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The role of COCO in ocular angiogenesis

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Résumé

Contexte: La néovascularisation pathologique oculaire entraîne plusieurs troubles de la cécité, y compris la dégénérescence maculaire néovasculaire liée à l'âge (nDMLA) et la rétinopathie de la prématurité (ROP). La nDMLA est la principale cause de cécité dans le monde industrialisé avec un impact socio-économique considérable (1-6). Les thérapies palliatives actuelles reposent sur la suppression du facteur de croissance de l'endothélium vasculaire (VEGF), prouvé sûr et efficace (7-19). Cependant, certains patients résistent aux injections intravitréennes mensuelles répétées d'anti-VEGF, et les conséquences à long terme comprennent une perte de vision supplémentaire et une atrophie géographique (10, 14, 17, 18, 20-27). Cliniquement, il existe un besoin de nouvelles cibles combinatoires ou alternatives dans les cliniques, permettant de réduire les doses d'anti-VEGF, de traiter et d'étendre un intervalle approprié personnalisé à chaque patient non répondeur, et de minimiser la charge des injections répétées (24, 25, 28, 29).

La famille TGF-β et sa sous-famille BMP sont cruciales dans l'angiogenèse oculaire physiologique dans un modèle de ROP, et pourraient être des cibles thérapeutiques potentielles chez les patients souffrant d'une nDMLA (30-66).

Hypothèse: COCO, un membre de la famille DAN, un modulateur connu des voies BMP et TGF-β, agit comme un facteur neurotrophique sur les progéniteurs de photorécepteurs humains en culture. Nous émettons l'hypothèse que les effets neurotrophiques et anti-angiogéniques de COCO pourraient être des approches thérapeutiques bénéfiques pour empêcher la néovascularisation dans la nDMLA ou la ROP.

Objectifs:

Objectif 1: Pour évaluer le rôle du COCO sur les maladies oculaires néovasculaires, nous proposons d'étudier ses effets sur la néovascularisation rétinienne et choroïdienne dans le développement et des modèles pathologiques de la DMLA et de la ROP lors d'injections intravitréennes.

Objectif 2: Pour comprendre le rôle physiologique et le mécanisme d'action du COCO, nous évaluerons les effets de COCO endogène au cours de l'angiogenèse et du développement vasculaire oculaire.

Conclusions: Nous avons découvert un nouvel inhibiteur de l'angiogenèse, COCO, un membre de la famille des protéines DAN. COCO abroge la migration et la prolifération des cellules endothéliales de la veine ombilicale humaine, en partie grâce à sa régulation des voies TGF-β et BMP et à une modification du métabolisme cellulaire et des gènes mitochondriaux.

Les injections intravitréennes de COCO suppriment la vascularisation rétinienne en cours du développement et dans un modèle expérimental de ROP. COCO inhibe de la même manière la néovascularisation choroïdienne dans un modèle de DMLA. De plus, COCO empêche l'angiogenèse rétinienne développementale sans affecter le système vasculaire mature. L'examen des souris Dand5 (COCO) KO a également montré un phénotype de développement rétinien léger à P12 ainsi qu'une exacerbation des touffes néovasculaires dans un modèle de rétinopathie induite par l'oxygène.

Impact: Nos données montrent que COCO inhibe la néovascularisation rétinienne et choroïdienne et pourrait être une nouvelle thérapie possible pour des maladies oculaires. Nos études fournissent des données sur les impacts de COCO sur le développement vasculaire rétinien, établissent ses caractéristiques moléculaires et déterminent son importance biologique.

Mots-clés: DMLA, ROP, nouvelle protéine anti-angiogénique, endothélium, antagoniste du TGF-β et de BMP, mitochondries

Abstract

Background: Ocular pathological neovascularization leads to several blinding disorders, including neovascular age-related macular degeneration (nAMD) and retinopathy of prematurity (ROP). nAMD, for instance, is the primary cause of blindness in the industrialized world with a tremendous socioeconomic impact (1-6). Current palliative therapies rely on suppressing vascular endothelial growth factor (VEGF), proven safe and effective (7-19). However, some patients are resistant to monthly repeated intravitreal injections of anti-VEGF, and long-term consequences comprise further vision loss and geographic atrophy (10, 14, 17, 18, 20-27). Clinically, there is a necessity for novel combinatory or synergistic therapeutic targets to reduce anti-VEGF treatment adherence. Novel personalized therapies to each non-responder patient may increase efficiency while minimizing the burden of repeated injections (24, 25, 28, 29). The TGF-β family, specifically the BMPs subfamily of proteins, is crucial in pathological ocular angiogenesis in a model of pre-retinal neovascularization. These alternative pathways could be potential therapeutic targets in nAMD patients as well (30-66).

Hypothesis: COCO, a member of the DAN family, is a known modulator of BMP and TGF-β pathways and acts as a neurotrophic factor on cultured human photoreceptor progenitors. We hypothesize that COCO's neurotrophic and anti-angiogenic effects could exert therapeutic benefits to preclude neovascularization in nAMD or ROP.

Objectives:

Aim 1: To assess the role of COCO on neovascular ocular diseases, we propose investigating its effects on retinal and choroidal neovascularization in the development and pathological models of AMD and ROP upon intravitreal injections.

Aim 2: To understand COCO's physiological role and mechanism of action, we evaluate the exogenous and endogenous effects of COCO during angiogenesis and ocular vascular development.

Conclusions: We discovered a novel inhibitor of angiogenesis, COCO, a DAN protein family member. COCO abrogated sprouting migration and cellular proliferation of human umbilical vein

endothelial cells, partly through its regulation of TGF-β and BMP pathways and cellular metabolism.

Intravitreal injections of COCO suppress retinal vascularization in development and an experimental model of ROP. COCO similarly inhibits choroidal neovascularization in an nAMD model. COCO prevents developmental retinal angiogenesis without affecting the mature vasculature. Examination of Dand5 (COCO) KO mice also shows a mild retinal developmental phenotype at P12 as well as an exacerbation of neovascular tufts in a model of oxygen-induced retinopathy.

Impact: Our data show that COCO inhibits retinal and choroidal neovascularization and could be of potential therapeutic use in treating neovascular ocular diseases. Our studies set grounds for understanding the role of COCO during retinal vascular development, characterizing its molecular features, and determining its biologic significance.

Keywords: AMD, ROP, novel anti-angiogenic protein, endothelium, TGF-β, and BMP antagonist, mitochondria

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List of Abbreviations

ADP: Adenosine diphosphate

αKG: alpha-ketoglutarate

AKT: AKT Serine/Threonine Kinase

ADHA1: Alcohol Dehydrogenase 1A

ALK: Activin receptor-like kinases

ALK1: activin receptor-like kinase 1

AMD: Age-related macular degeneration

AM: Anophtalmia-microphtalmia

AMH: anti-Müllerian hormone

ATP: Adenosine triphosphate

AVM: arteriovenous malformaitons

BAMBI: BMP And Activin Membrane-Bound Inhibitor

BBB: Blood-brain barrier

BCA:Bicinchoninic Acid

bFGF : Basic fibroblast growth factor

BMP: bone morphogenetic protein

BM: Bruch's membrane

BRB: blood-retinal barrier

cAMP: cyclic adenosine monophosphate

CATT: Comparison of Age-Related Macular Degeneration Treatment Trials

CBC: CREB-binding protein

CD31: a cluster of differentiation 31

CDC42: Cell Division Cycle 42

Cdh5CreERT2: Endothelial-specific Cadherin 5

CDK1:cyclin-dependent kinase 1

CDKN1A: p21 Cip 1

CER1: Cerberus

CERL2: Cerberus like 2

CNV: choroidal neovascularisation

CollIV: collagen type IV

CoSMAD4: the SMAD transcriptional factors and proteins consist of common

CPT1A: carnitine palmitoyltransferase 1A

CBP: CREB-binding protein

dapB: 4-hydroxy-tetrahydrodipicolinate reductase

DAN: Differential screening-selected gene Aberrative in Neuroblastoma

DCFDA: 20,70–dichlorofluorescein diacetate

CREB: cAMP response element-binding protein

DEseq2: differential expression sequencing

2-DG: 2-deoxy-D-glucose

Dll-4: Delta-like 4

DMEM: Dulbecco's Modified Eagle Medium

EBM: Endothelial Cell Growth Basal Medium

EC: Endothelial Cells

ECAR: Extracellular acidification rate

ECM: extra cellular membrane

EdU: 5-Ethynyl-2´-deoxyuridine

ENG:Endoglin

ERG:electroretinogram

ERK: Extracellular regulated kinases

ETC: electron transport chain

FABP4: fatty acid-binding protein 4

FADH2: flavin adenine dinucleotide

FAO: Fatty acid oxidation

FBS: Fetal Bovine Serum

FGF: fibroblast growth factors

FIH: Factor-inhibiting

FITC: Fluorescein isothiocyanate

FKBP12: Prolyl Isomerase

FOXG1: foxhead box G1

FOXO:foxhead box O

GDP: guanosine diphosphate

GDF: growth differentiation factor

GREM1: Gremlin 1

GSEA: Gene Set Enrichment Analysis

GTP: guanosine triphosphate

GLUT1*:* Glucose transporter 1

HEY1: Hes Related Family BHLH Transcription Factor With YRPW Motif 1

HHT: hereditary hemorragic telangiectasia

HIF-1α: hypoxia-inducible factor-1

HIFs 1,2,3: Hypoxia-inducible factors 1,2,3

HK2: hexokinase 2

HRMECs: Human Retinal Microvascular Endothelial Cells

HUVEC: Human Umbilical Vein Cells

HRP: *Horseradish Peroxidase*

ID:DNA-binding protein

IGF-1: insulin-like growth factor-1

IGF2: insulin-like growth factor 2

IL-1: Interleukin 1

INL: inner nuclear layer

IPL: inner plexiform layer

I-SMADs: inhibitory SMADs

IsoB4: Isolectin B4

IVI: Intravitreal injection

LRP5/6: lipoprotein receptor-related protein-5 and 6

JAGG1: Jagged 1

MAP-kinase: mitogen-activated protein kinase

MTCO1: Mitochondrially Encoded Cytochrome C Oxidase I

NBL1: Neuroblastoma Suppressor of Tumorigenicity

NAC: N-acetyl cysteine

NAD: Nicotinamide adenine dinucleotide

NADKD1: mitochondrial NAD kinase 1

NADP: Nicotinamide adenine dinucleotide phosphate

nAMD: neovascular AMD

NDUFV2: NADH dehydrogenase

NHDF: Normal Human Dermal Fibroblasts

NO: nitric oxide

NOS: nitrous oxide systems

NOS3 : nitric oxide synthase

NOX1 : NADPH Oxidase

NRP1-NRP2: Neuropilins1, 2

3NP: 3-Nitropropionic acid

OCR: oxygen consumption rates

OCT: Optical coherence tomography

OCT compound: Optimal cutting temperature

OCTA: Optical coherence tomography angiography

OIR: oxygen-induced retinopathy

ONL: outer nuclear layer

OPL: outer plexiform layer

OPHS: oxidative phosphorylation

PH3: phospho-histone 3

PAH: pulmonary arterial hypertension

PlGF: placental growth factor

PDGF: platelet-derived growth factor

PDH: pyruvate dehydrogenase

PDK1: Pyruvate Dehydrogenase Kinase 1

PECAM-1: Platelet endothelial cell adhesion molecule

PKM2: pyruvate kinase M2

PFA: paraformaldehyde

PFKFB3: phosphofructokinase-2/fructose-2,6-bisphosphatase

PHDs: prolyl hydroxylase domain

POU2F1: Oct-1

QPC-KO: respiration in mitochondria

RGC: Retinal ganglion cell

RhO: Ras homologous

Robo: Roundabout

ROP: Retinopathy of prematurity

ROS: reactive oxygen species

RNFL: Retinal nerve fiber layer

RPE: Retinal pigmented epithelium

R-SMADs: receptor SMADs

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM: standard error of the mean

SERPINE1: serpin family E member 1

SFRP2: secreted frizzled-related protein 2

SLIT2: Slit Guidance Ligand 2

SMAD: Mothers Against Decapentaplegic Homolog

SMAD6 and SMAD7: I-Smads

SMCS: Smooth muscle cells

SOST: Sclerostin

TβRI-TβRIII: TGF-β types I-III

TCA: tricarboxylic acid

TGF-β: transforming growth factor-beta

TGF**α**: transforming growth factor-alpha

TNF**α**: tumor necrosis factor-alpha

Trp63: transcription factor p63

UPR: unfolded protein response

USAG: uterine sensitization-associated gene-1

UQCRC1: Ubiquinol-Cytochrome C Reductase Core Protein

Uqcrq: Ubiquinol-Cytochrome C Reductase Complex III Subunit VII

VCP: Transitional Endoplasmic Reticulum ATPase

Vhl:Von Hippel–Lindau protein

VE(Cadherin): Vascular endothelial cadherin

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor receptor

WNT: Wingless-related integration site

ZO: zonula occludens

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Chapter I: A literature review

This chapter summarizes the current literature on ocular angiogenesis and the pathways of interest.

1.1. The retina

The retina is a light-sensitive tissue located at the back of the eye and belongs to the central nervous system. The primary retina's function is to convert light into electrical impulses by specialized photosensitive neurons, enabling vision.

1.1.1 Anatomy of the retina

The anatomy of the retina differs among species, and its organization into cellular layers and their specific function permits visual acuity. Anatomically, the retina is composed of the innermost neural retina and the RPE, separating the retina from the choroid.

The primary function of the retina is to convert the light signal into an electrical signal allowing the brain to process it as an image. The retina is highly organized in multiple cellular layers: pigmented layer, photoreceptor layer, membrane limitans externa, outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), retina ganglion layer, stratum opticum, and membrana limitans interna. All different cells of the retina participate in the process of vision with specific functions. Ultimately, the five main neuronal and glial cell types most preserved across vertebrate species for its physiological relevance are photoreceptors, horizontal cells, bipolar cells, retinal ganglion cells (RGC), and Müller cells (Fig. 1).

The photoreceptors are responsible for sensing the light; they lay adjacent to the retinal pigmented epithelium (RPE) layer, which is accountable for their proper survival and maintenance by releasing neurotrophic factors such as vascular endothelial growth factor (VEGF). Rods and cones are the two main types of photoreceptors. The photoreceptors extend from the

photoreceptor layer, with the nuclei in the outer nuclear layer, into the outer plexiform layer, where the synapsis connect**s** with the horizontal and the bipolar cells. The horizontal cells moderate the synaptic signal among photoreceptors and bipolar cells (Fig. 1). The abundance of photoreceptors, their morphology, and the horizontal cell networks do vary across species. In the IPL, bipolar cells interact with retinal ganglion and amacrine cells. The axons of the RGC, forming the optic nerve, are the only neural cells extending their axons to the brain's visual center.

In primates, the region of the retina with the highest concentration of photoreceptors (enriched in cones) and responsible for central vision is the macula. The macula's center is known as the fovea and represents the retina's thinner outer nuclear layers, nourishing the choriocapillaris beneath. When observing the vasculature by fundus angiography, the macula appears avascular.

1.1.2. The development of the retina

The specialized neurons that form the vertebrate retina develop at distinct embryonic and post-natal development stages. Some cell types, such as amacrine, horizontal, RGC, and cones, develop within the first days after birth, while rods, bipolar cells, and glia develop after the first week before the eye opens (67).

In vivo microscopy has provided insight that the genesis of the retina initiates embryonically in the dendrites of the RGCs, horizontal cells, and cone photoreceptors to a great extent before birth. In the mouse embryonic retina, for example, amacrine cells initiate at embryonic day (E) 16 and are complete before the postnatal day (P) 3 (68, 69). The mouse rods start developing and E16 until the postnatal period. The last to start their genesis are bipolar cells and Müller cells, which mature in the first postnatal week (70-72). The axons of bipolar cells can form an IPL alone without counting on Müller cells, amacrine cells, and RGCs (73).

The genesis of the retina is coordinated by neuronal cues that dictate the lamination of the layers and the synaptic transmission. Analogously to neuronal development, blood vessels invade the retina promptly to provide an energetic supply and comply with the high metabolic demand.

Angiogenesis, or the creation of blood vessels, is primarily originated in the yolk sac, which is differentiated from the mesoderm. The formation of blood vessels occurs through a phenomenon known as sprouting, in which mature endothelial cells (ECs) migrate and proliferate to create novel vascular branches further stabilized by mural cells or pericytes. In the eye, these neovascular mechanisms are crucial during development and disease (74-77).

At birth, most non-primate mammals present an avascular retina, and the vasculature matures around the first month. (78, 79). The vasculature of the retina develops in humans during the final trimester of pregnancy and completes upon birth. The first plexus to invade the retina is the superficial one, and it branches to originate the intermediate and deep plexuses (Fig. 1) via sprouting following neurotrophic gradients and guidance cues. Initially, the hyaloid plexus vasculature and the choriocapillaris provide the retina with blood supply before the vessels populate the retina throughout the ocular formation. The physiological regression of the vessels of the hyaloid plexus, the distinction of the neuronal layers, and the ultimate growth of thicker retina create a hypoxic milieu favorable for vascular development (80). Three planar plexuses of the vasculature mature during mammalian evolution of the retina with non-vascular and vascular regions (Fig. 1).

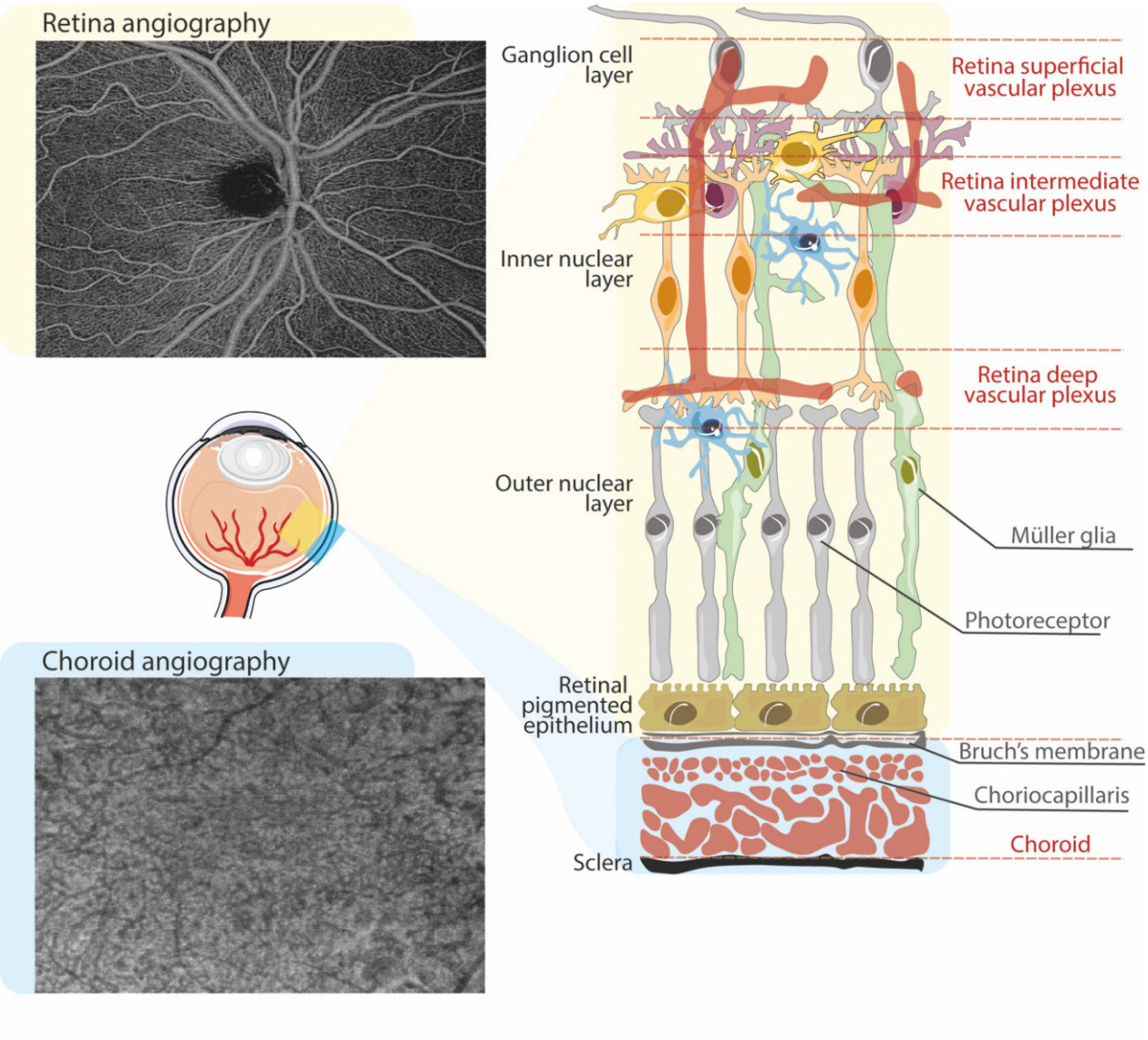


Fig.1 Schematic illustration depicting the different vascular plexus of the retina and cell types kindly provided by Dr. Sergio Crespo-Garcia. Angiography of the retina and choroid of a healthy adult human, kindly provided by Dr. Marisse Masis Solano.

1.1.3. The neurovascular unit

The neurovascular unit consists of the physical and biochemical interaction of neurons with vascular and mural cells necessary for maintaining the homeostasis of the retina. During disease, this homeostasis is dysregulated and the neurovascular unit, uncoupled.

A functionally stable vasculature requires several cell types: ECs, perivascular or mural cells such as pericytes, glial cells such as astrocytes and Müller cells, and ultimately immune cells as macrophages or microglia. The coordinated function of these cellular elements is accomplished through the phenomenon known as neurovascular coupling.

ECs display specific differences between tissues based on their location, but their main goal remains to constitute a barrier based on tight junctions that enable the so-called blood-retina barrier (81). Retinal endothelial cells form the blood-retina barrier in tight junctions, choroidal endothelial cells are fenestrated, while Human Umbilical Vein endothelial (HUVECs) are primary vein cells from the umbilical cord. Cultured endothelial cells from different vascular beds show several phenotype similarities; some differences have been noted, which could be associated with the tight blood barrier of Human Retinal Microvascular Endothelial Cells HRMECs (82).

The principal migrating tip cell leads to avascular areas in nascent vessels while the stalk cells proliferate and form the vessel lumen upon maturation (83, 84). The ECs present a dynamically exchangeable plastic phenotype between stalk, tip, and phalanx cells (83, 85). Proper enrollment of mural cells around the ECs is crucial for nascent blood vessel growth, function, and stability throughout developmental angiogenesis. Indeed, the absence of mural cells on the vasculature predisposes to a lack of vascular remodeling, vessel instability, and apoptosis. In mature blood vessels, mural cells are crucial preserving the blood vessel dormancy or quiescence (86).

The astrocytes are solely present in the vascularized retina. Preceding angiogenesis, astrocytes arise from the head of the optic nerve and are eventually located at the RGC layer together with the end feet of Müller cells. Adhesion molecules and growth factors facilitate astrocyte patterning as a guiding cue. Growth factors are secreted upon retinal hypoxia, causing the endothelial cells to proliferate and the retina to vascularize (87). Astrocytes and Müller cells create a VEGF gradient to coordinate angiogenesis, modulating endothelial cell proliferation (87, 88). The differentiation and growth of neurons create hypoxia, to which photoreceptors, Müller cells, and RPE express growth and generate a gradient of cytokine towards the outer retina (89).

1.1.4. VEGF and VEGF-dependent pathways in angiogenesis

VEGF gradient in the retina is the primary driver of both physiological and pathological angiogenesis. The VEGF, part of the platelet-derived-growth factor subfamily (PDGF), comprises VEGF-A (usually denoted as VEGF), VEGF-B, VEGF-C, VEGF-D, and the placental growth factor (PlGF) (90). VEGFs bind principally to kinase receptors: Flt1 or VEGR1, VEGFR2, and VEGFR3 (91).

VEGF-A, identified as a vascular permeability factor specific to endothelial cells, is expressed upon hypoxia and activation of numerous factors, including reactive oxygen species (ROS) (92-96). In ECs, the signaling of VEGF/ VEGFR2 has significant functions, inducing several pathways like Phosphoinositide 3-kinases (PI3-kinase)–Akt pathway and the mitogen-activated protein kinase (MAP-kinase) (97), which trigger the migration and proliferation of ECs, impede apoptosis and augment the vessel permeability, cause degradation of the ECM, and extension of filopodia (98). VEGFR1 acts principally as a decoy receptor with a greater affinity to VEGF-A than VEGFR2. VEGF-A has different splicing isoforms, and the achieved distance depends on which one is secreted, guides the nascent vasculature, and is crucial for the vascular patterning of the brain and the retina (99).

Multiple studies demonstrated that VEGF is crucial in angiogenesis during the formation of the retina. During development, astrocytes express VEGF-A, whereas ECs express VEGFR1 and VEGFR2 [9]. Müller cells had augmented VEGF expression in a murine model of ischemic retinopathy, and suppression with a soluble VEGFR decreases retinal angiogenesis (100)**.** Various transgenic mice models show the relevance in angiogenesis of VEGFs and VEGFRs via impacting the vascular retina phenotype (93, 99, 101-142). The canonical VEGF pathway is crucial in physiological angiogenesis, where it is overactivated by the pathological state. In neurons of the retina, the expression of VEGFR2 is also vital (130). A study emphasized the importance of neurons in establishing the accessible quantity of VEGF for angiogenesis (143). The neurotrophic role of the VEGFs and VEGFRs is also crucial since they regulate the survival of neurons (112, 120, 130, 131, 144-147). In physiological conditions, RGCs highly express VEGF-B, which is

neuroprotective but does not impact physiological or pathological angiogenesis (144). The different isoforms of VEGF create a diverse gradient based on their affinity to the extracellular matrix (ECM). The astrocytes and Müller cells generate a VEGF gradient in the murine retina, leading to the outgrowth of tip endothelial cells.

Multiple VEGF-dependent signaling pathways are relevant in developmental murine angiogenesis (148-163). During specification of arterial ECs, VEGF signals initiate the Notch pathway when the ligands Delta-like (Dll) and Jagged (Jagg) bind to Notch receptors (164, 165). A defective Notch signaling affects the differentiation of arteries and veins throughout vessel growth (166). Notch signaling triggers a transcription factor that decreases the expression of genes related to veins, such as growth differentiation factor (GDF) while increasing the genes related to artery specification such as keratin (167). Multiple other factors promote ECs proliferation and sprouting, such as FGFs (168), or the vasculature's stability, such as PDGFs (162, 163).

1.2. Pathological angiogenesis

Pathological angiogenesis consists of the growth of new vessels, generally due to the stimuli of growth factors such as VEGF. Several diseased angiogenic conditions require treatments to prevent neovascularization, such as the anti-VEGF drugs.

1.2.1. Pathological angiogenesis in ocular disease

Globally, ocular pathological angiogenesis is one of the leading causes of blindness and a socio-economic burden for healthcare systems [1-6]. In recent years, intravitreal injections of anti-VEGFs have been the standard of palliative therapy for neovascular ocular diseases [7-19].

Pathological neovascularization or dysfunctional angiogenesis causes several sight-threatening ocular pathologies such as retinopathy of prematurity (ROP) and neovascular age-related

macular degeneration (nAMD) (169-171). In ROP, for instance, hyperoxia decreases VEGF levels, and the retina vasculature regresses, leaving ischemic regions (172, 173). The use of anti-VEGFs therapy should consider that some VEGF levels are necessary to maintain blood vessel survival (104, 157, 174, 175). The majority of FDA-authorized anti-angiogenic treatments inhibit VEGF for neovascular ocular pathologies. Novel combinatory therapies of anti-angiogenic drugs targeting different pathways directed to additional cell types other than the ECs alone could help augment the current therapy efficiency. The treatment that targets VEGF/VEGFR2 signaling could be toxic while signaling through PlGF/VEGR1 solely affects pathological angiogenesis without compromising the physiological (93, 176, 177). Thus, there is a need to identify novel pathways for efficient and safe therapeutic approaches. Nowadays, multiple clinically approved therapy strategies target either a single VEGF or several of its isoforms, VEGFRs, or their downstream pathways (100).

1.2.2. Age-related macular degeneration (AMD)

AMD, present in two forms, primarily affects the macula of the retina and leads to loss of central vision, influencing patients' quality of life. AMD is mainly associated with age and also with other environmental and genetic factors (178). Worldwide, AMD affects 196 million people, and it is projected that by 2030, there will be 243 million due to an increase in the aging population (179). Estimates also show that more than 10.4 million people have vision impairment or blindness due to AMD (179). The rising number of AMD patients legally blind implicates a tremendous public health burden with socioeconomic consequences.

As mentioned above, AMD is a complex aging disease caused by multiple factors: numerous risk genes, dysregulated complement, and several pathways involving lipid, inflammation, and angiogenesis (180, 181). Studies from several continents show a higher occurrence of AMD in white ethnicity, a significant risk factor being older than sixty, and a higher prevalence in women

than men (182-184). Environmental aspects such as unhealthy diet and smoking can advance the disease from early to late phase (185).

Clinical guidelines classify AMD pathology into the early and the late phase. The early characteristic of AMD is the presence of drusen, deposits of lipids in the space among the Bruch's membrane, and RPE, which is altered at this stage of the disease. Patients with an early form of AMD do not have impaired vision. The late AMD stage subdivides in dry AMD, also known as geographic atrophy and neovascular (n) AMD. The nAMD is accountable for most of the cases of blindness, although it is the less recurrent form. The distinctive feature of nAMD is choroidal neovascularization underneath the macula, initiating many pro-inflammatory and pro-angiogenic pathways upon impairment of the RPE and outer retinal layer. In nAMD, the nascent irregular blood vasculature arises beneath the RPE, disrupting the Bruch's membrane and occupying the subretinal space. This process causes hemorrhages, lesions, and fibrosis, culminating in detachment of the retina, which results in lasting damage to the macula and vision (186).
The complex of choroidal neovascularization includes multiple distinctive injuries such as fluid accumulation, hemorrhage of the retina in different locations, detachment of RPE, deposits of lipid, and fibrosis which defining nAMD. Optical coherence tomography (OCT) allows the visualization of the details and characteristics of both neovascularization and drusen.

Fluorescein angiography distinguishes three distinct types of nAMD groups depending on the neovascular degree of infiltration in the retina (187-190). The primary loss of atrophic RPE, extended photoreceptors, and consequently, the choriocapillaris degeneration arises into AMD's late-stage and leads to geographic atrophy (191). Fluorescein angiography evaluates the position and the leakiness of the pathological choroidal neovascularization to diagnose nAMD. OCT angiography (OCTA) spots the choroidal vasculature while not discriminating against the leakage (192). These imaging techniques help both the diagnosis and the clinical classification and progression of the phases of the disease. Late-stage AMD can take weeks to months to develop to neovascular while transitioning from years to decades into atrophic.

1.2.2.1. Anti-VEGF therapy in nAMD

Anti-VEGF therapy is considered the most successful therapy for neovascular AMD and a standard of care through personalized periodical injections [24, 25]. VEGF is the main pro-angiogenic factor triggering choroidal neovascularisation; thus, intravitreal injection of anti-VEGF is the primary therapy for nAMD (193). Most patients necessitate frequent recurrent injections and consistent follow-up, given the ten percent being resistant to anti-VEGFs treatments and sporadic complications that could cause lasting vision loss (10, 14, 17, 18, 20-23, 30).

Nowadays, there is no effective treatment for the atrophic stage of this disease. In nAMD trials, the primary anti-VEGF drug was the aptamer pegaptanib, ligating bigger VEGF isoforms and $VEGF^{165}$ (194). A fragment of an antibody, ranibizumab, extensively used for nAMD, ligates every isoform of VEGF-A. The off-labeled used bevacizumab, a similar drug, also has this ability (195). The Comparison of Age-Related Macular Degeneration Treatment Trials (CATT) regarding the use of bevacizumab and ranibizumab demonstrated similar visual acuity outcomes (196). There is a preference for using off-label drugs in the clinic, given the benefit related to cost-efficiency. Recently, clinicians have started to utilize the novel recombinant protein, aflibercept, composed of VEGFR1 and VEGFR2 sequences, suppressing every VEGF-A and VEGF-B isoforms as well as PIGF. The trial of the VEGF trap-eye demonstrated that extending the intravitreal injection of aflibercept to two months has equivalent reduced fluid accumulation and maintenance of visual acuity outcomes similar to once-a-month ranibizumab (22). In most patients, clinicians can extend aflibercept injection for up to two months (197). First trials argued the requirements of once-a-month injections of anti-VEGF to preserve vision. More recent trials demonstrated that this therapeutic schedule is associated with marginally improved outcomes as patients required an average of eight intravitreal injections in the initial year (10, 22, 198, 199).

Even though most patients responded after three months of the first dose, approximately 1 out of 8 AMD patients responded late to the treatment (200). Given the temporary efficacy of anti-VEGFs, clinicians prefer to personalize treatment frequency to each case (24, 25). In multiple

countries, the anti-VEGF treatment has become a standard of care for nAMD patients. This allows the combination of flexible periods established on clinical outcomes of vision and remarking beneficial results up to two years (201-203). However, long-term adherence to treatment decreases efficiency (204). After five years in the Comparison of the AMD Treatment Trial (CATT), visual acuity decreased in twenty percent of patients, and half of the eyes injected with anti-VEGFs displayed fibrosis (205-207). Fibrosis results from disproportionate wound healing and creates permanent injury to photoreceptors (208). A retrospective of the CAAT study demonstrates that more than fifteen percent of AMD patients will develop geographic atrophy (26). A seven-year follow-up trial shows diminished visual acuity in thirty-seven percent of nAMD patients, macular atrophy area increased, and loss of visual acuity due to geographic atrophy (7, 27). The prompt diagnosis of nAMD will avoid undertreating the eyes and additional vision deterioration over time (27).

