Pulmonary Hypertension

The MicroRNA-328 Regulates Hypoxic Pulmonary Hypertension by Targeting at Insulin Growth Factor 1 Receptor and L-Type Calcium Channel-α1C

Lei Guo, Zhaoping Qiu, Liuping Wei, Xiufeng Yu, Xu Gao, Shulin Jiang, Hai Tian, Chun Jiang, Daling Zhu

Abstract—Chronic hypoxia is the most common cause of secondary pulmonary hypertension, for which the mechanisms are still unclear. Recent studies implicated an important role for microRNAs (miRNAs) in hypoxia-mediated responses in various cellular processes, including cell apoptosis and proliferation. Therefore, we hypothesized that these regulatory molecules might be implicated in the etiology of hypoxic pulmonary hypertension. Here we show that miRNA-328, a posttranscriptional regulator, was drastically downregulated in the pulmonary artery (PA) after a hypoxic assault. PA rings, Western blot, quantitative real-time PCR, in situ hybridization, and luciferase assay were used to investigate the role of miRNA-328 in hypoxic pulmonary hypertension. We found that hypoxia produced a significant inhibition of miRNA-328 expression, which was involved in PA vasoconstriction and remodeling. Overexpressing miRNA-328 in the transgenic mice remarkably decreased the right ventricular systolic pressure and PA wall thickness under both normoxia and hypoxia. MiRNA-328 inhibited L-type calcium channel-α1C expression through a miRNA-328 binding site within the 3′ untranslated region of L-type calcium channel-α1C. The L-type calcium channel-α1C inhibition attenuated the PA response to KCl. Furthermore, miRNA-328 suppressed the insulin growth factor 1 receptor, ultimately leading to apoptosis of pulmonary arterial smooth muscle cells. The posttranscriptional repression of L-type calcium channel-α1C and insulin growth factor 1 receptor was further confirmed by luciferase reporter assay. These results showed that miRNA-328, an important protecting factor, plays a significant role in PA constriction and remodeling by regulating multiple gene targets in hypoxic pulmonary hypertension. (Hypertension. 2012;59:1006-1013.)

Key Words: pulmonary hypertension ■ miR-328 ■ vasoconstriction ■ apoptosis ■ hypoxia

Pulmonary hypertension (PH) is a challenge to modern medicine. More than 40 000 people die of primary PH each year, and more fatalities are attributed to the secondary PH. The PH is characterized by a component of abnormal pulmonary vasoconstriction and structural remodeling of the small pulmonary arteries, which undergo suppressed apoptosis and enhanced proliferation in pulmonary arterial smooth muscle cells (PASMCs).1 Both processes bring about a progressive increase in pulmonary arterial resistance, which, when fully developed, culminates in the right ventricular failure and death.2,3 Although progress has been made over the past decade, the cellular and molecular mechanisms for PH are still unclear.

MicroRNAs (miRNAs), single-stranded RNAs 19 to 25 nucleotides in length, regulate several pathways, including the development, hematopoiesis, organogenesis, apoptosis, cell proliferation, and tumorigenesis. As a class of naturally occurring small and noncoding RNA molecules, miRNAs bind to the 3′ untranslated region of target mRNAs and either block the translation or initiate the transcript degradation.4 Recently, misexpression of miRNAs has been implicated in many cardiovascular diseases, including PH, but their molecular role in these pathologies has not yet been fully uncovered.

In this study, we examined the role of microRNA-328 (miR-328) in hypoxic PH (HPH). We profiled the genomewide miRNA expression in rat pulmonary artery (PA) of hypoxic rats. Several altered miRNAs were suggested, in which miR-328 was identified as a strong candidate responsible for hypoxic pulmonary vasoconstriction (HPV) and...
vessel remodeling. Therefore, miRNAs and miRNA-mediated gene silencing may contribute to abnormal smooth muscle proliferation and contractility in HPH.

Materials and Methods
For detailed Material and Methods, please see the online-only Data Supplement. Two-way ANOVA was used for the analysis of the results using the transgenic mice exposed to hypoxia and normoxia. For other experiments statistical analysis was performed with 1-way ANOVA followed by the Dunnett test, where appropriate. Differences were considered to be significant at \( P \leq 0.05 \).

Results

Altered MiRNA Expression in the PA of the Hypoxia Rat
Hypoxic rat subjected to a hypoxic assault with 12% \( \text{O}_2 \) for 9 days had been made according to a report by Ma et al.\(^5,6\) MicroRNA expression of rat PAs was analyzed with an RNA/cDNA-based microarray screening. Eight microRNAs were identified to be \( \geq 2 \)-fold upregulated in hypoxic PAs. Another 3 miRNAs were markedly downregulated in hypoxic PAs (Figure S1A, available in the online-only Data Supplement). The results of microarray-based screening were further confirmed by quantitative real-time PCR, in which 7 upregulated and 2 downregulated miRNAs were validated (Figure S1B). To determine how these differentially expressed miRNAs might contribute to HPH, we searched for their potential regulatory targets using algorithms based on miRNA-mRNA complementarity and its evolutionary conservation (TargetScan and PicTar). Interestingly, some candidate target mRNAs of miR-328 were involved in vasomotor remodeling functions in HPH. Thus, we first did the research regarding the role of miR-328 on HPH.

Our results showed that hypoxia downregulated miR-328 expression compared with normoxia (Figure S1B). Furthermore, we compared the miR-328 expression in PAs between PH patients and normal persons, and miR-328 showed a significant downregulation in PAs from PH patients (Figure S1C).

