BMP9 induces the calcification of vascular smooth muscle cells

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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 mineralisation, 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 Na_2HPO_4/NaH_2PO_4 for 14d. Calcium deposition was confirmed by alizarin red staining and quantitative HCL leaching. Tissue non-specific alkaline phosphatase (TNAP) activity was determined by measuring the cleavage of p-nitrophenyl phosphate and corrected for protein content. Semi quantitative PCR was performed to examine the expression of BMP receptors. RT-qPCR and western blot analysis assessed gene and protein expression, respectively. Fluorescent immunocytochemical staining confirmed the translocation of p-Smad1/5/8 to the nucleus. siRNA was used to knockdown Smad4 in VSMCs. Serum concentrations of BMP9 were determined by ELISA.

Results:

VSMC calcification in vitro is associated with increased Bmp9 expression

High Pi (3mM) induced increase in VSMC calcium deposition at day 7 and day 14, as revealed by alizarin red staining (Fig.1A) and HCL leaching (Fig.1B). A significant increase in mRNA expression of *Runx2* (Fig.1C) , *Bmp2* (Fig.1D) and *PiT-1* (Fig.1E) was seen by 14 d. The up-regulation of these osteogenic markers in VSMCs cultured in calcifying medium confirms the validity of this *in vitro* model to study AMC. *Bmp9* mRNA expression was also significantly increased at 14 d in VSMCs cultured in calcifying medium (Fig.1F) .

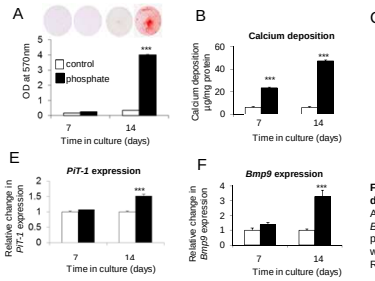

Figure 1: *In vitro* calcification of murine aortic VSMCs cultured for 14 days under calcifying conditions. Quantification of calcification by (A) Alizarin red staining and (B) HCL leaching (n=3). Fold change in (C) *Runx2*, (D) *Bmp2* (E) *PiT-1* and (F) *Bmp9* mRNA expression in VSMCs cultured with high phosphate (3mM P_i) or control (1mM P_i) medium (n=5). VSMCs were treated with control (white bar) or high phosphate containing (filled bar) medium. Results are presented as mean+/- S.E.M. ***P<0.001 compared with control.

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 *PiT-1* (Fig. 2F). Notably, the up-regulation of the osteocyte gene *Sost* was also induced by 5ng/ml BMP9 (Fig. 2H). Furthermore, a concomitant reduction in the mRNA expression of the mineralisation inhibitor *Mgp* was observed following treatment of VSMCs with 50ng/ml BMP9 (Fig. 2G).

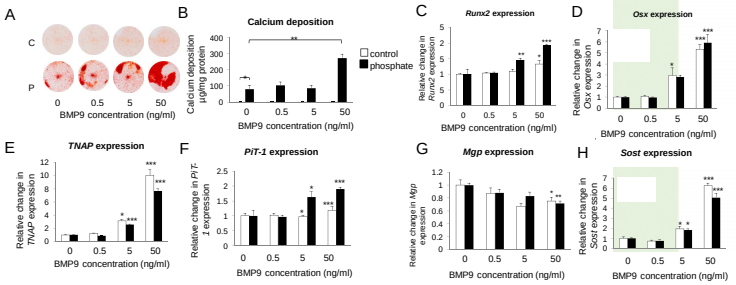

Figure 2: Effect of BMP9 treatment on the *in vitro* calcification of VSMCs. VSMCs were incubated with BMP9 (0.5-50ng/ml) in high phosphate (P) (3mM Pi) or control (C) (1mM Pi) medium for 9 days. Calcium content was (A) visualised with alizarin red staining and (B) quantified by HCL leaching (µg/mg protein) (n=3). Fold change in the mRNA expression of osteogenic/osteocyte markers (C) *Runx2*, (D) *Osx*, (E) *TNAP*, (F) *PiT-1*, (G) *Mgp* and (H) *Sost* (n=4). Results are presented as mean +/- S.E.M. *P<0.05; **P<0.01; ***P<0.001 compared with corresponding 0ng/ml BMP9 treatment.

