Role for miR-204 in human pulmonary arterial hypertension

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Pulmonary arterial hypertension (PAH) is characterized by enhanced proliferation and reduced apoptosis of pulmonary artery smooth muscle cells (PASMCs). Because microRNAs have been recently implicated in the regulation of cell proliferation and apoptosis, we hypothesized that these regulatory molecules might be implicated in the etiology of PAH. In this study, we show that miR-204 expression in PASMCs is down-regulated in both human and rodent PAH. miR-204 down-regulation correlates with PAH severity and accounts for the proliferative and antiproliferative phenotypes of PAH-PASMCs. STAT3 activation suppresses miR-204 expression, and miR-204 directly targets SHP2 expression, thereby SHP2 up-regulation, by miR-204 down-regulation, activates the Src kinase and nuclear factor of activated T cells (NFAT). STAT3 also directly induces NFATc2 expression. NFAT and SHP2 were needed to sustain PAH-PASMC proliferation and resistance to apoptosis. Finally, delivery of synthetic miR-204 to the lungs of animals with PAH significantly reduced disease severity. This study uncovers a new regulatory pathway involving miR-204 that is critical to the etiology of PAH and indicates that reestablishing miR-204 expression should be explored as a potential new therapy for this disease.

Abbreviations used: ChIP, chromatin immunoprecipitation; iPASMC, iPAH, idiopathic PAH; MCT, monocrotaline; mRNA, microRNA, miRNA, messenger RNA; NFAT, nuclear factor of activated T cells; PA, pulmonary artery; PAAT, PA acceleration time; PAEC, PA endothelial cell; PAH, pulmonary arterial hypertension; PASMC, PA smooth muscle cell; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; scRNA, small interfering RNA; TLDA, TaqMan low density array; small interfering RNA; siRNA, coiled-coil–containing protein kinase; seq, sequencing; siRNA, platelet-derived growth factor; qRT-PCR, quantitative RT-PCR; ROCK, Rho-associated, coiled-coil–containing protein kinase; seq, sequencing; siRNA, small interfering RNA; TLDA, TaqMan low density array; TMRM, tetramethylrhodamine methyl ester; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UTR, untranslated region; VEGF, vascular endothelial growth factor.

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PASMCs has been linked to the activation of both the tyrosine
kinase Src (Wong et al., 2005) and a STAT3/miR-17-92
microRNA (miRNA) secondary to IL-6 exposure, suggest-
ing the implication of miRNAs in the etiology of PAH
(Brock et al., 2009).

miRNAs are small noncoding RNAs (21–23 nt) that are
now known to be important regulators of gene expression.
They form imperfect RNA–RNA duplexes and use their
seed region to interact with messenger RNAs (mRNAs),
mainly in the 3′ untranslated region (UTR; Khan et al.,
2009). This interaction leads to a negative posttranscriptional
regulation of the relevant mRNAs. Recently, misexpression of
miRNAs has been implicated in many cardiovascular diseases,
including pulmonary hypertension (Latronico and Condorelli,
2009; Mishra et al., 2009; Zhang, 2009; Caruso et al.,
2010), but their molecular role in these pathologies has not been
uncovered yet.

RESULTS
miR-204 is aberrantly expressed in human PAH-PASMCs
To determine whether miRNAs are aberrantly expressed in
human PAH, PASMCs were isolated from distal PAs of two
nonfamilial PAH patients (two idiopathic PAH [iPAH] pa-
tients A and B) based on the World Health Organization
(WHO) classification and two control patients (A and B) and
cultured as previously described (passage 3 and less; McMurtry
et al., 2005). The expression of 377 different miRNAs was
measured. Seven miRNAs were aberrantly expressed in
PAH-PASMCs compared with control PASMCs (miR-204,
-450a, -145, -302b, -27b, -367, and -138; Fig. S1 A). Among
them, only the miR-204 level was down-regulated (Fig. S1 A).
miR-204 down-regulation between control versus PAH
was confirmed by quantitative RT-PCR (qRT-PCR) in
PASMCs isolated from three PAH (all from group 1 based on
WHO classification patients A–C) compared with control
PASMCs isolated from five control patients (A–E). Note that
no significant differences in miR-204 expression was found
among the control patients and the PAH patients (Fig. S1 B).
Therefore, for the rest of the study, all five control PASMC
and the three PAH-PASMC cell lines were used for every
cell-based experiment.

Interestingly, in retinal epithelial cells and several cancer
cells, miR-204 down-regulation has been associated with en-
hanced cell proliferation and membrane potential depolariza-
tion (Lee et al., 2010; Wang et al., 2010), which are both
aspects also seen in PAH-PASMCs (Bonnet et al., 2006,
2007b). We recently showed in several cancer cells and PAH-
PASMCs (Bonnet et al., 2007a,b) that this pro-proliferative
phenotype was associated in part with the activation of the
Src–STAT3 (accounting for BMPR2 down-regulation; Wong
et al., 2005) and NFAT pathways (Bonnet et al., 2007b). This
suggests a putative link between miR-204 down-regulation,
NFAT activation, and cell proliferation. Thus, miR-204 is
likely implicated in PAH, and a role in the sustainability of the
PAH-PASMC pro-proliferative and antiapoptotic phenotype
is possible. Therefore, the current study will be focused on the
role of miR-204 in the etiology of PAH. Interestingly, using
in silico and microarray gene expression analyses, we observed
that among the 461 predicted targets of miR-204 (TargetScan
5.1), only 165 were increased by artificial miR-204 inhibition
in control human PASMCs (n = 2 patients; Fig. S1 C). In ac-
cordance with the pro-proliferative and antiapoptotic pheno-
types seen in PAH, several Src–STAT3- and NFAT-related
genes were identified (Fig. S1 C).

