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Inhibition of farnesyl pyrophosphate synthase prevents angiotensin II-induced cardiac fibrosis *in vitro*

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Summary

Farnesyl pyrophosphate synthase (FPPS)-catalysed isoprenoid intermediates are important for the activation of Ras homologue gene family, member A (RhoA) in angiotensin (Ang) II-induced cardiac fibrosis. This study was designed to investigate the specific role of FPPS in the development of cardiac fibrosis. We demonstrated that FPPS expression was elevated in both *in-vivo* and *in-vitro* models of Ang II-mediated cardiac fibrosis. FPPS inhibition by zolendronate and FPPS knock-down by a silencing lentivirus decreased the expression of cardiac fibrosis marker genes, including collagen I, collagen III and transforming growth factor (TGF)-β1. FPPS inhibition was reversed by geranylgeraniol (GGOH) and mimicked by RhoA knock-down with siRhoA. The antagonistic effect of GGOH on the zolendronate-mediated modulation of RhoA activation in Ang II-stimulated cardiac fibroblasts was demonstrated by a pull-down assay. Furthermore, FPPS knock-down also prevented RhoA activation by Ang II *in vitro*. In conclusion, FPPS and RhoA may be part of a signalling pathway that plays an important role in Ang II-induced cardiac fibrosis *in vitro*.

Keywords: cardiac fibrosis, farnesyl pyrophosphate synthase, RhoA

Introduction

Cardiac fibroblast is a major cell type in the heart [1,2] that plays a vital role in the development of cardiac fibrosis through synthesis of the extracellular matrix (ECM) [3–5], a process that requires factors such as collagen I, collagen III and transforming growth factor-β1 (TGF-β1), which are the marker genes of cardiac fibrosis [6,7]. Cardiac fibrosis can be induced by various stimuli [8–10], including angiotensin II (Ang II) [11,12]. Ang II acts through several signalling pathways in cells, some of which involve small G proteins, including Ras homologue gene family, member A (RhoA)[13–19], as described in Fig. 1.

RhoA switches between an inactive guanosine diphosphate (GDP)-bound form and an active guanosine triphosphate (GTP)-bound form [20–23]. RhoA must first undergo geranylgeranylation, which is the attachment of 20-carbon geranylgeranyl groups to its C-terminal cysteine residues, in order to be localized to the membrane, followed by its interaction with effector molecules such as Rho-associated kinase (ROCK) to trigger downstream cellular functions [24]. FPPS is a key enzyme in the mevalonate pathway [25,26]. FPPS catalyses the biosynthesis of isoprenoid intermediates, which are vital for the activity and function of RhoA [6,27].

Our previous studies have demonstrated the importance of FPPS both in *in-vitro* [27] and *in-vivo* [28,29] models of cardiac hypertrophy responses that are associated with Ang II. FPPS inhibition by alendronate or FPPS knock-down abolished Ang II-mediated *in-vitro* [24,27] and *in-vivo* [28,29] cardiac hypertrophy. Recent studies have suggested that RhoA is involved in *in-vitro* [30] and *in-vivo* [18] cardiac fibrosis. Overexpression of RhoA in the heart led to the development of heart failure with bradycardia [31], impaired contractile function and induction of interstitial fibrosis in *in-vivo* models [18].

In this study, we aimed to evaluate the expression of FPPS in Ang II-induced cardiac fibrosis in both *in-vitro* and *in-vivo* models. Then, we assessed the effects of FPPS inhibition or silencing on cardiac fibrosis formation, and studied the signalling pathway involving FPPS and RhoA *in vitro*.

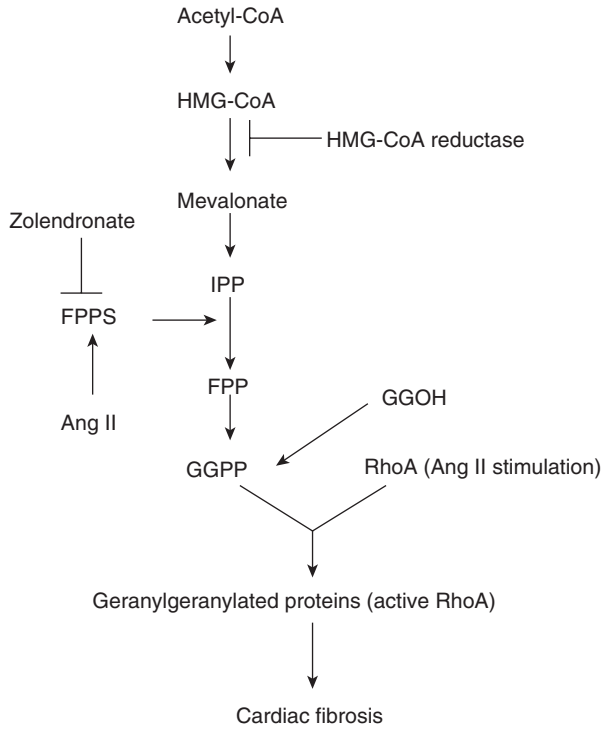

Fig. 1. The anti-fibrotic effect of zolendronate (Zol) via regulation of isoprenoid synthesis and Ras homologue gene family, member A (RhoA) activation in neonatal cardiac fibroblasts. Cell-permeable geranylgeraniol (GGOH) is converted to geranylgeranyl-pyrophosphate (GGPP) in neonatal cardiac fibroblasts.

