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

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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 (TG)2 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 aortic 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.

Keywords

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]

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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.[5,9,10–12] P_i generation and sodium-dependent P_i uptake, bone morphogenetic protein (BMP)-2 and Wnt signaling, and certain oxidized proatherogenic 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 PP_i help physiologically hold SMC chondro–osseous differentiation and artery calcification in check.[8,13] In this light, hydrolysis of PP_i 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),[6,14] 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 (TG)2 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 protein 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 thrombin[17,21,23,24] 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_i donor treatment and BMP-2 and P_i 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 background[27] 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.[3] 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 IA and IB in the online data supplement).

Formation of multicellular nodules that calcified (assessed by von Kossa or Alizarin red S staining for deposited P_i and Ca^{2+}, respectively) was blunted in $TG2^{-/-}$ SMCs, which here were removed from laminin and stimulated with 50 μg/mL ascorbate and the P_i donor/TNAP substrate β-glycerolphosphate (2.5 mmol/L) (Figure 2A through 2C). We also observed attenuated calcification by $TG2^{-/-}$ SMCs, which was partially rescued by exogenous, soluble recombinant wild-type TG2 (100 ng/mL), an effect shared by the K173L GTP binding site mutant of TG2[18] and the FXIIIA TG isoenzyme but not by TG catalytic site dead C277G TG2 mutant (Figure 2D).[18] FXIIIA is expressed by adventitial macrophages,[28] but we verified absence of FXIIIA expression by cultured aortic SMCs (supplemental Figure II).

In P_i-stimulated calcification, [29] $TG2^{+/+}$ but not $TG2^{-/-}$ SMCs developed increased mRNA for the transcription factor Msx2, which promotes maintenance of multipotentiality and osteoblastogenesis but suppresses chondrogenesis,[10] of TNAP, of Pit-1, of the osteoblast master transcription factor runx2, and of the chondrocyte-specific extracellular matrix constituent aggrecan (Figure 3, top). In contrast, $TG2^{-/-}$ SMCs developed increased mRNA expression of the artery calcification inhibitors OPN, MGP, and OPG (Figure 3, bottom). Extracellular PP_i and PP_i-generating nucleotide pyrophosphatase phosphodiesterase-specific activity were not significantly altered in $TG2^{-/-}$ SMCs, but TNAP-specific enzyme activity more than doubled in $TG2^{+/+}$ while remaining unchanged in $TG2^{-/-}$ SMCs over 10 days in culture (not shown). OPN (at days 1 to 7) and OPG (at days 1 to 17) were markedly increased in the conditioned media of $TG2^{-/-}$ relative to $TG2^{+/+}$ SMCs stimulated to calcify (Figure 4A and 4B). In the absence of either stimulation by a P_i donor or of further culture, freshly isolated $TG2^{-/-}$ mouse aortic SMCs and aortas demonstrated minimal differences relative to $TG2^{+/+}$ samples for mRNA of the same promoters and inhibitors of calcification, except for more OPG in $TG2^{-/-}$ aortas (supplemental Figures III and IV).

To rule out compensatory effects attributable to germline TG2 depletion 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 P_i 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 mR-NAs 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 P_i-Stimulated Calcification in Aortas in Organ Culture

To validate the physiological and translational significance of deficient P_i-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 P_i and first stained for chondrocyte-specific type IX/XI collagen expression,[8] which we observed to be induced in $TG2^{+/+}$ but not in $TG2^{-/-}$ explants (Figure 8A). Induced ^{45}Ca incorporation and free Ca^{2+} both were suppressed in $TG2^{-/-}$ aortas (Figure 8B and 8C), and the TG2 catalytic site-specific irreversible inhibitor Boc-DON-Gln-Ile-Val-OMe[34] (at 10 μmol/L) inhibited P_i-induced calcification by $TG2^{+/+}$ aortic explants by ≈50% under conditions in which $TG2^{-/-}$ aortic explants demonstrated ≈75% less calcification (supplemental Figure VII).

Discussion

In this study, we demonstrated that TG2 is essential for P_i-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 P_i donor treatment. P_i 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, P_i that is taken up by sodium-dependent cotransport via Pit-1[36] (and in some conditions Pit-2 in mineralization-competent cells including SMCs[37]) 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 P_i donor but also to BMP-2, whose expression is elevated in atherosclerotic lesions.[39] BMP-2 promotes calcification by SMCs and myofibroblasts in part through Msx2 and Wnt signaling pathways.[10] However, BMP-2 also induces Pit-1 expression in osteoblastic cells, a mechanism essential to driving matrix calcification.[40] We observed Pit-1 mRNA and P_i-generating TNAP mRNA expression and enzyme activity to be suppressed in $TG2^{-/-}$ SMCs.

