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Régulation de l'angiogenèse physiologique et tumorale par le système chémérine

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Regulation of physiological and tumoral angiogenesis by the chemerin system

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Abstract

Angiogenesis is an essential and complex process, by which new blood vessels grow from preexisting ones, as a tightly regulated response of endothelial cells (ECs) to a combination of pro- and anti-angiogenic factors. The establishment of a functional blood vessel network is initiated by the formation of a primitive vascular plexus, from which new blood vessels sprout, coordinately expand, and branch. Redundant vessel branches are then selectively removed through vessel regression, a process called pruning.

Chemerin was originally isolated from inflammatory exudate fluids and found to be a ligand for the orphan G protein-coupled receptor, ChemR23/CMKLR1. Active chemerin is a chemoattractant factor for immature dendritic cells, natural killer cells and macrophages, by acting through CMKLR1. Bioactive chemerin generation and infiltration by CMKLR1-expressing cells have been described in various human diseases. We and others have identified both pro- and anti-inflammatory activities of the chemerin/CMKLR1 system according to the experimental setting. Our laboratory reported the antitumor activity of chemerin in a chemical model of skin carcinogenesis, and more recently, in two tumor graft models. These anti-tumoral properties were reported as independent from leukocyte recruitment. Instead, a reduction of the neoangiogenesis process was identified as the driving mechanism explaining the delay in tumor growth.

The role played by chemerin during angiogenesis remains controversial. Pro-angiogenic, but often moderate effects of chemerin were reported. In this thesis, we investigated the role of chemerin on the neonatal development of the retinal vascular network in mice, a well-established model. We used for this purpose a mouse line allowing the controlled overexpression of a bioactive form of chemerin in keratinocytes, which results in a significant increase of chemerin bioactivity in the whole body. We observed in these mice a reduction in the density of the retinal vascular network from day 6 after birth and this phenotype persisted to adulthood. The extension of the network was however not affected, nor the proliferation of endothelial cells or the number of tip cells. There was rather an increase in the number of empty sleeves and of apoptotic endothelial cells, pointing towards an increase in vessel regression during the remodeling phase of the angiogenesis process. These anti-angiogenic effects of

chemerin are mediated by CMKLR1, as they were abrogated in CMKLR1 knock-out mice. The anti-angiogenic effects of chemerin were confirmed in a model of oxygen-induced retinopathy, as well as in the hind limb ischemia model, and indications of vascular regression were also found in tumor grafts. A PTEN/AKT/FOXO1 pathway was identified as mediating the effects of chemerin on endothelial cells.

Chemerin binds to three receptors, CMKLR1, GPR1 and CCRL2. The role of CCRL2 in angiogenesis remains largely unknown. We demonstrated that mice invalidated for CCRL2 display a reduction of the retinal vascular network similar to that observed in chemerin-overexpressing mice. This phenotype seems to be due to an increase in blood chemerin levels.

Following the identification of chemerin as a negative regulator of angiogenesis, we investigated the consequence of chemerin overexpression in lymphangiogenesis. We demonstrated that lymphangiogenesis and lymph node metastasis was not affected in chemerin-overexpressing mice.

Taken together, the experiments presented in this thesis suggest that the anti-angiogenic and anti-tumoral effects of chemerin may qualify the chemerin/CMKLR1 system as a potential therapeutic target for future anti-cancer therapies, but also for diseases in which excessive angiogenesis contribute to the pathogenesis. In this frame, the expression of the non-signaling chemerin receptor CCRL2 should be considered.

Résumé

L'angiogenèse est la formation de nouveaux vaisseaux sanguins à partir des vaisseaux préexistants. L'établissement d'un réseau de vaisseaux sanguins fonctionnels est initié par la formation d'un plexus vasculaire primitif, à partir duquel de nouveaux vaisseaux sanguins se développent de manière coordonnée et se ramifient. Les branches de vaisseaux redondantes sont ensuite sélectivement supprimées par régression des vaisseaux, un processus appelé élagage.

La chémérine est une protéine multifonctionnelle initialement caractérisée par le laboratoire hôte comme un facteur chimioattractant pour certaines populations de leukocytes. Son principal récepteur fonctionnel est ChemR23/CMKLR1. Notre groupe a identifié un mécanisme original par lequel la chémérine retarde la progression tumorale, en inhibant la vascularisation efficace des tumeurs et leur croissance. Le but de mon projet de thèse est d'étudier plus précisément comment la chémérine affecte l'angiogenèse, *in vitro* et *in vivo*, en utilisant un ensemble de modèles génétiques établis ou nouvellement développés chez la souris. Dans l'ensemble, nous visons à valider le système chémérine comme cible thérapeutique dans le cadre du cancer humain.

Nous avons démontré que les souris surexprimant la chémérine développent en effet un réseau vasculaire rétinien moins dense, et cette particularité perdure jusqu'à l'âge adulte. Nous avons pu déterminer que ce n'est pas la formation du réseau qui est affectée, mais bien la stabilité des néovaisseaux lors de la phase de maturation de ce réseau. Il en résulte une nette augmentation de l'apoptose des cellules endothéliales et une régression accrue de segments vasculaires du réseau. L'expression de chémérine protège partiellement de l'angiogenèse excessive observée dans un modèle de rétinopathie, réduit le temps de récupération de la perfusion suite à la ligature de l'artère fémorale et perturbe distribution de collagène de type IV autour des cellules endothéliales, signe d'un manque de maturation et de l'instabilité de ces vaisseaux.

Enfin, nous avons étudié les cascades de signalisation impliquées dans ces effets anti-angiogéniques. Par l'utilisation d'inhibiteurs spécifiques, nous avons mis en évidence, en réponse à la chémérine, une augmentation de l'activité de PTEN, une diminution de l'activité

de la cascade PI3K/AKT et une augmentation de l'activité du facteur de transcription FOXO1, un régulateur bien connu du métabolisme et de la survie des cellules endothéliales.

La chémérine se lie à trois récepteurs, CMKLR1, GPR1 et CCRL2. Il a été démontré qu'uniquement CMKLR1 et GPR1 induisaient une signalisation en aval après leur liaison à la chémérine. CCRL2 est exprimé sur plusieurs types cellulaires et son expression est rapidement augmentée suite à des stimuli inflammatoires. Cependant, son rôle dans l'angiogenèse reste largement méconnu. Nous avons démontré que les souris invalidées pour CCRL2 présentent une réduction du réseau vasculaire rétinien similaire à celle observée chez les souris surexprimant la chémérine. Ce phénotype semble être dû à une augmentation des taux sanguins de chémérine.

Suite à l'identification de la chémérine comme régulateur négatif de l'angiogenèse, nous avons étudié la conséquence de la surexpression de la chémérine dans la lymphangiogenèse. Nous avons démontré que la lymphangiogenèse et les métastases ganglionnaires n'étaient pas affectées chez les souris surexprimant la chémérine.

Les résultats présentés dans cette thèse suggèrent que les effets anti-angiogéniques et antitumoraux de la chémérine pourraient qualifier le système chémérine/CMKLR1 comme une cible thérapeutique potentielle pour de futures thérapies anticancéreuses, mais aussi pour des maladies dans lesquelles une angiogenèse excessive contribue à la pathogenèse. Dans ce contexte, l'expression du récepteur non-signalant de la chémérine CCRL2 doit être prise en compte.

Author's Declaration

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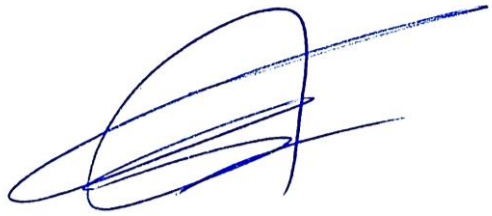
The research work in this thesis that was performed in in accordance with European guidelines and local regulations and approved by the local ethics committee (Commission d'Ethique du Bien-Etre Animal, CEBEA) of the ULB Medical School. All efforts were made to minimize suffering.

Cyrine Ben Dhaou



« Prier est sans doute chose convenable et excellente, mais, tout en invoquant les dieux, il faut s'aider soi-même... »

Hippocrates

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****

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Thank you God for this blissful life

À ma Famille,

Mama, Papa, Ons et Anas,

C’est à vous que je dois tout ce que je suis et espère être…

À la mémoire de mes grands-parents

List of Abbreviations

α-SMA	alpha smooth muscle actin
ACC	adrenocortical carcinoma
ACKR	atypical chemokine receptor
AKT	protein kinase B
ALK1	activin receptor-like kinase 1
Ang	angiopoietin
Asn	asparagine
ATP	adenosine triphosphate
BAL	bronchioalveolar lavage
BEC	blood vascular endothelial cell
BMI	body mass index
BMP9	bone morphogenetic protein 9
CBP	CREB-binding protein
CCL	CC chemokine ligand
CCM	cerebral cavernous malformation
CCR	CC chemokine receptor
CCRL2	CC chemokine receptor-like 2
CMKLR1	Chemokine like receptor 1
CNS	central nervous system
CRP	C reactive protein
CSC	cancer stem cell
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptors
DARC	Duffy antigen receptor for chemokines
DC	dendritic cell
DLL4	Delta like canonical Notch ligand 4
DSS	dextran sodium sulphate
EAE	experimental autoimmune encephalomyelitis

EC50	50% effective concentration
EC	endothelial cell
ELM	external limiting membrane
ELR	Glu-Leu-Arg motif
EMT	endothelial to mesenchymal transition
EPO	erythropoietin
ERK	extracellular signal-regulated kinase
FFA	free fatty acid
FGF	fibroblast growth factor
FIH	factor inhibiting HIF
FIZZ-1	found in inflammatory zone protein
GCL	ganglion cell layer
GFAP	glial fibrillary acidic protein
GPCR	G protein-coupled receptor
GPR1	G protein-coupled receptor 1
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HPLC	high-performance liquid chromatography
HRE	hypoxia response element
HUVEC	human umbilical vein endothelial cell
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IGF	insulin-like growth factor
IL	interleukin
ILM	inner lining membrane
INL	inner nuclear layer
i-NOS	inducible nitric oxide synthase
IPL	inner plexiform layer
KLF2	Krüppel-like factor 2
LEC	lymphatic endothelial cell
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase

MEKK3	mitogen-activated protein kinase kinase kinase 3
MMP	matrix metalloproteinase
MS	multiple sclerosis
NG2	neural/glial antigen 2
NK	natural killer
NO	nitric oxide
NSCLC	non-small cell lung cancer
OIR	oxygen-induced retinopathy
ONL	outer nuclear layer
OPL	outer plexiform layer
OVA	ovalbumin
P38	p38 mitogen-activated protein kinase
PCOS	polycystic ovary syndrome
pDC	plasmacytoid dendritic cell
PDGF	platelet-derived growth factor
PHD	prolyl-hydroxylase domain
PI3K	phosphatidylinositol 3-kinase
PlGF	placental growth factor
PKB	protein kinase B
PPARγ	peroxisome proliferator-activated receptor gamma
PR	photoreceptor layer
Prox1	prospero homeobox protein 1
PTB	phosphotyrosine-binding
RA	retinoic acid
RARRES2	retinoic acid receptor responder 2
RGC	retinal ganglion cell
RNFL	retinal nerve fiber layer
ROCK	rho-associated protein kinase
ROP	retinopathy of prematurity
RPE	retinal pigment epithelium
SCCOT	squamous cell carcinoma of the oral tongue
SH2	Src homology-2

SMC	smooth muscle cell
SREBP-2	sterol regulatory element-binding protein 2
SRF	serum related factor
ST2	stromal cells
TGFβ	transforming growth factor-β
TIE	tyrosine kinase with immunoglobulin-like and EGF-like domains
TIG-2	tazarotene-induced gene 2
TLR	toll-like receptor
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VO	vessel obliteration

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Preface

A brief walk with cancer: History and actuality

"And for 3000 years and more, humanity has been knocking at the door of the medical profession for a cure".

-Fortune, March 1937

What else might it be! This illness is cancer! Described in 1600 BC for the first time, in an antique Egyptian papyrus (the earliest medical report). Nowadays, in the 21st century, cancer remains a major problem in our societies. Global cancer estimates have increased to 19.3 million new cases and 10 million fatalities in 2020. In other terms, worldwide, one in 6 females and one in 5 males will develop cancer during their lifetime, and one in 11 females and one in 8 males and will die because of it. Cancer is an economic illness as well. $170 billion is spent on managing cancer patients.

From radical surgery and mastectomy to discovering chemotherapy and radiotherapy, cancer has seen some improvement in treatment, but not enough to significantly reduce cancer mortality rates. Its aggressive and heterogeneous character makes it difficult to control. Therefore, more research is needed to better understand the cancer initiation and progression process, providing more personalized and effective treatments.

And behind that giant?!

"Stimulating the legs of cancer crab and it is not as red as cellulitis but with a trend to blackness, heat and greenness "

-Avicenna,
View on Cancer from his Canon, 1294

Following Hippocrates' suggestion that any illness has natural causes, Galien proposed that cancer is caused by black bile stagnation. Then, with the virus discovery, the researchers have stated that all cancers are virus induced. This theory is remains true for certain cancers. Today, we know that behind this giant initiation stands angiogenesis.

The excessive growth of blood vessels is a characteristic of all malignant tumors, and it is a major phenotype in arthritis, psoriasis and macular degeneration affecting the onset of blindness. Insufficient angiogenesis occurs in chronic injuries of diabetic patients, and failure to recover often leads to amputations of affected limbs.

My PhD thesis: A step towards better understanding cancer and angiogenesis-dependent diseases

My PhD objective was to study the role of chemerin, our protein of interest, on normal and pathological angiogenesis. This thesis allowed the identification of the chemerin system as an important regulator of angiogenesis.

In the hope that the results I present in this PhD thesis will open up new horizons for cancer research and bring us a step closer to conquering the so-called "Emperor of All Maladies", Cancer.

I. Introduction

This is not an exhaustive review of the literature. In my introduction, I will attempt to contextualize my doctoral work and to highlight related research studies and knowledge.

A. Physiological angiogenesis

Leonardo da Vinci had postulated that the vascular system grew like a tree out of a seed (heart) by sprouting roots (liver capillary mesh-work) and a trunk with major branches (aorta) (Risau, 1997). The cardiovascular system includes the heart, arteries, arterioles, capillaries, veins, and venules. The structure of a blood vessel varies according to its function. For example, arteries have thick vascular walls to deal with high blood pressure, while veins contain valves to prevent low-pressure blood reflux. Large vessels carry blood and small vessels, especially the capillaries, exchange gases and metabolites through the vessel wall and provide a bridge for immune surveillance. This is the first organ system to become functional within the embryo. The vascular system was initially described as a connected tube system by William Harvey in 1628 (Carmeliet, 2005). A few decades later, another class of vessels, the lymphatic vessels were discovered by Caspar Aselius. Lymphatic vessels are responsible for regulating homeostasis in the body by transporting fluid from tissues back to the blood circulation.

All blood vessels are covered by one layer of endothelial cells. Described as the foundation stones of the vascular system, they have important roles in physiological processes, including the regulation of blood flow and blood pressure, but are also implicated in several pathological processes including atherosclerosis, hypertension, and cancer (Khazaei et al., 2008). A large part of our knowledge of the physiology and function of endothelial cells has developed from *in vitro* studies of human umbilical vein endothelial cells (HUVECs). Endothelial cell culture was initially reported over 40 years ago (Jaffe et al., 1973).

The vascular system is now known to be partially formed by **vasculogenesis**, before the heart starts to beat, and later by **angiogenesis**. The importance of exploring the secrets of blood and lymphatic vessel development has increased with the more accurate knowledge of their role in physiological and pathological conditions.

1. Vasculogenesis

In the embryo, the first vessels are generated by the *de novo* formation of blood vessels from dispersed precursor cells, angioblasts, which build blood islands that later fuse to create a primitive plexus of vessels (Carmeliet and Jain, 2011b). This process is known as

vasculogenesis. The first primordial vessels are usually radial, with a uniform diameter, and have a low capillary density. Once circulation begins, the vascular network transforms into arteries and veins to form a functional circulatory loop. Various growth factors, such as transforming growth factor-β (TGFβ), platelet-derived growth factor (PDGF) and fibroblast growth factors (FGF) are needed for vasculogenesis, but the most critical pathways for this event seem to be the vascular endothelial growth factor (VEGF) and angiopoietin systems (Figure 1). Moreover, the initial development of the vascular system appears to be genetically programmed (Carmeliet, 2005). Vasculogenesis occurs mostly in the embryo but can also contribute to the formation of new vessels after birth, such as in hypoxic conditions.

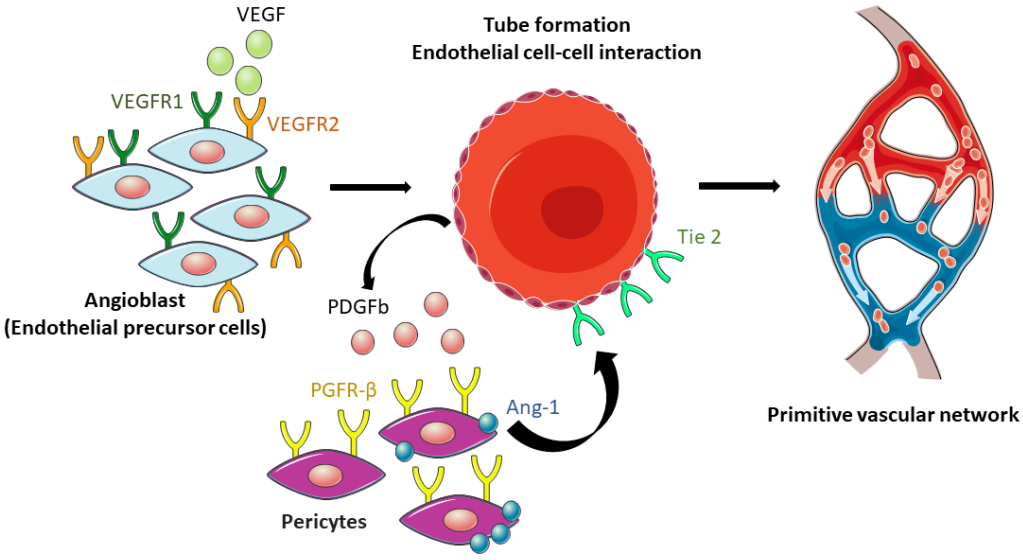

Figure 1. Vessel wall assembly.

Under the action of VEGF, angioblasts (mesenchymal cells) constitutively expressing VEGF receptors 1 (VEGFR-1 or Flt-1) and 2 (VEFR-2 or Flk-1) proliferate and differentiate into endothelial cells (via VEGFR-2) which then assemble into capillary structures (via VEGFR-1). PDGF-B, secreted by angiogenic endothelial cells (ECs), acts as the best characterized growth factor for the recruitment of PDGFR-β -expressing pericytes. Angiopoietins (Ang) and their TIE receptors control the association of ECs and pericytes. Yet, their molecular mechanism-of-action is much less well understood. TIE-2, expressed by ECs, is activated by pericyte-expressed Ang-1, which contributes to the maintenance of a quiescent EC phenotype, while Ang-2, released by activated ECs, destabilizes the interactions between ECs and pericytes by antagonizing the effect of Ang-1 on TIE-2. Adapted from Carmeliet, 2005.

2. Angiogenesis

The term angiogenesis is derived from the Greek term “angêion” (vessel) and “genesis” (birth or emergence) and defines the formation of new blood (hemangiogenesis) or lymph (lymphangiogenesis) vessels from existing ones.

Angiogenesis is a complex, and narrowly regulated process that involves the interaction of several types of cells. In adulthood, most blood vessels remain quiescent, except in the vascularization of the female reproductive tract (Carmeliet, 2005). However, endothelial cells retain the capacity to activate and quickly divide in response to physiological stimuli. When these stimuli are excessive, pathological conditions may appear, especially in malignant, ocular and inflammatory disorders (Carmeliet, 2005; Ellenberg et al., 2010).

a) Mature blood capillary: Endothelial quiescence

Structural differences in the capillary endothelium were first reported in the 1960s by electron microscopy (Florey, 1966) (Figure 2).

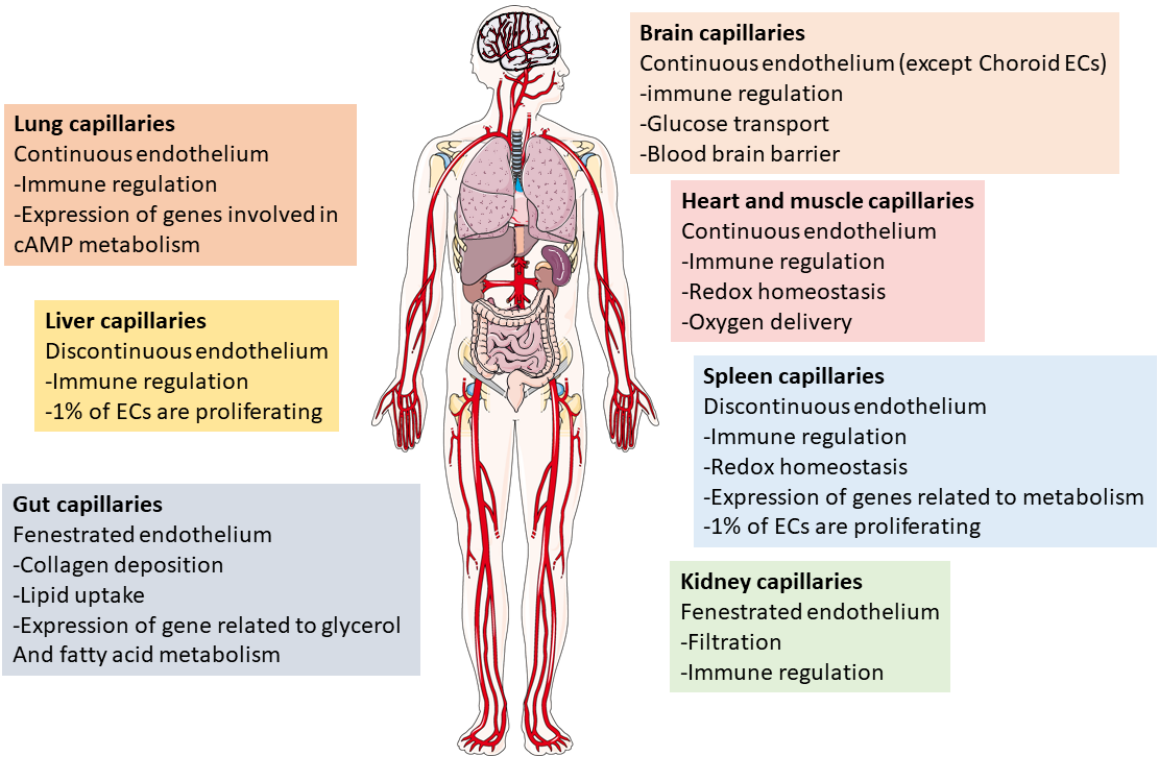

Figure 2. Endothelial heterogeneity in health.

There are three types of capillaries: continuous, fenestrated and sinusoids. The capillary type of an organ depends on its functions. Most organs have continuous capillaries forming barriers (lung, brain, skin and heart), with closely connected endothelial cells surrounded by a continuous basal membrane. This architecture allows the diffusion of water, small solutes, and liposoluble materials, while preventing the passage of cells and pathogens. In contrast, fenestrated capillaries have intracellular pores (windows) with a diaphragm, and are found in the renal glomeruli, exocrine glands, endocrine glands, and intestinal mucosa. While these fenestrae increase the permeability to fluids and solutes, they do not increase the permeability to macromolecules. Sinusoids are fenestrated capillaries with gaps between endothelial cells, and a thinner basal membrane than in the continuous or fenestrated endothelium. They are found within the bone marrow, spleen and liver. The gaps found in sinusoids facilitate the selective exchange of material. Adapted from Florey, 1966.

This 'quiescent' endothelium performs extensive active work, starting from the secretion of paracrine and endocrine factors to the maintenance of the barrier. Remarkably, little attention has been given to what controls this 'active quiescence' and what keeps vascular normalcy in physiological conditions. Quiescent ECs receives autocrine, endocrine, and paracrine signaling inputs (Figure 3).

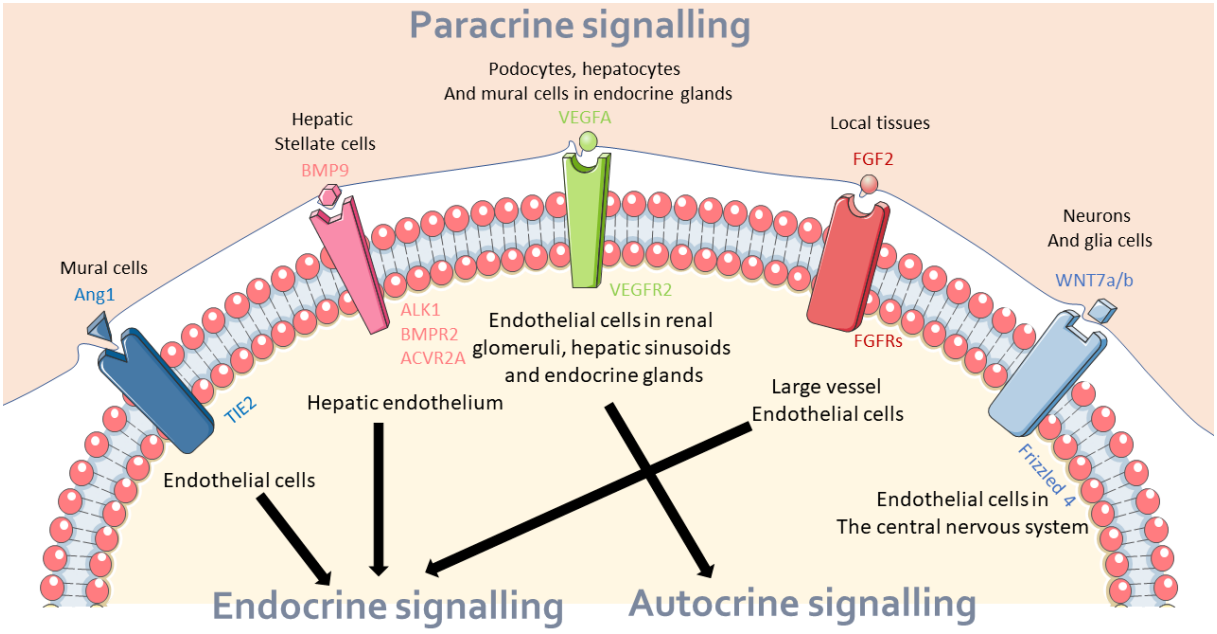

Figure 3. Tissue specificity of signaling pathways implicated in endothelial quiescence.

Paracrine stimulation provides the endothelium with a tissue-specific micro-environment. VEGF signaling is essential to the development and maintenance of fenestrated endothelium. Paracrine WNT signaling is essential to the formation and maintenance of the continuous endothelial lining, which is crucial to the overall vascular integrity and that of the blood-brain barrier. TGFβ autocrine signaling is a signature of endothelial cell dysfunction. Adapted from Ricard et al., 2021.

While numerous functions of the quiescent endothelium are organ-specific, other functions are common to all quiescent endothelial cells. For example, in the healthy quiescent endothelium, the TGFβ signaling pathway is inhibited. Activation of the TGFβ pathway is associated with the development and progression of disease states and with the loss of normal endothelial cell fate via endothelial to mesenchymal transition (EMT) (Figure 4).

In fact, the endothelial ability to avoid the activation of TGFβ signaling is closely associated with its ability to resist the development of a disease. The increased sensitivity of endothelial cells to TGFβ activation may be a risk factor for some vascular diseases (atherosclerosis). The capacity to understand the endothelial cells sensitivity, both genetically and functionally, would allow a better risk assessment and the development of therapies preventing disease development. The importance of controlling TGFβ signaling is further illustrated by various signaling cascades that inhibit TGFβ signaling in endothelial cells, including VEGF-ERK, FGF-ERK, BMP9-ALK1 and cerebral cavernous malformations (CCM)-MEKK3 pathways (Figure 4).

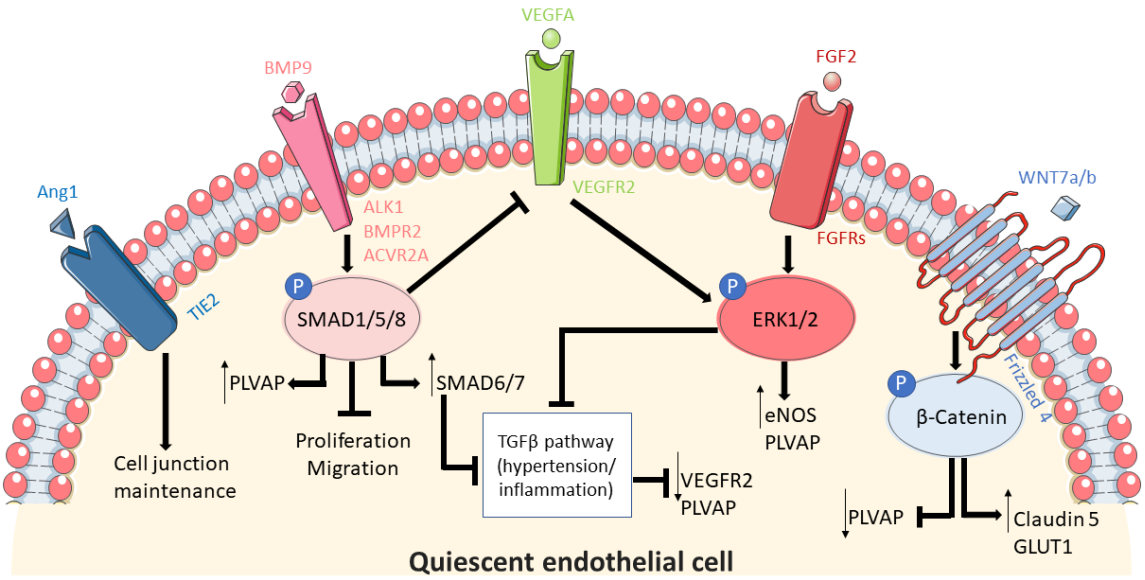

Figure 4. Signaling crosstalk in quiescent endothelial cells.

The existence of certain signaling cascades suggests that controlling TGFβ signaling is crucial to maintain cellular homeostasis and that abnormalities in this pathway may trigger specific diseases. Adapted from Ricard et al., 2021.

b) Angiogenic switch

To activate quiescent vascular endothelial cells, important interactions among various cells and molecules are needed. It is now widely accepted that angiogenesis is 'off' when concentrations of pro-angiogenic molecules are balanced by the action of anti-angiogenic molecules. Angiogenesis occurs when the balance moves towards a pro-angiogenic state, with a dominating effect of stimulating growth factors (Figure 5). This is called the "angiogenic switch", specifically in the context of cancer progression. Triggering events, tilting the switch towards angiogenesis, include hypoxia, inflammation, oncogene activation, and mechanical stress.

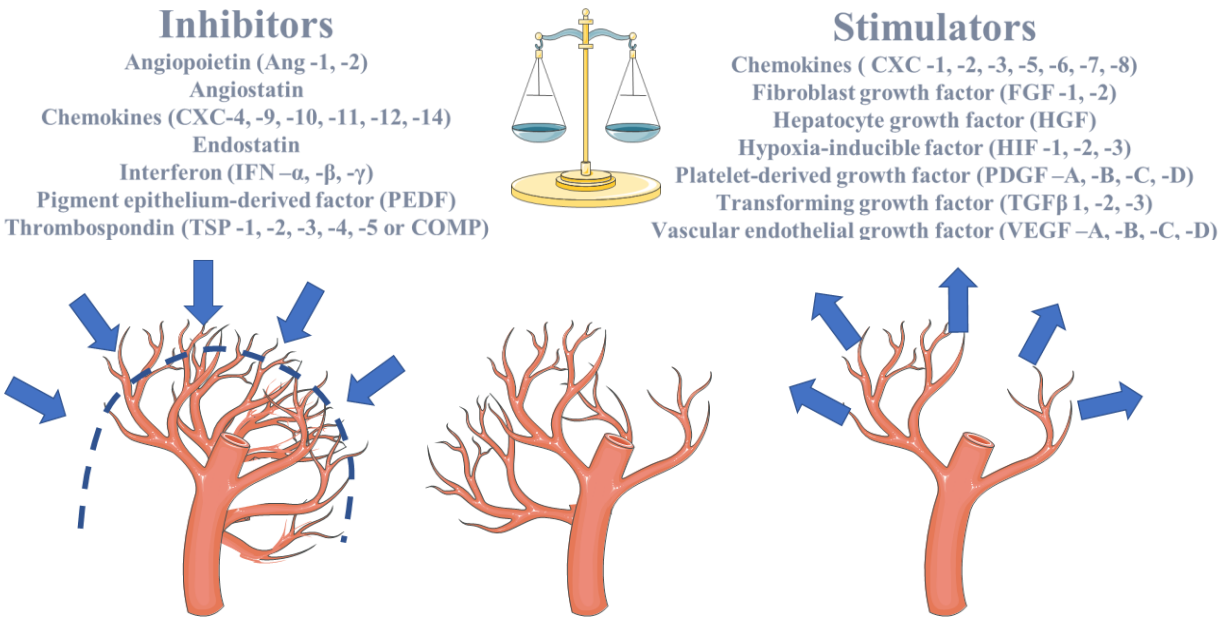

Figure 5. The balance between pro-angiogenic and angiostatic factors determines the "angiogenic switch".

Several endogenous inhibitory and stimulatory factors have been identified, which regulate the balance between the pro- and antiangiogenic states. Adapted from Zetter, 2008.

c) Main players in angiogenesis

• Environmental factors

✓ Hypoxia

Hypoxia refers to a too low oxygen pressure in tissues. This may be due to an overconsumption of O_2 by the tissue and / or to a mechanically insufficient supply of blood to the tissue. Hypoxia is a major stimulus for sprouting angiogenesis and the transcription factor hypoxia-inducible factor 1 (HIF-1) is at the heart of this phenomenon. HIF-1 is an αβ heterodimer that was originally recognized as a transcription factor mediating the hypoxia-inducible transcription of erythropoietin. Both HIF-α and HIF-β exist as a series of isoforms. HIF-β subunits are constitutive nuclear proteins, whereas HIF-α subunits are inducible by hypoxia (Pugh and Ratcliffe, 2003) (Figure 6). Among the three HIF-α isoforms, HIF-1α and HIF-2α are closely related.

Both HIF-1α and HIF-2α can interact with hypoxia response elements (HREs) in promoter regions of genes to induce transcriptional activity (Tian et al., 1997). The third isoform, HIF-3α, appears to be involved in the negative regulation of this response (Makino et al., 2001).

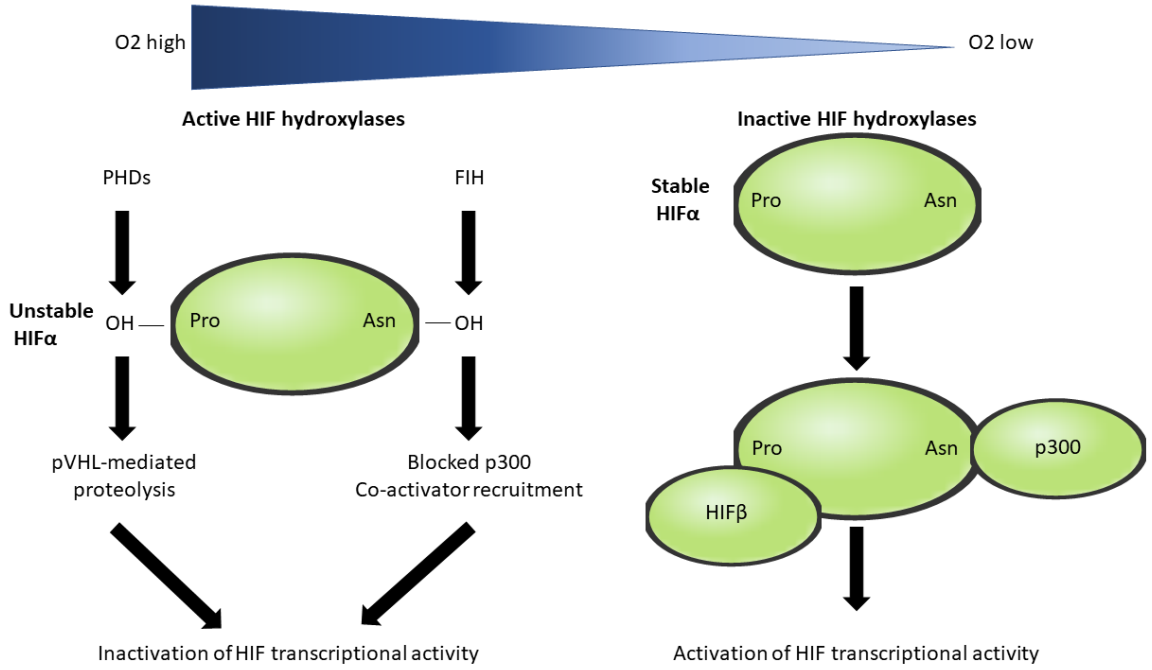

Figure 6. The control of HIF expression.

Under normoxic conditions, an enzyme, HIF prolyl-hydroxylase domain (PHD), hydroxylates conserved proline residues of HIF-1α, allowing the interaction with the von Hippel-Lindau E3 ubiquitin ligase complex. This complex transfers ubiquitins to lysine side chains of HIF-1α, targeting the protein for proteosomal destruction. In addition, the asparaginyl hydroxylase FIH (Factor Inhibiting HIF) hydroxylates HIF-1α on asparagine residue Asn-851, which blocks the recruitment of the CBP / p300 co-activator to the complex and thereby the transcriptional activity of HIF-1α. Under hypoxic conditions, PHD is inhibited because it uses oxygen as a cosubstrate, allowing HIF-1α to evade inactivation. HIF-1α forms a heterodimer in the nucleus with the HIF-1β subunit and, in interaction with the CBP / p300 coactivator, activates the transcription of different target genes containing a hypoxia response element (HRE) or a similar sequence in their promoter. These target genes encode different proteins able to improve the oxygen supply, such as VEGF, erythropoietin (EPO), inducible nitric oxide synthase (i-NOS), etc. Adapted from Tian et al., 1997; Makino et al., 2001 and Pugh and Ratcliffe, 2003.

✓ Inflammation

Broadly speaking, inflammation is the body's reaction to infection or external stimulation, and is characterized by pain, swelling, redness and heat. In addition to defending the body from pathogens, inflammation may promote angiogenesis as part of a wound healing response.

During inflammation, leukocytes enter the affected site by a process of leukocyte rolling, activation, adhesion, and extravasation from pre-existing vessels, followed by migration to the site of injury (Springer, 1994). These cells express early response inflammatory mediators such as IL-1α, IL-1β and TNF-α (Han et al., 2001) and further promote inflammation. TNF-α acts by upregulating VCAM-1 and ICAM-1 in endothelial cells, further enhancing leukocyte extravasation. Ang-2 amplifies the effects of TNF-α at early stages of the inflammatory response, when TNF-α concentrations are still low (Imhof and Aurrand-Lions, 2006). This interplay between Ang-2 and TNF-α is an example of the crosstalk between inflammation and angiogenesis. In addition, the proinflammatory mediators IL-1β and IL-1α enhance endothelial cell proliferation by stimulating the expression of VEGF. From a clinical standpoint, ocular diseases such as pterygium (Talghini and Shenasi, 2013), diabetic retinopathy (Capitão and Soares, 2016) and choroidal neovascularization all involve components of inflammation and angiogenesis. Altogether, inflammation is a strong inducer of angiogenesis, and the two processes are mutually interdependent.

• Pro-angiogenic factors

Endothelial cells, fibroblasts, immune cells (lymphocytes, polymorphonuclear neutrophils and macrophages) release proangiogenic agents, recapitulated in Table 1, in response to hypoxic or inflammatory situations (Mirabelli, 2019).

Protein Family	Component Proteins	Receptors
VEGF	VEGF-A VEGF-B VEGF-C VEGF-D VEGF-E PIGF	VEGFR-1 (FLT1) VEGFR-2 (KDR, FLK1) VEGFR-3 (FLT4)
FGF	FGF-1 (acidic FGF) FGF-2 (basic FGF)	FGFR-1 FGFR-2 FGFR-3 Integrins Heparin sulphate proteoglycans

PDGF	PDGF-A PDGF-B PDGF-C PDGF-D	PDGFR-α PDGFR-β
Ang	Ang-1 Ang-2 Ang-3 Ang-4	TIE-2 TIE-1
HGF	-	c-Met
HIF	HIF-1α HIF-1β	HIF-1β Aryl hydrocarbon receptor nuclear translocator
IGF	IGF-1 IGF-2	IGF-2 IGF2R
TGFβ	TGF-β1 TGF-β2 TGF-β3	TGFBR-1 TGFBR-2
MMP	MMP1-23	-
TNF	TNF1-18	TNFR1 TNFR2

Table 1. Angiogenic triggers involved in angiogenesis.

Chemical stimuli modulating the mechanism of angiogenesis are mainly VEGF, FGF, PDGF, Ang, hepatocyte growth factor (HGF), HIF, insulin-like growth factors (IGF), TGFβ, MMP, and tumor necrosis factors (TNF).

✓ VEGF

HIFs upregulate many genes. The induction of VEGF is however the most striking, increasing up to thirty-fold within minutes (Carmeliet, 2003). VEGFs, found in higher eukaryotes, are a family of soluble secretory glycoproteins. They bind to tyrosine kinase receptors (VEGFR), or co-receptors expressed by endothelial cells, and modulate various functions of these cells, including metabolic homeostasis, cell proliferation and migration, and tubulogenesis. The VEGF signaling pathway is the main regulator of angiogenesis, vasculogenesis and lymphangiogenesis. There are six members of the VEGF family: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF) (Barratt et al., 2018; Dehghani et al.,

2018; Yang et al., 2018). VEGF-A is considered as the principal inducer of angiogenesis by signaling through the VEGFR-2 receptor (Ferrara et al., 2003). The VEGF-A gene can generate by alternative splicing seven proangiogenic and five anti-angiogenic isoforms in human, including VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206} (the subscript indicating the number of amino acid residues) (Woolard et al., 2009; Gu et al., 2013; Eswarappa et al., 2014; Dehghani et al., 2018; Yang et al., 2018). Another isoform, VEGF-Ax, was also identified in 2014 (Eswarappa et al., 2014). VEGF-A_{165} was the first isoform characterized and remains the most extensively investigated in terms of function, signaling, expression and pathological roles. VEGF-B is largely found in cardiac and skeletal muscle, regulating the vascular remodeling of the lung after chronic hypoxia. VEGF-C and VEGF-D are key lymphangiogenesis regulators, while VEGF-E is a virus-encoded homolog, with no corresponding gene in the human genome (Wise et al., 2012; Barratt et al., 2018; Rauniyar et al., 2018; Stacker and Achen, 2018). PlGF is expressed at high level in placenta, thyroid, and lung. Its exact role remains uncertain, some studies showing that it can act as an endogenous competitive inhibitor.

VEGFR-1 is expressed by monocytes, macrophages, hematopoietic stem cells, vascular smooth muscle cells, and leukemic cells. VEGFR-2 is a high-affinity receptor for VEGF expressed in vascular endothelial cells, endothelial progenitors and megakaryocytes. VEGFR-3 is expressed in lymphoid endothelial cells (Failla et al., 2018; Melincovici et al., 2018; Yang et al., 2018). Upon activation by VEGF, cross-phosphorylation occurs within receptor dimers on multiple tyrosine residues along the cytoplasmic domain of each monomer (Schlessinger, 2000). The phosphotyrosines are crucial for the recruitment of a variety of signaling proteins containing Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains. The complexes formed lead to the activation of a variety of signaling cascades, including the Ras/MAP kinase and PI3K/PKB pathways, promoting respectively the proliferation and survival of the cells.

VEGFs can also interact with other proteins, including neuropilins, heparan sulphate proteoglycans and some integrins. The non-tyrosine kinase receptors, neuropilin-1 and neuropilin-2, bind VEGF isoforms selectively and guide the migration of endothelial cells during angiogenesis by enhancing VEGFR-1 and VEGFR-2 functions (Melincovici et al., 2018). Maintaining physiological levels of VEGFs is essential, since high concentrations have been linked to aberrant angiogenesis and other pathological conditions (Laakkonen et al., 2019).

✓ Angiopoietins

Angiopoietins (Ang) are a family of proteins consisting of Ang1, Ang2, Ang3, and Ang4 (Isidori et al., 2016) implicated in angiogenesis and vasculogenesis. Ang1 and Ang2 are the major isoforms regulating vascular homeostasis (Gnudi, 2016). Angiopoietins binds a tyrosine kinase receptor, Tie2, expressed by endothelial cells and certain myeloid cells. Tie1 is an orphan receptor, poorly characterized and presumed to act as a modulator of the Tie2 activity.

✓ FGF

Fibroblast growth factors (FGF) are secreted by vascular endothelial cells, damaged cardiac myocytes and stem cells. They regulate the survival, proliferation, differentiation and migration of ECs. FGFs stimulate the secretion of proteases such as plasminogen activator and metalloproteinases, leading to the degradation of the extracellular matrix. They promote as well vessel maturation (Henning, 2016; Inampudi et al., 2018). FGF-1 and FGF-2 mediate the angiogenic effects of the FGF family and are key factors in wound healing. They stimulate the proliferation of fibroblasts and endothelial cells, which will produce the granulation tissue necessary for the healing process (Henning, 2016; Inampudi et al., 2018; Shoeibi et al., 2018).

✓ HGF

Hepatocyte growth factor (HGF) was initially found to be a mitogen for adult rat hepatocytes. HGF is a pleiotropic factor signaling through its receptor, c-Met (Libetta et al., 2016). HGF is secreted as a glycoprotein precursor activated by proteolytic cleavage. The active molecule is an αβ heterodimer, with the α chain binding the receptor, and the β chain necessary for its activation. c-Met is a tyrosine kinase receptor expressed by bone marrow-derived endothelial progenitors, smooth muscle cells and endothelial cells. Under physiological conditions, c-Met activates different signaling pathways regulating tissue homeostasis. Additionally, tissue hypoxia activates c-Met transcription, which contributes to the angiogenesis process (Kato, 2017).

