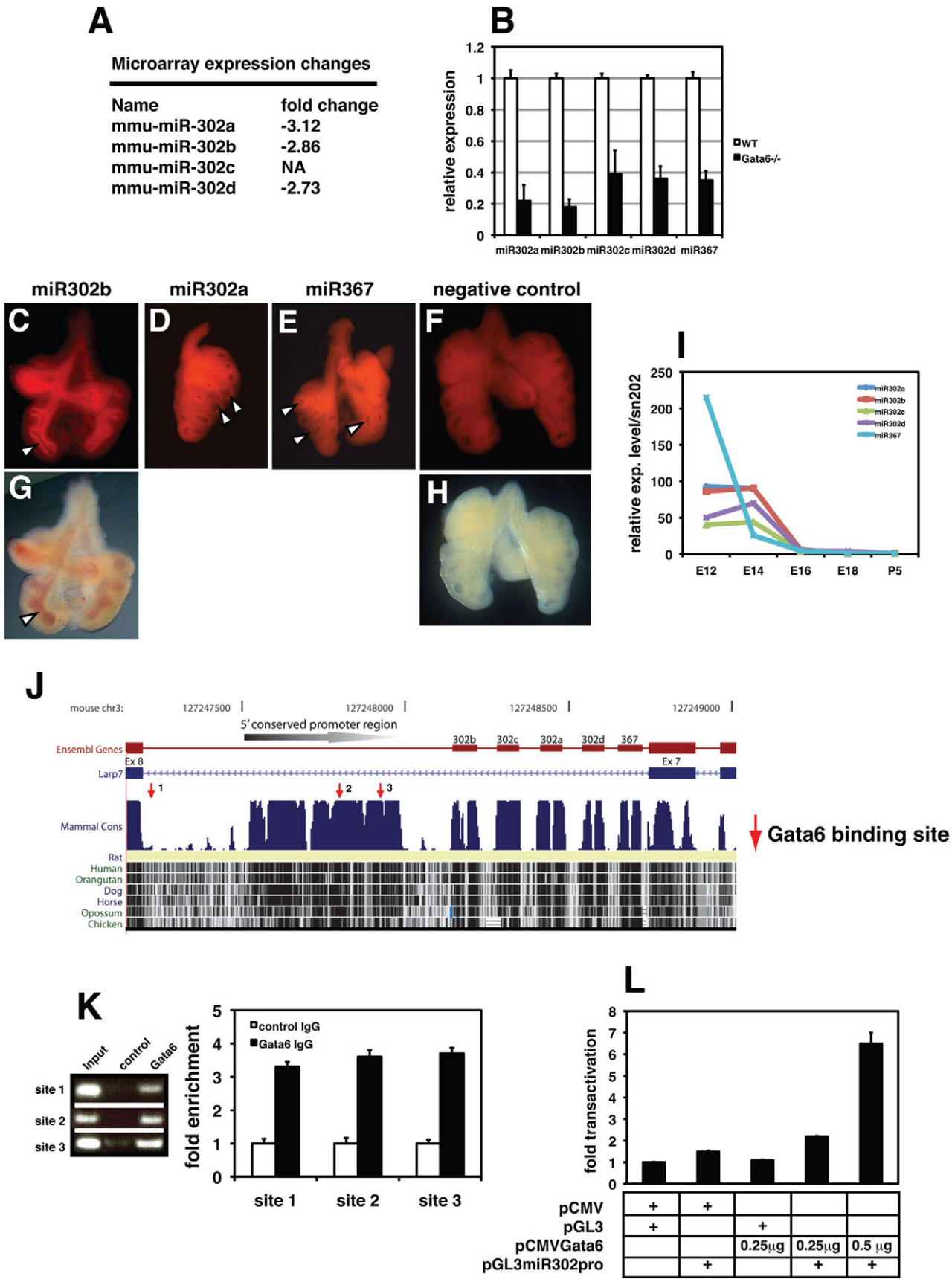

Fig. 1. See next page for legend.

Fig. 1. Gata6 regulates transcription of the *miR302/367* cluster during lung development. (**A**) Microarray analysis revealed decreased expression of several members of the *miR302/367* cluster, including *miR302a*, *miR302b* and *miR302d* in *Gata6$^{flox/flox}$:Sftpc-rtTA:tetO-cre* mutant mouse lungs at E14.5. (**B**) Expression of *miR302/367* was assessed in Gata6 mutant and control littermate lungs at E12.5 by Q-PCR. Data are mean±s.e.m. (**C-E**,**G**) Whole-mount in situ hybridization of *miR302b*, *miR302a* and *miR367* LNA probes showing the expression of *miR302/367* in epithelial cells at E14.5. (**F**,**H**) Control in situ hybridization in the absence of a primary probe. (**I**) Relative expression of *miR302/367* cluster members during lung development by RT-QPCR. (**J**) Cross-species conservation of *miR302/367* 5′ proximal genomic region showing the putative Gata6 DNA-binding sites (red arrows). (**K**) ChIP assays showing binding of Gata6 to sites 1, 2 and 3 in the *miR302/367* promoter region. Data are mean±s.e.m. (**L**) Gata6 transactivation of the *miR302/367* promoter in a dose-dependent manner. Data are mean±s.e.m.

PCR analysis using the oligonucleotides listed in Table S1 in the supplementary material. MicroRNA microarray analysis was performed with RNA isolated from E14.5 lungs from *Sftpc-rtTA:tetO-cre* and *Gata6$^{flox/flox}$:Sftpc-rtTA:tetO-cre* mutant littermates using three samples of each genotype using Exiqon *Mus musculus* miRNA array 2.0. Data analysis was performed as previously described (Zhang et al., 2008; Cohen et al., 2009). miRNAs that exhibited changes of 1.5-fold or greater in *Gata6$^{flox/flox}$:Sftpc-rtTA:tetO-cre* lungs versus *Sftpc-rtTA:tetO-cre* lungs were analyzed further. The microarray results have been deposited in the Gene Expression Omnibus database and the accession number is GSE25874.

Generation of *miR302/367* gain- and loss-of-function mouse models

The 3.7 kb human *SFTPC* promoter has been described previously (Liu et al., 2002; Yang et al., 2002). For the overexpression studies, the mouse 690 bp of *miR302/367* cluster DNA was cloned downstream of the human *SFTPC* promoter and upstream of a SV40 polyadenylation sequence, resulting in *Sftpc:miR302/367*. For the decoy or sponge *miR302/367* inhibitor construct, seven copies of a *miR302b* target sequence, which contains an identical seed sequence to *miR302a*, *miR302c* and *miR302d*, along with seven copies of a *miR367* target sequence were cloned downstream of a EGFP cDNA. This was in turn cloned between the human *SFTPC* promoter and the SV40 polyadenylation sequence, resulting in *Sftpc:miR302/367^{decoy}*. Both transgenic cassettes were excised from the resulting plasmids, purified and injected into FVBN fertilized oocytes. Transgenic positive embryos were identified by PCR using the primers in Table S1 in the supplementary material.

