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## \*Angiotensin converting enzyme-induced activation of local angiotensin signaling is required for ascending aortic aneurysms in fibulin-4 deficient mice

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### Abstract

Aortic aneurysms are life-threatening and often associated with defects in connective tissues and mutations in smooth muscle cell (SMC) contractile proteins. Despite recent advances in understanding altered signaling in aneurysms of Marfan syndrome, the underlying mechanisms and options for pharmacological treatment for other forms of aneurysms are still under investigation. We previously showed in mice that deficiency in the fibulin-4 gene in vascular SMCs (*Fbln4<sup>SMKO</sup>*) leads to loss of the SMC contractile phenotype, hyperproliferation and ascending aortic aneurysms. Here, we report that abnormal upregulation of angiotensin converting enzyme (ACE) in SMCs and subsequent activation of angiotensin II (AngII) signaling is involved in the onset of aortic aneurysms in *Fbln4<sup>SMKO</sup>* mice. In this model, aneurysm formation was completely prevented by inhibition of the AngII pathway with losartan or captopril within a narrow therapeutic window during the first month of life, even though the altered mechanical properties of blood vessel walls were not reversed by the pharmacological treatment. The

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therapeutic effects of losartan in *Fbln4<sup>SMKO</sup>* mice do not require the AngII receptor type 2 (Agtr2) but likely require both type 1a (Agtr1a) and 1b (Agtr1b) receptors. The results indicate that fibulin-4 is a vascular matrix component required for regulation of local angiotensin signaling, aortic aneurysms, and development and maintenance of the SMC phenotype.

## Introduction

Aortic aneurysms are associated with extrinsic or intrinsic defects of the blood vessel wall, including compromised vessel integrity and alterations of contractile proteins in vascular smooth muscle cells (SMCs) (reviewed in (1,2)). Dysregulation of TGF $\beta$  has been shown to be a central mechanism of the pathology in various forms of thoracic aortic aneurysms in humans (3-5), a mouse model of Marfan syndrome (*Fbn1<sup>C1039G/+</sup>*) and *Fbln4* hypomorphic mice (6,7). Inhibition of the angiotensin II (AngII) receptor type 1 (Agtr1) by angiotensin receptor blockade (ARB) was suggested to inhibit transcription of TGF $\beta$  in Marfan mice (8) or lower blood pressure in *Fbln4* hypomorphic mice (9), and was effective in attenuation of the aneurysm phenotype in both mouse models (8,9). However, a fundamental question remains whether the endogenous angiotensin system plays a causal role in aneurysm formation and whether ARB therapy can be applied to all congenital aortic aneurysms as a universal therapy.

Fibulin-4 localizes to microfibrils of elastic fibers and possesses multiple functions pertaining to vascular development, including binding to elastin and elastin cross-linking enzymes to assist in the formation of elastic fibers and maintenance of a SMC contractile phenotype (10-13). Mutations in *Fbln4* in humans cause cutis laxa with various systemic vascular defects, including arterial tortuosity, aneurysms and stenosis (14-16). We have previously shown that proliferation of SMCs precedes aneurysm formation, with a marked increase of phosphorylated (p-) ERK1/2 in the ascending aorta of mice that have a deletion of *Fbln4* specifically in vascular SMCs (termed *Fbln4<sup>SMKO</sup>*) (13), suggesting that soluble factor(s) may be involved in the initiation and/or progression of aneurysmal lesions. Here, we used the *Fbln4<sup>SMKO</sup>* mouse model to further explore mechanisms underlying aneurysm development associated with defects in vascular matrix proteins.

## Results

### ACE is upregulated in vascular SMCs of the aneurysmal wall in *Fbln4<sup>SMKO</sup>* mice

To search for signaling pathways involved in proliferative changes of SMCs with elevated p-ERK1/2 in aneurysms of *Fbln4<sup>SMKO</sup>* mice, we performed qPCR analyses on *Fbln4<sup>SMKO</sup>* aortas at 1 month of age. We found that transcripts of angiotensin converting enzyme (*Ace*) and endopeptidase (*Enpep*) were increased 2.5-3 times ( $P=0.0001$  and  $P=0.0008$  for *Ace* and *Enpep*, respectively) whereas renin (*Ren*) was decreased by half in the *Fbln4<sup>SMKO</sup>* ascending aorta ( $P=0.0039$ , Fig. 1A). *Ace* and *Enpep* were also upregulated in the descending aorta where aneurysms did not develop (Fig. S1). No differences in *Ace* expression between wild-type and mutants were detected in the lung, but a 1.5-fold increase in *Ace* expression was observed in the kidney of *Fbln4<sup>SMKO</sup>* mice (Fig. S2). Amounts of angiotensinogen (*Agt*), a precursor for angiotensin I and substrate for renin, were comparable between the liver of *Fbln4<sup>SMKO</sup>* and wild-type (Fig. S2). Consistent with qPCR results, high amounts of ACE were detected by immunostaining of aneurysmal wall SMCs in mutant aorta, whereas in the wild-type aorta, ACE staining was confined to endothelial cells (Fig. 1B). Upregulation of ACE was apparent in 14-day-old *Fbln4<sup>SMKO</sup>* aorta and it persisted throughout adult life (Fig. 1C, Fig. S3). ACE was higher in ascending aorta compared to descending aorta in wild-type and *Fbln4<sup>SMKO</sup>* mice (Fig. 1D). ACE expression in primary SMCs from *Fbln4<sup>SMKO</sup>* aortas, however, was comparable to that of SMCs from

wild type aortas, suggesting that loss of *Fbln4* was not sufficient to induce ACE in vitro (Fig. S4), and that additional mechanical or soluble factors from other cell types or disruption of cell-matrix connections may need to be present.

We asked if the local upregulation of ACE resulted in upregulation of Ang II in the mutant aorta. Ang II levels were increased 2-fold in *Fbln4<sup>SMKO</sup>* aorta relative to wild-type aorta (Fig. 1Ea), whereas in the kidney, Ang II levels were comparable between genotypes (Fig. 1Eb). This result also indicated that upregulation of Ace resulted in a net increase in local AngII levels despite upregulation of *Enpep*, which cleaves AngII to Ang III. Because we previously reported that the vessel wall of *Fbln4<sup>SMKO</sup>* aorta was thicker and contained immature SMCs (13), we performed proliferation assays in 1-month *Fbln4<sup>SMKO</sup>* aorta using bromodeoxyuridine (BrdU). In the mutant aorta, nearly 6% of cells were positive for BrdU compared to less than 1% in the wild-type aorta ( $P=0.0039$ , Figs. 1Fa-c). Vessel area was increased in *Fbln4<sup>SMKO</sup>* aorta compared to wild-type ( $P=0.0063$ , Fig. 1Fd), and cell number per vessel area ( $\text{mm}^2$ ) was comparable between genotypes (Fig. 1Fe), indicating that the increase in vessel area reflects an increase in cell number in the mutant aortic wall (Fig. 1Fd-e). These data suggest that local production of AngII due to increased ACE expression in SMCs causes upregulation of local AngII-mediated signaling (e.g., ERK1/2 (17)) leading to hyperproliferation of SMCs in the *Fbln4<sup>SMKO</sup>* ascending aorta.

