Online data supplement:

Materials and Methods:

Mice studied, and mouse SMC, aorta, and aortic ring organ culture studies: All animal procedures were humanely performed according to an institutionally reviewed protocol. In vitro analyses used tissues of congenic $TG2^{+/+}$ mice and congenic $TG2^{-/-}$ mice, originally on a hybrid C57BL6 / 129SVJ background (1) and crossed for more than 9 generations onto C57BL6. Primary SMCs were isolated at 2 months of age from mouse aortas, from which adventitia was removed and the aorta cut open to expose the endothelial layer (2). Tissues from 3 animals were pooled for digestion with 1 mg/ml collagenase I (Worthington Biochemical) for 10 min to remove remaining adventitia and endothelium, followed by placement in medium containing 2 mg/ml collagenase I, 25% elastase, and 20% FCS for 1.5 hours. Washed cells were plated in M231 medium (Cascade Biologics) containing SMC growth supplement (bFGF, EGF, insulin, 5% FCS) and staining for SM-actin (>95% positive) and VWF (< 1% positive) verified specificity of each SMC isolate. SMCs, on tissue culture plates coated with 1 $\mu g/cm^2$ murine laminin (Sigma-Aldrich) to promote maintenance of contractile differentiation state, were expanded for two passages before experimentation. Additionally, we maintained human aortic SMCs (Cascade Biologics) on laminin for fewer than 10 passages before we induced matrix calcification by adding both 2.5 mmol/L β-glycerolphosphate and 50 μg/ml ascorbic acid, or adding 10 ng/ml of recombinant BMP-2 (R&D Systems), to SMCs in growth medium. To detect calcifying multicellular nodules in SMC cultures, 0.5% Alizarin Red S, pH 4.0 was used to visualize deposited Ca^{2+} whereas the von Kossa method revealed deposited P_i. Multicellular von Kossa positive nodules were counted at 40X magnification and analyzed from 50 fields for each experimental condition.

Cultures of 2-3 mm aortic rings (3) were performed in the aforementioned SMC growth medium supplemented with 2.5 mmol/L sodium P_i and 7U/ml alkaline phosphatase for 7-9 days. To measure calcification, SMCs and aortic ring cultures were decalcified in 0.6 N HCl for 24 hours, and free calcium determined colorimetrically by a stable interaction with phenolsulphonethalein (Bioassay Systems) (4),

corrected for total protein concentration (SMCs) or dry weight (aortic rings). Alternatively, aortic ring explants were treated with 0.3 μCi/ml ^{45}Ca for 24 hours prior to collection and the incorporated ^{45}Ca was quantified by liquid scintillation counting (3). Where indicated, aortic ring explants were incubated with the TG2 catalytic site-specific and irreversible inhibitor Boc-DON-Gln-Ile-Val-OMe (5) from N-Zyme BioTec.

Where indicated, we studied freshly isolated whole aortas. To do so, the whole aorta (in replicates of 8 animals) was removed with adventitia and endothelial layer intact, and the aorta was extensively minced prior to isolation of total RNA using Trizol. For studies of freshly isolated SMCs without further culture, the whole aorta was dissected out, the adventitia was removed and the aorta was cut open to expose the endothelial layer. The aortas were then washed 3 times, followed by a 10 minute digestion in 1 mg/ml collagenase I in 1% FCS, DMEM high glucose and then digested with continuous agitation at 37^{o}C in 2 mg/ml collagenase I and 1.25 Units of elastase in 20% FCS, DMEM high glucose with 20% FCS for 1- 1.5 hours, followed by centrifugation and 3 washes in DMEM high glucose supplemented with 1% FCS. These SMC samples, from 3 digested aortas per tube and in replicates of 8, were examined by RNA isolation followed by qPCR.

Quantitative RT-PCR (qPCR): Total RNA was isolated, and for quantitative RT-PCR, 1 μl of a 5-fold dilution of the cDNA from reverse transcription reactions was amplified using the LightCycler FastStart DNA MasterPlus SYBR Green I kit (Roche Diagnostics) with addition of 0.5 μM of each primer (designed using Roche proprietary software) in the LightCycler 2.0 (Roche Diagnostics), with primers and accession numbers listed in Table 1. Relative quantification of the target gene and reference (GAPDH) analysis determined the normalized target gene: GAPDH mRNA copy ratios by the LightCycler Software (Version 4.0).