Central to the effects of P_i in chondro–osseous cell differentiation are regulation of mitogen-activated protein kinase signaling and modulation of expression of multiple genes that regulate calcification, exemplified by rapid induction of OPN.[38] We observed the OPN induction response of $TG2^{-/-}$ SMCs to P_i donor treatment to be increased. However, exaggerated OPN expression also was observed in $TG2^{-/-}$ SMCs without donor treatment. We did not P_i measure P_i uptake by TG2-deficient SMCs in this study. It remains possible that basal sodium-dependent P_i uptake mediated by Pit-1 (or sodium-independent P_i uptake[37]) 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,[17,27] 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,[1] and osteoblasts grown on type I collagen crosslinked by TG2 differentiate more quickly than on native untreated collagen.[41] Furthermore, TG2 transamidation of OPN promotes OPN dimerization and crosslinking to collagen[42] and alters the capacity of OPN to regulate calcium-containing crystal deposition in the extracellular matrix.[43] 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.[44]

Adhesion to the basement membrane protein laminin (or to collagen I fibrils) normally promotes retention of contractile SMC differentiation.[3] 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,[45] 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.[46] However, fibronectin-binding integrin coreceptor activity, GTPase signaling activity, and phospholipase Cδ1 binding by TG2[18,20,47] each could modulate differentiation of SMCs. For example, guanine nucleotide-bound TG2 binds the cytoplasmic tail peptide GFFKR motif in α integrin subunits, including α1, -5, -V, and -IIb, and thereby inhibits fibroblast migration.[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 P_i 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[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[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,[48] and increased RhoA activity stimulates chondrogenesis.[49] We did not test whether SMC intracellular TG2 accounted for incomplete reconstitution by exogenous TG2 of calcification by $TG2^{-/-}$ 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[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[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[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.[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,[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,[16] 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.[51] 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.[25,52,53] Significantly, atorvastatin promotes endothelial TG2 expression.[54] It would be of interest to assess the role of TG2 in stabilization of atherosclerotic plaques by statins.

SMCs are heterogeneous within atherosclerotic lesions.[55,56] Furthermore, TG2 catalytic activity can become deficient via decreased TG2 expression, increased TG2 proteolysis, and increased conversion of TG2 to the GTP-bound form.[20] 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 vasoconstriction[1] and limits the size and possibly necrotic core expansion of atherosclerotic lesions,[25] 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_i-induced aortic explant calcification buttress the translational potential of our findings for arterial calcification, particularly that associated with hyperphosphatemia in chronic kidney disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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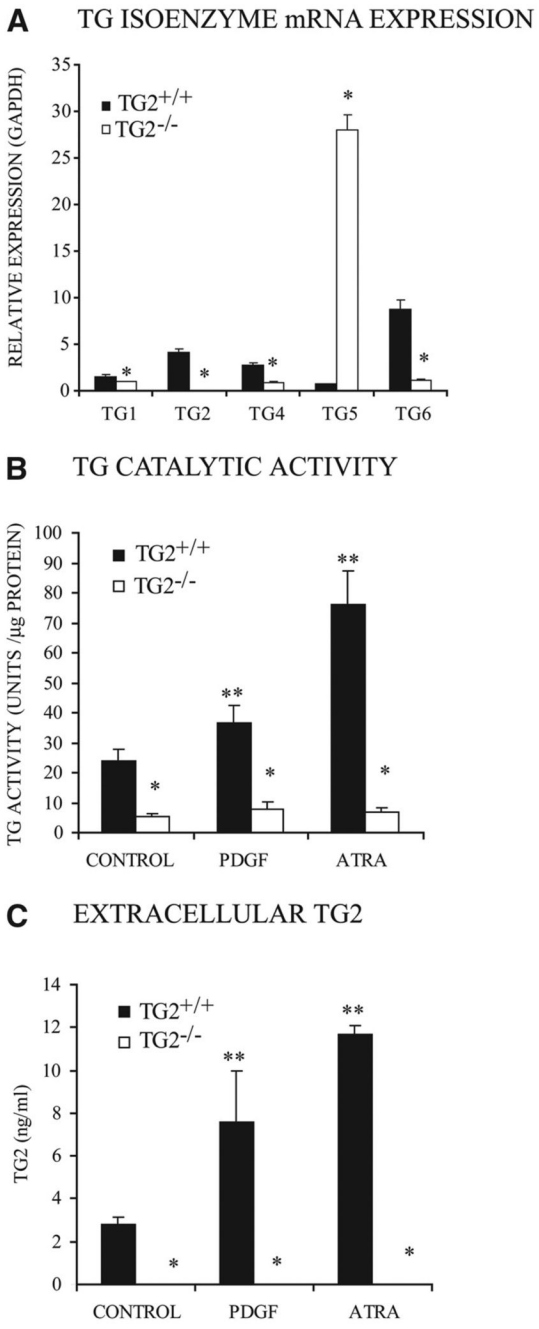

