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Abteilungsleiter: Prof. Dr. med. vet. Hellmut Augustin

NCK1/2 are specific mediators of migration in Pericytes and promising targets in ischemic retinopathies

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Steffen Emil Künzel
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Dekan: Herr Prof. Sergij Goerdt, Dr. med.
Doktorvater: Herr Prof. Hellmut Augustin, Ph.D.
Co-Betreuer: Herr Prof. Alexandre Dubrac, Ph.D.
Co-Betreuer: Frau Prof. Anne Eichmann, Ph.D.

To my family

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Abbreviations

α-SMA α Smooth Muscle Actin

ACTA Actin α, Smooth Muscle

AKT RAC-α Serine/Threonine-Protein Kinase

ALK Activin Receptor-Like Kinase

AMD Age-related Macular Degeneration

ANG Angiopoietin

BB Blocking Buffer

BBB Blood Brain Barrier

BRB Blood Retina Barrier

BRVO Branch Retinal Vein Occlusion

BSA Bovine Serum Albumine

BM Basement Membrane

CDC42 Cell Division Control Protein 42

CNS Central Nervous System

COL4 Collagen 4

CRVO Central Retinal Vein Occlusion

CSPG Chondroitin Sulfate Proteoglycan

CT Control

DMEM Dulbecco's Modified Eagle's Medium

DR Diabetic Retinopathy

DM Diabetes Mellitus

EC(s) Endothelial Cell(s)

ECM Extracellular Matrix

ECGM Endothelial Cell Growth Medium

EGM Endothelial Growth Media

EM Electron Microscopy

ERG ETS Transcription Factor

ERK Extracellular Signal-Regulated Kinase

Ex Embryonic Day x

FBS Fetal Bovine Serum

FN Fibronectin

GFP Green Fluorescent Protein

HBMEC(s) Human Brain Microvascular Endothelial Cell(s)

HBVPC(s) Human Brain Vascular Pericyte(s)

HBVSMCs) Human Brain Vascular Smooth Muscle Cell(s)

HHT Hereditary Hemorrhagic Telangiectasia

HIF Hypoxia-inducible Factor

HUVEC(s) Human Umbilical Vein Endothelial Cell(s)

HV Hyaloid Vessel(s)

IB Isolectin

KO Knockout

mTmG Membrane tagged tdTomato/membrane tagged EGFP

MYH11 Myosin Heavy Chain

NCK Non-Catalytic Region Of Tyrosine Kinase

NG2 Neural/glial antigen 2

NPDR Non Proliferative Diabetic Retinopathy

NVT(s) Neovascular Tuft(s)

OIR Oxygen-Induced Retinopathy

PAK P21 Activated Kinase

PBS Phosphate-Buffered Saline

PC(s) Pericyte(s)

PCR Polymerase Chain Reaction

PDGF Platelet-derived Growth Factor

PDGFR Platelet-derived Growth Factor Receptor

PDR Proliferative Diabetic Retinopathy

Px Postnatal Day x

RAC Ras-Related C3 Botulinum Toxin Substrate 1

RBC(s) Red Blood Cell(s)

ROP Retinopathy of Prematurity

RT Room Temperature

s.e.m. Standard Error of the Mean

SMAD Mothers Against Decapentaplegic Homolog

SMC(s) Smooth Muscle Cell(s)

TGF Transforming Growth Factor

TXF Tamoxifen

VEGF Vascular Endothelial Growth Factor

VEGFR Vascular Endothelial Growth Factor Receptor

1 Introduction

1.1 Clinical perception of ischemic retinopathies and AMD

Age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), central and branch retinal vein occlusion (CRVO, BRVO), and retinopathy of prematurity (ROP) are significant causes of blindness in the western world and worldwide (Chakravarthy et al., 2010, Duh et al., 2017, Li et al., 2017, Karia, 2010, Shah et al., 2016). They cause major life restrictions in patients and economic damage due to visual impairment (Zhang et al., 2017, Ke et al., 2006, Hopley et al., 2003). Important risk factors include the genetic background and patient age which can not be influenced in a preventive manner. Thus, therapeutic measures play a crucial role (Armstrong & Mousavi, 2015, Lima et al., 2016). Current medical therapies are limited to a small number of substance classes due to a confined understanding of the disease processes. Although the above-mentioned diseases display individual and distinctive hallmarks and characteristics, pathological vessel abnormalities play a crucial role in all of them. In fact, there is a substantial body of literature documenting the hope of finding novel therapeutic targets in the field of vascular biology, with so far limited success (Shah et al., 2016, Hwang et al., 2015, Sapieha et al., 2010), compare Table 1.

1.1.1 Economic and clinical relevance

The exudative late-stage manifestation of AMD as well as proliferative DR are common causes of irreversible vision loss in the developed world. Approximately 196 million people will suffer from AMD in 2020 worldwide, increasing to an estimated number of 288 million in 2040 (Wong et al., 2014). While the exudative late-stage manifestation of AMD is mainly affecting elderly people, DR is the leading cause of new blindness in patients younger than 75 and in people suffering from Diabetes Mellitus (Lee et al., 2015, Jonasson et al., 2011). Of an estimated 285 million people with Diabetes Mellitus (DM) worldwide, approximately one third have signs of DR and of these, a further one third of DR is vision-threatening (Lee et al., 2015). In fact, up to 80% of patients with a 20-year history of DM develop DR, whereas the majority of these cases are patients suffering from non-proliferative DR (Kertes & Johnson, 2010). AMD and DR massively impair life quality in patients and lead to tremendous economic damage (Compare also Table 1).

Many AMD and DR patients do not experience any visual warning signs. However, as the disease processes, patients are likely to suffer visual symptoms that constrain eye functionality and life quality (Arroyo, 2019, Duh et al., 2017).

Characteristic symptoms for early and intermediate AMD include distorted vision in the form of mild metamorphopsia, slow recovery of visual function after exposure to bright light (i.e. impaired dark-adaptation), as well as unspecific visual symptoms (Mehta, 2015, Goodman et al., 2012).
The exudative late-stage manifestation of AMD, which is caused by choroidal or oc-

casionally retinal neovascularization (i.e. retinal angiomatous proliferation), typically leads to severe metamorphopsia and loss of visual acuity. Without treatment, the exudative late-stage manifestation leads to fibrosis of the macula, resulting in legal blindness. With regard to activities of daily life, patients complain mostly about a loss of reading ability and the ability to recognize faces. The peripheral vision and mobility under high luminance conditions is relatively unaffected (Arroyo, 2019, García-Layana et al., 2017, Philip Storey, 2014).

DR can be classified as non-proliferative and proliferative DR. First, during the so-called non-proliferative phase, patients do not suffer from any symptoms and regularly have good visual acuity and a normative visual field. However, non-proliferative DR can also be complicated by diabetic macular edema leading to metamorphopsia and loss of visual acuity (Heng et al., 2013, Fraser, 2018).
Proliferative DR, hallmarked by vitreoretinal neovascular membranes (neovascularization of the disc [NVD], neovascularization elsewhere [NVE]) and neovascularization of the iris [rubeosis iridis]) may lead to irreversible loss of vision. Sight threatening complications of proliferative DR include vitreous haemorrhages, tractive retinal detachments, secondary glaucoma as well as diabetic macular edema (Duh et al., 2017, Solomon et al., 2017, Fraser, 2018).

CRVO and BRVO constitute the second most common retinal vascular disorder. In 2015, the global prevalence of any retinal vein occlusion, BRVO and CRVO in people aged 30-89 years was 0.77%, 0.64% and 0.13%, equivalent to an overall of 28.06 million, 23.38 million and 4.67 million affected people (Song et al., 2019). Patients regularly do not suffer from any symptoms, but can present with photophobia, redness of eyes, painful blind eye, and decreased vision, whereas the vision loss can be sudden or gradual (Klein R; Moss SE; Meuer SM; Klein BE, 2008, Karia, 2010, McAllister Franzco, 2011, Brogan et al., 2018).

