Université de Montréal

Role of the BMP9/ALK1 pathway in the regulation of pathological and VEGF-mediated angiogenesis

par
Kalonji Ntumba

Programme de Sciences biomédicales
Faculté de médecine

Thèse présentée à la Faculté de médecine
en vue de l'obtention du grade de PhD
en sciences biomédicales

Janvier, 2019

Résumé

L'angiogenèse est définie comme la formation de nouveaux capillaires à partir des vaisseaux sanguins pré-existants. Elle contribue à l'extension du réseau vasculaire et assure ainsi l'efficacité des échanges gazeux et du transport des cellules, nutriments, métabolites et molécules de signalisation vers les tissus. L'angiogenèse par bourgeonnement passe par la spécification d'une cellule endothéliale en cellule meneuse et la formation d'un réseau de cellules suiveuses à la base du bourgeon vasculaire. Toute perturbation de ce mode de néovascularisation génère des vaisseaux fortement tortueux, immatures et non-étanches qui soit affectent les fonctions physiologiques des organes en causant ainsi des pathologies potentiellement fatales, ou accélèrent la progression des conditions telles que le cancer. Dans l'oeil, l'angiogenèse pathologique des vaisseaux choroïdiens et rétiniens cause une perte de la vue. Particulièrement, la dégénérescence maculaire liée à l'âge (DMLA) de type néovasculaire, une maladie oculaire caractérisée par le bourgeonnement anormal de la choroïde dans l'espace sous-rétinien, représente la cause majeure de cécité au sein des populations des pays industrialisés. Les thérapies conventionnelles contre la DMLA humide reposent sur l'usage des médicaments qui ciblent la signalisation du facteur de croissance de l'endothélium vasculaire (VEGF). Bien que démontrant des résultats cliniques, ces traitements anti-VEGFs sont invasifs et présentent multiples effets secondaires. Par ailleurs, ils n'induisent aucun effet chez une portion des patients traités. De ces faits, il existe présentement un grand besoin de thérapies alternatives aux anti-VEGFs. De façon intéressante, la protéine de morphogénèse osseuse 9 (BMP9), qui active son récepteur "activin receptor-like kinase 1" (ALK1), régule l'angiogenèse développementale des vaisseaux rétiniens de l'oeil de la souris. Par ailleurs, les mutations au sein du BMP9, de son récepteur ALK1 ou de ses intermédiaires de signalisation sont associées à la morphogénèse anormale des vaisseaux qui contribue ultimement à la pathogénèse de diverses maladies néovasculaires. De façon additionnelle, le récepteur ALK1 au BMP9 est restreint à la cellule endothéliale; contrairement à ceux des ligands angiogéniques tels que le VEGF, exprimés par une diversité de cellules. Par ailleurs, au sein de cette cellule, le BMP9 contribue à la régulation des phénotypes meneur et suiveur qui sont induits par le VEGF et requis pour

le déroulement de l'angiogenèse par bourgeonnement. De ces faits qui précèdent, nous avons émis l'hypothèse du rôle du BMP9 dans la régulation de la néovascularisation pathologique relative à la DMLA humide. Ainsi, les travaux de la présente thèse déterminent spécifiquement l'effet du BMP9 sur l'angiogenèse pathologique à l'aide des modèles oculaires pertinents à la DMLA humide et examine aussi sa base mécanistique. Les travaux de cette thèse démontrent l'effet anti-angiogénique du BMP9 sous les conditions expérimentales de néovascularisation choroïdienne induite au laser (CNV) et de rétinopathie induite par l'oxygène (OIR). Ils montrent aussi les effets régulateurs de la signalisation du BMP9 sur les voies de signalisation endothéliales du VEGF et de Notch, respectivement de façon dépendante de VEGFR1 et de JAG1. En somme, les présentes études démontrent les effets anti-angiogéniques du BMP9 sur la néovascularisation pathologique relative à la DMLA humide et identifient les facteurs moléculaires qui contribuent à son action inhibitrice du bourgeonnement vasculaire induit par le VEGF.

Mots-clés: BMP9, ALK1, angiogenèse, VEGF, Notch, VEGFR1.

Abstract

Angiogenesis is defined as the formation of new capillaries from existing blood vessels. It extends the vasculature and thereby sustains the efficient exchange of gases and transport of cells, nutrients, metabolites and signalling molecules to tissues. Sprouting angiogenesis proceeds through the selective specification of an endothelial cell into a leading tip cell and the formation of stalk cells at the base of the sprout. A disturbance in this modality of neovascularisation leads to highly tortuous, immature and leaky vessels that either impair the physiological functions of organs, thereby causing life-threatening diseases, or accelerate the progression of conditions such as cancer. In the eye, the pathological angiogenesis of choroidal and retinal vessels specifically results in vision loss. Particularly, the neovascular form of the age-related macular degeneration (AMD), an ocular disease characterized by the abnormal sprouting of the choroidal network into the subretinal space, represents the leading cause of blindness in populations of industrialized countries. Conventional therapies against wet AMD are based on drugs that target the signaling of the vascular endothelium growth factor (VEGF). Despite their clinical achievements, the anti-VEGFs treatments are invasive and show multiple adverse effects. Moreover, they are not effective in a portion of treated patients. Thus, there currently is a substantial need of therapy alternatives to anti-VEGFs. Interestingly, the bone morphogenetic protein 9 (BMP9), that activates its activin receptor-like kinase 1 (ALK1) transducer, regulates the developmental angiogenesis of the mouse eye retina vasculature. Moreover, mutations in BMP9, its receptor ALK1 or its signaling mediators correlate with the abnormal vessel morphogenesis that ultimately drives the pathogenesis of various neovascular diseases. Additionally, the BMP9-specific receptor ALK1 is restricted to endothelial cells; in contrast to those of neovascularisation-inducing ligands such as VEGF, expressed by a range of cells. Particularly within these cells, BMP9 contributes to regulate the VEGF-induced tip/stalk phenotypes required for sprouting angiogenesis. Given the aforementioned, we hypothesized the role of BMP9 in regulating the pathological angiogenesis associated with wet AMD. Thus, the studies from the current thesis specifically determine the effect of BMP9 on pathological NV using ocular models relevant to AMD and further investigate its mechanical basis. The current work

demonstrates the antiangiogenic effects of BMP9 under experimentally induced oxygen-induced retinopathy (OIR) and laser-induced choroid neovascularisation (CNV) conditions. Moreover, this thesis shows the regulatory effects of BMP9 signaling on the VEGF and Notch endothelial pathways, respectively in VEGFR1 and JAG1 -dependent manners. Collectively, the current studies demonstrate the anti-angiogenic effects of BMP9 on pathological NV associated with wet AMD and identify the molecular players that mediate its inhibitory action on VEGF-mediated sprouting.

Keywords: BMP9, ALK1, angiogenesis, VEGF, Notch, VEGFR1.

Table of Contents

List of figures

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

List of abbreviations

ActR/ACVR: Activin receptor

ADAM: a Disintegrin and Metalloprotease

ALK: Activin receptor-like kinase

AMD: age-related macular degeneration

ANG: Angiopoietin

BM: basement membrane

BMP: bone morphogenetic protein

BMPER: BMP -binding endothelial regulator

BMPR: BMP receptor

cDNA: coding DNA

CNV: laser-induced choroidal neovascularisation

co-Smad: common Smad

COX: Cyclooxygenase

c-Raf: cellular-rapidily accelerated fibrosarcoma protein

CSL: CBF1, Su(H), Lag-1

DLL: Delta-like ligand

DNA: deoxyribonucleic acid

EC: endothelial cell

ECM: extra-cellular matrix

ENG: Endoglin

ERG: ETS-related gene

ERK: extracellular signal-regulated kinase

ESM-1: endothelial specific molecule 1

ETS: E26 transformation-specific

FAK: focal adhesion kinase

FBS: foetal bovine serum

FGF: fibroblast growth factor

FLK-1: fetal liver kinase 1

FLT: Fms-like tyrosine kinase

HES: hairy and enhancer of split

HEY: hairy/enhancer-of- split-related with YRPW motif

HGF/SF: hepatocyte growth factor/scatter factor

HHT: hereditary hemorrhagic telangiectasia

HIF: hypoxia-inducible factor

HLH: helix–loop–helix

HLX: H2.0-like homeobox

HRE: HIF response element

HUVEC: human umbilical vein endothelial cell

IF: immuno-fluorescence

IsoB4: Isolectin GS IB4

JAG: Jagged

KDR: kinase-insert domain receptor

LFNG: lunatic Fringe

LNR: Lin-12 Notch repeats

MAML: Mastermind-like protein

MAPK: mitogen-activated protein kinase

MEK: mitogen/extracellular signal-regulated kinase-

MINT: Msx2 interacting nuclear target/

MMP: matrix metallo-proteinase

mRNA: messenger RNA

NCoR/CIR: nuclear receptor co-repressor protein/ corepressor interacting with $RBP_{J\kappa}$

NEXT: Notch extracellular truncation

NICD: Notch intracellular domain

NO: nitric oxide

NRE: Notch response element

NRP: Neuropilin

NV: neovascularisation

OIR: oxygen-induced retinopathy

PA: Plasminogen activator

PBS: phosphate buffered saline

PCR: Polymerase chain reaction

PDGF: platelet-derived growth factor

PDGFR: platelet-derived growth factor receptor

PF: platelet factor

PI3K: phosphatidylinositol 3 kinase

PKB (Akt): protein kinase B

PlGF: placenta growth factor

PLXN: Plexin

PTK2/FAK: protein-tyrosine kinase 2

qPCR: real-time quantitative PCR

$RBP_{J\kappa}$: recombination signal-binding protein 1 for J kappa

RGC: retina glial cells

RGM: repulsive guidance molecule

ROP: retinopathy of prematurity

RPE: retinal pigment epithelium

R-Smad: receptor-regulated Smad

SEMA: Semaphorin

SHARP: SMRT/HDAC1-associated repressor protein

SHB: Src homology 2 domain-containing adaptor protein B

si-RNA: silencing RNA

Smad: Sma (small body size) / mothers against decapentaplegic

SMRT: Silencing-mediator for retinoid/thyroid hormone receptors

SMURF: Smad ubiquitination regulatory factor

T_1R: type I receptor

T_2R: type II receptor

TACE: TNFα-converting enzyme

TGF: transforming growth factor

TGFβR: TGFβ receptor

TIMP: tissue inhibitors of metalloproteinase

TNFα: tumor necrosis factor-alpha

TSP: Thrombospondin

UNC: Uncoordinated

uPAR: Urokinase-type Plasminogen activator receptor

VEGF: vascular endothelial growth factor

VEGFR: VEGF receptor

VHL: von Hippel-Lindau protein

VSMC: vascular smooth muscle cells

Remerciements

J'aimerai remercier mon directeur de recherche le Dr Larrivée pour avoir permis la réalisation de cette aventure. Loin d'apprendre la science, j'ai pu admirer en toi un côté naturel. Il y a six ans, nous avions commencé ce cheminement en empruntant notre première pipette, sans se douter qu'elle mènerait à l'état actuel de connaissances professionnelles et personnelles.

Je remercie le jury (les Drs Casimiro Gerarduzzi, Pierre Hardy, Arnold Ludwig Hayer et Heather Melichar) et tous les membres de mon comité de suivi (les Drs Dan Bernard, Isabelle Royal et Martin Sirois) pour les corrections et discussions. Nos rencontres ont toujours été des moments de re-évaluation de connaissance, de challenge et d'épanouissement.

Je remercie les membres de mon laboratoire: Erika, Claire, Naoufal, Cindy, Natalija, Ali et Isaac. Nos échanges étaient des moments d'apprentissage, particulièrement ceux en franglais.

Je remercie aussi tous les amis et collègues qui ont contribué à ce travail de diverses façons.

Je termine en remerciant sincèrement toute ma famille pour son soutien. J'ai eu souvent l'impression que nous réalisions ces expériences ensembles.

CHAPTER I - Introduction

1. Physiological neovascularisation

1.1. Definitions

Angiogenesis is defined as the formation of new capillaries from existing blood vessels [1]. Angiogenesis differs from embryonic vasculogenesis where endothelial precursors, the angioblasts, derive from multipotent mesodermal cells and further differentiate into a primitive capillary network [2]–[7] (Fig.1). In most developmental and physiological conditions, angiogenesis occurs via vessel sprouting where tip endothelial cells (ECs)**,** migrating from pre-existing vessels**,** are followed by proliferative ECs to create new branches and thereby extend the vasculature [3], [8], [9]. Angiogenesis can also proceed through intussusception, in which pre-existing vessels split to expand the vasculature [10], [11]. Other angiogenesis modalities that characterize cancer include vessel co-option or vascular mimicry whereby tumor cells can either incorporate the surrounding vasculature, line pre-existing vessels or generate tumor endothelium from putative cancer stem-like cells [10], [11]. Ultimately, the newly established sprout capillaries should allow an efficient perfusion and a supply of tissues in nutrients and oxygen while clearing metabolic waste products. Thus, following a correctly tuned sprouting angiogenesis, the new branches undergo extension, acquisition of a lumen, interconnecting anastomosis and maturation. The maturation steps of neovascularisation consist of recruitment of mural cells [12]–[16], strengthening of cell–cell junctions [17], pruning of excessive vessels [18], deposition of a basement membrane and formation of a

lumen [19]. Subsequently in adults, angiogenesis of the predominantly quiescent vasculature enables the establishment of vascular networks that contribute to physiological or pathological responses under biological, injury or illness conditions [20]. Such processes include fetal growth, wound healing and tissue repair, menstrual cycle, cancer, ischemia and various inflammatory diseases [20]–[23].

Angiogenesis consists of multiple cellular responses that are spatially and temporally regulated by growth factors and microenvironmental cues [24]–[26]. These responses can be grouped in three main stages. First, angiogenic stimuli cause an increase in vessel permeability, subsequent to the mural cell detachment, and proliferation of endothelial cell (EC) [27]. Second, the proteolysis of the basement membrane and matricellular components promotes the stroma invasion of the neighboring tissue by the migratory EC [25], [28] and the elongation of the new capillary sprout. Third, migrated ECs trigger the lumen formation in the sprout multicellular structure to form the capillary channel. Finally, the capillary is stabilized through the formation of the basement membrane and the establishment of adherens junctions.

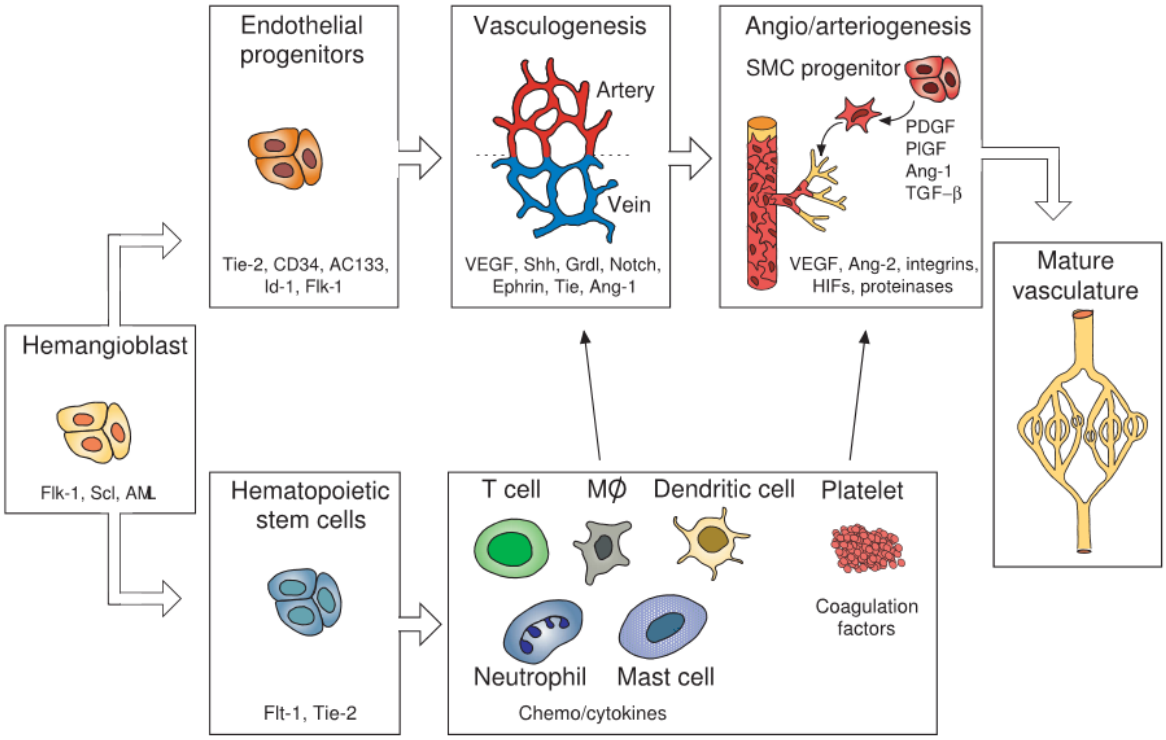


Fig.1 Primitive formation of blood vessels

Arterial and venous endothelial cells result from the differentiation of endothelial progenitors and form the primitive capillary plexus (vasculogenesis). New vessels sprout from this network (sprouting angiogenesis) and mature after SMC recruitment. Hematopoietic stem cell-derived leukocytes and platelets indirectly contribute to angiogenesis. Adapted from Carmeliet, 2003.

1.2. Histology of the vascular system

The vasculature of higher organisms is a closed circulatory system of arteries, veins and interconnecting capillaries that transports gases, nutrients, metabolites, cells and signalling molecules to tissues [2]. Arteries and arterioles allow the blood to flow from the heart to the organs, whereas veins and venules drain back the peripheral blood to the heart. Capillary beds, arterioles and venules constitute the microvasculature sites where metabolic exchange and most intercellular communication occur [29], [30].

Histologically, large arteries and veins are characterized by a lining of ECs, a basement membrane (BM), extra-cellular matrix (ECM) proteins and layers of mural cells. These components are organised to limit or connect the intima, the media and the adventitia vascular layers [2], [29]–[31].

The tunica intima

The intima is the innermost layer of blood vessels. It consists of a BM lined by endothelial cells on the apical side of the vessel [29]. The BM contains collagens, laminins and the platelet-recruiting von Willebrand factor [32]–[34]; whereas ECs exhibit distinct organ-specific molecular and functional properties that give rise to substantial heterogeneity in the vascular network [2], [29], [35]–[42]. These EC properties are linked to cell-intrinsic developmental pathways and transcriptional programs [42]–[46] that are regulated by microenvironment signals, such as growth factors [45], [47]–[50], mechanical forces [44], [51]–[54], metabolic stimuli [55]–[60], cell–matrix and cell–cell interactions [2]. Thus, the endothelium made by ECs constitutes a continuous, fenestrated

or discontinued layer that differentially contributes to the normal development and function of vessels and various organs. For instance, ECs from the endothelium assume organ-dependent functions such as protection [61], preferential metabolite absorption including the transendothelial transport at the blood–brain barrier (BBB) [43], [62]–[67], endocytic clearance, size and charge selective filtration [42], homing and entry of immune cells [68]–[70].

The tunica media

The media is the thickest layer of arteries and mainly consists of the differentiated mesenchymal mural cells such as the vascular smooth muscle cells (VSMC) [71], [72], the pericytes in capillaries [29], [73], [74] and hepatic stellate cells. The mural cells interact and interplay with ECs during angiogenesis processes [11], [75]. VSMCs are concentrically organized in multilayers attached to elastic lamellae through dense plaques of focal adhesion complexes in large vessels, or a loosely dispersed monolayer within the ECM in small vessels [2], [73], [76], [77]. They are essential for tone of vessels, as well as blood pressure regulation in large arteries [2], [62], [63]. Pericytes wrap capillaries and venules, make contacts with the endothelium and regulate the stability of vessels and the formation of vascular barriers such as BBB [2]. The media of elastic arteries is particularly enriched with ECM elastin and connecting fibrillins that mechanically support their conduction of blood influx and proliferation of VSMCs [78]–[82]. Moreover, the media in muscular arteries particularly characterized by concentric sheets of VSMCs that contribute to their sphincter function [29], [76], [77], [83].

The tunica adventitia

The adventitia is the most external layer of vessels and specifically thick in veins. It is a fibroelastic layer, at the junction of vessels and the surrounding connective tissues, characterized by the self-perfusing capillary network called vasa vasorum [29].

1.3. Sprout-specific tip and stalk cells

Sprouting angiogenesis mainly proceeds through dynamic vessel extensions called sprouts. These are microvascular structures formed by an endothelial tip cell leading a group of following endothelial stalk cells. In addition to the phalanx phenotype of quiescent EC cells, EC plasticity implies the pivotal but transient tip and stalk phenotypes that contribute to the fine tuning of angiogenesis [84]. All three EC subtypes interact during capillary sprouting [85]. Tip cells are specialized migratory endothelial cells characterized by filopodial protrusions and directed by extracellular guidance signals [12] (Fig.2). During angiogenesis initiation, the vascular endothelial growth factor (VEGF) gradients trigger the downstream activation of ETS factors that induce the membrane-bound Delta-like ligand (DLL) 4, a tip cell marker [19]. Furthermore, DLL4 expression in tip cells mediates the transactivation of Notch and expression of Notch targets in adjacent stalk cells. The adequate ratio between these tip and stalk cells is required for the regulation of sprouting and branching patterns [86]–[89]. It is controlled by the interplay between DLL4-Notch signaling and the VEGF pathway; at the level of crosstalks that regulate and dynamically re-evaluate the tip/stalk specification between adjacent ECs during sprouting [90]–[92].

1.4. Regulation of angiogenesis

During angiogenesis, ECs are exposed to cues that regulate vessel formation. These are molecules that specifically mediate processes such as the basal membrane disintegration, EC differentiation, cell-cell junction dynamics, migration, proliferation, apoptosis, anastomosis, lumen formation and mural cell recruitment (Fig.2). These processes can be grouped in angiogenesis stages that are characterized by various molecular markers. First, angiogenic factors such as VEGF or fibroblast growth factor (FGF) bind to EC receptors and activate angiogenic cascades [93], [94]. Matrix metallo-proteinases (MMP) and urokinase-type plasminogen activator receptor (uPAR) degrade the basement membrane and the ECM. This enables the formation and elongation of sprouts via the migration and proliferation of ECs. Tube formation and remodeling follow upon the mediation by regulators such as integrins. Finally, factors such as the transforming growth factor (TGF)-β and Angiopoietin (ANG)-1 mediate vessel maturation that involves pericytes and smooth muscle cells [95]–[100].

The state of the balance between the regulators of angiogenesis determines its progression [101]–[103]. Thus, angiogenic regulators could be manipulated clinically to treat conditions such as ocular angiogenesis [104], hemangiomas [105], tumor growth and metastasis [106]–[109], psoriasis [110], rheumatoid arthritis [111], atherosclerosis [112], ischemia [113], or in wound healing and reconstructive surgery [114]. Overall, angiogenesis mediators comprise signaling inducers or inhibitors involved in complex

angiogenic cascades, as well as proteolytic enzymes lysing extracellular matrix components [22], [23], [101].

1.4.1. Proteolytic enzymes

Proteolytic enzymes comprise the MMPs and plasminogen activator (PA) system [28], [115]–[117]. MMPs are extracellular or membrane-bound molecules that target different substrates such as collagen, gelatin, laminin, fibronectin, proteoglycans and pro-MMPs. Moreover, the cooperative activity of MMPs and PA system is required in the proteolysis of the basement membrane and matricellular components [25].

1.4.2. Angiogenesis inducers

The VEGF group of proteins are members of the Platelet-derived growth factor (PDGF) family that constitute key inducers of angiogenesis, along with the FGF family members [93]. They regulate EC proliferation, migration, survival and differentiation [21], [25], [118]–[120]. Other mediators recruited by these agonists include molecules such as ANG-1, TGF-β, hepatocyte growth factor/scatter factor (HGF/SF), tumor necrosis factor-alpha (TNFα), interleukin-1/8, angiogenin, ephrins, integrins and cyclooxygenase-2 (COX-2) [110], [121]–[123].

1.4.3. Angiogenesis inhibitors

Various cells such as platelet or mesenchymal cells inhibit EC proliferation/migration, induce their apoptosis, inhibit blood vessel maturation or counter MMPs or uPA activity. They contribute to the modulation of angiogenesis along with antiangiogenic factors. The antiangiogenic molecules include thrombospondin-1/2 (TSP-1/2), angiostatin/kringle 5 (plasminogen fragment), endostatin (collagen XVIII fragment), vasostatin (calreticulin fragment), tumstatin, platelet factor-4 (PF4), antiangiogenic

antithrombin III, prolactin 16-kD fragment, fragment of SPARC, 2-methoxyestradiol, tissue inhibitors of metalloproteinase (TIMPs), interferon-alpha/beta/gamma (IFN α/β/γ), interleukin-12 (IL-12), IP-10, ANG-2 [99], [100], [124]–[129].

1.4.4. Guidance signals in angiogenesis

Similarly to the exploration of surroundings by axonal growth cones, tip cells sense guidance cues that include molecules such as Ephrins, Semaphorins (SEMA), Slits and Netrins. These guidance proteins are evolutionarily conserved between ECs and axons or shared in the instance of VEGF. Moreover, migrating ECs express axon-specific guidance receptors. These include Neuropilins (NRP), Plexins and EPH receptors binding SEMA and/or Ephrins; the ROBO4 specific to Slit proteins; and uncoordinated (UNC)5B for Netrin proteins. Most ligands of the guidance system are transmembrane proteins that generally regulate cell-cell dependent vessel morphogenesis or segregation patterning. Moreover, their ligand-receptor complex can generate a bidirectional signaling. For instance, the interaction between EPH receptors and Ephrins generates forward and reverse signaling respectively in EPHB4- and EphrinB2- expressing cells. During angiogenesis, the reverse signaling of guidance molecules such as Ephrin-B2 induces filopodia extension in tip-cells through a VEGF receptor (VEGFR)-2 internalization mechanism or promote the recruitment of mural cells and bone-marrow-derived endothelial progenitor cells. During vasculogenesis, the segregation pattern of arterial and venous territories results from repulsive actions of respective Ephrin-B2 and EPHB4. In tumours, the EPHB4 receptor has prevailingly a pro-angiogenic effect that correlates directly with its upregulation. Guidance molecules such as EPH-A2 and Ephrin-A1 contribute to vessel growth and tumor resistance to anti-VEGFs. Ephrin-A1 increases in

tumors during treatment with VEGF blockers. Moreover, most SEMA are repulsive cues and show antiangiogenic activity. These include SEMA3-A, B, D, E, F and SEMA4A that inhibit tumour angiogenesis. In contrast, SEMA3C and SEMA4D favor tumour angiogenesis. Overall, the guidance system contributes to refining the formation of vessels during angiogenesis. In fact, the loss of cues or their receptors results in the inhibition of vascularisation due to defective VEGF signaling or erroneous navigation as observed in mice and embryos deficient in regulators such as PLXN-D1 or NRP-1.

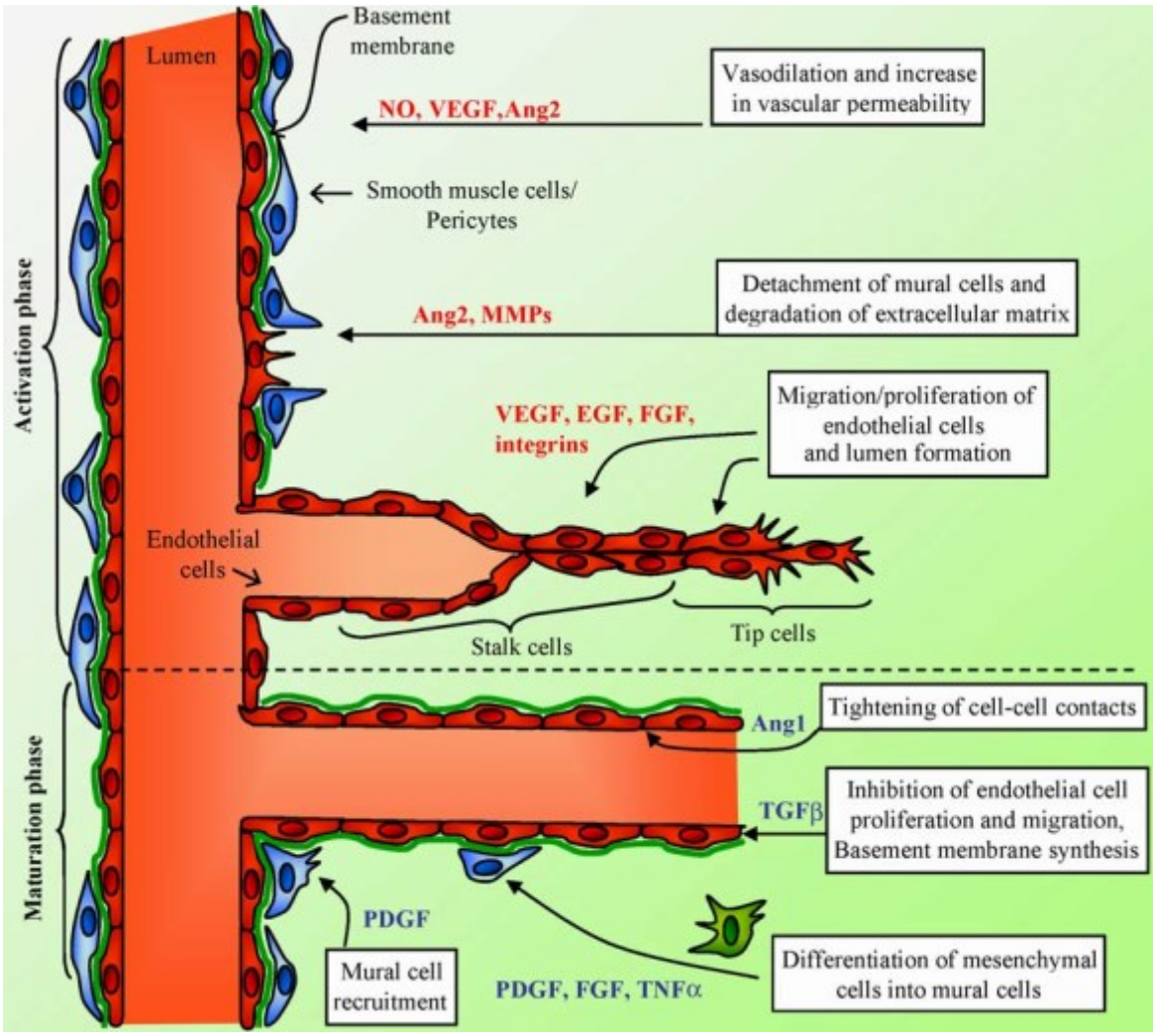


Fig.2 Regulators of activation and maturation phases of sprouting angiogenesis

Signaling molecules and their receptors contribute to angiogenesis by inducing changes on the perivascular cells, the basement membrane, the extracellular matrix (ECM) and endothelial cells during the activation and maturation phases. The elongating sprout consists of a migratory tip cell leading proliferative stalk cells. Adapted from David et al, 2009.

2. VEGF in sprouting angiogenesis

2.1. VEGF expression

VEGF represents a hallmark of angiogenesis and vasculogenesis as it plays a critical role in these processes [25]. For instance, the expression of VEGF and its receptors (VEGFRs) is important during foetal development as their inactivation in a knockout mouse model results in vasculogenesis defects at early stages of development and embryo death [94]. Moreover, the paracrine VEGF-A expressed by Muller cells, retina glial cells (RGC) and reactive astrocytes is the main sprouting clue for vessel in the retina and the choroid [130]–[133].

VEGF is normally synthesized in response to local hypoxia that originates from vascular compression or ischemia. In fact, hypoxia causes oxidative stress, by increasing mitochondrial superoxide production, and stimulates the hypoxia-inducible factor (HIF)-1α. HIF-1α binds to its response element (HRE) within the VEGF promoter and induces VEGF expression [25].

VEGF can also be expressed in well-oxygenated and non-ischemic endothelium in growing collaterals. Thus, ischemia is not a VEGF stimulus during arteriogenesis. Under these conditions, factors such as mechanical forces (shear stress and cell stretch) stimulate VEGF synthesis. The steep pressure gradient between pre- and post-occlusive regions in arteries can lead to increased pulsatile fluid shear stress (FSS), expression of all nitric oxide (NO) synthase (NOS) isoforms, production of NO and induction of VEGF and monocyte chemoattractant protein 1 (MCP-1) in SMCs, monocytes or T-cells. These

events result in the proliferation of endothelial and VSMCs and thereafter lead to the outward remodeling of pre-existent inter-connecting arterioles in collateral vessels following an arterial occlusion [134].

Additionally, a variety of stimuli may regulate VEGF expression. These include tumor promoters, transformation, P53 mutation, growth factors, hormones and inflammatory mediators. For instance, VEGF expression can be regulated positively by oncoproteins such as *Ras* and negatively by tumor suppressors such as von Hippel-Lindau (VHL) [135]. Moreover, VEGF expression can be induced by the cyclooxygenase-2 (COX-2)-increased prostaglandin (PG)-E2, estrogens, thyroid-stimulating hormone (TSH), IL-1 and TGF-β [25], [136].

2.2. VEGF subfamily and biological effects

VEGFs represent a subfamily of proteins that belong to the PDGF-like proteins, a family that displays a motif of conserved cystine knot core with backbone loops between successive cystine residues [137]. VEGFs contain a homology domain of eight characteristically spaced cysteines. Despite of their common domain, VEGFs have different physical and biological properties. Thus, they selectively bind VEGFRs as well as the SEMA-specific NRPs and, thereby, differentially regulate various cell responses including angiogenesis [138]–[146] (Fig.3). The VEGF subfamily consists of seven members that include VEGF -A, -B, -C, -D, -E, -F and the placenta growth factor (PlGF) [138], [147].

VEGF-A expression and level are crucial during embryonic development as well as physiological and pathological conditions. In fact, homozygous and heterozygous *VEGF-A* knockout mice die on embryonic days E8–E9 and E11–E12 respectively, during embryogenesis [138], [147], [148]. VEGF-A regulates vascularisation in a variety of conditions including embryogenesis, corpus luteum formation, tumor growth, wound healing and compensatory angiogenesis in the heart [148]. However, its overexpression results in large, dilated and leaky vessels [149]–[151]. VEGF-A mainly binds to VEGFR-1 and VEGFR-2, as well as NRP-1 and NRP-2 [139]. It induces several angiogenic processes including proliferation, sprouting, migration and tube formation); as well as EC survival via the activation of phosphatidyl-inositol-3 kinase and BCL-2 anti-apoptotic pathways [141]–[145], [148]. Moreover, VEGF-A was discovered as the vascular

permeability factor secreted by carcinoma cell lines where it mediates the extravasation of fluid and plasma proteins, including fibrin, that might also contribute to the enhanced migration of ECs in extracellular matrix [152]. Additionally, VEGF-A causes vasodilatation through the induction of endothelial (e)-NOS and increase of NO production [153]. VEGF enhances the permeability of skin blood vessels and also stimulates the production of ascites [152], probably via cGMP-mediated increase of vesico-vascular organelles, fenestrations and transcellular gaps, following VEGFR2-induced and NO-mediated activation of guanylyl cyclase (GC) [148].

At least six different isoforms of VEGF-A polypeptides of different sizes (121, 145, 165, 183, 189 and 206 amino acids) are known to exist. VEGF-A isoforms also have distinct but overlapping functions in angiogenesis due to their differential binding to heparan sulphate and NRPs [138], [147], [148]. For instance, VEGF-A121 does not bind to heparin or extracellular matrix while VEGF-A165 has moderate heparin binding ability. Knockout studies in mice have suggested VEGF165 as the isoform responsible for most VEGF-A effects. VEGF-A145 also contains a heparin binding domain and elements that enable the binding of VEGF-A145 to the extracellular matrix. VEGF-A189 and VEGF-A206 bind heparin more strongly and are sequestered both in the extracellular matrix and at the cell surface. These two isoforms are probably less active than either VEGF-A121 or VEGF-A165 *in vivo*. Only three VEGF-A splice forms are soluble and include VEGF-A121, VEGF-A145, and VEGF-A165 while the VEGF-A183, VEGF-A189 and VEGF-A206 are the matrix bound forms. With exception of the placental VEGF-A 145 and 206, the VEGF-A isoforms can be simultaneously expressed by various VEGF producing cell [138], [147], [148].

Finally, aside from ECs, VEGF-A also has effects on other cell types. VEGF-A is mitogenic for retinal pigment epithelium (RPE) and Schwann cells; and a neuro-protective factor for hypoxic motor neurons that can regulate amyotrophic lateral sclerosis [144], [148]. Its role has been reported during vascular smooth muscle cell (VSMC) proliferation and migration [145]. VEGF-A hematopoietic effects have also been reported. It induces colony formation of mature granulocyte-macrophage progenitor cells, regulates hematopoietic stem cell survival via an autocrine mechanism and promotes monocyte chemotaxis [146]. It also induces procoagulant activity in ECs and monocytes via its stimulation of coagulation factor [148], [154].

PlGFs were first identified in placenta and have later been found to be expressed in the heart and lungs [148]. The PlGF gene encodes four PlGF1-4 isoforms [155], [156]. Unlike PlGF -2 and -4, PlGF -1 and -3 lack heparin-binding domains and represent two diffusible isoforms. Additionally, their carboxy termini also lack the NRP1/2-binding insert of 21 basic amino acids [148], [156]. In general, PlGFs have direct amplifying effects on EC VEGF signaling and angiogenesis via the induction of their own signaling or the increase of VEGF availability [157]–[160]. More specifically, they either activate VEGFR1 [155] to transphosphorylate VEGFR2, bind VEGFRs to signal through them, heterodimerize with VEGF-A to bind VEGFR1/VEGFR2, or simply displace the trapped VEGF-A from VEGFR-1 to increase its availability for VEGFR2 [159]. For instance, PlGF2 has been reported to induce angiogenesis through VEGFR1-dependent mechanisms; whereas overexpression of soluble VEGFRs 1 and 2 inhibited its effects [148], [158], [160], [161].

VEGF-B gene promoter, similarly to VEGF-A, also contains binding sites for SP1 and AP-2 transcription factors. But in contrast to the latter, it contains no site for HIF-1 [162]. Therefore hypoxia does not appear to induce VEGF-B expression [148]. Its two isoforms are the abundantly expressed heparin-binding VEGF-B_{167} and the diffusible VEGF-B_{186}. VEGF-B can bind VEGFR-1 and NRP-1 or heterodimerizes with VEGF-A but doesn't bind VEGFR -2 or -3 [138], [148], [163]. VEGF-B precise function in angiogenesis is not well-known despite few reports on its weak role in coronary collateral formation and inflammatory angiogenesis in arthritis [149], [151], [164], [165].

The VEGF-C proteolytic activation in the extracellular space by proteases generates a homodimeric protein with high affinity for both VEGFR-2 and VEGFR-3 respectively in blood vessel permeability or angiogenesis and lymphangiogenesis [149], [151], [166]–[168]. In adults, it has a limited expression in organs such as the heart, small intestine, placenta, ovary and the thyroid gland and can induce mitogenesis, migration and survival of ECs [148].

VEGF-D gene expresses a glycoprotein with half homology to VEGF-C, later processed at N- and C- terminal ends. It has been proposed to have similar affinities and functions as VEGF-C as it binds to VEGFR2/3 [168] with roles in vitro, in vivo or tumor angiogenesis [145], [149], [169]; while it is suggested to be dispensable or redundant in lymphangiogenesis [170]–[172].

VEGF-E originates from the sheep and goat infecting parapox/orf virus [173]. Its variants depend on the virus strain and specifically bind to VEGFR-2 and NRP-1. They induce EC mitogenesis, vascular permeability and a strong angiogenic response [148], [174], [175].

VEGF-F is of snake (viper) venom origin and has 50% homology with VEGF-A 165 [176]. It has two isoforms, Vammin and VR-1, that respectively have 110 and 109 residues and specifically bind to VEGFR2 [177]. VEGF-F C-terminal end has a heparin-binding region and a peptide that selectively blocks VEGF-A165 activity [178].

2.3. VEGF receptors

VEGFs act through structurally related VEGF receptors (VEGFRs) on ECs and various types of cells (Fig.3). In amphibians and mammals, three full-length receptors VEGFR1-3 and a soluble (s) VEGFR-1/Fms-like tyrosine kinase (FLT)1 are encoded by three VEGFR genes that regulate blood and lymph vessel formation [179]; in contrast to the single VEGFR homolog from invertebrates [180], [181]. These FLT family receptors share 7-Immunoglobulin (Ig)-like domains in the extracellular region and a kinase insert in the middle of the kinase domain. Unlike their related 5 Ig-type PDGFR receptors, VEGFR kinase inserts domains do not have tyrosine-X-X-methionine recognition motif for the binding of its phosphorylated protein to the phosphatidylinositol 3 kinase (PI3K) SH2 domain [182], [183]. Therefore, VEGF stimulation doesn't usually highly activate PI3K-Akt and Ras pathways in comparison to PDGF [181].

VEGFR2, also termed Kinase-insert domain receptor (KDR) or Fetal liver kinase (FLK)-1, is a specific tyrosine kinase receptor and the main transducer of VEGF signaling in ECs [184]. It binds to VEGF-A, -C and –D to induce EC proliferation, migration and vasodilatation. VEGFR2-mediated migration of EC might be integrin-dependent, as it forms a complex with integrin $\alpha_V\beta_3$ upon binding VEGF-A [148], [185].

Aside from ECs, other cells also express VEGFR2. These include circulating endothelial progenitor cells, pancreatic duct cells, retinal progenitor cells and megakaryocytes [148]. Through its consensus sites, ETS-1 has been shown to be essential in the transcription of VEGFR2 in cooperation with HIF2α [186]–[188].

VEGFR1, also known as FLT-1, binds VEGF-A with higher affinity than VEGFR2, as well as VEGF-B and PlGF [181], [189]–[192]. VEGF and PlGF trapping by VEGFR1 and sVEGFR1 negatively regulates angiogenesis. For instance, sVEGFR1 maintains the cornea avascularity in the eye, forms a trophoblastic barrier against the abnormal fusion of foetal umbilical capillaries and maternal vessels, induces preeclampsia symptoms such as hypertension and proteinuria, by preventing vasodilatation and scavenging VEGF [181], [193]–[197]. Moreover, *FLT-1* TK-deficient *($FTK^{-/-}$)* mice are healthy with normal angiogenesis. However, VEGFR-1 only transmits weak mitogenic signals in ECs and complexes with the highly signaling VEGFR-2 [198]. Additionally, VEGF-induced signaling of FLT1 TK is involved in tumour growth, metastasis, chronic arthritis, bone marrow formation and neuron physiology [148], [199]–[203].

VEGFR-3, also known as FLT4, mediates lymphangiogenesis upon binding to its ligands VEGF -C and –D and becomes restricted to lymphatic ECs in adults [148], [204].

NRP -1 and -2, two receptors of class 3 repulsive SEMA3 and VEGF coreceptors, have roles in angiogenesis [205]–[207], but also in neuronal development and immune system [181], [208]. NRP-1 recognizes VEGF-A165, VEGF-B and PlGF whereas NRP-2 is specific to VEGF-A165, VEGF- C and PlGF [2], [206]. During angiogenesis, the NRP-1 co-receptor form complexes with VEGFR-1 and thereby potentiates the interaction of free VEGF with VEGFR-2; as shown by its overexpression in chimeric mice that resulted in excessive capillaries and blood vessels formation, hemorrhages and cardiac malformations [181].

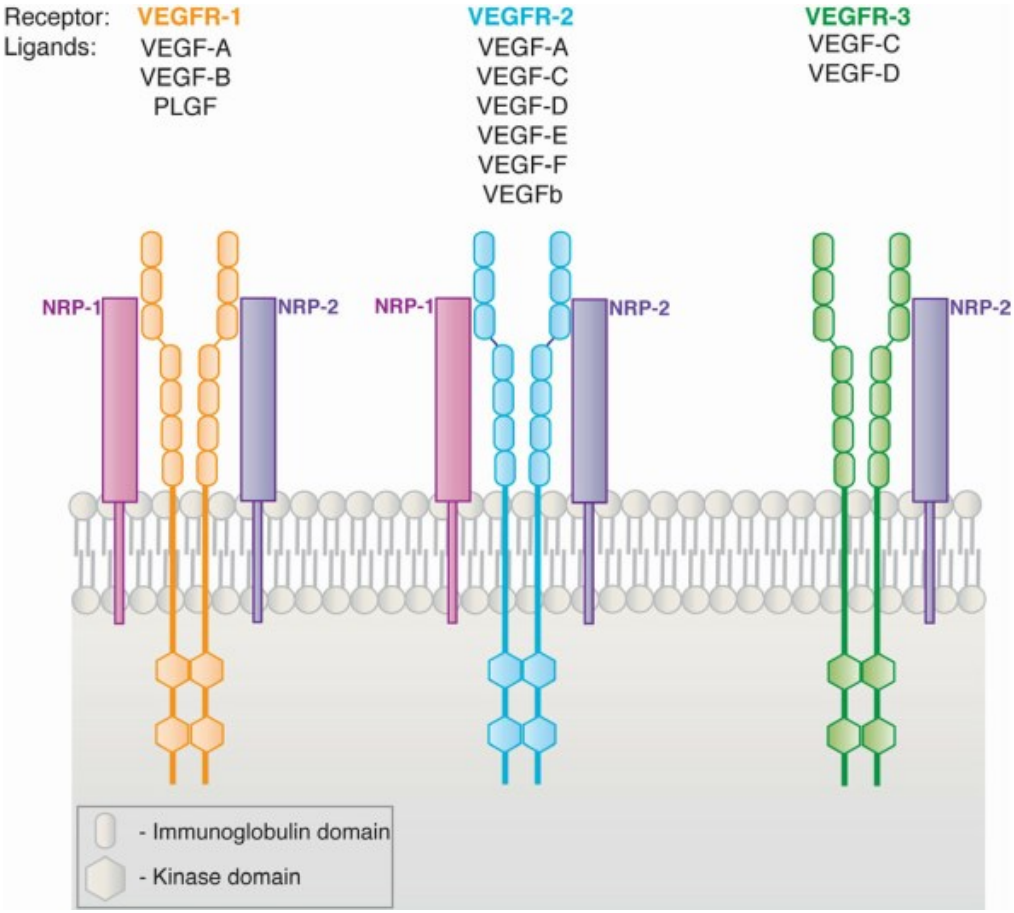


Fig.3 VEGF subfamily members, receptors and co-receptors

VEGF receptors are shown with their binding ligands (top) and interacting co-receptors (side). Adapted from Patel-Hett and D'Amore, 2011.

2.4. VEGF signaling

2.4.1. VEGF activity and pathways

Most of VEGF angiogenic effects result from VEGFR2. Upon ligand binding, VEGFR2 dimerizes, changes conformation and becomes activated by autophosphorylation on tyrosine residues. Phosphorylation of VEGFR2 leads to the recruitment of adaptors such as SHB and the activation of mediators involved in several downstream signaling pathways, mostly the Ca^{2+}-rheum-cRaf-MEK-MAPK1/3 phosphorylation cascade [25]. Aside from MAPK, activated effectors include PLC-γ producing the signaling diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) for Protein Kinase (PK) C activation; Phosphatidylinositol 3-kinase (PI3K) that regulates the PIP3-mediated activation of Akt1/PKB; and Src that regulates PTK2/FAK1 [182]. These effectors often lead to the induction of targets genes involved in the reorganisation of Actin, survival, migration and proliferation of ECs (Fig.4).

