Smooth Muscle Cells Give Rise to Osteochondrogenic Precursors and Chondrocytes in Calcifying Arteries

Mei Y. Speer, Hsueh-Ying Yang, Thea Brabb, Elizabeth Leaf, Amy Look, Wei-Ling Lin, Andrew Frutkin, David Dichek, Cecilia M. Giachelli

Abstract—Vascular calcification is a major risk factor for cardiovascular morbidity and mortality. To develop appropriate prevention and/or therapeutic strategies for vascular calcification, it is important to understand the origins of the cells that participate in this process. In this report, we used the SM22-Cre recombinase and Rosa26-LacZ alleles to genetically trace cells derived from smooth muscle. We found that smooth muscle cells (SMCs) gave rise to osteochondrogenic precursor- and chondrocyte-like cells in calcified blood vessels of matrix Gla protein deficient (MGP<sup>−/−</sup>) mice. This lineage reprogramming of SMCs occurred before calcium deposition and was associated with an early onset of Runx2/Cbfa1 expression and the downregulation of myocardin and Msx2. There was no change in the constitutive expression of Sox9 or bone morphogenetic protein 2. Osterix, Wnt3a, and Wnt7a mRNAs were not detected in either calcified MGP<sup>−/−</sup> or noncalcified wild-type (MGP<sup>+/+</sup>) vessels. Finally, mechanistic studies in vitro suggest that Erk signaling might be required for SMC transdifferentiation under calcifying conditions. These results provide strong support for the hypothesis that adult SMCs can transdifferentiate and that SMC transdifferentiation is an important process driving vascular calcification and the appearance of skeletal elements in calcified vascular lesions. (Circ Res. 2009;104:733-741.)

Key Words: genetic fate mapping ▪ lineage reprogramming ▪ Runx2/Cbfa1 ▪ smooth muscle cells ▪ vascular calcification

Vascular calcification refers to the abnormal deposition of calcium phosphate salts in blood vessels, myocardium, and cardiac valves. Vascular calcification can be life-threatening, as in the case of generalized infantile arterial calcification, calcific uremic arteriolopathy, and calcific valve disease. In atherosclerotic lesions, calcification is mainly found in the intima of blood vessels as dispersed punctate or patchy crystals associated with the necrotic core of atheromas (intimal calcification) and has been shown to positively correlate to the atherosclerotic plaque burden and the increased risk of myocardial infarction. Calcium phosphate salts deposit also in the media of blood vessels, known as Monckeberg’s medial sclerosis (medial calcification), and is prevalent in aging and patients with chronic kidney disease and type 2 diabetes mellitus. Medial calcification in these patients can occur independently of intimal calcification and or atherosclerotic lesions and features linear calcium phosphate deposits along the elastic lamina and, when advanced, circumferential mineral deposits throughout the media. Medial calcification results in increased vessel wall stiffness and decreased vessel compliance and therefore leads to increased arterial pulse wave velocity and pulse pressure that eventually affects coronary artery perfusion and heart function. Consequently, medial calcification-associated loss of arterial compliance may at least partially underlie increased coronary ischemic syndromes including myocardial infarction and left ventricular hypertrophy in diabetes and chronic kidney disease population.

Although previously considered a degenerative, uncontrolled process, the presence of bone-related proteins, cells of osteoblast and chondrocyte morphology, and outright bone- and cartilage-like tissue in calcified lesions has underscored the active, cell-mediated nature of vascular calcification. These findings have also led to the important questions of what cell type(s) give rise to the skeletal elements found in vascular calcification and what mechanisms regulate vascular calcification.

Smooth muscle cells (SMCs) are the predominant cell type found in the arterial wall and are essential for the structural and functional integrity of the vessel. Unlike most cell types that undergo terminal differentiation, SMCs retain substantial phenotypic plasticity in response to injurious stimuli in the local microenvironment. For example, SMCs convert from a quiescent, contractile phenotype to a proliferative, synthetic phenotype following arterial injury and in atherosclerotic disease. In calcified blood vessels, direct apposition of
calcification to medial SMCs that expressed bone and cartilage marker proteins, alkaline phosphatase, bone sialoprotein, and type II collagen has been reported. 4,5 Molecules regulating osteoblastic and chondrocytic differentiation, such as Runx2/Cbfa1, bone morphogenetic protein (BMP)2, Mx2, osterix, and Sox9, were also identified in calcified lesions of blood vessels. 5,9,11,12 In addition, cultured vascular SMCs are induced to calcify by addition of supraphysiological levels of phosphate. Concomitant with the onset of calcification, elevated phosphate levels induced cultured SMCs to undergo an osteochondrogenic phenotype change characterized by the loss of SMC markers (SM22α and SM α-actin) and gain of osteochondrogenic markers (Runx1/Cbfa1, osteopontin, osteocalcin, and alkaline phosphatase). 13 Similar phenotypic changes were also triggered in vivo via adenoviral expression of transforming growth factor (TGF)-β1 in arterial endothelium. Increased expression of TGF-β1 in arterial endothelium caused cartilaginous metaplasia in the underlying media of rats. 14 Finally, electron microscopic and immunohistochemical studies identified putative transitional cells, termed “myochondrocytes,” that showed hybrid SMC and chondrocyte properties in human and mouse atherosclerotic lesions. 9

