Atherosclerosis 253 (2016) 124–127

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Endothelial-mesenchymal transition in atherosclerotic lesion calcification

Kristina I. Boström [a, b, **], Jiayi Yao [a], Pierre J. Guihard [a], Ana M. Blazquez-Medela [a], Yucheng Yao [a, c, *]

[a] Division of Cardiology, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095-1679, USA
[b] The Molecular Biology Institute at UCLA, Los Angeles, CA 90095-1570, USA
[c] Jonsson Comprehensive Cancer Center at UCLA, Los Angeles, CA 90095, USA

ARTICLE INFO

Article history:
Received 20 May 2016
Received in revised form
2 August 2016
Accepted 31 August 2016
Available online 2 September 2016

Keywords:
Endothelial-mesenchymal transition
Atherosclerotic lesion calcification
Sex-determining region Y-box 2
Serine protease
Endothelial cells

ABSTRACT

Background and aims: Endothelial-mesenchymal transitions (EndMTs) in endothelial cells (ECs) contribute to vascular disease.
Methods: We used *ApoE*$^{-/-}$ mice fed a high-fat/high-cholesterol diet.
Results: We reported evidence of EndMT in atherosclerotic lesions contributing to calcification. Stem cell and mesenchymal markers, including sex-determining region Y-box 2 (Sox2), were upregulated in aortic ECs of fat-fed *ApoE*$^{-/-}$ mice. Limiting Sox2 decreased marker expression and calcification in *ApoE*$^{-/-}$ aortas. Furthermore, a complex of serine proteases was upregulated in *ApoE*$^{-/-}$ aortic ECs. Blockade of these proteases reduced expression of Sox2 and atherosclerotic lesion calcification.
Conclusions: Together, our data suggest that EndMTs contribute to atherosclerotic lesion calcification.

1. Introduction

Vascular calcification is a common feature of atherosclerosis [1–4], associated with increased plaque burden and poor clinical prognosis [5]. Vascular calcification is a regulated process that requires systemic and local factors, responding to proatherogenic oscillatory shear stress, oxidative stress and proinflammatory cytokines [4,6–8]. Bone morphogenetic proteins (BMPs) are important pro-calcific factors, found at high levels in calcified atherosclerotic lesions [9,10]. Overexpression of a BMP inhibitor, matrix Gla protein (MGP), limits atherosclerotic lesion calcification in *ApoE*$^{-/-}$ mice [11]. An inhibitor of BMP type I receptor kinases also reduces the lesion calcification in *Ldlr*$^{-/-}$ mice [12]. Recently, it was found that excess BMP activity triggers EndMT allowing the endothelium to contribute cells to the calcifying process [10,13,14].

EndMT is a process through which ECs transit into mesenchymal stem cells and gain multipotency [15], prior to differentiating into various cell lineages [13]. EndMT has been shown in normal development, such as neural crest formation and cardiogenesis [16,17]. In disease, EndMTs contribute to cardiac and renal fibrosis [18,19], fibrodysplasia ossificans progressive [20], cancer progression [21] and pulmonary hypertension [22]. Although EndMTs have been found to occur in atherosclerotic lesions [23], it is still unclear whether EndMTs contribute to atherosclerotic lesion calcification.

2. Materials and methods

2.1. Animals

ApoE$^{-/-}$ (B6.129P2-ApoEtm1Unc/J) mice were obtained from the Jackson Laboratory. Genotypes were confirmed by PCR [24] and experiments were performed with generations F4-F6. All mice were fed a standard chow diet (Diet 8604, HarlanTeklad Laboratory). At 8–10 weeks of age, all *ApoE*$^{-/-}$ mice were switched to a high-fat/high-cholesterol diet (Western diet) (Research Diets, New Brunswick, NJ, diet #D12108, containing 21% fat, 1.25% cholesterol) for 16 weeks. Diisopropylfluorophosphate (DFP) (Sigma-Aldrich),

* Corresponding author. Division of Cardiology, David Geffen School of Medicine, UCLA, Jonsson Comprehensive Cancer Center, UCLA, Box 951679, Los Angeles, CA 90095-1679, USA.
** Corresponding author. Division of Cardiology, David Geffen School of Medicine, UCLA, Jonsson Comprehensive Cancer Center, UCLA, Box 951679, Los Angeles, CA 90095-1679, USA.
E-mail addresses: kbostrom@mednet.ucla.edu (K.I. Boström), yyao@mednet.ucla.edu (Y. Yao).

http://dx.doi.org/10.1016/j.atherosclerosis.2016.08.046
0021-9150/

serpina1 (Origene) or Sox2 shRNA were injected via tail vein or retro-orbital injection (20–50 ng/g, daily) as in previous studies [13]. Injections in *ApoE*$^{-/-}$ mice were started at 16–18 weeks of age, and continued for 8 weeks. The studies were reviewed and approved by the Institutional Review Board and conducted in accordance with the animal care guideline set by the University of California, Los Angeles. The investigation conformed to the National Research Council, Guide for the Care and Use of Laboratory Animals, Eighth Edition (Washington, DC: The National Academies Press, 2011).

2.2. RNA analysis

Real-time PCR analysis was performed as previously described [25]. Primers and probes for mouse *Sox2*, Kruppel-like factor 4 (*Klf4*), snail family zinc finger 2 (*Slug* or *Snail2*), stem cell antigen 1 (*Sca1*), cluster of differentiation (*CD)10, CD44, CD71, CD90*, *c-kit* (also referred to as *CD117*), and all elastases and kallikreins were obtained from Applied Biosystems as part of Taqman® Gene Expression Assays.