Materials and methods

Animals

Eight-week-old Sprague–Dawley (SD) rats were purchased from Zhejiang University experimental animal centre and housed in a pathogen-free laboratory at Sir Run Run Shaw Hospital of Zhejiang University. The study was conducted in accordance with the guidelines and requirements from the Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th edition, 2011) and the Institutional Animal Care and Use Committee of Zhejiang University.

Cell culture

Neonatal cardiac fibroblasts were prepared from the ventricles of 1–3-day-old SD rats obtained from Zhejiang University experimental animal centre, as described previously [32]. Each heart was cut into small segments and digested by 0·1% collagenase type II and 0·12% trypsin at 37°C. The digestion was performed eight times for 5 min each. The cardiac fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) (HyClone, Thermo Scientific, Logan, UT, USA) in an environment containing 5% CO_2 for 1 h. The adherent cells were collected and plated in dishes. Cardiac fibroblasts were cultured until they reached 80–90% confluence. Cells in passages 2–4 were used for our experiments.

In-vitro and *in-vivo* models of cardiac fibrosis

Neonatal cardiac fibroblasts were cultured for 24 h in serum-free medium (SFM) and then treated with or without 0·1 μM Ang II (Sigma Chemical, St Louis, MO, USA) for 48 h. SD rats that were infused with Ang II were used as the *in-vivo* cardiac fibrosis model. Ang II was administered at a rate of 65 ng/min for 14 days via a subcutaneously implanted osmotic mini-pump (Alzet, model 2002; Durect Corp., Cupertino, CA, USA) [33].

RNA extraction and quantitative real-time–polymerase chain reaction (qRT–PCR)

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) from cardiac fibroblasts or from the left ventricles of SD rats using a standard protocol [34]. cDNA synthesis was performed with 1 μg of total RNA using the miScript II RT Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. qRT–PCR and data analysis were performed with the ABI 7500 cycler (Applied Biosystems, Carlsbad, CA, USA). α-Tubulin was used as the endogenous control for mRNA expression. The primers that we designed were as follows: FPPS forward, 5′-TGACTG AGGATGAACTGGGACA-3′, reverse, 5′-GAGGAAGAAAG CCTGGAGCA-3′; collagen I forward, 5′-GAGCCTAACC ATCTGGCATCT-3′, reverse, 5′-AGAACGAGGTAGTCTTT CAGCAAC-3′; collagen III forward, 5′-GAGCGGAGAAT ACTGGGTTGAT-3′, reverse 5′-GGTATGTAATGTTCTGGG AGGC-3′; and α-tubulin forward, 5′-GACAGGATGCAGA AGGAGATTACT-3′, reverse, 5′-TGATCCACATCTGCTGG AAGGT-3′.

Western blots

Total protein from cardiac fibroblasts that were cultured in six-well plates was extracted in a radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) [34]. Protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Beyotime). Equal amounts of protein (20 μg) were separated on 10 or 12% (for RhoA analysis) sodium dodecyl sulphate polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk–Tris-buffered saline and Tween 20 (TBST) and incubated overnight with primary antibodies at 4°C, followed by 1 h of incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature. The bands were visualized with an enhanced chemiluminescence reagent

(Amersham, Haemek, Israel) on a LAS-4000 image reader system (Fujifilm, Tokyo, Japan). To ensure equal protein loading, the α-tubulin protein was used as the endogenous control.

Anti-collagen I, anti-collagen III and anti-TGF-β1 antibodies were purchased from Abcam (Cambridge, UK). The anti-FPPS antibody was purchased from Epitomics (Burlingame, CA, USA). The anti-α-tubulin antibody was purchased from Beyotime Institute of Biotechnology (Beyotime).

In-vitro cell proliferation assay

Cell proliferation was assessed by a colorimetric procedure using the Cell Counting Kit-8 (Dojindo, Shanghai, China), according to the manufacturer's protocol [34]. The absorbance was measured at 490 nm. Five duplicate wells were tested per experiment for each sample.

RNA interference by lentiviral delivery of short hairpin RNA

A lentiviral short hairpin RNA construct against FPPS (shFPPS) was designed according to a previous study [24] and synthesized by Genechem Co. (Shanghai, China). The shFPPS sequence was as follows: 5′-GACAGCTTTCT ACTCTTTC-3′. Infections with the shFPPS-containing lentivirus and the control lentivirus were performed at a multiplicity of infection of 50, according to the manufacturer's instructions. After 48 h of infection, successful expression of shFPPS was validated by microscopic detection of GFP fluorescence, and FPPS knock-down was verified by Western blotting.

RhoA pull-down assay

RhoA activity was assessed using a RhoA activation assay kit according to the manufacturer's protocol (Cytoskeleton, Denver, CO, USA). Cardiac fibroblasts were infected with the lentiviruses for 24 h, cultured in SFM for another 24 h, and then stimulated by 1 μM Ang II for 15 min [24] before the addition of lysis buffer. Total protein concentration was equalized for all samples, and α-tubulin was used as an endogenous control for Western blot. The expression of both active RhoA and total RhoA was analysed by Western blotting.