Matrix Gla protein (MGP) is a calcification inhibitor that accumulates at the border of calcified areas and normal media of blood vessels, and appears to act locally to limit calcium phosphate deposition in the vessel wall. 4,8,15,16 Because MGP requires vitamin K-dependent γ-carboxylation for activation, undercarboxylated MGP, attributable mainly to vitamin K insufficiency and/or long-term warfarin treatment, accelerates the development of vascular calcification. 7,17 In addition, polymorphisms of the MGP gene are associated with increased risk of myocardial infarction and cardiovascular mortality in chronic kidney disease and hemodialysis patients. 18 Mutation of the MGP gene causes excessive arterial calcification as seen in human autosomal recessive condition, Keutel syndrome, and the MGP mutant mouse (MGP−/−). 16,19

To provide definitive evidence that SMCs contribute to the development of skeletal elements seen in calcified vascular media, we undertook a genetic fate mapping approach in MGP−/− mice. MGP−/− mice develop calcification of the arterial media with predominance of the elastic lamellae in elastic and muscular arteries, such as aortas, carotids, and coronary arteries. Calcification in these mice is associated with profound changes in cell differentiation as arterial SMCs are replaced by chondrocyte-like cells undergoing progressive mineralization. There are no fatty streaks or atherosclerotic plaques in the affected arteries of MGP−/− mice. MGP deficiency also causes aortic valve calcification, peripheral pulmonary artery stenosis, and skeletal defects including abnormal cartilage and bone calcification and nasal hypoplasia, but no ectopic calcification was found in the myocardium and other SM tissues of these mice. 13,16,20 Thus, using MGP−/− mice as an arterial medial calcification model in this report, we genetically labeled SMCs with the SM22-Cre recombines (SM22-Cre) 21 and the Cre reporter Rosa26-LacZ (R26R-LacZ) 22 alleles during embryonic development of the mice. This lineage tracing approach permits a direct test of whether vascular SMCs can undergo lineage reprogramming and contribute to the development of skeletal elements in calcified blood vessels.

**Materials and Methods**

MGP mutant mice generated in the C57BL/6j background were kind gifts from Dr Karsenty. 16 To generate MGP mutant mice in which cells of SM origin were genetically marked by LacZ transgenes, MGP heterozygotes (MGP+/−) were bred respectively to SM22α-Cre recombinase transgenic mice (gift from Dr Her, University of Texas Southwestern Medical Center, Dallas) and Rosa26 Cre reporter transgenic mice (gift from Dr Soriano, Fred Hutchinson Cancer Research Center, Seattle, Wash) to produce SM22α-Cre+/−:MGP+/− and R26R-LacZ+/−:MGP+/−. The F1 offspring were inbred to produce male SM22α-Cre+/−:MGP−/− and female R26R-LacZ+/−:MGP+/− mice which were then used as breeders to produce SM22α-Cre+/−:R26R-LacZ+/−:MGP−/− experimental mice and SM22α-Cre+/−;R26R-LacZ+/−:MGP−/+ controls. Mice were maintained in a specific pathogen-free environment, and genotypes were determined. 16,21,22 One- to 8-week-old mice were euthanized by lethal intraperitoneal injection of Nembutal (0.3 mg/g) for necropsy. A total of 52 mice were examined for these studies. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Tissues dissected from SM22α-Cre+/−;R26R-LacZ+/−:MGP−/− and SM22α-Cre+/−;R26R-LacZ+/−:MGP−/+ were stained with X-gal before processing and embedding in paraffin. Five-micron sections were used for histochemical and immunohistochemical analyses.

An expanded Materials and Methods is available in the online data supplement at http://circres.ahajournals.org.

**Results**

**Characterization of MGP Mutant Mice Carrying SM22-Cre and R26R-LacZ Transgenes**

As described in Figure 1A, mice carrying both SM22-Cre and R26R-LacZ transgenes delete the floxed stuffer sequence exclusively in SM22α-positive cells during embryonic development, generating intracellular β-galactosidase activity. Because Cre recombination occurs at the level of genomic DNA and is irreversible, β-galactosidase expression persists in the SM22α-expressing cells irrespective of subsequent down-regulation of SMC lineage proteins including SM22α. As shown in Figure 1C, MGP−/− mice hemizygous for R26R-LacZ and SM22-Cre transgenes had blue SMCs in the arterial media. Outside the vasculature, with rare exceptions (eg, occasional β-galactosidase positive cells in the outer fibrous layer of the epiphysial perichondrium), β-galactosidase expression was confined to SM-rich tissue (Figure 1D through 1H) and to a lesser extent, cardiomyocytes that transiently express SM22α early in development (data not shown). No β-galactosidase–positive cells were found in the BM (Figure 1H). In addition, calcification of blood vessels in MGP−/− mice did not affect β-galactosidase expression in cells of the vascular media (Figure 1I and 1J). The homogeneity of X-gal staining indicated excellent Cre excision efficiency (Figure 1C through 1J), identical to the findings in floxed tgfbr2 mice. 23 Finally, tissues from mice carrying only the R26R-LacZ transgene did not stain with X-gal (Figure 1B and 1K).