miR-204 expression is decreased in human PAH
and correlates with PAH severity
To investigate the expression pattern of miR-204 in normal
and pulmonary hypertensive lungs, we examined miR-204
expression levels in (a) lung biopsies from 8 individuals with
nonfamilial PAH compared with biopsies from 8 individuals
without pulmonary hypertension, (b) lungs from 6 mice with
hypoxia-induced pulmonary hypertension compared with 5
control littermates, and (c) lungs from 5 rats with monocrota-
line (MCT)-induced pulmonary hypertension compared with
10 control littermates (Fig. 1 A). We found decreased levels of
miR-204 in human and rodent pulmonary hypertensive lung
tissues compared with normotensive lung samples. To charac-
terize whether down-regulated miR-204 levels were specific
to the lung in rats with pulmonary hypertension, we com-
pared organ–specific levels of miR-204 between normal
and pulmonary hypertensive rats (Fig. 1 B). Even if we were
able to detect minimal amounts of miR-204 in most organs,
miR-204 levels were only down-regulated in the lung and
PAs but not in the aorta, liver, heart, and kidney in rats
3 wk after MCT injection (pulmonary hypertensive rats)
compared with non–pulmonary hypertensive rats (Fig. 1 B).
To test whether miR-204 down-regulation correlated
with disease progression, we studied humans, mice, and rats
with varying degrees of PAH. In both human subjects and rodents,
miR-204 levels in the lung correlated directly with the severity
of PAH, as measured by pulmonary vascular resistance in hu-
mans and mean PA pressure in rodents (Fig. 1 C). Our results
indicate that levels of miR-204 correlate with the severity
of PAH in humans and experimental pulmonary hypertension.

miR-204 is confined to PASMCs in the lung
To determine the lung distribution of miR-204, miR-204
expression was measured by qRT-PCR in rat bronchi, veins,
and PAs. Our results indicate that miR-204 is in majority ex-
pressed within PAs but not in veins or bronchi tissue (Fig. S2 A).
To determine the cell type distribution of miR-204 within
PAs, miR-204 expression was measured in both cultured
passage 3 and less) human PASMCs and PA endothelial cells
(PAECs). Our results indicate that within PAs, miR-204 is
mostly confined to the PASMCs as control-cultured PASMCs
expressed seven times more miR-204 compared with control
PAECs (Fig. S2 B).

miR-204 expression level is a potent biomarker for PAH
Finally, because miRNAs are currently used in humans as a
biomarker of cancer (Ferracin et al., 2010), to further
Diminution of miR-204 level promotes PASMC proliferation and resistance to apoptosis

To study the effect of miR-204 on PASMC proliferation and apoptosis in vitro, cultured human PAH-PASMCs were either exposed to 10% FBS to promote proliferation or 0.1% FBS to promote apoptosis (Bonnet et al., 2007b). When compared with control PASMCs containing a high level of miR-204, PAH-PASMCs displayed a higher cell proliferation rate and resistance to induced apoptosis (Fig. 2 A). The implication of miR-204 in regulating PASMC proliferation and apoptosis was confirmed in control PASMCs, in which miR-204 inhibition increased proliferation and resistance to apoptosis to levels similar to those seen in PAH-PASMCs (Fig. 2 A).

confirm the implication of miR-204 in PAH, we measured miR-204 expression level in human buffy coat isolated from non-pulmonary hypertensive patients and patients with PAH (Table S1). We previously showed that human PAH-PASMCs and cells from the buffy coat of PAH patients have a lot of similarities in term of activated pathways. For example, both have activated NFAT (Bonnet et al., 2007b); thus, miR-204 expression in the buffy coat could parallel miR-204 expression in PASMCs. Indeed, as in PAH-PASMCs, miR-204 expression was significantly decreased in patients with PAH (Fig. S2 C). This result is of a great clinical interest as it confirms the implication of miR-204 in PAH and suggests that miR-204 can become a reliable biomarker of PAH.

**Figure 1.** Correlation between miR-204 expression and PAH severity. (A) miR-204 is decreased in human, mouse, and rat PAH lungs. qRT-PCR analysis of miR-204 expression in human lungs with PAH (n = 8), mouse lungs with hypoxia-induced pulmonary hypertension (n = 6), and rat lungs with MCT-induced pulmonary hypertension (n = 5) compared with human (n = 8), mouse (n = 10) and rat (n = 5) control (Ctrl) lungs. (B) miR-204 is mainly expressed in the distal PAs. RT-PCR analysis of miR-204 expression in several rat organs with MCT-induced pulmonary hypertension (n = 5) compared with control rats (n = 5). (C) miR-204 down-regulation correlates with PAH severity. qRT-PCR analysis of miR-204 expression in the lungs from healthy subjects (n = 8) and from patients with varying severity of PAH (n = 3), in mouse lungs with varying severity of hypoxia-induced pulmonary hypertension (n = 3), and in rat lungs with varying severity of MCT-induced pulmonary hypertension (n = 3) compared with control animals (n = 5). (D) miR-204 expression in the lungs from healthy subjects (n = 8) and from patients with varying severity of PAH (n = 3), in mouse lungs with varying severity of hypoxia-induced pulmonary hypertension (n = 3), and in rat lungs with varying severity of MCT-induced pulmonary hypertension (n = 3) compared with control animals (n = 5). Statistical significance is compared with control group. In all experiments, the level of miR-204 is relative to the control RNA U6. Data are expressed as means ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Altering miR-204 level promotes the activation of the pro-proliferative and antiapoptotic Src–STAT3–NFAT pathway in PAH–PASMCs

The increase in PASMC proliferation and resistance to apoptosis observed in PAH has been linked to the activation of the Src–STAT3–BMPR2 (Wong et al., 2005; Brock et al., 2009) and NFAT pathways (Bonnet et al., 2007b). The putative implication of miR-204 in these pathways was thus investigated. As expected, we observed an increase in the activity of Src (increased phosphorylated Src [p-Src] / Src ratio; Fig. 2 B), STAT3 (increased p-STAT3 / STAT3 ratio and p-STAT3 nuclear translocation; Fig. 2 C and Fig. S3 A), and NFATc2 (increased expression, nuclear translocation, and luciferase activity; Fig. 2 D and Fig. S3 A) in PAH-PASMCs. An increase in miR-204 level altered the activation of Src, STAT3, and NFATc2 in PAH-PASMCs, whereas a decrease of miR-204 promoted them in control PASMCs (Fig. 2 and Fig. S3). These findings demonstrate that a down-regulation of miR-204 leads to the activation of the Src–STAT3–NFAT pathway in PAH-PASMCs. Activation of STAT3 was also confirmed in lung biopsies from PAH patients (Fig. S3 B), whereas NFATc2 activation in PAH lungs has been previously shown by our group (Bonnet et al., 2007b).

