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miR181a protects against angiotensin II-induced osteopontin expression in vascular smooth muscle cells

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Abstract

Objective—Osteopontin (OPN) is a multifunctional protein found in abundance in atherosclerotic plaques. Angiotensin II (Ang II) promotes atherosclerosis by inducing adhesion and migration of vascular smooth muscle cells (VSMCs). MicroRNAs (miRNAs) are critical regulators of protein expression. However, the relationship between Ang II, miRNAs and OPN has yet to be fully explored.

Methods and results—Using cultured VSMCs, we found that Ang II increased cellular OPN protein expression 4 h after treatment by 420 ± 54% ($p < 0.03$) in a translation dependent manner. Sequence analysis revealed a putative binding site for mir181a and raised the possibility that miR181a is a potential regulatory mechanism for OPN expression. We demonstrated that Ang II decreased miR181a expression by 52 ± 7% ($p < 0.0001$) and overexpressing miR181a inhibited Ang II induced increases in OPN protein expression by 69 ± 9% ($p < 0.05$). Furthermore, we demonstrated that miR181a is functionally important in that overexpression of miR181a inhibited VSMCs adhesion to collagen in response to Ang II as compared to controls by 36 ± 4%. ($p < 0.05$)

Conclusions—These results demonstrate that miR181a regulates OPN expression and that altering miR181a expression may be a novel therapeutic approach to modulate OPN protein expression.

Keywords

Angiotensin II; MicroRNA; Translational control; Osteopontin

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Disclosures
None.

Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.01.037.

1. Introduction

Atherosclerosis is widely accepted as a chronic inflammatory disease characterized by endothelial dysfunction, vascular inflammation, and the accumulation of lipids and cellular debris within the vessel wall [1]. The renin-angiotensin system has been implicated in the pathogenesis of atherosclerosis through its primary effector molecule angiotensin II (Ang II) [2]. During pathological conditions, chronic exposure of cardiovascular tissues to Ang II affects the growth, migration and adhesion of smooth muscle cells [3,4]. Several *in vivo* studies have demonstrated that Ang II can induce the expression of adhesion molecules and increase production of reactive oxygen species (ROS), which initiate a potent inflammatory response resulting in the release of growth factors, cytokines and chemokines within the aortic wall [3,5–7].

Under normal conditions, osteopontin (OPN) is not expressed in the arterial wall. However, in the setting of atherosclerosis, hypertension and vascular injury, OPN expression is dramatically increased [8–13]. Furthermore, Ang II-induced atherosclerosis is attenuated in $OPN^{-/-}$ mice, suggesting that OPN is a potential mediator in the development of Ang II-induced pathologies [9,14]. OPN is a negatively charged, secreted, glycosylated phosphoprotein expressed in multiple tissues and cell types including osteoblasts, macrophages, vascular smooth muscle cells (VSMCs) and T cells [8]. OPN contains an arginine-glycine-aspartic acid (RGD) motif that is recognized by integrins (primarily $\alpha v\beta 3$ integrin, as well as $\alpha v\beta 1$ and $\alpha v\beta 5$), which mediates adhesion, proliferation, and migration in VSMCs [15–19]. Furthermore, OPN has been implicated as a proinflammatory protein, serving as a chemoattractant for both VSMCs and macrophages, while inducing activation in the latter. Given the pleiotropic response of OPN signaling in a vast array of cells, it is important to fully understand the mechanisms responsible for the regulation of OPN by Ang II.

Recently, it has been shown that microRNAs (miRNAs) represent a novel regulatory mechanism for protein expression. miRNAs are a class of highly conserved endogenous single stranded RNA nucleotides (~22nt) that bind preferentially to the 3′ untranslated region (3′UTR) of target eukaryotic mRNA and inhibit protein translation in mammalian cells. It has become increasingly evident that these small, endogenous RNAs play a crucial role in many biological processes including development, differentiation, proliferation, and apoptosis [20,21]. The goal of the present study is to understand the relationship between Ang II, miR181a and OPN protein levels in VSMCs as it relates to vascular dysfunction. We propose a model whereby miR181a modulates Ang II-induced OPN expression. Ang II-induced increases in OPN promote cell adhesion, contributing to the migratory actions of VSMCs, which is crucial in the pathophysiology of atherosclerosis.

2. Materials & methods

2.1. Cell culture

VSMCs enzymatically derived from rat aortas were generously provided by Dr. Kathy K. Griendling PhD, Emory University, Atlanta GA, were obtained as previously described [22]. Prior to experimental stimulation, cells were serum-starved for 48 h and stimulated with Ang II (Sigma) for the indicated time points. In some cases, cells were pretreated with 100 μM 5,6-dichloro-1-β-D-ribobenzimidazole (DRB) (Sigma) to inhibit transcription. Mouse aortic vascular smooth muscle cells (MASMs) were prepared from $OPN^{-/-}$ or $OPN^{+/+}$ mice (age 6–8 weeks) obtained from Jackson Laboratories as previously described [23]. Sub-cultured MASMs were maintained in DMEM containing 100 units/mL penicillin, 100 μg/mL streptomycin and 10% FBS.

2.2. Quantitative real time-polymerase chain reaction (qRT-PCR)

RNA was extracted from VSMCs, using the RNeasy kit (Qiagen) according to their protocol and quantified using a spectrophotometer. cDNA was prepared and purified from using standard Invitrogen protocol with primers unique for rat OPN (Qiagen). All RNA was normalized to 18S (Invitrogen) unless otherwise noted.

2.3. Protein extraction and western blotting

20 μg of protein lysed in radio immunoprecipitation assay buffer (RIPA) were loaded onto 7.5% SDS polyacrylamide gels (BioRad) and subsequently blotted onto nitrocellulose (Amersham). Membranes were blocked with 5% milk (Santa Cruz). The osteopontin antibody was obtained from R&D Systems (Minneapolis, MN) and used at a 1:1000 dilution. Erk and Phospho-erk antibodies were acquired from Cell Signaling. Blots were analyzed using densitometry (U-Scan-It) and normalized to β-actin (Cell Signaling) at a 1:2000 dilution.