✓ PDGF

The platelet-derived growth factor (PDGF) family comprises four different polypeptide chains, namely PDGF-A, PDGF-B, PDGF-C, and PDGF-D, which act as dimers stabilized by disulfide bonds. One heterodimer (PDGF-AB) and 4 homodimers (PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD) were identified (Manzat Saplacan et al., 2017; Medamana et al., 2017). These factors bind to two tyrosine kinase receptors, PDGFR-α and PDGFR-β (Manzat Saplacan et al., 2017; Medamana et al., 2017; Lee and Li, 2018). The extracellular domain of PDGFRs contains five immunoglobulin-like domains (Lee and Li, 2018). PDGFR-αα is activated by all PDGF ligands except PDGF-DD, PDGFR-ββ is activated by PDGF-BB and PDGF-DD, and PDGFR-αβ is activated by all PDGF ligands except PDGF-AA. The receptors signal mainly through the Ras/MAP-kinase pathway (Manzat Saplacan et al., 2017). PDGFs were originally isolated from platelets and considered as major mitogens for mesenchymal cells, including fibroblasts, glial cells, and smooth muscle cells. They are stored in platelet granules and released upon degranulation, as a response to thrombin or other stimuli (Thiagarajan et al., 2017; Lee and Li, 2018). PDGFs were later shown to be involved in a broader range of functions, including vascular development, by promoting recruitment, proliferation and survival of vascular mural cells. Besides megakaryocytes, they are expressed at low levels by a variety of other cells, including monocytes, macrophages, lymphocytes, mast cells, fibroblasts, pericytes, endothelial cells, and tumor cells (Medamana et al., 2017; Thiagarajan et al., 2017; Lee and Li, 2018).

✓ Pro-angiogenic chemokines

All identified ELR^+ chemokines promote angiogenesis (Strieter et al., 2005). CXCR2 is expressed on endothelial cells and is the main receptor for ELR^+ chemokines (Strieter et al., 2005). Also expressed by human endothelial cells, CXCR1 is a second CXC receptor mediating the angiogenic responses. It binds CXCL8 and CXCL6 (Wolf et al., 1998). Blocking antibodies for either CXCR1 or CXCR2 reduces chemotaxis of human microvascular dermal endothelial cells (Salcedo et al., 2000), and the blockage of both receptors reinforces this effect. CXCR1 affects early steps of chemotaxis, such as the appearance of stress fibers. CXCR2 affects later steps of chemotaxis such as cell retraction (Schraufstatter et al., 2001).

Nomenclature	ELR motif	Receptor	Reference
CXCL1	+	CXCR2	(Martins-Green et al., 1991)
CXCL2	+	CXCR2	(Cao et al., 1995)
CXCL3	+	CXCR2	(Luan et al., 1997)
CXCL5	+	CXCR2	(Arenberg et al., 1998)
CXCL6	+	CXCR1, 2	(Van Coillie et al., 2001)
CXCL7	+	CXCR2	(Ahuja and Murphy, 1996)
CXCL8	+	CXCR1, 2	(Koch et al., 1992)
CXCL12	-	CXCR4, 7	(Nagasawa et al., 1996)
CCL2	N/A	CCR2, 4	(Goede et al., 1999)
CCL11	N/A	CCR3	(Salcedo et al., 2000)
CCL16	N/A	CCR1	(Strasly et al., 2004)

Table 2. Angiogenic chemokines and their receptors.

The distinction between the ELR^+ and ELR^- CXC chemokines is a functional one. All identified ELR^+ chemokines stimulate angiogenesis.

• Anti-angiogenic factors

✓ Angiostatin

Angiostatin is a fragment of plasminogen, generated by various protease combinations. Angiostatin inhibits endothelial cell proliferation and migration, tube formation, leukocyte transmigration, expression of MMPs, and tumor cell invasion. However, it promotes endothelial cell apoptosis and the production of anti-angiogenic factors, such as thrombospondin-1, and it attenuates VEGF expression. The mode of action of angiostatin on endothelial cells is not completely understood. Different mechanisms have been proposed, including through the binding to ATP synthase, integrins and angiomotin (Kanno, 2019).

✓ Endostatin

Endostatin, generated by the proteolytic activity of MMP-7, is a C-terminal fragment of type XVIII collagen, (Tanabe et al., 2018). It has extensive anti-angiogenic activities on endothelial cells, including the inhibition of MMP expression. MMP-2, MMP-9, and MMP-13 are the

targets for the inhibitory activity of endostatin (Poluzzi et al., 2016). As angiostatin, it also inhibits proliferation and migration of vascular endothelial cells, and promotes their apoptosis. These anti-angiogenic activities have been attributed to its binding to α5 and αv-integrins and to VEGFR2 (Poluzzi et al., 2016; Tanabe et al., 2018).

✓ Thrombospondin

Thrombospondin-1, known as a glycoprotein, is one of the five paralogs of thrombospondins. It is the main constituent of platelet α-granules (Lord et al., 2017). Thrombospondin-1 inhibits the migration, proliferation, survival of endothelial cells and tubulogenesis. Thrombospondin can interact with numerous extracellular matrix proteins and cell surface adhesion proteins. Its anti-angiogenic effects are mainly attributed to its interactions with CD36, CD47, and integrins on endothelial cells. It may also displace VEGF from its complex with heparan sulfate (Lawler and Lawler, 2012). Several studies have shown that high concentrations of thrombospondin-1 can also promote angiogenesis (Ren et al., 2006; Lawler and Lawler, 2012).

✓ Anti-angiogenic chemokines

ELR^- chemokines include CXCL4, CXCL9, CXCL10, CXCL11 and CXCL14. The first anti-angiogenic chemokine described was CXCL4 (Taylor and Folkman, 1982; Maione et al., 1990). CXCL4 exists as two non-allelic variants, CXCL4 and CXCL4L1, which differ only by three amino acids. Studies on human microvascular endothelial cells have shown that CXCL4L1 inhibits bFGF- and CXCL8-driven chemotaxis more effectively than CXCL4 (Struyf et al., 2004). How CXCL4 and CXCL4L1 affect angiogenesis is poorly understood. They were shown to inhibit EC chemotaxis as well as cell cycle progression (Gupta and Singh, 1994). These two effects are mediated in part by the capacity of CXCL4 to inhibit the binding of the two proangiogenic factors VEGF and bFGF to their respective receptors (Sato et al., 1990; Gengrinovitch et al., 1995; Perollet et al., 1998). CXCL4 can heterodimerize with bFGF, preventing the homodimerization of this factor, which is required for receptor binding (Perollet et al., 1998; Jouan et al., 1999). CXCL4 can also inhibit the binding of $VEGF_{165}$ but not of the $VEGF_{121}$ isoform to VEGFRs (Gengrinovitch et al., 1995). The $VEGF_{165}$ and $VEGF_{121}$ isoforms are distinguished by their capacity to bind heparan sulphates. While $VEGF_{161}$ efficiently associates with heparan sulphates and remains bound to the extracellular

matrix, $VEGF_{121}$ does not (Ferrara, 2010). This could indicate that CXCL4 specifically inhibits the binding of $VEGF_{165}$ to its receptor by interfering with heparan sulphates. However, CXCL4 mutants unable to bind heparin still inhibit angiogenesis (Gengrinovitch et al., 1995), and CXCL4 inhibits $VEGF_{121}$-induced endothelial proliferation, while it does not bind this factor. These observations suggest that CXCL4 might inhibit VEGF signalling via a mechanism that does not involve direct binding to either VEGF ligands or their receptors.

The main receptor for antiangiogenic ELR^- CXC chemokines is CXCR3. It can bind CXCL4, CXCL9, CXCL10 and CXCL11 (Keeley et al., 2008). It exists in three different splice isoforms, CXCR3A, CXCR3B and CXCR3-alt. While CXCR3A is primarily responsible for the recruitment of lymphocytes (Loetscher et al., 1996; Moser and Loetscher, 2001), CXCR3B is expressed by endothelial cells (Salcedo et al., 2000; Romagnani et al., 2001; Lasagni et al., 2003). CXCR3B was shown to mediate the antiangiogenic effects of CXCL10 in a melanoma context (Yang and Richmond, 2004). There is however no CXCR3B form in mice and CXCL10 was shown to inhibit endothelial cell proliferation independently of CXCR3 function (Campanella et al., 2010). Together, these studies demonstrate that the mode of action of the antiangiogenic chemokine CXCL10 is context- and organism-dependent.

d) Mechanisms of angiogenesis

• Sprouting angiogenesis

The formation of vascular sprouts involves several successive steps strictly regulated by different factors. (a) First, the activation of endothelial cells promotes the degradation of the basement membrane common to ECs and pericytes by matrix metalloproteinases (MMPs). (b) Tip cells are selected from the parent vessel. Each tip cell becomes a migratory leading cell and blocks its adjacent cells to adopt a leading cell fate through a process of lateral inhibition. (c) During the sprout extension phase, the tip cell migrates along the chemotactic path, followed by stalk cells. Tip cells do not divide, while stalk cells proliferate and elongate the stalk of the sprout and form the lumen (De Smet et al., 2009). (d) Finally, a lumen forms within the sprout and connects to the luminal space of the parent vessel. These sprouts connect to other vessels through a process called anastomosis (Figure 7). VEGF-A, the most important regulator of sprouting angiogenesis, particularly following hypoxia, induces a signaling cascade that

activates endothelial cells and regulates them to become tip cells. Tip cells become more reactive to VEGF by overexpressing its receptors and thereby navigating the growing sprout along the growth factor gradient (De Smet et al., 2009). The tip cells are guided by thin extensions called filopodia. Stalk cells establish junctions with neighboring cells and synthesize basement membrane components that will stabilize the new vessel (Phng and Gerhardt, 2009). As endothelial cells switch from active sprouting to quiescence, they adopt a "phalanx" phenotype, looking like a phalanx formation by ancient Greek soldiers. They become immobile and non-proliferative, which contributes to the integrity and stability of vessels (Bautch, 2009). To form a functional loop, tip cell filopodia from different sprouts connect, subsequently increasing the cell-cell contacts and establishing a perfused vessel.

Live imaging studies using zebrafish have shown that the development of cell junctions is a highly stereotyped process (Herwig et al., 2011; Kochhan et al., 2013; Lenard et al., 2015). Filopodia from migrating tip cells express junctional proteins such as VE-cadherin (Lenard et al., 2013; Phng et al., 2013). Filopodia from adjacent tip cells make and break contacts many times during initial interactions, after which VE-cadherin molecules concentrate at the contact point between filopodia and a ring shaped junction is formed, creating a small luminal pocket at this site (Blum et al., 2008; Herwig et al., 2011; Lenard et al., 2013). Then, filopodia in excess retract, the membrane contacts of the anastomosis expand, concentrating apical markers such as podocalyxin, and upregulating the expression of junctional molecules on the cell surface. The various mechanisms by which the lumen and perfused tubes form are called type I and type II anastomosis, examined in detail by Betz et al. (Betz et al., 2016).

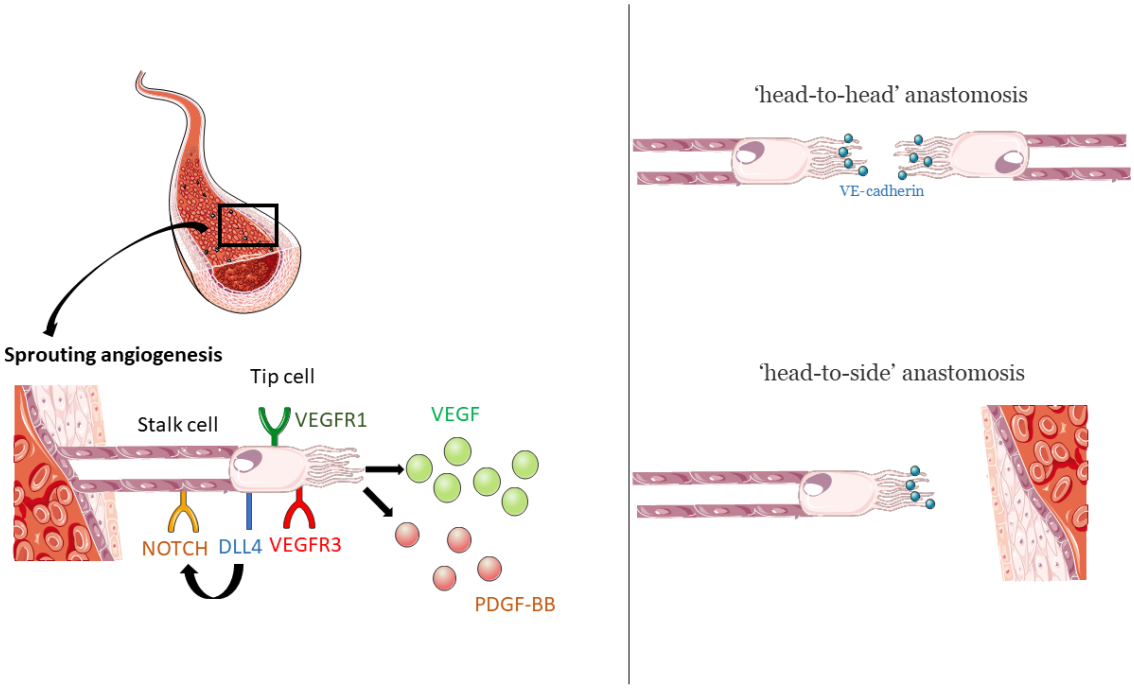

Figure 7. Mechanisms of angiogenesis: sprouting angiogenesis.

A crosstalk between the VEGF and Delta-like-4 (DLL4)/Notch pathways regulates tip and stalk cell selection. As a result of VEGF stimulation, tip cells produce the DLL4 ligand, PDGF-B, VEGFR-2 and VEGFR-3/Flt-4, thereby blocking Notch signaling, enhancing sprouting, branching, migratory capacity and filopodia formation in tip cells. DLL4 secreted by tip cells activates Notch signaling in the stalk cells, suppressing tip cell formation by inhibiting the expression of VEGFR-2 and VEGFR-3, and inducing the expression of VEGFR-1 (decoy for VEGF). Tip cells secrete matrix degrading proteases, acquire motile and invasive phenotypes and extend numerous filopodia that guide new blood vessel formation towards the VEGF gradient. Stalk cells follow the tip cells and branch out from the parent vessel. Stalk cells, compared to tip cells, are more proliferative and have fewer filopodia. Vascular anastomosis refers to the development of a junction between two new sprouts ('head-to-head' anastomosis), or a sprout and an existing blood vessel ('head-to-side' anastomosis). Adapted from Betz et al., 2016.

• Intussusceptive angiogenesis

Intussusceptive angiogenesis involves the maturation of vascular networks through the formation of translaminar pillars within the capillaries, thus dividing a vessel into two parallel vessels without proliferation of vascular endothelial cells (Figure 8). The pillars are then covered by pericytes and myofibroblasts, which provide collagen to the vessel wall and stabilize them (Burri et al., 2004). Intussusception was first described by Caduff et al in 1986 when they found tiny holes in the vasculature of the developing lungs (Caduff et al., 1986). Since then, intussusceptive angiogenesis has received attention as a significant mode of growth and

remodeling during vascular development (Burri et al., 2004; Styp-Rekowska et al., 2011). Intussusception occurs quickly, within a few hours or sometimes minutes, and requires little energy, because it does not involve cell proliferation or basal membrane degradation. The occurrence of sprouting or intussusception depends essentially on metabolic and hemodynamic factors. In contrast to sprouting angiogenesis, which is driven by angiogenic factors, intussusception is stimulated by higher levels of shear stress (mechanical forces from blood flow) (Styp-Rekowska et al., 2011) or by a drop in angiogenic factors (Hlushchuk et al., 2008). So far, intussusceptive angiogenesis has been identified in many developing organs, such as the retina, but also as a vascular defense mechanism to escape anti-angiogenic therapies in tumors (Hlushchuk et al., 2008).

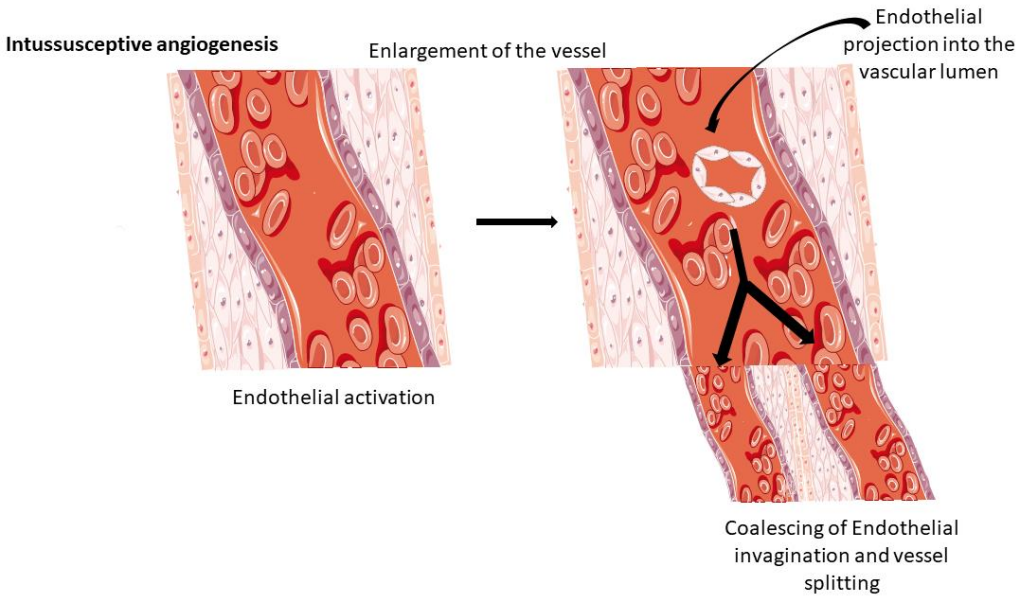

Figure 8. Mechanisms of angiogenesis: Intussusceptive angiogenesis.

Intussusceptive angiogenesis is the result of the enlargement of the capillary plexuses, without requiring much energy. Adapted from Burri et al., 2004 and Styp-Rekowska et al., 2011.

• Looping angiogenesis

Looping angiogenesis involves the expansion of translocated vessels by biomechanical forces (Benest and Augustin, 2009). By the action of contracting myofibroblasts in the region of a wound, surrounding blood vessels incorporate in the lesion during the repair process, as perfused, functional vessels. The recruited vessels are mature in terms of perfusion, presence

of smooth muscle cells, and presence of a basement membrane. The invasion of vessels is inhibited by blocking matrix contractility (Kilarski et al., 2009).

e) Vessel maturation and regression

• Vessel maturation

Perivascular, or mural cells, were initially described histologically as cells closely associated to the endothelial layer of blood vessels and are found in all organs throughout the body. This is a phenotypically diversified family of cells whose roles vary with the anatomic location and function of the vessel (Bergers and Song, 2005). They can be divided into two major categories: vascular smooth muscle cells and pericytes, although there is considerable heterogeneity within each of these groups.

Vascular smooth muscle cells are associated with arteries and veins and are separated from the endothelial layer by the basement membrane and the inner elastic lamina. They adopt a stellate shape, express strongly α-smooth muscle actin (α-SMA), and line up in a circumferential pattern around the vessel. Pericytes, on the other hand, are associated with capillaries and are embedded in the same basement membrane as the endothelial layer. They are elongated and flat in shape and are oriented along the long axis of the vessel, with many finger-like projections extending through the basement membrane and making direct contacts with endothelial cells (Bergers and Song, 2005). These connections have a peg and socket arrangement, which allow direct signaling between pericytes and the endothelial layer. A single pericyte can interact with several endothelial cells. In skeletal muscles, there is approximately a 1:100 ratio between pericytes and endothelial cells (Armulik et al., 2011), whereas in the brain and retina, the ratio is 1:1 and pericytes play a major role in maintaining the blood brain barrier (Hirschi et al., 1999, 2003; Armulik et al., 2005). Their positioning, in contact with multiple endothelial cells, allows to coordinate the response of a population of endothelial cells along the vessel, as intercellular signaling can be propagated to multiple cells in the endothelial layer through a single pericyte (Armulik et al., 2005; Bergers and Song, 2005).

A debate exists regarding the characteristics that define a perivascular cell, and thus a precise definition remains elusive. Therefore, no single specific marker exists for defining this cell

population. Instead, a set of cell surface markers are typically used and each of these may have variable expression depending on the anatomical location and the angiogenic state of the cell (Attwell et al., 2016; Vanlandewijck et al., 2018). Common markers that are expressed in perivascular cells include neuron-glial antigen-2 (NG2), CD146, α-SMA, and platelet derived growth factor receptor-β (PDGFR-β). Endothelial and leukocyte-specific markers, such as CD31, VE-cadherin, von Willebrand factor and CD45, are absent from pericytes (Cuevas et al., 1984; Gerhardt and Betsholtz, 2003; Bergers and Song, 2005; Alvino et al., 2018).

Vessel maturation is a process involving the recruitment of mural cells, including pericytes and smooth muscle cells (Hirschi and D'Amore, 1996; Darland and D'Amore, 2001; Jain, 2003). PDGF is crucial for recruiting both cell types. VEGF promotes platelet-derived growth factor (PDGF-B) expression by endothelial cells and this allows mural cell engagement (Benjamin et al., 1998; Hellstrom et al., 2001). In turn, the production of angiopoietin-1 by pericytes reduces the sensitivity of endothelial cells to VEGF, thereby promoting the acquisition of a resting phenotype.

- ## Capillary remodeling and regression

While we have learned much about early stages of vascular development, we still know relatively little on how to achieve the ultimate anatomic and functional organization. Since all processes of neovascularization initially lead to the formation of excessive vasculature, pruning is necessary to restore the vascular density to its optimal value in order to reach effective tissue perfusion.

According to studies dealing with developmental angiogenesis (Hughes and Chang-Ling, 2000, 20; Potente et al., 2011; Franco et al., 2015), and in adults (Modlich et al., 1996), various modes of capillary remodeling have been described. Apoptosis of endothelial cells contributes to hyaloid vessel regression (Mitchell et al., 1998), and pupillary membrane vessel regression (Lang et al., 1994, 199; Meeson et al., 1996, 1999). Endothelial cell migration as another mechanism was observed in the remodeling of the zebrafish midbrain network (Chen et al., 2012). Vessel perfusion is a key factor in vessel maintenance. Shear stress mediates vasodilation by the upregulation of nitric oxide (NO)-synthase and superoxide dismutase (Dekker et al., 2005) and promotes endothelial cell survival through the Akt pathway and Krüppel-like factor 2 (KLF2) activation. Inhibition of VEGF signaling favors capillary

regression, which involves cessation of flow and endothelial cell apoptosis (Baluk et al., 2004; Baffert et al., 2006). Another signaling axis involved in capillary remodeling is Wnt signaling, which regulates migration, proliferation, and survival of endothelial cells (Clevers and Nusse, 2012). Angiopoietins and their TIE receptors constitute another axis involved in capillary remodeling and regression (Augustin et al., 2009) (Figure 9).

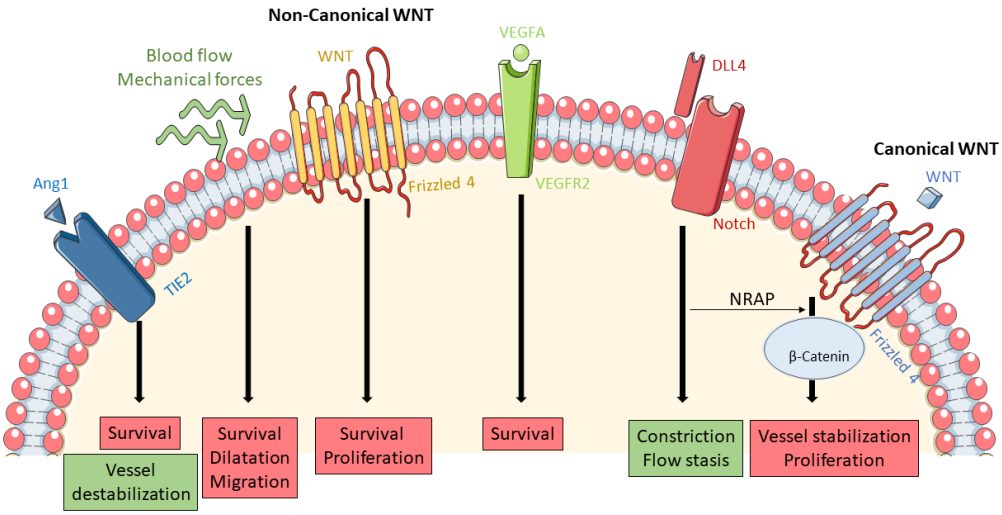

Figure 9. Signaling pathways controlling vessel pruning and regression.

Ang and TIE signaling, blood flow and mechanical forces, WNT signaling, VEGF signaling and DLL4 and Notch signaling are involved in capillary remodeling and regression. Adapted from Korn and Augustin, 2015.

Franco and co-workers have analyzed vascular pruning in the postnatal mouse retinal vasculature and propose a four-step model for vessel regression (Figure 10).

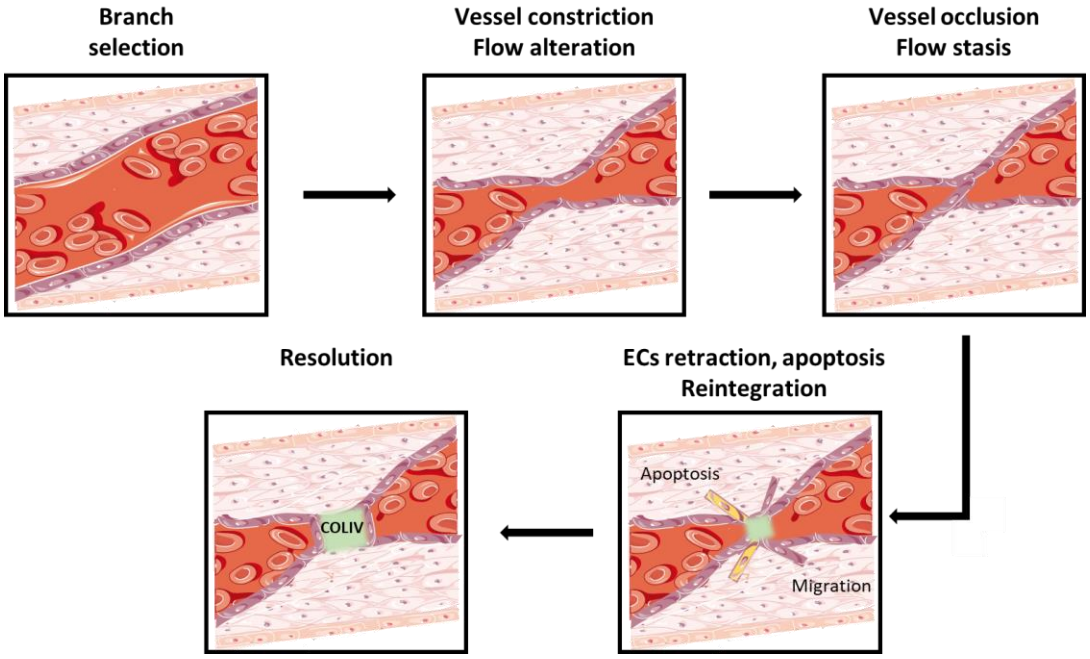

Figure 10. Multistep nature of vessel pruning and regression processes.

The four-step model for vessel regression consists of branch selection, lumen stenosis, ECs retraction, and resolution. Adapted from Korn and Augustin, 2015.

B. Modeling angiogenesis

Many models have been developed over the years to study the different aspects of angiogenesis, *in vitro*, *ex vivo* and *in vivo*.

Most *in vitro* models use human umbilical vein endothelial cells (HUVEC). However, over 19 types of endothelial cells (uterine, cardiac, dermal and pulmonary amongst others) are now available, facilitating the study of different vascular endothelial subtypes (Staton et al., 2009). Current *in vitro* models include two-dimensional cultures of tissue-specific vascular endothelial cells on collagen, and three dimensional cultures in matrigel (Poulaki, 2011). Endothelial cells can be cultured alone or in co-culture with fibroblasts or pericytes for studying cellular interactions. Advantages of *in vitro* models include the possibility of manipulating cell growth, proliferation, migration and interactions by using different media and cocktails of pro- or anti-angiogenic factors. The major disadvantages, however, are the artificial environment, the absence of blood flow, and of complex physiological interactions that occur *in vivo*.

Ex vivo models include the aortic ring assay, the *vena cava* explant assay and the foetal mouse metatarsal assay. In these *ex vivo* models, segments, discs, or sections of the specific tissue are cultured in three dimensional matrices *in vitro* and monitored for microvascular outgrowth for 5 to 14 days. The aortic ring assay was introduced by Nicosia & Ottinetti in 1990 and is currently the most used organ culture model (Nicosia and Ottinetti, 1990). An advantage of the aortic ring model is the presence of non-endothelial cells (pericytes and other supporting cells) able to contribute to vessel growth or maturation, hence mimicking an *in vivo* context. The cells can also be transfected to study the function of specific genes and proteins (Masson V et al., 2002; Go and Owen, 2003). In addition, the conditions of the assay can easily be modified and there is no interference of inflammatory responses that may be encountered in *in vivo* models (Blacher et al., 2001). Disadvantages include the lack of blood flow and of surrounding tissues found *in vivo* and the relative difficulty in the quantification of the angiogenesis response. To overcome species-related issues, human placental umbilical and saphenous veins have also been used in organ culture models. Whether these assays are truly representative of *in vivo* angiogenesis in humans remains to be determined (Staton et al., 2009).

In vivo models include the development of the vasculature in zebrafish embryos and in the mouse retina (Cao et al., 2008), choroidal neovascularization, regenerative angiogenesis (Huang et al., 2003) and the oxygen-induced retinopathy (OIR) model. The rat hind limb ischemia model is important for addressing the remodeling of an existing network following an acute obliteration. It mimics the pathological situations of stroke and myocardial infarction (Paskins-Hurlburt and Hollenberg, 1992; Cao, 2010). An additional model system of angiogenesis is corneal angiogenesis. The cornea is naturally avascular, and all vessels that develop into the cornea are the result of the experimental stimuli.

1. Retinal structure and development

The retina is the most inner layer of the eye. It detects light and transmits visual signals to the brain via the optic nerve. This highly organized tissue controls sophisticated and precise crosstalk between neural, vascular, and glial cells during development in order to fulfill its function.

Retinal development is a complex process that begins with an evagination of the anterior central nervous system (CNS). The retina first originates from the diencephalon as an optic pit at around 23 weeks of gestation in human (O'Rahilly and Müller, 1994). The tissue then develops into an outer retinal pigment epithelium (RPE) and the inner neural retina. The retina is morphologically and functionally structured in different layers (Figure 11).

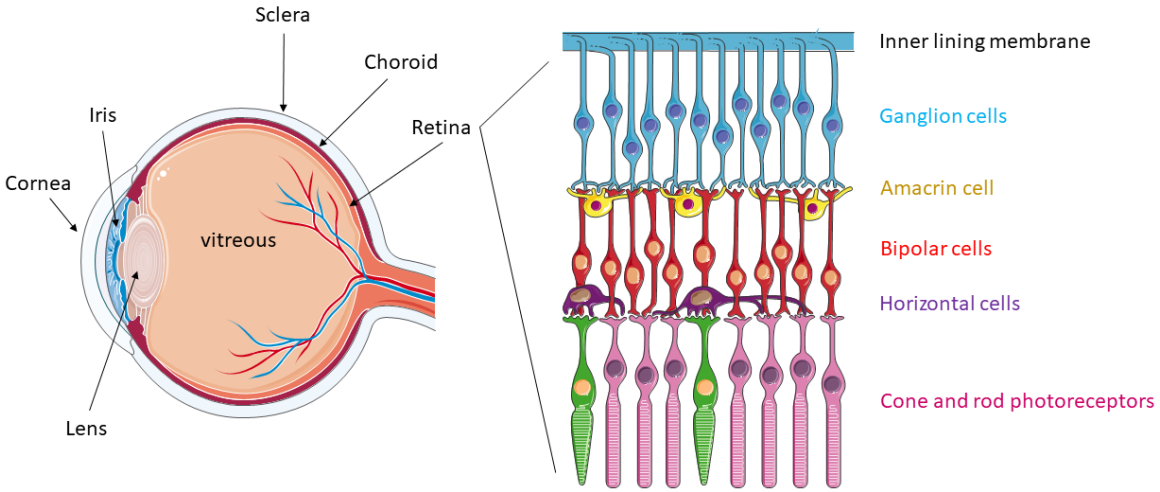

Figure 11. Diagram of a human eye showing the various layers of retinal cells.

The retina consists of 10 layers from the inner to the outer part:
1- Inner Lining Membrane (ILM) is formed by the end feet of the Müller cells.
2- Retinal Nerve Fiber Layer (RNFL) contains axons of ganglion cells and retinal vessels.
3- Ganglion Cell Layer (GCL) mainly composed of the nuclei of ganglion cells.
4- Inner Plexiform Layer (IPL) where bipolar, amacrine and ganglion cells interact.
5- Inner Nuclear Layer (INL), contains the nuclei of bipolar, horizontal, amacrine and Müller cells.
6- Outer Plexiform Layer (OPL) where photoreceptor cells, bipolar cells and horizontal cells interact.
7- Outer Nuclear Layer (ONL) contains the nuclei of photoreceptor cells.
8- External Limiting Membrane (ELM) is formed by the contacts of Müller cells with each other and with photoreceptor cells.
9- Photoreceptor Layer (PR) contains cones and rods.
Adapted from Yang et al., 2021.

Three layer compose the inner neuronal retina: the ganglion cell layer (GCL), the inner nuclear layer (INL) composed of amacrine, bipolar and horizontal cells and the outer nuclear layer (ONL) containing rods and cone cells. The development of ganglion cells, cone photoreceptors and horizontal cells precedes that of rod photoreceptors, bipolar and Müller cells, which differentiate later (Carter-Dawson and LaVail, 1979; Rapaport et al., 2004).

The development of the foveal region is initiated by the migration of ganglion cells to surrounding areas between the 24th and 26th weeks of gestation in human. Other cell types, including amacrine and bipolar cells, are then progressively lost with the continuous thinning of GCL and INL, and thickening of ONL; eventually delineating a small area comprising only cone photoreceptors and Müller cells (Hendrickson and Kupfer, 1976; Hendrickson and Yuodelis, 1984; Hendrickson, 1992).

Photoreceptors express the photon-activated opsins, which belong to the G protein-coupled receptor family. Rod cells contain rhodopsin, detect dim light and are mostly located outside of the fovea (Curcio et al., 1990). Cone photoreceptors are concentrated in the fovea and detect colors; they are divided into three subtypes expressing opsins responding to either red, green, or blue light. A third class of photoreceptors, photosensitive ganglion cells, was suggested to contribute to the control of circadian rhythms (Foster et al., 1991).

There are three types of glial cells in the retina: microglial cells, Müller cells and astrocytes.

The microglia are important for its macrophagic function after retinal injury. Activation of microglial cells is often associated with retinal degeneration and photoreceptor apoptosis (Zeiss and Johnson, 2004; Zeng et al., 2005). This activation requires an interaction with other glial cells and neurons for the phagocytosis of cellular debris (Dick et al., 2003).

Müller cells are the specialized and predominant glial cell type in the retina. Spanning its entire thickness, Müller cells extend from the vitreous edge to the subretinal space and are essential for normal retinal function. These glial cells are crucial for the mechanical and metabolic support of retinal neurons, their synaptic activity and proper function (Bringmann et al., 2006, 2009). They are also responsible for waste removal and ion exchange that maintains retinal homeostasis (Bringmann et al., 2004).

After retinal injury or neuronal stress, Müller cells proliferate and change shape, resulting in a drastic increase in the expression of the intermediate filament component, glial fibrillary acidic protein (GFAP). Activated Müller cells have also been shown to change their ion transport properties, and may contribute to neuronal survival and pathological angiogenesis by secreting VEGF (Rutka and Smith, 1993; Reichenbach et al., 1997). The process of gliosis protects and

repairs neurons but may also contribute to disease development (Sahel et al., 1990; Amin et al., 1997; Ridet et al., 1997; Streit et al., 1999).

Müller cells have also the potential to serve as neural progenitor/stem cells in mature retina. After a retinal lesion, they can migrate to the injury site and differentiate into specific neurons both in fish and mammalian species (Fischer and Reh, 2001; Ooto et al., 2004; Bernardos et al., 2007; Roesch et al., 2008).

Astrocytes migrate from the brain to the optic nerve and have a symmetrical stellate morphology at the periphery and elongated shape in the central retina near the optic nerve head (Stone and Dreher, 1987). Astrocytes are distributed unevenly in retina and located close to the ganglion cell and nerve fiber layers.

The hyaloid vascular system that originates from the optic cup contributes the initial blood supply to the developing eye and it fully regresses by approximately 34 weeks of gestation in human. The development of the retinal vasculature begins at the 16th week of gestation and reaches its completion at term, around the 40th week (Roth, 1977).

In the developing retina, hypoxia in retinal astrocytes leads to the expression of VEGF resulting in an angiogenesis process adapted to meet the oxygen demand of the tissue. The retinal vasculature network, containing arteries and veins, spreads across the whole retina with three parallel and inter-connected plexus (Fruttiger, 2007). Its development begins with the formation of the superficial plexus. Subsequent vessel branching and sprouting forms the deep vascular plexus, above the outer plexiform layer (OPL) that lies between the inner and outer nuclear layers. Later, an intermediate vascular plexus (IPL) forms at the inner boundary of the inner nuclear layer (Provis, 2001; Saint-Geniez and D'Amore, 2004).

Prior to the development of retinal vessels, retinal astrocytes migrate from the optic nerve and establish a network forming a template for the superficial vasculature (Stone and Dreher, 1987; Watanabe and Raff, 1988; Fruttiger et al., 1996). Retinal ganglion cells (RGCs) produce PDGF-A that interacts with PDGFR-α expressed on astrocytes and hence govern in part the astrocytic template (Fruttiger et al., 1996). Vessel formation follows the astrocytic template and hypoxic conditions drive VEGF expression by astrocytes, thereby stimulating angiogenesis.

C. Angiogenesis in diseases

Hypoxia is the major driving force for angiogenesis in diseases (Shi and Fang, 2004; Rouhi et al., 2010). In tumors, hypoxia is in part caused by inadequate perfusion (Chouaib et al., 2012). VEGF-A is the major target gene activated by transcriptional factors (mainly HIF-1α) as a result of hypoxia and promoting angiogenesis (Lin et al., 2004). In ocular pathologies such as proliferative diabetic retinopathy, hypoxia, caused by capillary occlusion, activates HIF-1α, which in turn promote the expression of angiogenic factors (Arjamaa and Nikinmaa, 2006; Hernández and Simó, 2007).

1. Tumor angiogenesis

Cancer encompasses a group of diseases still lacking effective cures. Conventional chemotherapies and radiotherapies kill essentially actively dividing cells. However, these treatments often have a relatively limited efficacy, because of the presence of cancer stem cells (CSCs) characterized by slow proliferation rates, thereby surviving and repopulating the tumor as soon as the treatment ceases. Along the last few decades, targeted therapies were developed, i.e. therapies specifically interfering with pathways contributing directly to the malignant phenotype and harming less stringently normal cells. Antiangiogenic therapies are part of this category of therapeutic approaches. In his Canon of Medicine, Avicenna already suggested that removing blood vessels going to the tumor could be a good therapeutic strategy to fight cancer. A thousand years later, Algire and his colleagues pointed out the importance of blood vessels in the growth of primary tumor (Algire, 1954). This discovery was confirmed by Judah Folkman's work in the early 1970s (Folkman, 1971). Indeed, Folkman suggested that, in order to survive and grow, tumors require the formation of new blood vessels, and proposed to starve tumors by inhibiting blood supply. Together with the identification of VEGF-A in the late 1980s, these observations led to the development of therapeutic strategies targeting tumor angiogenesis (Leung et al., 1989; Ferrara et al., 2004).

Tumor angiogenesis is a relatively chaotic process that leads to the formation of a morphologically abnormal vasculature. Vessels are often tortuous with irregular branching patterns and lack the normal hierarchical arrangement of artery-arteriole-capillary (Konerding et al., 1999). They also exhibit a poor stability due, in part, to defective pericyte coverage

(Morikawa et al., 2002). These structural changes affect the blood flow, with certain vessels having bidirectional flow and others with no blood flow at all. Functionally, tumor vessels are permeable to large macromolecules. They are inefficient in delivering oxygen to the tumor and removing waste products. This results in the creation of a hypoxic and acidic tumor microenvironment. These abnormalities are due essentially to the permanent dominance of pro-angiogenic factors such as VEGF in the tumor microenvironment, and the absence of maturation of the newly formed vessels.

Abnormalities in the tumor vasculature are believed to contribute to tumor progression. Fragile, permeable vessels can allow tumor cells to enter the circulation and spread to distant sites. Deficient pericyte coverage has been linked to increased metastatic potential in human cancers (Yonenaga et al., 2005). Correcting these abnormalities in mouse models can suppress tumor invasion and metastases (Mazzone et al., 2009). The abnormal vasculature reduces the efficiency of drug delivery to poorly perfused regions of the tumor. Hypoxia itself leads to the resistance to radiotherapies and some chemotherapeutic agents that require the presence of oxygen. Therefore, blocking VEGF signaling can “normalize” the blood flow and improve drug delivery (Jain, 2005). This concept is supported by mouse models in which a single dose of bevacizumab (a monoclonal antibody targeting VEGF) decreased microvessel density, vascular permeability and interstitial pressure and enhanced intratumorally perfusion (Dickson et al., 2007). Combining bevacizumab with chemotherapeutic agents was described to improve the prognostic of patients with advanced colorectal cancer (Hurwitz et al., 2004). This led to two conflicting schools of thought about anti-angiogenic strategies (Carmeliet and Jain, 2011b). On one hand, the destruction of tumor vessels may result in tumor shrinkage due to oxygen starvation, on the other hand, the normalization of the vascular network may limit the metastatic spread and improve the response to conventional anticancer treatments.

2. Retinopathy of prematurity

Ocular diseases caused by pathological neovascularization affect millions of patients in the United States alone. Prematurely born infants may exhibit a proliferative ocular disease, the retinopathy of prematurity (ROP). It was initially described by Terry et al. and called "retrolental fibroplasia" (Patz and Kinsey, 1971; Kinsey et al., 1977). ROP occurs approximately 32 weeks after conception, irrespective of the gestational age at birth (Flynn,

1992). The hyperoxic extrauterine environment disturbs normal vasculogenesis and angiogenesis, and contributes to retinal vaso-obliteration (Kushner et al., 1977). Subsequently, the resulting hypoxia leads to compensatory pathological neovascularization and gliosis, which eventually lead to retinal detachment and vision loss (Chan-Ling et al., 1992).

Over 50% of preterm infants weighing less than 1250 g at birth have signs of ROP and approximately 10% of infants develop severe ROP (National Eye Institute, https://nei.nih.gov/health/rop/rop). ROP develops in 60% of premature infants born between 28 and 31 weeks of gestation and in 80% of infants born before 28th week (Good and Early Treatment for Retinopathy of Prematurity Cooperative Group, 2004). There are about 400-600 cases of total vision loss and 1,100-1,500 cases of severe visual impairment resulting from ROP each year in the USA (National Eye Institute, https://nei.nih.gov/health/rop/rop). However, severe ROP leading to vision loss occurs in only a few percentage of infants and ROP regresses spontaneously in most cases (Ju et al., 2013).

Besides low gestational age and low birth weight, other potential risk factors of ROP include male sex, white race, bronchopulmonary dysplasia, hypoxia, sepsis, acidosis, oxygen therapy and maternal use of beta blocker during pregnancy (Shohat et al., 1983; Ben Sira et al., 1988; Bossi and Koerner, 1995). ROP developing in the most central part of retina is associated with the worst severity, and can progress rapidly within two weeks of disease onset (Morizane, 1976; Quiram and Capone, 2007).

Unlike humans, the retinal vascular development in rodents starts after birth making it easily suitable for developmental studies. Thanks to the immature postnatal retinal vessels at birth, models of oxygen-induced proliferative retinopathy were established in the mid-1990's in rodents. The oxygen-induced retinopathy (OIR) model has been used to investigate the mechanisms of proliferative retinopathy. This model is the most frequently cited in ophthalmology (Smith et al., 1994; Hartnett, 2010) (Figure 12).

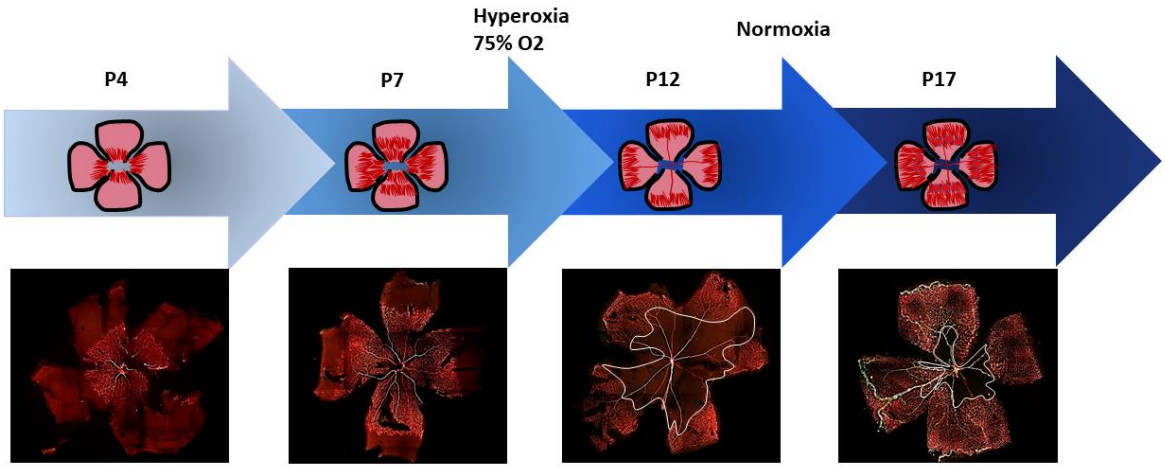

Figure 12. Oxygen-induced retinopathy model.

