



MicroRNA 16 modulates epithelial sodium channel in human alveolar epithelial cells

Prasanna Tamarapu Parthasarathy, Lakshmi Galam¹, Bao Huynh¹, Asfiya Yunus¹, Toaa Abuelenen, Annie Castillo, Gurukumar Kollongod Ramanathan, Ruan Cox Jr., Narasaiah Kolliputi^{*}

Division of Allergy and Immunology, Department of Internal Medicine, Morsani College of Medicine, University of South Florida, 12901 Bruce B. Downs Blvd., MDC 19, Tampa, FL 33612, United States

ARTICLE INFO

Article history:

Received 1 August 2012

Available online 23 August 2012

Keywords:

Acute lung injury

Hyperoxia

Pulmonary edema

Ion transport

Ion channels

ABSTRACT

Acute lung injury (ALI) is a devastating disease characterized by pulmonary edema. Removal of edema from the air spaces of lung is a critical function of the epithelial sodium channel (ENaC) in ALI. The molecular mechanisms behind resolution of pulmonary edema are incompletely understood. MicroRNA's (miRNA) are crucial gene regulators and are dysregulated in various diseases including ALI. Recent studies suggest that microRNA-16 (miR-16) targets serotonin transporter (SERT) involved in the serotonin (5-HT) transmitter system. Alterations in serotonin levels have been reported in various pulmonary diseases. However, the role of miR-16 on its target SERT, and ENaC, a key ion channel involved in the resolution of pulmonary edema, have not been studied. In the present study, the expression patterns of miR-16, SERT, ENaC and serotonin were investigated in mice exposed to room air and hyperoxia. The effects of miR-16 overexpression on ENaC, SERT, TGF- β and Nedd4 in human alveolar epithelial cells were analyzed. miR-16 and ENaC were downregulated in mice exposed to hyperoxia. miR-16 downregulation in mouse lung was correlated with an increase in SERT expression and pulmonary edema. Overexpression of miR-16 in human alveolar epithelial cells (A549) suppressed SERT and increased ENaC β levels when compared to control-vector transfected cells. In addition, miR-16 over expression suppressed TGF β release, a critical inhibitor of ENaC. Interestingly Nedd4, a negative regulator of ENaC remained unaltered in miR-16 over expressed A549 cells when compared to controls. Taken together, our data suggests that miR-16 upregulates ENaC, a major sodium channel involved in resolution of pulmonary edema in ALI.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Acute lung injury (ALI) is a devastating disease characterized by flooding of alveolar spaces with a protein-rich fluid that impairs pulmonary gas exchange, leading to arterial hypoxemia and respiratory failure [1]. Hyperoxic acute lung injury is a well-established animal model to study human ALI and acute respiratory distress syndrome (ARDS) [2,3]. Alveolar epithelial injury is a major contributor to alveolar flooding, because the epithelial barrier is much less permeable under normal conditions than the endothelial barrier [4]. Injury to alveolar epithelial cells can also disrupt normal epithelial fluid transport, impairing the removal of edema fluid from the alveolar space [1,5]. Earlier reports have shown that reduced alveolar fluid clearance is a characteristic feature of ALI.

However, these factors for this impair in epithelial fluid transport have not been well studied. The removal of lung edema fluid from the airspace occurs via an active transport-dependent sodium concentration gradient across the distal lung epithelium [6,7].

Epithelial sodium channel (ENaC) is the main force to drive sodium ions for transepithelial reabsorption and for formation of ion gradients to drive fluid out of alveolar spaces. ENaC is composed of α , β , and γ subunits. Several studies have found that ENaC is the major determinant of alveolar fluid clearance across the alveolar epithelium. Earlier studies have demonstrated that serotonin (5-HT) significantly inhibits amiloride-sensitive sodium transport across rat and human lung alveolar epithelial cell monolayers via a receptor-independent inhibition of ENaC activity [8]. As 5-HT also significantly inhibits the amiloride-sensitive fraction of the alveolar fluid clearance in mice, 5-HT can be considered as an endogenous inhibitor of ENaC. Increased levels of 5-HT have been reported in various pulmonary diseases, and inappropriate function of the 5-HT transmitter system further aggravates the disease condition. Therefore, any molecule that modulates the 5-HT transmitter system and upregulates ENaC show considerable therapeutic promise.

Abbreviations: ALI, acute lung injury; miR-16, microRNA-16; ENaC, epithelial sodium channel; SERT, serotonin transporter; TGF β , transforming growth factor β ; 5-HT, serotonin.

^{*} Corresponding author. Fax: +1 813 974 8575.

E-mail address: nkollipu@health.usf.edu (N. Kolliputi).

¹ These authors contributed equally to this work.

