Supplemental Material

Research Design and Methods

Cell Culture: Aortic SMCs from 11 week old male ZL or ZO were cultured as previously described [1]. Four pairs of ZL and ZO aortic SMCs were isolated separately. Aorta without endothelium was transferred to DMEM with collagenase (3 mg/ml) for 30 min at 37°C and the adventitia was stripped off. The medial layer was minced into 1-2 mm pieces with scissors, and a solution of collagenase and elastase (1 mg/ml) was added and agitated by pipette every 15 min over one to two hours until a single cell suspension was achieved. The solution was centrifuged and cells were resuspended in DMEM supplemented with penicillin/streptomycin and 10% heat-inactivated FBS. Cells were sub-cultured by detachment with trypsin (0.025%, EDTA) and plating at a 1:4 split ratio. SMC were confirmed by α-smooth muscle actin positive staining. Cells from passages 1 to 4 were used.

Amplex red assay for ROS production [2]: Cells were grown on 12 well dishes. Cells were treated as described and washed once with DMEM without phenol red and 0.2% FBS, containing amplex red (50 μmol/L, Invitrogen), horse-radish peroxidase (2 U/mL) and 0.2% FBS with or without the presence of PEG-catalase (150 U/ml). After 45 min the supernatant was transferred to 96-well plates and H_2O_2-dependent oxidation of amplex red was measured by a microplate fluorimeter (excitation 540 nm, emission 580 nm). The data was calculated by subtracting the value obtained in the presence of catalase from the value without catalase.

Detection of mRNA levels for NADPH oxidase components by real time quantitative PCR: Total cellular RNA was isolated from cells using TRIzol according to the manufacturer's protocol. The first-strand complementary DNA (cDNA) was synthesized using Takara RNA PCR kit (Japan). Real time PCR was performed with synthetic gene-specific primers, according to the following schedule: denaturation, annealing, and extension at 95ºC, 55ºC and 72ºC for 30 seconds, 30 seconds, and 1 minute, respectively, for 40 cycles, and GAPDH acts as an internal control. The sequences of primers used here are listed in Online Table 1.

RNA interference [2]: To directly test the effect of Nox4, Nox4 small interfence RNA (siRNA) was applied to knockdown Nox4 in mRNA level. Scrambled control siRNA or Nox1 siRNA acted as controls. The siRNAs specific for rat Nox1 and Nox4 were synthesized by Invitrogen. The siRNA sequence for Nox1 is: 5'-GGUCGUGAUUACCAAGGUU-3'. The siRNA sequence for Nox4 is: 5'-GACCUGACUUUGUGAACAU-3'. When cells reached 80% confluence, they were washed with PBS, and cultured in DMEM containing siRNA (60 nmol/L) without serum and antibiotics. Cells were switched to DMEM containing 0.2%FBS 6 h later for 3 days.

Application of transforming growth factor-β1 (TGF-β1) in cultured aortic ZL SMCs: SMCs were seeded into 60 mm cell culture dishes in DMEM with 10% FBS. When they reach 80% confluence, cells were switched to media containing 0.2% FBS overnight. Human TGF-β1 (0.5 ng/mL, Sigma) was applied to cells; isotype matched irrelevant IgG was administered as a control. Three days later, cells were lysed in lysis buffer (Cell Signaling) containing 2 mM PMSF for immuno-blotting. For cell migration assays, ZL SMCs were seeded into 6-well cell culture plates in DMEM with 10% FBS. When cells were 80% confluent, cells were treated with TGF-β1 as described above for 3 days. In separate experiments, ZL SMCs were transfected with Nox4 siRNA for 6 h as mentioned above and then were switched to DMEM containing 0.2% FBS and TGF-β1 for 3 days.

Application of anti-TGF-β1 or SB203580 to cultured ZO aortic SMCs: ZO SMCs were seeded into 6-well cell culture plates in DMEM with 10% FBS. When cells were 80% confluent, anti-TGF-β1 monoclonal antibody (0.5 μg/mL, Sigma) or SB203580 (5 μM, Promega) was applied in DMEM with 0.2% FBS for 3 days. As a control treatment,

isotype matched irrelevant IgG was administered instead of anti-TGF-β1, and DMSO (0.1%) was applied instead of SB203580.

