UNIVERSITÀ DEGLI STUDI DI PADOVA

UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

SCUOLA DI DOTTORATO DI RICERCA IN BIOSCIENZE

INDIRIZZO GENETICA E BIOLOGIA MOLECOLARE DELLO SVILUPPO

CICLO: XXII

TESI DI DOTTORATO

ROLE OF MIR-99A AND MIR-99B IN MESENCHYMAL CELL PROLIFERATION AND DIFFERENTIATION

DIRETTORE DELLA SCUOLA : CH.MO PROF. TULLIO POZZAN

COORDINATORE D'INDIRIZZO: CH.MO PROF. PAOLO BONALDO

SUPERVISORE: CH.MO PROF. RODOLFO COSTA

CORRELATORE: CH.MO PROF. DAVID WARBURTON

DOTTORANDO: DOTT. GIANLUCA TURCATEL

CONTENTS

ABSTRACT

Micrornas are small 18-22 nucleotides long RNAs with pivotal roles in normal biological processes and diseases. They act as translational repressor inhibiting the expression of the specific messenger RNA. Lung development is highly coordinated by the interaction between the mesenchyme and the epithelium. Proper lung growth and development is achieved by temporo-spatial changes of expression of specific morphogenic and transcriptional factors.

However the role of mirnas on the lung development still remains unknown. Only few recent researches have shown that the mirnas of the mir-17-92 family of mirnas are necessary for normal lung development. Mir-17- 92 cluster of mirnas is indispensable for growth and branching of lung epithelium. We identified two micrornas mir-99a and mir-99b strongly expressed by embryonic lung mesenchyme and mesothelium respectably. Moreover their expression increases during lung development reaching the highest expression in the adult lung. We found that mir-99a and mir-99b are negative regulators of cell proliferation and they stimulate the differentiation of mesenchymal cells into smooth muscle cells. We identified mTOR gene as major target since down-regulation of mTOR with a specific sirna has the same outcome of the over-expression of mir-99a and mir-99b. We found also that mir-99a and mir-99b affect cell migration. We validated other targets SMARCA5, BAZ2A, CALM, PAM but the single contribution of these genes on the mir-99a and mir-99b role in the biological processes is still not defined and it will be clarified in future studies.

ABSTRACT IN LINGUA ITALIANA

I microrna sono piccoli RNAs di lunghezza di circa 18-22 nucleotidi con un ruolo molto importante nei normali processi biologici e nella patogenesi di varie malattie. I microrna sono inibitori della traduzione di specifici RNA messaggeri. Lo sviluppo embrioniale del polmone e' cordinato dalla interazione tra mesenchima ed epitelio e grazie all'azione di precisi cambiamenti temporali e spaziali di espressione di specifici fattori di transcrizione e fattori morfogenici.

Il ruolo dei microrna nello sviluppo embryonale del polmone non e' completamente conosciuto. Sino ad oggi solo la famiglia di microrna appartenente al gruppo mir-17-92 e' stata identificata avente un ruolo indispensabile for lo sviluppo normale del polmone. In particolare il gruppo di micorna della famiglia mir-17-92 e' regola proliferazione ed espansione dell'epitelio polmonare embrionale. In questo progetto di ricerca abbiamo studiato due micrornas espressi dal mesenchima e dal mesotelio polmonare, mir-99a e mir-99b. Inoltre abbiamo evidenziato che inibiscono la proliferatione cellulare e stimolano la differenziazione del cellule mesenchimale in cellule muscolari lisce. Il processo di identificazione dei bersagli di mir-99a e mir-99b ha portato a scoprite il gene MTOR come principale bersaglio e come bersagli secondati SMARCA5, BAZ2A, CALM, PAM. Il singolo ruolo di questi bersagli nel quadro generale della funzione di mir-99a and mir-99b sara' l'oggetto di future ricerche.

INTRODUCTION

1. LUNG DEVELOPMENT

The formation of the lung occurred relatively recently in evolution, representing a singular solution for terrestrial survival of vertebrates. From the comparison of the genomes of different species it has become apparent that a big part of the genome has been devoted to control gene transcription and that species diversity is generated by the differential control of gene expression, rather than by differences in the number of genes. Thus more than 20% of the human genome encodes for transcriptional factors regulating proteins [1].
Organ specification and morphogenesis depend on both tissue-selective and ubiquitous transcription factors and genes that work in interacting networks. Proper lung growth and development is achieved by temporo-spatial changes of expression of specific morphogenic and transcriptional factors [1, 2].

1.1 Mouse embryonic lung development

Mouse lung formation initiates on day 9.5 post-coitum (E9.5) from the laryngotracheal groove and involves mesenchymal-epithelial cell interactions, which include paracrine growth factor stimulation, that induce cellular proliferation, migration, and differentiation [3, 4]. The airway tree develops through a process known as branching morphogenesis, which results by repeated evagination processes of foregut endoderm-derived epithelial cells into the surrounding splanchnic mesoderm, resulting in formation of the respiratory

bronchioles and the terminal alveolar sacs, which are integrated within the endothelial capillary bed [5]. Lung development is divided histologically into five stages: embryonic, pseudoglandular, canalicular, saccular and alveolar phase [1, 3].

1- The **embryonic phase** starts at E9.5 to E11.5 in mouse (3 to 8 weeks of human pregnancy**,**) and begins with the formation of the laryngo-tracheal grove from the ventral portion of the pharynx. Next, from the caudal extremity a bud forms to create the true lung primordium. The lung primordium divides into two daughters buds which will become the two main bronchi.

2 – The **pseudo-glandular phase** starts at E11.5 and ends at E16.5 (5 to 17 weeks of human pregnancy).The entire air conducting bronchial tree up to the terminal bronchioli are set up during this phase and an undifferentiated primordial system forms. This primordial air-conducting tree is initially lined by cuboidal epithelium, which is the precursor of ciliated epithelium and secretory cells. The process which results in the formation of the bronchial tree is called branching morphogenesis [5]. The branching process is remarkably stereotyped and elegant: the air conducting tree is generated by geometrically simple local modes of branching used in three different orders throughout the lung [5].

3 – The **canalicular phase** follows the pseudo-glandular phase and ends at the embryonic stage E17.5 (16 to 25 weeks of human pregnancy). During this phase the canaliculi start to form at the end of the terminal bronchi, which will become the respiratory part of the lung (pulmonary parenchyma). The canalicular stage is also characterized by marked amplification of the vascular tree.

4 – In the **saccular phase** (or terminal sac stage) (E17.5 to P5 in mouse, 24 weeks to late fetal period in human) the number of terminal sacs and their vascularization expand. Continued thinning of the stroma brings the capillaries into apposition with the prospective alveoli. Type I and type II cells start to appear.

5 – Lungs become mature during the **alveolar stage** (late fetal period to childhood in human, P5 to P-28 in mouse): the terminal sacs develop into mature functional alveolar ducts and alveoli.

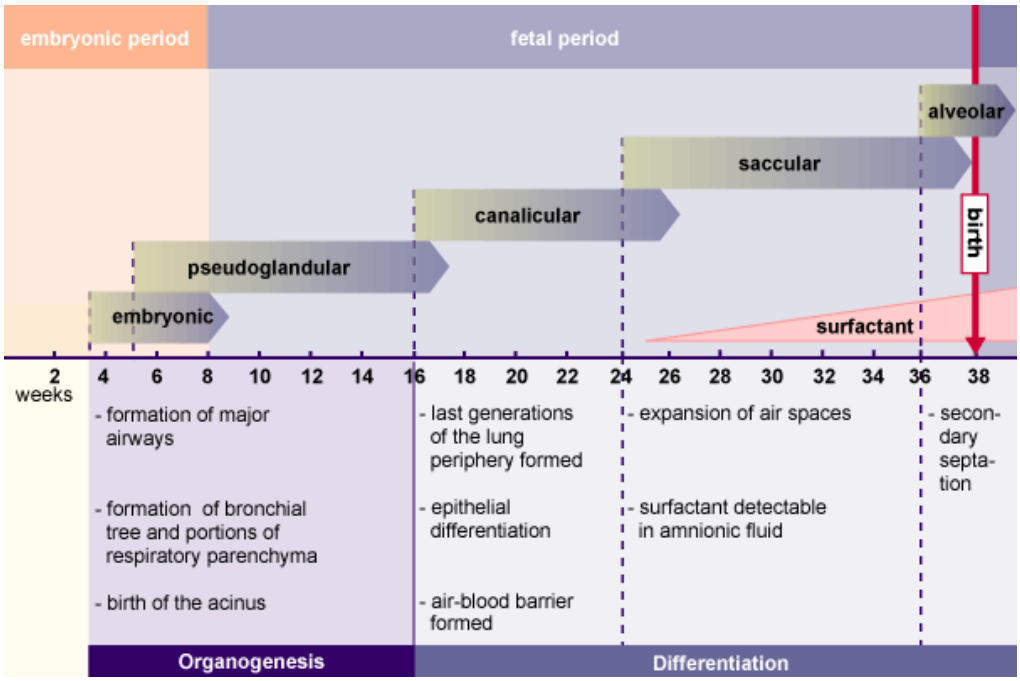


Figure 1 - Overview of human lung development*. (Figure from www.embryology.ch)*

1.2 Lung cells

The adult lung is composed of different cell types. Epithelial cell lineages are arranged in a distinct proximo-distal pattern in the airways. The larynx is lined with squamous epithelium and the upper airways are lined with ciliated columnar cells and mucus secreting cells. The lower airways are lined with Clara cells. The alveoli are lined with alveolar type I and II epithelial cells (AEC1 and 2). AEC1 cells are the structural cells of the lung in the lung while AEC2 cells produce the surfactant associated proteins necessary for to promoter the gas exchange in the alveoli. Moreover AEC2 represents the progenitor cells population of the epithelium and after injury they can transdifferentiate into AEC1 cells.

In the upper airways the pulmonary neuro-endocrine (PNE) cells are also situated in small foci surrounded by other epithelial cells.
The pulmonary interstitium contains several specialized lineages of mesenchymal cells including fibroblasts, myofibroblasts and smooth muscle cells [3].
Lung cells develop following a specific temporal pattern: until the E14 stage the epithelium is co-expressing several lineage markers as surfactant associated proteins (SP)-C, SP-A, Clara cell (CC-10) and calcitonin gene-related peptide (cGRP). The epithelium seems to be multipotential. Later in gestation lung epithelial cells start to following different differentiative fates: Clara cells start to express CC-10, AEC2 cells SP-C and SP-B and PNE cells cGRP [3].

1.2.1 Smooth muscle cells in lung

Another group of cells with a key role in the lung development, physiology and diseases is constituted by the smooth muscle cells. Smooth muscle cells perform many functions including synthesis of matrix and tissue contractions and their activities result altered in various pathologies such as LAM (lymphangioleiomyomatosis), asthma, hypertension [6]. Lung has mainly two different populations of smooth muscle cells: the airway smooth muscle cells which surround the airways and the vascular smooth muscle cells which are part of the vascular system [6, 7].
Airway smooth muscle cells develops from the distal mesenchymal cells population that, at the initiation of the pseudoglandular phase begin to migrate to surround the embryonic epithelium where they elongate and express smooth muscle cells markers. The appearance of bronchial smooth muscle cells precedes the appearance of vascular smooth muscle cells. Vascular smooth muscle cells originate part from migration of adjacent airway smooth muscle cells and part from undifferentiated mesenchymal cells [6, 8]. Recently findings showed

that mesothelium also contributes to development of vascular smooth muscle cells [9].

Studies on smooth muscle cells have put in evidence that mechanical tension, cell shape and basal membrane are key regulators of smooth muscle cell differentiation and proliferation [8, 10, 11]. Multiple signaling molecules and transcriptional factor regulate smooth muscle cell proliferation and differentiation during normal lung development. Figure 2 shows a schematic diagram of the complex network underling smooth muscle cells development.

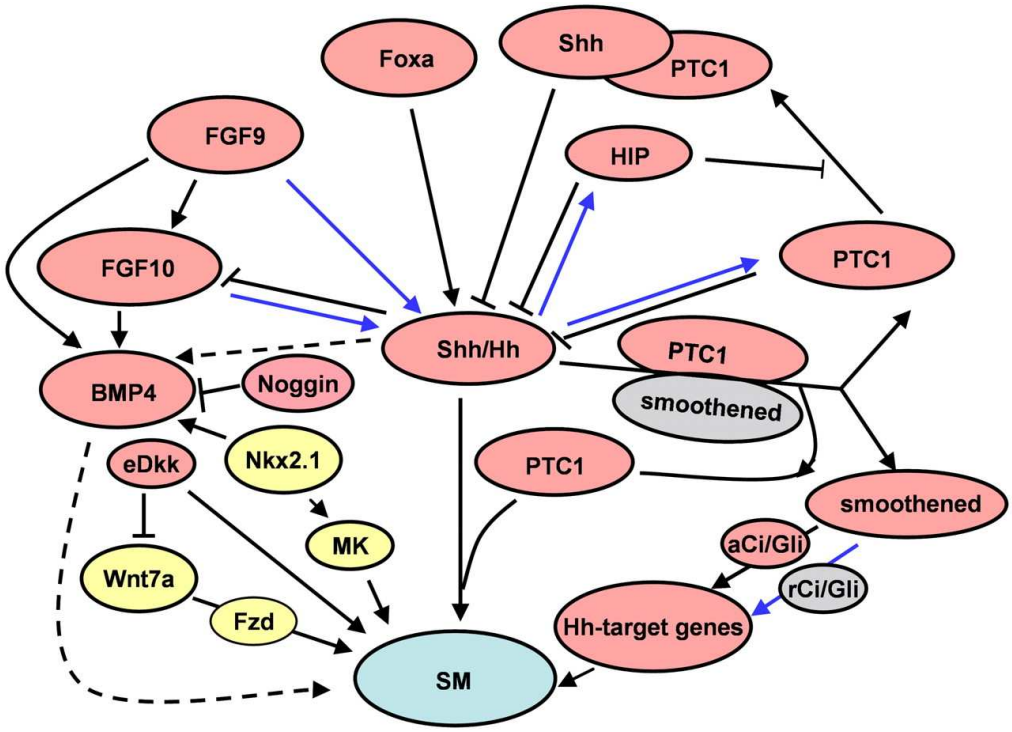


Figure 2 - Signaling pathway regulating lung smooth muscle cells development. *(figure from "Embryological origin of airway smooth muscle cells", Proc. Am. Thorac. Soc.,2007)*

1.3 Morphogenic molecules in lung development

Lung growth is initiated and developed through changes in specific gene expression. The activity and expression level of relevant transcription factors determines the morphogenetic process which is instructed in a precise temporo-spatial order.

The molecular regulators of lung development are not limited to the transcription factors that directly modulate gene expression in the cell nucleus. Peptide growth factors and cytokines as well as their related intracellular signaling components mediate cell-cell interaction. Extra cellular matrix provides important environmental cues for developing lung tubules to branch and cells to differentiate. The specifics of all these integrated networks of regulatory mechanisms are still being explored, but the interaction between epithelium and mesenchyme compartments has long been known to play a critical role during airway branching morphogenesis and lung maturation. The list of the signaling molecules and transcriptional factors involved in lung development is long. Transcriptional factors important for lung development can be classified into at least four groups: forkhead box transcription factors, Nkx homeodomain transcription factors, retinoic acid receptors and GLI family of zinc finger transcription factors.

Many autocrine or paracrine factors regulate and coordinate lung growth at the right time and right place. Many of those factors are peptide growth factors, including FGF, EGF, TGFβ, IGF, PDGF, SHH [1-4, 12-14].

The table 1 (supporting materials, p.52) reports a list of key lung molecular regulators.

1.4 Small non-coding RNA and lung development

Recent studies have highlighted the importance of small non-coding RNAs in lung development. Brigitte Hogan research group generated a transgenic mouse to over-express the mir-17-92 cluster of mirnas in the embryonic lung epithelium. The over-expression of all these micrornas increased epithelial cell proliferation and caused expansion of the number of epithelial progenitor cells expressing Sox9, a marker of lung epithelial progenitor cells [15]. On the other hand the knock-out of these micrornas induces lung hypoplasia [16]. Finally, down-

regulation of MiR-17 and its paralogos, miR-20a, and miR-106b, affects epithelial buds branching and E-Cadherin distribution in epithelial lung bud cells [17]. These recent works highlight for the fist time the role of mirnas in the regulation of lung development, but still the specific role of the remaining hundreds of unstudied mirnas remains unknown.