### Losartan and captopril effectively prevent the aneurysm phenotype in *Fbln4<sup>SMKO</sup>* mice

To examine the possible cause-effect relationship of the AngII pathway and aneurysm formation in *Fbln4<sup>SMKO</sup>* mice, we performed pharmacological experiments with captopril (ACE inhibitor, 75 mg/l in drinking water ad libitum), losartan (ARB, 0.6 g/l ad libitum) and propranolol ( $\beta$ -adrenergic receptor blocker, 0.5 g/l ad libitum) as previously described (8). Each drug was administered from mid-gestation by giving it to pregnant females, and continued until 3 months of age, when all animals were sacrificed and analyzed. Large aneurysms observed in untreated *Fbln4<sup>SMKO</sup>* mice were completely prevented by either losartan or captopril, whereas propranolol showed only modest inhibitory effects on aneurysm formation (Fig. 2A). Similarly, internal elastic lamina (IEL) perimeter and total vessel area were completely normalized with losartan or captopril, whereas propranolol treatment only resulted in mild reduction of IEL perimeter and no effects on the total vessel area (Fig. 2B, Table S1). Histologically, hyperproliferation and disarray of SMCs in the aortic wall were also completely prevented by losartan or captopril (Fig. 2C). Morphologically, SMCs in these treated animals displayed a more normal spindle cell shape with flattened nuclei, and vessel wall thickness was decreased and similar to control. Although the amount of collagen was decreased, elastic fibers remained irregular, and numerous disruptions were observed even after drug treatment (arrows in Fig. 2C). These findings indicate that ligand-dependent activation of Agtr1 is responsible for hyperproliferation of SMCs, and inhibition of AngII signaling by ACE inhibitor or ARB is equivalent and sufficient to prevent aneurysms, but without complete reversal of elastic fiber defects in the aneurysmal wall.

### Postnatal days 7 to 30 are a critical time window for therapeutic intervention in mice

To determine the critical time window for the therapeutic effect of AngII inhibition, we used losartan to further examine the time course for prevention of the aneurysmal phenotype. We first examined early pathological changes in the *Fbln4<sup>SMKO</sup>* aorta at postnatal days (P)1, P7, and P14. The ascending aorta was macroscopically indistinguishable between mutant and wild-type at P1 and P7 (Fig. S5A, Fig. 3A), whereas slight dilatation was noted at P14 (arrows in Fig. 3A). The aorta remained histologically normal at P1 in the mutants (Fig. S5B), but cellularity increased by P7, especially in the subendothelial layer and near the adventitia (Fig. 3B, arrowhead). By P14, SMCs were morphologically abnormal, and the

proliferation of SMCs extended throughout the medial layer in the mutant aorta (Fig. 3B). These data indicated that the microscopic changes began around P7, and suggested that P7 may be a target time point for postnatal drug treatment.

We set up drug treatment protocols (Fig. 3C) in which losartan was administered from P7 to P90, P30 to P90, or P7 to P45, and the ascending aorta was evaluated at P90. Aneurysms were completely prevented if losartan was administered starting at P7, even if treatment stopped at P45 (Fig. 3C and 3D). In contrast, administration of losartan from P30 to P90 did not prevent aneurysm development. These data indicate that the optimal therapeutic window is between P7 and P30, and that transient inhibition of AngII signaling during the early postnatal period is sufficient to prevent aneurysm development at least until 90 days of age.

### Blood pressure and vessel mechanical properties are not completely normalized by losartan treatment in *Fbln4<sup>SMKO</sup>* mice

Hemodynamic changes occur during the transition from embryonic to adult circulation, and growth-related increases in blood pressure and body weight are prominent between P7 and P30 during mouse development (18). It is plausible that intrinsic defects in SMCs and the resultant altered response to increasing hemodynamic stress in the elevated AngII microenvironment trigger aneurysm formation in *Fbln4<sup>SMKO</sup>* mice. To gain insights into hemodynamic contributions to aneurysm formation, we measured blood pressure in untreated animals and those treated with losartan, propranolol or captopril from P7 to P43 ± 2 (Fig. 4A-D, Table S2). Average arterial systolic pressures were similar in untreated control (104 ± 1 (SEM) mmHg) and *Fbln4<sup>SMKO</sup>* mice (107 ± 3 (SEM) mmHg, Fig. 4A); however, there was a trend toward lower average diastolic pressures in *Fbln4<sup>SMKO</sup>* mice (Fig. 4B), and thus pulse pressures were increased 50% in untreated *Fbln4<sup>SMKO</sup>* mice compared to control ( $P=0.004$ , Fig. 4D). This marked increase in pulse pressure in the mutants was similar to what has been observed in *Fbln5*-null (19) and *Fbln4* hypomorphic mice (9), in which elastic laminae are severely disrupted. Captopril decreased systolic blood pressures about 20% in *Fbln4<sup>SMKO</sup>* mice (88 ± 3 (SEM) mmHg,  $n=5$ ,  $P=0.006$ , Fig. 4A) and eliminated the increase in pulse pressure observed in untreated *Fbln4<sup>SMKO</sup>* mice (Fig. 4D). Losartan did not affect systolic pressures in *Fbln4<sup>SMKO</sup>* mice (104 ± 2 (SEM) mmHg,  $n=7$ , Fig. 4A), thus pulse pressures remained higher in losartan-treated *Fbln4<sup>SMKO</sup>* mice. Propranolol showed no effects on blood pressures in control or *Fbln4<sup>SMKO</sup>* mice (Fig 4A-D). Because both captopril and losartan prevented the aneurysm phenotype, but only captopril had significant effects on systolic and pulse pressure in *Fbln4<sup>SMKO</sup>* mice, these data suggest that hemodynamic changes are not required to prevent aneurysms in *Fbln4<sup>SMKO</sup>* mice.

The mechanical stress on the vessel wall is determined both by hemodynamic forces, such as blood pressure, and the mechanical properties of the wall, which are illustrated by pressure-diameter and pressure-compliance relationships. Pressure-diameter curves showed large starting diameters in *Fbln4<sup>SMKO</sup>* aorta (~ 2-fold greater than control at zero pressure), indicating the presence of aneurysms in *Fbln4<sup>SMKO</sup>* ascending aorta. These diameters were significantly larger than control animals at all pressures tested ( $P < 0.001$ , Fig. 4E, Table S3). Compliance of the *Fbln4<sup>SMKO</sup>* ascending aorta, defined as the percent change in outer diameter with each 25 mmHg pressure step, was decreased 60-80% in the mid- to high pressure range (75-175 mmHg) compared to the control aorta ( $P < 0.001$ , Fig. 4F, Table S3), indicating that the vessel wall is stiffer, similar to elastin heterozygous mutants (20). Thus, the compliance or stiffness of *Fbln4<sup>SMKO</sup>* vessels is similar to that of other mouse models with mild to severe elastic fiber defects that do not develop aortic aneurysms, suggesting that mechanical changes alone are not sufficient to induce aneurysm formation.

