FEBS Letters 588 (2014) 4677–4685

journal homepage: www.FEBSLetters.org

MiR-20a regulates the PRKG1 gene by targeting its coding region in pulmonary arterial smooth muscle cells ☆

Yan Zeng [a,b], Yanping Pan [a], Hongtai Liu [a], Kang Kang [a], Yike Wu [a], Gang Hui [c], Wenda Peng [b], Ramaswamy Ramchandran [d], J. Usha Raj [d], Deming Gou [a,d,*]

[a] Shenzhen Key Laboratory of Microbial Genetic Engineering, College of Life Sciences, Shenzhen University, Shenzhen, Guangdong 518060, China
[b] Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Optoelectronic Engineering, Shenzhen University, Shenzhen, Guangdong, China
[c] Department of Chest Surgery, Peking University Shenzhen Hospital, Shenzhen, Guangdong 518000, China
[d] Department of Pediatrics, University of Illinois at Chicago, Chicago, IL 60612, USA

ARTICLE INFO

Article history:
Received 3 September 2014
Revised 22 October 2014
Accepted 29 October 2014
Available online 13 November 2014

Edited by T. Dalmay

Keywords:
MicroRNA
cGMP-dependent protein kinase
Coding region
Pulmonary arterial smooth muscle cells
Hypoxia

ABSTRACT

Chronic hypoxia triggers pulmonary vascular remodeling, which is associated with de-differentiation of pulmonary artery smooth muscle cells (PASMC). Here, we show that miR-20a expression is up-regulated in response to hypoxia in both mouse and human PASMC. We also observed that miR-20a represses the protein kinase, cGMP-dependent, type I (PRKG1) gene and we identified two crucial miR-20a binding sites within the coding region of PRKG1. Functional studies showed that miR-20a promotes the proliferation and migration of human PASMC, whereas it inhibits their differentiation. In summary, we provided a possible mechanism by which hypoxia results in decreased PRKG1 expression and in the phenotypic switching of PASMC.
© 2014 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Pulmonary arterial hypertension (PAH) is a devastating, life-threatening disease characterized by pulmonary vascular remodeling, leading to a progressive increase in pulmonary vascular resistance, right ventricular failure and ultimately death [1]. Although the specific mechanisms responsible for the development of PAH remain unclear, pulmonary vascular remodeling involving abnormal proliferation, migration and dedifferentiation of pulmonary arterial smooth muscle cells (PASMC) from a contractile, differentiated phenotype to a synthetic, undifferentiated phenotype, is the key feature of PAH pathology [2]. A number of factors play roles in the abnormal proliferation and dedifferentiation of PASMC among which hypoxia is an important stimulus.

Zhou et al. have demonstrated that the decreased expression levels of smooth muscle cell contractile markers in hypoxia were strongly related to a decrease in cGMP-dependent protein kinase (PKG) expression and that this effect of hypoxia can be reversed by PKG overexpression [3]. PKG is a serine/threonine-specific protein kinase in the nitric oxide/cGMP signaling pathway and plays critical roles in regulating cardiovascular and neuronal functions in addition to relaxing smooth muscle tone, preventing platelet aggregation, and modulating cell growth [4]. Mammalian PKG exists in two major forms: PKG-1, a soluble enzyme consisting of α and β isoforms derived from alternative splicing from one gene, that differ in the amino-terminal domain (through residues 89 and 104, respectively) and therefore contain identical catalytic domains; and PKG-2, a membrane-associated form derived from another gene [5,6]. Type 1 PKG, encoded by PRKG1 gene, is the only PKG expressed in vascular smooth muscle cells [7]. Boerth et al. [7] and Lincoln et al. [8] have described the roles of PKG in regulation of aortic smooth muscle cell phenotype and in vascular disease. PRKG1-transfected smooth muscle cells (SMC) express several-fold higher levels of the contractile protein α-SMA and have a lower proliferation rate relative to untransfected SMC [9]. We have

☆ This study was supported in part by National Natural Science Foundation of China No. 81170047 and 81370151 (to D.G.); National Basic Research Program of China (973 Program, 2012CB124701) and Shenzhen overseas high-level talents innovation program No. YFZZ20111009 (to D.G.).
Author contributions: D.G. and Y.Z. conceived the study and directed the experiments. J.R. and R.R. guided the manuscript revision. H.L. and Y.P. conducted experiments on sample preparation. Y.P., K.K., Y.W. and Y.Z. performed the experiments. G.H. and W.P. contributed reagents/materials/analysis tools. Y.Z. and D.G. analyzed the data and coauthored the paper. All authors approved the manuscript.
* Corresponding author at: College of Life Sciences, Shenzhen University, Nanhai Ave 3688, Shenzhen, Guangdong 518060, China. Fax: +86 755 2653427.
E-mail address: dmgou@szu.edu.cn (D. Gou).

http://dx.doi.org/10.1016/j.febslet.2014.10.040
0014-5793/© 2014 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

reported that both in short-term and chronic hypoxia PKG expression and activity are reduced in pulmonary vascular smooth muscle cells [5,10]. However, it remains unclear how the functional and transcriptional responses of PKG to hypoxia are mediated.

MicroRNA (miRNA), a class of evolutionarily conserved small non-coding RNAs, have been identified as essential mediators in a variety of cellular processes [11,12]. miR-20a belongs to the miR-17–92 cluster, which is a conserved gene encoding seven miRNA and is located on chromosome 13q31 in the intron of chr13orf25 [13]. This cluster is highly expressed in embryonic stem cells [14] and plays a role in many malignancies, including B-cell lymphomas [15], prostate carcinoma [16], gastric cancer [17], and lung cancer [18]. There is a growing mass of evidence suggesting that miR-20a is involved in cell proliferation and cell cycle regulation [19,20]. A recent study reported that miR-20a, via the STAT3-miR-17/92 pathway, participates in the regulation of morphogenetic protein receptor type 2 (BMPR2), which is a key determinant of idiopathic familial pulmonary hypertension [21]. Subsequent work revealed that antagomiR directed against miR-20a could restore functional BMPR2 signaling and prevent vascular remodeling in hypoxia-induced pulmonary hypertension [22].

It is now widely accepted that miRNA modulate the expression of their target genes in mammalian cells by forming imperfect duplexes at the 3′-UTR of mRNA [23,24]. More recently, it has been demonstrated that miRNA can also regulate target mRNA by binding to its coding region [25,26] and several studies have depicted the functional role of mammalian miRNA-mRNA interaction within the coding regions [26–29]. Here we demonstrate that human miR-20a can regulate PRKG1 expression post-transcriptionally through its interaction with two conserved sites within the protein coding sequence and play a pro-proliferation, pro-migration and anti-differentiation role in PASMC.

2. Materials and methods

2.1. Cell culture

HPASMC were maintained in SmGM-2 BulletKit Media (Lonza, Basel, Switzerland) containing 5% FBS, growth factors, and antibiotics. Hypoxia was achieved by using a special hypoxia incubator with a gas mixture of 5% CO_2 and balance nitrogen to keep oxygen concentrations at 3%.