**SMCs Give Rise to Osteochondrogenic Precursors and Chondrocytes in Calcifying Arteries of MGP−/− Mice**

MGP−/− mice develop arterial medial calcification that has features similar to human calcified vessels. 13,16,20 As shown in
Figure I (A through D in the online data supplement), SMCs of noncalcified 1-week-old MGP\(^{-/-}\) vessels showed expression of SMMHC, SM22\(^{\alpha}\) and SM\(^{\alpha}\)-actin genes, demonstrating a normal SMC differentiation in this mutant strain. In calcified blood vessels of 4-week-old mice (Figures 1I, 1J, and 2E, dark brown and black), medial cells did not express SMC lineage proteins, SMMHC (Figure 2F versus brown in Figure 2B) and SM22\(^{\alpha}\) (Figure 2G versus brown in Figure 2C) but gained expression of the osteochondrogenic protein osteopontin (Figure 2H and 2D, brown). Because these medial cells stained blue with X-gal (marking them as cells that expressed SM22\(^{\alpha}\) at an earlier time point) and because they were present at the precise locations that were occupied by X-gal positive, SMC marker–expressing cells in noncalcified arteries, they appeared to have undergone a dramatic phenotypic change consistent with transdifferentiation from SMCs to osteochondrogenic progenitor cells. Moreover, in older mice, blue-staining type II collagen–expressing chondrocyte-like cells were often observed in the calcified media (Figure 3A and 3B, arrows). Of 11 calcified aortas, 9 contained chondrocyte-like cells in the calcified aortic media (82%), as identified by morphology and type II collagen staining (Figure 3D, brown). Importantly, nearly all of these cells also expressed \(\beta\)-galactosidase (Figure 3D, blue), suggesting strongly that they differentiated in situ from SMCs. Finally, X-gal, type II collagen antibody, and nuclear fast red triple-stained sections were used to quantify the proportion of chondrocyte-like cells that were derived from SMCs. Of 617 type II collagen–positive cells counted in calcified aortic media, 599 cells were stained blue by X-gal (97%), supporting the notion that osteochondrogenic precursor- and chondrocyte-like cells observed in the calcified MGP\(^{-/-}\) vessels derive from SMCs that transdifferentiate in situ.

To understand whether there was an increased turnover of medial cells in the MGP\(^{-/-}\) arteries, which would support circulating and/or residential multipotent mesenchymal pro-
genitors as possible sources of the observed osteochondrogenic precursor- and chondrocyte-like cells, we stained the arteries for active caspase-3 (apoptotic cells) and PCNA (proliferating-cell nuclear antigen) (proliferating cells). All sixteen stained MGP−/− aortas (1-week- to 8-week-old) showed only rare active caspase-3–positive cells in either adventitia or outer layer of the media (supplemental Figure II, B through D). Very few PCNA-positive cells were occasionally seen in the neointima and adventitia of the MGP−/− vessels (supplemental Figure II, G and H).

To further determine whether bone marrow (BM)-derived progenitors make a significant contribution to the osteochondrogenic precursor- and chondrocyte-like cells that appear in arteries of MGP−/− mice, we attempted to engraft green fluorescent protein (GFP)-expressing BM cells into MGP−/− neonates. Because MGP−/− mice start to develop vascular calcification at ≈2 weeks old and do not survive lethal irradiation, we used a nonablative neonatal BM transplantation strategy. The engraftment rate of the MGP−/− chimeras was low (<0.5% in peripheral blood versus 10% by Soper et al24), although GFP-positive cells were easily detected in thymus and spleen of recipients (supplemental Figure III, A and B). We also found GFP-positive cells in the aortae of recipients but these cells were rare (2 in the 2-week-old aorta and 1 in the 5-week-old aorta), and were all positive for CD45, identifying them as inflammatory cells (supplemental Figure III, D through F). Interpretation of this study is limited by a low engraftment rate (most likely attributable to use of a nonablative approach and noncongenic BM donors) and the possibility that only certain subpopulations of BM progenitor cells successfully engrafted. Therefore, the BM transplant study does not alone exclude a role for BM-derived cells as a source of osteochondrogenic precursor- and chondrocyte-like cells that appear in calcifying arteries of MGP−/− mice. However, combined with the genetic fate mapping study and the apoptosis and proliferation studies of the MGP−/− vessels that showed very low turnover rate of artery wall cells, the results of the BM transplantation study support our conclusion that BM-derived progenitors do not make a significant contribution to osteochondrogenesis in calcified MGP−/− vessels.

Runx2/Cbfa1 Is an Early Marker of SMC Transdifferentiation, and Its Upregulation Precedes Matrix Calcification

To identify potential regulators of SMC transdifferentiation in vivo, we extracted RNA from mildly calcified carotids of 2-week-old MGP−/− mice and measured expression of genes associated with differentiation of SMCs, osteoblasts, and
chondrocytes. As shown in Figure 4, the SMC master transcription coactivator, myocardin, and its target genes, SMMHC and SM22α, were downregulated in MGP⁻/⁻ arteries compared to wild-type counterparts. In contrast, the osteochondrogenic transcription factor, Runx2/Cbfa1, was highly upregulated in calcified arteries. BMP2, a potent inducer of ectopic calcification, and Sox9, a transcription factor required for chondrocyte differentiation, were present in equal amounts in MGP⁻/⁻ (calcified) and MGP⁺/+ (non-calcified) arteries. On the other hand, expression of Msx2, an inhibitor of chondrocytic differentiation, was decreased in MGP⁻/⁻ compared to MGP⁺/+ arteries. No detectable expression of osteoblast differentiation factors, osterix, Wnt3a, or Wnt7a was observed in either MGP⁻/⁻ or MGP⁺/+ vessels.

