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MicroRNA-449a levels increase by several orders of magnitude during mucociliary differentiation of airway epithelia

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MicroRNAs of the miR-34/449 family mediate cell cycle arrest and tumor suppression. Here we show that the expression of microRNA miR-449a, unlike its paralog miR-34a, is highly tissue specific and largely restricted to pulmonary and testicular tissue. MiR-449a levels in the murine lung are particularly high shortly before and after birth, coinciding with terminal differentiation of lung epithelia. Strikingly, miR-449a is upregulated by more than 1,000-fold when epithelial cells from human airways are lifted from a liquid environment to air, allowing them to undergo mucociliary differentiation. The induction of miR-449a occurs in parallel to its host gene CDC20B and the transcription factor FoxJ1. Exposure to tobacco smoke induces a moderate further increase in the levels of miR-449a, and also miR-34a, in differentiated airway epithelia. We propose that miR-449a can serve as an exquisitely sensitive and specific biomarker for the differentiation of bronchial epithelia. Moreover, miR-449a may actively promote mucociliary differentiation through its ability to block cell cycle progression, and it may conribute to a first line of defence against genotoxic stress by its proapoptotic functions.

Introduction

The differentiation of airway epithelia represents one of the most dramatic changes in cell function that occurs shortly before and after birth, essential for the survival of the organism. Epithelial cells within the lung must differentiate in a highly ordered fashion[1] to allow proper ventilation, ensure gas exchange, prevent the loss of fluid, contribute to mucociliary clearance and host defence and avoid the accumulation of toxic substances from the environment. The development of the lung involves the activity of several transcription factors in a sequential and cell-specific manner.[2] However, our knowledge about post-transcriptional master regulators behind this process remains incomplete at this stage.

To gain insight into the differentiation process of bronchial epithelia, it has been recapitulated in a cell culture setting. Primary airway epithelial cells (AECs) can be obtained from human donors and maintained in culture. When such a cell monolayer is lifted from a liquid environment to the interface between liquid and air, it starts a mucociliary differentiation program that reflects the physiological processes occurring in the lung.[3] By analyzing the associated changes in mRNA levels, a number of differentially regulated genes were previously identified.[4] Among those, the transcription factor FoxJ1 was suspected to represent one of the regulatory factors that govern downstream genes triggering differentiation.[2,5,6] Little is known about other master regulators that act at the same stage of differentiation.

MicroRNAs represent a novel class of regulators for gene expression. Their best-described mechanism of action consists in their interaction with target mRNAs, resulting in reduced translation and/or decreased mRNA stability.[7] Some microRNAs are ubiquitously distributed, whereas others are expressed in a highly tissue-specific manner.[8] Interestingly, the microRNA processing enzyme Dicer is required for proper pulmonary development,[9] arguing that microRNAs are essential in this process. Moreover, microRNAs were found to be differentially regulated when lungs develop.[10-15] To our knowledge, however, no microRNA species were previously found associated specifically with mucociliary differentiation of airway epithelia.

MicroRNAs 449a and 449b represent two related microRNAs, expressed form the same locus within their host gene CDC20B on human chromosome 5. They belong to the same family as microRNAs 34, a-c, and all members of this family are capable of inducing cell cycle arrest and apoptosis. While miR-34a-c is inducible through the tumor suppressor p53,[16] miR-449 can be activated by the cell cycle regulatory transcription factor E2F1.[17,18] However, the tissue specificities of these processes are

