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SOX9 and myocardin counteract each other in regulating vascular smooth muscle cell differentiation

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ABSTRACT

Transdifferentiation of vascular smooth muscle cells (VSMC) into chondrogenic cells contributes significantly to vascular calcification during the pathogenesis of atherosclerosis. However, the transcriptional mechanisms that control such phenotypic switch remain *unclear*. This process is characterized by the induction of Sox9 and Col2a1 genes accompanied by the repression of myocardin (Myocd) and SMC differentiation markers such as SM22, SM α-actin and SM-MHC. Here we explore the regulatory role of SOX9, the master regulator for chondrogenesis, in modulating SMC marker gene expression. qRT-PCR and luciferase assays show that over-expression of SOX9 inhibits SMC gene transcription and promoter activities induced by myocardin, the master regulator of smooth muscle differentiation. Such suppression is independent of the CArG box in the SMC promoters but dependent on myocardin. EMSA assay further shows that SOX9 neither participates in SRF (serum response factor) binding to the CArG box nor interacts with SRF, while co-immunoprecipitation demonstrates an association of SOX9 with myocardin. Conversely, myocardin suppresses SOX9-mediated chondrogenic gene Col2a1 expression. These findings provide the first mechanistic insights into the important regulatory role of SOX9 and myocardin in controlling the transcription program during SMC transdifferentiation into chondrocytes.

1. Introduction

Smooth muscle cells (SMCs) are highly plastic and have the capacity to convert from the differentiated contractile phenotype to a variety of synthetic dedifferentiated states exhibiting enhanced proliferation, migration, inflammation, chondrogenesis and osteogenesis during the pathogenesis of vascular diseases such as atherosclerosis [1,2]. Smooth muscle cell (SMC) differentiation is marked by the expression of a set of contractile proteins including smooth muscle α-actin (Acta2), smooth muscle myosin heavy chain (Myh11), and SM22 (Tagln or SM22α). In response to vascular injury, SMCs undergo phenotypic modulation: this is characterized by the downregulation of SMC contractile genes.

Osteo/chondrocytic conversion of SMCs in calcified atherosclerotic plaques is accompanied by the induction of bone and cartilage differentiation regulators, including Cbfa1 (RUNX2), SOX9 and MSX2 [3,4]. Previous studies have characterized the role of RUNX2 and MSX2 in promoting osteogenic conversion of VSMCs by repressing SRF/myocardin-mediated SMC differentiation [5–8]. However, the regulatory roles of SOX9 in SMC phenotypic switching remain not understood.

Serum response factor (SRF), a widely expressed MADS box containing transcription factor, binds to the CArG [CC(A/T)$_6$GG] box in the promoters of SMC genes as well as in the promoters of early growth genes [9]. SRF cooperates with a variety of transcriptional coactivators and corepressors to regulate gene expression in response to different signals [10–14]. Among them, myocardin is the most potent coactivator of SRF in transactivating cardiac and SMC specific gene transcription [9,11]. Myocardin is specifically expressed in smooth and cardiac muscle lineages throughout embryonic development and adulthood; myocardin interacts with SRF and/or other transcriptional factors to induce SMC gene transcription in CArG box-dependent and independent manners [11,15].

Abbreviations: VSMCs, vascular smooth muscle cells; SRF, serum response factor; Myocd, myocardin; SOX9, SRY-type high mobility group box 9; qRT-PCR, quantitative reverse-transcription PCR.

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It has been demonstrated that SMCs give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries; this process is accompanied by increased expression of SOX9 [3,7]. However, the functional role of SOX9 in SMC phenotypic modulation during artery calcification has not been determined. We recently showed that carotid injury induces prominent medial chondrogenic differentiation; this is accompanied by the upregulation of Sox9 and Col2a1 transcription and the downregulation of myocardin and SMC marker gene transcription in medial SMCs in $SM22^{-/-}$ mice [16]. Since Sox9 and myocardin are master transcriptional regulators for chondrogenesis and myogenesis respectively, the interplay of SOX9 and myocardin is likely to have a critical role in directing the transcription program switch from myogenesis to chondrogenesis. The goal of the present study is to determine the regulatory role of SOX9 on myocardin-mediated SMC gene transcription, and to explore underlying molecular mechanisms.

