

Endothelial NADPH oxidase-2 promotes interstitial cardiac fibrosis and diastolic dysfunction through pro-inflammatory effects and endothelial-mesenchymal transition.

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Brief title: Endothelial NOX2 enhances cardiac fibrosis

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ABSTRACT

Objectives. To investigate the effect of endothelial dysfunction on development of cardiac hypertrophy and fibrosis.

Background. Endothelial dysfunction accompanies cardiac hypertrophy and fibrosis but its contribution to these conditions is unclear. Increased NADPH oxidase-2 (NOX2) activation causes endothelial dysfunction.

Methods. Transgenic mice with endothelial-specific NOX2 overexpression (TG) and wild-type littermates received chronic AngII infusion (1.1mg/kg/day, 2 weeks) to induce hypertrophy and fibrosis.

Results. TG had similar systolic hypertension and hypertrophy to wild-type but developed greater cardiac fibrosis and evidence of isolated left ventricular diastolic dysfunction ($P<0.05$). TG myocardium had more inflammatory cells and VCAM-1-positive vessels than wild-type after AngII (both $P<0.05$). TG microvascular endothelial cells (EC) treated with AngII recruited 2-fold more leukocytes than wild-type in an *in vitro* adhesion assay ($P<0.05$). However, inflammatory cell NOX2 *per se* was not essential for the pro-fibrotic effects of AngII. TG mice showed a higher level of endothelial-mesenchymal transition (EMT) than wild-type after AngII infusion. In cultured EC treated with AngII, NOX2 enhanced EMT as assessed by the relative expression of fibroblast versus endothelial-specific markers.

Conclusion. AngII-induced endothelial NOX2 activation has profound pro-fibrotic effects in the heart *in vivo*, leading to a diastolic dysfunction phenotype. Endothelial NOX2 enhances EMT and has pro-inflammatory effects. This may be an important mechanism underlying cardiac fibrosis and diastolic dysfunction during increased renin-angiotensin activation.

Key Words: angiotensin II, diastolic dysfunction, endothelium, endothelial-mesenchymal transition, NADPH oxidase

Abbreviations

AngII, angiotensin II; α SMA, α -smooth muscle actin; BM, bone marrow; CMEC, coronary microvascular endothelial cells; EC, endothelial cell; EMT, endothelial-mesenchymal transition; NOX, NADPH oxidase; ROS, reactive oxygen species; TG, transgenic; VCAM-1, vascular cell adhesion molecule-1

INTRODUCTION

Endothelial dysfunction accompanies conditions such as hypertension that predispose to adverse cardiac remodeling, and predicts future cardiovascular morbidity.(1) As well as modulating vascular function, the endothelium has direct effects on cardiac function.(2-4) Pioneering work by Brutsaert and colleagues(3) demonstrated that cardiac endothelial cells directly influence myocardial contraction, a concept later confirmed in humans *in vivo*.(5) The importance of endothelial-cardiomyocyte crosstalk has been demonstrated in several disease settings.(3,6) However, the role of endothelial dysfunction in the pathogenesis of adverse cardiac remodeling caused by increased renin-angiotensin activation remains unclear.

A major driver of endothelial dysfunction is the reactive oxygen species (ROS)-generating enzyme, NADPH oxidase-2 (NOX2), which is activated by angiotensin II (AngII) and other agonists.(7) NOX2 is expressed in endothelium as well as cardiomyocytes, adventitial fibroblasts, inflammatory cells and conduit vascular smooth muscle cells. Vascular NOX2 expression and activity are increased in hypertension where it modulates intracellular signaling pathways that promote vascular remodeling, contributes to vasodilator dysfunction through superoxide-mediated inactivation of nitric oxide, and may have pro-inflammatory effects.(7)

To investigate the pathophysiologic roles of endothelial NOX2, we generated a transgenic mouse model with endothelial-specific NOX2 overexpression.(8) This model has a 2-fold increase in endothelial NOX2 protein levels and displays augmented NOX2 activation in response to AngII. Here, we used this model to investigate the effects of endothelial NOX2 activation on the development of cardiac hypertrophy, fibrosis and dysfunction during chronic AngII elevation.

METHODS

Detailed methods are provided in the online supplement.

Animal experiments complied with UK Home Office regulations. Male transgenic mice with endothelium-targeted NOX2 overexpression (TG) (8) were compared with wild-type littermates. Chimeric mice were generated by bone marrow (BM) transplantation.(9) AngII (1.1mg/kg/day) or vehicle were infused via osmotic minipumps. Blood pressure was measured by radiotelemetry or tail-cuff plethysmography.(10) Left ventricular pressure-volume relations and echocardiography were performed as described.(11) Cardiac fibrosis was quantified in Picrosirius Red-stained paraffin sections while immunostaining was performed in cryosections. LV homogenate NADPH oxidase activity was measured using lucigenin-enhanced chemiluminescence.(12)

In vitro leukocyte adhesion to coronary microvascular endothelial cells (CMEC) from TG or wild-type mouse hearts was studied in a flow chamber,(13) with or without AngII (0.1μmol/L, 4h). To assess endothelial-mesenchymal transition (EMT), cultured human aortic EC were infected with Ad.βGal or Ad.NOX2 virus, then treated with 0.1μmol/L AngII or vehicle for up to 5 days. Co-culture experiments with EC and fibroblasts were performed in Transwell dishes.

Data are expressed as mean±SEM. Comparisons were made by repeated measures ANOVA, 2-way ANOVA with Bonferroni post-hoc testing, 1-way ANOVA or Student's t-test as appropriate. P<0.05 was considered significant.

RESULTS

Effects of endothelial NOX2 overexpression on AngII-induced hypertension and cardiac hypertrophy.

Chronic AngII infusion for 14 days caused similar systolic hypertension in TG and wild-type mice (Supplementary Fig. 1A). Left ventricular NADPH oxidase activity increased in both AngII-treated groups, with a significantly greater rise in TG (Supplementary Fig. 1B, supplementary Fig. 2). AngII infusion induced a similar increase in left ventricle/body weight

ratio or cardiomyocyte cross-sectional area in TG and wild-type mice (Supplementary Fig. 1C,D).

Endothelial NOX2 overexpression potentiates AngII-induced cardiac fibrosis.

The extent of AngII-induced fibrosis was significantly (~2-fold) greater in TG than wild-type hearts (Supplementary Fig. 1E). TG also had significantly higher pro-collagen I mRNA expression than wild-type (Supplementary Fig. 1F). Representative examples of the fibrosis and myocyte hypertrophy are shown in Fig. 1.

LV diastolic dysfunction with preserved systolic function in endothelial NOX2 TG.

TG had significantly lower echocardiographic left ventricular end-diastolic dimensions than wild-type after chronic AngII infusion (Supplementary Fig. 3). However, there were no significant differences in indices such as interventricular septal thickness or ejection fraction. More detailed assessment was undertaken by pressure-volume analysis. Systolic function assessed by end-systolic elastance or $LV\ dP/dt_{max}/EDV$ was similar in AngII-treated wild-type and TG groups (Supplementary Fig. 4A,B). Left ventricular end-diastolic volume increased in wild-type mice after AngII but decreased in TG (Supplementary Fig. 4C). Left ventricular end-systolic volume tended to increase in wild-type after AngII treatment but fell significantly in TG (Supplementary Fig. 4D). As a result, both stroke volume and stroke work were significantly lower in AngII-treated TG versus wild-type (Supplementary Fig. 4E, Table 1). Isovolumic left ventricular relaxation time-constant and left ventricular end-diastolic pressure were similar in wild-type and TG groups (Table 1). However, left ventricular diastolic stiffness assessed by the end-diastolic pressure volume relationship was significantly higher in AngII-treated TG versus wild-type hearts (Supplementary Fig. 4F). Although there was no difference in systolic BP, arterial elastance was significantly higher in AngII-treated TG (Table 1). Supplementary Fig. 4G,H show representative steady-state pressure-volume loops and the response to preload reduction in wild-type and TG. These indicate that TG

hearts were smaller and stiffer than wild-type after chronic AngII, indicating a phenotype of isolated diastolic left ventricular dysfunction with preserved systolic function.

Endothelial NOX2 enhances myocardial inflammatory cell infiltration.

To investigate mechanisms underlying the increased fibrosis in TG, we assessed myocardial inflammatory cell infiltration. TG hearts had significantly more CD45⁺ inflammatory cells and Mac3⁺ cells (macrophages) after AngII infusion than wild-type (Fig. 2, Supplementary Fig. 5). AngII treatment increased connective tissue growth factor and osteopontin mRNA levels but levels were similar in wild-type and TG (Supplementary Fig. 6). There was no difference between groups in TGFβ, TNFα, MCP-1 or endothelin-1 mRNA levels.

To confirm that the effects of endothelial NOX2 on inflammatory infiltration and fibrosis were ROS-dependent, mice were treated with the antioxidant N-acetylcysteine concurrent with AngII infusion. BP and cardiac hypertrophy were similar in wild-type and TG treated with N-acetylcysteine (Supplementary Fig. 7). N-acetylcysteine markedly reduced CD45⁺ cell number in both wild-type and TG AngII-treated groups, and prevented the development of fibrosis (Supplementary Fig. 7).

NOX2 enhances endothelial activation and leukocyte-endothelial interaction.

We assessed the interaction between inflammatory cells and NOX2-overexpressing endothelial cells in an *in vitro* adhesion assay under flow conditions. Leukocyte attachment to TG and wild-type CMEC was low at baseline, but after AngII treatment (0.1 μM, 4 h) significantly more leukocytes attached to TG CMEC (Fig. 2C). TG CMEC had significantly higher vascular cell adhesion molecule-1 (VCAM-1) protein expression than wild-type after AngII treatment (Fig. 2D). In line with this, LV sections of TG treated with chronic AngII had more VCAM-1 positive blood vessels than wild-type (Fig. 2E, Supplementary Fig. 8). There was no difference in capillary density between TG and wild-type hearts (Fig. 2F).

Inflammatory cell NOX2 is not essential for AngII-induced cardiac fibrosis.

Since NOX2 is abundantly expressed in inflammatory cells, we investigated whether NOX2 in these cells was required for the pro-fibrotic response to AngII. We used BM transplantation to create chimeric mice with different NOX2 genotypes in BM and resident tissue cells: (a) wild-type recipient mice with wild-type BM (WT:WT); (b) wild-type recipients with BM from global NOX2 knockout mice (KO:WT); (c) global NOX2 knockout mice with wild-type BM (WT:KO); (d) NOX2 TG with TG BM (TG:TG); (e) NOX2 TG with wild-type BM (WT:TG); and (f) wild-type mice with TG BM (TG:WT). Successful alteration of NOX2 genotype after BM transplantation was confirmed by assessing ROS production in recipient BM and peripheral blood mononuclear cells (Supplementary Fig. 9A).