Further, the anti-VEGF therapy effectiveness needs to be assessed on the subtype of nAMD, since up to date, trials were done mainly on classical choroidal neovascularization (209, 210). Multiple trials examining double antagonism would benefit these nAMD patients, late responders, and the long-term side effects of the anti-VEGFs. Thus, there is collective clinical interest to improve the combination of drugs to postpone the advancement from early to late AMD.

1.2.3. Retinopathy of Prematurity (ROP)

ROP affects premature babies under a specific weight and gestational week. It is divided into five different phases and, depending on severity, can lead to blindness.

In physiological conditions, the retina initiates to vascularize at the sixteenth gestational week and completes entirely by the fortieth gestational week. Premature neonates with low weight and gestational age under thirty-two weeks at birth have atypical or postponed retinal vascular development and are vulnerable to injury arising in ROP (211). Upon the initial damage, the vasculature restarts to develop, deprived of onward direction and establishes ridges that can

reverse in the vitreous, culminating in hemorrhages and detachment of the retina caused by unrestricted neovascularization (212-214). In most ROP neonates, the vasculature will regress without treatment, while the most at risk are prematurely born under twenty-five weeks.

Clinical guidelines classify the ROP into two diverse stages. During phase I, hyperoxia stops vascular progression; and in phase II, hypoxia triggers the vessel's development. Several signaling pathways are involved in both stages (215). Premature babies are subjected to high oxygen at birth, leading to higher oxygen tensions than those observed in utero and promoting vasoconstriction that obstructs the choroidal and retinal vascular blood flow (216).

The oxidative damage defines phase I, promoting the expression of anti-angiogenic molecules and suppressing pro-angiogenic factors like VEGF and insulin growth factor I (IGF-I) (217-219). Precisely hyperoxia impairs tissue growths which secondarily reduce GH/IGF-1 while, on the other hand, intermittent hypoxia can reduce IGF-1 (220).

After hyperoxia, the oxidative injury forms ROS, causing damage to the microvasculature and apoptosis of ECs, while antioxidants can prevent it (221-224). The EC apoptosis is associated with novel capillaries and decreases VEGF levels (225-228).

Once neonates are relocated to room air from hyperoxia, hypoxia triggers the retinal vascular proliferation in Phase II of ROP. Hypoxia promotes VEGF levels increase, causing neovascularization of the retina and incursion in the vitreous typical of ROP and the oxygen-induced retinopathy (OIR) model (147, 229). VEGF expression in the inner retina decreases in hyperoxia and augments in room air. In the hyperoxic phase of the OIR model, VEGF intravitreal injection prevents EC apoptosis and rescues the retina blood vessels (229, 230). Studies show the importance of the hypoxia-mediated VEGF increase by stimulating the HIF-1α transcriptional factor in ROP (230-236). HIF-1α controls the stability and action of p53, which regulates multiple redox genes of the cells and is involved in the mitochondrial apoptosis pathway induced in several models of ischemia in the retina (237-239).

At low levels, IGF-I suppresses neovascularization of the retina, while high levels of IGF-I induce it in ROP (240). In ROP, neonates have low levels of retina IGF-I, and this period of low IGF-1 relates to the disease gravity (241).

In ROP, the physiological development of the retinal vasculature is interrupted, leaving the peripheric area empty. The saturation of oxygen augments from the placenta to room air upon birth in prematurity, causing the delay of physiological retinal vessel development. In this ROP Phase I, the central retina is vascularized while the periphery is avascular, showing no discrimination among the two areas (242, 243).

More than six weeks after birth, the avascular peripheral retina becomes thicker and partially developed and requires more oxygen for its metabolism. The environment of the retina changes to hypoxic, corresponding to Phase II of ROP when the vasculature develops again. This phase requires monitoring the patient and treatment; if the neovascularization advances, retinal detachment is possible. In Phase II of ROP, the vasculature restarts to develop centrifugally, while in certain neonates, the severity of the hypoxia in the periphery produces pathological levels of VEGF, causing abnormal retinal vasculature (244, 245).

ROP divides from stages one to stage five of disease development depending on the degree of the avascular retina. Using fundoscopy, it is possible to diagnose the various stages based on the three different avascular zones, determined by the diametric distance from the optic nerve head and fovea. Stage three would affect five percent of neonates necessitating therapy, especially if they have enlargement of the central retinal vasculature. Early well-timed therapy can prevent the detachment of the retina that can occur at stages 4 and 5, linked with reduced lasting consequences. Preterm neonates can have aggressive posterior ROP, which does not proceed by stage and develops extremely fast in pathological vessels.

1.2.3.1 ROP therapies

Several palliative treatments have been utilized depending on the different phases and degree of severity of ROP, with anti-VEGF drugs being predominant. Initially, half of the premature infants with ROP were treated with cryotherapy (246, 247). Laser photocoagulation of the retina substituted cryotherapy in ROP therapy (248-251). Both treatments obliterate the avascular retina to diminish pro-angiogenic factors generation in hypoxia. In most patients, it prevents the progress of the ROP to late stages, and the therapy is harmless. However, in some cases, laser therapy could augment myopia (252).

In Phase I, the augmented growth of retinal vessels would decrease the avascular peripheral retina later and restrain the pro-angiogenic factors, thus preventing Phase II. During ROP Phase I, it has been shown that retinal vessels can be normalized with supplements of IGF-1, crucial for physiological development, the weeks after birth (253-256). The indirect outcome of supplementing IGF-1/IGFBP3 is that despite augmenting its systemic levels, it did not affect the rate or severity of ROP but the rate of bronchopulmonary dysplasia (257-259). These clinical trials were completed [258], and ongoing studies are now investigating whether IGF-1/IGFBP3 can improve lung and brain outcomes (clinical trial NCT01096784).

Anti-VEGFs are safe for Phase II of ROP and beneficial some days upon therapy (244). When the pathological vasculature progress supplementing with IGF-1 could exacerbate the ROP. A large clinical trial, BEAT-ROP, compared intravitreal injections of anti-VEGF to the laser therapy in ROP, finding less recurrence of proliferation in the anti-VEGF treated babies (260). The advantage of bevacizumab is that it stops ROP progression in the majority of premature and does not damage the avascular retina like laser therapy (260). This study would require a long-term follow-up to examine the avascular retina beyond the fifty-four postmenstrual week and evaluate systemic effects upon anti-VEGF therapy (261-264). The correct ideal dosing of the bevacizumab in ROP needs to be confirmed due to its potential systemic impact on the development of other organs

(265-268). Studies show similar visual acuity among ROP patients treated with the laser than bevacizumab; the benefit is less myopia in ROP therapy with anti-VEGF (252, 269, 270).

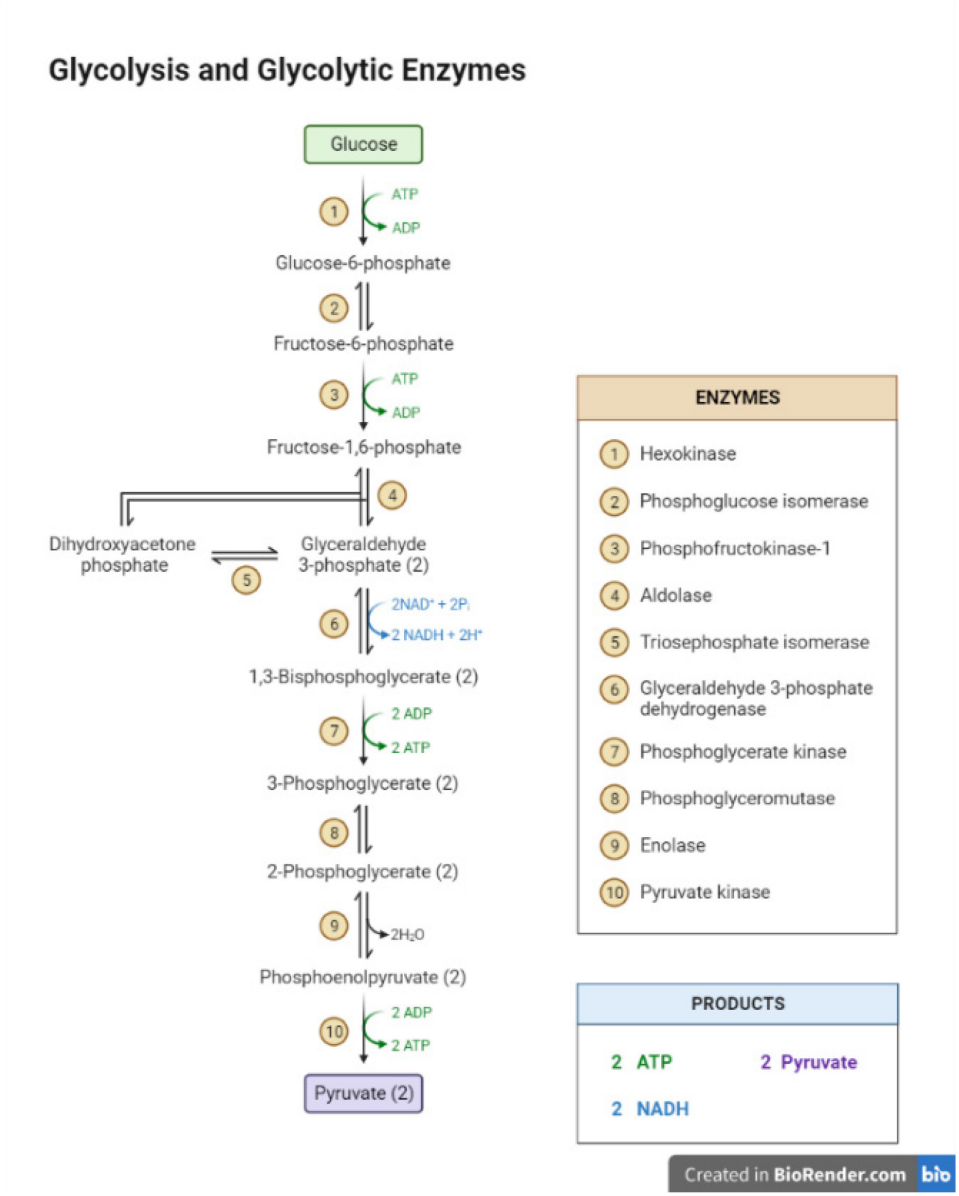
In animal models, lower and higher doses of anti-VEGF were similar in regulating the pathological angiogenesis, while the lower permit better peripheral vascularization (271). Ranibizumab**,** due to shorter systemic retention than bevacizumab**,** might be advantageous in ROP (199, 268, 272-274). The CARE-ROP clinical trial on ranibizumab is beneficial due to faster systemic clearance timing and the advantages of using half of the adult dosing to decrease side effects; it seems equally effective (10, 15, 275, 276). In premature babies, combination therapy of anti-VEGF and laser photocoagulation could be beneficial to avoid the recurrence generated by VEGF upon months of initially successful therapy (261, 277).

1.3. Aerobic and anaerobic respiration

Glycolysis occurs in the cytosol in both aerobic and anaerobic respiration and does not require oxygen. Aerobic respiration, which necessitates oxygen, includes glycolysis, pyruvate decarboxylation, tricarboxylic acid cycle (TCA), and oxidative phosphorylation. The Glut transporters first shuttle the glucose into the cytoplasm. In the first step of glycolysis, the glucose is retained in the cytoplasm through phosphorylation by hexokinase using ATP. The second step of glycolysis produces the first ATP molecule. In the third step, the final product of glycolysis leads to the production of two of each of the following molecules: pyruvate, ATP, NADH, water, hydrogen ions, and four free electrons. The four electrons with the two hydrogen ions produced by glycolysis are used by Nicotinamide Adenine Dinucleotide (NAD), forming the two NADH. The generation of ATP through glycolysis is named substrate-level phosphorylation.

Glycolysis generates two pyruvates that are shuttled to the mitochondrial matrix and, through pyruvate decarboxylation, are transformed into two acetyl CoA, which enter TCA or Krebs cycle from the NADH and the $FADH_2$ necessary for the electron transport chain.

The pyruvate decarboxylation also releases two CO_2 and two electrons, which will form one NADH. The pyruvate dehydrogenase complex, composed of three enzymes, regulates the pyruvate decarboxylation through its activation or inactivation. In resting conditions, inhibition of pyruvate dehydrogenase complex to convert pyruvate into Acetyl CoA prevents the TCA and the electron transport chain (ETC), thus consequently, ATP production.

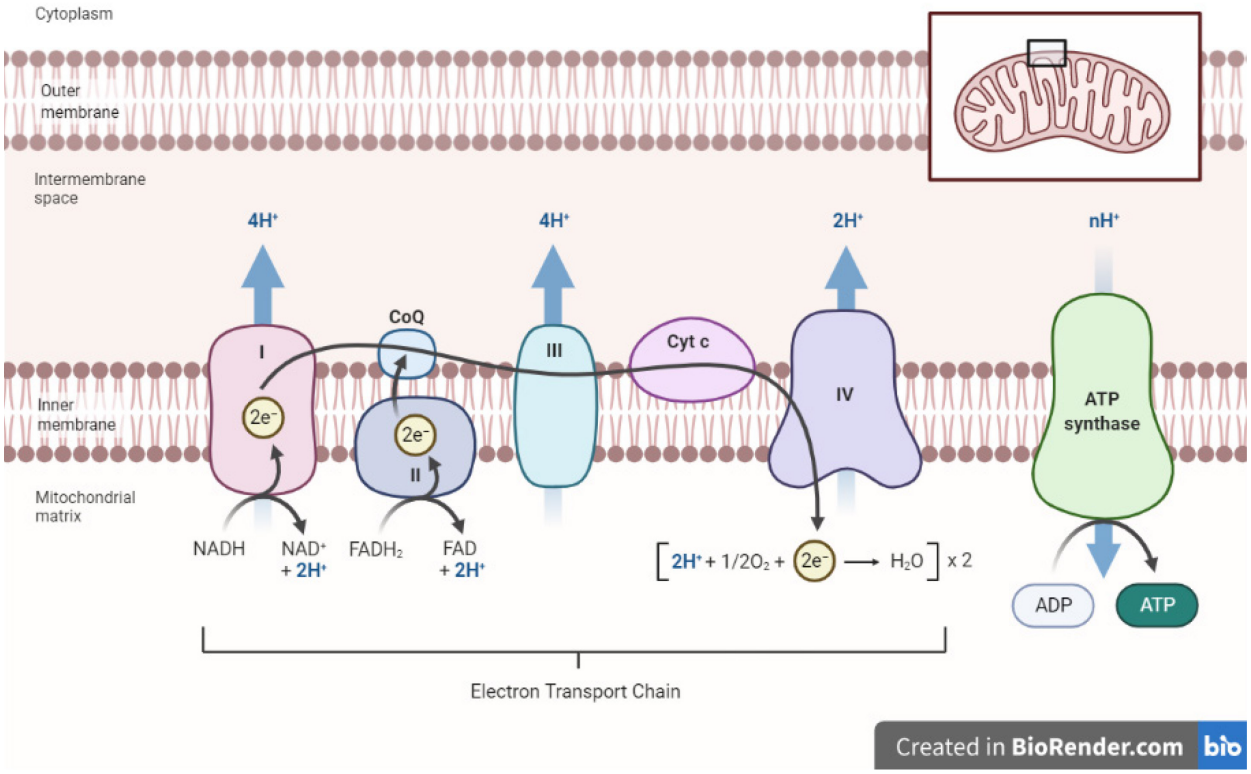


The TCA cycle occurs twice for each glucose; thus, with the initial two pyruvates, the final product six NADH, two $FADH_2$, four CO_2, two Guanosine-5'-triphosphate (GTP), and eight electrons. The NADH and $FADH_2$ carry the electrons generated during the TCA cycle into the inner membrane of the mitochondria to the electron transport chain (ETC).

The four multiprotein complexes of the ETC located in the inner mitochondrial membrane permit the electrons' movement by their gradient, creating a proton gradient across the two sides of the membrane, which is utilized to generate ATP during oxidative phosphorylation. The NADH dehydrogenase or complex I accept electrons from the NADH glycolysis and TCA, which are transferred to the coenzyme Q and oxidize NADH to NAD. Succinate reductase or complex II oxidase $FADH_2$ from the TCA into FAD. Cytochrome c oxidoreductase or complex III accepts electrons from ubiquinone and transfers them to cytochrome c, which relocates electrons to complex IV. Coenzyme Q or ubiquinone, located in the inner mitochondrial membrane, shuttles the electrons from complexes I or II to complex III; thus, this process reduces ubiquinone to ubiquinol. Cytochrome C reductase or complex IV reduces oxygen to water. Complex I, III, and IV pump protons into the matrix space of the mitochondria.

Complex V or ATP synthetase uses the proton energy to produce ATP, released into the mitochondrial matrix. Oxidative phosphorylation characterizes as the phosphorylation of ADP to ATP by the ETC. In this context, one NADH and one $FADH_2$ produce three and two ATP, respectively. Finally, one glucose molecule undergoes all the steps of aerobic respiration produces thirty-six ATP (278). The ETC oxidizes the NADH into NAD+, which is reutilized by glycolysis, pyruvate decarboxylation, and TCA.

In anaerobic respiration, the cell relies on glycolysis and lactic acid fermentation. Glycolysis is the only mechanism used to produce two ATP from one glucose. Glycolysis requires NAD^+ regeneration through lactic acid fermentation to take place in anaerobic conditions. Hypoxia leads to excessive production of pyruvate and NADH with a concomitant lack of NAD^+. The lactate dehydrogenase can reduce the pyruvate and NADH into lactic acid, decreasing cellular PH and NAD^+, which is reutilized for glycolysis. This reaction is reversible.



1.3.1. Endothelial cell metabolism

Several recent studies demonstrated the importance of endothelial cell metabolism in regulating angiogenesis. The endothelial cell metabolism drives angiogenesis and primarily produces ATP through aerobic glycolysis(279-284)(279-284)(279-284)(279-284) [278-295]. Modulation of glycolytic enzymes cause reduced angiogenesis, affecting the proliferation and migration of ECs *in vitro* and *in vivo* (281, 285-287) and decreased ocular pathological angiogenesis (284).

The ECs present a dynamically exchangeable phenotype between stalk, tip, and phalanx cells that play a crucial role in the developing vasculature (83, 288). Notably, multiple studies show control of murine retinal angiogenesis through modulation of metabolites and metabolic pathways, including glycolysis, oxidative phosphorylation, fatty acid oxidation (FAO), pentose phosphate

pathway, glutamine, serine, proline, and transcriptional factors such as FOXO and Myc depending on the different ECs phenotypes of the phalanx, tip, and stalk cells (279-284, 288-294).
The analysis of single-cell data obtained from choroidal neovascularization upon laser burn in the mice model of nAMD shows the dysregulation of several metabolic pathways such as retinol, amino acid, glucose transport, ATP synthesis, TCA, and oxidative phosphorylation (295).

1.3.2. The role of mitochondria in angiogenesis

Mitochondria are essential organelles for energy and ATP production, fueling cellular migration, proliferation, growth, and angiogenesis. Processes occurring in the mitochondria, such as tricarboxylic acid cycle (TCA) and fatty acid oxidation (FAO), induce endothelial cell proliferation and sprouting, while oxidative phosphorylation restricts ROS production (279-284, 289-294).
Damage of the complex III of the mitochondria decreases the TCA cycle, leading to diminished proliferation and altering angiogenic genes, causing defective signaling (279). In human umbilical vein endothelial cells (HUVECs), the ratio of NAD^+/NADH sustain the proliferation of ECs and complex III in regenerating NAD^+ without affecting migration (279, 296-298).
In mice with ECs-specific conditional ablation of a Ubiquinol-Cytochrome C Reductase Complex III Subunit VII (Uqcrq) subunit of the mitochondrial complex III, reduced respiration leads to decreased proliferation and post-natal angiogenesis of the retina with no changes in migration (279, 299, 300). The gene set enrichment analysis (GSEA) of RNA-sequencing on ECs of lungs from these knock-out pups showed augmented proliferation genes as cell cycle targets, glycolysis, mTOR, MYC, mitochondrial metabolism, and stress response (279, 301). Specific removal in endothelial cells or overexpression of MYC affects mitochondria, glycolysis, and proliferation in the developing retina. FOXO1 also represses MYC and maintains the endothelium quiescent through metabolic suppression (66, 302-306).

ROS released from the mitochondrial ETC can lead to multiple signaling pathways such as inflammatory response, DNA damage, iron homeostasis, cell survival, and differentiation (302). More specifically, the first three complexes of the mitochondria produce ROS, such as peroxide and superoxide anions (279, 307). Complex III discharges ROS into both intermembrane space

and the matrix of the mitochondria, while complexes I and II only into the matrix (308). The ubiquinone of mitochondrial complex III produces augmented ROS, and its signaling stabilizes HIF-1α, thus necessitates a functional mitochondrial ETC (309-316). HIFs control VEGF to promote the development of the new vasculature, the insulin-like growth factor 2 (IGF2) that triggers the proliferation and survival of cells, and the transport of glucose permitting the production of ATP (317-319).

1.4. TGF-β signaling in angiogenesis

TGF-β signaling can have distinct angiogenic properties, depending on expression levels and the tissue context in development, physiology, and disease.

1.4.1. TGF-β in developmental angiogenesis

Mutations of the TGF-β signaling family members in humans and mice are linked to vascular development defects.

Although VEGF signaling is the most studied pathway related to angiogenesis, the signaling of transforming growth factor β (TGF-β) controls multiple pathways relevant to vascular development and disease. Indeed, several studies show the importance of TGF-β through the downregulation of several signaling members. Contingent on the tissue and expression levels, the signaling of TGF-β during vessel development could exert either pro-angiogenic or anti-angiogenic properties (320).

The superfamily of TGF-β comprises bone morphogenetic proteins (BMPs), the three TGF-β1, -β2, and -β3, three primary receptors of TGF-β types (TβRs), seven activin receptor-like kinases (ALK) among other members (321-324). The TGF-β ligation to its receptors triggers intracellular signaling of the transcription factor mothers against decapentaplegic homolog (SMAD) that translocate to the nucleus. The SMAD binding with fox headbox O (FOXO), cause the arrest of the

cell cycle (321). TGF-β also limits the inhibition factors playing a role in the expression of c-Myc (325-327). TGF-β can bind ALK1, distinctively expressed in ECs, signaling by SMAD1/5/8, promoting angiogenic molecules like ID1 (328-331). Multiple antagonist proteins act on the canonical signaling of TGF-β, including foxhead box G1 (FOXG1) and PI3K through AKT suppresses the complex of SMADs-FOXO (325).

The role of TGF-βs in angiogenesis is varied; early studies show both that TGF-βs suppress proliferation and migration in ECs and further enhances proliferation (332, 333). The ratio of expression of TGF-βs elucidates the initial inconsistency in the studies since low levels have been proposed to enhance angiogenesis while higher levels may suppress ECs and vasculature development (334). ECs release of TGF-βs on mesenchymal cells causes the differentiation of pericyte and vascular smooth muscle cells (322).

TGF-β autoregulates its expression and regulates several angiogenic growth factors, some collagens, and integrins (335). Furthermore, both TGF-β and fibroblast growth factor (FGF2) induce VEGF expression in ECs (336, 337). TGF-β stabilizes FGF2 through restraining factors responsible for migration and capillaries development and, on the other hand, during the remodeling of capillaries**,** promotes apoptosis of ECs. VEGF-A acts as a pro-survival factor through the p38MAPK pathway (336, 338-340).

TGF-β controls angiogenesis through diverse receptors, such as ALK1 and ALK5, which rely on contrary signaling pathways for the development and proliferation of the vasculature (330). The ratio of ALK1 and ALK5 establishes the effects of TGF-β and necessitates the co-receptor endoglin (ENG) (341). The ALK1/Smad1/5/8 produces anti-angiogenic signaling while ALK5/Smad2/3 pathway promotes vasculature development through the secretion of ECM proteins (330, 342).

Multiple genetically engineered mice models of TGF-β family members are embryonically lethal due to defects in developmental angiogenesis (343-352). Furthermore, the importance of TGF-β

signaling in the central nervous system (CNS) and retinal developmental angiogenesis has also been shown through defects observed in KO animals (Table 1).

Table 1. **TGF-β pathway in CNS and retina developmental angiogenesis**

Gene	Mice Model	Age	Phenotype	Ref.
Itgα	*αv-/-*	E12.5-P0	Lethal, defective vessels of the brain	(353)
Itgβ	*β3/β5*$^{-/-}$	E12.5	No brain vessel abnormalities	
	itgβ8$^{Nestin\text{-}Cre}$ *itgβ8* $^{tie2\text{-}cre}$	P28	Embryo hemorrhages compensate in adults brain no hemorrhages of the brain	(354)
	itgβ8$^{-/-}$	P21-P30	50% lethal, endorse neuroblast migration by vessels	(355)
	Itgβ6/β8$^{-/-}$	E16.5-18.5	Brain vessel defects, similar to *Tgfb1*$^{-/-}$ *and Tgfb3*$^{-/-}$	(356)
	itgβ8$^{-/-}$ *Nestin*$^{Cre/+;}$*β8f*$^{lox/flox}$	P14	Retinal defects in the growth of the secondary plexus. Pathological retinal angiogenesis	(357)
TGFBR2	*Tgfbr2* ^{iECKO}	P14	Retina defects in the growth of the deep plexus and decreased pSmad2	(358)
TGFBR2 **ALK5** **Itgav**	*Tgfbr2* ^{iECKO} *Alk5* ^{iECKO} *Nestin*$^{-Cre}$*Itgav*$^{f/f}$	E11.5-13.5	Lethal brain vessel abnormalities β8 trigger TGF-βs controls angiogenesis in the developing brain	(359)
TGFBR2	*Alk1*$^{Cre/+}$*;Tgfbr2*$^{fl/fl}$ *Alk1*$^{-Cre}$*;Nrp1*$^{fl/fl}$*x* *Nestin*$^{-Cre}$*;β8*$^{fl/fl}$	E11.5-16.5	β8 triggers TGF-β signaling while Nrp1 reduces in ECs Deficient TGF-β by NRP1 or β8 augmented vascular branching and sprouting	(360)
Itgβ **SMAD4**	*itgb8*$^{Cre\text{-}}$ *smad4* $^{Cre\text{-}}$		Dysplasia of novel vessels	(361)

Multiple human vascular diseases are linked to mutations of proteins involved in TGF-β pathways, such as hereditary hemorrhagic telangiectasia (HHT), characterized by mutations of the genes Alk1 and endoglin and with arteriovenous malformations (AVM) in various organs (362). Families with a mutation in the genes of *ITGAV* and *ITGB8* are also inclined to hemorrhages of the brain (363).

1.4.2. TGF-β expression in the human retina

Various cell types of the human retina express TGF-β highlighting its relevance in ocular development and function. In the human eye's vitreous, aqueous humor, and posterior segment, while TGF-β2 is prevalent, the three TGF-β isoforms are also present (364-366).

The main isoform present in monkeys is TGF-β2 in the outer photoreceptor layer and the RPE choroid complex, with significantly higher concentration than the neural retina (364, 367, 368). In murine eyes without TGF-β2, the embryo morphogenesis of the eye is disrupted, contrary to TGF-β1 and TGF-β3 (369).

Table 2 shows the expression of TGF-βs in different cell types of the posterior segment of the human eye.

Table 2. **Expression of TGF-βs in the posterior segment of the human eye**

Cell type	**TGF-β1**	**TGF-β2**	**TGF-β3**	**Ref.**
Choriocapillaries's endothelium	Present	-		(370, 371)
Choroidal histiocytes	-		Present	
Choroidal vessels' connective tissue	-	Present	-	
Choroidal stroma	-	Present	-	
Ganglion cells	Present	-		
Microglia	Present			
Müller glia cells	Present	Present	Present	
Pericytes of superficial retinal vessels	Present		-	
Photoreceptors	Present	Present		
Photoreceptors' mitochondria	-		Present	
Photoreceptors' outer layer		Present		
Retinal Pigmented Epithelial (RPE) cells	Present		-	(367, 368, 372)
Smooth muscle cells (SMCs)	Present		-	(370, 371)
Vitreous hyalocytes	Present			

1.4.3. TGF-β in nAMD

TGF-β expression and its downstream signaling contribute to nAMD. Thus TGF-β has been proposed as a therapeutic target [32-42]. TGF-β acts as a pro-angiogenic factor in nAMD by inducing inflammation facilitated by macrophages, the proliferation of the choroidal ECs, or the VEGF-A release from RPE (34, 373-375). The levels of TGF-β1, although the least expressed isoform in the vitreous humor, augment in nAMD patients (60, 376). The nAMD patients' RPE has also enhanced the expression of TGF-β (377, 378). nAMD patients, upon anti-VEGF therapy, have reduced TGF-β2 levels in the aqueous humor compared to controls (379-381).

Experimentally, the most established model for studying nAMD is laser-induced choroidal neovascularization (CNV) in the mouse. In this model, an argon laser disrupts the Bruch's membrane and the RPE, triggering the choriocapillaris neovascularization into the subretinal space accompanied by inflammation. Unfortunately, the model only resembles the angiogenic of nAMD without the chronic age-related inflammation and senescence features. Furthermore, the macula is absent in rodents, so the neural retina laser injury is superior in nAMD patients (382, 383). The laser injury enhances the protein levels of TGF-β1 and TGF-β2 in the CNV in mice (42, 376, 384). Injection o TGF-β inhibitors decreased VEGF-A expression and CNV volume (42, 385, 386).

Other studies demonstrated that TGF-β2 produced by Müller cells suppresses the proliferation of ECs of the retina (387). TGF-β2 acts as a RPE pro-survival in human aged eyes and protects the Bruch's membrane of AMD patients (388). Table 3 shows the role of TGF-β family members in ocular pathological angiogenesis.

Table 3. **TGF-β pathway in ocular pathological angiogenesis**

Gene	Rodent Models	Age	Phenotype	Ref.
SMOC	*SMOC1*$^{+/-}$	Postnatal	Undeveloped retinal vasculature, through ALK1	(389)
LRG1	*Lrg1*$^{-/-}$ laser CNV		Diminished vasculature of the choroid and retina	(373)
TGF-β1	*ꞵB1-TGF-ꞵ1*	Postnatal	Trigger choriocapillaris atrophy, no CNV	(390, 391)
TGF-β2	*Tgfb2*$^{-/-}$	E12.5	Persistent vasculature of the vitreous	(392)
TGF-βR2	*Tgfbr2*$^{\Delta eye}$ *Tgfbr2*$^{\Delta EC}$	2-6 months	Augmented retinal VEGF-A expression and CNV phenotype of wet AMD (not in *Tgfbr2*$^{\Delta RPE)}$	(393)
	OIR, injection stem cells	P13-P17	TGF-β1 repressed unnecessary retinal angiogenesis	(394)
	IVI hTGF-β1		Impede retinal damage trigger by a component of drusen, mimic early AMD	(395) (396)

1.4.4. BMPs, a subfamily of TGF-β proteins

The bone morphogenetic proteins (BMPs) secreted proteins, a main subfamily of TGF-β, were first described as inducers of cartilage and bone growth. BMPs play different roles in the organism, such as cell proliferation, apoptosis, morphogenesis, development, and vascular pathologies (397-399).

BMPs, like other members of the TGF-β superfamily, have seven preserved cysteines, of which six form disulfide bridges assuring arrangement in the cysteine knot (400). The remaining cysteine forms disulfide bonds among the two dimers essential for its function can either be homodimers or heterodimers such as BMP2/BMP4, permitting the connections among diverse paths (323, 401-403).

Upon ligation with BMPs, the BMP receptor I (-RI) and BMP-RII receptors or (ALKs) also arrange into either homomeric or heteromeric complexes to signal (323). For example, BMPR-IA (ALK3) binds BMP2, and BMPR-IB (ALK6) ligate BMP4 with significant affinity. These receptors can activate signaling through both the canonical or non-canonical SMAD pathways (404-406). BMP signals through the heteromeric complex SMAD1/5/8, whereas TGF-β by SMAD2/3 carry the signal to the nucleus (403, 406-409). The non-canonical, SMAD independent signaling involves ERK, mitogen-activated protein kinases p38/MAPK, regulating various cellular responses such as differentiation, growth, survival, and apoptosis (408, 410).

Several extracellular antagonists of BMPs have been described, including noggin, chordin, ventroptin, follistatin, and FLRG. The extracellular antagonists of BMP also include the differential screening-selected gene and members aberrative in neuroblastoma (DAN) family, containing cysteine knot motifs similar to the superfamily of TGF-β which and directly ligate and preclude BMPs -receptor complex cooperation (398, 411, 412).

The heterodimerization among different BMPs, their antagonists, and receptors permits the interaction and downstream signaling through various pathways crucial in regulating the action of BMPs. Thus, this balance among activity and suppression of BMPs, receptors, and antagonists depends also on the different spatiotemporal expressions in development and adults (413).

1.4.5. BMP4 in the human eye

BMPs are of importance for the development and function of the human eye. Conserved mutations in the BMP4 gene led to variable abnormalities of ocular development, among which retinal dystrophy (54). Thus, BMP4 has a function in the development and preserving the retina (54). BMP4 expression in the developing eye shows from dorsally to ventrally changes upon maturation, demonstrating its signaling necessity during adulthood (54). The non-Mendelian genetics and inconsistent ocular developmental pathologies indicate a complex multigenic and

environmental interaction (54). Further studies of human families identified BMP4 truncation in variable ocular phenotypes, including a bilateral detachment of the retina (55, 56).

1.4.6. BMP in retina development and angiogenesis

BMPs are crucial in regulating angiogenesis, ocular development, and pathologies [43-53]. Mutations of several family members in both humans and mice, such as BMP4, lead to retinal defects during ocular development [54-58]. BMPs and their regulators have been proposed as therapeutic targets in ROP, nAMD, and geographic atrophy [52, 64-66].

Knockout mice of multiple BMPs, BMP-Rs, and BMP antagonists in the eye demonstrate their importance in the morphogenesis of anterior and posterior eye segments (43-51, 57, 414-429).

Table 4 summarizes various endothelial-specific knockout crucial BMPs, BMPRs, and downstream targets during vascular and retina developmental angiogenesis.

