Transglutaminase 2 Is Central to Induction of the Arterial Calcification Program by Smooth Muscle Cells

Kristen A. Johnson, Monika Polewski, Robert A. Terkeltaub

Abstract—Arterial calcification is a phenotype of vascular repair in atherosclerosis, diabetes, hyperphosphatemic renal failure, and aging. Arterial calcification is modulated by transition of arterial smooth muscle cells (SMCs) from contractile to chondro–osseous differentiation programmed in response to increases in P_i, bone morphogenetic protein-2, and certain other stimuli. Transglutaminase (TG2) release modulates tissue repair, partly by transamidation-catalyzed covalent crosslinking of extracellular matrix substrates. TG2 regulates cultured SMC differentiation, resistance artery remodeling to vasoconstriction, and atherosclerotic lesion size. Here, TG2 expression was required for the majority of TG activity in mouse and human arterial SMCs. TG2−/− SMCs lost the capacity for P_i donor–induced formation of multicellular bone-like nodules and for increased expression of the type III sodium–dependent P_i cotransporter Pit-1 and certain osteoblast and chondrocyte genes (tissue-nonspecific alkaline phosphatase, the osteoblast master transcription factor runx2, and chondrocyte-restricted aggrecan), and for P_i donor– and bone morphogenetic protein-2–induced calcification. Uniquely in TG2−/− SMCs, P_i donor treatment increased expression of the physiological SMC chondro–osseous differentiation and calcification inhibitors osteoprotegerin, matrix Gla protein, and osteopontin. Conversely, TG2−/− SMCs, unlike wild-type SMCs, failed to maintain contractile differentiation on laminin. Exogenous catalytically active TG2 augmented calcification by TG2−/− SMC in response to P_i donor treatment. TG2 expression also drove P_i-stimulated calcification of mouse aortic ring organ cultures, which was suppressed by the TG2 catalytic site-specific inhibitor Boc-DON-Gln-Ile-Val-OMe (10 μmol/L). Our results suggest that TG2 release in injured arteries is critical for programming chondro–osseous SMC differentiation and calcification in response to increased P_i and bone morphogenetic protein-2. (Circ Res. 2008;102:529-537.)

Key Words: smooth muscle cells • atherosclerosis • osteopontin • matrix Gla protein • osteoprotegerin • laminin

Arterial smooth muscle cells (SMCs) mediate complex vascular repair and remodeling processes.1 SMCs are phenotypically plastic and are drawn out of a contractile differentiation by stimuli, including biomechanical forces, changes in vascular tone, extracellular matrix modifications, thrombotic factors, and inflammatory and growth factors exemplified by platelet-derived growth factor (PDGF).2 The transition of contractile to synthetic SMCs modulates repair of arterial injuries via the capacity to migrate, proliferate, remodel the extracellular matrix, modulate inflammation, and promote thrombosis.3

Normal artery SMC populations also contain cells that undergo phenotypic transition to calcifying osteoblastic and chondrocytic cells,4–8 a potentiality shared in the diseased artery by pericytes, adventitial myofibroblasts, and vascular stem cells.5,8,10–12 P_i generation and sodium-dependent P_i uptake, bone morphogenetic protein (BMP)-2 and Wnt signaling, and certain oxidized phospholipidic lipids are recognized to drive intraarterial chondro–osseous differentiation and calcification.4–12 SMC generation by NPP1 and transport by the murine progressive ankylosis gene (ANK) of the basic calcium phosphate crystal growth and chondrogenesis inhibitor PPi, help physiologically hold SMC chondro–osseous differentiation and artery calcification in check.5,13 In this light, hydrolysis of PPi to P_i is a major activity of tissue-nonspecific alkaline phosphatase (TNAP), and TNAP expression is linked to SMC chondro–osseous differentiation that promotes calcification.8 Arterial calcification also is physiologically limited by SMC expression of the potent BMP-2 inhibitor (MGP),14 the potent basic calcium phosphate crystal growth inhibitor and mineral resorption promoter osteopontin (OPN),5,14 and osteoprotegerin (OPG), an inhibitor of RANKL and TRAIL signaling and potential modulator of the bone–vascular axis.15,16

