

# Osteopontin is indispensable for AP1-mediated angiotensin II-related miR-21 transcription during cardiac fibrosis

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## Aims

Osteopontin (OPN) is a multifunctional cytokine critically involved in cardiac fibrosis. However, the underlying mechanisms are unresolved. Non-coding RNAs are powerful regulators of gene expression and thus might mediate this process.

## Methods and results

OPN and miR-21 were significantly increased in cardiac biopsies of patients with myocardial fibrosis. Ang II infusion via osmotic minipumps led to specific miRNA regulations with miR-21 being strongly induced in wild-type (WT) but not OPN knockout (KO) mice. This was associated with enhanced cardiac collagen content, myofibroblast activation, ERK-MAPkinase as well as AKT signalling pathway activation and a reduced expression of Phosphatase and Tensin Homologue (PTEN) as well as SMAD7 in WT but not OPN KO mice. In contrast, cardiotropic AAV9-mediated overexpression of OPN *in vivo* further enhanced cardiac fibrosis. *In vitro*, Ang II induced expression of miR-21 in WT cardiac fibroblasts, while miR-21 levels were unchanged in OPN KO fibroblasts. As pri-miR-21 was also increased by Ang II, we studied potential involved upstream regulators; Electrophoretic Mobility Shift and Chromatin Immunoprecipitation analyses confirmed activation of the miR-21 upstream-transcription factor AP-1 by Ang II. Recombinant OPN directly activated miR-21, enhanced fibrosis, and activated the phosphoinositide 3-kinase pathway. Locked nucleic acid-mediated miR-21 silencing ameliorated cardiac fibrosis development *in vivo*.

## Conclusion

In cardiac fibrosis related to Ang II, miR-21 is transcriptionally activated and targets PTEN/SMAD7 resulting in increased fibroblast survival. OPN KO animals are protected from miR-21 increase and fibrosis development due to impaired AP-1 activation and fibroblast activation.

## Keywords

Osteopontin • Cardiac fibrosis • microRNA • Angiotensin II • miR-21

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## Translational perspective

Osteopontin (OPN) is a pleiotropic cytokine, which has been shown to be a pivotal factor in myofibroblast activation in cardiac fibrosis, thereby acting as a strong driver of heart failure development in humans. MicroRNAs (miRNAs) are under intense investigation as powerful regulators of various diseases. First phase I and II clinical trials using miRNA inhibitors have been initiated. We here show, that OPN is essential in the activation of AP-1 and subsequent transcription of miR-21 in cardiac fibrosis related to Ang II. OPN null mice are protected from miR-21 increase and fibrosis development due to impaired AP-1 activation and fibroblast activation. In the future, these findings may result in miRNA therapeutic approaches to treat patients with cardiac remodelling, in which levels of OPN and miR-21 are increased.

## Introduction

Cardiac fibrosis is characterized by activation/proliferation of cardiac fibroblasts and excessive matrix deposition, including collagen.<sup>1</sup> It is associated with a variety of injurious insults of different causes to cardiac tissue, which culminates in destruction of physiological tissue architecture and progressive organ dysfunction, ultimately resulting in heart failure.<sup>1</sup> A major role in this process has been attributed to various growth factors, proteolytic enzymes, angiogenic factors, and fibrogenic cytokines.<sup>1,2</sup> Osteopontin (OPN) is a pleiotropic cytokine that is involved in the recruitment and retention of macrophages and T cells to sites of inflammation.<sup>3</sup> It has been shown to be a pivotal factor in myofibroblast activation in cardiac fibrosis,<sup>4</sup> thereby acting as a strong driver of heart failure development in humans.<sup>5</sup> Classical mediators of acute inflammation [tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin 1- $\beta$  (IL-1 $\beta$ )] as well as fibrogenic cytokines [angiotensin II (Ang II), transforming growth factor- $\beta$  (TGF- $\beta$ )] strongly induce OPN expression.<sup>3</sup> OPN has thus been implicated as a key factor in the development of interstitial fibrosis.<sup>6,7</sup> Moreover, OPN was identified as a strong independent predictor of mortality in patients with chronic heart failure.<sup>8</sup> However, the underlying mechanisms of OPN transcriptional regulation with regard to fibrosis development in the heart are not well defined. MicroRNAs (miRNAs) are under intense investigation as powerful regulators of various diseases with potential critical impact on disease initiation and/or progression. MiRNAs represent small non-coding RNA transcripts with a length of  $\sim 22$  nucleotides, which through post-transcriptional binding of the 3'-untranslated region (UTR) of mRNA targets lead to the repression of gene/protein expression and/or translational inhibition of protein synthesis.<sup>9</sup> In the present study, we analysed the transcriptional regulation of Osteopontin-related fibrogenic miRNA expression in response to Ang II *in vivo* using OPN wild-type (WT) and knockout (KO) mice as well as *in vitro* in primary cardiac fibroblasts. The *in vivo* role of OPN was further characterized by cardiac overexpression of OPN with adeno-associated viral vectors of serotype 9 (AAV9). Cardiac biopsies of patients with myocardial fibrosis related to aortic stenosis were analysed concerning OPN and fibrogenic miRNA expression.

## Methods

### Myocardial biopsies of patients

Patients with myocardial fibrosis related to aortic stenosis ( $n = 15$ ) as well as healthy controls ( $n = 5$ ) were included for myocardial biopsies. Patients with aortic stenosis were recruited at the Department of

Cardiology, Würzburg University. Healthy tissue was obtained from AMS Biotechnology (USA). Patient characteristics and echocardiographic data are displayed in Table 1. The study was approved by the institutional review committee. Patients gave written informed consent.

### Cell culture and reagents

Adult mouse cardiac fibroblasts were isolated from OPN WT and KO mice by enzymatic digestion as described previously with modifications.<sup>10</sup> Primary mouse cardiac fibroblasts were cultured in DMEM

**Table 1** Demographic, clinical, and laboratory characteristics of patients

	Total	Male	Female
Number of patients (%)	15	8 (53)	7 (47)
Age (years; mean + SD)	78 (4.3)	77 (3.7)	78 (5.2)
Concomitant disease			
Arterial hypertension (n; %)	11 (73)	7 (47)	4 (27)
Diabetes mellitus (n; %)	7 (47)	5 (33)	2 (13)
Renal insufficiency (n; %)			
Myocardial infarction (n; %)	3 (20)	2 (13)	1 (6)
Stroke (n; %)	1 (6)	0 (0)	1 (6)
Cancer (n; %)	1 (6)	1 (6)	0 (0)
Concomitant drug treatment			
ARB (n; %)	3 (20)	1 (6)	2 (13)
ACE-inhibitors (n; %)	5 (33)	4 (27)	1 (6)
Beta-blocker (n; %)	8 (53)	4 (27)	4 (27)
Aldosterone antagonist (n; %)	9 (60)	4 (27)	5 (33)
Calcium channel blocker (n; %)	1 (6)	0 (0)	1 (6)
Diuretics (n; %)	4 (27)	2 (13)	2 (13)
Statins (n; %)	11 (73)	6 (40)	5 (33)
Acetylsalicylic acid (n; %)	7 (47)	4 (27)	3 (20)
Septum (mm)	8 (53)	4 (27)	4 (27)
Posterior wall (mm)	12 (0.3)	13 (0.2)	12 (0.3)
LVEDd (mm)	12 (0.3)	13 (0.3)	12 (0.2)
AOA (cm <sup>2</sup> )	46 (0.9)	50 (1)	44 (0.6)
dPmean (mmHg)	0.8 (0.2)	0.9 (0.2)	0.8 (0.1)
LVEF (%)	40 (1.8)	40 (1.6)	39 (2.2)
	63 (1.2)	61 (1.4)	63.5 (0.9)

ACE-inhibitors, angiotensin-converting enzyme inhibitors; AOA, aortic opening area; ARB, angiotensin-receptor blockers; dPmean, change in mean pressure; LVEDd, left-ventricular end-diastolic diameter; n, number of patients included.