ROP is a disease that affects mainly premature babies undergoing postnatal high oxygen therapies (Table 1). Approximately 50-70 % of children whose weight is less than 1.250 g at birth suffer from ROP, which may subsequently lead to severe visual impairment or even blindness, often causing lifelong disabilities (Hussain N et al., 1999). Although the field of neonatology has massively progressed due to significant discoveries, ROP remains a severe complication in the group of the youngest patients (Kaiser et al., 2001, Hwang et al., 2015, Fledelius & Jensen, 2011).

The significant risk factors for AMD are race, family history, genetic background, age, and to a much lower degree smoking (Armstrong & Mousavi, 2015, Kim et al., 2019a, Marshall & Roach, 2013). Risk correlated factors for DM Type 1 and DR are a combination of genetic and environmental factors, but accurate characterization of these factors and their weights remains unclear. Risk factors for Type 2 DM are mainly genetic background and overweight (Bellou et al., 2018, Fareed et al., 2017, Issaka et al., 2018, Hussain et al., 2004). Causes for CRVO and BRVO are systemic vascular

diseases and glaucoma (Yau et al., 2008, Appiah & Trempe, 1989, Kolar, 2014). ROP is observed in premature children. Factors correlating with premature birth are from a versatile range with only very few being preventable (Liu et al., 2005, Isaza et al., 2013).
It is not my intention at this point to further discuss or evaluate risk factors for the named diseases which can be reviewed elsewhere. Nevertheless, the current experts of the field adopted the view that the majority of these risk factors can not or only very hardly be targeted. Thus, therapists and patients around the globe have high hopes in future therapeutic opportunities for AMD, DR, and OIR (Table 1).

1.1.2 Current medical therapeutic standards and their limitations

Ischemic retinas experience high hypoxic stress levels, which subsequently results in the overexpression of vascular growth factors as a responsive regulatory process (Kermorvant-Duchemin et al., 2010, Aiello et al., 1994). These include VEGF-A, PDGF-B, TGFβ, and others (Pfeiffer et al., 1997, Boulton et al., 1998, Paques et al., 1997, Mori et al., 2002). The elevated levels of expressed growth factors lead to pathological neovascularization, which can be observed in so-called Neovascular Tuft Formations (NVTs) and numerous vascular sprouts (Pierce et al., 1995b, Dubrac et al., 2016). Those newly-formed vessels are weak, likely to bleed and to leak proteins and blood below the macula and retina, which results in irreversible damage to the photoreceptors and the characteristic visual symptoms (Osborne et al., 2004, Unoki et al., 1994).

This current understanding of pathological vascular sprouting in ischemic retinopathies led to the identification of medications that specifically target the mentioned signal cascades, especially the VEGF pathway. In fact, anti-VEGF-A therapies have been a major innovation and proven very useful to prevent vision loss and even promote vision gain in a subset of patients suffering from AMD, DR, and other ischemic retinopathies (Osaadon et al., 2014, Kovach et al., 2012). Today, these medications are established and promising therapeutic measures in ischemic retinopathies (Jardeleza et al., 2009, Nicholson & Schachat, 2010). Besides surgical interventions, laser treatment and photodynamic therapy, the leading medical treatment of AMD and DR is limited to only three drugs in Germany: Bevacizumab (off label use), Ranibizumab and Aflibercept (Neubauer et al., 2007, Ziemssen et al., 2015, Deissler et al., 2014).

Bevacizumab and Ranibizumab are neutralizing monoclonal antibodies against VEGF-A, the primary known driver of sprouting angiogenesis. Ranibizumab is the Fab-fragment of Bevacizumab, and since both are functioning through the same mechanism, no significant difference in terms of effectivity strength or side effects could be observed (Berg et al., 2015, Wells et al., 2015, Schmucker et al., 2012, Takeda et al., 2007). Aflibercept is highly similar to the above-mentioned proteins as it consists of the extracellular parts of VEGF receptors as well as the FC portion of the igG1 immunoglobulin. Based on the phase III trials for Aflibercept, it is assumed that Aflibercept treatment every 2 months is not inferior to monthly Ranibizumab in AMD (Gillies

et al., 2019, W. Stewart, 2011).
After injection in the vitreal body, the above-mentioned substances decrease VEGF-A-mediated signaling cascades and correct the pathologically elevated levels of the growth factor. Thus, they can potentially help to reduce neovascularization, bleeding, and macular edema in diseased retinas (Brown et al., 2013, Rosenfeld et al., 2005).

Unfortunately, long-term observations reveal that anti-VEGF-A therapies are only partially successful. Some of the treated patients still suffer from significant vision loss and intravitreally injected anti-VEGF-A agents do not enable the retina to rejuvenate to a full-healthy tissue (Jardeleza et al. (2009) and Fig. 1). Furthermore, intravitreal anti-VEGF-A treatment potentially causes severe side-effects. These include stroke, retinal detachment, eye pain, subconjunctival hemorrhage (bloodshot eye), vitreous floaters, irregularity or swelling of the cornea, inflammation of the eye, and visual disturbances (Ueta et al., 2011, Pieramici & Rabena, 2008, Tolentino, 2011). Specific side-effects of the antibody treatment are also described and include neuronal toxicity and other ocular complications (Thaler et al., 2008). Side effects can be observed between 1,4 and 26 % in of treated patients (Falavarjani & Nguyen, 2013).

Other anti-VEGF-A therapeutic drugs are approved, but do not play a role in the treatment of ischemic retinopathies today. The aptamer Pegaptanib is approved, but not available, at the moment. It has been predecessor of the currently established therapies (Ng et al., 2006). The Protein Kinase C-β-inhibitor Ruboxistaurin is currently not approved for medical treatments (Nakamura et al., 2010, Gardner & Antonetti, 2006). The efficacy is significantly lower than the firstly-mentioned drugs (PKC-DRS Study Group, 2005).
In summary, the treatment options for ischemic retinopathies are limited to only very few substances. These do not provide perfect results and bear an increased risk for severe side-effects. The limited therapeutic options underline the urgent need for new pharmaceutical targets in neovascular retinal diseases.

However, some important and individual features have to be respected in term of the treatment and terminology of the mentioned diseases.
PDR, CRVO, BRVO, and ROP, but not AMD, are usually summarized under the term "ischemic retinopathy", as they display avascular areas in the retinal periphery with elevated levels of VEGF-A, as further discussed below (Al-Shabrawey et al., 2013, Sapieha et al., 2010, Aiello et al., 1994, Stefansson et al., 1990). This leads to macular edema in PDR, CRVO, BRVO, and vitreo-retinal neovascularizations in PDR, CRVO, BRVO, and ROP, respectively (Boyer et al., 2014, Tasman & Brown, 1989, Hikichi et al., 1996, Quinlan et al., 1990). Macular Edema can be treated with anti-VEGF-A therapies and steroids, and is usually reversible and well treatable (Grover et al., 2008, Antcliff et al., 2001, Cunningham et al., 2008, Wells et al., 2015, Rosenfeld & Feuer, 2018). Vitreo-retinal neovascularizations are an indication for laser therapy of the peripheral retina (Mirshahi & Roohipoor, 2008, Stefansson et al., 1990). Otherwise severe vitreal bleeding and tractive retinal detachment have to be expected (Spraul &

Grossniklaus, 1997, Rice et al., 1991). Both are treatable with par-plana vitrectomy - a commonly employed technique in vitreoretinal surgery that includes removal of the vitreous body and enables access to the posterior segment. Severe retinal damage is a common consequence (Pautler, 2010, Shi & Huang, 2012).

In contrast, from a clinical point of view, AMD does not fall under the generic term ischemic retinopathy. AMD is characterized by choroidal neovascularization and, in special cases, by RAP lesions (Jia et al., 2014, Liu et al., 2007). Both of them cannot be observed in any of the other mentioned diseases. Interestingly, animal experiments confirm, that an elevation of VEGF-A as sole stimulus is not sufficient to induce choroidal neovascularization (Schwesinger et al., 2001). Most probably, it is a senescence of the Bruchs membrane, that leads to choroidal neovascularizations in AMD (Majji et al., 2000, Wang et al., 2016). In vice versa, vitreo-retinal neovascularizations and proliferations, that characterize the above-mentioned diseases as ischemic retinopathies are not described in AMD. Despite the fact, that AMD is strictly speaking - not considered as an ischemic retinopathy, the pathophysiology displays an ischemic character (Xuan et al., 1999, Yoshida et al., 2010, Ng & Adamis, 2005). The topic is approached through the eyes of a vascular biologist. Thus, whenever the generic term ischemic retinopathies is mentioned in this dissertation, it includes AMD (compare Table 1).