2.4. miRNA isolation and quantification

Total RNA including small RNAs were extracted from cultured cells using miRVana miRNA isolation kit (Ambion, Invitrogen) and reverse transcribed for semi-quantification by standard real-time qRT-PCR (Applied Biosystems). MicroRNA expression was determined using the TaqMan microRNA assay kit for hsa-miR181a (Applied Biosystems), with RNU6B RNA used as an internal control. Fold change was calculated using the previously reported $2^{-\Delta\Delta CT}$ method [24].

2.5. miR181a overexpression

Double-stranded, human/mouse/rat specific miR181a mimetic RNA oligonucleotides were synthesized by Applied Biosystems and prepared according to the manufacturer's instructions. For transfection, VSMCs were grown to 80% confluence with DMEM (Hyclone) supplemented with 10% calf serum. 2×10^6 cells were transfected with 50 pmoles of a miR181a mimetic -AACAUUCAACGCUGUCGGUGAGU- or negative control (catolog #AM171100) both purchased from Applied Biosystems using electroporation (AMAXA, Lonza) and recovered in OptiMEM (Invitrogen) according to the manufacturer's specifications. Twenty-four hours post transfection, cells were serum deprived for 48 h and then treated with 100 nM of Ang II (Sigma). Protein was then harvested, quantified as described and analyzed for OPN expression by Western blot analysis.

2.6. Adhesion assay

VSMCs were seeded at a density of 5×10^4 cells/plate on precoated collagen type I coverslips (Fisher) for 1hr at 37 °C in serum free media. Non-adherent cells were aspirated and the adherent cells were washed in phosphate-buffered solution (PBS) and fixed with 4% paraformaldehyde for 10 min. Cells were then permeabilized using 0.2% Triton X and quenched with 50 nm NH_4Cl, followed by immunostaining with Phalloidin (Invitrogen) and 4′-6-diamidino-2-phenylindole (DAPI/vectasheild) to visualize the nuclei. Images were acquired using an Olympus microscope at 10× magnification or confocal microscopy at 20× as indicated. Images were taken of 5 random fields per dish per experiment and quantified.

2.7. Statistical analysis

All data were reported as mean ± SEM for at least three independent experiments. Statistical significance for quantitative experiments was assessed with either a student *t*-test or analysis of variance (ANOVA, 2-tailed) with Bonferonni's post-hoc analysis. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Ang II increases OPN protein expression in VSMCs

To determine whether OPN expression was regulated by Ang II, we stimulated cultured VSMCs with Ang II and found that OPN mRNA expression increased at a later time point, 12 h after Ang II administration (Fig. 1A). Conversely, we observed early increases in OPN cellular protein expression as early as 2 h after Ang II treatment. By 4 h of Ang II treatment, OPN protein expression had increased by 420 ± 54% over baseline in the absence of any detectable increase in OPN mRNA expression ($p < 0.03$, Fig. 1B). OPN protein expression further increased for at least 24 h after treatment as shown in Fig.1B. These data demonstrated that OPN at the mRNA and protein level was upregulated in response to Ang II stimulation in cultured VSMCs. Interestingly, these studies established that OPN protein expression was increased prior to any change in OPN mRNA expression suggesting that a mechanism exists for Ang II-mediated increases in OPN that is independent of changes in mRNA expression.

3.2. Early increases in Ang II-induced OPN expression are transcriptionally independent

To determine if Ang II-induced increases in OPN protein levels observed at the earlier time points after Ang II treatment are mediated through a post-transcriptional mechanism, we used 5,6-dichloro-1-β-D-ribobenzimidazole (DRB) to block transcription prior to stimulation with Ang II. We treated VSMCs with Ang II in the presence of 100 μMDRB or vehicle control. We found that there was no significant difference between the DRB and vehicle treated cells until 6 h after Ang II treatment, suggesting that the earlier increase in OPN protein expression was due to an increase in translation, not transcription (Fig. 2A). Furthermore, Ang II did not increase OPN mRNA stability as shown by comparing DRB treated cells in the presence and absence of Ang II (Fig. 2B). These data demonstrate that OPN mRNA expression was unchanged at the early time points after Ang II treatment and that early Ang II-induced increases in OPN protein levels at the earliest time points are likely the result of increased translation of pre-existing OPN mRNA. This represents a novel mechanism for Ang II regulation of OPN protein expression.

3.3. Ang II decreases miR181a expression

We next sought to determine a potential role for miRNAs in the regulation of OPN protein levels. We performed an extensive literature and database search for miRNAs that target the 3′UTR of OPN as a potential mechanism for modifying Ang II induced OPN expression. Search results from three miRNA databases (TargetScan.org, miRWalk and microRNA.org) indicated the presence of a putative binding site for miR181a within the 3′UTR of human OPN. Therefore, our goal was to assess whether Ang II regulates miR181a expression, as a potential mechanism for OPN regulation. Alignment of OPN mRNA for human (NM_001040058.1), mouse (NM_001204201.1) and rat (NM_012881.2) species revealed and evolutionarily conserved miR181a binding site within the 3′UTR of all transcripts (–GAAUGUA-Fig. 3A).

In order to determine if miR181a is regulated by Ang II, we examined the effect of Ang II on miR181a in cultured VSMCs. We found that miR181a expression was decreased by 52 ± 7% ($p < 0.0001$) after treatment with Ang II (Fig. 3B). These data are consistent with the hypothesis that miR181a is a potential mediator through which Ang II modulates OPN protein levels.

Overexpression of miR181a inhibits Ang II induced OPN protein expression

To test whether Ang II-induced translation dependent increases in OPN protein expression at 4 h were potentially mediated by decreased miR181a expression, we used a miR181a

mimetic, which allows for overexpression of miR181a. As shown in Fig. 4A, overexpression of miR181a in VSMCs inhibited Ang II-induced increases in OPN protein expression at 4 h by 69 ± 9% ($p < 0.05$) compared to non-transfected control and negative miRNA treated cells stimulated with Ang II for 4 h. The level of inhibition of OPN by miR181a is similar to levels observed in serum deprived VSMCs (control band, Fig. 4A). After 24 h of Ang II stimulation, we still observed inhibition of OPN by overexpressing miR181a, suggesting a prolonged inhibitory effect (data not shown). To verify that signaling pathways in miR181a transfected cells maintained their ability to properly signal in response to Ang II stimulation of the AT1 receptor, we used a previously reported [25] assay to assess phosphorylation of Erk1/2, 5 min after Ang II stimulation. As shown in Fig. 4B, miR181a overexpression did not interfere with Ang II signal transduction as evidenced by robust increases in phosphorylation of Erk1/2 at 5 min, suggesting that AT1 receptor signaling was intact.

