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KLF2 Mediates the Suppressive Effect of Laminar Flow on Vascular Calcification by Inhibiting Endothelial BMP/SMAD1/5 Signaling

Juan Huang,* Yujie Pu,* Hongsong Zhang,* Liping Xie, Lei He, Cheng-Lin Zhang, Chak Kwong Cheng, Yingsong Huo, Song Wan, Shaoiliang Chen, Yuhong Huang, Chi Wai Lau, Li Wang, Yin Xia, Yu Huang, Jiang-Yun Luo

RATIONALE: Vascular calcification in arterial intima is closely associated with atherosclerosis. Endothelial cells (ECs) sense blood flow and respond to the mechanical cues generated by different flow patterns. Laminar flow induces an anti-atherosclerotic EC phenotype whereas disturbed flow exerts an atheroprone effect. However, the contribution of blood flow to calcification in atherosclerotic arteries remains to be evaluated.

OBJECTIVE: We aim to investigate whether blood flow plays a determinant role in the distribution of vascular calcification and the underlying mechanisms involved.

METHODS AND RESULTS: Computed tomography angiography analysis of human coronary arteries (n=48) shows that calcification preferentially develops at flow perturbated sites. Similar phenomenon was observed in calcified human aortic valves and mouse arteries. Nonuniform shear stress produced in Y-shaped slide simulating live conditions in branched arteries promotes calcification in human umbilical vein ECs. The expression of KLF2 (Krüppel-like factor 2), a transcription factor inducible by laminar flow, is reduced in ECs of calcified human aortic valves and in endothelial calcification model, suggesting that KLF2 downregulation is likely involved in intimal calcification. Indeed, KLF2 silencing induces endothelial-to-mesenchymal transition and accelerates osteoinduction in both human aortic ECs and human umbilical vein ECs. EC-specific KLF2 knockdown promotes whereas AAV9 (adeno-associated virus serotype 9)-mediated EC-KLF2 overexpression ameliorates vascular calcification in ApoE−/− mice. Global mRNA profiling in human umbilical vein ECs reveals that KLF2 inhibits the BMP (bone morphogenetic proteins)/SMAD1/5 pathway which is critical in vascular calcification. Furthermore, KLF2 mediates laminar flow-induced inhibition of the BMP/SMAD1/5 pathway. By contrast, disturbed flow-induced activation of BMP/SMAD1/5 pathway is suppressed by KLF2 overexpression. Mechanistically, KLF2 binds to the promoters of BMP4, BMPER, and SMAD1 to directly regulate their expression in ECs.

CONCLUSIONS: Vascular calcification prefers to occur at branched or bifurcated areas of vasculature. Laminar flow inhibits vascular calcification through KLF2-mediated inhibition of endothelial BMP/SMAD1/5 signaling. Targeting KLF2 may represent a novel therapeutic approach against vascular calcification.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: endothelial cells • vascular calcification
Vascular calcification is a widely used indicator for the severity of atherosclerosis. The prevalence of calcification in the coronary artery correlates roughly with age, and 60% of the population over 60 years old have calcific vasculopathy. The only available therapeutic options for treating cardiovascular calcification are invasive transcatheter procedures or surgeries which, however, cannot fully meet the increasing medical need. The cellular and molecular mechanisms underlying vascular calcification remain to be further explored, and the development of new therapeutic strategies is an urgent need to tackle this human health problem.

Although vascular calcification can develop in both the intimal and medial layers of the arteries with some common pathomechanisms, the atherosclerotic calcification primarily targets the intimal layer of arteries. Endothelial cells (ECs) are not only critical for atherogenesis but also believed as a source of osteoprogenitor cells that initiate intimal calcification. ECs can undergo a transdifferentiation process named endothelial-to-mesenchymal transition (EndMT) to become pluripotent and are committed to osteogenic differentiation guided by various stimuli, such as BMPs (bone morphogenetic proteins). To better understand the pathogenesis of vascular calcification, it shall be valuable to further elucidate the role of ECs in this pathological process.

Multiple factors participating in the developmental, inflammatory, and metabolic processes are involved in intimal calcification. Among them, BMPs were demonstrated to play a causal role in vascular calcification.

Novelty and Significance

What Is Known?

- Vascular calcification is a common complication of cardiovascular diseases. Patients with coronary artery calcification have a higher risk of cardiovascular events.
- Oscillatory shear stress induces, whereas laminar shear stress inhibits, endothelial-to-mesenchymal transition (EndMT). Laminar shear stress also induces a sustained upregulation of KLF2 (Krüppel-like factor 2) in endothelial cells. Oscillatory shear stress induces BMP (bone morphogenetic proteins)-4 production and SMAD1/5 phosphorylation in endothelial cells.
- Activation of the BMP/SMAD1/5 pathway plays a critical role in the initiation of EndMT and vascular calcification.

What New Information Does This Article Contribute?

