Unbalanced apoptosis is a major cause of structural remodelling of vasculatures associated with PAH (pulmonary arterial hypertension). miRNAs (microRNAs) regulate the expression of several proteins that are important for cell fate, including differentiation, proliferation and apoptosis. It is possible that these regulatory RNA molecules play a role in the development of PAH. To this hypothesis, we studied the effect of several miRNAs on the apoptosis of cultured PASMCs (pulmonary artery smooth muscle cells) and identified miR-138 to be an important player. miR-138 was expressed in PASMCs, and its expression was subjected to regulation by hypoxia. Expression of exogenous miR-138 suppressed PASMC apoptosis, prevented caspase activation and disrupted Bcl-2 signalling. The serine/threonine kinase Mst1, an amplifier of cell apoptosis, seemed to be a target of miR-138, and the activation of the Akt pathway was necessary for the anti-apoptotic effect of miR-138. Therefore the results of the present study suggest that miR-138 appears to be a negative regulator of PASMC apoptosis, and plays an important role in HPVR (hypoxic pulmonary vascular remodelling).

Key words: apoptosis, hypoxic pulmonary vascular remodelling, microRNA, pulmonary arterial hypertension.

INTRODUCTION

PAH (pulmonary arterial hypertension) is a malignant pulmonary disease characterized by elevated pulmonary arterial pressures leading to right ventricular failure and death. A major pathogenic process in the development of PAH is the remodelling of pulmonary vessels involving apoptosis and regeneration of PASMCs [PA (pulmonary artery) SMCs (smooth muscle cells)]. Although these events also occur in healthy lungs, a subtle balance between them is normally achieved without permanent damage to the structure and function of pulmonary vasculature [1–3]. Unbalanced vascular remodelling can cause a structural change in the pulmonary vessels, persistent vasoconstriction and PAH [4–8].

Hypoxia, a well-known trigger event of PAH, has effects on pulmonary vascular remodelling. Although the mechanisms underlying hypoxic PAH are still not fully understood, experimental evidence suggests that hypoxia has an anti-apoptotic effect that can misbalance the remodelling of pulmonary vasculature [9,10]. The resulting excessive regeneration of PASMCs and prolongation of their viability may produce hypertrophy of vascular smooth muscles, thickening of the vascular wall, narrowing of vessel inner diameters and an increase in the perfusion resistance. These pathophysiological alterations in turn can worsen tissue hypoxia. Thus a cascade of events is triggered by hypoxia, leading to adverse vasoconstriction and PAH.

The molecular and cellular basis for the hypoxic vascular remodelling of PAs is unclear. One potential mechanism is the alteration of mRNA stability by miRNAs (microRNAs). miRNAs are endogenous non-coding small RNAs that negatively regulate gene expression by targeting mRNAs. Such post-transcriptional regulation affects a variety of physiological and pathological processes, including cell proliferation and apoptosis, underlying tumorigenesis and several cardiovascular diseases [11–18]. In a recent study, we screened a number of miRNAs in PAs with hypoxic exposure. We found seven miRNAs to be up-regulated and two miRNAs (miR-328 and miR-290) to be down-regulated. The latter two were studied in detail, and our previous results suggested that miR-328 acts on PASMC remodelling and PA constriction [16]. In contrast, how the other seven miRNAs function in hypoxia-induced PH (pulmonary hypertension) remains unclear. Therefore we performed the present study to investigate how these hypoxia-augmented miRNAs affect apoptosis of PASMCs after the cells were challenged with hypoxia, and which intracellular signalling mechanisms are critical.

MATERIALS AND METHODS

Animal use

Animal care and use complied to the Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1996),
and were approved by the Institutional Animal Care and Use Committee of Harbin Medical University [Protocol (2009)-11]. Adult male Wistar rats (180–200 g) were from the Experimental Animal Center of Harbin Medical University.

Induction of the HPVR (hypoxic pulmonary vascular remodelling) rat model and tissue collection

HPVR rat models were induced by raising the animals in a hypoxic environment (12 % O₂) for 9 days [19]. After this time period, rats were anaesthetized with pentobarbital sodium (50 mg/kg of body weight intraperitoneally) [20]. When animals were sufficiently anaesthetized, the chest was surgically opened. Then the heart and lungs were removed together and placed in ice-cold PBS solution for the preparation of tissue collection [21]. The lungs were harvested and processed for immunohistochemistry and in situ hybridization. The PAs were collected for qRT (quantitative real-time reverse-transcription)-PCR and Western blot assay.

Localization of miRNAs by in situ hybridization

In situ hybridization was performed using a detection kit from Boster Bio-engineering on sections of paraffin-embedded lung tissues. The digoxigenin-labelled probe for miro-138 was designed and synthesized by Sangon Biotech. Morphometric analysis was analysed with Image software (Image Pro Plus).

Computational prediction of miRNA targets

We used three established miRNA target-prediction algorithms including TargetScan5.1, miBase and miRGene prediction analysis to identify the candidate miRNAs that were potentially targeted by the apoptosis-related genes.

Cell isolation and cultivation

The intrapulmonary arteries were de-endothelialized and then gently digested with enzymatic solution: 0.15 % type II collagenase ( Worthington) and 0.15 % BSA in PBS solution for 1 h at 37 °C. The digested PASMCS were then cultured in complete DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10 % (v/v) FBS (fetal bovine serum), 1 % (w/v) streptomycin and 1 % (w/v) penicillin for 3–5 days in a humidified incubator with 5 % CO₂ at 37 °C. Passages 2–4 were used for further experiments. Before each experiment, the cells were incubated in serum-free low-glucose DMEM for 24 h to stop growth. For hypoxic cultivation, the cells were grown in a Tri-Gas incubator (HF100; Heal Force) providing an atmosphere of 92 % N₂/5 % CO₂/3 % O₂ for 24 or 48 h as described previously [22].