To further investigate the time course of SMC transdifferentiation in relation to arterial calcification, immunohistochemistry for Runx2/Cbfa1 was performed in arteries of 1- to 8-week-old MGP⁻/⁻ mice. As shown in Figure 5, Runx2/Cbfa1 was selectively localized to the nucleus of the majority of arterial medial cells in all MGP⁻/⁻ mice examined by 2 weeks of age (Figure 5A, brown). Staining of adjacent sections with an antibody recognizing β-galactosidase confirmed the SM lineage of these cells (Figure 5B, brown). A small number of Runx2/Cbfa1 positive cells were sometimes observed in the adventitia (Figure 5A, brown), but these cells were not β-galactosidase positive (Figure 5B), indicating that they were not of SM origin. Of particular interest, many of the Runx2/Cbfa1-β-galactosidase positive cells coexpressed SM22α at this time point (Figure 5C, brown), suggesting that they were transitional cells in an early stage of transdifferentiation to osteochondrogenic progenitors. Finally, the process of SMC transdifferentiation appeared to start before mineral deposition, because no arterial medial calcification could be detected in adjacent sections by von Kossa (Figure 5D) or Alizarin red S (data not shown) staining.

In contrast to the findings in 2-week-old mice, MGP⁻/⁻ arteries of 4- to 5-week-old mice were substantially calcified (Figure 5E, dark brown and black) and had much less Runx2/Cbfa1 expression. Only 2 of 5 mice showed a few Runx2/Cbfa1-positive cells in the calcified area (Figure 5F, brown) at this age. Runx2/Cbfa1 staining was exclusively in β-galactosidase–positive cells (smooth muscle [SM] origin) of the vessel (Figure 5F, blue and brown). By 6 to 8 weeks of age, MGP⁻/⁻ arteries no longer expressed Runx2/Cbfa1 either in the media or in the adventitia (Figure 5G), despite high levels of calcification, increased expression of the Runx2/Cbfa1 downstream target gene osteopontin (Figures 2H and 3C), and the presence of chondrocyte-like cells (Figure 3B and 3D). No Runx2/Cbfa1 staining was detected in 1-week-old MGP⁻/⁻ arteries (Figure 5H) or in MGP⁺/+ arteries at all ages examined (data not shown). Thus, temporal expression of Runx2/Cbfa1 correlated with early stages of SMC transdifferentiation, and preceded matrix calcification.

**Phosphorylation of Erk1/2 Is Required for SMC Transdifferentiation**

To study the potential mechanisms of SMC transdifferentiation, we isolated aortic medial cells from wild-type mice carrying SM22-Cre and R26R-LacZ transgenes. Cultures were induced to undergo calcification (Figure 6A) with elevated phosphate as previously described. Under these conditions, SMCs downregulated the expression of SM lineage markers, SM22α and SM α-actin (Figure 6B), and upregulated the expression of osteochondrogenic markers, osteopontin, Runx2/Cbfa1, and alkaline phosphatase (Figure 6C), a phenomenon that was also observed in clonal populations of mouse SMCs (data not shown). Thus, these calcifying cell culture experiments, performed with wild-type SMCs, seemed to reproduce the SMC transdifferentiation observed in calcified MGP⁻/⁻ arteries.

Because extracellular signal-regulated kinases (Erks) have been implicated in the regulation of SMC and osteoblast differentiation, we focused on the early stages of calcification and used this in vitro calcification model to examine the role of Erks in SMC transdifferentiation. Phosphorylation

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**Figure 4.** Expression of genes associated with differentiation of SMCs, osteoblasts, and chondrocytes in mouse arteries. Total RNA was extracted from 2 to 4 carotids of 2-week-old MGP⁻/⁻ or MGP⁺/+ mice. Total RNA (1 μg) of these pooled samples was reverse transcribed to cDNA. Various gene expression levels were determined by RT-PCR using specific primers as listed in supplemental Table I. Total RNA (1 μg) extracted from cementoblasts was used as positive controls for osteochondrogenic genes. Expression levels of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were determined to assure equal loading. Data shown represent 1 of the 2 preparations; similar results were achieved in another independent preparation. WT indicates wild type; KO, knockout; +Ctrl, positive control of osteochondrogenic genes.
of Erk1/2 was augmented in calcifying SMCs that were treated with high phosphate for 1 to 7 days. The increase of phosphorylated Erk1/2 levels occurred before a decrease in the levels of SMC lineage marker (Figure 7A). Furthermore, inhibition of Erk phosphorylation by the MEK inhibitor U0126 prevented the down regulation of SMC lineage markers (Figure 7B) and the SMC-specific transcription coactivator myocardin (Figure 7C) in calcifying SMCs. Moreover, Erk1/2 activation was accompanied by an increase of Runx2/Cbfa1. Upregulation of Runx2/Cbfa1 mRNA levels in calcifying SMCs was measured by quantitative real-time PCR. The increase in Runx2/Cbfa1 expression was confirmed by immunohistochemistry in calcifying arteries (Figure 5). U0126 prevented the down regulation of RUNX2/Cbfa1 in calcifying SMCs (Figure 7C).