Next, we attempted to identify the mechanism responsible for down-regulating miR-204 in PAH–PASMCs. By stimulation experiments, we found that miR-204 expression is down-regulated by PDGF, endothelin-1, and angiotensin II, which are all well known to be involved in the pathogenesis of pulmonary hypertension (Zhao et al., 1996; Archer and Rich, 2000). Because PDGF, endothelin-1, and angiotensin II signaling is mainly mediated by STAT3 (Yellaturu and Rao, 2003; Banes-Berceli et al., 2007), the effect of STAT3 inhibition by small interfering RNA (siRNA) on miR-204 was also investigated. We observed that siSTAT3 abolished the down-regulation of miR-204 seen in PAH-PASMCs (Fig. 3 A). Finally, we found that miR-204 expression was inversely correlated with STAT3 activation, i.e., the higher STAT3 was activated, the stronger miR-204 was down-regulated (Fig. 3 B). The coding sequence of miR-204 lies within intron 6 of the human TRPM3 (transient receptor potential melastatin 3; Wang et al., 2010). A previous study revealed that miR-204 and TRPM3 share the same regulatory motif for transcription and are derived from a single transcription unit (Wang et al., 2010). This was confirmed in PAH and in control PASMCs exposed to pro-PAH factors (Fig. 3 C). To further demonstrate the implication of STAT3 in the regulation of miR-204 expression, we performed promoter region analysis of TRPM3 using ENCODE (encyclopedia of DNA elements) chromatin

mimics were added when indicated. Smooth muscle actin (SM-actin) was used as a loading control. Representative Western blots are shown. (D) miR-204 down-regulation in PAH–PASMCs increases NFAT expression and activation in human PASMCs. NFATc2 mRNA expression (left) and activity (right) were measured by qRT-PCR (left) and luciferase assay (right) in PASMCs from control or PAH patients treated either with control or miR-204 antagonist. Error bars represent mean value ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
immunoprecipitation (ChIP) sequencing (seq; ChIP-seq) data for the STAT family of transcription factors and identified three putative STAT-binding sites located nearby the promoter region of TRPM3 (Fig. S3 C). We therefore tested whether STAT3 can bind directly to these regulatory sites. ChIP using p-STAT3 antibody followed by PCR brought direct support for a STAT3–TRPM3 interaction (Fig. 3 D). These data suggest that STAT3 may diminish the TRPM3/miR-204 gene locus, leading to the activation of Src and NFAT.

In human vascular smooth muscle cells, the down-regulation of TRPM3 promotes IL-6 production by an undiscovered mechanism (Naylor et al., 2010). As TRPM3 is down-regulated in PAH (Fig. 3 C) and IL-6 has been reported to be increased in PAH (Humbert et al., 1995), TRPM3 might be implicated in the etiology of PAH and thus could account for the PAH phenotype. Nonetheless, TRPM3 inhibition using siRNA in control PASMCs did not mimic the PAH phenotype (no changes in [Ca2+], Dym, and PASMC proliferation and apoptosis; Fig. S4). These effects were not associated with miR–204. Indeed, as miR–204 is localized within an intronic region of TRPM3, siTRPM3 did not affect miR–204 levels (Fig. S4). Moreover, we demonstrate that miR–204 effects are not mediated by TRPM3 as within 48 h, ectopic increases of miR–204 inhibit the Src–STAT3 axis, decreasing PAH-PASMC proliferation, resistance to apoptosis, and IL-6 secretion without restoring TRPM3 expression, whereas miR–204 down-regulation in control PASMCs mimics PAH without decreasing TRPM3 (Fig. S4, A and B; Wang et al., 1999). The fact that miR–204 regulates IL-6 secretion (Fig. S4 B) and increases Src activity (Fig. 2) in PAH-PASMCs (which had been linked to several pathophysiological processes seen in PAH such as cell proliferation [Steiner et al., 2009] and migration and K+ channel inhibition [Wong et al., 2005], as well as BMPR2 down-regulation [Wong et al., 2005]) suggests that miR–204 down-regulation might indirectly down-regulate BMPR2. We showed a significant up-regulation of BMPR2 in both human PAH-PASMCs and in PAs from PAH rats treated with miR–204 mimic (Fig. S5, A and B). This could result from the inhibition of STAT3 by the increase in miR–204, which would block the previously described STAT3-dependent BMPR2 down-regulation (Brock et al., 2009) because siSTAT3 also increases BMPR2 expression in human PAH-PASMCs (Fig. S5, A and B).