Recently, microRNAs (miRNAs) emerged as a major class of gene expression regulators implicated in deregulation of ion channel genes leading to channelopathies [9]. The consequence of miRNAs in fine-tuning gene expression shows that changes in the abundance of a single miRNA can affect the levels of expression of several different functional proteins [10,11]. Thus, it is possible that a single dysregulated miRNA can push cells into an injured or inflammatory state. Recently, microRNA-16 (miR-16) has been reported to selectively target the serotonin transporter (SERT) involved in the 5-HT transmitter system [12]. However, miRNAs have never been studied in regulation of amiloride sensitive channels in ALI and role of miR-16 on its targets has not yet been elucidated. In this study, we investigated the role of miR-16 on ENaC expression and its target SERT in human lung alveolar epithelial cells. The results indicate that miR-16 modulates ENaC protein and SERT expression in alveolar epithelial cells. In addition, miR-16 suppressed the expression of transforming growth factor β (TGF β), an inhibitor of ENaC and a critical mediator of ALI [13]. miR-16 mediated upregulation of ENaC is independent of Nedd4 (E3 ubiquitin protein ligase) pathway. Targeting SERT by miR-16 can be considered as a novel therapeutic approach to modulate ENaC expression and restore alveolar fluid balance in acute lung injury.

2. Materials and methods

2.1. Cell culture and transfection

Human alveolar epithelial cells (A549) derived from human lung adenocarcinoma were obtained from American Type Culture Collection (ATCC, Manassas, VA), and were maintained in complete Dulbecco's modified Eagle medium (Invitrogen, Grand Island, NY) as per vendor instructions. Constructs expressing miR-16 and control vector were purchased from Add gene (Cambridge MA) and plasmid isolation was performed using miniprep plasmid isolation kits (Qiagen, Valencia, CA). Briefly, 2×10^5 cells were suspended in complete DMEM and seeded in a 6 well plate. At 70% confluency, cells were transfected with miR-16 plasmid or control-vector using Lipofectamine (Invitrogen). Complete DMEM was added to the A549 cells, 4 h post-transfection. Serotonin, at final concentration of 1 or 2 mM, was added to the respective wells.

2.2. Reagents and antibodies

Antibodies directed to ENaC α , β , γ and SERT were obtained from Alpha Diagnostics Int. (San Antonio, TX). Antibodies against β -actin and GAPDH were obtained from Cell signaling technology Inc., (Boston, MA). Serotonin hydrochloride and HRP-conjugated secondary antibodies were obtained from Sigma (St. Louis, MO).

2.3. Mice

The Animal Care and Use Committee at the University of South Florida approved all animal procedures. C57BL/6J mice were purchased from Harlan Laboratories (Indianapolis, IN). In all experiments, mice aged 7–9 weeks, were exposed to 100% oxygen as described earlier [14].

2.4. Quantitative RT-PCR

Total mRNA was isolated using the Trizol method [15]. Equal amounts of RNA were reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and cDNA was amplified using qPCR performed on a Biorad CFX96 real-time PCR detection system as described earlier [16]. All primers were purchased from Integrated DNA Technologies (Coralville, IA) and were

used at final concentrations of 100 nM. All experiments were performed in triplicate. Primers used were: mouse SERT (forward): 5'TCGCCCAGGACAACATCACCTGGAC3', (reverse): 5'TATGTGATG AAAAGGAGGCTGG3'; mouse18S(forward): 5'ACCTGGTTGATCCTGC CAGTAG3', (reverse): 5'TTAATGAGCCATTCCGAGTTTC-3'; human-SERT(forward): 5' CAGCGTGTGAAGATGGAGAAG3', (reverse): 5'TGGGATAGAGTCCCGTGTGT3'; human GAPDH (forward):5'CGGAGTCAACGGATTGGTTCGTAT3', (reverse): 5' AGCCTTCTCCATGGTGGTGAAGAC3'.

2.5. Isolation of mouse RNA and quantification of miR-16

Total RNA enriched with small RNAs was extracted from mouse lung homogenates, using mirVana™ miRNA isolation kit (Ambion, Foster City, CA). miR-16 levels were measured using the TaqMan microRNA reverse transcription kit (Applied Biosystems, Carlsbad, CA).

2.6. Western blot

Cells were lysed with RIPA buffer and protein concentrations were determined with a Bicinchoninic Acid Assay kit (Pierce, Rockford, IL). Protein lysates were prepared for SDS–PAGE by adding 1/4 volume of $4 \times$ SDS–PAGE sample buffer (100 mM Tris–Cl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and the mixture was heated at 95 °C for 5 min. Thirty micrograms of the protein was separated via SDS–PAGE and transferred onto a PVDF membrane by electrophoresis (Immobilon P; Millipore, Bedford, MA). After blocking with TBS Tween buffer (10 Mm Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat dry milk for 1 h, the membranes were incubated overnight at 4 °C with blocking solution containing the indicated antibody (diluted 1:1,000–1:2,000). Membranes were washed and incubated with a suitable HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) as mentioned previously [15]. HRP activity was detected using an ECL kit according to the manufacturer's instructions (Pierce, Rockford, IL). Densitometric analysis of the immunoblots are added in the supplemental section.