3.5. miR181a overexpression inhibits Ang II-mediated VSMC adhesion

To further explore whether miR181a modulation of OPN protein levels results in a physiologic impact, we examined the effect of modulation of miR181a expression on cell adhesion. Previous reports have suggested that the absence of OPN leads to decreased adhesion of VSMCs to collagen substrates [19,26]. Therefore, we investigated whether the inhibition of Ang II induced OPN with a miR181a mimetic resulted in changes in cellular adhesion. To first assess the contribution of OPN to adhesion, we isolated and cultured MASMs from age matched $OPN^{-/-}$ or wildtype (WT) mice and plated them onto collagen coated coverslips. We observed that $OPN^{-/-}$ MASMs exhibited decreased adherence to collagen as compared to WT MASMs at 30 min by 63 ± 9% ($p < 0.05$) and at 1hr by 61.9 ± 9% ($p < 0.001$) respectively (Fig. 5A). These data demonstrate that OPN contributes significantly to cell adhesion and thus provides a physiologic target to study the functional role of miR181a in a cell culture model.

We used this strategy to assess the importance of miR181a dependent decreases in OPN expression on Ang II mediated VSMC adhesion to collagen. We found that in control cells, Ang II increased cell adhesion by 68 ± 6% compared to non-stimulated controls (Fig. 5B top panels). In contrast, we observed that VSMCs transfected with miR181a mimetic and stimulated with Ang II for 24 h significantly abolished Ang II induced adhesion (Fig. 5B lower panels). In line with previous reports that suggest OPN regulates β1 expression, we show a modest decrease in β1 integrin expression with miR181a overexpression, suggesting that decreased OPN and β1 integrin expression collectively reduce adhesion of VSMCs to collagen (data not shown). We also have demonstrated that mir181a does not alter cell viability or proliferation (supplemental Figs. 2 and 4). Collectively, these data support our hypothesis that miR181a mediated regulation of OPN has a functional impact on VSMCs as evidenced by the inhibition of adhesion of VSMCs.

4. Discussion

It has been postulated that angiotensin II plays a central role in the pathogenesis of atherosclerosis, a well-documented inflammatory disease [3,27,28]. One mechanism by which Ang II promotes inflammation is through the upregulation of proinflammatory molecules, such as osteopontin. MicroRNAs function in part as critical regulators of protein expression by binding to the 3′UTRs of target mRNAs, resulting in inhibition of protein translation in mammalian cells. As such, miRNAs have been implicated in the pathogenesis of vascular disease and have been suggested as potential therapeutic targets. The goal of our study was to evaluate the ability ofmiR181a to regulate Ang II induced OPN protein expression in VSMCs. We showed that in VSMCs, Ang II increased OPN cellular protein expression in a translation-dependent manner. We also found that miR181a, predicted to bind OPN's 3′UTR, was expressed in VSMCs and that its expression was down regulated

by Ang II. We observed that overexpressing miR181a in VSMCs inhibited Ang II induced OPN expression in cultured VSMCs. We further demonstrated thatmiR181a overexpression had a functional impact on Ang II induced VSMCs adherence to collagen substrates. We therefore propose a novel mechanism for the regulation of Ang II induced OPN expression attributable to modulation of miR181a.

Clinical studies have previously reported that OPN is upregulated in atherosclerotic plaques and injured arterial walls, implicating OPN in the pathogenesis of atherosclerosis [9]. This may in part be due to OPNs ability to promote macrophage and immune cell activation, as well as smooth muscle cell adhesion, migration and proliferation. Since OPN is capable of mediating adhesion, migration and proliferation, greater insight into the mechanisms of regulation of OPN protein expression may help to define novel therapeutic targets.

Our studies suggest that Ang II mediated transcriptional regulation of OPN occurs after 6 h and that the early increases in OPN cellular protein expression by Ang II occur solely by translation dependent mechanisms. It is likely that a more complex time-dependent mechanism of action exists for regulation of OPN protein expression at later time points. Ishida and colleagues previously reported early increases in OPN mRNA, peaking between 8 and 12 h, followed by later increases in secreted OPN protein in response to Ang II [8]. To our knowledge, our data are the first report that Ang II increases non-secreted OPN protein levels in VSMCs through a translation dependent manner following exposure to Ang II. We were able to exploit this mechanism of regulation, as a means for modulating OPN protein expression at the posttranscriptional level.

After confirming that Ang II increased cellular OPN protein expression in VSMCs, we performed database searches for miRNAs capable of binding to the 3′UTR of OPN. Bhattacharya et al. used a luciferase based reporter system and serial deletion of components of the 3′UTR to demonstrate that miR181a targets the human OPN 3′UTR [29]. We were able to identify the miR181a binding site across several species including human, mouse and rat, suggesting an evolutionarily conserved importance for mir181a. Using qRT-PCR, we were able to demonstrate that not only was miR181a expressed in unstimulated VSMCs, but that Ang II reduced miR181a expression.

Further investigation of the relationship between miR181a, OPN and Ang II, has allowed us to demonstrate a novel mechanism by which miR181a is capable of inhibiting OPN protein synthesis in the presence of Ang II in VSMCs. Our findings are supported by previous observations demonstrating that overexpression of miR181a plays an inhibitory role in the regulation of OPN protein expression in human hepatocellular cancer cell lines [29]. The conservation of this regulatory mechanism by which miR181a inhibits Ang II induced OPN protein expression in VSMCs and in non-stimulated hepatocellular cancer cells suggest that miR181a may be an important and necessary regulatory mechanism for OPN in multiple cell types. At present, the mechanism by which Ang II modulates miR181a expression remains to be elucidated. We speculate a mechanism by which Ang II decreases expression and binding of miR181a to OPN's 3′UTR. A decrease of miR181a binding by Ang II could permit active translation of OPN mRNA through RNA conformational changes that alter the affinity of RNA binding proteins (RBPs) to the 3′UTR. It has previously been established that RBPs and miRNAs work in concert to promote translation or repression of mRNA expression [30,31]. It is plausible that miR181a acts to recruit RBPs that repress translation, but in the presence of Ang II, miR181a expression is repressed and no longer bound to OPN, RBPs are no longer recruited, and translation occurs freely. Regardless of the mechanism, it is clear that overexpression of miR181a is a potential mechanism for the regulation of OPN translation.

