BMB
Reports

BMB Rep. 2023; 56(3): 160-165
www.bmbreports.org

Inhibition of the Semaphorin 4D-Plexin-B1 axis prevents calcification in vascular smooth muscle cells

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Vascular calcification is common in cardiovascular diseases including atherosclerosis, and is associated with an increased risk of pathological events and mortality. Some semaphorin family members play an important role in atherosclerosis. In the present study, we show that Semaphorin 4D/Sema4D and its Plexin-B1 receptor were significantly upregulated in calcified aorta of a rat chronic kidney disease model. Significantly higher Sema4D and Plexin-B1 expression was also observed during inorganic phosphate-induced calcification of vascular smooth muscle cells. Knockdown of Sema4D or Plexin-B1 genes attenuated both the phosphate-induced osteogenic phenotype of vascular smooth muscle cells, through regulation of SMAD1/5 signaling, as well as apoptosis of vascular smooth muscle cells, through modulation of the Gas6/Axl/Akt survival pathway. Taken together, our results offer new insights on the role of Sema4D and Plexin-B1 as potential therapeutic targets against vascular calcification. [BMB Reports 2023; 56(3): 160-165]

INTRODUCTION

Vascular calcification, defined as the pathological deposition of calcium phosphate in the vasculature, is typically observed in elderly individuals, as well as in patients with atherosclerosis, chronic renal failure, and diabetes mellitus (1). Importantly, it is associated with an increased risk of cardiovascular disease and mortality (1). Vascular smooth muscle cells (VSMCs) play a pivotal role in the intervening calcification of blood vessels owing to their transdifferentiation into osteoblast-like cells or chondrocyte-like cells (2). This process is altered by disturbed mineral homeostasis, inflammation, oxidative stress, and an imbalance between promoters and inhibitors (3). Most dangerously, the calcification of VSMCs augments the risk of plaque instability and rupture in atherosclerosis (4).

Semaphorins belong to a large family of membrane-bound and secreted proteins originally identified in the nervous system as mediators of axonal guidance (5). Semaphorin 4D (Sema4D) was initially identified in immune cells as the CD100 antigen and binds to two types of receptors, the high-affinity Plexin-B1 receptor and low-affinity CD72 C-type lectin receptor (6). Sema4D-Plexin-B1 is a multifunctional ligand-cell surface receptor structure found in several tissues, including neurons, lymphocytes, vascular endothelial cells, and tumor cells, with a role played in different physiological and pathological situations (7). Sema4D is the first documented member of the semaphorin family to be involved in the development of atherosclerosis (8). This phenomenon has been proved based on the previous observations that Sema4D and its receptor signaling promotes monocyte adhesion to the vascular endothelium, intraplaque angiogenesis, platelet activation, and thrombus formation (9-12).

In the present study, we investigated the role of Sema4D in phosphate-induced VSMC calcification and the calcified aorta of a chronic kidney disease (CKD) animal model, and its underlying mechanism in VSMCs.

RESULTS

Sema4D and Plexin-B1 expression increases during aortic calcification in a CKD model

To elucidate the role of Sema4D in vascular calcification *in vivo*, we used an adenine diet-induced CKD vascular calcification rat model (13). Blood biochemical parameters of CKD rats are summarized in Supplementary Table 1. Histological assessment by hematoxylin and eosin staining revealed a clear and neat arrangement of VSMCs in sham animals, but a disordered smooth muscle arrangement in the aortic tissue of CKD rats (Fig. 1A). Von Kossa and alizarin red S (ARS) staining disclosed an extensive medial calcification in the aortas derived from CKD rats compared to those from sham animals

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https://doi.org/10.5483/BMBRep.2022-0165

Received 20 October 2022, Revised 1 November 2022, Accepted 26 November 2022, Published online 1 February 2023

Keywords: Plexin-B1, Semaphorin 4D, Vascular calcification, Vascular smooth muscle cells

ISSN: 1976-670X (electronic edition)