AngII infusion caused similar increases in BP and hypertrophy among groups (Supplementary Fig. 9B,C). AngII-stimulated cardiac fibrosis was significantly lower in global NOX2 KO versus wild-type, as reported previously(12) (Supplementary Fig. 10). WT:WT and KO:WT mice both had similar fibrosis to un-transplanted wild-type mice (Supplementary Fig. 10A,B). In contrast, WT:KO mice had significantly less fibrosis, similar to un-transplanted knockout mice (Supplementary Fig. 10A,B). Thus, knockout BM cells did not significantly alter AngII-induced fibrosis. Fibrosis in NOX2 TG:TG was similar to that in WT:TG while transplantation of TG BM to wild-type mice did not increase the level of fibrosis to that in the TG:TG group (Supplementary Fig. 10A right-hand panels, Supplementary Fig. 10B). Thus, TG BM cells did not significantly alter AngII-induced fibrosis. Taken together, these results indicate that NOX2 in BM cells is dispensable for the pro-fibrotic effects of AngII and it is NOX2 in the resident tissue cells that is essential. Furthermore, endothelium-targeted NOX2 overexpression does not significantly alter the effects of BM cells as compared to wild-type.

NOX2 promotes endothelial-mesenchymal transition (EMT).

Recent studies indicate that EMT is an important contributor to cardiac fibrosis during chronic pressure overload.(14) We therefore investigated whether enhancing NOX2 activity in endothelial cells affected EMT. Hearts of AngII-treated TG had significantly higher levels of fibroblast-specific α -smooth muscle actin (α SMA) and collagen-1 than wild-type while levels of endothelial-specific CD31 were significantly lower (Fig. 3A). Myocardial sections of AngII-treated TG showed increased evidence of EMT, as indicated by co-localization of endothelial-specific and mesenchymal markers, compared with wild-type (Fig. 3B).

To further assess the effects of endothelial NOX2, we studied cultured human aortic EC with adenoviral-mediated overexpression of NOX2 or β -galactosidase control, and treated with AngII. AngII caused a modest increase in fibroblast-specific markers (FSP-1, α SMA) and a concomitant decrease in endothelial-specific markers (CD31, CD144) in control EC. This transition from endothelial- to fibroblast-specific expression was enhanced in NOX2-overexpressing cells (Fig. 4). NOX2 overexpression *per se* caused a small switch from endothelial to fibroblast-specific expression in the absence of AngII. Supplementary Fig. 11A shows representative examples of co-expression of endothelial-specific and mesenchymal markers in NOX2-overexpressing cells after AngII. Cellular morphology was significantly different after 5 days of AngII treatment, with NOX2-overexpressing cells showing a more elongated fibroblast phenotype (Supplementary Fig. 11B).

Paracrine effects of endothelial cells on fibroblasts?

It is feasible that NOX2 elevation in EC affects fibroblasts through the direct effects of ROS or other released factors. To assess this possibility, we performed experiments in which human aortic EC overexpressing NOX2 or β -galactosidase were co-cultured with fibroblasts (see Supplementary Methods). Experiments were performed with and without AngII. No difference was found between groups in the fibroblast expression of pro-collagen I mRNA or in markers of myofibroblast transformation (Supplementary Fig. 12).

DISCUSSION

We investigated the specific effects of endothelial NOX2 activation on AngII-induced cardiac remodeling, taking advantage of a recently developed mouse model with endothelium-targeted overexpression of NOX2.(8) The 2-fold increase in endothelial NOX2 protein in this model has no basal effects but enhances AngII-stimulated increases in NOX2 activity. In the present study, we found that *in vivo* AngII-induced endothelial NOX2 activation (a) enhances the development of cardiac fibrosis independent of cardiomyocyte hypertrophy, and leads to a phenotype of left ventricular diastolic dysfunction with preserved systolic function; (b) promotes myocardial inflammatory cell infiltration through increased endothelial expression of VCAM-1 (i.e. endothelial activation), although NOX2 in inflammatory cells is dispensable for the pro-fibrotic effects; (c) enhances EMT which contributes to the cardiac fibrosis. Collectively, these data suggest that NOX2-driven endothelial dysfunction and activation may be an important mechanism that promotes cardiac fibrosis and left ventricular diastolic dysfunction in pathologic settings of renin-angiotensin system activation.

Endothelium-cardiomyocyte crosstalk

The cardiac endothelium forms a strategic interface between circulating blood and myocardial tissue. Crosstalk between cardiac EC and cardiomyocytes plays important roles in normal cardiac development.(3,4) The cardiac endothelium also influences contractile function in the adult heart, for example through paracrine factors such as nitric oxide and endothelin.(2) Reciprocal signaling between cardiomyocytes and the myocardial microvasculature has been found to be important during chronic pressure overload. For example, signaling from EC to cardiomyocytes through the neuregulin 1-ErbB2 axis promotes cardiomyocyte survival during pressure overload,(15) and angiogenic factors released by cardiomyocytes in the chronically overloaded heart regulate myocardial capillary

density.(11,16) While these studies support the potential for endothelial dysfunction to affect cardiomyocyte growth, survival and contractile function, the effects on cardiac fibrosis in the heart under stress are unclear.

Pro-inflammatory effects of endothelial NOX2

Here, we demonstrate that endothelial dysfunction has a profound ROS-dependent impact on the development of cardiac fibrosis, independent of cardiomyocyte hypertrophy. One mechanism underlying these effects may be the pro-inflammatory properties of activated endothelium. The healthy endothelium is anti-inflammatory and anti-thrombotic but in pathologic settings such as renin-angiotensin system activation, it becomes activated and promotes increased interaction with circulating inflammatory cells.(17) Previous studies suggested that NOX2 is involved in cytokine- and AngII-induced EC activation and the expression of adhesion molecules such as VCAM-1,(18,19) and in enhanced monocyte binding to ECs under oscillatory shear stress.(20) Here, we found that NOX2-overexpressing CMEC had a larger increase in VCAM-1 expression after AngII stimulation than wild-type, which was associated with greater endothelial-leukocyte adhesion in an *in vitro* flow assay. Consistent with a similar pro-inflammatory action *in vivo*, TG myocardium had more VCAM-1-positive blood vessels and inflammatory cells after AngII treatment than wild-type. These results clearly indicate that AngII-induced endothelial NOX2 activation augments an inflammatory response in the heart *in vivo*. Whether other cell types (e.g. vascular smooth muscle cells, pericytes) are also involved is an interesting question.

In addition to EC, NOX2 is abundant in inflammatory cells where it is suggested to contribute to oxidative stress, for example in atherosclerosis.(21) NOX2 activation in inflammatory cells is involved in AngII-induced vascular hypertrophy.(22) It was therefore possible that NOX2 activation in infiltrating inflammatory cells (as well as the endothelium) might be important in the development of cardiac fibrosis. To dissect the specific contribution

of inflammatory cell NOX2 activation, we generated chimeric mice in which the BM comprised NOX2-competent or -deficient cells. These series of studies demonstrated that NOX2 activation in infiltrating inflammatory cells is dispensable for the development of AngII-induced fibrosis and that it is NOX2 in recipient heart cells that is essential for the pro-fibrotic effects. This result suggests that although NOX2 may be important for certain functions of inflammatory cells (e.g. phagocytosis, LDL oxidation in atherosclerosis [21]), it is not essential for all functions and is dispensable in the setting of AngII-induced cardiac pathology.

Role of EMT

Another mechanism by which endothelial dysfunction might promote cardiac fibrosis is through EMT. The transition of epithelial and endothelial cells to a mesenchymal phenotype is well recognized to be important in cardiac development. In a landmark study, Zeissberg et al.(14) showed that EMT makes a significant contribution to pathologic cardiac fibrosis during chronic pressure overload. EC production of endothelin-1 has been reported to contribute to EMT in the diabetic heart.(23) We therefore investigated whether endothelial NOX2 activation affects EMT. We found evidence of significantly increased EMT in TG hearts after chronic AngII as compared with wild-type. More definitive evidence that endothelial NOX2 promotes EMT was obtained in cultured EC where NOX2 significantly enhanced the transition from endothelial to fibroblast phenotype after AngII treatment. These results strongly suggest that NOX2-dependent increase in EMT may be a crucial mechanism that enhances cardiac fibrosis in response to chronic AngII treatment.

We also tested the possibility that paracrine effects of EC on fibroblasts, for example through the direct effects of ROS released by EC, may contribute to fibrosis. *In vitro* co-culture experiments with EC and fibroblasts did not provide evidence in support of this

possibility. However, a possible contribution of such a mechanism *in vivo* cannot be excluded.

Isolated diastolic dysfunction

A striking functional consequence of endothelial NOX2 overexpression on the response to chronically elevated AngII was a phenotype of LV diastolic dysfunction with preserved systolic function, resulting in smaller and stiffer hearts and reduced stroke volumes in TG compared with wild-type. This was documented in TG by the gold-standard method of *in vivo* pressure-volume analysis, which demonstrated significantly increased left ventricular diastolic passive stiffness in the AngII-treated TG versus wild-type group whereas load-independent indices of systolic function were similar in the two groups. We found no significant difference between the groups in parameters of active left ventricular relaxation (e.g. the isovolumic relaxation time-constant), suggesting that cardiomyocyte relaxation was probably similar in the two groups. This contractile dysfunction phenotype is consistent with, and fully explainable by, the increased fibrosis observed in TG independent of differences in cardiomyocyte hypertrophy. The use of an endothelium-targeted transgenic model allowed us to study the role of endothelial dysfunction/activation independent of other changes that occur in the remodeling heart during increased renin-angiotensin system activation. As mentioned earlier, NOX2 is also expressed in cardiomyocytes and previous studies in cultured cells and *in vivo* models have shown that cardiomyocyte NOX2 activation may contribute to the development of AngII-induced cardiac hypertrophy.(12) No difference was found in extent of hypertrophy between wild-type and TG in the current study, consistent with the fact that NOX2 was overexpressed specifically in EC. Therefore, the phenotype of diastolic left ventricular dysfunction with preserved systolic function found in the current model was the consequence specifically of endothelial dysfunction/activation.

The clinical syndrome of heart failure with preserved ejection fraction is currently receiving significant attention due to the high prevalence of the condition, controversy regarding its etiology and pathogenesis, and the lack of effective therapies.(24) Whilst simple extrapolations cannot be made from the current study in a murine model to the clinical setting, the occurrence of a contractile dysfunction phenotype that apparently closely resembles the cardiac dysfunction found in human heart failure with preserved ejection fraction is of great interest. It is notable that previous human studies suggested that inflammation contributes to enhanced fibrosis in such patients,(25) consistent with the findings of increased inflammatory cell infiltration, fibrosis and diastolic dysfunction in our study. The current results raise the intriguing possibility that endothelial dysfunction/activation and downstream pathways such as inflammation and EMT may be important mechanisms contributing to the development of heart failure with preserved ejection fraction. If so, this could provide new therapeutic options to tackle this prevalent condition.

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FIGURE LEGENDS

Figure 1. Representative myocardial sections showing cardiac hypertrophy and fibrosis.

Top, cardiomyocyte borders outlined using wheat-germ agglutinin. Scale bars 50 μm .

Bottom, Picrosirius Red-stained sections. Scale bars 100 μm . Sal = saline.

Figure 2. Inflammatory cell infiltration *in vivo* and endothelial-leukocyte interaction *in vitro*.

(A,B) Mean number of CD45⁺ and Mac3⁺ cells/field, respectively. (C) Number of leukocytes adherent to WT or TG EC with or without AngII treatment (0.1 μM , 4 hr) in an *in vitro* assay. (D) VCAM-1 protein levels in WT and TG EC treated with AngII.

