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Effect of BMP-9 on endothelial cells and its role in atherosclerosis

Hamid Hassanisaber[1], Leonie Rouleau[2], Nathalie Faucheux[1,3]

[1]Cell-Biomaterial Biohybrid Systems, Department of Chemical and Biotechnological Engineering, Universite de Sherbrooke, 2500 boulevard de l'Universite, Sherbrooke, QC, Canada, J1K 2R1, [2]Department of Mechanical Engineering, Universite de Sherbrooke, 2500 boulevard de l'Universite, Sherbrooke, QC, Canada, J1K 2R1, [3]Clinical Research Centre, Centre Hospitalier Universitaire de Sherbrooke, 12e Avenue N., Sherbrooke, QC, Canada, J1H 5N4

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1. ABSTRACT

Atherosclerosis is an inflammatory disease involving dysfunction of endothelial cells (EC) and enhanced permeability of the endothelium to oxidized low-density lipoprotein and the transmigration of monocytes from the blood to the intima where they are transformed into foam cells after lipid engulfment. Changes in the composition of the basement membrane leading to increased fibronectin deposition also occur and modify EC-extracellular matrix (ECM) mechanotransduction. The release of lipids due to foam cell apoptosis, as well as the migration of vascular smooth muscle cells from the media to the intima and their proliferation, increase the stiffness of arteries at later stages of atherosclerosis. EC dysfunction also involves other factors, including soluble cytokines and growth factors (GF) such as bone morphogenetic proteins (BMP). BMP-9 is a potent circulatory GF which has been shown to affect EC behavior. However, to date, few studies have investigated its role in atherosclerosis. The present review describes the histology and homeostasis of arteries by explaining EC function/dysfunction and discusses BMP-9 effect on EC behavior, considering factors engaged in the development of atherosclerosis.

2. INTRODUCTION

The maintenance of the proper functioning of the body is a complicated process known as homeostasis. Cardiovascular homeostasis is necessary for controlling the balance of vital parameters in blood vessels, including pressure, volume, temperature, and pH (1). It ensures the transport of hormones, oxygen, nutrients, and metabolic products in the blood circulatory system (2). The functionality of ECs, which line the interior wall of blood vessels and form the endothelium, is thus crucial for cardiovascular homeostasis. For example, the integrity of the endothelium is required to conserve the selective migration of leukocytes such as monocytes into the intima during inflammatory responses and to reduce the risk of thrombosis (3, 4).

EC injury and dysfunction can result in atherosclerosis, an inflammatory disease that paves the way for cardiovascular disease (CVDs) (2, 5-8). CVDs are the leading cause of mortality worldwide, with cancer being the second (9). Heart disease is the second leading cause of death in Canada, and heart failure costs the Canadian economy $3 billion a year (10, 11). Many GFs, cytokines, and chemokines influence EC function. Of these, BMPs have an impact on EC survival and proliferation (12). BMP-9 is of particular interest as the active form is present in blood plasma at high concentrations (13, 14). It can regulate the effects of several factors known to be involved in EC dysfunction during atherosclerosis. These factors range from soluble molecules to ECM components and cells, including oxidized low-density lipoprotein (ox-LDL), soluble cytokines such as TNF-alpha and VEGF, basement membrane (BM)/ECM proteins such as fibronectin (FN), wall shear stress (WSS), and vascular smooth muscle cells (VSMCs) (15-26). A review of currently available literature on the roles played by BMP-9 in EC function/dysfunction is thus of great interest and will also provide the scientific community with insights into the potential roles of BMP-9 in the genesis and development of atherosclerosis.

The present review thus provides a brief INTRODUCTION to arterial histology and the location of atherosclerotic plaque as well as vascular cell functions. EC-ECM and EC-EC interactions are explained by describing the molecules involved in these interactions, including integrins, adhesion receptors, and vascular endothelial VE-cadherin as they play a crucial role during EC inflammatory responses and the progression of atherosclerosis. The structures, the canonical Smad and MAPK signaling pathways induced by BMP family members, and their crosstalk with the Wnt and Notch pathways are addressed. Lastly, a comprehensive summary of the effect of BMP-9 on EC function (quiescence, sprouting/vessel formation, and inflammation) and on atherosclerotic factors is provided.

2.1. Histology of arteries

2.1.1. Tunica

Arteries, which carry blood away from the heart, are comprised of three main layers or tunica. The innermost layer is the tunica intima (or interna). The next layer is the tunica media, while the outermost layer is the tunica adventitia (Figure 1) (2). The tunica intima is covered with a monolayer of ECs (the endothelium), which is in direct contact with the blood flow. The intima is also comprised of a BM, which is a layer of connective tissue in the sub-endothelial region that is part of the ECM connected to ECs (2, 27). The connective tissue provides the intima with structural flexibility (2). The tunica media is mainly responsible for adjusting the size of the lumen by contracting/releasing VSMCs, which are highly specialized spindle-shaped cells whose main function is contraction beneath the intima to preserve blood pressure (2). ECs partially modulate this process by secreting NO, a free radical with short half-life that induces the constriction of VSMCs. NO production can be increased by various stimuli such as histamine and WSS through the upregulation of eNOS, the enzyme responsible for NO production (4, 28). The tunica adventitia provides structural support for arteries and is rich in fibrillar type I and III Collagen (Coll) and elastin (29, 30). Coll in the

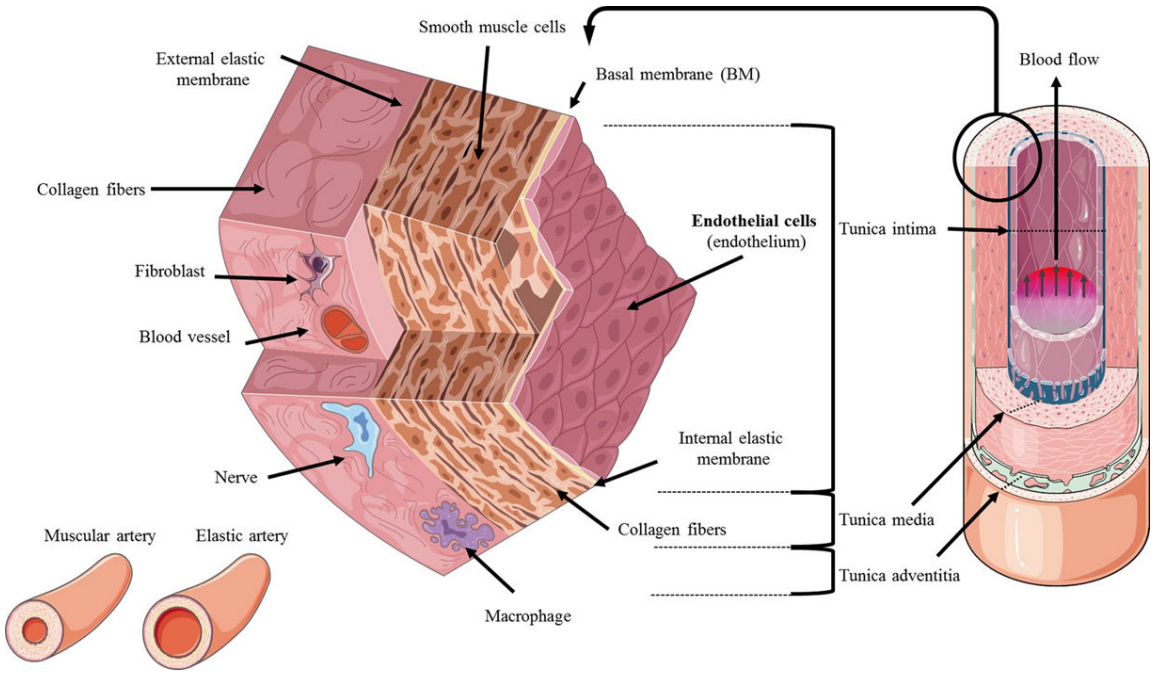

Figure 1. Histology of elastic and muscular arteries, the figure is adapted with permission from (2) [https://creativecommons.org/licenses/by/4.0/]. [The figure was created using Servier Medical Art. https://smart.servier.com.].

adventitia provides a supporting framework whereas elastin is responsible for the uniform distribution of stress that enables arteries to better resist mechanical forces (31). The adventitia fixes arteries in their location by providing linkages with the surrounding connective tissue (2). Arteries can be categorized into two major forms based on the thickness and composition of these three layers: elastic and muscular (2). Large diameter arteries (> 10 mm) are considered elastic because they contain higher levels of elastin in the tunica. They are located closer to the heart and are thus subject to higher pressures. The mechanical properties of elastic arteries allow them to resist changes in blood pressure caused by systole and diastole (~120/80 mmHg) (2). The walls of muscular arteries are generally the thicker of the two forms. In regions more distant from the heart, there is not much need for arteries to expand as the blood pressure drops. These arteries thus have fewer elastic fibers in their intima and higher numbers of VSMCs in their media and are smaller in diameter than elastic arteries (0.1 mm to 10 mm) (2, 32).