2. Materials and methods

2.1. Plasmids

The mammalian expression vector plasmids pcDNA3.1-Myc-myocardin, pcDNA3-Flag-SOX9, pCGN-HA-SRF and pCGN-VP16-SRF were described previously [11,17,18]. The luciferase reporter plasmids controlled by the promoters of Sm22, Myh11, Aclp, 4× Sm22CArGnear, and 4× FosCArG were described previously [19–21]. The luciferase reporter controlled by the Col2a1 promoter (pCol2a1-luc) and the 4× SOX9 binding sites promoter (4× SOX9-site-luc) were described previously [22,23].

2.2. Cell culture, transfection and luciferase assays

C3H10T1/2 or 10T1/2 (a mouse embryonic mesenchymal progenitor cell line), PAC1 (a rat arterial smooth muscle cell line) and COS7 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL), at 37 °C with 5% CO_2. Plasmid transfection was performed using Lipofectamine Reagent (Invitrogen) according to the manufacturer's instruction. For luciferase assay, 50 ng of each transcription factor, 50 ng of the reporter plasmid, and 1 ng of renilla reporter plasmid (Promega) were transiently transfected into 10T1/2 or PAC1 cells for 24 h. The total amount of plasmid DNA among samples was kept equal by adding corresponding control vector plasmids. Dual luciferase assays (Promega) was conducted 24 h after transfection. Luciferase reporter activities were determined by the firefly luciferase activities normalized to the activity of internal control renilla luciferase activities using the dual luciferase assay system described by the manufacturer (Promega, Madison, WI). All assays were performed at least three times independently.

2.3. RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

Total RNA extraction was performed using RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. cDNAs were reverse transcribed from 1 μg of isolated total RNA using Superscript II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) was subsequently performed on StepOne Plus system (Applied Biosystems) using Fast Sybr Green Master Mix (Applied Biosystems). The primers for qRT-PCR are listed in the Table. The relative transcript quantity was determined using the $\Delta\Delta C_T$ method adopted in Stepone Software 2.0 data analysis tool. Gapdh was used as normalization control. All assays were repeated at least two times independently. The primers used for qRT-PCR are listed in Table 1.

Table 1
Primer sequences used for QPCR. Primer sequences for Gapdh, Tagln (Sm22), Acta2 and Myh11 used in QPCR analysis are listed. Note: all primers are the same for mouse C3H10T1/2 and Rat PAC1 cell lines, except for Tagln. +/− denotes sense/anti-sense strand.

Species	Gene	Strand	Primer Sequence (5′-3′)
Mouse	Gapdh	+	TGAATACGGCTACAGCAACAGGGT
		−	TTGTGAGGGAGATGCTCAGTGTTG
	Tagln	+	TCCTTCCAGTCCACAAACGACCAA
		−	TTTGGACTGCACTTCTCGGCTCAT
	Acta2	+	GAGAAGCCCAGCCAGTCG
		−	ATCTTTTCCATGTCGTCCCAGTTG
	Myh11	+	AACGCCCTCAAGAGCAAACTCAGA
		−	TCCCGAGCGTCCATTTCTTCTTCA
Rat	Tagln	+	TCCTTCCAGCCCACAAACGACCAA
		−	CTTGGACTGCACTTCACGGCTCAT

2.4. Electrophoretic mobility shift assay (EMSA)

The plasmids containing Flag-SOX9 and HA-SRF were transfected into COS7 cells using Lipofectamine Reagent (Invitrogen). Seventy two hours after transfection nuclear protein extraction was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). IRdye700 fluorescence labeled probe containing the SM22α proximal CArG box (the CArGnear box) with flanking sequences (CArG box underlined; 5′-ACTTGGTGTCTTTCCCCAAA TATGGAGCCTGTGTGGAGTGA-3′). The oligos were synthesized by Integrated DNA Technologies Inc. The nuclear protein, anti-SRF (sc-335x, Santa Cruz) or anti-SOX9 (sc-20095x, Santa Cruz) and the probe were incubated and EMSA was conducted using the Odyssey EMSA Buffer Kit according to the manufacturer's instruction. Protein/DNA complexes were separated on 6% DNA Retardation Gel (Invitrogen), and detected by Odyssey Infrared Imaging System.

