MicroRNA-127 modulates fetal lung development

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Submitted 8 June 2008; accepted in final form 11 March 2009

Bhaskaran M, Wang Y, Zhang H, Weng T, Baviskar P, Guo Y, Gou D, Liu L. MicroRNA-127 modulates fetal lung development. Physiol Genomics 37: 268–278, 2009. First published March 17, 2009; doi:10.1152/physiolgenomics.90268.2008.—MicroRNAs (miRNAs) are small endogenous RNAs and are widely regarded as one of the most important regulators of gene expression in both plants and animals. To define the roles of miRNAs in fetal lung development, we profiled the miRNA expression pattern during lung development with a miRNA microarray. We identified 21 miRNAs that showed significant changes in expression during lung development. These miRNAs were grouped into four distinct clusters based on their expression pattern. Cluster 1 contained miRNAs whose expression increased as development progressed, while clusters 2 and 3 showed the opposite trend of expression. miRNAs in cluster 4 including miR-127 (miR-127) had the highest expression at the late stage of fetal lung development. Quantitative real-time PCR validated the microarray results of six selected miRNAs. In situ hybridization demonstrated that miR-127 expression gradually shifted from mesenchymal cells to epithelial cells as development progressed. Overexpression of miR-127 in fetal lung organ culture significantly decreased expression of miRNAs that were significantly changed during this process. Studies have suggested the important roles of miRNAs in lung cancer. It has been found that the decrease of let-7 expression is correlated with an increased death rate in patients with lung cancers (48). The expression of miRNAs in lung cancers has already been profiled. The results demonstrated the correlation of miRNA expression with the prognosis of lung adenocarcinoma patients (61). Studies on expression of important molecules in miRNA processing, namely, members of the Argonaute (Ago) gene family, in the embryonic day (E)11.5 lung have shown that Ago1 and Ago2 are enriched in branching regions, suggesting that miRNAs may play important roles in lung development (25). Inactivation of Dicer, a key component in miRNA processing, was found to cause the inhibition of lung epithelial branching (13). It was reported recently that transgenic overexpression of the miR-17-92 cluster results in the promotion of proliferation and the inhibition of differentiation of epithelial progenitor cells in developing lungs (27).

Rat lung development can be divided into five stages (4, 64). In the first 13 days, lobar division takes place. This is called the embryonic phase. Following the embryonic phase is the glandular or pseudoglandular phase (13–18 days), in which epithelial tubes of air passages are formed but have little or no lumen. In the canalicular phase (18–20 days), bronchioles are produced and a lumen can be recognized in many tubules. With a further thinning of the interstitium and a flattening of the epithelium, alveolar ducts and air sacs are formed in the saccular phase (20 days to full term). Some epithelial cells begin to synthesize and secret pulmonary surfactant. The final stage happens after birth and is termed the alveolar phase, in which true alveoli are formed.

Each of the five developmental stages is coordinated by a multitude of signaling molecules and pathways (54). Some of the well-studied signaling molecules include fibroblast growth factors, transforming growth factors (TGFs), retinoids, Wnt genes, and Sonic hedgehog (34, 36, 52, 55). However, little is known about what the role of miRNAs is in this process and how they regulate lung development by modulating these signaling pathways. In addition, the temporal and spatial expression patterns of miRNAs in rat lung development are still not known.

In this study, we used a miRNA microarray platform developed in our laboratory (53) to profile the expression of miRNAs at different stages in rat lung development. There were 21 miRNAs that were significantly changed during this process. Some of these miRNAs were selected and validated by real-
time PCR. The spatial expression patterns of selected miRNAs were determined by in situ hybridization. We selected miR-127 for further study based on its expression pattern. miR-127 overexpression in an fetal lung organ culture at an earlier stage resulted in lesser and defective terminal bud formation and uneven development of the lung. These results demonstrate a critical role of miR-127 in fetal lung development.