Atherosclerotic plaques preferentially develop in the arterial tree in curves, branching points, and bifurcations, which are associated with recirculating eddies and changes in WSS direction and magnitude (33). These alterations in WSS can affect EC function and can trigger the development of atherosclerosis (24, 33-36). Although atherosclerosis occurs mainly in large and midsize arteries, it can also occur in peripheral arteries (2).

2.2. EC-BM interactions and EC-EC contacts

ECs can recognize and interact with BM proteins through specific transmembrane receptors called integrins. Human ECs express integrins, including alpha5 beta1, alphav beta3, alpha1 beta1, alpha2 beta1, alpha3 beta1, alpha6 beta1, and alphav beta5 (37, 38). Integrin/ECM interactions influence EC behavior such as cell survival, proliferation, and migration (39-44).

2.2.1. Integrins

Integrins are composed of non-covalently linked heterodimeric alpha and beta subunits (45). Their interactions with specific ECM proteins induce the recruitment of cytoplasmic structural proteins (talin, vinculin, and kindlin) and the activation of FAK and Src signaling proteins by an outside-in mechanism (Figure 2) (46). However, the activation state of integrins is regulated by inside-out signaling. For example, talin binding to the cytoplasmic tail of resting integrins activates the integrins by forcing the extracellular segments of the alpha and beta subunits away from cell membrane where they can bind to their ECM ligands (45-47).

Integrin activation can be followed by clustering and the formation of nascent adhesions. These adhesions can develop into FXs that can mature to form FAs (48). FAs contain both structural

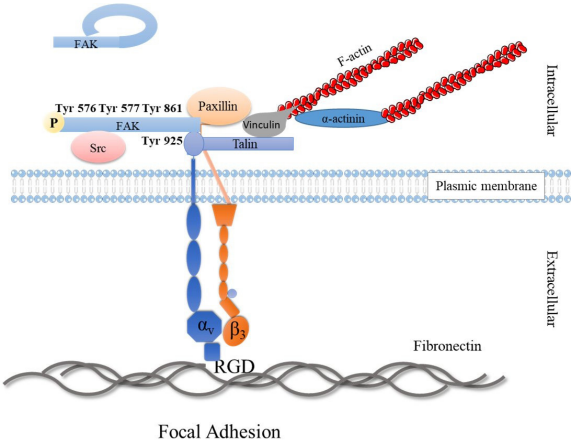

Figure 2. Organization of a focal adhesion, the figure is adapted with permission from (53). [The figure was created using Servier Medical Art. https://smart.servier.com.].

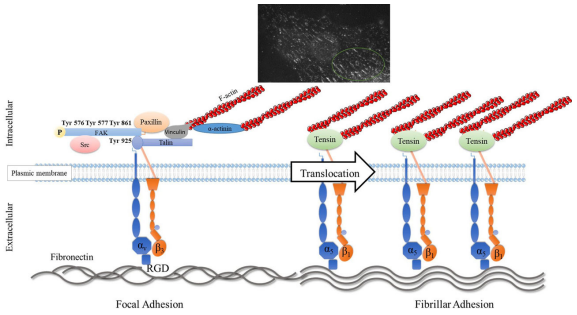

Figure 3. Formation of fibrillar adhesions, adapted with permission from (67). [The figure was created using Servier Medical Art. https://smart.servier.com.].

cytoplasmic proteins (e.g., vinculin) and cytoplasmic signaling proteins (e.g., FAK) (40). FAs also connect the actin cytoskeleton to the ECM (45, 46, 49, 50). When signaling proteins come into the vicinity of FAs, they induce various signaling transduction pathways, including Rho GTPase (51). These pathways can ultimately affect the arrangement of the actin cytoskeleton (41). Certain soluble factors such as VEGF and TNF-alpha can induce the rearrangement of the actin cytoskeleton to form stress fibers. This is associated with EC activation (16, 23, 52).

2.2.2. Composition of the BM in normal and pathological conditions

The ECM is responsible for various tasks, including maintaining the structural integrity of vessels and modulating cell functions through signaling pathways (53, 54). The BM is a specialized ECM with an amorphous, dense, sheet-like structure (55, 56). In physiological conditions, the BM of the endothelium contains Coll IV, laminin, nidogens, and perlecan (56). However, in atherosclerotic lesions, the BM is enriched in FN and Coll I (57).

2.2.2.1. Fibronectin

FN is a glycoprotein composed of two quite similar subunits linked at their C-terminal extremities by disulfide bridges. This dimeric protein (~440-500 kDa) contains approximately 2300 amino acids that are mainly divided into three repeating modules, i.e., homology sequence types I (12), II (2), and III (15-17) (58, 59). FN can bind to other proteins such as Coll and fibrin as well as to glycosaminoglycans such as heparin (60-62). The FNIII-10 module is an important motif as it contains the RGD-loop that binds to many integrins in ECs, including alpha5 beta1 and alphav beta3 (63, 64). The FNIII-9 module contains a synergy site (proline-histidine-serine-arginine-asparagine sequence, PHSRN) that, upon interacting with the FNIII-10 module, can significantly enhance the affinity of alpha5 beta1 integrin for FN (64-66). The alpha5 beta1 integrin can also translocate from the periphery (FA) to the center of cells to form fibrillar adhesions, which allow the cell to transform soluble FN into insoluble fibrils by inducing FN molecule stretching (Figure 3) (67, 68). This also unmasks cryptic binding sites that are mostly available at the N-terminal domain, especially FNIII modules such as III1-2 (68-70). Raitman *et al.* used CHO-677 cells to identify the key role played by heparin/heparan sulfate binding sites in FN fibrillogenesis (71).

By binding to alpha5beta1 integrin, FN can also regulate the role of ox-LDL in changing ECs into a pro-inflammatory phenotype by enhancing the activation of a pro-inflammatory transcription factor (NF-kappaB) and the expression of VCAM-1 (15). It also induces the Rho signaling pathway, reducing the integrity of the EC barrier by disrupting AJs (23).

2.2.2.2. Collagen

Members of the collagen family are the most ubiquitous ECM proteins (54). So far, 27 Coll proteins have been identified. Coll proteins usually contain a triple helix of polypeptide chains (54, 72). For example, two identical alpha1(I)-chains and one alpha2(I)-chain make up the triple helix of Coll I, the most abundant Coll protein in the body (54,73). Coll I maintains the structural integrity of the ECM. It also contains an RGD sequence that can be recognized by alpha1 beta1 and alpha2 beta1 integrins on ECs and that can influence actin stress fiber formation (73-75). Rother *et al.* used PAECs seeded on a 3D hydrogel scaffold made of Coll I to show that this interaction can promote EC proliferation (76). Coll I also interacts with other ECM proteins (FN, Coll V) and glycosaminoglycans (54, 58, 62, 73, 77).

2.3. EC-EC adhesion and vascular permeability

The passage of circulating cells such as leukocytes and soluble molecules such as LDL from

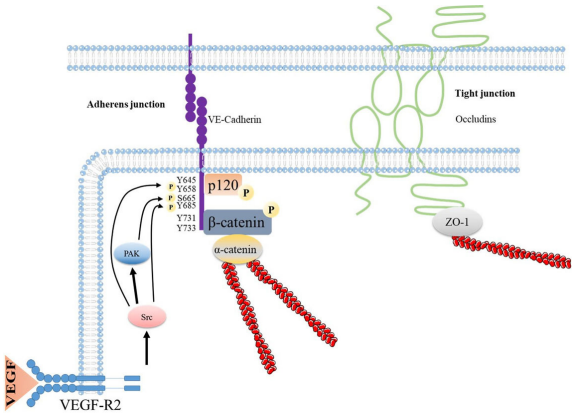

Figure 4. Tight and adherens junctions and phosphorylation of VE-cadherin by VEGF signaling, adapted with permission from (79-82). [The figure was created using Servier Medical Art. https://smart.servier.com.].

the blood to the intima depends on the integrity of the endothelium. Injuries to or dysfunctions of the EC barrier affect vascular homeostasis by disrupting the selectivity and effectiveness of the barrier (8, 78). The main cell-cell contacts in ECs include AJs, TJs, and GJs (Figure 4).

The present review focuses primarily on AJs because of the key role they play in vascular permeability (4, 83-86). However, more information on TJs and GJs can be found in the articles by Radeva and Waschke (2018) and Komarova *et al.* (2017) (87, 88).