Figure 3. A primary STAT3 activation by circulating pro-PAH factors accounts for miR–204 down-regulation in PAH-PASMCs. (A) siSTAT3 increases miR–204 expression in PAH-PASMCs. miR–204 level measured by qRT-PCR in PAH treated with control siRNA (siRNA ctrl) or siSTAT3 as indicated (n = 3). (B) STAT3 activation and miR–204 expression are inversely correlated in PASMCs. Analysis of the correlation between STAT3 activation (measured by the pY705-STAT3/STAT3 ratio monitored by Western blot) and miR–204 expression (measured by qRT-PCR; n = 2 experiments/patient in three PAH and five control patients). (C) Pro-PAH factors decrease miR-204 and TRPM3 expression similarly by a STAT3-dependent mechanism in control PASMCs. miR–204 (top) and TRPM3 (middle) expression were measured by qRT-PCR performed on control cells treated with the pro-PAH factors PDGF, endothelin–1 (ET–1), or angiotensin II (All) as indicated (n = 3 experiments/patient in three PAH and five control patients). (bottom) Analysis of the similarities between miR-204 and TRPM3 pattern of expression measured by qRT-PCR in control, PAH, and PAH treated with siSTAT3 as indicated. (D) p-STAT3-binding sites were detected downstream of the TRPM3 gene. ChIP-PCR experiments studying STAT3-binding sites upstream (Up1) and downstream (Dw1 and Dw2) on TRPM3 genes. The OR8J1 gene was used as a negative control, whereas the VEGF gene was used as a positive control. Graphs represent means ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Among JAK2, SHP2, and SHC, only SHP2 was up-regulated in PAH-PASMCs (Fig. 4 A and Fig. S5 C). Moreover, increasing miR-204 level in PAH-PASMCs decreased SHP2, whereas miR-204 inhibition in control PASMCs cells increased SHP2 expression (Fig. 4 A). These results suggest that SHP2 may be the primary target of miR-204 in PAH. To test this hypothesis, we performed a reporter assay in which the luciferase reporter gene was under control of the SHP2 3’ UTR. We observed that both point mutations that abrogate the binding site of miR-204 in SHP2 3’ UTR as well as the sequestration of miR-204 with a specific inhibitor increase the expression of the luciferase reporter, confirming the implication of miR-204 in the regulation of SHP2 mediated through its 3’ UTR (Fig. 4 B).

Finally, as expected, the SHP2-dependent activation of Src activation by miR-204 promotes STAT3 and NFAT activation in PAH-PASMCs

From in silico analysis using TargetScan 5.1, neither STAT3 nor NFATc1, -c2, or -c3 (three NFAT isoforms activated in PAH-PASMCs; Bonnet et al., 2007b) is a predicted target of miR-204. But because a decrease in miR-204 expression increases STAT3 and NFAT activation (Fig. 2, B and C), miR-204 may be indirectly implicated in both STAT3 and NFAT activation. STAT3 activation mostly results from the activation of either the JAK2 or Src pathways (Gharavi et al., 2007; Cheranov et al., 2008; Li et al., 2008). Interestingly, among all the predicted targets of miR-204, JAK2 as well as two Src activators (SHP2 [Wu et al., 2006] and SHC [Src homology 2 domain containing; Sato et al., 2002]) were identified.

Figure 4. SHP2 up-regulation by miR-204 promotes activation of the Src–STAT3–NFAT axis in PAH-PASMCs. (A) SHP2 is up-regulated by miR-204 in PAH-PASMCs. Total and phosphorylated JAK2 (left), SHC1 (middle), and SHP2 (right) protein expression was monitored by Western blot (n = 3 independent experiments) in PASMCs from three PAH and five control patients. (right) miR-204 antagonist (Inh) and mimic along with appropriate controls (Ctrl) were added as indicated. SM-actin, smooth muscle actin. (B) miR-204 directly targets the SHP2 3’ UTR. (left) Binding sites of miR-204 found in the 3’ UTR of SHP2. Mutations introduced into the luciferase reporter are shown in red. (right) Relative firefly luciferase activity derived from the SHP2 3’ UTR and SHP2 3’ UTR mutated reporter constructs monitored after transfection in control PASMCs (n = 5). Control and miR-204 inhibitor (n = 3) were added as indicated. L.U., luciferase unit. (C) STAT3 regulates NFATc2 expression. NFATc2 mRNA level (left) relative to 18S measured by qRT-PCR in PASMCs from control and PAH patients treated when indicated with control or STAT3 siRNA (n = 3 qRT-PCR/patient in three PAH and five control patients). ChIP-PCR experiments (right) studying STAT3 binding on genes encoding the indicated NFAT isoforms (NFATc1, -c2, and -c3). The OR8J1 gene was used as a negative control, whereas the VEGF gene was used as a positive control. Graphs represent means ± SEM (*, P < 0.05; **, P < 0.01).
increases miR-204 level in PAH-PASMCs decreases [Ca\textsuperscript{2+}]i proliferation to the level seen in PAH-PASMCs, whereas the siSHP2, the decrease in [Ca\textsuperscript{2+}]i resulting from increasing miR-204 in control PASMCs (Fig. 5 B). Similarly to SHP2 inhibition, the down-regulation of K+ channels (Platoshyn et al., 2000; Bonnet et al., 2007b) has been linked to mitochondrial hyperpolarization. We observed that miR-204 inhibition in control PASMCs hyperpolarizes $\Delta \Psi_m$ to a level similar to that observed in PAH-PASMCs (Fig. 5 D), whereas either increasing the miR-204 level or using SHP2 siRNA, Src inhibitor PP2, or STAT3 siRNA in PAH-PASMCs depolarizes $\Delta \Psi_m$ to a level similar to that measured in control PASMCs (Fig. 5 E). Finally, mitochondrial depolarization induced by up-regulating miR-204 expression in PAH-PASMCs increases serum starvation–induced apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL]; Fig. 5 F). As for [Ca\textsuperscript{2+}], $\Delta \Psi_m$ was not affected by TRPM3 inhibition (Fig. S4).

