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# Signalling pathways and vascular calcification

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## 1. ABSTRACT

Vascular calcification is a major risk factor for cardiovascular morbidity and mortality. A full understanding of the signalling pathways mediating vascular calcification is crucial not just because of the importance of this pathology in disease, but also for exploring potential therapeutic targets. Clinically there is a need to develop therapies to prevent or even reverse calcification *in situ*ations of atherosclerosis, chronic kidney disease, diabetes, and aging. In this brief review, we intend to explore the initial triggers, which are commonly related to calcification in different disease scenarios and examine the downstream signalling pathways that instigate the process of vascular calcification. In particular, we try to dissect these pathways and also examine cross-talk between different signalling pathways. Our focus is the vascular smooth muscle cell (VSMC) as it is ultimately the phenotypic modulation of these cells that may drive the calcification process.

## 2. INTRODUCTION

Vascular calcification refers to the deposition of calcium salts in the neointima of atheromatous plaques or in the media of vascular beds and it is a major risk factor for cardiovascular morbidity and mortality. Vascular calcification is prevalent in patients with atherosclerosis, type II diabetes, chronic kidney disease (CKD), and aging.

Extensive studies have shown that vascular calcification is an active, cell-regulated process very similar to bone mineralisation and many of the key regulators of bone mineralisation are active in cardiovascular calcification. It is well known that vascular smooth muscle cells (VSMCs) retain multi-potential capability and can transform into osteo/chondrocytic-like cells (1). A panel of bone differentiation markers have been detected in calcified areas in the vessel wall, and these are commonly used as markers to indicate the phenotypic conversion of VSMCs to osteoblast-like cells (2-4). These bone-related proteins

