Regulation and function of miR-214 in pulmonary arterial hypertension

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Abstract: Dysregulation of microRNAs (miRNAs) can contribute to the etiology of diseases, including pulmonary arterial hypertension (PAH). Here we investigated a potential role for the miR-214 stem loop miRNA and the closely linked miR-199a miRNAs in PAH. All 4 miRNAs were upregulated in the lung and right ventricle (RV) in mice and rats exposed to the Sugen (SU) 5416 hypoxia model of PAH. Further, expression of the miRNAs was increased in pulmonary artery smooth muscle cells exposed to transforming growth factor β1 but not BMP4. We then examined miR-214$^{-/-}$ mice exposed to the SU 5416 hypoxia model of PAH or normoxic conditions and littermate controls. There were no changes in RV systolic pressure or remodeling observed between the miR-214$^{-/-}$ and wild-type hypoxic groups. However, we observed a significant increase in RV hypertrophy (RVH) in hypoxic miR-214$^{-/-}$ male mice compared with controls. Further, we identified that the validated miR-214 target phosphatase and tensin homolog was upregulated in miR-214$^{-/-}$ mice. Thus, miR-214 stem loop loss leads to elevated RVH and may contribute to the heart failure associated with PAH.

Keywords: Sugen 5416 hypoxia model, transforming growth factor β1, microRNA-199, right ventricular hypertrophy.

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Pulmonary arterial hypertension (PAH) is a disease characterized by narrowing of the small pulmonary arteries, leading to vascular remodeling, an elevation in pulmonary artery pressure, right ventricular hypertrophy (RVH), and heart failure.[1] Current therapies for PAH aim to reverse the endothelial dysfunction and vasoconstriction observed.[2] However, despite these therapies, PAH mortality rates remain high, and the 3-year survival of patients is only 54.9%.[3]

Changes in the pulmonary vasculature are the primary cause of PAH; however, right ventricle (RV) function is a major determinant of the severity of symptoms and prognosis of pulmonary hypertension. Many therapies in development for PAH are focused on targeting the RV since heart failure is the ultimate cause of mortality in PAH.[4] PAH is predominant in females, with female : male ratios of 1.4–4.1 : 1.[5] Sexual dimorphism has also been observed in RV failure. Female PAH patients exhibit improved RV ejection fraction and survival compared with men.[6] This could be due at least in part to the protective effect of estrogen on RV function.[7,8]

MicroRNAs (miRNAs) are involved in multiple cellular responses during normal development and disease; they act as post-transcriptional regulators to fine-tune protein synthesis. Evidence has emerged for a key role for miRNA in regulation of the cellular processes involved in PAH. We previously demonstrated that a range of miRNAs are dysregulated in rats exposed to models of PAH.[9] Later studies have shown that miR-21, the miR-143/145 cluster, miR-27a, the miR-17-92 cluster, miR-124, miR-150, miR-138, miR-190, miR-204, miR-206, miR-210, and miR-328 play a role in the development of PAH.[10] Multiple miRNAs could potentially be targeted in concert as therapeutics in PAH.[11]

MicroRNA miR-214 is transcribed as a bicistronic primary transcript, which is processed to generate 4 separate mature miRNAs: the main strands miR-199-5p and miR-214-3p and their passenger strands miR-199-3p and miR-214-5p. Previously, miR-199-5p and miR-214-3p were shown to be significantly upregulated at the mature miRNA level in both the hypoxia and the moncrotaline rat models of PAH.[9] In cardiac tissue, miR-214 is a marker of stress,[12] and expression increases in RVH, heart failure, and ischemic injury in the heart.[13-15] The role of miR-214 in the heart is controversial, as one study suggests that miR-214 stem loop is protective in ischemia reperfusion injury,[13] whereas another suggests that miR-214 ablation is protective in heart failure.[15] Deletion of miR-214 leads to increased fibrosis, apoptosis, and decreased contractility after ischemic injury in the heart, a result of target derepression of the sodium/calcium exchanger member 1 (*NCX1*), leading to calcium overloading of

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cardiomyocytes.[13] However, inhibition of miR-199/214 using antimiRs in mice is protective through target derepression of peroxisome proliferator-activated receptor delta (*PPARδ*) and restoration of mitochondrial fatty acid oxidation in the heart.[15]

MicroRNA miR-214 also has functions in tissues other than the heart. It can inhibit angiogenesis through targeting the KH domain containing RNA-binding (*QKI*) transcript in a retinal developmental angiogenesis model in mice.[16] It can also function as both a tumor suppressor and an oncogene in various types of human cancer through influencing proliferation, migration, and apoptosis.[17-20] Therefore, though its effects may be varied, miR-214 can influence processes important in PAH in a cell type–specific manner.

MicroRNA miR-214 is induced by transforming growth factor beta 1 (TGF-β1)[21] and is upregulated in kidney and liver fibrosis.[21-24] While in the heart, miR-214 ablation can be protective or deleterious, depending on the injury; in the kidney, miR-214 knockout is protective against fibrosis.[25] TGF-β1 is a critically important mediator of pathophysiological events in PAH and fibrosis.[26-29] Many processes in PAH and fibrosis are known to be driven primarily by TGF-β1 along with a host of other cytokines and growth factors.[29] It is probable that these processes involve complex and interrelated molecular pathways in which miRNAs play an important role.

Because miR-214 is implicated in many of the processes associated with PAH pathology, we characterized the miR-214 knockout (miR-214$^{-/-}$) mouse. We used the Sugen (SU) 5416 hypoxia model to induce PAH and assessed the pathological hallmarks of disease. Despite observing no effect on vascular remodeling and RV systolic pressure (RVSP) in the miR-214$^{-/-}$ mice, we found a significant damaging effect on RVH in male but not in female miR-214$^{-/-}$ mice.