- Vascular calcification preferentially develops at the bifurcations of coronary arteries in patients and vascular regions with disturbed flow in vascular calcification-related mouse models. Nonuniform shear stress promotes mineral deposits in endothelial cells.
- The shear stress-sensitive transcription factor KLF2 protects against vascular calcification in vivo.
- KLF2 deficiency promotes vascular calcification in atherosclerotic ApoE−/− mice and induces EndMT and osteogenic differentiation in endothelial cells.
- KLF2 negatively regulates BMP/SMAD1/5 signaling which promotes EndMT and vascular calcification. KLF2 mediates the inhibitory effect of laminar shear stress on the BMP pathway and its overexpression reverses oscillatory shear stress-induced activation of the BMP/SMAD signaling in endothelial cells.

Vascular calcification is an independent risk factor for life-threatening cardiovascular disorders. The present study demonstrates a novel link between hemodynamic forces and vascular calcification and reveals that flow-sensitive KLF2 works against vascular calcification. KLF2 gene silencing induces EndMT and accelerates osteogenic stimuli-induced chondral/osteogenic transdifferentiation, subsequently resulting in calcium phosphate deposits in endothelial cells. Mechanistically, KLF2 negatively regulates the BMP/SMAD1/5 signaling which promotes EndMT and vascular calcification. BMP4, BMPER, and SMAD1 were identified as novel targets regulated by KLF2. Thus, these findings suggest that targeting the KLF2-BMP/SMAD1/5 signaling cascade may hold promise as a novel therapeutic strategy against vascular calcification.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BMPs</td>
<td>bone morphogenetic proteins</td>
</tr>
<tr>
<td>DF</td>
<td>disturbed flow</td>
</tr>
<tr>
<td>ECs</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>EndMT</td>
<td>endothelial-to-mesenchymal transition</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>HAECs</td>
<td>human aortic endothelial cells</td>
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<tr>
<td>HUVECs</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>KLF2</td>
<td>Krüppel-like factor 2</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LSS</td>
<td>laminar shear stress</td>
</tr>
<tr>
<td>OM</td>
<td>osteogenic medium</td>
</tr>
<tr>
<td>OSS</td>
<td>oscillatory shear stress</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecular</td>
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BMP ligands BMP2, BMP4, and BMP6 act through receptor-regulated SMAD1/5 proteins to regulate expression of osteogenic genes in multiple cell types, paving the way for the initiation of calcification. Inhibition of BMPs is effective in preventing vascular calcification although future human studies are required to validate this beneficial effects. It, however, remains incompletely understood for the regulatory mechanisms of BMPs signaling in vascular calcification.

In addition to the biochemical factors, mechanical force generated by blood flow is likely to be a key contributor to intimal calcification, although the exact mechanism is still unclear. Many studies, including our previous reports, demonstrated that oscillatory shear stress (OSS) generated by disturbed flow (DF) induces inflammation and oxidative stress in ECs, while laminar shear stress (LSS) is anti-inflammatory and promotes a quiescent and healthy endothelial phenotype. KLF2 (Krüppel-like factor 2), a transcription factor mainly expressed in ECs, is highly sensitive to LSS. KLF2 mediates the expression of approximately half of the genes responsive to LSS and plays a protective role in ECs owing to its anti-inflammatory and antithrombotic properties. However, the role of KLF2 in vascular calcification has yet to be uncovered.

In the present study, we observed a site-specific distribution of vascular calcification in human coronary arteries and revealed a close association between blood flow patterns and vascular calcification. We demonstrated that transcription factor KLF2 mediates the effect of mechanical forces against atherosclerotic calcification. Our unbiased transcriptional profiling results show that KLF2 inhibits the BMP/SMAD1/5 pathway in ECs, thereby protects against vascular calcification.

**METHODS**

**Data Availability**

All supporting data are available within the article and its Data Supplement. For details on the experimental procedures, see the Materials and Methods section in the Data Supplement.

**RESULTS**

**Site-Specific Distribution of Coronary Arteries Calcification in Patients**

We initially observed that coronary artery calcification prefers to occur at arterial branching points, where flow pattern is disturbed. This phenomenon led us to question whether hemodynamic forces may have a determinant role in the distribution of calcification. To answer our curiosity, 48 patients were enrolled and the coronary artery calcification was evaluated by coronary computed tomography angiography. Anatomically, the left coronary artery, starting with the left main coronary artery, then branches into the left anterior descending and the left circumflex arteries. The calcification in the bifurcations and nonbifurcations was calculated in these 4 arteries (Figure 1A). The statistical analysis shows that calcification is more likely to develop at bifurcated sites compared with other regions, clearly revealing the site-specific distribution of vascular calcification (Figure 1B through 1F). The patients were further divided into 4 groups according to their calcification scores. Strikingly, the calcification score of the bifurcations is significantly higher than nonbifurcations in all 4 groups of patients (Figure IA through ID in the Data Supplement). These clinical data suggest a strong association of vascular calcification progression with DF in patients.