The integrity of AJs is closely associated with VE-cadherin, a transmembrane adhesion protein (83, 85). Cadherins are classified as type I and type II. Type II cadherins lack the histidine-alanine-valine (HAV) motif, which prevents cadherin-mediated cell aggregation (89, 90). VE-cadherin is a type II cadherin and contains an extracellular cadherin domain (EC-domain) and a cytoplasmic tail (C-terminus). The EC-domain of VE-cadherin mediates calcium ions dependent hemophilic binding between ECs, whereas its C-terminus can bind to catenins and stabilize AJs (85, 91, 92). Cortical actin filaments aligned with the cell surface are associated with AJs through VE-cadherin/catenin complexes (beta, alpha, and gamma-catenin). Quiescent ECs in the endothelium contain continuous VE-cadherin/catenin complexes at the cell periphery (23, 83). Intense tyrosine phosphorylation of VE-cadherin has been reported in non-attached and migrating ECs with disrupted AJs (91, 93-96). In contrast, VE-cadherin/beta-catenin complex formation increases when the cytoplasmic tail of VE-cadherin is phosphorylated on a serine (83).

Soluble cytokines such as TNF-alpha and GFs such as VEGF can enhance endothelial permeability by phosphorylating VE-cadherin. This results in a weaker EC barrier by inhibiting AJs and increasing leukocyte recruitment/extravasation through the endothelium as a result of an immune response (4, 27, 97-99). However, the maintenance of cortical actin filaments can inhibit these EC barrier (AJ) disruptions (100). In addition to soluble GFs and cytokines, mechanical cues such as WSS in bovine aortic ECs can affect the stability of AJs by increasing the tyrosine phosphorylation of VE-cadherin (101, 102).

3. EC FUNCTIONS

3.1. Blood fluidity

ECs maintain the non-adhesive nature of the lumen in order to maintain blood fluidity in vessels. ECs have antithrombotic (preventing thrombosis or local blood clotting), anticoagulant (preventing the activation of the coagulation cascade), and fibrinolytic (hydrolyzing fibrin) activities (4, 103). For example, ECs synthesize thrombomodulin (TM), which prevents blood coagulation by binding to thrombin, which is responsible for turning soluble fibrinogen into an insoluble fibrin network (2). ECs also produce PGI2 and NO, which limit platelet activation and aggregation and thus clotting (4, 103). They also synthesize plasminogen activating factor, which is responsible for the conversion of plasminogen into plasmin, the protein involved in fibrin cleavage (103).

However, in damaged vessels, ECs can secrete other factors that can cause blood clotting (104). For example, vWF is synthesized by ECs following vascular injury to provide needed clot formation. This occurs as result of vWF-mediated platelet-collagen and platelet-platelet adhesion leading to platelet aggregation at the injury site (105-108).

In addition to the roles played by ECs in the regulation of hemostasis, ECs also play a role in vascular tone. This occurs mainly via NO-mediated EC-induced contraction of VSMCs, which alters the luminal cross-section and, in turn, blood pressure (4, 109, 110).

3.2. Inflammatory response

Inflammation (acute and chronic) weakens the EC barrier and causes an increase in the migration of circulating leukocytes through the endothelium as part of the immune response (4). Leukocyte tethering to the EC monolayer is essential for the proper functioning of the innate and adaptive immune responses (83). Local inflammatory cues in particular can initiate the recruitment of neutrophils and monocytes (which are leukocytes of the innate immune system) from the blood stream that can, in turn, initiate the adaptive immune response and prevent the progression of infections

(111). The inflammatory response relies on interactions between ECs and leukocytes that are promoted by the expression of E-selectin, ICAM-1, VCAM-1, and MCP-1 by ECs (80). An increase in the expression of these proteins notably occurs in response to soluble TNF-alpha via NF-kappaB activation (8, 24, 98, 99, 112).

Transcellular (through a cell) and paracellular (between cells) transport mediate the passage of isolated circulatory leukocytes once they adhere to ECs (80, 83). Paracellular passage, also known as leukocyte diapedesis, occurs when leukocytes squeeze through EC-EC junctions whereas transcellular passage occurs via the cytoplasm of ECs (vesicular system) (83). EC-EC junctions normally reassemble quickly after paracellular transport to maintain the integrity of the EC barrier (83). Leukocytes use the transcellular pathway to pass through approximately 40% of cultured human umbilical vein ECs (HUVEC: a common EC line for *in vitro* models) (111). PECAM-1 (or CD31) is also intimately involved in leukocyte extravasation and is expressed on EC, monocyte, and neutrophil membranes (4). PECAM-1 is initially concentrated at EC-EC junctions to protect endothelial integrity. However, it can facilitate leukocyte extravasation through homophilic (i.e., CD31 on the leukocyte surface) or heterophilic (alphav beta3 integrin on the neutrophil surface) binding (4, 113).

3.3. Angiogenesis

Angiogenesis is a process that results in the production of new vessels and normally occurs following metabolic modifications in the body such as changes in oxygen supply (114, 115). In contrast, vasculogenesis is a process related to embryonic development by which new vessels are formed as result of the differentiation of mesodermal cells into endothelial progenitor cells (114). ECs play an essential role in forming new vessels during angiogenesis, which is initiated by the activation and migration of ECs (116). EC activation can occur as result of various events, including changes in the gradient of soluble cytokines secreted by monocytes/neutrophils (e.g., TNF-alpha) and soluble GFs secreted by macrophages, parenchymal cells, cardiomyocytes, skeletal muscle fibers, and hepatocytes (e.g., VEGF) (114, 115, 117). When ECs are activated, the integrity of the endothelium weakens under the influence of NO produced by ECs, which paves the way for the other steps required for the formation of new vessels such as the dilation of pre-existing vessels, the removal of the BM by MMPs, and the migration of ECs to form tip/stalk cells (16, 115, 118-120). During angiogenic sprouting, ECs separate into two distinct phenotypes: (a) tip cells, which are migrating polarized cells that acquire long filopodia that, in turn, direct new sprouts, and (b) stalk cells, which are proliferating cells that follow tip cells to form the lumen of new sprouts (121). VEGF activates VEGF-Receptor 2 (VEGFR2) in ECs and triggers a selection process in which ECs form distinct tip or stalk cells. The Notch signaling pathway inhibits VEGFR2 activation, which is common in stalk cells (121).

For the new tubes to mature and stabilize, ECs secrete PDGF, which causes the differentiation of adjacent mural cells into VSMCs in large vessels and pericytes in small vessels. The maturation process includes the deposition of proteins such as laminin that are required for EC quiescence (121, 122).

Angiogenesis plays an important role in the development of atherosclerosis as new vessels in atherosclerotic lesions contribute to plaque growth. However, the fragility of new vasculature can undermine plaque stability, resulting in thromboembolism (123).

3.4. EC dysfunction in atherosclerosis

Atherosclerosis is a chronic inflammatory disease that has a causal association with EC activation (5, 7, 8). Many risk factors are associated with the activation of ECs and atherosclerosis, including hyperlipidemia, smoking, chronic infections, diabetes, aging, and genetic predisposition (6, 24). EC activation weakens the EC-EC junction, which enhances the passage of circulating LDL into sub-endothelial regions. This is followed by a higher expression of adhesion molecules (VCAM-1, ICAM-1, MCP-1, P-selectin), an increase in the extravasation of leukocytes such as monocytes (8, 23), and an increase in pattern-recognition receptors (scavenger and toll-like receptors) on macrophages in sub-endothelial regions (8). When scavenger receptors engulf LDL oxidization products and accumulate cholesterol buildups, this leads up to the formation of foam cells, which are predominantly present in fatty streaks in the core of atherosclerotic plaque. LDL oxidization and heat shock protein 60 (HSP 60) increase the activation of toll-like receptors and the production of proteases by macrophages (8). MMPs and cysteine proteases enhance the risk of plaque rupture by disrupting Coll fibers (6, 8). The expression of MMPs also depends on endothelial-to-mesenchymal transition (EndMT), which involves the TGF-beta family and hypoxic conditions (124-126). Evrard *et al.* used a model of atherosclerotic mice to show that EndMT-derived fibroblasts make up to 9% of intimal plaque (125). An accumulation of MMP-secreting EndMT-derived fibroblasts may be a possible cause of atheroma plaque destabilization (125).