METHODS

RNA extraction, miRNA, and mRNA expression

Total RNA from pulmonary artery smooth muscle cells (PASMCs) was obtained using the miRNeasy kit (Qiagen, Hilden). Complementary DNA for miRNA analysis was synthesized from total RNA using specific stem-loop reverse-transcription primers (Life Technologies, Paisley). The mRNA/miRNA expression was assessed using specific primers by quantitative real-time polymerase chain reaction (qRT-PCR; Life Technologies) and normalized to a housekeeper. For gene expression, β-2-microglobulin (B2M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for mouse and human samples, respectively. For miRNA control, the small RNA U6 (mouse), U87 (rat), and RNU48 (human) were used. Results were expressed as fold change relative to the relevant control. The qRT-PCRs were run in triplicate, and results are presented as the mean ± standard error mean of samples. The miR-214 target genes for analysis were selected on the basis of previous knowledge of validated target genes.

Cell culture and stimulation

Single-donor human PASMCs were obtained from PromoCell (Heidelberg). Cells were used between passages 1 and 8. PASMCs were grown to 80% confluency in 6-well plates and quiesced in Dulbecco's modified eagle medium (DMEM) with 0.2% fetal bovine serum (FBS) for 24 hours before being stimulated or placed in a hypoxic chamber (5% O, 5% CO_2, 90% N_2) for 48 hours. The Smad signaling pathway was assessed by recombinant human TGF-β1 (0.1–10 ng/mL), BMP4 (50 ng/mL; R&D Systems, Minneapolis, MN), and the commercially available activin-like kinase (ALK) 5 specific inhibitor SB525334 (1 nm; Tocris Bioscience, Bristol, UK). Cells were quiesced in 0.2% FBS for 24 hours, followed by stimulation for 72 hours, and then the medium was changed to fresh stimulation medium for 48 hours (5 days stimulation in total).

Disease modeling in vivo

All protocols and surgical procedures were approved by the local animal care committee. Animal experiments were conducted in accordance with the Animals Scientific Procedures Act 1986 (United Kingdom). For the 2- and 3-week hypoxia models, the development of chronic hypoxic PAH in 8-week-old C57BL/6 mice was achieved with hypobaric hypoxia, as previously described.[30]

Wild-type (WT) littermate control and miR-214$^{-/-}$ and mice were used in this study (kindly gifted by Eric Olson, University of Texas Southwestern Medical Center).[13] PAH was established in male and female 8-week-old miR-214 WT and knockout mice by serial injection (days 0, 7, and 14) with the vascular endothelial growth factor Flk-1/KDR receptor inhibitor Semaxanib (SU 5416, 20 mg/kg subcutaneously; Sigma-Aldrich, Poole, UK) in combination with 21 days of continuous exposure to hypoxia. PAH was established in Wistar Kyoto rats by 1 injection (day 0) with SU 5416 (20 mg/kg subcutaneously) in combination with 2 weeks of continuous exposure to hypoxia, followed by 3 weeks in normoxic conditions.[31,32]

Hemodynamic measurements

RVSP and RV systemic pressure measurements were taken on day 21 before euthanasia, RVH assessment, and tissue harvest. Systemic arterial pressure was recorded via a cannula placed in the carotid artery, as previously described.[30] RVSP was measured under isoflurane (1.5% O_2) anaesthesia via a needle advanced into the RV transdiaphragmatically. RVH was determined as the ratio of the RV to the left ventricle plus septum (LV + S) weight.

Immunohistochemistry

Lungs were fixed in a 4% paraformaldehyde solution for 18 hours and embedded in paraffin. For lung α-smooth muscle actin (SMA) immunohistochemistry, anti-α-SMA (1 : 100; Abcam, Cambridge, MA) and control immunoglobulin G were used for detection. For remodeling analysis, distal vessels were assessed (≤80 μm), and a ratio of vascular wall thickness : vascular diameter was determined and used to define the extent of pulmonary vascular thickness and remodeling (10 distal pulmonary arteries were quantitatively analyzed from 6 animals per group).

For hematoxylin and eosin staining of the RV, sections were incubated with hematoxylin solution (Sigma-Aldrich) for 5 minutes and then rinsed in water, washed in 95% alcohol, and counterstained

in eosin Y solution (Sigma-Aldrich). For picrosirius red staining of the RV, sections were stained with Weigert's hematoxylin and solution B (Sigma-Aldrich) for 10 minutes at room temperature. Slides were washed and incubated in the dark with Sirius red F3B (0.1% w/v; Sigma-Aldrich) for 4 hours.

In situ hybridization

Detection of miR-214 in lung and RV tissue was carried out as previously described.[33] In brief, after sodium citrate antigen retrieval and blocking (50% formamide, 4× saline sodium citrate [SSC], 2.5× Denhardt's solution, 2.5 mg/mL salmon DNA, 0.6 mg/mL yeast transfer RNA, 0.025% sodium dodecyl sulfate, and 0.1% blocking reagent), 5-μm sections were incubated in blocking buffer overnight with 40 nM of miR-214 or scramble miRCURY LNA Detection probe, labeled with 3′ and 5′ digoxigenin (DIG; Exiqon, Denmark) at 60°C. After stringency washing with SSC buffer and phosphate-buffered saline (PBS) and blocking (1% blocking reagent and 10% fetal calf serum in PBS), immunodetection was performed with an anti-DIG antibody (Roche Applied Science, Indianapolis, IN) diluted 1 : 500 overnight. In order to stain miR-214, BM purple solution (Roche Applied Science, Mannheim) was added to each section respectively and left at room temperature for 5 hours.