(PAA) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO<sub>2</sub>. HL-1 cells were cultured in Claycomb Medium (Sigma-Aldrich, St Louis, MO, USA, catalogue no. 51800C) with 10% FBS (Sigma-Aldrich, catalogue no. 12103C), penicillin/streptomycin (100 U/mL:100 µg/mL; Sigma-Aldrich, catalogue no. P4333), 0.1 mM norepinephrine (Sigma-Aldrich, catalog no. A0937), 2 mM L-glutamine (Sigma-Aldrich, catalogue no. G7513) at 37°C in 5% CO<sub>2</sub>. Primary mouse cardiac fibroblasts and HL-1 cells were grown to a confluence of 80%. The cells were starved for 24 h in DMEM containing 1% FBS before they were stimulated with 100 nM Ang II.

## Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was used to detect protein–DNA interactions. We applied the MAGnify™ Chromatin Immunoprecipitation System according to the manufacturer's instructions (Life technologies). Samples were subjected to either immunoprecipitation with 5 µg RNA polymerase II CTD repeat YSPTSPS (phospho S2) antibody (Abcam, Cambridge, UK) or control mouse IgGs o/n at 4°C. For ChIP primer-design, we first identified 2000–2500 bp upstream promoter regions of candidate target genes by Ensembl Genome Browser (<http://www.ensembl.org/index.html>). Then we screened the promoter region for potential AP-1 binding sites by the use of ALLGEN-Promo and selected appropriate primer pairs that amplify potential AP-1 binding sites. Subsequent PCR analysis of chipped DNA fragments was done by mixing 2.5 µL sample, 2.5 µL 4 µM appropriate primer pairs, 10 µL HotStarTaq Mix (Qiagen) and applying the following protocol: 94°C 10 min, [94°C 1 min, 57°C 30 s, 72°C 1 min] × 33, 72°C 10 min, 4°C hold. The oligonucleotide primer sequence is given in Supplementary material online, Table S1.

## Protein analysis

Protein expression was investigated by western blot analysis using 10–40 µg of total protein. Tissue was homogenized, cells were pelleted. Cell lysis was performed (Cell lysis buffer, Cell Signaling Technology, Danvers, MA, USA) and protein electrophoresis initiated. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% milk in TBS-Tween, and probed overnight at 4°C with the following primary antibodies: SMAD7 (rabbit anti-mouse, Invitrogen, USA), PTEN (rabbit anti-mouse, Abcam), Foxo3a (rabbit anti-mouse, Cell signaling Technology), phospho-AKT (ser) (rabbit anti-mouse, Cell signaling Technology), AKT (rabbit anti-mouse, Cell signaling Technology), phospho-ERK (44/42) (rabbit anti-mouse, Cell signaling Technology), ERK (rabbit anti-mouse, Cell signaling Technology). Antibody binding was visualized by chemiluminescence (Super-Signal West Pico Chemiluminescent, Thermo Scientific, Rockford, IL, USA). Rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich) was used as an internal loading control and for normalization of protein quantification. Immunoblots were scanned and quantified using ImageJ densitometry software. Recombinant OPN was purchased at RnD systems (USA). The phosphoinositide-3-kinase (PI3-kinase) inhibitor wortmannin was obtained from Sigma-Aldrich.

## Scratch assay of primary fibroblasts

Primary cardiac fibroblasts isolated from WT animals were treated with 0, 0.5, 1, and 2 µg/mL recombinant OPN (RnD systems, USA) immediately after a scratch in the cell monolayer was generated with a 100 µL tip. In certain experiments, miR-21 was silenced using locked nucleic acids (LNAs) targeting miR-21 (LNA-21) and compared with cells transfected with LNAs targeting a mismatch sequence (LNA-MM) for 72 h. The cells were photographed at 0, 4, 8, 12, and 24 h with a Nikon Ti 90 microscope (Germany). Subsequently, the migrated cell area was calculated.

## microRNA/RNA isolation, miRNA/mRNA reverse transcription–polymerase chain reaction, and global transcriptome analysis

RNA isolation was performed with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For detection of miRNAs in samples, different TaqMan miRNA assays (Applied Biosystems) were applied. The small RNA molecule RNU-6B was amplified as a control. Reverse transcription–polymerase chain reaction analysis was performed in an ICycler (Bio-Rad). MiRNA expression analysis was performed in hearts of OPN WT and KO mice subjected to Ang II infusion using the Mouse Genome Wide microRNA 384-well qRT–PCR Array (Biocat, Germany) according to the manufacturer's instructions. Reverse transcription was performed with total RNA using oligoDT primers (Bio-Rad). Amplified cDNA was used as a template for quantitative PCR. Reverse transcription–polymerase chain reaction analysis was performed in an ICycler (Bio-Rad) with SYBR green mastermix. The specific primers used in our study are depicted in Supplementary material online, Table S1. For detection of OPN, we used primers provided by Qiagen (Hilden, Germany) of undisclosed sequence.

## microRNA target prediction

The miRNA databases and target prediction tools miRBase (<http://microrna.sanger.ac.uk/>), PicTar (<http://pictar.mdc-berlin.de/>), and TargetScan (<http://www.targetscan.org/index.html>) were used to identify potential miRNA targets. We focused on targets predicted by at least two prediction databases and containing a miR-21-8mer seed match in the respective 3'UTR region.

## Transfection assays

Transient liposomal transfection of small inhibitory RNAs (siRNAs) or miRNAs was performed according to the manufacturers' instructions. Briefly, cells were split 1 day before transfection to reach 60–70% confluence on the day of transfection. Specific siRNAs/miRNAs and control siRNA/miRNA and Lipofectamine 2000 (Invitrogen) were mixed separately and incubated for 5 min with Opti-MEM I media (Invitrogen). Complexes were added together and incubated for 20 min. Media were changed to antibiotic-free media before the addition of liposomal siRNA complexes (final concentration 150 nmol/L for siRNA and 100 nmol/L for miRNAs). Cells were incubated for 4 h before the media were changed to fresh media. Silencing of proteins or miRNA targets was monitored for 48 h (siRNA) or 72 h (miRNAs) after transfection by western blot analysis.

## Animal experiments

In the Ang II model of cardiac stress, OPN WT and OPN KO mice (each  $n = 6$ ) were implanted with osmotic minipumps according to the manufacturers' instructions (Alzet). Briefly, an incision was made in the mid-scapular region, and an osmotic minipump (Alzet, model 2002; 0.5 µL/h for 14 days) was implanted subcutaneously. The implanted minipumps contained Ang II (Sigma-Aldrich) dissolved in water, and the infusion rate was 1.5 mg/kg/day. Sham-operated mice underwent an identical surgical procedure. Ang II minipump-implanted mice were either treated with an LNA targeting a scrambled sequence or miR-21 (Exiqon) at Day 0 and Day 7 (each dose 20 mg/kg) via intraperitoneal injection. After 2 weeks, cardiac function and dimension were measured and the hearts were removed for biochemical analysis. Cardiotropic OPN-AAV9 or CTR-AAV9 vectors (for generation see Supplementary material online, Methods) were injected by puncture of the retroorbital venous plexus 2 weeks prior to implantation of osmotic minipumps. Sufficient overexpression of OPN *in vivo* at the study endpoint was assessed by western blot (rabbit anti-mouse OPN antibody, Abcam, UK).

Immunostainings for inflammatory cell influx was performed using the following primary antibody: monoclonal rat anti-mouse CD45 (BD Pharmingen, BD Biosciences, Santa Cruz, CA, USA).