In mice, exposure to high oxygen concentrations (75%) from postnatal day (P) 7 to 12 was found to halt vessel growth in central retinal and generate an area of vaso-obliteration (VO), reminiscent of the first phase of retinopathy of prematurity (ROP). Upon returning to room air, the relative hypoxia activates HIF-1 and instigates retinal vascular proliferation. Neo-vascularization peaks at P17 (phase II). Shortly thereafter, part of the new vessels spontaneously regress, and the vascular network of the retina turns back to normal by P25. The quantification of VO at P12 and neovessels at P17 provides an accurate representation of phases I and II of proliferative retinopathy in mice. Adapted from Quiram and Capone, 2007; Hartnett, 2010.

D. Lymphatic vascular system

The lymphatic vascular system is a regulator of fluid homeostasis, immune functions, and lipid metabolism. In the mouse, the lymphatic vasculature develops at E9.5, after blood circulation is established. Lymphatic endothelial cells (LEC) progenitors differentiate from blood vascular endothelial cells (BECs) in the embryonic cardinal vein. In the skin, LEC progenitors, which are derived from the hemogenic endothelium, organize into primitive lymphatic clusters that contribute to the formation of the network (Martinez-Corral et al., 2015) (Figure 13).

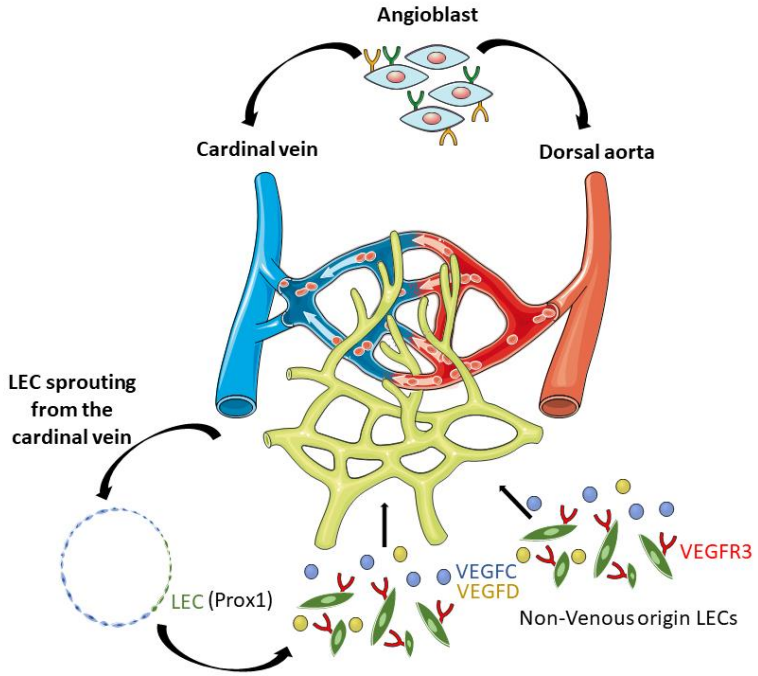

Figure 13. Origin of lymphatic endothelial cells and assembly of the vasculature.

LEC progenitors expressing the transcription factor prospero homeobox protein *1 (Prox1) migrate away from the cardinal vein to form the first primitive lymphatic vessel, the dorsal peripheral longitudinal vessel in the jugular region of the embryo. Several factors are implicated in lymphangiogenesis. Some act directly on LECs such as VEGF-C, VEGF-D and angiopoietins, others, such as FGF and inflammatory cytokines, may act indirectly through the regulation of VEGF-C expression. During embryogenesis and lymphangiogenesis in the adult, VEGF-C is the key regulator of lymphatic endothelial development, acting through its receptor VEGFR-3. Large lymphatics are covered by SMCs and contain valves that prevent backflow. Adapted from Martinez-Corral et al., 2015.*

In adults, lymphatic vessels are quiescent but may begin to sprout following stimulatory signals related to inflammation, malignancies, or trauma (Cursiefen et al., 2006). The coupling of angiogenesis and lymphangiogenesis illustrates a major function of the lymph vascular system, which is to maintain fluid homeostasis by collecting leaking fluid from capillary blood vessels and returning it to the blood circulation. Unlike blood vessels, lymphatic vessels can be blind-ended and display bidirectional flow, transporting fluid, macromolecules, and immune cells from the tissue back to the circulation. Lymphatic capillaries are composed by a thin monolayer wall without supporting pericytes or smooth muscle cells. They are irregular and have a variable lumen diameter. An increase in interstitial pressure stretches the extracellular matrix and opens the lining lymphatic endothelial cells, allowing fluid and macromolecules to enter the vessel lumen. Lymphatic capillaries transport the fluid to larger collecting lymphatic vessels connected to the blood circulation mainly through the junction between the thoracic duct and

the left subclavian vein (Norrmén et al., 2011). As immune cells, such as antigen-presenting cells, are transported in the lymph from tissues to regional lymph nodes, lymph vessels have an important role in immune functions (Yamagami and Dana, 2001; Cursiefen et al., 2006). Lymphatic vessels also play a critical role in cancer by disseminating tumor cells to lymph nodes and other metastatic sites (Cao, 2005).

E. The chemerin system

The heart of my doctoral thesis is to study the role of chemerin in angiogenesis and cancer progression, and therefore I will give in the next section an overview of chemerin and its three receptors.

1. Chemerin

a) Historical overview and structure of chemerin

The gene encoding chemerin was first discovered in 1997 as overexpressed in the skin and psoriatic lesions of patients treated with the retinoid tazarotene and named tazarotene-induced gene 2 (TIG2) or retinoic acid receptor responder 2 (RARRES2). In this initial description, the authors reported TIG2 expression in several tissues by Northern blotting (liver, pancreas, small intestine, ovary and prostate) (Nagpal et al., 1997) suggesting that it may be involved in many physiological and pathophysiological processes. TIG2 expression was also reported to be induced during osteoclast differentiation (Adams et al., 1999). In particular, ST2 stromal cells were reported to express TIG2 when treated with a combination of vitamin D and dexamethasone (Adams et al., 1999). Later, chemerin was reported to be expressed in a larger range of tissues including liver, skin, lung, pituitary, ovary, kidney and white adipose tissue (Wittamer et al., 2003; Bozaoglu et al., 2007). The main sources of chemerin are thought to be liver and white adipose tissue, although platelets have also been reported to release chemerin during activation (Bondue et al., 2011b).

The structure of chemerin is predicted to contain a cystatin-like fold with an amino-terminal α-helical domain followed by an antiparallel β-pleated sheet, and a disordered carboxyl-terminus (Zabel et al., 2006b). The structure is stabilized by three disulphide bonds. Other proteins with

similar overall 3D structures include cystatins, cathelicidin precursors and kininogens (Lehrer and Ganz, 2002; Zabel et al., 2006b).

b) Chemerin bioactivity regulation by proteolytic cleavage

Human chemerin is synthesized as a 163 amino acid pre-pro form (Nagpal et al., 1997) containing a 20 amino acid signal peptide. A pro-chemerin form of 143 amino acids is secreted and its concentration in blood is in the range of 90-200 ng/ml (Rourke et al., 2013). Under resting conditions, the majority of chemerin present in blood is the biologically inactive pro-form (Zhao et al., 2011a). Activation of chemerin requires the proteolytic cleavage of 6 or 7 amino acids from the C-terminus of the pro-form (Wittamer et al., 2003), and a range of proteases were characterized to regulate the chemerin bioactivity (carboxypeptidases, cathepsins, elastase, Factor XIIIa, Factor XIa, chymase, plasmin, and tryptase) (Wittamer et al., 2005; Zabel et al., 2005). Bioactive chemerin is generated in the frame of inflammatory reactions by neutrophil proteases (elastase and cathepsin G), but also as a result of tissue lesions by proteases of the coagulation and fibrinolytic cascades (Wittamer et al., 2005; Guillabert et al., 2008; Kulig et al., 2011). A range of active and inactive isoforms can be generated according to the proteases present (Table 2). Staphopain B, a cysteine protease from *Staphylococcus aureus* can also generate active chemerin from prochemerin, suggesting a potential role of chemerin in host defense mechanisms (Kulig et al., 2007). In accordance with its proteolytic processing, the C-terminal part of chemerin is key in its signaling activity and short peptides derived from the carboxyl terminus retain the ability to activate chemerin receptors (Wittamer et al., 2004). A nonapeptide named chemerin-9 (YFPGQFAFS) displays an EC_{50} of 7 nM for CMKLR1, while full size chemerin has an EC_{50} of 0.2 nM. The generation of variants of this peptide identified the four aromatic residues, the glycine and the terminal carboxyl group as essential elements for its bioactivity (Wittamer et al., 2004).

Chemerin isoforms are usually named according to the number of its last amino acid (Table 3). The precursor is prochemerin^{163}. The most active human isoform on CMKLR1 is chemerin^{157}, while chemerin^{156} is a bit less active (Wittamer et al., 2005; Guillabert et al., 2008). The chemerin^{154}, chemerin^{155} and chemerin^{158} isoforms, which can be found *in vivo*, are inactive

(Wittamer et al., 2004; Guillabert et al., 2008; Du et al., 2009). The first description of chemerin in biological samples (ascites fluid) involved the chemerin^{157} isoform (Wittamer et al., 2003).

Protease	Cleavage substrate	Product	C-terminal sequence	Bioactivity	Reference
Elastase	Chemerin^{163}	Chemerin^{157}	…PGQFAFS	High	(Wittamer et al., 2005)
Cathepsin-G	Chemerin^{163}	Chemerin^{156}	…PGQFAF	High	(Wittamer et al., 2005)
Proteinase-3	Chemerin^{163}	Chemerin^{155}	…PGQFA	Inactive	(Guillabert et al., 2008)
Mast cell chymase	Chemerin^{156} Chemerin^{157}	Chemerin^{154}	…PGQF	Inactive	(Guillabert et al., 2008)
Cathepsins K, L	Chemerin^{163}	Chemerin^{157}	…PGQFAFS	High	(Kulig et al., 2011)
Mast cell tryptase	Chemerin^{163}	Chemerin^{158} Chemerin^{155}	…PGQFAFSK …PGQFA	Inactive Inactive	(Zabel et al., 2005)
Plasmin	Chemerin^{163}	Chemerin^{158}	…PGQFAFSK	Inactive	(Zabel et al., 2005)
Factors XIIa, VIIa	Unconfirmed	Unconfirmed	Unconfirmed	n/a	(Zabel et al., 2005)
uPA, tPA	Unconfirmed	Unconfirmed	Unconfirmed	n/a	(Zabel et al., 2005)
Carboxypeptidases B and N	Chemerin158	Chemerin157	…PGQFAFS		(Zabel et al., 2005)

Table 3. Proteolytic cleavage of human chemerin by different proteases.

The neutrophil proteases cathepsin G and elastase generate respectively the active isoforms chemerin^{156} and chemerin^{157} from pro-chemerin. In contrast, neutrophil proteinase-3 generates the inactive chemerin^{155} isoform from prochemerin. The active chemerin^{157} and chemerin^{156} forms are inactivated by mast cell chymase, which generates the chemerin^{154} isoform. Carboxypeptidases B and N cleave chemerin^{158} (inactive) to chemerin^{157} (the most active isoform). All mouse isoforms are 1 amino acid shorter than the rat/human forms.

c) Regulation of chemerin expression

Chemerin expression may be regulated by several factors including free fatty acids, insulin, steroid hormones, and inflammatory cytokines (TNF-α, IL-1β, Interferon-γ). As mentioned earlier, the combination of dexamethasone and vitamin D was first found to induce expression

of chemerin in human skin (Nagpal et al., 1997). This is potentially due to PPAR-γ binding sites located in the chemerin promoter region (Muruganandan et al., 2011). Retinoic acid (RA) was reported to control chemerin expression during the development of intestinal epithelial cells (Maheshwari et al., 2009). Induction of chemerin expression and secretion by RA was also described in murine endothelial cells. RA treatment increased bioactive chemerin expression and was able to induce the transmigration of dendritic cells across an endothelial cell monolayer (Gonzalvo-Feo et al., 2014).

Chemerin expression seems to be regulated also by metabolic factors. In an *in vitro* system, free fatty acids (FFAs) induced chemerin expression in 3T3-L1 cells during adipocyte differentiation. FFAs increase also the activity of sterol regulatory element-binding protein 2 (SREBP-2), a transcription factor activated during cholesterol shortage and binding to sterol regulatory elements in the promoter of genes involved in cholesterol synthesis (Horton et al., 1998). Cholesterol depletion induced SREBP-2 activation and an increase in chemerin synthesis and knockdown of SREBP-2 in differentiating adipocytes ablated FFA-induced chemerin expression (Bauer et al., 2011). These studies implicate SREBP-2 as an important inducer of chemerin during adipocyte differentiation as well as a possible link between cholesterol levels and chemerin expression.

Chemerin was increased in women suffering from polycystic ovary syndrome (PCOS) (Tan et al., 2009). Interestingly, insulin treatment significantly increased chemerin levels in these patients while metformin (an anti-diabetic drug) significantly decreased its expression (Tan et al., 2009). These data suggest that excess insulin may drive chemerin expression *in vivo*.

Lastly, pro-inflammatory cytokines are also capable of increasing chemerin expression. TNF-α treatment of 3T3-L1 adipocytes induced a significant increase in chemerin production (Parlee et al., 2010). The same study found that TNF-α treatment increased chemerin levels in the plasma of wild type mice and this effect was abrogated in animals lacking the TNF receptor (Parlee et al., 2010). Interestingly, TNF-α did not seem to increase chemerin expression in the liver, suggesting that during inflammation, the majority of chemerin may be produced by the white adipose tissue (Parlee et al., 2010). Banas et al. reported similar findings in the skin, where IL-1β increased chemerin expression while cytokines associated with psoriasis, like IL-17 and IL-22, decreased chemerin expression (Banas et al., 2015). In a similar set of

experiments using adipocytes, IL-1β increased chemerin expression by ~1.3 fold. However, these studies were not confirmed by *in vivo* experiments (Kralisch et al., 2009). Interferon-γ and TNF-α both induced chemerin expression in fibroblast-like synoviocytes from rheumatoid arthritis patients (Kaneko et al., 2011). It should be noted however, that Gonzalvo-Feo et al. observed no increase in chemerin expression in lymphatic or vascular murine endothelial cells in response to TNF-α, interferon-g, IL-1β or LPS stimulation (Gonzalvo-Feo et al., 2014). Surprisingly, another study using human tubular epithelial cells, reported that TNF-α and interferon-γ treatment decreased overall chemerin production (De Palma et al., 2011).

It seems therefore that chemerin expression can be regulated by a large variety of factors *in vivo* or *in vitro*, but the effects and mechanisms vary according to the cell type, and the amplitude of the effects is often limited. In addition, while transcriptional regulation of chemerin may be important in some contexts, proteolytic regulation of chemerin bioactivity likely plays a much more important role.

2. Chemerin receptors

a) CMKLR1

The first chemerin receptor to be identified was ChemR23/CMKLR1. It was first cloned as an orphan G protein-coupled receptor (Gantz et al., 1996) and found to share homology with receptors of chemoattractant factors, such as C5a, C3a and formyl-peptides (Joost and Methner, 2002). Its expression was initially reported on monocyte-derived dendritic cells and macrophages (Samson et al., 1998). Chemerin has been shown to be the natural ligand of ChemR23/CMKLR1 in 2003 (Meder et al., 2003; Wittamer et al., 2003). Wittamer et al used a recombinant cell line expressing human CMKLR1 as a screening assay and identified a specific bioactivity in fractions of inflammatory fluids such as ascites associated with ovarian tumors, synovial fluid from arthritic joints as well as in spleen extracts. The active product was purified by HPLC and identified as the product of the TIG2/RARRES2 gene by mass spectrometry (Wittamer et al., 2003). This ligand of the ChemR23 receptor was therefore named chemerin. Meder et al. took a similar approach, and identified chemerin as the active component in human hemofiltrates after HPLC fractionation and mass spectrometry (Meder et al., 2003).

CMKLR1 was later shown to be expressed by a larger range of cell types, including myeloid and plasmacytoid DCs, NK cells, macrophages, microglial cells, adipocytes, chondrocytes, endothelial cells and some epithelial cells (Bondue et al., 2011b; Rourke et al., 2013). Its expression in mouse dendritic cells is decreased by inflammatory stimuli such as LPS, interferon-γ and other TLR ligands but increased by TGF-β (Zabel et al., 2006b; Hart and Greaves, 2010). CMKLR1 mediates the chemotactic effects of chemerin. The activation of CMKLR1 triggers various signaling cascades shared by most G_i-coupled GPCRs. These include the activation of phospholipase C and the release of intracellular calcium, the inhibition of adenylate cyclase and of cAMP production, the phosphorylation of ERK1/2 and p38 MAPkinases, and the activation of phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Zabel et al., 2006a; Hart and Greaves, 2010; Bondue et al., 2011b). Rourke et al. showed that CMKLR1 activates also the transcription factor serum related factor (SRF) in a RhoA- and rho-associated protein kinase (ROCK)-dependent manner (Rourke et al., 2015).

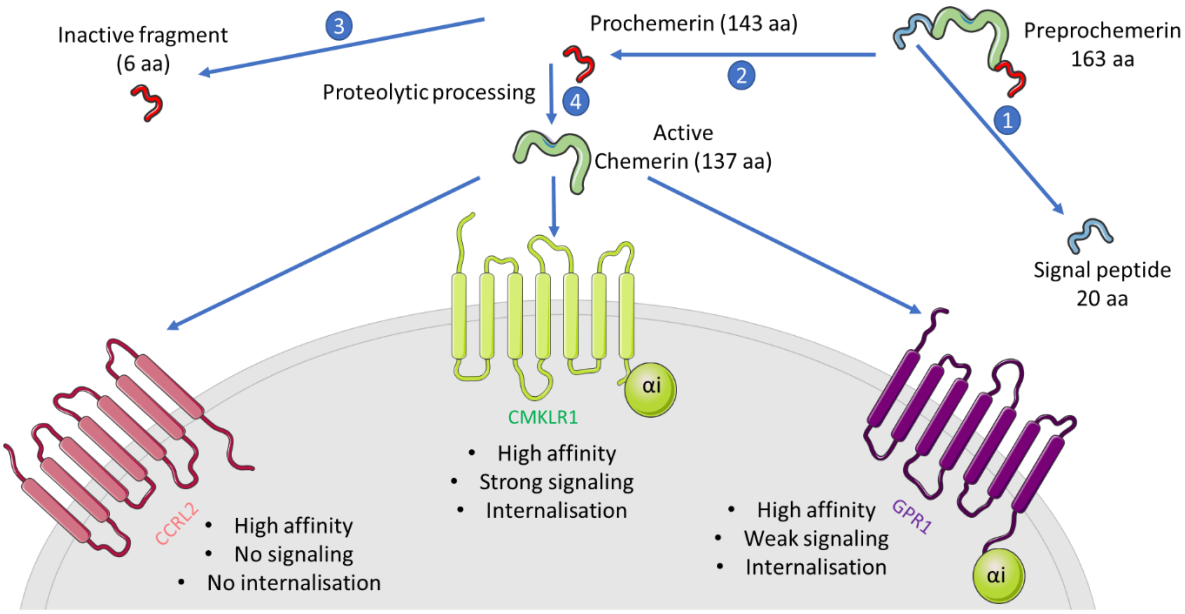

Figure 14. Schematic representation of the biological role of chemerin and chemerin receptors.

CMKLR1 activation induced MAP kinase and ERK 1/2 signaling which converged with the RhoA and ROCK pathway at the activation of SRF. Adapted from Bondue et al., 2011b.

Herova et al. reported expression of CMKLR1 by human monocytes and at higher level by macrophages. CMKLR1 expression and functional response to chemerin was also influenced by the polarization of macrophages. In contrast to the previous murine studies, pro-

inflammatory signals like interferon-g and LPS promoted M1 polarization and increased expression of CMKLR1. Macrophages polarized toward M2 by the Th2 cytokines IL-4 and IL-13 downregulated CMKLR1 expression and did not respond to chemerin. TGFβ had no effect on CMKLR1 expression (Zabel et al., 2006a; Hart and Greaves, 2010; Herová et al., 2015). These observations suggest possible differences in the regulation of CMKLR1 between mice and humans (Herová et al., 2015).

CMKLR1 was reported to constitute a receptor for the pro-resolving eicosanoid resolvin E1 (Arita et al., 2005) but these data were not confirmed by other groups (Bondue et al., 2011b).

b) GPR1

GPR1 is G protein-coupled receptor that shares high sequence similarity with CMKLR1 (Bondue et al., 2011b). It was first recognized as a receptor for chemerin by Barnea et al. through the development of a screening assay to identify novel ligands for orphan G protein-coupled receptors (Barnea et al., 2008). They found that both full-length chemerin and the chemerin-9 peptide could activate GPR1 in a β-arrestin recruitment assay, with EC_{50} values of 240 pM and 1 nM respectively. The authors also demonstrated the ability of chemerin to induce calcium mobilization in GPR1-transfected cells, although to a much lesser degree than for CMKLR1.

GPR1 is expressed mainly in the central nervous system, but also in white adipose tissue, skin, and muscle (Bondue et al., 2011b; Rourke et al., 2014; Banas et al., 2015). The physiological role of this receptor remains unclear, however animals lacking GPR1 have been reported to exhibit glucose intolerance and decreased insulin secretion in response to glucose, implicating GPR1 in glucose homeostasis (Rourke et al., 2014). GPR1 may also play a role in host defense, as GPR1 expression in skin is increased in response to IL-1β and bacteria such as Staphylococcus aureus and E.coli, although the importance of this receptor in this context requires further investigation (Banas et al., 2015).

GPR1 signaling was confirmed to be rather weak through the classical pathways dependent on G proteins, while β-arrestin recruitment and receptor internalization are as efficient as for CMKLR1 (De Henau et al., 2016). Rourke et al. described the activation of a

RhoA/ROCK/serum response factor (SRF) pathway by GPR1 with some differences between the murine and human receptors. For example, both human and mouse GPR1 activated the pathway, but this was dependent on ERK1/2 and P38 for the mouse but not the human receptor (Rourke et al., 2015). It should be noted that no signaling studies on GPR1 were performed so far using primary cells, and the overexpression of GPR1 in recombinant systems might overestimate the signaling properties of the receptor. It remains therefore possible that GPR1 would act purely as a decoy receptor.

c) CCRL2

C-C Motif Chemokine Receptor Like (CCRL2 or L-CCR) is upregulated in murine macrophages after LPS stimulation (Shimada et al., 1998). Chemerin does not trigger signaling following binding to CCRL2 and does not promote receptor internalization, in contrast to chemokine decoy receptors such as ACKR2 (Figure 14). Animals lacking CCRL2 displayed decreased dermal inflammation in a model of skin inflammation induced by 2,4-dinitrophenol. The exact mechanism by which CCRL2 modulates these responses was not clear. However, cells expressing CCRL2 were shown to bind chemerin and could induce calcium flux in cells expressing CMKLR1 (Zabel et al., 2008).

CCRL2 was subsequently reported to play a role in the ovalbumin (OVA)-induced model of asthma. CCRL2 KO mice challenged with OVA displayed reduced numbers of leukocytes (particularly eosinophils and lymphocytes) as well as lower levels of Th2 cytokines in the bronchioalveolar lavage (BAL) fluid (Otero et al., 2010). Antigen-loaded DCs displayed impaired trafficking to draining lymph nodes in response to an OVA challenge in these mice, and levels of Th2 cytokines such as IL-5 and IL-13 were reduced in lymph nodes from CCRL2 KO mice challenged *ex vivo* with OVA. However, the trafficking of DCs to the lungs remained unaffected (Otero et al., 2010).

In addition, animals lacking CCLR2 were reported to display a reduction of CMKLR1-expressing NK cell recruitment to the lungs following a challenge with LPS (Monnier et al., 2012). CCRL2 KO animals displayed higher chemerin levels in the blood both in naive conditions and after an LPS challenge. CCRL2 expression was increased in the liver after LPS treatment but not in the lungs, in which the expression was constitutively high. The authors

postulated that CCRL2 functions acted by concentrating chemerin locally and presenting it to CMKLR1 expressing cells in line with their previous report (Zabel et al., 2008). CCRL2 may also be important in other pathologies such as ischemic brain injury. Douglas et al. observed a decrease in cell death and smaller infarct volumes in mice lacking CCRL2 as compared to wild-type animals (Douglas et al., 2013). CCRL2 was rapidly induced in brain slices from wild type animals after ischemia but the mechanism by which CCRL2 aggravates the phenotype is unclear (Douglas et al., 2013).

Increased CCRL2 expression was reported in glioblastoma and liver carcinoma. Expression of CCRL2 in glioma cell lines promoted cell migration in a transwell invasion assay, although the precise mechanism was not identified (Yin et al., 2012).

3. The role of chemerin in inflammatory diseases

Chemerin has long been implicated in a range of inflammatory diseases. For example, chemerin was reported to play a role in the recruitment of pDCs and NK cells to psoriatic lesions (Vermi et al., 2005; Skrzeczyńska-Moncznik et al., 2009). Chemerin levels were also found increased in the blood of patients with psoriasis compared to healthy controls (Nakajima et al., 2010) and the treatment of psoriasis patients with the anti-TNF-α drug Inflixamab reduced the levels of chemerin in parallel to CRP and resistin (Gisondi et al., 2013).

Chemerin was also implicated in inflammatory bowel disease (IBD), although the literature is contradictory. Higher chemerin blood levels in patients suffering from IBD (ulcerative colitis and Crohn's disease) were reported in a large cohort of 230 patients while no difference with the control group was found in another, also only 24 patients were analyzed (Weigert et al., 2010b; Waluga et al., 2014). In this latter study, corticosteroid treatment had no effect on serum chemerin level while effectively decreasing resistin and visfatin (Waluga et al., 2014). Murine models of IBD were also conflicting. Increased chemerin expression was reported in the colon of animals in a model of dextran sodium sulphate (DSS)-induced colitis as well as in colon biopsies from patients suffering from ulcerative colitis (Lin et al., 2014). Animals receiving chemerin during DSS challenge displayed an increased histological score for colon inflammation as well as increased inflammatory cytokine production from cultured tissue explants. This was attributed to a skewing of intestinal macrophages toward an M1

inflammatory phenotype, as chemerin-treated animals displayed decreased levels of M2 macrophage markers such as Arginase-1, FIZZ-1 and IL-10. Neutralizing endogenous chemerin with a polyclonal antibody significantly decreased the disease severity (Lin et al., 2014).

In a subsequent study, Dranse et al. reported a similar increase in chemerin expression in the colon of animals in the DSS-induced colitis model. Total chemerin levels were increased in the serum while the ratio of bioactive chemerin to total chemerin was increased. However, systemic chemerin levels did not correlate with disease severity, leading the authors to suggest that local chemerin levels rather than systemic levels reflect more accurately the disease severity (Dranse et al., 2015). In contrast to the study by Lin et al., no difference in disease severity was observed under chemerin treatment using the same dose, route of administration and genetic background (Dranse et al., 2015). Further studies will therefore be required to address this discrepancy.

Several studies have examined the role of chemerin in the pathology of rheumatoid arthritis (RA) and osteoarthritis. A first study demonstrated that human chondrocytes express both chemerin and its receptor CMKLR1. Treatment of chondrocytes from RA patients with recombinant human chemerin[157] activated the PI3K-Akt and ERK1/2 pathways and increased the expression of various inflammatory mediators including IL-6, IL-8, TNF-α, MMP-9 and IL-1α (Berg et al., 2010). Similar observations were made in a subsequent study using fibroblast-like synoviocytes (FLS) from rheumatoid arthritis patients. Both chemerin and CMKLR1 were expressed by these cells and chemerin promoted Akt and ERK1/2 phosphorylation and expression of IL-6 and CCL2 (Kaneko et al., 2011). Eisinger et al. confirmed chemerin and CMKLR1 expression by synoviocytes, and an increase in CCL2 expression under chemerin treatment, but no increase in IL-6 or MMP-9 or Akt phosphorylation (Eisinger et al., 2012).

Increased chemerin levels were reported in the plasma of patients with rheumatoid arthritis (Ha et al., 2014), and anti-TNF-α therapy decreased these levels in correlation with clinical score and serum IL-6 (Herenius et al., 2013). Treatment of mice with TNF-α was previously shown to increase serum chemerin levels (Parlee et al., 2010) and treatment of RA patients with a humanized anti-IL-6 monoclonal antibody significantly decreased serum chemerin levels (Makrilakis et al., 2015). These data suggest a mechanism by which both TNF-α and IL-6

promote the expression of chemerin *in vivo*, and a potential contribution of chemerin to the disease phenotype.

In experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis in human (MS), a pro-inflammatory role of chemerin was also suggested. Expression of chemerin in the CNS of animals experiencing EAE was higher than in naive controls, and DCs and macrophages infiltrating the central nervous system were shown to express CMKLR1. Mice lacking CMKLR1 also displayed significantly reduced clinical signs of the disease (Graham et al., 2009). The same authors identified in a subsequent study a small molecule antagonist of CMKLR1, called α-NETA. In the EAE model, this compound delayed the disease onset and decreased its severity as assessed by histological and clinical scores. Antagonist treatment also decreased the recruitment of T cells, B cells, DCs and macrophages to the CNS, as well as of NK cells to the spleen (Graham et al., 2014). These data suggest a potential role of the chemerin/CMKLR1 axis in MS as well, although the pathology in human is more complex and there is so far no direct association between chemerin and MS in human patients.

Taken together, these data suggest that chemerin is a biomarker of inflammatory state, and possibly a driver of inflammation, in the context of rheumatoid arthritis, psoriasis, lupus nephritis and potentially MS. Its expression is consistently increased in the context of inflammatory diseases and, in some cases, decreased by anti-inflammatory therapies. It remains to be conclusively proven however, whether chemerin is directly driving inflammation in these pathologies or whether its expression is increased as a byproduct of inflammation. Potent CMKLR1 agonists and antagonists would be of interest for clarifying this.

4. Anti-inflammatory effects of chemerin

Besides the likely pro-inflammatory role of chemerin in certain pathologies and disease models, there is also a body of evidence suggesting that chemerin can exert anti-inflammatory effects. Luangsay et al. investigated the role of chemerin in a model of acute lung inflammation induced by intratracheal instillation of bacterial lipopolysaccharide (LPS). LPS treatment resulted in a marked increase of neutrophils and inflammatory mediators such as CXCL1, IL-6, TNF-α and IL-1β, in the lungs and the bronchoalveolar lavage (BAL) fluid. Co-administration of chemerin dramatically reduced the neutrophil counts and cytokine concentrations. These anti-

inflammatory effects were dependent on CMKLR1 as chemerin treatment of animals lacking this receptor no longer exerted any anti-inflammatory effects. Furthermore, CMKLR1 KO animals also displayed increased neutrophil recruitment to the lungs after challenge with LPS alone, as compared to wild-type animals, indicating a possible role for endogenous chemerin in the regulation of inflammatory responses in the lung (Luangsay et al., 2009).

In a model of viral pneumonia caused by the pneumonia virus of mice (PVM), the mouse counterpart of the human respiratory syncytial virus (RSV), CMKLR1 KO animals displayed increased neutrophil recruitment to the lungs, an increased expression of inflammatory mediators and a much more severe clinical picture (Bondue et al., 2011a). These animals also exhibited impaired pDC recruitment, decreased type 1 interferon production and delayed viral clearance. Adoptive transfer experiments established that the anti-inflammatory role of the chemerin/CMKLR1 system in this model was mediated by non-leukocyte populations (Bondue et al., 2011a). As a possible explanation for this, CMKLR1 was reported to be expressed by human endothelial cells and chemerin to exert anti-inflammatory effects on these cells (Yamawaki et al., 2012), suggesting that endothelial cells might be involved in the chemerin anti-inflammatory effects in these lung models (Yamawaki et al., 2012). However, these observations were not reproduced by Neves et al. who reported that chemerin could exert pro-inflammatory effects directly on human microvascular endothelial cells, increasing expression of CCL2, TNF-α and VCAM-1 as well as adhesion of THP-1 monocytes (Neves et al., 2015).

Chemerin was also reported to play an anti-inflammatory role in the context of allergic airway inflammation. In the OVA model of asthma in mice, intranasal chemerin administration reduced the recruitment of $CD4^+$ T cells, eosinophils and inflammatory DCs to the lungs following the OVA challenge (Zhao et al., 2014). Chemerin also decreased the production of Th2 cytokines in the lung, including IL-4 and IL-13, as well as of the T cell chemoattractants CCL17 and CCL22. The mechanism by which chemerin decreases inflammatory DC recruitment was proposed to involve a reduction of CCL2 production by activated lung epithelial cells, highlighting a novel mechanism by which chemerin may exert its anti-inflammatory actions in this tissue (Zhao et al., 2014). A further confirmation of the anti-inflammatory actions of chemerin in the context of allergic airway inflammation was reported by Doyle et al. who developed lipidated analogues of the chemerin-9 nonapeptide in order to overcome the short half-lives of this peptide *in vivo* (Wittamer et al., 2004; Shimamura et al., 2009). This lipidated

peptide displayed anti-inflammatory effects in the OVA model in mice (Doyle et al., 2014; Zhao et al., 2014). Altogether, these reports suggest the potential interest of CMKLR1 agonists mimicking the effects of chemerin in the treatment of allergic asthma and other lung inflammatory states.

5. The function of chemerin as an adipokine

Chemerin was proposed to have important functions as an adipokine. A first report by Goralski et al. described high chemerin expression in white adipose tissue. Maturing adipocytes were reported to express both chemerin and CMKLR1. The chemerin that was secreted was demonstrated to be active by the ability of supernatants of differentiating adipocytes to induce migration of CMKLR1-expressing cells. Chemerin acted in an autocrine/paracrine manner during adipocyte differentiation, and knockdown of chemerin by small hairpin RNAs in 3T3-L1 cells impaired the differentiation process (Goralski et al., 2007). This work was closely followed by several other papers reporting similar findings. Bozaoglu et al. reported that chemerin was increased in the adipose tissue of P. Obesus mice, which are obese and develop type 2 diabetes. In the same study, plasma chemerin levels were measured in type 2 diabetes and control patients. There was no significant difference between the two groups, but a correlation between chemerin, body mass index (BMI) and other parameters of the metabolic syndrome was found (Bozaoglu et al., 2007). Roh et al. also reported chemerin and CMKLR1 expression in differentiating adipocytes and confirmed the importance of chemerin signalling during this process (Roh et al., 2007).

Chemerin was also suggested to affect insulin sensitivity. Takahashi et al. reported that chemerin increases insulin signaling and insulin-stimulated glucose uptake by adipocytes, suggesting a possible role of chemerin in obesity and diabetes (Takahashi et al., 2008). A subsequent study reported that chemerin rather impairs insulin signaling in skeletal smooth muscle cells, suggesting cell type-specific metabolic effects (Sell et al., 2009). Glucose intolerance was later shown in chemerin-deficient mice, and this was attributed to a decrease in glucose-dependent insulin secretion and increased glucose production by the liver. In contrast, chemerin-overexpressing mice displayed higher insulin secretion, increased insulin sensitivity and improved glucose uptake, compared to wild type animals (Takahashi et al., 2011). These effects of chemerin are mediated in part by pancreatic β-cells, which were demonstrated to

express chemerin, CMKLR1 and GPR1. Chemerin was also shown to promote insulin secretion from β-cells *in vitro* (Takahashi et al., 2011). Extending these findings, Wargent et al. reported that animals lacking CMKLR1 display impaired glucose tolerance and that chemerin promotes glucose uptake by adipocytes in the presence of insulin in a CMKLR1-dependent manner (Cash et al., 2008; Wargent et al., 2015). Rourke et al. [232][232][232][232][232][232][1] reported that animals lacking GPR1 also display glucose intolerance and decreased insulin sensitivity, suggesting that both CMKLR1 and GPR1 may be important for the effects of chemerin on glucose homeostasis. In contrast, Rouger et al. reported that animals lacking CMKLR1 display no differences in glucose tolerance or insulin sensitivity (Rouger et al., 2013). Hence, the exact role of chemerin and its receptors on glucose homeostasis remain unclear, with some studies proposing chemerin driving metabolic dysfunction while others supporting a protective function by increasing insulin sensitivity and glucose uptake.

Numerous human studies have reported increased chemerin levels in patients with metabolic and cardiovascular diseases, although the functional role of chemerin in these contexts remains unclear (Bozaoglu et al., 2009; Sell et al., 2009; Weigert et al., 2010a; Landgraf et al., 2012; Ji et al., 2014).

6. Chemerin effects on the vascular system

The positive effect of chemerin on angiogenesis has been described in several studies mainly *ex vivo* on HUVEC-based models, but also *in vivo* (Bozaoglu et al., 2010; Kaur et al., 2010; Nakamura et al., 2018). One of these studies reported a positive but moderate effect of chemerin on the formation of tubes in a coculture of ECs and fibroblasts (Bozaoglu et al., 2010). Curiously, these effects were only observed for chemerin concentrations well below the EC_{50} for CMKLR1, while the concentration near the EC_{50} (10 ng/ml, around 0.5 nM) was ineffective. Another report described positive effects of chemerin on the proliferation of HUVECs and the formation of tubes in Matrigel, although the active concentrations in these two assays were quite discordant (Kaur et al., 2010). A later report described positive effects of chemerin *in vivo*, in Matrigel plugs and the mouse corneal angiogenesis assay, as well as in the rat aortic ring assay, while confirming the previously reported activities on EC migration, proliferation and tube formation *in vitro* (Nakamura et al., 2018).

Full-length chemerin and the chemerin-9 peptide were shown to cause the contraction of isolated rat and human arteries and to potentiate the effect of established vasoconstrictors such as endothelin-1 (Lobato et al., 2012; Watts et al., 2013; Kostopoulos et al., 2014; Neves et al., 2015; Darios et al., 2016; Kennedy et al., 2016; Ferland et al., 2017; Hanthazi et al., 2019). Importantly, a healthy endothelium clearly influences the physiological effects of chemerin on vessels. The chemerin-induced contraction was significantly increased when the endothelium is removed (Watts et al., 2013; Kennedy et al., 2016). In endothelial cells, chemerin increased the production of reactive oxygen species and decreased that of nitric oxide (Neves et al., 2014, 2015; Hanthazi et al., 2019). Nitric oxide synthase inhibitors increased the magnitude of contraction caused by chemerin-9, (Watts et al., 2013). These data suggest that chemerin acts on endothelial cell receptors thereby limiting the direct contractile effect. It is important to understand how chemerin affects the physiology of endothelial cell as dysfunction of endothelial cells is a hallmark of cardiovascular diseases.

7. Chemerin in cancer

In cancer, chemerin's functions depend on the context. Chemerin is upregulated in some cancer types, such as neuroblastoma, glioblastoma, squamous cell carcinoma of the esophagus, mesothelioma, and squamous cell carcinoma of the oral tongue (SCCOT) (Mohr et al., 2004; Yamaguchi et al., 2011; Wang et al., 2014; Farsam et al., 2016; Kumar et al., 2016; Zhang et al., 2017). Chemerin is downregulated in most cancer types, such as acute myeloid leukemia (AML), adrenocortical carcinoma (ACC), breast cancer, Ewing sarcoma, hepatocellular carcinoma, melanoma, non-small cell lung cancer (NSCLC), prostate cancer, and squamous cell carcinoma of the skin (Lin et al., 2011; Pachynski et al., 2012; Alholle et al., 2013; Farsam et al., 2016; Liu-Chittenden et al., 2017). Both tumor suppression and tumor promoting effects have been reported in the literature.

In a murine graft model using B16 melanoma cells, Pachynski et al. reported a delay in the growth of tumors expressing bioactive chemerin. Exogenous chemerin treatment also decreased the tumor size. The infiltration by DCs, T cells and NK cells was increased in chemerin-expressing tumors and the anti-tumoral effect was attributed to the recruitment of NK cells. Indeed, RAG KO animals, which lack T and B cells, displayed the same responses to chemerin

treatment as wild type animals while chemerin had no effect on tumor growth when NK cells were depleted (Pachynski et al., 2012).

II. Aims and Objectives

Previous results of our research group have shown that CMKLR1 KO mice develop spontaneous skin tumors. In a chemical carcinogenesis model (DMBA/TPA), chemerin-overexpressing mice developed fewer papillomas and both the growth of papillomas and their evolution to carcinomas was considerably slower (Dubois-Vedrenne et al., 2019). This protecting effect was mediated by CMKLR1. More recently, our group confirmed that expression of chemerin in tumor cell lines (Lewis lung carcinoma, LLC, and B16 melanoma) resulted in a delay in tumor growth when grafted to syngenic C57Bl6 mice (Dubois-Vedrenne et al., 2021). Tumor growth was also impaired in mice overexpressing chemerin in keratinocytes, showing that the growth delay is independent from the site of expression (tumor cells or host). In all these models, the effect of chemerin was dependent on the presence of CMKLR1. In contrast to the previous report by Pachynski (Pachynski et al., 2012), the recruitment of leukocytes at various stages of tumor development was unaffected by chemerin expression, and a similar growth delay was observed in NSG (NOD-scid IL2rγnull) mice, showing that immune defenses are not involved in the protective effect. Rather, the number of blood vessels was smaller in these tumors and an angiogenesis signature was decreased in RNAseq analyses. Chemerin seems therefore to inhibit angiogenesis in this tumoral context, in turn creating hypoxia and promoting apoptosis and necrosis of tumoral cells (Dubois-Vedrenne et al., 2021).

We believe that our protein of interest, chemerin can act as a negative regulator of angiogenesis. My PhD subscribes in the continuity of this work and proposes further results supporting this hypothesis.

Considering these statements, the main objectives of this work were:

(i) The characterization of the mouse chemerin/CMKLR1 system in other pathological situations and physiological context of angiogenesis.

(ii) The investigation of the role of CCRL2 in angiogenesis.

(iii) The characterization of the mouse chemerin/CMKLR1 system in lymphangiogenesis.

III. Results

A. Characterization of the mouse chemerin/CMKLR1 system in angiogenesis

1. Objectives of Article 1

a) General context

Angiogenesis is the formation of new blood vessels from preexisting ones, as a response of endothelial cells (EC) to a combination of pro- and anti-angiogenic factors (Carmeliet and Jain, 2000; Geudens and Gerhardt, 2011; Betz et al., 2016). Vessels sprout, expand, and branch from a primitive vascular plexus. Vessel branches in excess are then selectively removed through a process called vessel regression (Coultas et al., 2005; Potente et al., 2011). Recent decades have seen the development of anti-angiogenic agents, as one type of targeted therapies in the frame of cancer. The efficiency of these agents, targeting essentially the VEGF axis, is however relatively limited. The work described in the present thesis intends to expand the range of factors involved in the regulation of the angiogenesis process. Besides its role as a leukocyte chemoattractant factor and adipokine, our protein of interest, chemerin, is shown to regulate angiogenesis in physiological and pathological contexts.

b) Chemerin Background

Chemerin is a small secreted chemotactic protein for monocytes/macrophages, myeloid and plasmacytoid dendritic cells, and NK cells (Bondue et al., 2011b). Chemerin binds to three G protein-coupled receptors (GPCR): CMKLR1, GPR1, and CCRL2 (Kennedy and Davenport, 2018). Several studies investigated the role of chemerin in various cancer types (Zheng et al., 2008; Goralski et al., 2019). In non-small-cell lung carcinoma (NSCLC) (Zhao et al., 2011b), squamous cell carcinoma of the skin (Zheng et al., 2008), and melanoma (Pachynski et al., 2012), chemerin was shown to be down regulated. In line with this observation, tumor growth was delayed when chemerin is expressed by cancer cells in different mouse models (Zhao et al., 2011; Treeck et al., 2019). Our host laboratory recently proposed a reduction of tumoral angiogenesis as a mechanism responsible for the anti-tumoral properties of chemerin (Zhao et al., 2011b; Treeck et al., 2019; Dubois-Vedrenne et al., 2021).

c) Working hypothesis

Our group proposed that, in mouse cancer graft models, the anti-tumoral activity of chemerin does not involve the recruitment of leukocyte populations but is mediated by the inhibition of the vascularization of the tumors, an effect mediated through CMKLR1 (Dubois-Vedrenne et al., 2021).

Therefore, in the first part of my thesis, I was interested in investigating the consequence of chemerin overexpression and invalidation of its main receptor CMKLR1 in physiological angiogenesis and in other pathological contexts involving neoangiogenesis or vascular remodeling.

We used the post-natal development of the vascular network in the mouse retina mouse as our main model of physiological angiogenesis. A mouse line, in which a bioactive form of chemerin is overexpressed by skin keratinocytes, under the control of the keratin 5 gene promoter (K5-Chem model) was used for this purpose. Next, we implemented a model of oxygen-induced retinopathy (mimicking the retinopathy of prematurity in humans), the hind limb ischemia model (involving the response to acute artery obliteration) and tumor graft models, in order to validate our results in different contexts. Finally, we initiated the identification of the molecular mechanisms and pathways mediating the anti-angiogenic effects of chemerin.

2. Article 1: Chemerin regulates normal angiogenesis and hypoxia-driven neovascularization

(Ben Dhaou et al., Angiogenesis, 2021)

Angiogenesis
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ORIGINAL PAPER

Chemerin regulates normal angiogenesis and hypoxia-driven neovascularization

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Abstract
Chemerin is a multifunctional protein initially characterized in our laboratory as a chemoattractant factor for leukocyte populations. Its main functional receptor is CMKLR1. We identified previously chemerin as an anti-tumoral factor inhibiting the vascularization of tumor grafts. We show here that overexpression of bioactive chemerin in mice results in a reduction of the density of the retinal vascular network during its development and in adults. Chemerin did not affect vascular sprouting during the post-natal development of the network, but rather promoted endothelial cell apoptosis and vessel pruning. This phenotype was reversed to normal in CMKLR1-deficient mice, demonstrating the role of this receptor. Chemerin inhibited also neoangiogenesis in a model of pathological proliferative retinopathy, and in response to hind-limb ischemia. Mechanistically, PTEN and FOXO1 antagonists could almost completely restore the density of the retinal vasculature, suggesting the involvement of the PI3-kinase/AKT pathway in the chemerin-induced vessel regression process.