Statistical analysis

Prior to experimental analysis, power calculations were utilized to ensure appropriately powered experiments. All qRT-PCR results are expressed as mean ± standard error of the mean (SEM) fold change, with all other results expressed as the mean ± SEM. All statistical calculations were carried out using GraphPad Prism or Excel. Student *t* tests were used when comparing two conditions, and a 2-way ANOVA with Bonferroni correction or a 1-way ANOVA followed by a Tukey post hoc test was used for multiple comparisons. Probability values of less than 0.05 were considered significant.

RESULTS

miR-214 is induced by TGF-β1 in PASMCs

The primary transcript of miR-214 (pri-miR-199/214) is located on chromosome 1 in humans and is transcribed together with miR-199, which is processed to generate 4 separate mature miRNAs (miR-214-3p, miR-214-5p, miR-199-3p, and miR-199-5p). The transcriptional start site for human pri-miR-199/214 is at position chr1:172113935 (GRCh37).[34] The locus contains an upstream region of ~2.4 kb with >60% homology to other mammalian species (ECR Browser).[35]

We first carried out in silico analysis of this putative promoter region using MatInspector (Genomatix software suite, http://www.genomatix.de). This revealed a number of putative binding sites for TGF-β1 responsive transcription factors, including several binding sites for Smad proteins (Fig. 1*A*). We tested the effect of TGF-β1 treatment in PASMCs and analyzed changes in the miR-199/214 axis by qRT-PCR, by assessing the primary (pri-) and mature forms of each miRNA. TGF-β1 increased the expression of pri-miR-199/214 in PASMCs (Fig. 1*B*). Correspondingly, TGF-β1 increased the expression of the 4 mature forms of the miR-199/214 cluster (Fig. 1*C*).

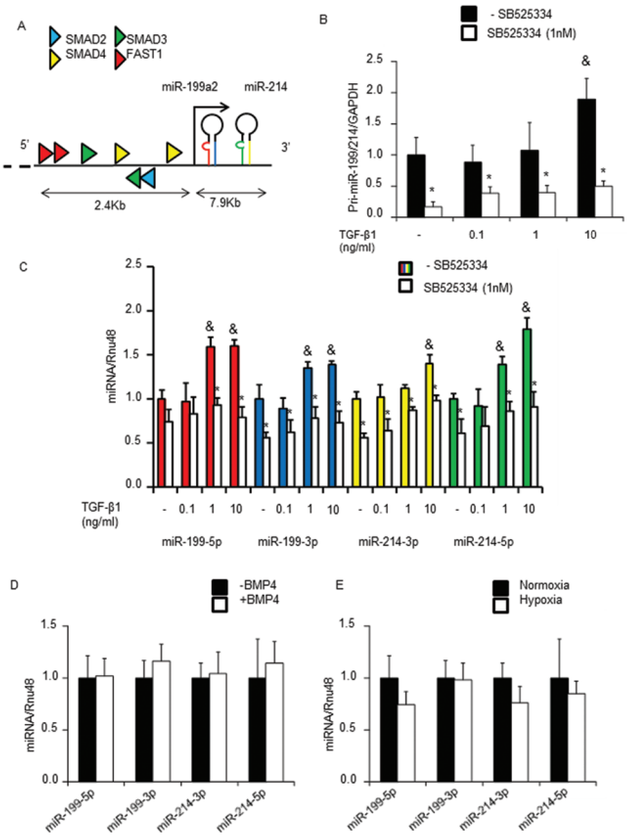

Figure 1. The miR-199/214 axis is induced by transforming growth factor beta 1 (TGF-β1) stimulation but not by BMP4 or hypoxia in pulmonary artery smooth muscle cells (PASMCs). *A*, SMAD sites are present in the primary (pri-) miR-199/214 promoter; these are indicated by triangles (blue, SMAD2; green, SMAD3; yellow, SMAD4; red, FAST1). *B*, PASMCs were treated with TGF-β1 with or without the specific activin-like kinase (ALK) 5 inhibitor SB525334 for 5 days (n = 3, duplicate). The pri-miR-199/214 expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). *C*, MicroRNA (miRNA) expression for miR-199-5p, miR-199-3p, miR-214-3p, and miR-214-5p. TGF-β1-only stimulation data were analyzed using a 1-way ANOVA with a Tukey post hoc test, significance relative to unstimulated control (ampersand, $P < 0.05$). A Student *t* test was used to compare samples stimulated with SB525334 to the equivalent unstimulated sample (relative to SB525334 unstimulated sample: asterisk: $P < 0.05$; relative to control unstimulated with TGF-β1: ampersand: $P < 0.05$). *D*, *E*, PASMCs were quiesced in Dulbecco's modified eagle medium with 0.2% fetal bovine serum for 24 hours and then treated with BMP4 (50 ng/mL) for 5 days (*D*) or exposed to hypoxic conditions for 48 hours after quiescence (*E*). MicroRNA expression was assessed by qRT-PCR (n = 3, duplicate). A Student *t* test was used for statistical analysis.

Treatment of PASMCs with a specific inhibitor of the TGF-β receptor *ALK5*, SB525334, completely abolished this effect (Fig. 1*B*, 1*C*). It has been shown that miR-214 is upregulated by hypoxia in cardiomyocytes,[15] but we found that hypoxia and BMP4 treatment did not affect the basal levels of the mature miRNAs in the miR-199/214 cluster in PASMCs (Fig. 1*D*, 1*E*).