MMPs such as MMP-9 can also favor the proliferation and migration of VSMCs by promoting anchorages between the cell surface and the matrix (77). VSMCs in the intima play two roles, depending on the stage of atherosclerosis. They induce plaque

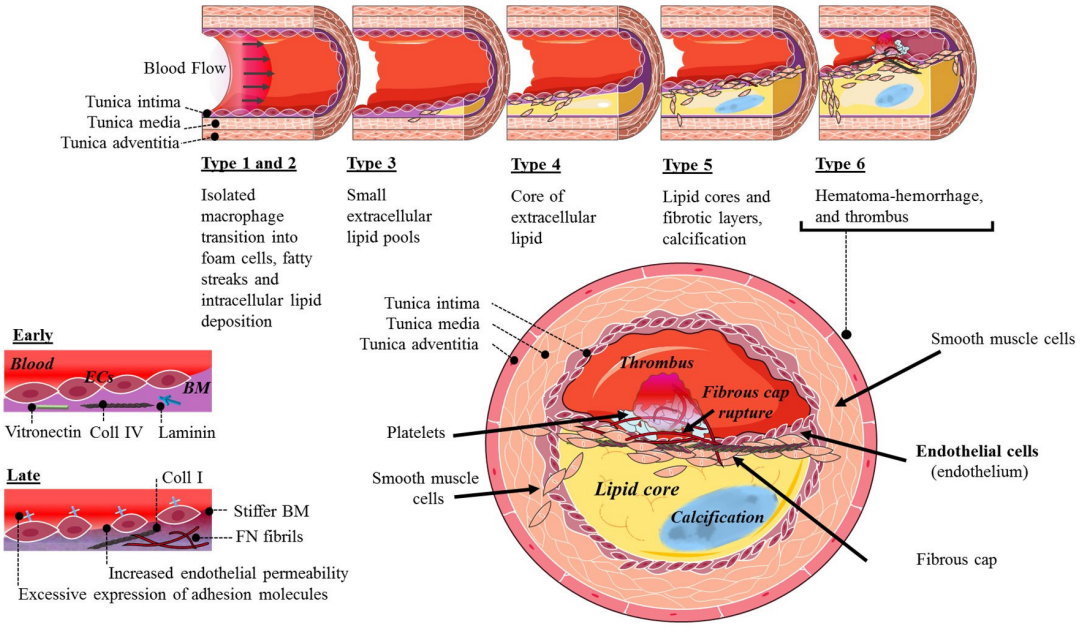

Figure 5. Atherosclerosis stages, adapted with permission from (133). [The figure was created using Servier Medical Art. https://smart.servier.com.].

formation by excessive proliferation in the early stage of atherosclerosis, and they contribute to plaque stabilization by reinforcing the fibrous cap in advanced lesions (127, 128). However, the engulfment of ox-LDL by VSMCs can alter their ability to synthesize and organize ECM proteins such as fibrillar FN and subsequent FN-induced Coll I fibrillogenesis (62, 129). To summarize, as atherosclerosis progresses, both ECs and VSMCs undergo phenotypic changes.

The calcification of VSMCs is also a key aspect of the development of atherosclerosis as it contributes to arterial stiffening and increases the chance of luminal thrombosis (e.g., coronary artery), potentially causing a stroke (130-132).

An increase in stiffness makes arteries less expandable when they need to dilate during systole to accommodate the surge of a large volume of blood from the left ventricle (stroke volume). This lack of compliance does not allow arteries to expand, resulting in higher resistance and thus higher blood pressure (2).

Atherosclerosis is classified into six stages or lesions depending on the gravity of the disease as defined by the American Heart Association (Figure 5) (133). The stages are as follows: 1. initial or type 1 lesions, which involve the transition of isolated macrophages into foam cells (fat-laden macrophages); 2. type 2 lesions, which are typified by the formation of fatty streaks under the endothelium due to the deposition of intracellular lipid rather than extracellular lipid build-up; 3. type 3 or intermediate lesions, which occur when small extracellular lipid pools appear; 4. type 4 or atheroma lesions, which have an extracellular lipid core; 5. type 5 or fibroatheroma lesions, which have lipid cores and fibrotic (excess fibrous connective tissue) layers; and, lastly, 6. type 6 or completed lesions, which cause surface defects, hematoma-hemorrhage, and thrombus (133). Type 1, 2, and 3 lesions are considered clinically silent, while type 4, 5, and 6 lesions can be clinically silent or can become clinically overt. Type 1 and 2 lesions mostly develop during the first decade of life while type 3 and 4 lesions progress during the third decade. Type 5 and 6 lesions occur during the fourth decade of life (6).

Atherosclerosis may lead to many cardiovascular conditions. Plaque formation on the arterial wall causes ischemia by disrupting the blood flow and the supply of nutrients and oxygen (hypoxia) (2, 134, 135). Hypoxia can be followed by the decay of associated tissues, which can have clinically grave consequences if it impairs the activity of the heart or brain (2). Plaque rupture accompanied by bleeding in the arterial wall may also occur and result in clot formation and thrombus (2). Clots in coronary arteries can occlude the blood stream and lead to stroke or myocardial infarction while clots in cerebral arteries can cause cerebrovascular accidents (CVA) (2). Normally, thrombus formation is more dangerous

than stenosis (narrowing) given the consequences of strokes (8).

The blockage of smaller arteries is another potential outcome of atherosclerosis. If plaques are damaged, debris and fragments can travel in the blood and obstruct smaller arteries along the way (6, 134, 135). Blockages in arteries carrying fresh blood to the legs can cause leg pain (claudication), changes in skin color, and sores and may lead to gangrene (6, 136).

Atherosclerosis lesions can also trigger aneurysms (abnormal swellings and localized dilations of arteries). Aneurysms can occur in the aorta, causing discomfort and pain, as well as in the brain (2,133-137).

4. BONE MORPHOGENETIC PROTEINS

The functions of the EC layer in blood vessels are affected by WSS patterns and values and, like all adherent cells, the behavior of ECs also depends on the composition and stiffness of the BM (23-25, 138). Cytokines in the EC microenvironment are also crucial players (16). BMPs are potent regulators of EC function (12, 14, 139). BMPs, BMP-9 in particular, not only affect EC behavior directly but also regulate the impact of other players on endothelium physiology, including FN, TNF-alpha, and VEGF (17, 18, 20, 22). It is thus important to determine how BMP-9 influences EC behavior during atherosclerosis, both directly and indirectly.

To date, 20 distinct BMPs have been identified, 14 of which are produced in the human body by various cell types such as osteoblasts, MSCs, and ECs (12, 140, 141). BMP-1, a C proteinase precursor of collagen composed of 730 amino acid residues, is a metalloproteinase, whereas other BMPs belong to the TGF-beta superfamily, which includes TGF-beta and activins (142-144).

4.1. BMP subgroups

Members of the BMP family can be divided into four main subgroups based on their amino acid sequence homology (145). The main BMP subgroups are: (1) the BMP-2/-4 subgroup (up to 83% amino acid sequence homology); (2) the BMP-5, -6, -7, -8, -8b subgroup; (3) the BMP-9 (growth/differentiation factor 2, GDF-2) and BMP-10 subgroup; and the BMP-12, -13, -14 subgroup (67, 146, 147).

TGF-beta family members share some common structural features such as a cysteine knot (six residues), a wrist epitope (four-turn alpha helix in the dimerization region), and a knuckle epitope (four antiparallel beta sheets emanating from a cysteine knot) (148, 149). The majority of BMPs are synthetized as ~500-amino-acid pre-pro-peptides with a signal peptide, a pro-domain, and a mature domain containing seven cysteines (13). The signal peptide (N-terminal) enables BMP secretion, while the pro-domain facilitates effective folding (150). The Arg-X-X-Arg sequence in the C-terminal domain of BMPs allows them to form homodimers or heterodimers (e.g., BMP-2/5, BMP-2/6, and BMP-9/10) through disulfide bridges and to become active signaling molecules (151-154). Human mature BMP-9, which consists of 110 amino acids, normally does not lose the N-terminal pro-domain (155). In contrast, mature BMP-2 (114 amino acids) does not contain the pro-domain (142). Molecular dynamics and thermodynamic simulations have revealed that the structural differences between BMP-2 and BMP-9 are responsible for binding to distinct receptors (156). Interestingly, both BMP-9 dimers, with or without the pro-domain, are active and can initiate BMP signaling pathways (13, 14).

BMPs were first discovered by Urist in 1965 when he observed *de novo* bone formation in rabbit models resulting from the differentiation of osteoprogenitor cells stimulated by decalcified components of the bone matrix (157). However, several studies suggest that some BMP subgroups are more efficient at stimulating *de novo* bone formation than others (158, 159). For example, Kang *et al.* injected murine myoblast C2C12 cells infected with recombinant adenoviral vectors encoding BMP (AdBMP) in mouse quadriceps and found that BMP-6 and BMP-9 promote more bone formation than BMP-2 and BMP-7 (159). However, the role of BMPs is not limited to bone formation as they also participate in other processes such as embryogenesis and vascular homeostasis (139, 160). In terms of vasculogenesis during embryonic development, it has been reported that human BMP-4 contributes to the growth of the posterior intermediate cell mass (ICM, blood island, including endothelial precursor cells) in zebrafish embryos while BMP-9 can affect embryonic vascular development by reducing the expression of the gene encoding Tmem100 in hPAECs (124, 161). Tmem100 is a vital player in mice embryo vascular development since Tmem100-null mice and endothelial-specific Tmem100 knockout mouse embryos do not survive due to the lack of EC differentiation (162).

4.2. BMP signaling pathways

BMPs act on cells by binding to type I and type II Ser/Thr kinase receptors, which leads to the activation of both the canonical Smad and the non-canonical TGF-beta-activated tyrosine kinase, TAK1, and MAPK signaling pathways.