Keywords CMKLR1 · ChemR23 · Retinal angiogenesis · Tumoral neoangiogenesis · Hind-limb ischemia model · Oxygen-induced retinopathy

Introduction

Angiogenesis is an essential and complex process, by which new blood vessels grow from preexisting ones, as a tightly regulated response of endothelial cells (EC) to a combination of pro- and anti-angiogenic factors [1–3]. The establishment of a functional blood vessel networks is initiated by the formation of a primitive vascular plexus, from which new blood vessels sprout, coordinately expand, and branch. Redundant vessel branches are then selectively removed through vessel regression, a process called pruning [4, 5]. This process is necessary for establishing the hierarchic vessel patterning during angiogenesis, and the removal of redundant or damaged vascular branches [6]. Mechanistically, vessel regression is regulated by hemodynamics and numerous signaling pathways [6].

Chemerin is a secreted protein of 16 kDa [7] initially characterized in our laboratory as a chemoattractant factor for immature myeloid and plasmacytoid dendritic cells (DC), macrophages, and natural killer (NK) cells [8]. It was later described as an adipokine regulating lipogenesis and lipid metabolism [9]. Chemerin is produced by many cell types, including fibroblasts, adipocytes, hepatocytes, and various epithelial cells as an inactive precursor, prochemerin. Prochemerin is processed into bioactive chemerin by the proteolytic removal of the last 6 or 7 C-terminal residues of the precursor [10]. While the main functional receptor of chemerin is CMKLR1 (also called ChemR23 or chemerin$_1$), GPR1 (chemerin$_2$), and CCRL2 were also described as chemerin receptors, with respectively limited and no signaling properties [11, 12]. CMKLR1 is coupled

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to the $G_{i/o}$ family of G proteins and to β-arrestin recruitment, and leads to inhibition of adenylate cyclase, release of intracellular Ca^{2+} and phosphorylation of ERK1/2 [11, 13, 14]. CMKLR1 is expressed by leukocyte populations (macrophages, DCs, NK cells) but also by endothelial cells, adipocytes, and smooth muscle cells [15–17].

We and others have identified chemerin as an anti-tumoral factor in different mouse models [18–20]. We recently proposed that, in mouse cancer graft models, the anti-tumoral activity of chemerin does not involve the recruitment of leukocyte populations but is mediated by the inhibition of the vascularization of the tumors, an effect mediated through CMKLR1 [21].

Hypoxia is the major trigger of tumor neovascularization. Hypoxic conditions promote the stabilization of the hypoxia-inducible transcription factor (HIF), which results in the upregulation of many genes, including those encoding vascular endothelial growth factors (VEGF) and their receptors. These factors stimulate the release of proteolytic enzymes by endothelial cells, as well as their proliferation and migration, leading to vascular sprouting [22]. Besides the essential role of angiogenesis during development, pathologic conditions in adults may promote neovascularization as a result of an acute or chronic hypoxic environment in tissues [23]. This hypoxia-triggered neovascularization response may be insufficient, such as in ischemic diseases, or rather excessive in proliferative retinopathies, such as the retinopathy of prematurity (ROP), in which aberrant neoangiogenesis is a common cause of vision loss and blindness [23–26].

Following the identification of chemerin as a negative regulator of angiogenesis in tumors, we investigated the consequence of chemerin overexpression and invalidation of its receptors in other physiological or pathological contexts involving neoangiogenesis or vascular remodeling. The development of the vascular retinal network in mice is a very robust model allowing to follow the different steps of the angiogenesis process. We observed in this system that chemerin favors the pruning of blood vessels once formed, as well as the apoptosis of endothelial cells. This results in a reduction of the density of the retinal vascular network, which persists until adulthood. Similar anti-angiogenic properties were also observed in a model of oxygen-induced retinopathy, mimicking the retinopathy of prematurity in human, and in the hind-limb ischemia model that investigates the vascular remodeling response to acute ischemia.

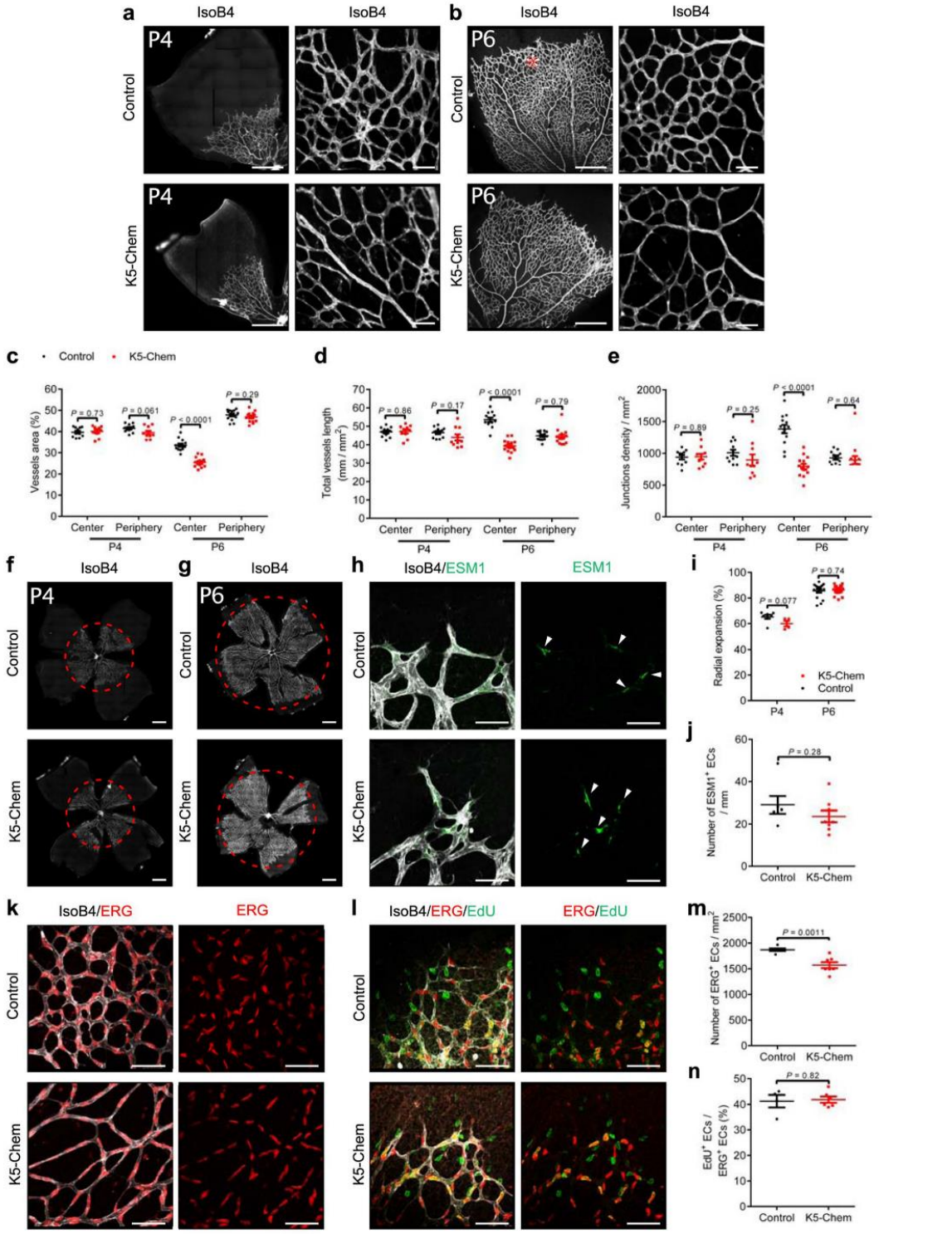
Fig. 1 Overexpression of chemerin decreases the density of the developing vessel retinal network, without affecting sprouting. **a, b** Representative images of retinas from mice overexpressing chemerin (K5-Chem) and their wild-type controls at post-natal days 4 (P4) and 6 (P6), stained with isolectin B4 (IsoB4). Scale bars: 500 µm in left panels, 50 µm in right panels. **c–e** Quantification of the vessels area, total vessels length, and junctions density, normalized for the surface, in the peripheral and central parts of the plexus ($n=11$ for P4 controls, $n=10$ for P4 K5-Chem, $n=13$ for P6 controls, $n=14$ for P6 K5-Chem). **f, g** Representative images of whole mount retinas from K5-Chem and control mice at P4 and P6, stained with IsoB4. Scale bars: 500 µm. **h** Representative confocal images of the angiogenic front of the retinal network at P6, stained with IsoB4 and for ESM1. White arrows point to endothelial tip cells expressing ESM1. Scale bars: 50 µm. **i** Radial expansion of the vascular network relative to the retina radius in K5-Chem and control mice ($n=7$ for P4 controls, $n=4$ for P4 K5-Chem, $n=16$ for P6 controls, $n=18$ for P6 K5-Chem). **j** Quantification of the number of $ESM1^+$ tip cells per mm of network front in K5-Chem ($n=8$) and control mice ($n=6$). **k** Representative confocal images of the remodeling zone of the retinal network from WT and K5-Chem mice at P6, stained with IsoB4 and for ERG. Scale bars: 50 µm. **l** Representative confocal images of the periphery of the retinal network from control and K5-Chem mice at P6, stained with isoB4 and for EdU and ERG. Scale bars: 50 µm. **m** Density of ERG^+ ECs in the remodeling plexus of K5-Chem ($n=7$) and control mice ($n=6$). **n** Quantification of EdU^+ ECs among ERG^+ cells at the network periphery in K5-Chem ($n=6$) and control mice ($n=4$). *P* values versus controls by two-tailed unpaired Student's *t*-test. All data are shown as mean ± SEM. Each point represents 1 animal

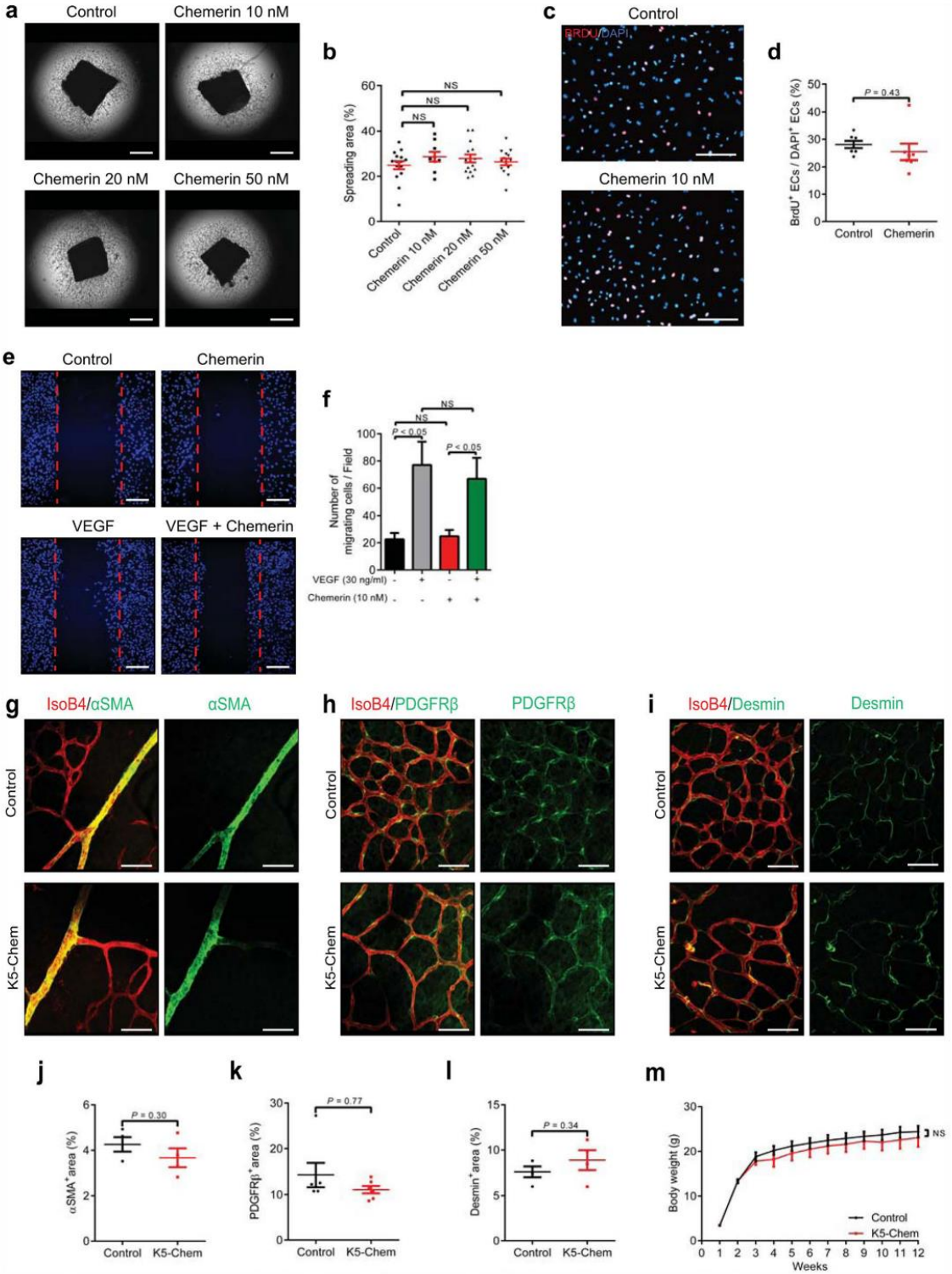
Results

Chemerin regulates blood vessel density in the postnatal retina in an EC sprouting-independent manner

In order to investigate the role of chemerin on physiological angiogenesis *in vivo*, we used a transgenic mouse model described earlier, in which a bioactive form of chemerin is overexpressed by skin keratinocytes, under the control of the keratin 5 gene promoter (K5-Chem model) [19]. In these mice, the levels of chemerin immunoreactivity and bioactivity are strongly elevated in the blood, affecting therefore all tissues in the body. The post-natal development of the vascular network in the retina of these mice was explored. This model is suitable to analyze the different phases of angiogenesis, including vessel sprouting, maturation, and remodeling/pruning [27].

The retinas of mice overexpressing chemerin (K5-Chem) showed a reduced density of the vascular network at postnatal day 6 (P6), while no differences were seen at postnatal day 4, when compared to control littermates (Fig. 1a, b). At P6, chemerin overexpression significantly decreased the relative vessels area (Fig. 1c), the total vessels length per surface unit (Fig. 1d) and the density of branch points (Fig. 1e) in the central part of the capillary plexus, compared


a
IsoB4
IsoB4
P4
Control
P4
K5-Chem
b
IsoB4
IsoB4
P6
Control
P6
K5-Chem
c
Control
K5-Chem
Vessels area (%)
P = 0.73
P = 0.061
P < 0.0001
P = 0.29
Center
Periphery
Center
Periphery
P4
P6
d
Total vessels length (mm / mm²)
P = 0.86
P = 0.17
P < 0.0001
P = 0.79
Center
Periphery
Center
Periphery
P4
P6
e
Junctions density / mm²
P = 0.89
P = 0.25
P < 0.0001
P = 0.64
Center
Periphery
Center
Periphery
P4
P6
f
IsoB4
P4
Control
K5-Chem
g
IsoB4
P6
Control
K5-Chem
h
IsoB4/ESM1
ESM1
Control
K5-Chem
i
Radial expansion (%)
P = 0.077
P = 0.74
K5-Chem
Control
P4
P6
j
Number of ESM1⁺ ECs / mm
P = 0.28
Control
K5-Chem
k
IsoB4/ERG
ERG
Control
K5-Chem
l
IsoB4/ERG/EdU
ERG/EdU
Control
K5-Chem
m
Number of ERG⁺ ECs / mm²
P = 0.0011
Control
K5-Chem
n
EdU⁺ ECs / ERG⁺ ECs (%)
P = 0.82
Control
K5-Chem


a
Control
Chemerin 10 nM
Chemerin 20 nM
Chemerin 50 nM
b
Spreading area (%)
NS
Control
Chemerin 10 nM
Chemerin 20 nM
Chemerin 50 nM
c
Control
BRDU/DAPI
Chemerin 10 nM
d
BrdU+ ECs / DAPI+ ECs (%)
P = 0.43
Control
Chemerin
e
Control
Chemerin
VEGF
VEGF + Chemerin
f
Number of migrating cells / Field
P < 0.05
VEGF (30 ng/ml)
Chemerin (10 nM)
g
IsoB4/αSMA
αSMA
Control
K5-Chem
h
IsoB4/PDGFRβ
PDGFRβ
i
IsoB4/Desmin
Desmin
j
αSMA+ area (%)
P = 0.30
k
PDGFRβ+ area (%)
P = 0.77
l
Desmin+ area (%)
P = 0.34
m
Body weight (g)
Control
K5-Chem
Weeks

◂**Fig. 2** Chemerin does not affect vessel sprouting nor pericyte coverage. **a** Representative images of the capillary network sprouting from aortic ring explants collected from WT mice and treated or not with 10, 20, or 50 nM chemerin. Scale bars: 500 µm. **b** Quantification of the area of capillary outgrowth ($n=15$ for controls, $n=9$, 15, and 14 for, respectively, 10, 20, and 50 nM chemerin). *P* values versus control by one-way ANOVA with Tukey's post hoc test. *NS* not significant. **c, d** Representative images and quantification of $BrDU^+$ cells in a culture of HUVECs treated or not with 10 nM chemerin and stained with DAPI ($n=7$). **e, f** Representative images and quantification of the migration of HUVEcs treated or not with VEGF and/or 10 nM chemerin and stained with DAPI in a scratch assay ($n=3$). Scale bars: 200 µm. *P* values by one-way ANOVA with Tukey's post hoc test. *NS* not significant. **g–i** Representative confocal images of the pericyte coverage on vessels from P6 retinas. The retinas were co-stained with IsoB4 and for either α-SMA, PDGFRβ, or desmin as pericyte markers. Scale bars: 50 µm. **j–l** Quantification of the area stained for α-SMA ($n=4$), PDGFRβ ($n=6$), and desmin ($n=4$) relative to the vascular area (%). **m** Weight curves of control and K5-Chem mice between 1 and 12 weeks after birth ($n=12$). *P* values versus control by 2-tailed unpaired Student's t-test. All data are shown as mean ± SEM. Each point represents 1 animal or one well

to control mice. However, at the periphery of the network, in the area located immediately behind the leading tip cells, (Fig. 1b, asterisk), the same parameters were not affected (Fig. 1c, d, e).

The extent of the network, as determined by the distance between the central artery and the periphery of the superficial vascular plexus was not modified either at P4 or P6 (Fig. 1f, g, red line, and Fig. 1i). In line with this observation, we did not detect changes in the number of cells labeled for endothelial cell-specific molecule 1 (ESM1), a tip cell marker [28, 29] (Fig. 1h, j). The decrease in vessel density was correlated with a decrease in the density of EC, as determined by the nuclear labeling for the EC-specific transcription factor ERG (Fig. 1k, m). We also evaluated the proliferation rate of endothelial cells at P6 by labeling nuclei for the ETS-related gene (ERG) product and measuring the percentage of cells labeled by 5-ethynyl-2'-deoxyuridine (EdU), injected into mice 2.5 h before sacrifice. The proportion of EdU^+ cells among ERG^+ cells was about 40 %, with no difference between mice overexpressing chemerin and their controls (Fig. 1l, n). These data suggested that the phenotype did not result from changes in vessel sprouting or endothelial cell proliferation, pointing instead toward increased regression during the remodeling phase.

To characterize further the influence of chemerin on the EC phenotype and try to delineate which steps of the angiogenesis process are affected in chemerin-overexpressing mice, we investigated EC sprouting *ex vivo* using the mouse aortic ring assay. Aortic rings were incubated with 30 ng/ml VEGF, in the presence or not of various concentrations of mouse recombinant chemerin (10, 20, and 50 nM). In this model, chemerin did not increase nor decrease the extent of vessel sprouts at any of the concentrations tested (Fig. 2a, b) thereby supporting that chemerin does not affect the sprouting step of angiogenesis. We also tested the effect of chemerin on the proliferation and migration of human umbilical vein endothelial cells (HUVEC). The cells were cultured for 24 h in the presence or absence of 10 nM chemerin and exposed for the last 3 h to BrdU. Chemerin did not affect, either positively or negatively, the ratio of $BrdU^+$ cells (Fig. 2c, d), demonstrating that chemerin has no effects on the proliferation rate of endothelial cells. In a scratch wound-healing assay, chemerin did not modify the migration of HUVECs promoted by VEGF-A (Fig. 2e, f).

We investigated further whether a defect in pericyte coverage might result from chemerin activity. Co-staining of endothelial cells by isolectin B4 (IsoB4), and of pericytes by antibodies directed at three markers, α-SMA (Fig. 2 g, j), PDGFR (Fig. 2h, k), and desmin (Fig. 2i, l), did not reveal obvious changes in the coverage of vessels by pericytes in retinas of K5-Chem mice, suggesting that this crucial step in vessel maturation is not affected [30].

Altogether, these data suggest a role of chemerin during developmental angiogenesis, possibly at the level of vessel remodeling rather than vessel sprouting and maturation. As previously reported [19], however, mice overexpressing chemerin develop normally to adulthood and appear grossly normal despite the alteration of developmental angiogenesis (Fig. 2m).

Chemerin promotes microvessel regression and EC apoptosis

During the regression phase of the retinal network development, redundant blood vessels constrict, and ECs retract. The basal lamina of the regressed vessels can be visualized as empty sleeves following staining for type IV collagen (ColIV) [6]. The number of empty sleeves was significantly higher in the retina of K5-Chem mice at post-natal day 6, in the more mature region of the plexus proximal to the optic nerve, and particularly in the vicinity of arteries (Fig. 3a, c, arrowheads), suggesting a role of chemerin in the control of the switch between vessel maintenance and vessel regression. No significant difference was seen in the vicinity of veins in the capillary plexus, neither close to the angiogenic front of the growing retinal network (Fig. 3c). As both EC apoptosis and migration to other vessel branches may contribute to the remodeling process [6, 31], we investigated whether overexpression of chemerin affected the rate of endothelial cell death. Interestingly, we observed a significant increase in the number of cleaved caspase-3^+ cells ($cCasp3^+$) (Fig. 3b, d, arrowheads), suggesting that chemerin action favors apoptosis of ECs, contributing thereby to the physiological remodeling process and supporting vessel pruning.

We then evaluated the effect of chemerin on HUVEcs in the tube formation assay. Chemerin did not influence the

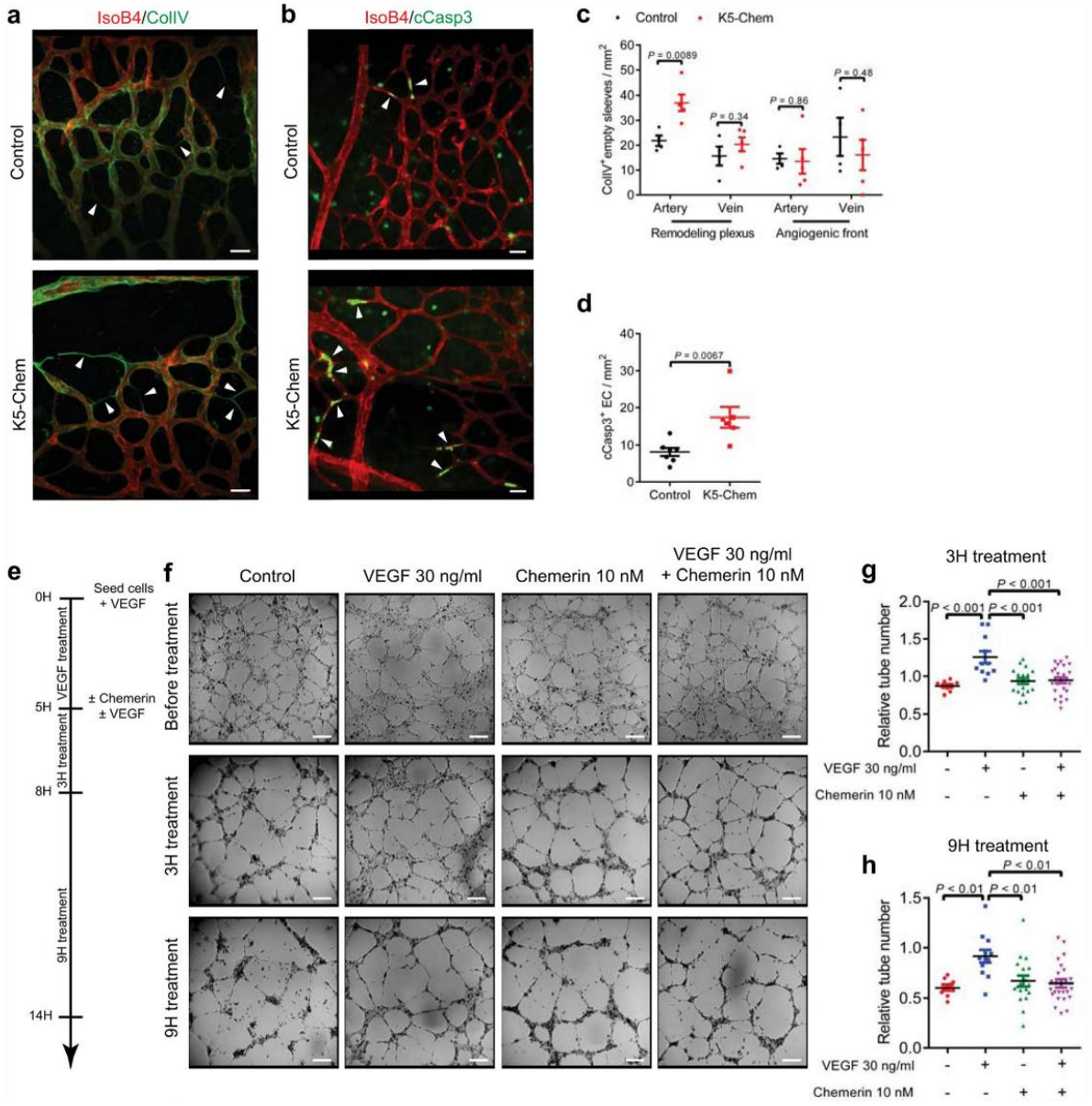


Fig. 3 Overexpression of chemerin promotes vessel regression and endothelial cell apoptosis. **a** Representative confocal images of the remodeling plexus of P6 retinas collected from control or K5-Chem mice, stained with IsoB4 and for collagen IV (ColIV). White arrows point to ColIV$^+$ IsoB4$^-$ empty sleeves. Scale bars: 20 µm. **b** Representative confocal images of the remodeling plexus of P6 retinas collected from control or K5-Chem mice, stained with IsoB4 and for cleaved caspase-3 (cCasp3). Apoptotic cells are indicated by white arrows. Scale bars: 20 µm. **c** Quantification of empty sleeves in the remodeling plexus and the angiogenic zone, in the vicinity of arteries or veins ($n=5$). **d** Quantification of cCasp3$^+$ IsoB4$^+$ cells in the remodeling plexus of K5-Chem ($n=6$) and control mice ($n=7$). **e** Diagram depicting the experimental timeline of the tube assay using HUVECs, allowing formation of tubes for 5 h in the presence of VEGF, and measuring the evolution of the network after 3 or 9 h in the presence or absence of 30 ng/ml VEGF and/or 10 nM chemerin. **f** Representative images of HUVEC tubes at 3 and 9 h in control conditions ($n=9$) or after treatment with 10 nM chemerin ($n=19$), 50 ng/ml VEGF ($n=11$), or the combination of both ($n=25$). Scale bars: 200 µm. **g, h** Quantification of the number of tubes 3 and 9 h after chemerin and/or VEGF treatment, normalized to the tube number before treatment (pool of 6 independent experiments). For **c**, **d**, *P* values versus controls by 2-tailed unpaired Student's t-test. For **g**, **h**, *P* values versus controls by one-way ANOVA with Tukey's post hoc test. All data are shown as mean ± SEM. Each point represents one animal or one well

formation of the tubes either positively or negatively (data not shown). However, when chemerin (10 nM) was added on pre-established tube networks formed in the presence of VEGF (Fig. 3e), it promoted a faster regression of the network after 3 and 9 h, counteracting the effects of VEGF (Fig. 3f–h). These data support a destabilization role of chemerin on established endothelial cell tubes.

CMKLR1 mediates the anti-angiogenic effects of chemerin

CMKLR1 is the main functional receptor of chemerin. Consistent with published data on tip-cell-enriched genes [32], CMKLR1 immunostaining decorated endothelial cells from the remodeling plexus and the angiogenic front in postnatal day 6 retinas (Fig. 4a, b). In order to determine the role of CMKLR1 in the phenotype of mice overexpressing chemerin, we used a mouse line genetically invalidated for this receptor that was described previously [15]. The vessels

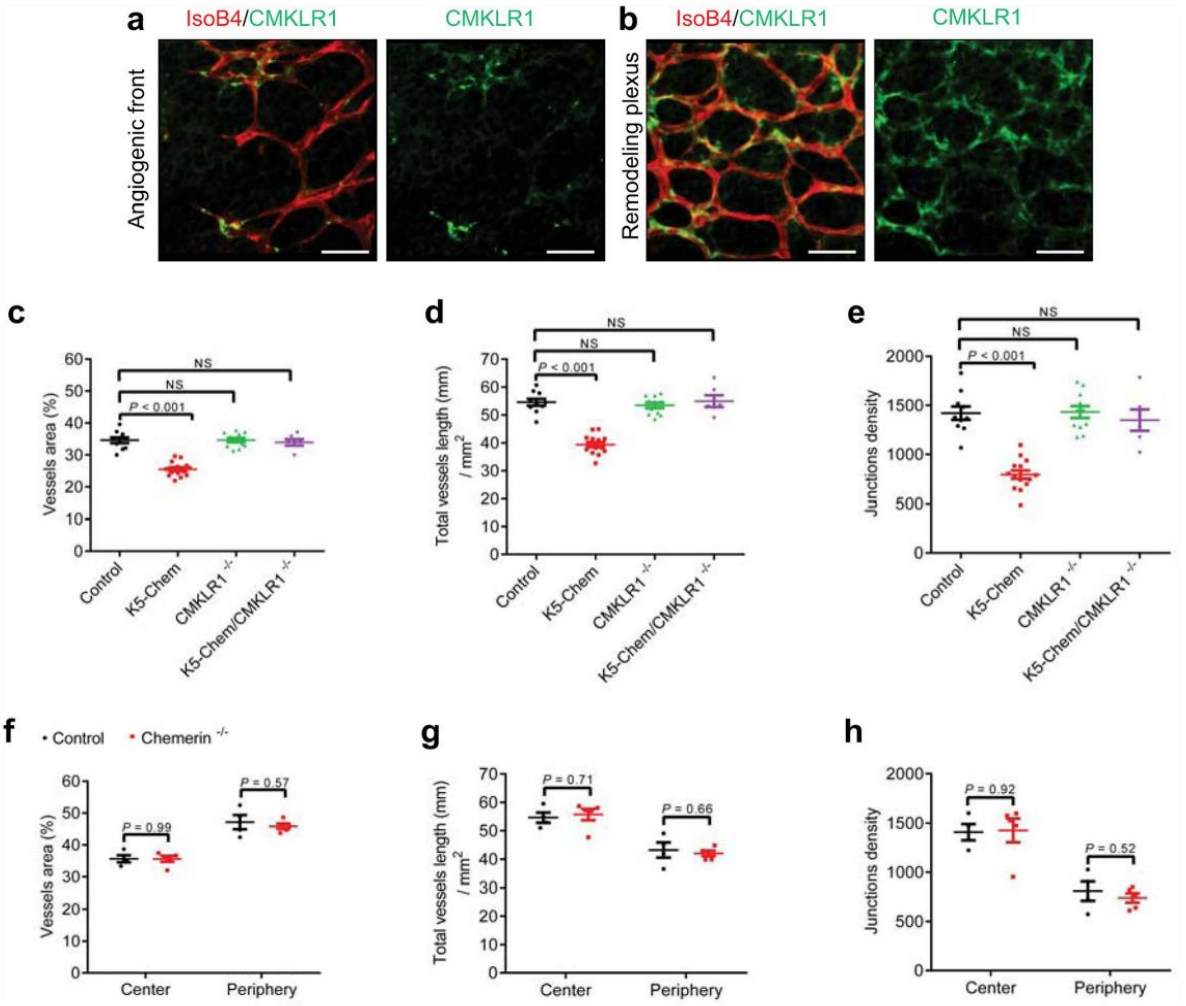


Fig. 4 CMKLR1 mediates the anti-angiogenic effects of chemerin. **a, b** Representative images of the retinas from P6 control mice, stained with IsoB4 (red) and for CMKLR1 (green) in the sprouting zone and remodeling plexus. Scale bars: 50 µm. **c–e** Quantification of the vessels area, total vessels length, and junction density in the remodeling plexus of P6 retinas of control mice and mice overexpressing chemerin and/or invalidated for CMKLR1 (n = 10 for controls, n = 14 for K5-Chem, n = 11 for CMKLR1$^{-/-}$, $n = 6$ for K5-Chem/CMKLR1$^{-/-}$). *P* values versus control by one-way ANOVA with Tukey's post hoc test. **f–h** Quantification of the vessels area, total vessels length, and junctions density in the remodeling and proliferative zones of the P6 retina from control ($n = 4$) and chemerin$^{-/-}$ mice ($n = 5$). P values versus control by 2-tailed unpaired Student's *t*-test. All data are shown as mean ± SEM. Each point represents 1 animal

area, the total vessels length, and the junctions density at P6 were not affected in K5-Chem/CMKLR1$^{-/-}$ pups compared with control littermates (Fig. 4c–e), indicating that the effects of chemerin on angiogenesis are entirely mediated by CMKLR1. Also, no changes were seen in CMKLR1$^{-/-}$ pups, nor in chemerin$^{-/-}$ pups (Fig. 4f–h) as compared to WT control mice, suggesting that the endogenous production of chemerin does not play a major role during the development of the retinal network in normal conditions.

The mature vascular network of adult mouse retinas was analyzed at 6 to 8 weeks. A lower density of vessels was observed in adult K5-Chem mice in the intermediate and deep layers, as compared to controls, while no differences were seen in the superficial layer (Fig. 5a–d; Supplementary Movies 1, 2). The vessels of the deep plexus and those connecting the intermediate and deep layers also appeared more tortuous in K5-Chem mice, as compared to the relatively straight vessels seen in control mice. These observations indicate the absence of compensatory mechanisms counteracting the consequences of chemerin overexpression during the late stages of retina development and growth. The ocular structure and retinal cell layers appeared otherwise normal in hematoxylin/eosin-stained sections of adult K5-Chem mice (Fig. 5e, f).

Altogether, these data show that overexpression of bioactive chemerin during retinal development results in a reduction of the vascular density originating early during retinal angiogenesis as a result of increased vessel pruning and EC apoptosis. These effects are mediated by CMKLR1, and their consequences persist throughout development and adulthood.

Chemerin decreases pathological angiogenesis in a model of oxygen-induced retinopathy

To determine whether CMKLR1 agonists might be relevant in ocular neovascular diseases, we used a mouse model of oxygen-induced retinopathy (OIR) that closely recapitulates

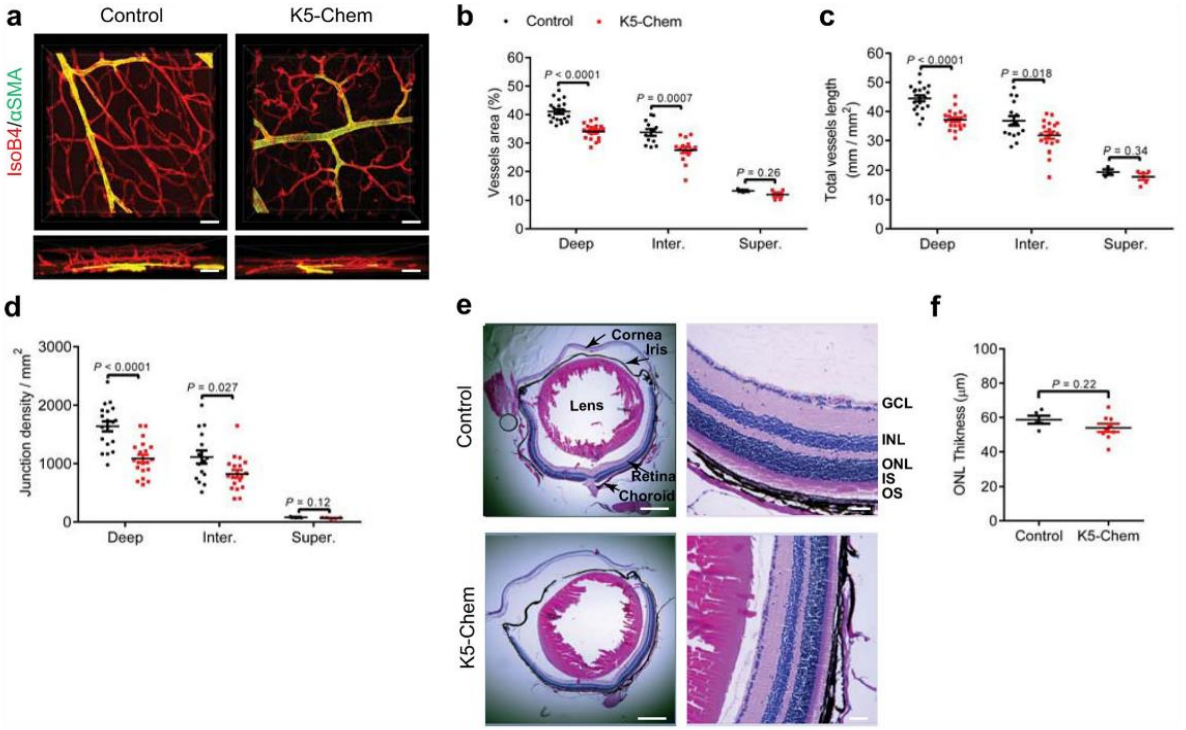


Fig. 5 The lower vascular density in the retina is maintained in adult mice overexpressing chemerin. **a** Representative 3D images of the vessel architecture in the retina of control and K5-Chem adult mice. Scale bars: 20 µm. **b–d** Quantification of the vessels area, total vessels length, and junctions density in the deep ($n=27$ for controls, $n=37$ for K5-Chem), intermediate ($n=15$ for controls, $n=19$ for K5-Chem), and superficial layer ($n=3$ for controls, $n=5$ for K5-Chem) of adult mice. Each point represents 1 field of view. **e** Representative images of H&E-stained sagittal ocular sections from 8-week-old control and K5-Chem mice. Scale bars: 500 µm in left panels, 50 µm in right panels. **f** ONL thickness was quantified in K5-Chem ($n=8$) and control mice ($n=5$). *GCL* ganglion cell layer, *INL* inner nuclear layer, *IS* inner segment, *ONL* outer nuclear layer, *OS* outer segment. *P* values versus controls by 2-tailed unpaired Student's *t*-test. All data are shown as mean ± SEM

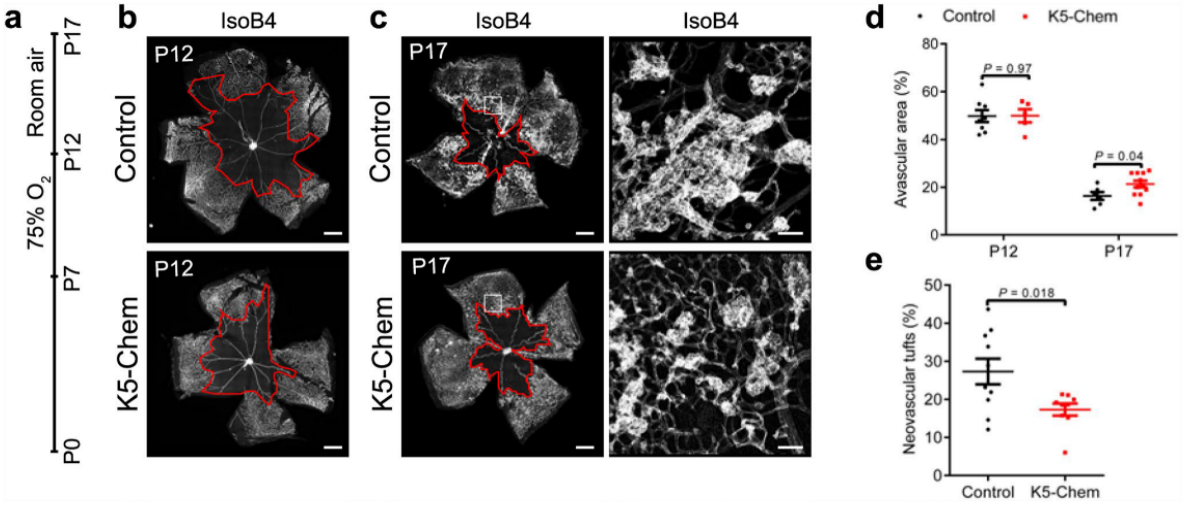


Fig. 6 Chemerin reduces neovascularization in a model of oxygen-induced retinopathy (OIR). **a** Diagram depicting the experimental timeline of the OIR protocol. Pups were placed in 75% oxygen (hyperoxia) from P7 to P12, then placed back under normal oxygen conditions (normoxia) until P17. **b**, **c** Representative images of the retinal vasculature stained with IsoB4 in control and K5-Chem mice at P12 and P17. Scale bars: 500 µm in left panels, 50 µm in right panels. The red line marks the limits of the avascular area. **d** Quantification of the avascular area in control and K5-Chem mice at P12 and P17 ($n=8$ for P12 controls, $n=5$ for P12 K5-Chem, $n=6$ for P17 controls, $n=11$ for P17 K5-Chem). **e** Quantification of the tufts area in control ($n=10$) and K5-Chem ($n=9$) mice. P values versus controls by 2-tailed unpaired Student's t-test. All data are shown as mean ± SEM. Each point represents 1 animal

the two phases of retinopathy of prematurity (ROP) [33]. Mouse pups were exposed to high oxygen levels (75%) from P7 to P12, to induce vessel obliteration (Fig. 6a). The return to normal oxygen levels after p12 results in a severe retinal hypoxia, increasing endothelial cell proliferation and promoting exuberant pathological neovascularization, as well as a gradual replacement of the lost vessels in central retina [34, 35]. The hyperoxia-induced vessel obliteration in the central capillary network was similar in K5-Chem and control mice at P12, suggesting that chemerin did not significantly affect this phase of the model (Fig. 6b, d). However, analysis of the retinal vasculature at P17 showed that pathological neovascularization, quantified by measuring the area of neovascular tufts [36], was reduced in the retinas of K5-Chem mice, as compared to controls (Fig. 6c, e). The avascular area at P17 was also slightly larger in K5-Chem pups compared to WT littermates (Fig. 6c, d). These data support the concept that chemerin prevents disease progression in a model of OIR, resembling ROP, reducing the severity of tuft formation without compromising the newly formed vasculature.

Chemerin inhibits ischemic tissue recovery and angiogenesis in a model of hind-limb ischemia

The functional role of chemerin in hypoxia-driven angiogenesis was further studied in the hind-limb ischemia model (HLI), in which neovascularization is induced by the acute disruption of blood supply in the hind limb and the resulting tissue ischemia and hypoxia [37]. After ligature of the right femoral artery, the recovery of blood flow in the paw was investigated up to 21 days later by laser Doppler imaging, using the left side as control. While WT mice exhibited nearly complete restoration of the blood flow (~80%) after 21 days, K5-Chem mice could only attain ~50% of the pre-HLI perfusion ratio (Fig. 7a, c). We also investigated the vessel density in the gastrocnemius muscle, by labeling endothelial cells with an anti-CD31 antibody. We observed that the capillary density was significantly lower in the injured limb of K5-Chem mice (Fig. 7b, d). These data reveal that chemerin inhibits the neovascularization process and the overall functional recovery in a model of acute ischemia.