Table 4. BMP pathway in vascular development and retinal angiogenesis

Gene	Mice Model	Age	Phenotype	Ref.
BMP4	*Bmp4$^{CKO\ optic\ vescicle}$*	E10.5	the optic vesicle develops into RPE	(57)
	Bmp4$^{+/-}$	P14	variable retinal phenotype, leaky hyaloid vessels	(57)
BMPR1	*Bmpr1b$^{-/-}$*	E15	necessary for axon guidance and retinal cell survival	(44, 45)
	Bmpr1a$^{-/fx}$ Bmpr1b$^{+/-}$; & Bmp4$^{-/-}$	E11.5	Both mutants lack FGF15 expression in the retina. *Bmpr1b$^{+/-}$* caused the irregular dorsoventral shape	(430)
ALK3 SMAD4	Alk3$^{EC/EC}$ Smad4$^{EC/EC}$	E10.5	Similar embryonic vascular deficiency phenotype	(52)
BMPR2	*Bmpr2^{iECKO}*	P5	decreased retinal vessel density and sprouting	(52)
ALK1	*Alk1^{iECKO}*		Increased vascular density	
ALK2	*Alk2^{iECKO}*		decreased retinal vascular density without sprouting	
ALK3	*Alk3^{iECKO}*		decreased retinal vessel density and sprouting	

SMAD1 SMAD5	Smad1/5^{diECKO}	P6	controls the growth and sprouting of tip cells and AVM	(66)
SMAD4	Smad4$^{i\Delta EC}$		retinal AVM	(430)
BMPER	Bmper$^{+/-}$ OIR	P14	Revascularization, increased pSMAD1/5/8 and ERK1/2	(52)

Several studies show that both BMP and WNT signaling pathways are essential during the development of the eye. The Wnt/β-catenin pathway controls several growth factors during ocular development. In keratocytes lacking β-catenin, enhanced BMP4 induces the phosphorylation of SMAD 1/5, ERK1/2 in post-natal murine and human corneal epithelial cells. Furthermore, in the cornea, β-catenin binds and represses the action of the BMP4 promoter (424). *In vitro,* secreted BMP4 and secreted frizzled-related protein 2 (SFRP2) are anti-proliferative on adult retina stem cells; targeting these factors *in vivo* could reactivate these stem cells in blind patients (431). In various animal models, a dorsoventral gradient of BMP4 is also required for the proper patterning of the retina, and intermediate apoptosis plays a neuroprotective for amacrine and bipolar cells and antagonizes the adult RPE (44, 45, 51, 58, 432-436).

BMP2 and BMP4 are pro-angiogenic and essential during the development of the blood vasculature (65, 437-439). *In vitro*, BMP4 promotes angiogenesis and controls capillary sprouting through ERK1/2 pathway rather than Smad1/5 (440). Knockout mice of *Bmp2* and *Bmp4* are lethal during embryogenesis caused by diminished cardiovascular development (441). Indeed, some animals developed a thinner INL with defects in the optic nerve head, RPE, and irregular lamination of the retina. Interestingly, in *Bmp4*$^{+/-}$ heterozygote eyes, the RGCs were fifty percent less abundant than in the wild type, but the photoreceptors were largely unaffected (57). Furthermore, in *Bmp4*$^{+/-}$ *heterozygote eyes,* the hyaloid vasculature was not regressed given the reduced number of macrophages, and vessels present closer to the retina and are leaky (57).

BMP4 binds ALK2, ALK3, and ALK6 in a context-dependent manner and obliterations of these receptors cause embryonic lethality. The ligands Bmp2/4 demonstrate specificity for Bmpr1a or ALK3, exclusively expressed on venous ECs. Alk3 signals mainly through downstream Smad1/5/8 signaling and controls venous identity during development (52). Given the autonomous role of

ALK3 during venous development, targeting ALK3 could be a potential antiangiogenic therapy (52).

Some studies investigate the selective endothelial removal of different BMPRs and their downstream SMADs in murine models of post-natal retinal angiogenesis (52, 66, 303-306). (52). BMPRs are expressed differentially in the vasculature of the retina (52). BMPR-II, for instance, is expressed in the entire vasculature yet intensified at the vascular front of the developing retina (52). Selective removal of Bmpr-II and *the* Alk3 specifically in ECs, decreased vessel branching and sprouting at the front (52). In retinal vessels, the endothelial-specific deletion of ALK2 and ALK3 are crucial for pro-angiogenic BMP signaling, and both are required for retinal morphogenesis (52). Thus, at the vessel front, the retinal sprouts need the signaling of ALK3-BMPR II (52).

As previously mentioned, the downstream signaling of ALK3 occurs mainly through Smad1/5/8. The role of Smad signaling during vascular development was studied using endothelium-specific Smad1/5 inducible double mutants. Lack of Smad1/5 in the endothelium regulated cell cycle progression, induced sprouting of tip cells, augmented vascular hyperdensity, limited vascular regression in the post-natal retina (66, 442). Furthermore, the Smad1/5 double mutant leads to arteriovenous malformation (AVM), similarly to endoglin (ENG), ALK1, and Smad4 mutants (66, 303-306, 442).

Taken together, these studies show that BMP4 regulates eye development by modulating the angiogenic response and the development and function of neuronal cells (52, 57, 66, 303-306, 442).

1.5. The DAN family members, BMPs antagonists

The Differential screening-selected gene Aberrative in Neuroblastoma (DAN) family members form a diverse group of secreted proteins that act as antagonists of BMP, TGF-β, and Wnt signaling molecules and are involved in establishing anterior-posterior patterning in vertebrates.

1.5.1. DAN family members and angiogenesis

Some DAN family protein members could modulate angiogenesis through their regulation of Wnt and VEGF pathways. The majority of the studies focus on the role of the DAN family members during development (443). Seven DAN family genes have been identified: Gremlin1 (Grem1), Gremlin2 or protein-related to Cerberus and DAN (Grem2 or PRDC), COCO (Grem3 or DAND5), NBL1 (Dan), Cerberus, Sclerostin (SOST), and uterine sensitization-associated gene-1 (USAG-1 or Wise or SOSTDC1) (444, 445). The seven DAN family members share eight cysteine knot motif and undergo phosphorylation and N-glycosylation (446, 447). One of the most studied members of the DAN family, Grem1 antagonizes BMP2, BMP4, and VEGFR2 with great affinity while it binds BMP7 with a lesser affinity (445, 448, 449).

The DAN antagonists have variable specificities for the diverse BMPs and ligate additional members of the TGF-β family, such as activin B and Nodal by Cerberus (450-452). In *Xenopus,* Cerberus also binds BMP4 and WNT (453). SOST and SOSTDC1 ligate the two receptors, low-density lipoprotein receptor-related protein-5 and 6 (LRP5/6), and suppress the Wnt signaling (451, 454-456). Structural studies show that SOST comprises a main paired cystine-knot domain similar to growth factors (457, 458). Crystallography of NBL1, Gremlin1, and Gremlin2 demonstrates a steady non-disulfide-connected homodimer and Gremlin2 complex with GDF5 (458-464). The functional significances of the antagonists' structures are complex to establish while conveying the differences among the members of the DAN family.

Most BMP antagonists include amino acids with positive charges, which are crucial for ligating the heparan sulfate and heparin, restraining BMP signaling, and establishing a gradient in development (465, 466). Numerous DAN family members ligate the heparan sulfate directly, such as SOST, Grem1, Grem2, and USAG-1 through the conserved central region for this binding (444, 455, 457, 462, 464, 467). Also, predictive docking models suggest that Grem1, Grem2, SOST, USAG1, and COCO ligate the heparins, contrary to DAN and Cerberus (445). Separate regions of Grem1 bind to BMP4 and heparin; thus, the ligation to the latter defines the local BMP gradient (462, 464, 465). For example, the complex Grem2-BMP2 and Grem2–GDF5 bind to heparin with

greater affinity than either protein alone (461, 464, 468, 469). Several antagonists use these heparin bonds to augment the endocytosis of the complex ligand-antagonist controlling the rapid degradation of the BMP signaling (445, 470, 471). Extracellular Gremlin facilitates the internalization of BMP4 by clathrin-dependent endocytosis, causing the degradation of both and further decrease the phosphorylation of SMADs (472, 473).

Interestingly, some DAN family members have roles beyond TGF-β ligand inhibition and can modulate VEGF signaling pathways. Grem1 plays a role in angiogenesis and interacts with VEGFR2. Its dimerization is critical for the interaction with VEGFR2 since it necessitates autophosphorylation for the intracellular cascade (449, 467, 474, 475). Grem1 acts as a covalent homodimer and a monomer, and while dimerization is necessary for inducing angiogenesis, it is unessential for antagonizing BMPs. Gremlin also triggers the establishment of the complexes VEGFR2/$\alpha_v\beta_3$ integrin (476). In ECs, Gremlin also initiates proinflammation signals, causing cAMP and ROS assembly, and activates CREB, which controls permeability and migration and causes macrophage extravasation from the endothelium (477). Finally, Grem1 facilitates the epithelial-mesenchymal transition through the SMAD pathway, promotes vessel remodeling of ECs in pulmonary arterial hypertension (PAH) and migration (478). Grem1 is a pro-angiogenic protein and functions in the fibrosis of multiple organs such as the eye and heart (429, 449, 479-482).

In bovine pericytes *in vitro* and a diabetic murine model, high glucose triggers gremlin expression through the MAPK pathway. By suppressing the signaling of BMP, Gremlin could have a function in diabetic retinopathy (483). BMP4 increased the human RPE release of VEGF in both time- and dose-dependent manners. BMP4 could be controlling, through this mechanism, the angiogenesis in diabetic retinopathy (484).

1.5.2. COCO, a member of the DAN family

COCO, a member of the DAN family, has been mainly studied in heart development and left-right patterning in mice and *Xenopus,* respectively.

COCO, also known as DAND5 or *Cerberus like 2* (CERL2), is the less studied member of the DAN family. The DAND5 gene localizes on the human chromosome 19p13.13, and deletions in this region link to ophthalmological dysfunctions like strabismus, myopia, and optic nerve dysfunction (485, 486). In humans, mutations in COCO lead to congenital heart diseases due to laterality defects (487). COCO include**s** cysteine motifs crucial for its biological function (444). A bioinformatic model of COCO structure based on structural homology is available (488).
COCO was initially identified in a screen of genes induced by SMAD7. It has a gradient-based maternal expression in the ectoderm that decays upon gastrulation in *Xenopus* (489). COCO's exclusive expression pattern and timing contrast to zygotically conserved post-gastrulation of the other secreted inhibitors of BMP, TGF-β, and Wnt (489). The human and murine COCO homologs are expressed in multipotent stem cells, indicating a comparable action in the embryogenesis of mammals (489).

COCO knock-out *(Dand5$^{-/-}$)* mice show that some mice die at P1, mainly caused by cardiac deficits. The wall of the left ventricular myocardium augments significantly in newborn *Dand5 $^{-/-,}$* which is independent of anomalies in the laterality (490). In *Dand5$^{-/-}$* at E13, elevated mitosis of the cardiomyocytes link with enhanced *Cyclin type D1* (Ccnd1) expression in the left heart ventricle, indicating a controlling function of COCO in cardiogenesis (490). At this time point, the left ventricle, compared to the right one, demonstrated an increased ratio of COCO expression, explaining its involvement in heart muscle development (490). In embryonic hearts *of Dand5$^{-/-}$*, show augmented pSmad2 and *Baf60c,* and the latter ATPase controls the extended stimulation of Nodal and TGF-βs signaling in postnatal hearts (490).

The establishment of the asymmetric left-right body axis is vital for the growth of vertebrate embryos, in which Nodal plays a central role (491). Loss of COCO, a Nodal antagonist, leads to abnormalities like heterotaxia where organs are misplaced, inverted, or present asymmetry right or left atrial isomerism (491). This defective left-right axis correlates with congenital heart diseases. Thus, the COCO regulation of Nodal is crucial for left-right laterality, as previously shown in *Xenopus* (491, 492). In humans, mutations of genes of the TGF-β pathways, such as

either deletion or duplication of TGFBR2, result in heterotaxia comparable to other genes involved in congenital heart diseases (493-495). In mice, targeted cardiac removal of TGFBR2 leads to atypical looping of the heart (496). In *Xenopus*, a feedback loop among COCO and Wnt also regulates the left-right axis (497).

COCO is a robust soluble antagonist of BMP4 and suppresses its downstream SMAD 1/5/8 signaling (498-500). COCO also inhibits Nodal, TGF-β, and Wnt pathways (501). In *Xenopous*, COCO ligates TGF-β1 and increases its binding to its ALK5 receptor (502).

1.5.3. COCO, a BMP antagonist, in the retina

Despite increasing evidence, little is known about the BMP antagonist, COCO, in the retina. The Human Protein Atlas show expression of COCO in various organs such as the heart, blood, brain, and retina (503). Cardiomyocytes are the cells showing the highest expression of Coco. In the retina, COCO is present in rod photoreceptors and the Müller cells (503). At a cellular level, the secreted COCO co-stains with mitochondria in several cell lines (504). COCO plays a role in neural cell fate differentiation (489). COCO potently induced human embryonic stem cell differentiation into cone photoreceptors by inhibiting Activin, TGF-β, BMP, and Wnt signaling (501).

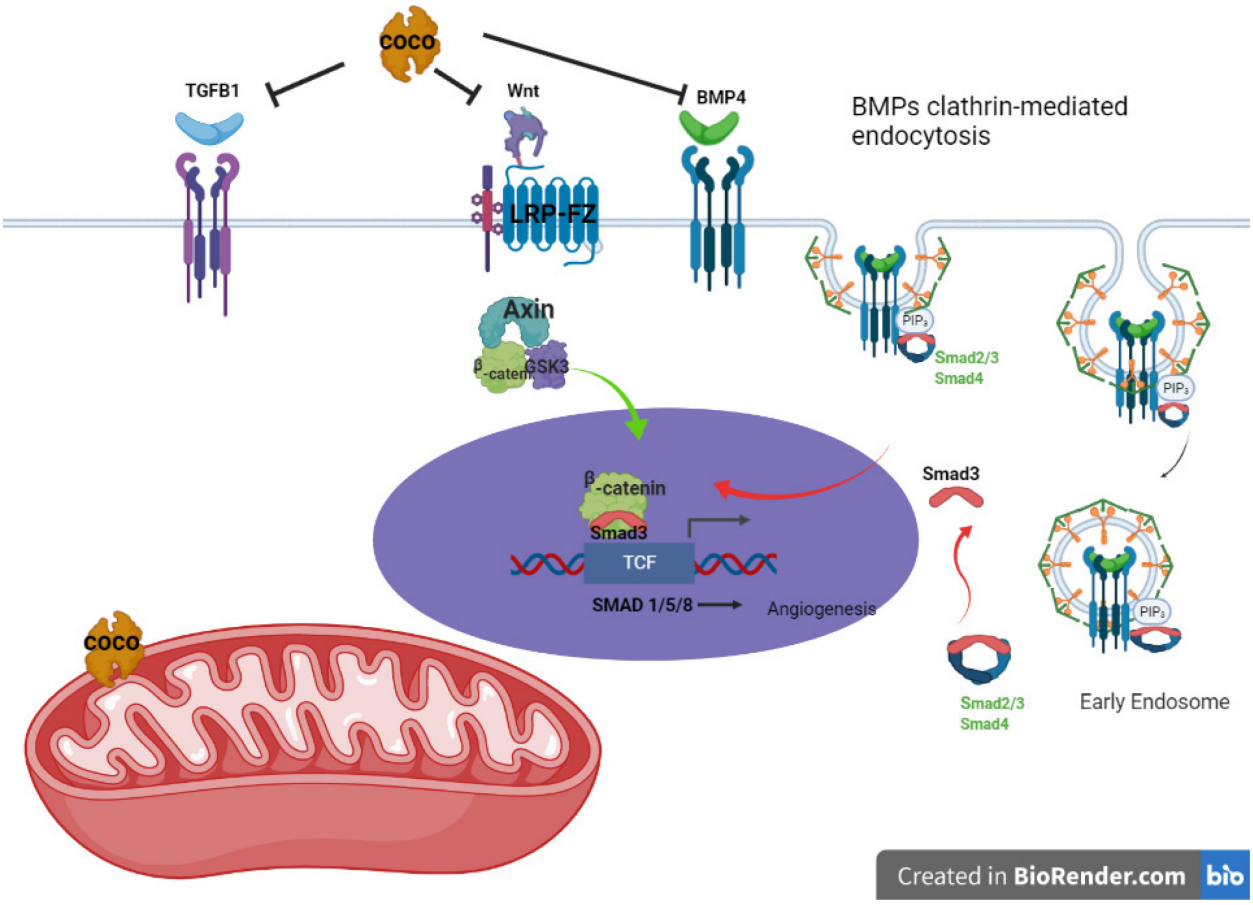


Fig. 4. Schematic combines knowledge from current literature on COCO and its binding partners. The DAN family member Gremlin1, similar in structure to COCO, ligate BMP4 and heparin; these lead to endocytosis in a clathrin-dependent manner and control the BMP signaling, suppress SMADs phosphorylation, and regulates intracellular signaling of BMP4 through Smad1/5/8 (498-500). TGF-βRs also internalize by caveolin-dependent mechanism. COCO ligates TGF-β1 /ALK5 receptor and inhibits Nodal, TGF-β, and Wnt pathways (501, 502). BMP4 ligates ALK3. Receptors BMPRII and ALK3, and the transcriptional Smad1/5 regulate retinal vascular development (66). COCO presents in two isoforms, localizes on mitochondria, and is secreted (503, 504).

Chapter II: Hypothesis

Globally, ocular vascular diseases like nAMD are the main reasons for damaged vision and blindness in the aging population (1-6). In the last decade, intravitreal injections of anti-VEGFs have transformed the therapy for those mentioned above ocular vascular diseases, demonstrating the safety and effectiveness of palliative care (7-19). However, despite its clinical success, a significant percentage of patients show resistance to anti-VEGF treatments (10, 20-22) and sporadic complications leading to vision loss (14, 17, 18, 23). Further, anti-VEGFs usually requires monthly injections given the temporary efficacy and clinician use the approach of treat and extend to establish a proper interval personalized to each patient (24, 25). Long-term intravitreal injections with anti-VEGF in clinical trials up to seven years show that nAMD patients develop further vision loss due to geographic atrophy (26, 27).

Therefore, there is a need for multiple complementary anti-angiogenic drugs to regulate the ocular neovascular pathologies that address these limitations of anti-VEGFs by targeting the non-responders and minimizing the burden of repeated injections (28, 29). Thus, additional or combination treatment that suppresses different angiogenic signaling pathways has a greater possibility to be therapeutically beneficial for ocular pathological neovascularization such as nAMD (30).

The TGF-β family, containing the BMPs subfamily, is responsible for several crucial functions such as proliferation, differentiation, development of neurons, fibrosis, and angiogenesis (31). The TGF-β pathway plays a role in the choroidal neovascularization of nAMD patients, and, as such, it could be a potential therapeutic target (32-36). Indeed, augmented intravitreal levels of TGF-β_1 correlated with retinal angiogenesis, triggers VEGF release in RPE, and regulates ECs proliferation (34, 37-42). In addition, BMPs are also necessary for ocular development and pathologies (43-51) and the regulation of angiogenesis (52, 53). For example, mutations in the BMP4 gene in humans cause variable abnormalities of ocular development, retinal dystrophy,

and bilateral retina detachment (54-56). Comparably, heterozygote BMP4 mice present a similar retinal phenotype with dystrophy related to anomalies of RPE and irregular leaky vessels (57, 58). Studies have proposed BMP4 as a therapeutic target in patients with nAMD and geographic atrophy. Other regulators of BMP signaling also control retinal angiogenesis in a model of ROP and are proposed as potential therapeutical targets (52, 64-66).

Based on the previous knowledge that TGF-β and BMPs are essential regulators of retinal and choroidal angiogenesis, we propose that the DAN family member COCO, which acts as a modulator of BMP and TGF-β signaling and possesses neurotrophic effects on cultured photoreceptors, could represent a beneficial therapeutic strategy to prevent neovascularization in nAMD or ROP.

To evaluate the efficacy of COCO for the treatment of neovascular ocular pathologies, we will investigate the effects of COCO delivery on retinal and choroidal neovascularization during development and in pathological models. Secondly, the development of therapeutic strategies targeting ocular neovascularization involving COCO requires a better understanding of its physiological role and mechanism of action. As such, we will also evaluate the cellular and molecular effects of exogenous and endogenous COCO during angiogenesis and ocular development. For the first time, our studies will provide a comprehensive examination of the impacts of COCO on retinal vascular development, establish its molecular features, and determine its biologic significance. This study will also offer steps towards the development of new therapies for neovascular ocular diseases.

Chapter III: COCO/Dand5 inhibits developmental and pathological ocular angiogenesis

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The author's contribution.

NP: manuscript writing, synopsis figure, literature review, experimental proposal, and analysis. *In vivo* experiments: breeding, maintaining the murine colony, intravitreal injections in pups and adult mice, retinal and choroidal dissection, immunostainings, cryosections, RNAscope, and microscopy. *Ex vivo* experiments: choroidal sprouting assay and analysis. *In vitro experiments:* scratch assay, polarity, mitochondrial co-localization immunostainings and microscopy, flow cytometry with analysis, western blot on COCO. RNA extraction, quantification, and transcriptomic analysis.

EH: *In vitro* metabolism and western blots on VEGF signaling. Quantification of the following: scratch assay, polarity, pH3, and cleaved caspase 3.

AB: Immunofluorescence on human retina and photoreceptors

FP: OIR and laser-CNV experiments

MB: Libraries and transcriptomics analysis

BL & GB: project conception, manuscript writing; guidance in designing of hypothesis and experiments.

Rational

Globally, ocular pathological angiogenesis is the leading cause of blindness, such as nAMD and ROP. Clinically effective intravitreal injection of anti-VEGF suppresses ocular angiogenesis arresting the nAMD progression. Some long-term side effects of the therapy and patients not responding to the treatment demonstrate the need for alternative and complementary targets to inhibit ocular neovascularization. Our study identifies a BMP antagonist, COCO, that suppresses ocular angiogenesis in mouse developmental and pathological angiogenesis models. As such, we propose to evaluate whether COCO could constitute a therapeutic tool to modulate retinal and choroidal angiogenesis. We evaluate its effects on angiogenesis during retinal development and pathological models and investigate the mechanistic basis of its effects on endothelial cells.

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COCO/DAND5 inhibits developmental and pathological ocular angiogenesis

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Abstract

Neovascularization contributes to multiple visual disorders including age-related macular degeneration (AMD) and retinopathy of prematurity. Current therapies for treating ocular angiogenesis are centered on the inhibition of vascular endothelial growth factor (VEGF). While clinically effective, some AMD patients are refractory or develop resistance to anti-VEGF therapies and concerns of increased risks of developing geographic atrophy following long-term treatment have been raised. Identification of alternative pathways to inhibit pathological angiogenesis is thus important. We have identified a novel inhibitor of angiogenesis, COCO, a member of the Cerberus-related DAN protein family. We demonstrate that COCO inhibits sprouting, migration and cellular proliferation of cultured endothelial cells. Intravitreal injections of COCO inhibited retinal vascularization during development and in models of retinopathy of prematurity. COCO equally abrogated angiogenesis in models of choroidal neovascularization. Mechanistically, COCO inhibited TGFβ and BMP pathways and altered energy metabolism and redox balance of endothelial cells. Together, these data show that COCO is an inhibitor of retinal and choroidal angiogenesis, possibly representing a therapeutic option for the treatment of neovascular ocular diseases.

Keywords angiogenesis; COCO; energy metabolism; ocular pathologies
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Introduction

Ocular neovascular diseases are a major cause of vision loss in the world. Age-related macular degeneration (AMD) is the third cause of blindness globally, but it is the primary cause in industrialized countries. Wet AMD arises from the abnormal growth of leaky blood vessels in the subretinal space, which disrupts the function of the heterogeneous cell populations that make up the retina, leading to a partial or complete loss of vision (Ambati & Fowler, 2012; Mitchell *et al*, 2018). While multiple signaling events contribute to the development and progression of pathological neovascularization, vascular endothelial growth factor (VEGF) has long been established as a primary driver of neovascular growth and angiogenesis (Ferrara, 2016; Apte *et al*, 2019). Treatments for neovascular diseases reflect the important role of VEGF in these pathologies. Multiple FDA-approved treatments that target VEGF signaling, including Lucentis, Eylea, and Macugen, have been developed and are in clinical use for the treatment of pathological neovascularization in the eye (Amadio *et al*, 2016). These agents have provided significant clinical benefits to patients afflicted with wet AMD, largely supplanting photodynamic therapy. However, despite great benefits, clinical studies have shown that not all patients respond to anti-VEGF treatments, which could be accounted by the fact that choroidal neovascularization (CNV) associated with AMD is a multifactorial condition whose pathogenesis involves inflammation, angiogenesis, and fibrosis (Kieran *et al*, 2012; Yang *et al*, 2016). Furthermore, all available anti-angiogenic monotherapies are directed specifically to VEGF, which is one of many pathways involved in neovascularization.

Although initially thought to be endothelial-specific, VEGF has been shown to target a variety of non-vascular cells such as neural stem cells, ependymal cells, and neurons including photoreceptors. Indeed, VEGF has been shown to have significant neurotrophic effects by protecting neurons from trauma or disease (Lange *et al*, 2016), although its effects on retinal function are still being debated. Studies using long-term delivery of VEGF inhibitors reported no adverse effects on photoreceptors and retinal function (Ueno *et al*, 2008; Miki *et al*, 2010). However, other studies have reported that VEGF has a survival role on Müller cells and photoreceptors, and that chronic depletion of VEGF results in photoreceptor loss and impaired retinal function (Saint-Geniez *et al*, 2008, 2009).

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Furthermore, deletion of *Vegfa* from the retinal pigmented epithelium results in an ablation of the choriocapillaris, as well as a loss of cone photoreceptor function (Marneros *et al*, 2005; Kurihara *et al*, 2012). Large multicenter clinical trials, which examined long-term anti-VEGF treatment in patients with AMD, also concluded that therapies that block VEGF could have an effect on the development and progression of geographic atrophy (Martin *et al*, 2012; Grunwald *et al*, 2014). Despite the conflicting evidence in the literature, attention has shifted in recent years to non-VEGF mechanisms of blood vessel formation in the context of providing alternatives to anti-VEGF therapies (Ferrara, 2016).

In addition to VEGF signaling, many other signaling pathways contribute to the development and stabilization of the retinal vasculature. Both canonical and non-canonical Wnt signals have been demonstrated to regulate the retinal vasculature (Zhou *et al*, 2014; Korn *et al*, 2014). Various bone morphogenetic proteins (BMPs) act as both pro- and anti-angiogenic factors (Ntumba *et al*, 2016; Lee *et al*, 2017; Akla *et al*, 2018). TGFβ and Notch signaling pathways are also well-established regulators of angiogenesis and can direct endothelial tip/stalk cell specification and metabolism (De Bock *et al*, 2013). Furthermore, TGFβ has been shown to have pro- and anti-angiogenic activities in neovascular AMD (Tosi *et al*, 2018). COCO (also known as DAND5 or CERL2) is a member of the DAN family (Bell *et al*, 2003). This family is composed of secreted proteins that act as antagonists of soluble BMP, TGFβ, and Wnt molecules (Bell *et al*, 2003). There are seven members of the DAN family: Sclerostin (SOST), uterine sensitization-associated gene-1 (USAG), Gremlin 2 (PRDC; GREM2), Dan (Neuroblastoma Suppressor of Tumorigenicity 1; NBL1), Cerberus (CER1), Gremlin 1 (GREM1), and COCO, which all contain a cysteine-rich DAN domain that is essential for their function (Nolan & Thompson, 2014). The DAN family members have been most widely studied for their roles during development. COCO in particular has been shown to be involved in establishing anterior–posterior patterning in vertebrates (Belo *et al*, 2017). Its inactivation in mice has been reported to lead to multiple laterality and cardiovascular defects and a significant proportion of animals die perinatally (Araujo *et al*, 2014). A recent study has shown that COCO is widely expressed in the retinal photoreceptor layer and that it is a potent inducer of human embryonic stem cell differentiation into cone photoreceptors through inhibition of Activin, BMP, and Wnt signaling (Zhou *et al*, 2015).

As the Wnt, TGFβ, and BMP families have all been implicated in both developmental and pathological retinal angiogenesis and knowing that COCO is expressed in the retina postnatally (Zhou *et al*, 2015), we postulated that COCO may be able to regulate angiogenesis. Furthermore, its positive effects on human photoreceptor development (Zhou *et al*, 2015) suggest that it may be a safe target for the treatment of ocular neovascular diseases. In this study, we report that COCO can inhibit both developmental and pathological angiogenesis in the eye. We further demonstrate that intra-ocular injection of COCO does not result in photoreceptor apoptosis or deleterious effects on the stability of mature blood vessels. Mechanistically, we found that exogenous COCO shows little effect on VEGF signaling but localizes to mitochondria and results in decreased ATP production and induction of reactive oxygen species (ROS) in Human Umbilical Vein Endothelial Cells (HUVECs). Our work identifies a novel inhibitor of retinal and choroidal angiogenesis with potential clinical applications for the treatment of neovascular ocular diseases.

Results

COCO inhibits angiogenesis by blocking endothelial cell proliferation and migration

To determine whether COCO affects sprouting angiogenesis in macro- and microvascular endothelial cells, HUVECs and Human Retinal Microvascular Endothelial Cells (HRMECs) were cultured in 3D fibrin gels and tube formation was induced in medium supplemented with 25 ng/ml VEGF-A_{165} (thereafter referred as VEGF), with or without increasing concentrations of COCO for 5 days as previously described (Larrivee *et al*, 2012). Quantification of endothelial tubes revealed a significant decrease in vascular tube area with increasing concentrations of COCO in both HUVECs (Fig 1A and D) and HRMECs (Fig 1B and E). The inhibitory effect of COCO on sprouting angiogenesis was also demonstrated using an *ex vivo* model of choroidal angiogenesis. Briefly, the choroidal tissues from C57BL/6J mice were isolated and cultured in Matrigel (Fig 1C). After treatment with or without COCO for 5 days, vascular sprouting area was evaluated. The sprouting area, normalized to controls, was reproducibly decreased in COCO-treated choroidal explants (Fig 1C and F). Together, these data show that COCO significantly inhibits sprouting angiogenesis in endothelial cells of macro- and microvascular origin as well as in choroidal explants.

We next evaluated the cellular mechanisms underlying the inhibitory effects of COCO on endothelial cell sprouting. Endothelial cell migration plays an essential role in neovascularization, as endothelial tip cells will need to migrate in response to VEGF, and constitutes one of the first steps of the angiogenic response (Gerhardt, 2008). To address the effects of COCO on migration, HUVECs were subjected to a wound healing assay. Briefly, a scratch was performed on a confluent monolayer of HUVECs, and wound closure was evaluated at the time of the scratch and again after 18 h. COCO significantly delayed wound closure compared with control treatment, indicating that COCO can prevent VEGF-induced cell migration (Fig 1G and H). Imaging of cells at the wound edge showed that COCO did not significantly affect polarization of the Golgi apparatus toward the leading edge, suggesting that COCO may not act through Rho GTPase Cdc42 and Rac, which are active at the leading edge of polarized cells and are central to polarity regulation (Raftopoulou & Hall, 2004; Fig EV1A–C).

To understand the possible role of COCO in endothelial cell proliferation, HUVECs cultured in complete medium were treated for 18 h in the presence or absence of recombinant COCO, followed by a 1-h EdU pulse. As shown in Fig 1I and J, HUVECs cultured in the presence of COCO showed reduced EdU incorporation when compared to controls. We also verified that the inhibitory effects of COCO on angiogenesis were not associated with increased apoptosis, as HUVECs cultured for 18 h in the presence of COCO did not show differences in the proportion of apoptotic cells (Fig 1K and L). Taken together, these data reveal that COCO displays anti-angiogenic activity by inhibiting endothelial sprouting, migration, and proliferation without affecting apoptosis.

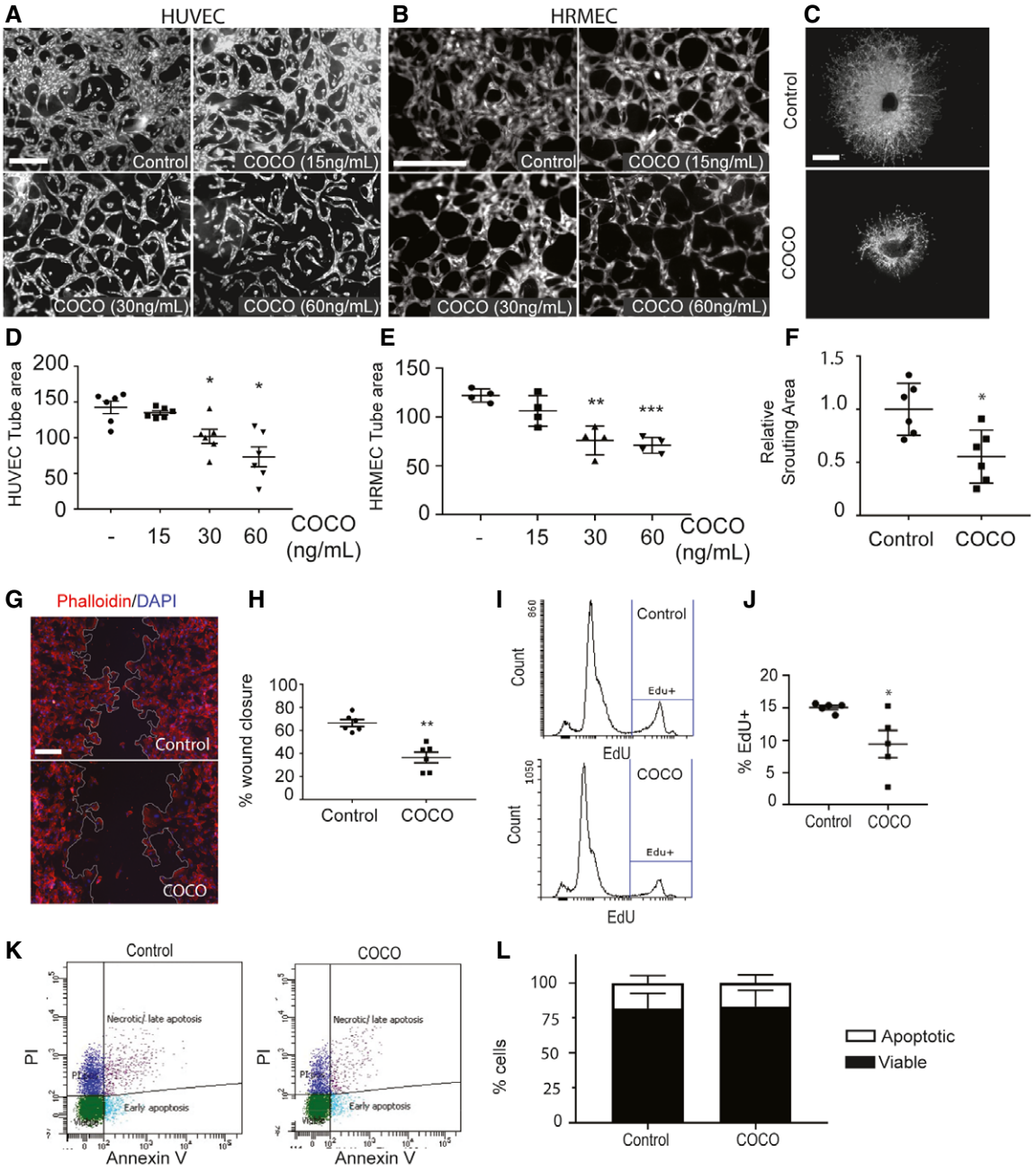

Figure 1. COCO inhibits sprouting angiogenesis.