2. MICRORNAS

2.1 Microrna biogenesis

MicroRNas (mirnas) are single stranded RNAs (ssRNAs) of 19-25 nucleotides in length that are generated from endogenous hairpin shaped transcripts [18]. Transcription of mirna genes yields primary transcripts, pri-mirna, that are usually several kilobases long and that contain a local hairpin structure. The stem loop structure is cleaved by nuclear Rnase III Drosha to release the precursor of mirna (pre-mirna) [18]. The pre-mirna then binds to Exportin 5 (EXP5), a member of the karyopherin family of nucleo-cytoplasmic factors: the complex pre-mirna- exportin 5 promotes promotes the nuclear export of the pre-mirna into the cytoplasm [19, 20]. In the cytoplasm the pre-mirna is further cleaved near the terminal loop by DICER, releasing the 22 bp mirna duplex. Dicer is a highly conserved protein that is found in almost all eukaryotic organisms. Some organisms contain multiple Dicer homologues, whereby different Dicer isotypes have distinct roles [21]. Following Dicer cleavage one strand of the mirna duplex is loaded into the “RNA-induced silencing complex” (RISC), the other strand, instead, is readily degraded. The two strands of the mirna duplex not have the same ability to be incorporated into the RISC: indeed the strand whose 5’ end is more weakly bound to the complementary strand is more readily incorporated into the RISC [22, 23]. The mirna loaded RISC can at this point interact with specific mRNA and alter their translation via binding to their 3’UTR portion [18, 24].

2.2 Microrna mode of action

Microrna regulate eukaryotic gene expression post-transcriptionally. Mirnas use base-pairing to guide the RISC to specific messages with fully or partially complementary sequences [25]. Microrna can direct the RISC to down-regulate gene expression by either of two posttranscriptional mechanisms: mRNA cleavage or translational repression. Once incorporated into the cytoplasmatic RISC, the mirna will specify cleavage if the mRNA has sufficient complementarity to the mirna (figure 3A), or it will repress productive translation if the mRNA does not have sufficient complementarity (figure 3B) to be cleaved but does have a different mirna complementary sites [24].

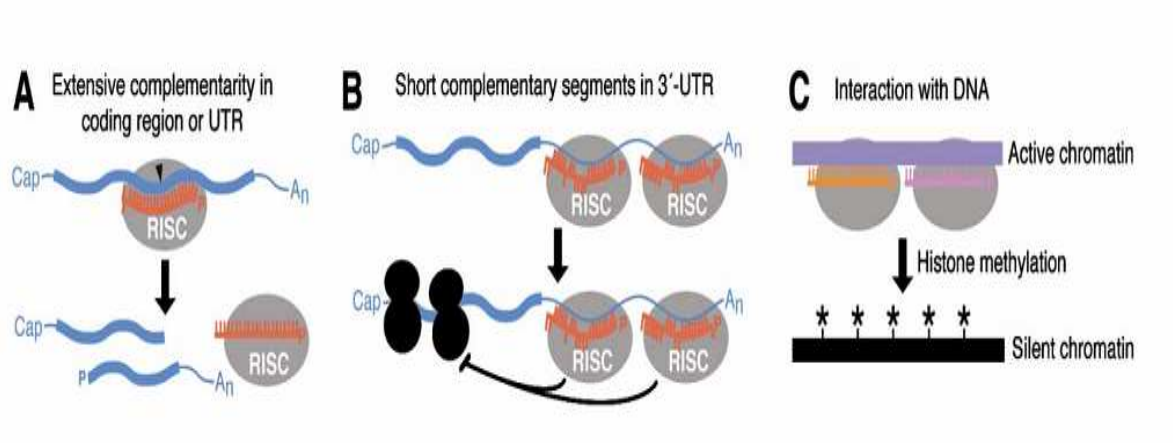


Figure 3 - Mirnas way of function. *Microrna inhibit expression of gene target: if the mirna sequence is extensively complementary to the target mRNA sequence the mirna loaded RISC degrades the target mRNA (3A). Otherwise the RISC inhibit the translation of the target mRNA (3B). Finally sirna can directly promotes DNA sequence (chromatin remodeling) (3C) (figure from "MicroRNAs: Genomics, Biogenesis, Mechanism, and Function", Cell, 2003).*

So far mirna have identified as functioning within the RISC complex. Argonaute proteins, which are the catalytic components of the RISC, and siRNAs have been found to be associated with DNA methylation and silencing in plants and chromatin remodeling in fungi which suggests the existence of a third mechanism of mirnas action in which they may directly effect DNA silencing (figure 3C) [24]. There are two

more considerations that support this last hypothesis: mature mirnas can be found inside the cell nuclei [26] and there is a nuclear import signal sequence inside some mirna sequences [27].

2.3 Micrornas target recognition

Mirnas inhibit gene expression by binding to the 3' UTR of the mRNA target. Residues from the 2nd to the 8th base are refereed to as the "seed sequence" of the mirna and is perfectly complementary to the target mRNA binding site. The "seed sequence rule" states that contiguous Watson-Crick base-pairing between the mirna seed sequence and the mRNA target is necessary for the mirna to function [25] (Fig1). This rule is commonly used to identify mirna targets by software such as PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/vert_42/). These softwares seek for putative targets using only the 3' UTR portion of the mRNA. But with the expansion of mirna studies, a more complex picture of mirna–target interaction has came to light: the mirna-target interaction often does not follow the seed sequence rule [28] mirnas can even inhibit the target mRNA translation via binding to both the 5'UTR [29] and the coding region [30] and finally mirnas can actually increase the translation of the target mRNA [29, 31]. With these new discoveries it appears clear that mirnas represent a new and complex family of regulators indispensable for the proper control of cellular processes.

2.4 Micrornas in biological processes and diseases

Despite the discovery of mirnas is relatively recent they have been shown to act in almost every biological pathway and thus control the entire biological process [32].

Micrornas are often deregulated in cancer: they participate in cancer progression and expansion and they are also involved in epithelial to mesenchymal transition. Because their involvement in cancer

pathogenesis, mirnas have been proposed and confirmed as prognostic markers for cancer bearing patients [33].

Mir-155 and the mirnas encoded by the mir-17-92 cluster were the first to be identified as over-expressed in many different solid tumors [34, 35] and thus was indicated by the term Oncomir [36]. Other examples of mirna deregulation in cancer are mir-21 [34], mir-15a and mir-16 for chronic lymphocytic leukemia [37], mir-221 and mir-222 in prostate cancer [38], mir-7 in lung cancer [39-41] and more.

The role of different mirnas on embryonic organ development has been highlighted too: mir-15 and mir-16 are key regulators of nodal signaling in xenopus [42]; Mir-214 regulates skeletal muscle cell differentiation and specification [43, 44]; mir-1 and mir-133 are necessary for normal heart development [45-48]; miRNAs have important roles in gene regulatory networks for proper brain development and adult neural plasticity [49, 50].

In conclusion, mirnas are a new class of gene expression regulators affecting multiple cellular and molecular processes in all organisms so far studied.

2.5 Alternative hypothesis of the mirna function in biological processes

Micrornas are highly abundant genes expressed in all organisms. Since every mirna can regulate hundred of genes, it is obvious to conclude that their lack should have a strong phenotype on normal embryo development. The total loss of mirnas can be achieved with the genetic knock-out of the DICER enzyme, which is necessary for the maturation of the pre-mirna to mature mirna. Unexpectedly, the total loss mirnas does not have a very severe affect on the development of the embryo. This observation has suggested alternative theories on the function of mirnas. It has been proposed that the main function of mirnas could be to buffer and to give robustness during the cellular processes. According to this view mirnas give robustness to genetic

pathways in at least two ways. First, miRNAs repress ‘leaky’ target mRNAs in tissues where they would be disruptive or deleterious. This is accomplished by high levels of miRNA expression in cells where targets are not transcribed. Second mirna can act post-transcriptionally to control genetic noise [51] this is carried out by miRNAs that are co-expressed with their target genes [52, 53].

MATERIALS AND METHODS

1. Cell lines

HELA, NMUMG and A549 cells were purchased from ATCC and maintained in DMEM, 10% FBS (Gibco/Invitrogen, Carlsbad, CA) and 1% antibiotics (Gibco/Invitrogen, Carlsbad, CA). NMuMG cells media were supplemented with insulin (10 ug/ml) (Sigma-Aldrich, St. Louis, MO). For serum stimulation cells were serum starved overnight and then pulsed with 10% FBS complete media for 30 minutes. MLE-15 (mouse epithelial lung) cells were a gift of Jeffrey Whittset (Cincinnati Children's Hospital Medical Center) and they were maintained in DMEM 10% FBS, penicillin and streptomycin (Gibco/Invitrogen, Carlsbad, CA).

2. Embryonic mesenchymal culture

Embryonic lungs were collected at E13.5 stage. The primordial trachea and main bronchi were removed and the embryonic lung lobs were digested in trypsin EDTA solution at 37℃ for 10 minutes. The cell suspension was then passed through a 100 μm cell strainer and cells were collected by centrifugation. The cells were resuspended in F12 media with 10% FBS and seeded in 6 wells plates and incubated for 1 hour to permit the mesenchymal cells to attach. Epithelial cells and debris were washed off with 5 washes of PBS. Finally F12 media supplemented with 0.5% of FBS and recombinant FGF9 (200 ng/ml) (R&D, Minneapolis, MN). To induce the mesenchymal cell to differentiate into smooth muscle cells were incubated with F12 media,

0.5% FBS without FGF9 for 48 hours. Mesenchymal cells were used at the first or second passage.

3. Whole mount in situ hybridization of micrornas

For whole mount *in situ* hybridization of miRNAs we used a modified protocol to take in consideration the short size of mature mirna (http://www.exiqon.com). Embryonic lungs were fixed in 4% paraformaldehyde (PFA) for 20 minutes and dehydrated in serial methanol (MeOH) washes and kept in 100% MeOH over night. The day after, lungs were dehydrated and washed in 100% TBS/Tween-20 0.1% (TBST) 3 times 5 minutes. Then lungs were digested with proteinase K (10 μg/ml) for 5 minutes, fixed again in 4% PFA for 20 minutes and washed with TBST 3 times for 5 minutes. The lungs were then transfered into hybridization buffer (HB) (Formamide 50%, SSC 5X, tRNA 0.5 mg/mL, SDS 1%, Heparin 0.05 mg/mL) for 1 hour at Tm-22℃ (where Tm is the melting temperature for the specific mirna probe) and than hybridized with the probe at Tm-22°C over night. The next day the lungs were washed with 100% HB at the same temperature as above for hybridization (Tm-22℃), 100% HM at the same temperature as above for hybridization very brief wash, 75% HM/25% 2 x SSC at hybridization temp,15 min, 50% HM/50% 2 x SSC at hybridization temp, 15 min, 25% HM/75% 2 x SSC at hybridization temp, 15 min, 2 x SSC at hybridization temp. 15 min, 0.2 x SSC, at hybridization temp, 2 x 30 min, 75% 0.2 x SSC/25% PBST (PBST= PBS 0.1% Tween 20) at RT 10 min, 50% 0.2 (or 0.05) x SSC/50% PBST at RT 10 min, 25% 0.2 (or 0.05) x SSC/75% PBST at RT 10 min, PBST at RT, 10 min, PBST/2% sheep serum/2mg:ml BSA at RT, several hrs. Finally lungs were incubated in antibody solution with HRP-anti-DIG antibody (1:5000) (Roche) overnight at +4℃. The color reaction was performed with BM Purple solution after 6 hours of

washes with PBST and 2 x 20 minutes washes with NTMT (NaCl 100mM, Tris pH9.5 100mM, Tween-20 0.1%).
For the sectioning stained embryonic lungs were embedded in resin and cut with a vibratome (thickness of section 7μm) (Leica).

4. CDNA retrotranscription and PCR

The RNA was extracted from tissue and cells culture pellets using TRIZOL reagent. 1 μg of RNA was retro-transcripted using the SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was amplified with Taq Polymerase (Invitrogen, Carlsbad, CA) in the presence of gene specific primers (Operon, Huntsville, AL) as indicated.

5. Micrornas retro-transcription and Real Time PCR

For mirnas quantification, TaqMan microRNAs Assay was used. TaqMan MicroRNA Assays use stem-looped primers that enable a two-step quantification of mirnas present in the sample. In the first step, stem-looped primers anneal to target mature mirnas and extend the length of the molecule by reverse transcription PCR. In the second step, a real-time PCR that involves a forward primer, a reverse primer, and a TaqMan probe quantifies the number of mature mirna molecules present in a sample based on fluorescent emission of a reporter dye. For the retro-transcription 10 ng of total RNA was used and retro-transcripted in 7.5 μl total volume reactions containing: 1.5 μl mirna specific primer (Applied Biosystem), 10 units of RNAase inhibitor, and 25 units of multiscribe reverse retro-transcriptase (Chen et al., 2005). Quantitative real-time PCR was carried out using the Roche Light Cycler 480 and the Light Cycler TaqMan Master Mix. Real Time PCR conditions were as follows: 95℃ for 10 minutes , 45 cycles: 95℃

15 seconds, 60℃ for 30 seconds. Mirnas specific pr imer and probe were supplied by Applied Biosystem.

6. Plasmid

Luciferase reporter (PGL4.13) and Renilla luciferase (PGL6.43) plasmids were bought from Promega (Promega, San Luis Obispo, CA).

7. Luciferase plasmids design

To confirm mir-99a and mir99b targets a luciferase assay was used. Part of the wild type 3'UTR (WT-3'-UTR) and mutated 3'UTR (MUT-3'UTR) of the hypothetical target gene messenger RNA, containing the putative mir-99a and mir-99b binding sites was amplified by PCR and inserted downstream to a luciferase reporter gene in a PGL4.13 plasmid (Promega, San Luis Obispo, CA) (Supplemental Figure 2). The mutated 3'UTR (MUT-3'-UTR) was obtained by inserting 4 point mutations in the mir-99a and mir-99b binding site, thus destroying the putative mirna/mRNA interaction. To create the 3'-UTR-MUT a PCR approach was used using specific primers covering the mir-99a and mir-99b binding sites bearing the mutated bases.
Table 3 (pag..) reports the sequence of the primers used for cloning the 3'-UTRs. To every primer was add a 5' overhang containing the XBA1 restriction site sequence. The WT and MUT-3'UTR was amplified by PCR, cut with the XBA1 restriction enzyme and ligated in the PGL4.13 (Promega,)plasmid downstream to the luciferase reporter gene.

8. Luciferase assay

Hela cells were seed in 24 well plates (80000 cells per well) and the next day transfected with 300 ng of the WT or MUT 3'UTR luciferase

reporter construct, together with a mir-99a, mir-99b or a scrambled precursor (final concentration 20 nM) (Dharmacon,). 50 ng of the luciferase plasmid PGL-6.43 (Promega, San Luis Obispo, CA) was co-transfected into each well and used as the transfection internal control. Cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega, San Luis Obispo, CA). The luciferase activity was normalized on the Renilla luciferase activity and expressed as true ratio between the luciferase activity of the WT-3'UTR plasmid and the MUT-3'UTR plasmid. Every luciferase assay experiment was repeated at least three times.

9. Antibodies

MTOR, p-S6K antibodies were purchased from Cell Signaling, ACTIN antibody from MP Biomedicals, CYCLIN D1, SM-22α, ROCK1, ROCK2 and E-Cadherin antibodies from Santa Cruz Biotechnology.

10. Western blot

Cells were seeded at 50% confluency and the next day transfected with mir-99a, mir-99b or a scrambled mirna precursor (final concentration 80nM) (Dharmacon) using lipofectamine 2000 (Gibco/Invitrogen, Carlsbad, CA). Fresh media was added 6 hour later. 72 hours post transfection cells were lysed with Ripa buffer (1XTBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide) (Sigma-Aldrich, St. Louis, MO), supplied with phosphatase and protease inhibitors (PMSF solution, sodium orthovanadate solution and protease inhibitor cocktail solution) (Sigma-Aldrich, St. Louis, MO). Cells were lysed in Ripa buffer in ice for 20 minutes. The lysate was centrifuged at 10000g for 10 minutes to remove debris. Total cellular proteins were quantified using the DC-CC

Protein assay (Biorad). B-ACTIN protein amount was measured in each western blot experiment and used as loading control. From each sample an equal amount of protein solution were supplemented with Loading Buffer 5X (250 mM Tris HCl pH 6.8, 10% SDS 30% Glycerol, 5% B-Mercaptoethanol, 0,02% Bromophenol blue), boiled for 1 minute and loaded on a SDS-PAGE gel (BIORAD). At the end of the run proteins were transferred to a PVDF 0.45 μm membrane (Millipore, Billerica, MA) and probed with the specific antibodies. Peroxide conjugated antibodies were used at the following concentrations: 1:10,000 for Anti-Mouse, 1:20,000 for Anti-Rabbit, and 1:120,000 for anti-Goat secondary antibodies. The blocking step was performed with 5% Dry Fat Milk (Santa Cruz Biotech., Santa Cruz, CA) in TBS-T. TBS-T was used as washing solution. Detection of antigens was performed using ECL Western Blotting detection Reagents (Amersham Biosciences/GE Healthcare, Buckinghamshire, UK), impressed on Biomax Light Film (GE Healthcare, Buckinghamshire, UK).

11. Proliferation assay

Cells were seeded in 96 well plates (5000 cells per well), and transfected the next day with the indicated mirnas at a final concentration of 80 nM using lipofectamine 2000 (Gibco/Invitrogen, Carlsbad, CA). 6 hour post transfection cell media was replaced with fresh media. 72 hours post transfection culture media was replaced with fresh containing 10% of Resazurin solution (R&D). Cell number was determined by measuring the absorbance of each well at 600 nm and 540nm according to the manufacture R&D instructions.

