THE FUNCTIONS OF MICRORNAS IN LUNG INJURY AND DEVELOPMENT

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2004

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2011

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AND DEVELOPMENT

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ABBREVIATIONS

3' UTR	3’ untranslated regions
AAV	adeno-associated virus
ABC	activated β-catenin
AD	Alzheimer’s disease
ADCY9	adenylate cyclase 9
AEC I	alveolar epithelial type 1 cells
AEC II	alveolar epithelial type 2 cells
AGO	argonaute
AKT2	v-akt murine thymoma viral oncogene homolog 2
ALI	acute lung injury
AP	alkaline phosphatase
APC	adenomatous polyposis coli
ARDS	acute respiratory distress syndrome
ARE	AU-rich element
AT1R	angiotensin II type I receptor
Aβ	amyloid beta-peptide
BACE	beta-site APP-cleaving enzyme
BASC	bronchioalveolar stem cell
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bHLH	basic helix-loop-helix
BMSC	bone marrow-derived stem cell
CCND1	cyclin D1
CCSP	Clara cell secretory protein
CF	cystic fibrosis
CFH	complement factor H
ChIP	immunoprecipitation
CK2α	Casein kinase 1 alpha
CLL	chronic lymphocytic leukemia
CMV	cytomegalovirus
COPD	chronic obstructive pulmonary disease

CoREST	REST corepressor 1
CSNK2A1	Casein kinase II, alpha 1 polypeptide
CT	threshold cycle
CTGF	connective tissue growth factor
DCL1	Dicer-like protein-1
Dll	delta-like
dsRBD	dsRNA binding domain
DUF283	domain of unknown function 283
DVL	dishevelled
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
FBS	fetal bovine serum
Fgf	fibroblast growth factor
Fgfr	Fgf receptor
FMR1	fragile X mental retardation protein
Foxa2	forkhead box A2
FZD	frizzled
FVC	forced vital capacity
GRB2	growth factor receptor bound protein 2
GRα	glucocorticoid receptor
GSK3	glycogen synthase kinase 3
HBSS	Hanks' balanced salt solution
HCC	hepatocellular carcinoma
HD	Huntington’s disease
HDAC4	histone deacetylase 4
HGF	hepatocyte growth factor
Hh	hedgehog
HOXD10	homeobox D10
HSD	honestly significant difference
Htt	Huntingtin
IL-8	interleukin-8
IPF	idiopathic pulmonary fibrosis
JAK2	Janus kinase 2
KGF	keratinocyte growth factor
LNA	locked-nucleic-acid
LRP5/6	low-density lipoprotein receptor related protein 5/6
MAPK	mitogen-activated protein kinase
MIF	migration inhibitory factor
MLCK	myosin light chain kinase
MMP	matrix metalloproteinases
MOI	multiplicity of infection

Mtpn	myotrophin
NAFLD	non-alcoholic fatty liver disease
NBT	nitro blue tetrazolium
NF-1	nuclear factor-1
NFIB	nuclear factor I/B
NRAS	neuroblastoma ras oncogene
NVC	non-viral control
OB	oligonucleotide/oligosaccharide-binding fold
OSI	organ specificity index
PAI-1	plasminogen activator inhibitor-1
PAS	periodic acid-Schiff staining
PAZ	Piwi-Argonaute-Zwille
PBST	phosphate buffered saline with Tween 20
PD	Parkinson's disease
PDK1	3'phosphoinositide-dependent protein kinase-1
PFU	plaque-forming units
PFV-1	primate foamy virus type I
PIK3C2A	phosphoinositide-3-kinase, class 2, α polypeptide
PMA	phorbol 12-myristate 13-acetate
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTBP1	polypyrimidine tract binding protein 1
QI	quality index
RA	retinoic acid
RAGE	advanced glycosylation end product-specific receptor
RAR	retinoic acid receptor
RISC	RNA-induced silencing complex
ROS	reactive oxygen species
RT	reverse transcription
SAM	significant analysis of microarray
SCP1	small C-terminal domain phosphatase 1
siRNA	short interfering RNA
Smo	sommthened
snoRNAs	small nucleolar RNAs
SNPs	single nucleotide polymorphisms
SOS1	son of sevenless homolog 1
SP	surfactant protein
SRF	serum response factor
TBS-T	Tris-buffered saline and Tween 20
Tbx	T-box transcription factor
TCF/LEF	T cell factor/lymphoid-enhancing factor
TGF	transforming growth factor
TH2	T helper-2

TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
TRBP	human immunodeficiency virus transactivating response RNA-binding protein
TTF-1	thyroid transcription factor-1
VC	virus control
VIG	Vasa intronic gene
Wg	wingless
WIF-1	Wnt inhibitor factor-1
YAP	yes-associated protein
α-SMA	α-smooth muscle actin
β-TRCP	beta-transducin repeat containing

CHAPTER I

INTRODUCTION

1.1 MicroRNAs

MicroRNAs (miRNAs) are ~22 nucleotide (nt) non-coding RNAs that participate in gene regulation. MicroRNAs confer their regulation at a post-transcriptional level. Thousands of identified mature microRNAs with highly conserved sequences exist in species ranging from plants to humans, suggesting that they are ancient players in gene regulation. Approximately one percent of the human genome is dedicated to microRNAs. According to a study *in silico*, more than half of the mammalian mRNAs are under selective pressure and are conserved targets of microRNAs [1]. The known functions of microRNAs include cell differentiation, proliferation, apoptosis, anti-viral defense, lipid and fat metabolism. They are also involved in cancer, diabetes, and many other diseases [2-4]. Improved software programs are now able to predict the targets of microRNAs in a more efficient manner, thus facilitating the elucidation of microRNA function. Furthermore, methods such as real-time PCR and microarray have been enhanced for studying microRNA expression. Using these tools, scientists are able to discover novel functions for microRNAs. It is likely that microRNAs have a role in virtually every aspect of gene regulation.

1.1.1 How are microRNAs generated?

MicroRNAs are now considered to be a general mechanism for gene regulation. The most important property of microRNA genes is that they are conserved throughout evolution, except for those found in viruses. More than half of the known mouse and human microRNAs have their homologs in puffer fish or zebrafish [5]. The locations of microRNA genes in the genome of these organisms are variable. Some exist individually and others are in clusters that are co-expressed polycistronically. Studies of genomic positions of microRNAs in mouse and human have revealed that many microRNA genes are in the intergenic regions. Some microRNA genes are located in introns of coding regions, either in sense orientation or reverse orientation. This means that microRNAs can be transcribed from their own promoters or derived from the transcript product of other genes. The biogenesis of microRNAs occurs in a stepwise fashion in the nucleus and the cytoplasm. Mature microRNAs are shortened RNAs that are the result of a series of cleavage processes that begins with a primary microRNA (pri-miRNA) [6, 7]. First, pri-miRNA is processed in the nucleus into a precursor microRNA (pre-miRNA) hairpin by Drosha, a double-stranded RNA (dsRNA)-specific ribonuclease [8-10]. Then, the hairpin RNAs are transported to the cytoplasm where they are digested into lengths of ~22 nt by another dsRNA-specific ribonuclease called Dicer [11]. This mature form of microRNA is now available to bind to a RNA-induced silencing complex (RISC).

1.1.1.1 Transcription of primary microRNAs

The pri-miRNAs are transcribed as long, double-stranded unstructured precursors with a 5’ cap and a 3’ poly(A) tail and are presumably dependent on the same regulatory machinery as encoded RNA destined for translation into protein [12, 13]. Many genes for microRNAs are separated from other genes, but some (~ one-third) are found within introns of protein-coding genes where they are likely controlled by a shared promoter [14]. As for transcription of microRNA genes, it remains unclear which RNA polymerase is responsible for the transcription. There are two polymerases possibly involved: Pol II is responsible for the transcription of

protein-coding genes, small nucleolar RNA genes, and some small nuclear RNA genes [15-17]. On the other hand, pol III transcribes small non-coding RNAs including tRNAs, 5S rRNA, some snRNAs, and other small RNAs [18]. Several studies have suggested that pol II may be the most important RNA polymerase engaged in microRNA gene transcription. First, the pri-miRNAs are longer than the ~70 nt pre-miRNAs and sometimes can be several thousand bases long. For example, pri-miR-21 is ~3433 nt in length. Therefore, it is too long for pol III- mediated transcription [13, 19]. Second, it is believed that the pri-miRNA gene sequences are flanked by promoters in the 5' region that are able to transcribe heterologous mRNAs [19]. Third, stretches with more than four U's, which terminate the transcription of pol III, widely exist in pri-miRNA sequences [20]. Forth, human pri-miRNAs are polyadenylated and capped, which are unique properties of pol II gene transcripts [21]. However, some new evidence indicates that, in some cases, microRNAs can also be transcribed by pol III. In the genome of mouse gamma herpes virus 68 (MHV68), microRNAs are embedded in the pol III tRNA primary transcripts and end with a stretch of oligo (T) [22]. The transcription of these microRNAs is largely unregulated as they are under the control of an unregulated tRNA-specific pol III promoter. From these observations we can speculate that both pol II and pol III may transcribe microRNA genes depending on the species and the evolutionary origins of these microRNAs.

1.1.1.2 Processing of primary microRNAs by Drosha

Ribonucleases serve as the post-transcriptional regulators that participate in the processing and decay of RNA. Thus, the rates of maturation and decay, along with transcription frequency, establish the steady-state level of an RNA. In the cell's nucleus, a specific ribonuclease of the RNase III endonuclease family, Drosha, enzymatically cuts the pri-miRNA into a smaller (~70 nucleotides) product [8, 23]. Drosha is able to do this with the help of a cofactor called DGCR8 or Pasha, which is a dsRNA binding protein that dimerizes with Drosha [24]. Drosha is composed of two tandem RNase III domains, a dsRNA binding domain, and an

N-terminal segment [25]. It is a dsRNA-specific endonuclease that produces the 2- nucleotide 3' overhang at the cleavage site [26]. Drosha cleaves both strands of pri-miRNA into an imperfect stem-loop structure, which predetermines one end of the mature microRNA. Mutagenesis studies revealed that the enzymatic activity of Drosha is dependent on cis-acting elements flanking the cleavage site, the length of the terminal loop, and the stem structure [8, 27]. However, the internal loop, or bulge, has little effect on the efficiency of Drosha. Recent knock-down studies have shown that Drosha cannot work without its partner, DGCR8 [28]. Human Drosha is found in two multi-protein complexes, with one being larger and containing several classes of RNA-associated proteins. The other smaller multi-protein complex is called a microprocessor, and contains both Drosha and DGCR8. The deletion of DGCR8 in humans has been shown to cause DiGeorge syndrome, a developmental disease characterized by specific cardiac malformations, facial abnormalities, and certain endocrine and immune anomalies [24]. Further *in vivo* and *in vitro* experiments have shown that this Drosha-DGCR8 microprocessor is necessary and sufficient for the processing of pri-miRNA to pre-miRNA [29]. The relative role for the larger Drosha multi-protein complex remains unknown. An alternative mechanism of pri-miRNA processing was discovered recently. Some introns, where intronic microRNAs are located, serve as pri-miRNAs. These introns mimic the structures of pre-miRNAs and enter the pre-miRNA processing directly without Drosha-mediated cleavage [30]. Another mechanism has also been discovered recently. It has been reported that some small nucleolar RNAs (snoRNAs) in various species can be processed into microRNA-like RNAs by Dicer, without involvement of Drosha [31-33]. As with mRNA, the processing of pri-miRNA is regulated post-transcriptionally. The terminal loops of some pri-miRNAs are highly conserved [34]. In addition, there is experimental evidence that the processing of pri-miRNA by Drosha and pre-miRNA by Dicer, which will be discussed in the next section, can be regulated via the loop structures [35, 36].

1.1.1.3 Export of precursor microRNAs to the cytoplasm

After being cleaved by Drosha, pre-miRNA is transported to the cytoplasm by Exportin-5, a nuclear export factor, in a Ran-GTP dependent manner [37, 38]. This type of nuclear export factor is known to shuttle transfer RNAs, short hairpin RNAs, and ribosomal RNAs to the cytoplasm [39]. Exportin-5 was first examined for its involvement with the nuclear transport of adenovirus VA1, a viral non-coding RNA with an 18 base pair stem [40]. The binding of Exportin-5 to pre-miRNA is specific because an RNA stem must be larger than 14 base pairs with a base-paired 5' end and a short 3' overhang in order for Exportin-5 to bind efficiently [41]. In addition, the binding of Exportin-5 to the pre-miRNAs *in vitro* prevents their degradation by a bacterial exonuclease, suggesting that the export factor provides protection of the pre-miRNA from nucleases as it is being transported out of the nucleus [41].

1.1.1.4 Maturation of microRNA in the cytoplasm

Once the pre-miRNA is transported into the cytoplasm, it is cleaved into an imperfect dsRNA duplex designated microRNA: microRNA*. Eventually, an extension of the stem-loop is cut off to yield the active ~22 nt microRNA. This process is carried out by Dicer, an RNase III endonuclease, which defines the other end of the mature microRNA [13, 42]. Dicer is an evolutionarily conserved enzyme found in mammals, worms, fungi, flies, and plants. It is composed of a helicase domain, a Piwi-Argonaute-Zwille (PAZ) domain, two RNase III motifs, a Domain of Unknown Function (DUF283), and a dsRNA binding domain (dsRBD) [42, 43]. Dicer forms an intramolecular dimer with its two RNase III domains, which generates an active catalytic site at the interface of the RNase III dimer for single dsRNA cleavage. This process is unlike Drosha because Dicer's second RNase III domain contains substitutions at a critical active site, which makes it partially inactive [44]. The crystal and NMR structures of the PAZ domain have been solved [45, 46]. It contains a five-stranded central β-barrel, which is a topological variation of the oligonucleotide/oligosaccharide-binding (OB) fold. The OB structure predominantly functions in single stranded nucleic acid binding. The PAZ domain has a weak but

consistent affinity for nucleic acids. Moreover, it has specificity for the 3' two-nucleotide overhang resulting from RNase III cleavage [46]. This suggests that the PAZ domain may make a major contribution to the substrate specificity of Dicer.

1.1.1.5 Species variation in microRNA maturation

There are two Dicer homologues in *Drosophila melanogaster*, Dicer1 and Dicer 2 [47]. Dicer1 plays an important role in pre-miRNA processing as Dicer1 deficiency blocks this process. A dsRNA binding protein, R3D1, forms a stable complex with Dicer1 and is necessary for pre-miRNA processing [48]. In contrast, Dicer2 does not have an effect on microRNA maturation but rather affects short interfering RNA (siRNA) production. It forms a complex with the dsRNA binding protein, R2D2, that initiates RISC assembly and enhances mRNA degradation mediated by RISC [47, 49, 50]. The orientation of this complex determines which siRNA strand is assembled into RISC [51]. The maturation of microRNA in plants is much different from that in animals as no Drosha homologue has been identified in plants thus far [52]. However, four Dicer homologues exist in the flowering plant, *Arabidopsis thaliana.* Two of these Dicer-like proteins are likely localized in the nucleus [53, 54]. Dicer-like protein-1 (DCL1) possibly performs both Drosha and Dicer-like activities for microRNA maturation inside the nucleus [55]. Interestingly, a miR162 target sequence is predicted near the middle of the DCL1 mRNA, suggesting that DCL1 is dependent on negative feedback regulation by a microRNA [56]. Therefore, *A. thaliana* as well as other plants that have existed over the past 100 million years utilizes redundant regulatory mechanisms for processing microRNA [57]. The complexity of microRNA maturation in animals is likely an evolutionary byproduct. Because cellular microRNAs have become increasingly complex in mediating post-transcriptional regulation, viruses have also evolved to encode microRNAs in order to successfully infect cells. Few studies have reported on the maturation of viral microRNA; however, it is generally thought to be similar to that of the host cell. Because microRNAs act at the mRNA level, it is not likely that viruses that possess RNA as their genetic

material would encode microRNAs. The more probable choices are DNA viruses, such as herpesviruses, that establish long term latent infections in cells. Other candidates include large lytic DNA viruses like human adenoviruses. Indeed, microRNAs have been found in both herpesviruses as well as human adenovirus [22, 58-60]. The pol II-transcribed product of most viral encoded microRNA genes is processed in the nucleus by Drosha and transported by Exportin-5 to the cytoplasm where it is processed by Dicer [22, 61, 62]. One of the arms of the processed microRNA is incorporated into RISC [63]. This maturation process may not be true in the case of MHV68, since MHV68 pre-miRNAs have an unusual short hairpin structure, which means the maturation and export mechanism may be different from that of the host cell [22]. Furthermore, studies on adenovirus have shown that the viral microRNA has the ability to mimic the stem loop structure of host microRNAs in order to bind and sequester Dicer [60]. The same study demonstrated that Dicer not only binds to viral pre-miRNA, but also cleaves it to yield a ~22 nt mature microRNA. Viral microRNAs not only target mRNAs that allow the virus to remain in the latent stage or to become lytic without being recognized by the host's immune system, but also target host mRNAs to escape recognition by the host immune system [61, 64, 65].

1.1.2 How do microRNAs work?

1.1.2.1 Formation of RISC

After pre-miRNA is processed into a microRNA: microRNA* duplex by Dicer, one of the RNA strands is incorporated into RISC for target recognition [66]. RISC has strand bias and always incorporates the microRNA strand with the less stable 5' end in comparison with the microRNA* strand [67]. The microRNA* strand is degraded in most cases. This strand bias may be the result of the relative ease of unwinding from one end of the duplex. A helicase likely carries out multiple trials from both ends in order to unwind the duplex. Occasionally, these trials lead to unwinding at the easier end of the complex, rendering a strong bias for the easier end. This

is supported by the finding that in rare cases where microRNAs have 5' ends with similar stability, both strands are incorporated into RISC [66]. RISC is composed of Dicer, Argonaute (AGO), PW182 (a P-body protein), the human immunodeficiency virus transactivating response RNA-binding protein (TRBP), fragile X mental retardation protein (FMR1), and other non-specified proteins [68-70]. TRBP is required for the recruitment of AGO2 to RISC [69]. AGO proteins bind to either microRNAs or siRNAs to create the core of the complex. They have both Ago and Piwi domains. The Ago domain, which is a PAZ domain, binds to the 2-nt overhang at the 3' end of RNA duplexes [46]. The Piwi domain mediates interactions between Argonaute proteins and Dicer [51]. Different Ago paralogs exist across species, and the Ago-associating proteins are also variable among species. Furthermore, variants of the AGO protein within the same animal can have different functions. For example, among RISC with any of the 4 human AGO proteins (AGO1-AGO4), only AGO2-associated RISC can direct the cleavage of target mRNA [71]. Drosophila AGO2 acts to cleave target mRNA, while AGO1 functions in translational repression [72]. Some of the other proteins that associate with AGO proteins are the Vasa intronic gene *(VIG*), an endonuclease like protein (TSN-1), and a homolog of fragile X mental retardation protein (dRXR) [73, 74]. Most AGO-associated proteins have unknown functions, but some are required to mediate mRNA cleavage [75]. It is likely that the different AGO homologues along with the variable associating factors allow for different subtypes of RISC in order to provide a specific response to a particular siRNA or microRNA. RISC has many diverse functions in both siRNA and microRNA mechanisms. It acts as an effector complex in mRNA cleavage, translation suppression, and heterochromatin formation [76, 77]. The details of the molecular mechanisms that make RISC work are still not clear. However, some observations have been made that will be discussed below.

1.1.2.2 Translational repression

MicroRNA-associated RISC can repress mRNA translation in a couple of ways. MicroRNAs in animals mostly suppress translation of their target mRNAs due to an imperfect base-pairing within 3' untranslated regions (UTRs) [78, 79]. By binding to the 3' UTR of the mRNA, the microRNA has the ability to inhibit translation by directly interfering with translation initiation factors or by disrupting poly (A) tail function [80-82]. However, polyribosomes co-purify with RISC, indicating that RISC may also interrupt the continuation of translation by forming a stable complex with polyribosomes [83, 84]. The distinction between translational repression and mRNA cleavage mediated by microRNA relies on the degree of complementarity between the microRNA/siRNA and its target. Plant microRNAs function in mRNA cleavage due to their near complete complementarity in base-pairing to their target mRNA [85-88]. When mammalian microRNAs have high enough complementarity with their target mRNA, they perform mRNA cleavage instead of translational repression as in the case of miR-196 and its target Hoxb8 [88, 89]. Although most plant microRNAs cleave and destroy their target mRNAs with nearly perfect complementarity between them, one plant microRNA, miR-172, acts as a translational suppressor [87, 90]. Furthermore, when the base-pairing between siRNA and its mRNA target does not have strong complementarity, siRNA can perform translation repression just as microRNAs do, suggesting that microRNAs and siRNAs are functionally interchangeable [91].

1.1.2.3 mRNA cleavage

Perfect base-pairing is thought to be the critical feature of microRNA-mediated mRNA cleavage, but it is not always sufficient in plants, suggesting the need for supplementary catalytic activity by RISC [90]. Regardless of the species, a perfect match between a microRNA and its target mRNA is required for efficient cleavage, especially considering the precise location of the cut between residues 10 and 11 of the microRNA [87, 92, 93]. Once the mRNA is efficiently cut,

the intact microRNA has the ability to cut other target mRNAs or guide mRNAs to mRNA processing bodies (P-bodies) for subsequent degradation [85, 93].

1.1.2.4 mRNA degradation

MicroRNAs can also act upon their target mRNAs by increasing the rate of mRNA degradation. By transfecting miR-1 and miR-124 into human cells and using microarray, Lim *et al.* [94] showed that a significant number of mRNAs are down-regulated in accordance with the microRNA expression. Another study reported that miR-16 contains an AU-rich sequence complementary to the AU-rich element (ARE) in the 3' untranslated region of unstable mRNAs, a sequence that is known to increase mRNA decay rates [95]. This finding suggests that microRNAs are required for ARE-RNA turnover. Furthermore, Bagga *et al.* [96] demonstrated that the expression of two of the earliest identified microRNAs, let-7 and lin-4, significantly decrease the levels of their target mRNAs. In addition, some studies have shown that microRNAs, such as miR-125 and let-7, can induce deadenylation of their target mRNAs, leading to rapid mRNA decay [97, 98]. All these studies provide evidence that microRNA can directly affect the turnover of their target mRNAs.

MicroRNAs are also associated with P-bodies. Cytoplasmic P-bodies are compartments in the cell that contain decapping enzymes and exonucleases for the degradation of mRNA. RISC, particularly AGO proteins, co-localize with P-bodies. Recent studies have shown that localization of targeted mRNAs to P-bodies is microRNA-dependent [99-101]. It is still not clear what functional capacity RISC possesses in P-bodies. Although P-bodies are primarily known for retaining targeted mRNAs for degradation, they contain several factors that are associated with translational repression [102]. Once the targeted mRNAs are sequestered in the P-bodies, they generally undergo deadenylation by removal of the 3' poly (A) tail, decapping by Dcp1/Dcp2, and degradation by an Xrn exonuclease [102, 103]. Deficits in the expression of the Xrn

exonuclease lead to an inefficient RNAi response [104, 105]. The microRNA-mediated degradation pathway is now known to be dependent upon the GW182 protein that interacts with the Piwi domain of AGO [106]. Taken together, this indicates that microRNAs can contribute to mRNA turnover by transporting mRNAs to P-bodies.

1.1.2.5 Activation of protein expression

In most cases, microRNAs inhibit the expression of target genes at the post-transcriptional level via the three mechanisms discussed above. However, inhibition is not always the case. It has been reported that microRNAs can actually activate expression of proteins in quiescent cells at cell-cycle arrest [107, 108]. The AU-rich element (ARE) in the 3' UTR of tumor necrosis factor-α (TNF-α) mRNA turns into a translation activating signal. The up-regulation of protein expression relies on recruitment of FMR1 and AGO2 to the 3' UTR.

1.1.3 How do scientists study microRNAs?

1.1.3.1 Expression studies of microRNAs

One of the prominent characters of microRNAs is that their expression is spatially and temporally regulated. Many microRNAs are highly expressed in certain organs or cell types and some are only expressed at certain stages during development. These regulations in expression patterns obviously coordinate with their functions; therefore, much research has concentrated on expression of microRNAs. The technology of microRNA expression detection ranges from simple to complex. One popular method uses small RNA cloning and sequencing to identify microRNA in tissues and cells [7, 109-113]. Although this method has potential to find novel microRNAs, the rates of discovery depends on the expression levels of the microRNAs. In general, cloning methods are not sensitive enough and are not suitable for high throughput microRNA expression profiling. Furthermore, it is possible that microRNAs have bias at various steps including ligation, reverse transcription, amplification and cloning. By far the most reliable

technique to detect expression of microRNA remains to be Northern blotting. After all, results from all other methods are always validated using Northern blots. For example, Sempere and colleagues [114] made an extensive effort to uncover the expression profiling of mammalian microRNAs using Northern blots and showed that some microRNAs are specifically expressed or highly enriched in certain organs. *In situ* hybridization has also been a reliable technique for evaluating the relative expression level as well as temporal and spatial patterns of microRNAs [115]. MicroRNA microarrays have been widely used to analyze microRNA expression. Krichevsky and colleagues [116] used a membrane spotted with antisense sequences to mature microRNAs. The RNA samples were end-labeled with gamma-^{32}P and hybridized to the membrane oligonucleotide array. Of the probed microRNAs, 20 percent were significantly changed during mammalian brain development. In most microRNA microarray platforms, glass slides are utilized for spotting antisense or sense probes. There are many variations of this technique that differ in several respects such as probe choice, RNA sample processing, and detection method. Some researchers use Cy3 or Cy5 labeled cRNA transcribed from cDNA of microRNA to hybridize with chips [117]. Others have developed their own microarray systems for microRNA detection using cDNAs from adapter ligation and reverse transcription of microRNAs [14, 118-121]. There are compelling advantages for using microRNA microarrays. They are very sensitive and, more importantly, high throughput. In some microarray designs, microRNAs with concentrations as low as the femto-mole range can be detected [121]. Also, microarrays are less time-consuming compared to Northern blots. However, the challenge in using microarrays is data analysis. One potential problem is data normalization, where housekeeping genes are not suitable for data normalization. Unlike DNA microarrays, in which there are between 10 to 30 thousand probes on the slide, microRNA microarrays only contain 200 to 300 probes. Furthermore, the overall microRNA expression levels may be variable in different tissues. Therefore, global normalization used for DNA microarrays may not be appropriate in some cases. Real-time PCR has been adapted to monitor microRNA expression. One such

adaptation uses stem-loop RT primers that are more efficient and specific than conventional primers [122]. It is now possible to analyze over 300 different gene profiles, which is comparable to the high throughput of microarrays [123]. Jiang et al. [124] successfully profiled the expression of 222 microRNA precursors in 32 cancer cell lines using real-time PCR. Their results showed that some microRNA precursors have significantly higher or lower expression in certain cell lines. They also reported that the expression of pre-miRNA parallels that of mature microRNA in most of the cells that they tested. Another study used real-time PCR to evaluate the expression profiling of several mature *Arabidopsis* microRNAs, where as little as 100 picograms of total RNA was used [125]. The increased sensitivity of real-time PCR surpasses that of both Northern blots and microarrays, making this technique desirable.

1.1.3.2 Function studies of microRNAs

Functional studies of microRNAs can be carried out by several approaches. The first approach is forward genetics which starts with a phenotype and moves toward the identification of a microRNA [126-128]. The second approach is reverse genetics where the study starts with a microRNA and assays the effect of the microRNA gene's disruption [129, 130]. Since most microRNA research begins with identification of a microRNA, another important approach to study microRNA function is to predict the microRNA's target gene(s) by computational algorithms and experimental validation.

1.1.3.2.1 Target prediction

It is crucial to identify microRNA targets in order to uncover the functions of microRNAs during in normal physiological and pathogenic conditions. As mentioned before, most plant microRNAs have nearly perfect complementarity with their target mRNA sequences leading to target mRNA cleavage, which makes target identification relatively easy. However, identification of target sequences in animal genomes is impossible by standard sequence comparison because

microRNAs are very short in length and their complementarity to target sequences is imperfect [131, 132]. For this reason, computational approaches for finding animal microRNA targets are very useful. Currently there are several web servers that contain computationally predicted targets for microRNAs across many species. Among them is the miRanda software (www.microrna.org) [133]. This program uses the miRanda algorithm to identify potential binding sites and then checks the potential target site in the 3' UTR to see whether the sites are conserved in orthologous transcripts. Another algorithm, PicTar, predicts microRNA targets in vertebrates and *Drosophila* species and has the ability to predict targets for single microRNAs and for combinations of microRNAs (http://pictar.bio.nyu.edu/) [134]. The TargetScan algorithm identifies mRNAs with conserved pairing to the 5' region of the microRNA and assesses the quantity and quality of these complementary sites (http://genes.mit.edu/targetscan) [131]. These programs have allowed for the creation of target databases that may potentially facilitate functional studies of microRNAs. One such database for predicted targets is TarBase (http://www.diana.pcbi.upenn.edu/tarbase), which contains target mRNAs tested in human, mouse, fruit fly, worm, and zebrafish [135]. Another such database is Argonaute (http://www.ma.uniheidelberg. de/apps/zmf/argonaute/interface/).

1.1.3.2.2 Target validation

After finding potential targets for microRNAs, the targets need to be validated by experimental methods. To test whether the target mRNA is cleaved by the microRNA, the target mRNA is reverse-transcribed, cloned and sequenced. The cleavage site should be at the site predicted according to the microRNA: mRNA duplex [93, 136]. To test whether the target is inhibited by translational repression, several methods have been used. The 3' UTR segment with predicted complementary sites or those with point substitutions that disrupt the base-pairing are fused with a reporter gene. If the microRNA inhibits translation, disruption of the base-pairing will abolish translation inhibition of the target mRNA. The correct unmutated 3' UTR segment

with the complementary sites is used concurrently as a control [90, 137]. Another method uses RNA inhibition to repress the activity of the microRNA to see if the effect on the target mRNA is real [138].

1.1.3.2.3 Over-expression and down-regulation of microRNAs

Studies also over-express microRNAs to elucidate their effect on their target mRNA and the physiological consequence on the cell [139, 140]. Over-expression of microRNAs should significantly reduce the expression of the target mRNA. To observe the reverse effect, microRNAs are blocked by a 2'-O-methyl oligoribonucleotide complementary to the microRNA [141, 142]. A screen of microRNAs involved in cell growth and death has been reported using a library of these modified oligonucleotides [143]. Krutzfeldt et al. [144] developed a new method to silence microRNAs *in vivo* with "antagomirs". Instead of using antisense 2'-O-methyl oligoribonucleotides, they designed chemically modified, cholesterol-conjugated antisense RNAs. Whether the microRNA is over-expressed or silenced, the target protein should have coincident expression with the microRNA. If the microRNA is over-expressed, the expression of target mRNA should be depressed. Conversely, if the microRNA's function is blocked, the translation inhibition should be released.

1.1.4 What do microRNAs do?

The previous section described the mechanisms of microRNA-mediated gene regulation. In this section, we will briefly discuss some of their physiological functions. MicroRNAs have been implicated in biological processes ranging from developmental timing to apoptosis. As scientists discover the role of microRNAs in even more processes, it will become clear just how important these tiny RNAs really are. One unique aspect of microRNAs is that they can synchronously inhibit a group of genes that are functionally related. Thus, microRNAs can work as an efficient molecular switch to turn on or turn off a biological process. For example, as

discussed below, let-7 targets a group of genes in *C. elegans*, including lin-14, lin-28, lin-41, lin-42, and daf-12 [145].

1.1.4.1 Developmental timing

Most of ous knowledge of the biogenesis and function of microRNAs stems from the paradigm provided by the discovery of lin-4 and let-7, which were identified as microRNAs implicated in the function of *C. elegans* developmental timing [126, 145]. Lin-4 and let-7 bind to multiple conserved sites in the 3' UTR of the lin-14 and lin-41 transcripts, respectively, through direct but imprecise base-pairing, thus inhibiting translation into protein [78, 126, 146]. In *C. elegans*, the down-regulation of the protein lin-14 at the end of the first larval stage initiates the second larval stage [126]. lin-41, on the other hand, regulates the developmental transition from the last larval stage to the adult stage [146]. Computational analysis reveals that let-7 is evolutionarily conserved throughout metazoans [147]. Lin-4 and let-7 also regulate two other genes, *lin-28* and *lin-57*, respectively [148, 149]. *Lin-28* encodes a RNA-binding protein that is important for neuronal differentiation of embryonic carcinoma cells [150] while *lin-57* encodes a protein responsible for the terminal differentiation of the hypodermis in *C. elegans* [149]. Since the original *C. elegans* experiments, the regulatory abilities of lin-4 and let-7 have been extended to flies and mammals [151]. In mice, these microRNAs inhibit expression of *lin-41*, which is involved in key developmental events such as limb formation [151]. Studies of three microRNAs in *Drosophila* (let-7, miR-125 (the lin-4 homolog) and miR-100) not only show their up-regulation during major points of development but also demonstrate their requirement for concurrent expression of a hormone in order to be functionally expressed [152]. Important mRNA targets responsible for developmental timing have also been found in *Arabidopsis*, suggesting that microRNA regulation in morphogenesis is a primitive mechanism [153, 154].

1.1.4.2 Cell proliferation and differentiation

In addition to guiding timing events, microRNAs have also been established as potent controllers of cell proliferation and differentiation. Although the division of cells is imperative for the growth of an organism, it can also be detrimental when occurring at inappropriate times. The latter is the hallmark of cancer, and several microRNAs have been shown to be up-regulated in tumors, a concept that will be discussed later along with microRNA-associated diseases. It is important to understand the significance of cell division during organismal growth beginning with stem cells. Mutation studies of *Drosophila* show that disruption of microRNA processing causes stem cells to be locked between the G1 and S phases, thus halting division [155]. A neuron-specific microRNA, miR-132, is a target of the transcription factor, cAMP-response element binding protein. It regulates neuronal growth by decreasing the levels of a GTPase-activating protein [156]. Another brain-specific microRNA, miR-134, is expressed in the synaptodendritic compartment of rat hippocampal neurons, where it is capable of down-regulating Limk-1, a protein responsible for spine development [157]. Regulatory roles of microRNAs are not limited to the brain. Adipose cell differentiation has been shown to be partially controlled by the expression of miR-143 [158]. Also, miR-1 and miR-133 are important regulators of skeletal muscle proliferation and differentiation [138]. Because cell growth and differentiation are such dynamic biological processes, it is no wonder that microRNA with its specific and fast-acting regulatory ability is vital for shaping these processes.

