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Transgenic over-expression of the microRNA *miR-17-92* cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells

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Abstract

The *miR-17-92* locus encodes a cluster of 7 microRNAs transcribed as a single primary transcript. It can accelerate c-Myc induced B cell lymphoma development and is highly expressed in many tumors, including lung tumors. However, the role of *miR-17-92* in development has not been well studied. From analysis of microRNAs during lung development, expression of the miR-17-92 cluster is high at early stages, but declines as development proceeds. We used the mouse surfactant protein C (Sftpc) promoter to over-express the cluster in embryonic lung epithelium. Transgenic lungs have a very abnormal lethal phenotype. They contain numerous proliferative epithelial cells that retain high levels of Sox9, a marker of distal progenitors. The differentiation of proximal epithelial cells was also inhibited. Furthermore, a significant increase in the number of neuroendocrine cell clusters was observed in the lungs of dead transgenic pups. We identify a tumor suppressor, Rbl2 which belongs to the Rb family, as a new target for *miR-17-5p*. Together, these studies suggest that *mir-17-92* normally promotes the high proliferation and undifferentiated phenotype of lung epithelial progenitor cells.

Keywords

miR-17-92; miRNAs; lung development; Rbl2; proliferation; progenitor epithelial cells

Introduction

MicroRNAs (miRNAs) are a class of small (~22bp), non-coding RNAs that regulate gene expression at a post transcriptional level. They are first transcribed as primary miRNAs by RNA polymerase II (Cai et al., 2004) and then cut by the RNase-III enzyme, Drosha, into ~70-nucleotide precursors (pre-miRNAs) (Lee et al., 2003; Lee et al., 2002), which are transported to the cytoplasm by Exportin 5 (Zeng and Cullen, 2004). Another enzyme, Dicer, then processes the pre-miRNAs to mature miRNAs (Bernstein et al., 2001; Hutvagner et al., 2001), which are recruited into the RNA-induced silencing complex (RISC) (Khvorova et al.,

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2003; Schwarz et al., 2003). Finally, this complex interferes with the translation and stability of target messenger RNAs (He and Hannon, 2004).

It is clear that miRNAs play an important role in development. Loss of *Dicer*, which completely blocks the processing of all miRNAs, causes embryonic lethality in both zebra fish (Giraldez et al., 2005) and mice (Bernstein et al., 2003). Hair follicle growth (Andl et al., 2006), skin morphogenesis (Yi et al., 2006) and angiogenesis (Yang et al., 2005) are also affected by deficiency of *Dicer*. Recently, it was reported that conditional deletion of *Dicer* in embryonic lung epithelium leads to inhibition of branching and increased apoptosis (Harris et al., 2006) suggesting that miRNAs play an important role in lung development. However, only a few miRNAs have been well studied for their function in mammalian development. For example, it was suggested that *miR-1*, regulated by serum response factors, controls the balance between differentiation and proliferation during cardiogenesis and muscle development by targeting Hand2 and HDAC4, respectively (Chen et al., 2006; Zhao et al., 2007; Zhao et al., 2005).

Here, we explore the function of a miRNA cluster, *miR-17-92*, in mouse lung development. *MiR-17-92* is a conserved gene which encodes 7 miRNAs. It also has two homologous miRNA clusters in the mouse genome (Fig. 1A). The human gene is located at chromosome 13q31, in the intron of chr13orf25. Amplification of this region has been reported in follicular lymphoma, B-cell lymphoma and in several lung and head and neck carcinomas (Knuutila et al., 1998). Recently, a large-scale analysis of the miRNA profiles of solid tumors showed up-regulation of the human *miR-17-92* cluster in many cancers, including lung cancer (Volinia et al., 2006) and small-cell lung cancer that is thought to arise from neuroendocrine cells (Hayashita et al., 2005). In addition to its upregulation in cancer cells recent studies showed that *miR-17-92* is highly expressed in embryonic stem cells, with expression levels decreasing during embryonic development in mice (Thomson et al., 2004). This is consistent with miRNA cloning data from pluripotential mouse embryonic stem (ES) cells (Houbaviy et al., 2003). Taken together, these results support the idea that high level of *miR-17-92* promote the proliferation of progenitor cells and inhibit their differentiation. However, little is known about the role of *miR-17-92* in the development of specific organs, including the lung.

In the mouse, lung development begins around embryonic days 9.5 (E9.5) when two primordial buds of epithelial endoderm cells surrounded by mesoderm grow out of the ventral foregut (Cardoso and Lu, 2006). Subsequent lung development can be divided into four stages, termed pseudoglandular, canalicular, terminal sac and alveolar. During the first two stages the distal tips of the lung buds contain a population of highly proliferative cuboidal epithelial cells. Descendants of these cells populate the more proximal tubules as the buds elongate. Beginning around E14, the proximal cells begin to differentiate into Clara, ciliated and neuroendocrine cells. During the saccular stage the distal epithelial cells give rise to the progenitors of the primitive alveoli which subsequently differentiate into specialized type I and type II cells. At the beginning of this study, we found that *miR-17-92* is highly expressed in the E11.5 lung and its level declines as development proceeds. We therefore hypothesized that *miR-17-92* is important for maintaining lung progenitor cells. In this study, we over-expressed the whole *miR-17-92* cluster in the distal epithelium of transgenic lungs from around E10.5, using the mouse surfactant protein C promoter. This results in a very abnormal lung phenotype, in particular the accumulation of large numbers of highly proliferative epithelial cells expressing markers of progenitors. In a search for potential targets of *miR-17-5p* that could account for this phenotype we have identified the tumor suppressor Rbl2. How this target may contribute to the network of genes potentially regulated by the cluster during lung development is discussed.

Materials and methods

Mice

A schematic diagram of the transgenic construct is shown in Fig. 3A. The mouse 6.5kb *Sftpc* promoter was kindly provided by Edward Morrisey, University of Pennsylvania. It drives transgene expression in the distal lung epithelium from about E10.5 (personal communication). The mouse 1.1kb *miR-17-92* cluster DNA was cloned by PCR from C57BL/6 mouse genomic DNA, using primer sets: sense, 5′ GTCAGAATTCCCCCTTGGGTATAAGCTGTAATT; anti-sense, 5′ GTCACTCGAGACCAACGAAAGCAATAGAAATCA (Restriction sites, EcoR I and Xho I, were added for cloning). The 0.4kb *SV40* small T intron and poly A were derived from plasmid pKC4 by Hind III and BamH I. A total of 8 transgenic embryos at E18.5 and 2 transgenic mice were generated by pronuclear injection of fertilized eggs of the FVB inbred strain at the Duke Transgenic Mouse Facility. Transgenic adults were crossed with ICR mice to obtain transgenic pups at P0.

MicroRNA In situ hybridization

Protocols for section and whole mount in situ hybridization were obtained from Exiqon (http://www.exiqon.com). Briefly, paraffin wax-embedded sections were dewaxed, pretreated with proteinase K (10 minutes at RT) and hybridized with Locked Nucleic Acid (LNA) probes (pre-designed oligos from Exiqon) in 50% formamide hybridization mix at 49°C. Probes were labeled with Digoxigenin (DIG) using DIG oligonucleotide 3′-end labeling kit, 2nd generation (Roche). Slides were then washed with 2×SSC and 2×SSC plus 50% formamide at 49 °C. The DIG-labeled probes were detected by alkaline phosphatase-coupled anti-DIG (Roche), followed by color development (Substrate: NBT, BCIP, Promega). Whole mount in situ hybridization also followed protocol from Exiqon. The proteinase K treatment for E11.5 embryos and E12.5 lungs was 30 and 10 minutes, respectively, at 37 °C. The antibody wash for E11.5 embryos and E12.5 lungs was 3 days and 6 hours respectively at 4°C.