Chemerin destabilizes blood vessels in tumor models

Chemerin was shown to display anti-tumoral properties in several mouse models [18, 20]. In a recent study, we reported that these properties could be attributed to an inhibitory effect on the vascularization of tumors, resulting in necrotic cell death [21]. We investigated further whether this anti-angiogenic effect in a tumoral context could be mediated by a destabilization of neovessels. We used B16-F0 melanoma and Lewis lung carcinoma (LLC), two models reported to progress in an angiogenesis-dependent manner [38, 39]. As reported previously [21], grafting

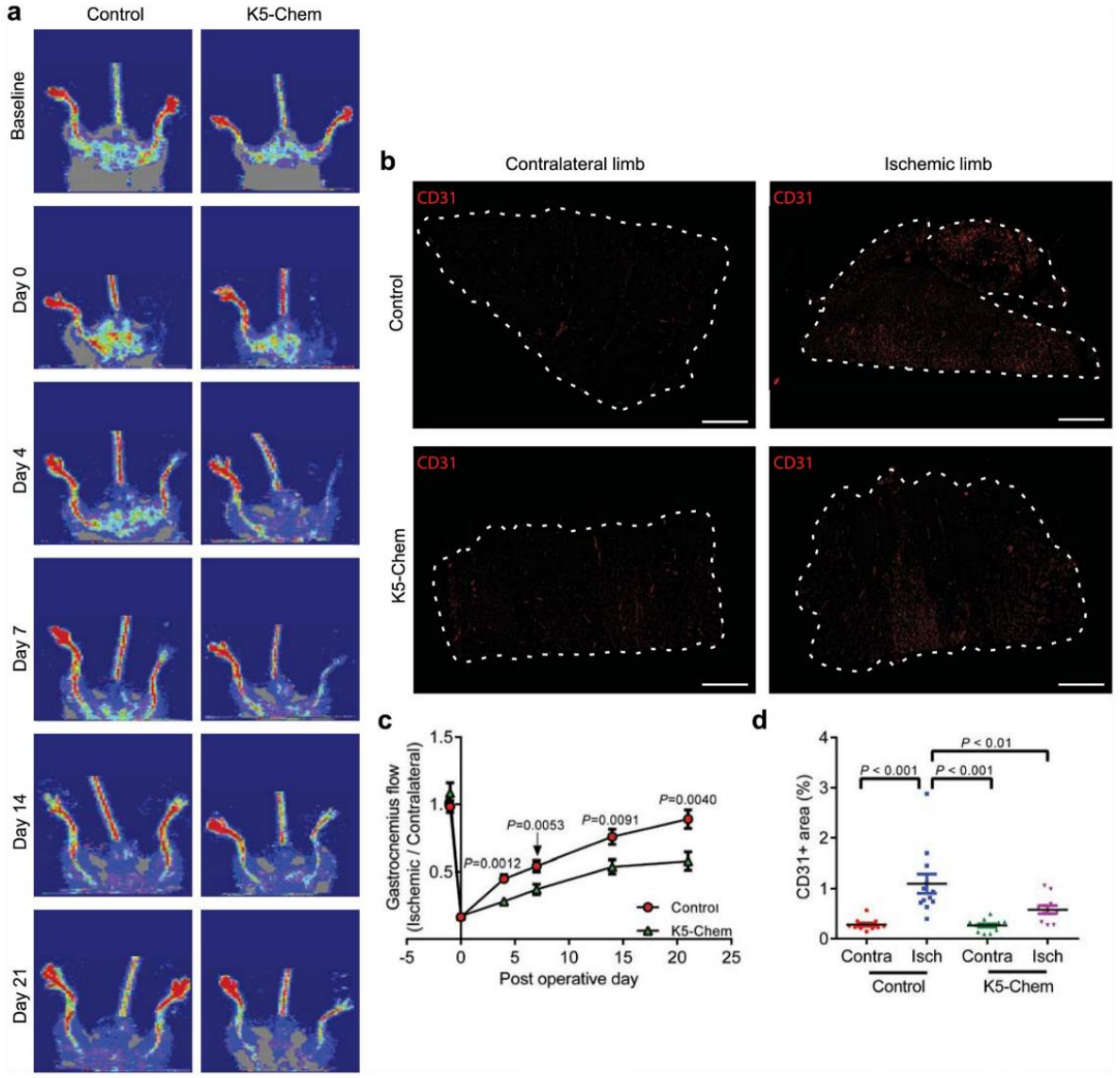


Fig. 7 Chemerin inhibits ischemic tissue recovery and angiogenesis in a model of hind-limb ischemia. **a** Representative laser Doppler images showing delayed reperfusion in the right hind limb of K5-Chem mice at days 4, 7, 14, and 21 following femoral artery ligation, as compared to control mice. **b** Representative images of gastrocnemius muscle sections stained for CD31. Scale bars: 1 mm. **c** Quantification of the distal flow in the gastrocnemius muscle by Doppler measurement in K5-Chem and control mice ($n = 15$). P values versus control by 2-tailed unpaired Student's t-test. **d** Quantification of $CD31^+$ area in the ischemic and contralateral limb of control and K5-Chem mice at day 21 ($n = 12$ for controls, $n = 13$ for K5-Chem, pool of 3 independent experiments). P values by one-way ANOVA with Tukey's post hoc test. All data are shown as mean ± SEM. Each point represents 1 animal

B16-F0 or LLC cells in K5-Chem mice resulted in smaller tumors than in control mice (Fig. 8a–c). Staining for the endothelial specific marker CD31 also confirmed the lower density of endothelial cells in B16 tumors harvested at day 9 from mice over-expressing chemerin (Fig. 8d–f). Staining for type IV collagen identified a higher area of ColIV-stained basal lamina associated with blood vessels in tumors from K5-Chem mice as compared to controls (Fig. 8d). In control tumors, ColIV staining lined closely the endothelial cells and had a smooth appearance, while in tumors from K5-Chem mice, ColIV was more widely distributed around the vessels, and the ratio of $ColIV^+$ to $CD31^+$ areas was increased (Fig. 8g). The same observations were made on 3D reconstitutions of cleared LLC

tumors (Supplementary Movies 3, 4). Such enlargement of ColIV staining around blood vessels in LLC tumors was previously attributed to a deregulation of blood vessel stability [40]. These data confirm in a tumoral model that chemerin favors regression of vessels resulting from neoangiogenesis. Chemerin invalidation had however no influence on B16 tumor growth (Fig. 8h, i).

The activity of chemerin involves the PTEN-AKT-FOXO1 axis

We next investigated the signaling pathways involved in EC regression that could be altered in K5-Chem ECs. Vessel regression or inefficient angiogenesis has been linked to hyper-activation of Notch signaling [41–43]. To test whether this pathway is involved in chemerin-induced vessel regression, we treated mice with the γ-secretase inhibitor DAPT. The resulting inhibition of Notch signaling in control mice led to increased EC density and sprouting at the angiogenic front (Fig. 9a). However, we still observed a decrease in vessels area, total vessels length, and junctions density in the remodeling plexus of K5-Chem mice (Fig. 9b–d), suggesting that the effect of chemerin is not mediated through the Delta/Notch pathway.

Signaling downstream of CMKLR1 was reported to result in phosphatase and tensin homolog (PTEN) activation in different cell types [44, 45], and the PI3K-AKT cascade is known to promote endothelial cell survival. We therefore hypothesized that enhanced PTEN activity in endothelial cells, resulting from chemerin stimulation, might contribute to the regression of neovessels. To test this hypothesis, pups were treated twice with the specific PTEN inhibitor bpV(HOpic) [46] before analysis of their retina at P6. The inhibitor did not modify the parameters of the vascular network in control mice, but completely reversed the effect of chemerin in K5-Chem mice (Fig. 9e–g). Forkhead box protein O1 (FOXO1) is well established as a negative regulator of EC metabolism, proliferation and survival [47, 48], and is inactivated by phosphorylation by AKT [49, 50]. As for PTEN inhibition, the treatment of pups by the FOXO1-specific inhibitor AS1842856 (AS), did not modify the structure of the vascular network in control P6 mice. In K5-Chem pups, however, the vessels area, total vessels length, and junctions density were reversed to control levels following the treatment (Fig. 9 h–j).

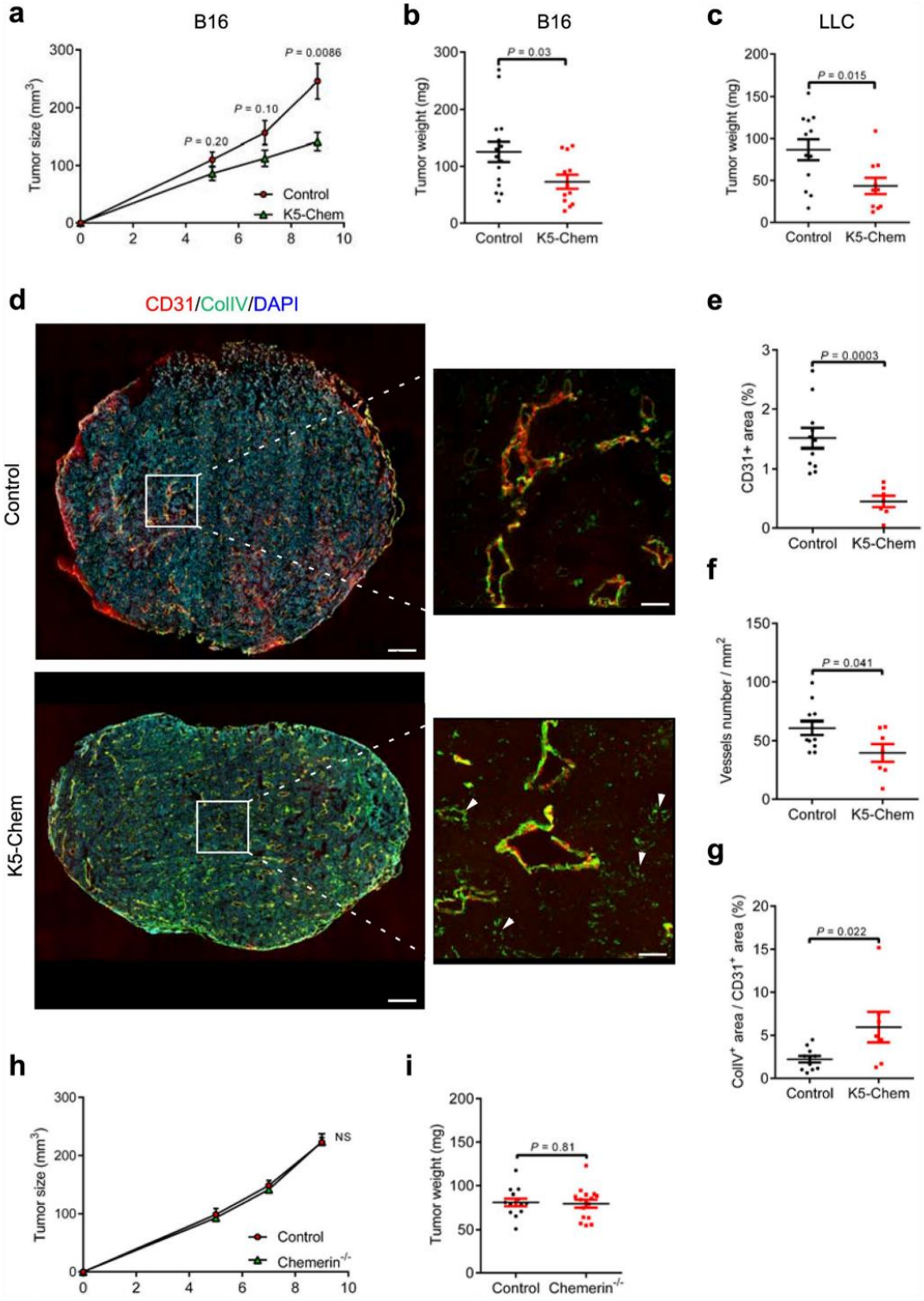
These results *in vivo* suggest that stimulation of CMKLR1 in endothelial cells results in the activation of PTEN, a downregulation of the PI3K-AKT pathway, and enhanced FOXO1 activity, favoring EC apoptosis and vessel regression. This hypothesis was tested further on primary cultures of human umbilical vascular endothelial cells (HUVECs). CMKLR1 expression by these cells was confirmed by immunofluorescence (Fig. 9k). To mimic the effects of hypoxia, the cells were treated with 100 µM $CoCl_2$, promoting the stabilization of HIF1α, and a sharp accumulation of the protein after 24 h (Fig. 9l, m). Chemerin had no effect on this HIF1α increase (Fig. 9n).

The amount and phosphorylation state of PTEN, AKT, and FOXO proteins, as representative elements of the PI3K/AKT pathway and its activation, were investigated by Western blotting. In "normoxic" conditions, treatment by 10 nM chemerin for 24 h did not affect the total level of each protein (quantification relative to a-actin not shown), nor their phosphorylation ratio. Treatment by 100 mM $CoCl_2$ ("hypoxic" conditions) for 24 h did not modify total protein levels but decreased significantly the proportion of Ser380/Thr382/Thr383-phosphorylated and inactive form of PTEN. There was also a tendency toward a decrease of the Ser473-phosphorylated (active) form of AKT and the phosphorylated and inactive form of FOXO1, without reaching statistical significance (Fig. 9o–r). Treatment with chemerin in these hypoxic conditions amplified the decrease in the phosphorylation state for all three proteins, reflecting a further activation of PTEN and FOXO1, and the inactivation of AKT. These results on HUVECs confirm therefore the ability of chemerin to inactivate the PI3K-AKT pathway through an activation of PTEN. These effects were however detectable only in conditions mimicking hypoxia, a context in which neovascularization occurs in vivo.

Discussion

Chemerin is a multifunctional protein. As a chemoattractant factor for various leukocyte populations, including macrophages, dendritic cells, and natural killer cells, it is endowed with pro- or anti-inflammatory properties according to the context and the disease model [10]. CMKLR1 is the main functional receptor of chemerin. Besides leukocytes, it is expressed by endothelial cells and smooth muscle cells, suggesting a range of potential roles in angiogenesis and the control of blood flow [12]. Several studies have proposed a positive action of chemerin on the angiogenesis process mainly *ex vivo* on HUVEC-based models, but also *in vivo* [16, 51, 52]. A contribution of chemerin as a vasoconstrictor molecule contributing to the control of vascular tone was proposed [53].

Chemerin was also described to display anti-tumoral properties in a number of cancer models, as well as pro-tumoral effects in some others [54]. In previous studies, we described the anti-tumoral properties of chemerin in several models of cancer [19, 21]. These effects were attributed to an inhibition of tumoral neoangiogenesis, leading to hypoxia and necrosis. These recent observations are in direct opposition with the conclusions of studies reported above, claiming


a
B16
Tumor size (mm3)
P = 0.0086
P = 0.10
P = 0.20
Control
K5-Chem
b
B16
Tumor weight (mg)
P = 0.03
Control
K5-Chem
c
LLC
Tumor weight (mg)
P = 0.015
Control
K5-Chem
d
CD31/ColIV/DAPI
Control
K5-Chem
e
CD31+ area (%)
P = 0.0003
Control
K5-Chem
f
Vessels number / mm2
P = 0.041
Control
K5-Chem
g
ColIV+ area / CD31+ area (%)
P = 0.022
Control
K5-Chem
h
Tumor size (mm3)
NS
Control
Chemerin-/-
i
Tumor weight (mg)
P = 0.81
Control
Chemerin-/-

◂**Fig. 8** Chemerin favors tumor vessel regression. **a** 1.10^6 B16-F0 cells were implanted into the left flank of control ($n=15$) and K5-Chem ($n=12$) mice (in 3 independent experiments). The tumor size was monitored with a caliper until day 9. **b** The weight of the tumors was measured following sacrifice at day 9. **c** 1.10^6 LLC cells were implanted into the left flank of mice and the weight measured after sacrifice at day 13 ($n=12$ for controls, $n=10$ for K5-Chem). **d** Immunofluorescence staining for CD31 and ColIV were performed on cryosections of B16 tumors collected from control and K5-Chem mice at day 9. Scale bars: 500 µm in left panels, 100 µm in right panels. **e–g** Quantification of the $CD31^+$ area, vessels number, and $ColIV^+$ area relative to the $CD31^+$ vascular area (%) in B16 tumors ($n=11$ for controls, $n=7$ for K5-Chem). **h** Control and chemerin$^{-/-}$ mice were grafted with B16 cells and the tumor size was measured. **i** The weight of the tumors was measured following sacrifice at day 9 ($n=14$ for controls, $n=16$ for chemerin$^{-/-}$). *P* values versus control by 2-tailed unpaired Student's *t*-test. All data are shown as mean ± SEM. Each point represents 1 animal

that chemerin has pro-angiogenic effects both *in vitro* and *in vivo* [16, 51, 52].

For the study of these tumoral models, we generated a transgenic mouse line in which a bioactive form of chemerin is expressed in the basal cells of the skin epithelium, resulting in a significant rise in circulating levels of the protein [19]. During the initial analysis of this mouse line, we did not identify obvious changes in the development of the animals, in the histology of tissues and organs, nor in the distribution of leukocyte populations in the skin or in primary and secondary lymphoid organs. In the present work, we used this genetic model of bioactive chemerin overexpression to explore the potential effect of chemerin on the development of the vascular network of the retina, and the resulting structure of this network in adults. Retinal angiogenesis became indeed a classical model to study neoangiogenesis *in vivo*, and to delineate the pathways involved in its regulation.

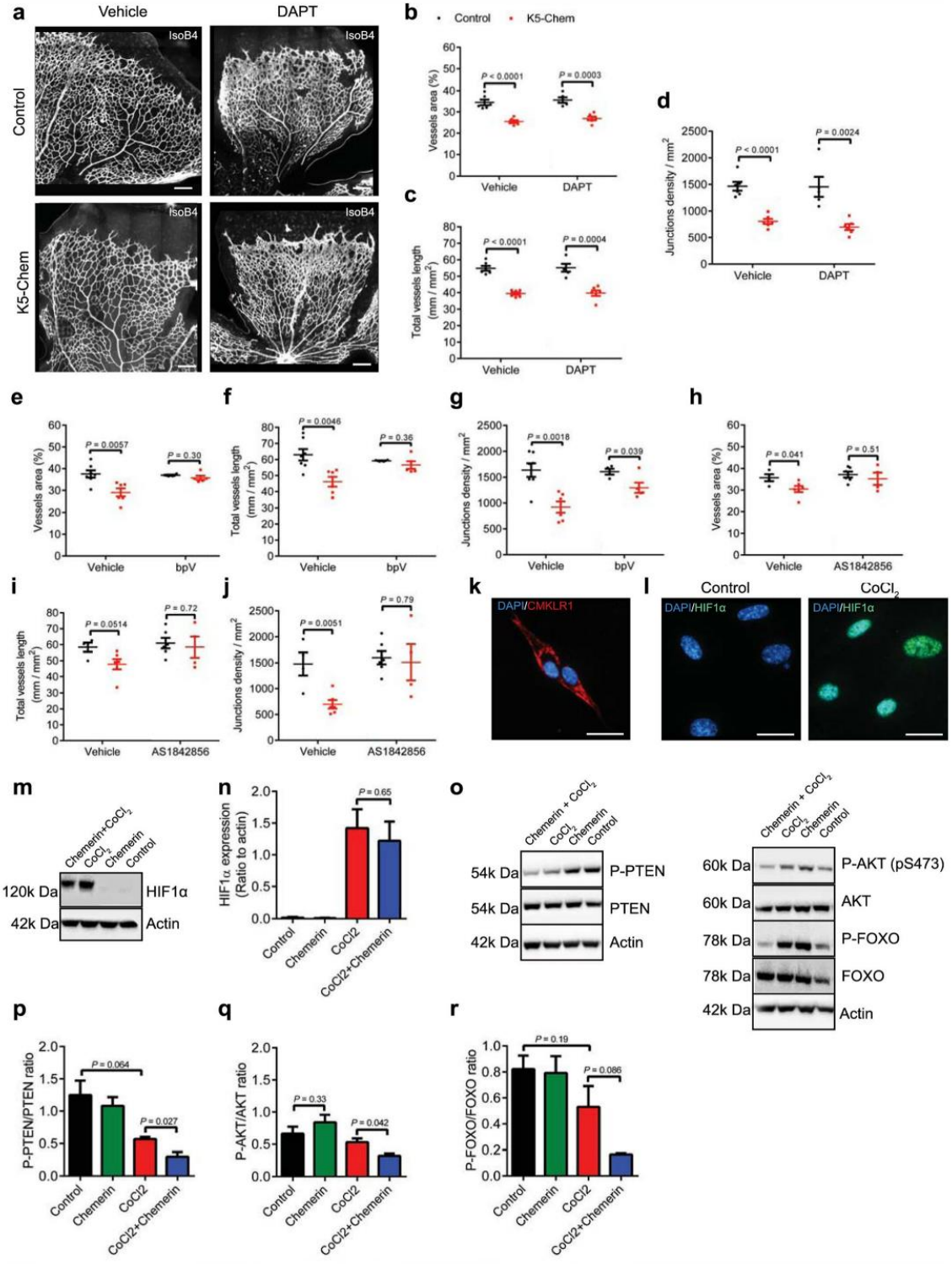
The retinal vascular network develops from the central vein and artery. A first network of capillaries develops by radial expansion toward the periphery of the retina during post-natal days 3 to 7. At the periphery of this growing network, classical neoangiogenesis occurs, driven by relative hypoxia and VEGF production, sprouting tip cells leading stalk cells in a collective migration paradigm. The central remodeling zone of the network is characterized by an adaptation to the local demand of oxygen, usually resulting in the regression of unnecessary vessels and a simplification of the plexus. In later days (P7–P15), sprouting vessels invade the outer parts of the retina, forming the deep and intermediate layers seen in adults. During the vessel remodeling phase, local differences in blood flow and shear stress determine which vessel segments are pruned away [55, 56]. This pruning process is driven essentially by EC migration [55–57], but EC apoptosis contributes to the removal of non-perfused segments during vessel maturation and the establishment of optimal vessel diameter in capillaries [58].

In the present study, we demonstrate that chemerin can contribute to the regulation of angiogenesis. We confirmed that CMKLR1, the main receptor of chemerin, is expressed by retinal endothelial cells, including during the development of the network. We show that chemerin does not affect the progression of the vascular network toward the retina periphery, the sprouting and proliferation of ECs, nor the coverage of vessels by pericytes. Instead, chemerin promotes the regression of vascular branches in the remodeling zone of the plexus and EC apoptosis, as demonstrated by the presence of empty sleeves and activated caspase 3-positive cells. In contrast to other situations affecting the early vascular network of the retina [59, 60], these changes were not compensated later in life, and adult mice were still characterized by a less dense vascular network, at least in the intermediate and deep layers. The overall organization of the retina did not appear to be affected by these modifications in the vascular network. All these changes were totally reversed in mice invalidated for CMKLR1, demonstrating the role of this receptor in the process, and eliminating the possibility of indirect or artefactual activities of chemerin through other and unidentified mechanisms.

The destabilization effects of chemerin could be reproduced *in vitro*, on HUVECs in the tube formation assay. While chemerin did not affect tube formation, it counteracted the effects of VEGF on the stability of the tubes, promoting their regression. In our hands, chemerin had no effects on neovessel sprouting in the aorta ring assay, nor on migration or proliferation of HUVECs in culture. It should be noted however that we reported recently a strong inhibitory effect of chemerin on the extent of the capillary network formed by HUVECs in the bead sprouting assay [21].

These observations contrast sharply with previous reports claiming a positive effect of chemerin on the angiogenesis process. One of these studies reported a positive but moderate effect of chemerin on the formation of tubes in a coculture of ECs and fibroblasts [51]. Curiously, these effects were only observed for chemerin concentrations well below the EC_{50} for CMKLR1, while the concentration near the EC_{50} (10 ng/ml, around 0.5 nM) was ineffective. Another report described positive effects of chemerin on the proliferation of HUVECs and the formation of tubes in Matrigel, although the active concentrations in these two assays were quite discordant [16]. A later report described positive effects of chemerin *in vivo*, in Matrigel plugs and the mouse corneal angiogenesis assay, as well as in the rat aortic ring assay, while confirming the previously reported activities on EC migration, proliferation, and tube formation *in vitro* [52]. We cannot at this point reconcile these observations with our own data, as similar assays were used with very different outcomes.

Following the observation that chemerin can regulate the physiological process of neoangiogenesis during the


a
Vehicle
DAPT
Control
K5-Chem
IsoB4
b
Control
K5-Chem
Vessels area (%)
P < 0.0001
P = 0.0003
Vehicle
DAPT
c
Total vessels length (mm / mm²)
P < 0.0001
P = 0.0004
d
Junctions density / mm²
P < 0.0001
P = 0.0024
e
Vessels area (%)
P = 0.0057
P = 0.30
bpV
f
Total vessels length (mm / mm²)
P = 0.0046
P = 0.36
g
Junctions density / mm²
P = 0.0018
P = 0.039
h
Vessels area (%)
P = 0.041
P = 0.51
AS1842856
i
Total vessels length (mm / mm²)
P = 0.0514
P = 0.72
j
Junctions density / mm²
P = 0.0051
P = 0.79
k
DAPI/CMKLR1
l
Control
CoCl2
DAPI/HIF1α
m
Chemerin+CoCl2
CoCl2
Chemerin
Control
120k Da
HIF1α
42k Da
Actin
n
HIF1α expression (Ratio to actin)
P = 0.65
Control
Chemerin
CoCl2
CoCl2+Chemerin
o
Chemerin + CoCl2
CoCl2
Chemerin
Control
54k Da
P-PTEN
54k Da
PTEN
42k Da
Actin
60k Da
P-AKT (pS473)
60k Da
AKT
78k Da
P-FOXO
78k Da
FOXO
42k Da
Actin
p
P-PTEN/PTEN ratio
P = 0.064
P = 0.027
q
P-AKT/AKT ratio
P = 0.33
P = 0.042
r
P-FOXO/FOXO ratio
P = 0.19
P = 0.086

◂**Fig. 9** Chemerin activity involves the PTEN-AKT-FOXO1 axis. **a** Representative images of retinas of P6 control and K5-Chem mice, injected intraperitoneally with DAPT or vehicle, after staining with IsoB4. Scale bars: 200 µm. **b–d** Quantification of the vessels area, total vessels length, and junctions density in the remodeling plexus of these retinas ($n=6$ for controls vehicle, K5-Chem vehicle and K5-Chem DAPT, $n=5$ for controls DAPT). **e–g** Quantification of the vessels area, total vessels length, and junctions density in the remodeling plexus of retinas from control and K5-Chem mice injected with bpV(HOpic) or vehicle ($n=7$ for controls vehicle, $n=6$ for K5-Chem vehicle, $n=4$ for controls bpV, $n=5$ for K5-Chem bpV). **h–j** Quantification of the vessels area, total vessels length, and junctions density in the remodeling plexus of retinas from control and K5-Chem mice injected with AS1842856 (AS) or vehicle ($n=4$ for controls vehicle and K5-Chem AS, $n=6$ for K5-Chem vehicle and controls AS). **k** Representative image of HUVECs stained for CMKLR1. Scale bar: 20 µm. **l** Representative images of HUVECs treated or not with $CoCl_2$ and stained by DAPI and for HIF1a. Scale bars: 20 µm. **m** Representative Western blot for HIF1a detection in HUVECs treated or not with $CoCl_2$ and/or 10 nM chemerin. **n** Quantification of HIF1a normalized to actin. **o** Representative Western blots of HUVECs treated or not with $CoCl_2$ and/or 10 nM chemerin. **p–r** quantification of the phosphorylation ratios of PTEN, AKT, and FOXO1. *P* values versus controls by 2-tailed unpaired Student's *t*-test. All data are shown as mean ± SEM. Each point represents 1 animal

development of retina, we investigated whether similar observations could be made in models of pathological neoangiogenesis. In the oxygen-induced retinopathy model, we observed indeed a slight delay in the recovery of a "normal" vascular network, but a significant reduction in pathological angiogenesis, reflected by the extent of vascular tufts. The OIR model simulates closely the retinopathy of prematurity, a blinding disease observed in some premature infants placed on oxygen support therapy to compensate their insufficient lung development [61]. OIR also mimics some aspects of proliferative diabetic retinopathy. Consequently, acting through CMKLR1 might be considered as a therapeutic avenue in these pathological situations involving excessive or inappropriate angiogenic responses in the retina. In the hind-limb ischemia model [62], a delay in the recovery of distal blood flow was observed in mice overexpressing chemerin, and this functional defect was correlated with the reduction in the number of vessels observed in the gastrocnemius muscle collected from the lesioned side. In complement to our observations on tumors reported elsewhere [21], we observed in LLC and B16 tumor grafts from chemerin-overexpressing mice a fuzzy and extended labeling for collagen IV around tumor vessels. Similar observations have been related to the lack of stability of the neovessels in other studies [40], suggesting that the reduction in tumor vascularization might be similarly due to decreased stability promoting vessel regression.

It should be noted that invalidation of the CMKLR1 gene or chemerin gene RARRES2 did not modify the development or adult structure of the retinal vascular network. This indicates that chemerin does not contribute significantly to the regulation of angiogenesis in normal conditions. Bioactive chemerin is generated as the result of proteolytic activity in the context of inflammatory reactions, tissue remodeling, and repair processes. Efficient concentrations are therefore likely not reached during the physiological development of the retina and other organs. However, the situation might be very different in neoangiogenesis taking place in the frame of pathological situations, such as acute or chronic inflammatory diseases, ischemia and reperfusion injuries, or tissue reconstruction following traumatic insults. All these situations involve the activation of protease cascades, which result in the activation of prochemerin. In all these pathological situations, chemerin might therefore contribute to the regulation of the neoangiogenesis process, preventing excessive angiogenesis in some cases, but also possibly impairing the recovery of adequate tissue perfusion in others. Our observations regarding the protective role of chemerin in the oxygen-induced retinopathy model, and the delayed recovery of perfusion in the hind-limb ischemia model support indeed this hypothesis.

We finally started to delineate the signaling cascades involved in the activity of chemerin on endothelial cells. CMKLR1 is coupled to the $G_{i/o}$ family of heterotrimeric G proteins, and the recruitment of β-arrestins, leading to the inhibition of cAMP accumulation, intracellular calcium release, and the activation of MAPK cascades including ERK1/2 and p38, as well as the PI3K/AKT pathway. Activation of ERK1/2, p38, and AKT have been reported in endothelial cells as a response to chemerin, and linked to the described effects on proliferation, migration, and survival [16, 52]. None of these pathways is an obvious candidate as a negative regulator of vessel stability. We therefore tested the potential implication of alternative pathways not necessarily linked to CMKLR1 but known to regulate vessel pruning. One of the candidates was the Delta/Notch pathway, but its inhibition by a γ-secretase inhibitor did not abrogate the effects of chemerin on the retinal network. Another candidate was FOXO1, described as a critical rheostat of vascular expansion [47, 48]. Endothelium-specific knockout of FOXO1 causes hyper-branching of retinal vasculature, while enhancing its activity reduces EC metabolism and proliferation and promotes apoptosis, thereby reducing vascular morphogenesis. FOXO1 is well known to be negatively regulated by the PI3K/AKT pathway, its phosphorylation impairing its nuclear translocation and hence its activity on transcription [49, 50]. Interestingly, chemerin was previously reported to increase PTEN activity in hepatocellular carcinoma cells, by disrupting a direct interaction with CMKLR1 leading to ubiquitination and degradation of the protein [44]. Another report described a transcriptional upregulation of PTEN promoted by chemerin in prostate and sarcoma tumor lines, mediated by the serum response factor (SRF) and early response 1 (EGR-1) transcription factors [45]. Although these studies do not deal with endothelial cells and describe quite

different routes toward PTEN activation, they prompted us to investigate PTEN as a possible upstream regulator of FOXO1 and angiogenesis. This hypothesis was tested by treating pups with selective inhibitors and investigating the consequences on retinal angiogenesis. Inhibitors of PTEN or FOXO1 did not modify the structure of the retinal network in control animals but restored a normal density in chemerin-overexpressing pups. These data suggest that a significant part of the effects of chemerin on endothelial cells *in vivo*, leading to their apoptosis and to vessel regression, is mediated through an activation of PTEN, the inhibition of the PI3K/AKT pathway, and a release of the FOXO1 activity.

These observations *in vivo* were complemented by *in vitro* experiments on HUVECs, confirming that chemerin can decrease the phosphorylation of PTEN, increasing its activity [63, 64], counteracting the activity of AKT, and preventing FOXO1 inactivation. All these observations were however made only in conditions mimicking hypoxia, and the total level of PTEN in the cells was unchanged, in contrast to what was described in tumor cell lines [44, 45].

Altogether, our data point toward a role of chemerin as a negative regulator of the PI3K-AKT pathway, particularly in hypoxic conditions in which neoangiogenesis occurs. This leads to an activation of the FOXO1 transcription factor, resulting in a modification of the balance between endothelial cell survival and neovessel stabilization on one side, and EC apoptosis and vessel regression on the other. These data suggest therapeutic applications of CMKLR1 agonists mimicking the effects of chemerin, with potential benefit in pathological situations involving inappropriate or excessive neoangiogenesis, including solid tumors, retinopathy of prematurity, diabetic retinopathy, and potentially arthritis in which destruction of the cartilage by the neoangiogenesis pathway triggered by inflammation plays a central role.

Methods

Mouse lines

C57BL/6J mice were purchased from Janvier. Mice overexpressing chemerin in keratinocytes under the control of the keratin K5 promoter (K5-Chem model) were described previously [19]. The CMKLR1 knockout mouse line was described previously [15]. The chemerin knockout mouse line (C57BL/6 N-*Rarres2*$^{tm1(KOMP)Vlcg}$/MbpMmucd) was obtained from the Mutant Mouse Resource and Research Center at University of California at Davis. All genetically modified mouse lines were bred on the C57BL/6J background. Mice were maintained in a specific pathogen-free facility with environmental enrichment and unlimited access to food and water. Experimental animals were used between day 2 and 10 weeks of age. Transgenic or knockout mice and their respective controls were littermates in all settings. Altogether, 419 mice were used in this study.

Antibodies and reagents for fluorescence microscopy and Western blotting

For fluorescence microscopy, the following primary antibodies were used: rabbit monoclonal anti-ERG (1:500, Abcam, ab110639), eFluor 660-conjugated mouse monoclonal anti-α-smooth muscle actin (α-SMA, 1:100, Invitrogen, 50-9760-82), rabbit polyclonal anti-cleaved caspase-3 (1:400, Cell Signaling, 9661), rabbit polyclonal anti-mouse collagen IV (1:400, Bio-Rad, 2150−1470), goat polyclonal anti-desmin (1:100, R&D Systems, AF3844), goat polyclonal anti-mouse PDGFRβ (1:100, R&D Systems, AF1042), mouse monoclonal anti-BrdU (1:100, BD Biosciences, 347580), PE-conjugated rat monoclonal anti-mouse CD31 (1:200, eBioscience, 12-0311-83), goat polyclonal anti-mouse ESM1 (1:100, R&D Systems, AF1999), rabbit monoclonal anti-HIF1α (1:100, Cell Signaling, 36169), mouse monoclonal anti-CMKLR1 (1:100, Santa Cruz Biotechnology, sc374570). Secondary antibodies used in immunofluorescence were Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 488-conjugated chicken anti-goat IgG, Cy3-conjugated donkey anti-mouse IgG (1:400, Life Technologies and Jackson Laboratories). Alexa Fluor 647-conjugated isolectin GS-IB_4 (IsoB4, 1:500, I32450, Invitrogen) was used to label endothelial cells and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg ml^{-1}, Life Technologies, D1306) for staining nuclei.

Primary antibodies used for Western blotting were rabbit polyclonal anti-AKT (1:3000, Cell Signaling, 9272), rabbit monoclonal anti-phospho-AKT (Ser473, 1:3000, Cell Signaling, 4060), rabbit monoclonal anti-FoxO1 (1:3000, Cell Signaling, 2880), rabbit polyclonal anti-phospho-FoxO1 (Thr24)/FoxO3a (Thr32) (1:3000, Cell Signaling, 9464), rabbit monoclonal anti-PTEN (1:3000, Cell Signaling, 9559), rabbit monoclonal anti-phospho-PTEN (Ser380/Thr382/383, 1:3000, Cell Signaling, 9549), rabbit monoclonal anti-HIF1a (1:2000, Cell Signaling, 36169), and rabbit polyclonal anti-a1 actin (1:3000, Sigma, A2066). The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit IgG (H + L) (1:3000, Cell Signaling Technology, 7074).

HUVEC cell culture and BrdU incorporation assay

HUVECs from a pool of donors were purchased from Lonza and used between passages 2 and 6. They were grown in EBM-2 medium containing EGM supplements (Lonza, CC-3121 and CC-4133) and 10% FBS. To assess the effects of chemerin on signaling cascades, we starved HUVECs

3 h in EBM-2 supplemented with 0.5% FBS and treated them with 10 nM chemerin and/or 100 µM $CoCl_2$ for 24 h. For the BrdU incorporation assay, HUVECs were plated on fibronectin-coated glass coverslips (10 µg/mL, Sigma, F1141), starved for 3 h in EBM containing 0.5% FBS. Recombinant human chemerin (R&D Systems, 2324-CM) was then added at the concentration of 10 nM, and 21 h later, 0.1 mg/ml BrdU (Invitrogen, B23151). The cells were collected 3 h later, stained with DAPI and an anti-BrdU antibody, and the percentage of $BrdU^+$ cells determined.

Fluorescence and light microscopy

Eyes were enucleated and fixed for 20 min in cold 4% paraformaldehyde in phosphate-buffered saline (PBS) [65]. Retinas were dissected in PBS and incubated overnight at 4 °C in TNBT (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 3% blocking reagent (ThermoFisher, 37580), 0.5% Triton X-100). Retinas were incubated overnight at 4 °C with IsoB4 and the indicated primary antibodies diluted in blocking buffer. The next day, retinas were washed three times in PBS and incubated with the corresponding secondary antibody for 2 h at room temperature (RT). Retinas were further washed and flat-mounted on glass slides using the FluorSave medium (Millipore, 345789).

HUVECs were seeded on 14 mm-diameter fibronectin-coated cover slides, starved for 3 h in endothelial cell basal medium (EBM-2) supplemented with 0.5% fetal bovine serum (FBS), and further treated with 10 nM chemerin and/or 100 µM $CoCl_2$ in the same medium for 24 h. The cells were fixed with cold 4% paraformaldehyde for 15 min, permeabilized by 0.1% Triton X-100 for 10 min, and blocked in 3 % bovine serum albumin (BSA, AmericanBIO, AB01088-00100). They were further incubated overnight at 4 °C with the indicated primary antibodies followed by 1 h incubation with appropriate secondary antibodies at RT.

Frozen sections were fixed in acetone for 10 min, washed in PBS, and blocked for 30 min with 5% BSA in PBS. The sections were incubated overnight with the indicated primary antibodies at 4 °C, washed and incubated with secondary antibodies for 2 h at RT.

Eyes were enucleated and fixed overnight in Davidson's fixative. After paraffin embedding, the eyes were cut into 5 µm sections and stained with hematoxylin and eosin. The thickness of the outer nuclear layer (ONL) was quantified with the Fiji software.

Western blotting

Total lysates from HUVECs were obtained by lysing the cells in RIPA buffer containing protease (Roche, 11836145001) and phosphatase (Sigma, 4906845001) inhibitors. The protein concentration was measured by the bicinchoninic acid (BCA) assay (Bio-Rad). Equal amounts of proteins were separated on NuPAGE 4–12% gradient Bis-Tris gels (ThermoFisher, NW04125BOX) and transferred onto nitrocellulose membranes. After blocking with nonfat dry milk, the membranes were incubated overnight at 4 °C with the indicated primary antibodies. An anti-a1 actin antibody was used as loading control. Following a 2-h incubation at room temperature with a horseradish peroxidase-conjugated antibody, bands were visualized by chemiluminescence using the Plus-ECL substrate (Perkin Elmer) and a Fusion Solo S imaging system (Vilber). The intensity of the bands was quantified by the Fiji software.

Tube formation assay

Tube formation assays were performed in µ-Slide Angiogenesis wells (Ibidi) using 10 µl of growth factor-reduced Matrigel (Corning, 356231) per well. Briefly, HUVECs were starved overnight in EBM-2 supplemented with 0.5% FBS, resuspended in the same medium containing 30 ng/ml VEGF (Peprotech, 450−32), seeded onto the polymerized Matrigel, and incubated for 5 h in a humidified chamber at 37 °C, 5% CO_2. New medium containing 30 ng/ml VEGF and/or 10 nM chemerin was then added, and images of one field per well were acquired 3 and 9 h later. All conditions were run in duplicates and the number of tubes was quantified manually in six independent experiments.

Wound-healing assay

HUVECs were seeded in a Culture-Insert 2 Well (Ibidi) at a density of 21×10^3 cells per insert. When confluents, the cells were starved for 6 h in EBM-2 supplemented with 0.5% FBS. The insert was then removed and fresh medium containing 30 ng/ml VEGF and/or 10 nM chemerin was added. After 24 h, cells were fixed with cold 4% PFA, stained with DAPI, and images of one field per well were acquired. All conditions were run in duplicates and the number of cells that migrated into the gap were counted manually in three independent experiments.

Aortic ring assay

Thoracic aortas were dissected from 3 mice per group and sectioned into segments (~500 µm long, > 6 segments per aorta), taking care not to damage the endothelium. The rings were embedded in Matrigel as previously described [33] and incubated in the presence of 30 ng/ml VEGF and various concentrations of recombinant mouse chemerin (0, 10, 20, and 50 nM). Each ring was photographed 5 days later. The area of endothelial sprouts surrounding the rings was analyzed with the Fiji software.

Pharmacological treatments

For inhibiting the Notch pathway, the γ-secretase inhibitor DAPT (Sigma, D5942) was solubilized at 1 µg/µl in 10 mM phosphate-buffered saline (PBS) containing 5% dimethyl sulfoxide (DMSO) and injected intraperitoneally (0.1 mg kg^{-1}) at post-natal days 4 and 5. The FoxO1 inhibitor AS1842856 (2.5 mg kg^{-1}, Calbiochem, 344355) solubilized in saline containing 5% DMSO or the PTEN inhibitor bpV(HOpic) (2 mg kg^{-1}, Millipore, 203701) solubilized in saline were injected subcutaneously in the vicinity of the eye at post-natal days 3 and 5. The vehicles were used as controls. Mice were sacrificed at day 6 and the retinas collected.

In vivo EdU labeling and detection

5-Ethynyl-2′-deoxyuridine (EdU, 100 µg/g body weight) was injected i.p. into P6 pups 2.5 h before sacrifice. EdU^+ cells in the retina were detected with the Click-iT EdU Alexa Fluor 488 Imaging Kit (ThermoFisher, 10337) according to the manufacturer's instructions. ECs were counterstained with IsoB4 and their nuclei by an anti-ERG antibody.

Oxygen-induced retinopathy (OIR) model

As previously described [34], pups were placed from P7 to P12 in 75% oxygen (hyperoxia) and returned at P12 to normal oxygen conditions (normoxia) until P17. Retinas were collected at P12 and P17.

Hind-limb ischemia model

Mice underwent unilateral femoral artery ligation as described [66]. Briefly, following anesthesia, the left femoral artery was exposed under a dissecting microscope. The proximal femoral artery and the distal portion of the saphenous artery were ligated, and an arteriectomy was performed. Blood flow was measured at day − 1, 0, 4, 7, 14, and 21 using a laser Doppler imaging system (Moor Instruments). On day 21, the mice were sacrificed, and the left and right gastrocnemius muscles were harvested, embedded in Tissue-TEK OCT compound, and frozen for cryosectioning.

Tumor models and imaging

Control and K5-Chem mice (6 to 8 weeks old) were injected subcutaneously with 10^6 Lewis lung carcinoma (LLC) or B16-F0 melanoma cells (American Type Culture Collection). The size of the tumors was monitored with a caliper, and LLC and B16 tumors were harvested and weighed after, respectively, 13 and 9 days. Tumors were either embedded in Tissue-Tek OCT compound or processed through the iDISCO protocol [67] for tissue clearing. Briefly, tumors were fixed for 24 h in cold 4% paraformaldehyde, dehydrated in an ascending methanol/water series (20 to 100%, 1 h each), transferred overnight in dichloromethane/methanol (2:1 v/v), and bleached overnight at 4 °C in methanol containing 5% H_2O_2 (Sigma, D216763). After rehydration through a methanol/water series (100–20%, 1 h each) and transfer to PBS containing 0.2% Triton X-100, the samples were incubated overnight at 37 °C in PBS containing 0.2% Triton X-100, 20% DMSO, and 0.3 M glycine (Sigma, G8898), then overnight at 37 °C in a blocking solution (PBS containing 0.2% Triton X-100, 10% DMSO, and 6% donkey serum), and washed overnight in PBS containing 0.2% Tween-20 (Sigma, P9416) and 10 mg/ml heparin (PTwH). The tumors were then incubated with primary antibodies diluted in PTwH containing 5% DMSO and 3% goat serum, for 5 to 6 days at 37 °C with gentle shaking on an oscillator, refreshing the primary antibodies once after 3 to 4 days. Tumors were washed four times for 1 h in PTwH, incubated for 3 to 4 days at 37 °C with gentle shaking with secondary antibodies diluted in PTwH containing 3% goat serum, and washed five times for 1 h in PTwH. Following dehydration through an ascending methanol/water series and incubation for 3 h in dichloromethane/methanol (2:1 v/v), the samples were incubated twice for 15 min in dichloromethane and finally in dibenzyl ether (Sigma, 108014). Imaging of the entire tumor was performed on a selective plane illumination microscope (Ultramicroscope II, LaVision Biotec) at 2.5× magnification, with a 25 µm step between tiles.

Morphometric analyses

Confocal images were acquired on a Zeiss LSM 780 NLO system fitted on an Axio Observer Z1 inverted microscope (Zeiss) equipped with a Chameleon Vision II 690–1064 nm multiphoton laser (Coherent Europe). Tiles were imaged with an Axio Imager Z1 fluorescence microscope (Zeiss).

For the quantification of vessels density, total vessels length, and branching points, images of the IsoB4-labeled capillary plexus were obtained with a 20× objective for each leaflet, in the remodeling zone and the proliferative zones of the network. The images were analyzed with the Angiotool software [68]. For each parameter, the mean of all values obtained for a retina was recorded as a data point.

Radial expansion was the distance between the optic disc and the network front, divided by the distance between the optic disc and the retina edge, determined on a reconstruction of the whole IsoB4-stained retina from tiles obtained with a 20x objective. The mean of values obtained for all leaflets of a retina was recorded as a data point.

The number of $ESM1^+$ ERG^+ cells was counted in Fiji on the leading edge of the network, on reconstructed images (20x) of the whole retina stained with IsoB4 and for ESM1 and ERG, and divided by the length of the angiogenic front.

The mean per retina was recorded as a data point. EC proliferation was estimated by counting the proportion of EdU^+ nuclei among ERG^+ nuclei on images (20×) taken in the proliferative zone of retinas stained with IsoB4 and for ERG and EdU. The mean per retina was recorded as a data point.

Pericyte coverage was determined in Fiji from images (20×) taken in the remodeling plexus, by dividing the area stained for α-SMA, desmin or PDGFRβ by the surface stained by IsoB4. The mean from the four retina leaflets was recorded as a data point.

For the quantification of basal membrane empty sleeves and apoptotic cells, reconstructed images (20×) of whole retinas stained with IsoB4, and for ColIV and cCasp3, were analyzed in Fiji. The number of empty sleeves ($ColIV^+$ and $IsoB4^-$) and $cCasp3^+$ cells were counted in the areas surrounding main arteries and first order arterial branches, or main veins and first order vein branches, in the remodeling and proliferative zones, and divided by the respective surface. Arteries and veins in damaged areas were excluded. For each parameter, the mean of all values obtained for a retina was recorded as a data point.