Expression of the miR-199/214 axis in mouse and rat models of PAH

TGF-β1 has an established role in PAH.[21,28,29] Therefore, we investigated expression of the pri-miR-199/214 transcript and mature miRNAs in mouse and rat models of PAH.

We sampled total lung and RV from WT mice exposed to hypoxia and SU 5416 for 21 days or rats exposed to hypoxia and SU 5416 for 14 days followed by normoxia for 21 days. These data were compared with normoxic controls to evaluate whether the miR-199/214 axis was altered during induction of PAH in these tissues. Expression levels for both strands of miR-199 and miR-214 were analyzed by qRT-PCR.

Levels of pri-miR-199/214 were upregulated in mouse lung and RV in response to hypoxia and SU 5416 injury in both the lung and the RV (Fig. 2*A*). Analysis of the miR-199/214 axis in the LV of the same animals did not reveal dysregulation of this miRNA axis (Fig. 2*B*). However, a significant upregulation of the mature miRNAs in the miR-199/214 cluster was demonstrated in the lung and RV in both mice and rats (Fig. 2*C*–2*F*). We also observed a significant increase in expression of the miR-199/214 axis in the lung and RV of mice exposed to hypoxia without SU 5416 for 3 weeks (Fig. 3*A*, 3*B*). Further, the significant increase in miR-199/214 axis expression observed in males was not detected in female lung and RV exposed to 3 weeks of hypoxia and SU 5416 injury (Fig. 3*C*, 3*D*).

We next performed in situ hybridization to localize which cells were expressing miR-214 within the lung and RV of control rats exposed to hypoxic and normoxic conditions. We observed that miR-214 was expressed in cardiomyocytes in the RV (Fig. 3*E*) and in the smooth muscle layer of vessels and bronchi in the lungs (Fig. 3*F*).

Quantification of PAH indices in WT and miR-214$^{-/-}$ male and female mice exposed to SU 5416 and normoxic or hypoxic conditions for 21 days

We compared hemodynamics in WT mice and miR-214$^{-/-}$ in response to SU 5416 and hypoxia treatment for 21 days in male and female mice in parallel and compared with littermate controls (Fig. 4*A*). Quantification of RV and systemic pressures was performed along with heart rate measurements.

In response to hypoxia and SU 5416, RVSP and RVH were significantly increased in both male and female mice (Figs. 4*B*, 4*C*, 5*A*, 5*B*). However, we observed that the increase in RVH in the male miR-214$^{-/-}$ mice was greater than that in the WT controls (Fig. 5*A*). However, no significant change was observed in females (Fig. 4*B*).

Knockout animals exposed to hypoxia demonstrated RVSP values comparable to those of hypoxic WT animals in both male and female mice (Figs. 4*C*, 5*B*). Similarly, comparable results were observed in remodeling analysis (Fig. 5*E*, 5*F*). Further, no difference in heart rate or systemic pressure was observed between groups (Figs. 4*D*, 4*E*, 5*C*, 5*D*). Therefore, our results show that miR-214 knockout has a significant effect on RVH in male mice, but other PAH indices were unaffected.

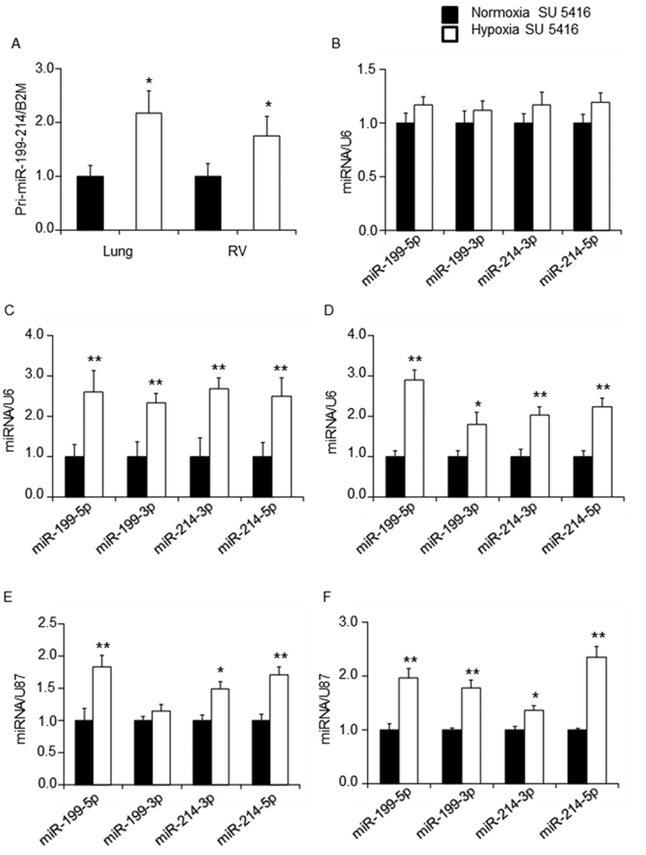

Figure 2. The miR-199/214 axis is induced in the lung and right ventricle (RV) but not in the left ventricle (LV) from mice exposed to the Sugen (SU) 5416 hypoxia model of pulmonary hypertension. *A*, The primary miR199/214 was quantified in mouse lung and RV. *B*, MiRNA expression was quantified by quantitative real-time polymerase chain reaction (qRT-PCR) in mouse LV exposed to the SU 5416 hypoxia (21 day) model of pulmonary hypertension. *C*–*F*, MicroRNA expression was quantified by qRT-PCR in mouse lung (*C*), mouse RV (*D*), rat lung (*E*), and rat RV (*F*) exposed to the SU 5416 hypoxia models of pulmonary hypertension. A Student *t* test was used for statistical analysis (asterisk: $P < 0.05$; two asterisks: $P < 0.01$; mouse tissues: $n = 6$; rat tissues: $n = 8$).

