Published in final edited form as:
FEBS Lett. 2015 January 2; 589(1): 172–176. doi:10.1016/j.febslet.2014.11.043.

MicroRNA-206 regulates surfactant secretion by targeting VAMP-2

Honghao Zhang[1], **Yujie Guo**[1,2], **Amarjit Mishra**[1], **Deming Gou**[3], **Narendranath Reddy Chintagari**[1], and **Lin Liu**[1,2,4]

[1]Lundberg-Kienlen Lung Biology and Toxicology Laboratory, Department of Physiological Sciences, Oklahoma State University, Stillwater, OK 73034

[2]Oklahoma Center for Respiratory and Infectious Diseases, Oklahoma State University, Stillwater, OK 73034

[3]Shenzhen Key Laboratory of Microbial Genetic Engineering, College of Life Sciences, Shenzhen University, Shenzhen, Guangdong, 518060, China

Abstract

Lung surfactant secretion is a highly regulated process. Our previous studies have shown that VAMP-2 is essential for surfactant secretion. In the present study we investigated the role of miR-206 in surfactant secretion through VAMP-2. VAMP-2 was confirmed to be a target of miR-206 by 3′-untranslational region (3′-UTR) luciferase assay. Mutations in the predicated miR-206 binding sites reduced the binding of miR-206 to the 3′-UTR of VAMP-2. miR-206 decreased the expression of VAMP-2 protein and decreased the lung surfactant secretion in alveolar type II cells. In conclusion, miR-206 regulates lung surfactant secretion by limiting the availability of VAMP-2 protein.

Introduction

MicroRNAs (miRNAs) are evolutionarily conserved small RNAs, typically 19–23 nucleotides, in eukaryotes. miRNAs are first transcribed as primary miRNAs (pri-miRNA) by RNA polymerase II and further processed by RNase III, Drosha, to a precursor miRNA (pre-miRNA). The pre-miRNA is transported into the cytoplasm by exportin 5, where it forms mature miRNA through the cleavage by Dicer. One of the mature miRNA strands incorporates into the RNA inducing silencing complex. This complex binds 3′-untranslated region (3′-UTR) of a target mRNA to cause protein translation repression or mRNA

[4]Correspondence should be addressed to Lin Liu, Department of Physiological Sciences, Oklahoma State University, 264 McElroy Hall, Stillwater, OK 74078, lin.liu@okstate.edu.

degradation. A miRNA can target multiple proteins and thus regulate various physiological and pathological processes [1].

Lung alveoli are the basic unit for gas exchange. They are lined by squamous alveolar epithelial type I and cuboidal type II cells. Alveolar type II cells synthesize, store and secrete a surface active lipid-rich substance called lung surfactant. The lung surfactant is stored in the lamellar bodies. Following stimulation of type II cells, the lamellar bodies fuse with plasma membrane, releasing their contents into the alveolar lumen. The secreted surfactant reduces the surface tension and prevents the collapse of lung alveoli. Lung surfactant deficiency leads to infant/neonatal respiratory distress syndrome [2].

A number of signaling cascades are important in lung surfactant secretion [2]. Lipid rafts and their constituent proteins also regulate surfactant secretion [3–5]. Our previous studies have demonstrated that the soluble N-ethylmelaimide-sensitive fusion protein attachment protein receptors (SNARE), SNAP-23 and syntaxin 2 [6], VAMP-2 [7], and other SNARE associated proteins including NSF and α-SNAP [8] are involved in surfactant secretion. Furthermore, Annexin A2 mediates the fusion of lamellar bodies with the plasma membrane by directly interacting with SNAP-23 [9]. We have previously shown that miR-375 and miR-150 modulate surfactant secretion by altering cytoskeleton reorganization in type II cells [10] and targeting purinergic ion-channel receptor (P2X7R) [11], respectively. However, the miRNAs targeting SNARE proteins in alveolar type II cells are unknown. In the present study, we examined the effects of miR-206 on VAMP-2 and lung surfactant secretion.

