

## ORIGINAL ARTICLE

# Phenylephrine induces elevated RhoA activation and smooth muscle $\alpha$ -actin expression in *Pkd2<sup>+/−</sup>* vascular smooth muscle cells

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The mechanisms underlying vascular complications in autosomal-dominant polycystic kidney disease (ADPKD) have not been fully elucidated. However, molecular components altered in *Pkd* mutant vascular smooth muscle cells (VSMCs) are gradually being identified. *Pkd2<sup>+/−</sup>* arterial smooth muscles show elevated levels of (1) phenylephrine (PE)-induced,  $\text{Ca}^{2+}$ -independent vasoconstriction and (2) smooth muscle  $\alpha$ -actin (SMA) expression. As these two processes are heavily influenced by RhoA signaling and by cellular filamentous-to-globular (F/G)-actin dynamics, we examined PE-induced changes in RhoA activation and the F/G-SMA ratio in wild-type (wt) and *Pkd2<sup>+/−</sup>* VSMCs; we further tested the hypothesis that the abnormal response to PE and the resultant elevation in the F/G-SMA ratio contribute to the exuberant SMA expression in *Pkd2<sup>+/−</sup>* VSMCs. GTP-RhoA and F/G-SMA in mouse aortic media and primary cultured VSMCs were determined using RhoA activation and *in vivo* F-to-G-actin assays. Myocardin-related transcription factor-A (MRTF-A) (SMA transcription coactivator) was localized by immunofluorescence, nuclear MRTF-A quantified by western analysis using nuclear extracts and SMA expression by luciferase reporter assay. PE induced a >3-fold higher RhoA activation in *Pkd2<sup>+/−</sup>* than in wt VSMCs and higher levels of downstream p-LIMK and p-cofilin. Moreover, *Pkd2<sup>+/−</sup>* VSMCs showed a higher baseline and PE-induced F/G-SMA ratio. The F/G-SMA elevation enhanced nuclear translocation of MRTF-A, which upregulated SMA transcription. In summary, PE-induced RhoA hyperactivation and defects in F-to-G SMA balance likely have a role in the abnormal vasoconstriction and SMA expression in *Pkd2<sup>+/−</sup>* arteries. These defects could potentially contribute to the genesis of vascular complications in ADPKD, thus providing new areas for further research and therapeutic targeting.

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## INTRODUCTION

Autosomal-dominant polycystic kidney disease (ADPKD) is a common (1:400–1000 live births) genetic disease with prominent vascular manifestations, including spastic vasoconstriction, abnormal arterial remodeling, early-onset hypertension and intracranial aneurysms. The mechanisms underlying these abnormalities have not been elucidated.

Autosomal-dominant polycystic kidney disease is caused by mutations to *PKD1* or *PKD2* gene, which encodes polycystin-1, a receptor-like protein with undefined ligand(s), and polycystin-2, a membrane protein that can function as a  $\text{Ca}^{2+}$  channel. Polycystins 1 and 2 interact, forming a signaling complex.<sup>1</sup> *Pkd2<sup>+/−</sup>* mouse, an orthologous model of ADPKD, carries a *Pkd2*-null allele and shows a reduced lifespan independent of kidney function.<sup>2</sup> Mineralocorticoids-induced hypertension in *Pkd2<sup>+/−</sup>* mice triggers a prominent vascular pheno-

type including irregular vessel wall thickness, intracranial aneurysmal dilations and sudden death likely due to vascular catastrophes.<sup>3</sup>

*Pkd2<sup>+/−</sup>* vascular smooth muscle cells (VSMCs), when compared to wild-type (wt), show a ~20% reduction in the basal intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ) and a ~30% elevation in the smooth muscle  $\alpha$ -actin (SMA) expression.<sup>3,4</sup> In response to adrenergic receptor agonist (phenylephrine, PE) stimulation, *Pkd2<sup>+/−</sup>* arterial strips generate an exaggerated (~200% higher) level of maximum contraction, yet a diminished (~50% less)  $[\text{Ca}^{2+}]_i$  increase than those in wt.<sup>4</sup> Such an elevated contraction-to- $[\text{Ca}^{2+}]_i$  ratio indicates a heightened,  $\text{Ca}^{2+}$ -independent mechanism of force generation ( $\text{Ca}^{2+}$  sensitization)<sup>5</sup> in *Pkd2<sup>+/−</sup>* VSMCs.

Smooth muscle  $\alpha$ -actin expression and smooth muscle  $\text{Ca}^{2+}$  sensitization, the two processes highly abnormal in *Pkd2<sup>+/−</sup>* VSMCs,

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are known to be heavily affected by a dynamic actin assembly/disassembly<sup>6,7</sup> and by the activation of Rho GTPase, RhoA.<sup>5,8</sup> Therefore, the defects observed in *Pkd2<sup>+/−</sup>* VSMCs infer alterations in actin dynamics and RhoA activation.

