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Alternative Inhibitors of Canonical Wnt Signaling

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Approaches to Inhibiting Calcification by Investigation of the Canonical Wnt Signaling Pathway

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Introduction

Cardiovascular diseases are one of the top causes of death in the United States, responsible for over 29% of deaths worldwide[1]. Myocardial infarctions, angina, and strokes take over 16 million lives every year, with the underlying mechanisms being currently investigated. One of the main underlying pathological inflammatory vascular diseases is atherosclerosis which accounts for most MIs and strokes[1]. Vascular calcification, which is the deposit of hydroxyapatite in the arterial wall causes increases of disease such as heart disease, stroke, and atherosclerotic plaque rupture[1]. One of the primary predictors of mortality is coronary arterial calcification (CAC) as men and women with type 1 diabetes are affected with CAC by their forties and fifties due to factors such as lifestyle factors, hormones, and menopause. Active inducers of calcification include hypercalcemia, inflammatory cytokines, uremic toxins, elevated inorganic phosphate, or hyperphosphatemia. This can be related to chronic kidney disease which is associated with advanced vascular calcification in chronic kidney disease (CKD)[2].

Chronic kidney is defined as when there is damage to the kidney or loss of function or at least of three months which are also reflected in the arterial stiffness used in diagnosis. Chronic kidney disease is usually linked to vascular calcification as the distinction between the two main layers of the arteries[3]. This occurs when the glomerular filtration rate decreases, and calcification increases with mineral disorders and the accumulation of both calcium and phosphate from the loss of kidney function[3]. This then leads to the calcification that can occur in the tunica intima or

the tunica media. Vascular calcification in the tunica intima is known as atherosclerosis while the calcification in the tunica media is known as arteriosclerosis that differs with various mechanisms. These mechanisms are associated with different inflammations and injuries to endothelial cells which are known to upstream the process of vascular calcification. Vascular calcification involves vascular smooth muscle cells (VSMC) undergoing a cellular mediated phenotypic switch into cells that resemble bone, characterized by an upregulation of osteogenic markers and a loss of smooth muscle markers. Runx2, one of the osteochondrogenic markers, is a transcription factor that contributes to osteoblast differentiation for calcifying the smooth muscle cells and could be the cause of this differentiation[4]. This transcription factor is also a Wnt signaling factor that regulates direct bone turnover and remodeling[4]. When these regulatory factors such as Runx2 and other osteochondrogenic markers become overwhelmed with injuries and damaged tissue, a buildup of hydroxyapatite crystals in the arterial layers, especially in the tunica media, which is thicker and contains more contractile tissue, promote hypertension, plaque buildup, and then erosion of the arterial tissue compliance. Within the tunica media, various regulatory bone formation and structural proteins that are in calcified medial arterial layers suggest an active process. Because 4regulatory proteins play a factor in calcification and have been investigated for its effects, looking into other factors that may play a part in calcification could inhibit parts of this process which could regulate vascular calcification[4]. Some of the inhibitors that have been studied extensively are proteins such as sclerostin which reduces calcification in chronic kidney disease and regulates adipocyte differentiation. However, more studies have shown other inhibitors in recent years such as stabilization of axin proteins, endostatin, a cancer reducing collagen, and other antagonists of the canonical Wnt signaling pathway have potential as a solution for inhibiting the progression of calcification. These novel

solutions will be investigated for their roles in the down regulation of the canonical Wnt pathway to minimize mechanical opening of arteries and treat the root of the vascular calcification.