Histology

Tissues were fixed in 4% paraformaldehyde, embedded in paraffin wax and sectioned at 5 μm intervals. Hematoxylin and Eosin staining was performed using standard procedures. Immunohistochemistry was performed using the following antibodies: mouse anti-phopho-histone3 (Cell Signaling Technology, Danvers, MA, USA; 1:200), goat anti-CC10 (Santa Cruz, Santa Cruz, CA, USA; 1:20), rabbit anti-SP-C (Chemicon, Billerica, MA, USA; 1:500), rabbit anti-Nkx2.1 (Santa Cruz, 1:50), mouse anti-Cdkn1a (Santa Cruz, 1:100), rabbit anti-Sox9 (Santa Cruz, 1:100), rabbit anti-Rbl2/p130 (Abcam, Cambridge, MA, USA; 1:50), rabbit anti-Par3 (Upstate, Billerica, MA, USA; 1:100), rabbit anti-Sox2 (Seven Hills Bioreagents, Cincinnati, OH, USA; 1:500). Slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Whole-mount in situ hybridization was performed using LNA probes and protocols obtained from Exiqon (Woburn, MA, USA).

Lung morphometry

Hematoxylin and Eosin-stained lung sections from the indicated genotypes were used for digital image capture. Horizontal, vertical and diagonal grid lines were overlaid on these images and used to count distance between alveolar septae. The mean linear intercept (MLI) was determined as previously described (Thurlbeck, 1967). Data are from six independent measurements from three samples of each indicated genotype. The thickness of the alveolar septa and airway lumen size were determined from six independent measurements using Image J software.

Luciferase assays

The firefly luciferase gene was derived from pGL3-basic vector (Promega, Madison, WI, USA) and cloned in to pcDNA3.1(–) vector. The DNA fragment containing full-length 3′ UTR for putative miR302/367 targets were inserted into pcDNA3.1(–) downstream of the luciferase cDNA. Mutations were introduced into the *miR302/367* binding sites using PCR-mediated site-directed mutagenesis. HEK293 cells were transfected using Fugene 6 reagent according to the manufacturer's instructions with both the luciferase reporter and an expression plasmid for *miR302/367*. Forty-eight hours after transfection, cell extracts were assayed for luciferase expression using a commercially available kit (Promega). Relative reporter activities are expressed as luminescence units normalized for β-galactosidase expression in the cell extracts.

Western blot analysis

Total protein extracts (20 μg) from transgenic lungs at E18.5 were resolved on SDS-PAGE gels and transferred to PVDF membranes for western blotting. Antibodies against Rbl2 (Abcam), Cdkn1a (Santa Cruz), Tiam1 (Abcam), Lis1 (Abcam), β-actin (Chemicon) and GAPDH (Chemicon) were used.

Cell transfection and chromatin immunoprecipitation (ChIP) assays

The upstream proximal promoter region of mouse *miR302/367* has been described previously. The region between –710 to –130 bp was subcloned into the pGL3 promoter luciferase reporter vector to generate pGL3-miR302/367.luc vector. This plasmid was transfected with a Gata6 expression plasmid into HEK293 cells using Fugene 6 and luciferase activities were determined using a commercially available kit (Promega). For ChIP assays, chromatin was extracted from 15 wild-type embryonic lungs at E13.5 using a ChIP assay kit (Upstate). Chromatin was immunoprecipitated with Gata6 antibody and purified chromatin was subjected to PCR using the oligonucleotides listed in Table S1 in the supplementary material.

RESULTS

Gata6 regulates transcription of *miR302/367* cluster during lung development

To address the mechanism of Gata6 regulation of early lung endoderm progenitor development, we performed a miRNA expression microarray screen analysis at E14.5 comparing RNA from *Gata6$^{flox/flox}$:Sftpc-rtTA:tetO-cre* mutant lungs with control lungs (*Sftpc-rtTA:tetO-cre*). This experiment identified decreased expression of three members of the *miR302/367* cluster, *miR302a/b/d*, suggesting that this cluster of miRNAs is a direct target of Gata6 regulation in the lung (Fig. 1A). Quantitative real-time PCR (Q-PCR) showed decreased expression of all five members of the *miR302/367* cluster in *Gata6$^{flox/flox}$:Sftpc-rtTA:tetO-cre* mutant lungs (Fig. 1A,B).

To determine the spatial and temporal pattern of expression of the *miR302/367* cluster, whole-mount in situ hybridization and Q-PCR was performed. As all five members are expressed from a single primary transcript, we determined expression of three members, *miR302a*, *miR302b* and *miR367*, to provide a representative expression pattern for the *miR302/367* cluster. In situ hybridization with locked nucleic acid (LNA) probes showed that *miR302a*, *miR302b* and *miR367* were highly expressed in the endoderm of the E12.5 lung (Fig. 1C-F). Expression of *miR302a* and *miR367* was also detected at lower levels in the mesenchyme of the E12.5 lung (Fig. 1E). Q-PCR analysis was performed on RNA isolated from mouse embryonic lungs at developmental

stages between E11.5 and postnatal day 5 for all five members of the *miR302/367* cluster. The expression of all five members of the cluster was greatest at E11.5 and decreased significantly after E14.5 (Fig. 1I). At E18.5 and postnatal stages, the expression of these miRNAs was very low to undetectable in the lung (Fig. 1I).

To determine whether the *miR302/367* cluster is a direct transcriptional target of Gata6, we analyzed the putative promoter region of *miR302/367* for Gata6-binding sites. The *miR302/367* cluster is found in intron eight of the *Larp7* gene and a previous study identified a ~500 bp promoter region upstream of *miR302/367* that contains binding sites for Oct4 and Sox2, which regulates *miR302/367* expression in embryonic stem cells (Card et al., 2008) (Fig. 1J). Analysis of this promoter region and flanking sequences identified three potential Gata6 DNA-binding sites that are conserved in mammals (Fig. 1J). Chromatin immunoprecipitation assays were performed to determine whether Gata6 bound these three sites in the embryonic lung. Both gel electrophoresis and Q-PCR studies showed robust association of Gata6 with all three DNA-binding sites (Fig. 1K). Moreover, Gata6 can efficiently transactivate this promoter in a dose-dependent manner when linked to a luciferase reporter (Fig. 1L). Together, these data indicate that the *miR302/367* cluster is expressed in the early lung endoderm and is a direct target of Gata6.

Abnormal lung development in *miR302/367* transgenic embryos

To assess the role *miR302/367* plays in lung development, gain-of-function experiments were performed using the human *SFTPC* promoter to overexpress *miR302/367* in early lung endoderm (Fig. 2A). *Sftpc:miR302/367* transgenics had larger lungs when examined at E18.5 (Fig. 2B). Histological analysis on E18.5 control lungs showed that the normal embryonic lung contains many small distal saccules or primitive alveoli, which are ready to open for respiration (Fig. 2C-E). By contrast, *Sftpc:miR302/367* transgenic lungs contain a thickened epithelial lining and decreased sacculation (Fig. 2F-H). Overexpression of all members of the *miR302/367* cluster in the transgenic lungs versus control littermates was confirmed by quantitative RT-PCR (Q-PCR) at E18.5 (Fig. 2I). Expression of Nkx2.1 highlights the disrupted nature of the distal airways and reveals a thickened epithelium in *Sftpc:miR302/367* transgenics (Fig. 2J,K). This change in airway morphology decreased the mean linear intercept between alveolar septae at E18.5 (Fig. 2L), and correlated with an increase in the average thickness of alveolar septa (Fig. 2M). These changes also result in decreased airway lumen size (see Fig. 4H). No *Sftpc:miR302/367* transgenics were ever found alive after the first day of life, indicating that they died in the perinatal period (data not shown).