## Statistical analysis

Average data are presented as mean and standard deviation (SD) unless otherwise stated. All statistical analyses were performed with the SPSS package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA, USA). Two-sided *P*-values <0.05 were considered statistically significant for all statistical procedures used. For statistical comparison of two groups in *in vitro* analyses, we used an unpaired two-tailed Student *t*-test; for the comparison of three or more groups, we used ANOVA followed by Tukey *post-hoc* tests. Figures were generated by using Adobe Illustrator software.

## Results

### Ang II induces osteopontin expression in cardiac cells and is elevated in humans with cardiac fibrosis due to aortic stenosis

First we treated cultured cardiac fibroblasts and cardiomyocytes with Ang II and found increased OPN mRNA (cardiac fibroblasts, *Figure 1A*; cardiomyocytes, *Figure 1F*) as well as enhanced secreted OPN in the supernatant (cardiomyocytes, *Figure 1G*). In line with this observation, myocardial tissue of patients with myocardial fibrosis related to aortic stenosis ( $n = 15$ ) when compared with control patients ( $n = 5$ ) showed increased levels of Collagen I and OPN (*Figure 1H–I*). Concomitant angiotensin receptor blocker (ARB) treatment in these patients lowered elevated levels of OPN (*Figure 1K*).

### Functional role of recombinant osteopontin in cardiac fibroblasts

The results of the pre-clinical and clinical studies described earlier about the potential role of OPN in cardiac fibrosis prompted us to better understand the underlying mechanism. We thus next assessed the functional role of recombinant OPN (rOPN) in cardiac fibroblasts *in vitro*. OPN dose-dependently induced fibroblast migration as assessed by a scratch assay (Supplementary material online, *Figure S1A–D* and *G*). In addition, rOPN upregulated various pro-fibrotic genes, including alpha smooth muscle actin ( $\alpha$ -SMA) (Supplementary material online, *Figure S2A*), connective tissue growth factor (CTGF) (Supplementary material online, *Figure S2B*) and TGF- $\beta$  (Supplementary material online, *Figure S2C*). Moreover, rOPN induced AKT phosphorylation by activation of phosphoinositide 3-kinase (PI3-kinase, Supplementary material online, *Figure S2E* and *F*), which could be blocked by the PI3-kinase inhibitor wortmannin (Supplementary material online, *Figure S2E* and *G*).

### Pro-fibrotic effects of Ang II-induced osteopontin *in vivo* and *in vitro*

In order to assess the *in vivo* relevance of OPN with respect to fibrosis development, we subjected OPN wild-type (WT) and OPN knock-out (OPN KO) mice to chronic Ang II infusion via osmotic mini pumps for 2 weeks (*Figure 2*). In both groups, Ang II increased blood pressure (Supplementary material online, *Table S2*). Expectedly, we found that Ang II induced fibrosis in WT hearts as assessed by

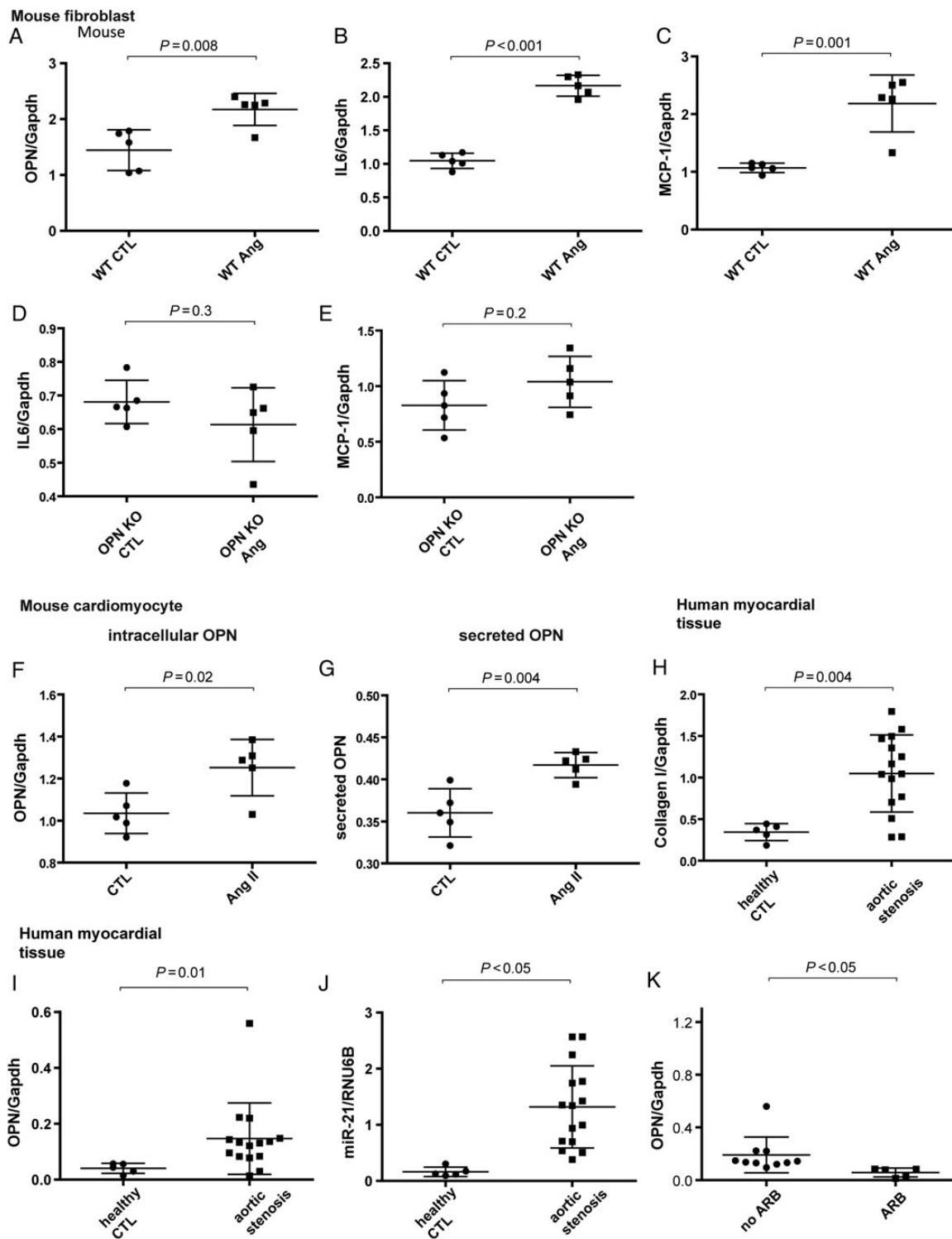
Sirius red staining (*Figure 2A–C*) and expression of the pro-fibrotic genes collagen I and  $\alpha$ -SMA (*Figure 2G* and *H*). In contrast, in OPN KO mice, fibrosis development as well as fibrotic gene expression was strongly attenuated (*Figure 2D–H*). Similarly, Ang II upregulated various pro-fibrotic genes in cultured WT fibroblasts, including Collagen I alpha 2, Collagen III, matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9), and  $\alpha$ -SMA (Supplementary material online, *Figure S3A–E*), while there were no changes in OPN KO fibroblasts (Supplementary material online, *Figure S3F–H*). Ang II infusion in mice increased cardiomyocyte size in both groups (WT and OPN KO mice, *Figure 3A–E*), but only increased heart weight/body weight ratio in WT mice (*Figure 3F*), suggesting a prominent anti-fibrotic effect of OPN deficiency specifically in cardiac fibroblasts. Furthermore, Ang II induced pro-survival and pro-fibrotic signalling pathways in WT, but not in OPN KO hearts as assessed by AKT, SMAD3, and ERK phosphorylation (*Figure 3G–J*). Interestingly, in areas of increased collagen content (Sirius red staining), the expression of OPN was also increased, indicating a direct role in fibrosis development (*Figure 3K* and *L*). In WT fibroblasts, Ang II enhanced ERK phosphorylation as well as AKT phosphorylation (Supplementary material online, *Figure S4A, B, F*, and *G*). In addition, Ang II led to the nuclear exclusion of Foxo3a, which is associated with enhanced cellular survival (Supplementary material online, *Figure S4D, J*, and *K*). None of these events occurred in OPN KO fibroblasts (Supplementary material online, *Figure S4C, E, H, I, L*, and *M*).