1.1.4.3 Apoptosis

Apoptosis (programmed cell death), is an integral part of animal tissue development. Apoptosis is an evolutionarily conserved process that allows animals to remove cells that are useless or that are detrimental to survival. Once apoptosis is activated, caspase proteins cleave both structural and functional elements of the cell. Therefore, cell death vs. survival depends largely on the control of active caspases in the cell. Because caspases are ubiquitous, it makes sense that microRNAs would play a role in their regulation. Indeed, in the *Drosophila* eye, the

absence of *miR-14* leads to an increase in the cell death effector, Drice, suggesting that miR14 is an inhibitor of apoptosis [159]. Likewise, the *bantam* gene encodes a microRNA that when over-expressed, suppressed apoptosis in the *Drosophila* retina. One of the targets for *bantam* was identified as the pro-apoptotic gene, *hid,* which possesses sequences complementary to *bantam* [127]. It has been known for quite some time that viruses must prevent apoptosis in order to survive in the host cell. Recently, it has been discovered that herpes simplex virus-1 inhibits apoptosis through a latency-associated microRNA (miR-LAT) that modulates TGF-β signaling [160]. By using microRNAs as well as proteins in the inhibition of apoptosis, viruses are able to survive as well as evade immune detection. As functional studies of microRNA continue, the list of targets involved in apoptosis will likely grow radically.

1.1.4.4 Regulation of signaling pathways

With more functions of microRNAs being identified experimentally, it is now clear that microRNAs regulate almost all conserved signaling pathways that are important in physiology and pathology. It has been reported that miR-21 activates the MAPK/ERK signaling pathway in cardiomyocytes by directly repressing expression of Spry1 and Spry2, which are negative regulators of the MAPK/ERK signaling pathway [161, 162]. The Notch signaling pathway mediates local cell-cell communication and is pivotal to proper pattern formation in development [163]. Two large families of Notch target genes in *Drosophila*, basic helix-loop-helix (bHLH) repressor genes and Bearded genes, contain conserved motifs (GY-box, Brd-box, and K-box), in their 3’ UTR. MicroRNAs from three different families (miR-2, miR-4, miR-7, miR-11, and miR-79) regulate the activities of these target genes and Notch signaling via binding to these motifs [164]. The TGF-β signaling pathway is known to stimulate epithelial-mesenchymal transition (EMT). It has been shown that the miR-200 family represses expression of the transcription factors ZEB1 and ZEB2, which are down-stream mediators of TGF-β signaling, resulting in the inhibition of EMT [165]. The Hedgehog (Hh) signaling pathway is another pivotal pathway in

development. It gives out signals for proper organogenesis of embryos. It has been reported that miR-125b and miR-326 inhibit Hh signaling by targeting to and repressing expression of Sommthened (Smo), which is an activator of the Hh signaling pathway. In addition, miR-324-5p also targets to Gli1, a downstream transcription factor in this pathway [166]. The Wnt signaling pathway will be discussed in detail in section 1.5. It has been reported that miR-8 regulates Wnt signaling in *Drosophila* at multiple levels. miR-8 inhibits the activity of this pathway by repressing expression of its targets, include Wingless (Wg), TCF, and CG32767, which is a positive regulator of this pathway [167].

1.1.5 Conclusions and perspectives

Since the discovery of the first microRNA in 1993, remarkable progress has been made in the area of identification, high throughput microRNA expression profiling, computational target prediction, and experimental confirmation. With the increasing knowledge through observation and confirmation of more microRNA-target relationships, the empirical parameters for target recognition will be modified and made increasingly powerful and unambiguous. However, questions still linger in the microRNA world. What are the exact mechanisms for microRNA-mediated cleavage of target mRNAs? What other proteins are involved in microRNA-mediated gene regulation? Most importantly, can synthetic microRNAs be used as a therapeutic intervention to diseases? These questions and many more will be the focus of future research for many laboratories. In the past few years, the understanding of microRNA function has increased dramatically. How widespread is the function of microRNAs? Perhaps clues will come from experiments that disrupt the function of Dicer homologues, AGO family proteins, or other important factors such as GW182 that have principal functions in microRNA function [168]. Although thousands of microRNAs have been identified in 39 species, only a small percentage of microRNAs have been tested for their function. Finding new functions for microRNAs is the new

frontier of molecular science. Many suspect that microRNAs will be revealed as the most important players in gene regulation: a tall order for a tiny string of nucleotides.

1.2 microRNAs and diseases

As discussed above, microRNAs are involved in the regulation of almost all developmental processes and cellular functions. It is therefore not surprising that the aberrant expression of microRNAs is closely linked with human diseases including cancers, central nervous system disorders, and liver, cardiovascular, and pulmonary diseases. The development of two powerful high-throughput microRNA detection techniques (solid-based microRNA microarray platform and liquid-based flow cytometry method) makes it possible to detect abnormal microRNA expression in diseases at a large scale [169, 170]. In this section, we mainly focus on those microRNAs whose functions in the pathogenesis of diseases have been confirmed by experimental evidence. Understanding the functions of microRNAs in diseases will provide potential therapeutic targets as well as prognostic and diagnostic tools.

1.2.1 MicroRNAs and cancers

MicroRNAs play important roles in cell proliferation, differentiation, and apoptosis. Their disrupted expression is associated with human cancers. Aberrant expression patterns of microRNAs have been profiled in a wide range of cancers, including ovarian, liver, pituitary, thyroid, lung, breast, stomach, prostate, colon, and pancreatic tumors [171-173]. The dysregulated microRNAs in different types of cancers have been summarized in several reviews [4, 173].

Although the aberrant expression of many microRNAs in cancer cells has been observed, there is a little evidence that microRNAs play a causal role in cancer pathogenesis. The study of miR-15 and miR-16 in chronic lymphocytic leukemia (CLL) is the first to establish a direct connection between microRNAs and tumorigenesis [174]. Hemizygous and/or homozygous deletions of the chromosomal region 13q14 are observed in most cases of CLL. It has been

suggested that there are some tumor suppressor genes located in this chromosome region [175]. In a study in 2002, miR-15 and miR-16 were found to be located within the lost region at 13q14 [174]. The expression of these two microRNAs are depleted or reduced in a majority of CLL. In many cases of CLL, the 13q14 loss is the sole genetic aberration. It is reasonable to conclude that the deletion or reduction of miR-15 and miR-16 is of pathogenic significance in CLL [174]. In later studies, more evidence was found to support this conclusion. Several oncogenes, such as BCL2, MCL1, CCND1, and Wnt3a are direct targets of miR-15 and miR-16. Over-expression of these two microRNAs leads to the inhibition of cell proliferation, the promotion of apoptosis, and the suppression of tumorigenesis [176, 177].

Another example is cluster miR-17-92, which has been intensively studied and is considered as an oncogene [178]. This cluster is located at chromosome 13q31, a region that is amplified in lymphomas and lung cancers [179]. The amplification of this region leads to the overexpression of cluster miR-17-92 in these cancers. Expression of the miR-17-92 cluster accelerates tumor development and inhibits apoptosis in lymphomas.

Metastasis is a major cause for death of cancer patients. MicroRNAs can function as a metastasis suppressor as well as an activator. It has been reported that miR-335, miR-126, and miR-206 are lost during human breast cancer cell metastasis. Restoration of these microRNAs suppresses metastasis by human breast cancer cells *in vivo* [180]. Silencing of miR-148a, miR-34b/c, and miR-9 by CpG island hypermethylation is frequently observed in metastasis formation in human primary malignancies, such as colon, lung, breast, and head and neck carcinomas and melanomas [181]. Furthermore, reintroduction of miR-148 and miR-34b/c inhibits cancer cell motility, growth, and metastasis, with the down-regulation of their target oncogenes, including C-MYC, E2F3, CDK6, and TGIF2 [181]. In contrast, it has been shown that miR-375 functions as a metastasis activator and stimulates cancer cell migration *in vitro* and *in vivo*, probably by repressing expression of its target CD44. miR-375 is significantly up-regulated in metastasis of

breast cancer, corresponding with down-regulation of CD44 [182]. Another example is miR-10b, which is up-regulated in metastatic breast carcinomas. miR-10b stimulates cancer cell migration and invasion by directly targeting homeobox D10 (HOXD10), which is a transcriptional repressor of a pro-metastatic gene, *RHOC* [183].

1.2.2 MicroRNAs and liver diseases

MicroRNAs are involved in different kinds of liver diseases, including viral hepatitis, drug-induced injury, non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, primary biliary cirrhosis, liver fibrosis, and hepatocellular carcinoma (HCC) [184]. For example, miR-122, a liver-specific microRNA, has been shown to enhance the replication of Hepatitis C virus [185]. miR-122 is also up-regulated in NAFLD. It may play a critical role in NAFLD since miR-122 is known to regulate the genes involved in fatty acid biosynthesis and the antagonist of miR-122 decreases the synthesis of hepatic fatty acid and cholesterol [186].

1.2.3 MicroRNAs and cardiovascular diseases

Some microRNAs have been shown to play roles in ventricular hypertrophy, fibrosis, heart failure, and arrhythmogenesis [187-189]. For example, the heart-specific microRNAs, miR-1 and miR-133, have been reported to have critical regulatory roles in cardiac hypertrophy [188]. Expression levels of these two microRNAs are down-regulated in both mouse and human models of cardiac hypertrophy and suppression of miR-133 can induce hypertrophy [188]. RhoA (a G-protein), Cdc42 (a kinase), and Nelf-A/WHSC2 (a nuclear factor) have all been confirmed as the targets of miR-133 in the regulation of cardiac hypertrophy. In addition, miR-1 exacerbates arrhythmogenesis by targeting *GJA1* and *KCNJ2*, which encode the K^+ channel subunit Kir2.1 and connexin 43, respectively [189]. Elimination of miR-1 relieves arrhythmogenesis. In contrast, expression of miR-195 is up-regulated during cardiac hypertrophy. It has been shown that overexpression of miR-195 leads to cardiac hypertrophy and heart failure *in vivo* [190].

1.2.4 MicroRNAs and central nervous system disorders

The aberrant expression patterns of microRNAs have been profiled in many neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD), Schizophrenia, and Parkinson's disease (PD) [191-198]. Some of the microRNAs that are important in pathogenesis of these neurodegenerative disorders will be discussed below.

AD is a neurodegenerative disease with progressive loss of cognitive function and memory. This disease is characterized by the deposit of amyloid beta-peptide (Aβ) in plaques, the production of which is controlled by beta-site APP-Cleaving Enzyme (BACE) [199]. A microarray analysis revealed that the expression patterns of three microRNAs (miR-9, miR-125b, and miR-128) are changed in AD compared with those in the age-matched controls [200]. miR-146a is up-regulated in AD brains and may regulate the inflammatory response in AD by targeting to complement factor H (CFH) [201]. Thirteen microRNAs are dysregulated in sporadic AD. The loss of miR-29a/b-1 is associated with increased expression of BACE1 [202]. miR-29a/b-1 can target to BACE1 and down-regulate the level of Aβ. The expression of BACE1 is also regulated by miR-107, whose expression is progressively decreased in the patients of AD [203].

miR-133b is specifically expressed in midbrain dopaminergic neurons and is deficient in the patients with PD because of the loss of dopaminergic neurons in midbrain tissues. Overexpression of miR-133b leads to reduction of the dopaminergic neuron maturation marker, DAT and dopamine release mediated by potassium-induced depolarization. miR-133b is regulated during maturation of dopaminergic neurons through a negative feedback circuit that involves the paired-like homeodomain transcription factor, Pitx3 [195]. In addition, it has been shown that miR-433 is involved in etiology of PD. One of the single nucleotide polymorphisms (SNPs) of *Fgf20* within the binding site for miR-433 in the 3'UTR abolishes the inhibition of

miR-433, resulting in an elevated level of Fgf20. Dysregulated Fgf20 subsequently enhances expression of α-synuclein, which accumulates in PD brains and has been confirmed to cause PD [204].

So far, there has been no evidence that microRNAs cause HD. However, some microRNAs, such as miR-9/miR-9*, are involved in this disease. REST is a transcription factor that represses expression of neuronal genes with RE1 repressor sequences. In neurons, it stays in the cytoplasm by binding to Huntingtin (Htt). Polyglutamine expansion in Htt disrupts this binding, resulting in nuclear translocation of REST and development of HD. In the nucleus, REST, together with the REST corepressor 1 (CoREST), inhibits expression of miR-9/miR-9* by binding to upstream RE1 sites. In turn, miR-9 targets REST and miR-9* targets CoREST [192].

1.2.5 MicroRNAs and lung diseases

In addition to lung cancer as mentioned previously, microRNAs also play important roles in many other chronic lung diseases, such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), asthma, and idiopathic pulmonary fibrosis (IPF) [205]. Most of these diseases are associated with chronic airway inflammation and/or systemic inflammation.

COPD is usually induced by cigarette smoking and/or chronic exposure to particulate matter. Alteration of microRNA expression has been reported in mice, rats, and humans exposed to cigarette smoke [206-208]. For example, miR-146a is down-regulated in COPD fibroblasts. It causes the degradation of COX-2 mRNA [209]. The reduced expression of miR-146a prolongs the half-time of COX-2 mRNA, resulting in the increase of the inflammatory mediator, prostaglandin E in COPD fibroblasts.

miR-21 is the most up-regulated microRNA in a mouse model of asthma [210]. Introduction of miR-21 inhibits the expression of its predicted target IL-12p35, indicating that miR-21 is involved in allergic airway inflammation.

miR-21 is also up-regulated in human IPF. An enhanced expression of miR-21 promotes TGF-β-induced fibrosis, while the inhibition of miR-21 reduces the severity of fibrosis [211]. let-7d is down-regulated in IPF lungs. TGF-β inhibits let-7d expression through SMAD3. The inhibition of let-7d causes increases in mesenchymal markers *in vitro* as well as alveolar septal thickening and increases in collagen *in vivo* [212]. miR-155 is another microRNA involved in IPF pathogenesis. Expression of miR-155 is up-regulated in the lungs of IPF patients and is correlated with the degree of lung fibrosis in a bleomycin-treated mouse model [213]. Induction of miR-155 expression causes fibroblast migration, probably by directly targeting angiotensin II type I receptor (AT1R), which has been indicated to contribute to fibrosis [214]. Expression of miR-29 is reduced in IPF and the bleomycin-treated mouse model. It has been shown that knockdown of miR-29 leads to an increase in genes associated with pulmonary fibrosis, including integrin, alpha 11, etc. [215].

miR-126 is significantly down-regulated in CF [216]. It can regulate the expression of TOM1 (the target of Myb1), which is a negative regulator of IL-1β and TNF-α induced signaling pathways [217]. Knockdown of TOM1 leads to an increase in NF-κB regulated IL-8 secretion [216]. These results indicate that miR-126 is involved in immune responses in CF lungs. In addition, miR-126 is also involved in pathogenesis of allergic asthma [218]. Expression of miR-126 is stimulated in an experimental allergic disease model, in which house dust mite induces allergic disease through activation of toll-like receptors (TLRs). miR-126 blockade results in suppressed asthmatic phenotype and diminished T helper-2 (TH2) lymphocyte responses [218].

1.2.6 MicroRNAs as potential therapeutic targets

With more knowledge of microRNA functions in diseases, microRNAs have the potential to be new therapeutic targets. Systemic delivery of adeno-associated virus (AAV) overexpressing miR-26a in a mouse model of hepatocellular carcinoma leads to inhibition of cancer cell

proliferation and induction of apoptosis [219]. Administration of the locked-nucleic-acid (LNA)-modified oligonucleotide complementary to miR-122 in mice reduces plasma cholesterol and increases hepatic fatty acid oxidation. The synthesis of hepatic fatty acid and cholesterol is also depressed [220]. Systemic delivery of the same antagonist of miR-122 by intravenous injection also effectively depletes mature miR-122 and reduces plasma cholesterol in African green monkeys [221]. These studies raise the possibility of use of this miR-122 inhibitor to treat NAFLD.

1.2.7 MicroRNAs as diagnostic and prognostic tools

MicroRNAs could be a powerful tool to diagnose and classify cancers. MicroRNA profiles have been demonstrated to be highly informative and more accurate than mRNA profiles in cancer diagnosis. MicroRNA profiles can reflect the developmental lineage and differentiation state of tumors. Because each organ has its own specific microRNA expression pattern, microRNA profiles can also be used to classify poorly differentiated tumors and to trace the tissue origin of tumors of unknown primary origin [170, 222]. In addition, the expression patterns of microRNAs can also be identified in serum, plasma, and saliva of patients, which makes early detection of colon and other occult cancers possible [223-227].

1.2.8 Anti-viral defense

Although microRNAs are implicated in diseases caused by malfunctions in the cellular machinery, they also serve an important role in preventing diseases caused by viruses. Scientists studying plants first proposed microRNAs as being able to induce post transcriptional gene silencing of viral mRNAs [228, 229]. In plants, microRNAs have an anti-viral capability with short-lived effects because evolved viral factors eventually inactivate the microRNAs [230]. In fact, many viruses have the capability to evade silencing by the host, but some viruses are better adapted for evading cellular machinery than others. In humans, for example, adenovirus can

block host microRNA biogenesis, thus squelching the very anti-viral microRNAs that are meant to stop adenovirus replication [231]. Also, tissue culture experiments show that the primate foamy virus type I (PFV-1) can escape silencing by *miR-32* by production of a silencing suppressor protein called Tas [232]. These observations suggest that host microRNA-mediated defense cannot always overcome viral attacks. However, these experiments do not account for the possibility of defense responses mounted by multiple microRNAs working together. A study of the hepatitis C virus demonstrates that the introduction of multiple siRNAs targeted to different areas of the viral genome prevents the virus from escaping siRNA-silencing [233]. Future experiments with microRNAs will likely produce similar results, considering that microRNAs are functionally similar to siRNAs. Due to the accelerated mutational activity of some viruses, microRNA-mediated anti-viral therapies are ideal. After all, the effectiveness of small RNAs is based on nucleic acid sequence, making the development of new therapeutic small RNAs expeditious, which is a far cry from conventional drug-based therapies.

1.3 Fetal lung development

1.3.1 Stages of fetal lung development

Development of the fetal lung can be divided into early stages and late stages. The early stages are those when the initiation of the air-conducting system occurs and the late stages are those when the gas exchange unit forms. The early stages include embryonic phase and glandular or pseudoglandular phase. The late stages include canalicular phase and saccular phase. In rats, the embryonic phase is from gestation day 0 to day 13, the glandular phase from gestation day 13 to day 18, the canalicular phase from day 18 to day 20, and the saccular phase from day 20 to full term.

1.3.2 Development of lung structure

In the early embryonic phase, lobar division takes place. A ventral diverticulum from the primitive esophagus gives rise to the origination of the lung. A pair of endodermal rudiments develop from this diverticulum and each primitive lung bud produces many branches by dichotomous division of terminal buds. Fetal lung development transits into glandular or pseudoglandular phase when air passage resembling tubes are formed. At this time, the lung appears like a gland under a microscope. During this phase, the pseudostratified epithelium of the lung buds is reduced to a columnar form and the proliferation of epithelial cells is very active. Meanwhile, there is little or no lumen in those epithelial tubes. At this stage, the primitive airway epithelium begins to differentiate and ciliated goblet, nonciliated goblet, and basal epithelial cell types can be recognized. Cartilage and smooth muscle cells are formed from mesenchymal cells [234]. With continued epithelial branching, the development transits into the canalicular phase marked by the production of bronchioles. In this phase, the mesenchymal tissue becomes thin and a lumen can be recognized in many tubules, as epithelial cells become more cuboidal. The formation of alveolar ducts and air sacs happens in the saccular phase. The mesenchymal tissue thins out further. Most of the epithelial cells flatten and others remain cuboidal. Many developing air sacs open. The flattened epithelial cells produce a thin air-blood barrier. At this stage, epithelial proliferation decreases and differentiation becomes more evident. Distinctive lamellar bodies can be observed in the cytoplasm of the cuboidal epithelial cells. These epithelial cells begin to synthesize and secrets pulmonary surfactant and are now defined as alveolar epithelial type 2 cells (AEC II). The markers for these cells include surfactant-related proteins, specific cytokeratins, and various membrane-associated proteins. Many AEC II lose the ability to synthesize surfactant and undergo a thinning process to become the squamous type 1 cells (AEC I). T1α and Aqp5 are the markers for AEC I [235]. There are also other differentiation markers widely used to identify different cell types. CD31 is a marker for endothelial cells and is expressed in all pulmonary endothelial cells [236]. α-smooth muscle actin and the smooth muscle myosin heavy-chain are reliable markers for smooth muscle cells [237, 238]. CC10 is a cell-

specific marker of airway Clara cells. Neuroendocrine cell markers include neuron-specific enolase, chromogranin A, and protein gene product 9.5 [239]. SP-A is a lung epithelial cell marker. Vimentin is a widely used mesenchymal marker.

1.3.3 Molecular regulation and signaling pathways

Before the lung separates from the foregut, TTF-1 (thyroid transcription factor-1) is the earliest known marker that exists in the endodermal cells committed to the prospective lung [240]. This region is marked by the coexpression of TTF-1 and FOXA2 [241, 242]. Outgrowth and branching of cells in this region require fibroblast growth factors (Fgf's) emanating from the heart, which arises from the mesoderm [243].

In the embryonic and pseudoglandular phase, Fgf10 in the mesenchyme and Fgfr2 in the endoderm are crucial for the formation of lung primordial buds [244, 245]. Gli2 and Gli3 are transcriptional effectors of the Shh signaling pathway and have been demonstrated to play important roles in the formation of the lung primordium [246-248]. It is believed that Shh at the bud tips limits bud outgrowth by inhibiting expression of Fgf10 when the bud grows toward the Fgf10-expressing mesenchyme. Retinoic acid (RA) and retinoic acid receptors (RARs) are critical in bud formation, as the expression of Fgf10 is dependent on RA [249, 250]. TTF-1 is required for peripheral branching morphogenesis [251]. The Wnt/β-catenin pathway regulates branching morphogenesis and peripheral airway cell differentiation [252, 253]. This pathway functions at a point upstream of N-myc, BMP4, and FGF signaling [253]. FOXA1 and FOXA2 play critical roles in branching and later cell differentiation. Deletion of both *Foxa1* and *Foxa2* inhibits the Shh signaling pathway [254]. Other protein factors that regulate branching morphogenesis include GATA-6, nuclear factor-1 (NF-1), and N-Myc [255-257]. There are also some important transcription factors identified in the pulmonary mesenchyme, which regulate branching morphogenesis, such as FOXF1, FOXM1B, POD1, HIF, GLI, T-BOX, and HOX family

members. T-box transcription factors (Tbx) like Tbx4 have also been implicated in the early stage of embryonic lung development [258]. Inhibition of TBX4 and TBX5 suppress the expression of FGF10 [259].

TTF-1, NF-1β, GATA-6, RB, ETS, SOX, and FOX family members play roles in controlling epithelial cell differentiation in the conducting airways. p63 is important in the formation of normal tracheobronchial epithelium and deletion of p63 results in the abnormal epithelium without basal cells [260]. FOXJ1 is required for ciliogenesis by regulating the expression of genes necessary for ciliogenesis, such as ezrin and calpastatin [261, 262]. The development of pulmonary neuroendocrine cells is influenced by several transcription factors, such as MASH1/HASH1, HES1, GFI1, and pRB [263, 264]. The members of SOX family are expressed in the developing lung include SOX2, 4, 9, 11, and 17. The ETS family members, including ETS-1, SPDEF, ELF-3, ESE-3, ERM, and PEA3, are also found in the fetal lungs. ERM and SPDEF bind to TTF-1 and regulate transcriptional target genes synergistically [265].

The transcriptional factors that regulate perinatal lung maturation in the saccular and alveolar phases include TTF-1, FOXA2, NFATc3, C/EBPα, and the glucocorticoid receptor (GRα). Deletion of *TTF-1, Cebpα, and Foxa2* genes influence the expression of some regulating genes, such as *Abca3, Scd-1, Pon-1, Kdap, Sftpa, Sftpb, Sftpc, Sftpd, Aq5, Scnn1g, Scl34a2, Vegfa, Lys,* and *Scgb1a1* [243]. Other partners of TTF-1 such as NF-1, NFATc3, and GATA-6 also play roles in the perinatal lung maturation in a similar way. Glucocorticoids increase surfactant lipid synthesis by increasing the expression of enzymes for lipid synthesis [266]. They can also influence the expression of epithelial sodium channels and Na^+-K^+-ATPase, resulting in the modulation of lung liquid resorption at birth [267]. Three members of the FOXP family (FOXP1, FOXP2, and FOXP4) are expressed in epithelial cells and repress activity of the CCSP and SP-C promoters [268].

Other transcription factors and signaling pathways also regulate the development of fetal lung, including E2F1, RB, SMADs, the TGF-β superfamily, BMPs, and the Notch signaling pathway. SMAD1 and SMAD7 positively regulate pulmonary branching morphogenesis, while SMAD2, 3, and 4 negatively regulate this process [269-272]. Inhibition of TGF-β type II receptor stimulates branching morphogenesis in lung organ cultures [270]. These observations indicate that the activation of TGF-β signaling pathway is inhibitory for branching morphogenesis [273]. In addition, TGF-β1 is a potent inhibitor of epithelial cell proliferation and differentiation *in vivo* [274].

Functions of the BMP signaling pathway in developing lungs are different from those of the TGF-β signaling pathway. BMP4 is highly expressed in the distal epithelium of branching airways and regulates epithelial proliferation and proximal-distal pattern formation during branching morphogenesis. A high level of BMP4 stimulates distal lung formation, while a low level of BMP4 initiates proximal differentiation [275].

The Notch signaling pathway modulates embryonic branching morphogenesis and cell differentiation. The Notch receptors are Notch1-Notch4, while the ligands include Jagged1, 2 and Delta-like 1, 3, 4 (Dll1, 3, 4). Expression of Notch1-Notch4, DLL1, and Jagged1 in the mouse lung is increased from E11.5 to adulthood [264, 276]. HES1 is a transcription factor and also a downstream effector of the Notch signaling pathway. HES1 is essential for Clara cell differentiation, while loss of HES1 results in aberrant lung neuroendocrine differentiation [264]. Silencing Notch-1 increases mouse lung branching morphogenesis on E11.5. In addition, silencing of Notch-1 or Jagged-1 results in an increase in the amount of neuroendocrine cells [277].

1.3.4 Epithelial-mesenchymal communications

The development of pulmonary epithelium is under the control of inductive stimuli from the mesenchyme, at least in the embryonic and glandular phases [278]. If the mesenchyme is removed from lung rudiments before branching happens, there is no branching and all further lung morphogenesis is blocked. Whether the pulmonary epithelium acquires a proximal or distal phenotype also depends on the instructive cues from its associated mesenchyme [279].On the other hand, platelet derived growth factor A (PDGF-A) secreted from lung epithelial cells provides survival signals to mesenchymal cells during alveolarization [280]. Taken together, communications between the epithelium and mesenchyme occur in both directions [281]. Epithelial-mesenchymal communications are carried out by short-range interactions mediated by soluble and diffusible signals. These signals have already been discussed in the section of molecular regulation, which include Fgf factors (e.g. Fgf10) and Fgfr receptors (e.g. Fgfr2), proteins in Shh signaling pathway (e.g. Shh and Gli2/3), components in the canonic Wnt pathway (e.g. Wnt7b, Wnt5a, and β-catenin), and members of the TGF-β superfamily (e.g. BMP4).

1.4 Acute lung injury and repair

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is characterized by acute onset of respiratory failure, non-cardiogenic edema, and hypoxemia [282, 283]. The definition for ALI and ARDS are the same except that ALI is characterized by less severe hypoxemia (a ratio of the partial pressure of arterial oxygen to that of inspired oxygen of ≤ 300). Based on the clinic disorders associated with the development of ALI/ARDS, there are two forms of ALI/ARDS. Primary ALI/ARDS is caused by direct lung injuries, for example, pneumonia, aspiration of gastric contents, pulmonary contusion, etc. Secondary ALI/ARDS results from indirect lung injuries, such as sepsis, acute pancreatitis, etc. [282].

There are two stages of ALI/ARDS: the acute/exudative phase and the reparative phase. The acute phase is characterized by diffuse damage of the alveolar-capillary barrier and influx of

protein-rich edema fluid. The later reparative phase is characterized by fibroproliferation and remodeling of the lung. In some patients with ALI/ARDS, the injury may be resolved and repaired completely, while in others, the injury cannot be repaired normally and progresses to fibrotic lung injury and often fibrosing alveolitis, with persistent hypoxemia, alveolar space filled with fibroblasts, decrease in lung compliance, and an increase in mortality [284-286].

1.4.1 Alveolar-capillary barrier damage

The alveolar-capillary barrier consists of the alveolar epithelium and the lung microvascular endothelium. It prevents the exchange of components between the alveolar space and the capillary. Influx of protein-rich edema fluid into the alveolar space results from increased permeability of the alveolar-capillary barrier [282, 287]. The endothelial injury in the acute phase of ALI/ARDS leads to an increase in vascular permeability and flux of fluid and proteins from pulmonary capillaries to the lung interstitium [288]. The ultra-structural basis of increased microvascular permeability lies in cell rounding, interruption of interendothelial junctions, and interendothelial gap formation [289, 290]. There are at least two mechanisms of increased permeability: (1) Inflammatory mediators induced during ALI activate RhoA by G-protein-coupled receptors, leading to actin polymerization and formation of stress fibers. Phosphorylation of myosin light chain by myosin light chain kinase (MLCK) activates myosin, providing the force required for cytoskeletal contraction [291]. (2) TNF-α destabilizes endothelial junctions and induces pulmonary vascular permeability by a MLCK independent mechanism. TNF-α induces phosphorylation of vascular endothelial cadherin by activating Src-family tyrosine kinase [292]. Tyrosine phosphorylation of cadherin impairs cadherin ectodomain hemophilic adhesion [293, 294].

As with endothelial injury, the epithelial integrity is also disrupted in ALI/ARDS. Ultra-structural alterations of alveolar epithelium in ALI/ARDS include cytoplasmic swelling, bleb

formation, necrosis, and denuding of epithelial cells, leaving a mainly intact basement membrane with an overlying hyaline membrane [284, 285, 288]. This disruption of lung epithelium contributes to pulmonary edema in several ways. First, the epithelial barrier is damaged, which means the fluid and proteins from the lung interstitium are able to move into the alveolar space. Second, upon injury, AEC II lose their ability to transport fluid. Thus the capacity to remove edema fluid from the alveolar space is impaired, which may contribute to the severity and duration of pulmonary edema [295, 296]. Besides pulmonary edema, epithelial injury has other consequences which lead to additional symptoms observed in ALI/ARDS. First, a major function of AEC II is to synthesize and secrete pulmonary surfactants. These functions are impaired after AEC II damage, contributing to the surfactant dysfunction observed in ALI/ARDS [297, 298]. Second, as described in more detail later, abnormal repair of the alveolar epithelium during the reparative phase may lead to fibrosis [299]. Finally, injured AEC II in bacterial pneumonia produce pro-inflammatory mediators, which may leak into blood circulation through the permeabilized alveolar epithelium, resulting in septic shock [300].

1.4.2 Inflammation

An inflammatory response is another hallmark of the acute phase of ALI/ARDS. It involves endothelial and epithelial cells, macrophages, monocytes, and neutrophils, as well as cytokines and chemokines secreted by these cells. Alveolar macrophages secrete pro-inflammatory cytokines, such as interleukin-8 (IL-8) and tumor necrosis factor α (TNF-α). These cytokines and various chemokines stimulate chemotaxis, which attracts and activates neutrophils. The activated neutrophils are recruited to the injured capillary endothelium and migrate into interstitial and alveolar spaces through the disrupted alveolar-capillary barrier. These activated neutrophils also release pro-inflammatory mediators, proteases, and reactive oxygen species (ROS), which causes further damage to the tissue. Neutrophils are the first immune cells to arrive on the scene after lung injuries. Circulating monocytes arrive at the injury site after neutrophils.

They migrate across the endothelium and transform into macrophages. As with alveolar macrophages and recruited neutrophils, these macrophages transformed from monocytes also produce inflammatory factors [282, 286, 301].

1.4.3 Resolution of ALI/ARDS

Resolution of ALI/ARDS includes several events. Alveolar edema fluid and proteins must be cleared from the alveolar space. Inflammation must be cleared and the inflammatory cells removed from alveolar space and interstitium. Injured endothelial and epithelial cells must also be repaired and the disrupted alveolar-capillary barrier must be restored. Functional alveolar structure must be restored for efficient gas exchange to occur.

The removal of alveolar edema is driven by active transport of sodium and chloride between alveolar spaces and nearby interstitium [302]. In order to keep the isosmotic stasis, water follows sodium by passive transportation into lung interstitium through aquaporins, which are transcellular water channels located primarily on AEC I [303].

Proteins in the alveolar edema fluid are cleared from the alveolar space in several different ways. The clearance of most of soluble proteins occurs through diffusion across alveolar epithelial cells, a process that is protein size dependent [304]. Some other proteins are cleared from alveoli through endocytosis or transcytosis by epithelial cells. This mechanism is mediated by receptors and is protein specific, but not size dependent. Some proteins are also degraded by enzymes on the surface of the alveolar epithelial cells with absorption of smaller fragments. In addition, alveolar macrophages can remove insoluble proteins from alveoli through endocytosis and degradation [304].

Inflammatory cells must be cleared from alveolar space to prevent further damage to endothelium and epithelium. Thus, the clearance of neutrophils is considered to be the first step in resolution of ALI/ARDS [305]. Apoptosis is thought to be the mechanism for neutrophil

clearance. The apoptotic neutrophils are then engulfed by macrophages via recognition of certain surface receptors (e.g. phosphatidylserine receptors), which switches the role of neutrophils from pro-inflammatory cytokine production to secretion of pro-resolution mediators (for example TGF-β) [305-308]. An alternative theory is that egression of neutrophils removes them from lung tissue, and they migrate into the airway lumen, where they were then undergo apoptosis and engulfment by macrophages. The mixture of secretions and exudates is removed by mucociliary escalator mechanisms [309].

The mechanism for resolution of endothelial damage is not well known. It is likely that endothelial cell migration and proliferation are involved in this process. It is also known that circulating endothelial cell progenitors participate in the repair of injured endothelium [310].

Based on current knowledge, the restoration of alveolar epithelium after injuries is mainly dependent on AEC II. AEC II are considered as the progenitors of alveolar epithelial cells. After injuries and loss of cell-cell contact, normally quiescent AEC II regain the ability to proliferate. AEC II migrate, proliferate, and trans-differentiate into AEC I cells. The denuded base membrane is then covered by AEC II and AEC I and the epithelium barrier is restored. In recent studies, researchers have found that there may be a population of bronchioalveolar stem cells (BASCs) located at the bronchioalveolar duct junction [311]. These BASCs can self-renew and maintain the population of bronchiolar Clara cells. However, whether these cells contribute to the re-epithelialization after epithelial injury is unknown. Several groups have reported that bone marrow-derived stem cells (BMSC) can differentiate into lung epithelial cells after injury [312]. However, none of them are conclusive. Restitution of alveolar epithelium has at least two roles. First, it restores the normal alveolar architecture and gas exchange function. Second, proliferation of AEC II facilitates the clearance of pulmonary edema from alveolar space by increasing the fluid-transport capacity.

