

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Chan, EC;Peshavariya, HM;Liu, GS;Jiang, F;Lim, SY;Dusting, GJ

Title:

Nox4 modulates collagen production stimulated by transforming growth factor  $\beta$ 1 in vivo and in vitro

Date:

2013-01-18

Citation:

Chan, E. C., Peshavariya, H. M., Liu, G. S., Jiang, F., Lim, S. Y. & Dusting, G. J. (2013). Nox4 modulates collagen production stimulated by transforming growth factor  $\beta$ 1 in vivo and in vitro. Biochemical and Biophysical Research Communications, 430 (3), pp.918-925. <https://doi.org/10.1016/j.bbrc.2012.11.138>.

Persistent Link:

<https://hdl.handle.net/11343/44024>

## Accepted Manuscript

Nox4 modulates collagen production stimulated by transforming growth factor  $\beta$ 1 *in vivo* and *in vitro*

Elsa C Chan, Hitesh M Peshavariya, Guei-Sheung Liu, Fan Jiang, Shiang-Yong Lim, Gregory J Dusting

PII: S0006-291X(12)02400-X

DOI: <http://dx.doi.org/10.1016/j.bbrc.2012.11.138>

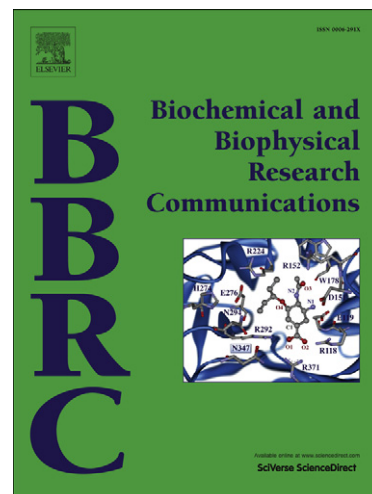
Reference: YBBRC 29706

To appear in: *Biochemical and Biophysical Research Communications*

Received Date: 27 November 2012

Please cite this article as: E.C. Chan, H.M. Peshavariya, G-S. Liu, F. Jiang, S-Y. Lim, G.J. Dusting, Nox4 modulates collagen production stimulated by transforming growth factor  $\beta$ 1 *in vivo* and *in vitro*, *Biochemical and Biophysical Research Communications* (2012), doi: <http://dx.doi.org/10.1016/j.bbrc.2012.11.138>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Nox4 modulates collagen production stimulated by transforming growth factor  $\beta$ 1 *in vivo* and *in vitro*

Elsa C Chan<sup>1\*</sup>, Hitesh M Peshavariya<sup>1</sup>, Guei-Sheung Liu<sup>1</sup>, Fan Jiang<sup>2</sup>, Shiang-Yong Lim<sup>3</sup>, Gregory J Dusing<sup>1</sup>

<sup>1</sup>Centre for Eye Research Australia (CERA), University of Melbourne, Victoria, Australia. <sup>2</sup>Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health, Qilu Hospital, Shandong University, Jinan, Shandong Province, China. <sup>3</sup>O'Brien Institute, Victoria 3065, Australia.

Address for correspondence: Elsa C Chan, CERA, 1/32, Gisborne Street, East Melbourne Victoria 3002, Australia.

Email: [elsa.chan@unimelb.edu.au](mailto:elsa.chan@unimelb.edu.au)

Phone: +613 9288 4018

Fax: +613 9416 0926

#### Abbreviations:

Ad-GFP, adenovirus expressing green fluorescent protein; Adv-LacZ, adenovirus expressing  $\beta$ -galactose; Adv-Nox4<sup>ΔNADPH</sup>, adenovirus carrying a dominant negative form of Nox4; Adv-Nox4i; adenovirus carrying siRNA targeting Nox4; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; MAPK, mitogen activated protein kinase; ROS, reactive oxygen species; TGF $\beta$ , transforming growth factor  $\beta$

## Abstract

The synthesis of extracellular matrix including collagen during wound healing responses involves signaling via reactive oxygen species (ROS). We hypothesized that NADPH oxidase isoform Nox4 facilitates the stimulatory effects of the profibrotic cytokine transforming growth factor (TGF)  $\beta_1$  on collagen production *in vitro* and *in vivo*. TGF $\beta_1$  stimulated collagen synthesis and hydrogen peroxide generation in mouse cardiac fibroblasts, and both responses were attenuated by a scavenger of superoxide and hydrogen peroxide (EUK-134). Furthermore, by expressing a dominant negative form of Nox4 (Adv-Nox4<sup>ΔNADPH</sup>) in fibroblasts, TGF $\beta_1$ -induced hydrogen peroxide production and collagen production were abrogated, suggesting that Nox4-dependent ROS are important for TGF $\beta_1$  signaling in collagen production. This was confirmed by the inhibitory effect of an adenovirus carrying siRNA targeting Nox4 (Adv-Nox4i) on TGF $\beta_1$ -induced collagen synthesis and expression of activated myofibroblasts marker smooth muscle alpha actin. Finally we used a mouse model of subcutaneous sponge implant to examine the role of Nox4 in the local stimulatory effects of TGF $\beta_1$  on collagen accumulation *in vivo*. TGF $\beta_1$ -induced collagen accumulation was significantly reduced when the sponges were instilled with Adv-Nox4<sup>ΔNADPH</sup>. In conclusion, Nox4 acts as an intermediary in the signaling of TGF $\beta_1$  to facilitate collagen synthesis.

**Keywords:** TGF $\beta$ ; Nox4; Collagen; Fibroblasts; Sponge implant

## Introduction

The synthesis of extracellular matrix is crucial for tissue repair and remodeling during normal wound healing responses. On the other hand, extensive accumulation of matrix proteins in fibrotic scars during pathological conditions such as pressure overload, myocardial infarction and lung inflammation tends to compromise normal tissue functions [1,2]. Fibroblasts and activated myofibroblasts identified in scarred tissues are responsible for matrix protein production [3,4]. Matrix protein collagen is predominantly found in fibrotic tissues and its synthesis is modulated by a profibrotic cytokine transforming growth factor  $\beta$  (TGF $\beta$ ) [1]. Indeed an exogenous application of TGF $\beta$  to incisional wounds accelerated collagen production and improved healing efficiency in rats [5]. In a clinical situation, there was a concomitant increase in gene expression of TGF $\beta$  and collagen 1 in endomyocardial biopsies from patients with dilated cardiomyopathy [6] while augmented expression of TGF  $\beta_1$  and its receptor was observed in human cardiac hypertrophy [7]. The intervention to block TGF $\beta$  might be considered as a strategy for suppressing matrix protein synthesis, but this would have undesired systemic side effects due to the multiple activity of TGF $\beta$  [8]. TGF $\beta$  has been shown to modulate a variety of biological and pathological responses such as vasculogenesis [9], inflammation [10] and cancer progression [11]. Therefore further characterization of the downstream signaling pathway of TGF $\beta$  in generation of matrix proteins might provide an alternative anti-fibrotic strategy for this large group of fibrotic disorders.