2.7. Quantification of serotonin and TGF β by ELISA

Serotonin levels in mouse lung homogenates were analyzed using ELISA as per the manufacturer's instructions (GenWay Biotech Inc., San Diego, CA). TGF β concentrations in cell supernatants were measured using the human TGF β ELISA kit (R&D Systems, Minneapolis, MN).

2.8. Statistical analysis

All experiments were performed in triplicate. A paired t-test was used to determine the statistical significance. A p -value of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Elevated lung serotonin and increased SERT expression were observed in hyperoxia exposed mice

It was shown that serotonin decreases alveolar epithelial fluid transport via a direct inhibition of epithelial sodium channel [8]. Many studies suggest that defects in alveolar epithelial fluid transport can lead to pulmonary edema [5]. High plasma serotonin levels are implicated in pulmonary disorders such as primary pulmonary hypertension and pulmonary fibrosis [17]. However,

serotonin levels have never been reported in hyperoxia-induced acute lung injury. To test the hypothesis that high serotonin levels may contribute to hyperoxia-induced ALI, we analyzed lung serotonin levels in mice under room air (normoxic) and hyperoxic (100% O₂) conditions. We found that exposure to hyperoxia causes a significant increase in lung serotonin levels when compared to room air controls (Fig. 1A). The increase in serotonin levels after hyperoxia exposure was highly significant ($p < 0.001$) when compared with room air controls. To assess the functional damage that occurs in ALI in terms of lung edema, we monitored the wet to dry ratio of lung tissue in mice exposed to hyperoxia and room air. The edematous lung damage caused by hyperoxia was highly significant ($p < 0.001$) compared with room air controls (Fig. 1B). These results clearly show that hyperoxia-induced lung edema is associated with elevated lung serotonin levels.

Polymorphisms in SERT expression and function can lead to various lung diseases like pulmonary arterial hypertension and chronic obstructive lung disease. As per our knowledge, role of SERT in ALI has not yet been elucidated. To determine whether

SERT expression is dysregulated in ALI, we measured SERT mRNA and protein expression levels in mice exposed to hyperoxia and room air (Fig. 1C and D, S2). SERT mRNA expression and protein levels were induced in mouse lungs exposed to hyperoxia when compared to normoxic controls.

3.2. miR-16 levels were downregulated in mice exposed to hyperoxia

Genetic factors that regulate SERT in acute lung injury are unknown. Recent studies suggest that SERT is a known direct target for miR-16 [12]. However, the role of miR-16 in hyperoxic ALI has not been studied. To test the hypothesis that miR-16 may contribute to dysregulated SERT expression in hyperoxia-induced ALI, we analyzed lung miR-16 levels after hyperoxia exposure. We found that exposure to hyperoxia caused a significant decrease in lung miR-16 levels (Fig. 2A) showing that increased SERT levels are associated with suppressed lung miR-16 levels in hyperoxia induced ALI.