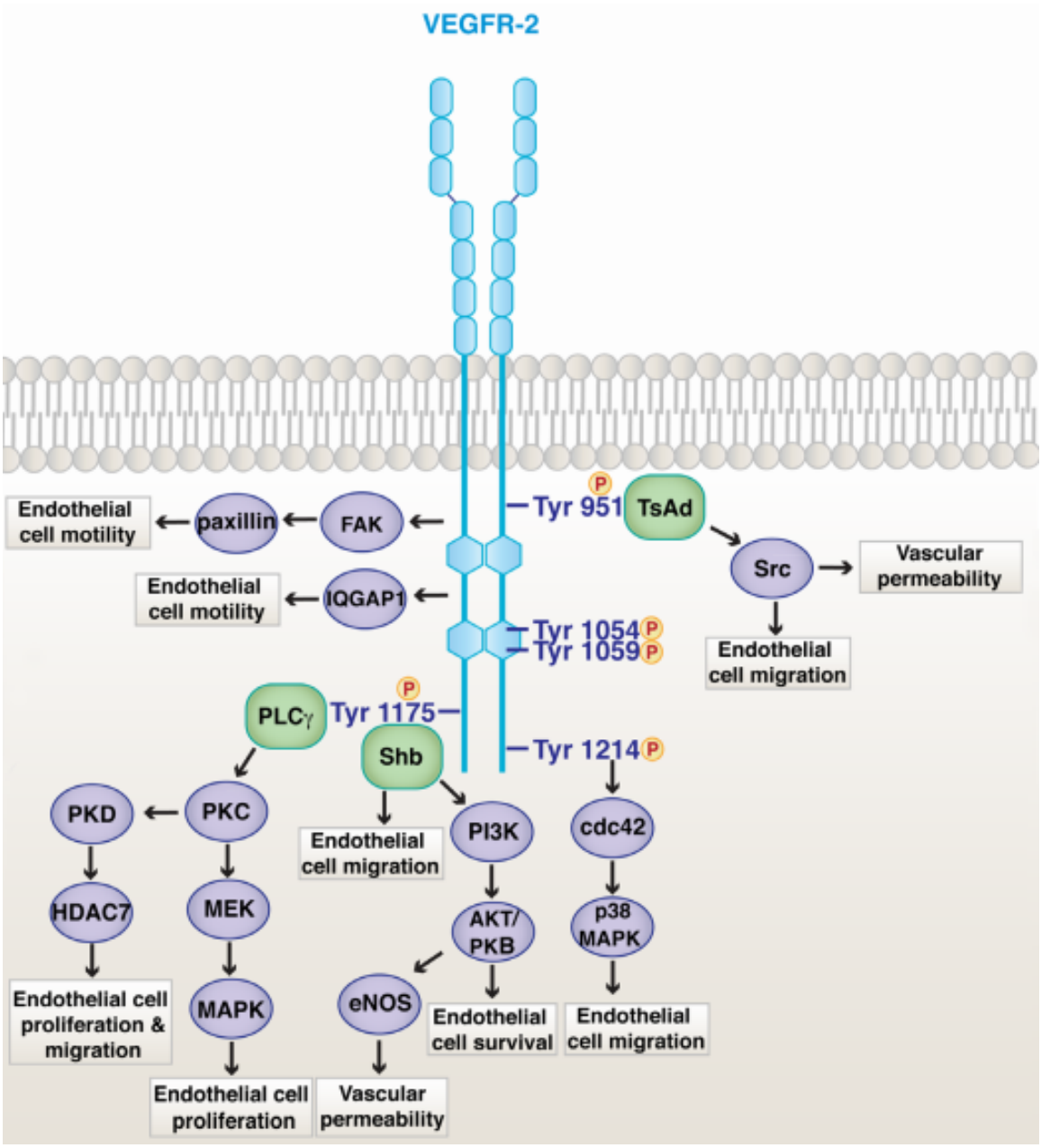


Fig.4 The VEGFR2 signaling cascade and its biological effects

The VEGFR2 receptor is represented with its phosphorylations sites (Tyr951, Tyr1054, Tyr1059, Tyr1175 and Tyr1214), binding proteins (green), downstream effectors (purple), as well as biological effects. Adapted from Patel-Hett and D'Amore, 2011.

2.4.2 Regulation of VEGF signaling

In addition to VEGF synthesis that modulates its bioavailability upstream of signaling, the downstream regulation of VEGF activity could be achieved at various levels such as receptors or mediators of its pathways. The fine downregulation of the VEGF signaling and associated pathways is necessary to establish a mature vessel network matching the tissue requirements. For instance, VEGFR1 negative role in VEGF signaling has generally been suggested to suppress proangiogenic signals in the early embryo [25]. Fong et al. observed that VEGFR1-null mutant mice die at embryonic stage E-8.5–9.0 due to the overgrowth and disorganization of blood vessels. Hiratsuka et al. also investigated whether the negative role of VEGFR1 involves the ligand-binding domain. Knock-out mice lacking the tyrosine kinase domain of VEGFR1 were basically healthy with nearly normal blood vessels but showed a defect in the migration of macrophages toward VEGF-A. Their results indicate that the ligand-binding and transmembrane domains of VEGFR1 are required together for a suppressive effect on angiogenesis during embryogenesis. However, in contrast to its negative role during embryogenesis, a positive role of VEGFR1 has been described in tumor growth, metastasis and inflammation. On the one hand, the tumor growth rate is rescued in VEGFR1 tyrosine kinase $(TK)^{-/-}$ mice in comparison to wildtype; on the other hand, this rate is oppositely affected by the overexpression of PlGF, a VEGFR1-specific ligand [209]. Moreover, some VEGF-induced proteins including HLX (H2.0-like homeobox) negatively regulate the sprouting of endothelial cells under normoxic conditions by balancing attractive with repulsive vessel guidance through the up-regulation of cues such as UNC5B [210].

3. VEGF and Notch cooperation during sprouting angiogenesis

3.1. Notch signaling and biological responses

The first relevant role of Notch signaling has been reported during embryonic vascularisation where it contributes to the arterial specification [211]. The Notch pathway is also critical in determining the developmental fate in invertebrates and vertebrates non-vascular cells [212], [213]. For instance, its developmental role has been well characterized in mouse induced-pluripotent [214] and embryonic stem cells [215], fibroblasts and neural progenitors [216]–[218]. In these cells, Notch activity results in the heterogeneously fated population of hairy and enhancer of split (HES1)-high and HES1-low cells [215], and reinforces or alleviates HES1-mediated repression of cell cycle [46] or fate-controlling genes [219]–[222] through its induction of ID proteins, along with Smad 1,5 [216], [223].

Mammals express four single-pass transmembrane Notch receptors (Notch 1-4) [224], [225]. On the cell surface, Notch receptors interact with five single-pass transmembrane ligands: Jagged (Jag) 1,2, Delta-like (DLL) 1,4 and the decoy DLL3 [219], [226]–[228] (Fig.5). In fact, post-transcriptional modifications such as glycosylation [229]–[233] and proteolytic cleavages at sites S1-4 [234]–[236] can regulate Notch activation [227]. Initially, the S1 Furin-mediated cleavage at the carboxyl end of the sequence RQRR (1651-1654) occurs during the ER/golgi secretory pathway and results in non-covalently attached amino- and carboxy- terminal segments in the cell membrane [237]–[241]. Regulatory proteins including Notchless, Numb and Deltex, as

well as the Notch1 S1 cleavage regulate the receptor availability at the membrane [242]. Upon ligand binding, juxta- and intra-membraneous cleavages sequentially occur at the extracellular and intracellular ends of Notch transmembrane domain. (TMD). In fact, the α secretase, a Disintegrin and Metalloproteinase (ADAM) 10 or 17/TACE cleaves Notch segments at S2 sites to release the Notch extracellular truncation (NEXT) fragment and a Nβ peptide [234]. Then, the ADAM-induced conformational change enables the γ secretase-mediated S3/S4 cleavages that in turn progressively generate the Notch intracellular domain (NICD) within the signal-receiving cell [227], [243], [244]. Upon cleavage, the NICD forms a transcriptional complex in which CSL (CBF1, Su(H), Lag-1) [245] and directs it to specific targets such as genes encoding the HES [246], [247] or hairy/enhancer-of- split-related with YRPW motif (HEY) proteins [19], [248], [249] (Fig.6). HES and HEY proteins are subfamilies of the basic helix–loop–helix (bHLH) transcription factors that subsequently regulate various genes, including the differential repression or upregulation of targets such as *VEGFR2* and VEGFR1 genes [219], [250]–[253]. Other Notch-upregulated targets include *JAG1*, *JAG2* and *UNC5B* genes [254].

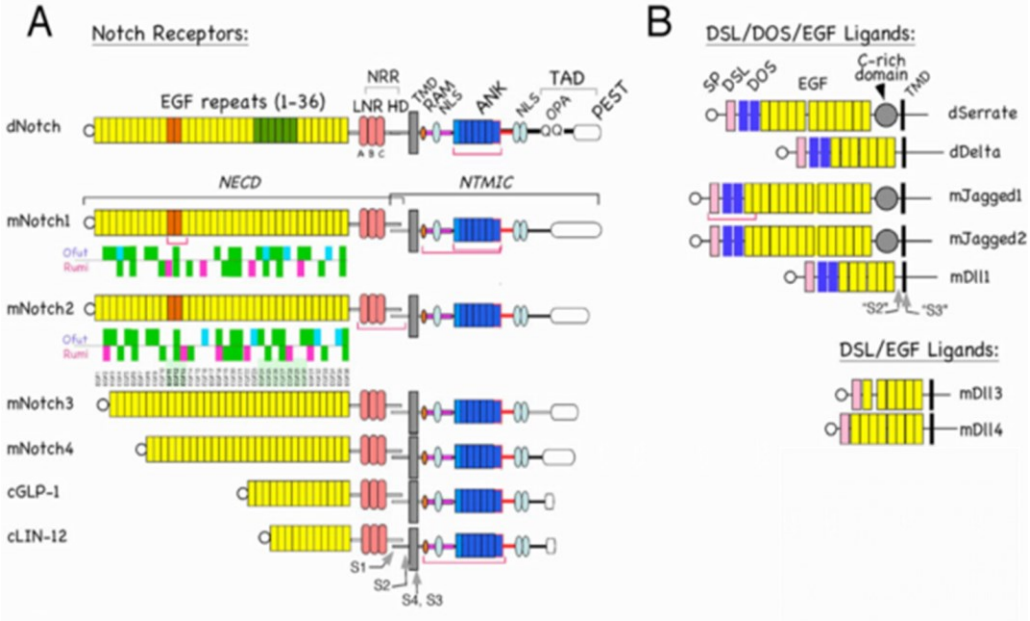


Fig.5 Domains of Notch receptors and ligands

A) The extracellular EGF-like repeats (yellow) of mammalian (m), *Drosophila* (d) and *C-elegans* (c) Notch1-4 are shown with their ligand interacting regions (orange and green). Underneath mNotch 1 and 2, their common (green) or unique sites of fucosylation (cyan) and glycosylation (magenta) are represented. The Lin-12 Notch repeats (LNR A, B and C) and the heterodimerization domain (HD) negative regulatory regions (NRR) next to EGF-like repeats are followed by S1-4 cleavage sites. B) Classical ligands found in Drosophila and mammalian contain DOS (blue), DSL (pink) and EGF (yellow) domains. TMD: transmembrane domain; RAM: $RBP_{Jκ}$-associated module; NLS: nuclear localization signal; ANK: Ankyrin repeats; TAD: transcriptional activation domain; PEST: proline, glutamate, serine, threonine degron domain. Adapted from Kopan and Ilagan, 2009.

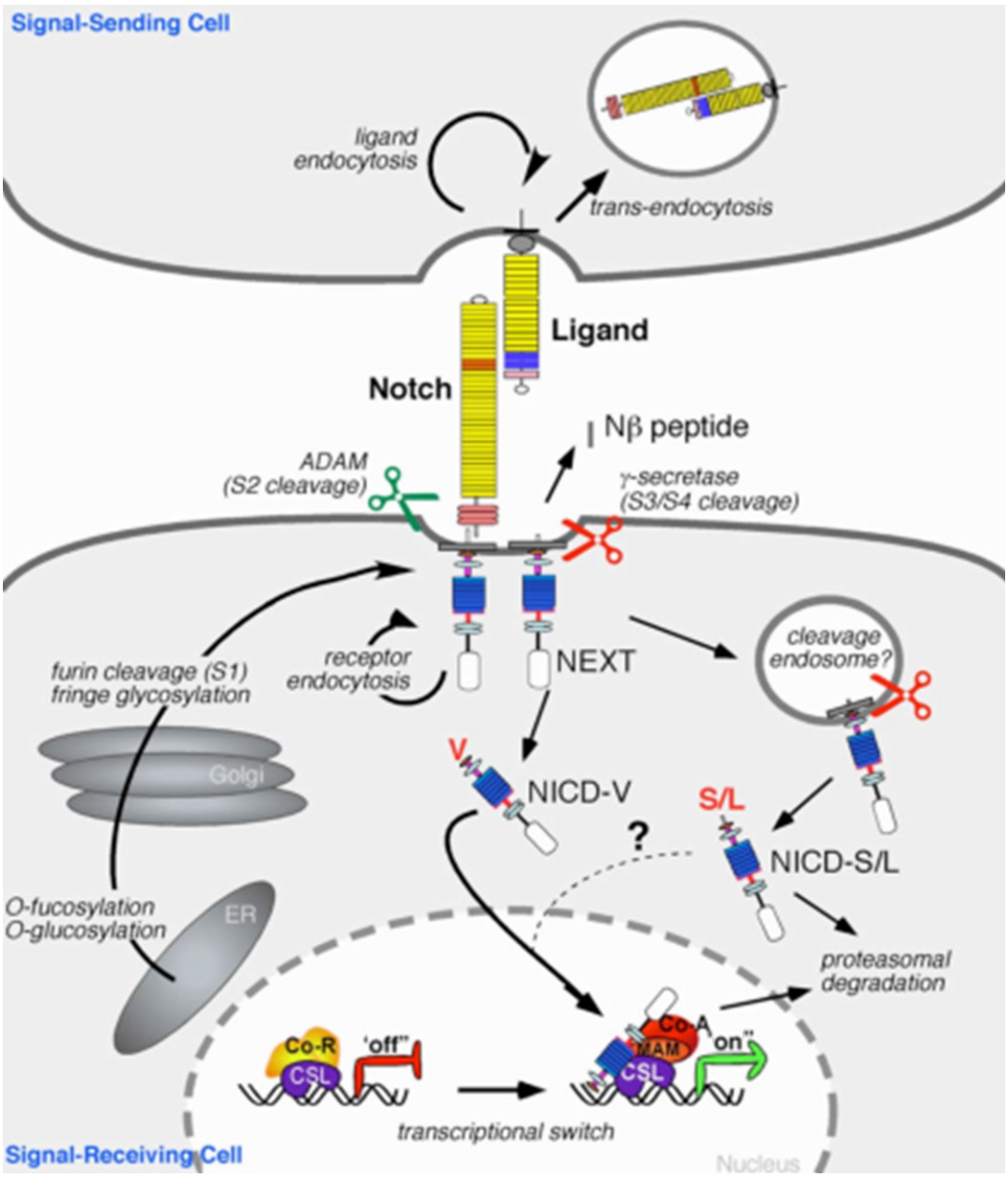


Fig.6 Proteolysis-mediated Notch signaling

The post-translational modifications and maturation of Notch include the O-fut- and Rumi- mediated glycosylation as well as Furin-catalyzed S1 proteolysis during the ER/Golgi pathway. In Fringe-expressing cells, the extension of O-fucose glycosylation alters the stimulatory ability of specific ligands of Notch. The processed receptor is expressed at the cell surface as a heterodimer held by non-covalent bonds. Notch is

transactivated by the binding of a ligand that induces its conformational change and exposes it to ADAM-mediated S2 cleavage that subsequently generates a Nβ peptide and a NEXT fragment. The later fragment is digested by the γ-secretase between TMD S3-S4 sites at the cell surface or in the endosomes to respectively produce stable or proteasome-destined NICD fragments. Upon the nuclear translocation of the stable NICD, its interaction with CSL displaces the ubiquitous CoR and HDACs from the repressor complex and recruits MAML and other co-activators to activate the transcription of Notch targeted genes. Adapted from Kopan and Ilagan, 2009.

.

3.2. Regulation of Notch signaling

The nuclear microenvironment mainly contributes to the regulation of NICD activity following its translocation [255]. On the one hand, intrinsic repressor complexes interfere with Notch activity by inhibiting the expression of its target genes. They include proteins such as NCoR/CIR, FLH1C/KyoT2 and the silencing assembly containing the SMRT/HDAC1-assosciated repressor protein (SHARP) and MINT/SPEN. Following the interaction of NICD with CSL/$RBP_{J\kappa}$ (recombination signal-binding protein 1 for Jκ), this repressor is displaced from its counterpart. Thus, the CSL repressor complex switches to an activator within its complex with NICD [219], [256]. Moreover, the NICD-CSL complex includes additional co-activators such as Skip, the Ankyrin- and CSL- binding Mastermind-like proteins (MAMLs) [256] and histone acetyl transferases (HATs) such as the MAML-acetyling P300 involved RNA polymerase-recruitment [257] (Fig.6). On the other hand, the Notch/CSL binding affinity doesn't prevent CSL interactions with other activators. The presence or absence of competing factors affect the amount of CSL or MAML co-activators available for NICD binding. Thus, extrinsic activators in the nucleus contribute to determine the type of responses induced by Notch in specific cellular conditions. For instance, the bHLH protein P48/PTF1a competes with NICD for binding at low concentrations of $RBP_{J\kappa}$. Moreover, when $RBP_{J\kappa}$ concentration is limiting, free NICD could be redirected to associate with other factors such as Smad, HIF1α or nuclear factor kappa B (NFκB). However, as there often is a stoichiometric excess of $RBP_{J\kappa}$ coupled to low nuclear concentration of NICD, no significant NICD should be

available to associate with other partners such as Smad under physiological conditions and *in vitro*.

3.3. Notch regulation of VEGF-induced tip/stalk phenotypes

In the angiogenic context, Notch has been reported as a relevant player that insures the smooth progression of sprouting. It collaborates with VEGF to prevent a dysregulated transformation of ECs by VEGF into tip cells [85], [86], [90], [213], and to retro-inhibit the VEGF responsiveness of selected tip cells on short-range intervals [11], [19], [84], [216]. Briefly, VEGF induces DLL4 that in turn triggers the transactivation of Notch in the neighboring cells and the expression of targeted proteins, including the Notch ligand JAG1 [86], [258]. Thus, VEGF and DLL4 establish a heterogenic population of tip senders and stalk receivers of Notch signals among ECs [19], [90], [213]. These opposite states of Notch signaling and their patterns of expressed proteins in neighboring ECs contribute to sustain their differential processing of VEGF cues by tip and stalk cells. On the one hand, the activation of Notch pathway in the stalk cells represses the transcription of own proangiogenic proteins, while it stimulates antiangiogenic effectors including VEGFR1. On the other hand, Notch-inactive tip cells become enriched in markers such as DLL4, VEGFR2, UNC5B, VEGFR3, Nidogen-1, uPAR, Integrin1, Apelin, Endothelial-specific molecule (ESM)-1, PECAM, CXCR4 and PDGFb [91], [219], [254]. Overall, the inactivity of endogenous Notch extends the VEGF responsiveness advantage of the initially exposed tip cell [213] and thereby favors its VEGF-directed migratory property at the leading edge of the sprout; in contrast to Notch-active stalk cells that only proliferate at the base of the sprout and respond less to VEGF (Fig.7).

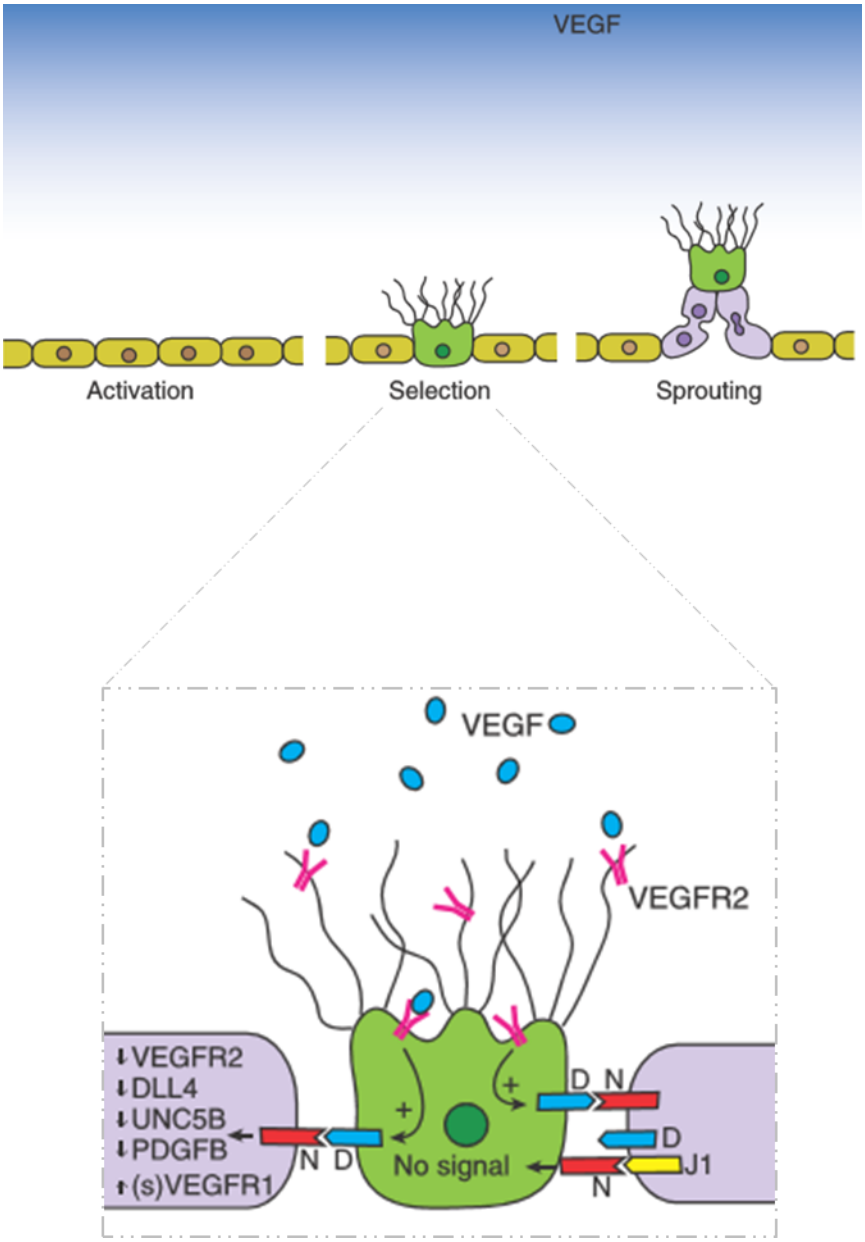


Fig.7 VEGF-Notch signaling during sprouting angiogenesis.

VEGF induces DLL4 in the selected tip cell that in turn transactivates Notch in adjacent stalk cells. Notch activation results into the lateral inhibition of tip markers and phenotype in stalk cells. Adapted from Geudens and Gerhardt, 2011.

4. BMPs in angiogenesis

During angiogenesis, the regulation of the tip/stalk phenotypes by the Notch-VEGF system also implies other proteins such as bone morphogenetic proteins (BMPs). In fact, there is an interplay between BMPs and VEGF-Notch pathway that originates from their ability to co-regulate target genes. This reciprocal relation is mainly based on the ability of BMP-induceable Smads to interact with the components of the Notch/VEGF signaling including NICD and some of their cytoplasmic or transcriptional binding partners or targets [19], [259], [260]. Overall, the BMP-Smad signaling contributes to the regulation of levels of Notch targets in ECs during sprouting [19].

4.1. Definitions and roles

BMPs represent a group of twenty or so proteins that play key roles in the regulation of biological processes including the maintenance of vascular homeostasis [74], [261], [262]. They belong to the transforming growth factor beta (TGF-β) superfamily [263], [264]. The BMP family includes the BMP proteins (Fig.8), most growth and differentiation factors (GDFs) and the anti-mullerian hormone (AMH). The other group from this superfamily, the TGFβ-like proteins, is composed of TGF-βs, activins, Nodals and some GDFs [2].

BMPs pertinence is reflected by the importance of processes they control. They were identified based on their properties to trigger ectopic bone and cartilage formation [265]–[268] following subdermal injection, whilst TGF-βs were first characterized based on the ability to confer a transformed phenotype. BMPs have also been found to regulate

cellular functions pertinent to organ development and physiology, alike members of the TGFβ superfamily [269] [270]. These cellular functions include differentiation, proliferation, axon guidance and apoptosis [270] [271]. Moreover, BMPs are clinically implicated in the treatment of bone disorders and injuries [271] or associated with tumor angiogenesis [272] and physiopathogenesis of various diseases [273]. Other BMP-associated pathologies include fibrosis [272], hereditary hemorrhagic telangiectasia (HHT) [273] or pulmonary arterial hypertension (PAH) where a counterbalance exists between their signaling and those of TGF-β [61].

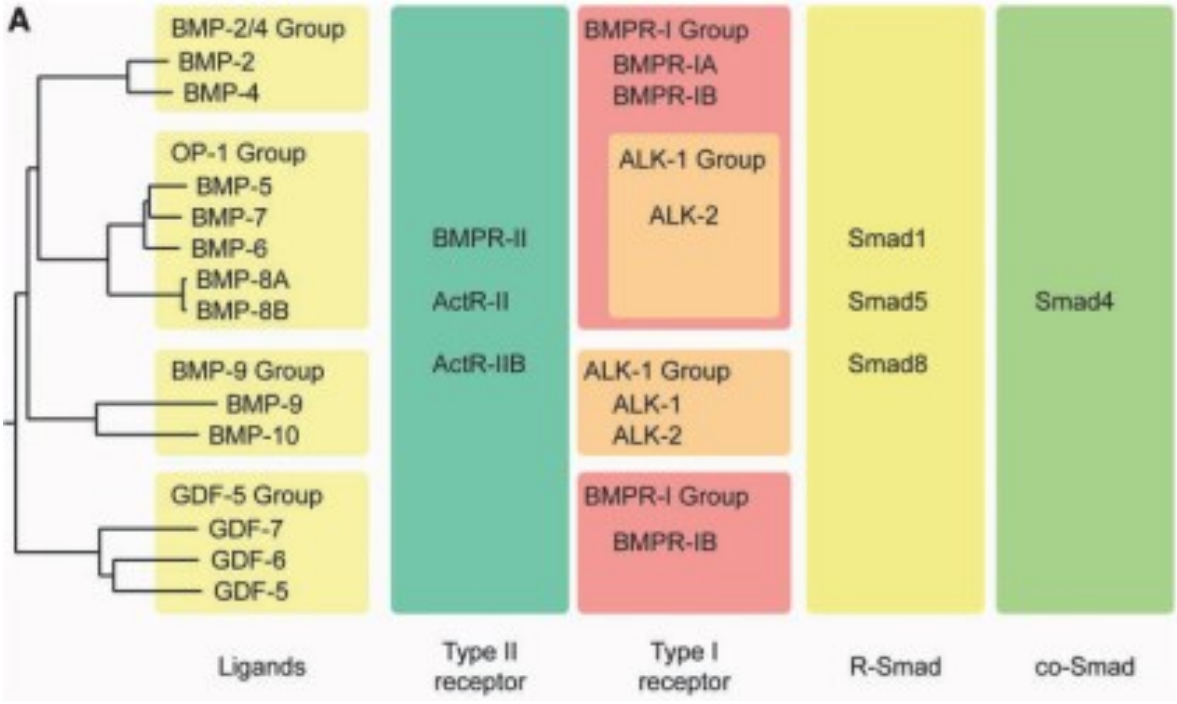


Fig.8 The relationship between BMPs, type I and II receptors and Smads

The phylogenetic tree of the BMP subgroups and the specificity of BMPs towards their receptors are presented. BMP-2/4 bind to BMPR-IA/IB. BMP-6/7 bind strongly to ALK-2 but weakly to BMPR-IB. BMP-9/10 bind to ALK-1 and ALK-2; whereas the GDF-5 group preferentially binds to BMPR-IB. Adapted from Miyazono and Morikawa, 2009.

4.2. BMP signaling

BMPs can interact with 7 type I receptors (T_1R) termed activin-like kinase (ALK 1-7), type II receptors (T_2R) and accessory receptors that transduce their biological signals [274] (Fig.8). Briefly, the BMPs bind to the constitutively active T2Rs that recruit and transphosphorylate ALK receptors in their Gly-Ser (GS) domains [275], [276]. The active ALKs specifically recruits and phosphorylates Smad 1,5,8 within the T1R/T2R heterocomplex [277]. Smads are well-conserved across various species and mediate signals from the TGF-β superfamily class of ligands. According to the current dogma, the BMPs and some GDFs mainly signal via Smad1/5/8, in contrast to TGF-βs, activins and Nodals that activate Smad2/3 [270], [274], [278]. However, some studies have reported the activation of Smad2 by BMP9 in pulmonary ECs [279]. The active Smad1/5,8 and Smad4 form complexes that translocate to the nucleus to interact with factors that regulate the transcription of BMP target genes [275] (Fig.9).

Among the seven T_1Rs, ALK -1, -2, -3 and -6 are highly specific to BMPs and signal via Smad1/5/8; with ALK2 exceptionally showing phosphorylation of Smad1/5 alone [277]. ALK -4, -5 and -7 have been shown to be activated by Activins and the TGF-β via Smad2/3 [277], [278]. Additionally, BMPs bind to T_2Rs including the BMP receptor (BMPR)-II and the Activin receptors (ACVR) -IIa and -IIb [280]. The other T_2R members such as TβR-II and MISR-II respectively bind TGFβ and the Mullerian inhibiting substance (MISR) [280].

Co-receptors such as the EC-specific Endoglin (ENG) and TGF-β type III receptor ($T\beta_3R$ or Betaglycan) are also components of the BMP-2,7,9, Activin-A and TGFβ-1/3 signaling pathways [281]–[283]. ENG is a membrane glycoprotein that generally requires a co-expression and association with corresponding T_2R receptors to bind to its ligands; except for its assembly with BMP2 that requires ALK3 or ALK 6 [281], [282]. ENG regulates BMP9/BMP10 signaling via ALK1-Smad1,5,8 as well as the TGF-β1 pathway via ALK5/TβRII-Smad2,3 [281]. Moreover, the co-receptor role of ENG during TGF-β1 activity can be mediated through sequential phosphorylations at its cytosolic $Ser^{634/635}$ and $Thr^{640/654}$ respectively by TβR-II and ALK1 [281], [284]–[286]. Except for pulmonary vessels and neovascularising tissues, ENG is downregulated in adult tissues in contrast to its wide expression throughout the developing embryo vasculature [277].

In addition to ENG, members of the glycosylphosphatidylinositol-anchored repulsive guidance molecule (RGM) family, such as RGMA, dragon and hemojuvelin (RGMC) [287], as well as the low-density lipoprotein receptor-related protein 1 (LRP-1) [288], have been characterized as co-receptors that potentiate BMP, but not TGF-β, signalling [74]. For instance, co-receptors RGMA and dragon are required for BMP2- and BMP12-mediated gene expression, whereas hemojuvelin is involved in regulating BMP-dependent iron homeostasis via hepcidin expression in liver. Another co-receptor named CRIPTO interacts with ALK4 during Nodal signaling [265], [289].

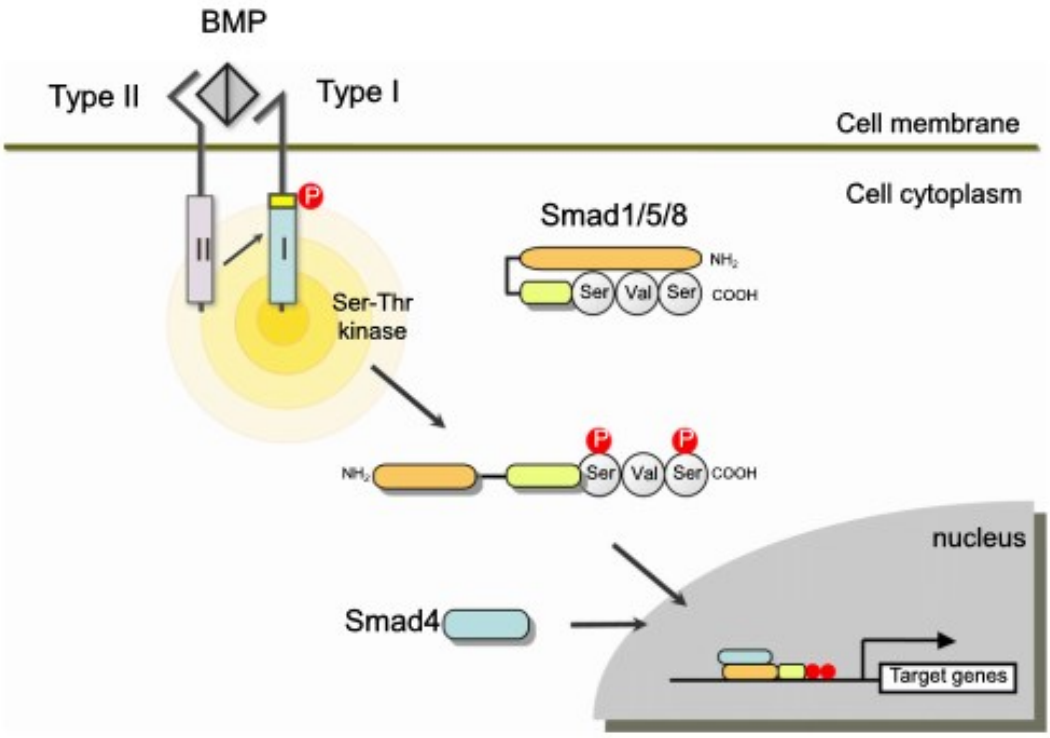


Fig.9 BMP canonical signaling.

Upon binding of BMPs to types I and II receptors on the cell surface, type II phosphorylates type I receptor kinase. Once active, the later kinase in turn phosphorylates two serine residues at the c-terminus of R-Smads (Smad1/5/8) that subsequently form heterocomplexes with Smad4 (co-Smad). The R-Smad-Smad4 complexes translocate to the nucleus where they drive the transcription of targeted genes. Adapted from Kokabu et al, 2012.

4.3. Regulatory features of the BMP pathways

Like other members of the family, BMPs and their receptors have domains and features that function and exist independently from the rest of the protein structure. They are characterized by protease-cleaved prodomains regions and disulfide bonds that regulate their activity [266]. Unlike T_2Rs, T_1Rs mainly feature the GS N-terminal domain formed by juxtamembrane repeats of the respective amino acids, and a neighboring Ser/Thr kinase domain [280]. Additionally, there are accessory differences between T_1R and T_2R ligand-binding extracellular domains that determines their orientation within the ligand-receptors complex [280]. T_1Rs also have an L45 loop located between kinase subdomains IV and V. This loop protrudes from the kinase domain to interact with the corresponding regions on receptor-activated Smads (R-Smad) [274], [290]. The L45 is conserved between subgroups of BMP T_1Rs and mediates their affinity in interacting with and phosphorylating Smad1/5,8 [81]–[84]. Thus, the type I receptor overall confers to the T_1R/T_2R heteromeric complex its signaling specificity [276], [291] toward Smad1/5/8 [292]–[295].

Structurally, Smads are characterized by two regions of homology named Mad-homology domains (MH) 1 and 2, respectively located at the amino and carboxy ends, and a proline-rich linker region. In their inactive configurations, the Smad MH1 and MH2 domains interact with each other. The MH2 domain serves as an effector domain in signal transduction whereas the MH1 recognizes the Smad binding element (SBE) in promoter regions of BMP or TGFβ targets [296]–[298]. Smad6 however negatively feeds

back on its inducer BMPs (Fig.10) [74], [299].

BMP-responsive elements (BRE) characterize BMP target genes. BREs contain 5'-GTCT-3' and palindromic GC-rich GGCGCC Smad-binding elements (SBE) that bind p-Smad1/5/8 and Smad4 [19], [221]. In addition to the inhibitory Smad6, BMP-targeted genes also comprise repressors of cell differentiation that bind their target DNA and promote cell cycle progression. These inhibitors include the bHLH transcription factor such as HEY proteins or ID1 [221], [222], [300].

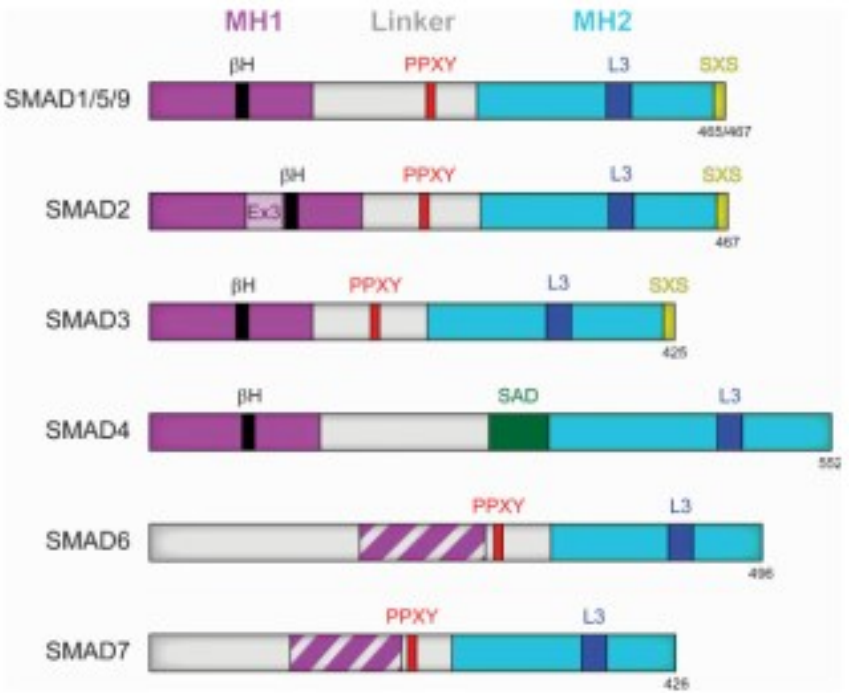


Fig.10 Structural domains of human Smads

Smads share Mad Homology domains (MH) 1 (purple) and 2 (blue) that mediate interactions with membrane, cytoplasmic or nuclear proteins. The MH2 domain contains a L3 loop that determines the specificity of R-Smad interaction with TGFβ-like versus BMP-like type I receptors and mediates the contacts with the SXS motif in oligomerized Smads. The C-terminal SSXS motif of R-Smads is phosphorylated at the last two serines by the type I receptor. The MH1 domain of the I-Smads 6 and 7 lacks the β-hairpin (βH) residues that is important for DNA interaction and is conserved between R-Smads and Smad4. The MH1 of most Smad2 isoforms contain an additional insert (Ex3) that interferes with their binding to DNA in native states. The linker region of Smads has a PPXY motif with transcriptional modulation role as it interacts with E3 ubiquitin ligases and the transcriptional modulators. In Smad4 however, the linker includes a Smad activation domain (SAD) essential for transcriptional activation. Adapted from Weiss and Attisano.

4.4. Non-classical BMP signaling

Upon ligand binding, BMPs and other members of the family can induce non-classical signaling pathways. Non-Smad pathways include BMP signaling via the mitogen-activated proteins kinases (MAPKs) such as P38, ERK, and JNK [301]–[303]; the phosphatidylinositol 3-kinase (PI3K)-Akt pathway and small Rho-like GTPases [74], [277], [304] (Fig.11). The non-canonical pathways of BMPs result from the crosstalk between their signaling and those of other ligands [74]. These BMP-crosstalking pathways includes those of growth factors or receptors such as VEGF, MAPK, JNK, PI3K, c-Src, P38, Notch, WNT, FGF and VE-cadherin, associated with vessel biology [305]. For instance, the non-Smad regulation of EC signaling and function by BMPs includes the mediation of BMP9 and ENG in inhibiting JNK activation [306] and trafficking of PI3K respectively [307]. BMP6 has been reported to induce ALK2 and BMPRII interaction with VE-cadherin [308] or c-Src phosphorylation that triggers VE-cadherin internalization [309], [310] and thereby the increased permeability. It either induces Smad or non-Smad pathways depending on the endocytic pathway of T_1R and/or T_2R. On the one hand, the clathrin-dependent endocytosis of T_1R and T_2R signal into the canonical activation of Smad. On the other hand, the caveolin-dependent internalisation specific to T_2R leads to the activation of non-Smad effectors such as P38-MAPK, JNK or possibly ERK [270] [277] [311].

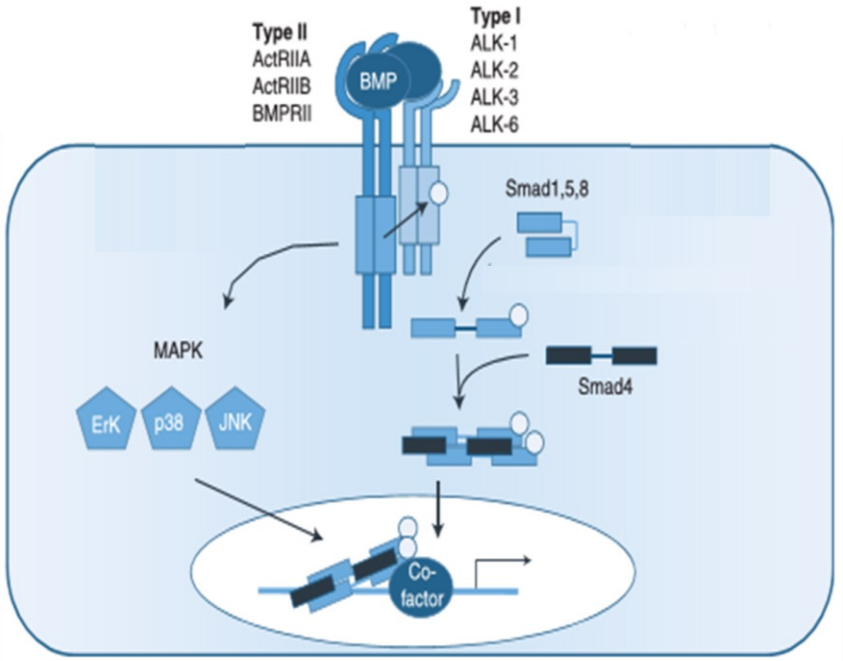


Fig.11 Canonical vs non-canonical BMP signaling

The heterotetrameric complex of type I and type II receptors forms upon the binding of specific BMPs. The stimulated receptor complex can either phosphorylate Smad1/5/8 that in turn propagate the signal via Smad4 binding and translocation (canonical BMP signaling); or mediate the activation of mitogen-activated proteins kinases (MAPKs) independently of Smad1/5/8 (non-canonical BMP signaling). Adapted from Goumans et al.

4.5. Regulation of the BMP pathways

Each level of the BMP pathway is tightly regulated to maintain a strict control of BMP signaling in cells and tissues [265], [312], given that they activate pathways that finely control developmental and physiological processes [313]. Generally, multiple mechanisms such as transcriptional regulation, protein degradation, post-translational modifications and subcellular localization [314] contribute to the modulation of the BMP pathways. Additional levels of regulation of BMPs or their receptors include the temporal or tissue-specific synthesis, the splicing of receptor RNA, the activation through the proteolytic cleavage of pro-regions of inactive precursors and the sequestration by ECM interfering molecules.

Specifically, the regulation of BMP pathways is mediated by extracellular antagonists, membrane-bound proteins and intracellular regulators that overall prevent the activation of R-Smads proteins or cause their degradation [313].

First, BMPs are secreted as larger propeptides and cleaved by proprotein convertases such as Furin. Mature BMPs form dimers that interact with BMPRI/II forming a hexameric complex [265], [315], [316]. The dimerization that generates active ligands requires a disulfide bridge at the seventh conserved cysteine outside of the monomer cystine knots [74], [311]. BMPs generally form homodimers; except BMP2 that generates more potent heterodimers with rather BMP -5, -6 or -7 than the corresponding monomer [74]. The cleaved prodomains remain non-covalently associated with their corresponding BMP dimers and don’t confer them latency, [74], [280], [317]–[319]. N- and O-

glycosylation posttranslational modifications additionally regulates BMPs secretion, half-life, receptor binding and functions [74], [320]. For instance, the glycosylation of BMPs and inhibitors proteins is likely to regulate their interaction with the ECM and thus modulates their diffusion and function [265], [316], [321], [322].

Competitive ligands or receptors inhibit BMP signaling either by associating directly with them to block their access to the receptors, or by competing with them for receptor binding [313]. Molecules such as Noggin, Chordin, Follistatin, Cerberus, the BMP-binding EC precursor-derived regulator (BMPER) differentially antagonize BMPs bio-availability [323]. For instance, Noggin binds with strong affinity to BMP-2, BMP-4, and BMP-7, with lower affinity to BMP-6, but not to BMP-9 or BMP-10 [74], [277], [324], [325]. Moreover XNR3 binds to the BMP receptors in competition to ligands [313] whereas the dominant negative BMP receptors or pseudoreceptors such as BAMBI prevent receptor activation [313], [326], [327]. Activins can also act as BMP inhibitors by sequestering the ACVR-II within inactive Activin-ALK2-ACVRII complexes whose wild-type ALK2 fails to induce Smad1/5/8 activation, [328].

Intracellularly and regardless of the constitutive kinase activity of T_2Rs, molecules such as the FK506-binding protein (FKBP) 12 can interact with T_2Rs and thereby sets a gatekeeper threshold on T_2R –induced T_1R activation in the absence of the ligand [74], [290].

Anchor proteins such as Endofin recruit and present Smad1 proteins to the BMP receptors for phosphorylation and mediate the receptor dephosphorylation via their protein phosphatase binding motif.

Smad6 and Smad7 are inhibitory Smads (i-Smads) that compete against r-Smads by binding to T_1R or recruiting protein phosphatases (PPA) [329]–[331], including their PPA1a recruit that dephosphorylates ALK-1 [74], [332]. Additionally, Smad6 competes by binding Smad4 and is BMP-specific, unlike Smad7. Thus, the competition between r-Smad1/5 and i-Smad6 for binding to the type I receptor may cause the kinetics of BMP-Smad1/5/8 phosphorylation to be relatively slow [265], [333]–[335]. Conversely, the methyltransferase PRMT1 methylates Smad6 on arginine, leads to its dissociation from the type I receptor, and eventually facilitates Smad1/5/8 phosphorylation and BMP signaling [265], [334].

The Smad ubiquitination regulatory factor (Smurf) 1, a recruited E3 ubiquitin ligase, timely adjusts BMP signaling. In fact, Smurf1 interacts with Smad 1 and 5 through their linker PPXY (PY) motif and thereby mediates the proteasome targeting of their basal rather than active level [270], [313] (Fig.12).

Unlike Smad downregulators, the protein associated with Smad1 (PAWS1) has been reported to alternatively mediate Smad4-independent BMP2/Smad1 signaling, following its phosphorylation by ALK3/BMPRIA and its binding to Smad1 [336]. Finally, transcriptional repressors such as BF-2 can inhibit BMP expression [313].

In summary, BMPs are substrates whose activity is temporally regulated by their trafficking, endocytosis or interaction with expressed modulators such as proteases or post-translation modulatory proteins. Furthermore, the spatial modulation of BMPs results from their compartmentalization and activation as determined by paracrine regulators and the ECM interactions. Moreover, the bioavailability of BMPs depends on their distribution/expression modality [337], [338] and diffusion [74], [318], [339], [340].

BMPs can act locally or circulate distantly from their site of expression, following their tightly regulated secretion and activation [74], [316], [324], [341], [342]. Among circulating BMPs are BMP4, BMP6, BMP9 and BMP10 [342], [343]. Finally, the combination of expressed type I, II and III receptors is cell- and time- specific, and thereby, spatially and temporally determines the tissue-specific sensitivity and responses to BMPs, based on the profile of available receptors and level of R-Smads. Overall, BMPs and their receptors specify, in concentration- , time- and tissue- dependent manners, distinct cell fates such as EC differentiation, proliferation and apoptosis [74], [316].