Small interfering RNA (siRNA) against RhoA

The siRNA against RhoA (siRhoA) was designed and synthesized by GenePharma Co. (Shanghai, China), and a negative control was designed with a randomly chosen nonsense sequence. The effective siRhoA sequence was as followed: forward, 5′-AUCCUAGUUGGGAACAAGATT-3′, reverse, 5′-UCUUGUCCCAACUAGGAUTT-3′. When the cells reached approximately 80–90% confluence in DMEM containing 10% FBS, they were detached and subcultured at $60–80 \times 10^4$ cells/well into six-well plates. After being cultured overnight, the cells were transfected with 50 nM siRNA. The cells were then cultured for another 24 h and then treated with Ang II for 48 h.

Statistics

All experiments were performed at least three times. The data were presented as the mean ± standard error of the mean (s.e.m.). Statistical analysis was conducted with SPSS version 20·0 software, using one-way analysis of variance (ANOVA) for multiple group comparisons or Student's *t*-test for two-group comparisons. $P < 0·05$ was considered statistically significant.

Results

Expression of FPPS in *in-vitro* and *in-vivo* models of Ang II-induced cardiac fibrosis

The *in-vivo* cardiac fibrosis model was established in SD rats that were infused with Ang II at a rate of 65 ng/min for 14 days via an osmotic mini-pump, and age-matched controls were included. When compared with the control group, the protein level of FPPS was enriched to approximately 1·4-fold (Fig. 2a) and the mRNA level of FPPS was at approximately 25-fold (Fig. 2b) in the experimental group.

The *in-vitro* model of cardiac fibrosis was established in cardiac fibroblasts that were stimulated with Ang II in culture. FPPS expression was up-regulated in a time- and concentration-dependent manner (data not shown). Notably, both the mRNA level and the protein level of FPPS peaked after 48 h of Ang II stimulation (Fig. 2c,d).

Taken together, the above results strongly suggest that FPPS up-regulation plays an important role in Ang II-induced cardiac fibrosis.

Proliferation of cardiac fibroblasts in response to Zol and Ang II

Cardiac fibroblast proliferation was measured using the cell counting kit-8 (CCK-8) assay. Treatment with 0–30 μM Zol alone for 48 h had no significant effect on cell proliferation (Fig. 3a). However, co-treatment with 30 μM Zol and Ang II inhibited proliferation compared with Ang II treatment alone (Fig. 3b).

Effects of FPPS inhibition and knock-down on the expression of collagen I, collagen III and TGF-β1 in Ang II-stimulated cardiac fibroblasts

Cells that have been cultured in SFM overnight were co-treated with Ang II and Zol for another 48 h before

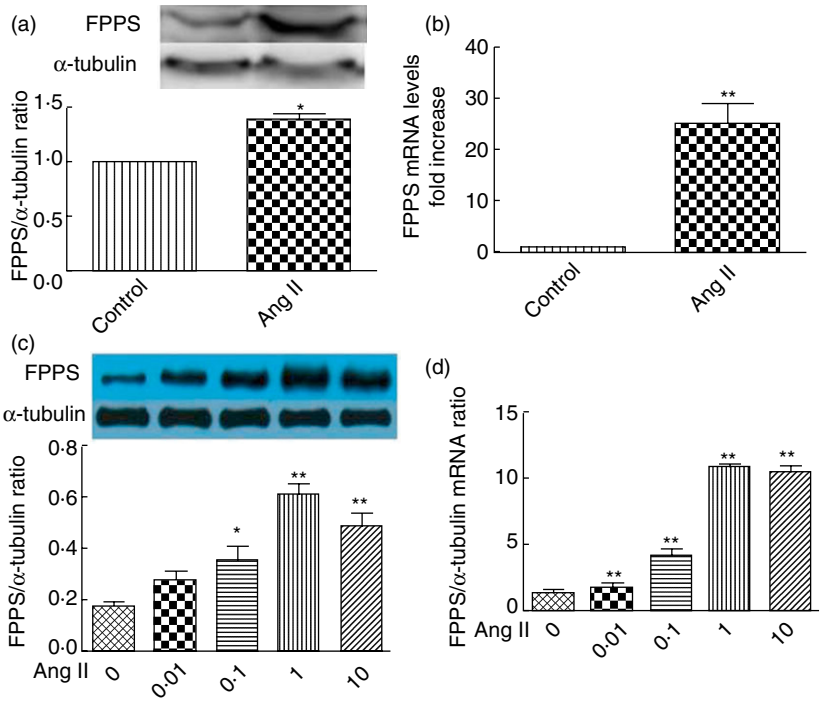

Fig. 2. Expression of farnesyl pyrophosphate synthase (FPPS) in *in-vitro* and *in-vivo* models of angiotensin (Ang) II-induced cardiac fibrosis. (a) FPPS protein expression in the *in-vivo* model; $n = 5$. $^{*}P < 0{\cdot}05$ *versus* control group. (b) FPPS mRNA expression in the *in-vivo* model; $n = 5$. $^{**}P < 0{\cdot}01$ *versus* control group. FPPS expression was up-regulated by Ang II in a concentration- and time-dependent manner in the *in-vitro* model. (c,d) FPPS protein and mRNA expression after 48 h of Ang II treatment; $n = 3$. $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ *versus* control group.

analysis. Collagen I, collagen III and TGF-β1 expression was decreased at both mRNA and protein levels in a Zol dose-dependent manner, and the effect was readily observable at 10 μM Zol (Fig. 4a–e).

