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Runx2/Cbfa1, But Not Loss of Myocardin, Is Required for Smooth Muscle Cell Lineage Reprogramming Toward Osteochondrogenesis

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ABSTRACT

Vascular calcification is a major risk factor for cardiovascular morbidity and mortality. Smooth muscle cells (SMCs) may play an important role in vascular cartilaginous metaplasia and calcification via reprogramming to the osteochondrogenic state. To study whether SM lineage reprogramming and thus matrix calcification is reversible and what the necessary regulatory factors are to reverse this process, we used cells isolated from calcifying arterial medias of 4-week-old matrix Gla protein knockout mice (MGP−/−SMCs). We found that vascular cells with an osteochondrogenic phenotype regained SMC properties (positive for SM22α and SM α-actin) and down-regulated osteochondrogenic gene expression (Runx2/Cbfa1 and osteopontin) upon culture in medium that favors SMC differentiation. Over time, the MGP−/− SMCs no longer expressed osteochondrogenic proteins and became indistinguishable from wild-type SMCs. Moreover, phenotypic switch of the restored SMCs to the osteochondrogenic state was re-induced by the pro-calcific factor, inorganic phosphate. Finally, loss- and gain-of-function studies of myocardin, a SM-specific transcription co-activator, and Runx2/Cbfa1, an osteochondrogenic transcription factor, revealed that upregulation of Runx2/Cbfa1, but not loss of myocardin, played a critical role in phosphate-induced SMC lineage reprogramming and calcification. These results are the first to demonstrate reversibility of vascular SMCs in the osteochondrogenic state in response to local environmental cues, and that myocardin-enforced SMC lineage allocation was not sufficient to block vascular calcification. On the other hand, Runx2/Cbfa1 was found to be a decisive factor identified in the process. J. Cell. Biochem. 110: 935–947, 2010.

KEY WORDS: CALCIFICATION; LINEAGE REPROGRAMMING; MYOCARDIN; Runx2/Cbfa1; SMOOTH MUSCLE CELLS

Vascular calcification has long been considered a benign complication arising from calcium phosphate salts deposited in blood vessels of the elderly and patients with atherosclerosis, diabetes mellitus, and chronic kidney disease. In recent decades, electron beam computed tomography (EBCT) has allowed rapid and sensitive measurement of vascular calcification to be correlated to a growing list of clinical events and cardiovascular risk [Puentes et al., 1995; Rumberger et al., 1995; Raggi, 2000; Moe et al., 2002; Reaven and Sacks, 2005]. In coronary arteries, calcification is positively correlated with atherosclerotic plaque burden [Rumberger et al., 1995], increased risk of myocardial infarction [Puentes et al., 1995], and increased risk of dissection following angioplasty [Fitzgerald et al., 1992]. Although its contribution to plaque instability is controversial [Huang et al., 2001], intimal calcification is believed to be a marker for plaques that have undergone rupture [Taylor et al., 2000]. Finally, vascular medial calcification is highly prevalent in dialysis patients and type II diabetic patients, and is an independent predictor of morbidity and mortality in this population [Lehto et al., 1996; Shanahan et al., 1999; Raggi, 2000; Moe et al., 2002].

A growing number of studies suggest that vascular calcification is an active, cell-mediated process that involves, in part, processes that recapitulate skeleton development and remodeling [Shanahan et al., 1999; Bobryshev, 2005; Aikawa et al., 2007; Neven et al., 2007]. In a report examining peripheral arteries of type II diabetic patients, medial calcification was observed in direct apposition to medial SMCs that express various bone and cartilage marker proteins, such as alkaline phosphatase, bone sialoprotein, bone Gla protein, type II collagen, and osteopontin [Shanahan et al., 1999]. Molecules regulating osteoblastic and chondrocytic differentiation including Runx2/Cbfa1 [Moe et al., 2002; Aikawa et al., 2007], BMP2 [Bostrom et al., 1993], Msx2 [Towler et al., 1998], osterix [Aikawa et al., 2007], and Sox9 [Tyson et al., 2003; Bobryshev, 2005], have also been identified in calcified vascular lesions. Moreover, osteochondrogenic differentiation and cartilaginous metaplasia are found in

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experimental animal and human calcified blood vessels [Shanahan et al., 1999; Bobryshev, 2005; Aikawa et al., 2007; Neven et al., 2007]. Our recent findings strongly suggest that lineage reprogramming of smooth muscle cells (SMCs) contributes to osteochondrogenic SMC differentiation [Steitz et al., 2001; Speer et al., 2009]. In addition, it is possible that multipotent mesenchymal progenitors (residing in the vessel wall and perivasculature and/or migrating from circulation) may also serve as sources of osteochondrogenic differentiation in vasculature.

Using a genetic fate mapping strategy, we identified lineage reprogramming of SMCs to osteochondrogenic precursors and chondrocytes in calcified arteries of MGP−/− mice [Speer et al., 2009]. Cells of SM origin were found to contribute to 97% of cartilaginous metaplasia and calcification of MGP−/− vessels as evidenced by the co-localization within a single cell of SM-specific transgene with the loss of SM lineage proteins and the gain of osteochondrogenic proteins. In this model, we also found that lineage reprogramming of SMCs toward osteochondrogenic precursors was likely the initial step of vascular calcification [Speer et al., 2009].

Our findings also linked SMC lineage reprogramming to a decreased activity of the SM transcription co-activator, myocardin, and the loss of its downstream target gene expression [Speer et al., 2009]. Myocardin was first discovered by Olson and colleagues as an extremely potent co-activator of serum response factor (SRF) and is responsible for its SM specificity [Wang et al., 2001; Du et al., 2003]. Targeted deletion of SRF binding domains of myocardin in mice leads to embryonic death in homozygotes due to severe vascular defects as a result of failure in SMC differentiation [Li et al., 2003]. Forced expression of myocardin in non-SM cells (e.g., ES cells) [Du et al., 2003] activates the expression of SM lineage genes, such as SM22α, SM α-actin, SM-MHC, and SM-calponin. These observations make myocardin an ideal candidate for studying whether down-regulation of SMC lineage gene expression may in itself drive osteochondrogenic phenotypic transition and/or matrix calcification.

Runx2/Cbfa1 is a known transcription factor required for skeletal formation and remodeling. Targeted deletion of Runx2/Cbfa1 in mice leads to immediate death after birth due to failure of skeletal mineralization that results in respiratory failure [Otto et al., 1997]. In addition, lack of mandibular condylar cartilage, deformity of Meckel's cartilage [Shibata et al., 2004], and abnormality of hypertrophic chondrocyte differentiation [Otto et al., 1997] are found in embryo and newborn homozygous Runx2/Cbfa1 null mice. Runx2/Cbfa1 is found in the vasculature specifically associated with the formation of cartilaginous metaplasia and calcification in human diabetic medial lesions, atherosclerotic intimal lesions, aortic valve disease, as well as vascular calcification of various mouse models [Moe et al., 2002; Aikawa et al., 2007; Neven et al., 2007; Speer et al., 2009]. It is important to note that Runx2/Cbfa1 is not normally expressed in the vasculature and its expression in diabetic and/or atherosclerotic lesions appears to precede overt calcification [Moe et al., 2002; Aikawa et al., 2007; Speer et al., 2009].

Whether SMC lineage reprogramming toward osteochondrogenesis is reversible and hence clinically modifiable, and what factors are critical in governing SMC lineage reprogramming are currently unknown. In the present study, we isolated SMCs from MGP−/− vessels that had undergone lineage reprogramming and were in the early stage of osteochondrogenesis. We found that upon isolation and culture, MGP−/− medial cells lost osteochondrogenic features and regained SMC properties. Loss- and gain-of-function studies identified that upregulation of Runx2/Cbfa1, but not loss of myocardin, appeared to be the decisive molecule for the SMC lineage reprogramming toward osteochondrogenesis and matrix calcification.