We then examined whether drug treatment altered the mechanical properties of the ascending aortas. Pressure-diameter curves were almost identical in control vessels

regardless of drug treatment. Propranolol-treated *Fbln4<sup>SMKO</sup>* aortas showed starting diameters about 2-fold larger than control aortas, similar to untreated *Fbln4<sup>SMKO</sup>* animals, indicating the presence of aneurysms (Fig. 4E). Losartan or captopril-treated *Fbln4<sup>SMKO</sup>* aortas showed approximately a 20% increase in starting diameter compared to controls but were smaller than untreated and propranolol treated *Fbln4<sup>SMKO</sup>* aortas for every pressure tested ( $P < 0.001$ ) and did not have aneurysms. Compliance of losartan- or captopril-treated *Fbln4<sup>SMKO</sup>* aortas was between that of control and untreated or propranolol-treated *Fbln4<sup>SMKO</sup>* in the low pressure range (0-50 mmHg); however, all the mutant aortas regardless of drug treatment showed reduced compliance in the high-pressure range (100-175 mmHg) compared to controls ( $P < 0.001$ , Fig. 4F). These data indicate that increased stiffness of the vessel wall is not completely reversed pharmacologically. Combined with the blood pressure data, these results show that changes in mechanical stress on the vessel wall are not the primary mechanism for aneurysm prevention in losartan and captopril-treated *Fbln4<sup>SMKO</sup>* mice.

### Elevated p-ERK1/2 and decreased expression of SMC differentiation markers are reversed by losartan

Recently, it was shown in *Fbn1<sup>C1039G/+</sup>* mice that elevated p-ERK1/2 driven by non-canonical TGF $\beta$ -signaling is responsible for aneurysm development (6). We therefore examined the effect of inhibiting the AngII-Agtr1 pathway on p-ERK1/2 levels in aneurysm lesions of the *Fbln4<sup>SMKO</sup>* aorta. Strong upregulation of p-ERK1/2 was observed in untreated *Fbln4<sup>SMKO</sup>* aorta, whereas marked downregulation of p-ERK1/2 was found in prenatal or postnatal losartan-treated groups and the prenatal captopril-treated group (Fig. 5A). In contrast, propranolol treated- or delayed losartan-treated groups, in which modest or no preventative effects were detected, showed persistently high levels of p-ERK1/2. These data suggest that increased p-ERK1/2 driven by strong AngII-Agtr1 signaling is associated with the development of aneurysms in *Fbln4<sup>SMKO</sup>* mice (Fig. 5A).

To compare signaling pathways implicated in aneurysm formation in congenital aortic aneurysm models, we examined p-Smad2/3, p-Smad1/5/8 and p-ERK1/2 by Western blot analysis. Our previous studies failed to detect increased Smad2/3 signaling in the aneurysmal wall (13); however, improved preparation of the aortic samples by complete removal of periaortic adipose tissues enabled us to detect elevation of p-Smad2/3 in aneurysm lesions of *Fbln4<sup>SMKO</sup>* mice. Losartan treatment effectively suppressed p-ERK1/2 and p-Smad2/3 levels to those of wild-type aorta (Fig. 5B), but the change in p-Smad1/5/8 levels was less prominent. qPCR analysis revealed that expression of SMC contractile genes was dramatically increased by losartan treatment compared to the untreated group (Fig. 5C). Among all contractile genes, *Myocd*, a master regulator of SMC differentiation, and *Myh11*, a marker of terminal SMC differentiation, returned to normal levels with losartan treatment (Fig. 5C). MYH11 protein levels showed a trend for increase, although not statistically significant, in losartan-treated animals (Fig. 5D). ACE protein was minimally decreased in losartan-treated animals (Fig. 5B), consistent with ACE being upstream of the AngII-Agtr1 pathway.

### Genetic ablation of *Agtr1a* alone is insufficient for prevention of ascending aortic aneurysms in *Fbln4<sup>SMKO</sup>* mice

We used a genetic approach to examine whether Agtr1-mediated pathways are essential for aneurysm formation in *Fbln4<sup>SMKO</sup>* mice. AngII is known to bind two types of G-protein coupled receptors, Agtr1 and Agtr2. In mice, two subtypes of Agtr1, Agtr1a and Agtr1b, are generated by duplication of Agtr1. Since Agtr1a is the predominant receptor in adult tissues (21), we used mice null for *Agtr1a* (*Agtr1a<sup>-/-</sup>* termed *1aKO*) and crossed them with *Fbln4<sup>SMKO</sup>* mice to generate *Agtr1a<sup>-/-</sup>;Fbln4<sup>SMKO</sup>* (termed *1aDKO*), then examined

aneurysm formation at 3 months compared with their littermates. Morphology of SMCs and elastic fibers in *1aDKO* aorta was comparable to that of *1aKO* aorta; however, the aortic wall was thicker in *1aDKO* mice compared to *1aKO* (Fig. 6A). Morphometric analysis showed that although 2 out of 10 *1aDKO* aortas had large IEL perimeters ( $> 0.6 \text{ mm}^2$ ), average IEL perimeter was improved compared to *Fbln4<sup>SMKO</sup>* animals (Fig. 6B). Nevertheless, the total vessel areas of *1aDKO* aortas were larger than control or *1aKO*. This result was in stark contrast to losartan-treated animals in which aneurysm formation was prevented completely (Fig. 2A), suggesting that Agtr1b may also contribute to aneurysm formation in *1aDKO* mice. This suggestion is supported by persistent elevation of p-ERK1/2 in the *1aDKO* aorta (Fig. 6A). These results indicate that the therapeutic effect of losartan was achieved by blocking both Agtr1a and Agtr1b.

### **Agtr2 is not required for losartan-mediated prevention of ascending aortic aneurysms in *Fbln4<sup>SMKO</sup>* mice**

In contrast to the defined intracellular signaling pathway and biological functions of Agtr1 in vivo, the role of Agtr2 has been elusive (22). Recently, deletion of *Agtr2* in *Fbn1<sup>C1039G</sup>* mice was shown to exacerbate aortic root enlargement and losartan was ineffective at preventing aneurysm formation in *Fbn1<sup>C1039G</sup>; Agtr2*-null mice, suggesting that Agtr2 functions as a protective receptor and acts antagonistically to Agtr1 in vivo (23). We therefore asked if Agtr2 is required for the therapeutic effect of losartan by rendering the AngII signal to exert protective effects through Agtr2 on SMCs in *Fbln4<sup>SMKO</sup>* mice. Deletion of *Agtr2* (*Agtr2<sup>Y/-</sup>*; termed as *2KO*) in *Fbln4<sup>SMKO</sup>* mice (*Fbln4<sup>SMKO</sup>; Agtr2<sup>Y/-</sup>*; termed *2DKO*) did not affect aneurysm formation (Fig. 6C). The media of the aortic wall was much thicker in the double mutants than in *2KO*, with hyperproliferation and disarray of SMCs observed at 3 months of age (Fig. 6C). Administration of losartan completely prevented the aneurysm phenotype in *2DKO* mice (Figs. 6C, D) and p-ERK1/2 concentrations were much lower in the losartan-treated group than in the untreated *2DKO* group (Fig. 6C), indicating that the therapeutic effect of losartan does not require the action of Agtr2 on SMCs. These results suggest that Agtr2 is a by-stander receptor in the process of aneurysm formation, and the protective effect of AngII-Agtr1 inhibition is independent of Agtr2 action in *Fbln4<sup>SMKO</sup>* mice.