2.2. Human serum collection

Serum samples were collected from healthy participants and patients with congenital heart disease-associated pulmonary arterial hypertension (CHD-PAH) at the Sun Yat-Sen Cardiovascular Hospital (Shenzhen, China). The blood samples were centrifuged at $3000 \times g$ for 10 min at 4 °C and stored at −80 °C. The study was approved by the ethics committee of Sun Yat-Sen Cardiovascular Hospital. All subjects who participated in the study provided written informed consent.

2.3. Quantitative RT-PCR

Total RNA isolation was performed as previously described [30]. Change in expression of miR-20a was determined using the S-Poly (T) method, as previously reported by us [31]. Sno202 and Sno44 were used as internal controls in mouse and human samples respectively. PRKG1 mRNA levels were determined using SoFast-EvaGreen Supermix (Bio-Rad, Hercules, CA) on a StepOnePlus Real-Time PCR System (Applied Biosystems), normalized to the internal control of ribosomal protein L19 (RPL19). Primers used for reverse transcription and quantitative PCR are as follows: miR-20a, RT primer (5-GTG CAG GGT CCG AGG TCA GAG CCA CCT GGG CAA TTT TTT TTT TTC TAC CT-3) and forward primer (5-CCG GGT AAA GTG CTT ATA GT-3); sno44 RT primer (5-GTG CAG GGT CCG AGG TCA GAG CCA CCT GGG CAA TTT TTT TTT TTA GTC AG-3) and forward primer (5-TGG CCT GGA TGA TGA TAA GCA-3); sno202 RT primer (5-GTG CAG GGT CCG AGG TCA GAG CCA CCT GGG CAA TTT TTT TTT TTC ATC AG-3) and forward primer (5-GTA CTT TTG AAC CCT TTT CCA T-3); PRKG1, sense (5-CAG GAA AAG TGT TTG GGG AA-3) and antisense (5-TCG GTA TGC TTG ATG AGT CCT-3); RPL19, sense (5-ATC ATC CGC AAG CCT GTG-3) and antisense (5-TGA CCT TCT CTG GCA TTC G-3).

2.4. MiR-20a inhibition and overexpression

To knockdown endogenous miR-20a, anti-miR-20a inhibitors and negative miRNA inhibitor control (Exiqon, Vedbaek, Denmark) were used to transfect cells with Lipofectamine 2000 (Invitrogen). Overexpression of miR-20a in HPASMC was generated using a lentiviral expression system (Clontech, Mountain View, CA). The pri-miR-20a was amplified from human genomic DNA with the forward primer (5-CAC CTC GAG CCT GCT ATT TCC TTC AA-3) and reverse primer (5-GAG AAT TCA GTA ACA GGA CAG TTT G-3). Cells stably overexpressing miR-20a or its negative control (without miRNA sequence) were generated by puromycin (1.5 μg/ml) selection.

2.5. Western immunoblot analysis

Total protein isolation and Western blot analysis were performed as previously described [30]. Protein bands were then probed with antibodies against MHC (ProteinTech, Chicago, IL), PKG (Stressgen Biotechnologies, Victoria, Canada), p-VASP (Cell Signaling Technology, Danvers, MA), β-tubulin, c-Myc, SM22 (Abcam, Cambridge, MA), calponin, α-SMA (Sigma Aldrich), smoothelin (Santa Cruz Biotechnology, Santa Cruz, CA) or DsRed2 (Clontech). Band intensities were quantified using the NIH-ImageJ software.

2.6. Luciferase reporter gene assay

Interaction of miR-20a with the PRKG1 promoter and 3′-UTR was analyzed by a dual reporter gene system, using a firefly luciferase construct (pGL3-Luc) and a reference Renilla luciferase construct (pRL-TK). PRKG1 promoter driven firefly luciferase vector was kindly provided by Dr. Tom Lincoln (University of South Alabama). PRKG1 3′-UTR was cloned from genomic DNA with the forward primer (5-CCG AAT TCT GTA TTT CTC TTA CCT GCT TC-3) and reverse primer (5-CAC ACT AGT GGA CTC AGT TTA ATT TGT GG-3). Firefly and Renilla luciferase activity was measured independently in cell extracts with a Lumat LB9508 luminometer (Berthold, Bad Wildbad, Germany).

2.7. Immunocytochemistry

Cells grown on chamber slides were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Pretreated cells were then incubated with a primary antibody against α-SMA (Sigma Aldrich). Indirect immunofluorescence was observed after incubation with a secondary Alexa 488-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), with DAPI stained for detecting nuclei.

2.8. Cell proliferation and migration assay

HPASMC proliferation and migration were determined by EdU incorporation assay and wound-healing assay, respectively. EdU

assay was performed by using an EdU Assay Kit (Ribobio, Guangzhou, China) according to the manufacturer's instructions. Wound-healing assay was performed as previously described [30]. Cell migration was assessed at 12 h by measuring the decreased width of the scratch. Data were presented as percentage of controls.

2.9. Statistical analysis

All data shown are mean values of at least three experiments, each performed in triplicate, with standard errors (SE). The differences between groups were analyzed using the double-sided Student's *t* test or One-way ANOVA and a *P* value less than 0.05 was considered significant.

3. Results

3.1. Hypoxia induces miR-20a expression in lung, pulmonary artery and serum

Mice were exposed to chronic hypoxia (10% O_2) for 3 weeks to induce pulmonary hypertension as previously described [32]. Expression of hypoxia-inducible factors (HIF) in lung tissue, endogenous marker for hypoxic effect identification, was analyzed by Western blotting. HIF-1α and HIF-2α expression increased with duration of exposure to hypoxia (Fig. 1A). Quantitative real-time PCR (qRT-PCR) assay revealed that miR-20a expression in the lungs increased with duration of exposure to hypoxia. At 1 and 2-weeks of hypoxia, miR-20a level was about 2-fold higher compared with normoxic controls (Fig. 1B). The relative expression of miR-20a was also assessed in isolated pulmonary arteries. Results showed that miR-20a levels were increased over 3-fold after 2-weeks hypoxic treatment compared with normoxic controls (Fig. 1C). It indicated that miR-20a might be involved in the pathological changes of pulmonary vascular in PAH. Serum miRNAs are currently regarded as promising biomarkers to be applied in clinical diagnosis of PAH [33]. As shown in Fig. 1D, upregulated serum miR-20a levels were found in CHD-PAH patients compared with healthy subjects by using qRT-PCR analysis. This result further highlighted miR-20a as a potential circulating biomarker at least for CHD-PAH.

3.2. MiR-20a is upregulated by hypoxia in HPASMC

To determine whether hypoxia-induced increase in expression of miR-20a was also observed in HPASMC in vitro, we maintained HPASMC either in normoxia (21% O_2) or hypoxia (3% O_2) for 48 h and relative miRNA levels were measured by qRT-PCR. Western blotting showed that HIF-1α and HIF-2α protein levels were increased as anticipated (Fig. 2A). The expression level of miR-20a was also upregulated about 2-fold at 48 h compared with normoxia control (Fig. 2B). We then measured the mRNA and protein levels of PRKG1. As shown in Fig. 2C and D, after 48 h of hypoxia, the mRNA level of PRKG1 was reduced by about 19% and PKG protein level by 36%.