MATERIALS AND METHODS

PREPARATION OF ARTERIAL SMCs FROM MGP−/− OR MGP+/+ MICE

Matrix Gla protein knockout (MGP−/−) mice were generated in a C57BL/6J background and were kind gifts from Dr. Karsenty [Luo et al., 1997]. MGP+/+ mice used as experimental controls were generated by interbreeding MGP+/− mice. The animals were maintained in a specific pathogen-free environment and genotypes were determined as previously described [Luo et al., 1997; Speer et al., 2009]. Animals were sacrificed by lethal intraperitoneal injection of nembutol (0.3 mg/g mouse). All protocols were approved by the Animal Use Committee, University of Washington. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Vascular SMCs were prepared from aortas of 4-week-old MGP−/− and +/+ mice as described previously. 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 min 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 min to 1 h with occasional gentle agitation, medial cells were released. The cell suspension was centrifuged at 800*g* for 5 min, 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 primary cultures and subcultures of 3–9 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.

GENERATION OF KNOCKDOWN CELL LINES VIA RNA INTERFERENCE

Nineteen nucleotide target sequences for mouse myocardin (5′GAACATTCTGCCGATGGAT3′) and Runx2/Cbfa1 (5′GAGTTTCACCTTGACCATA3′) were selected from the encoding region of the respective mRNA and synthesized by MWG Biotech Inc. The annealed double stranded oligonucleotides of each gene were inserted into *Hin*d III and *Bgl* II sites of pSUPER retroviral vector. Positive clones having 64-bp insert were sequenced to confirm the sequence identity. The recombinant pSUPER containing either

mouse myocardin or Runx2/Cbfa1 siRNAs were then used to transiently transfect phoenix ecotropic packaging cells (the Nolan laboratory, Stanford University, CA) via calcium-phosphate precipitation. At 48-h post-transfection, virus-containing culture media was collected and used to infect mouse vascular SMCs at passage 2 to establish myocardin knockdown (MyosiRNA) and Runx2/Cbfa1 knockdown (Runx2 siRNA) stable cell lines. Transduction of the pSUPER-containing non-specific oligonucleotide was used to create control SMC lines (OligoCT). All cell lines were selected with puromycin to achieve pure populations. To determine the knockdown efficiency and specificity of the myocardin and Runx2/Cbfa1 siRNAs, myocardin and Runx2/Cbfa1 mRNA levels, the respective family members, and the respective downstream target genes were evaluated.

FORCED-EXPRESSION OF MYOCARDIN IN MOUSE VASCULAR SMCs

Rat myocardin cDNA was obtained from Dr. Joe Miano (University of Rochester). Identity of the cDNA was confirmed by DNA sequence analysis. An adaptor sequence containing the start codon was added to the 5′ of the insert and the complete cDNA was subcloned into the retroviral vector pLXIN (BD Biosciences, Palo Alto, CA). The recombinant pLXIN containing rat myocardin cDNA was then used in retroviral transduction to generate a stable cell line that forcedly expresses rat myocardin in a MyosiRNA SMC line (MyosiRNA-rMyocardin) as previously described. pLXIN vector backbone was used to generate a control SMC line (MyosiRNA-pLXIN). All cell lines were selected with 1 mg/ml G418 to achieve pure populations.

WESTERN BLOT ANALYSIS AND IMMUNOCYTOCHEMICAL STAINING

Protein lysates were prepared from freshly isolated SMCs or SMC monolayers of indicated passages 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 of the same specimens was used as sample loading controls.

To immunostain cultured SMCs, freshly isolated SMCs were seeded at a density of 1×10^4/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.

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.), monoclonal mouse anti-human desmin (D33, DAKO), 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 β-tubulin (ab6046, Abcam Inc.).

TAQMAN REAL-TIME QUANTITATIVE RT-PCR AND CONVENTIONAL RT-PCR

Total RNA was extracted from freshly isolated mouse aortic SMCs, subcultured SMCs of indicated passages, or various SMC lines using RNeasy Mini kit. The contaminating genomic DNA was digested by RNase-free DNase I (Qiagen). Two hundred nanograms to 1 μg total RNA was used to synthesize first-strand cDNA using Omniscript (Qiagen) at 37°C for 1 h and the cDNA was used as template to determine the expression levels of various genes via Taqman real-time quantitative PCR using an ABI Prism 7000 (Applied Biosystems). To avoid PCR amplification of any residual genomic DNA, probe sequences spanned exon-exon junction of gene. 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. Expression level of the desired gene was normalized to 18s ribosomal RNA of the same sample and expressed as fold of this calibrator. The expression levels of rat myocardin were determined by conventional RT-PCR. Thirty cycles of PCR were used to allow exponential amplification of the gene and the sample GAPDH expression levels were used as internal loading controls. Primer and probe sequences used for conventional and Taqman real-time quantitative RT-PCR analyses are listed in Table I.

CALCIUM QUANTIFICATION

Cell cultures were rinsed with PBS and decalcified with 0.6 mmol/L HCl at 4°C for 24 h. Levels of calcium released from cell cultures were 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.

TABLE I. Primers and Probes Used to Detect Various Genes and Transcription Factors

Genes	Sense primers (5′–3′)	Antisense primers (5′–3′)	Probes (5′–3′′)
Runx2/Cbfa1	CGGGCTACCTGCCATCAC	GGCCAGAGGCAGAAGTCAGA	CGTATTTCAGATGATGACACTG
Runx1	GGCAGGCAACGATGAAAACT	CATGGCCGCGGTAGCA	CTCGGCAGAACTGAGAA
Runx3	TCCGCTGTCATGAAGAACCA	GGCCCACGAATCGAAGGT	TGGCCAGGTTCAAC
Osterix	GGTTCTCTCCATCTGCCTGACT	CAGGGGACTGGAGCCATAGT	CTGCTTGAGGAAGAAG
Osteopontin	TGAGGTCAAAGTCTAGGAGTTTCC	TTAGACTCACCGCTCTTCATGTG	TTCTGATGAACAGTATCCTG
Myocardin	CCACCCCAGACATCAAATCC	TGCATCATTCTTGTCACTTTCTGA	ACAATCCAGGATCTCACTC
SM22α	GACTGACATGTTCCAGACTGTTGAC	CAAACTGCCCAAAGCCATTAG	TGAAGGTAAGGATATGGCAGC
Rat myocardin	CCAACAGTTCCGGAGATAACC	ACTTGGGGAGGATGGTGGTT	
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	

STATISTICAL ANALYSIS

Data, shown as means ± SD, significance between groups was analyzed with Student's *t*-test or ANOVA followed by Fisher's PLSD. Data were considered statistically significant at a P value of <0.05. The association of two variables, shown as correlation coefficient, was considered statistically significant at a P value of <0.05.

RESULTS

OSTEOCHONDROGENIC MEDIAL CELLS ISOLATED FROM MGP−/− VESSELS REGAIN SMC LINEAGE PROPERTIES IN CULTURE

Our previous studies revealed that SM22α-expressing smooth muscle cells were the major contributors to the osteochondrogenic precursors and chondrocytes observed in calcifying arteries of MGP−/− mice [Speer et al., 2009]. To further study the reversibility of the SMC lineage reprogramming, we isolated arterial medial cells from the aortas of 4-week-old MGP−/− mice. At this age, the majority of the medial SMCs had differentiated to osteochondrogenic precursors as identified by the Runx2/Cbfa1 expression and the loss of SM lineage markers, but not yet to collagen type II-expressing chondrocyte-like cells [Speer et al., 2009]. Each batch of the medial cells was isolated and pooled from the aortic media tissue of five to six animals. Newly isolated cells were used to determine the expression levels of SM lineage proteins and osteochondrogenic marker proteins as described in the methods section.