**MATERIALS AND METHODS**

Isolation of RNA from rat lungs. Whole lungs were isolated from rat fetuses on gestational days 16, 19, and 21 (E16, E19, and E21) and from newborn, 6-day-old, 14-day-old [postnatal day (P)0, P6, and P14], and 2-mo-old adult (AD) rats. For each time point, there were three independent biological replicates (each from different rats). All procedures in this study followed the protocols approved by the Oklahoma State University Animal Care and Use committee. For the fetal lungs, pregnant Sprague-Dawley rats were killed with CO₂. Fetuses were removed from the uterus, and the lungs were isolated from these fetuses. For pup and adult lungs, male Sprague-Dawley rats were anesthetized before death and isolation of the lungs. Immediately after isolation, the lungs were rinsed with DMEM and then homogenized in the Lysis/Binding Buffer from the mirVana miRNA Isolation Kit (Ambion, Austin, TX). Enriched small RNA and total RNA from the lungs were isolated with the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s instructions.

miRNA microarrays. The miRNA microarrays were performed on an in-house platform developed in our laboratory as described previously (53). There are three identical blocks on each slide and six identical probes for each miRNA in each block. The NCode miRNA Labeling System (Invitrogen, Carlsbad, CA) was used to generate labeled miRNA molecules for hybridization to the microarrays. Six hundred nanograms of RNA was used in each labeling reaction. One-fifth of the labeled RNA was used for each hybridization. Small RNAs from the lungs at certain time points were cohybridized with the common reference. All the lung samples. To each block, one labeled sample (Alexa Fluor 3 or Alexa Fluor 5) was cohybridized with the common reference labeled with the other dye (Alexa Fluor 5 or Alexa Fluor 3). Dye swaps were performed to eliminate dye bias. The hybridized slides were scanned with ScanArray Express (PerkinElmer Life and Analytical Sciences, Boston, MA), and the images were analyzed with GenePix 5.0 pro (Axon Instruments, Union City, CA). The data were calculated, normalized, and qualified as described previously (53). The signal from each spot was normalized to the average signal of the whole block. The highest and lowest signals from the six identical probes in the same block were excluded from further analysis. The geometric average of the other four signals was considered to be the signal of that particular miRNA. The quality of the signals was assessed with RealSpot software. The miRNAs with an average quality index (QI) < 1 were filtered. The qualified data were then analyzed with Significant Analysis of Microarrays software (SAM; Stanford University, http://www-stat.stanford.edu/~tibs/SAM/) (multiclass response), and any miRNAs significantly changed between any two time points were picked up (q < 0.05). The miRNAs that passed the SAM test were clustered and viewed with Cluster (K-means clustering) and TreeView software (http://rana.lbl.gov/EisenSoftware.htm).

Quantitative real-time PCR for miRNA. Quantitative real-time PCR (qRT-PCR) for miR-18, miR-20a, miR-29a, and miR-351 was performed with the mirVana qRT-PCR miRNA Detection Kit (Ambion) and that for miR-195 and miR-351 was performed with TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA) per the company’s protocols. In brief, total RNA was isolated from rat lungs at different time points of development (E16, E19, E21, P0, P6, P14, and AD) with the mirVana miRNA Isolation Kit (Ambion). TURBO DNA-free (Ambion) was used to remove DNA contamination. Three biological replicates were performed at each time point. For qRT-PCR using the mirVana qRT-PCR miRNA Detection Kit, 50 ng of RNA was used in each reverse transcription reaction with miRNA specific mirVana reverse transcription (RT) primers. Duplicate RT reactions were performed for RNA from each biological replicate and no template controls. The reactions were incubated in 96-well plates at 37°C for 30 min and then at 95°C for 10 min. PCR Master Mix (15 µl) was added to each RT reaction. qRT-PCR was performed on an Applied Biosystems 7500 Real-Time PCR System. The reactions were incubated at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Dissociation analysis was performed after amplification to identify the characteristic peak for the specific PCR product. The threshold cycle (CT) was determined for each miRNA. RT and PCR for U6 snRNA were also performed in each plate as an endogenous control. The comparative CT method was used, and the relative amount of each miRNA to U6 snRNA was calculated with the equation 2^-ΔΔCT. For qRT-PCR with TaqMan MicroRNA Assays, 75 ng of total RNA was used as template in each RT reaction with the miRNA-specific RT primers. The reactions were incubated on ice for 5 min, followed by 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. For each PCR reaction, 1.33 µl of RT product was used as a template. The PCR reaction was incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. All PCR reactions were run in duplicate. RT and PCR for 18S in each sample were also performed as an endogenous control with TaqMan Ribosomal RNA Control Reagents (Applied Biosystems). Data analysis was done as described above. For miR-136 and miR-337, we adopted the method of Shi and Chiang (43), with a few modifications. Briefly, 5 µg of total RNA was polyadenylated with poly(A) polymerase (Ambion) at 37°C for 1 h. RT was performed with a poly(T) adaptor, GCCGACGACGAATTTAACGACTCACTATAGG TTTTTTTTTTTT. Real-time PCR was performed with a universal reverse primer, GCCGACGACGAATTTAACGACTC, and a forward primer with the same sequence as the mature miRNA.