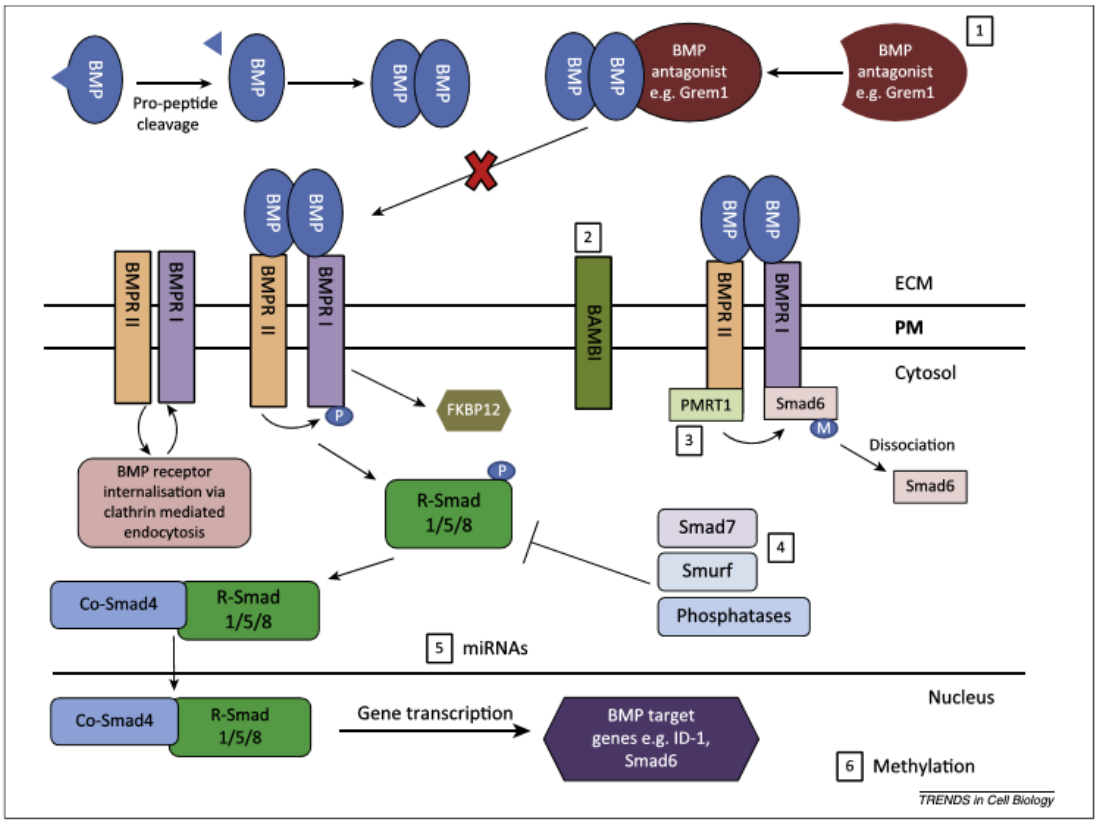


Fig.12 Regulation of BMP signaling

The extracellular regulation of BMP signaling occurs via the processing of ligands into mature dimers or the binding of antagonists such as Gremlin (Grem1) and Noggin (1). At the level of the plasma membrane the pseudoreceptor BMP and Activin membrane bound inhibitor (BAMBI) downregulates this pathway (2). Juxtamembrane constraints on receptor-mediated Smad1/5/8 phosphorylation occur via FK-binding protein 12 (FKBP12) binding and inhibitory Smad6 binding (relieved by the PRMT1 methyltransferase) (3). The intracellular regulation occurs via Smad7, phosphatases and ubiquitin ligases such as Smurf (4). Additionally, the BMP-mediated gene expression is modulated at levels of miRNAs (5) and methylation (6). Adapted from Brazil et al, 2015.

4.6. Vascular roles of BMPs

Most BMPs and their signaling mediators have been reported to play a role in vascular biology processes such as the development of endothelial and mural cells; as well as their responses to hypoxia, shear stress and inflammation [11], [74]. For instance, hypoxic conditions promote the proliferation and migration of the pulmonary artery SMCs (PASMCs) by inducing HIF1α that inhibits the expression of ALK3 along with BMPRII and interferes with the phosphorylation of Smad1,5. Additionally, the hypoxia-mediated repression of BMPER in the hypoxic retina ultimately results in BMP enhanced signaling and revascularisation [74], [344]. Moreover, during hypoxic conditions, BMP9 protects pulmonary microvascular ECs from apoptosis by mediating the activation of the BMPRII-ALK1 axis [345]. Furthermore, BMP-induced Smad4 contributes to mediate angiogenic effects as its nuclear translocation corelates with the presence of cell-specific transcriptional modulators that differentially repress *FLK1/KDR* within Smad complex [294], [346], [347].

Additionally, mutations or dysregulation in BMPs, their receptors or coreceptors lead to vascular diseases such as HHT/Rendu-Osler, PAH and preeclampsia [74], [277], [348], [349].

Specifically, *ENG, ACVRL1, Smad4* and *BMP9* mutations are associated with various HHT types [350]–[353]. HHT is an autosomal dominant disorder characterized by hemorrhages and associated with vascular lesions and arterio-veinous malformations (AVM), mucosal and skin telangiectasia as well as pulmonary, cerebral and hepatic

malformations [277]. Furthermore, alike TGF-β that promotes VSMC differentiation [354], the deficiency of ENG or ALK1 impairs mural cell development and has been the aim for preclinical studies that use their respective antibodies to inhibit tumor angiogenesis [11], [355].

Moreover, *BMPR2, ALK1* and *Smad8* mutations cause PAH, a condition characterized by occlusions in the pulmonary artery vasculature followed by severe hypertension and right heart failure [74], [277], [356]–[358]. Both BMPRII and ENG levels are also affected by hypoxia during PAH, as well as HHT, indicating that their levels play pathogenic roles in the associated oxidative stress and metabolic dysfunction [74], [359]. Moreover, the increase of ENG and its promotion of ALK1 signaling has been reported in hypoxic ECs [360].

Dysregulated levels of sENG and sVEGFR1 cause preeclampsia during pregnancy, a condition characterized by a high systemic blood pressure that causes the maternal, foetal or neonatal death [277].

Furthermore, BMP9 and BMP10 have their high affinity receptors ALK1 and BMPRII selectively or particularly enriched in ECs, whereas their low affinity receptor ACVRIIb is restricted to some cells including ECs [74].

Overall, BMPs contribute to regulate and determine the fates of vascular cells including ECs, surrounding pericytes and VSMCs during hypoxia and pathological conditions. Thus, BMPs specifically modulate EC and mural cell responses such as migration, proliferation or apoptosis; and thereby regulate vessel sprouting, tubulogenesis and vascular remodeling.

4.7. Implications of BMP9/ALK1 axis in angiogenesis

4.7.1. BMP9 expression and structure

BMP9 is encoded by the *GDF2* gene and expressed in the liver where abundant transcripts were determined in non-parenchymal Kupffer cells (KC), hepatic stellate cells, and liver endothelial cells (LEC). Like other BMPs, the proteolytical processing of the encoded BMP9 preproprotein is mainly intracellular and generates each subunit of the disulfide-linked homodimer [74], [361]. BMP9 pro-domain remains non-covalently associated to the mature homodimer and rapidly separates from the ligand upon biding to its receptor [361]. However, as the pro-region alone is inactive but does not inhibit BMP9 biological activity, both BMP9 and pro-BMP9 have equivalent signaling and cell-growth activity [271], [361] (Fig.13). BMP9 folding is driven by a characteristic cysteine knot with three pairs of disulfide bonds. Its conformation is of a hand with a concave palm side and two parallel β-sheets fingers. Alike other BMPs, its α-helix on the concave side binds T_1R whereas its convex interface interacts with T_2R.

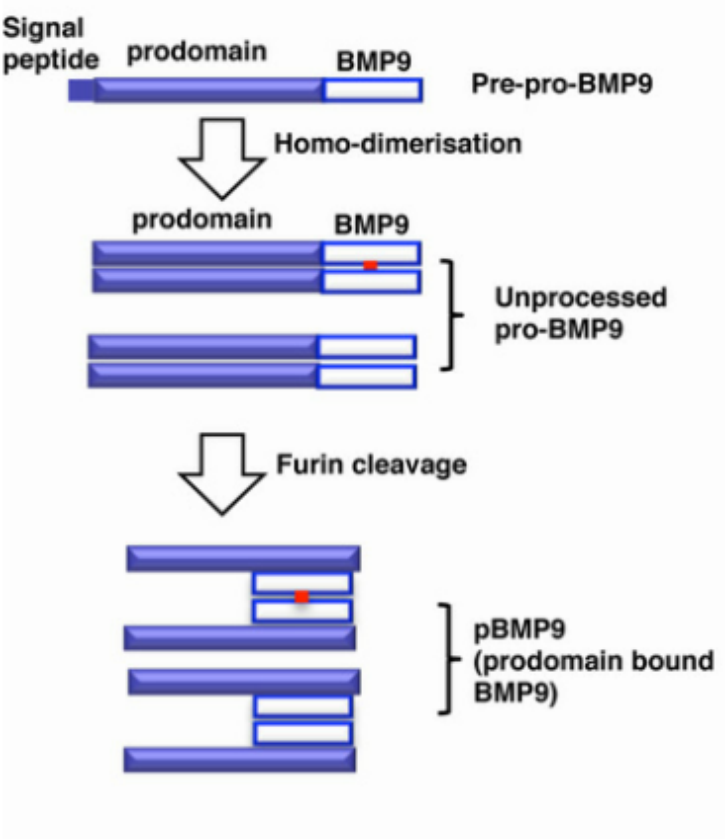


Fig.13 Schematic diagram of BMP9 processing

BMP9 is synthesized as an immature pre-pro-protein containing the signal peptide and the prodomain. The signal peptide is cleaved during secretory pathway prior to the protein hodimerization. The resulting dimer is processed through Furin proteolysis to generate the mature BMP9 dimer that can be non-covalently linked to the prodomain. Adapted from Li et al, 2016.

4.7.2. BMP9 signaling activity

BMP9 signals through the type I activin receptor-like kinase 1 (ALK1/ACVRL1) [65], [105], [106] in complex with BMPRII or ACVRIIA [277] (Fig.14). ALK1 additionally binds BMP10 [362]. However, BMP9 binds ALK1 with much higher affinity compared to that of BMP10 or other BMPs for their receptors. and, thereby, minimizes the BMP redundancy towards ALK1 [277], [280], [363]. In endothelial cells, BMP9 signaling through Smad1 requires the coreceptor ENG. BMP 9 and 10 also bind to ALK2 via ACVRIIB or to ALK6 under certain conditions [277]. However, in its early studies BMP-9 was reported to bind receptors on the liver-derived HepG2 cell line [364], [365].

The BMP9-specific receptor ALK1 is enriched in ECs [271], [363], [343]. Along with other ALKs, ALK1 structurally shows the N-terminal signal sequence, the hydrophilic and Cys-rich domain for ligand binding, the hydrophobic and single-pass transmembrane region, and the C-terminal and intracellular kinase domain. Despite the little sequence homology in the extracellular domains, the cysteine residues are conserved between ALKs. ALK 1-4 form a subfamily that shares a sequence homology between 60-79%. Moreover, the sequence identity between ALKs and ACVR II and IIB is somewhat maintained to 40%. In contrast to all other T_1Rs, ALK1 misses the residue Phe85 involved in the hydrophobic interaction between BMPs and corresponding T_1Rs [277]. Additionally, ALK1 has been demonstrated to bind TGFβ1 and TβR-II in ECs where it inhibits TGFβ1-dependent transcriptional activation of ALK5 [96], [97]. Thus, ALK-1 has been reported as a lateral inhibitor of TGFβ/ALK5 signaling pertinent to vasculogenesis and angiogenesis. However, the overall ALK1 signaling is independent of

TGFβ1/ALK5 axis as demonstrated by Shao et al that ALK5 suppression does not interfere with the induction of Smad1/5/8 phosphorylation by BMP9/ALK1 activity in bovine aortic ECs [366]. Accordingly, ALK5 expression is very low in ECs in contrast to perivascular cells from where it would rather regulate angiogenesis based on a paracrine and context–dependent mode of action of TGF-β1 [273], [367], [368], in addition to its stimulatory effect on VEGF expression [366].

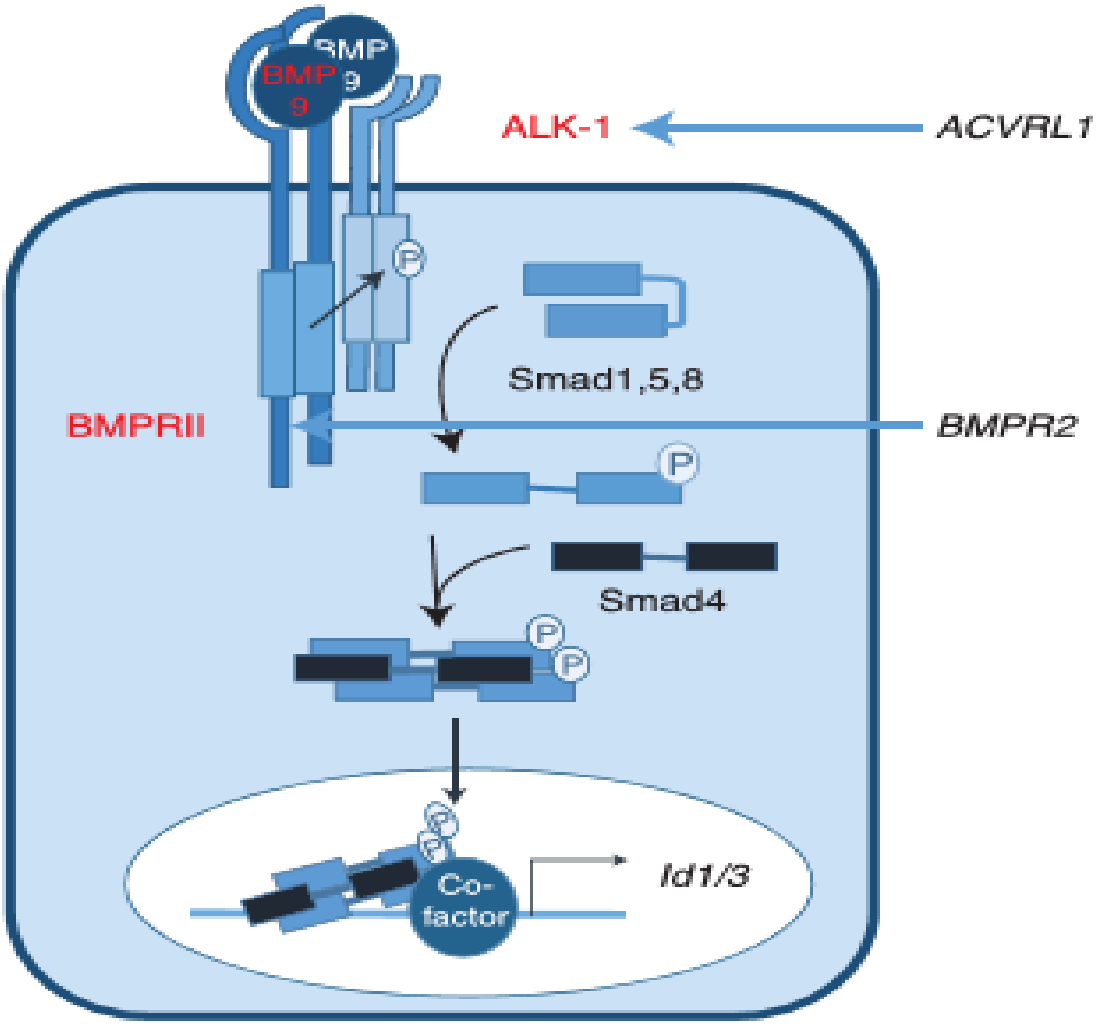


Fig.14 Components of the BMP9/Smad pathway

Representation of BMP9 signaling showing its binding to its strong interaction with the heterocomplex of type I ALK1 and type II BMPRII receptors. This complex activates R-Smad1/5/8 by phosphorylation and allows their interaction with Smad4. The nuclear translocation of the R-Smads/coSmad complex leads to the expression of target genes such as *Id1/3*. Adapted from Goumans et al, 2017

4.7.3. BMP9 biological responses

Originally, BMP-9 has been described to be involved in cartilage and bone development. It has also been reported to play a role in the differentiation of cholinergic central nervous system where it drives in the synthesis and trafficking of acetylcholine (ACh) [369], [370]. Moreover, BMP9 regulates the iron homeostasis-controlling peptide hepcidin, in a Smad4-dependent signaling, via the binding to its co-receptor and hepcidin-inducing hemojuvelin/hemochromatosis type 2 protein (HJV/HFE2/RGMC) [371]. Furthermore, BMP9 has been associated with tumorigenesis modulation in osteocarcinoma (OS) [372]. Additional BMP-9 roles have been reported in the regulation of transcription of several genes involved in glucose or fatty acid metabolism, and thereby in the decrease of glucose level in cultured cells or the reduction of glycemia in diabetic mice [373].

Nowadays, BMP9 has emerged as a crucial circulating factor for endothelial function and pathogenesis of vascular diseases [96], [97], [343], [366], [367]. Its receptor ALK1 is enriched in ECs including microvascular cells where BMP9 inhibits Actin reorganisation, cellular migration and proliferation through its induction of Smad1/5/8 phosphorylation or ERK/JNK-dependent signaling [301], [374]. Specifically, the BMP9/ALK1 signaling has been reported to suppress VEGF expression in ECs of aortic vessels [110]. Additionally, ALK1 contributes to the specification of the arterio-venous identity during the early development of vessels and thereby prevents the appearance of arteriovenous malformations (AVMs)/shunts in the newly formed endothelial tubes of the embryo [375]. Moreover, mutations in BMP9 and ALK1 are associated with HHT AVMs

during the embryonic development or the postnatal neovascularisation [74], [277]. Some studies have suggested a hemodynamics-mediated reinduction of ALK1 in preexisting and new arteries, as well as its role in arteriogenesis and arterial remodeling rather than venous during wound healing [96], [97] and tumor angiogenesis [272]. Furthermore, the BMP9/ALK1 axis has been reported to collaborate with Notch in countering VEGF-induced signaling and regulating the formation, branching and remodeling of neovessels. BMP9 inhibits VEGF-mediated processes in ECs and favors the phalanx state by activating the BMPR2-ALK1 complex; in contrast to TGFβ that has a bifunctional effect on angiogenesis. For instance, BMP9 interferes with VEGF expression [366] and additionally represses the transcription of its (co)receptors and effectors including KDR, NRP1, FLT4, ERK, Akt; while it stimulates the expression of VEGF negative regulators such as FLT1 or Notch target genes including *HEY1* and *HEY2*.

Overall, BMP9 shows a preferential affinity for the EC-specific ALK1 receptor and collaborates with various EC pathways such as VEGF and Notch. Additionally, BMP9 and its signaling components including ALK1, ENG and Smads are relevant to maintaining the vessel morphogenesis. Thus, the aforementioned properties of the BMP9 signaling axis contribute to sustaining its emerging roles in vascular biology, and physiological [250] or pathological angiogenesis occurring during ocular, systemic or cancer conditions [368].

5. Clinical implications of angiogenesis

5.1. Pathological angiogenesis

Uncontrolled or insufficient angiogenesis may lead to several angiogenic or ischemic disorders affecting various systems or organs, including the eye. On the one hand, during the pathological angiogenesis, also termed neovascularisation (NV), there is a balance shift of angiogenesis regulators towards its activators. In atherosclerosis for instance, local hypoxia and overexpression of VEGF mediate the NV that contributes to the growth and rupture of atherosclerotic plaques; whereas in benign hemangiomas of infancy, there is an excessive expression of both VEGF and bFGF leading to a mix of normal and abnormal vessels [25]. In rheumatoid arthritis, there is an excessive production of angiogenic factors by infiltrated macrophages and immune or inflammatory cells that leads to the formation of a vascularised pannus in joints [111]. In psoriasis, an autoimmune-mediated condition where faulty signals induce the overproduction of skin cells, along with the angiogenic IL8, while the antiangiogenic thrombospondin 1 (TSP-1) expression is downregulated [110]. Angiogenesis also contributes to accelerating the growth and spread of cancer. On the other hand, ischemic vasculopathies result from an insufficiency in angiogenesis and can lead to heart attacks, stenosis, ununited fractures, neurodegenerative disease, peripheral blood circulation deficiencies and baldness [20], [25], [113], [376]–[378].

5.2. Neovascular AMD and retinal vasculopathies

5.2.1. The retinal and choroidal vessels of the eye

The neovascular diseases of the eye result from the abnormal or excessive formation of its vessels. Neovessels that invade the outer retina and the subretinal space originate from the retinal or the choroidal networks [379], [380].

The retinal network first arises during development and consists of superficial and deep plexi [381]. The superficial plexus of the retina extends outward and, in human, also vertically towards the deep retina. Their deeper plexus develops from the sprouting of the inner plexus. In addition to an avascular outer retina, the human retina is characterized by an avascular perifovea macula [379], [381]. Both avascular regions are limited by a continuously interconnected arcade of bordering capillaries [381]. The retina comprises other cell types that associate or not with its mature vascular endothelium. Neuronal and glial cells are organized into layers that directionally transmit light from the outer to the inner retina [379]. On the one hand, photoreceptors reside in the outer retina and possess light-capturing segments located away from vessels; whereas the retinal pigment epithelial (RPE) cells tightly line the Bruch's membrane [379], [381]. On the other hand, mature retinal vessels associate with other cells such as pericytes, smooth muscle cells, astrocytes, Muller cells and dendritic cells (microglia) [382]. Astrocytes, microglia and pericytes respectively appear prematurely, concurrently or subsequently to the retina vasculature [382].

In contrast to retina vessel layers, the choroid is fenestrated network that is separated from the retina by the Bruch's membrane and the RPE (Fig.15). The tightly joined RPE of the choroid and the retinal ECs constitute the two components of the blood-retina barrier (BRB) required to protect the photoreceptor integrity by selectively restricting any leakage of fluid or electrolytes in the subretinal space or the outer retina [379], [383]. Any leakage or disturbance in electrolyte concentration disrupts photoreceptor transmission or survival and thereby degrades vision

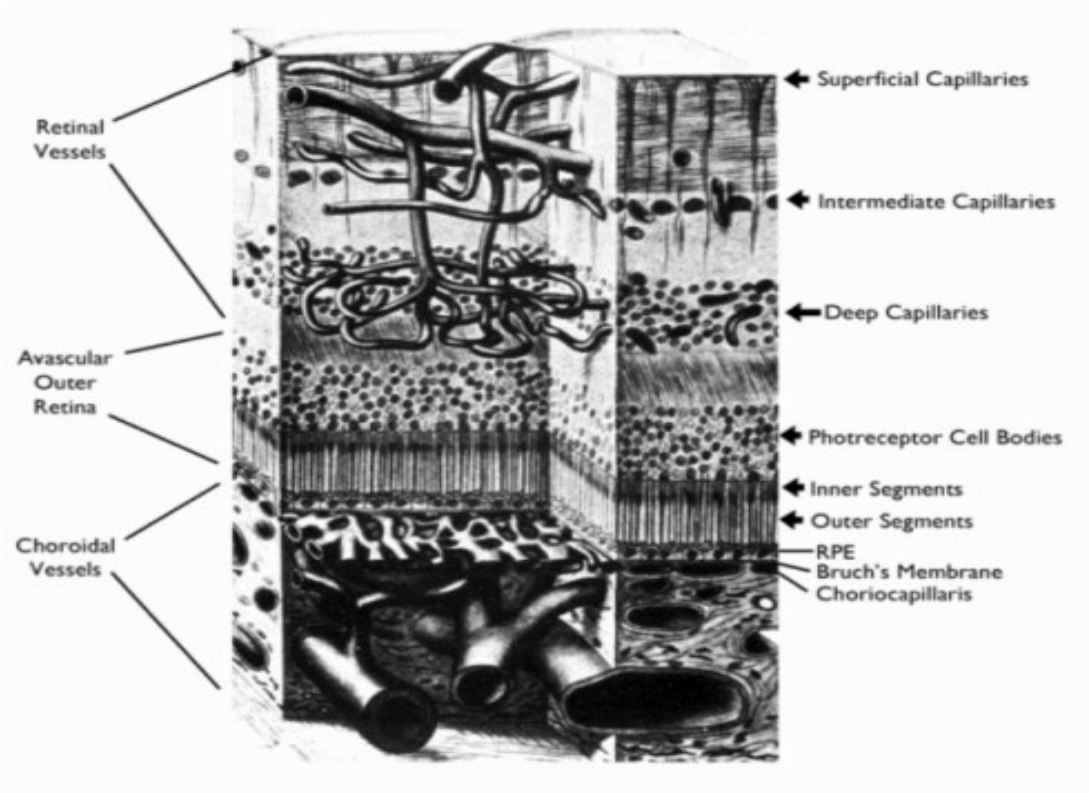


Fig.15 Retinal and choroidal vessel networks.

The superficial capillary bed of the retina is either branched by arterioles or connected to the venules that are respective branches of the central artery or vein of the retina. This superficial network sends branches that partially penetrate the retina to form the intermediate and deep capillaries. The remaining outer third of the retina is avascular and consists of cell bodies and photoreceptor inner and outer segments. The neuro-retina sits on the retinal pigmented epithelium (RPE) that layers the Bruch's membrane (BM).and acts as a fluid barrier between the photoreceptor and the underneath choroid capillary network. The choriocapillaris is fenestrated and allows the plasma to extravate to the BM. Adapted from Campochiaro, 2013.

5.2.2. Age-related Macula Degeneration (AMD)

The neovascular diseases of the eye mostly consist of the pathologies of the posterior segment of the eye such as the wet Age-related Macular Degeneration (AMD) and the retinal neovascularisations. Among them, the neovascular AMD affects the central retina and represents the main cause of blindness in the aging population of industrialized countries. Its pathogenesis is sustained by multiple factors including age, sex, smoking, genetics and reactive oxygen species (ROS). The dry AMD is avascular and represents the most common form of the disease. This form is characterized by dysfunctional RPE depositions that cause the scaring of the retina and the macula detachment. However, it often progresses to a severe atrophic geography and a neovascular AMD. During the wet AMD, neovessels originate from the choroid network, pass through the Bruch's membrane and RPE to invade the subretinal space and photoreceptors [384]. The subretinal outgrowth of choroidal vessels leads to photoreceptor damage and death. The photoreceptor death in wet AMD also results from other retinal damages including the subretinal hemorrhage, detachment of the retinal pigmentary epithelium (RPE) and fibrovascular scarring [148], [385]. Thus, wet AMD represents a severe form with the highest incidence of vision loss. Unlike ischemic retinopathies and despite of its significantly high levels of VEGF-A in the vitreous of patients [386], there is no clear evidence of retinal or choroidal ischemia associated with the induction of VEGF in AMD subretinal NV [148], [272], [379], [387]. However, HIF-1 still has an important contribution in wet AMD as mice lacking a hypoxia response element in the VEGF promoter significantly fail to develop NV at Bruch's membrane

rupture sites [379], [387]. The increased oxidative stress in RPE and photoreceptors during AMD has been reported to contribute to high levels of HIF-1, as mitochondrial reactive oxygen species stabilize HIF-1 through their inhibition of prolyl hydroxylases activity [379], [387]–[390]. Thus, oxidative stress promotes [391] whereas antioxidant vitamins and zinc significantly prevents [392] choroidal NV in dry AMD patients. Apart from oxidative stress in RPE and photoreceptors, inflammation [393] or other hypoxia-mediated factors such as SDF-1, FGFs and PDGF-B may contribute to the AMD-related sprouting of choroidal vessels into the subretinal space [130], [394], [395]. Thus, despite of the differential dynamics in the pathogenesis of retinal and subretinal NV, there are key overlaps in their contributing mediators such as VEGF, HIF and ROS [379].

5.2.3. Retinal vasculopathies

In addition to AMD, the retinal vasculopathies or ischemic retinopathies are ocular NV that also affect the retina function and thereby vision. They consist of conditions such as retinopathy of prematurity (ROP), retinal angiomatous proliferation (RAP), proliferative diabetic retinopathy (PDR), diabetic macular edema (DME) and retinal vein occlusions [379]. In RAP, there is an additional outgrowth of the retina capillary bed that anastomoses with the choroidal vessels in the subretinal space. Retinal vasculopathies commonly damage the vessels and result in ischemia [379]. Despite of high concentrations of factors such FGFs 1 and 2 in the retina and their angiogenic activity [130], recent studies report VEGF to be the main mediator of ischemic retinopathies [25], [131], [396], [397]. Moreover, VEGF induces RAP in contrast to FGF2 transgenic mice that do not develop RAP or a spontaneous phenotype [398]–[400].

5.3. Anti-VEGFs treatments

The extent of abnormal angiogenesis is a key parameter in the prognosis of various NV conditions and retinopathies. A relation exists between the progression or recurrence of NV conditions, the measurements of neovessels and the concentration of angiogenic proteins (biologically active FGF or VEGF) [25], [93], [101], [102], [123], [401]–[404]. Thus, targeting the molecular markers that specifically mediate neovessel formation has been considered as the basic approach in the therapy against these pathologies [405]–[411]. Most of approved antiangiogenic drugs against NV conditions specifically inhibit VEGF signaling. In the context of ophthalmic diseases, anti-VEGFs such as anti-$VEGFA_{165}$ aptamer pegaptanib (Macugen ribonucleic acid), ranibizumab (Lucentis) hFab fragment of a monoclonal antibody against $VEGFA_{165}$, and VEGF-Trap (Aflibercept) were approved for AMD treatment [25], [181], [412], [413]. The neutralizing antibody fragment particularly favors the partial recovery of visual activity, in addition to slowing down NV progression [181]. Additional antiangiogenic drugs in trials target various cellular mechanisms including the EC pathways and biological responses (Table 3) [25]. Moreover, a photodynamic therapy agent called Visudyne (QLT Therapeutics/CIBA Vision) had shown effectiveness for treating macular degeneration and was the first FDA-approved treatment the vascular diseases of the eye in 2004 [413].

Table 3: Antiangiogenic strategies (adapted from [25])

Inhibited angiogenic process/target	Drugs
ECM remodeling	- cartilage-derived TIMP, uPA Ab
Adhesion molecules	- $\alpha_v\beta_3$ Ab or peptides
EC biological responses	- endogenous inhibitors (endostatin, angiostatin, Ang-2) - nitric oxide synthase inhibitors
Ligand	- Bevacizumab (Avastin), pegaptanib (Macugen aptamer) - bFGF Ab, antisense-bFGF - sFLT-1 receptor
Kinase activity	- Imatinib (Glivec), Sunitinib (Sutent), sorafenib (Nexavar), pazopanib (Votrient)

6. Hypothesis and research objectives

The disturbance of vessel quiescence and integrity subsequent to a dysregulated angiogenesis often result in organ-specific pathologies. In wet AMD, the subretinal neovascularisation of the eye causes vision loss. Drugs targeting the VEGF pathway such as VEGF/VEGFR-neutralizing antibodies, VEGF-trap/VEGFR-derived peptides and small molecule kinase inhibitors have been successfully developed as clinical treatments against NV diseases [181]. However, despite of clinical achievements, the anti-VEGFs show multiple adverse effects. On the one hand, their systemic side-effects include hypertension, haemorrhages, proteinuria and toxicity [181]. On the other hand, the ocular burden of anti-VEGFs includes macula and retina degeneration or oedema, conjunctival bleeding, ocular pain, increase of intraocular pressure, vitreous detachment, cataract, presence of vitreal floaters, reduction in visual acuity, death of photoreceptors and local infection resulting from invasiveness and frequency of intravitreal injections [181], [414]. Attempts to minimize these side effects by administrating lower doses of anti-VEGFs have often resulted in suboptimal concentrations and hence their reduced efficacy. Moreover, in some patients anti-VEGFs present bystander effects associated with their impaired delivery, penetration or selectivity [25], [181], [414]. Overall, the invasiveness and the lack of efficacy or site-specific action of anti-VEGFs treatments represent the major drawbacks for their use in conventional therapies of ocular NV diseases. Thus, efficiently targeting neovessels, in a specific and non-aggressive manner, using novel endothelial markers constitutes an interesting approach for improving the treatment of ocular neovascular diseases.

Interestingly, the plasma molecule BMP9 could selectively regulate angiogenesis as it activates the EC-specific receptor ALK1; in contrast to neovascularisation-triggering molecules, including VEGF, that present a range of cellular targets [277]. In fact, the stimulation of ALK1 by its high affinity ligand BMP9 modulates the developmental angiogenesis in the mouse eye [250] and cooperates with Notch in regulating the VEGF-induced tip/stalk specification [220]. Moreover, mutations in BMP9 or its signaling partners such as ALK1, ENG and Smad4 are associated with vessel morphogenesis pathologies such as HHT. Furthermore, BMP9 co-receptor sENG, inhibits the hypoxia-induced NV and vasodilatation in the placenta; while promoting, along with the VEGF receptor sFLT-1, the degree of preeclampsia [181]. Therefore, we hypothesize that BMP9 signaling would specifically inhibit the VEGF-mediated sprouting and thereby modulate the pathological angiogenesis that sustains the deleterious effects of wet AMD. Thus, this thesis specifically investigates the potential antiangiogenic effects of the BMP9/ALK1 axis during wet AMD and further examines their mechanistic basis. Overall, a deeper understanding of the BMP9/ALK1 axis in ECs and NV models would provide a useful insight to its emerging role of its components either as new markers for the diagnosis of neovascular AMD; or as agents for an innovative treatment of AMD, free of adverse effects of inhibiting the neurotrophic VEGF.

CHAPTER II - BMP9/ALK1 inhibits pathological angiogenesis in models of wet AMD

Published article:

BMP9/ALK1 inhibits neovascularisation in mouse models of age-related macular degeneration

Kalonji Ntumba[1], Naoufal Akla[2], S. Paul Oh[5], Anne Eichmann[6,7]and Bruno Larrivée [1,3,4,8*]

[1]Department of Biomedical Sciences, [2]Department of Biochemistry, [3]Department of Molecular Biology and [4]Department of Ophthalmology, Maisonneuve-Rosemont Hospital Research Center, University of Montreal, Montreal, Quebec, Canada. [5]Department of Physiology and Functional Genomics, University of Florida, Gainesville, FL. [6]Yale Cardiovascular Research Center, New Haven, CT. [7]Inserm U970, Paris Cardiovascular Research Center, Paris, France, [8]Department of Biological Sciences, Université du Québec à Montréal, Montréal, Quebec, Canada.

Keywords: Age-related Macular Degeneration, Ocular Pathologies, Angiogenesis, BMP signaling

Abstract

Age-related macular degeneration (AMD) is the leading cause of blindness in aging populations of industrialized countries. The drawbacks of inhibitors of vascular endothelial growth factor (VEGFs) currently used in the treatment of AMD include resistance and potential serious side-effects They require the identification of new therapeutic targets to modulate angiogenesis. BMP9 signaling through the endothelial ALK1 serine-threonine kinase receptor modulates the response of endothelial cells to VEGF and promotes vessel quiescence and maturation during development. Here we show that BMP9/ALK1 signaling inhibits neovessel formation in mouse models of pathological ocular angiogenesis relevant to AMD. Activating ALK1 signaling in laser-induced choroidal neovascularisation (CNV) and oxygen-induced retinopathy (OIR) inhibited neovascularisation and reduced the volume of vascular lesions. ALK1 signaling was also found to interfere with VEGF signaling in endothelial cells whereas BMP9 potentiated the inhibitory effects of antibody-mediated VEGFR2 signaling blockade, both in OIR and laser-induced CNV. Together, the data show that targeting BMP9/ALK1 efficiently prevents the growth of neovessels in AMD models and introduce a new approach to improve the therapy based on conventional anti-VEGFs.

Introduction

AMD is the leading cause of blindness in the aging populations of western industrialized countries [415], [416]. The dry form of AMD is characterized by the development and accumulation of drusen associated with environmental, physiological and genetic factors including smoking, age, gender, inflammation and family history [416], [417]. Dry AMD can progress to neovascular/wet AMD, a more advanced form of the disease. Wet AMD accounts for about 10–20% of AMD cases, but is responsible for 80–90% of the severe loss of central vision associated with AMD. This advanced form is characterized by pathological choroidal neovascularisation (CNV) below the retinal pigmented epithelium (RPE) or in the subretinal space [384], [417], [418]. Ultimately, plasma or blood leakage from the pathological neovessels into surrounding tissues lead to the formation of fibrovascular scars in the retina, photoreceptor dysfunction or death and AMD-associated visual loss [418].

Paracrine VEGF induces the progression of AMD towards its neovascular form. It represents a contributory factor in the initiation of angiogenesis as it directs the migration of the tip cell, a specialized endothelial cells (EC) that guides vessel outgrowth towards hypoxic or inflamed tissue and leads the EC stalk cells forming the base of the sprout [12]. The tip/stalk phenotype is controlled by the expression of the DLL4 ligand in cells exposed to a VEGF gradient, and the subsequent transactivation of Notch in adjacent cells [86], [419]. The differential signaling of these pathways further ensures the selection of the initial Notch-inactive cell as a single tip cell leading the neovesssels as opposed to the Notch-active stalk cells forming the base of the sprout [420]. Ultimately,

the inhibition of VEGF-mediated sprouting by Notch promotes the maturation and quiescence of the capillary network [419].

VEGF signaling inhibitors are conventionally used for the treatment of wet AMD [421]. Since 2004, approved antagonists of VEGF signaling have successively included antibodies and small molecule inhibitors targeting VEGF and its receptors [422]–[425]. The intravitreal administration of these agents has shown therapeutic benefits. However, the adverse effects and the inefficacy of VEGF inhibitors in 7-15% of ocular NV patients are two major drawbacks of these treatments [426]. Secondary effects of VEGF inhibitors, both systemic and localized, include increased intra ocular pressure, cataract, retinal detachment, endophthalmitis, photoreceptor cell death and thinning of the inner neuronal layer of the retina [427], [428]. Thus, the unmet needs of anti-VEGF therapy require the identification of new targets to efficiently treat wet AMD.

Multiple pathways including those triggered by Bone Morphogenetic Proteins (BMPs) and Notch, have been shown to modulate VEGF-directed neovascularisation, and may also regulate the maturation or quiescence of immature and leaky AMD vessels [250], [366], [419]. BMPs, as members of the TGF-β superfamily, have been shown to crosstalk with the VEGF and Notch pathways during developmental angiogenesis [250], [366]. For instance, BMP9, a high affinity ligand for the endothelial-specific receptor activin receptor-like kinase 1 (ALK1) [271], [343], has been demonstrated to prevent VEGF and bFGF induced sprouting angiogenesis in vitro [363], and developmental retinal neovascularisation in vivo, via the activation of downstream Smad 1,5 pathways [363]. Mutations in ALK1, its co-receptor Endoglin (ENG) or the common effector Smad4 are involved in the pathogenesis of hereditary hemorrhagic telangiectasia (HHT)

[350], [351], [429], [430], a vascular condition characterized by arteriovenous malformations (AVM). BMP9 has been shown to modulate the expression of markers sustaining the tip cell phenotype and to promote the maturation phase of developmental angiogenesis [250]. ALK1 has been reported to collaborate with Notch to counter VEGF-induced signaling and regulate the formation and stabilization of retinal blood vessels [271]. ALK1 and ALK5 signaling are suppressed in tip cells through the guidance receptor Neuropilin-1 (NRP-1), but signal in stalk cells in cooperation with Notch to promote stalk cell behavior [220], [250], [431]. As such, the ALK1 signaling pathway could have important clinical implications for anti-angiogenic treatments for AMD patients. The present work investigates the role of the BMP9/ALK1 axis in the formation of pathological neovessels in models of ocular angiogenesis.

Materials and Methods

Mice and Adenoviruses

Adenoviruses were cloned and produced as previously reported by Larrivée *et* al.[250] C57BL/6 WT mice were purchased from The Jackson Laboratory. Cdh5-CreERT2 mice were provided by Ralf Adams (Max Planck Institute for Molecular Biomedicine). ALK1flox mice were kindly provided by Paul S Oh (University of Florida). All animals were manipulated according to the institutional guidelines as defined by the Canadian Council on Animal Care (CCAC).

Oxygen-induced retinopathy

C57BL/6J mouse pups at postnatal day (P)7 and their fostering mothers (CD1, Charles River) were submitted to 75% oxygen in oxycycler chamber for 5 days. Pups were then returned to normoxia and administered 50 µl ($1x10^8$ CFU) of either control, BMP9/ALK1Fc-expressing viruses and/or 50 µg/ g of body weight of DC101 (InVivoMAb, BioXcell Fermentation) or IgG isotype (intraperitonal [I.P.] injections). Eyes were enucleated at P17 and processed for immunostaining.

Laser-induced choroid neovascularisation

Eight weeks old C57BL/6J mice were anesthetized with a ketamine/xylazine mix prior to applying a photocoagulating laser (400mW intensity, 0.05s exposure time). Four spots were burned around the optical nerve. Mice received $2x10^8$ CFU I.P. injections of either control, BMP9/ALK1Fc-expressing viruses and/or 50 µg DC101/ g of body weight. Eyes were enucleated after 14 days and processed for immunostaining.

Immunofluorescence staining

Ocular globes were initially fixed for 15 min in 4% paraformaldehyde (PFA). Retinas or choroids were collected after eyes dissection in phosphate buffered saline (PBS) and blocked 1h in PBS 3% BSA 0.1% Triton X-100. Fixation was prolonged in 1% PFA overnight for choroid extraction or eyes sectioning. Prior to sectioning, eyes were maintained in sucrose gradients (10-30%), cryo-preserved in a matrix gel and sliced in 14 µm sections on a cryostat (Leica CM3050S). Staining with either FITC-labeled isolectin GS IB4 (Life technologies corporation), rhodamine phalloidin (Cedarlane Laboratories) or goat anti-mouse ALK1 primary (R&D systems) and anti-goat secondary (Life technologies) antibodies were performed on whole and/or sectioned retinas/choroids. Retinas and choroids were then mounted in fluoromount aqueous medium (Sigma-Aldrich).

Quantification of Retinal Vaso-obliteration and Neovascularisation

Neovascularisation was quantified using imageJ/Swift_NV as previously described by Stahl *et* al [432]. Briefly, the composite flatmount image was divided into four quadrants and assigned a manual threshold value based on fluorescence intensity such that only the areas of greatest intensity (corresponding to neovascularisation) are shown as a "neovascularisation" map. The individual maps for each quadrant are then combined to give a neovascularisation overlay for the entire retinal flat mount. The area of the overlay is then compared to the overall area of the retina, without any avascular areas subject to vaso-obliteration, to obtain percent neovascularisation for each individual retina. The vaso-obliterated area was quantified by measuring the central retinal area devoid of blood vessels and comparing it to the whole retinal surface area. For each flatmount,

quantification was performed by two independent graders in a masked fashion, and the average of their measurements was used for subsequent analysis.

ELISA

The BMP9 levels in mouse serum were determined by ELISA using BMP-9 Duoset kit (R&D systems) according to the instructions of the manufacturer. Optical density was measured and corrected at 450nm and 570nm respectively using a plate reader (Tecan).

Real-time PCR

Eyes from OIR or control mice were dissected and lysed in 1% β-mercapto-ethanol RLT buffer. Total RNA extraction and cDNA synthesis were performed using the RNeasy extraction (Qiagen) and iscript (BioRad) kits respectively. Real-time amplifications of various target genes (*ALK1*, *ALK2*, *BMPR2*, *ENG* and *ALK3*) were performed on 7500 Fast Real-Time PCR System (Applied Biosystems) using corresponding primers. Primers were obtained from QIAGEN (Quantitect Primer Assays).

Sprouting Assays

Sprouting assays were performed as previously described by Larrivée *et* al. Briefly, after siRNA transfection with control or VEGFR2 siRNA (25 pmol of RNAimax, Life Technologies), HUVECs (250,000 cells/well in 6-well plates) were resuspended in 300 µl fibrinogen solution (2.5 mg/ml fibrinogen, Sigma-Aldrich) in EBM-2 (Lonza) supplemented with 2% FBS and 50 µg/ml aprotinin (Sigma-Aldrich), and plated on top of a pre-coated fibrin layer (400 µl fibrinogen solution clotted with 1 U thrombin (Sigma-Aldrich) for 20 min at 37°C). The second layer of fibrin was clotted for 1 hr at 37°C. NHDF cells (250,000 cells/well), in EBM-2 supplemented with 2% FBS and 25 ng/ml

VEGF, were then plated on top of the fibrin layers. Cultures were incubated at 37°C, 5% CO_2.

Statistical analyses

All data are shown as mean ± standard error of the mean (SEM). Statistical analyses were performed for all quantitative data using Prism 6.0 (Graph Pad). Statistical significance for paired samples and for multiple comparisons was determined by Student's t test and ANOVA, respectively. Data were considered statistically significant if the p value was less than 0.05.

Results

BMP9 receptor expression in Pathological Retinal and Choroidal Neovascularisation:

To evaluate the involvement of BMP9 signaling in pathological angiogenesis in the retina, we first examined the expression of genes involved in BMP9 signaling in retinal ECs from mice subjected to oxygen-induced retinopathy (OIR). P7 mouse pups were subjected to OIR and mRNA was harvested from retinas at P17, correlating with timing of maximal pathological neovascularisation. P17 littermates not subjected to OIR were used as controls. Transcripts corresponding to BMP receptors (*ALK1, ALK2, ALK3, BMPR2 and ENG*) were detected in both groups, but levels of *ALK1* were significantly increased in OIR retinas compared to controls, suggesting differential use during pathological retinal angiogenesis (Fig. 1A). The expression of *ALK1* in the retinal endothelium was confirmed by immunofluorescence staining in retinas subjected to OIR, showing expression in pathological vessels and in vascular tufts in particular (Fig. 1B). In contrast to receptor expression, the levels of circulating BMP9 remained unchanged in the plasma of mice subjected to OIR. This was observed both after the vaso-obliteration (P12) and neovascularisation phases (P17), suggesting that changes in receptor expression, and not of the circulating ligand, were associated with OIR-induced angiogenesis (Fig. 1C).

The expression of BMP9 receptors in the choroid-sclera complex of mice undergoing CNV was also evaluated. Eight-week old mice were subjected to laser photocoagulation by applying 10 to 15 laser spots per eye, and choroids were harvested after 2 weeks. Gene expression analysis showed a significant increase in *ALK1* and *ENG* levels in animals

with CNV compared to control animals (Fig. 1D). Together, these observations show that the BMP9 receptor ALK1 is significantly enriched in pathological vessels of the retina and the choroid.

ALK1 Signaling during Normal and Pathological Retinal Angiogenesis:

As BMP9/ALK1 signaling is a potent inhibitor of developmental retinal angiogenesis [271], we investigated whether modulation of ALK1 signaling could affect neovascularisation in a pathological model of retinal angiogenesis. To manipulate ALK1 signaling in vivo, we used an adenoviral delivery approach to modulate the circulating levels of BMP9, using either adenoviral particles encoding BMP9 or the ligand trap ALK1Fc as previously described [21]. This strategy resulted in either elevated (BMP9 Ad) or reduced (ALK1Fc Ad) levels of circulating BMP9 (Supp. Fig. 1A). Changes in circulating BMP9 levels in mice were associated with either increased (BMP9) or decreased (ALK1Fc) levels of Smad1/5,8 phosphorylation (Supp. Fig. 1B), demonstrating that ALK1 downstream signaling could be modulated in vivo through this approach. We evaluated whether changes in circulating BMP9 levels could affect the vaso-obliteration phase of OIR, which occurs from P7 to P12 due to hyperoxia. P7 pups were injected with BMP9 or control adenoviral particles and subjected to 75% oxygen for 5 days to trigger vessel regression. Retinas from pups sacrificed at P12 revealed no differences in the vaso-obliterated retinal area under high or low BMP9 levels (Fig. 2A,C).

The effects of BMP9/ALK1 signaling on pathological neovascularisation was then evaluated by injecting adenoviral particles in P12 pups which had been subjected to 75% oxygen from P7 to P12. Analysis of P17 retinas using ImageJ/Swift_NV quantification of

neovascular tufts [432] showed that elevated BMP9 levels significantly decreased pathological retinal angiogenesis (Fig. 2B,D), consistent with the observations during developmental angiogenesis [420]. Even though we did not observe significant changes in retinal avascular area, elevated BMP9 levels were associated with increased occurrence and size of neovascular tufts, a key feature of pathological retinal vessels (Fig. 2E). Conversely, inhibition of ALK1 signaling during the neovascularisation phase of OIR through delivery of the ALK1Fc trap or through genetic ablation of ALK1 in the endothelium using Cdh5Cre-ERT2-ALK1 floxed mice resulted in increased pathological blood vessel and tuft formation in OIR mice (Fig. 2B,D,E, Fig. 3A,B). The retinal pathological phenotype was more severe in Cdh5Cre-ERT2-ALK1 floxed mice than that of ALK1Fc-treated mice. This relative difference may reflect a partial blockade of ALK1 ligands by ALK1Fc in contrast to the nearly complete deletion of ALK1 in the retinal endothelium of Cdh5Cre-ERT2-ALK1 floxed mice (Supp. Fig. 2). Together, these data indicate that the activation of ALK1 signaling using BMP9 prevents retinal neovascularisation but doesn't affect the regression of pre-existing vessels in pathological models of retinal angiogenesis.