Induction of an apoptotic model of PASMCS

The apoptosis of PASMCS was induced by SD (serum deprivation) or H₂O₂ administration. Briefly, after transfection, cells were kept in SD and cultured under normoxic or hypoxic conditions for another 24 h before qRT-PCR or Western blot assay and 48 h before apoptosis detection. For H₂O₂ administration, cells were transfected and switched to complete DMEM [10 % (v/v) FBS] containing 100 μM H₂O₂ under normoxic conditions for the next 24–48 h and were prepared as described above.

Transfection of oligonucleotides

After growth arrest, PASMCS were transfected with a 2 μg mixture of different groups of oligonucleotides using X-tremeGene siRNA (small interfering RNA) Transfection Reagent (Roche) according to the manufacturer’s instructions. Subsequently, the cells underwent SD or were switched to complete DMEM [10 % (v/v) FBS] under normoxic (21 % O₂/5 % CO₂/balance N₂) or hypoxic (3 % O₂/5 % CO₂/balance N₂) growth conditions for another 24–48 h [23–25].

Aptosis detection

Cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl-2H-tetrazolium bromide] assay, and mitochondrial membrane potential was measured with the JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) probe. Fragmented DNA of the apoptotic PASMCs was measured using the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) assay, and chromatin condensation was examined using Hoechst 33258 staining. Caspase-3 activity and Western blotting were performed to evaluate the caspase cascade and activity of Bcl-2 family.

qRT-PCR analysis

Analysis and quantification of miro-138 and Mst1 (serine/threonine kinase 4) mRNA expression levels using qRT-PCR were performed with high-capacity CDNA Reverse Transcription Kit and Fast SYBR® Green Master Mix (Applied Biosystems) following the manufacturer’s instructions. Primers specific for miRNAs and Mst1, RNU6B and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), were purchased from Sangon Biotech (Table 1). qRT-PCR was performed on a thermocycler ABI Prism 7500 fast (Applied Biosystems) for 40 cycles. The fold increase relative to control samples was determined by the 2−ΔΔCt method [26]. Expression level of target gene mRNA was determined using qRT-PCR utilizing the total RNA from PAs or PASMCs. RNU6B and GAPDH were used as an internal control for miro-138 and Mst1 respectively.

Western blot assay

Proteins extracted from PASMCs and PAs were detected using a standard Western blotting protocol. An antibody specific for β-actin was used as an internal control.

Additional details and the other methods are supplied in the Supplementary Online Data (at http://www.biochemj.org/bj/452/bj4520281add.htm).

Statistical analysis

The composite data were expressed as means ± S.E.M. Comparisons of data were accomplished by one-way ANOVA followed by Dunnett’s test. The differences between means were considered significantly different at P ≤ 0.05.

RESULTS

Effect of hypoxia on miro-138 expression and distribution in HPVR rat model

The rat model of HPVR was induced by consecutive hypoxic exposure for 9 days, and shown by the morphological change of PAs using H/E (haematoxylin and eosin) staining. Compared with normal rats, PAs obtained from rats exposed to hypoxia showed medial thickening by 36.0 ± 0.1 % (n = 3 animals for each group) (Figures 1A and 1B).

In our previous study, we identified seven miRNAs that were up-regulated by hypoxia in PAs [16]. To determine their roles in HPH (hypoxic PH), we first tested their expression patterns...
miR-138 and hypoxia-induced pulmonary vascular remodelling

Table 1  Oligonucleotide sequences of the primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Accession number</th>
<th>Primers (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-184</td>
<td>M0000929</td>
<td>GGGTGACGAGAACTGATAA (forward); CAGTGCGTGCGTGAAT (reverse)</td>
</tr>
<tr>
<td>miR-146a</td>
<td>M0000919</td>
<td>GGGTGAGAACTGATCCCA (forward); CAGTGCGTGCGTGAAT (reverse)</td>
</tr>
<tr>
<td>miR-138</td>
<td>M0000912</td>
<td>GGGAGCTGGTGTTGTGAATCAA (forward); CAGTGCGTGCGTGAAT (reverse)</td>
</tr>
<tr>
<td>miR-194</td>
<td>M0000937</td>
<td>GGGTGTAACGCGACTCC (forward); CAGTGCGTGCGTGAAT (reverse)</td>
</tr>
<tr>
<td>miR-190</td>
<td>M0000933</td>
<td>GGGTGATTTGTGGATAATAG (forward); CAGTGCGTGCGTGAAT (reverse)</td>
</tr>
<tr>
<td>RNU6B</td>
<td>NR_002752</td>
<td>GCTTCGGCAGCACATATACTAAAAT (forward); CGCTTCACGAATTTGCGTGTCAT (reverse)</td>
</tr>
<tr>
<td>Mst1</td>
<td>NM_001107800</td>
<td>GCTATTTGATATGTTTGGCTGTAA (forward); CACGGCAGTGGAGGAAGCT (reverse)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>CCTGGAGAAACCTGCCAAGTAT (forward); CTCGGCCGCTGCTT (reverse)</td>
</tr>
</tbody>
</table>