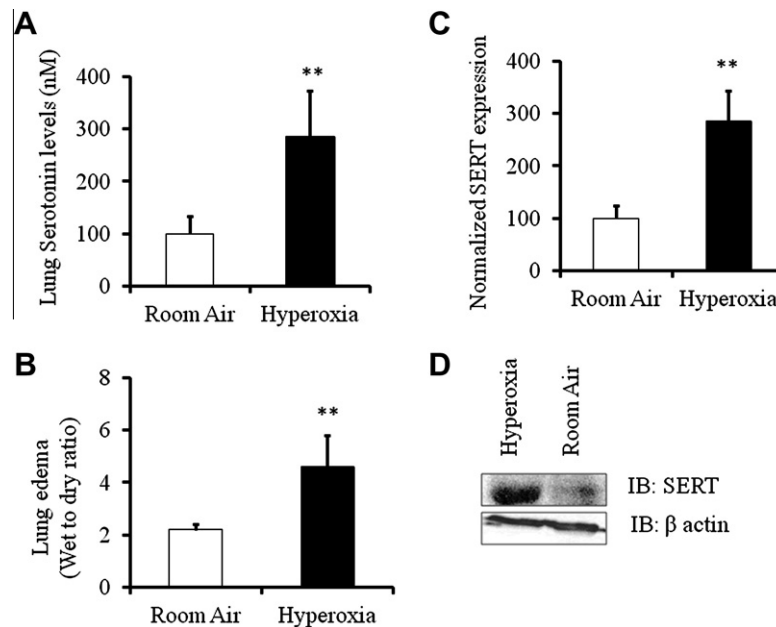


Fig. 1. Elevated serotonin and SERT expression in mice exposed to hyperoxia (100% O₂). (A) Lung serotonin levels in mice exposed to room air and hyperoxic conditions. Lung homogenates were obtained from mice and subjected to capture ELISA to assess serotonin concentrations. (B) Lung edema was measured using wet to dry ratio of mouse lungs exposed to hyperoxia and room air. (C) Quantitative RT-PCR analysis to measure SERT mRNA expression in lung homogenates of mice exposed to hyperoxia and their room air controls. SERT mRNA was measured using reverse transcription and qPCR with SERT primers. 18S rRNA was used as internal control (D) Relative SERT protein expression in mice lung homogenates was measured using Western blot analysis. ** $P < 0.001$ compared with room air controls.

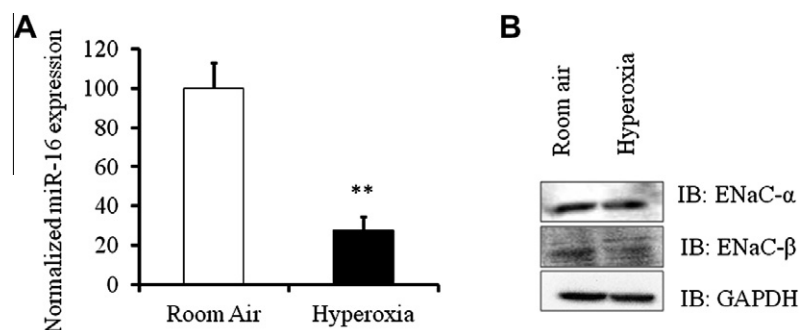


Fig. 2. Expression levels of miR-16 and ENaC in hyperoxia induced ALI mouse models. (A) miR-16 levels were analyzed by qRT-PCR in lung homogenates of mice exposed to hyperoxia and room air. Total RNA from the lung was isolated, reverse transcribed and analyzed for miR-16 using miR-16 specific assay primers by SYBR green method. miR-16 expression was normalized to 18S. (B) ENaC- α and ENaC- β protein levels in mouse lung homogenates exposed to normoxia and hyperoxia were measured by western blot analysis. Lung homogenates ($n = 4$) were subjected to immunoblotting with the indicated antibodies. ** $P < 0.001$ compared with room air controls.

3.3. Effect of hyperoxia on lung epithelial sodium channel (ENaC) expression levels

Elevated serotonin decreases alveolar epithelial fluid transport via a direct inhibition of the epithelial sodium channel [8]. Active vectorial Na⁺ transport through the distal lung epithelium is essential to maintain a physiological lung fluid balance [18]. Vectorial fluid and ion transport are altered in ALI. Clinical data and experimental studies provided a potential role of ENaC expression in fluid clearance and active ion transport [19]. We therefore analyzed the levels of ENaC expression in hyperoxia induced ALI mice. ENaC protein expression in mouse lung homogenates was analyzed by western blot (Fig. 2B, S3). When mice were exposed to hyperoxic conditions, there was a significant decrease in lung ENaCβ subunit compared to normoxic controls. ENaCα subunit expression was slightly decreased in hyperoxia induced ALI mice when compared to normoxic controls. Our results suggest that hyperoxia alters ENaCα expression and decreases ENaCβ expression.

3.4. Over expression of miR-16 down regulates SERT in the presence of serotonin in A549 cells

We next evaluated if overexpression of miR-16 can down regulate SERT in the presence of serotonin. To test this hypothesis, A549 cells were cultured in complete DMEM for 24 h and were treated with different concentrations of serotonin. Cells treated with 1 or 2 mM serotonin, showed significant increase in SERT mRNA levels by 10–40-fold (Fig. 3A). Surprisingly, cells treated with 4 mM serotonin did not show an increase in SERT mRNA, which may be due to a feedback inhibition of these transporters at higher serotonin concentrations. We next transfected A549 cells with miR-16 or control-vector and then treated cells with 1 or 2 mM serotonin. SERT mRNA and protein expression were analyzed by qRT-PCR and western blot analysis respectively. Down regulation of SERT mRNA and protein was observed in cells containing miR-16 treated

with 1 or 2 mM serotonin (Fig. 3B and C, S4). These results demonstrate that miR-16 targets SERT and downregulates its expression in A549 cells post-serotonin treatment.