### Pro-inflammatory effects of Ang II-induced osteopontin *in vivo* and *in vitro*

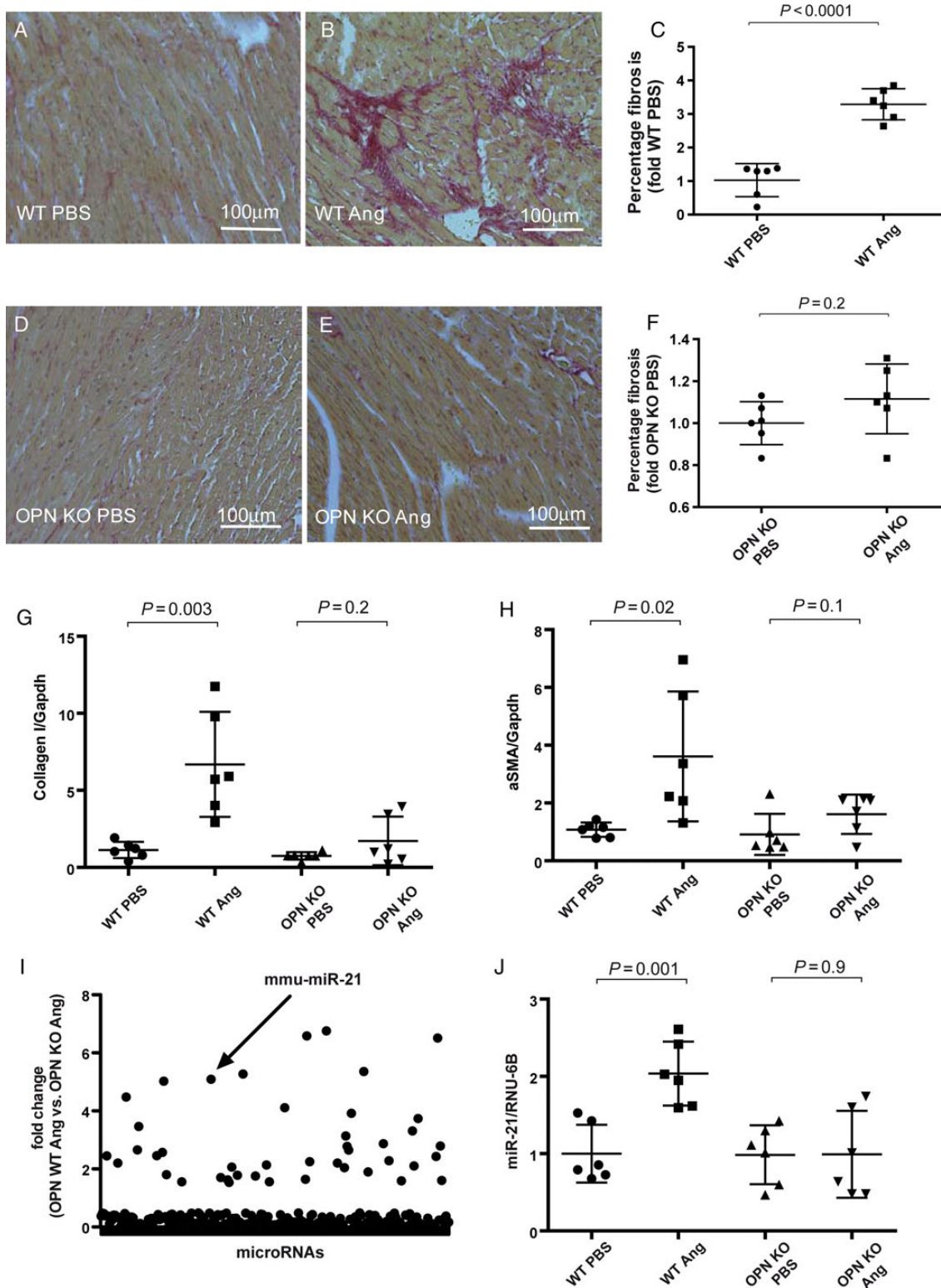
In order to further elucidate the mechanism of fibrosis development by Ang II, we assessed its potential pro-inflammatory role, since inflammation has been shown to be an important driver of fibrosis. We found that Ang II upregulated the expression of the inflammatory mediators interleukin-6 (IL-6), OPN, and macrophage chemoattractant protein-1 (MCP-1) in WT, but not in OPN KO fibroblasts (*Figure 1A–E*). *In vivo*, Ang II infusion induced the infiltration of CD45<sup>+</sup>-leucocytes (Supplementary material online, *Figure S5A–C*) as well as the mRNA expression of macrophage inflammatory protein 2-alpha (MIP-2 $\alpha$ ), IL-1 $\beta$ , TNF- $\alpha$ , and MCP-1, while none of these changes occurred in OPN KO animals (Supplementary material online, *Figure S5D–G*).

### MiRNA expression analysis in osteopontin wild-type and osteopontin knock out animals

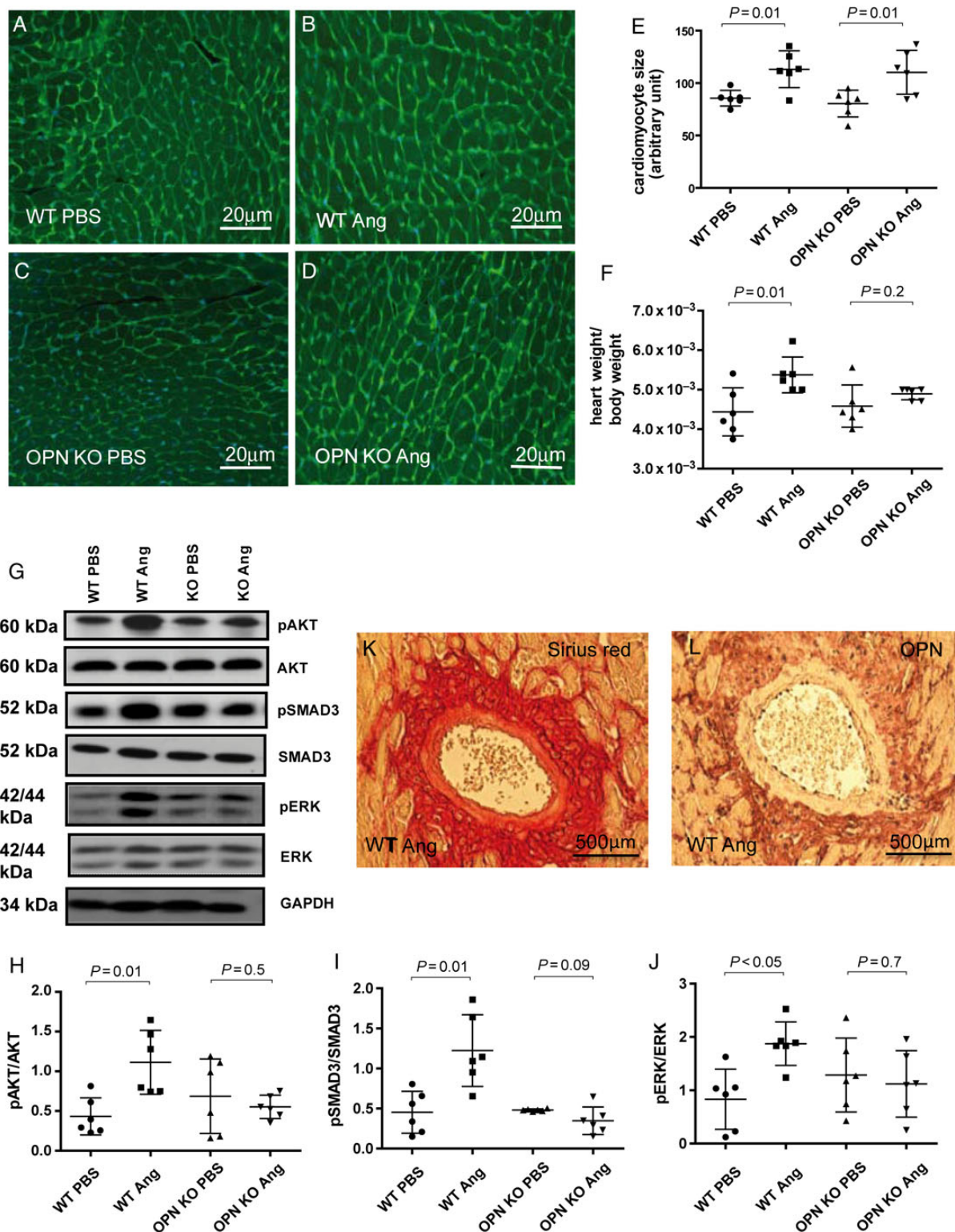
In order to further investigate the underlying mechanisms of fibrosis development, we assessed the deregulation of miRNAs (miRs), a class of short regulatory RNA molecules. A global miRNA expression profiling in hearts of WT and OPN KO animals subjected to Ang II infusion revealed a number of miRNAs to be deregulated (*Figure 2I*). MiR-21 was one of the top upregulated miRNAs in WT hearts compared with OPN KO hearts. The 10 most highly up-regulated miRNAs of the array are shown in Supplementary material online, *Table S3*. MiR-21 has previously been shown to serve as master regulator of fibrosis development.<sup>1,10</sup> The results of the global expression analysis were confirmed by independent qRT-PCR