Vascular Calcification

Atherosclerosis, once believed to be a passive process, is now understood to be an active process that involves various cell types, organ systems, molecular mechanisms, and other pathological conditions such as type 2 diabetes and hypertension. This process is characterized by the buildup of lipids, inflammatory cells, and other fibrous elements in the arterial walls that can lead to intimal thickening and reduction of blood flow clinically manifesting as angia or strokes[4]. Vascular calcification occurs through various mechanisms such as the loss of regulating bone formation, activation of bone formation, nucleational complexes, and cell death that elevates calcium and phosphate levels in the arteries[4]. This can be further be separated into intimal vs medial calcification based upon the location in the artery within the tunica media or the tunica intima as shown in Figure 1. As stated previously, calcification in the tunica intima is referred to as atherosclerosis while the tunica media is referred to as arteriosclerosis with an onset mechanism distinguishing the two processes[5]. Calcification in the tunica intima is associated with inflammation that occurs after injury to the endothelial cells which makes atherosclerosis. This is one of the most common calcification locations as many lifestyle risk factors trigger this process to create a pro-inflammatory environment which encourages adhesion of lipids and foam cells after an injury to the endothelium[6]. There is also adhesion of monocytes, platelets, and lymphocytes which leads to plaque formation in the inner layer of the arterial wall while also recruiting inflammatory cytokines to mediate this process[6]. Contrary to this, arteriosclerosis is in the tunica media which is made up of vascular smooth muscle cells. Arteriosclerosis is when the tunica media layer hardens and calcifies and stiffens the arterioles in the body over a period of

time[5]. While there has been extensive research on atherosclerosis, arteriosclerosis has not been as understood as much since there are differing views on the root cause such if only smooth muscle cells are involved in differentiation or if the myofibroblasts that are involved with injury of the heart differentiate as well[7]. It is imperative to further understand the vascular smooth muscle cells involved and their phenotypes and processes since it can lead to further findings and prevent further mortality rates caused by cardiovascular problems.

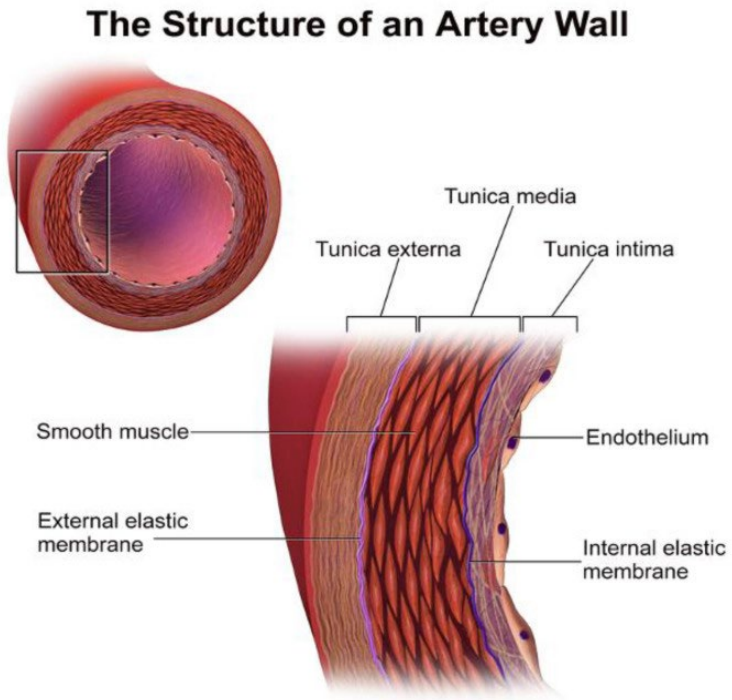

Figure 1. The structure of an artery wall and the components that are associated with vascular calcification. Vascular smooth muscle cells are in the tunica media while endothelial cells are in the tunica intima[12].

VSMC Phenotypes

For many years, smooth muscle cell cells with the potential to undergo activation, dedifferentiation, and trans differentiation had been regarded as a single synthetic, proliferative phenotype[8]. However, in recent years, there has been a discovery that there are a diverse number of phenotypes that are involved in vascular calcification[4]. Understanding vascular smooth muscle cells and their phenotypes lead to the knowledge of the differentiation and mineralization of vascular smooth muscle cells (VSMCs) to osteoblast-like cells. Recent scRNA-seq analysis has provided information on vascular smooth muscle cell phenotypic characterization for classification[8]. Different phenotypes such as contractile, mesenchymal, fibroblast-like, macrophage-like, osteogenic-like, and adipocyte-like phenotypes have been found to exist that mediate the VSMC phenotype switching[8]. While external stimuli such as TGF-β and heparin help in promoting and maintaining VSMC contractile phenotypes, exposure to these factors can lead to upregulation of structural extracellular matrix genes that may inhibit VSMC proliferation. The change in the composition of extracellular matrix proteins such as elastin and collagen fibers can trigger vascular stiffening in atherosclerosis accompanied by fibrosis and increased calcification which can also affect mesenchymal-like VSMCs. Mesenchymal-like VSMCs are normally characterized by the ability to proliferate and self-renew and can differentiate along multiple lineages.