## **Discussion**

In this study, we have shown that loss of one ECM protein produced by vascular SMCs is sufficient to drive upregulation of ACE in the aortic wall of *Fbln4<sup>SMKO</sup>* mice. Because angiotensinogen expression in the liver and ACE expression in the lung were comparable between controls and *Fbln4<sup>SMKO</sup>* mice and AngII concentration in the aorta was increased in *Fbln4<sup>SMKO</sup>* mice, it is likely that local conversion of AngI to AngII contributes to the cellular changes in the mutant aorta. This is consistent with the previous report showing that local ACE serves as a key rate-limiting enzyme for AngII-mediated vascular hypertrophy in the rat carotid artery overexpressing the *Ace* gene (24). It is noteworthy that *Fbln4<sup>SMKO</sup>* aorta shows marked proliferation and disarray of SMCs. We speculate that the absence of intact elastic laminae and thus loss of physical barriers within the vessel wall further facilitate migration of SMCs across the lamellar unit and expansion of immature SMCs in the *Fbln4<sup>SMKO</sup>* aorta.

Upregulation of ACE is associated with various pathological conditions, including hypertension, atherosclerosis and diabetic nephropathy (reviewed in (25,26)). Although the endothelium is a prominent source of ACE in the vasculature, ACE has also been shown to be upregulated in SMCs of diseased vessels in humans and animal models, such as neointima induced by wire denudation injury, percutaneous transluminal coronary

angioplasty (27,28), and small pulmonary arteries in hypoxia-induced pulmonary hypertension (29). Interestingly, SMCs explanted from thoracic aneurysms of patients carrying *MYH11* mutations show high levels of *ACE* transcripts (30). The exact mechanism of *ACE* upregulation in SMCs has not been completely understood; however, soluble factors such as bFGF and endothelin were suggested to mediate upregulation of *ACE* (31,32). Mechanical forces such as shear stress and pulsatile pressure, as well as hypoxia, can also increase expression of *ACE* in SMCs (33,34). Our study provides evidence that loss of fibulin-4 in the context of vessel wall is sufficient for upregulation of *ACE* in SMCs in vivo. The resulting activation of local AngII signaling caused ascending aortic aneurysms in *Fbln4<sup>SMKO</sup>* mice, as evidenced by the complete prevention of aneurysm formation through perturbation of this pathway either at the level of AngII production or receptor activation. The results also suggest that fibulin-4 is required to prevent overactivation of local AngII-induced pathways that result in loss of the SMC phenotype but may be largely overcome by early interruption of AngII signaling.

Our study allowed us to determine the critical therapeutic time window for prevention of aneurysm development in vivo. Inhibition of AngII-mediated pathway by losartan effectively suppressed local proliferative changes in SMCs if treatment was initiated at P7, and these effects persisted even after withdrawal of losartan at P45. In contrast, after P30, mutant SMCs did not respond to losartan and upregulation of p-ERK1/2 continued. Thus, a critical therapeutic time window is between P7 and P30, and proliferation of mutant SMCs is dependent on AngII signaling during this period. The mechanism underlying the irreversible change in SMCs after P30 was not examined in this study; however, it is possible that AngII activates alternative signaling pathways that independently sustain the abnormal SMC phenotype in *Fbln4<sup>SMKO</sup>* aorta and/or vessel microenvironment containing high AngII may induce chromatin modification to alter the response to losartan and/or suppress SMC differentiation markers.

Results from genetic studies with angiotensin II receptor-deficient mice demonstrated that inhibition of *Agtr1a* alone is not sufficient to achieve complete prevention of aneurysms in *Fbln4<sup>SMKO</sup>* mice. The fact that losartan completely prevented aneurysmal phenotypes indicated that *Agtr1b* also mediates pathological signals in the *Fbln4<sup>SMKO</sup>* aorta. *Agtr1a* is the dominant receptor in adult tissues in mice, and *Agtr1b* expression is limited to a few organs, including adrenal glands, testis and brain (21). Upregulation of *Agtr1b*, however, was shown to be responsible for augmenting glomerular lesions in the absence of *Atgr1a* in murine autoimmune nephritis (35). Therefore, it is possible that AngII-mediated signaling through *Agtr1b* participates in the development of aneurysms in *1aDKO* aorta and that losartan effectively prevents the aneurysm formation by blocking both *Agtr1a* and *Agtr1b* in *Fbln4<sup>SMKO</sup>* mice. Generation of triple knockout mice for *Agtr1a*, *Agtr1b*, and *Fbln4* in a SMC specific manner will provide information on the role of *Agtr1* receptors in the development of aneurysms in *SMKO* mice. Our results also show that *Agtr2* is not required for mediating the protective action of losartan in *Fbln4<sup>SMKO</sup>* mice, unlike its critical role described in *Fbn1<sup>C1039G</sup>* mice (23). Prevention of the aneurysm phenotype by captopril also confirms that blocking AngII signals mediated by both *Agtr1* and *Agtr2* is an effective strategy in prevention of aneurysm formation in *Fbln4<sup>SMKO</sup>* mice and provides evidence against an antagonistic interaction between *Atgr1* and *Agtr2* in the development of aortic aneurysms in *Fbln4<sup>SMKO</sup>* mice. This finding is of particular importance in designing a strategy to stimulate *Agtr2* as a potential treatment for cardiovascular diseases (36). Recently, direct stimulation of *Agtr2* by a non-peptide agonist Compound 21 (C21) was shown to reduce arterial stiffness independent of blood pressure in rats treated with nitric oxide synthase inhibitor (37). C21-mediated beneficial effects were also reported on scar size and cardiac function in rat myocardial infarction (MI) model (38), as well as on perivascular fibrotic changes in stroke-prone rats (39), while another study in a mouse MI

model did not support a protective effect of Agtr2 stimulation (40). Our animal model will be useful to determine the effect of Agtr2 agonists in aneurysm progression in vivo.

A protective association between ACE inhibitors and risk of aneurysm rupture was reported in a large population-based case-control study in abdominal aortic aneurysm patients, in which an ACE inhibitor, but not ARB or other anti-hypertensive reagents, decreased the risk of aneurysm rupture independent of lowering blood pressures (41). In the study targeted to Marfan patients, an ACE inhibitor reduced arterial stiffness and aortic root diameters compared to placebo-treated group (42) or  $\beta$  blocker (43). The clinical trials with a larger cohort are currently underway to test the effects of ARB on Marfan patients (44,45). Our in vivo results provide further support for the idea that inhibition of AngII-Agtr pathway by ACE inhibitors or ARBs are equally effective choices for the treatment of ascending aortic aneurysms during the early postnatal period in human ascending aortic aneurysm patients and warrant further investigation.