Adenovirus application: ZO SMCs were seeded into 6-well cell culture plates in DMEM with 10% FBS. When 80% confluent, cells were infected with lacZ, SERCA wild type (WT), or SERCA C674S adenovirus (1-10 pfu/cell) in DMEM with 0.2% FBS for 3 days. The SERCA C674S and wild type adenoviral constructs were published [3]. The virus titer was adjusted to yield the same amount of SERCA protein assessed by immunoblot. The infection had no notable effect on cell morphology.

Wounded monolayer migration assay in SMCs: The detailed methods were published [4]. Briefly, SMCs were seeded into 6-well cell culture plates in DMEM with 10% FBS. When they reach 80% confluent, different treatments were applied. DETA NONOate (300 μmol/L) was used as NO donor. Cells were allowed to migrate for 6 h after wounding.

Western blot analysis: Total SERCA (IID8 919, Affinity Bioreagent; 1:1000), SERCA C674SO_3H (Bethyl laboratory, Inc; 1:1000) [5], SERCA-294, 295 nY (Bethyl laboratory, Inc; 1:1000) [6], Nox4 (Norus; 1:1000), TGF-β1 precursor (Sigma; 1:1000), TGF-β1 (Novus;1:1000), Smad2/3 (Cell signaling; 1:1000) and phospho-Smad2 (Ser245/250/255, Cell signaling, 1:1000) were detected. Alpha-actin (Sigma; 1:5000) or GAPDH (Santa Cruz Biotechnology; 1:2000) were assessed for loading controls.

Adenoviral Nox4 short hairpin RNA (shRNA) constructs: Target sequences were identified using the Dharmacon website (www.dharmacon.com). Target sequences (AGACCTGGCCAGTATATTA) were chosen against rat Nox4 (GenBank accession number NM_053524), spanning regions of the mRNA transcript that are 100% identical in the rat and mouse. These sequences were chemically synthesized as complementary oligonucleotides (Integrated DNA Technologies), annealed and ligated into the pAdTrack-HP vector at the *Bgl*II and *Hind*III sites. pAd-HP/shNox4 constructs were sequenced for verification (Center for Functional Genomics, Rensselaer, NY). The oligonucleotides sequences are (5'-3'): GATCCCCAGACCTGGCCAGTATATTATTCAA GAGATAATATACTGGCCAGGTCTTTTTTGGAAA (top), AGCTTTTCCAAAAAAGA CCTGGCCAGTATATTATCTCTTGAATAATATACTGGCCAGGTCTGGG (bottom). These constructs were then linearized with *Pme*I and electroporated into AdEasier-1 cells which contain the AdEasy-1 plasmid. Successful recombinants were cloned and verified by restriction enzyme analysis, and the viral plasmids were linearized with *Pac*I and transfected into 293 cells with Effectene. After 8 days, cells were lysed in viral resuspension buffer (25mM Tris-HCl pH 8.0, 22.5mM NaCl) and subjected to two rounds of amplification in 293 cells.

SMAD reporter assay: HEK293T cells were transfected with SMAD reporter plasmid mix using Lipofectamine 2000 (Invitrogen) in 12 well plates according to the protocol. The SMAD reporter (SABiosciences) is a mixture of an inducible SMAD-responsive luciferase construct and a constitutively expressing Renilla construct (40:1). At 8 hours post transfection, media was changed and the cells were allowed to recover for 24 hours. Cells were then incubated with or without TGF-β1 (5 ng/ml) for 18 hours. In some group, SB203580 (5 μM) was applied 1h before TGF-β1 treatment. Cells were harvested and lysed 48 h after SMAD reporter transfection, and dual luciferase assays were performed according to the manufacturer's protocols (Promega). Luciferase activity was measured using a Turner ED-20e Luminometer and calculated as relative luciferase activity (firefly luciferase/*Renilla* luciferase.

Animal surgical procedure: 11 week old male obese Zucker rats were purchased from Charles River Laboratories (Boston, MA). All animal procedures were performed under aseptic conditions and this protocol was approved by Institutional Animal Care and Use Committee at the Boston University Medical Center. All rats arrived and were acclimatized for 3 days. The rats were anesthetized with intraperitoneal ketamine and

xylazine (90 mg/kg Ketamine + 4 mg/kg Xylazine). Balloon catheter injury of the left common carotid artery (CCA) was accomplished by denuding the endothelium with a 2Fr Fogarty balloon catheter (Edwards Lifesciences) that was introduced through a skin incision and via the external carotid artery. Inflation and retraction of the balloon catheter were repeated three times. Adenoviral Nox4 shRNA or GFP ($5x10^8$ pfu) was introduced into the lumen, and the CCA was incubated for 20 minutes without blood flow. Then the external carotid artery was tied off and viral solution was flushed out when blood flow was resumed. The right CCA underwent the same procedure without balloon catheter injury and adenovirus application. Vascular remodeling and immunohistochemistry was evaluated following euthanasia of the animal.