In situ hybridization for miRNA. In situ hybridization for miRNA was done with 5’ DIG-labeled LNA probes (Exiqon, Woburn, MA). Paraffin-embedded rat lung tissues were dewaxed in xylene and rehydrated in descending grades of alcohol. The slides were then washed in PBS (pH 7.5) and permeabilized by incubating for 10 min in proteinase K (Ambion) for 5 min at 37°C. The slides were again washed in 0.2% glycine, fixed in 4% paraformaldehyde, rinsed in PBS, and prehybridized in hybridization buffer (50% formamide, 5× SSC, 0.1% Tween 20, 9.2 mM citric acid, 50 µg/ml heparin, and 500 µg/ml yeast RNA, pH 6) in a humidified chamber. The 5’ DIG-labeled LNA probes were then added to the sections at a 20 nM concentration and incubated overnight at the hybridization temperature [21°C lower than the melting temperature (Tm) values of the specific probes]. The slides were rinsed in 2× SSC and washed three times for 30 min in 50% formamide, 2× SSC solution at the same hybridization temperature. This was followed by blocking with 2% sheep serum, 2 mg/ml BSA in PBS + 0.1% Tween 20 (PBST) and incubation with anti-DIG-AP Fab fragments antibody (1:2,000) (Roche Applied Sciences, Indianapolis, IN) overnight at 4°C in a humidified chamber. After washing in PBST and AP buffer (in mM: 100 Tris-HCl, pH 9.5, 50 MgCl₂, and 100 NaCl, with 0.1% Tween 20), the color reaction was carried out by incubation in 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) color solution (Roche Applied Sciences, Indianapolis, IN) with 1 mM levamisole for 6–24 h at room temperature. The color reaction was stopped after observation of sufficient development of blue precipitate by washing with PBST. The slides were then mounted, coverslipped, and observed under a Nikon E-600 microscope.

Construction of miR-127 overexpression adenoviral vector. Rat miR-127 with the flanking sequences (~200 base pairs at each end) was amplified from rat genomic DNA with the primers 5’-CCCT-GTGCACCTGAGAACTCCAG-3’ and 5’-AGAATTCTTTAGG-
CATTAAGTGCGCTCCAGACCCC-3' and digested by Sall and EcoRI digestion. The digested PCR product was cloned into a modified pENTR/CMV-EGFP vector (12) between enhanced green fluorescent protein (EGFP) stop codon and SV40 poly(A) terminal sequences through XhoI and EcoRI restriction sites. Cytomegalovirus (CMV)-driven EGFP was included in the vector for the purpose of monitoring transfection efficiency. The empty vector of the CMV-driven EGFP was used as a vector control. The CMV-EGFP-miRNA in the pENTR vector was moved into an adenoviral vector by the Gateway technique (Invitrogen). Obtained adenoviral vectors were linearized by PacI and used to transfect HEK293A cells. Adenovirus was amplified by reinfecting HEK293A cells. Titer of virus was determined by making a series of dilutions of viral stock, infecting HEK293A cells, and counting for virus-infected cells and is expressed as plaque-forming units (PFU) per milliliter.