For the OIR model, IsoB4-stained retinas were imaged (20×) and reconstructed. The avascular area and surface of neovascular tufts were quantified in Fiji and divided by the total retinal area.

Frozen sections from tumors or gastrocnemius muscle, stained by DAPI and for CD31 and/or ColIV, were imaged (20×) as tiles and reconstructed. The $CD31^+$ area, normalized to the total surface of the tissue, was measured in Fiji. For tumors, the $ColIV^+$ area was measured and normalized to the $CD31^+$ area. Two to three sections per sample were analyzed and the mean per mouse was recorded as a data point.

Statistics and artwork

All data are presented as mean ± SEM. Statistical analysis was performed using the Instat or GraphPad Prism 7 softwares. The unpaired two-tailed Student's *t*-test was used to determine statistical significance between two groups. For comparison of more than two groups, we performed one-way or two-way ANOVA followed by Tukey's post hoc test. Reproducibility was ensured by performing several independent experiments. Analyses were performed blinded, without knowing the genotype of animals, the treatment received, or experimental conditions. All experimental data collected from transgenic or knockout animals were compared with those obtained from littermate controls. For the retinal parameters and OIR experiments, the body weight of mice was measured, and outliers that were over two standard deviations away from the mean of their littermates were excluded. In all experiments, animals were age-matched and distributed randomly into groups. Graphs were generated in GraphPad Prism 7 and figures assembled in Adobe Illustrator.

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Author contributions VW and MP designed the study. CBD performed experiments, with contributions of KM and MF to immunofluorescence experiments, AA and BDB to the hind-limb ischemia model, and AR, MA, and NB to Western blot experiments. CBD wrote the initial draft. MP supervised the study, acquired funding, and wrote the final draft. All authors edited and approved the manuscript.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest M.P. is C.E.O. of the biotech company Gepeceron. All other authors declare no conflict of interests.

Ethical Approval All animal experiments were conducted in accordance with European guidelines and local regulations and approved by the local ethics committee (Commission d'Ethique du Bien-Etre Animal, CEBEA) of the ULB Medical School (N° 660 and 707 N). All efforts were made to minimize suffering.

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3. Summary

In the above section, I presented the main results of my thesis just published in Angiogenesis.

In this work, we identified chemerin as an anti-angiogenic factor. We showed that CMKLR1, the main receptor of chemerin, is expressed by retinal endothelial cells, including during the development of the network. Mice overexpressing chemerin display a reduction in the density of the vascular network of the retina. Chemerin does not affect the progression of the vascular network toward the retina periphery, the sprouting and proliferation of ECs, nor the coverage of vessels by pericytes. Instead, chemerin promotes the regression of vascular branches in the remodeling zone of the plexus and EC apoptosis, as demonstrated by the presence of empty sleeves and activated caspase 3-positive cells. In the oxygen-induced retinopathy model, we showed a significant reduction in pathological angiogenesis, reflected by the extent of vascular tufts. In the hind limb ischemia model, we showed a delay in the recovery of the distal blood flow in chemerin-overexpressing mice, and this functional defect was correlated with the reduction in the number of vessels observed in the gastrocnemius muscle collected from the lesioned side. In LLC and B16 tumor grafts from chemerin-overexpressing mice, we confirmed the reduction in the number of neovessels, and morphological features suggesting maturation defects in these tumor vessels. Finally, we showed that the stimulation of CMKLR1 in endothelial cells in hypoxic conditions results in the activation of PTEN, a downregulation of the PI3K-AKT pathway, and enhanced FOXO1 activity.

Taken together, our data point toward a role of chemerin as a negative regulator of the PI3K-AKT pathway, particularly in hypoxic conditions in which neoangiogenesis occurs. This leads to an activation of the FOXO1 transcription factor, resulting in a modification of the balance between endothelial cell survival and neovessel stabilization on one side, and EC apoptosis and vessel regression on the other side.

B. Essential role of CCRL2 in angiogenesis

1. Objectives of Article 2

In the previous article, we characterized the effects of chemerin in different angiogenesis paradigms. Although the majority of chemerin activities are thought to be mediated through the CMKLR1 receptor, CCRL2 is also expressed by endothelial cells. CCRL2 does not trigger any known intracellular signaling pathway, but binds chemerin with high affinity and shows a low level of constitutive endocytosis that is not enhanced following chemerin binding. Several studies have shown that CCRL2 controls the bioavailability of chemerin by increasing its local concentration and presenting it to CMKLR1-expressing cells (Zabel et al., 2008; Monnier et al., 2012; Gonzalvo-Feo et al., 2014). The role of CCRL2 in the angiogenesis process was not studied so far.

Hence, in this part, I was interested in investigating the consequences of CCRL2 loss of function on angiogenesis.

Thus, we used the model of the post-natal development of the retinal vascular network to study physiological angiogenesis and the model of oxygen-induced retinopathy to study pathological angiogenesis in CCRL2$^{-/-}$ mice.

2. Article 2: CCRL2 modulates physiological and pathological angiogenesis during retinal development

(Ben Dhaou et al., Frontiers in cell and developmental biology, 2021)

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CCRL2 modulates physiological and pathological angiogenesis during retinal development

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Abstract

Chemerin is a multifunctional protein involved in the regulation of inflammation, metabolism, and tumorigenesis. It binds to three receptors, CMKLR1, GPR1 and CCRL2. CMKLR1 is a fully functional receptor mediating most of the known activities of chemerin. CCRL2 does not seem to couple to any intracellular signaling pathway and is presently considered as an atypical receptor able to present the protein to cells expressing CMKLR1. CCRL2 is expressed by many cell types including leukocyte subsets and endothelial cells, and its expression is strongly upregulated by inflammatory stimuli. We recently reported that chemerin can negatively regulate the angiogenesis process, including during the development of the vascular network in mouse retina. The role of CCRL2 in angiogenesis was unexplored so far. In the present work, we demonstrate that mice lacking CCRL2 exhibit a lower density of vessels in the developing retina and this phenotype persists in adulthood, in a CMKLR1-dependent manner. Vascular sprouting was not affected, while vessel pruning, and endothelial cell apoptosis were increased. Pathological angiogenesis was also reduced in CCRL2$^{-/-}$ mice in a model of oxygen-induced retinopathy. The phenotype closely mimics that of mice overexpressing chemerin, and the concentration of chemerin was found elevated in the blood of newborn mice, when the retinal vasculature develops. CCRL2 appears therefore to regulate the distribution and concentration of chemerin in organs, regulating thereby its bioactivity.

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Introduction

C-C Motif Chemokine Receptor Like (CCRL2, also known as CRAM or HCR in human and L-CCR in mouse), was first identified in 1998 as a potential chemokine receptor upregulated in murine macrophages after LPS stimulation (Fan et al., 1998; Shimada et al., 1998). CCRL2 is expressed in most leukocyte populations, including monocytes, macrophages, DCs, neutrophils, $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, and mast cells (Brouwer et al., 2004; Oostendorp et al., 2004; Zabel et al., 2008; Otero et al., 2010). Its expression is strongly upregulated in monocytes/macrophages, DCs and neutrophils by pro-inflammatory stimuli, such as LPS, TNF-α, interferon-γ or CD40 ligand (Patel et al., 2001; Migeotte et al., 2002; Galligan et al., 2004; Hartmann et al., 2008). CCRL2 expression was also described in other cell types, particularly in inflammatory conditions, including microglial cells, astrocytes, airway epithelial cells, hepatic stellate cells, adipocytes, and endothelial cells (Oostendorp et al., 2004; Zabel et al., 2008; Monnier et al., 2012). CCRL2 shares high sequence similarities with receptors for inflammatory CC chemokines, such as CCR1, CCR2, CCR3 and CCR5 (Shimada et al., 1998) and its gene is located in the same cluster as these receptors' genes on human chromosomal segment 3p21. However, similarly to the atypical chemokine receptors ACKR1 (DARC) and ACKR2 (D6), CCRL2 lacks the DRYLAIV motif that is essential for G protein coupling and chemotactic responses (Shimada et al., 1998).

CCRL2 was reported to bind the chemokines CCL2, CCL5, CCL7, CCL8 (Biber et al., 2003) as well as CCL19 (Leick et al., 2010), but these observations were not confirmed (Zabel et al., 2008; Del Prete et al., 2013; De Henau et al., 2016). Rather, CCRL2 was shown to bind with high affinity a non-chemokine chemoattractant factor, chemerin, without triggering any signaling (Zabel et al., 2008).

Chemerin has two other receptors, CMKLR1 (also known as $chemerin_1$ or ChemR23) and GPR1 ($chemerin_2$) and is so far the only commonly accepted ligand of CCRL2 (Wittamer et al., 2003; Barnea et al., 2008; Kennedy and Davenport, 2018). The three chemerin receptors have very different functional properties. CMKLR1 is a classical G_i-coupled receptor, with full signaling capabilities through the inhibition of adenylate cyclase, and the activation of phospholipase C, PI3K/AKT and ERK1/2 pathways. Chemerin promotes also efficient β-arrestin recruitment and rapid internalization of this receptor. CMKLR1 is expressed in monocytes/macrophages, myeloid and plasmacytoid dendritic cells and natural killer (NK) cells, and chemerin is a potent chemoattractant factor for these cell populations. The recruitment of these leukocyte subsets can contribute to inflammation and the mounting of adaptive immune responses, but anti-inflammatory properties have also been described in several disease models (Bondue et al., 2011). Besides leukocytes, CMKLR1 is also expressed by endothelial cells and vascular smooth muscle cells, suggesting potential roles in angiogenesis and the control of vascular tone (Kennedy and Davenport, 2018). CMKLR1 mediates most of the known biological effects of chemerin, including the chemotactic activity, the modulation of glucose and lipid metabolism, and the regulation of tumorigenesis and angiogenesis.

GPR1 is instead a receptor characterized by a very weak coupling to G protein-mediated pathways, while promoting strong β-arrestin recruitment and internalizing robustly in response to chemerin (Barnea et al., 2008; De Henau et al., 2016). It is expressed mostly in the central nervous system, but also in skeletal and smooth muscle cells and adipocytes. Very little is known regarding the precise contribution of GPR1 to the functional activities of chemerin.

While the only accepted ligand of CCRL2 is chemerin, it does not trigger any known signaling through this receptor. In addition, CCRL2 does not recruit β-arrestin, and chemerin does not modify the slow basal recycling of the receptor between the plasma membrane and its intracellular stores (Zabel et al., 2008; De Henau et al., 2016; Mazzotti et al., 2017). CCRL2 appears therefore as an atypical receptor,

with properties different from those of atypical chemokine receptors (ACKR) that mediate ligand scavenging and transcellular transport (Fra et al., 2003; Bachelerie et al., 2014; Bonecchi and Graham, 2016). CCRL2 was proposed to act as a chemerin binding site, and because the binding to CCRL2 and CMKLR1 involves different domains of chemerin, it has the unique ability to display chemerin at the surface of cells, thereby favouring its interaction with CMKLR1 expressed by neighbouring cells (Zabel et al., 2008). In particular, the C-terminus of chemerin, which is essential for the activation of CMKLR1, does not contribute to the interaction with CCRL2. Although unable to signal by itself, CCRL2 can therefore influence the bioactivity of chemerin by exposing the ligand and modifying the local concentration and distribution of chemerin in tissues. In this frame, expression of CCRL2 was shown to influence inflammatory states and tumorigenesis in different experimental models in mice (Monnier et al., 2012; Del Prete et al., 2019; Schioppa et al., 2020; Al Delbany et al., 2021). CCRL2 was also shown to modulate the activity of other receptors such as CXCR2, by forming heterodimers with these receptors (Del Prete et al., 2017).

As in other cell populations, CCRL2 is strongly upregulated by inflammatory stimuli in endothelial cells (Monnier et al., 2012). This upregulation contributes to the recruitment from the bloodstream of leukocytes displaying CMKLR1, particularly NK cells (Del Prete et al., 2019; Schioppa et al., 2020). However, despite the close interconnection between inflammation and angiogenesis, the role of CCRL2 in angiogenesis was not investigated so far. Chemerin was described to regulate angiogenesis in different ways. Several reports described pro-angiogenic properties of the chemerin-CMKLR1 pathway in tube formation assays using human umbilical vein endothelial cells (HUVEC) *in vitro* (Bozaoglu et al., 2010; Kaur et al., 2010) and more recently in *in vivo* models in mice (Nakamura et al., 2018). Chemerin was also reported to display anti-tumoral properties in mouse models (Zabel et al., 2008; Dubois-Vedrenne et al., 2019), and we identified rather an inhibitory effect on tumoral angiogenesis as the driving mechanism (Dubois-Vedrenne et al., 2021). The anti-angiogenic properties of chemerin were confirmed in the model of neonatal development of the mouse retinal vascular network (Ben Dhaou et al., 2021), in which chemerin favored blood vessel regression and endothelial cell apoptosis, thereby reducing the density of the network. Similar observations were made in the oxygen-induced retinopathy and hind limb ischemia models (Ben Dhaou et al., 2021). An anti-angiogenic effect of chemerin was also observed in the bead-sprouting assay using HUVECs, while we could not confirm the pro-angiogenic properties in tube-forming assays described previously (Dubois-Vedrenne et al., 2021).

Considering the properties of chemerin in the angiogenesis process, the expression of CCRL2 by endothelial cells and its known ability to modulate the bioactivity of chemerin, we investigated whether CCRL2 could influence physiological and pathological angiogenesis using the developing mouse retina as a model. We observed in CCRL2$^{-/-}$ mice a reduction in the density of the vascular network as a consequence of vessel pruning and EC apoptosis. This phenotype recapitulates the observations made previously in mice overexpressing chemerin, and the blood concentration of this ligand was found to be elevated in CCRL2-deficient mice. This work highlights the important role of CCRL2 as a regulator of chemerin activity in physiological contexts *in vivo*.

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Materials and Methods

Mouse lines

C57BL/6J mice were obtained from Janvier. The CCRL2 (Otero et al., 2010) and CMKLR1 (Luangsay et al., 2009) knockout mouse lines were described previously and bred on the C57BL/6J background. Mice were housed in a specific pathogen-free (SPF) facility with environmental enrichment and unlimited access to food and water. Experiments were held on animals aged from two days to ten weeks. Groups of control (+/+) and knockout (-/-) mice were made from littermates generated by crosses between heterozygous (+/-) animals. Experiments were made in accordance with European guidelines and Belgian regulations. They were approved by the local ethics committee (Commission d'Ethique du Bien-Etre Animal, CEBEA) of the ULB Medical School.

Light and fluorescence microscopy

For light microscopy, eyes were collected, fixed in Davidson's fixative for 16 hours, embedded in paraffin, cut into 5 µm sections and stained with hematoxylin and eosin.

For fluorescence microscopy, eyes were collected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at 4°C (Pitulescu et al., 2010). After dissection in PBS, retinas were incubated in 10 mM Tris HCl, pH 7.4, 150 mM NaCl, 3% blocking reagent (ThermoFisher, 37580), 0.5% Triton X-100 (TNBT) at 4°C for 16 hours, then overnight at 4°C with Alexa Fluor 647-conjugated isolectin GS-IB_4 (IsoB4, 1:500, I32450, Invitrogen) and primary antibodies diluted in TNBT. Primary antibodies were rabbit polyclonal anti-mouse collagen IV (1:400, Bio-Rad, 2150-1470), rabbit polyclonal anti-cleaved caspase 3 (1:400, Cell Signaling, 9661), rabbit monoclonal anti-ERG (1:500, Abcam, ab110639) and goat polyclonal anti-mouse ESM1 (1:100, R&D Systems, AF1999). Retinas were washed three times in PBS and incubated with secondary antibodies (Alexa Fluor 488-conjugated donkey anti-rabbit IgG or Alexa Fluor 488-conjugated chicken anti-goat IgG) for 2 h at room temperature (RT). Retinas were washed and mounted on glass slides in FluorSave medium (Millipore, 345789).

Oxygen-induced retinopathy (OIR) model.

Mice were housed in a 75% oxygen environment from P7 to P12, and back to normoxic conditions afterwards (Campochiaro and Hackett, 2003). Retinas were collected at the end of the hyperoxic period (P12) and after 5 days of normoxia (P17).

Morphometric analyses

Confocal images were acquired on a Zeiss LSM 780 NLO microscope (Zeiss) equipped with a Chameleon Vision II 690-1064 nm multiphoton laser (Coherent Europe). Parameters of the retinal vascular network (vessels density, total vessels length, junctions density) were analyzed by the Angiotool software (Zudaire et al., 2011) on images of IsoB4-stained retinal leaflets obtained with a 20x objective. These parameters were measured in different fields located in the remodeling or proliferative zones of the network and normalized for the surface analyzed. For each of these parameters, a data point was considered as the mean of all values obtained for a retina.

For the determination of the radial expansion of the network, images of whole retinas stained with IsoB4 were reconstructed from tiles obtained with a 20x objective, the distances separating the network front and the retinal edge from the optic disc were measured and the ratio calculated. A data point was considered as the mean of values obtained for all leaflets of a retina.

For the quantification of endothelial cells and tip cells, the retinas were stained with IsoB4 and for ESM1 and ERG, and reconstructed images (20x) of whole retinas were built. The density of ERG^+ cells was calculated in Fiji, as well as the linear density of $ESM1^+$ ERG^+ cells along the angiogenic front of the network. A data point was considered as the mean of all values obtained for a retina.

Empty sleeves were visualized by staining endothelial cells with IsoB4 and basal membranes for ColIV. Images of whole retinas were reconstructed from tiles (20x) and analyzed in Fiji. The density of empty sleeves ($ColIV^+$ $IsoB4^-$) was calculated in the remodeling and proliferative zones, either in the vicinity of main arteries and first order arterial branches, or around main veins and first order vein branches. Damaged areas were excluded. For each parameter, a data point was considered as the mean of all values obtained for a retina. The number of apoptotic endothelial cells was counted in the vicinity of main arteries and first order arterial branches on retina quadrants stained with IsoB4 and for cleaved caspase 3 and divided by the surface.

For the oxygen-induced retinopathy (OIR) model, retinas were stained with IsoB4 and imaged at 20x. The Fiji software was used to determine the area devoid of vasculature and that of neovascular tufts, which were divided by the total surface of the retina. For the determination of vascular parameters of mouse retinas, including in the OIR model, the body weight was measured, and mice over two standard deviations from the mean were not considered.

Immunoassays

Chemerin concentrations were assessed in the plasma of $CCRL2^{-/-}$ and control pups, using a mouse chemerin DuoSet ELISA kit (R&D Systems, DY2325), following the manufacturer's instructions.

Statistics

Data are presented as mean ± SEM. Statistical significance was calculated by unpaired two-tailed Student's t-test, using Instat or Graphpad Prism 7 softwares. For all experiments, knockout and control animals were littermates, and age-matched animals were randomly distributed into groups. The genotype and experimental conditions were not known at the time of data collection.

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Results

CCRL2 regulates the density of blood vessels in the developing retina

Considering the role played by chemerin during the development of the vascular network in the post-natal mouse retina, we investigated the potential role of the non-functional chemerin receptor, CCRL2, in the same process. This *in vivo* model of physiological angiogenesis allows to monitor the various mechanisms contributing to angiogenesis, such as endothelial cell sprouting, pruning and vessel maturation (Fruttiger, 2007). The retina of mice deficient for CCRL2 ($CCRL2^{-/-}$) and their control littermates were analyzed at various time-points. At post-natal day 6 (P6), the density of the vascular network under development was lower in $CCRL2^{-/-}$ mice than in control littermates (Fig. 1A). The various parameters recorded, including the surface of vessels (Fig. 1B), cumulative vessels length (Fig. 1C) and number of branch points (Fig. 1D), normalized for the analyzed area, were significantly lower in the central part of the network in $CCRL2^{-/-}$ retina. The same parameters were however not affected in the peripheral part of the network, behind the tip cell zone (Fig. 1A, asterisk 1B, C, D).

The progression of the network from the central artery and vein to the periphery of the retina was not affected $CCRL2^{-/-}$ mice. Indeed, the distance separating the superficial vascular front from the central artery was unaffected at P6 (Fig. 1A, red line, and Fig. 1E). Also, no changes were seen in the linear density of tip cells, stained for the endothelial cell-specific molecule 1 (ESM1) marker (del Toro et al., 2010; Rocha et al., 2014) along the network front (Fig. 1F, H). However, the density of endothelial cells, determined after nuclear staining for the transcription factor ERG, was lower in the central part of the plexus (Fig. 1G, I). These observations suggested that CCRL2 loss-of-function did not affect EC sprouting, but rather increased vessel regression during the remodeling phase of vascular development, a phenotype similar to that observed previously in mice overexpressing chemerin (Ben Dhaou et al., 2021).

CCRL2 loss-of-function favors vessel regression and endothelial cell apoptosis

The normal development of the vascular network in the retina of newborn mice involves a phase of regression, during which newly formed blood vessels, generated in excess during the sprouting phase, are removed by a process involving vessel constriction, as well as endothelial cell migration and apoptosis. Vessels undergoing regression are seen as empty sleeves after staining of their basal lamina for type IV collagen (ColIV) (Korn and Augustin, 2015). In the remodeling zone of P6 retinas from $CCRL2^{-/-}$ mice, empty sleeves were significantly more numerous, particularly around arteries (Fig. 2A, B, arrowheads), while no differences were found around veins in the remodeling plexus, or close to the angiogenic front of the network (Fig. 2C). Apoptotic cells, stained for cleaved caspase 3, were also more abundant in the remodeling zone of retinas from P6 $CCRL2^{-/-}$ mice (Fig. 2B, D).

The retinal phenotype is maintained in adult $CCRL2^{-/-}$ mice and involves CMKLR1

Changes observed during development of the retinal vascular network are frequently compensated later. We therefore analyzed the parameters of the vascular network in the mature retina of adult mice aged 6 to 8 weeks. The vessel density was found to be lower in the deep layer of the retinal network in adult $CCRL2^{-/-}$ mice, as compared to WT littermates, while no changes were observed for the intermediate and superficial layers (Fig. 3A, C, D, E). The morphology of vessels in the deep plexus appeared also different in $CCRL2^{-/-}$ mice. They were more tortuous than the vessels of control mice. Vessels connecting the intermediate and deep layers were also less organized. These observations, which mimic what was seen previously in chemerin-overexpressing mice (Ben Dhaou et al., 2021)

show that no compensatory mechanisms counteract the consequences of CCRL2 loss-of-function, after the initial stages of retinal development.

CMKLR1 is the main functional receptor of chemerin. To determine the contribution of CMKLR1 in the phenotype of CCRL2$^{-/-}$ mice, we analyzed the retina of mice knocked out for both receptors. The vessels area, total vessels length, and junctions density were not modified in adult CCRL2$^{-/-}$/CMKLR1$^{-/-}$ mice compared to WT controls (Fig. 3B, F, G, H), indicating that CMKLR1 is involved in the retinal phenotype of CCRL2$^{-/-}$ mice. The histological aspect of retinas of adult CCRL2$^{-/-}$ and CCRL2$^{-/-}$/CMKLR1$^{-/-}$ mice and their various cell layers were otherwise normal on sections stained by hematoxylin and eosin (Fig. 3I).

CCRL2 loss-of-function protects from aberrant angiogenesis in the oxygen-induced retinopathy model

The consequences of CCRL2 loss-of-function on the physiological angiogenesis of retina prompted us to evaluate whether the lack of CCRL2 might be protective in a model of pathological angiogenesis. The mouse oxygen-induced retinopathy (OIR) model is mechanistically very similar to an ocular disease frequent in premature infants, the retinopathy of prematurity (ROP), with a phase of vessel obliteration caused by hyperoxia, followed by the exuberant proliferation of abnormal vessels as a result of hypoxia (Scott and Fruttiger, 2010). Following exposure to 75% oxygen from P7 to P12, mouse pups were returned to normoxic conditions at P12 (Fig. 4A) (Smith et al., 1994; Campochiaro and Hackett, 2003). The consequences of hyperoxia were similar in CCRL2$^{-/-}$ and control mice at P12, with a marked regression of blood vessels in the central part of the retina (Fig. 4B, E), suggesting that CCRL2 does not contribute significantly to this phase of the model. In contrast, the surface occupied by neovascular tufts, the main component of pathological neovascularization formed as a result of hypoxia (Connor et al., 2009), was considerably larger in the retinas of CCRL2$^{-/-}$ mice at P17, as compared to WT littermates (Fig. 4C, D). The retinal surface remaining avascular was also somewhat larger in CCRL2$^{-/-}$ pups at P17 (Fig. 4C, E). These observations indicate that CCRL2 loss-of-function is protective against excessive neovascularization in the mouse OIR model, reducing the formation of vascular tufts.

Chemerin is elevated in the blood of CCRL2$^{-/-}$ mice

The concentration of chemerin was assayed by ELISA in the plasma of P6 mice, during the development of the vascular network of the retina (Fig. 5A). Plasma chemerin levels were significantly increased in CCRL2$^{-/-}$ mice, suggesting that CCRL2 is involved in the regulation of circulating levels of chemerin, thereby controlling its bioactivity in naive conditions.

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Discussion

In previous studies, we demonstrated that chemerin displays anti-angiogenic properties in a number of physiological and pathological settings. We showed that chemerin can inhibit the formation of sprouts in the bead sprouting assay *in vitro* using human endothelial cells (HUVEC) and inhibit the vascularization and growth of tumor grafts in mice (Dubois-Vedrenne et al., 2021). *In vivo* as well, we demonstrated that high chemerin levels in mice can reduce the density of the vascular network in the developing retina, through the pruning of vascular branches and apoptosis of endothelial cells (Ben Dhaou et al., 2021). All these effects were mediated by CMKLR1.

In the present work, we investigated the consequences of CCRL2 loss-of-function on the post-natal development of the vascular network in the mouse retina. This model became over the recent years a classical model of angiogenesis, allowing to study *in vivo* the various steps of the process in a physiological context, with solid and reproducible parameters. We showed that, in the absence of CCRL2, the growth of the vascular network from the center to the periphery of the retina is not affected, while a higher number of vascular branches regressed in the remodeling zone of the plexus, a process accompanied by EC apoptosis. As a result, the density of the vascular network was decreased all along the developmental stages. In other situations affecting early steps in the formation of the vascular network in mouse retina, the changes are compensated at later stages and the adult retina appears unaffected (Ishida et al., 2003; Kubota et al., 2009). The retinas of adult CCRL2$^{-/-}$ mice kept however a lower density in the deep layers of its vascular network, showing that in this case, the developmental change was not compensated later in life.

This phenotype was almost identical to that observed in mice overexpressing bioactive chemerin in basal keratinocytes of the skin and presenting high levels of chemerin in their blood (Ben Dhaou et al., 2021). The retinal phenotype of CCRL2$^{-/-}$ mice was completely reversed in animals knocked-out as well for the CMKLR1 receptor, demonstrating that the anti-angiogenic consequences of CCRL2 loss-of-function involves an hyperactivation of the chemerin/CMKLR1 pathway. In line with this, the chemerin levels were found elevated in the blood of CCRL2$^{-/-}$ mice at post-natal day 6, during the developmental phase of the retinal vascular network. Such increase was also described previously in adult CCRL2$^{-/-}$ mice under naive conditions (Monnier et al., 2012).

We showed previously that chemerin can also temper pathological neoangiogenesis in the oxygen-induced retinopathy model (Ben Dhaou et al., 2021). We observed here, in CCRL2$^{-/-}$ mice, a significant reduction in the formation of vascular tufts as compared to control mice, and a moderate delay in recovering a normal vascular network. This phenotype is also reminiscent of that observed for chemerin-overexpressing mice. CCRL2 can therefore influence pathological angiogenesis as well, playing in this case a detrimental role by dampening the activity of endogenous chemerin.

It should be noted that CCRL2 loss-of-function affects the efficiency of retinal angiogenesis, while CMKLR1 deficiency does not. This means that in the presence of physiological levels of CCRL2 expression, endogenous chemerin does not affect the angiogenesis process, and this can likely be attributed to the buffering activity of CCRL2, and/or its ability to remove the ligand from the circulation and the extracellular medium. CCRL2 might therefore play important roles in controlling the activity of chemerin in both directions. In the context of physiological angiogenesis, CCRL2 protects the network under development from the anti-angiogenic properties of chemerin. In inflammatory situations however, CCRL2 is strongly upregulated in various cell types, including endothelial cells. In this context, the concentration of chemerin bound to CCRL2 might instead increase locally the bioactivity of the ligand, modifying the trafficking of leukocytes but also potentially the angiogenesis process generally associated with inflammatory states.

Since our CCRL2 KO model is constitutive, it is not known whether a specific expression site is responsible for the modulation of the angiogenesis process. CMKLR1 is expressed by endothelial cells and chemerin can directly affect the properties of these cells. Indeed, chemerin inhibited the formation of vessel sprouts by HUVECs in the bead sprouting assay *in vitro*, in the absence of other cell types expressing CMKLR1 (Dubois-Vedrenne et al., 2021). CCRL2 is also expressed by endothelial cells and the endothelium certainly contributes to the regulation of chemerin activities, particularly in the frame of leukocyte trafficking. However, many other cell types expressing CCRL2, including leukocyte subsets, adipocytes and epithelial cells, may also play the role of chemerin sink. Cell-type specific knock out lines might help delineate whether CCRL2 inactivation in one of these cell populations plays a dominant role in the elevation of the circulating chemerin levels that leads to its anti-angiogenic effects.

Chemerin is a chemoattractant factor for leukocyte populations but is structurally unrelated to the large family of chemokines. Two of the chemerin receptors, CMKLR1 and GPR1, are evolutionary closer to receptors for complement peptides ($C5a_1$, C3a), formylpeptides (FPR1, FPR2, FPR3) and leucotrienes (BLT_1, BLT_2) than to chemokine receptors. CCRL2 however is structurally related to receptors for inflammatory chemokines. Many chemokines and chemokine receptors were described to regulate angiogenesis positively or negatively, in addition to their roles in leukocyte chemoattraction and immunity (Keeley et al., 2011; Bosisio et al., 2014). The new functions attributed to chemerin, CMKLR1 and CCRL2 are therefore reminiscent of the dual activities of several chemokine systems and provide further links between inflammation and angiogenesis. Chemokines encompass two main subfamilies, according to the relative position of the first two cysteines involved in the formation of disulfide bonds, CC and CXC-chemokines (Zlotnik and Yoshie, 2012). The CXC subfamily itself is divided into ELR^+ and ELR^- chemokines following the presence or absence of this amino acid motif in their N-terminal domain. ELR^+ CXC-chemokines, including CXCL1 to 3, and CXCL5 to 8 are pro-angiogenic. These properties are at least partially mediated by a direct effect on endothelial cells that express their common receptor, CXCR2 (Keeley et al. 2011). These chemokines are indeed able to promote proliferation and migration of endothelial cells *in vitro*. However, part of the angiogenic effects *in vivo* can also be mediated through the recruitment of leukocyte populations, such as neutrophils, which in turn may release cytokines stimulating the production of pro-angiogenic factors through the HIF pathway. Some ELR^- CXC-chemokines, including CXCL4 and CXCL9 to 11 display anti-angiogenic properties. These effects may also involve direct effects on endothelial cells as well as indirect effects through the recruitment of leukocyte populations that in turn inhibit angiogenesis (Bosisio et al., 2014). CXCL9, CXCL10 and CXCL11 act through their common receptor CXCR3, which is expressed by endothelial cells. CXCL4 was described to act through CXCR3 as well, but is also affecting endothelial cells in a CXCR3-independent manner.

The ELR^- chemokine CXCL12 and its receptors CXCR4 and CXCR7 constitute also a pro-angiogenic system, that in contrast to the other chemokines, contributes to physiological angiogenesis during development. Indeed, CXCL12 or CXCR4 KO mice display developmental defects of the vascular bed in the gut and kidney (Tachibana et al. 1998). Atypical chemokine receptors (ACKR) were also shown to influence angiogenesis by modulating the activity of chemokines and their receptors (Bosisio et al., 2014). In this regard, the chemerin system appears to behave in a very similar way to chemokine systems, controlling the trafficking of specific leukocyte subpopulations, but also modulating angiogenesis, through a direct action on endothelial cells, but also indirectly through the specific effects of each leukocyte subset they recruit. These various activities contribute to the intricate relationships linking inflammation and angiogenesis.

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366 Altogether, our data support the important role of CCRL2 as a modulator of the chemerin/CMLKLR1
367 axis, and of the effects of this system on inflammation, tumorigenesis and angiogenesis. CMKLR1
368 agonists are being considered for their potential therapeutic use in some inflammatory diseases and
369 cancer types. However, these strategies should also take into account the role CCRL2 may play on the
370 chemerin/CMKLR1 axis, and the consequences of local CCRL2 expression in this context.

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Author Contributions

M.P. and C.B.D. designed the study. C.B.D. performed experiments. A.D.P. and S.S. generated the CCRL2 KO line. C.B.D. wrote the initial draft. M.P. supervised the study and wrote the final draft. M.P. and S.S. acquired funding. All authors edited and approved the manuscript

Conflict of Interest

M.P. is C.E.O. of the biotech company Gepeceron. All other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Legend to Figures

Figure 1. CCRL2 loss-of-function reduces the density of the vascular network in the developing mouse retina. **A** Retinas from CCRL2-/- and control mice at post-natal day 6 (P6), stained with isolectin B4 (IsoB4). Scale bars: 500 µm in left panels, 50 µm in right panels. **B-D** Vessels area, total vessels length and junctions density, relative to the surface, in the angiogenic and remodeling zones of the vascular plexus (n = 14 for controls, n = 11 for CCRL2-/-). **E** Radial expansion of the vascular network (% of retinal radius) in control and CCRL2-/- mice (n = 12 for controls, n = 7 for CCRL2-/-). **F** Angiogenic zone of the retinal network of P6 mice, stained with IsoB4 and for ESM1. Endothelial tip cells positive for ESM1 are indicated by white arrows. Scale bars: 50 µm. **G** Remodeling zone of the retinal network of P6 mice in control and CCRL2-/- mice, stained with IsoB4 and for ERG. Scale bars: 50 µm. **H** Linear density of ESM1+ tip cells at the front of the vascular network in control (n = 11) and CCRL2-/- mice (n = 6). **I** Density of ERG+ endothelial cells in the remodeling zone of the vascular plexus in the retina of control (n = 6) and CCRL2-/- mice (n = 5). Mean ± SEM, 2-tailed unpaired Student's t test. Each point represents one animal.

Figure 2. CCRL2 loss-of-function promotes vessel regression. A, B Confocal images of the remodeling part of the vascular plexus in retinas collected from control or CCRL2-/- mice at P6, stained with IsoB4 and for collagen IV (ColIV, **A**) or with IsoB4 and for cleaved caspase 3 (cCasp3, **B**). ColIV+ IsoB4– empty sleeves and apoptotic cells are indicated by white arrows. Scale bars: 20 µm. C Density of empty sleeves in the remodeling and angiogenic zones of the vascular plexus, respectively around arteries and veins (n = 7). Each point represents one retina. **D** Density of cCasp3+ apoptotic endothelial cells in areas surrounding arteries in the remodeling zone of the plexus from a retinal quadrant (n = 8 for controls and 7 for CCRL2-/-). Mean ± SEM, 2-tailed unpaired Student's t test.

Figure 3. The low density of the vascular network persists in the retina of adult CCRL2-/- mice, and is CMKLR1-dependent. A, B Deep layer of the vascular network from adult control, CCRL2-/- and CCRL2-/-/CMKLR1-/- retinas, stained with IsoB4. Scale bars: 50 µm. **C-E** Vessels area, total vessels length and number of junctions (normalized for the surface) in the deep (n = 29 for controls, n = 38 for CCRL2-/-), intermediate (n = 14 for controls, n = 40 for CCRL2-/-) and superficial layers (n = 3 for controls, n = 12 for CCRL2-/-) of the vascular plexus from adult mouse retinas. **F-H** Vessels area, total vessels length and junctions density in the deep (n = 52 for controls, n = 37 for CCRL2-/-/CMKLR1-/-), intermediate (n = 8 for controls, n = 6 for CCRL2-/-/CMKLR1-/-) and superficial layers (n = 8 for controls, n = 4 for CCRL2-/-/CMKLR1-/-) of the retinal vascular plexus from WT or CCRL2-/-/CMKLR1-/- adult mice. **I** H&E-stained sagittal sections of eyes from 8-weeks-old control, CCRL2-/- and CCRL2-/-/CMKLR1-/- mice. Scale bars: 500 µm in upper panels, 50 µm in lower panels. GCL, ganglion cell layer; INL, inner nuclear layer; IS, inner segment, ONL, outer nuclear layer; OS, outer segment. Mean ± SEM, 2-tailed unpaired Student's t test. Each point represents one field of view.

Figure 4. CCRL2 loss-of-function protects against aberrant neovascularization in the oxygen-induced retinopathy (OIR) model. A Experimental timeline of the OIR protocol, in which mouse pups are exposed to 75% oxygen from P7 to P12, then to normoxia until P17. **B, C** Retinal vascular network of control and CCRL2-/- mice stained with IsoB4 at P12 and P17. Scale bars: 500 µm in left panels, 50 µm in right panels. The avascular area is surrounded by the red line. **D** Relative area of

vascular tufts in control and CCRL2-/- mice (n = 6). **E** Relative avascular area in control and CCRL2-/- mice at P12 and P17 (n = 7 for P12 controls, n = 5 for P12 CCRL2-/-, n = 8 for P17 controls, n = 6 for P17 CCRL2-/-). Mean ± SEM, 2-tailed unpaired Student's t test. Each point represents one animal.

Figure 5. CCRL2 regulates circulating chemerin levels in vivo. Chemerin immunoreactivity measured by ELISA in the plasma of control and CCRL2-/- mice at postnatal day 6 (n = 12 for controls, n = 6 for CCRL2-/-). Mean ± SEM, 2-tailed unpaired Student's t test. Each point represents one animal.

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Figure 1

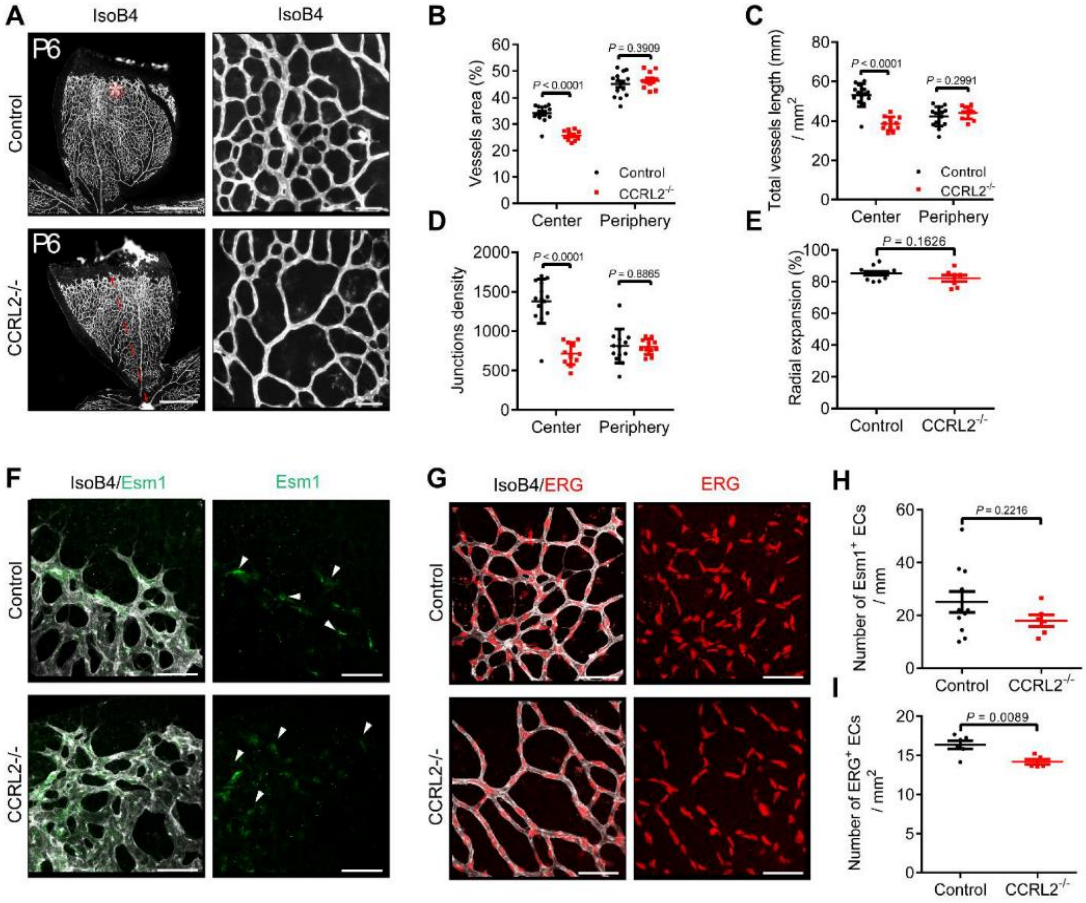

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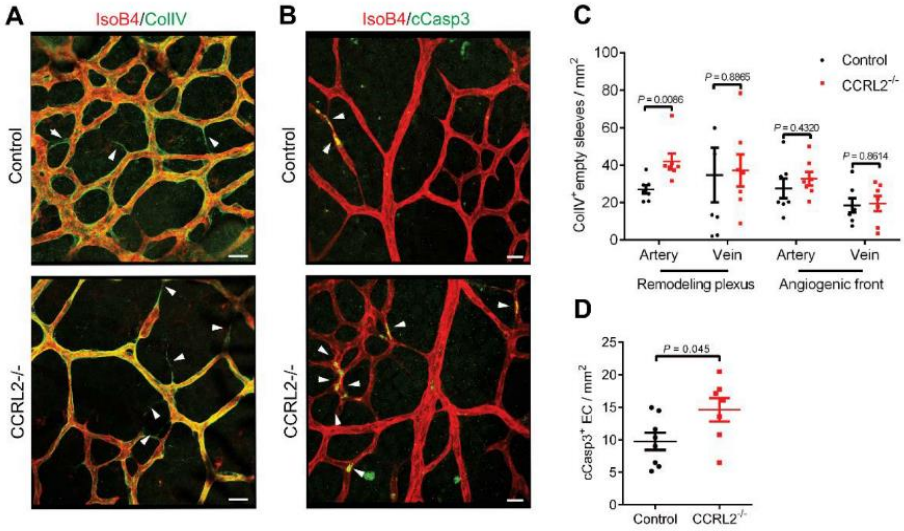
Figure 2



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588 **Figure 3**

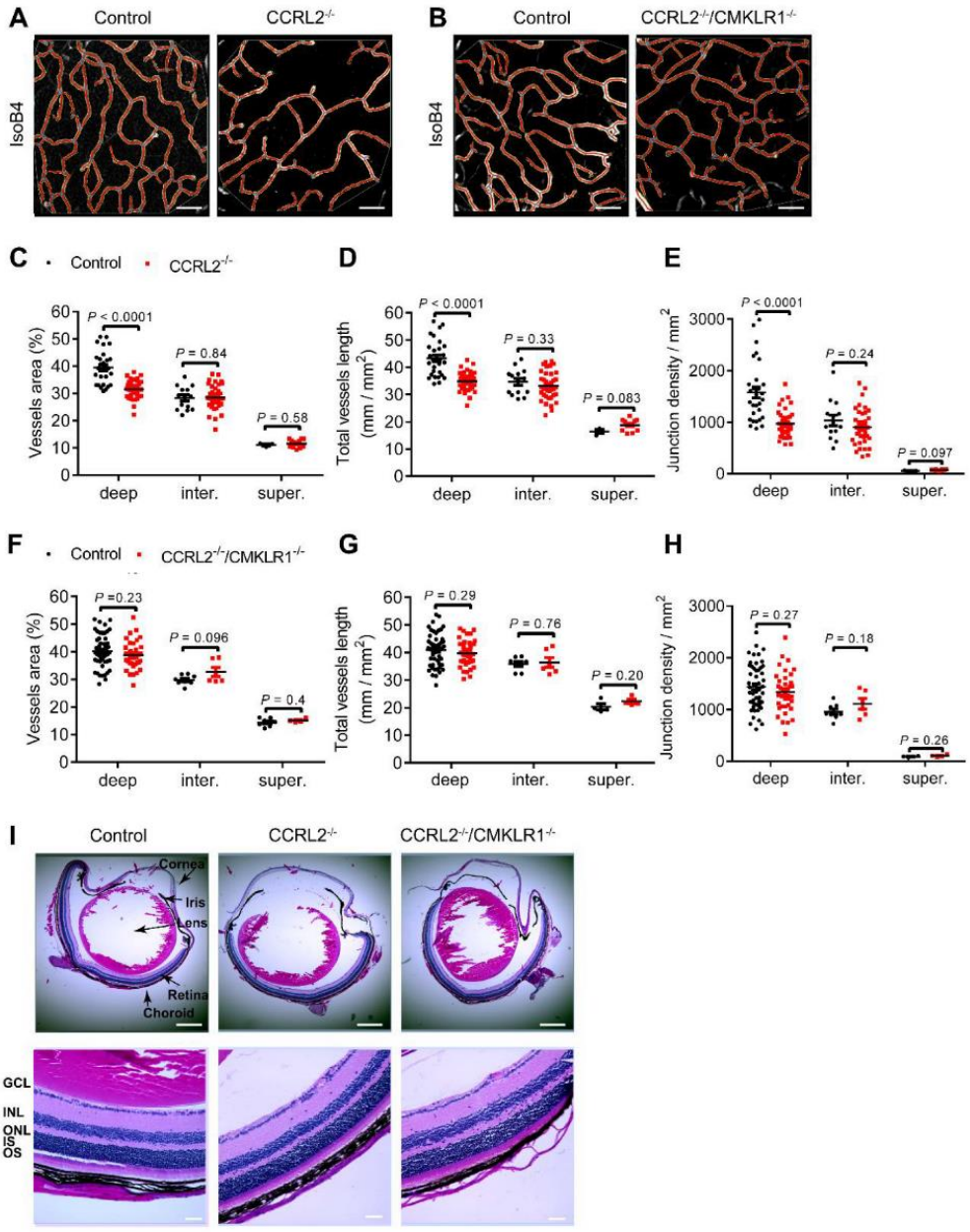

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Figure 4

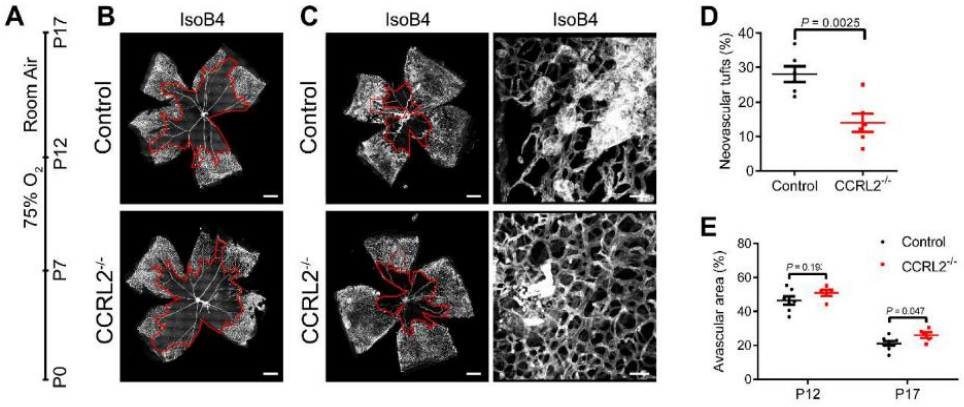

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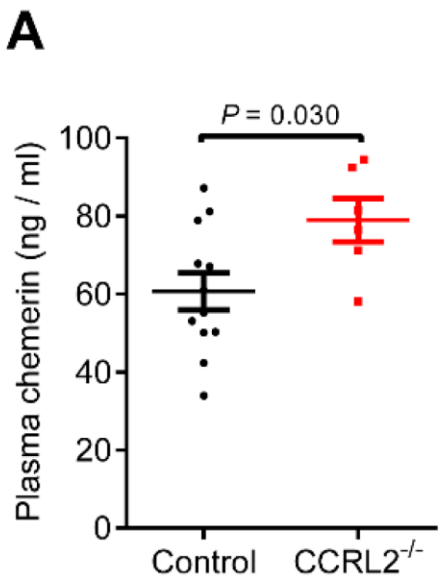
Figure 5



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3. Summary

This work allowed the identification of CCRL2 as a new regulator of angiogenesis. We demonstrated that CCRL2 plays a role in both normal and pathological angiogenesis. We showed that CCRL2$^{-/-}$ mice have a phenotype almost identical to that observed in mice overexpressing bioactive chemerin in basal keratinocytes of the skin and presenting high levels of chemerin in their blood. Knockout of CCRL2 reduced angiogenesis, without affecting the sprouting of new vessels but by promoting the regression of vascular branches in the remodeling zone of the plexus.