Tissue processing and Immunohistochemistry: Aorta and CCA were fixed in 10% buffered formalin acetate and embedded in paraffin. For CCA injured groups, about 0.5 cm length of the middle part of the whole injured artery were used for data analysis. The sham and injured CCA were embedded together for each animal. After removal of paraffin and rehydration, tissue sections (5 μm thick) were treated with 10 mmol/L citric acid (pH 6.0) and heated in a microwave (2 min, 3 times at 700 W) to recover antigenicity. Nonspecific binding was blocked with 10% normal goat or horse serum in phosphate-buffered saline (PBS, pH 7.4) for 30 min before incubation with individual primary antibodies. Primary antibodies against smooth muscle 22α actin (SM22α actin, 1:250), total SERCA (1:50), SERCA C674-SO_3H (1:100), TGF-β1 (1:25) and Nox4 (1:100) were used. The appropriate IgG isotype acted as negative control. The secondary antibody, a biotinylated anti-mouse or anti-rabbit IgG secondary antibody was used at 1:200. Vector Red alkaline phosphatase substrate or DAB peroxidase substrate (Vector Laboratory) was used to visualize positive immuno-reactivity. Staining of aortic and CCA smooth muscle was scored on a scale of 0-4 by 3 experienced people that were blinded to sample identity. The staining of injured CCA was normalized by its sham CCA of each animal. Hematoxylin/Eosin (HE) staining was routinely done to evaluate the lesion thickness. The intima and media thickness were measured separately along three cross lines (2, 4 and 6 o'clock) and the mean values were used for analysis.

Data analysis: Data are expressed as means ± SEM. Statistics were analyzed with SPSS 13.0 as indicated for each experiment, and statistical significance was accepted for a P value less than 0.05.

Online Table I. The primer sequence for real-time quantitative PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Nox1	AACAACAGCACTCACCAATG	TCAAGAAGGAAGCAAAGGG
Nox2	AAATGGATCGCATCTGTGTGAC	TGGCCACACTAACAGTGATTTAGAG
Nox4	TCAACTGCAGCCTGATCCTTT	CTGTGATCCGCGAAGGTAAG
$p22^{phox}$	CCAATTCCAGTGACAGATGAG	GGGAGCAACACCTTGGAAAC
$p47^{phox}$	CGACCATCCGCAATGCA	ACGCTGTTGCGGCGATAG
$p67^{phox}$	AGCTCCGGCTGGAACACACTA	TGGCCACACTAACAGTGATTTAGAG
rac1	GTAAAACCTGCCTGCTCATC	GCTTCGTCAAACACTGTCTTG
GAPDH	AACCCATCACCATCTTCCAGG	GGGGCATCAGCGGAAGG