Overexpression of miR-127 in fetal lung organ culture. E14 embryos were dissected from timed pregnant Sprague-Dawley rats. Fetal lungs were isolated from each fetus by removing the surrounding tissues with 21- to 24-gauge needles and placed in Hanks' balanced salt solution (HBSS). They were then cultured on 0.4-μm-pore size culture inserts (Millipore, Billerica, MA) and placed in six-well tissue culture plates for 2 days. Each well contained 1.5 ml of serum-free chemically defined BGJb medium (Fitton-Jackson modification; Invitrogen) with 0.2 mg/ml ascorbic acid, 50 U/ml penicillin, and 50 μg/ml streptomycin. The day of isolation (E14) was denoted as day (D)0, and the next 2 days of culture were denoted as D1 and D2, respectively. The adenoviruses containing EGFP and miRNA overexpression sequence or empty vector with EGFP only (virus control) were added to the inserts on D0 at a dose of 4.7 × 10^7 PFU/fetal lung. As a blank control, an equal amount of DMEM was added on the fetal lung. Excess liquid, if any, in the insert was removed with a pipette after 3 h. Half of the initial dose of virus was added on D1 to maintain the overexpression as tissue mass increased. The lung was photographed on each day with a digital camera mounted onto a Nikon-E 600 microscope at the same magnification for every lung.

Morphometric analysis of lung. The morphometric analyses of fetal lungs were done on the images taken on D0, D1, and D2 at the same magnification with MetaVue software (Molecular Devices, Downingtown, PA). The images were coded, and analyses were performed in a blinded manner by two investigators. The number of terminal buds was counted by enlarging the images on the MetaVue software and counting those buds that were at the periphery of the lung. The number of terminal buds for each lung was divided by the number of terminal buds present at the time of isolation as a normalization procedure. The terminal and internal bud sizes were measured with the software at the longest diameter for each bud. The average bud sizes were calculated by randomly selecting at least 30 internal or terminal buds from each lung and calculating the average for each lung. Data were pooled from all lungs that received the same treatment. The data from at least 10 lungs obtained from fetuses of 3 different dams were used to measure each parameter.

Statistical analysis. Statistical analysis of microarray data was done as described previously (53). One-way ANOVA was performed for the real-time PCR study pertaining to miR-127 overexpression in fetal lungs, followed by Dunnett’s multiple-comparison test for comparison between individual treatments. For all other studies, a paired t-test was done between the virus control and miR-127 overexpression groups. A P value of < 0.05 was considered significant. All values are presented as means ± SE.

RESULTS

miRNA expression profile during lung development. To detect the miRNA expression profile during rat lung development, we used the miRNA microarray platform developed in our laboratory (53). The microarray contained probes for 227 nonredundant RNAs: 177 rat miRNAs, 5 human miRNAs, 31 mouse miRNAs, and 14 other kinds of RNAs and controls. Small RNAs of rat lungs from seven time points of lung development (E16, E19, E21, P0, P6, P14, and AD) were cohybridized onto the slides with the common reference, which consisted of equal amounts of enriched small RNAs from each time point. Three biological replicates and dye swaps were performed for each sample. One hundred seven miRNAs passed the quality test by RealSpot, with an average QI > 1 (53). Statistical analysis was performed with SAM. Twenty-one miRNAs were shown to have significant changes between at least one pair of the seven time points (q < 0.05). To identify miRNAs with similar expression patterns, these significantly changed miRNAs were then grouped into four clusters with Cluster software by K-means clustering (Fig. 1). The number of clusters was chosen to best reflect different expression patterns after comparing different numbers of clusters. Cluster 1 included let-7b, miR-29a, miR-23a, miR-22, and miR-195. Cluster 2 included miR-298, miR-341, miR-130b, and miR-92. Cluster 3 included miR-17, miR-214, miR-106b, miR-93, miR-290, miR-20a, miR-17-5p, and miR-18. Cluster 4 consisted of miR-127, miR-210, miR-19b, and miR-351. The
expression patterns of the identified miRNAs are shown in the line charts in Fig. 2. In cluster 1, the miRNA expression increased gradually from fetal to adult lungs (E16 to AD). The miRNAs in clusters 2 and 3 decreased from E16 to AD, a large part of which markedly decreased from E16 to E19. In cluster 4, all the miRNAs peaked at some point between E16 and P6. For example, miR-127 reached maximum on E21 and miR-351 reached maximum on E19.