3.3. MiR-20a modulates the expression of PKG

To determine whether there is a direct relationship between decreased PKG and increased miR-20a expression in hypoxia, we investigated the effects of miR-20a overexpression and knockdown on PKG expression in HPASMC. Cells were transfected with recombinant miRNA-expressing lentivirus or chemically synthesized miRNA inhibitors. Transfection with miR-20a lentiviral particles resulted in a 9-fold increase in miR-20a level relative to its scramble control (miR-con) (Fig. 3A). On the other hand, inhibitors against miR-20a (anti-20a) significantly decreased miR-20a expression by about 90% (Fig. 3B). Overexpression of miR-20a led to a significant decrease to about 50% in PKG protein levels (Fig. 3C). Inhibition of miR-20a resulted in an increased expression of PKG in HPASMC by 1.7-fold compared to negative control (Fig. 3D). The results demonstrate that PKG is a likely downstream target of miR-20a.

3.4. MiR-20a regulates PRKG1 gene by directly targeting its coding region

To understand how miR-20a affects the expression of PKG, we measured PRKG1 3′-UTR activity when miR-20a was overexpressed using a luciferase assay. Luciferase reporter gene assay was performed in a 293T cell line. Enhanced expression of miR-20a

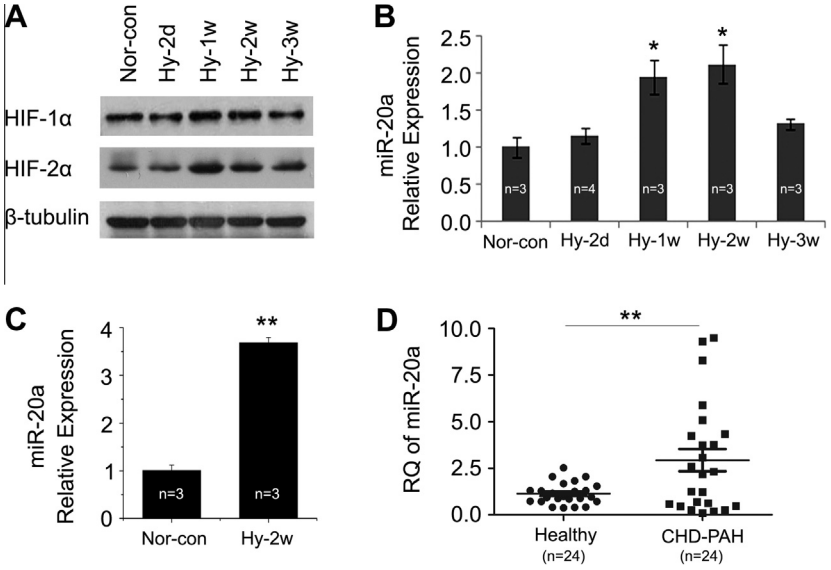

Fig. 1. Induction of miR-20a by hypoxia in mouse lungs. (A) Western blot of lung HIF-1α and HIF-2α from control mice in normoxia (Nor-con), and mice exposed to hypoxia for 2 days (Hy-2d), 1 week (Hy-1w), 2 weeks (Hy-2w) and 3 weeks (Hy-3w). β-tubulin levels served as an internal control. (B) Quantitative RT-PCR assay of miR-20a in mouse lungs during the time course of exposure to hypoxia ($n = 3$ or 4 for each time point). Data were analyzed using the ΔΔCt method by normalizing to endogenous sno202 and shown as mean ± SE, $^{*}P < 0.05$ vs Nor-con (One-way ANOVA). (C) The relative expression of miR-20a in pulmonary arteries of hypoxia-induced mice (2 weeks). Data are shown as mean ± SE, $^{**}P < 0.01$ (student's *t* test). (D) miR-20a expression is measured in the serum samples of CHD-PAH patients ($n = 24$) and is compared to the expression in the healthy controls ($n = 24$). $^{**}P < 0.01$ (student's *t* test).

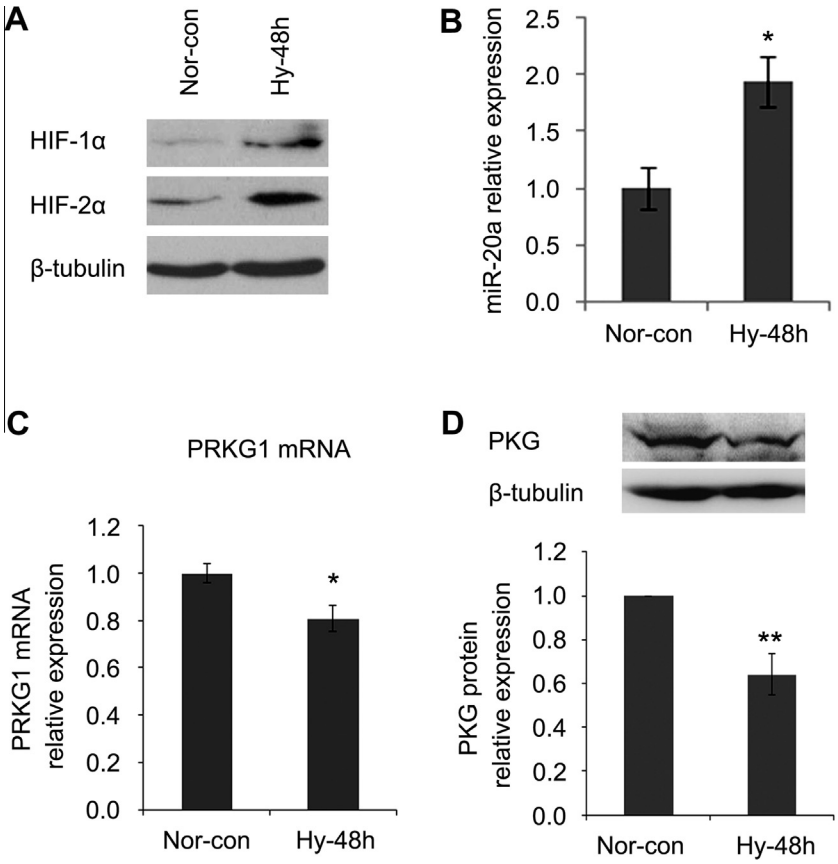

Fig. 2. Hypoxia causes increased miR-20a and decreased PKG expression in HPASMC. (A) Immunoblots of HIF-1α, HIF-2α and β-tubulin (as loading control) protein levels in HPASMC incubated in either normoxia or hypoxia for 48 h. (B) The relative quantity of miR-20a was estimated by real-time PCR using sno44 levels for normalization. (C) The relative mRNA levels of PRKG1 under normoxic or hypoxic conditions were normalized to RPL19 levels. Data are shown as mean ± SE relative to normoxia controls. (D) Representative Western blot for hypoxia-induced decrease in PKG, with β-tubulin as a loading control. Protein bands were quantified and bar chart showing relative protein levels was derived from triplicate samples. Data are shown as mean ± SE, $^{*}P < 0.05$ (student's t test).

had no influence on 3′-UTR activity of PRKG1 (Fig. 4A). Next, a PRKG1 promoter-driven luciferase assay was performed to identify whether an indirect miRNA–mRNA interaction exists. As shown in Fig. 4B, miR-20a did not have any effect on PRKG1 promoter activity. Next, we scanned through the entire PRKG1 gene transcript for potential miR-20a binding sites. By using RNA22 software (IBM) for analysis, we found two putative binding sites within the amino acid coding sequence (CDS) of PRKG1. As shown in the schematic diagram (Fig. 4C), the two predicted binding sites were located at 517–539 bp and 1058–1080 bp downstream of the transcriptional initiation site. Furthermore, the two miR-20a target sequences, especially the 'seed region' in the coding sequence of PRKG1, were found to be highly conserved among six species (Fig. 4D).