Figure legends

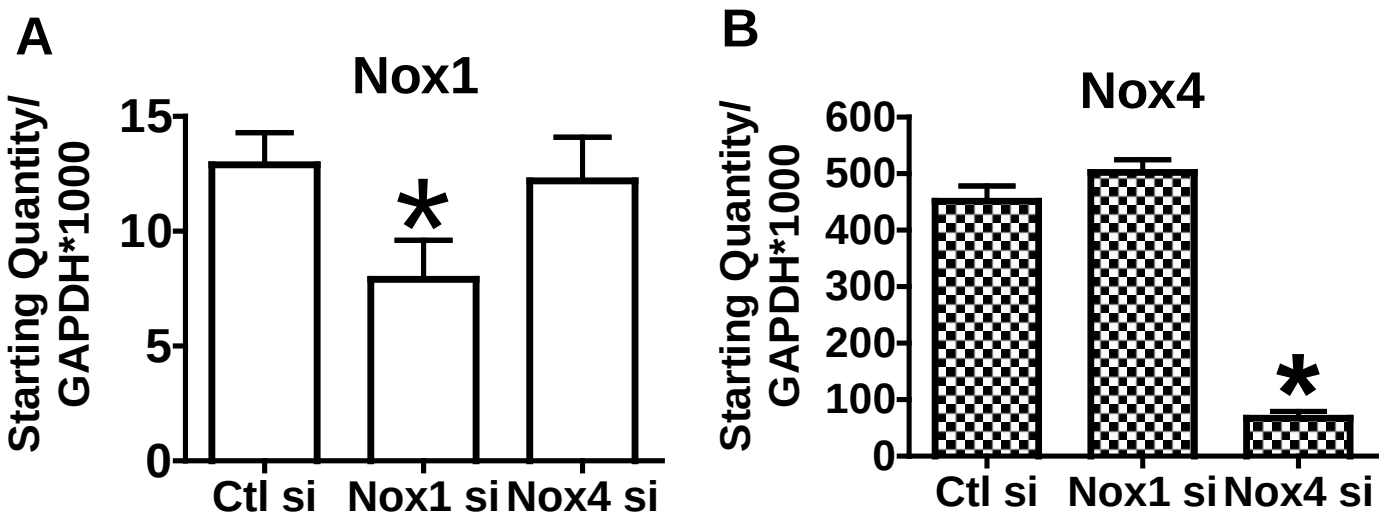
Online Figure I. A. Knockdown of Nox1 by Nox1 siRNA in obese Zucker rat (ZO) SMCs. **P*<0.05 compared with control siRNA, N=3, one-way ANOVA. B. Knockdown of Nox4 by Nox4 siRNA in ZO SMCs. **P*<0.05 compared with control siRNA, N=3, one-way ANOVA.

Online Figure II. A. Application of TGF-β1 increases ROS production in ZL SMCs. **P*<0.05 compared with the IgG control, N=3, paired *t*-test. B. Blockade of TGF-β1 by

anti-TGF-β1 antibody decreases ROS production in ZO SMCs. *P<0.05 compared with the IgG control, N=3, paired *t*-test.
Online Figure III. The Smad reporter assay in HEK293T cells. TGF-β1 **(5** ng/ml) and SB203580 (5 μM) was applied for 18h before Smad reporter assay which Smad reporter gene was transfected for 48 h.
Online Figure IV. Immunohistochemical staining of ZL and ZO aorta. A shows representative staining for TGF-β1, Nox4, total SERCA and SERCA-C674SO_3H in ZL and ZO aorta. The bar indicates 100 micron. B. The bar graph summarizes staining scores on 4 animals in each group. Both Nox4 and SERCA-C674SO_3H are significantly increased in ZO aorta compared with ZL aorta, while TGF-β1 showed similar tendency, but did not reach statistical significance. *P<0.05 compared with ZL, Student *t*-test. There was no staining with non-immune IgG isotype control primary antibody (data not shown).
Online Figure V. Knockdown of Nox4 by adenoviral Nox4 shRNA compared with GFP control in ZO SMCs.

Reference List

(1) Grainger DJ, Hesketh TR, Metcalfe JC, Weissberg PL. A large accumulation of non-muscle myosin occurs at first entry into M phase in rat vascular smooth-muscle cells. *Biochem J.* 1991;277 (Pt 1):145-51.
(2) Tong X, Schroder K. NADPH oxidases are responsible for the failure of nitric oxide to inhibit migration of smooth muscle cells exposed to high glucose. *Free Radic Biol Med.* 2009;47:1578-83.
(3) Ying J, Tong X, Pimentel DR, Weisbrod RM, Trucillo MP, Adachi T, Cohen RA. Cysteine-674 of the sarco/endoplasmic reticulum calcium ATPase is required for the inhibition of cell migration by nitric oxide. *Arterioscler Thromb Vasc Biol.* 2007;27:783-90.
(4) Tong X, Ying J, Pimentel DR, Trucillo M, Adachi T, Cohen RA. High glucose oxidizes SERCA cysteine-674 and prevents inhibition by nitric oxide of smooth muscle cell migration. *J Mol Cell Cardiol.* 2008;44:361-9.
(5) Ying J, Sharov V, Xu S, Jiang B, Gerrity R, Schoneich C, Cohen RA. Cysteine-674 oxidation and degradation of sarcoplasmic reticulum Ca(2+) ATPase in diabetic pig aorta. *Free Radic Biol Med.* 2008;45:756-62.
(6) Xu S, Ying J, Jiang B, Guo W, Adachi T, Sharov V, Lazar H, Menzoian J, Knyushko TV, Bigelow D, Schoneich C, Cohen RA. Detection of sequence-specific tyrosine nitration of manganese SOD and SERCA in cardiovascular disease and aging. *Am J Physiol Heart Circ Physiol.* 2006;290:H2220-H2227.