OPN is a multifunctional protein involved in regulating a myriad of processes. We choose to evaluate OPN in the setting of adhesion because of a series of studies implicating both Ang II and OPN in cell adhesion. Ang II has been shown to promote adhesion of cardiac fibroblast to collagen substrates through an integrin mediated mechanism in which PKC phosphorylates the β1 integrin, causing it to translocate to the cell surface [32]. In addition, We intraub et al. previously demonstrate that OPN facilitates VSMC adhesion to collagen [19]. Using these data as a precursor for our functional assessment of miR181a on OPN, we have shown that miR181a overexpression in the setting of Ang II stimulation reduces the ability of VSMCs to adhere to collagen substrates. We intraub et al. have further demonstrated that reduced adhesion of OPN deficient VSMCs to collagen is partially due to decreased expression of α1 and β1 collagen binding integrins. We observe a similar trend in that VSMCs deficient in OPN also have a modest decrease in β1 integrin expression.

The fact that overexpression of miR181a inhibits Ang II induced increases in OPN protein in VSMCs suggest a role for miR181a in cardiovascular disease through modulation of OPN–mediated adhesion, which is essential for migration and proliferation of VSMCs. It should be noted that at baseline, intracellular OPN protein expression in VSMCs is low, making it difficult to observe a reduction in OPN levels when overexpressing miR181a. Additionally, no effect on OPN protein expression was noted in VSMCs transfected with a miR181a inhibitor, lending support to an RBP-based mechanism of de-repression. Without Ang II, translation would not be initiated, regardless of the miRNA/RBP relationship, potentially explaining why antagonizing miR181a has no effect on OPN protein expression in non-stimulated cells. Nonetheless, the miR181a mediated inhibition of Ang II-induced OPN expression is valuable to the current understanding of miRNAs and vascular disease progression. The ability of miR181a to reduce OPN protein expression and function in the setting of Ang II may make miR181a a novel therapeutic target for atherosclerosis.

The ability of various miRNAs to inhibit Ang II's effect on the vasculature has recently been evaluated in human endothelial cells, suggesting a role for miRNAs in inflammation and migration [33]. Overexpression of miR155 and miR-221/222 was shown to block Ang II activation of Ets-1(endothelial transcription factor) and subsequent increases in inflammatory genes such as VCAM, MCP-1 and FLT-1 [33]. miRNA regulation of Ang II induced protein expression in endothelial cells was successful, in part due to the down regulation of the AT1 receptor by miR155. Here we clearly demonstrate reduction of specific Ang II mediated effects through miRNA delivery. However, our mechanism appears to be more specific to OPN and independent of AT1 receptor down regulation.

In conclusion, we report a novel mechanism of OPN regulation in VSMCs involving miR181a mediated post-transcriptional silencing. Our data indicate that miR181a activity is an important mechanism by which Ang II induced OPN expression is inhibited in the vasculature during exposure to Ang II. The ability of miR181a to have a functional impact on Ang II induced VSMC adhesion, underscores the contribution of miRNAs to disease pathology. Modulation of OPN levels using miR181a may have implications for the treatment of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Tesanovic S, Vinh A, Gaspari TA, Casley D, Widdop RE. Vasoprotective and atheroprotective effects of angiotensin (1–7) in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2010; 30:1606–1613. [PubMed: 20448208]
2. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am J Physiol Cell Physiol. 2007; 292:C82–C97. [PubMed: 16870827]
3. Weiss D, Sorescu D, Taylor WR. Angiotensin II and atherosclerosis. Am J Cardiol. 2001; 87:25C–32C.
4. Faries PL, Rohan DI, Wyers MC, et al. Vascular smooth muscle cells derived from atherosclerotic human arteries exhibit greater adhesion, migration, and proliferation than venous cells. J Surg Res. 2002; 104:22–28. [PubMed: 11971673]
5. Hansson GK, Robertson AK, Soderberg-Naucler C. Inflammation and atherosclerosis. Annu Rev Pathol. 2006; 1:297–329. [PubMed: 18039117]
6. Libby P. Inflammation in atherosclerosis. Nature. 2002; 420:868–874. [PubMed: 12490960]
7. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation. 2002; 105:1135–1143. [PubMed: 11877368]
8. Abe K, Nakashima H, Ishida M, et al. Angiotensin II-induced osteopontin expression in vascular smooth muscle cells involves Gq/11, Ras, ERK, Src and Ets-1. Hypertens Res. 2008; 31:987–998. [PubMed: 18712054]
9. Cho HJ, Cho HJ, Kim HS. Osteopontin: a multifunctional protein at the cross-roads of inflammation, atherosclerosis, and vascular calcification. Curr Atheroscler Rep. 2009; 11:206–213. [PubMed: 19361352]
10. Asou Y, Rittling SR, Yoshitake H, et al. Osteopontin facilitates angiogenesis, accumulation of osteoclasts, and resorption in ectopic bone. Endocrinology. 2001; 142:1325–1332. [PubMed: 11181551]
11. Chakraborty G, Jain S, Behera R, et al. The multifaceted roles of osteopontin in cell signaling, tumor progression and angiogenesis. Curr Mol Med. 2006; 6:819–830. [PubMed: 17168734]
12. Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. J Clin Invest. 1993; 92:1686–1696. [PubMed: 8408622]
13. Ikeda T, Shirasawa T, Esaki Y, Yoshiki S, Hirokawa K. Osteopontin mRNA is expressed by smooth muscle-derived foam cells in human atherosclerotic lesions of the aorta. J Clin Invest. 1993; 92:2814–2820. [PubMed: 8254036]
14. Bruemmer D, Collins AR, Noh G, et al. Angiotensin II-accelerated atherosclerosis and aneurysm formation is attenuated in osteopontin-deficient mice. J Clin Invest. 2003; 112:1318–1331. [PubMed: 14597759]
15. Standal T, Borset M, Sundan A. Role of osteopontin in adhesion, migration, cell survival and bone remodeling. Exp Oncol. 2004; 26:179–184. [PubMed: 15494684]
16. Rangaswami H, Bulbule A, Kundu GC. Osteopontin: role in cell signaling and cancer progression. Trends Cell Biol. 2006; 16:79–87. [PubMed: 16406521]
17. Li JJ, Han M, Wen JK, Li AY. Osteopontin stimulates vascular smooth muscle cell migration by inducing FAK phosphorylation and ILK dephosphorylation. Biochem Biophys Res Commun. 2007; 356:13–19. [PubMed: 17336930]
18. Yue TL, McKenna PJ, Ohlstein EH, et al. Osteopontin-stimulated vascular smooth muscle cell migration is mediated by beta 3 integrin. Exp Cell Res. 1994; 214:459–464. [PubMed: 7925641]
19. Weintraub AS, Schnapp LM, Lin X, Taubman MB. Osteopontin deficiency in rat vascular smooth muscle cells is associated with an inability to adhere to collagen and increased apoptosis. Lab Invest. 2000; 80:1603–1615. [PubMed: 11092521]
20. Plasterk RH. Micro RNAs in animal development. Cell. 2006; 124:877–881. [PubMed: 16530032]