One of the transcription factors that regulates this phenotype is KLF4 which results in a phenotypic switch from contractile to mesenchymal-like that migrates into the media and intima to support tissue repair which is an important factor to vascular calcification[8]. In various vascular models, KLF4 has shown to induce VSMC differentiation into a mesenchymal-like VSMC type[8]. This can be reversed by TGF-β, which maintains the contractile VSMC phenotype[8]. In response to injury, fibroblast-like phenotype is observed following vascular injury during aortic aneurysm

formation to determine if there is an upregulation of genes that are involved in adhesion, ECM organization, proliferation, and deposition of collagen which contributes to vascular calcification[8]. This is important for aortic aneurysms and calcification since the abundance and differentiation of these stem cells contribute to aortic fibrosis and stiffness which is a major part of arteriosclerosis[8]. It also is crucial to understand the phenotypic switch in the physiological arteriogenesis process factors such as blood flow hemodynamic forces and inflammation are associated with the damage and inflammation in the arteries[9]. Macrophage-like VSMCs are valuable to various stages of inflammation and healing to clear pathogens, especially in atherosclerosis where macrophages are known to clear the vascular wall from becoming foam cells[4]. Osteogenic-like mesenchymal stem cells are important since the role of osteoblasts are to generate bone tissue. They usually exist within the body naturally for the regulation and maintenance of existing bones, but osteoblast like cells also is involved with the calcification and stiffening of the artery. This can occur due to the inflammation and damage to the cardiovascular system which recruits the turnover of mesenchymal stem cells. Both chondrocytes and osteoblast can be formed from mesenchymal VSMCs contributing to the bone-like formation in the arteries[8]. The deposition of calcium and phosphate can trigger VSMCs to switch to phenotypes that can contribute to vascular calcification as shown in figure 2. With calcification of the intimal layer, it is normally associated with arterial obstruction while calcification of the medial layer is usually associated with vessel stiffening that leads to heart failure[4]. Osteogenic-like phenotypes are characterized by a loss of contractile markers and an increase in calcification markers, such as Runx2[8]. Expression of Runx2 is one of the main determinants of the osteogenic-like VSMC phenotype which will be important to understanding the Wnt signaling pathway and its role in osteogenesis[8]. Adipocyte-like VSMC phenotypes have been described in

a single scRNA-seq studies have been adapted to regulate thermogenesis from PCR testing on mice with induced vascular calcification[4]. These VSMC phenotypes are important for various circumstances but are especially important to responses in vascular injury[8].

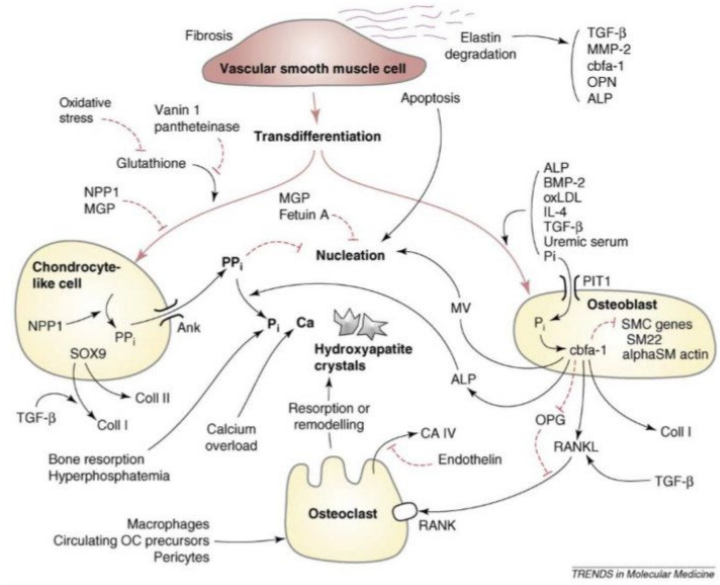

Figure 2. The pathways leading to the differentiation and mineralization of VSMCs to osteoblast-like cells.[12]