12. Cytofluorimetry and cell cycle analysis

Cells were seeded in 6 well plates at 40% confluency and, the next day, transfected with the indicated siRNA or mirna (80 nM final

concentration) using lipofectamine 2000 (Gibco/Invitrogen, Carlsbad, CA). Fresh media was added 6 hours after transfection. After 72 hours cells were trypsinized, washed with PBS and fixed in ice with cold 70%/30% ethanol/PBS solution for 30 minutes. Cells were then washes with PBS and resuspended in propidium iodium (20 μg/ml) solution containing RNAse (200 μg/ml) for 30' at room temperature.
Cellular DNA content was determined with a FACScalibur instrument (Becton Dickinson).

13. Migration Assay: scratch assay

Cells were seed in 12 well plates at 50% confluent and the next day transfected with mir-99a, mir-99b or a scramble mirna at a final concentration of 80 nM. Media was changed 6 hours later. 72 hours post transfection, cell layer was scratched with a p200 tip pipette. The healing process was followed for the next 24 hours. The ability of the cells to close the wound was expressed as true ratio between the width of the wound at 24 hours post scratch and the wound width right after the scratch was done.

14. Migration Assay: transwell migration assay

Cells were seed in 12 well plates at 50% confluent and the next day transfected with mir-99a, mir-99b or a scramble mirna at a final concentration of 80 nM. Media was changed 6 hours later. 72 hours post transfection cells were trypsinized and counted. For the different mirnas, the same number of cells resuspended in DMEM media with 0.5 % of FBS was seeded on the top a matrigel transmigration chamber. The bottom part of the chamber was filled with DMEM with 10% FBS or with TFG-β 2ng/ml to attract the cells to migrate throught the membrane. The number of cells that had migrated was determined with crystal violet staining 24 hours later.

15. SIRNAs and mirna mimics and knock-down probe

SIRNA for mTOR and control sirna were bought from Applied Biosystem and used at a final concentration of 80 nM. Lipofectamine 2000 (Invitrogen) was used for the transfection. Cells were seeded in 24 well plates at 50% confluent and the next day transfected with the indicate sirna at a final concentration of 80 nM. Media was changed 6 hours later. 72 hours post transfection cells were collected and the protein extracted with RIPA buffer. Mirna mimic precursors were bought from Dharmacon. LNA (locked nucleic acid) 99b-knock-down and control probes were bought from Exiqon.

16. Software

For the identification of mir-99a and mir-99b targets, online bio-informatical tools were used: PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/).

RESULTS

1. Mir-99a and mir-99b are highly expressed by embryonic lung

A real time PCR approach was used to identify mirnas expressed during normal lung development [17]. RNAs from embryonic and adult murine lungs were collected at different stage of development and real time was used to quantify the expression of specific mirna. We identified mir-99a and mir-99b as two among the highest expressed mirnas in the embryonic lung. Their expression was quite constant during the embryonic stages, but increases reaching the peak of expression in the adult lung (Supplemental Figure 1). A study from a different lab confirmed [15] our expression data on mir-99a and mir-99b. The sequence of the mature mir-99a and mir-99b is:

MIR-99a: 5'-A**ACCCGUA**GAUCCGAUCUUGUG-3'

MIR-99b: 5'-C**ACCCGUA**GAACCGACCUUGCG-3'

Mir-99a and mir-99b share the 82% of the sequence (only 4 bases are different) and the entire seed sequence (highlighted in green).

To detect where mir-99a and mir-99b are expressed in the embryonic lung, we used whole mount situ hybridization on embryonic lungs at and E12.5 (Figure 4). Mir-99a is mostly expressed by the distal mesenchyme. Mir-99b instead is expressed by the distal mesenchyme and strongly by the mesothelium.

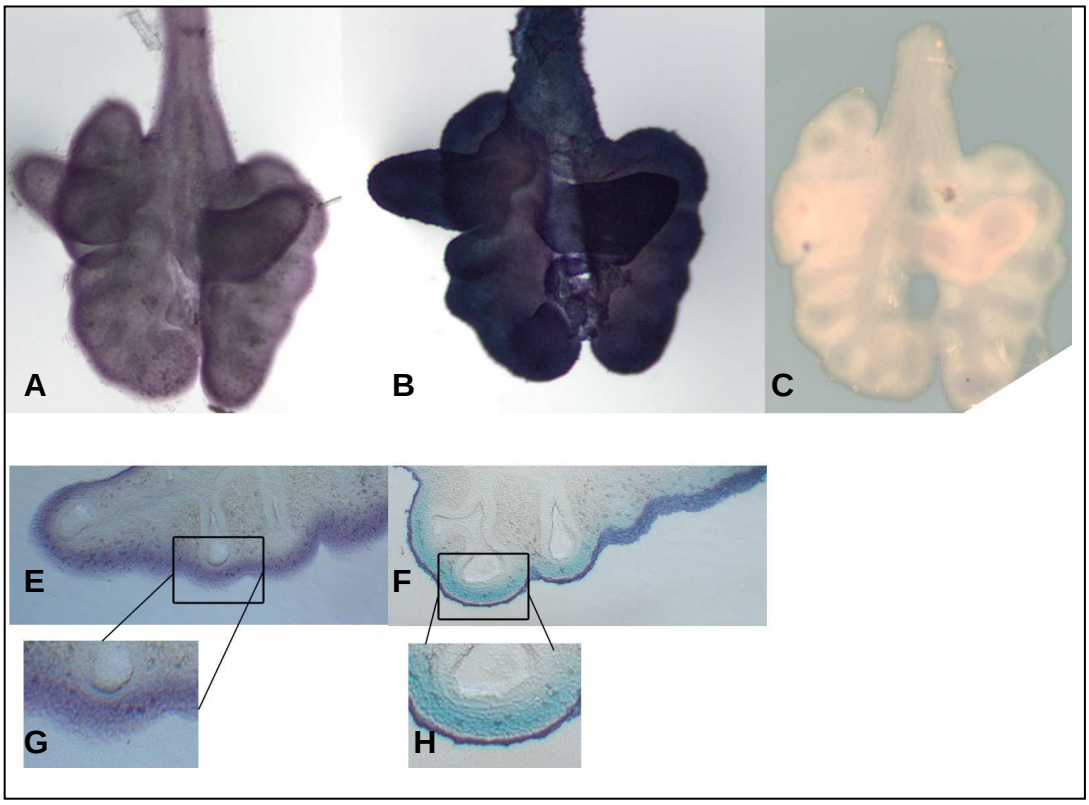


Figure 4 - Mir-99a and mir-99b expression. *Mir-99a (A, E, G) and mir-99b (B, F, H) are mostly expressed by lung mesenchyme. Mir-99b is strongly expressed by the mesothelium too. C probe negative control staining.*

2. Mir-99a and mir-99b expression increases during the differentiation of lung mesenchymal cells

Since the expression of both mir-99a and mir-99b increase with lung development and they mostly expressed by the lung mesenchyme, we determined if their expression changes during differentiation of lung mesenchymal cells into smooth muscle cells. We isolated embryonic lung mesenchymal cells and kept them in culture *in vitro* with media containing FGF-9, which maintains mesenchymal cells in a progenitor state [54]. After removal of FGF-9 from the culture media, embryonic lung mesenchymal cells spontaneously differentiate into smooth muscle cells [54]. We determined if, during this process of mesenchymal cell differentiation, the expression of mir-99a and mir-99b changes. Figure 5 shows that expression of mir-99a and mir-99b

increases when FGF-9 is removed from the media concomitantly with the increased expression of α-SMA. Thus we concluded that mir-99a and mir-99b may have a specific role in smooth muscle cells differentiation.

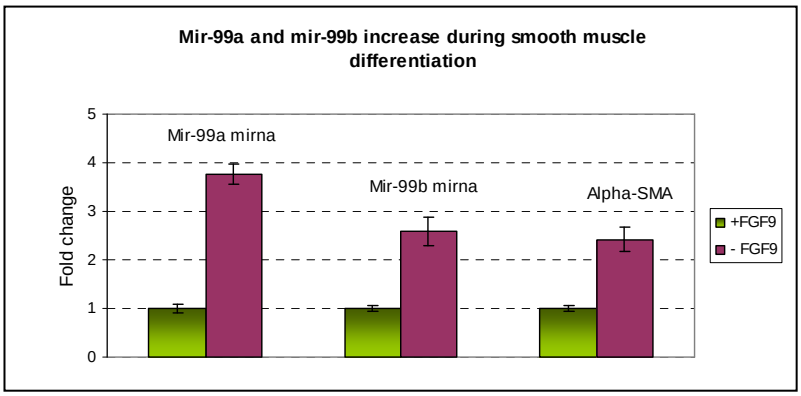


Figure 5 - Mir-99a and mir-99b expression increase during smooth muscle cells differentiation. *During the differentiation induced by FGF9 withdrawal for 48h, mesenchymal cells differentiate into smooth muscle cells and the expression of mir-99a and mir-99b increases. The expression of α-SMA is showed to confirm the differentiation process (p<0.01).*

3. Mir-99a and mir-99b target mTOR gene

We found the mir-99a and mir-99b expression increase during the differentiation of lung embryonic mesenchymal cells into smooth muscle cells. We used PicTar software to determine if mir-99a and mir-99b targets are involved in smooth muscle cells differentiation. We found that mTOR gene is a predicted target and has been involved specifically in smooth cell differentiation [55]. To validate mTOR gene as target we used two approaches: luciferase test and western blot. In the luciferase test the 3'UTR of mTOR gene is cloned downstream a luciferase reporter gene and transfected in cells together with mir-99a, mir-99b or a scrambled mirna. If the mirna binds to the 3'UTR of mTOR we predict a decreased luciferase activity when the specific mirna is over-expressed (details in Material and Methods). Next we pursued a western blot assay to confirm that over-expression of mir-99a and mir-

99b results in the down-regulation of the mTOR protein. Figures 6A and 6B show the luciferase test and western blot results.

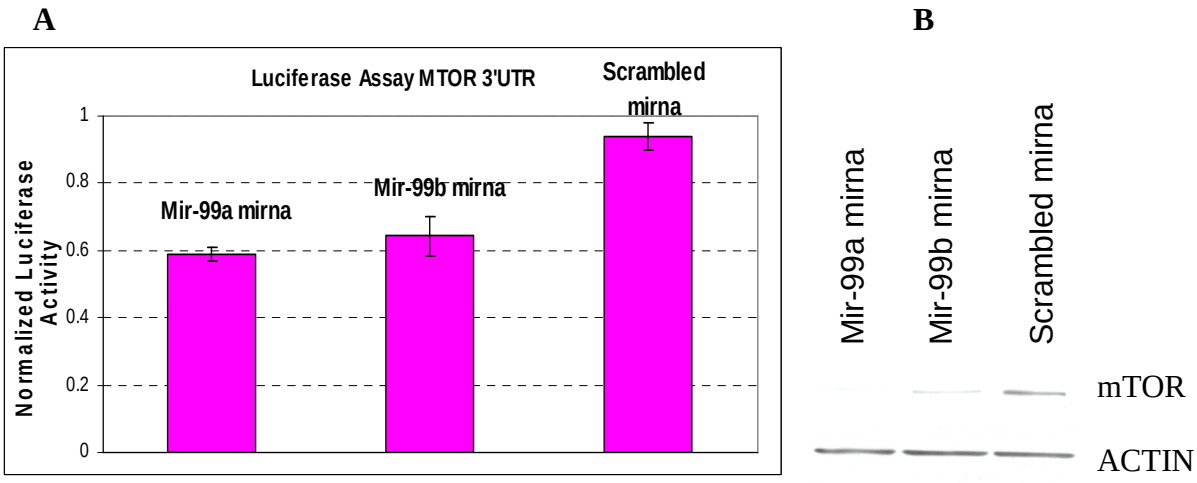


Figure 6 - Mir-99a and mir-99b target mTOR gene: luciferase test (A) and western blot (B). *Luciferase test was used to confirm that mir-99a and mir-99b bind to the mTOR 3'UTR (A). Mir-99a and mir-99b over-expression in Hela cells induces down-expression of mTOR protein (B).*

4. Mir-99a and mir-99b induce expression of smooth muscle cell marker SM-22-α

Mir-99a and mir-99b expression increases during the differentiation of lung embryonic mesenchymal cells into smooth muscle induced by FGF-9 withdrawal. Moreover we identified mTOR as a target that has a role involved in smooth muscle cells differentiation [55]. Thus we determined if the over-expression of these mirnas promotes smooth muscle cells differentiation of mesenchymal cells. We used primary lung embryonic mesenchymal culture supplemented with FGF9. As depicted in figure (4) the over-expression of mir-99a enforces the expression of Transgelin (SM-22-α), a smooth muscle cell marker [56], by the mesenchymal cells (Figure 7). We then confirmed that mir-99a and mir-99b have a pro-differentiation effect toward the smooth muscle cell phenotype with two other cell lines: A549 and NMUMG. These

cells are epithelial cells but they become mesenchymal after treatment with TGF-β, by undergoing epithelial to mesenchymal transition (EMT) [57, 58].

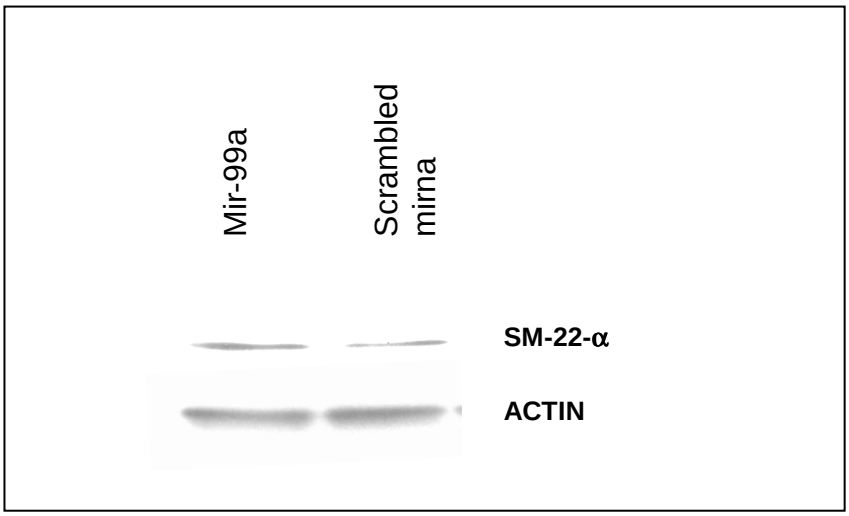


Figure 7 - Over-expression of mir-99a in lung embryonic mesenchymal cells enforces SM-22-α expression.

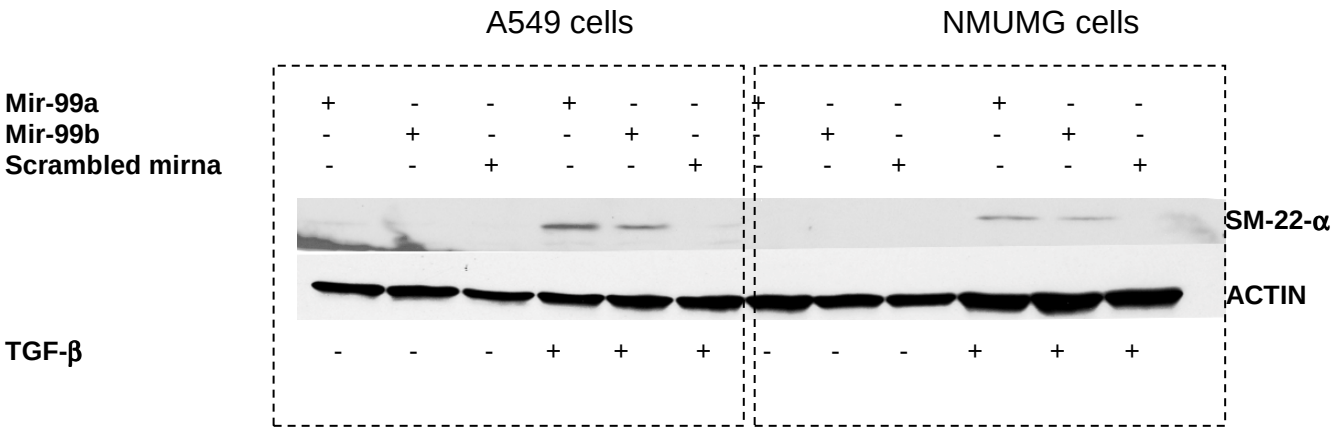


Figure 8 - Over-expression of mir-99a and mir-99b induces expression of the smooth muscle marker SM-22-α in A549 and NMUMG cells if they are treated with TGF-β.