TG2 mRNA knockdown: Wild type and site mutant human TG2 and FXIIIA cDNA in pcDNA4/HisMax were used to generate recombinant His-tagged TG2 and FXIIIA (6). The shRNA specific for human TG2 (5'-GAGCGAGATGATCTGGAAC-3'(1116-1132)) was ligated into pSilencer 4.1-CMV (Ambion). Transient transfection of human aortic SMCs was performed using a Nucleofector

apparatus (AMAXA), employing the transfection reagent Fugene 6 (Roche), with ~60% transfection efficiency.

Immunohistochemistry: Sections (10 μm) of aortic ring cultures were fixed for 5 minutes in 4% paraformaldehyde, and to detect Types IX / XI collagen, sections were then permeabilized in 0.1% Triton X-100 for 5 minutes and blocked with Peroxoblock (Invitrogen) for 30 seconds and then 5% goat serum, 0.5% casein and 0.5% BSA for 30 minutes. AEC was used to detect the positive antigen staining using reagents from the Histostain Plus kit (Invitrogen). Identical methods in non-permeabilized cells were used to detect OPN, TG2 and type I collagen immunocytochemically. All light microscopy images were visualized on a Nikon microscope using the 4X and 10X objective lenses and with 10X binoculars, and Nikon digital camera images were captured using ACT-2U software. The JPEG images were cropped and arranged using Adobe Photoshop and Illustrator software.

Assays of TG2, PP_i, Alkaline phosphatase, OPN, OPG: TG transamidation activity was determined via incorporation of added biopentylamine (2 mmol/L) into 20 mg/ml casein. TG2 was quantified after binding to Immuno Module plates (Nunc) using biotin-labeled TG2-specific antibody CUB7402 (Neomarkers) and direct ELISA. Alkaline phosphatase and nucleotide pyrophosphatase phosphodiesterase specific activity in cell lysates was determined by colorimetric substrate assay, extracellular PP_i (normalized to cell DNA), and OPN were assayed in conditioned media collected from SMCs or aortic cultures as described (2). OPG ELISA was performed on conditioned media collected from SMCs according to the manufacturer's instructions (R&D Systems).

Analysis of FXIIIA protein expression: As a positive control for cellular FXIIIA expression, we used human chondrocytic CH-8 cells (7) and employed immunocytochemical staining for FXIIIA, as described (8).

Statistical Analyses: Error bars represented SD. Where indicated one-way analysis of variance ANOVA with Tukey's post hoc multiple comparison test (alternatively know as Tukey's HSD test) was performed. Where indicated, an independent samples t-test with Bonferroni correction addressed the differences in

mRNA levels between samples of mice of the distinct wild type and knockout genotypes and we limited those analyses to data for individual days in the time course.

Online Data Supplement References:

1. Nanda N, Iismaa SE, Owens WA, Husain A, Mackay F, Graham RM. Targeted inactivation of Gh/tissue transglutaminase II. *J Biol Chem.* 2001;276:20673-8.

2. Johnson K, Polewski M, van Etten D, Terkeltaub R. Chondrogenesis mediated by PP_i depletion promotes spontaneous aortic calcification in NPP1-/- mice. *Arterioscler Thromb Vasc Biol.* 2005;25:686-91.

3. Lomashvili KA, Cobbs S, Hennigar RA, Hardcastle KI, O'Neill WC. Phosphate-induced vascular calcification: role of pyrophosphate and osteopontin. *J Am Soc Nephrol.* 2004;15:1392-401.

4. Jono S, McKee MD, Murry CE, Shioi A, Nishizawa Y, Mori K, Morii H, Giachelli CM. Phosphate regulation of vascular smooth muscle cell calcification. *Circ Res.* 2000;87:E10-7.

5. Madi A, Karpati L, Kovacs A, Muszbek L, Fesus L. High-throughput scintillation proximity assay for transglutaminase activity measurement. *Anal Biochem*. 2005;343:256-62.

6. Johnson KA, Terkeltaub RA. External GTP-bound transglutaminase 2 is a molecular switch for chondrocyte hypertrophic differentiation and calcification. *J Biol Chem.* 2005;280:15004-12.

7. Burton DW, Foster M, Johnson KA, Hiramoto M, Deftos LJ, Terkeltaub R. Chondrocyte calcium-sensing receptor expression is up-regulated in early guinea pig knee osteoarthritis and modulates PTHrP, MMP-13, and TIMP-3 expression. *Osteoarthritis Cartilage*. 2005;13:395-404.