Effect of Hypoxia on MiR-328 Expression and Distribution
The morphology of pulmonary vessels was examined with hematoxylin-eosin stain to show potential correlations of the morphological changes with remodeling. Wall thickening was found in medium-sized PAs obtained from rats exposed to hypoxia for 9 days and in PH patients (Figure 1A and 1B). The precise location of miR-328 in PA was examined using in situ hybridization in the lung tissue sections from normoxic and hypoxic rats and normal human and PH patients. The sections were stained with Dig-labeled miR-328 riboprobes. As shown in Figure 1C and 1D, miR-328 was majorly localized in PASMCs. Meanwhile, the miR-328 was also distributed in smooth muscle cells of the vein and trachea (Figure S2A). Furthermore, miR-328 expression was downregulated by hypoxia, as shown by the weaken staining in PASMCs from hypoxic rats (Figure 1E). In addition, the downregulation of the miR-328 expression was confirmed in PH patients (Figure 1E).

To determine whether the downregulation of the miR-328 expression by hypoxia is specific, the effect of hypoxia on miR-328 expression in thoracic aorta, mesenteric artery, and brain artery was tested by quantitative real-time PCR. We found that miR-328 was significantly decreased in PA but not altered in thoracic aorta, mesenteric artery, and brain artery (Figure S2B).

Effect of MiR-328 on Pulmonary Vasoconstriction
To explore the biological effect of miR-328, the right ventricular systolic pressure in the transgenic mice was measured. We found that the right ventricular systolic pressure was remarkably decreased in miR-328–overexpressed transgenic mice under both normoxia and hypoxia (Figure 2A through 2C). Furthermore, The PA tension from transgenic mice was significantly reduced (Figure S3A). To ascertain the role of miR-328 on PA constriction, the PAs were dissociated from normoxic rats and then transfected with adenovirus vector for overexpressing miR-328 (Adv-miR-328) or Adv-pDC-316 (an empty adenovirus vector used as control). The response of PAs to KCl (30–50 mmol/L) was tested after transfection for 48 hours. It was found that the miR-328 transfection augmented the KCl-induced constriction of endothelium-intact PA rings (Figure S3B). These findings were not a coincidence with our hypothesis that miR-328 downregulation contributed to HPV. We speculated that Adv-miR-328 may be transacted into endothelium cells but not into PASMCs. To clarify this, the endothelium was removed in the PAs transfected with Adv-miR-328. No significant difference in constriction response was observed between control and miR-328 transfection PA rings (Figure S3C). However, in endothelium-denuded PA rings transfected with Adv-miR-328 for 48 hours, the KCl-induced constriction of PA rings was clearly blunted (Figure S3D), suggesting that hypoxia-induced downregulation of miR-328 occurs in PASMCs and contributes to HPV.

Elevation of Ca\(^{2+}\) influx in PASMCs plays an important role in HPV. To identify the effect of miR-328 on [Ca\(^{2+}\)]\text{c} in PASMCs, we use laser scanning confocal microscopy to investigate the influx of extracellular calcium induced by KCl. Our results indicated that miR-328 could significantly repress the influx of extracellular calcium induced by KCl. In contrast to miR-328, antisense oligonucleotides against miR-328 (AMO-328) could significantly increase the Ca\(^{2+}\) influx (Figure S4), suggesting that the calcium current change induced by miR-328 was involved in HPV.

Effect of MiR-328 on PASMC Apoptosis
The lung tissues from transgenic mice were dissociated. Hematoxylin-eosin stain was used to show potential correlation of the morphological changes with remodeling. The results showed that the overexpression of miR-328 could significantly attenuate wall thickness under both normoxia and hypoxia (Figure 2D through 2F). To test the effect of miR-328 on cell fate, rat PASMCs were divided into 6 groups, control, \( \text{H}_2\text{O}_2 \), miR-328, AMO-328, miR-328 plus AMO-328, and negative control, all of which were subjected to transfection procedures. The \( \text{H}_2\text{O}_2 \) group was used here as a positive control of apoptosis. Apoptotic cells were determined by 3-(4, 5)-dimethylthiaziazole dye reduction.
Figure 1. The effect of hypoxia on microRNA (miRNA) 328 distribution in pulmonary artery (PA). A, The morphology of PA from normoxia and hypoxic rats was examined with hematoxylin-eosin stain. B, Summarized data showing that the wall thickness in pulmonary hypertension (PH) human and rats was higher compared with control group. C, MiR-328 expression and localization in PAs from
Overexpression of miR-328 significantly induced cell apoptosis, whereas AMO-328 could largely prevent the apoptosis (Figure S5A). Furthermore, there was a reverse correlation between different amounts of miR-328 and growth in PASMCs (Figure S5B).

The alteration of nuclei conformation visualized by acridine orange staining was applied to determine the percentage of apoptotic cells in PASMCs. As shown in Figure S5C, the percentage of apoptotic cells was increased in PASMCs transfected with Adv-miR-328. TUNEL assay was undertaken to determine whether miR-328 participated in DNA fragmentation of PASMCs. As shown in Figure S5D, the number of TUNEL-positive cells was significantly increased after serum defect and miR-328 for 48 hours. In contrast, AMO-328 significantly decreased the number of TUNEL-positive cells induced by miR-328. Because caspase 3 plays an important role in the process of apoptosis, the expressions of procaspase 3 and cleaved caspase 3 in PASMCs were examined. MiR-328 suppressed the procaspase 3 protein expression, which was significantly alleviated when transfected with AMO-328 (Figure S6A through S6C). By contrast, miR-328 increased the expression of cleaved caspase 3 protein (Figure S6D). Moreover, transfection of cells with miR-328 increased cleaved caspase 3 activity, an effect prevented by coapplication of AMO-328 (Figure S6E).