1.4.4 Factors and pathways in epithelial repair

The restitution of alveolar epithelium involves multiple factors, such as cytokines, chemokines, growth factors, matrix components, and cell surface proteins. Several signaling pathways are also involved in epithelial wound healing after ALI/ARDS.

Epidermal growth factor (EGF) family proteins, including EGF and transforming growth factor-α (TGF-α) participate in epithelial repair. These EGF factors and their receptors are up-regulated in response to lung injury. They promote proliferation, migration, and spreading of AEC II and are likely to contribute to epithelial recovery *in vivo* after ALI/ARDS [313-316]. Some members of the fibroblast growth factor (FGF) family are also involved in alveolar epithelial repair, including keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and FGF-10. KGF is a mitogen for AEC II and promotes AEC II migration and adhesion as well as proliferation [317-321]. Instillation of KGF into the lung leads to hyperplasia of AEC II, which differentiate into AEC I or undergo apoptosis [322, 323]. Similar to KGF, it has been shown *in vitro and in vivo* that HGF is a mitogen for rat AEC II [324, 325]. In addition to promoting epithelial repair, FGF-10 can prevent and attenuate epithelial cell DNA damage induced by asbestos, H_2O_2, and mechanical stretch through activating the MAPK/ERK pathway [326-328]. Inflammatory mediators are another group of factors that regulate epithelial repair. However, their functions are context-sensitive. For example, pro-inflammatory interleukin IL-1β has been shown to stimulate the repair of injured rat AEC II [329]. Because cell spreading and migration are important in epithelial restitution, it is reasonable to anticipate that the coordination between integrin and the underlying matrix are important for epithelial repair. It has been shown that migration of rat AEC II on fibronectin and type I collagen depends on different combinations of integrin subunits [330]. Several matrix metalloproteinases (MMP) have been shown to be involved in remodeling of the extracellular matrix, thus facilitating lung epithelial cell migration in lung epithelial repair. These MMPs includes MMP-1, MMP-7, and MMP-9 [331-333]. Rho

GTPases, which are involved in the increase of endothelial permeability in the acute phase of ALI/ARDS, regulate cytoskeletal remodeling and wound healing of epithelial cells in the reparative phase. For example, as mentioned previously, RhoA is responsible for actin bundle organization and formation of stress fiber and large focal adhesions. Expression of constitutively activated RhoA inhibits epithelial cell recovery from injury by inhibiting attachment and migration of AEC II [320].

The signaling pathways that are involved in epithelial repair after ALI/ARDS include sonic hedgehog (Shh) signaling pathway, mitogen-activated protein kinase (MAPK) pathways, Wnt pathway, and TGF-β signaling pathway. The Shh pathway is activated in epithelial cells during airway epithelial repair from acute injury and is implicated in epithelial-mesenchymal transition (EMT) [334]. MAPK signaling pathways are well known to be important in cell proliferation, differentiation, and migration. However, the studies of MAPK functions in epithelial repair after ALI/ARDS are controversial. Their functions depend on the context. For example, macrophage migration inhibitory factor (MIF) induces phosphorylation of ERK1/2 and proliferation of AEC II through binding to CD74. However, MIF cannot activate ERK1/2 in AEC I that lack CD74 [335]. TGF-β signaling pathway is another one that plays important and multiple roles in repair. It regulates cell proliferation, differentiation, apoptosis and extracellular matrix production [336]. It is also well known for its capability to stimulate epithelial-mesenchymal transition (EMT), which is discussed below in detail. The target genes induced by TGF-β signaling include connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA), collagen 1A2, and plasminogen activator inhibitor-1 (PAI-1) through Smad-dependent signaling [337]. Some of these targets, such as CTGF and α-SMA, are used as markers of EMT. TGF-β can stimulate migration and scratch wound repair in AEC II [338, 339]. In addition, it has been shown that TGF-β promotes AEC II repair after hyperoxic injury [339]. The Wnt signaling pathway is critical in fetal lung development and is discussed in detail in section 1.5. It is also involved in

injury healing in ALI/ARDS as repair from injury is considered to recapitulate ontogeny. The target genes of Wnt signaling include several MMPs, such as MMP2, MMP3, MMP7, and MMP9 [340]. As discussed previously, these MMPs are pivotal to tissue remodeling, cell migration, and repair from injury. Proteolytic degradation of extracellular matrix facilitates migration of fibroblasts to the site of lesion.

1.4.5 Abnormal repair and fibrosis

After the acute phase of ALI/ARDS, some patients have a rapid resolution of lung damage. The inflammation is cleared, the denuded base membrane is re-epithelialized and re-endothelialized, and the interrupted alveolar-capillary barrier is restored. However, in some patients, the disorders cannot be resolved and progress to fibrotic lung injury or fibrosing alveolitis. The alveolar space is filled with mesenchymal cells and proliferating fibroblasts, together with new blood vessels [341]. The content of collagen types I and II and fibronectin is increased in the lungs of these patients [342]. The decision whether ALI/ARDS results in a rapid resolution of abnormal fibroproliferative repair is made at the early point of the disorder and depends on the integrity of alveolar-capillary barrier base membrane [282]. If the base membrane is intact, the functional alveolar-capillary barrier can be reestablished with re-epithelialization and re-endothelialization on the intact base membrane. However, if the integrity of base membrane is disrupted by severe lung injury and prolonged inflammation, the alveolar structure is lost. Without the intact base membrane and alveolar structure, the proliferation and migration of AEC II, endothelial cells, and fibroblasts lead to alveolar fibrosis [343].

1.5 Wnt/β-catenin signaling pathway

Since the first Wnt ligand, Wnt-1/int-1, was discovered by Nusse and Varmus in 1982 [344], the Wnt signaling pathway has been demonstrated to be a key regulatory mechanism for cell proliferation and cell fate determination during embryogenesis and in adult tissue

homeostasis. The Wnt signaling pathway is a pleiotropic pathway that can be divided into two categories: the canonical Wnt pathway in which Wnt signaling goes through its receptors Frizzleds to β-catenin, and the non-canonical Wnt pathways which do not require β-catenin involvement.

1.5.1 Wnt ligands

Wnts are a family of secreted cysteine-rich glycolipoproteins that have conserved sequences in all metazoan animals. There are 19 Wnt ligands in mammals. They have a highly conserved distribution of cysteines and contain an N-terminal signal peptide for secretion. Although they are secreted proteins, Wnt proteins are palmitoylated and thus more hydrophobic than predicted from their amino acid sequences [345]. The glycosylations and lipid modifications are indispensable for the activity and secretion of Wnt proteins. For example, glycosylation is required for active Wnt3a secretion [346]. Wnt3a without glycosylation is retained in endoplasmic reticulum (ER), while Wnt3a with glycosylation is present in both ER and Golgi. Two types of palmitoylations of Wnt3a have been reported. One is palmitoylation at cysteine 77, which is required for Wnt3a to bind with its receptors and to activate β-catenin signaling [346]. The other lipid modification happens at serine 209. Wnt3a mutated at this site cannot be secreted from cells and accumulates in the ER [347].

The palmitoylations and the hydrophobic character of Wnt proteins raise an issue for their diffusion and distribution in the aqueous extracellular space. For long range Wnt signaling, Wnt proteins may form multimers to bury hydrophobic parts inside, or bind with lipoprotein particles [348, 349].

1.5.2 Activation and inactivation of Wnt/β-catenin signaling

Wnt/β-catenin is inactivated in the absence of Wnt ligands. Cytoplasmic β-catenin is phosphorylated and degraded by the destruction complex. The destruction complex contains Axin,

adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3), and casein kinase 1 alpha (CK1α) amongst other factors. The scaffolding protein Axin binds with GSK3, CK1α, and β-catenin using different domains, which bring these core components into proximity. β-catenin is phosphorylated at serine 45 by CK1α and then at threonine 41, serine 37, and serine 33 successively by GSK3 [350]. In addition, GSK3 and CK1 phosphorylate Axin and APC, which enhance their binding with β-catenin and further promotes β-catenin phosphorylation. β-catenin phosphorylation at the N-terminus creates a binding site for β-TRCP (beta-transducin repeat containing protein), causing the ubiquitination of β-catenin by an E2 ligase. The ubiqutinated β-catenin is then degraded by proteosomes [350, 351].

In the canonical Wnt/β-catenin pathway, Wnt ligands bind to seven-pass transmembrane receptors, Frizzled (FZD) and the co-receptors LRP5/6 (low-density lipoprotein receptor related protein 5/6) [351, 352]. This binding leads to the activation of Dishevelled (DVL) and phosphorylation of LRP. The binding of Wnt with FZD and LRP recruits Axin to the membrane. Wnt signaling activation inhibits β-catenin phosphorylation by GSK3, through Axin-GSK3 dissociation, inhibition of GSK3, or Axin degradation [351]. The stabilized β-catenin then accumulates in the nucleus to form complexes with transcription factors TCF/LEF (T cell factor/lymphoid-enhancing factor) and regulates the expression of downstream β-catenin dependent genes, such as cyclin D1 and c-myc.

1.5.3 Wnt/β-catenin signaling pathway and fetal lung development

Given that the Wnt/β-catenin signaling pathway is important in the regulation of cell proliferation and differentiation, it is not surprising that Wnt/β-catenin signaling pathway plays important roles in fetal lung development. It has been reported that Wnt3a represses growth and proliferation of the lung, while knockdown of Wnt signaling results in more branches and cell proliferation [353]. However, it has also been shown that Wnt/β-catenin signaling is required for

the formation of the distal airways, but not proximal airways [252]. Without β-catenin in the epithelial cells, the proximal lung tubules grow and differentiate normally. However, the peripheral airways fail to develop normally and the mice die at birth of respiratory failure. Another study has shown that this regulation of the proximal-distal differentiation of airway epithelium by Wnt/β-catenin signaling is in part through N-myc, BMP4, and Fgf signaling [253]. The differentiation of the respiratory epithelium is also regulated by the Wnt/β-catenin signaling pathway [354]. An activated form of β-catenin causes ectopic differentiation of alveolar type II-like cells in conducting airways. Goblet cell hyperplasia and air space enlargement are also observed in early postnatal lungs with activated β-catenin. The mesenchymal proliferation and vascular development in the lung are regulated by Wnt7b [235]. Wnt7b is expressed in the airway epithelium and knockout of Wnt7b leads to perinatal death due to respiratory failure. Wnt7b knockout causes defects in both mesenchymal proliferation and the smooth muscle component of the major pulmonary vessels.

1.5.4 Cross-talk between the Wnt signaling pathway and other pathways in fetal lung development

Although the Wnt signaling pathway plays an important role in fetal lung development as discussed above, it does not function independently. Some studies have shown that the Wnt signaling pathway is involved in cross-talk with other signaling pathways, such as the TGF-β, Shh, and FGF pathways, which makes their regulation more specific and versatile.

The Wnt signaling pathway interacts with the TGF-β signaling pathway in the regulation of cell differentiation during fetal lung development [355]. Inactivation of a BMP type I receptor disrupts lung morphogenesis, cell proliferation and differentiation. In the meantime, the Wnt signaling pathway is stimulated, probably through down-regulation of Wnt inhibitor factor-1 (WIF-1) after Alk3 inactivation [356]. On the other hand, the Wnt/β-catenin pathway functions

upstream of BMP4. Activation of the Wnt signaling pathway with stabilized β-catenin in lung epithelial cells also activates BMP4, resulting in inhibition of cell differentiation [357]. Meanwhile, inhibition of this pathway with Wnt inhibitor Dkk1 or deletion of β-catenin depresses expression of BMP4 [253].

The non-canonical Wnt pathway stimulated by Wnt5a interacts with the Shh signaling pathway and FGF signaling. It has been shown that loss of Wnt5a increases expression of Shh and its receptor Ptc, while overexpression of Wnt5a in lung epithelium inhibits the activity of epithelial Shh signaling and increases expression of Fgf10 in the mesenchyme [358, 359].

1.6 Purpose and significance of this research

microRNAs are a group of small RNAs that regulate their target gene expression at the post-transcriptional level. The functions of microRNAs have been studied extensively in recent years. They cover almost every aspect of cell physiology and are extensively involved in human diseases. Studies on microRNA functions help us to understand gene regulation in physiology and pathology from a novel perspective. The discoveries shed light on development of new therapeutic interventions for human diseases.

The purpose of the first part of my study (Chapter 2) was to set up an efficient high-throughput method to profile expression patterns of microRNAs. When I started my study, little was known about microRNA expression in the lung, as well as microRNA expression profiles in various processes in the lung, such as alveolar epithelial trans-differentiation, fetal lung development, and recovery after injuries. Characterization of the microRNA expression profile was still a great challenge at that time. In order to resolve this problem, I set up a microRNA microarray platform and profiled the expression of microRNAs in different organs of the rat. A novel data mining method was used in microarray data analysis. The method was tailored according to the unique characters of our microRNA microarray slides. I further verified the

microRNA microarray data with microRNA Northern blots, which showed that the microRNA microarray was surprisingly sensitive and specific. The data were extremely reliable and reproducible. This microRNA microarray platform was then used in my later studies.

In the second part of my study (Chapter 3), we selected miR-127 from our microarray data for further study. We found that it was most highly expressed at the late stage of fetal lung development and the expression of miR-127 shifted from mesenchymal cells to epithelial cells during this development process. We further found that overexpression of miR-127 led to disruption of fetal lung branching morphogenesis.

The goal of the third part of my study (Chapter 4) was to figure out how microRNAs regulate alveolar epithelial trans-differentiation. In this part, I profiled the expression of microRNAs during alveolar epithelial trans-differentiation with the microRNA microarray platform described in Chapter 2. The result showed that miR-375 was significantly down-regulated during alveolar epithelial trans-differentiation, which was verified with qRT-PCR. Furthermore, I demonstrated that miR-375 interrupted alveolar epithelial trans-differentiation through inhibiting the Wnt/β-catenin signaling pathway by directly targeting FZD8. This is a new mechanism that regulates alveolar epithelial trans-differentiation. This discovery helps to understand the mechanism of the recovery process after lung injury from a new angle and may provide a new target for therapy.

The fourth part of my study (Chapter 5) focused on microRNA functions in fetal lung development. qRT-PCR showed that miR-124a was down-regulated during fetal lung development. I demonstrated that miR-124a inhibited epithelial cell maturation during late stages of fetal lung development. miR-124a and its targets NFIB, could be potential therapeutic targets to improve epithelial maturation in the lungs of premature babies.

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CHAPTER II

IDENTIFICATION OF RAT LUNG-SPECIFIC MICRORNAS BY MICRORNA MICROARRAY: VALUABLE DISCOVERIES FOR THE FACILITATION OF LUNG RESEARCH

2.1 Abstract

An important mechanism for gene regulation utilizes small non-coding RNAs called microRNAs (miRNAs). These small RNAs play important roles in tissue development, cell differentiation and proliferation, lipid and fat metabolism, stem cell function, exocytosis, diseases and cancers. To date, relatively little is known about functions of microRNAs in the lung except in the setting of lung cancer.

In this study, we utilized a rat microRNA microarray containing 216 microRNA probes, printed in-house, to detect the expression of microRNAs in the rat lung compared to the rat heart, brain, liver, kidney and spleen. Statistical analysis using the Significant Analysis of Microarray (SAM) and Tukey Honestly Significant Difference (HSD) tests revealed 2 microRNAs (miR-195 and miR-200c) that were expressed specifically in the lung, and 9 microRNAs co-expressed in the lung and another organ. Twelve selected microRNAs were verified by Northern blot analysis.

The identification of lung-specific microRNAs from this work will facilitate functional studies of microRNAs during normal physiological and pathophysiological processes of the lung.

2.2 Introduction

MicroRNAs (miRNAs) include a large group of regulatory, non-coding small RNAs that measure ~22 nucleotides (nt) in length [1, 2]. In animal cells, microRNAs are first transcribed from genes by the RNA polymerase, pol II, as primary microRNAs, which are then cleaved by an RNase III enzyme, Drosha, into hairpin-like precursor microRNA (pre-miRNA) [3]. The pre-miRNAs are transported into the cytoplasm with the help of a protein called Exportin 5 [4]. In the cytoplasm, the pre-miRNAs are cut into double-stranded RNA duplexes by another RNase III enzyme, Dicer [5]. Typically, one of the strands becomes mature microRNA and is incorporated into a RISC complex with other components for target recognition [6]. The RISC complex then binds to its target mRNA through base pairing and carries out its functions. Customarily, in the target mRNAs of an animal microRNA, there are multiple complementary sites, which correspond to the same microRNA. This cooperative action makes inhibition more efficient [7, 8]. On the other hand, one microRNA usually inhibits multiple target mRNAs [9, 10]. This property enables microRNA to regulate many genes in a pathway or physiological process at the same time. The action of microRNAs includes cleavage of target mRNA, translational inhibition, and mRNA deadenylation [11-15]. Several studies have shown that the degree of complementarity between the microRNA and its target determines the mode of how a microRNA works [11, 13-16]. Since the discovery of the first microRNA, *lin-4* [9], much progress has been made in the elucidation of microRNA mechanisms [1, 2, 9, 17-20]. However, only a handful of microRNAs have been studied for their precise functions. The microRNAs in animals function in tissue development, cell differentiation, apoptosis, fat and lipid metabolism, exocytosis, stem cell division and differentiation, diseases and cancers [17, 21-33]. These known functions may represent just a small part of a much bigger picture. One third of the genes in the human genome are predicted to be microRNA targets [34]. With the continuing discovery of new microRNA

functions, it is possible that microRNAs will be associated with the regulation of almost every aspect of cell physiology.

Spatial and temporal expression patterns of microRNAs can provide clues for their possible functions. Profile studies have already shown that many microRNAs are specifically expressed in certain organs, cell types, and developmental stages. In a recent study, microRNA expression patterns among different pancreas cell types were compared [28]. One of the detected microRNAs in the study was identified as a pancreas islet-specific microRNA, which was later demonstrated to function in insulin secretion [28].

In order to study the expression profiling of microRNAs in mice and humans, several high throughput platforms have been developed. Microarrays on either membranes or slides are widely used for this purpose [35-43]. Various probe designs and labeling methods have also been utilized. Some groups have applied these microarray methods in an effort to perform the expression profiling of microRNAs in different tissues and cell types in humans and mice. Real-time PCR has also been used to detect the expression of pre-mRNAs and mature microRNAs [44, 45].

The purpose of this study is to identify microRNAs that are expressed specifically in the rat lung or co-expressed in the lung and one of the five other organs in the rat. In addition, this study aims to set up a reliable, in-house microRNA microarray platform for lung research. Although the expression profiling of microRNAs in human and mouse organs has been described by some groups, the microRNAs expression profile in rat organs has not been extensively studied. Furthermore, relatively little is known about the detailed functions of microRNAs in the lung, except in lung cancers. For this study, we designed a probe set for rat microRNAs as well as human and mouse microRNAs that possess conservative sequences in the rat genome, based on the fact that microRNAs are highly conserved in animals and in plants [46-51]. The

hybridizations were performed with slides printed in our laboratory. The reliability of the microarray platform was tested before other experiments were performed. As a result, our microarray allowed us to identify lung-specific microRNAs in the rat that are likely to facilitate studies of microRNA functions in the lung.

2.3 Material and Methods

2.3.1 Microarray fabrication

Sequences of 217 mature microRNAs were downloaded from the microRNA registry (Wellcome Trust Sanger Institute). Among these, 177 microRNAs were from rat, and 40 non-redundant conservative ones were from human or mouse. Sequences of some of these human and mouse microRNAs did not match their corresponding sequence in the rat genome exactly and were modified in accordance with those in the rat genome. The probes for the microRNAs had two copies of antisense sequences (34–50 nt) (Fig. II1A). Probe sets, which contained 5' amino modified C6 oligos were synthesized by Sigma-Genosys (Woodlands, TX) at 100 μM concentration and suspended in 3 × SSC buffer. The oligos were diluted to 25 μM with 3 × SSC prior to use. The probes were then printed onto epoxy-coated slides (CEL Associates, Pearland, TX) with an OmniGrid 100 array (GeneMachine, San Carlos, CA) at 65% humidity and then incubated for 48 hours at the same humidity. Each slide contained three identical blocks in a landscape orientation. Within each block every probe was printed 6 times in 3 separate pairs (Fig. II1B). The oligo set also contained one probe for U6 and 3 probes for tRNAs as positive controls as well as one probe for a plant microRNA as a negative control.

2.3.2 Tissue sample and small RNA extraction

Four male Sprague-Dawley rats (200 g, Charles River Laboratories, Inc., Wilmington, MA) were anaesthetized with intraperitoneal injection of Ketamine (40 mg/kg body weight) and Xylazine (8 mg/kg body weight). Six organs (lung, heart, brain, kidney, liver and spleen) were

collected and powdered in liquid nitrogen. Small RNA was enriched from the powdered samples using the *mir*Vana™ miRNA isolation kit from Ambion (Austin, TX), according to the manufacturer's protocol. First, 200 mg powder was homogenized in 2 ml Lysis/Binding Buffer. Then, one-tenth of the volume of microRNA homogenate additive was added to the homogenate and a volume of acid-phenol: chloroform was used to extract RNA. One-third volume of 100% ethanol was added to the aqueous phase, and the sample was passed through a filter cartridge. Two-thirds volume of 100% ethanol was then added to the filtrate, and the sample was passed through a second filter cartridge. The second filter cartridge was subsequently washed once with Wash Solution 1 and then twice with Wash Solutions 2 and 3. Afterwards, the small RNA was eluted with 95 ℃ nuclease-free water. Total RNA for Northern blots was also extracted from these organs by the aforementioned protocol. Following organic extraction, one and one-fourth volumes of 100% ethanol was added to the aqueous phase. The lysate/ethanol mixture was then passed through a filter cartridge. The cartridge was washed, and the total RNA was eluted with water as described above. The concentration of RNA was determined by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Tech., Rockland, DE). The quality of enriched small RNA was determined on a denaturing 15% polyacrylamide gel, and the quality of total RNA was tested on a 1% agarose gel.

2.3.3 MicroRNA labeling and microarray hybridization

The labeling and hybridization of microRNA were performed with the 3 DNA array900 microRNA direct kit (Genisphere, Hatfield, PA), according to the manufacturer's protocol. Enriched small RNA (120 ng) was used for each hybridization. First, the microRNA was tailed with poly A by PAP enzyme (poly (A) polymerase). Then the capture sequence was ligated to the tailed microRNA. Tagged microRNA was purified with the MinElute PCR Purification Kit (Qiagen, Valencia, CA). All of the small RNA samples were separately labeled with Cy3 or Cy5 capture sequence. After labeling and purification, equal amounts of small RNA from all the

samples, labeled with the same dye, were pooled together as a common reference. The hybridization was performed as previously described [52]. To each block, one labeled sample was hybridized along with a common reference labeled with the other dye. Dye-swap was performed to eliminate dye bias. Tagged microRNA hybridizations were performed at 52 ℃ overnight, and then the slide was washed 15 min in pre-warmed 2 × SSC, 0.2% SDS, followed by 12 min in 2 × SSC at room temperature and finally for 12 min in 0.2 × SSC at room temperate. The 3 DNA hybridization was performed at 62 ℃ for 4 h, and then the slide was washed and dried.

2.3.4 Microarray data analysis

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, Inc. Union City, CA). The signal from each spot was normalized to the average signal of the whole block. The highest and lowest signals from the 6 identical probes in the same block were excluded from the data analysis. The geometric average of the other 4 signals was considered to be the signal of that particular microRNA. The ratio of the sample signal to the reference signal was log2 transformed. A quality test was performed with the software, Realspot, developed in our laboratory [53]. The microRNAs with an average quality index of <1 were filtered. The microRNAs that passed the quality test were analyzed with SAM (Significant Analysis of Microarray) in order to choose microRNAs that were significantly changed between different organs ($q < 0.01$) [54]. These microRNAs were then subject to the Tukey Honestly Significant Difference (HSD) test ($p < 0.05$) [52]. The organ specificity index (OSI) was also used to determine the relative specificity of microRNA in organs. The OSI was defined as the correlation coefficient of microRNA expression between a microRNA and a putative microRNA whose expression levels were given the value of 1,000 in prominent organs and a value of 0 in other organs [52].

2.3.5 Northern blot analysis

Total RNA from the same organ was pooled together. The probe sequences were exactly the same as the antisense sequences to microRNAs, except that those with sequences which started with C were capped with G or T at the 5' end to increase ^{32}P labeling efficiency. RNA samples were denatured at 95°C for 4 minutes. 15 μg total RNA was separated on a 15% denaturing PAGE gel at 100 V for 2 h in 1 × TBE buffer. The RNA was then transferred to a Hybond-N^+ membrane (Amersham, Piscataway, NJ) using a Trans-blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA) at 20 ~ 25 V for 1 hour using 0.25 × TBE as a transfer buffer. Membranes were UV crosslinked with a 120 mJ burst and then baked at 80 ℃ for 1 hour. For each sample, 20 pmol antisense oligonucleotide probes were end labeled with γ^{32}P dATP (>7000 Ci/mmol, MP Biomedical, Irvine, CA) using T4 polynucleotide kinase (NEB, Ipswich, MA) for 4 hours at 37°C. The reactions were stopped with 2 μl 0.5 M EDTA. The probes were then purified with a G-25 MicroSpin column (Amersham). Pre-hybridization and hybridization were carried out at 30 ℃ using ULTRAhyb-Oligo hybridization buffer (Ambion), according to the manufacturer's manual. After hybridization the membranes were washed twice with 2 × SSC 0.5% SDS for 30 minutes at 30 ℃. The membranes were then exposed to a phosphor screen overnight and scanned with the Personal Molecular Imager® FX (Bio-Rad). U6 was probed as a loading control and only exposed to the phosphor screen for 5 to 10 minutes.

2.4 Results

2.4.1 Reproducibility and specificity of the microRNA microarray platform

In order to evaluate the reliability of the microRNA microarray, we tested the reproducibility of the data from multiple hybridizations (Table II1). First, equal amounts of enriched microRNAs from each of the 6 organs were pooled as the common reference. Then, microRNA from each organ (Cy3) was co-hybridized with the common reference (Cy5). The

maximum and minimum probe signals in the six replicates from each hybridization were excluded from data analysis. The microRNA signal was then calculated as the geometric average of the remaining 4 probe signals. The correlation coefficient of the microRNA signals of the common references (Cy5) between two hybridizations was calculated in order to assess the reproducibility of the microarray hybridization. The results are shown in Table II1. The correlation coefficients between two hybridizations were 0.89 – 0.98. For comparison, we also used one spot per block for calculations, where the correlation coefficients were 0.83 to 0.95. The results suggest that our arrays are highly reproducible.

To test the specificity of the microarray, we used mismatched probes for two microRNAs, *rno-miR-16* and *rno-miR-324-5p*, which were included in the control oligos provided with the labeling kit. The probes with one, two and three nucleotide mismatches were denoted with m1, m2 and m3, respectively. The microarray could not reliably distinguish those with only one mismatch. However, the signals from probes with two mismatches were significantly decreased, >20 fold less than those from probes with perfect base pairing (Fig. II2). From these results, we concluded that our microarray could differentiate between microRNAs with two or more nucleotide differences.

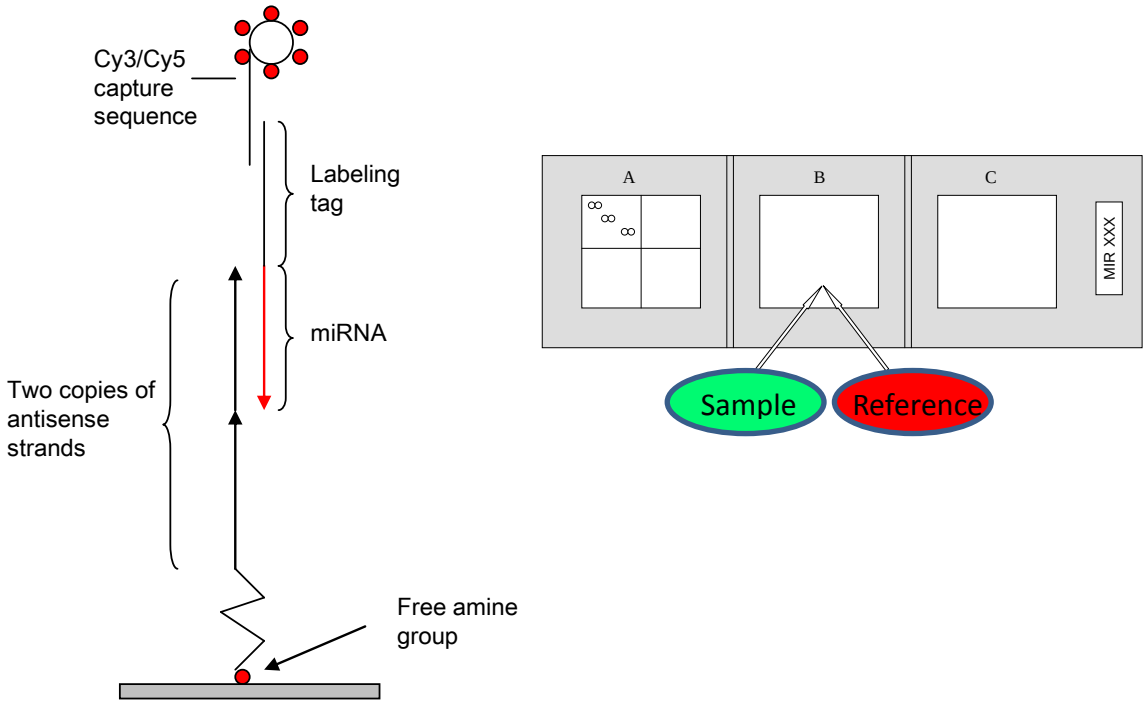


Fig. II1 microRNA microarray fabrication and hybridization strategy. (A) The probes were two copies of antisense oligos of mature microRNA. The 5' ends of the probes were amino modified. The probes were linked to epoxy-coated slides covalently. MicroRNA samples were tagged and hybridized to the slides. (B) Each slide contained three identical blocks and each block was hybridized with two-colored paired samples. There were 6 duplicated probes for each microRNA as shown in block A. MIR xxxx, series number.

Table II1: The correlation coefficients of common reference signals between two hybridizations

	lung	heart	brain	liver	kidney
heart	0.984393				
brain	0.980202	0.973659			
liver	0.973702	0.97738	0.965574		
kidney	0.96804	0.971573	0.965264	0.978328	
spleen	0.895349	0.893531	0.92511	0.917216	0.925989

Cy3-labeled organ and Cy5-labeled common reference (pooled samples from 6 organs) were co-hybridized. The correlation coefficients were calculated from the log2 transformed average signals of the common reference between two hybridizations.

2.4.2 Identification of lung-specific microRNA

In order to identify microRNAs that are prominently expressed in the rat lung or co-expressed in the lung and another organ, microRNA samples from 6 organs of 4 rats were co-hybridized with the common reference. A dye flip was subsequently performed by switching the dyes labeling the organ samples and the common reference. After scanning the slides, spots qualities were evaluated using the Realspot software [53]. Any microRNAs with average QI's no larger than 1 in any of the six organs were eliminated from data analysis. Out of 216 microRNAs, only 127 passed the Realspot quality test. The qualified microRNA signals were then tested by Significant Analysis of Microarray (SAM) to eliminate microRNAs that did not have significant changes between any of the six organs [54]. After the SAM test, another 21 microRNAs were eliminated, leaving 106 microRNAs for further study. Any microRNAs that were prominent in one organ or co-expressed in two organs were identified by Tukey Honestly Significant Difference (HSD) analysis ($P < 0.05$) (Tables II2, II3 and Fig. II3). Two microRNAs (*rno-miR-195* and *rno-miR-200c*) were identified as being expressed specifically in the rat lung. There are 5 and 3 nucleotide differences between *miR-200c,* and *miR-200a* and *miR-200b*, respectively. Therefore, our arrays were able to detect the difference between the isoforms. The numbers of the prominently expressed microRNAs in the heart, brain, liver, kidney and spleen were 6, 13, 5, 2 and 18, respectively. The numbers of co-expressed microRNAs are shown on the lines between the two organs in Fig. II3. The lung had more microRNAs co-expressed in the heart than with any other organ. This finding is likely due to the relationship between the lung and the heart in organogenesis.

After HSD analysis, we calculated the organ specificity index (OSI) for organ-microRNAs that passed the SAM test. There were 2, 5, 18, 5, 1, and 16 microRNAs prominently expressed in the lung, heart, brain, liver, kidney and spleen, respectively using OSI >0.90 as a criterion (Table II2). We also noted organ specificity if the microRNA expression in one organ

was at least two fold of that in all other organs. According to this two-fold definition, similar results were obtained (Table II2), showing the number of microRNAs exclusively expressed in only one of the 6 organs to be 2 (lung), 5 (heart), 14 (brain), 5 (liver), 2 (kidney), and 15 (spleen).

Table II2: Organ-specific microRNAs identified by HSD test, OSI test and two-fold criteria.

Lung (2)			Heart (6)			Brain (13)			Liver (5)			Kidney (2)			Spleen (18)		
	OSI	2 fold		OSI	2 fold		OSI	2 fold		OSI	2 fold		OSI	2 fold		OSI	2 fold
mo-miR-195	0.957	✓	mmu-miR-1	0.999	✓	mmu-miR-9*	0.999	✓	mo-miR-101b	0.998	✓	mo-miR-10a	0.975	✓	mmu-miR-17-5p	0.983	✓
mo-miR-200c	0.973	✓	mmu-miR-133b	0.998	✓	mo-miR-103	0.902		mo-miR-122a	1.000	✓	mo-miR-10b	0.899		mmu-miR-207	0.973	✓
			mo-miR-133a	0.999	✓	mo-miR-107	0.967	✓	mo-miR-192	0.956	✓	mo-miR-200b†	0.900	✓	mo-miR-106b	0.911	
			mo-miR-208	0.999	✓	mo-miR-124a	1.000	✓	mo-miR-194	0.914	✓				mo-miR-142-5p	0.976	✓
			mo-miR-22	0.975	✓	mo-miR-127	0.995	✓	mo-miR-31	0.994	✓				mo-miR-150	0.992	✓
			mo-miR-27a	0.886		mo-miR-128a	1.000	✓							mo-miR-15b	0.895	
						mo-miR-128b†	0.964	✓							mo-miR-16	0.857	
						mo-miR-130a	0.940	✓							mo-miR-17	0.971	✓
						mo-miR-132	0.999	✓							mo-miR-187	0.990	✓
						mo-miR-137†	0.958	✓							mo-miR-191	0.944	✓
						mo-miR-138	0.980	✓							mo-miR-20	0.982	✓
						mo-miR-181a	0.925	✓							mo-miR-206	0.998	✓
						mo-miR-218	0.982	✓							mo-miR-210	0.997	✓
						mo-iR-221†	0.916								mo-miR-297	0.997	✓
						mo-miR-29b	0.981	✓							mo-miR-328	0.980	✓
						mo-miR-29c†	0.906								mo-miR-333	0.997	✓
						mo-miR-9	1.000	✓							mo-miR-346	0.995	✓
															mo-miR-93	0.944	✓

The numbers in the parenthesis are the quantities of the microRNAs identified by HSD test. The listed microRNAs were identified by HSD except those marked by "†". The symbol, "√" indicates the organ-specific microRNAs that were identified by two-fold criteria. An OSI of >0.9 was considered as organ-specific.