**The STAT3–miR-204–Src–STAT3–NFAT axis is activated in the PAH animal model**

We next tested the contribution of miR-204 in the MCT-injected rat model of PAH (Frasc et al., 1999; Bonnet et al., 2007b). We observed that miR-204 down-regulation parallels PAH development, confirming that the decreasing of miR-204 level correlates with PAH progression and severity (Fig. 6, A and E). To further study the timing of activation of the STAT3–miR-204–Src–STAT3–NFAT axis in the progression of PAH, rats were sacrificed at various intervals after the injection of MCT. Before sacrifice, pulmonary arterial pressure was measured directly by right heart catheterization in closed chest rats. We observed an increase in STAT3 activation ($\leq$1 wk; Fig. 6 C) preceding miR-204 down-regulation (occurring 2 wk after MCT injection). Once miR-204 is down-regulated ($\geq$2 wk), SHP2 is increased and STAT3 activation is pushed up to reach and sustain a maximal level from week 2–4 (Fig. 6, B and C). Once STAT3 activation becomes maximal, NFAT gets activated ($\geq$3 wk; Fig. 6 D), increasing pulmonary arterial pressure (Fig. 6 E). Therefore, the...
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lowing NFAT-dependent PASMC proliferation and resistance to apoptosis and increasing PA remodeling and pressures.

To test whether restoration of miR-204 level can reverse symptoms of PAH in the rat model, synthetic miR-204 RNA molecules were selectively delivered to the lung of

time course analysis confirms our in vitro data supporting the idea that the activation of STAT3 occurs before the decrease in miR-204, which thereby amplifies the activation of STAT3 and NFAT. These results confirmed the initial implication of STAT3 in the attenuation of miR-204 in PAH. Once miR-204 is down-regulated, STAT3 activation is further increased and maintained over a long period of time, thus al- nylon activation.

Figure 5. miR-204 restoration decreases [Ca²⁺], and depolarizes mitochondrial membrane potential. (A–C) Analysis of [Ca²⁺], (Fluo3-AM) and cell proliferation (PCNA nuclear localization) of PASMCs from PAH and control (Ctrl) patients. miR-204 antagonir (Inh) and mimics, VIVIT (NFAT competitor peptide), and PP2 (Src inhibitor) compared with PP3, its negative control, STAT3 siRNA, SHP2 siRNA, and appropriate controls for each treatment were added as indicated (n = 50–150 cells/patient in three PAH and five control patients). (D–F) Analysis of the mitochondrial membrane potential (ΔΨm; TMRM) and serum starvation-induced apoptosis (TUNEL staining) of PASMCs from PAH and control patients. miR-204 antagonir (Inh) and mimics, VIVIT (NFAT competitor peptide), PP2 (Src inhibitor), STAT3 siRNA, SHP2 siRNA, and appropriate controls for each treatment were added as indicated (n = 50–150 cells/patient in three PAH and five control patients). F.U., fluorescence unit. Graphs represent means ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Figure 6. miR-204 level decreases within distal PAs of rats injected with MCT during the fourth week of PAH development. (A) miR-204 expression relative to U6 measured by qRT-PCR in distal PAs of rats. (B) SHP2 protein expression was quantified in distal PAs by immunofluorescence (F.U., fluorescent unit) on lung sections (n = 5 measurements by rat in five rats per time point). (C and D) STAT3 and NFAT activation were measured by the percentage of cells presenting p-STAT3 and NFAT nuclear localization, respectively, in distal PAs of rats (n = 5 measurements by rat in 10 rats per time point). (E) Mean PA pressure measured by right catheterization in closed chest rats (n = 5 rats per group). Graphs represent means ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
MCT-induced PAH (MCT-PAH) rats 10–15 d after MCT injection (when endogenous miR-204 down-regulation reached its peak and PAH was established) by intratracheal nebulization. To verify the tissue distribution of nebulized miR-204, we measured miR-204 mRNA levels in several tissues by qRT-PCR, and we analyzed by immunofluorescence the distribution of the mimic DY547 labeled (control of transfection; Fig. S7 A). Our results revealed that nebulized synthetic miR-204 is essentially localized to intraparenchymal resistance PAs and therefore has limited, if any, detrimental effect.

A longitudinal study to assess the efficacy of our treatment was performed for 2 wk using noninvasive measurements (Doppler echocardiography). We observed that the local delivery of synthetic miR-204 in MCT-PAH rats reduced pulmonary arterial pressure (Fig. 7 A), as assessed by the PA acceleration time (PAAT), a Doppler parameter linked to PA pressure (PAAT being inversely correlated to PA pressure; Fig. S7 B). In addition, synthetic miR-204 decreased right ventricle wall thickness (Fig. S7 B) when compared with MCT-PAH rats treated with nonspecific synthetic RNA molecules. In an opposite manner, miR-204 antagonir nebulization in control rats induced PAH development within 3 wk (Fig. 7 A and Fig. S7 B), whereas antagonir negative control nebulized to two animals had no effects (not depicted). To determine whether synthetic miR-204 delivery can reduce PA remodeling in MCT-PAH animals, we measured medial wall thickness. We observed that animals treated with the synthetic miR-204 displayed a significant reduction in medial thickness of small (≤300 µm) and medium-sized (≤600 µm) PAs (Fig. 7 B). A significant decrease of SHP2, p-STAT3, NFATc2 activation, PASMC proliferation (as assessed by PCNA distribution), and resistance to apoptosis (TUNEL) was also observed in rats treated with synthetic miR-204 (Fig. 7 C and Fig. S8).