As shown in Figure 1A, lysate of aortic medial cells isolated from MGP wild type mice (MGP+/+ SMC) was strongly positive for the

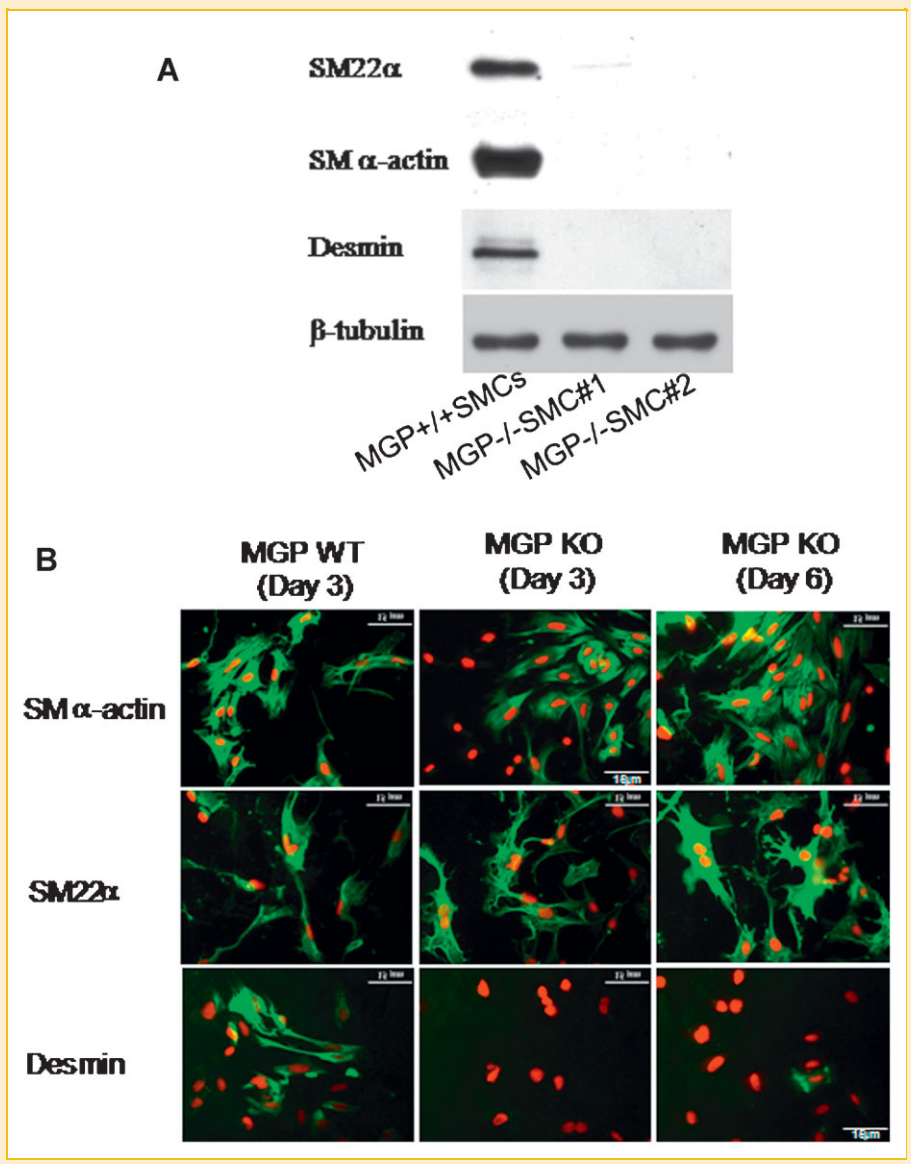

Fig. 1. Medial cells isolated from calcifying MGP−/− vessels regained SM lineage gene expression in culture. Aortic medial cells were isolated from 4-week-old MGP−/− and MGP+/+ mice respectively as described. A: Lysates prepared from freshly isolated medial cells were blotted for SM lineage marker proteins. Data shown were from two MGP−/− SMC preparations, each pooled from five to six aortas. B: Freshly isolated SMCs were cultured in DMEM medium supplemented with 20% FBS. At days 3 and 6 of the primary culture, the cells were fixed and stained immunocytochemically for SM α-actin, SM22α, and desmin. Similar results were achieved in another independent experiment.

SM lineage proteins SM22α, SM α-actin, and desmin, while two batches of cell lysate prepared from MGP−/− mice (MGP−/− SMC) were negative or had barely detectable levels of all SM lineage proteins examined. These results recapitulate the in vivo findings in calcified MGP−/− vessels [Steitz et al., 2001; Speer et al., 2009]. Interestingly, upon culturing these medial cells in DMEM medium supplemented with 20% FBS, the cells started to regain SM α-actin, SM22α, and low levels of desmin expression (Fig. 1B) as determined immunohistochemically. By day 6 of the primary culture, the majority of cells (>90%) were positive for SM α-actin and SM22α. No obvious cell death was observed when the cultures were stained with 0.2% trypan blue (data not shown). In addition, this in-culture restoration of SM lineage protein expression was maintained in all subsequent subcultures tested (data not shown).

Concomitant with the studies of SM lineage protein expression of the MGP−/− SMCs, we examined expression levels of osteochondrogenic marker proteins in these specimens. Using the same cell lysates as Figure 1A, we found elevated levels of alkaline phosphatase protein in both batches of the MGP−/− SMC lysate (Fig. 2A). Consistent with the Western blot findings, alkaline phosphatase was high in the cytoplasm of primary MGP−/− SMCs (Fig. 2C) versus wildtype counterparts (Fig. 2B), and not detectable in the subcultures of any passages tested (data not shown and Fig. 3C, −Pi panel). To determine the expression levels of Runx2/Cbfa1 and osteopontin, we extracted total RNA from either newly isolated or cultured SMCs of indicated passages and measured the Runx2/Cbfa1 and osteopontin mRNA levels via quantitative RT-PCR. As shown in Figure 2D, Runx2/Cbfa1 expression of newly isolated MGP−/− SMCs was about five times higher than the wild type counterpart, and osteopontin was over 500-fold greater (Fig. 2E). In contrast to the SM lineage gene expression, culture of the MGP−/− SMCs in growth medium sharply diminished the expression of Runx2/Cbfa1 and osteopontin. Differences between the MGP−/− and MGP+/+ SMCs for Runx2/Cbfa1 mRNA were insignificant after passage 2; while for osteopontin, the expression in MGP−/− SMCs was decreased by 12.7-fold at passage 2 and to the levels seen in the MGP+/+ SMC levels at passage 4. The findings that MGP−/− SMCs lost osteochondrogenic marker proteins (alkaline phosphatase, Runx2/Cbfa1, and osteopontin) and regained SM lineage proteins (SM22α and SM α-actin) upon isolation and in vitro culturing in medium containing FBS strongly suggests a reversibility of osteochondrogenic differentiation of SMCs, a potential target for vascular calcification prevention and therapy.