A, B Representative images of HUVECs (A; $n = 6$) and HRMECs (B; $n = 4$) sprouting in a fibrin gel with VEGF (25 ng/ml) in the presence or absence of COCO. Scale bar, 75 μm.

C Representative images of choroidal explants cultured for 5 days in the presence or absence of COCO. Scale bar, 500 μm.

D, E Quantification of tube surface area of micrographs shown in (A, B). Results are presented as mean ± SEM; statistical analyses were performed using Mann–Whitney test. (D: $*P = 0.0119$ (– vs. COCO 30 ng/ml); $*P = 0.0108$ (– vs. COCO 60 ng/ml); (E: $**P = 0.0013$ (– vs. COCO 30 ng/ml); $***P = 0.00026$ (– vs. COCO 60 ng/ml)).

F Quantification of sprouting surface area of micrographs shown in (C; $n = 6$). Results are presented as mean ± SEM, statistical analyses were performed using Mann–Whitney test. $*P = 0.0107$.

G COCO decreases scratch wound migration after 18 h. Scale bar, 150 μm.

H Quantification of wound closure. Results are presented as mean ± SEM, statistical analyses were performed using Mann–Whitney test. $**P = 0.0010$; ($n = 6$).

I COCO decreases EdU incorporation in cultured HUVECs.

J Quantification of EdU incorporation. Results are presented as mean ± SEM, statistical analyses were performed using Mann–Whitney test. $*P = 0.0317$; ($n = 5$).

K COCO does not induced apoptosis in HUVECs.

L Quantification of apoptotic (Annexin V-positive) and viable (Annexin V-negative) cells ($n = 4$).

COCO inhibits retinal neovascularization

As COCO prevents angiogenic sprouting in cultured endothelial cells, we evaluated whether it could inhibit retinal vascular development. Newborn mouse pups (P1) received intravitreal (ivt) injections of recombinant COCO, and retinas were harvested after 4 days (P5) (Fig 2A). Delivery of exogenous COCO resulted in a significant inhibition of blood vessel development (Fig 2B). Compared with PBS-injected eyes, a pronounced reduction in vessel area (area covered by vessels) and microvessel density (ratio of vessel area to vascularized area) was detected in the retinas of COCO-injected eyes (Fig 2C). The altered vascular pattern was associated with a reduced number of vascular branch points, resulting in a significant reduction in vascular network complexity in COCO-injected retinas. The inhibition of COCO on retinal neovascularization was also as pronounced as that of a VEGF inhibitor (mouse Flt1Fc; R&D systems) (Fig 2B and C). The retinal vasculature of COCO-injected eyes displayed reduced endothelial cell proliferation (Fig EV2A–C) but showed no change in apoptosis (Fig EV2D and E). Blood vessels constrict in the course of vessel regression and endothelial cells retract, leaving behind empty basement membrane sleeves (Korn & Augustin, 2015). However, the retinal vasculature of COCO-treated eyes did not show differences in the number of empty type IV collagen (CollIV) basement membrane sleeves (Fig 2D and E), suggesting that COCO does not affect the switch between vessel maintenance and regression. In spite of its effects on endothelial cells and retinal vascular outgrowth, COCO injections also did not affect retinal pericyte coverage (Fig 2F and G) or photoreceptor apoptosis (Fig 2H and I). Together, these data show that exogenous COCO impairs developmental angiogenesis in the retina and is associated with reduced endothelial cell proliferation.

Long-term delivery of COCO does not adversely affect photoreceptors

Patients affected by ocular neovascular diseases such as retinopathy of prematurity and wet AMD are typically treated with VEGF inhibitors to control pathological angiogenesis (Amadio *et al*, 2016). While VEGF inhibitors show good clinical efficacy for the prevention of neovascularization, concerns have been raised since studies have suggested that chronic inhibition of VEGF could adversely affect non-vascular cells in the eye (Nishijima *et al*, 2007; Lv *et al*, 2014). We therefore addressed the long-term effects of COCO on photoreceptors and the neural retina. Newborn pups (P1) received weekly injections of COCO or Flt1Fc for 4 weeks (Fig 3A). While there was a mild decrease in the density of retinal vessels in Flt1Fc-injected animals, a striking reduction in blood vessel formation was observed in the retinas of mice that received COCO (Fig 3B and C). Likewise, the number of photoreceptor nuclei, which did not display visible pyknosis, and the thickness of the outer nuclear layer were unaffected in COCO-injected eyes (Fig 3D and E). Thus, even though COCO strongly suppresses retinal neovascularization, it does not appear to compromise photoreceptor survival.

COCO delivery does not affect mature established vessels in the retina

It is noteworthy that COCO had limited effect on mature retinal vessels, suggesting that COCO may block angiogenesis only in the presence of pro-angiogenic stimuli. This was confirmed *in vivo* by the observation that COCO inhibition did not affect mature established vessels, when injected in 8-week-old mice for 5 days (Fig 4A). As opposed to newborn pups, which undergo retinal vascular development, a five-day treatment in adult mice showed no difference in the retinal vasculature between PBS and COCO-treated eyes (Fig 4B and C), indicating that COCO mediates its effects by preventing the growth of newly formed vessels, rather than inducing the regression of pre-existing vessels, which is consistent with the absence of changes in empty collagen sleeves (Fig 2D). We extended these findings by injecting adult mice with COCO over a one month-period (Fig 4D). As opposed to newborn pups, which displayed an important reduction in retinal blood vessels outgrowth and density after COCO treatments (Fig 2B and C), no significant effects were observed in adult mice following long-term injections of COCO (Fig 4E and F).

COCO inhibits pathological neovascularization

The effects of COCO on postnatal developmental angiogenesis led us to evaluate its effects on pathological angiogenesis by subjecting mouse pups to oxygen-induced retinopathy (OIR). Briefly, P7 pups were placed in 75% oxygen for 5 days, leading to vaso-obliteration of the retinal vascular plexus (Stahl *et al*, 2010). At P12, pups were returned to room air, which stimulates retinal angiogenesis, and leads to the formation of a pathological vascular retinal network characterized by neovascular tufts (Fig 5A). Treatment of COCO at P12 significantly reduced pathological neovascularization in retinas harvested at P17. While revascularization and the avascular region of the central part of the retina was not affected by COCO, the amount and size of neovascular tufts were significantly reduced in the eyes injected with COCO compared with PBS treatment (Fig 5B–D). As with developmental retinal angiogenesis, the anti-angiogenic effects of COCO were similar to those observed with Flt1Fc.

The effects of COCO on CNV were also evaluated by subjecting mice to laser-induced CNV, a model which recapitulates the CNV occurring in wet AMD patients (Lambert *et al*, 2013). Briefly, 8-week-old mice were subjected to laser impact, followed by intravitreal injections of either COCO, Flt1Fc, or PBS, and CNV was detected 14 days later by staining choroid-sclera whole mounts with IsoB4 (blood vessels) and phalloidin (RPE) (Fig 5E). We observed a significant decrease in the area of CNV in mice treated with COCO and Flt1Fc compared with controls (Fig 5F and G). Together, these observations show that the inhibitory effect of COCO on retinal and choroidal neovascularization is similar to that observed with Flt1-Fc treatment.

COCO does not directly alter VEGF signaling in primary endothelial cells

VEGF is one of the main drivers of angiogenesis, and inhibition of this pathway has been the main target of anti-angiogenic therapies. Gremlin, a member of the Dan family, has previously been shown to directly interact with VEGFR2 and promote its activity (Mitola *et al*, 2010). We therefore evaluated whether VEGF signaling was modulated following COCO treatment. HUVECs were starved overnight in 1% FBS in the presence or absence of COCO, followed by

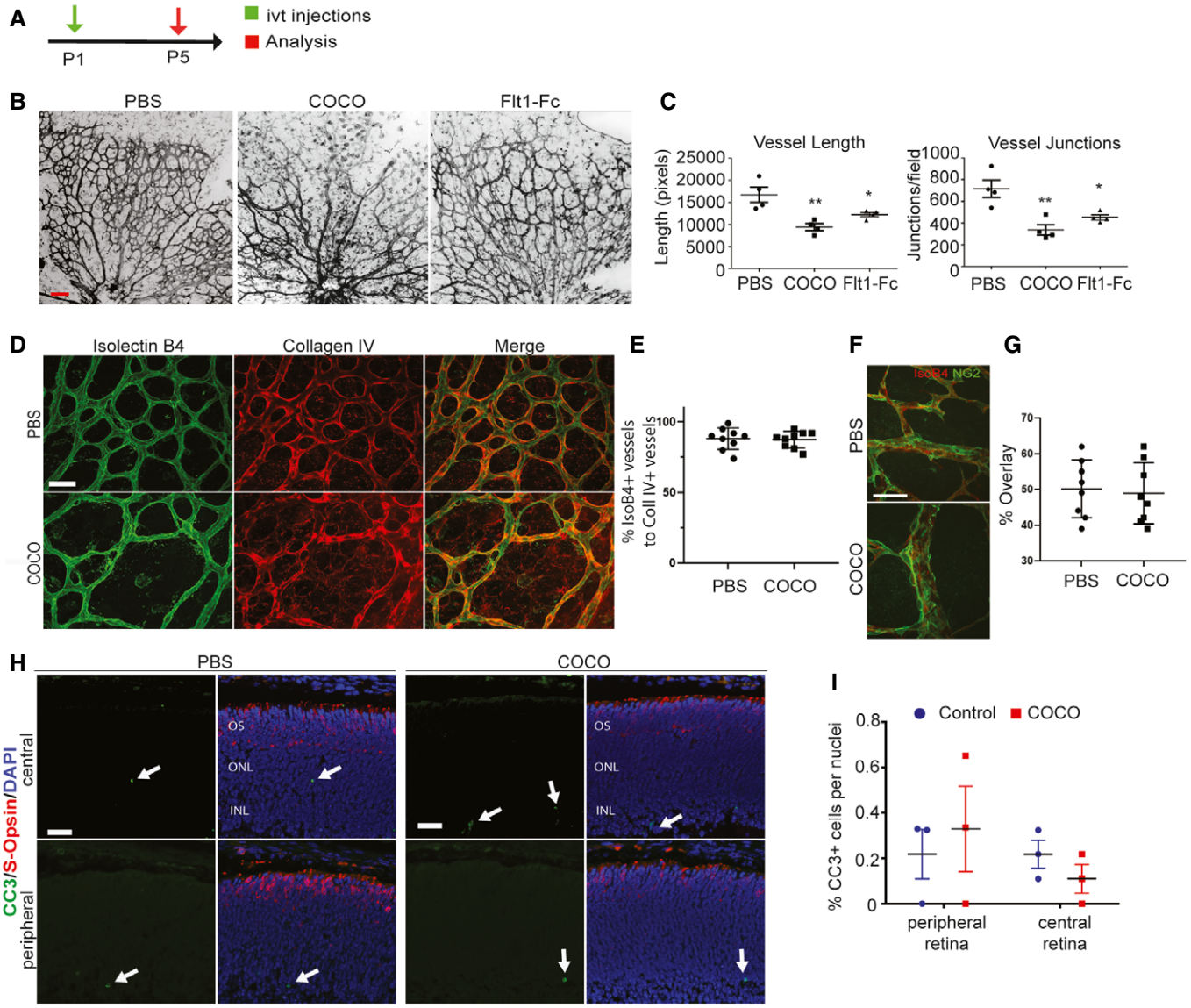

Figure 2. COCO inhibits developmental retinal angiogenesis.

A Schematic of the experimental strategy to assess early formation of the retinal vasculature (P1–P5).

B Retinal flat mounts of P5 mice injected with PBS, COCO, or Flt1Fc are stained with IB4 (negative images of the fluorescent signal). Scale bar, 100 μm.

C Quantification of vascular length and number of branchpoints (*P*-values are vs. PBS treatment). Vessel length: $^{**}P = 0.0059$ (PBS vs. COCO); $^{*}P = 0.0169$ (PBS vs. Flt1Fc). Vessel junctions: $^{**}P = 0.0063$ (PBS vs. COCO); $^{*}P = 0.0159$ (PBS vs. Flt1Fc); ($n = 4$ mice/group).

D COCO does not increase empty collagen IV sleeves. Scale bar, 50 μm.

E Quantification of % of IsoB4 + vessels to Coll IV + vessels; ($n = 9$).

F Visualization of pericyte coverage (NG2:green; IsoB4:red) in PBS- or COCO-injected retinas. Scale bar, 50 μm.

G Quantification of percentage of IsoB4 vascular staining covered by NG2 staining (% overlay) ($n = 8$).

H COCO injections (P1) do not result in apoptosis in P5 retinas. Scale bar, 40 μm.

I Quantification of apoptotic cells in control and COCO-injected mice ($n = 3$). Results are presented as mean ± SEM and statistical significance was analyzed by Mann–Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$.

VEGF stimulation for up to 60 min. Immunoblotting analysis of key signaling regulatory events did not reveal changes in VEGF-induced phosphorylation of VEGFR downstream signaling molecules (Fig 6A and B). In HUVECs cultured in the presence of COCO, VEGFR2 phosphorylation at Y1175 was not significantly altered, nor was the phosphorylation of Akt, Erk, and p38 in response to VEGF stimulation (Fig 6A and B). The expression of VEGF receptors was also not altered following COCO stimulation for up to 24 h (Fig 6C and D). Taken together, these data suggest that the inhibitory effects of COCO do not appear to be a direct consequence of altered VEGF signaling in endothelial cells, although it remains a possibility that downstream cellular events may similarly be modulated by both

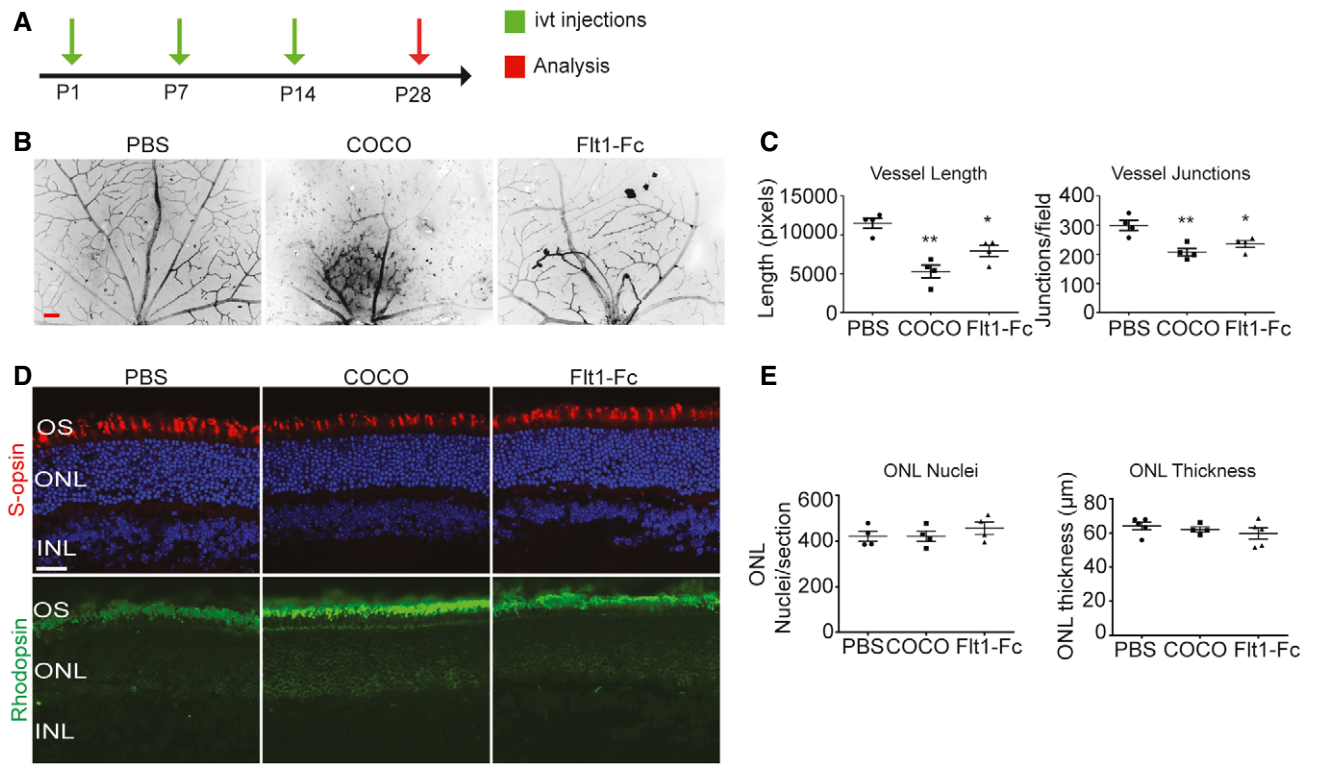

Figure 3. Chronic injections of COCO decrease blood vessel development but do not result in retinal apoptosis.

A Schematic of the experimental strategy to assess early formation of the retinal vasculature (P1–P28).

B Retinal flat mounts of P28 mice injected with PBS, COCO, or Flt1Fc are stained with IB4 (negative images of the fluorescent signal). Scale bar, 100 µm.

C Quantification of vascular length and number of branchpoints (*P*-values are vs. PBS treatment). Vessel length: $^{**}P = 0.0019$ (PBS vs. COCO); $^{*}P = 0.0128$ (PBS vs. Flt1Fc). Vessel junctions: $^{**}P = 0.0073$ (PBS vs. COCO); $^{*}P = 0.0302$ (PBS vs. Flt1Fc); ($n = 4$).

D COCO injections (P1, P7, and P14) do not result in ONL thinning in P28 retinas. Scale bar, 40 µm.

E Quantification of apoptotic cells (cleaved caspase-3; CC3) in control and COCO-injected mice (*P*-values are vs. PBS treatment). ($n = 4$). Results are presented as mean ± SEM and statistical significance was analyzed by Mann–Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

factors. We also evaluated whether COCO could potentiate the anti-angiogenic effects associated with VEGF inhibition. *In vitro*, we show that treatment with a sub-optimal concentration of COCO (50 ng/ml) can potentialize the anti-angiogenic effects of Flt1Fc in a sprouting experiment (Fig EV3A and B), although no such potentiation was observed at higher COCO concentrations (75–300 ng/ml COCO), suggesting that the concentration window required to observe an additive effect between COCO and a VEGF inhibitor is relatively narrow. *In vivo*, combination of COCO (50 ng/ml) with Flt1Fc did not show an additive effect on ocular neovascularization (Fig EV3C and D). This lack of additive effects between VEGF and COCO on retinal vascular development may reflect that COCO and VEGF still share common downstream cellular events in endothelial cells that are limiting or tightly regulated. Alternatively, COCO likely also acts on non-vascular cells in the retina, which may indirectly mask the effects of an anti-VEGF on the retinal endothelium.

Expression of COCO in cultured endothelial cells and in human and mouse retina

To fully understand the multifaceted mechanism of the anti-angiogenic effects of COCO in retinal angiogenesis, we evaluated its endogenous expression in the retina. Western blot analysis of adult human retina tissue showed a 36 kDa band, consistent with the presence of a COCO dimer (Fig 7A; Nolan & Thompson, 2014). COCO was also abundantly detected in human pluripotent stem cell-derived cone photoreceptors (Fig 7B). Immunostaining of adult human retinas revealed the presence of COCO in inner segments of photoreceptors (a mitochondria-rich region), at or close to the outer plexiform layer, and in the ganglion cell layer (Fig 7C). We also confirmed the pattern of COCO expression using the highly sensitive RNAscope *in situ* hybridization technique for visualizing *Dand5* transcripts in mouse retinal sections (Fig 7D). *Dand5* transcripts were detected throughout the neural retina and were also present in the photoreceptor nuclear layer, as shown by co-localization with visual-arrestin immunostaining, in agreement with previous findings (Zhou *et al*, 2015; Fig 7E). In addition, *Dand5* was widely expressed in inner nuclear layer, composed of horizontal, bipolar, and amacrine cells, as well as in the ganglion cell layer. Finally, we next evaluated the expression of COCO in cultured endothelial cells. While immunostaining of unstimulated HUVECs showed no significant COCO immunoreactivity (Fig EV4), HUVECs exposed to recombinant COCO for 5 h displayed COCO immunoreactivity in mitochondria, as shown by co-localization with a mitochondria-

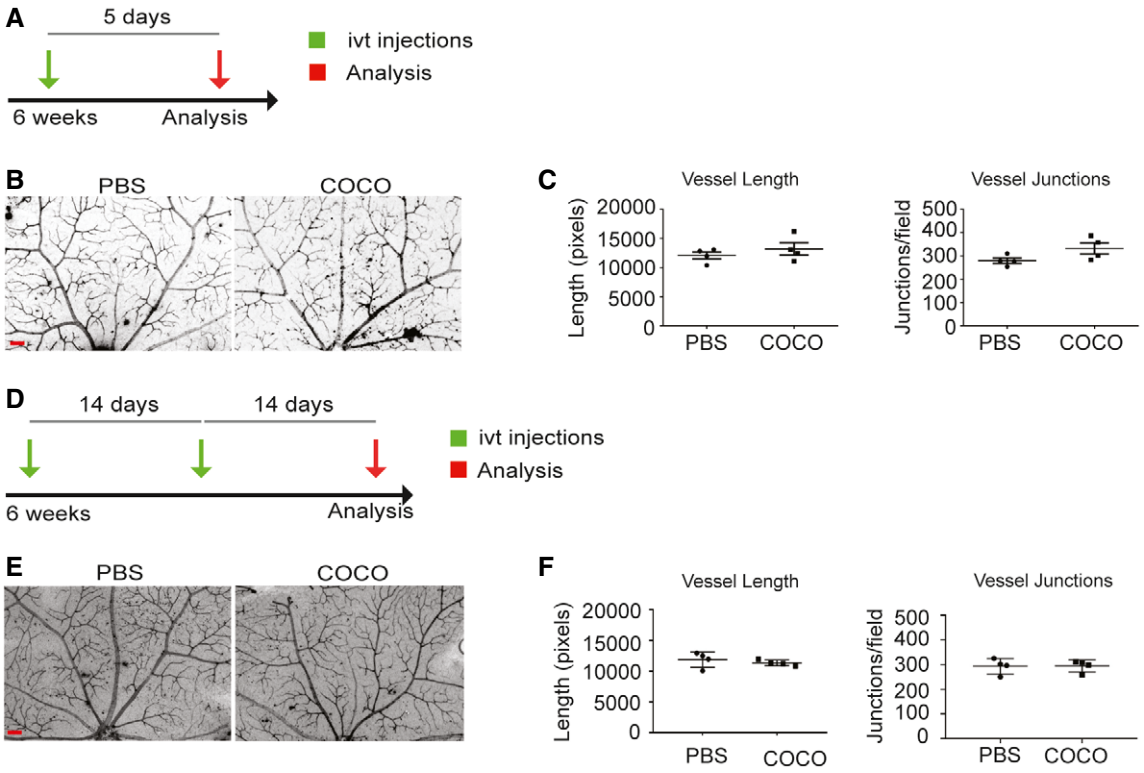

Figure 4. COCO injections do not induce vascular regression in adult mice.

A Schematic of the experimental strategy to assess injections of COCO in the adult retinal vasculature.
B Retinal flat mounts of adult mice injected with PBS or COCO for 5 days are stained with IB4 (negative images of the fluorescent signal). Scale bar, 100 μm.
C Quantification of vascular length and number of branchpoints; (n = 4).
D Schematic of the experimental strategy to assess repeated injections of COCO in the adult retinal vasculature.
E Retinal flat mounts of adult mice injected with PBS or COCO for 28 days are stained with IB4. Scale bar, 90 μm.
F Quantification of vascular length and number of branchpoints; (n = 4). Results are presented as mean ± SEM and statistical significance was analyzed by Mann–Whitney test.

specific antibody (Fig 7F), consistent with data from the human protein atlas which detected COCO expression in the mitochondria (https://www.proteinatlas.org/) (Thul *et al*, 2017). These data suggest possible uptake and transport to mitochondria of exogenously added COCO.

COCO alters the redox and glycolytic balance of endothelial cells

To further explore the mechanisms underlying the effects of COCO on endothelial cells, we performed transcriptomic analysis of HUVECs stimulated with COCO for 16 h (Fig 8A). Among the most dysregulated genes ($Log_2Fc \geq \pm 1$; Pvalue ≤ 0.05) (Fig 8B), we found changes for several transcripts in regard to mitochondrial metabolic function and energy metabolism, such as Type 2 NADH dehydrogenase (*NDUFV2*), mitochondrial NAD kinase 1 (*NADKD1*), Ubiquinol-Cytochrome C Reductase Core Protein (*UQCRC1*), Oct-1 (*POU2F1*), and Transitional Endoplasmic Reticulum ATPase (*VCP*) among others (Fig 8B; Wang & Jin, 2010; Xu *et al*, 2017; Zhang *et al*, 2018; Parzych *et al*, 2019). Further gene set enrichment analysis (GSEA) also showed that COCO induced a significant down-regulation of pathways involved in TGFβ and BMP signaling in HUVECs, consistent with published data showing that COCO acts as an antagonist of BMP and TGFβ signaling (Fig 8C). A significant increase in genes associated with generation of ROS was also found in COCO-treated HUVECs (Fig 8C). Given the changes that we observed in genes associated with mitochondrial metabolic function and ROS production, combined with the observation that TGFβ and BMP signature pathways, which have been involved in endothelial cell metabolism (Rodríguez-García *et al*, 2017; Lee *et al*, 2018), were decreased in COCO-treated cells, we addressed the effects of COCO on endothelial cell ROS generation and energy metabolism.

To better explore endothelial cell metabolism following COCO stimulation, we measured oxygen consumption rates (OCR) and extracellular acidification rate (ECAR), an indicator of glycolysis, in HUVECs stimulated with COCO for 1, 2, and 24 h. Treatments with 2-Deoxyglucose (2-DG), a potent inhibitor of glycolysis, severely decreased ECAR and, to a lesser extent oxygen consumption in HUVECs (Fig 9A). While we observed that COCO slightly decreased basal OCR at 2-h following stimulation, COCO-treated cells exhibited significantly reduced ECAR compared with control cells, suggesting decreased glycolytic capacity (Fig 9A). However, both OCR and ECAR levels were similar 24 h following COCO treatments, suggesting that these changes are transient. Endothelial cells rely primarily

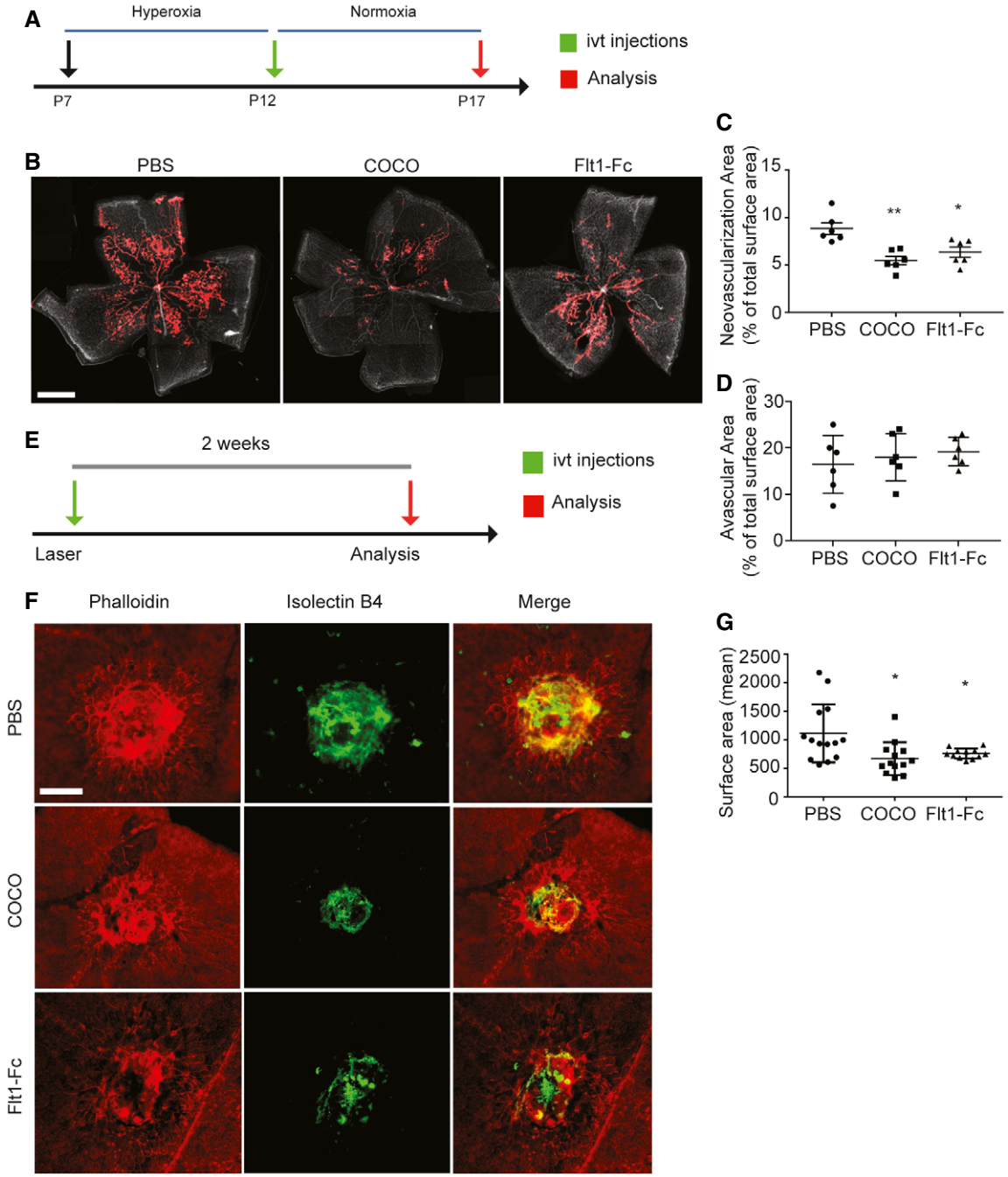

Figure 5. COCO inhibits retinal and choroidal pathological angiogenesis.

A Schematic of the experimental strategy to assess the effects of COCO in the Oxygen-induced Retinopathy model.

B Retinal flat mounts of P17 OIR mice injected with PBS, COCO, or Flt1Fc are stained with IB4. Red areas highlight vascular tufts. Scale bar, 500 μm.

C Quantification of neovascular tuft area in P17 OIR. $^{**}P = 0.0021$ (PBS vs. COCO); $^{*}P = 0.0122$ (PBS vs. Flt1Fc); ($n = 6$).

D Quantification of central avascular area in P17 OIR; ($n = 6$).

E Schematic of the experimental strategy to assess the effects of COCO in the Laser-induced Choroidal Neovascularization (CNV) model.

F Choroidal flat mounts of adult mice subjected two weeks prior to laser-CNV injected with PBS, COCO or Flt1Fc stained with IB4 (green) and phalloidin (red). Scale bar, 100 μm.

G Quantification of CNV surface area. $^{*}P = 0.0210$ (PBS vs. COCO); $^{*}P = 0.0113$ (PBS vs. Flt1Fc); ($n = 12$–14 burns from 5 animals/group). Results are presented as mean ± SEM and statistical significance was analyzed by Mann–Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$.