Materials and Methods

Reagents

Fetal bovine serum (FBS), trypsin-EDTA, Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, non-essential amino acids, ligase for cloning, pENTR plasmid, and Lipofactamine 2000 were purchased from Invitrogen Life Technologies (Carlsbad, CA). Enhanced chemiluminescence reagent was from Amersham Pharmacia (Arlington Heights, IL). Polyclonal rabbit anti-VAMP-2 and anti-VAMP-8 antibody were from Synaptic System (Goettingen, Germany). Polyclonal rabbit anti-β-actin, horse serum and protease inhibitor cocktail were from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-rabbit IgG was from BioRad Laboratories (Hercules, CA). Restriction enzymes were from New England Biolab (Ipswich, MA) unless mentioned. Luciferase reporter plasmid, pGL3 (firefly luciferase) and passive lysis buffer were purchased from Promega (Madison, WI). Poly A polymerase and 18S rRNA primers were from Ambion (Austin, TX). The minElute reaction cleanup kit was from Qiagen (Valencia, CA).

Cell culture

HEK 293A cells and A549 cells were cultured at 37°C in DMEM supplemented with 10% FBS and 1% non-essential amino acids. Media were changed on alternate days. Cells were sub-cultured every 3 days. PC12 cells were cultured in DMEM with 10% horse serum and 5% FBS. Media were changed every 3 days, and cells were sub-cultured every week.

Construction of miRNA overexpression plasmids and adenoviral vectors

The miRNA overexpression vectors (pENTR-miRNA) contained the CMV promoter, followed by an enhanced green florescent protein (EGFP) tag, a mature miRNA with flanking sequences (~0.5 kb), and the SV40 polyA terminal sequence. The miRNAs were amplified from human genomic DNA and inserted into the pENTR vector through Xho I and EcoR I sites as previously described [12]. The EGFP expression enabled us to monitor transfection efficiency. The empty vector of CMV-driven EGFP was used as a vector control. The CMV-EGFP-miRNA in pENTR vector was switched into an adenovirus vector by Gateway technique exactly as described by the supplier. Adenoviral vectors were then linearized by PacI before they were used to transfect 293A cells. The virus was amplified by reinfecting HEK 293A cells. Tilter of virus was determined in HEK 293A cells. Mature miR-206 is conserved between rat and human species.

Construction of VAMP-2 reporter vector

The 3′-UTR region of VAMP-2 was amplified from rat cDNA by using specific primers (forward: 5′cacctctagaGAGAATTCGGCCTCTCCCCCACC-3′; and reverse: 5′gatctagaGATCTAGAAATCTGGATGCGCCACA-3′). Then 3′-UTR region (1-1729, numbered after the stop codon of the VAMP-2 gene) was cloned into the pGL3 vector through EcoR I and Xba 1 sites. The reporter construct (pFLuc-3′-UTR-VAMP-2) contained the SV40 promoter, a firefly luciferase reporter gene, the 3′-UTR of VAMP-2 and the SV40 poly A terminal sequence. The binding sites for miR-206 on 3′-UTR of VAMP-2 are conserved among vertebrates.

Dual luciferase assay

Fifty ng of miRNA overexpression plasmid, 5 ng of the reporter construct, pFLuc-3′-UTR-VAMP-2, and 1 ng of pRL-TK *Renilla* luciferase normalization vector were transfected into HEK 293A cells or A549 cells using Lipofectamine. After being cultured for 48 hours, cells were lysed by the addition of 35 μl of passive lysis buffer. Seven microliters of lysate were used for determining dual luciferase activities. The firefly luciferase activity was normalized against *Renilla* luciferase activity.

Western Blotting

Cells were solubilized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 and protease inhibitors). Proteins were separated by 12% of SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with specific antibodies. The primary antibodies were used at the following dilutions: anti-VAMP-2 (1:200), anti-β-actin (1:2000) and anti-VAMP-8 (1:1000). Secondary antibody was used at 1:2000 dilution. Protein bands were visualized by enhanced chemiluminiscence.

Detection of miRNA expression

miRNA expression was determined as described [13]. Briefly, 1 μg of total RNA was polyadenylated using poly A polymerase. The RNA product was purified by mini Elute kit. The RNA was reverse-transcribed into cDNA using the Poly(T) adapter (GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN). PCR was

performed using an universal reverse primer (GCGAGCACAGAATTAATACGACTCAC) and a miRNA-specific forward primer (miR-206: TGGAATGTAAGGAAGTGTGTG; U2: GTTGGAATAGGAGCTTGCTCCGTCC). PCR conditions were: 95°C for 2 min, 95°C, and 35 cycles of 95°C for 30 s, 35°C for 30 s and 72°C for 30 s. The PCR products were separated on 2% agarose gel.