2.5. Western blotting (WB)

Equal amount of proteins were loaded onto Bis–Tris Mini Gel (Invitrogen) for SDS–PAGE separation. Proteins were then transferred from the gel to PVDF membrane using XCell II™ Blot Module (Invitrogen). The membrane was blocked in 5% non-fat milk at room temperature for 1 h before incubating with primary antibody. Anti-Myc (Santa Cruz), anti-HA (Abcam), anti-flag (Sigma), anti-SOX9 (Santa Cruz), anti-Sm22 (Abcam) and anti-GAPDH (Santa Cruz) were used for target protein detection. The membrane was then washed and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions. The blots were exposed to HyBlot CL film (Denville) for signal visualization.

2.6. Co-immunoprecipitation (Co-IP)

Co-IP assays were carried out as described previously [24]. Briefly, COS7 cells were co-transfected with expression plasmids encoding myc-tagged myocardin or SOX9 as indicated. Forty eight hours after transfection, nuclear protein was harvested and Co-IP assays were performed using the nuclear complex Co-IP kit as described by the manufacturer (Active Motif). One hundred micrograms of nuclear protein extracts were incubated with 50 μl of anti-Myc beads (Sigma) or 3 μg control rabbit IgG in 500 μl low salt IP buffer (Active Motif) overnight at 4 °C. Fifty microliters of anti-rabbit IgG beads (True Blot) were added to control IgG sample for an additional 1 h with rocking and then immobilized complexes from both anti-myc and anti-rabbit IgG beads were washed six times with the low salt IP buffer with or without BSA. The immu-

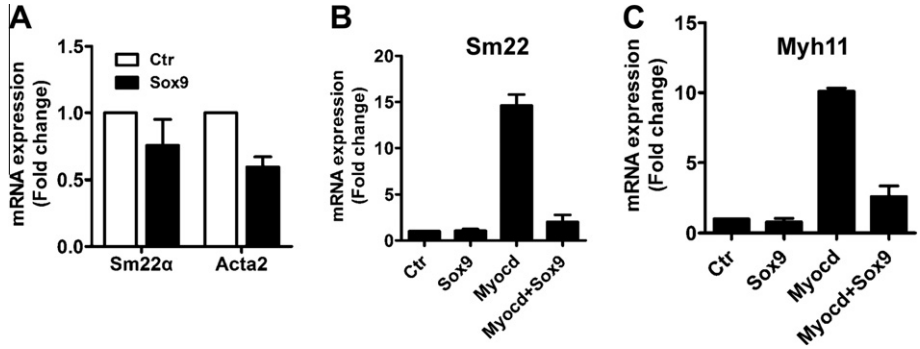

Fig. 1. SOX9 represses Myocd-induced smooth muscle gene transcription by qPCR assays. PAC1 cells were transiently transfected with Sox9 (A). 10T1/2 cells were transiently transfected with myocardin and/or Sox9 as indicated. The mRNA expression of Sm22 (B) and Myh11 (C) was determined by QPCR. Gapdh mRNA was used as normalization control. Error bars indicate standard error of the mean (SEM). Ctr: Control.