**Uniform LSS Protects Against Vascular Calcification**

DF is present in the curvature of the aortic arch and branch origin sites, while laminar flow is present in other sites. The observations in patients suggest a positive correlation between DF and vascular calcification. To further explore such correlation, ApoE−/− mice were fed a western diet (MP Biomedicals, 02960404) for 20 weeks to induce calcification. OsteoSense 680 fluorescence reflectance imaging shows that strong nanoparticle-derived signals were yielded in the arteries of ApoE−/− mice, suggesting a successful induction of vascular calcification (Figure 2A). Fluorescence intensity is higher in the aortic arch than that in the descending thoracic aorta. In the abdominal aorta and carotid artery, calcification preferentially develops in the branching points (Figure 2A and 2B). The descending aorta has a very low level of calcification, and this calcification also appears to correlate with intercostal branching points that experience DF in vivo. Furthermore, Alizarin red staining showed that calcification preferentially develops at the aortic arch and arterial branching sites in all the frozen sections of the brachiocephalic arteries, aortic arch, and thoracic aortas (Figure 2C). In the aortic arch, the calcium deposit mainly occurred at the inner curvature but not the outer curvature (Figure 2C). Similar observation was also made in the mouse model of vitamin D3 overload-induced arterial media calcification (Figure IE through IG in the Data Supplement), indicating that flow patterns are likely to have a critical role in the geometric distribution of vascular calcification regardless of the types of calcification.

Arterial branches redirect blood flow in a manner that exposes regions to low and nonuniform shear stress. This phenomenon was simulated in cell culture using a Y-shaped chamber slide that contains both straight and branching regions. To further determine the important role of flow patterns in vascular calcification, human umbilical vein ECs (HUVECs) were subjected to flow in this Y-shaped chamber attached to a fluidic unit/pump system that continuously perfuses the cell surface with culture medium. The flow rate was 8.79 mL/min,
equivalent to a shear stress of 20 dyne/cm² along the straight segment of the channel. The shear stress in the regions between branching points was 10 dyne/cm² due to flow division. In agreement with in vivo observations, calcium deposition was detected in the branching areas that experienced nonuniform shear stress, and almost no calcium deposition was found in the straight regions that were exposed to uniform LSS (Figure 2D and 2E). Therefore, calcium deposit was impacted by the uniformity of shear stress and uniform LSS suppresses calcification in ECs.

Flow-Sensitive KLF2 Is Repressed in Cardiovascular Calcification

Consistent with previous reports, we also observed that calcification primarily occurs at the aortic side of the calcified aortic valves from patients undergoing surgical valve replacement for severe aortic valve stenosis (Figure IH in the Data Supplement). On the ventricular side, shear stress is unidirectional pulsatile while it is low and reciprocating on the aortic side without a clear direction. Thus, there is a spatial correlation between the local hemodynamic environment and calcific lesions on the aortic side of the valve. To understand the underlying molecular basis for such correlation, we focus on the role of KLF2, a master regulator of shear stress-responsive genes in ECs, in contribution to the site-specific effect of blood flow on calcification. Importantly, the Alizarin red staining and immunostaining of human aortic valve specimen showed that the KLF2 level is reduced in ECs from the calcified area compared with the noncalcified area (Figure 3A and 3B), suggesting that the repressed expression of endothelial KLF2 is likely to be involved in cardiovascular calcification. To test this hypothesis, we detected the KLF2 expression and found a reduction of KLF2 level in both in vitro and in vivo models of vascular calcification (Figure 3C).
regulating KLF2 expression, LSS at 12 dyn/cm² or OSS at 0.5±4 dyn/cm² were applied to HUVECs. The mRNA level of KLF2 was induced rapidly (within 3 hours) by LSS and remained elevated at 24 hours (Figure 3D). KLF2 expression was also higher in rat aortic endothelium exposed to LSS, as compared with OSS (Figure 3E).

**KLF2 Silencing Promotes EndMT and Accelerates Osteoinduction in ECs**

Recent studies have uncovered that OSS induces EndMT to drive the progression of atherosclerosis.\(^{14,15}\) Given KLF2 is not inducible by OSS, whether the absence of KLF2 induction by OSS has any impact on EndMT is not explored. To test this, we silenced KLF2 expression in HUVECs and observed that KLF2 silencing caused morphological changes in HUVECs which acquire an appearance like fibroblast or mesenchymal cells (Figure IVA and IVB in the Data Supplement). Consistent with this morphological switch, the gene expression data showed reduced mRNA levels of EC markers (PECAM1 [platelet endothelial cell adhesion molecule-1] and CDH5) but elevated levels of smooth muscle cell markers (ACTA2, CNN1, and TAGLN), mesenchymal markers (VIM and FN1) as well as EndMT transcription factor SNAI1 in KLF2-silenced HUVECs and human aortic endothelial cells (HAECs) (Figure IIIA and IIIB in the Data Supplement). The changes in the gene expression were confirmed by Western blotting data (Figure 4A). The process of vascular calcification is generally quite similar to bone formation and many cellular participants in bone formation are also involved the progression of vascular calcification.\(^{16}\) The present results strongly suggest that KLF2 silencing promotes the transition of ECs to mesenchymal cells, which could be subsequently triggered to differentiate into osteoblasts or chondrocytes during the progression of vascular calcification.\(^{17}\) To further investigate whether EndMT induced by KLF2 silencing promotes