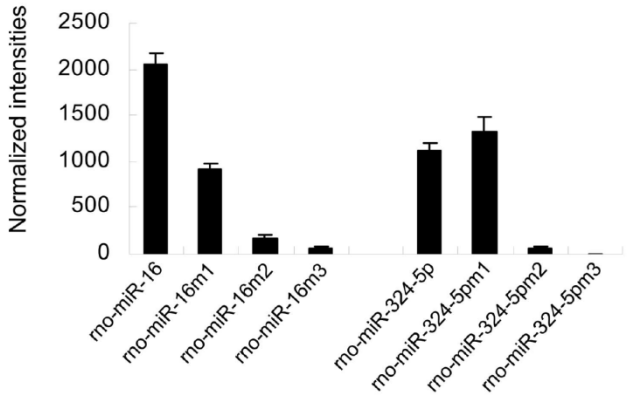
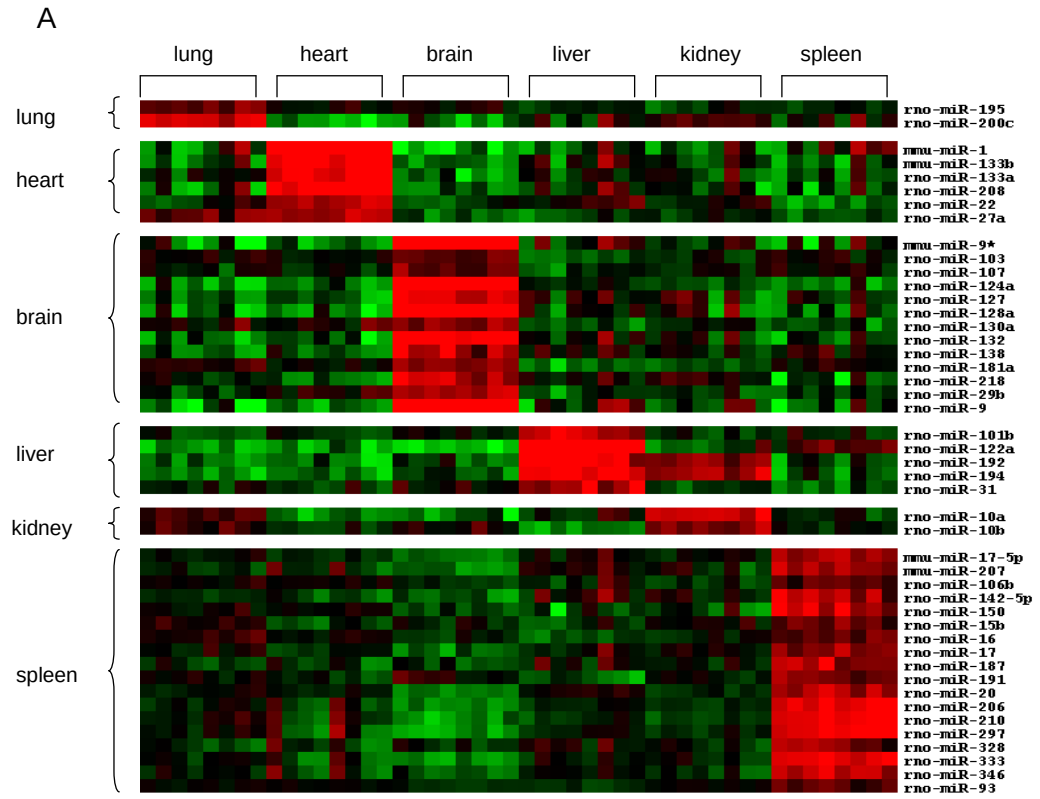


Fig. II2. Specificity of microRNA microarray hybridization. The control oligos in the 3DNA array900 microRNA direct kit were ligated with Cy5 capture sequence and hybridized onto one block. The mismatched probes had one (m1), two (m2) or three (m3) mismatched nucleotides with the corresponding control oligos. The signals from the slide were processed with the method described in Materials and Methods. Data shown were means ± S.E. from 3 hybridizations.

TABLE II3: microRNAs co-expressed in two organs. The listed microRNAs co-expressed in two organs were identified by HSD except those marked with "†". The symbol " √ " indicates the microRNAs that were identified by 2 fold criteria.

lung-heart		lung-brain		lung-kidney		heart-brain		heart-spleen		brain-liver		liver-kidney		liver-spleen	
	2-fold		2-fold		2-fold		2-fold		2-fold		2-fold		2-fold		2-fold
mmu-miR-322-5p†	√	rno-miR-125a†	√	rno-miR-10a		rno-miR-29b	√	mmu-miR-1†	√	rno-miR-128b	√	rno-miR-192	√	rno-miR-122a	√
rno-miR-126	√					rno-miR-29c†	√					rno-miR-194	√		
rno-miR-143	√														
rno-miR-145	√														
rno-miR-23a	√														
rno-miR-23b	√														
rno-miR-24															
rno-miR-27a	√														
rno-miR-27b															



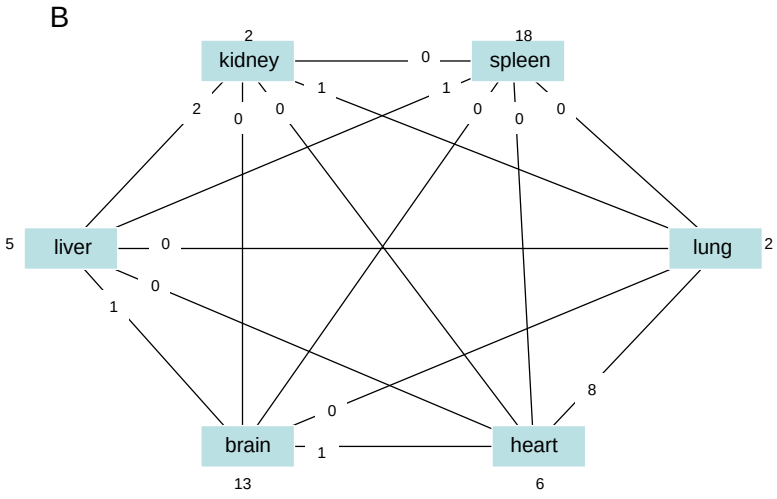


Fig. II3 Organ-specific microRNAs. (A) Hot maps. The microRNA samples from 6 organs were co-hybridized with the common reference. The signals were processed as described in Materials and Methods. The normalized data were subjected to the SAM test [67]. The microRNAs that passed SAM were further tested by the HSD test ($P < 0.05$) to identify organ-specific microRNAs. Each column represents one hybridization and each row represents one microRNA. The figure was drawn by Treeview. Red represents positive values, green negative values, and black zero. The number of microRNAs for each organ is: Lung 2; heart 6; brain 13; liver 5; kidney 2 and spleen 18. (B) Summary of differently expressed microRNAs among 6 organs. The number beside each organ represents the number of microRNAs that are expressed significantly higher in this organ than any other organs ($P < 0.05$). The number on the line between any two organs is the quantity of microRNAs expressed significantly higher in these two organs than in other organs ($P < 0.05$).

2.4.3 Confirmation by Northern blot

To further investigate the reliability of our microarray data, 12 microRNAs were selected for Northern blot confirmation (Fig. II4). Total RNA was extracted from the lung, heart, brain, liver, kidney and spleen from 4 rats. These were the same tissues from the rats utilized for small RNA extraction for the microarray experiment. Total RNA samples from the respective organs were pooled for the experiment. The intensities of the blots were normalized to U6 snRNA and the normalized intensities from Northern blots were compared with those from the arrays (Fig. II5). Among the 12 selected microRNAs, two were lung-specific (*miR-195* and *miR-200c*), one was kidney-specific (*miR-10a*), and three were co-expressed in the lung and heart (*miR-126*, *miR-143* and *miR-145*) as determined by HSD, OSI and two-fold criteria. Most microRNAs had similar expression patterns from microarray analysis and Northern blots (Fig. II4 a-c). In a few cases, the Northern blot showed a higher expression in comparison with the microarray, including *microRNA-195* in heart and *microRNA-145* in kidney and spleen. We also selected additional microRNAs for verification: 3 microRNAs that had high expression in the lung and/or heart (Fig. II4d and II5d) and 33 microRNAs that were expressed in most of the 6 organs (Fig. II4e and II5d). Again, the results exhibited the consistency of the expression patterns between the microRNA array and Northern blot analysis. The correlation coefficients of 7 microRNAs between microarray and Northern blot were higher than 0.9. Although there were few discrepancies, it is clear that the microarray data agreed with the Northern blot data.

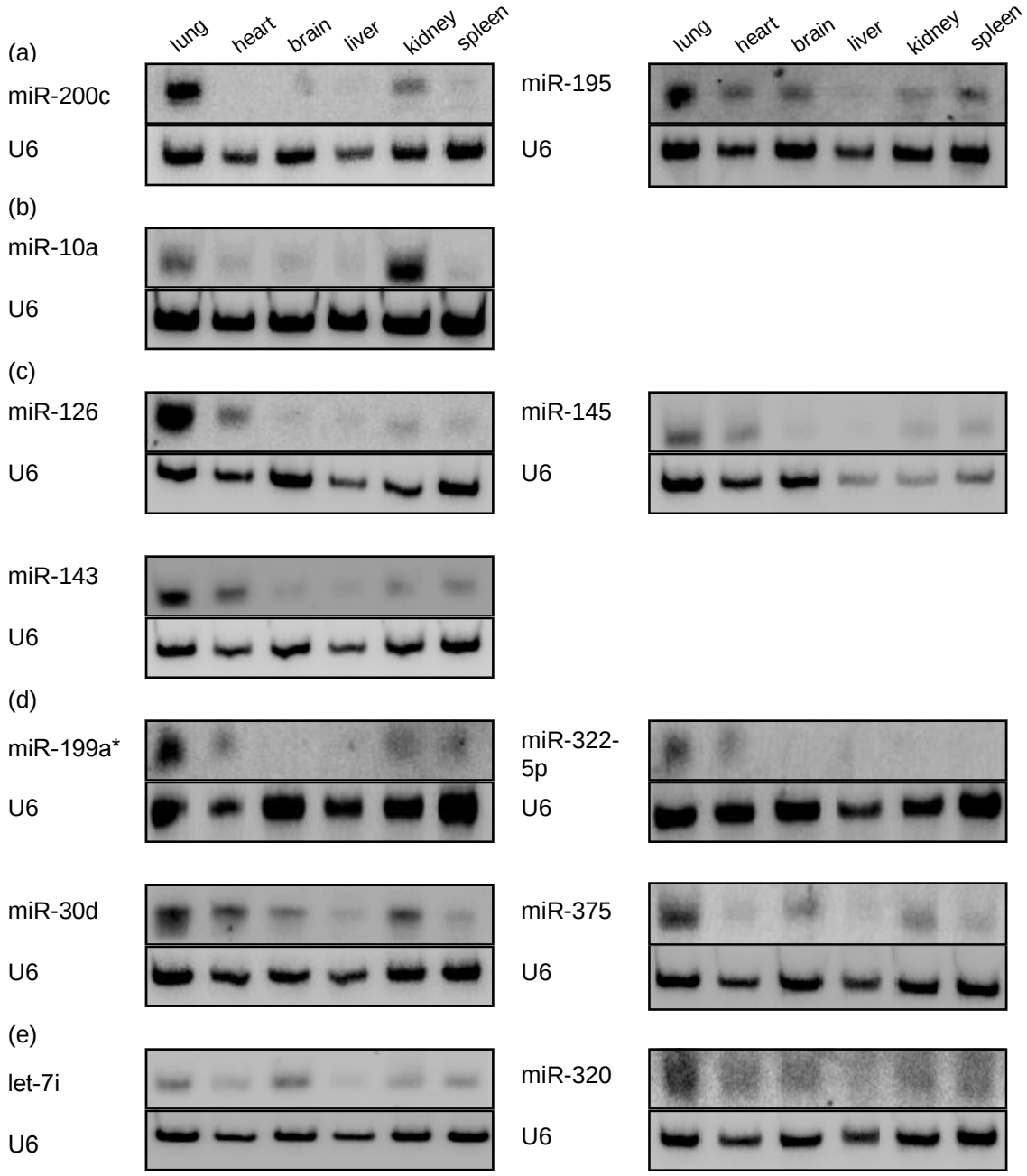


Fig. II4 Northern blot validation. 15 μg total RNA was separated on a denaturing 15% PAGE gel. The 32P labeled probes for the microRNA were hybridized to the membrane overnight. The U6 snRNA was probed as the loading control after the hybridization of microRNA probes. (a) lung-specific microRNAs; (b) kidney-specific microRNAs; (c) co-expressed microRNAs in the lung and heart identified by HSD; (d) microRNAs highly expressed in the lung and/or the heart. (e) commonly expressed microRNAs.

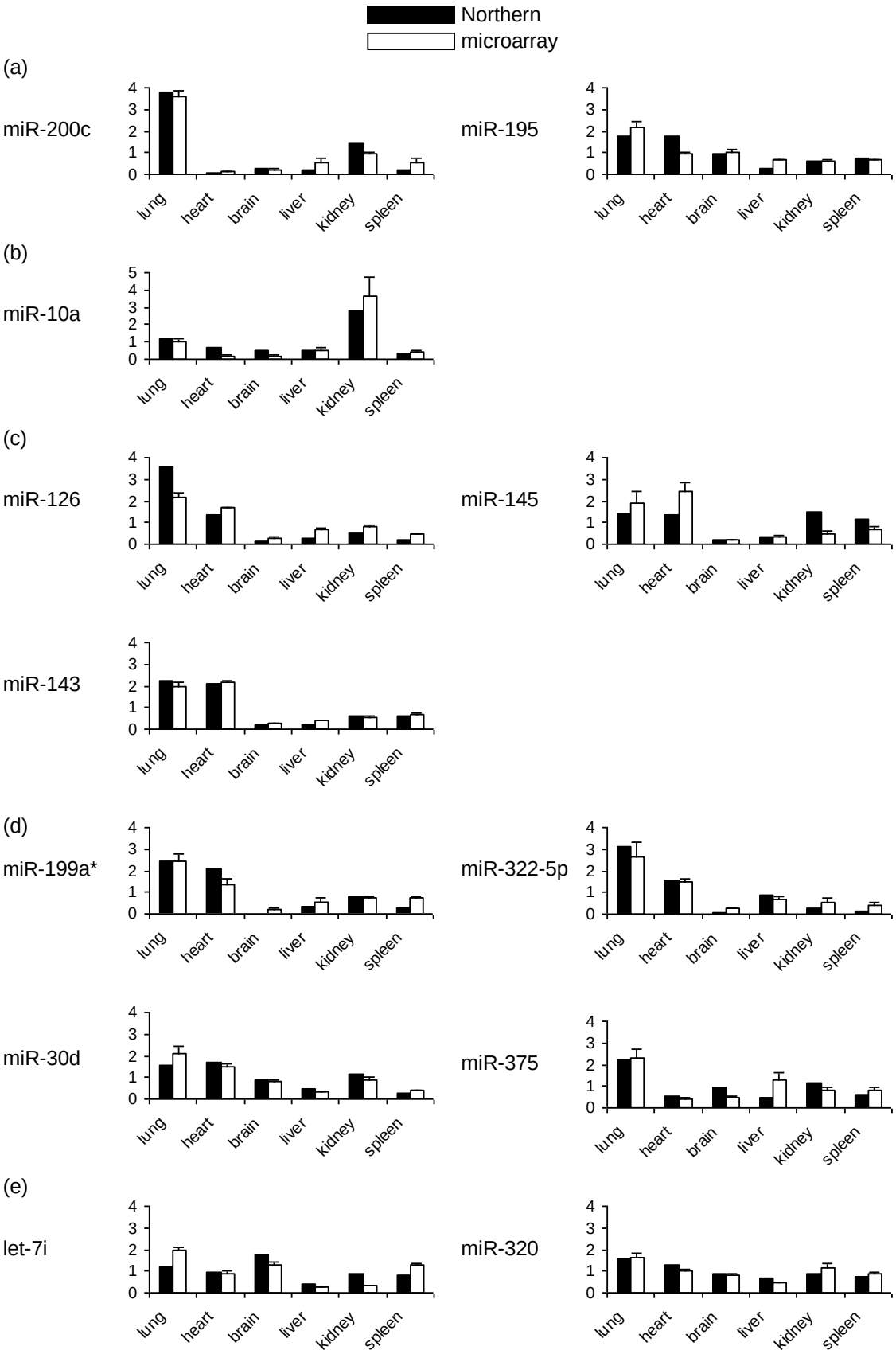

Northern
microarray
(a)
miR-200c
miR-195
(b)
miR-10a
(c)
miR-126
miR-145
miR-143
(d)
miR-199a*
miR-322-5p
miR-30d
miR-375
(e)
let-7i
miR-320
lung
heart
brain
liver
kidney
spleen

Fig. II5 Comparison between results from the microarray and Northern blots. The Northern blots in Figure 4 were quantitated by the Personal Molecular Imager® FX and normalized to U6. Y axes represent the normalized intensities in the Northern blots or the normalized ratios in the microarrays divided by the average of those from 6 organs. The microarray data shown are means ± S.E. from 8 hybridizations. (a) lung-specific microRNAs; (b) kidney-specific microRNAs; (c) co-expressed microRNAs in the lung and heart identified by HSD; (d) microRNAs highly expressed in the lung and/or the heart. (e) commonly expressed microRNAs.

2.5 Discussion and conclusion

In this study, we designed a microRNA microarray system in our laboratory and tested its reliability. This method was used to compare the microRNA expression patterns of six different rat organs, namely the lung, heart, brain, liver, kidney and spleen. We identified 2 microRNAs (*miR-195* and *miR-200c*) that were distinctively expressed in the rat lung, 8 microRNAs that were co-expressed in the lung and heart and 1 microRNA that was co-expressed in the lung and kidney. The reliability of our microarray was confirmed by the high consistence between the microarray and Northern blot analysis.

Several groups have developed microRNA microarray platforms to detect the profiling of microRNAs. However, our platform has several unique features. First, we used two-channel co-hybridization. We also pooled all of the samples as the common reference. Additionally, we performed the dye swap to eliminate the effect of dye bias. The approaches are similar to those used in DNA microarrays. Conversely, most of the other platforms use a single channel platform. Some groups have utilized synthesized oligos as the common reference in their microarrays [37, 43], but the DNA-RNA hybridization may differ from DNA-DNA hybridization. Second, we have 6 replicate spots in our microarray, making us able to exclude the maximum and minimum values while being able to calculate the geometric average signal from the remaining 4 replicates. This method significantly increased the reproducibility of the data. Third, we printed slides in-house with 3 identical blocks in each slide. This procedure allowed us to hybridize 6 samples (3

pairs) on a single slide. We have previously demonstrated that this approach significantly reduces the variance and increases the efficiency [52].

When comparing our data with previous studies, there are similarities as well as differences. For example, only one of the microRNAs we identified as lung-specific, *miR-200c*, has been reported by other groups [37]. Also, *miR-195* was previously reported to be expressed higher in the spleen than in the lung [37], but both our microarray and Northern blot results show *rno-miR-195* expressing much higher in the lung than in the spleen. A few factors could have led to these discrepancies. First, the microRNAs printed on our microarray slides were different from others. Some platforms did not contain all of the 217 microRNAs that we printed on our slides. For example, *mmu-miR-375* is a newly identified microRNA expressed highly in the lung. This particular microRNA was not included on microarray slides in the other studies. Therefore, our study is supplemental and concurrent with other microRNA profiling studies. Second, the sample origins, hybridization conditions, and data analysis methods were different among different research groups. Our microRNA samples were extracted from rat organs and those of other groups were extracted from human and mouse organs. Although this may cause some differences, most microRNAs identified thus far are conserved among species. The use of different hybridization conditions and normalization methods may have also caused some inconsistencies in sensitivity and specificity. However, the methods and conditions used in our study are highly reproducible. Third, some microRNAs that were lung-specific were not highly expressed. This may have caused a disagreement with other microarray platforms. Indeed, when we compared the highly expressed microRNAs that we found in the rat brain to those identified by other groups, 11 out of 13 were consistent with others [35, 37, 43, 55], suggesting that our results are reliable and comparable to other microRNA microarray platforms.

There are some differences between the results from the microarray and the Northern blots. The most obvious reason for the variation was that the hybridization conditions and the

normalization methods were different between the two. The hybridization temperatures and buffer affected the sensitivity and specificity of the assays. The assumption for the microarray normalization was that the total amount of microRNA was consistent between samples from different organs. Realistically, this assumption was not true in some cases. The signals from the Northern blots were normalized to the signals of U6 snRNA with the assumption that the amount of U6 snRNA was the same as the amount of total RNA from the different organs. It is virtually impossible to provide the exact same amount of RNA between samples.

Among the 12 microRNAs confirmed by the Northern blots, none has a known function except *mmu-miR-375* [28]. The expression of *mmu-miR-375* was reported to be limited to the pancreatic β cells, although we also detected it in the rat lung. It has been reported to regulate the secretion of insulin. Neither the secondary transduction signals nor the actin filament network are affected by *mmu-miR-375*. *Mtpn* was validated as a target gene of *mmu-miR-375*. *Mtpn* was reported to form a complex with CapZ which regulates actin polymerization [56]. In the lung, the alveolar epithelial type II cells secrete surfactant through exocytosis, which helps to reduce the surface tension of the alveolar sacs and facilitate the normal function of gas exchange. The mechanism of the secretion of surfactant in the lung is similar to that of the secretion of insulin in the pancreas. We suspect that *mmu-miR-375* works in both of these exocytosis processes. We may find some hints as to the mechanism of exocytosis in the lung if we find more targets of *miR-375* or any of the components that interact with these targets.

Two well-known microRNAs, *miR-1* and *miR-133*, have highly specific expression in cardiac and skeletal muscle tissue [55]. In our study, we also identified these microRNAs as having heart-specific expression. These two microRNAs are clustered together in the mouse genome and both of them modulate muscle proliferation and differentiation. *miR-1* promotes myogenesis by targeting histone deacetylase 4 (*HDAC4*), while *miR-133* promotes myoblast proliferation by inhibiting serum response factor (*SRF*) [57].

A brain-specific microRNA, *miR-9*, has been identified by our microarray as well as by microarrays from other groups. It has been shown to affect neural lineage differentiation in ES cells. STAT3, which is a member of the signal transducer and activator of transcription family, is believed to be involved in this function [27]. In presenilin-1 null mice, *miR-9* has been shown to be down-regulated, leading to severe brain developmental defects [38].

The liver-specific microRNA, *miR-122*, likely modulates the hepatitis C virus by facilitating replication of the viral RNA. Mutational analysis and ectopic expression studies have revealed that *miR-122* interacts with the 5' non-coding region of the viral genome [58]. This suggests that *miR-122* may be a target for antiviral interaction [58]. In addition, *miR-122* is a key regulator of cholesterol and fatty-acid metabolism in the adult liver by regulating plasma cholesterol levels, fatty-acid oxidation, hepatic fatty-acid synthesis as well as cholesterol synthesis [22].

Among the spleen-specific microRNAs identified, five of them belong to the *mir17* microRNA cluster, which comprise *miR-17*, *miR-18*, *miR-19a*, *miR-19b*, *miR-20*, *miR-25*, *miR-92*, *miR-93*, *miR-106a*, and *miR-106b* [59]. Among these 5 microRNAs, *miR-17-5p*, *miR-17* and *miR-20* belong to one of the *mir17* microRNA clusters, the *mir-17–92* cistron, which is one with well characterized cancer association. The *mir-17–92* polycistron is located at 13q31, a genomic locus that is often amplified in cancers. The substantial increase in the expression of microRNAs from this cistron has been reported in human B-cell lymphomas and human lung cancers [60, 61]. However, the prominent expression and function of these microRNAs in the spleen are not known.

There are few studies concerning the functions of microRNAs in the lung. Several recent studies have given rise to a great interest in this field of research. The reduction in the expression of *let-7* in human lung cancers is correlated to increased death rates in patients [62].

Experimentally, over-expression of *let-7* can inhibit lung cancer cell growth *in vitro*. This discovery shows that *let-7* may have potential clinical value in treating lung cancers. Inactivation of Dicer results in the defect of epithelial branching [63]. This defect is independent of the requirement for Dicer in cell survival and does not stop the epithelial growth [63]. In the E11.5 lung, Ago1 and Ago2 are enriched in the branching regions, which undergo the most dynamic changes during lung remodeling. This discovery suggests that microRNAs regulate processes responsible for the biogenesis of the lung [64]. Another study shows that the decrease in Dicer expression is associated with the poor prognosis in lung cancer patients [65]. The microRNA expression profiles in lung cancers correlate with the prognosis of lung adenocarcinoma patients [66].

In summary, we designed a reliable microRNA microarray platform that is low in cost and easy to update with highly reproducible results. The expression profiling of microRNAs in 6 rat organs was detected with this platform. The expression patterns of lung-specific and lung co-expressed microRNAs were confirmed by Northern blot analysis. Our platform adds to the implementation of detecting microRNA profiles, as no other microarray platform has been made for the detection of rat microRNA profiles. Furthermore, our microarray platform contains several recently discovered microRNAs, making it supplementary to other platforms. When applied, our study of the expression patterns of microRNAs in the lung should shed light on the functions of microRNAs in lung physiology as well as lung pathophysiology.

Authors' contributions

The manuscript has been published in BMC Genomics. Yang Wang carried out microRNA microarray printing, hybridization, data analysis and northern blot and drafted the manuscript. Tingting Weng participated in the array printing and data analysis. Deming Gou participated in northern blot analysis. Zhongming Chen participated in data analysis.

Narendranath Reddy Chintagari participated in sample collection. Lin Liu conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. Heidi Stricker for editorial assistance. This work was supported by NIH R01 HL-052146, R01 HL-071628 and R01 HL-083188 (To LL).

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CHAPTER III

MIR-127 MODULATES FETAL LUNG DEVELOPMENT

3.1 Abstract

MicroRNAs (microRNAs) 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 microRNAs in fetal lung development, we profiled the microRNA expression pattern during lung development with a microRNA microarray. We identified 21 microRNAs that showed significant changes in expression during lung development. These microRNAs were grouped into four distinct clusters based on their expression pattern. Cluster 1 contained microRNAs whose expression increased as development progressed, while clusters 2 and 3 showed the opposite trend of expression. MicroRNAs in cluster 4 including microRNA-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 microRNAs. *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 the terminal bud count, increased terminal and internal bud sizes, and caused unevenness in bud sizes, indicating improper development. These findings suggest that miR-127 may have an important role in fetal lung development.

Keywords: *in situ* hybridization, microarray, lung morphometry

3.2 Introduction

microRNAs (microRNAs) are a class of small RNAs (~21–24 nt) that regulate the expression of their target genes at the posttranscriptional level [1, 2]. In animal cells, they are first transcribed from microRNA genes in the genome as primary microRNAs (pri-miRNAs) and then processed by an RNase III enzyme, Drosha, into ~70-nt premature microRNAs (pre-miRNAs) with hairpin structures [3]. With the help of Exportin 5, pre-miRNAs are then transported into the cytoplasm, where they are cleaved by another RNase III enzyme, Dicer [4, 5]. The cleavage results in double-stranded RNA duplexes. Usually, one strand of the duplex becomes mature microRNA [6]. Mature microRNAs are then recruited into nucleoprotein complexes called RNA-induced silencing complexes (RISC). Based on the pairing of microRNAs and their target sites, the complexes can negatively regulate the expression of their genes in three ways [7]: they can cleave the messenger RNAs; they can inhibit the translation of the messenger RNAs; and they can accelerate deadenylation of the messenger RNAs, leading to the acceleration of their degradation [8-12]. In rare cases, microRNAs can activate translation [13, 14]. MicroRNAs are important regulatory molecules that modulate various biological processes including cellular physiology, developmental timing, cell fate determination, apoptosis, lipid and fat metabolism, insulin secretion, and progression of various cancers [15-17].

The functions of microRNAs in various aspects of lung biology are less studied but are subjects of several recent investigations. Studies have suggested the important roles of microRNAs 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 [18]. The expression of microRNAs in lung cancers has already been profiled. The results demonstrated the correlation of microRNA expression with the prognosis of lung adenocarcinoma patients [19]. Studies on expression of important molecules in microRNA 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 microRNAs may play important roles in lung development [20]. Inactivation of Dicer, a key component in microRNA processing, was found to cause the inhibition of lung epithelial branching [21]. 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 [22].

Rat lung development can be divided into five stages [23, 24]. 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 secrete 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 [25]. Some of the well-studied signaling molecules include fibroblast growth factors, transforming growth factors (TGFs), retinoids, Wnt genes, and Sonic hedgehog [26-29]. However, little is known about what the role of microRNAs is in this process and how they regulate lung development by modulating these signaling pathways. In addition, the temporal and spatial expression patterns of microRNAs in rat lung development are still not known.

In this study, we used a microRNA microarray platform developed in our laboratory [30] to profile the expression of microRNAs at different stages in rat lung development. There were 21 microRNAs that were significantly changed during this process. Some of these microRNAs were

selected and validated by real-time PCR. The spatial expression patterns of selected microRNAs 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.

3.3 Materials and methods

3.3.1 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 P0, 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 with Ketamine (40 mg/kg body weight) and Xylazine (8 mg/kg body weight) before euthanization 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 microRNA Isolation Kit (Ambion) according to the manufacturer's instructions.

3.3.2 MicroRNA microarray

The microRNA 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 microRNA in each block. The NCode microRNA Labeling System (Invitrogen, Carlsbad, CA) was used to generate labeled microRNA 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 co-hybridized with the common reference, which was pooled equally from small RNAs of all the lung samples. To each block, one labeled sample (Alexa Fluor 3 or Alexa Fluor 5) was co-hybridized 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 [30]. 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 microRNA. The quality of the signals was assessed with RealSpot software. The microRNAs 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 microRNAs significantly changed between any two time points were picked up ($q < 0.05$). The microRNAs that passed the SAM test were clustered and viewed with Cluster (K-means clustering) and TreeView software (http://rana.lbl.gov/EisenSoftware.htm).

3.3.3 Quantitative real-time PCR for microRNA.

Quantitative real-time PCR (qRT-PCR) for miR-18, miR-20a, miR-29a, and miR-351 was performed with the mirVana qRT-PCR microRNA 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 microRNA 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 microRNA Detection Kit, 50 ng of RNA was used in each reverse transcription reaction with microRNA 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 ℃ for 30 min and then at 95 ℃ 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 ℃ for 3 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ 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 microRNA. 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 microRNA to U6 snRNA was calculated with the equation $2^{-(C_T miRNA - C_T U6)}$. For qRT-PCR with TaqMan MicroRNA Assays, 75 ng of total RNA was used as template in each RT reaction with microRNA-specific RT primers. The reactions were incubated on ice for 5 min, followed by 16 ℃ for 30 min, 42 ℃ for 30 min, and 85 ℃ 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 ℃ for 15 s and 60 ℃ 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 ℃ for 1 h. RT was performed with a poly(T) adaptor, GCGAGCACAGAATTAATACGACTCACTATAGG TTTTTTTTTTTTVN. Real-time PCR was performed with a universal reverse primer, GCGAGCACAGAATTAATACGACTCAC, and a forward primer with the same sequence as the mature microRNA.

3.3.4 *In situ* hybridization for microRNA.

In situ hybridization for microRNA 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 ℃. 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 ℃ 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 ℃ in a humidified chamber. After washing in PBST and AP buffer (in mM: 100 Tris ⊣HCl, pH 9.5, 50 $MgCl_2$, 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.

3.3.5 Construction of miR-127 overexpression adenoviral vector.

Rat miR-127 with flanking sequences (~200 base pairs at each end) was amplified from rat genomic DNA with the primers 5′-CCTTGTCGACCTCGAGAACCTCCAG-3′ and 5′-

AGAATTCTTAGGCATTAAGTGGCTCCAGACCC-3′ and digested by SalI and EcoRI digestion. The digested PCR product was cloned into a modified pENTR/CMV-EGFP vector [31] 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-microRNA 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.

3.3.6 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 D0, and the next 2 days of culture were denoted as D1 and D2, respectively. The adenoviruses containing eGFP and microRNA 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.

3.3.7 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.

3.3.8 Statistical analysis.

Statistical analysis of microarray data was done as described previously [30]. 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 $\pm$ SE.

3.4 Results

3.4.1 MicroRNA expression profile during lung development.

To detect the microRNA expression profile during rat lung development, we used the microRNA microarray platform developed in our laboratory [30]. The microarray contained probes for 227 non-redundant RNAs: 177 rat microRNAs, 5 human microRNAs, 31 mouse microRNAs, 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 co-hybridized 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 microRNAs passed the quality test by RealSpot, with an average QI > 1 [32]. Statistical analysis was performed with SAM. Twenty-one microRNAs were shown to have significant changes between at least one pair of the seven time points ($q < 0.05$). To identify microRNAs with similar expression patterns, these significantly changed microRNAs were then grouped into four clusters with Cluster software by K-means clustering (Fig. III1). 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 microRNAs are shown in the line charts in Fig. III2. In cluster 1, the microRNA expression increased gradually from fetal to adult lungs (E16 to AD). The microRNAs 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 microRNAs peaked at some point between E16 and P6. For example, miR-127 reached maximum on E21 and miR-351 reached maximum on E19.