By combining hemodynamic and mechanical studies with pharmacological experiments, we were able to assess the relationship between mechanical stress on the vessel wall and aneurysm phenotypes. Although our data indicate that loss of integrity of the vessel wall is coupled with cellular changes in SMCs, the aneurysm phenotype can be prevented without completely reversing increased pulse pressures (for losartan-treated animals) or the altered mechanical properties of the aorta, including decreased compliance and increased stiffness. The absence of pressure changes in *Fbln4<sup>SMKO</sup>* mice with losartan treatment implies that signaling through Agtr2 is sufficient to maintain blood pressure in these mice and provides further evidence against the complete dependence on pressure changes for the aneurysm prevention. Our results show that aneurysm in our model is a disease affecting SMC phenotypes and ECM-cell interactions, rather than a condition resulting solely from increased hemodynamic forces and/or mechanical defects of the vessel wall, and that intervention to alter SMC phenotypes by captopril or losartan leads to effective prevention of the aneurysm formation.

Although fibulin-4 is expressed in both ascending and descending aorta, the aneurysms resulting from a lack of this protein are confined to the ascending aorta, suggesting that SMCs in this region may possess unique characteristics that confer susceptibility to external growth signals in addition to the direct influence of outflow pressure. One possibility is that SMCs with a diverse embryonic origin may contribute to the differential response to AngII signals in the vessel wall. SMCs are known to be derived from the neural crest in the ascending aorta (46) and are distinct from SMCs in the descending/ abdominal aorta derived from the somite (47). Our data indicating that ACE expression is higher in the ascending aorta than descending aorta support this possibility. Regional differences in ACE activity were also reported in chick embryos, demonstrating significantly increased ACE activity in the ascending aorta compared to the abdominal aorta (48). Furthermore, endothelial cells incubated with conditioned media harvested from neural crest-derived SMCs exhibited a marked increase in ACE activity compared with those from mesenchyme-derived SMCs. Therefore, basal ACE activity may be regulated in a cell lineage-dependent manner and may contribute to the regional difference in formation of aortic aneurysms.

In summary, our results highlight the AngII pathway as a cause of ascending aortic aneurysms in *Fbln4<sup>SMKO</sup>* mice and provide a basis for the effective prevention of aneurysms with postnatal administration of ACE inhibitor and/or ARB. Our study underscores differences in the pathological basis of aortic aneurysms and suggests that therapeutic regimens need to be tailored according to underlying disease mechanisms in human patients. The limitations of this study include a lack of observations of the aneurysm phenotype over an extended period of time after early losartan treatment, which would have enabled us to



evaluate the long-term effects of losartan on aneurysm prevention in *Fbln4<sup>SMKO</sup>* mice. We recognize that our mouse model is a cell-type specific inactivation of *Fbln4*, whereas reported human patients have systemic inactivation of *FBLN4* (14-16) and, as expected, symptoms are more severe than the present mouse model. Thus, additional information regarding tissue/serum levels of fibulin-4 in a large cohort of aortic aneurysms and the correlation with clinical features and severity of the disease will be valuable. In addition, results from this mouse model of aneurysm development indicate that further investigations regarding intracellular signaling pathways downstream of AngII-Agtr that are responsible for induction of proliferative changes in SMCs and identification of specific SMC populations activated by Ang II signals are needed.

## Materials and Methods

### Mice

*Fbln4<sup>SMKO</sup>* (*Fbln4<sup>fl/KO</sup>* containing SM22 $\alpha$ -Cre transgene) and *Agtr2* knockout mice were generated and described previously (13) (49). *Agtr1a* knockout mice were purchased from Jackson Laboratory (B6.129P2-*Agtr1atm1Unc/J*). Comparisons of the phenotype between animals were performed on the same genetic background, and wild-type littermates containing *Cre* transgenes were used as controls (Tables S1-S3). Both female and male mice were used in this study. All mice were kept on a 12 h/12 h light/dark cycle under specific pathogen free conditions, and all animal protocols were approved by the IACUC of the University of Texas Southwestern Medical Center.

### Histology and immunohistochemistry

Aortas were harvested and perfusion fixed in 4% paraformaldehyde and embedded in paraffin. Five- $\mu$ m sections were stained with Hematoxylin & Eosin (H&E) for routine histology and Masson-Trichrome and Hart's staining for detection of collagen fibers and elastic fibers, respectively. Immunostaining was performed with antibodies for ACE (1:100, Santa Cruz) and p-ERK1/2 (1:100, Cell Signaling) as previously described (13). Minimum of 4 animals were analyzed in each experiment.

### Western blot analysis

Aortas were harvested and perivascular adipose tissues were thoroughly removed. Wild-type aortas (n=2-6) and *Fbln4<sup>SMKO</sup>* aortas (n=1-4) were pooled in each experiment and minced in 2x lammelli buffer or RIPA buffer containing 1% protease inhibitor (Sigma) and 1% phosphatase inhibitor (Sigma), then boiled at 95°C for 10 min, and subjected to SDS-PAGE. SMCs were prepared from P45 wild-type and *Fbln4<sup>SMKO</sup>* aortas as previously described (13) and passage number 5 was used for protein extraction. Proteins were transferred to a Western PVDF membrane (Millipore, Inc.) and immunoblotted with antibodies to phosphorylated ERK1/2 (1:1000, Cell Signaling), ACE (1:1000, Santa Cruz), or GAPDH (1:3000, Cell Signaling). Membranes were then incubated with anti-mouse or anti-rabbit HRP-conjugated secondary antibody, as appropriate (1:3000, Bio-Rad) and visualized with a chemoluminescence kit (Santa Cruz Biotechnology, Inc.). Quantification of Western blots was performed with Scion NIH IMAGE Software (National Institutes of Health).

### Quantitative PCR

RNA was isolated from aortas at the indicated ages with an RNeasy Plus Micro Kit (QIAGEN) and 1  $\mu$ g of total RNA was subjected to reverse transcription reactions with superscript III reverse transcriptase (Invitrogen). SYBR Green was used for amplicon detection, and gene expression was normalized to the expression of housekeeping genes  $\beta$ 2-microglobulin ( $\beta$ 2M) and GAPDH. PCR reactions were carried out in triplicate in a CFX96

real-time PCR detection system (Bio-Rad) with one cycle of 3 min at 95°C, then 40 cycles of 10 sec at 95°C and 30 sec at 60°C. mRNA concentrations were determined with the ddCt method and expressed relative to WT controls. Wild-type aortas (n=4-6) and *Fbln4<sup>SMKO</sup>* aortas (n=1-3) were pooled and analyzed in each experiment. Primer sequences are provided in Table S4.