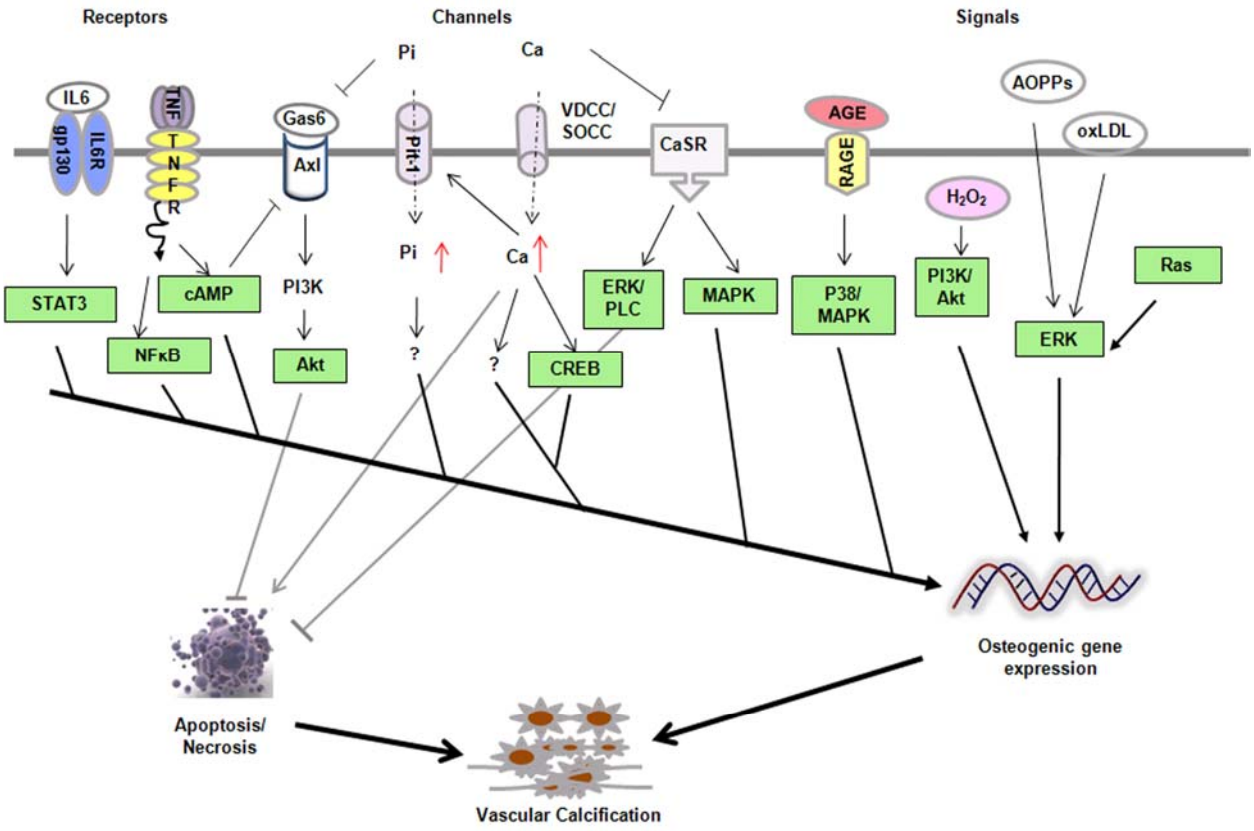


**Figure 1.** Diagram showing the complex signalling pathways that have been implicated in VSMC calcification. Multiple factors via their specific receptors and channels, are able to activate signalling pathways that act to induce osteogenic phenotypic modulation in VSMCs and/or apoptosis, both contributing to the progression and/or regulation of VSMC calcification. These signalling pathways include STAT3, NF-kappaB, MAPK, and PI3K/Akt pathway.

include transcription factors, such as Runx2 (5), osterix, and Msx2 (6), factors that may contribute to the mineralisation process including alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OC), as well as inhibitors of osteochondrogenic mineralisation, such as osteopontin (OPN) (7), matrix gamma-carboxyglutamic acid (MGP) (8), and osteoprotegerin (OPG) (9). In addition to VSMC phenotypic modulation, apoptosis is also a key event in initiating the calcification cascade in the vessel wall (10). During the past decade, a number of excellent reviews have provided insight into the complexity of the mechanistic events that promote vascular calcification and have focused on the impact of inflammation, mineral imbalance and loss of calcification inhibitors (11-16).

In this brief review, we intend to provide an update on what we have learned from the recent literature about the process of vascular calcification focusing on the signalling pathways involved in the osteogenic differentiation of VSMCs and also exploring the role of apoptosis as an initiating event in calcification. Five initial triggers, prevalent in different disease scenarios, are evaluated and discussed in the context of the downstream signalling pathways they activate. In particular, we try to dissect these pathways and discuss how cross-talk between them may elaborate the osteogenic response of VSMCs to these stimuli.

## 3. SIGNALLING NETWORKS AND HUBS IN VASCULAR CALCIFICATION- FROM THE EXTRACELLULAR MILIEU TO THE NUCLEUS

### 3.1. Environmental triggers

Systemic mineral imbalance and local disturbances of calcium and phosphate metabolism, chronic inflammation, high glucose, and oxidative stress have been suggested to contribute to the development of vascular calcification in multiple disease scenarios (14). (Figure 1)

#### 3.1.1. Hyperphosphatemia

Hyperphosphatemia is an important contributor to vascular calcification and commonly seen in patients with CKD, metabolic syndrome and aging. For example, serum inorganic phosphate (Pi) levels can typically exceed 2 mmol/L in dialysis patients (17), compared to the normal range of 1.0 to 1.5 mmol/L. CKD patients are two to five times more likely to develop vascular calcification (18, 19). However, the molecular mechanisms underlying the process of Pi-induced calcification are still under investigation (20).

Elevated extracellular Pi promotes human VSMC mineralisation in a dose-dependent manner (21). Several studies have shown that elevated Pi can induce the expression of osteogenic differentiation markers in VSMCs, such as osteocalcin, OPN and Runx2, although to different extents (1, 21-23). It is also known that extracellular Pi is transported into VSMCs *via* both sodium-dependent (Pit-1/Pit-2) and sodium-independent phosphate cotransporters (Pit-2) (24). Pit-1 mRNA levels are increased in the calcified aorta of uremic rats suggesting it may be upregulated in response to elevated phosphate (25). *In vitro*, Pit-1 knockdown can attenuate Pi-induced VSMCs mineralisation, and this can be rescued *via* over-expression of Pit-1(26). This data suggests that Pit-1 plays an important role in Pi-induced VSMC phenotypic transition. However, the link between elevated intracellular Pi and up-regulation of bone transcriptional factors is still missing. Recently a conditional Pit-1 knockout mouse was generated (27), and this mouse model might help to uncover the role of Pi and its transporter, Pit-1, in VSMC mineralisation. Meanwhile, some of the intracellular signalling pathways activated by phosphate are beginning to be elucidated.

Accumulated intracellular Pi can be extruded from VSMCs in matrix vesicles which bud off from the plasma membrane, and are then deposited in the extracellular matrix where they can initiate extracellular nucleation of hydroxyapatite crystals. Under physiological conditions, these hydroxyapatite crystals form and grow in the vessel wall. However, normally this crystal nucleation and growth can be inhibited by several factors, including pyrophosphate (PPi) and OPN (28). Extracellular PPi not only suppresses hydroxyapatite crystal growth, but also concurrently provides a reservoir for the generation of pro-mineralising Pi. PPi is generated by ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1) (29, 30), which is localized on the endoplasmic reticulum, on the plasma membrane, and in matrix vesicles. In addition, a transmembrane protein, ankylosis, is responsible for exporting intracellular PPi from the cell (31). It has been shown that the balance between extracellular Pi and PPi is regulated at least in part, *via* the protein kinase A (PKA) pathway (32). PKA up-regulates ALP, which cleaves the inhibitor PPi to generate Pi and upregulate Enpp1 expression. Therefore, a persistently activated PKA pathway could promote vascular calcification.

