Role of miR206 in Genistein-Induced Rescue of Pulmonary Hypertension in Monocrotaline Model

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Abstract

Pulmonary hypertension (PH) is a progressive lung disease associated with proliferation of smooth muscle cells and constriction of lung microvasculature leading to increased pulmonary arterial pressure, right ventricular failure and death. We have previously shown that genistein rescues preexisting established PH by significantly improving lung and heart function. Here, we have examined the role of microRNAs (miRs) in the rescue action of genistein in monocrotaline (MCT)-induced PH in rats. Our microRNA-microarray analysis on the lung samples from control, PH and Genistein-rescue group revealed that miR206 which was robustly upregulated to approximately 11 fold by PH was completely normalized to control levels by Genistein treatment. Next, we examined whether knockdown of miR206 could reverse preexisting established PH. PH was induced in male rats by 60 mg/kg of MCT and rats received three intratracheal doses of either miR206 antagomir (10 mg/kg body weight) or scrambled miR control at days 17, 21 and 26. Knockdown of miR206 resulted in significant improvement in the cardiopulmonary function as right ventricular pressure was significantly reduced to 38.6±3.61 mmHg from 61.2±5.4 mmHg in PH and right ventricular hypertrophy index was decreased to 0.35±0.04 from 0.59±0.037 in PH. Knockdown of miR206 reversed PH-induced pulmonary vascular remodeling in vivo and was associated with restoration of PH-induced loss of capillaries in the lungs and induction of vascular endothelial growth factor A (VEGFA) expression. In conclusion, miR206 antagomir therapy improves cardiopulmonary function and structure and rescues preexisting severe PH in MCT-rat model possibly by stimulating angiogenesis in the lung.

Keywords: Pulmonary hypertension, right heart failure, Genistein, microRNA, angiogenesis.
Introduction

Pulmonary arterial hypertension (PAH) is a cardiopulmonary disease associated with severe pulmonary vascular remodeling, progressive increase in pulmonary artery (PA) pressure leading to right ventricular (RV) hypertrophy, and eventually right heart failure and death (14; 38). Many therapies are designed to target the vascular changes and inflammation associated with the etiology of PAH (22; 24). We have previously shown that Genistein, a natural soybean-derived phytoestrogen, with much higher affinity for estrogen receptor beta (ERβ) than -alpha is very effective in rescuing pre-existing advanced pulmonary hypertension (PH) in rats induced by MCT (20).

MiRs are single-stranded non-coding RNA molecules that can regulate gene expression through binding to the 3’ untranslated regions of their target genes, and thereby degrading mRNA transcripts or blocking protein translation (3; 4; 19). There is growing evidence for the involvement of many miRs in the progression of PH. For example, downregulation of microRNA-204 (miR204) in both human and rodent models has been associated with the pro-proliferative and antiapoptotic phenotypes of pulmonary smooth muscle cells (10). MiR145 has been reported to be dysregulated in mouse models of PH and patient samples and its downregulation is protective against the development of PAH (7). MiR21 has been shown to play an important role in the pathogenesis of chronic hypoxia-induced pulmonary vascular remodeling (23). In the search for potential miR candidates that can be regulated by Genistein in its rescue action of PH in the MCT-rat model, we found that miR206 which is expressed at very low levels in healthy lungs, was robustly upregulated by ~11 fold in MCT-induced PH in rats and Genistein treatment was associated with restoration of miR206 expression to control levels. Many studies have shown that disordered angiogenesis may contribute to the progression of
Regeneration of pulmonary vasculature and overexpression of proangiogenesis factors have been shown to ameliorate the effects of PH and may play potential therapeutic role in this disease(6; 44; 45). To explore further whether normalization of miR206 levels in the lungs of MCT-rats could play a protective role, we performed the knockdown of miR206 in the lungs of PH animals and found that miR206 downregulation is sufficient to rescue advanced pre-existing PH. Downregulation of miR206 was associated with an increase of vascular endothelial growth factor (VEGFA) and angiogenesis. Thus it is likely that the rescue action by knockdown of miR206 was accompanied with an increase in angiogenesis through induction of VEGFA.