4.2.1. Canonical Smad pathway

The canonical BMP pathway (Figure 6) is referred to as Smad because of the similarity of

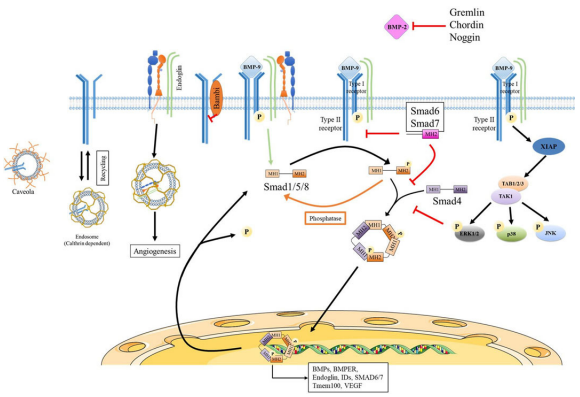

Figure 6. Canonical and non-canonical BMP signaling and regulation, adapted with permission from (53). [The figure was created using Servier Medical Art. https://smart.servier.com.].

the amino acid sequences of these proteins to the *Caenorhabditis elegans* SMA protein (small worm) and the MAD (Mothers Against Decapentaplegic) protein, which is a member of the basic helix-loop-helix leucine zipper family that influences the transcriptional activities of BMPs (163-165). BMPs trigger the association of type I and type II Ser/Thr receptors into hetero-tetrameric complexes (166). Type I Ser/Thr kinase receptors interact with the wrist epitopes of BMPs, whereas Ser/Thr kinase type II receptors interact with the knuckle epitopes (167). The binding of BMPs to type I and II receptors can also occur separately, i.e., where type I and type II receptors are not associated. However, BMPs have a higher affinity for hetero-tetrameric complexes (168).

Upon BMP binding, the Gly-Ser repeats of type I receptors are phosphorylated by Ser/Thr kinase type II receptors and, in turn, phosphorylate the intracellular receptor-regulated Smad 1/5/8 (R-Smad) (169). Phosphorylated R-Smad can then interact with the common-partner Smad (Co-Smad, Smad-4) molecule to create a hetero-dimeric complex that translocates into the nucleus (170). The transcriptional activity of Smad complexes can regulate the expression of numerous genes, including those encoding molecules involved in EC functions such as eNOS, MCP-1, VCAM-1, ICAM-1, E-selectin (CD62E), NF-kappaB, VEGF, endothelin-1, Id-1, Id-2, and BMPER (19-22, 124, 171-178).

4.2.1.1. Ser/Thr kinase receptors of BMPs and type III co-receptors

Type I and II Ser/Thr kinase receptors are composed of a short extracellular domain with a cysteine residue, a single transmembrane domain, and a Ser/Thr kinase in the intracellular domain. To date, seven type I (activin receptor-like kinases 1 to 7, ALK-1-7) and five type II (type 2 BMP receptor, BMPRII, type 2 activin receptor, ActRIIA, type IIB activin receptor, ActRIIB, TGF-betaRII, and anti-Mullerian hormone receptor type II, AMHRII) receptors have been identified (179, 180). ALK-1 and ALK-5 (TGF-beta receptor, TbetaRI) are both expressed in ECs, including HAEC, HUVECs and HMEC-1 cells (20, 180-184). However, ALK-1 is expressed preferentially in ECs while ALK-5 is ubiquitously expressed (182). Both BMP-9 and BMP-10 bind to ALK-1 in HMVEC-d, BOEC, and HAEC cells (22, 185). Johnson *et al.* analyzed the genetic profiles of five families and found that patients suffering from a genetic vascular dysplasia called Osler-Rendu-Weber syndrome or hereditary hemorrhagic telangiectasia present several mutations in the *ALK-1* gene (186).

Type III co-receptors are also influential in the BMP pathway (187). This subgroup of BMP receptors is composed of GPI-anchored RGM proteins such as the Dragon protein, which is expressed by COS-1 fibroblast cells (188-190) and the transmembrane protein ENG, which is expressed in ECs (190-193). BMP-9 strongly binds to ENG in ECs such as HMECs. Moreover, the ENG/integrin alpha5 beta1 complex can increase the surface density of ALK-1 by favoring endocytic recycling in MEECs (20).

4.2.1.2. Smad

Smad proteins have two conserved MAD-homology domains, one in the N-terminal (MH1, except for the N-terminals of Smad6 and 7) and one in the C-terminal (MH2). MH1 and MH2 are linked together by a linker domain (194, 195). The Type I Ser/Thr kinase receptor phosphorylates intracellular R-Smad on two Ser residues (Ser-Val-Ser motif, Ser-Met-Ser for Smad2) in the C terminus (169, 170). This pSer-X-pSer motif directs the association of R-Smad and the basic pocket in the Smad4 MH2 domain (170). The beta-hairpins in the MH1 domains of R-Smad and Smad4 are involved in binding to the DNA molecule (170). The MH2 domain of R-Smad also interacts with co-factors such as FoxH, FoxO, Mixer, OAZ, and Smad4, which can attach to DNA molecules and can thus also mediate the transcriptional activity of Smads (170). Both R-Smad and I-Smad also have a PY motif (polyproline-tyrosine rich motif) in the linker domain that paves the way for ubiquitination by Smurf ubiquitin ligases after its recognition by the Smurf WW domain. Ubiquinated Smads are then broken down by proteasome complexes (170, 196).

4.2.1.3. Regulation of Smad pathway activation

Many events can disrupt BMP signaling. BMPRI and BMPRII can both be internalized by endocytosis and become unavailable for BMP binding.

Endocytosis can also increase BMPRI availability by recycling it back to the cell membrane (12, 167). Some pseudo-receptors such as BAMBI (BMP and activin membrane-bound inhibitor) are receptors without the intercellular Ser/Thr kinase phosphorylation domain required for Smad signaling (197, 198).

BMP signaling can be inhibited by extracellular and intracellular factors. Extracellular BMP antagonists such as noggin, chordin, and Grem1 can inhibit the BMP signaling pathway by binding to BMP dimers and preventing their recognition by BMP receptors. Noggin can inhibit BMP-2, BMP-4, and BMP-7 but has no effect on the ability of BMP-9 to act on cells directing by example the differentiation of mesenchymal stem cells into osteoblasts (180, 199, 200). In the same way, BMP-3 can inhibit the bone formation induced by BMP-2, BMP-6, and BMP-7 in mice but has no effect on BMP-9 (159).

BMPs can also downregulate their own canonical pathways by triggering the expression of intracellular Smad6/7. The binding of I-Smad to the intracellular domains of Ser/Thr kinase type I receptors prevents the phosphorylation of R-Smad, which blocks BMP signal transduction (201). Various intracellular phosphatases such as PP1, dullard protein, pyruvate dehydrogenase phosphatase (PDP), phosphatase methyltransferase 1 (PPM1), and small C-terminal domain phosphatase 1/2 (SCP 1/2) can also dephosphorylate and thus inactivate R-Smad (202). For example, it has been shown that PP1 inhibits ALK-1/TGF-beta-induced Smad1/5 activation after 6 h in MEECs (203).

4.2.2. MAPK pathways

MAPK pathways are engaged in many aspects of cell functions, including survival, proliferation, and differentiation (204). These pathways involve three main cascades consisting of p38 (4 isoforms alpha, beta, gamma, and delta), extracellular signal-regulated kinase (ERK, 8 isoforms ERK1 to ERK8), and c-Jun NH_2-terminal kinase (JNK, 3 isoforms, JNK1-JNK3) (Figure 6) (205). The activation of the MAPK pathway following BMP stimulation is related to the type I Ser/Thr kinase receptor interacting with the X chromosome-linked inhibitor of apoptosis (XIAP), TGF-beta-activated kinase 1/MAP3K7 binding protein 1 (TAB1), and TGF-beta-activated kinase 1 (TAK1) (206).

MAPKs such as ERK1/2, CDKs (cyclin-dependent kinase), and other kinases can phosphorylate the linker region of R-Smad, inhibiting their translocation to the nucleus (207, 208). For example, ERK1/2 activation by BMP-9 in MSCs can in turn inhibit the phosphorylation of Smad1/5/8 on the C-terminal MH2 domain (170, 206).

4.3. Other signaling pathways involved in BMP signal transduction

4.3.1. Wnt pathways

Wingless-type (Wnt) pathways are involved in the control of EC differentiation from hematopoietic precursors during embryonic development and, especially, during the vascularization of the central nervous system (209). Wnt pathways also contribute to cell migration and polarity and cellular responses to shear stress (210). They are classified into at least three major signal transduction cascades: one is beta-catenin-dependent (canonical) while two, the Wnt/calcium and Wnt/planar cell polarity (PCP) pathways, are beta-catenin-independent (non-canonical) (211).