Wnt Signaling Cascade

Canonical Wnt signaling pathway occurs when canonical Wnt signals are transduced through receptors called frizzled family receptors and LRP5 coreceptor to the β-catenin signaling cascade[11]. Wnts are 19 molecules in a family in mammals that consist of 350-400 amino acids. Specifically, the frizzled family is a seven transmembrane receptor of Wnts that range from 500 to 700 amino acids that have cysteine-rich domain in the amino terminal extracellular region. This is highly essential for the activation of the canonical Wnt pathway[11]. The cascade is activated when the extracellular Wnt ligands binds to these receptors that associate with the LRP5 and LRP6 of the low-density lipoprotein receptor family as shown in figure 3[11]. When the

Wnt binds to the frizzled receptor, the signal recruits cytoplasmic phosphoprotein Disheveled to the plasma membrane that diverges the pathway into three separate branches, canonical being the branch studied the most[5]. Once the Dsh is activated, the inhibition of the GSK3 enzyme activates a series of events that lead to the degradation of β-catenin[11]. The stabilized β-catenin then translocate into the nucleus that is poorly understood. It is proposed that it may likely "piggyback" other factor to translocate where Axin undergoes nuclear shuttling. This then upregulates β-catenin and translocate to form a transcriptional complex with LEF-1/TCF DNA-binding transcription factors that upregulates those genes[11]. During canonical Wnt signaling, the Wnt ligands bind to the cell membrane which then inhbits the β-catenin destruction complex which results in translocation to the nucleus for transcription of the Wnt target genes being induced[11].

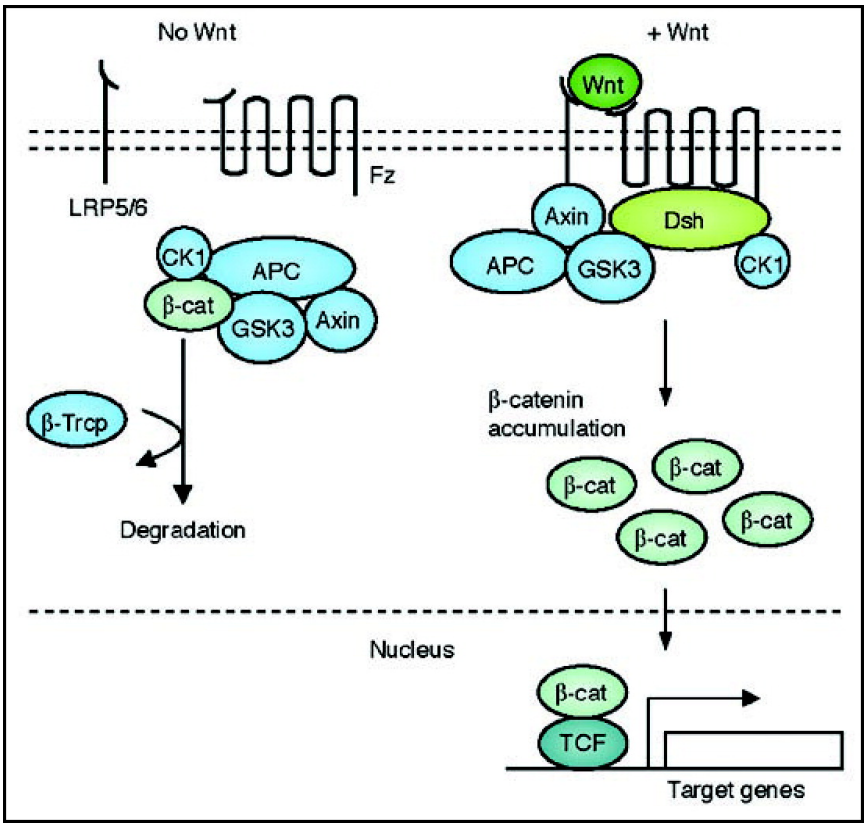

Figure 3. The canonical Wnt signaling cascade that is heavily involved with the upregulation of vascular calcification transcription factors[12].