Similarly, the expression of procaspase 3 protein was decreased by miR-328 in transgenic mice under both normoxia and hypoxia. MiR-328 overexpression in a mouse model can rescue hypoxia-induced increases in procaspase 3 expression (Figure S7A and S7B). These results suggest that miR-328 upregulation leads to PASMC apoptosis.

In addition, we also observed that miR-328 decreased proliferation cell nuclear antigen protein expression in transgenic mice (Figure S7C and S7D). However, the mechanism still needs to be explored.

Repression of L-Type Calcium Channel-α1C and Insulin Growth Factor 1 Receptor Expression by MiR-328

Based our observations above, miR-328 decreased vasoconstriction and induced apoptosis. It is possible that these actions result from the regulation of distinct apoptotic and constriction factors by miR-328. To address this issue, we used a computation- and bioinformatics-based approach to predict the putative targets related to apoptosis and constriction using TargetScan hosted by the Wellcome Trust Sanger Institute. These explorations lead to the identification of 2 candidate targets of miR-328, insulin growth factor 1 receptor (IGF-1R) and L-type calcium channel α1C (CaV1.2; Figures 3A and 4A). To prove that IGF-1R and CaV1.2 are indeed repressed posttranscription by miR-328, we determined the effect of the miR-328 on the protein expression. Western blot analysis showed that the levels of IGF-1R and CaV1.2 proteins were lowered markedly in PAs from miR-328–overexpressed transgenic mice. Percentage changes of CaV1.2 expression in transgene-positive mice exposed to normoxia and hypoxia were lower than that in transgene-negative mice. Therefore, miR-328 overexpression in the mouse model can rescue hypoxia-induced increases in IGF-1R and CaV1.2 expression (Figure S7E through S7H). In rat PASMCs, overexpression of miR-328 also inhibited IGF-1R and CaV1.2 protein expression, which could be abolished by AMO-328 (Figures 3B through 3D and 4B through 4D). We subsequently investigated the effect of the miR-328 on mRNA levels of IGF-1R and CaV1.2 in PASMCs. The IGF-1R but not CaV1.2 mRNA level was repressed by miR-328 (Figure S8A and S8B).

Verification of Interactions Between MiR-328 and Their Target Genes

We placed the 3’ untranslated regions of IGF-1R and CaV1.2 into the 3’ untranslated region of a luciferase reporter plasmid to construct chimeric vectors. Transfection of the chimeric constructs to HEK293 cells (human embryonic kidney cell line) resulted in smaller luciferase activity relative to transfection of the chimeric plasmid alone, suggesting that IGF-1R and CaV1.2 were the target genes of miR-328 (Figure S9A and S9B).

Comparison of MiR-328 Expression Levels Under Various Conditions

The expression of miR-328 was measured in the PASMCs and PAs transfected with Adv-miR-328. Our data showed an ≈3-fold change in PASMCs (Figure S10A) and 4-fold increases in the miR-328 level in PAs (Figure S10B). Successful delivery of miR-328, AMO-328, and negative control to the cells was further verified by comparing the miR-328 levels 48 hours after transfection in cultured PASMCs. Transfection resulted in ≈2- to 3-fold increases in miR-328 levels (Figure S10C). Our data were collected at 48 hours after transfection because that was when the plateau level was reached. Furthermore, the expression of miR-328 in transgenic mice under normoxia and hypoxia was shown in Figure S10D. Moreover, the transfection efficiency of adenovirus vector in PASMCs was further verified by calculating the percentage of cells expressing green fluorescent protein (Figure S10E). These results proved the feasible of all of the experiments.

Discussion

PH is a progressive disease of the small pulmonary arteries of which the mechanisms have been studied for several decades. Chronic hypoxia has been shown to be an important reason for PH involving multiple molecular signaling pathways. However, the mechanism remains elusive. In the present study, we show that the miR-328 was downregulated in PAs.
Figure 2. The effect of microRNA (miRNA) 328 on vasoconstriction and vessel remodeling in transgenic mice. 

A, The right ventricular systolic pressure (RVSP) measurement. B, Summarized data showing that the RVSP was increased by hypoxia. C, Percentage changes of L-type calcium channel α1C (CaV1.2) expression in both genotypes exposed to normoxia and hypoxia. D, The morphology of pulmonary arteries (PA) from transgenic mice was examined with hematoxylin-eosin stain. Scale bars are 50 μm (×10), 20 μm (×20), and 10 μm (×40). E, Summarized data showing the wall thickness was increased by hypoxia. F, Percentage changes of wall thickness in both genotypes exposed to normoxia and hypoxia. n=6. **P<0.01, *P<0.05. Nor (-) indicates normoxia; Hyp (●), hypoxia; Tg (-), transgene-negative mice; Tg (+), transgene-positive mice.
from hypoxic rats and PH patients, affecting HPV and hypoxic pulmonary vessel remodeling by targeting Cav1.2 and IGF1R, leading to HPH.