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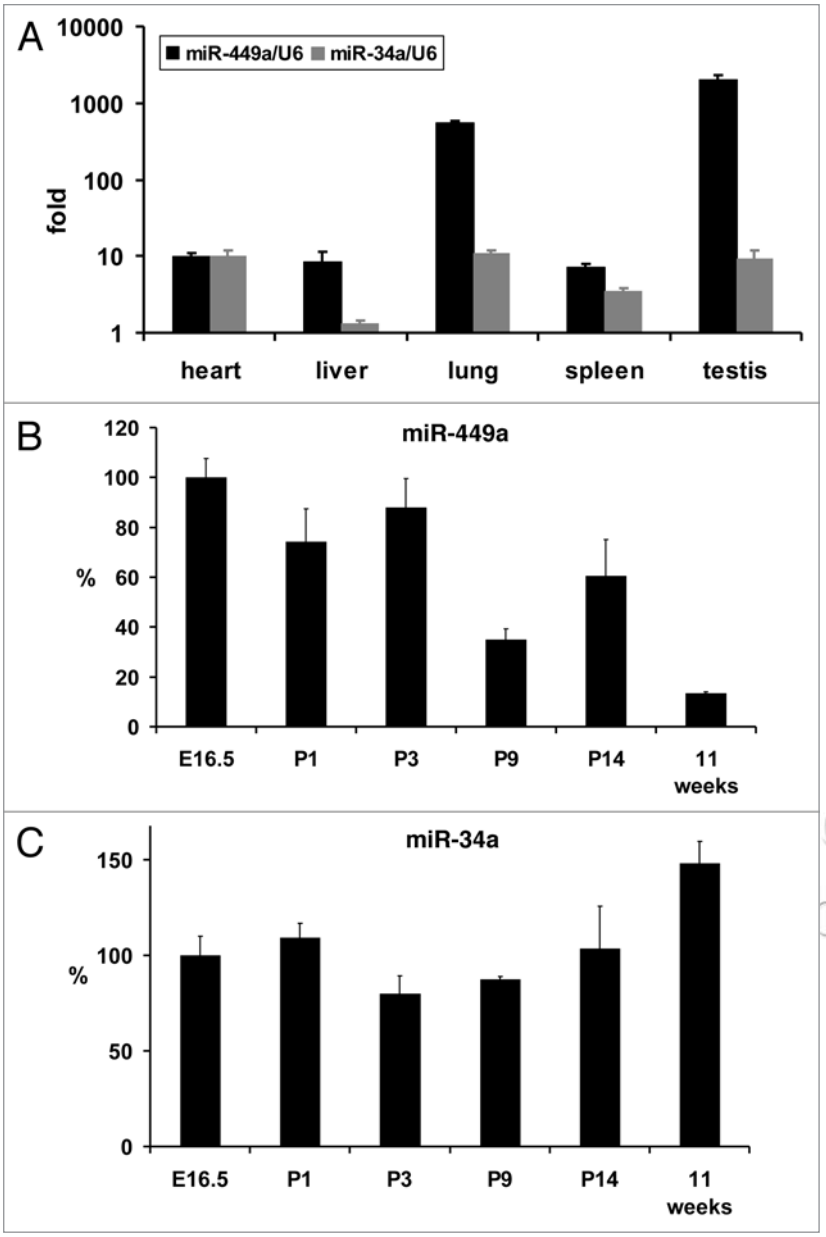

Figure 1. miR-449a expression levels in murine tissue. RNA was extracted from the indicated tissues obtained from C57BL/6 mice, followed by quantitative RT-PCR to determine the levels of microRNAs miR-34a and miR-449a. (A) Tissues were obtained from adult mice, 11 weeks of age. (B) Lung tissue was prepared from mouse embryos at stage E16 (shortly before birth), day 1, 3, 9 and 14 after birth, and 11 weeks after birth, followed by quantification of miR-449a and (C) miR-34a.

largely unknown. Here we show that miR-449a is a highly specific marker of differentiated bronchial epithelial cells.

Results

miR-449a is highly abundant in lung tissue, in particular around birth. To gain insight into the expression patterns of miR-449a and miR-34a, we assessed the levels of both microRNA species in murine tissues by quantitative RT-PCR. Whereas miR-34a showed a broad distribution, the miR-449a was largely restricted to testes and lung (**Fig. 1A**), in accordance with our previous findings.[17] In these tissues, miR-449a levels appear to exceed those of miR-34a (**Fig. S1**). To assess the temporal pattern of miR-449a expression in the lung, we extracted RNA form murine pulmonary tissue shortly before and after birth. As shown in **Figure 1B**, miR-449a was highly expressed in the lungs of mouse embryos at stage E16 (i.e., three days before term) and then steadily decreased. In contrast, the levels of miR-34a remained largely unchanged. We conclude that miR-449a is specifically expressed in pulmonary tissue, but mostly during the time around birth.