### **BrdU proliferation assay**

Wild-type (n=4) and *Fbln4<sup>SMKO</sup>* (n=4) mice at 1 month of age were given 100 mg/kg 5-bromo-2'-deoxyuridine (BrdU) by IP injection once per day on four consecutive days. Aortas were harvested 1 hr after the final BrdU injection and embedded in OCT (Tissue-Tek). Tissue sections (10 µm thick) were fixed in 4% paraformaldehyde for 10 min and treated with 2N HCl containing 0.5% Triton-X-100 for 1 hr, followed by TBS pH 8.4 for 5 min to neutralize HCl. Sections were blocked for 30 min and incubated with rat anti-mouse BrdU antibody (Abcam, 1:100) overnight followed by Alexa 488-conjugated donkey anti-rat antibody (Invitrogen, 1:200) for 1 hr. Sections were mounted with DAPI and photographed using a Zeiss Axio Observer.Z1 microscope and Axiovision software. BrdU+ nuclei and total nuclei were counted in 4 high-powered fields (40x) for each tissue sample and expressed as a percentage of BrdU+ cells. Vessel areas were measured using ImageJ software (NIH).

### **Tissue AngII measurement**

Aortas from 3-month old wild-type (n=16) and *Fbln4<sup>SMKO</sup>* mice (n=14) were pooled and processed for tissue extraction. Kidneys from 3-4 month old wild-type (n=6) and *Fbln4<sup>SMKO</sup>* (n=6) mice were individually processed. Tissues were boiled in 2 ml of water for 20 min to deactivate endogenous peptidases. The solution was then adjusted to 1N CH<sub>3</sub>COOH and homogenized. After centrifugation (2,600 g × 40 min), the supernatant was loaded onto OASIS HLB Extraction Cartridges (Waters), washed with water/0.1% TFA, and eluted with 100% CH<sub>3</sub>CN/0.1% TFA. The eluates were lyophilized and dissolved in the EIA buffer of the Angiotensin II ELISA kit (SPI-BIO). Angiotensin II levels were measured in duplicate according to the manufacturer's instructions.

### **Pharmacological experiments**

Losartan (0.6 g/l in drinking water, ad libitum, provided by Merck Inc.), captopril (75 mg/l in drinking water, ad libitum, Sigma-Aldrich) and propranolol (0.5 g/l in drinking water, ad libitum, Sigma-Aldrich) were administered during the indicated periods. The numbers of animals used in each group are provided in each graph. For prenatal treatment, drugs were administered to the pregnant mother and then given to the mother through the period of lactation until weaning.

### **Morphometric analysis**

Cross sections of the aorta were stained with Hart's stain, and images were digitally captured with a Leica DM2000 microscope. Morphometric analysis was performed with Scion NIH IMAGE Software (National Institutes of Health). At least three sections were taken from different levels of ascending and descending aorta, and measurements were done three times per section by a blinded examiner.

### **Blood pressure measurement**

The mice used for this experiment were all male, except for one female included for losartan treatment both in control and *Fbln4<sup>SMKO</sup>* groups (6-8 weeks). For drug-treated animals, losartan (LSRT), captopril (CPT), or propranolol (PPL) was administered from P7 to P43 ± 2, then the drugs were removed and animals were acclimated for 5 days before blood

pressure measurement. The animals were anesthetized with 1.5% isoflurane, and body temperature was maintained with a feedback controlled heating pad. Arterial pressure was measured with a catheter (1.2 F, Scisense, Inc.) inserted in the ascending aorta through the right common carotid artery.

### Mechanical testing

Mechanical testing was performed on the ascending aorta with a pressure myograph (Danish Myotechnology), as previously described (18). Briefly, each aorta was mounted in the test system in physiologic saline at 37 °C, stretched to its approximate in vivo length and preconditioned. Each aorta was then pressurized for three cycles from 0 – 175 mmHg in steps of 25 mmHg with 12 sec/step. Compliance was defined as the percent change in outer diameter with each pressure step.

### Statistical analysis

One-way ANOVA with the Bonferroni post hoc test was used for comparison of drug effects (Figs. 2B, 3D, 4, 5C) and *Agtr1a* allelic difference (Fig. 6B) on aneurysm phenotypes, and two-tailed Student *t*-test was used for the rest of the statistical analysis. Bars are mean ± SEM and  $P < 0.05$  was considered significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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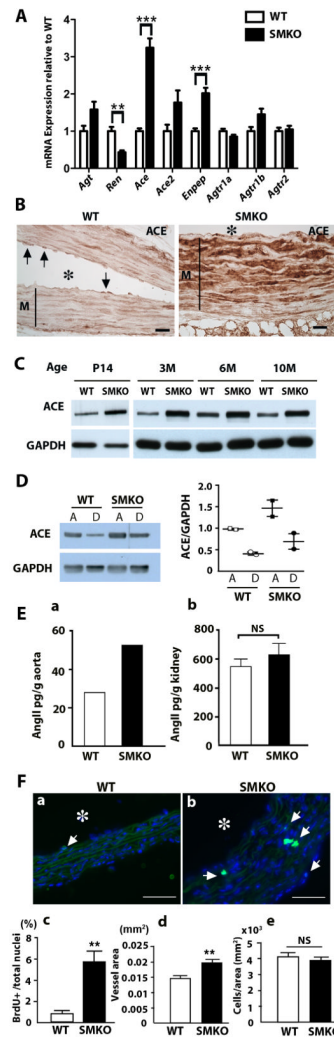
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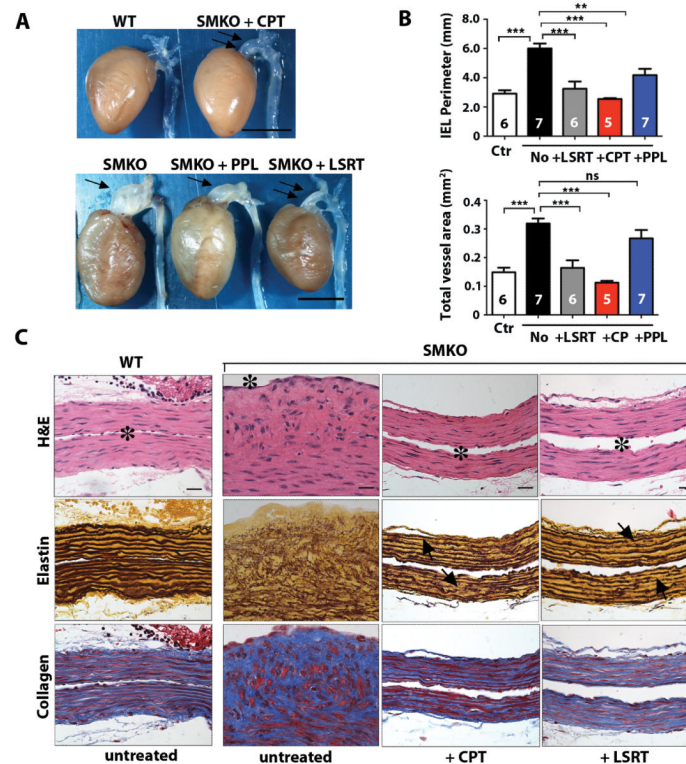
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**One Sentence Summary:** Upregulation of angiotensin converting enzyme (ACE) in the aortic wall causes aneurysms in fibulin-4 deficient mice, and the aneurysm phenotype can be prevented by losartan or captopril without reversal of mechanical properties in the absence of angiotensin II receptor type 2 (Agtr2).