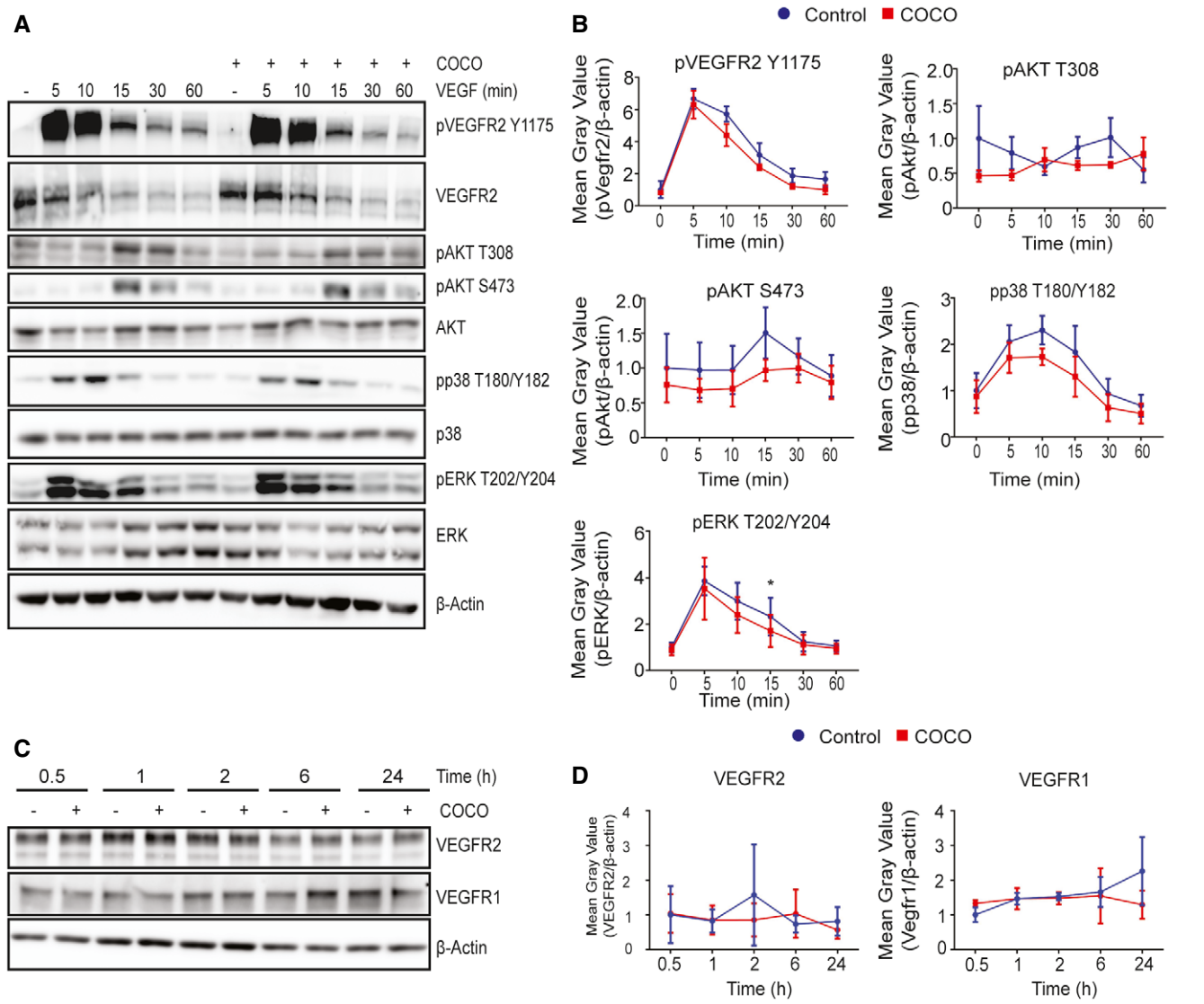

Figure 6. COCO does not impair VEGF signaling in cultured endothelial cells.

A Western blot analysis of HUVECs treated 24 h prior with COCO and stimulated with VEGF for up to 60 min. Immunoblots are representative of 4 independent experiments.

B Densitometric analysis of Western blot experiments. *$P = 0.0457$; ($n = 4$).

C COCO treatment does not alter VEGF receptor expression in HUVECs. Immunoblots are representative of 3 independent experiments.

D Densitometric analysis of Western blot experiments; ($n = 3$). Results are presented as mean ± SEM and statistical significance was analyzed by two-way ANOVA. *$P < 0.05$.

Source data are available online for this figure.

on glycolysis as their main energy source and ATP production during angiogenesis (Draoui *et al*, 2017). Glucose uptake and metabolism are increased during angiogenesis to meet this energy demand. As glycolytic flux was reduced HUVECs, we also tested whether glucose uptake was altered in HUVECs treated with COCO. Analysis of glucose uptake in HUVECs revealed a rapid but transient decrease in glucose transport 1 h after COCO treatment (Fig 9B), which correlated with decreased ECAR. Total ATP levels were also assessed after 1, 6, and 24 h of treatment with COCO. ATP levels were rapidly reduced after COCO treatment and reached a minimum of 25% of baseline levels after 1 h although these levels went up after 6 h (Fig 9C), indicating that COCO leads to a significant but transient inhibition of ATP production. Altogether, these data suggest that COCO transiently impairs glycolytic capacity and ATP production in endothelial cells, which may be reflected in the decreased proliferative and migrating capacity of endothelial cells treated with COCO.

The NAD+/NADH redox couple is known as a regulator of cellular energy metabolism such as glycolysis and mitochondrial oxidative phosphorylation (Xiao *et al*, 2018). As a co-factor for a

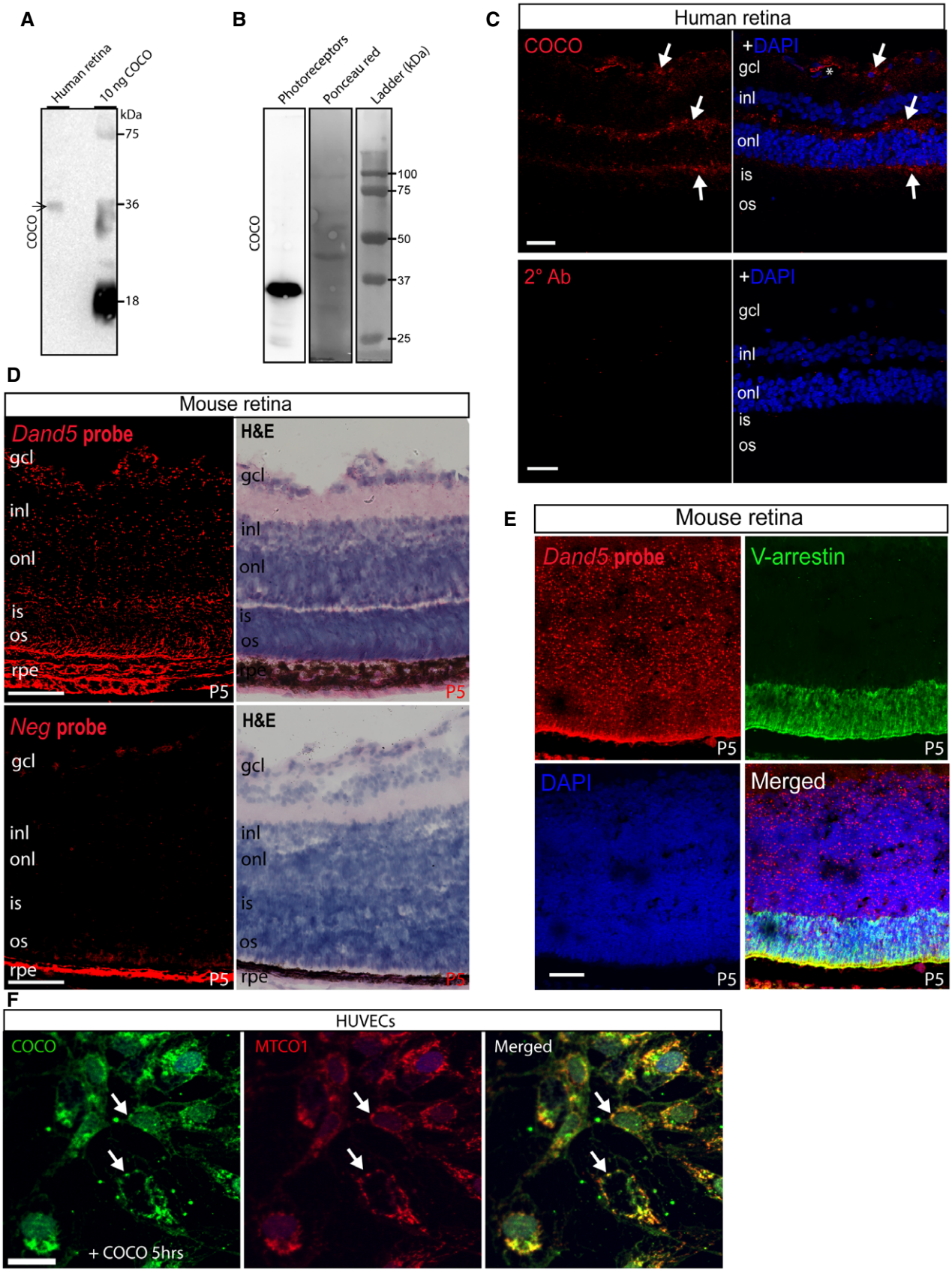

Figure 7.

Figure 7. COCO is expressed in human and mouse retina and localizes to mitochondria in COCO-exposed HUVECs.

A Western blot of whole adult human retina extracts incubated with an anti-human COCO antibody and revealing a unique band at ~ 36 kDa (arrow). Recombinant human COCO was used as positive control.

B Western blot of photoreceptors produced from human embryonic stem cells incubated with an anti-mouse COCO antibody and revealing a unique band at ~ 36–38 kDa (arrow). Western blots are representative of 3 independent experiments.

C Immunofluoresence analysis of adult human retina sections with an anti-mouse COCO antibody. Specific immunoreactivity was observed in multiple areas (arrows) when compared with sections only exposed to the secondary antibody. Scale bar, 40 μm.

D RNAscope *in situ* hybridization (*Dand5*, top; Negative probe (*dapB*); down) and hematoxylin staining of P5 mouse retinas. Scale bar, 40 μm.

E Dual RNAscope *in situ* hybridization and visual-arrestin immunohistochemistry of P5 mouse retinas. Scale bar, 40 μm. Images are representative of 4 animals.

F Immunofluoresence analysis of HUVECs exposed to COCO for 5 h prior to fixation. Exogenously added COCO was detected using an anti-human COCO antibody, showing co-localization with human mitochondria (MTCO1 antibody). Scale bar 25 μm.

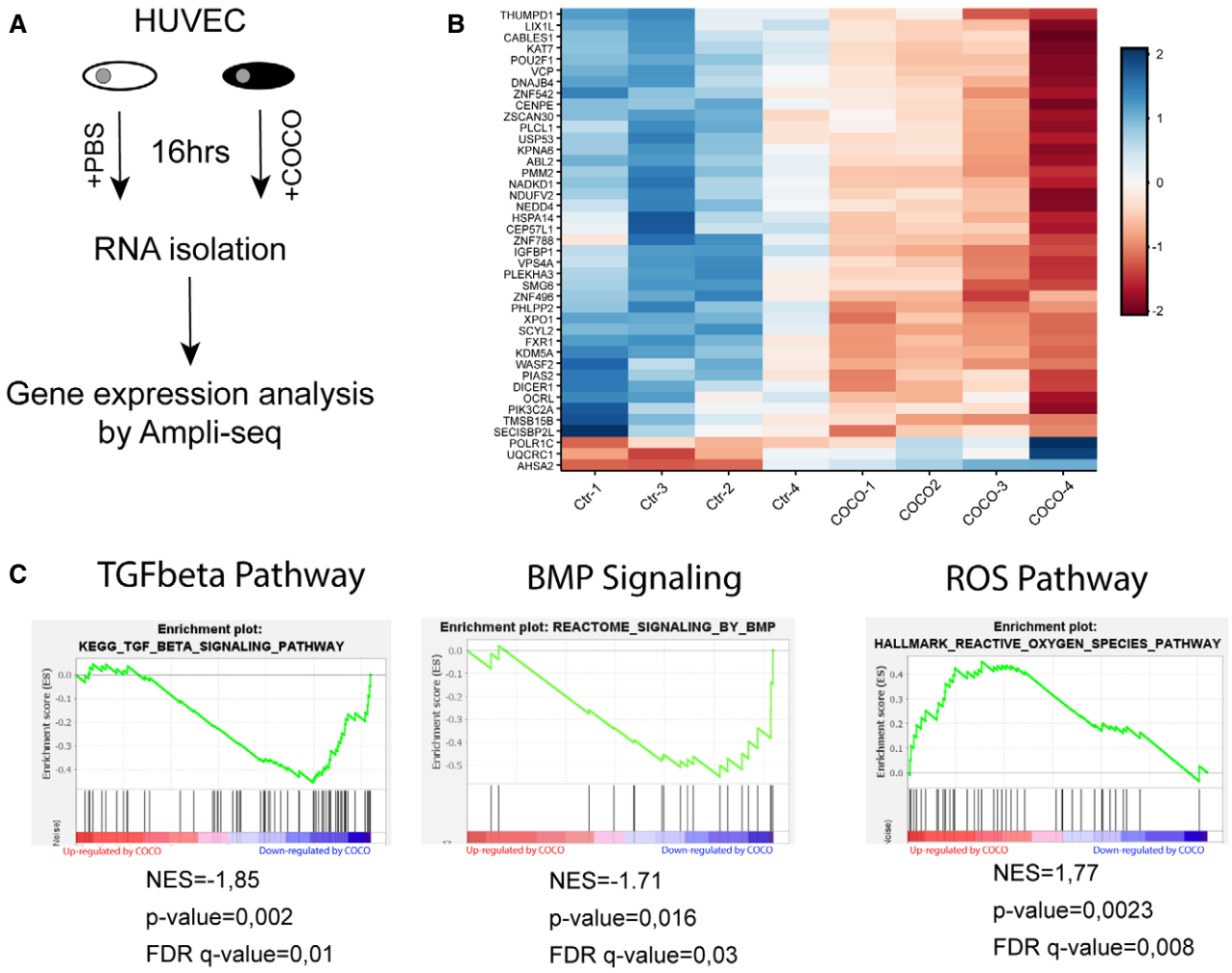

Figure 8. Transcriptional changes associated with COCO stimulation in ECs.

A Scheme of the protocol to determine differential gene expression after treatment of HUVECs 16 h with COCO.

B Differential gene expression heatmap generated by DESeq2 of top-altered genes of HUVECs treated with COCO compared with PBS-treated control cells (n = 4 independent samples/group).

C GSEA pathway analysis of control vs. COCO-treated HUVECs for TGFβ, BMP, or ROS pathways.

variety of oxidoreductase enzymes, NAD(H) mainly functions in biodegradation reactions and energy generation. We therefore evaluated whether decreased ATP production in the presence of COCO was associated with alterations in the ratio of NAD+ and its reduced form. HUVECs treated with COCO displayed a significant decrease in the NAD+/NADH ratio (Fig 9D). NAD+, required for the ATP-generating steps of glycolysis, is regenerated from NADH by mitochondrial NADH dehydrogenase or lactate dehydrogenase

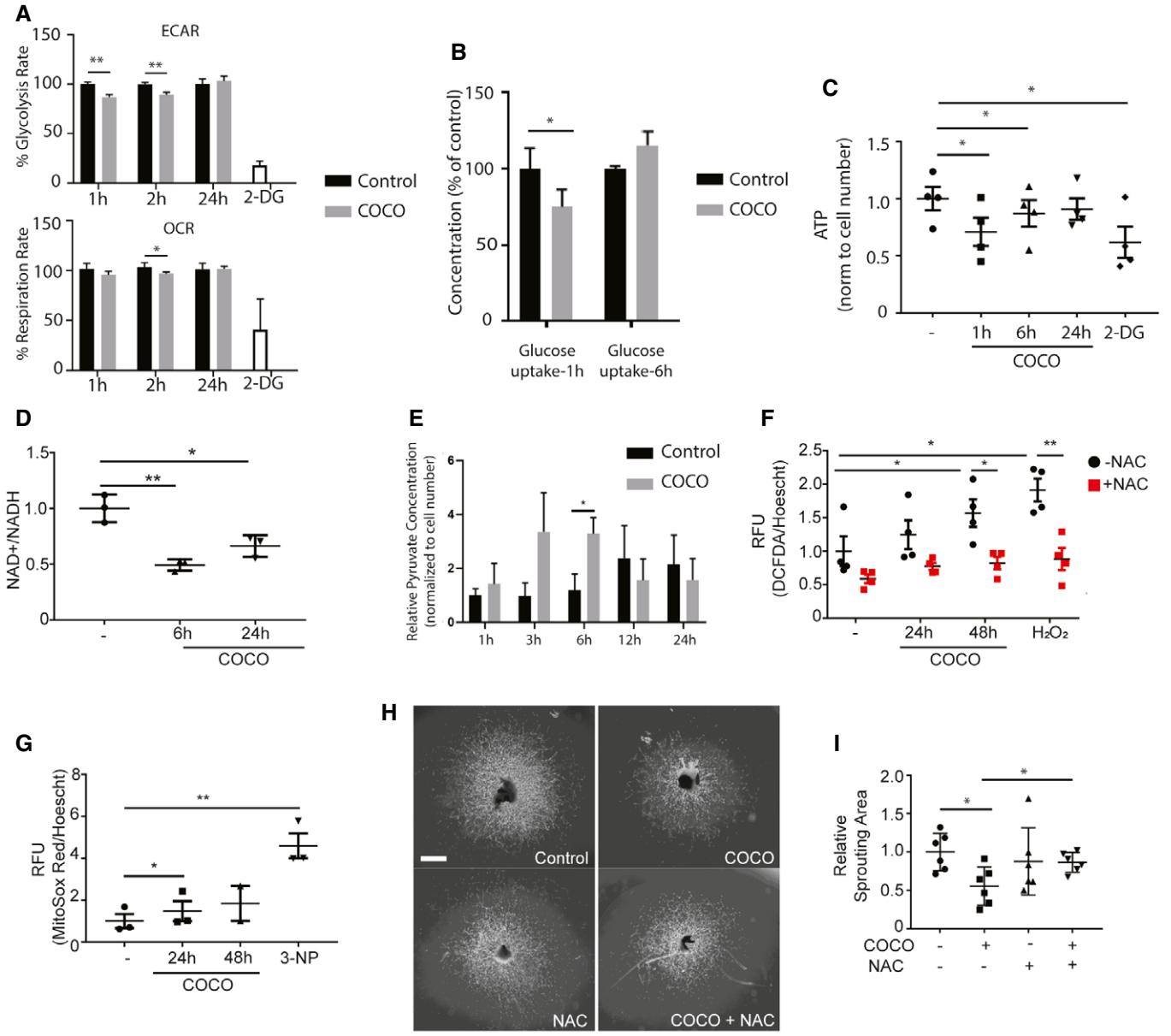

Figure 9. COCO alters the energy metabolism of endothelial cells.

A Seahorse analysis of mitochondrial respiration (OCR) and glycolysis (ECAR) in HUVECs stimulated with COCO for 1, 2, and 24 h. $^{**}P = 0.0032$ for ECAR, 1 h; $^{**}P = 0.0079$ for ECAR, 2 h; $^{*}P = 0.0255$ for OCR, 2 h; ($n = 5$).

B Media glucose levels (BioNova analysis) in HUVECs cultured with COCO for 1 or 6 h, expressed as % of initial levels $^{*}P = 0.0459$; ($n = 4$).

C Evaluation of ATP content in HUVECs stimulated with COCO for 1, 6, or 24 h. $^{*}P = 0.0120$ (– vs. 1 h); $^{*}P = 0.0109$ (– vs. 6 h); $^{*}P = 0.0219$ (– vs. 2-DG); ($n = 4$).

D Determination of NAD+/NADH ratio in HUVECs for 6 or 24 h with COCO. $^{**}P = 0.0028$ (6 h); $^{*}P = 0.0207$ (24 h); ($n = 3$).

E Evaluation of pyruvate content in HUVECs stimulated with COCO for 1, 3, 6, 12, or 24 h. $^{*}P = 0.0244$; ($n = 4$).

F Determination of cellular ROS using DCFDA fluorescence in HUVECs treated with COCO and/or N-acetylcystein. $^{*}P = 0.0343$ (– vs. 48 h-NAC); $^{*}P = 0.0114$ (– vs. H_2O_2-NAC); $^{*}P = 0.0252$ (48 h-NAC vs. 48 h + NAC); $^{**}P = 0.0067$ (H_2O_2-NAC vs. H_2O_2 + NAC); ($n = 4$).

G Determination of mitochondrial superoxide using MitoSox fluorescence in HUVECs treated with COCO. $^{*}P = 0.0372$ (– vs. 24 h); $^{*}P = 0.0137$ (– vs. 3-NP); ($n = 3$).

H Representative images of choroidal explants cultured for 4 days in the presence or absence of COCO and/or N-acetylcystein. Scale bar, 500 μm.

I Quantification of sprouting surface area of micrographs shown in (H). $^{*}P = 0.0126$ (– vs. COCO-NAC); $^{*}P = 0.0200$ (COCO-NAC vs. COCO + NAC); ($n = 6$). Results are presented as mean ± SEM and statistical significance was analyzed by Mann–Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$.

(Stambaugh & Post, 1966). Accordingly, we observed a significant increase in accumulation of intracellular pyruvate after 6 h of treatment with COCO (Fig 9E). Taken together, these data show that COCO treatments lead to an accumulation of pyruvate and decreased NAD+ regeneration, associated with a reduction in overall glucose consumption.

The build-up of NADH together with low ATP production has been associated with increased mitochondrial ROS generation. Indeed, the build-up of NADH due to reduced ATP synthesis and consequent lowered respiration rate decreases the NAD+/NADH ratio and leads to $O_2^{\bullet-}$ formation by complex I in the mitochondrial matrix (Kudin *et al*, 2004; Kussmaul & Hirst, 2006). Given that GSEA analysis revealed a significant increase in ROS-related genes (Fig 8C), levels of ROS were also evaluated in HUVECs treated for up to 48 h with 60 ng/ml COCO using the ROS probe 2′,7′–dichlorofluorescin diacetate (DCFDA), which generates fluorescence proportional to the amount of oxidized DCFDA to DCF. We observed that HUVECs cultured with COCO for 48 h showed increased levels of ROS, which were quenched by concomitant treatment with N-acetyl cysteine (NAC) (Fig 9F). Mitochondrial-specific superoxide production in HUVECs treated with COCO was also assessed using MitoSox Red dye, and we found that COCO significantly increased mitochondrial superoxide production after 24-h exposure (Fig 9G). Taken together, these data suggest that COCO stimulation may impair glucose-dependent energy production, which in turn may overload complex I with NADH leading to decreased ATP production and enhanced complex I production of ROS (Zorov *et al*, 2014).

The redox system is heavily involved in endothelial cell function and dysfunction. Indeed, while modest and controlled generation of ROS in endothelial cells is required for numerous vital signaling pathways involved in cell survival, proliferation, activation, stress response, cell motility, vasodilation, and angiogenesis, high levels of oxidants have inhibitory effects on endothelial cell function (Jerkic *et al*, 2012; Kim & Byzova, 2014) and have been associated with increased senescence and DNA damage. To evaluate whether antioxidants could modulate the effects of COCO on sprouting angiogenesis, choroidal explants were cultured with or without COCO, in the presence or absence of NAC. As previously described, control choroidal explants cultured in the presence of VEGF displayed numerous endothelial sprouts after 5 days of culture. Accordingly, the addition of COCO resulted in a significant inhibition of the choroidal sprouting area. The addition of NAC significantly abrogated the inhibitory effects of COCO on vascular sprouting, suggesting that increased ROS generation may also play a significant role in the anti-angiogenic effects of COCO (Fig 9H and I).

Discussion

We have identified COCO, a secreted antagonist of BMP, TGFβ, and Wnt ligands, as a potent inhibitor of neovascularization in the eye. Injection of COCO during developmental retinal angiogenesis delayed formation of new blood vessels, and the effect of COCO on blood vessel formation appeared to be as potent as that of a VEGF inhibitor, Flt1Fc. In experimental models of choroidal neovascularization and vascular retinopathy, COCO also displayed potent inhibitory effects on angiogenesis. Importantly, COCO was shown to act specifically on developing blood vessels, as intravitreal delivery in adult mice did not result in obvious morphological changes on the mature vasculature. Also, long-term delivery of COCO, while having a potent effect on newly formed blood vessels, did not adversely affect photoreceptors. Few studies have looked at the effects of Dan family members during vascular development. Dan family members have been shown to act as antagonists of BMP, TGFβ, and Wnt signaling molecules. As these pathways play critical roles in vascular development, it is not unexpected that COCO, by antagonizing their signaling, may interfere with the angiogenic process. Specifically, COCO has previously been shown to inhibit BMP4 and Wnt1 in a dose-dependent manner in cultured photoreceptors (Zhou *et al*, 2015). In microvascular endothelial cells, BMP4, along with BMP2, have been shown to induce tube formation as well as promote migration (Rothhammer *et al*, 2007). Wnt1 has also been shown to induce the proliferation and survival of human endothelial cells, is expressed in developing blood vessels, and exerts salutary effects on postnatal endothelial progenitors (Masckauchan *et al*, 2005; Gherghe *et al*, 2011). By interfering with Wnt, BMP, and TGFβ signaling, COCO may therefore interfere with blood vessel development by altering several components of the angiogenic response, including proliferation and migration of endothelial cells.

One of the more surprising findings of this study was the observation that COCO affects energy metabolism in endothelial cells. Several studies have highlighted the importance of metabolic regulation in the endothelium and shown the critical importance of metabolic and glycolytic pathways in driving the angiogenic process (De Bock *et al*, 2013; Treps *et al*, 2016; Cantelmo *et al*, 2016; Eelen *et al*, 2018). Experimental data show that glycolytic ATP is a driver for endothelial cell rearrangements in the sprout by enhancing filopodia formation (Eelen *et al*, 2018) and that inhibition of the glycolytic flux blocks both physiological and pathological angiogenesis (Treps *et al*, 2016). Our data therefore argues that COCO, which reduces endothelial cell metabolic activity, may lead to a quiescent phalanx cell-like phenotype (Potente *et al*, 2011). The mechanisms by which COCO alters the energy metabolism and redox homeostasis of endothelial cells remain to be established. Our transcriptomics data reveal that several genes associated with mitochondrial function are altered in COCO-treated HUVECs, such as *NDUFV2*, a subunit of mitochondrial complex I, and *UQCRC1*, a component of the mitochondrial complex III, which could play a role in the effects of COCO on cellular metabolism and ROS production. Indeed, decreased expression or activity of *NDUFV2* has been associated with impaired complex I activity, correlated with reductions in ATP synthesis and mitochondrial membrane potential, while disruption of *UQCRC1* results in decreased complex III formation and activity, mitochondrial membrane potential, and ROS formation (Ogura *et al*, 2012; Shan *et al*, 2019; Wang *et al*, 2019; Chen *et al*, 2020). Therefore, COCO may alter endothelial metabolism and ROS production in part by modulating the expression of genes involved in mitochondrial function, although the rapid onset of the effects of COCO on glucose uptake and ATP production (starting 1 h after stimulation) is also suggestive of mechanisms that are independent from the modulation of gene expression. Indeed, the observation that exogenously added COCO rapidly triggers metabolic changes and localizes to the mitochondria in endothelial cells seems to suggest that it may also interact with unidentified cell surface or intracellular mediators involved in mitochondrial function. The interaction of Dan family members with cell surface receptors is indeed not unprecedented. Gremlin, a BMP antagonist structurally related to COCO, has previously been shown to interact with VEGFR2, although its function as an agonist or antagonist of VEGF signaling is still being debated (Mitola *et al*, 2010; Grillo *et al*, 2016; Dutton *et al*, 2019). Furthermore, Gremlin has also been shown to interact with Slit2 and block

Robo signaling (Tumelty *et al*, 2018), which is an important mediator of vascular development in the retina (Rama *et al*, 2015). Gremlin-BMP2 complex have also been shown to have a high affinity to heparan sulfates, whose presence on the cell surface and in the extracellular environment is critical to many physiological processes including angiogenesis (Kattamuri *et al*, 2017). Further studies investigating the interactions of COCO with alternative binding partners and the implications of its intracellular transport and its presence in the mitochondria will bring a better understanding of its role in cell metabolism and vascular development. Taken together, our data indicate that COCO may alter the endothelial glycolytic flux, whereby redox balance between NADH and NAD+ is perturbed and may result in decreased ATP production and increased ROS generation. This balance in energy production likely plays a significant role in the anti-angiogenic effects of COCO.

The effects of COCO on endothelial cell metabolism could also be linked to its ability to sequester Wnt, TGFβ, and/or BMP ligands. Indeed, studies have previously demonstrated that Wnt signaling is associated with altered glycolytic metabolism through PDK1-mediated inhibition of pyruvate flux to mitochondrial respiration (Pate *et al*, 2014). Wnt-driven Warburg metabolism characterized by increased reliance on glycolysis for energy production has also been reported in several cell lines (Esen *et al*, 2013; Pate *et al*, 2014). Furthermore, BMP signaling can also regulate glucose metabolism in chondrocytes through the upregulation of the glucose transporter Glut1 (Lee *et al*, 2018). Pulmonary hypertension, defined by altered BMP signaling, is also associated with endothelial dysfunction, an imbalance of proliferation and apoptosis, and an altered glycolytic metabolic profile (Tuder *et al*, 2013; Goumans *et al*, 2018). Similarly, depending on the context, TGFβ stimulation can also modulate mitochondrial respiration, glycolysis, and ROS generation (Abe *et al*, 2013; Bernard *et al*, 2015; Soukupova *et al*, 2017). As such, COCO may act in part by interfering with Wnt, TGFβ, and/or BMP signaling in endothelial cells, leading to alterations in energy production. Given that we report a significant inhibition of TGFβ pathway, along with a reduction in the expression of several genes involved in BMP signaling in COCO-treated HUVECs (Fig 8), we anticipate that COCO may modulate the energy metabolism of endothelial cells at least in part through the sequestration of TGFβ and/or BMP ligands. Notably, COCO appeared to act independently of BMP9-BMP10/Alk1 signaling, as shown by the observation that COCO retained its anti-angiogenic properties in Alk1ΔEC mice (Fig EV5), which lack Alk1 specifically in the endothelium. This does not preclude however that COCO could act by inhibiting the signaling of other BMP ligands, such as BMP2 and BMP4 (Mausner-Fainberg *et al*, 2019).

We show here a novel effect of COCO delivery in retinal vascular development. While we demonstrate the anti-angiogenic effects of exogenous COCO, the contributions of endogenous COCO in retinal angiogenesis remain to be demonstrated. Its inhibitory effects on neo-vessels, combined with its expression in the neural retina, may suggest a role for the prevention of angiogenesis in the outer retina and the maintenance of the photoreceptor avascular privilege. Our study has also important implications for the development of therapies targeting neovascular diseases in the eye, as we describe a novel inhibitor of pathological retinal and choroidal angiogenesis that apparently acts independently of VEGF signaling. Importantly, work is under progress to test whether chronic intra-ocular injection of COCO at therapeutic concentrations can lead to local and peripheral toxic effects in animals. While the mechanisms underlying the effects of COCO on blood vessels remain to be fully elucidated, our data show that its modulation of metabolism may play an important role in its effects on endothelial cells. Future studies will help decipher the mechanisms by which COCO affects the formation of new blood vessels.

Materials and Methods

Mice, tissue samples, and reagents

This study was conducted according to the CIUSS-de-l'Est-de-l'Ile-de-Montreal institutional guidelines and the Declaration of Helsinki. Eyes from human donors were provided by our local Eye Bank (Banque de tissus oculaires pour la recherche en vision; Centre de recherche du CHU de Québec-Université Laval; Quebec City, Quebec, Canada). Adult (3-month-old) and P1 to P17 pups mice C57BL/6J (Jax Mice) or Alk1ΔEC mice (Aspalter *et al*, 2015) (kindly provided by Paul S Oh (University of Florida)) were used in this study. All animals were housed and bred in a normal experimental room and exposed to a 12 h light/dark cycle with free access to food and water. All animal procedures were conducted under the regulation of Canadian federal and institutional guidelines (Protocol #2020-1921). Human Umbilical Vein Endothelial Cells (HUVECs; Promocell) were cultured in EndoGro-VEGF medium (EMD Millipore). Human Retinal Microvascular Endothelial Cells (HRMECs; Cell Systems, Kirkland, USA) were grown in EGM-2 microvascular medium (Lonza). Cells were routinely tested for mycoplasma contamination. COCO (R&D systems; Cat#3047-CC) was resuspended according to the instructions of the manufacturer at a stock concentration of 100 μg/ml.

Intravitreal Injections

Animals were anaesthetized with isofluorane. A 10 μl Hamilton syringe with a glass-pulled capillary was inserted with a 45° injection angle into the vitreous. When accessing the role of COCO developmentally, animals were injected either at P1 (final concentration COCO: 100 ng/ml in 5 μl vitreous; or similar volume PBS) or P3 to evaluate vascular growth (Schmucker & Schaeffel, 2004). During the neovascularization phase of OIR, animals were injected at P12 (2 μl) before sacrifice at P17 for quantification of neovascularization. For adult mice and laser-induced neovascularization, intravitreal injections were performed under a surgical microscope. Mice were anaesthetized with isofluorane. Pupils were dilated using 1% tropicamide and a 33-gauge needle was inserted from the limbus with a 45° injection angle into the vitreous.

Choroidal sprouting assays

Choroidal sprouting assays were performed as previously described (Shao *et al*, 2013). Following the removal of neuroretina from the posterior pole, the complex consisting of the retinal pigment epithelium (RPE)-choroid-sclera was collected, cut in 16 explant fragments, and cultured in Matrigel (BD biosciences) in 24-well plate. Explants were stimulated at day 1 and day 3 with COCO (100 ng/ml) and

sprouts were imaged at day 5. Quantification of sprouting area was performed using software analysis (Fiji/ImageJ).

Oxygen-induced retinopathy

C57BL/6J mouse pups at postnatal day (P)7 and their fostering mothers (CD1, Charles River) were subjected to 75% oxygen in an oxycycler chamber for 5 days. Pups were then returned to normoxia at P12 and administered 100 ng/ml (final concentration) of recombinant human COCO intravitreally or similar volume of vehicle in the contralateral eye. Eyes were enucleated at P17 and processed for immunostaining.

Laser-induced choroid neovascularization

Three-month-old C57BL/6J mice were anesthetized with a ketamine/xylazine mix prior to applying a photocoagulating laser (400 mW intensity, 0.05 s exposure time). Four spots were burned around the optical nerve. Mice received 100 ng/ml (final concentration) of recombinant human COCO intravitreally or similar volume of vehicle in the contralateral eye. Eyes were enucleated after 14 days and processed for immunostaining.