Methods

Animals and treatments

Male Sprague-Dawley rats (250-300g) received a single SC injection of MCT (60 mg/kg; Sigma) at day 0 to induce PH or saline as controls. MCT was dissolved in 1N HCl, the pH was adjusted to 7.4, and diluted with PBS before injection. Genistein was dissolved in a mixture of DMSO (Part number: 20684, Thermo scientific) and polyethylene glycol (PEG) (Part number: B21992, Alfa Aesar). MiR206 antagomir and scramble oligonucleotides were dissolved in ultrapure water. MCT-rats were assigned to receive either Genistein (1 mg/kg per day, SC) or vehicle (100µl of a mixture containing 1.25% DMSO and 98.75% PEG) from day 21 to day 30. For miR206 knockdown study, MCT-rats were assigned to receive either miR206 inhibitor oligonucleotide (10 mg/kg body weight in 50 µl PBS (n=5-6), mature miR206 sequence, 3'-UGGAAUGUAAGGAAGUGUGUGG -5') or scramble miR oligonucleotide (n=5-6) on days 17, 21 and 26. MiR206 antagomir or scramble miR oligonucleotides were delivered through the oropharyngeal cavity into the trachea by intratracheal instillation. Knockdown of miR206 in the
lungs was confirmed by QRT-PCR. Protocols received institutional review and committee approval. The investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

**Echocardiographic assessments and cardiopulmonary hemodynamic measurements**

A VisualSonics Vevo 2100 echocardiogram device with a 30-MHz linear transducer was used to perform the pulmonary pulsed wave Doppler echocardiography of pulmonary artery (PA) flow. The probe was placed in a parasternal long axis position to visualize the PA outflow tract. Pulsed Flow Doppler imaging was then overlaid to observe the dynamics of blood flow through the PA valve. Pulmonary artery acceleration time (PAAT) was determined by calculating time taken from the start of flow to maximal velocity using echocardiogram software (Vevo 2100 version: 1.5.0). The right ventricular systolic pressure was measured by direct right heart catheterization at day 30. Rats were anesthetized by Ketamine (80 mg/kg) and Xylazine (5 mg/kg) intraperitoneally and were placed on a controlled warming pad to keep the body temperature constant at 37°C. After tracheotomy was performed, a cannula (18G, Biovalve) was inserted, and the animals were mechanically ventilated. After a midsternal thoracotomy, rats were placed under stereomicroscope (Zeiss, Hamburg, Germany) and a pressure-conductance catheter (model 1.4F Millar SPR-671) was introduced via the apex into the RV and positioned towards the pulmonary valve. The catheter was connected to a signal processor (AD Instruments) and RV pressures were recorded digitally with a recording speed of 1k/sec. The maximum rate of rise of RV pressure (dP/dt\text{max}), the maximum isovolumetric rate of relaxation (-dP/dt\text{min}) and the heart rates were directly calculated from the recordings.
Gross histological evaluation

The RV wall, the left ventricular wall and the interventricular septum were dissected and the ratio of the right ventricle to left ventricle plus septum weight [RV/(LV+IVS)] was calculated as an index of RV hypertrophy.

Gene expression and microRNA assessment

Total RNA was purified from whole lungs using the Trizol method. Two micrograms of total RNA was reverse transcribed to cDNA using the Omniscript RT kit (Qiagen) according to the manufacturer’s instructions (Qiagen). Quantitative real-time reverse transcriptase–polymerase chain reaction (QRT-PCR) was performed using iQ SYBR Green Supermix (Part number: 1708884, Bio-Rad). The vascular endothelial growth factor A (VEGFA) gene expression was assessed using gene specific primers. GAPDH was used as a reference control for normalization. Levels of gene expression in each sample were determined with the comparative Ct method using Bio-Rad CFX manager 2.1 software. The primer sequences were 1) VEGFA: F-5’ACACGGTGGTGGAAGAAGAG-3’; R:5’CAAGTCTCTGGGGACAGAA3’;
2)GAPDH: F:5’CCCTGCACCACAACTCGTTAG-3’; R:5’-ATGACCTTGCCCACAGCTTGA-3’. For gene expression, cycling parameters were: 2 minutes at 50°C, 20 seconds at 95°C, 40 cycles: 1 second at 95°C and 20 seconds at 60°C. For miR studies, total RNA was isolated using the mirVana™ miRNA Isolation Kit (Life technologies: Part number: AM1560) according to the manufacturer’s instructions. A microarray screen of miRNA expression in the total lung tissue of rats was performed with the use of non-Affymetrix single-channel arrays (MirBASE 17.0 MicroRNA Array, Ocean Ridge Biosciences). The miRNA microarray (miRBase17 version) contained 8817 spots, which include: mammalian miRNAs, spiking control and negative controls. Each probe was represented as three spots on the microarray. Microarray data analysis
was performed by Ocean Ridge Biosciences. Briefly, the data was filtered to select probes where the average signal for at least one treatment group was above the average detection threshold (the threshold was calculated from the formula: Threshold=$\log_2(5\times\text{STD EV(Background)}+10\%\text{Trim Mean(Negative Controls)})$. The saturated probes were then removed from the filtered list.