	ROP	DR	AMD
Age of first symptoms	32-45 weeks after fertilization	Middle-age	Older people
Epidemiology	Up to 56 % of premature newborns with weight <1.500g	Leading cause of blindness in patients aged 20 to 64	More than 6 million people worldwide (2015), prevalence higher in Europe than in Asia/ Africa
Risk Factors	- Prematurity and correlated risk factors - High oxygen levels after birth - Infections - Cardiac defects	- Diabetes Mellitus, Metabolic Syndrome and related diseases - Down's Syndrome protective	- Aging - Family history - Genetics (polymorphisms are described) - factors associated with metabolic syndrome (Obesity, high cholesterol, smoking, hypertension and others)
Symptoms	See prognosis	- NPDR: - no early warning symptoms - no or mild symptoms - complicated symptoms possible - PDR: - blurred vision - blood floats in vis. field - loss of clear vision - severe complications	- Early / interm.: - distorted vision - impaired dark-adaption - unspecific visual symptoms - Late: - severe metamorphopsia - loss of visual acuity - "legal blindness"
Therapy	– VEGF-Control (by laser/ anti-VEGF): Zone I ROP: any stage with "plus disease"*/ stage 3 - no "plus disease"/ Zone II ROP: stage 2 or 3 with "plus disease" – Surgery is indicated, when retinal detachment occured	– PDR/ NPDR with macular edema: anti-VEGF or steroids – PDR: Laser – Surgery is indicated, when retinal detachmend occured (PDR)	- Modification of risk factors (smoking, atherosclerosis, dietrary modifications), antiox./ mineral suppl. - Intermediate AMD: avoiding of progression with AREDS supplement - Intravitreal injection of anti-VEGF agents, when neovascular AMD - Special forms of AMD (e.g. PCV): PDT
Prognosis	Blindness (Depending on disease stage, stage 1 and 2 do not lead to blindness), Refractive errors, Squint, Ablyopia, Ret. detach., Glaucoma	Under treatment good prognosis (up to 95% of patients keep vision)	Therapy aims to slow down symptom progression. Treatments do not reverse symptoms.

Table 1. Overview of disease characteristics of ROP, DR, and AMD.

Table 1: **Overview of disease characteristics of ROP, DR, and AMD.**
While each disease is characterized by individual properties, such as patient age, progression, risk factors and symptoms, all three of them resemble one another in the high clinical relevance, strain of suffering, and overlapping treatment options.

References:
ROP: Age of first symptoms, Risk Factors, Symptoms and Prognosis: Bashinsky (2017), Jandeck et al. (2003), Hellström et al. (2013); Therapy: Jandeck (2008), Hartnett (2014), Dani et al. (2012), Solarte et al. (2010);
DR: Kopp & Schorr (2015), Duh et al. (2017), Mohamed et al. (2007), Wang & Lo (2018), Sabanayagam et al. (2019); AMD: Disease et al. (2016), Berufsverband der Augenärzte Deutschland e.V. (BVA) & Deutsche Ophthalmologische Gesellschaft (DOG) (2015), Al-Zamil & Yassin (2017), Hernández-Zimbrón et al. (2018), Solomon et al. (2019), Browning et al. (2004), Jonas et al. (2017)

1.1.3 Therapeutic challenges and translational studies focusing on Pericytes

The development of anti-VEGF-A drugs was a clinical success and a landmark finding in the field. However, even though much efforts have been made to improve anti-VEGF-A therapies, the results are still not the best possible, and it is crucial to find novel targets for a better life of patients suffering from ischemic retinopathies and AMD. However, the apparent solution - blocking pathological angiogenesis by blocking VEGF-A - is not as easy as it might be assumed and reaches certain limitations (Simó & Hernández, 2008, Nicholson & Schachat, 2010, Sankar et al., 2018, Finger et al., 2016).
Firstly, the currently approved substances target a single growth factor which is predominantly influencing one cell type - ECs (discussed in more detail below). Studies reveal that numerous signal cascades in different cell types from a broad range of background and tissues are dysregulated in ischemic retinopathies (Paques et al., 1997, Nadal et al., 2002, Praidou et al., 2010, Hammes, 2005). Thus, it should not be assumed that a monocausal solution is a key to success in ischemic retinopathies. This multi-dimensional dysregulation is still an obstacle in the field. An entire resetting of affected cell types and individual signaling cascades to healthy levels has not been reported in humans, yet. The current understanding should encourage the search for more elaborated therapy targets aside from established cell types and signaling cascades, such as ECs and their major regulator VEGF-A.
A crucial second hurdle in the treatment of ischemic retinopathies is the parallel appearance of hypersprouting vessels and - in contrast to this - avascular areas in diseased retinas (Dubrac et al., 2016, Selvam et al., 2018), as visualized in Fig. 1 B and C . VEGF-A is not only crucial for sprouting angiogenesis, but also for vessel stability and integrity in disease and health (Von Degenfeld et al., 2006, Cross & Claesson-Welsh, 2001, Gu et al., 2003). Thus, anti-VEGF-A treatments do not only block pathological hypersprouting, but impair revascularization of avascular retinal areas (Tokunaga et al., 2014, Nakao et al., 2012). This leads to the crucial challenge to invent an intervention that simultaneously normalizes hypersprouting vessels as well as inciting the vascularization of avascular retinal areas. There are only very few substance classes described with this capability at this point, e.g. Kim et al. (2017).