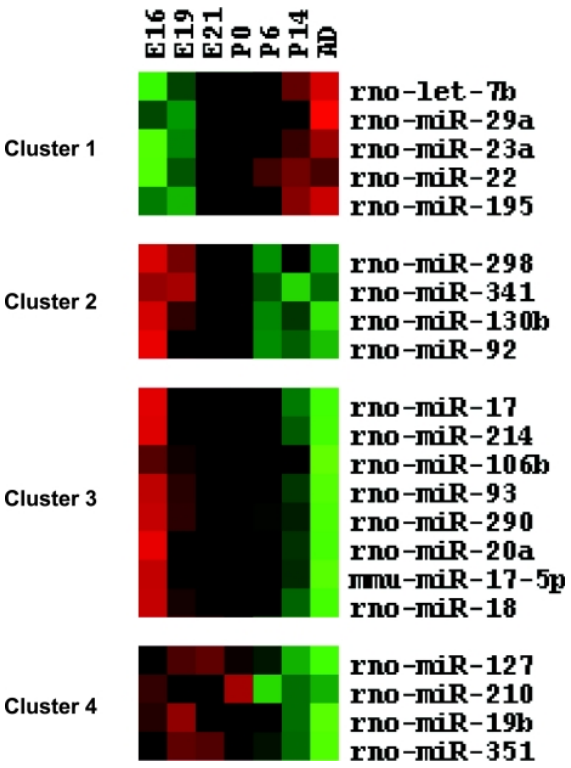

Fig. III1 **Cluster analysis of microRNAs significantly changed during rat lung development.** MicroRNAs from lungs on gestational days 16, 19 and 21 (E16, E19, and E21) or postnatal days 0, 6, and 14 (P0, P6, and P14) or adult lungs (AD) were cohybridized with the common reference. Normalized data were subjected to Significant Analysis of Microarrays (SAM) test to identify microRNAs whose expression was significantly changed during this process ($q < 0.05$). The identified microRNAs were grouped into 4 clusters by *K*-means clustering and viewed by TreeView. Each column represents 1 stage, and each row represents 1 microRNA. Each value of expression is the average of 6 replicates and is then $\log_2$ transformed. Red represents positive values, green negative values, and black zero.

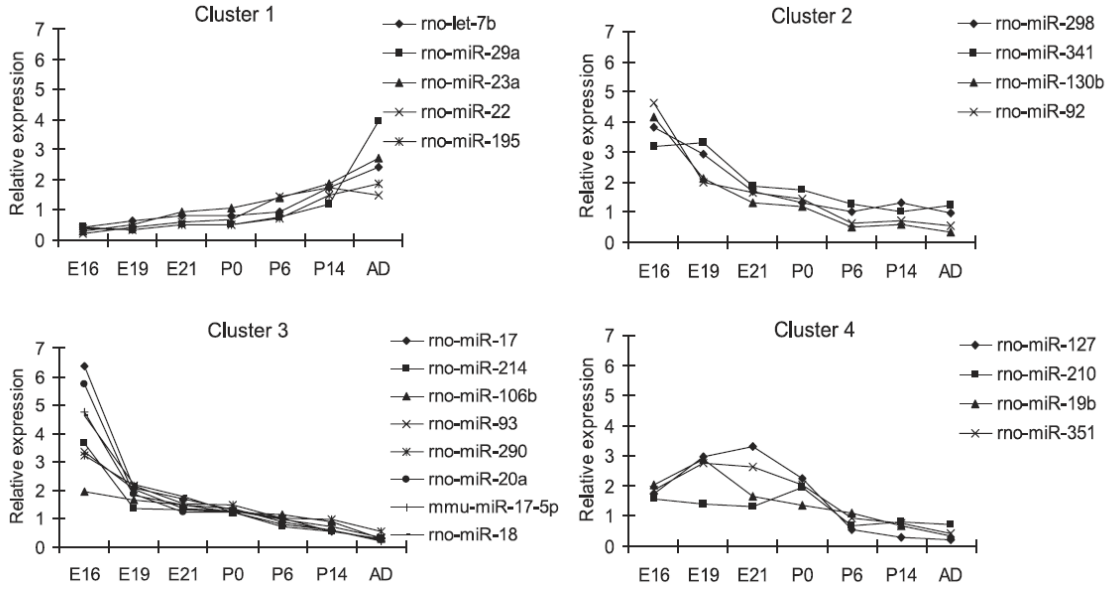

Fig. III2 **microRNA expression patterns during rat lung development.** The relative expression of microRNAs in each cluster of Fig. 1 is plotted against each stage of development. The relative expression level is the ratio of the sample signal to the common reference signal. The data shown are the means of 6 replicates.

3.4.2 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 microRNAs from each of three clusters. We chose microRNAs from clusters 1, 3, and 4 because the expression of cluster 2 resembled cluster 3 in the general trend. We chose two microRNAs from each cluster based on high fold changes, their expression in lungs, and functional studies in other systems. miR-29a and miR-195 (Fig. III3, A and B) were chosen to represent cluster 1, while miR-18 and miR-20a (Fig. III3, C and D) represented cluster 3 and miR-127 and miR-351 (Fig. III3, E and F) represented cluster 4. All of these microRNAs followed the same trend of expression as seen in the microarray experiment, thus validating our microarray platform.

The expression profiles of microRNAs 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. III3, G and H).

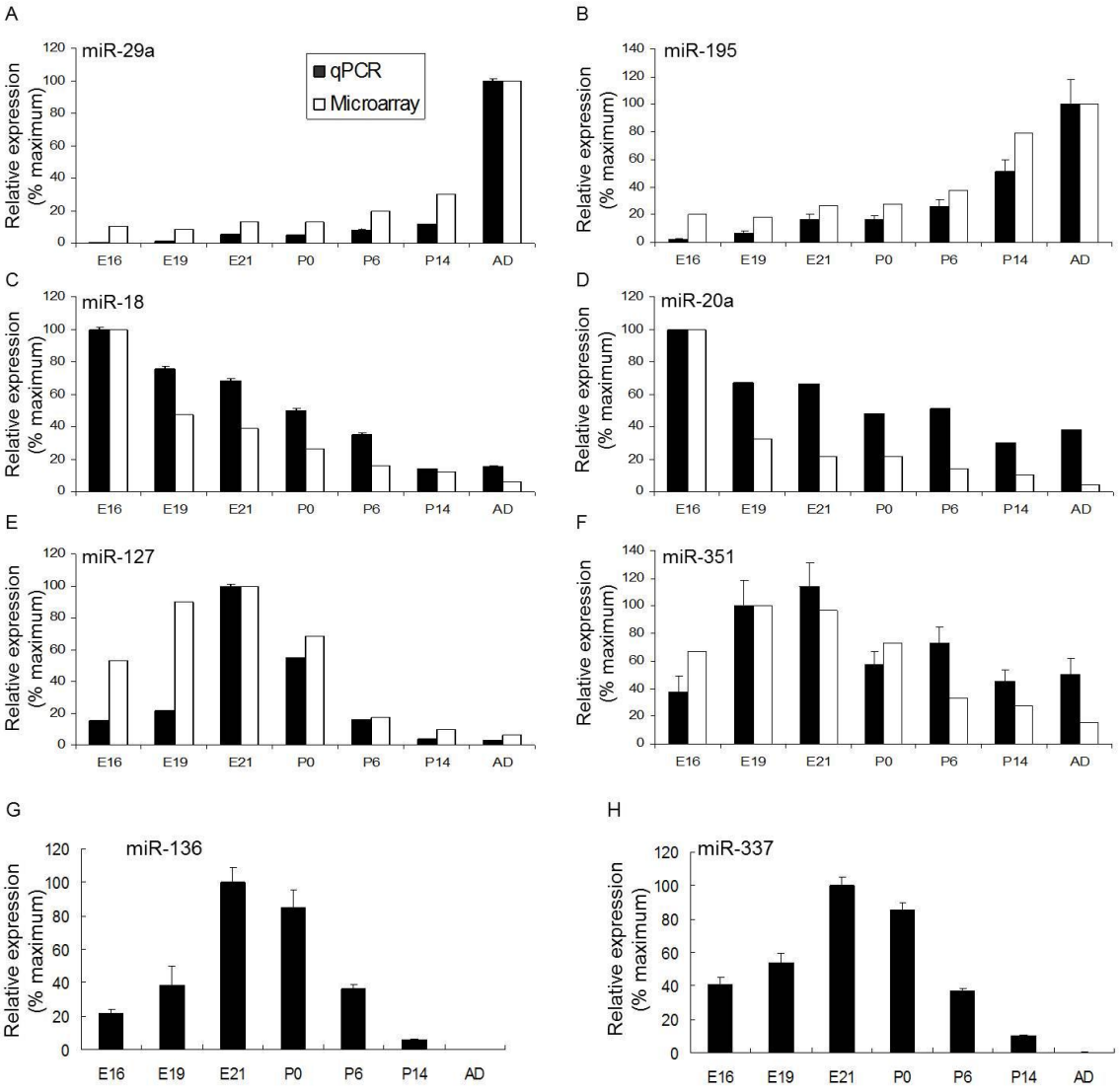

Fig. III3 **Validation of microRNA microarray results by quantitative real-time PCR (qRT-PCR).** Total RNA was extracted from fetal lungs at E16, E19 and E21 and lungs at P0, P6, P14, and AD. MicroRNA levels were measured by real-time PCR. Relative expression levels of miR-29a (A), miR-195 (B), miR-18 (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.

3.4.3 Cellular localization of microRNAs.

In situ hybridization using 5′ DIG-labeled LNA probes was done to determine the spatial expression pattern of selected microRNAs 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. III4A). miR-20a expression followed the trend of expression consistent with the microarray and qRT-PCR data (Fig. III4A). 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 microRNA targets was used as a negative control. No signals were detected on E16 sections (Fig. III4A). 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. III4B). 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).

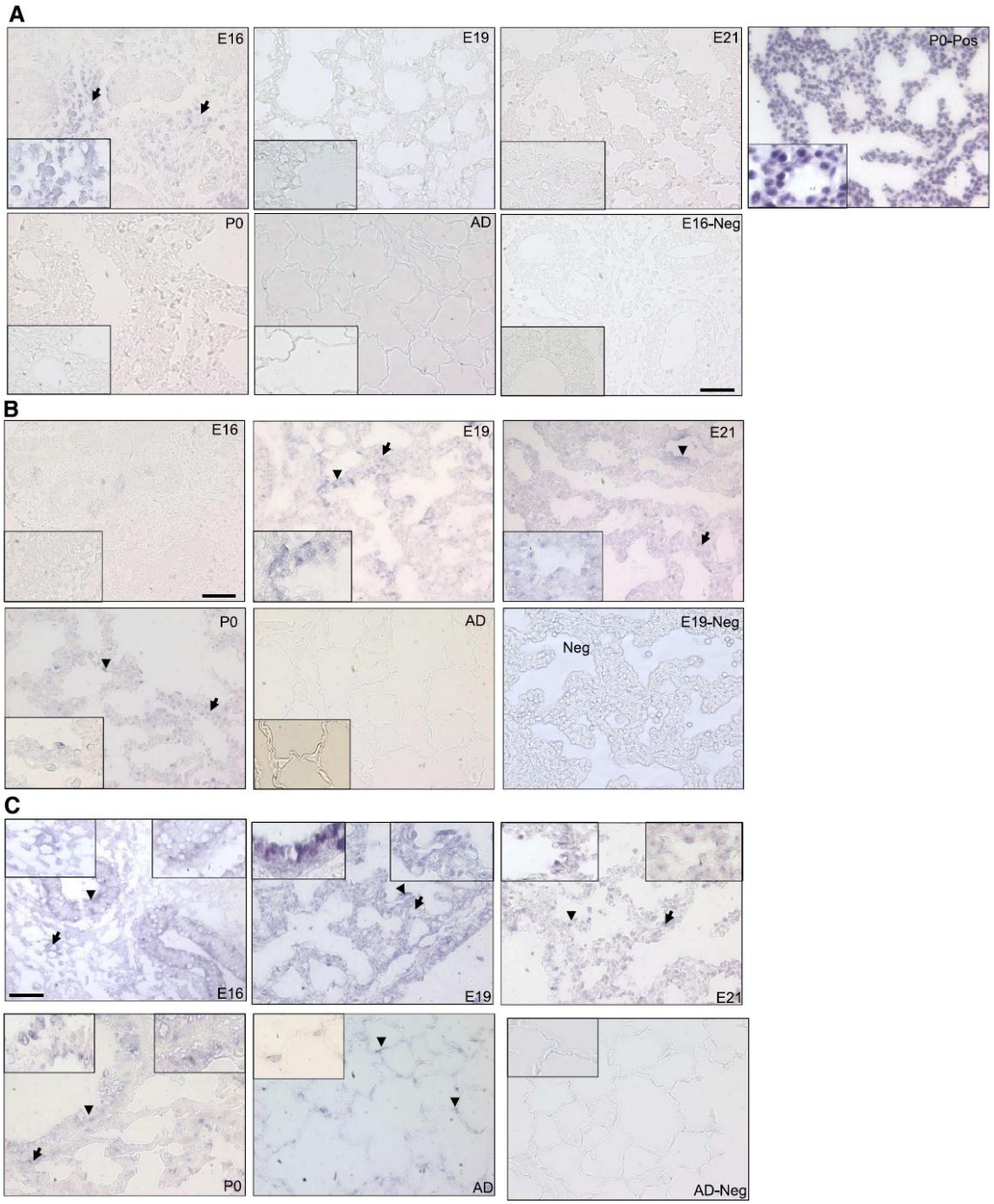

Fig. III4 ***In situ* hybridization for microRNAs.** *In situ* hybridization was carried out in dewaxed and rehydrated fetal rat lung tissue sections at E16, E19 and E21 and lungs at P0 and AD. The sections were hybridized with 5' DIG-labeled LNA probes against miR-20 (A), miR-127 (B), and miR-351 (C). A probe with scrambled sequence unrelated to known microRNAs 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. Scale bars, 40 μm.

The miR-351 expression pattern also corroborated the qRT-PCR and microarray data (Fig. III4C). 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. III4C), 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).

3.4.4 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. III5A). The dose of the virus was standardized by examining the intensities and even distribution of GFP along the whole lung. Since there was a significant increase in the lung tissue mass as a function of culture time we added half the initial dose of virus on D1, and this gave a 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. III5B). 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 resulted in a larger terminal bud size compared with blank control and virus control (Fig. III6, A and B). Variability in the sizes of 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. III5C). The average internal bud size was higher for miR-127, as in the case of terminal buds compared with controls (Fig. III5D). 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. III5E). 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.

3.5 Discussion

In the present work, we described the microRNA expression profile during lung development and identified four clusters of microRNAs that showed specific trends of expression. Expression levels of 21 microRNAs were found to be significantly changed during the course of lung development. The microRNA microarray results were validated by qRT-PCR analysis and *in situ* hybridization of the selected microRNAs. 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.

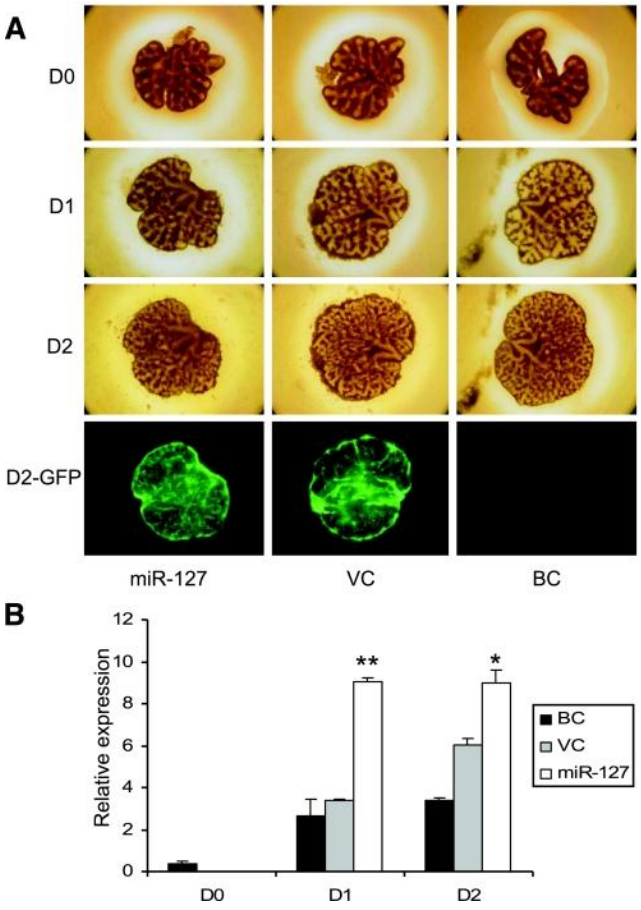

Fig. III5 **miR-127 overexpression in fetal lung culture.** A: E14 fetal lungs were cultured in an insert. miR-127 overexpression adenovirus or virus control (VC) was added to the culture on day 0 (D0), and culture continued for 2 days (D1 and D2). The blank control (BC) was treated with medium alone. 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.

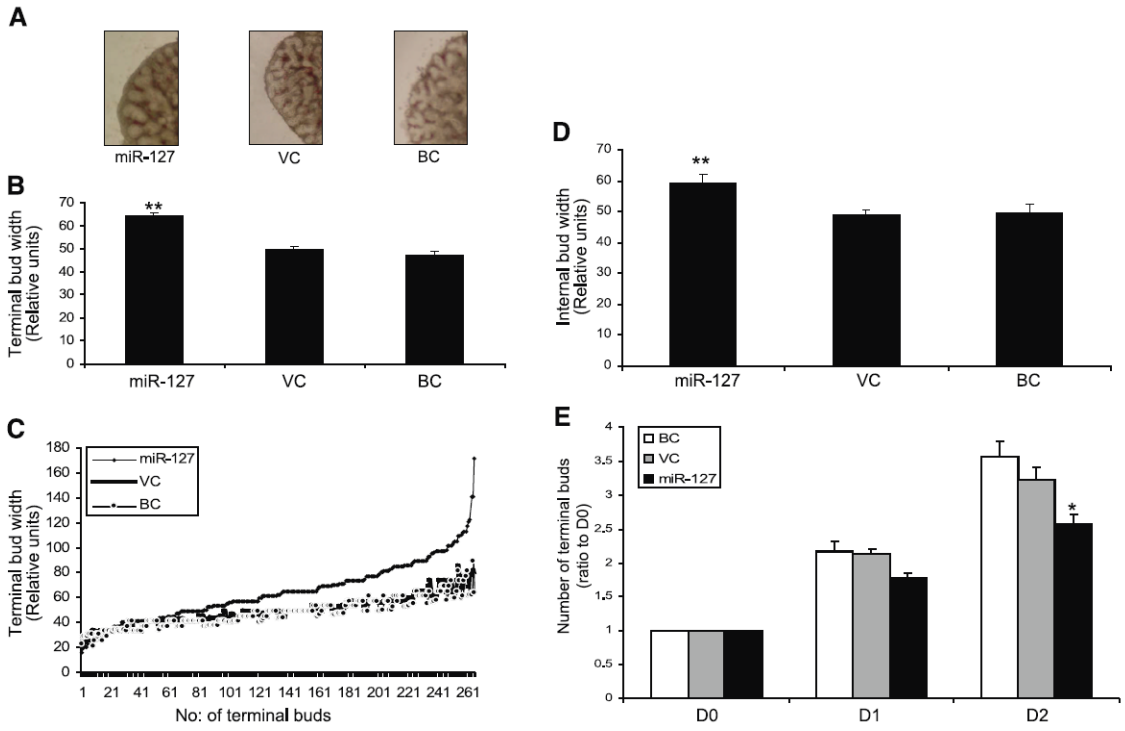

Fig. III6 **Effect of miR-127 overexpression on fetal lung development.** E14 fetal lungs cultured in inserts were treated with miR-127 overexpression adenovirus or VC or BC for 2 days. Images were taken at the end of culture. A: enlarged image from Fig. 5A. B: terminal bud width was measured with MetaVue software. The width of each bud was measured at its longest diameter and expressed in relative units. C: variability in terminal bud width. Terminal bud width values were arranged in ascending order in each treatment and plotted against the number of buds to demonstrate variability in terminal bud size. Data were obtained from >250 terminal buds from at least 10 fetuses obtained from 3 mothers. D: average internal bud width was calculated with MetaVue software and the same measuring parameter for terminal buds. The value is expressed in relative units. E: no. of terminal buds formed at the end of D2 in miR-127-overexpressed lungs was compared with VC and BC after normalizing with the terminal buds on D0. Number of terminal buds was counted in a blinded manner by at least 2 different individuals, and the relative number at the end of D2 was expressed as a ratio to number of terminal buds on D0. At least 25 terminal or random internal buds from each lung were used for the respective analyses. Error bars represent SE. *P < 0.05 vs. VC, **P $\leqslant$ 0.02 vs. VC.

microRNAs have rapidly emerged as one of the key regulatory molecules that control various biological processes ranging from development to disease. Various microRNAs have been implicated in regulating developmental timing and controlling left/right neuronal asymmetry in [33, 34], insulin secretion [35], lipid metabolism [36], proliferation and apoptosis [37, 38], stem cell division [39, 40], and B-cell differentiation [41]. Involvement of microRNAs in progression of various cancers has been extensively investigated [18, 19, 42-45], but studies on their role in the normal physiology of the lung have been very limited. Some important proteins involved in microRNA processing, such as Ago1, Ago2, and Dicer, have been shown to be important to lung morphogenesis [20, 21]. These discoveries suggest the importance of microRNAs in the lung. Our previous study [30] showed that two microRNAs, namely, miR-195 and miR-200c, are specifically expressed in the lung. It has been shown that the expression levels of some microRNAs are changed after lipopolysaccharide-induced inflammation, and miR-146a can regulate the inflammatory response in lung alveolar epithelial cells [46, 47].

Williams et al. [48] compared microRNA expression between fetal (pooled from 18–29 weeks) and adult (2 time points: 1 fetal and 1 adult) human lungs with real-time PCR. They found that 13 microRNAs were up-regulated in human fetal lungs and 8 microRNAs 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 microRNAs were expressed at higher levels in neonatal mouse lungs, and thirty microRNAs were more highly expressed in the adult mouse lungs. In our studies, we mainly focused on the dynamic changes in microRNA 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 similar to these in rat lungs from our present studies. In addition to the microRNAs that increase or decrease from early lung development to adult, we

also identified a microRNA cluster in which their expression was the highest in the later stage of fetal lung development, compared with the 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 has been observed previously [22, 48]. 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 [49]. Overexpression of the miR-29 family in lung cancer cell lines has been shown to inhibit tumorigenicity both *in vitro* and *in vivo* [50]. 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 [51]. Its low expression during lung development may be an important factor that helps in the controlled proliferation and differentiation of cells in the lung. This cluster also contained let-7b, a member of the let-7 family known to regulate developmental timing in Drosophila [52].

Clusters 2 and 3 contained microRNAs 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 microRNAs. A recent study in mouse embryonic lung development has shown a similar trend of expression for these three microRNAs from E11.5 to adult lungs [22]. 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 proliferation and inhibit differentiation of lung epithelial progenitor cells [22, 53-55]. 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 [56]. A recent study has shown that miR-20a has an important role in the regulation of E2F2 and E2F3 expression [57]. 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 anti-apoptotic 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 pro-apoptotic E2F1 network [58]. The anti-apoptotic 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 [55]. These studies have concentrated on the effect of microRNAs 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 microRNAs 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 [59].

Cluster 4 contained miR-127 and miR-351, which showed the highest expression just before and after birth in the saccular-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 [23, 60]. The other members of this microRNA cluster in the rat genome including miR-136 and miR-337 had the same expression patterns as miR-127, indicating that these microRNAs 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 microRNAs 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 [61, 62]. 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 [63]. With treatment with chromatin-modifying drugs miR-127 expression was up-regulated, and this, in turn, inhibited the expression of its target, the proto-oncogene 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 at low levels 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 up-regulation at the later stage of fetal lung development.

Together, our results have demonstrated the reliability of a microRNA microarray platform to identify the microRNA profile during fetal lung development. We have also confirmed the expression profile and localization of selected microRNAs and have demonstrated that miR-127 overexpression results in defective fetal lung development. Since microRNAs 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 microRNAs regulate multiple mechanisms of control of lung development.

Authors' contributions

Yang Wang and Dr. Manoj Bhaskaran co-operated on this project and contributed equally to this study. Yang Wang collected the fetal lung samples and performed microRNA microarray and data analysis. Yang Wang and Dr. Manoj Bhaskaran set up microRNA qRT-PCR and *in situ* hybridization techniques and verified microRNA expression with qRT-PCR together. Dr. Manoj Bhaskaran overexpressed miR-127 in fetal lung organ cultures and performed the morphometric study. Yang Wang and Manoj Bhaskaran drafted the manuscript together. Honghao Zhang, Tingting Weng, Pradyumna Baviskar, and Deming Gou helped in sample collection, vector construction, and data analysis. Lin Liu conceived of this study, and participated in experiment design and coordination and helped to draft the manuscript. This paper has been included previously in Dr. Manoj Bhaskaran's dissertation.

Grants

This work was supported by National Heart, Lung, and Blood Institute R01-HL-052146, R01-HL-071628, and R01-HL-083188 (to L. Liu) and American Heart Association (AHA)

Beginning Grant-in-Aid 0865162F (to D. Gou). Y. Wang and T. Weng were supported by AHA predoctoral fellowships 0810016Z and 0610143Z.

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CHAPTER IV

MIR-375 REGULATES ALVEOLAR EPITHELIAL CELL TRANS-DIFFERENTIATION BY INHIBITING WNT/β-CATENIN PATHWAY

4.1 Abstract

Alveolar epithelial cell trans-differentiation is a process by which type II alveolar epithelial cells (AEC II) trans-differentiate into type I alveolar epithelial cells (AEC I) during lung recovery after various injuries, in which AEC I are damaged. This process is critical for lung tissue repair. MicroRNAs (microRNAs) are a group of small RNAs that regulate gene expression at the post-transcriptional level. They have the potential to regulate almost every aspect of cell physiology. However, whether alveolar epithelial cell trans-differentiation is regulated by microRNAs is completely unknown. In this study, we identified miR-375 as the most down-regulated microRNA during alveolar epithelial cell trans-differentiation using a microRNA microarray platform. We also found that miR-375 was highly expressed in the lung, particularly in AEC II. The overexpression of miR-375 with an adenoviral vector inhibited alveolar epithelial trans-differentiation as indicated by an increase in the AEC II marker, SP-C, and decreases in the AEC I markers, T1α and RAGE. This effect occurs via inhibition of the Wnt/β-catenin pathway

through direct targeting of Frizzled 8. Silencing of β-catenin mimicked miR-375 effects on alveolar epithelial cell trans-differentiation, while stabilized β-catenin blocked the effect of miR-375. In summary, our results demonstrate that miR-375 can regulate alveolar epithelial cell trans-differentiation by the inhibition of the Wnt/β-catenin pathway through direct targeting of Frizzled 8. The discovery of these components in the regulation of trans-differentiation may provide new targets for therapeutic intervention to benefit lung recovery from injuries.

Key words: miR-375, AEC trans-differentiation, Wnt/β-catenin signaling pathway, Frizzled 8

4.2 Introduction

The discovery of microRNAs (miRNAs) has opened up new avenues of research into regulation of gene expression and mechanism of diseases. MicroRNAs are a group of endogenous noncoding regulatory RNAs. They are about 22 nt long and regulate the expression of their target genes at the post-transcriptional level by cleavage of a target mRNA, translational inhibition, and mRNA deadenylation [1-4]. So far, more than one thousand microRNAs have been discovered in humans. The known functions of microRNAs in animals have covered almost every aspect of cell physiology, including regulation of development timing, cell proliferation and differentiation, apoptosis, fat and lipid metabolisms, and exocytosis. MicroRNAs are also known to be involved in the pathogenesis of cancers, diabetes, and other diseases [5-7]. According to computational analysis, a majority of mammalian mRNAs are under selective pressure to be conserved targets of microRNAs [8].

miR-375 has previously been reported to be a pancreatic islet-specific microRNA. It can regulate insulin secretion and pancreatic islet development [9-11]. The identified targets of miR-375 include 3’phosphoinositide-dependent protein kinase-1 (PDK1) and myotrophin (Mtpn). Recently, miR-375 has been shown to be a proliferation inhibitor and a tumor suppressor. The involved targets include yes-associated protein (YAP), Janus kinase 2 (JAK2), and PDK1 [12-

14]. We have previously reported that miR-375 is expressed in the rat lung [15]. The question of what is the function of miR-375 in the lung is of particular interest to us.

The epithelium of the lung is composed of cuboidal type II alveolar epithelial cells (AEC II) and squamous type I alveolar epithelial cells (AEC I). AEC II are multifunctional cells involved in surfactant synthesis and secretion, fluid transport, and recovery from lung injury [16]. The main functions of AEC I are gas exchange and fluid transport [17]. AEC I can also protect lung epithelium from hyperoxic injury [18].

During the saccular phase of lung development, columnar epithelial cells differentiate into AEC II, which contain distinctive lamellar bodies in the cytoplasm. As the air sacs expand, AEC I begin to derive from AEC II and undergo a thinning process. The squamous type I epithelium and the capillary endothelium form a thin air-blood barrier. Under a variety of disease conditions, AEC I are damaged and AEC II proliferate. Some of these AEC II keep their morphologic characteristics, while others trans-differentiate into AEC I [19-21]. Radioactive tracing experiment after injury reveals that tritiated thymidine is first incorporated in AEC II and is subsequently observed in AEC I, which further confirms the AEC II as progenitor cells of AEC I [22, 23]. When cultured in plastic dishes, AEC II gradually lose their morphologic characteristics and the ability to synthesize and secrete surfactant. On the other hand, these cells acquire the characteristics of AEC I, which include the squamous appearance and expression of all known AEC I markers such as T1α and advanced glycosylation end product-specific receptor (RAGE) [24-27]. This is a well-established *in vitro* model that mimics the alveolar epithelial cell trans-differentiation *in vivo*. We have previously reported that TGF-β regulates the alveolar epithelial cell trans-differentiation through the Smad pathway [27]. However, the functions of microRNAs in the regulation of this trans-differentiation process are completely unknown.

The Wnt/β-catenin signaling pathway plays a critical role in the regulation of lung epithelial fate determination. In the canonical Wnt/β-catenin pathway, Wnt ligands bind to transmembrane receptors, Frizzled (FZD) and the co-receptors LRP5/6 [28, 29]. This binding leads to the activation of Dishevelled (DVL), which results in the inhibition of β-catenin phosphorylation by glycogen synthase kinase-3β (GSK-3β) and degradation of β-catenin. The stabilized β-catenin then translocates into the nucleus, binds with TCF/LEF (T cell factor/lymphoid-enhancing factor) transcriptional factors, and regulates the expression of downstream β-catenin dependent genes, such as cyclin D1 and c-myc. In respiratory epithelial cells, Wnt/β-catenin signaling is necessary for branching morphogenesis and distal airway cell specification [30-32]. Some Wnt ligands including Wnt5a and Wnt7b are expressed in the lung epithelium [33-35]. FZD8 is also found in the lung epithelium [36]. A recent study has demonstrated that Wnt/β-catenin promotes trans-differentiation in the alveolar epithelial cell culture model [37].

In this study, we performed microRNA expression profiling during alveolar epithelial cell trans-differentiation using a microRNA microarray platform. miR-375 was significantly down-regulated during this process. Furthermore, we provided evidence that miR-375 can regulate alveolar epithelial trans-differentiation through the Wnt/β-catenin pathway by direct targeting of FZD8.

4.3 Materials and Methods

4.3.1 Isolation of AEC II

AEC II were isolated from male Sprague-Dawley rats (200-250 g) as previously described [27]. All the animal experiments in this study followed the protocols approved by the Oklahoma State University Animal Care and Use committee. In brief, the rat lungs were perfused and isolated. They were then lavaged, and digested with elastase. The lung lobes were chopped

and incubated with DNase. The mixture was then filtered through a 160-μm, 37-μm, and 15-μm nylon mesh in sequence. The cells were incubated in rat IgG-coated plastic dishes twice for 30 min each. Cells were then pelleted and resuspended in complete medium (MEM supplemented with 10% FBS). The cell purity was > 90% as determined by the modified Papanicolaou staining.

4.3.2 Alveolar epithelial trans-differentiation

Freshly isolated AEC II were seeded into 35-mm tissue culture dishes at a density of 0.8 $\times 10^6$/dish. Cells were treated with different conditions after seeding and cultured for 1-5 days with medium changed on Day 1 and every other day.

4.3.3 RNA Extraction

Small RNA and total RNA were extracted from freshly isolated AEC II and cultured AEC II using mirVana™ miRNA isolation kit (Ambion, Austin, TX) following the manufacturer's instructions. For quantitative real-time PCR (qRT-PCR), total RNA was treated with TURBO DNA-*free*™ (Ambion) to eliminate genomic DNA contamination.

4.3.4 Fetal lung isolation

Whole lungs were isolated from rat fetuses on gestational day 16, 19, 21 (E16, E19, E21), new born (P0), postnatal day 6 and 14 (P6 and P14), and adult (AD) rats. For fetal lungs, pregnant Sprague-Dawley rats were sacrificed with CO_2 and the lungs were isolated from the fetuses. For pup and adult lungs, male rats were anesthetized with Ketamine (40 mg/kg body weight) and Xylazine (8 mg/kg body weight) and sacrificed before isolation of the lungs. The lungs were homogenized immediately after isolation and total RNA was extracted as described above.

4.3.5 MicroRNA microarray

microRNA expression profiling was performed as previously described with a microRNA microarray platform developed in our lab [15]. For each sample, 600 ng enriched small RNA was labeled with NCode microRNA Labeling system (Invitrogen, Carlsbad, CA). In each hybridization, labeled RNA recovered from 120 ng small RNA was co-hybridized with a common reference which came from a pool of equal amounts of all the samples. Dye swaps were performed to eliminate dye bias. After hybridization, the scanned images were analyzed with GenePix 5.0 pro (Axon Instruments, Inc. Union City, CA). The data was analyzed as previously described [15]. The signal from each spot was normalized to the average signal of the whole block and the highest and lowest signals from the 6 identical probes in the same block were excluded from further analysis. The geometric average of the remaining 4 signals was calculated as the signal of that particular microRNA. The signals were qualified with Realspot software [38] and all the microRNAs that passed the quality test were subjected to SAM analysis (Significant Analysis of Microarrays, Stanford University, http://www-stat.stanford.edu/~tibs/SAM/).

4.3.6 qRT-PCR

qRT-PCR was performed with TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. In each RT reaction, 75 ng of total RNA was used as the template. The reactions were incubated on ice for 5 min, 16 ℃ for 30 min, 42 ℃ for 30 min, and 85°C for 5 min. The RT products were diluted 1:3 and 5 µl of diluted RT product was used as the template in each PCR reaction. The PCR reactions were incubated at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 60 s. qRT-PCR for 18S rRNA was also performed for each sample as an endogenous control. Each PCR reaction was performed in duplicate. The comparative CT method was used and the relative expression of microRNA was calculated with the equation $2^{-(C_T\text{microRNA}-C_T18s)}$.

4.3.7 *In situ* hybridization

In situ hybridization for miR-375 was performed with 5' DIG-labeled LNA probes (Exiqon, Woburn, MA), following the manufacturer's instructions. The scrambled sequence for the negative control is GTGTAACACGTCTATACGCCCA. In brief, paraffin-embedded adult lung tissues were dewaxed in xylene and rehydrated. The slides were permeabilized by incubating with Proteinase K for 10 min at 37 ℃. The reaction was stopped with 0.2% glycine. Then, the slides were fixed with 4% paraformaldehyde and prehybridized in hybridization buffer for 1 h. After that, the tissues were incubated with 20 nM 5' DIG-labeled probes at 60 ℃ overnight in a humidified chamber. After stringent rinses, they were blocked with blocking buffer, followed by incubation with anti-DIG-AP Fab fragments antibody (1:2000, Roche Applied Sciences, Indianapolis, IN) at 4 ℃ overnight. The color reaction was carried out in 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) color solution (Roche Applied Sciences). After washing, the slides were mounted and observed under a Nikon E-600 microscope.