Representative Western blots at the top, mean data below. (E) Number of VCAM-1 positive vessels in myocardial sections from WT and TG. (F) Capillary density in myocardial sections from WT and TG (capillaries/ mm^2). * $P < 0.05$ by 2-way ANOVA; $n = 6-8/\text{group}$.

Figure 3. Enhanced endothelial-mesenchymal transition in AngII-treated TG hearts.

(A) Representative Western blots and mean data for CD31, smooth muscle actin (SMA) and collagen 1 (Coll) levels in cardiac homogenates from AngII-treated WT or TG mice. Actin was used as a loading control. *, $P < 0.05$; $n = 4/\text{group}$. (B) Myocardial sections co-stained for CD31 (red) and SMA (green) and imaged by confocal microscopy. Yellow color indicates co-localisation of endothelial and mesenchymal markers. Scale bar 10 μm . Panels to the right show pixel-by-pixel fluorescence intensities of the two fluorophores along the line indicated in the top panel (TG AngII) and confirm a significant overlapping pattern between CD31 and SMA, indicative of endothelial-mesenchymal transition.

Figure 4. Effect of NOX2 on endothelial-mesenchymal transition in cultured endothelial cells.

(A) Representative Western blots for CD144, CD31, FSP1, smooth muscle actin (SMA) and NOX2 levels in EC overexpressing NOX2 or β -gal and treated with AngII (100 nmol/L) or vehicle (Con). GAPDH was a loading control. (B) Mean data for CD144 and SMA protein levels. Similar results were observed for CD31 and FSP1. *, $P < 0.05$; $n = 4/\text{group}$.

Table 1. Cardiac function assessed by LV pressure-volume loops in WT and TG mice.

	WT Saline	WT ANGII	TG Saline	TG ANGII
Heart rate (bpm)	552±9	509±17	543±14	543±12
LVEDP (mmHg)	7.5±0.7	5.0±0.8	6.9±1.1	8.1±2.3
dP/dtmax (mmHg/min)	7804±459	8348±365	7497±529	8098±435
dP/dtmin (mmHg/min)	-6497±406	-6654±417	-6600±544	-6843±604
Stroke volume (μL)	16.7±0.9	22.8±1.7**	15.4±1	16.3±1.6**
End systolic volume (μL)	17.8±1.1	21.9±3.6	17.4±1.4	13.7±0.9‡‡
End diastolic volume (μL)	29.5±1.7	39.9±3.7**	29.0±1.4	25.3±1.5‡‡‡
Ejection fraction (%)	53±1	57±5	52±4	60±4
Cardiac output (mL/min/g)	105.5±8.1	112.0±11.5	91.2±5.0	82.3±8.2‡
Stroke work (mmHg.μl/g)	14472±1280	17216±1888	12365±865	11569±1324‡
Ea (mmHg/μL)	5.7±0.2	4.6±0.4	5.9±0.4	7.0±1.0‡‡
Tau-Weiss (ms)	7.4±0.4	6.2±0.6	7.4±0.7	7.8±1.2
dP/dtmax/EDV	261.9±39.6	258.8±32.0	273.9±73.0	334.8±71.1
Ees	4.28±0.38	8.91±0.69***	4.78±0.40	8.91±1.28***
EDPVR	0.22±0.03	0.25±0.04	0.20±0.02	0.46±0.11** ‡
Number	10	10	9	8

Ea, arterial elastance; EDV, end-diastolic volume; Ees, end-systolic elastance; EDPVR, end-diastolic pressure volume relation. **,*** = P<0.01, 0.001 respectively for effect of AngII within the group. #,##,### = P<0.05, 0.01, 0.001 respectively for comparison of TG AngII group versus WT AngII group.