It is well-established that apoptosis and vesicle release from VSMCs are crucial initiating events in vascular calcification (10, 33). Growth arrest-specific gene 6 (Gas6) is a member of the vitamin K-dependent protein family and is a secreted protein, binding to a membrane receptor tyrosine kinase, Axl. The Gas/Axl-PI3K/Akt signalling pathway acts to block VSMC apoptosis induced by serum starvation (34). Son *et al* (35) have shown that elevated Pi can also induce VSMC apoptosis *via* down-regulation of the Gas6-Axl interaction. Furthermore, in the presence of elevated Pi, the phosphorylation of essential survival signals Akt, Bcl2, and Bad was reduced and caspase 3 was activated. Recombinant Gas6 attenuated Pi-induced apoptosis and calcification (36), suggesting that Gas6/Axl signalling pathway might be involved in Pi-induced vascular calcification.

In addition, both *in vitro* and *in vivo* studies have shown that the ERK pathway is involved in Pi-induced vascular calcification with Pi induced ERK activation leading to up-regulation of Glvr-1/-2, a sodium-dependent phosphate transporter, and subsequent crystal formation (37, 38). P38-MAPK may also contribute to Pi-induced calcification in addition to ERK pathway (39, 40).

### 3.1.2. Hypercalcemia

Hypercalcemia, or the elevation of calcium levels in the blood, has been associated with increased coronary artery calcification (41). Calcium not only plays vital roles in VSMC contraction, but also serves as an important messenger controlling VSMC phenotype transition in response to environmental cues (42). Extracellular calcium can enter VSMCs *via* voltage-dependent $Ca^{2+}$ channels (VDCCs) and/or voltage-independent cation channels, such as store-operated $Ca^{2+}$ channels (SOCCs). Increased intracellular $Ca^{2+}$ can also result from release of $Ca^{2+}$ from intracellular stores *via* both the ryanodine receptor or the inositol 1,4,5-trisphosphate [InsP(3)]) receptor (43). $Ca^{2+}$-induced gene expression can be mediated by $Ca^{2+}$-dependent phosphorylation of the transcription factor $Ca^{2+}$/cAMP response element (CRE)-binding protein (CREB) (44). Moreover, long-term elevated calcium treatment of VSMCs *in vitro* induced expression of Pit-1 (45). In addition, calcium overload drives mitochondrial-dependent cell death (both apoptotic and necrotic) (46).

Recently, the calcium-sensing receptor (CaSR) and its signalling pathways have been shown to play an important role in the initiation and progression of vascular calcification (47). The extracellular CaSR is a cell surface protein belonging to the family of G protein-couple receptors, which is mainly present in tissues involved in systemic calcium homeostasis, such as parathyroid, thyroid, kidney, bone and gastrointestinal tract (48). In addition, the CaSR has also been shown to be present in VSMCs, and its expression is downregulated in atherosclerotic, calcified lesions (49, 50). Binding of extracellular calcium or other CaSR agonists to the extracellular domain of the receptor triggers a number of intracellular signalling pathways, including PLC, PLA2, MAPK and protein kinases (51), and enables the cells to respond to small changes in extracellular ionized calcium concentrations. It is noteworthy that the MAPK pathway, which is activated by signals from the extracellular matrix and parathyroid hormone (PTH), plays a crucial role in the induction of Runx2 activity, resulting in the induction of osteoblastic differentiation (52-54). In the arteries of uremic patients, who usually suffer from hypercalcaemia and hyperphosphataemia, CaSR expression is reduced compared with that of non-uremic subjects. Activated CaSR leads to the up-regulation of ERK1/2 phosphorylation, and the CaSR/ERK1/2/PLC pathway is important for VSMC survival, proliferation and protection against apoptosis (55). A recent study has shown that elevated $Ca^{2+}$ can down-regulate CaSR expression *in vitro*

while over-expression of a dominant-negative CaSR enhances mineral deposition by VSMCs (50). Taken together, these observations suggest that a functional CaSR in VSMC is important in preventing mineralisation. However, the connection between specific $Ca^{2+}$ signals and transcriptional control of gene expression in the process of VSMC osteogenic differentiation needs to be further explored.