Figure 1.

TG isoenzyme expression, TG catalytic activity, and TG2 release in cultured mouse SMCs. We cultured aliquots of 5×10^4 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 released 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.

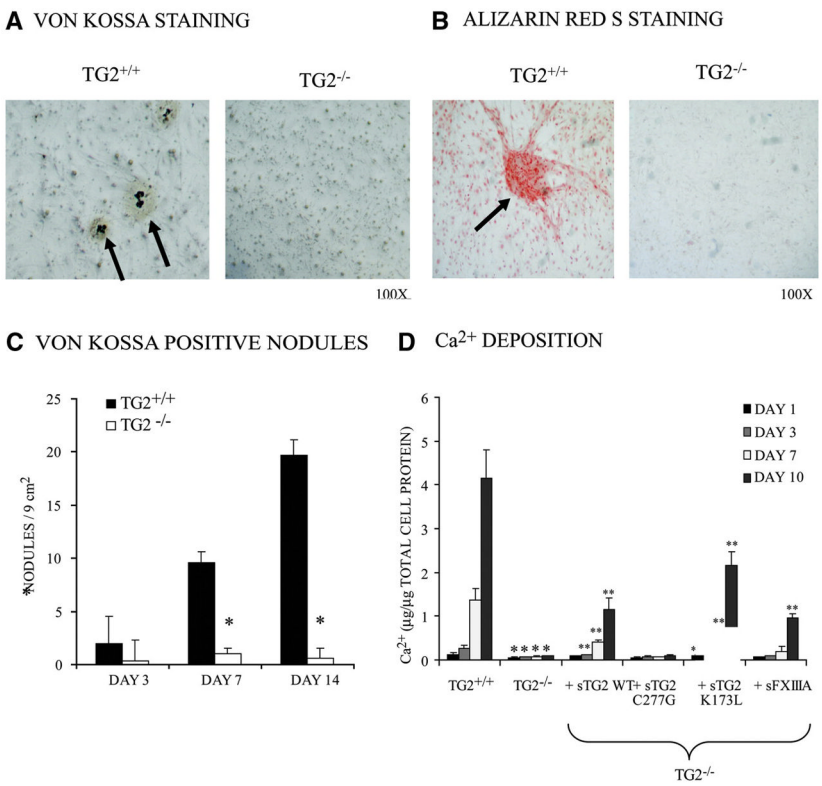

Figure 2.

Decreased chondro–osseous nodule formation and matrix calcification by $TG2^{-/-}$ SMCs. Second-passage mouse aortic SMCs (5×10^4 per well in 24-well plate) (A through C) or 1×10^5 per well in a 12-well plate (D) were cultured in media for up to 14 days with added 2.5 mmol/L β-glycerolphosphate and 50 μg/mL ascorbate to stimulate calcification. A and B, SMCs were fixed with 4% paraformaldehyde for 10 min and then washed with H_2O (A) or PBS (B). A, To detect P_i deposited in the matrix, fixed cultures were stained with von Kossa and counterstained with nuclear fast red (day 14 results shown). B, To visualize matrix Ca^{2+} deposition, Alizarin red S staining was performed (day 7 results shown). A and B are representative of 4 experiments, cells pooled from 12 animals each genotype, each experiment. C, Each 24-well dish (assessed as in A) was examined microscopically for the total number of von Kossa–positive nodules found per 9-mm^2 well. Data were pooled from 4 experiments, replicates of 3. *P<0.001 for $TG2^{-/-}$ vs $TG2^{-/-}$ by independent samples t test 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) TG2, or WT FXIIIA TG isoenzyme for 1 to 10 days. Ca^{2+} deposition was determined after decalcification in 0.6 N HCl using phenolsulphonphthalein to bind free Ca.[2] 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 post hoc Tukey test.