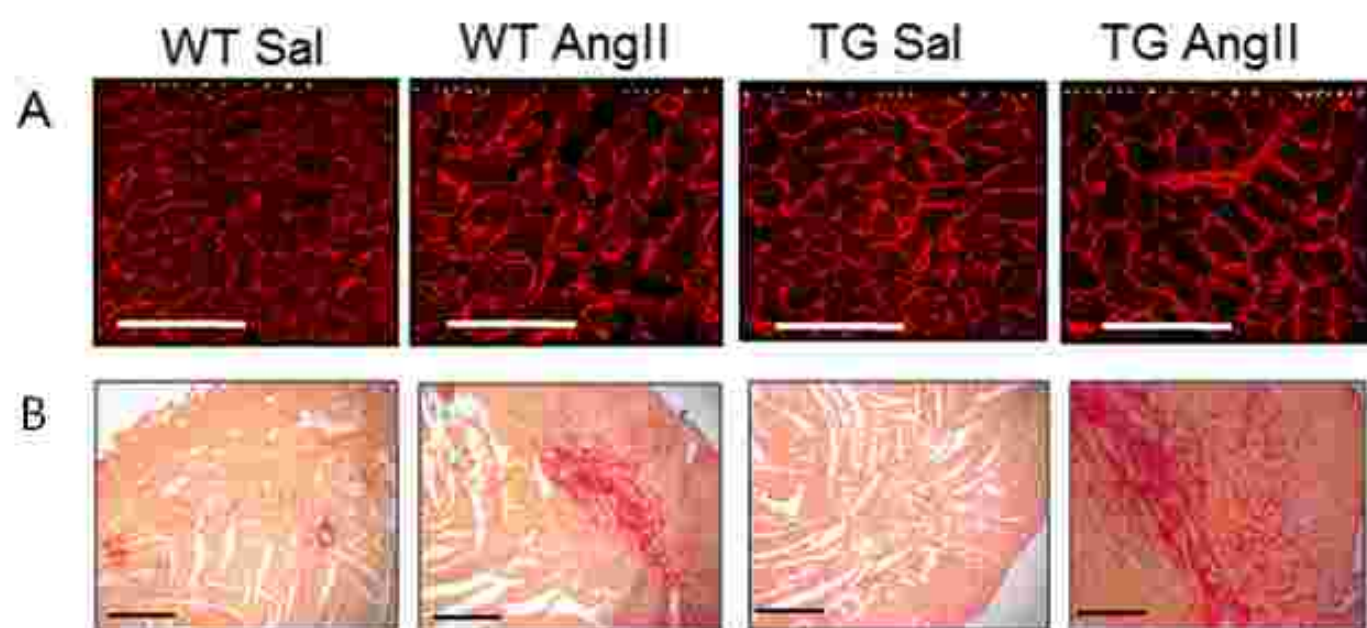


Fig. 1

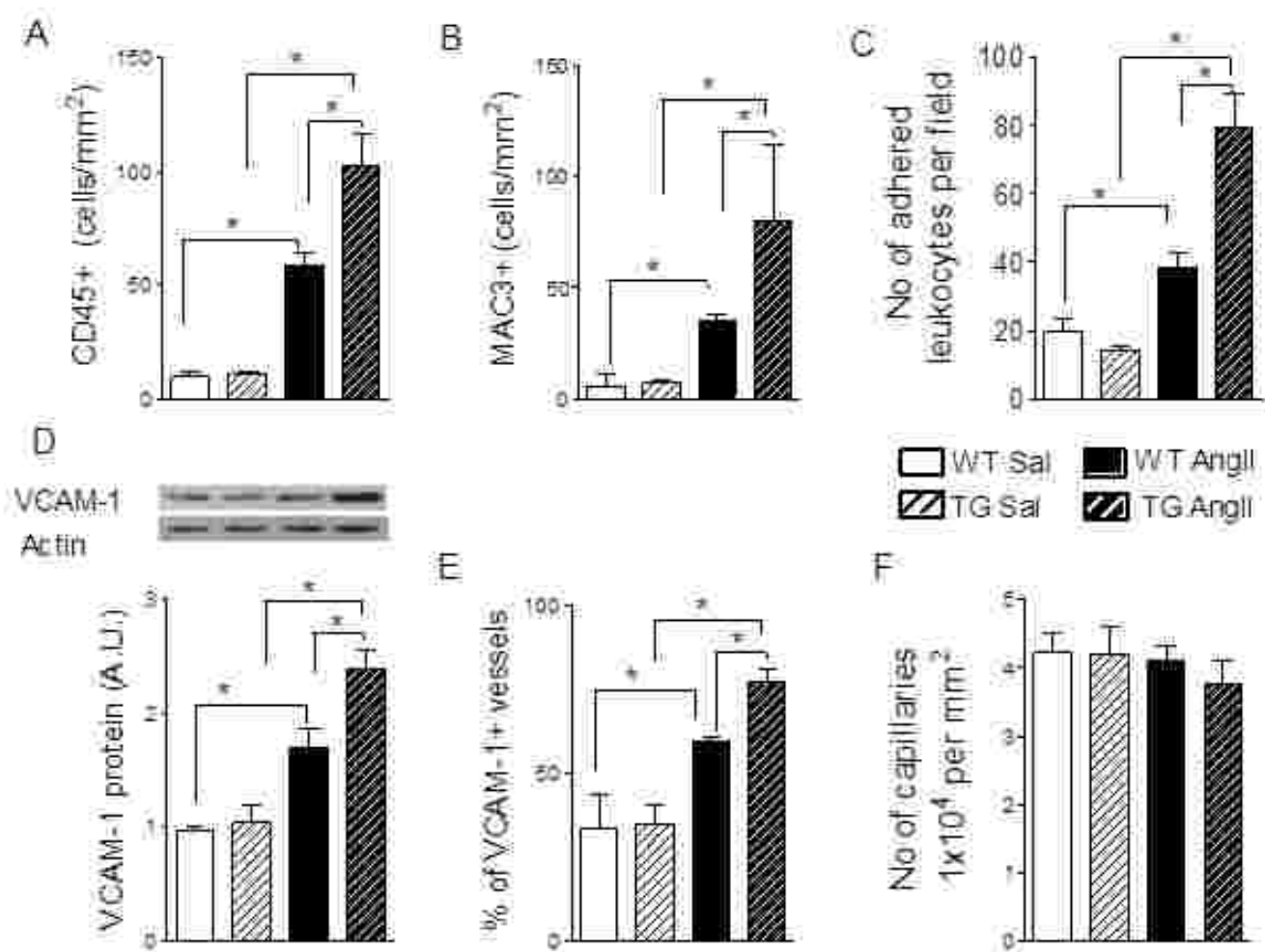


Fig. 2

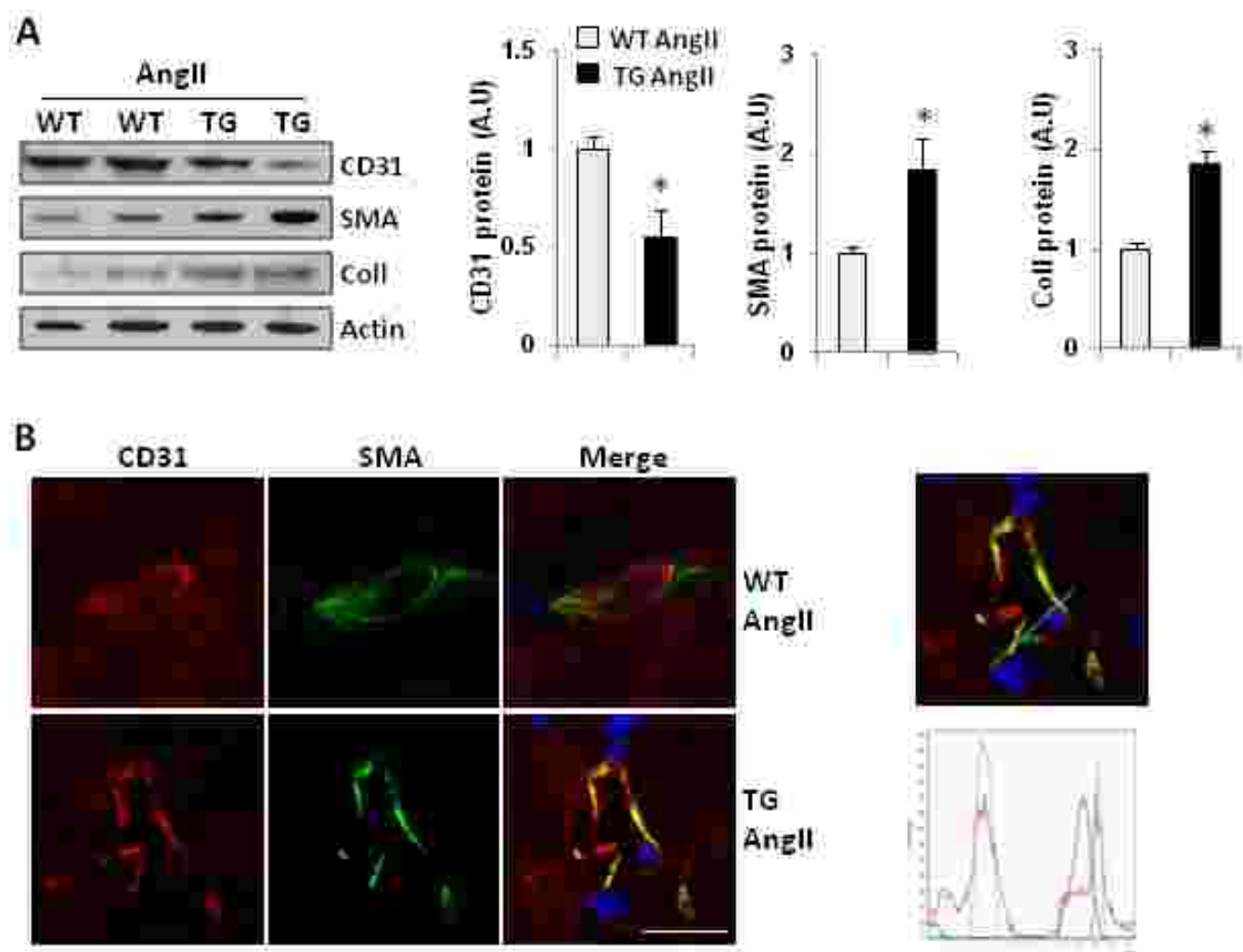


Fig. 3

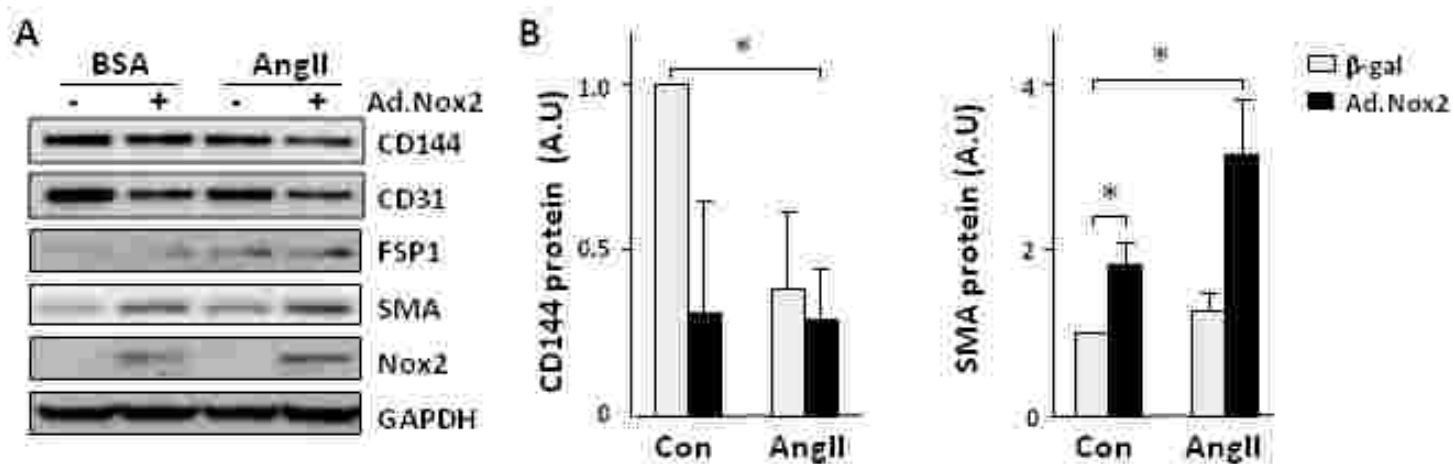


Fig. 4

SUPPLEMENTAL MATERIAL

Endothelial NADPH oxidase-2 promotes interstitial cardiac fibrosis and diastolic dysfunction through pro-inflammatory effects and endothelial-mesenchymal transition.

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METHODS

Animal studies.

All procedures were performed in accordance with the 'Guidance on the Operation of the Animals' (Scientific Procedures) Act, 1986 (UK Home Office). Transgenic mice with endothelium-targeted overexpression of Nox2 (TG) and global Nox2 knockout mice, both on a C57/BL6 background, were described previously.^{1,2} Male Nox2 TG were compared with matched wild-type littermates (WT).

Chimeric mice were generated by bone marrow (BM) transplantation as previously described.³ 6-8 week old mice received a lethal dose of whole-body irradiation (10Gy, 15min) and 24 hours later, 0.5×10^7 BM cells isolated from donor mice were administered by tail vein injection. Donor mice were killed by cervical dislocation, their femurs were removed aseptically and a single cell suspension was prepared by flushing the marrow cavities. Cells were washed twice in HBSS and resuspended 1×10^7 cells per ml. Mice were allowed to recover for at least 4 weeks before further study.

AngII (1.1mg/kg/day) or saline vehicle was infused via osmotic minipumps (Model 1002, Alzet, Cupertino, CA) that were implanted subcutaneously under isoflurane anesthesia. Osmotic pumps containing saline were used as controls.

Blood pressure was measured either by ambulatory radiotelemetry (PA-C10, Data Sciences International) or tail-cuff plethysmography, as described previously.⁴ Tail-cuff

plethysmography was performed after 3 training sessions in conscious restrained mice, using a Kent Scientific system (XBP 1000; Torrington, CT).

LV pressure-volume relations were measured with a 1.4F micromanometer/microconductance catheter system (SPR-839, Millar Instruments, Houston, TX) introduced retrogradely into the LV via the right carotid artery under 2% isoflurane anesthesia.⁵ Body temperature was maintained at 37°C to ensure heart rates of approximately 500 bpm. After stabilization, pressure volume loops were obtained via a Aria pressure-volume system (Millar, Houston, Tex) coupled to a Powerlab/8SP with Chart Software (ADInstruments, UK). Analysis was conducted using Chart and pressure volume analysis software (PVAN 3.3 Millar Instruments). The correction factor (alpha) was obtained from the ratio of cardiac output obtained from echocardiographic measurements (product of aortic blood flow and aortic cross sectional area) and the catheter cardiac output. Parallel conductance was obtained from a series of loops obtained during iv bolus injection of hypertonic saline via the jugular vein.

Echocardiography was performed under 2% isoflurane anesthesia at heart rates >400 bpm, using either a Sonos 5500 system with a 15 MHz linear transducer (Philips, Bothell, WA) or a Vevo 2100 with a 30MHz linear array transducer (Visualsonics, Toronto, CA). Temperature was maintained at 37°C and heart rates were over 400 bpm. Two-dimensional and M-mode images were obtained in parasternal long axis and short axis views respectively, the latter being measured at the level of the papillary muscles.

Histology and immunohistochemistry.

Hearts were arrested in diastole by perfusion with 5M KCL at 115 mmHg. Tissue was fixed in PFA (1%) and underwent normal histological preparation into paraffin blocks and onto slides. 6 µm sections were then deparaffinized and rehydrated through a series of EtOH concentrations. Cardiomyocyte area and cardiac fibrosis were quantified in sections stained with wheat-germ agglutinin and Picrosirius Red respectively.⁵ The collagen content was quantified as the percentage of total LV area under polarized light. Cardiomyocyte membranes and capillaries were co-stained using Wheat Germ Agglutinin (WGA) and Isolectin B4, conjugated to TRITC and FITC respectively (Vector RL-1022; Vector, USA). Confocal images were obtained on a Leica laser scanning confocal microscope (TCS-SP5) at

640x magnification. The numbers of capillaries per mm² were calculated from 10 different fields (~90 capillaries/field) using AxioVision v4.6 (Carl Zeiss, Germany).

Cryosections were used for immunostaining with antibodies against CD45 (BD, UK), Mac3 (Abcam), CD31/PECAM-1 (Chemicon International), α -SMA (Abcam) and VCAM-1 (R&D). 4'-6-Diamidino-2-phenylindole (DAPI; Sigma) was used for nuclear staining. Sections were then incubated with HRP-labeled secondary antibody and streptavidin-HRP (Perkin Elmer) signal amplification was used. Visualization was conducted by incubation with diaminobenzidine (DAKO kit SK4100, Vector Laboratories) followed by Harris counterstaining. CD45 or MAC3-positive cells were counted using semi-automated software (AxioVision v4.6, Carl Zeiss, Germany) and expressed as counts per mm² averaged over 10 fields. VCAM-1 positive blood vessels were counted and expressed as % of the total number of blood vessels per heart section.

Leukocyte adhesion assay.

Coronary microvascular endothelial cells (CMEC) were isolated from hearts of 6-8 week old mice and used at passages 2-3.⁴ A flow chamber (Glycotech, Maryland) was placed onto confluent CMEC which had been incubated with or without AngII (0.1 μ M, 4h). BM cells isolated from WT mice and labeled with Celltracker™ (Invitrogen) were added to the perfusate which superfused at a rate of 1.5 dynes/cm² for 30 min.⁶ Non-bound leukocytes were washed away before visualization using an inverted microscope. The number of recruited leukocytes was counted in 8-10 fields from 3 separate isolations.

EndMT assays.

Human aortic endothelial cells (HAEC) were purchased from ATCC and maintained in gelatine-coated flasks in medium M199 supplemented with 10% FBS (Invitrogen), 1 ng/ml β -endothelial cell growth factor (Sigma), 3 μ g/ml endothelial cell growth supplement from bovine neural tissues (Sigma), 2.5 μ g/ml thymidine (Sigma), 10 U/ml heparin (Sigma) and 100 U/ml penicillin-streptomycin in a 5% CO₂ humidified incubator. HAECs were infected with Ad. β Gal or Ad.NoX2 virus at MOI=20 for 6 hr in the presence of complete growth medium, then incubated with human endothelial serum free medium (Invitrogen) in the absence or presence of 100 nmol/L AngII. The medium was refreshed every day.

Immunofluorescent staining was performed 48 hr post virus infection while Western blotting was performed 5 days post infection.