Figure 8 shows that the over-expression of mir-99 and mir-99b brings to expression of SM-22-α only when A549 and NMUMG cells are

treated with TGF-β, thus when they are mesenchymal cells. The over-expression of mirnas in the epithelial state of those cells does not induce expression of SM-22-α.

mTOR is a target of mir-99a and mir-99b. We next determined if down-regulation of mTOR alone can promote the expression of SM-22-α in A549 cells treated with TGF-β.

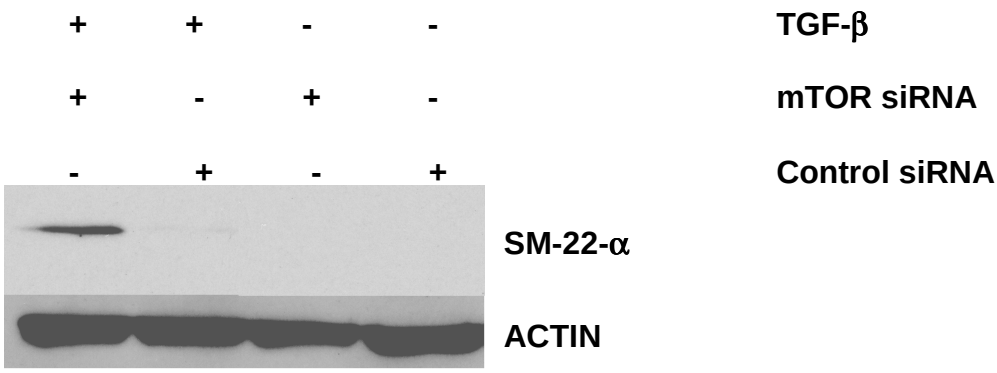


Figure 9 - MTOR down-regulation induces the expression of SM-22-α in TFG-β treated with a549 cells.

As shown in figure 9 the down-regulation of mTOR with a specific sirna induces the expression of SM-22-α in TGF-β treated A549 cells as seen previously with mir-99a and mir-99b over-expression. Taken these data together we concluded that mir-99a and mir-99b may induce smooth cells differentiation via inhibition of mTOR gene expression.

5. Mir-99a and Mir-99b inhibit both mesenchymal and epithelial cell proliferation

Since the expression of mir-99a and mir-99b expression increases during lung development, we determined if they are involved in cells proliferation via inhibition of mTOR gene expression. To test this hypothesis we over-expressed mir-99a and mir-99b in mesenchymal

NMUMG cells (pre-stimulated with TGF-β). Cytofluorimetry analysis, western blot, and proliferation assay confirmed that mir-99a and mir-99b inhibit the proliferation of NMUMG mesenchymal cells (Figure 10).

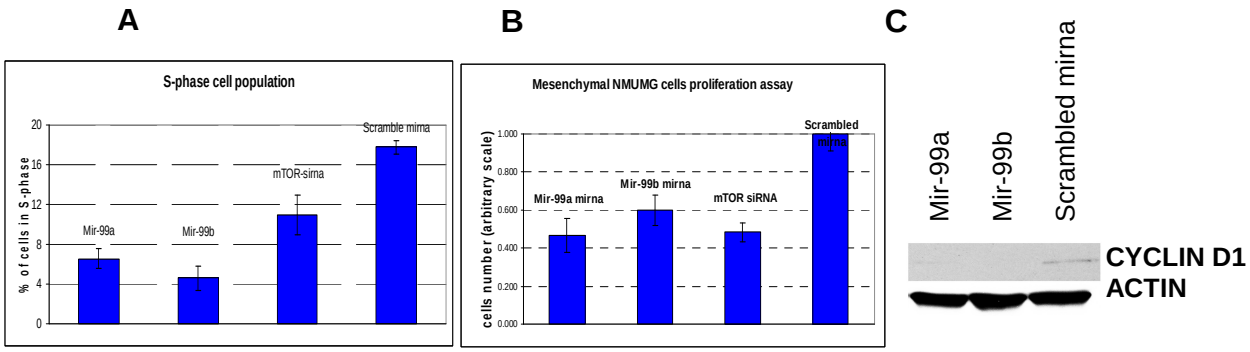


Figure 10 - Mir-99a and mir-99b inhibits proliferation of mesenchymal cells. *The over-expression of mir-99a or mir-99b in TGF-β treated NMUMG cells inhibits their proliferation, as assayed by cell cycle analysis (A) and proliferation assay (B). Cyclin D1 is down-regulated by mir-99a and mir-99b (C). (p<0.05).*

Mir-99a and mir-99b expression is not restricted to mesenchymal cells. In fact all the epithelial cell lines we tested were positive for the expression of mir-99a and mir-99b as assayed by real-time PCR (data not shown). We used lung epithelial cells (MLE-15) to determine if mir-99a and mir-99b affect epithelial cell proliferation as they do mesenchymal cells. Figure 5 shows that the over-expression of mir-99a and mir-99b induce a significant depletion of cells in S-phase (data at 72h post-transfection). Western blot confirmed that cyclin D1 is down-regulated when mir-99a and mir-99b are over-expressed in MLE-15 cells.

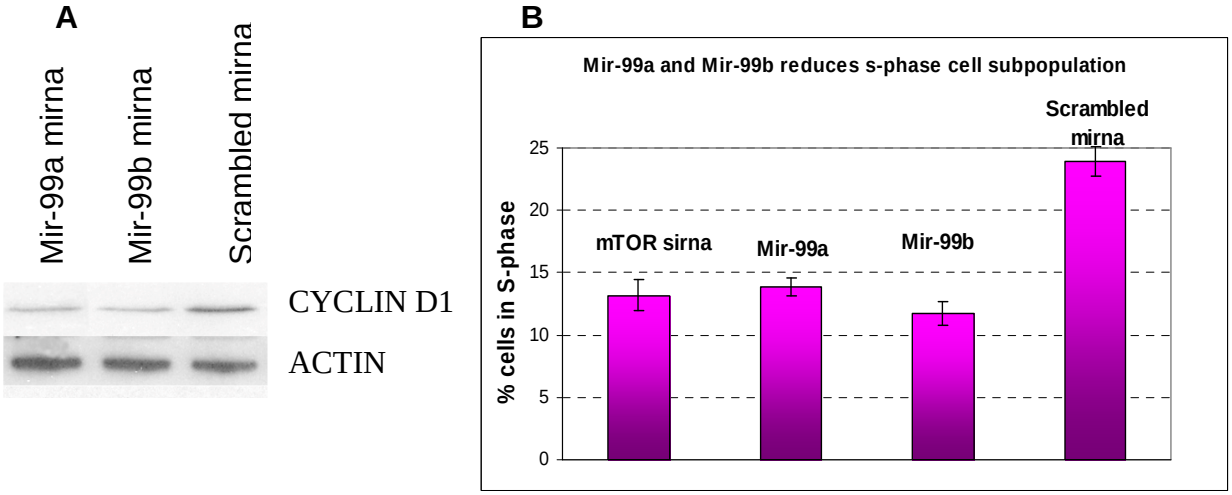


Figure 11 - Mir-99a and mir-99b inhibit epithelial cell proliferation. *Over-expression of mir-99a and mir-99b inhibits cell proliferation of mouse lung epithelial (MLE-15) cells. The percentage of cells in S-phase significantly decreases in mir-99a and mir-99b mimic treated MLE-15 cells compared with the scrambled mirna treated MLE-15 cells. In accordance with the cytofluorimetry analysis cyclin D1 decreased in mir-99a and mir-99b treated MLE-15 cells. mTOR sirna was used as a positive control*.

The data above confirmed that mir-99a and mir-99b are negative regulators of proliferation of both epithelial and mesenchymal cells in vitro. Similar results were obtained with mTOR sirna, which led us to conclude that mir-99a and mir-99b affect cell proliferation via inhibition of mTOR gene.

6. Mir-99a and mir-99b inhibit the serum induced proliferative stimulus

We identified mir-99a and mi-99b as negative regulators of cell proliferation in both epithelial and mesenchymal cells. We next confirmed that they block the proliferative stimulus induced by serum. A549 cells were transfected with mir-99a, mir-99b or a scramble mirna, serum starved and pulsed with media containing 10% of serum for 30 minutes. Phosphorylation of S6K was analyzed by western blot. Figure 12 shows that mit-99a and mir-99b inhibit the phosphorylation and thus activation of S6K kinase induced by serum compared with a scrambled mirna.

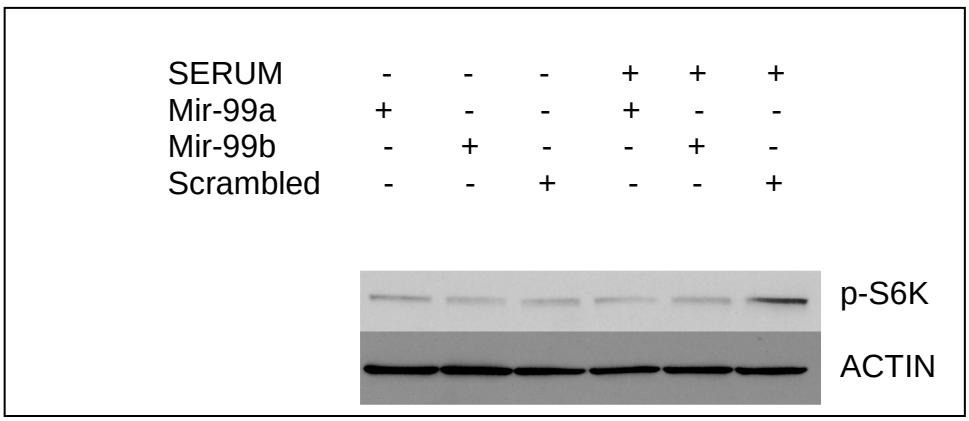


Figure 12 - Mir-99a and mir-99b inhibit the activation of S6K kinase induced by serum.

S6K protein is a direct downstream target of mTOR [59]. The decreased phosphorylation of S6K further confirmed the inhibitory effect of mir-99a and mir-99b on cell proliferation and mTOR pathway.

7. Mir-99a and mir-99b affect cell migration

We found that mir-99a and mir-99b affect cell proliferation. We next determined if they affect one other important aspect of the cell, cell migration. We over-expressed mir-99a and mir-99b in A549 cells. 72 hours later cells were trypsinized and put on the top of a Matrigel Transwell Migration chamber. We used as chemoattractant TGF-β (2ng/ml) and serum containing media (10% FBS). The number of migrated cells through the membrane was measured after 24h with a colorimetric assay. Mir-99a and mir-99b increased the migration of A459 cells toward serum and TGF-β containing media compared with a scrambled mirna (Figure 13).

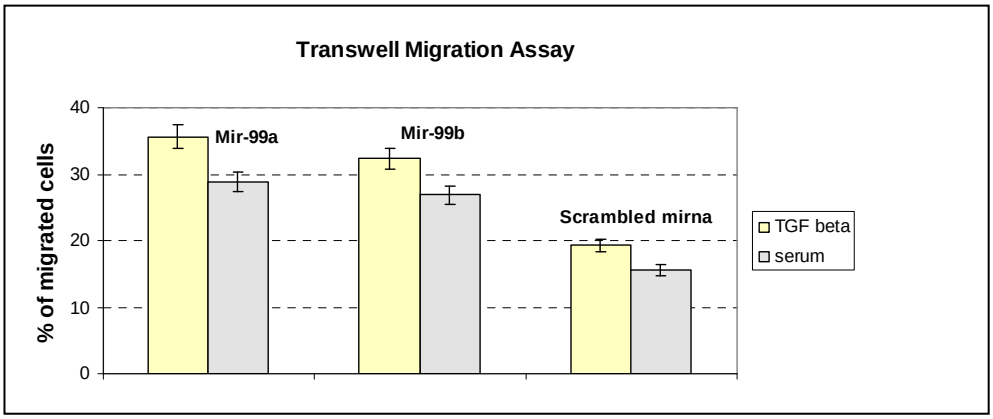


Figure 13 - Mir-99a and mir-99b increase the migration of A549 cells in a Transwell Migrational assay. (p<0.05)

We further confirmed that mir-99a and mir-99b stimulate cell migration using a different migration assay in NMUMg cells: the wound repair assay. This time we used also a mTOR sirna as reference. NMUMG

cells were transfected with mir-99a, mir-99b or a scramble mirna; after 72 hours the cells layer was scratched with a p200 pipet tip and the wound closure was measured 24 h later. Figure 6 shows that both mir-99a and mir-99b accelerate wound closure (Figure 14-A) compared with a scramble mirna. They also down-regulate E-CADHERIN and ROCK-2 (Figure 14-B).

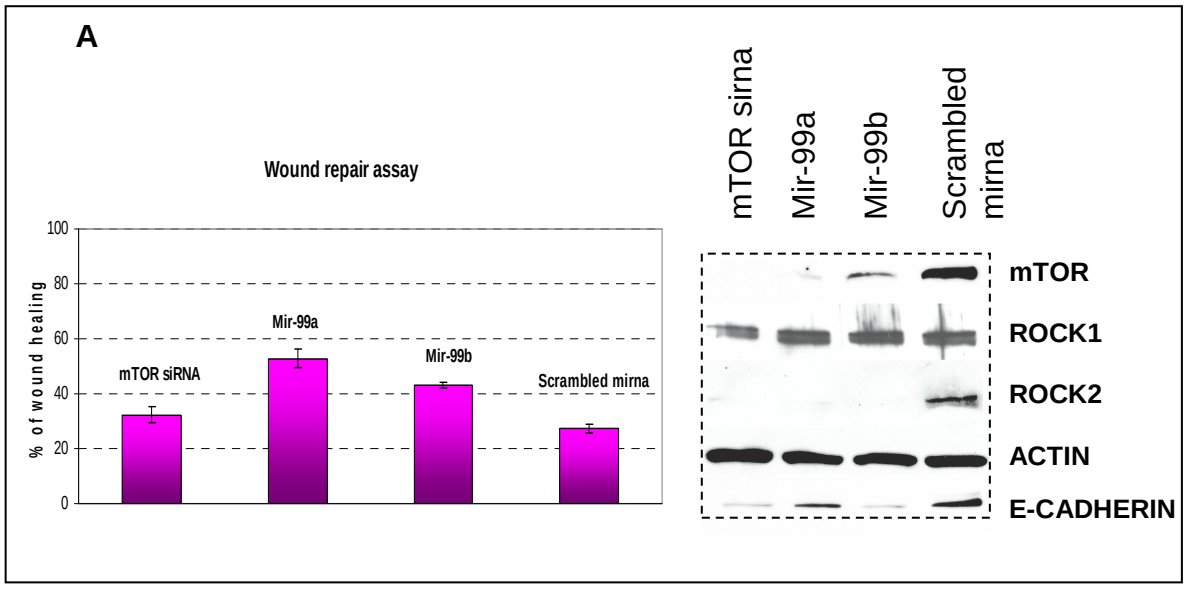


Figure 14 - Mir-99a and mir-99b increase the migration ($p<0.05$) of NMUMG cells and down-regulate E-cadherin and ROCK2.

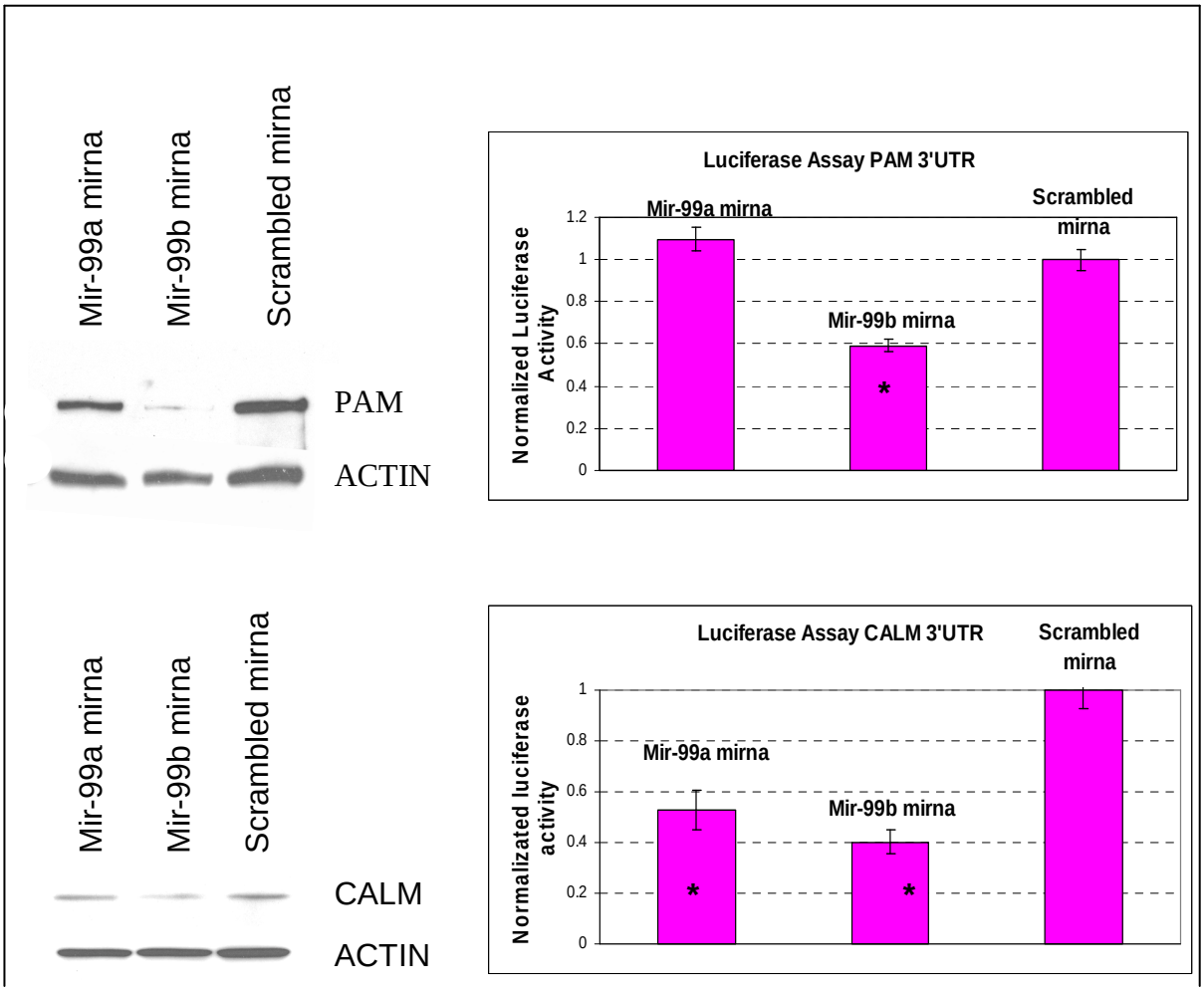
Mir-99a and mir-99b down-regulate E-cadherin presumably via inhibition of mTOR.