Figure 1  Functional screening of hypoxia up-regulated miRNAs in PASMCs

(A and B) Characterization of the rat model of HPVR. Sections of lung tissues from rats exposed to normoxic (Nor) or hypoxic (Hyp) conditions were identified using H/E staining. Scale bars are 200, 100 or 50 μm as indicated. (B) The ratios of intimal-to-medial areas of the vessel. (C) qRT-PCR verification of the expression of miRNAs in PASMCs cultured under normoxic and hypoxic conditions. (D) Effects of several miRNAs on cell viability under hypoxic conditions. These miRNAs were overexpressed or inhibited by using the double-stranded Mims and the Amos complementary to the mature miRNAs. Ctl, control. *P < 0.05, **P < 0.01. (A) and (B), n = 3 animals for each group; (C) and (D), n = 3–6 separate experiments.

in PASMCs under hypoxic conditions. Our results confirmed that only miR-138, miR-146a, miR-184 and miR-190 were expressed and up-regulated by hypoxia in PASMCs (Figure 1C).

Subsequently, we studied the function of these miRNAs in PASMC apoptosis under hypoxic conditions. They were overexpressed or inhibited by using the double-stranded Mims (miRNA mimics) and the Amos (antisense oligonucleotides) complementary to the mature miRNAs (Table 2). PASMC apoptosis was induced by SD after transfection with Mims or Amos. The MTT assay showed that a transfection with either miR-138 or Amo-138 significantly affected the cell viability under hypoxic conditions, whereas none of the other miRNAs had any significant effect (Figure 1D). Moreover, using algorithms based on miRNA–mRNA complementarity and its evolutionary conservation (TargetScan, miRBase and miRGene), we found that miR-138 targets candidate mRNAs known to be involved in regulating cell apoptosis in HPH. These findings suggest that miR-138 seems to play a role in HPH via an anti-apoptosis of PASMCs.
Table 2  Sequences of miRNAs, anti-miRNAs and NC
FAM, 6-carboxyfluorescein; 2′-Ome; 2′-O-methyl.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′→3′)</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-184</td>
<td>UGGACGGAGACUGAUAGGCU (sense); ACCCUUAUCAGUCUCUGUCCA (antisense)</td>
<td>2′-Ome</td>
</tr>
<tr>
<td>Amo-184</td>
<td>ACCCUUAUCAGUUCUCUGUCCA</td>
<td>2′-Ome</td>
</tr>
<tr>
<td>miR-146a</td>
<td>UGAGACUGAUACUCAUGGCUU (sense); AACCCAUGAAUCAGUUCUCCA (antisense)</td>
<td>2′-Ome</td>
</tr>
<tr>
<td>Amo-146</td>
<td>AACCCAUGAAUCAGUUCUCCA</td>
<td>2′-Ome</td>
</tr>
<tr>
<td>miR-138</td>
<td>AGCCGUGUUGUGAAGUACCGCU (sense); CGGCCUGAUUCACAACACCAGCU (antisense)</td>
<td>2′-Ome</td>
</tr>
<tr>
<td>Amo-138</td>
<td>CGGCCUGAUUCACAACACCAGCU</td>
<td>2′-Ome</td>
</tr>
<tr>
<td>miR-190</td>
<td>UGAUAUGUUUGUAUAUUAGGU (sense); CUAAUAUAUCAAACAUAUCAUU (antisense)</td>
<td>2′-Ome</td>
</tr>
<tr>
<td>Amo-190</td>
<td>ACCUAAUAUAUCAAACAUAUCA</td>
<td>2′-Ome</td>
</tr>
<tr>
<td>NC</td>
<td>UUCUCGGAACUGUACAGCU (sense); ACUGACAGBUCGGAGAATT (antisense)</td>
<td>5′-FAM</td>
</tr>
<tr>
<td>NC-FAM</td>
<td>UUCUCGGAACUGUACAGCU (sense); ACUGACAGBUCGGAGAATT (antisense)</td>
<td>5′-FAM</td>
</tr>
</tbody>
</table>

Figure 2  Effect of miR-138 on PASMC apoptosis

(A) Representative fluorescent images showing miR-138 relieved the increase in TUNEL-positive cells induced by SD. The bar graph shows quantitative analysis of TUNEL-positive cell content, which was calculated as the ratio of TUNEL-positive cells to the total number of PASMCs among different groups. Scale bars are 100 μm. (B) Representative fluorescent images showing miR-138 prevented chromatin condensation induced by SD. Typical nuclei groups represented typically normal or abnormal nuclei morphs. The bar graph shows quantitative analysis of abnormal nuclei content, which was calculated as the ratio of abnormal nuclei to the total number of nuclei among different groups. Scale bars are 100 μm. A, Amo-138; Ctl, control; M, miR-138. *P < 0.05 and **P < 0.01 (n = 3–6 separate experiments).