RNA extraction and Northern blot

Lungs were collected and immediately frozen in liquid nitrogen and stored at −80°C. RNA was extracted by Trizol (Invitrogen) following the product manual. The Northern blot protocol was derived from Exiqon (http://www.exiqon.com). Briefly, total RNA was separated by 15% polyacrylamide gel electrophoresis with 0.5×TBE. After 1.5h transfer to a Hybond-N+ nylon membrane (Amersham) by semi-dry transfer cell (Bio-Rad), the membrane was prehybridized and hybridized in PerfectHyb Plus Hybridization Buffer (Sigma) at 49°C for LNA probes or room temperature (RT) for DNA probes. Probes were labeled with ^{32}P γ-ATP by T4 Polynucleotide Kinase (New England BioLabs). The membrane was washed with 10×SSC at RT for autoradiography.

MiRNA microarray and Affymetrix analysis

MiRNA microarray was performed essentially as described (Thomson et al., 2004). For the Affymetrix analysis, total RNA (10 μg) was extracted from three normal and three transgenic lungs using Trizol (Invitrogen) and RNeasy Mini Kit (Qiagen) and assessed for quality with an Agilent Labon-a-Chip 2100 Bioanalyzer (Agilent Technologies). All the reactions were carried out at Duke Microarray Facility. In brief, probes for hybridization were prepared from total RNA according to standard Affymetrix protocols. First strand cDNA was synthesized using a T7-linked oligo-dT primer, followed by second strand synthesis. An in vitro transcription reaction was performed to generate the cRNA containing biotinylated UTP and CTP, which was subsequently fragmented chemically at 95°C for 35 minutes. The fragmented, biotinylated cRNA was hybridized in MES buffer (2-[N-morpholino]ethanesulfonic acid) containing 0.5 mg/ml acetylated bovine serum albumin to Affymetrix GeneChip Mouse 430A

arrays at 45°C for 16 h, according to the Affymetrix protocol (http://www.affymetrix.com). Arrays were washed and stained with streptavidin-phycoerythrin (SAPE; Molecular Probes Inc, Eugene, USA). Signal amplification was performed using a biotinylated anti-streptavidin antibody (Vector Laboratories) at 3 μg/ml. This was followed by a second staining with SAPE. Normal goat IgG (2 mg/ml) was used as a blocking agent. All data are available in Figure S1 and Table S1.

MiRNA cloning

The miRNA cloning was performed as described (Lau et al., 2001), except that the 50μg total RNA extracted by Trizol from lung samples was first enriched for the small RNA fraction by mirVana miRNA Isolation Kit (Ambion) before cloning.

Immunohistochemistry

Tissues for immunohistochemistry were fixed in 4% freshly prepared paraformaldehyde in PBS at 4°C overnight, embedded in paraffin wax and sectioned at 7μm. After rehydration, the endogenous enzyme activity was quenched by 2% H_2O_2 in methanol (30 minutes). Before the blocking and antibody reaction in 2% BSA, 5% serum and 0.1% Triton X-100 in PBS, different antigen retrievals were performed for different antibodies, as follows: Mouse anti-BrdU (Sigma, 1:500): 2N HCl at 37 °C, 20 minutes and then 0.05% trypsin/PBS at room temperature (RT), 5 minutes; rabbit anti-proSurfactant protein C (Chemicon, 1:200): 0.05% trypsin/PBS at RT, 5 minutes; mouse anti-Nkx2.1 (NeoMakers, 1:500): microwave antigen retrieval with 10mM pH6.0 sodium citrate. Rabbit anti-CC10 (kindly provided by Barry Stripp, 1:10000), goat anti-Scgbla1 (CCSP/CC10) (kindly provided by Barry Stripp, 1:10000), mouse anti-β tubulin (BioGenex, 1:1000), rabbit anti-CGRP (Penlabs, 1:1000), rabbit anti-Sox9 (kindly provided by Dr. Francis Poulat, 1:2000), rabbit anti-Nmyc (SantaCruz, 1:100) and rabbit anti-Rbl2/p130 (Abcam, 1:500) did not need antigen retrieval. Secondary antibodies for the color reactions were purchased from Jackson ImmunoResearch and Molecular Probes. Signal was amplified by ABC kit (Vector) and color was developed by peroxidase substrate kit DAB (Vector).

Luciferase assay

A schematic diagram of the luciferase reporter construct is shown in Fig. 3A. The *firefly luciferase* gene, derived from pGL3-Basic vector (Promega), was cloned into pcDNA3.1(+) by Hind III and EcoR I. *Rbl2* 3′UTR fragment was cloned by PCR from C57BL/6 mouse genomic DNA and inserted into pcDNA3.1(+) behind *firefly luciferase* using EcoR I and Xho I. The primer sets for cloning are: sense, 5′ AGTCGAATTCTCTTCCTCTACTTAGTGCTTAC; anti-sense, 5′ AGTCCTCGAGGATAGTTTCTTTGGGATAACTAC. The point mutation procedure followed the instruction manual of QuickChange II Site-Directed Mutagenesis Kit (Stratagene). The primer sets for point mutations are: site 1 sense: 5′ AAGGAACCTGTGTAATTGGTGGGTGTTTATGGCTGTAAGATGCATAG; site 1 anti-sense: 5′ CTATGCATCTTACAGCCATAAACACCCACCAATTACACAGGTTCCTT; site 2 sense: 5′ GGGTTTGCTGTATATTTATGATGGGTGTTTCTATGGTGTGAACTTTGGTAG; site 2 anti-sense: 5′ CTACCAAAGTTCACACCATAGAAACACCCATCATAAATATACAGCAAACCC. 3×10^4 Hela cells were seeded per well in 24-well plate the day before transfection and then cells were transfected with 50ng firefly luciferase reporter vector, 50ng Renilla luciferase control vector pRL-SV40 (Promega) and 15 pmol Pre-miR miRNA Precursor Molecules (Ambion) per well by Lipofectamine 2000 (Invitrogen). 24h after transfection, luciferase signal was detected by Dual-Luciferase Reporter Assay System (Promega).

Results

The miR-17-92 cluster is expressed in both the endoderm and mesoderm of the developing lung

To determine which miRNAs are expressed in the developing lung, we carried out miRNA microarray analysis of total RNA from lungs of different embryonic stages and the adult (Fig. S1). We found that about 37% (73/198) of miRNAs have no or very low expression at any stage. By contrast, 36% (72/198) and 11% (22/198) of miRNAs have highest expression in the adult or early embryonic (E11.5) lung, respectively. Of the remainder, 11 show a peak at E17.5, the end of the canalicular stage, and the others show either two peaks of expression or little change during lung development.

The subclass of miRNAs shown by microarray analysis to be expressed at highest levels during early development included 6 members of the *miR-17-92* cluster (Fig. 1B). This result was confirmed by Northern blot analysis (Fig. 1C). We also carried out miRNA cloning from small RNA extracted from lungs at E11.5 and E17.5. We found that more than 10% of the total small RNA clones come from the *miR-17-92* cluster at E11.5 (Table 1). Five members of the cluster, *miR-17-5p*, *miR-18*, *miR-19b*, *miR-20a* and *miR-92*, have relatively high abundance, while *miR-19a* has very low abundance and *miR-17-3p* is not represented at all. As expected, the abundance of all six miRNAs decreases at E17.5, but the fold decrease differs among the cluster members. For example, *miR-19b* decreases 11 fold in abundance while the decrease in *miR-17-5p* is less than 2 fold. Another homologous miRNA cluster, *miR-106a-363* which also contains *miR-19b* and *miR-92* (Fig. 1A), exists on chromosome X. However, no clone of *miR-106a*, *miR-18X*, *miR-20b* and *miR-363* was found at E11.5 or E17.5, suggesting that this cluster is not active in the lung.

In situ hybridization with LNA probes to three members of the cluster, *miR-17-5p*, *miR-19b* and miR-20 supports the conclusion that the gene is widely, but not uniformly expressed in the E11.5 embryo (Fig. 2A–C). All three miRNAs have high expression levels in the central and peripheral nervous system and dorsal root ganglia, somite mesoderm and in many internal organs, including the lung, intestine, and stomach. However, the liver has only very weak expression (Fig. 2H). In the E11.5 and E12.5 lungs, the three members are expressed in both the endoderm and mesoderm (Fig. 2C–G, I–L). In the endodermal epithelial cells, the miRNAs appear to locate most strongly to the apical cytoplasm (Fig. 2F, I, K).