Immunohistochemistry

Ocular globes were initially fixed for 15 min in 4% paraformaldehyde (PFA). Retinas or choroids were collected after eyes dissection in PBS and blocked 1 h 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), phospho-histone H3 (Abcam), Collagen IV (Abcam), or cleaved caspase-3 (Cell Signaling) antibodies were performed on whole and/or sectioned retinas/choroids (see Table EV1 for the list of antibodies). Retinas and choroids were then mounted in fluoromount aqueous medium (Sigma-Aldrich). Quantitative analysis of tufts, vaso-obliterated, or vessel areas were performed using ImageJ/Swift_NV as previously described (Ntumba *et al*, 2016). Neovascular tuft formation was quantified by comparing the number of pixels in the affected areas with the total number of pixels in the retina. The avascular area in the retina was measured in the same way.

For HUVEC immunostaining, cells stimulated for 5 h with recombinant COCO were subsequently acid-washed to strip the surface-bound molecules. Cells were fixed for 15 min in 4% PFA and blocked 1 h in PBS 3% BSA 0.1% Triton X-100, followed by overnight incubation with primary antibodies (anti-hCOCO, Sigma; anti-MTCO1, Abcam). Cells were then washed, incubated with secondary reagents for 1 h at room temperature, washed and mounted. Slides were then mounted with fluoromount containing DAPI (Sigma) and visualized by confocal microscopy (Olympus Fluoview).

Endothelial cell sprouting assays

Sprouting assays were performed as previously described (Larrivee *et al*, 2012). Briefly, HUVECs or HRMECs (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 h at 37°C. NHDF cells (250,000 cells/well), in EBM-2 supplemented with 2% FBS and 25 ng/ml VEGF, with or without COCO, were then plated on top of the fibrin layers. Cultures were incubated at 37°C, 5% CO_2. Quantification of sprouting area was performed using software analysis (Fiji/ImageJ).

Transcriptome analysis by AmpliSeq

Transcriptomic analysis of HUVEC cells treated with or without COCO for 16 h, in quadruplicate, was performed using the Ion AmpliSeq Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, mRNA from 10 ng of total RNA was reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) and amplified with Ion AmpliSeq HIFI Mix together with primers from the Ion AmpliSeq Transcriptome Human Gene Expression Core Panel simultaneously targeting over 20,000 RefSeq genes. Primer sequences were partially digested with FuPa Reagent and then barcoded using Ion Xpress Barcodes (Thermo Fisher Scientific). Purification was carried out by AMPure XP Reagent (Beckman Coulter). Libraries concentrations were defined by qPCR using Ion Library Quantification kit (Thermo Fisher Scientific). Libraries were pooled together for emulsion PCR, carried out using the Ion Chef Instrument (Thermo Fisher Scientific). Purified Ion Sphere Particles were loaded on Ion P1 Chip. The sequencing was performed on Ion Proton system (Thermo Fisher Scientific). Ion Torrent software, Torrent Suite v5.12 (Thermo Fisher Scientific), was used for base calling, alignment to the human reference genome (hg19) and quality control. Raw reads were then analyzed automatically using the AmpliSeqRNA plugin to generate gene-level expression values. Differential gene expression was determined using DEseq2 (version 3.11) package. Gene set enrichment analysis (GSEA) was performed using the GSEA software using pre-defined gene sets based on prior biological knowledge (version 4.1).

Scratch assays

Confluent HUVEC monolayers were grown in 6-well plates. Cells were starved 18 h in EBM-2 medium with 1% FBS. A horizontal wound was created using a sterile 200 μl pipette tip. Next, the cells were washed with EBM2 at 37°C and incubated in EBM-2 supplemented with VEGF-A (25 ng/ml) with or without COCO (60 ng/ml) at 37°C for 16 h. Pictures of scratch wounds were taken just before stimulation (time 0) and after 16 h. Migration % was calculated using ImageJ software.

Flow cytometry

Sub-confluent HUVECs were cultured overnight in starvation medium (EBM2-1%FBS) in the presence or absence of 60 ng/ml COCO, followed by VEGF stimulation for 1 h. HUVECs were subjected with a pulse of 5-ethynyl-2′-deoxyuridine (EdU) for 1 h,

and flow cytometry analysis of EdU incorporation was performed as previously described (Oubaha *et al*, 2016). Detection of apoptotic cells was performed using a dead cell apoptosis kit (Cell Signaling) according to the instructions of the manufacturer.

Western blotting

Cells were washed with cold PBS and extracted in Laemmli's buffer, followed by sonication. Samples were run on SDS–PAGE gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% Bovine Serum Albumin (BSA) and probed with primary antibodies overnight at 4°C. HRP-conjugated secondary antibodies (Vector Laboratories) were used to detect primary antibodies. Antibodies are listed in Table EV1. Densitometric band intensity quantification of detected immunoblotting protein-antibodies complexes was done using ImageJ software.

RNAscope *in situ* hybridization

In situ hybridization was performed on paraformaldehyde-fixed, OCT-embedded sections as directed by the manufacturer (Advanced Cell Diagnostics, Hayward, CA, USA). We used RNAscope Probe-Mm-Dand5 (NPR-0006197) to detect mouse *Dand5*. The *Dand5* probe was designed to target 53–1,175 of mouse *Dand5* (NR_033145.1). Negative control sections were probed for bacterial dihydrodipicolinate reductase mRNA (dapB).

ROS measurement

HUVECs were plated into a black 96-well plate and treated with COCO, H_2O_2, or 3-NP. Media was removed after specific times and replaced with complete media containing 20 μM 2,7-Dichlorofluorescin diacetate (Sigma, D6883) for 30 min or 5 μM MitoSox Red (Life Technologies M36008) for 5 min at 37°C. Media was removed and cells were washed once with PBS. Fluorescence was measured at Excitation/Emission 485/535 nm (DCFDA) or 544/590 nm (MitoSox Red). After the initial reading, cells were incubated with Hoescht and fluorescence was detected at 355/460 nm. DCFDA and MitoSox fluorescence was normalized to Hoescht for each well.

NAD+/NADH enzymatic cycling assay

Evaluation of cellular NAD+/NADH levels were performed as previously described (Kato *et al*, 1973; Lin *et al*, 2001). HUVECs plated into 100 mm dishes were treated with COCO for various time points. NAD+ and NADH were extracted using ice cold alkali (0.5 M NaOH, 1 mM EDTA) and acidic buffers (0.1 M HCl), respectively. Extracts were heated at 60°C for 30 min and buffers were neutralized with either the NADH (100 mM Tris–HCl pH 8.1, 0.05 M HCl) or NAD+ (0.4 M Tris) neutralization buffers. To attain measurable quantities of NAD+ or NADH, an amplifying cycling assay was performed. Extracts and NAD+ standards were incubated with cycling reagent (67 mM Tris–HCl pH 8, 200 mM EtOH, 1.3 mM beta-mercaptoethanol, 0.01% BSA, 2 mM oxaloacetic acid, 0.5 μg/ml malate dehydrogenase, 5 μg/ml alcohol dehydrogenase) for 1 h at room temperature. All samples were heated for 5 min at 100°C to stop enzymatic reactions then cooled on ice. For detection, extracts were incubated in an indicator buffer (50 mM 2-amino-2-methyl-propanol pH 9.9, 200 μM NAD+, 10 mM glutamate, 0.04% BSA, 5 μg/ml malate dehydrogenase, 2 μg/ml glutamate oxaloacetate transaminase) for 10 min at room temperature. Then, 100 μl if sample was transferred to a black 96-well plate. Fluorescence was detected using a plate reader (TECAN) at an excitation of 365 nm and emission of 460 nm. Standard curves were generated and the concentration of NAD+ and NADH were calculated from the standard curve.

ATP detection

HUVECs were plated into 100 mm dishes and treated with COCO for various time points. ATP was measured by luminescence (ATP Detection Assay Kit; Cayman Chemicals).

Seahorse analysis

The Seahorse analyzer XF96 (Agilent, Santa Clara, USA) was used to continuously monitor OCR and ECAR. Two days prior to the experiment, 2,000 cells/ well were seeded in a XF96 cell culture plate in EndoGro medium (EMD Millipore) and cultivated at 37°C in humidified atmosphere with 5% CO_2. Cells were then treated with COCO for 1, 2, and 24 h. One day prior to the experiment, 200 μl of XF calibrant was added to each well of the utility plate and the XF cartridge was incubated overnight at 37°C in a humidified atmosphere in a non-CO_2 incubator. Just before readings were performed, cells were washed twice with unbuffered XF DMEM

The paper explained

Problem

Neovascular age-related macular degeneration (AMD) is a significant cause of vision loss in aging populations. Current therapies for neovascular AMD focus on the inhibition of a protein, Vascular Endothelial Growth Factor (VEGF), to block the growth of blood vessels in the eye. While therapies targeting VEGF have been shown to slow or stop the progression of AMD, there are still patients that show limited response to anti-VEGF drugs, and some adverse effects have also been reported after long-term treatments. Therefore, there is a need to identify alternative targets to block neovascularization in the eye in order to develop new therapeutic approaches.

Results

We have identified a protein, COCO, that can block the growth of blood vessels in the eye. Using murine models of developmental and pathological neovascularization, we have shown that COCO delays the growth of new blood vessels in the retina and the choroid, without affecting pre-existing mature vessels.
Mechanistically, using endothelial cells and cultures of choroidal explants, we have been able to demonstrate that COCO prevents the migration and proliferation of endothelial cells, in part by decreasing the signaling of TGFβ and BMPs, a family of genes known to regulate vascular growth, and by limiting energy metabolism and promoting reactive oxygen species production.

Impact

Our study identifies a new function for COCO, a factor that could be used in the clinics to limit the growth of pathological blood vessels in the eye, and could lead to the development of new therapies for the treatment of neovascular eye diseases.

assay medium (containing 5 mM glucose and 2 mM glutamine, pH 7.4) and incubated for 1 h in a humidified non-CO_2 incubator. Inhibitor compounds were prepared as per the instructions of the Seahorse XF Cell Energy Phenotype Test Kit (#103325-100). Oligomycin and FCCP were added together to the injection port of the XF cartridge to yield final concentrations of 1 and 1.5 μM, respectively. After 15 min equilibration time, baseline OCR and ECAR were assessed every minute (after 3 min mixing, 2 min wait, 3:30 min measure). Five measurements were taken under stressed conditions following the compound injection. Following ECAR and OCR measurements, cells were lysed and protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher Scientific) for normalization. Data are presented as percentage of untreated cells.

Metabolite measurements

HUVECs were cultured for up to 48 h in the presence or absence of COCO. Culture media was collected at 1, 3, 6, 12, 24, and 48 h, centrifuged at 16,260 *g* for 5 min, aliquoted, and stored at −80°C. Media was analyzed for glucose concentration using a BioProfiler 400 Analyzer (BioNova). For measurements of pyruvate, the culture media was analyzed using the Pyruvate Colorimetric/Fluorometric Assay Kit (BioVision) as per the manufacturer's instructions.

Statistical analyses

Data analyses were performed in a blinded fashion. In animal studies, sample size was determined based on previous experiments performed in our laboratory, accounting for potential side effects of injections (weight loss, ocular inflammation). Animals were excluded from studies only if they displayed severe weight loss (over 20% body weight), or if they showed signs of ophthalmic inflammation following intravitreal injections. No randomization was used; however, we ensured that mice of similar weight and size were used for each group before treatments. All data are shown as mean ± standard error of the mean (SEM). Statistical analyses were performed for all quantitative data using Prism 6.0 (GraphPad). Statistical significance for paired samples and for multiple comparisons was determined by Mann–Whitney test and ANOVA, respectively. Normality and homogeneity were proved with Shapiro–Wilk test. Data were considered statistically significant if the *P* value was less than 0.05.

Data availability

The datasets produced in this study are available in the following databases: Microarray: Gene Expression Omnibus GSE160099 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160099).

Expanded View for this article is available online.

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Author contributions
Manuscript writing: BL, NP, EH, and GB; Guidance in designing of hypothesis and experiments: BL and GB; *In vivo* experiments, flow cytometry, choroidal sprouting assay, RNAscope, and immunostainings: NP; Transcriptomics analysis: NP, AF, and MB; *In vitro* metabolic experiments and VEGF signaling experiments: EH; Immunofluorescence on human retina and photoreceptors: AB; OIR and laser-CNV experiments: FP; Project conception: BL and GB.

Conflict of interest
G.B. and A.F. are co-founders and shareholders of StemAxon™. G.B. and B.L. are inventors on patent application (U.S. Provisional Application No. 62/879,755) that covers the use of COCO for the treatment of ocular neovascularization. The rest of authors declare no conflict of interest.

For more information
i https://www.proteinatlas.org/
ii https://crhmr.ciusss-estmtl.gouv.qc.ca/en
iii http://visionnetwork.ca/

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Expanded View Figures

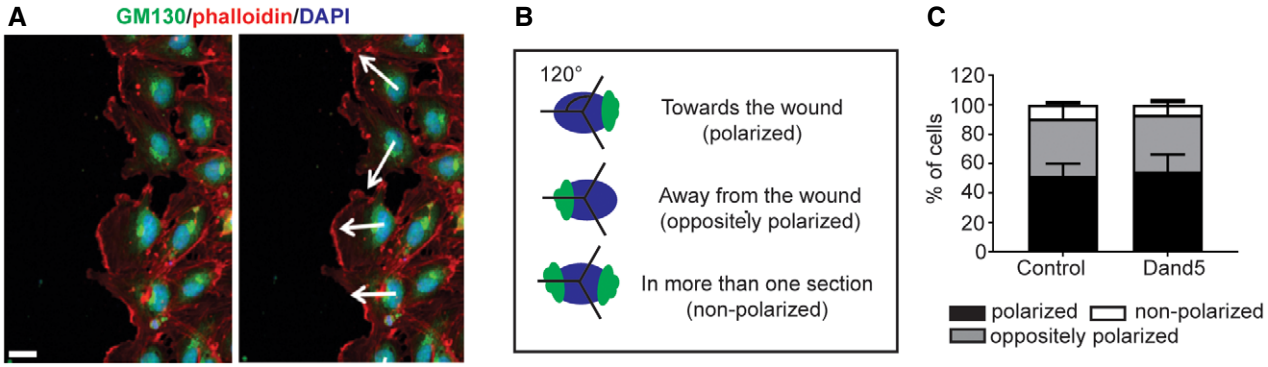


Figure EV1. COCO treatment does not affect front-rear polarity.

A Golgi (GM130) orientation of COCO-treated HUVECs. Scale bar, 20 μm.
B Schematic of the polarization quantification strategy.
C Quantification of cell polarity in COCO-treated HUVECs (n = 3; 100 cells/experiment).

Figure EV2. Effect of COCO on retinal proliferation and apoptosis.

A Schematic of the experimental strategy to assess proliferation and apoptosis of the retinal vasculature.
B, C Pictures and quantification of retinas injected 24 h prior with recombinant COCO, stained with IsoB4 and phospho-HistoneH3 antibody. ***P = 0.0007; (n = 11).
D, E Pictures and quantification of retinas injected 24 h prior with recombinant COCO, stained with IsoB4 and cleaved caspase 3 antibody; (n = 3–5 mice/group). Scale bars, 100 μm. Results are presented as mean ± SEM and statistical significance was analyzed by Mann–Whitney test. ns: not significant.

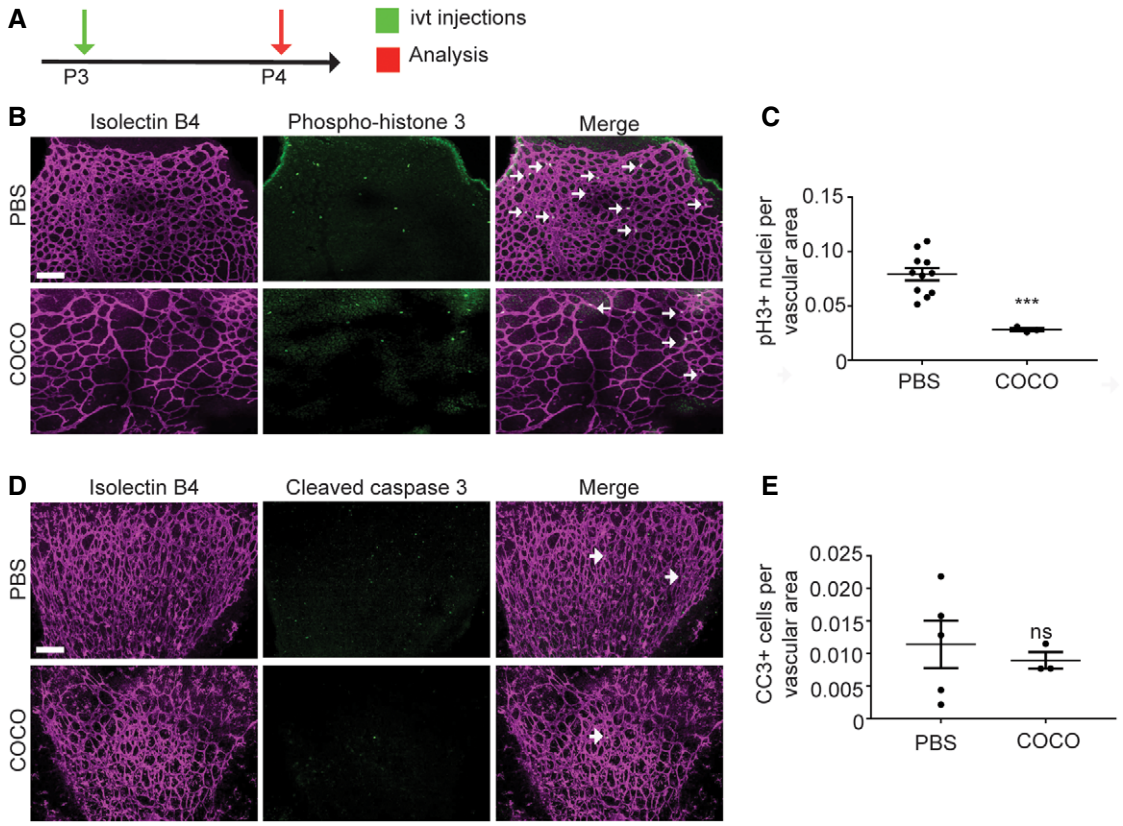


Figure EV2.

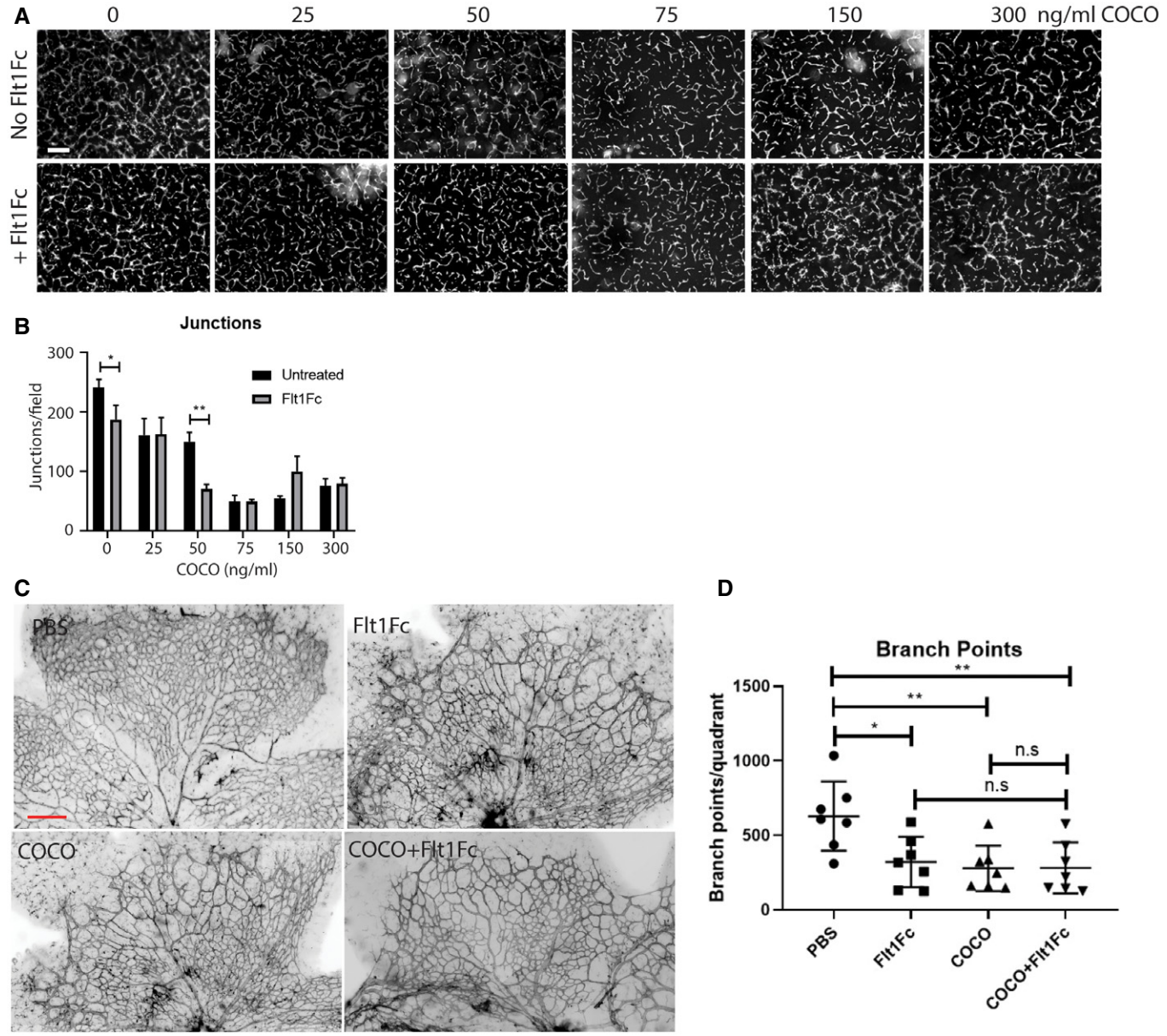
Figure EV3. Effect of the combination of COCO and VEGF inhibition on angiogenesis.

A Representative images of HUVECs sprouting in a fibrin gel with or without Flt1Fc (100 ng/ml) in the presence or absence of COCO. Scale bar, 75 μm.

B Quantification of the number of junctions/field of images shown in (A). *P = 0.0378 (Untreated vs. Flt1Fc); **P = 0.0069 (COCO50 ng/ml vs. COCO50 ng/ml + Flt1Fc); (n = 3; 5 pictures/experiment).

C Retinal flat mounts of P5 mice injected with PBS, COCO, or Flt1Fc alone or in combination are stained with IB4 (negative images of the fluorescent signal). Scale bar, 100 μm.

D Quantification of number of branchpoints. Results are presented as mean ± SEM and statistical significance was analyzed by two-way ANOVA. *P = 0.0151 (PBS vs. Flt1Fc); **P = 0.0060 (PBS vs. COCO); **P = 0.0079 (PBS vs. COCO + Flt1Fc); n.s. = non significant; (n = 7).


A
0
25
50
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No Flt1Fc
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C
PBS
Flt1Fc
COCO
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Figure EV3.

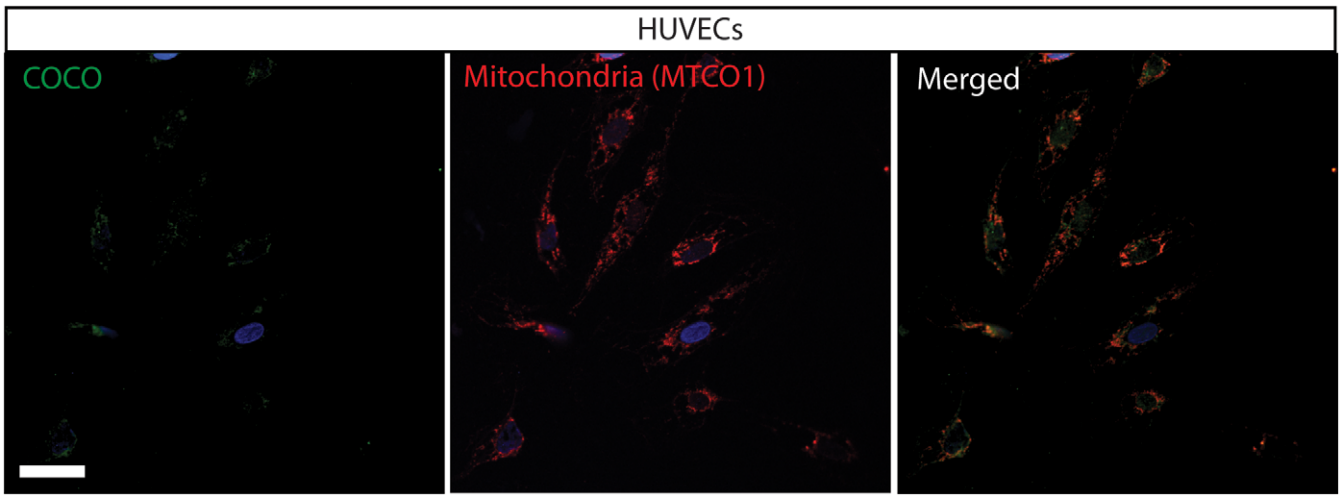

Figure EV4. COCO immunostaining in unstimulated HUVECs.

COCO and MTCO1 immunofluoresence staining of unstimulated HUVECs. Scale bar 25 μm.

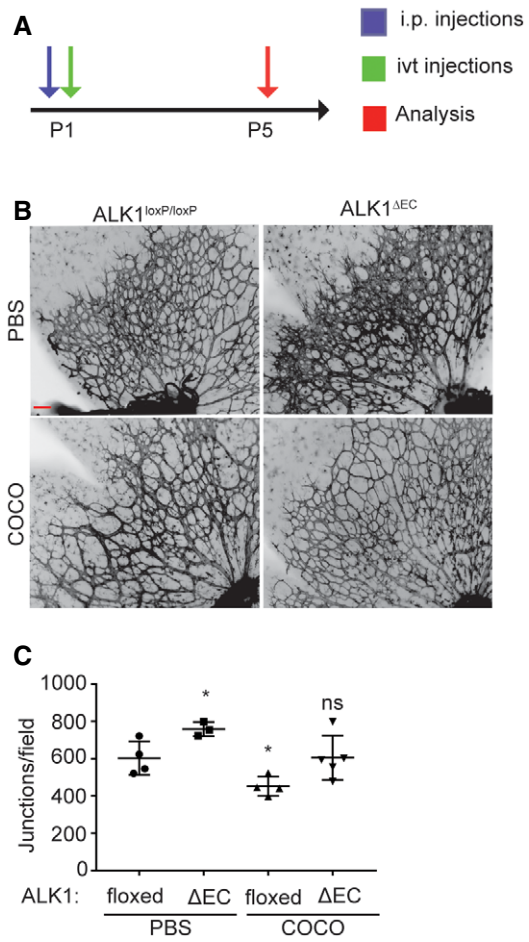

Figure EV5. Effect of COCO delivery on retinal development in control ($Alk1^{loxP/loxP}$) or Alk1ΔEC mice.

A Schematic of the experimental strategy to assess early formation of the retinal vasculature in tamoxifen-inducible Alk1ΔEC mice.

B Retinal flat mounts of P5 mice. Scale bar, 100 μm.

C Quantification of number of branchpoints. $N = 4$ animals/group. Results are presented as mean ± SEM and statistical significance was analyzed by Mann–Whitney test. *$P = 0.0126$ for ΔEC(PBS); *$P = 0.0152$ for floxed (COCO); ns: not significant; ($n = 3$–5/group).

Chapter IV: Characterization of ocular angiogenesis in Dand5$^{-/-}$ mice

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(This is a publication in preparation)

NP: manuscript writing, literature review, proposing experiments, and experimental analysis. *In vivo* experiments: importing, breeding, maintaining the murine colony, genetic observation, murine phenotyping of all the organs, retinal dissection in pups and adult mice, cryosections, immunostainings, and microscopy. OCT, RNA extraction, quantification for RNA sequencing. *Ex vivo* experiments: Ampli-sequencing from choroidal sprouting assay, RNA extraction, quantification, libraries, and ongoing analysis.

Sergio Crespo-Garcia: OCT experiments.

Marisse Masis Solano: Analysis of OCT.

Manuel Buscarlet: Analysis of Ampli-sequencing from choroidal sprouting assay.

Gael Cagnone: single-cell RNA-sequencing analysis.

Jose A Belo: Developed Dand5$^{-/-}$ mice.

BL & GB: project conception, manuscript writing; guidance in designing hypothesis and experiments, analysis.

Rational

In the study presented in the previous chapter, we have demonstrated that intravitreally injected COCO, an antagonist of TGF-β, BMPs, and WNT, suppresses the ocular angiogenesis and could be of interest for the development of therapeutic approaches to treat neovascular ocular diseases. Despite this promise, the fundamental processes by which COCO mediates these biological effects remain largely unknown. Low success rates during therapeutics development are due, in part, to the poorly defined mechanism of action and the absence of molecular markers of therapeutic activity. As such, the development of novel druggable strategies targeting ocular neovascularization involving COCO requires a better understanding of its physiological role and mechanism of action. Therefore, we propose to better understand the physiological role of COCO in the eye during development and in post-natal life. To do so, we analyze the retinal phenotype of COCO KO (*Dand5*$^{-/-}$) mice in physiological and pathological conditions to gain new insights into the function of endogenous COCO.

ABSTRACT

We have previously shown that COCO (*dand5*) is a potent inhibitor of retinal and choroidal angiogenesis expressed in the developing retina. However, its function in ocular development has not been characterized. We hypothesized that COCO acts as a negative regulator of vascular development in the eye and that inhibition of its expression may exacerbate an angiogenic response. Using *Dand5*$^{-/-}$ mice, we observed that while there were no significant alterations in the formation of the superficial vascular plexus in KO mice, there was an exacerbation of the angiogenic response in the formation of the deep vascular plexus at P12 that was resolved in adults. Furthermore, we showed that *dand5* is up-regulated in oxygen-induced retinopathy and that *Dand5*$^{-/-}$ mice display increased neovascular tuft formation in this model. Thus, our findings suggest that COCO expression in the retina could partially modulate endothelial cells' angiogenic response at specific stages of vascular development, in part by regulating the cellular responses to TGF-β and BMP signals.

INTRODUCTION

Pathological neovascularization occurs in a spectrum of ocular disorders such as age-related macular degeneration (AMD) and retinopathy of prematurity (ROP). One of the underlying causes of vision loss in proliferative retinal diseases is the increased vascular permeability leading to retinal edema, vascular fragility resulting in hemorrhage, or fibrovascular proliferation with retinal detachment. In ocular vaso-proliferative diseases, deregulated growth factors lead to excessive angiogenesis and vascular leak, inducing edema, hemorrhage, and compromising vision. Although neovascularization tends to occur at a relatively late stage in the course of many ocular disorders, it is an attractive target for therapeutic intervention since it represents a final common pathway in processes that are multifactorial in etiology and is the event that typically leads directly to visual loss (1-6). Identification of these angiogenesis regulators has enabled the development of novel therapeutic approaches. Inhibition of VEGF with blocking antibodies is used in AMD patients with macular edema to prevent vision loss. However, some patients are refractory to treatment, and most patients develop resistance to VEGF inhibition over time. Identifying pathways that contribute to angiogenesis besides VEGF, which may be activated upon VEGF inhibition, is critical for developing novel therapeutics [7-19].

Members of the Differential screening-selected gene Aberrative in Neuroblastoma (DAN) family form a diverse group of antagonists initially identified as Bone Morphogenetic Protein (BMP) inhibitors. DAN family members are expressed during development, having a significant role in limb bud formation and digitation, kidney formation and morphogenesis, and left-right axis specification. DAN family members have been associated with several pathologies during post-natal life, including pulmonary and renal fibrosis and cancer (445, 448, 449). COCO is a member of the DAN family, composed of secreted proteins that act as antagonists of BMP, TGF-β, and Wnt signaling molecules and establish anterior-posterior patterning in vertebrates (501, 502). Seven DAN family genes have been identified: Nbl1, SOST, USAG-1/Wise, COCO (DAND5), Gremlin, Cerberus, and Gremlin-2. Recent breakthroughs have shown that COCO is widely expressed in the retinal photoreceptor layer and that it is a potent inducer of cone photoreceptor differentiation and maintenance. Notably, COCO also displays pro-survival activities on human cones in vitro (501). In addition, we have previously demonstrated that COCO inhibits sprouting,

migration, and cellular proliferation of cultured endothelial cells. Intravitreal injections of COCO inhibited retinal vascularization during development and in models of ROP, with activity equal or greater than that observed with VEGF inhibition. Mechanistically, COCO inhibits TGF-β and BMP pathways and alters the metabolism of endothelial cells, although the mechanisms by which COCO affects cellular energy expenditure remain unknown (505).

In the study presented in the previous chapter, we have demonstrated that intravitreally injected COCO, an antagonist of TGF-β, BMPs, WNT, suppresses the ocular angiogenesis and could be of interest for the development of therapeutic approaches to treat neovascular ocular diseases. Despite this promise, the fundamental processes by which COCO mediates these biological effects remain largely unknown. Low success rates during therapeutics development are due, in part, to the poorly-defined mechanism of action and the absence of molecular markers of therapeutic activity. As such, the development of therapeutic strategies targeting ocular neovascularization involving COCO requires a better understanding of its physiological role and mechanism of action. Therefore, we propose to better understand the physiological role of COCO in the eye during development and in post-natal life. To do so, we analyze the retinal phenotype of COCO KO (*Dand5-/-*) mice in physiological and pathological conditions to gain new insights into the function of endogenous COCO.

METHODS

Animals

Dand5$^{-/-}$ mice (provided by J.A. Belo) were previously described (506) and were maintained in the animal research facility in Maisonneuve-Rosemont Hospital. The Animal Research Ethic committee of the research center approved all protocols.