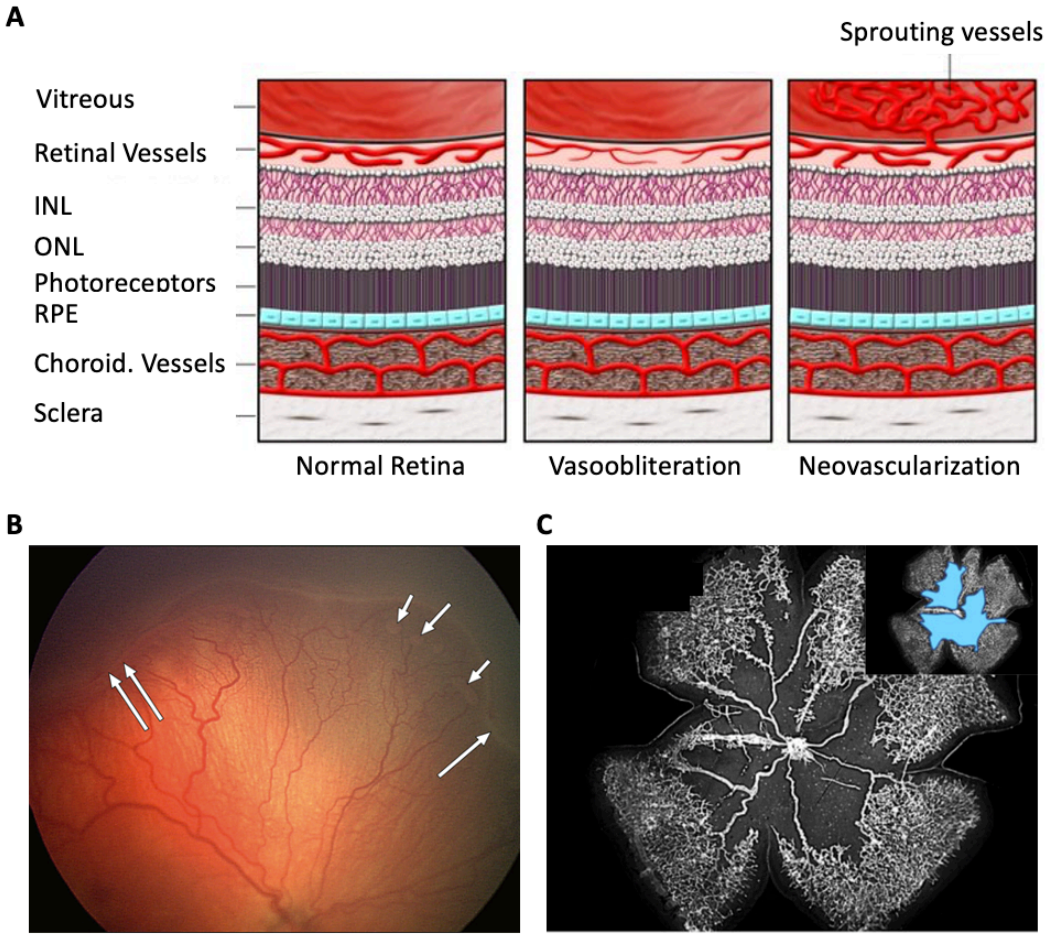


Figure 1: **Vasoobliteration and pathological hypersprouting in ischemic retinopathies and AMD.**
A Schematic depiction of the retinal layers in health and disease. Left, Normal Retina: Retinal vessels appropriately perfuse the retina adjacent to the retinal ganglion cell layer next to the vitreous body. Center, Vasoobliteration: During the first phase of ROP, massive vessel obliteration occurs. The retina is only scarcely perfused by vessels. Right, Neovascularization: This phase is characterized by pathological sprouting angiogenesis toward the vitreous of the retina. Newly formed vessels are weak and prone to bleeding, and edema. The choroidal vascular network adjacent to the sclera is more likely affected in AMD. *INL, inner nuclear layer. ONL, outer nuclear layer, RPE, retinal pigment epithelium.*
B Fundus photography of ROP patient (stage 2). Avascular, under-perfused retinal areas occur next to pathological hypersprouting. Transitional retina is indicated by long arrows. Isolated neovascular tufts of new vessels ("popcorn") are indicated by short arrows and are a hallmark finding in ROP patients.
C Retinal flatmount and vessel staining of P17 WT mouse retina following the OIR protocol (described below). Notably, avascular area in the center is present next to hypersproutive vessels at the outer edges. Dense white spots in the vasculature represent NVTs. Blue area in top-right inset marks avascular area. *retinal diameter: 6,3 mm*

References:
A: from Sapieha et al. (2010), B: from International Comittee for the Classification of ROP (2005), C: from Dubrac and Künzel et. al. (2018)

There are novel therapeutics in the pipeline of drug development, some of them already in clinical trials, but with limited success so far. For the sake of clarity and as it is of particular importance for my current work, I will focus on the only clinical trial focusing on PDGFs and Pericytes at this point. I will sideline elaborated other therapeutic developments, such as CRISPR/Cas9 and stem cell solutions as they approach the topic from a completely different direction.

As discussed in more detail later in this dissertation, several recent studies support the hypothesis, that pathological activation of PCs and their primary regulator PDGF-B are crucial drivers of pathological sprouting angiogenesis in retinopathies (Hammes, 2005, Ejaz, 2008, Hall, 2006, Beltramo & Porta, 2013, Addison et al., 1970). Although the mentioned studies do not agree on a specific function or role of PCs during ischemic retinopathies, they led to the development of Fovista (pegleranib), a pegylated aptamer against PDGF-B, an anti-PDGF-B agent (Bahadorani & Singer, 2017). Phase 1 and 2 studies of Fovista in combination with Ranibizumab (Lucentis, anti-VEGF-A) were promising (Jaffe et al., 2016, 2017, Boyer et al., 2014, Tolentino et al., 2015). In contrast, the Phase 3 clinical trial could not show a superiority of Fovista (pegleranib) in combination with the established anti-VEGF-A therapy compared to anti-VEGF-A monotherapies (Ophthotech Company, 2016). A benefit in terms of best-corrected visual acuity in the treated patients could not be observed. An increase in adverse effects in patients treated with Fovista in addition to anti-VEGF-A therapies was mainly related to the injection procedure. However, problems were reported in terms of patient selection and selection of the primary endpoint, so that it is questionable, whether Fovista would have a benefit using another study design (Rosenfeld & Feuer, 2018). At this point, it remains unclear whether the mentioned study drives a nail in the coffin of anti-PDGF-B therapies in ischemic retinopathies. A better understanding of the role of PDGF-B and PCs in pathological angiogenesis in retinopathies is urgently needed to evaluate their potential importance for future treatments.

1.2 Vascular network

A highly branched, complex system of interconnected blood vessels builds the vascular network in humans and vertebrates (Fig. 2). Blood vessels are tube-formed structures, which act as transport channels guaranteeing the convey of blood to the periphery of the body, and an efficient flow back to the heart, respectively (Natalia Foley, 2010). The blood flow ensures the transportation of O2, CO2, nutrients, hormones, and metabolic wastes throughout the body and guarantees the high demands of the complex body functions and architecture (Tucker & Mahajan, 2019, Alberts et al., 2002, Ramasamy, 2017, Cai et al., 2015, Wells, 2006). These demands include cell-level metabolism, regulation of pH, temperature, protection against infectious harms, and many more. All blood vessels together combined with the heart as a pump organ build the circulatory system (Ihrcke et al., 1993, Yoon et al., 2012, Charkoudian, 2003).

Vascular defects cause some of the most common reasons for disease and death in the western world and worldwide. Dysfunction, degeneration, and malformation of blood vessels are involved in a various number of conditions, such as hypertension, aneurysms, myocardial infarctions, stroke, ischemic retinopathies, and also cancer progression including metastatic spread is depending on blood vessel development. Thus, understanding and maintaining a healthy vascular system is a major concern in clinic and laboratories around the world.

1.2.1 Vessel hierarchy

The circulatory vessel system splits into a small and a large circuit (Natalia Foley, 2010). The small circuit connects the lung as a gas exchanger of the body with the heart (Marini et al., 2018). The large circuit guarantees blood flow from the heart to the whole body, including essential organs such as the brain, kidney, liver, and many more (Burch, 1983). Both loops intersect at the heart, where the blood of the two circuits is transferred to one another (Fig. 2). Furthermore, both of the loops segment into an arterial and a venous part. The different requirements of arteries and veins of the large and small circuit result in the very different vessel wall morphologies and capabilities (Pries et al., 1995, Anwar et al., 2012, Deng & Guidoin, 2016, Rhoades & Bell, 2012).

The arterial part of the large loop transports oxygen- and nutrient-rich blood from the heart to different organs (Ku & Woodruff, 1997, Weiss & Sinha, 1978, P Bose, 1935, Jaffe et al., 2016, Harrop, 1919). This process is crucial for various body functions and underlies complex regulatory mechanisms that guarantee a much higher blood pressure in this part of the system in comparison to the venous and capillary vessels (Haeren et al., 2018, Belz, 1995, Westerhof et al., 2009).
After the exchange of gas and metabolites in the organs, the blood is led back to the heart through the venous system (Fig. 2). Blood of the venous system of the large circuit contains high levels of CO2 as a waste-product - and much lower oxygen levels (Harrop, 1919).
To avoid ambiguities: Artery should not be understood as vessel containing high lev-

els of oxygen, which is only valid for arteries of the large circuit. The pulmonary arteries of the small circuit contain low levels of oxygen, and the pulmonary veins are the vessels with higher oxygen levels (Marini et al., 2018, Soria et al., 2016). The term artery should be understood as vessel leading away from the heart, as all arteries are leading away from the heart (Fig. 2). The only exception to this are coronary arteries which supply the heart muscle with oxygen and nutrients. They are the only arteries leading back to the heart (Villa et al., 2016).