**Figure 1** Ang II-induced inflammation and fibrosis: angiotensin II induces the mRNA expression of osteopontin (A), interleukin-6 (B), as well as macrophage chemoattractant protein-1 (C) in wild-type fibroblasts, while interleukin-6 (D) and macrophage chemoattractant protein-1 (E) levels remain unchanged in osteopontin knockout fibroblasts in response to angiotensin II. Angiotensin II induces the mRNA expression of osteopontin *in vitro* in cardiomyocytes (F) as well as the secretion of osteopontin in the cell-culture supernatant of cultured cardiomyocytes (G).  $n = 5$  independent experiments. Expression of collagen I (H), osteopontin (I), as well as miR-21 (J) in cardiac biopsies of patients with myocardial fibrosis related to aortic stenosis ( $n = 15$ ) compared with healthy control patients ( $n = 5$ ). Concomitant angiotensin-receptor blocker treatment lowers elevated levels of osteopontin in biopsies of patients with myocardial fibrosis (K).



**Figure 2** Ang II-induced fibrosis and miRNA expression in osteopontin wild type and knockout mice: Sirius red staining in paraffin-embedded sections of cardiac tissue of osteopontin wild type mice following vehicle-infusion (2 weeks, PBS, A) and Ang II infusion (B) as well as quantification of results (C) and osteopontin knockout mice following vehicle-infusion (D) and Ang II infusion (E) as well as quantification of results (F). Expression of Collagen I (G) and alpha smooth muscle actin ( $\alpha$ -SMA, H) mRNA following Ang II or vehicle infusion. A global miRNA screen in hearts of osteopontin wild type and osteopontin knockout mice following Ang II infusion (I). MiR-21 expression in osteopontin wild type and knockout animals (J).  $n = 6$  animals per group and analysis.



**Figure 3** Effects of osteopontin *in vivo*: wheat-germ agglutinin staining in osteopontin wild type (A and B) and knockout (C and D) mice to visualize cell membranes of cardiomyocytes. Cardiomyocyte cell size was counted and quantified (E). Heart weight/body weight ratio in osteopontin wild type and knockout mice (F). Downstream signalling pathways *in vivo*: phosphorylation of AKT (G and H), SMAD2,3 (G and I) and ERK (G and J) *in vivo*. In areas of increased perivascular fibrosis (Sirius red staining, K), osteopontin expression (brown staining) is also increased (L).  $n = 6$  animals per group and analysis.

analysis in WT and OPN KO hearts (Figure 2J). In myocardial tissue of patients with aortic stenosis compared with healthy control patients miR-21 expression was also highly increased (Figure 1J). Intriguingly, OPN expression displayed a close correlation with that of miR-21, underlining its significance in human cardiac fibrosis (data not shown,  $P < 0.01$ ,  $r = 0.5$ ). In cultured cardiac fibroblasts isolated from WT mice, Ang II also upregulated miR-21 expression (Supplementary material online, Figure S6A), while the level of miR-21 was unchanged in cardiac fibroblasts isolated from OPN KO animals (Supplementary material online, Figure S6B). While OPN stimulated enhanced migratory capacity of cardiac fibroblasts in a dose-dependent way (Supplementary material online, Figure S1A–D and G), miR-21 silencing blocked pro-migratory OPN effects (Supplementary material online, Figure S1E, F, and H). We next aimed to elucidate the potential transcriptional activation of miR-21 in cardiac fibrosis related to Ang II. First, we found the expression of primary miR-21 (pri-miR-21) to also be induced by Ang II in WT cells, suggesting direct transcriptional activation (Supplementary material online, Figure S6C). Transcriptional activation of miR-21 by Ang II was further underlined by employing a miR-21 promoter primer encompassing the AP-1 binding site after ChIP using an RNA polymerase II antibody (Supplementary material online, Figure S6D). It was further shown that Ang II treatment induced binding of an AP-1 oligonucleotide in electrophoretic mobility shift analysis in WT fibroblasts, but not in OPN KO fibroblasts (Supplementary material online, Figure S6E). MiR-21 expression (Supplementary material online, Figure S1D) and AP-1 oligonucleotide binding (Supplementary material online, Figure S6F) was also induced by rOPN. MiR-21 expression induced through rOPN could be lowered by PI3 kinase inhibition with wortmannin, suggesting Akt to be upstream from miR-21 (Supplementary material online, Figure S2H). Ang II-induced miR-21 upregulation could be blocked by concomitant use of the ARB losartan (Supplementary material online, Figure S3I). MiR-21 led to increased proliferation (Supplementary material online, Figure S6G) and reduced apoptosis (Supplementary material online, Figure S6H) of cardiac fibroblasts.

### Adeno-associated viral vectors of serotype-9-mediated overexpression of osteopontin and miR-21 silencing *in vivo*

In order to further identify the pro-fibrotic role of OPN *in vivo*, we generated cardiotropic OPN-AAV9 vectors for *in vivo* use (Figure 4). OPN-AAV9 alone increased both OPN and miR-21 expression (Figure 4A–C). OPN-AAV9 together with concomitant Ang II infusion significantly increased tissue fibrosis when compared with control CTL-AAV9-treated (with Ang II) and sham-operated animals (Figure 4D–F and I). Fibrosis development was highly attenuated by use of an LNA targeting miR-21 (LNA-21, Figure 4G–I). The reduction of fibrosis was more pronounced in CTL-AAV9-treated animals than in OPN-AAV9-treated animals.