Actin assembly/disassembly in VSMCs, reflected by the ratio of filamentous-to-globular (F/G)-actin, is critical for modifying cellular morphology and vascular contraction and remodeling.<sup>9</sup> In this process, the disassembly of F-actin is rate limiting and is tightly regulated by several ubiquitously expressed regulatory molecules including Ca<sup>2+</sup> and RhoA. Ca<sup>2+</sup>, ranging from ~50 nM to 10 μM, has been shown to activate actin-severing gelsolin superfamily proteins,<sup>10,11</sup> thereby promoting F-actin disassembly and reducing F-actin. On the contrary, RhoA through its downstream ROCK-LIMK-cofilin<sup>12,13</sup> and diaphanous-related formin (mDia1)<sup>14</sup> elevates cellular F-actin. Thus, Ca<sup>2+</sup> reduces while RhoA signaling elevates the F/G-actin ratio.

The cellular F/G-actin ratio has been shown to influence the expression of smooth muscle differentiation-related proteins at the level of transcription.<sup>7,15</sup> The transcription of this group of genes is promoted by the binding of serum response factor (SRF) to the CArG boxes within their promoters.<sup>16</sup> Such SRF-CArG binding is vastly enhanced by the myocardin family of SRF coactivators. These coactivators include myocardin, myocardin-related transcription factor (MRTF)-A and MRTF-B. MRTFs, not myocardin, shuttle between the cytoplasm and the nucleus in an F/G-actin-sensitive manner.<sup>17,18</sup> Monomeric G-actin binds and traps MRTFs in the cytoplasm,<sup>18</sup> thereby limiting their nuclear translocation. Thus, F/G-actin reduction (high in G-actin pool) attenuates, while its elevation (low in G-actin pool) augments MRTF nuclear translocation and MRTF-SRF-CArG target gene expression, including the expression of SMA.<sup>19</sup>

In this study, we explored the mechanisms underlying the elevated SMA expression in *Pkd2<sup>+/−</sup>* VSMCs. We found that, when compared with wt, *Pkd2<sup>+/−</sup>* arterial smooth muscle contained a significantly higher proportion of F-SMA. Moreover, PE stimulation induced a higher level of RhoA activation and a greater F/G-SMA ratio in *Pkd2<sup>+/−</sup>* VSMCs. The elevated F/G-SMA ratio triggered an excessive MRTF-A nuclear translocation, resulting in an augmented SMA expression in *Pkd2<sup>+/−</sup>* VSMCs, compared with those in wt VSMCs.

## METHODS

### Animals and VSMCs culture

Inbred wt and *Pkd2<sup>+/−</sup>* littermates (3–5 month olds)<sup>3</sup> were used for the generation of primary VMSC culture,<sup>20</sup> and animal procedures approved by the Institutional Animal Care and Use Committee.

### Antibodies and reagents

Antibodies for PC2 (sc-25749), Myocardin (sc-33766), MRTF-A (sc-32909), MRTF-B (sc-47282) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); for TATA binding protein (TBP) (ab62125) from Abcam (Cambridge, MA, USA), and for SMA (clone 1A4) from Sigma (St Louis, MO, USA). Unless specified, all reagents were purchased from Sigma.

### Indirect immunofluorescence

Immunostaining of serum-starved (~48 h) VSMCs (passages 3–5) were previously reported.<sup>20</sup> Negative controls, in which the primary antibody was replaced by normal serum, were used in parallel for each primary antibody.

### Total cellular SMA and F/G-SMA quantification

The tunica media layers isolated from the descending aortas of wt and *Pkd2<sup>+/−</sup>* male littermates and serum-starved VSMCs were used. Total cellular SMA was quantified as described.<sup>3</sup> For F/G-SMA quantification, an F-actin/G-actin *in vivo* Assay Kit (cat. no. BK037, Cytoskeleton, Denver, CO, USA) was used following the manufacturer's protocol and detected with specific SMA antibody.

### Determination of RhoA activation

Using a RhoA activation assay kit (cat. no. BK036, Cytoskeleton), GTP-bound RhoA was quantified following the manufacturer's protocol. Briefly, VSMCs were lysed and homogenized by sonication. Supernatants were incubated with rhotekin-Rho binding domain at 4 °C (~1 h) on a rotator. Bead-precipitated proteins and total cell lysate were fractionated and immunoblotted with antibody against RhoA.

### Nuclear protein extraction and quantitative western analysis

Nuclear proteins were extracted from wt and *Pkd2<sup>+/−</sup>* VSMCs at 0 and 24 h of PE (5 μM) stimulation, using Nuclear Extraction Kit (cat. no. 2900, Millipore, Billerica, MA, USA) according to the manufacturer's recommendation. The nuclear protein concentrations were determined, and samples of equal protein content were fractionated and analyzed by western blotting. Briefly, polyvinylidene fluoride membranes were incubated with the primary antibodies, MRTF-A (1:500) and TBP (1:1000 as a loading control), overnight at 4 °C. Membranes were then washed and incubated with appropriate secondary antibodies. Blots were developed using Chemiluminescent HRP Substrate (Thermo Scientific, Rockford, IL, USA).

### Measurement of SMA promoter activity

Serum-starved (~48 h) wt and *Pkd2<sup>+/−</sup>* VSMCs were transfected with a rat α-SMA-luciferase<sup>17</sup> (a generous gift from Dr Nemenoff) and CMV-β-galactosidase (to monitor the transfection efficiency) using Opti-MEM medium (Invitrogen, Carlsbad, CA, USA). This rat SMA promoter has been effectively used in mouse cells.<sup>21</sup> After transfection, the cells were allowed to recover overnight before PE stimulation. The luciferase assay was carried out using a Promega kit (Madison, WI, USA) following a standard protocol.