Involvement of Wnt and Runx2 in Osteogenesis

As stated before, Runx2 is one of the main determinants of the osteogenic-like VSMC phenotype which will be important to understanding the Wnt signaling pathway and its role in osteogenesis. Osteogenesis is programmed by different defined transcriptional programs regulated by various physiological cues[13]. Osteogenic differentiation is an expression of the transcription factor Runx2 that determines the osteogenic phenotype and changes vascular smooth muscle cells into osteoblasts. Runx2 also binds to downstream receptors that regulate bone genes and β-catenin which regulates bone development including Type 1 collagen, osteopontin, etc[13]. This can promote calcification when inducing Runx2 and other osteogenic markers because the expression and upregulation of Runx2 can promote smooth muscle cells to differentiate into osteoblasts[13]. With the abundance of atherosclerotic plaque, Runx2 is often elevated and upregulated in the arterial walls which promotes the phenotypic switch in patients with atherosclerosis and CKD[13]. Studies have been conducted to quantify how Runx2 expression triggers β-catenin by expressing Runx2 to different degrees in the cytoplasm demonstrating how it can translate over to vascular smooth muscle cells to differentiate into osteoblasts[22]. This also proves how the expression of Runx2 is modulated by canonical Wnt signaling which can result into the inhibition of chondrocyte differentiation and directs smooth muscle cells into often becoming osteoblasts instead[14] .The process usually often occurs during the embryonic development of establishing the development and functions of the body which can occur when there is injury to the cardiovascular system resulting in vascular calcification[15].

Role of Wnt in Vascular Calcification

Wnt is an important factor in key processes in the body, especially when it involves cardiac development and angiogenesis[16]. There is more research and studies that have proven how important Wnt signaling is in atherosclerosis and especially in vascular calcification[16]. In these studies, specifically the Wnt ligands and their receptors play a significant role in calcification as well as other atherosclerotic events that contribute to this pathway[16]. One of the most studied pathways is the canonical Wnt signaling pathway that has been demonstrated to increase adhesion of endothelial cells since it is one of the signs of atherosclerosis[16]. There have been studies that have demonstrated the relationship between canonical Wnt signaling with ligands Wnt1, Wnt2, and Wnt3a and how it affects arterial smooth muscle cell proliferation contributing to the intimal thickening stage[16]. Canonical Wnt signaling has also been directly associated with foam cell formation which also contributes to calcification[16].

Vascular calcification is associated with the upregulation of Wnt signaling that involves BMP2. BMP2 expression is activated by pathogenic stimuli and has also demonstrated the regulation of osteogenic pathways that contribute to vascular calcification[17]. One-way BMP2 regulates osteogenic gene expression is through the induction of transcription factor Msx2, which regulates craniofacial mineralization and promotes osteogenic differentiation. This transcription factor also contributes to vascular calcification. It enhances Wnt signaling though upregulation of other Wnt ligands and downregulates dickkopf homologue, which is a canonical Wnt pathway antagonist[17]. Msx2 and Wnt balance each other to regulate expression of both pathways and does so in a bidirectional way[17].

Wnt signaling also plays a role in bone development and homeostasis that maintains a highly proliferative state for embryonic skeletal development. When there is loss of Wnt

coreceptor LRP5, there is a reduced bone mass, fragility, and overall decrease in bone mass and reduced osteoblast proliferation[17]. This suggests the dependence for LRP5 to regulate osteogenesis. In studies, mice that lack LRP5 had a decrease in calcification in the aortic valve and had a decreased osteoblast apoptosis which demonstrates how the high bone mass phenotype occurs due to increased osteoblast cell count. LRP5 has also been shown to affect the genetic variation in exons 10 and 18 which may be able to modulate Wnt signaling as well[17]. With the study of atherosclerosis, results have shown mutations in these receptors that impairs Wnt signaling that demonstrates the genetic link between LRP5, Wnt signaling, and coronary artery disease.