### MiR-21 targets involved in increased fibroblast survival *in vivo* and *in vitro*

We identified Phosphatase and tensin homolog (PTEN) and SMAD family member 7 (SMAD7) as targets of miR-21 in WT animals (Figure 5A–C) and WT cardiac fibroblasts (Supplementary material

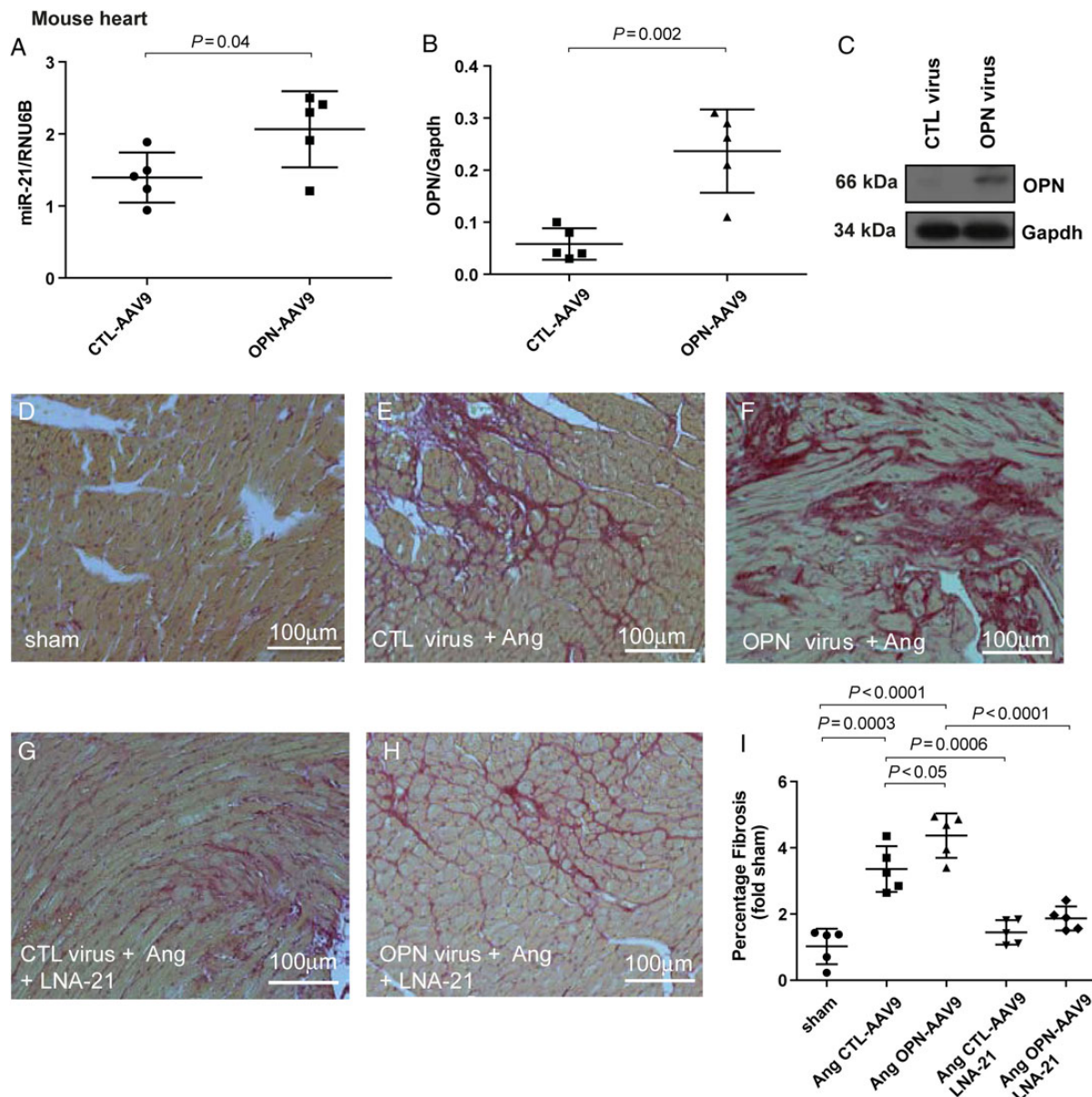
online, Figure S7A, C, and E). Programmed cell-death protein 4 (PDCD4) was tested as an additional target, but could not be confirmed in our study (Figure 5A and D for animals and Supplementary material online, Figure S7A and D for cells). MiR-21 silencing *in vivo* by LNA-21 treatment normalized the expression of PTEN and SMAD7 (Figure 5A–C). None of the aforementioned targets were regulated in OPN KO animals (Figure 5E–H) and OPN KO fibroblasts *in vitro* (Supplementary material online, Figure S7B and F–H), most likely related to unaltered levels of miR-21, further underlining the major regulatory role of OPN with regard to fibrosis development.

## Discussion

We here show that OPN is essential in transcriptional activation of miR-21 in Ang II-induced cardiac fibrosis. The proposed mechanism of Ang II action and the role of OPN with respect to cardiac fibrosis are shown in Figure 6. The results are as follows: (i) OPN and miR-21 are increased in myocardial biopsies of patients with myocardial fibrosis related to aortic stenosis; (ii) OPN KO mice are protected from Ang II-induced cardiac fibrosis through a mechanism involving AP-1-mediated miR-21 transcription and subsequent pro-fibrotic action; (iii) miR-21 targets PTEN and SMAD7, thereby leading to activation of WT fibroblasts; (iv) Ang II induces OPN expression in WT fibroblasts; (v) OPN KO fibroblasts show impaired activation and expression of pro-fibrotic genes, unaltered levels of miR-21 and targets in response to Ang II; (6) Ang II leads to phosphorylation of ERK and AKT as well as nuclear Foxo3a exclusion, culminating in enhanced fibroblast survival; (vii) OPN is secreted from cardiomyocytes by Ang II stimulation; (viii) recombinant/secreted OPN leads to transcription of miR-21 through AP-1 activation, induces fibrotic gene expression and survival of WT fibroblasts, partly through PI3-kinase activation; (ix) *in vivo* overexpression of OPN by cardiotropic OPN-AAV9 amplifies Ang II-induced cardiac fibrosis; (x) the use of an LNA targeting miR-21 ameliorates Ang II-induced cardiac fibrosis.

Several experimental and clinical studies have highlighted the pro-fibrotic role of OPN in cardiovascular disease.<sup>6,7,11</sup> Plasma levels of OPN are elevated in essential hypertension, and in patients with coronary artery disease and re-stenosis.<sup>12–14</sup> OPN levels are predictive of adverse cardiac events in patients with chronic stable angina.<sup>15</sup> Kato *et al.*<sup>14</sup> could show that pre-procedural OPN predicts re-stenosis in patients undergoing percutaneous coronary intervention. OPN has been implicated as a key factor in the development of atherosclerosis,<sup>12–18</sup> and its expression was reported to be closely related to arterial smooth muscle cell proliferation both *in vitro* and *in vivo*.<sup>19,20</sup> OPN-transgenic mice develop marked atherosclerosis.<sup>18</sup> The results of our study confirm a major role of OPN in cardiovascular disease. Furthermore, we here unravel the underlying mechanisms of fibrosis development with regard to OPN. We show that OPN is essential in activating the transcription factor AP-1, which subsequently induces miR-21 transcription and regulation of anti-fibrotic targets in Ang II-induced fibrosis. The transcription factor AP-1 is a heterodimer composed of the subunits c-fos, the protein families of c-Jun, activating transcription factor, and Jun dimerization protein.<sup>1,21</sup> The expression of AP-1 is induced by various cytokines and growth factors and activates genes responsible for cellular differentiation and proliferation.<sup>1,21</sup> The transcription of fibrosis-associated miRNAs, such as miR-21, is partly regulated by AP-1.<sup>1,22</sup>