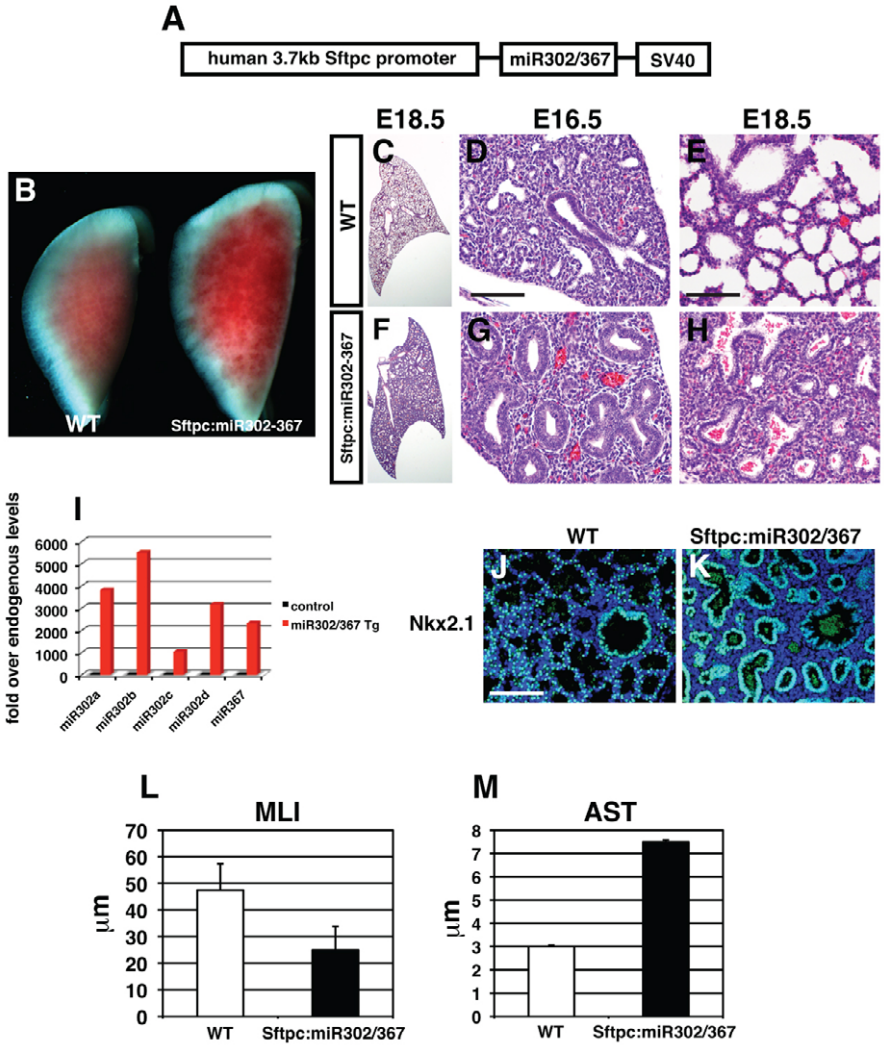

Fig. 2. Abnormal lung phenotype in *miR302/367* gain-of-function transgenic embryos. (**A**) Schematic of *miR302/367* transgenic construct (*Sftpc:miR302/367*) containing the 3.7 kb human *SFTPC* promoter and the *miR302/367*-coding region. (**B**) Whole-mount picture of wild-type and *Sftpc:miR302/367* mouse mutant lungs at E18.5. (**C-H**) Hematoxylin and Eosin-stained sections of left lobe from both the wild-type (C-E) and *Sftpc:miR302/367* transgenic (F-H) lungs. (**I**) Quantitative real-time PCR analysis showing increased expression of all five members of the *miR302/367* cluster in *Sftpc:miR302/367* transgenic lungs versus wild-type lungs at E18.5. (**J**,**K**) Condensed pattern of Nkx2.1 expression revealing abnormal airway development in *Sftpc:miR302/367* transgenic lungs. (**L**,**M**) Mean linear intercept (L) and alveolar septal thickness (M) were calculated for *Sftpc:miR302/367* transgenic lungs and wild-type littermates at E18.5. Scale bars: 100 µm. Error bars indicate s.e.m.

The *miR302/367* cluster regulates lung endoderm progenitor proliferation and differentiation

Given the severe disruption in endoderm development in *Sftpc:miR302/367* transgenics, we performed immunohistochemical staining for Sox2 and Sox9 to determine the effect of increased expression of *miR302/367* on lung endoderm progenitors. In the developing lung, Sox2 is normally expressed in endoderm progenitors of the proximal airways and is absent in the distal endoderm (Fig. 3A) (Que et al., 2007). Conversely, Sox9 is expressed at the highest levels in the distal lung endoderm progenitors and expression in distal progenitors decreases significantly so that by E16.5, Sox9 is detected in only a small subset of cells in the terminal distal endoderm (Fig. 3B) (Okubo et al., 2005; Lu et al., 2007). In *Sftpc:miR302/367* transgenics, Sox2-expressing progenitors are expanded into the distal airways (Fig. 3C). Moreover, there is an increase in the number of $Sox9^{+}$ endoderm progenitors in the distal airways of *Sftpc:miR302/367* transgenics (Fig. 3D). Q-PCR confirms the increased expression of Sox2 and Sox9 in transgenic lungs, as well as an additional marker of distal endoderm progenitors, Id2 (Fig. 3E). These data show that increased expression of *miR302/367* leads to expansion of both proximal and distal endoderm progenitors within the distal airway endoderm.

To assess whether the *miR302/367* cluster influences endoderm differentiation in the lung, immunostaining for markers of proximal airway Clara epithelial cells (CC10) and distal epithelial cells (SP-C) was performed at E18.5. In *Sftpc:miR302/367* transgenic lungs, both CC10 and SP-C expression is significantly downregulated (Fig. 3F-I). Q-PCR confirms these results and also shows reduction of additional markers of lung epithelial differentiation and maturation, including SP-A, SP-B and Aqp5 (Fig. 3J). Together, these data indicated that increased expression of *miR302/367* promotes expansion of both proximal and distal lung progenitors but inhibits their subsequent differentiation.