**DISCUSSION**

Although a previous study has reported that several miRNAs were aberrantly expressed in PAH (Caruso et al., 2010), our study is the first providing a mechanistic approach to their local delivery. We observed that the local delivery of synthetic miR-204 in MCT-PAH rats reduced pulmonary arterial pressure (Fig. 7 A), as assessed by the PA acceleration time (PAAT), a Doppler parameter linked to PA pressure (PAAT being inversely correlated to PA pressure; Fig. S7 B). In addition, synthetic miR-204 decreased right ventricle wall thickness (Fig. S7 B) when compared with MCT-PAH rats treated with nonspecific synthetic RNA molecules. In an opposite manner, miR-204 antagonir nebulization in control rats induced PAH development within 3 wk (Fig. 7 A and Fig. S7 B), whereas antagonir negative control nebulized to two animals had no effects (not depicted). To determine whether synthetic miR-204 delivery can reduce PA remodeling in MCT-PAH animals, we measured medial wall thickness. We observed that animals treated with the synthetic miR-204 displayed a significant reduction in medial thickness of small (≤300 µm) and medium-sized (≤600 µm) PAs (Fig. 7 B). A significant decrease of SHP2, p-STAT3, NFATc2 activation, PASMC proliferation (as assessed by PCNA distribution), and resistance to apoptosis (TUNEL) was also observed in rats treated with synthetic miR-204 (Fig. 7 C and Fig. S8).

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**DISCUSSION**

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implication in the etiology of human PAH. Despite the fact that several other miRNAs are aberrantly expressed in PAH-PASMCs, we focused our study on miR-204, which was the only one down-regulated, and because putative mRNA targets predicted in silico (TargetScan 5.1) were members of pathways implicated in cell proliferation and resistance to apoptosis, including Src (Wong et al., 2005), STAT3 (Shibata et al., 2003), and NFAT (Fig. S1; Bonnet et al., 2009). Because miR-204 is expressed seven times more in PASMCs than PAECs, we focused our research on the role of miR-204 in PAH-PASMC proliferation and resistance to apoptosis, although we cannot exclude a role of miR-204 in PAH-PAECs, which are also implicated in PAH etiology (Jurasz et al., 2010).

Other miRNAs have been implicated in vascular diseases, but their implication remains elusive. For example, although a recent study has shown that miR-21-1 up-regulation was implicated in vascular neointimal lesions (Ji et al., 2007), miR-21 has been shown to be down-regulated in lung tissue of MCT rats and unchanged in chronic hypoxia (Caruso et al., 2010). Interestingly, in our human PAH-PASMCs, miR-21 is unchanged, suggesting that miR-21 might not necessarily be an important player in the etiology of PAH. Interestingly, in the Caruso et al. (2010) study, miR-204 is down-regulated by 45% and 40% in both chronic hypoxic and MCT rat lungs, respectively, confirming our findings in PASMCs and human lungs.

The role of miR-204 in vascular tissues remained unknown. A recent study performed in retinal epithelial cells and several cancer cells has demonstrated that the down-regulation of miR-204 is associated with enhanced PDGFβ expression, cell proliferation, and the down-regulation of K+ channels, which in turn depolarizes epithelial cell membrane potential (Wang et al., 2010). These findings are consistent with ours and with the already known pathophysiological processes of PAH, reinforcing the importance of miR-204 in the etiology of PAH. Indeed, increased PDGFβ has been reported in PAH (Barst, 2005), and this is consistent with STAT3 activation (PDGFβ being an activator of STAT3; Yu et al., 2003), PASMC proliferation, and resistance to apoptosis (Bonnet et al., 2009).

miR-204 is encoded within the TRPM3 gene. Although, transient receptor potential cation channels have been implicated in PAH (Yu et al., 2004), the role of TRPM3 remains unknown. In our study, we demonstrate that miR-204 effects are not mediated by TRPM3. First, ectopic increases of miR-204 inhibit the SHP2–Src–STAT3 axis, decreasing PAH-PASMC proliferation, resistance to apoptosis, and IL-6 secretion without restoring TRPM3 expression (Fig. S4, A and B). Second, miR-204 down-regulation in control PASMCs mimics PAH, without decreasing TRPM3 (Fig. S4 A). Third, TRPM3 inhibition (without affecting miR-204 levels) did not induce a PAH phenotype in control PASMCs (Fig. S4 C).

Recently, a study performed in human PASMCs linked the down-regulation of BMPR2 to the activation of an IL-6–STAT3–miR-17–92 axis (Brock et al., 2009). In their model, IL-6 activates STAT3, thereby increasing the miR-17–92 cluster expression and down-regulating BMPR2. Despite the fact that miR-17–92 cluster expression is unchanged in our PAH-PASMCs, our study offers a new perspective on the mechanism of BMPR2 down-regulation in PAH. The fact that miR-204 regulates IL-6 secretion (Fig. S4 B) and increases Src activity (Fig. 2) in PAH-PASMCs suggests that miR-204 down-regulation might indirectly down-regulate BMPR2. This is supported by preliminary data showing a significant up-regulation of BMPR2 in both human PAH-PASMCs and in PAs from PAH rats treated with miR-204 mimic (Fig. S5).

One major strength of the present study is the clear demonstration of a mechanism for the origin of the mRNA deregulation in ~10 PAH patients that we provide for the first time in the field of vascular diseases. We show that a primary STAT3 activation by circulating pro-PAH factors such as endothelin-1, PDGF, and angiotensin II (which all increase at the onset of PAH; Archer and Rich, 2000) accounts for the down-regulation of miR-204 in control PASMCs. This finding was confirmed by both promoter analysis and ChIP-PCR analysis, which show a direct binding of STAT3 near the miR-204 gene (within TRPM3; Fig. 3 D). Moreover, we showed that the activation of STAT3 in vivo in PAs of rats injected with MCT precedes miR-204 down-regulation (Fig. 6 C). Once miR-204 is down-regulated, the Src activator SHP2 is directly up-regulated and IL-6 (Fig. S4 B) and PDGF production (Wang et al., 2010) increased, reinforcing STAT3 activation via Src and allowing NFAT activation. This could explain the sustained pro-proliferative and antiapoptotic phenotype of cultured PAH-PASMCs. The activation of such a mechanism is in accordance with a recently published RNA profiling study performed in 18 iPAH patients (Rajkumar et al., 2010). A reanalysis of the data available for this study confirms the down-regulation of TRPM3 as well as the up-regulation of both SHP2 (also known as PTPN11) and NFATc2. Although we provide evidence that STAT3 increases NFAT expression by binding to the NFAT genes promoter region, the activation of NFAT by STAT3 requires the activation of either the Ca2+-calcinurin pathway or the activation of other NFAT activators like Pim-1. In our model, [Ca2+]i, is indeed increased, which could participate in calcineurin activation. Moreover, Pim-1 is a protooncogene that is regulated by STAT3 and that increases in vascular diseases (Katakami et al., 2004). It is therefore possible that STAT3 activation not only accounts for NFAT expression but also promotes NFAT activation by up-regulating Pim-1 expression (Rainio et al., 2002). Finally, we measured miR-204 expression in buffy coats of 13 PAH patients and 7 control donors and showed that as in PAH lungs, miR-204 is down-regulated, suggesting that miR-204 could be a good PAH biomarker.