8. Johnson K, Hashimoto S, Lotz M, Pritzker K, Terkeltaub R. Interleukin-1 induces pro-mineralizing activity of cartilage tissue transglutaminase and factor XIIIa. *Am J Pathol.* 2001;159:149-63.

Table 1: Primers designed for qPCR analyses. All primers were from the murine sequences.

F, Forward primer; R, Reverse primer.

NAME	SEQUENCE	ACCESSION NUMBER Designed / BLAST result
Aggrecan F	5'-TTCCATCTGGAGGAGAGGG-3'	NM_007424
Aggrecan R	5'-ATCTACTCCTGAAGCAGATGTC-3'	
Elastin F	5'-GGAGCAGTACCTGGATCG-3'	NM_007925
Elastin R	5'-TCCTGCTACTCTACCGGGA-3'	
Factor XIIIA F	5'-CCGTTACACCATCACAGCTTA-3'	M14354
Factor XIII R	5'-AGCTGACCCATGTACTCG-3'	
GAPDH F	5'-CATCCCAGAGCTGAACG- 3	DQ403054 /NM_199472
GAPDH F	5'-CTGGTCCTCAGTGTAGCC-3'	
MGP F	5'- GTGGCAACCCTGTGCTAC-3'	NM_008597
MGP R	5'- CAGGCTTGTTGCGTTCC-3'	
MSX2 F	5'- GAGCCCGGCAGATACTC-3'	NM_013601
MSX2 R	5'- CCCGCTCTGCTATGGAC-3'	
Myocardin F	5'-GTATTCATCCAAAGATGACTGGTTTAC-3'	NM_145136
Myocardin R	5'-AGTCATTTGCTGCTTCACT-3'	
Myosin Light Chain Kinase (MLCK)-210 F	5'- TGGGAAGACACTCAAGACCAC-3'	NM_139300
MLCK-210 R	5'- ACAAACCTCGGTCCTCAG-3'	
Notch 3 F	5'-AGCCATGTCAGCATGGAG-3'	X74760 / NM_00876
Notch 3 R	5'-CTGTCTGCTGGCATGGGATA-3'	
OPG F	5'-ACCTTGAGGGCCTGAT-3'	NM_008764
OPG R	5'- ACCTTGAAGGGCCTGAT-5'	
OPN F	5'-CTTCCAAGCAATTCCAATGAAAG-3'	AF515708/ NM_009263
OPN R	5'-TGTGTACTAGCAGTGACGG-3'	
mPit-1 F	5'-CACTCATGTCCATCTCAGACT-3'	NM_015747
mPit-1 R	5'-CGTGCCAAAGAAGGTGAAC-3'	
RUNX2 F	5'-TTTGGACATTTGGAACATTTCTTAGTGTA-3'	NM_009820
RUNX2 R	5'- TCACATTATGCCTGAAGGAATTGAG-3'	
SM A2 actin F	5'-CCATTTGGAAACGAACGCTT-3'	NM_007392

SM A2 actin R	5’-TGACAGGACGTTGTTAGCATAGA-3’	
SM MHC F	5’-ACAGAGAACACAAAGAAAGTCATAC-3’	L25860 / NM_013607
SM MHC R	5’-GAATGAACTTGCCAAAGCGAG-3’	
TG1 F	5’-TTCGCTACCCGTACCGTCA-3’	AF186373
TG1 R	5’-CTTCATCCAGCAGTCGTT-3’	
TG2 F	5’-ATTGGCAGTGTGGACATTC-3’	AF076928
TG2 R	5’-TCGTGGGCGGAGTTGTA-3’	
TG4 F	5’-CCCATCTATTTGACCATAACTTTGAA-3’	NM_177911
TG4 R	5’-GCGAGAAACACCCTTGATT-3’	
TG5 F	5’-AGGCAGGATTCTGGAGAATATG-3’	NM_028799
TG5 R	5’-GGGCCACAGCAGTAGAG-3’	
TG6 F	5’-TCCGAGTCAATGTGAGCG-3’	NM_177726
TG6 R	5’-GTCTTCTGTCAGGTCTCCTTTGTA-3’	
TNAP F	5’- CCTCAAAGGCTTCTTCTTGC-3’	NM_007431
TNAP R	5’- GTCCATCTCCACTGCTTCA-3’	
Type I collagen F	5’- CCCTGGTATGACTGGCTT -3’	NM_007743
Type I collagen R	5’- GACCACGAATCCCTTCCT -3’	