The lentiviral infection efficiency was assessed based on GFP expression (Fig. 4f). Treatment with shFPPS following 48 h of Ang II stimulation significantly reduced the mRNA and protein expression of collagen I, collagen III and TGF-β1 (Fig. 4g–n).

Regulation of the anti-fibrotic effect of Zol by geranylgeranylated proteins in cardiac fibroblasts

To determine whether the anti-fibrotic effect of Zol could be reversed by the addition of geranylgeranylated or farnesylated proteins, we co-treated Ang II-stimulated cardiac fibroblasts with 10 μM Zol and 10 μM geranylgeraniol (GGOH) or farnesol (FOH). Interestingly, the inhibitory effect of Zol on the expression of collagen I, collagen III and TGF-β1 was reversed partially by GGOH, but not by FOH (Fig. 5a–h).

Effect of siRhoA on Ang II-induced fibrotic responses in cardiac fibroblasts

Given that GGOH partially reversed the inhibitory effect of Zol on Ang II-induced fibrosis and that RhoA is a geranylgeranylated protein, we sought to investigate the role of RhoA in cardiac fibrosis using an siRNA against RhoA. We first validated that siRhoA effectively inhibited RhoA expression in the cardiac fibroblasts (Fig. 6a). We then demonstrated that Ang II could no longer induce collagen I, collagen III and TGF-β1 expression when the cells were pretreated with siRhoA (Fig. 6b–i).

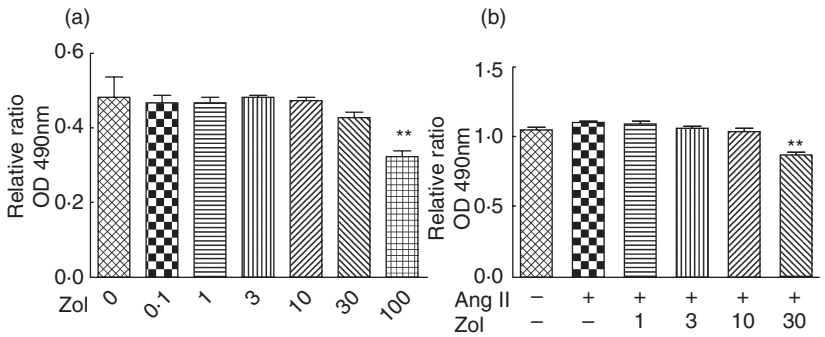

Fig. 3. Proliferation of cardiac fibroblasts in response to zolendronate (Zol) and angiotensin (Ang) II. (a) Proliferation of cardiac fibroblasts in response to 48 h of Zol (0–30 μM) treatment alone; $n = 5$. $^{**}P < 0{\cdot}01$ *versus* control group. (b) Proliferation of cardiac fibroblasts in response to 48 h of Ang II (0·1 μM) and Zol (μM) co-treatment; $n = 5$. $^{**}P < 0{\cdot}01$ *versus* Ang II group; $^{\#}P < 0{\cdot}05$ *versus* control group.