Besides the blood vessel network, the blind-ending, unidirectional lymphatic network is another essential vascular system. The lymphatic network distinguishes from the blood vessel network through its morphology and function (Kerjaschki, 2014, Padberg et al., 2017, Wang & Oliver, 2010). Lymphatic vessels are not transporting blood, but lymph, a fluid containing infection-fighting white blood cells, electrolytes, proteins, and chylomicrons. The lymphatic system helps the body to get rid of toxins, waste, and other unwanted materials (Hansen et al., 2015, Santambrogio, 2018, Huxley & Scallan, 2011, Schwager & Detmar, 2019). The lymph of the whole body is gathered and subsequently lead back to the blood network via the thoracic duct, which ends in the root of the neck (Phang et al., 2014).
The lymphatic network is not at the center of my work. To avoid misunderstandings: all wordings and formulations in this dissertation such as vessel, circulation, EC, and similar expressions refer to the blood vessel network unless further specified. Interested readers of the lymphatic system should look for relevant literature elsewhere.

1.2.2 Vessel structure

Blood vessels are hollow organs, formed in a tube shape (Fig. 3 + 4). The wall of bigger blood vessels consists of three layers: The Tunica intima which is in direct contact to blood, the Tunica media and the Tunica externa, which is also called adventitia (Tucker & Mahajan, 2018, Sicard, 2018).

The Tunica intima is essential for gas and fluid exchange between blood and vessel tissue (Gartner & Hiatt, 2014, Milutinović et al., 2019). The particular morphology is depending on the vessel type. The Tunica intima of veins and arterioles contains a single layer of ECs. In contrast to this, arteries usually use a supporting layer of elastin-rich collagen, fibroblasts, or SMCs depending on their location and function (Milutinović et al., 2019). The Tunica intima is of particular interest in the pathology of aneurysms when a laceration in the intima allows blood to flow between the different vessel layers potentially causing life-threatening complications (Siasos et al., 2015). ECs of this layer experience shear stress, which is massively depending on their location (Chistiakov et al., 2017). It is of great interest in current studies about atherosclerosis and similar pathologies.

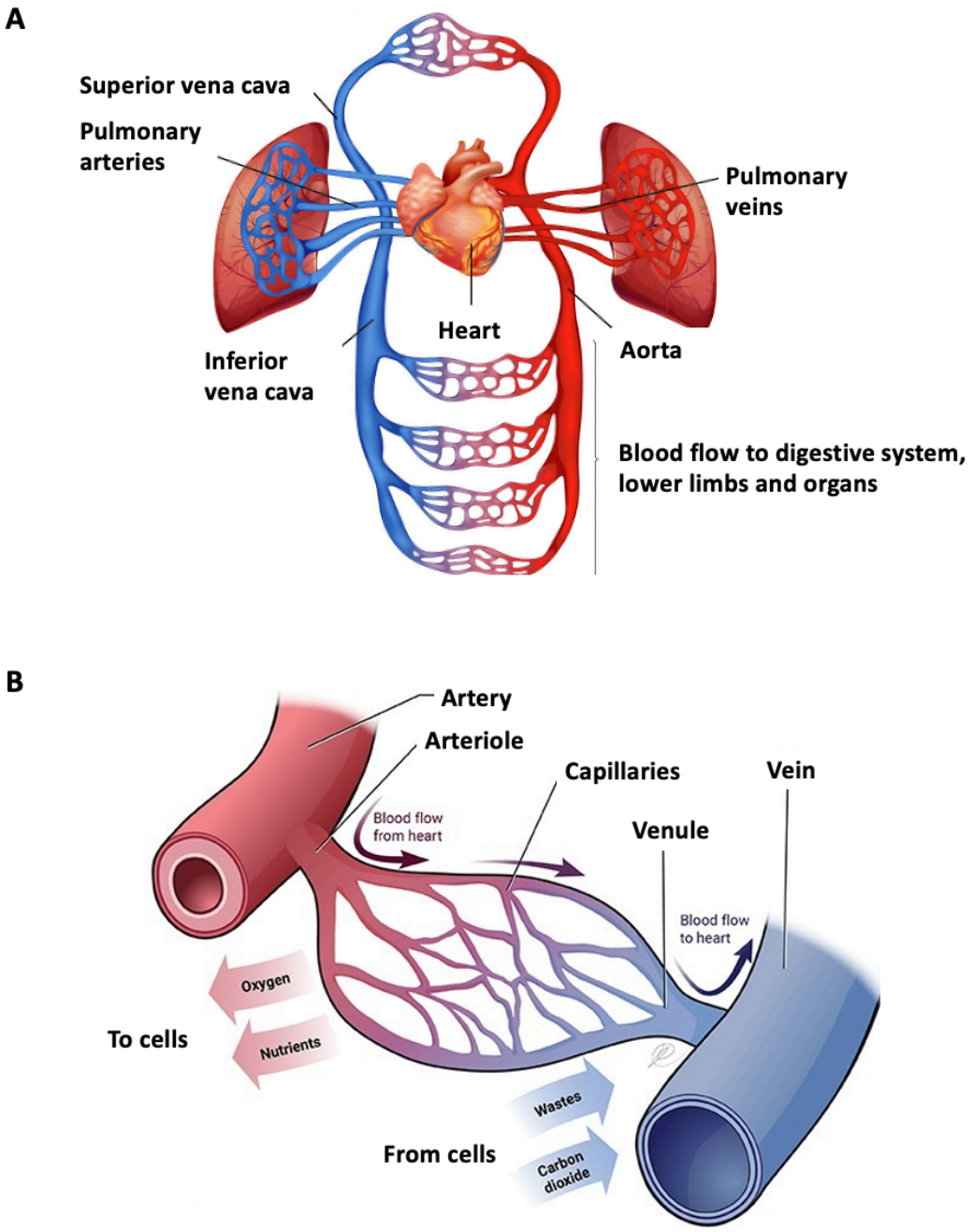


Figure 2: **The human circulatory system and vessel hierarchy.**
A Schematic demonstrating the blood flow in the human circulatory system. Please note the division into a large and small circuit, and the arterial and venuous parts of each. Colours indicate level of oxygen: blue=low, red=high.
B Schematic showing the vessel hierarchy and the exchange of nutrients, waste and gases in the human body.

References:
A: from Hall (2006), B: from Evan & Mandira (2017)

The Tunica media is shaped very differently depending on the specific organ and position in the circulatory system (Tonar et al., 2015, Tucker & Mahajan, 2018), as visualized in Fig. 3 and Fig. 4. It can potentially consist of SMCs, elastic tissue, and collagen (Owens et al., 2004, Žaloudíková et al., 2019, Tonar et al., 2016). In the smallest arteries, it principally consists of only very few layers of SMCs in fine bundles surrounding the vessels (Fig. 3 + 4). Larger arteries, as the iliac, femoral, and carotid, utilize elastic fibers and collagen to form so-called lamellae which stretch in close connection to smooth muscle fibers (Toda et al., 1984). The Tunica media of the largest arteries close to the heart are usually built up of elastic fibers which guarantee a Windkessel function to support adequate blood flow to the periphery (Westerhof et al., 2009). The elastic fibers are typically embedded in a neighborhood of approximately five to seven layer of SMCs (Misra et al., 2017). In general, more distal and smaller arteries have more smooth muscle fibers than more prominent arteries close to the heart (Bacakova et al., 2018). These play a crucial role blood flow and pressure regulation (McCurley et al., 2012, Hill et al., 2015). Different organs are depending on precise and specific blood pressure and flow regulation (Meng et al., 2019, Mensah et al., 2002). To achieve this, blood vessels have to decrease or enlarge the vessel diameter, thereby regulating vessel resistance, blood flow, and pressure. This regulatory process is called vessel contraction or dilatation and is strictly mediated by the vegetative nervous system (Joyner et al., 2010). In larger arteries, it is well understood, that SMCs of the Tunica media play a crucial role in this process (Zhu et al., 2019a, Tykocki et al., 2017). Since this regulation of the vessel diameter is happening in afferent feeding vessels, there are more SMCs in arteries than in veins (Bacakova et al., 2018, Kim et al., 2004). Although this mechanism is well-understood in larger vessels, it remains unclear which cell types contribute to vessel contractility in smaller vessels (Zhu et al., 2019b, Mazzoni et al., 2015, Bandopadhyay et al., 2001, Finney & Orr, 2018). While Hill et al. (2015) state, that PCs are not capable to contract the vessel, Cheng et al. (2018), Hamilton et al. (2010) and Alarcon-Martinez et al. (2019) argue the opposite. Notably, Alarcon-Martinez et al. (2019) observe, that contractility is mediated by α-SMA, a protein that is used for identification of SMCs - so a purely matter of cell definition? It is not yet sufficiently determined, whether and to what extent PCs are potential vessel contractors (Hashitani & Mitsui, 2019, Armulik et al., 2011).