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**Figure 2. Uniform laminar shear stress protects against vascular calcification.**

A. Calcification in the aortas of ApoE\(^{-/-}\) mice visualized by Osteosense 680 staining after tail vein injection 24 h before euthanization and imaging. n=5 for normal diet, n=6 for western diet. B. The statistic results showing vascular calcification primarily develops at the branch points or the curvatures. C. Representative Alizarin red staining of frozen sections of the brachiocephalic arteries, aortic arch, and thoracic aortas in ApoE\(^{-/-}\) mice. In the aortic arch, mineral deposits were increased in inner curvature compared with outer curvature. D. Schematic illustration of the Ibidi Y-shaped chamber slide. E. Images corresponding to the regions experiencing nonuniform shear stress show increased microcalcifications indicated by near-infrared fluorescence calcium tracer in human umbilical vein endothelial cells (HUVECs). Bar, 50 μm. Four independent experiments were performed with similar results. The P value was calculated by a Mann-Whitney U test or an unpaired 2-tailed Student t test in B, as indicated by \(P_U\) or \(P_t\), respectively.
Huang et al. KLF2 Inhibits Calcification by Suppressing BMP Signaling

osteogenesis, we cultured KLF2-silenced HUVECs in an osteogenic medium (OM) and found that these cells expressed elevated levels of osteogenic markers, including MSX2 (Msh Homeobox 2), RUNX2 (Runt-related transcription factor 2), and Osterix, indicating that KLF2 deficiency accelerates the osteogenic/chondrogenic transdifferentiation (Figure 4B). In line with this, both the Osteosense 680 and Alizarin red staining results showed that KLF2 deficiency increased the calcium deposition in HUVECs and HAECs cultured in OM (Figure 4C through 4F; Figure IVC and IVD in the Data Supplement).

Endothelial-Specific Klf2 Knockdown Increases Vascular Calcification in ApoE−/− Mice Fed a Western Diet

To determine the role of endothelial KLF2 in vascular calcification, we achieved EC-specific Klf2 knockdown (Klf2-KD) in ApoE−/− mice using CRISPR/Cas9-based in vitro genome editing approach (Figure V in the Data Supplement). Briefly, EC-enhanced AAV9 (adeno-associated virus serotype 9)-mediated Klf2-sgRNA was delivered to Cas9+ ApoE−/− and Cdh5+ Cas9+ ApoE−/− mice via tail vein injection (Figure 5A). The Klf2 knockdown efficiency was validated by markedly diminished Klf2 mRNA level in the lung ECs and aorta (Figure 5B and 5C). The KLF2 target gene, Nos3, was also down-regulated in Cdh5+ Cas9+ ApoE−/− mice compared with control mice, suggesting the functional reduction of endothelial KLF2. The mice were fed a western diet for 18 weeks to induce calcification. Metabolic parameters, including body weight, blood glucose, plasma calcium, and phosphate level, as well as lipid profile did not differ between Klf2-KD; ApoE−/− and ApoE−/− mice (Table II in the Data Supplement). As shown by a near-infrared fluorescent calcium tracer, Klf2-KD; ApoE−/− mice displayed increases of vascular calcification (Figure 5D and 5E). Moreover, Klf2-KD; ApoE−/− mice exhibited significantly elevated calcium content in aortas compared with ApoE−/− mice (Figure 5F), thus indicating that knockdown of Klf2 in ECs promotes vascular calcification in vivo. These results also suggest that KLF2 deficiency
might be one of the mechanisms that explain why the coronary artery calcification is much greater in vascular branching points exposing constantly to DF in patients.