Real-time PCR validation of microarray results. We wanted to validate our microarray results with a more sensitive and quantitative method. qRT-PCR was done to confirm the trends of expression exhibited by miRNAs from each of three clusters. We chose miRNAs from clusters 1, 3, and 4 because the expression of cluster 2 resembled cluster 3 in the general trend. We chose two miRNAs from each cluster based on high fold changes, their expression in lungs, and functional studies in other systems. miR-29a and miR-195 (Fig. 3, A and B) were chosen to represent cluster 1, while miR-18 and miR-20a (Fig. 3, C and D) represented cluster 3 and miR-127 and miR-351 (Fig. 3, E and F) represented cluster 4. All of these miRNAs followed the same trend of expression as seen in the microarray experiment, thus validating our microarray platform.

The expression profiles of miRNAs miR-136 and miR-337, other members of the miR-127 family, were also examined. qRT-PCR demonstrated that miR-136 and miR-337 had the same expression patterns as miR-127 (Fig. 3, G and H).

Cellular localization of miRNAs. In situ hybridization using 5' DIG-labeled LNA probes was done to determine the spatial expression pattern of selected miRNAs in tissue sections from different stages of lung development. We chose miR-20a for in situ hybridization because it is a member of the miR-17-92 cluster, which has been shown to be important in fetal lung development. The selection of miR-127 and miR-351 was because of their high expression at the late stage of fetal lung development and our interests in this stage of lung development. A U6 probe was used as a positive control. Positive signals were observed in nuclei of all the cells on P0 sections (Fig. 4A). miR-20a expression followed the trend of expression consistent with the microarray and qRT-PCR data (Fig. 4A). Expression was seen on E16 and not in other stages of development. Mesenchymal and epithelial cells were identified by immunostaining with pancytokeratin (epithelial cell marker) and vimentin (mesenchymal marker) on lung tissue sections adjacent to those used for in situ hybridization (data not shown). The signal on E16 was confined mainly to cells of mesenchymal origin in the interstitium, although some staining of epithelial cells was also noted. A probe that contained a scrambled sequence and had no known miRNA targets was used as a negative control. No signals were detected on E16 sections (Fig. 4A). The general trend of miR-127 expression for in situ hybridization and real-time PCR is similar, although the microarray data at E19 are less consistent with the in situ hybridization (Fig. 4B). E19 sections showed miR-127 expression in both epithelial and mesenchymal cells, but more in mesenchymal cells. In E21 sections, the expression shifted more toward the epithelial regions lining the airways. P0 lungs showed weaker signal intensity than E21, but the trend of expression was the same as on E21. Adult lung sections did not give any signals for miR-127. There was no signal from the lining of blood vessels (data not shown).

The miR-351 expression pattern also corroborated the qRT-PCR and microarray data (Fig. 4C). E16 sections showed miR-351 expression in both epithelial and mesenchymal cells, but more in epithelial cells lining the future terminal airways and alveoli. Expression was highest at the E19 stage, when miR-351 was seen both in epithelial cells and mesenchymal cells. The expression was strongest in the epithelial cells lining the terminal bronchioles (Fig. 4C), while it was absent in the...
lining of blood vessels. E21 showed the same pattern of expression, although much weaker than E19. Also, the expression shifted more toward epithelial cells lining the alveoli than mesenchymal cells. The weakening of the signal continued to the P0 stage, and signal was seen mainly in epithelial cells lining the alveoli and terminal air spaces. In adult tissue sections, the signal was exclusive and specific in alveolar type II cells and could not be detected in other cells. In all stages, no signal was detected from the lining of blood vessels (data not shown).

Effect of miR-127 overexpression on fetal lung development. miR-127 was selected for further functional studies because of its interesting expression trend. miR-127 was expressed at E19, E21, and P0, the period immediately before birth, and the period directly after birth. We decided to overexpress miR-127 at an earlier stage of development (E14) in an in vitro fetal lung culture model to see whether it causes any changes of fetal lung development. An adenoviral vector that overexpressed miR-127 was used to transduce E14 fetal lungs cultured for 2 days as described in MATERIALS AND METHODS. miR-127 overexpression and its effect on lung branching morphogenesis were visualized on each day (Fig. 5A).