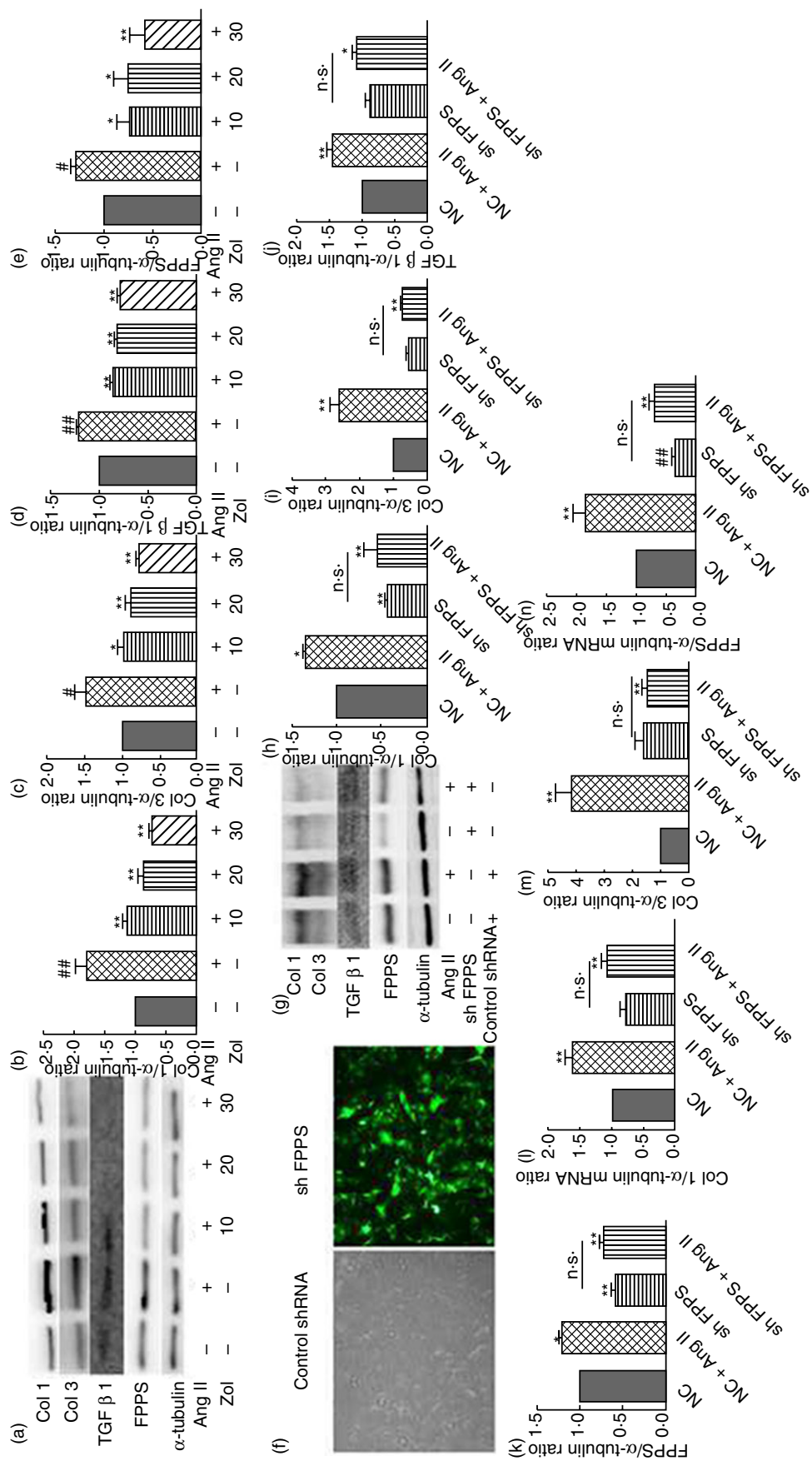

Fig. 4. Effect of farnesyl pyrophosphate synthase (FPPS) inhibition and knock-down on angiotensin (Ang) II-mediated expression of collagen I, collagen III and transforming growth factor (TGF)-β1 in cardiac fibroblasts. (a) Western blot analysis of the expression of cardiac fibrosis marker genes (collagen I, collagen III and TGF-β1) and FPPS in the presence of Ang II (0·1 μM) and different concentrations of zolendronate (Zol) (μM). (b–e) Relative expression levels of collagen I, collagen III, TGF-β1 and FPPS protein are normalized to α-tubulin and expressed as the mean ± standard error of the mean (s.e.m.), $n = 3$. $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ *versus* Ang II group, $\#P < 0{\cdot}05$ and $\#\#P < 0{\cdot}01$ *versus* control group. (f) Effect of FPPS knock-down on cardiac fibroblasts. The transduction efficiency of the lentivirus was at a multiplicity of infection of 50 in neonatal cardiac fibroblasts. (g) Effect of FPPS knock-down on the protein expression of collagen I, collagen III, TGF-β1 and FPPS. (h–k) Relative protein expression levels of collagen I, collagen III, TGF-β1 and FPPS are normalized to α-tubulin and expressed as the mean ± s.e.m., $n = 3$. $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ *versus* NC group, $\#\#P < 0{\cdot}01$ *versus* NC + Ang II group, NS for shFPPS group *versus* shFPPS + Ang II group. (l–n) Relative mRNA expression levels of collagen I, collagen III and FPPS are normalized to α-tubulin and expressed as the mean ±s.e.m., $n = 3$. $^{**}P < 0{\cdot}01$ *versus* NC group, $^{++}P < 0{\cdot}01$ *versus* NC + Ang II group, not significant (n.s.) for shFPPS group *versus* shFPPS + Ang II group.