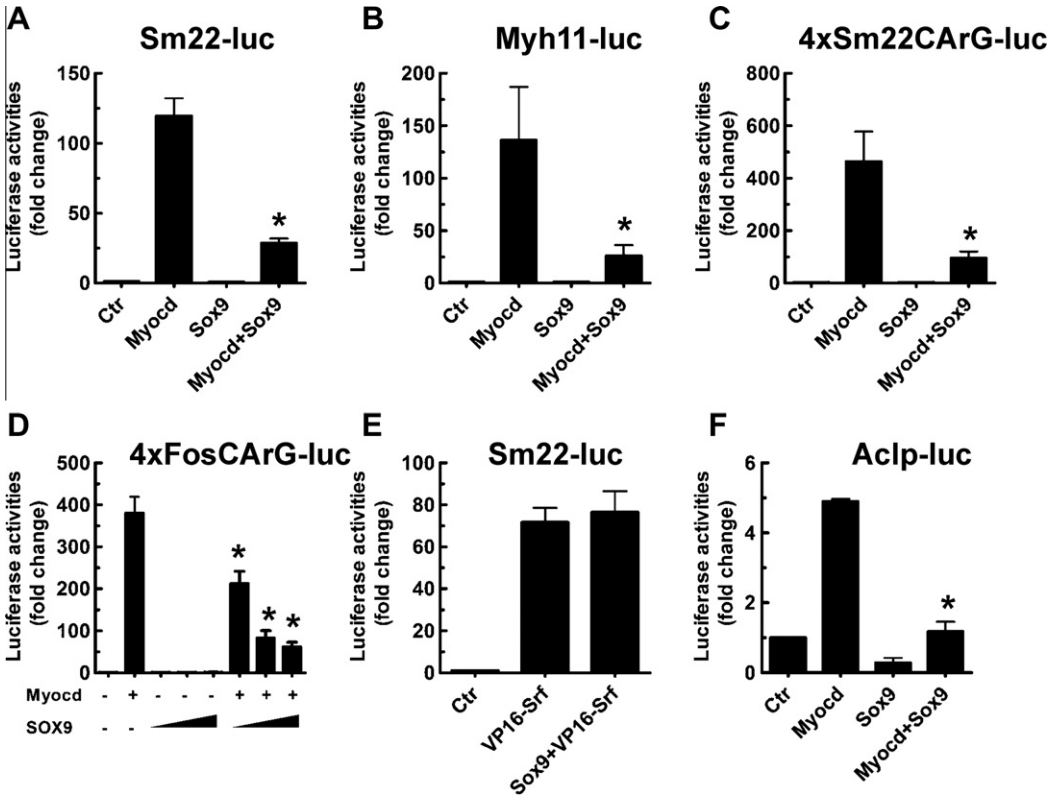

Fig. 2. SOX9 suppresses myocardin-induced SMC gene promoter activities by the luciferase assays. Myocardin or Sox9 were co-transfected with indicated luciferase reporter of the Sm22 promoter (A) or the Myh11 promoter (B) or the promoter containing 4xSm22CArGnear boxes (C), or 4xFosCArG boxes (D), or the Aclp promoter (F) into 10T1/2 cells. For 4xFosCArG-luc, Myocd:SOX9 ratio is 3:1, 1:1, and 1:3, respectively. For the activation of the Sm22 promoter by VP16-SRF and Sox9 (E), the ratio of Sox9 and VP16-SRF is kept at 1:1. Error bars represent standard error of the mean (SEM). * indicates $p < 0.05$ versus control (Myocd).

noprecipitated proteins were then mixed with 45 μl 2× SDS sample buffer and analyzed by WB as indicated.

2.7. Statistics

The data represent the means ± SEM (the standard error of the mean). $P < 0.05$ is considered statistically significant. One-way ANOVA was used for data analysis.

3. Results and discussion

3.1. SOX9 suppresses SMC marker gene transcription

SOX9, a key transcriptional regulator for chondrocyte differentiation, is highly expressed during SMC osteochondrogenic conversion [3,7,25,26]. We recently showed that carotid injury-induced medial chondrogenic differentiation in SM22$^{-/-}$ mice is accompanied by the upregulation of Sox9 and Col2a1 transcription and the concomitant downregulation of myocardin and SMC marker gene transcription [16]. It is therefore reasonable to propose that SOX9 inhibits SMC differentiation while promoting chondrocytic differentiation in VSMCs. To test this hypothesis, we examined whether SOX9 suppresses endogenous SMC gene transcription in VSMCs. After transfecting SOX9 gene into SMC line PAC1 cells, we found by qRT-PCR assays that SOX9 overexpression repressed mRNA expression of Sm22 and Acta2, smooth muscle differentiation markers (Fig. 1A). 10T1/2 cells, a mesenchymal cell line, are known to express SMC contractile genes in response to myocardin overexpression [27]. To examine whether SOX9 modulates myocardin-mediated SMC gene transcription, we transfected Sox9 and Myocd

individually and in combination into 10T1/2 cells. As expected, introduction of myocardin greatly enhanced expression of Sm22 (Fig. 1B) and Myh11 (Fig. 1C) while co-transfection of SOX9 with Myocd dramatically compromised myocardin-mediated expression of Sm22 (Fig. 1B) and Myh11 (Fig. 1C). These results suggest that SOX9 inhibits myocardin-induced transcription of SMC marker genes.