For miR quantification by QRT-PCR, 10 nanograms of total RNA was used to prepare cDNA using TaqMan® MicroRNA Reverse Transcription Kit (Part number: 4366596, Life technologies) and TAQMAN specific probe for miR206 (Part number: 4427975; Assay ID: 000510, Life technologies). U6 small nucleolar RNA (Part number: 4427975; Assay ID: 0019730, Life technologies) was used as a reference control for normalization. QRT-PCR was performed using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Part number: 4324018, Life technologies). The QRT-PCR cycling parameters were: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles: 15 seconds at 95°C and 1 minute at 60°C). Data was analyzed using Bio-Rad CFX manager 2.1 software.

**Immunohistochemistry, imaging and quantification**

Whole lungs were isolated and inflated manually using a syringe by perfusing 4% paraformaldehyde (PFA) in 0.1 M Na2HPO4 and 23 mM NaH2PO4 (pH 7.4) through trachea. Isolated perfused lungs were fixed in 4% PFA for 4 hours on ice. The tissue was then immersed in ice-cold 20% sucrose overnight to cryoprotect, mounted using OCT, and transversal 4-μm sections were obtained with a cryostat (Microm HM525, Thermo Scientific). Standard Hematoxylin/Eosin (Sigma) stainings were performed according to manufacturer’s protocol and images were acquired with a light microscope (Nikon). For immunohistochemistry, tissue section slides were fixed by submerging tissue in 100% acetone for 5 minutes and then air-dried. Tissue sections were washed three times for 10 minutes each in PBS and Triton X-100 (0.2%).
To block background, tissue was incubated with a blocking solution containing 10% normal goat serum in PBS/0.2% Triton X-100 for 60 minutes at room temperature. Tissue was then incubated overnight at 4°C with primary antibody in PBS/0.2% Triton X-100/1% normal goat serum. The primary antibodies were against either alpha smooth muscle actin (Sigma A2547) for the assessment of arteriole wall thickening or Von Willebrand factor (Abcam ab6994) for the assessment of vessel density. The tissue was then washed three times for 10 minutes each in PBS/0.2% Triton X-100 and incubated with DAPI for 15 min at room temperature followed by secondary antibody in PBS/0.2% Triton X-100/1% normal goat serum for 1h at room temperature. The tissue was washed three times for 10 minutes each in PBS/0.2% Triton X-100 and mounted on microscope slides with ProLong antifade reagent (Molecular Probes, Eugene, OR). The images were acquired using a laser-scanning confocal microscope (Nikon eclipse Ti inverted confocal microscope) and processed using Nikon NIS Elements advanced research analysis version 4.20.01)

Pulmonary arteriolar medial wall thickness was quantified using ImageJ software by measuring the maximum thickness of arteriolar walls. About 50-100 arterioles per group were analyzed (n=5-6 rats/group, 5-10 arterioles/slides and 2 slides per animal). The number of micro-vessels per high power filed (60X magnification) was quantified by measuring vWF positive micro-vessels with a lumen less than 20 micrometers as we published previously(37). A total of 50 high power magnification images from 5-6 animals per group were used for quantification. Sections were randomly selected and the observers were blinded.

**Statistical Analysis**

Means were compared across groups using one-way analysis of variance (ANOVA) for three or more groups or t tests for two groups for data that followed the normal distribution and met
variance homogeneity. Significance was assessed using the Tukey criterion for pairwise mean comparisons under the ANOVA model. Normality was assessed using the Kolmogorov-Smirnov test. Computations were carried out using SPSS SigmaStat for Windows, version 3.0 (SPSS Inc, Chicago Ill). $P$ values $< 0.05$ were considered significant. The microRNA-microarray statistical analysis was performed by Ocean Ridge Biosciences. The detectable probes were analyzed using NIA Array Analysis software for ANOVA. Significant probes were selected based on criteria of $P<0.05$ and False Detection Rate $<0.1$ from a one-way ANOVA test.