Fig. 3. Validation of miRNA microarray results by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from fetal lungs at E16, E19 and E21 and lungs at P0, P6, P14, and AD. miRNA levels were measured by real-time PCR. Relative expression levels of miR-29a (A), miR-195 (B), miR-18 (C), miR-20a (D), miR-127 (E), miR-351 (F), miR-136 (G), and miR-337 (H) are expressed as % of maximum expression. Error bars represent SE; n = 3 independent preparations, each assay performed in duplicate. Microarray data shown are averages from 6 replicates.
Fig. 4. In situ hybridization for miRNAs. In situ hybridization was carried out in dewaxed and rehydrated fetal rat lung tissue sections at E16, E19 and E21 and lungs at P0 and AD. The sections were hybridized with 5’ DIG-labeled LNA probes against miR-20 (A), miR-127 (B), and miR-351 (C). A probe with scrambled sequence unrelated to known miRNAs was used as a negative control (Neg), and a probe for U6 was used as a positive control (Pos). Positive signals were visualized as dark blue/purple color. Arrowheads denote signals from epithelial cells; arrows denote signals from mesenchymal cells; insets represent enlarged images. Scalebars, 40 μm.
in a much narrower range (20 – 80 relative units) (Fig. 5). The blank control or blank control showed a size variation that fluctuated 10 and 180 relative units, while those treated with virus showed a size variation that fluctuated unevenly between terminal bud sizes. The sizes of the terminal buds from the lungs of 10 fetal lungs per treatment in ascending order of their individual sizes. The sizes of the terminal buds was also plotted on a graph by arranging the various terminal bud sizes from at least 250 terminal buds from 10 fetal lungs per treatment in ascending order of their individual sizes. The sizes of the terminal buds from the lungs treated with miR-127 seemed to fluctuate unevenly between almost 10 and 180 relative units, while those treated with virus control or blank control showed a size variation that fluctuated in a much narrower range (20 – 80 relative units) (Fig. 5C). The average internal bud size was higher for miR-127, as in the case of terminal buds compared with controls (Fig. 5D). We defined internal buds as those buds that were not at the periphery of the developing lung. They were enclosed and not tubular, and they budded out from the secondary tubular formations in the developing lung. The terminal bud count, another important indicator of proper lung branching, showed that miR-127 overexpression decreased the number of terminal buds (Fig. 5E). Overall, miR-127 overexpression resulted in larger terminal buds, yet in lesser numbers, that showed a high amount of variability between bud sizes, and this trend was reflected in internal buds, too. These results clearly demonstrate that miR-127, if overexpressed at an early stage, causes defective lung branching morphogenesis.

**DISCUSSION**

In the present work, we described the miRNA expression profile during lung development and identified four clusters of miRNAs that showed specific trends of expression. Expression levels of 21 miRNAs were found to be significantly changed during the course of lung development. The miRNA microarray results were validated by qRT-PCR analysis and in situ hybridization of the selected miRNAs. The overexpression of miR-127 in a fetal lung organ culture system caused defective lung development characterized by decreased terminal bud counts and varied bud sizes.

miRNAs have rapidly emerged as one of the key regulatory molecules that control various biological processes ranging from development to disease. Various miRNAs have been implicated in regulating developmental timing and controlling left/right neuronal asymmetry in *Caenorhabditis elegans* (18, 37), insulin secretion (35), lipid metabolism (8), modulating proliferation and apoptosis (3, 7), stem cell division (14, 42), and B-cell differentiation (60). Involvement of miRNAs in progression of various cancers has been extensively investigated (2, 17, 28, 46, 48, 61), but studies on their role in the physiology of the lung have been very limited. Some important proteins involved in miRNA processing, such as Ago1, Ago2, and Dicer, have been shown to be important to lung morphogenesis (13, 25). These discoveries suggest the importance of miRNAs in the lung. Our previous study (53) showed that two miRNAs, namely, miR-195 and miR-200c, are specifically expressed in the lung. It has been shown that the expression levels of some miRNAs are changed after lipopolysaccharide-induced inflammation, and miR-146a can regulate the inflammatory response in lung alveolar epithelial cells (30, 33).