Alveolar type II cells isolation and tissue collection

All of the animal studies were approved by the Institutional Animal Care and Use Committee at the Oklahoma State University. The Sprague-Dawley rats (180–200 g) were anesthetized with ketamine and xylazine via intraperitoneal injection. Lung, heart, muscle and brain were collected for RNA isolation. Alveolar type II cells were isolated from the rats as previously described [7]. The lungs were perfused, lavaged, digested with elastase and minced. The cell suspension was filtered through 160-, 37- and 15-μm nylon meshes. The cells were incubated with rat IgG-coated petri dishes to remove alveolar macrophages. The unattached cells were collected by centrifugation and re-suspended in DMEM. The purity of alveolar type II cells was more than 90% and viability of cells was more than 95%. The isolated type II cells were cultured on an air-liquid interface as described [14]. For lung surfactant secretion, the cells were labeled with 0.6 μCi of [^{3}H] choline overnight. The cells were washed and then stimulated with 100 μM ATP and 0.1 μM PMA for 2 hrs. The lipids in the media and cells were extracted and [^{3}H] radioactivities were counted [15]. Surfactant secretion was calculated as (dpm in medium)/(dpm in medium + dpm in cells) x100%.

Statistical analysis

Student t-test was used for statistical analysis and $P<0.05$ was considered significant.

Results

Identification of the miRNAs that target VAMP-2

Several computational methods have been developed to predict the miRNAs that target the 3′-UTR of a specific mRNA. Online software tools, Targetscan and Pictar were used to identify miRNAs that regulate VAMP-2. The tools identified several miRNA which could potentially regulate VAMP-2 expression. We then constructed miRNA overexpression vectors. The expression of EGFP and miRNA were under the control of a cytomegalovirus (CMV) promoter. For confirming the binding of miRNAs to VAMP-2, dual luciferase assays were employed. The 3′-UTR region of VAMP-2 was fused to downstream of firefly luciferase gene in pGL3 plasmid. Overexpression of miR-206 decreased luciferase activity in HEK 293A cells by 50% (Fig. 1A). We did not observe any significant differences in luciferase activities following the overexpression of miR-28, miR-367, miR-493, miR-409, miR-185 and miR-34b. Similar results were observed in lung epithelial cell line, A549 cells (Fig. 1B).

Localization of binding sites for miR-206

We examined whether the effect of miR-206 on VAMP-2 expression was through the binding of miR-206 to the 3′-UTR of VAMP-2 in HEK 293A cells. We mutated the binding sites in the 3′-UTR with three base mutations in its seed sequence (Fig. 2A). Mutations at

the first binding site of miR-206 still decreased luciferase activity by 50%. However, when the second binding site was mutated, miR-206-mediated repression was attenuated (Fig. 2B). Similar results were obtained when both the binding sites were mutated. Virus control and the unrelated miRNA, miR-34b did not affect the luciferase activities of all mutants. Thus, the second binding site, but not the first binding site, was the essential site for the binding of miR-206 with 3′-UTR region of VAMP-2 mRNA. These results were confirmed in human lung epithelial A549 cells (Fig. 2C).

Differential expression of miR-206 in tissues

The expression of miR-206 was studied in various tissue including lung, heart, muscle and brain. The expression pattern was also examined in two diverse secretory cell types *i.e.*, PC12 and alveolar type II cells. Both PC-12 and alveolar type II cells are secretory cells. PC-12 is a cell line derived from a transplantable rat pheochromocytoma of adrenal medulla and secretes catecholamines. Alveolar type II cells isolated from the lung and secrete lung surfactant. miR-206 was abundantly expressed in the lung, skeletal muscle and alveolar type II cells (Fig. 3). However, the expression of miR-206 was minimal in brain and PC12 cells.