INORGANIC PHOSPHATE INDUCES OSTEOCHONDROGENIC DIFFERENTIATION AND CALCIFICATION OF RESTORED MGP−/− SMCs

To further study whether the switch of osteochondrogenic properties (non-cultured) to SMC properties (in culture) of MGP−/− SMCs is reversible upon exposure to pro-calcific factors, subcultured MGP−/− SMCs that had restored SM properties were treated with elevated levels of inorganic phosphate, a stimulus we have previously shown to induce osteochondrogenic phenotype change and calcification of vascular SMC in vitro [Steitz et al., 2001]. Unlike wild type SMCs [Speer et al., 2009], confluent MGP−/− SMCs cultured in normal phosphate-containing media spontaneously developed a mild calcification with time (Fig. 3A, open bars; $P < 0.01$). When the cells were cultured in high phosphate media, a time-dependent increase in matrix calcification was observed (Fig. 3A, solid bars). From days 4 to 10, high phosphate induced almost twice as much matrix calcification compared to normal phosphate media. By day 15, high phosphate increased MGP−/− SMC calcification fourfold. To analyze cell phenotypes under various culture conditions, we extracted RNA from parallel dishes and determined the expression levels of osteopontin, an osteochondrogenic marker. As shown in Figure 3B, osteopontin expression was slightly increased in MGP−/− SMCs that developed mild matrix calcification under normal phosphate conditions, while high phosphate greatly induced osteopontin expression in a time-dependent manner. Using data from both groups, a positive correlation of osteopontin expression levels with the extent of culture calcification was found ($r = 0.960$, $P < 0.0001$). In addition, we determined the levels of SM lineage proteins, SM α-actin and SM22α, and the bone marker protein, alkaline phosphatase, in MGP−/− SMC cultures via Western blot analyses. As shown in Figure 3C, elevated phosphate inhibited the expression of SM lineage genes, SM α-actin and SM22α, and induced the expression of alkaline phosphatase of MGP−/− SMCs. Taken together, these results demonstrate a plasticity and reversibility of vascular SMCs phenotype in response to the local environmental cues. In response to pro-calcific cues, such as activated BMP2 in vivo (presumably due to MGP deficiency) [Bostrom et al., 2001] or high phosphate in vitro [Steitz et al., 2001; Speer et al., 2009], SMCs differentiated toward osteochondrogenesis; while SM properties were restored in cells in response to anti-calcific cues or removal of environmental pro-calcific cues.

LOSS- AND GAIN-OF-FUNCTION STUDIES OF SMC TRANSCRIPTIONAL CO-ACTIVATOR MYOCARDIN

Myocardin plays an important role in the SRF-dependent transcriptional program of SMCs and is required for vascular SMC development and differentiation [Du et al., 2003; Li et al., 2003]. Our previous studies also link vascular calcification with reduced expression of myocardin and its downstream target genes. Down-regulation of myocardin and its target genes in MGP−/− vessels occurred prior to calcification and persisted throughout the development of calcification in the vasculature [Speer et al., 2009]. To further study whether loss of SM lineage gene expression was sufficient to drive SMC lineage reprogramming and calcification, we inhibited myocardin gene expression in wild type SMCs via small interfering RNA (siRNA). As shown in Figure 4A, myocardin mRNA levels in the knockdown SMCs (MyosiRNA) were reduced by 99.6% as compared to the control cell line (OligoCT). Reduction of myocardin mRNA by siRNA led to a sharp decrease in expression of SM lineage proteins (Fig. 4B). Myocardin knockdown did not affect the expression levels of Runx2/Cbfa1 and osteopontin, nor did spontaneous matrix calcification occur when the MyosiRNA SMCs were cultured in normal phosphate media for over 15 days (data not shown). Finally, challenging the MyosiRNA SMCs with high phosphate media induced matrix calcification. However, there was no significant difference between the MyosiRNA SMCs and the OligoCT control cells cultured in the presence of elevated phosphate

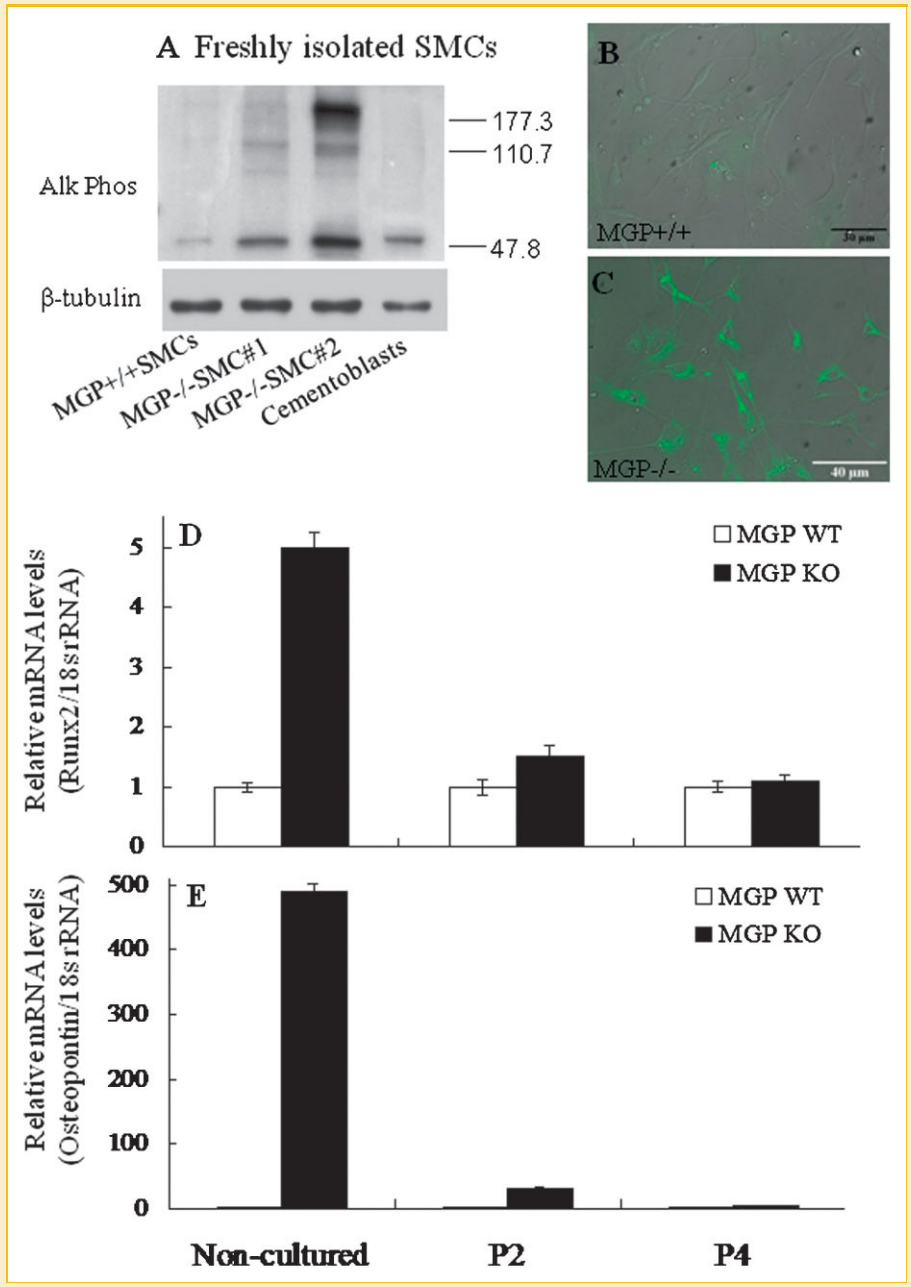

Fig. 2. Medial cells isolated from calcifying MGP−/− vessels lost osteochondrogenic properties in culture. A: Immunoblotting for tissue-nonspecific alkaline phosphatase (Alk Phos). Cell lysates were the same as those used for Figure 1A. Lysate of mouse cementoblasts (OCCM30) was used as a positive control. B,C: Immunocytochemical staining for Alk Phos. Cells were at day 3 of primary culture. D,E: Expression of Runx2/Cbfa1 (D) and osteopontin (E) in non-cultured and subcultured MGP−/− SMCs. Aortic SMCs were isolated from 4-week-old MGP−/− and MGP+/+ mice and cultured as described. Total cellular RNA was extracted either from freshly isolated SMCs (non-cultured) or from cultured SMCs of 2 (P2) and 4 (P4) passages. The expression of Runx2/Cbfa1 and osteopontin was determined by quantitative RT-PCR. RNA extracted from non-cultured SMCs was from eight aortas, while RNA extracted from cultured SMCs of indicated passages were from triplicate dishes. Data shown were normalized to 18s ribosomal RNA of the samples, and are expressed as folds of the MGP+/+ SMC counterparts.

levels (Fig. 4C; $P > 0.05$). Thus, it appeared that downregulation of myocardin mRNA, while decreasing SM lineage markers, did not affect the ability of SMC to mineralize their matrices under elevated phosphate conditions.