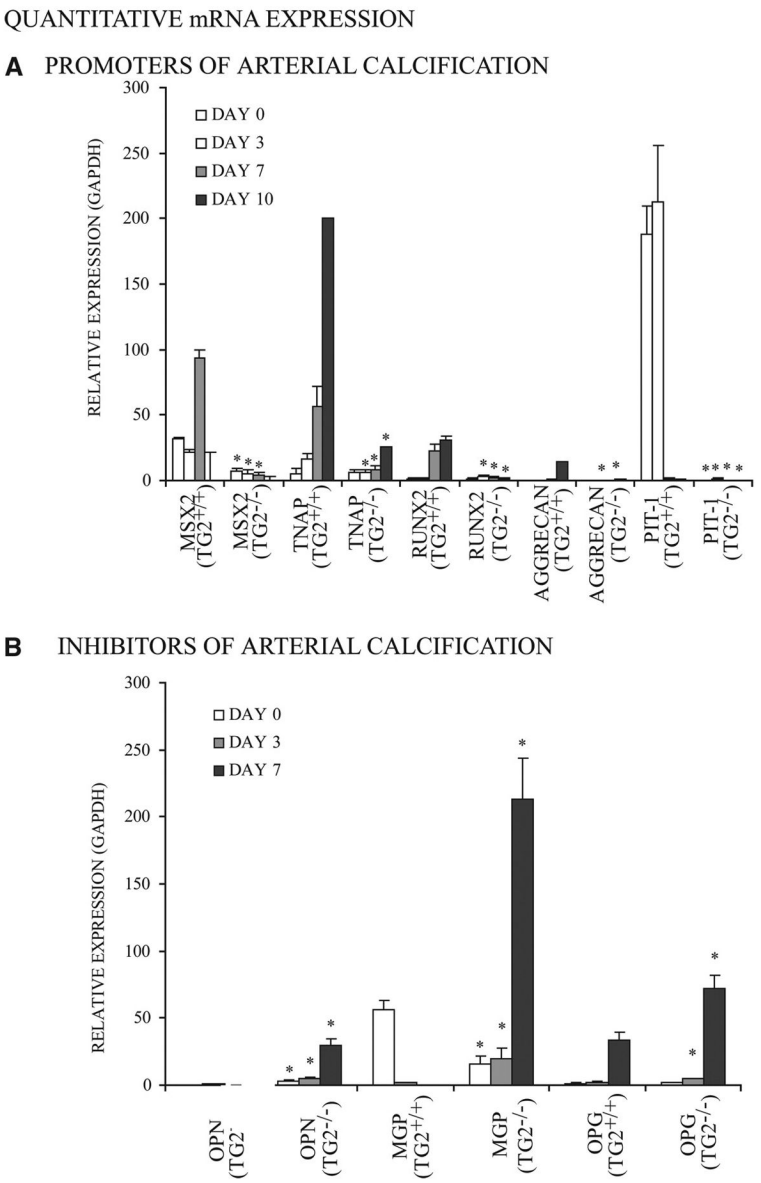

Figure 3.

Changes in calcification regulatory gene expression in $TG2^{-/-}$ SMCs in response to P_i donor treatment. For quantitative PCR, total RNA was collected from 5×10^4 second-passage SMCs in media with 2.5 mmol/L β-glycerolphosphate and 50 μg/mL ascorbate added at day 0, with results expressed relative to GAPDH. A, Decreased expression of chondro–osseous and calcification-promoting genes (TNAP, Msx2, runx2, aggrecan, Pit-1) in $TG2^{-/-}$ SMCs. Data were pooled from 3 experiments, replicates of 2. *P<0.005 for $TG2^{+/+}$ vs $TG2^{-/-}$ by independent samples t test with Bonferroni correction. B, Increased expression of physiological arterial calcification inhibitors (MGP, OPN, OPG) in $TG2^{-/-}$ SMCs. Data were pooled from 3 experiments, replicates of 2. *P<0.001for $TG2^{+/+}$ vs $TG2^{-/-}$ by independent samples t test with Bonferroni correction.