Recently, the generation of ROS from NADPH oxidase has been implicated in TGF $\beta_1$ -induced collagen and fibronectin expression in cultured mouse [12] and human [13]

cardiac fibroblasts and human lung fibroblasts [2]. Liu et al. [14] recently showed the inhibitory effect of oral administration of a NADPH oxidase inhibitor apocynin on pressure overload-induced collagen deposition and TGF $\beta$  expression in the myocardium of rabbits. Others demonstrated a reduction in interstitial fibrosis in the myocardium of Nox2 type NADPH oxidase-deficient mice following myocardial infarction [15] or pressure overload [16] in comparison to wildtypes. Targeted deletion of NADPH oxidase isoform Nox4 with tracheal administration of siRNA also suppressed bleomycin-induced lung fibrosis in mice [2]. However the precise role of NADPH oxidase in mediating TGF $\beta$ -induced collagen production is poorly defined. We therefore hypothesized that TGF $\beta$ -induced collagen synthesis is dependent on NADPH oxidase isoform Nox4-derived ROS generation in cultured mouse cardiac fibroblasts. We then used a murine model of sponge implant [17] to investigate whether an intervention targeting NADPH oxidase isoform Nox4 affects the local stimulatory effects of TGF  $\beta_1$  on collagen accumulation *in vivo*.

## Materials and Methods

### Cell culture

Cardiac fibroblasts were isolated from male mice (C57B/L6, 16-20 weeks old) and cultured and characterized as previously described [18]. Briefly, freshly excised mouse cardiac ventricles were minced and digested in serum free Dulbecco's Modified Eagle Medium (DMEM from Invitrogen, USA) containing collagenase I (1 mg/mL, Sigma-Aldrich, Australia) and trypsin (2 mg/mL, Sigma-Aldrich) at 37°C. The supernatant was

transferred to culture medium (DMEM supplemented with 10% fetal calf serum, penicillin (100 U/mL, Invitrogen) and streptomycin (100 µg/mL, Invitrogen) every 20–25 min, and cardiac fibroblasts collected from 3 digestive cycles were pelleted, resuspended in fresh culture medium, and cultured at 37 °C in 5% CO<sub>2</sub>. Cells cultured from passages 2-3 were used.

### **Treatment with pharmacological inhibitors**

Fibroblasts (10<sup>5</sup> cells/cm<sup>2</sup>) seeded in 6-well or 12-well plates were serum starved overnight prior to TGFβ<sub>1</sub> stimulation (10 ng/mL for 24 h). To examine a role of ROS generation and TGFβ signaling on collagen production, a mimetic of superoxide dismutase and catalase (EUK-134, 1 µM) and an inhibitor of TGFβ type 1 receptor ALK5 (SB431542, 10 µM) were applied 1 h before TGFβ<sub>1</sub> treatment. Treated cells were then harvested at the indicated time point as shown in Fig. S1 and subjected to Amplex® Red assay (Invitrogen) for hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) determination, RNA isolation, and Sircol assay (BiColor, UK) for soluble collagen production.

### **Adenovirus infection with Adv-Nox4<sup>ΔNADPH</sup> and Adv-Nox4i**

To suppress the ROS generating capacity of Nox4, we infected fibroblasts with adenoviruses carrying a dominant-negative mutant of Nox4 lacking the NADPH binding domain (Adv-Nox4<sup>ΔNADPH</sup>). To knock down Nox4 gene expression, we used adenoviral vectors expressing RNA interference targeting human Nox4 nucleotides 418–436 from the start codon (Adv-Nox4i). Adenovirus expressing β-galactose (Adv-LacZ) and GFP (Adv-GFP) was used as a control for Adv-Nox4<sup>ΔNADPH</sup> and Adv-Nox4i respectively.

Fibroblasts were infected in reduced serum medium (Opti-MEM, Invitrogen, USA) for 24 h at a multiplicity of infection (MOI) of 2500. Fibroblasts were then incubated in culture medium for 24 h and then serum-deprived overnight before TGF $\beta$ <sub>1</sub> stimulation (10 ng/mL for 24 h) prior to Amplex® Red assay, RNA isolation, and Sircol assays (Fig. S1).

### **Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) measurement**

Extracellular H<sub>2</sub>O<sub>2</sub> was detected using Amplex® Red assay as previously described [19]. Briefly, fibroblasts (10<sup>5</sup> cells/cm<sup>2</sup>) were seeded in 12-well plates overnight and then subjected to inhibitor treatment or adenovirus infection as described in Fig. S1. Following treatments, trypsinised cells were then suspended in Krebs-HEPES buffer (HBSS, in mM: NaCl 98.0, KCl 4.7, NaHCO<sub>3</sub> 25.0, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.5, D-glucose 11.1 and Hepes-Na 20.0) containing Amplex® Red reagent (10 mM). Fluorescence was then measured with excitation and emission at 480nm and 530nm respectively, using a Polarstar microplate reader (BMG Labtech, Germany) at 37°C. Fluorescence values were normalized to cell numbers determined by Alamar Blue cell viability assays as according to manufacturer's instructions (Life Technologies, USA).

### **Gene expression detected by real time-PCR**

Cells (10<sup>5</sup> cells/cm<sup>2</sup>) seeded in 6-well plates were subjected to inhibitor treatment or adenovirus infection (Fig. S1). Cells were then collected in 0.5 mL TriReagent and total RNA was extracted according to the manufacturer's protocol (Ambion, Austin, TX, USA). Total RNA was reverse-transcribed to cDNA (100 ng) using high capacity cDNA



reverse transcription kit (no. 4374996, Applied Biosystems, USA) at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. Real-time PCR reactions were performed in a 7300 real-time PCR system (Applied Biosystems) using TaqMan Universal PCR master mix and predesigned gene-specific probe and primer sets (TaqMan Gene Expression Assays, Life Technologies) for mouse Nox2 (Mm00432775\_m1), Nox4 (Mm00479246\_m1), collagen 1 $\alpha$ 2 (Mm01165187\_m1) and human Nox4 (Hs00418356\_m1 for determining Nox4 expression in cells infected with Ad-Nox4<sup>ANADPH</sup> illustrated in Fig. 3A). 18s (rat: Forward primer: 5-CGGCTACCACATCCAAGGAA-3; reverse primer: 5-GCTGGAATTACCGCGGCT-3; probe: 5-VIC-TGCTGGCACCAGACTTGCCCTCTAMRA-3 and human: Hs99999901\_s1 for human Nox4; purchased from Life Technologies) was used as the housekeeping gene.

### **Sircol collagen assay for acid-soluble collagen determination**

Sircol collagen assay was used to measure the release of newly synthesized acid-soluble collagen in the conditioned media [12,20]. Cells (10<sup>5</sup> cells/cm<sup>2</sup>) seeded in 6-well plates were treated with inhibitors or infected with adenoviruses (Fig. S1). The release of collagen in media was then determined using Sircol collagen assay according to manufacturer's instruction (Bicolor, UK) and normalized to the amount of proteins from cells using Bradford assays as described previously [21].