Recently, the multifunctional protein transglutaminase (TG2) has been implicated as a regulator of calcification by chondrocytes and osteoblasts.17–19 TGs, by calcium-dependent transamidation (EC2.3.2.13), covalently crosslink a broad array of substrate proteins with available glutamine and lysine residues (such as collagen I, fibronectin, laminin,
and OPN).20 Producing protease-resistant isopeptide bonds, TGs directly mediate stabilization of extracellular matrices,20 TG2 also mediates cultured chondrocyte maturation to terminal hypertrophic differentiation and the capacity of chondrocytes to calcify the matrix in response to retinoic acid and certain inflammatory cytokines.17,18,21 TG2, although lacking signal peptide, is released by cells.18,20 Exogenous nanomolar TG2 is sufficient to promote chondrocyte hypertrophy,18 and direct effects of extracellular TG2 on cell differentiation have been linked to consequences of TG2-catalyzed pericellular matrix crosslinking.20,22

Resistance artery remodeling induced by chronic vasoconstriction is driven by extracellular TG2 and blocked by suppressing TG catalytic activity.1 TG2 expression and release are induced in vitro by nitric oxide, the nitric oxide–derived oxidant peroxynitrite, retinoic acid signaling, certain inflammatory cytokines, and thrombin17,21,23,24 and in vivo in macrophages and SMCs in atherogenesis.25,26 TG2 limits both atherosclerotic lesion size and necrotic core expansion.25

Calcification decreases artery wall compliance, and arterial calcification is linked to excess mortality in hyperphosphatemic renal failure, diabetes mellitus, and atherosclerosis.5 Here, we identify that TG2 is critical for programming calcification by cultured SMCs in response to P, donor treatment and BMP-2 and P, donor–induced calcification in aortic ring explants in organ culture.

Materials and Methods

An expanded Materials and Methods section that contains details on mouse aortic SMC and explant isolation and culture, immunohistochemical experiments, RT-PCR, data collection, and statistics is available in the online data supplement at http://circres.ahajournals.org.

Animals

In vitro analyses used tissues of congenic TG2+/+ mice and congenic TG2−/− mice, originally on a hybrid C57BL6/129SVJ background27 and crossed for more than 9 generations onto C57BL6 background.

Murine SMC Studies

Primary SMCs were isolated at 2 months of age from mouse aortas,8 and cells were carried on laminin for 2 passages before experimentation, unless otherwise indicated.

Results

TG2 Critically Mediates Induction of Both Chondro–Osseous Differentiation and Calcification by Cultured SMCs

SMC pericellular matrix alterations in diseased arteries promote changes in basal SMC contractile differentiation, mimicked by placing arterial SMCs in culture without a fibrillar collagen, laminin, or endothelial cell substratum.5 Here, we removed third-passage mouse aortic SMCs from laminin for 72 hours in culture and observed mRNA for TG2 and several TG isoenzymes to be expressed by normal cultured SMCs, with TG5 mRNA upregulated in TG2−/− SMCs (Figure 1A). TG2 expression was required for presence of the majority (∼80%) of TG catalytic activity in unstimulated mouse aortic SMCs, and PDGF and all-trans retinoic acid (ATRA) failed to induce TG activity in TG2−/− SMCs, unlike the case for TG2+/+ SMCs (Figure 1B). Under these conditions, TG2 release into conditioned medium was stimulated by PDGF and ATRA in wild-type SMCs, but TG2 was undetectable in both conditioned medium (Figure 1C) and cell lysates (not shown) of TG2−/− SMCs. In freshly isolated primary mouse SMCs and aortas, we observed basal expression of several TG isoenzymes in congenic TG2−/− and TG2+/+ mice, with upregulated TG5 mRNA in freshly isolated TG2−/− SMCs and aortas (Figure 1A and 1B in the online data supplement).