Online Data Supplement Figures:

Online Data Supplement Figure 1. Expression profiles of TG isoenzymes in freshly isolated SMCs from aortas without further culture at Day 0, and in freshly isolated aortas. A, Using Trizol, and as described in detail in the Methods, we isolated RNA from SMCs freshly removed by collagenase digestion from aortas and not subjected to plating or further cell culture ("Day 0"), or B, from digested whole aortas with adventitia attached. After reverse transcription, qPCR was performed for the indicated mRNAs, with the target mRNAs individually quantified relative to GAPDH mRNA. A, Expression profile of SMCs at Day 0, replicates of 8, *$p<0.005$ by independent samples t-test with Bonferroni correction. B, Expression profile of freshly isolated aortas, replicates of 8, *$p<0.05$ by independent samples t-test with Bonferroni correction.

Online Data Supplement Figure 2. Lack of FXIIIA expression by mouse aortic SMCs. A, For qPCR analysis of FXIIIA expression, total RNA was collected from aliquots of 5×10^4 second passage SMCs or from human chondrocytic CH-8 cells carried in a 24 well plate. FXIIIA relative to GAPDH mRNA The qPCR analyzed data pooled from 3 experiments done in replicates of 2, *$p<0.05$ for $TG2^{+/+}$ vs $TG2^{-/-}$ SMCs and **$p<0.05$ for TG2 in SMCs vs TG2 in chondrocytes by independent samples t-test with Bonferroni correction for multiple comparisons. B, For immunocytochemistry of FXIIIA, aliquots of 1×10^5 $TG2^{-/-}$ SMCs or chondrocytic CH-8 cells were cultured for 24 hours prior to fixation with 4% paraformaldehyde, and FXIIIA expression detected as described in the Methods. Representative of 2 experiments.

Online Data Supplement Figure 3. The mRNA expression of selected promoters of arterial calcification (and the chondrocyte-specific marker aggrecan) in freshly isolated SMCs and aortic explants. A, Using Trizol, we isolated RNA from SMCs freshly removed by collagenase digestion from aortas and not subjected to plating or further cell culture ("Day 0"), or B, from digested whole aortas with adventitia attached. The qPCR was performed as above for the indicated panel of genes (TNAP, MSX2, RUNX2,

Aggrecan, Pit-1). n= 8. No significant genotype-dependent differences were observed by independent samples t-test with Bonferroni correction.

Online Data Supplement Figure 4. The mRNA expression of selected inhibitors of arterial calcification in freshly isolated SMCs and aortic explants. A, Using Trizol, we isolated RNA from SMCs freshly removed by collagenase digestion from aortas and not subjected to plating or further cell culture ("Day 0"), or B, from digested whole aortas with adventitia attached, and qPCR was performed as above for the indicated panel of genes (MGP, OPN, and OPG). Done in replicates of 8, *$p<0.05$ by independent samples t-test with Bonferroni correction.

Online Data Supplement Figure 5. Loss of contractile differentiation and gain of type I collagen and OPN expression in $TG2^{-/-}$ SMCs plated on laminin and with no P_i donor treatment administered. Primary SMCs were isolated from 3 congenic $TG2^{+/+}$ and $TG2^{-/-}$ mice each and plated on a tissue culture plate pre-coated with 1 µg/ml of laminin. SMCs were grown for 5 days in M231 media supplemented 5% FCS, until they reached confluence, and RNA isolated using Trizol, and reverse-transcribed and analyzed by qPCR for the mRNAs indicated. Data pooled from 3 experiments, *$p<0.05$ by independent samples t-test with Bonferroni correction.

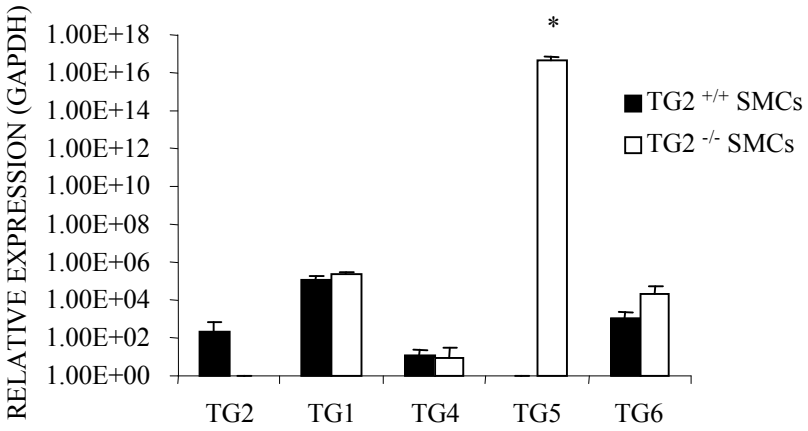
Online Data Supplement Figure 6. The mRNA expression profiles of genes involved in contractile and synthetic differentiation in freshly isolated SMCs ("Day 0") and freshly isolated aortic explants. For these studies, the samples in panels A and B were prepared and analyzed as described above. n=8. No significant genotype-dependent differences were observed by independent samples t-test with Bonferroni correction.