Abnormal lung development in miR-17-92 transgenic embryos and new born pups

To test the function of miR-17-92 in lung development, we over-expressed the whole cluster in the lung epithelium of transgenic embryos using the mouse surfactant protein C promoter (Fig. 3A). In total, 10 transgenic founders were obtained, which were *Sftpc-miR-17-92* positive by PCR. Of these, 8 were collected at E18.5 and 5 of them showed an obvious hypercellularity phenotype in the lung (Fig. 3C,E). The 3 that showed no phenotype did not express the transgene, as judged by microRNA microarray analysis (Fig. S2). Two female founders survived and were crossed with wild type males. One mother delivered normal pups. In situ hybridization suggested that this founder did not express the transgene (data not shown). However, the pups from the other mother showed an abnormal lung phenotype. Of a total of 64 pups, 27 (42%) were transgenic. Of these pups, 7 were born dead with a very abnormal lung phenotype. There was no air in their lungs, which suggests that they died within minutes of birth. The remainder were sacrificed at P0 and all showed an abnormal lung phenotype by histological analysis (Fig. 3F,H). When the lungs of the surviving female were examined, they were normal. From this we conclude that this transgenic female survived because she was mosaic for the transgene.

The normal E18.5 embryonic lung contains many small distal saccules or primitive alveoli, which are ready to open for respiration (Fig. 3B,D). After birth, air fully enters the lung, expanding the alveoli (Fig. 3F,H). By contrast, transgenic lungs contain few normal alveoli and are packed with numerous epithelial cells (Fig 3C,E,G,I). To assess the level of transgenic expression, we carried out microarray of lungs from one litter (six transgenic and six normal pups) born to the mosaic transgenic mother. All six miRNAs were clearly up-regulated (Fig. 3L). Among cluster members, *miR-17-5p* and *miR-20a* are up-regulated by about 5 fold, while the increases in *miR-18*, *miR-19a*, *miR-19b* and *miR-92* appear less. Considering that we extracted the RNA from the whole lung lobe, the actual increase of expression in epithelial cells is likely to be higher than the value from the array. Although these pups all inherit the same transgene, the level of expression of the cluster still varies between individuals (Fig. S3).

In situ hybridization confirmed the over-expression of miRNAs in the transgenic lungs. *MiR-17-5p* is hardly detected in the normal P0 lung (Fig. 3J), while in the transgenic lung, a strong signal was observed in the epithelial cells. Significantly, the miRNA appears to be concentrated in the apical cytoplasm (Fig. 3K), as seen with the endogenous miRNAs at E11.5 and E12.5.

Elevated proliferation of epithelial cells in transgenic lungs

To address whether the hypercellularity of the transgenic lung involves epithelial cells, we carried out immunohistochemistry with antibody to Nkx2.1, which marks all lung epithelial cells. The transgenic lung has a significant higher percentage of Nkx 2.1 positive cells than the wild type (Fig. 4A, B).

To look at cell proliferation, we injected BrdU to the pregnant mother at E18.5 and collected the embryos one hour later. When sections of a lung with a strong phenotype were analyzed the percentage of total cells that were BrdU labeled was about 4-fold higher than in the lung of a control, non-transgenic littermate (Fig. 4C, D). Significantly, no cell death was observed by cleaved caspase 3 staining in either transgenic or normal lungs (data not shown). Control samples (E13.5 normal digits) examined at the same time ensured that apoptotic cells would have been detected.

Epithelial cell differentiation in transgenic lungs

We used immunohistochemistry to examine P0 transgenic lungs for differentiation of proximal and distal epithelial cells. The proximal airways (intralobular bronchi and bronchioles) of transgenic lungs contain Clara cells (positive for Scgb1a1/CC10), ciliated cells (positive for β-tubulin) and neuroendocrine cells (positive for CGRP) (Fig. 5A–F). Significantly, lungs of the dead pups, which had the most severe phenotype and did not contain air, contain about three times more neuroendocrine cell clusters compared with non transgenic control pups collected just before birth (Fig. 5F,M).

The distal region of transgenic lungs was packed with large numbers of epithelial cells and showed few primitive alveoli. These cells still maintain high levels of Sox9 (Fig. 5H). This transcription factor is normally highly expressed in the distal epithelial cells before E16.5 and is virtually undectectable in the P0 lung (Okubo et al., 2005;Perl et al., 2005). The transgenic distal epithelial cells also express high levels of Nmyc by immunohistochemistry (Fig. 5I,J). This finding was confirmed by microarray analysis of RNA extracted from transgenic and control lungs which shows a ~3 fold increase of Nmyc mRNA (Fig. 5N). Furthermore, few cells in the transgenic distal lung have strong Sp-C staining (Fig. 5L). Many of the distal cells have weak signal, which is not easily distinguishable from the background.

Analysis of lungs from E18.5 embryos also showed an abnormal phenotype that varied between individuals. In some transgenic embryos, expression of the differentiated cell marker, Scgb1a1, was significantly reduced (Fig. 6E). In two, only a very few weak positive cells were seen in the smaller bronchi and bronchioles (Fig. 6F). Similar patterns were also seen for β-tubulin staining (data not shown). The distal epithelial cells of these transgenic lungs were Sox9 positive as seen in the transgenic P0 lungs (Fig. 6H,I). The difference between E18.5 and P0 lungs suggests that not only is the proliferation of distal progenitor cells enhanced by overexpression of miR-17-92, but also the differentiation of proximal cells is delayed.

Evidence that miR-17-5p targets retinoblastoma-like 2 (Rbl2/p130)

To elucidate the possible mechanism causing the phenotype of *miR-17-92* transgenic lungs, we first used three well known miRNA target programs (TargetScan, MiRanda and PicTar) to predict 100 potential targets. Then we overlapped these 100 genes with 9,497 genes downregulated in the transgenic lungs derived from Affymetrix array. From this we obtained 65 candidate targets. Among them, Rbl2 was selected for analysis for three reasons: First, Rbl2 was predicted with a high score by three programs to have two *miR-17-5p* family target sites in the 3′UTR. Second, microarray analysis showed that the mRNA of *Rbl2* is down-regulated by about 10% in the transgenic lungs compared with controls (Fig. 7D). Such a small downregulation is consistent with the behavior of mRNAs targeted by miRNAs (Lim et al., 2005). Third, conditional deletion of all three genes of the Rb family in the the lung results in epithelial hyperplasia and an increase in neuroendocrine clusters (Wikenheiser-Brokamp, 2004). We therefore linked the 3′UTR of *Rbl2* to firefly luciferase driven by the CMV promoter (Fig. 7A), and transfected this reporter construct into HeLa cells. Co-transfection with a synthetic *miR-17-5p* precursor gave a decrease in the luciferase signal by about 20% compared with a control miRNA precursor. Mutation of the 3 nucleotides within the seed sequences of both putative target sites in the 3′UTR abolished this downregulation (Fig. 7C). To test whether the Rbl2 protein is regulated by miR-17-5p *in vivo*, we performed immunohistochemistry of transgenic and normal lungs using antibody to Rbl2. This showed that the level of Rbl2 in transgenic lung epithelial cells was significantly reduced compared with non-transgenic controls (Fig. 7H).

Discussion

In this paper we have identified a total of 198 miRNAs differentially expressed during mouse lung development. We have focused on the *miR-17-92* cluster which is highly expressed in both the epithelium and mesenchyme at E11.5 and declines gradually until it is barely detectable in the adult. We hypothesized that high level of *miR-17-92* cluster help to maintain lung progenitor cells in a proliferative and undifferentiated state. To test this hypothesis we generated transgenic embryos which maintain this high expression level in the epithelium throughout development. Both hyperproliferation and diminished differentiation of distal epithelial cells were observed in the transgenic lungs. Our finding that *miR-17-5p* regulates the novel target, Rbl2, can partially explain the abnormal phenotype.