![Figure 1. TG isoenzyme expression, TG catalytic activity, and TG2 release in cultured mouse SMCs. We cultured aliquots of 5×10⁴ third-passage congenic TG2+/+ and TG2−/− mouse aortic SMCs per well (in a 24-well plate) for 72 hours. A, Expression patterns of TG isoenzymes. Here, and in quantitative PCR experiments below, SYBR green–based mRNA copy quantification compared individual target mRNA to GAPDH mRNA copy levels for each experiment. Data were pooled from 4 experiments, replicates of 3. *P<0.001 for TG2+/+ vs TG2−/− by independent samples t test with Bonferroni correction. B, TG catalytic-specific activity in cell lysates. C, TG2 release into SMC conditioned media in response to 10 ng/mL PDGF or 10 nmol/L ATRA, assayed by ELISA. For B and C, Data were pooled from 4 experiments, replicates of 3. *P<0.001 for TG2+/+ vs TG2−/−. **P<0.001 for increases induced by PDGF and ATRA by ANOVA with post hoc Tukey test.](http://circres.ahajournals.org)
Formation of multicellular nodules that calcified (assessed by von Kossa or Alizarin red S staining for deposited Pi, and Ca\(^{2+}\), respectively) was blunted in TG2\(^{-/-}\) SMCs, which here were removed from laminin and stimulated with 50 \(\mu\)g/mL ascorbate to stimulate calcification. Data were pooled from 4 experiments, replicates of 3. **P<0.01 for TG2\(^{-/-}\) vs TG2\(^{-/+}\) with recombinant isoenzymes, ***P<0.01 for TG2\(^{-/-}\) vs TG2\(^{-/+}\) by ANOVA with Bonferroni correction. D. To assess TG2 structure/function in calcification, SMCs were cultured with 100 ng/mL recombinant soluble wild-type (WT) TG2 (sTG2), TG catalytic site (C277G), GTP binding site mutant (K173L), or TF FXIIIA TG isozyme for 1 to 10 days. Ca\(^{2+}\) deposition was determined after decalcification in 0.6 N HCl using phenolsulphonphthalein to bind free Ca. Data were pooled from 4 experiments, replicates of 3. **P<0.01 for TG2\(^{-/-}\) vs TG2\(^{-/+}\) by independent samples t test with Bonferroni correction. E. To assess TG2 structure/function in calcification, SMCs were cultured with 100 ng/mL recombinant soluble wild-type (WT) TG2 (sTG2), TG catalytic site (C277G), GTP binding site mutant (K173L), TG isozyme for 1 to 10 days. Ca\(^{2+}\) deposition was determined after decalcification in 0.6 N HCl using phenolsulphonphthalein to bind free Ca. Data were pooled from 4 experiments, replicates of 3. **P<0.01 for TG2\(^{-/-}\) vs TG2\(^{-/+}\) by independent samples t test with Bonferroni correction.

To rule out compensatory effects attributable to germline TG2 deletion that limited calcification by SMCs, we used short hairpin RNA transfection in human aortic SMCs to knock down TG2 expression by >80%, associated with ~55% to 65% loss of total TG catalytic activity in the 72 hours after transfection (Figure 5A and 5B). The acquired TG2 depletion blunted calcification in response to both the Pi donor and BMP-2 (10 ng/mL) in human SMCs (Figure 5C). TG2 “gain of function” in human SMCs via treatment with nanomolar (100 ng/mL) recombinant soluble TG2\(^{18}\) suppressed OPN production by >50% (Figure 6A). Hence, we qualitatively assessed OPN expression by immunocytochemistry for TG2\(^{-/-}\) mouse SMCs plated on 1 \(\mu\)g/cm\(^2\) murine laminin to promote maintenance of contractile differentiation state, with or without additional precoating of the plate with TG2 (100 ng/mL for 10 min), followed by 4 washes with PBS. Under these conditions, where no TG2 was provided by SMCs, there was substantial TG2 retention in the laminin
matrix and the TG2 pretreatment of the laminin substratum suppressed OPN expression (Figure 6B).