Overexpression of miR-206 leads to repression of VAMP-2 expression

To determine the effect of miR-206 on VAMP-2 expression, PC12 cell line was used. The cell line was chosen because of abundant amount of VAMP-2 protein, with minimal expression of miR-206. PC12 cells were infected with adenovirus overexpressing miR-206. The RT-PCR confirmed the overexpression of mature miRNAs following transduction of cells (Fig. 4A). Overexpression of miR-206 decreased the expression of VAMP-2 protein when compared to controls (Fig. 4B).

Overexpression of miR-206 inhibits surfactant secretion

VAMP-2 is one of SNARE partners involved in exocytosis. Since miR-206 regulated VAMP-2 expression, we investigated the effects of miR-206 on lung surfactant secretion. Overexpression of miR-206 in primary alveolar type II cells did not affect the expression of VAMP-2 mRNA (Fig. 4C). However, the VAMP-2 protein was reduced (Fig. 4D). Virus control did not affect the expression of VAMP-2. The miR-206 did not affect expression of VAMP-8. Furthermore, the overexpression of miR-206 significantly inhibited surfactant secretion (Fig. 4E).

Discussion

Lung surfactant secretion is regulated by many proteins including SNAREs. We have previously shown that miR-375 and miR-150 regulate surfactant secretion by altering cytoskeleton organization and by targeting P2X7 receptors, respectively [10;11]. In the present study we identified miR-206 as another miRNA that regulates surfactant secretion through directly targeting VAMP-2.

Several studies have demonstrated that miRNAs have various roles in exocytosis. miR-375 has been reported to reduce insulin secretion by targeting myotropin [16]. miR-9 inhibits glucose- or potassium-elicited insulin secretion by increasing Granuphilin/Slp4 level

through the reduction of Onecut-2 transcription factor [17]. miR-124 and miR-96 also modulate insulin secretion [18]. miR-142-3p increases FcεRI-mediated degranulation from mast cells [19]. A number of miRNAs directly target SNAREs. For example, syntaxin-1a has been confirmed as a direct target of miR-29a [20]. miR-153 targets SNAP-25 and thus regulates neurotransmission [21].

Our studies revealed that miR-206 was abundantly expressed in the lung, especially in alveolar type II cells. Our results are in conformity with previous reports [22;23]. Our current studies support a role of miR-206 in lung surfactant secretion since the overexpression of miR-206 reduced the lung surfactant secretion. This effect is likely through VAMP-2 because miR-206 down-regulated the expression of VAMP-2 protein in alveolar type II cells. However, miR-206 did not decrease the expression of VAMP-2 mRNA, suggesting that miR-206 regulates VAMP-2 expression post-transcriptionally. Since VAMP-2 is localized on the lung lamellar bodies [7], the miR-206-mediated decrease in VAMP-2 protein could limit the availability of VAMP-2 for the formation of SNARE complex, a prerequisite for membrane fusion, thus suggesting that miR-206 plays a key role in lung surfactant secretion.

Targetscan search did not find any conserved miR-206 binding sites on the 3′-UTR of SNAP-23, syntxain 2, NSF and α-SNAP. However, one poorly conserved binding site of miR-206 was found in the NSF 3-UTR. Thus, it is possible that miR-206 also targets NSF or other proteins involved in lung surfactant secretion.

miR-206 plays various roles in several organs and cells. In the lung, miR-206 is down-regulated in bronchopulmonary dysplasia (BPD) and is associated with the development of BPD [24]. Interestingly, miR-206 is also reduced in the hypoxic lungs and contributes to pulmonary hypertension [25]. miR-206 inhibits hypertrophy and differentiation of myogenic cells by targeting histone deacetylase 4 [26;27] and also regulates muscle regeneration via Hmgb3[28]. miR-206 represses lipogenesis in hepatocytes and enhances cholesterol efflux in human macrophages via controlling liver X receptor α [29;30]. miR-206 slows the progression of amyotrophic lateral sclerosis, a neurodegenerative diseases [31] and contributes to the escalation of voluntary alcohol consumption by inhibiting BDNF [32]. The current finding of miR-206 regulation of lung surfactant secretion adds an expanding list of new functions of miR-206.

Acknowledgments

This work was supported by the National Heart, Lung and Blood Institute under Award Number R01 HL116876, the National Institute of General Medical Sciences under Award number P20GM103648 (Molecular Biology Core), and American Heart Association Southwest affiliate under Award number GRNT7450069.