For analysis of retinal vascular development, mice were sacrificed at post-natal day 5 (P5), P10, P12, and P28. Ocular globes were harvested, and retinas were dissected. For IsolectinB4 immunostaining, dissected retinas were blocked for 1h in PBS 3% BSA 0.1% Triton X-100 and labeled overnight with FITC-labeled isolectin GS IB4 (Life technologies corporation). Retinal flat mounts were visualized by confocal microscopy. For assessing retinal vascular layers, Z-stacks were acquired. The vessel branch points and length were assessed using the Angiogenesis Analyzer application (ImageJ) from whole-mount retinas.

Oxygen-induced retinopathy

Dand5 WT and *Dand5* $^{-/-}$ mouse pups at postnatal day (P)7 and their fostering mothers (CD1, Charles River) were subjected to 75% oxygen in oxycycler chamber for 5 days. Pups were then returned to normoxia at P12. Eyes were enucleated at P17 and processed for immunostaining as previously described. For quantitative analysis of COCO expression, retinas were harvested at P17, followed by RNA isolation using a RNeasy mini kit according to the manufacturer's instructions. Quantitative PCR was performed using primers obtained from QIAGEN (Quantitect Primers Assays).

Spectral-domain Optical Coherence Tomography (SD-OCT)

Mice were subjected to pupil dilation with Mydriacyl (Alcon, Mississauga, ON, Canada) and anesthetized intraperitoneally with a mix of 10% ketamine and 4% xylazine (10µl/g body weight). Animals were then placed on a custom-made platform coupled with an OCT Heidelberg Spectralis (Heidelberg Engineering, Germany). Images were obtained with a 30º lens and using the IR-OCT mode. Eyes were kept moisturized throughout the experiment, and no contact lenses were utilized during the acquisition. The retina was imaged in the temporal region adjacent to the optic nerve head. A total of 24 B-scans were acquired in each eye evaluated using an RT of 20 frames.

Optical Coherence Tomography Image Processing

Images were curated with custom-made software using MATLAB (v9.10; R2021a). In brief, a median filter (3x3 pixel) was applied to reduce noise, and each image was then converted into grayscale. Automated segmentation (mask and then calculated the distance for the first and last change in contrast) permitted to delimit the retina between both the retinal nerve layer (RNFL) and the choroidal-scleral interphase (CSI). The distance between the two segmented layers was calculated longitudinally in each of the 24 B-scans and then averaged. The computed mean values of both eyes of the animal were combined and averaged.

Transcriptomic analysis of OIR retinas

Transcriptomic differences between normoxic and OIR cell types were analyzed using NCBI's Gene Expression Omnibus (accession no. GSE150703) as previously described (507).

Statistical analysis

Statistical significance was evaluated with the unpaired t-test using GraphPad Prism software. P-values less than 0.05 were considered to be significant.

RESULTS

Role of endogenous COCO in retinal vascular development

We have previously shown that exogenous COCO modulates vessel branching during retinal physiological and pathological angiogenesis (505). While COCO expresses in the neural retina during development, the role of endogenous COCO in ocular angiogenesis and visual function remains unknown. The retinal model of developmental angiogenesis is widely used to study developmental angiogenesis, where blood vessels emerge from the optic nerve and sprout towards the periphery during the first week of life. The role of endogenous COCO in sprouting retinal angiogenesis was investigated in control and *dand5* KO mice five days after birth (P5) during the superficial retinal vascular plexus development (Fig. 1). Despite the previous observation that intravitreal delivery of exogenous COCO significantly delays retinal angiogenesis at P5 (505), no significant differences were observed between WT and Dand5$^{-/-}$ animals at this stage of retinal vascular development (Fig. 1A, B). Between P7 and 10, the superficial retinal vessels dive into the retina to form two additional layers of vessels. In the outer plexiform layer, the deep vascular plexus is complete by P14, while the intermediate plexus forms by P21 in the inner plexiform layer.

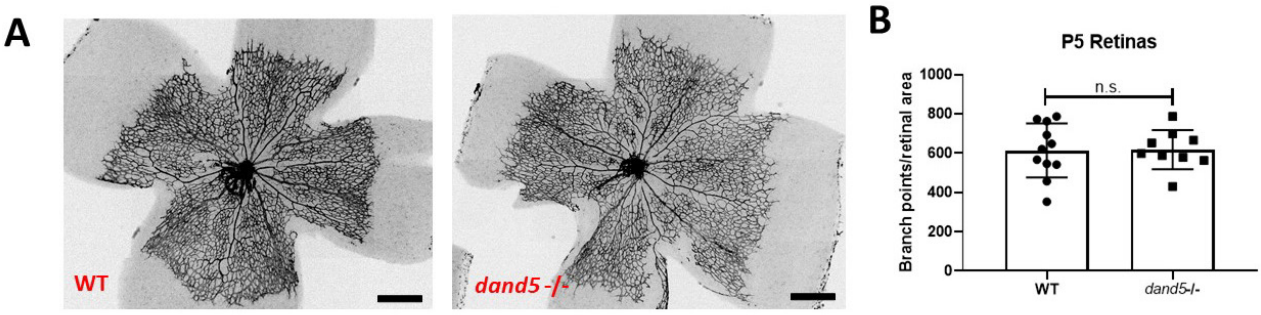

Figure-1. Retinal vascular phenotype of *dand5* KO mice at P5 (A) P5 retinas from WT and *dand5* KO littermates stained with IsoB4. (B) Quantification of vascular branch points. (n=11 animals/group). Scale Bars: 1000 μm

Furthermore, while the overarching superficial network still exhibited an unaltered density at this stage in mutants, the deep vascular plexus in dand5 mutants exhibited increased branch points compared with controls (Fig. 2 A, B).

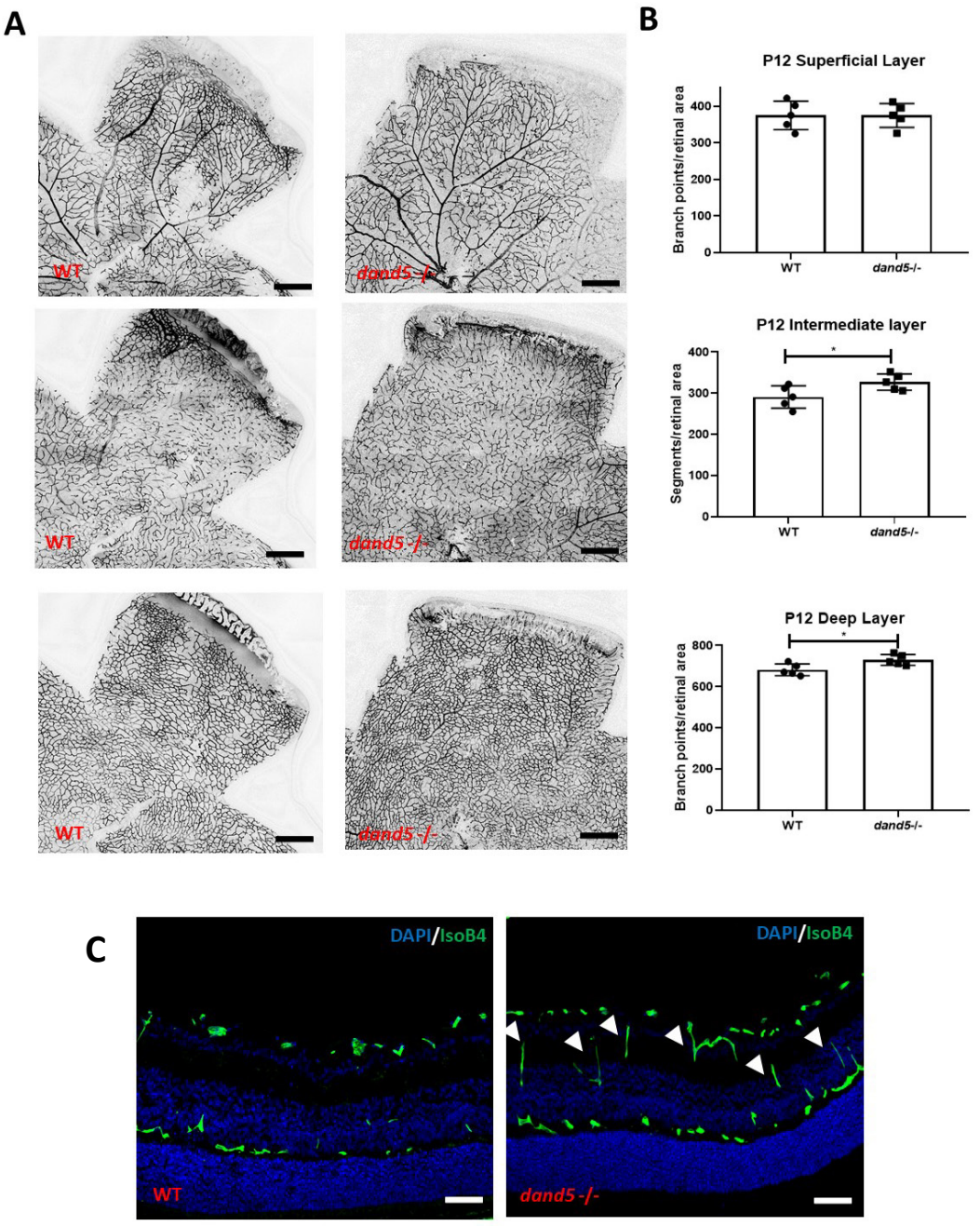

Figure-2. Retinal vascular phenotype of *dand5* KO mice at P12 (A) Representative images of P12 retinal superfical (top), intermediate (middle) and deep (bottom) vascular layers. Scale Bars: 200 µm (B) Quantification of the retinal vasculature of individual retinal layers (n=5 animals/group). *: P<0.05. (C) Cross-sections of P10 retinas from WT and *dand5* -/- mice were stained with IsoB4, showing increased vessel sprouting in deeper retinal layers (arrows: diving vessels). Scale Bars: 40 µm.

However, when adult retinas were examined at P28, no differences were observed between control and Dand5$^{-/-}$ animals, suggesting a resolution of the transient phenotype observed at the formation stage of retinal intermediate and deep vascular plexus (Fig. 3 A, B). Furthermore, no differences in retinal thickness were detected in adult Dand5 $^{-/-}$ mice compared to WT animals, as assessed by spectral-domain optical coherence tomography (SD-OCT) (Fig. 3 C, D). Taken together, these data suggest that, while COCO does not appear to be required for the formation of the superficial vascular retinal plexus during development, its deletion may result in a significant, but transient, exacerbation of the angiogenic response during the formation of the intermediate and deep retinal vascular plexus, although the phenotype appears to resorb in adult mice.

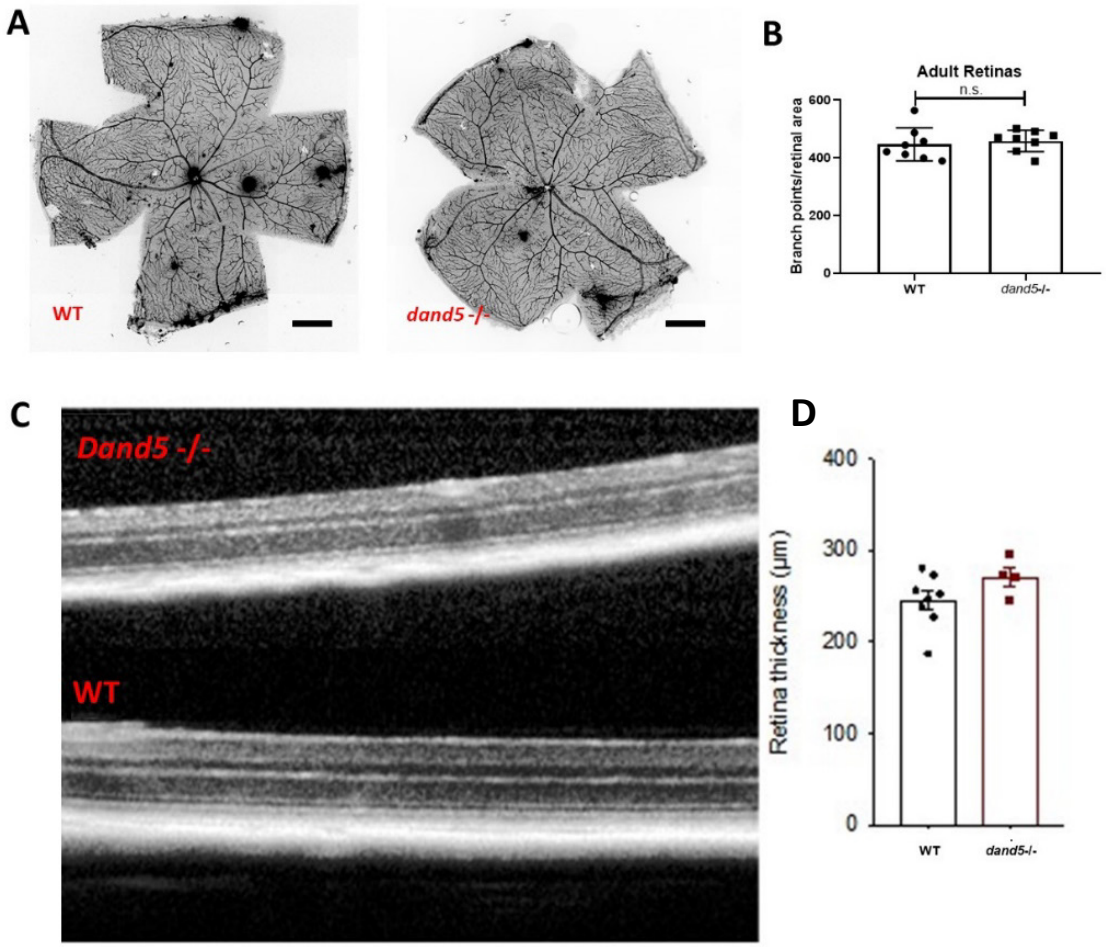

Figure-3. Retinal vascular phenotype of adult *dand5* KO mice (A) P28 retinas from WT and *dand5* KO littermates stained with IsoB4. Scale Bars: 500 µm. (B) Quantification of vascular branch points. (C) Illustrative OCT images depicting a single B-scan of the retina of a wild-type or *dand5*$^{-/-}$ knock-out mice. (D) Quantification of the retinal thickness in wild-type or Dand5$^{-/-}$ knock-out reveals no significant differences.

Exacerbated response to OIR in Dand5$^{-/-}$ mice

Our previous data showed COCO broad expression in the neural retina and potent inhibition of pathological neovascularization in a pathological model of oxygen-induced retinopathy (OIR) (505). We, therefore, asked whether COCO expression in the retina could be modulated in OIR, and whether its deletion could affect the pathological vascular features observed in this model. C57/Bl6 mice were subjected to OIR as previously described, and mRNA was harvested from retinas at P17. As controls, mRNA was used from retinas collected from littermate mice that were not subjected to OIR. Quantitative PCR analysis revealed a significant upregulation of COCO in retinas subjected to OIR compared to normoxic mice (Fig. 4A). These data were also confirmed by analyzing single-cell sequencing data (Fig. 4B) from published data evaluating gene expression differences between control and OIR retinas (507), showing that COCO was up-regulated predominantly in retinal astrocytes and ganglion cells under OIR.

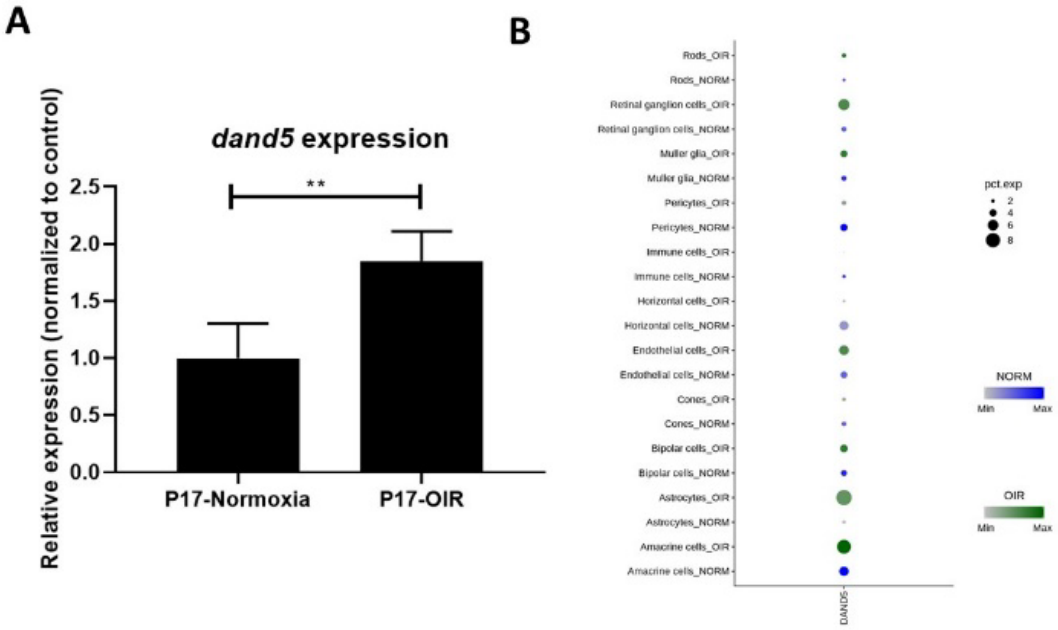

Figure-4. Dand5 expression is upregulated in pathological retina. (A) Quantitative PCR analysis of P17 retinas confirm *dand5* overexpression in OIR retinas. **: P<0.01. (B) Cell populations from control and OIR P17 reveals that in OIR, several retinal cell populations (astrocytes, amacrine cells, retinal ganglion cells) express higher levels of *dand5* (analyzed from GSE150703).

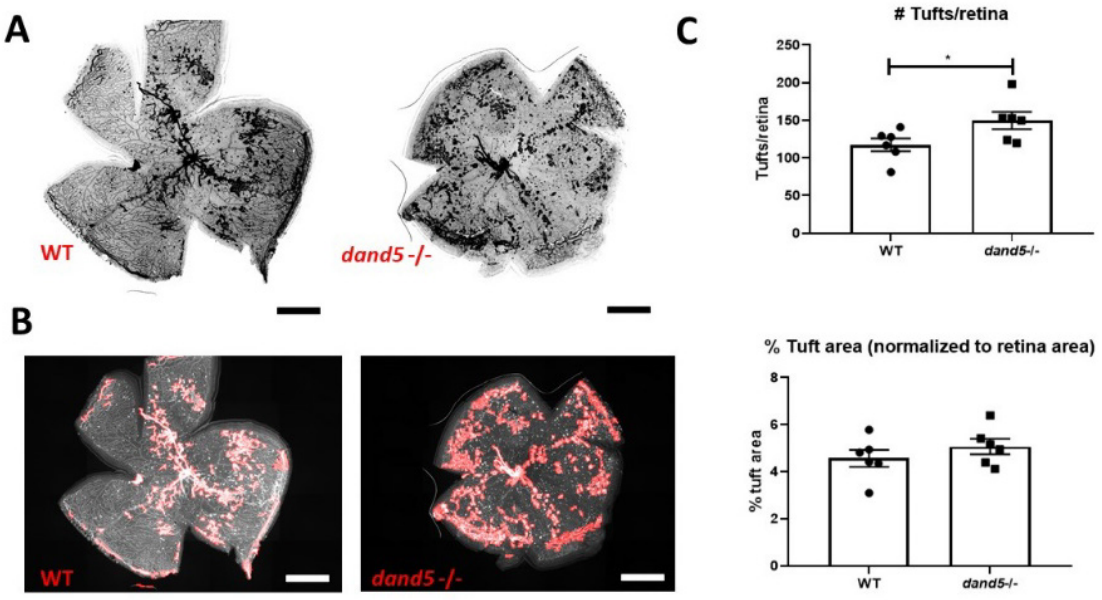

Figure-5. Exacerbation of OIR neovascular tufts in *dand5* KO mice (A) Representative images of P17 retinas harvested from WT and *dand5*-/- mice subjected to OIR. (B) Quantification of neovascular tufts using Swift_NV, shown in pink. Scale Bars: 1000 µm. (C) Quantification of the number of tufts per retina (top) and percentage of retinal area covered by neovascularization (bottom) in WT and *dand5*-/- OIR retinas (n=6 animals/group). *: $P<0.05$.

We then evaluated whether COCO played a role in developing neovascular pathological features by subjecting WT and Dand5$^{-/-}$ littermates to OIR. Retinas from Dand5$^{-/-}$ mice exhibited a small but significant exacerbation of the number of pathological neovascular tuft formation compared to WT littermates (Fig. 5). Together, these data would suggest that endogenous COCO expression is increased in pathological conditions and that COCO could play a significant role in preventing excessive neovascularization in OIR.

DISCUSSION

Studies have revealed that COCO is essential in establishing left-right asymmetry during axis formation and heart development during embryogenesis (508); its effect on ocular development has not been shown. Recent evidence demonstrates that i) the protein COCO acts as an inhibitor of retinal and choroidal angiogenesis both in vitro and in vivo, ii) that exogenous COCO promotes the differentiation of cone photoreceptors from embryonic stem cells and the growth and survival of photoreceptors in vitro and iii) that is expressed in the developing retina, we postulated that COCO might significantly contribute to ocular development.

To assess the contribution of COCO in the development of the eye, we used Dand5$^{-/-}$ mice. We observed that the offspring of Dand5 $^{+/-}$ **i**ntercrosses were not born according to the correct Mendelian ratio, with KO animals being lower than expected (Table I).

Table I. Genotype of *dand5* offspring from *dand5* +/- intercrosses

#1-223	# Female	%Female	#Male	% Male	TOTAL	% TOTAL
WT	24	**29%**	25	**27.17%**	49	**28.16%**
HET	46	**56%**	54	**58.69%**	100	**57.47%**
KO	12	**14.6%**	13	**14.13%**	25	**14.37%**

These data show the requirement of COCO during embryonic development that is likely related to its described roles in left/right axis patterning and heart development. Of the mouse pups that were born, we examined the vascular phenotype of the retina at multiple stages of blood vessel development. Retinal vessels in mice develop in a highly stereotyped manner, with the first vessels originating at the optic nerve head and spreading over the retina's inner surface, forming a dense network after seven days. After the vascular network has spread across the entire retina,

vessels start to sprout downward into the inner plexiform layer, establishing a second vascular network parallel to the first. No vascular differences were observed at post-natal day 5 (P5) between WT and Dand5$^{-/-}$ mice. Given our previous observations that exogenous COCO is a potent inhibitor of retinal angiogenesis and expressed in the retina at this stage, we could have expected to observe increased retina vascularization. Our previous observation could partially explain this lack of vascular phenotype at P5 that COCO does not modulate endothelial cells' response to VEGF. Indeed, at this stage of retinal vascular development, angiogenesis is primarily driven by astrocyte-derived VEGF (509). It is likely that COCO, expressed by photoreceptors and neuronal cells in the deeper layers of the retina, does not affect this process.

By contrast, at P12, COCO appeared to affect the formation of the outer deep vascular plexus significantly. Interestingly, the TGF-β pathway plays a crucial role in forming the deep retinal vascular layers, in contrast to the superficial layer, which appears to rely on VEGF and NOTCH signaling. Studies have shown that the lack of TGF-β signaling severely impairs the development of the secondary vascular plexus of the retina and is associated with increased proliferation of endothelial cells, leading to the formation of hemorrhagic vessels (510, 511). Given our observations that COCO can block TGF-β pathways in endothelial cells, we postulate that COCO produced by neuronal cells in the retina contributes to TGF-β signals regulation. These signals are required for the proper formation and patterning of the deep vascular plexus of the retina by controlling the migration, proliferation, and metabolic profile of diving endothelial cells (512). In addition to TGF-β signaling, Wnt significantly affects the development of the deeper vascular layers of the retina, with mutations in norrin resulting in the lack of deep retinal vascular layers (513). As such, COCO could also regulate the diving of retinal vessels by sequestering Wnt ligands and preventing their signaling.

Several modulators of TGF-β signaling are overexpressed in the mouse OIR model and other hyperoxia-induced animal models, suggesting sensitivity to oxygen alteration (514). Our data and analysis of previously published single-cell sequencing (507) show that COCO is expressed at higher levels in OIR mice and that deletion of *Dand5* results in an exacerbation of the pathological features of OIR. As COCO is a multifactorial protein with described ability to modulate BMP4, TGF-β, Wnt-1, it is difficult to determine the mechanism by which it modulates pathological

angiogenesis. All of the previously mentioned factors modulate pathological angiogenesis in the OIR model (515-517). Therefore, upregulation of COCO in OIR may partly serve as negative feedback to regulate these signaling pathways. In addition, endothelial cells and microglia in OIR are highly glycolytic, and inhibition of glycolysis could be associated with decreased pathological angiogenesis (518). Given that our previous study showed that COCO impaired ATP production and glycolysis in endothelial cells, the increased tuft formation observed in *Dand5*$^{-/-}$ mice may be a consequence of altered energy metabolism in the retinas of these mice (505).

In summary, we find that the retinas of *Dand5*$^{-/-}$ mice present exacerbated development of the secondary vascular plexus but that this phenotype resorbs in adulthood. Furthermore, lack of Dand5 expression was associated with increased tuft formation in OIR. Future studies will help uncover its mechanism of action and develop possible new therapies to target pathological angiogenesis in the eye.

Mechanistically, transcriptional profiling using RNAseq of retinas and choroidal explants treated with COCO revealed modulation of TGF-β and BMP signaling and pathways related to oxidative phosphorylation (data ongoing analysis).

Chapter V: Discussion

This chapter discusses our findings on COCO in ocular angiogenesis contextualized with the current literature.

5.1. COCO/DAND5 inhibits developmental and pathological ocular angiogenesis

This subchapter discusses the main findings in our publication in EMBO Molecular Medicine. (505).

Ocular neovascular pathologies such as nAMD are the principal cause of blindness in aging patients worldwide. nAMD is characterized by the uncontrolled growth of blood vessels in the retina, leading to blindness. Intravitreal injections of anti-VEGF are the primary standard of care for nAMD. However, efficacy is generally accomplished only with high adherence, and a percentage of patients do not respond to treatment. Therefore, there is a need for complementary anti-angiogenic therapies that target alternative pathways in ocular neovascular disease.

Our study recognizes a member of the DAN family, COCO, as an inhibitor of ocular angiogenesis (Fig. 1). During developmental retinal angiogenesis, intravitreal injections of COCO delayed the formation of novel blood vessels as effectively as the Flt1-Fc, a VEGF inhibitor. COCO also showed an anti-angiogenic effect in pathological retinal and choroidal neovascularization murine models of oxygen-induced retinopathy (OIR) and laser-induced choroidal neovascularization (CNV), which mimic ROP and nAMD, respectively. Notably, COCO effects appeared to be specific to newly formed vasculature since intravitreal injections in adult eyes did not affect mature retinal vessels. Furthermore, repeated injections of COCO did not perturb photoreceptors.

The TGF-β, BMPs, WNT signaling pathways are crucial in both developmental and pathological angiogenesis. Some antagonists of these pathways are proposed as a novel potential target for pathological ocular angiogenesis. Some members of the DAN family, known BMPs antagonists, such as Gremlin, while mainly studied during development, also play a role in angiogenesis. Since

Gremlin shows relatively high homology with COCO, we could expect a function of COCO through antagonism of their signal. COCO can inhibit TGF-β, BMP4, and Wnt1 in cultured human photoreceptors in a dose-dependent manner (501). In the microvasculature, BMP4 and BMP2 trigger migration and the formation of endothelial tubes, while Wnt1 stimulates the survival and proliferation of human ECs. Furthermore, Wnt1 is expressed in forming blood vessels and induces postnatal endothelial progenitors (519). Therefore, the interaction of BMP, TGF-β, and Wnt signaling pathways with COCO could delay the formation of novel vasculature and alter ECs migration and proliferation processes, affecting the angiogenic pathway.

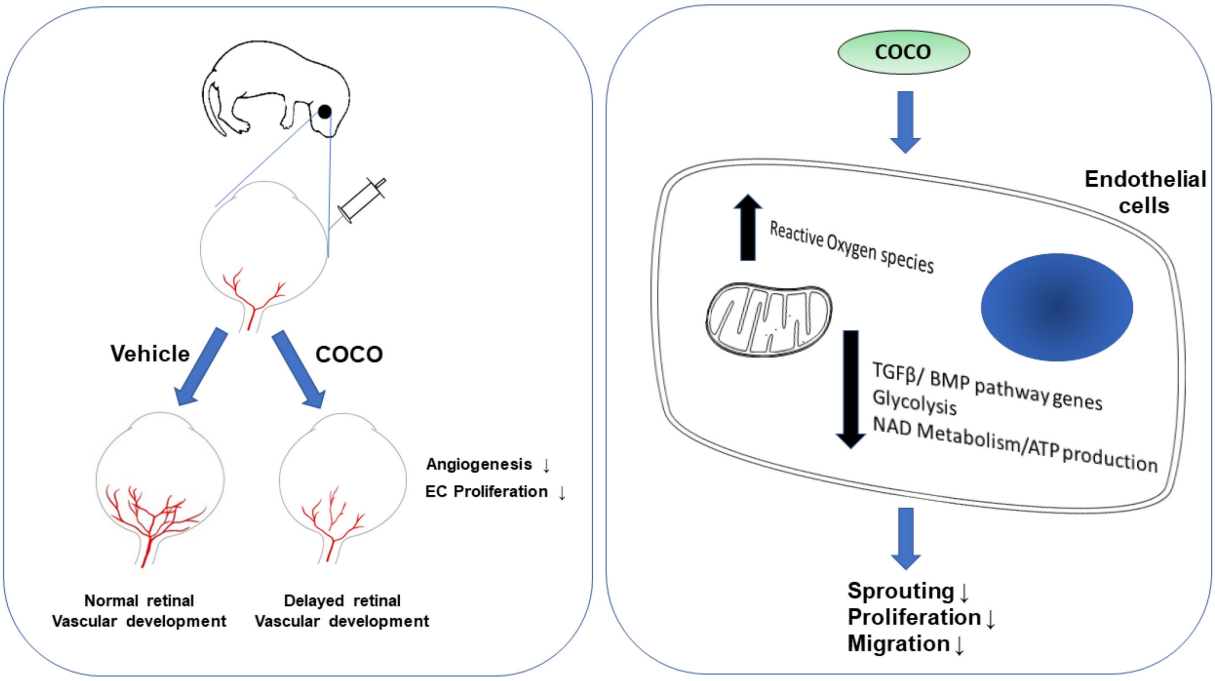

Fig. 1. Synopsis figure from the paper COCO/DAND5 inhibits developmental and pathological ocular angiogenesis (505). Left. A model of exogenous Coco inhibition of angiogenesis and proliferation upon intravitreal injections in neonatal mice. Right. In HUVECs, exogenous COCO protein inhibits TGF-β and BMPs signaling pathways, sprouting, proliferation, migration, genes related to mitochondria metabolism, and glycolysis while increasing ROS (505).

Several pathways might explain why COCO did not affect revascularization during OIR. Wnt signaling plays a significant role in pathological angiogenesis during OIR. Indeed, during OIR, WNT receptor Frizzled 4 (Fzd4) is found specifically in pathologic neovascular tufts, and mice lacking Wnt signaling show decreased levels of pathologic neovascularization in retinopathy [512]. As such, COCO may show a more significant effect on neovascular tufts due to its capacity to interfere with WNT signaling. Its effects on revascularization would be limited, as we have shown that it does not significantly interfere with VEGF signaling, which drives the revascularization process.

Several molecules inhibit pathological choroidal and retinal neovascularisation in murine models of laser-induced CNV and OIR, respectively. For example, the Ca^{2+}-Calmodulin-Dependent Protein Kinase II (CAMKII) inactivates SMAD/TGF-β signaling (520) and regulates BMP4 and TGF-β1 (521). COCO bind to both proteins (502). The two different isoforms of CAMKII knock-out mice similarly decreased laser-induced CNV while had a diverse effect on OIR. The revascularization in the OIR was suppressed in the CAMKIIδ KO, while increased in the CAMKIIγ KO (522). The murine CAMKIIγ KO isoform was similar to the IGFR1 endothelial cell knock-out, while CAMKIIδ to the KO of FGFR in endothelial cells (522, 523).

As shown in figure 4, COCO (Dand5) expression is enhanced in OIR in the retina compared to normoxia in mice at P17. These changes in COCO expression in different cell types during OIR might also be why revascularization is not affected in OIR. The glial cells and retinal neurons control revascularization in the OIR model [128].

Intravitreal injections of exogenous COCO dose were sufficient to inhibit neovascularization in the OIR context while possibly not sufficient to affect revascularization during OIR. Similarly, in dogs using different concentrations of VEGF trap, lower doses of the trap suppress neovascularization and regression of preexisting tufts yet not affecting revascularization [268].

We previously found that COCO inhibits TGF-β [503]; our second publication in preparation shows COCO's relevance during the development of the deep outer layer. In line with the recent

findings that non-canonical TGF-β signaling is crucial, the retinal revascularization in OIR (512) might explain why it did not affect retinal revascularization in OIR.

Our data suggest that COCO alters the metabolism of ECs. Furthermore, the human protein atlas and our results show that COCO localizes to the mitochondria. In ECs, metabolism is crucial to regulate the dynamics between proliferation and migration in angiogenesis. Further, mitochondria regulate the balance between glycolysis and oxidative phosphorylation controlling angiogenesis (281, 524). The essential function of the mitochondria is ATP production through oxidative phosphorylation, mitochondrial NAD^+ biosynthesis, which is vital for vision, and signaling by ROS release, which stabilizes hypoxia. Furthermore, glycolytic ATP drives the rearrangement of ECs in sprouting through increased filopodia formation, and suppression of glycolysis prevents both physiological and pathological angiogenesis (524).