The Tunica externa is also called adventitia and connects the outer vessel surface with the environment, thereby guaranteeing stability (Stenmark et al., 2013), as visualized in Fig. 3 and 4. This layer is of lower interest in pathologies with the exception for the vitamin deficiency disease Scurvy (De Luna et al., 2003).

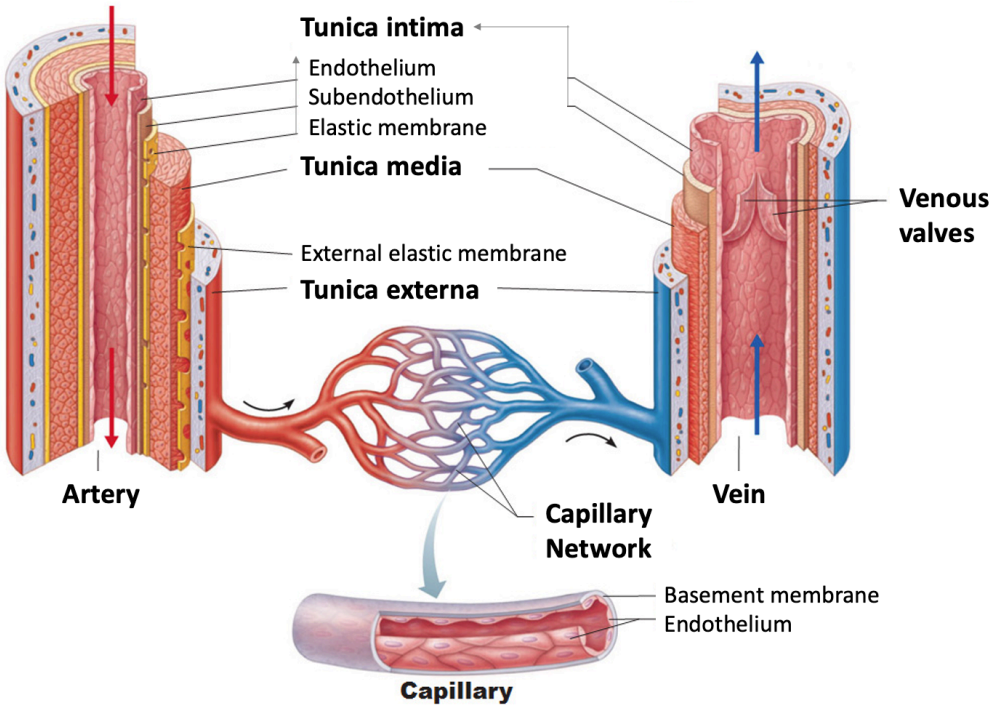


Figure 3: **Schematic of vessel wall morphology.**
Vessel wall morphology of arteries, veins and capillaries. Note, that the artery vessel wall is much thicker compared to the one of veins and capillaries. Veins can be characterized by venous valves. Capillaries typically consist of a single layer of ECs surrounded by a Basement membrane.

from Antranik (2020)

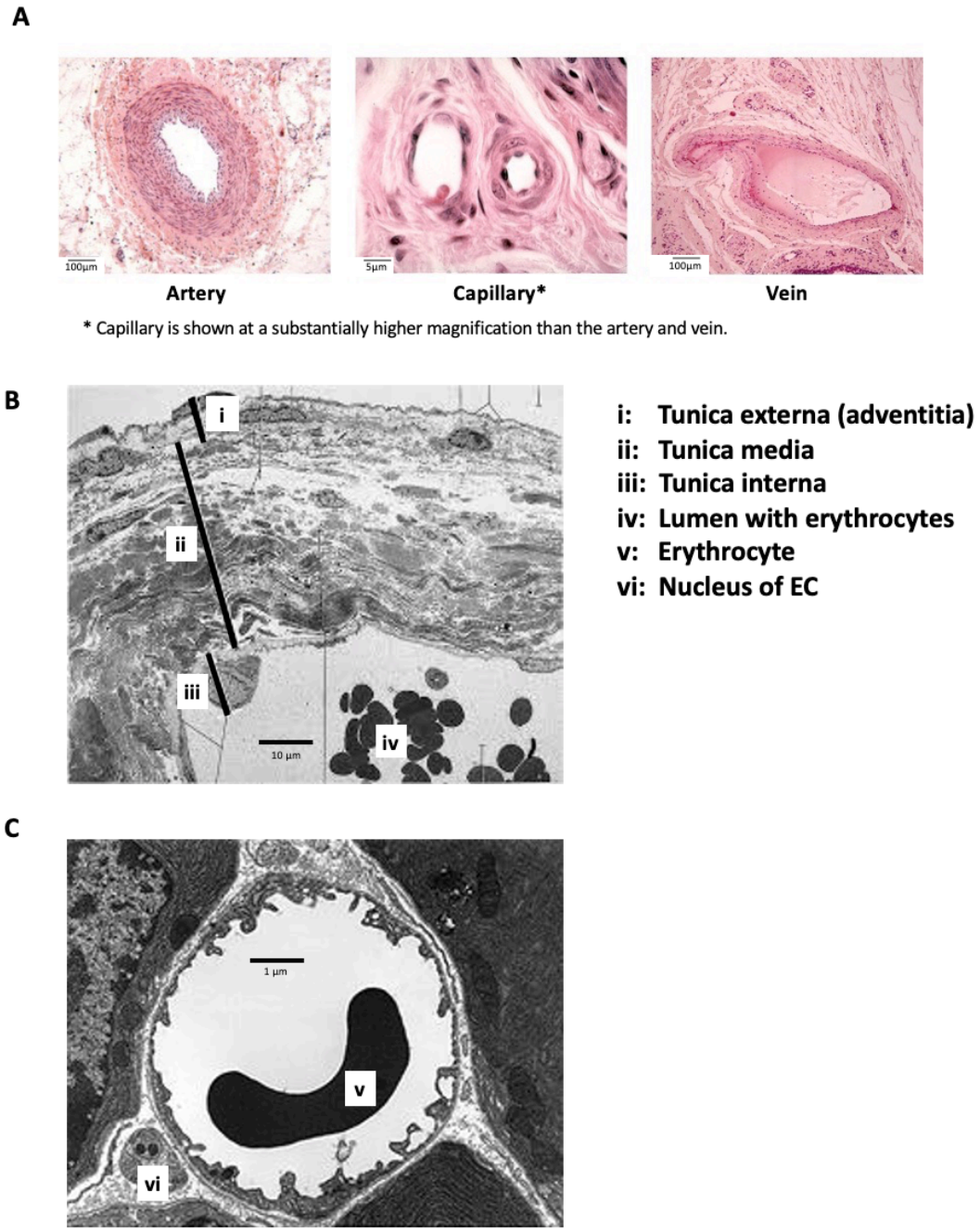


Figure 4: **Vessel wall formation of different vessels.**
A Histological slices showing the vessel wall morphology of artery, capillary and vein. The media wall in arteries is typically built up by a thick layer of SMCs or elastic fibers to guarantee an efficient and sufficient blood pressure. Veins are characterized by a single layer of ECs surrounded by a tunica media that is similar to the one of arteries but with a much thinner diameter. Magnification indicated by scale bars.
B EM image of an artery. Scale bars indicate magnification.
C EM image of a capillary. Scale bars indicate magnification.