**AAV9-Mediated EC-KLF2 Overexpression Ameliorates Vascular Calcification in ApoE<sup>−/−</sup> Mice**

To test the potential of KLF2 as a therapeutic target in vascular calcification, we overexpressed *Klf2* specifically in ECs of *ApoE<sup>−/−</sup>* mice through EC-enhanced AAV9-mediated *Klf2* expression under the control of EC-specific *Cdh5* promoter (AAV-Cdh5-Klf2). After gene delivery, the *ApoE<sup>−/−</sup>* mice were fed with a western diet for 18 weeks (Figure 6A). *ApoE<sup>−/−</sup>* mice receiving AAV-Cdh5-Klf2 exhibited reduced vascular calcification as detected with a near-infrared fluorescent calcium tracer (Figure 6B and 6C). Moreover, *ApoE<sup>−/−</sup>* mice injected with AAV-Cdh5-Klf2 had significantly reduced calcium content in aortas in comparison with vector-injected mice (Figure 6D). As expected, Klf2 and its target gene Nos3 in the aorta were upregulated in mice injected with AAV-Cdh5-Klf2 compared with the control (Figure 6E). The reduced expression of osteogenic markers in aortic tissues was also detected, including Runx2, Msx2, and Osterix in *ApoE<sup>−/−</sup>* mice with KLF2 overexpression (Figure 6F). In addition, KLF2 overexpression inhibits osteogenic differentiation of ECs (Figure VI in the Data Supplement). Taken together, these results demonstrate that endothelial KLF2 overexpression ameliorates vascular calcification.
KLF2 Inhibits Calcification by Suppressing BMP Signaling

To explore the underlying mechanisms for KLF2-mediated inhibition of vascular calcification, we analyzed the mRNA profiles in HUVECs transduced with Ad-GFP (green fluorescent protein) or Ad-KLF2. The results ranked the TGF-β signaling pathway as one of the top pathways regulated by KLF2 (Figure 7A). BMPs constitute a subgroup of the TGF-β superfamily and play an important role in EndMT and vascular calcification.3,6,7 To verify whether the BMP/SMAD1/5 pathway is inhibited by KLF2, we performed quantitative polymerase chain reaction analysis, and the results showed that KLF2 overexpression downregulated the mRNA expression of BMP2, BMP4, and BMP6 (Figure 7B; Figure VIIIIA in the Data Supplement). The expression of BMPER, an endothelial BMP antagonist that protects against atherosclerotic lesion formation and calcification,18 was upregulated by KLF2 overexpression (Figure 7B; Figure VIII in the Data Supplement). Conversely, KLF2 knockdown increased expression of BMP2/4 (HUVECs, Figure VIIIIB in the Data Supplement) and BMP4/6 (HAECs, Figure 7C) but decreased BMPER expression. To test the link between the BMP/SMAD1/5 signaling and shear stress, we firstly used a quantitative polymerase chain reaction array to detect the expression profile of BMP ligands and antagonists in HUVECs maintained at static or exposed to LSS conditions (Figure 7D). LSS decreased the expression of BMP4 but not BMP2 or BMP6 (Figure 7D). Except for BMPER which is highly upregulated, other expression of other BMP antagonists was statistically not different or reduced in HUVECs exposed to LSS (Figure 7D). We next, examined the dynamic regulation of the BMP/SMAD1/5 pathway by different flow patterns, LSS at 12 dyn/cm² or OSS at 0.5±4 dyn/cm², respectively, applied...
to HUVECs or HAECs for 24 hours and found that both BMP4 and BMPER are mechanosensitive in ECs (Figure 7E and 7F).

Interestingly, BMP4 and BMPER are mainly expressed in the endothelium of the mouse aortic wall (Figure II in the Data Supplement). Consistent with in vitro results, AAV-Cdh5-Klf2 administration reduced the BMP4 expression but elevated the BMPER level in the mouse aortas with atherosclerotic calcification (Figure 7G and 7H). To determine the role of KLF2 in flow-mediated regulation of BMP4 and BMPER expression, we knocked down KLF2 in LSS-treated HUVECs and HAECs and found that LSS-induced suppression of BMP/SMAD1/5 signaling was abolished (Figure 7I; Figure IX in the Data Supplement). In addition, KLF2 overexpression reversed the OSS-induced activation of BMP/SMAD1/5 signaling (Figure 7J; Figure XB through XD in the Data Supplement). To investigate the mechanisms of KLF2-mediated regulation of BMP4 and BMPER expression, we performed chromatin immunoprecipitation to determine whether KLF2 binds to the promoters of BMP4 and BMPER. KLF2 overexpression in HVUECs indeed enhanced the enrichment of KLF2 binding to the promoters of BMP4 (−1025 to −918 bp and −465 to −355 bp upstream of transcription start sites) and BMPER (−636 to −400 bp and −387 to −269 bp upstream of transcription start sites) compared with mock control cells (Figure 7K and 7L). These results reveal that KLF2 can directly regulate BMP4 and BMPER expression through a transcriptional mechanism.