Figure 5. Runx2/Cbfa1 expression in MGP−/− arteries of various ages. Aortas were dissected from 1-week-old (H), 2-week-old (A through D), 4-week-old (E and F), and 6-week-old (G) SM22α-Cre+/R26R−/−:MGP−/− mice. Adjacent sections were stained immuno-histochemically for Runx2/Cbfa1 (A and F through H), β-galactosidase (B), and SM22α (C), and for mineral by von Kossa (D and E). B and C were counterstained with nuclear fast red, D and E were counterstained with Methyl green, and F and G were prestained with X-gal. Arrows designate Runx2/Cbfa1-positive cells that originate from SM lineage (β-galactosidase positive). Inset, Higher-power magnification of the boxed region shows colocalization of β-galactosidase (blue) and Runx2/Cbfa1 (brown). L indicates lumen; M, media; Ad, adventitia.

Figure 6. SMC calcification and transdifferentiation in culture on exposure to elevated inorganic phosphate. SMCs were cultured in DMEM culture medium containing basal (1.0 mmol/L) or high (3.0 mmol/L) inorganic phosphate levels. At day 5, calcium content of the cultures was determined as described in Materials and Methods. Data shown are means±SD (n=3) (A). SMCs treated as in A were double stained by X-gal and antibodies specific for SMC lineage proteins (B) and bone tissue–associated proteins (C). Similar results were achieved in another independent experiment. ALP indicates alkaline phosphatase.
fying SMCs was also inhibited by U0126 (Figure 7D). Therefore, the early molecular events that likely initiate the process of SMC calcification are inhibited by U1026. Because prolonged treatment with U1026 was toxic to SMC, we were unable to determine whether prolonged blockade of MEK in this model would prevent SMC calcification.

Discussion

The cellular origins and mechanisms controlling development of ectopic cartilage and bone in diseased blood vessels are largely unknown. Decades of studies have raised 2 possibilities: transdifferentiation from mature SMCs or differentiation from immature multipotent mesenchymal progenitors that reside within the vessel wall or migrate from the circulation. In this report, we used a genetic fate mapping strategy to identify the origin of the cells that give rise to osteochondrogenic precursor- and chondrocyte-like cells observed in the calcifying blood vessels of MGP-/- mice. SMCs of the vascular media are labeled with β-galactosidase during embryonic development. Coexistence within a single vascular medial cell of β-galactosidase activity and osteochondrogenic or chondrocytic markers along with simultaneous loss of SM lineage markers provides strong evidence supporting lineage reprogramming of SMCs to osteochondrogenic precursors and chondrocytes. According to this reasoning, our experiments reveal that the majority of the osteochondrogenic precursor- and chondrocyte-like cells observed in the calcifying blood vessels of MGP-/- mice were derived from SMCs. This conclusion is supported by localization of Runx2/Cbfa1, osteopontin, and type II collagen expression within β-galactosidase–positive cells.

Previous studies have implicated multipotent mesenchymal progenitors as possible sources of skeletal elements observed in vascular calcification. Demer and colleagues have identified a clonal population of bovine arterial medial cells, termed calcifying vascular cells (CVCs). The CVCs lack SMC marker proteins and display pericyte-like properties early in culture. With time in culture these CVC spontaneously form calcifying nodules and develop osteoblastic features.

More recently, the CVCs have been shown to undergo additional developmental fates, including chondrogenesis, leiomyogenesis, and stromagenesis, depending on the culture conditions. Thus, the CVCs behave like pericytes, a cell type that has long been postulated as a reservoir of multipotent stem cells in adult vasculature and can be induced to differentiate into multiple lineages, including osteoblasts.

In our studies of MGP-/- medial calcification, osteochondrogenic cells seen in the calcified arterial media were unlikely to be derived from pericytes or multipotent mesenchymal progenitors because no β-galactosidase activity was found in the BM (Figure 1H) or in the vascular adventitia of the SMC22α-Cre:R26R-LacZ mice at any age examined (see also). In addition, no cells expressing the mesenchymal/hematopoietic stem cell markers Sca-1 and CD34 were observed in the calcifying MGP-/- vessels (data not shown). Moreover, a hypothesis that attributed chondrogenesis to intramural migration of extramural precursor cells would need to account for the simultaneous near-total disappearance of resident SMCs from vascular media. This seems highly unlikely as supported by the rare occurrence of apoptotic cells and the low number of both proliferating cells in calcified MGP-/- arteries (supplemental Figure II) and engrafted GFP-expressing cells in the MGP-/- neonatal chimeric arteries (supplemental Figure III). Nevertheless, our studies cannot exclude rare events and although they overwhelmingly support a major role for SMC lineage reprogramming in arterial medial calcification of MGP-/- mice, they do not completely exclude limited contribution of non-SM
MGP

\[ \rightarrow \]

\[ \text{Pi} \]

BMP2

\[ \uparrow \text{Erk1/2 phosphorylation} \]

\[ \text{Runx2/Cbfa1} \]

\[ \rightarrow \text{SM lineage genes (myocardin, SM22α, SMα-actin)} \]

Osteochondrogenic precursors

+Sox9

Mx2

Osteoblasts

Chondrocytes

Figure 8. Proposed mechanisms of SMC transdifferentiation in calcified arteries. Loss of MGP with subsequent release from inhibition of BMP2, or exposure to elevated phosphate, leads to increased BMP2 and phosphorylation of Erk1/2 in SMCs. Erk1/2 activation increases Runx2/Cbfa1 and decreases myocardin and SMC lineage markers to generate the osteochondrogenic precursor state. In the presence of high Sox9 and the absence of Mx2, Wnts, and osterix, osteochondrogenic precursors preferentially differentiate toward a chondrocytic lineage.