### Statistical analysis

Data are expressed as mean ± s.e. Student's *t*-test and one-way or two-way analysis of variance with Bonferroni's *post-hoc* analysis were used for comparisons between different groups. *N* denotes the number of animals or independently generated cell cultures. A *P*-value of <0.05 was considered significant.

## RESULTS

### *Pkd2<sup>+/−</sup>* arterial smooth muscle contained a higher level of filamentous SMA

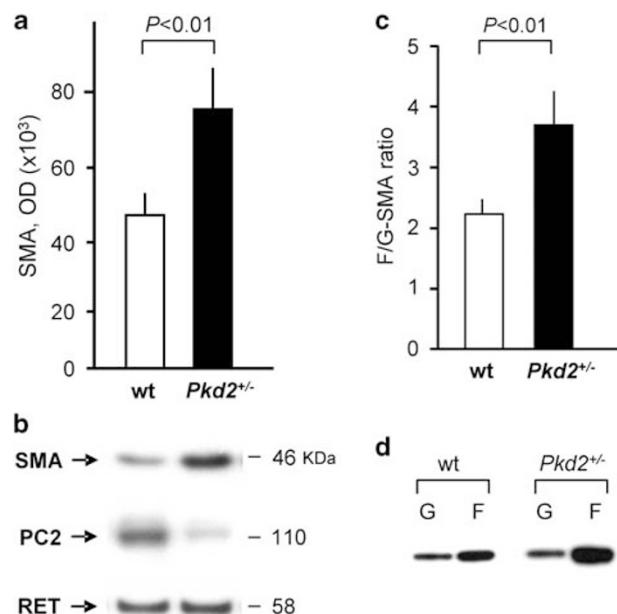
*Pkd2<sup>+/−</sup>* aortic smooth muscle media contains a higher level of total cellular SMA than that in wt.<sup>4</sup> Here, we examined total cellular SMA in primary cultured (passage ≤5) wt and *Pkd2<sup>+/−</sup>* VSMCs. As shown in Figures 1a and b, similar to that in the aortic media, *Pkd2<sup>+/−</sup>* VSMCs contained a higher level of SMA than that in wt ( $75.2 \pm 10.1$  vs.  $45.7 \pm 6.69$  OD; *P*<0.01, *n*=3).

We further examined the proportion of F-SMA and G-SMA in both freshly dissected wt and *Pkd2<sup>+/−</sup>* aortic media and primary cultured VSMCs. As shown in Figures 1c and d, *Pkd2<sup>+/−</sup>* aortic media had a higher F/G-SMA ratio than that in wt ( $3.80 \pm 0.71$  vs.  $2.24 \pm 0.37$ ; *P*<0.01, *n*=3). This difference was conserved in primary cultured VSMCs (data not shown).

### PE induced elevations in RhoA signaling and F/G-SMA ratio in *Pkd2<sup>+/−</sup>* VSMCs

*Pkd2<sup>+/−</sup>* arterial smooth muscle responds to PE stimulation with an heightened Ca<sup>2+</sup>-independent force generation.<sup>4</sup> RhoA is well known to have a major role in this process. To determine the level of RhoA activation, we stimulated wt and *Pkd2<sup>+/−</sup>* aortic strips (endothelial-denuded and preloaded to their optimal length<sup>4</sup>) with PE (5 μM) for 3 min, the time point of maximum contraction,<sup>4</sup> and quantified the activated GTP-RhoA. Although barely detectable under non-stimulated condition (data not shown), the level of GTP-RhoA after PE stimulation was significantly elevated in both wt and *Pkd2<sup>+/−</sup>* arteries, but it was much higher in *Pkd2<sup>+/−</sup>* arteries ( $4.28 \pm 0.62$  OD) than in wt ( $1.37 \pm 0.39$  OD; *P*<0.01,

(n=12 artery pairs) (Figure 2a). This observation suggests that RhoA hyperactivation likely contributes to the generation of Ca<sup>2+</sup>-independent force, as previously reported<sup>4</sup> in *Pkd2<sup>+/−</sup>* arteries.



**Figure 1** *Pkd2<sup>+/−</sup>* vascular smooth muscle cells (VSMCs) contain a higher level of smooth muscle  $\alpha$ -actin (SMA) and a higher ratio of filamentous-to-globular (F/G)-SMA. (a) Densitometric analysis of SMA bands from quantitative western analysis using total cell lysates (n=3). (b) Representative western blots hybridized with antibodies against PC2, SMA and calreticulin (RET). (c) Ratios of F/G-SMA in wild-type (wt) and *Pkd2<sup>+/−</sup>* VSMCs (n>3). (d) Representative blot of G-SMA and F-SMA.