### 3.1.3. Chronic inflammation

A chronic inflammatory state is commonly seen in atherosclerotic, diabetic, and CKD patients and is associated with vascular calcification (56-58). Clinical studies have shown that inflammatory cytokines, such as IL-6, IL-8, and TNF-alpha, are dysregulated in the uremic milieu and appear to influence the risk for CVD in CKD patients (59). Serum Pi and Ca x P also directly correlate with IL-6 in CKD patients (57) suggesting dysregulated mineral metabolism may act to drive inflammation and calcification in this patient group. Studies of the direct effects of these cytokines on VSMCs also support this notion.

#### 3.1.3.1. TNF super family

Tumour necrosis factor-alpha (TNF-alpha) is mainly produced by activated macrophages in response to oxidized LDL, bacterial infection and it can also be released from damaged extracellular matrix. Alternate origins of TNF-alpha in the vasculature include immune cells (T&B cells, NK cells) and VSMCs. Tintut Y *et al* (60) were the first to demonstrate that TNF-alpha promotes calcifying vascular cell (CVC) mineralisation *in vitro* by causing increased expression and activity of ALP *via* the cAMP pathway. Furthermore, TNF-alpha enhances DNA binding of the osteoblast specific transcription factor (Osf2), as well as activated protein 1 (AP1), and cAMP responsive element binding protein, transcription factors which are all important in osteoblastic differentiation. TNF-alpha also augmented Pi-induced calcification and this process was associated with AMP-activated protein kinase (AMPK)-dependent Gas6 expression and Akt signalling; a VSMC survival signal described previously (61). More recently, TNF-alpha has been shown to enhance Msx2 expression in a dose- and time-dependent manner *via* the NF-kappaB pathway (62). Since the ALP promoter contains an Msx2-response element, it has been suggested that TNF-alpha directly induces Msx2 *via* the NF-kappaB pathway, and this leads to downstream activation of ALP and subsequent bio-mineralisation.

OPG is another member of the TNF superfamily that serves as a decoy receptor for receptor activator of NF-kappaB ligand (RANKL). In animal models knockout of OPG leads to vascular calcification and osteoporosis and serum OPG levels are associated with the extent of vascular calcification in hemodialysis patients (63). Thus the RANKL/OPG signalling pathway seems crucial to the processes regulating both vascular calcification and bone turnover, although the mechanisms have not been fully defined. Knowledge concerning this pathway has been reviewed recently by Shao *et al* (64).

#### 3.1.3.2. IL-6

IL-6 acts both as a proinflammatory/proatherogenic cytokine and anti-inflammatory cytokine. VSMCs in the tunica media of many blood vessels can produce IL-6 as a pro-inflammatory cytokine while IL-6's role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-alpha and IL-1, as well as *via* activation of IL-1R alpha and IL-10. IL-6 signals through a cell-surface type I cytokine receptor complex consisting of the ligand-binding IL-6R alpha chain, and the signal-transducing component gp130 (also called CD130). The binding of IL-6 to its receptor triggers the gp130 and IL-6R proteins to form a complex, thus activating a downstream signalling cascade through janus kinases (JAKs) and signal transducers and activators of transcription (STATs) (65). It has been shown that IL-6 induces increased STAT3 phosphorylation and ALP activity, leading to CVCs undergoing osteoblastic differentiation (40).

In summary, both local and systemic effects of proinflammatory cytokines have been shown to play an important role in vascular calcification and further studies to elucidate their cellular source and signalling pathways will be invaluable in minimizing the cycle of inflammation and calcification in ageing and disease (64).

### 3.1.4. Oxidative stress

Reactive oxygen species (ROS) play a critical role in the pathobiology of arterial mineralisation. Vascular cells can generate ROS, such as hydrogen peroxide ($H_2O_2$), *via* multiple enzymatic systems including vascular NAD(P)H oxidases, mitochondria, xanthine oxidase, and uncoupled endothelial nitric-oxide synthase. $H_2O_2$ promotes the phenotypic switch of VSMCs from contractile to osteogenic (66) with $H_2O_2$-treated VSMCs showing dramatic increases in ALP, OC, and Runx2 expression, and decreased expression of VSMC markers. Knockdown of Runx2 blocks this $H_2O_2$-induced calcification. Furthermore, activation of the PI3K/AKT pathway plays an important role in oxidative stress-induced VSMC calcification by increasing DNA binding of Runx2 and its transcriptional activation.