Online Data Supplement Figure 7. Decreased calcification in $TG2^{+/+}$ mouse aortic ring explant cultures treated with the TG2 catalytic site-specific inhibitor Boc-DON-Gln-Ile-Val-OMe. Whole $TG2^{+/+}$ aortas

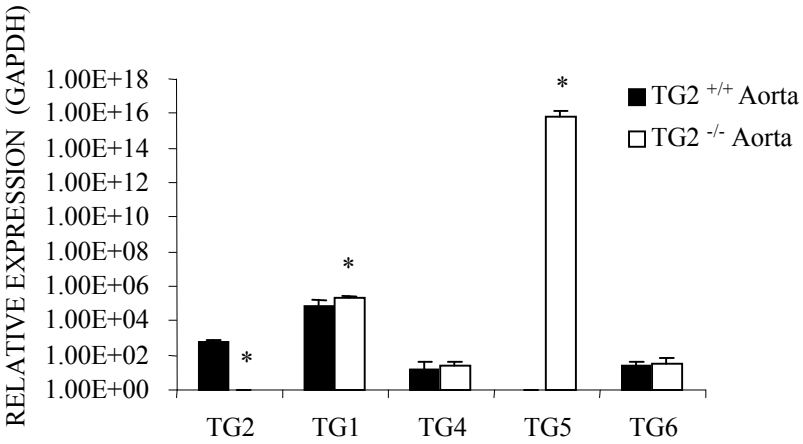
were cut into 2-3 mm rings and cultured in media with added 2.5 mmol/L NaP_i and 7U/ml alkaline phosphatase for 9 days, with the indicated concentrations of Boc-DON-Gln-Ile-Val-OMe present where denoted. Cultured $TG2^{-/-}$ aortic rings were compared under the same conditions, with results presented on the far right side bar. Free Ca^{2+} deposition/ mg dry weight was determined by phenolsulphonephthalein binding, as above. Data pooled from 3 experiments, with a total of 18 replicates per condition. *$p<0.001$ relative to the control samples of $TG2^{+/+}$ aortic explants without Boc-DON-Gln-Ile-Val-OMe treatment, assessed by ANOVA with post hoc Tukey test.