(Fig. 1A). This was confirmed by a significantly greater calcified area relative to the that observed in the aortas of CKD rats (Fig. 1B). Next, we investigated the levels of Sema4D and Plexin-B1 in calcified aortic tissues from CKD rats. Immunohistochemistry showed significantly higher Sema4D and Plexin-B1 expression in aortic tissues of CKD rats than in those of sham animals (Fig. 1C), which was confirmed by western blotting (Fig. 1D). At the same time, serum analysis using an enzyme-

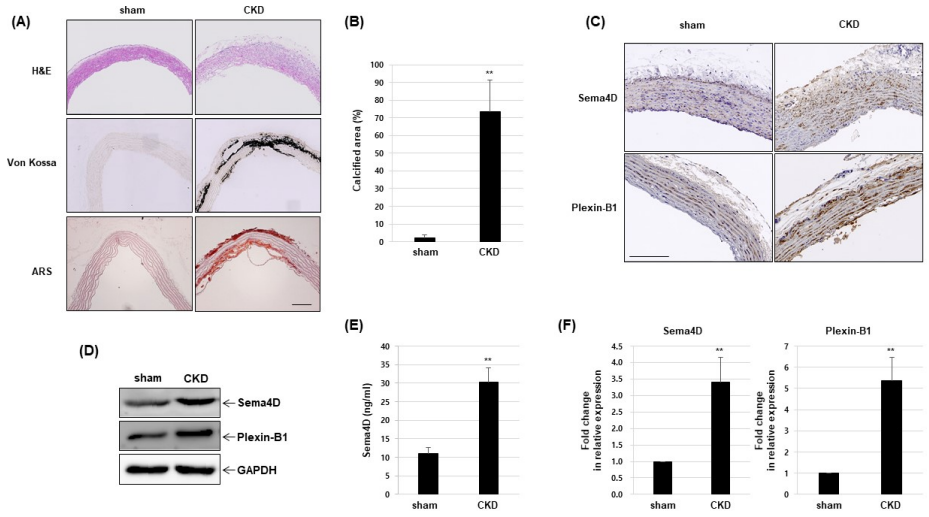

Fig. 1. Sema4D and Plexin-B1 expression in calcified aorta of CKD rats. (A) Representative photomicrographs of the abdominal aortas stained by hematoxylin and eosin, von Kossa, and ARS. Scale bar: 50 μm. (B) Percentage of calcified area was calculated as the ratio of von Kossa-positive area versus total tissue area. **P < 0.01 vs. sham. (C) Representative immunohistochemistry photomicrographs showing Sema4D and Plexin-B1 localization. (D) Sema4D, Plexin-B1, and β-actin levels examined by western blotting. (E) ELISA measurements revealing Sema4D serum levels. **P < 0.01 vs. sham. (F) Quantitative real-time PCR results revealing Sema4D and Plexin-B1 mRNA levels. **P < 0.01 vs. sham.