Results

Genistein-induced rescue of PH is associated with normalization of miR206 in the lungs of MCT treated rats

To identify the potential miRs involved in the rescue action of Genistein, microRNA microarray (non affymetrix) was performed in the lungs of controls, PH and Genistein rescue group at day 30 in MCT model. The expression of 366 miRs was successfully detected. Multiple miRs were differentially regulated between the control and the PH group. Among these miRs, miR206 was upregulated the most by 11 fold in the PH compared to the CTRL lungs. The known regulated miR, miR21 was increased $\sim$3.8 fold in PH vs. control lungs. Additionally, miR322 was downregulated by $\sim$2.6 fold; miR503 by $\sim$3.6 fold in PH vs. control lungs whereas miR124 remained unchanged (Fig. 1). Genistein treatment was associated with normalization of PH-induced changes of miR206, miR21, miR322 and miR503 (Fig. 1A). However, Genistein was not able to prevent the induction of miR451 in PH (Fig. 1A). MiR124 whose expression was not modified in PH, was however induced by Genistein treatment (Fig. 1A). These data suggest that Genistein treatment is associated with selective changes in specific miR expressions previously
implicated in PH. In this study our main focus was on miR206 because of its robust induction in the lung tissue compared to other miRs regulated by Genistein in MCT-induced PH. The inhibition of miR206 by Genistein treatment was further confirmed by QRT-PCR (Fig. 1B). In addition, miR206 is predominantly a skeletal muscle specific miR and is expressed at very low levels in the lungs of healthy rats compared to miR21, miR322 and miR451 (Fig. 1C). Therefore, the aberrant expression of miR206 in the lungs of MCT-induced PH could aggravate lung injury.

Knockdown of miR206 rescues MCT-induced PH in rats by improving cardiac and pulmonary structure and function

To test whether restoration of miR206 level in MCT treated rats can reverse symptoms of PH, synthetic miR206 antagomir RNA molecules were selectively delivered to the lung by intratracheal instillation at days 17, 21 and 26 in the MCT model (Fig. 2A). Synthetic miR206 antagomir delivery resulted in a significant knockdown of miR206 in the lungs of miR206-KD group compared to scrambled miR control (0.07±0.02 relative expression units in miR206-KD group vs. 0.7±0.2 in SCR-CTRL, p<0.05, Fig. 2B). MiR206 antagomir delivery effectively rescued pre-existing PH as the RV systolic pressures were significantly lower than the PH group (38.6±3.61 mmHg in miR206-KD group vs. 61.2±5.4 in PH, p<0.01) (Fig. 2C). Also, knockdown of miR206 decreased RV hypertrophy index in MCT rats (0.35±0.04 in miR206-KD vs. 0.59±0.037 in PH, p<0.001, Fig. 2D). The scrambled miR control (SCR-CTRL) had no effect in RV systolic pressures and RV hypertrophy index as these values were not significantly different than the PH group (RVSP: 62.7±7.0 mmHg in SCR-CTRL group vs. 61.2±5.4 in PH, p<0.32; RV hypertrophy index: 0.58±0.03 mmHg in SCR-CTRL group vs. 0.59±0.04 in PH, p<0.98), suggesting the specificity of miR206 antagomir oligonucleotides.
Knockdown of miR206 also prevented the increased arteriolar muscularization in the lungs of the PH group in the MCT model (4.3±0.4 µm in miR206 KD group vs. 7.2±0.5 µm in PH, p<0.001). No significant difference was observed in arteriolar muscularization between scramble miR control and PH groups: 7.01±0.46 in SCR-CTRL vs. 7.2±0.5 in PH, p<0.1) (Fig.3A,B). Additionally, miR206 knockdown considerably improved the pulmonary artery flow as evident in the Doppler echocardiography images and pulmonary artery acceleration time (PAAT) parameter (29.6±0.5 ms in miR206 KD group vs. 18.3±0.6 ms in PH, p<0.001) (Fig.3C,D). Scrambled miR control had no effect on PAAT as this parameter was not significantly different than the PH group (19.1±0.6 ms in SCR-CTRL group vs. 18.3±0.6 ms in PH, p<0.73). The heart rates between all four groups of animals were similar (Fig. 3E). MiR206 knockdown also significantly reduced the maximum rate of rise of RV pressure (dP/dt max) (1358.8±153 ms in miR206 KD group vs. 2322.6±339.2 ms in PH, p<0.05), and the maximum isovolumetric rate of relaxation (-dP/dt min) (-1036.2±148.3 in miR206 KD group vs. -1887.3±117 in PH, p<0.001) compared to PH (Fig. 3F,G). No significant difference was observed in (dP/dt max) and (-dP/dt min) between scramble miR control and PH groups (dP/dt max): 2480±193.6 in SCR-CTRL group vs. 2322.6±339.2 in PH, p<0.716; (-dP/dt min): -2022±170 in SCR-CTRL group vs. -1887.2±117 in PH, p<0.898).