QUANTITATIVE mRNA EXPRESSION

A. TG ISOENZYME mRNA EXPRESSION IN SMCS AT DAY 0

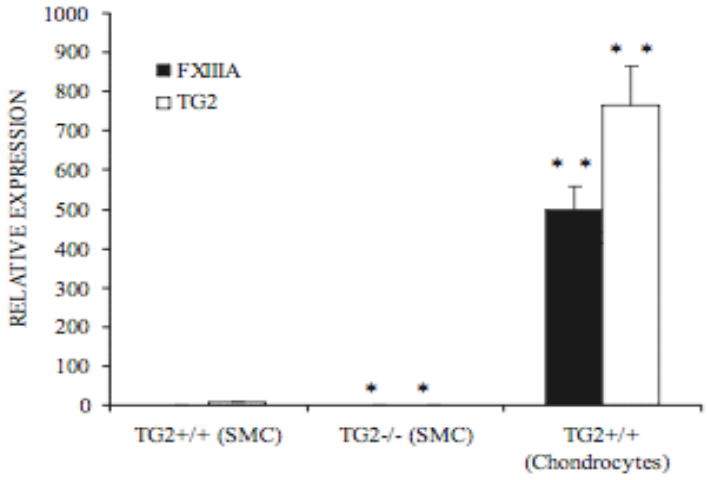


B. TG ISOENZYME mRNA EXPRESSION IN FRESHLY ISOLATED AORTAS

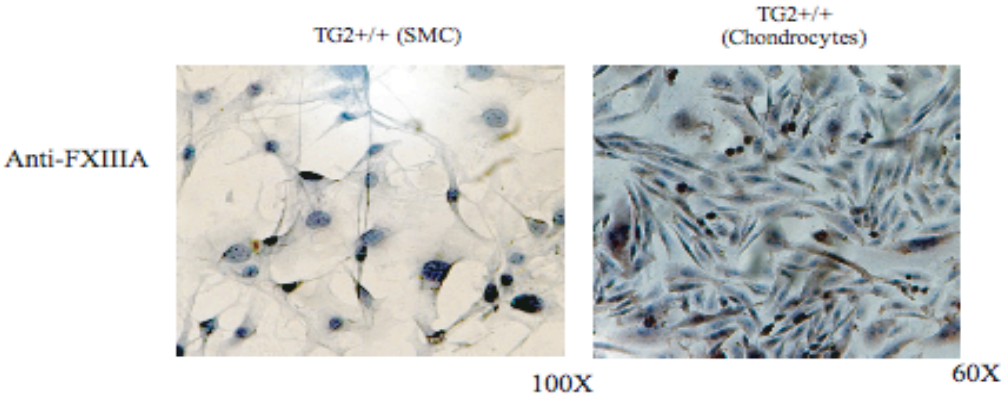


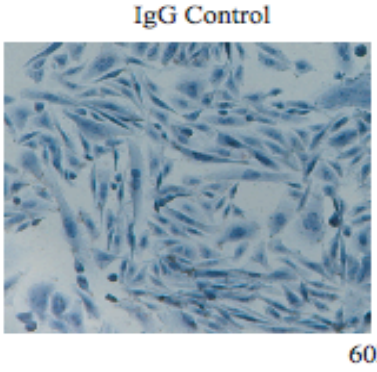
Online Supplemental Figure 1

A. mRNA EXPRESSION



B. FXIIIA PROTEIN EXPRESSION

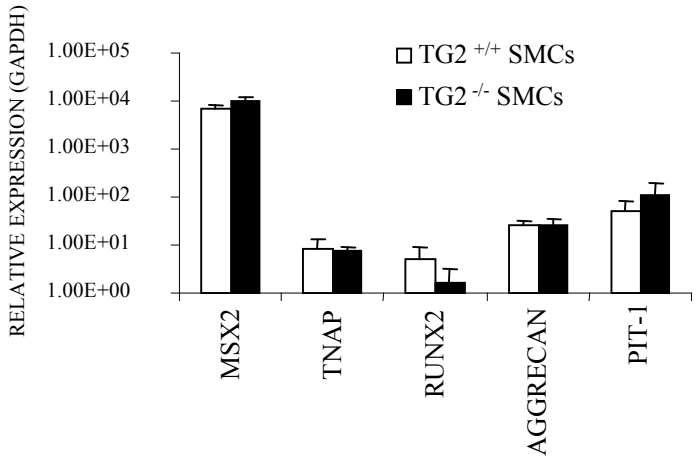




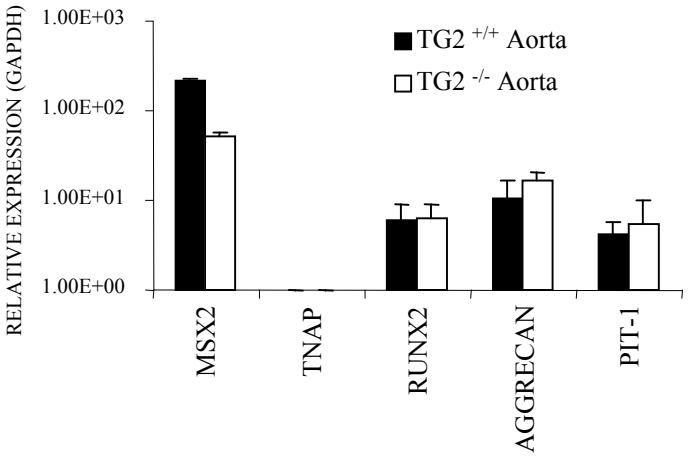