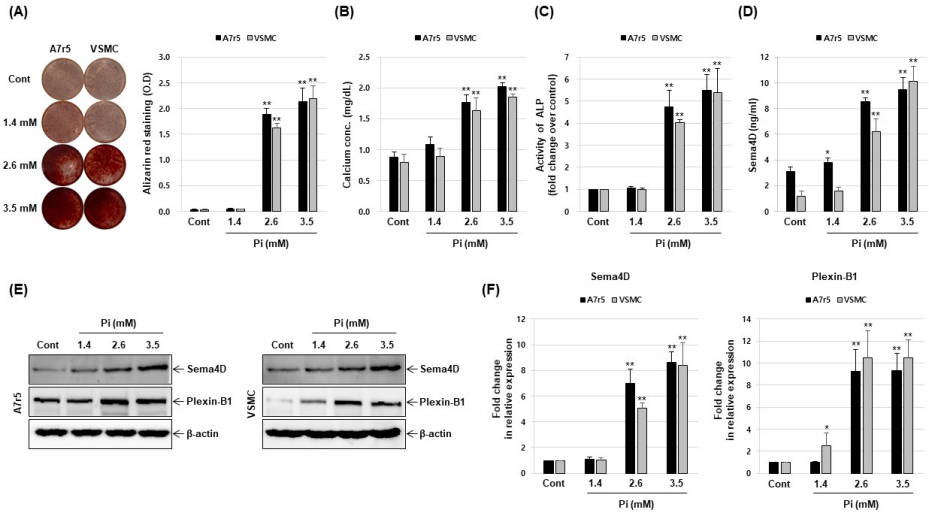

Fig. 2. Sema4D and Plexin-B1 expression during VSMC calcification. Confluent A7r5 and primary VSMCs were exposed to 1.4, 2.6, and 3.5 mM Pi for 7 days with medium change every 2 or 3 days. (A) Absorbance measurements showing the degree of mineralization in calcium deposits within VSMCs stained by ARS. **P < 0.01 vs. Cont. (B) Calcium content was measured by colorimetric calcium assay. **P < 0.01 vs. Cont. (C) Alkaline phosphatase activity was measured and normalized to protein content for quantitative analysis. **P < 0.01 vs. Cont. (D) ELISA results showing Sema4D levels in cell culture medium. *P < 0.05, **P < 0.01 vs. Cont. (E) Sema4D, Plexin-B1, and β-actin levels examined by western blotting. (F) Quantitative real-time PCR revealing Sema4D and Plexin-B1 mRNA levels. *P < 0.05, **P < 0.01 vs. Cont.

linked immunosorbent assay (ELISA) showed that the secreted Sema4D protein were also elevated in the CKD rats compared with the sham rats (Fig. 1E). Finally, quantitative real-time polymerase chain reaction (PCR) demonstrated a prominent increase in the Sema4D and Plexin-B1 mRNA expression in the thoracic aortas from CKD rats (Fig. 1F).

Elevated expression of Sema4D and Plexin-B1 in phosphate-induced VSMC calcification *in vitro*

Inorganic phosphate (Pi) stimulates the calcification of VSMCs *in vitro* (14). Thus, we analyzed whether elevated Pi promoted VSMC calcification under our established experimental conditions. As expected, addition of 2.6 and 3.5 mM Pi caused calcium deposition (Fig. 2A); whereas 1.4 mM Pi, equivalent to the human physiological serum phosphate level, failed to induce calcification. Calcium content and alkaline phosphatase (ALP) activity, a molecular marker of vascular calcification, in VSMCs were also markedly elevated under calcifying conditions compared to normal conditions (Fig. 2B, C). Next, we estimated the levels of soluble Sema4D protein in VSMC calcification stimulated by Pi. Estimation of soluble Sema4D revealed a gradual increase in the amount of secreted Sema4D with the severity of Pi-induced VSMC calcification (Fig. 2D). Western blotting and quantitative real-time PCR confirmed the elevated protein (Fig. 2E) and mRNA (Fig. 2F) expression of Sema4D and Plexin-B1 in Pi-induced VSMC calcification.

Sema4D and Plexin-B1 silencing attenuates osteogenic differentiation during Pi-induced vascular calcification

To assess whether inhibition of Sema4D and Plexin-B1 alleviated Pi-induced VMSC calcification, we silenced the Sema4D and Plexin-B1 expressions with small interfering RNA (siRNA). The siRNA knockdown efficiency was confirmed by a decrease in Sema4D and Plexin-B1 protein and mRNA levels (Supplementary Fig. 1). Importantly, their silencing reduced the calcium deposition significantly even under calcifying conditions (Fig. 3A), as well as calcium content in VSMCs (Fig. 3B). Similarly, the increased activity of ALP was markedly inhibited by the Sema4D and Plexin-B1 knockdown (Fig. 3C). Next, we evaluated the effect of targeted silencing on the expression of contractile and osteogenic markers. As shown in Fig. 3D, E and Supplementary Fig. 2, the increased protein expression of Runx2, a master transcription factor of osteoblast differentiation, was suppressed by Sema4D and Plexin-B1 siRNA; whereas the decreased level of calponin, a marker of the contractile phenotype, was completely reversed. In addition, Sema4D and Plexin-B1 silencing upregulated the calponin mRNA and downregulated the Runx2 mRNA expressions in VSMCs subjected to Pi-induced osteogenic differentiation (Fig. 3F). Previous reports along with ours have reported that the phosphorylation of SMAD1/5 activates osteogenic gene markers such as Runx2 during osteogenic differentiation of VMSCs (15, 16). Accordingly, we investigated whether the knockdown of Sema4D and Plexin-B1 altered the phosphorylation of SMAD1/5 in calcified VSMCs. Indeed, silencing of these two proteins blocked the increased