It is interesting to note, however, that mir-99a and mir-99b also affect cell migration in a MTOR independent manner since the only mTOR down-regulation with sirna does not alter cell migration of these cells (Figure 14-A), which is in accordance with previous studies [60]. Thus mir-99a and mir-99b alter cell migration by targeting a different target that we plan to identify in future studies.

8. Mir-99a and mir-99b target other genes connected with mTOR pathway

Micrornas are known to target multiple genes simultaneously. We identified mTOR as a principal target of mir-99a and mir-99b. Next we used PicTar (http://pictar.mdc-berlin.de/cgi-bin) and TargetScan (http://www.targetscan.org/) softwares to look at other targets. We found and validated that mir-99a and mir-99b target multiple genes: PAM (MYCBP2), BAZ2A, SMARCA5 and CALM. The validation of BAZ2A as a target of mir-99a and mir-99b is not complete because we could not obtain an antibody for BAZ2A that works on western blot analysis.

Even though mir-99a and mir-99b share 89% of the sequence and have the same seed sequence it was interesting to find some targets that are onlymir-99b specific (CALM, PAM) (Figure 15).



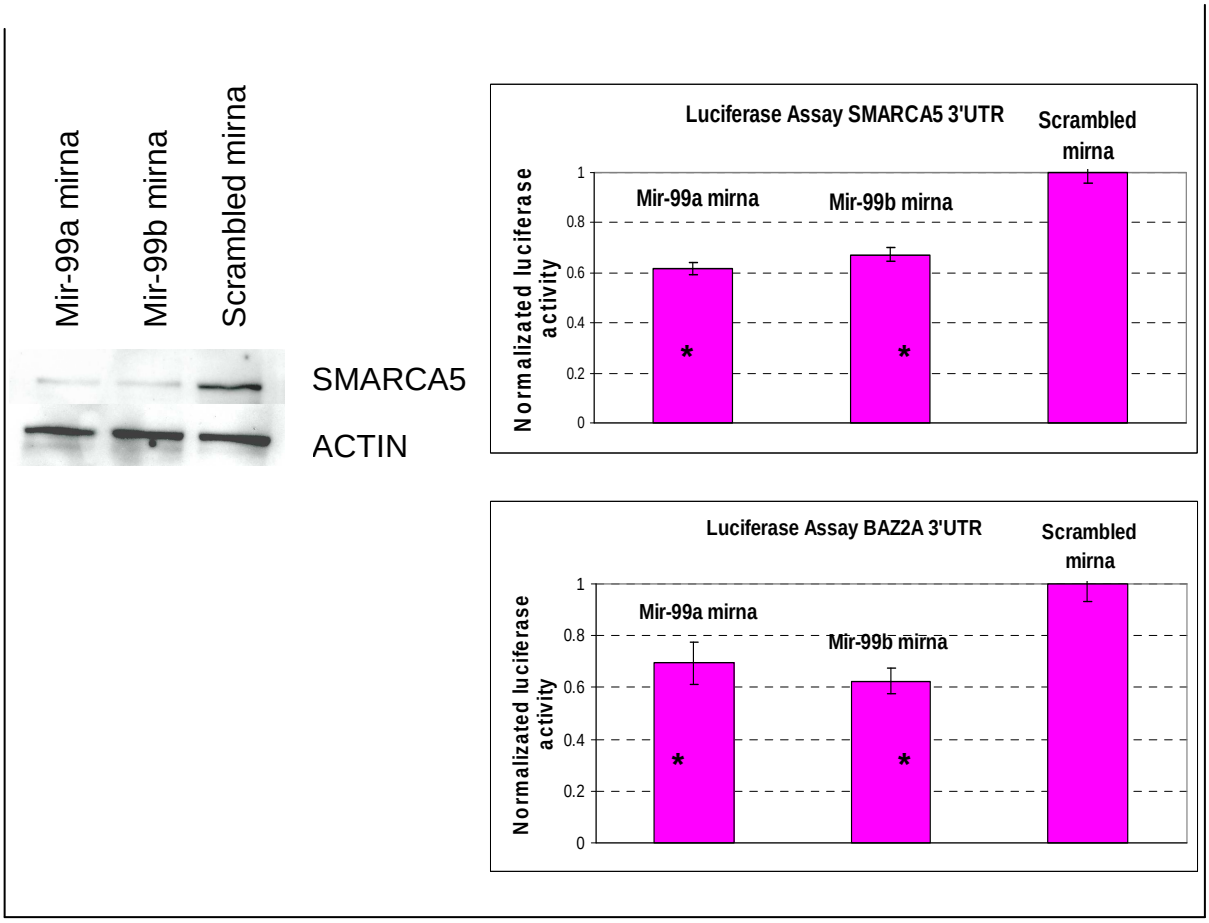


Figure 15 - Identification of mir-99a and mir-99b targets. *Luciferase and western blot assays confirmed mir-99a targets SNF2H and BAZ2A and mir-99b targets CALM, PAM, SNF2H and BAZ2A. (*=p<0.01)*

It was also interesting to find that the some of validated target genes are connected directly to the mTOR pathway itself which others are not. PAM is ubiquitin E3 ligase of Tuberin protein (TSC2), as well as a positive regulator of mTOR itself [61]. CALM has also been found to be associated with TSC2. BAZ2A and SMARCA5 are involved in the expression of the ribosomal RNA but no connection has been found directly between them and the mTOR pathway. However the downstream mTOR genes P6K and 4E-BP regulate transcription and translation of genes involved in ribosome biogenesis. It would be interesting to determine if S6K and 4E-BP interact with BAZA and SMARCA5. This would place BAZ2A and SMARCA5 as new

downstream regulators of mTOR. This hypothesis will be tested in our future research.

Moreover we confirmed that mir-99a and mir-99b are not targeting the following genes even if they are predicted to be targets by PicTar and/or TargetScan software: FGFR3, ICMT, INSM1, CDW92, CDK7, HOXA1, OGT, ZZEF1 (data not shown). Some of the remaining mir-99a and mir-99b predicted targets are brain specific genes (PHOX2B, HS3ST2) and thus were not taken into consideration. Only a few of the predicted target genes have not been analyzed (Supplemental Tables 2A-B).

9. Mir-99b may target TSC2 gene on the CDS region

We found that mir-99b selectively targets PAM gene. PAM, also called MYCBP2 is an ubiquitin E3 ligase which adds ubiquitin to TSC2 protein, promoting its degradation. With the intent to further confirm MYCBP2 as a specific target of mir-99b, we quantified the TSC2 protein level. Unexpectedly the over-expression of mir-99b does not bring about TSC2 over-expression, but instead brings about its down-regulation (Figure 16). Even though TSC2 was not a predicted target of mir-99a and mir-99b, we used Miranda software which is able to find mirna binding sites on mRNA: we thus found that mir-99a and mir-99b have many binding sites on the coding sequence of TSC2 mRNA. Moreover the free energy (ΔG, which represents the strength of the binding interaction) of binding interaction for those sites is lower for mir-99b than mir-99a: this further supports the hypothesis that TSC2 is a specific target of mir-99b. Research made by other groups showed that mirnas can affect translation of target mRNAs by binding directly to the coding sequence.

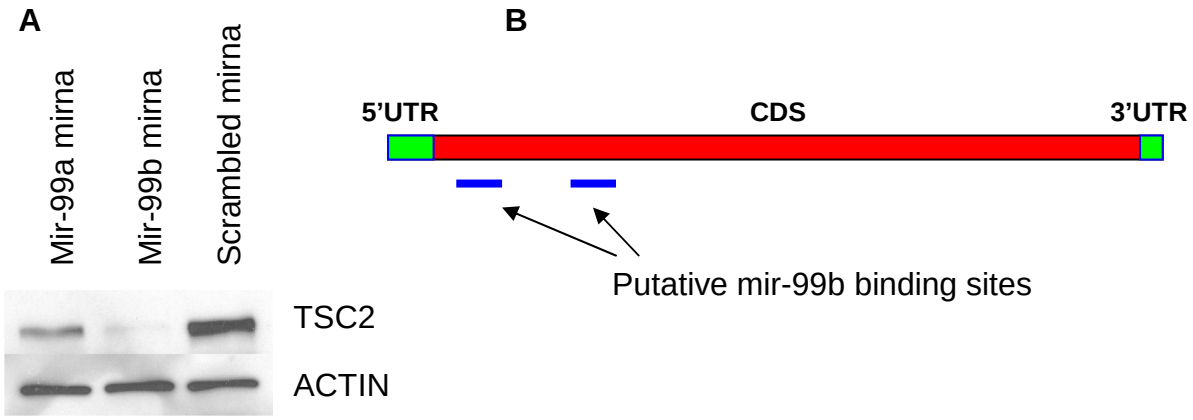


Figure 15 - Over-expression of mir-99b induces down-regulation of TSC2 protein (A). TSC2 coding sequence region has two strong binding sites for mir-99b (450:458 and 690-700 bases of the mRNA sequence) (B). The binding sites reported are the one the lowest free energy, the strongest.

In Figure 16-A we confirmed that the down-regulation of TSC2 is not due to changes of the level of TSC2 messenger, supporting the hypothesis that mir-99b targets TSC2 mRNA. In Figure 16-B we repeated the same experiment as above including this time a knock-down-LNA probe for mir-99b to block its activity. The block of mir-99b activity results in the increase of TSC2 protein expression.

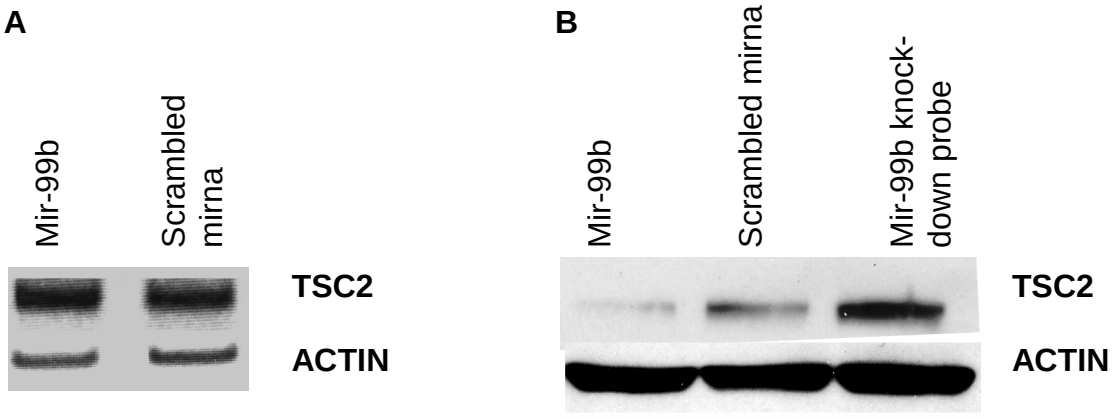


Figure 16. Mir-99b does not affect the level of the TSC2 mRNA level while the down-regulation of mir-99b induces over-expression of TSC2 protein.

The validation of TSC2 as a specific target of mir-99b via binding to the coding sequence still needs to be confirmed and it will be part of our future studies. Nevertheless this is an interesting result which shows the great complexity and diversity of the mirna regulatory network.

DISCUSSION

Mirnas are key regulators of biological processes and diseases. Their role in the lung development is still unclear. To date only the mir-17-92 family of mirnas has been identified as important regulator for normal lung development. In fact it has been shown that mir-17-92 family of mirnas has a key role for the proliferation and branching of the lung embryonic epithelium [15] *in vivo* and in *vitro*. The aim of the current research was to identify other mirnas highly expressed in the embryonic lung and thus with a putative role in the lung development. A real-time PCR assay was used. Many micrornas are expressed in the embryonic lung [15, 17], but we focused our attention on mir-99a and mir-99b. The decision was made in part because the number of putative their target genes is very small (Supplemental Table-2A, 2B). Expression analysis at different stage of lung development revealed that their expression changes during lung maturation: it is quite stable in the embryonic lung and increases to the highest level of expression in the adult lung.

We next used in situ hybridization on E12.5 embryonic lungs to identify where mir-99a and mir-99b are expressed in the embryonic lung. Mir-99a is expressed by the sub-epithelial mesenchyme while mir-99b is strongly expressed by the mesothelium and lesser by the sub-epithelial mesenchyme. These results revealed that mir-99a and mir-99b are mesenchymal specific mirnas at least in the embryonic stage E12.5. Since the mir-17-92 family of mirnas is expressed mostly by the epithelium, mir-99a and mir-99b are the first and only mirnas so far found expressed by the embryonic lung mesenchyme.

The dynamic expression of both mir-99a and mir-99b fits with their role in in cell proliferation and differentiation.

1. Mir-99a and mir-99b stimulate mesenchymal cell to differentiate into smooth muscle cells

To elucidate the putative role of mir-99a and mir-99b on mesenchymal cell differentiation we used primary embryonic lung mesenchymal culture. Since both mirnas are expressed in the mesenchymal compartment and their expression increases with lung maturation, we sought for a possible connection between mir-99a and mir-99b in the mesenchymal cell differentiation process. Embryonic mesenchymal lung cells can be easily separated from the epithelium compartment, using their ability to attach to tissue culture plates faster than epithelial cells [54]. Moreover isolated mesenchymal cells can be kept in a progenitor state by supplying FGF9 in the culture media [54]. FGF9 withdrawal is then known to stimulate the differentiation of embryonic lung mesenchymal cells into smooth muscle cells [54]. We used real time PCR to determine that mir-99a and mir-99b expression increases during the differentiation of mesenchymal cells into smooth muscle cells. This means that they may have a role in the smooth muscle differentiation process. We next found that over-expression of mir-99a and mir-99b in different type of mesenchymal cells induces their differentiation into smooth muscle cells as suggested by the onset of expression of SM-22-α.

These data identified that mir-99a and mir-99b have a key role in the process of smooth muscle cell differentiation. Moreover the use of the NMUMG and A549 cell lines, able to undergo EMT showed that mir-99a and mir-99b over-expression can't induce the expression of SM-22-α when they are epithelial. This means that mir-99a and mir-99b alone are not sufficient to promote epithelial cells to undergo EMT and next smooth muscle cell differentiation.

2. Mir-99a and mir-99b inhibit cell proliferation

Increased expression of mir-99a and mir-99b during lung development suggests thus could be due to a possible role of these in cell proliferation. We found that over-expression of mir-99a and mir-99b inhibits cell proliferation. Cyclin D1 is down-regulated and the population of cells in S-phase is diminished by mir-99a and mir-99b over-expression. This inhibitory effect was found in different cell types: epithelial murine lung MLE-15 cells, NMUMG as well as human carcinoma lung A549 cells.

3. Targets identification of mir-99a and mi-99b

Mirnas bind to the 3'UTR of the mRNA target via the seed sequence, the 7 nucleotides (2^{nd} to 8^{th} nucleotide) at the 5' portion of their sequence. Because of the short length of the binding sequence, only 7 bases, every mirna usually targets thousands of genes simultaneously. Instead the seed sequence of mir-99a and mir-99b seems to be very particular and uncommon. In fact the number of predicted genes targeted is very small: only 33 are predicted by PicTar (Table 2A and 2B) and 35 by TargetScan (table not reported). The relatively low number of predicted targets was of great help for the identification and validation of the mir-99a and mir-99b, targets and thus for understanding of their role in biological processes. Moreover, some putative targets are not expressed in the lung such as PHOX2B, and they were therefore not taken into consideration in our studies.

