Transcription Factor SOX18 Is Expressed in Human Coronary Atherosclerotic Lesions and Regulates DNA Synthesis and Vascular Cell Growth

Marta García-Ramírez, José Martínez-González, Josep O. Juan-Babot, Cristina Rodríguez, Lina Badimon

Objective—SOX18, a member of the SOX gene family (SRY-like 3-hydroxy-3-methylglutaryl box gene), is a transcription factor expressed in the development of blood vessels during embryogenesis. We analyzed SOX18 expression in human coronary atherosclerotic lesions and investigated its potential function in vascular cells.

Methods and Results—In advanced human coronary atherosclerotic lesions, SOX18 immunostaining was localized in endothelial cells (on the luminal surface, in vasa vasorum, and in intimal neovessels) and in vascular smooth muscle cells (VSMCs) scattered in the intima, colocalizing with proliferating cell nuclear antigen. In cell cultures, SOX18 was mainly localized in subconfluent and denuded areas. Significant SOX18 mRNA levels (by Northern blot analysis and reverse transcription–polymerase chain reaction) were detected in cell cultures from human umbilical vein endothelial cells and human VSMCs. Antisense SOX18 inhibited DNA synthesis ([3H]thymidine incorporation) and vascular cell growth. Antisense SOX18 also significantly reduced VSMC regrowth after injury in an in vitro model of wound repair.

Conclusions—Our results indicate that SOX18 is involved in vascular cell growth and suggest that this transcription factor may play a role in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2005;25:2398-2403.)

Key Words: vascular biology ■ gene expression ■ endothelial function ■ atherosclerosis ■ growth factors ■ SOX18
Coronary Artery Sampling and Preservation

Human coronary arteries were obtained from hearts removed during transplant operations performed at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). Immediately after surgical excision, coronary arteries were dissected and processed for either immunohistochemical analysis or the isolation of VSMCs, as described earlier.21 The specimens for immunohistochemistry were immersed in fixative solution (4% paraformaldehyde/0.1 mol/L phosphate-buffered saline, pH 7.4). After overnight treatment, vessels were sectioned into blocks and embedded in paraffin. The specimens for cell culture were immersed in cell culture maintenance medium and processed for VSMC isolation as described.21 The reviewer institutional Committee on Human Research of the Hospital of Santa Creu i Sant Pau approved the research protocol for this study.

Immunohistochemical Analysis

Paraffin-embedded specimens were cut into 5-μm-thick serial sections, placed on poly-L-lysine–coated slides, deparaffinized, and stained with Masson’s trichrome or processed for immunohistochemistry. Characterization of the lesions in Masson’s trichrome–stained sections was performed according to American Heart Association criteria. In brief, consecutive sections were deparaffinized and treated for nonspecific binding, and the presence of SOX18 and other cell markers was assessed. The primary antibodies used were as follows: anti-SOX18 (sc-20100, Santa Cruz Biotechnology Inc); anti-α-smooth muscle actin (α-SMA; clone 1A4, Dako), a marker for VSMCs; anti-proliferating cell nuclear antigen (PCNA; clone PC10, Zymed laboratories Inc.), a marker of cell proliferation; anti-CD34 (QBEnd/10, Novocastra Laboratories Ltd) or anti-CD31 (clone JC/70A, Dako) as EC markers; and a monoclonal antibody clone HAM56, Dako) specific for macrophages. After incubation with the primary antibody (1 hour; each antibody was tested in 4 sections from every lesion), sections were washed and then incubated with the appropriate biotinylated secondary antibodies (1/200, from Vector). Finally, sections were incubated with avidin-biotin complex (ELITE, Vector), and 3,3’-diaminobenzidine was used as the substrate for peroxidase, as described.21 SOX18 immunostaining was assessed by 2 investigators simultaneously using a double-headed light microscope. The extent of staining was graded according to a semiquantitative scale of 0 to ++++; 0, no staining detected; +, weak staining; ++, moderate staining; and ++++, extensive staining.