Abnormal endoderm-mesoderm signaling in *Sftpc:miR302/367* transgenic lungs

Paracrine signaling between the lung endoderm and mesoderm is crucial for development of pulmonary smooth muscle (Shu et al., 2002; Weaver et al., 2003; Miller et al., 2004). To assess lung

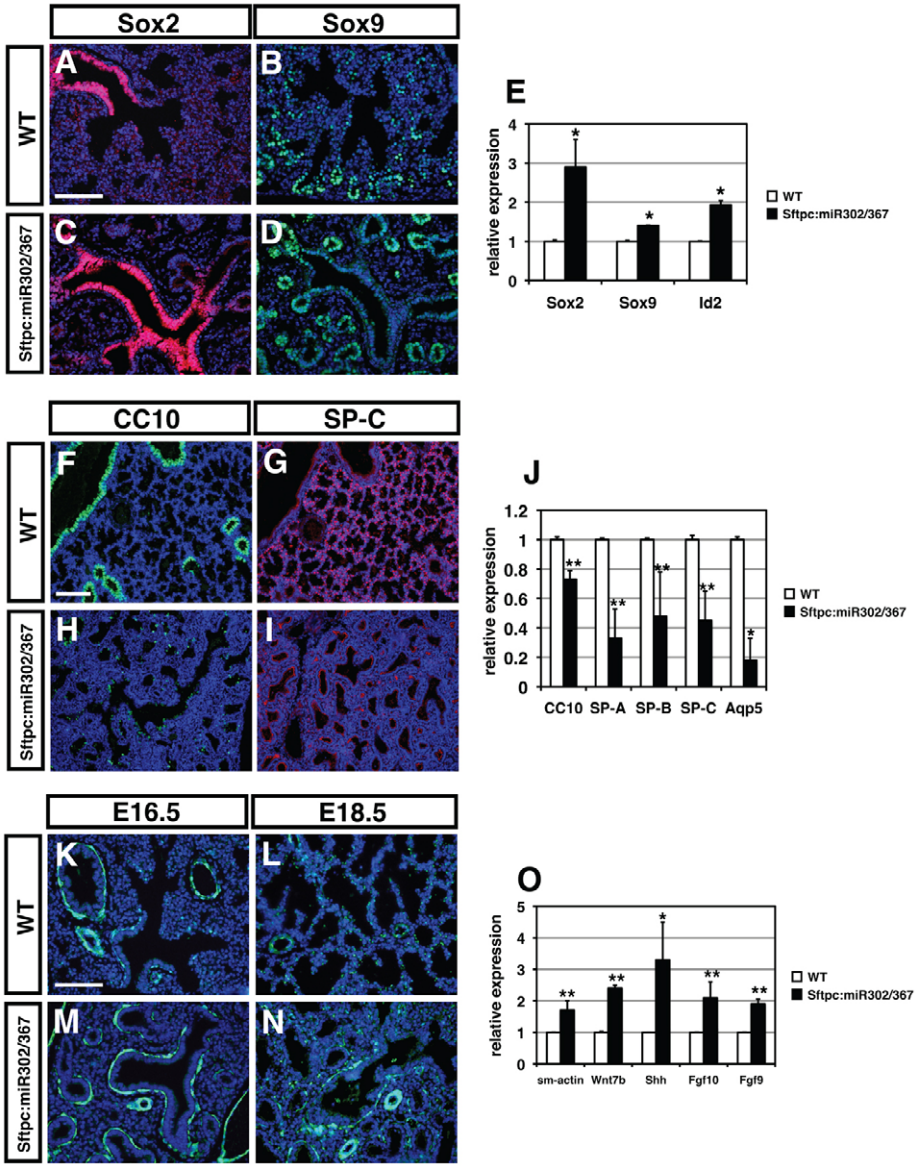

Fig. 3. Expansion of lung endoderm progenitors and effects on mesenchymal differentiation in *Sftpc:miR302/367* transgenic lungs. (**A-D**) Immunostaining showing the expression of Sox2 and Sox9 in wild-type *miR302/367* transgenic mouse lungs at E16.5. (**E**) Q-PCR for Sox2, Sox9 and Id2 at E18.5 in wild-type and *Sftpc:miR302/367* transgenic lungs. Data are mean±s.e.m. (**F-I**) Immunostaining showing decrease expression of CC10 and SP-C in *Sftpc:miR302/367* transgenic lungs at E18.5. (**J**) Q-PCR for CC10, SP-A, SP-B, SP-C and Aqp5 in wild-type and *Sftpc:miR302/367* transgenic lungs at E18.5. Data are mean±s.e.m. (**K-N**) Increased expression of SM22α in *Sftpc:miR302/367* transgenic lungs compared with their littermate controls by immunostaining. (**O**) Increased expression of sm-αactin, Wnt7b, Shh, Fgf9 and Fgf10 in *Sftpc:miR302/36*, as measured by Q-PCR. Data in E,J,O are mean±s.e.m. Statistical significance of quantitative changes for Q-PCR studies $*P<0.01$, $**P<0.05$. Scale bars: 100 μm.

mesodermal differentiation in *Sftpc:miR302/367* transgenic lungs, we used immunohistochemistry to analyze smooth muscle 22α (SM22α) expression. In the normal lungs, SM22α-positive smooth muscle cells encircle the endoderm of the proximal airways and the blood vessels, but are absent from the advancing tips of distal airways (Fig. 3K,L) (Tollet et al., 2001). However, *Sftpc:miR302/367* transgenic lungs displayed a dramatic increase in SM22α-positive staining around the primitive alveolar structures (Fig. 3M,N). Q-PCR also confirmed the upregulation of smooth muscle α-actin (α-SMA) expression in transgenic lungs (Fig. 3O). Expression of several other key signaling molecules and transcription factors known to be important for pulmonary smooth muscle development, including Wnt7b, Shh, Fgf10 and Fgf9, was significantly increased in transgenic lungs (Fig. 3O). These data show that increased *miR302/367* expression and the resulting expansion of lung endoderm progenitors leads to increased endoderm-mesoderm signaling that promote smooth muscle development in the lung.

miR302/367 regulates lung progenitor proliferation through repression of *Cdkn1a* and *Rbl2*

The increased numbers of lung endoderm progenitors in *Sftpc:miR302/367* transgenics suggested that these miRNAs promotes proliferation of these progenitors. Phospho-histone H3 (PO4-H3) immunostaining showed a significant increase in proliferation in the developing endoderm of *Sftpc:miR302/367* transgenics (Fig. 4A-C). Careful analysis of the Hematoxylin and Eosin-stained slides showed an increase in mitotic figures in the epithelium of *Sftpc:miR302/367* transgenics at E16.5 (Fig. 4D,E). This increase in proliferation correlates with the formation of a multi-layered epithelium that lines the airways of *Sftpc:miR302/367* transgenics, as shown by Nkx2.1 expression (Fig. 4F,G). This multi-layered epithelial lining also decreases the average airway lumen size (Fig. 4H).