3.2. SOX9 inhibits myocardin-mediated SMC gene promoter activities

We further investigated whether SOX9 affects the transactivational activities of Myocd on the SMC gene promoters by using luciferase reporter assays in C3H10T1/2 cells. Co-transfection with SOX9 gene significantly repressed Myocd-activated promoters of Sm22α (Fig. 2A) and Myh11 (Fig. 2B). To evaluate whether the inhibitory effect of Sox9 acts through the SRF/CArG boxes, we utilized 4× *Sm22*CArG-luc which contains four copies of the proximal *Sm22* CArG box (Fig. 2C): forced expression of SOX9 drastically suppressed Myocd-induced activation of 4× *Sm22*CArG-luc (Fig. 2C). A similar repressive effect was observed for 4× *cFos*-CArG-luc, which contains four copies of the CArG box in the c-Fos promoter (Fig. 2D). This result suggests that Sox9 may suppress Myocd transactivational activities by inhibiting SRF–CArG complexes. Unexpectedly, SOX9 could not repress the VP16-SRF-activated *Sm22* promoter (Fig. 2E). VP16 serves as a transactivation domain fused to SRF to activate SRF-dependent transcription. This result suggests that SOX9 suppression on SMC gene expression may be Myocd-dependent, but not SRF/CArG box-dependent.

This conjecture was supported by the fact that SOX9 significantly reduced Myocd activation of the Aclp promoter, which is known to be regulated independent of the SRF/CArG box [20](Fig. 2F). Therefore, SOX9 may suppress Myocd-induced SMC gene expression, most likely through a mechanism targeting Myocd instead of the SRF/CArG box. Previous studies showed that Myocd could transactivate promoter activities independent of the SRF/CArG box by interacting with other transcription factors bound on the promoter [15].

3.3. SOX9 does not participate in SRF binding to the CArG box but associates with myocardin

Accumulating evidence supports the notion that the SRF–CArG association is subject to both positive and negative signals to regulate SMC gene transcription [10–12,28,29]. To determine whether SOX9 inhibits SMC gene expression via intervening SRF association with CArG box, we performed EMSA assay utilizing nuclear extract from COS7 cells transfected with SOX9 and/or SRF with a fluorescence labeled CArGnear box probe derived from the Sm22 proximal CArG box sequence. As shown in Fig. 3A, the SRF/CArG complex was identified: it is indicated by an arrow. The SRF/CArG complex was abolished in the presence of anti-SRF antibody, but not by anti-Sox9 antibody nor rabbit IgG (Fig. 3A, lane 6–8 compared with lane 1). However, increased Sox9 overexpression had no effects on the SRF/CArG complex formation (Fig. 3A, lane 3–5 compared with lane 1). Moreover, we did not find any new complex formed when SOX9 was co-transfected with SRF (Fig. 3A, lane 3–5 compared with lane 7). These results suggest that Sox9 does not interfere with SRF binding to CArG box, and that it is also unlikely to interact directly with SRF. Consistent with this speculation, we failed to detect interaction between Sox9 and SRF using either anti-Flag resin or anti-HA antibody for immunoprecipitation in COS cells transfected with HA-SRF and flag-SOX9 plasmids (Fig. 3C).

Since Sox9 significantly suppresses myocardin-mediated promoter activities (Fig. 2), we assessed the possibility that Sox9 may interact with myocardin to exert its repressive effect. Co-