**Failure of Stimulated Induction of Chondro–Osseous Differentiation and Calcification by TG2-Deficient SMCs Is Not Attributable to Enhanced Retention of Contractile Differentiation**

We next tested whether failure of stimulated induction of chondro–osseous differentiation in TG2/−/− SMCs was attributable to enhanced retention of contractile differentiation or predisposition to synthetic differentiation, given that OPN and MGP expression are associated with SMC synthetic differentiation.3,30,31 We confirmed TG2/+/−/− SMCs to robustly express the prerequisite for spreading type I collagen and to spread when on fibronectin but to retain contractile differentiation on laminin (Figure 7).32 In contrast, TG2/−/− SMCs robustly expressed type I collagen and spread both on fibronectin and laminin (Figure 7). Primary TG2/−/− SMCs cultured on laminin for 5 days also developed decreased expression of the contractile differentiation associated mRNAs Notch-3, myocardin, and smooth muscle α-actin and myosin heavy chain, whereas expression increased for collagen I and OPN expression and the stereotypic synthetic differentiation marker myosin light chain kinase (MLCK) 210-kDa isoform (supplemental Figure V).3 There were only minimal TG2 deficiency–related differences among these same markers of contractile and synthetic differentiation in freshly isolated aortic SMCs without further culture and in whole aortas (supplemental Figure VIA and VIB).

**TG2 Promotes P3- Stimulated Calcification in Aortas in Organ Culture**

To validate the physiological and translational significance of deficient P3-induced calcification in TG2/−/− SMCs, we adapted a rat aortic ring organ culture model to mouse samples.33 We cultured aortic 2- to 3-mm ring explants for 7...
days in medium supplemented with 7 U/mL alkaline phosphatase and 2.5 mmol/L sodium Pi and first stained for chondrocyte-specific type IX/XI collagen expression,8 which we observed to be induced in TG2/+/H11001/H11001 explants (Figure 8A). Induced 45Ca incorporation and free Ca2+/H11001 both were suppressed in TG2/H11002/H11002 aortas (Figure 8B and 8C), and the TG2 catalytic site-specific irreversible inhibitor Boc-DON-Gln-Ile-Val-OMe34 (at 10⁻⁹ mol/L) inhibited Pi-induced calcification by TG2/+/H11001/H11001 aortic explants by ≥50% under conditions in which TG2/H11002/H11002 aortic explants demonstrated ≥75% less calcification (supplemental Figure VII).

**Discussion**

In this study, we demonstrated that TG2 is essential for Pi-induced programming of SMC transition to chondro-osseous differentiation and that TG2 played a central role in calcification by cultured SMCs and aortic ring explants in organ culture. Remarkably, with TG2 deficiency, there was failure to upregulate several genes associated with chondro-osseous differentiation, coupled with sharply increased expression of the physiological artery calcification inhibitors MGP, OPN, and OPG in response to Pi donor treatment. Pi functions as a raw material for deposition of crystalline basic calcium phosphate within SMC-derived matrix vesicles and in the “hole regions” of fibrillar type I collagen.35 Additionally, Pi, that is taken up by sodium-dependent cotransport via Pit-1³⁵ (and in some conditions Pit-2 in mineralization-competent cells including SMCs³⁷) functions as a signaling molecule and growth regulator in osteogenic development.³⁸ Importantly, the calcification response of TG2-deficient SMCs was attenuated in response to not only a Pi donor but also to BMP-2, whose expression is elevated in atheroscle-
calcification. We observed Pit-1 mRNA and Pi-generating pathways. However, BMP-2 also induces Pit-1 expression myofibroblasts in part through Msx2 and Wnt signaling mediated by Pit-1 (or sodium-independent Pi uptake) could be altered in TG2-deficient SMCs, for example, by loss of TG2 effects on cytosolic and plasma membrane protein–protein interactions in putative macromolecular complexes that move P_i. Even so, the capacity of exogenous catalytically active TG2 and FXIIIa to partially restore calcification responses of TG2−/− SMCs to P_i donor treatment and the observed partial suppression of calcification in aortic explants by a peptide-based TG2-specific catalytic site inhibitor are noteworthy. Our observations, combined with the grossly normal skeletal development of TG2−/− mice, argue that paracrine and autocrine effects of secreted, catalytically active TG2 on SMCs promote transition to calcifying chondro–osseous nodule formation in SMCs.