Accumulation of advanced oxidation protein products (AOPPs) is common in uremia and diabetes and these are a marker/source of oxidative stress, which are also potent mediators of inflammation (67-69). You *et al* have demonstrated that AOPPs directly increase calcium deposition and expression of Runx2 and OPN, while concomitantly decreasing SM-alpha-actin expression in human VSMCs and this is dependent on the ERK pathway (70). Advanced glycation end products (AGE) also induce calcification of VSMCs by acting through the receptor for AGE (RAGE) and the p38 MAPK signalling pathway (71). Oxidized LDL (oxLDL) can also enhance beta-glycerophosphate-induced ALP activity in bovine VSMCs *via* the MAPK/ERK signalling pathway and downstream activation of osterix expression (72). Importantly OxLDL also contributes to low-grade vascular inflammation *via* up-regulation of TNF-alpha and can also enhance BMP2 expression further promoting vascular calcification.

### 3.1.5. Aging

Medial calcification is often found in elderly people and age is often associated with increased vascular calcification. In atherosclerotic plaques, a population of senescent VSMCs has been identified, and defined by increased senescence-associated beta-galactosidase activity and elevated expression of the cyclin-dependent kinase inhibitors (73, 74). Senescent VSMCs are associated with oxidative DNA damage, impaired DNA repair, and telomere shortening (74, 75). More interestingly, senescent VSMCs have been shown to have increased levels of ALP activity, collagen I, and Runx2 expression (76). Microarray data has also shown that senescent VSMCs have increased levels of BMP2, as well as increased inflammatory factors (such as IL-1, IL-8, and TNF-alpha) and decreased MGP, suggesting that VSMCs adopt an osteoblastic procalcific phenotype during senescence (77). The role of BMP2 signalling in senescence-induced calcification needs to be further validated as contrary to the elevated levels of BMP2 in one study Nakano-Kurimoto *et al* showed that BMP2 signalling was down- regulated in senescent VSMCs (76).

Importantly, aging is also associated with increased IL-6 and a dysregulation of inflammatory cytokines (65) with aged vessels showing increased expression of inflammatory markers (78). For example, injury-induced senescent VSMCs had increased levels of IL-1beta, ICAM-1, MMP-9, TNF-alpha, and these were activated *via* the NF-kappaB pathway (79). Constitutive activation of Ras also promotes VSMC senescence and expression of proinflammarotry cytokines, such as IL-1, IL-6, and IL-8, *in vitro* and *in vivo* (80). Conversely, functional inhibition of Ras can suppress expression of proinflammatory molecules *in vivo* (81). Given the fact that secreted inflammatory cytokines are crucial mediators of senescence (82) and vascular calcification, DNA damage and inflammation may play a synergistic role in promoting medial calcification associated with VSMC premature senescence and aging.

## 3.2. Secondary responses

Following the initial environmental triggers, some of which are described above, VSMC osteogenic differentiation and calcification likely proceed through a number of selected signalling pathways. These pathways, which are all crucial for bone development, form a complex interaction network with multiple levels of crosstalk and feedback that mean there is significant heterogeneity in the 'osteogenic' phenotype of VSMCs in association with calcification in different disease states. Indeed the pathways activated are dependent on factors such as the origin of the cells, with both circulating, adventitial and medial-derived cells being implicated in driving calcification and the pathway that has been activated. In many situations this is likely to be a stochastic response to multiple and varied stimuli. The resultant calcification is very rarely true bone rather it is dystrophic mineral deposition due to the activation of inappropriate pathways in dysfunctional cells. (Figure 2)

### 3.2.1. TGF beta signalling pathway

Transforming growth factor beta (TGF beta), which has three isoforms (TGF beta-1/2/3), is a multifunctional cytokine and a potent growth inhibitor for a wide variety of cells. TGF beta family ligands bind to the type II receptor (TGFBR2), which recruits and phosphorylates a type I receptor. The type I receptor then phosphorylates receptor-regulated SMADs (R-SMAD2/3) which can bind the coSMAD, SMAD4. R-SMAD/coSMAD complexes transport to and accumulate in the nucleus, where they act as transcription factors to orchestrate multiple cellular changes including apoptosis, extracellular matrix synthesis, G1 arrest in the cell cycle, and immunosuppression.

TGF beta is capable of promoting VSMC differentiation, matrix formation, and regulating vascular calcification (83). TGF beta1 has been shown to directly activate expression of the SM differentiation marker SM22 in 10T1/2 cells, as well as Smad3, which can directly bind to the SM22 promoter in association with the serum response factor (SRF) complex (84). TGF beta2 is increased in the diabetic aorta, and in this context TGF beta was found to inhibit VSMC calcium transients *via* down-regulation of the IP3 receptor, leading to impaired vascular function (85). More importantly, TGF beta not only impairs IP3R-mediated calcium release, but also uncouples ER-mitochondrial calcium communication, affecting energy metabolism in the mitochondrial matrix (86). Kanno *et al* reiterated the important role of TGF beta signalling in the process of vascular calcification (87). They showed that nitric oxide reduced expression of the TGF beta receptor, ALK5, decreased phosphorylation of Smad2/3 and consequently expression of plasminogen activator inhibitor-1 *via* a cGMP-dependent pathway, resulting in the inhibition of calcification.