miR-449a levels sharply increase upon differentiation of airway epithelial cells. Since mucociliary epithelia are a major component of the respiratory tract, we speculated that they represent the site of high miR-449a levels. To test this, we employed a system to recapitulate AEC differentiation in vitro. Human AECs were lifted to the liquid air interface and thereby induced to fully differentiate.[3] At several time points, RNA was extracted to quantify microRNA levels. Before airlift, miR-449a was barely detectable by RT-PCR. Strikingly, however, miR-449a levels sharply increased to 100-fold higher levels within seven days, and more than 1,000-fold within the following week (**Fig. 2A**). In parallel, the host gene CDC20B (containing the miR-449a encoding region within an intron) was upregulated (**Fig. 2B**), as reported previously,[4] although not as dramatically as miR-449a. A similar pattern was also seen for a previously described putative master regulator of AEC differentiation, FoxJ1 (**Fig. 2B**) and the generation of a seal with high electrical resistance was observed in parallel, indicating the generation of a differentiated cell monolayer (**Fig. 2C**). Similar results were obtained with the AECs form a different individual (**Sup. Fig. 2**). In contrast, the levels of miR-34a remained largely unchanged (**Fig. 2A**). Curiously, the levels of E2F1 mRNA also remained unaffected (**Fig. 2B**), in contrast to the mutual regulation of E2F1 and miR-449a that we previously described for cells of different origin that undergo DNA damage.[17] Continuous incubation of AECs without airlift resulted in a far less pronounced increase of miR-449a levels, and also did not lead to detectably augmented FoxJ1 mRNA (**Sup. Fig. 3**). In conclusion, miR-449a represents a highly specific marker for differentiated AECs, perhaps acting as a master regulator of the AEC-specific gene expression program. To our knowledge, and in comparison to other differentially regulated genes,[4] the increase in miR-449a represents the strongest change in gene expression levels that occurs upon AEC differentiation.

miR-449a levels further increase upon exposure of airway epithelia to tobacco smoke. We had previously characterized

miR-449a as upregulated in response to DNA damage,[17] and similar findings were reported for miR-34a.[16] To assess this in the context of airway epithelia, we exposed a differentiated AEC monolayer to tobacco smoke.[19] As shown in **Figure 3A**, this procedure increased the levels of miR-449a even further, although the additional upregulation was moderate as compared to that induced by AEC differentiation. This increase was also found for the mRNA encoding E2F1 (**Fig. 3B**), a transcription factor that targets miR-449a™,[17,18] possibly explaining the upregulation of miR-449a. Similarly, miR-34a levels were increased under such circumstances (**Fig. 3A**), perhaps as a result of the known p53-responsiveness of miR-34a.[16] Other mRNA species were quantitated as controls and were found largely unchanged (**Sup. Fig. 4**). This is in contrast to a number of microRNA species that were previously found downregulated by tobacco smoke exposure.[20] We conclude that miR-449a is further regulated by genotoxic stress, as it occurs in response to exposure of AECs to tobacco smoke. Unlike the differentiation-associated upregulation of miR-449a, the induction of miR-449a by genotoxicity may be a result of E2F1 activation.

Discussion

Our results identify miR-449a as an exquisitely specific biomarker of differentiated airway epithelia. To our knowledge, this is the first description of a microRNA that displays strong specificity for mucociliary differentiation, although several microRNAs, including miR-449a, were reported to be expressed in lung tissue depending on the developmental stage.[14,15] MiR-449a undergoes the strongest induction of gene expression that was so far observed during AEC differentiation. Finally, this observation raises the possibility that miR-449, perhaps in addition to FoxJ1, serves as a master regulator to ensure the proper differentiation of this vital cell population.

Previous studies have used microarray hybridization to identify changes in mRNA levels that occur in the course of AEC differentiation in an air liquid interface.[4] Strikingly, one of the most differentially expressed genes found in this study was CDC20B. miR-449 is encoded by a gene locus that resides within the second intron of CDC20B, and the mRNA of CDC20B and miR-449 were previously found coregulated.[17,18] We therefore assume that at least some of the regulatory mechanisms that govern miR-449 upregulation upon AEC differentiation apply to its host gene CDC20B as well. However, it is remarkable that the upregulation of miR-449a was even stronger than that of CDC20B in our assays (**Fig. 2**). We cannot fully rule out that this may be due to differences in PCR efficiency. However, it is tempting to speculate that AEC differentiation is associated with increased efficiency of miR-449a processing from its precursor RNA, explaining the additional increase.