Immunofluorescence analysis of parafformaldehyde–fixed sections was used to analyze colocalization of PCNA (rabbit polyclonal antibody sc-7907, Santa Cruz Biotechnology Inc) with vascular cell markers. Immunocytochemical Analysis

Cells were cultured in chamber slides (Costar), fixed with 4% formaldehyde, and processed for SOX18 immunostaining as described.21 Horseradish peroxidase–conjugated rabbit anti-mouse antibodies (Dako) were used as secondary antibodies. In control experiments with isotype-matched nonspecific antibodies, no immunocytochemical staining was detected. Results were evaluated with an Olympus Vanox AHBT3 microscope, and images were digitized by a Sony DSC-S500 camera.

Polymerase Chain Reaction

Cells seeded in 6-well plates (106 cells/well) were grown in corresponding media until confluent. Then the cells were serum deprived: VSMCs in medium 199/0.2% FCS for 48 hours; PAECs in medium 199/0.2% FCS for 24 hours; and HUVECs in medium 199/5% FCS for 12 hours before treatment with serum or human recombinant VEGF-A (rhVEGF165, R&D Systems). After treatment, cells were processed and total RNA was isolated that was analyzed with the Ultraspec system (Biotex) according to the manufacturer’s recommendations. SOX18 mRNA levels were analyzed by polymerase chain reaction (PCR) with the PCR Dig labeling mix (Roche Molecular Biochemicals) as described.21 Specific SOX18 oligonucleotides were designed within the human SOX18 sequence (GenBank NM018419). The oligonucleotides used to generate a 583-bp cDNA were used as probes in Northern blot experiments as follows: 5’-CCG-AGTTGACGAGCTCCTC-3’ and 5’-AAGGAGAGCGTTGCTTAAAAA-3’. The oligonucleotides used in reverse transcription (RT)—PCR analysis were as follows: 5’-CCGAGTCCGACGAGCTCCTC-3’ and 5’-GAGGTGACGAGCTGTAATAA-3’. RT–PCR analyses were performed with 28 cycles of denaturation at 94°C for 30 seconds; annealing at 61°C for 1 minute; and polymerization at 72°C for 1 minute. PCR products were resolved by electrophoresis on agarose gels and transferred to nylon membranes (Nyttran Plus, Schleicher & Shuell) by a standard capillary technique. Blots were UV cross-linked. Detection of digoxigenin-labeled nucleic acids was performed with an anti-digoxigenin antibody linked to alkaline phosphatase and disodium 3-(4-methoxy-spiro[1,2-dioxetano-3,2',-5'-(chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenylphosphate used as the substrate. Results were normalized to glyceraldehyde 3-phosphate dehydrogenase as previously described.24

SOX7 and SOX17 mRNA levels were determined by real-time PCR. In brief, RNA was reverse-transcribed with use of a Taqman RT kit (Applied Biosystems) with random hexamers. Assay-on-Demand (Applied Biosystems) of Taqman fluorescent real-time PCR primers and probes were used for SOX7 (Hs00846731_s1) and SOX17 (Hs00751752_s1). Glyceraldehyde 3-phosphate dehydrogenase (4326317E) was used as an endogenous control.

Northern Blot Analysis

Total RNA was obtained as indicated earlier and analyzed by Northern blotting as described.23 A SOX18 583-bp cDNA obtained by RT-PCR with specific SOX18 primers (see previous section) and cloned into the pGEM-T easy vector (Promega) was used as a probe. This probe recognizes a SOX18 region that shares no significant homology with other members of the SOX family to avoid cross-hybridization with SOX-related genes. Hybridization and washes of blots were carried out under high-stringency conditions.25 To estimate transcript size, RNA ladders (Invitrogen) were used.