Analysis of fibrosis, hypertrophy, and miR-214 targets in in vivo samples

Previous studies have shown that fibrosis was increased in an ischemia reperfusion model in the heart but decreased in a model of kidney fibrosis in miR-214$^{-/-}$ mice.[13,21,25] In order to assess whether a change in fibrosis was important in the RV phenotype observed, we assessed expression of collagen, type I, alpha 1 (*COL1A1*) and collagen, type 3, alpha 1 (*COL3A1*) in male RV. We found no significant change in expression level between groups (Fig. 6*A*, 6*B*). There was no change in fibrosis between the groups, as assessed by picrosirius red staining (Fig. 6*C*). We carried out expression analysis for myosin, heavy chain 7, cardiac muscle, beta (*MYH7*) and myosin, heavy chain 11 (*MYH11*) in order to add further evidence to our observation that the RV : LV + S ratio was increased in miR-214$^{-/-}$

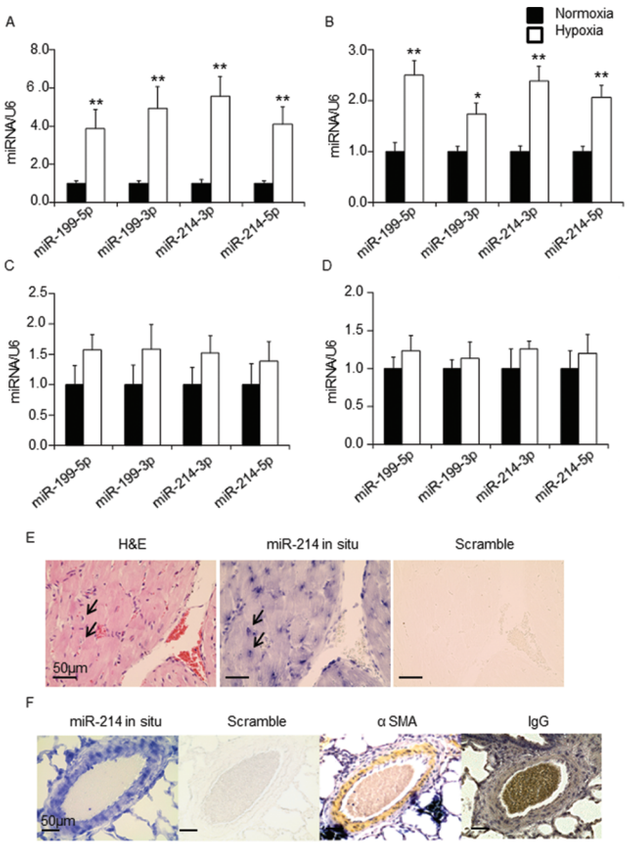

Figure 3. The miR-199/214 axis is induced in the lung and right ventricle (RV) in male mice exposed to hypoxia for 3 weeks but not in female mice exposed to hypoxia and Sugen (SU) 5416 for 21 days. *A–D*, MicroRNA (miRNA) expression was quantified by quantitative real-time polymerase chain reaction (qRT-PCR) in male mouse lung (*A*) and mouse RV (*B*) exposed to the 3-week hypoxia model of pulmonary hypertension or female mouse lung (*C*) and mouse RV (*D*) exposed to hypoxia and SU 5416 for 21 days. A Student *t* test was used for statistical analysis (asterisk: $P < 0.05$; two asterisks: $P < 0.01$; $n = 6$). *E*, *F*, In situ hybridization shows miR-214-3p localization in rat RV (*E*) and rat lung (*F*). Paraffin sections were rehydrated and incubated with an anti-miR-214-3p or scramble probe as a negative control. For colocalization, α-smooth muscle actin (*αSMA*) was detected in the same samples using an immunohistochemistry assay, with nonimmune isotype-immunoglobulin G (IgG) antibody as a negative control. Hematoxylin and eosin (H&E) stain was used to identify cardiomyocytes. Magnification ×40 ($n = 5$).

mice. Mutations in these hypertrophy markers are associated with hypertrophic cardiomyopathy.[36] Both of these are modulated in hypertrophy, and MYH7 is upregulated while MYH11 is downregulated.[37] We found that expression of *MYH11* decreased while *MYH7* increased in hypoxia. Furthermore, *MYH7* and *MYH11* levels were significantly different between the miR-214$^{-/-}$ group and the WT (Fig. 6*D*, 6*E*). These data were indicative of increased RVH in the miR-214$^{-/-}$ group. Taken together, these results suggested that the increased RVH in miR-214$^{-/-}$ mice was due to hypoxia and SU 5416-induced hypertrophy rather than fibrosis or pressure overload.

Target gene analysis was performed for previously validated miR-214 targets on mRNA extracted from male RV. We found that expression of the phosphatase and tensin homolog (*PTEN*), which has been shown to have a role in RVH,[38] was increased in the RV of miR-214$^{-/-}$ mice (Fig. 7*A*). This panel included *NCX1*, which has been shown to cause fibrosis and apoptosis in the heart.[13] No significant regulation was observed in hypoxia between the miR-214$^{-/-}$ and WT mice for *NCX1* or any of the other targets (Fig. 7). However, we did observe that cAMP (cyclic monophosphate) responsive element binding protein 1 (*CREB1*) and apolipoprotein C-III (*APOC3*) had significantly increased expression in the miR-214$^{-/-}$ normoxic group compared with that in the WT normoxic group (Fig. 7*D*, 7*E*).