MiRNA expression patterns in the lung

We found that miRNAs expressed in the developing lung fall into several distinct subclasses. The majority, including *miR-1* and *Let-7* have highest expression in the adult. In other systems, miR-1 has been shown to promote the differentiation and maintain the differentiated state of cardiac and skeletal muscle (Chen et al., 2006; Zhao et al., 2007), while *Let-7* limits cell growth in culture by targeting Ras (Johnson et al., 2005). It is possible that in the lung these miRNAs have a similar function. Significantly, Fgf signaling is important for the proliferation of early lung progenitor epithelial cells (White et al., 2006) and the regulation of FGF signaling mainly depends on the Ras/MAP kinase pathway (Thisse and Thisse, 2005). So the low level of

Let-7 may facilitate high proliferation of lung progenitors in early development, while the adult lung needs a high level of *Let-7* to slow down cell growth by targeting Ras. Loss of *Let-7* could help to elevate levels of Ras in lung cancer (Johnson et al., 2005).

MiR-17-92 cluster expression

Among the miRNAs most highly expressed in the embryonic lung were some (but not all) members of the *miR-17-92* cluster. This cluster encodes seven miRNAs within a 1kb region and is believed to be transcribed as one primary transcript. However, both the microarray and microRNA cloning data show that the expression level of individual cluster members varies, as reported previously (Mineno et al., 2006). Differences in the efficiency of post-transcriptional processing steps, mediated by Drosha (Han et al., 2006), Exportin5 and Dicer (Lund and Dahlberg, 2006), may account for this. However, it is still difficult to explain why the abundance of miRNAs within the cluster does not change co-ordinately between E11.5 and E17.5. One possibility is that unknown processing mechanisms exist that affect some members of the cluster but not others. This hypothesis is supported by evidences for post-transcriptional regulation of some miRNAs (Obernosterer et al., 2006; Thomson et al., 2006; Wulczyn et al., 2007).

Relationship between miR-17-92 cluster expression and Nmyc function in the developing lung

The *miR-17-92* cluster was recently reported to be up-regulated by the proto-oncogene Myc (Dews et al., 2006; O'Donnell et al., 2005). Myc is a member of proto-oncogene family (Myc, Lmyc and Nmyc), which encodes basic helix-loop-helix leucine zipper (bHLHz) proteins. One gene regulated by Myc is *E2f1*, which is targeted by *miR-17-5p* and *miR-20a*. Since E2f1 can also induce Myc expression (Matsumura et al., 2003), it was suggested that the negative regulation of E2f1 by *miR-17-5p* and *miR-20a* could dampen Myc-E2f1 reciprocal activation (O'Donnell et al., 2005).

In the lung, Nmyc is specifically expressed in the epithelium, while *Myc* is transcribed only in the mesenchyme. Over-expression of Nmyc in the lung epithelium under the control of human *Sftpc* regulatory elements caused elevated cell proliferation and inhibition of differentiation, with a phenotype very similar to the phenotype of *miR-17-92* cluster transgenic mice (Okubo et al., 2005). So it was surprising that no significant up-regulation of the *miR-17-92* cluster was observed in the *NmycEGFP* transgenic lungs (Fig. S4) despite the structural and functional similarity between Myc and Nmyc. On the contrary, levels of Nmyc are still high in *Sftpc-miR-17-92* transgenic lungs. We do not know whether the high level of Nmyc is due to the large number of undifferentiated, Nmyc expressing epithelial cells or to higher Nmyc expression per cell, or both. At least, it suggests that high levels of the *miR-17-92* cluster does not negatively regulate Nmyc expression. This conclusion is supported by evidence of augmentation of Myc-induced tumor formation by co-over-expression of the *miR-17-92* cluster (Dews et al., 2006; He et al., 2005).

Possible mechanisms underlying the abnormal cell proliferation and differentiation phenotype of transgenic lungs

Up to now, several genes have been identified as targets of members of the *miR-17-92* cluster by in vitro assays and therefore need to be considered as candidate targets in the developing lung. These include *E2f1* (targeted by *miR-17-5p/20a*), *Pten* (targeted by *miR19*), *Ncoa3* (targeted by *miR-17-5p*), *Thbs1* (thrombospondin, targeted by *miR19*) and *Ctgf* (connective tissue growth factor, targeted by *miR-18*) (Dews et al., 2006; Hossain et al., 2006; Lewis et al., 2003; O'Donnell et al., 2005). Among them, Ctgf is not apparently expressed in the embryonic lung (Ivkovic et al., 2003) so it is not considered further here. Of the remaining candidates, *Ncoa3*, which encodes nuclear receptor co-activator 3, is amplified in several cancers and acts

as a proto-oncogene (Torres-Arzayus et al., 2004). *Ncoa3* null mutant mice are smaller than normal and cells derived from mutant embryos show decreased proliferation in response to insulin-like growth factor 1 (Wang et al., 2000). It therefore appears unlikely that downregulation of this protein would be a major cause underlying the hyperproliferative phenotype reported here in *miR-17-92* transgenic lungs.

Another potential target, *E2f1*, encodes a transcription factor that promotes cell proliferation by transactivating genes required for G1 to S transition (DeGregori et al., 1997; Johnson et al., 1993). Deceased levels of E2f1 results in reduced apoptosis (Field et al., 1996; Holmberg et al., 1998). Since neither the normal developing lung nor transgenic lungs show evidence of apoptosis, it is unlikely that *miR-17-92* functions to promote expansion of the progenitor cells through inhibition of apoptosis by downregulating E2f1.

Pten is a well known tumor suppressor gene that normally restricts cell proliferation. Embryos lacking *Pten* die before E10.5, while heterozygous mutant mice show increased tumor incidence or hyperplasia in many tissues, including the mammary gland, prostate and lymphoid system (Di Cristofano et al., 1998; Freeman et al., 2006). Although a lung phenotype was not reported in these heterozygotes, it is possible that downregulation of Pten could contribute to the abnormal phenotype seen here. Microarray analysis showed the *Pten* mRNA level is downregulated by 20–30% in the transgenic lungs (Table S1).

Thbs1 is an extracellular calcium binding protein with multiple functions (Adams and Lawler, 2004). At E11.5, *Thbs1* mRNA is detected in distal epithelial cells (Liu and Hogan, 2002) and Thbs1 protein is localized to the basal lamina of both the embryonic and adult lung epithelium (O'Shea and Dixit, 1988; Wight et al., 1985). In addition, Thbs1 is secreted by alveolar macrophages and type II cells (Sage et al., 1983; Yehualaeshet et al., 1999). Mice homozygous null for *Thbs1* have hyperplasia in many tissues, including lung bronchial epithelium (Crawford et al., 1998). However, this phenotype may in large part be secondary to strong inflammation (Lawler et al., 1998). It is therefore unclear whether down regulation of the gene during lung development affects proliferation and differentiation.

Here, we provide evidence that another important tumor suppressor, Rbl2, is a target of *miR-17-5p*. Rbl2 belongs to the Rb pocket protein family, which also contains Rb and Rbl1/p107. All three members share extensive structural homology and can inhibit E2f responsive promoters (Classon and Dyson, 2001). Rbl1 and Rbl2 can at least partially compensate for loss of Rb function in vivo (Lipinski and Jacks, 1999). Studies using both in situ hybridization and Northern blot have shown that expression of Rbl2 increases during late embryonic development and are maintained at relatively high levels in the adult lung (Chen et al., 1996; Jiang et al., 1997; Pertile et al., 1995). In addition, Rbl2 mRNA is transcribed in both endoderm and mesoderm of the lung (Jiang et al., 1997). Our immunohistochemistry data shows that Rbl2 protein more abundant in non-transgenic epithelial cells compared to transgenic (Fig. 7E–H). Taken together, all these observations support Rbl2 is a direct target of miR-17-5p during normal lung development.