Determination of DNA Synthesis

[3H]thymidine incorporation into DNA was used as an index of cell proliferation. HUVEC DNA synthesis was assessed as described.24 Cells under basal growth conditions (10% FCS for 12 hours) were incubated with 10 ng/mL VEGF-A in medium containing 0.5 μCi/mL of [3H]thymidine and incubated for an additional 24 hours. Similarly, arrested human coronary VSMCs (0.2% FCS for 48 hours) were stimulated with 10% human serum in medium containing [3H]thymidine, and DNA synthesis was determined as described.24 The effect of AS phosphorothioate ODNs against SOX18 (0.2 to 10 μmol/L) on VSMC DNA synthesis was assessed. The AS ODN used
was AS-SOX18 (5'-CGCGCATCTCTGCAT-3') complementary to nucleotides 111 to 125 of human SOX18 mRNA (GenBank accession No. NM018419). As controls, the corresponding sense sequence (SE-SOX18) and a random oligonucleotide (random) were used. Cells were preincubated with ODNs for 6 hours to assess their effect on both basal and stimulated DNA synthesis. The ODNs did not produce any effect on cell morphology, cell apoptosis (assessed by staining with Hoechst 33258 colorant), or cell viability as analyzed by measuring mitochondrial dehydrogenase activity with use of a commercial kit (XTT-based assay for cell viability, Roche).

### Quantification of Cell Growth

Cell growth was evaluated by cell counting. Growth-stimulated cells were treated with 10 μmol/L ODNs (AS-SOX18 or SE-SOX18), after 72 hours were dissociated with trypsin/EDTA, and then counted with a microscope counting chamber (hemocytometer).

### In Vitro SMC Injury

The ability of AS-SOX18 ODNs to inhibit VSMC wound repair after mechanical injury was assessed in human coronary VSMCs in culture as described. In brief, confluent growth-arrested human coronary VSMCs were denuded with a scraper and incubated in medium with 10% human serum in the presence or absence of 10 μmol/L AS-SOX18 ODNs for an additional 72 hours. SE-SOX18 ODNs were used as controls. Cells were fixed and stained with methylene blue. Images were digitized by a Sony DXC-S500 camera, and cell number in the denuded zone was determined.

### Statistical Analysis

Results are expressed as mean±SEM. The Stat View II (Abacus Concepts) statistical package for the Macintosh computer was used for all analysis; multiple groups were compared by 1-factor ANOVA, followed by Fisher’s protected least significant difference test to assess specific group differences.

### Results

#### SOX18 Immunohistochemical Staining in Coronary Arteries and Vascular Cells

In human coronary artery early lesions, SOX18 immunostaining was virtually absent and rare in intermediate lesions (Table I and Figure 1, available online at http://atvb.ahajournals.org). By contrast, in human coronary advanced atherosclerotic lesions (Figure 1A), SOX18 immunoreactivity was detected in the luminal endothelium and in the vasa vasorum, colocalizing with EC markers CD34 (Figure 1B and 1C) and CD31 (data not shown). In addition, SOX18 was detected in neovessels and in cells expressing α-SMA scattered in the media (Figure 2; Figure III, available online at http://atvb.ahajournals.org). By contrast, in human coronary advanced atherosclerotic lesions, SOX18 immunoreactivity was virtually absent and rare in intermediate lesions (Figure 1A). SOX18 immunoreactivity was detected in both endothelial luminal surface (CD31-positive cells) and VSMC-rich areas (α-SMA-positive cells; Figure 2; Figure III, available online at http://atvb.ahajournals.org).

#### Effect of SOX18 Inhibition on Vascular Cell Proliferation

In VSMCs, AS-SOX18 (10 μmol/L) significantly reduced SOX18 mRNA levels in both control (unstimulated) and growth-stimulated cells (Figure 5A). SE-SOX18 or random sequences did not produce any effect. AS-SOX18 also strongly inhibited serum-induced VSMC DNA synthesis in a dose-dependent manner (IC50<1 μmol/L; Figure 5B). Similarly, AS-SOX18 inhibited both SOX18 mRNA levels and cell number in the denuded zone was determined.

#### SOX18 in Vascular Cells in Culture

In ECs and VSMCs in culture, SOX18 immunostaining was detected in subconfluent areas and in cell monolayers that had been denuded (Figure 3). In Northern blot experiments with a cDNA probe homologous to the human SOX18 generated by RT-PCR and under high-stringency conditions, we detected a single transcript corresponding to the size of SOX18 (1.6 kb). In both growing HUVECs and VSMCs (Figure 4A), SOX18 mRNA levels were transiently induced by mitogenic stimuli (VEGF or serum) in both HUVECs and VSMCs (Figure 4B). SOX18 mRNA levels also were induced by mitogenic stimulus in PAECs (data not shown).
DNA synthesis in HUVECs (Figure IV, available online at http://atvb.ahajournals.org). AS-SOX18 also inhibited VSMCs (30.3% inhibition versus control cells, \( P < 0.005 \)) and EC (33.7% inhibition versus control cells, \( P = 0.002 \)) growth, whereas SE-SOX18 did not produce any effect.