TG-induced transamidation of both extracellular matrix and plasma membrane proteins likely contributes substantially to SMC chondro–osseous differentiation and calcification, because treatment of type I collagen with TG2 promotes collagen compaction, and osteoblasts grown on type I collagen crosslinked by TG2 differentiate more quickly than on native untreated collagen. Furthermore, TG2 transamidation of OPN promotes OPN dimerization and crosslinking to collagen and alters the capacity of OPN to regulate calcium-containing crystal deposition in the extracellular matrix. The physiological significance of the relationship between TG2 and OPN in calcified arteries was recently highlighted by the discovery that most of the OPN extracted from MGP−/− mouse aortas is polymerized in association with TG2 expression and isopeptide bond formation.

Adhesion to the basement membrane protein laminin (or to collagen I fibrils) normally promotes retention of contractile SMC differentiation. However, cultured TG2−/− SMCs developed synthetic differentiation on laminin. Significantly, TG2 transamidation of RhoA places RhoA in a constitutively active state, and RhoA maintains SMC contractile differentiation partly by suppressing the activity of Akt, a serine/threonine kinase downstream of phosphatidylinositol 3-kinase that is stimulated by multiple receptor tyrosine kinases, functions as a cell survival and growth promoter, and also inhibits calcification by SMCs. However, fibronectin-binding integrin coreceptor activity, GTPase signaling activity, and phospholipase Cβ1 binding by TG2 transamidation of SMCs. For example, guanine nucleotide-bound TG2 binds the cytoplasmic tail peptide

Figure 6. Exogenous TG2 modulates OPN expression by SMCs. A, Aliquots of 5×10^4 human aortic SMCs were cultured for 72 hours with and without 100 ng/mL recombinant soluble WT TG2 (sTG2), and conditioned media were assayed for OPN by ELISA. Data were pooled from 3 experiments. *P<0.001 by independent samples t test. B, Where indicated, recombinant WT sTG2 (100 ng/mL) was added to laminin-coated coverslips for 10 min at 37°C, followed by 4 washes in PBS to remove TG2 unincorporated into the laminin substrate, followed by plating and culturing of aliquots of 1×10^5 TG2−/− SMCs for 24 hours before fixation with 4% paraformaldehyde. Immunocytochemistry assessed TG2 and OPN. Data are representative of 3 experiments.

Figure 7. TG2−/− SMCs fail to retain contractile differentiation on laminin. Aliquots of 1×10^5 second passage mouse aortic SMCs were cultured for 72 hours on 1 μg/cm² laminin (to maintain contractile differentiation) or fibronectin (to promote synthetic differentiation). After 24 hours, SMCs were fixed and immunohistochemically stained for type I collagen. Data are representative of 5 experiments.
GFFKR motif in α integrin subunits, including α1,-5,-V, and -Iib, and thereby inhibits fibroblast migration.\textsuperscript{47}

In this study, we complemented SMC culture studies with aortic organ culture experiments that yielded very similar results in response to stimulation with a Pi donor. Removal of SMCs from their physiological extracellular matrix for cell culture experiments herein was informative for SMC responses to matrix alterations and stress, but, significantly, we observed few robust differences between normal and freshly isolated TG2-deficient aortas and aortic SMCs for the chondro–osseous, and contractile and synthetic differentiation, markers and regulators studied. Thus, primary functions of TG2 in the aorta and other vessels\textsuperscript{1,28} likely become evident when arteries remodel in response to injury.