DISCUSSION

We demonstrated upregulation of the miR-199/214 axis in response to TGF-β1 in vitro and in response to mouse and rat models of PAH in vivo. We observed differential effects of genetic ablation of the miR-214 stem loop on the PAH phenotype between heart and lung, and this effect was sex specific. The increased RVH in the miR-214$^{-/-}$ mice was not dependant on derepression of *NCX1*, which has been shown to increase fibrosis and apoptosis in the heart.[13] However, *PTEN*, which has been implicated in RVH,[38] was

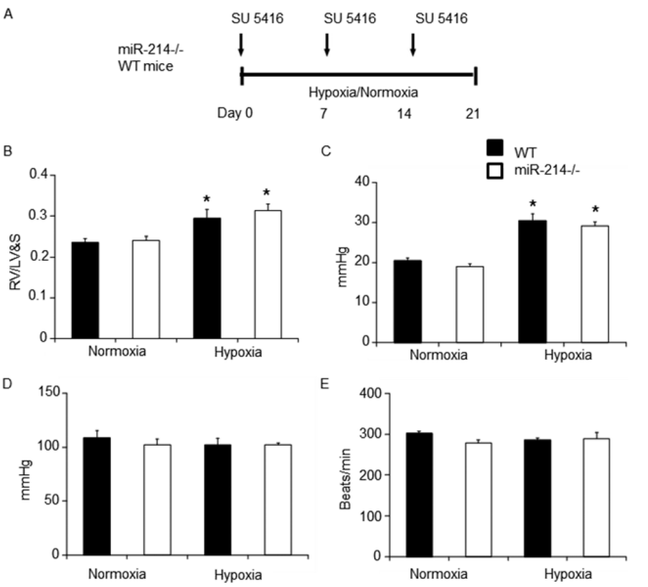

Figure 4. Quantification of pulmonary arterial hypertension indices in miR-214$^{-/-}$ and wild-type (WT) female mice exposed to Sugen (SU) 5416 and hypoxic or normoxic conditions for 21 days. *A*, WT and miR-214$^{-/-}$ littermate mice were exposed to SU 5416 plus 21-day chronic hypoxia or normoxia. *B–E*, Quantification of right ventricular (RV) hypertrophy (*B*), RV systolic pressure (*C*), systemic pressure (*D*), and heart rate (*E*) in female mice (n = 8–10 per group). Pressures and tissue were taken after 21 days in normoxic or hypoxic conditions. Data were analyzed using a 2-way ANOVA followed by a Bonferroni post hoc test (asterisk: $P < 0.05$); significance is expressed relative to WT normoxic. LV&S: left ventricle and septum.

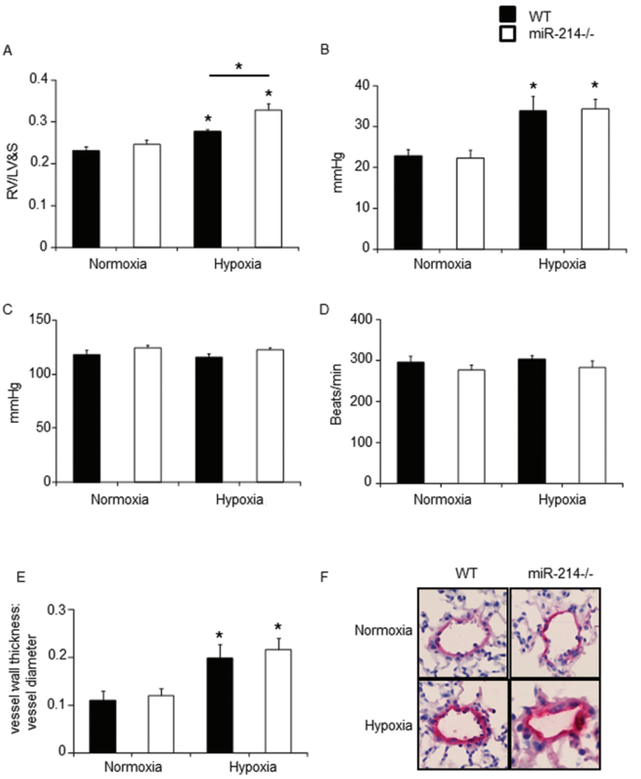

Figure 5. Quantification of pulmonary arterial hypertension indices in miR-214$^{-/-}$ and wild-type (WT) male mice exposed to Sugen (SU) 5416 and hypoxic or normoxic conditions for 21 days. *A–D*, Quantification of right ventricular (RV) hypertrophy (*A*), RV systolic pressure (*B*), systemic pressure (*C*), and heart rate (*D*) in male mice (n = 8–10 per group). Pressures and tissue were taken after 21 days in normoxic or hypoxic conditions. *E*, *F*, Pulmonary arterial remodeling quantification (*E*; n = 6 per group) and representative pictures stained with smooth muscle actin (*F*); magnification ×40. Data were analyzed using a 2-way ANOVA followed by a Bonferroni post hoc test (asterisk: $P < 0.05$); significance is expressed relative to WT normoxic unless comparison is shown. LV&S: left ventricle and septum.

identified as a target in RV and may play an important role in RVH in our model. This data demonstrates that miR-214 is regulated in response to PAH stimuli and acts in a tissue- and sex-specific manner.