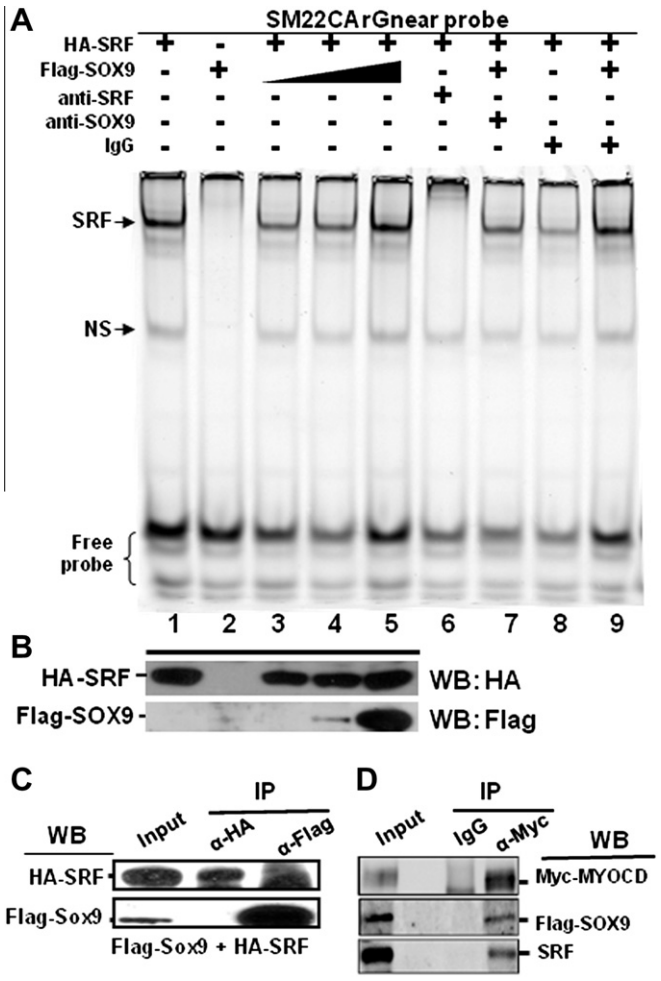

Fig. 3. SOX9 does not interfere SRF binding to the CArG box but interacts with myocardin. (A) COS7 cells were transiently transfected with HA-SRF or Flag-SOX9 as indicated for isolating nuclear extracts. EMSA was performed by incubating nuclear exacts and indicated antibodies with IRDye700 fluorescence labeled Sm22CArGnear (the proximal CArG box) probe. NS: non-specific. (B) The expression of SRF and SOX9 in nuclear extracts was determined by Western blotting (WB) using anti-HA and anti-Flag antibodies. (C) COS7 cells were co-transfected with HA-SRF and Flag-SOX9 plasmids. Anti-Flag and anti-HA antibodies were used for immunoprecipitation (IP) followed by WB using anti-Flag and anti-HA antibodies as indicated to detect HA-SRF and Flag-SOX9 in the immunoprecipitates. (D) COS7 cells were co-transfected with Myc-myocardin and Flag-SOX9 plasmids. Anti-Myc antibodies were used for immunoprecipitation (IP) followed by WB using anti-myocardin, anti-SOX9 and anti-SRF antibodies respectively. IP with IgG served as a negative control.

immunoprecipitation showed that SOX9 was detected in Myocd immunoprecipitates in COS cells cotransfected with Flag-SOX9 and Myc-myocrdin plasmids (Fig. 3D). SRF is known to interact with myocardin. As a positive control, the endogenous SRF was also detected in the Myocd immunoprecipitates. Taken together, these results suggest that the inhibitory effect of SOX9 on SMC gene transcription is through interaction with myocardin instead of the classical SRF–CArG box complex. This conclusion is consistent with the above luciferase assays (Fig. 2) and EMSA assays (Fig 3A).

3.4. Myocardin represses SOX9-mediated Col2a1 expression

As chondrogenesis and myogenesis feature distinct transcriptional machineries, it is reasonable that these two sets of machineries might counteract each other during VSMC chondrogenic

conversion. Consistent with this notion, during the transdifferentiation of SMCs into chondrogenic cells, expression of Myocd decreases when SOX9 expression is increased [16]. We further propose that Myocd may suppress SOX9 mediated transcriptional activation of chondrogenic gene such as type II collagen Col2a1. To this end, we conducted the luciferase reporter assay using two different Col2a1 promoter vectors harboring SOX9 binding sites in either PAC1 or 10T1/2 cells respectively. Overexpression of Sox9 increased both Col2a1 and 4xSOX9 sites promoter activities (Fig. 4A–B). In contrast, Myocd significantly suppressed SOX9-transactivated Col2a1 promoters (Fig. 4A–B).