### **Murine subcutaneous sponge implant**

Animal study has been conducted in accordance with St Vincent's Hospital Animal

Ethics Committee guidelines (Melbourne, Victoria, Australia) and the Australian National Health and Medical Research Council guidelines for the care and health of animals. Polyvinyl alcohol sponge discs (8 mm diameter with 1 mm thickness, PVA Unlimited, Warsaw, IN, USA) immersed with Adv-LacZ ( $1 \times 10^8$  pfu/ $\mu$ L), Adv-LacZ+TGF $\beta_1$  (10 ng/mL), Adv-Nox4 <sup>$\Delta$ NADPH</sup> ( $1 \times 10^8$  pfu/ $\mu$ L) or Adv-Nox4 <sup>$\Delta$ NADPH</sup>+TGF $\beta_1$  (10 ng/mL) were placed subcutaneously in the dorsal skin of a male C57B/L6 mouse (16-20 weeks). Two weeks after sponge implant, animals were euthanized and sponges were harvested, cleaned of connective tissues and fixed in 4% paraformaldehyde overnight. Paraffin embedded sections (4  $\mu$ m) were stained with Masson trichrome to identify collagen deposition. The collagen content was assessed by video microscopy under 20x magnification with the computer-assisted stereological toolbox (CAST) system (Olympus, Albertsturd, Denmark) [22]. Fifty randomly selected fields with 12 points per field were counted. Collagen counts were then estimated by dividing the number of points in each of selected fields that fell randomly on collagen by the total number of points counted on a blinded manner. To identify endothelial cells and fibroblasts, sections were subjected to enzymatic-mediated antigen retrieval with 0.1% Proteinase K (pH 7.8) at 37 °C for 3 min or to heat-mediated antigen retrieval in the citrate buffer (pH 6 at 90°C for 30 min) respectively. Endogenous peroxidase activity was quenched with 3% H<sub>2</sub>O<sub>2</sub>. The sections were blocked with total protein block solution (Dako) or 10% swine serum (Dako) respectively for 30 min, and then incubated with rat anti-mouse CD31 for endothelial cells (1:150, 0.5 mg/mL, MEC13.3/#553370, BD Biosciences Pharmingen) or rabbit polyclonal vimentin antibody for fibroblasts (1:900, 4 mg/mL, ab7783, Abcam) for 1 h. Sections were then incubated with biotinylated rabbit anti-rat IgG for CD31 (1:200,

Dako) or swine anti-rabbit for vimentin (1:400; Vector Laboratories) for 30 min, followed by avidin-biotinylated-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) and detected by DAB chromogen (DAKO). Sections were counterstained with hematoxylin and mounted in DPX (VWR International, Poole, UK). Rat IgG and rabbit IgG (Dako) were used as negative controls.

### Statistical analysis

Data are expressed as mean  $\pm$  standard error of the mean (SEM). The mean data were analyzed with one-way analysis of the variance (ANOVA) followed by post-hoc Tukey analysis. A value of  $P < 0.05$  was regarded as statistically significant. All values are normalized to values obtained from corresponding control conditioned media without TGF $\beta_1$  treatment.

## Results

### Profibrotic effect of TGF $\beta_1$ is mediated by ROS in cardiac fibroblasts

TGF- $\beta_1$  (10 ng/mL) significantly increased collagen 1 mRNA level (Fig. 1A) and the release of newly synthesized collagen in the media (Fig. 1B). Such response was accompanied by an increase in H<sub>2</sub>O<sub>2</sub> generation (Fig. 1C). To clarify whether TGF $\beta_1$  effects were mediated via TGF $\beta$ /activin signaling, we co-treated the cells with TGF $\beta_1$  and the inhibitor of TGF $\beta$  type 1 receptor/ALK5 (SB431542, 10  $\mu$ M). SB431542 inhibited the stimulatory effects of TGF $\beta_1$  on collagen 1 mRNA expression (Fig. 1A), soluble collagen release (Fig. 1B) and H<sub>2</sub>O<sub>2</sub> production (Fig. 1C), confirming that TGF $\beta_1$

responses required ALK5 activation. To examine the role of ROS generation, fibroblasts were pretreated with a superoxide dismutase and catalase mimetic EUK-134 (1  $\mu$ M) to scavenge  $H_2O_2$ . EUK-134 abolished the induction of  $TGF\beta_1$  on collagen 1 gene expression (Fig. 1A), soluble collagen accumulation (Fig. 1B) and  $H_2O_2$  generation (Fig. 1C).

### **$TGF\beta_1$ upregulated Nox4 gene expression**

A previous study showed that NADPH oxidase was a major source of ROS generation in human cardiac fibroblasts [13] and we found that ROS production was implicated in  $TGF\beta_1$ -driven responses. We therefore determined which NADPH oxidase isoform was involved.  $TGF\beta_1$  did not affect Nox2 gene expression (Fig. 1D) but it significantly upregulated Nox4 mRNA expression (Fig. 1E) in cardiac fibroblasts. In contrast, Nox1 was not detected in mouse cardiac fibroblasts. Furthermore, Nox4 upregulation by  $TGF\beta_1$  was inhibited by co-treatment with SB431542 (Fig. 1E).

### **Suppressing Nox4 activity and gene expression reduced $TGF\beta_1$ mediated responses**

We next infected fibroblasts with an adenovirus carrying dominant-negative mutant of Nox4 lacking the NADPH binding domain (Adv-Nox4 <sup>$\Delta$ NADPH</sup>) to suppress ROS generation activity of Nox4. Over 90% of fibroblasts were infected with a control adenovirus carrying LacZ (Adv-LacZ) at a MOI 2500 (Fig. S2A). Furthermore, fibroblasts infected with Adv-Nox4 <sup>$\Delta$ NADPH</sup> (at MOI 2500) showed a remarkable elevation of dominant-negative mutant form of Nox4 mRNA (Fig. 2A), confirming a successful infection of fibroblasts with Adv-Nox4 <sup>$\Delta$ NADPH</sup>. Suppressing the ROS generating capacity

of Nox4 with Adv-Nox4<sup>ΔNADPH</sup> abolished the stimulatory effect of TGFβ<sub>1</sub> on H<sub>2</sub>O<sub>2</sub> production (Fig. 2B), collagen 1 gene expression (Fig. 2C) and release of soluble collagen (Fig. 2D). We also used an adenovirus carrying interference RNA against Nox4 (Adv-Nox4i) to suppress Nox4 gene expression and found that TGFβ<sub>1</sub>-induced responses were also inhibited. Following an infection of Ad-Nox4i, basal Nox2 gene expression (Fig. S3A) was not affected while Nox4 gene expression (Fig. S3B) was markedly reduced in cardiac fibroblasts, indicating the specificity of Adv-Nox4i. Adv-Nox4i significantly attenuated TGFβ<sub>1</sub>-induced H<sub>2</sub>O<sub>2</sub> generation (Fig. S3C), collagen 1 gene expression (Fig. S3D) and the myofibroblast differentiation marker smooth muscle alpha actin (Fig. S3E). In summary, TGFβ<sub>1</sub>-induced collagen synthesis required Nox4-derived H<sub>2</sub>O<sub>2</sub> production.

#### **Suppressing Nox4 activity attenuated TGFβ<sub>1</sub> mediated fibrogenesis *in vivo***

To substantiate that TGFβ<sub>1</sub>-induced collagen production was dependent on Nox4 activity *in vivo*, we used a murine subcutaneous sponge model to test whether Adv-Nox4<sup>ΔNADPH</sup> treatment affect collagen deposition in sponges instilled with TGFβ<sub>1</sub> (10 ng/mL). X-gal blue staining of Adv-LacZ treated sponges revealed the presence of adenovirus even after 14 days of sponge implant (Fig. S2B), confirming the presence of adenoviruses throughout the 14-day experimental period. Examination of Masson trichrome stained section (Fig. 3A) showed collagen deposition (in blue) and cell infiltration throughout the sponge matrix (identified in black). Higher magnification of the trichrome stained section revealed collagen accumulation around sponge matrix and blood vessels with red blood cells within the lumen (Fig. 3A). To characterize the cell types infiltrated the sponges,

sections were stained with vimentin for fibroblasts [23] and CD31 for endothelial cells. Vimentin-positive fibroblasts (Fig. 3B) and CD-31-positive endothelial cells (Fig. 3C) were detected in the interstices of sponge matrix. Quantification of collagen content in trichrome stained sections showed increases in collagen contents in sponges instilled with TGF $\beta_1$  (Fig. 4A, 4B and 4E). This response was abrogated by Adv-Nox4 <sup>$\Delta$ NADPH</sup> (Fig 4C, 4D and 4E).