The identity of transcription factors that regulate CDC20B/miR-449 in the context of AEC differentiation remains elusive. We initially suspected a regulatory function of FoxJ1 in this context, but neither did we identify consensus FoxJ1 binding sites within the CDC20B promoter, nor did our unpublished observations reveal increased miR-449a levels in response to FoxJ1 overexpression.

It is very possible that miR-449a not only marks differentiated AECs but also serves as an active regulator of this differentiation process. MiR-449a is a potent inducer of cell cycle arrest, and this may contribute to terminal differentiation. In addition, miR-449a reduces the levels of the histone deacetylase Sirt1,[17] a pleiotropic regulator of transcription, possibly contributing to a differentiation-associated gene expression program. An active

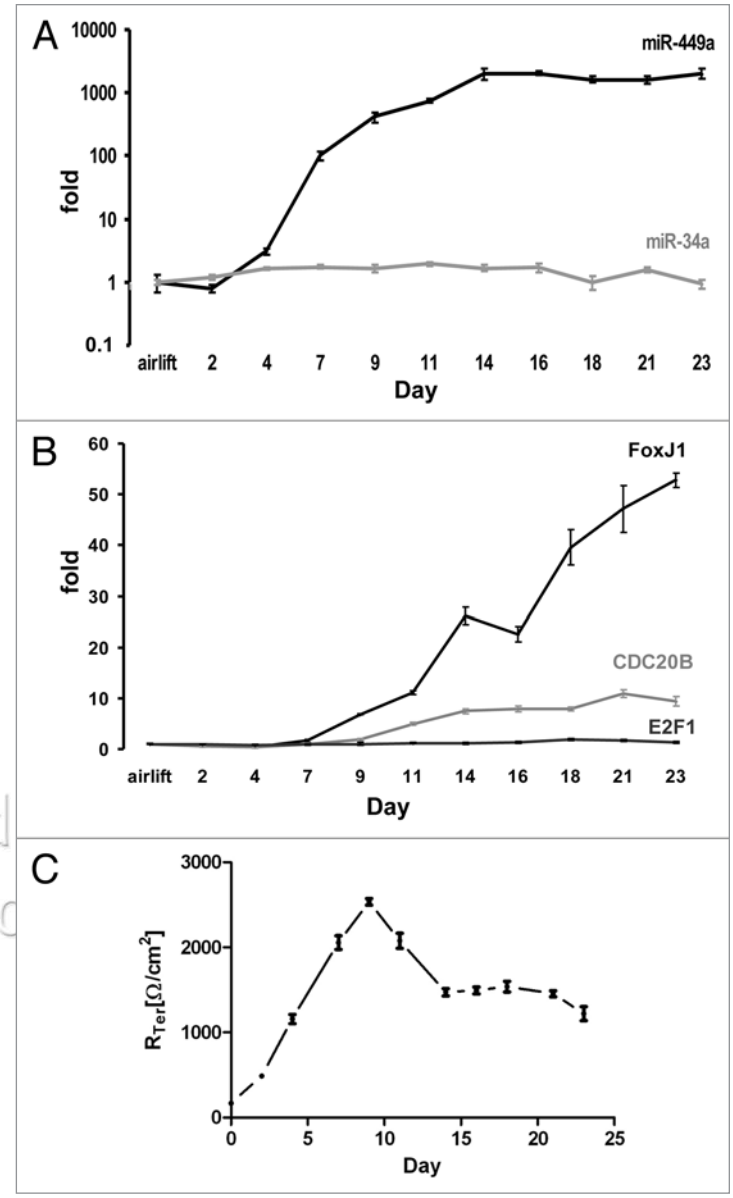

Figure 2. Strong miR-449a induction in differentiating human airway epithelial cells. Airway epithelial cells (AECs) were cultivated for three days in liquid media and lifted to the interface between the media and the air. At the indicated time points (days after airlift), samples of the cells were harvested to prepare RNA, followed by RT-PCR to quantitate specific RNA species. (A) The levels of microRNAs 34a and 449a were quantified. (B) The abundance of the indicated mRNA species was assessed. (C) The electrical resistance of the cell monolayer was measured to ensure full differentiation.