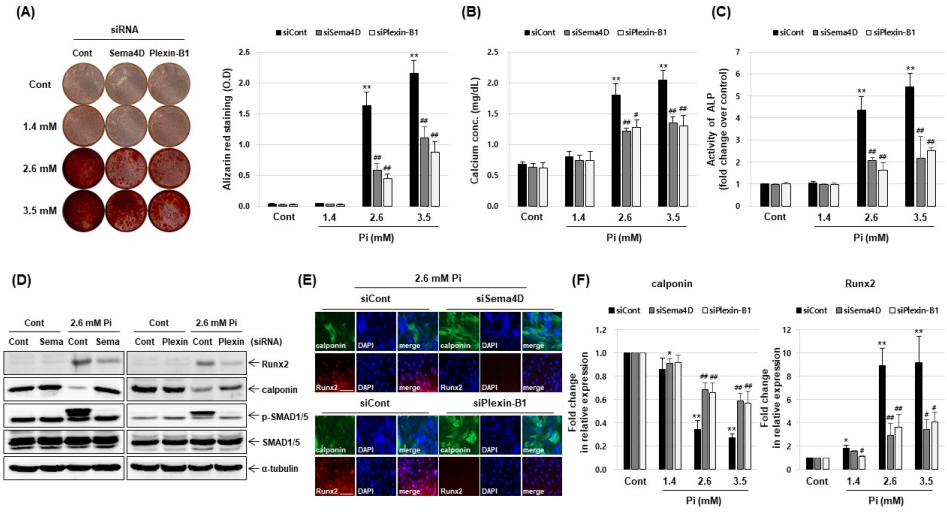

Fig. 3. Effect of Sema4D and Plexin-B1 silencing in phosphate-induced osteogenic differentiation of VSMCs. A7r5 cells were transfected with Sema4D siRNA, Plexin-B1 siRNA or negative control siRNA for 24 h, and then cultured in calcification medium (2.6 mM Pi). After 5 days, VSMC calcification was determined by ARS staining (A), calcium content assay (B), and ALP activity assay (C). $^{**}P < 0.01$ vs. Cont; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. siCont. (D) Runx2, calponin, phospho-SMAD1/5, SMAD1/5, and α-tubulin levels examined by western blotting. (E) Immunocytochemistry photomicrographs showing the expression of Runx2 (red) and calponin (green). DAPI staining (blue) denotes nuclear DNA. (F) Quantitative real-time PCR results revealing Runx2 and calponin mRNA levels. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Cont; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. siCont.