Cell slides were fixed with methanol at room temperature for 20min; washed twice with PBS, 5min each; permeabilized with 0.01% Triton X-100/PBS for 15 min, and then washed with PBS three times for 5min each. Slides were blocked with diluted normal donkey serum (1:20 in PBS) at room temperature for 30 min, incubated with rabbit anti-VE-cadherin (1:100 in diluted donkey serum, 2158, Cell Signaling) and mouse anti-alpha smooth muscle actin (1:100 in diluted donkey serum, ab683, Abcam) at 4°C overnight, followed by washing three times with PBS, 10min each. The slides were then incubated with donkey anti-Rabbit IgG-Alexa569 (1:1000 in diluted donkey serum, DAKO) and donkey anti-mouse IgG-Alexa488 (1:1000 in diluted donkey serum, DAKO) at room temperature for 45 min, followed by washing three times with PBS, 10 min each. The slides were counterstained with DAPI at room temperature for 5min, followed by washing twice with PBS, 5min each. Fluorescent mounting medium and coverslip were applied and images were taken under an Olympus 1X81-2 fluorescent microscope with Velocity software by PerkinElmer.

Endothelial cell-fibroblast co-culture

HAECs were infected with Ad.βGal or Ad.NoX2 virus (MOI=20) as described above for EMT assays. 24 hrs post-infection, the HAECs were detached with trypsin and seeded into the inserts of 100 mm² Transwell culture dishes (Sigma) in EC medium. In parallel, human F153 fibroblasts (ATCC) were seeded in 100 mm² dishes in F-12/DMEM medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. Six millilitre human endothelial serum free medium (SFM, Life Technologies) with or without 100 nmol/L AngII was added into the HAECs-containing inserts and F153-containing dishes, respectively. One HAEC-containing insert was put into one F153-containing dish to form a co-culture system. 24 hrs later, the cells were collected separately. One third of the F153 cells were used to extract cellular total RNA for qPCR analyses with primer sets of 5'-GCTCGTGGAAATGATGGTGC-3' and 5'-ACCTGGGGACCTTCAGAG-3' for pro-collagen I and 5'-CACAACTGGGACGACATGGAG-3' and 5'-TTCATGAGGTAGTCAGTCTGG-3' for β-actin as loading control. Two-thirds of the cells were lysed for Western blot analysis.

ROS measurement

NADPH oxidase activity in homogenised left ventricle (50µg) was assessed by lucigenin (5 µM)-enhanced chemiluminescence on a plate luminometer (Lucy 1, Rosys Anthos, Wals, Austria) as previously described.⁷ All samples were analyzed in triplicate and the level of superoxide production was calculated from the average area under the curve. In some experiments, one of the following inhibitors was pre-incubated with the cell homogenate for 20 minutes prior to addition of NADPH: the flavoprotein inhibitor, diphenyleneiodonium (DPI, 10 µM); a NOS inhibitor, N^G-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 µM); a mitochondrial electron transport chain inhibitor, rotenone (50 µM); a superoxide scavenger, tiron (10 mM); a putative NADPH oxidase inhibitor, apocynin (10 µM); or a xanthine oxidase inhibitor, oxypurinol (100 µM).

ROS production in dihydroethidine-loaded BM mononuclear cells was quantified by flow cytometry.

Real-time RT-PCR

Quantification of mRNA expression was conducted using the Applied Biosystems 7000 sequence detection system (Applied Biosystems, UK) with SYBR Green and the comparative Ct method, with GAPDH levels used for normalization. RNA was isolated using an SV total RNA isolation kit (Promega, UK) and cDNA synthesised using avian myeloblastosis virus reverse transcriptase (AMV; Promega, UK) at 42°C for 90 minutes. Forward and reverse primer sequences were as follow (all 5'-3'): GAPDH: 5'-CGTGCCGCCTGGAGAA, 5'-CCCTCAGATGCCTGCTTCAC; Actin, 5'-CTGGCACCCAGCACAATG, 5'-GCCGATCCACACGGAGTACT; Procollagen I, 5'-CCTCAGGGTATTGCTGGACAAC, 5'-TTGATCCAGAAGGACCTTGTTG; MCP1, 5'-TGTAGTTTTTGTCCACCAAGCTCAAG, 5'-GTAGGTTCTGATCTCATTTGGTTCC

Western blot

Cells (75ml flask) were scratched off in the presence of medium and collected by centrifugation at 1000rpm at 4°C for 5min. The cell pellets were washed twice with ice cold PBS and resuspended in 200µl lysis buffer (20mM Tris-Cl, pH7.5, 120mM NaCl, 1mM EDTA, 1% Triton X-100 plus protease inhibitors) followed by sonication for 6 sec and incubation on ice for 45 min. Supernatant was recovered after spinning down at 10,000rpm at 4°C for

5min and protein concentration was detected using Bio-Rad reagents. 25 µg cell lysate was applied to SDS-PAGE, followed by transfer to PVDF membrane and incubation with primary antibodies against FSP1 (ab41532, Abcam), α-SMA (ab683, Abcam), PECAM-1 (CD31, sc-1506, Santa Cruz), CD144/ VE-cadherin (Santa Cruz), Nox2 (BD), GAPDH (sc-25778, Santa Cruz) and HRP-conjugated secondary antibody (DAKO). The bound antibodies were revealed by ECL reagents (DAKO) and X-ray film.

Statistics

Data are expressed as mean±SEM. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc testing, 1-way ANOVA or Student's t-test as appropriate. Analyses were performed on GraphPad Prism (v4.03 for Windows, San Diego, CA). P<0.05 was considered significant.

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Supplementary Figure 1. Effect of endothelial NOX2 on AngII-induced cardiac hypertrophy and fibrosis. (A) Systolic blood pressure (SBP) after saline or AngII (1.1mg/kg/day) infusion. Average SBP \pm SEM over a 12-hour dark period (active) is shown. *, $P < 0.05$ AngII vs saline by repeated measures ANOVA. $n = 6$ /group. (B) LV NADPH oxidase activity in wild-type and TG treated with saline or AngII. (C) LV/body weight (BW) ratio. (D) Cardiomyocyte area. (E) % fibrosis. (F) LV collagen I mRNA levels. * $P < 0.05$, *** $P < 0.001$ by 2-way ANOVA; $n = 6-8$ /group.

Supplementary Figure 2: NADPH-dependent superoxide generation in LV homogenates. Lucigenin (5 μ M)-enhanced chemiluminescence was measured in LV homogenate from WT and TG mice with saline or AngII infusion. The chemiluminescence signal was measured over 15 min in the presence or absence of DPI (10 μ M), L-NAME (100 μ M), Tiron (20 mM), oxypurinol (100 μ M), rotenone (100 μ M) or apocynin (100 μ M). Results were quantified as area under the curve and presented as arbitrary integrated light units. Data is shown for the effects of inhibitors in the AngII-treated TG group. The inhibitors had similar effects in the other 3 groups. **, $P < 0.01$ by 1-way ANOVA followed by Dunnett's post hoc test, comparing to control. $n \geq 6$ /group.

Supplementary Figure 3: Echocardiographic indices in WT and TG mice treated with AngII or saline. (A) Interventricular septal diameter. (B) LV end diastolic diameter (LVEDD). (C) LV

end systolic diameter (LVESD). (D) Ejection fraction. (E) Heart rate (HR). **, $P < 0.05$ by 2-way ANOVA. $n = 12/\text{group}$.

Supplementary Figure 4. Left ventricular function assessed by pressure-volume analysis.

(A) Ees, end-systolic elastance. (B) LV dp/dt_{\max} normalized by end-diastolic volume (EDV). (C) LV EDV. (D) LV end-systolic volume (ESV). (E) Stroke work (SW). (F) End-diastolic pressure volume relation (EDPVR). (G) Representative steady-state pressure-volume loops from wild-type (WT) and TG mice. (H) Representative pressure-volume loops during acute preload reduction in AngII-treated WT and TG. Dashed lines at top left and bottom right of the loops show the ESPVR and EDPVR respectively. * $P < 0.05$ AngII vs. respective saline group; # $P < 0.05$ WT AngII vs. TG AngII by 2-way ANOVA; $n = 12/\text{group}$.

Supplementary Figure 5. Inflammatory cell infiltration *in vivo*. (A,B) Representative sections stained for CD45⁺ and Mac3⁺ cells, respectively. Scale bars 100 μm .

Supplementary Figure 6: Expression of inflammatory cytokines and pro-fibrotic factors in WT and TG heart. mRNA levels of the genes shown were measured by real-time PCR in LV from WT and TG treated with saline or AngII. β -actin levels were used for normalization. * $P < 0.05$ compared with respective saline control. $n \geq 6/\text{group}$.

Supplementary Figure 7: Effects of N-acetyl cysteine (NAC) on blood pressure, cardiac hypertrophy and fibrosis. (A) Systolic blood pressure (SBP) measured by tail cuff plethysmography. (B) Cardiac fibrosis in LV sections. (C) LV/BW ratio. (D) CD45⁺ cells in LV sections. NAC (5g/L) was administered in the drinking water for the whole duration of 2 week AngII infusion. * $P < 0.05$ vs saline treatment by repeated measure ANOVA. $n = 6/\text{group}$ for BP and morphometric data; $n = 4/\text{group}$ for histological data.

Supplementary Figure 8: Expression of VCAM-1 in LV sections. Representative examples of VCAM-1 positive blood vessels in LV sections from WT and TG mice chronically treated with AngII.

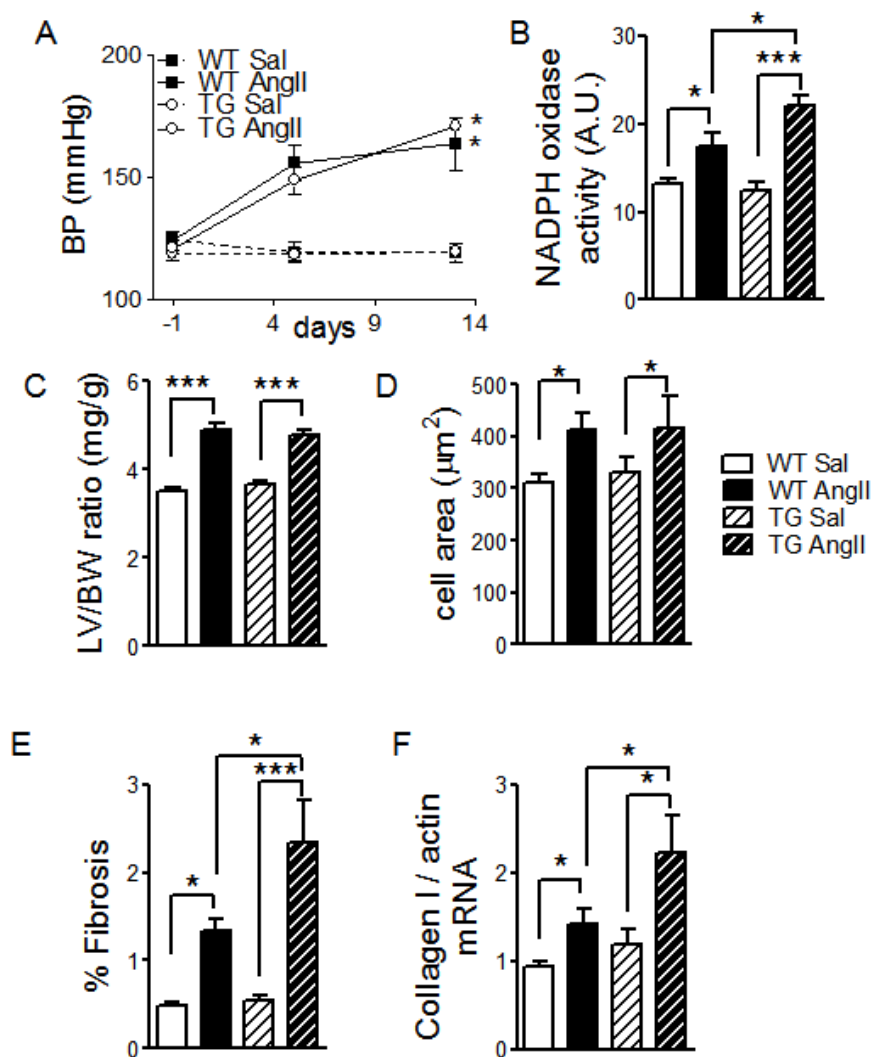
Supplementary Figure 9: Effects of bone marrow (BM)-derived cells on blood pressure and cardiac hypertrophy. Bone marrow chimeric mice were generated as described in the text.