References:
A: from Maffey (2019), B: from Young et al. (2013), C: from Howard (2018)

The focus of this dissertation is on the microvasculature. Microvessels stand out because of their small diameter and their unique functions (Wang et al., 2016). Larger vessels convey blood from one organ to another (e.g., aorta and more prominent arteries carry blood from the heart to the kidneys) and consist of solid and thick vessel walls. The microvessels, in contrast, distribute blood within the tissues and are crucial for oxygen and nutrient exchange (discussed above). In fact, the majority of oxygen and nutrient exchange happens at the level of capillaries (Levick & Michel, 2010). They comprise afferent arterioles, capillaries, and venules, and are the smallest blood vessels of the human body with a diameter of less than $10\ \mu m$ (Gould et al., 2017). Usually, they build up a close network, the capillary bed. The density of this network depends on the metabolic activity of the surrounding tissue (Adair & Montani, 2010). Exchanged substances between capillaries, the surrounding tissues, and interstitial fluids are oxygen, glucose, water, and waste products. Waste products comprise carbon dioxide, uric acid, lactic acid, urea, and creatinine (Wiszniak & Schwarz, 2014).

Capillaries can be further classified in continuous, fenestrated, and sinusoid capillaries depending on their wall structure (Mescher, 2009, Augustin & Koh, 2017).
Continuous capillaries are tube-shaped structures that are closed in all directions and in full-length (Gould et al., 2017). The ECs of this kind of capillaries do not contain any gaps or pores (Pavelka & Roth, 2010a). This kind of capillaries predominantly supplies the CNS, and since the retina anatomically belongs to the CNS, this is the type of capillary I am looking at during the experiments performed in the mouse retina (Sarin, 2010). The continuity of the capillaries allows the stable formation of the Blood-Brain Barrier (BBB) and the Blood-Retina Barrier (BRB), respectively (Daneman & Prat, 2015). The continuous capillary type can also be found in skeletal muscles, fingers, gonads, and skin, but with much more transport vehicles involved than in the capillaries of the BBB and BRB (Daneman & Prat, 2015, Mescher, 2009, Deng et al., 1999).
In contrast to this, fenestrated capillaries describe capillaries with numerous pores that allow smaller molecules to pass through (Pavelka & Roth, 2010b). Fenestrated capillaries are predominantly supplying endocrine glands, pancreas, and the glomeruli of the kidney (Tucker & Mahajan, 2019).
Sinusoid capillaries are a special form of vessels of the liver, spleen, bone marrow, lymph nodes and glands. They describe capillaries with numerous gaps in its EC wall, allowing for the exchange of bigger substances (Gartner & Hiatt, 2014).

In all three capillary subgroups, the vessel wall formation is determining the extent of substance and fluid exchange over the vessel wall. The ECs of continuous capillaries of the CNS are extraordinarily tight and only small molecules can diffuse through the vessel wall (Gartner & Hiatt, 2014). Fenestrated or sinusoid capillaries represent the opposite: molecules and other substances can get in and out in a much more accessible manner (Stan et al., 2012). There are multiple formula and models to calculate which substance can cross the vessel wall to which extent. The Starling equation takes different parameters in account to calculate the net flux. These include the hy-

drostatic and oncotic pressure in each of the two, the capillary and the surrounding interstitium, the filtration coefficient, the reflection coefficient, and the proportionality constant (Vicaut, 1996).

Capillaries play a crucial role in different diseases. A reduced number of capillaries is characteristic for patients with coronary heart disease (Sanchez-Garcia et al., 2018). In contrast, pathological hypersprouting can be observed in retinopathies, various cancers, and plays a role in tumor growth and metastasis (Armulik et al., 2011).

1.2.3 The mouse retina as valuable angiogenesis model

The formation and maturation of a blood vessel with its particular cells and functional requirements is a highly complex process. In vitro strategies aiming to copy the physiological outgrowth of a vascular network on a plate are challenging and often not satisfactory, yet (Sun et al., 2015). Thus, in vivo experiment are an essential and indispensable tool for vascular biologists. The mouse species and in particular the mouse retina are powerful and valuable models to explore the maturation and expansion of blood vasculature.

There are many reasons in basic science to perform experiments in mice; some of them apply exceedingly to vascular biology. The close phylogenetic relation of mouse and human, as well as the short reproduction time and established methods for gene manipulation, make mice an optimal animal species for the study of blood vessels (Cannarozzi et al., 2007, Finn, 1963, Sauer, 1998). The short time of mouse gestation and the young age of the examined animals reduces the study period for developmental experiments to a maximum of very few weeks from crossing the parental animals to the visualization of tissue samples. This makes mouse experiments useful and reduces potential animal suffering. Parental animals generally survive developmental experiments of their offspring (Kim et al., 2019a).

Mouse experiments have been used in vascular biology for decades. Numerous genes that are involved in vascular development are expressed ubiquitously in many cell types of the organism. Due to early embryonic mortality of genetic modifications in the majority of these crucial genes, it was a scientific challenge to study the postnatal vasculature of mice targeted in these core signal cascades (Miquerol et al., 2000, Ishii et al., 2006, Kulkarni et al., 1993). In recent decades, this hurdle was cleared by the development of inducible and cell-specific gene knockouts, or a combination of both (Claxton et al., 2008, Sauer, 1998). Creation of transgenic mouse lines is a fast, cheap, and efficient research method. It is much more sophisticated than decades ago (Czarnek & Bereta, 2016). Furthermore, immunological methods improved, and specific signal cascades can be targeted on the level of monoclonal antibodies or by adeno- and retroviruses, today (Singh et al., 2018, Lentz et al., 2012, Lundstrom, 2018).

The vascularization of the retina is of particular interest for my dissertation and future

therapies in ophthalmology for three reasons.
First, the murine retinal layer can be easily visualized in a two-dimensional manner similar to a disk, and the entire organ vasculature can be examined in a single flat-mounted preparation (Fig. 5 A). Methods to do so are established and sophisticated for many years (discussed above). Sample collection, preparation, and visualization of retinas are fast and uncomplicated procedures due to the thin retinal layer (Ogawa & Okajima, 2018, Ivanova et al., 2013). The first methods in animals were developed more than a half-century ago (Michaelson, 1948). Although the majority of studies include systemic interventions, secondary consequences from other organs on the retina can be ruled out, e.g., by intraocular injections of drugs or *Cre*-recombinase-activating substances like Interferon or Tamoxifen (Yardeni et al., 2011, Vacca et al., 2015, Harvey et al., 2002). Furthermore, the retinal outgrowth of blood vessels appears to be highly stereotypic and well-organized compared to more complex systems, e.g., tumors (see Fig. 5 A). Thus, crucial insights and findings in the retina can be easily compared between mutants and wildtype animals or even between different genetically modified mice (Carmeliet, 2000)).
The second reason pro retina is, that the mouse retinal blood vessel development is unique, as it begins after birth when the majority of other vessel networks is already fully developed (Stahl et al., 2010), visualized in Fig. 5 A . This allows targeting specific signal cascades postnatally.
Third and probably most important, the overall goal of my project is to find novel targets for better treatments of retinopathies. Considering the substantial heterogeneity of blood vessels and PCs in different organs of the mammal body, it is just natural to have a closer look at retinal vascularization. However, this heterogeneity also implies, that it is highly questionable whether the findings of the retina can be applied to other organs (Augustin & Koh, 2017, Pu et al., 2018, Aird, 2012, Marcu et al., 2018). Furthermore, retinal vessels anatomically belong to the CNS. Since major vascular diseases manifest in the brain, it is very likely that scientific findings in the retina can be valuable for a better understanding of aneurysms, stroke, Alzheimer's disease, and other vascular brain diseases.