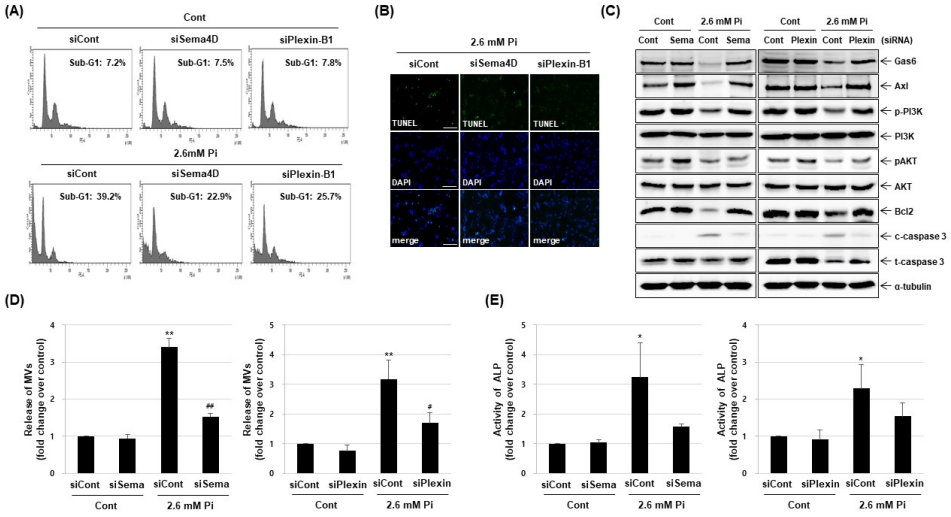

Fig. 4. Effect of Sema4D and Plexin-B1 knockdown on apoptosis and matrix vesicle release in calcified VSMCs. A7r5 cells were transfected with Sema4D siRNA, Plexin-B1 siRNA or negative control siRNA for 24 h, and then cultured in calcification medium (2.6 mM Pi) for 5 days. (A) Flow cytometry results identifying propidium iodide-positive cells undergoing apoptosis. (B) Representative photographs showing TUNEL-positive apoptotic nuclei (green). DAPI staining (blue) denotes nuclear DNA. Scale bar: 50 μm. (C) Changes in Gas6, Axl, total-/phospho-PI3K, total-/phospho-AKT, Bcl2, and total/cleaved caspase-3 levels as determined by western blotting. (D, E) Matrix vesicles were isolated by collagenase digestion, after which ALP activity was measured and normalized to vesicle total protein content. *P $<$ 0.05, **P $<$ 0.01 vs. Cont; $^{\#}$P $<$ 0.05, $^{\#\#}$P $<$ 0.01 vs. siCont.

expression of phospho-SMAD1/5 (Fig. 3D).

Sema4D and Plexin-B1 knockdown suppresses VSMC apoptosis and matrix vesicle release during vascular calcification

Given that apoptosis plays a pivotal role in the initiation of vascular calcification (17), we assessed the extent of apoptosis in VSMCs using propidium iodide staining and the TUNEL assay. Compared with non-transfected cells, Sema4D and Plexin-B1 knockdown significantly inhibited Pi-induced apoptosis in VSMCs, with the proportion of apoptotic cells decreasing by 16.3% or 13.5%, respectively (Fig. 4A). Likewise, the proportion of TUNEL-positive apoptotic cells was significantly reduced when calcified VSMCs were treated with Sema4D and Plexin-B1 siRNA (Fig. 4B and Supplementary Fig. 3). Next, we checked the effect of Sema4D and Plexin-B1 knockdown on the expression of pro-apoptotic and anti-apoptotic markers in calcified VSMCs. Sema4D and Plexin-B1 knockdown blocked the increase in cleaved caspase-3 induced by 2.6 mM Pi (Fig. 4C), while significantly stimulating Bcl2 in calcified VSMCs (Fig. 4C). Pi is known to downregulate the Gas6/Axl/PI3K/Akt survival pathway, leading to VSMC apoptosis and calcification (18). We found that the exposure to 2.6 mM Pi markedly downregulated the expression of Gas6 and Axl; however, this effect was abolished by Sema4D and Plexin-B1 silencing (Fig. 4C). Because the Gas6/Axl survival pathway is PI3K/Akt-dependent (19), we examined the effect of Sema4D and Plexin-B1 knockdown on the PI3K/Akt pathway in calcified VSMCs. As expected, silencing of Sema4D and Plexin-B1 blocked the Pi-induced dephosphorylation of PI3K and Akt (Fig. 4C). VSMC apoptosis favors the calcification of VSMCs by promoting the release of calcifying membrane-bound matrix vesicles (20). Therefore, to confirm the inhibitory action of Sema4D and Plexin-B1 silencing on the release of these vesicles, the matrix vesicles were isolated from calcified VSMC lysates by collagenase digestion. The release of matrix vesicles was enhanced in the presence of 2.6 mM Pi, but was significantly reversed by Sema4D and Plexin-B1 silencing (Fig. 4D). Finally, Sema4D and Plexin-B1 knockdown successfully counteracted the increased ALP activity observed within matrix vesicles released from calcified VSMCs (Fig. 4E).