Williams et al. (56) compared miRNA expression between fetal (pooled from 18 – 29 wk) and adult (2 time points: 1 fetal and 1 adult) human lungs with real-time PCR. They found that 13 miRNAs were upregulated in human fetal lungs and 8 miRNAs in human adult lungs. These authors also performed similar studies in newborn and adult mouse lungs (3 postnatal time points: 1-, 14-, and 60-day-old mice). Fourteen miRNAs were expressed higher in the mouse neonatal lungs and thirty miRNAs in the mouse adult lungs. In our studies, we mainly focused on the dynamic changes in miRNA expression because rat fetal lung develops with selected time points that pertain to different stages of development (E16, pseudoglandular; E19, canalicular; E21, saccular; P0, P6, and P14, alveolar; and AD). The changes of let-7b, miR-23a, miR-29a, and miR-195 in mouse lungs and miR-214 and miR-29a in human lungs were constant and more even GFP expression than a single treatment on D0. The same treatment regimen was followed for both virus control and blank control. The overexpression was confirmed by real-time PCR (Fig. 5B). It was seen that there was a significant increase in miR-127 overexpression on D1 and D2. An increase in the endogenous expression of miR-127 was also noted as the culture progressed. miR-127 overexpression adenovirus or virus control (VC) was added to the culture on day 0 (D0), and culture continued for 2 days (D1 and D2). The blank control (BC) was treated with medium alone. Images and green fluorescent protein (GFP) fluorescence were taken at D0 – D2. Each treatment was carried out in at least 10 lungs from 3 different mothers in 3 separate experiments. B: qRT-PCR to quantify overexpression of miR-127. Total RNAs from D1 and D2 of the fetal lung culture were isolated. miR-127 levels were measured by qRT-PCR. Error bars represent SE; n = 3 independent preparations, each assay performed in duplicate. *P < 0.05 vs. VC, **P ≤ 0.02 vs. VC.
similar to these in rat lungs from our present studies. In addition to the miRNAs that increase or decrease from early lung development to adult, we also identified a miRNA cluster in which their expression was the highest in the later stage of fetal lung development compared with early stage of fetal lung development and adult lungs.

The first cluster we identified included miR-29a and miR-195. Their expression remained low during all stages of fetal lung development and was high in adult lung. The higher expression of miR-29a in both mouse and human adult lungs and miR-195 in adult mouse lungs compared with fetal or newborn stages have been observed previously (27, 56). miR-29a showed a similar trend of expression during development of the brain, i.e., low in embryonic brain tissue and high in adult cortex and striatum (20). Overexpression of the miR-29 family in lung cancer cell lines has been shown to inhibit tumorigenicity both in vitro and in vivo (9). miR-195, on the other hand, has been identified as a key regulator of cardiac growth and function. The overexpression of miR-195 in cardiomyocytes led to abnormal cardiac remodeling and heart failure (49). Its low expression during lung development may be an important factor that helps in the controlled proliferation and differentiation of cells in the lungs. This cluster also contained let-7b, a member of the let-7 family known to regulate developmental timing in Drosophila (32).

Clusters 2 and 3 contained miRNAs whose expression decreased as development progressed. Interestingly, cluster 3 contained miR-17-5p, miR-18, and miR-20a, all of which are encoded by the miR-17-92 cluster, a conserved gene that encodes seven miRNAs. A recent study in mouse embryonic lung development has shown a similar trend of expression for these three miRNAs from E11.5 to adult lungs (27). Our in situ hybridization of miR-20a indicated its expression mainly in the mesenchymal region at E16. The expression rapidly disappeared as development progressed. Analysis of predicted targets of the miR-17-92 cluster showed that almost 58% of their predicted targets were transcription factors, regulators of nucleotide or nucleic acid metabolism or cellular protein metabolism, all of which are key features in driving the developmental process in the right direction.