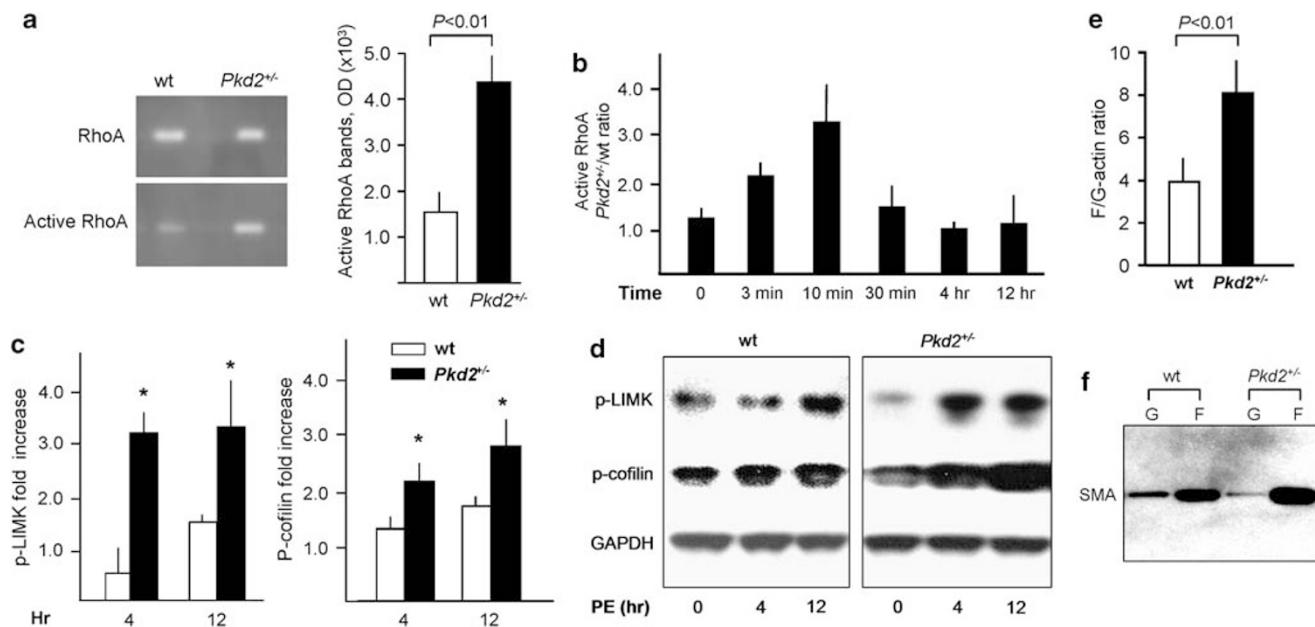
We further investigated PE-induced RhoA activation in primary cultured VSMCs at multiple time points. With sustained PE (5  $\mu$ M) stimulation, both wt and *Pkd2<sup>+/−</sup>* VSMCs developed RhoA activation, peaked at ~10 min. Significantly, *Pkd2<sup>+/−</sup>* VSMCs developed an approximately threefold higher peak RhoA activation than that in wt (Figure 2b).

RhoA activation has been shown to sequentially activate its downstream ROCK-LIMK. Active phospho-LIMK (p-LIMK) in turn phosphorylates cofilin. We examined p-LIMK and p-cofilin in PE-stimulated VSMCs. As shown in Figures 2c and d, after 4 and 12 h of stimulation, *Pkd2<sup>+/−</sup>* VSMCs developed higher levels of p-LIMK and p-cofilin than those in wt. A similar temporal pattern of sequential activation in Rho-LIMK-cofilin has been reported in endothelial cells (induced by shear stress)<sup>22</sup> and myofibroblasts (induced by force).<sup>17</sup>

Once phosphorylated, cofilin becomes inactivated and loses its ability to bind and to sever F-actin. Consistent with the p-cofilin elevation, the F/G-actin ratio was increased after PE stimulation from  $3.80 \pm 0.71$  (without PE stimulation) to  $7.98 \pm 0.99$  ( $n>3$ ,  $P<0.01$ ) in *Pkd2<sup>+/−</sup>* and from  $2.24 \pm 0.37$  to  $3.95 \pm 0.64$  ( $n>3$ ,  $P<0.01$ ) in wt VSMCs (Figures 2e and f). *Pkd2<sup>+/−</sup>* VSMCs showed a much more robust percentage elevation in the F/G-SMA ratio ( $115.8 \pm 12.9\%$ ) than that in wt ( $75.8 \pm 11.2\%$ ,  $P<0.01$ ).

#### PE-induced F/G-SMA elevation enhanced MRTF-A nuclear translocation in *Pkd2<sup>+/−</sup>* VSMCs

The F/G-actin ratio has been shown to influence MRTF nuclear translocation. We examined both MRTF-A and MRTF-B in our cultured VSMCs; we noted that MRTF-B immunofluorescence signal was substantially weaker (barely notable) than that of MRTF-A, and we could not appreciate a difference in the signal intensity of MRTF-B between wt and *Pkd2<sup>+/−</sup>* VSMCs with or without PE stimulation (data not shown).