## Discussion

Excessive accumulation of collagen in tissues during wound healing can be deleterious affecting normal tissue functions in diseases such as myocardial infarction [24]. In the present study, we demonstrated that Nox4 is an effector that facilitates TGF $\beta_1$ -induced collagen production in cultured cardiac fibroblasts. Furthermore, we showed for the first time that suppressing Nox4 with Adv-Nox4 <sup>$\Delta$ NADPH</sup> attenuated the stimulatory effect of TGF $\beta_1$  on collagen accumulation in sponges in mice. Given the limited therapeutic options for fibrotic disorders, targeting Nox4 could be a potential therapeutic strategy to treat pathological tissue fibrosis under the influence of TGF $\beta_1$  such as myocardial infarction.

NADPH oxidase is a multimeric enzyme system dedicated to ROS production and Nox4 has recently been implicated in the modulation of fibrogenic responses in murine models of pressure overload [16] and bleomycin-induced lung injury [2]. Accumulation of interstitial collagen in hypertrophic heart induced by aortic constriction was found to be reduced in mice with cardiac-specific deletion of Nox4 when compared to wildtypes

[16]. Little is known about how Nox4 affects the production of extracellular matrix such as collagen *in vivo*, apart from several *in vitro* studies illustrating a role of Nox4 signaling in the profibrotic activity of TGF $\beta$  [2,13]. TGF $\beta_1$  is a profibrotic cytokine implicated in fibrotic disorders [25] and it is also a profibrotic mediator of fibrosis-promoting peptides angiotensin II and aldosterone [1]. We therefore employed an *in vivo* murine subcutaneous sponge implantation model [17] to investigate this aspect. After 14 days post-implantation of sponges, immunohistochemical characterization of sponges demonstrated an invasion of fibroblasts and formation of blood vessels lining positive for the endothelial marker CD31. Therefore this model allows us to explore matrix protein deposition by infiltrated cells including fibroblasts in angiogenesis during wound healing response.

Deposition of collagen in the pressure overload model can be complicated by the contribution of other redox-sensitive profibrotic factors such as angiotensin II or shear stress [26]. On the other hand, the subcutaneous sponge model allows an instillation of TGF $\beta_1$  in sponges to examine the local stimulatory effect of TGF $\beta_1$  on collagen accumulation without undesired systemic effects. We demonstrated for the first time that suppressing Nox4 activity with Ad-Nox4<sup>ANADPH</sup> attenuated collagen synthesis driven by exogenous application of TGF $\beta_1$  *in vivo*. The efficiency of the adenovirus construct on blocking Nox4-mediated ROS generation has been confirmed in our systems and other previous studies [27,28]. This novel finding agrees with a recent study by Hecker et al. [2], who showed the inhibitory effect of tracheal administration of Nox4 siRNA on bleomycin-induced collagen accumulation in mouse lungs. TGF $\beta_1$  upregulation had been

implicated in bleomycin-induced lung injury but the level was not determined by Hecker et al. [2]. On the other hand, our study provides evidence that Nox4 is involved in the local stimulatory effects of TGF $\beta$ <sub>1</sub> on collagen accumulation *in vivo*.

Suppressing Nox4 with either Ad-Nox4<sup>ΔNADPH</sup> or Ad-Nox4i in cardiac fibroblasts suppressed the stimulatory effect of TGF $\beta$ <sub>1</sub> on H<sub>2</sub>O<sub>2</sub> production, collagen synthesis and expression of contractile smooth muscle alpha actin, indicating that Nox4-dependent ROS production facilitates TGF $\beta$ <sub>1</sub>-mediated profibrotic responses. In the present study, although ROS inducing enzymes Nox2 and Nox4 were both detected in mouse cardiac fibroblasts, we found that TGF $\beta$ <sub>1</sub> specifically stimulated Nox4 gene expression without affecting Nox2. The specific stimulatory effect of TGF $\beta$ <sub>1</sub> on Nox4 has also been demonstrated in human cardiac fibroblasts [13] and other cell types including mouse endothelial cells (Schroder et al. 2011). Amara et al. [29] recently showed that the gene and protein expression of Nox4, but not other Nox isotypes, was upregulated in pulmonary fibroblasts derived from patients with idiopathic pulmonary fibrosis, suggesting that upregulation of Nox4 would have a role in fibrotic responses.

TGF $\beta$ <sub>1</sub> is classically known to activate Smad2/3 proteins to induce cellular events leading to collagen production in fibroblasts [30]. Medina et al [31] recently found that TGF $\beta$  augmented protein expression of ALK5 and phosphorylation of Smad2 and Smad3 in intestinal myofibroblasts. The use of siRNA targeting these proteins also led to a reduction in collagen 1 gene expression [31], implicating TGF $\beta$ /ALK5/Smad pathway in collagen gene upregulation. We found that TGF $\beta$ -induced Nox4 gene upregulation and



ROS generation were inhibited by ALK5 inhibitor, suggesting that Nox4 activation occurs downstream of Smad. One way that ROS generation modulates collagen synthesis is by inducing phosphorylation of redox sensitive mitogen activated protein kinase (MAPK) involved in collagen synthesis. Hu et al. [12,20] demonstrated that overexpressing TGF $\beta$  in mouse cardiac fibroblasts induced phosphorylation of both p38 and p44/42 MAPKs and collagen production. The induced phosphorylation of MAPKs were inhibited by non-selective NADPH oxidase inhibitors apocynin and diphenylene iodonium [12,20], implicating a role of NADPH oxidase in modulating redox sensitive MAPKs to facilitate TGF $\beta$ <sub>1</sub>-mediated collagen synthesis. On the other hand, Cucoranu et al. [13] showed that Nox4 activation prolonged the phosphorylation of Smads 2/3 in human cardiac fibroblasts. Therefore TGF $\beta$ <sub>1</sub>-induced ROS generation through Nox4 activation may also act as a feed forward mechanism to maintain Smad activation, thereby facilitated collagen production in mouse cardiac fibroblasts. It should be noted that the decrease in collagen synthesis could be partly attributable to a reduction in the transformation of fibroblasts to activated myofibroblasts characterized by an expression of smooth muscle alpha actin [4]. Transformation of resident fibroblasts to activated myofibroblasts, is mediated by TGF $\beta$  signaling and is a key process during tissue fibrosis [32]. Activated myofibroblasts identified in healing wound has been shown to potentiate collagen production and participate in the contractile phase of tissue remodeling [4].

In summary, we provided the novel finding that Nox4 signaling facilitates TGF $\beta$ <sub>1</sub>-induced collagen synthesis *in vivo* in mice. Tissue fibrosis found in injured tissues such as myocardial infarction represents a large group of disorders that have limited treatment

options (Leask 2010). Targeting TGF $\beta$ <sub>1</sub> downstream via the profibrotic mediator Nox4 could be a potential anti-fibrotic therapy. Interestingly, Chablais and Jazwinska [23] recently identified the requirement of TGF $\beta$ /Smad signaling in the formation of collagen rich scars for the subsequent cardiac regeneration from an infarcted myocardium of zebrafish. Blocking TGF $\beta$  signals with SB431542 impaired the collagen synthesizing capacity of fibroblasts and myofibroblasts identified in infarcted zone during repair as well as proliferation of cardiomyocytes during the regeneration phase [23]. Whether Nox4 participates in TGF $\beta$ <sub>1</sub>-mediated scar-based repair and cardiac regeneration awaits further investigation but characterization of such signaling pathway may benefit regeneration of tissues for repairing fibrotic organs such as hearts following myocardial infarction.