Usage of Sclerostin in Wnt signaling

Sclerostin is a protein that is encoded by the SOST gene, which is detected in many organs including bone, cartilage, kidney, liver, heart, and fetal skin[18]. Sclerostin in the human body is normally produced by osteocytes and cemetocytes that has been recently detected in the aorta of patients who undergo aortic valve replacement and is normally upregulated in calcifying VSMCs and calcified valvular plaques[18]. Osteoprotegerin is one of the decoy receptors that is an inhibitor of bone resorption and acts as a Wnt target gene[18]. Sclerostin antagonizes the canonical Wnt signaling pathway which also in return regulate Osteoprotegerin[18]. With this, the overexpression of sclerostin in osteocytes increase intracortical remodeling and osteoclasts that is accompanied by a decrease in osteoprotegerin expression[18]. Wnt/β-catenin signaling has been shown to inhibit osteoclast formation directly with sclerostin as a key player on osteoclast differentiation[9]. Another role that sclerostin demonstrates is how it interacts with members of BMP and Wnt pathways such as BMP4, BMP2, and Lrp[18]. It has been shown that the binding of sclerostin has reduced calcification in CKD and regulates adipocyte differentiation[18]. This is done when the

canonical Wnt signaling pathway binds to a low-density lipoprotein receptor-related protein LRP 5/6 which can also inhibit osteoblast differentiation[18]. The mediation of sclerostin is most closely associated with the regulation of bone acquisition with the intracortical remodeling and inhibition of osteoclast formation[20]. There is still future research that is to be done for the role of Wnt β-catenin independent roles of sclerostin and how it affects pathways in vascular calcification[9].

Inhibition of Wnt Signaling through Stabilization of Axin

Axin is one of the most important scaffolding proteins that is responsible for the formation of the β-catenin destruction complex[21]. Stabilization of axin protein is regulated by ubiquitin-proteasome system, and modulation of Axin is impactful for Wnt/β-catenin signaling[21]. In the Wnt/ β-catenin signaling pathway, the signaling pathway that has an important role in embryonic development and adult tissue homeostasis regulates the transcription cofactor β-catenin[21]. When Wnt is absent β-catenin binds to the β-catenin destruction complex that also contains the protein Axin. Axin directly interacts with cofactor β-catenin which is responsible for the destruction complex that controls the protein level that Axin has an impact on the Wnt/β-catenin signaling[21]. There have been CRISPR testing that is done to discover the unknown regulators of Wnt/ β-catenin signaling[21]. The testing was done in HEK293T cells that expressed Cas9 and a transcription report that has multiple TCF binding sites. These were treated with a conditioned medium that then showed a positive regulator of Wnt/β-catenin singaling, USP7[21]. Since Axin is a key scaffolding protein in the β-catenin destruction complex, USP7 has been crucial for its negative role in Wnt/ β-catenin signaling. USP7 has been shown to enhance the degradation of Axin1 through a knockout[21]. Since USP7 negatively regulates Wnt signaling, there could be a possibility that it could inhibit Wnt induced osteoblast differentiation. By using CRISPR, p53

abundance is knocked out to observe the Wnt3a-induced accumulation of active β-catenin in MSCs. Studies have recently shown that treatment of the USP7 inhibitor Almac4 increased accumulation of active β-catenin without exogenous Wnt since it inhibits the differentiation though attenuating Wnt/ β-catenin signaling[22]. This suggests that USP7 could also affect Wnt-induced osteoblast differentiation since Almac4 is an alkaline phosphatase marker of osteoblast differentiation[23]. Axin is an antagonist of Wnt signaling and further CRISPR testing could reveal more regulators that may help regulate β-catenin and may inhibit calcification as a result[22].

Role of Endostatin as Potential Inhibitor of Wnt Signaling

Endostatin is a fragment of collagen XVIII that has antiangiogenic activity[24]. Angiogenesis is the formation of new capillaries which is one of the primary processes responsible for tumor neovascularization[24]. This depends on the effects of pro and antiangiogenic molecules which is a recombinant ES that inhibits tumor growth in several animal models. It has been useful for human cancer treatment in clinical trials and high levels of endostatin has been shown to potentially inhibit endothelial cell migration and stop the cell cycle by inhibiting TCF-dependent transcription[24]. There have been new studies where endostatin is not only being tested for usefulness in cancer, but also its effects on Wnt signaling by testing phenotypes that are characteristic of embryos where Wnt signaling has been inhibited by depletion of β-catenin[24]. Doses of injected endostatin have been used into frog dorsal vegetal blastomeres that contribute to dorsal development[24]. The endostatin appears to affect the secondary body axis and overexpresses GSK3 which is a well characterized inhibitor of β-catenin. Endostatin also demonstrates properties that activates a molecular pathway that can lead to β-catenin degradation. Endostatin also regulates β-catenin by stabilizing upstream components of the Wnt