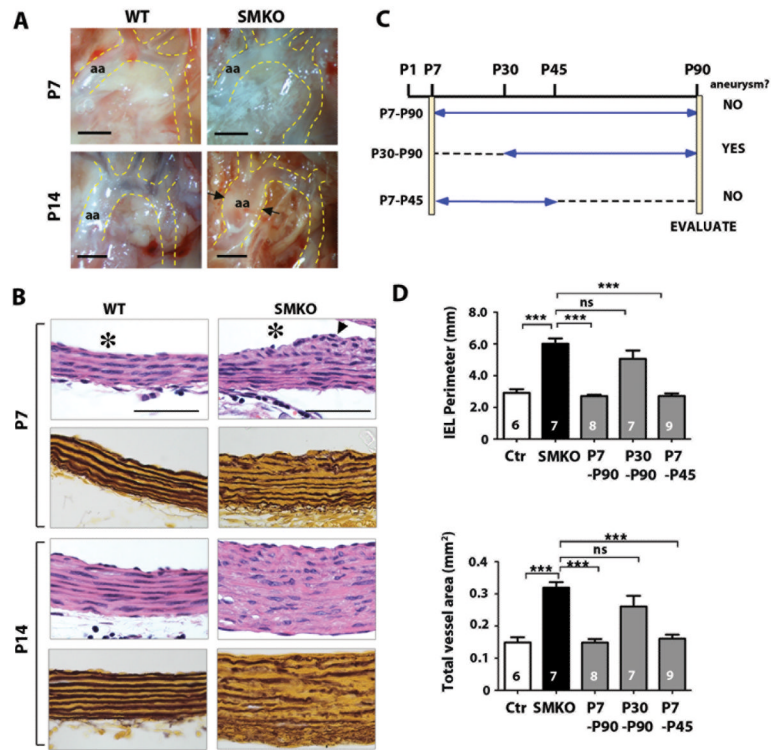


**Fig. 1.** Upregulation of ACE in the ascending aorta of *Fbln4<sup>SMKO</sup>* (SMKO) mice. **(A)** qPCR analysis of mRNA from aortas of wild-type (WT, n=4) and SMKO (n=4) mice at 1 month of age. Two-tailed Student *t*-test. Bars are mean  $\pm$  SEM. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  **(B)** ACE immunostaining of the aorta from WT and SMKO mice at 3 months of age. Arrows indicate ACE expressed in endothelial cells in the WT aorta. Asterisk indicates lumen and M indicates medial layer. Bar is 20  $\mu$ m. **(C)** Western blot showing ACE upregulation in the mutant mice throughout postnatal and adult life. **(D)** Western blot showing ACE expression in the ascending (A) and descending (D) aortas of WT and SMKO mice (1-3 month). Experiment was repeated with a different set of animals and quantified (right). **(E)** Tissue Ang II concentration determined by enzyme immunoassay. **a**, Entire thoracic aortas from WT (n=16) and SMKO (n=14) mice were pooled and analyzed. **b**, Kidneys from WT (n=6) and SMKO (n=6) mice were separately analyzed. Two-tailed Student *t*-test. Bars are mean  $\pm$  SEM. NS: not significant. **(F)** BrdU proliferation assay. **a-b**, Cross sections of WT (a, n=4) and SMKO (b, n=4) aortas were examined. Arrows indicate BrdU-positive cells. Asterisk indicates lumen. Bar is 50  $\mu$ m. **c**, Percentage of BrdU+ cells per total nuclei. **d-e**, Comparison of vessel area (d) and cell numbers per vessel area (e). Two-tailed Student *t*-test. Bars are mean  $\pm$  SEM. \*\*  $P < 0.01$ .

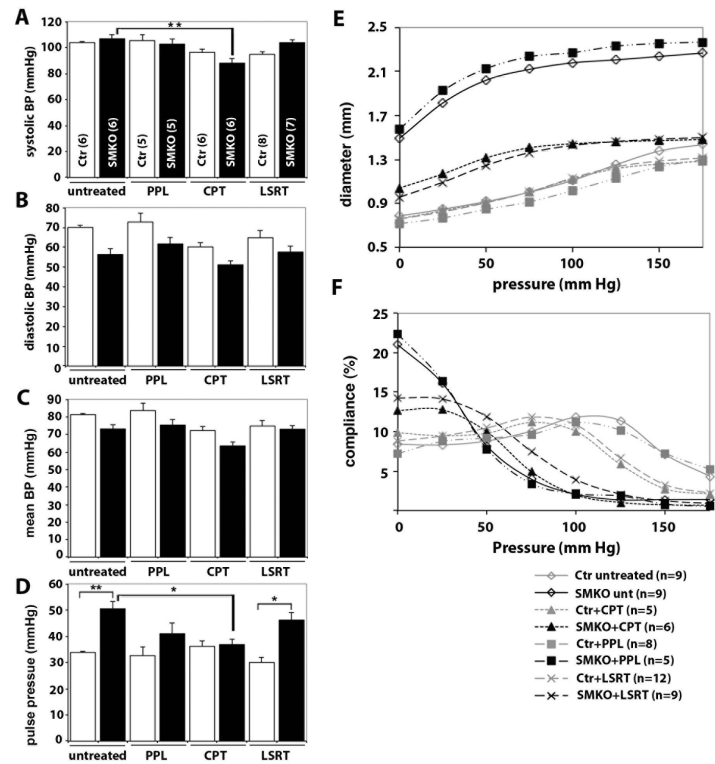




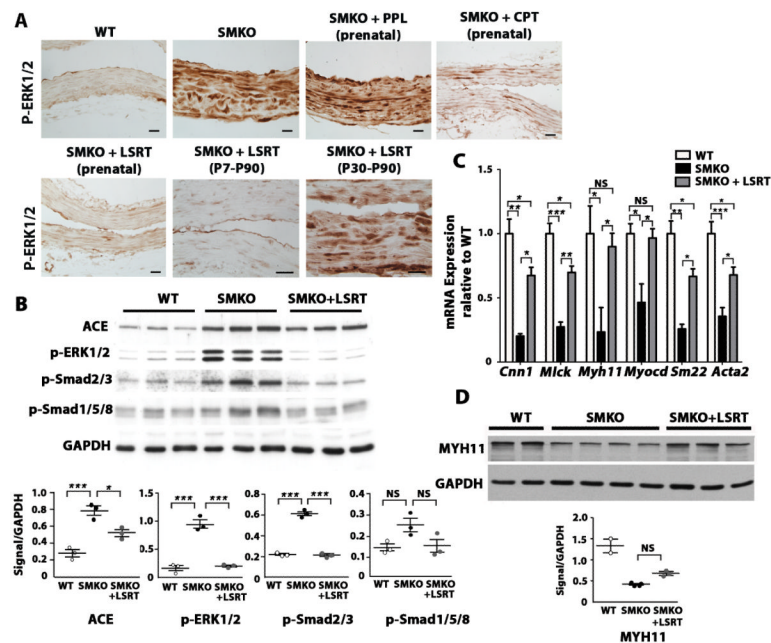
**Fig. 2.** Pharmacological prevention of aneurysms in *SMKO* mice. **(A)** Gross morphology of the ascending aorta in WT, untreated *SMKO*, and *SMKO* mice prenatally treated with captopril (CPT), propranolol (PPL) or losartan (LSRT). A large aneurysm (single arrows) is detected in *SMKO* and PPL-treated *SMKO* aorta. CPT or LSRT treatment prevents aneurysm formation (double arrows). Bars are 5 mm. **(B)** Morphometric analysis of cross sections of the ascending aorta from control (Ctr), untreated, LSRT, CPT or PPL-treated *SMKO* mice. Internal elastic lamina (IEL) perimeter and total vessel area are shown. One-way ANOVA. Bars are mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*\*  $P < 0.001$ . Number of animals is indicated on each bar. **(C)** Histological images of the ascending aorta from WT, untreated *SMKO*, CPT or LSRT-treated *SMKO* mice stained with H&E, Hart's stain (for elastin) and Masson-Trichrome stain (for collagen). Lumen is indicated by asterisk. Fuzzy appearance and disruption of elastic fibers is detectable even after CPT or LSRT treatment (arrows). Bars are 20  $\mu$ m.