The non-canonical Wnt/calcium pathway can be activated by the binding of Wnt ligands (a family of 19 secreted glycoproteins to date) such as Wnt-5a or Wnt-4 to Frizzled (Fz) receptors, which activates the disheveled protein, leading to the stimulation of phospholipase C and an increase in the concentration of intracellular calcium ions. These calcium ions in turn activate calmodulin-dependent protein kinase II and protein kinase C. The activation of the Wnt/PCP pathway depends on the activation of small G proteins such as Rac and Rho GTPases by Wnt-Frizzled receptor recognition (212).

The canonical Wnt pathway is activated in ECs by the binding of some Wnt ligands such as Wnt1, Wnt-3/3a, and Wnt-7a/7b to a complex composed of Fz receptors and LDL receptor-related protein 5 (LRP5) or LRP6 co-receptor (212). This Wnt-receptor interaction leads to the inactivation of glycogen synthase kinase-3beta (GSK3beta) by favoring, for example, its entrapment in multivesicular bodies (213, 214). Canonical Wnt activation induces the accumulation of cytoplasmic beta-catenin that can then translocate into the nucleus to regulate the expression of targeting genes by displacing the transcription repressor Groucho and interacting with DNA-bound T cell factor/lymphoid enhancer-binding factor (TCF/LEF). When the Wnt ligand is missing, GSK3beta remains active and forms a complex with Axin, APC, and CK1alpha. CK1alpha and GSK3beta can phosphorylate beta-catenin at its N-terminus, inducing its ubiquitination by E3 ubiquitin ligase beta TrCP and its degradation in the proteasome (215, 216).

GSK3beta can strongly influence the BMP canonical pathway by phosphorylating the linker domain of Smad1/5 (Figure 7), leading to Smad1/5 ubiquitination and subsequent proteasomal degradation (207, 213, 217). The canonical Wnt pathway can also control angiogenesis during embryonic development by stopping the progression of the cell cycle via crosstalk with the Notch pathway (218).

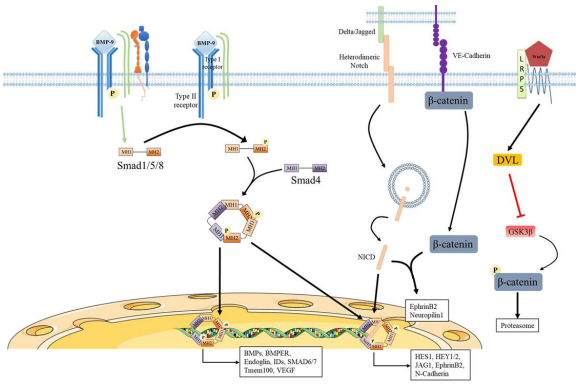

Figure 7. Crosstalk between BMP-9 and the Notch, Wnt, and VE-cadherin signaling pathways, adapted with permission from (53). [The figure was created using Servier Medical Art. https://smart.servier.com.].

4.3.2. Notch pathway

The Notch pathway plays a key role in angiogenesis by favoring stalk ECs involved in the sprouting step (218). It was also recently found to be involved in the endothelial-to-mesenchymal transition, a phenomenon observed during embryonic cardiogenesis and atherosclerosis progression (126, 219, 220). Signaling via the Notch pathway is initiated in cells by the activation of transmembrane Notch receptors (Notch 1-4) by specific membrane-bound Delta-like (Dll-1, Dll-3, Dll-4) and Jagged (Jagged-1, Jagged-2) ligands. Dll and Jagged ligands can also initiate signaling via the Notch pathway in neighboring cells by transactivating their transmembrane receptors, leading to the release of the NICD due to receptor cleavage by gamma-secretase complexes (221). NICD is then translocated to the nucleus where it promotes the expression of targeting genes of the *hairy enhancer of split* (Hes)/*Hairy-related* (Hey) family or the gene encoding decoy receptor VEGFR1 in ECs. In contrast, the Notch pathway can also induce a downregulation of VEGFR2 and VEGFR3 gene expression in stalk ECs, blocking VEGF signaling (222-225).

Interestingly, the number of Dll ligands/Notch receptors expressed by ECs changes during inflammation. For example, the expression of Dll4 by ECs is involved in communication between ECs and macrophages (226). Dll4 and Notch1 act in synergy with BMP-9 to control vascular homeostasis, especially EC quiescence (Figure 6) (227). Laminar shear stress can also influence the expression of Dll4 and its Notch transmembrane receptor, increasing the nuclear translocation of NICD in ECs (210). In addition, a non-canonical Notch pathway was recently discovered in ECs that uses the transmembrane domain of Notch to regulate AJ and EC permeability (228).

5. ROLE OF BONE MORPHOGENETIC PROTEINS IN VASCULAR HOMEOSTASIS

BMPs such BMP-2, BMP-4, BMP-9, and BMP-10 can affect the survival, proliferation, and function of ECs (12, 19, 229-231). In the present review, we focus on the effect of BMP-9 on EC function since there is ample evidence of its effects on vascular homeostasis (Table 1) (18, 20, 232-236).

5.1. Effect of BMP-9 on EC proliferation

BMP-9 is mainly synthetized in the liver by hepatocytes and intrahepatic biliary epithelial cells, approximately 60% of which circulate in an active form in blood plasma (14). Its EC_{50} for Smad1/5/8 activation, as measured by the expression of ID1 promoter-derived (BRE), is as low as 50 pg/mL (2 pM). Healthy flow shear stress (12 dynes/cm2) can reduce the EC_{50} to 3.5 pg/mL (25, 185). In addition, BMP-9 can induce the phosphorylation of both Smad1 and Smad2 in HPAEC, HAEC, and HMEC-1 cells (175). Despite the fact that BMP-9 and BMP-10 share sequence homology, BMP-10 circulates in blood plasma in an inactivated form (206, 237).

In addition, many studies have suggested that BMP-9 does not act in the same way on cells as BMPs from other subgroups (13, 14, 156, 238). For example, BMP-9, but not BMP-2 and BMP-4, is able to significantly induce ET-1 in HPAECs (176, 177). ET-1 is a potent peptide involved in the process of blood vessel narrowing and vascular remodeling (177, 239).

Yoshimatsu *et al.* reported that 65% of the decrease in HDLEC proliferation after 48 h can be attributed to a BMP-9 treatment (1 ng/mL), suggesting that BMP-9 has an inhibitory effect on the formation of new lymphatic vessels (240). On the other hand, blocking BMP-9 (10 ng/mL) with the recombinant human ALK-1 extracellular domain/Fc (ALK-1ecd) increases the proliferation of HMVEC-d cells (185). The influence of BMP-9 on EC proliferation appears to depend on the type II receptors involved. BMP-9 inhibits DNA synthesis in HPAECs by binding to ALK1 and BMPRII receptors, while blocking the type II receptor ActR-II by siRNA transfection does not alter the ability of BMP-9 to inhibit growth (175).

5.2. Effect of BMP-9 on vessel formation and sprouting

The capacity of ECs to form new vessels following a treatment with VEGF can be altered by BMP-9 (17, 18, 184). For example, 7-day parallel stimulations with BMP-9 (100 ng/mL) and VEGF (50 ng/mL) in *ex vivo* mouse fetus models show that BMP-9 inhibits vascularization (17).

Table 1. BMP-9/EC interactions: cell proliferation, angiogenesis, and inflammation