Using multiple databases, including TargetScan, MiRanda and PicTar (Krek et al., 2005; Grimson et al., 2007; Betel et al., 2008), a large subset of miR302/367 target genes associated with cell proliferation according to their gene ontology annotation was identified for further characterization. Potential miR302/367 targets included Cdkn1a, Rbl2, Kat2b, Mnt, Mecp2 and Pten (see Table S2 in the supplementary material). As miRNAs are known to decrease the stability and steady-state levels of mRNAs (Guo et al., 2010), Q-PCR was used to assess the expression of these proliferation associated factors. These analyses found that expression of all but Mnt were downregulated in *Sftpc:miR302/367* transgenic lungs (Fig. 4I). Rbl2 and Cdkn1a were further selected for analysis because of their importance in the regulation of the cell cycle, as well as in malignant tumor progression (Tanaka et al., 2001; Russo et al., 2003; Wikenheiser-Brokamp, 2004; Simpson et al., 2009). Western blots and immunohistochemistry demonstrated significant reduction in protein expression of Rbl2 and Cdkn1a in *Sftpc:miR302/367* transgenic lungs (Fig. 4J-N). Luciferase reporter constructs were generated using the 3′ UTR regions of Rbl2 and Cdkn1a cloned downstream of the luciferase cDNA and were used to determine whether expression of *miR302/367* could repress Rbl2 and Cdkn1a expression via conserved miR302/367-binding sites (Fig. 4O). Expression of *miR302/367* inhibited expression of these luciferase reporters in these assays and mutation of the miR302/367-binding site resulted in alleviation of this inhibition (Fig. 4P,Q). These data indicate that miR302/367 promotes progression through the cell cycle and increased proliferation in early lung progenitors by repressing the cell cycle inhibitors Cdkn1a and Rbl2.

Regulation of endoderm progenitor apical-basal polarity by miR302/367

Gross examination of E16.5-E18.5 transgenic lung sections revealed numerous layers of irregularly packed endoderm cells lining the developing airways of *Sftpc:miR302/367* transgenic lungs (Fig. 4; Fig. 5A,C). This contrasted with the control lung endoderm cells in which the nuclei of the single-layer endoderm are orderly aligned perpendicularly to the basement membrane (Fig. 4; Fig. 5B,D). To determine whether the altered epithelial morphology influenced the epithelial apical-basal polarity in transgenic lungs, we examined the localization of Par3, an integral member of the Par-aPKC complex (Lin et al., 2000; Hirose et al., 2002; Patalano et al., 2006). As expected, Par3 expression was mostly restricted to the apical and tight junctions in control lung endoderm (Fig. 5A,C). Strikingly, transgenic lung endoderm displayed a disorganized apical layer, suggesting that the apical surface of cells is highly variable, with many of the cells found in the airway lumen exhibiting increased Par3 expression throughout their plasma membranes (Fig. 5B,D). Moreover, increased expression of Par3 was observed in the cells directly adjacent to the basement membrane of the airways (Fig. 5D). Examination of the potential target list for miR302/367 revealed Tiam1 and Lis1 as predicted targets of miR302/367 (see Table S2 in the supplementary material). The Rac activator Tiam1, a T-lymphoma invasion and metastasis-inducing protein, acts as a crucial component of the Par complex in regulating neuronal and epithelial apical-basal polarity (Chen and Macara, 2005; Mertens et al., 2005; Nishimura et al., 2005). Lis1 (β-subunit of platelet-activating factor acetylhydrolase) is essential for proliferation and the precise control of mitotic spindle orientation in both neuroepithelial stem cells and radial glial progenitor cells (Yingling et al., 2008; Yamada et al., 2010). Expression of both *Tiam1* and *Lis1* mRNA is decreased in *Sftpc:miR302/367* transgenic lungs (Fig. 5E). Western blot analysis shows decreased expression of Tiam1 and Lis1 proteins (Fig. 5F). To further assess whether Tiam1 and Lis1 are direct targets of miR302/367, we cloned the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for miR302a and miR367 binding was found in the 3′ UTR region of *Tiam1* and *Lis1*, respectively (Fig. 5G,H). When expressed, *miR302/367* can mediate repression through the 3′ UTR regions of *Tiam1* and *Lis1* (Fig. 5G,H). Mutations of the conserved miR302a or miR367-binding sites within these 3′ UTR regions abolishes this repression (Fig. 5G,H). Thus, downregulation of *miR302/367* later in lung development allows for establishment of proper apical-basal polarity of a single-layered endoderm by regulating Tiam1 and Lis1.

Loss of miR302/367 function results in decreased lung endoderm proliferation with enhanced differentiation

To determine the necessity of *miR302/367* in lung endoderm development, we generated a transgenic loss-of-function model using a ‘sponge’ or decoy construct to inhibit *miR302/367* function. Previous studies both in vivo and in vitro have shown the usefulness of miRNA decoys in competitively inhibiting miRNA function (Care et al., 2007; Ebert et al., 2007; Cohen, 2009; Ma et al., 2010). We generated a transgenic construct using the human *SFTPC* promoter driving a EGFP cDNA with two tandemly arrayed miRNA-binding sites in the 3′ UTR

region, each of them containing seven repetitive sequences complementary to *miR302a-d* and *miR367* seed sequences with mismatches at positions 9-12 for enhanced stability (Fig. 6A) (Ebert et al., 2007).

Sftpc:miR302/367^{decoy} transgenic lungs appeared slightly smaller at E18.5 (Fig. 6B,C). Hematoxylin and Eosin -stained sections showed that, as expected, the distal airways of wild-type embryos had begun the process of alveolarization with a thinning

