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## Endothelial-mesenchymal transition in atherosclerotic lesion calcification

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### Abstract

**Background and aims**—Endothelial-mesenchymal transitions (EndMTs) in endothelial cells (ECs) contribute to vascular disease.

**Methods**—We used *ApoE*<sup>-/-</sup> 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*<sup>-/-</sup> mice. Limiting Sox2 decreased marker expression and calcification in *ApoE*<sup>-/-</sup> aortas. Furthermore, a complex of serine proteases was upregulated in *ApoE*<sup>-/-</sup> 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.

### Keywords

Endothelial-mesenchymal transition; atherosclerotic lesion calcification; sex-determining region Y-box 2; serine protease; endothelial cells

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### CONFLICT OF INTEREST

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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## 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*<sup>-/-</sup> mice (11). An inhibitor of BMP type I receptor kinases also reduces the lesion calcification in *Ldlr*<sup>-/-</sup> 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.

## MATERIALS AND METHODS

### Animals

*ApoE*<sup>-/-</sup> (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, Harlan Teklad Laboratory). At 8 to 10 weeks of age, all *ApoE*<sup>-/-</sup> 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. Diisopropyl fluorophosphate (DFP) (Sigma-Aldrich), serpinal (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*<sup>-/-</sup> mice were started at 16 to 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).

### 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.

## 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).  $\beta$ -actin (1:5000 dilution; Sigma-Aldrich) was used as loading control.

## Quantification

Lesion calcification was quantified as previously described (13).

## Statistical analysis

Data were analyzed for statistical significance by ANOVA with *post hoc* Tukey's analysis. The analyses were performed using GraphPad Instat®, version 3.0 (GraphPad Software). Data represent mean  $\pm$  SD.  $p < 0.05$  was considered significant, and experiments were performed a minimum of three times.

## RESULTS

### Induction of Sox2 and EndMTs in calcified atherosclerotic lesions

Our previous studies showed increased BMP activity in atherosclerotic lesions in *ApoE*<sup>-/-</sup> mice (9). Here, we confirmed high expression of BMP in aortic ECs isolated from *ApoE*<sup>-/-</sup> mice fed a western diet for 16 weeks. We found significant induction of BMP ligands BMP4 and 6, receptors activin receptor-like kinase (ALK) 1, 2 and 3, BMP type II receptor and inhibitors matrix gla protein (MGP), noggin and chordin (Figure 1A). Enhanced BMP activity has been reported to induce EndMTs and allow ECs to undergo osteogenic differentiation (10, 14, 20). Therefore, we examined stem cell and mesenchymal markers, and endothelial and osteogenic markers in aortic ECs isolated from the fat-fed *ApoE*<sup>-/-</sup> mice. We found strong induction of *Sox2*, *Klf4*, *c-kit*, *Slug*, *Sca1*, *CD10*, *CD44*, *CD71*, and *CD90* (Fig. 1B), and *osterix*, *Cbfa1*, *osteocalcin* and *osteopontin* (Fig. 1C) with reduction of endothelial markers *VE-cadherin*, von Willebrand factor (*vWF*) and *Flk1* (Fig. 1D). Immunostaining showed that Sox2 and the osteogenic marker Cbfa1 co-localized with vWF in calcified atherosclerotic lesions (Fig. 1E and F), suggesting that EndMTs occur in atherosclerotic calcification.

### Limiting Sox2 reduced EndMTs and lesion calcification

Since depletion of Sox2 reduces EndMTs (13), we treated *ApoE*<sup>-/-</sup> mice with *Sox2* shRNA and fed them a western diet for 8 weeks before examining the expression of stem cell and mesenchymal markers and lesion calcification. We found that treatment with *Sox2* shRNA decreased both marker expression and lesion calcification in the fat-fed *ApoE*<sup>-/-</sup> mice (Fig. 1G–I). There was no significant change in serum lipid levels and size of atherosclerotic lesions, as measured in the aortic sinuses (data not shown), although the study was not powered for lesion size but for calcification. The results suggest that Sox2 is a mediator of EndMTs in lesion calcification.