The precise location of miR-138 in PAs was further examined using in situ hybridization with digoxigenin-labelled miR-138 probes in lung tissue sections from normoxic and hypoxic rats. miR-138 was mainly expressed in PASMCs, and its expression level was augmented by hypoxia, as shown by the strength of staining in PASMCs from hypoxic rats (Supplementary Figure S1 at http://www.biochemj.org/bj/452/bj4520281add.htm).

miR-138 suppressed mitochondria-mediated caspase-dependent apoptosis in PASMCs

Our TUNEL assay, which measures DNA cleavage in apoptotic cells, showed that the number of TUNEL-positive cells was significantly increased after SD (Figure 2A). Such an effect was attenuated by an overexpression of miR-138 in the cells. Under the SD conditions, apoptotic cells had a classical appearance of chromatin condensation, which is one of the most important criteria and is used to identify apoptotic cells. These nuclear morphological changes were also diminished in the miR-138-transfected cells compared with the SD groups. Meanwhile, co-transfection with Amo-138 partly reversed the effect of miR-138 on apoptosis (Figure 2B). To assess the components of the apoptotic cascade involved in the miR-138-mediated anti-apoptosis, we measured the enzymatic activity of caspase-3 and tested caspase activation, accompanied by a proteolytic cleavage of the unprocessed form (procaspase), using Western blot assay. We found that the miR-138 transfection reduced the SD-activated...
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Figure 3 Effect of miR-138 on the expression or activity of caspase-3 (CASP3) and -9 (CASP9)
Western blot analysis of proCASP3 (A), proCASP9 (B) and CASP9 (D) expression in PASMCs. (C) Regulation of CASP3 activity by miR-138. A, Amo-138; Ctl, control; M, miR-138. *P < 0.05, **P < 0.01 (n = 3–6 separate experiments). Molecular masses in kDa are indicated on the Western blots.

Figure 4 miR-138 suppressed apoptosis by disrupting Bcl-2 signalling
(A) miR-138 attenuated mitochondrial potential reduction in apoptotic PASMCs. A change in the colour from green to red indicates a shift in the fluorescence ratio correlating with an increase in mitochondrial depolarization. Scale bars are 100 μm. (B–E) miR-138 increased/decreased the activity of Bcl-2/Bad. The activity of the Bcl-2 family in different groups was measured by the expression of Bcl-2/phospho-Bcl-2 and Bad/phospho-Bad. A, Amo-138; Ctl, control; M, miR-138; pBad, phosphorylation of Bad at Ser136; pBcl-2, phosphorylation of Bcl-2 at Ser70. *P < 0.05, **P < 0.01 (n = 3–6 separate experiments). Molecular masses in kDa are indicated on the Western blots.
and control cells were co-transfected with miR-138/NC and the pcDNA3.1 vector/pGL3-vector. (mouse (Mmu). (rats were identified using qRT-PCR. (conditions of SD since it is one of the most widely used and experiments, we focused on the response of PASMCs under induced apoptosis ([Supplementary Figures S2–S4 at http://www.

miR-138 co-transfection partially reversed the effect of miR-138 on caspase activities (Figure 3).

Caspase activity (such as caspase-9 activation) is known to induce mitochondrial damage during apoptosis. Therefore we measured mitochondrial potential in PASMCs using the JC-1 probe, which aggregates in the intact mitochondria in non-apoptotic cells emitting orange–red fluorescence and distributes widely in apoptotic cells emitting green fluorescence as the monomeric form at 488 nm. In SD-treated cells, the aggregated JC-1 within normal mitochondria was dispersed to the monomeric form and S5B at http://www.biochemj.org/bj/452/bj4520281add.htm).

Our computational analysis showed a 6-nt match to the miR-138 seed region in the 3′-UTR (3′-untranslated region) of the serine/threonine kinase Mst1 that is highly conserved among rat, human and mouse. (Figure 5A). To test whether miR-138 directly targeted the 3′-UTR of Mst1, we performed luciferase assays using 3′-UTR sequence fragments containing the predicted target of miR-138 and its mutated version inserted downstream of a luciferase reporter (Supplementary Figures S5A and S5B at http://www.biochemj.org/bj/452/bj4520281add.htm). As shown in Figure 5(B), transient transfection of HEK (human embryonic kidney)-293 cells with Mst1 3′-UTR and miR-138 (Supplementary Figure S5C) resulted in down-regulation of luciferase activity compared with Mst1 3′-UTR and NC co-transfected cells, which was abrogated when the predicted miR-138 binding site was mutated. The miR-138 or NC co-transfected with empty vectors generated similar luciferase activities, proving the stable luciferase assay system (Figure 5B).

Western blot analysis showed that the expression pattern of Mst1 and its cleavage product in PAs from normoxic and hypoxic conditions, we examined lysates from PASMCs when miR-138 was either overexpressed or antagonized, and found that there was an inverse correlation between miR-138 and Mst1 protein expression. The expression of Mst1 and its cleavage product at the protein level were clearly suppressed in the miR-138-transfected PASMCs compared with control samples, and this reduction was efficiently prevented by co-transfection with Amo-138 (Figures 6A and 6B), suggesting that miR-138 mediated Mst1 repression via a step after translation initiation, or repressed translation by sequestering the mRNA into a complex where it was not accessible to the ribosomes.

As the Mst1 signalling pathway is known to link the activation of phospho-Akt/Akt with apoptotic signalling in some cell types, we examined the activation of Akt signalling. A marked increase in Akt phosphorylation at Ser 473 was found in PASMCs.
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overexpressing miR-138, which was blocked by co-transfection with Amo-138 (Figures 6A and 6C), suggesting that miR-138 activates Akt signalling by negatively regulating Mst1 at the post-transcriptional level.

We also found that the effect of miR-138 on activation of Akt can be blocked by LY294002, an antagonist of the Akt pathway, administration, providing a link between miR-138 and Akt (Figure 7A). Meanwhile, the miR-138-induced inhibition of the cleavage of procaspase-3 and up-regulation of Bcl-2 expression during apoptosis was also suppressed by LY294002 treatment (Figures 7B and 7C). These data strongly suggest that the Akt signalling pathway is involved in the suppression of PASMC apoptosis by miR-138.