### 3.2.2. The BMP signalling pathway

Bone morphogenetic proteins (BMPs) are a group of cytokines with great osteogenic capacity. Like TGF beta, BMP signalling is also mediated by receptor-regulated R-Smad transcription factors, Smad1/5/8, and the common-mediator coSmad, Smad4. In addition, cofactors (such as ATF2, AP-1, AML) with specific DNA binding sites, are crucial for BMPs to recognize specific target genes such as the bone transcription factors, Runx2 (88) and Msx (89). Furthermore, it has been shown that BMP-induced Msx1and Msx2 can form a complex with SRF and myocardin, leading to the inhibition of expression of VSMC marker genes, suggesting that BMP signalling pathways play an important role in VSMC phenotype transition (90).

BMP2 has been most frequently associated with vascular calcification. BMP2 secretion has been shown to progressively increase during calcification and factors present in uremic serum can enhance its secretion when compared to normal serum (91). Oxidative stress, inflammation, hyperlipidemia and hyperglycemia can also trigger BMP2 expression in the vasculature (92-94). BMP2 participates in the process of VSMC calcification *via*

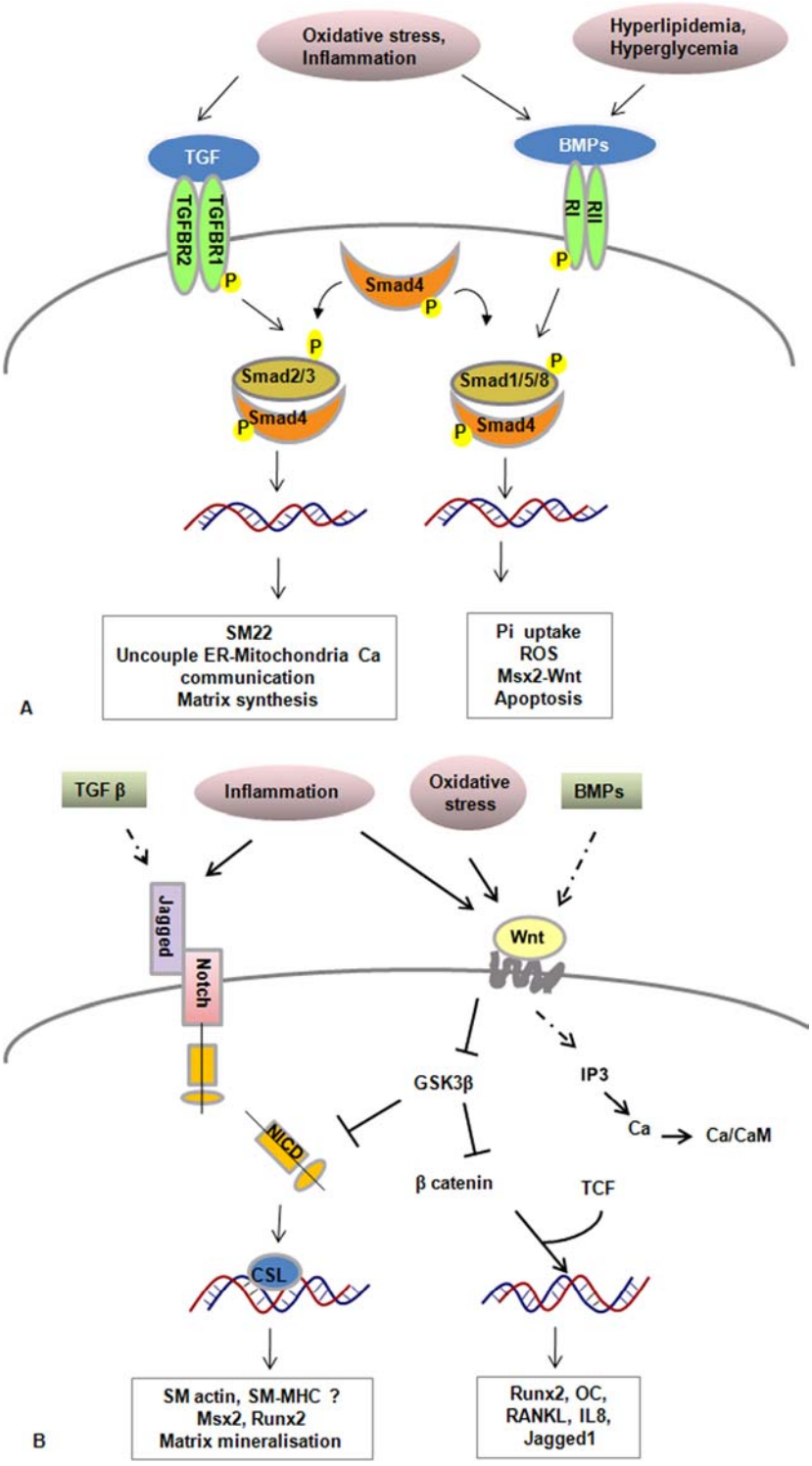


**Figure 2.** Major signalling pathways implicated in bone differentiation have also been implicated in inducing osteogenic differentiation of VSMCs. These pathways include signalling *via* the TGF superfamily (a) as well as Wnt and Notch signalling pathways (b). Note that these signalling pathways are activated by initial triggers however crosstalk between a number of these pathways has been demonstrated. As shown in (b), the BMP pathway can activate Wnt signalling, while both TGF beta and Wnt signalling have been shown to regulate Notch signalling. GSK3beta can phosphorylate NICD, leading to its degradation.

diverse effectors. For example, Li *et al* (95) have shown that BMP2 enhanced phosphate uptake *via* up-regulation of Pit-1 in VSMCs, leading to increased calcification. BMP2-treated VSMCs demonstrated elevated ROS levels, generated by NADPH oxidase and this increased ROS up-regulated the transcription factors Sp1 and NF-kappaB subunit p50, resulting in an up-regulation of Runx2 (96). The inhibitory effect of MGP on vascular calcification is finely tuned by BMP2 availability (97) and BMP2 can induce apoptosis in pulmonary VSMCs (98). Last but not the least, BMP2 is also a upstream factor in the Wnt signalling pathway, and the BMP2-Msx2-Wnt axis plays an important role in TNF alpha induced vascular calcification (99).