To determine whether the observed decrease in PRKG1 expression by miR-20a overexpression was dependent on the two putative target sites, we first constructed an cMyc-tagged wild type PRKG1 overexpression vector and then performed non-sense mutation as indicated in Fig. 4D (named as Mut 1 or Mut 2) to disrupt the recognition sites of miR-20a. The wild type and the two mutant types (Mut 1 and Mut 2) of cMyc-PRKG1 vectors were then co-transfected with miR-20a overexpression vector or control vector into the cells. Another control vector of pDsRed2-C1 expressing red fluorescent protein was also included to normalize the transfection efficiency. Importantly, mutated sites did not affect the amino acid of the fusion proteins, as verified by sequencing and immunoblot analysis (data not shown). Transient transfection and Western blot analysis revealed that either Mut 1 or Mut 2 could partly abrogate the miR-20a-mediated suppression of cMyc-PKG protein. However, Mut 1 mutation seemed to reduce the effect of miR-20a much more than Mut 2 based on the quantified protein bands (Fig. 4E). These results strongly suggest that miR-20a downregulates PRKG1 gene by interacting with the two binding sites within its coding region.

3.5. MiR-20a effects the proliferation, migration and phenotype of HPASMC

Adequate level of PKG expression is necessary to maintain vascular smooth muscle in a well differentiated contractile phenotype. Decreased PKG levels lead to increased cell proliferation and a dedifferentiated phenotype [3,34,35]. Therefore we investigated whether miR-20a was involved in regulating proliferation, differentiation, as well as migration of HPASMC, which are critical for vascular remodeling in the development of PAH. EdU incorporation assay was carried out to assess the effect of miR-20a overexpression or knockdown on cell proliferation. As show in Fig. 5A and B, the relative EdU positive cells in HPASMC that overexpressed miR-20a was significantly higher than in control cells (increased by 24% vs miR-con). Conversely, miR-20a silencing reduced the ratio of EdU positive cells by about 14% compared with anti-con. In addition, results showed the reverse impact of PKG, relative to miR-20a, on HPASMC proliferation (Fig. 5C and D).

Cell migration was assessed through a wound-healing assay. As shown in Fig. 6A and B, the decrease in the width of the scratched wound was larger in miR-20a transfected cells than that in control cells after 12 h, and silence of miR-20a led to a decrease in wound

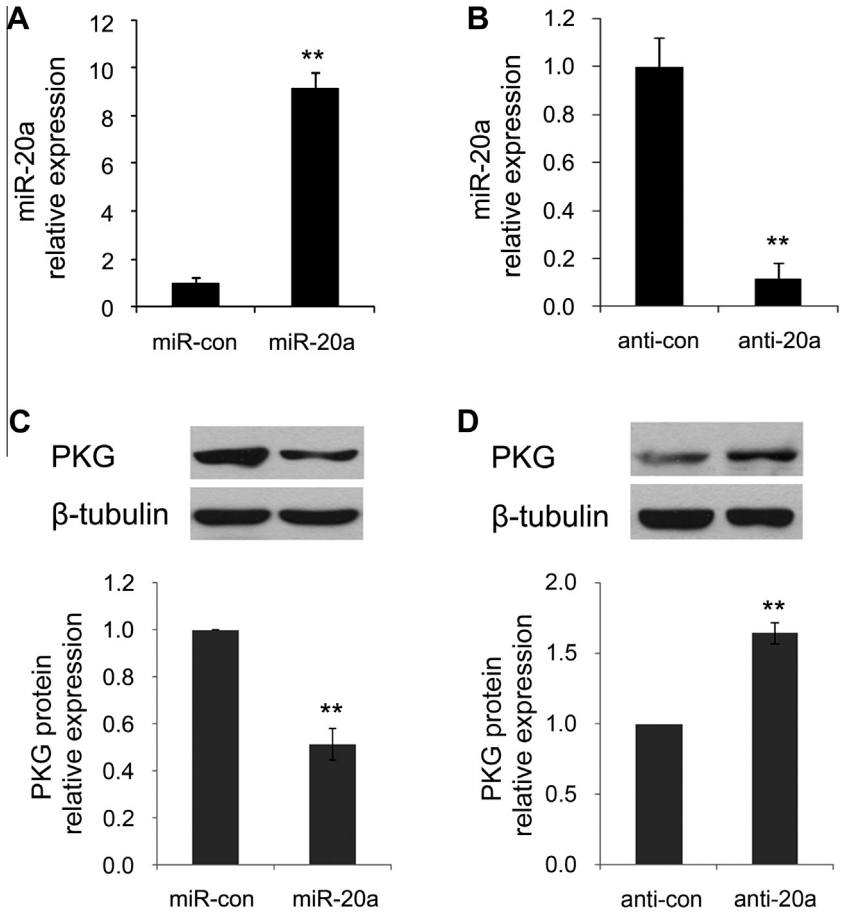

Fig. 3. MiR-20a represses the endogenous expression of PKG. (A) Quantitative real-time PCR showing increased expression of miR-20a in HPASMC transfected with recombinant lentiviral particles expressing miR-20a (miR-20a), compared with its vector control (miR-con). (B) Relative miR-20a levels measured by quantitative PCR in cells transfected with inhibitors against miR-20a (anti-20a) and its scramble control (anti-con). (C) Western blot showing miR-20a overexpression leading to decreased protein level of PKG (upper panel). Fold change is normalized to β-tubulin and shown in bar chart at the bottom. (D) Western blot analysis showing miR-20a knockdown increases PKG protein expression in HPASMC (upper panel). Relative quantification of PKG protein is shown at the bottom. Data are shown as mean ± SE, $^{**}P < 0.01$ (student's *t* test).

healing compared with anti-con transfection. The influence of PKG on cell migration was also analyzed. Results showed that enhanced expression of PKG significantly blocked the migration of HPASMC, while PKG inhibition conversely accelerate this process (Fig. 6C and D).

The expression of contractile phenotype markers were determined by Western blotting and immunocytochemistry. As shown in Fig. 7A and B, increased expression of miR-20a significantly reduced the protein levels of calponin, SM22, α-SMA, smoothelin and MHC. In contrast, miR-20a inhibition augmented smooth muscle restricted proteins expression, especially calponin and α-SMA. The level of PKG-mediated phosphorylation of vasodilator stimulated phosphoprotein (p-VASP) implied miR-20a could also cause repression on PKG activities. Meanwhile, PKG overexpression and knockdown induced changes were also analyzed to confirm its functional relevance to miR-20a. Immunocytochemistry staining for α-SMA revealed an expression pattern consistent with the findings from immunoblotting. MiR-20a overexpression revealed more cells with a round, relaxed appearance and with a low fluorescent signal for α-SMA. Transfection with miR-20a inhibitors led to cells with a high fluorescent signal for α-SMA (Fig. 7C and D).