Knockdown of miR206 is associated with induction of VEGFA and stimulation of angiogenesis in the lungs

Stimulation of angiogenesis in the lungs by overexpression of angiogenic factor VEGFA has been shown to attenuate the development and progression of pulmonary arterial hypertension (6). Since VEGFA is a well studied target of miR206 we examined whether knockdown of miR206
can stimulate neoangiogenesis in the lungs (32). Fig. 4A shows Hematoxylin-Eosin as well as immnuohistochemistry of thin lung sections (4 μm) with anti-von Willebrand antibody and DAPI. Vessel density of micro-vessels up to 20 μm in the lungs was drastically reduced in PH group and miR206-KD significantly increased the number of micro-vessels in MCT treated rats (Fig. 4A). Quantification of blood vessels shows ~3 fold reduction in the micro-vessels in PH (5.6±0.4 vessels per high-power field in PH vs. 16.7±0.8 in CTRL, p<0.001). MiR206-KD led to a significant ~3-fold induction of micro-vessels compared to PH group (17.6±0.8 vessels per high-power field in miR206-KD vs. 5.6±0.4 vessels in PH, p<0.001) (Fig. 4B). Scrambled miR control had no effect on micro-vessel density, as this parameter was not significantly different than PH group (4.9±0.4 vessels per high-power field in SCR-CTRL group vs. 5.6±0.4 vessels in PH, p>0.05, Fig. 4B). Correspondingly, VEGFA transcripts were downregulated in the lungs of PH animals by ~3 fold compared to the controls (1.3±0.4 in PH vs. 5.2±0.7 in CTRL, p<0.05). MiR206-KD was associated with increase in the expression of VEGFA in the PH animals by ~3-fold above the controls and ~6 fold above PH, thus reinforcing a potential anti-angiogenic role of miR206 (8.06±1.5 in miR206-KD vs. 1.3±0.4 in PH, p<0.001) (Fig. 4C).

Discussion

Previously we have shown that Genistein therapy after the establishment of PH rescues preexisting advanced PH by restoring cardiopulmonary function and structure in rats(20). In this report, we have explored the role of downstream effector miRNA candidates regulated by Genistein. Among the shortlisted miRs we found that miR206 is the most robustly upregulated miR in the lungs of PH rats induced by MCT (~11 fold). Rescue of PH by Genistein treatment was associated with normalization of miR206 in the lungs of PH similar to levels in control
group. More importantly, we found that knockdown of miR206 in the lungs of PH rats was sufficient to rescue pre-existing PH. Reduction of pulmonary arteriolar thickness and pulmonary arterial pressures (Fig 2, 3A-D) by miR206 KD, resulted in reduced RV afterload as the RV was no longer required to generate high contractile forces and thus both dP/dt(max) and (min) were reduced to levels similar in CTRL (Fig 3F,G). In addition, miR206 KD was associated with induction of VEGFA expression and increased angiogenesis in the lungs (Fig. 4).

Role of miR206 in muscle development and function

MiRs are non-coding RNA molecules that belong to a class of highly conserved regulatory molecules. They participate in the regulation of various physiological and pathological processes including apoptosis, growth, differentiation and development as well as many cancers, cardiomyopathies and other diseases. MiR206 belongs to the miR1 family, which includes miR1/206 and miR133a/b based on their sequence conservation. Unlike most miRs that are ubiquitous, this family of miRs is expressed in tissue specific manner. These miRs are muscle-specific and very well characterized in terms of their function and influence on many muscle-related myopathies. Despite the similarity in their sequence and expression, these miRs have very distinct targets and promoter differences for transcriptional activation. As a result, these miRs differ in their biological roles. MiR206 is primarily expressed in the skeletal muscle(21) and is regulated by many transcription factors(29; 30). In skeletal muscle, miR206 promotes myogenic differentiation by targeting several genes(28). MiR206 was also shown to inhibit proliferation and induce differentiation by repressing an enzyme involved in DNA synthesis(17). These studies highlight the role of miR206 in myogenesis.
Pathological role of miR206