**Figure 2** *Pkd2<sup>+/−</sup>* aortic smooth muscles and vascular smooth muscle cells (VSMCs) show a higher level of phenylephrine (PE)-induced RhoA-LIMK-cofilin activation. (a) Representative blots of total and GTP-bound RhoA and densitometric analysis of active RhoA in wild-type (wt) and *Pkd2<sup>+/−</sup>* aortic smooth muscle strips stimulated by PE (5  $\mu$ M). (b) The ratios of GTP-bound RhoA in *Pkd2<sup>+/−</sup>* and wt VSMCs responding to PE (5  $\mu$ M) at 0 (control), 3, 10, and 30 min, and at 4 and 12 h. (c) Activate, p-LIMK and p-cofilin in wt and *Pkd2<sup>+/−</sup>* VSMCs responding to PE (5  $\mu$ M) stimulation for 4 and 12 h (n=3). (d) Representative western blots hybridized for p-LIMK and p-cofilin. (e) Filamentous-to-globular (F/G)-SMA ratios in wt and *Pkd2<sup>+/−</sup>* VSMCs stimulated with PE (5  $\mu$ M) for 12 h (n>3). (f) Representative blot of F-actin and G-actin in VSMCs after PE stimulation for 12 h. \*P≤0.05.

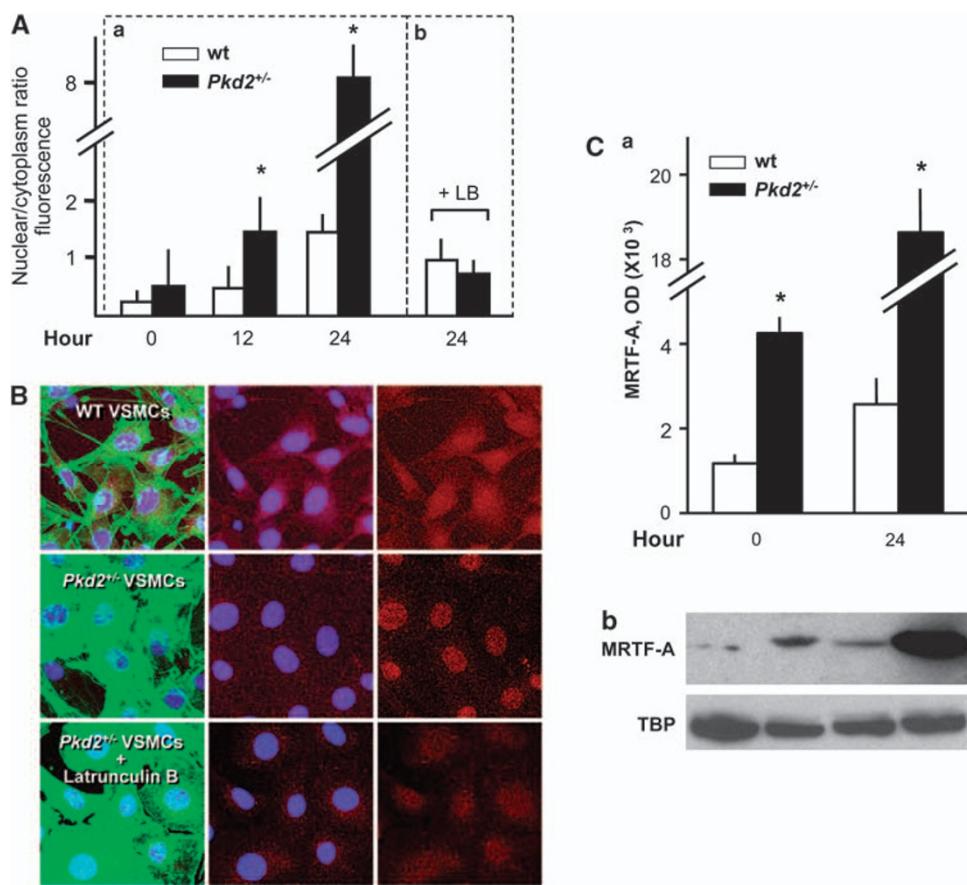
not shown). However, these results could have been caused by the weakness of the signals.

MRTF-A is a key coactivator for the SRF-CArG-mediated SMA expression,<sup>23</sup> and its nuclear translocation is sensitive to F/G-actin in NIH3T3 cells.<sup>18</sup> We examined the distribution of MRTF-A in wt and *Pkd2<sup>+/−</sup>* VSMCs at 0, 12 and 24 h after PE stimulation. The cells from each time point (in triplicate) were fixed and immunostained with an MRTF-A antibody, and the nuclear-to-cytoplasmic fluorescence intensity ratios in the cells from 10 randomly selected confocal fields ( $\times 20$ ) in each sample were obtained. As shown in Figures 3A-a and B (the first and second rows), the fluorescence ratios of nuclear-to-cytoplasmic MRTF-A in *Pkd2<sup>+/−</sup>* VSMCs were significantly higher after 12 and 24 h of PE stimulation; a trend of elevation was also noted at the basal condition (0 h).