4. Discussion

In this study, we have described a new mechanism by which PKG is post-transcriptionally downregulated in PASMC during hypoxia. We have also characterized some of the biological effects of miR-20a overexpression and knockdown in PASMC. Our data demonstrate that hypoxia results in increased expression of miR-20a, which leads to repression of PKG by the direct targeting of the PKG gene coding region by miR-20a, resulting in increased proliferation, migration and dedifferentiation.

MiRNA have long been shown to be important regulators of gene expression at the post-transcriptional level. In plants, miRNA primarily associate with protein coding regions by extensive base pairing. In contrast, in animals, miRNA have been shown to inhibit mRNA translation and to decrease the stability of mRNA by binding to the sequences in the 3′-UTR region [36]. However, there are a few studies that have shown the functional role of mammalian miRNA–mRNA interactions within the gene coding region [26,28,29]. Here we have discovered another example of CDS-associated miRNA regulation. We have shown that miR-20a significantly represses PKG protein expression in HPASMC. But we found no interaction between miR-20a and the PKG gene promoter or the 3′-UTR region, although there is a poorly conserved miR-20a binding site predicted in the PKG 3′-UTR. By using computational analysis and site mutation assays, we found two binding sites, located in the coding region of PRKG1 gene, are responsible for miR-20a recognition.

In mammals, miRNA typically bind to their targets with short binding sequences making it difficult to identify the binding sites based on the sequence alone. So, prediction of miRNA-mRNA interactions commonly relies on the evolutionary conservation of target sites [37,38], a method that is more important for sequences

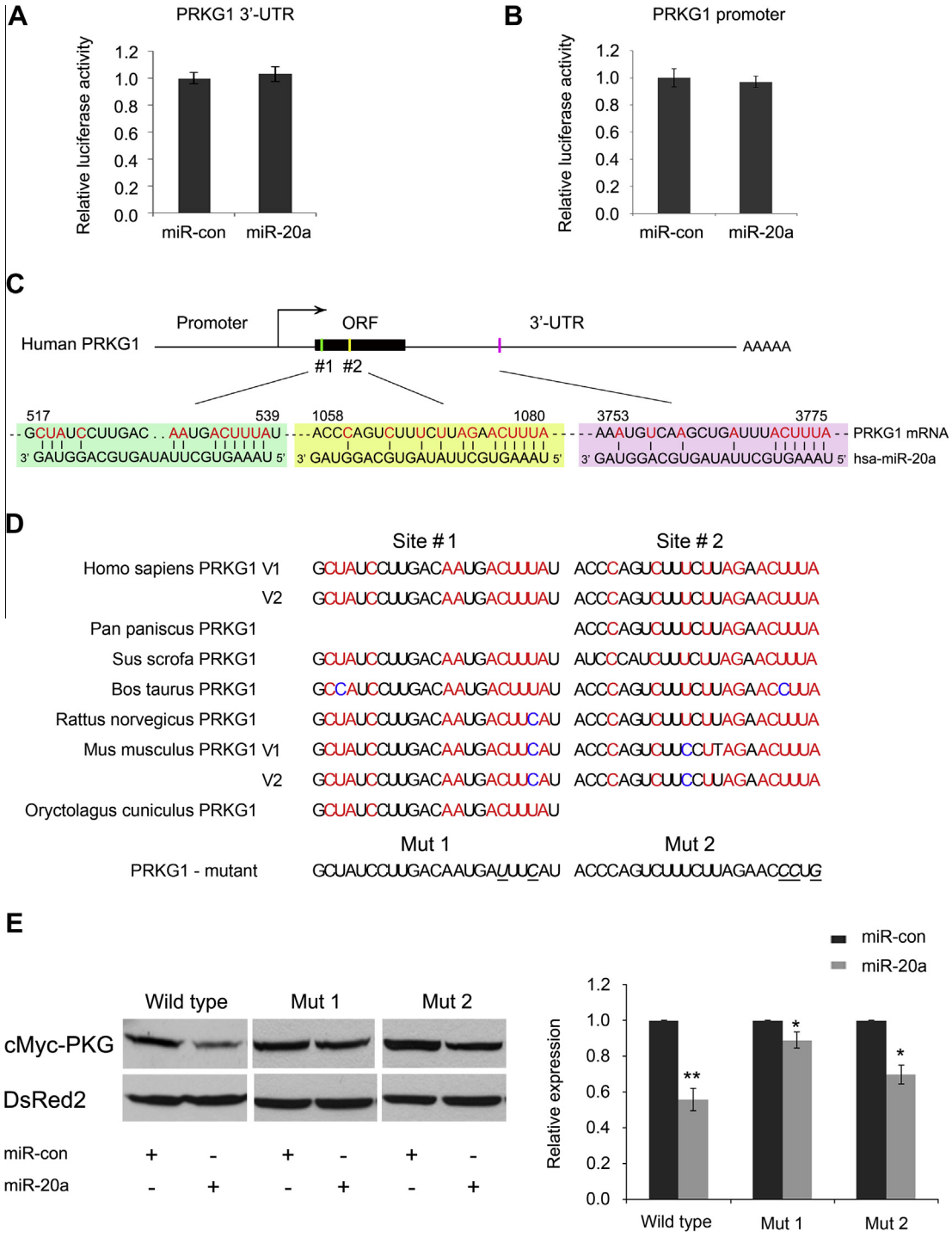

Fig. 4. MiR-20a targets the coding region of PRKG1. (A) Luciferase reporter assay to assess interaction between miR-20a and 3′-UTR of PRKG1. (B) Luciferase assay of PRKG1 promoter activity following miR-20a overexpression in 293a cells. (C) Nucleotide sequence of the human PRKG1 transcript containing the potential binding sites for miR-20a. There were two putative binding sites (site #1 and #2) predicted within the coding region and one binding site in the 3′-UTR. (D) Sequence conservation of the two predicted miR-20a binding sites within coding region of PRKG1 in six species. Red: paired bases; blue: A: C pair; underlined: mutant bases. (E) Western blot showing the effects of mutation of the binding site on miR-20a recognition. Wild type or the two mutated PRKG1 constructs (pcDNA3-cMyc-PRKG1-wt, pcDNA3-cMyc-PRKG1-mut1 or pcDNA3-cMyc-PRKG1-mut2) were co-transfected with miR-20a or its control overexpression vector along with pDsRed2-C1 vector. Exogenous cMyc-PKG protein levels were immunoblotted with cMyc antibody and quantified using co-transfected DsRed2 level for normalization. The fold change in protein expression is shown in bar chart on the right (mean ± SE). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs miR-con.

within the coding region [26]. In this study, the two miR-20a binding sites within PRKG1 coding region are highly conserved between variant transcripts, and among many species (chimpanzee, boar, cattle, rat, mouse, and rabbit), suggesting that these sequences are precisely retained through evolution and have a great potential for miRNA interaction.