The miR-17-92 cluster has been found to be overexpressed in lung cancers and has been demonstrated to promote prolif-
eration and inhibit differentiation of lung epithelial progenitor cells (15, 26, 27, 29). This cluster has also been demonstrated to influence the translation of the E2F family of transcription factors, which are important in regulation of the cell cycle and apoptosis (31). A recent study has shown that miR-20a has an important role in the regulation of E2F2 and E2F3 expression (47). The same study found that E2F1, E2F2, and E2F3 could bind directly to the promoter of the miR-17-92 cluster and act as an autoregulatory feed loop mechanism. miR-20a also seemed to have an antiapoptotic role in this study, in which its overexpression decreased apoptosis in a prostate cancer cell line and its inhibition caused increased cell death. Another group found that E2F3 was the primary E2F family member that bound to the promoter of miR-17-92. They have proposed that the miR-17-92 cluster is also pro-proliferative because it shifts the transcriptional balance more toward the proliferative E2F3 network than the proapoptotic E2F1 network (57). The antiapoptotic role of miR-20a and miR-17-5p was further confirmed in another study in which their inhibition caused increased apoptosis in lung cancer cells (29). These studies have concentrated on the effect of miRNAs on cancer cell lines. Their role in affecting the expression of E2F factors in normal cells of a developing organ has not been studied. Since controlled cell death, proliferation, and differentiation go hand in hand in the development of any organ system and since these miRNAs seem to regulate these processes, we believe that they have a critical role in regulating lung development as well. This view is strengthened by another study in which the members of the miR-17-92 family were also implicated in the promotion of adipocyte differentiation by negatively regulating Rb2/p130, the retinoblastoma genes that also interact with E2F transcription factors (51).

Cluster 4 contained miR-127 and miR-351, which showed the highest expression just before and after birth in the sacculo-alveolar stage. Many dramatic events including differentiation of alveolar epithelial type I and type II cells, the initiation of formation of alveoli, and progressive decrease in the interstitial tissue occur in these stages (4, 38). The other members of this miRNA cluster in the rat genome including miR-136 and miR-337 had the same expression patterns as miR-127, indicating that these miRNAs may be under the transcriptional control of the same promoter and transcription factors. In situ hybridization showed that both miR-127 and miR-351 tend to shift from the mesenchymal compartment of the developing lung to the epithelial cells, which may indicate a role for these miRNAs in the cellular reorganization process and differentiation of alveolar epithelial cells or mesenchymal to epithelial transition.

miR-127 is embedded in a CpG island and remains methylated in most tissues except sperm. It shows an imprinted expression in the mouse (40, 41). The functional studies on miR-127 so far have identified it as a potential tumor suppressor whose expression goes down in cancer cell lines and in a significant number of primary tumors (39). With treatment with chromatin-modifying drugs miR-127 expression was upregulated, and this, in turn, inhibited the expression of its target, the protooncogene BCL6. Modulation in the miR-127 expression pattern in the context of organ development has not yet been reported. The overexpression of miR-127 in E14 fetal lung cultures significantly affected normal branching and terminal bud formation, indicating its role in fetal lung development.

miR-127 expression was lower in the early than the late stage of fetal lung development. We chose to overexpress it at an earlier stage. The rationale for our approach was twofold: 1) if miR-127 differential expression is important in lung development, overexpressing it at a stage where it is supposed to be expressed less should alter the lung development process and 2) during the later stage of fetal lung development, the branching in the in vitro fetal lung organ culture becomes so complicated that it is almost impossible to count the individual branches and to perform morphometric analysis. Thus examining the effects of the reduction of miR-127 at the later stage of development on lung development is not practical with in vitro organ culture. However, it is noteworthy that the present approach has its limitation, i.e., the overexpression of miR-127 at an earlier stage does not define specific roles of miR-127 upregulation at the later stage of fetal lung development.

Together, our results have demonstrated the reliability of a miRNA microarray platform to identify the miRNA profile during fetal lung development. We have also confirmed the expression profile and localization of selected miRNAs and have demonstrated that miR-127 overexpression results in defective fetal lung development. Since miRNAs are believed to have multiple targets and because there are many signaling pathways that are involved in the lung development process, it is likely that the miRNAs regulate multiple mechanisms of control of lung development.

**REFERENCES**


38. Wang Q, Li YC, Wang J, Kong J, Qi Y, Quigg RJ, Li X. miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tu...