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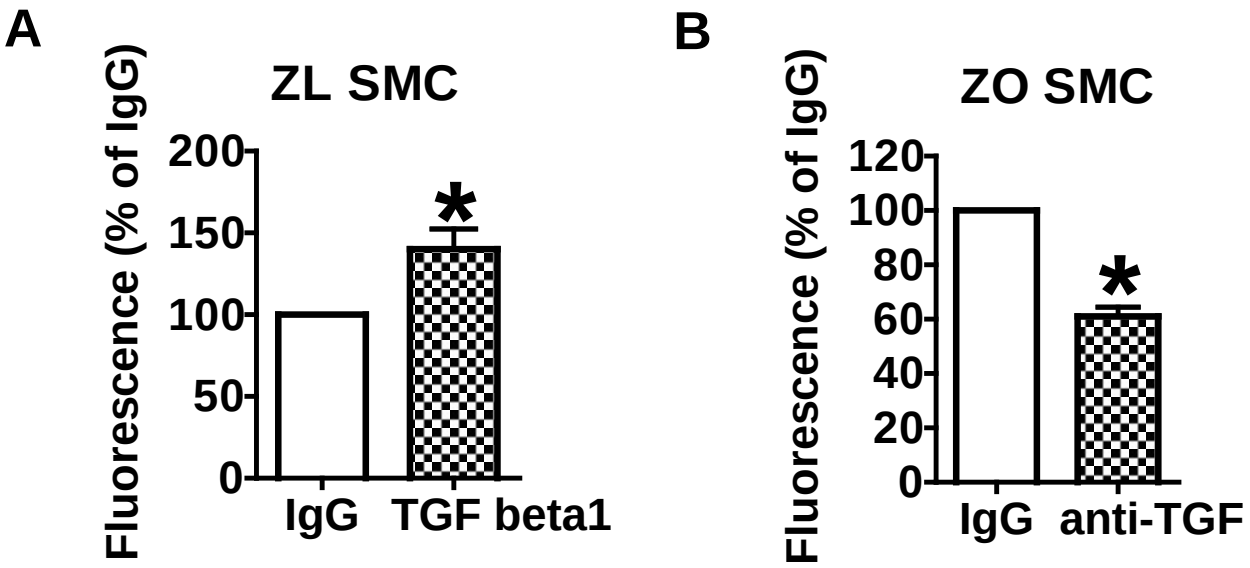

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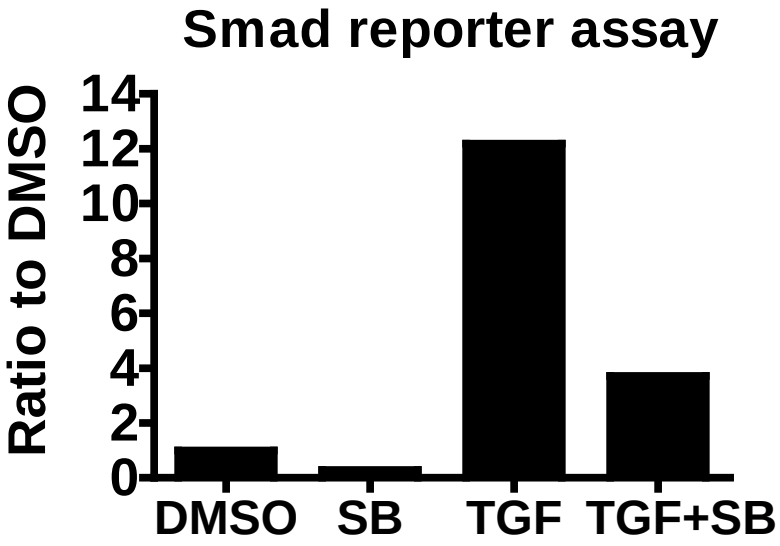