**SMAD1 Mediates KLF2 Silencing-Induced Osteogenic Differentiation of HAECs and HUVECs**

SMAD1/5 are the core transcriptional regulators of the BMP pathway. We found that the phosphorylation level of SMAD1/5 in the calcified arteries was significantly elevated (Figure XA in the Data Supplement).
RNA-sequencing, quantitative polymerase chain reaction and Western blotting results all show that KLF2 overexpression in HUVECs downregulated the expression of SMAD1 but not SMAD5 or SMAD4 (a common SMAD complexed with the phosphorylated SMAD1/5; Figures XIA and XIC and XIVA in the Data Supplement). The inhibited expression of SMAD1 by KLF2 is also observed in KLF2-overexpressed HAECs (Figure 8A and 8B). OSS-induced upregulation of SMAD1 level was reversed by KLF2 overexpression (Figure XIVB in the Data Supplement). The chromatin immunoprecipitation experiments show that the binding of KLF2 to the promoter of SMAD1 was increased in KLF2 overexpressed HUVECs (Figure 8C). Confocal imaging of SMAD1 reveals that KLF2 decreased the nuclear content of SMAD1 (Figure XIIA in the Data Supplement). Cytoplasmic/nuclear fractionation experiments further show that both nuclear SMAD1 and chromatin-bound SMAD1 were reduced in KLF2 overexpressed HUVECs (Figure XIIB in the Data Supplement). The enrichment of KLF2 binding to the predicted KLF2 binding sites in the promoter regions of BMP4 (K) and BMPER (L) was quantified by quantitative polymerase chain reaction (qPCR) with primers listed in Table V in the Data Supplement. n=3–4. *P<0.05.

Statistical analysis was performed using the Mann-Whitney U test in C, E, F, K, L and an unpaired 2-tailed Student t test in B, G, and H.

**Figure 7.** KLF2 (Krüppel-like factor 2) inhibits the activation of BMP (bone morphogenetic proteins)/SMAD1/5 signaling by regulating BMP4 and BMPER.

A, KEGG (the Kyoto Encyclopedia of Genes and Genomes) enrichment pathway analysis for mRNA profile in human umbilical vein endothelial cells (HUVECs) with KLF2 overexpression. B, KLF2 overexpression downregulates BMP2/4/6 and upregulates BMPER expression in HAEc. n=6. C, KLF2 knockdown increases BMP4/6 and decreases BMP2 and BMPER level in HAECs. n=5–6. D, Heatmap showing the gene expression of BMP ligands and antagonists in HUVECs exposed to laminar shear stress (LSS) for 24 h. Oscillatory shear stress (OSS) upregulates BMP4 (E) expression but downregulates BMPER (F) expression vs LSS in HUVECs (n=4) and HAECs (n=3–5). G–H, Relative mRNA expression of Bmp4 and Bmper in aortas of Apoe−/− mice injected with AAV-Cdh5-Vector or AAV-Cdh5-Klf2 and fed a western diet for 16 wk. n=8. I, Western blotting analysis showing that laminar flow (LF)-induced expression of BMPER was abolished by KLF2 silencing. KLF2 mediated the regulation of OSS on BMP4, BMPER and pSMAD1/5 in HAECs. J, HAECs were transduced with Ad-GFP (green fluorescent protein) and Ad-KLF2 for 24 h followed by exposure to OSS for 16 h. Western blotting analysis showing that KLF2 overexpression abolishes OSS-induced activation of BMP/SMAD1/5 signaling. K–L, Chromatin immunoprecipitation (ChIP) assays were performed by using anti-KLF2 or rabbit IgG as an isotype control in HUVECs overexpressed with KLF2. The enrichment of KLF2 binding to the predicted KLF2 binding sites in the promoter regions of BMP4 (K) and BMPER (L) was quantified by quantitative polymerase chain reaction (qPCR) with primers listed in Table V in the Data Supplement. n=3–4. *P<0.05.
Importantly, the p-SMAD1/5 level was induced by OM treatment (Figure 8F) in both HUVECs and HAECs, suggesting that KLF2 silencing is likely to create a cellular condition similar to OM treatment. To determine the role of SMAD1 in mediating the expression of osteogenic genes, SMAD1 was silenced (Figure XIIIA and XIIIB in the Data Supplement) in HUVECs and HAECs with KLF2 knockdown, we found SMAD1 silencing reversed KLF2 silencing-induced expression of osteogenic markers (Figure 8G through 8I), indicating that SMAD1 mediates KLF2 deficiency-induced osteogenic differentiation of ECs.

**DISCUSSION**

Previous reports showed that aortic valve calcification occurs in a side-specific manner. The fibrosa side where the flow pattern is mainly disturbed is preferentially calcified.19–21 However, whether DF plays a role in the vascular calcification has not been defined. Here, we present clinical evidence that DF is positively associated with vascular calcification in human coronary arteries. We also show that vascular calcification preferentially develops at the curvature or the branching points of the artery in both atherosclerotic calcification in ApoE−/− mice and high-dosage of vitamin D3-induced medial calcification in C57 mice. Flow experiments in a Y-shaped chamber slide simulating the flow patterns at arterial branches show that mineralization is mainly detected at the branching points, suggesting that low and nonuniform shear stress promotes the osteogenic differentiation of ECs. Our study thus defines a causal role of flow disturbance in the development of vascular calcification.