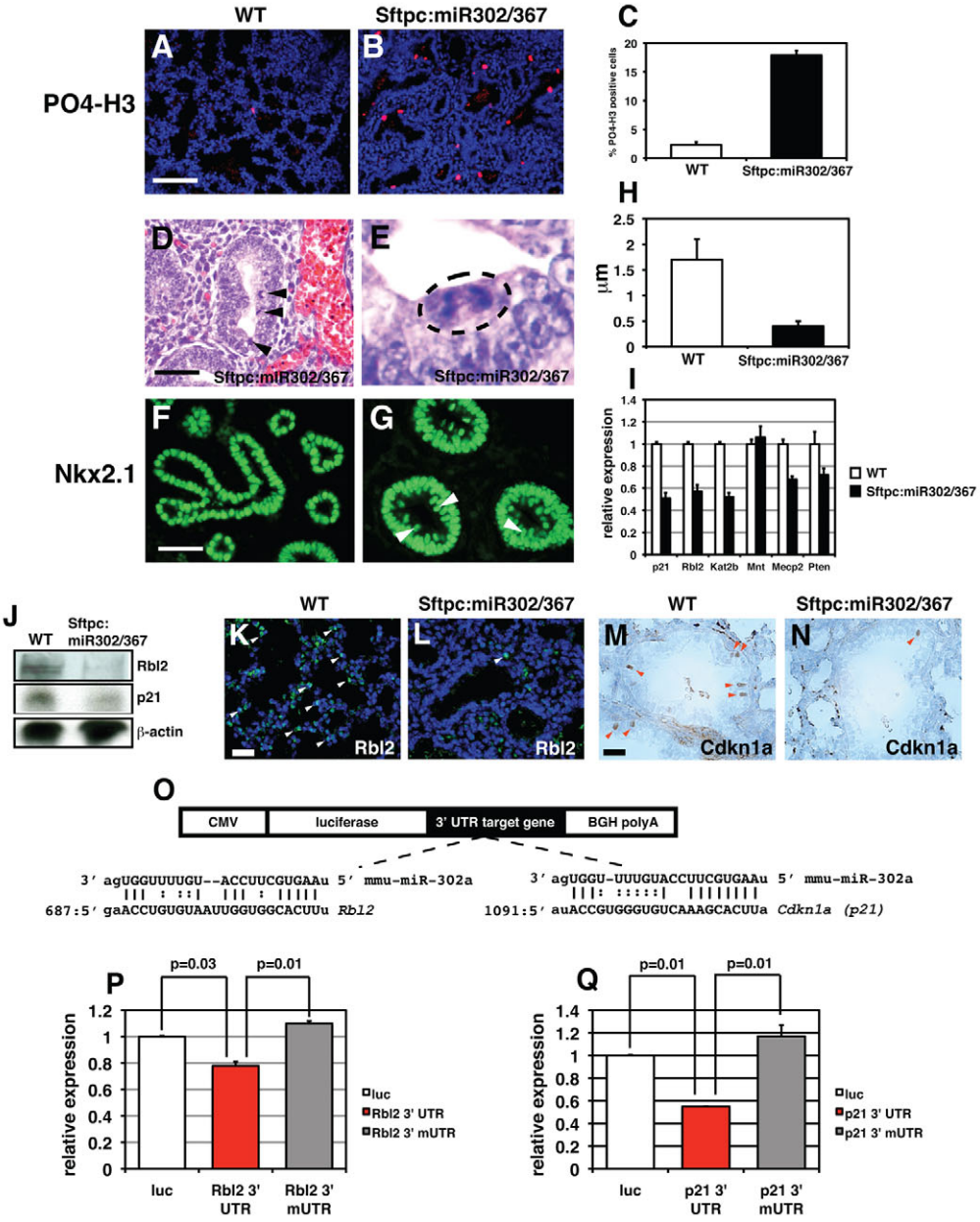

Fig. 4. Lung progenitor proliferation is controlled by miR302/367 through regulation of Cdkn1a and Rbl2. (**A**,**B**) PO4-H3 immunostaining showing increased proliferation in the epithelium of *Sftpc:miR302/367* transgenic mouse lungs at E18.5. (**C**) Quantitation of PO4-H3-positive cells. Data are mean±s.e.m. (**D**,**E**) Increased mitotic figures in the epithelium of *Sftpc:miR302/367* transgenic lungs at E16.5 (arrowheads and dotted outline). (**F**,**G**) Presence of a multi-layered epithelium lining the airways of *Sftpc:miR302/367* transgenic lungs at E16.5 as denoted by Nkx2.1 immunostaining (arrowheads indicate Nkx2.1-positive cells within the airways). (**H**) Average airway lumen size was decreased in *Sftpc:miR302/367* transgenic lungs at E16.5. Error bars indicate s.e.m. (**I**) Decreased expression of putative miR302/367 target genes related to cell proliferation measured by Q-PCR. All quantitative changes are significant (P<0.01) in I, with the exception of Mnt expression. Data are mean±s.e.m. (**J**) Decreased expression of Rbl2 and Cdkn1a by western blot in *Sftpc:miR302/367* transgenic lungs. (**K-N**) Decreased expression of Rbl2 and Cdkn1a in *Sftpc:miR302/367* transgenic lungs by immunostaining (arrowheads indicate positively stained cells). (**O**) Schematic of 3′ UTR luciferase reporters with alignment of miR302/367 target sequences in Rbl2 and Cdkn1a mRNAs. (**P**) *miR302/367* expression can repress luciferase expression through the *Rbl2* 3′ UTR and mutation of this site abolishes this repression. (**Q**) *miR302/367* expression can repress luciferase expression through the Cdkn1a 3′ UTR and mutation of this site abolishes this repression. Statistical significance of *miR302/367* repression on 3′ UTR regions in O and P are shown. Data are mean±s.e.m. Scale bars: 100 μm in A,B; 50 μm in D-G,K-N.

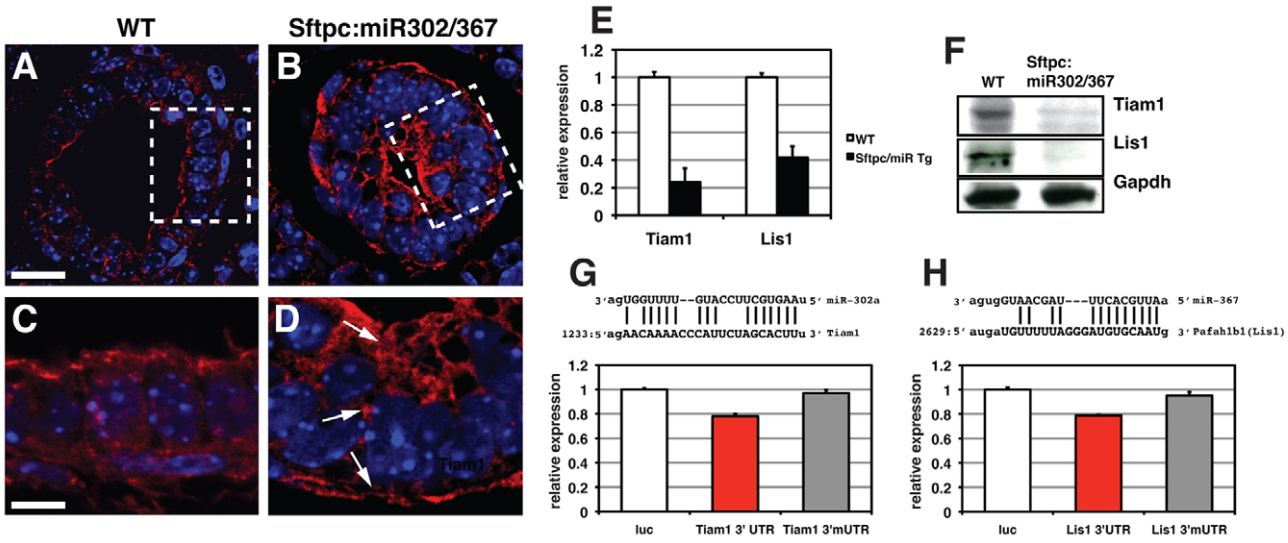

Fig. 5. miR302/367 controls lung endoderm progenitor cell polarity through regulation of *Tiam1* and *Lis1*. (**A-D**) Expression of Par3 reveals disrupted apical-basal polarity in *Sftpc:miR302/367* transgenic endoderm. Arrows indicate increased expression of Par3 surrounding the multi-layered endoderm, as well as in the basal lateral and basal surface of the outer layer of endoderm in *Sftpc:miR302/367* transgenics. Boxed regions in A and B are shown at higher magnification in C,D. (**E**) Decreased expression of Tiam1 and Lis1 in *Sftpc:miR302/367* transgenic lungs at E16.5, as measured by Q-PCR. Data are mean±s.e.m. (**F**) Decreased expression of Tiam1 and Lis1 in *Sftpc:miR302/367* transgenic lungs at E16.5, as measured by western blot analysis. (**G**) Alignment of miR302/367 target sequence in the 3′ UTR of Tiam1 and data showing that overexpression of *miR302/367* can repress this 3′ UTR region when linked to a luciferase reporter and that mutation of the *miR302/367* binding site abolishes this repression. (**H**) Alignment of miR302/367 target sequence in the 3′ UTR of Lis1 and data showing that overexpression of *miR302/367* can repress this 3′ UTR region when linked to a luciferase reporter and that mutation of the miR302/367-binding site abolishes this repression (G). Statistical significance of *miR302/367* repression on 3′ UTR regions in G and H are shown. Data in G,H are mean±s.e.m. Scale bars: 50 μm in A,B; 25 μm in C,D.