### Serine proteases were upregulated in calcified lesions

We previously showed that a complex of serine proteases, including elastase 1, 2 and kallikrein 1, 5, and 6, regulate Sox2 in EndMTs (13). Therefore, we examined the expression of elastases and kallikreins, and found a strong induction of *elastase 1, 2* and *kallikrein 1, 5, 6* in the aortic ECs of fat-fed *ApoE*<sup>-/-</sup> mice (Fig. 2A, right panel). Induced elastase 1, as an example of the group, co-localized with vWF and Sox2 in calcified lesions (Fig. 2B and C), supporting that activation of elastases and kallikreins also occurs in ECs in atherosclerotic lesions.

### Inhibition of serine proteases decreased calcification in atherosclerotic lesions

We then determined whether protease inhibition reduced Sox2, EndMTs, and calcification by using the inhibitors diisopropylfluorophosphate or serpin1, similar to our previous study (13). We treated *ApoE*<sup>-/-</sup> mice for 8 weeks with diisopropylfluorophosphate or serpin1, respectively, together with western diet. We found that protease inhibition reduced both marker expression and lesion calcification in the fat-fed *ApoE*<sup>-/-</sup> mice (Fig. 2D–F). No significant changes in serum lipid levels or size of atherosclerotic lesions were detected (data not shown). The data suggest that the activation of elastases and kallikreins triggers Sox2 expression and EndMTs in ECs in atherosclerosis and contributes to the lesion calcification.

Thus, the same mechanism previously detected in MGP-deficient mice is also activated in the calcification of atherosclerotic lesions.

## DISCUSSION

We previously found EndMTs contributing to aortic calcification in mice lacking MGP, a vascular BMP inhibitor, where BMP activation is increased due to lack of BMP inhibition (13). In this study, we explored whether similar EndMTs occur in atherosclerotic lesions in fat-fed *ApoE*<sup>-/-</sup> mice. MGP, as a BMP inhibitor, is induced in aortic endothelium of fat-fed *ApoE*<sup>-/-</sup> mice (Fig. 1) (9). Our studies suggest that increased BMP activity is the primary force to drive EndMTs leading to calcification in both atherosclerotic lesions and *Mgp*<sup>-/-</sup> mice. However, the mechanism by which high BMP activity is achieved differs between atherosclerotic lesions where increased expression of BMPs and BMP receptors enhances BMP signaling, and *Mgp*<sup>-/-</sup> mice where BMP signaling increases due to less BMP inhibition (9, 13). Furthermore, MGP is induced in atherosclerotic lesions (9), but not enough to overcome BMP induction. Enhancement of the MGP response does suppress the excessive BMP activity (9). In addition, inhibition of serine proteases or reduction of Sox2 expression, both of which decreased calcification in *Mgp*<sup>-/-</sup> mice (13), reduced EndMTs and calcification in *ApoE*<sup>-/-</sup> mice. However, we did not detect any decrease in the atherosclerotic lesions from these interventions. It may be due to the limited size of the study, which was focused on and powered for changes in calcification. Overall, the results suggest that the same mechanism promoting calcification in *Mgp*<sup>-/-</sup> mice is activated in atherosclerotic lesion.