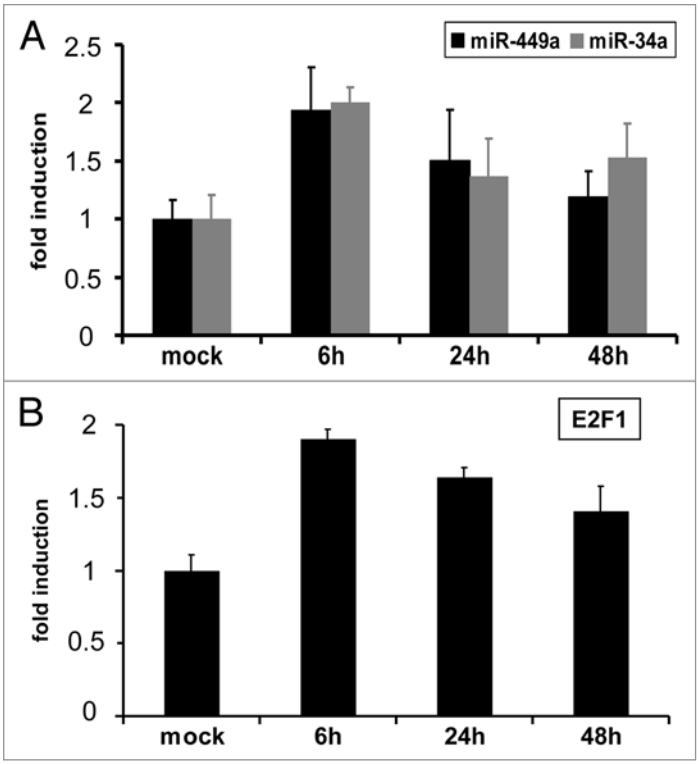

Figure 3. Further induction of miR-449a in airway epithelial cells exposed to tobacco smoke. A fully differentiated monolayer of AECs was obtained as described in the legend to Figure 2. At 21 days after airlift, the cells were exposed to cigarette smoke for 15 min (=3 cigarettes) or mock-treated, followed by RNA analysis. (A) microRNA-449a and -34a levels and (B) E2F1 mRNAs were quantitfied by RT-PCR.

function of miR-449a in mucociliary cell differentiation would be in agreement with its high expression levels shortly before and after birth, when the demand for such a differentiation program is highest. It would also correspond to the previous finding that CDC20B, the host gene of miR-449a, is upregulated upon AEC injury,[21] perhaps facilitating the regeneration of bronchial epithelia.

Besides differentiation, miR-449a is also capable of inducing apoptosis, and this activity may help miR-449a to provide a first line defence mechanism upon genotoxic stress (**Fig. 3**), virus infection[22] or asthma.[23] The further upregulation of miR-449a upon smoke exposure was moderate, but it may still contribute to the elimination of cells with damaged DNA and thus prevent the occurrence of malignancies. MiR-34a acts as a general effector of p53-induced apoptosis in a broad range of tissues.[16] In contrast, its cousin miR-449a appears to exert a comparable tumor suppressive role specifically in airway epithelia.

Materials and Methods

RNA extraction, reverse transcription (RT) and quantitative PCR (qPCR). C57BL/6 mice (B6N, n = 3 per group) were used for tissue analysis. Animals were handled in accordance with the German Animal Protection Law and with the permission of the local government.

Total RNA was isolated from AECs using the miRVana kit (Ambion/Applied Biosystems), or from murine tissues using TRIzol (Invitrogen).