Recently, the expression and role of miR206 in other tissues and diseases has also been identified. Many studies have highlighted the tumor suppressor role of miR206. Expression levels of miR206 are negligibly low in human rhabdomyosarcoma, a soft tissue sarcoma arising from skeletal muscle progenitors. Reexpression of miR206 in rhabdomyosarcoma cell lines significantly decreased proliferation, promoted apoptosis and activated myogenic program. These studies suggest that repression of miR206 could participate in rhabdomyosarcoma development due to aberrant cell proliferation and migration (39; 43). In agreement, loss of miR206 has been shown to correlate with increased cell proliferation, migration, tumorogenesis and apoptosis (34; 41). MiR206 expression is markedly decreased in ERα-positive human breast cancer tissues and its overexpression in breast cancer cells results in growth inhibition (18). MiR206 targets Notch3 signaling and activate apoptosis in HeLa cells (31). Role of miR206 is also highlighted in the progression of amyotrophic lateral sclerosis and in neuromuscular synaptic regeneration in mice (40). It is evident from these works that although miR206 is highly tissue specific, it plays a regulatory role in other tissues as well via multiple targets, and its differential expression is associated with many diseases.

Controversial role of miR206 in PH

MiR206 is a skeletal muscle specific miR, and as observed from our microarray data, the control lungs have a very low copy number of miR206. However, after monocrotaline insult the expression of miR206 is increased by ~11 fold in the lung tissue. Interestingly, this aberrant induction of miR206 is strikingly suppressed by Genistein treatment. Even though multiple miRs including miR206, miR21, miR322, miR503 were dysregulated in PH and genistein normalized
their expression, knockdown of miR206 alone is sufficient to rescue pre-existing PH. This could potentially be due to drastic regulation of miR206 in PH compared to other miRs.

In contrast to our observation, two recent studies have shown downregulation of miR206 in the hypoxia model of PH (16; 42). In the study by Jalali et al, it was shown that pulmonary smooth muscle cells isolated from hypoxic PH mice have decreased levels of miR206 compared to control mice. Gain of miR206 inhibited proliferation, migration, contraction and increased apoptosis in these cells possibly by downregulation of Notch3 signaling (16). Although the other study by Yue et al shows that miR206 is downregulated in the lungs of hypoxic rat model of PH, contrary to their expectation gain of miR206 resulted in a significant increase in PASMC proliferation in cultured PASMCs and the expression of its bonafide target HIF-1α remained unchanged (42). These two studies in hypoxia indicate that the exact role and function of miR206 is quite complex and is possibly influenced by multiple factors including transcriptional regulation, effect of other miRs and different microenvironment. In addition, nature of insult (monocrotaline vs. hypoxia), interaction of different cell types in lungs and temporal regulation of miR206 in the pathogenesis of the disease could contribute to differences observed in PH from different models. Therefore, it is not surprising that miR206 expression is highly induced in the lungs of MCT model as shown in our study whereas an opposite trend is observed in the hypoxia model as shown in the studies by Yue et al and Jalali et al (16; 42). A recent study comparing the expression patterns of 350 miRs in MCT-rat and hypoxia-rat models of PH demonstrates time- and insult-dependent differences in the expression of miRs within the same species. Interestingly, only a few miRs including let7f, miR22, and miR30c (downregulated), miR322 and miR451 (upregulated) were consistently regulated across both MCT and hypoxia models. Notably, miR21 and let7a were significantly downregulated only in MCT-treated rats.
These differences in the miR modulation are reflective of the distinct directions of pathogenesis in these two models that arise in part from the nature of insult.

Our study is an attempt to understand the complex role of miR206 in PH. However, it is limited in its scope as it employs only MCT-rat model of PH. Similar to most of the animal models of PH studied till date, MCT-rat model also has limitations in mimicking human PAH. Nonetheless many aspects of this model are pertinent to human diseases especially in the context of inflammation (13) and it is an extensively used model in PH studies. Our findings highlight the role of miR206 in MCT model of PH, however, the role of miR206 in human PAH remains to be explored.