EC Function	Treatment	System Type	Cell / Specie	Method Highlights	Effect of Treatment	Other Observations	References
Proliferation	BMP-9 (10 ng/mL, 48 h)	*in vitro*	HMVEC-d	5% FBS	BMP-9 ┤ Proliferation compared to control		(185)
	BMP-9 (1 ng/ml, 48 h) + VEGF (30 ng/mL, 48 h)	*in vitro*	MESEC	Serum-free	BMP-9 + VEGF → Proliferation compared to VEGF alone	BMP-9 + VEGF → Pro-angiogenic ↑mRNA coding VEGFR2, Tie2, VEGF, compared to VEGF alone in mouse embryonic stem cells	(19)
	BMP-9 (1 ng/ml, 48 h) + VEGF (30 ng/mL, 48 h) + ALK-1-Fc (30 ng/mL, 48 h)	*in vitro*	MESEC	Serum-free	BMP-9 + ALK-1-Fc + VEGF ┤ Proliferation compared to BMP-9 + VEGF		(19)
	BMP-9 (10 ng/mL, 72 h) + bFGF (30 ng/mL, 72 h)	*in vitro*	BAEC	Serum-free	BMP-9 + bFGF ┤ Proliferation compared to bFGF alone		(17)
Migration	BMP-9 (10 ng/mL, 24 h) +/- bFGF (30 ng/mL, 24 h)	*in vitro*	BAEC		BMP-9 ┤ Migration compared to bFGF		(17)
	BMP-9 (10 ng/mL, 48 h)	*in vitro*	HMVEC-d	0.5% FBS	BMP-9 ┤Migration compared to control		(185)
Migration	BMP-9-transduced cells vs. pWzl retrovirus treaded cells	*in vitro*	BMP-9-SVEC, Wzl-SVEC	0.5% BSA, Coll coating	BMP-9-SVEC → Migration, compared to pWzl-SVEC with little BMP-9 mRNA expression	BMP-9 (5 ng/mL, 24 h) ┤ activation of gene encoding E-selectin in HMVEC-d, compared to control	(233)
	BMP-9 (2 ng/mL, 168 h), +/- control Fc protein (20 µg/ml, 168 h)	*ex vivo*	Mice & MS1 EC	Serum-free, Matrigel	BMP-9 ┤Migration, compared to control Fc protein		(246)
Sprouting	BMP-9 (10 ng/mL, 144 h) + VEGF (25 ng/mL, 144 h)	*in vitro*	HUVEC	2% FBS, 3D fibrin gel	BMP-9 + VEGF ┤ Sprouting compared to VEGF alone		(184)
	anti-BMP-9 (RAP-041)	*in vitro*	HUVEC	Complete medium+ 0.1% methylcellulose, Coll I	anti-BMP-9 had no effect on sprouting, compared to control		(246)
	anti-BMP-9 (RAP-041) + VEGF-A (50 ng/mL)	*in vitro*	HUVEC	Complete medium + 0.1% methylcellulose, Coll I	anti-BMP-9 + VEGF-A ┤Sprouting, compared to VEGF-A alone		(246)
	anti-BMP-9 (18h) + VEGF (50 ng/ml, 18h)	*in vitro*	HUVEC	EGM2 + 2% FCS + 0.1% methylcellulose, Coll I	anti-BMP-9 + VEGF ┤Sprouting, compared to VEGF alone	anti-hALK-1 (40 µg/mL, 18 h) + VEGF ┤HUVEC sprout lengths, compared to VEGF alone	(247)
	BMP-9 (0.5 mg/mL, 16 h)	*in vivo*	Postnatal mice		BMP-9 ┤ Hyper-sprouting compared to control		(184)

Cord formation	anti-BMP-9 (RAP-041, 10 µg/mL, 16 h)	*in vitro*	HUVEC	4% FBS, Matrigel plug	anti-BMP-9 ┤ Cord formation, compared to control		(246)
Angiogenesis	BMP-9 (100 ng/mL, 168 h)	*ex vivo*	Metatarsals of mouse foetuses		BMP-9 ┤ Angiogenesis compared to control		(17)
	BMP-9 (100 ng/mL, 168 h) + VEGF (50 ng/mL, 168 h)	*ex vivo*	Metatarsals of mouse foetuses		BMP-9 + VEGF ┤ Angiogenesis compared to VEGF alone		(17)
	BMP-9 (4 ng/mL, 48 h)	*ex vivo*	Mouse embryos		BMP-9 → Angiogenesis compared to control		(19)
	Endo-MO (blocking ENG translation, 5 ng/nL, 48 h)	*in vivo*	Zebrafish embryos		Endo-MO ┤ Angiogenesis compared to control		(20)
	BMP-9 (55 ng, 24 h)	*in vivo*	Chick embryos		BMP-9 ┤ Angiogenesis compared to control		(232)
	BMP-9 (20 ng, 168 h) +/- FGF-2 (200 ng, 168 h)	*in vivo*	Balb-C mice		BMP-9 ┤ Angiogenesis compared to FGF-2		(232)
	BMP-9 (10 ng/mL, 168 h) + FGF-2 (1 µg/mL, 168 h)	*in vivo*	BALB/c mice	Matrigel	BMP-9 + FGF-2 → Angiogenesis compared to FGF-2 alone		(19)
	BMP-9 (BMP-9 encoding lentivirus, 35 days)	*in vivo*	BALB/c nude mice	Subcutaneously inoculation of BxPC3 human pancreatic adenocarcinoma cells, infected with lentiviruses encoding BMP-9 or GFP (control)	BMP-9 → Angiogenesis, compared to control		(19)
Angiogenesis	BMP-9 (adenoviral over-expression)	*in vivo*	Postnatal mice	BMP-9 adenovirus injection vs. control siRNA adenovirus injection	BMP-9 ┤ Angiogenesis compared to control		(184)
	BMP-9 (2 ng/mL, 168 h) +/- TGF-beta (2 ng/mL, 48 h)	*in vivo*	Mice	Subcutaneously injected Matrigel plugs	BMP-9 + TGF-beta had no effect on angiogenesis compared to control		(246)
	BMP-9 (2 ng/mL, 168 h) + TGF-beta (2 ng/mL, 48 h) +/- VEGF-A (300 ng/mL, 168 h) +/- bFGF-2 (700 ng/mL, 168 h) +/- control Fc protein (20 µg/ml, 168 h)	*in vivo*	Mice	Subcutaneously injected Matrigel plugs	BMP-9 + TGF-beta ┤ Angiogenesis compared to BMP-9 + TGF-beta + VEGF-A + bFGF-2 + control Fc		(246)

Inflammation	BMP-9 (5 ng/mL, 16 h) + TNF-alpha (0.05 ng/mL, 4 h)	*in vitro*	HAEC BOEC		BMP-9 + TNF-alpha → Inflammation compared to TNF-alpha alone and control		(22)
	BMP-9 (5 ng/mL, 4 h)	*in vitro*	PAEC HMEC	Serum-free	BMP-9 ┤ Inflammation compared to control	BMP-9 ┤ IL-6 and MCP-1 gene expression	(124)
	BMP-9 (5 ng/mL, 16 h) + LPS (100 ng/mL, 4 h)	*in vitro*	BOEC HPAEC		BMP-9 + LPS → Inflammation compared to LPS alone and control		(243)
	BMP-9 (50 µg/kg, 240 h)	*in vivo*	Rat pups	Hyperoxia	BMP-9 ┤ Inflammation compared to control		(124)

Abbreviations: activin receptor (ActR), bovine aortic endothelial cell (BAEC), basic fibroblast growth factor (bFGF), bone morphogenetic protein (BMP), bone morphogenetic protein receptor (BMPR), blood outgrowth endothelial cell (BOEC), bone morphogenetic protein□responsive element Luciferase (BRE-Luc), bovine serum albumin (BSA), collagen (Coll), endothelial cell (EC), endothelial cell growth medium (EGM2), endoglin (ENG), endothelial selectin (E-selectin), fetal bovine serum (FBS), fragment crystallizable (Fc), fetal calf serum (FCS), green fluorescent protein (GFP), human aortic endothelial cell (HAEC), human activin receptor-like kinase (hALK), dermal human microvascular endothelial cells (HMEC-d, HMVEC-d), human pulmonary artery endothelial cell (hPAEC), human umbilical vein endothelial cell (HUVEC), DNA-binding protein inhibitor (ID), interleukin (IL), lipopolysaccharide (LPS), intercellular monocyte chemoattractant protein-1 (MCP-1/CCL2), mouse embryonic-stem-cell-derived endothelial cell (MESEC), messenger RNA (mRNA), mouse microvascular endothelial cell (SVEC), MILE SVEN 1 (MS1), phosphorylated Smad (pSmad), stromal-derived factor 1 (SDF1, CXCL12), small interfering (siRNA), tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor (VEGFR), Signs: [→ increase/activation] [↑ increase/more] [┤ block/decrease]

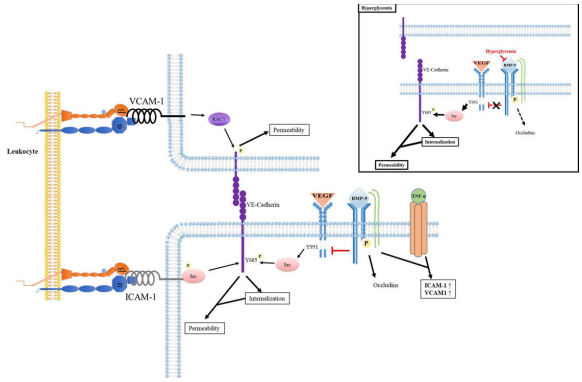

Figure 8. Effect of BMP-9 on risk factor or inflammation involved in atherosclerosis, adapted with permission from (244). [The figure was created using Servier Medical Art. https://smart.servier.com.].

The anti-angiogenesis effect of BMP-9 on ECs has been reported in several studies (17, 20, 232, 241). Larrivee *et al.* reported a 9-fold decrease in the number of tip cells in mice retina 16 h after a BMP-9 (10 ng/mL) treatment by intraocular injection (184). They also showed that VEGFR1 mRNA levels were 4-fold higher in HUVECs treated for 24 h with BMP-9 (10 ng/mL) and that there was a 0.5-fold decrease in VEGF-induced HUVEC tube formation, indicating that BMP-9 has an inhibitory effect on VEGF-mediated sprouting (184).