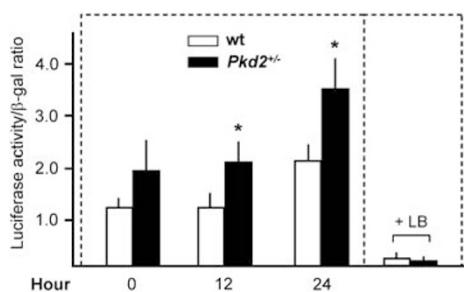
The elevation of the F/G-SMA ratio in *Pkd2<sup>+/−</sup>* VSMCs at basal condition (0 h) could potentially influence the degree of MRTF-A nuclear translocation. Consistent with this assumption, our immunofluorescence data at time 0 h showed a trend of elevated nuclear MRTF-A (Figure 3A-a). We further quantified nuclear-located MRTF-A by quantitative western analysis using nuclear extracts from wt and *Pkd2<sup>+/−</sup>* VSMCs. As shown in Figure 3C, the nuclear-localized

MRTF-A in *Pkd2<sup>+/−</sup>* VSMCs at time 0 h was higher than that in wt VSMCs ( $1.19 \pm 0.21$  vs.  $3.99 \pm 0.81$  OD,  $P < 0.01$ ,  $n=3$ ), in line with an elevated F/G-SMA ratio (less G-SMA mediated cytosolic MRTF-A trapping) and a higher SMA expression (promoted by nuclear-located MRTF-A) at non-stimulated condition (Figure 1). Moreover, consistent with the immunofluorescence results (Figures 3A and B), the nuclear-located MRTF-A was dramatically elevated in *Pkd2<sup>+/−</sup>* VSMCs ( $18.7 \pm 3.06$ ) after PE stimulation (Figure 3C), much more than that in wt VSMCs ( $3.25 \pm 0.53$ ,  $P < 0.001$ ,  $n=3$ ).

To further determine whether the elevated F/G SMA ratio (Figures 2e and f) in *Pkd2<sup>+/−</sup>* VSMCs positively influenced nuclear translocation of MRTF-A, we repeated the experiments in the same cells pretreated with latrunculin B (LB, 0.5  $\mu$ M), a molecule that depolymerizes F-actin and has been frequently used to study F-actin-mediated effects.<sup>24</sup> As shown in Figures 3A-b and B (the bottom row), F-SMA depolymerization sharply reduced the nuclear translocation of MRTF-A and eliminated the difference in its distribution between wt and *Pkd2<sup>+/−</sup>* VSMCs. These results are consistent with a scenario in which an imbalance of F/G-SMA in *Pkd2<sup>+/−</sup>* VSMCs results in a relative reduction in the cytosolic MRTF-A trapping.



**Figure 3** Phenylephrine (PE)-induced filamentous-to-globular (F/G)-SMA elevation results in an excessive myocardin-related transcription factor-A (MRTF-A) nuclear translocation in *Pkd2<sup>+/−</sup>* vascular smooth muscle cells (VSMCs). **(A-a)** The fluorescence ratios of nuclear-to-cytoplasmic MRTF-A at 0 (control), 12 and 24 h of PE stimulation in wild-type (wt) and *Pkd2<sup>+/−</sup>* VSMCs ( $N > 3$ ,  $P < 0.01$  by two-way ANOVA). \*Significant difference by post-hoc analysis. **(A-b)** The ratios of nuclear-to-cytoplasmic MRTF-A in wt and *Pkd2<sup>+/−</sup>* VSMCs pretreated with latrunculin B (LB) (0.5  $\mu$ M). **(B)** Representative confocal images, taken with  $\times 100$  oil immersion lens, of MRTF-A fluorescence labeling in wt and *Pkd2<sup>+/−</sup>* VSMCs, and *Pkd2<sup>+/−</sup>* VSMCs pretreated with LB (0.5  $\mu$ M) and stimulated with PE (5  $\mu$ M) for 24 h. The cells were co-immunostained with an MRTF-A antibody (the secondary antibody conjugated to Texas-Red) and SMA antibody (the secondary antibody conjugated to Alexa Fluor 488). 4',6-Diamidino-2-phenylindole stained for the nuclei. **(C)** Nuclear-located MRTF-A, quantified by western analysis of nuclear extracts, were significantly elevated in *Pkd2<sup>+/−</sup>* VSMCs both at time 0 h and 24 h after 5.0  $\mu$ M PE stimulation. **(a)** Bars represent densitometric analysis of MRTF-A bands from quantitative western analysis using nuclear extracts ( $n=3$ ). **(b)** Representative blot, showing the corresponding MRTF-A in nuclear extracts of wt and *Pkd2<sup>+/−</sup>* VSMCs at 0 and 24 h of PE stimulation. TATA binding protein (TBP) was used as a loading control.



**Figure 4** Higher levels of smooth muscle  $\alpha$ -actin (SMA) promoter activity in *Pkd2<sup>+/−</sup>* than in wild-type (wt) vascular smooth muscle cells (VSMCs). (a) Luciferase activities, represented by the increment of luciferase to  $\beta$ -galactosidase ratios in wt and *Pkd2<sup>+/−</sup>* VSMCs at basal condition (time 0 h) and after 12 and 24 h of phenylephrine (PE; 5  $\mu$ M) stimulation. (b) SMA transcriptions in wt and *Pkd2<sup>+/−</sup>* VSMCs pretreated with latrunculin B (LB) (0.5  $\mu$ M) and stimulated with PE (5  $\mu$ M) for 24 h. \* $P \leq 0.05$ .