(A) Superoxide production measured by flow cytometry in dihydroethidine (DHE)-loaded BM cells from chimeric mice that previously received bone marrow transfer (BMT) of WT cells (left) or Nox2 knockout (KO) cells KO (right). Phorbol ester (PMA) was used to stimulate ROS production. (B) Systolic blood pressure (SBP) measured by tail cuff plethysmography in the different experimental groups. (C) Cardiac hypertrophy assessed by LV/body weight (BW) ratio. *, $P < 0.05$ for the comparisons shown; $n = 6$ /group.

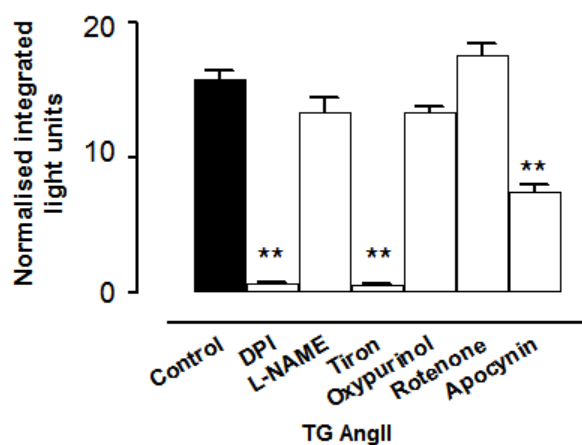
Supplementary Figure 10. Effect of NOX2 in bone marrow-derived inflammatory cells on AngII-induced fibrosis. (A) Representative Picrosirius Red-stained LV sections. Scale bars 100 μm . (B,C) Bottom, mean % fibrosis. See text for details of experimental groups. Horizontal lines above the columns in panel B denote significance at $P < 0.05$ level (*) by 1-way ANOVA; $n \geq 5$ /group.

Supplementary Figure 11. Effect of NOX2 on endothelial-mesenchymal transition in cultured endothelial cells. (A) Representative confocal images of EC stained for CD144 and SMA. Arrow indicates cell expressing both endothelial and mesenchymal markers. (B) Representative phase contrast images of EC after AngII treatment. Arrow indicates cell with typical elongated fibroblastic phenotype.

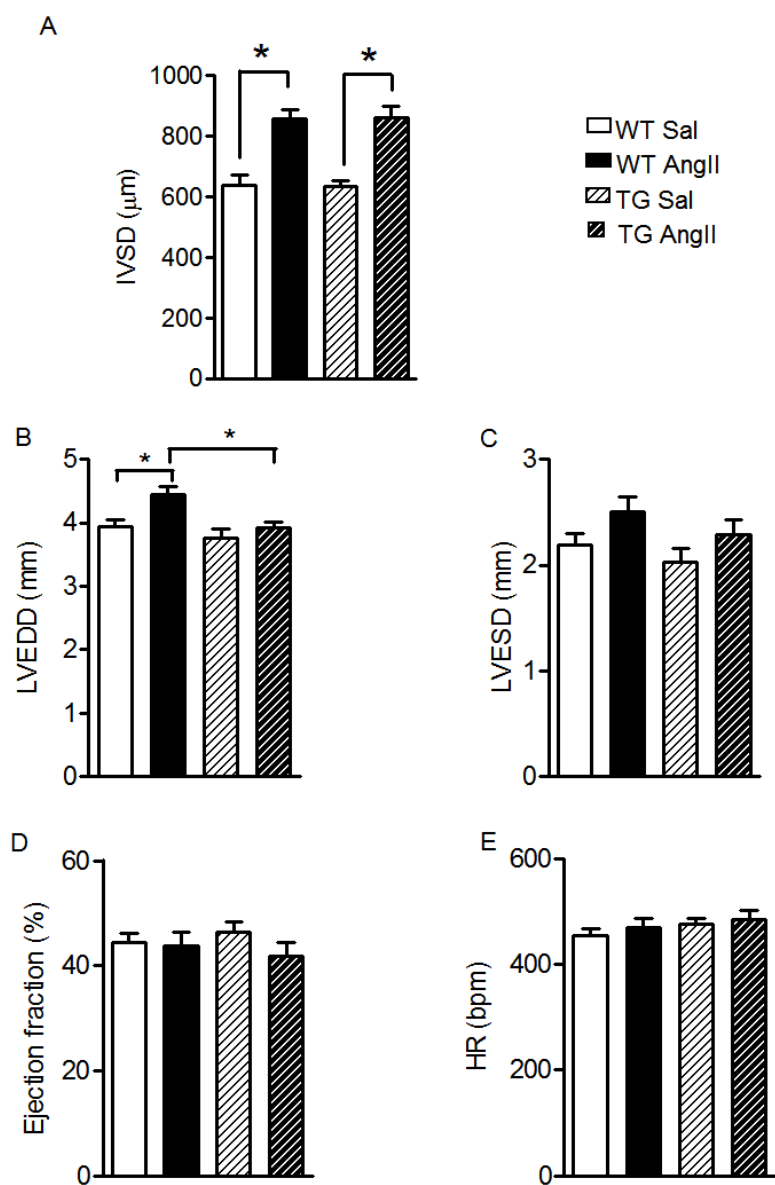
Supplementary Figure 12: Effects of endothelial NOX2 overexpression on fibroblasts in Transwell co-culture. Endothelial cells were infected with Ad.NOX2 or Ad. β -Gal and experiments were performed with or without AngII (100 nmol/L). (A) Fibroblast pro-collagen 1 mRNA expression levels. (B) Representative Western blots showing markers of myofibroblast transformation, smooth muscle actin (SMA) and SM22. (C, D) Mean data for experiment shown in panel B. $n = 4$ /group.