The AS-SOX18 ODNs used in these experiments were specific for SOX18, and they did not modify mRNA levels corresponding to SOX7 and SOX17, as determined by real-time PCR (SOX7, 100 ± 17% in serum-stimulated VSMCs versus 110 ± 24% in serum-stimulated VSMCs plus AS-SOX18 ODNs; SOX17, 100 ± 21% in serum-stimulated VSMCs versus 105 ± 25% in serum-stimulated VSMCs plus AS-SOX18 ODNs).

**Discussion**

SOX18 is a transcription factor previously known to be involved in blood vessel development and embryogenesis,\(^{12,26}\) which has also been related to lymphatic vessel pathologies.\(^{27}\) In the present study, we show that SOX18 is expressed in primary cultures of ECs and VSMCs and in human coronary advanced atherosclerotic lesions. In addition, we show that in vascular cells in culture, inhibition of SOX18 expression prevents DNA synthesis and reduces SMC wound healing.

Recent evidence suggests that the activation of vascular cells in processes such as intimal thickening or neovascularization requires coordinate regulation by multiple genes that control cell cycle entry, cell migration and proliferation, and cell synthetic activity, among other functions.\(^{7-9}\) Here we show that the SRY-related HMG box factor, SOX18, a gene previously identified in fetal brain and expressed in fetal and...
SOX18 has been identified in VSMCs, both in human atherosclerotic arterial septa of adult hearts, this is the first time that SOX18 has been identified in ventricles and the interventricular septa of adult hearts, is expressed in both ECs and VSMCs from human coronary artery advanced arterosclerotic lesions, colocalizing at least in a part with a marker of cell proliferation, whereas cell culture studies indicate that SOX18 could play an important role in vascular cell growth. Taken together, the results of our studies suggest that SOX18 could play a more important role in atherogenesis in those processes that involve cell growth, such as arterial intimal thickening and neovascularization. In fact, lesion neovascularization and arterial intimal thickening are closely associated in advanced lesions, because multiple transcription factors are involved in the regulation of VCAM-1 in cells from advanced lesions, because neovessels are required to ensure the viability and growth of intimal cells (hyperplasia). Finally, Hosking et al recently showed that vascular cell adhesion molecule-1 (VCAM-1), an adhesion molecule overexpressed in atherosclerotic vessels from both human and animal models of hypercholesterolemia, is a target of SOX18 in several cell lines. We did not observe any effect of AS-SOX18 on VCAM-1 mRNA levels in HUVECs and VSMCs (unpublished results); however, because multiple transcription factors are involved in the regulation of VCAM-1 in a cell-specific manner, more focused studies are needed to elucidate the relative role of SOX18 in the transcription rate of VCAM-1 in cells from human adult vessels.

Transcription factors involved in development are critical in the regulation of cell proliferation, differentiation, and migration. In recent years, some of these transcription factors, such as the homeobox genes, have been shown to be key players in cardiovascular system development during embryogenesis and in disease states such as atherosclerosis. Furthermore, HMG box genes, transcription factors more related to the SOX family, regulate vascular development, and the induction of angiogenesis during development and the induction of angiogenesis during development.