3.5. Novelty and significance of this study

In summary, this study identifies the antagonism between Sox9 and Myocd as central regulators in controlling SMC and chondrogenic gene transcription in SMCs in response to injury (Fig. 4C). SMCs have the potential to differentiate into multi mesenchymal lineages such as osteoblastic, chondrocytic and adipocytic cells controlled by their distinct lineage-specific key transcriptional regulators [3,4,30,31]. The present study provides new mechanistic insights into the counteracting roles of master regulators of SMCs and chondrocytes in SMC phenotypic modulation. This work expands and reinforces our recent observation showing that, in response to vascular injury, the expressions of Sox9 and chondrogenic marker genes are up regulated concomitantly with the down regulation of myocardin and myogenic marker genes [16].

Here, we demonstrate that SOX9 and Myocd oppose each other to modulate the transcription of SMC and chondrocyte marker genes. Interestingly, the inhibitory effect of SOX9 on SMC markers expression is independent of SRF–CArG box; SOX9 associates with myocardin to suppress SMC gene transcription. This is in contrast to the findings by Tanaka et al., where Runx2 repression on Myocd activity was attributed to direct interaction with SRF [5]. It should be noted that SOX9 and RUNX2 play important roles in cartilage and bone development: SOX9 is required to establish osteochondroprogenitor cell lineage while RUNX2 is required for subsequent osteoblast differentiation [3,8,32–35].

In view of the similarity of VSMC osteochondrogenic conversion and bone/cartilage development, it is likely that SOX9-mediated suppression of SMC differentiation might be crucial for the initiation of an osteochondrogenic conversion. We speculate that, as the transdifferentiation proceeds, SOX9 (Myocd-dependent) might cooperate with Runx2 (SRF dependent) to drive SMC phenotypic conversion. As chondrocyte hypertrophy proceeds, the expression of Sox9 gradually decreases while the expression of Runx2 increases, leading to osteogenesis and vascular calcification. Therefore, the present study provides the first characterization of the molecular mechanisms of VSMC chondrogenic conversion. The molecular mechanisms that mediate the interplay of SOX9, Runx2 and myocardin in VSMC osteochondrogenic conversion during vascular calcification will be investigated in further studies.

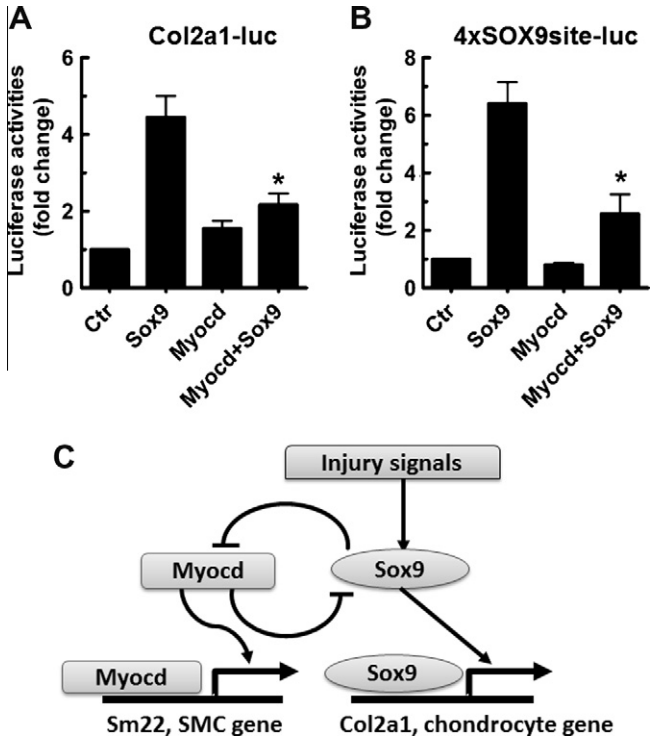

Fig. 4. Myocardin represses SOX9-induced Col2a1 promoter activities by the luciferase assays. PAC1 or 10T1/2 cells were transiently transfected with myocardin or SOX9 as indicated, along with luciferase reporter controlled by the Col2a1 promoter in PAC1 (A) and the 4xSOX9 sites promoter in 10T1/2 cells (B). Error bars represent standard error of the mean (SEM). * indicates $p < 0.05$ versus control (Sox9). (C) A model of antagonistic effect between SOX9 and Myocd during SMC chondrogenic transdifferentiation.

Acknowledgments

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