### **Acknowledgments**

We thanked Professor John F Keaney from the University of Massachusetts Medical School, USA for providing Adv-Nox4i and Dr Barry Goldstein from Thomas Jefferson University, Philadelphia, USA for providing Adv-Nox4<sup>ANADPH</sup>. HMP is supported by Post-Doc Fellowship (PF 11M 6093) from National Heart Foundation Australia. F.J. is supported by a grant from Natural Science Foundation of China (grant number 81070164). The O'Brien Institute acknowledges the Victorian State Government's Department of Innovation, Industry and Regional Development's Operational Infrastructure Support Program.

## References

- [1] A. Leask, Potential therapeutic targets for cardiac fibrosis: TGFbeta, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation, *Circ Res* 106 (2010) 1675-1680.
- [2] L. Hecker, R. Vittal, T. Jones, R. Jagirdar, T.R. Luckhardt, J.C. Horowitz, S. Pennathur, F.J. Martinez, V.J. Thannickal, NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury, *Nat Med* 15 (2009) 1077-1081.
- [3] T.A. Wynn, Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases, *J Clin Invest* 117 (2007) 524-529.
- [4] B. Hinz, Formation and function of the myofibroblast during tissue repair, *J Invest Dermatol* 127 (2007) 526-537.
- [5] T.A. Mustoe, G.F. Pierce, A. Thomason, P. Gramates, M.B. Sporn, T.F. Deuel, Accelerated healing of incisional wounds in rats induced by transforming growth factor-beta, *Science* 237 (1987) 1333-1336.
- [6] M. Pauschinger, D. Knopf, S. Petschauer, A. Doerner, W. Poller, P.L. Schwimmbeck, U. Kuhl, H.P. Schultheiss, Dilated cardiomyopathy is associated with significant changes in collagen type I/III ratio, *Circulation* 99 (1999) 2750-2756.
- [7] G. Li, R.K. Li, D.A. Mickle, R.D. Weisel, F. Merante, W.T. Ball, G.T. Christakis, R.J. Cusimano, W.G. Williams, Elevated insulin-like growth factor-I and transforming growth factor-beta 1 and their receptors in patients with idiopathic hypertrophic obstructive cardiomyopathy. A possible mechanism, *Circulation* 98 (1998) II144-149; discussion II149-150.
- [8] L. Deelman, K. Sharma, Mechanisms of kidney fibrosis and the role of antifibrotic therapies, *Curr Opin Nephrol Hypertens* 18 (2009) 85-90.
- [9] E. Pardali, M.J. Goumans, P. ten Dijke, Signaling by members of the TGF-beta family in vascular morphogenesis and disease, *Trends Cell Biol* 20 (2010) 556-567.
- [10] C.B. Schmidt-Weber, K. Blaser, Regulation and role of transforming growth factor-beta in immune tolerance induction and inflammation, *Curr Opin Immunol* 16 (2004) 709-716.
- [11] M. Tian, J.R. Neil, W.P. Schiemann, Transforming growth factor-beta and the hallmarks of cancer, *Cell Signal* 23 (2011) 951-962.
- [12] C. Hu, A. Dandapat, L. Sun, J.A. Khan, Y. Liu, P.L. Hermonat, J.L. Mehta, Regulation of TGFbeta1-mediated collagen formation by LOX-1: studies based on forced overexpression of TGFbeta1 in wild-type and lox-1 knock-out mouse cardiac fibroblasts, *J Biol Chem* 283 (2008) 10226-10231.
- [13] I. Cucoranu, R. Clempus, A. Dikalova, P.J. Phelan, S. Ariyan, S. Dikalov, D. Sorescu, NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts, *Circ Res* 97 (2005) 900-907.
- [14] Y. Liu, H. Huang, W. Xia, Y. Tang, H. Li, C. Huang, NADPH oxidase inhibition ameliorates cardiac dysfunction in rabbits with heart failure, *Mol Cell Biochem* 343 (2010) 143-153.

- [15] Y.H. Looi, D.J. Grieve, A. Siva, S.J. Walker, N. Anilkumar, A.C. Cave, M. Marber, M.J. Monaghan, A.M. Shah, Involvement of Nox2 NADPH oxidase in adverse cardiac remodeling after myocardial infarction, *Hypertension* 51 (2008 ) 319-325.
- [16] J. Kuroda, T. Ago, S. Matsushima, P. Zhai, M.D. Schneider, J. Sadoshima, NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart, *Proc Natl Acad Sci U S A* 107 (2010) 15565-15570.
- [17] E.N. Unemori, L.S. Beck, W.P. Lee, Y. Xu, M. Siegel, G. Keller, H.D. Liggitt, E.A. Bauer, E.P. Amento, Human relaxin decreases collagen accumulation in vivo in two rodent models of fibrosis, *J Invest Dermatol* 101 (1993) 280-285.
- [18] S.Y. Lim, D.J. Hausenloy, S. Arjun, A.N. Price, S.M. Davidson, M.F. Lythgoe, D.M. Yellon, Mitochondrial cyclophilin-D as a potential therapeutic target for post-myocardial infarction heart failure, *J Cell Mol Med* 15 (2011) 2443-2451.
- [19] G. Liu, H. Peshavariya, H. M, A. Brewer, C. Chang, E.C. Chan, G. Dusting, Microphthalmia-associated transcription factor modulates expression of NADPH oxidase type 4: a negative regulator of melanogenesis, *Free Rad Biol Med* Accepted (2012).
- [20] N.K. Malavia, J.D. Mih, C.B. Raub, B.T. Dinh, S.C. George, IL-13 induces a bronchial epithelial phenotype that is profibrotic, *Respir Res* 9 (2008) 27.
- [21] H. Peshavariya, F. Jiang, C.J. Taylor, S. Selemidis, C.W. Chang, G.J. Dusting, Translation-linked mRNA destabilization accompanying serum-induced Nox4 expression in human endothelial cells, *Antioxid Redox Signal* 11 (2009) 2399-2408.
- [22] F. Jiang, G. Zhang, I. Hashimoto, B.S. Kumar, S. Bortolotto, W.A. Morrison, G.J. Dusting, Neovascularization in an arterio-venous loop-containing tissue engineering chamber: role of NADPH oxidase, *J Cell Mol Med* 12 (2008) 2062-2072.
- [23] F. Chablais, A. Jazwinska, The regenerative capacity of the zebrafish heart is dependent on TGFbeta signaling, *Development* 139 (2012) 1921-1930.
- [24] A. Leask, TGFβ, cardiac fibroblasts, and the fibrotic response, *Cardiovasc Res* 74 (2007) 207-212.
- [25] A. Biernacka, M. Dobaczewski, N.G. Frangogiannis, TGF-beta signaling in fibrosis, *Growth Factors* 29 (2011) 196-202.
- [26] D.J. Grieve, J.A. Byrne, A. Siva, J. Layland, S. Johar, A.C. Cave, A.M. Shah, Involvement of the nicotinamide adenosine dinucleotide phosphate oxidase isoform Nox2 in cardiac contractile dysfunction occurring in response to pressure overload, *J Am Coll Cardiol* 47 (2006 ) 817-826.
- [27] S.R. Datla, H. Peshavariya, G.J. Dusting, K. Mahadev, B.J. Goldstein, F. Jiang, Important role of Nox4 type NADPH oxidase in angiogenic responses in human microvascular endothelial cells in vitro, *Arterioscler Thromb Vasc Biol* 27 (2007) 2319-2324.
- [28] K. Mahadev, H. Motoshima, X. Wu, J.M. Ruddy, R.S. Arnold, G. Cheng, J.D. Lambeth, B.J. Goldstein, The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H<sub>2</sub>O<sub>2</sub> and plays an integral role in insulin signal transduction, *Mol Cell Biol* 24 (2004) 1844-1854.
- [29] N. Amara, D. Goven, F. Prost, R. Muloway, B. Crestani, J. Boczkowski, NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFbeta1-induced fibroblast differentiation into myofibroblasts, *Thorax* 65 (2010) 733-738.