SOX18 is known to be involved in vascular development and the induction of angiogenesis during wound healing and repair of skin tissues. Indeed, SOX18 is found in keratinocytes and in ECs from capillaries within the granulation tissues, showing an expression pattern similar to that of VEGFR2 (Flk-1). Here we show that SOX18 is expressed in primary cultures of HUVECs and human coronary VSMCs. In fact, in both cell types, we detected significant levels of the transcript corresponding to SOX18 by Northern blotting (a less-sensitive technique than the RT-PCR–based technique used to analyze SOX18 mRNA levels throughout the study). Although high SOX18 mRNA levels were previously identified in ventricles and the interventricular septa of adult hearts, this is the first time that SOX18 has been identified in VSMCs, both in human atherosclerotic lesions and in culture. In cell cultures, SOX18 immunostaining was mainly localized in subconfluent areas and cell monolayers (either from ECs or VSMCs) that had undergone denudation, suggesting its involvement in cell growth. In fact, inhibition of SOX18 expression with specific oligonucleotides decreases both DNA synthesis and the growth of vascular cells. In addition, inhibition of SOX18 expression by AS oligonucleotides also reduced VSMC wound repair. We detected expression of SOX7 and SOX17 in human vascular cells; however, AS-SOX18 specifically inhibited SOX18 mRNA levels. Therefore, our results do not support the concept of functional redundancy among these 3 SOX family members in adult vascular cells. SOX18, together with SOX7 and SOX17, forms group F within the SOX family. They share significant structural identity and similar expression patterns, in particular during embryogenesis. Based on these similarities and in the absence of an apparent phenotype in SOX18-deficient mice, it has been suggested that these transcription factors could be functionally redundant. However, neither compensatory induction nor other compensatory mechanisms previously described in other genes playing overlapping functions has been demonstrated among these SOX proteins. In fact, SOX7 and SOX17 expression levels were not analyzed in SOX18-knockout mice. Finally, it should be taken into account that in our model, we modulated (not suppressed) SOX18 expression in short-term experiments, and it is possible that the potential compensatory mechanism among SOX proteins does not work in this early setting.

In summary, immunohistochemical studies have identified SOX18 immunostaining in ECs and VSMCs from human coronary artery advanced arterosclerotic lesions, colocalizing at least in a part with a marker of cell proliferation, whereas cell culture studies indicate that SOX18 could play an important role in vascular cell growth. Taken together, the results of our studies suggest that SOX18 could play a more important role in atherogenesis in those processes that involve cell growth, such as arterial intimal thickening and neovascularization. In fact, lesion neovascularization and arterial intimal thickening are closely associated in advanced lesions, because multiple transcription factors are involved in the regulation of VCAM-1 in cells from advanced lesions, because neovessels are required to ensure the viability and growth of intimal cells (hyperplasia). Finally, Hosking et al recently showed that vascular cell adhesion molecule-1 (VCAM-1), an adhesion molecule overexpressed in atherosclerotic vessels from both human and animal models of hypercholesterolemia, is a target of SOX18 in several cell lines. We did not observe any effect of AS-SOX18 on VCAM-1 mRNA levels in HUVECs and VSMCs (unpublished results); however, because multiple transcription factors are involved in the regulation of VCAM-1 in a cell-specific manner, more focused studies are needed to elucidate the relative role of SOX18 in the transcription rate of VCAM-1 in cells from human adult vessels.

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Figure 6. AS oligonucleotides against SOX18 inhibit wound repair after mechanical injury of human coronary VSMC monolayers. Arrested VSMCs were scraped and then incubated for 72 hours in the absence [Control(−)] or presence of 10% human serum [Control(+)] or human serum plus 10 μmol/L oligonucleotides (AS-SOX18 or SE-SOX18, 10 μmol/L). A, Representative photomicrograph of cells subjected to this procedure. B, Number of cells in the denuded zone were counted and represented graphically (n=3 experiments performed in triplicate). *P<0.05 vs control(−); †vs control(+) or cells stimulated with serum and treated with SE-SOX18.
cell migration and proliferation. Recently, the SOX gene family has emerged as a potential set of genes implicated in diverse pathophysiologic functions. Our results suggest that SOX18 could play an important role in vascular cell growth. Future experiments designed to demonstrate the in vivo role of SOX18 in processes involving cell proliferation, such as neovascularization and arterial intimal thickening, will help to determine whether this gene could be regarded as a new target to prevent atherosclerosis progression.

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**Figure I.** SOX18 is not present in early human atherosclerotic lesions. A, Masson trichromic staining of an early human coronary artery lesion. B, high-power view of a serial section immunostained with anti-SOX18 antibodies. (Bar = 100 µm).