During the process of pulmonary vascular remodeling, the dedifferentiation, migration and proliferation of PASMC are essential events happened in the early stage. Sheikh et al. have described the program of distal arteriole muscularization in hypoxia-induced PAH mice that encompasses SMC dedifferentiation, distal migration, proliferation, and then redifferentiation that ultimately lead

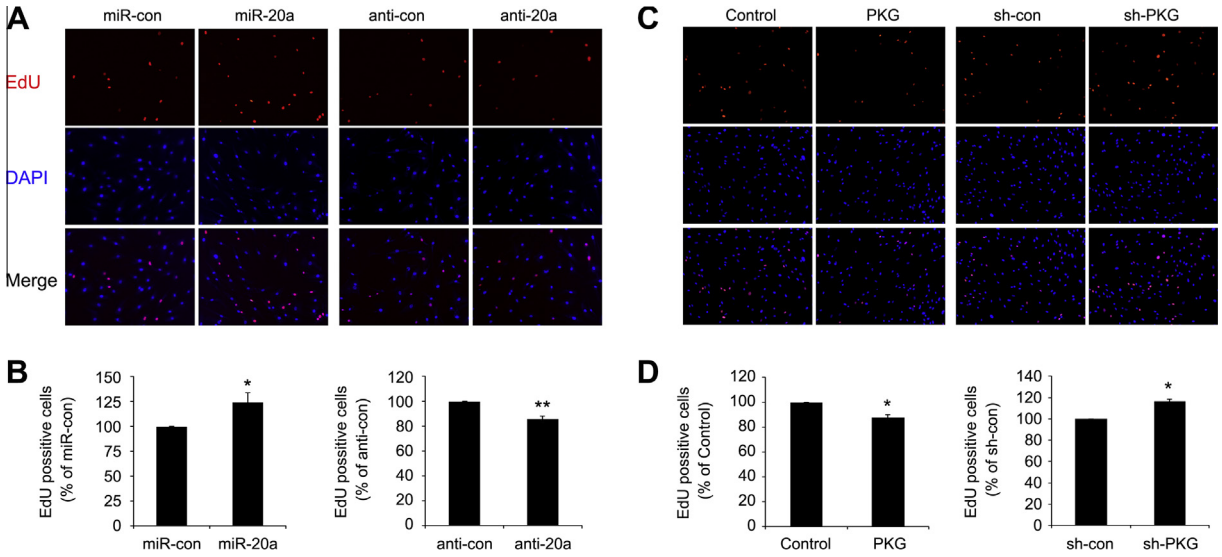

Fig. 5. MiR-20a promotes proliferation of HPASMC. (A) Representative images of EdU labeling showing proliferation of HPASMC either with miR-20a overexpression or knockdown. DAPI staining was used to identify all the cells. (B) Bar chart showing the relative EdU positive cells after 24 h. (C) Representative images of EdU labeling showing proliferation of HPASMC either with PKG overexpression or knockdown. Cells are transfected with recombinant lentiviral particles expressing PKG (PKG) or its vector control (Control), or shRNA against PKG (sh-PKG) and its scramble control (sh-con). (D) Relative EdU positive cells are shown in bar chart (mean ± SE). Data are derived from three independent experiments, with nine visual fields in triplicate samples. $^{*}P < 0.05$ and $^{**}P < 0.01$ (student's t test).

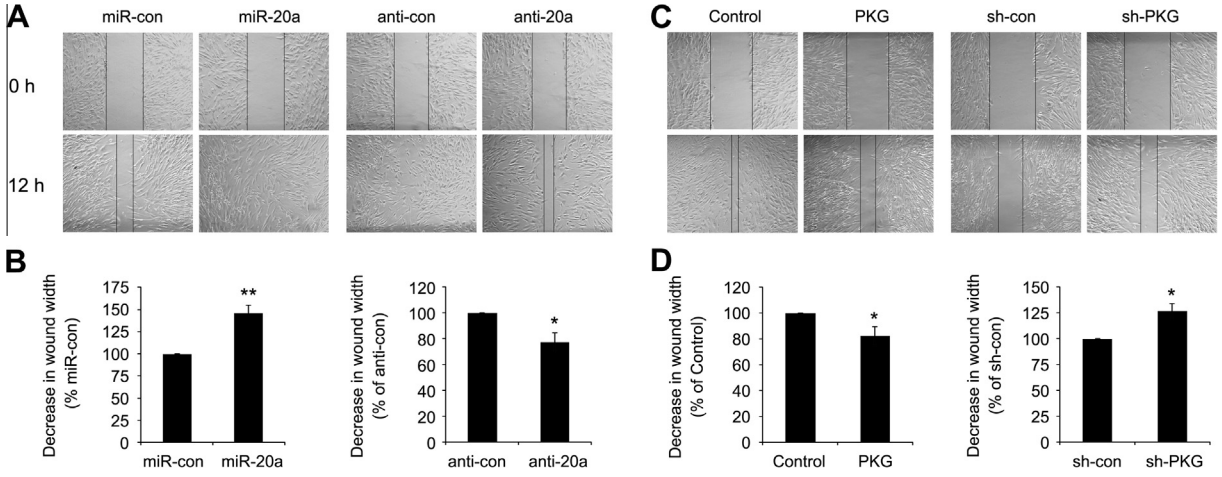

Fig. 6. MiR-20a promotes migration of HPASMC. (A) Representative images of wound-healing assay showing migration of HPASMC either with miR-20a overexpression or knockdown. (B) Bar chart showing relative decreased wound width after 12 h. (C) Representative images of wound-healing assay showing migration of HPASMC either with PKG overexpression or knockdown. (D) Bar chart of relative decreased wound width after 12 h. Data (mean ± SE) are derived from an average of three independent experiments, with three visual fields in triplicate samples. $^{*}P < 0.05$ and $^{**}P < 0.01$ (student's t test).

to muscularization of distal arteriole by differentiated and mature SMCs [39]. In our study, miR-20a levels in mouse lung showed marked increase from 1 to 2 weeks of hypoxia, and then returned to that of normoxia by 3 weeks. Therefore, the upregulation of miR-20a was almost consistent with the acceleration of SMC proliferation in response to hypoxia.

Brock et al. have reported their studies on the role of miR-20a in hypoxia-induced pulmonary hypertension in mouse model [22]. Pulmonary arterioles stained for α-SMA showed that antagomiR-20a injections significantly reduces the vessel occlusion caused by hypoxia and lessens the thickness of the vessel wall. They also demonstrated that the proliferation of cultured PASMC was repressed by antogomiR-20a transfection, which was accompanied by decreased protein level of cell cycle inhibitor p21. They demonstrated that the specific inhibition of miR-20a could restore functional levels of BMPR2 both in vivo and in vitro. Dysregulation of the BMPR2 is a hallmark feature that has been described in several forms of pulmonary hypertension [40–42]. It is also reported to be a potent inducer of contractile phenotype and mediate induction of contractile genes [43], and inhibition of PASMC growth and migration [44,45]. In this study, we showed that miR-20a could modulate the proliferation, migration and differentiation of human PASMC via specific repression of PRKG1. Our current results together with existing data in the literature provide strong evidence to support the specific impact of miR-20a in the pathological changes of PASMC in PAH.

PKG has long been shown to promote the maintenance of the contractile phenotype of smooth muscle cell and to slow cell proliferation. We have previously reported that in hypoxia PKG mRNA and protein levels are decreased but we had not determined the mechanism for this decrease. Hypoxia-induced increase in miR-20a may partly account for this effect.