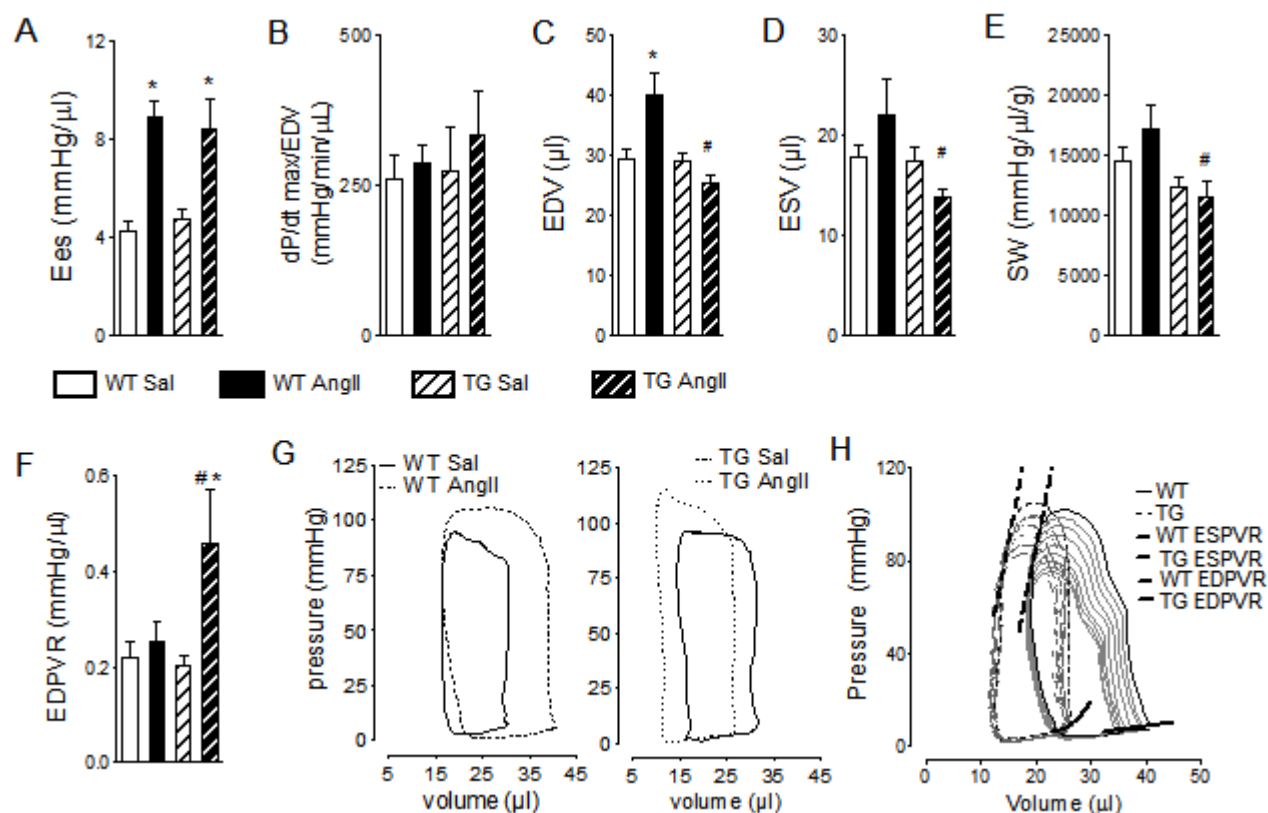
Supplementary Fig. 1



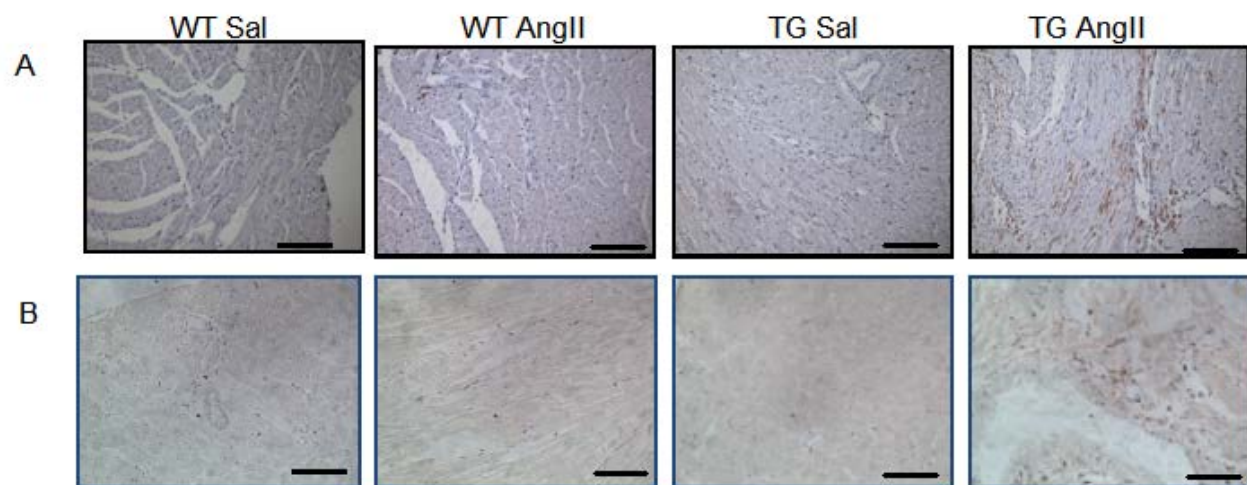
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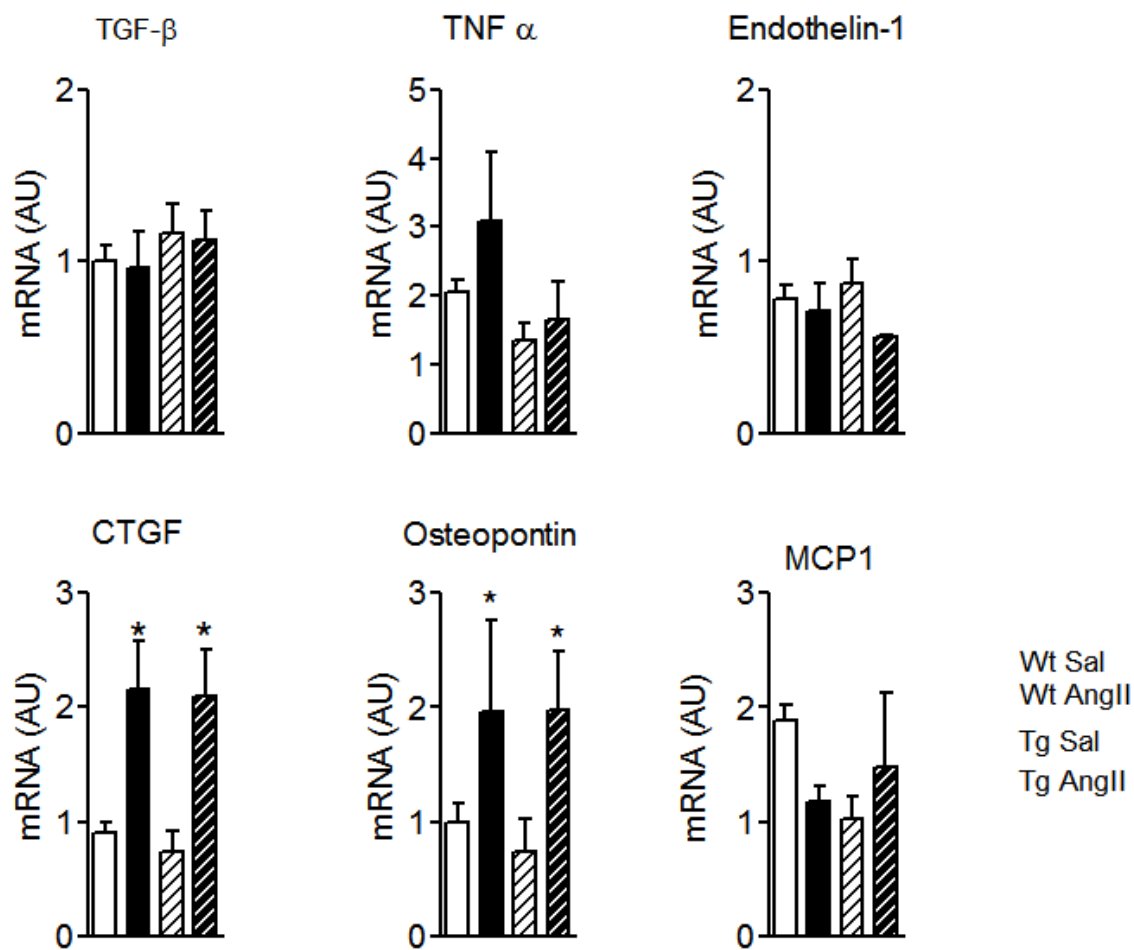
Supplementary Fig. 3



Supplementary Fig. 4

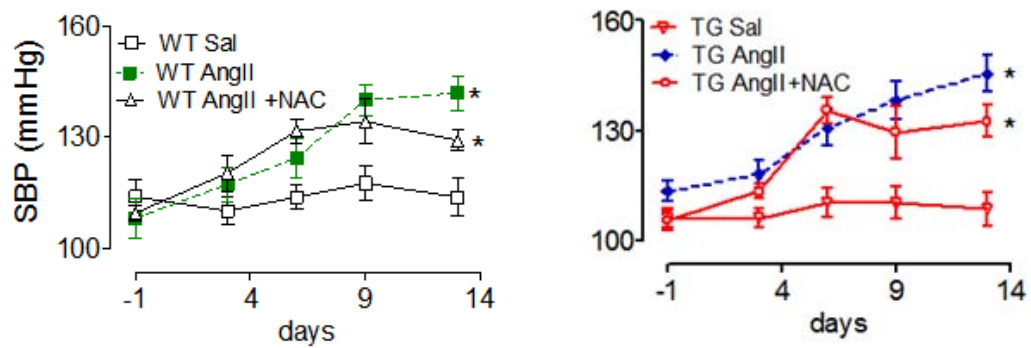


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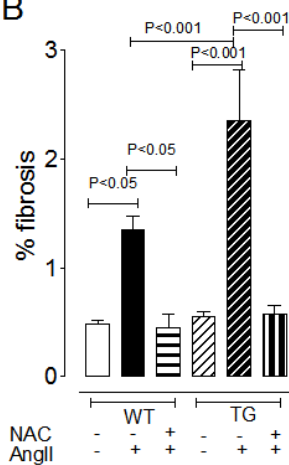