21. Bushati N, Cohen SM. microRNA functions. Annu Rev Cell Dev Biol. 2007; 23:175–205. [PubMed: 17506695]
22. Griendling KK, Taubman MB, Akers M, Mendlowitz M, Alexander RW. Characterization of phosphatidylinositol-specific phospholipase C from cultured vascular smooth muscle cells. J Biol Chem. 1991; 266:15498–15504. [PubMed: 1651335]
23. Ohmi K, Masuda T, Yamaguchi H, et al. A novel aortic smooth muscle cell line obtained from p53 knock out mice expresses several differentiation characteristics. Biochem Biophys Res Commun. 1997; 238:154–158. [PubMed: 9299470]
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods. 2001; 25:402–408. [PubMed: 11846609]
25. Ishida M, Ishida T, Thomas SM, Berk BC. Activation of extracellular signal-regulated kinases (ERK1/2) by angiotensin II is dependent on c-Src in vascular smooth muscle cells. Circ Res. 1998; 82:7–12. [PubMed: 9440699]
26. Weintraub AS, Giachelli CM, Krauss RS, Almeida M, Taubman MB. Autocrine secretion of osteopontin by vascular smooth muscle cells regulates their adhesion to collagen gels. Am J Pathol. 1996; 149:259–272. [PubMed: 8686750]
27. Berk BC, Haendeler J, Sottile J. Angiotensin II, atherosclerosis, and aortic aneurysms. J Clin Invest. 2000; 105:1525–1526. [PubMed: 10841510]
28. Touyz RM. Reactive oxygen species and angiotensin II signaling in vascular cells – implications in cardiovascular disease. Braz J Med Biol Res. 2004; 37:1263–1273. [PubMed: 15273829]
29. Bhattacharya SD, Garrison J, Guo H, et al. Micro-RNA-181a regulates osteopontin-dependent metastatic function in hepatocellular cancer cell lines. Surgery. 148:291–297. [PubMed: 20576283]
30. van Kouwenhove M, Kedde M, Agami R. MicroRNA regulation by RNA-binding proteins and its implications for cancer. Nat Rev Cancer. 2011; 11:644–656. [PubMed: 21822212]
31. Ma F, Liu X, Li D, et al. MicroRNA-466l upregulates IL-10 expression in TLR-triggered macrophages by antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation. J Immunol. 2010; 184:6053–6059. [PubMed: 20410487]
32. Hein L. Angiotensin II and cell-matrix adhesion: PKCepsilon is essential. Cardiovasc Res. 2005; 67:6–8. [PubMed: 15922318]
33. Zhu N, Zhang D, Chen S, et al. Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. Atherosclerosis. 215:286–293. [PubMed: 21310411]