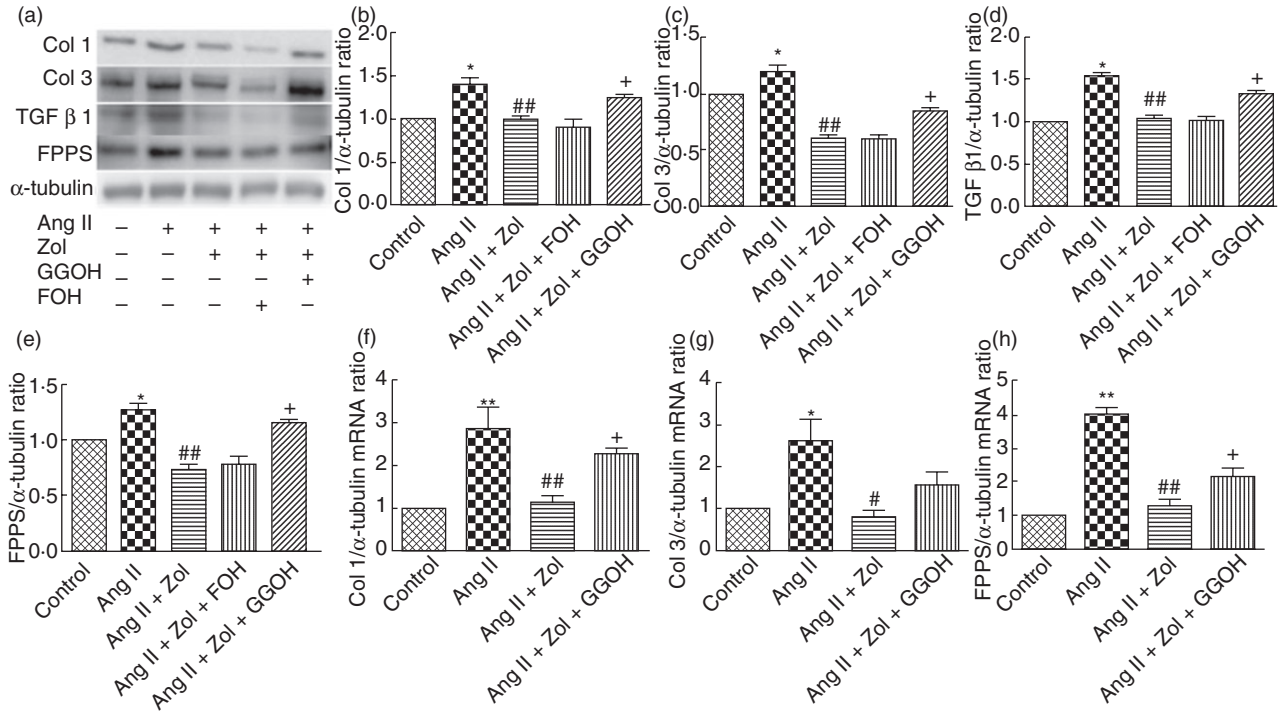

Fig. 5. Attenuation of the anti-fibrotic effect of zolendronate (Zol) by geranylgeranylated protein in cardiac fibroblasts. (a) Western blot analysis of the expression of cardiac fibrosis marker genes [collagen I, collagen III and transforming growth factor (TGF)-β1] and farnesyl pyrophosphate synthase (FPPS) in presence of angiotensin (Ang) II (0·1 μM) and Zol (10 μM) together with farnesol (FOH) (10 μM) or geranylgeraniol (GGOH) (10 μM). (b–e) Relative protein expression levels of collagen I, collagen III, TGF-β1 and FPPS and normalized to α-tubulin and expressed as the mean ± standard error of the mean (s.e.m.), $n = 3$. $^{*}P < 0{\cdot}05$ *versus* control group, $^{\#}P < 0{\cdot}05$ and $^{\#\#}P < 0{\cdot}01$ *versus* Ang II group, $^{+}P < 0{\cdot}05$ *versus* Ang II + Zol group. (f–h) Relative mRNA expression levels of collagen I, collagen III and FPPS are normalized to α-tubulin and expressed as the mean ± s.e.m., $n = 3$. $^{*}P < 0{\cdot}05$ *versus* control group, $\#P < 0{\cdot}05$ and $\#\#P < 0{\cdot}01$ *versus* Ang II group, $^{+}P < 0{\cdot}05$ *versus* Ang II + Zol group.

Effect of FPPS inhibition and knock-down on RhoA activity in cardiac fibroblasts

The active form of RhoA was elevated to 120% of the control group after a 15-min treatment with Ang II (Fig. 7a,b). Zol markedly reduced RhoA activation to 60% of the control group. However, this inhibitory effect was almost completely reversed in the presence of GGOH (Fig. 7a,b). Furthermore, the level of active RhoA could not be elevated by Ang II when the cells were pretreated with shFPPS (Fig. 7c,d).

These results suggest that RhoA activation is partially attenuated by FPPS inhibition via the inhibition of geranylgeranylation *in vitro*.

Discussion

Ang II, the main effector peptide of the RAS, is a known activator of *in-vivo* [35] and *in-vitro* [36] cardiac fibrosis. Using *in-vivo* models, we demonstrated that the FPPS expression was increased significantly in the fibrotic myocardium that has been stimulated by Ang II. Under the given experimental conditions, Ang II successfully induced a fibrotic response [11,12] and also evoked the time- and concentration-dependent elevation of FPPS expression in cardiac fibroblasts. This result suggests that FPPS might play an important role in the Ang II-mediated *in-vitro* cardiac fibrotic response.