To determine whether Mst1 mediated the miR-138-dependent suppression of caspase-dependent apoptosis, we inhibited Mst1 (si-Mst1) in Amo-138-transfected PASMCs under hypoxic SD conditions and measured apoptosis. The effect of Amo-138 on caspase-dependent apoptosis under this condition was determined using a caspase inhibitor, ZDK [Z-VA-D-FMK (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone)]. As predicted, ZDK or si-Mst1 partially rescued the decrease in cell viability caused by Amo-138 (Figure 8A). Also, no effect of ZDK on miR-138 and Mst1 expression was found in PASMCs, whereas si-Mst1 reversed the Amo-138-induced strengthening of Mst1 and its cleavage expression (Figures 8B and 8C). These data thus suggest that the miR-138-mediated suppression of caspase-dependent apoptosis appears to target at Mst1.

miR-138-mediated hypoxia-induced anti-apoptosis in PASMCs

As hypoxia is likely to lead to the suppression of VSMC (vascular smooth muscle cell) apoptosis, we examined further the effect of miR-138 on apoptosis under hypoxic conditions. As predicted, hypoxic exposure inhibited cell apoptosis (increased cell viability, decreased cleavage of procaspase-3, enhanced Bcl-2 expression and activated phospho-Akt) (Figure 9). Using SD, all these hypoxic effects were relieved and miR-138 overexpression could reverse these attenuations. Amo-138 also attenuated the effect of miR-138 on apoptosis (Figures 9A–9C). Without SD induction, Amo-138 still inhibited the effect of hypoxia on

Figure 6 miR-138 activated phospho-Akt/Akt by targeting at Mst1

The expression pattern (A) and quantitative analysis (B and C) of Mst1 and phospho-Akt were analysed by Western blotting. A, Amo-138; Ctl, control; M, miR-138; pAKT, phosphorylation of Akt at Ser473.* P < 0.05, **P < 0.01 (n = 3–6 separate experiments). Molecular masses in kDa are indicated on the Western blot.

Figure 7 miR-138 suppressed apoptosis through the Akt pathway

Western blot analysis of phospho-Akt/Akt (A), proCASP3 (procaspase-3) (B) and Bcl-2 (C) in PASMCs. A, Amo-138; Ctl, control; LY, LY294002; M, miR-138; pAKT, phosphorylation of AKT at Ser473.* P < 0.05, **P < 0.01 (n = 3–6 separate experiments). Molecular masses in kDa are indicated on the Western blots.

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PASMCs (Figures 9D–9G), confirming the role of miR-138 in mediating this hypoxia-induced anti-apoptosis.

**Hypoxic induction of miR-138 in PASMCs was HIF (hypoxia-inducible factor)-1α-dependent**

HIFs are a family of transcription factors that are activated in response to hypoxia, regulating gene expression and the cellular response to hypoxia [27]. We asked whether the induced expression of miR-138 was indeed triggered by HIF. We used HIF1α and HIF2α siRNA oligonucleotides to suppress the HIF pathway and test the expression levels of miR-138 under these conditions. After stable knockdown of HIF1α and HIF2α (Figures 10A and 10B), the PASMCs transfected with siRNA scramble controls had a robust induction of miR-138 expression under hypoxic conditions. However, this induction was abolished not by siRNAs against HIF2α, but by siRNAs against HIF1α (Figure 10C), implying a HIF-1α-dependent regulatory mechanism.

**DISCUSSION**

In the present study, we have shown evidence for the role for miR-138 in regulating PASMC apoptosis in HPH, and found the underlying mechanisms. The importance of miRNAs in pathological processes is being recognised, especially in cardiovascular disease. A number of miRNAs have been implicated in signal transduction pathways relevant to PH. For example, miR-21 in PH has been shown to target proteins that regulate Bcl-2 and Akt signalling pathways and subsequent cell proliferation and apoptosis [28]. miR-21 and miR-204 have been shown to participate in PH by regulating cell-cycle inhibitors and the Src/Stat3 (signal transducer and activator of transcription 3) cascade [29,30]. There is evidence suggesting the role of miR-328 and miR-214 in regulating the expression of ion channels (CACNA1C and CACNB1) and eNOS (endothelial nitric oxide synthase), candidate regulators of vascular tones [31,32]. Moreover, Albimsson et al. [33] have shown that, in the Dicer-depleted mouse line, the loss of miRNAs in SMCs results in a dramatic decrease in blood pressure due to loss of contractile function, phenotypic modulation of SMCs and vascular remodelling, indicating an important role of miRNAs in differentiation of VSMCs. All these previous studies suggest the importance of miRNAs in vascular cell fate and the consequential effect on PH. In the present study, we have focused on miR-138, and shown its effect on PASMC viability under hypoxic conditions.

The results of the present study indicate that miR-138 suppresses caspase-dependent cell apoptosis and attenuates mitochondrial depolarization in apoptotic PASMCs. This effect exists under hypoxic conditions. According to bioinformatics-based analysis and luciferase assay, Mst1 has been identified to be a direct target of miR-138 involvement in HPVR. Mst1 is a 487-amino-acid protein that contains two cleavage sites between the regulatory and catalytic domains, which may be selectively cleaved to generate catalytically active enzymes of 36 kDa [34]. It has been shown that the activation of Mst1, in both cleaved and full-length forms, is associated with the mechanotransduction pathway for cell apoptosis. Activated Mst1 and its cleavage products have been proved to be novel inhibitors of Akt through binding to Akt1 in the cytoplasm and the nucleus following activation of an apoptotic signal [35–38]. Thus Mst1 may suppress the PI3K (phosphoinositide 3-kinase)/Akt pathway during apoptosis by inhibiting Akt [39]. In the present study, hypoxia and miR-138 can decrease the expression of both cleaved and full-length Mst1. Moreover, both ZDK and si-Mst1 can partially rescue the decrease of cell viability caused by Amo-138. Although lacking direct imaging analysis, the results of the MTT assay suggest that the miR-138-induced suppression of PASMC apoptosis is mediated by targeting Mst1. The luciferase reporter and Western blot assays further validated Mst1 to be a direct target of miR-138 at the post-transcriptional level.
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Figure 9  miR-138 mediated hypoxia-induced anti-apoptosis in PASMCs