of the distal epithelium, whereas the airways of *Sftpc:miR302/367*^{decoy} were more narrow and condensed, and the epithelium had not begun alveolarization-mediated thinning (Fig. 6D,E). Immunostaining for Nkx2.1 expression reveals this narrowing of the airway lumen in *Sftpc:miR302/367*^{decoy} transgenics (Fig. 6F,G; see Fig. S1 in the supplementary material). Of note, the lung endoderm in *Sftpc:miR302/367*^{decoy} transgenics remained as a single layer, unlike the gain-of-function *Sftpc:miR302/367* transgenics (Fig. 6G; see Fig. S1 in the supplementary material). Q-PCR confirms the downregulated expression of all members of the *miR302/367* cluster, whereas the expression of another miRNA expressed in the lung, *miR200b* (Gregory et al., 2008; Dong et al., 2010), is not significantly affected (Fig. 6H). These data suggest that expression of the *miR302/367* decoy results in a specific inhibition of *miR302/367* expression and activity in vivo.

In contrast to the enhanced lung endoderm progenitor proliferation observed in *Sftpc:miR302/367* lungs, *Sftpc:miR302/367*^{decoy} transgenic lungs displayed reduced cell proliferation (Fig. 6I-K). To assess whether lung endoderm differentiation was affected by loss of *miR302/367* expression during lung development, expression of CC10, SP-C, SP-B, SP-A and Aqp5 was assessed by Q-PCR. These data show that expression of markers of lung endoderm differentiation and maturation were increased in *miR302/367* decoy transgenic lungs at E18.5 (Fig. 6L). Moreover, expression of the miR302/367 targets Rbl2 and Cdkn1a was also increased in *Sftpc:miR302/367*^{decoy} transgenics. Together, these data show that miR302/367 coordinates lung endoderm progenitor behavior by targeting multiple cellular processes, including proliferation, differentiation and apical-basal polarity, which are required for formation of a single-layered mature epithelium essential for postnatal lung function (Fig. 7).

DISCUSSION

How miRNAs affect tissue specific progenitor cell behavior is poorly understood. In this report, we show that a single miRNA cluster, *miR302/367*, can coordinate multiple aspects of lung endoderm progenitor behavior by regulating cell proliferation and polarity, which in turn affects overall endoderm differentiation in the developing lung. miR302/367 regulates a core set of cell cycle and cell polarity genes, and in doing so affects the ability of lung endoderm progenitors to exit their highly proliferative and undifferentiated state and differentiate into the single-layered mature lung epithelium, which is required for proper postnatal respiration. These studies highlight the potent ability of miRNAs to control cellular processes by coordinately regulating multiple cellular pathways that affect progenitor cell behavior in endoderm-derived tissues.

The mechanisms promoting differentiation of multipotential anterior foregut endoderm down the organ-specific pathways is only partially understood. Key signaling pathways, such as Wnt and Fgf signaling, are important during early stages of lung specification and endoderm differentiation. Previous studies have shown that Gata6 helps to regulate early lung endoderm differentiation through regulation of Wnt signaling (Zhang et al., 2008). Gata6 expression levels decrease during lung development and its regulation of *miR302/367* is likely to influence the overall proliferation of early lung progenitors through suppression of the cell cycle inhibitors Cdkn1a and