**Role of angiogenesis and VEGFA in PH**

Pulmonary hypertension is associated with complex vascular remodeling including pulmonary vasoconstriction, excessive proliferation of endothelia cells and dysregulated angiogenic signaling. Angiogenesis factors including vascular endothelial growth factor (VEGF) and its receptor VEGF receptor 2 (VEGFR) are important factors for the maintenance, function and survival of endothelial cells (12; 15). More importantly, VEGF-driven angiogenesis has been shown to be critical in the normal lung alveolar development with both gain and loss of function studies (35). The development of rodent models in which PH is induced by multiple insults through combination of VEGFA receptor inhibition by specific antagonist (SU5416) and chronic hypoxic exposure support the view that VEGF/VEGFR-2 signaling contribute to pulmonary vascular remodeling in the pathogenesis of PH(9; 33). Cell based gene transfer of VEGFA has been shown to minimize the development and progression of pulmonary arterial hypertension by preventing the loss of existing vessels or by inducing the development of new blood vessels within the lung(6). Several studies have highlighted the fact that vascular remodeling in PAH
patients is not only restricted to lungs and surrounding vasculature but also affects the peripheral tissues (2; 26). This systemic angiogenic impairment of vasculature was shown to be associated with dysregulation of miR126 in the skeletal muscle in PAH through VEGF/ERK pathway, further confirming the role of VEGFA signaling in PH (27). MiR206 has been shown to directly target VEGFA in muscle and therefore negatively regulates angiogenesis(32). Lung angiogenesis in MCT model of PH has been shown to be drastically attenuated(1; 25; 37). In agreement with previous studies, Dutly et al using fluorescent microangiography, a highly quantitative tool to study lung microvascular changes in animal models of PH, demonstrated a marked decrease in the number of vessels in MCT-treated animals (11). In addition to miR206, other miRs have also been shown to regulate pulmonary vascular remodeling in PH. For example, miR-130/301 family has been shown to regulate vasomotor tone in PH by controlling the expression of the master protein peroxisome proliferator-activated receptor γ (PPAR-γ). PPAR-γ in turn facilitates cross talk between pulmonary vascular endothelial and smooth muscle cells via regulating many downstream vasoactive factors including endothelin-1(5). Therefore, multiple miRs can regulate pulmonary vascular signaling in PH via different vasoactive factors.

In this study, we observe that miR206-KD in the lungs of PH-rats induced by MCT is associated with a significant induction and restoration of VEGFA expression to the levels observed in controls. Also, high levels of miR206 were associated with a decrease in angiogenesis. However, it is possible that our quantification of micro-vessels may have been affected if the lung volumes were different after the lung injury. Such an injury could affect lung volume and thus the number and thickness of vessels. A bigger lung would have thinner walls and fewer proximal vessels. Although, we cannot distinguish between bronchial or pulmonary micro-vessels with von Willebrand factor staining, we do observe a significant global increase in angiogenesis in the
lung with miR206 knockdown. It is quite possible that among many factors, angiogenesis could be a contributing factor for overall improvement in cardiopulmonary function with miR206 knockdown. MiR206 knockdown in MCT treated animals resulted in the rescue of pre-existing PH. This was evident from various functional parameters including reduction of thickening of lung arterioles and increase in angiogenesis. The pressure changes in the right ventricle were also substantially normalized to control levels in these animals suggesting an overall improvement in the RV function. It is possible but not exclusive that many of the improved functional parameters were contributed by an increase in angiogenesis.

**Possible regulatory mechanisms of miR206 by Genistein**

Our previous studies have shown that both estrogen and Genistein rescue PH and they most likely act through ER-beta receptor pathway(20). In this report, we have explored miRs as one of the potential downstream mechanisms for the rescue action of Genistein in PH. Although our in vivo data clearly suggests that Genistein treatment is associated with inhibition of miR206 in the MCT-induced model of PH in rats, whether this inhibition is through ER-beta pathway or through Genistein’s tyrosine kinase inhibitory effect is not clear. It is possible that both these pathways culminate in the net restoration of miR206 levels by Genistein. The contribution of other potential mechanisms of Genistein action, for example, increases in endothelial NO synthase levels and NO-mediated PA relaxation could not be ruled out as the indirect factors for modulation of miR206 levels. In addition, we cannot rule out the possibility that downregulation of miR206 expression upon Genistein treatment could be a result of improvement in the cardiopulmonary function of the animals and not a direct consequence of Genistein action. Therefore, Genistein rescue and miR206 downregulation could ultimately converge to rescue pre-existing PH in the MCT model. Exploring the direct or indirect action of both monocrotaline
and Genistein on the transcriptional regulation of miR206 as well as its downstream targets warrants further investigation to understand the model-specific transcriptional regulation of miR206 and potential link between Genistein and miR206 regulation.