Cell types or circulating BM-derived precursors to the population of osteochondrogenic cells and chondrocytes that appear in MGP−/− arteries. Moreover, we cannot assume that the lineage reprogramming shown in this model would also explain observations in other vascular calcification models.9,12,14,30,31

MGP is a 10-kDa protein containing 5 γ-carboxyglutamic acid residues. It is normally expressed at high levels in cartilage and SM, and serves as a calcification inhibitor in cartilage and vasculature.25 Part of this inhibitory effect has been attributed to its capacity to bind and inhibit BMP2, a major regulator of Runx2/Cbfa1-dependent osteochondrogenic differentiation and SM lineage genes, SMMHC, SM22α, and SMα-actin, and because 1-week-old MGP−/− arteries showed normal SMC differentiation as evidenced by expression of the SM lineage genes, SMMHC, SM22α, and SMα-actin, and because 1-week-old MGP−/− arteries showed no expression of osteochondrogenic genes such as Runx2/Cbfa1, transdifferentiation of SMCs toward osteochondrogenic precursor–chondrocyte-like cells in the calcifying MGP−/− vessels is unlikely to be due simply to a lack of functional MGP. It is proposed that lack of MGP gene expression in SMCs of MGP−/− mice leaves BMP2 activity unopposed, resulting in Runx2/Cbfa1 expression and inhibited myocardin expression, and thus transdifferentiation of SMCs to osteochondrogenic precursors (Figure 8). Our data suggest that differentiation of these precursors preferentially down the chondrocyte path is favored by low levels of Mx2, an inhibitor of chondrogenesis,26 expression of Sox9, a chondrocyte differentiation factor,26 and lack of expression of osterix and Wnts, factors that are required for osteoblastic differentiation and prevention of osteoblast differentiation to chondrocytes respectively.32

Our finding of endochondral differentiation in calcifying MGP−/− vessels differs from the osteoblastic differentiation described by Towler and colleagues31 in arteries of diabetic, atherosclerotic, LDL receptor knockout (LDLr−/−) mice. This is likely attributable to differences in signaling pathways present in calcifying arteries of MGP−/− versus LDLr−/− mice. In LDLr−/− mice, hyperlipidemia induced upregulation of aortic BMP2 expression and promoted adventitial Mx2-Wnt signaling, which triggered mural CVCs and other resident osteoprogenitors to proceed down the osteoblast differentiation path via tumor necrosis factor-α–dependent signals.31 In agreement with this model, hyperlipidemic CMV-Msx2 transgenic LDLr−/− mice exhibited marked cardiovascular calcification. Intraperitoneal administration of BMP2 enhanced aortic Mx2 expression and canonical Wnt signaling in the tunica media of the blood vessels of TOPGAL mice (Wnt signaling reporters).31 In contrast, calcifying MGP−/− vessels were characterized by Sox9 expression, downregulation of Mx2 expression, and no detectable expression of osterix, Wnt3a, or Wnt7a. Thus, chondrocytic versus osteoblastic differentiation is likely to depend on the local signaling milieu, which can differ substantially depending on the underlying disease and/or deficiency state.

Our in vitro studies suggest that transdifferentiation of SMCs is initiated by activation of the Erk1/2 signaling pathway, suppression of the SMC master transcription coactivator myocardin, and induction of the osteochondrogenic transcription factor Runx2/Cbfa1 (Figure 8). Because BMP2 provokes phosphate uptake and SMC phenotypic transition toward osteochondrogenic progenitors33 and is known to induce Erk1/2 signaling,27 BMP2 and elevated phosphate appear to share a common downstream signaling pathway for induction of SMC transdifferentiation. In support of these possibilities, Olson and coworkers reported that platelet-derived growth factor-BB–mediated inhibition of SMC-specific gene expression was attributable to the Erk1/2-dependent phosphorylation of the transcriptional repressor Elk1. Phosphorylation of Elk1 promoted its binding to SRF and prevented the association of SRF with myocardin and thus inhibited the expression of SM-specific genes.28 Consistent with these findings, Erk phosphorylation was associated with injury- or fibronectin-induced phenotypic modulation of vascular SMCs.34,35 Finally, Erk1/2 is important in osteoblast differentiation, as identified by its essential role in expression of osteogenic genes including Runx2/Cbfa1, osteopontin, osteocalcin, and bone sialoprotein, as well as its function in Runx2/Cbfa1–dependent skeletal development.27,29

Our findings suggest a crucial role for SMCs in mediating the onset and development of vascular medial calcification especially under conditions of MGP deficiency. The osteochondrogenic state of SMCs may be exquisitely designed to repair and/or adapt to a calcifying microenvironment, with enhanced expression of a number of calcification regulatory molecules. Understanding the mechanisms that control SMC
transdifferentiation to osteochondroprogenitors and subsequent vascular calcification may help developing novel strategies that prevent or reverse vascular calcification.

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Disclosures

None.

References


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Materials and Methods

Nonablative Neonatal Marrow Transplantation of MGP Mutant Mice

Neonatal bone marrow chimeras of MGP-/- mice were generated as described previously. Briefly, MGP heterozygotes were backcrossed to C57BL/6 mice for five generations to obtain MGP mutants with an estimated 96.9% C57BL/6 genomic background. MGP+/- mice were then timed-mated to produce the offspring recipients.