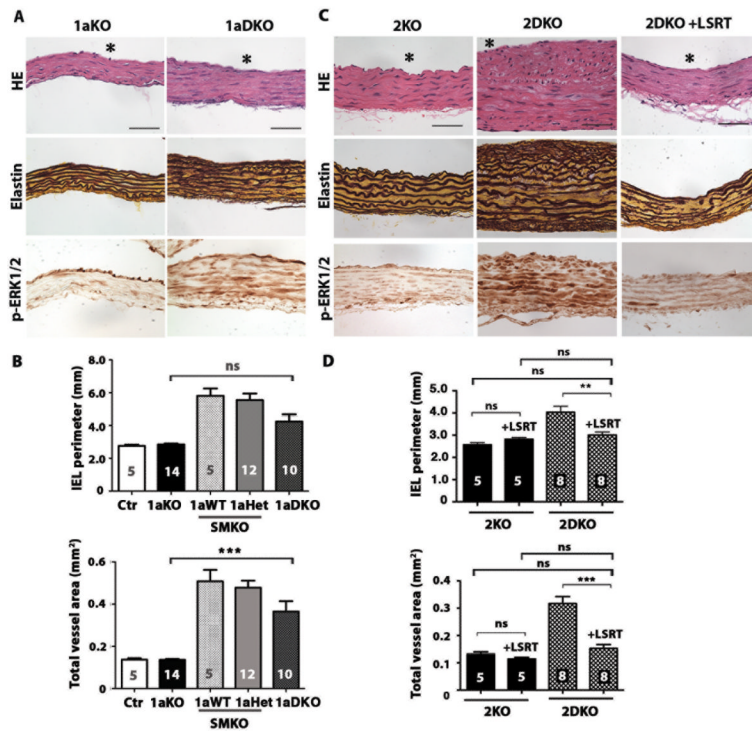
**Fig. 3.** Determining a critical time window for therapeutic intervention. **(A)** Macroscopic changes in the *SMKO* ascending aorta at early neonatal stages. Arrows indicate aneurysm. aa; ascending aorta. Bars are 1 mm. **(B)** Cross sections of ascending aorta at P7 and P14 stained with H&E and Hart's (elastin). Bars are 50 μm. Lumen is indicated by asterisk. **(C)** Postnatal losartan administration protocol. All animals were evaluated at 3 months of age. **(D)** Morphometric analysis of aortas from *SMKO* mice treated with LSRT at various time points according to the above protocol. One-way ANOVA. Bars are mean ± SEM. \*\*\*  $P < 0.001$ .



**Fig. 4.** Blood pressure and vessel biomechanics in *SMKO* mice. (**A-D**) Average systolic blood pressures (BP), diastolic blood pressures, mean blood pressures and pulse pressures in untreated or propranolol (PPL), captopril (CPT), or losartan (LSRT)-treated animals. Control (Ctr, white bars) and *SMKO* (black bars) are shown. Number of animals tested is indicated in parentheses. One-way ANOVA. Bars are mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.005$ . (**E**) Pressure-diameter curves for the ascending aorta in control (gray symbols) and *SMKO* (black symbols) untreated and drug-treated animals. (**F**) Pressure-compliance curves for the ascending aorta in control (gray symbols) and *SMKO* (black symbols) untreated and drug-treated animals. In E and F, oneway ANOVA. Error bars are not included for clarity.



**Fig. 5.** The effects of AngII-Agtr1 inhibition on SMCs in aneurysmal lesions. **(A)** Changes in p-ERK1/2 concentration in aneurysmal lesions after pharmacological intervention for the indicated time periods. Bars are 20  $\mu$ m. **(B)** Western blot showing the effect of prenatal treatment with LSRT on the concentrations of p-ERK1/2, p-Smad2/3, p-Smad1/5/8, and ACE in mutant aortas. Quantification is shown below. One-way ANOVA. Bars are mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*\*  $P < 0.001$ . **(C)** qPCR analysis of SMC differentiation marker genes in ascending aortas of wild type ( $n=3$ ) and SMKO aortas ( $n=2-3$ ), with and without prenatal losartan treatment. One-way ANOVA. Bars are mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.005$ , \*\*\*  $P < 0.001$ . **(D)** Western blot comparing MYH11 levels in WT and mutant aorta with and without LSRT treatment. Housekeeping protein GAPDH is shown as a loading control. Quantification is shown below. One-way ANOVA. Bars are mean  $\pm$  SEM. Animals were analyzed at 3 months of age.

**Fig. 6.**

Evaluation of the role of *Agtr1a* and *Agtr2* in formation of aortic aneurysms. (A) Histological images of the ascending aorta from *Agtr1a*-null (*1aKO*) and *Agtr1a*-null;*Fbln4<sup>SMKO</sup>* (*1aDKO*) mice at 3 months of age stained with H&E, Hart's (for elastin) and immunostained with anti-pERK1/2. Lumen is indicated by asterisk. Bars are 50  $\mu$ m. (B) The effect of the loss of an *Agtr1a* allele in *Fbln4<sup>SMKO</sup>* mice on IEL perimeter and total vessel area. One-way ANOVA. Bars are mean  $\pm$  SEM. \*\*\*  $P < 0.001$ . (C) Histological images of the ascending aorta from *Agtr2*-null (*2KO*) and *Agtr2*-null;*Fbln4<sup>SMKO</sup>* (*2DKO*) mice at 3 month of age stained with H&E, Hart's and immunostained with anti-pERK1/2. Bars are 50  $\mu$ m. (D) The effect of prenatal treatment with LSRT on IEL perimeter and total vessel area in *Agtr2*-null (*2KO*, black column) and *Agtr2*-null; *Fbln4<sup>SMKO</sup>* (*2DKO*, shaded column) mice evaluated at 3 months of age. One-way ANOVA. Bars are mean  $\pm$  SEM. \*\*  $P < 0.005$  and \*\*\*  $P < 0.001$ .