Coding sequences for miR-199 and miR-214 stem loops are highly conserved and are separated by ~7.2 kb on murine and human chromosome 1. Previously, miR-214-3p was shown to regulate fibrosis, angiogenesis, and proliferation, which are also regulated by TGF-β1.[39] In agreement with previous reports, we demonstrated that miR-214-3p was regulated by TGF-β1.[21] Here, we established that TGF-β1 induced transcription of pri-miR-199/214 and expression of all 4 mature miRNAs. We identified an increase in expression of the miR-199/214 axis in RV and lung from mice and rats exposed to the SU 4516 hypoxia model of PAH. Therefore, stimuli that lead to PAH could induce expression of the miR-199/214 axis. We investigated the effect of miR-214-3p/5p loss in vivo, but potentially the whole miR-199/214 axis could be important in PAH, since these coregulated miRNAs could have a protective role in PAH. It has been shown that miR-199 can target HIF-1α, leading to reduced endothelin-1 expression.[40] It would be interesting to investigate whether a miR-199 and miR-214 knockout mouse would have a more pronounced PAH phenotype.

We performed studies that assessed chronic knockdown of miR-214 using knockout male and female mice. The female miR-214$^{-/-}$ mice displayed high RVSP and RVH in hypoxia, similar to WT hypoxic mice, whereas the male miR-214$^{-/-}$ mice had increased RVH compared with the WT hypoxic group (Figs. 4, 5). Therefore, although genetic knockdown of miR-214 in this setting had no significant effect on the development of PAH under the experimental conditions tested in females, miR-214 knockdown in males could progress RVH.

PAH is a condition with a sex bias at the clinical level.[41] Clinical as well as experimental data show a significant difference be-

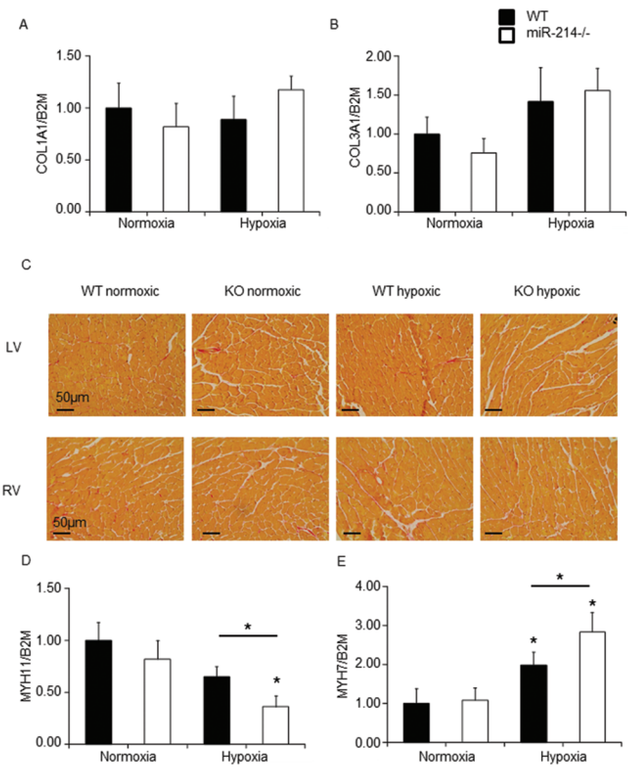

Figure 6. Analysis of fibrosis and hypertrophy in male miR-214$^{-/-}$ (KO) and wild-type (WT) right ventricle (RV). Gene expression is assessed in mouse RV using specific probes and normalized to housekeeper (B2M). *A*, *B*, *COL1A1* (*A*) and *COL3A1* (*B*) expression levels were quantified in miR-214$^{-/-}$ and WT RV exposed to normoxic or hypoxic conditions. *C*, Fibrosis was measured using picrosirius red staining; magnification × 40 (n = 5). *D*, *E*, Expression levels of hypertrophy markers *MYH11* (*D*) and *MYH7* (*E*). Data were analyzed using a 2-way ANOVA followed by a Bonferroni post hoc test relative to WT normoxic unless comparison is shown. Asterisk: $P < 0.05$ (n = 6). LV: left ventricle.

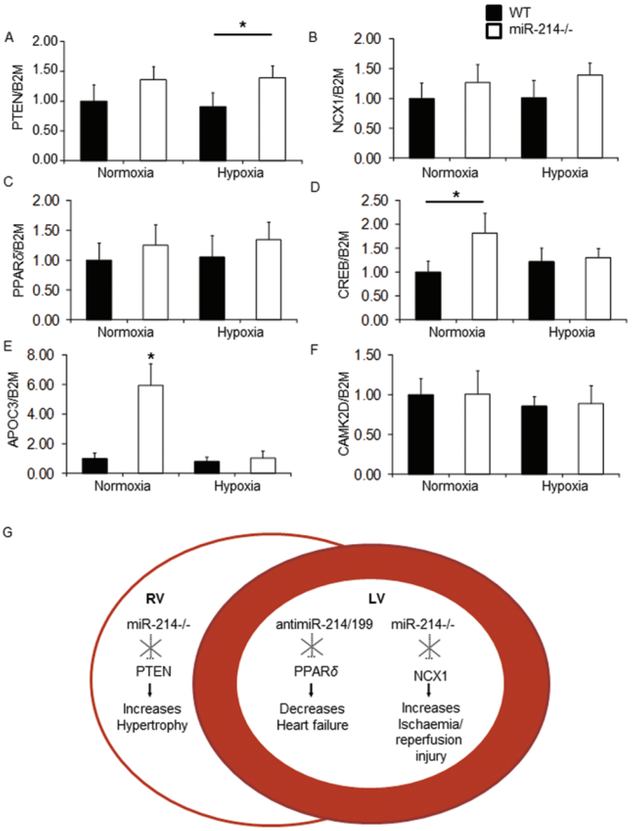

Figure 7. Target gene analysis of miR-214$^{-/-}$ mice and the effect of miR-214 on right ventricle (RV) and left ventricle (LV) in hearts with different types of injury. *A–F*, Target gene expression in male RV by quantitative real-time polymerase chain reaction for *PTEN* (*A*), *NCX1* (*B*), *PPARδ* (*C*), *CREB* (*D*), *APOC3* (*E*), and *CAMK2D* (*F*). Data were analyzed using a 2-way ANOVA followed by a Bonferroni post hoc test; significance is expressed relative to WT normoxic unless comparison is shown (asterisk: $P < 0.05$; $n = 6$). *G*, MicroRNA miR-214 can target alternate targets between different models of heart disease. *PTEN*, *PPARδ*, and *NCX1* are the targets identified in pulmonary arterial hypertension RV hypertrophy, heart failure, and ischemia reperfusion injury, respectively.