pathway Dsh, axin, and GSK3[24]. It can also inhibit Wnt signaling depending on Glypican 1, since cells with Glypican 1 fail to down-regulate TCF reporter activity in response to ES. Because Wnt signaling is also involved with endothelial cells and increases cell proliferation, endostatin has the potential to also show that endothelial cell migration and cell cycle progression depend on the activation of TCF responsive genes by regulation of endostatin[25]. Although it is currently a cancer treatment, it may prove useful to inhibit Wnt signaling as endostatin has been tested many times already as an angiogenesis inhibitor[26].

Other antagonists of Wnt Signaling

There are a variety of potential canonical Wnt signaling pathway inhibitors that are being investigated that could be future possibilities of treating vascular calcification[27]. There are secreted Wnt inhibitors that antagonize Wnt signaling by preventing ligand-receptor interactions. Dkks are a small family of conserved secreted glycoproteins that are embryonic head inducers and Wnt antagonists[27]. They specifically inhibit the Wnt/β-catenin signaling cascade that can inhibit both canonical and noncanonical Wnt signaling by the Dkks binding to Fz and preventing signal transduction[27]. The sFRP protein is also one of the largest families of secreted Wnt inhibitors and resemble ligand-binding CRD domain of the frizzled family of Wnt receptors that binds to Frzb and then to Wnt1 to inhibit Wnt/ β-catenin signaling[27]. Cerberus is also another protein that behaves as an antagonist of the transforming growth factor family[28]. The Cerberus protein functions as a multivalent growth factor antagonist by binding to Wnt proteins via independent sites[28]. The expression of Cerberus is activated by nodal-related signals in endoderm by Spemann-organizer factors that repress signaling by BMP and Wnt[29].

Conclusion

In conclusion, while the canonical Wnt signaling pathway is one of the most studied pathways that contribute to vascular calcification, inhibiting this pathway is still being investigated for treatment of diseases such as atherosclerosis and chronic kidney disease. Vascular smooth muscle cells display a wide range of phenotypes in two states of contractile and synthetic. High phosphate and calcium environments can induce calcification in VSMCs and direct them towards an osteogenic phenotype. When this trans differentiation occurs, it is due to a loss of VSMC markers and an increase in osteogenic markers, such as Runx2. Runx2 is a target gene of Wnt signaling which involves proteins such as Dsh, Fzd, and Axin. Currently, there are studies being conducted to understand what inhibits canonical Wnt signaling and if there could be any possible treatments that could be brought to clinical trials. Right now, the investigation of Axin stabilization and endostatin have been tested the most as endostatin is a current cancer treatment and could be tested for atherosclerosis. Furthermore, if this proves to be effective with other studies of inhibitors of Wnt signaling, there could be a possibility of treatment for vascular calcification since the information for the Wnt signaling pathway has been explored. As a result, additional research and testing is needed for understanding of these alternative Wnt inhibitors discussed. For example, experiments that have been conducted in studies utilized the effect of endostatin on β-catenin activity. This is a mediator of Wnt signaling and is known to induce a secondary axis when overexpressed in Xenopus embryos. An injection of β-catenin RNA and endostatin into a ventral blastomere of embryos would demonstrate inhibition of the Wnt pathway by the lessoning number of endothelial cells. Also, additional testing of Axin as a negative regulator has been conducted in observing patterns during mouse embryogenesis and organogenesis. These expressions overlapped with several Wnt genes and through the inducing of Wnt signaling and the promoter and intron of a DNA fragment, the binding sites of Tcf/LEF

greatly diminished, suggesting a negative feedback loop to limit the intensity of the Wnt-initiated signal. This literature review has summarized the current understanding of vascular calcification, the canonical Wnt pathway, and has explored some options to inhibiting Wnt signaling. To our knowledge, there is not a clinically tested option for inhibiting Wnt signaling and treating calcification, but if these potential options are studied and tested, the future holds possibilities for combating this deadly disease.

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