### 3.2.3. Wnt Signalling pathway

Wnts are a large family of conserved secreted carbohydrate and lipid-modified proteins that are involved in cell differentiation, proliferation, and maturation (100). Wnt proteins bind to cell-surface receptors of the Frizzled family and lipoprotein receptor-related protein (LRP)-5/6, causing the receptors to activate the Dishevelled (DSH) family proteins, which in turn inhibit Axin/APC/GSK3β complex activity, ultimately resulting in the accumulation of beta-catenin. Beta-catenin translocates to the nucleus where it interacts with the TCF/LEF family of transcription factors to promote specific gene expression (101). Many proteins involved in vascular calcification are known Wnt target genes, including Runx2, OC, RANK ligand, Jagged1, and IL-8. (http://www.stanford.edu/~rnusse/pathways/targets.html). In addition to this canonical pathway, there exist beta-catenin-independent pathways, such as the planar cell polarity pathway, $Ca^{2+}$/protein kinase A pathway, G protein/protein kinase C/JNK signalling pathway, and Src/ERK pathway (102). Whether or how these pathways are involved in vascular calcification needs to be further investigated.

It has been shown that Wnt signalling is downstream of Msx2, which up-regulates Wnt ligands and down-regulates dickkopf homologue1 (Dkk1), an antagonist of LRP5/6, thus promoting osteogenic differentiation (103). The Wnt signalling pathway is also active in cardiovascular calcification. Towler's group have identified the importance of Msx2-Wnt signalling in vascular calcification in response to TNF-alpha and oxidative stress, using $LDLR^{-/-}$ high-fat fed mouse, a model of diabetic medial arterial calcification (99,104-106). This beta-catenin/TCF-mediated transcriptional response also stimulates chondrogenic and inhibits adipogenic differentiation of pericytes, stem-cell like VSMCs that are present in the adventitia of the vessel wall (107).

### 3.2.4. Notch signalling pathway

The Notch family is made up of four transmembrane receptor proteins (Notch 1-4), and five ligands (Delta-like1, 3, 4 and Jagged1, 2), which also locate on the cell surface. The interaction between ligands and receptors, which can also occur *via* heterotypic cell interactions, leads to the cleavage of the receptor. This releases an intracellular domain (NICD), which migrates to the nucleus, complexes with the CSL (CBF1/RBP-Jkappa, Su(H), Lag-1) transcription factor, and activates transcription of target genes containing CSL binding elements. The Notch direct target genes include Hes (hairy/enhancer of split) and Hrt (hairy-related, also referred to as Hey, CHF, HESR) family and play roles in mediating the development and maintenance of the cardiovascular system (108). Apart from the canonical pathway, Notch has also been shown to have close interactions with other signalling pathways, such as NF-kappaB, TGF beta, R-Ras, and Wnt signalling pathways (109-111). For example, GSK 3beta has been shown to bind and phosphorylate Notch, leading to Notch IC degradation, therefore the Wnt pathway can regulate Notch signalling directly (112). Notch family members have also been implicated in governing cell fate and playing an important role in the control of VSMC phenotype and the development of the cardiovascular system and cardiovascular diseases including valve calcification (113).