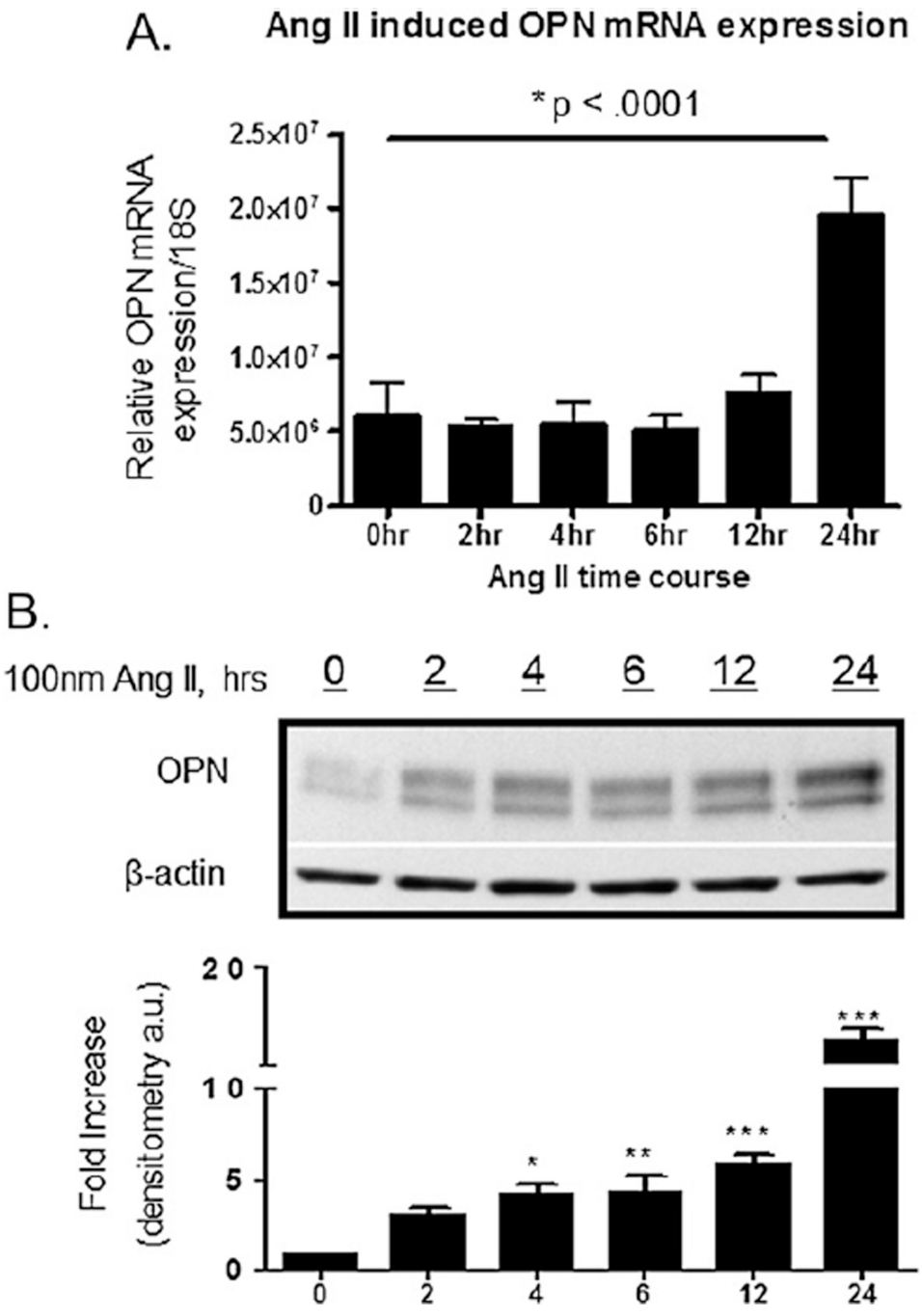

Fig. 1.

Ang II induces OPN protein expression. A. In VSMCs, Ang II increased OPN mRNA expression after 12 h, normalized to 10^6 copies of 18 s ($n = 6$ $p < 0.001$) B. In VSMCs Ang II increased OPN protein expression beginning 4 h after treatment by 420 ± 54% ($n = 3$ $p < 0.0001$) in cultured VSMCs and continuing through 24 h.

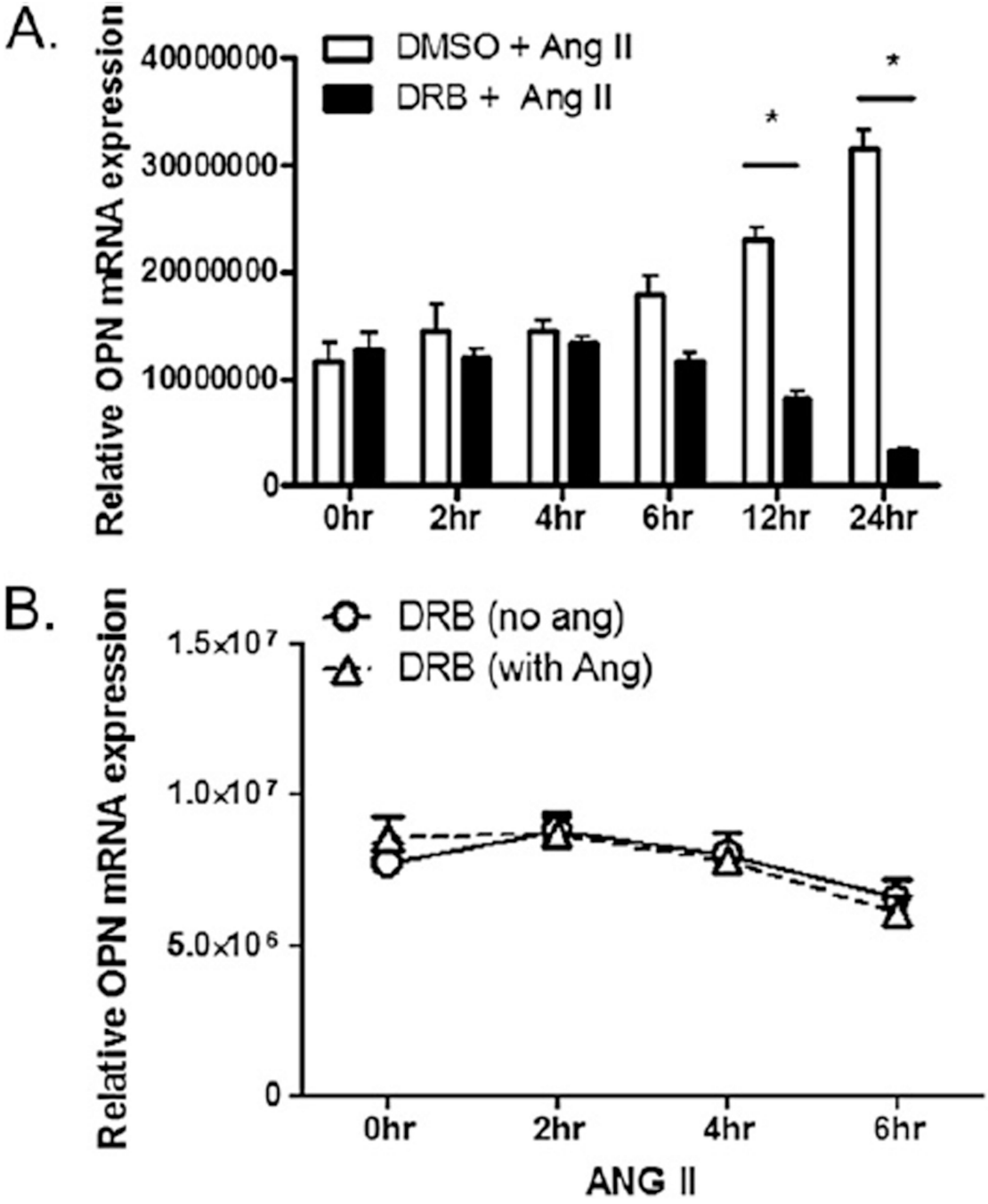

Fig. 2.