(A–C) miR-138 mediated hypoxia-induced suppression of PASMC apoptosis. After transfection, cells were kept in SD under hypoxic conditions for 24 h and prepared for Western blot analysis as before. Cells cultivated in complete DMEM [10% (v/v) FBS] under normoxic and hypoxic conditions after growth arrest served as normoxic and hypoxic control respectively. (D–F) Amo-138 relieved hypoxia-induced suppression of PASMC apoptosis. The cells were transfected with different groups and switched into complete DMEM [10% (v/v) FBS] under hypoxic conditions for another 24–48 h. The expression of procaspase3, Bcl-2 and phospho-Akt (Ser473)/Akt in different groups was detected by Western blotting. (G) Effect of miR-138 on cell viability under hypoxic conditions.

Previous studies have shown the effects of miR-138 on cell viability in carcinogenicity. The deregulation of miR-138 is frequently associated with a variety of cancers, including HCC (hepatocellular carcinoma), ATC (anaplastic thyroid carcinoma) and CML (chronic myeloid leukaemia) [40–42]. Thus the regulation of the expression of miR-138 may be a potential tumour suppression mechanism in different carcinomas.

In the present study, we have examined the signalling pathway underlying the miR-138-regulated anti-apoptosis of PASMCs. We found that miR-138 induces Akt phosphorylation that leads to the cleavage of procaspase-3 and up-regulation of Bcl-2.

The present study has implicated a central role of the transcription factor HIF in regulating gene expression under hypoxic conditions [43,44]. Also, previous studies have shown that regulation of hypoxia-induced miRNAs is HIF-dependent [45,46]. As hypoxic HIF activity is controlled primarily through post-translational modification and stabilization of HIF1α and HIF2α subunits [47], therefore we used HIF1α and HIF2α siRNA to expose the mechanisms underlying this hypoxia-induced miR-138 modulation. Our results show that the hypoxic regulatory mechanism of miR-138 is transcriptionally mediated via HIF-1α. Further studies are still needed to reveal how HIF-1α regulates miR-138 expression under hypoxic conditions. Precisely analysing the location of the HIF DNA-binding sites or adjacent transcripts regulated by HIF-1α conferring miR-138 regulation will be required. There are more than 300 predicted targets of miR-138, some of which are relevant to the apoptotic process [e.g. CCNL2 (cyclin L2), ROCK (Rho kinase) and RARA (retinoic acid receptor α)]. Obviously, other unidentified miR-138 targets may contribute to the pathological process of HPVR. Nevertheless, the aberrant expression of the global miRNAs in HPH suggests that post-transcriptional gene regulation by miRNAs is an important step in HPH pathogenesis.

A number of miRNAs induced during hypoxia have been identified. Interestingly, reports about these altered miRNAs are different. It has been reported that miR-210 is strongly induced by HIF-1α and has multiple effects in different cell types. For example, in HUVECs (human umbilical vein endothelial cells), miR-210 expression results in increased tubulogenesis and increased cell migration through repression of Ephrin-A3. In stromal cells, miR-210 increases osteoblastic differentiation by repression of Acrv1B while promoting cell migration and invasion in human HCC cells under hypoxic conditions [48–50]. In addition, the roles of other hypoxia-inducible miRNAs (e.g. miR-34a, miR-200b, miR-20a etc.) are indicated in different cellular types [51–53]. Although progress has been made...
regarding the role of hypoxia-induced miRNAs, their roles in HPH have not been fully understood. Therefore the results from the present study illustrating the role of hypoxia-induced miR-138 in PASMCs constitute a significant step towards the understanding of the molecular mechanisms of HPH.

In conclusion, the present study provides new evidence showing that miR-138 plays an important role in the vascular remodelling of HPH. miR-138 represses Mst1 expression, which, in turn, results in the activation of the Akt signalling pathway functioning as a negative regulator of PASMC apoptosis via a mitochondria-mediated caspase-dependent mechanism. Therefore the stabilization of the miR-138 level may be a novel strategy for clinical treatment of HPH in the future.

AUTHOR CONTRIBUTION

Shanshan Li conceived and designed the project, and performed experiments, data analysis and interpretation, wrote the paper and provided financial support. Yajuan Ran conceived and designed the project, and performed experiments, data analysis and interpretation. Daling Zhu conceived and designed the project, and performed experiments, data analysis and interpretation, and provided financial support.

ACKNOWLEDGEMENTS

We thank Dr Chun Jiang at Georgia State University, Atlanta, GA, U.S.A. for his comments on this paper prior to submission.

FUNDING

This study was supported by the National Natural Science Foundation of China [grant numbers 30370578 and 31071007], the Science and Technique Foundation of Harbin [grant numbers 2008AAS0097 and 2008RFXXS092 (to D.L.Z.)], the National Natural Science Foundation of China [grant number 8110036] and the Science Foundation of Health Department of Heilongjiang Province [grant number 2009-250 (to S.S.L.)].