DISCUSSION

Vascular calcification is often accompanied by loss of bone mineral density in patients with CKD (21). This apparent calcification paradox was first documented by the frequent association between atherosclerotic vascular calcification and osteoporosis in postmenopausal women (22). However, the underlying mechanism and common regulators of the bone-vascular axis have not yet been established. Sema4D, expressed in osteoclasts during osteoclastogenesis, inhibits the bone formation by interacting with Plexin-B1, which is expressed by osteoblasts (23). Not surprisingly, serum Sema4D levels correlate with the changes in osteoporosis in postmenopausal women (24). In this study, Sema4D was shown to be crucial for the

regulation of phosphate-induced calcification in VSMCs and calcium deposition in the aortas. Inflammation leads to a common microenvironment for vascular mineralization and bone demineralization (25). Inflammatory cytokines trigger vascular calcification through ossification of VSMCs and endothelial-mesenchymal transition, as well as bone loss through increased bone resorption and decreased bone formation (26). In addition, we confirmed the increased levels of pro-inflammatory genes, including the interleukins IL-6 and IL-8, monocyte chemoattractant protein-1, and tumor necrosis factor-α, in calcified Pi-induced VSMCs. Soluble Sema4D levels in serum and body fluids have shown a positive correlation with the disease severity in chronic inflammatory disorders such as rheumatoid arthritis (27). The findings shown in the present study, together with previous observations, suggest that Sema4D may act as a common regulator linking bone disorders and vascular calcification.

Emerging evidence suggests that Sema4D-Plexin-B1 signaling is involved in the endothelial dysfunction of the vasculature. Sema4D regulates pathological retinal neovascularization and endothelial permeability via Plexin-B1 (28). Sema4D expressed in endothelial cells mediated monocytes adhesion to the endothelial cells, a critical step in vascular inflammation, by interaction with Plexin-B1 (10). Additionally, Sema4D controls migration, as well as pro-angiogenic and pro-inflammatory responses in the endothelium (9, 29). In comparison, little attention has been paid to the potential role of Sema4D-Plexin-B1 in the vascular dysfunction of VSMCs, the predominant vascular cell type. The present results provide first-time evidence that Sema4D-Plexin-B1 signaling is crucial for the regulation of mineralization and apoptosis in VSMCs during vascular calcification. Growing evidence suggests that communication between vascular endothelial cells and VSMCs is important for osteogenic differentiation of VSMCs during vascular calcification. Receptor activator of nuclear factor kappa beta-ligand stimulates osteoblastic activity of VSMCs by inducing the release of BMP-2 from endothelial cells in a paracrine manner (30). Conditioned medium from endothelial cells or co-culture with endothelial cells reinforces the calcification of VSMCs through the upregulation of IL-8 or matrix metalloproteinases (31, 32). Future studies will determine whether Sema4D expressed in endothelial cells mediates the osteogenic differentiation of VSMCs when coupled to Plexin-B1.

Hyperphosphatemia, a key risk factor associated with atherosclerotic calcification in CKD, leads to excessive reactive oxygen species (ROS) production in patients and CKD animal models (33). Increased ROS generation triggers the cellular activation of osteo/chondrogenic signaling pathways, which are implicated in osteogenic conversion of VSMCs (34). Proper modulation of the redox status through inhibition or elimination of ROS production represents a potential therapeutic strategy in the prevention of VSMC calcification and the associated risk of cardiovascular disease (35). In this study, we observed that silencing of Sema4D or Plexin-B1 decreased the Pi-induced ROS generation in VSMCs (data not shown). Therefore, it is possible that blocking of the Sema4D-Plexin-B1 axis could protect against ROS-mediated vascular calcification and these possibilities are under investigation.

In conclusion, the present study identified a novel function for the Sema4D-Plexin-B1 axis in vascular calcification. Moreover, we demonstrate that the inhibition of Sema4D and Plexin-B1 attenuates the osteoblastic differentiation in VSMCs through SMAD1/5 signaling, as well as VSMC apoptosis through Gas6/Axl/Akt signaling. These findings provide new evidence supporting the use of Sema4D-Plexin-B1 as a therapeutic target of vascular calcification in atherosclerosis or CKD.

MATERIALS AND METHODS

Further detailed information is provided in the Supplementary Information.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Research Foundation of Korea funded by the Korean government (MSIT, NRF-2018R1A5A2023879 and 2021R1A2C1003687).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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