Recently, some studies have reported that miRNAs are associated with remodeling and the development of hypertrophy and failure in the heart and vasculature.7–9 Meanwhile, several miRNAs were shown to be aberrantly expressed and involved in the etiology of PH.10–12 Interestingly, reports about these altered miRNAs are different. It had been reported that microRNA-21 was increased in human PASMCs exposed to hypoxia and promoted to hypoxia-induced cell proliferation and migration,10 whereas another report showed that microRNA-21 was unchanged in human PH-PASMCs and chronic hypoxic rats.11,12 Moreover, microRNA-204 downregulation in both human and rodent PH correlates with PH severity and accounts for the proliferative and antiapoptotic phenotypes of PH-PASMCs.11 However, neither microRNA-21 nor microRNA-204 was altered in the hypoxic rat model of our present study. A possible explanation is that the models used for screening altered miRNAs were different. Recent reports mainly focused their scanning on cultured human PASMCs or lung tissues from different PH rats, whereas we analyzed the miRNA dysfunction on PAs from hypoxic rats.

Although great progress has been made regarding the role of miRNAs in PH, this research has not fully clarified the mechanism of miRNAs in HPH, especially in HPV. Here, we focused our study on miR-328, which is of higher smooth muscle cell specificity in lung tissue compared with other altered miRNAs (unpublished data). Its putative mRNA targets predicted in silico (TargetScan and PicTar) were implicated in both HPV and hypoxic pulmonary vessel

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**Figure 3.** The effect of microRNA (miRNA) 328 on insulin growth factor 1 receptor (IGF-1R). A, The sequences showing the unique sites of miRNA::mRNA complementarity between miR-328 and IGF-1R. B, Western blot analysis of IGF-1R expression in pulmonary arteries from hypoxic and normoxic rats. C, Western blot analysis of IGF-1R expression in pulmonary arterial smooth muscle cells (PASMCs) transfected with adenovirus vector. D, Western blot analysis of IGF-1R expression in PASMCs transfected with sequences. n=5, **P<0.01, *P<0.05. Adv-miR-328 indicates adenovirus vector for overexpressing miR-328; Adv-pDC-316, an empty adenovirus vector used as control; AMO, antisense oligonucleotides against miR-328; NC, negative control.**
remodeling. Furthermore, we found that hypoxia only decrease miR-328 expression in PA but not the thoracic aorta, mesenteric artery, and brain artery. This phenomenon showed that the role of miR-328 on HPV and hypoxic pulmonary vessel remodeling is specific.

Our results identify the miR-328 could attenuate PA vasoconstriction and found CaV1.2 as a potential mRNA target of miR-328. Many studies indicated that CaV1.2 is an essential regulator of PA constriction. In the present study, we found that both hypoxic exposure and exogenous miR-328 decreased CaV1.2 expression. Moreover, the calcium flux in cultured PASMCs can be inhibited by miR-328. Although lacking dynamic electrophysiology, data from confocal microscopy somehow reveals the role for miR-328 on L-type calcium channel activity, because the L-type calcium channel is the principal Ca$^{2+}$ entry pathway in smooth muscle cells. Luciferase assay identified CaV1.2 as a direct target of miR-328. Taken together, these results thus provided direct evidence that chronic hypoxia induced PA constriction and increased CaV1.2 translation via miR-328.

Another major strength of the present study is that it identifies the role of miR-328 on PA remodeling. We found that the
overexpression of miR-328 attenuated the PA remodeling. Furthermore, miR-328 attenuating the PA remodeling was related to PASMC apoptosis. According to bioinformatics-based analysis and luciferase assay, IGF-1R was identified as the direct target of miR-328 involving remodeling. IGF-1R signaling involves autophosphorylation and subsequent tyrosine phosphorylation of Shc and insulin receptor substrates 1 through 4. Insulin receptor substrate serves as a docking protein and can activate multiple signaling pathways, including phosphatidyl inositol 3-kinase, Akt, and mitogen-activated protein kinase. The activation of these signaling pathways induces differential biological actions, including cell growth, differentiation, migration, and survival. We confirm that miR-328 induces PASMC apoptosis through repression of IGF-1R. Future studies should be addressed that show which downstream factors of IGF1R pathway are activated.

Our study also shows that different amounts of miR-328 overexpression in the mouse model may show different reactions on hypoxia, and miR-328 overexpression (=3 times) may be too low to rescue the effect of hypoxia (data not shown). However, the expression of miR-328 in transgenic mice is transferred and overexpressed over 6 times; it can rescue hypoxia-induced PA vasoconstriction and remodeling. However, the mechanism underlying a different sensibility of a different degree of miR-328—overexpression transgenic mice to hypoxia was still unclear.

In conclusion, our data provide new evidence that miR-328 plays a dominant role in the vasoconstriction and remodeling of HPH. The inhibitory effect of miR-328 on vasoconstriction is attributable to a block of CaV1.2. IGF-1R is also the target of miR-328, which is involved in apoptosis. These studies are an effort to establish molecular and ionic mechanisms of HPH and provide the potential target for treatment of HPH.

Perspectives

MicroRNAs are important mediators in cell proliferation and apoptosis. Although recent studies have shown that several miRNAs were altered and involved in the pathogenesis of PH, the mechanisms of miRNAs in HPH, especially in HPV, have not been fully clarified. Here, this study found that miR-328 was downregulated in PAs from PH patients and rats. In addition, miR-328 downregulated the IGF-1R and CaV1.2 translation and functionally antagonized the negative effect of IGF-1R on PASMC apoptosis and the positive effect of CaV1.2 on PA constriction. In view of these observations, we reveal a novel regulatory pathway, that miR-328 may be an important player in PH etiology and may provide a novel therapeutic insight for PH treatment, because miR-328 could modulate both processes of PH. Future experiments are needed to uncover whether reestablishing the miR-328 level can protect humans from PH, so that we can ultimately design specific therapies in the clinic to turn the process off.