Donor UBC-GFP mice (C57BL/6, ~ 6 week old) were injected intraperitoneally with one dose 5-fluorouracil (0.15 mg/g body weight) 3 days prior to bone marrow cell collection in order to eliminate cycling cells and thus enrich the nucleated stem cell content of the marrow. The enriched marrow cells were harvested from femurs of the UBC-GFP donors and the marrow erythrocytes were removed by incubating with Gey’s solution. Finally, the enriched nucleated GFP marrow cells were transplanted intravenously to the MGP mutant neonatal recipients through the superficial temporal vein on the first and third days after birth. The recipients were not irradiated. Each recipient neonate was injected with 20 µl containing 2 – 3 × 10^6 GFP marrow cells per day, and a total of 13 neonates were injected to generate neonatal bone marrow chimeras. The MGP chimeras were genotyped on day 10 after birth and 2 MGP-/- chimeras were identified. Tissues of MGP-/- chimeras were collected at 2 weeks and 5 weeks after birth. The engraftment rate of the MGP chimeras was evaluated by flow cytometry of peripheral blood and immunohistochemical staining of thymus and spleen for GFP.
Tissue Preparation, Histochemical and Immunohistochemical Staining

Tissues dissected from SM22α-Cre+/0:R26R-LacZ+/0:MGP−/− and SM22α-Cre+/0:R26R-LacZ+/0:MGP+/+ mice were fixed with Methyl Carnoy's fixative and embedded in paraffin. Tissues dissected from neonatal MGP−/− bone marrow chimeras were fixed with 10% buffered formalin and embedded in paraffin. Five-micrometer sections were used for histochemical and immunohistochemical analyses.² Haemotoxylin and eosin stain was used to visualize morphology; Alizarin red S and von Kossa stain were used to detect mineral; antibodies recognizing SM myosin heavy chain (SMMHC; ab683, Abcam), SM22α (ab10135, Abcam), SM α-actin (1A4, Sigma), osteopontin (AF808, R&D systems), Runx2/Cbfa1 (MAB2006, R&D systems), type II collagen (AB761, Chemicon), CD45 (30-F11, BD Pharmingen), CD34 (ab8158, Abcam), Sca1 (AF1226, R&D Systems), and green fluorescent protein (A11122, Invitrogen) were used to detect SMCs, osteochondrogenic cells, chondrocytes, inflammatory cells, hematopoietic stem cells, and GFP-positive cells of the tissues; antibody recognizing cleaved caspase-3 (9664, Cell Signaling Technology, Inc.) was used to detect apoptotic cells; antibody recognizing proliferating cell nuclear antigen (PCNA; M0879, DAKO) was used to detect proliferating cells. Sections were counterstained with either methyl green or nuclear fast red (Vector).

Vascular SMC Preparation

Vascular SMCs were prepared from aortas of 4-week-old MGP+/+:SM22α-Cre+/0:R26R-LacZ+/0 transgenic mice as described previously.³ Briefly, the media was
carefully stripped from the thoracic and upper part of abdominal aorta under a dissection microscope and cut into 1-mm pieces. The media pieces were first treated with 1 mg/mL collagenase for 20 minutes to remove residual endothelial and adventitial cells, rinsed with culture medium, and then dispersed in a mixture of 1 mg/mL collagenase and 0.5 mg/mL elastase in culture medium containing 12.5% FBS. After incubation at 37°C for 40 minutes to 1 hour with occasional gentle agitation, medial cells were released. The cell suspension was centrifuged at 800g for 5 minutes, and the cell pellet was washed and resuspended in DMEM culture medium containing 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL fungizone, and 20% FBS. Aortic SMCs were seeded at a density of 1 × 10^5 cells/mL for primary culture, and split 1:2 at confluency. Cells used for the experiments were from 3rd – 9th passages. Subcultured SMCs were maintained in DMEM culture medium containing 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL fungizone, and 10% FBS.³

**Western Blot Analysis and Immunocytochemical Staining**

Protein lysates were prepared from SMC monolayers using 0.1 mol/L Tris-HCl buffer, pH 6.8, supplemented with 2% SDS, 2 µg/mL pepstatin, 2 µg/mL leupeptin, 2 µg/mL aprotinin, and 1 mmol/L PMSF. Protein content of the lysates was measured by the Micro BCA assay (Pierce Rockford). Equal amounts of the protein from each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a PVDF membrane (Perkin Elmer). Proteins of interest were then blotted using specific antibodies, biotin-streptavidin amplification, and Western Blot
Chemiluminescence detection (Perkin Elmer, CA). β-tubulin blotting was used as sample loading controls (data not shown).

To immunostain cultured SMCs, cells were seeded at a density of $1 \times 10^4$/mL/well into permanox chambers (Lab-Tek chamber slide, Nalgen Nunc Int Corp). At the indicated culture time, the cells were fixed with cold methanol or 10% formalin. Proteins of interest were detected using specific antibodies as described.3

Antibodies used for Western blot analysis and immunocytochemistry were as follows: monoclonal mouse anti-human smooth muscle α-actin (1A4, Sigma), polyclonal goat anti-SM22α antibody (ab10135, Abcam Inc.), polyclonal goat anti-mouse OPN (AF808, R&D Systems), monoclonal rat anti-human Runx2/Cbfa1 (MAB2006, R&D systems), monoclonal mouse anti-human alkaline phosphatase (B4-78, tissue non-specific, Developmental Studies Hybridoma Bank, University of Iowa, cross react with mouse tissue non-specific alkaline phosphatase), and polyclonal rabbit anti-human p44/42 MAP kinase and phosphor MAP kinase (9100, Cell Signaling Technology, Inc.).