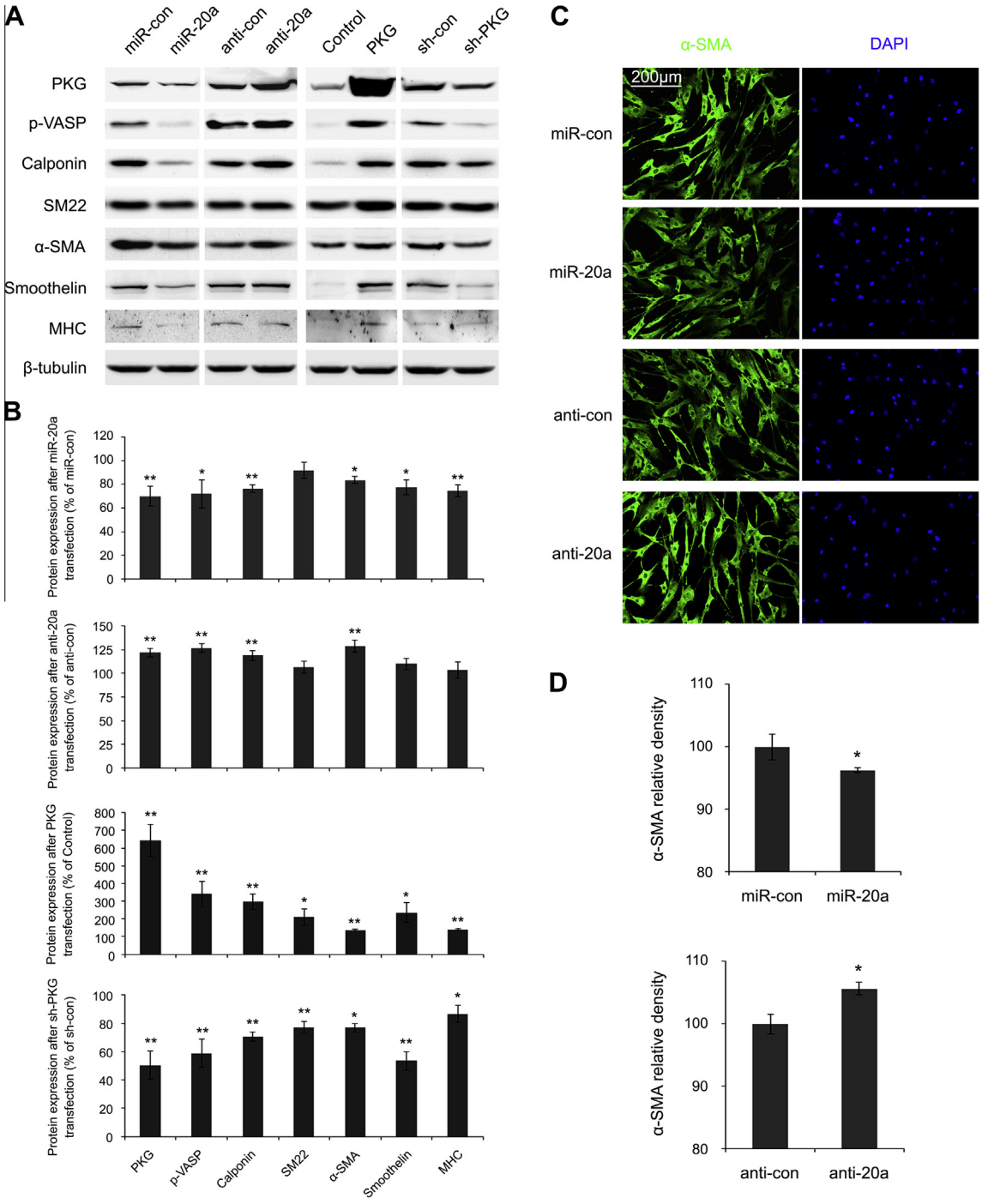

Fig. 7. MiR-20a downregulates the contractile protein expression in HPASMC. (A) Western blot of smooth muscle contractile proteins in miR-20a mimic or inhibitor transfected HPASMC (left panel), and in HPASMC with PKG overexpression or knockdown (right panel). β-tubulin levels served as an internal control. (B) Protein bands were quantified by normalization to β-tubulin and bar chart showing the relative protein levels in each manipulated group (vs. miR-con, anti-con, Control, and sh-con, respectively). Data (mean ± SE) were derived from four independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ (One-way ANOVA). (C) Immunocytochemistry showing effect of miR-20a overexpression or knockdown on α-SMA expression in HPASMC, with miR-con and anti-con included as a reference. DAPI staining was used to identify cell nuclei. Scale bar, 200 μm. (D) Relative α-SMA density is quantified and shown in bar chat. Data are derived from five visual fields in triplicate samples (mean ± SE). $^{*}P < 0.05$ (student's *t* test).

Acknowledgements

We would like to thank Thomas Lincoln, Ph.D. (University of South Alabama) for kindly providing the PRKG1 promoter luciferase reporter.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.10.040.