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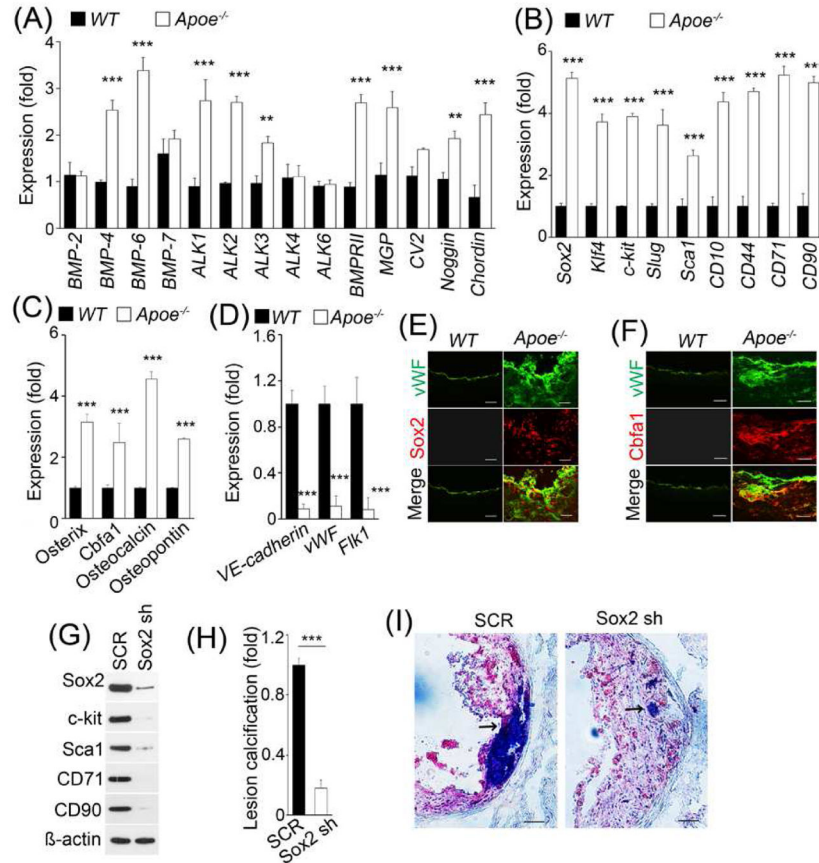
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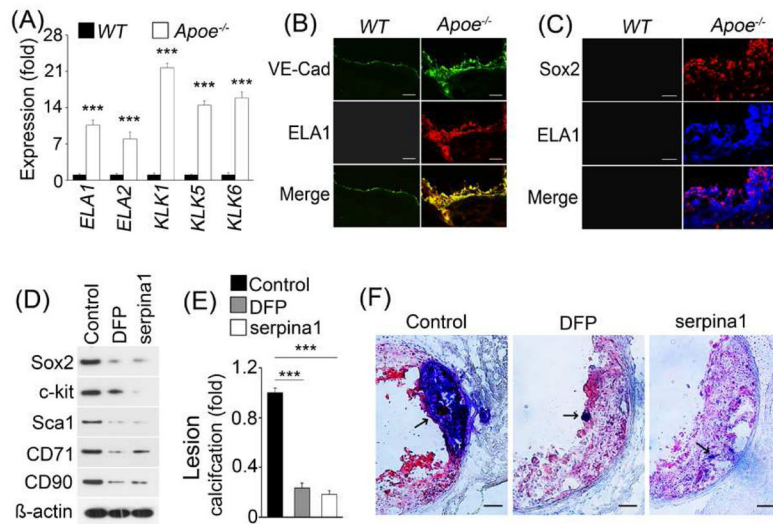
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- Endothelial-mesenchymal transitions (EndMTs) in atherosclerotic lesions contribute to calcification.
- Stem cell and mesenchymal markers, including sex-determining region Y-box 2 (Sox2), are upregulated in aortic endothelial cells (ECs) of fat-fed *ApoE*<sup>-/-</sup> mice.
- Limiting Sox2 decreases the stem cell and mesenchymal markers and calcification in *ApoE*<sup>-/-</sup> aortas.
- A complex of serine proteases is upregulated in *ApoE*<sup>-/-</sup> aortic ECs.
- Blockade of these proteases reduces expression of Sox2 and atherosclerotic lesion calcification.



**Fig. 1. Sox2 and EndMTs in atherosclerotic lesion calcification of *ApoE*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> 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.





**Fig. 2. Elastases and kallikreins in EndMTs in calcified atherosclerotic lesions of *ApoE*<sup>-/-</sup> mice** (A) Expression of elastases and kallikreins in isolated aortic ECs of wild type (WT) and *ApoE*<sup>-/-</sup> mice as shown by real-time PCR. (B–C) Elastase (ELA) 1 co-localized with EC marker VE-cadherin (VE-Cad) (B), and Sox2 (C) in calcified atherosclerotic lesions. (D–F) *ApoE*<sup>-/-</sup> mice were fed a western diet and treated with control, diisopropylfluorophosphate (DFP) and serpina1. Immunoblotting showed the expression of Sox2, c-kit, Sca1, CD71 and CD90 in aortic tissues (n=5) (D). The volume of calcification in atherosclerotic lesions was examined (n=5) (E). Representative Oil Red O stained aortic sinus sections (F). Arrows indicate calcification. Scale bar (b, c, f), 100 μm. \*\*\**p*<0.001.