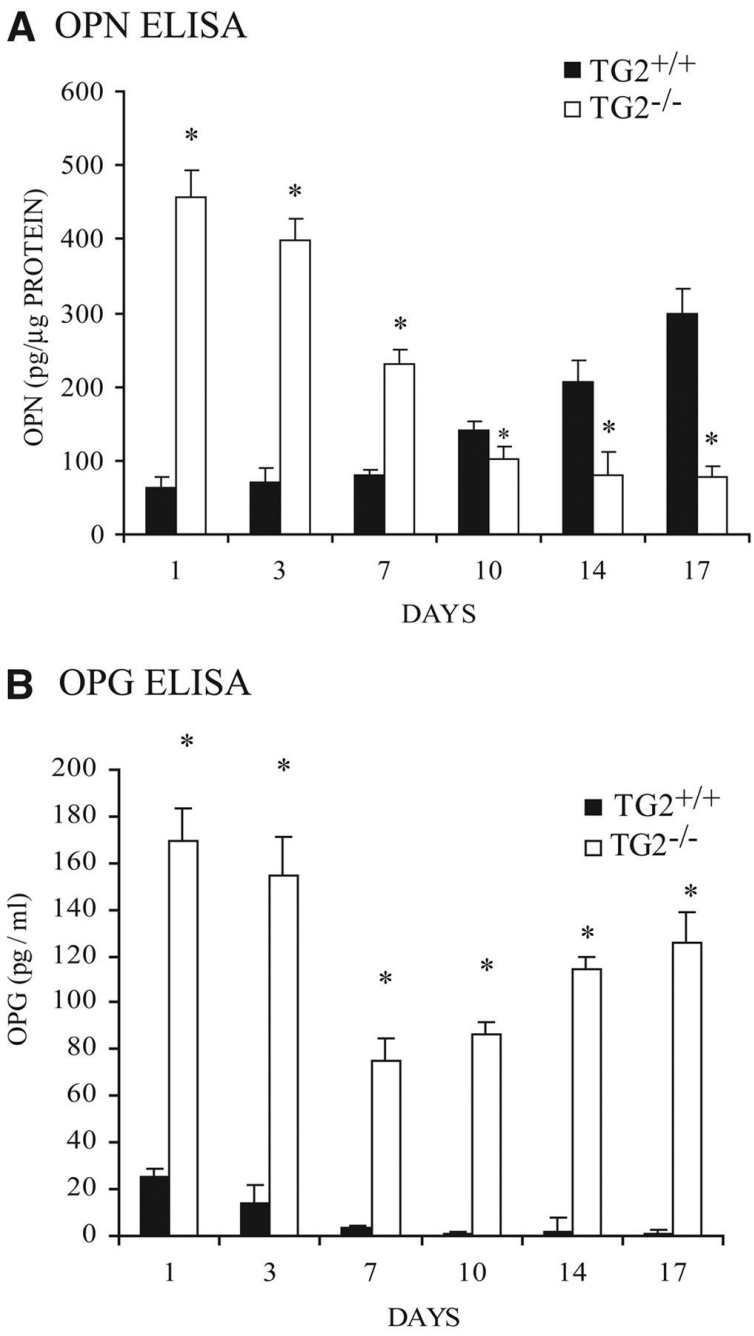

Figure 4.

TG2 modulates extracellular OPN and OPG levels. A and B, Aliquots of 5×10^4 second-passage SMCs per well in 24-well plates were cultured up to 17 days with added 2.5 mmol/L β-glycerolphosphate and 50 μg/mL ascorbate, and conditioned media were collected for OPN (A) and OPG (B) ELISA. Data were pooled from 3 experiments, replicates of 3. *P<0.001 for $TG2^{+/+}$ vs $TG2^{-/-}$ by independent samples t tests with Bonferroni correction.