4.3.8 Construction of adenoviral vectors

The pre-miRNA sequence of hsa-miR-375 with flanking sequences of 350 nt on both the 5' and 3' ends was PCR-amplified from human genomic DNA with the following primers: forward primer cacctcgagGCACAGCCTCTCCCACCCGTA and reverse primer gagaattcCGTGTCAGCCGCAGATGCGT. The sequence was inserted into the pENTR plasmid (Invitrogen), downstream of the CMV-GFP [39, 40]. The insert was then switched to the adenoviral vector through LR recombination. After digestion by Pac I, the vector was transfected into 293A cells to produce adenovirus overexpressing miR-375 (Ad-miR-375). A control virus was also constructed from an empty vector with GFP only. For the β-catenin silencing adenovirus vector (si-β-catenin), we used a new method developed in our laboratory [41]. Four short hairpin RNAs were driven by four different promoters: mU6, hU6, H1, and 7SK. The four small RNA sequences for β-catenin silencing were: GGACCAGGTGGTCGTTAATAA, GTGGATTCCGTACTGTTCTAC, GAATGCCGTTCGCCTTCATTA, and

ACTGTTGGATTGATCCGAAAC. The expression cassette was then cloned into the adenovirus vector as described above [41]. A vector expressing four non-relevant siRNA sequences was used as a control (si-control). For the Ad-ΔGSK-β-catenin adenoviral vector, the eGFP-ΔGSK-β-catenin expression sequence was transferred from pEGFP-C1 vector into a pENTR vector. This sequence contained mutations at four GSK3β phosphorylation sites (Ser33, Ser37, Thr41, and Ser45) [42]. The pEGFP-C1 vector expressing ΔGSK-β-catenin was a gift of Dr. Angela Barth from Stanford University.

4.3.9 3'-UTR luciferase assay

The 3'-UTR of rat FZD8 was PCR-amplified and cloned into pGL3 vector downstream of a firefly luciferase reporter gene. The following primers for PCR were used: forward, CGCGAATTCCTGAACGGAAGCCCAGAAG, and reverse, GACTCTAGAGCTGCTGTTAGTGTAAGTGGC. The forward primer includes an EcoRI restriction site and the reverse primer contains an XbaI restriction site. One day after plating 293A cells in a 96-well plate, 50 ng microRNA overexpression plasmid pENTR-miR-375 was co-transfected into 239A cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) together with 5 ng of the reporter construct and 0.4 ng control pRL-TK, which contains a *Renilla* luciferase gene (Promega). The cells were harvested and luciferase activities were detected 48 h post-transfection using Dual Luciferase Reporter Assay System (Promega). In brief, 35 μl passive lysis buffer was applied to each well. After shaking, 7 μl cell lysate was taken for the dual-luciferase reporter assay. Firefly luciferase activity and *Renilla* luciferase activity were measured by the FLUOstar OPTIMA microplate fluorometer (BMG LABTECH, Offenburg, Germany).

4.3.10 Western blotting

The following primary antibodies were used in Western blotting and immunocytochemistry: mouse monoclonal anti-T1α (1:2000) from Dr. Mary Williams (Boston

University), mouse monoclonal anti-PCNA (ab29, 1:5000) from AbCam (Cambridge, MA), mouse monoclonal anti-ABC (#05-665, 1:500) from Millipore (Billerica, MA), mouse monoclonal anti-β-catenin (#610154, 1:2000) from BD (Franklin Lakes, NJ), rabbit polyclonal anti-CSNK2A1 (#2656, 1:1000) from Cell Signaling (Danvers, MA), goat polyclonal anti-FZD8 (sc-33504, 1:200) from Santa Cruz (Santa Cruz, CA), rabbit polyclonal anti-PDK1 (#3062, 1:1000) from Cell Signaling, and rabbit polyclonal anti-β-actin from Sigma (A-2066, 1:2000). For Western blots, 35 μg of protein was loaded for each sample. After incubation with primary antibodies, the membranes were washed with Tris-Buffered Saline and 0.05% Tween 20 (TBS-T) and incubated with horseradish peroxidase-conjugated anti-mouse or rabbit secondary antibodies (1:2000) for 1 h. The target proteins were visualized and quantitated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and Molecular Imager VersaDoc MP 5000 System (Bio-Rad), respectively.

4.3.11 Immunocytochemistry

The cultured cells were fixed in 4% paraformaldehyde, permeabilized with 0.4% Triton X-100, and then blocked in 10% FBS. The cells were incubated with mouse anti-T1α (1:250) or rabbit polyclonal anti-SP-C (Santa Cruz, sc-13979, 1:100) antibodies at 4 ℃ overnight followed by incubation with Alexa 546-conjugated anti-mouse or anti-rabbit secondary antibodies (1:250). After washing, the cells were examined under a Nikon Eclipse E600 fluorescence microscope.

4.3.12 Chromatin immunoprecipitation assay

The ChIP assay was performed with EZ-ChIP™ Chromatin immunoprecipitation kit from Millipore (Billerica, MA). Briefly, freshly isolated AEC II or cultured cells were fixed in 1% formaldehyde. The crosslinked DNA was then sheared into ~200-1000 base pair fragments with sonication. One percent of the sheared DNA was set aside as an input control. Anti-β-catenin antibody or normal mouse IgG was incubated with sheared DNA at 4 ℃ overnight with rotation.

Protein G Agarose was then added. Protein/DNA complexes were then eluted from the agarose and the crosslinking then was reversed to free DNA. Purified DNA was then analyzed with PCR using the primers flanking LEF1 biding site in the CCND1 promoter. The GAPDH promoter without LEF1 biding sites was used as a negative control. The following primers were used: CCND1 forward TTCTCTGCCCGGCTTTGAT, CCND1 reverse CACAGGAGCTGGTGTTCCATG, GAPDH forward GTGCAAAAGACCCTGAACAATG, and GAPDH reverse GAAGCTATTCTAGTCTGATAACCTCC.

Statistical Analysis- All experiments were performed with at least 3 independent replicates. The statistical analysis of microarray data was carried out as described in the microRNA microarray section. For all the other assays, student t-test was used and $p<0.05$ was considered significant. All values were shown as means $\pm$ S.E.

4.4 Results

4.4.1 MicroRNA expression profiling during alveolar epithelial trans-differentiation

In order to determine which microRNAs are important in the regulation of alveolar epithelial cell trans-differentiation, we utilized the microRNA microarray platform developed in our laboratory to profile the expression of microRNAs during this process. Enriched small RNAs from freshly isolated AEC II and those cultured in plastic dishes for 1~5 days (D0, D1, D3, and D5) were co-hybridized on the array slides together with the common reference. Among all the up-regulated and down-regulated microRNAs, miR-375 is of particular interest. This microRNA has previously been reported to be a pancreas specific microRNA and a regulator of insulin secretion [9]. However, we have previously reported that it is also highly expressed in the lung [15]. The expression of miR-375 was decreased significantly during alveolar epithelial trans-differentiation. On Day 1, the expression of miR-375 was decreased 2-fold. On Day 3 and Day 5, it was decreased 10-fold and 16-fold, respectively. This result was confirmed by qRT-PCR (Fig.

IV1a). According to qRT-PCR, this decrease of miR-375 was much larger (100-fold on Day 5). We also determined the expression levels of other microRNAs by qRT-PCR (Fig. IV1b). The miR-351 decreased markedly starting on day1 while miR-346 was unchanged on day 1 and decreased to about ~50% of starting levels at day 3 and day 5. miR-20a showed a modest increase on day 1 and a decrease on day 5. miR-21 had a 2-fold increase on Day 3. miR-27b and miR-34a exhibited only a modest decrease on Day 1. The expression patterns of these microRNAs suggest that expression of microRNAs undergoes dynamic changes during alveolar epithelial cell trans-differentiation.

4.4.2 miR-375 is enriched in AEC II

In order to determine the cellular location of miR-375, *in situ* hybridization using 5' DIG-labeled LNA probe for miR-375 was done in adult lung tissue sections. A probe with a scrambled sequence was used as a negative control. Positive signals were observed in different areas of the lung, including airway and alveolar epithelium and mesenchyme. More signals were observed at the corners of the alveolar spaces where AEC II exist (shown by the arrow) (Fig. IV2a). In order to confirm the enrichment of miR-375 in AEC II, the relative expression of miR-375 in AEC II and the whole lung was measured using qRT-PCR. The relative expression level of miR-375 was 2.8-fold higher than that in the whole lung (Fig. IV2b).

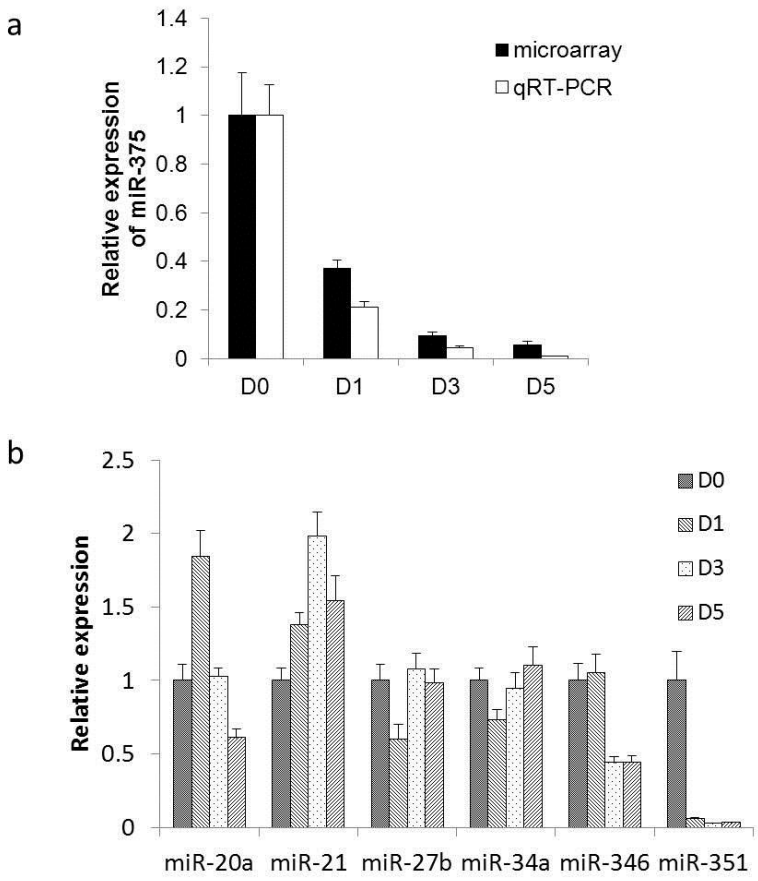
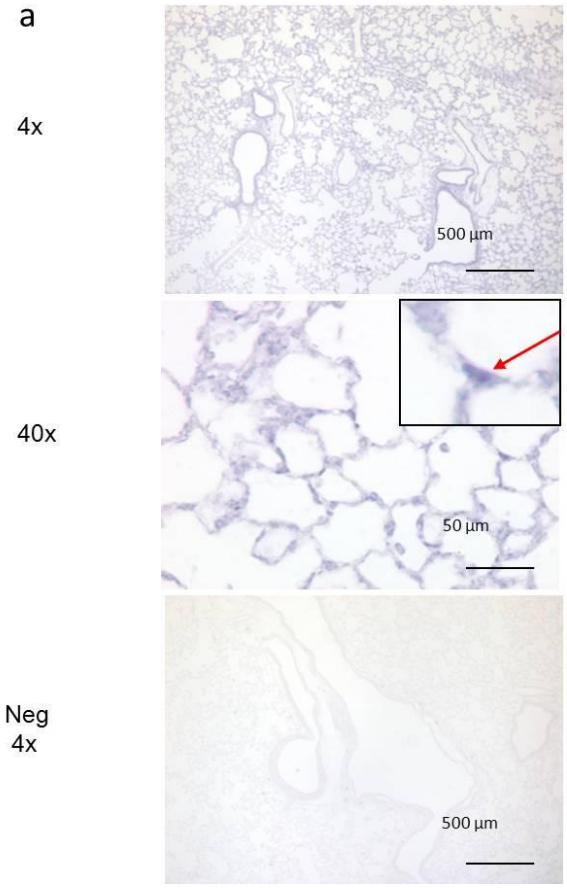


Fig. IV1 **The expression pattern of miR-375 and other microRNAs in the alveolar epithelial cell trans-differentiation.** Freshly isolated AEC II were cultured for 0, 1, 3, or 5 days (D0, D1, D3, or D5) on plastic dishes. (a) Relative expression of miR-375 was determined by both microarray and qRT-PCR. The expression level of miR-375 at each time point was expressed as the fraction of that on D0. Data shown are means ± S.E from 3 independent cell preparations and technical replicates of 4 (microarray) or 2 (qRT-PCR). (b) Relative expression of miR-20a, 21, 27b, 34a, 346, and 351 was determined by qRT-PCR. Data shown are means ± S.E. from 3 independent cell preparations.



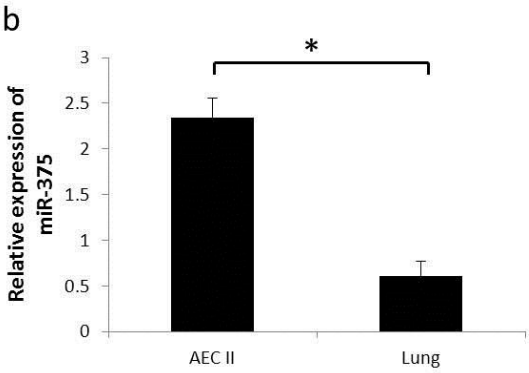


Fig. IV2 **Localization of miR-375 in the lung.** *(a)* Detection of miR-375 in adult lung by *in situ* hybridization. Paraffin-embedded adult lung tissues were hybridized with a 5' DIG-labeled LNA probe containing the complementary sequence to miR-375. The expression pattern of miR-375 was assessed under different magnification (4x and 40x). Different areas showed positive signals, including the epithelium of the alveoli and airways. A 5'DIG-labed LNA probe containing a scrambled sequence was hybridized to the lung tissues as a negative control (Neg). The arrow points to a type II cell. *(b)* Detection of the expression of miR-375 in isolated AEC II and the whole lung by qRT-PCR. The expression levels of miR-375 were normalized to 18S rRNA. Data shown are means ± S.E. from 3 independent replicates. * $p<0.0001$.

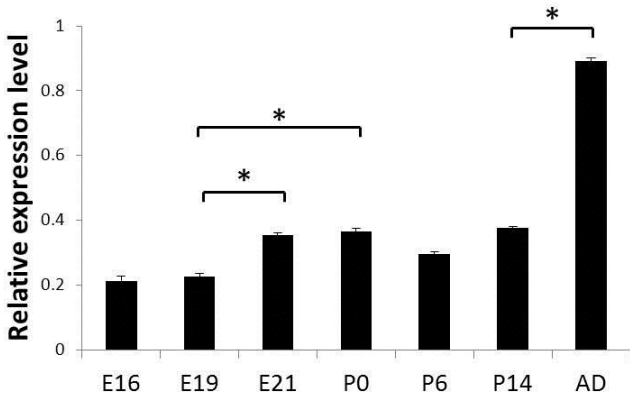


Fig. IV3 **The expression pattern of miR-375 during fetal lung development.** Whole lungs were isolated from rat fetuses on gestational day 16, 19, 21 (E16, E19, E21), new born (P0), postnatal day 6 and 14 (P6 and P14), and adult (AD) rats. The expression levels of miR-375 were detected with qRT-PCR. Expression data were normalized to 18S rRNA. * $p<0.001$. Data shown are means ± S.E. from 3 independent replicates.

4.4.3 Expression patterns of miR-375 in lung development

In order to study the functions of miR-375 in alveolar epithelial trans-differentiation, we also determined the expression of miR-375 during fetal lung development that involves trans-differentiation using qRT-PCR. The development of the fetal lung can be divided into 4 stages: the embryonic phase, the glandular or pseudoglandular phase, the canalicular phase, and the saccular phase. We selected different time points that represent different stages of lung development and determined the expression of miR-375 at these time points. In rat, the canalicular phase starts on Day 18 and ends on Day 20. The saccular phase lasts from Day 20 to full term. AEC II first appear in the transition from the canalicular to the saccular phase. During the saccular stage, the differentiation of AEC II becomes more evident. As determined by qRT-PCR, the expression of miR-375 increased at this late stage (E21 and P0), which could result from more differentiated AEC II in the lung. The amount of miR-375 increased two-fold from P14 to AD. In the adult lung, the percentage of AEC II is much higher than that in the fetal lung, which could explain why the expression of miR-375 is much higher in adult lungs than in fetal lungs.

4.4.4 miR-375 inhibits alveolar epithelial cell trans-differentiation

The expression of miR-375 is significantly decreased during alveolar epithelial trans-differentiation. It is reasonable to expect that the decrease of endogenous miR-375 is important for this process. In order to study the function of miR-375 in this process, an adenovirus overexpressing miR-375 was used to transduce AEC II. The overexpression efficiency was assessed by eGFP signaling on Day 4 and qRT-PCR for miR-375 on Day 2. Nearly 100% of the cells were expressing eGFP (Fig. IV4a). The expression of miR-375 was increased more than 40-fold, compared with that in cells transduced with the control virus (Fig. IV4b).

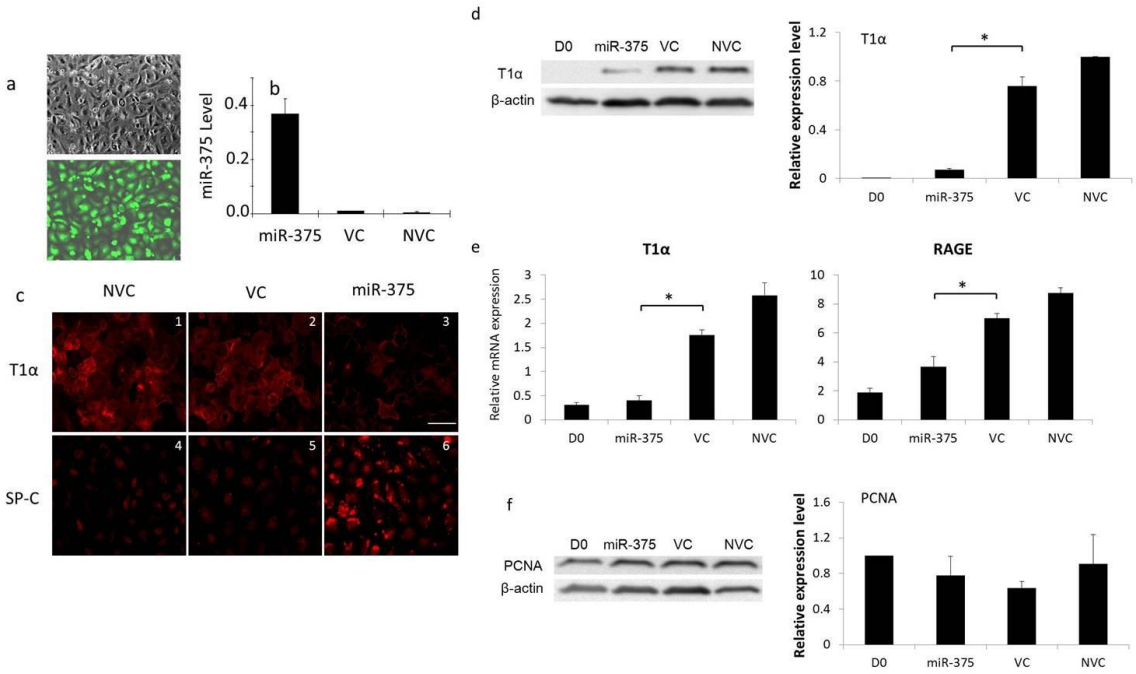


Fig. IV4 **Effect of miR-375 overexpression on the trans-differentiation of AEC II to AEC I.** Freshly isolated AEC II were transduced with a miR-375 overexpression virus or a virus control (VC) (multiplicity of infection or MOI=100) or none (NVC) and cultured for 4 days if not mentioned otherwise. (a) Bright-field and GFP fluorescence of miR-375-transduced cells. (b) miR-375 levels on Day 2 revealed by real-time PCR. Data are normalized to 18S rRNA. (c) Immunofluorescence of T1α and SP-C using anti-T1α and anti-SP-C antibodies. Because of GFP, double-labeling is not practical. (d) The expression of T1α was detected and quantitated with Western blots. Data were normalized to β-actin. (e) The mRNA levels of T1α and RAGE were determined by qRT-PCR. (f) The expression of PCNA was detected and quantitated with Western blots. Data were normalized to β-actin. Data shown are means ± S.E. from 3 independent cell preparations. * $p<0.001$.

The effect of miR-375 overexpression on alveolar epithelial cell trans-differentiation was monitored after culturing for 4 days. T1α, a marker protein for AEC I, and SP-C, a marker protein for AEC II were detected with immunofluorescence. In non-virus and virus controls, SP-C disappeared and T1α increased significantly, indicating that AEC II trans-differentiated into AEC I (Fig. IV4c). In cells overexpressing miR-375, T1α was significantly decreased and SP-C was increased in comparison with controls (Fig. IV4c). The expression level of T1α was further determined using Western blots. The amount of T1α in cells overexpressing miR-375 decreased significantly compared with that in cells transduced with the control virus ($p<0.001$). The control virus had no effects on T1α expression (Fig. IV4d). In addition, the mRNA levels of T1α and RAGE, an AEC I marker were dramatically increased during trans-differentiation and were significantly inhibited by miR-375 (Fig. IV4e). These results suggest that alveolar epithelial trans-differentiation is inhibited by miR-375. The effect of miR-375 on cell proliferation was also determined by detecting the expression of the proliferation marker, PCNA. The expression level of PCNA was not changed by miR-375 overexpression (Fig. IV4f), indicating that miR-375 does not affect cell proliferation.

4.4.5 miR-375 inactivates Wnt/β-catenin pathway

It has been shown that the Wnt/β-catenin signaling pathway can promote alveolar epithelial cell trans-differentiation *in vitro* [37]. We tested whether this pathway was affected by miR-375. In freshly isolated AEC II, there was almost no β-catenin detected. After culturing for 4 days, there was an abundance of β-catenin protein (Fig. IV5a). Interestingly, the mRNA level of β-catenin did not change during culture (Fig. IV5c). The overexpression of miR-375 slightly but not significantly decreased the total amount of β-catenin. The active form of β-catenin (ABC) can be detected with the antibody that recognizes β-catenin dephosphorylated on Ser37 or Thr41. Activated β-catenin (ABC) was decreased significantly after miR-375 overexpression (Fig. IV5b).

Cyclin D1 (CCND1) is a direct transcriptional target of the β-catenin/LEF-1 pathway. The expression of CCND1 is regulated by this pathway through a LEF-1 binding site in the CCND1 promoter [43]. We further used the chromatin immunoprecipitation (ChIP) assay to detect the binding of β-catenin to the promoter of CCND1 during the process of trans-differentiation after transduction of miR-375 or control virus (VC). Fractions of the sheared DNA samples were set aside as input controls. The rest of the DNA was precipitated with the β-catenin antibody or mouse normal IgG. The purified DNAs from the precipitations were amplified using the primers for LEF1 binding site and the flanking sequences of the CNND1 promoter. As expected, the overexpression of miR-375 eliminated the association between β-catenin and the promoter of CCND1 (Fig. IV5d). β-catenin did not bind to the GAPDH promoter that does not have the LEF1 binding sites. The negative control using normal IgG did not yield signals. These data provided evidence that miR-375 can inhibit the canonical Wnt/β-catenin pathway.

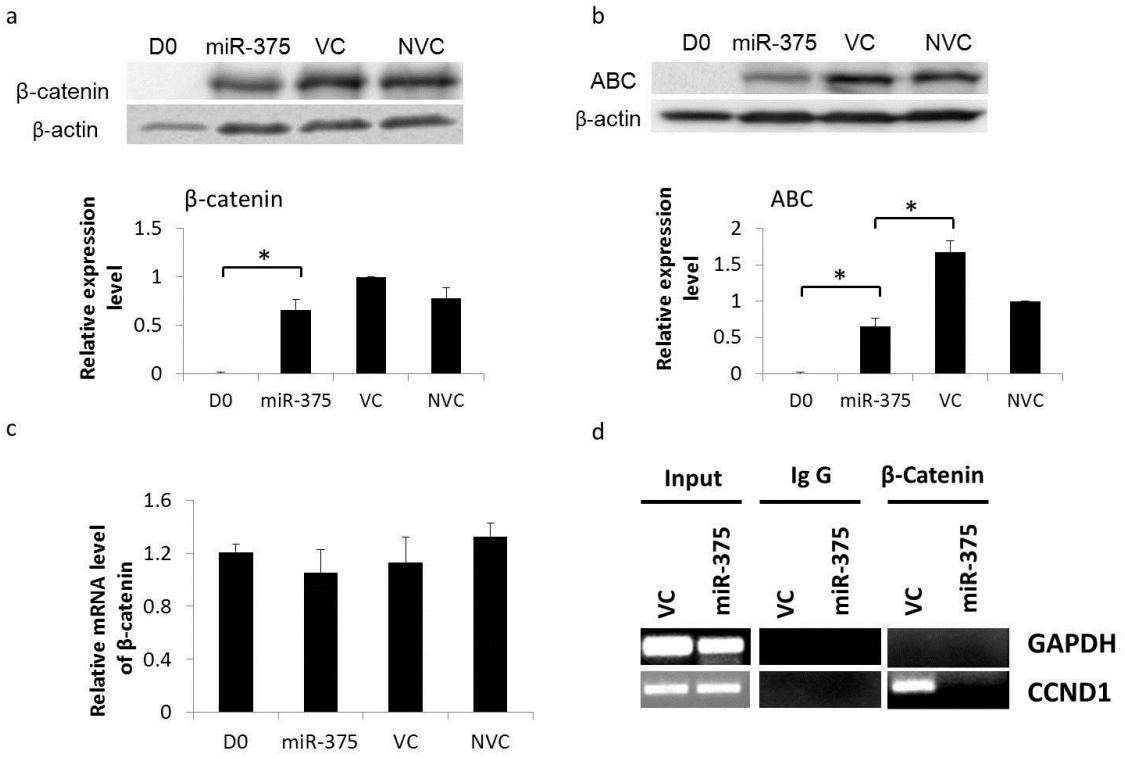


Fig. IV5 **Effect of miR-375 on the Wnt/β-catenin pathway.** Freshly isolated AEC II (D0) were transduced with Ad-miR-375 (miR-375) or control virus (VC) (MOI=100), or none (NVC) and cultured for 4 days. The relative amounts of total β-catenin and activated β-catenin (ABC) were detected with Western blots (a, b) and qRT-PCR (c) after miR-375 overexpression in the alveolar epithelial cell trans-differentiation. Data shown are means ± S.E. from 3 independent replicates. * $p<0.01$. (d) ChIP assays were used to detect the association of β-catenin with the promoter of CCND1 in AEC II transduced with Ad-miR-375 or the virus control and cultured for 4 days. Mouse normal IgG was used as a negative control. The promoter of GAPDH was used as another negative control.

4.4.6 Knockdown of β-catenin inhibits alveolar epithelial cell trans-differentiation

In order to test whether the inhibition of alveolar epithelial trans-differentiation by miR-375 is caused by inhibition of the Wnt/β-catenin pathway, we decided to knockdown β-catenin to block this pathway. We utilized a novel method to construct an adenovirus vector that expresses 4 shRNAs targeting to β-catenin (si-β-catenin) [41]. AEC II were treated with si-β-catenin, or control virus, on D0 with a MOI of 100. The cells were collected on Day 4 and the expression of β-catenin was detected by Western blot. As shown in Fig. IV6, the expression of β-catenin was reduced by more than 90%. The silencing of β-catenin led to the decrease in T1α, indicating the inhibition of alveolar epithelial cell trans-differentiation.

4.4.7 Stabilized β-catenin blocks the effect of miR-375

In order to demonstrate that the regulation of miR-375 on the alveolar epithelial cell trans-differentiation occurs through down-regulation of activated β-catenin, we transduced AEC II with adenovirus Ad-ΔGSK-β-catenin, which expressed a stabilized form of β-catenin without GSK3β phosphorylation sites (ΔGSK-β-catenin) [42]. The expression of ΔGSK-β-catenin was confirmed by Western blots with ABC antibody (Fig. IV7a). The expression of T1α was decreased by miR-375 overexpression in the absence of ΔGSK-β-catenin. In the cells treated with Ad-ΔGSK-β-catenin, the expression of T1α was no longer affected by miR-375 (Fig. IV7b). These data showed that expression of stabilized β-catenin and thus constitutively activation of Wnt/β-catenin pathway blocked the effect of miR-375 on AEC trans-differentiation.

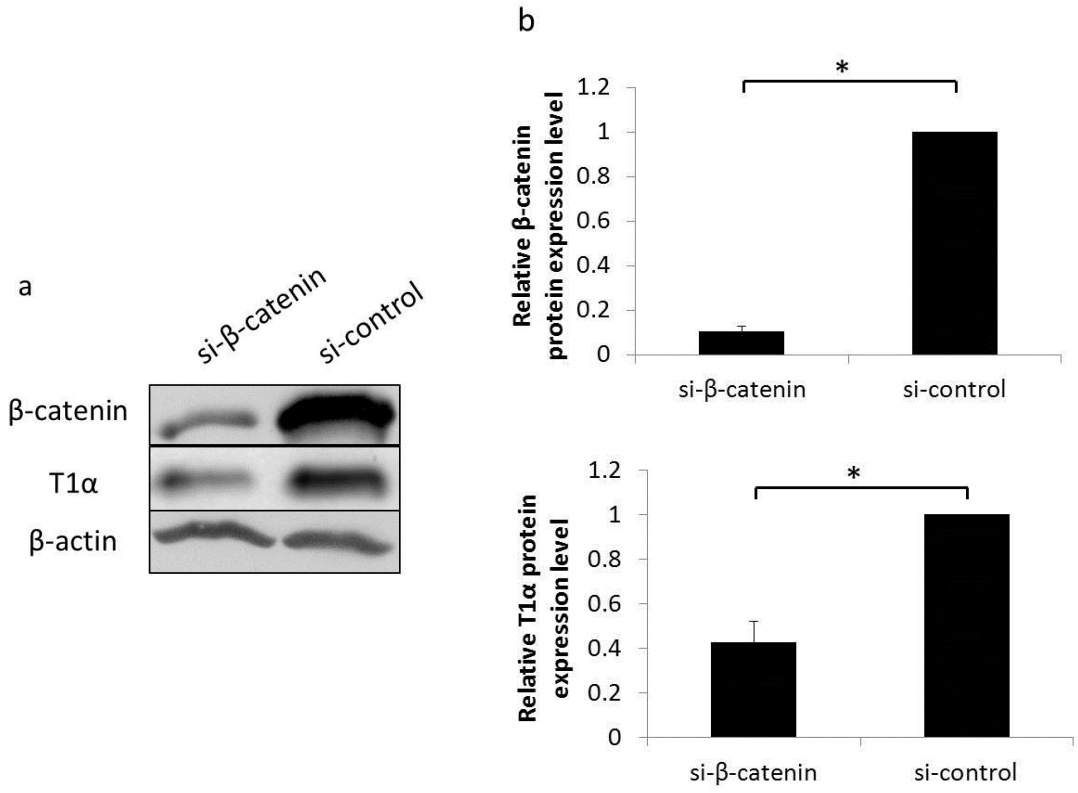


Fig. IV6 **Effect of silencing β-catenin on AEC trans-differentiation.** AEC II were transduced with β-catenin silencing virus (si-β-catenin) or control virus (si-control) and then cultured for 4 days. The expression of β-catenin and T1α was detected with Western blots. The signals were quantitated and the expression levels were shown as the fractions of those in the si-controls. Data shown are means ± S.E. from 3 independent replicates. * $p<0.01$ vs. si-control.

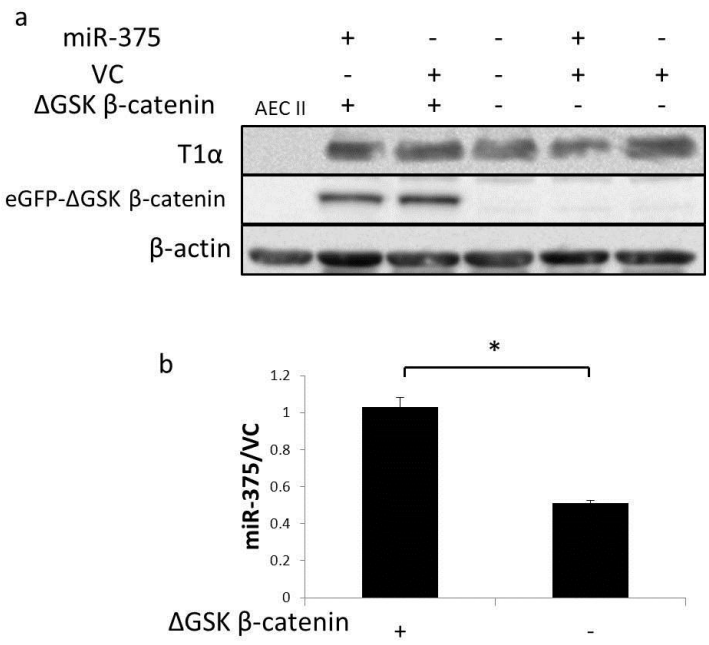


Fig. IV7 **Effect of stabilized β-catenin on the trans-differentiation.** Freshly isolated AEC II were transduced with Ad-miR-375 or virus control with or without the expression of eGFP-ΔGSK β-catenin as indicated in the figure (Ad-miR-375 MOI=50 and Ad-ΔGSK β-catenin MOI=5). Cells were then cultured for 4 days. (a) The expression of T1α and eGFP-ΔGSK β-catenin was detected by Western blots using anti-T1α and anti-ABC antibodies, respectively. (b) The Western blots were quantitated. The expression of T1α with miR-375 overexpression was normalized to that in the virus control. Data shown were means ± S.E. from 4 independent experiments. * $p<0.001$.