**Figure II.** SOX18 immunostaining is not detected in macrophage-rich areas. High power view of serial sections corresponding to a human coronary artery lesion immunostained using anti-SOX18 antibodies (sc-20100) and HAM56 monoclonal antibody (a marker of macrophages). (Bar = 50 µm).

**Figure III.** PCNA positive cells in the endothelial luminal surface and in smooth muscle cell-rich areas are endothelial and smooth muscle cells. Upper panel, co-localization of PCNA (red) with CD31 (green, endothelial cell marker) in the endothelial luminal surface. Lower panel, co-localization of PCNA (red) with α-SMA (green, smooth muscle cell marker) in a smooth muscle cell-rich area. Arrow heads indicate co-localization. (Bar = 25 µm).

**Figure IV.** Antisense oligonucleotides against SOX18 inhibit DNA synthesis in human endothelial cells (HUVEC). A, SOX18 mRNA levels in unstimulated cells (-VEGF) and cells stimulated with 10 ng/mL VEGF-A (+ VEGF) treated or not with oligonucleotides (10 µmol/L). B, HUVEC were exposed to increasing concentration of antisense SOX18 ODNs (AS-SOX18; 0.2 to 10 µmol/L) and DNA synthesis ([³H]thymidine incorporation) was assessed in the absence (white bars) or presence (black bars) of 10 ng/mL VEGF-A. The effect of AS-SOX18, sense SOX18 (SE-
SOX18) and Random ODNs (10 μmol/L) is also shown in both basal and VEGF-A-stimulated conditions (n=3 experiments performed in quadruplicate). \( P<0.05: \ast, \text{ vs. Control; } \dagger, \text{ vs. VEGF-A-treated cells; } \ddagger, \text{ vs. Control and VEGF-A treated cells.} \)
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**CD31**

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<td>Control</td>
<td>AS-SOX18</td>
<td>SE-SOX18</td>
<td>AS-SOX18</td>
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<td>DNA synthesis</td>
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<td>(% of Controls)</td>
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† ‡
Online figure legend

**Figure I.** SOX18 is not present in early human atherosclerotic lesions. A, Masson trichromic staining of an early human coronary artery lesion. B, high-power view of a serial section immunostained with anti-SOX18 antibodies. (Bar = 100 \( \mu m \)).

**Figure II.** SOX18 immunostaining is not detected in macrophage-rich areas. High power view of serial sections corresponding to a human coronary artery lesion immunostained using anti-SOX18 antibodies (sc-20100) and HAM56 monoclonal antibody (a marker of macrophages). (Bar = 50 \( \mu m \)).

**Figure III.** PCNA positive cells in the endothelial luminal surface and in smooth muscle cell-rich areas are endothelial and smooth muscle cells. Upper panel, co-localization of PCNA (red) with CD31 (green, endothelial cell marker) in the endothelial luminal surface. Lower panel, co-localization of PCNA (red) with \( \alpha \)-SMA (green, smooth muscle cell marker) in a smooth muscle cell-rich area. Arrow heads indicate co-localization. (Bar = 25 \( \mu m \)).

**Figure IV.** Antisense oligonucleotides against SOX18 inhibit DNA synthesis in human endothelial cells (HUVEC). A, SOX18 mRNA levels in unstimulated cells (-VEGF) and cells stimulated with 10 ng/mL VEGF-A (+ VEGF) treated or not with oligonucleotides (10 \( \mu \)mol/L). B, HUVEC were exposed to increasing concentration of antisense SOX18 ODNs (AS-SOX18; 0.2 to 10 \( \mu \)mol/L) and DNA synthesis ([\( ^3 \)H]thymidine incorporation) was assessed in the absence (white bars) or presence (black bars) of 10 ng/mL VEGF-A. The effect of AS-SOX18, sense SOX18 (SE-
SOX18) and Random ODNs (10 µmol/L) is also shown in both basal and VEGF-A-stimulated conditions (n=3 experiments performed in quadruplicate). \( P<0.05 \): *, vs. Control; †, vs. VEGF-A-treated cells; ‡, vs. Control and VEGF-A treated cells.