3.5. Overexpression of miR-16 induces epithelial sodium channel-β expression in alveolar epithelial cells

A majority of patients with increased permeability and edema in ALI have impaired alveolar epithelial fluid transport associated with dysregulation of epithelial sodium channel expression [5,6]. It has been reported that higher serotonin levels can decrease alveolar epithelial fluid transport by inhibition of the epithelial sodium channel [8]. Since, our data showed miR-16 mediated modulation of SERT in epithelial cells, we hypothesized that miR-16 may regulate ENaC expression in epithelial cells. To test this hypothesis, A549 cells were transfected with miR-16 or control plasmid, and 24 h post-transfection and post-serotonin treatment, the cells were assayed for ENaC expression. The effect of miR-16 on different ENaC subunits in A549 cells was examined by western blot analysis (Fig. 4A, S5, S6). Transfection of miR-16 into A549 cells resulted in a significant increase of ENaC-β subunit when compared to control. However, ENaC-γ expression was unaltered in miR-16 transfected, post-serotonin treated A549 cells when compared to controls. ENaC-α expression was decreased in miR-16 transfected cells, which may be due to a significant increase in ENaC-β subunits. These two subunits complement each other to form a functional epithelial sodium channel. These results demonstrate that miR-16 has the ability to induce ENaC expression in human alveolar epithelial cells.

3.6. miR-16 alters TGFβ levels in human alveolar epithelial cells in vitro

TGFβ has been shown to be a critical mediator of ALI [1]. TGFβ is known to decrease ENaC expression and alveolar sodium transport

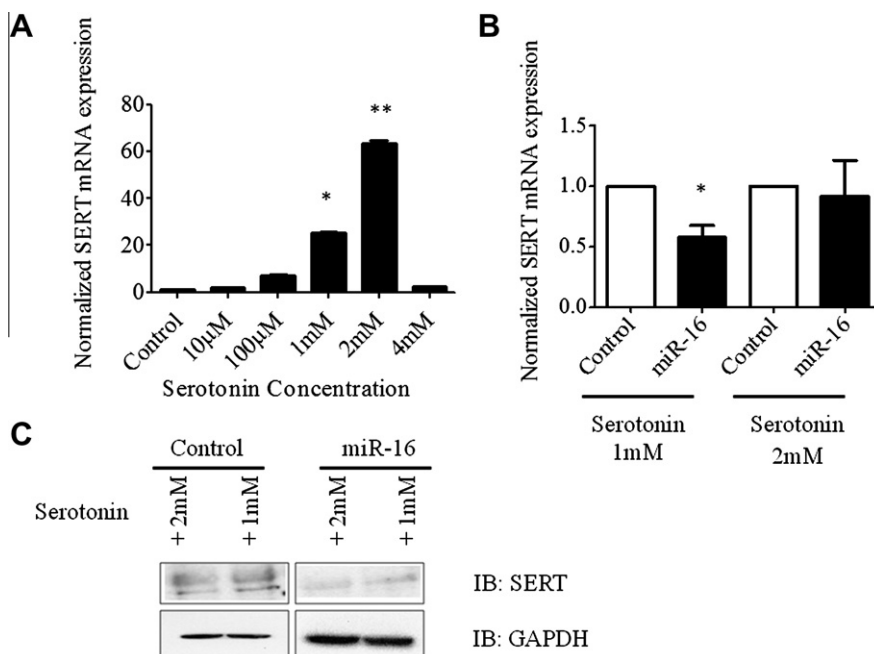


Fig. 3. Over expression of miR-16 downregulated SERT expression in A549 cells in vitro. (A) Serotonin transporter expression varied dose dependently with the addition of different concentrations of serotonin. A549 cells were treated with increasing concentrations of serotonin and were subjected to qRT-PCR to assess SERT mRNA expression levels. SERT mRNA expression was normalized to GAPDH. (B) qRT-PCR analysis of SERT in miR-16 overexpressed A549 cells post-treated with serotonin. A549 cells were transfected with miR-16; 4 h post-transfection, cells were treated with either 1 or 2 mM serotonin, and 24 h later, RNA was isolated and subjected to qRT-PCR analysis. All experiments were repeated at least 3 independent times. (C) SERT protein expression was analyzed in miR-16 overexpressed A549 cells post-treated with serotonin. A549 cells were transfected with miR-16 or control plasmid and post-treated with serotonin for 24 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. **P* < 0.01 and ***P* < 0.001 compared with control.

