**BMP9 induces the calcification of vascular smooth muscle cells**

**Introduction:**
Arterial Medial Calcification (AMC) is highly correlated with elevated serum phosphate levels and cardiovascular mortality in patients with End Stage Renal Disease (ESRD). The process of AMC shares many similarities with that of physiological skeletal mineralization, and involves the deposition of hydroxyapatite crystals in arteries. However, the cellular mechanisms responsible have yet to be fully elucidated. BMP9 has been shown to exert direct effects on both bone development and vascular function. In the present study we have investigated the role of BMP9 in vascular smooth muscle cell (VSMC) calcification.

**Methods:**
Murine VSMCs were cultured in calcifying medium containing 3mM NaHPO4 and 144g CaCl2. Calcium deposition was confirmed by alizarin red staining and quantified by HCL leaching.

**Results:**
VSMC calcification in vitro is associated with increased BMP9 expression.

**BMP9 directly modulates VSMC calcification:**
Studies were undertaken to investigate whether BMP9 promotes vascular calcification. A significant increase in calcium deposition was observed following BMP9 treatment at 50 ng/ml, as determined by alizarin red staining (Fig. 2A) and HCL leaching (Fig. 2B). Furthermore, a minimum concentration of 5ng/ml BMP9 treatment induced a significant increase in the mRNA expression of the osteogenic markers Runx2 (Fig. 2C), Osterix (Fig. 2D), TNAP (Fig. 2E) and PTHrP (Fig. 2F). Notably, the up-regulation of the osteocyte gene Sox5 was also induced by 5ng/ml BMP9 (Fig. 2H). Furthermore, a concomitant reduction in the mRNA expression of the mineralization inhibitor Mmp1 was observed following treatment of VSMCs with 50ng/ml BMP9 (Fig. 2G).

**BMP9 induces VSMC calcification through a TANAP-dependent mechanism:**
Further studies were undertaken to establish whether BMP9 modulates VSMC calcification through a TANAP-dependent mechanism. Our data revealed that a minimum concentration of 5ng/ml BMP9 was required to induce TNAP activity in VSMCs (Fig. 3A). Furthermore, co-treatment with the TNAP inhibitor 2,5-Dimethoxy-4-(quinolin-3-yl) benzoxazetidinone (DNB, 3μM) significantly reduced the pro-calcificatory effects of BMP9 (Fig. 3B).

**BMP9 signals through the ALK1 receptor to promote VSMC calcification:**
The profile of BMP receptors expressed in murine VSMCs was examined using RT-PCR. Strong bands were obtained using primers for ALK1, ALK2, BMPR1A, ActRIIB and ActRIIB (Fig. 4A). BMP9 preferentially binds to the type 1 BMP receptor ALK1. Therefore, we next sought to examine the effect of inhibiting ALK1 signaling on BMP9-induced VSMC calcification, using a soluble chimeric protein (ALK1-Fc). ALK1-Fc (250ng/ml) significantly inhibited BMP9-induced TNAP activity (Fig. 4B) and markedly reduced the pro-calcificatory actions of BMP9 on VSMCs (Fig. 4C).

**BMP9-mediated calcification of VSMCs is Smad4 dependent:**
Smad4 is a well-known co-receptor and binds to BMP ligands. To further investigate the role of Smad4 in VSMC calcification, we determined the effect of BMP9 on calcification in Smad4-knockdown VSMCs. Transfection of VSMCs with Smad4 siRNA resulted in an 80% reduction of Smad4 mRNA with a comparable decrease in protein expression at 48 h post-transfection, which was sustained at 72 h (Fig. 5A & B). Transfection of VSMCs with Smad4 siRNA significantly inhibited BMP9-induced TNAP activity (Fig. 5C) and markedly reduced the pro-calcificatory actions of BMP9 on VSMCs (Fig. 5D).

**Increased serum BMP1 in CKD dialysis patients:**
Serum BMP9 levels were compared in CKD patients and dialysis serum from children with CKD. Intriguingly, BMP9 was markedly elevated in serum from dialysis patients (234% increase; P<0.001; Fig. 7A). Whilst no correlation between serum BMP-9 concentration and calcium/phosphate concentration was noted, a significant correlation (Pearson correlation = 0.712, P<0.05; Fig. 7B) was observed between dialysis time and BMP9 concentration in patients receiving haemodialysis, suggesting that this highly osteogenic BMP may contribute to the accelerated calcification associated with dialysis.

**Conclusions:**
BMP9 appears to play a critical role in arterial medial calcification and may represent a novel potential therapeutic target.

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