2.3. Immunoblotting

Immunoblotting was performed as previously described [13]. Blots were incubated with specific antibodies to c-kit (200 ng/ml; Cell Signaling Technology), Sca1 (200 ng/ml; Merck Millipore), CD90 (both 200 ng/ml; Abcam), CD71 (1:200; ThermoFisher). β-actin (1:5000 dilution; Sigma-Aldrich) was used as loading control.

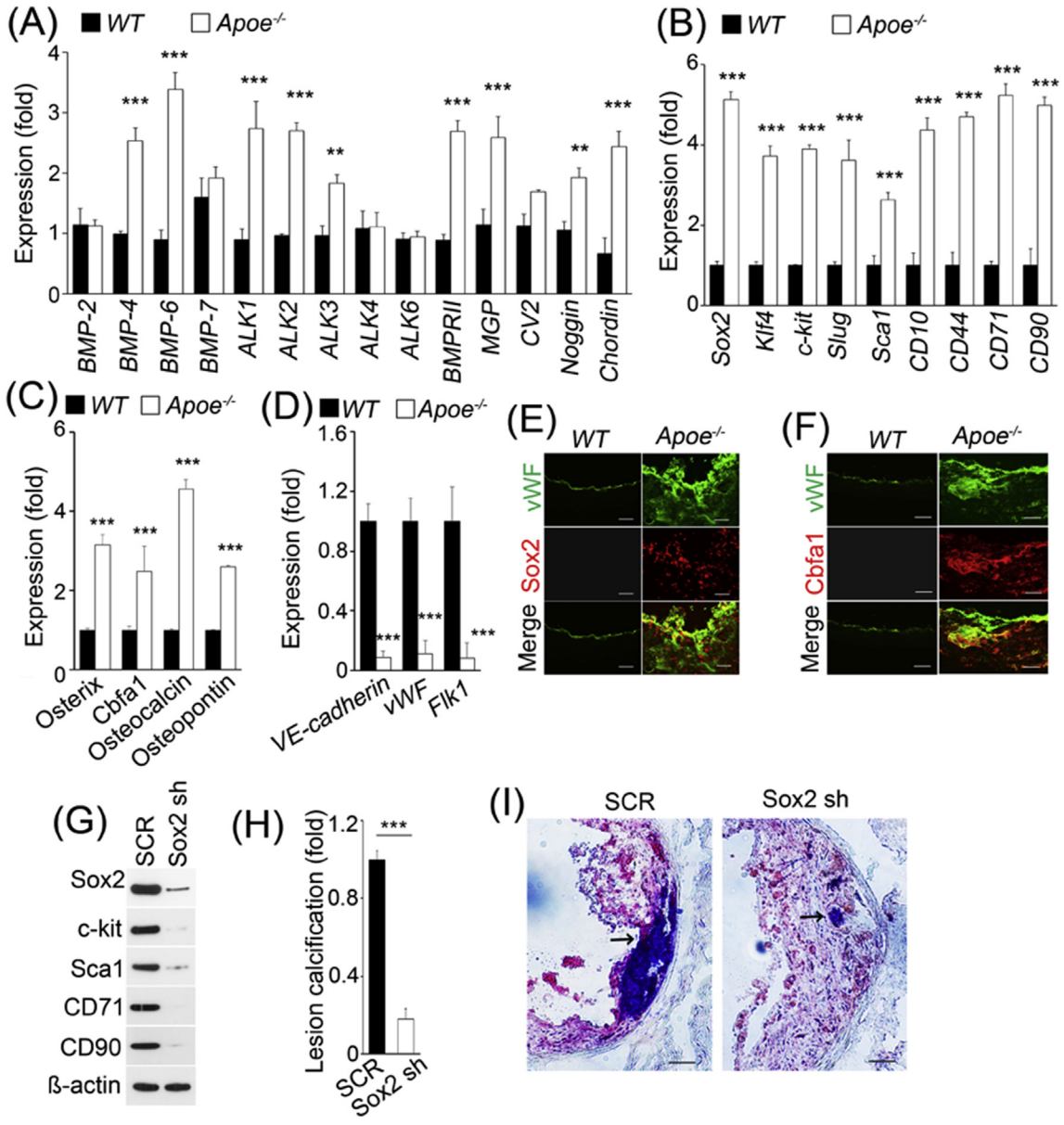

Fig. 1. Sox2 and EndMTs in atherosclerotic lesion calcification of *ApoE*$^{-/-}$ mice. (A–D) Expression of BMP components (A), stem cell and mesenchymal markers (B), osteogenic markers (C) and EC markers (D) in isolated aortic ECs of wild type (WT) and *ApoE*$^{-/-}$ mice, as determined by real-time PCR. (E–F) The EC marker vWF co-localizes with Sox2 (E) and bone marker Cbfa1 (F) in calcified atherosclerotic lesions. (G–I) *ApoE*$^{-/-}$ mice were fed a western diet and treated with scrambled shRNA (SCR) or *Sox2* shRNA (sh) for 16 weeks. Aortic expression of Sox2, c-kit, Sca1, CD71 and CD90 was examined by immunoblotting (G). The volume of calcification in atherosclerotic lesions was examined (n = 5) (H). Representative Oil Red O stained aortic sinus sections (I). Arrows indicate calcification. Scale bar (b, c, f), 100 μm ***$p < 0.001$.