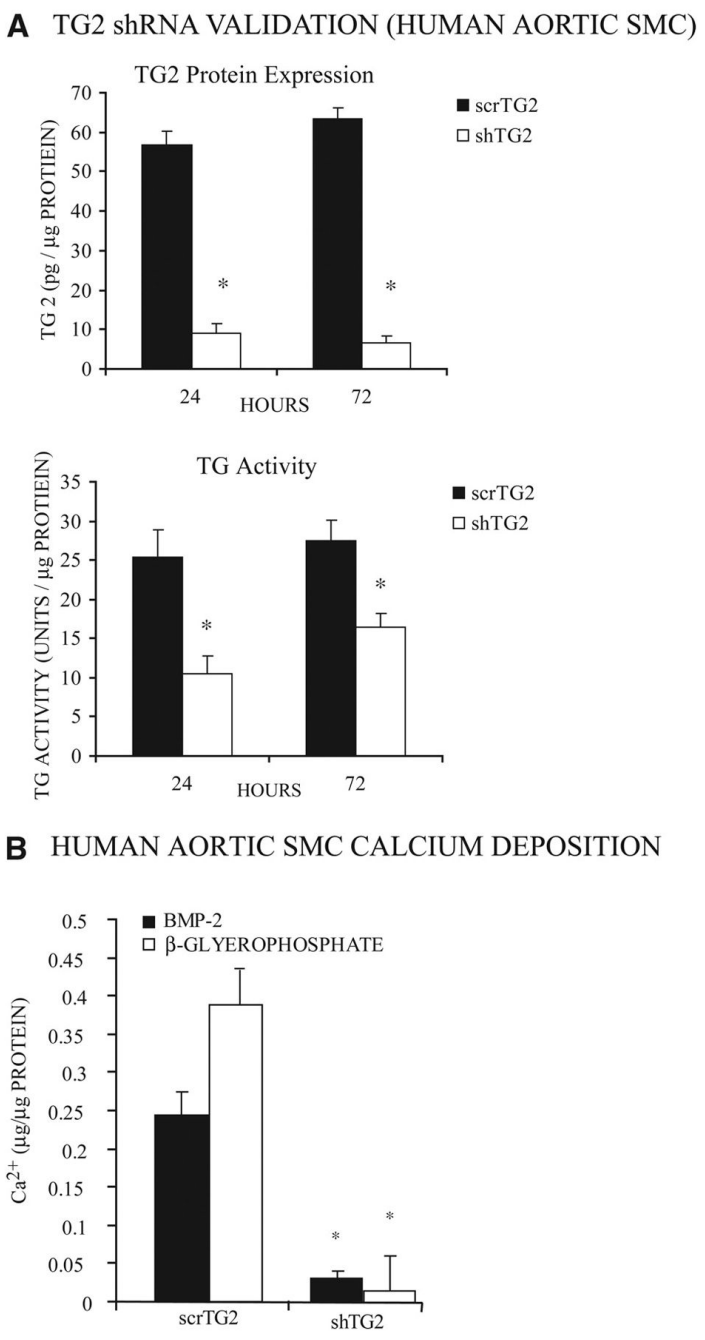

Figure 5.

Both BMP-2– and P_i donor–induced calcification are inhibited in human aortic SMCs. A, To verify the effectiveness of TG2 depletion by short hairpin RNA (shTG2) and scrambled control (scrTG2) cDNA transfections, human aortic SMC lysates from aliquots of 3×10^5 cells/well in a 6-well plate were collected 24 and 72 hours after transfection. Total cellular TG2 protein determined by sandwich ELISA was normalized to total protein. Additionally, TG catalytic activity was determined. Data were pooled from 3 experiments, replicates of 3. *P<0.001 by independent samples t tests with Bonferroni correction. B, Human aortic SMCs were transfected with shTG2 and scrTG2, and after 12 hours, 10 ng/mL BMP-2, or 2.5 mmol/L β-glycerolphosphate and 50 μg/mL ascorbate, was added for 72 hours, followed by determination

of Ca^{2+} deposition as above. Data were pooled from 3 experiments, replicates of 3. *P<0.001 by independent samples *t* test with Bonferroni correction.