**Conclusion**

In conclusion, this study shows that in the lungs of MCT-induced PH rats, Genistein treatment is associated with the differential regulation of several miRs. Among these miR206 expression shows steep upregulation in PH and its expression is completely restored by Genistein treatment. Knockdown of miR206 by specific antagonir is sufficient to rescue advanced PH in MCT-rat model concomitant with an increase in angiogenesis and decrease in arteriolar thickening in the lungs.

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**Conflict(s) of Interest/Disclosure(s)**

None


Figure legends

**Figure 1:** MiR206 is strongly upregulated in the lungs in MCT-PH and Genistein therapy restores lung miR206 levels. (A) Heatmap generated from the microRNA microarray analysis in the lungs of CTRL, PH and Genistein-rescue rats in the MCT-model of PH (n = 4 rats per group). (B) Relative lung miR206 expression is confirmed using qRT-PCR analysis in MCT model in control, PH, Genistein vehicle control (GEN-VEH) and Genistein group (n = 6-7 animals per group). (C) Relative copy number of miR206 and other known miRs in PH including miR21, miR322, and miR451 in the lungs of healthy controls (n = 5 rats per group). *P<0.01, ^P<0.001.

**Figure 2:** Knockdown of miR206 rescues PH by improving cardiopulmonary function and structure. (A) Experimental protocol for MCT model: male rats were injected with monocrotaline (MCT) or phosphate-buffered saline (PBS) at day 0 (arrowhead). The MCT injected animals were left untreated to develop severe PH (PH group), received miR206 inhibitor (10 mg/kg body weight, miR206-KD group) or scrambled miR oligonucleotide (10 mg/kg body weight, SCR-CTRL group) on days 17, 21 and 26. All of the rats were sacrificed at day 30. (B) Relative miR206 expression in the lungs of SCR-CTRL versus miR206-KD group at day 30 (n = 5-6 animals per group), (C) RV systolic pressure (RVSP) (n = 5-7 animals per group). (D) The RV hypertrophy index (RV/ (LV + IVS) where IVS is interventricular septum and LV is the left ventricular wall (n = 5-6 rats per group). *P<0.05, ¦P<0.01, ^P<0.001.

**Figure 3:** Knockdown of miR206 improves pulmonary hemodynamics and structure. (A) Alpha-smooth muscle actin staining for lung arterioles in male rats. (B) Quantification of arteriole wall thickness (50-100 arterioles per group were analyzed from n=5-6 rats/group). (C) Echocardiographic images of pulse-wave doppler in male rats.(D) Quantification of pulmonary
artery acceleration time (PAAT) calculated from the pulse-wave doppler in male rats (n = 5-6 rats per group). (E) Quantification of heart rate (breaths per minute) in male rats (n = 5-6 rats per group). (F) Quantification of the maximum rate of rise of RV pressure (dP/dt_max) and (G) the maximum isovolumetric rate of relaxation (-dP/dt_min) in male rats (n = 5-6 rats per group, #P<0.01, ^P<0.001).

**Figure 4:** Knockdown of miR206 is associated with stimulation of angiogenesis and vascular endothelial growth factor (VEGFA) transcripts. (A) Representative Hematoxylin-Eosin images of lung sections (left), and confocal images immunostained for von Willebrand Factor (red) and DAPI for nuclei (blue) at lower magnification (middle) and at higher magnification (right). White arrows show representative examples of micro-vessels that were used for quantification (B) Quantification of vessels per high power field (HPF) in control (CTRL), pulmonary hypertension (PH), Scrambled miR control (SCR-CTRL) and miR206 knock down (miR206-KD). #P<0.001 (20 high power magnification images from 3-4 animals per group). (C) QRT-PCR assessment of transcript levels of VEGFA in the lung tissues of CTRL, PH, SCR-CTRL and miR206-KD groups in MCT rat model of PH. *P<0.05, ^P<0.001 (n = 5-6 rats per group).

**Figure 5.** MiR206 knockdown therapy rescues PH and induces angiogenesis in the lung. Pulmonary hypertension in MCT-rat experimental model of PH results in elevated levels of miR206. Robust expression of miR206 is associated with downregulation of VEGFA and decreased angiogenesis in the lung tissue. MiR206 knock down therapy results in restoration of miR206 expression to healthy controls values and is associated with induction of angiogenesis by upregulating VEGFA expression.
Fig. 1, Sharma et al.
Fig. 2, Sharma et al.
Fig. 3, Sharma et al.
Figure 4, Sharma et al
Fig. 5, Sharma et al.