Tian *et al.* reported that the effect of BMP-9 on sprouting may also involve FN via an integrin alpha5 beta1/ENG/ALK1 complex (20). They used murine embryonic endothelial cells (MEEC+/+ ENG wild-type and -/- ENG knockout cells) cultured on FN (40 µg/mL) to show that Smad1/5 activation mediated by BMP-9 (2 ng/mL) is ENG-dependent (20). The results of this study suggest that the blockade of BMP-9/ENG signaling contributes to abnormal disrupted intersegmental vessel (ISV) sprouting. In the same way, when type I receptor ALK-1 expression is disrupted by small interfering RNA, the Smad1/5-mediated BMP-9 anti-angiogenic effect is blocked (175).

In contrast, Tachida *et al.* reported that BMP-9 (10 ng/mL) mediates the upregulation of pro-angiogenesis signaling (i.e., a 5-fold increase in HEY1 mRNA levels) in co-cultures of HASMCs and HAECs (242). However, the authors did not perform any experiments to determine whether there is an increase in angiogenesis.

5.3. Effect of BMP-9 on inflammation

BMP-9 influences the role of ECs in inflammation (Figure 8) (22, 124). Chen *et al.* reported that there is a downregulation of MCP-1 mRNA, which is involved in ECs monocyte recruitment, in HPAECs (~5 fold) and HMECs (~2 fold) in *in vitro* cultures following a BMP-9 treatment (5 ng/mL, 4 h) (124). Unlike the anti-inflammatory role of BMP-9 mentioned above, Mitrofan *et al.* reported that a pre-treatment with BMP-9 (5 ng/mL) 16 h prior to adding TNF-alpha (0.05 ng/mL for 4 h) upregulates the mRNA and protein levels of adhesion molecules (E-selectin, ICAM-1, VCAM-1) (22). The upregulation of the adhesion proteins was followed by an increase in monocyte recruitment (~1.6 fold) in HAECs. An increase in monocyte recruitment (~1.4 fold) has also been reported in BOECs (22). The discrepancy in the effect of BMP-9 on monocyte recruitment reported by Mitrofan *et al.* and Chen *et al.* may be related to the TNF-alpha used in the later study (22, 124). Appleby *et al.* also observed an increase (~2-fold) in neutrophil

recruitment in BOECs and HPAECs following a pre-treatment with BMP-9 (5 ng/mL, 16 h), although only when an endotoxin (LPS, 100 ng/mL, 4 h) was added to the cell culture (243).

5.4. Effect of BMP-9 on factors involved in atherosclerosis

The effect of many players involved in the development of atherosclerosis, ranging from risk factors to soluble cytokines, may also be influenced by BMP-9 (Figure 8). Akla *et al.* recently reported that an increase in D-glucose concentration (from 5 to 11 mM) can block the Smad 1/5 activation induced by BMP-9 (1 ng/mL) bound to type 1 receptor (ALK-1) (244). D-glucose at 11 mM also significantly reduced the expression of ALK-1 at the cell membrane. Interestingly, hyperglycemia had no effect on BMP-9/ALK-1 signaling for BMP-9 concentration >1 ng/mL. Using C57BL/6J mice with or without induced diabetes mellitus (blood glucose >17 mM), Akla *et al.* also found that the concentration of BMP-9 in blood was similar in control and diabetic mice. In contrast, a decrease in circulating BMP-9 levels has been observed in the plasma of newly diagnosed Type 2 diabetes mellitus patients, and may be linked to insulin resistance (245). BMP-9 can also prevent hyperglycemia-induced vascular permeability as shown by Akla *et al* both *in vitro* (HUVECs with D-glucose at 25 mM) and *in vivo* (mouse model with induced diabetes mellitus) (244). Thus, BMP-9 signaling plays a crucial role in the regulation of glucose homeostasis and diabetes diseases and vice-versa, diabetes being a major risk factor for the development of atherosclerosis.

Furthermore, BMP-9 signaling appears to be associated with other major atherosclerosis risk factors and with atherosclerotic complications. For instance, in advanced lesions, fibrous cap formation in atherosclerotic plaques requires the formation of new vessels while BMP-9 can be an anti-angiogenic factor (123, 184). The inhibition of cap formation makes plaque less stable in complicated lesions (127, 128). Furthermore, the matrix calcification induced by VSMCs (also called calcification of VSMCs), a phenomenon involved in advanced atherosclerotic lesions, is intensified by *in vitro* BMP-9 treatments, further indicating that BMP-9 signaling is involved in the progression of atherosclerosis (26, 132).

6. CONCLUSION

Many studies have shown that BMP-9 markedly affects EC behavior, either directly or by regulating the effects of other factors such as VEGF. BMP-9 may thus have the capacity to influence the progression of atherosclerosis. During the genesis of atherosclerosis, BMP-9 combined with TNF-alpha may increase the expression of adhesion molecules that facilitate leukocyte extravasation and, in turn, foam cell formation. BMP-9 may also regulate plaque development and growth by blocking angiogenesis. However, this anti-angiogenetic property can enhance the risk of plaque rupture in more advanced plaques, where BMP-9 may also promote VSMC calcification. These observations suggest that BMP-9 may play a complex role in the progression of atherosclerosis in the early and advanced stages. The involvement of BMP-9 in atherosclerosis needs to be investigated further using systems that study the effect of a combination of atherosclerosis-related factors. For instance, BMP-9 treatments in a dynamic system (WSS) of an EC/VSMC co-culture with a mixed FN/Coll coating could pave the way for designing more effective *in vitro* models.

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Abbreviations: AJ, Adherens junctions; ALK, Activin receptor-like kinase; AMHR, Anti-Mullerian hormone receptor; APC, Adenomatous polyposis coli; BAEC, Bovine aortic endothelial cell; BAMBI, BMP and activin membrane-bound inhibitor; bHLHZIP, Basic helix-loop-helix leucine zipper; BM, Basal/basement membrane; BMP, Bone morphogenetic protein; BMPER, Bone morphogenetic protein binding endothelial regulator; BOEC, Blood outgrowth endothelial cell; BRE, BMP response element; CDKs, Cyclin-dependent kinase; Coll, Collagen; CHOC, Chinese hamster ovary cell; CK1alpha, Casein kinase 1alpha; CVD, Cardiovascular disease; Dll, Delta-like ligand; EC, Endothelial cell; EC-domain, Extracellular cadherin domain; ECM, Extracellular matrix; ENG, Endoglin; ERK, Extracellular signal-regulated kinase; EndMT, Endothelial-to-mesenchymal transition; eNOS, Endothelial NO synthase; ET, Endothelin; FAK, Focal adhesion kinase; FA, Focal adhesion; FN. Fibronectin; FX, Focal complex; GF, Growth factor; GJ, Gap junctions; GSK3beta, Glycogen synthase kinase-3beta; GPI, Glycosylphosphatidylinositol; HAEC, Human aortic endothelial cell; HASMC, Human aortic smooth muscle cell; HAV, Histidine-alanine-valine; HDLEC, Human dermal lymphatic endothelial cell; HMEC, Human microvascular endothelial cell; HMVEC, Human dermal microvascular endothelial cell; hPAEC, Human pulmonary artery endothelial cell; HUVEC, Human umbilical vein endothelial cell; ICAM, Intercellular adhesion molecule; Id. Inhibitor of differentiation; ISV, Disrupted intersegmental vessel; LEF, Lymphoid enhancer-binding factor; MAPK, Mitogen-activated protein kinase; MCP, Monocyte chemoattractant protein; MEEC, Murine embryonic endothelial cell; MMP, Matrix metalloproteinase; MSC, Mesenchymal stem cell; NF, Necrosis factor; NICD, Notch intracellular domain; NO, Nitric oxide ; ox-LDL, Oxidized low-density lipoprotein; PDGF, Platelet-derived growth factor; PAEC, Porcine aortic endothelial cell; PECAM, Platelet/endothelial cell adhesion molecule; PGI2, Prostacyclin; PHSRN, Proline-histidine-serine-arginine-asparagine sequence; PP, Protein phosphatase; RGM, Repulsive guidance molecule; Src, Steroid receptor coactivator; TCF, T cell factor; TGF, Transforming growth factor; TJ, Tight junction; Tmem, Transmembrane protein; TM, Thrombomodulin; TNF, Tumor necrosis factor; VCAM, Vascular cell adhesion protein; VEGF, Vascular endothelial growth factor ; VSMC, Vascular smooth muscle cell; vWF, Von Willebrand factor; Wnt, Wingless-type; WSS, Wall shear stress

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Send correspondence to: Nathalie Faucheux, Cell-Biomaterial Biohybrid Systems, Department of Chemical and Biotechnological Engineering, Universite de Sherbrooke, 2500 boulevard de l'Universite, Sherbrooke, QC, Canada, J1K 2R1, Tel: 819-821-8000 ext 61343, Fax: 819-821-7955, E-mail: Nathalie.Faucheux@usherbrooke.ca