BMP9 induces VSMC calcification through an TNAP-dependent mechanism

Further studies were undertaken to establish whether BMP9 modulates VSMC calcification through an TNAP-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-N-(quinolin-3-yl) benzenesulfonamide (DNB, 3µM) significantly reduced the pro-calcificatory effects of BMP9 (Fig. 3B).

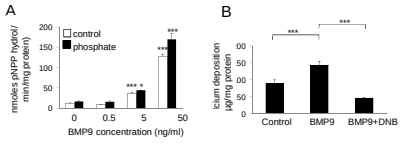

Figure 3: Effect of TNAP inhibition on BMP9-induced VSMC calcification. (A) Quantification of TNAP activity (mean moles pNPP hydrol/min/mg protein in VSMCs incubated with BMP9 (0.5-50ng/ml) in high phosphate (3mM P_i) or control (1mM P_i) medium for 4 days (n=5). *P<0.05; ***P<0.001 compared with 0ng/ml BMP9 treatment. (B) Calcium content was quantified by HCL leaching (µg/mg protein) in VSMCs incubated with BMP9 (50ng/ml) in high phosphate (3mM P_i) in the presence/absence of the TNAP inhibitor DNB (3µM). *P<0.05; **P<0.01; ***P<0.001 compared with control. Results are presented as mean +/- S.E.M.

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*, *BMPR-II*, *ActR-IIA* and *ActR-IIB* (Fig. 4A). BMP9 preferentially binds with 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).

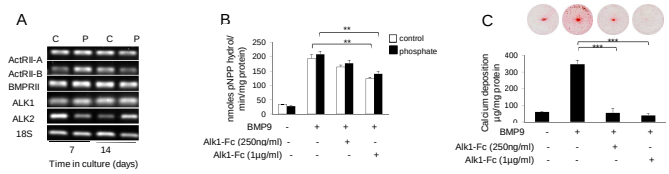

Figure 4: Effect of ALK-1 inhibition on BMP9-induced VSMC calcification. (A) Expression of BMP receptors in VSMCs cultured in high phosphate (3mM Pi) or control (1mM Pi) medium for 14 days. (B) Quantification of TNAP activity (mean moles pNPP hydrol/min/mg protein in VSMCs incubated with BMP9 (50ng/ml) in high phosphate (3mM Pi) or control (1mM Pi) medium in the presence/absence of ALK1-Fc (250ng/ml and 1µg/ml) for 4 days (n=5). (C) Calcium content was quantified by HCL leaching (µg/mg protein) in VSMCs incubated with BMP9 (50ng/ml) in high phosphate (3mM Pi) in the presence/absence of the ALK1-Fc (250ng/ml and 1µg/ml). Results are presented as mean+/- S.E.M. **P<0.01; ***P<0.001.

BMP9 induces VSMC calcification through activation of the Smad signalling pathway

Signal transduction studies were completed to disclose the BMP9 initiated signaling mechanism by which this ligand induces VSMC calcification. BMP9 (0.5-50 ng/ml) markedly induced phosphorylation of Smad1/5/8 following treatment for 10, 30 and 60 min (Fig. 5A & B). Immunofluorescent staining confirmed the translocation of Smad1/5/8 to the nucleus following BMP9 exposure (Fig. 5C). Concomitantly, the phosphorylation of Smad2, Smad3 and Erk1/2 was weakly induced by BMP9 (Fig. 5A & B).