Transcriptional repression reveals a translation-dependent mechanism. A. Ang II did not increase OPN mRNA expression until after 6 h as compared to DMSO controls. ($n = 3$ $p < 0.05$) we observed baseline OPN mRNA expression that did not increase with Ang II until 12 h after treatment, normalized to 2 μg of RNA ($n = 3$ $p < 0.001$) B. Ang II did not further stabilize OPN mRNA before 6 h as shown by comparing DRB in the presence and absence of Ang II at the indicated time points.

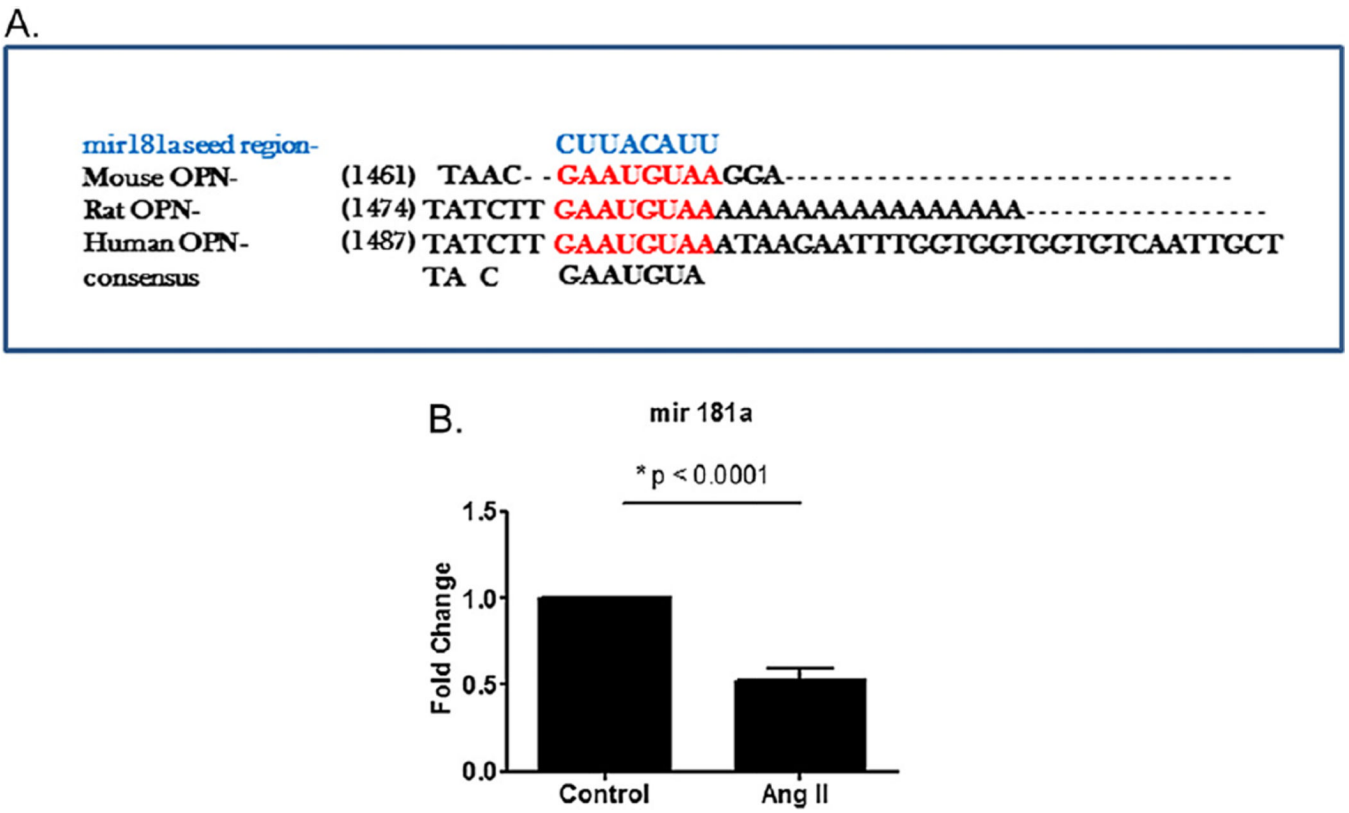

Fig. 3.

miR181a is predicted to bind the 3′UTR (untranslated region) of OPN and is regulated by Ang II. A. A putative miR181a binding site -GAAUGUA- showed evolutionary conservation within the 3′UTR of OPN in human, mouse and rat species. B. Ang II decreased miR181a expression by 52 ± 7% ($n = 6$ $p < 0.0001$) in VSMCs.