**Reverse Transcription-PCR and Taqman Real-time Reverse Transcription-PCR**

Total RNA was extracted from either 2-week-old mouse carotids or SMC monolayers using RNeasy Mini kit. The contaminating genomic DNA was digested by RNase-free DNase I (Qiagen). 1 μg total RNA was used to synthesize first-strand cDNA using Omniscript (Qiagen) at 37°C for 1 hour, and the cDNA produced was used to determine
genes associated with differentiation of SMCs, osteoblasts, and chondrocytes by reverse transcription-PCR (RT-PCR) using the primers listed in Table 1. Thirty cycles of PCR were used to allow exponential amplification of the desired genes.

Myocardin and Runx2/Cbfa1 mRNA levels were quantified by Taqman real-time RT-PCR using ABI Prism 7000 (Applied Biosystems). To avoid PCR amplification of any residual genomic DNA, probe sequences spanned exon-exon junction of the genes. Sequences used in the Taqman real-time PCR assay were: myocardin forward primer, 5’ CCACCCCGAGCACTCAAATCC 3’, myocardin reverse primer, 5’ TGCATCATTCTTGTCACTTTCTGA 3’, myocardin probe, FAM (6-carboxyfluorescein)-ACAATCCAGGATCTCACTC-MGB (minor groove binder); Cbfa1 forward primer, 5’ CGGGCTACCTGCCATCAC 3’, Cbfa1 reverse primer, 5’ GGCCAGAGGCAGAAGTCAGA 3’, Cbfa1 probe, FAM-CGTATTTCAGATGATGACACTG-MGB. To control sample loading, 18s ribosomal RNA was used as calibrator. Sample 18s ribosomal RNA was determined using Taqman® Ribosomal RNA Control Reagents from ABI. The expression levels of myocardin and Runx2/Cbfa1 were normalized to 18s ribosomal RNA of the same samples and expressed as fold of this calibrator.

**X-gal staining**

Tissues dissected from SM22α-Cre+/0:R26R-LacZ+/0 mice were stained using a β-gal stain kit from Specialty Media prior to tissue processing and paraffin embedding. SMCs
isolated from aortas of SM22α-Cre+/0;R26R-LacZ+/0 mice were stained using the cultured cell β-gal stain kit from the same vendor.

**Calcium Quantification**

Cell cultures were rinsed with PBS and decalcified with 0.6 mmol/L HCl at 4°C for 24 hours. Calcium released from the cell cultures was determined colorimetrically by the o-cresolphthalein complexone method as described previously (Sigma calcium diagnostic kit). Calcium amount was normalized to cellular protein of the culture and expressed as µg/mg cellular protein.

**Statistical Analysis**

Data, shown as means ± SD, were analyzed with Student’s t-test or ANOVA to determine the significance of differences. Data were considered to be statistically significant at a p value < 0.05.
Figure legends

**Online Figure I. Differentiation of vascular SMCs in MGP-/- mice.** Aortas were dissected from 1-week-old SM22α-Cre+/0:R26R+/0:MGP-/- mice. Cells of SMC origin were stained by X-gal (A) before embedding and sectioning. Adjacent sections were stained for SM lineage proteins, SMMHC (B), SM22α (C), and SM α-actin (D). Note the normal SMCs differentiation in non-calcified arteries of this mutant strain (brown). L =lumen; M=media; Ad=adventitia.

**Online Figure II. Apoptosis and proliferation in MGP-/- arteries of various ages.** Aortas of 1- to 8-week-old MGP-/- mice were stained for apoptosis and proliferation using antibodies recognizing active caspase-3 (A – E) and PCNA (F – I). Eleven out of sixteen aortas were negative for active caspase-3 staining, whereas the others showed only rare positive cells in either the adventitia or outer layer of the media. A and F, MGP+/+ aorta (2 week old), B – D and G – H, MGP-/- aortas (B and G, 2 week old, others 5-6 week old). C was pre-stained with X-gal. E, thymus, positive control for active caspase-3 stain. I, intestine, positive control for PCNA stain. Arrows designate active caspase-3 or PCNA positive cells. L =lumen; M=media; Ad=adventitia.

**Online Figure III. Evaluation of engraftment and homing of the circulating GFP marrow cells of the MGP-/- chimeras.** Thymus (A), spleen (B and C), and aortas (D – F) of 2-week-old MGP-/- chimera mouse were stained for GFP-positive cells (A, B, D,
and E) and CD45-positive cells (C and F). Note GFP-positive cells were mostly found in thymus and spleen. Only two GFP-positive cells were found in aorta, and these also stained positively for the leukocyte marker, CD45 (E vs F). Arrows designate GFP-positive cells that stained also for CD45. L = lumen; M = media; Ad = adventitia.

Online Figure I

Online Figure II
Online Table I. Primers used to detect various gene and transcription factors associated with differentiation of SMCs, osteoblasts, and chondrocytes.

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<tr>
<th>Genes</th>
<th>Sense primers (5’-3’)</th>
<th>Antisense primers (5’-3’)</th>
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References