ALK1 signaling and CNV:

The formation of choroidal neovessels invading the subretinal space is the hallmark of neovascular AMD. The effects of BMP9/ALK1 signaling on retinal neovascularisation led us to investigate whether it could also prevent choroidal angiogenesis. To test this, we examined the effects of modulating the ALK1 signaling on laser-induced CNV progression in C57BL/6 mice. Eight-week old mice subjected to laser impact were I.P. injected with adenoviral BMP9 and ALK1Fc adenoviral particles, and CNV was detected

14 days later by staining choroid-sclera whole-mounts with IsoB4 (blood vessels) and phalloidin (RPE). We observed a significant decrease in the area of CNV in mice treated with BMP9 compared with controls (Fig. 4). However, even though there was a small increase in CNV in ALK1Fc treated mice, these changes were not significant, which suggest that choroidal vessels may be less sensitive to ALK1 inhibition than retinal vessels. Together, these observations show that stimulating the BMP9-mediated ALK1 signaling negatively regulates pathologic choroidal angiogenesis, while inhibiting it does not exacerbate CNV lesions.

Additive effects of ALK1 signaling modulation and VEGFR2 inhibition in OIR and CNV:

Current treatments for neovascular AMD focus on the inhibition of angiogenesis through blockade of VEGF. Previously published data suggest that ALK1 signaling modulates the response of endothelial cells to VEGF [250]. Indeed, stimulation of endothelial cells with BMP9 alters the expression of VEGF receptors, and endothelial tip cell markers downstream of VEGF (Supp Fig.3A) in HUVECs. In response to BMP9, levels of VEGFR1 were increased (8.9 fold) in HUVECs while VEGFR2 expression fell by 47%. This was also observed in the retinas of mice which received intraocular injections of BMP9 (500 ng), which showed an overall decrease in tip cell markers such as ANG2 and Apelin, while the expression of VEGFR1 and the stalk cell marker Jagged1 were increased (Supp Fig. 3B). We also observed a non-significant down-regulation of VEGFR2 expression in BMP9-injected retinas. This likely reflects the fact that, in contrast to ALK1-specific ECs, VEGFR2 remains expressed by additional cell types in the retina (photoreceptors, neurons). Together, these data suggest that BMP9 could alter the responses of ECs to VEGF signaling in part by modulating the levels of VEGF

receptors, thereby leading to an overall decrease VEGF-induced angiogenesis. Based on its effects on VEGF signaling, we investigated whether BMP9 could potentiate the effects of VEGFR2 inhibition on endothelial sprouting. Using a fibrin co-culture assay previously described [271], we observed that combination of BMP9 signaling potentiated the effects of VEGFR2 inhibition on endothelial sprouting, resulting in a 74% decrease in endothelial tube formation (Supp. Fig. 3C,D). These data led us to evaluate whether modulation of ALK1 signaling could potentiate the effects of VEGFR2 inhibition in models of pathological ocular angiogenesis.

Using the neutralizing DC101 antibody, we investigated the effects of blocking VEGFR2 signaling alone or together with BMP9 on OIR-induced neovascularisation. Compared to controls, both BMP9 and DC101 by themselves suppressed neovascularisation in OIR mice, as was previously reported for DC101 [433]. The inhibitory efficacy was slightly, but not significantly higher for BMP9 than DC101. However, a combination of BMP9 and DC101 showed a greater inhibitory efficacy on neovascularisation (51.5% inhibitory efficacy) than BMP9 or DC101 alone (Fig. 5). Additionally, while BMP9 or DC101 treatments alone did not affect the avascular area of OIR retinas, the combination of both factors caused a significantly increased avascular area (Fig. 5B). Interestingly, while Alk1Fc treatment by itself increased neovascular tuft formation in OIR, combination of Alk1Fc with DC101 completely suppressed neovascularisation, suggesting that hypervascularisation associated with Alk1 inhibition is dependent on VEGFR2 signaling. The combinatory effects of BMP9 and DC101 were also assessed on CNV formation. Mice subjected to laser burns received either BMP9 adenoviral particles or DC101, alone or in combination. Again, both BMP9 and DC101 significantly reduced CNV (Fig. 6). A

combination of both factors significantly increased the inhibitory effects over individual treatments. Together, these data suggest that BMP9/ALK1 signaling could potentiate the effects of VEGF inhibitors used to modulate pathological neovascularisation in wet AMD.

Discussion

Current therapies to treat ophthalmic diseases are mostly centered on the inhibition of a single factor, VEGF. The aims of anti-VEGF treatments are to counteract pathological neovascularisation and disease progression, to arrest visual impairment and, in the best case, to gain the recovery of vision. Some molecules targeting VEGF are currently used in ophthalmology, and many more are under investigation in clinical trials for either AMD, ROP, or other eye diseases characterized by neovascularisation. While VEGF blocking agents have provided good clinical benefits, a number of patients show poor responses to these drugs and some concerns have been raised regarding the long term use of VEGF inhibitors [434]. It has been proposed that pan-VEGF blockade is responsible of increasing geographic atrophy in AMD patients, a gradual complication characterized, among others, by choriocapillaries and RPE atrophy, photoreceptors death, and leading to a progressive visual loss [418], [435]. It is therefore of great clinical interest to identify novel targets that could complement or replace current treatments. Numerous signaling pathways have been shown to modulate VEGF activity, and therefore could be targeted to improve the therapeutic benefits of current anti-angiogenic therapies. Among those, signaling components of the Notch, Wnt and Ephrin/Eph families have been found to be differentially regulated in pathological vessels and could represent new targets to prevent neovascularisation in ocular diseases [436]–[439].

In this study, we have examined the involvement of ALK1 signaling in pathological models of ocular neovascularisation, in the backdrop of its established role in physiological angiogenesis in the developing retina [250]. We found that ALK1 was up-

regulated in the endothelium of mice undergoing pathological angiogenesis, which may act as a feedback ‘‘brake’’ or negative regulator to restrain VEGF-induced angiogenesis. As such, inhibition of ALK1 signaling using ALK1Fc or genetic deletion of *ALK1* significantly increased neovascularisation and vascular tuft formation. However, the activation of ALK1 in pathological vessels is likely suboptimal, as we show that exogenous BMP9 can reduce pathological angiogenesis in OIR and CNV lesions.

The mechanisms that drive pathological neovascularisation, a process characterized by the formation of vascular tufts growing towards the vitreous, are distinct from those driving healthy revascularisation of the avascular region of OIR retinas [440]. The tuft formation is often referred to as ‘pathological angiogenesis’, while the closure of the avascular area as revascularisation. While the efficient revascularisation reduces hypoxia and desirably prevents tissue damage, neovascular tufts are characterized by newly forming vascular sprouts that fail to regenerate the capillary network and instead cause outgrowths towards the vitreous. It is intriguing that the two processes are usually inversely correlated; when healthy vascular regeneration is increased, neovascular tufts are reduced [440]. However, the mechanisms underlying this effect are not entirely clear. Our data show that modulation of ALK1 signaling does not appear to affect the avascular area of OIR retinas (revascularisation),but is highly efficient at reducing the appearance of vascular tufts (pathological neovascularisation), suggesting that ALK1 agonists would prevent pathological neovascularisation without adversely affecting normal revascularisation. The effect of BMP9 on vascular tuft formation may reflect its ability to modulate VEGF signaling, which is the main driver of tuft formation.

During retinal angiogenesis, ALK1 signaling maintains homeostasis by offering a counterbalance to proangiogenic pathways, such as the VEGF-mediated one , since it contributes to normalizing the specification of ECs into stalk and tip cells and to properly remodeling the vasculature [250], [441]. Consistent with this notion, BMP9 decreased VEGFR2 expression and increased VEGFR1 expression in ECs, likely altering the sensitivity of VEGF-VEGFR2 signaling. These changes, combined with the effects of ALK1 signaling on the expression of endothelial tip and stalk cell markers, likely contribute to the BMP9-promoted reduction of pathological angiogenesis.

Recently, numerous classes of ALK1 inhibitors have been developed for the prevention of tumor angiogenesis [442], [443]. These inhibitors have been shown to prevent tumor angiogenesis and increase the anti-angiogenic effects of VEGF inhibitors. These results may at first seem to contradict ours, which didn't show inhibition of retinal and choroidal angiogenesis with ALK1Fc. Some discrepancies in ALK1 signaling in tumor angiogenesis may be explained in part by the cell context, the dose, ALK1 cross-talks with other signaling pathways, the micro-tumor environment, as well as the stage of cancer at the time of treatment. In line with this supposition, tumor vessels are known to behave aberrantly and are notably more tortuous and leakier than vessels from tissues such as the retina.

We did however observe in OIR that the combination of ALK1Fc with DC101 abrogated the appearance of neovessels caused by ALK1 inhibition. These data provide evidence that ALK1 regulates angiogenesis at least partially through the modulation of VEGF signaling. This is in line with a study showing that treatment with bevacizumab, an antibody that binds and neutralises human VEGF, decreases the number of dysplastic

vessels in the brain of mice deficient for ALK1 [444]. In addition, we also show that BMP9/ALK1 signaling results in changes in VEGFR1 expression in ECs. This suggest that in blood vessels stimulated with BMP9, higher VEGFR1 levels may contribute to decreased VEGF signaling through the negative regulation of VEGF bioavailability [445]. These data, along with studies showing that BMP/Smad signaling modulates Notch activity in ECs [220], [250] demonstrate that ALK1 is an important regulator of the response of ECs to angiogenic signals, and that modulators of its signaling may affect the response of blood vessels to VEGF. Therefore, the approach aiming to the regulation of ALK1 activity may lead to the development of novel therapeutic strategies to overcome the resistance to VEGF antagonists in diseases such as AMD. Based on the present data, ALK1 agonists could also be of interest for the treatment of a variety of ischemic retinopathies including ROP and diabetic retinopathy. Arguably, components of the BMP9/ALK1 signaling may be implicated as positive effectors of the desired quiescence of EC but also eventual mediators of lateral side-effects on non-vascular retina cells. Thus, future studies on targets of ALK1 signaling might be needed to limit this possibility.

Overall, the results from the current study reveal BMP9 as an effective and potent inhibitor of pathological neovascularisation associated with wet AMD. Moreover, BMP9 agonists represent promising complements that would lower the conventional dose of anti-VEGF agents required to achieve an equivalent therapeutic index. Thus, the BMP9 and anti-VEGF combined therapy would limit the aforementioned adverse effects commonly associated with anti-VEGFs alone.

Acknowledgments

We would like to thank Elisabeth Andrienssen for help with the Laser-CNV model. The work described in this paper was supported by the Foundation Fighting Blindness, The Vision Health Research Network of the FRQS and Hopital Maisonneuve-Rosemont Foundation. B.L. was supported by a New Investigator Award from the Heart and Stroke Foundation of Canada.

Figure legends

Figure-1. Expression of components ALK1 signaling during pathological ocular neovascularisation.

(A) qRT-PCR analysis of P17 retinas from pups subjected to OIR revealed the expression of transcripts corresponding to components of the canonical BMP9 signaling (n=4 control mice and 4 OIR mice). (B) ALK1 immunofluorescence staining of OIR retinas at P17 shows specific expression of ALK1 in blood vessels. Arrowheads show vascular tufts. Scale Bar: 20 μm. (C) BMP9 ELISA of plasma from mice subjected to OIR collected at P12 (after vaso-obliteration) (n=3 control and n=3 OIR) and P17 (neovascularisation phase) (n=3 control and n=4 OIR). (D) qRT-PCR of choroid-sclera complexes subjected to laser-CNV of genes involved in BMP9 signaling (n=4 mice per group). All histograms represent mean ± standard error of the mean. $*P < 0.05$.

Figure-2. Perturbations of ALK1 signaling influence neovascularisation but not vaso-obliteration during OIR.

(A) Effect of BMP9 and ALK1Fc on the vaso-obliterative phase of OIR. P12 retinas of mice injected with control, BMP9 or ALK1Fc subjected to 75% oxygen from P7 to P12. Scale bar: 500 μm. (B) Effect of BMP9 and ALK1Fc on the neovascularisation phase of OIR. C57/Bl6 mice were subjected to OIR followed by I.P. injections of adenoviral constructs at the onset of neovascularisation (P12) and morphometric analyses at P17

following IsoB4 staining. Scale bar: 500 μm. (C) Quantification of vaso-obliterated areas in P12 retinas using ImageJ/Swift (n=4 controls, n=4 BMP9 and n=3 ALK1Fc). (D) Quantification of neovascular and vaso-obliterated areas in P17 retinas subjected to OIR using ImageJ/Swift (n=6 control, n=8 BMP9 and n=5 ALK1Fc). (E) Wholemount IsoB4 and Smooth muscle actin staining of wild-type P17 OIR retinal vessels after treatment with control, ALK1Fc or BMP9 adenovirus Scale bar: 100 μm. All histograms represent mean ± standard error of the mean. $*P < 0.05$, $***P < 0.005$.

Figure-3. Genetic deletion of ALK1 in the endothelium worsens OIR-induced neovascularisation.

(A) IsoB4 staining of P17 retinas from ALK1-flox and Cdh5Cre-ALK1 flox mice subjected to OIR. Injections of tamoxifen were performed at P12, at the onset of neovascularisation. Scale bar: 500 μm. (B) High magnification of the retinal vasculature shown enlarged vessels accompanied by neovascularisation following deletion of ALK1. Scale bar: 50 μm. (C) Quantification of neovascular and vaso-obliterated areas in P16 retinas subjected to OIR using ImageJ/Swift (n=4 ALK1 fl/fl, n=4 Cdh5CreERT2-ALK1f/f).

Figure-4. ALK1 signaling regulates neovascularisation in mice subjected to laser-CNV.

Phalloidin (red) and IsoB4 staining of choroid-sclera complexes two weeks after laser burn and treatment with adenoviral particles. Graph shows quantification of neovascular area following laser-burn. (n=8 control, n=8 BMP9 and n=6 ALK1Fc). All histograms represent mean ± standard error of the mean. ∗∗∗P < 0.005. Scale bar: 75 μm.

Figure-5. Potentiation of the anti-angiogenic effect of VEGFR2 inhibition by BMP9.

(A) Representative images of P17 OIR retinas subjected to VEGFR2 inhibition and/or treatment with BMP9 or Alk1Fc adenoviruses. Neovascular areas are highlighted in red. (B) Quantification of neovascular and avascular areas in P17 retinas subjected to OIR using ImageJ/Swift (n = 5 control adenovirus, n = 3 control IgG, n = 6 BMP9, n = 7 DC101, n = 5 BMP9+DC101, n = 4 Alk1Fc, n = 4 Alk1Fc+DC101). All histograms represent mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.005. Scale bar: 500 μm.

Figure-6. Effects of BMP9 and VEGFR2 blockade on CNV.

(A) Phalloidin (red) and IsoB4 staining of choroid-sclera complexes two weeks after laser burn and treatment with adenoviral particles. (B) Quantification of neovascular area following laser-burn (n=4 animals/group). All histograms represent mean ± standard error of the mean. ∗∗P < 0.01, ∗∗∗P < 0.005. Scale bar: 75 μm.

Supplementary figure-1. Evaluation of BMP9 circulating levels in animals treated with adenoviral particles.

(A) BMP9 ELISAs from plasma of P17 mice subjected to OIR and injected with adenoviruses at P12. (n=5 mice/group). (B) Western blot analysis of lung tissue from P17 mice injected five days prior with control, Alk1Fc or BMP9 adenoviruses. Membranes were probed with anti pSmad1/5,8 or Actin antibodies. Protein samples from 3 animals per group were tested. Histogram represents mean ± standard error of the mean. $**P < 0.01$.

Supplementary figure-2. Tamoxifen injection leads to inhibition of ALK1 expression in OIR retinas of Cdh5CreERT2-ALK1 f/f mice.

IsoB4 and ALK1 immunostaining of P17 retinas of mice subjected to OIR. Injections of tamoxifen were performed at P12, at the onset of neovascularisation. Scale bar: 75 µm

Supplementary figure-3. BMP9 modulates the response of endothelial cells to VEGF.

(A) qRT-PCR analysis of tip and stalk cell markers in HUVECs treated with VEGF with or without BMP9 for 3 hours (n=3 repeats). (B) qRT-PCR from retinas of C57/Bl6 P5 mice 6 hours after intravitreal injections of BMP9 (500 ng) show modulation of expression of tip and stalk cell markers (n= 3 PBS control and n=4 BMP9). (C) Representative images of HUVEC sprouting assays following transfection with VEGFR2 siRNA and treatment with BMP9 (10ng/mL). (D) Quantification of tube density using

software analysis (ImageJ) (n=3 experiments). All histograms represent mean ± standard error of the mean. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.005. Scale bar: 75 μm.

Figures

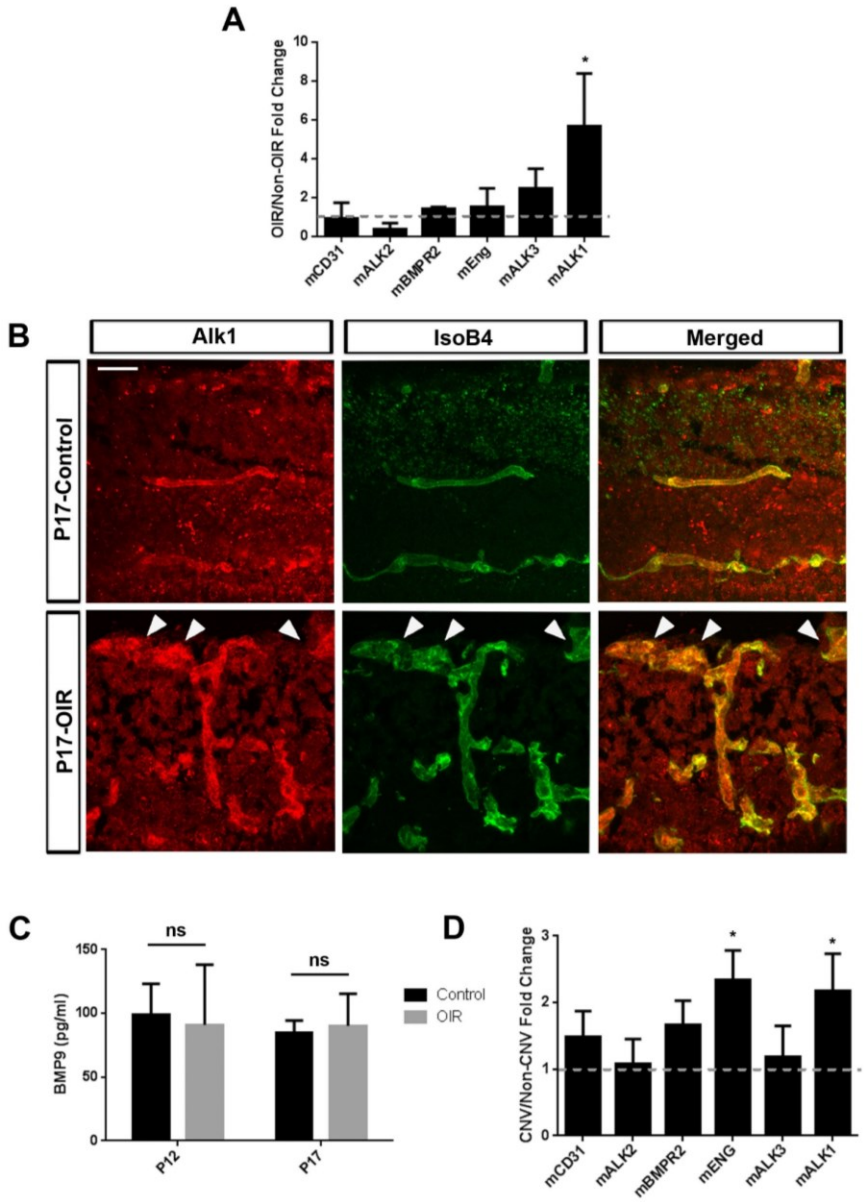


Fig.1 Expression of components ALK1 signaling during pathological ocular neovascularisation (OIR).

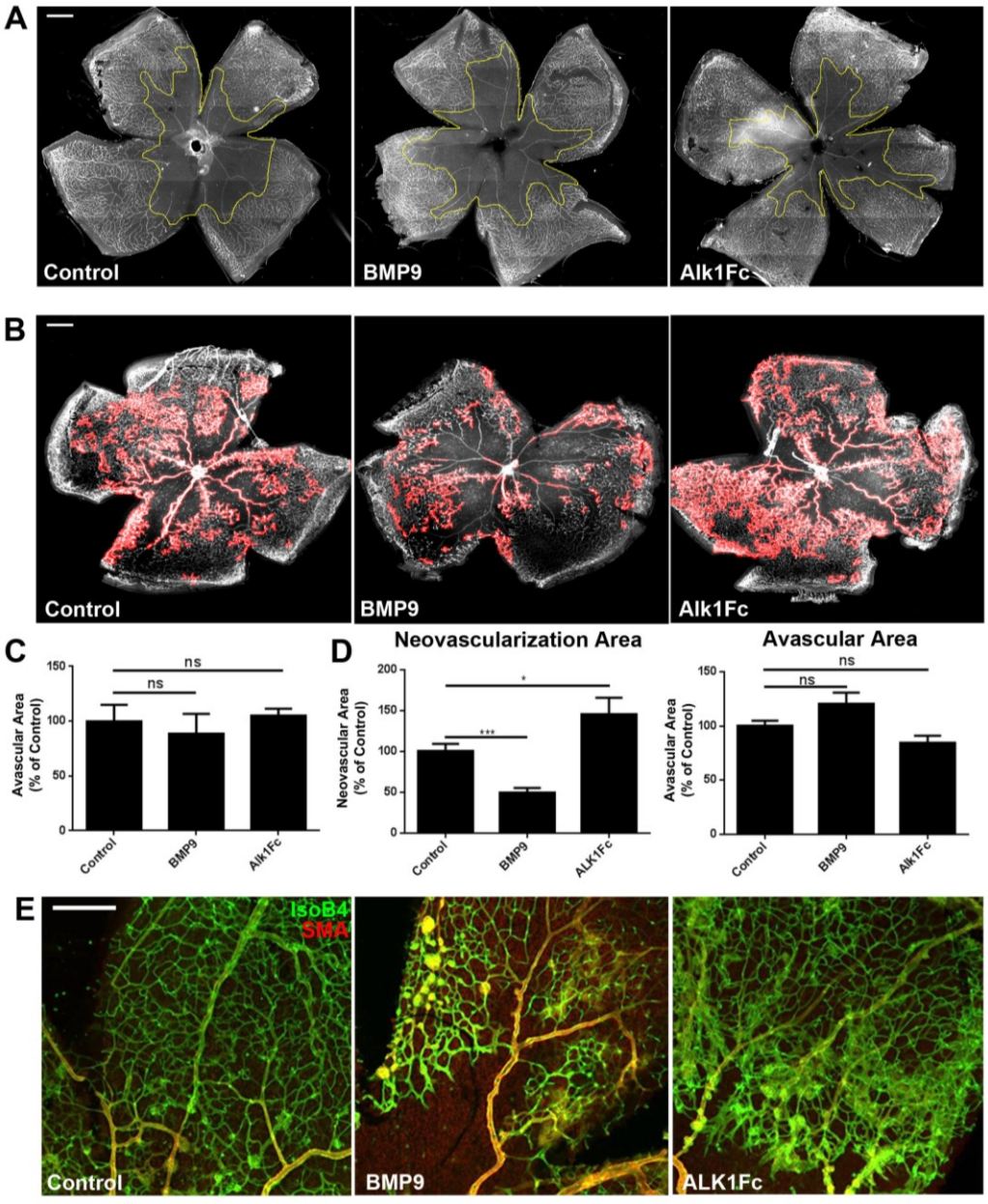


Fig.2 Perturbations of ALK1 signaling influence neovascularisation but not vaso-obliteration during OIR

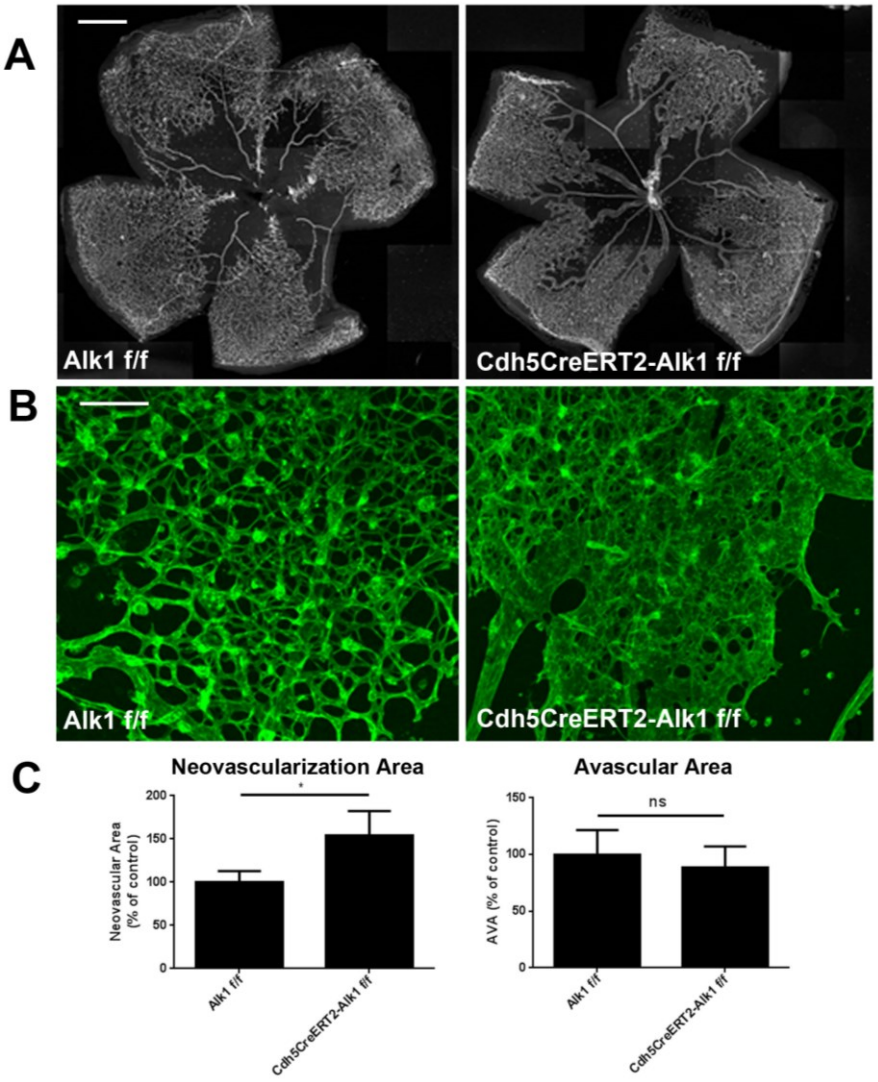


Fig.3 Genetic deletion of ALK1 in the endothelium worsens OIR-induced neovascularisation

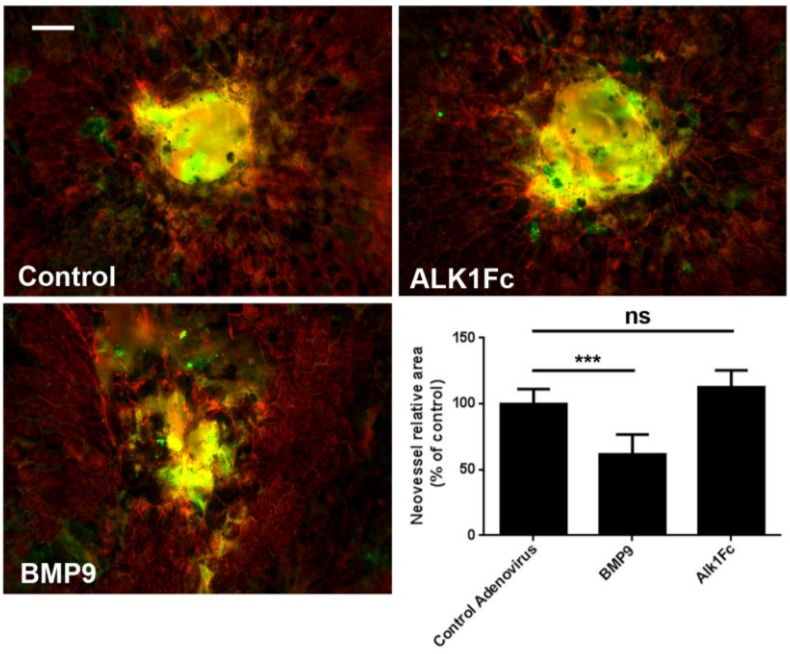


Fig.4 ALK1 signaling regulates neovascularisation in mice subjected to laser-CNV

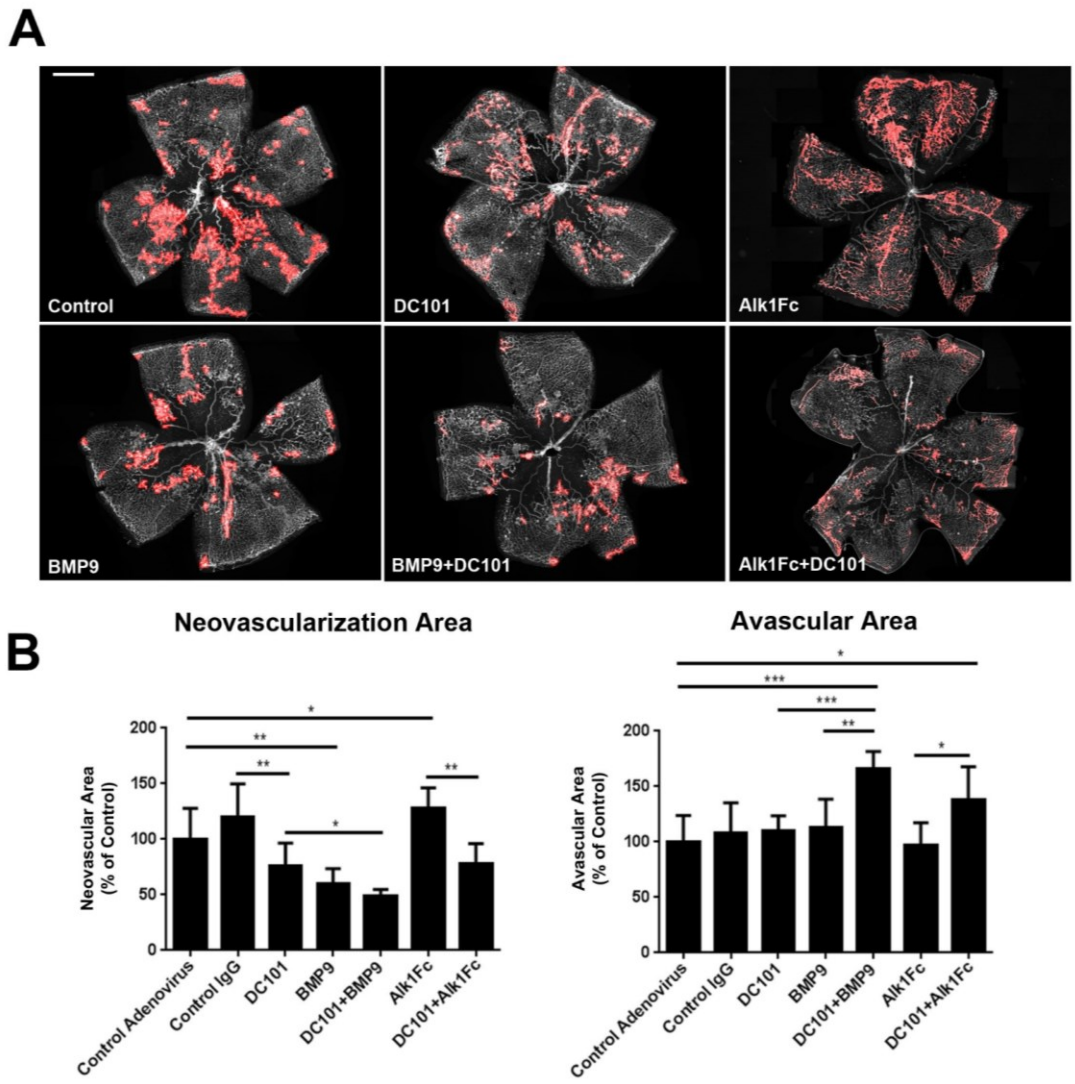


Fig.5 Potentiation of the anti-angiogenic inhibitory effects of VEGFR2 by BMP9 (OIR)

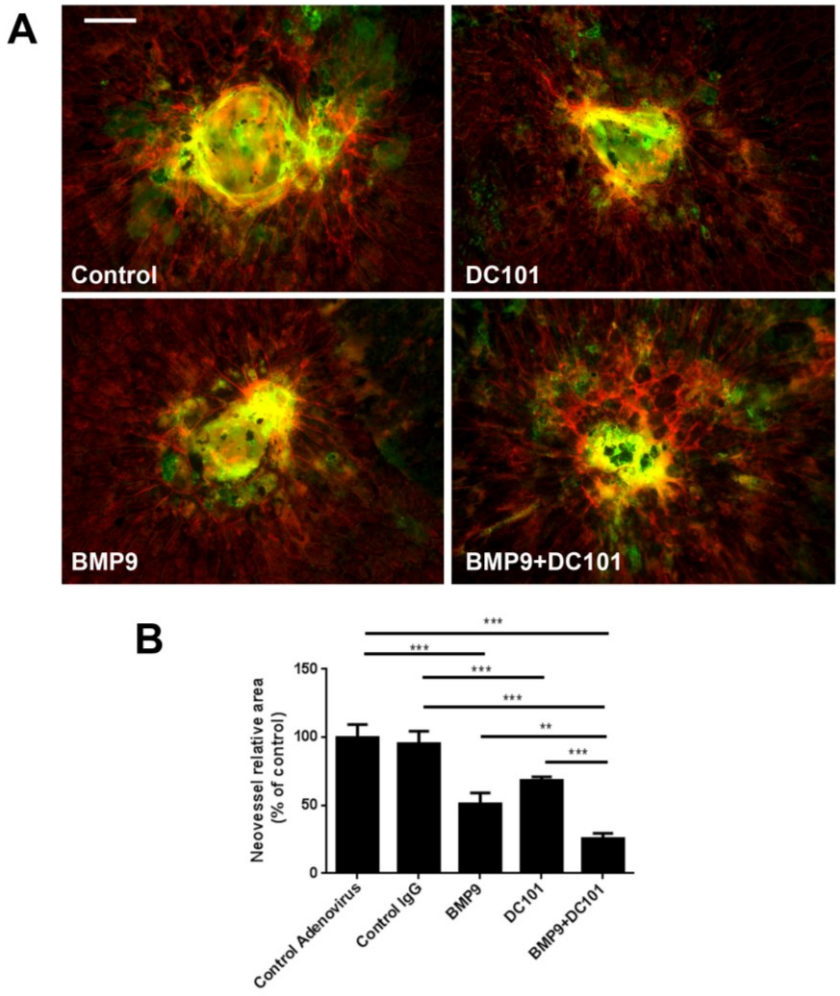
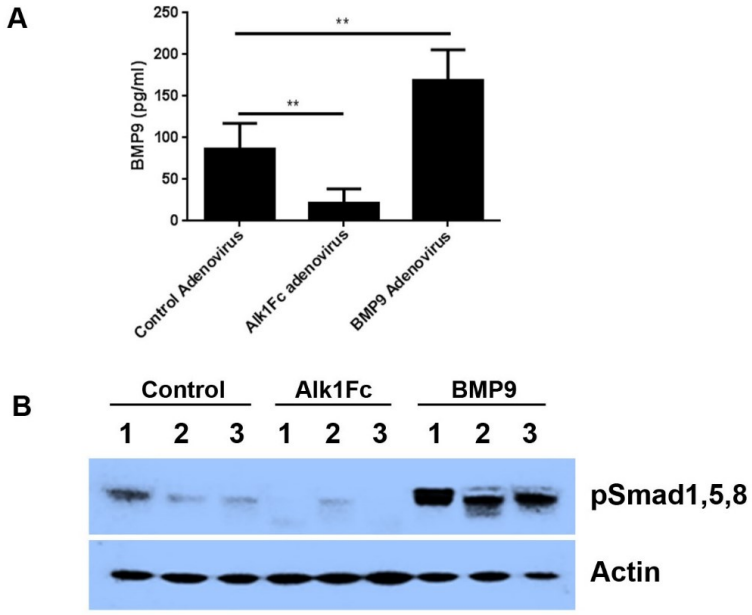
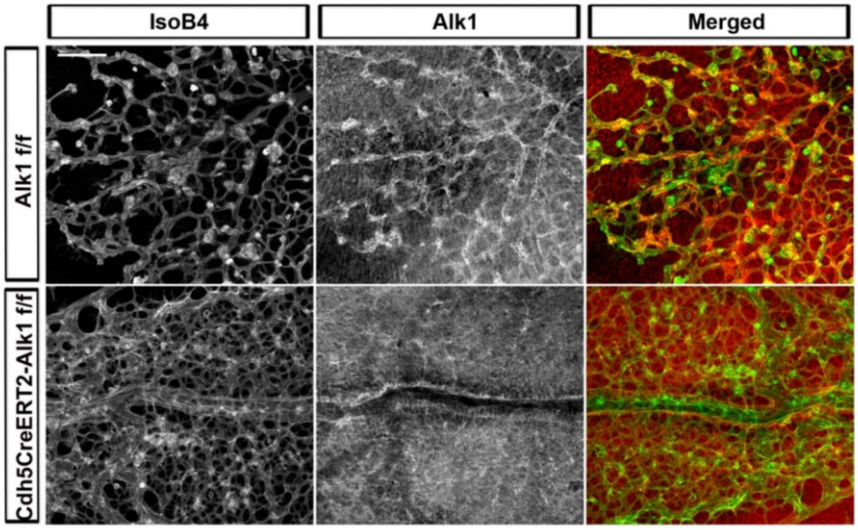


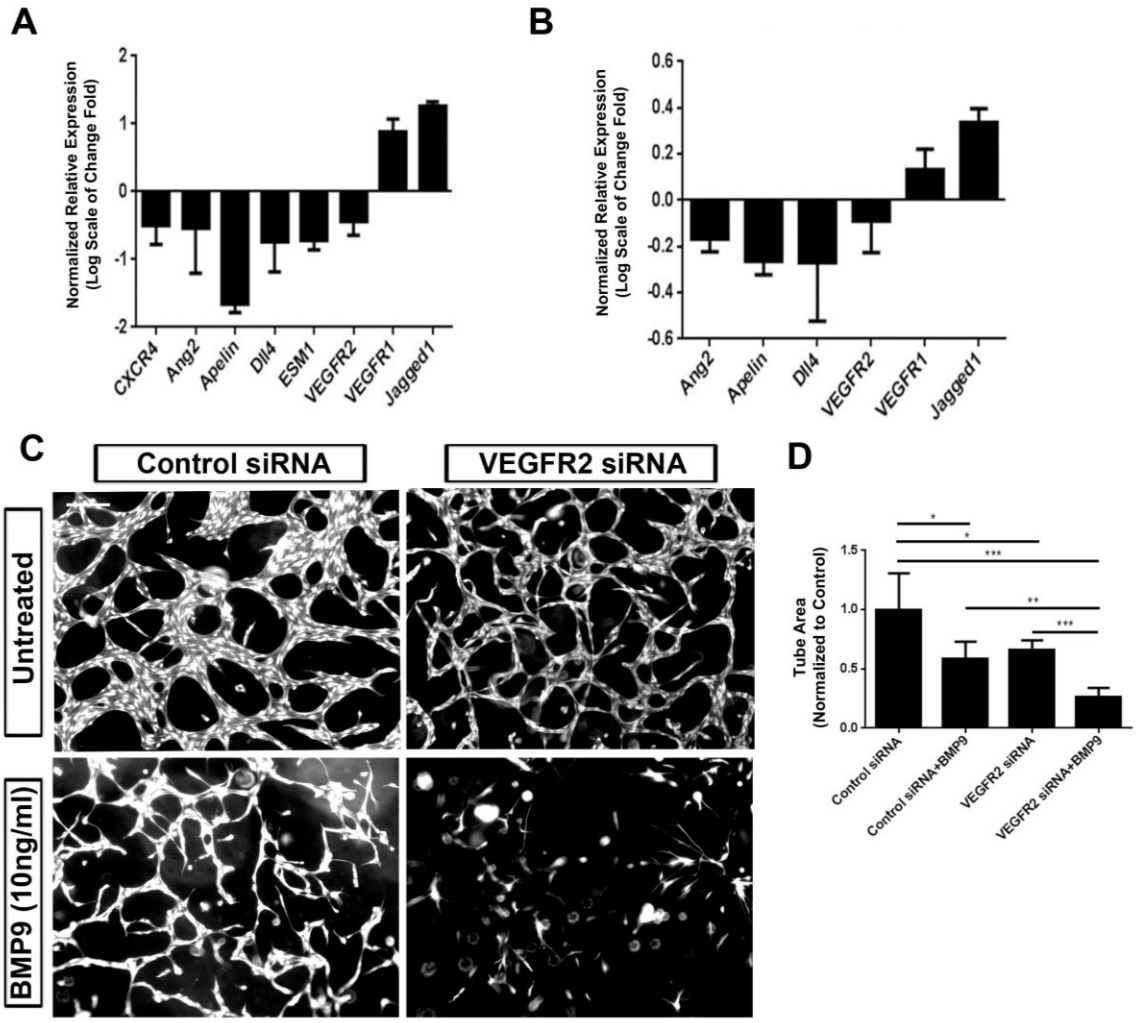
Fig.6 Effects of BMP9 and VEGFR2 blockade on CNV



Supp. fig.1 Evaluation of BMP9 circulating levels in animals treated with adenoviral particles



Supp. fig.2 Tamoxifen injection leads to inhibition of ALK1 expression in OIR retinas of Cdh5CreERT2-ALK1 f/f mice.



Supp. fig.3 BMP9 modulates the response of endothelial cells to VEGF

CHAPTER III – BMP9 signaling crosstalks with VEGF and Notch pathways to induce vessel quiescence

Article in preparation:

BMP9 induction of VEGFR1 crosstalks with VEGF and Notch pathways to induce vessel quiescence

Kalonji Ntumba[1,2], Isaac Legault-Navarette[2,4] and Bruno Larrivée [1,2,3*]

[1]Department of Biomedical Sciences, [2]Department of Ophthalmology and [3]Department of Molecular Biology, Maisonneuve-Rosemont Hospital Research Centre, University of Montreal, Montreal, Quebec, Canada. [4]Department of Biochemistry, University of Sherbrooke, Montreal, Quebec, Canada, Quebec, Canada.

Key words: Angiogenesis, BMP, VEGF, Notch, VEGFR1, JAG1

Abstract

VEGF-mediated sprouting of blood vessels depends on the formation of the tip and stalk phenotypes. The cooperation of VEGF and Notch pathways is essential to regulate these transient fates of endothelial cells (ECs). We and others have previously shown that BMP9 signaling cooperates with Notch to regulate sprouting angiogenesis. However, the molecular basis of this cooperation remains poorly characterized. Here we demonstrate that BMP9 signaling directly and indirectly regulates Notch and VEGF activities to modulate sprouting angiogenesis. The phosphorylation analysis of transducers downstream of VEGF revealed that BMP9 significantly inhibited its signaling effectors, including ERK, Akt, c-Jun and eNOS. Protein and mRNA levels of VEGFR1 and key Notch signaling components such as JAG1 were found to be increased by BMP9 in mouse retina and HUVEC *in vitro*. We have demonstrated that BMP9-induced expression of VEGFR1 correlated with the inhibition of both VEGF-controlled pathways and tip cell formation. Overall, our results indicate that BMP9 signaling induces the quiescence of ECs in sprouting vessels through its differential regulation of VEGF and Notch pathways.

Introduction

Angiogenesis is defined as the formation of new capillaries from existing blood vessels [446]. It mainly occurs via vessel sprouting, a modality in which a migratory tip cell leads a group of stalk cells to form new branches that extend the vascular network [3], [84], [447]. During the sprouting angiogenesis, endothelial cell (ECs) respond to VEGF cues by expressing various targets including DLL4 [87], [448]. The tip phenotype is selectively induced in DLL4-expressing cells but laterally inhibited in neighboring ECs where these ligands transactivate Notch [419]. The tip cell at the leading edge of the angiogenic sprout migrates toward the VEGF gradient, whereas the group of proximal stalk cells proliferate at its base to form the lining of the new vessel [419], [449]. The tip and stalk phenotypes establish a mosaic pattern of ECs in the nascent sprout. On the one hand, VEGF confers a directional migration advantage to the filopodia-expressing tip cells enriched in markers such as DLL4, ESM-1, VEGFR2/3 and uPAR [254]. On the other hand, the proliferating stalk cells at the base of the nascent sprout, show characteristic Notch activity resulting in the expression of its targets including VEGFR1 and JAG1 receptors. Thus, VEGF induces DLL4 and its own transducers in the tip cell, whereas Notch stimulates the expression of the VEGF-inhibiting receptor VEGFR1 and its own ligand JAG1. This cross-induction of ligands or receptors results into an interplay between the DLL4-inducing VEGF and the JAG1/VEGFR1-inducing Notch pathways. The VEGF-Notch interconnection may give rise to a negative feedback loop on the tip cell that alleviates its lateral inhibition on stalk cells. Thus, the VEGF-Notch interplay would transiently reset the phenotypes of neighboring ECs and would periodically

contribute to renew their competition for the tip position. Overall, VEGF and Notch pathways cooperate during sprouting to quantitatively balance and dynamically regulate the selection of a tip cell among ECs [250].

Aside from VEGF and Notch pathways, the morphogenetic protein (BMP) 9 and its activin receptor-like kinase 1 (ALK1/ACVRL1) constitute an endothelium-specific axis that also contributes to the biological responses of endothelial cells and to the modulation of the tip/stalk markers [250]. BMP9 is a TGF-β superfamily member that specifically signals through the EC-enriched ALK1 type I receptor in complex with dimers of type II receptors. These include the BMP receptor (BMPR 2) and activin receptors (ActR2 A and B). Upon BMP9 binding, ALK1 and BMPR2 or ActR2A/B form a complex that further induces the phosphorylation of receptor-regulated Smad (R-Smad) 1/5/8. Activated R-Smads bind co-Smad and translocate to the nucleus where they act together as part of a transcriptional assembly that interacts with Smad Binding Elements (SBE) in target genes. Moreover, the BMP9/ALK1 signaling crosstalks with the VEGF and Notch pathways at various levels as they share common signaling components. In fact, BMP9-activated Smads, VEGF and Notch co-regulate various mediators and targets either independently or through downstream complexes. For instance, VEGFR1, JAG1, DLL4, VEGFR2, UNC5B, HES1, HEY1, HEY2, Smad1/5 constitute crossroads between BMP9, VEGF or Notch signaling activities [250], [248], [450], [300]. These signaling components constitute either common targets or connectors between these pathways; and some of them have been reported to share promoter-associated responsive elements for Smad (SBE), Notch (NRE) and VEGF-controlled transcription factors. As BMP9, VEGF and Notch signaling activities interplay through hierarchical or feedback regulations, a

refined integration of these pathways by ECs is thus critical to coordinate their biological responses and sprouting phenotypes during angiogenesis.

Furthermore, BMP9 has been associated with physiological angiogenesis as well as vascular diseases. For instance, BMP9 inhibits the developmental vascularisation of the eye in mice [250] and the pathological angiogenesis in models of retina diseases [451]. Mutations in BMP9 and ALK1 have also been reported in hereditary hemorrhagic telangiectasia (HHT) 2 and 4 [351], [430].