The mechanism by which the absence of CCRL2 reduces angiogenesis remains unclear. Animals lacking CCRL2 were shown to have higher circulating chemerin levels in the blood, and this may be due to the absence of chemerin scavenging by CCRL2. The erythrocyte lineage was shown to express CCRL2 at high level, and red blood cells might therefore play the role of a chemerin sink. This hypothesis should however be tested further.

C. Characterization of the mouse chemerin/CMKLR1 system in lymphangiogenesis

1. Objectives of Article 3

Angiogenesis and lymphangiogenesis are critical processes that allow tumor progression and the formation of metastases, which is the main cause of mortality in cancer patients (Hanahan and Weinberg, 2011; Reddy et al., 2012; Massagué and Obenauf, 2016). The ability to colonize distant sites is correlated with the formation of new blood and lymphatic vessels in different types of cancer (Achen et al., 2005; Balsat et al., 2011). Most studies are focused on the angiogenesis process, and the lymphatic system often remains the forgotten part of the tumor vascularization. Tumor-associated lymphangiogenesis is however of high significance, since the draining lymph nodes (LNs) constitute the first site of metastasis in many human malignancies, such as melanoma and various carcinomas. Lymph node metastases are often of poor prognosis for the patients. Encouraging data have been reported for the combination of anti-angiogenic and anti-lymphangiogenic agents (Tampellini et al., 2016). As vascular angiogenesis, lymphangiogenesis is a complex biological process regulated by a large panel of molecules including growth factors, their cell surface receptors, integrins, proteases, and their inhibitors.

After studying the role of the chemerin system in the regulation of angiogenesis, the objective of this work is to investigate the impact of chemerin on the lymphangiogenesis process.

In this work, we used the B16F10 melanoma cell line because of its great ability to invade the lymphatic system.

2. Article 3: Chemerin does not affect lymphangiogenesis and lymph node metastasis in a B16F10 melanoma graft model

In this section, I present some results of my thesis in the form of an article.

However, kindly note that it was not submitted so far

Chemerin does not affect lymphangiogenesis and lymph node metastasis in a B16F10 melanoma graft model

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Abstract

Lymphatic vessels are critical for the maintenance of tissue fluid homeostasis and their dysfunction contributes to several human diseases. Chemerin has emerged as a key player in the recruitment of leukocyte populations and regulates thereby inflammatory reactions. Yet, little is known regarding its roles in angiogenesis, and almost nothing is known about its involvement in lymphangiogenesis. The aim of this work was to investigate whether chemerin could play a role in lymphangiogenesis. We found here that, following subcutaneous tumor implantation in the ear, the metastatic process to lymph nodes as well as normal and tumor-induced lymphangiogenesis were not affected by chemerin in mice overexpressing a bioactive form of the protein.

Introduction

Clinicopathologically, lymphatic metastasis is the most common route of metastasis from original sites (primary tumors) to lymph nodes (secondary tumors), accounting for more than 60% of tumor metastatic incidences. Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is an integral part of both normal developmental and numerous pathological processes, ranging from inflammation and wound healing to cancer [1]. After decades of extensive study, anti-angiogenic drugs have recently entered the clinic, and with concomitant use of chemotherapy they can significantly prolong patient survival [2]. Although both blood and lymphatic endothelial cells originate from common developmental precursors, their structures and functions are quite different [3]. Unlike blood capillaries, lymphatic capillaries are formed by endothelial cells with discontinuous basal lamina and overlapping intercellular junctions, and they lack mural cells [4]. Lymphatic vessels are essential for the maintenance of tissue fluid balance, immune surveillance, and absorption of fatty acids and are thus involved in pathological responses such as tumor metastasis and inflammation [5, 6]. Nevertheless, the molecular mechanisms regulating lymphangiogenesis, the formation of new lymphatic vessels from pre-existing ones, have been explored far less than those of angiogenesis. For example, whereas tumor metastasis to regional lymph nodes is used clinically as a prognostic tool and a guide to treatment, the molecular mechanisms remain unclear [7]. Because of the identification of lymphatic endothelial cell-specific markers, however, considerable progress has been made over the past several years [3].

Chemerin is a secreted protein of 16 kDa [8], originally isolated from inflammatory exudate fluid. Chemerin is produced by many cell types, including fibroblasts, adipocytes, hepatocytes and various epithelial cells as an inactive precursor, prochemerin. The carboxyl terminus can be cleaved by different proteases to generate active chemerin isoforms [9]. Active chemerin is a chemoattractant for immature myeloid and plasmacytoid dendritic cells (DC), macrophages, and natural killer (NK) cells [10].

We and others have identified chemerin as an anti-tumoral factor in different mouse models [11–15]. We also identified chemerin as a regulator of the physiological process of neoangiogenesis during the development of retina. we demonstrate that chemerin can contribute to the regression of vascular branches in the remodeling zone of the plexus and EC apoptosis [15].

Following the identification of chemerin as a negative regulator of angiogenesis, we investigated the consequence of chemerin overexpression in lymphangiogenesis.

Results

Normal patterning of ear lymphatic capillaries in adult K5-Chem mice

In order to investigate the role of chemerin in the patterning of the lymphatic vasculature in adult skin in vivo, we used a transgenic mouse model described earlier, in which a bioactive form of chemerin is overexpressed by skin keratinocytes, under the control of the keratin 5 gene promoter (K5-Chem model) [12]. In these mice, the levels of chemerin immunoreactivity and bioactivity are strongly elevated in the blood, affecting therefore all tissues in the body. The ear skin of these mice was explored. This model is suitable because of the simple and reproducible dissection for whole mount immunohistochemical analysis. The outer and inner parts of the ear skin were peeled away from the intervening cartilage (Fig. 1a). Our high-resolution whole-mount immunohistochemistry of the ear skin was performed with antibodies to lymphatic-specific hyaluronan receptor (LYVE-1) and α smooth muscle actin (αSMA) as a VSMC marker (Fig. 1b). We decided to focus our analysis on the outer ear skin because of the ease of dissection and clear αSMA staining compared to the inner ear skin. Morphometric analyses confirmed that the average area of LYVE-1+ cells and the average area α-SMA+ cells were not significantly affected in K5-Chem mice vs controls (Fig. 1c, d, e). These data suggest that chemerin don't affect lymphatic capillaries.

Normal patterning of ear lymphatic capillaries in adult CMKLR1-/- and CCRL2-/- mice

CMKLR1 is the main functional receptor of chemerin. In order to determine the role of CMKLR1 in the patterning of the lymphatic vasculature, we used a mouse line genetically invalidated for this receptor that was described previously [16]. The average area of LYVE-1+ cells and the average area αSMA+ cells were not significantly affected in CMKLR1-/- mice vs controls (Fig. 1a, c).

Following the observation that CCRL2-/- have the same phenotype than the overexpression of chemerin concerning the regulation of angiogenesis [15, 17], we investigated whether lymphatic vessels could be affected in CCRL2-/- mice. Similar to the observation made in k5-Chem mice, the average area of LYVE-1+ cells and the average area αSMA+ cells were not significantly affected in CMKLR1-/- mice vs controls (Fig. 1a, c).

These results confirms that the system chemerin/CMKLR1/CCRL2 is not implicated in lymphatic vessel formation.

Chemerin delays tumor growth

Chemerin was shown to display anti-tumoral properties in several mouse models [11, 14]. In a recent study, we reported that these properties could be attributed to an inhibitory effect on the vascularization of tumors, resulting in necrotic cell death [13, 15]. We further quantified the impact of chemerin on tumoral development by inoculating B16F10 tumor cells expressing the firefly luciferase enzyme (B16F10Luc+ cells) which allowed for in vivo follow-up of tumor over time. As expected, bioluminescence signal in K5-Cem mice was significantly decreased 12 days after injection (Fig. 3a, b). Grafting B16F10 or LLC cells in K5-Chem mice resulted in smaller tumors than in control mice (Fig. 3c, d). These results confirm the anti-tumoral effect of chemerin as previously reported.

Chemerin does not induce morphological changes in mouse ears following B16F10 tumor cell graft

The growth of B16F10Luc+ tumors did not induce atrophy, necrosis, desquamation of the ear skin up to 12 days after injection (Fig. 4a), neither in control mice nor in K5-Chem mice. The ear thickness of the uninjected ear was about 0.5 mm, while it increased to about 1 mm for the injected ear, both in control and K5-Chem mice (Fig. 4b, c). This increase in ear thickness, following tumor cell graft, involved the stratum corneum, stratum granulosum, and stratum spinosum of the epithelium, as well as the connective tissue infiltrated by leukocytes. The thickness of the uninjected ear was significantly lower in the contralateral ear of K5-Chem mice compared to control mice, suggesting that the size of the growing tumor could affect the contralateral ear. These results suggest that the inflammatory reaction secondary to the development of the tumor in the ear injected with B16F10 melanoma cells is not affected by chemerin overexpression.

Chemerin does not affect lymphangiogenesis in ears receiving a B16F10 cell graft

Melanoma is one of the tumors that metastasize mainly through invasion of the regional lymph nodes [18–21].We investigated further whether chemerin have any anti-metastatic effect. We used B16F10Luc+ melanoma to explore their metastatic potential. Quantification of the luciferase signal from the lymph nodes showed no difference between controls and K5-Chem mice at this stage of tumor progression (Fig. 6b).

On day 12, metastases were found to be present in regional cervical lymph nodes of 40% of mice bearing ear melanomas. This percentage was the same in K5-Chem mice (Fig. 6c). It appeared that the metastasis was no different between K5-Chem mice and their control (Fig. 6d). These data suggest that chemerin does not affect lymph node metastasis.

Chemerin does not affect the metastatic potential of B16F10Luc+ cells to regional lymph nodes

Melanoma is one of the tumors that metastasizes mainly through invasion of the regional lymph nodes [19–22]. We investigated whether chemerin, besides inhibiting tumor growth, could also exhibit an anti-metastatic effect. The metastatic potential of B16F10Luc+ melanoma cells to regional lymph nodes was explored following the graft into ears. Quantification of the luciferase signal emitted from the lymph nodes showed no difference between control and K5-Chem mice at any stage of tumor progression (Fig. 6b).

On day 12, regional cervical lymph nodes were collected following the sacrifice of animals, and metastases were found to be present in 40% of mice bearing ear melanomas. This percentage was the same in control and K5-Chem mice (Fig. 6c, d). These data suggest that chemerin does not affect lymph node metastasis in this setting.

Discussion

In adults, lymphangiogenesis occurs during the formation of the corpus luteum and during wound healing and may be abundant in association with pathological processes [22]. Thus, lymphangiogenesis typically occurs at sites of inflammation induced by factors produced by macrophages and granulocytes. The process contributes to reduction of tissue edema and activation of immune responses by facilitating fluid drainage and the transport of dendritic cells and macrophages [23, 24]. The lymphatic system is also a main route of tumor dissemination and metastasis. Tumor invasion to regional lymph nodes is often the first sign of tumor metastasis and a marker of poor prognosis. Stimulation of growth and dilation of preexisting lymphatic vessels by the tumor facilitates tumor spread via the lymphatics [21, 25] and the contribution of intratumoral lymphangiogenesis may depend on tumor type [26–28].

The current model for the development of the mammalian lymphatic vascular system conforms to the centrifugal theory originally proposed by Florence Sabin over 100 years ago [29].According to this model, lymphatic endothelial cells (LECs) differentiate from blood endothelial cells (BECs) and migrate from veins to form lymph sacs during embryogenesis. Sprouting from these sacs gives rise to an immature lymphatic network which remodels into a hierarchal pattern of capillaries and valved collecting vessels [3]. The centrifugal theory for mammals has been supported by the expression pattern of molecular markers of LECs [30], lineage tracing experiments [31], and by the unique mutant phenotypes of genetically modified mice, reviewed in [32]. Despite advances, the molecular mechanisms driving the expansion of the lymphatic network remain largely unknown.

Chemerin is a chemoattractant and a member of adipokines. Studies have reported that chemerin plays important roles in regulating angiogenesis, cell proliferation and migration, inflammation, chemotaxis, glucose, and lipid metabolism [9, 12, 13, 15, 33–36]. Therefore, exploring the possible roles of chemerin in lymphangiogenesis may lead to a better understanding of tumor progression and metastasis, and will be helpful in improving cancer therapy.

In the present work, we used a genetic model of bioactive chemerin overexpression [12] to explore the potential effect of chemerin on the lymphangiogenesis and lymph node metastasis.

LYVE-1 is a hyaluronan receptor highly expressed by lymphatic capillaries but not by collecting lymphatic vessels or valves [37, 38]. We show that the density of lymphatic capillaries in K5-Chem mice is not affected in physiological context.

It should be noted that invalidation of the CMKLR1 gene or CCRL2 did not modify the area occupied by lymphatic vessels in the ear. This confirms that the chemerin system don’t affect lymphatic capillaries in normal conditions.

B16F10 mouse melanoma implantation in the ear allowed the evaluation of lymphangiogenesis and the spontaneous metastatic development of B16F10 tumors into local cervical lymph nodes. Ears are composed of a thin epidermis, arranged as 2 to 3 viable layers of keratinocytes, and a thicker dermis, with a low density of elongated fibroblasts. The ear

also encloses striated muscle fibers between the dorsal dermis and the cartilage [39]. The thin (approximately 200 mm) ear allows a synchronous examination of B16F10 induced reaction of all its tissue component.

Several mouse models of cancer have also supported an anti-tumoral role of chemerin, including in the B16 melanoma model [11]. We confirmed here that B16F10 melanoma cells injected in our model of K5-Chem mice grow less efficiently than in control mice. However, we demonstrate that chemerin don't affect lymphangiogenesis in the mouse ear.

Metastasis development in regional lymph nodes is a frequent and early event in melanomas, since a biopsy of sentinel lymph nodes is performed to establish a diagnosis of the melanoma stage [40]. In this study we don't show any correlation between primary tumor and lymph node metastasis.

All together, these observations suggest that the function of chemerin in lymphatic vessels is different from its function in blood vessels. Chemerin is not required neither for the expansion neither for the specification or maturation, of the lymphatic vasculature.

Methods

Animals and cell lines

C57BL/6J mice were purchased from Janvier. Mice overexpressing chemerin in keratinocytes under the control of the keratin K5 promoter (K5-Chem model) [12] invalidated for CMKLR1 [16] or CCRL2 [41] were described previously. Mice were maintained in a specific pathogen-free facility with environmental enrichment and unlimited access to food and water. Experimental animals were used between 6 and 8 weeks of age.

All animal experiments were conducted in accordance with European guidelines and local regulations and approved by the local ethics committee (Commission d'Ethique du Bien-Etre Animal, CEBEA) of the ULB Medical School. All efforts were made to minimize suffering.

B16F10 Red-FLuc (B16F10Luc+) mouse melanoma cells (Perkin Elmer, BW124734) were grown in RPMI-1640 (Life Technologies) supplemented with 10% fetal bovine serum (Gibco), 1% sodium pyruvate (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2. The cells were first harvested from culture by trypsinization and washed three times with cold serum-free medium before injection.

Tumor graft model

B16F10Luc+ cells (4×10^5 cells in 50 µl) were injected between the dorsal epithelium and the cartilage in the center of the ears using a 30 g needle, under anesthesia by ketamine and xylazine. The size of the tumors was monitored with a caliper and the tumor volume was estimated by the formula $V = (\text{length} \times \text{width}^2)/2$.

In vivo follow-up was performed at day 2,5 9 and 12 by bioluminescence imaging. Mice were anesthetized with isoflurane (4% for induction, then 2% for maintenance), were shaved to decrease light absorption and scattering, and injected s.c. with D-luciferin (20 mg/ml solution in 0.9% NaCl, 150 mg/kg body weight, VivoGlo, Promega). Mice were imaged in a PhotonImager Optima (Biospace Lab, France) for at least 25 min, and images were analyzed by the M3Vision software (Biospace Lab). The signal intensity was collected individually for the ear and lymph node areas for a 5 min period within the plateau.

At day 12 after the graft, mice were perfused under anesthesia through the left ventricle with 10 mL of 2% paraformaldehyde and tumors were harvested and weighed. Macroscopic pictures of the ears (Fig. 4a) were captured on a Leica M80 stereomicroscope.

Fluorescence and light microscopy

The skin of adult mouse ears was dissected, fixed overnight at 4 °C in 4% paraformaldehyde in phosphate buffered saline (PBS), and washed 3 times in PBS. Cartilage, fat tissue, and hairs were removed before staining. The samples were incubated overnight at 4°C in 10 mM Tris HCl, pH 7.4, containing 150 mM NaCl, 3% blocking reagent (ThermoFisher, 37580), and 0.5% Triton X-100 (TNBT), then overnight at 4°C with anti-αSMA (1:100, Invitrogen, 53-9760-82) and anti LYVE-1 (1:100, Invitrogen, 50-0443-80) antibodies diluted in TNBT. The next day, samples were washed three times in PBS and flat-mounted on glass slides using the FluorSave medium

(Millipore, 345789). Pictures and tiles were imaged with an Axio Imager Z1 fluorescence microscope (Zeiss).

The lymphatic vessel density and αSMA coverage were quantified on at least three regions in the periphery of the outer skin layer of each ear by the Angiotool software [41]. The fluorescence threshold level was set to minimize background.

Ears from tumor-grafted mice were harvested, fixed overnight at room temperature in Davidson fixative, embedded in paraffin, and 5 µm sections were stained with hematoxylin and eosin (HE). The thickness of the ears was measured at 6 positions on images taken at x20 magnification, using the Fiji software.

Analysis of metastasis

The two largest superficial cervical lymph nodes (Fig. 6a) were collected 12 days after the tumor graft, fixed in 4% PFA and examined macroscopically for the presence of metastases.

Statistical analyses

All data are presented as mean ± SEM. Statistical analysis was performed using the Instat or Graphpad Prism 7 software. The unpaired two-tailed Student's t-test was used to determine statistical significance between two groups. Reproducibility was ensured by performing several independent experiments. Analyses were performed blinded, without knowing the genotype of animals or experimental conditions. All experimental data collected from knockout animals were compared with those obtained from littermate controls. In all experiments, animals were age-matched and distributed randomly into groups.

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Author contributions

M. Parmentier and C. Ben Dhaou designed the study. C. Ben Dhaou performed experiments with the contribution of L Larbanoix for bioluminescence imaging. C. Ben Dhaou wrote the initial draft. M. Parmentier supervised the study and wrote the final draft. M. Parmentier acquired funding. All authors edited and approved the manuscript.

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Competing Interests

M.P. is CEO of the Biotech Company Gepeceron, a spin-off of the Université Libre de Bruxelles. All other authors declare no conflict of interests.

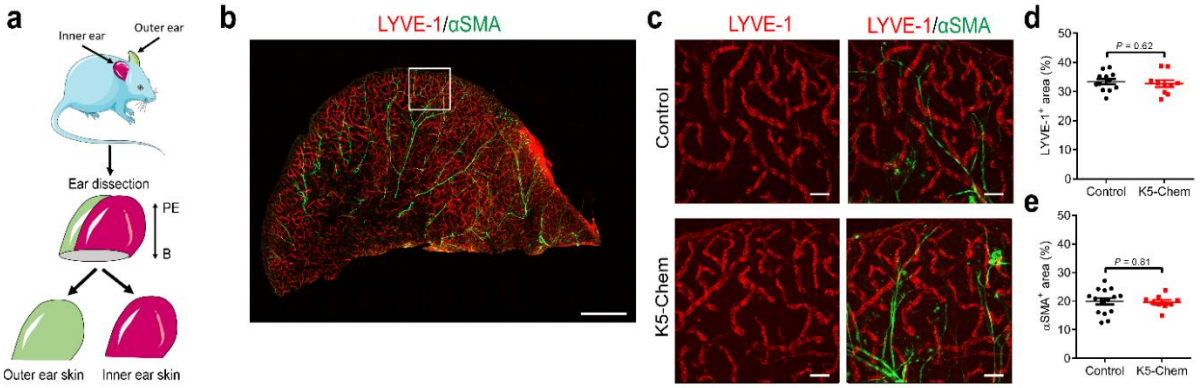

Figure 1. Overexpression of chemerin does not affect the lymphatic vasculature in mouse ears. a Diagram of the experimental procedure for adult ear skin whole-mount staining. a The orientation of base (B)-periphery (PE) axis is shown. **b** Representative image of whole mount outer ear skin, stained for the lymphatic-specific hyaluronan receptor LYVE-1 and α-SMA as a pericyte marker. Scale bar: 2 mm. **c** Representative images of the periphery of the outer ear skin, stained for LYVE-1 and α-SMA. Scale bars: 200 µm. **d** Quantification of the LYVE-1 area, normalized to the surface, in the peripheral part of the outer ear skin of K5-Chem (n = 10) and control mice (n = 12). **e** Quantification of the α-SMA area, normalized to the surface, in the peripheral part of the outer ear skin of K5-Chem (n = 9) and control mice (n = 15). P values versus controls by two-tailed unpaired Student's t test. All data are shown as mean ± SEM. Each point represents 1 field of view.

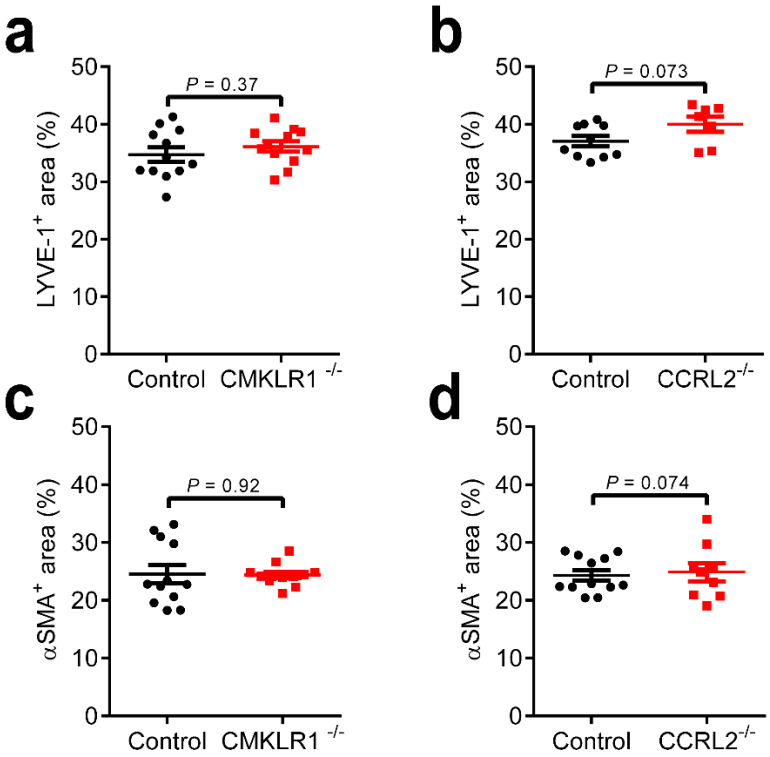

Figure 2. The knockout of the functional chemerin receptor CMKLR1 or of the non-signaling receptor CCRL2 does not affect the lymphatic vasculature in the mouse ear. a Quantification of the LYVE-1 area, normalized to the surface, in the peripheral part of the outer ear skin in CMKLR1-/- and control mice (n = 12). **b** Quantification of the LYVE-1 area, normalized to the surface, in the peripheral part of the outer ear skin in CCRL2-/- (n = 7) and control mice (n = 11). **c** Quantification of the α-SMA area, normalized to the surface, in the peripheral part of the outer ear skin in CMKLR1-/- and control mice (n = 12). **d** Quantification of the α-SMA area, normalized to the surface, in the peripheral part of the outer ear skin in CCRL2-/- (n = 9) and control mice (n = 12). P values versus controls by two-tailed unpaired Student's t test. All data are shown as mean ± SEM. Each point represents 1 field of view.

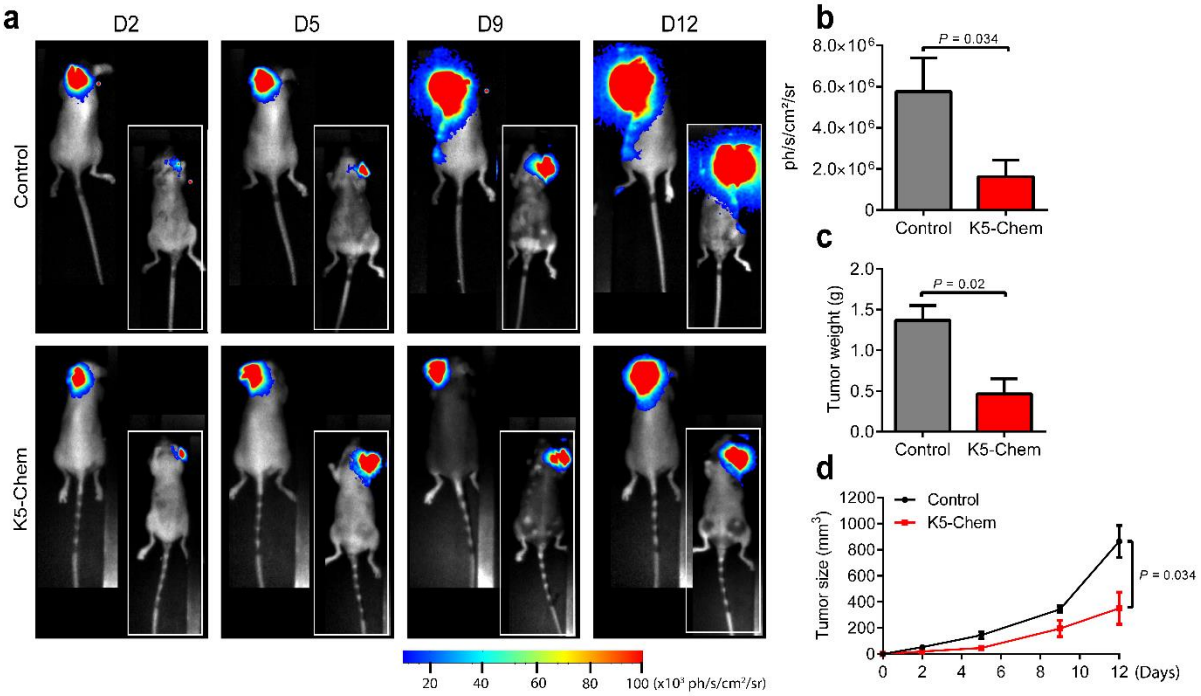

Figure 3. Overexpression of chemerin delays tumor growth. a A suspension of 4×10^5 B16F10Luc+ tumor cells in 50 µl was injected under the dorsal skin layer of the ear (intradermal injection) of control and K5-Chem mice. Representative images of the dorsal view of the bioluminescence data generated until 12 days post-injection. Insets show the ventral view. **b** Bioluminescence signal detection at day 12 following injection (n = 5 for controls, n = 6 for K5-Chem). **c** The weight of the tumors was measured following sacrifice at day 12 (n = 5 for controls, n = 6 for K5-Chem). **d** The tumor size was monitored with a caliper until day 12. P values versus control by 2-tailed unpaired Student's t test. All data are shown as mean ± SEM. Each point represents 1 animal.

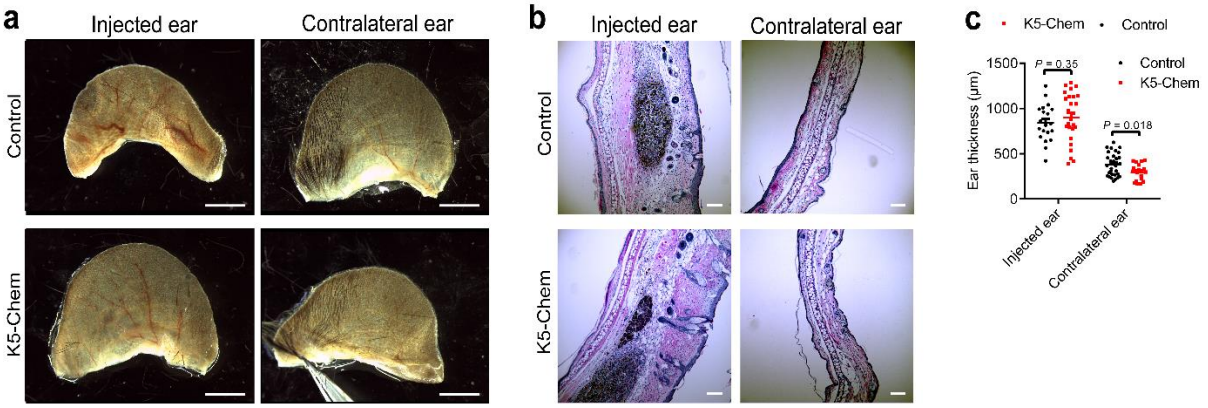

Figure 4. Overexpression of chemerin does not promote macroscopic changes in the mouse ear after B16F10Luc+ tumor cell injection. a Representative images of the dorsal view of control and K5-Chem ears injected with B16F10Luc+ tumor cells and their contralateral ear, 12 days after injection. Scale bars: 2 mm. **b** Representative images of H&E-stained sagittal sections of ears injected with B16F10Luc+ tumor cells, and the contralateral ear, collected from control and K5-Chem mice. Scale bars: 100 µm. **c** The ear thickness was quantified in K5-Chem (n = 26 for injected ear and n = 18 for contralateral ear) and control mice (n = 20 for injected ear and n = 30 for contralateral ear). P values versus controls by 2-tailed unpaired Student's t test. All data are shown as mean ± SEM. Each point represents 1 measurement.

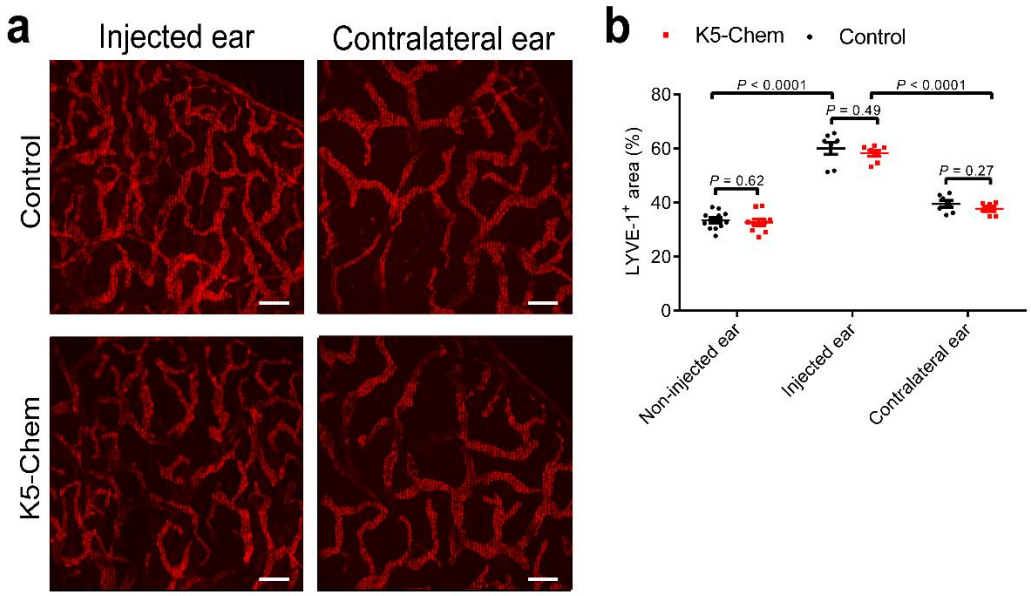

Figure 5. Overexpression of chemerin does not affect lymphangiogenesis. a Representative images of the peripheral part of the outer skin of injected and contralateral ears, stained for LYVE-1, collected from control and K5-Chem mice. Scale bars: 200 µm. **b** Quantification of the LYVE-1 area, normalized to the surface, in the peripheral part of the outer ear of K5-Chem (n = 10 for non-injected ear, n = 7 for injected ear and n = 6 for contralateral ear) and control mice (n = 12 for non-injected ear, n = 7 for injected ear and n = 7 for contralateral ear). P values versus controls by 2-tailed unpaired Student's t test. All data are shown as mean ± SEM. Each point represents 1 field of view.

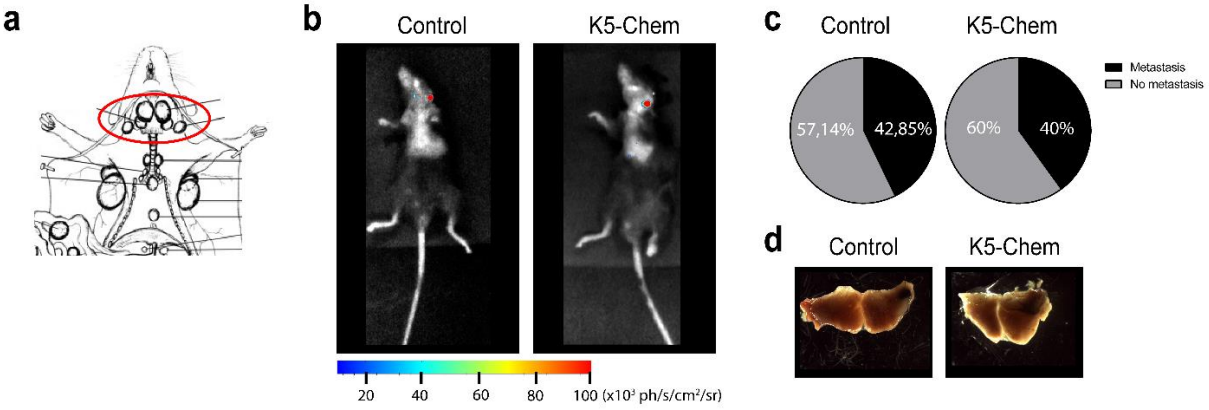

Figure 6. Overexpression of chemerin does not affect lymph node metastasis. a Localization of sampled lymph nodes (red). **b** Representative images of the bioluminescence data generated from lymph nodes at day 12 post B16F10Luc+ tumor cell injection. **c** Quantification of the percentage of control (n = 7) and K5-Chem mice (n = 5) with cervical lymph node metastases on day 12. **d** Representative images of cervical lymph nodes from control and K5-Chem mice.

IV. General discussion, conclusions and perspectives

This work leaves unanswered questions and opens up a number of perspectives for future research

Improving our understanding of the biology of angiogenesis could reveal new targets for treating the diseases involving deregulations of this complex process. I have started my introduction by shedding light on the crucial but still elusive part of the angiogenic process: the regression of newly formed blood vessels following the phase of vessel sprouting. In fact, in all situations of physiological angiogenesis, the migration of endothelial cells and their organization into new vessels is followed by a phase of vessel maturation and regression resulting in a perfused network that meets the metabolic requirement of the tissue and preserves homeostasis (Egginton and Gaffney, 2010). While the current research is focused on models of pathological angiogenesis with the aim of finding a molecule that would stop the process, very little is known about the mechanisms and endogenous anti-angiogenic factors that control and limit excessive vessel formation (Cuevas and Boudreau, 2009). This is the context in which we studied chemerin and its role in angiogenesis.

So, what did we learn from my thesis work about the properties of chemerin in the angiogenesis process? And how relevant is this work for angiogenesis-dependent diseases?

I will discuss, for each section, some hanging questions and potential future experiments that may help validating the proposed hypotheses to better elucidate the roles of the chemerin system in angiogenesis.

Role of the chemerin system in a physiological context

Previous results of our research group have shown that the expression of bioactive chemerin by B16 melanoma and Lewis lung carcinoma (LLC) cells, or in basal keratinocytes of the host mice, delays tumor growth *in vivo* (Dubois-Vedrenne et al., 2021). It was observed that chemerin inhibits the angiogenesis process in growing tumors and that CMKLR1 mediates this anti-tumoral effect of chemerin. Following these observations, we were interested in identifying the role of the chemerin system in physiological angiogenesis. Indeed, the mechanisms and pathways involved in the activity of chemerin might be easier to identify in a well controlled physiological context than in the heterogeneous and disorganized tissue of tumors. We turned to the development of the vascular network in the mouse retina for this purpose. This process takes place in newborn animals and is therefore easily accessible. It also occurs in well defined successive phases, starting by the expansion of a two-dimensional plexus from the central veins

and arteries. Simple image analysis routines allow therefore to characterize the network at different steps by a number of solid and reproducible parameters. These characteristics explain why the developping mouse retina became one of the most studied angiogenesis model used to determine *in vivo* the role of various genes and pathways.

We confirmed that CMKLR1, the main receptor of chemerin, is expressed by retinal endothelial cells, including during the development of the network. We showed that neither overexpression of chemerin nor the lack of CCRL2 affected the progression of the vascular network toward the retina periphery, the sprouting and proliferation of ECs, or the coverage of vessels by pericytes.

We show that the overexpression of chemerin increased vessel regression as well as endothelial cell apoptosis in the remodeling zone of the plexus. *In vitro* experiments have clearly shown that the role of chemerin in vessel regression is completely independent from the recruitement of immune cells.

In the context of angiogenesis, several lines of evidence indicate that endothelial cell apoptosis contributes to vascular regression in a number of cases. This involves the deletion of extra cells from the network, thereby improving the efficiency of the regression process or clearing away isolated vessel segments remaining after vessel regression (Claxton and Fruttiger, 2003). It is important to know that this interpretation arises primarly from the analysis of normal retinal angiogenesis. The question remains whether this will hold true in the frame of vascular growth in other tissues.

Other lines of evidence do not support the role of EC apoptosis in the pruning of angiogenic vessels. There was no correlation between vessel regression and EC apoptosis during retinal angiogenesis in the rat (Hughes and Chang-Ling, 2000), and whereas most apoptotic ECs are found in regressing vessels during retinal angiogenesis in mice (Watson et al., 2016), only 5% of all regressing vessels actually contain apoptotic ECs (Franco et al., 2015). In many mutant mouse lines in which retinal angiogenesis is affected, a direct correlation between EC apoptosis and vessel regression is not observed. Furthermore, time-lapse imaging studies in zebrafish have shown that EC apoptosis is not common during vessel pruning (Chen et al., 2012; Kochhan et al., 2013; Lenard et al., 2015).

After these observations, a natural question comes to mind: If it's not apoptosis then what is it?

The differences in blood flow between neighboring vessels favor instead the migration of ECs out of the vessel with the lower shear, leading to its regression (Chen et al., 2012; Lenard et al., 2015). Therefore, EC migration appears as the main mechanism of vessel regression during adaptive vessel pruning, and this is highly conserved across species and vessel networks. It is therefore possible that chemerin promotes more EC apoptosis than what is usually associated with vessel pruning in most physiological contexts.

I suggest to investigate the role of chemerin on the effects of blood flow and shear stress in the developing retina. For this purpose, I propose to analyze the alignment of endothelial cells, a hallmark of endothelial response to flow, by using CD31 as a major endothelial marker exposed on plasma membranes (Hahn and Schwartz, 2009). By labeling CD31, the contours of the cells and their orientation become clearly visible, both in large and small arteries, and we could analyze the EC alignment by measuring the average angle between the long axis of individual cells and the direction of the vessel, which represents the direction of the flow in the vessel (Boriushkin et al., 2019). The comparison of retinas collected from mice overexpressing or not chemerin would allow to determine whether chemerin modifies the response of endothelial cells to flow and shear stress.

We showed also that, in contrast to other situations affecting the early vascular network of the retina (Ishida et al., 2003; Kubota et al., 2009), these changes were not compensated later in life, and adult mice were still characterized by a less dense vascular network, at least in the intermediate and deep layers. The overall organization of the retina did not appear to be affected by these modifications in the vascular network and all these changes were totally reversed in mice lacking CMKLR1, demonstrating the role of this receptor in the process. These data contrast with previous reports in the literature claiming a pro-angiogenic effect of chemerin (Bozaoglu et al., 2010; Kaur et al., 2010; Nakamura et al., 2018), observations that we could not reproduce.

Following the study of physiological angiogenesis, I went back to models involving angiogenesis in pathological contexts.

Role of chemerin in the model of Oxygen-Induced Retinopathy

Angiogenesis is controlled by a balance of pro- and anti-angiogenic factors. When this equilibrium comes out of balance, pathological neovascularization (NV) may be induced. This is a common factor in several retinal diseases, including the wet form of age-related macular degeneration (AMD), retinopathy of prematurity (ROP) and diabetic retinopathy (DR) (Campochiaro, 2013). These diseases of the retina are the leading causes of severe visual impairment and blindness in developed countries. The oxygen-induced retinopathy (OIR) model is commonly used to study retinal diseases involving NV, since it shares many hallmarks with human ischemic retinopathies (Scott and Fruttiger, 2010; Stahl et al., 2010; Vessey et al., 2011; Vähätupa et al., 2016).

What we actually showed is that chemerin did not significantly affect the first phase of the OIR model, in which hyperoxia induces vessel obliteration in the central capillary network. However, an essential information is missing. EC apoptosis is not key in all vessel pruning sutuations, it has been reported in the case of hyperoxia-induced retinal vaso-obliteration (Alon et al., 1995). The importance of apoptosis in vessel regression in this model is supported by one study showing that retinal vaso-obliteration (Wang et al., 2011) is inhibited in mice lacking genes essential for the apoptotic process. Therefore, chemerin might favor EC apoptosis in the normal physiological context of retinal development, while it may be unable to enhance this mechanism in pathological contexts in which EC apoptosis takes already an important role.

We showed also that the overexpression of chemerin induced a slight delay in the recovery of a “normal” vascular network and a significant reduction in pathological angiogenesis after hypoxia. It is hard at this stage to define the precise mechanism causing vessel regression in mice overexpressing chemerin. Further studies and investigations are needed to better understand if apoptosis combined with shear stress in K5-Chem mice constitute the cause of vessel regression, or are rather one of the the consequences of vessel regression.

Role of chemerin in the model of hind limb ischemia

The model of retinal development during the first weeks of life is not representative of the mechanisms supporting arteriogenesis (Gerhardt and Betsholtz, 2003). Arterial growth and

repair mechanisms are however of fundamental interest since arteries are the main vessels affected in the most frequent cardiovascular diseases in humans. The mouse hind limb is perfused by the femoral artery, which gives rise to the A. profunda femoris (Limbourg et al., 2009). This A. profunda femoris divides into collateral vessels that pervade the semimembranosus and semitendinosus muscles of the proximal limb and later reconnect to the femoral artery closer to the knee (Limbourg et al., 2009). Ligature of the femoral artery causes a sudden increase of the blood flow and significant hemodynamic changes in the collaterals (Heil et al., 2006). The mouse model of hind limb ischemia recapitulates the development of collaterals in peripheral arterial diseases in humans (Hirsch et al., 2006). Although collateral development occurs mostly independently from ischemia, ligation of the femoral artery induces ischemia distally in the gastrocnemius (GC) muscle of the calf, which triggers an angiogenic response (Heil et al., 2006). The advantage of this model is that it allows to evaluate, at the same time, arteriogenesis, angiogenesis and ischemic perfusion after a relatively simple surgical intervention.

We showed that chemerin-overexpressing mice exhibited a delay in the recovery of distal blood flow and a reduction in the number of vessels in the gastrocnemius muscle in the hind limb ischemia model. Following these results, it would be interesting to investigate the impact of chemerin specifically on the arteriogenesis process. This could be done by performing the morphometric assessment of collateral artery growth in the semimembranosus muscle on tissue sections.