We then further explored the role of FPPS in Ang II-induced fibrosis in neonatal cardiac fibroblasts. First, 0–30 μM Zol treatment for 48 h inhibited cell proliferation in the presence of 0·1 μM Ang II, but not in the absence of Ang II. Secondly, Zol dose-dependently blocked the expression of collagen I, collagen III and TGF-β1 in Ang II-treated cardiac fibroblasts. This result was confirmed by RNA interference against FPPS, which also significantly reduced the mRNA and protein levels of collagen I, collagen III and TGF-β1 in Ang II-treated cells. Furthermore, GGOH, but not FOH, reversed Zol-mediated inhibition of collagen I, collagen III and TGF-β1 under Ang II stimulation. Provided that GGOH is metabolized to geranylgeranyl-pyrophosphate (GGPP) in cells [37,38] and that RhoA is a geranylgeranylated protein [39], the above results suggested that the protective effect of FPPS inhibition on cardiac fibrosis *in vitro* was mediated, at least partly, through the suppression of GGOH synthesis, therefore indicating that

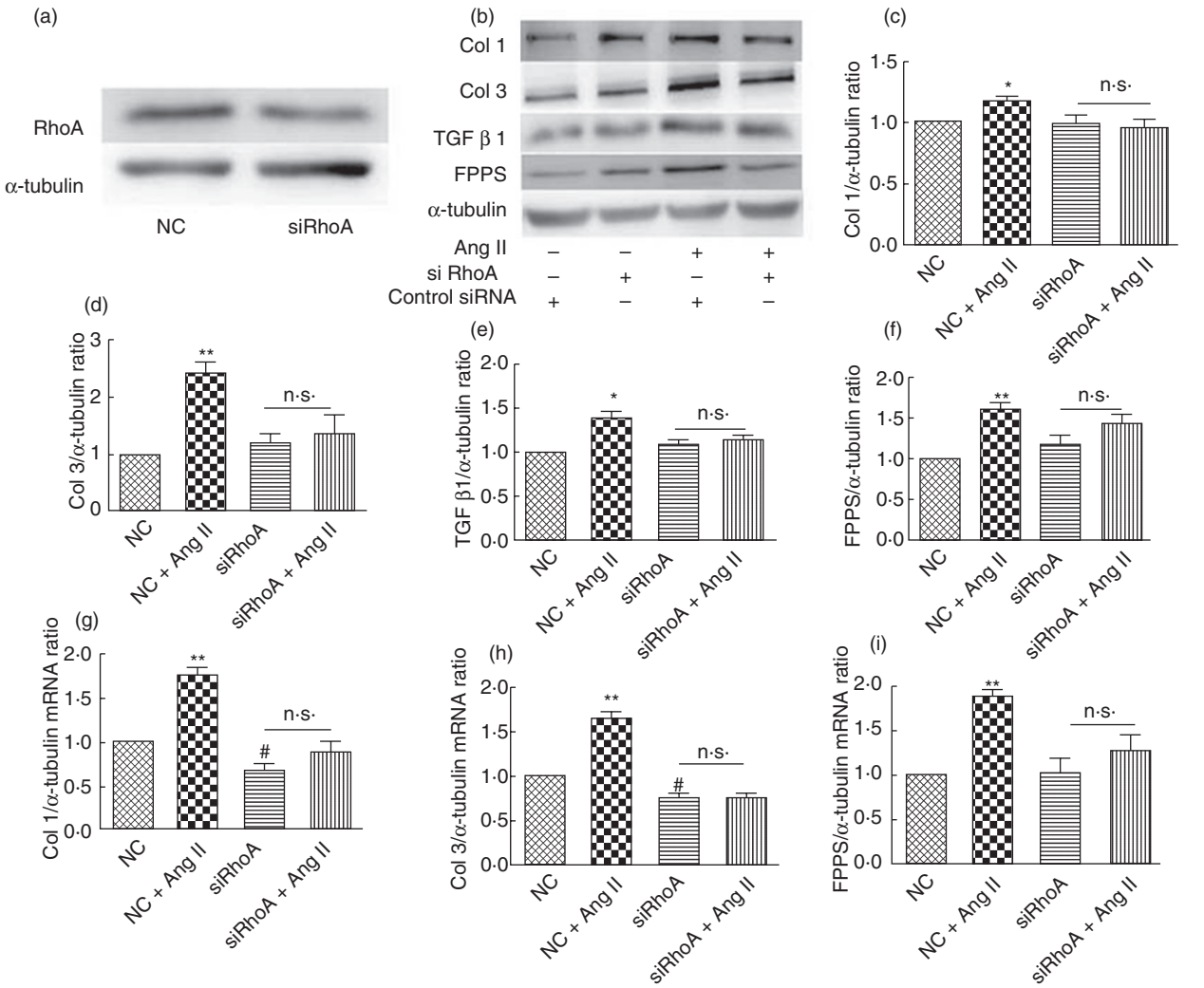

Fig. 6. Effect of Ras homologue gene family, member A (RhoA)silencing by siRhoA on angiotensin II (Ang II)-induced fibrotic response in cardiac fibroblasts. (a) Effect of siRhoA on the protein level of RhoA in cardiac fibroblasts. (b) Western blot analysis of farnesyl pyrophosphate synthase (FPPS) protein expression in response to siRhoA and Ang II. (c–f) Relative protein expression level of collagen I, collagen III, transforming growth factor (TGF)-β1 and FPPS are normalized to α-tubulin and expressed as the mean ± standard error of the mean (s.e.m.), $n = 3$. $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ *versus* NC group, NS for siRhoA group *versus* siRhoA + Ang II group. (g–i) Relative mRNA expression levels of collagen I, collagen III and FPPS are normalized to α-tubulin and expressed as the mean ± s.e.m., $n = 3$. $^{**}P < 0{\cdot}01$ *versus* NC group, $\#P < 0{\cdot}05$ *versus* NC group, not significant (n.s.) for siRhoA group *versus* siRhoA + Ang II group.

the down-regulation of geranylgeranylated RhoA (Fig. 1) might also be part of the protective response to *in-vitro* cardiac fibrosis formation.