- [30] A. Leask, D.J. Abraham, TGF-beta signaling and the fibrotic response, *Faseb J* 18 (2004) 816-827.
- [31] C. Medina, M.J. Santos-Martinez, A. Santana, M.C. Paz-Cabrera, M.J. Johnston, M. Mourelle, A. Salas, F. Guarner, Transforming growth factor-beta type 1 receptor (ALK5) and Smad proteins mediate TIMP-1 and collagen synthesis in experimental intestinal fibrosis, *J Pathol* 224 (2011) 461-472.
- [32] C.A. Souders, S.L. Bowers, T.A. Baudino, Cardiac fibroblast: the renaissance cell, *Circ Res* 105 (2009) 1164-1176.

**Figure 1. Effect of TGF $\beta$ <sub>1</sub> on collagen and hydrogen peroxide production and NADPH oxidase mRNA expression in mouse cardiac fibroblasts.** TGF $\beta$ <sub>1</sub> (T, 10 ng/mL) stimulated collagen 1 mRNA expression (A), release of soluble collagen in the culture media (B) and hydrogen peroxide generation (C). All responses were attenuated by an inhibitor of TGF receptors I activine link kinase SB431542 (SB, 10  $\mu$ M) and a superoxide dismutase and catalase mimetic EUK-134 (EUK, 1  $\mu$ M). Nox2 mRNA expression was unaffected by TGF $\beta$ <sub>1</sub> (D). TGF $\beta$ <sub>1</sub>-induced Nox4 mRNA upregulation was abrogated by SB431542 (E). \*P<0.05 from control; #P<0.05 from TGF $\beta$ <sub>1</sub> treatment alone, n= 4-8 experiments.

**Figure 2. Suppressing Nox4 with Ad-Nox4 <sup>$\Delta$ NADPH</sup> reduced TGF $\beta$ <sub>1</sub> driven responses in mouse cardiac fibroblasts.** Cardiac fibroblasts expressing Adv-Nox4 <sup>$\Delta$ NADPH</sup> (2500 MOI) showed a massive increase in Nox4 gene expression compared to control and LacZ treated cells (A). The stimulatory effect of TGF $\beta$ <sub>1</sub> (10 ng/mL) on hydrogen peroxide production (B), collagen 1 mRNA expression (C), release of soluble collagen in the culture media (D) was abolished by Adv-Nox4 <sup>$\Delta$ NADPH</sup> (MOI 2500). \*P<0.05 from control or Adv-LacZ, #P<0.05 from Adv-LacZ with TGF $\beta$ <sub>1</sub> treatment, n= 4 experiments.

**Figure 3. Histological examination of subcutaneous sponge implant in mouse.** Higher magnification of the box insert in (A), (B) and (C) is shown on the adjacent panels. Masson trichrome staining of a cross section (4  $\mu$ m) of sponges (A) and a higher magnification image revealed collagen accumulation (in blue) and a blood vessel

containing red blood cells (RBC) in the interstices of sponge matrix (in black). Immunohistochemical staining of sponge sections with vimentin for identifying fibroblasts (B) and CD-31 for characterizing blood vessels (C). Positive immunohistochemical staining is shown in brown (B, C).

**Figure 4. Effect of Adv-Nox4<sup>ΔNADPH</sup> on collagen deposition in a murine sponge model.** Masson trichrome staining of cross section (4 μm) of sponges implanted subcutaneously in mouse for 14 days. Sponges were treated with a control adenovirus (Adv-LacZ, 1x10<sup>8</sup> pfu) or a dominant negative mutant form of Nox4 (Adv-Nox4<sup>ΔNADPH</sup>, 1x10<sup>8</sup> pfu) to suppress its ROS generation capacity. Co-incubation of TGFβ<sub>1</sub> (10 ng/mL) in the sponges augmented collagen deposition and this response was impaired by Adv-Nox4<sup>ΔNADPH</sup>. Scale bar represents 50 μm). A bar graph summarizes the degree of collagen deposition in TGFβ<sub>1</sub> instilled sponges treated with Adv-LacZ and Adv-Nox4<sup>ΔNADPH</sup>. Collagenous connective tissue (arrow) is shown in blue while PVA sponge matrix is shown in black. \*P<0.05 from Adv-LacZ and #P<0.05 from Adv- LacZ +TGF, n= 6 experiments.