Online Supplemental Figure 2

QUANTITATIVE mRNA EXPRESSION

A. PROMOTERS OF ARTERIAL CALCIFICATION IN SMCS AT DAY 0



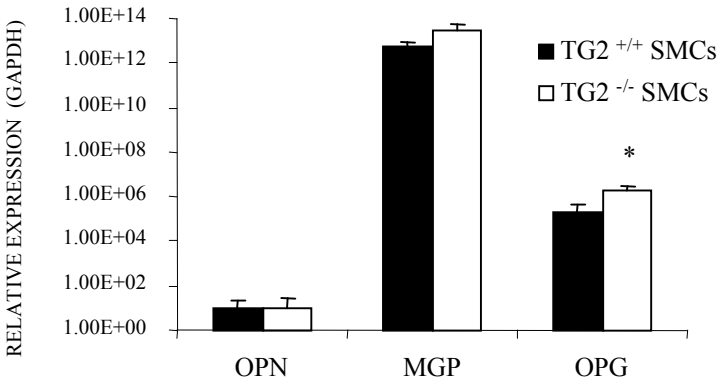
B. PROMOTERS OF ARTERIAL CALCIFICATION IN FRESHLY ISOLATED AORTAS



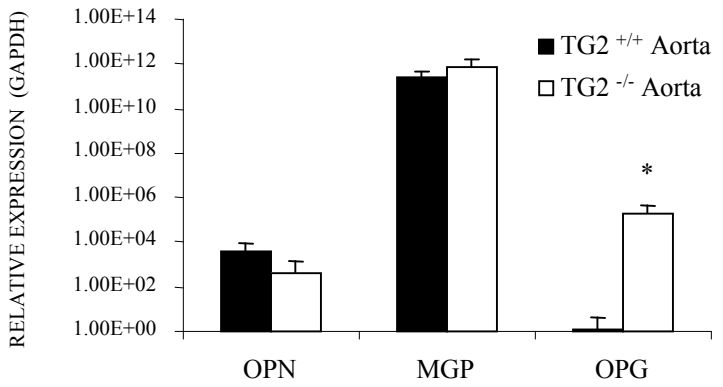
Online Supplemental Figure 3

QUANTITATIVE mRNA EXPRESSION

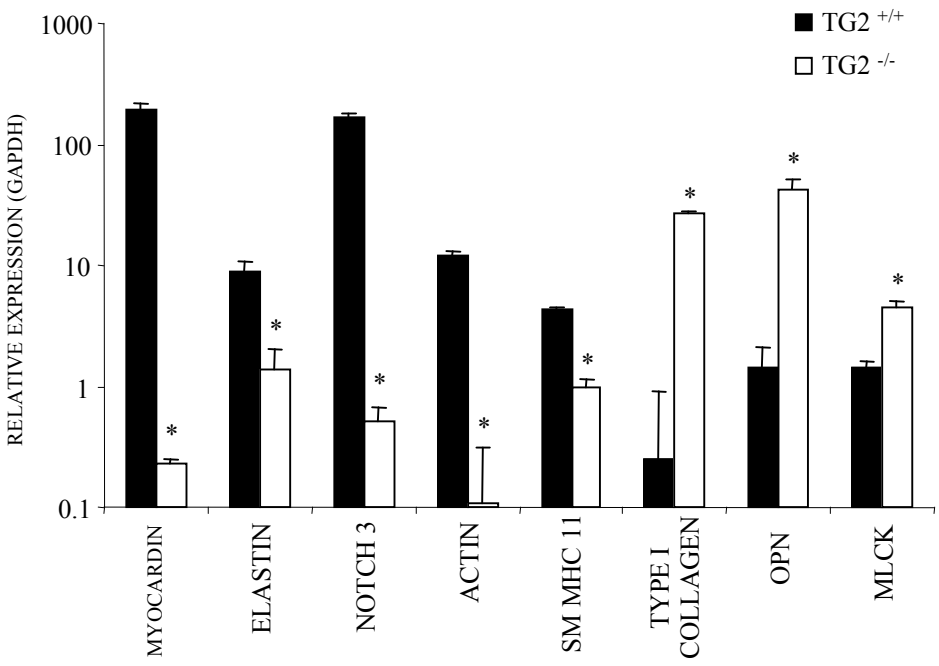
A. INHIBITORS OF ARTERIAL CALCIFICATION IN SMC AT DAY 0