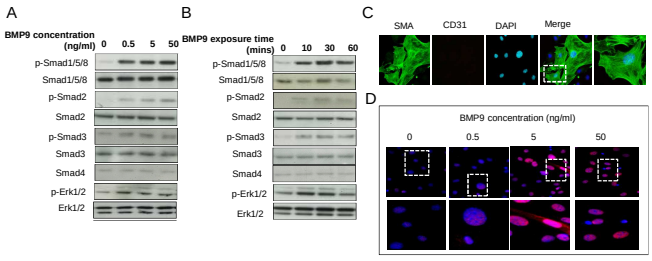

Figure 5: BMP9 induces VSMC calcification through activation of the Smad signalling pathway. Effect of (A) BMP9 concentration (0.5-50ng/ml) and (B) BMP9 (50ng/ml) exposure time (10-60min) on the phosphorylation (p) of Smad1/5/8, Smad2, Smad3 and Erk1/2 compared with total Smad1/5/8. Immunofluorescence staining of murine primary VSMCs demonstrates (C) positive staining with the smooth muscle cell marker Smooth Muscle Actin (SMA; Green) and negative staining with the endothelial cell marker, CD31 (Red) and (D) BMP9-induced (0.5-50ng/ml) nuclear translocation of phosphorylated Smad1/5/8, (Red). Areas within white markings are shown under increased magnification.

BMP9-mediated calcification of VSMCs is Smad4 dependent

Smad1/5/8, Smad2 and Smad3 form complexes with the common-partner Smad, Smad 4. These complexes translocate and accumulate in the nucleus and regulate the transcription of target genes. Therefore, to directly test whether BMP9 promotes VSMC calcification through a Smad signaling mechanism, we examined the effect of Smad4 siRNA knockdown on BMP9-induced calcification of 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 to 96 h (Fig. 6A & B). Transfection of VSMCs with Smad4 siRNA significantly inhibited BMP9-induced TNAP activity (Fig. 6C) and markedly reduced the pro-calcificatory actions of BMP9 on VSMCs (Fig. 6D).

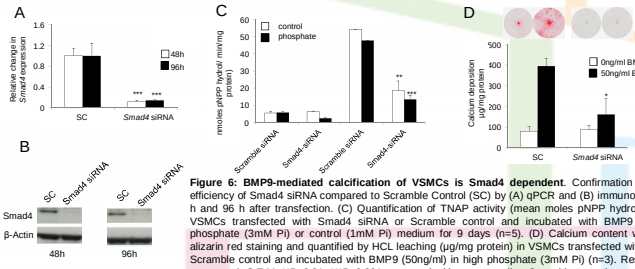

Figure 6: BMP9-mediated calcification of VSMCs is Smad4 dependent. Confirmation of the knockdown efficiency of Smad4 siRNA compared to Scramble Control (SC) by (A) qPCR and (B) immunoblotting analysis 48 h and 96 h after transfection. (C) Quantification of TNAP activity (mean moles pNPP hydrol/min/mg protein in VSMCs transfected with Smad4 siRNA or Scramble control and incubated with BMP9 (50ng/ml) in high phosphate (3mM Pi) or control (1mM Pi) medium for 9 days (n=5). (D) Calcium content was visualised with alizarin red staining and quantified by HCL leaching (µg/mg protein) in VSMCs transfected with Smad4 siRNA or Scramble control and incubated with BMP9 (50ng/ml) in high phosphate (3mM Pi) (n=3). Results are presented as mean+/- S.E.M. **P<0.01; ***P<0.001 compared with corresponding Scramble control.

Increased serum BMP9 in CKD dialysis patients

Next we sought to compare BMP9 levels in predialysis 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 with dialysis time and BMP9 concentration in patients receiving haemodialysis, suggesting that this highly osteogenic BMP may contribute to the accelerated calcification associated with dialysis.

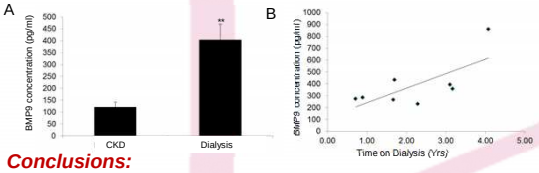

Figure 7: Assessment of BMP9 in serum from CKD dialysis patients. (A) BMP9 concentrations (pg/ml) in predialysis and dialysis serum from children with CKD. (B) Correlation of BMP9 serum concentrations (pg/ml) with dialysis time (yrs).

Conclusions:

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

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