Given the aforementioned roles of BMP9 and its crossroads with VEGF and Notch pathways, we hypothesized that the BMP9/ALK1 axis would modulate the VEGF and Notch signaling activities, induce the quiescence of vessel endothelium and ultimately inhibit the sprouting angiogenesis. We aimed to investigate the mechanistic basis of BMP9 antiangiogenic activity by examining its effects on the tip and stalk phenotypes as well as its interplay with VEGF and Notch pathways during sprouting. Thus, the present study specifically investigates BMP9-mediated effects on signaling components of VEGF and Notch pathways in the context of sprouting.

Material and methods

1) Material and reagents

Primary HUVECs were purchased from Lonza (cat # cc-2517). The recombinant human (rh) BMP-9 (cat# 3209-BP/CF), ALK1-Fc (cat# 370-AL) and VEGF-165 (cat# 293-VE-010/CF) were from R&D systems. The iScript cDNA synthesis kit (cat # 1708840) and qPCR polymerase (cat# 1725121) kits were obtained from Bio-Rad whereas the RNA extraction kit was purchased from Qiagen (cat# 74134). The human primers for qPCR were ordered from Invitrogen (*DLL4*, *JAG1*, *HLX* and *Nidogen*), Quantitect (*FLT1*, *Apelin*, *LFNG*, *HES1*, *HEY1* and *HEY2*) and IDT (*KDR* and *Actin*). The antibody to $_{pY1175}$VEGFR2, $_{pY951}$VEGFR2, VEGFR2, $_{pT202/Y204}$ERK1/2, ERK1/2, $_{p}$Smad1/5, Smad1/5/9, $_{pS473}$Akt, Akt, $_{pY397}$FAK, FAK Smad4, cleaved Notch1 $_{(Val1744, D3B8)}$ were ordered from Cell signaling, The antibodies to VEGFR1 (cat# AF321 and AF471) and ESM1 (cat# AF1810) were from R&D systems. The ALK1 $_{(ab37807)}$, Actin, CD144, JAG1$_{(ab7771)}$, LFNG$_{[EPR10391(B)]}$, NRP1$_{[EPR3113]}$, Erg$_{[EPR3864]}$ antibodies were purchased from Abcam. Alexa 488 (cat# A-11055) and 647 (cat# A-31573) -conjugated secondary antibodies were ordered from Thermo Scientific; whereas Horse radish peroxidase-labeled antibodies to goat (PI-9500), mouse (PI-2000) and rabbit (PI-1000) IgG were obtained from Vector. IsolectinB4 conjugated to Alex fluor 488/647 (Sigma, cat # L2895) were purchased from Sigma. Control and silencing RNAs (si-RNA) si-*FLT1*, si-*ALK1*, si-*Smad4*, si-*JAG1* were ordered from Qiagen. CJ57/BL6 mice were from Jackson.

2) Cell culture and transfection:

HUVECs were seeded to reach a density of $2x10^5$ cells/well on a 6-well plate in EndoGRO basal medium (Millipore, cat# SCME-BM) completed with EndoGRO supplement kit SCME002-S (containing rhVEGF, rhEGF, rhFGF, rhIGF-1, ascorbic acid, hydrocortisone hemisuccinate, heparine sulfate, L-glutamine and FBS). For transfection, the plated HUVECs were exposed to 25 nmol of si-RNAs from transfection mixes prepared as per the protocol of the lipofectamine RNAiMAX kit (ThermoFisher, cat # 13778030). 24 hours later, transfected and non-transfected cells were starved 18h in 10ng/mL rhBMP-9 0.1% FBS EndoGRO basal medium. These cells were either concomitantly or subsequently treated with 25ng/mL of VEGF-165 depending on their processing for phosphoarray, Western blot or qPCR (below).

3) Western blotting and phospho-kinase array

Control and BMP9-treated (detailed above) HUVECs were subsequently stimulated or not with 25ng/mL of VEGF-165 for 15 min. Cell lysates were collected and incubated over night at 4ºC with capture antibody-labeled and blocked membranes, as per protocols of the proteome profiler human phospho-kinase (cat # ARY003B) and phospho-receptor tyrosine kinase (RTK) (cat # ARY001B) array kits. The membranes were subsequently washed and successively stained with the detection antibody cocktail A or B and the streptavidin-horse radish peroxidase (HRP) from the array kits. Each membrane was exposed to 1-2 mL of freshly prepared mix of chemi

reagents 1 and 2 prior to development on a luminescent image analyzer (Fuji, cat # LAS-3000 IDX4).

4) Real-time PCR

Control and BMP9-treated (detailed above) HUVECs were stimulated or not with 25ng/mL of VEGF-165 for 8h. Cell lysates were collected in RLT and the total RNA extracted using the RNAeasy kit reagents and protocol on a thermal cycler (Bio-Rad, cat # C1000 Touch). The cDNAs were synthesized as per the iScript kit protocol. Targeted sequences of *DLL4*, *JAG1*, *HLX*, *Nidogen*, *FLT1*, *Apelin*, *LFNG*, *HES1*, *HEY1* and *HEY2*, *KDR* and *Actin* genes were amplified on the ABI instrument (Applied Bio-systems, 7500 real-time PCR system) set to temperature cycles of 95ºC (10sec), 60ºC (35sec) and 72ºC (30s).

5) Immunofluorescence staining

P(4) mice were injected intravitreally with BMP9 (500 ng) or ALK1Fc (100 ng) in phosphate buffered saline (PBS). Eyes were collected at P(5) in 4% PFA for 15min and dissected in PBS. Retinas were then blocked 2h in 0.1% Tx-100 3% BSA in PBS and stained O/N with 1:400 (v/v) IsoB4 conjugated to Alex fluor 488 or 647 and primary antibodies detected with Alexa-labeled secondary antibodies.They were flat-mounted in fluoroshield (Sigma-Aldrich, cat# F6182,) and imaged by fluorescence microscopy.

Results

BMP9 decreases the endothelial tip cell conversion in developing retinas

A previous study by Larrivée et *al.* (2012) has shown that BMP9 overexpression is a potent inhibitor of retinal angiogenesis and that treatment of cultured endothelial cells (HUVECs) results in the inhibition of endothelial tip cell markers (DLL4, Apelin). Therefore, we evaluated the consequences of BMP9 delivery on tip cell conversion in developing retinas. P4 mice received intravitreal injections of BMP9 and retinas were harvested 24 hours later. To evaluate the number of tip cells in the developing vasculature, retinas were stained with an antibody directed against Endothelial cell-specific molecule 1 (ESM), a marker highly expressed by tip cell during sprouting. Our results showed that BMP9 treatment significantly reduced the number of ESM1-expressing cells *in vivo*, in contrast to mice which were injected with only PBS (Fig. 1A). Conversely, injections of ALK1Fc resulted in a significant increase of ESM-1-positive cells. Interestingly, while ESM-1-positive tip cells were found mainly at the leading edge of growing capillaries in PBS-injected animals, they were found throughout the whole retinal plexus in ALK1Fc-injected pups, suggesting that loss of ALK1 signaling interferes with the Notch-induced inhibition of the tip cell phenotype that occurs at the tip/stalk cell interface. Together, these data confirm that ALK1 prevents neovascularisation by preventing the switch of endothelial cells to the tip cell phenotype.

BMP9 prevents the VEGF-induced expression of tip cell markers

VEGF drives neovascularisation by inducing the stalk/tip cell switch, which implies the differential expression of subsets of genes which confers a migratory phenotype to endothelial cells. Previous studies have shown an enrichment in genes involved in Notch signaling (DLL4), pericyte recruitment (PDGF-B) and basement membrane degradation (uPAR, Nidogen-1 and Nidogen-2). We next evaluated whether BMP9 could block the VEGF-induced expression of tip cell markers. BMP9 significantly reduced both basal and VEGF-stimulated mRNA levels of *DLL4, Apelin, HLX* and *Nidogen-2 in vitro* (Fig. 2). These effects suggest that BMP9 signaling can interfere with the VEGF signaling cascade inducing the tip cell markers. Thus, consistent with its previous inhibitory effect on tip cell conversion, BMP9 represses the expression of the tip cell markers DLL4, Apelin, HLX and Nidogen-2 in a VEGF-dependent manner. Overall, these results indicate that BMP9 interferes with the migratory tip cell switch by blocking the effects of VEGF and, thus, would ultimately inhibit angiogenesis sprouting and lead to a quiescent vasculature.

As BMP9 interferes with the VEGF-induced expression of endothelial tip cell markers, we explored the consequences of BMP9 stimulation on VEGF signaling in endothelial cells. Previous studies have indicated a crucial role in VEGF-dependent Akt/mTOR, p38 and ERK signaling on tip cell conversion. Thus, BMP9 effects on VEGF signaling was investigated by determining how the co-treatment with BMP9 affects downstream signaling mediators. A screening of VEGF signaling mediators by phosphoarray revealed a significant inhibition of multiple VEGF-dependent pathways in the presence of BMP9 (Fig. 3). Indeed, we observed a significant reduction in VEGF-

dependent VEGFR2, ERK1/2, Akt1/2/3$_{y308}$, c-Jun, eNOS, EPHA2, EPHB4, STAT3, RSK1/2/3, FAK and P70 S6 kinases phosphorylation in the presence of BMP9. These data were confirmed by immunoblotting and demonstrated that BMP9 blocked the phosphorylation of VEGF signaling mediators as well as the phosphorylation of VEGFR2 on two distinct tyrosines involved in VEGF-induced proliferation and migration (Y1175) and permeability (Y951) (Fig. 4). Together, these data demonstrate that BMP9/ALK1 signaling can interfere with VEGF signaling in endothelial cells. Interestingly, the inhibition of VEGFR2 phosphorylation suggests that BMP9 inhibition occurs early in the VEGF signaling cascade.

BMP9-induced expression of VEGFR1 leads to the inhibition of VEGF signaling

BMP9-mediated inhibition of VEGFR2 activity indicated that it could act upstream at the level of VEGF receptors to inhibit VEGF signaling. One of the main regulators of VEGF signaling is the VEGF receptor VEGFR1, which has been shown to act as a ligand trap for VEGF. Indeed, numerous studies have previously shown that both the membrane-bound and soluble versions of VEGFR1 limit the bioavailability of soluble VEGF, thereby limiting angiogenesis. We thereby questioned whether VEGFR1 could prevent VEGFR2 phosphorylation and VEGF downstream signaling by regulating the expression of VEGFR1. Quantitative PCR analysis revealed that VEGFR1 mRNA was significantly upregulated in the presence of BMP9, in contrast to VEGFR2 and NRP1 expression, which was not significantly altered (Fig. 5A). An increased expression of VEGFR1 was confirmed at the protein level by immunoblotting (Fig. 5B). We also evaluated whether BMP9 could upregulate VEGFR1 expression in vivo. Mice (P4) were injected with recombinant BMP9 (500ng) and retinas were harvested 24 hours later. Immunofluorescent staining of the retinal vasculature revealed weak expression of VEGFR1 in animals injected with PBS; whereas a significant increase in VEGFR1 expression was detected throughout the vasculature of mice injected with BMP9 (Fig. 5C). As VEGFR1 acts as a potent sink of VEGF, its induction by BMP9 would increase the trapping of VEGF and decrease its availability for VEGFR2 binding and phosphorylation. Therefore, the VEGFR1-mediated reduction of VEGF availability would then decrease VEGFR2 activation and would ultimately cause a partial lack in the

phosphorylation of effectors downstream of VEGF signaling such ERK, FAK, Akt and eNOS.

As VEGF signaling mainly directs the formation of tip cells and their migration during sprouting, the overexpression of VEGFR1 would be one of plausible mechanisms through which BMP9 inhibits angiogenesis. We therefore evaluated the contribution of VEGFR1 in mediating the effects of BMP9 on VEGF signaling. To address this, HUVECs were transfected with control or VEGFR1 siRNA. Twenty-four hours post-transfection, HUVECs were treated with BMP9 overnight before stimulation with VEGF for 15 minutes. Immunoblotting revealed a 70% decrease in VEGFR1 expression. In control siRNA-treated cells, BMP9 reduced VEGF-induced phosphorylation of several signaling molecules, including VEGFR2, ERK and Akt as previously shown (Fig. 3, 4). As expected, the silencing of VEGFR1 resulted in exacerbated VEGF-induced phosphorylation of these signaling molecules (Fig. 6). Furthermore, the inhibition of VEGFR1 expression also impaired the inhibitory effects of BMP9 on the phosphorylation of effectors downstream of VEGF signaling (Fig. 6). These results therefore indicate that the BMP9-induced expression of VEGFR1 mediates the downregulation of VEGF signaling in ECs and could represent a mechanism through which BMP9 suppresses neovascularisation.

BMP9/ALK1 signaling leads to Notch cleavage and signaling

Studies have shown that Notch is a potent regulator of tip/stalk cell conversion. The induction of Notch signaling by its ligand DLL4 was also shown to regulate VEGF signaling via VEGFR1 up-regulation and VEGFR2 down-regulation. While it has previously been shown that BMP9 can directly regulates the expression of Notch canonical targets including the bHLH proteins of the HES/HEY family, its direct effects on Notch cleavage and signaling have not been investigated. Given the role of BMP9 on tip/stalk cell conversion and its effects on VEGF signaling, we evaluated whether BMP9 could directly mediate the activation of Notch signaling in ECs. HUVECs were stimulated with BMP9, VEGF or VEGF+BMP9 for up to 24 hours, and the expression of Notch downstream targets were examined by quantitative PCR (Fig. 7A). While VEGF stimulation alone did not significantly modulate the expression of HES1, HEY1 and HEY2, BMP9 rapidly induced robust expression of these factors, independently of VEGF stimulation. Interestingly, while HES1 and HEY1 were rapidly induced and reached an expression plateau, HEY2 was gradually increased over a period of 24 hours. The expression of HEY1 and HEY2 proteins was confirmed by Western (Fig. 7B). Smad signaling could account for the direct increase of Notch targets such as HEY1 and HEY2 in the presence of BMP9, as Smad-responsive elements have been described in the promoter region of these genes. As no such elements have been described for other Notch-responsive genes, BMP9 may additionally potentialize Notch targets indirectly through Smads or in a Smad-independent manner.

Interactions between the BMP and Notch pathways have also been reported. Indeed, it has been previously demonstrated that some TGF-β family members can lead

to Notch cleavage and inhibition. As such, we explored the possibility that BMP9 could lead to Notch cleavage, and thereby ultimately reinforce its signalling and effects on the angiogenic response. Thus, Notch activity was then determined following BMP9 treatments. P4 mice were injected with BMP9 and retinas were harvested at P5. Immunofluorescent staining with a cleaved Notch antibody of the developing retinal vasculature revealed a salt and pepper pattern of Notch activation at the growing edge of capillaries in PBS-injected animals. Strikingly, Notch cleavage was observed in a majority endothelial cells of retinas injected with BMP9 (Fig. 8A), and the vasculature displayed a significant reduction in the number of tip cells, as revealed by decreased number of filopodia. Similarly, an increased cleavage of Notch was observed in HUVECs treated for 24 hours with BMP9 (Fig. 8B). Interestingly, Notch activation in HUVECs in the presence of BMP9 was only observed after 24 hours culture, and not at shorter time points (data not shown), suggesting that BMP9 may lead to Notch cleavage through the transcriptional regulation of modulators of Notch signaling.

Notch activation would require signaling through its ligands DLL4 and JAG1. Quantitative PCR analysis of Notch ligands in HUVECs, revealed that BMP9 induced robust expression of Jagged1 (Fig.9A). While, as previously reported, VEGF induced expression of DLL4, BMP9 stimulation resulted in a significant decrease in DLL4 expression in the presence or absence of VEGF. Notch glycosylation by members of the fringe glycosyltransferases has also been reported to regulate the affinity of Notch for its ligands and modulate its signaling. Indeed. Lunatic Fringe (LFNG) has been shown to increase DLL4/Notch interaction and lead to Notch cleavage. Our data showed that LFNG was significantly induced by BMP9 and may play a role in Notch cleavage and

activation. Together, these data show that BMP9 up-regulates Jagged1 and LFNG, which may in turn lead to Notch cleavage and activation.

So far, results suggest that BMP9 might operate through more than one mechanism to upregulate Notch signaling components LFNG, DLL4, JAG1, its targets HES1 proteins, and ultimately VEGFR1. Interestingly, Jagged1 represents a crossroad between BMPs and Notch since it can be induced by both Smad1/5-4 and Notch intracellular domain (NICD) complexes. Thus, JAG1 expression in response to BMP9 and its contribution in mediating BMP9 effects were of particular interest. First, JAG1 expression was evaluated *in vivo* in the retinas of mice injected with BMP9. Immunofluorescent staining of Jagged1 protein levels showed higher expression in the retinal endothelium of mice injected with BMP9 in comparison to PBS controls (Fig. 10A). To evaluate the specific contribution of Jagged1 in mediating BMP9 effects on Notch activation, a loss of function approach by siRNA knockdown was used. The specific knockdown of *JAG1* caused a loss of the BMP9-induced activation of Notch1 after 24 hours of stimulation. These data suggest that Jagged1 expression is required to mediate the BMP9-induced activation of Notch1 (Fig. 10B). Furthermore, knocking down the genes of BMP9 signaling components ALK1 and Smad4 also caused a loss of BMP9-induced activation of Notch1. These results suggest that BMP9 canonical signaling through ALK1 and Smad4 leads to required JAG1 expression and thereby Notch activation.

Discussion

Sprouting angiogenesis depends on the coordinated establishment of tip and stalk phenotypes through the cooperation of VEGF and Notch pathways. VEGF signaling through VEGFR2 triggers the endothelial tip cell formation, whereas VEGFR1 expression and Notch activation in neighbouring stalk cells inhibit the VEGF pathways to control angiogenesis. The current study investigates BMP9 antiangiogenic effects on VEGF-mediated sprouting.

On the one hand, BMP9 upregulated both transcription and synthesis of VEGFR1 HEY1 and HEY2. On the other hand, these BMP9-induced expressions correlate with Smad1/5/8 phosphorylations. Thus, the BMP9 signaling through Smad1/5/8 mediate the transcription of *VEGFR1*, *HEY1* and *HEY2* genes. Accordingly, Smads have been reported to bind directly to their promoters since they contain SBE for Smad transcriptional complexes [220]. Furthermore, the increased expression of VEGFR1 by BMP9 concomitantly reduced the phosphorylation of VEGFR2 sites including its Tyr 951 and 1175. This reverse correlation between VEGFR1 expression and VEGFR2 phosphorylation suggests that the BMP9-induced VEGFR1 interferes with VEGF signaling through inhibition of VEGFR2, similarly to the VEGF lateral inhibition observed in stalk cells. In addition to increasing the level of VEGFR1, BMP9-induced HEY1 and HEY2 also act as repressors of *VEGFR2* gene.

Along with VEGFR1 expression, Notch transactivation represents a second event that contributes to the lateral inhibition of VEGF pathways in stalk cells. Thus, Notch

prevents the stalk-to-tip conversion and thereby controls the VEGF-dependent sprouting of stalk cells [219]. In fact, BMP9-increased expression of JAG1 leads to Notch activation as knocking down *JAG1* neutralised the BMP9 effects on Notch. Thus, JAG1 was specifically required by BMP-9 to mediate its activation of Notch and thereby to indirectly drives the synthesis of its targets such as HES1, LFNG, HEY1,2. and VEGFR1. Moreover, BMP9-induced expression of JAG1 and LFNG *in vivo* and *in vitro* ultimately contributes to the transactivation of Notch in a double positive and intercellular Notch-JAG1/LFNG feedback. This double feedback between neighbor ECs potentially results in the amplification of additional Notch targets such as members of the HLH family including HES1, HEY1, HEY2 [250]. Accordingly, Smad1/5 have been reported to cooperate with Notch to transcriptionally increase stalk cell marker genes such as *VEGFR1*, *JAG1, HES1, HEY1* and *HEY2* [250]. Except *HES1*, all of these Notch targets have been demonstrated to additionally bind the BMP9-induceable Smad1/5 through direct interactions at their promoter SBE [300]. Thus, they are directly regulated by Smad1/5-Smad4 and NICD complexes downstream of BMP9 and Notch signaling. Therefore, BMP9 signaling downregulates VEGF activity in tip cells through VEGFR1 transcription following its direct binding to Smad1/5. Moreover, BMP9-induced Smad1/5 bind and induce HEY1 and HEY2 independently or cooperatively with Notch via Smad-NICD complex. In turn HEY1 and HEY2 potentiate the direct transcription of VEGFR1 by Smad1/5; while, significantly or not, downregulating VEGFR2. Furthermore, BMP9-induced JAG1 amplify Notch activity and thereby the expression of stalk markers including HES1.

Overall, ECs integrate BMP9, VEGF and Notch pathways at levels of HES1, HEY1, HEY2, VEGFR1 and JAG1. The hierarchical and reciprocal interactions between these mediators support the model of VEGFR1 and JAG1 as angiogenic check points induced by BMP9 signaling to induce Notch activity and inhibit VEGF signaling. These differential regulations of VEGF and Notch pathways by BMP9 inhibit the tip/stalk specification and ultimately lead to its antiangiogenic effects. Taken together, the BMP9 signaling leads to the modulation of EC responsiveness to VEGF sprouting cue and thereby induces the quiescence of the vascular network.

Figure legends

Fig. 1 BMP9 interferes with retinal vessel sprouting (IF). A) Immunofluorescence staining of retina flatmounts: P4 mice were intra-vitreously injected with BMP9 (500 ng/mL), ALK1Fc (500ng/mL) or PBS for 48h. Dissected retina were stained with Isolectin GS IB4 (IsoB4)-Alexa fluor 647 conjugate, anti-ESM1 (detected with Alexa fluor 488-conjugated secondary antibody) and anti-Erg (detected with Alexa fluor 350-conjugated secondary antibody). B) Ratio quantification of ESM1-positive cells over nuclei.

Fig.2 BMP9 inhibits the expression tip cell markers (qPCR). cDNAs were synthesized from 500ng RNA extracted from lysates of starved HUVECs treated for 8h with BMP9 (10ng/mL) and/or VEGF (25ng/mL). The real-time amplification of indicated genes was performed by qPCR with the respective primers.

Fig.3 BMP9 decreases the phosphorylation of effectors downstream of VEGF (phospho-array). A) Phospho-arrays of HUVEC lysates. Control and BMP9-treated (10ng/mL for 18h) HUVECs were subsequently stimulated or not with 25ng/mL of VEGF-165 for 15 min. Array membranes spotted with respective capture antibodies were incubated with cell lysates and subsequently stained with the detection antibody cocktail (biotin- and streptavidin- conjugated) as per protocols of the proteome profiler human phospho-kinase and phospho-receptor tyrosine kinase (RTK) array kits. The membranes

were developed on an Image analyzer. B) Quantification of membrane spots intensity (n=2).

Fig.4 BMP9 downregulates the activity of VEGF downstream pathways (immunoblot). HUVECs were treated with BMP9 (10 ng/ml; 16 hours) followed by a 15 minutes stimulation with VEGF (10 ng/ml). The cell lysates were processed for immunoblotting using the specified antibodies.

Fig.5 BMP9 regulates the expression VEGF receptors. A) Real-time PCR. cDNAs were synthesized from 500ng RNA extracted from lysates of starved HUVECs treated for 8h with BMP9 (10ng/mL) and/or VEGF (25ng/mL). The real-time amplification of indicated genes was performed on a thermocycler using *VEGFR1*, *VEGFR2* and *NRP1* primers respectively. B) Western blots. Untreated and BMP9-treated HUVECs (10 ng/mL) were lysed after 16 hours. The cell lysates were processed for immunoblotting using the antibodies against total VEGFR2, VEGFR1 or NRP1. C) Retina flatmounts. P4 mice were injected with BMP9 (500 ng/mL) or PBS for 48h. Dissected retina were stained with IsoB4-Alexa fluor 488 conjugate and anti-VEGFR1 antibody (detected with Alexa fluor 647-conjugated secondary antibody).

Fig.6 VEGFR1 contributes to BMP9-mediated inhibition of VEGF signaling. A) Representative immunoblots of HUVECs transfected with control or VEGFR1 siRNA. Twenty-four hours post-transfection, cells were treated with BMP9 (10 ng/ml; 16 hours) followed by a 15 minutes stimulation with VEGF (10 ng/ml). Proteins were processed for immunoblots using the specified antibodies. B) Quantification of immunoblots band intensity (n=3).

Fig.7 BMP9 upregulates Notch targets. A) qPCR. cDNAs were synthesized from 500ng RNA extracted from lysates of starved HUVECs treated between 0-24h with BMP9 (10ng/mL) and/or VEGF (25ng/mL). The real-time amplification by qPCR was performed on cDNAs of specific time-points using the respective primers of indicated genes. B) Western blots. Untreated and BMP9-treated HUVECs (10 ng/mL) were lysed after 16 hours. The cell lysates were processed for immunoblotting using the antibodies against HEY1 and HEY2.

Fig.8 BMP9 increases Notch cleavage. A) Immuno-fluorescence of retina flatmounts. P4 mice were intra-vitreously injected with BMP9 (500 ng/mL) or PBS for 48h. Dissected retina were stained with CD144-Alexa fluor 488 conjugate and cleaved Notch antibody (detected with Alexa fluor 647-conjugated secondary antibody). They were flatmounted in DAPI-containing fluoroshield. B) Western blots. Untreated and BMP9-treated HUVECs (10 ng/mL) were lysed after 16 hours. The cell lysates were processed for immunoblotting using the antibodies against cleaved Notch.

Fig.9 BMP9 modulates the expression of Notch regulators. A) qPCR. cDNAs were synthesized from 500ng RNA extracted from lysates of starved HUVECs treated between 0-24h with BMP9 (10ng/mL) and/or VEGF (25ng/mL). The real-time amplification was performed by qPCR on cDNAs of specific time-points using the primers of respective genes. B) Western blot. Untreated and BMP9-treated HUVECs (10 ng/mL) were lysed after 16 hours. The cell lysates were processed for immunoblotting using the antibodies against JAG1 and LFNG.

Fig.10 JAG1 expression is required for BMP9-mediated activation of Notch.

A) Immuno-fluorescence of retina flatmounts. P4 mice were intra-vitreously injected with BMP9 (500 ng/mL) or PBS for 48h. Dissected retina were stained with IsoB4-Alexa fluor 488 conjugate and JAG1 antibody (detected with Alexa fluor 647-conjugated secondary antibody). They were flatmounted in fluoroshield. B) Representative immunoblots of HUVECs transfected with control, *ALK1*, *Smad4* or *JAG1* siRNA. Twenty-four hours post-transfection, cells were treated with BMP9 (10 ng/ml for 16 hours). Proteins were processed for immunoblots using the specified antibodies.

Figures

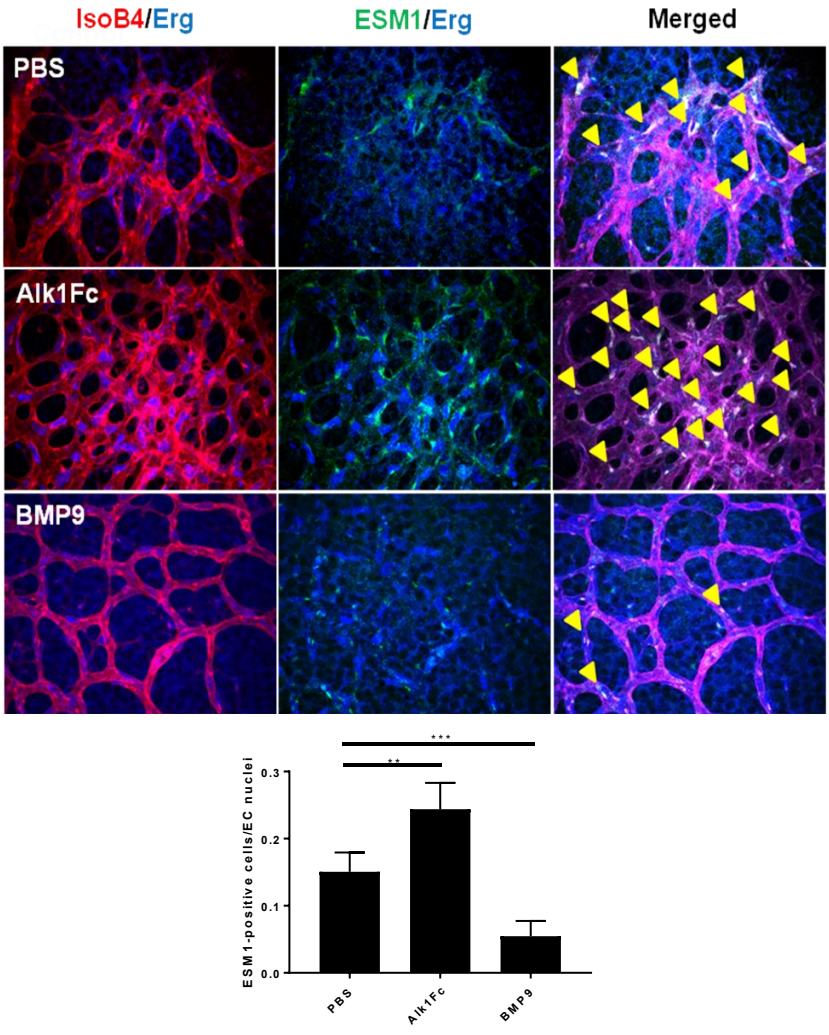


Fig.1 BMP9 interferes with the sprouting of retina vessels (IF).

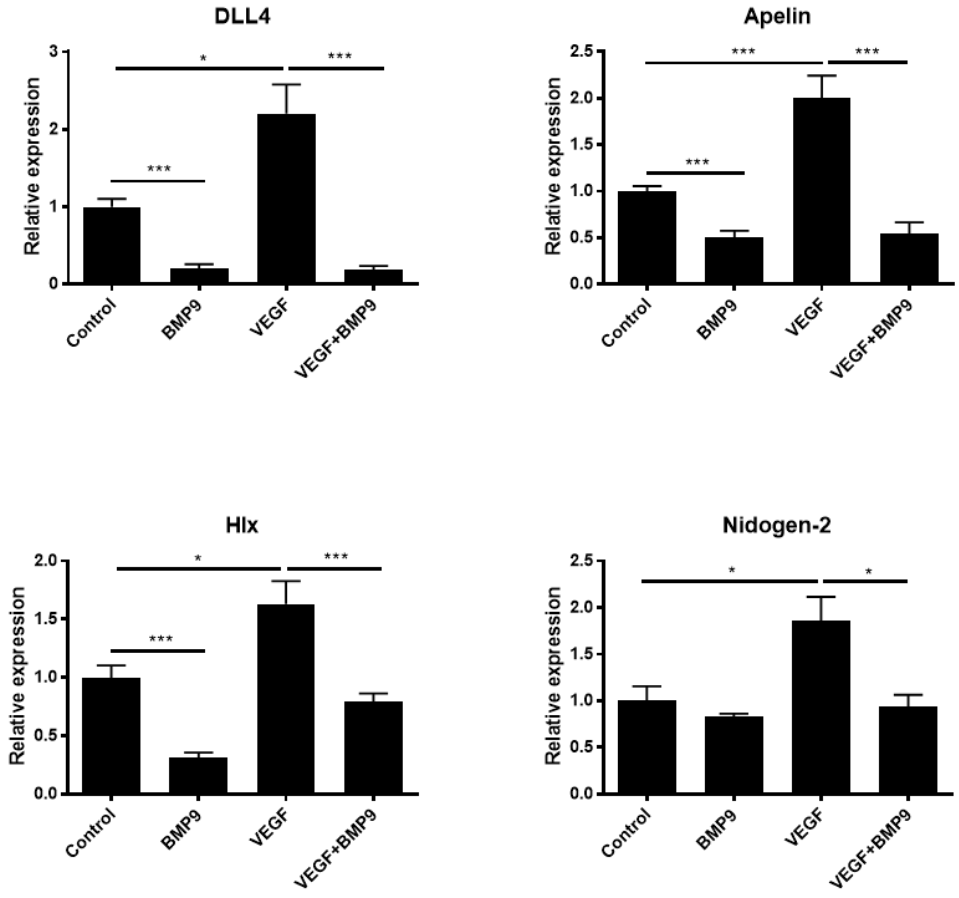


Fig.2 BMP9 inhibits the expression of tip cell markers (qPCR).

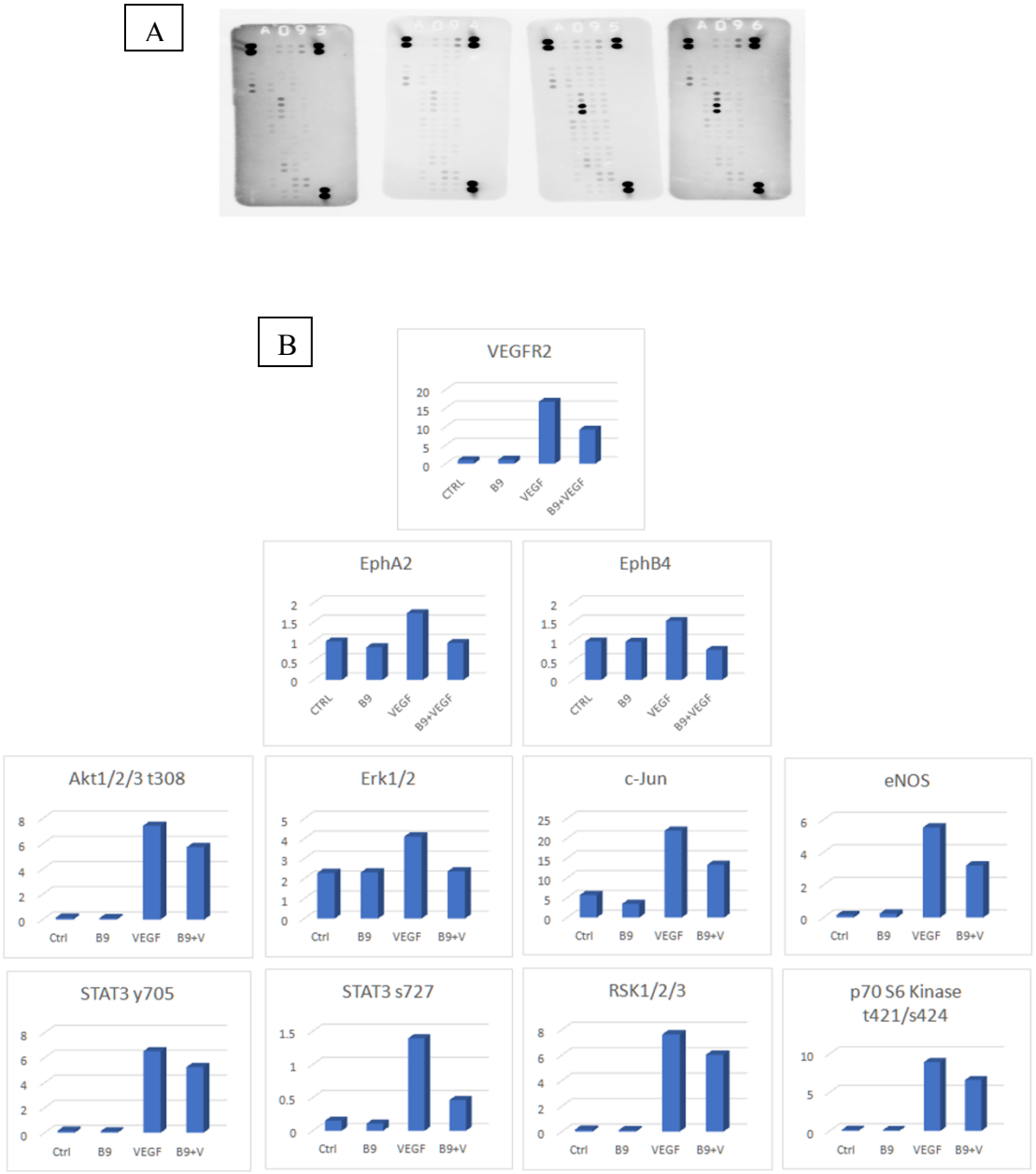


Fig.3 BMP9 decreases the phosphorylation of effectors downstream of VEGF (phospho-array).

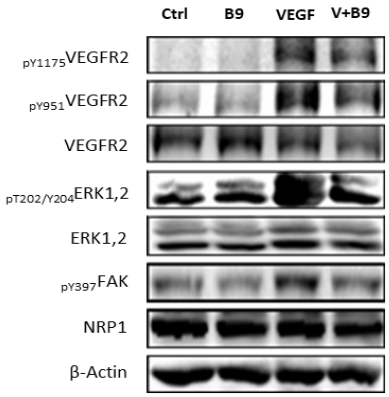


Fig.4 BMP9 downregulates the activity of effectors downstream of VEGF (immunoblotting).

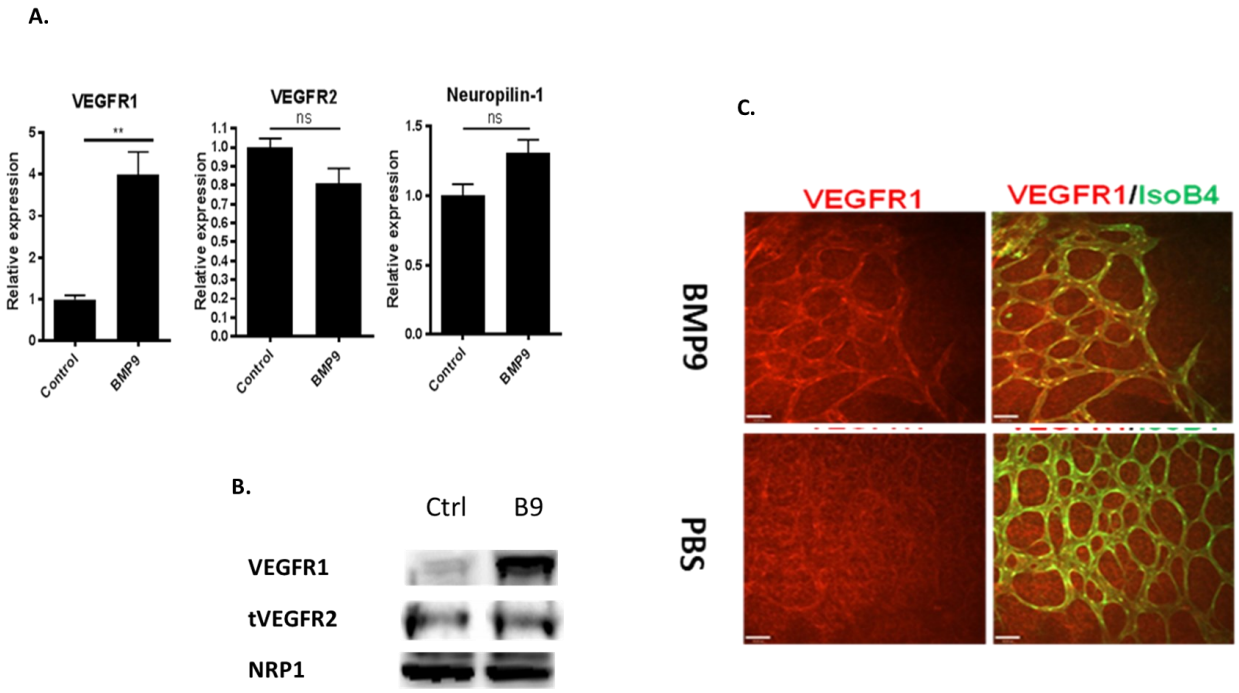


Fig.5 BMP9 regulates the expression VEGF receptors.

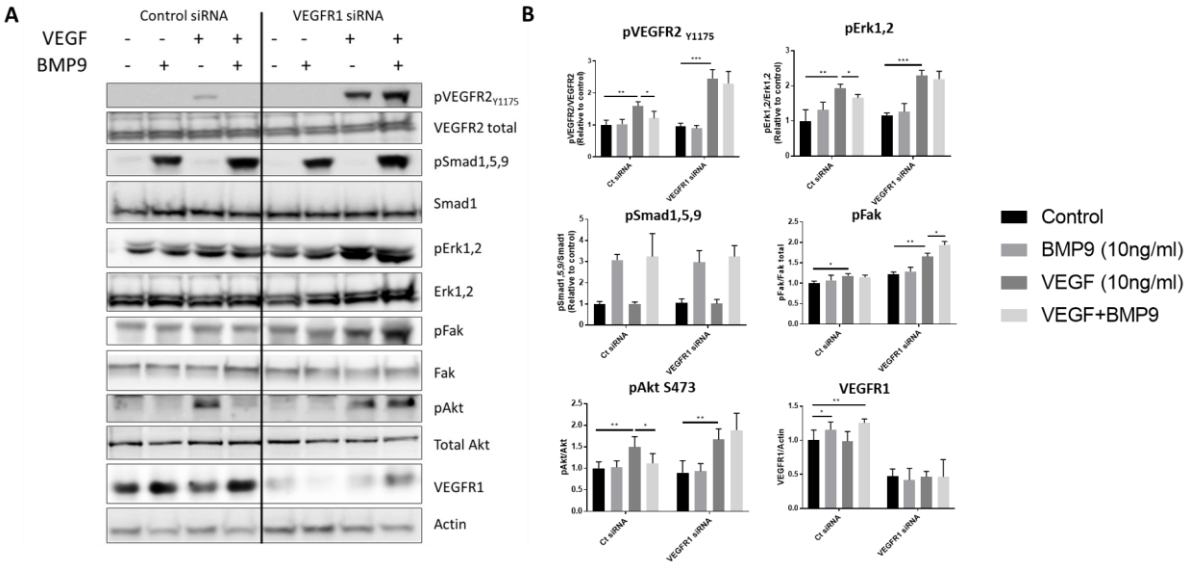


Fig.6 VEGFR1 contributes to BMP9-mediated inhibition of VEGF signaling (immunoblotting).

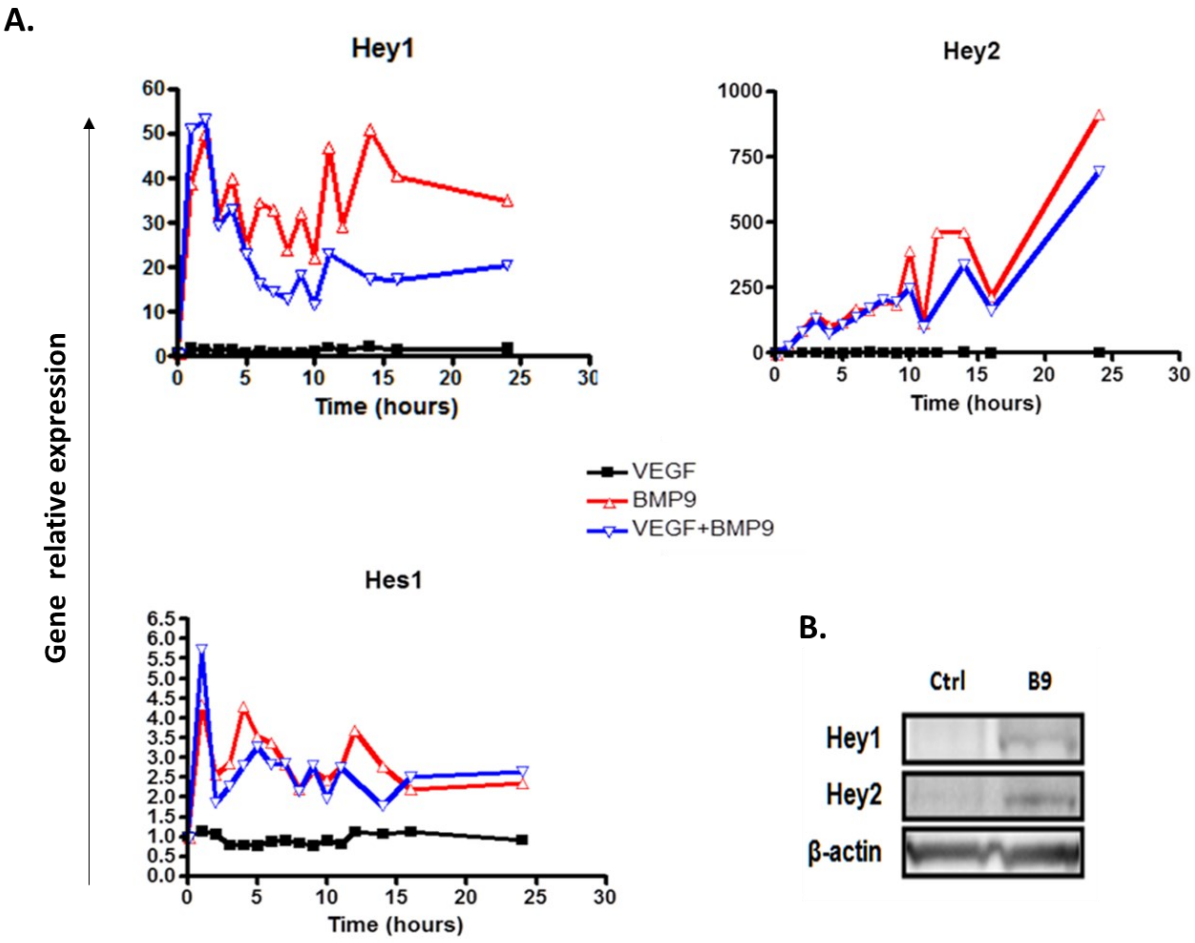


Fig.7 BMP9 upregulates Notch targets.

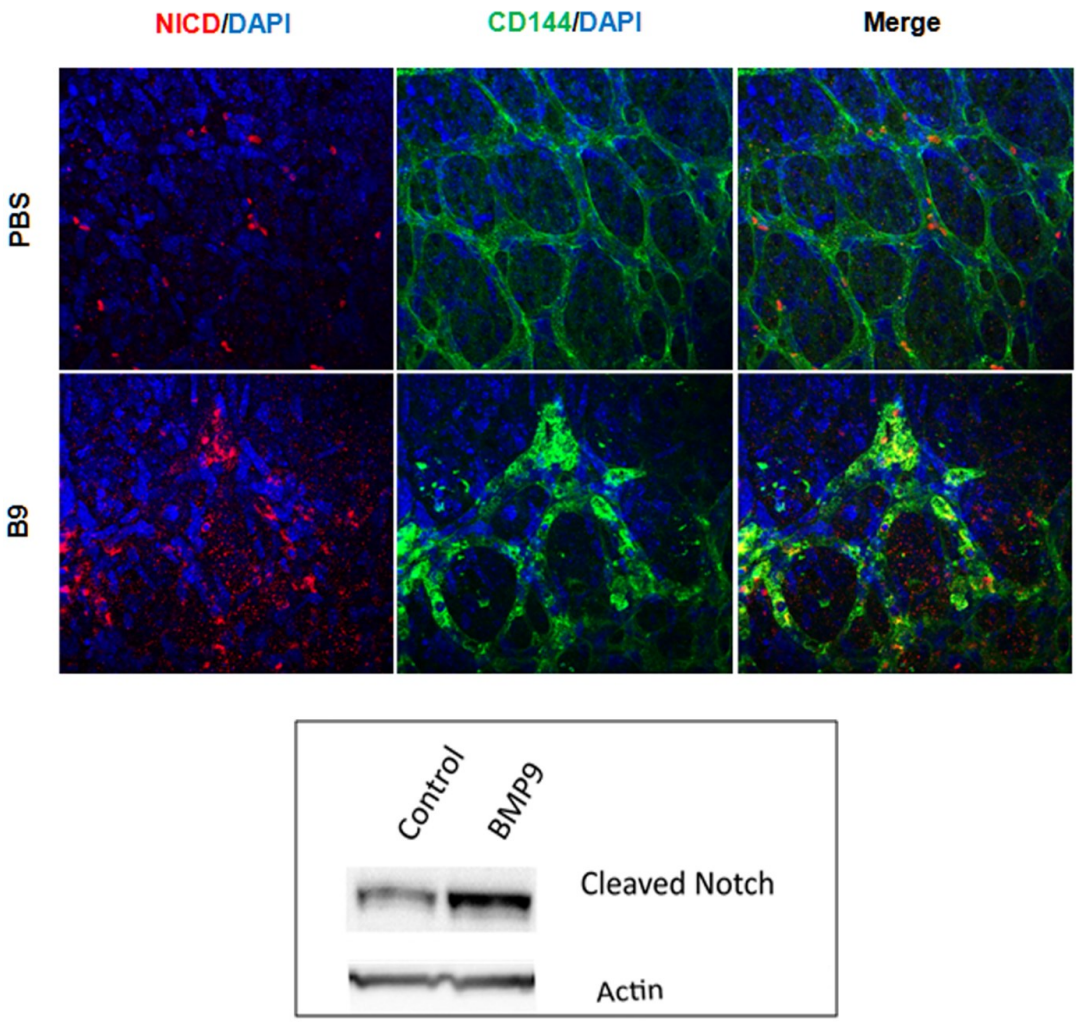


Fig.8 BMP9 increases Notch cleavage.