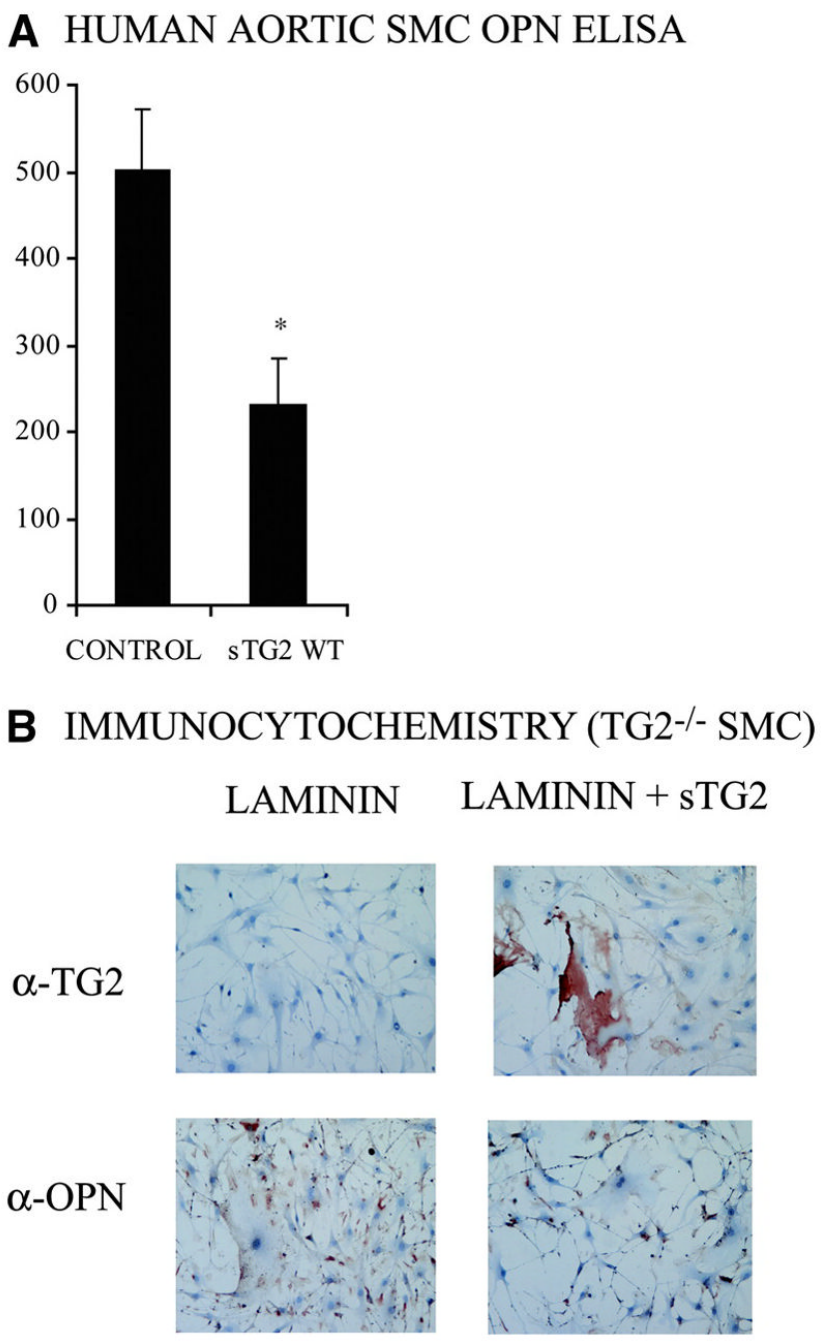

Figure 6.

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 substratum, 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.

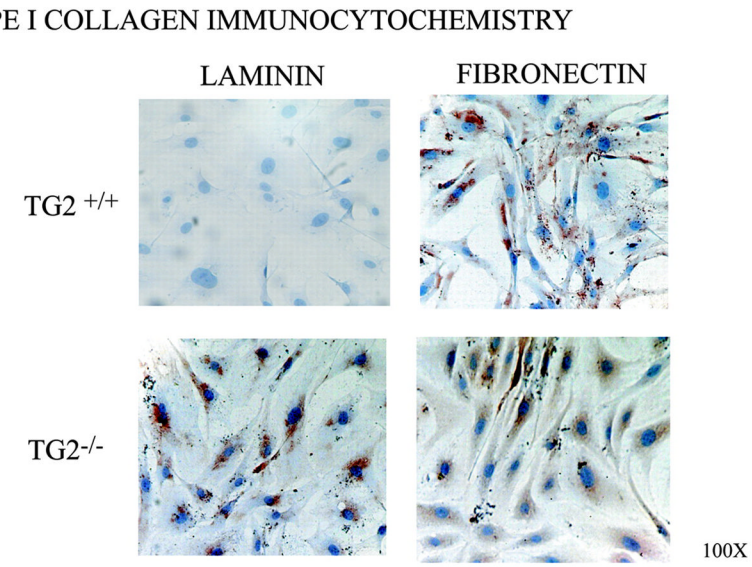

Figure 7.

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/cm^2 laminin (to maintain contractile differentiation) or fibronectin (to promote synthetic differentiation). After 24 hours, SMCs were fixed and immuno-histochemically stained for type I collagen. Data are representative of 5 experiments.