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Disclosures

None.

References

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The miR-328 regulates hypoxic pulmonary hypertension by targeting at IGF-1R and CaV1.2
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Supplemental Methods

Animals and lung tissues preparation

Adult male Wistar rats with a mean weight of 200 g were from the Experimental Animal Center of Harbin Medical University, which is fully accredited by the Institutional Animal Care and Use Committee (IACUC). About 150 rats were used in this experiment. Twelve-hour light exposure cycles, standard rat chow, and water ad libitum were provided to all rats. Adult male Wistar rats were randomized to 9 days of normal and hypoxic environments with fractional inspired oxygen (FiO2) 0.21 and 0.12, respectively as previously described 1. Normoxic rats were kept in the same room adjacent to the hypoxic chamber. At the end of the 9 days exposure period, we anesthetized each rat with pentobarbital injection (120 mg/kg, i.p.), opened the thorax and removed the heart and lungs to the flat plate.

Human sample preparation and pulmonary artery (PA) dissection

We obtained the human tissues from the Second Affiliated Hospital of Harbin Medical University under the procedures approved by the Ethnic Committee for Use of Human Samples of the Harbin Medical University. The human normal lung tissues were from death human of road accident and HPH lung tissues were form PH patients. Fresh lung tissues were immediately placed in cold oxygenated Krebs solution (in mM: NaCl 116, KCl 4.2, CaCl2 2.5, NaH2PO4 1.6, MgSO4 1.2, NaHCO3 22, and D-glucose 11, pH 7.4). And the distal pulmonary arteries were microdissected free of surrounding tissue under microscope. After dissection, the PAs of each sample were divided into two separate parts, one for measuring viability, the rest for preparing for other studies. The response of PAs to KCl was used to test vessel viability. The viability of PAs will be well if their tension is significantly increased induced by KCl, indicating that the rest of these samples can be used in subsequent experiments.

MicroRNA microarray analysis

Fresh lung tissues were prepared from four normoxic rats and four hypoxic rats. Total RNA of PAs was extracted by using TRizol (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. Five μg of total RNA from each sample was labeled and hybridized on microRNA microarray chips (Exiqon Company, Denmark) as previously described 2.

Cell culture

Primary cultured pulmonary artery smooth muscle cells (PASMCs) were prepared from Wistar rats as previously described 3. HEK293 were cultured in Dulbecco’s Modified Eagle Medium (DMEM). The cultures were supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin.

Construction of miR-328 transgenic mice

The construction procedures of miR-328 transgenic mice were similar to the study reported by Debra L. Baker 4. The vector containing premiR-328 sequence and smooth muscle-myosin heavy chain (SM-MHC) promoter was obtained by recombinant DNA technology. After digested, separated and purified, the DNA fragment containing the premiR-328 sequence was prepared. Eggs from mated sexually immature female mice (C57BL/6, 4 ~ 5 weeks old) were individually microinjected with the DNA fragment. Then the injected eggs were bilaterally implanted into the oviduct of pseudopregnant female mice. Transgene presence was identified by PCR analysis of genomic DNA isolated from mice tail tissue, with primer pairs 5’-CCTTACCACCATAGACCT-3’ and 5’-CTGTAGATCTTCTCTCCT-3’. The PCR conditions were as follows: 2 minutes at 94°C; 35 cycles of 20 seconds at 94°C, 7 seconds at 60°C, and 20 seconds at 72°C; and 5 minutes at 72°C. The transgenic mouse line carrying a mismatched premiR-328 sequence was used as a negative control in following experiments.

Construction of adenoviral vector

The construction procedures were as follow. Rno–miR-328 precursor DNA (5’-GGATCCgACCCCGTCCCGCGTCCCGGAGTCCCTCTCTTTTCGTAGATGTCGGGGGAGAGACGGGACAGGGGGTCATTttttttAAGCTT-3’) synthesized by GenScript (Nanjing, PR China) was first cloned into adenovirus shuttle plasmid pDC316-EGFP-U6 (Microbix Biosystems Inc, Canada). The empty vector of the pDC316-EGFP-U6 was used as a vector control. The combinant pDC316-EGFP-premiR-328 shuttle plasmid was identified by PCR and then cotransfected with the rescue adenovirus genomic plasmid pBHGlox E1.3Cre
into HEK 293 cells by liposome reagent. The recombinant adenovirus vector containing premiR-328 gene (Adv-premiR-328) was generated by site specific recombination and confirmed by PCR, and then Adv-premiR-328 was propagated in HEK 293 cells and purified. Adenovirus was amplified by reinfecting HEK293 cells. Titer of virus was determined by making a series of dilutions of viral stock, infecting HEK293 cells, and counting for virus-infected cells and is expressed as plaque-forming units (PFU) per milliliter. The infective titer of Adv-premiR-328 was 10^6 PFU/ml. The terminal titer of Adv-premiR-328 used in research was 10^5 PFU/ml.

Synthesis of miRNAs and sequences of miR-328 inhibitors
MiR-328 was synthesized by Integrated DNA Technologies (IDT). The sequences of miR-328 inhibitors (AMOs; anti-miRNA oligonucleotides) are 5'-ACGGAA GGGCAGAGAGGGCCAG-3'. [The sequences of miR-328 are identical in human and rat].