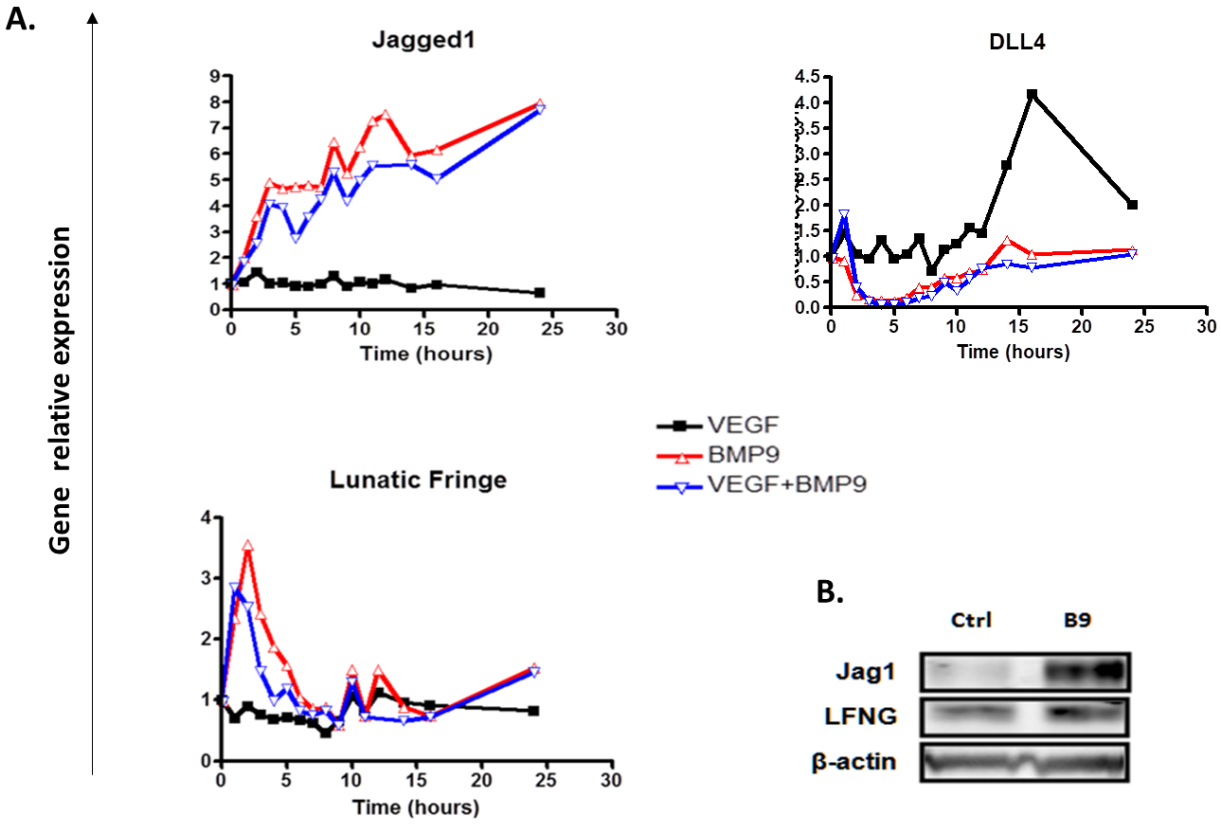


Fig.9 BMP9 modulates the expression of Notch regulators.

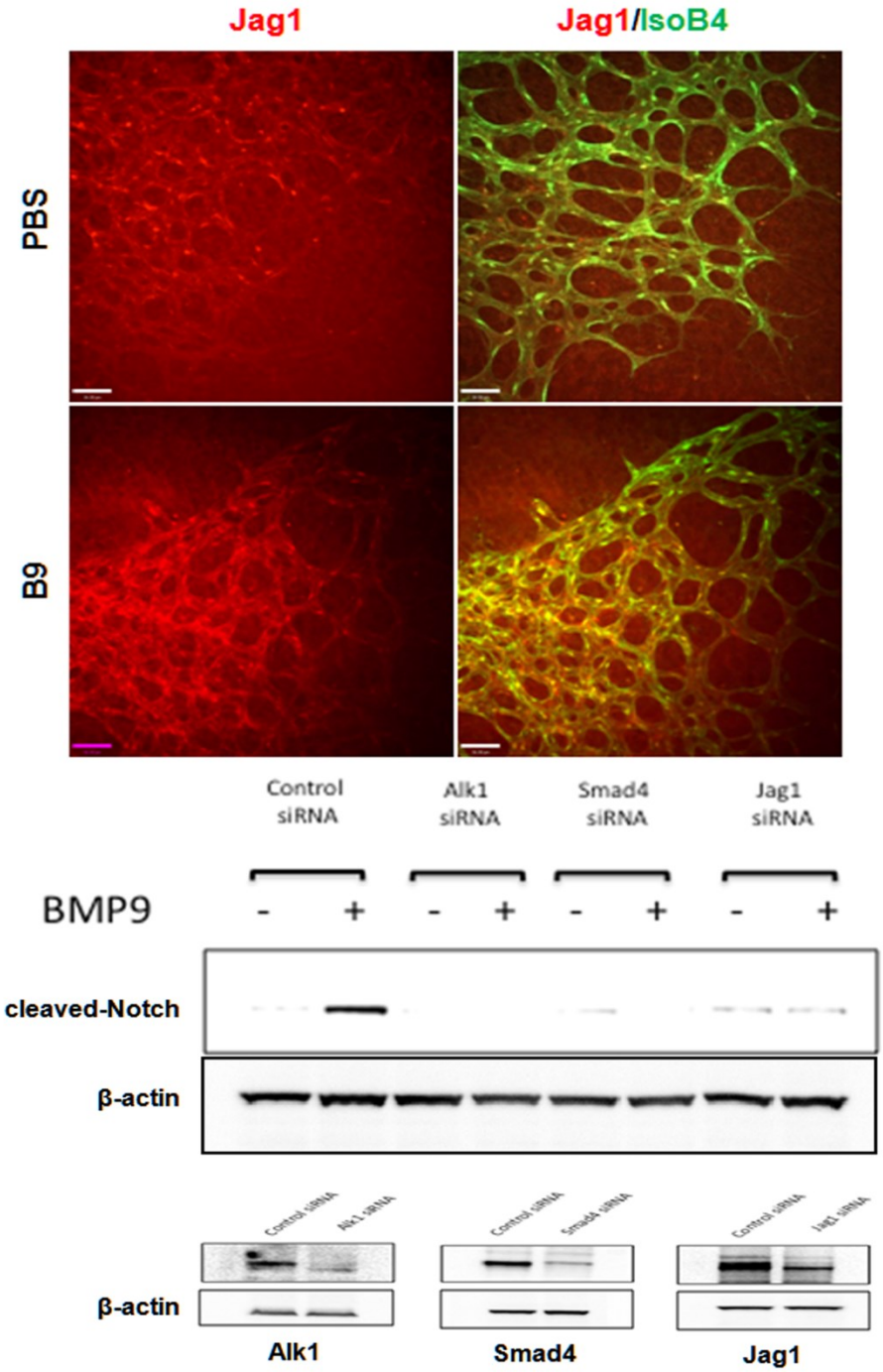


Fig.10 JAG1 expression is required for BMP9-mediated activation of Notch.

CHAPTER IV – Discussion

Angiogenic pathways crosstalk within ECs to regulate the formation of transient tip and stalk phenotypes. Their efficient collaboration is required to maintain the smooth progress of EC biological responses and related physiological processes such as sprouting. For instance, VEGF mediates tip cell selection by inducing DLL4-Notch transactivation that prevents stalk cells directed migration. Additionally, the differential regulation of VEGF receptor levels in tip and stalk cells as well as Notch-induced oscillatory targets generate fluctuating phenotypes that continuously compete for the VEGF-mediated tip selection [84], [452].. Thus, VEGF and Notch pathways cooperation is essential to selectively regulate the formation of tip cells. In fact, any loss in Notch functionality leads to VEGF-mediated hypersprouting features including ectopic filopodia, the dysregulated vessel branching with elevated tip markers [86], [419], [436] as well as the distributed fusions of adjacent tip-like cells in sheets of coalescing vessels [220]; subsequent to DLL4 blockade [453] and to heterozygosity or morpholino knockdown of Notch signaling components [454], [455]. Apart from the VEGF-Notch crosstalk, the BMP9/ALK1 axis also adds its level of regulation within EC to efficiently regulate vessel sprouting. Taken together, this thesis has investigated the effects of BMP9 on pathological angiogenesis and the mechanisms through which the BMP9/ALK1 axis crosstalks with VEGF and Notch pathways in ECs to induce vessel quiescence.

1. BMP9 regulates pathological angiogenesis

Pathological angiogenesis results from EC aberrant responses to signaling that mainly involve the VEGF pathways. Our results on ocular models of neovascularisation (NV) indicate that BMP9 inhibits the overgrowth of retinal and choroidal vessels under OIR and CNV conditions [451]. Thus, BMP9 shows an anti-angiogenic effect on the these vessel networks under conditions of experimental NV. Moreover, BMP9 shows a higher anti-angiogenic effect compared to an anti-VEGF, DC101, and furtherly potentiates it as their combination results in increased efficacy compared to their individual actions. Thus, BMP9 could be combined with lower doses of anti-VEGFs to reduce their concentration- and leakage- dependent local or systemic adverse effects. Additionally, BMP9 could be used to prevent the vaso-obliteration in the early phase of ischemic retinopathies such as ROP, since it reduces the vaso-obliteration of retina vessels associate with OIR. Furthermore, given that ALK1 expression and signaling are respectively EC-specific and non-apoptotic, BMP9 wouldn't show the secondary effects of anti-VEGFs on established vessels and VEGF-dependent neurons. In fact, BMP9 shows first no significant effects on the retina revascularisation under OIR conditions. Secondly, the localised expression of its receptor ALK1 generates the BMP9 specificity towards EC, in contrast to the VEGF receptors expressed on neurons and various cell types.

2. BMP9 canonical signaling induces Notch-mediated stalk markers

Our results show that BMP9 increases the activity of Notch as well as the expression of the stalk markers HEY1, HEY2, HES1, JAG1 and VEGFR1. The effects on these stalk markers are mediated by BMP9 canonical signaling via Smads. First, BMP9 activates Smad1/5, as revealed by their concomitant phosphorylation, during its upregulation of VEGFR1. In fact, the *FLT1* promoter contains an SBE region that directly binds to Smads. Secondly, the classical components of BMP9 signaling such as ALK1 and Smad4 are required for its induction of Notch activation. Indeed, knocking-down ALK1 and Smad4 with corresponding siRNAs interferes with the BMP9-mediated upregulation of Notch. However, BMP9-stimulated Smads require JAG1 prior to Notch activation as revealed by JAG1 knock-down. Thus, the active Smad complexes bind to the SBE sequence of *JAG1* in order to transactivate Notch. Subsequently, the JAG1-stimulated Notch induces its targets, including HES1 and related proteins, VEGFR1 and JAG1 itself.

Overall, BMP9 signals through ALK1 to activate Smads that in turn directly or indirectly amplify the stalk markers in ECs. On the one hand, BMP9 stimulates the formation of Smad transcriptional complexes that bind to SBE regions of *HEY1*, *HEY2*, *JAG1* and *VEGFR1* genes to induce their proteins. On the other hand, BMP9-induced Smads do not bind to *Notch* and *HES1* directly but rather stimulate Notch activation as well as the expression of its targets, including HES1, through their binding to *JAG1*. However, other studies have reported additional Smad1/5-based mechanisms in their

induction of HES1 via the synthesis of IDs. Together, these data show that BMP9 increases the stalk markers HEY1/2, HES1, JAG1 and VEGFR1 in ECs through Smad signaling mechanisms.

3. Cross-talks between BMP9/Smad1/5 and Notch pathways amplify VEGFR1

The BMP9 and Notch pathways crosstalk at various levels where they converge to regulate common targets. In fact, our results show that BMP9 specifically induces HEY1 and HEY2 that are both converging points of Smads and Notch transcriptional complexes. In addition, BMP9 induces JAG1, another junction that positions BMP9 signaling upstream of the Notch pathway. Indeed, JAG1 expression is required for BMP9-mediated activation of Notch, along with the activity of the ALK1/Smad4 axis. Moreover, this activation of Notch by BMP9-induced JAG1 subsequently leads to the loop that amplifies its targets such as HES1. Thus, BMP9-activated Smad1/5 induce the Notch targets HEY1, HEY2 and HES1 either directly or indirectly via JAG1-mediated activation. These junction components are specific HLH proteins that collectively regulate the synthesis of VEGFR1, a key stalk marker and regulator of VEGF signaling. Therefore, in addition to the direct upregulation of VEGFR1 by BMP9-induced Smad1/5, the BMP9 signaling indirectly amplifies VEGFR1 levels through its induction of the Notch targets HEY1, HEY2 and HES1. Accordingly, the Smad1/5 and Notch signaling cooperativity has been reported in other studies where the absence of Smad1/5 impairs Notch signaling in stalk cells and furtherly causes a loss of the stalk cell-enriched transcripts ID1-3, HES1, HEY1, JAG1 and VEGFR1; instead, it generates excessive tip features such as enrichment of VEGFR2/3 and DLL4 [220]. Collectively, these data

indicate that BMP9 activity synergizes with Notch signaling at HEY1, HEY2, JAG1 and HES1 crossroads to potentiate its Smad1/5-mediated direct upregulation of VEGFR1.

4. BMP9 signaling induces EC quiescence through differential regulation of VEGF and Notch pathways

Previous results have reported that HES1 and HEY1 differentially regulate the transcription of VEGFR2/3, DLL4, VEGFR1 and JAG1 [215], [251], [252]. Our results show that BMP9 and Notch pathways converge at HEY1, HEY2 and HES1 to induce VEGFR1, either directly via Smad activation or indirectly via JAG1 transcription. Thus, our data indicate a direct activity of BMP9 signaling on VEGFR1 synthesis that is additionally potentiated by its synergy with Notch signaling. Moreover, as VEGFR1 essentially constitutes a VEGF trap, its direct and indirect increase by BMP9-induced Smads and Notch targets leads to the inhibition of VEGF signaling. Therefore, BMP9 increases VEGFR1 as a checkpoint to modulate VEGF pathways. Furthermore, the BMP9-induced HEY1, HEY2 and HES1 concomitantly inhibit the expression of VEGFR2; despite the non-significant reduction of VEGFR2 transcription or synthesis as revealed by its mRNA and protein levels. However, the higher affinity of VEGFR1 toward VEGF is enough to conceal any persistent VEGFR2. Thus, on the one hand, BMP9-increased VEGFR1 inhibits VEGF signaling in tip cells; whereas on the other hand, BMP9-induced JAG1 reactivates their Notch. Together, the decrease of VEGF pathways and the reactivation of Notch by BMP9 alleviates the VEGF-DLL4-Notch lateral inhibition that regulates the tip selection [86], [219]. Thereby, the inhibition of tip cell formation by BMP9 through VEGFR1 and JAG1 induces the quiescence of ECs. Accordingly, other studies have reported that JAG1 peptides inhibit cellular division [456]–[458] and rescue the EC specification during ALK1Fc-mediated hypersprouting

[250]. Overall, our data indicate that BMP9 abrogates sprouting angiogenesis by maintaining ECs in a non-proliferative and quiescent phalanx-like state. Specifically, BMP9-induced quiescence results from the inhibition of tip cell formation; as BMP9 oppositely regulates Notch and VEGF pathways through their converging or check points such as HEY1, HEY2, JAG1, HES1 and VEGFR1 (Fig.1).

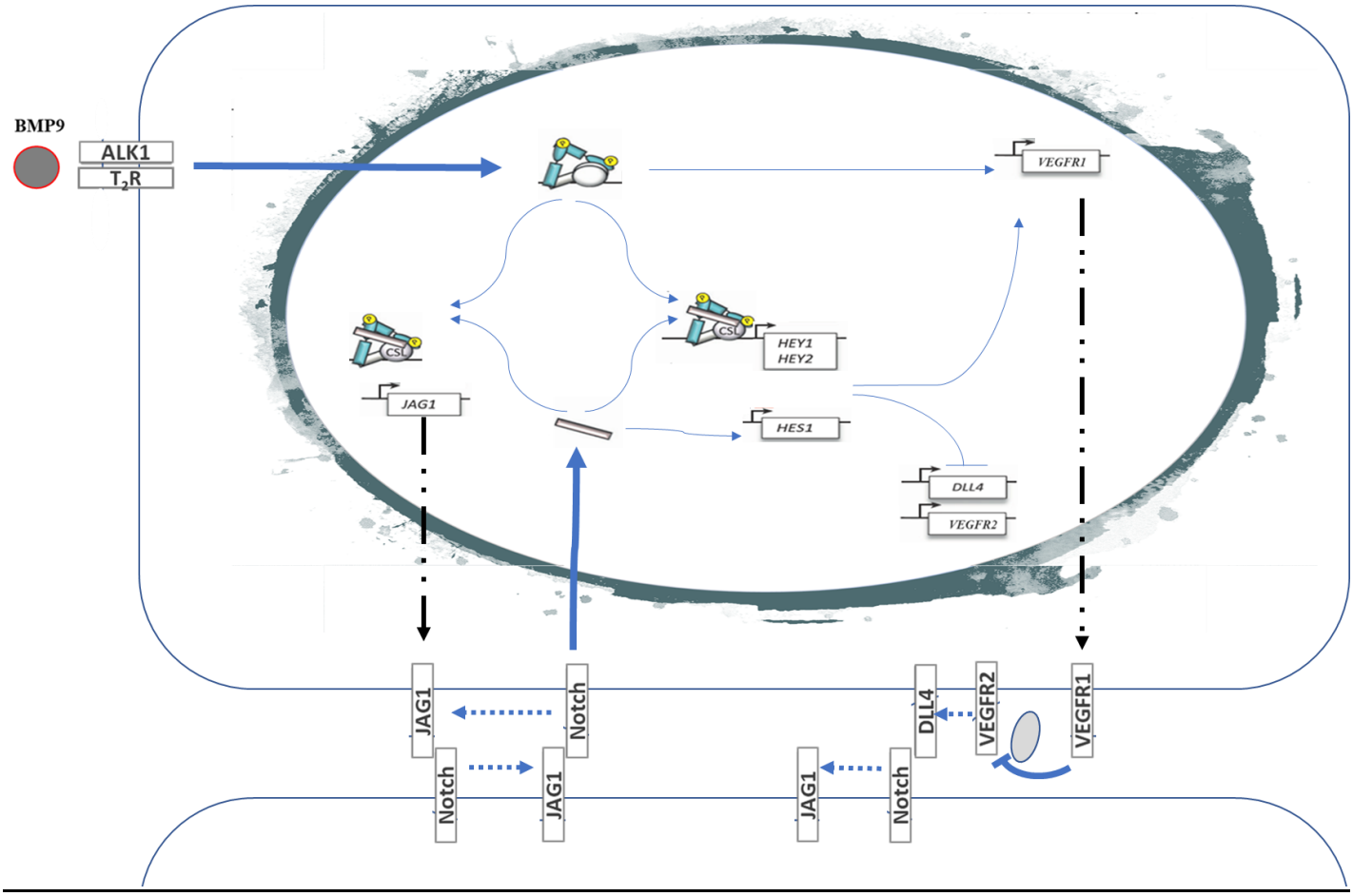


Fig.1 BMP9 signaling modulates VEGF and Notch through upregulation of VEGFR1 and JAG1

BMP9-induced Smad complex upregulates VEGFR1 through direct binding to its gene. Active Smad complex indirectly mediates Notch activity by inducing JAG1 that in turn stimulates the JAG1-Notch double positive feed-back loop. Cleaved Notch, directly or cooperatively with Smad complex, induces HES-related proteins that subsequently potentiate VEGFR1 transcription while inhibiting VEGFR2. Increased expression of VEGFR1 by Smad and Notch targets leads to the inhibition of VEGF signaling.

Taken together, the data from this thesis show that BMP9, VEGF and Notch pathways crosstalk at the level of VEGFR1, JAG1 and HES-related proteins to integrate their redundant or opposite activities within ECs. Ultimately, the BMP9-mediated increase of VEGFR1 and JAG1 differentially regulates VEGF and Notch pathways. On the one hand, the interplay between BMP9 and VEGF pathways through VEGFR1 results in the inhibition of VEGF signaling and downstream effectors of tip cell selection. On the other hand, the crosstalk between BMP9 and Notch pathways at the level of JAG1 promotes Notch activities that in turn enable the conversion of tip cells back to phalanx cells. Ultimately, the coordinated regulations of VEGF and Notch signaling by BMP9 reverse the migratory or proliferative phenotype of tip and stalk cells; and thereby sustain its antiangiogenic effect on sprouting blood vessels. Overall, the ECs coordinate the signaling activities of BMP9, Notch and VEGF during angiogenesis to induce the quiescence of blood vessels. Thus, the diversity of interactions between BMP9, Notch and VEGF pathways generates a flexibility of antiangiogenic therapy targets for the treatment of the pathological neovascularization-associated conditions such as AMD, ROP or DME. Furthermore, investigating the interplay between BMP9 and other angiogenic pathways might provide additional insights on its anti-angiogenic properties.

CHAPTER V – Conclusion

Sprouting angiogenesis is required during various physiological and developmental processes including foetal growth, wound healing, tissue repair and menstrual cycle. A disturbance in this modality of vascularisation mostly leads to pathological vessels that cause the dysfunction of organs or accelerate the progression of cancer. The data from this thesis show that the BMP9/ALK1 pathway inhibits the pathological angiogenesis in ocular models of CNV and OIR. Thus, the BMP9/ALK1 axis emerges as a promising target for the treatment of wet AMD and neovascular diseases. BMP9 also presents higher anti-angiogenic effects at its circulating level in comparison to the VEGF receptor-inhibiting compound DC101 and potentiates its effects when they are combined. Moreover, BMP9 effects are selective to neovessels, in contrast to the reported action of conventional anti-VEGFs. Accordingly, the EC-enriched expression of the receptor ALK1 would promote the specificity BMP9 towards ECs, in contrast to VEGF receptors that are expressed on photoreceptors and various cell types. Therefore, the low dosage and the specificity of BMP9 favor it to be an efficient anti-angiogenic candidate with reduced adverse effects. Mechanistically, BMP9 restores the vessel quiescence by inhibiting the VEGF pathways and thereby interfering with their induction of ECs specification into tip cells. Specifically, BMP9 directly and indirectly induces VEGFR1 expression while it activates Notch in ALK1-, Smad4- and JAG1-dependent manner. Globally, the current studies indicate that BMP9 inhibits the pathological angiogenesis by selectively promoting the quiescence of sprouting vessels through its opposite regulations of VEGF and Notch pathways in tip and stalk ECs. Consequently, the activation of BMP9-mediated pathways, alone or in combination with

reduced doses of anti-VEGFs, represents a promising therapeutic approach free of the adverse burden of conventional anti-angiogenic drugs for patients suffering from wet AMD or neovascular diseases.

References

[1] W. Risau and V. Lemmon, “Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis,” *Dev. Biol.*, vol. 125, no. 2, pp. 441–450, Feb. 1988.

[2] M. Potente and T. Mäkinen, “Vascular heterogeneity and specialization in development and disease,” *Nat. Rev. Mol. Cell Biol.*, vol. 18, no. 8, pp. 477–494, Aug. 2017, doi: 10.1038/nrm.2017.36.

[3] A. Szymborska and H. Gerhardt, “Hold Me, but Not Too Tight-Endothelial Cell-Cell Junctions in Angiogenesis,” *Cold Spring Harb. Perspect. Biol.*, vol. 10, no. 8, Aug. 2018, doi: 10.1101/cshperspect.a029223.

[4] K. Alitalo and P. Carmeliet, “Molecular mechanisms of lymphangiogenesis in health and disease,” *Cancer Cell*, vol. 1, no. 3, pp. 219–227, Apr. 2002, doi: 10.1016/S1535-6108(02)00051-X.

[5] N. Paffett-Lugassy *et al.*, “Heart field origin of great vessel precursors relies on nkx2.5-mediated vasculogenesis,” *Nat. Cell Biol.*, vol. 15, no. 11, pp. 1362–1369, Nov. 2013, doi: 10.1038/ncb2862.

[6] A. F. Siekmann, C. Standley, K. E. Fogarty, S. A. Wolfe, and N. D. Lawson, “Chemokine signaling guides regional patterning of the first embryonic artery,” *Genes Dev.*, vol. 23, no. 19, pp. 2272–2277, Oct. 2009, doi: 10.1101/gad.1813509.

[7] K. Proulx, A. Lu, and S. Sumanas, “Cranial vasculature in zebrafish forms by angioblast cluster-derived angiogenesis,” *Dev. Biol.*, vol. 348, no. 1, pp. 34–46, Dec. 2010, doi: 10.1016/j.ydbio.2010.08.036.

[8] Y. Blum, H.-G. Belting, E. Ellertsdottir, L. Herwig, F. Lüders, and M. Affolter, “Complex cell rearrangements during intersegmental vessel sprouting and vessel fusion in the zebrafish embryo,” *Dev. Biol.*, vol. 316, no. 2, pp. 312–322, Apr. 2008, doi: 10.1016/j.ydbio.2008.01.038.

[9] L. Sauteur *et al.*, “Cdh5/VE-cadherin promotes endothelial cell interface elongation via cortical actin polymerization during angiogenic sprouting,” *Cell Rep.*, vol. 9, no. 2, pp. 504–513, Oct. 2014, doi: 10.1016/j.celrep.2014.09.024.

[10] R. Wang *et al.*, “Glioblastoma stem-like cells give rise to tumour endothelium,” *Nature*, vol. 468, no. 7325, pp. 829–833, Dec. 2010, doi: 10.1038/nature09624.

[11] P. Carmeliet and R. K. Jain, “Molecular mechanisms and clinical applications of angiogenesis,” *Nature*, vol. 473, no. 7347, pp. 298–307, May 2011, doi: 10.1038/nature10144.

[12] H. Gerhardt *et al.*, “VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia,” *J. Cell Biol.*, vol. 161, no. 6, pp. 1163–1177, Jun. 2003, doi: 10.1083/jcb.200302047.

[13] T.-H. Chun *et al.*, “MT1-MMP-dependent neovessel formation within the confines of the three-dimensional extracellular matrix,” *J. Cell Biol.*, vol. 167, no. 4, pp. 757–767, Nov. 2004, doi: 10.1083/jcb.200405001.

[14] C. A. Franco *et al.*, “Serum response factor is required for sprouting angiogenesis and vascular integrity,” *Dev. Cell*, vol. 15, no. 3, pp. 448–461, Sep. 2008, doi: 10.1016/j.devcel.2008.07.019.

[15] M. Graupera *et al.*, “Angiogenesis selectively requires the p110alpha isoform of PI3K to

control endothelial cell migration," *Nature*, vol. 453, no. 7195, pp. 662–666, May 2008, doi: 10.1038/nature06892.
[16] A. C. Aplin, E. Fogel, and R. F. Nicosia, "MCP-1 promotes mural cell recruitment during angiogenesis in the aortic ring model," *Angiogenesis*, vol. 13, no. 3, pp. 219–226, Sep. 2010, doi: 10.1007/s10456-010-9179-8.
[17] E. Dejana, F. Orsenigo, C. Molendini, P. Baluk, and D. M. McDonald, "Organization and signaling of endothelial cell-to-cell junctions in various regions of the blood and lymphatic vascular trees," *Cell Tissue Res.*, vol. 335, no. 1, pp. 17–25, Jan. 2009, doi: 10.1007/s00441-008-0694-5.
[18] S. Claxton and M. Fruttiger, "Oxygen modifies artery differentiation and network morphogenesis in the retinal vasculature," *Dev. Dyn. Off. Publ. Am. Assoc. Anat.*, vol. 233, no. 3, pp. 822–828, Jul. 2005, doi: 10.1002/dvdy.20407.
[19] K. Beets, D. Huylebroeck, I. M. Moya, L. Umans, and A. Zwijsen, "Robustness in angiogenesis: notch and BMP shaping waves," *Trends Genet. TIG*, vol. 29, no. 3, pp. 140–149, Mar. 2013, doi: 10.1016/j.tig.2012.11.008.
[20] P. Carmeliet, "Angiogenesis in health and disease," *Nat. Med.*, vol. 9, no. 6, pp. 653–660, Jun. 2003, doi: 10.1038/nm0603-653.
[21] A. Hoeben, B. Landuyt, M. S. Highley, H. Wildiers, A. T. V. Oosterom, and E. A. D. Bruijn, "Vascular Endothelial Growth Factor and Angiogenesis," *Pharmacol. Rev.*, vol. 56, no. 4, pp. 549–580, Dec. 2004, doi: 10.1124/pr.56.4.3.
[22] C. G. Colpaert *et al.*, "Inflammatory breast cancer shows angiogenesis with high endothelial proliferation rate and strong E-cadherin expression," *Br. J. Cancer*, vol. 88, no. 5, pp. 718–725, Mar. 2003, doi: 10.1038/sj.bjc.6600807.
[23] J. Folkman, "Angiogenesis in cancer, vascular, rheumatoid and other disease," *Nat. Med.*, vol. 1, no. 1, pp. 27–31, Jan. 1995, doi: 10.1038/nm0195-27.
[24] J. Folkman, "Clinical applications of research on angiogenesis," *N. Engl. J. Med.*, vol. 333, no. 26, pp. 1757–1763, Dec. 1995, doi: 10.1056/NEJM199512283332608.
[25] S. Y. Yoo and S. M. Kwon, "Angiogenesis and Its Therapeutic Opportunities," *Mediators Inflamm.*, 2013, doi: 10.1155/2013/127170.
[26] M. Klagsbrun and M. A. Moses, "Molecular angiogenesis," *Chem. Biol.*, vol. 6, no. 8, pp. R217-224, Aug. 1999, doi: 10.1016/S1074-5521(99)80081-7.
[27] M. S. Pepper, "Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis," *Arterioscler. Thromb. Vasc. Biol.*, vol. 21, no. 7, pp. 1104–1117, Jul. 2001, doi: 10.1161/hq0701.093685.
[28] P. Mignatti and D. B. Rifkin, "Plasminogen activators and matrix metalloproteinases in angiogenesis," *Enzyme Protein*, vol. 49, no. 1–3, pp. 117–137, 1996, doi: 10.1159/000468621.
[29] J. A. Eble and S. Niland, "The extracellular matrix of blood vessels," *Curr. Pharm. Des.*, vol. 15, no. 12, pp. 1385–1400, 2009.
[30] J. Di Russo *et al.*, "Vascular laminins in physiology and pathology," *Matrix Biol. J. Int. Soc. Matrix Biol.*, vol. 57–58, pp. 140–148, 2017, doi: 10.1016/j.matbio.2016.06.008.
[31] A. E. Forbes and J. J. Going, "Color Atlas of Histology," *J. Anat.*, vol. 215, no. 4, p. 472, Oct. 2009, doi: 10.1111/j.1469-7580.2009.01120.x.
[32] D. J. Prockop and K. I. Kivirikko, "Collagens: molecular biology, diseases, and potentials for therapy," *Annu. Rev. Biochem.*, vol. 64, pp. 403–434, 1995, doi: 10.1146/annurev.bi.64.070195.002155.

[33] S. Katsuda and T. Kaji, “Atherosclerosis and extracellular matrix,” *J. Atheroscler. Thromb.*, vol. 10, no. 5, pp. 267–274, 2003, doi: 10.5551/jat.10.267.
[34] J. Myllyharju and K. I. Kivirikko, “Collagens, modifying enzymes and their mutations in humans, flies and worms,” *Trends Genet. TIG*, vol. 20, no. 1, pp. 33–43, Jan. 2004, doi: 10.1016/j.tig.2003.11.004.
[35] R. Kalluri, “Basement membranes: structure, assembly and role in tumour angiogenesis,” *Nat. Rev. Cancer*, vol. 3, no. 6, pp. 422–433, Jun. 2003, doi: 10.1038/nrc1094.
[36] V. S. LeBleu, B. Macdonald, and R. Kalluri, “Structure and function of basement membranes,” *Exp. Biol. Med. Maywood NJ*, vol. 232, no. 9, pp. 1121–1129, Oct. 2007, doi: 10.3181/0703-MR-72.
[37] R. V. Iozzo, “Basement membrane proteoglycans: from cellar to ceiling,” *Nat. Rev. Mol. Cell Biol.*, vol. 6, no. 8, pp. 646–656, Aug. 2005, doi: 10.1038/nrm1702.
[38] F. G. Giancotti and E. Ruoslahti, “Integrin signaling,” *Science*, vol. 285, no. 5430, pp. 1028–1032, Aug. 1999, doi: 10.1126/science.285.5430.1028.
[39] M. A. Arnaout, B. Mahalingam, and J.-P. Xiong, “Integrin structure, allostery, and bidirectional signaling,” *Annu. Rev. Cell Dev. Biol.*, vol. 21, pp. 381–410, 2005, doi: 10.1146/annurev.cellbio.21.090704.151217.
[40] Y. Wallez and P. Huber, “Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis,” *Biochim. Biophys. Acta*, vol. 1778, no. 3, pp. 794–809, Mar. 2008, doi: 10.1016/j.bbamem.2007.09.003.
[41] H. Methe, S. Hess, and E. R. Edelman, “Endothelial immunogenicity--a matter of matrix microarchitecture,” *Thromb. Haemost.*, vol. 98, no. 2, pp. 278–282, Aug. 2007.
[42] W. C. Aird, “Endothelial cell heterogeneity,” *Cold Spring Harb. Perspect. Med.*, vol. 2, no. 1, p. a006429, Jan. 2012, doi: 10.1101/cshperspect.a006429.
[43] G. Coppiello *et al.*, “Meox2/Tcf15 heterodimers program the heart capillary endothelium for cardiac fatty acid uptake,” *Circulation*, vol. 131, no. 9, pp. 815–826, Mar. 2015, doi: 10.1161/CIRCULATIONAHA.114.013721.
[44] K. L. Marcelo, L. C. Goldie, and K. K. Hirschi, “Regulation of Endothelial Cell Differentiation and Specification,” *Circ. Res.*, vol. 112, no. 9, pp. 1272–1287, Apr. 2013, doi: 10.1161/CIRCRESAHA.113.300506.
[45] M. Corada, M. F. Morini, and E. Dejana, “Signaling pathways in the specification of arteries and veins,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 34, no. 11, pp. 2372–2377, Nov. 2014, doi: 10.1161/ATVBAHA.114.303218.
[46] X. L. Aranguren *et al.*, “COUP-TFII orchestrates venous and lymphatic endothelial identity by homo- or hetero-dimerisation with PROX1,” *J. Cell Sci.*, vol. 126, no. Pt 5, pp. 1164–1175, Mar. 2013, doi: 10.1242/jcs.116293.
[47] M. Simons and A. Eichmann, “Molecular controls of arterial morphogenesis,” *Circ. Res.*, vol. 116, no. 10, pp. 1712–1724, May 2015, doi: 10.1161/CIRCRESAHA.116.302953.
[48] V. Kohli, J. A. Schumacher, S. P. Desai, K. Rehn, and S. Sumanas, “Arterial and venous progenitors of the major axial vessels originate at distinct locations,” *Dev. Cell*, vol. 25, no. 2, pp. 196–206, Apr. 2013, doi: 10.1016/j.devcel.2013.03.017.
[49] L. Le Guen *et al.*, “Ccbe1 regulates Vegfc-mediated induction of Vegfr3 signaling during embryonic lymphangiogenesis,” *Dev. Camb. Engl.*, vol. 141, no. 6, pp. 1239–1249, Mar. 2014, doi: 10.1242/dev.100495.
[50] T. Kamba *et al.*, “VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature,” *Am. J. Physiol. Heart Circ. Physiol.*, vol. 290, no. 2, pp. H560-576, Feb.

2006, doi: 10.1152/ajpheart.00133.2005.
[51] A. Sabine, C. Saygili Demir, and T. V. Petrova, “Endothelial Cell Responses to Biomechanical Forces in Lymphatic Vessels,” *Antioxid. Redox Signal.*, vol. 25, no. 7, pp. 451–465, 01 2016, doi: 10.1089/ars.2016.6685.
[52] A. Sabine *et al.*, “FOXC2 and fluid shear stress stabilize postnatal lymphatic vasculature,” *J. Clin. Invest.*, vol. 125, no. 10, pp. 3861–3877, Oct. 2015, doi: 10.1172/JCI80454.
[53] N. Baeyens, C. Bandyopadhyay, B. G. Coon, S. Yun, and M. A. Schwartz, “Endothelial fluid shear stress sensing in vascular health and disease,” *J. Clin. Invest.*, vol. 126, no. 3, pp. 821–828, Mar. 2016, doi: 10.1172/JCI83083.
[54] J. M. Tarbell, S. I. Simon, and F.-R. E. Curry, “Mechanosensing at the vascular interface,” *Annu. Rev. Biomed. Eng.*, vol. 16, pp. 505–532, Jul. 2014, doi: 10.1146/annurev-bioeng-071813-104908.
[55] D. L. Ramírez-Bergeron, A. Runge, K. D. C. Dahl, H. J. Fehling, G. Keller, and M. C. Simon, “Hypoxia affects mesoderm and enhances hemangioblast specification during early development,” *Dev. Camb. Engl.*, vol. 131, no. 18, pp. 4623–4634, Sep. 2004, doi: 10.1242/dev.01310.
[56] B. W. Wong *et al.*, “The role of fatty acid β-oxidation in lymphangiogenesis,” *Nature*, vol. 542, no. 7639, pp. 49–54, 02 2017, doi: 10.1038/nature21028.
[57] N. N. Pavlova and C. B. Thompson, “The Emerging Hallmarks of Cancer Metabolism,” *Cell Metab.*, vol. 23, no. 1, pp. 27–47, Jan. 2016, doi: 10.1016/j.cmet.2015.12.006.
[58] C. Jang *et al.*, “A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance,” *Nat. Med.*, vol. 22, no. 4, pp. 421–426, Apr. 2016, doi: 10.1038/nm.4057.
[59] F. Végran, R. Boidot, C. Michiels, P. Sonveaux, and O. Feron, “Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-κB/IL-8 pathway that drives tumor angiogenesis,” *Cancer Res.*, vol. 71, no. 7, pp. 2550–2560, Apr. 2011, doi: 10.1158/0008-5472.CAN-10-2828.
[60] G.-X. Ruan and A. Kazlauskas, “Lactate engages receptor tyrosine kinases Axl, Tie2, and vascular endothelial growth factor receptor 2 to activate phosphoinositide 3-kinase/Akt and promote angiogenesis,” *J. Biol. Chem.*, vol. 288, no. 29, pp. 21161–21172, Jul. 2013, doi: 10.1074/jbc.M113.474619.
[61] J. LeCouter *et al.*, “Angiogenesis-independent endothelial protection of liver: role of VEGFR-1,” *Science*, vol. 299, no. 5608, pp. 890–893, Feb. 2003, doi: 10.1126/science.1079562.
[62] A. Armulik *et al.*, “Pericytes regulate the blood-brain barrier,” *Nature*, vol. 468, no. 7323, pp. 557–561, Nov. 2010, doi: 10.1038/nature09522.
[63] A. Armulik, G. Genové, and C. Betsholtz, “Pericytes: developmental, physiological, and pathological perspectives, problems, and promises,” *Dev. Cell*, vol. 21, no. 2, pp. 193–215, Aug. 2011, doi: 10.1016/j.devcel.2011.07.001.
[64] C. E. Hagberg *et al.*, “Vascular endothelial growth factor B controls endothelial fatty acid uptake,” *Nature*, vol. 464, no. 7290, pp. 917–921, Apr. 2010, doi: 10.1038/nature08945.
[65] R. Kivelä *et al.*, “VEGF-B-induced vascular growth leads to metabolic reprogramming and ischemia resistance in the heart,” *EMBO Mol. Med.*, vol. 6, no. 3, pp. 307–321, 2014, doi: 10.1002/emmm.201303147.
[66] M. H. Dijkstra *et al.*, “Lack of cardiac and high-fat diet induced metabolic phenotypes in

two independent strains of Vegf-b knockout mice," *Sci. Rep.*, vol. 4, p. 6238, Aug. 2014, doi: 10.1038/srep06238.
[67] Z. Zhao, A. R. Nelson, C. Betsholtz, and B. V. Zlokovic, "Establishment and Dysfunction of the Blood-Brain Barrier," *Cell*, vol. 163, no. 5, pp. 1064–1078, Nov. 2015, doi: 10.1016/j.cell.2015.10.067.
[68] A. Aspelund, M. R. Robciuc, S. Karaman, T. Makinen, and K. Alitalo, "Lymphatic System in Cardiovascular Medicine," *Circ. Res.*, vol. 118, no. 3, pp. 515–530, Feb. 2016, doi: 10.1161/CIRCRESAHA.115.306544.
[69] M. Lee *et al.*, "Transcriptional programs of lymphoid tissue capillary and high endothelium reveal control mechanisms for lymphocyte homing," *Nat. Immunol.*, vol. 15, no. 10, pp. 982–995, Oct. 2014, doi: 10.1038/ni.2983.
[70] J.-P. Girard, C. Moussion, and R. Förster, "HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes," *Nat. Rev. Immunol.*, vol. 12, no. 11, pp. 762–773, Nov. 2012, doi: 10.1038/nri3298.
[71] S. Sinha, D. Iyer, and A. Granata, "Embryonic origins of human vascular smooth muscle cells: implications for in vitro modeling and clinical application," *Cell. Mol. Life Sci. CMLS*, vol. 71, no. 12, pp. 2271–2288, Jun. 2014, doi: 10.1007/s00018-013-1554-3.
[72] Y. Cao, H. Li, F.-T. Mu, O. Ebisui, J. W. Funder, and J.-P. Liu, "Telomerase activation causes vascular smooth muscle cell proliferation in genetic hypertension," *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.*, vol. 16, no. 1, pp. 96–98, Jan. 2002, doi: 10.1096/cj.01-0447fje.
[73] P. Lacolley, V. Regnault, P. Segers, and S. Laurent, "Vascular Smooth Muscle Cells and Arterial Stiffening: Relevance in Development, Aging, and Disease," *Physiol. Rev.*, vol. 97, no. 4, pp. 1555–1617, Oct. 2017, doi: 10.1152/physrev.00003.2017.
[74] M.-J. Goumans, A. Zwijsen, P. Ten Dijke, and S. Bailly, "Bone Morphogenetic Proteins in Vascular Homeostasis and Disease," *Cold Spring Harb. Perspect. Biol.*, vol. 10, no. 2, Feb. 2018, doi: 10.1101/cshperspect.a031989.
[75] W. Risau, "Mechanisms of angiogenesis," *Nature*, vol. 386, no. 6626, pp. 671–674, Apr. 1997, doi: 10.1038/386671a0.
[76] K. P. Dingemans, P. Teeling, J. H. Lagendijk, and A. E. Becker, "Extracellular matrix of the human aortic media: an ultrastructural histochemical and immunohistochemical study of the adult aortic media," *Anat. Rec.*, vol. 258, no. 1, pp. 1–14, 01 2000, doi: 10.1002/(SICI)1097-0185(20000101)258:1<1::AID-AR1>3.0.CO;2-7.
[77] J. Thyberg, K. Blomgren, J. Roy, P. K. Tran, and U. Hedin, "Phenotypic modulation of smooth muscle cells after arterial injury is associated with changes in the distribution of laminin and fibronectin," *J. Histochem. Cytochem. Off. J. Histochem. Soc.*, vol. 45, no. 6, pp. 837–846, Jun. 1997, doi: 10.1177/002215549704500608.
[78] S. M. Mithieux and A. S. Weiss, "Elastin," *Adv. Protein Chem.*, vol. 70, pp. 437–461, 2005, doi: 10.1016/S0065-3233(05)70013-9.
[79] A. K. Baldwin, A. Simpson, R. Steer, S. A. Cain, and C. M. Kielty, "Elastic fibres in health and disease," *Expert Rev. Mol. Med.*, vol. 15, p. e8, Aug. 2013, doi: 10.1017/erm.2013.9.
[80] C. M. Kielty, "Elastic fibres in health and disease," *Expert Rev. Mol. Med.*, vol. 8, no. 19, pp. 1–23, Aug. 2006, doi: 10.1017/S146239940600007X.
[81] A. Patel, B. Fine, M. Sandig, and K. Mequanint, "Elastin biosynthesis: The missing link in tissue-engineered blood vessels," *Cardiovasc. Res.*, vol. 71, no. 1, pp. 40–49, Jul. 2006,

doi: 10.1016/j.cardiores.2006.02.021.
[82] B. S. Brooke, S. K. Karnik, and D. Y. Li, “Extracellular matrix in vascular morphogenesis and disease: structure versus signal,” *Trends Cell Biol.*, vol. 13, no. 1, pp. 51–56, Jan. 2003, doi: 10.1016/s0962-8924(02)00007-7.
[83] E. P. Moiseeva, “Adhesion receptors of vascular smooth muscle cells and their functions,” *Cardiovasc. Res.*, vol. 52, no. 3, pp. 372–386, Dec. 2001, doi: 10.1016/s0008-6363(01)00399-6.
[84] L. Jakobsson *et al.*, “Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting,” *Nat. Cell Biol.*, vol. 12, no. 10, pp. 943–953, Oct. 2010, doi: 10.1038/ncb2103.
[85] F. De Smet, I. Segura, K. De Bock, P. J. Hohensinner, and P. Carmeliet, “Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 29, no. 5, pp. 639–649, May 2009, doi: 10.1161/ATVBAHA.109.185165.
[86] M. Hellström *et al.*, “Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis,” *Nature*, vol. 445, no. 7129, pp. 776–780, Feb. 2007, doi: 10.1038/nature05571.
[87] N. W. Gale *et al.*, “Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 45, pp. 15949–15954, Nov. 2004, doi: 10.1073/pnas.0407290101.
[88] A. Duarte *et al.*, “Dosage-sensitive requirement for mouse Dll4 in artery development,” *Genes Dev.*, vol. 18, no. 20, pp. 2474–2478, Oct. 2004, doi: 10.1101/gad.1239004.
[89] L. T. Krebs, J. R. Shutter, K. Tanigaki, T. Honjo, K. L. Stark, and T. Gridley, “Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants,” *Genes Dev.*, vol. 18, no. 20, pp. 2469–2473, Oct. 2004, doi: 10.1101/gad.1239204.
[90] R. Blanco and H. Gerhardt, “VEGF and Notch in Tip and Stalk Cell Selection,” *Cold Spring Harb. Perspect. Med.*, vol. 3, no. 1, p. a006569, Jan. 2013, doi: 10.1101/cshperspect.a006569.
[91] J. J. Tung, I. W. Tattersall, and J. Kitajewski, “Tips, Stalks, Tubes: Notch-Mediated Cell Fate Determination and Mechanisms of Tubulogenesis during Angiogenesis,” *Cold Spring Harb. Perspect. Med.*, vol. 2, no. 2, Feb. 2012, doi: 10.1101/cshperspect.a006601.
[92] A. Eichmann and M. Simons, “VEGF signaling inside vascular endothelial cells and beyond,” *Curr. Opin. Cell Biol.*, vol. 24, no. 2, pp. 188–193, Apr. 2012, doi: 10.1016/j.ceb.2012.02.002.
[93] M. S. Pepper, N. Ferrara, L. Orci, and R. Montesano, “Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro,” *Biochem. Biophys. Res. Commun.*, vol. 189, no. 2, pp. 824–831, Dec. 1992, doi: 10.1016/0006-291x(92)92277-5.
[94] N. Ferrara *et al.*, “Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene,” *Nature*, vol. 380, no. 6573, pp. 439–442, Apr. 1996, doi: 10.1038/380439a0.
[95] Y.-G. Chen and J. Massagué, “Smad1 Recognition and Activation by the ALK1 Group of Transforming Growth Factor-β Family Receptors,” *J. Biol. Chem.*, vol. 274, no. 6, pp. 3672–3677, Feb. 1999, doi: 10.1074/jbc.274.6.3672.
[96] S. P. Oh *et al.*, “Activin receptor-like kinase 1 modulates transforming growth factor-β1 signaling in the regulation of angiogenesis,” *Proc. Natl. Acad. Sci.*, vol. 97, no. 6, pp. 2626–2631, Mar. 2000, doi: 10.1073/pnas.97.6.2626.