To further validate these findings, we utilized a rat myocardin cDNA in gain-of-function studies. This cDNA encodes a functional rat myocardin that is not targeted by the mouse myocardin siRNA. The rat cDNA insert was cloned into a retroviral vector and used to infect MyosiRNA SMC. Expression of rat myocardin in mouse MyosiRNA SMCs was confirmed by RT-PCR (Fig. 5A; MyosiRNA-rMyocardin vs. OligoCT and MyosiRNA-pLXIN). As determined by the upregulation of mouse SM22α mRNA levels, transcriptional activity of rat myocardin in MyosiRNA SMC was increased 50-fold compared to OligoCT SMCs and 20-fold compared to MyosiRNA-

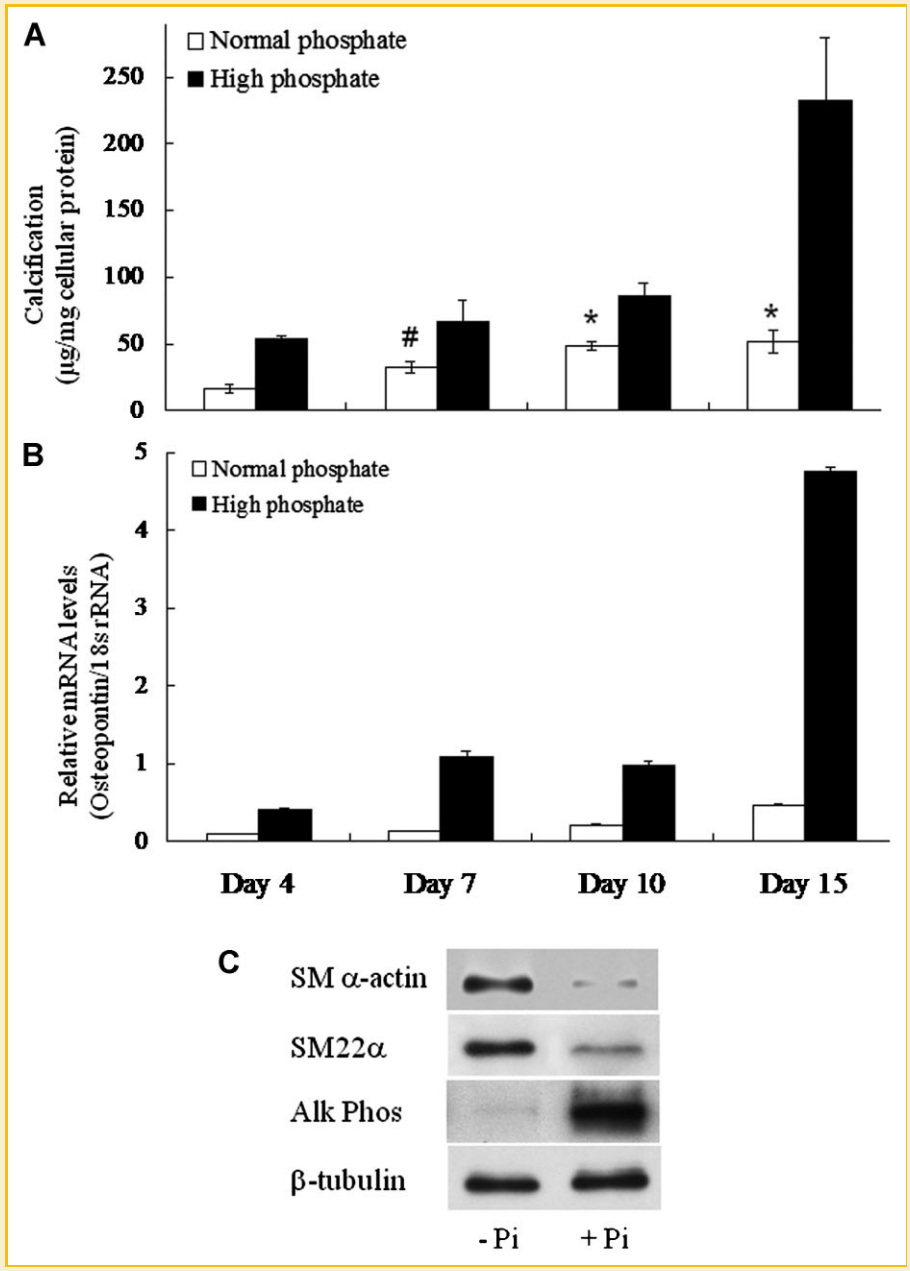

Fig. 3. Inorganic phosphate induced osteochondrogenic differentiation and calcification of restored MGP−/− SMCs. MGP−/− SMCs at passage 8 were cultured in normal or 3.0 mmol/L inorganic phosphate (high phosphate) for indicated days. A: Calcium amounts of the cultures were determined as described in Materials and Methods Section. Data shown are means ± SD, n = 3. Significant increase compared with day 4 cells cultured in normal phosphate medium, $^{\#}P < 0.01$, $^{*}P < 0.005$. B: RNA was extracted from parallel dishes of A and osteopontin expression was determined as described. Data shown were normalized to 18s ribosomal RNA of the samples and are presented as means ± SD, n = 3. C: Immunoblotting for SM α-actin, SM22α, and Alk Phos. day 10 cultures of MGP−/− SMCs in the presence (+Pi) or absence (−Pi) of 3.0 mmol/L inorganic phosphate. Similar results were achieved in another independent experiment.

pLXIN SMCs (Fig. 5B). Next, we examined whether forced-expression of myocardin would impact the calcification susceptibility of SMCs. Four cell lines with various expression levels and transcriptional activities of myocardin were cultured in normal or high phosphate media for 4 and 9 days, and SMC calcification was determined as described. As shown in Figure 5C, there was no significant difference in calcium amount between the cell lines, cultured with or without high phosphate, during any of the days sampled. Additionally, forced-expression of myocardin did not affect the expression levels of osteochondrogenic transcription factor Runx2/Cbfa1 (Fig. 5D). Thus, our studies indicate that manipulating SMC phenotype via myocardin expression levels does not modulate the calcification ability of SMCs. Therefore, enhancing SMC phenotype is not sufficient to modulate osteochondrogenic differentiation and the calcification ability of SMCs with or without high phosphate challenge.