Measurement of right ventricular systolic pressure (RVSP)
All transgenic mice were male, aged 2-3 months, weighed 22-26 g. Transgene-positive mice and transgene-negative littermates were randomized into normoxic group (FiO2 0.21, 9 days) and hypoxic group (FiO2 0.12, 9 days). RVSP was measured from six mice of each group by right heart catheterization, according to Song Y 5. Before catheterization, mice were anesthetized with 35 mg/kg pentobarbital sodium by intraperitoneal injections. If further anesthesia was necessary, repeat doses of pentobarbital sodium (17.5 mg/kg) were administered. A 1.2 French Pressure Catheter (Scisense Inc.) was connected to the Scisense FA-404 recorder. When the right jugular vein was exposed, the catheter was inserted into the vein, then advanced into superior vena cava, and finally into right ventricular. RVSP was continuously recorded for 45 minutes.

Tension studies of PA rings
PAs were dissected according to the method of human PA dissection described above. PA tension studies were carried out in a manner as previously published 6. Tension data was relayed from the pressure transducers to a signal amplifier (600 series eight-channel amplifier, Gould Electronics). Data was acquired and analyzed with CODAS software (DataQ Instruments, Inc.).

Histology
The lung tissues were obtained from anesthetized rats or mice, sliced into tissue blocks, and immersed in 4% paraformaldehyde for overnight fixation. Fixed tissues were then dehydrated, cleared, and embedded in paraffin wax. The tissues were cut into 5 µm thick sections and stained with hematoxylin and eosin (H&E). Results are expressed as the wall thickness. The sections were viewed with an Eclipse 600 Nikon microscope and photographed with a digital camera. Morphometric analysis was analyzed with image software (Image Pro Plus).

Non-isotopic in situ hybridization
Dig-labeled probes were designed according to the miR-328 mature sequences of rat and synthesized by Invitrogen Inc. The sequences of Dig-labeled probes against miR-328 mRNA were: 5'-ACGGAAGGGCAGAGAGGGCCAG-3'. In situ hybridization was performed using a detection kit on sections of 4% paraformaldehyde-fixed (containing 0.1% diethylpyrocarbonate) of lung tissues according to the manufacturer’s instructions.

Transfection of miRNAs and luciferase assay
DNA fragments of the 3' untranslated regions (3'-UTRs) of CaV1.2 mRNA and IGF1R mRNA containing the putative miR-328 binding sequence were synthesized by Invitrogen, respectively. These fragments were then respectively cloned into the multiple cloning sites downstream the luciferase gene (HindIII and SpeI sites) in the pMIR-REPORTTM luciferase miRNA expression reporter vector (Ambion, Inc.), as described elsewhere. After 24 hours starvation in serum-free medium, HEK293 cells (1-10^5 per well) were transfected with 1 µg miR-328 or 1 µg PGL3-target DNA (firefly luciferase vector) and 0.1 µg PRL-TK (TK-driven Renilla luciferase expression vector), with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Luciferase activities were measured 48 hours after transfection with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507) 7,8.
MTT assay for cell viability
PASMCs were cultured in 96 well culture clusters (about 1×10^4 per well), and then the cells were transfected with miR-328 or AMO with lipofectamine 2000. The cells cultured in complete medium were considered as control. The cells were incubated for 4 h in a medium containing 0.5% 3-[4,5-dimethylthiazol-2-yl] -2,5 –diphenyl- tetrazolium bromide (MTT), the yellow mitochondrial dye. The amount of blue formazan dye formed from MTT is proportional to the number of survival cells. The MTT reaction was terminated by adding DMSO to the medium followed by incubation for 10 min at room temperature. The absorbance was read at 540 nm in a spectrophotometer.

Nuclear morphology determination
Cells were washed with PBS for two times, and stained with acridine orange (AO) for 8 min at 24 °C. The AO-stained cells were imaged with a fluorescent microscope under 488 nm laser excitation and 405 nm emission. For each well, 15-25 shot were randomly selected to determine the percentage of apoptotic cells in total cells based on the morphological characteristics of apoptosis. Cells with nuclear crenation, nuclear condensation and nuclear fractionation were defined as apoptotic cells.

TUNEL
Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays were performed with the one step TUNEL kit according to the manufacturer’s instructions. Cells grown in 6 well culture clusters were permeabilized with 0.1% Triton X-100 for 2 min on ice followed by TUNEL for 1 h at 37 °C. The FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscope by using 488 nm excitation and 530 nm emission. The cells with green fluorescence were defined as apoptotic cells.

Measurement of [Ca2+]i fluorescence intensity (FI).
PASMCs were plated on 1-cm diameter glass coverslips, divided into five groups, control, miR-328, AMO-328, miR-328 plus AMO-328, NC. After transfected for 48 hours, the [Ca2+]i was measured with the fluorescent indicator Fluo-3-acetoxymethyl ester. Briefly, PASMCs was loaded 5-10 µM Fluo 3-AM (dissolved in DMSO with 20% pluronic acid) for 30–45 min at 37 °C. Cells were then washed three times with normal Tyrode's solution (containing: NaCl 135 mM, KCl 5.4 mM, CaCl2 1.8 mM, MgCl2 1.2 mM, NaH2PO4 0.33 mM, glucose 10 mM, HEPES 5 mM; pH 7.35) to remove extracellular Fluo 3-AM. The [Ca2+]i FI was detected at 10 s intervals and excited at 488 nm, while collecting emitted light at 530 nm by confocal laser scanning microscopy(Olympus, Japan). After 20 s baseline recording, cells were exposed to 120 mM KCl in order to trigger extracellular Ca2+ entry and then recorded for 280 s. Image analysis was performed offline using Fluview-FV300 (Olympus, Japan) to select cell regions from which FI was extracted and further analyzed using Excel (Microsoft, USA) and Origin Version 7.5 software (OriginLab Corporation, UK). [Ca2+]i changes were assessed by FI normalizing to initial FI (FI/F0).