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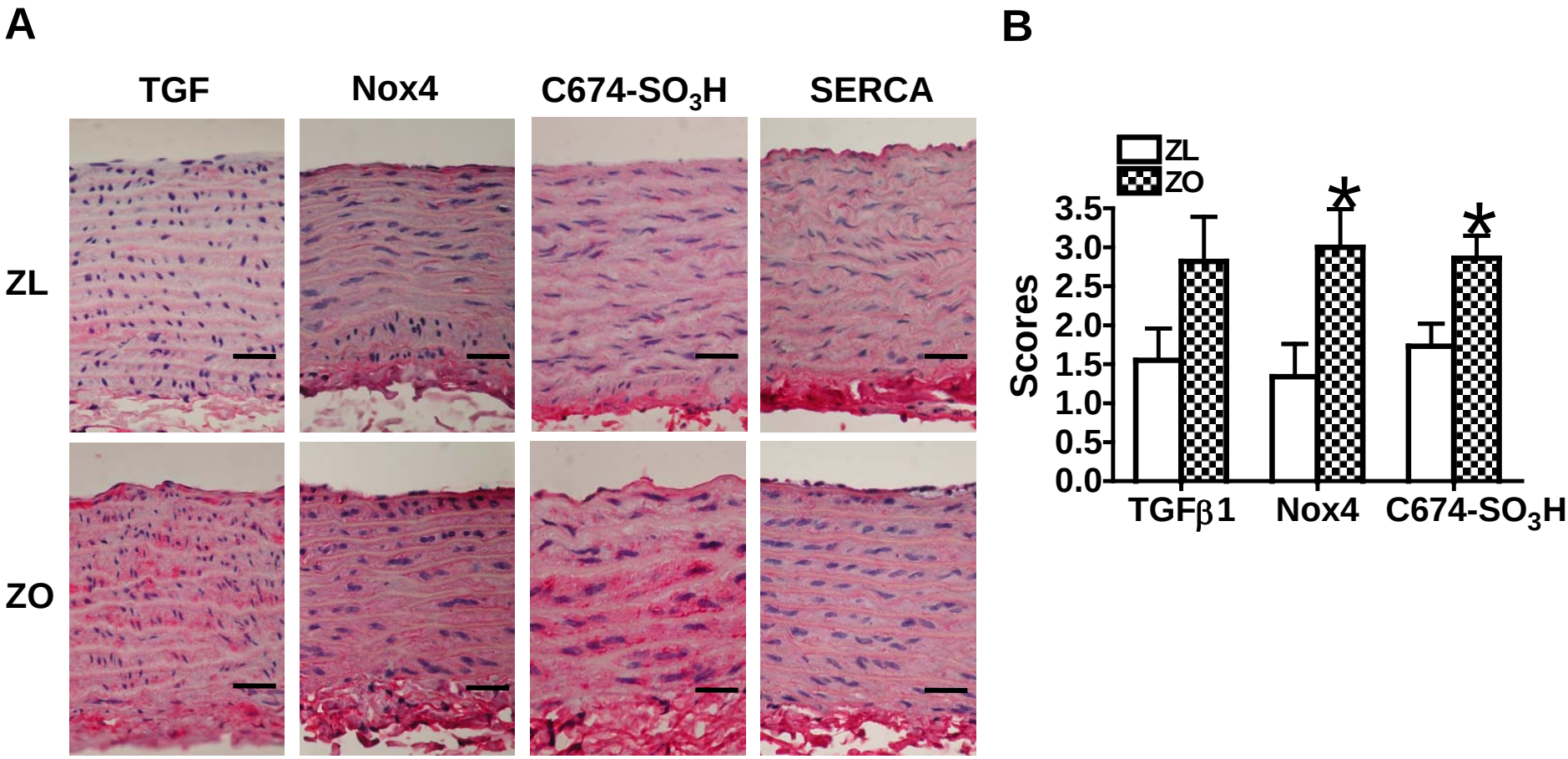

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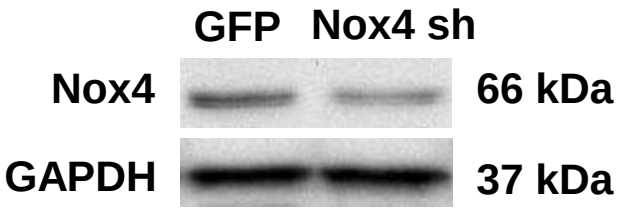

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