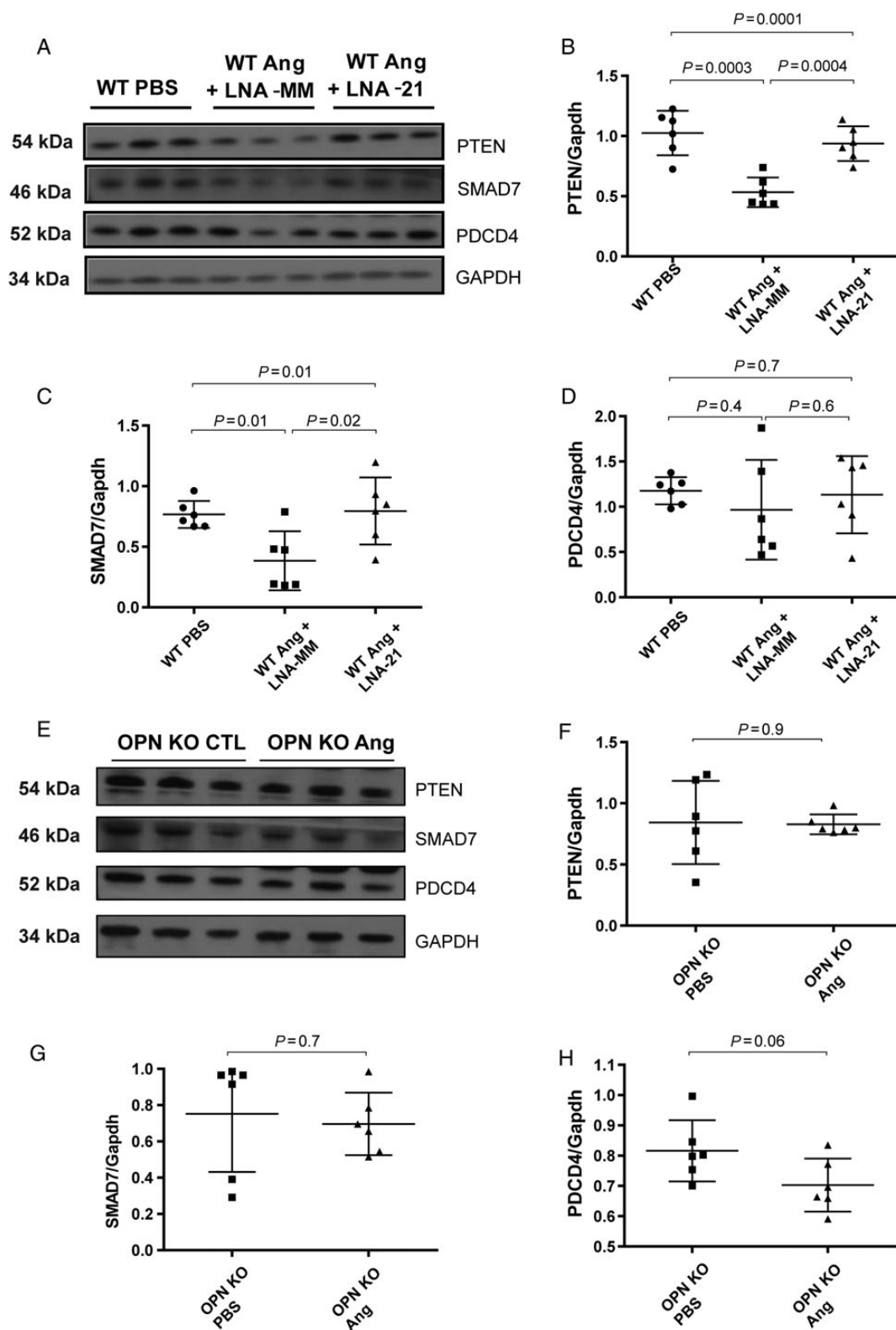




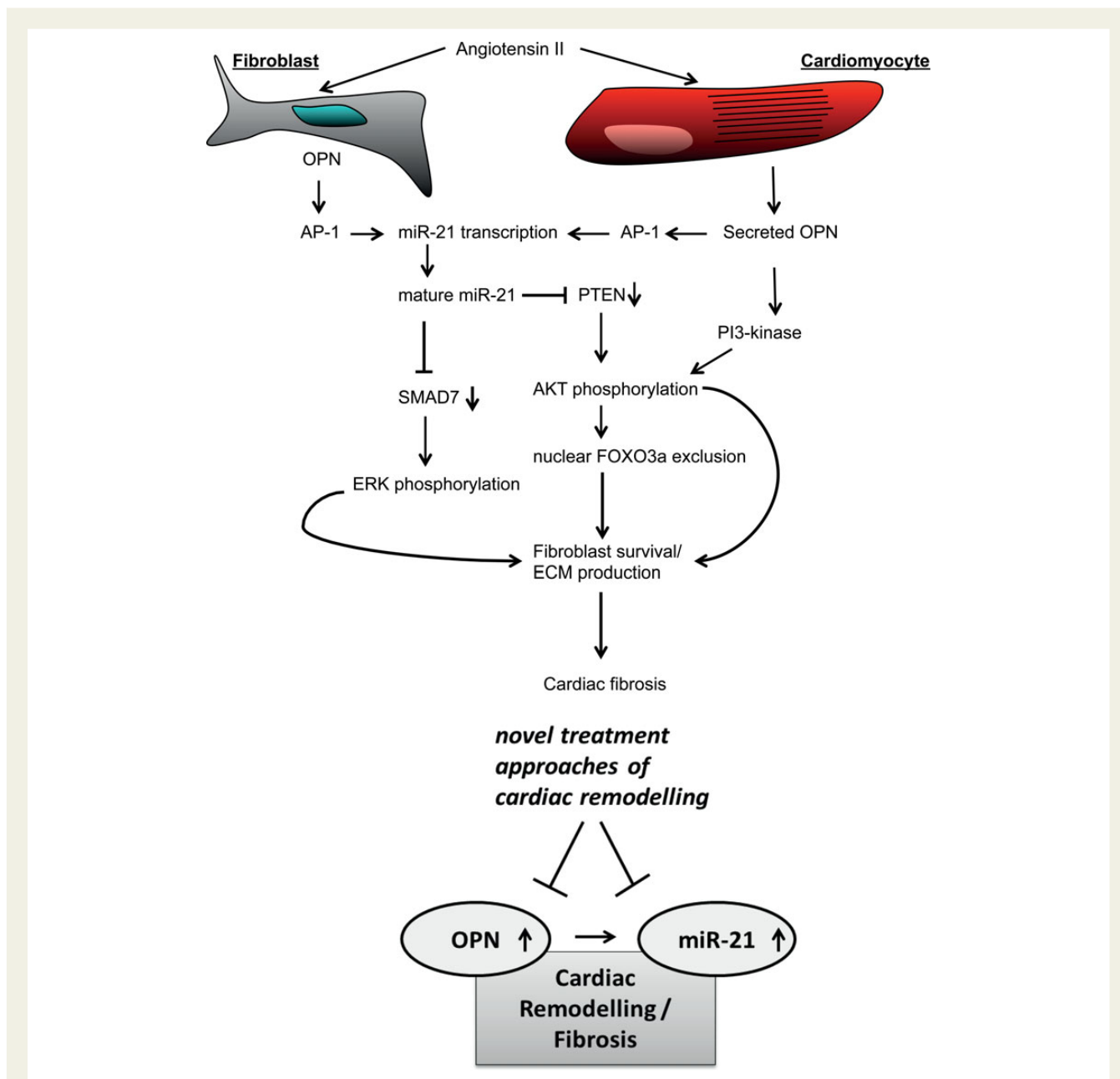
**Figure 4** MiR-21 expression in mice following cardiotropic osteopontin-AAV9 injection (A) as well as osteopontin protein expression in hearts (B and C) compared with CTL-AAV9. Sirius red staining in paraffin-embedded sections of cardiac tissue of sham-operated mice (D), mice subjected to CTL-AAV9 and Ang II (E), osteopontin-AAV9 and Ang II (F), CTL-AAV9 and Ang II as well as locked-nucleic acid treatment targeting miR-21 (LNA-21, G), osteopontin-AAV9 and Ang II as well as LNA-21 (H), and quantification of results (I).  $n = 5$  animals per group and analysis.