[97] T. Seki, J. Yun, and S. P. Oh, "Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling," *Circ. Res.*, vol. 93, no. 7, pp. 682–689, Oct. 2003, doi: 10.1161/01.RES.0000095246.40391.3B.
[98] M.-J. Goumans, G. Valdimarsdottir, S. Itoh, A. Rosendahl, P. Sideras, and P. ten Dijke, "Balancing the activation state of the endothelium via two distinct TGF-β type I receptors," *EMBO J.*, vol. 21, no. 7, pp. 1743–1753, Apr. 2002, doi: 10.1093/emboj/21.7.1743.
[99] J. Folkman and M. Klagsbrun, "Angiogenic factors," *Science*, vol. 235, no. 4787, pp. 442–447, Jan. 1987, doi: 10.1126/science.2432664.
[100] A. B. Roberts and M. B. Sporn, "Regulation of endothelial cell growth, architecture, and matrix synthesis by TGF-beta," *Am. Rev. Respir. Dis.*, vol. 140, no. 4, pp. 1126–1128, Oct. 1989, doi: 10.1164/ajrccm/140.4.1126.
[101] M. L. Iruela-Arispe and H. F. Dvorak, "Angiogenesis: a dynamic balance of stimulators and inhibitors," *Thromb. Haemost.*, vol. 78, no. 1, pp. 672–677, Jul. 1997.
[102] K. M. Dameron, O. V. Volpert, M. A. Tainsky, and N. Bouck, "Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1," *Science*, vol. 265, no. 5178, pp. 1582–1584, Sep. 1994, doi: 10.1126/science.7521539.
[103] L. Holmgren, M. S. O'Reilly, and J. Folkman, "Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression," *Nat. Med.*, vol. 1, no. 2, pp. 149–153, Feb. 1995, doi: 10.1038/nm0295-149.
[104] K. A. Neely and T. W. Gardner, "Ocular Neovascularization," *Am. J. Pathol.*, vol. 153, no. 3, pp. 665–670, Sep. 1998.
[105] A. N. Haggstrom *et al.*, "Prospective study of infantile hemangiomas: clinical characteristics predicting complications and treatment," *Pediatrics*, vol. 118, no. 3, pp. 882–887, Sep. 2006, doi: 10.1542/peds.2006-0413.
[106] M. Volm, R. Koomägi, and J. Mattern, "Prognostic value of vascular endothelial growth factor and its receptor Flt-1 in squamous cell lung cancer," *Int. J. Cancer*, vol. 74, no. 1, pp. 64–68, Feb. 1997, doi: 10.1002/(sici)1097-0215(19970220)74:1<64::aid-ijc11>3.0.co;2-i.
[107] M. Tomisawa *et al.*, "Expression pattern of vascular endothelial growth factor isoform is closely correlated with tumour stage and vascularisation in renal cell carcinoma," *Eur. J. Cancer Oxf. Engl. 1990*, vol. 35, no. 1, pp. 133–137, Jan. 1999, doi: 10.1016/s0959-8049(98)00278-0.
[108] H. M. Sowter, A. N. Corps, A. L. Evans, D. E. Clark, D. S. Charnock-Jones, and S. K. Smith, "Expression and localization of the vascular endothelial growth factor family in ovarian epithelial tumors," *Lab. Investig. J. Tech. Methods Pathol.*, vol. 77, no. 6, pp. 607–614, Dec. 1997.
[109] H. Yoshiji, D. E. Gomez, M. Shibuya, and U. P. Thorgeirsson, "Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer," *Cancer Res.*, vol. 56, no. 9, pp. 2013–2016, May 1996.
[110] B. J. Nickoloff, R. S. Mitra, J. Varani, V. M. Dixit, and P. J. Polverini, "Aberrant production of interleukin-8 and thrombospondin-1 by psoriatic keratinocytes mediates angiogenesis," *Am. J. Pathol.*, vol. 144, no. 4, pp. 820–828, Apr. 1994.
[111] D. J. Peacock, M. L. Banquerigo, and E. Brahn, "Angiogenesis inhibition suppresses collagen arthritis," *J. Exp. Med.*, vol. 175, no. 4, pp. 1135–1138, Apr. 1992, doi: 10.1084/jem.175.4.1135.
[112] E. R. O'Brien *et al.*, "Angiogenesis in human coronary atherosclerotic plaques," *Am. J. Pathol.*, vol. 145, no. 4, pp. 883–894, Oct. 1994.

[113] M. Fernandez, F. Vizzutti, J. C. Garcia-Pagan, J. Rodes, and J. Bosch, “Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice,” *Gastroenterology*, vol. 126, no. 3, pp. 886–894, Mar. 2004, doi: 10.1053/j.gastro.2003.12.012.
[114] P. Martin, “Wound healing--aiming for perfect skin regeneration,” *Science*, vol. 276, no. 5309, pp. 75–81, Apr. 1997, doi: 10.1126/science.276.5309.75.
[115] H. Nagase, “Activation mechanisms of matrix metalloproteinases,” *Biol. Chem.*, vol. 378, no. 3–4, pp. 151–160, Apr. 1997.
[116] V. M. Kähäri and U. Saarialho-Kere, “Matrix metalloproteinases and their inhibitors in tumour growth and invasion,” *Ann. Med.*, vol. 31, no. 1, pp. 34–45, Feb. 1999, doi: 10.3109/07853899909019260.
[117] J. Westermarck and V. M. Kähäri, “Regulation of matrix metalloproteinase expression in tumor invasion,” *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.*, vol. 13, no. 8, pp. 781–792, May 1999.
[118] R. G. Rubio and A. P. Adamis, “Ocular Angiogenesis: Vascular Endothelial Growth Factor and Other Factors,” *Dev. Ophthalmol.*, vol. 55, pp. 28–37, 2016, doi: 10.1159/000431129.
[119] W. H. Burgess and T. Maciag, “The heparin-binding (fibroblast) growth factor family of proteins,” *Annu. Rev. Biochem.*, vol. 58, pp. 575–606, 1989, doi: 10.1146/annurev.bi.58.070189.003043.
[120] G. Conn *et al.*, “Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 87, no. 7, pp. 2628–2632, Apr. 1990, doi: 10.1073/pnas.87.7.2628.
[121] H. U. Wang, Z.-F. Chen, and D. J. Anderson, “Molecular Distinction and Angiogenic Interaction between Embryonic Arteries and Veins Revealed by ephrin-B2 and Its Receptor Eph-B4,” *Cell*, vol. 93, no. 5, pp. 741–753, May 1998, doi: 10.1016/S0092-8674(00)81436-1.
[122] H. Seno *et al.*, “Cyclooxygenase 2- and prostaglandin E(2) receptor EP(2)-dependent angiogenesis in Apc(Delta716) mouse intestinal polyps,” *Cancer Res.*, vol. 62, no. 2, pp. 506–511, Jan. 2002.
[123] M. Onimaru *et al.*, “Fibroblast growth factor-2 gene transfer can stimulate hepatocyte growth factor expression irrespective of hypoxia-mediated downregulation in ischemic limbs,” *Circ. Res.*, vol. 91, no. 10, pp. 923–930, Nov. 2002, doi: 10.1161/01.res.0000043281.66969.32.
[124] P. R. Lawler and J. Lawler, “Molecular Basis for the Regulation of Angiogenesis by Thrombospondin-1 and -2,” *Cold Spring Harb. Perspect. Med.*, vol. 2, no. 5, May 2012, doi: 10.1101/cshperspect.a006627.
[125] S. L. Richey and T. E. Hutson, “Angiopoietins and Non–Vascular Endothelial Growth Factor Antiangiogenic Targets in Advanced Renal Cell Carcinoma,” *Cancer J.*, vol. 19, no. 4, p. 307, Aug. 2013, doi: 10.1097/PPO.0b013e31829d5d15.
[126] M. A. Sovak, M. Arsura, G. Zanieski, K. T. Kavanagh, and G. E. Sonenshein, “The inhibitory effects of transforming growth factor beta1 on breast cancer cell proliferation are mediated through regulation of aberrant nuclear factor-kappaB/Rel expression,” *Cell Growth Differ. Mol. Biol. J. Am. Assoc. Cancer Res.*, vol. 10, no. 8, pp. 537–544, Aug. 1999.
[127] M. S. O’Reilly *et al.*, “Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma,” *Cell*, vol. 79, no. 2, pp. 315–328,

Oct. 1994, doi: 10.1016/0092-8674(94)90200-3.
[128] K. Norrby, “Mast cells and angiogenesis,” *APMIS Acta Pathol. Microbiol. Immunol. Scand.*, vol. 110, no. 5, pp. 355–371, May 2002, doi: 10.1034/j.1600-0463.2002.100501.x.
[129] M. S. O’Reilly *et al.*, “Endostatin: an endogenous inhibitor of angiogenesis and tumor growth,” *Cell*, vol. 88, no. 2, pp. 277–285, Jan. 1997, doi: 10.1016/s0092-8674(00)81848-6.
[130] A. Baird, F. Esch, D. Gospodarowicz, and R. Guillemin, “Retina- and eye-derived endothelial cell growth factors: partial molecular characterization and identity with acidic and basic fibroblast growth factors,” *Biochemistry*, vol. 24, no. 27, pp. 7855–7860, Dec. 1985, doi: 10.1021/bi00348a001.
[131] L. P. Aiello *et al.*, “Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders,” *N. Engl. J. Med.*, vol. 331, no. 22, pp. 1480–1487, Dec. 1994, doi: 10.1056/NEJM199412013312203.
[132] N. Kwak, N. Okamoto, J. M. Wood, and P. A. Campochiaro, “VEGF is major stimulator in model of choroidal neovascularization,” *Invest. Ophthalmol. Vis. Sci.*, vol. 41, no. 10, pp. 3158–3164, Sep. 2000.
[133] J.-P. Tong and Y.-F. Yao, “Contribution of VEGF and PEDF to choroidal angiogenesis: a need for balanced expressions,” *Clin. Biochem.*, vol. 39, no. 3, pp. 267–276, Mar. 2006, doi: 10.1016/j.clinbiochem.2005.11.013.
[134] W. Schaper, “Collateral circulation,” *Basic Res. Cardiol.*, vol. 104, no. 1, pp. 5–21, Jan. 2009, doi: 10.1007/s00395-008-0760-x.
[135] O. Iliopoulos, A. P. Levy, C. Jiang, W. G. Kaelin, and M. A. Goldberg, “Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 93, no. 20, pp. 10595–10599, Oct. 1996, doi: 10.1073/pnas.93.20.10595.
[136] M. Houssier *et al.*, “CD36 deficiency leads to choroidal involution via COX2 down-regulation in rodents,” *PLoS Med.*, vol. 5, no. 2, p. e39, Feb. 2008, doi: 10.1371/journal.pmed.0050039.
[137] N. L. Daly and D. J. Craik, “Bioactive cystine knot proteins,” *Curr. Opin. Chem. Biol.*, vol. 15, no. 3, pp. 362–368, Jun. 2011, doi: 10.1016/j.cbpa.2011.02.008.
[138] T. Tammela, B. Enholm, K. Alitalo, and K. Paavonen, “The biology of vascular endothelial growth factors,” *Cardiovasc. Res.*, vol. 65, no. 3, pp. 550–563, Feb. 2005, doi: 10.1016/j.cardiores.2004.12.002.
[139] M. Klagsbrun, S. Takashima, and R. Mamluk, “The role of neuropilin in vascular and tumor biology,” *Adv. Exp. Med. Biol.*, vol. 515, pp. 33–48, 2002, doi: 10.1007/978-1-4615-0119-0_3.
[140] D. R. Senger, S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, and H. F. Dvorak, “Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid,” *Science*, vol. 219, no. 4587, pp. 983–985, Feb. 1983, doi: 10.1126/science.6823562.
[141] C. A. Bootle-Wilbraham, S. Tazzyman, W. D. Thompson, C. M. Stirk, and C. E. Lewis, “Fibrin fragment E stimulates the proliferation, migration and differentiation of human microvascular endothelial cells in vitro,” *Angiogenesis*, vol. 4, no. 4, pp. 269–275, 2001, doi: 10.1023/a:1016076121918.
[142] J. Kroll and J. Waltenberger, “A novel function of VEGF receptor-2 (KDR): rapid release of nitric oxide in response to VEGF-A stimulation in endothelial cells,” *Biochem. Biophys. Res. Commun.*, vol. 265, no. 3, pp. 636–639, Nov. 1999, doi: 10.1006/bbrc.1999.1729.
[143] H. P. Gerber, V. Dixit, and N. Ferrara, “Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells,” *J. Biol.*

Chem., vol. 273, no. 21, pp. 13313–13316, May 1998, doi: 10.1074/jbc.273.21.13313.
[144] H. Takahashi and M. Shibuya, "The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions," *Clin. Sci. Lond. Engl. 1979*, vol. 109, no. 3, pp. 227–241, Sep. 2005, doi: 10.1042/CS20040370.
[145] S. Bhardwaj, H. Roy, T. Heikura, and S. Ylä-Herttuala, "VEGF-A, VEGF-D and VEGF-D(DeltaNDeltaC) induced intimal hyperplasia in carotid arteries," *Eur. J. Clin. Invest.*, vol. 35, no. 11, pp. 669–676, Nov. 2005, doi: 10.1111/j.1365-2362.2005.01555.x.
[146] H.-P. Gerber *et al.*, "VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism," *Nature*, vol. 417, no. 6892, pp. 954–958, Jun. 2002, doi: 10.1038/nature00821.
[147] N. Ferrara, H.-P. Gerber, and J. LeCouter, "The biology of VEGF and its receptors," *Nat. Med.*, vol. 9, no. 6, pp. 669–676, Jun. 2003, doi: 10.1038/nm0603-669.
[148] H. Roy, S. Bhardwaj, and S. Ylä-Herttuala, "Biology of vascular endothelial growth factors," *FEBS Lett.*, vol. 580, no. 12, pp. 2879–2887, May 2006, doi: 10.1016/j.febslet.2006.03.087.
[149] S. Bhardwaj *et al.*, "Angiogenic responses of vascular endothelial growth factors in periadventitial tissue," *Hum. Gene Ther.*, vol. 14, no. 15, pp. 1451–1462, Oct. 2003, doi: 10.1089/104303403769211664.
[150] T. T. Rissanen, J. Rutanen, and S. Ylä-Herttuala, "Gene transfer for therapeutic vascular growth in myocardial and peripheral ischemia," *Adv. Genet.*, vol. 52, pp. 117–164, 2004, doi: 10.1016/S0065-2660(04)52004-7.
[151] T. T. Rissanen *et al.*, "VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses," *Circ. Res.*, vol. 92, no. 10, pp. 1098–1106, May 2003, doi: 10.1161/01.RES.0000073584.46059.E3.
[152] D. R. Senger, S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, and H. F. Dvorak, "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid," *Science*, vol. 219, no. 4587, pp. 983–985, Feb. 1983, doi: 10.1126/science.6823562.
[153] A. Ando, A. Yang, H. Nambu, and P. A. Campochiaro, "Blockade of nitric-oxide synthase reduces choroidal neovascularization," *Mol. Pharmacol.*, vol. 62, no. 3, pp. 539–544, Sep. 2002, doi: 10.1124/mol.62.3.539.
[154] M. Clauss *et al.*, "Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration," *J. Exp. Med.*, vol. 172, no. 6, pp. 1535–1545, Dec. 1990, doi: 10.1084/jem.172.6.1535.
[155] S. Ylä-Herttuala and K. Alitalo, "Gene transfer as a tool to induce therapeutic vascular growth," *Nat. Med.*, vol. 9, no. 6, pp. 694–701, Jun. 2003, doi: 10.1038/nm0603-694.
[156] M. Tjwa, A. Luttun, M. Autiero, and P. Carmeliet, "VEGF and PlGF: two pleiotropic growth factors with distinct roles in development and homeostasis," *Cell Tissue Res.*, vol. 314, no. 1, pp. 5–14, Oct. 2003, doi: 10.1007/s00441-003-0776-3.
[157] H. Roy *et al.*, "Adenovirus-mediated gene transfer of placental growth factor to perivascular tissue induces angiogenesis via upregulation of the expression of endogenous vascular endothelial growth factor-A," *Hum. Gene Ther.*, vol. 16, no. 12, pp. 1422–1428, Dec. 2005, doi: 10.1089/hum.2005.16.1422.
[158] A. Ahmed, C. Dunk, S. Ahmad, and A. Khaliq, "Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen--a review," *Placenta*, vol. 21 Suppl A, pp. S16-24, Apr. 2000, doi: 10.1053/plac.1999.0524.

[159] M. Autiero *et al.*, “Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1,” *Nat. Med.*, vol. 9, no. 7, pp. 936–943, Jul. 2003, doi: 10.1038/nm884.
[160] A. Luttun *et al.*, “Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1,” *Nat. Med.*, vol. 8, no. 8, pp. 831–840, Aug. 2002, doi: 10.1038/nm731.
[161] F. Pipp *et al.*, “VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism,” *Circ. Res.*, vol. 92, no. 4, pp. 378–385, Mar. 2003, doi: 10.1161/01.RES.0000057997.77714.72.
[162] B. Enholm *et al.*, “Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia,” *Oncogene*, vol. 14, no. 20, pp. 2475–2483, May 1997, doi: 10.1038/sj.onc.1201090.
[163] A. D. Nash, M. Baca, C. Wright, and P. D. Scotney, “The biology of vascular endothelial growth factor-B (VEGF-B),” *Pulm. Pharmacol. Ther.*, vol. 19, no. 1, pp. 61–69, 2006, doi: 10.1016/j.pupt.2005.02.007.
[164] D. Bellomo *et al.*, “Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia,” *Circ. Res.*, vol. 86, no. 2, pp. E29-35, Feb. 2000, doi: 10.1161/01.res.86.2.e29.
[165] A. W. Mould *et al.*, “Vegfb gene knockout mice display reduced pathology and synovial angiogenesis in both antigen-induced and collagen-induced models of arthritis,” *Arthritis Rheum.*, vol. 48, no. 9, pp. 2660–2669, Sep. 2003, doi: 10.1002/art.11232.
[166] B. Enholm *et al.*, “Adenoviral expression of vascular endothelial growth factor-C induces lymphangiogenesis in the skin,” *Circ. Res.*, vol. 88, no. 6, pp. 623–629, Mar. 2001, doi: 10.1161/01.res.88.6.623.
[167] M. J. Karkkainen *et al.*, “Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins,” *Nat. Immunol.*, vol. 5, no. 1, pp. 74–80, Jan. 2004, doi: 10.1038/ni1013.
[168] M. Jeltsch *et al.*, “Hyperplasia of lymphatic vessels in VEGF-C transgenic mice,” *Science*, vol. 276, no. 5317, pp. 1423–1425, May 1997, doi: 10.1126/science.276.5317.1423.
[169] D. O. Bates and S. J. Harper, “Regulation of vascular permeability by vascular endothelial growth factors,” *Vascul. Pharmacol.*, vol. 39, no. 4–5, pp. 225–237, Nov. 2002, doi: 10.1016/s1537-1891(03)00011-9.
[170] S. A. Stacker *et al.*, “VEGF-D promotes the metastatic spread of tumor cells via the lymphatics,” *Nat. Med.*, vol. 7, no. 2, pp. 186–191, Feb. 2001, doi: 10.1038/84635.
[171] M. G. Achen *et al.*, “Localization of vascular endothelial growth factor-D in malignant melanoma suggests a role in tumour angiogenesis,” *J. Pathol.*, vol. 193, no. 2, pp. 147–154, Feb. 2001, doi: 10.1002/1096-9896(2000)9999:9999<::AID-PATH757>3.0.CO;2-G.
[172] M. E. Baldwin *et al.*, “Vascular endothelial growth factor D is dispensable for development of the lymphatic system,” *Mol. Cell. Biol.*, vol. 25, no. 6, pp. 2441–2449, Mar. 2005, doi: 10.1128/MCB.25.6.2441-2449.2005.
[173] D. J. Lyttle, K. M. Fraser, S. B. Fleming, A. A. Mercer, and A. J. Robinson, “Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus,” *J. Virol.*, vol. 68, no. 1, pp. 84–92, Jan. 1994.
[174] M. Shibuya, “Vascular endothelial growth factor receptor-2: its unique signaling and specific ligand, VEGF-E,” *Cancer Sci.*, vol. 94, no. 9, pp. 751–756, Sep. 2003, doi: 10.1111/j.1349-7006.2003.tb01514.x.

[175] L. M. Wise *et al.*, “Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, no. 6, pp. 3071–3076, Mar. 1999, doi: 10.1073/pnas.96.6.3071.
[176] Y. Yamazaki, K. Takani, H. Atoda, and T. Morita, “Snake Venom Vascular Endothelial Growth Factors (VEGFs) Exhibit Potent Activity through Their Specific Recognition of KDR (VEGF Receptor 2),” *J. Biol. Chem.*, vol. 278, no. 52, pp. 51985–51988, Dec. 2003, doi: 10.1074/jbc.C300454200.
[177] K. Suto, Y. Yamazaki, T. Morita, and H. Mizuno, “Crystal structures of novel vascular endothelial growth factors (VEGF) from snake venoms: insight into selective VEGF binding to kinase insert domain-containing receptor but not to fms-like tyrosine kinase-1,” *J. Biol. Chem.*, vol. 280, no. 3, pp. 2126–2131, Jan. 2005, doi: 10.1074/jbc.M411395200.
[178] Y. Yamazaki, Y. Tokunaga, K. Takani, and T. Morita, “Identification of the heparin-binding region of snake venom vascular endothelial growth factor (VEGF-F) and its blocking of VEGF-A165,” *Biochemistry*, vol. 44, no. 24, pp. 8858–8864, Jun. 2005, doi: 10.1021/bi050197d.
[179] K. Yaniv, S. Isogai, D. Castranova, L. Dye, J. Hitomi, and B. M. Weinstein, “Live imaging of lymphatic development in the zebrafish,” *Nat. Med.*, vol. 12, no. 6, pp. 711–716, Jun. 2006, doi: 10.1038/nm1427.
[180] P. Duchek, K. Somogyi, G. Jékely, S. Beccari, and P. Rørth, “Guidance of cell migration by the Drosophila PDGF/VEGF receptor,” *Cell*, vol. 107, no. 1, pp. 17–26, Oct. 2001, doi: 10.1016/s0092-8674(01)00502-5.
[181] M. Shibuya, “Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases,” *J. Biochem. (Tokyo)*, vol. 153, no. 1, pp. 13–19, Jan. 2013, doi: 10.1093/jb/mvs136.
[182] T. Takahashi and M. Shibuya, “The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-gamma pathway and partially induces mitotic signals in NIH3T3 fibroblasts,” *Oncogene*, vol. 14, no. 17, pp. 2079–2089, May 1997, doi: 10.1038/sj.onc.1201047.
[183] M. Shibuya and L. Claesson-Welsh, “Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis,” *Exp. Cell Res.*, vol. 312, no. 5, pp. 549–560, Mar. 2006, doi: 10.1016/j.yexcr.2005.11.012.
[184] I. Zachary and G. Gliki, “Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family,” *Cardiovasc. Res.*, vol. 49, no. 3, pp. 568–581, Feb. 2001, doi: 10.1016/s0008-6363(00)00268-6.
[185] H. Hutchings, N. Ortega, and J. Plouët, “Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation,” *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.*, vol. 17, no. 11, pp. 1520–1522, Aug. 2003, doi: 10.1096/fj.02-0691fje.
[186] G. Elvert *et al.*, “Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1),” *J. Biol. Chem.*, vol. 278, no. 9, pp. 7520–7530, Feb. 2003, doi: 10.1074/jbc.M211298200.
[187] A. Kappel, T. M. Schlaeger, I. Flamme, S. H. Orkin, W. Risau, and G. Breier, “Role of SCL/Tal-1, GATA, and ets transcription factor binding sites for the regulation of flk-1 expression during murine vascular development,” *Blood*, vol. 96, no. 9, pp. 3078–3085, Nov. 2000.

[188] A. Kappel, V. Rönicke, A. Damert, I. Flamme, W. Risau, and G. Breier, "Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice," *Blood*, vol. 93, no. 12, pp. 4284–4292, Jun. 1999.
[189] C. de Vries, J. A. Escobedo, H. Ueno, K. Houck, N. Ferrara, and L. T. Williams, "The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor," *Science*, vol. 255, no. 5047, pp. 989–991, Feb. 1992, doi: 10.1126/science.1312256.
[190] A. Luttun *et al.*, "Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1," *Nat. Med.*, vol. 8, no. 8, pp. 831–840, Aug. 2002, doi: 10.1038/nm731.
[191] K. Aase *et al.*, "Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect," *Circulation*, vol. 104, no. 3, pp. 358–364, Jul. 2001, doi: 10.1161/01.cir.104.3.358.
[192] A. Sawano, T. Takahashi, S. Yamaguchi, M. Aonuma, and M. Shibuya, "Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor," *Cell Growth Differ. Mol. Biol. J. Am. Assoc. Cancer Res.*, vol. 7, no. 2, pp. 213–221, Feb. 1996.
[193] S. E. Maynard *et al.*, "Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia," *J. Clin. Invest.*, vol. 111, no. 5, pp. 649–658, Mar. 2003, doi: 10.1172/JCI17189.
[194] K. Koga *et al.*, "Elevated serum soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in women with preeclampsia," *J. Clin. Endocrinol. Metab.*, vol. 88, no. 5, pp. 2348–2351, May 2003, doi: 10.1210/jc.2002-021942.
[195] R. J. Levine *et al.*, "Circulating angiogenic factors and the risk of preeclampsia," *N. Engl. J. Med.*, vol. 350, no. 7, pp. 672–683, Feb. 2004, doi: 10.1056/NEJMoa031884.
[196] S. Zhang *et al.*, "Podocyte-specific VEGF down-regulation and pathophysiological development," *IUBMB Life*, vol. 62, no. 9, pp. 677–683, Sep. 2010, doi: 10.1002/iub.368.
[197] D. Veron *et al.*, "Acute podocyte vascular endothelial growth factor (VEGF-A) knockdown disrupts alphaVbeta3 integrin signaling in the glomerulus," *PloS One*, vol. 7, no. 7, p. e40589, 2012, doi: 10.1371/journal.pone.0040589.
[198] K. Huang, C. Andersson, G. M. Roomans, N. Ito, and L. Claesson-Welsh, "Signaling properties of VEGF receptor-1 and -2 homo- and heterodimers," *Int. J. Biochem. Cell Biol.*, vol. 33, no. 4, pp. 315–324, Apr. 2001, doi: 10.1016/s1357-2725(01)00019-x.
[199] S. Hiratsuka *et al.*, "MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis," *Cancer Cell*, vol. 2, no. 4, pp. 289–300, Oct. 2002, doi: 10.1016/s1535-6108(02)00153-8.
[200] R. N. Kaplan *et al.*, "VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche," *Nature*, vol. 438, no. 7069, pp. 820–827, Dec. 2005, doi: 10.1038/nature04186.
[201] M. Murakami *et al.*, "Signaling of vascular endothelial growth factor receptor-1 tyrosine kinase promotes rheumatoid arthritis through activation of monocytes/macrophages," *Blood*, vol. 108, no. 6, pp. 1849–1856, Sep. 2006, doi: 10.1182/blood-2006-04-016030.
[202] M. Muramatsu, S. Yamamoto, T. Osawa, and M. Shibuya, "Vascular endothelial growth factor receptor-1 signaling promotes mobilization of macrophage lineage cells from bone marrow and stimulates solid tumor growth," *Cancer Res.*, vol. 70, no. 20, pp. 8211–8221, Oct. 2010, doi: 10.1158/0008-5472.CAN-10-0202.

[203] M. Kerber *et al.*, “Flt-1 signaling in macrophages promotes glioma growth in vivo,” *Cancer Res.*, vol. 68, no. 18, pp. 7342–7351, Sep. 2008, doi: 10.1158/0008-5472.CAN-07-6241.
[204] A. Kaipainen *et al.*, “Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, no. 8, pp. 3566–3570, Apr. 1995, doi: 10.1073/pnas.92.8.3566.
[205] M. Simons, E. Gordon, and L. Claesson-Welsh, “Mechanisms and regulation of endothelial VEGF receptor signalling,” *Nat. Rev. Mol. Cell Biol.*, vol. 17, no. 10, pp. 611–625, 2016, doi: 10.1038/nrm.2016.87.
[206] Y. Xu *et al.*, “Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3,” *J. Cell Biol.*, vol. 188, no. 1, pp. 115–130, Jan. 2010, doi: 10.1083/jcb.200903137.
[207] W. Zheng *et al.*, “Notch restricts lymphatic vessel sprouting induced by vascular endothelial growth factor,” *Blood*, vol. 118, no. 4, pp. 1154–1162, Jul. 2011, doi: 10.1182/blood-2010-11-317800.
[208] R. O. Pritchard-Jones *et al.*, “Expression of VEGF(xxx)b, the inhibitory isoforms of VEGF, in malignant melanoma,” *Br. J. Cancer*, vol. 97, no. 2, pp. 223–230, Jul. 2007, doi: 10.1038/sj.bjc.6603839.
[209] I. Appelmann, R. Liersch, T. Kessler, R. M. Mesters, and W. E. Berdel, “Angiogenesis Inhibition in Cancer Therapy,” in *Angiogenesis Inhibition*, Springer, Berlin, Heidelberg, 2010, pp. 51–81.
[210] J. Testori *et al.*, “The VEGF-regulated transcription factor HLX controls the expression of guidance cues and negatively regulates sprouting of endothelial cells,” *Blood*, vol. 117, no. 9, pp. 2735–2744, Mar. 2011, doi: 10.1182/blood-2010-07-293209.
[211] N. Villa, L. Walker, C. E. Lindsell, J. Gasson, M. L. Iruela-Arispe, and G. Weinmaster, “Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels,” *Mech. Dev.*, vol. 108, no. 1, pp. 161–164, Oct. 2001, doi: 10.1016/S0925-4773(01)00469-5.
[212] S. Artavanis-Tsakonas, M. D. Rand, and R. J. Lake, “Notch Signaling: Cell Fate Control and Signal Integration in Development,” *Science*, vol. 284, no. 5415, pp. 770–776, Apr. 1999, doi: 10.1126/science.284.5415.770.
[213] R. C. A. Sainson *et al.*, “Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis,” *FASEB J.*, vol. 19, no. 8, pp. 1027–1029, Mar. 2005, doi: 10.1096/fj.04-3172fje.
[214] X. Kang *et al.*, “Nox2 contributes to the arterial endothelial specification of mouse induced pluripotent stem cells by upregulating Notch signaling,” *Sci. Rep.*, vol. 6, Sep. 2016, doi: 10.1038/srep33737.
[215] T. Kobayashi and R. Kageyama, “Hes1 regulates embryonic stem cell differentiation by suppressing Notch signaling,” *Genes Cells*, vol. 15, no. 7, pp. 689–698, Jul. 2010, doi: 10.1111/j.1365-2443.2010.01413.x.
[216] H. Hirata *et al.*, “Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop,” *Science*, vol. 298, no. 5594, pp. 840–843, Oct. 2002, doi: 10.1126/science.1074560.
[217] Y. Masamizu *et al.*, “Real-time imaging of the somite segmentation clock: Revelation of unstable oscillators in the individual presomitic mesoderm cells,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 5, pp. 1313–1318, Jan. 2006, doi: 10.1073/pnas.0508658103.
[218] H. Shimojo, T. Ohtsuka, and R. Kageyama, “Oscillations in notch signaling regulate

maintenance of neural progenitors," *Neuron*, vol. 58, no. 1, pp. 52–64, Apr. 2008, doi: 10.1016/j.neuron.2008.02.014.
[219] L. Jakobsson, K. Bentley, and H. Gerhardt, "VEGFRs and Notch: a dynamic collaboration in vascular patterning," *Biochem. Soc. Trans.*, vol. 37, no. Pt 6, pp. 1233–1236, Dec. 2009, doi: 10.1042/BST0371233.
[220] I. M. Moya *et al.*, "Stalk Cell Phenotype Depends on Integration of Notch and Smad1/5 Signaling Cascades," *Dev. Cell*, vol. 22, no. 3, pp. 501–514, Mar. 2012, doi: 10.1016/j.devcel.2012.01.007.
[221] Z. Zebedee and E. Hara, "Id proteins in cell cycle control and cellular senescence," *Oncogene*, vol. 20, no. 58, pp. 8317–8325, Dec. 2001, doi: 10.1038/sj.onc.1205092.
[222] J. D. Norton and G. T. Atherton, "Coupling of Cell Growth Control and Apoptosis Functions of Id Proteins," *Mol. Cell. Biol.*, vol. 18, no. 4, pp. 2371–2381, Apr. 1998.
[223] S. Yoshiura, T. Ohtsuka, Y. Takenaka, H. Nagahara, K. Yoshikawa, and R. Kageyama, "Ultradian oscillations of Stat, Smad, and Hes1 expression in response to serum," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 27, pp. 11292–11297, Jul. 2007, doi: 10.1073/pnas.0701837104.
[224] H.-T. Cheng *et al.*, "Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron," *Dev. Camb. Engl.*, vol. 134, no. 4, pp. 801–811, Feb. 2007, doi: 10.1242/dev.02773.
[225] L. T. Krebs *et al.*, "Notch signaling regulates left-right asymmetry determination by inducing Nodal expression," *Genes Dev.*, vol. 17, no. 10, pp. 1207–1212, May 2003, doi: 10.1101/gad.1084703.
[226] J. Cordle *et al.*, "A conserved face of the Jagged/Serrate DSL domain is involved in Notch trans-activation and cis-inhibition," *Nat. Struct. Mol. Biol.*, vol. 15, no. 8, pp. 849–857, Aug. 2008, doi: 10.1038/nsmb.1457.
[227] R. Kopan and Ma. X. G. Ilagan, "The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism," *Cell*, vol. 137, no. 2, pp. 216–233, Apr. 2009, doi: 10.1016/j.cell.2009.03.045.
[228] H. Komatsu *et al.*, "OSM-11 facilitates LIN-12 Notch signaling during Caenorhabditis elegans vulval development," *PLoS Biol.*, vol. 6, no. 8, p. e196, Aug. 2008, doi: 10.1371/journal.pbio.0060196.
[229] N. Haines and K. D. Irvine, "Glycosylation regulates Notch signalling," *Nat. Rev. Mol. Cell Biol.*, vol. 4, no. 10, pp. 786–797, Oct. 2003, doi: 10.1038/nrm1228.
[230] R. Rampal, K. B. Luther, and R. S. Haltiwanger, "Notch signaling in normal and disease States: possible therapies related to glycosylation," *Curr. Mol. Med.*, vol. 7, no. 4, pp. 427–445, Jun. 2007, doi: 10.2174/156652407780831593.
[231] P. Stanley, "Regulation of Notch signaling by glycosylation," *Curr. Opin. Struct. Biol.*, vol. 17, no. 5, pp. 530–535, Oct. 2007, doi: 10.1016/j.sbi.2007.09.007.
[232] I. Visan, J. S. Yuan, J. B. Tan, K. Cretegny, and C. J. Guidos, "Regulation of intrathymic T-cell development by Lunatic Fringe- Notch1 interactions," *Immunol. Rev.*, vol. 209, pp. 76–94, Feb. 2006, doi: 10.1111/j.0105-2896.2006.00360.x.
[233] N. Vodovar and F. Schweisguth, "Functions of O-fucosyltransferase in Notch trafficking and signaling: towards the end of a controversy?," *J. Biol.*, vol. 7, no. 2, p. 7, 2008, doi: 10.1186/jbiol68.
[234] J. J. Peschon *et al.*, "An essential role for ectodomain shedding in mammalian development," *Science*, vol. 282, no. 5392, pp. 1281–1284, Nov. 1998, doi:

10.1126/science.282.5392.1281.
[235] C. Brou *et al.*, “A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE,” *Mol. Cell*, vol. 5, no. 2, pp. 207–216, Feb. 2000, doi: 10.1016/s1097-2765(00)80417-7.
[236] J. S. Mumm *et al.*, “A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1,” *Mol. Cell*, vol. 5, no. 2, pp. 197–206, Feb. 2000, doi: 10.1016/s1097-2765(00)80416-5.
[237] M. J. Malecki *et al.*, “Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes,” *Mol. Cell. Biol.*, vol. 26, no. 12, pp. 4642–4651, Jun. 2006, doi: 10.1128/MCB.01655-05.
[238] R. Kopan, E. H. Schroeter, H. Weintraub, and J. S. Nye, “Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 93, no. 4, pp. 1683–1688, Feb. 1996.
[239] S. Jarriault, C. Brou, F. Logeat, E. H. Schroeter, R. Kopan, and A. Israel, “Signalling downstream of activated mammalian Notch,” *Nature*, vol. 377, no. 6547, pp. 355–358, Sep. 1995, doi: 10.1038/377355a0.
[240] T. Lieber, S. Kidd, E. Alcamo, V. Corbin, and M. W. Young, “Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei,” *Genes Dev.*, vol. 7, no. 10, pp. 1949–1965, Oct. 1993.
[241] F. Logeat *et al.*, “The Notch1 receptor is cleaved constitutively by a furin-like convertase,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 95, no. 14, pp. 8108–8112, Jul. 1998.
[242] W. R. Gordon *et al.*, “Effects of S1 Cleavage on the Structure, Surface Export, and Signaling Activity of Human Notch1 and Notch2,” *PLOS ONE*, vol. 4, no. 8, p. e6613, Aug. 2009, doi: 10.1371/journal.pone.0006613.
[243] E. H. Schroeter, J. A. Kisslinger, and R. Kopan, “Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain,” *Nature*, vol. 393, no. 6683, pp. 382–386, May 1998, doi: 10.1038/30756.
[244] M. D. Rand *et al.*, “Calcium depletion dissociates and activates heterodimeric notch receptors,” *Mol. Cell. Biol.*, vol. 20, no. 5, pp. 1825–1835, Mar. 2000, doi: 10.1128/mcb.20.5.1825-1835.2000.
[245] O. Y. Lubman, M. X. G. Ilagan, R. Kopan, and D. Barrick, “Quantitative dissection of the Notch:CSL interaction: insights into the Notch-mediated transcriptional switch,” *J. Mol. Biol.*, vol. 365, no. 3, pp. 577–589, Jan. 2007, doi: 10.1016/j.jmb.2006.09.071.
[246] J. Lee, J. M. Basak, S. Demehri, and R. Kopan, “Bi-compartmental communication contributes to the opposite proliferative behavior of Notch1-deficient hair follicle and epidermal keratinocytes,” *Dev. Camb. Engl.*, vol. 134, no. 15, pp. 2795–2806, Aug. 2007, doi: 10.1242/dev.02868.
[247] C. J. Fryer, J. B. White, and K. A. Jones, “Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover,” *Mol. Cell*, vol. 16, no. 4, pp. 509–520, Nov. 2004, doi: 10.1016/j.molcel.2004.10.014.
[248] F. Itoh *et al.*, “Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells,” *EMBO J.*, vol. 23, no. 3, pp. 541–551, Feb. 2004, doi: 10.1038/sj.emboj.7600065.
[249] G. Bai *et al.*, “Id sustains Hes1 expression to inhibit precocious neurogenesis by releasing negative autoregulation of Hes1,” *Dev. Cell*, vol. 13, no. 2, pp. 283–297, Aug. 2007, doi: 10.1016/j.devcel.2007.05.014.

[250] B. Larrivée *et al.*, "ALK1 Signaling Inhibits Angiogenesis by Cooperating with the Notch Pathway," *Dev. Cell*, vol. 22, no. 3, pp. 489–500, Mar. 2012, doi: 10.1016/j.devcel.2012.02.005.
[251] T. Kobayashi, H. Mizuno, I. Imayoshi, C. Furusawa, K. Shirahige, and R. Kageyama, "The cyclic gene Hes1 contributes to diverse differentiation responses of embryonic stem cells," *Genes Dev.*, vol. 23, no. 16, pp. 1870–1875, Aug. 2009, doi: 10.1101/gad.1823109.
[252] A. M. Henderson, S. J. Wang, A. C. Taylor, M. Aitkenhead, and C. C. Hughes, "The basic helix-loop-helix transcription factor HESR1 regulates endothelial cell tube formation," *J. Biol. Chem.*, vol. 276, no. 9, pp. 6169–6176, Mar. 2001, doi: 10.1074/jbc.M008506200.
[253] T. Kobayashi and R. Kageyama, "Hes1 oscillation: making variable choices for stem cell differentiation," *Cell Cycle Georget. Tex*, vol. 9, no. 2, pp. 207–208, Jan. 2010, doi: 10.4161/cc.9.2.10478.
[254] R. del Toro *et al.*, "Identification and functional analysis of endothelial tip cell-enriched genes," *Blood*, vol. 116, no. 19, pp. 4025–4033, Nov. 2010, doi: 10.1182/blood-2010-02-270819.
[255] J. J. Mack and M. L. Iruela-Arispe, "NOTCH regulation of the endothelial cell phenotype," *Curr. Opin. Hematol.*, vol. 25, no. 3, pp. 212–218, May 2018, doi: 10.1097/MOH.0000000000000425.
[256] J. J. Wilson and R. A. Kovall, "Crystal structure of the CSL-Notch-Mastermind ternary complex bound to DNA," *Cell*, vol. 124, no. 5, pp. 985–996, Mar. 2006, doi: 10.1016/j.cell.2006.01.035.
[257] K. Jin *et al.*, "Acetylation of Mastermind-like 1 by p300 Drives the Recruitment of NACK to Initiate Notch-Dependent Transcription," *Cancer Res.*, vol. 77, no. 16, pp. 4228–4237, 15 2017, doi: 10.1158/0008-5472.CAN-16-3156.
[258] S. Weijzen *et al.*, "The Notch ligand Jagged-1 is able to induce maturation of monocyte-derived human dendritic cells," *J. Immunol. Baltim. Md 1950*, vol. 169, no. 8, pp. 4273–4278, Oct. 2002, doi: 10.4049/jimmunol.169.8.4273.
[259] O. Korchynskyi and P. ten Dijke, "Identification and Functional Characterization of Distinct Critically Important Bone Morphogenetic Protein-specific Response Elements in the Id1 Promoter," *J. Biol. Chem.*, vol. 277, no. 7, pp. 4883–4891, Feb. 2002, doi: 10.1074/jbc.M111023200.
[260] X. Guo and X.-F. Wang, "Signaling cross-talk between TGF-beta/BMP and other pathways," *Cell Res.*, vol. 19, no. 1, pp. 71–88, Jan. 2009, doi: 10.1038/cr.2008.302.
[261] T. Katagiri and T. Watabe, "Bone Morphogenetic Proteins," *Cold Spring Harb. Perspect. Biol.*, vol. 8, no. 6, Jun. 2016, doi: 10.1101/cshperspect.a021899.
[262] M. Morikawa, R. Derynck, and K. Miyazono, "TGF-β and the TGF-β Family: Context-Dependent Roles in Cell and Tissue Physiology," *Cold Spring Harb. Perspect. Biol.*, vol. 8, no. 5, May 2016, doi: 10.1101/cshperspect.a021873.
[263] K. N. Retting, B. Song, B. S. Yoon, and K. M. Lyons, "BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation," *Dev. Camb. Engl.*, vol. 136, no. 7, pp. 1093–1104, Apr. 2009, doi: 10.1242/dev.029926.
[264] N. Ni and Q. Li, "TGFβ superfamily signaling and uterine decidualization," *Reprod. Biol. Endocrinol. RBE*, vol. 15, Oct. 2017, doi: 10.1186/s12958-017-0303-0.
[265] D. P. Brazil, R. H. Church, S. Surae, C. Godson, and F. Martin, "BMP signalling: agony and antagony in the family," *Trends Cell Biol.*, vol. 25, no. 5, pp. 249–264, May 2015, doi: 10.1016/j.tcb.2014.12.004.

[266] J. E. de Larco and G. J. Todaro, “Growth factors from murine sarcoma virus-transformed cells,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 75, no. 8, pp. 4001–4005, Aug. 1978.
[267] M. R. Urist, “Bone: formation by autoinduction. 1965,” *Clin. Orthop.*, no. 395, pp. 4–10, Feb. 2002, doi: 10.1097/00003086-200202000-00002.
[268] P. B. Yu *et al.*, “BMP type I receptor inhibition reduces heterotopic [corrected] ossification,” *Nat. Med.*, vol. 14, no. 12, pp. 1363–1369, Dec. 2008, doi: 10.1038/nm.1888.
[269] H. Shiratori and H. Hamada, “TGFβ signaling in establishing left–right asymmetry,” *Semin. Cell Dev. Biol.*, vol. 32, pp. 80–84, Aug. 2014, doi: 10.1016/j.semcdb.2014.03.029.
[270] A. von Bubnoff and K. W. Cho, “Intracellular BMP signaling regulation in vertebrates: pathway or network?,” *Dev. Biol.*, vol. 239, no. 1, pp. 1–14, Nov. 2001, doi: 10.1006/dbio.2001.0388.
[271] Brown et al., “Crystal Structure of BMP-9 and Functional Interactions with Pro-region and Receptors,” Accessed: Jun. 13, 2018. [Online]. Available: http://www.jbc.org/content/280/26/25111.long.
[272] S. I. Cunha *et al.*, “Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis,” *J. Exp. Med.*, vol. 207, no. 1, pp. 85–100, Jan. 2010, doi: 10.1084/jem.20091309.
[273] S. O. Park *et al.*, “ALK5- and TGFBR2-independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2,” *Blood*, vol. 111, no. 2, pp. 633–642, Jan. 2008, doi: 10.1182/blood-2007-08-107359.
[274] K. Miyazono, Y. Kamiya, and M. Morikawa, “Bone morphogenetic protein receptors and signal transduction,” *J. Biochem. (Tokyo)*, vol. 147, no. 1, pp. 35–51, Jan. 2010, doi: 10.1093/jb/mvp148.
[275] J. L. Wrana, L. Attisano, R. Wieser, F. Ventura, and J. Massagué, “Mechanism of activation of the TGF-β receptor,” *Nature*, vol. 370, no. 6488, pp. 341–347, Aug. 1994, doi: 10.1038/370341a0.
[276] J. Massagué, “TGF-β Signal Transduction,” *Annu. Rev. Biochem.*, vol. 67, no. 1, pp. 753–791, Jun. 1998, doi: 10.1146/annurev.biochem.67.1.753.
[277] L. David, J.-J. Feige, and S. Bailly, “Emerging role of bone morphogenetic proteins in angiogenesis,” *Cytokine Growth Factor Rev.*, vol. 20, no. 3, pp. 203–212, Jun. 2009, doi: 10.1016/j.cytogfr.2009.05.001.
[278] S. Mazerbourg and A. J. W. Hsueh, “Genomic analyses facilitate identification of receptors and signalling pathways for growth differentiation factor 9 and related orphan bone morphogenetic protein/growth differentiation factor ligands,” *Hum. Reprod. Update*, vol. 12, no. 4, pp. 373–383, Aug. 2006, doi: 10.1093/humupd/dml014.
[279] P. D. Upton, R. J. Davies, R. C. Trembath, and N. W. Morrell, “Bone morphogenetic protein (BMP) and activin type II receptors balance BMP9 signals mediated by activin receptor-like kinase-1 in human pulmonary artery endothelial cells,” *J. Biol. Chem.*, vol. 284, no. 23, pp. 15794–15804, Jun. 2009, doi: 10.1074/jbc.M109.002881.
[280] D. Yadin, P. Knaus, and T. D. Mueller, “Structural insights into BMP receptors: Specificity, activation and inhibition,” *Cytokine Growth Factor Rev.*, vol. 27, pp. 13–34, Feb. 2016, doi: 10.1016/j.cytogfr.2015.11.005.
[281] R. I. Koleva *et al.*, “Endoglin Structure and Function DETERMINANTS OF ENDOGLIN PHOSPHORYLATION BY TRANSFORMING GROWTH FACTOR-β RECEPTORS,” *J. Biol. Chem.*, vol. 281, no. 35, pp. 25110–25123, Sep. 2006, doi: 10.1074/jbc.M601288200.