4.4.8 FZD8 is a target of miR-375 in the Wnt/β-catenin pathway

The web-based software TargetScan and PicTar predicted FZD8 as a target of miR-375. To verify the prediction, we produced a reporter construct by fusing the 3'-UTR of FZD8 with a firefly luciferase reporter gene. The microRNA-375 overexpression plasmid and the luciferase reporter construct were transfected into 293A cells together with a control vector pRL-TK, which contains a *Renilla* luciferase gene. As shown in Fig. IV8a, the luciferase activity of the reporter construct containing the 3'-UTR of FZD8 was significantly decreased by miR-375.In order to confirm that endogenous FZD8 is regulated by miR-375, we examined the effects of miR-375 on the expression of FZD8 during alveolar epithelial cell trans-differentiation. The protein level of FZD8 was decreased significantly by miR-375 as shown in Fig. IV8b. The mRNA level of FZD8 was also decreased by 60% after miR-375 overexpression compared to cells infected with the control virus (Fig. IV8c). Altogether, miR-375 inhibits the expression of FZD8 at both protein and mRNA levels.

CSNK2A1 (casein kinase II, alpha 1 polypeptide) is another predicted target of miR-375 in the canonical Wnt signaling pathway. However, the protein expression of CSNK2A1 was also not affected by miR-375 overexpression (Fig. IV8d). These data ruled out the possibility that miR-375 regulates Wnt/β-catenin signaling through CSNK2A1 during alveolar epithelial cell trans-differentiation.

It has been reported that PDK1 is a direct target of miR-375 in gastric carcinomas [14]. In order to test whether it is the same in our case, we also examined the expression of PDK1 during AEC trans-differentiation (Fig. IV8e). The expression of PDK1 was not changed during the trans-differentiation (from D0 to D4), and was not decreased by miR-375 overexpression. These data indicate that PDK1 is not the target of miR-375 in AEC probably because of differences in the cell environment.

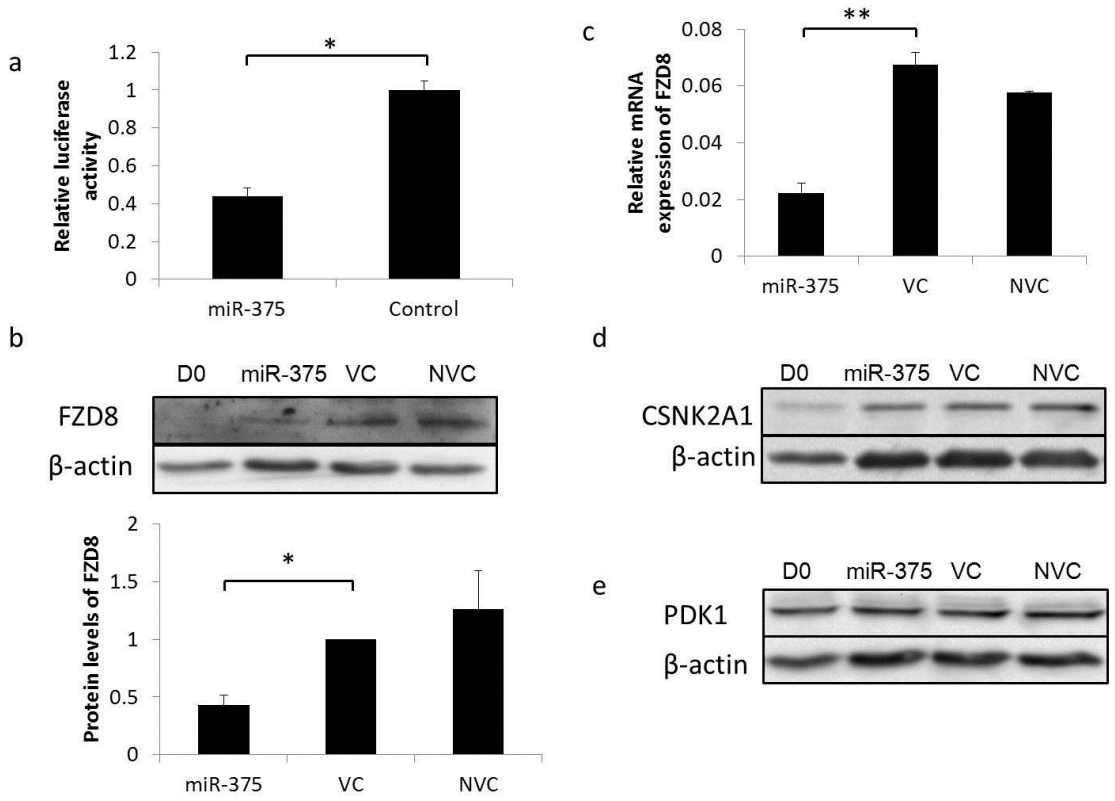


Fig. IV8 **Identification of target genes.** (a) Luciferase assay. 3'-UTR of FZD8 was fused with the firefly luciferase gene. miR-375 overexpression plasmids were co-transfected with the luciferase-3'-UTR constructs into 293A cells. Luciferase activity was determined 48 h post-transfection. The firefly luciferase activity was first normalized to the Renilla luciferase activity and then expressed as a ratio of the control plasmid. (b,c) Effect of miR-375 on the expression of endogenous FZD8. AEC II were transduced with Ad-miR-375 or virus control (MOI=100) and then cultured for 4 days. The protein and mRNA levels of FZD8 were detected with Western blots (b) and qRT-PCR (c). The expression levels of CSNK2A1 (d) and PDK1 (e) were detected with Western blots. Data represent means ± S.E. from at least 3 replicates. * $p<0.01$, ** $p<0.001$. The blots shown are representatives of 3 independent replicates.

4.4.9 miR-375 is down-regulated in IPF

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive interstitial lung disease, characterized by fibrosis and excessive deposition of collagen in the pulmonary interstitium [44]. IPF is also characterized by AEC II hyperplasia [44, 45]. A role of alveolar epithelial injury, AEC II proliferation and trans-differentiation in IPF pathogenesis and progression has been demonstrated [44]. In this study, we determined the expression of miR-375 in lung samples from IPF patients using qRT-PCR. The expression of miR-375 was decreased in severe IPF (50-80% forced vital capacity (FVC) and <50% FVC) compared to less severe IPF (>80%) (Fig. IV9). This result is consistent with our discovery that miR-375 is decreased during AEC II trans-differentiation.

4.5 Discussion

In this study, we carried out microRNA expression profiling during alveolar epithelial cell trans-differentiation by using the microRNA microarray platform. Among all the microRNAs whose expression levels were changed during this process, miR-375 was one of the most down-regulated microRNAs. Further studies demonstrated that miR-375 inhibited the process of trans-differentiation when it was over-expressed in isolated AEC II. miR-375 also inactivated the Wnt/β-catenin pathway, whereas constitutively activated β-catenin reversed the miR-375 effects, indicating that miR-375 acts up-stream of β-catenin. FZD8 was identified as a direct target of miR-375. Together, these data indicate that miR-375 inhibits alveolar epithelial cell trans-differentiation through the inactivation of Wnt/β-catenin pathway, by direct targeting of FZD8 (Fig. IV10).

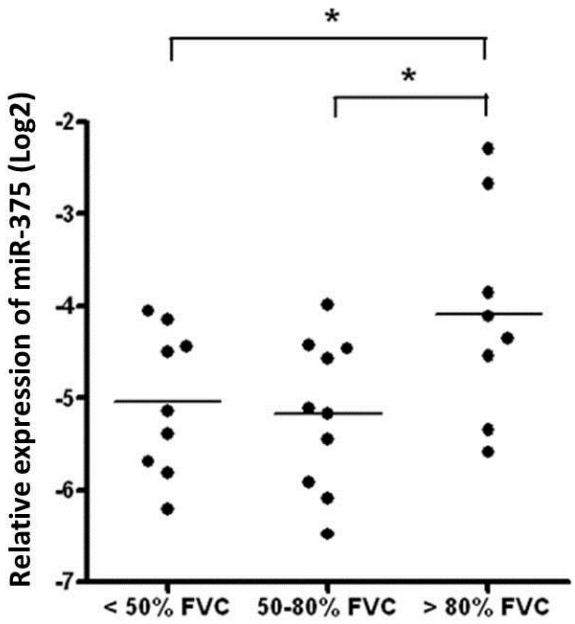


Fig. IV9 **The expression of miR-375 is down-regulated in IPF.** The expression of miR-375 in the lungs of patients with different stages of IPF was determined with qRT-PCR. n=10 for <50% FVC, n=10 for 50-80% FVC, and n=8 for >80% FVC. *p<0.05.

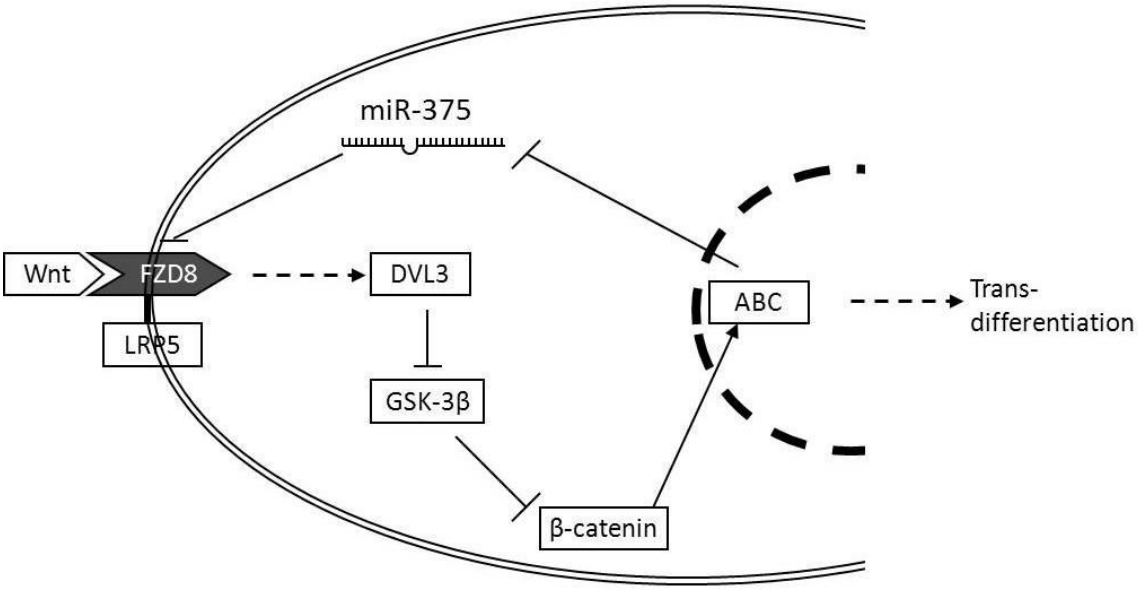


Fig. IV10 **miR-375 inhibits alveolar epithelial cell trans-differentiation through inactivation of the Wnt/β-catenin pathway.**

To our knowledge, this is the first report studying the function of miR-375 in the lung epithelial cell differentiation. When miR-375 was first identified, it was reported to be expressed only in pancreatic islets but not in other tissues [9]. However, we have shown that miR-375 is highly expressed in the rat lung in comparison with heart, liver, kidney, spleen, and brain [15]. This result was verified by Northern blots. In this study, we discovered that miR-375 was enriched in AEC II, and that the expression of miR-375 was dramatically down-regulated during trans-differentiation of AEC II to AEC I. In addition, miR-375 expression in fetal lungs was lower than that in the adult lung. All of the expression data suggest a potential role of miR-375 in the lung.

There have been several studies regarding the function of miR-375 in the pancreas. miR-375 suppresses insulin secretion by direct targeting of the mRNA of *myotrophin (Mtpn)* [9]. miR-375 not only regulates the function of the pancreatic islet but also affects the development of pancreatic islets. Knockdown of miR-375 results in aberrant formation of the pancreas islet [10]. In addition, miR-375 is reported to maintain normal pancreatic α and β cell mass and therefore normal glucose homeostasis [46].

In spite of all these studies in the pancreas, there are few functional studies of miR-375 in other organs. It has been reported that the expression of miR-375 is down-regulated in hepatocellular tumors [47, 48]. An expression ratio of miR-221:miR-375 showed a high sensitivity and specificity for head and neck squamous cell carcinoma, indicating that miR-375 may be used as a diagnostic marker for certain cancers [47].

We have demonstrated that miR-375 inhibits pulmonary surfactant secretion from AEC II. This inhibition is carried out via the reorganization of cytoskeleton, rather than effects on surfactant synthesis or the formation of lamellar bodies [40]. The result is reminiscent of the function of miR-375 in the regulation of insulin secretion in pancreas islets [9].

The Wnt signaling pathway has been proven to have widespread roles in tissue differentiation and organogenesis [49] including fetal lung development. This pathway regulates branching morphogenesis and epithelial cell differentiation [30, 31]. Wnt7b and Wnt5a induce lung branching morphogenesis through epithelial-mesenchymal communications, possibly via N-myc, BMP4, and FGF signaling. An activated form of β-catenin causes ectopic differentiation of AEC II-like cells in airways, indicating a critical role for the Wnt/β-catenin signaling pathway in the differentiation of lung epithelial cells [32].

A recent study, which used the same AEC II culture model that we did, has shown the pivotal function of the β-catenin pathway during alveolar epithelial trans-differentiation [37]. Constitutive β-catenin signaling cannot be detected in adult AEC II *in vivo* or in freshly isolated AEC II. This signaling is activated after the lung is subjected to bleomycin-induced injury or during the culture of AEC II. Activation of the Wnt/β-catenin pathway promotes AEC trans-differentiation, while forced inhibition of this pathway leads to an increase in cell death [37]. In our study, we also found that this pathway was activated during the process of trans-differentiation and that silencing of β-catenin inhibited trans-differentiation. Total β-catenin protein could not be detected in freshly isolated AEC II and increased markedly in cultured AEC II. However, the mRNA level of β-catenin did not change in freshly isolated and cultured AEC II. This could result from detachment of β-catenin from cell-cell adhesion complex and degradation by proteasomes during AEC II isolation.

It has been reported that miR-8 negatively regulates the Wnt/Wingless pathway at multiple levels by directly targeting *wntless* and *CG32767* and by repressing the protein level of TCF in *Drosophila* [50]. The depression of Wnt signaling by miR-8 leads to promotion of adipogenesis in mammals [50]. Canonical Wnt signaling is important for osteoblast differentiation. miR-29a modulates osteoblast differentiation by directly targeting to the negative regulators of Wnt/β-catenin signaling, namely Dkk1, Kremen2, and sFRP2. On the other hand,

the miR-29a promoter activity is under the regulation of the canonical Wnt pathway [51]. Activation of the canonical Wnt pathway induces miR-29a transcription, which subsequently down-regulates Wnt antagonists and induces Wnt signaling even further.

Although we are the first to report that the Wnt/β-catenin pathway is regulated by miR-375, we are not the only group that is interested in the relation between miR-375 and this pathway. The down-regulation of miR-375 is associated with β-catenin mutations in hepatocellular tumors [48], suggesting that the activation of β-catenin signaling represses miR-375. Our results showed that miR-375 can inhibit the activation of the Wnt/β-catenin pathway. During trans-differentiation, miR-375 is down-regulated and β-catenin signaling is activated, which could lead to further depression of miR-375. It is quite likely that miR-375 regulates AEC trans-differentiation through this positive feedback loop. In this process, miR-375 may work as a switch of trans-differentiation. Once miR-375 decreases at the beginning of trans-differentiation, the Wnt/β-catenin signaling is activated, which leads to further depression of miR-375 expression and prevents the process from going backward.

In investigating the mechanism of how miR-375 regulates alveolar epithelial cell trans-differentiation through the Wnt/β-catenin pathway, we identified FZD8 as a target of miR-375. FZD8 is one of the Wnt receptors. The binding of Wnt ligands to the FZD8 receptor leads to the stabilization of cytoplasmic β-catenin and activation of the canonical Wnt pathway. Whole-mount RNA *in* situ hybridizations have shown that FZD8 is highly expressed throughout the epithelium during the early stage of mouse lung development [36]. We demonstrate in this study that miR-375 down-regulates the mRNA and protein levels of FZD8 during alveolar epithelial cell trans-differentiation, leading to the inactivation of the Wnt/β-catenin pathway.

FZD8 is not likely to be the only target of miR-375. TargetScan software predicts 141 and 117 targets for human and rat miR-375, respectively. However, it should be noted that many

of these predictions cannot be verified experimentally. Furthermore, the relationship between microRNAs and targets is also cell content- and developmental stage-dependent. One of the components of Wnt/β-catenin signaling, CSNK2A1 (casein kinase II), is another predicted target of miR-375; PDK1 has been experimentally verified as a direct target of miR-375 in gastric carcinomas [11]. However, both CSNK2A1 and PDK1 protein levels were unchanged during the trans-differentiation of type II cells to type I cells and thus these factors are not likely targets of miR-375 in alveolar epithelial cells. Other targets identified in the literature, including YAP, HuD, RASD1 and Mtpn are not obviously related to Wnt/β-catenin signaling [12, 52, 53].

Alveolar epithelial cell trans-differentiation is involved in the pathogenesis of IPF and progression. It has been reported that the Wnt/β-catenin pathway is significantly activated in AEC II from IPF patients [54, 55]. Increased Wnt/β-catenin signaling induces alveolar epithelial cell proliferation and probably trans-differentiation. In other words, a process similar to alveolar epithelial cell trans-differentiation is involved in IPF. There is a negative correlation between the expression of miR-375 and activation of Wnt/β-catenin signaling in IPF, which further indicates that miR-375 may modulates alveolar epithelial cell trans-differentiation through the regulation of the canonical Wnt signaling pathway in the diseased state.

Alveolar epithelial cell trans-differentiation is very important for the recovery of injured alveolar epithelium resulting from a variety of disease conditions. Thus, the findings from this study may shed light on the recovery from lung injuries. The discovery that down-regulation of miR-375 and activation of the Wnt/β-catenin pathway are necessary for this process may provide potential targets for therapeutic intervention. In addition, it is also well known that abnormal activation of Wnt signaling resulting from several different genetic defects causes cancers [56]. It is possible that the inhibition of Wnt signaling by miR-375 can be used as a therapeutic method to treat cancers.

In summary, we identified a new function of miR-375 in alveolar epithelial cell trans-differentiation. The regulation of AEC trans-differentiation by miR-375 is through the inhibition of the canonical Wnt pathway by direct targeting of FZD8. This discovery may provide potential targets for therapeutic intervention in the recovery from lung injuries.

Acknowledgements

We thank Dr. Mary Williams (Boston University) and Dr. Angela Barth (Stanford University) for kindly providing anti-T1α antibodies and ΔGSK-β-catenin construct. This study utilized biological specimens and data provided by the Lung Tissue Research Consortium (LTRC), supported by the National Heart, Lung, and Blood Institute (NHLBI). This work was supported by the National Institutes of Health [HL071628, HL087884, and HL095383 (LL)]. Yang Wang, Tingting Weng, and Yujie Guo were supported by American Heart Association predoctoral fellowships [08100162], [06101432], and [09PRE2300211].

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CHAPTER V

MIR-124 REGULATES FETAL PULMONARY EPITHELIAL CELL MATURATION BY TARGETING NFIB

5.1 Abstract

MicroRNAs are a family of small non-coding RNAs that regulate the expression of their target proteins at the post-transcriptional level. Their functions cover almost every aspect of cell physiology and are involved in lung diseases, liver diseases, cardiovascular diseases, and cancers. However, the functions of microRNAs in fetal lung development remain largely unknown. In this study, we discovered that the expression of miR-124 was dramatically down-regulated during fetal lung development, which corresponds to the increase in expression of surfactant proteins. Overexpressing miR-124 led to the inhibition of fetal lung epithelium maturation in both fetal lung organ culture and fetal lung epithelial cell culture as indicated by the decrease in surfactant protein expression and the increase in the cellular glycogen pool. miR-124 also modulates several important signaling pathways, including the NFκB and Wnt signaling pathways. We further determined that NFIB, which is known to regulate fetal lung epithelium maturation, is a direct target of miR-124 by 3' UTR reporter assay and endogenous NFIB expression. In conclusion, miR-124 inhibits fetal epithelium maturation through the inhibition of NFIB.

5.2 Introduction

The development of the rat lung can be divided into 5 stages: the embryonic stage (0-13 days), glandular stage (13-18 days), the canalicular stage (18-20 days), the saccular stage (20 days to full term), and the alveolar stage (after birth) [1]. In the embryonic stage, a pair of lung rudiments arise from the primitive esophagus. In the glandular stage, the air passage tubes are formed by dichotomous branching of the terminal buds, the lung appears like a gland, and the epithelium switches from pseudostratified cells to columnar cells. At the canalicular stage, the mesenchymal tissue becomes thin and bronchioles appear, epithelial cells become cuboidal, and glycogen content also increases. In the saccular stage, some cells flatten and other cells remain cuboidal, the cuboidal cells lose glycogen and begin to synthesize surfactant, and alveolar ducts and air sacs form. The formation of true alveoli occurs in the alveolar stage after birth. The transcriptional control of fetal lung development has been studied for a long time. However, the functions of microRNAs in the regulation of epithelial development and maturation are still largely unknown.

MicroRNAs are a group of small RNAs (~22 nt) that regulate protein expression at the post-transcriptional level. They bind to the mRNA of target genes directly and usually down-regulate their expression by cleavage of the target mRNA, translational inhibition, or mRNA deadenylation [2-5]. To date, thousands of microRNAs have been discovered in humans and other animals. According to a study *in silico*, more than half of the mammalian mRNAs are under selective pressure and are conserved targets of microRNAs [6]. So far, the known functions of microRNAs have covered almost every aspect of cell physiology, including cell proliferation and differentiation, apoptosis, and lipid and fat metabolism. MicroRNAs are implicated in cancer, diabetes, and many other diseases [7-9].

There have been some reports on microRNA functions in fetal lung development. Conditional deletion of Dicer, an enzyme required for the maturation of microRNAs, in lung epithelium resulted in branching arrest, indicating the important role of microRNAs in fetal lung morphogenesis [10]. The miR-17-92 cluster is also important in fetal epithelial development [11]. The cluster is highly expressed at early stages of development and down-regulated at later stages. Overexpression of this cluster in fetal lung epithelium results in increased epithelial progenitor cell proliferation and inhibition of differentiation. In addition, another study has shown that miR-17-92 null mice develop severely hypoplastic lungs [12]. In our previous study, we showed that the expression levels of 21 microRNAs change during different stages of fetal lung development, which suggests the importance of microRNAs in the regulation of lung development [13]. We further found that overexpression of miR-127 results in the disruption of fetal lung morphogenesis, with a decrease in the number of terminal buds but an increase in the bud size. However, in spite of these studies, the functions of microRNAs in lung development are still largely unknown.

miR-124 is preferentially expressed in the brain [14]. It has been reported that miR-124 drives the gene expression profile toward that of the brain [15]. It helps to define and maintain the cell specificities in the brain. At least two mechanisms are involved in this regulation. First, miR-124 directly targets PTBP1 (polypyrimidine tract binding protein 1), which is a global repressor of nervous system-specific pre-mRNA splicing. miR-124 inhibits the expression of PTBP1, leading to the differentiation from progenitor cells to neuronal cells [16]. Another mechanism involves SCP1 (small C-terminal domain phosphatase 1), which is an anti-neural phosphatase. During neurogenesis, miR-124 depresses the expression of SCP1 and antagonizes the REST/SCP1 pathway [17]. However, so far, no one has reported the functions of miR-124 in lung development.

In this study, we discovered that the expression of miR-124 was significantly down-regulated during fetal lung development. Overexpression of miR-124 inhibited fetal lung epithelium maturation as indicated by decreased expression of surfactant proteins (SP-A, SP-B, and SP-C) and increased amount of glycogen pool. Several signaling pathways were affected by miR-124 overexpression. In addition, we identified nuclear factor I/B (NFIB), a transcriptional factor known to regulate fetal lung epithelium maturation, as a direct target of miR-124. Taken together, we demonstrated that miR-124 inhibited fetal lung epithelium maturation by directly targeting NFIB.

5.3 Materials and methods

5.3.1 qRT-PCR

Total RNAs were isolated from fetal lung explants and cells using the mirVana™ miRNA isolation kit (Ambion, Austin, TX) and then treated with TURBO DNA-free™ (Ambion) to eliminate any genomic DNA contamination. For each reverse transcription reaction, 1 μg of total RNA was reverse-transcribed into cDNA using MMLV reverse transcriptase. Real-time PCR was performed using SYBR Green master mix from Eurogentec (Seraing, Belgium) on an ABI 7500 Fast system (Applied Biosystems, Foster City, CA). The primers used in this study are listed in Table V1.

Table V1: Primers for qRT-PCR. SP-A/B/C, surfactant protein A/B/C; T1α, podoplanin; CCSP, Clara cell secretory protein; eGFP, enhanced green fluorescent protein; FAS, fatty acid synthase.

SP-A forward	GATCAAACATCAGATTCTGCAAACA
SP-A reverse	TCCTGCTCTGGTACACATCTCTTTA
SP-B forward	AATGACCTGTGCCAAGAGTGTG
SP-B reverse	AGGACCAGCTTGTTCAGCAGAG
SP-C forward	AGCTCCAGGAACCTACTGCTACAT
SP-C reverse	AGGACTTGGCCTGGAAGTTCTT
T1α forward	GCCATCGGTGCGCTAGAAGATGATCTT
T1α reverse	GTGATCGTGGTCGGAGGTTCCTGAGGT
CCSP forward	CTAATTATGAGGCAGCCCTGAAG
CCSP reverse	GTCTCCTGTGGGAGGGTATCC
eGFP forward	CTGCTGCCCGACAACCA

eGFP reverse	GAACTCCAGCAGGACCATGTG
FAS forward	TCAGAGGTTACACTGTGTTAGGTGTTG
FAS reverse	CCCATCCCTGAGCAGATGAA
18s forward	TCCCAGTAAGTGCGGGTCATA
18s reverse	CGAGGGCCTCACTAAACCATC

MicroRNA qRT-PCR for miR-124 was performed with the TaqMan® MicroRNA Assay kit (Applied Biosystems). In each reverse transcription reaction, 75 ng of total RNA was used as template for cDNA synthesis. The reactions were kept on ice for 5 min and then incubated at 16 ℃ for 30 min, 42 ℃ for 30 min, and 85 ℃ for 5 min. The PCR reactions were carried out at 95 ℃ for 10 min, and then 40 cycles of 95 ℃ for 15 s and 60 ℃ for 60 s. The expression of 18S rRNA was also detected as an internal control. The relative expression of miR-124 was calculated with the formula $2^{-(C_T\text{microRNA}-C_T18s)}$.

5.3.2 *In situ* hybridization

In situ hybridization of miR-124 was performed with 5' DIG-labeled LNA probes from Exiqon (Woburn, MA). A probe with a scrambled sequence (GTGTAACACGTCTATACGCCCA) was used as a negative control. Paraffin sections of rat fetal lung tissues were dewaxed in xylene and rehydrated. The sections were then permeabilized with 10 μg/ml of Proteinase K at 37 ℃ for 5 min, followed by incubation in 0.2% Glycine to stop the reaction. The sections were then fixed in 4% paraformaldehyde and prehybridized in hybridization buffer for 2 h. After that, the sections were hybridized with 20 nM probes in hybridization buffer at 60 ℃ overnight. After stringent washes, the sections were blocked in blocking buffer for 1 h and then incubated with anti-DIG-AP Fab fragments (1:2000, Roche Applied Sciences, Indianapolis, IN) at 4 ℃ overnight. After washing with Phosphate Buffered Saline with 0.05% Tween 20 (PBST) and Alkaline Phosphatase (AP) buffer, the sections were incubated in 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) color solution (Roche Applied Sciences) until desired blue color developed.

5.3.3 Adenovirus construction

The construction of miR-124 overexpression adenoviral vector (Ad-miR-124) was performed as previously described [13]. The pre-miRNA sequence of miR-124-1 with flanking sequences at both the 5' and the 3' end was cloned from human genomic DNA. The primers used were as follows: forward primer CACCTCGAGCACACGCACCGTCTACACTTC, reverse primer GAGAATTCTATTTGCACAGGCGGGAACTAC. This sequence was then inserted into the pENTR vector (Invitrogen, Carlsbad, CA) downstream of the CMV-GFP through XhoI and EcoRI restriction sites and switched to the adenoviral vector plasmid through LR recombination. This plasmid was digested by Pac I and transfected into 293A cells to produce adenovirus. Virus containing cells and medium were harvested and the crude adenoviral lysate was prepared by three freeze/thaw cycles and centrifugation. The adenoviral stock was amplified by infecting 293A cells with 100 μl of crude adenoviral lysate. In order to titer the amplified adenovirus stock, the virus stock was diluted 10^4 times with serial dilutions. 293A cells were infected with the diluted virus and the infected cells with eGFP signals were counted 48 h after infection. The virus titer is expressed as plaque-forming units (PFU) per ml. The virus made from empty pENTR vector with CMV-GFP was used as a control.

5.3.4 Fetal lung organ culture

For timed pregnancy, the day on which vaginal plugs were discovered was denoted as gestational Day 0. Female Sprague-Dawley timed pregnant rats on gestational Day 15 were euthanized with CO2. Fetal lungs were dissected from fetuses without surrounding tissues. They were then transferred to transwell inserts (Millipore, Billerica, MA) in 6-well plates. Each well contained 1.5 ml culture medium. The culture medium consisted of BGJb medium supplemented with 0.2 mg/ml ascorbic acid, 50 U/ml penicillin, and 50μg/ml streptomycin. Three hours later, the medium were removed and replaced with fresh culture medium. Adenovirus (10^8 pfu) was

pre-incubated with 0.1 mg/ml of protamine for 15 min. The total volume of the mixture was 40 µl. After the incubation, the mixture was added to each fetal lung explants. This day was denoted as D0. Medium with viruses was removed 24 h later and culture medium was changed every day until sample collection at day 5 (D5). The second virus treatment at the same dose was given to each fetal lung explant on D2. The protocols used in the animal experiments in this study were approved by the Oklahoma State University Animal Care and Use Committee.

5.3.5 Fetal AEC II isolation and culture

Fetal alveolar epithelial type II cells (AEC II) were isolated as described by Batenburg et al [18]. Timed pregnant Sprague-Dawley rats on gestational day 18 were euthanized with CO_2. Ten to fourteen fetal lungs were dissected from the fetuses and chopped into 1-mm^3 pieces. Cells were dissociated by digestion with 10 ml of a solution consisting of 1 mg/ml Collagenase, 1 mg/ml Trypsin, and 0.4 mg/ml DNase I in MEM for 10 min at 37 ℃. The lung tissue was then pelleted by spinning at 70 g for 1 min and the supernatant was collected. The digestion was repeated 3 times. The supernatant was collected and pooled together. The resulting cell suspension was filtered through 160- and 37-µm nylon filters. Cells were then seeded in a 20-cm plastic dish and incubated for 45 min x 4 times to remove fibroblasts. The cell suspension was then filtered through a 15-µm nylon filter. Two million fetal AEC II were plated on 500 µl Matrigel (BD Matrigel™ Basement Membrane Matrix High Concentration, Growth Factor Reduced, Cat # 354263, Lot # 99301, 18.7 mg/ml, 1:1 dilution) in each well of a 6-well plate and infected with adenovirus expressing miR-124 (Ad-miR-124) or control virus with a multiplicity of infection (MOI) of 100. The medium used on Day 0 was MEM supplemented with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and 1% Penicillin/Streptomycin (Invitrogen, penicillin 5000 IU/ml, streptomycin 5mg/ml). On the second day, the medium was changed to the defined medium which consisted of DMEM supplemented with d-biotin (100 µg/L), ethanolamine (30 µg/L), phosphoethanolamine (70 µg/L), putrescine/bitane-1,4-diamile

(20 mg/L), transferrin (5 mg/L), $CuSO_4$ (25 μg/L), $FeCl_3$ (1 mg/L), $ZnCl_2$ (30 μg/L), $MnCl_2$ (10 μg/L), Na selenite (10 μg/L), 1% MEM non-essential amino acids (Invitrogen), and 1% penicillin/streptomycin. Cells were cultured on Matrigel for 2 days. After that, Matrigel was dissolved by incubation with 1 ml of Dispase (50 caseinolytic units/ml, BD Biosciences, Bedford, MA) for 1 h. Cells were then collected for RNA extraction with the mirVana™ microRNA isolation kit.

5.3.6 Anthrone assay

Glycogen content was determined as previously described [19, 20]. Briefly, cultured fetal lung explants were boiled in 100 μl 30% KOH for 30 min. The resulting slurry was diluted with 1.5 ml H_2O and mixed completely. A 400-μl aliquot of this diluted sample was then mixed with 800 μl of 0.2% anthrone reagent in 95% H_2SO_4 in a cold water bath. The mixture was boiled for 10 min and cooled down to room temperature in a cold water bath. The OD was read at 620 nm with a UV spectrophotometer.

5.3.7 Pathway screening

Pathway analysis was performed with Cignal™ Reporter Assays (SABiosciences, Frederick, MD). Nine pathways known to be involved in development, including Notch, Wnt, Myc/Max, NFκB, TGF-β, C/EBP, cAMP/PKA, MAPK/ERK, and MAPK/JNK, were screened using reporter luciferase assays. In brief, HEK 293T cells were transfected with 50 ng pathway reporter vector and 100 ng microRNA overexpression plasmid or control plasmid, with or without corresponding stimuli. The cells were harvested 48 h post-transfection and the firefly and *Renilla* luciferase activities were determined using Dual Luciferase Reporter Assay System (Promega) by the FLUOstar OPTIMA microplate fluorometer (BMG LABTECH, Offenburg, Germany). Firefly signals were normalized with *Renilla* signals.

5.3.8 3' UTR luciferase assay

The 3’ untranslated region (3’ UTR) of rat GNAI3 (G protein α inhibiting polypeptide 3), Foxa2 (forkhead box A2), SOS1 (son of sevenless homolog 1), NRAS (neuroblastoma ras oncogene), AKT2 (v-akt murine thymoma viral oncogene homolog 2), GRB2 (growth factor receptor bound protein 2), PIK3C2A (phosphoinositide-3-kinase, class 2, α polypeptide), NFIB, and ADCY9 (adenylate cyclase 9) were PCR-amplified from rat genomic DNA with Advantage® 2 Polymerase Mix (Clontech, Mountain View, CA) and cloned into pmirGLO Dual-Luciferase microRNA Target Expression Vector (Promega, Madison, WI) using NheI and SalI restriction sites except for ADCY9, for which SacI and SalI were used. The primers used for PCR were listed in Table V2.

Table V2 Primers for pmirGLO dual-luciferase microRNA target expression vector construction. The restriction sites were underlined.