For detection of mRNA, 1 μg of total RNA was incubated with primers (random nonamers and anchored oligodT primers) and M-MuLV Reverse Transcriptase at 42°C for 1 h. qPCR was then performed in the following mix: Tris-HCl (75 mM), $(NH_4)_2SO_4$ (20 mM), Tween-20 (0.01%), $MgCl_2$ (3 mM), Triton X-100 (0.25%), SYBR Green I (1:80,000), dNTPs (0.2 mM), Taq-polymerase [20 U/ml] and 250 nM of each primer. The primer set for 18S rRNA was 5'-AAC TGA GGC CAT GAT TAA GA and 5'-GGA ACT ACG ACG GTA TCT GA, for 36B4 5'-GAT TGG CTA CCC AAC TGT TG and 5'-CAG GGG CAG CAG CCA CAA A, for ATR 5'-GCC GCT CCG ATC GTG TAC and 5'-TTT GTA TGC TCT GTG ATA ACC TTG TTT, for CDC20B 5'-AAC TTT GCG AAG AGG CTGTC and 5'-TCT TTC TCA GGC GGT GTC TT, for CDK6 5'-AGA CCC AAG AAG CAG TGT GG and 5'-AAG GAG CAA GAG CAT TCA GC for E2F1 5'-CGG TGT CGT CGA CCT GAA CT and 5'-AGG ACG TTG GTG ATG TCA TAG ATG, for FoxJ1 5'-GCC CAG GAC CAG AAT CGC T and 5'-GGA AGA CGC GGA GCA ATG AAA CAC, and for mtRNR2a 5'-CAT AAG CCT GCG TCA GAT CA and 5'-CCT GTG TTG GGT TGA CAG TG.[24] mRNA expression levels were normalized to 36B4.

Stem-loop qRT-PCR for mature microRNAs was done using the TaqMan MicroRNA assays (Applied Biosystems) as described.[25,26] microRNA expression levels were normalized to U6. The standard curve method was used for the relative quantification of gene expression.

Isolation of human primary airway epithelial cells. Human primary bronchial epithelial cells (HBEC) were isolated from large airways resected during surgery and cultivated as submersed or air liquid interface (ALI) cultures as described previously.[3] Donors underwent lung transplantation due to pulmonary fibrosis. Results did not differ between cells from the donors. The protocol was approved by the ethics committee of the University of Marburg and informed consent was obtained from the patients. Briefly large airways were digested enzymatically with protease 14 (Sigma, Steinheim, Germany). The cells were expanded in airway epithelial cell growth medium supplemented with growth factors (Promocell, Heidelberg, Germany) and preserved in liquid nitrogen.

Establishment of air-liquid interface cultures and cigarette smoke exposition. HBECs were seeded in cell culture plates and grown until they reached 70–80% confluence. For the establishment of ALIs 2,5–3 x 10^5 cells/well were seeded in transwell six-well culture plates (Corning Life Science, Netherlands) in airway epithelial cell growth medium supplemented with growth factors (Promocell, Heidelberg, Germany) and 1% of a mixture of Penicillin and Streptomycin (Gibco, Paisley). The cells were incubated for three days in a standard cell culture incubator. The apical medium was removed and the basolateral medium replaced by differentiation medium (DMEM/HamF12, 1:1) containing 2% Ultroser G serum substitute (Pall Life Science,

Dreieich, Germany). The cells were regarded as fully differentiated after reaching a transepithelial resistance greater than 1,000 Ohms/cm^2 as measured by an epithelial ohmmeter (EVOM, World Precision Instruments, Sarasota, FL). For control experiments without airlift, HBECs were seeded in 6-well plates at a density of 1 x 10^5 in airway epithelial cell growth medium supplemented with growth factors (Promocell, Heidelberg, Germany).

Tissue cultures were exposed to volatile cigarette smoke (CS) as described previously.[19] Briefly, tissue cultures were exposed to CS for 15 min (=3 cigarettes). After the exposure, the medium of the cultures was replaced immediately. Control cultures were incubated in the exposure chamber for the same time period without burning a cigarette.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/LizeCC9-22-sup.pdf