tween males and females in cardiovascular responses.[1,5,7,8] Previous studies have shown different pathophysiology between male and female mice in a transgenic mouse model.[8,42,43] Furthermore, male PAH patients have decreased RV function compared with females,[6] and male rodents and swine develop more severe RVH when exposed to chronic hypoxia compared with females.[42,44] These significant sex differences in adaptation to hypoxia may reflect the different sensitivity of males and females to oxygen deprivation and other stresses.

The incidence of PAH is greater in women, and a role has been confirmed for estrogen in disease pathogenesis.[45-47] Male but not female mice had increased expression of the miR-199/214 axis in the lung and RV in SU 4516 hypoxia-induced PAH (Figs. 2*C*, 2*D*, 3*C*, 3*D*). It has been demonstrated previously that estrogen can inhibit the expression of miR-214.[48] Therefore, increased levels of estrogen in females may prevent the expression of miR-214 in response to induction of PAH. This upregulation of miR-214 in males may account for some of the differences observed between the male and female mice. Potentially, the increase in miR-214 observed in males exposed to hypoxia may have a protective effect in the heart; miR-214 induction does not occur in females, and thus female knockout mice may be more resilient to miR-214 ablation. Part of this may be due to the inverse correlation between miR-214 and estrogen levels.[48] Hence, pathway redundancy in females may compensate for the fluctuations in miR-214 mediated by estrogen.

It has been shown previously that miR-214$^{-/-}$ mice have increased fibrosis in the heart, a result of target derepression of the sodium/calcium exchanger *NCX1*, leading to calcium overloading of cardiomyocytes.[13] However, in our study, we did not observe any change in expression levels of *NCX1* or fibrosis (Figs. 6*A*–6*C*, 7*B*). Our data suggest that miR-214$^{-/-}$ mice have increased RVH and reduced RV function because of worsening of hypoxia and SU 4516 hypoxia-induced RVH by targets such as *PTEN*.

Our data, taken together with previous work, demonstrate that miR-214 has specific effects in PAH-induced RVH compared with whole heart in heart failure/ischemia reperfusion injury. MicroRNA miR-214 can target alternate targets between different models of heart disease, since NCX1 and *PPARδ* were not targeted by miR-214 in RV from our PAH model but were found to be targets in whole heart in other studies.[13,15] However, analysis of miR-214 target genes revealed derepression of *PTEN* in the RV of miR-214$^{-/-}$ male mice (Fig. 7*A*, 7*G*) but not in female mice (not shown). Increased levels of *PTEN* have been demonstrated in RVH.[38] *PTEN* has opposing effects on PAH in the lung vasculature and RVH.[38,49] Further, *PTEN* has also been shown to increase proliferation, migration, and invasion; in addition, it can cause apoptosis of PASMCs.[20,50] Potentially, derepression of *PTEN* in the lung of miR214$^{-/-}$ mice may have initially induced increased remodeling but may have caused apoptosis in PASMCs in the remodeled vessels at later stages of the model. This may account for the effect observed on RVH but not on RVSP or remodeling. Differential effects of PTEN in a tissue-specific manner may allow miR-214 to mediate cell type–specific effects in response to the same regulatory stimuli. However, further targets will be important in this model, and additional studies are warranted to fully explore the miR-214 targets.

The current paradigm suggests that RVH is secondary and proportional to pulmonary remodeling; however, it has been found that pulmonary vascular remodeling and RVH can be dissociated and are not always directly proportional in PAH.[51] However, both increased pulmonary vascular resistance and hypoxia can affect RVH, and hypoxia can have a direct effect on the heart.[52,53] This study shows that in miR-214$^{-/-}$ mice, the mouse SU 4516 hypoxia model can increase RVH nonproportionally relative to RVSP. Pathways involved in the response of the heart to hypoxia may be modulated by miR-214; this could be due to the target *PTEN*, which has been shown to modulate responses to hypoxia.[54]

These data have implications for therapeutics, since at the stage of PAH diagnosis vascular remodeling has occurred and leads to in-

creased pulmonary vascular resistance and hypoxia in the heart. Currently, remodeling cannot be reversed; however, the response of the RV to hypoxia could be targeted. The miR-214 targets that may be beneficial in the RV may not be in the pulmonary circulation; therefore, specific delivery of miR-214 to the RV using adeno-associated virus vectors could be an option as a therapeutic strategy.

Taken together, the results of this study demonstrate that the miR-199/214 axis is induced in PAH. Moreover, genetic deletion of the miR-214 stem loop increases the development of RVH induced by transient exposure to hypoxia and SU 4516 injury in male mice. However, genetic deletion of miR-214 stem loop has no effect on PAH in female mice. These data suggest that PAH pathology in the lung vasculature and heart is mediated via distinct pathways and identifies another potential source of sex-specific variation.

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