In conclusion, we herein provide the first evidence that aberrantly expressed miRNAs play a critical role in the etiology of human PAH. We demonstrate both in vitro and in vivo that miR-204 can be therapeutically targeted, leading to a decrease of proliferation, vascular remodeling, and PA blood pressure and thus represents a new therapeutic approach for
PAH. Moreover, we have preliminary evidence that miR-204 might regulate the RhoA–ROCK pathway in PAH-PASMCs (another important component of PAH; Fig. S6 D; Doggrell, 2005). Although other experiments are required to identify the exact mechanism, Bregenon et al. (2009) and Kimura and Eguchi [2009] have previously demonstrated a role of SHP2 in the RhoA–ROCK pathway. This constitutes the basis for a further investigation. Our study proposes that the therapeutic modulation of a single miRNA (miR-204) may affect many pathways simultaneously associated with PAH to achieve clinical benefit. Compared with currently used therapies that target a single protein (ET-1 receptor blockers, PDE5 inhibitor, etc.), the regulation of hundreds of targets in multiple pathways by miRNAs may reduce the emergence of drug resistant as currently seen in PAH because many simultaneous mutations would be required to subvert the effects of miRNA expression. However, at the same time, miRNA-based therapies will require thorough preclinical validation as these broad effects may, in some cases, have toxic consequences. Nonetheless, this was not observed in our rats.

In summary, we provide a comprehensive model (Fig. S8 E) linking miRNA abnormal expression to already known pathophysiological processes in PAH, including NFAT activation, BMPR2 down-regulation, IL-6 production, the Rho pathway, PASMC proliferation, and resistance to apoptosis (Cowan et al., 2000; Sakao et al., 2005; Bonnet and Archer, 2007; Bonnet et al., 2007b; Tudor et al., 2007). Thus, our study does not only demonstrate the importance of miRNAs in PAH but also suggests that reestablishing the miR-204 level might represent a novel therapeutic approach for human PAH.

MATERIALS AND METHODS

Ethics. All experiments were performed in accordance with the Université Laval’s Ethics and Biosafety Committee (protocol number 20142) and the Centre Hospitalier Universitaire de Québec’s Ethics Committee. The investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (publication no. 85–23, revised 1996) and with the principles outlined in the Declaration of Helsinki.

Human tissue samples. See Table S1. All patients gave informed consent before the study. Normal lung tissues (controls) were obtained during lung explants. All patients had right catheterization that confirmed pulmonary arterial pressure >25 mmHg. Age- and gender-matched healthy controls (n = 3) or malignant (n = 5) tumors. Only the healthy parts of the lungs were used in this study. All the PAH tissues were from open lung biopsies or malignant tumors. Only the healthy parts of the lungs were used in this study. All the PAH tissues were from open lung biopsies or malignant tumors (n = 5).

Cell culture. We used cells in the first to third passage. PAH-PASMCs were obtained as described previously (McMurray et al., 2005) from ~1,500-μm-diameter small PAs from two males with PAH (31- and 48-yr-old patients A and B) and one female with PAH group 1 (fopus; 54-yr-old patient C) from lung explants. All patients had right catherization that confirmed pulmonary hypertension (mean pulmonary arterial pressure >25 mmHg). Age- and sex-matched control PASMCs (three males A, B, and C; 45, 21, and 64 yr old; and two females D and E 17 and 35 yr old), and PAECs were purchased from Cell Application USA. PASMCs were grown in high-glucose DMEM supplemented with 10% FBS (Invitrogen) and 1% antibiotic/antimotic (Invitrogen; Bonnet et al., 2007a). NFAT was inhibited by 4 μM VIVIT as previously described (Bonnet et al., 2007b). Control PASMCs were exposed to 30 ng/ml PDGF, 10 nM endothelin-1, 200 nM angiotensin II, or 100 ng/ml TNF (all from EMB Canada). The Src inhibitor PP2 effects were compared with its negative control inhibitor PP3 (4-amino-7-phenylpyrazol [3,4-d] pyrimidine; 10 μM for 48 h). For each experiment, we used a proper control (n = 4; or fopus). For each experiment, we used a proper control (n = 5). For each experiment, we used a proper control (n = 5).

DNA microarrays. DNA microarray experiments were performed using the Whole Human Genome microarray kit (Agilent Technologies). The arrays were scanned using a dual-laser DNA microarray scanner (Agilent Technologies), and the data were extracted from images using the Feature Extraction software. For the control versus PAH patient comparison, RNAs extracted from two control patients were hybridized on Cy3, and RNAs extracted from two PAH patients were hybridized on Cy5. For the miR-204 inhibition experiment, RNAs extracted from control PASMCs treated for 48 h with 200 nM miR-204 antagonist (Thermo Fisher Scientific) were hybridized with Cy3, whereas PASMCs treated with antagonist negative were hybridized on Cy5. Data were yellow subtracted and normalized within the array using the LOESS normalization before significant modulation assessment using the Empirical Bayesian method within the package limma in Bioconductor. Genes listed as targets of miR-204 in TargetScan 5.1 and having a level of expression ≥100 in log2 base and being up-regulated after miR-204 inhibition were considered as miR-204 targets in our model. Microarray data have been deposited in GEO (Acc no. GSE21284).

qRT-PCR. To measure miR-204 expression, the mirVana kit (Applied Biosystems) was used to extract total RNA from PAH-PASMCs or control PASMCs. Stem-loop qRT-PCR for mature miRNAs was performed on a real-time PCR system (ABI 7900; Applied Biosystems). Regular qRT-PCR was performed as previously described (Bonnet et al., 2007b).