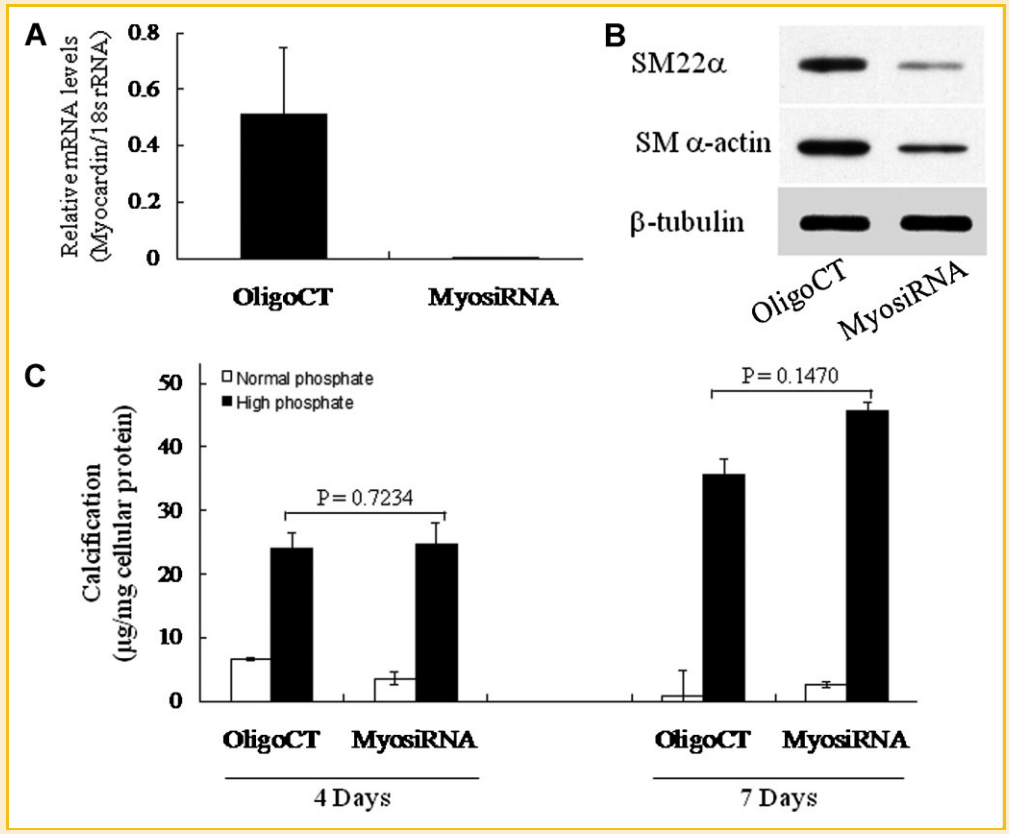

Fig. 4. Loss of myocardin transcription activity was unlikely to enhance calcification ability of vascular SMCs. SMCs were isolated from wild type mice and the myocardin gene expression was manipulated via siRNA (MyosiRNA). Non-specific oligo was used to generate a control cell line (OligoCT). A: Myocardin expression as determined by quantitative RT-PCR. Data shown were normalized to 18s ribosomal RNA of the samples and are presented as means ± SD, n = 3. B: Immunoblotting for SM22α and SM α-actin of the cell lines. C: Myocardin knockdown and SMC calcification. MyosiRNA and OligoCT SMCs were cultured in normal or high phosphate media for 4 and 7 days. Calcium amounts of the cultures were determined as described. Data shown are means ± SD, n = 3. Similar results were achieved in another two independent experiments.

Runx2/Cbfa1 IS THE DECISIVE MOLECULE THAT IS REQUIRED FOR SMC CALCIFICATION

Runx2/Cbfa1 is a critical transcription factor involved in bone and cartilage development [Komori et al., 1997; Otto et al., 1997; Shibata et al., 2004]. Our previous genetic fate mapping studies found an early onset expression of this gene in association with the onset of vascular SMC lineage reprogramming and prior to the calcium phosphate deposition in MGP−/− vessels [Speer et al., 2009]. To study the role of Runx2/Cbfa1 in vascular SMC lineage reprogramming and calcification, we manipulated Runx2/Cbfa1 gene expression via siRNA. We first tested the knockdown efficiency and specificity of the siRNA in a mouse osteoblast cell line, MC3T3. As shown in Figure 6A, Runx2/Cbfa1 expression in the knockdown cells (Runx2 siRNA) was reduced by 99% as compared to the control cell line (OligoCT). Reduction of the Runx2/Cbfa1 gene expression led to a sharp decrease in the mRNA levels of its downstream target gene, osterix (Fig. 6B). No effect of the Runx2 siRNA on other Runx family members (Runx1 and Runx3) was observed (Fig. 6C,D). The Runx2/Cbfa1 siRNA sequence was cloned into a retroviral vector and used to generate a stable vascular SMC knockdown line. Using these cells, we examined whether knockdown of the Runx2/Cbfa1 gene would alter the susceptibility of SMCs to matrix calcification. Runx2 siRNA and OligoCT SMC lines were cultured in the presence of normal or high phosphate for 4 and 7 days and SMC calcification was determined as described. As shown in Figure 6E, high phosphate induced matrix calcification of OligoCT SMCs in a time-dependent manner. Knockdown of the Runx2/Cbfa1 gene expression in SMCs led to a complete blockage of the phosphate-induced matrix calcification at 4 days and by 61.4% at 7 days as compared to the OligoCT SMC cultures ($P < 0.001$), identifying a crucial role of Runx2/Cbfa1 in phosphate-induced SMC calcification.

DISCUSSION

Our previous genetic fate mapping studies have identified the origin of cells that give rise to osteochondrogenic precursor- and chondrocyte-like cells in calcifying blood vessels of MGP−/− mice. Vascular medial SMCs were labeled with transgene β-galactosidase at the genetic level via SM22α-Cre and R26R-LacZ alleles during embryogenesis. 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 reprogram-

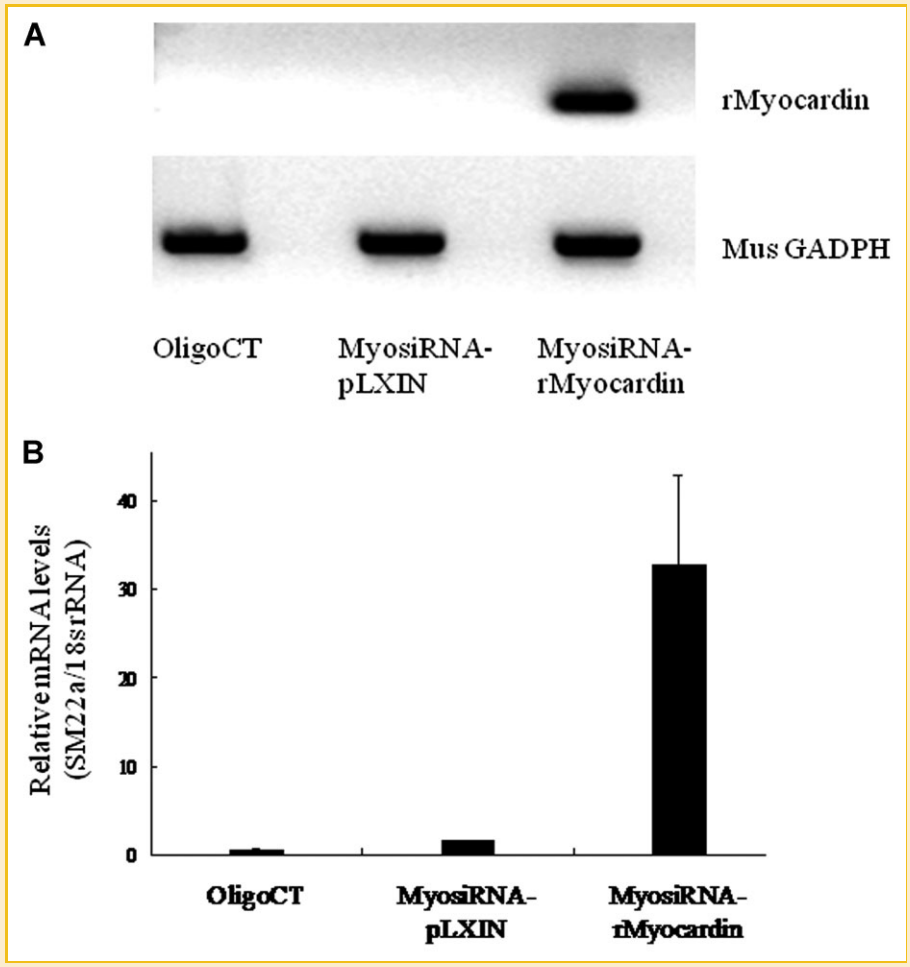

Fig. 5. Forced-expression of myocardin did not modulate calcification ability of vascular SMCs. Rat myocardin cDNA was transduced into MyosiRNA SMCs (MyosiRNA-rMyocardin) as described. Vector backbone was used to generate a control cell line (MyosiRNA-pLXIN). A: RT-PCR analysis of rat myocardin expression. Total RNA was isolated from various cell lines and myocardin expression was determined using primers specific for rat myocardin. Mouse GADPH was amplified as an internal control. B: Transcriptional activity of rat myocardin in mouse cell lines was determined using primers and probes specific for mouse SM22α. Data shown were normalized to 18s ribosomal RNA and are presented as means ± SD, n = 3. C: Myocardin expression and SMC calcification. Four cell lines with various expression levels of myocardin were cultured in normal or high phosphate media (2.6 mM) for 4 and 9 days. Calcium of the cultures was determined as described. Data shown are means ± SD, n = 3. Similar results were achieved in another independent experiment. D: Runx2/Cbfa1 expression in MyosiRNA-pLXIN and MyosiRNA-rMyocardin cell lines. Cells were cultured in growth medium for 2, 4, and 6 days. Total RNA was extracted and the expression levels of Runx2/Cbfa1 were determined using quantitative RT-PCR as described in Materials and Methods Section.