Recently, pocket proteins have been shown to inhibit neuroendocrine cell fate in the lung. Conditional deletion of *Rb* or the whole *Rb* family, including *Rbl2*, leads to epithelial cell hyperplasia and to an increase in both the number and size of neuroendocrine clusters (Wikenheiser-Brokamp, 2004). Loss of *RBL2* gene is correlated with small cell lung cancer (Baldi et al., 1996; Helin et al., 1997; Xue Jun et al., 2003), which is considered to have a neuroendocrine cell origin. A significant increase in neuroendocrine cell clusters is also observed in our *miR-17-92* transgenic P0 lungs, which supports the idea that pocket proteins could be miRNA targets. Although the whole Rb family has been predicted to be targeted by *miR-17-5p*, only the highest scored Rbl2 showed significant down-regulation in our luciferase

assay (data not shown). Whether knocking down Rbl2 alone can also lead to hypercellularity of neuroendocrine cells is unclear, since no obvious phenotype has been reported in *Rbl2* null mutant mice (Cobrinik et al., 1996).

In conclusion, we cannot expect to explain the phenotype of Spc-miR-17-92 transgenic lungs by any single target gene and there are likely more targets which have not yet been discovered. To address which target or targets are most important for the regulation of progenitor cell proliferation and differentiation during lung development is a topic for future research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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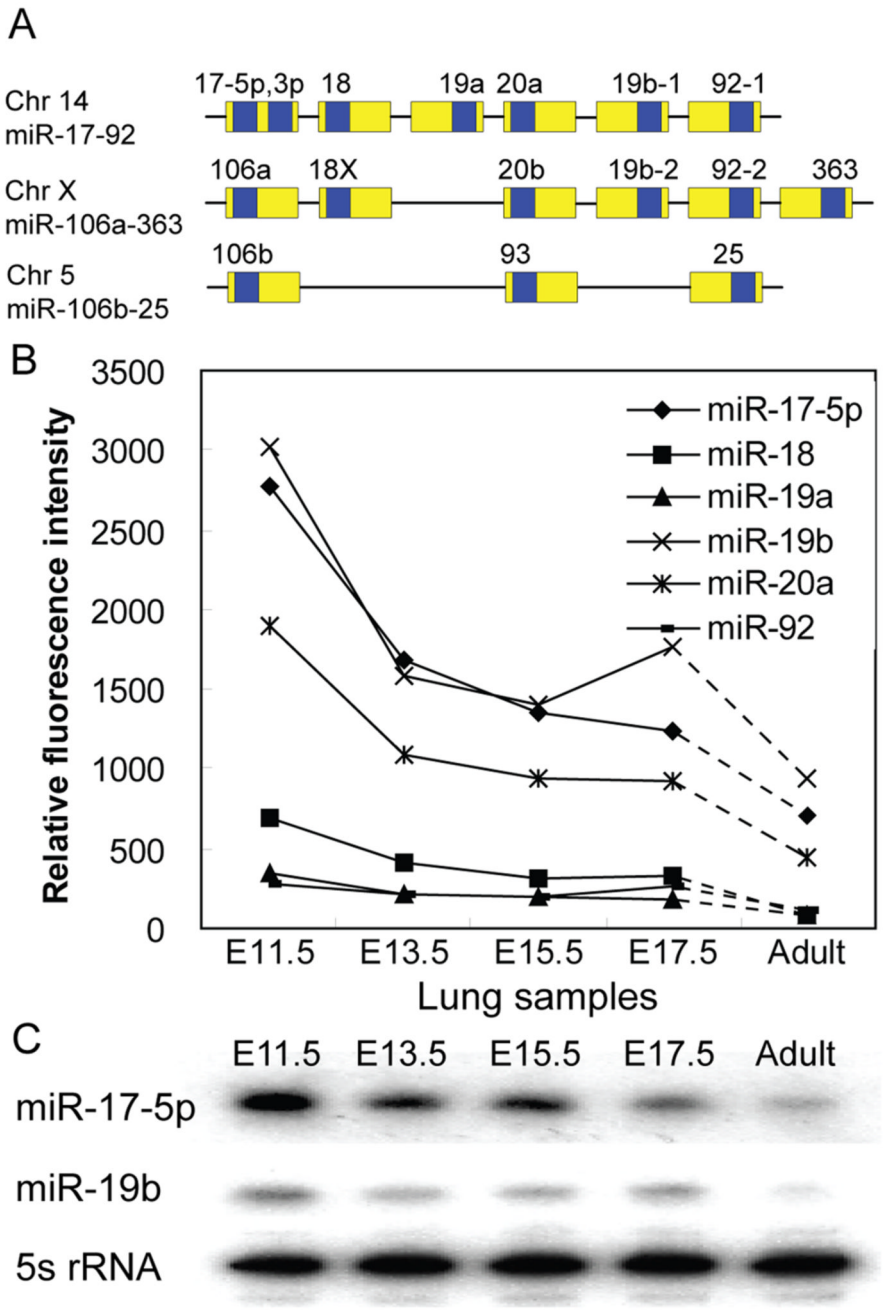

Figure 1.

Relative expression of *miR-17-92* cluster members during lung development. (A) Genomic organization of three homologous mouse miRNA clusters (*miR-17-92*, *miR106a-363* and *miR-106b-25*) (yellow boxes, pre-miRNAs; blue boxes, mature miRNAs). Note that *miR-18X* is a predicted miRNA, which has not yet been cloned. MicroRNA microarray (B) and Northern blot (C) show the relative expression levels of *miR-17-92* cluster members in developing lungs. Five different developmental stages are shown: E11.5, E13.5, E15.5, E17.5 and adult (about 12 weeks old).

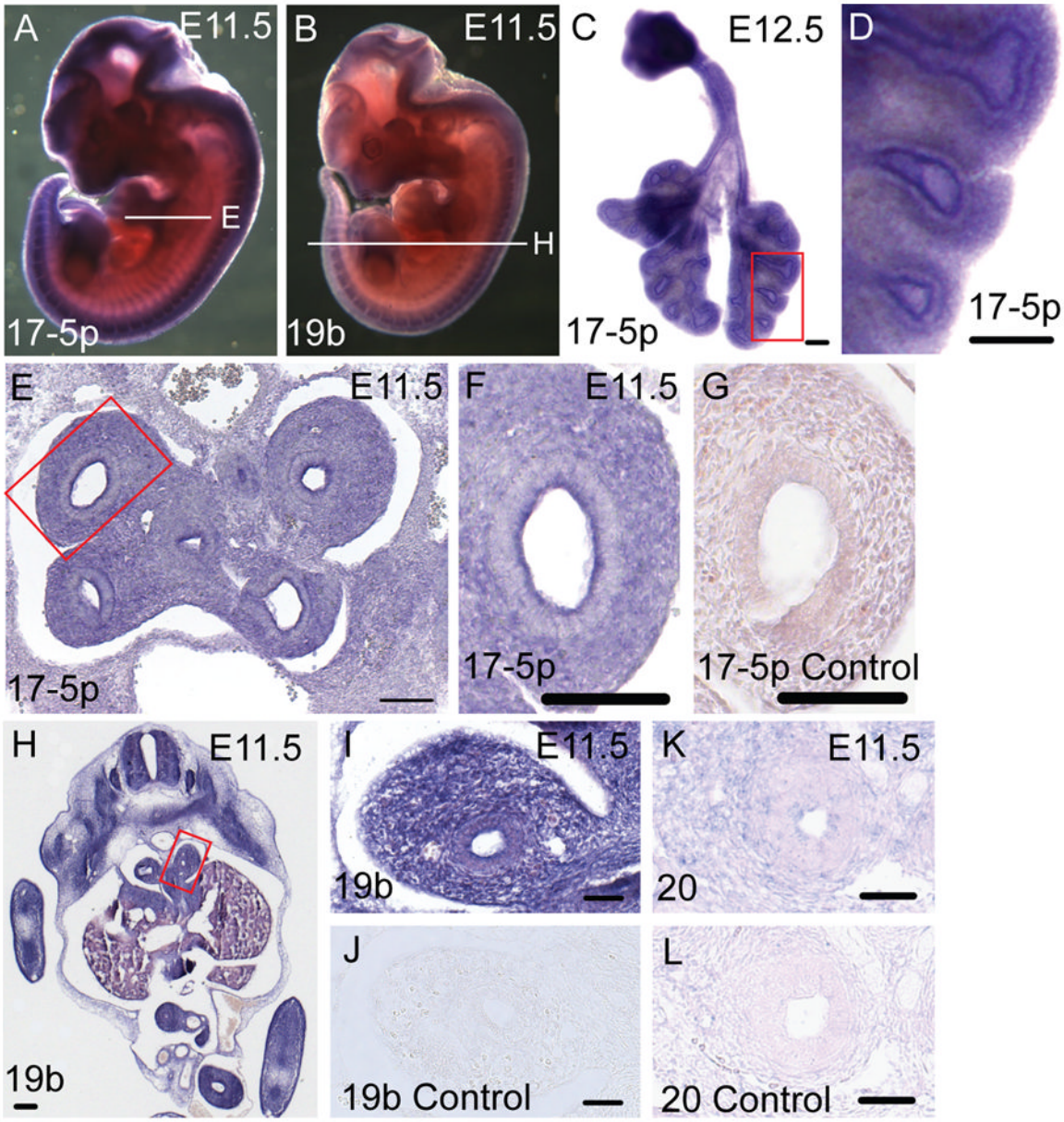

Figure 2.