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Received 24 April 2012/26 February 2013; accepted 13 March 2013
Published as BJ Immediate Publication 13 March 2013, doi:10.1042/BJ20120680

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SUPPLEMENTARY ONLINE DATA

microRNA-138 plays a role in hypoxic pulmonary vascular remodelling by targeting Mst1

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MATERIALS AND METHODS

Materials

Antibodies against procaspase-3, caspase-9, Bcl-2, phospho-Akt (Ser473), Akt, HIF-1α and β-actin were purchased from Santa Cruz Biotechnology. Polyclonal antibodies against Mst1 were purchased from Cell Signaling Technology. Anti-HIF-2α antibody was purchased from Boste Company. Antibodies against phospho-Bcl-2 (Ser61) and Bad, LY294002 [PI3K (phosphoinositide 3-kinase) inhibitor], ZDK (caspase inhibitor), JC-1 probe, the TUNEL cell apoptosis detection kit, Hoechst 33258 and caspase-3 activity kit were obtained from Beyotime Institute of Biotechnology. The antibody against phospho-Bad (Ser136) was purchased from Bioword Technology. Enhanced chemiluminescence reagents were from Amersham International. All other reagents were purchased from common commercial sources.

Histological staining

The lung tissues were harvested from rat models and infused with 4% paraformaldehyde overnight for fixation after washing thoroughly with PBS. The fixed tissues were stained with H/E and viewed with a Nikon Eclipse microscope (TS100). Results were described as the ratios of intima to media.

Synthesis of miRNAs and anti-miRNAs

To regulate the expression of miRNAs in PASMCs, double-stranded Mins and Amos, whose ends were bridged by a methylene bridge between the 2′-O- and the 4′-C atoms, were synthesized by GenePharma. A scrambled RNA was used as the NC. The transfection efficiency was evaluated using NC-FAM [5′-FAM (carboxyfluorescein)-labelled scrambled oligonucleotides] (GenePharma) (Table 2 of the main text).

Synthesis of anti-Mst1 oligonucleotides (si-Mst1) and anti-HIF1 oligonucleotides (si-HIF1α and si-HIF2α)

To rescue the effect of miR-138 on PASMCs, the siRNAs (sense, 5′-GGCACACGUGACUGUAUGUTT-3′; antisense, 5′-GUCAUGUAUGUUGCUGCGCTT-3′) that targeted rat HIF1α (GenBank® accession number NM_023090) mRNAs (sense, 5′-GGGCCGUUCAAUUAUUAUCUUGUTT-3′; antisense, 5′-ACAAGUUCCAGUGU-CCTT-3′) and that targeted rat HIF2α (GenBank® accession number NM_023522) mRNAs (sense, 5′-GGACCAGACUGA-AUCUUGUTT-3′; antisense, 5′-ACAAGUUCAGUCUGCCTT-3′) were synthesized by GenePharma and are named si-HIF1α and si-HIF2α respectively.

A non-targeted control siRNA (sense, 5′-UUUCGGAACGUGUCACGUTT-3′; antisense, 5′-ACGUGACAGUCCG-AGAATT-3′) served as a NC.

MTT assay

The cells were incubated for 4 h in the medium with MTT at a final concentration of 0.5 mg/ml. The reaction was terminated by adding 150 μl of DMSO to the medium. The absorbance was read at 540 nm in a spectrophotometer.

Mitochondrial depolarization assay

Mitochondrial membrane potential was measured with the JC-1 probe to detect the mitochondrial function. Mitochondrial membrane potential was determined by relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using Olympus fluorescent microscope (BX51) at 488 nm excitation. Mitochondrial depolarization was indicated by an increase in the green/red fluorescence intensity ratio.

TUNEL assay

The TdT-mediated TUNEL method was performed to label the 3′-end of fragmented DNA of the apoptotic PASMCs. The cells cultured in a 6-well plate were treated as mentioned before, fixed with 4% paraformaldehyde phosphate buffer saline for 1 h at room temperature, rinsed with PBS and then permeabilized by 0.1% Triton X-100 for 2 min on ice followed by TUNEL working solution for 1 h at 37°C. The FITC-labelled TUNEL-positive cells were imaged by a Olympus fluorescent microscope (BX51) at 488 nm excitation and 530 nm emission. The cells with green fluorescence were defined as apoptotic cells.

Measurement of caspase-3 activity

Caspase-3 activity was measured by cleavage of its substrate Ac-DEVD-pNA [acetyl-Asp-Glu-Val-Asp pNA (p-nitroanilide)] and producing yellow pNA. The absorbance of yellow pNA was measured using a spectrometer at 405 nm. The specific caspase-3 activity, which was normalized for total proteins of cell lysates, was then expressed as fold of the baseline caspase-3 activity of control cells.

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Figure S1  Location and expression of hypoxia-induced miR-138

(A) Localization of miR-138 by in situ hybridization in lung tissues from normoxic and hypoxic rats. Scale bars are 100 and 50 μm as indicated. (B) Quantitative analyses of positive staining per vascular area. Hyp, hypoxia; Nor, normoxia. *P < 0.05 (n = 3 animals for each group).

Figure S2  Representative fluorescent images of miR-138 revealed the increase of TUNEL-positive cells induced by H₂O₂ administration

The bars represent quantitative analysis of TUNEL-positive cell content among different groups. Scale bars are 100 μm. A, Amo-138; Ctl, control; M, miR-138. *P < 0.05, **P < 0.01 (n = 3–6 separate experiments).