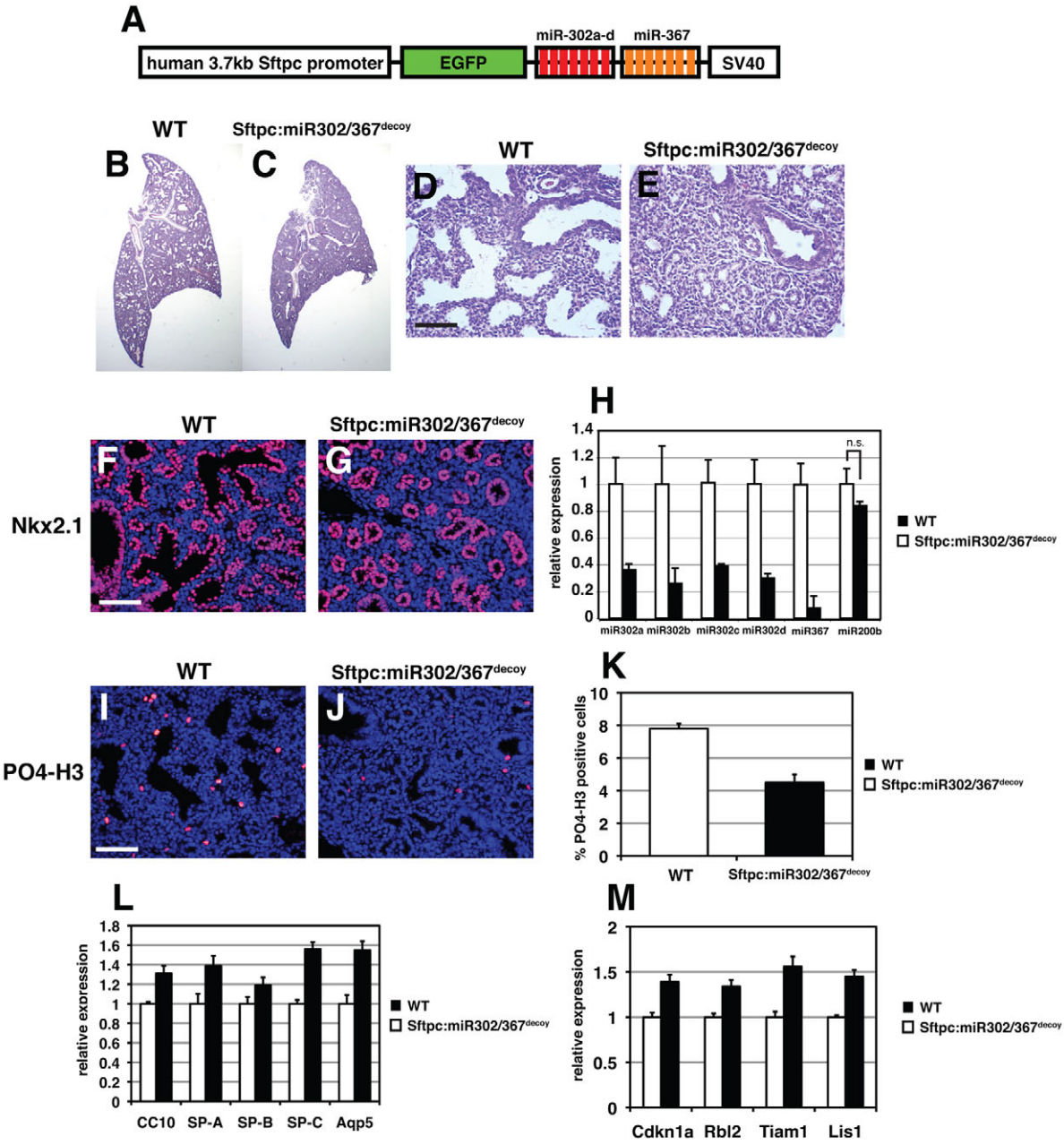

Fig. 6. Loss of miR302/367 function in lung endoderm progenitors leads to decreased proliferation and enhanced differentiation. (**A**) Schematic diagram of decoy or 'sponge' *Sftpc:miR302/367*^{decoy} construct. (**B-E**) Hematoxylin and Eosin staining of *miR302/367* decoy transgenic lungs at E16.5. (**F**,**G**) Altered expression pattern of Nkx2.1, showing disruption in airway branching of *Sftpc:miR302/367*^{decoy} transgenics. (**H**) All members of the *miR302/367* cluster were significantly downregulated in *Sftpc:miR302/367*^{decoy} transgenic lungs at E14.5, whereas expression of *miR200b* was not significantly affected. All changes in H are significant (P<0.01) except for *miR200b* expression, which is not significant (n.s.). Data are mean±s.e.m. (**I**,**J**) Decreased cell proliferation in *Sftpc:miR302/367*^{decoy} transgenic lungs as shown by PO4-H3 immunostaining. (**K**) Quantitation of cell proliferation shown in I and J, which is statistically significant (P<0.01). Data are mean±s.e.m. (**L**) Increased expression of CC10, SP-A, SP-B, SP-C and Aqp5 in *Sftpc:miR302/367*^{decoy} transgenic lungs at E18.5 by Q-PCR. Data are mean±s.e.m. (**M**) Increased expression of *Cdkn1a*, *Rbl2*, *Tiam1* and *Lis1* in *Sftpc:miR302/367*^{decoy} transgenic lungs at E18.5. Data are mean±s.e.m. All expression changes in L and M are statistically significant (P<0.05). Scale bars: 100 μm.

Rbl2. Gata6, along with Gata4, are important regulators of early foregut endoderm development. Both Gata6/4 have been shown to regulate cell proliferation in the lung, heart, liver and intestine (Ketola et al., 2004; Zhao et al., 2005; Haveri et al., 2008; Zhang et al., 2008; Agnihotri et al., 2009; Singh et al., 2010). Previous studies have also shown a correlation between Gata factor and Wnt signaling activity in both the developing lung and heart (Zhang et al., 2008; Tian et al., 2010). Given the combined pro-proliferative abilities of miR302/367 and the Wnt pathway, one of the major roles of Gata factor function in development is likely to promote proliferation of tissue-specific progenitors through regulation of these pathways.

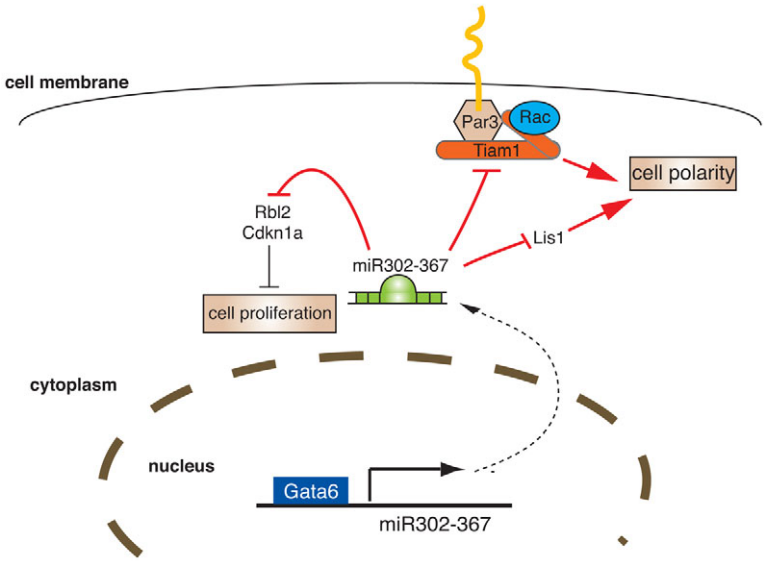

Fig. 7. Model of miR302/367 function in mouse early lung endoderm development. miR302/367 regulates proliferation, differentiation and apical-basal polarity of lung endoderm progenitors through targeting Cdkna1, Rbl2, Tiam1 and Lis1.

Little is known about the ability of miRNAs to regulate tissue-specific progenitor populations. In the skin, miR203 promotes differentiation of skin basal progenitor cells by repressing the important skin progenitor maintenance factor p63 (Lena et al., 2008; Yi et al., 2008; Wei et al., 2010). However, less is known in the lung. Loss of Dicer in early lung endoderm results in defective differentiation of early lung endoderm and increased apoptosis (Harris et al., 2006). However, which miRNAs play an important role in regulating early lung development is less clear. One of the few miRNAs that have been studied in lung development is miR17-92. Increased *miR17-92* expression leads to increased lung progenitor proliferation, similar to *miR302/367* expression (Lu et al., 2007). Conversely, loss of miR17-92 activity leads to lung hypoplasia (Ventura et al., 2008). Like miR302/367, miR17-92 also targets Rbl2 (Lu et al., 2007). Thus, miRNAs such as miR302/367 and miR17-92 may play a crucial role in the regulation of early lung progenitors by targeting a core set of factors that negatively regulate the cell cycle. This could directly or indirectly antagonize progenitor differentiation during early development and allow for the generation of the proper number of endoderm progenitors. As expression of *miR302/367* decreases, the progenitors exit this highly proliferative state to differentiate into mature lung epithelium.

The ability of miR302/367 to regulate lung endoderm progenitor apical-basal polarity suggests that this attribute of mature polarized epithelium is required to be repressed in an early progenitor state to allow for rapid cell proliferation. Previous studies have shown that the combined activity of miR338-3p and miR451 is required for proper apical-basal polarity in colon cancer cell lines (Tsuchiya et al., 2009). miR29a has also been shown to play an important role in maintaining apical-basal polarity of epithelial cells in culture (Gebeshuber et al., 2009). However, little was known about whether or how miRNAs affected epithelial polarity in vivo. The loss of apical-basal polarity upon overexpression of *miR302/367* in the lung points to an important correlation between high proliferation rates and the ability of epithelial cells to maintain a motile and less polarized phenotype.

miR302/367 is highly expressed in embryonic stem cells and is a target of the pluripotency factors Oct4 and Sox2 (Card et al., 2008). Interestingly, Sox2 and other Sox factors play key roles in early lung development by regulating lung progenitor expansion and differentiation in a spatially specific manner (Que et al., 2009). Loss of Sox2 in the lung leads to decreased proximal airway epithelial development characterized by loss of the secretory cell lineages (Tompkins et al., 2009). Increased Sox2 leads to the converse phenotype with increased secretory lineage expansion at the expense of the more distal progenitor compartment and upon extended overexpression can lead to lung cancer (Lu et al., 2010). Our data suggest that increased levels of miR302/367 lead to an increase in both Sox2- and Sox9-positive progenitors. Future studies into whether *miR302/367* is associated with lung cancer might shed more light onto the multiple roles for this miRNA cluster.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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