MiR-17-92 expression in embryos and lungs. (A,B) E11.5 mouse embryos. (C,D) E12.5 lungs. (E-L) Cross section of E11.5 embryos and lungs. In situ hybridization of *miR-17-5p*, *miR-19b* and *miR-20* were performed using LNA probes (whole mount: A–D; paraffin section: E–L). (D,F,I) High magnification of regions boxed in (C), (E) and (H) respectively. The negative control for in situ hybridization was done without the probes. (C, D, E, H) Scale bar = 200μm; (F, G, I, J, K, L) Scale bar = 50μm.

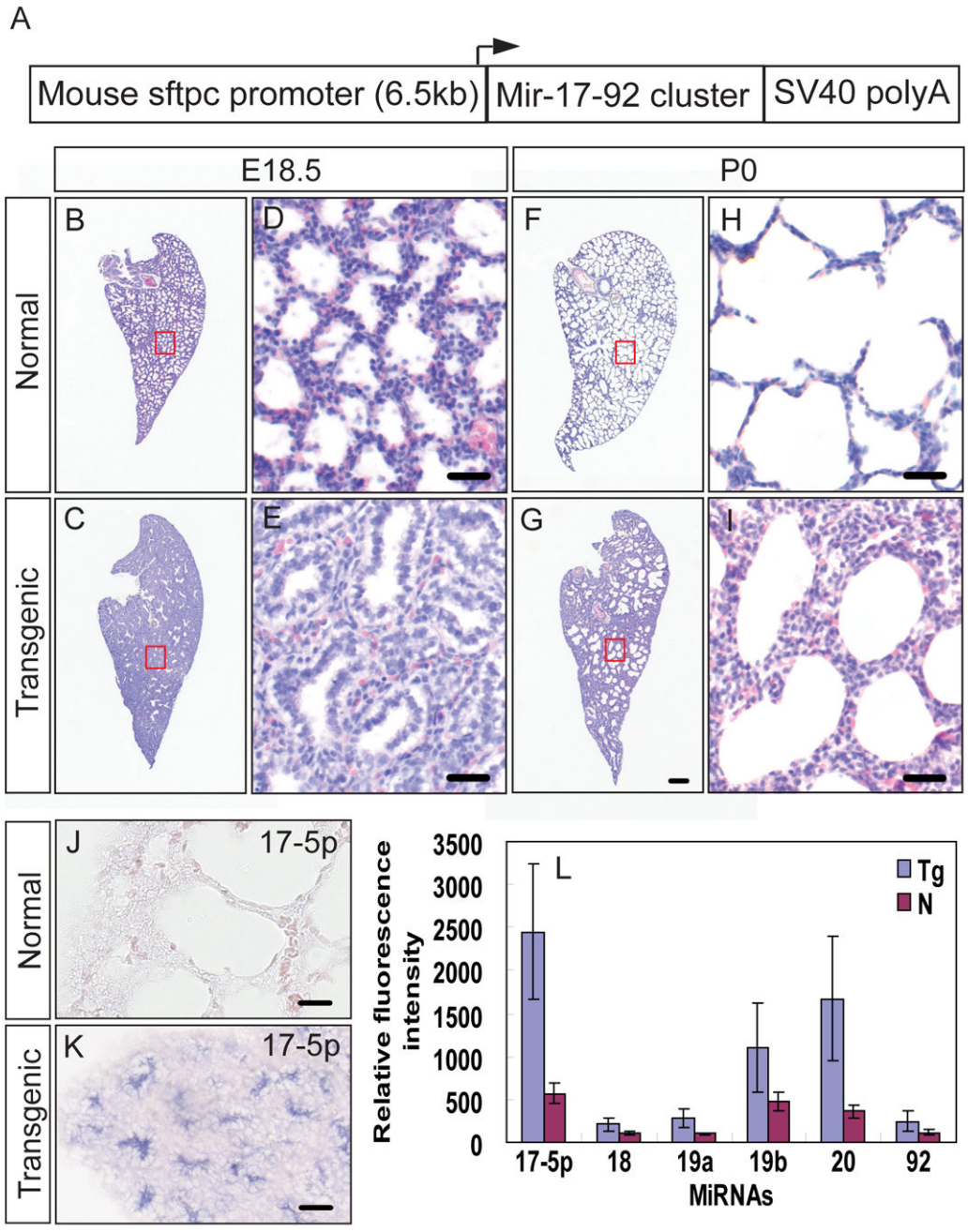

Figure 3.

Abnormal lung phenotype observed in *miR-17-92* cluster transgenic embryos and newborn pups. (A) Schematic illustration of *miR-17-92* cluster transgenic construct. The cluster contains all six miRNA precursors and 133bp genomic flanking region at both ends. (B–I) H&E stained sections of left lobe from both the normal and transgenic lungs. (D,E,H,I) The enlarged pictures of (B), (C), (F) and (G) respectively. (J,K) MiR-17-5p in situ hybridization of normal and transgenic P0 lungs. (L) Microarray analysis shows that the expression levels of all six miRNAs within the cluster are increased in the transgenic lungs. Tested RNA was extracted from P0 lungs six transgenic and six normal pups from one litter of the transgenic mosaic founder. (B,C,F,G) Scale bar = 200μm; (D,E,H,I) Scale bar = 100μm; (J,K) Scale bar = 15μm.