ming of SMCs toward osteochondrogenesis [Speer et al., 2009]. In the current report, we isolated cells from calcifying arterial medias of 4-week-old MGP−/− mice. We found that SMCs isolated from the MGP−/− vessels at this age had undergone lineage reprogramming and expressed osteochondrogenic markers (Runx2/Cbfa1, osteopontin, and alkaline phosphatase) [Sasaki et al., 2000] but not SM lineage proteins (SM22α, SM α-actin, and desmin), similar to previous in situ observations [Steitz et al., 2001; Speer et al., 2009]. Intriguingly, these cells, once in culture and removed from procalcific environmental cues, regained SM lineage markers and down-regulated osteochondrogenic gene expression. During prolonged culture in media that favors SMC differentiation, MGP−/− SMCs no longer expressed osteochondrogenic marker proteins and became indistinguishable from wildtype SMCs. Moreover, the phenotypic switch to the osteochondrogenic state together with enhanced susceptibility to matrix calcification was re-induced when cells were treated with the procalcifying stimulus, elevated phosphate, consistent with findings of wild type SMCs of human, bovine and mouse aorta [Jono et al., 2000; Steitz et al., 2001; Speer et al., 2009]. These results are the first to demonstrate a plasticity and reversibility of vascular SMCs that undergo lineage reprogramming toward bone and cartilage progenitors.

MGP−/− mice do not develop hyperphosphatemia [data not shown and Murshed et al., 2004]. Loss of the MGP gene expression in SMCs of the MGP−/− vessels results in activation of bone morphogenetic protein-2 (BMP-2) [Zebboudj et al., 2003], a potent osteoinductive factor that initiates also chondrogenic lineage differentiation [Schmitt et al., 2003; Zebboudj et al., 2003]. As a consequence, BMP-2 upregulates sodium-dependent phosphate cotransporter, Pit-1 in vascular SMCs, enhances phosphate uptake

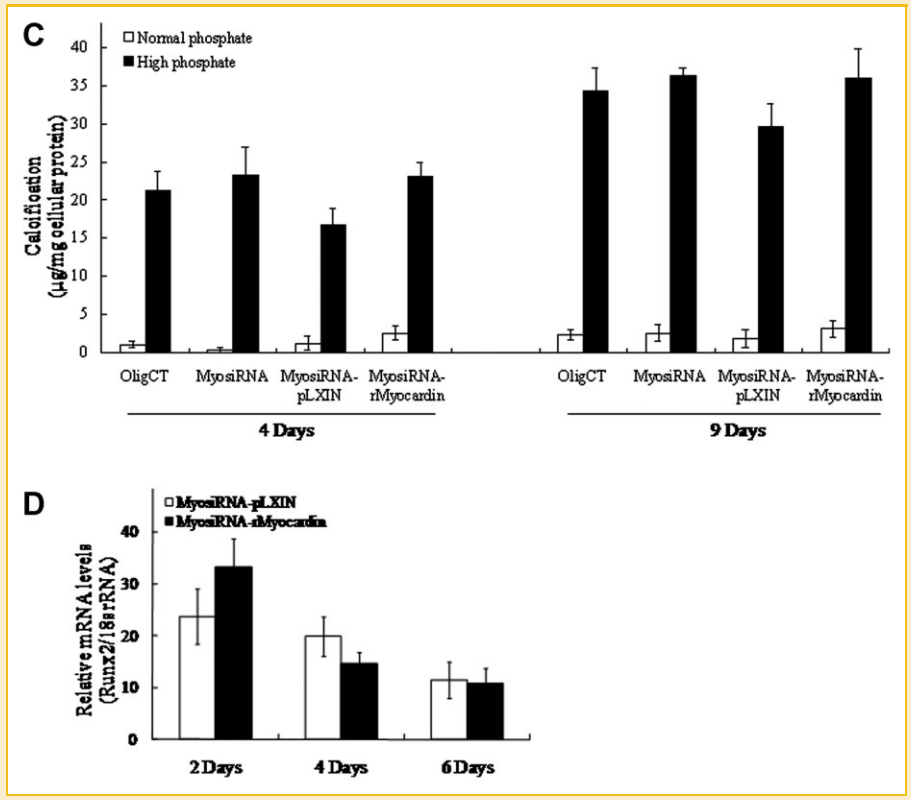

Fig. 5. (*Continued*)

by the cells, and promotes lineage reprogramming of the SMCs toward osteochondrogenesis and matrix calcification [Li et al., 2008]. Conversely, removing SMCs from the MGP−/− matrices and culturing in FBS-supplemented medium is likely to restore MGP, thereby allowing the cells to regain SMC properties (Fig. 1). This is likely due mainly to the compensation of serum MGP that binds to and inactivates BMP-2 activity in culture [Zebboudj et al., 2002] and thus allowed SMCs to differentiate according to their original fate decision. In an elegant study of Karsenty and colleagues, MGP expression was targeted to mouse liver via ApoE-MGP transgenic alleles to allow a 6–10 folds increase of MGP in circulation [Murshed et al., 2004]. Interestingly, increased circulating MGP levels did not rescue vascular calcification nor affect bone mineralization of MGP−/− mice. However, addition of the ApoE-MGP mouse sera that contained high levels of MGP to wild type osteoblasts in culture completely inhibited matrix calcification [Murshed et al., 2004]. These findings, together with the reversibility of the osteochondrogenic MGP−/− SMCs once they were in contact with the MGP-enriched medium, suggest that delivery of MGP to the loci of osteochondrogenesis is critical for MGP to counteract BMP-2 activity and thus inhibit the osteochondrogenic differentiation of the cells locally.

The absence of SMC lineage marker proteins in calcified blood vessels of human and various animal models has long been reported and is associated with the appearance of osteochondrogenic proteins [Shanahan et al., 1999; Bobryshev, 2005; Neven et al., 2007; Speer et al., 2009]. Loss of SMC lineage proteins in medial SMCs also occurred in normal vessels with enhanced transforming growth factor-β1 (TGF-β1) activity. In that work, Schulick et al. [1998] delivered active TGF-β1 to uninjured rat arteries via adenoviral gene delivery and found that the underlying arterial medial SMCs lost their lineage markers. Concomitantly ~10–25% of intimal and medial cells were chondrocyte-like. In addition, vascular SMCs cultured in high phosphate media were found to undergo phenotypic transition as characterized by the loss of SMC-specific proteins and the gain of osteochondrogenic proteins [Steitz et al., 2001; Hayashi et al., 2006]. Our in vivo time course study of MGP−/− vessels showed that reduced expression of myocardin and its downstream transcriptional targets (as identified by decreased expression of SM22α and loss of SMMHC were concomitant with the gain of an early marker, the osteochondrogenic transcription factor, Runx2/Cbfa1, and occurred prior to vascular calcification [Speer et al., 2009]. Thus, SMC lineage gene down-regulation was correlated with osteochondrogenesis and matrix calcification.