GNAI3 forward	TCAGCTAGCTGTGGCCTTTTTTGCTAGGAGAC
GNAI3 reverse	TCAGTCGACGACAATCTTCAGACAGCTTTGG
SOS1 forward	TCTGCTAGCGACCTAAGCTGAGCCAAGAGAATAC
SOS1 reverse	TCAGTCGACTGTGGGCTATATAAGGCATTTT
NRAS forward	TCAGCTAGCAAGGACCCTTTAAAAGTTCTGT
NRAS reverse	TCAGTCGACGGTTTGAAGAATCATTAATCAC
AKT2 forward	ATAGCTAGCTCTGCCACCACAGGACACAGCAT
AKT2 reverse	TCAGTCGACCTAAGGCTTCCTTGTTCCACAC
GRB2 forward	TCAGCTAGCTTAAAGAAAGTGAAAAGTTGAG
GRB2 reverse	TCAGTCGACTTATTCACAGTTAATCACTACC
PIK3C2A forward	TATGCTAGCTGTTGACTCCTACCAATTCCAA
PIK3C2A reverse	ATAGTCGACGAAGTCACATTCAATTCACTGA
NFIB forward	TCAGCTAGCTGGTTCCTTTTCAAGTGTCAAA
NFIB reverse	TCAGTCGACGGTCAATTAAAACAAACAAACA
ADCY9 forward	TCAGAGCTCCTCTGCTTGTCCAAACACAATA
ADCY9 reverse	TCTGTCGACTCAGCTGTGTCCTTTGCAAACT

For each 3’ UTR, 50 ng of microRNA overexpression plasmid pENTR-miR-124 was transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) together with 5 ng of the pmirGLO reporter construct. The cells were harvested 48 h post-transfection and the firefly and *Renilla* luciferase activities were detected as described above. Empty pmirGLO reporter plasmid was also used for normalization purpose. For data analysis, first, the firefly luciferase activities were normalized to the *Renilla* luciferase activities. The fold changes of the normalized luciferase

activities with or without miR-124 overexpression were then divided by that of the pmirGLO empty vector.

5.3.9 Western blotting

The following primary antibodies were used in Western blotting: mouse monoclonal anti-PIK3C2A (Santa Cruz, sc-136298, 1:200), rabbit polyclonal anti-SOS1 (Santa Cruz, sc-256, 1:200), rabbit polyclonal anti-NFIB (Active Motif, Carlsbad, CA, 39091, 1:1000), rabbit polyclonal anti-SP-C (Santa Cruz, sc-13979, 1:500), and rabbit polyclonal anti-β-actin from Sigma (A-2066, 1:2000). For Western blots, 35 μg of protein was loaded into each well. The membranes were incubated with primary antibodies at 4 ℃ overnight. After washing with Tris-Buffered Saline and 0.05% Tween 20 (TBS-T), they were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:2000) for 1 h. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and visualized and quantitated with Molecular Imager VersaDoc MP 5000 System (Bio-Rad, Hercules, CA).

5.4 Results

5.4.1 miR-124 is down-regulated during fetal lung development

We measured the expression levels of miR-124 at different stages of fetal lung development, including gestational day 16, 19, and 21 (E16, E19, and E21), postnatal day 0, 6, and 14 (P0, P6, and P14), and 2 months after birth (AD), with microRNA qRT-PCR (Fig. V1a). The expression of miR-124 was remarkably decreased from E16 to E19 and continued to decrease gradually from E19 to adult. The localization of miR-124 in E16 fetal lung was determined by microRNA *in situ* hybridization. As shown in Fig. V1b, miR-124 was mainly expressed in the epithelial cells of E16 lungs.

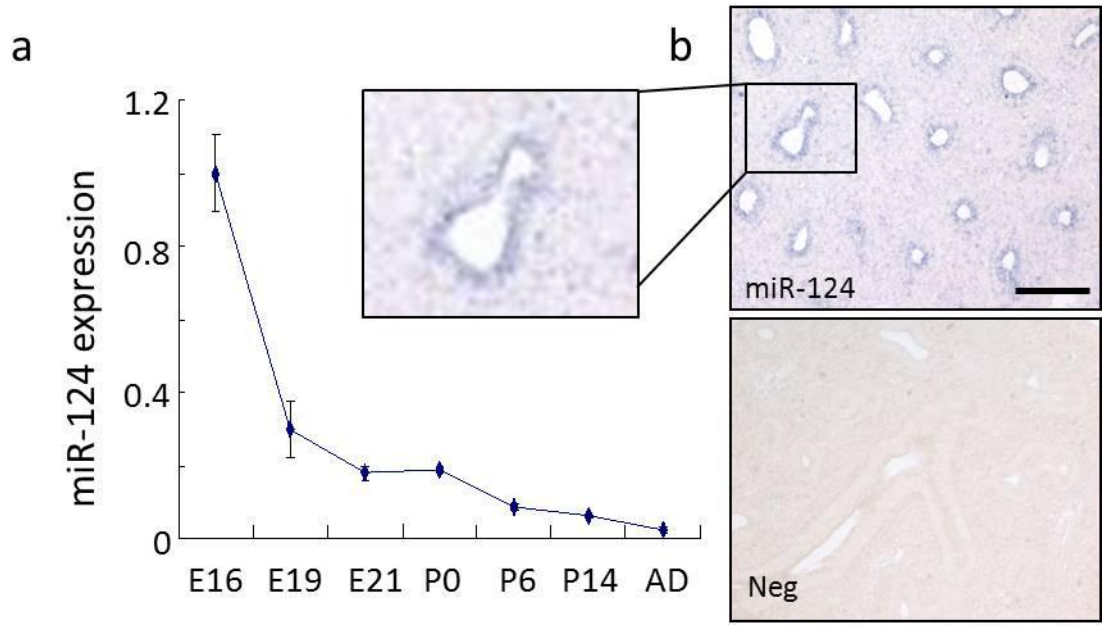

Fig. V1 **The expression of miR-124 during rat fetal lung development.** a) The mRNA expression patterns of miR-124 at different stages of fetal lung development (E16, E19, E21, P0, P6, P14, and AD) were determined using qRT-PCR. Data shown are means ± S.E. from 3 independent replicates. b) *In situ* hybridization for miR-124 in E16 fetal lung. Neg: negative control using a scrambled sequence. Scale bar: 100 μm.

5.4.2 miR-124 inhibits alveolar epithelial cell maturation.

In order to identify the functions of miR-124 during fetal lung development, we overexpressed miR-124 ectopically with an adenovirus in fetal lung organ culture. To improve the transduction efficiency, we pre-incubated adenovirus with protamine, which neutralizes the charges on the viruses and cells, and has been shown to improve the adenoviral transduction efficiency [21].

Fetal lungs on gestational day 15 were transduced with miR-124 overexpressing adenovirus (Ad-miR-124) in the presence of with 0.1 mg/ml of protamine and cultured for 5 days. The expression of miR-124 was detected with qRT-PCR (Fig.V2a). Viral overexpression increased the expression of miR-124 more than 10-fold.

In order to determine whether miR-124 influences the alveolar epithelial cell differentiation, we then determined the mRNA expression of several cell marker genes. The expression levels of SP-A, SP-B, SP-C and FAS, an important enzyme in surfactant synthesis, were significantly decreased in the miR-124 treated lungs (Fig. V2b). Western blots revealed that the SP-C protein was also decreased by miR-124 overexpression (Fig. V2c). However, the alveolar epithelial type I cell (AEC I) marker, T1α and the Clara cell marker, CCSP were not significantly changed.

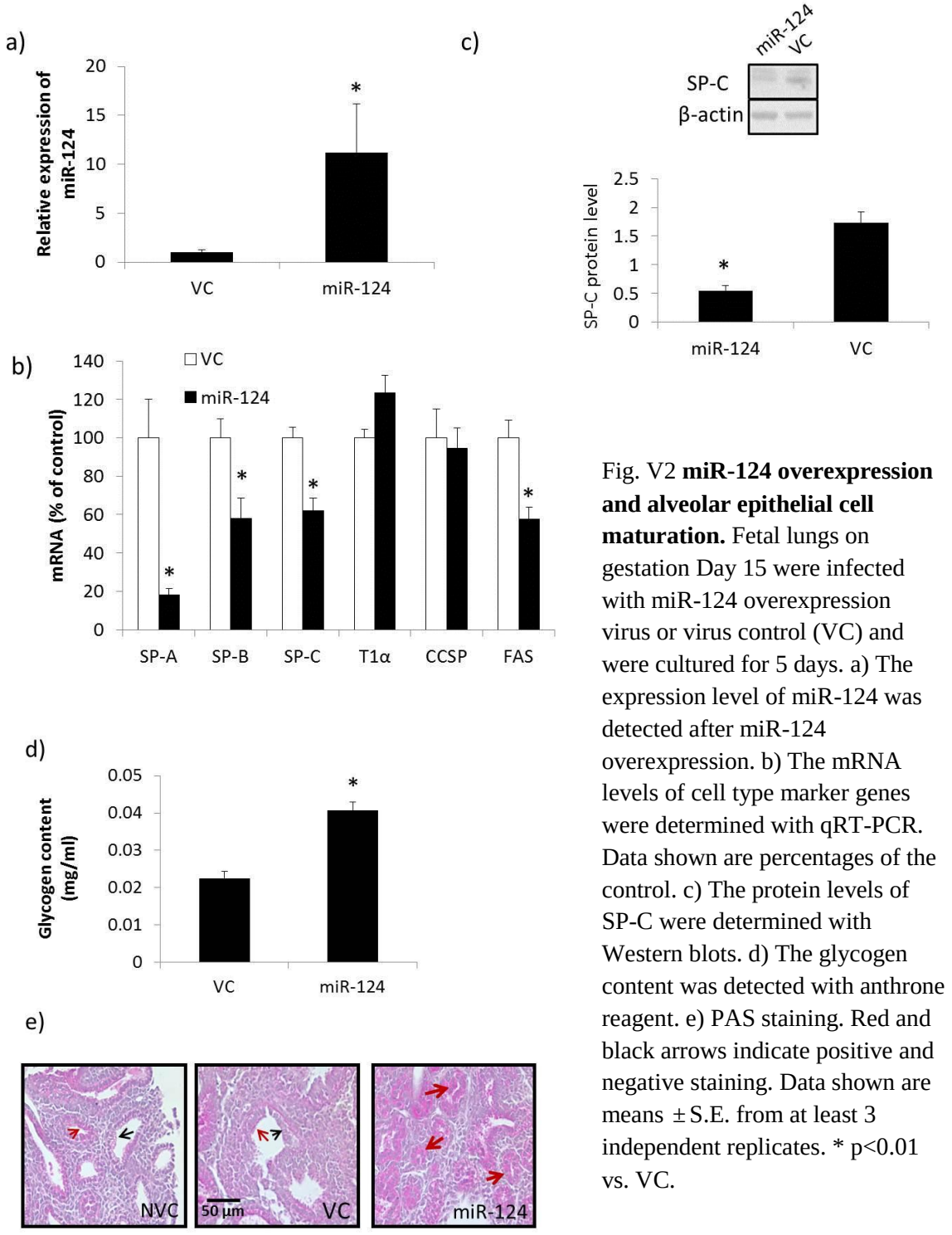

Fig. V2 **miR-124 overexpression and alveolar epithelial cell maturation.** Fetal lungs on gestation Day 15 were infected with miR-124 overexpression virus or virus control (VC) and were cultured for 5 days. a) The expression level of miR-124 was detected after miR-124 overexpression. b) The mRNA levels of cell type marker genes were determined with qRT-PCR. Data shown are percentages of the control. c) The protein levels of SP-C were determined with Western blots. d) The glycogen content was detected with anthrone reagent. e) PAS staining. Red and black arrows indicate positive and negative staining. Data shown are means ± S.E. from at least 3 independent replicates. * $p<0.01$ vs. VC.

Glycogen content was also measured to confirm the inhibition of AEC II maturation (Fig. V2d). During AEC II maturation, glycogen disappears from the pre AEC II. We performed an anthrone assay to measure the glycogen content in the fetal lung organ culture. The glycogen content was higher in the Ad-miR-124 transduced fetal lungs (Fig. V2d). In addition, the glycogen was also detected with periodic acid-Schiff (PAS) staining. The amount of glycogen was significantly increased in the Ad-miR-124 treated lungs (Fig. V2e). Taken together, these results indicate that miR-124 inhibited AEC II maturation, while AEC I and Clara cells were not affected.

5.4.3 miR-124 inhibits the expression of surfactant proteins in fetal AEC II culture.

In order to determine whether miR-124 inhibits AEC II maturation without the participation of mesenchyme, we isolated fetal AEC II cells from pregnant rats on gestational day 18 and transduced them with Ad-miR-124 or control virus in the presence of 0.1 mg/ml of protamine. It has been reported that isolated fetal rat AEC II can differentiate in defined medium on a basement membrane matrix, with formation of lamellar bodies and the expression of surfactant proteins [22]. The isolated fetal AEC II were cultured on Matrigel for 2 days and the expression of SP-A, SP-B, and SP-C was detected with qRT-PCR (Fig. V3). Consistent with results from fetal lung organ culture, the expression of SP-A, SP-B, and SP-C was significantly decreased. The decrease of surfactant proteins indicated the inhibition of AEC II maturation. These results indicate that miR-124 can inhibit fetal AEC II maturation without participation of mesenchyme.

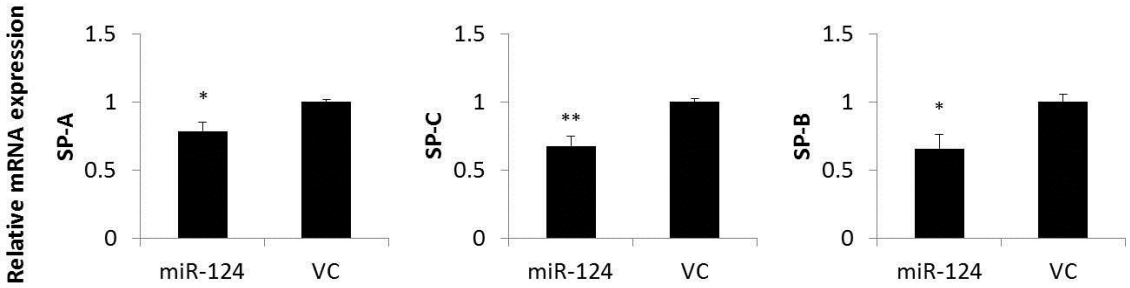

Fig. V3 **Expression of surfactant proteins in cultured fetal AEC II.** E18 fetal AEC II were infected with Ad-miR-124 or control virus (VC) (MOI= 100) and cultured on Matrigel for 2 days. The mRNA expression of SP-A, SP-B, and SP-C were detected with qRT-PCR. Data shown are mean ± S.E. from 3 independent replicates. (*p<0.05, ** p<0.005 vs. VC)

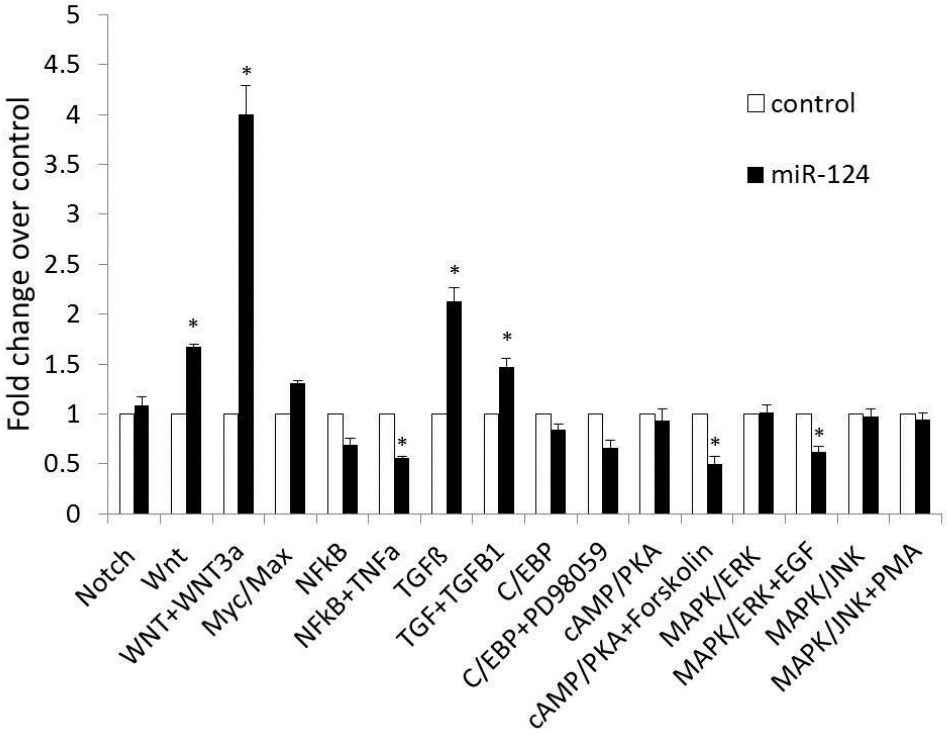

Fig. V4 **Luciferase assays for screening of pathways affected by miR-124.** The activities of 9 development pathways after miR-124 overexpression with or without stimuli were determined using luciferase reporter assays in HEK 293T cells. The activities were expressed as fractions of the control using an empty overexpression vector. Data shown are means ± S.E. * p<0.005.

5.4.4 miR-124 inhibits NFκB, cAMP/PKA and MAP/ERK pathways

In order to figure out how miR-124 regulates epithelium maturation in fetal lung development, we screened several development pathways with reporter vectors to identify which pathways are regulated by miR-124. The Wnt, NFκB, TGF-β, C/EBP, cAMP, MAPK/ERK, and MAPK/JNK pathways were stimulated with Wnt3a conditioned medium, tumor necrosis factor α (TNFα, 0.01 ng/μl), transforming growth factor, beta 1 (TGF-β1, 0.005 ng/μl), PD98059 (2.67 ng/μl), forskolin (1 ng/μl), epidermal growth factor (EGF, 0.1 ng/μl), and phorbol 12-myristate 13-acetate (PMA, 0.01 ng/μl), respectively. The luciferase reporter assays revealed that NFκB, cAMP/PKA, and MAPK/ERK pathways under stimulated conditions are significantly depressed by miR-124, while the Wnt and TGF-β pathways were stimulated by miR-124 at both basal and stimulated states (Fig. V4).

According to target prediction with TargetScan (http://www.targetscan.org/) and pathway analysis with DAVID (http://david.abcc.ncifcrf.gov/), some predicted targets of miR-124 were nested in miR-124-depressed pathways, including GNAI3 and ADCY9 in the cAMP/PKA pathway, SOS1, NRAS, and GRB2 in the MAPK/ERK pathway, and AKT2 and PIK3C2A in the upstream of the NFκB pathway. The 3' UTR dual luciferase assays revealed that SOS1 and PIK3C2A were significantly inhibited by miR-124 and all others were not affected (Fig. V5a). However, when we overexpressed miR-124 in MLE15 cells, the protein levels of SOS1 and PIK3C2A were not changed (Fig. V5b), suggesting that these two proteins are not targeted by miR-124 in lung epithelial cells.

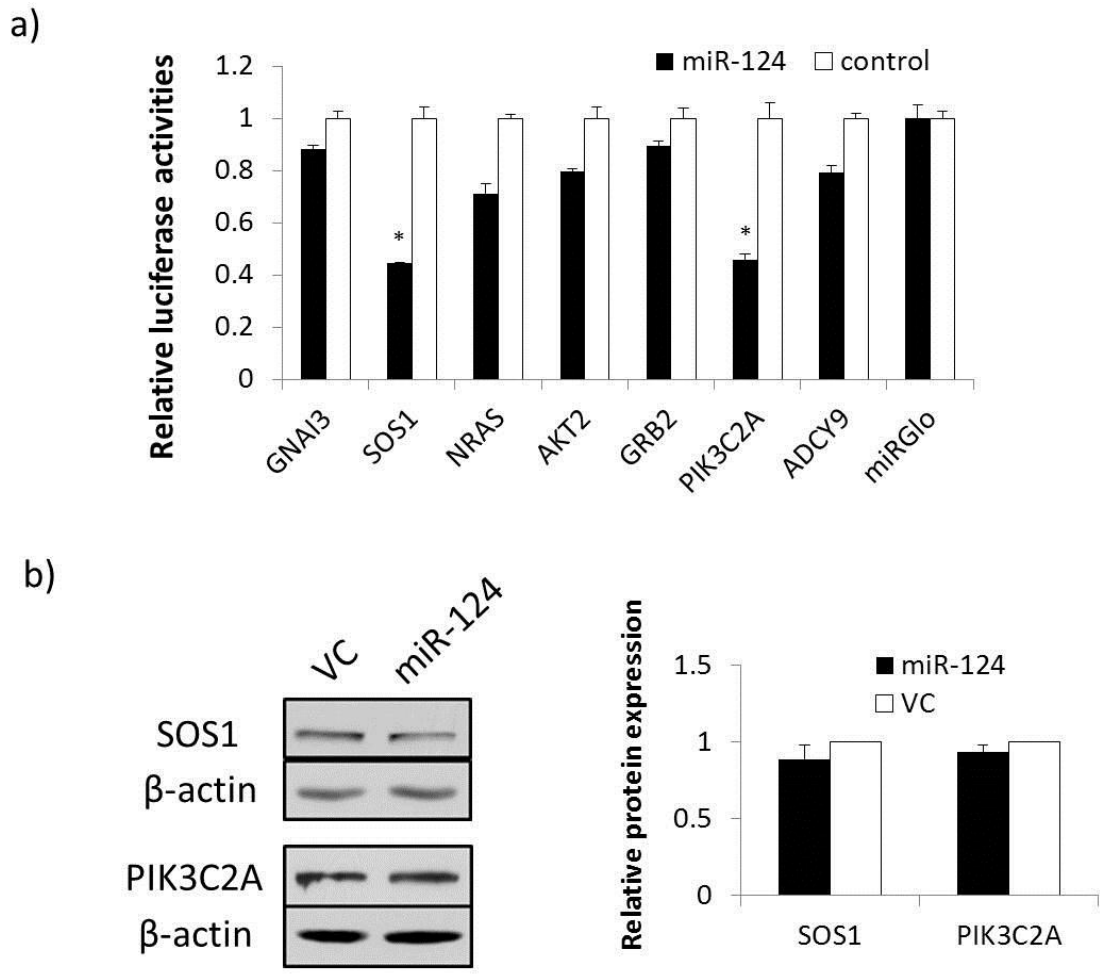

Fig. V5 **Verification of predicted targets.** a) Luciferase assays for 3' UTR verification. HEK 293T cells were transfected with miRGlo reporter constructs containing the 3'UTR of predicted targets of miR-124, together with the miR-124 overexpression pENTR plasmid or the control plasmid. Luciferase activities were determined 48 h post-transfection. Data shown were means ± S.E. from 3 replicates and are expressed as fractions of the control. * $p<0.001$ vs. control. miRGlo, empty miRGlo vector without any 3' UTR sequence. b) MLE15 cells were transduced with Ad-miR-124 or control virus (VC) and were collected 72 h after transduction. The protein levels of SOS1 and PIK3C2A were measured with Western blots. Blots shown are representatives of 6 independent replicates. Blots were quantitated and data shown are means ± S.E. and are represented as fractions of VC.

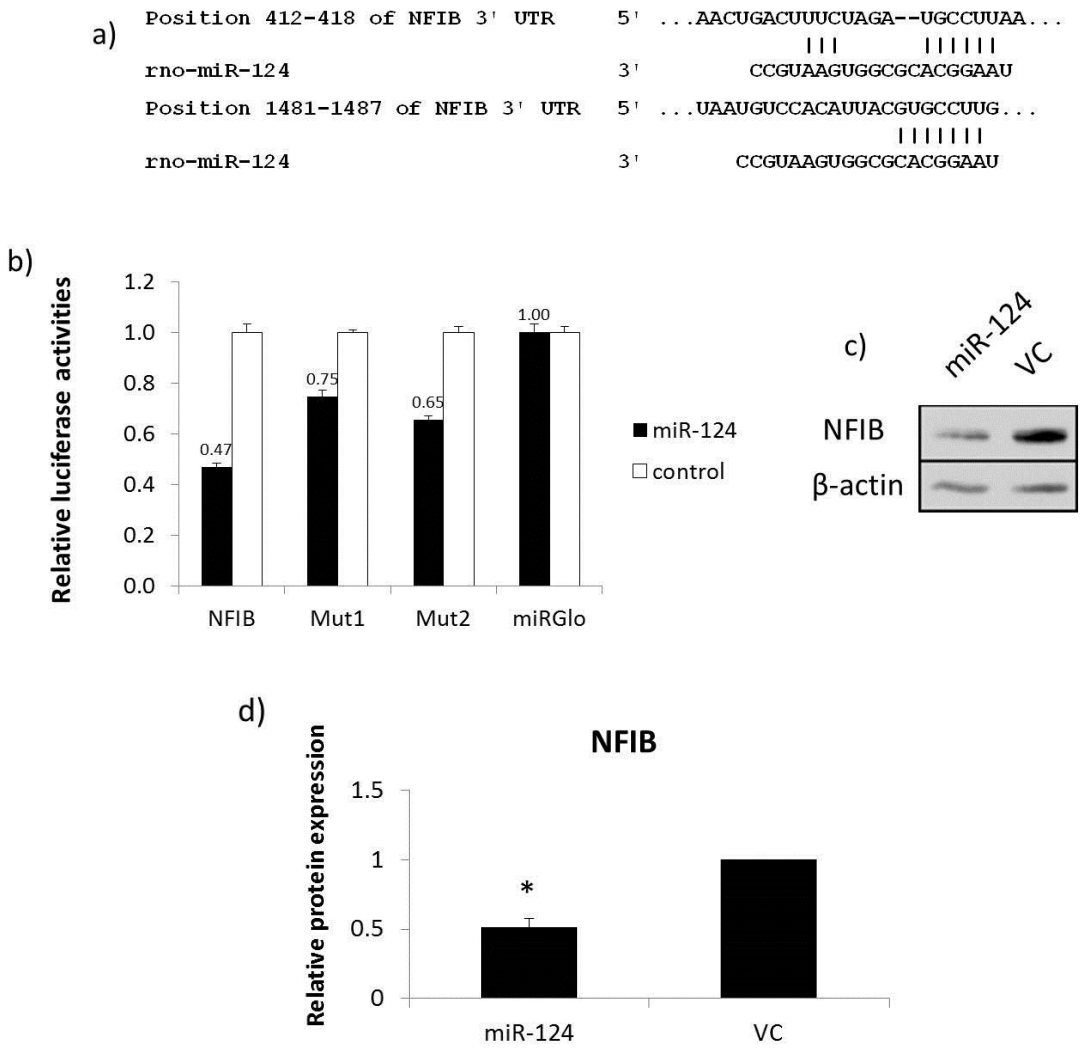

Fig. V6 **miR-124 inhibits expression of NFIB at the protein level.** a) Schematic of 2 putative miR-124 binding sites in the 3' UTR of NFIB. b) 3'UTR luciferase assay. pmirGLO luciferase reporter vectors with NFIB 3'UTR (NFIB), mutated 3' UTR at the first or second binding sites (Mut1 and Mut2), or without any 3' UTR insert (miRGlo) were transfected into HEK 293T cells. pENTR-miR-124 (124) or pENTR-Control (control) was co-transfected with the reporter vectors. Luciferase activities were determined 48 h after transfection and are expressed as fractions of the control. Data shown are normalized with miRGlo and are means ± S.E. from 6 replicates. c) MLE15 cells were transduced with Ad-miR-124, or virus control (VC). Cells were collected 72 h after treatment. The protein levels of NFIB were determined with Western blots. d) The blots were quantitated. Data shown are means ± S.E. from 6 independent replicates.

5.4.5 miR-124 directly inhibits the expression of NFIB

It has been reported that NFIB can regulate epithelium maturation in fetal lung development [23]. TargetScan predicts NFIB as a target of miR-124, with 2 conserved binding sites (Fig. V6a). It is possible that miR-124 inhibits fetal lung epithelium maturation through the depression of NFIB. In order to confirm this hypothesis, we performed luciferase reporter assays with NFIB 3' UTR. As shown in Fig. V6b, the luciferase activity of the reporter vector with NFIB 3' UTR was decreased by about 50%. Furthermore, we mutated the 2 predicted binding sites in NFIB 3' UTR in order to identify whether they are responsible for the inhibition of expression. If binding site 1 or site 2 was mutated, the luciferase activity was decreased 25% and 35%, respectively. This result suggests that both binding sites are functional in the inhibition of NFIB expression (Fig. V6b). When miR-124 was overexpressed in MLE15 cells with adenovirus Ad-miR-124, the protein level of NFIB was also decreased by 50% (Fig. V6c and V6d).

5.5 Discussion

In this study, we found that miR-124 was predominantly expressed in epithelial cells in fetal lung on gestation day 16 and is down-regulated during fetal lung development. Overexpression of miR-124 inhibits pulmonary epithelium maturation as indicated by the decrease in surfactant proteins, FAS, and increase of glycogen content. In addition, we identified NFIB as a direct target of miR-124.

To our knowledge, this is the first report studying the function of miR-124 in fetal lung development. In addition, we are the first to identify NFIB as a direct target of miR-124. miR-124 is preferentially expressed in brain and retina [24-26]. Most of the studies on miR-124 focused on the neural system. It has been reported that miR-124 plays important roles in neurogenesis, neuronal differentiation, plasticity, and microglia quiescence [16, 17, 27-37]. miR-124 is also involved in brain cancers, including medulloblastoma and glioblastoma [38, 39]. In addition,

miR-124 has been shown to be involved in intracellular signaling in pancreatic beta cells, hepatocellular carcinoma, haematological malignancies, cervical cancer, oral squamous cell carcinoma, acute lymphoblastic leukemia, and gastrulation of embryonic stem cells [40-46]. Many proteins have been identified as targets of miR-124, including Foxa2, CDK6, ROCK2, EZH2, CREB, ITGB, NeuroD1, C/EBP-α, Sox9, Lhx2, JAG1, IκBζ, SLUG, and IQGAP1 [30, 32, 33, 35-38, 43, 44, 46, 47]. In this study, we added NFIB to this growing list of miR-124 targets.

Lung epithelial cell differentiation becomes more evident at the saccular stage of lung development. At this stage, cuboidal epithelial cells begin to synthesize pulmonary surfactant. The increase of surfactant protein expression corresponds with the decrease of miR-124 at this period. As indicated by *in situ* hybridization, miR-124 is mainly expressed in epithelial cells in E16 lungs. This suggests that the disappearance of miR-124 from epithelial cells may be necessary for epithelial cell maturation. Our data support this assumption, as ectopic expression of miR-124 in fetal lung organ culture inhibits epithelium maturation. The decrease of miR-124 and synthesis of surfactant proteins also corresponds with the increase of NFIB in the epithelial cells. The decrease of miR-124 in epithelial cells is critical for the increase of NFIB and thus epithelial cell maturation.

Nuclear factor I (NFI) is a family of transcription factors that bind to the consensus palindromic binding site $TTGGCN_5GCCAA$ in double-stranded DNA as homo- and hetero-dimers [48-50]. In vertebrates, there are 4 NFI genes (*Nfia*, *Nfib*, *Nfic*, and *Nfix*) [51, 52]. These genes have unique but overlapping expression patterns. Depending on the cellular context and alternative splicing of NFI mRNAs, NFI can activate or depress the expression of target genes [53]. The importance of NFI proteins in development has been demonstrated with knockout mice. Loss of *Nfia* results in perinatal lethality and defects in brain development [54]. *Nfib* deficient mice die early after birth with severe lung hypoplasia and defects in lung maturation [55, 56]. These mice also show defects in brain development similar to *Nfia* deficient mice. NFIB is

expressed predominantly in mesenchymal cells at early stages of fetal lung development and is expressed in both epithelium and mesenchyme at late stages. Loss of *Nfib* affects the expression of a number of genes involved in extracellular matrix and cell adhesion. Loss of *Nfib* also leads to a decreased expression of pulmonary surfactant proteins, including SP-A, SP-B, and SP-C, and the increase of fibroblast growth factor 10 (Fgf10) [23, 56]. Fgf10 is expressed in mesenchymal cells and is known to regulate lung epithelial cell proliferation and differentiation. Overexpression of Fgf10 in the embryonic lung results in interruption of fetal lung branching morphogenesis and distal epithelial cell differentiation [57, 58]. *Nfic* null mice showed defects in tooth root development [59].

From the results of the development pathway study, it is clear that miR-124 has important roles in development. It dramatically affects activity of the Wnt, NFκB, TGF-β, cAMP/PKA, and MAPK/ERK signaling pathways. However, no components in the NFκB, cAMP/PKA, or MAPK/ERK pathways can be confirmed as direct targets of miR-124. It is quite possible that miR-124 regulates these pathways indirectly or by actions on unidentified targets. Since we have identified the transcriptional factor, NFIB as a direct target of miR-124, whether NFIB can regulate these pathways is of particular interest in the future study.

In summary, we demonstrated that miR-124 was down-regulated during late stages of fetal lung development and NFIB was a direct target of miR-124. miR-124 regulates fetal lung epithelium maturation. These results will help to understand fetal lung development from a new perspective and may provide potential targets for therapeutic intervention to promote epithelial cell maturation in premature infants.

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VITA

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Thesis: THE FUNCTIONS OF MICRORNAS IN LUNG INJURY AND DEVELOPMENT

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Scope and Method of Study:

The purpose of this study is to figure out what are the functions of microRNAs in lung development and injury. First, a microRNA microarray was set up to profile expression pattern of microRNAs in various biological processes in the lung. Lung specific microRNAs were identified using this method. Expression patterns of microRNAs in fetal lung development and alveolar epithelial cell trans-differentiation were also uncovered with this platform. The expression profiles were then verified with Northern blots and microRNA qRT-PCR. Expression of miR-127 in the fetal lung reach peak at the late stage of fetal development. Overexpression of miR-127 interrupted fetal lung morphogenesis. miR-375 is significantly decreased during alveolar epithelial cell trans-differentiation. Overexpression of miR-375 interrupted this process by inhibiting the Wnt/β-catenin signaling pathway. Expression of miR-124 is decreased during fetal lung development. miR-124 inhibits fetal alveolar epithelial cell maturation, as indicated by a decrease in surfactant protein expression and an increase in glycogen pool.

Findings and Conclusions:

1. Two miRNAs (miR-195 and miR-200c) are expressed specifically in the lung and nine miRNAs are co-expressed in the lung and another organ.
2. miR-127 has the highest expression at late stages of fetal lung development.
3. Expression of miR-127 shifts from mesenchymal cells to epithelial cells during fetal lung development.
4. miR-127 inhibits branching morphogenesis.
5. miR-375 is decreased during alveolar epithelial cell trans-differentiation.
6. The Wnt/β-catenin pathway is activated during alveolar epithelial cell trans-differentiation.
7. miR-375 reduces trans-differentiation by inhibiting the Wnt/β-catenin pathway through directly targeting FZD8.
8. miR-124 is down-regulated during fetal lung development.
9. miR-124 inhibits fetal epithelium maturation by directly targeting NFIB.

ADVISER'S APPROVAL: Dr. Lin Liu