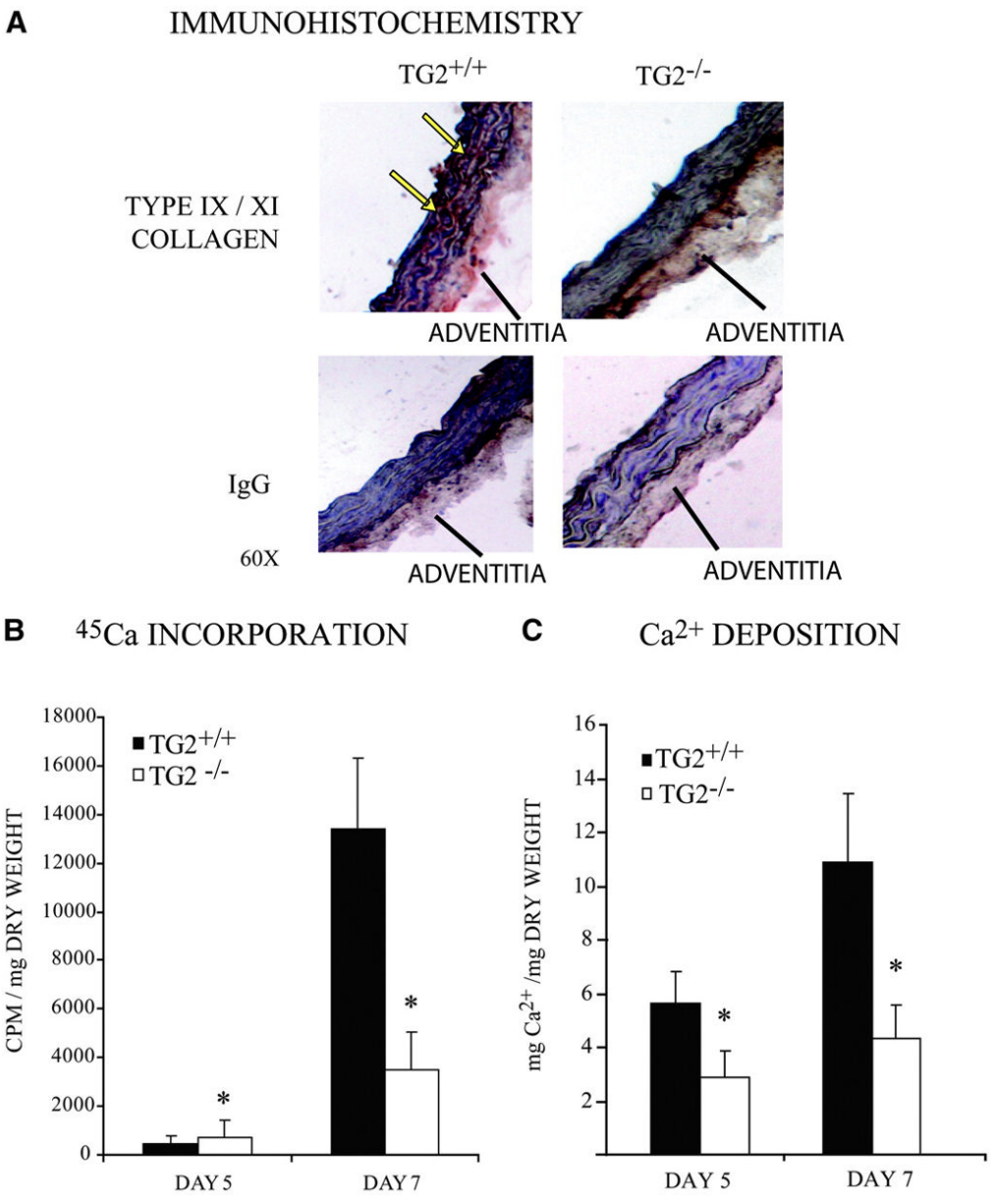

Figure 8.

Decreased calcification in $TG2^{-/-}$ mouse aortic ring explant cultures. Isolated whole aortas were cut into 2- to 3-mm slices and cultured in growth media supplemented with 2.5 mmol/L NaP_i and 7 U/mL alkaline phosphatase. A, Frozen sections were immunohistochemically stained for chondrocyte-specific type IX/XI collagen. B, Aortic cultures were incubated as above for 4 and 6 days before addition of 0.3 μCi/mL ^{45}Ca for 24 hours. Aortas were collected on days 5 and 7, washed 3 times with PBS, dried, and weighed, and incorporated ^{45}Ca cpm was quantified. Data were pooled from 10 animals, replicates of 3. *P<0.005 by independent samples t test with Bonferroni correction. C, Free Ca^{2+} deposition per milligram of dry weight in aortic ring explants was determined by phenolsulphonphthalein binding as above. Data were pooled from 12 animals, 2 aortic cultures per time point. *P<0.001 by independent samples t test with Bonferroni correction. Data were pooled from 5 animals, replicates of 3.