3.1. MTOR gene as principal target

The first target to be validated was mTOR gene. MTOR is a key regulator of cell proliferation and differentiation, and since mir-99a and mir-99b affect the same biological processes, mTOR was a good candidate to be a MASTER TARGET GENE. The mTOR protein

belongs to two different complexes, MTORC1 and MTORC2 (Figure 17). MTORC1 regulates cell proliferation through the two main downstream targets S6K and 4E-BP. S6K and 4E-BP regulate transcription and translation of TOP-mRNA genes, which encode for rRNA and mRNA important for ribosomal biogenesis [62]. MTORC2 complex affects cell motility and cell shape through regulation of the RHO-GTPase pathway [59].

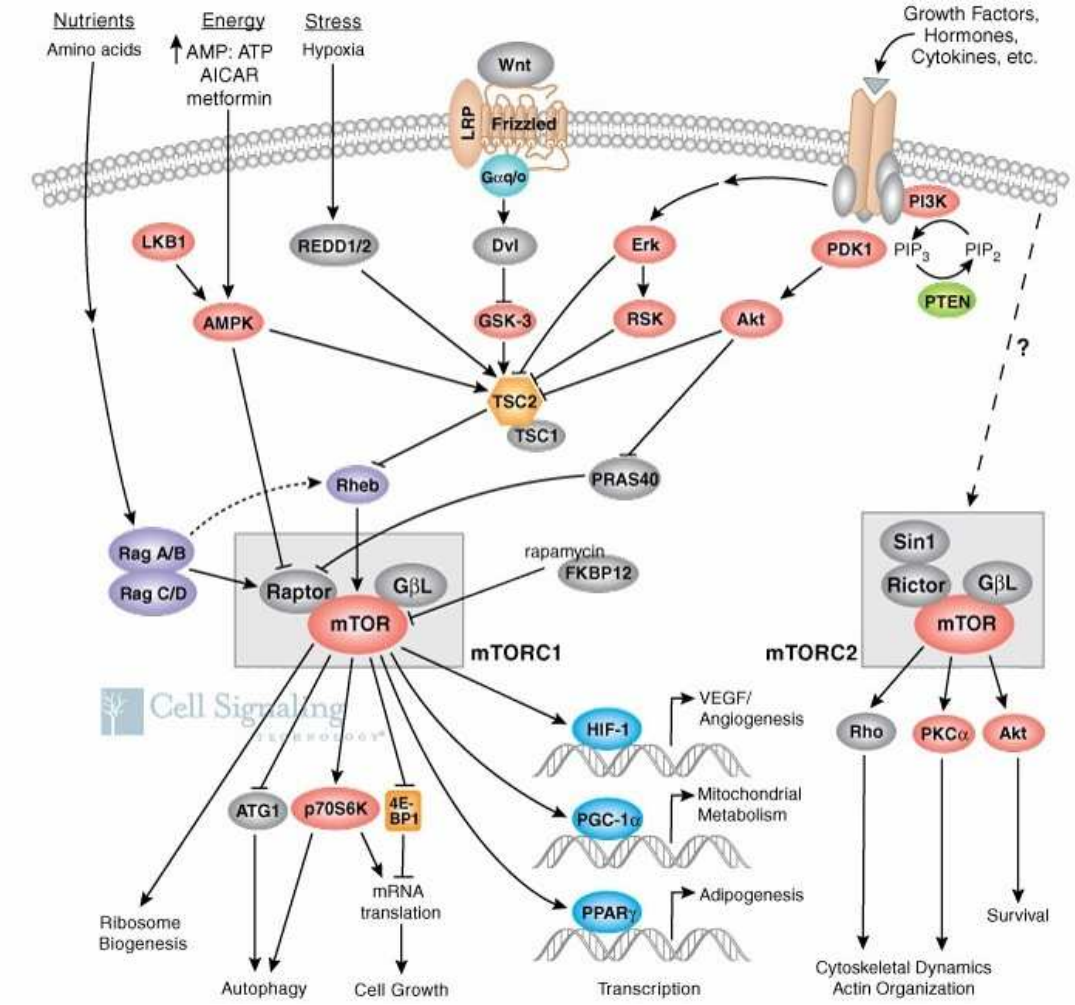


Figure - 17. Overview of mTOR pathway. *mTOR pathway showing the two mTOR complexes mTORC1 and MTORC2, regulating cell proliferation and cytoskeleton respectively.*

mTOR gene was validated as target of both mir-99a and mir-99b by luciferase test and western blot, which confirmed that they bind to the 3'UTR mTOR and inhibit the RNA messenger translation.

The delivery of a sirna active against mTOR inside cells induces a similar inhibition of cell proliferation as that observed with the over-expression of mir-99a and mir-99b, which implies that they act on the same common pathway. Moreover, mir-99a and mir-99b negatively regulate cyclin-D1 level, which is a major mTOR down-stream regulated gene.

mTOR is involved in cell differentiation. It has been reported that mTOR inhibition with rapamycin induces cell to differentiate into smooth muscle cells [55]. Similarly we found that mir-99a and mir-99b over-expression as well as sirna mediated down-regulation of mTOR in mesenchymal cells induce the expression of the adult smooth muscle cell marker, SM-22-α.

Finally, we confirmed that mir-99a and mir-99b affects the mTOR complex 2 in the same way an mTOR sirna does and thus affecting the Rho-GTPase which was confirmed by the confirmed by the down-regulation of ROCK2 protein. We also found that mir-99a and mir-99b over-expression as well as the down-regulation of mTOR protein with a specific sirna result in the down-regulation of E-cadherin. These data taken together led us to define mTOR as a major target of mir-99a and mir-99b because multiple experiments showed that the over-expression of mir-99a and mir-99b has a molecular footprint very similar to the mTOR sirna knock-down and rapamycin inhibitor. On the other hand we found that mir-99a, mir-99b and mTOR sirna affect cell migration in differently manner. The down-regulation of mTOR with a specific sirna doesn’t affect epithelial cell migration which is in accordance with previously studies [60]. Mir-99a and mir-99b over-expression instead increases cells migration. We hypothesize that mir-99a and mir-99b are targeting a gene different than mTOR, with a specific role in cell migration.

Beside the role of mir-99a and mir-99b on cell migration which is still not completely understood, we determined that mir-99a and mir-99b have a negative role in cells proliferation and they stimulate the differentiation of mesenchymal cells into smooth muscle cells by targeting mTOR gene.

4. Mir-99a and mir-99b target SNF2H and BAZ2A gene

We validated that mir-99a and mir-99b target, besides mTOR, also SMARCA5 and BAZ2A: these two genes are involved in chromatin remodeling. Together they form a complex called NoRC (nucleolar remodeling complex). The NoRC is a nucleolar protein complex involved in remodeling the chromatin at the rDNA locus. These genes may thus be involved in cell proliferation acting on ribosome biogenesis. Mir-99a and mir-99b may thereafter affect proliferation not only by inhibiting mTOR but also by inhibiting ribosome biogenesis. This hypothesis will be tested in future studies.

5. Mir-99b target specifically PAM and CALM gene

A commonly accepted rule for mirnas studies is that they bind to target mRNAs mainly via base pairing of the seed sequence: thus mirnas with the same seed sequence should have the same targets. Different research groups have shown though that mirnas can target mRNAs even if the seed sequence is not totally complementary to the target, thus giving importance to the rest of the mirna sequence in targeting processes.
Even though mir-99a and mir-99b share 82% of the sequence and they have the same seed sequence we found at least two genes specifically targets of mir-99b only: PAM and CALM. Luciferase test and western blot confirmed that only mir-99b can repress the expression of CALM and PAM proteins. Conversely mir-99a has no effect on their

expression. It would be interesting to determine in future the role of this selective target discrimination in biological processes and any connection with the differential expression pattern of mir-99a and mir-99b in the embryonic lung.

6. Other mir-99a and mir-99b targets

We conclude this section on target validation with the list of genes that we excluded as not real targets of mir-99a and mir-99b, even if they are predicted to be so in silico by PicTar and/or TargetScan softwares: FGFR3, ICMT, INSM1, CDW92, CDK7, HOXA1. Western blot and/or luciferase test was used to show that the over-expression of mir-99a and mir-99b do not affect their protein level (data not shown).

CONCLUSION

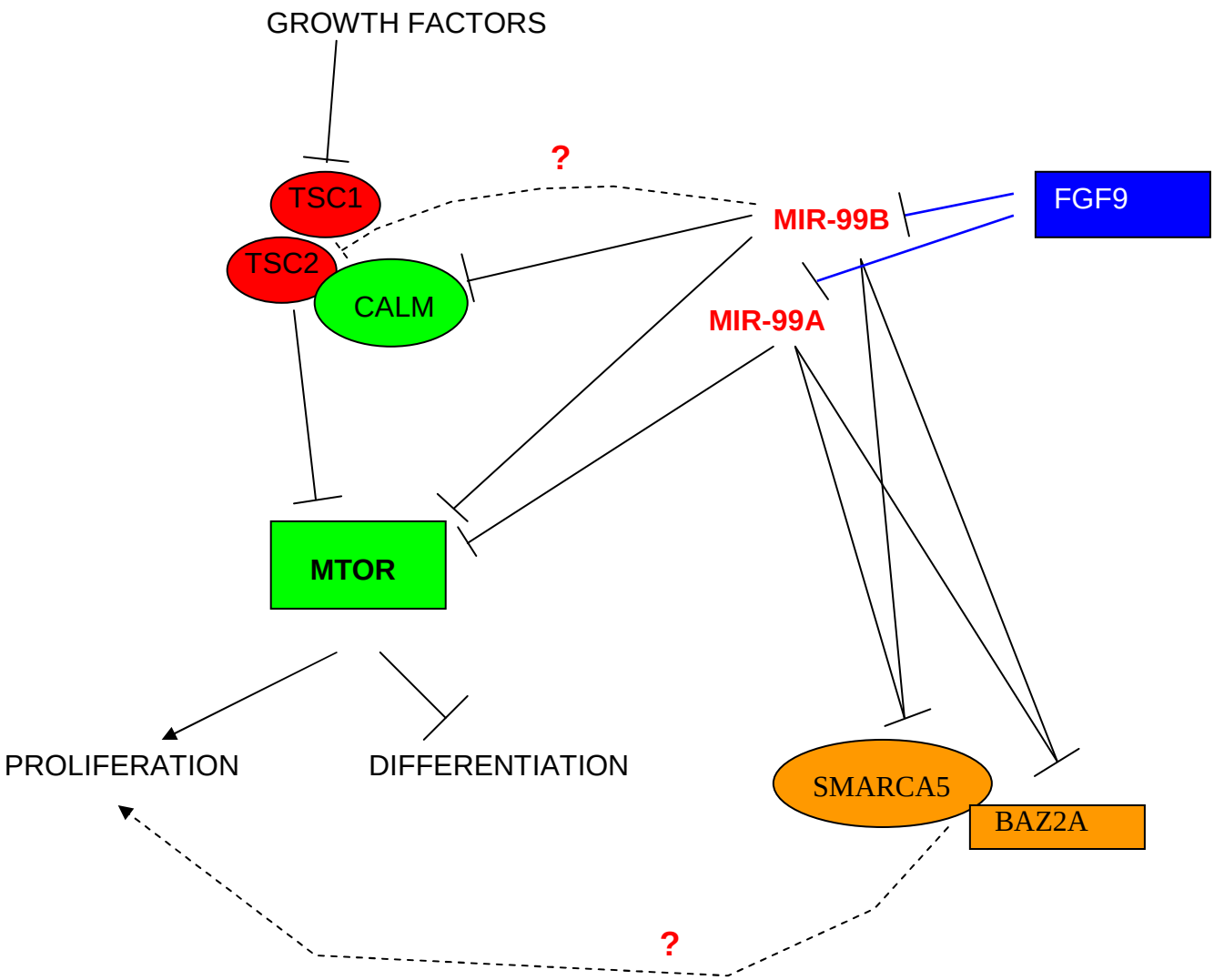
Mirnas are key regulators of biological processes and diseases. Herein we focused our research on mir-99a and mir-99b. We identified mir-99a and mir-99b as important regulators of cell proliferation and smooth muscle cell differentiation via direct inhibition of mTOR gene expression. However the spectrum of transcriptional roles of these mirnas in cellular biological processes remains incompletely understood, because other targets have been already identified and their specificity role still needs to be elucidated. Since each mirna has its own wide network of regulated genes, the complete understanding of each microrna function can be very complicated. Even though the number of predicted mir-99a and mir-99b targets is relatively small we did find a special case of target-microrna interaction. TSC2 gene does appear to be a specific target of mir-99b, but the identified binding sites are not within the 3'UTR portion of the mRNA but in the coding sequence. Thus, fully understanding of mirna function becomes even more complex. It is clear that mirnas have a key role in cellular processes and that complete identification of the targets is a vital step in understanding their activity. Microrna represent a relatively new class of regulatory genes and research on their activity is just at beginning.

The significance of the two microrna we studied, mir-99a and mir-99b, is high. The mTOR pathway is deregulated in cancer and it is also a specific pathway altered in LAM disease. Thus mir-99a and mir-99b may be druggable targets for these diseases. Previously research with mirna and mice showed that the injection intravenously of mirnas is feasible [63], and gives good delivery to the target cells. Thus the use

of mir-99a and mir-99b as drug is feasible and it will be the main research project in our future studies.

Finally mTOR is targeted by Rapamycin which is commonly used as immunosuppressive. So far we have not addressed any putative role of mir-99a and mir-99b in suppressing the immune system, but this is another aspect to keep in mind for their eventual use in medicine.

MIR-99A and MIR-99B / MTOR PATHWAY CROSS TALK DIAGRAM



SUPPLEMENTAL MATHERIALS

Supplemental figure 1 - Expression of mir-99a and mir-99b during murine lung development

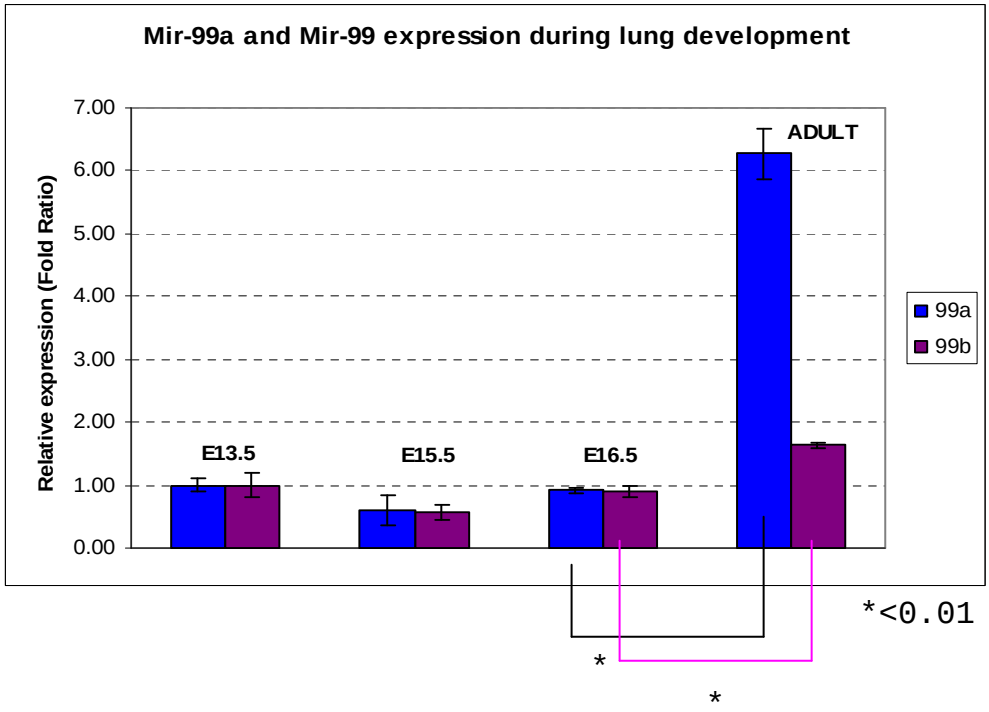


Table 1 - List of transcription factors associated with abnormalities in lung morphogenesis or function