#### F/G-SMA elevation augmented SMA expression in *Pkd2<sup>+/−</sup>* VSMCs

The finding of F/G-SMA-sensitive MRTF-A nuclear translocation suggested that a higher F/G-SMA ratio in *Pkd2<sup>+/−</sup>* VSMCs might positively regulate SMA expression by MRTF-SRF activation. This possibility was investigated by using a luciferase reporter assay in both wt and *Pkd2<sup>+/−</sup>* VSMCs, both at the baseline and after PE stimulation.

The cells were transfected with a luciferase reporter construct driven by a SMA promoter (containing two CArG elements known to be critical for SMA expression<sup>17</sup>) and a  $\beta$ -gal vector as a loading control. The level of SMA promoter activity was examined at time 0 h and after 12 and 24 h of PE stimulation. The results presented here were derived from five independent transfections at each time point. Control vector transfection generated negligible signal at all time points (data not shown). As shown in Figure 4a, luciferase activities at both basal and after PE stimulation, represented by the increment of luciferase-to- $\beta$ -gal signal ratio, were higher in *Pkd2<sup>+/−</sup>* than in wt VSMCs (by two-way analysis of variance), consistent with a higher level of SMA expression in *Pkd2<sup>+/−</sup>* VSMCs.

To further determine that the imbalance of F-SMA to G-SMA was attributable to the higher level of SMA expression in *Pkd2<sup>+/−</sup>* VSMCs, we repeated the assay after the cells were pretreated with LB (0.5  $\mu$ M  $\times$  30 min) to disrupt F-SMA and then stimulated with PE (5  $\mu$ M  $\times$  24 h) at the continual presence of LB. As shown in Figure 4b, LB treatment eliminated the differential SMA promoter activity in wt and *Pkd2<sup>+/−</sup>* VSMCs. Collectively, these results strongly suggest that the higher F/G-SMA ratio in *Pkd2<sup>+/−</sup>* VSMCs positively regulates SMA expression.

#### DISCUSSION

This study shows that (i) *Pkd2<sup>+/−</sup>* VSMCs contain a higher level of total cellular SMA and a higher ratio of F/G-SMA, (ii) *Pkd2<sup>+/−</sup>* VSMCs respond to PE stimulation with a higher level of RhoA-LIMK-cofilin signaling and an escalated elevation in the ratio of F/G-SMA and (iii) PE-induced F/G-SMA elevation amplifies SMA expression by promoting MRTF-A nuclear translocation and SMA transcriptional activation.

A striking and consistently observed abnormality in *Pkd2<sup>+/−</sup>* VSMCs in this study is that they contain a higher ratio of F/G-SMA than that in wt, both at basal condition (by ~70%) and after PE stimulation (by >100%). We propose that such an F-SMA to G-SMA imbalance could potentially be associated with  $[Ca^{2+}]_i$  reduction<sup>3,4</sup> and RhoA hyperactivation (during PE stimulation) in *Pkd2<sup>+/−</sup>* VSMCs.

$Ca^{2+}$  is known to activate gelsolin, the principal member of the gelsolin family, F-actin severing proteins.<sup>11,25</sup> Gelsolin contains six highly homologous repeats, G1–G6.<sup>26</sup>  $Ca^{2+}$  binds G4–G6 and causes a large-scale conformational change, allowing the high-affinity binding between G1–G3 and the lateral aspect of F-actin. Such binding weakens longitudinal actin–actin contact, resulting in F-actin severing.<sup>25</sup> Gelsolin then caps the fast-growing barbed ends of the severed filaments and prevents the G-actin reuptake, leading to a regional reduction in F-actin and a reciprocal elevation in G-actin.  $Ca^{2+}$  has been shown to activate gelsolin progressively in concentrations ranging from ~50 nM to several micromolars.<sup>11</sup> Although confirmatory study (that is, to examine the F/G ratio and gelsolin activity under controlled levels of  $[Ca^{2+}]_i$ ) is necessary, the current data infer that the  $[Ca^{2+}]_i$  deficiency in *Pkd2<sup>+/−</sup>* VSMCs both at basal state and after PE stimulation<sup>3,4</sup> could restrain the gelsolin activity, favoring a net increase in cellular F-actin.

Active RhoA, while minimum without stimulation, was increased in both wt and *Pkd2<sup>+/−</sup>* VSMCs after PE stimulation. Significantly, *Pkd2<sup>+/−</sup>* VSMCs showed a threefold higher maximum RhoA activation than that in wt VSMCs (Figures 2a and b). In several cellular systems, active RhoA has been shown to promote actin polymerization through its downstream ROCK-LIMK-cofilin and mDia1 pathways.<sup>12–14</sup> Although both pathways are important in this process, they are not significantly redundant.<sup>5</sup> Our observation of an augmented elevation in p-LIMK and p-cofilin is consistent with a hyperactivation of ROCK-LIMK-cofilin signaling in *Pkd2<sup>+/−</sup>* VSMCs, leading to a shift in F/G-SMA balance toward more F-SMA. The precise etiology underlying the RhoA hyperactivation upon PE stimulation in *Pkd2<sup>+/−</sup>* VSMCs needs further investigation. In this regard, G-protein-coupled receptors could be an attractive candidate for further investigation, as previous studies have shown that *Pkd* mutations are associated with an aberrant hyperactivation in G-protein-coupled receptor signaling cascades (in non VSMCs).<sup>27–29</sup> It should also be noted that LIMK, in addition for being an effector of RhoA-ROCK, can be phosphorylated by other mechanisms such as Rac-PAK1 (p21-activated kinase 1) and Cdc42-PAK4. However, these pathways are independent and nonredundant to the RhoA-ROCK pathway.<sup>30</sup> Although concomitant activations of such nonredundant pathways could not be excluded, our results indicate the existence of RhoA-ROCK hyperactivation in *Pkd2<sup>+/−</sup>* VSMCs.