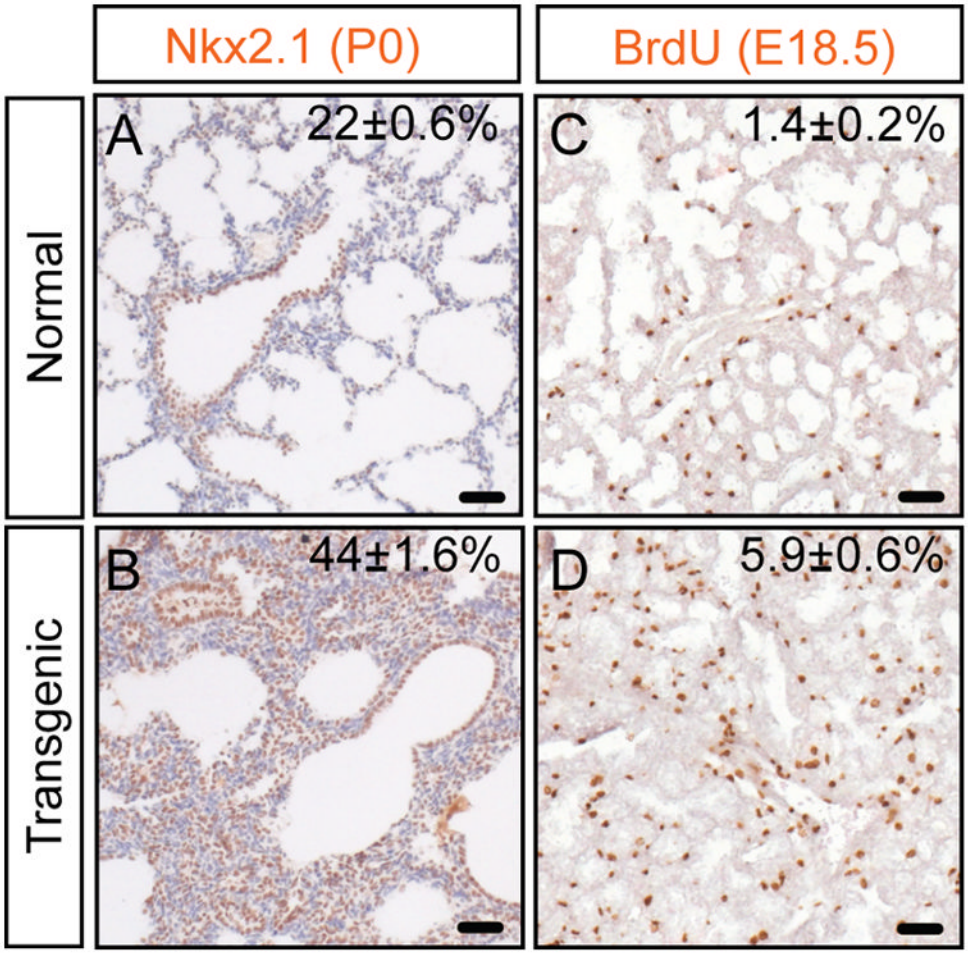

Figure 4.
Elevated number and proliferation of epithelial cells in the transgenic lungs. (A,B) Nkx 2.1 (Titf1) immunostaining. (C,D) BrdU immunostaining. The percentage of BrdU and Nkx 2.1 positive cells in the total cells of the lung are shown in the top left corner of each digram. Scale bars = 50μm.

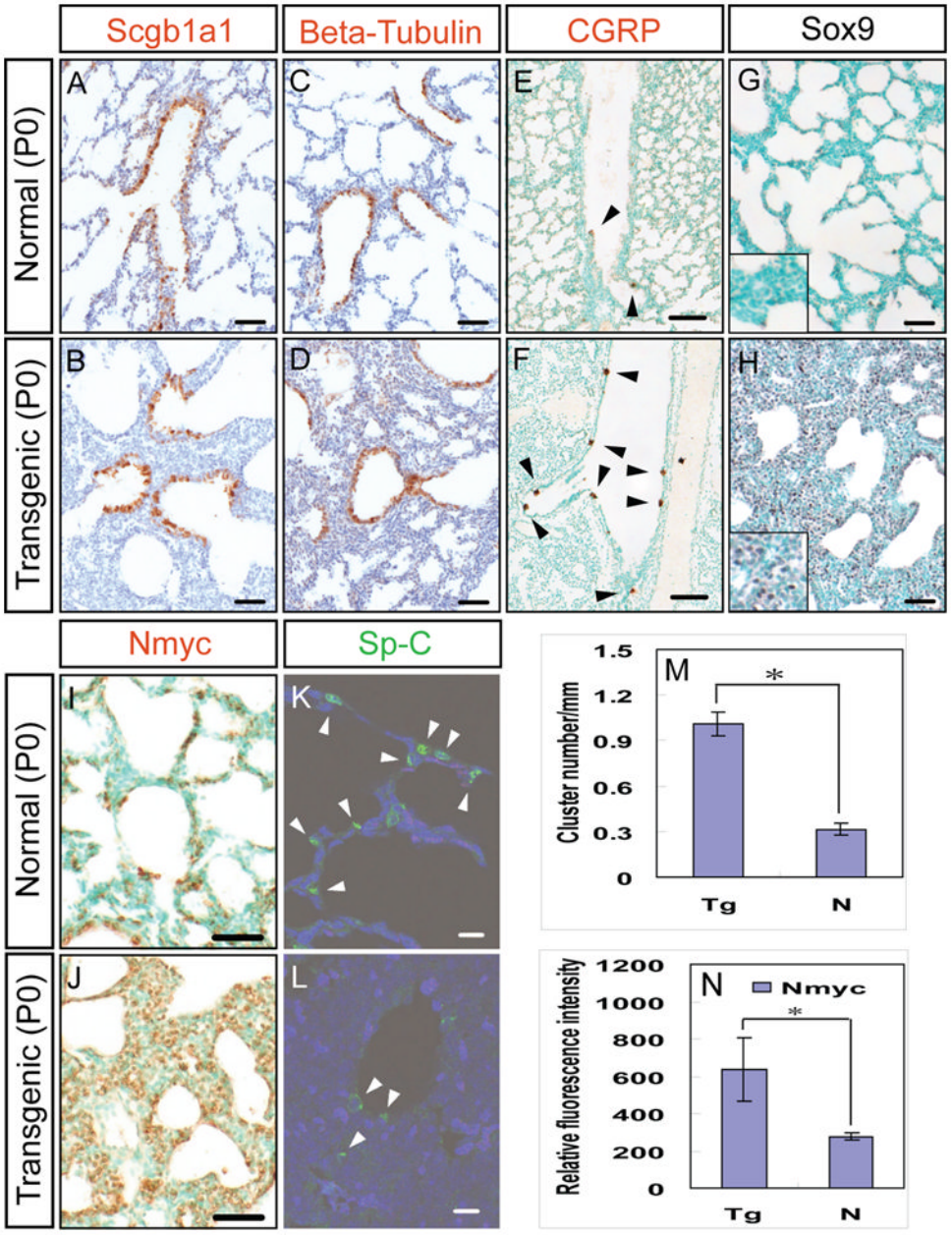

Figure 5.

Epithelial progenitor cells persist in transgenic P0 lungs. (A,B) Scgb1a1/CC10, (C,D) β-tubulin, (E,F) CGRP, (G,H) Sox9, (I,J) Nmyc and (K,L) Sp-C were detected by immunostaining. The black arrowheads in (E) and (F) mark the CGRP positive neuroendocrine cell clusters. White arrowheads mark the Sp-C positive cells. (M) CGRP stained sections from the lungs of three dead pups and three prenatal embryos were counted for CGRP cluster numbers per millimeter luminal surface of bronchus. Tg: 1.01±0.08/mm, N: 0.32±0.04/mm. P<0.0002. (N) Increase of Nmyc mRNA was confirmed by microarray analysis. Three transgenic and three normal lungs were tested in the Affymatrix array. P<0.023. (A–B,D–J) Scale bars = 50um. (E,F) Scale bars = 100um. (K,L) Scale bars = 5μm. Tg: transgenic; N: normal.