PART 1

	Family	Location	Downstream Genes	Lung Morphogenesis/ Defect	Disorder	Reference Nos.
HIF1A	bHLH	M, E, enth	Vegf	Vascularization	Die at midgestation(−/−)	201, 280, 281
HIF2A	bHLH	M, E, enth	Vegf, Sftpb	Sacculation/ vascularization	RD/atelectasis(−/−)	29, 44, 65, 242
N-MYC/MYCN	bHLH	dE	Sftpa, Sftpb, Aqp5, Sox9, Rog	Structure/branching	PN(−/−, OE)	161, 162, 181
POD1/TCF21	bHLH	M	Scgb1a1, Bmp4, Sftpc	Branching	PN(−/−)	194
ASCL1/MASH1	bHLH	PNEC	Calca, Dll1	PNEC	PNEC absent(−/−)	25, 95
HES1	bHLH	E(noPNEC)	Ascl1, NeuroD, Notch1	No PNEC	Unknown	95
CREB1	bZIP	SAEC	Sftpd	Sacculation	RD/atelectasis(−/−)	2, 200
CEBPA	bZIP	Type II & AM	Surfactant protein	Sacculation	RD/atelectasis(−/−)	10, 14, 57, 147, 179, 223
CEBPB	bZIP	Lung	Scgb1a1, Cyp2b1	Unknown	COPD, CB	13, 15
CEBPD	bZIP	Lung	Scgb1a1, Cyp2b1	Unknown	Unknown	13
SREBP-1c/SREBF1	bZIP	Type II	Fasn, Scd1	Normal	Lipid metabolism	33, 148, 285
ATF2	bZIP	Lung	Sftpb	Unknown	RD/atelectasis(−/−)	16, 141, 237
JUNB	bZIP	Lung	Sftpb	Unknown	Unknown	163, 207
C-JUN	bZIP	Lung	Sftpb	Unknown	Unknown	207, 259
JUNDI	bZIP	H292	Sftpb	Unknown	Unknown	72, 207
NRF2/NFE212	bZIP	Unknown	Nqo1, Ugt1a6	Normal	AL1, inflammation, emphysema	32, 90
FOXA1	Fox	E	Sftpa, Sftpb, Sftpc, Sftpd, Scgb1a1	Differentiation	Normal function/differentiation delay(−/−)	16, 17, 19, 123, 244
FOXA2	Fox	E	Sftpa, Sftpb, Sftpd, Scgb1a1, Wnt7b, Titf1	Goblet cell/ alveolarization	RD/atelectasis(−/−)	42, 88, 244–246, 252, 290, 291
FOXF1	Fox	dM	Gli3, Fgf10, Tbx	Vascularization/ alveolarization	PN, RD, hemorrhage, AL1(−/−)	42, 82, 100–108, 127
FOXJ1	Fox	Cillated E	Dnahc11, Sftpb, Sftpc, Tubb, Nodal, Pitx2, Ezrin/Vit2	Cillogenesis/L-R asymmetry	PN, PostN, autoimmune(−/−)	38, 86, 128, 232, 279, 285
FOXM1	Fox	M	Cyclin, Flk1/Kdr, Pecam1	Vascularization	Air space enlargement (−/−)	99, 100, 110, 111, 275
FOXP1	Fox	pE, dE	Scgb1a1, Sftpc	Normal	Unknown	125, 137, 215, 247
FOXP2	Fox	dE	Scgb1a1, Sftpc	Unknown	Unknown	125, 137, 215
FOXP4	Fox	pE, dE	Scgb1a1	Unknown	Unknown	125, 137
HOXA5	Homeo	M	Sftpb, Sftpc, Titf1, Foxa2, Nmyc/Mycn	Sacculation	RD/atelectasis(−/−)	5
HOXB2, 3, 4, 5	Homeo	M	Unknown	Unknown	Unknown	22, 23
TTF-1/TITF1	Homeo	E	Sftpa, Sftpb, Sftpc, Sftpd, Scgb1a1, Bmp4, Wnt7b	Structure/ differentiation	RD/hypoplasia(−/−)	24, 51, 112
CUTL1	Homeo	E	Unknown	Sacculation/ alveolarization	RD	64
HOP/HOD	Homeo	E	Surfactant protein	Structure	Hemorrhage/alveolar disruption(−/−)	277
PROP1	Homeo	Pituitary gland	Titf1	Sacculation	RD/atelectasis(−/−)	175
PITX2	Homeo	Left bud	Unknown	L-R asymmetry	Situs inverses(−/−)	113, 278
GR/NR3C1	NR	E	Unknown	Sacculation	RD/atelectasis(−/−)	43, 115, 192, 195
ERbeta/ESR2	NR	E(adult)	Sftpc, Pdgf, Gm-csf/ Csf2	Alveolarization	Surfactant accumulation(−/−)	187
RAR	NR	E	Sftpb, elastin	Structure/agenesis	PN/hypoplasia(−/−)	149, 151, 263
LXR/NR1H	NR	?	Unknown	Normal	Foam cell accumulation(−/−)	204
VDR	NR	E	Unknown	Normal	Inflammation(−/−)	177, 260
PPAR	NR	E(adult)	Sftpb, Gata3	Normal	Inflammation(−/−)	153, 261, 271
GATA6	Zinc	dE	Sftpc, Scgb1a1, Foxa2, Aqp5, Foxp2, Wnt7b, Titf1	Differentiation/ sacculation	PN or RD(−/−)	27, 117, 133, 166, 210, 252, 266
SP1	Zinc	bE(adult)	Sftpb	Unknown	Unknown	105, 145
SP3	Zinc	bE(adult)	Scgb1a1	Sacculation	RD(−/−)	26, 105
LKLF/KLF2	Zinc	Lung	Unknown	Branching	Die at midgestation(−/−)	248
GLI1	Zinc	M, E	Foxm1	PNEC/normal	Normal(−/−)	182, 228, 250
GLI2	Zinc	M	Gli1, Ptch	Structure	Hypoplasia/agenesis/die at birth(−/−)	76, 168, 182
GLI3	Zinc	M	Unknown	Structure	Hypoplasia/agenesis/extra digits(−/−)	76, 127, 168, 182

Table 1 - List of transcription factors associated with abnormalities in lung morphogenesis or function

PART 2

	Family	Location	Downstream Genes	Lung Morphogenesis/Defect	Disorder	Reference Nos.
ZIC3	Zinc	Cerebellum (adult)	Nodal, Pitx2	L-R asymmetry	Pulmonary reversal or isomerism(−/−)	4, 193
PLAGL2	Zinc	Lung	Sftpc	Unknown	Unknown	209
GFI1	Zinc	PNEC	Unknown	PNEC	PNEC hyperplasia	109
STAT1	STAT	E(adult)	Unknown	Normal	Pulmonary fibrogenesis, asthma	202, 243
STAT3	STAT	E(adult)	Sftpa, Sftpb	Normal	ALI	85, 208, 264, 269
STAT4	STAT	E(adult)	Unknown	Normal	Inflammation, COPD	59, 224, 229
STAT6	STAT	E(adult) & more	Unknown	Normal	Inflammation, asthma	1, 21, 122, 160
HDAC	CoA	Lung	Tnf, I/8, Mmp9, Sftpa	Unknown	Asthma, COPD	92–94, 277
NCOR/SMRT	CoA	Lung	Sftpa	Unknown	Unknown	92
SRC1/TIF2/NCOA	CoA	E, M	Sftpa, Sftpb	Sacculation	RD/atelectasis(−/−)	98, 146, 173, 276
P300/EP300	CoA	E, M	Scgbia1, Sftpc, Sftpd	Sacculation	RD(mut)	8, 106, 213
CBP/CREBBP	CoA	E, M	Sftpa, Sftpb, Sftpd	Sacculation	Atelectasis(mut)	92, 106, 173, 213, 276
CARM1	CoA	Lung	Unknown	Sacculation	PN/atelectasis	262
MRG15/MORF4L1	CoA	Lung	Unknown	Sacculation	PN/atelectasis	284
PARP1/PARP2	CoA	E, AM	Sftpb	Unknown	Unknown	140
BR22/TAP26	CoA	E(adult)	Sftpb	Unknown	Unknown	270, 273
TAZ/WWTR1	CoA	E	Sftpc	Unknown	Unknown	135
RCD1	CoA	Lung	Unknown	Branching	Unknown	83
CATENIN/CTNNB1	CoA	E, M	Sftpa, Sftpb, Sftpc, Vegf, Pecam, Foxa2, Nmyc/Mycn, Bmp4	Branching/goblet cell	Pulmonary tumor/pulmonary fibrosis/ALI	40, 60, 169, 170, 181, 183, 214, 287
TCF/LEF	HMG	E, M	Sftpb, Cdx1, Atoh1	Submucosal glands	ALI/no submucosal gland(−/−)	60, 62, 63, 180, 227
SOX2	HMG	E	Unknown	Unknown	Unknown	90, 183
SOX7	HMG	Lung	Unknown	Unknown	Unknown	225
SOX9	HMG	E, M	Unknown	Normal	Normal	190
SOX11	HMG	E, M	Unknown	Sacculation	RD/atelectasis	219
SOX17	HMG	E, M?	Foxa1, Foxa2	Branching, PNEC	Focal epithelial hyperplasia (OE)	183, 216
SMAD1	SMAD	E, M	Sftpc	Branching	Inflammation	36, 211
SMAD2	SMAD	E	Unknown	Branching	ALI/inflammation/ pulmonary fibrosis	150, 240, 287
SMAD3	SMAD	E	Sftpb	Branching/alveolarization	ALI/pulmonary fibrosis/emphysema	36, 123, 240, 287, 289
SMAD4	SMAD	E, M	Unknown	Branching	ALI	240, 287
SMAD5	SMAD	Lung	Unknown	Branching	Unknown	211
SMAD6	SMAD	Vessel E	Unknown	Unknown	COPD	70, 220, 288
SMAD7	SMAD	daE	Sftpc	Branching	ALI/inflammation/ pulmonary fibrosis/ COPD	150, 172, 220, 240, 288
PU.1/SPI1	ETS	AM	Cd32/Fcgr2a, M-csfr/ Csflr	Alveolar macrophage	Alveolar proteinosis	212
ETS1	ETS	Lung	Caveolin-1/Cav1	Unknown	Unknown	108
ELF3	ETS	Lung	Unknown	Unknown	Unknown	108
ESE3/EHF	ETS	Lung	Unknown	Unknown	Unknown	108
SPDEF	ETS	E	Foxj1	Goblet cell differentiation	Inflammation	108; Park, unpublished data
PEA3	ETS	E, M	Aqp5, caveolin-1/ Cav1	Normal	Unknown	108, 134
ERM	ETS	dE	Sftpc, caveolin-1/ Cav1	Branching	RD	108, 129, 134
NF1	Nf1	M, E	Sftpa, Sftpb, Sftpc, Sftpd, Aqp1, ENaC/Scnn1	Sacculation	PN/RD/atelectasis	7, 8, 78, 222
RB1	Rb	Lung	Calca	PNEC	PNEB hyperplasia	258
TBX1	T-box	E	Unknown	Unknown	DiGeorge's syndrome	34, 97, 130, 152
TBX4/5	T-box	M	Fgf10	Branching	Unknown	31, 34
NFATC3(w/CNB1)	NFAT	bE	Sftpa, Sftpb, Sftpc, Sftps, Abca3	Sacculation	RD	48
NFKB	Rel	M	Sftpa	Unknown	Inflammation	91, 92
B-MYB/MYBL2	Myb	dbE	Sftpa	Unknown	Unknown	28
E2F1	E2F	Lung?	Unknown	Structure(W/Rb) (sacculation)	Unknown	121, 135
RUNX3	Runt	D	Ccr7	Dendritic cell	Inflammation	66, 67

Supplemental Table 2A - Mir-99a predicted targets according to PicTar software

Rank	human Refseq Id	PicTar score	microRNAs with Anchor sites	predicted sites for all microRNAs embedded in the UCSC genome browser	annotation
1	NM_003601	4.76	hsa-miR-99a	Genome browser	Homo sapiens SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5 (SMARCA5), mRNA.
2	NM_013449	4.56	hsa-miR-99a	Genome browser	Homo sapiens bromodomain adjacent to zinc finger domain, 2A (BAZ2A), mRNA.
3	NM_003383	3.82	hsa-miR-99a	Genome browser	Homo sapiens very low density lipoprotein receptor (VLDLR), mRNA.
4	NM_006041	3.55	hsa-miR-99a	Genome browser	Homo sapiens heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1 (HS3ST3B1), mRNA.
5	NM_006043	3.07	hsa-miR-99a	Genome browser	Homo sapiens heparan sulfate (glucosamine) 3-O-sulfotransferase 2 (HS3ST2), mRNA.
6	NM_004496	3.01	hsa-miR-99a	Genome browser	Homo sapiens forkhead box A1 (FOXA1), mRNA.
7	NM_015630	2.92	hsa-miR-99a	Genome browser	Homo sapiens enhancer of polycomb homolog 2 (Drosophila) (EPC2), mRNA.
8	NM_004958	2.87	hsa-miR-99a	Genome browser	Homo sapiens FK506 binding protein 12-rapamycin associated protein 1 (FRAP1), mRNA.
9	NM_012154	2.87	hsa-miR-99a	Genome browser	Homo sapiens eukaryotic translation initiation factor 2C, 2 (EIF2C2), mRNA.
10	NM_018569	2.80	hsa-miR-99a	Genome browser	Homo sapiens chromosome 4 open reading frame 16

					(C4orf16), mRNA.	
11	NM_031866	2.71	hsa-miR-99a	Genome browser	Homo sapiens frizzled homolog 8 (Drosophila) (FZD8), mRNA.	
12	NM_002196	2.60	hsa-miR-99a	Genome browser	Homo sapiens insulinoma-associated 1 (INSM1), mRNA.	
13	NM_005522	2.51	hsa-miR-99a	Genome browser	Homo sapiens homeo box A1 (HOXA1), transcript variant 1, mRNA.	
14	NM_018211	2.43	hsa-miR-99a	Genome browser	Homo sapiens hypothetical protein FLJ10770 (KIAA1579), mRNA.	
15	NM_080546	2.27	hsa-miR-99a	Genome browser	Homo sapiens CDW92 antigen (CDW92), mRNA.	
16	NM_173822	2.24	hsa-miR-99a	Genome browser	Homo sapiens hypothetical protein MGC39518 (MGC39518), mRNA.	
17	NM_153620	2.22	hsa-miR-99a	Genome browser	Homo sapiens homeo box A1 (HOXA1), transcript variant 2, mRNA.	
18	NM_021038	2.14	hsa-miR-99a	Genome browser	Homo sapiens muscleblind-like (Drosophila) (MBNL1), transcript variant 1, mRNA.	
19	NM_207297	2.12	hsa-miR-99a	Genome browser	Homo sapiens muscleblind-like (Drosophila) (MBNL1), transcript variant 7, mRNA.	
20	NM_003924	2.00	hsa-miR-99a	Genome browser	Homo sapiens paired-like homeobox 2b (PHOX2B), mRNA.	
21	NM_032505	1.97	hsa-miR-99a	Genome browser	Homo sapiens T-cell activation kelch repeat protein (TA-KRP), mRNA.	
22	NM_000142	1.97	hsa-miR-99a	Genome browser	Homo sapiens fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism) (FGFR3), transcript variant 1, mRNA.	
23	NM_021643	1.94	hsa-miR-99a	Genome browser	Homo sapiens tribbles homolog 2 (Drosophila)	

					(TRIB2), mRNA.	
24	NM_032283	1.80	hsa-miR-99a	Genome browser	Homo sapiens zinc finger, DHHC domain containing 18 (ZDHHC18), mRNA.	
25	NM_015113	1.76	hsa-miR-99a	Genome browser	Homo sapiens zinc finger, ZZ type with EF hand domain 1 (ZZEF1), mRNA.	
26	NM_021090	1.70	hsa-miR-99a	Genome browser	Homo sapiens myotubularin related protein 3 (MTMR3), transcript variant 3, mRNA.	
27	NM_012405	1.69	hsa-miR-99a	Genome browser	Homo sapiens isoprenylcysteine carboxyl methyltransferase (ICMT), transcript variant 1, mRNA.	
28	NM_003605	1.48	hsa-miR-99a	Genome browser	Homo sapiens O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT), transcript variant 3, mRNA.	
29	NM_206835	1.47	hsa-miR-99a	Genome browser	Homo sapiens TNF receptor-associated factor 7 (TRAF7), transcript variant 2, mRNA.	
30	NM_019885	1.39	hsa-miR-99a	Genome browser	Homo sapiens cytochrome P450, family 26, subfamily B, polypeptide 1 (CYP26B1), mRNA.	
31	NM_005808	1.37	hsa-miR-99a	Genome browser	Homo sapiens CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like (CTDSPL), mRNA.	
32	NM_021116	0.69	hsa-miR-99a	Genome browser	Homo sapiens adenylate cyclase 1 (brain) (ADCY1), mRNA.	