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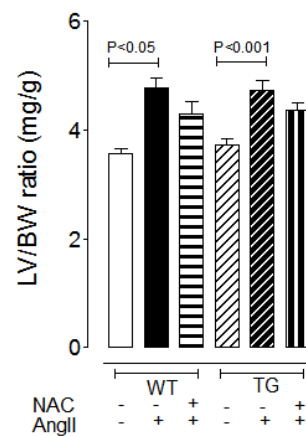
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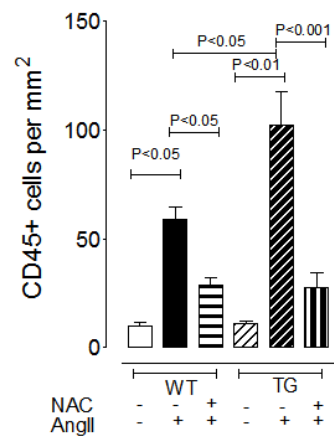
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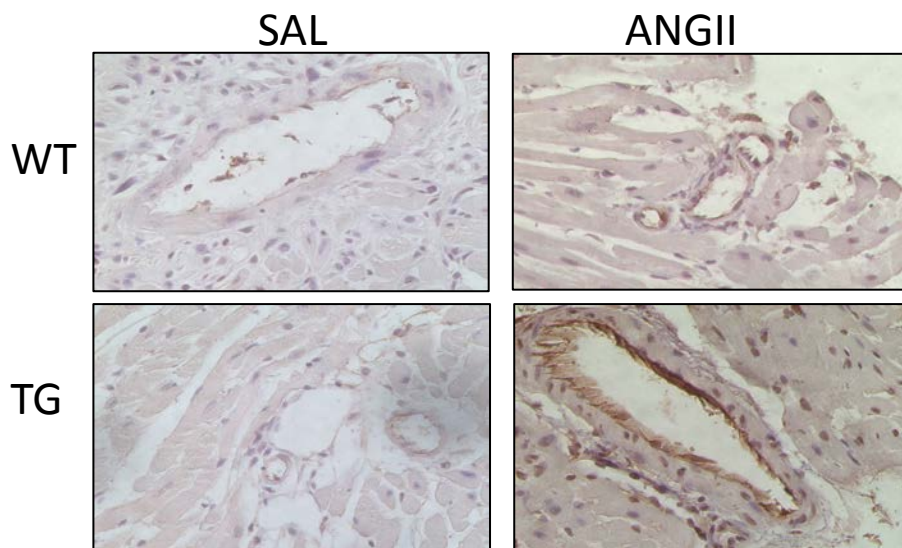
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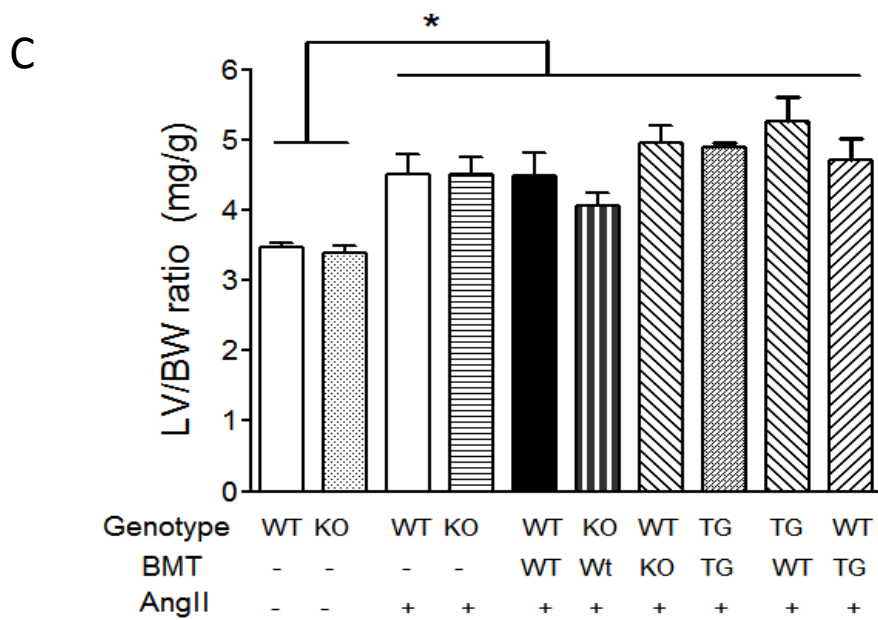
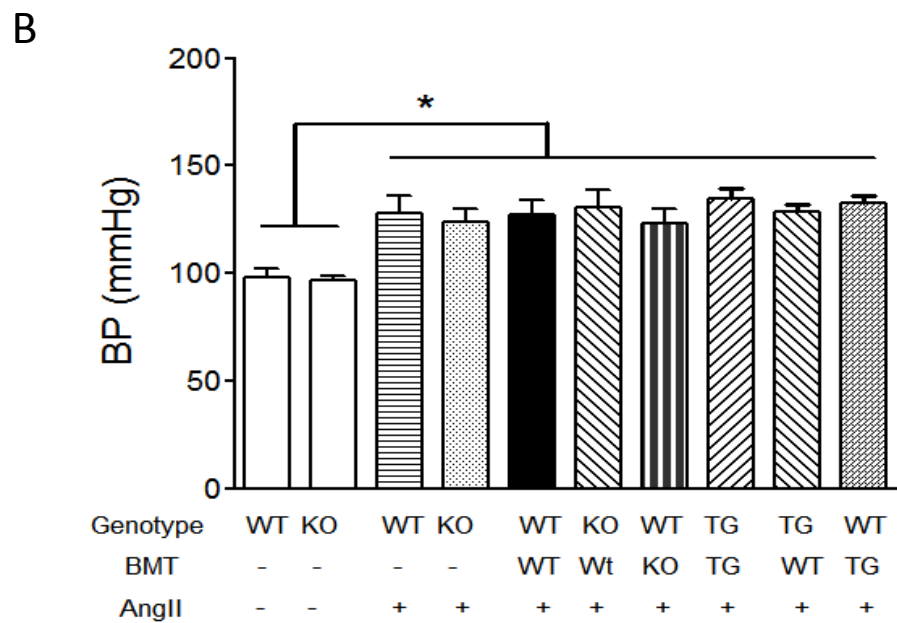
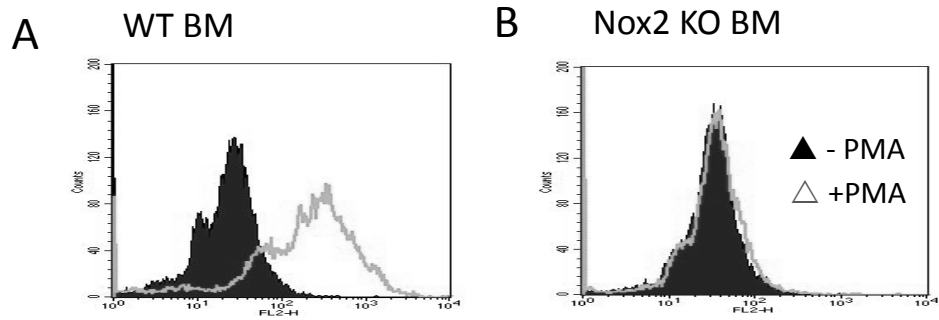
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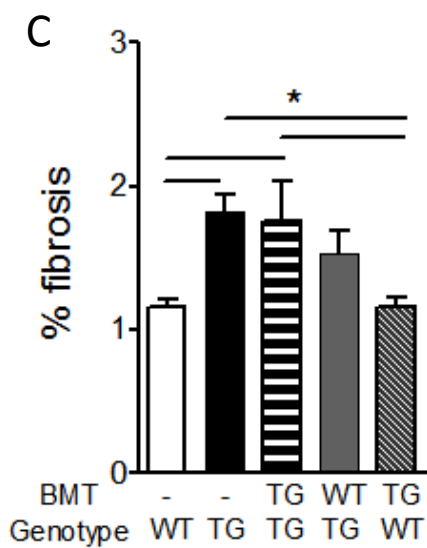
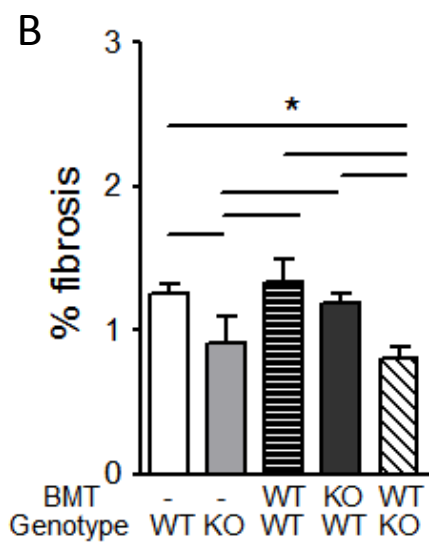
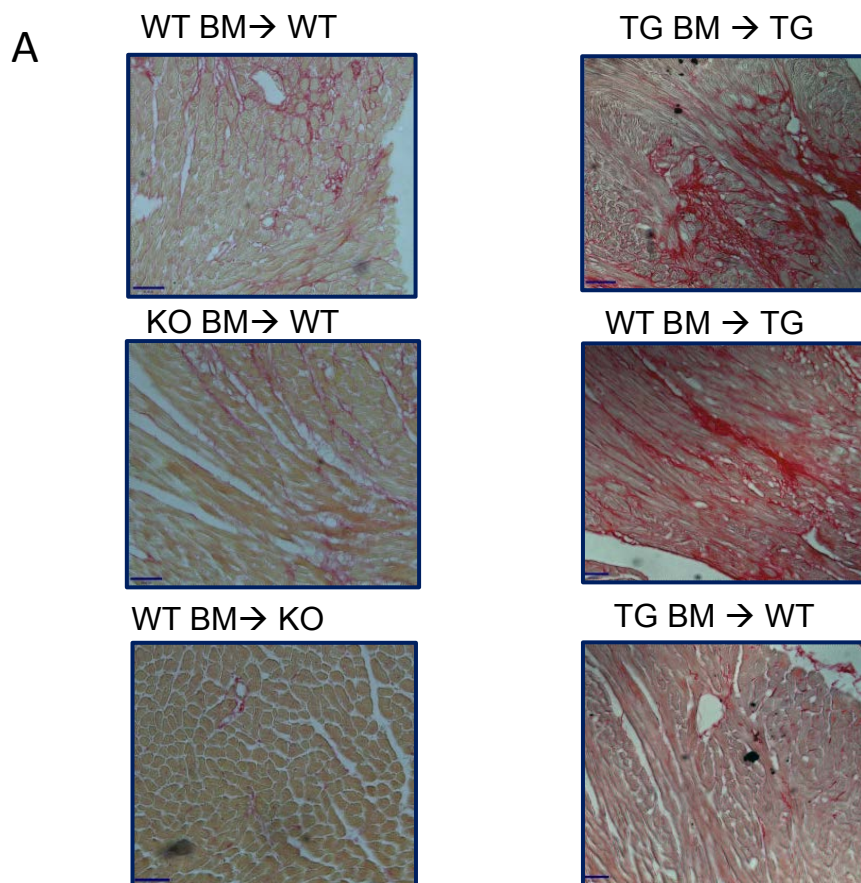


Supplementary Fig. 7

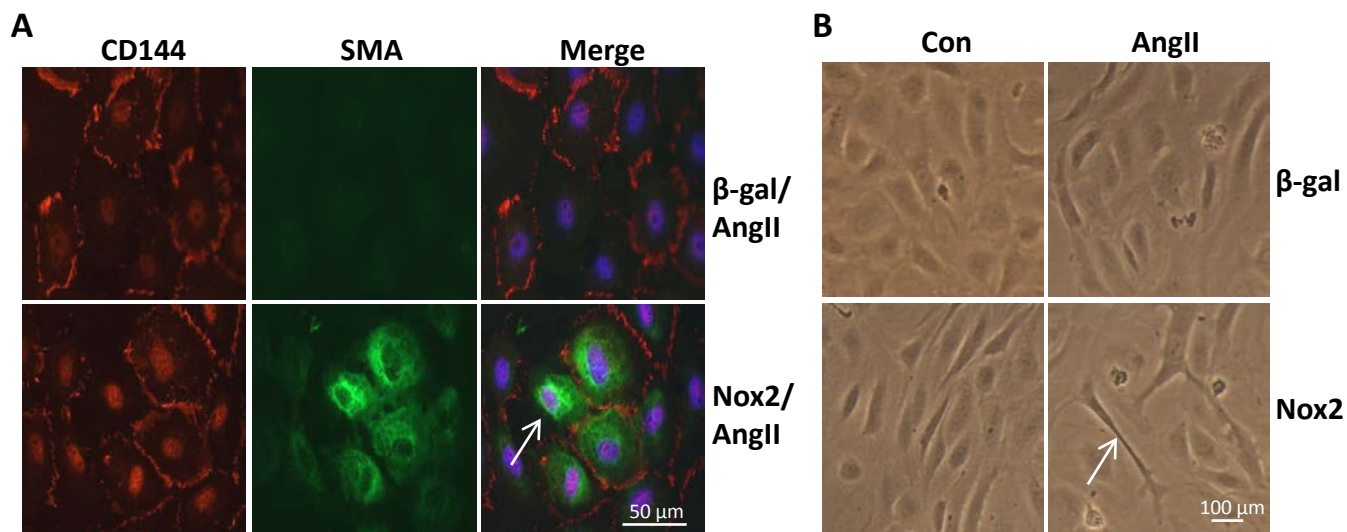


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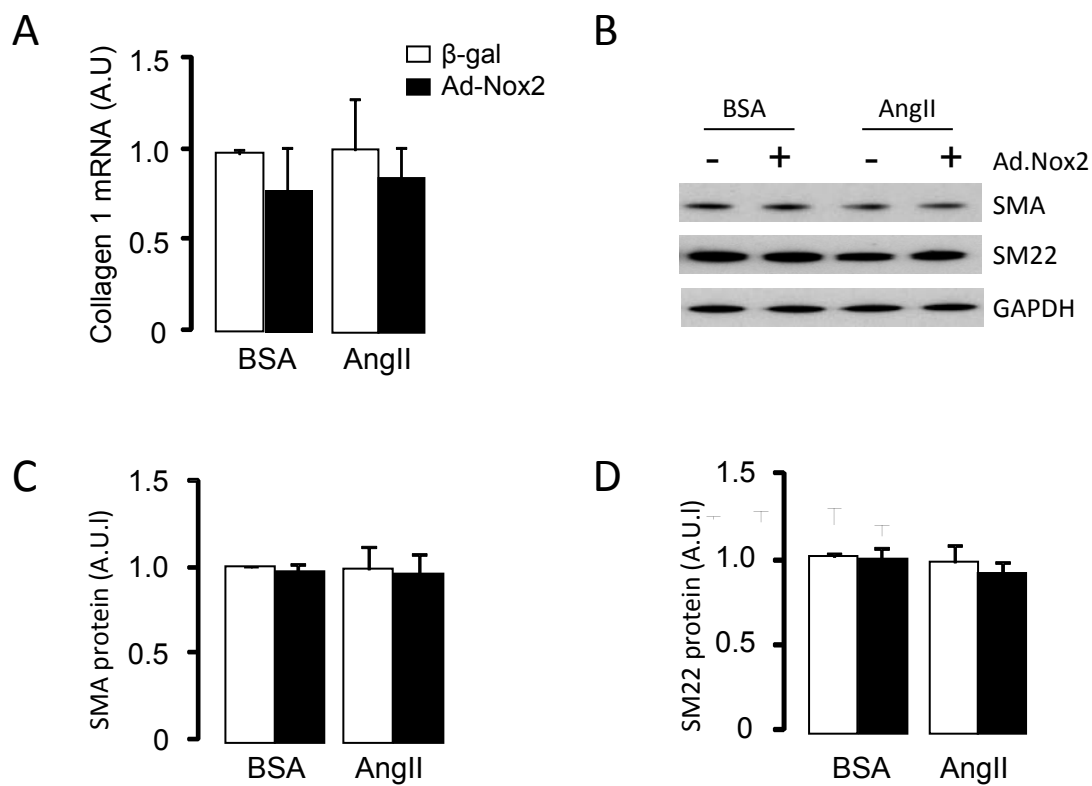




Supplementary Fig. 10



Supplementary Fig. 11



Supplementary Fig. 12



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Corresponding Author: Prof. Shah

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