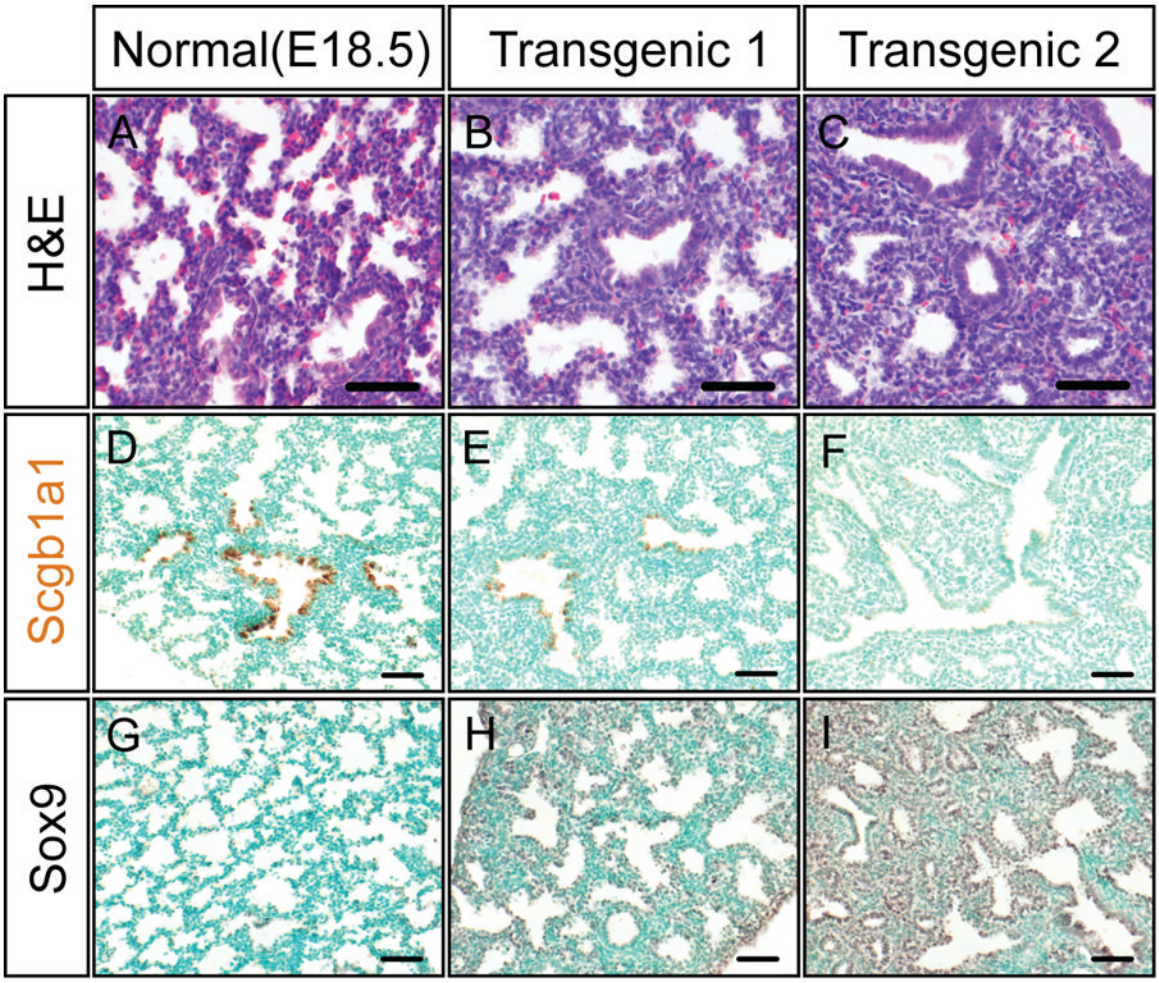

Figure 6.
Differentiation of proximal epithelial cells is inhibited in transgenic embryos. (A–C) H&E staining of normal and two transgenic E18.5 lungs. (D–F) Scgb1a1/CC10 immunostaining. (G–I) Sox9 immunostaining. Scale bars = 50μm.

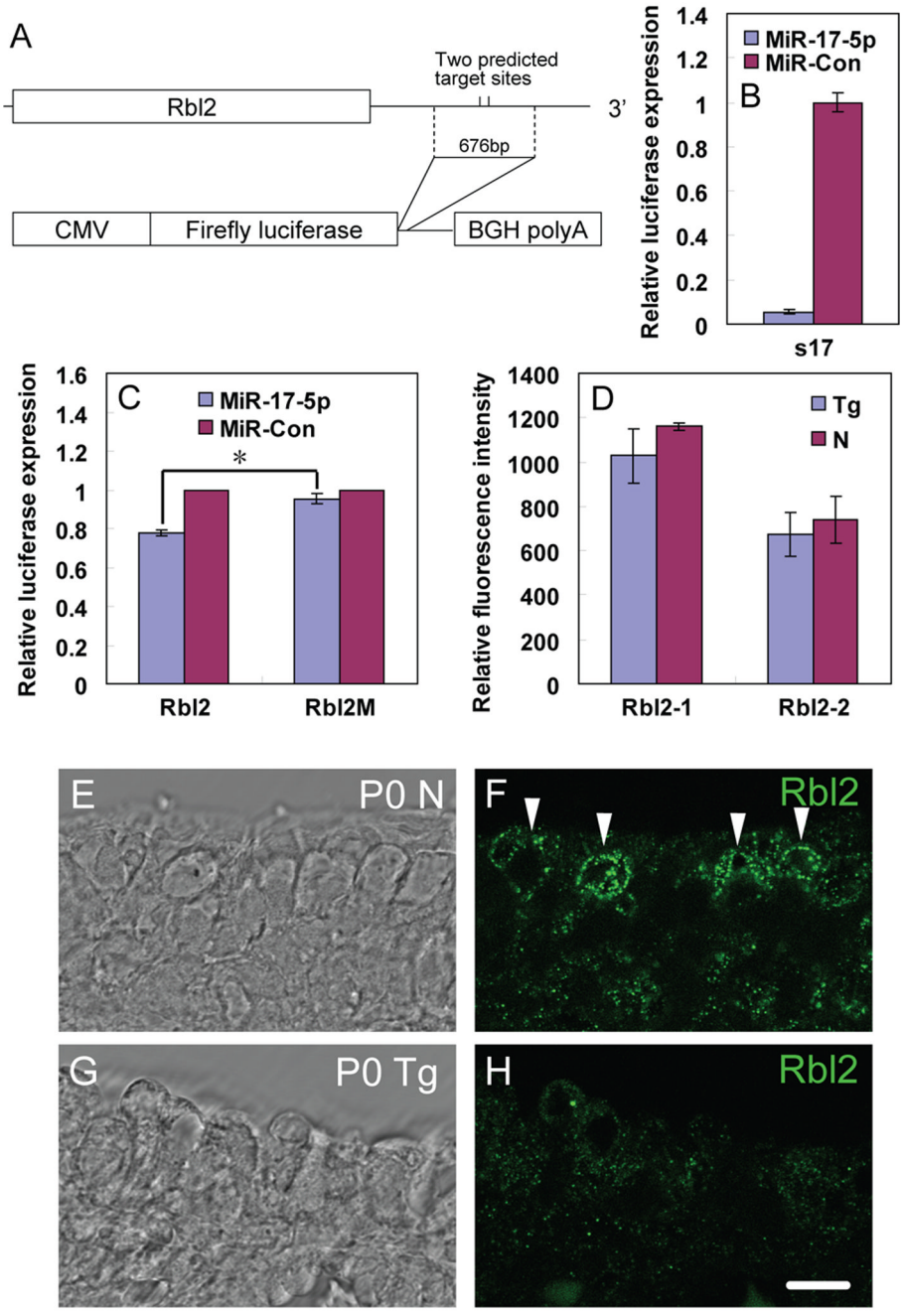

Figure 7.

Evidence that the 3′UTR of *Rbl2* is targeted by *miR-17-5p*. (A) Schematic diagram of luciferase assay. 676bp of *Rbl2* 3′UTR containing two predicted target sites were cloned into the 3′ end of the *firefly luciferase* gene. (B) Positive control assay was performed using a sensor construct, s17, in which three perfect miR-17-5p anti-sense fragments were cloned to the 3′end of firefly luciferase. n=3. The data from the control wells, which have the control miRNA (miR-Con), is normalized to 1. (C) *Rbl2* and *Rbl2M* 3′UTR were tested in the luciferase assay. *Rbl2M* is the *Rbl2* 3′UTR fragment with 3 point mutations in each predicted target site. Firefly luciferase reportor construct, renilla luciferase internal control vector and miRNA were co-transfected to Hela cells. P<0.002, n=9. (D) *Rbl2* mRNA level is slightly decreased in transgenic lungs by

microarray analysis. Two probes, Rbl2-1 and Rbl2-2, show similar ~10% down-regulation in transgenic lungs. Three transgenic and three normal P0 lung RNA samples were tested in the array. (E) Section through normal P0 lung interlobular airway. DIC image. (F) Immunofluorescence image using antibody to Rbl2. The white arrowheads mark the epithelial cells expressing high levels of the protein in the nuclear and perinuclear locations. (G) Section through transgenic P0 lung airway. (H) Immunofluorescence image of the same section of (G). Note lower levels of Rbl2 protein. Tg: transgenic; N: normal. Scale bar: 5μm.

Table 1

miR	E11.5	E17.5
17-5p	2.2%	1.9%
18	2.0%	1.1%
19a	0.1%	0.1%
19b	1.1%	0.1%
20	3.5%	0.7%
92	1.6%	0.7%