Overexpression of RhoA in the heart leads to the development of heart failure with bradycardia [31], impaired contractile function and induction of interstitial fibrosis in *in-vivo* models [18]. Pitavastatin, an inhibitor of a key enzyme upstream of FPPS in the mevalonate pathway, inhibits RhoA activity and effectively prevents the development of cardiac fibrosis [40]. In our study, RhoA silencing by siRhoA inhibited Ang II-induced expression of collagen I, collagen III and TGF-β1 in cardiac fibroblasts. Ang II has been shown to markedly increase RhoA activity in cardiac fibroblasts [41]. RhoA is activated in the hearts of AngII-stimulated cardiac fibrosis models *in vivo*, as shown by Yang *et al.* [29]. We confirmed this finding by showing that a 15-min treatment with Ang II was able to promote RhoA activity, which was reversible by Zol. In turn, Zol activity was reversed by GGOH. Notably, administration of Zol decreased the level of active RhoA but not total RhoA. In addition, FPPS knock-down had the same effect, indicating that FPPS might specifically target the activation of RhoA in Ang II-treated cardiac fibroblasts. Our results highlight the importance of FPPS in the RhoA signalling pathway during the development of cardiac fibrosis in response to Ang II *in vitro*.

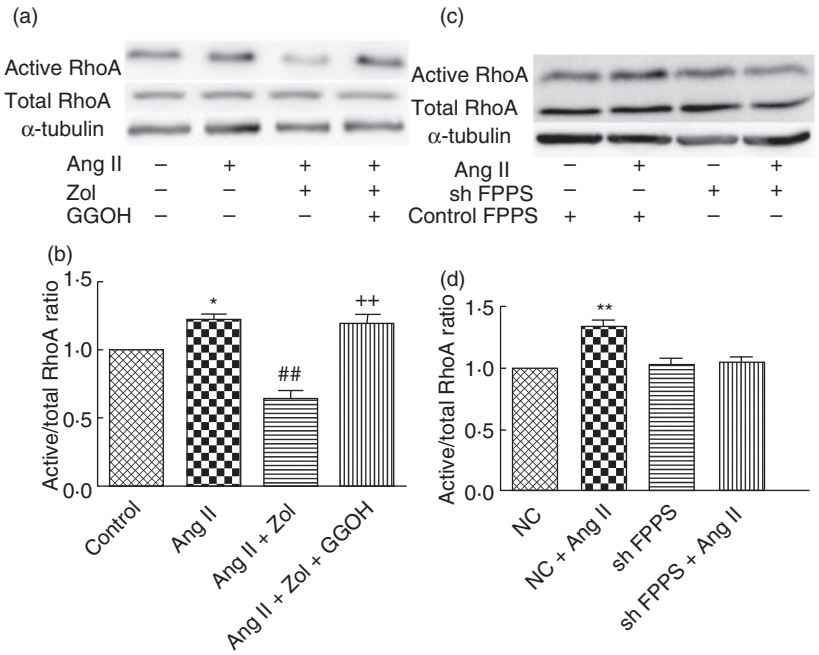

Fig. 7. Effect of farnesyl pyrophosphate synthase (FPPS) inhibition and knock-down on Ras homologue gene family, member A (RhoA)activity in cardiac fibroblasts. (a) Serum-starved cardiac fibroblasts in the absence or presence of 10 μM zolendronate (Zol) and 10 μM geranylgeraniol (GGOH) were incubated with 1 μM Ang II for 15 min. The protein levels of active RhoA (upper panel) and total RhoA (middle panel) in the cell lysates were measured by Western blot. (b) Relative protein expression levels of active and total RhoA are normalized to α-tubulin and expressed as the mean ± standard error of the mean (s.e.m.), $n = 3$. $^{*}P < 0{\cdot}05$ *versus* control group, $^{\#\#}P < 0{\cdot}01$ *versus* angiotensin (Ang) II group, $^{++}P < 0{\cdot}01$ *versus* Ang II + Zol group. (c) Effect of FPPS-silencing lentivirus on active RhoA expression. (d) Relative protein expression levels of active and total RhoA are normalized to α-tubulin and expressed as the mean ± s.e.m., $n = 3$. $^{**}P < 0{\cdot}01$ *versus* NC group.

In conclusion, FPPS/RhoA signalling is vital to the production of fibrotic collagen in Ang II-stimulated cardiac fibrosis *in vitro*.

A limitation of the study is that we could not evaluate directly the effect of FPPS suppression on isoprenoid intermediates because of our experimental conditions. Although this study proposed several possible reasons why sustained FPPS inhibition prevents cardiac fibrosis *in vitro*, other mechanisms should be explored in future studies. In addition, *in-vivo* study will need further investigation.

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Disclosures

None declared.

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