References

1. Sayed D, Abdellatif M. MicroRNAs in development and disease. Physiol Rev. 2011; 91:827–887. [PubMed: 21742789]
2. Andreeva AV, Kutuzov MA, Voyno-Yasenetskaya TA. Regulation of Surfactant Secretion in Alveolar Type II cells. Am J Physiol Lung Cell Mol Physiol. 2007; 293:L259–L271. [PubMed: 17496061]

3. Chintagari NR, Jin N, Wang P, Narasaraju TA, Chen J, Liu L. Effect of cholesterol depletion on exocytosis of alveolar type II cells. Am J Respir Cell Mol Biol. 2006; 34:677–687. [PubMed: 16439800]
4. Chintagari NR, Gou D, Liu L. Knockdown of flotillin-2 inhibits lung surfactant secretion by alveolar type II cells. Cell Res. 2008; 18:701–703. [PubMed: 18458680]
5. Chintagari NR, Mishra A, Su L, Wang Y, Ayalew S, Hartson SD, Liu L. Vacuolar ATPase Regulates Surfactant Secretion in Rat Alveolar Type II Cells by Modulating Lamellar Body Calcium. PLoS One. 2010; 5:e9228. [PubMed: 20169059]
6. Abonyo BO, Gou D, Wang P, Narasaraju T, Wang Z, Liu L. Syntaxin 2 and SNAP-23 are required for regulated surfactant secretion. Biochemistry. 2004; 43:3499–3506. [PubMed: 15035620]
7. Wang P, Howard MD, Zhang H, Chintagari NR, Bell A, Jin N, Mishra A, Liu L. Characterization of VAMP-2 in the lung: implication in lung surfactant secretion. Cell Biol Int. 2012; 36:785–791. [PubMed: 22571236]
8. Abonyo BO, Wang P, Narasaraju TA, Rowan WH III, McMillan DH, Zimmerman UJ, Liu L. Characterization of alpha-Soluble N-Ethylmaleimide-Sensitive Fusion Attachment Protein in Alveolar Type II Cells: Implications in Lung Surfactant Secretion. Am J Respir Cell Mol Biol. 2003; 29:273–282. [PubMed: 12663329]
9. Wang P, Chintagari NR, Gou D, Su L, Liu L. Physical and Functional Interactions of SNAP-23 with Annexin A2. Am J Respir Cell Mol Biol. 2007; 37:467–476. [PubMed: 17575076]
10. Zhang H, Mishra A, Chintagari NR, Gou D, Liu L. Micro-RNA-375 inhibits lung surfactant secretion by altering cytoskeleton reorganization. IUBMB Life. 2010; 62:78–83. [PubMed: 20014235]
11. Weng T, Mishra A, Guo Y, Wang Y, Su L, Huang C, Zhao C, Xiao X, Liu L. Regulation of lung surfactant secretion by microRNA-150. Biochem Biophys Res Commun. 2012; 422:586–589. [PubMed: 22595456]
12. Bhaskaran M, Wang Y, Zhang H, Weng T, Baviskar P, Guo Y, Gou D, Liu L. MicroRNA-127 modulates fetal lung development. Physiol Genomics. 2009; 37:268–278. [PubMed: 19439715]
13. Shi R, Chiang VL. Facile means for quantifying microRNA expression by real-time PCR. Biotechniques. 2005; 39:519–525. [PubMed: 16235564]
14. Jin N, Narasaraju T, Kolliputi N, Chen J, Liu L. Differential expression of GABA(A) receptor pi subunit in cultured rat alveolar epithelial cells. Cell Tissue Res. 2005; 321:173–183. [PubMed: 15912403]
15. Liu L. Regulation of lung surfactant secretion by phospholipase A_2. J Cell Biochem. 1999; 72:103–110. [PubMed: 10025671]
16. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M. A pancreatic islet-specific microRNA regulates insulin secretion. Nature. 2004; 432:226–230. [PubMed: 15538371]
17. Plaisance V, Abderrahmani A, Perret-Menoud V, Jacquemin P, Lemaigre F, Regazzi R. MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. J Biol Chem. 2006; 281:26932–26942. [PubMed: 16831872]
18. Lovis P, Gattesco S, Regazzi R. Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. Biol Chem. 2008; 389:305–312. [PubMed: 18177263]
19. Yamada Y, Kosaka K, Miyazawa T, Kurata-Miura K, Yoshida T. miR-142-3p enhances FcepsilonRI-mediated degranulation in mast cells. Biochem Biophys Res Commun. 2014; 443:980–986. [PubMed: 24361879]
20. Bagge A, Dahmcke CM, Dalgaard LT. Syntaxin-1a is a direct target of miR-29a in insulin-producing beta-cells. Horm Metab Res. 2013; 45:463–466. [PubMed: 23315993]
21. Wei C, Thatcher EJ, Olena AF, Cha DJ, Perdigoto AL, Marshall AF, Carter BD, Broadie K, Patton JG. miR-153 regulates SNAP-25, synaptic transmission, and neuronal development. PLoS One. 2013; 8:e57080. [PubMed: 23451149]
22. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet. 2006; 38:228–233. [PubMed: 16380711]

23. Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z. The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med. 2007; 13:486–491. [PubMed: 17401374]
24. Zhang X, Xu J, Wang J, Gortner L, Zhang S, Wei X, Song J, Zhang Y, Li Q, Feng Z. Reduction of microRNA-206 contributes to the development of bronchopulmonary dysplasia through up-regulation of fibronectin 1. PLoS One. 2013; 8:e74750. [PubMed: 24040336]
25. Yue J, Guan J, Wang X, Zhang L, Yang Z, Ao Q, Deng Y, Zhu P, Wang G. MicroRNA-206 is involved in hypoxia-induced pulmonary hypertension through targeting of the HIF-1alpha/Fhl-1 pathway. Lab Invest. 2013; 93:748–759. [PubMed: 23628900]
26. Winbanks CE, Beyer C, Hagg A, Qian H, Sepulveda PV, Gregorevic P. miR-206 represses hypertrophy of myogenic cells but not muscle fibers via inhibition of HDAC4. PLoS One. 2013; 8:e73589. [PubMed: 24023888]
27. Winbanks CE, Wang B, Beyer C, Koh P, White L, Kantharidis P, Gregorevic P. TGF-beta regulates miR-206 and miR-29 to control myogenic differentiation through regulation of HDAC4. J Biol Chem. 2011; 286:13805–13814. [PubMed: 21324893]
28. Maciotta S, Meregalli M, Cassinelli L, Parolini D, Farini A, Fraro GD, Gandolfi F, Forcato M, Ferrari S, Gabellini D, Bicciato S, Cossu G, Torrente Y. Hmgb3 is regulated by microRNA-206 during muscle regeneration. PLoS One. 2012; 7:e43464. [PubMed: 22912879]
29. Zhong D, Huang G, Zhang Y, Zeng Y, Xu Z, Zhao Y, He X, He F. MicroRNA-1 and microRNA-206 suppress LXRalpha-induced lipogenesis in hepatocytes. Cell Signal. 2013; 25:1429–1437. [PubMed: 23499676]
30. Vinod M, Chennamsetty I, Colin S, Belloy L, De PF, Schaider H, Graier WF, Frank S, Kratky D, Staels B, Chinetti-Gbaguidi G, Kostner GM. miR-206 controls LXRalpha expression and promotes LXR-mediated cholesterol efflux in macrophages. Biochim Biophys Acta. 2014; 1841:827–835. [PubMed: 24603323]
31. Williams AH, Valdez G, Moresi V, Qi X, McAnally J, Elliott JL, Bassel-Duby R, Sanes JR, Olson EN. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. Science. 2009; 326:1549–1554. [PubMed: 20007902]
32. Tapocik JD, Barbier E, Flanigan M, Solomon M, Pincus A, Pilling A, Sun H, Schank JR, King C, Heilig M. microRNA-206 in rat medial prefrontal cortex regulates BDNF expression and alcohol drinking. J Neurosci. 2014; 34:4581–4588. [PubMed: 24672003]

Highlights

- VAMP-2 was identified as a target of miR-206.
- miR-206 decreased the expression of VAMP-2 protein.
- miR-206 reduced lung surfactant secretion in alveolar type II cells.