Role of chemerin in the model of tumor graft

Regression of established microvessels is associated with remnant basement membrane sleeves from which ECs have been lost (Mancuso et al., 2006). In line with previous observations (Dubois-Vedrenne et al., 2021), we observed a growth delay for LLC and B16 tumors grown in chemerin-overexpressing mice. We also showed in these tumors a markedly increased labelling of collagen IV^+ and changes in the structure of neovessels, characterized by fuzzy and extended labeling for collagen IV, a feature previously associated with vessel instability.

As I mentioned in the introduction, there are two conflicting schools regarding the mechanisms and benefits of anti-angiogenic therapies: the destruction of tumor vessels or the normalization of the vascular network (Carmeliet and Jain, 2011b).

Since we still have vessels, the next question is: Is chemerin requiered for vessel normalization ?

As previously mentioned, a structurally and functionally abnormal vasculature is a hallmark of most cancers. Although normal angiogenesis appears as a very controlled process, tumor vessels are, unlike normal vessels, chaotic in their organization and permeable, and the vascular network of tumors is altogether poorly functional. The increase in permeability may be due in part to the absence of pericyte coverage and defects in the vascular basement membrane. Successful anticancer treatments by conventional chemotherapy or radiotherapy are strongly dependent on an adequate blood supply, which is required to distribute drugs properly to all parts of the tumor or supply the oxygen necessary for the production of free radicals (Carmeliet and Jain, 2011a). Considering this, Jain et al. speculate that, instead of inhibiting the formation of new blood vessels in the tumor, anti-angiogenic agents normalize the disorganized tumor vascularization (Jain, 2005). Consequently, there is a restored balance between pro- and anti-angiogenic molecules, a higher pericyte coverage and a restoration of the basement membrane, leading to improved blood circulation and improved efficiency of chemotherapy and radiotherapy.

A better understanding of how chemerin affects the vascular network in tumors is therefore desirable to determine potential therapeutic benefits of CMKLR1 agonists. I suggest to investigate the vessel functionality by injecting tumor-bearing mice with lectin-fluorescein isothiocyanate (FITC) to label perfused vessels, or dextran-FITC to measure leaky vessels, and then stain B16-F0 and LLC tumor sections for CD31 to identify all vessels (perfused and nonperfused) so that the percentage of perfused and leaky vessels could be determined.

All of these observations converged to confirm the anti-angiogenic properties of the chemerin/CMKLR1 system in various contexts.

Role of CCRL2 in angiogenesis

We identified CCRL2 as a modulator of the chemerin/CMLKLR1 axis in angiogenesis. We showed that CCRL2 loss of function reduced the formation of vascular tufts in the oxygen-induced retinopathy model, and significantly delayed the recovery of a "normal" vascular network. It is interesting to note that the phenotype of these CCRL2 KO mice was almost identical to that observed in mice overexpressing bioactive chemerin in basal keratinocytes of the skin. In line with this, we showed that the chemerin blood levels were elevated in these mice, pointing toward a contribution of CCRL2 to the removal of chemerin from the extracellular compartments.

Can erythrocytes act as a sink for chemerin ?

CCRL2 is an atypical GPCR, which is apparently unable to stimulate signalling cascades in response to ligand binding. It lacks the DRYLAIV sequence at the boundary between the third transmembrane domain and the second intracellular loop, and this highly conserved motif amongst chemokine receptors is essential for G protein coupling and intracellular signalling. A recent study investigating the transcriptome of single cells during early development has shown that CCRL2 is expressed at the highest level in the erythroid lineage (Pijuan-Sala et al., 2019). The presence of CCRL2 on the plasma membrane of mature erythocytes has not been reported so far. I suggest therefore to investigate further the expression of the CCRL2 gene by erythrocyte precursors and the presence of the protein on circulating erythrocytes. With their large surface, this cell population could indeed play a significant role as a chemerin sink, as it does for various chemokines through the presence of the atypical chemokine receptor ACKR1 (DARC/Duffy) in their membrane. This could explain the higher concentration of chemerin observed in CCRL2 KO mice, and the retinal phenotype of these mice (Ben Dhaou et al., 2021a). To test further this hypothesis, we could test the expression of CCRL2 in erythocyte precursors isolated from human and mouse bone marrow, and test the binding of chemerin to mature erythrocytes by using an iodinated of GFP-fusion tracer in a binding assay. We could also test wether erythrocytes pre-incubated with chemerin are able to activate CHO-K1 cells expressing CMKLR1, demonstrating a chemerin-presenting role of these cells.

Role of the chemerin system in lymphangiogenesis

We showed that the chemerin system does not seem to play a role in lymphangiogenesis and the development of lymph node metastases. We should keep in mind that this might be model-dependent. Additional investigations in different contexts of lymphangiogenesis might therefore be performed to confirm our observations. As an example, another interesting experiment would be to use whole mouse mammary glands which provide a particularly clear view of the organization of the lymphatic vasculature (Betterman et al., 2012).

In our tumor model, we oberved a rapid lymphatic spread when cells are directly injected in the ear, which does not allow to monitor subtle changes in the efficiency of the metastatic process. The injection method might therefore be modified. I used to inject 4×10^5 cells in 50 µl directly in the ear. A more controlled way would be to implant in the mouse ear B16-F10 melanoma cells encapsulated in a biomaterial, which would prevent their rapid lymphatic spread (García-Caballero et al., 2017) and would allow to evaluate more precisely the consequences ofalterations of the chemerin system.

Signaling pathways mediating the anti-angiogenic effects of chemerin

We started to investigate the mechanisms by which the chemerin/CMKLR1 system affects angiogenesis. None of the classical pathways activated by CMKLR1 are obvious candidates for promoting the apoptosis of endothelial cells and the regression of newly formed tubules. Studies on tumor cell lines have reported that activation of CMKLR1 could lead the upregulation of PTEN actrivity through different pathways (Li et al., 2018; Rennier et al., 2020). We therefore investigated whether the PTEN/PI3K/AKT pathway might be involved in the effects of chemerin on endothelial cells. The use of several inhibitors of this pathway *in vivo* and *in vitr*o, suggests indeed that this might be the case. The inhibition of the PI3K-AKT pathway by chemerin, particularly in hypoxic conditions in which neoangiogenesis occurs, favors the activity of FOXO1, and this transcription factor is well known for promoting endothelial cell apoptosis and vessel regression. Additional experiments will however be necessary to confirm these observations and identify precisely by which mechanism the chemerin/CMKLR1 system controls the activity of PTEN in endothelial cells.

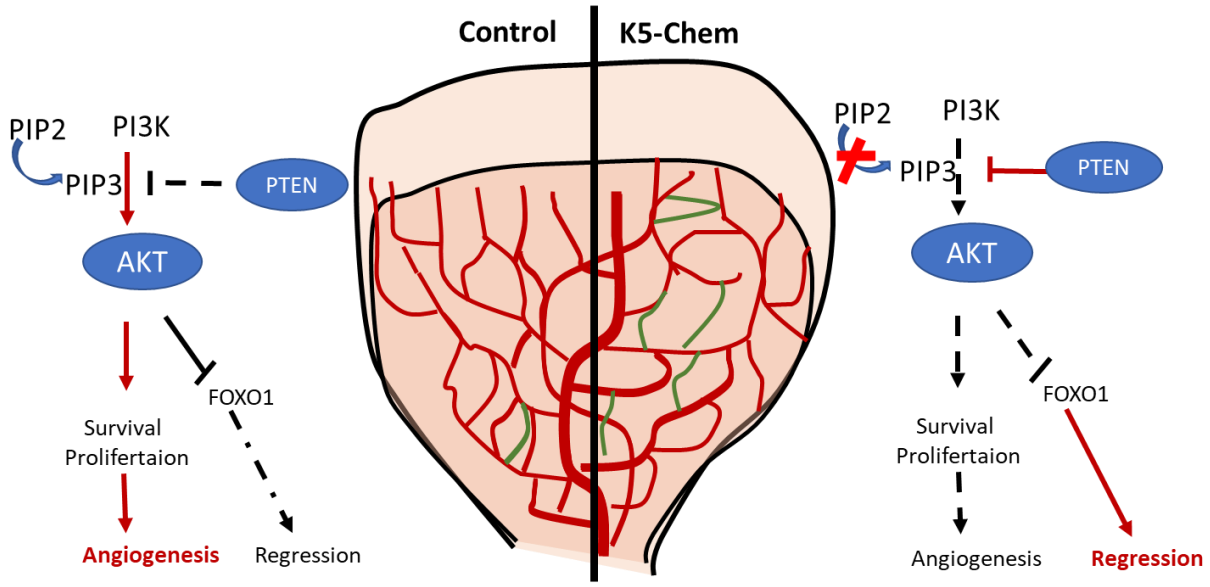

Figure 15: Model for signaling pathways mediating chemerin roles in endothelial cells.

Chemerin seems to act as a negative regulator of the PI3K-AKT pathway. This effect is mediated through an activation of PTEN, the inhibition of the PI3K/AKT pathway, and a release of the FOXO1 activity, resulting in a modification of the balance between endothelial cell survival and neovessel stabilization on one side, and EC apoptosis and vessel regression on the other. The precise mechanism by which CMKLR1 regulates PTEN activity in endothelial cells is presently unknown.

Which are the genes and pathways regulated by chemerin in endothelial cells ?

It has been shown that chemerin binding to CMKLR1 results in the transcriptional activation of the serum response factor (SRF) (Rourke et al., 2015). SRF is known to promote expression of its target gene *EGR-1* (early growth response 1) that in turn activates PTEN expression (Virolle et al., 2001; Horita et al., 2011). PI3K pathway activation can also decrease SRF levels and as a consequence EGR-1 transcription (Shin et al., 2006). There is therefore a potential positive feedback loop involving PTEN, AKT, SRF and EGR-1, and this pathway may mediate the effects of the chemerin/CMKLR1 system on endothelial cells. This hypothesis should be tested further by analysing the expression of the different factors involved in HUVECs treated or not with chemerin. RT-qPCR could be used to quantify the corresponding transcripts, while Western blotting would be used to detect the proteins and their phosphorylation status (AKT, PTEN). It might be useful to investigate the effect of chemerin in different conditions of culture of the HUVECs, including in the tube formation and bead sprouting assays, as these conditions are closer to the *in vivo* conditions of angiogenesis.

During vessel regression, blood vessels contract and EC retract, leaving behind empty basement membrane sleeves (Korn and Augustin, 2015) and leading to the upregulation of vasoconstrictory genes (Lobov et al., 2011). As we demonstrated that chemerin controls the switch between vessel maintenance and vessel regression, I suggest to investigate further the expression of a set of genes involved in vasoconstriction and EC survival, namely the endothelin receptor type A (*Ednra*), angiotensinogen (*Agt*), the apoptosis-related gene *Stat2* and the EC survival signaling receptor TEK/TIE2 (Augustin et al., 2009). More broadly, RNAseq experiments could be performed to determine the full transcriptome of endothelial cells and its modification upon chemerin stimulation. More particularly, the most interesting approach would be to run single cell RNA sequencing experiments on endothelial cells isolated from mice. The single cell format would allow to detect transcriptional changes in the different populations of endothelial cells.

Could CMKLR1 agonists be used as therapeutic agents ?

Provided the anti-angiogenic properties of chemerin, agonists of CMKLR1 could be considered as potential therapeutic agents. Yet, chemerin is a medium size protein and short peptides such as chemerin-9 (YFPGQFAFS, EC_{50}: 5 nM) have a very short half-life *in vivo*. A peptide analog (yYFPsQFa-Tic-S) was shown to display higher stability than chemerin-9. Other peptides have been described in a patent application (WO2013117581A1): Ac-Y-Cha-PGMYAFf and Ac-Y-Cha-Hyp-G-Cit-Fa-Tic-S have EC_{50} of 1-2 nM and a half-life in human serum of about 25 hours. We could use these peptides in *ex vivo* settings and in mouse models *in vivo* to evaluate their efficiency in tumoral, inflammatory and angiogenesis settings.

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In conclusion, this PhD work allowed us to reveal the importance of the chemerin system in the regulation of angiogenesis. The development of anti-angiogenic treatments is still limited by the incomplete knowledge of essential stop-signals that temper the growth of the vasculature and promote blood vessel regression. In this frame, our data suggest possible therapeutic applications of CMKLR1 agonists mimicking the effects of chemerin, with potential benefits in cancer and in other diseases involving excessive angiogenesis.

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VI. Appendix

A. Abstract and Poster at the Gordon Research Conferences (GRC) and the Gordon Research Seminar (GRS) on Vascular Cell Biology, Ventura, CA United States. January 19-25, 2019

Regulation of tumor angiogenesis by Chemerin and its receptor ChemR23

Cyrine Ben Dhaou[1] and Marc Parmentier[1]

[1] I.R.I.B.H.M, Université Libre de Bruxelles, Campus Erasme, 808 route de Lennik, B-1070 Brussels, Belgium.

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Neo-angiogenesis, the outgrowth of new blood vessels from preexisting ones, is a key process involved in development and tissue repair, but also in various pathological situations including cancer. The mouse retina has been used extensively over the past decades to study both physiological and pathological angiogenesis.

Chemerin is a small, secreted protein acting mainly through its functional receptor ChemR23/CMKLR1, but binding also to two other receptors, GPR1 and CCRL2. Chemerin was initially described as a chemoattractant factor for leukocyte populations, but many other properties have been described over the years for what is now considered as a multifunctional protein, including the control of lipid and glucose metabolism, or the regulation of vascular smooth muscle cell tone.

Here, we show that ChemR23, CCRL2 and GPR1 are all expressed in the developing retina and are involved in the development of retinal blood vessels. Mice over-expressing bioactive chemerin in keratinocytes under control of the keratin K5 promoter exhibit a decreased density of the vascular network in the adult and developing retina, particularly in the deep layer. No developmental defect was seen however in ChemR23-deficient mice. Our results unveil a novel role for chemerin in the development of retinal vasculature and may suggest a potential role in diseases involving retinal neoangiogenesis.

This abstract was peer-reviewed. The poster was presented at both GRC and GRS. It contains the preliminary part of the work presented in the first article.

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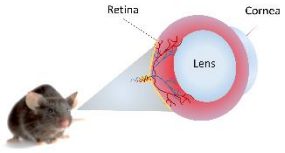

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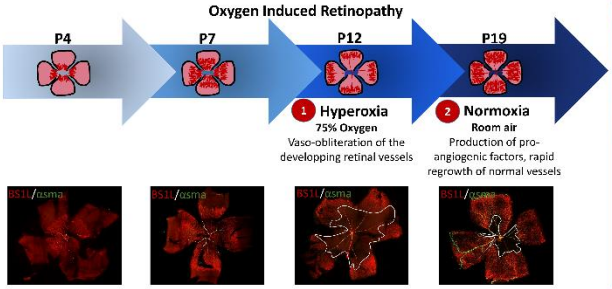

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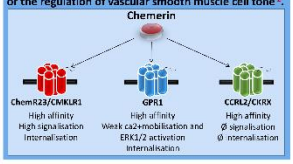
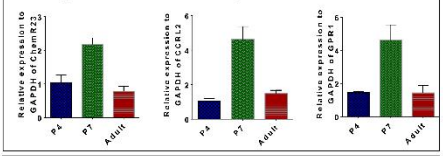
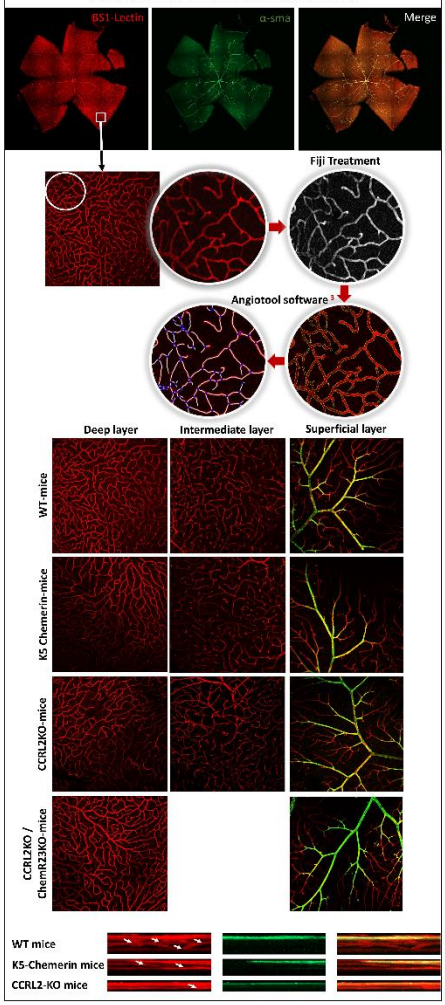


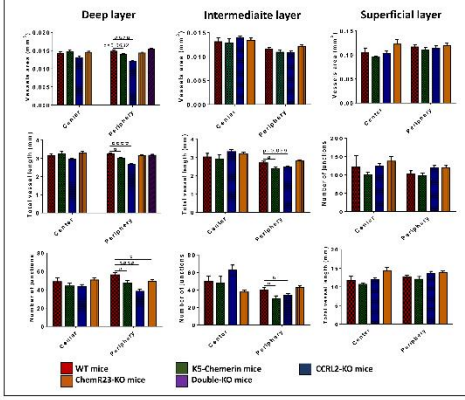
Expression of chemerin receptors in the mice retina



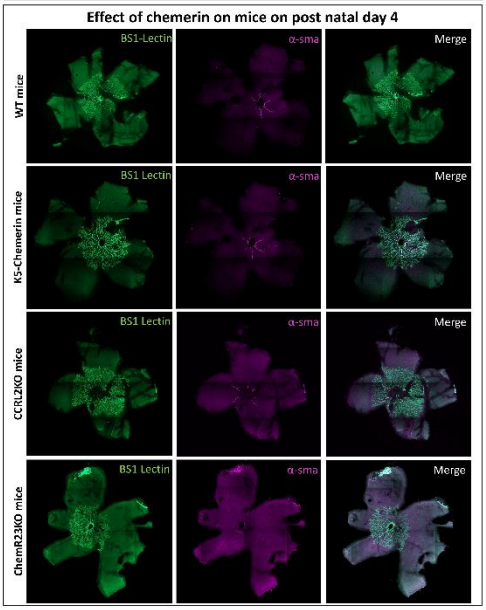
Effect of chemrin on adult mouse retina



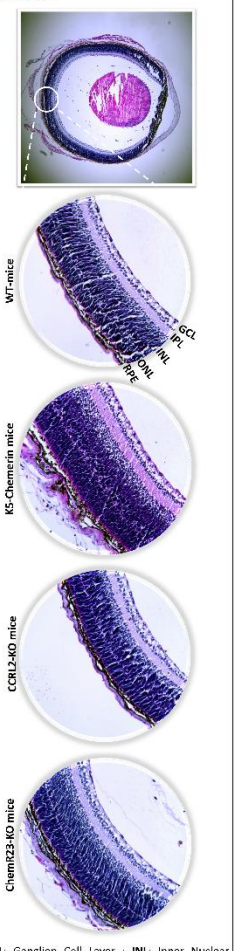
Chemerin decrease retinal vasculature in adult mice retina



Effect of chemerin on mice on post natal day 4



Effect of chemerin on mice on post natal day 7



GCL: Ganglion Cell Layer ; **INL**: Inner Nuclear Layer ; **ONL**: Outer Nuclear Layer ; **IPL**: Inner Plexiform Layer ; **RPE**: retinal pigment epithelium

Here, we show that ChemR23, CCRL2 and GPR1 are all expressed in the developing retina and are involved in the development of retinal blood vessels. Mice over-expressing bioactive chemerin in keratinocytes under the control of keratin K5 promoter exhibit a decreased density of the vascular network in the adult and developing retina, particularly in the deep layer. However, no developmental defect was seen in ChemR23-deficient mice. Our results unveil a novel role for chemerin in the development of retinal vasculature and may suggest a potential role in diseases involving retinal neo-angiogenesis.

References
[1] Gariano R, Gardner T. Retinal angiogenesis in development and disease. Nature. 2005; 438:960-6.
[2] Wittamer V, Grégoire F, Robberecht P, Vassart G, Communi D, Parmentier M. The C-terminal nonapeptide of mature chemerin activates the chemerin receptor with low nanomolar potency. J Biol Chem. 2004; 279:9956-9962.
[3] Zudaire E, Gambardella L, Kurcz C, Vermeren S. A Computational Tool for Quantitative Analysis of Vascular Networks. PloS One. 2011.

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B. Abstract and Poster at the Télévie Cancer Seminar, Université de Liège, Gembloux, Belgium. January 28th, 2019

Chemerin : A negative regulator of angiogenesis

Cyrine Ben Dhaou[1,2] and Marc Parmentier[1].

[1]WELBIO and I.R.I.B.H.M., Université Libre de Bruxelles, Campus Erasme, 808 route de Lennik, B-1070 Brussels, Belgium. cyrine.ben.dhaou@gmail.com

[2]University of Tours, INRA Centre Val-de-Loire UMR-85, CNRS UMR-1247, Physiologie de la Reproduction et Comportements, FRANCE.

Chemerin is a multifunctional protein characterized initially in the host laboratory as a chemoattractant factor for leukocyte populations. While its main functional receptor is ChemR23/CMKLR1, GPR1 and CCRL2 have also been described as chemerin receptors, despite their signaling properties appear limited. Chemerin and CMKLR1 were shown to display anti-tumoral properties in animal models and our group identified an original mechanism by which chemerin interferes with cancer progression by inhibiting the efficient vascularization of the tumors and their growth.

Here, we demonstrate that chemerin don't affect the sprouting angiogenesis *in vivo*, the post-natal development of the retinal blood vessel network, *ex vivo* aorta ring assay, and *in vitro*, migration assay. The signals that prune the exuberant vascular growth of tissue repair are still ill defined. We show that chemerin selectively targeted endothelial cells but not pericytes, mediates the regression of newly formed blood vessels and endothelial cell death by the activation of caspase 3

The efficacy of chemerin in inducing rapid regression of neovessels could open up new avenues of research to identify factors that support tumor angiogenesis and provides a novel strategy to selectively induce regression of nascent neovessels and without affecting normal stabilized vasculature.

The poster contains the first part of the work presented in the first article.

Chemerin : A negative regulator of angiogenesis

Cyrine BEN-DHAOU and Marc PARMENTIER
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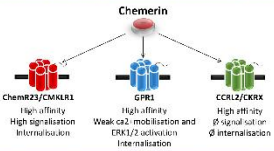
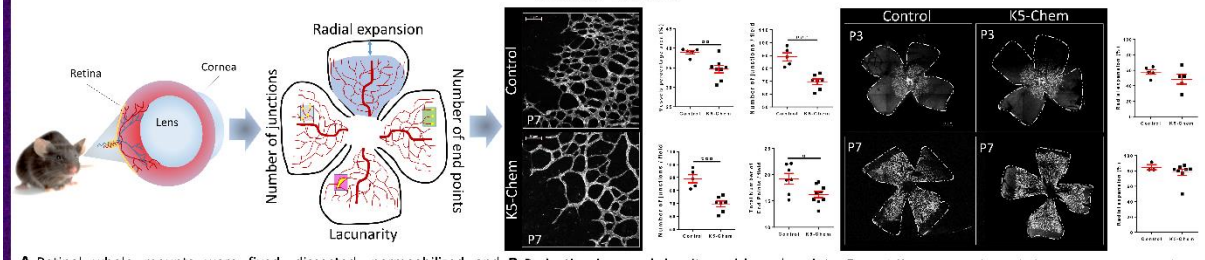
Neo-angiogenesis, the outgrowth of new blood vessels from preexisting ones, is a key process involved in development and tissue repair, but also in various pathological situations including cancer.
The field of angiogenesis research has primarily focused on the mechanisms of sprouting angiogenesis. Yet vascular networks formed by vessel sprouting subsequently undergo extensive vascular remodeling to form a functional and mature vasculature. This "trimming" includes distinct processes of vascular pruning, the regression of selected vascular branches. In some situations complete vascular networks may undergo physiological regression[1].
Chemerin is a small secreted protein acting mainly through its functional receptor ChemR23/CMKLR1, but binding also to two other receptors, GPR1 and CCRL2. Chemerin was initially described as a chemoattractant factor for leukocyte populations, but many other properties have been described over the years for what is now considered as a multifunctional protein, including the control of lipid and glucose metabolism, or the regulation of vascular smooth muscle cell tone[2].



Aim : address a fundamental and unexplored biologic question
Does chemerin affect sprouting angiogenesis and vascular pruning during normal and pathologic angiogenesis?

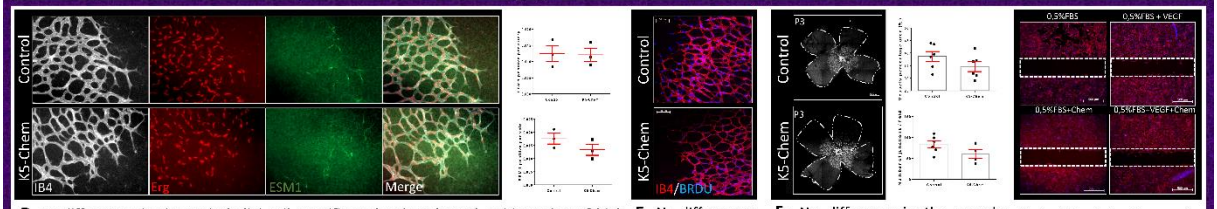
Methods & Results



A-Retinal whole mounts were fixed, dissected, permeabilized and stained. Angiotool Software was used for quantification.

B-Reduction in vessel density and branch points at P7.

C-No differences in the radial expansion at P3 and P7.

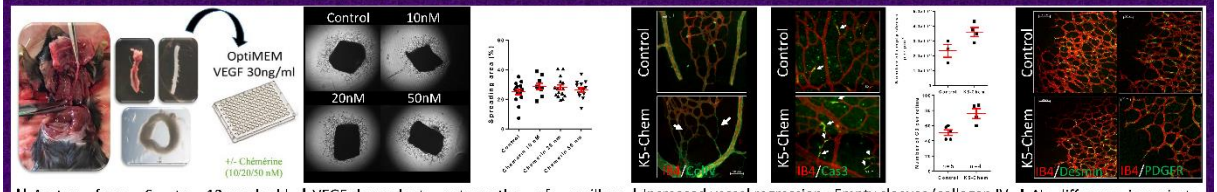


D-No differences in the Endothelial cell–specific molecule 1 (Esm1), a biomarker of high vascular endothelial growth factor A (VEGF-A) signaling.

E- No differences in BRDU staining.

F- No difference in the vascular density at postnatal day 3.

G-Endothelial cells equally accelerate wound closure.

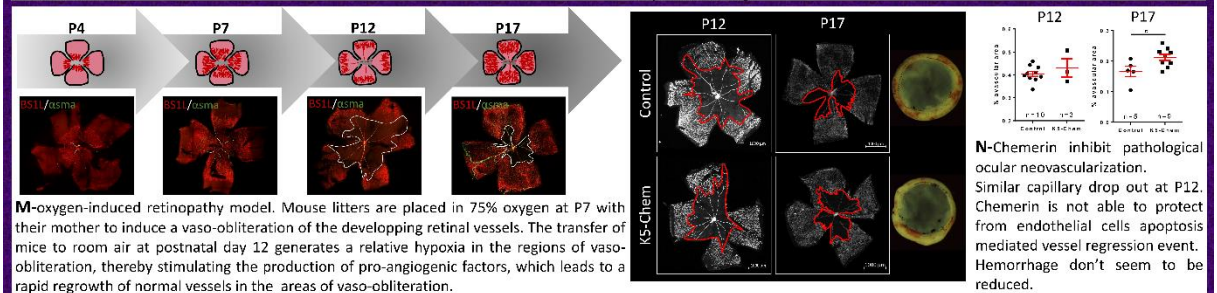


H-Aortae from 6- to 12-week-old C57BL/6 mice were dissected, cleaned from fat tissue, and transversely cut into 1 mm rings. Rings were mounted in 100 mL of Matrigel.

I-VEGF-dependent outgrowths of capillary sprouts from the aortic rings were observed in both control and chemerin treated mice.
No significant difference in the spreading area.

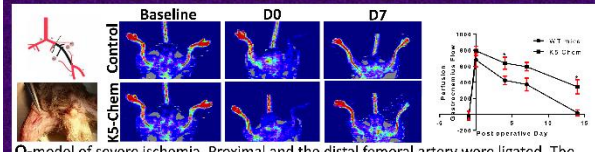
J-Increased vessel regression : Empty sleeves (collagen IV positive and isolectin B4 negative) were increased in the remodeling plexus of K5 chemerin mice.
K-Increased endothelial cell apoptosis : activated caspase 3 staining.

L-No difference in pericyte coverage.



M-oxygen-induced retinopathy model. Mouse litters are placed in 75% oxygen at P7 with their mother to induce a vaso-obliteration of the developping retinal vessels. The transfer of mice to room air at postnatal day 12 generates a relative hypoxia in the regions of vaso-obliteration, thereby stimulating the production of pro-angiogenic factors, which leads to a rapid regrowth of normal vessels in the areas of vaso-obliteration.

N-Chemerin inhibit pathological ocular neovascularization.
Similar capillary drop out at P12. Chemerin is not able to protect from endothelial cells apoptosis mediated vessel regression event. Hemorrhage don't seem to be reduced.



O-model of severe ischemia. Proximal and the distal femoral artery were ligated. The perfusion of the ligated and the control limb was measured with LDI.

P-Over 14 days, K5 chemerin mice showed delayed functional outcomes in the gastrocnemicus and the semimembranosus. No difference in the Tarlov score.

Conclusion



In physiological context :
- Chemerin decrease the vessel density
- Chemerin don't affect sprouting angiogenesis
- Chemerin increase vessel regression
- Chemerin don't affect the vessel stability

In pathological context :
- Chemerin inhibit pathological ocular neovascularization.
- Chemerin inhibit arteriogenesis.

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C. Abstract and Oral Communication at the 2nd PhD Research Symposium in Health Sciences and Biomedicine, Madrid, Spain. May 21st, 2021

Chemerin regulates normal angiogenesis and hypoxia-driven neovascularization

Cyrine Ben Dhaou[1,2], Kamel Mandi[1], Michaël Frye[1], Angela Acheampong[3], Ayoub Radi[1], Benjamin De Becker[3], Mathieu Antoine[1], Nicolas Baeyens[4], Valérie Wittamer[1], Marc Parmentier[1].

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[3] Erasme Hospital, Université Libre de Bruxelles, Route de Lennik 808, 1070 Anderlecht

[4] Laboratoire de Physiologie et Pharmacologie, Université Libre de Bruxelles, Campus Erasme, 808 route de Lennik, B-1070 Brussels, Belgium

Chemerin is a multifunctional protein initially characterized in our laboratory as a chemoattractant factor for leukocyte populations. Its main functional receptor is CMKLR1. We identified previously chemerin as an anti-tumoral factor inhibiting the vascularization of tumors. We show here that overexpression of bioactive chemerin in mice results in a reduction of the density of the retinal vascular network. Chemerin did not affect vascular sprouting during the post-natal development of the network, but rather promoted endothelial cell apoptosis and vessel pruning. This phenotype was reversed to normal in CMKLR1-deficient mice, demonstrating that the destabilizing role of chemerin on the retinal network is mediated by this receptor. We also demonstrate that the neoangiogenesis process in a model of pathological proliferative retinopathy, and in response to hind limb ischemia, is significantly reduced in mice overexpressing chemerin. Mechanistically, PTEN and FOXO1 antagonists could almost completely restore the density of the retinal vasculature. These results suggest that inhibition of the PI3-kinase/AKT pathway is involved in the chemerin-induced vessel regression process.

This abstract was peer-reviewed and published in IBJ Clinical Pharmacology. The oral presentation contained a section of the work presented in the first article.

D. Abstract and Poster at 9ème congrès de la Société Française d'Angiogenèse, Nantes, France. November 16-17, 2021

Chemerin regulates normal angiogenesis and hypoxia-driven neovascularization

Cyrine Ben Dhaou[1,2], Kamel Mandi[1], Michaël Frye[1], Angela Acheampong[3], Ayoub Radi[1], Benjamin De Becker[3], Mathieu Antoine[1], Nicolas Baeyens[4], Valérie Wittamer[1], Marc Parmentier[1].

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This abstract was peer-reviewed. The poster contained the work presented in the first article.

Chemerin : A negative regulator of angiogenesis

Cyrine Ben Dhaou[1,2], Kamel Mandi[1], Mickaël Frye[1], Nicolas Baeyens[4], Valérie Wittamer[1], Marc Parmentier[1]
[1]WELBIO and I.R.I.B.H.M., Université Libre de Bruxelles, Campus Erasme, 808 route de Lennik, B-1070 Brussels, Belgium.
[2]Physiologie de la Reproduction et des Comportements, INRA Val-de-Loire UMR-85, CNRS UMR-1247, University of Tours, France.

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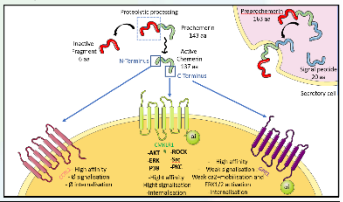
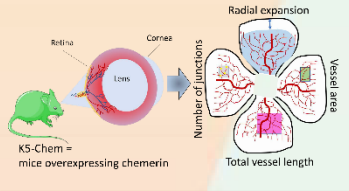
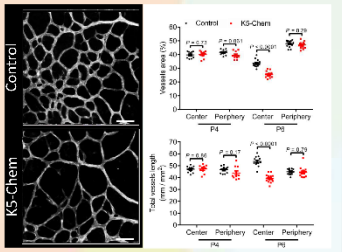


Aim : Does chemerin affect sprouting and vascular pruning during normal and pathologic angiogenesis?

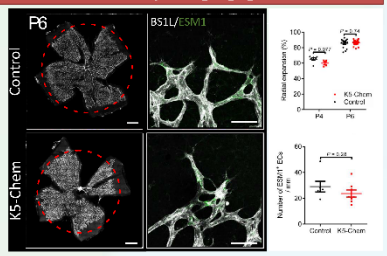
A- Retinal whole mounts staining



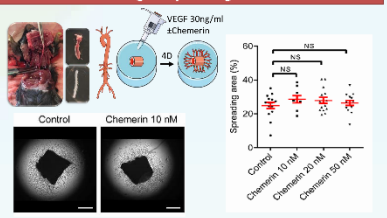
B- Reduction in vessel density at P6



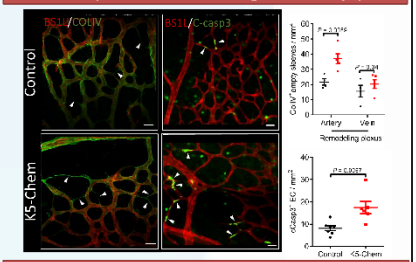
C- No differences in sprouting angiogenesis *in vivo*



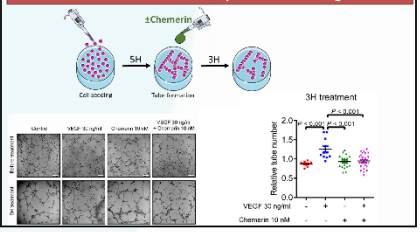
D- *Ex vivo* Aortic ring assay - No significant difference



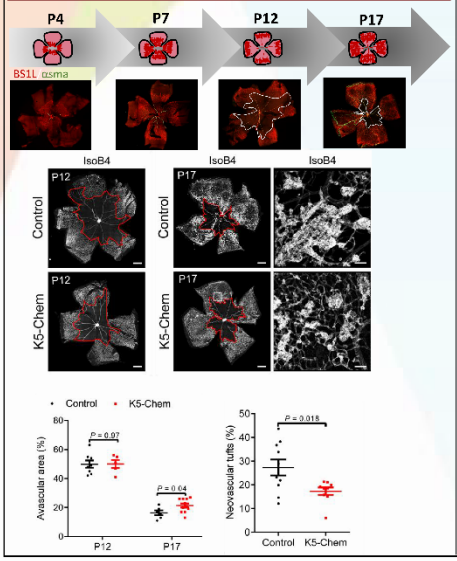
E- Chemerin promotes microvessel regression and apoptosis



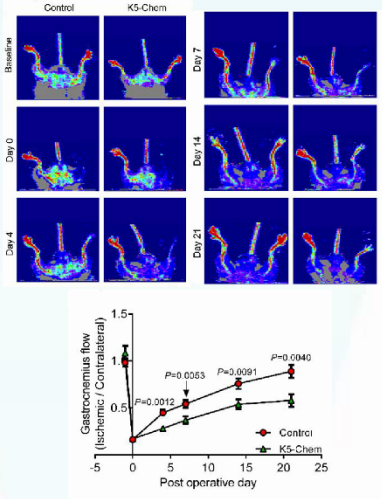
F- *In vitro* Tube formation assay - Microvessel regression



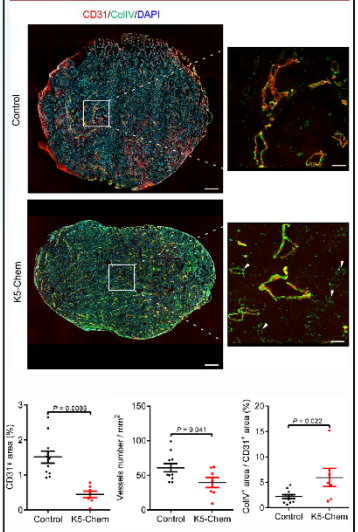
G- Oxygen-Induced Retinopathy model - Chemerin inhibit pathological ocular neovascularization



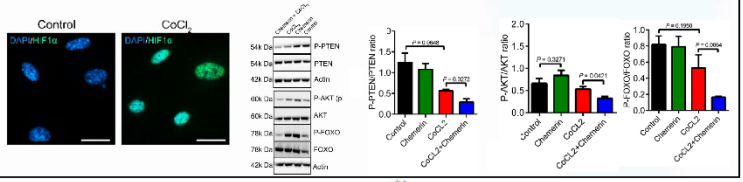
G- Hind Limb Ischemia model - Chemerin inhibits ischemic tissue recovery and angiogenesis in a model of HLI



H- Tumor graft model - Chemerin destabilizes blood vessels in tumor models



I- The activity of chemerin involves the PTEN-AKT-FOXO1 axis



Conclusion

In physiological context :
Chemerin did not affect vascular sprouting during the post-natal development of the network, but rather promoted endothelial cell apoptosis and vessel pruning.

In pathological context :
Chemerin inhibited neoangiogenesis in a model of pathological proliferative retinopathy, and in response to hind limb ischemia.

The activity of chemerin involves the PTEN-AKT-FOXO1 axis.

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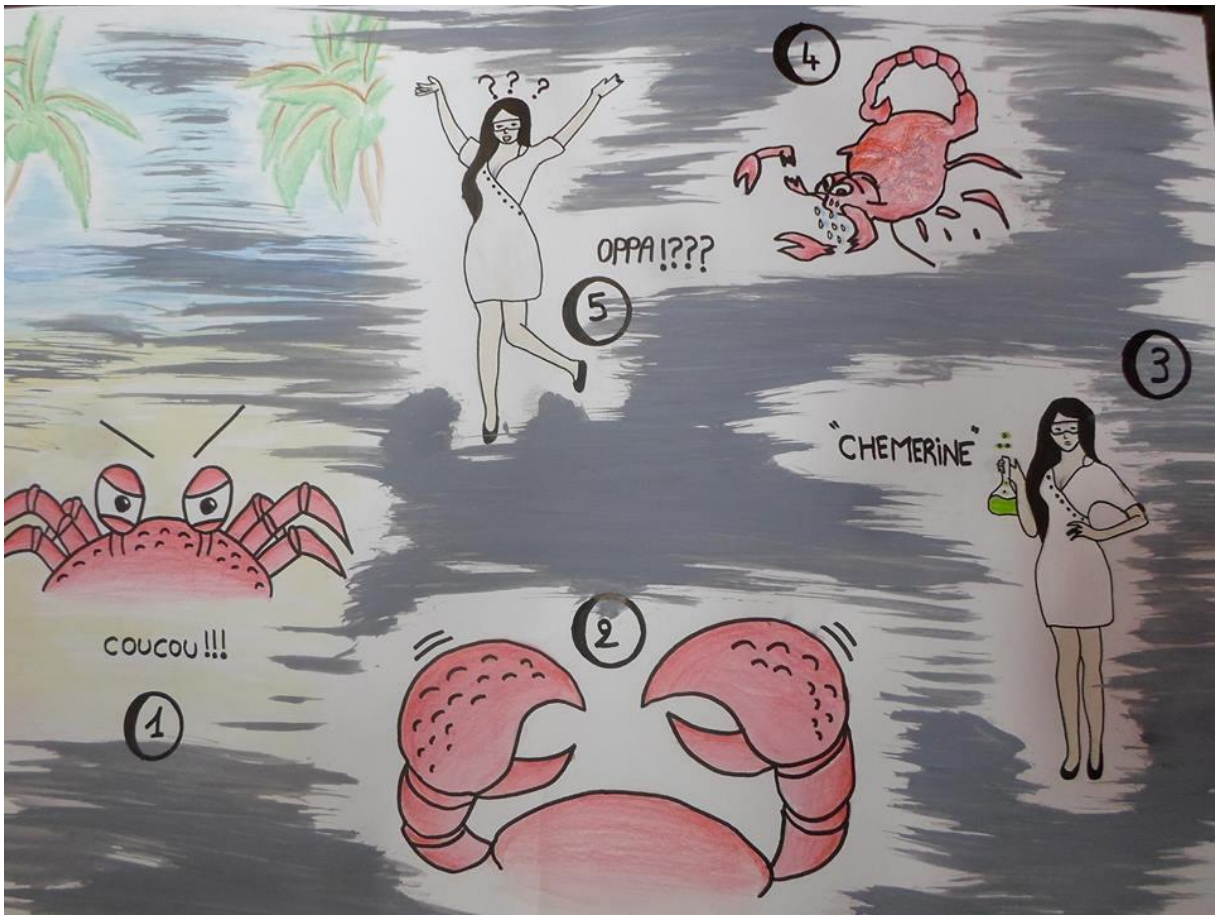
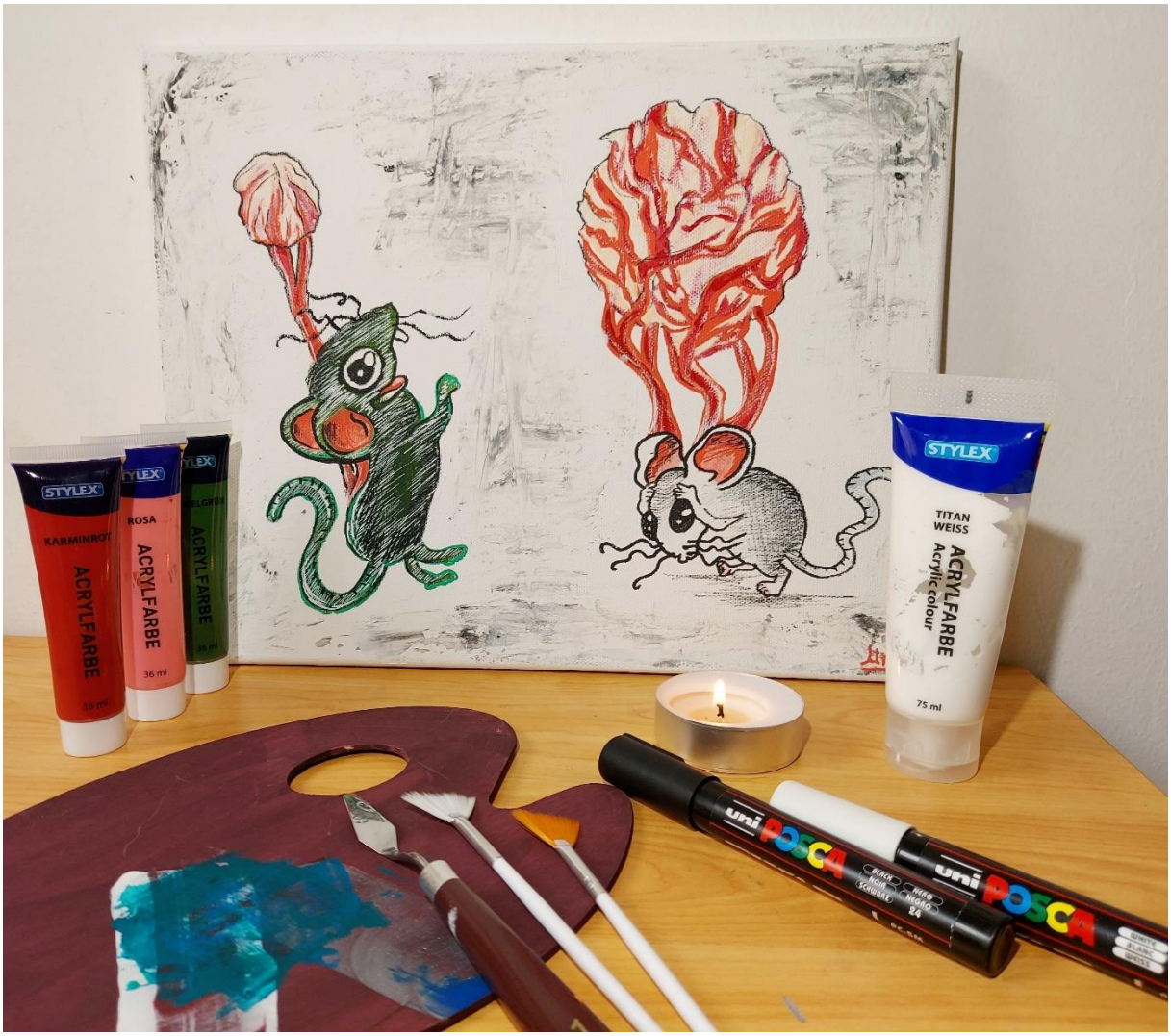


MT180 – ULB – 2018



MT180 – ULB – 2021 - Trinquons à la chémérine contre le cancer