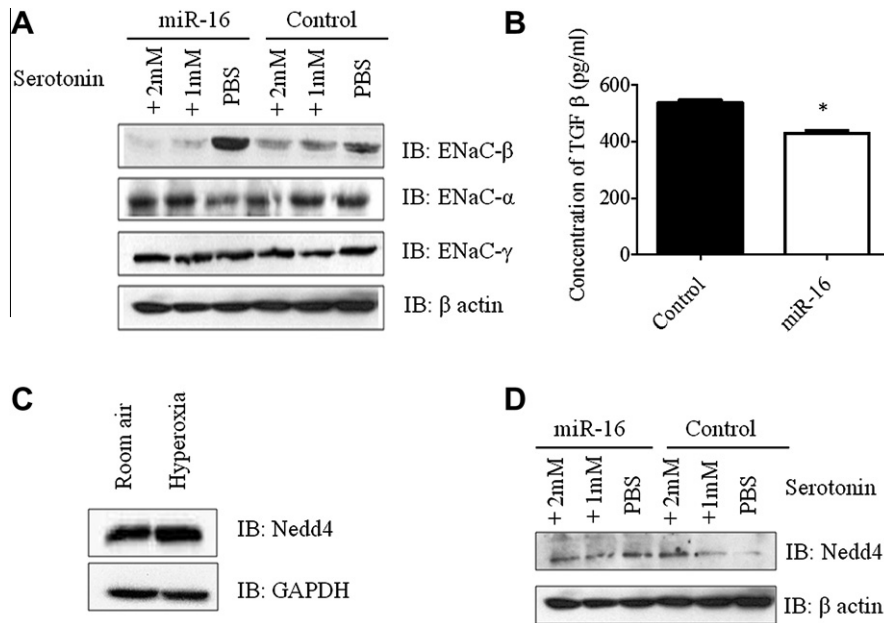


Fig. 4. miR-16 overexpression in A549 cells modulates ENaC and TGF-β expression via Nedd4 independent pathway. (A) Immunoblot analysis of different subunits of ENaC in miR-16 and control plasmid transfected A549 cells. A549 cells transfected with miR-16 or control plasmid were post-treated with or without serotonin and protein lysates were analyzed for ENaC-α, -β and -γ by immunoblot. (B) TGF-β concentration in cell supernatants was analyzed by capture ELISA. Human alveolar epithelial cells were transfected with miR-16 or control plasmid and 24 h post-transfection, the supernatants were subjected to ELISA. (C) Mice were exposed to hyperoxia and room air, and lung homogenates were analyzed for Nedd4 levels by immunoblot. (D) Nedd4 immunoblot in A549 cells transfected with miR-16 or control plasmid in the presence or absence of serotonin treatment post-transfection. **P* < 0.01 compared with control.

in epithelial cells [13]. Since our results showed that miR-16 modulates ENaC expression, we next evaluated the effect of miR-16 on TGFβ levels in A549 cells. To check whether miR-16 can modulate TGFβ expression, TGFβ levels were analyzed in culture supernatants from A549 cells transfected with miR-16 or control plasmid. There was a significant reduction of TGFβ levels in miR-16 transfected A549 cells compared to controls (Fig. 4B). Increased ENaC-β expression as observed earlier in miR-16 transfected human alveolar epithelial cells (Fig. 4A) may thus be associated with a significant decrease in TGFβ levels.

3.7. miR-16 mediated ENaC upregulation is independent of Nedd4

Target scan revealed several targets for miR-16. One among them is Nedd4 binding partner 1 (N4BP1) [20]. It has been recently reported that N4BP1 acts as an expressed interactor and monoubiquitinylation substrate of Nedd4. Nedd4 has been implicated in the regulation of fluid and electrolyte homeostasis by controlling the surface abundance of ENaC subunits [21]. We checked the levels of Nedd4 in lungs of mice exposed to hyperoxia and normoxia. Nedd4 was upregulated in mice exposed to hyperoxia compared to their room air controls (Fig. 4C, S6). This showed an association with decreased ENaC subunits in mice exposed to hyperoxia. To further determine if miR-16-mediated ENaC expression is through Nedd4 modulation, we monitored the expression levels of Nedd4 in miR-16 overexpressed A549 cells and controls post-treated with 1 or 2 mM serotonin. Levels of Nedd4 were slightly reduced in miR-16 transfected A549 cells post-treated with 2 mM serotonin when compared to controls (Fig. 4D, S6). Our data suggest that miR-16 mediated upregulation of ENaC is independent of Nedd4.

4. Discussion

In this study we have shown that miR-16 suppression in ALI is correlated with increased serotonin levels. Serotonin (5-HT)

significantly inhibits amiloride-sensitive sodium transport across rat and human lung alveolar epithelial cell monolayers via a receptor-independent inhibition of epithelial sodium channel (ENaC) expression [8]. It is known that SERT is a key player for 5-HT mediated effects in alveolar epithelial transport. Therefore, inhibitors of SERT offer considerable therapeutic promise. miR-16 is known to target SERT and overexpression of miR-16 in A549 cells downregulated its target SERT and upregulated ENaCβ in the presence of serotonin. For the first time, a biological explanation for an increase in lung SERT levels, during ALI by means of downregulation of miR-16 (S1) and effects of overexpression of miR-16 on ENaC is provided in this study.