ChIP-PCR. In brief, control PASMC asynchronously growing cells were treated with endothelin at 10 nM. Cross-links were generated with 1% formaldehyde, and chromatin was extracted in lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 0.2% SDS, and 5 mM Na-butyrate). Chromatin was then sheared by sonication (Bioruptor; Diagenode) on ice to a mean length of 750 bp. After pre-clearing with a mix of protein A/G-Sepharose beads (4°C for 1 h), 80 μg chromatin was used for immunoprecipitation with appropriate antibodies (10 ml p-Sat3 [Tr7/95; 9131; Cell Signaling Technology] and 10 μg normal rabbit IgG [I-1000; Vector Laboratories]) in 50 μl of protein A Dynabeads (Invitrogen) was added and incubated for >1 h. Beads were extensively washed, and immunoprecipitated complexes were eluted in buffer E (50 mM Na bicarbonate and 1% SDS). Cross-links were reversed overnight at 65°C. Samples were treated with proteinase K, and the DNA was extracted using phenol-chloroform. Quantitative real-time PCR was performed using SYBR green I (LightCycler 480; Roche). Enrichment for a specific DNA sequence was calculated using the comparative Ct method. The numbers presented with standard errors are based on two biological repeats (cells/chromatin/immunoprecipitation). Primers used in the PCR reactions (Table S2) were analyzed for specificity, linearity range, and efficiency to accurately
evaluate occupancy (percentage of immunoprecipitation/input). Vascular endothelial growth factor (VEGF) primers were used as positive control, whereas OR8J1 primers were used as negative control.

Confocal microscopy. NEAT-1 and -c2 and STAT3 nuclear translocation assays were performed using antibodies (1:250; Abcam) as previously described (Bonnet et al., 2007b). TMRM, TUNEL, PCNA, and Fluor-3 were measured as previously described (Bonnet et al., 2009; Bonnet et al., 2007b).

Transfection and luciferase assay for different 3’ UTR constructions. The 3’ UTRs of each gene of interest were cloned and inserted in the psi-CHECK2 plasmid immediately downstream from the stop codon of firefly luciferase. Once ready, cells were transfected with the reporter plasmid with 200 nM of unrelated small RNA duplex (mimic control; Invitrogen), miR-luciferase. Once ready, cells were transfected with the reporter plasmid with CHECK2 plasmid immediately downstream from the stop codon of firefly luciferase.

The presence of an interaction between miR-204 and target mRNA would reduce the firefly luciferase activity (normalized to Renilla luciferase activity measured as previously described (Bonnet et al., 2009; Bonnet et al., 2007b).

In vivo model rats. Male rats were injected s.c. with a 60–mg/kg MCT solution (Todorovich-Hunter et al., 1988). PAH was assessed by hemodynamic measurements (using Swan-Ganz catheters) and echocardiography (using Vevo 2100, VisualSonics), which were performed as previously described (Bonnet et al., 2007b). In vivo, rats with established PAH (measured by Echo-Doppler) were nebulized with miR-204 mimic (mature sequence, 5’-UUUCCUUUGUCAUCUAGCCU-5’) or mimic negative (20 µM once a week for 2 wk). In vivo nebulization (in vitro) was used as transfection agent according to the manufacturer’s instructions. Transfection efficiency and tissue distribution were assessed by qRT-PCR. Tissue distribution was assessed using fluorescent distribution of the commercially available DY547-labeled mimic control (Thermo Fisher Scientific).

Chronic hypoxic mice model. Mice were placed for 2–3 wk in normobaric hypoxic chambers maintained with 5.5–liter min⁻¹ flow of hypoxic air (10% O₂ and 90% N₂). Chambers were opened twice a week for cleaning and replenishment of food and water. Oxygen concentration was continuously monitored with blood gas analyzers. Soda lime was used to lower carbon dioxide concentration.

Statistical analysis. Values are expressed as fold change or mean ± SEM. Unpaired Student’s t tests were used for comparisons between two means. For comparisons between more than two means, we used one-way analysis of variance followed by a Dunn’s test. A p-value <0.05 was considered statistically significant. Vascular endothelial growth factor (VEGF) primers were used as positive control, whereas OR8J1 primers were used as negative control.

Online supplemental material. Fig. S1 shows seven miRNAs that are aberrantly expressed in human PAH-PASMCs compared with control PASMCs. Fig. S2 represents the measurements of miR-204 level in the pulmonary vasculature and buffy coat. Fig. S3 shows that miR-204 down-regulation in PAH-PASMCs promotes the activation of STAT3 and NFAT. Fig. S4 shows that the miR-204 effect is independent of TRPM3 expression. Fig. S5 shows that the miR-204 mimic molecule restores BMPR2 expression in PAH. Fig. S6 shows that a decrease of miR-204 level activates the Src–STAT3 axis that corresponds to the miR-204 binding site in position 2, 4, and 6 from the 3’ UTR sequence that the miR-204 mimic molecule restores BMPR2 expression in PAH. Fig. S7 shows that the miR-204 mimic molecule restores BMPR2 expression in PAH-PASMCs, whereas OR8J1 primers were used as negative control. Fig. S8 shows that the validation of miR-204 mimic/antagomir transfection and siRNA effects on PASMCs. Table S1 lists patients providing tissue. Table S2 lists primers used for ChIP–real-time PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101812/DC1.

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