Our data demonstrate that COCO decreases ECs metabolism, leading to a quiescent EC phalanx phenotype (524, 525). However, the mechanisms of action by which COCO affects the metabolism of ECs remain uncharacterized. COCO could change the formation of ROS and metabolism of ECs partially through the control of gene expression implicated in the functioning of mitochondria. Our transcriptomic analysis shows dysregulation of several genes related to mitochondrial function upon treatment with COCO in HUVECs. Some examples are *NDUFV2* and *UQCRC1* subunits of mitochondrial complex I and III, respectively, and several ATPases, which could partially explain the impact of COCO on ROS production and alteration of the metabolism. Diminished *NDUFV2* correlates with a reduction in the action of mitochondrial complex I and the synthesis of ATP, whereas alteration of *UQCRC1* leads to the lessened activity of complex III and ROS production (526-529). At the same time, the immediate effects of COCO, initiated one hour upon stimulation, on glycolysis and formation of ATP also suggest a transcriptionally independent mechanism. Exogenous COCO starts rapid metabolic alteration and colocalizes to the mitochondria in ECs. Gremlin, a member of the COCO same family, acts together with VEGFR2 even though its role in this signaling pathway is still controversial (449, 475, 530). Gremlin networks with crucial proteins for the formation of retina vessels (531, 532). The complex Gremlin-BMP2 has a high affinity for heparan sulfates, central to angiogenesis (468). Additional

studies need to elucidate COCO bindings interactions and localization to the mitochondria to understand its function during vessel development and metabolism. Our experiments show that COCO could modulate the flux of glycolysis in ECs and that the altered equilibrium among NADH/NAD$^+$ may lead to enhanced ROS production and diminished ATP generation. This energetic equilibrium may impact the anti-angiogenic function of COCO significantly.

The effect of COCO on ECs metabolism may also be due to its described interaction with TGF-β, BMP, and Wnt ligands. Wnt pathways link to changes in glycolysis by PDK1-facilitated pyruvate suppression of mitochondrial respiration (533). The Wnt-induced Warburg metabolism is based on augmented dependence on glycolysis for energy production (533, 534).

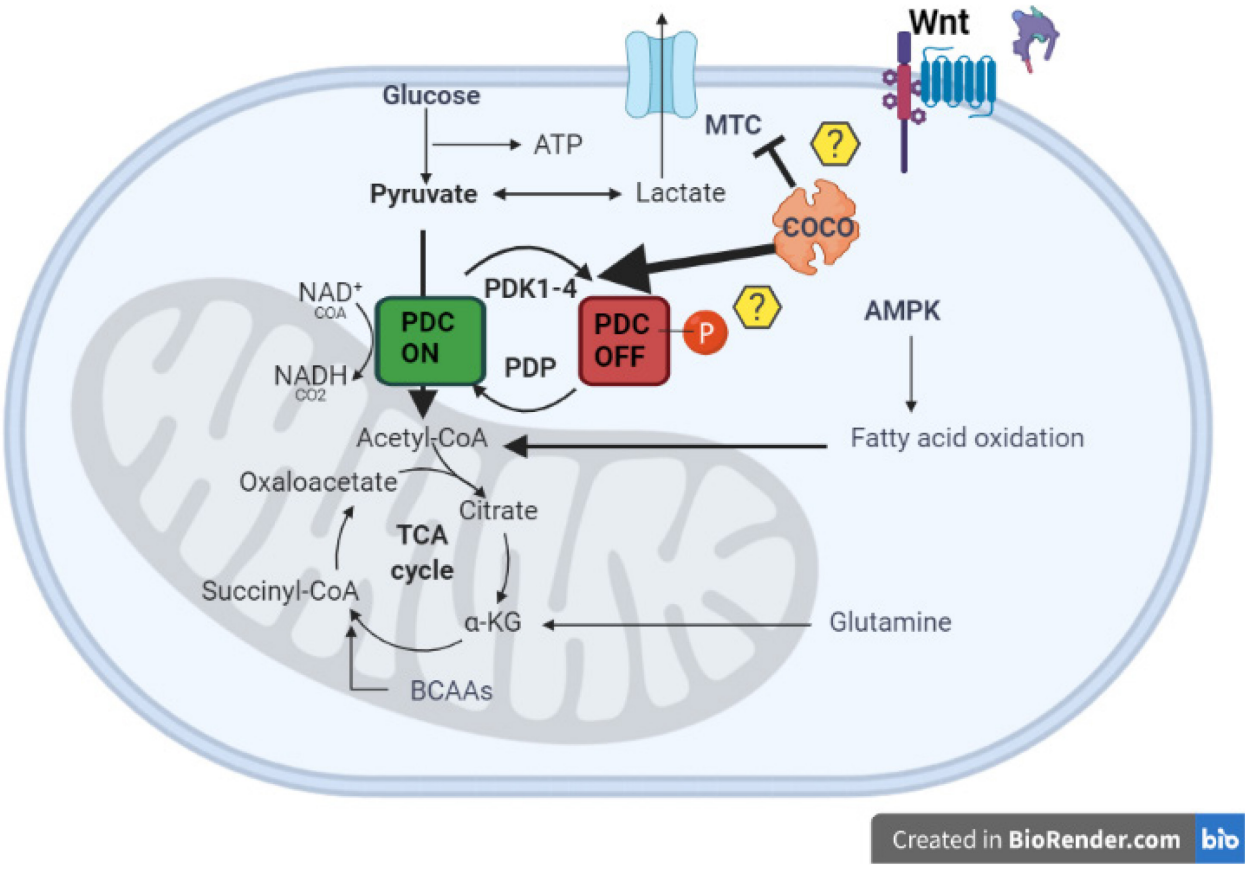

Fig. 2. COCO contributes to pyruvate accumulation in endothelial cells. The scheme represents a proposed pathway of action of COCO on PDKs and MTC regulation.

In the mitochondria, the pyruvate dehydrogenase complex (PDC) converts the pyruvate into acetyl-CoA and initiates the entry into the Krebs cycle. The pyruvate dehydrogenase phosphatase (PDP) and the pyruvate dehydrogenase kinase 1-4 (PDK1-4) regulate PDC activity by activating or suppressing it, respectively. PDK1-4 enzymes prevent, consequently, aerobic respiration. In HUVECs stimulated with COCO, the accumulation of pyruvate could be due to the inhibiting action of the PDKs on the PDC, as this would impede the conversion of pyruvate to acetyl-CoA. On the other hand, hypoxia can trigger PKD1 and PDK3, which would induce the switch of oxidative respiration to glycolysis. Interestingly, Wnt regulates both PDK1 and monocarboxylate transporter 1 (MTC1) (533, 535).

The observation of COCO stimulation leads to an accumulation of pyruvate, which may implicate the modulation of the lactate receptor G protein-coupled receptor 81 (GPR81) through the monocarboxylate cotransporter (MTCs)/basigin, more specifically, MCT1. The MCTs transport the lactate across the cellular membrane. In the murine and human retina, MCT1, MCT3, MCT4 are expressed at the membrane of RPE, and the protein basigin is crucial for their proper localization [537, 538]. Notably, the rod-derived cone viability factor (RdCVF) promoting cone survival through glycolysis binds basigin coupled to both GLUT1 and MTC1 [539]. Furthermore, in ECs of the brain, suppression of MTC1 stimulate lactate generation and inhibits its uptake, leading to reduced angiogenesis and proliferation [540]. Indeed, increased lactate generation, mainly produced by glycolysis, should be eliminated to regenerate NAD^+. Müller cells transport lactate to photoreceptors and RGCs (536). In the retina, lactate acts as pro-survival factor of Müller cells through the MCTs and GPR81 expressed by Müller cells and RGCs (537, 538). In Müller cells, the augmented extracellular lactate levels result in positive feedback, increasing its uptake (537). A low glucose concentration promotes a switch from glycolysis to oxidative phosphorylation. Consequently, mitochondria engage pro-survival signaling of Müller glia through GPR81 (537, 539). In the absence of glucose, the expression of GPR81 augments, strengthening the signaling effects of lactate (537). The enhanced expression of GPR81 reduces cAMP (540), while cAMP suppresses MTCs through endocytosis in cerebrovascular endothelial cells (541). In diabetic retinopathy, the disruption of the BRB through elevated glucose correlates with augmented cAMP in RPE; decreasing cAMP can reverse the damage (542).

BMP signaling can also control glucose metabolism by the upregulation of its transporter in chondrocytes and pathological settings (543-545). In a context-dependent manner, the triggering of TGF-β signaling alters glycolysis, ROS production, and mitochondrial respiration (546-548). Thus, COCO could partially act through TGF-β, BMP, and Wnt pathways in ECs, causing metabolism modulation. Since our RNA sequencing data show significant suppression of TGF-β and BMP signaling pathways in HUVECs with exogenous Coco, we expect that Coco could partially regulate ECs metabolism by binding with selected ligands of these two pathways. While it emerged that COCO operates in a manner independent of BMP9-BMP10/Alk1 pathway, given that intravitreal injection did not show effect in the absence of endothelial-specific Alk1ΔEC mice, it could likely prevent BMP4 or BMP2 ligands, as shown in other models (549).

5.2. Insights into the molecular mechanisms of COCO on angiogenesis

This subchapter examines different genes found through transcriptomic analyses in context with the present knowledge.

Previously, we evaluated the most significantly dysregulated genes, and pathways in HUVECs treated with COCO (Table 1) (505). Some of the most enriched genes, such as *KDM5A*, are implicated in VEGF, BMP signaling, and cellular proliferation (550, 551). *KDM5A,* a protein involved in retinoblastoma, also controls the translational machinery that regulates the expression of the p53 protein (552). TGF-β1, which interacts with COCO, also regulates *TMSB15B*, involved in cell migration (553). ALK4 is the receptor through which different TGF-β ligands regulate the left-right axis in *Xenopus* (554, 555). *VPS4A*, an ATPase that facilitates the β-catenin exosome release and localization. I*GFBP1* is pro-angiogenic, signaling through the p38 MAPK pathway (556, 557).

Table 1. Summary of the most dysregulated genes COCO-treated in HUVECs in the GSEA (505)

Related Pathway	Genes arise from a GSEA analysis heat map of HUVECs COCO involved in several pathways
VEGF	*KDM5A, NEDD4, PLCL1*
WNT	*SCYL2, XPO1, VPS4A*
TGF-β/ BMP	*KDM5A, TMSB15B, ALK4*
Cell cycle motility junctions	*KDM5A, ABL2, CABLES1, CENPE CEP57L1, DNAJB4, KPNA6, IGFBP1, PHLPP2, TMSB15B, USP53, WASF2*
Mitochondria / ATP	*AHSA2P, DNAJB4, VPS4A, NADKD1, NDUFV2, UQCRC1, VCP*
N-glycosylation	*PMM2*

5.2.1. Endocytosis of BMPs antagonists

The common mechanism of endocytosis regulates the BMPs concentration availability in a timely manner, such as BMP4 and their antagonists, such as the DAN family member Gremlin.

BMP4 is crucial for the physiological development of the eye, and studies using the *Bmp*$^{+/-}$ mutant mouse demonstrated multiple pathological features such as retina dysplasia and vascular permeability (57). Transcriptomic data on HUVECs treated with COCO, demonstrated dysregulation of pathways related to the receptor complex at the plasma membrane, phosphatidylinositol phosphates (PIPs), early endosome, and clathrin-dependent endocytosis (505). There is evidence that COCO displays similarities with Gremlin, another DAN family member antagonist of BMPs, and can bind BMP4 (558). Therefore, extracellular Gremlin and other BMPs modulators may facilitate the internalization of BMP4 by clathrin-dependent endocytosis, causing the degradation of both factors in a dose-dependent manner in ECs, postnatal ventricular cardiomyocytes, and embryonic fibroblasts (472, 473, 559). This mechanism contributes to the regulation of intracellular BMP4 signaling (472, 560-563). The clathrin-dependent internalization of BMP4, BMPR-I, and BMPR-II is coherent with the mechanism utilized by receptors of BMP and TGF-β (472, 564-567). Furthermore, the two BMPRs colocalize in the late endosome (472, 568). Interestingly, Gremlin has a high affinity for BMP4 and can regulate the phosphorylation of SMADs (472). TGF-βRs internalize through caveolin-dependent

to degrade the proteosome since the clathrin-dependent is necessary for initiating the SMADs (564, 565). Thus, endocytosis is part of the broad mechanism of suppressing the function of BMPs as an approach to control the temporally available concentration (472). These data hint at COCO as a possible modulator of the angiogenic response by endocytosis of BMP4, a well-known pro-angiogenic factor.

5.2.2. Effect of mitochondrial metabolism and cross-link with TGF-β and BMPs pathway

In retinal angiogenesis TGF-β, BMPs pathways converge onto mitochondrial metabolism important downstream factors such as FOXO, MYC, and STATs.

We demonstrated that COCO modulates common genes of the mitochondrial ETC using diverse *in vitro* GSEA pathway analyses of HUVECs treated with COCO show dysregulation of cAMP genes, and transcriptomic supported these data showing significantly altered MYC targets. The mitochondria have enzymes implicated in the production and degradation of cAMP, and PKA targets multiple of its proteins. The signaling of the mitochondrial cAMP/PKA also controls the homeostasis of the cell (569). cAMP/PKA signaling also controls the mitochondrial complex I stability (569).

Angiogenesis necessitates the synchronization, through MYC, of the metabolic respiration of mitochondria and glycolysis. Indeed, ECs lacking MYC leads to altered proliferation, mitochondrial function, and glycolysis (570). *In vivo*, the lack of complex III of the mitochondria also triggers an increase in levels of mTORC1 and MYC (279). Importantly, FOXO and MYC are crucial in regulating retinal angiogenesis (570, 571).

More research is needed to elucidate the function of Myc in controlling BMP4 expression since *Myc* knock-down reduces the cAMP enhancer for BMP4 expression in ECs. Furthermore,

conserved binding sites of *Myc* in the promoter region of BMP4 in different orthologous species have been reported (572, 573). The pro-survival BMP7 is also a target of MYC (574, 575).

In diverse ECs from distinct species, shear stress suppresses the expression of BMP4 at a transcriptional level, decreased by reduction of the cyclic adenosine monophosphate (cAMP)/ *cAMP-dependent protein kinase* (PKA) pathway (576). Shear stress triggers integrin β, causing increased levels of intracellular cAMP. In human coronary arterial ECs, suppression of PKA prevents the phosphorylation and initiation of transcriptional regulator cAMP response element-binding protein (CREB) by cAMP (576). The ligation of pCREB to the CRE regulates the transcription of BMP4. Indeed, the cAMP/PKA pathway reduces BMP4 transcription (576). The decapentaplegic *dpp* gene in *Drosophila*, homologous to the mammalian BMP4, is crucial for embryonic dorsoventral differentiation, and PKA suppresses its expression during limb formation (577, 578). The regulation mechanisms of BMP4 expression reliant on PKA it is conserved during evolution (576).

BMP4 promotes the SMAD1/5/8 pathway, the expression of (inhibitor of DNA-binding 1) Id1 and Bcl-xL due to suppression of cytochrome C secretion by mitochondria. BMP4 survival role links it to the Bcl-xL anti-apoptotic action through the SMAD pathway (579).

Transcriptomic of COCO in HUVECs also demonstrates an alteration of the TGF-β and JAK/Stat Pathways (505). STAT3 affects the mitochondria and consequently controls ATP and ROS production (569, 580). In pathological conditions, STAT3 can also crosstalk with SMAD3, thus altering the TGF-β response (581).

The cysteine bond formation and NAD mitochondrial crosstalk with ROS in angiogenesis (582). In human mitochondria, NADK is crucial for proper vision (583).

In conclusion, our data suggest that COCO could interfere with the angiogenic process by mitochondrial function, promoting ROS production, inhibiting ATP. Thus, we expect that COCO could be interfering with BMP4 signaling also in the retina and through this mechanism suppresses angiogenesis.

5.2.3. Mechanisms involved in the model of retinopathy of prematurity

Our result on OIR Dand5$^{-/-}$ mice from the publication in preparation from chapter IV, *"Characterization of Ocular Angiogenesis in Dand5$^{-/-}$ mice"* are examined with IGFBP, TGF-β, and WNT pathways relevant during transcriptomic.

Our data demonstrate differential development of intermediate and outer deep vascular plexus in *Dand5$^{-/-}$* mice at P12 compared to controls. At this developmental stage of the deep vascular plexus formation, both TGF-β and Wnt ligands and their signaling pathways play an essential role (512, 584, 585). These signaling pathways are also involved in pathological angiogenesis in the eye. Indeed, our data also show that COCO is overexpressed in OIR retinas and that *Dand5$^{-/-}$* mice display increased tuft formation in OIR. Several possible mechanisms could explain the exacerbation of OIR pathological features in the absence of COCO. TGF-β is affected in a model of retinopathy of prematurity and humans [511]. The absence of regulators of TGF-β signaling, such as integrin *Itgb8* expressed mainly by Müller glia*,* damages the retinal deep vascular plexus and correlates with augmented ECs proliferation causing hemorrhages (357). Müller cells release several growth factors implicated in retinal physiological and pathological angiogenesis (586). In HUVECs, COCO suppresses TGF-β pathways (505). Given that in the retina rod, photoreceptors and Müller cells, among others, produce COCO, they could modulate TGF-β signaling and regulate the metabolism of proliferating and migrating ECs forming the retinal deep vascular plexus (512). COCO could also modulate the deep retinal vasculature by inhibiting several Wnt ligands such as Norrin and blocking its pathway. Autosomal dominant Familial Exudative Vitreoretinopathy (FEVR) arises from a mutation in *Fz4,* damaging retinal angiogenesis, and present defects in the Norrin *Fz4* pathway (584, 587). In mice, Norrin and its receptor Fz4 lead to comparable vascular abnormalities by activating Lrp5/Wnt signaling, which is impaired in disease. Mice lacking Fz4 have impaired migration of ECs and delayed hyaloid vessel regression; furthermore, the retinal vasculature appears fenestrated in the NFL, and retinal hemorrhages are observed (584).
Notably, a study shows the link between metabolism and Wnt signaling during developmental and pathological retinal angiogenesis, which could be a potential mechanism of action of COCO.

Müller cells highly express the GPR81 receptor, MTC1, Norrin and generate most of the lactate. The GPR81 receptor and the lactate produced by hypoxia through Norrin release allow proper oxygenation and promote the development of the inner retinal vessels. Lactate is vital during angiogenesis, particularly for revascularization, stimulating restoration of the vessels of the inner plexus in OIR. Indeed, in GPR81 deficient mice, the augmented expression of Norrin compensates for the vascularization of the retina, while Norrin suppression in Müller cells disrupts the migration of ECs (585).

Oxidative stress plays a crucial role in ROP (588). As previously mentioned, glutathione pathways are altered following COCO treatments, and in OIR mice, the absence of the glutathione peroxidase regulating ROS exacerbated the pathological features of retinopathy (589). BMP4 modulates the oxidative stress triggered by RPE, and as such, COCO could also interfere with this process by modulating BMP4 signaling (61). We have also shown that the TGF-β pathway is altered in HUVECs treated with COCO (505). COCO binds TGF-β1 in *Xenopus* (502). In OIR mice, TGF-β1 and SMAD4 are overexpressed (517).

Another crucial pathway involved in retinopathy of prematurity is the IGF pathway. Our RNAseq data with HUVECs treated with COCO also show the dysregulation of NEDD4 and IGFBP1 and altered IGFB levels in retinas treated with COCO (Table 1). IGFBP-rP1 suppresses retinal angiogenesis by preventing ERK signaling and VEGF expression in the mouse model of OIR, and it has been proposed as a therapeutical target for pathological neovascularization of the retina (590). Studies show that the p53/ IGFR1 axis was increased in oxygen-induced retinopathy (591). The localization of IGFR1 also changes upon OIR (592). Indeed, OIR induces the activation of Müller cells, and COCO is expressed in these cells. Thus, we could have expected a change in COCO expression in this model. Our retina data show differentially expressed LRP, a Wnt receptor. In the OIR model, Lrp5 augments the pathological neovascular model linked to the expression of Claudin 5 (593). The Wnt/β-catenin signaling in Müller cells and ECs enhance the restoration of the vessels upon OIR, also linking the effect to IGF1 (594).

Together, our data show that COCO stimulation modulates several pathways that could exacerbate the pathological features of OIR and in *Dand5*$^{-/-}$ mice, such as GPR81, MTC1, IGF, integrins, and claudins, in addition to the already known Wnt, TGF-β1 and BMP4. However, more studies will be required to elucidate the role of COCO in OIR fully.

5.3. Conclusion

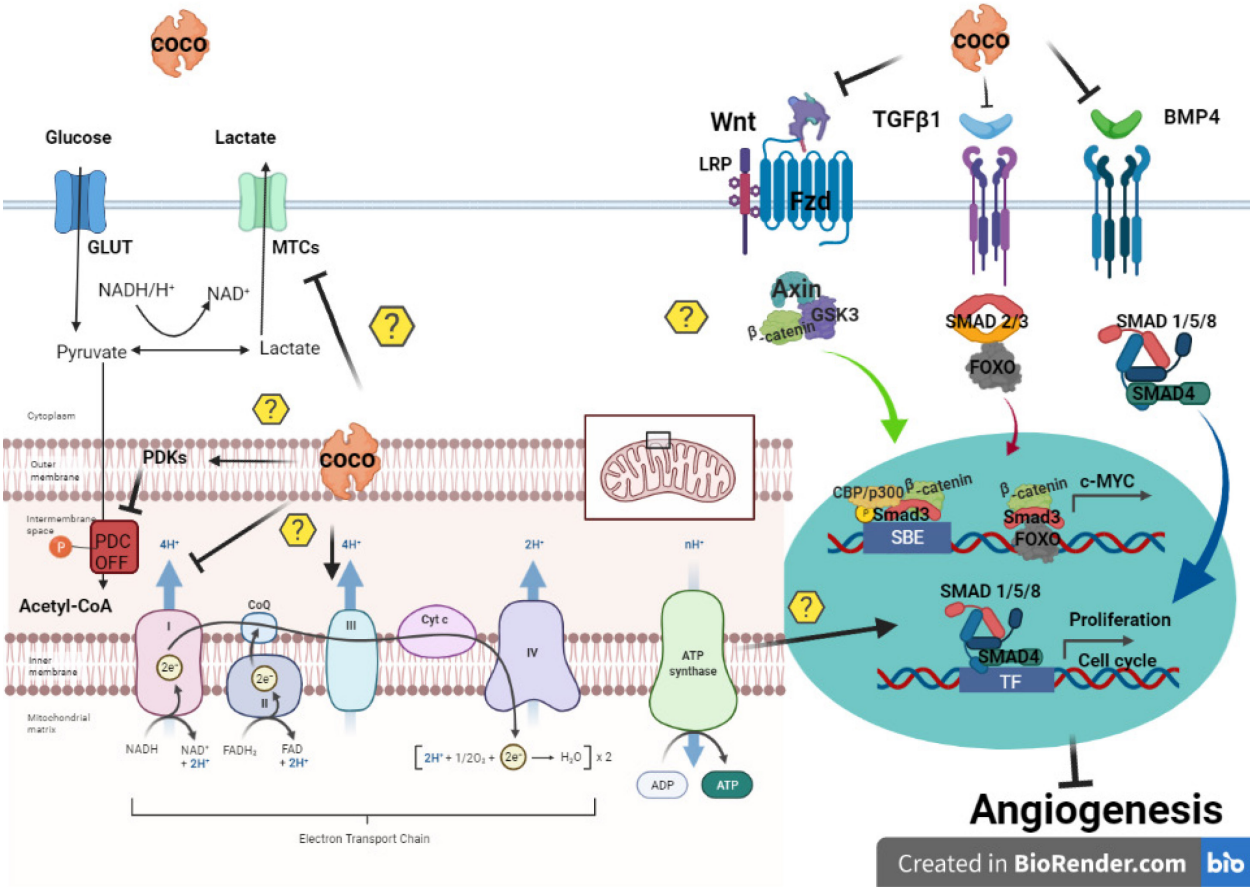

Fig. 3. A simplified hypothetical COCO working model integrates current literature knowledge with our findings. COCO by binding TGF-β, WNT, BMP decrease angiogenesis might occur transcriptionally through the Smad1/5/8, essential during retinal angiogenesis (303). Internalization of exogenous COCO onto the mitochondria might regulate oxidative

phosphorylation, thus controlling NAD^+/NADH ratio, ATP, and ROS levels through complexes I and III, which present dysregulated in transcriptomics. Indeed, complex III is crucial in retinal angiogenesis (279). Accumulation of pyruvate might arise through the inactivation of PDH by PDKs, impeding its conversion into Acetyl CoA or suppression of lactate export by regulating the MTCs [539]. PDK and MTC1 are regulated by Wnt (533, 535). Furthermore, FOXO1 and cMYC, downstream of TGF-β, are the primary switch regulators between glycolysis and oxidative phosphorylation, thus promoting endothelial quiescence and are crucial in retinal angiogenesis (570).

Our study identifies exogenous COCO, a BMP antagonist, as an anti-angiogenic protein during developmental angiogenesis in the OIR model of retinopathy of prematurity and the laser-induced CNV model of neovascular AMD. Mechanistically, exogenous COCO suppresses migration and proliferation in HUVECs, possibly by antagonizing TGF-β, BMP, Wnt ligands and/or internalization and transport to the mitochondria, where it could exert its anti-angiogenic effect by changes of the metabolism. Notably, metabolic pathways including glycolysis and mitochondrial oxidative phosphorylation are modulated by TGF-β, BMP, Wnt downstream transcriptional factors such as Smad1/5, FOXO1, MYC, STAT to control retinal angiogenesis (303, 570).

In retinas of wild-type mice during OIR, COCO is expressed at higher levels compared to normoxic controls. *Dand5*$^{-/-}$ mice also show an excess of neovascular tufts without revascularization. Interestingly, at P12, *Dand5*$^{-/-}$ mice display alterations of the outer deep vascular plexus observed in several mice mutants of the TGF-β and Wnt family members (512, 585).

More studies are required to elucidate the detailed mechanism of action of both endogenous and exogenous COCO. Further ongoing comparative RNA-Seq analysis performed on COCO-treated HUVECs and *ex vivo* choroidal sprouting assay and retinas from eyes intravitreally injected with COCO show multiple common pathways of interest such as mitochondrial metabolism, TGF-β, BMP pathway, Wnt genes, together with IGF/p53 axis.

5.4. Future directions

The current project has provided fundamental grounds for understanding COCO in ocular angiogenesis, yet several questions remain unanswered. One of the first leads to follow is to determine further the role molecular mechanism of COCO. It is of relevance to decipher the structure of the protein and identify its binding partners. Finally, a must requisite will be to run a pre-clinical assessment of its therapeutic activity for neovascular ocular diseases.

Firstly, uncovering the detailed COCO molecular mechanism of action in ocular angiogenesis would require the tissue-specific deletion of COCO and rescue experiments, given the interplay of multiple pathways involved. The specific deletion of COCO in Müller cells, rod photoreceptors, and mitochondria would clarify the metabolic impact on angiogenesis and visual function. Targeted deletion of COCO in retinal cells would impact its neuroprotective activity towards cone photoreceptors. Crossing these tissue-specific mutants with KO mice of the BMP4, TGF-β1, and Wnt family members would be interesting to assess if the phenotype observed in *Dand5*$^{-/-}$ mice arises from inadequate signaling by members of these families. Specifically, loss of BMP4, TGF-β1, and Wnts would be interesting to explore further during retinal vascular development in the context of the outer deep plexus layer and also during OIR. Combinatory intravitreal injection with COCO and Wnt1 were performed during development (data not shown).

Secondly, identifying the binding partners of COCO in the retina and their downstream signaling targets would shed light on the crosstalk among the various developmental angiogenic pathways involved in the migration, proliferation, and metabolic activities of endothelial cells. Several in-depth and interdisciplinary studies are required to provide insight.

Finally, it would also be of great therapeutic interest to assess the potential pre-clinical benefits of COCO in other ocular diseases, such as diabetic retinopathy. Furthermore, although challenging to study, it is essential to develop potential drugs to assess the homo-and hetero-dimerization of COCO and their differences in biological activity. It is complementary to assessing the dose-dependent effects of exogenous COCO and its potential synergistic activity when combined with other anti-angiogenic drugs in pre-clinical models of ocular pathologies.

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APPENDIX

PEER-REVIEWED PUBLICATIONS INCLUDED IN THE THESIS

1. **N. Popovic,** E. Hooker, A. Barabino, A. Flammier, F. Provost, M.G. Bernier, B. Larrivée." COCO/DAND5 inhibits developmental and pathological ocular angiogenesis "**.** EMBO Molecular Medicine **Febr. 2021**

PUBLICATIONS IN PREPARATION INCLUDED IN THE THESIS

1. **N.Popovic,** S. Crespo-Garcia, M. Masis Solano, M. Buscarlet, G. Cagnone, J.A Belo, G. Bernier, B.Larrivée Characterization of Ocular Angiogenesis in Dand5$^{-/-}$ mice.

PEER-REVIEWED PUBLICATIONS NOT INCLUDED IN THE THESIS

1. Viallard C, Audiger C., **Popovic N**, Akla N., Legault-Navarette I., Melichar H., Costantino S., Lesage S., Larrivée B. "Alk1 signaling *promotes the normalization of tumor blood vessels".* Oncogene. **Febr. 2020**
2. Akla N., Viallard C, **Popovic N**, Lora Gil C, Sapieha P, Larrivée B. *"BMP (Bone Morphogenetic Protein) 9/Alk1 (Activin-Like Kinase Receptor Type I) Signaling Prevents Hyperglycemia-Induced Vascular Permeability. "*Arterioscler Thromb Vasc Biol. **Jun 2018**
3. Wilson AM, Shao Z, Grenier V, Mawambo G, Daudelin JF, Dejda A, Pilon F, **Popovic N**, Boulet S, Parinot C, Oubaha M, Labrecque N, de Guire V, Laplante M, Lettre G, Sennlaub F, Joyal JS, Meunier M, Sapieha P. „*Neuropilin-1 expression in adipose tissue macrophages protects against obesity and metabolic syndrome.“* Science Immunology **March 2018**
4. Oubaha M., Miloudi K., Dejda A., Guber V., Mawambo G., Germain M.-A., Bourdel G., **Popovic N.,** Rezende F., Kaufman R.J., Mallette F.A.*, Sapieha P.* *Therapeutic inhibition of the senescence-associated secretory phenotype prevents pathological retinal angiogenesis.* Science Translational Medicine. **Oct. 2016**

PUBLICATIONS IN PREPARATION NOT INCLUDED IN THE THESIS:

1. NP Preliminary results produced for Acceleron company data. Popovic N, Larrivée B. "Effect of BMP9 inhibitors on choroidal neovascularization." 2019

Correction to Chapter III: "COCO/Dand5 inhibits developmental and pathological ocular angiogenesis."

Pages of EMBO Molecular Medicine published in February 2021 are as follow:

Comment: *It was not clear to this reviewer as to selection of COCO dose for experiments. Were COCO concentrations used based on patho-physiologic retinal values?*

Response: Page 3. Figure 1A. The COCO concentration dose was based on its previously reported optimal effects on photoreceptor differentiation; for endothelial cells, we did a dose-response of its activity on endothelial cell sprouting, as shown in Fig. 1A.

Comment: Page 4, paragraph COCO inhibits retinal neovascularization. "*Compared with PBS injected eyes, a pronounced reduction in vessel area (area covered by vessels) and microvessel density (ratio of vessel area to vascularized area) was detected in the retinas of COCO-injected eyes (Fig 2C)"* 2C is length and junctions, not density.

Response: Indeed, Figure 2C quantifies vascular length and junctions.

Comment: Page 4, paragraph COCO inhibits pathological neovascularization. "*Briefly, 8-week-old mice were subjected to laser impact, followed by intravitreal injections of either COCO, Flt1Fc, or PBS, and CNV was detected 14 days later by staining choroid-sclera whole mounts with IsoB4 (blood vessels) and phalloidin (RPE) (Fig 5E)."* Phalloidin stains f-actin actin is not specific to the RPE.

Response: Indeed, phalloidin stains f-actin and not specific RPE.

Comment: Page 5 Figure 2. B , D, F. The diameter seems bigger in the COCO condition (in B D and F). A compensation from the fact that the vessels have less grown?

Answer: Yes, it is very likely to be a compensation upon vascular inhibition.

Comment: Page 6. *Figure 3E. Legend Fig 3E does not correspond to figure 3E.*

Response: Indeed, legend Fig 3E. Quantification of the number of nuclei per section and thickness in the ONL of figure D.

Comment: Page 7. *Fig EV4 and Fig 7F. These data suggest possible uptake and transport to mitochondria of exogenously added COCO." Interesting. What made you think of this experiment?*

Response: Our phenotypic results in intravitreally injected COCO mice show decreased proliferation and angiogenesis; also, HUVECs treated with COCO demonstrated suppressed migration and proliferation. Mitochondria are essential for the movement of the cells. Metabolic respiration drives these phenotypic changes; indeed, among other metabolic processes, the switch between glycolysis and mitochondrial oxidative phosphorylation is crucial in the anti-angiogenic process. Thus, colocalizing exogenous COCO is essential to unravel its molecular mechanism associated with the phenotypic changes observed. Finally also the indication of COCO co-localization to the mitochondria by the human protein atlas.

Comment: Page 8. *Figure 5F. Not sure I understand the rational for the phalloidin staining here.*

Response: Phalloidin allows evaluating the size of the laser-induced lesion.

Comment: *If a low dose but not a higher dose of COCO augments effects of anti-VEGF, is this a potentiation or additive effect till maximum impact on sprouting?*

Response: At this point, it is not possible to speculate whether COCO has an additive or synergistic effect when combined with anti-VEGF, as this would require more doses of both COCO and anti-VEGF to be tested. We suppose that low doses of both COCO and anti-VEGF would show more significant clinical benefits by potentiating the anti-angiogenic effect while minimizing the potential effects of both factors. In supplementary figure EV3, the additive, in this case, the synergetic effect of 50 ng/ml of both COCO and anti-VEGF in a HUVEC sprouting assay.