As discussed previously, inflammation plays a pivotal role in vascular calcification and it has been shown that macrophages express all four Notch receptors. Studies have shown that the expression of Dll4, a Notch ligand, was increased in response to proinflammatory stimuli such as lipopolysaccharide, and IL-1beta, resulting in macrophage activation. These activated macrophages may cross-talk with adjacent VSMCs while they infiltrate into the atherosclerotic plaque, and thus activate the Notch signalling pathway in VSMCs. However, it is quite controversial as to whether Notch signalling promotes or inhibits VSMC differentiation. For example, it has been found that the Jagged1-RBP-Jkappa pathway induces SM-myosin heavy chain (SM-MHC) expression, which is independent of the myocardin-serum response factor-CArG complex (114). The Notch downstream transcription factor, CSL, directly binds a conserved cis element in the SM actin promoter, resulting in the activation of SM alpha-actin gene expression (115). Others have shown that over-expression of a constitutively active NICD (both Notch 1 IC and Notch 3 IC) resulted in a significant down-regulation of alpha-actin, calponin, myosin, and smoothelin expression (116). Blockade of Notch3 signalling facilitated the VSMC switch to an inflammatory state (117). These discrepancies may be due to the availability of certain cofactors which are restricted temporally or spatially (118) and further work is required to determine the role of Notch signalling in mediating VSMC phenotype in different disease states.

A recent study detected that Notch 1 and its ligand Jagged1 were highly expressed in calcified atherosclerotic plaques, and co-localised with Msx2 and Runx2, but were not present in normal vessels (119). Furthermore, they also demonstrated that over-expression of NICD significantly increased ALP activity and matrix mineralisation in human aortic VSMCs, *via* Msx2. There is a RBP-Jkappa binding site in Msx2 promoter region, thus Notch/RBP-Jkappa signalling can directly regulate Msx2 expression, providing a novel Notch-RBP-Jkappa-Msx2

signalling pathway in vascular calcification. However, Notch signalling alone cannot execute VSMC mineralisation, other triggers, such as Pi, are also required.

## 4. CONCLUSION AND PERSPECTIVES

The search for understanding of the mechanisms of vascular calcification has been an area of intense study. Given the complexity of the signalling pathways activated in response to different culprits, one might think of the signalling network as a spider's web in which movement of one knot leads to the movement of many others. Elucidating how these pathways signal and cross talk, and how the resulting VSMC phenotype mediates calcification is the greatest challenges for future studies.

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**Abbreviations:** VSMC: vascular smooth muscle cell, CKD: chronic kidney disease, ALP: alkaline phosphatise, BSP: bone sialoprotein, OC: osteocalcin, OPN: osteopontin, MGP: matrix gamma-carboxyglutamic acid, OPG: osteoprotegerin, Pi: inorganic phosphate, PPi: pyrophosphate, Enpp1: ectonucleotide pyrophosphatase/ phosphodiesterase 1, PKA: protein kinase A, Gas6: Growth arrest-specific gene 6, VDCCs: voltage-dependent $Ca^{2+}$ channels, SOCCs: store-operated $Ca^{2+}$ channels, CREB: $Ca^{2+}$/cAMP response element (CRE)-binding protein, CaSR: calcium-sensing receptor, PTH: parathyroid hormone, TNF-alpha: Tumour necrosis factor-alpha, CVC: calcifying vascular cell, Osf2: osteoblast specific transcription factor, AP1: activated protein 1, AMPK: AMP-activated protein kinase, RANKL: receptor activator of NF-kappaB ligand, JAKs: janus kinases, STATs: signal transducers and activators of transcription, ROS: Reactive oxygen species, $H_2O_2$: hydrogen peroxide, AOPPs: advanced oxidation protein products, AGE: Advanced glycation end products, oxLDL: Oxidized LDL, TGF beta: Transforming growth factor beta, TGFBR2: Transforming growth factor beta receptor 2, SRF: serum response factor, BMPs: Bone morphogenetic proteins, LRP: lipoprotein receptor-related protein, DSH: Dishevelled, Dkk1: dickkopf homologue1, NICD: Notch intracellular domain,

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