Figure S3  miR-138 suppressed the cleavage of procaspase-3 and -9 in apoptotic PASMCs

(A–C) Cleavage activation of procaspase-3 or -9 were detected using Western blotting using an antibody against procaspase-3 and -9 or caspase-9. A, Amo-138; Ctl, control; M, miR-138; proCASP, procaspase. *P < 0.05 (n = 3–6 separate experiments). Molecular masses in kDa are indicated on the Western blots.
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Figure S4  Effect of miR-138 on the activity of Bcl-2/Bad

(A–D) Expression of Bcl-2/phospho-Bcl-2 and Bad/phospho-Bad in different groups was detected using Western blot analysis. A, Amo-138; Ctl, control; M, miR-138; pBad, phosphorylation of Bad at Ser136; pBcl-2, phosphorylation of Bcl-2 at Ser70. *P < 0.05, **P < 0.01 (n = 3–6 separate experiments). Molecular masses in kDa are indicated on the Western blots.

Dual luciferase reporter gene construction

A fragment of the Mst1 (AATCCATGCTCTCTGTGTTAGA-CGGAGGTCTCTTCCACCAGCTGACACTGACCCAACT-TGGAA) containing the predicted binding site for rno-miR-138 was synthesized and subcloned downstream of the Renilla luciferase reporter gene of the pGL3-promoter luciferase expression reporter vector (Invitrogen) and named as pGL3-Mst1 3′-UTR reporter (Figure S3).

A mutated fragment of the Mst1 (AATCCATGCTCTCTGTGTTAGA-CGGAGGTCTCTTCCACCCAAGCTGACACTGACCCAACT-TGGAA) was synthesized and subcloned downstream of the Renilla luciferase reporter gene of the pGL3-promoter luciferase expression reporter vector and named as Mut pGL3-Mst1 3′-UTR reporter (Figure S4).

Synthesis of miR-138 expression vector

The precursor dsDNA (double-stranded DNA) encoding mature rno-miR-138 was synthesized and subcloned into pcDNA™6.2-GW/EmGFPMiR expression vector (Invitrogen) and named as pcDNA6.2-miR-138 (Figure S5). pcDNA™6.2-GW/EmGFPMiR expression vector containing a scrambled miRNA was used as a NC (pcDNA6.2-NC).

Luciferase assay

Mst1 3′-UTR or mutant Mst1 3′-UTR and NC or miR-138 were co-transfected into HEK-293 cells. A scrambled miRNA was used as a NC for miR-138. Luciferase activities were measured with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507). Briefly, HEK-293 cells, chosen on the basis of their low endogenous expression of miRNAs, were grown to 90% confluence in white 6-well plates in DMEM supplemented with 10% (v/v) FBS at 37°C under 5% CO2. Cells were co-transfected with different constructs, which were divided into seven groups: (i) 250 ng of pGL3-Mst1 3′-UTR, 750 ng of pcDNA3.1(+) vector and 25 ng of pRL-TK (TK-driven Renilla luciferase expression vector); (ii) 250 ng of pGL3-Mst1 3′-UTR, 750 ng of pcDNA6.2-NC and 25 ng of pRL-TK; (iii) 250 ng of pGL3-Mst1 3′-UTR, 750 ng of pcDNA6.2-miR-138 and 25 ng of pRL-TK; (iv) 250 ng of Mut pGL3-Mst1 3′-UTR, 750 ng of pcDNA6.2-miR-138 and 25 ng of pRL-TK; (v) 250 ng of pGL3-vector, 750 ng of pcDNA3.1(+) vector and 25 ng of pRL-TK; (vi) 250 ng of pGL3-vector, 750 ng of pcDNA6.2-miR-138 and 25 ng of pRL-TK; and (vii) 250 ng of pGL3-vector, 750 ng of pcDNA6.2-NC and 25 ng of pRL-TK, for 5 h in serum and antibiotics-free Opti-MEM with Lipofectamine™ 2000. A total of 25 ng of pRL-TK was transfected for normalization and as an internal control for transfection efficiency. A scrambled miRNA (NC) was used as a negative control for miR-138. pcDNA3.1(+) vector (Invitrogen) was used as a NC of the pcDNA™6.2-GW/EmGFPMiR expression vector. The empty vectors cotransfected with/without NC or miR-138 (v–vii) served as NCs of the luciferase assay system. Following cultivation in DMEM [10% (v/v) FBS] for another 48 h, luciferase activities were measured with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507).
Figure S5  Expression vector construts

(A) Illustration of the construction of the pGL3-promoter luciferase expression reporter vector carrying the 3′-UTR of Mst1. A fragment of the Mst1 containing the predicted binding site for rno-miR-138 was subcloned downstream of the Renilla luciferase reporter gene of the pGL3-promoter luciferase expression reporter vector. (B) Schematic illustration of the construction of the pGL3-promoter luciferase expression reporter vector carrying the mutated 3′-UTR of Mst1. A fragment of the Mst1 containing the predicted binding site for rno-miR-138 was mutated by three sites (labelled in grey) and subcloned into the downstream of the Renilla luciferase reporter gene of the pGL3-promoter luciferase expression reporter vector. (C) Schematic illustration of the construction of the pcDNA6.2-GW/EmGFPMiR expression vector carrying miR-138. The precursor DNA encoding miR-138 was inserted into pcDNA6.2-GW/EmGFPMiR expression vector (Invitrogen) named as pcDNA6.2-miR-138.