Limitations of this study included lack of study of signal transduction mechanisms. We did not determine whether transamidation and deamidation of extracellular matrix proteins\textsuperscript{20} by TG2 modulates matrix–cell communication, SMC condensation into nodules, and hydroxyapatite growth to promote calcification. Significantly, crosslinking of fibronectin by TG2 on the cell surface promotes activation of RhoA\textsuperscript{48} and increased RhoA activity stimulates chondrogenesis.\textsuperscript{49} We did not test whether SMC intracellular TG2 accounted for incomplete reconstitution by exogenous TG2 of calcification by TG2\textsuperscript{−/−} SMCs, let alone the only partial inhibition of aortic explant calcification by micromolar Boc-DON-Gln-Ile-Val-OMe. SMC transdifferentiation to osteoblasts and chondrocytes has been described previously\textsuperscript{4} but is not universally accepted. It remains possible that expansion of small numbers of pericytes, vascular stem cells, and adventitial myofibroblasts within SMC preparations and their transition to osteoblastic differentiation\textsuperscript{5,9,11,12} contributed to calcification events described. We did not explore why differences appear to exist between TG isoenzyme expression patterns in small mesenteric arteries\textsuperscript{28} and, described here, in the aorta and whether this relates to vascular calcification. We also have not assessed direct impact on differentiation and function of TG2-deficient SMCs of TG5, a TG isoenzyme first discovered in epidermis that regulates keratinocyte differentiation but also is expressed outside of the skin.\textsuperscript{50} Last, we have not yet extended these studies to in vivo analyses of arterial calcification.

Our results provide further evidence for TG2 modulating the nature of the SMC differentiation response and phenotypic features of arterial responses to injury,\textsuperscript{1,28} such as patterns of intima and media repair and remodeling. Our
findings reveal that indirect effects complement direct effects of TG2 on SMCs in artery repair. These include regulation of expression of OPG, an inhibitor of both atherosclerotic lesion progression and calcification, and suppression of the expression of OPN, which promotes matrix metalloproteinase-9 activation, induces oxidative stress and matrix metalloproteinase-2 expression in SMCs, and is proatherogenic. Effects of exogenous TG2 (and FXIIIA) on SMCs here are significant because both SMCs and cells that interact with SMCs, including endothelial cells and macrophages, could release TG2 in normal and diseased arteries, and activated macrophages can release FXIIIA. Significantly, atorvastatin promotes endothelial TG2 expression. It would be of interest to assess the role of TG2 in stabilization of atherosclerotic plaques by statins.

SMCs are heterogeneous within atherosclerotic lesions. Furthermore, TG2 catalytic activity can become deficient via increased TG2 expression, increased TG2 proteolysis, and increased conversion of TG2 to the GTP-bound form. Therefore, our results may point to a new paradigm in which differential effects of clones of TG2-sufficient versus TG2-deficient SMCs modulate the phenotype of arterial repair in atherosclerosis and other forms of arterial injury. In essence, although TG2 mediates arterial remodeling to vasocostriction and limits the size and possibly necrotic core expansion of atherosclerotic lesions, robust TG2 release in the course of artery wall repair has the potential to promote calcification. The normal developmental phenotype of TG2−/− mice and the capacity of pharmacological TG inhibition specific for TG2 to inhibit P-induced aortic explant calcification buttress the translational potential of our findings for arterial calcification, particularly that associated with hypertension in chronic kidney disease.

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Disclosures
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References


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In an article by Johnson et al (Circ Res. 2008;102:529–537), a portion of Figure 3B became obscured when the authors converted file formats. The authors regret this error. The corrected figure is published below, and the corrected article is available online at http://circres.ahajournals.org/cgi/content/full/102/5/529.

**Correction**

QUANTITATIVE mRNA EXPRESSION

A  PROMOTERS OF ARTERIAL CALCIFICATION

B  INHIBITORS OF ARTERIAL CALCIFICATION
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 µg/cm² 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°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, PPi, 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 PPi (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:


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

F, Forward primer; R, Reverse primer.

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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 x 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 1x10^5 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 Pi 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$_4$ 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
Online Supplemental Figure 2

A. mRNA EXPRESSION

B. FXIIIA PROTEIN EXPRESSION
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

![Bar chart showing relative expression of various genes in TG2+/+ and TG2-/- SMcs.]

B. QUANTITATIVE PCR ANALYSIS OF FRESHLY ISOLATED AORTAS

![Bar chart showing relative expression of various genes in TG2+/+ and TG2-/- aortas.]

Online Supplemental Figure 6
Online Supplemental Figure 7

mg Ca^{2+} / mg DRY WEIGHT

Control  | BOC 1μM | BOC 10 μM | TG2 /-

*