Figure 1

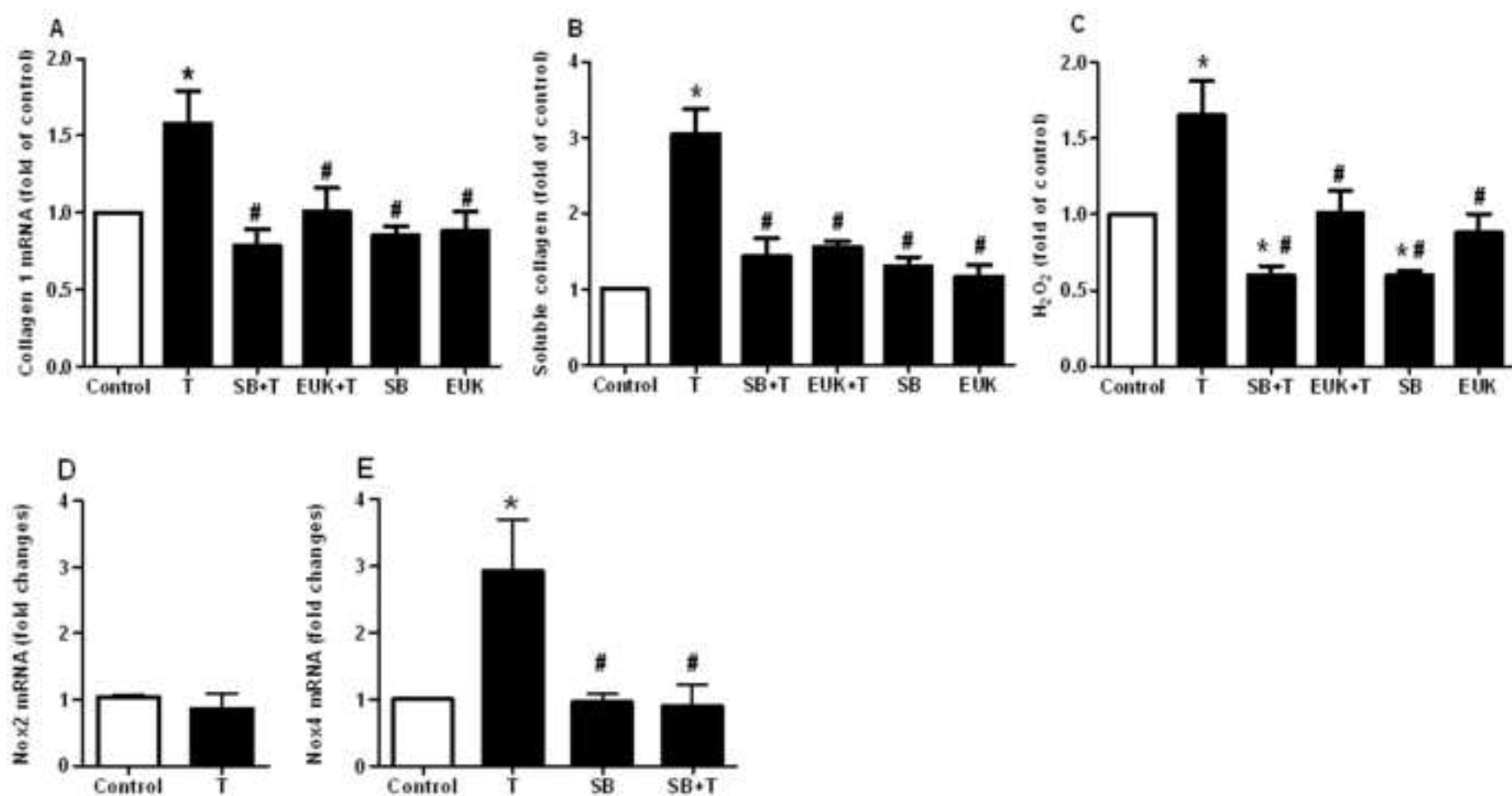




Figure 2

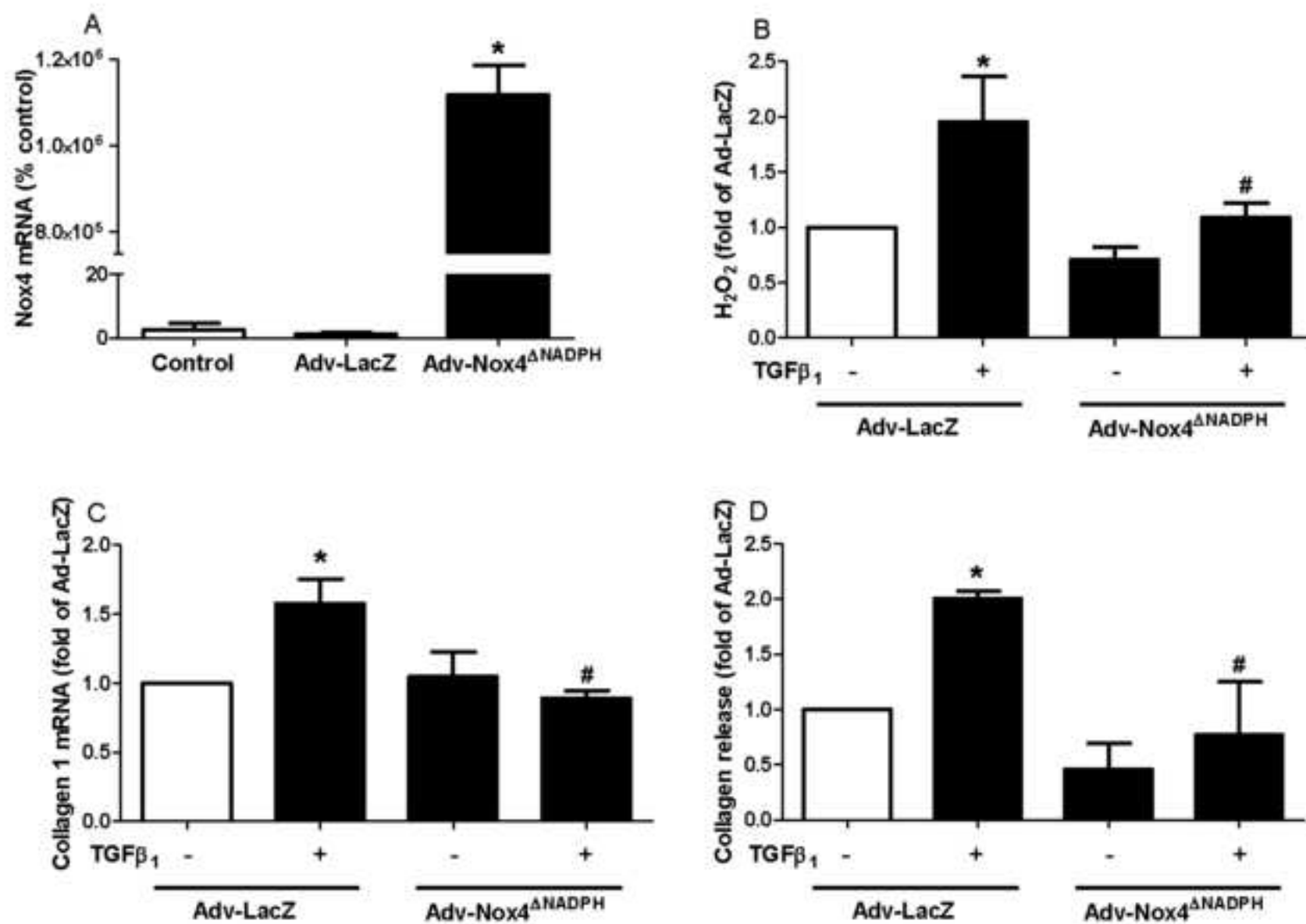




Figure 3

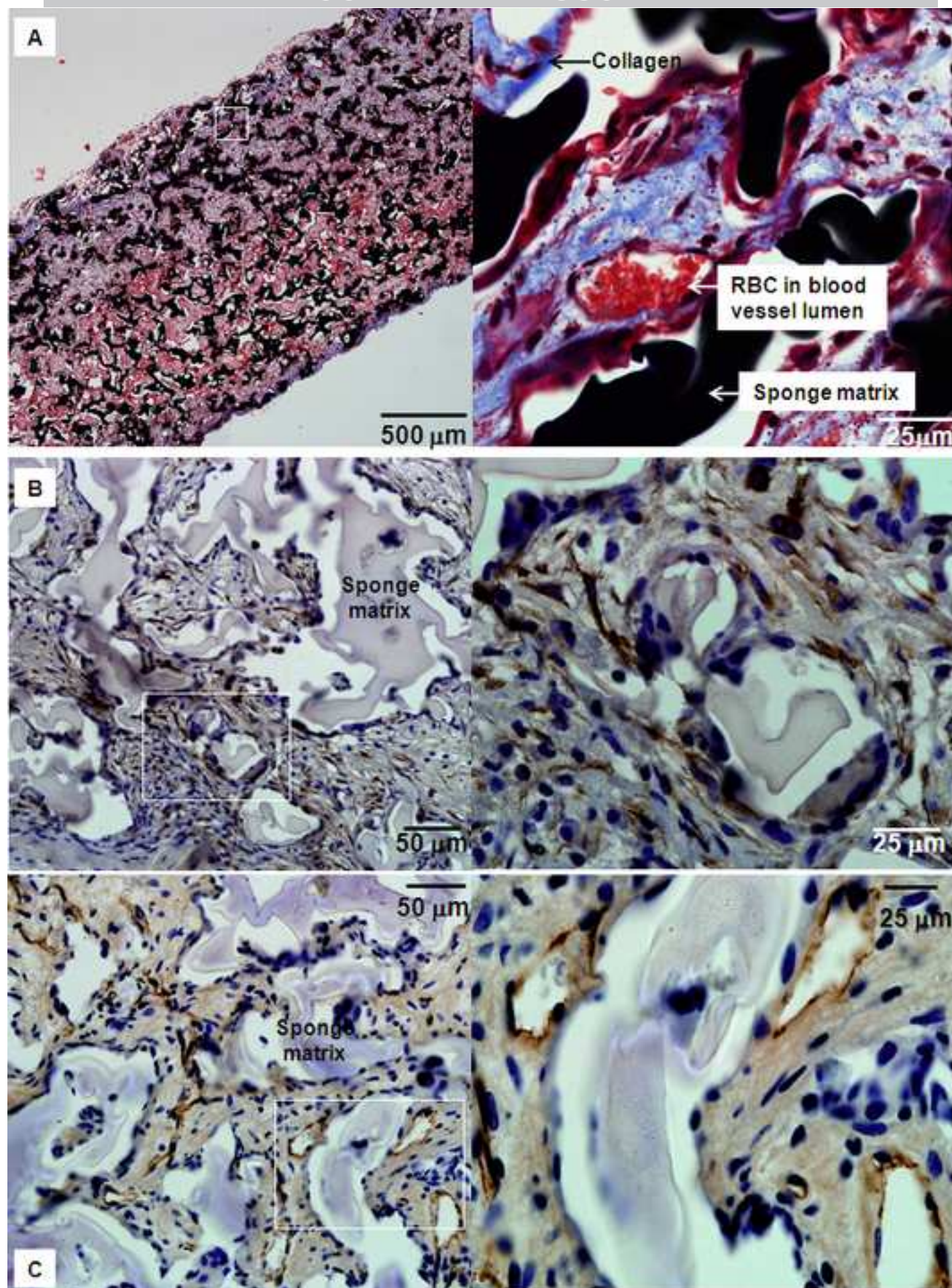
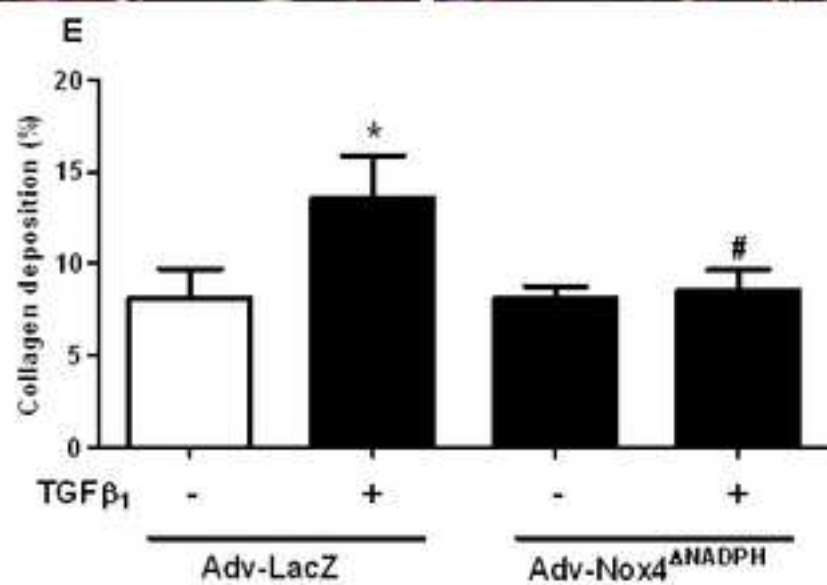
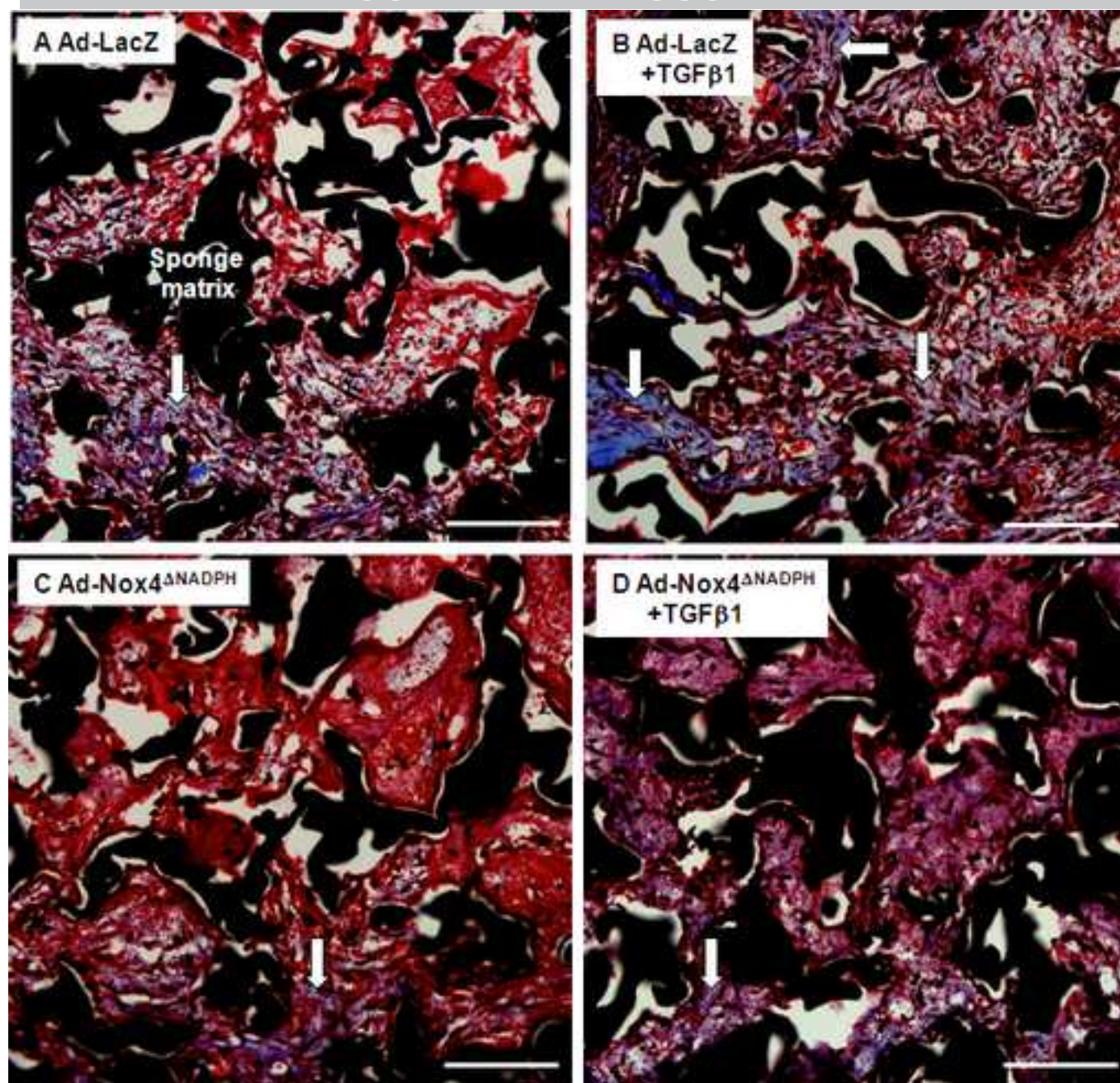




Figure 4

ACCEPTED MANUSCRIPT



- We examined the role of Nox4 on collagen accumulation *in vivo* and *in vitro*
- TGF $\beta$ <sub>1</sub>-induced collagen accumulation in sponges cotreated with Adv-Nox4<sup>ΔNADPH</sup> implanted in mice
- Suppressing Nox4 reduced collagen accumulation *in vivo*
- Inhibiting Nox4 gene expression and ROS release reduced collagen synthesis *in vitro*
- Nox4 acts as an intermediary effector to facilitate TGF $\beta$ -induced collagen synthesis