KLF2 is a flow-sensitive transcription factor mainly expressed in ECs of the vascular wall. The present study demonstrates that endothelial KLF2 suppresses vascular calcification. Several lines of experimental data substantiate our conclusions: (1) KLF2 silencing promoted EndMT and accelerated osteoinduction, (2) endothelial-specific...
KLF2 knockdown increased while EC-specific KLF2 overexpression ameliorated vascular calcification in \textit{ApoE}{\textasciitilde}/\textit{−/−} mice, and (3) KLF2 level in ECs was reduced in the calcified area of the human aortic valves compared with the noncalcified region. In KLF2 overexpressed \textit{ApoE}{\textasciitilde}/\textit{−/−} mice, the body weight, plasma levels of calcium, phosphate, triglycerides, and HDL (high-density lipoprotein) cholesterol were unaffected by transfection of AAV-\textit{Cdh5-Klf2} (Table III in the Data Supplement). However, the plasma levels of LDL (low-density lipoprotein) cholesterol and total cholesterol were slightly but significantly reduced (Table III in the Data Supplement). Since ECs resident in the liver are not few, AAV-\textit{Cdh5-Klf2}-mediated increase of KLF2 level in liver ECs may affect cholesterol metabolism. Therefore, it cannot be ruled out that the reduction of plasma LDL cholesterol level may also contribute albeit to a lesser degree to the overall inhibitory effect of KLF2 overexpression on vascular calcification. Nevertheless, the present results demonstrate that KLF2 overexpression is effective to suppress vascular calcification, and endothelial KLF2 could be a useful therapeutic target against cardiovascular calcification.

The BMP/SMAD1/5 signaling plays a critical role in EndMT and vascular calcification.\textsuperscript{3,6,22} BMPs are potent osteogenic differentiation factors originally identified in the bone tissue. The expression of BMPs in the calcified vasculature is upregulated, and BMP4 treatment in ECs induces EndMT and acquisition of a stem cell-like phenotype.\textsuperscript{17} Loss of MGP, a BMP inhibitor (Matrix Gla protein) in ECs leads to EndMT and significantly elevates phosphorylation of SMAD1/5.\textsuperscript{17} In addition, knockout of MGP in mice facilitates the transdifferentiation of ECs into osteoprogenitor cells to contribute to vascular calcification.\textsuperscript{3,6} In the present study, we found that BMPER but not MGP is flow-sensitive (Figure VII in the Data Supplement). BMPER is an endothelial BMP modulator known to function as an inhibitor of vascular inflammation by modulating leukocyte adhesion and migration.\textsuperscript{23} \textit{ApoE}{\textasciitilde}/\textit{−/−} mice lacking one allele of \textit{Bmpr1a} (\textit{Bmpr1a}{\textasciitilde}/\textit{−/−}) exhibit more severe atherosclerotic lesion formation and calcification compared with \textit{Bmpr1a}{\textasciitilde}/\textit{ApoE}{\textasciitilde}/\textit{−/−} mice, suggesting that BMPER is an endogenous inhibitor of vascular calcification.\textsuperscript{18} In addition, prolonged treatment of BMP small molecule inhibitor LDN-193189 in \textit{Ldr}{\textasciitilde}/\textit{−/−} mice prevents against arterial calcification,\textsuperscript{7} suggesting that BMP inhibition could become a promising therapy for vascular calcification.

Consistent with the previous report,\textsuperscript{24,26} our time-course study shows that LSS inhibits the BMP4 expression whereas OSS promotes it. BMP4 produced in ECs by OSS simulates ICAM (intercellular adhesion molecule)-1 expression, monocyte adhesion, and subsequent atherosclerotic plaque development.\textsuperscript{24,26} Altered shear stress also induces the upregulation of BMP4 in porcine aortic valve leaflets, resulting in endothelial expression of VCAM (vascular cell adhesion molecule)-1 and ICAM-1.\textsuperscript{19} By contrast, BMP inhibitors including noggin, follistatin, and BMPER inhibit OSS-induced monocyte adhesion and inflammatory response.\textsuperscript{19,26} ECs have been shown to coexpress BMP antagonists (ie, MGP, follistatin, and noggin) along with BMP4 to minimize the inflammation in response to OSS, acting as a negative feedback mechanism.\textsuperscript{26} However, the present study identified that BMPER was downregulated by OSS, suggesting the complex regulatory mechanism of BMP4 and BMP antagonists in ECs exposed to OSS condition. In contrast to LSS, OSS leads to a sustained activation of SMAD1/5 in ECs.\textsuperscript{26} Consistent with our observation that p-SMAD1/5 level is significantly upregulated in calcified mouse arteries, a previous study reported that endothelial SMAD1/5 is highly activated in plaques of human coronary arteries.\textsuperscript{27} It was also reported that p-SMAD1/5 level increases in vasa vasora ECs at early stages of atherosclerosis and remains sustained in advanced lesions.\textsuperscript{26} Given the importance of the BMP/SMAD1/5 signaling in vascular calcification, its suppression by LSS through KLF2 is most likely to account for the inhibitory effect of LSS on vascular calcification.