[282] N. P. Barbara, J. L. Wrana, and M. Letarte, “Endoglin Is an Accessory Protein That Interacts with the Signaling Receptor Complex of Multiple Members of the Transforming Growth Factor-β Superfamily,” *J. Biol. Chem.*, vol. 274, no. 2, pp. 584–594, Jan. 1999, doi: 10.1074/jbc.274.2.584.
[283] S. Cheifetz *et al.*, “Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells.,” *J. Biol. Chem.*, vol. 267, no. 27, pp. 19027–19030, Sep. 1992.
[284] P. Lastres, J. Martín-Perez, C. Langa, and C. Bernabéu, “Phosphorylation of the human-transforming-growth-factor-beta-binding protein endoglin.,” *Biochem. J.*, vol. 301, no. Pt 3, pp. 765–768, Aug. 1994.
[285] H. Yamashita, H. Ichijo, S. Grimsby, A. Morén, P. ten Dijke, and K. Miyazono, “Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor-beta.,” *J. Biol. Chem.*, vol. 269, no. 3, pp. 1995–2001, Jan. 1994.
[286] C. B. Schmidt-Weber, M. Letarte, S. Kunzmann, B. Rückert, C. Bernabéu, and K. Blaser, “TGF-{beta} signaling of human T cells is modulated by the ancillary TGF-{beta} receptor endoglin,” *Int. Immunol.*, vol. 17, no. 7, pp. 921–930, Jul. 2005, doi: 10.1093/intimm/dxh272.
[287] E. Corradini, J. L. Babitt, and H. Y. Lin, “The RGM/DRAGON family of BMP co-receptors,” *Cytokine Growth Factor Rev.*, vol. 20, no. 5–6, pp. 389–398, Dec. 2009, doi: 10.1016/j.cytogfr.2009.10.008.
[288] X. Pi *et al.*, “LRP1-dependent endocytic mechanism governs the signaling output of the bmp system in endothelial cells and in angiogenesis,” *Circ. Res.*, vol. 111, no. 5, pp. 564–574, Aug. 2012, doi: 10.1161/CIRCRESAHA.112.274597.
[289] C. Yeo and M. Whitman, “Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms,” *Mol. Cell*, vol. 7, no. 5, pp. 949–957, May 2001, doi: 10.1016/s1097-2765(01)00249-0.
[290] M. Huse, Y. G. Chen, J. Massagué, and J. Kuriyan, “Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12,” *Cell*, vol. 96, no. 3, pp. 425–436, Feb. 1999.
[291] R. Derynck and X. H. Feng, “TGF-beta receptor signaling,” *Biochim. Biophys. Acta*, vol. 1333, no. 2, pp. F105-150, Oct. 1997.
[292] P. Makkar, R. P. R. Metpally, S. Sangadala, and B. V. B. Reddy, “Modeling and analysis of MH1 domain of Smads and their interaction with promoter DNA sequence motif,” *J. Mol. Graph. Model.*, vol. 27, no. 7, pp. 803–812, Apr. 2009, doi: 10.1016/j.jmgm.2008.12.003.
[293] J. Massagué and D. Wotton, “Transcriptional control by the TGF-β/Smad signaling system,” *EMBO J.*, vol. 19, no. 8, pp. 1745–1754, Apr. 2000, doi: 10.1093/emboj/19.8.1745.
[294] R. Derynck, Y. Zhang, and X.-H. Feng, “Transcriptional Activators of TGF-β Responses: Smads,” *Cell*, vol. 95, no. 6, pp. 737–740, Dec. 1998, doi: 10.1016/S0092-8674(00)81696-7.
[295] L. Attisano and J. L. Wrana, “Smads as transcriptional co-modulators,” *Curr. Opin. Cell Biol.*, vol. 12, no. 2, pp. 235–243, Apr. 2000, doi: 10.1016/S0955-0674(99)00081-2.
[296] Y. Shi, Y.-F. Wang, L. Jayaraman, H. Yang, J. Massagué, and N. P. Pavletich, “Crystal Structure of a Smad MH1 Domain Bound to DNA: Insights on DNA Binding in TGF-β Signaling,” *Cell*, vol. 94, no. 5, pp. 585–594, Sep. 1998, doi: 10.1016/S0092-8674(00)81600-1.
[297] L. Zawel *et al.*, “Human Smad3 and Smad4 Are Sequence-Specific Transcription Activators,” *Mol. Cell*, vol. 1, no. 4, pp. 611–617, Mar. 1998, doi: 10.1016/S1097-

2765(00)80061-1.
[298] S. Dennler, S. Itoh, D. Vivien, P. ten Dijke, S. Huet, and J.-M. Gauthier, “Direct binding of Smad3 and Smad4 to critical TGFβ-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene,” *EMBO J.*, vol. 17, no. 11, pp. 3091–3100, Jun. 1998, doi: 10.1093/emboj/17.11.3091.
[299] A. Ishisaki *et al.*, “Differential inhibition of Smad6 and Smad7 on bone morphogenetic protein- and activin-mediated growth arrest and apoptosis in B cells,” *J. Biol. Chem.*, vol. 274, no. 19, pp. 13637–13642, May 1999, doi: 10.1074/jbc.274.19.13637.
[300] M. Morikawa *et al.*, “ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif,” *Nucleic Acids Res.*, vol. 39, no. 20, pp. 8712–8727, Nov. 2011, doi: 10.1093/nar/gkr572.
[301] L. David, C. Mallet, B. Vailhé, S. Lamouille, J.-J. Feige, and S. Bailly, “Activin receptor-like kinase 1 inhibits human microvascular endothelial cell migration: potential roles for JNK and ERK,” *J. Cell. Physiol.*, vol. 213, no. 2, pp. 484–489, Nov. 2007, doi: 10.1002/jcp.21126.
[302] J. Guicheux, J. Lemonnier, C. Ghayor, A. Suzuki, G. Palmer, and J. Caverzasio, “Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation,” *J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res.*, vol. 18, no. 11, pp. 2060–2068, Nov. 2003, doi: 10.1359/jbmr.2003.18.11.2060.
[303] S. Gallea *et al.*, “Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells,” *Bone*, vol. 28, no. 5, pp. 491–498, May 2001, doi: 10.1016/s8756-3282(01)00415-x.
[304] C. Gamell *et al.*, “BMP2 induction of actin cytoskeleton reorganization and cell migration requires PI3-kinase and Cdc42 activity,” *J. Cell Sci.*, vol. 121, no. Pt 23, pp. 3960–3970, Dec. 2008, doi: 10.1242/jcs.031286.
[305] Y. E. Zhang, “Non-Smad Signaling Pathways of the TGF-β Family,” *Cold Spring Harb. Perspect. Biol.*, vol. 9, no. 2, Feb. 2017, doi: 10.1101/cshperspect.a022129.
[306] L. Long *et al.*, “Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension,” *Nat. Med.*, vol. 21, no. 7, pp. 777–785, Jul. 2015, doi: 10.1038/nm.3877.
[307] N. Y. Lee, B. Ray, T. How, and G. C. Blobe, “Endoglin promotes transforming growth factor beta-mediated Smad 1/5/8 signaling and inhibits endothelial cell migration through its association with GIPC,” *J. Biol. Chem.*, vol. 283, no. 47, pp. 32527–32533, Nov. 2008, doi: 10.1074/jbc.M803059200.
[308] A. Benn, C. Bredow, I. Casanova, S. Vukičević, and P. Knaus, “VE-Cadherin facilitates BMP-induced endothelial cell permeability and signaling,” *J. Cell Sci.*, Nov. 2015, doi: 10.1242/jcs.179960.
[309] A. K. Lagendijk and B. M. Hogan, “VE-cadherin in vascular development: a coordinator of cell signaling and tissue morphogenesis,” *Curr. Top. Dev. Biol.*, vol. 112, pp. 325–352, 2015, doi: 10.1016/bs.ctdb.2014.11.024.
[310] S. M. Weis and D. A. Cheresh, “Pathophysiological consequences of VEGF-induced vascular permeability,” *Nature*, vol. 437, no. 7058, pp. 497–504, Sep. 2005, doi: 10.1038/nature03987.
[311] C. Sieber, J. Kopf, C. Hiepen, and P. Knaus, “Recent advances in BMP receptor

signaling," *Cytokine Growth Factor Rev.*, vol. 20, no. 5–6, pp. 343–355, Dec. 2009, doi: 10.1016/j.cytogfr.2009.10.007.
[312] E. Gazzerro and E. Canalis, "Bone morphogenetic proteins and their antagonists," *Rev. Endocr. Metab. Disord.*, vol. 7, no. 1–2, pp. 51–65, Jun. 2006, doi: 10.1007/s11154-006-9000-6.
[313] W. Wang, F. V. Mariani, R. M. Harland, and K. Luo, "Ski represses bone morphogenic protein signaling in Xenopus and mammalian cells," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 97, no. 26, pp. 14394–14399, Dec. 2000.
[314] D. S. Wilkinson *et al.*, "A Direct Intersection between p53 and Transforming Growth Factor β Pathways Targets Chromatin Modification and Transcription Repression of the α-Fetoprotein Gene," *Mol. Cell. Biol.*, vol. 25, no. 3, pp. 1200–1212, Feb. 2005, doi: 10.1128/MCB.25.3.1200-1212.2005.
[315] Y. Cui, F. Jean, G. Thomas, and J. L. Christian, "BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development," *EMBO J.*, vol. 17, no. 16, pp. 4735–4743, Aug. 1998, doi: 10.1093/emboj/17.16.4735.
[316] D. B. Constam, "Regulation of TGFβ and related signals by precursor processing," *Semin. Cell Dev. Biol.*, vol. 32, pp. 85–97, Aug. 2014, doi: 10.1016/j.semcdb.2014.01.008.
[317] K. Miyazono, U. Hellman, C. Wernstedt, and C. H. Heldin, "Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization.," *J. Biol. Chem.*, vol. 263, no. 13, pp. 6407–6415, May 1988.
[318] G. Sengle, R. N. Ono, K. M. Lyons, H. P. Bächinger, and L. Y. Sakai, "A new model for growth factor activation: type II receptors compete with the prodomain for BMP-7," *J. Mol. Biol.*, vol. 381, no. 4, pp. 1025–1039, Sep. 2008, doi: 10.1016/j.jmb.2008.06.074.
[319] H. Jiang *et al.*, "The Prodomain-bound Form of Bone Morphogenetic Protein 10 Is Biologically Active on Endothelial Cells," *J. Biol. Chem.*, vol. 291, no. 6, pp. 2954–2966, Feb. 2016, doi: 10.1074/jbc.M115.683292.
[320] S. Saremba, J. Nickel, A. Seher, A. Kotzsch, W. Sebald, and T. D. Mueller, "Type I receptor binding of bone morphogenetic protein 6 is dependent on N-glycosylation of the ligand," *FEBS J.*, vol. 275, no. 1, pp. 172–183, Jan. 2008, doi: 10.1111/j.1742-4658.2007.06187.x.
[321] T. Miyazaki, S. Miyauchi, A. Tawada, T. Anada, S. Matsuzaka, and O. Suzuki, "Oversulfated chondroitin sulfate-E binds to BMP-4 and enhances osteoblast differentiation," *J. Cell. Physiol.*, vol. 217, no. 3, pp. 769–777, doi: 10.1002/jcp.21557.
[322] Q. Hang *et al.*, "Asparagine-linked glycosylation of bone morphogenetic protein-2 is required for secretion and osteoblast differentiation," *Glycobiology*, vol. 24, no. 3, pp. 292–304, Mar. 2014, doi: 10.1093/glycob/cwt110.
[323] M. Moser *et al.*, "BMPER, a novel endothelial cell precursor-derived protein, antagonizes bone morphogenetic protein signaling and endothelial cell differentiation," *Mol. Cell. Biol.*, vol. 23, no. 16, pp. 5664–5679, Aug. 2003, doi: 10.1128/mcb.23.16.5664-5679.2003.
[324] L. David *et al.*, "Bone morphogenetic protein-9 is a circulating vascular quiescence factor," *Circ. Res.*, vol. 102, no. 8, pp. 914–922, Apr. 2008, doi: 10.1161/CIRCRESAHA.107.165530.
[325] K. Song *et al.*, "Identification of a key residue mediating bone morphogenetic protein (BMP)-6 resistance to noggin inhibition allows for engineered BMPs with superior agonist activity," *J. Biol. Chem.*, vol. 285, no. 16, pp. 12169–12180, Apr. 2010, doi: 10.1074/jbc.M109.087197.

[326] D. Onichtchouk *et al.*, “Silencing of TGF-beta signalling by the pseudoreceptor BAMBI,” *Nature*, vol. 401, no. 6752, pp. 480–485, Sep. 1999, doi: 10.1038/46794.
[327] N. Guillot *et al.*, “BAMBI regulates angiogenesis and endothelial homeostasis through modulation of alternative TGFβ signaling,” *PloS One*, vol. 7, no. 6, p. e39406, 2012, doi: 10.1371/journal.pone.0039406.
[328] S. J. Hatsell *et al.*, “ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A,” *Sci. Transl. Med.*, vol. 7, no. 303, p. 303ra137, Sep. 2015, doi: 10.1126/scitranslmed.aac4358.
[329] P. Kavsak *et al.*, “Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation,” *Mol. Cell*, vol. 6, no. 6, pp. 1365–1375, Dec. 2000, doi: 10.1016/s1097-2765(00)00134-9.
[330] T. Ebisawa *et al.*, “Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation,” *J. Biol. Chem.*, vol. 276, no. 16, pp. 12477–12480, Apr. 2001, doi: 10.1074/jbc.C100008200.
[331] G. Murakami, T. Watabe, K. Takaoka, K. Miyazono, and T. Imamura, “Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads,” *Mol. Biol. Cell*, vol. 14, no. 7, pp. 2809–2817, Jul. 2003, doi: 10.1091/mbc.e02-07-0441.
[332] G. Valdimarsdottir, M.-J. Goumans, F. Itoh, S. Itoh, C.-H. Heldin, and P. ten Dijke, “Smad7 and protein phosphatase 1alpha are critical determinants in the duration of TGF-beta/ALK1 signaling in endothelial cells,” *BMC Cell Biol.*, vol. 7, p. 16, Mar. 2006, doi: 10.1186/1471-2121-7-16.
[333] J. Xu *et al.*, “Arginine Methylation Initiates BMP-Induced Smad Signaling,” *Mol. Cell*, vol. 51, no. 1, pp. 5–19, Jul. 2013, doi: 10.1016/j.molcel.2013.05.004.
[334] X.-H. Feng and R. Derynck, “Specificity and versatility in tgf-beta signaling through Smads,” *Annu. Rev. Cell Dev. Biol.*, vol. 21, pp. 659–693, 2005, doi: 10.1146/annurev.cellbio.21.022404.142018.
[335] J. Massagué, J. Seoane, and D. Wotton, “Smad transcription factors,” *Genes Dev.*, vol. 19, no. 23, pp. 2783–2810, Dec. 2005, doi: 10.1101/gad.1350705.
[336] J. Vogt *et al.*, “Protein associated with SMAD1 (PAWS1/FAM83G) is a substrate for type I bone morphogenetic protein receptors and modulates bone morphogenetic protein signalling,” *Open Biol.*, vol. 4, p. 130210, Feb. 2014, doi: 10.1098/rsob.130210.
[337] T. G. W. Letteboer *et al.*, “Genotype-phenotype relationship in hereditary haemorrhagic telangiectasia,” *J. Med. Genet.*, vol. 43, no. 4, pp. 371–377, Apr. 2006, doi: 10.1136/jmg.2005.035451.
[338] G. Lesca *et al.*, “Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia: data from the French-Italian HHT network,” *Genet. Med. Off. J. Am. Coll. Med. Genet.*, vol. 9, no. 1, pp. 14–22, Jan. 2007, doi: 10.1097/gim.0b013e31802d8373.
[339] X. Wang, R. E. Harris, L. J. Bayston, and H. L. Ashe, “Type IV collagens regulate BMP signalling in Drosophila,” *Nature*, vol. 455, no. 7209, pp. 72–77, Sep. 2008, doi: 10.1038/nature07214.
[340] F. Ramirez and D. B. Rifkin, “Extracellular microfibrils: contextual platforms for TGFbeta and BMP signaling,” *Curr. Opin. Cell Biol.*, vol. 21, no. 5, pp. 616–622, Oct. 2009, doi: 10.1016/j.ceb.2009.05.005.
[341] M. Bidart *et al.*, “BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain,” *Cell. Mol. Life Sci. CMLS*, vol. 69, no. 2, pp. 313–324, Jan. 2012, doi: 10.1007/s00018-011-0751-1.

[342] B. Herrera and G. J. Inman, “A rapid and sensitive bioassay for the simultaneous measurement of multiple bone morphogenetic proteins. Identification and quantification of BMP4, BMP6 and BMP9 in bovine and human serum,” *BMC Cell Biol.*, vol. 10, p. 20, Mar. 2009, doi: 10.1186/1471-2121-10-20.
[343] L. David, C. Mallet, S. Mazerbourg, J.-J. Feige, and S. Bailly, “Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells,” *Blood*, vol. 109, no. 5, pp. 1953–1961, Mar. 2007, doi: 10.1182/blood-2006-07-034124.
[344] I. Moreno-Miralles, R. Ren, M. Moser, M. E. Hartnett, and C. Patterson, “Bone morphogenetic protein endothelial cell precursor-derived regulator regulates retinal angiogenesis in vivo in a mouse model of oxygen-induced retinopathy,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 31, no. 10, pp. 2216–2222, Oct. 2011, doi: 10.1161/ATVBAHA.111.230235.
[345] M. Ciumas *et al.*, “Bone morphogenetic proteins protect pulmonary microvascular endothelial cells from apoptosis by upregulating α-B-crystallin,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 33, no. 11, pp. 2577–2584, Nov. 2013, doi: 10.1161/ATVBAHA.113.301976.
[346] S. Itoh, F. Itoh, M. J. Goumans, and P. Ten Dijke, “Signaling of transforming growth factor-beta family members through Smad proteins,” *Eur. J. Biochem.*, vol. 267, no. 24, pp. 6954–6967, Dec. 2000.
[347] J. Choi, L. Dong, J. Ahn, D. Dao, M. Hammerschmidt, and J.-N. Chen, “FoxH1 negatively modulates flk1 gene expression and vascular formation in zebrafish,” *Dev. Biol.*, vol. 304, no. 2, pp. 735–744, Apr. 2007, doi: 10.1016/j.ydbio.2007.01.023.
[348] R. C. Trembath *et al.*, “Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia,” *N. Engl. J. Med.*, vol. 345, no. 5, pp. 325–334, Aug. 2001, doi: 10.1056/NEJM200108023450503.
[349] W. Li, R. M. Salmon, H. Jiang, and N. W. Morrell, “Regulation of the ALK1 ligands, BMP9 and BMP10,” *Biochem. Soc. Trans.*, vol. 44, no. 4, pp. 1135–1141, 15 2016, doi: 10.1042/BST20160083.
[350] K. A. McAllister *et al.*, “Endoglin, a TGF-β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1,” *Nat. Genet.*, vol. 8, no. 4, pp. 345–351, Dec. 1994, doi: 10.1038/ng1294-345.
[351] D. W. Johnson *et al.*, “Mutations in the activin receptor–like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2,” *Nat. Genet.*, vol. 13, no. 2, pp. 189–195, Jun. 1996, doi: 10.1038/ng0696-189.
[352] C. J. Gallione *et al.*, “A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4),” *Lancet Lond. Engl.*, vol. 363, no. 9412, pp. 852–859, Mar. 2004, doi: 10.1016/S0140-6736(04)15732-2.
[353] W. L. Wooderchak-Donahue *et al.*, “BMP9 mutations cause a vascular-anomaly syndrome with phenotypic overlap with hereditary hemorrhagic telangiectasia,” *Am. J. Hum. Genet.*, vol. 93, no. 3, pp. 530–537, Sep. 2013, doi: 10.1016/j.ajhg.2013.07.004.
[354] R. K. Jain, “Molecular regulation of vessel maturation,” *Nat. Med.*, vol. 9, no. 6, pp. 685–693, Jun. 2003, doi: 10.1038/nm0603-685.
[355] E. Pardali, M.-J. Goumans, and P. ten Dijke, “Signaling by members of the TGF-beta family in vascular morphogenesis and disease,” *Trends Cell Biol.*, vol. 20, no. 9, pp. 556–567, Sep. 2010, doi: 10.1016/j.tcb.2010.06.006.
[356] L. Anderson *et al.*, “Bmp2 and Bmp4 exert opposing effects in hypoxic pulmonary

hypertension," *Am. J. Physiol. - Regul. Integr. Comp. Physiol.*, vol. 298, no. 3, pp. R833–R842, Mar. 2010, doi: 10.1152/ajpregu.00534.2009.
[357] Z. Deng *et al.*, "Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene," *Am. J. Hum. Genet.*, vol. 67, no. 3, pp. 737–744, Sep. 2000, doi: 10.1086/303059.
[358] K. B. Lane *et al.*, "Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension," *Nat. Genet.*, vol. 26, no. 1, pp. 81–84, Sep. 2000, doi: 10.1038/79226.
[359] L. W. van Laake *et al.*, "Endoglin has a crucial role in blood cell-mediated vascular repair," *Circulation*, vol. 114, no. 21, pp. 2288–2297, Nov. 2006, doi: 10.1161/CIRCULATIONAHA.106.639161.
[360] F. Tian *et al.*, "Endothelial cells are activated during hypoxia via endoglin/ALK-1/SMAD1/5 signaling in vivo and in vitro," *Biochem. Biophys. Res. Commun.*, vol. 392, no. 3, pp. 283–288, Feb. 2010, doi: 10.1016/j.bbrc.2009.12.170.
[361] Y. Kienast *et al.*, "Rapid Activation of Bone Morphogenic Protein 9 by Receptor-mediated Displacement of Pro-domains," *J. Biol. Chem.*, vol. 291, no. 7, pp. 3395–3410, Feb. 2016, doi: 10.1074/jbc.M115.680009.
[362] H. Neuhaus, V. Rosen, and R. S. Thies, "Heart specific expression of mouse BMP-10 a novel member of the TGF-beta superfamily," *Mech. Dev.*, vol. 80, no. 2, pp. 181–184, Feb. 1999.
[363] M. Scharpfenecker *et al.*, "BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis," *J. Cell Sci.*, vol. 120, no. 6, pp. 964–972, Mar. 2007, doi: 10.1242/jcs.002949.
[364] J. J. Song, A. J. Celeste, F. M. Kong, R. L. Jirtle, V. Rosen, and R. S. Thies, "Bone morphogenetic protein-9 binds to liver cells and stimulates proliferation," *Endocrinology*, vol. 136, no. 10, pp. 4293–4297, Oct. 1995, doi: 10.1210/endo.136.10.7664647.
[365] A. F. Miller, S. A. K. Harvey, R. S. Thies, and M. S. Olson, "Bone Morphogenetic Protein-9 AN AUTOCRINE/PARACRINE CYTOKINE IN THE LIVER," *J. Biol. Chem.*, vol. 275, no. 24, pp. 17937–17945, Jun. 2000, doi: 10.1074/jbc.275.24.17937.
[366] E. S. Shao, L. Lin, Y. Yao, and K. I. Bostrom, "Expression of vascular endothelial growth factor is coordinately regulated by the activin-like kinase receptors 1 and 5 in endothelial cells," *Blood*, vol. 114, no. 10, pp. 2197–2206, Sep. 2009, doi: 10.1182/blood-2009-01-199166.
[367] T. Seki, K.-H. Hong, and S. P. Oh, "Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development," *Lab. Investig. J. Tech. Methods Pathol.*, vol. 86, no. 2, pp. 116–129, Feb. 2006, doi: 10.1038/labinvest.3700376.
[368] S. I. Cunha and K. Pietras, "ALK1 as an emerging target for antiangiogenic therapy of cancer," *Blood*, vol. 117, no. 26, pp. 6999–7006, Jun. 2011, doi: 10.1182/blood-2011-01-330142.
[369] I. López-Coviella, B. Berse, R. Krauss, R. S. Thies, and J. K. Blusztajn, "Induction and maintenance of the neuronal cholinergic phenotype in the central nervous system by BMP-9," *Science*, vol. 289, no. 5477, pp. 313–316, Jul. 2000.
[370] I. López-Coviella, B. Berse, R. S. Thies, and J. K. Blusztajn, "Upregulation of acetylcholine synthesis by bone morphogenetic protein 9 in a murine septal cell line," *J. Physiol. Paris*, vol. 96, no. 1–2, pp. 53–59, Mar. 2002.
[371] J. Truksa, H. Peng, P. Lee, and E. Beutler, "Bone morphogenetic proteins 2, 4, and 9

stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 27, pp. 10289–10293, Jul. 2006, doi: 10.1073/pnas.0603124103.
[372] Z. Xie, J. Xu, L. Peng, Y. Gao, H. Zhao, and Y. Qu, "miR-149 promotes human osteocarcinoma progression via targeting bone morphogenetic protein 9 (BMP9)," *Biotechnol. Lett.*, vol. 40, no. 1, pp. 47–55, Jan. 2018, doi: 10.1007/s10529-017-2445-8.
[373] C. Chen *et al.*, "An integrated functional genomics screening program reveals a role for BMP-9 in glucose homeostasis," *Nat. Biotechnol.*, vol. 21, no. 3, pp. 294–301, Mar. 2003, doi: 10.1038/nbt795.
[374] S. Lamouille, C. Mallet, J.-J. Feige, and S. Bailly, "Activin receptor–like kinase 1 is implicated in the maturation phase of angiogenesis," *Blood*, vol. 100, no. 13, pp. 4495–4501, Dec. 2002, doi: 10.1182/blood.V100.13.4495.
[375] L. D. Urness, L. K. Sorensen, and D. Y. Li, "Arteriovenous malformations in mice lacking activin receptor-like kinase-1," *Nat. Genet.*, vol. 26, no. 3, pp. 328–331, Nov. 2000, doi: 10.1038/81634.
[376] A. Helisch and W. Schaper, "Arteriogenesis: the development and growth of collateral arteries," *Microcirc. N. Y. N 1994*, vol. 10, no. 1, pp. 83–97, Jan. 2003, doi: 10.1038/sj.mn.7800173.
[377] H. Kamihata *et al.*, "Improvement of collateral perfusion and regional function by implantation of peripheral blood mononuclear cells into ischemic hibernating myocardium," *Arterioscler. Thromb. Vasc. Biol.*, vol. 22, no. 11, pp. 1804–1810, Nov. 2002, doi: 10.1161/01.atv.0000039168.95670.b9.
[378] M. Heil *et al.*, "Blood monocyte concentration is critical for enhancement of collateral artery growth," *Am. J. Physiol. Heart Circ. Physiol.*, vol. 283, no. 6, pp. H2411-2419, Dec. 2002, doi: 10.1152/ajpheart.01098.2001.
[379] P. A. Campochiaro, "Ocular Neovascularization," *J. Mol. Med. Berl. Ger.*, vol. 91, no. 3, pp. 311–321, Mar. 2013, doi: 10.1007/s00109-013-0993-5.
[380] G. D. Hildebrand and A. R. Fielder, "Anatomy and Physiology of the Retina," Accessed: Aug. 05, 2018. [Online]. Available: /paper/Anatomy-and-Physiology-of-the-Retina-Hildebrand-Fielder/01ffa1607f70f8ed851a6b744eb0de0b7353ebd2.
[381] R. F. Gariano, "Special features of human retinal angiogenesis," *Eye Lond. Engl.*, vol. 24, no. 3, pp. 401–407, Mar. 2010, doi: 10.1038/eye.2009.324.
[382] R. F. Gariano, "Cellular mechanisms in retinal vascular development," *Prog. Retin. Eye Res.*, vol. 22, no. 3, pp. 295–306, May 2003.
[383] K. Bharti, "Patching the retina with stem cells," *Nat. Biotechnol.*, vol. 36, no. 4, pp. 311–313, Apr. 2018, doi: 10.1038/nbt.4118.
[384] L. A. Yannuzzi *et al.*, "Retinal Angiomatous Proliferation in Age-Related Macular Degeneration," *Retina*, vol. 21, no. 5, pp. 416–434, Oct. 2001.
[385] A. D. Kulkarni and B. D. Kuppermann, "Wet age-related macular degeneration," *Adv. Drug Deliv. Rev.*, vol. 57, no. 14, pp. 1994–2009, Dec. 2005, doi: 10.1016/j.addr.2005.09.003.
[386] A. N. Witmer, G. F. J. M. Vrensen, C. J. F. Van Noorden, and R. O. Schlingemann, "Vascular endothelial growth factors and angiogenesis in eye disease," *Prog. Retin. Eye Res.*, vol. 22, no. 1, pp. 1–29, Jan. 2003, doi: 10.1016/s1350-9462(02)00043-5.
[387] S. A. Vinores *et al.*, "Implication of the hypoxia response element of the Vegf promoter in mouse models of retinal and choroidal neovascularization, but not retinal vascular

development," *J. Cell. Physiol.*, vol. 206, no. 3, pp. 749–758, Mar. 2006, doi: 10.1002/jcp.20525.
[388] N. S. Chandel, E. Maltepe, E. Goldwasser, C. E. Mathieu, M. C. Simon, and P. T. Schumacker, "Mitochondrial reactive oxygen species trigger hypoxia-induced transcription," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 95, no. 20, pp. 11715–11720, Sep. 1998, doi: 10.1073/pnas.95.20.11715.
[389] N. S. Chandel *et al.*, "Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing," *J. Biol. Chem.*, vol. 275, no. 33, pp. 25130–25138, Aug. 2000, doi: 10.1074/jbc.M001914200.
[390] H. Lu, C. L. Dalgard, A. Mohyeldin, T. McFate, A. S. Tait, and A. Verma, "Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1," *J. Biol. Chem.*, vol. 280, no. 51, pp. 41928–41939, Dec. 2005, doi: 10.1074/jbc.M508718200.
[391] A. Dong *et al.*, "Oxidative stress promotes ocular neovascularization," *J. Cell. Physiol.*, vol. 219, no. 3, pp. 544–552, Jun. 2009, doi: 10.1002/jcp.21698.
[392] Age-Related Eye Disease Study Research Group, "A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8," *Arch. Ophthalmol. Chic. Ill 1960*, vol. 119, no. 10, pp. 1417–1436, Oct. 2001, doi: 10.1001/archopht.119.10.1417.
[393] Y. Oshima *et al.*, "Increased expression of VEGF in retinal pigmented epithelial cells is not sufficient to cause choroidal neovascularization," *J. Cell. Physiol.*, vol. 201, no. 3, pp. 393–400, Dec. 2004, doi: 10.1002/jcp.20110.
[394] R. Lima e Silva *et al.*, "The SDF-1/CXCR4 ligand/receptor pair is an important contributor to several types of ocular neovascularization," *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.*, vol. 21, no. 12, pp. 3219–3230, Oct. 2007, doi: 10.1096/fj.06-7359com.
[395] N. Jo *et al.*, "Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization," *Am. J. Pathol.*, vol. 168, no. 6, pp. 2036–2053, Jun. 2006, doi: 10.2353/ajpath.2006.050588.
[396] D. Shweiki, A. Itin, D. Soffer, and E. Keshet, "Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis," *Nature*, vol. 359, no. 6398, pp. 843–845, Oct. 1992, doi: 10.1038/359843a0.
[397] K. H. Plate, G. Breier, H. A. Weich, and W. Risau, "Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo," *Nature*, vol. 359, no. 6398, pp. 845–848, Oct. 1992, doi: 10.1038/359845a0.
[398] N. Okamoto *et al.*, "Transgenic mice with increased expression of vascular endothelial growth factor in the retina: a new model of intraretinal and subretinal neovascularization," *Am. J. Pathol.*, vol. 151, no. 1, pp. 281–291, Jul. 1997.
[399] T. Tobe *et al.*, "Evolution of neovascularization in mice with overexpression of vascular endothelial growth factor in photoreceptors," *Invest. Ophthalmol. Vis. Sci.*, vol. 39, no. 1, pp. 180–188, Jan. 1998.
[400] H. Yamada *et al.*, "Cell injury unmasks a latent proangiogenic phenotype in mice with increased expression of FGF2 in the retina," *J. Cell. Physiol.*, vol. 185, no. 1, pp. 135–142, Oct. 2000, doi: 10.1002/1097-4652(200010)185:1<135::AID-JCP13>3.0.CO;2-Y.
[401] R. Vang and F. A. Tavassoli, "Risk for subsequent development of breast cancer," *Am. J. Surg. Pathol.*, vol. 27, no. 2, pp. 268–271; author reply 271-274, Feb. 2003, doi:

10.1097/00000478-200302000-00020.
[402] N. Weidner, J. P. Semple, W. R. Welch, and J. Folkman, “Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma,” *N. Engl. J. Med.*, vol. 324, no. 1, pp. 1–8, Jan. 1991, doi: 10.1056/NEJM199101033240101.
[403] J. P. Dales *et al.*, “Long-term prognostic significance of neoangiogenesis in breast carcinomas: comparison of Tie-2/Tek, CD105, and CD31 immunocytochemical expression,” *Hum. Pathol.*, vol. 35, no. 2, pp. 176–183, Feb. 2004, doi: 10.1016/j.humpath.2003.10.008.
[404] K. Sfiridaki *et al.*, “Angiogenesis-related cytokines, RANKL, and osteoprotegerin in multiple myeloma patients in relation to clinical features and response to treatment,” *Mediators Inflamm.*, vol. 2011, p. 867576, 2011, doi: 10.1155/2011/867576.
[405] S. Rezzola *et al.*, “In vitro and ex vivo retina angiogenesis assays,” *Angiogenesis*, vol. 17, no. 3, pp. 429–442, Jul. 2014, doi: 10.1007/s10456-013-9398-x.
[406] R. Auerbach, W. Auerbach, and I. Polakowski, “Assays for angiogenesis: a review,” *Pharmacol. Ther.*, vol. 51, no. 1, pp. 1–11, 1991, doi: 10.1016/0163-7258(91)90038-n.
[407] G. W. Cockerill, J. R. Gamble, and M. A. Vadas, “Angiogenesis: models and modulators,” *Int. Rev. Cytol.*, vol. 159, pp. 113–160, 1995, doi: 10.1016/s0074-7696(08)62106-3.
[408] D. Ribatti and A. Vacca, “Models for studying angiogenesis in vivo,” *Int. J. Biol. Markers*, vol. 14, no. 4, pp. 207–213, Dec. 1999.
[409] K. Norrby, “In vivo models of angiogenesis,” *J. Cell. Mol. Med.*, vol. 10, no. 3, pp. 588–612, Sep. 2006, doi: 10.1111/j.1582-4934.2006.tb00423.x.
[410] R. K. Jain, K. Schlenger, M. Höckel, and F. Yuan, “Quantitative angiogenesis assays: progress and problems,” *Nat. Med.*, vol. 3, no. 11, pp. 1203–1208, Nov. 1997, doi: 10.1038/nm1197-1203.
[411] T. Shih and C. Lindley, “Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies,” *Clin. Ther.*, vol. 28, no. 11, pp. 1779–1802, Nov. 2006, doi: 10.1016/j.clinthera.2006.11.015.
[412] C. M. Andreoli and J. W. Miller, “Anti-vascular endothelial growth factor therapy for ocular neovascular disease,” *Curr. Opin. Ophthalmol.*, vol. 18, no. 6, pp. 502–508, Nov. 2007, doi: 10.1097/ICU.0b013e3282f0ca54.
[413] R. D. Jager, W. F. Mieler, and J. W. Miller, “Age-related macular degeneration,” *N. Engl. J. Med.*, vol. 358, no. 24, pp. 2606–2617, Jun. 2008, doi: 10.1056/NEJMra0801537.
[414] M. T. Bolinger and D. A. Antonetti, “Moving Past Anti-VEGF: Novel Therapies for Treating Diabetic Retinopathy,” *Int. J. Mol. Sci.*, vol. 17, no. 9, Sep. 2016, doi: 10.3390/ijms17091498.
[415] L. S. Lim, P. Mitchell, J. M. Seddon, F. G. Holz, and T. Y. Wong, “Age-related macular degeneration,” *The Lancet*, vol. 379, no. 9827, pp. 1728–1738, May 2012, doi: 10.1016/S0140-6736(12)60282-7.
[416] C. Bowes Rickman, S. Farsiu, C. A. Toth, and M. Klingeborn, “Dry Age-Related Macular Degeneration: Mechanisms, Therapeutic Targets, and Imaging,” *Investig. Opthalmology Vis. Sci.*, vol. 54, no. 14, p. ORSF68, Dec. 2013, doi: 10.1167/iovs.13-12757.
[417] L. Nivison-Smith, R. Milston, M. Madigan, and M. Kalloniatis, “Age-Related Macular Degeneration: Linking Clinical Presentation to Pathology,” *Optom. Vis. Sci.*, vol. 91, no. 8, pp. 832–848, Aug. 2014, doi: 10.1097/OPX.0000000000000281.
[418] F. G. Holz, E. C. Strauss, S. Schmitz-Valckenberg, and M. van Lookeren Campagne, “Geographic Atrophy: Clinical Features and Potential Therapeutic Approaches,”

Ophthalmology, vol. 121, no. 5, pp. 1079–1091, May 2014, doi: 10.1016/j.ophtha.2013.11.023.
[419] S. Suchting *et al.*, "The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 9, pp. 3225–3230, Feb. 2007, doi: 10.1073/pnas.0611177104.
[420] L.-K. Phng and H. Gerhardt, "Angiogenesis: A Team Effort Coordinated by Notch," *Dev. Cell*, vol. 16, no. 2, pp. 196–208, Feb. 2009, doi: 10.1016/j.devcel.2009.01.015.
[421] D. G. Duda, "Molecular Biomarkers of Response to Antiangiogenic Therapy for Cancer," *ISRN Cell Biol.*, vol. 2012, pp. 1–11, 2012, doi: 10.5402/2012/587259.
[422] D. M. Brown *et al.*, "Ranibizumab versus Verteporfin for Neovascular Age-Related Macular Degeneration," *N. Engl. J. Med.*, vol. 355, no. 14, pp. 1432–1444, Oct. 2006, doi: 10.1056/NEJMoa062655.
[423] F. Semeraro, F. Morescalchi, S. Duse, E. Gambicorti, M. R. Romano, and C. Costagliola, "Systemic thromboembolic adverse events in patients treated with intravitreal anti-VEGF drugs for neovascular age-related macular degeneration: an overview," *Expert Opin. Drug Saf.*, pp. 1–18, May 2014, doi: 10.1517/14740338.2014.911284.
[424] E. Agosta *et al.*, "Pharmacogenetics of antiangiogenic and antineovascular therapies of age-related macular degeneration," *Pharmacogenomics*, vol. 13, no. 9, pp. 1037–1053, Jul. 2012, doi: 10.2217/pgs.12.77.
[425] A. M. Keating and D. S. Jacobs, "Anti-VEGF Treatment of Corneal Neovascularization," *Ocul. Surf.*, vol. 9, no. 4, pp. 227–238, Oct. 2011, doi: 10.1016/S1542-0124(11)70035-0.
[426] B. L. Nesmith, M. Ihnen, and S. Schaal, "Poor Responders to Bevacizumab Pharmacotherapy in Age-Related Macular Degeneration and in Diabetic Macular Edema Demonstrate Increased Risk for Obstructive Sleep Apnea:," *Retina*, vol. 34, no. 12, pp. 2423–2430, Dec. 2014, doi: 10.1097/IAE.0000000000000247.
[427] M. Saint-Geniez *et al.*, "Endogenous VEGF Is Required for Visual Function: Evidence for a Survival Role on Müller Cells and Photoreceptors," *PLoS ONE*, vol. 3, no. 11, p. e3554, Nov. 2008, doi: 10.1371/journal.pone.0003554.
[428] A. W. Scott and S. B. Bressler, "Long-term follow-up of vascular endothelial growth factor inhibitor therapy for neovascular age-related macular degeneration:," *Curr. Opin. Ophthalmol.*, vol. 24, no. 3, pp. 190–196, May 2013, doi: 10.1097/ICU.0b013e32835fefee.
[429] S. Cole, M. Begbie, G. Wallace, and C. Shovlin, "A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5," *J. Med. Genet.*, vol. 42, no. 7, pp. 577–582, Jul. 2005, doi: 10.1136/jmg.2004.028712.
[430] P. Bayrak-Toydemir *et al.*, "A fourth locus for hereditary hemorrhagic telangiectasia maps to chromosome 7," *Am. J. Med. Genet. A.*, vol. 140A, no. 20, pp. 2155–2162, Oct. 2006, doi: 10.1002/ajmg.a.31450.
[431] I. M. Aspalter *et al.*, "Alk1 and Alk5 inhibition by Nrp1 controls vascular sprouting downstream of Notch," *Nat. Commun.*, vol. 6, Jun. 2015, doi: 10.1038/ncomms8264.
[432] A. Stahl *et al.*, "Computer-aided quantification of retinal neovascularization," *Angiogenesis*, vol. 12, no. 3, pp. 297–301, 2009, doi: 10.1007/s10456-009-9155-3.
[433] H. Huang, J. Shen, and S. A. Vinores, "Blockade of VEGFR1 and 2 Suppresses Pathological Angiogenesis and Vascular Leakage in the Eye," *PLoS ONE*, vol. 6, no. 6, p. e21411, Jun. 2011, doi: 10.1371/journal.pone.0021411.
[434] K. Ghasemi Falavarjani and Q. D. Nguyen, "Adverse events and complications associated with intravitreal injection of anti-VEGF agents: a review of literature," *Eye*, vol.

27, no. 7, pp. 787–794, Jul. 2013, doi: 10.1038/eye.2013.107.
[435] I. Bhutto and G. Lutty, “Understanding age-related macular degeneration (AMD): Relationships between the photoreceptor/retinal pigment epithelium/Bruch’s membrane/choriocapillaris complex,” *Mol. Aspects Med.*, vol. 33, no. 4, pp. 295–317, Aug. 2012, doi: 10.1016/j.mam.2012.04.005.
[436] I. B. Lobov *et al.*, “Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 9, pp. 3219–3224, Feb. 2007, doi: 10.1073/pnas.0611206104.
[437] J. Chen *et al.*, “Wnt signaling mediates pathological vascular growth in proliferative retinopathy,” *Circulation*, vol. 124, no. 17, pp. 1871–1881, Oct. 2011, doi: 10.1161/CIRCULATIONAHA.111.040337.
[438] C. Ehlken *et al.*, “Therapeutic interference with EphrinB2 signalling inhibits oxygen-induced angioproliferative retinopathy,” *Acta Ophthalmol. (Copenh.)*, vol. 89, no. 1, pp. 82–90, Feb. 2011, doi: 10.1111/j.1755-3768.2009.01609.x.
[439] N. Umeda, H. Ozaki, H. Hayashi, and K. Oshima, “Expression of ephrinB2 and its receptors on fibroproliferative membranes in ocular angiogenic diseases,” *Am. J. Ophthalmol.*, vol. 138, no. 2, pp. 270–279, Aug. 2004, doi: 10.1016/j.ajo.2004.04.006.
[440] A. Scott and M. Fruttiger, “Oxygen-induced retinopathy: a model for vascular pathology in the retina,” *Eye Lond. Engl.*, vol. 24, no. 3, pp. 416–421, Mar. 2010, doi: 10.1038/eye.2009.306.
[441] N. Ricard *et al.*, “BMP9 and BMP10 are critical for postnatal retinal vascular remodeling,” *Blood*, vol. 119, no. 25, pp. 6162–6171, Jun. 2012, doi: 10.1182/blood-2012-01-407593.
[442] L. J. A. C. Hawinkels *et al.*, “Activin Receptor-like Kinase 1 Ligand Trap Reduces Microvascular Density and Improves Chemotherapy Efficiency to Various Solid Tumors,” *Clin. Cancer Res.*, vol. 22, no. 1, pp. 96–106, Jan. 2016, doi: 10.1158/1078-0432.CCR-15-0743.
[443] S. I. Cunha *et al.*, “Endothelial ALK1 Is a Therapeutic Target to Block Metastatic Dissemination of Breast Cancer,” *Cancer Res.*, vol. 75, no. 12, pp. 2445–2456, Jun. 2015, doi: 10.1158/0008-5472.CAN-14-3706.
[444] E. J. Walker, H. Su, F. Shen, V. Degos, K. Jun, and W. L. Young, “Bevacizumab attenuates VEGF-induced angiogenesis and vascular malformations in the adult mouse brain,” *Stroke J. Cereb. Circ.*, vol. 43, no. 7, pp. 1925–1930, Jul. 2012, doi: 10.1161/STROKEAHA.111.647982.
[445] N. C. Kappas *et al.*, “The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching,” *J. Cell Biol.*, vol. 181, no. 5, pp. 847–858, Jun. 2008, doi: 10.1083/jcb.200709114.
[446] S. Patel-Hett and P. A. D’Amore, “Signal Transduction in Vasculogenesis and Developmental Angiogenesis,” *Int. J. Dev. Biol.*, vol. 55, no. 0, pp. 353–363, 2011, doi: 10.1387/ijdb.103213sp.
[447] S. Isogai, N. D. Lawson, S. Torrealday, M. Horiguchi, and B. M. Weinstein, “Angiogenic network formation in the developing vertebrate trunk,” *Dev. Camb. Engl.*, vol. 130, no. 21, pp. 5281–5290, Nov. 2003, doi: 10.1242/dev.00733.
[448] J. R. Shutter *et al.*, “Dll4, a novel Notch ligand expressed in arterial endothelium,” *Genes Dev.*, vol. 14, no. 11, pp. 1313–1318, Jun. 2000.
[449] M. Montero-Balaguer, K. Swirsding, F. Orsenigo, F. Cotelli, M. Mione, and E. Dejana,

"Stable Vascular Connections and Remodeling Require Full Expression of VE-Cadherin in Zebrafish Embryos," *PLoS ONE*, vol. 4, no. 6, Jun. 2009, doi: 10.1371/journal.pone.0005772.
[450] Y. Fu *et al.*, "Differential Regulation of Transforming Growth Factor β Signaling Pathways by Notch in Human Endothelial Cells," *J. Biol. Chem.*, vol. 284, no. 29, pp. 19452–19462, Jul. 2009, doi: 10.1074/jbc.M109.011833.
[451] K. Ntumba, N. Akla, S. P. Oh, A. Eichmann, and B. Larrivée, "BMP9/ALK1 inhibits neovascularization in mouse models of age-related macular degeneration," *Oncotarget*, vol. 7, no. 35, pp. 55957–55969, Aug. 2016, doi: 10.18632/oncotarget.11182.
[452] K. Bentley, G. Mariggi, H. Gerhardt, and P. A. Bates, "Tipping the Balance: Robustness of Tip Cell Selection, Migration and Fusion in Angiogenesis," *PLoS Comput. Biol.*, vol. 5, no. 10, Oct. 2009, doi: 10.1371/journal.pcbi.1000549.
[453] J. Ridgway *et al.*, "Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis," *Nature*, vol. 444, no. 7122, pp. 1083–1087, Dec. 2006, doi: 10.1038/nature05313.
[454] J. D. Leslie, L. Ariza-McNaughton, A. L. Bermange, R. McAdow, S. L. Johnson, and J. Lewis, "Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis," *Dev. Camb. Engl.*, vol. 134, no. 5, pp. 839–844, Mar. 2007, doi: 10.1242/dev.003244.
[455] A. F. Siekmann and N. D. Lawson, "Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries," *Nature*, vol. 445, no. 7129, pp. 781–784, Feb. 2007, doi: 10.1038/nature05577.
[456] J. H. Baek, J. Hatakeyama, S. Sakamoto, T. Ohtsuka, and R. Kageyama, "Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system," *Dev. Camb. Engl.*, vol. 133, no. 13, pp. 2467–2476, Jul. 2006, doi: 10.1242/dev.02403.
[457] M. Ishibashi, S. L. Ang, K. Shiota, S. Nakanishi, R. Kageyama, and F. Guillemot, "Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects," *Genes Dev.*, vol. 9, no. 24, pp. 3136–3148, Dec. 1995.
[458] J. Hatakeyama *et al.*, "Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation," *Dev. Camb. Engl.*, vol. 131, no. 22, pp. 5539–5550, Nov. 2004, doi: 10.1242/dev.01436.