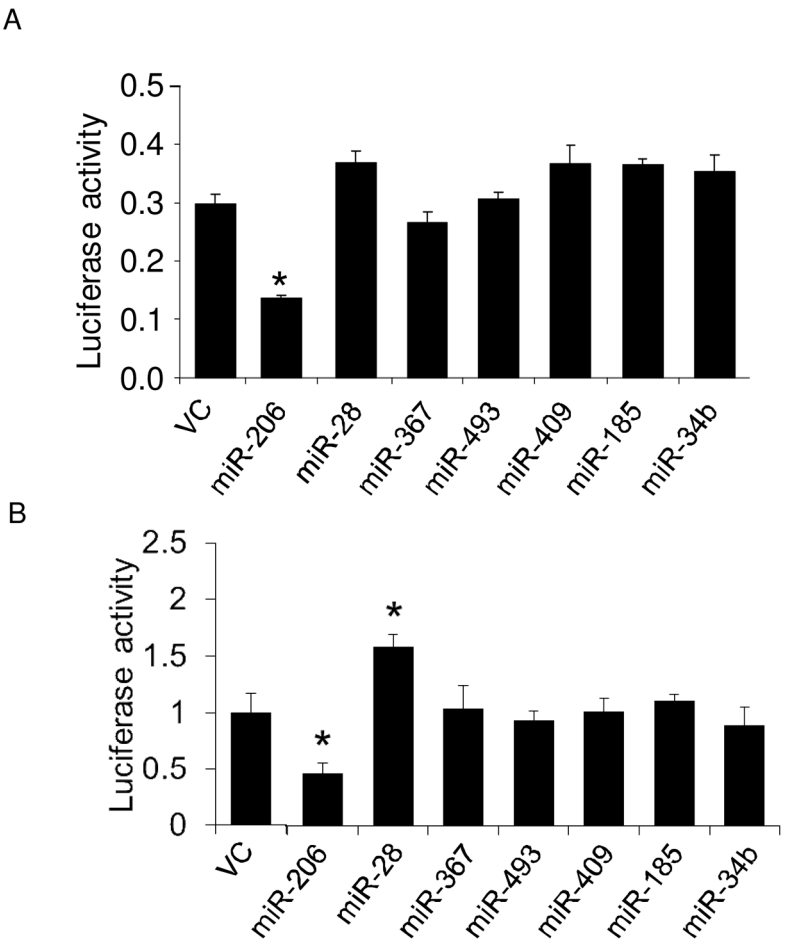

Fig. 1. Screening of miRNAs that regulate 3′-UTR activity of VAMP-2

HEK 293A cells (A) and A549 cells (B) were transfected with pENTR-miRNA (50 ng), pFLuc-3′-UTR- VAMP-2 (5 ng), and normalization plasmid pRL-TK (1 ng) for 48 hrs and the dual luciferase activities assayed. The firefly luciferase activity was normalized with pRL-TK activity. An empty vector control (VC) was used as a control. The results were expressed as % of VC. * $p<0.05$ v.s. VC (n=3).

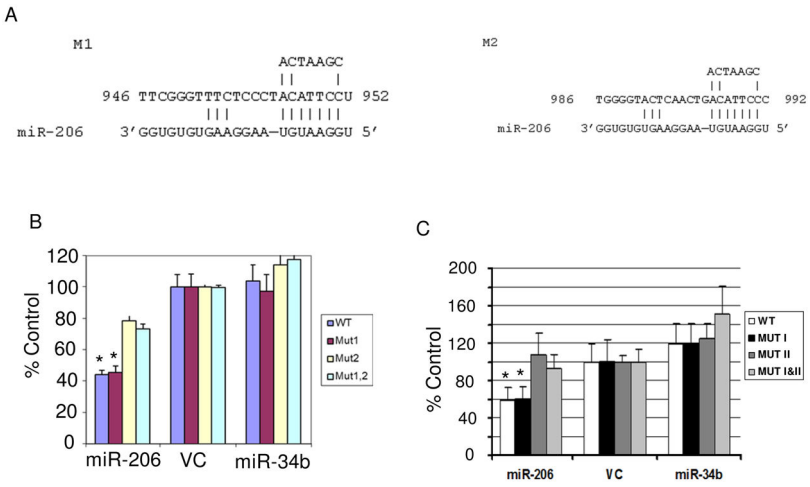

Fig. 2. Analysis of miRNA binding sites on the 3′-UTR of VAMP-2

(A) Binding sites of miR-206 on 3′-UTR region of VAMP-2. (B, C) Luciferase assay was performed as described in Fig. 1 in HEK 293A (B) and A549 cells (C) with wild-type, mutations in the first, second, or both binding sites (Mut1, Mut2, Mut1,2) of 3′-UTR reporter. Data shown were means ± SE (n=3). The results were expressed as % of VC. * $p<0.05$ v.s. VC (n=3).