Our mechanistic study reveals that KLF2 inhibits the SMAD1 expression through a transcriptional regulatory mechanism. However, the present results cannot exclude the possibility that KLF2 can affect SMAD1/5 protein stability. An elevation of SMAD5 protein but not mRNA expression in \textit{LSS}{\textasciitilde}/\textit{−/−} mice lacking \textit{Bmpr1a} suggests that increased SMAD5 protein level under this scenario is likely due to increased level of SMAD1, which can be targeted by SMAD ubiquitination-related factors (Smurf) for proteasome-mediated degradation.\textsuperscript{29} Smurf1 was known to inhibit osteoblast differentiation and bone formation through inactivation of RUNX2.\textsuperscript{30} Future studies on how KLF2 may regulate the activity of Smurf1/2 shall shed some light on the regulation of SMAD1/5 protein stability in calcified arteries, although it is currently beyond the scope of this study.

To highlight the translational significance and clinical relevance of our findings, we found that simvastatin inhibits the BMP/SMAD1/5 pathway through upregulating KLF2 expression in HUVECs (Figure X in the Data Supplement). Currently, the therapeutic effect of statins on vascular calcification is still disputed. Puri et al\textsuperscript{31} reported that statins promote coronary atheroma calcification, while other studies showed that statins inhibit cardiovascular calcification by reducing inflammation and inhibiting osteogenic pathways in myofibroblasts.\textsuperscript{32,33} In the present study, we found that simvastatin reverses OM-induced suppression of KLF2, and inhibits \textit{RUNX2} expression and osteinduction in HUVECs cultured in OM (Figure XV in the Data Supplement), indicating statins treatment in vivo may have suppressive effects on EndMT and osteogenic differentiation of ECs. Vascular calcification is associated with atherosclerotic plaque stability and can be classified as microcalcification (<50 μm in diameter) and macrocalcification (>50 μm in diameter).
Most imaging techniques used in the clinical studies of calcification generally have a lower resolution threshold of 30 to 50 µm, limiting their ability to identify high-risk microcalcifications.\(^\text{34}\) While it remains unclear whether calcification promotes plaque stability or instability, the ability to control calcification is highly likely to be clinically important. Whether statins have an inhibitory effect on the initiation of microcalcification in patients is still unknown. In the future study, it is worthwhile determining whether statins treatment inhibits cardiovascular calcification in vivo and whether this effect is KLF2 dependent in KLF2 deficient mice.

In contrast to the present finding that KLF2 inhibits osteogenic differentiation of ECs, KLF2 in osteoblast is found to promote osteoblast differentiation via its interaction with RUNX2.\(^\text{25}\) KLF2 is a transcription factor that has both the transactivation and transrepression the expression of downstream genes through binding to different cofactors.\(^\text{36,37}\) In the study by Hou et al.,\(^\text{36}\) they identified osteoblastic KLF2 was found to physically interacts with RUNX2 to increase its expression and transactivacy, thereby promoting the osteogenic differentiation of osteoblasts. However, in normal ECs, the expression of RUNX2 is very low. We, therefore, speculate that it would be very difficult for KLF2 to form a complex with RUNX2 in normal ECs. Although there is also a such interaction exists during the osteogenic differentiation process of ECs, the pathophysiological significance of such interaction is still unknown. It is likely that KLF2 is a negative regulator of RUNX2 protein stability during osteogenic differentiation of ECs. However, more experiments are needed to address this possibility.

Several limitations should not be neglected when interpreting the in vivo data in this study. First, although our data confirm that the local hemodynamic environment and calcific lesions on the aortic side of the valve are closely correlated, the relative contribution of DF to the development of calcification on aortic side of the valve is not determined. Moreover, other anatomic and developmental factors that also contribute to valve calcification remain to be determined. Second, inflammatory and senescent ECs secrete macrovesicles containing BMP2 and calcium that can promote calcification in vascular smooth muscle cells.\(^\text{38,39}\) Vascular smooth muscle cell-specific BMP2 overexpression promotes vascular calcification in ApoE\(^\text{−/−}\) mice.\(^\text{2}\) The inhibition of BMP4 expression subsequent to KLF2 overexpression might not only inhibit EndMT and EC-derived calcification but may also suppress chondrocyte and osteoblasts differentiation of smooth muscle cells. More detailed mechanistic study is required in future study.

In conclusion, the present study in both in vitro and in vivo as well as in patients uncovers that nonuniform shear stress and DF promote vascular calcification. KLF2 inhibits the BMP/SMAD1/5 signaling to mediate the protective role of LSS against vascular calcification.

**REFERENCES**

KLF2 Inhibits Calcification by Suppressing BMP Signaling

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