Previous studies have shown that TGF-β a critical mediator of ALI, significantly decreased ENaC-α mRNA and protein expression [13]. The inhibitory effect of TGF-β1 on sodium uptake and ENaC-α expression in rat alveolar type II (ATII) cells was mediated by activation of the MAPK, ERK1/2 pathway. Serotonin is also known to induce TGFβ [22] and increased TGFβ levels are known to suppress ENaC expression. To determine whether miR-16 mediated effect of ENaC upregulation is due to alteration of TGF-β, we checked the levels of TGF-β in miR-16 overexpressed alveolar epithelial cells. Our study shows that increased ENaC expression by miR-16 is associated with a significant decrease in TGF-β levels. Therefore miR-16 mediated ENaC expression is further dependent on TGF-β downregulation.

The importance of Nedd4-2 in regulating ENaC expression and distal lung fluid absorption near term and at birth was also recently demonstrated by artificially removing Nedd4-2 using specific siRNA [23]. In that report, it was demonstrated that reducing Nedd4 expression led to an increased lung fluid absorption rate, and thus it was suggested that Nedd4 plays an important role in regulating ENaC expression and lung fluid absorption at birth. However, factors that modulate Nedd4 expression in ALI are not clear. Since miR-16 is a known target for N4BP1, a Nedd4 binding partner, we checked the levels of Nedd4 in miR-16 overexpressed A549 cells. Our results show that overexpression of miR-

16 did not significantly suppress Nedd4 in epithelial cells. However, a slight decrease in Nedd4 levels was observed in miR-16 overexpressed cells post-treated with 2 mM serotonin. Therefore, miR-16 modulation of ENaC, which we observed in our study, may be independent of the Nedd4-2 pathway.

In conclusion, we have provided evidence that overexpressed miR-16 is an endogenous inhibitor of SERT and we also provided the functional effects of miR-16 on ENaC upregulation, which is a key protein involved in alveolar fluid clearance in ALI. Based on our results, we propose that exogenous miR-16 provides a therapeutic strategy to induce ENaC expression by SERT suppression and may have use as a therapeutic to treat ALI and other diseases involving pulmonary edema.

Acknowledgments

This work was funded by the American Heart Association National Scientist Development Grant 09SDG2260957 and National Institutes of Health R01 HL105932 to NK and the Joy McCann Culverhouse Endowment to the Division of Allergy and Immunology. Authors thank Dr. Brenda Flam for critical reading and editing this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2012.08.063>.