B. INHIBITORS OF ARTERIAL CALCIFICATION IN FRESHLY ISOLATED AORTAS

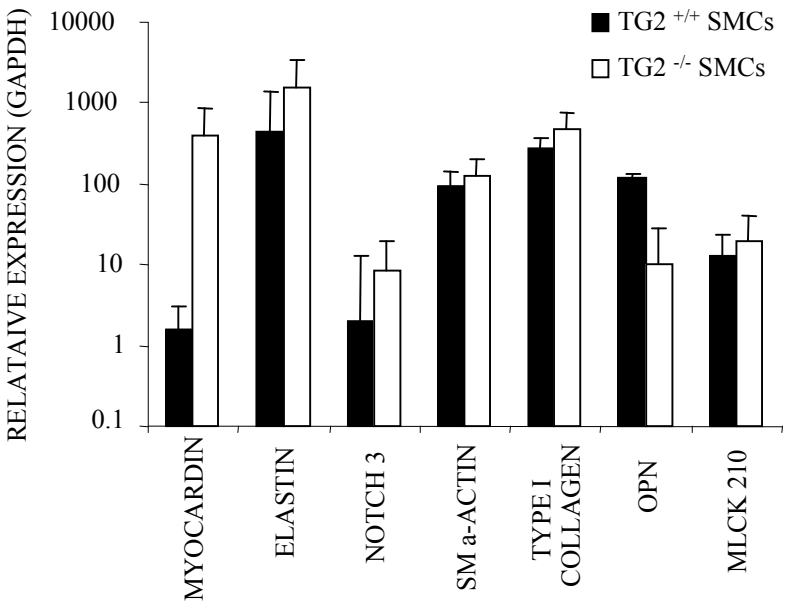


Online Supplemental Figure 4

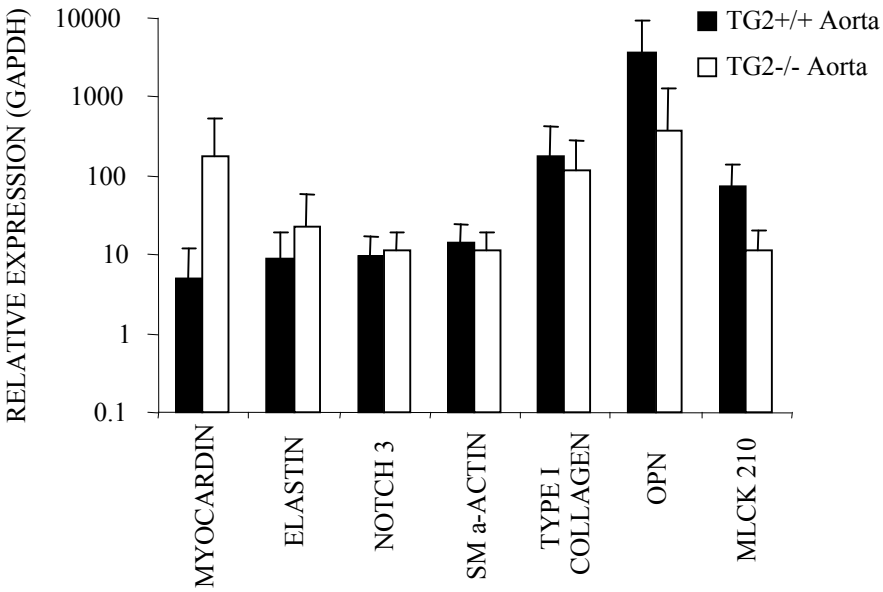


Online Supplemental Figure 5

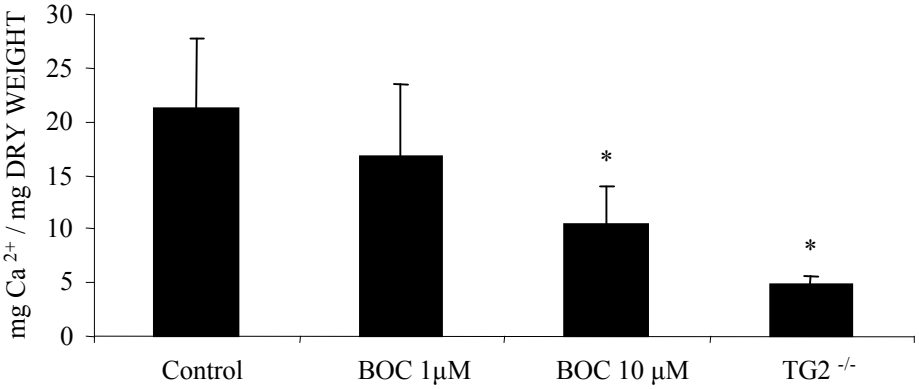
A. QUANTITATIVE PCR ANALYSIS OF SMCS AT DAY 0



B. QUANTITATIVE PCR ANALYSIS OF FRESHLY ISOLATED AORTAS



Online Supplemental Figure 6



Online Supplemental Figure7