Quantification of mRNA and miRNA levels
For quantification of CaV1.2 and IGF-1R transcripts, conventional real-time RT-PCR was carried out with total RNA samples extracted from PASMCs after transfection. The mirVanaTM qRT-PCR miRNA Detection Kit (Ambion) was used in conjunction with real-time PCR with SYBR Green I for quantification of miR-328 transcript, as detailed elsewhere 7, 8.

Western blot analysis
The protein samples were extracted from PASMCs cells, with the procedures essentially the same as described in detail elsewhere 7, 8. Protein samples (~50 µg) were fractionated by SDS-PAGE (7.5-10% polyacrylamide gels). The primary antibodies against CaV1.2, IGF1R, Procaspase-3, Cleaved caspase-3, PCNA were used, with β-actin as an internal control.

Measurement of caspase-3 activity
Caspase-3 activity was measured by cleavage of chromogenic caspase substrates, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Aspp-nitroanilide), a caspase-3 substrate. The absorbance of the substrate was measured at 405 nm after cleavage by caspase-3. The optical density value at 405 nm was thus used as indication for the amount of caspase-3. The protein samples were prepared as indicated in Western blot analysis. Then
approximate 50 mg of total proteins were added to the reaction buffer containing Ac-DEVD-pNA (2 mM), incubated for 2 h at 37 °C, and the absorbance of yellow pNA cleaved from its corresponding precursors was measured using a spectrometer at 405 nm. The specific caspase-3 activity, normalized for total proteins of cell lysates, was then expressed as fold of the baseline caspase activity of control cells cultured in DMEM with 20% FBS.

Supplemental References
Supplemental Figure and Figure Legends

Fig S1

A: The table showing microRNAs expression analyzed with an RNA/cDNA-based microarray screening.

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B: Gene chip results were validated with qRT-PCR in PAs of rats cultured under normoxia, hypoxia. n = 4, **P < 0.01, *P < 0.05.

C: Compared the miR-328 expression in PAs from normal persons and PH patients. n = 6, *P < 0.05. PAs indicates pulmonary arteries; qRT-PCR, quantitative Real-Time PCR; PH, pulmonary hypertension; Nor, normoxia; Hyp, hypoxia.