Supplemental Table 2B - Mir-99b predicted targets according to PicTar software

Rank	human Refseq Id	PicTar score	microRNAs with Anchor sites	predicted sites for all microRNAs embedded in the UCSC genome browser	annotation
1	NM_003601	4.76	hsa-miR-99b	Genome browser	Homo sapiens SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5 (SMARCA5), mRNA.
2	NM_013449	4.56	hsa-miR-99b	Genome browser	Homo sapiens bromodomain adjacent to zinc finger domain, 2A (BAZ2A), mRNA.
3	NM_001743	4.48	hsa-miR-99b	Genome browser	Homo sapiens calmodulin 2 (phosphorylase kinase, delta) (CALM2), mRNA.
4	NM_015057	4.02	hsa-miR-99b	Genome browser	Homo sapiens MYC binding protein 2 (MYCBP2), mRNA.
5	NM_006041	3.55	hsa-miR-99b	Genome browser	Homo sapiens heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1 (HS3ST3B1), mRNA.
6	NM_021643	3.11	hsa-miR-99b	Genome browser	Homo sapiens tribbles homolog 2 (Drosophila) (TRIB2), mRNA.
7	NM_006043	3.07	hsa-miR-99b	Genome browser	Homo sapiens heparan sulfate (glucosamine) 3-O-sulfotransferase 2 (HS3ST2), mRNA.
8	NM_080546	2.88	hsa-miR-99b	Genome browser	Homo sapiens CDW92 antigen (CDW92), mRNA.
9	NM_004958	2.87	hsa-miR-99b	Genome browser	Homo sapiens FK506 binding protein 12-rapamycin associated protein 1 (FRAP1), mRNA.
10	NM_012154	2.87	hsa-miR-99b	Genome browser	Homo sapiens eukaryotic translation initiation factor

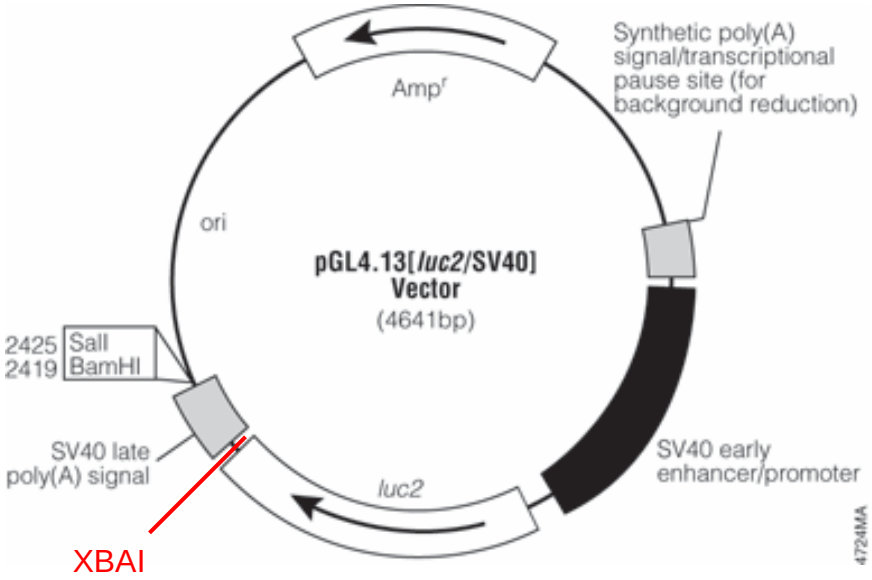
					2C, 2 (EIF2C2), mRNA.
11	NM_018569	2.80	hsa-miR-99b	Genome browser	Homo sapiens chromosome 4 open reading frame 16 (C4orf16), mRNA.
12	NM_152470	2.72	hsa-miR-99b	Genome browser	Homo sapiens chromosome 18 open reading frame 23 (C18orf23), mRNA.
13	NM_031866	2.71	hsa-miR-99b	Genome browser	Homo sapiens frizzled homolog 8 (Drosophila) (FZD8), mRNA.
14	NM_002196	2.60	hsa-miR-99b	Genome browser	Homo sapiens insulinoma-associated 1 (INSM1), mRNA.
15	NM_018211	2.52	hsa-miR-99b	Genome browser	Homo sapiens hypothetical protein FLJ10770 (KIAA1579), mRNA.
16	NM_005522	2.51	hsa-miR-99b	Genome browser	Homo sapiens homeo box A1 (HOXA1), transcript variant 1, mRNA.
17	NM_153620	2.22	hsa-miR-99b	Genome browser	Homo sapiens homeo box A1 (HOXA1), transcript variant 2, mRNA.
18	NM_021038	2.14	hsa-miR-99b	Genome browser	Homo sapiens muscleblind-like (Drosophila) (MBNL1), transcript variant 1, mRNA.
19	NM_173510	2.12	hsa-miR-99b	Genome browser	Homo sapiens hypothetical protein FLJ33814 (FLJ33814), mRNA.
20	NM_207297	2.12	hsa-miR-99b	Genome browser	Homo sapiens muscleblind-like (Drosophila) (MBNL1), transcript variant 7, mRNA.
21	NM_032505	1.97	hsa-miR-99b	Genome browser	Homo sapiens T-cell activation kelch repeat protein (TA-KRP), mRNA.
22	NM_000142	1.97	hsa-miR-99b	Genome browser	Homo sapiens fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism) (FGFR3), transcript variant 1, mRNA.

23	NM_015113	1.76	hsa-miR-99b	Genome browser	Homo sapiens zinc finger, ZZ type with EF hand domain 1 (ZZEF1), mRNA.
24	NM_021090	1.70	hsa-miR-99b	Genome browser	Homo sapiens myotubularin related protein 3 (MTMR3), transcript variant 3, mRNA.
25	NM_012405	1.69	hsa-miR-99b	Genome browser	Homo sapiens isoprenylcysteine carboxyl methyltransferase (ICMT), transcript variant 1, mRNA.
26	NM_019885	1.39	hsa-miR-99b	Genome browser	Homo sapiens cytochrome P450, family 26, subfamily B, polypeptide 1 (CYP26B1), mRNA.
27	NM_005808	1.37	hsa-miR-99b	Genome browser	Homo sapiens CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like (CTDSPL), mRNA.
28	NM_177979	1.27	hsa-miR-99b	Genome browser	Homo sapiens chordin (CHRD), transcript variant 3, mRNA.
29	NM_206835	1.16	hsa-miR-99b	Genome browser	Homo sapiens TNF receptor-associated factor 7 (TRAF7), transcript variant 2, mRNA.
30	NM_177978	0.95	hsa-miR-99b	Genome browser	Homo sapiens chordin (CHRD), transcript variant 2, mRNA.
31	NM_021116	0.69	hsa-miR-99b	Genome browser	Homo sapiens adenylate cyclase 1 (brain) (ADCY1), mRNA.

Supplemental Table 3 – Sequence of the primers used for cloning the 3'UTR of the mirna target genes.

Gene name	Forward and reverse primers	Size of the 3'UTR cloned
mTOR (NM_020009.1)	For 5'-CCTTGTCTGTGCTTCCAGTG-3' Rev 5'-ACGGGTGAGGTAACAGGATG-3'	382 bp
PAM (NM_207215.2)	For 5'-CAGATCCTTTGTCTATGGAGAGG-3' Rev 5'-TACAGGACAAACATTGATAGCTTTA-3'	530 bp
CALM (NM_007589.4)	For 5'-TGAAGTAACATGTTGCATGTGG-3' Rev 5'-TTGGAAAACAAATATACAACTTGG-3'	420 bp
BAZ2A (NM_054078.2)	For 5'-GCCCTGAACATGCTGCTT-3' Rev 5'-TGCATAATATAAAGTCAATTCAAA-3'	1360 bp
SMARCA5 (NM_053124.2)	For 5'-GCATTTTTGTCTTATAATCACTAACTG-3' Rev 5'-AAAGGCCATTCATCCAACAA-3'	589 bp

Supplemental figure 2 – pGL4.13 vector map



REFERENCES

1. Maeda, Y., V. Dave, and J.A. Whitsett, *Transcriptional control of lung morphogenesis.* Physiol Rev, 2007. **87**(1): p. 219-44.
2. Mendelson, C.R., *Role of transcription factors in fetal lung development and surfactant protein gene expression.* Annu Rev Physiol, 2000. **62**: p. 875-915.
3. Warburton, D., et al., *The molecular basis of lung morphogenesis.* Mech Dev, 2000. **92**(1): p. 55-81.
4. Cardoso, W.V., *Lung morphogenesis revisited: old facts, current ideas.* Dev Dyn, 2000. **219**(2): p. 121-30.
5. Metzger, R.J., et al., *The branching programme of mouse lung development.* Nature, 2008. **453**(7196): p. 745-50.
6. Low, R.B. and S.L. White, *Lung smooth muscle differentiation.* Int J Biochem Cell Biol, 1998. **30**(8): p. 869-83.
7. Stevens, T., et al., *Lung vascular cell heterogeneity: endothelium, smooth muscle, and fibroblasts.* Proc Am Thorac Soc, 2008. **5**(7): p. 783-91.
8. Badri, K.R., Y. Zhou, and L. Schuger, *Embryological origin of airway smooth muscle.* Proc Am Thorac Soc, 2008. **5**(1): p. 4-10.
9. Que, J., et al., *Mesothelium contributes to vascular smooth muscle and mesenchyme during lung development.* Proc Natl Acad Sci U S A, 2008. **105**(43): p. 16626-30.
10. De Langhe, S.P., et al., *Levels of mesenchymal FGFR2 signaling modulate smooth muscle progenitor cell commitment in the lung.* Dev Biol, 2006. **299**(1): p. 52-62.
11. De Langhe, S.P., et al., *Formation and differentiation of multiple mesenchymal lineages during lung development is regulated by beta-catenin signaling.* PLoS One, 2008. **3**(1): p. e1516.
12. Cardoso, W.V. and J. Lu, *Regulation of early lung morphogenesis: questions, facts and controversies.* Development, 2006. **133**(9): p. 1611-24.
13. Cardoso, W.V., *Molecular regulation of lung development.* Annu Rev Physiol, 2001. **63**: p. 471-94.
14. Shi, W., S. Bellusci, and D. Warburton, *Lung development and adult lung diseases.* Chest, 2007. **132**(2): p. 651-6.
15. Lu, Y., et al., *Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells.* Dev Biol, 2007. **310**(2): p. 442-53.
16. Ventura, A., et al., *Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters.* Cell, 2008. **132**(5): p. 875-86.
17. Carraro, G., et al., *miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through*

MAPK14 and STAT3 regulation of E-Cadherin distribution. Dev Biol, 2009. **333**(2): p. 238-50.
18. Kim, V.N., *MicroRNA biogenesis: coordinated cropping and dicing.* Nat Rev Mol Cell Biol, 2005. **6**(5): p. 376-85.
19. Yi, R., et al., *Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs.* Rna, 2005. **11**(2): p. 220-6.
20. Yi, R., et al., *Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs.* Genes Dev, 2003. **17**(24): p. 3011-6.
21. Kim, V.N., J. Han, and M.C. Siomi, *Biogenesis of small RNAs in animals.* Nat Rev Mol Cell Biol, 2009. **10**(2): p. 126-39.
22. Schwarz, D.S., et al., *Asymmetry in the assembly of the RNAi enzyme complex.* Cell, 2003. **115**(2): p. 199-208.
23. Khvorova, A., A. Reynolds, and S.D. Jayasena, *Functional siRNAs and miRNAs exhibit strand bias.* Cell, 2003. **115**(2): p. 209-16.
24. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
25. Brodersen, P. and O. Voinnet, *Revisiting the principles of microRNA target recognition and mode of action.* Nat Rev Mol Cell Biol, 2009. **10**(2): p. 141-8.
26. Politz, J.C., E.M. Hogan, and T. Pederson, *MicroRNAs with a nucleolar location.* Rna, 2009. **15**(9): p. 1705-15.
27. Hwang, H.W., E.A. Wentzel, and J.T. Mendell, *A hexanucleotide element directs microRNA nuclear import.* Science, 2007. **315**(5808): p. 97-100.
28. Reinhart, B.J., et al., *The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans.* Nature, 2000. **403**(6772): p. 901-6.
29. Orom, U.A., F.C. Nielsen, and A.H. Lund, *MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation.* Mol Cell, 2008. **30**(4): p. 460-71.
30. Duursma, A.M., et al., *miR-148 targets human DNMT3b protein coding region.* Rna, 2008. **14**(5): p. 872-7.
31. Vasudevan, S., Y. Tong, and J.A. Steitz, *Switching from repression to activation: microRNAs can up-regulate translation.* Science, 2007. **318**(5858): p. 1931-4.
32. Migliore, C. and S. Giordano, *MiRNAs as new master players.* Cell Cycle, 2009. **8**(14): p. 2185-6.
33. Yanaihara, N., et al., *Unique microRNA molecular profiles in lung cancer diagnosis and prognosis.* Cancer Cell, 2006. **9**(3): p. 189-98.
34. Volinia, S., et al., *A microRNA expression signature of human solid tumors defines cancer gene targets.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2257-61.
35. Croce, C.M., *Causes and consequences of microRNA dysregulation in cancer.* Nat Rev Genet, 2009. **10**(10): p. 704-14.

36. Uziel, T., et al., *The miR-17~92 cluster collaborates with the Sonic Hedgehog pathway in medulloblastoma.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2812-7.
37. Calin, G.A., et al., *Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15524-9.
38. Galardi, S., et al., *miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1.* J Biol Chem, 2007. **282**(32): p. 23716-24.
39. Esquela-Kerscher, A., et al., *The let-7 microRNA reduces tumor growth in mouse models of lung cancer.* Cell Cycle, 2008. **7**(6): p. 759-64.
40. Takamizawa, J., et al., *Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival.* Cancer Res, 2004. **64**(11): p. 3753-6.
41. Kumar, M.S., et al., *Suppression of non-small cell lung tumor development by the let-7 microRNA family.* Proc Natl Acad Sci U S A, 2008. **105**(10): p. 3903-8.
42. Martello, G., et al., *MicroRNA control of Nodal signalling.* Nature, 2007. **449**(7159): p. 183-8.
43. Juan, A.H., et al., *Mir-214-dependent regulation of the polycomb protein Ezh2 in skeletal muscle and embryonic stem cells.* Mol Cell, 2009. **36**(1): p. 61-74.
44. Flynt, A.S., et al., *Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate.* Nat Genet, 2007. **39**(2): p. 259-63.
45. Yang, B., et al., *The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2.* Nat Med, 2007. **13**(4): p. 486-91.
46. Terentyev, D., et al., *miR-1 overexpression enhances Ca(2+) release and promotes cardiac arrhythmogenesis by targeting PP2A regulatory subunit B56alpha and causing CaMKII-dependent hyperphosphorylation of RyR2.* Circ Res, 2009. **104**(4): p. 514-21.
47. Mishima, Y., C. Stahlhut, and A.J. Giraldez, *miR-1-2 gets to the heart of the matter.* Cell, 2007. **129**(2): p. 247-9.
48. Mishima, Y., et al., *Zebrafish miR-1 and miR-133 shape muscle gene expression and regulate sarcomeric actin organization.* Genes Dev, 2009. **23**(5): p. 619-32.
49. Makeyev, E.V., et al., *The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing.* Mol Cell, 2007. **27**(3): p. 435-48.
50. Kefas, B., et al., *The neuronal microRNA miR-326 acts in a feedback loop with notch and has therapeutic potential against brain tumors.* J Neurosci, 2009. **29**(48): p. 15161-8.
51. Raser, J.M. and E.K. O'Shea, *Noise in gene expression: origins, consequences, and control.* Science, 2005. **309**(5743): p. 2010-3.
52. Hornstein, E. and N. Shomron, *Canalization of development by microRNAs.* Nat Genet, 2006. **38 Suppl**: p. S20-4.

53. Peterson, K.J., M.R. Dietrich, and M.A. McPeek, *MicroRNAs and metazoan macroevolution: insights into canalization, complexity, and the Cambrian explosion.* Bioessays, 2009. **31**(7): p. 736-47.
54. del Moral, P.M., et al., *Differential role of FGF9 on epithelium and mesenchyme in mouse embryonic lung.* Dev Biol, 2006. **293**(1): p. 77-89.
55. Martin, K.A., et al., *The mTOR/p70 S6K1 pathway regulates vascular smooth muscle cell differentiation.* Am J Physiol Cell Physiol, 2004. **286**(3): p. C507-17.
56. Li, L., et al., *SM22 alpha, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis.* Circ Res, 1996. **78**(2): p. 188-95.
57. Miettinen, P.J., et al., *TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors.* J Cell Biol, 1994. **127**(6 Pt 2): p. 2021-36.
58. Kasai, H., et al., *TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT).* Respir Res, 2005. **6**: p. 56.
59. Wullschleger, S., R. Loewith, and M.N. Hall, *TOR signaling in growth and metabolism.* Cell, 2006. **124**(3): p. 471-84.
60. Lamouille, S. and R. Derynck, *Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway.* J Cell Biol, 2007. **178**(3): p. 437-51.
61. Maeurer, C., et al., *Sphingosine-1-phosphate induced mTOR-activation is mediated by the E3-ubiquitin ligase PAM.* Cell Signal, 2009. **21**(2): p. 293-300.
62. Iadevaia, V., et al., *All translation elongation factors and the e, f, and h subunits of translation initiation factor 3 are encoded by 5'-terminal oligopyrimidine (TOP) mRNAs.* Rna, 2008. **14**(9): p. 1730-6.
63. Hendrickson, D.G., et al., *Systematic identification of mRNAs recruited to argonaute 2 by specific microRNAs and corresponding changes in transcript abundance.* PLoS One, 2008. **3**(5): p. e2126.