References

1. Metzger RJ, Klein OD, Martin GR, Krasnow MA. The branching programme of mouse lung development. Nature 2008; 453:745-50.
2. Maeda Y, Dave V, Whitsett JA. Transcriptional control of lung morphogenesis. Physiol Rev 2007; 87:219-44.
3. Bals R, Beisswenger C, Blouquit S, Chinet T. Isolation and air-liquid interface culture of human large airway and bronchiolar epithelial cells. J Cyst Fibros 2004; 3:49-51.
4. Ross AJ, Dailey LA, Brighton LE, Devlin RB. Transcriptional profiling of mucociliary differentiation in human airway epithelial cells. Am J Respir Cell Mol Biol 2007; 37:169-85.
5. Brody SL, Yan XH, Wuerffel MK, Song SK, Shapiro SD. Ciliogenesis and left-right axis defects in forkhead factor HFH-4-null mice. Am J Respir Cell Mol Biol 2000; 23:45-51.
6. Chen J, Knowles HJ, Hebert JL, Hackett BP. Mutation of the mouse hepatocyte nuclear factor/forkhead homolog 4 gene results in an absence of cilia and random left-right asymmetry. J Clin Invest 1998; 102:1077-82.
7. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004; 5:522-31.
8. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 2007; 129:1401-14.
9. Harris KS, Zhang Z, McManus MT, Harfe BD, Sun X. Dicer function is essential for lung epithelium morphogenesis. Proc Natl Acad Sci USA 2006; 103:2208-13.
10. Bhaskaran M, Wang Y, Zhang H, Weng T, Baviskar P, Guo Y, et al. MicroRNA-127 modulates fetal lung development. Physiol Genomics 2009; 37:268-78.
11. Wang Y, Weng T, Gou D, Chen Z, Chintagari NR, Liu L. Identification of rat lung-specific microRNAs by micoRNA microarray: valuable discoveries for the facilitation of lung research. BMC Genomics 2007; 8:29.
12. Williams AE, Moschos SA, Perry MM, Barnes PJ, Lindsay MA. Maternally imprinted microRNAs are differentially expressed during mouse and human lung development. Dev Dyn 2007; 236:572-80.
13. Zhang H, Mishra A, Chintagari NR, Gou D, Liu L. Micro-RNA-375 inhibits lung surfactant secretion by altering cytoskeleton reorganization. IUBMB Life 62:78-83.
14. Dong J, Jiang G, Asmann YW, Tomaszek S, Jen J, Kislinger T, et al. MicroRNA Networks in Mouse Lung Organogenesis. PLoS One 5:10854.
15. Lu Y, Okubo T, Rawlins E, Hogan BL. Epithelial progenitor cells of the embryonic lung and the role of microRNAs in their proliferation. Proc Am Thorac Soc 2008; 5:300-4.
16. Hermeking H. p53 enters the microRNA world. Cancer Cell 2007; 12:414-8.
17. Lize M, Pilarski S, Dobbelstein M. E2F1-inducible microRNA 449a/b suppresses cell proliferation and promotes apoptosis. Cell Death Differ 17:452-8.
18. Yang X, Feng M, Jiang X, Wu Z, Li Z, Aau M, et al. miR-449a and miR-449b are direct transcriptional targets of E2F1 and negatively regulate pRb-E2F1 activity through a feedback loop by targeting CDK6 and CDC25A. Genes Dev 2009; 23:2388-93.
19. Beisswenger C, Platz J, Seifart C, Vogelmeier C, Bals R. Exposure of differentiated airway epithelial cells to volatile smoke in vitro. Respiration 2004; 71:402-9.
20. Schembri F, Sridhar S, Perdomo C, Gustafson AM, Zhang X, Ergun A, et al. MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. Proc Natl Acad Sci USA 2009; 106:2319-24.
21. Heguy A, Harvey BG, Leopold PL, Dolgalev I, Raman T, Crystal RG. Responses of the human airway epithelium transcriptome to in vivo injury. Physiol Genomics 2007; 29:139-48.
22. Wang Y, Brahmakshatriya V, Zhu H, Lupiani B, Reddy SM, Yoon BJ, et al. Identification of differentially expressed miRNAs in chicken lung and trachea with avian influenza virus infection by a deep sequencing approach. BMC Genomics 2009; 10:512.
23. Williams AE, Larner-Svensson H, Perry MM, Campbell GA, Herrick SE, Adcock IM, et al. MicroRNA expression profiling in mild asthmatic human airways and effect of corticosteroid therapy. PLoS One 2009; 4:5889.
24. Pirngruber J, Shchebet A, Schreiber L, Shema E, Minsky N, Chapman RD, et al. CDK9 directs H2B monoubiquitination and controls replication-dependent histone mRNA 3'-end processing. EMBO Rep 2009; 10:894-900.
25. Braun CJ, Zhang X, Savelyeva I, Wolff S, Moll UM, Schepeler T, et al. p53-Responsive micrornas 192 and 215 are capable of inducing cell cycle arrest. Cancer Res 2008; 68:10094-104.
26. Scheel AH, Beyer U, Agami R, Dobbelstein M. Immunofluorescence-based screening identifies germ cell associated microRNA 302 as an antagonist to p63 expression. Cell Cycle 2009; 8:1426-32.