We also show that secreted/recombinant OPN induces a significant increase of migration and proliferation of fibroblasts, thereby promoting the further production of extracellular matrix and ultimately interstitial fibrosis. This is due in part to the activation of pro-survival phosphoinositide-3-kinase/Akt signalling as shown in our study and by others.<sup>23–25</sup> Furthermore, we show that OPN itself activates AP-1, miR-21, and pro-fibrotic genes. These findings are in line with previous data showing that OPN expression in skin fibroblasts is responsible for inflammation-associated fibrosis.<sup>26</sup> Delivery of OPN antisense oligodeoxynucleotides into mouse skin wounds leads to accelerated healing and reduced granulation tissue

formation and scarring.<sup>26</sup> Macrophage-derived platelet-derived growth factor (PDGF)-BB seems to be responsible for the OPN expression in wound fibroblasts.<sup>26</sup> We show that Ang II induces inflammation in WT fibroblasts and hearts of WT mice, while it is absent in OPN KO fibroblasts and mice. Ang II treatment is associated with enhanced fibroblast survival *in vitro* by the regulation of PTEN through miR-21. Reduced PTEN levels result in an activation/phosphorylation of AKT and a downstream inactivation of Foxo3a by nuclear exclusion, thus promoting fibroblast survival.<sup>27</sup> SMAD7 was identified as an additional target of miR-21, thereby promoting the fibrosis process. We show that Ang II activates the TGF- $\beta$



**Figure 5** miR-21 targets in osteopontin wild type and knockout mice: Protein expression as well as densitometric quantification of PTEN (A and B), SMAD7 (A and C), and PDCD4 (A and D) in wild type mice subjected to Ang II and locked-nucleic acid treatment targeting miR-21 (LNA-21) or control mismatch LNA (LNA-MM) treatment. Protein expression as well as densitometric quantification of PTEN (E and F), SMAD7 (E and G), and PDCD4 (E and H) in osteopontin knockout mice subjected to Ang II infusion. *n* = 6 animals per group and analysis.



**Figure 6** Proposed scheme of angiotensin II-induced osteopontin expression: angiotensin II leads to the expression of osteopontin in cardiac fibroblasts, which induces AP-1-mediated transcription of miR-21. Mature miR-21 targets and down-regulates PTEN, which subsequently leads to phosphorylation of AKT and nuclear exclusion of FOXO3a. In addition, miR-21 targets SMAD7, which results in phosphorylation of ERK. In cardiomyocytes, angiotensin II leads to the secretion of osteopontin, which induces the aforementioned mechanisms. Moreover, secreted osteopontin activates the PI3-kinase pathway as well as AKT phosphorylation. Taken together these mechanisms results in fibroblast survival and deposition of extracellular matrix (ECM).

signalling pathway. The interrelation between Ang II and TGF- $\beta$  is established.<sup>28</sup> TGF- $\beta$  binds to type II receptor, which activates the type I receptor kinase, which, in turn, phosphorylates the receptor-regulated SMADs SMAD2 and SMAD3. The SMAD complexes translocate to the nucleus and function as transcriptional regulators of target genes. The inhibitory SMAD7 binds to activated type I receptor, thereby preventing phosphorylation of SMAD2/3, or recruits the ubiquitin ligases Smurf1 and Smurf2 to induce proteasomal degradation of the receptor complexes.<sup>29–30</sup>

Intriguingly, we also found OPN and miR-21 to be increased in myocardial biopsies of patients with aortic stenosis. Moreover, miR-21 and OPN showed a strong degree of correlation. Elevated levels of OPN can be lowered by ARB treatment in humans. This is in line with previous results by our group.<sup>31</sup> Similarly, miR-21 and OPN expression in cultured fibroblasts can be blocked by pre-treatment with the ARB losartan. These findings are well in line with recent experimental data. In a study involving male Japanese white rabbits fed on a high-cholesterol diet, treatment with olmesartan decreased macrophage

accumulation and OPN expression in aortic valve leaflets.<sup>32</sup> Knockdown of OPN in vascular smooth muscle cells by siRNA reduced Ang II-induced mediators of inflammation.<sup>33</sup> These results indicate that Ang II-induced inflammation and subsequent fibrosis is at least in part mediated by OPN. Our results confirm a regulatory effect of ARB treatment on OPN.

OPN deficiency reduced the number of infiltrating CD45<sup>+</sup>-leucocytes in response to Ang II-infusion in our study. The importance of OPN in leucocyte chemotaxis was exemplified by the impaired recruitment of OPN  $-/-$  neutrophils in experimental colitis, which was restored after the administration of exogenous OPN.<sup>34</sup> The increased level of OPN would support a role for osteopontin in an autocrine feedback loop, which could potentiate the disease process by promoting the further accumulation of monocytes and macrophages to the site of inflammation with subsequent increase in fibrosis.

Intriguingly, AAV9-mediated overexpression of OPN *in vivo* specifically in the heart using cardiotropic OPN-AAV9 further enhanced Ang II-related fibrosis underlining the *in vivo* significance of OPN with regard to fibrosis development. Our results are in line with previous studies using adenoviral constructs to overexpress OPN in hepatocytes with respect to liver fibrosis.<sup>35</sup>

We were previously able to show that in cardiac fibrosis in response to cardiac pressure overload, miR-21 is specifically enriched in cardiac fibroblasts promoting fibroblast survival and growth factor secretion.<sup>10</sup> In our study, miR-21 silencing *in vivo* by using an LNA targeting miR-21 prevented the development of Ang II-induced cardiac fibrosis. Supplementary material online, Table S3 lists the 10 most highly upregulated miRNAs in our array. For instance miR-181a was also among the top deregulated miRNAs. MiR-181a has previously been shown to be a TGF- $\beta$  regulated miRNA.<sup>36</sup> Therefore, it cannot be entirely excluded that other miRNAs also contribute to OPN-mediated fibrosis. However, we believe that our data clearly demonstrate a pathophysiological link between deregulated OPN expression, miR-21 induction, and cardiac fibrosis development.

In conclusion, we show that Ang II-induced cardiac fibrosis is mediated by activation of the transcription factor AP-1 and subsequent miR-21 regulation. MiR-21 targets the anti-survival and anti-fibrotic targets PTEN and SMAD7, ultimately resulting in fibroblast proliferation. OPN KO mice are protected from Ang II-induced cardiac fibrosis through impaired activation of AP-1 and miR-21.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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**Conflict of interest:** T.T. filed and licensed patents about the role of miR-21 in fibrosis.

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## CARDIOVASCULAR FLASHLIGHT

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### Unique coronary artery anomaly: three separate coronary artery ostia within one coronary sinus

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A 69-year-old patient was presented at our cardiac care unit with acute inferior infarction, for which she underwent primary PCI.

Cardiac catheterization revealed unique coronary artery anatomy, with an aberrant left main coronary artery (LMCA), and a septal branch both deriving from the right sinus Valsalva, but with separate ostia from the right coronary artery (Panels A and B; Supplementary material online, Videos S1 and S2). The LMCA passes the pulmonary artery anteriorly, after which it bifurcates into the left anterior descending artery and the ramus circumflex.

A septal branch passes in between the aorta and the right ventricular outflow tract. Cardiac CT confirmed this coronary arterial course (Panel C).

The origin of the LMCA from the right aortic sinus of Valsalva is the least common coronary anomaly (incidence: 0.15%), and is notorious for its malign course between the great vessels. A benign course (LMCA anterior to the pulmonary trunk), as was the case in our patient, remains even more rare. Indeed, the fact that three coronary arteries derive from a single sinus, but with three separate ostia seems unique.

The benign course of coronary anomaly explains the fact that our patient had remained without symptoms throughout her life, and presented with acquired ischaemic heart disease at the age of 69. There is no intention to correct her anomaly as no harm is expected from it in the future.

Supplementary material is available at *European Heart Journal* online.