References

- [1] J.F. Pittet, M.J. Griffiths, T. Geiser, N. Kaminski, S.L. Dalton, X. Huang, L.A. Brown, P.J. Gotwals, V.E. Kotliansky, M.A. Matthay, D. Sheppard, TGF- β is a critical mediator of acute lung injury, *J. Clin. Invest.* 107 (2001) 1537–1544.
- [2] V. Bhandari, R. Choo-Wing, C.G. Lee, Z. Zhu, J.H. Nedrelow, G.L. Chupp, X. Zhang, M.A. Matthay, L.B. Ware, R.J. Homer, P.J. Lee, A. Geick, A.R. de Fougerolles, J.A. Elias, Hyperoxia causes angiotensin 2-mediated acute lung injury and necrotic cell death, *Nat. Med.* 12 (2006) 1286–1293.
- [3] N. Kolliputi, A.B. Waxman, IL-6 cytoprotection in hyperoxic acute lung injury occurs via PI3K/Akt-mediated bax phosphorylation, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 297 (2009) L6–L16.
- [4] A.B. Waxman, N. Kolliputi, IL-6 protects against hyperoxia-induced mitochondrial damage via bcl-2-induced bax interactions with mitofusins, *Am. J. Respir. Cell Mol. Biol.* 41 (2009) 385–396.
- [5] Y. Berthiaume, M.A. Matthay, Alveolar edema fluid clearance and acute lung injury, *Respir. Physiol. Neurobiol.* 159 (2007) 350–359.
- [6] M.A. Matthay, H.G. Folkesson, C. Clerici, Lung epithelial fluid transport and the resolution of pulmonary edema, *Physiol. Rev.* 82 (2002) 569–600.
- [7] T. Geiser, K. Atabai, P.H. Jarreau, L.B. Ware, J. Pugin, M.A. Matthay, Pulmonary edema fluid from patients with acute lung injury augments in vitro alveolar epithelial repair by an IL-1 β -dependent mechanism, *Am. J. Respir. Crit. Care Med.* 163 (2001) 1384–1388.
- [8] A. Goolaerts, J. Roux, M.T. Ganter, V. Shlyonsky, A. Chraïbi, R. Stephane, F. Mies, M.A. Matthay, R. Naeije, S. Sariban-Sohraby, M. Howard, J.F. Pittet, Serotonin decreases alveolar epithelial fluid transport via a direct inhibition of the epithelial sodium channel, *Am. J. Respir. Cell Mol. Biol.* 43 (2010) 99–108.
- [9] E. Elvira-Matlot, X. Jeunemaitre, J. Hadchouel, Regulation of ion transport by microRNAs, *Curr. Opin. Nephrol. Hypertens.* 20 (2011) 541–546.
- [10] M. Malumbres, MiRNAs versus oncogenes: the power of social networking, *Mol. Syst. Biol.* 8 (2012) 569.
- [11] M. Abdellatif, Differential expression of microRNAs in different disease states, *Circ. Res.* 110 (2012) 638–650.
- [12] A. Baudry, S. Mouillet-Richard, B. Schneider, J.M. Launay, O. Kellermann, MiR-16 targets the serotonin transporter: a new facet for adaptive responses to antidepressants, *Science* 329 (2010) 1537–1541.
- [13] J. Frank, J. Roux, H. Kawakatsu, G. Su, A. Dagenais, Y. Berthiaume, M. Howard, C.M. Canessa, X. Fang, D. Sheppard, M.A. Matthay, J.F. Pittet, Transforming growth factor- β 1 decreases expression of the epithelial sodium channel α ENaC and alveolar epithelial vectorial sodium and fluid transport via an ERK1/2-dependent mechanism, *J. Biol. Chem.* 278 (2003) 43939–43950.
- [14] N. Kolliputi, L. Galam, P. Tamarapu Parthasarathy, S.M. Tipparaju, R.F. Lockey, NALP-3 inflammasome silencing attenuates ceramide-induced transepithelial permeability, *J. Cell. Physiol.* 227 (2012) 3310–3316.
- [15] N. Kolliputi, R.S. Shaik, A.B. Waxman, The inflammasome mediates hyperoxia-induced alveolar cell permeability, *J. Immunol.* 184 (2010) 5819–5826.
- [16] S.A. Aljbran, R. Cox Jr, P. Tamarapu Parthasarathy, G. Kollongod Ramanathan, V. Rajanbabu, H. Bao, S.M. Mohapatra, R. Lockey, N. Kolliputi, Enhancer of zeste homolog 2 induces pulmonary artery smooth muscle cell proliferation, *PLoS One* 7 (2012) e37712.
- [17] P. Egermayer, G.I. Town, A.J. Peacock, Role of serotonin in the pathogenesis of acute and chronic pulmonary hypertension, *Thorax* 54 (1999) 161–168.
- [18] D.C. Eaton, J. Chen, S. Ramosevac, S. Matalon, L. Jain, Regulation of Na^+ channels in lung alveolar type II epithelial cells, *Proc. Am. Thorac. Soc.* 1 (2004) 10–16.
- [19] D.C. Eaton, M.N. Helms, M. Koval, H.F. Bao, L. Jain, The contribution of epithelial sodium channels to alveolar function in health and disease, *Annu. Rev. Physiol.* 71 (2009) 403–423.
- [20] A. Oberst, M. Malatesta, R.I. Aqeilan, M. Rossi, P. Salomoni, R. Murillas, P. Sharma, M.R. Kuehn, M. Oren, C.M. Croce, F. Bernassola, G. Melino, The Nedd4-binding partner 1 (N4BP1) protein is an inhibitor of the E3 ligase itch, *Proc. Natl. Acad. Sci. USA* 104 (2007) 11280–11285.
- [21] A. Dinudom, K.F. Harvey, P. Komwatana, J.A. Young, S. Kumar, D.I. Cook, Nedd4 mediates control of an epithelial Na^+ channel in salivary duct cells by cytosolic Na^+ , *Proc. Natl. Acad. Sci. USA* 95 (1998) 7169–7173.
- [22] J.S. Grewal, Y.V. Mukhin, M.N. Garmovskaya, J.R. Raymond, E.L. Greene, Serotonin 5-HT $_2$ A receptor induces TGF- β 1 expression in mesangial cells via ERK: proliferative and fibrotic signals, *Am. J. Physiol.* 276 (1999) F922–F930.
- [23] T. Li, S. Koshy, H.G. Folkesson, IL-1 β -induced cortisol stimulates lung fluid absorption in fetal guinea pigs via SGK-mediated Nedd4-2 inhibition, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 296 (2009) L527–L533.