References

[1] Rabinovitch, M. (2007) Pathobiology of pulmonary hypertension. Annu. Rev. Pathol. 2, 369–399.
[2] Humbert, M. et al. (2004) Cellular and molecular pathobiology of pulmonary arterial hypertension. J. Am. Coll. Cardiol. 43, 13S–24S.
[3] Zhou, W., Dasgupta, C., Negash, S. and Raj, J.U. (2007) Modulation of pulmonary vascular smooth muscle cell phenotype in hypoxia: role of cGMP-dependent protein kinase. Am. J. Physiol. Lung Cell. Mol. Physiol. 292, L1459–L1466.
[4] Munzel, T., Feil, R., Mulsch, A., Lohmann, S.M., Hofmann, F. and Walter, U. (2003) Physiology and pathophysiology of vascular signaling controlled by guanosine 3′,5′-cyclic monophosphate-dependent protein kinase [corrected]. Circulation 108, 2172–2183.
[5] Negash, S., Gao, Y., Zhou, W., Liu, J., Chinta, S. and Raj, J.U. (2007) Regulation of cGMP-dependent protein kinase-mediated vasodilation by hypoxia-induced reactive species in ovine fetal pulmonary veins. Am. J. Physiol. Lung Cell. Mol. Physiol. 293, L1012–L1020.
[6] Sellak, H., Choi, C.S., Dey, N.B. and Lincoln, T.M. (2013) Transcriptional and post-transcriptional regulation of cGMP-dependent protein kinase (PKG-I): pathophysiological significance. Cardiovasc. Res. 97, 200–207.
[7] Boerth, N.J., Dey, N.B., Cornwell, T.L. and Lincoln, T.M. (1997) Cyclic GMP-dependent protein kinase regulates vascular smooth muscle cell phenotype. J. Vasc. Res. 34, 245–259.
[8] Lincoln, T.M., Dey, N.B., Boerth, N.J., Cornwell, T.L. and Soff, G.A. (1998) Nitric oxide–cyclic GMP pathway regulates vascular smooth muscle cell phenotypic modulation: implications in vascular diseases. Acta Physiol. Scand. 164, 507–515.
[9] Stegemann, J.P., Dey, N.B., Lincoln, T.M. and Nerem, R.M. (2004) Genetic modification of smooth muscle cells to control phenotype and function in vascular tissue engineering. Tissue Eng. 10, 189–199.
[10] Ramchandran, R., Pilipenko, E., Bach, L., Raghavan, A., Reddy, S.P. and Raj, J.U. (2012) Hypoxic regulation of pulmonary vascular smooth muscle cyclic guanosine monophosphate-dependent kinase by the ubiquitin conjugating system. Am. J. Respir. Cell Mol. Biol. 46, 323–330.
[11] Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.
[12] Ambros, V. (2004) The functions of animal microRNAs. Nature 431, 350–355.
[13] Lu, Y., Thomson, J.M., Wong, H.Y., Hammond, S.M. and Hogan, B.L. (2007) Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. Dev. Biol. 310, 442–453.
[14] Thomson, J.M., Parker, J., Perou, C.M. and Hammond, S.M. (2004) A custom microarray platform for analysis of microRNA gene expression. Nat. Methods 1, 47–53.
[15] He, L. et al. (2005) A microRNA polycistron as a potential human oncogene. Nature 435, 828–833.
[16] Pesta, M. et al. (2010) Importance of miR-20a expression in prostate cancer tissue. Anticancer Res. 30, 3579–3583.
[17] Liu, R. et al. (2010) A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis. Eur. J. Cancer 47, 784–791.
[18] Matsubara, H. et al. (2007) Apoptosis induction by antisense oligonucleotides against miR-17-5p and miR-20a in lung cancers overexpressing miR-17-92. Oncogene 26, 6099–6105.
[19] O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V. and Mendell, J.T. (2005) C-Myc-regulated microRNAs modulate E2F1 expression. Nature 435, 839–843.
[20] Pickering, M.T., Stadler, B.M. and Kowalik, T.F. (2009) MiR-17 and miR-20a temper an E2F1-induced G1 checkpoint to regulate cell cycle progression. Oncogene 28, 140–145.
[21] Brock, M. et al. (2009) Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. Circ. Res. 104, 1184–1191.
[22] Brock, M. et al. (2012) AntagomiR directed against miR-20a restores functional BMPR2 signalling and prevents vascular remodelling in hypoxia-induced pulmonary hypertension. Eur. Heart J.
[23] Filipowicz, W., Bhattacharyya, S.N. and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9, 102–114.
[24] Bushati, N. and Cohen, S.M. (2007) MicroRNA functions. Annu. Rev. Cell Dev. Biol. 23, 175–205.
[25] Tay, Y., Zhang, J., Thomson, A.M., Lim, B. and Rigoutsos, I. (2008) MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 455, 1124–1128.
[26] Forman, J.J., Legesse-Miller, A. and Coller, H.A. (2008) A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. Proc. Natl. Acad. Sci. U.S.A. 105, 14879–14884.
[27] Elcheva, I., Goswami, S., Noubissi, F.K. and Spiegelman, V.S. (2009) CRD-BP protects the coding region of betaTrCP1 mRNA from miR-183-mediated degradation. Mol. Cell 35, 240–246.
[28] Qin, W., Shi, Y., Zhao, B., Yao, C., Jin, L., Ma, J. and Jin, Y. (2010) MiR-24 regulates apoptosis by targeting the open reading frame (ORF) region of FAF1 in cancer cells. PLoS ONE 5, e9429.
[29] Yi, C. et al. (2011) MiR-143 enhances adipogenic differentiation of 3T3-L1 cells through targeting the coding region of mouse pleiotrophin. FEBS Lett. 585, 3303–3309.
[30] Sarkar, J., Gou, D., Turaka, P., Viktorova, E., Ramchandran, R. and Raj, J.U. (2010) MicroRNA-21 plays a role in hypoxia-mediated pulmonary artery smooth muscle cell proliferation and migration. Am. J. Physiol. Lung Cell. Mol. Physiol. 299, L861–L871.
[31] Kang, K. et al. (2012) A novel real-time PCR assay of microRNAs using S-Poly(T), a specific oligo(dT) reverse transcription primer with excellent sensitivity and specificity. PLoS ONE 7, e48536.
[32] Gou, D. et al. (2012) MiR-210 has an antiapoptotic effect in pulmonary artery smooth muscle cells during hypoxia. Am. J. Physiol. Lung Cell. Mol. Physiol. 303, L682–L691.
[33] Wei, C. et al. (2013) Circulating miRNAs as potential marker for pulmonary hypertension. PLoS ONE 8, e64396.
[34] Lincoln, T.M., Wu, X., Sellak, H., Dey, N. and Choi, C.S. (2006) Regulation of vascular smooth muscle cell phenotype by cyclic GMP and cyclic GMP-dependent protein kinase. Front. Biosci. 11, 356–367.
[35] Dey, N.B., Foley, K.F., Lincoln, T.M. and Dostmann, W.R. (2005) Inhibition of cGMP-dependent protein kinase reverses phenotypic modulation of vascular smooth muscle cells. J. Cardiovasc. Pharmacol. 45, 404–413.
[36] Llave, C., Kasschau, K.D., Rector, M.A. and Carrington, J.C. (2002) Endogenous and silencing-associated small RNAs in plants. Plant Cell 14, 1605–1619.
[37] Krek, A. et al. (2005) Combinatorial microRNA target predictions. Nat. Genet. 37, 495–500.
[38] Lewis, B.P., Burge, C.B. and Bartel, D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20.
[39] Sheikh, A.Q., Lighthouse, J.K. and Greif, D.M. (2014) Recapitulation of developing artery muscularization in pulmonary hypertension. Cell Rep. 6, 809–817.
[40] Lane, K.B., Machado, R.D., Pauciulo, M.W., Thomson, J.R., Phillips 3rd, J.A., Loyd, J.E., Nichols, W.C. and Trembath, R.C. (2000) Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. Nat. Genet. 26, 81–84.
[41] Machado, R.D. et al. (2009) Genetics and genomics of pulmonary arterial hypertension. J. Am. Coll. Cardiol. 54, S32–S42.
[42] Atkinson, C., Stewart, S., Upton, P.D., Machado, R., Thomson, J.R., Trembath, R.C. and Morrell, N.W. (2002) Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. Circulation 105, 1672–1678.
[43] Davis-Dusenbery, B.N., Chan, M.C., Reno, K.E., Weisman, A.S., Layne, M.D., Lagna, G. and Hata, A. (2011) Down-regulation of Kruppel-like factor-4 (KLF4) by microRNA-143/145 is critical for modulation of vascular smooth muscle cell phenotype by transforming growth factor-beta and bone morphogenetic protein 4. J. Biol. Chem. 286, 28097–28110.
[44] Long, L., Crosby, A., Yang, X., Southwood, M., Upton, P.D., Kim, D.K. and Morrell, N.W. (2009) Altered bone morphogenetic protein and transforming growth factor-beta signaling in rat models of pulmonary hypertension: potential for activin receptor-like kinase-5 inhibition in prevention and progression of disease. Circulation 119, 566–576.
[45] Dewachter, L. et al. (2009) Bone morphogenetic protein signalling in heritable versus idiopathic pulmonary hypertension. Eur. Respir. J. 34, 1100–1110.