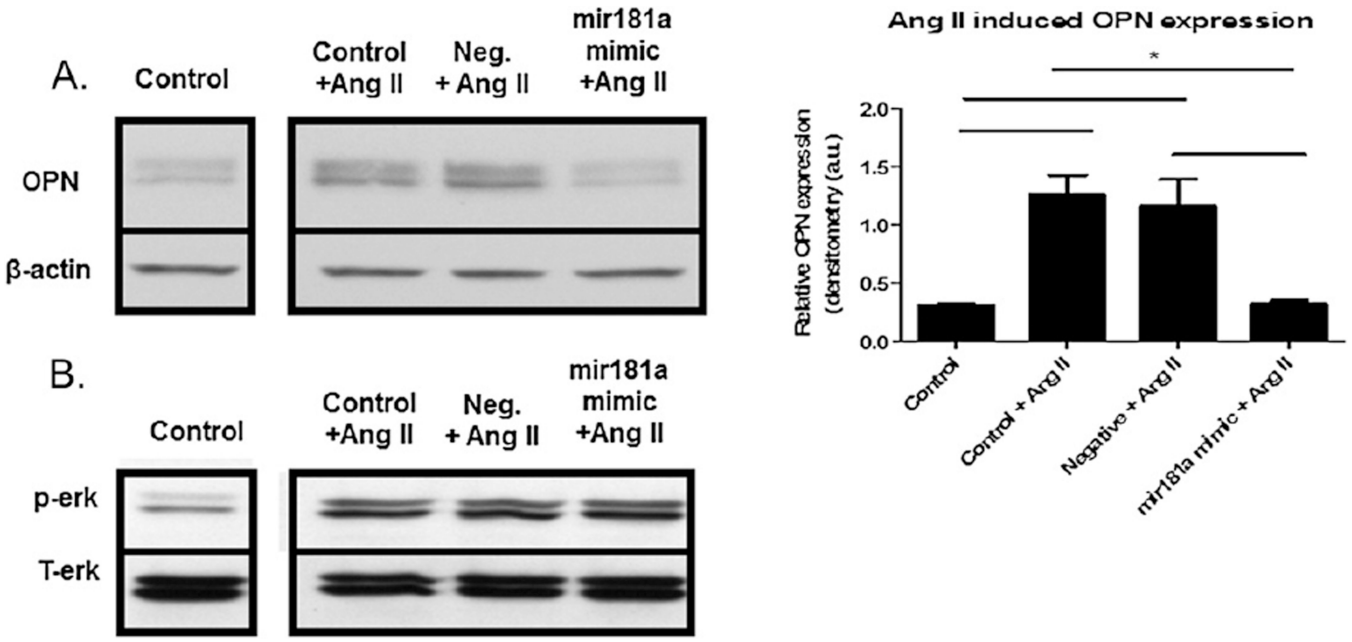

Fig. 4.

Overexpressing miR181a negatively regulates Ang II induced OPN expression. A. miR181a overexpression inhibited Ang II induced OPN expression at 4 h in VSMCs by 69 ± 9% (n = 3 $p < 0.001$) B. Ang II was still capable of downstream signaling in miRNA overexpressed cells as evidenced by increased phosphorylation of erk1/2–5 min after stimulation with Ang II.

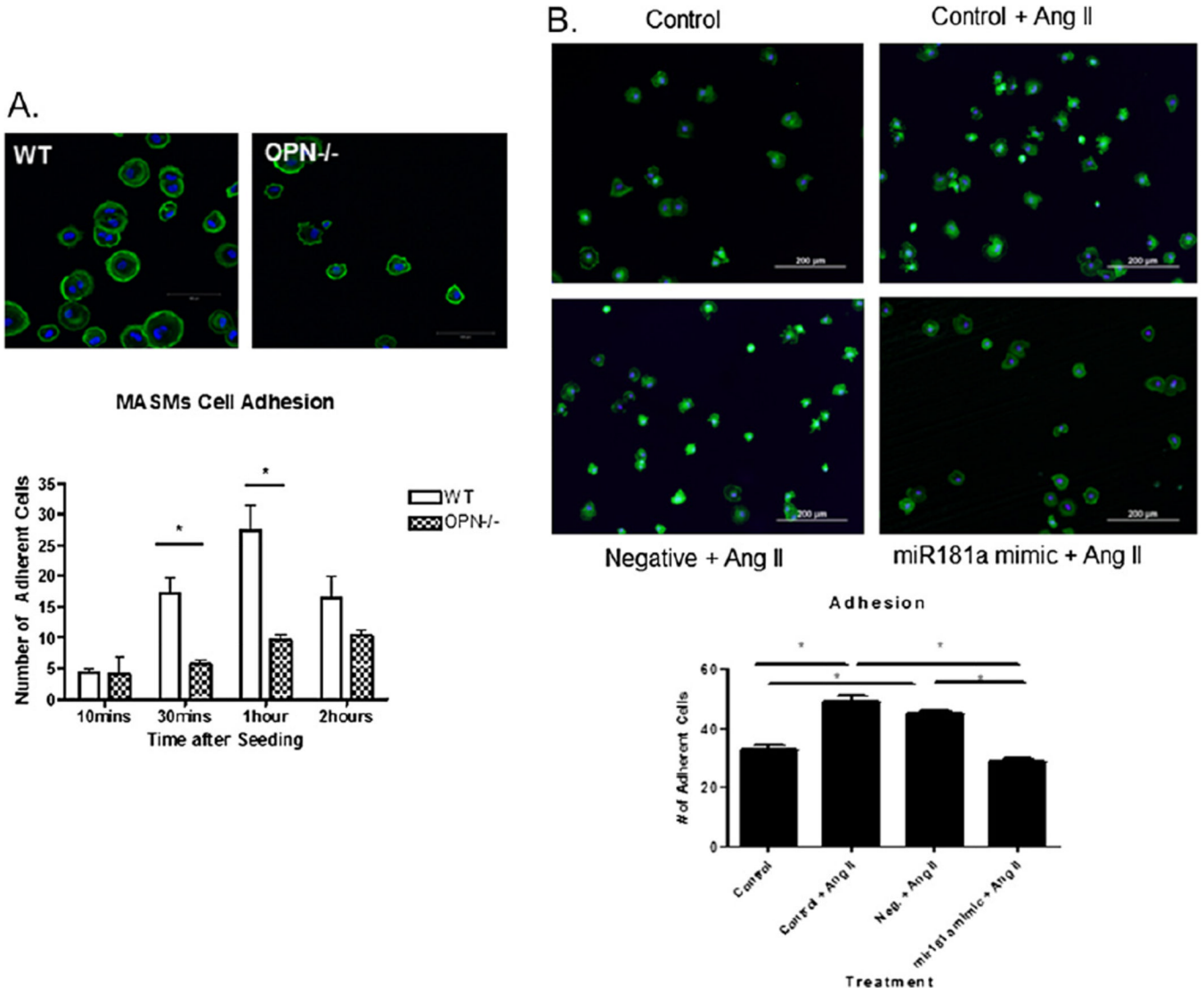

Fig. 5.

miR181a overexpression has a functional impact on VSMCs adhesion. A. OPN$^{-/-}$ MASMs exhibited decreased adhesion collagen coated coverslips as compared to WT controls at 30 min and 1 h ($n = 3$ $p < 0.05$ and $p < 0.001$ respectively. Images are representative images from 5 fields per experiments at 1hr. B. Ang II induced adhesion of VSMCs to type I collagen at 24 h (top panels). Ang II treated VSMCs with miR181a mimetic reduced adhesion of VSMCs to baseline levels (Bottom panels) ($n = 3$ $p < 0.05$). Green = Phalloidin Blue = DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)