Our current studies tested whether loss of SMC lineage gene expression was necessary or sufficient for gain of osteochondrogenic markers and/or vascular calcification. We manipulated SMC specific gene expression via forced-expression and RNA interference of the SMC specific transcription co-activator myocardin and determined whether these would affect the osteochondrogenic phenotypic switch and/or matrix calcification. We found that

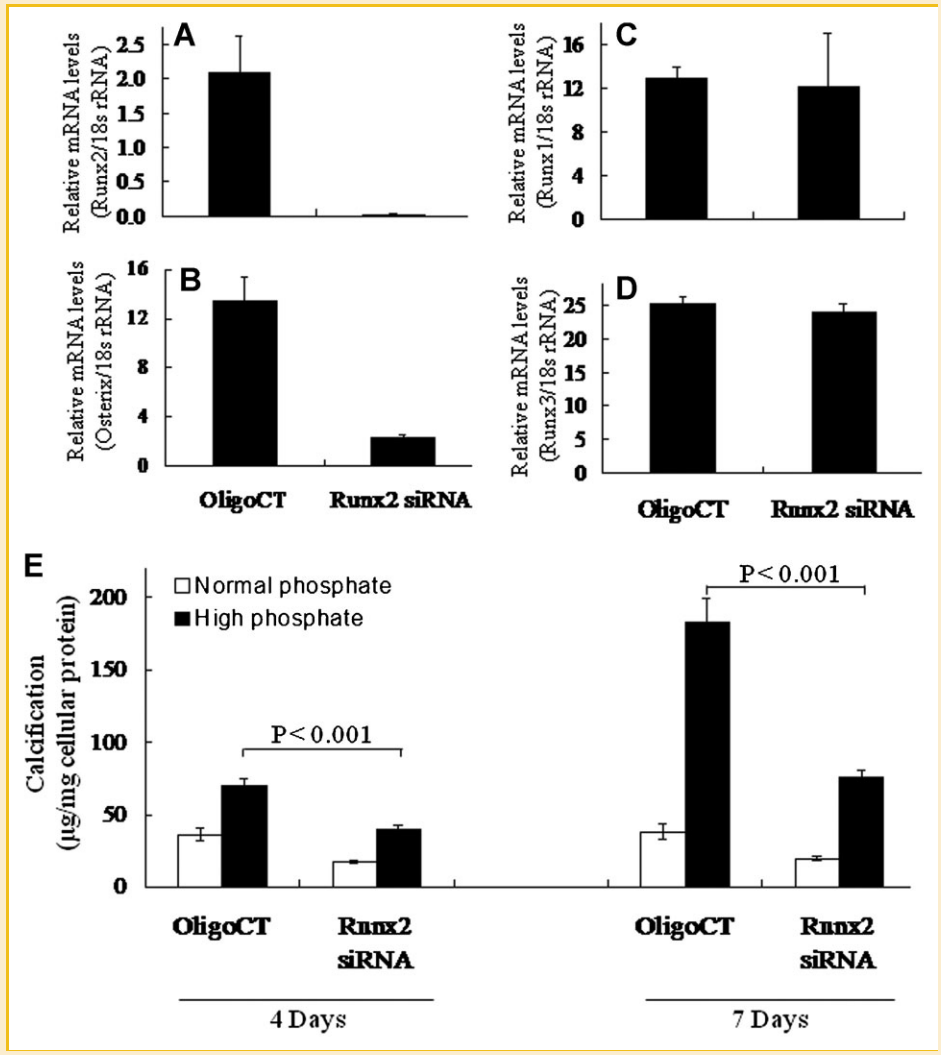

Fig. 6. Loss of Runx2/Cbfa1 expression in SMCs prohibited the phosphate-induced SMC calcification. A: MC3T3 cells were transduced with recombinant Runx2/Cbfa1 siRNA construct (Runx2 siRNA) and used to characterize the knockdown efficiency and specificity of the sequence. Non-specific oligo was used to generate a control cell line (OligoCT). Expression levels of Runx family members (Runx1, Runx2, and Runx3) and Runx2 downstream target gene, osterix, were determined by quantitative RT-PCR. Data shown were normalized to 18s ribosomal RNA of the samples and are presented as means ± SD, n = 3: B. Runx2 siRNA sequence characterized in A was used to generate stable SMC lines of Runx2 siRNA and OligoCT using wild type mouse aortic SMCs. The cells were cultured in normal or high phosphate media for 4 and 7 days. Calcium level of cultures was determined as described. Data shown are means ± SD, n = 3. Similar results were achieved in another independent experiment.

changing myocardin expression levels and transcription activity did not modulate the ability of SMCs to differentiate toward an osteochondrogenic state or regulate the process of matrix calcification. A near complete knockdown of myocardin expression did not promote osteochondrogenic differentiation (as identified by the expression levels of Runx2/Cbfa1, osteopontin, and osterix; data not shown) or matrix calcification (Fig. 4C). Moreover, enhancing SMC phenotype via forced-expression of myocardin was not sufficient to alter osteochondrogenic differentiation and calcification of SMCs in response to high phosphate (Fig. 5C). These are the first studies to show that loss of myocardin is not sufficient or necessary for SMC calcification.

Lack of a requirement for SM lineage down-regulation during osteochondrogenic differentiation and calcification of vascular SMCs is supported by re-evaluating the findings of Hayashi et al. These investigators treated rat vascular SMCs with BMPs to achieve a phenotypic switch of SMCs to an osteochondrogenic state, and found that Msx transcription factors (Msx1 and Msx2) reduced the myocardin-dependent promoter activities, for example, SM22α and caldesmon, via the formation of a ternary complex with SRF and myocardin. Msx1 and Msx2 thereby inhibited the binding of SRF or SRF/myocardin to the CArG box motif of the SMC-specific promoters [Hayashi et al., 2006]. Likewise, Tanaka et al. [2008] found a similar role of Runx2/Cbfa1 in myocardin-mediated differentiation of vascular SMCs. Similar to Msx transcription factors, Runx2/Cbfa1 repressed SMC differentiation via interacting with SRF to compete with its binding by myocardin, and thus prohibited the formation of the SRF/myocardin ternary complex.

While Runx2/Cbfa1 expression is associated with cartilaginous metaplasia and calcification of blood vessels in vivo [Bobryshev,

2005; Aikawa et al., 2007; Speer et al., 2009], in vitro studies using a cultured SMC calcification model reveal an important role of Runx2/Cbfa1 in SMC phenotypic change and calcification. An induced Runx2/Cbfa1 expression along with SMC phenotypic change and calcification has been reproduced by various researchers [Steitz et al., 2001; Moe et al., 2003; Chen et al., 2006; Hofbauer et al., 2006]. The expression of Runx2/Cbfa1 in human vascular SMCs is associated with elevated phosphate levels and is dependent on the activity of type III sodium-dependent phosphate cotransporter, Pit-1. Inhibition of Pit-1 by RNA interference reduces cellular phosphate uptake and thus the reduction of Runx2/Cbfa1 expression and SMC phenotype change [Li et al., 2006]. A vital role of Runx2/Cbfa1 in high phosphate-mediated SMC phenotype change and calcification is confirmed in this study. Through gene silencing we showed that Runx2/Cbfa1 is the key regulatory factor that promotes SMC lineage reprogramming and matrix calcification in the context of high phosphate, an important contributor to the vascular calcification in patients with chronic kidney disease [Block et al., 2007].

Interestingly, in a mouse model of atherosclerosis and chronic renal failure, vascular calcification was reversed by sevelamer carbonate, a phosphate binder that reduces phosphate load [Mathew et al., 2007]. In that study, high phosphate induced osteochondrogenic SMC differentiation and matrix calcification, which could be reversed with the phosphate binder, sevelamer. These findings, together with our findings identifying Runx2 as a key factor for SMC calcification, provide a strong basis for the notion that vascular calcification may be therapeutically treated, and potentially reversed.

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