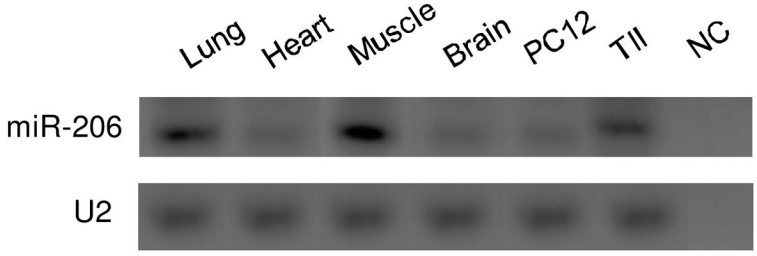

Fig. 3. Expression of miR-206 in tissues and cells

Total RNA was isolated from lung, heart, skeletal muscle, brain, PC12 cells and alveolar type II cells (TII). All of the tissues and cells were from the rats. The RNA was polyadenylated and reverse-transcribed. miRNAs were amplified and separated by agarose gel electrophoresis. Sample without the addition of poly A polymerase was used as negative control (NC).

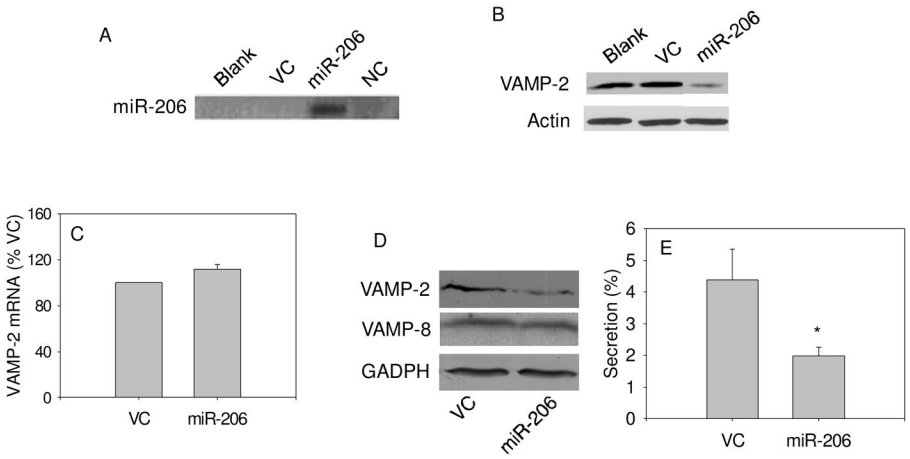

Fig. 4. Effect of miR-206 overexpression on VAMP-2 expression and surfactant secretion

(A, B) PC12 cells were transduced with an adenovirus expressing a miRNA at a MOI of 50 for 5 days. The representative images (A) indicating overexpression of miRNAs in PC12 cells using RT-PCR. Western blot analysis (B) for VAMP-2 in PC12 overexpressing miR-206. Blots were reprobed with β-actin as a loading control. (C–E) Type II cells were cultured on an air-liquid interface. The cells were transduced with miR-206 overexpressing adenovirus (MOI: 100) on day 3. An adenovirus without miR-206 at the same MOI was used as a virus control (VC). The cells were cultured until day 7. The mRNA (C) and protein levels (D) of VAMP-2 were determined by real-time PCR and Western blot, respectively. (E) For measurement of surfactant secretion, cells cultured for 7 days were stimulated with 100 μM ATP and 0.1 μM PMA for 2 hrs. The released of [^{3}H]-labeled PC was measured for surfactant secretion. The basal surfactant secretion was 1.25 ± 0.39. *P<0.05 v.s. VC from 3 cell preparations (Student's t-test).