Active RhoA is also well-known to underlie the force generation by  $Ca^{2+}$  sensitization of myofilaments, in which active RhoA-ROCK inhibits MLC (myosin light chain)-phosphatase, leading to an accumulation of p-MLC that enhances cross-bridging of actin-myosin, hence a heightened force generation.<sup>5</sup> The exaggerated PE-induced RhoA activation in *Pkd2<sup>+/−</sup>* VSMCs is consistent with the previous findings of higher PE-induced p-MLC and  $Ca^{2+}$ -independent vasoconstriction in *Pkd2<sup>+/−</sup>* conduit (aortic) and resistance (mesenteric) arteries.<sup>4</sup>

It has been suggested that RhoA-enhanced vasoconstriction couples to a long-term contractile protein expression, mediated through actin polymerization<sup>17</sup> and MRTF nuclear translocation.<sup>18</sup> In our VSMCs, MRTF-A is expressed in a much higher level than that of MRTF-B. We questioned whether the elevated F/G-SMA ratio in *Pkd2<sup>+/−</sup>* VSMCs could influence MRTF-A nuclear translocation and SMA expression. By both indirect immunofluorescence and the luciferase reporter assay, we showed that the elevation of F/G-SMA ratio was associated with a higher level of nuclear translocation of MRTF-A (Figure 3A) as well as SMA transcriptional activation (Figure 4a). It is noted that even without PE stimulation, SMA protein content in *Pkd2<sup>+/−</sup>* VSMCs is higher than that in wt VSMCs, in line with the findings of higher

F/G-SMA ratio and higher nuclear-localized MRTF-A. However, without stimulation, cellular GTP-RhoA was barely detectable in serum-starved *Pkd2<sup>+/−</sup>* VSMCs, thus unlikely to have exerted a substantial effect. This assumption was supported by the observation of a non-substantial (~20%) reduction in SMA expression (mRNA determined by real-time reverse transcription-PCR) in non-stimulated *Pkd2<sup>+/−</sup>* VSMCs pretreatment with a ROCK inhibitor (Y-27632, 20 μM × 4 h) (Q Qian, unpublished data). We suspect that the diminished basal [Ca<sup>2+</sup>]<sub>i</sub> in *Pkd2<sup>+/−</sup>* VSMCs, through altering the F/G-SMA ratio, has possibly exerted a role in the enhanced SMA expression in non-stimulated condition, although confirmatory studies are necessary.

The causal relation between the F/G ratio and MRTF-A nuclear translocation/SMA expression in *Pkd2<sup>+/−</sup>* VSMCs was further supported by the results generated from experiments in which the cells were pretreated with an F-actin depolymerizer, LB. The reduction in F-SMA blunted the differences in MRTF-A nuclear localization and in SMA expression between wt and *Pkd2<sup>+/−</sup>* VSMCs (Figures 3A-b and 4b). These findings strongly suggest that in *Pkd2<sup>+/−</sup>* VSMCs, F/G-SMA elevation compromises the cytoplasmic G-SMA-mediated MRTF-A trapping, resulting in an excessive MRTF-A nuclear translocation and SMA transcription.

Although the results of F/G-SMA-induced SMA expression are convincing, we cannot absolutely rule out the possibility that LB itself might have influenced SMA expression, independent of F/G-SMA in our primary cultured VSMCs. However, this seems unlikely because previous studies have shown that LB treatment in constitutively polymerizing mutant actin had no appreciable effect on actin transcriptional activation.<sup>24,31</sup>

The altered F/G-SMA balance could compromise the plasticity of VSMCs, which may affect the regulations of vascular lumen diameter and resistance. The F/G-SMA imbalance may also affect VSMC migration, vesicular trafficking and exocytosis of matrix metalloproteinases, processes that are important for vascular remodeling.<sup>32</sup> In addition, elevated SMA expression and F-SMA facilitate contractile force generation. Taken together, it seems likely that these defects in *Pkd2<sup>+/−</sup>* VSMCs could alter vasoconstriction and remodeling, which underlie the vascular abnormalities in ADPKD.

In summary, the elevated F/G-SMA ratio, likely associated with [Ca<sup>2+</sup>]<sub>i</sub> reduction and RhoA hyperactivation, positively regulates SMA expression by promoting MRTF-A nuclear translocation in *Pkd2<sup>+/−</sup>* VSMCs. To the best of our knowledge, this is the first investigation on the defects in actin regulation and RhoA activation in *Pkd* mutant VSMCs. These results suggest that targeting the regulations of SMA filaments and Rho signaling has potential for modifying ADPKD-associated vasculopathy.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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