Figure S1. Altered miRNA expression in the PAs from hypoxic rats. A: The table showing microRNAs expression analyzed with an RNA/cDNA-based microarray screening. B: Gene chip results were validated with qRT-PCR in PAs of rats cultured under normoxia, hypoxia. n = 4, **P < 0.01, *P < 0.05. C: Compared the miR-328 expression in PAs from normal persons and PH patients. n = 6, *P < 0.05. PAs indicates pulmonary arteries; qRT-PCR, quantitative Real-Time PCR; PH, pulmonary hypertension; Nor, normoxia; Hyp, hypoxia.
Figure S2. The effect of hypoxia on miR-328 specificity expression. A: miR-328 expression and localization in SMCs of vein and trachea through in situ hybridization. Scale bars are 50 µm. B: In contrast to the PA, hypoxia had no significant effect on miR-328 expression in mesentery artery, thoracic aorta and brain artery. n = 5, **P < 0.01, *P < 0.05. SMCs indicates smooth muscle cells; Nor, normoxia; Hyp, hypoxia; PA, pulmonary artery; MA, mesentery artery; BA, brain artery; AO, aorta artery.
Figure S3. The effect of miR-328 on pulmonary vessel tension. A: The measurement of PA tension from transgenic mice. n = 3, *P < 0.05. B: The response of PA to KCl (30-50 mmol/L) was tested after transfection with Adv-miR-328. n = 3, *P < 0.05. C: The response of endothelium-denuded PA to KCl (30-50 mmol/L) was tested after transfection with Adv-miR-328. n = 3, *P < 0.05. D: The response of PA to KCl (30-50 mmol/L) was tested after denuding endothelium and transfection with Adv-miR-328. n = 3, *P < 0.05. PA indicates pulmonary artery; Adv-miR-328, adenovirus vector for overexpressing miR-328; Adv-pDC-316, an empty adenovirus vector used as control; WT, wild type mice; Tg (-), transgene-negative mice; Tg (+), transgene-positive mice.
Figure S4. The effect of miR-328 on Ca$^{2+}$ influx induced by KCl in PASMCs. The [Ca$^{2+}$]$_i$ FI was detected and recorded for 300 s at 10 s intervals. Cells were exposed to 120 mM KCl to trigger extracellular Ca$^{2+}$ entry after 20s recording. A. control (n=22 cells from 5 rats). B. miR-328 (n=13 cells from 5 rats). C. AMO-328 (n=14 cells from 5 rats). D. miR-328 plus AMO-328 (n=8 cells from 5 rats). E. NC (n=10 cells from 5 rats). F. the AUC of the Ca$^{2+}$ response induced by KCl. *P < 0.05. PASMCs indicates pulmonary arterial smooth muscle cells; FI, fluorescence intensity; F0, initial FI; FI/F0, FI normalizing to initial FI, AMO, antisense oligonucleotides against miR-328; NC, negative control; AUC, area under the curve; T(s), Time (seconds).
Figure S5. The effect of miR-328 on PASMCs apoptosis. A: Overexpression of miR-328 induced significantly cell apoptosis measured by MTT. n = 6, **P < 0.01, *P < 0.05. B: There is a reverse correlation between different amount of miR-328 and growth in PASMCs, which measured by MTT. n = 6, r = -0.99306, **P < 0.01, *P < 0.05. C: MiR-328 induced nuclei conformation visualized by Acridine orange staining. n = 4, **P < 0.01, *P < 0.05. Scale bars are 50 µm. D: Cells undergoing apoptosis were positively stained with TUNEL reagent and were shown in green. n = 4, **P < 0.01, *P < 0.05. Scale bars are 50 µm. PASMCs indicates pulmonary arterial smooth muscle cells; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; MTT, 3-(4, 5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide; SD, serum defect; AMO, antisense oligonucleotides against miR-328; NC, negative control; Adv-miR-328, adenovirus vector for overexpressing miR-328; Adv-pDC-316, an empty adenovirus vector used as control.
Figure S6. The effect of miR-328 on caspase-3 expression and activity. A: Western blot analysis of procaspase-3 expression in PAs from hypoxic and normoxic rats. n = 4, **P < 0.01, *P < 0.05. B: Western blot analysis of procaspase-3 expression in PASMCs transfected with adenovirus vector. n = 4, **P < 0.01, *P < 0.05. C: Western blot analysis of procaspase-3 expression in PASMCs transfected with sequences. n = 4, **P < 0.01, *P < 0.05. D: Western blot analysis of cleaved Caspase-3 protein levels with and without miR-328 treatment. n=5, *P < 0.05. E: Regulation of Caspase-3 activity by miR-328. Transfection was performed with Lipofectamine 2000 and measurements were made 48 hours after transfection. n=6 for each group. n=5 experiments for each group. *P < 0.05. Procasp-3 indicates Procaspase-3; Cleaved casp-3, Cleaved caspase-3; Casp 3, caspase-3; PASMCs, pulmonary arterial smooth muscle cells; Adv-miR-328, adenovirus vector for overexpressing miR-328; Adv-pDC-316, an empty adenovirus vector used as control; AMO, antisense oligonucleotides against miR-328; NC, negative control.
Figure S7. The expression of procaspase-3, PCNA, IGF1R and CaV1.2 from PAs of transgenic mice under normoxia and hypoxia. A: Western blot analysis of procaspase-3 expression. B: Percent changes of procaspase-3 expression in both genotypes exposed to normoxia and hypoxia. C: Western blot analysis of PCNA expression. D: Percent changes of PCNA expression in both genotypes exposed to normoxia and hypoxia. E: Western blot analysis of IGF1R expression. F: Percent changes of IGF1R expression in both genotypes exposed to normoxia and hypoxia. G: Western blot analysis of CaV1.2 expression. H: Percent changes of CaV1.2 expression in both genotypes exposed to normoxia and hypoxia. n = 4, **P < 0.01, *P < 0.05. Nor indicates normoxia; Hyp, hypoxia; Tg (-), transgene-negative mice; Tg (+), transgene-positive mice; Procasp-3, Procaspase-3; PCNA, proliferation cell nuclear antigen; IGF1R, insulin growth factor 1 receptor; CaV1.2, L-type Calcium channel alpha 1C.
**Figure S8.** The effect of miR-328 on IGF-1R and CaV1.2 mRNA expression. A: Effects of miR-328 on IGF-1R mRNA levels in PASMCs transfected with sequence, as determined by qRT-PCR. B: Effects of miR-328 on mRNA levels of CaV1.2 in PASMCs transfected with sequence, as determined by qRT-PCR. n = 5, **P < 0.01. AMO indicates antisense oligonucleotides against miR-328; NC, negative control.

**Figure S9.** Verification of IGF-1R (A), CaV1.2 (B) as cognate targets of miR-328. Data on luciferase reporter activities showed the interaction between miR-328 and IGF-1R and CaV1.2 3'-UTRs. n = 5, **P < 0.01. IGF1R indicates insulin growth factor 1 receptor; CaV1.2, L-type Calcium channel alpha 1C; 3'-UTR, 3'-untranslational region; NC, negative control.
Figure S10. Comparison of miR-328 expression levels under various conditions, measured by quantitative Real-Time PCR (qRT-PCR). A: miR-328 levels in PASMCs with transfection of adenovirus vector determined by qRT-PCR. n = 4. **P < 0.01. B: miR-328 levels in PA with transfection of adenovirus determined by qRT-PCR. n = 4. **P < 0.01. C: miR-328 levels in PASMCs with transfection of sequence determined by qRT-PCR. n = 4. **P < 0.01. D: The expression of miR-328 in transgenic mice under normoxia and hypoxia were measured by qRT-PCR. n = 4. **P < 0.01. E. PASMCs transfected of adenovirus vector were observed to express GFP by fluorescence microscope. AMO indicates antisense oligonucleotides against miR-328; NC, negative control; Adv-miR-328, adenovirus vector for overexpressing miR-328; Adv-pDC-316, an empty adenovirus vector used as control; WT, wild type mice; Tg (-), transgene-negative mice; Tg (+), transgene-positive mice.