1.2.4 Vasculogenesis and sprouting angiogenesis in the mouse retina

Gas and nutrients exchange of the embryonic eye are guaranteed by diffusion from hyaloid vessels (Saint-Geniez & D'Amore, 2004). This primary hyaloid network is formed in the primitive vitreous body between embryonic days (E) 10.5 and E13.5, and recedes on postnatal days (P) 4 to P8. At P10, only very few HV vessels persist and the vitreous body is mostly avascular. Simultaneously to the HV regression, the retinal vasculature is beginning to grow at P0 in response to a deprivation of oxygen in the superficial (or inner) layer of the retina (Stahl et al., 2010, Connolly et al., 1988), as visualized in Fig. 5.
During vascularization of an organ, two processes potentially play an essential role. The first one is characterized by vasculogenesis, which describes the de novo formation of vessels from mesoderm-derived vascular endothelial precursor cells, so-called

angioblasts (Risau & Flamme, 1995, Flamme et al., 1997, Swift & Weinstein, 2009, Adams & Alitalo, 2007). Angioblasts are attracted by astrocytes, which populate the retina prior to the blood vessel network (Fig. 5 B). Hence, angioblasts migrate to the location of future vessels, where they differentiate into ECs, forming the primitive lumenized vascular plexus (Fruttiger, 2002, Gariano, 2003, Lutty et al., 2010).
The ECs lining the vascular tubes are crucial for sprouting angiogenesis, which is the second step of retinal vascularization. Sprouting angiogenesis is different to vasculogenesis. It describes a process of blood vessel formation, in which no angioblasts are involved, but proliferating and migrating ECs that extend the vascular network from preexisting vessels (Flamme et al., 1997, Chan-Ling et al., 2004). This extension of the blood vessels occurs from the central optic nerve head, over the inner surface, toward the edge of the retina (Fig. 5 A). Beginning at P0, the network reaches half of the retinal surface at P4-P5 and the edge at P7. Subsequently, the blood vessels start to dive in deeper retinal layers in an almost right angle building two more vascular networks (Fig. 5 A). The retinal blood vessel remodeling and maturation finishes not before P21 (Connolly et al., 1988, Stahl et al., 2010).

1.2.5 Hypoxia-induced retinal sprouting angiogenesis

Hypoxia-induced sprouting angiogenesis is observed in growing tissues, and pathological conditions such as wound healing, tumors, and retinopathy in which the oxygen and nutrient supply is not sufficiently available and newly formed blood vessels are urgently required (Butturini et al., 2019, Carmeliet, 2005, Uddin et al., 2016). Key triggers mediating this nutrient and oxygen paucity to angiogenesis are cytokines and proangiogenic factors that are expressed and activated under shortage conditions. The major known proangiogenic factor is the polypeptide VEGF-A (Gerhardt et al., 2003).

One key question is how tissues sense and respond to low oxygen levels. Impaired tissue oxygenation leads to a well-defined molecular response. Critical factors in this response are hypoxia inducible factors (HIFs), which are transcription factors activated at low oxygen concentrations and control the expression of a large number of genes (Balamurugan, 2016), inter alia the vascular active agents VEGF-A, PDGF-B and TGF-B (Schito et al., 2012, Mingyuan et al., 2018, Du et al., 2018, Basu et al., 2011). HIFs themselves are activated under the control of the tumor suppressor Von-Hippel-Lindau (VHL) protein complex (Zhang & Zhang, 2018). VHL interacts with hydroxylated HIF-α subunits and mediates a proteasomal degradation pathway of HIF under physiological conditions. Oxygen-dependent Elongin-BC9 enzymes hydroxylate a proline residue on HIF1-α, which promotes binding of Hif1-α to VHL (Haase, 2009, Okumura et al., 2012). Under hypoxic conditions, prolyl hydroxylation fails, and HIF1-α is spared (Ohh et al., 2000). Thus, degradation is reduced, and the HIFs are stabilized, thereby having a stronger impact on gene expression, especially increasing the secretion levels of VEGF-A (Kurihara et al., 2014). Kaelin, Semenza, and Ratcliffe were awarded the Nobel prize in Medicine or Physiology in 2019 for their discoveries of HIF and related signaling cascades.

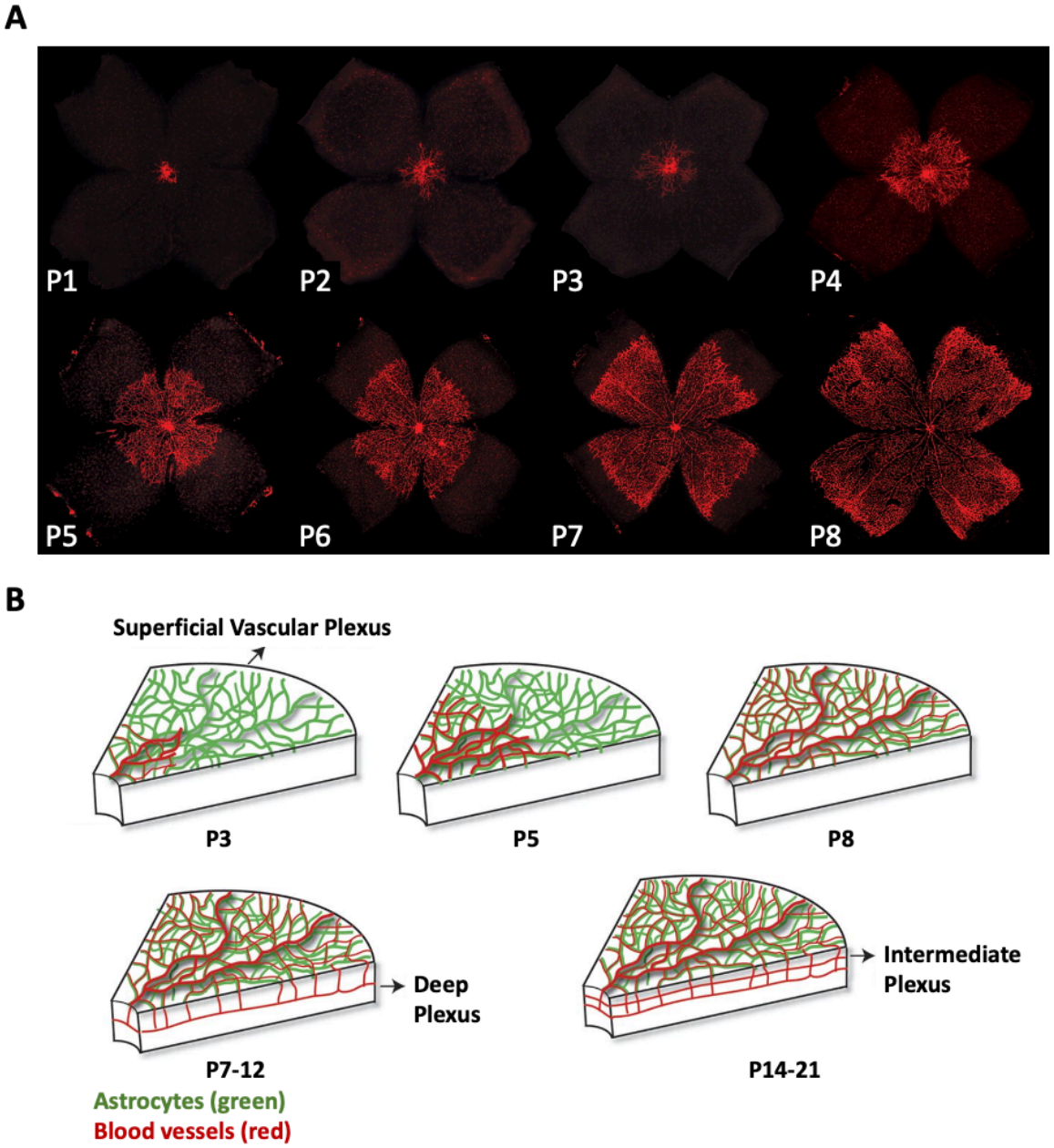


Figure 5: **The developing blood vasculature of the mouse retina after birth.**
A Isolectine B4 (IB4) staining of flat-mounted mouse retinas at different time points after birth, P1-P8. *Magnificaion not indicated in original publication.*
B The interplay between ECs and astrocytes during sprouting angiogenesis. *Vegf-a* is expressed in a preformed network of astrocytes. Note: Deeper vascular plexi are formed between P7 and P21 independently of astrocytes.

References:

A: from Stahl et al. (2010), B: from Sapieha (2012)

1.2.6 VEGF-A as major driver of angiogenesis

The vascular endothelial growth factor family is the major driver of sprouting angiogenesis (Gerhardt et al., 2003). In human and mice six secreted ligands of the VEGF family can be detected (VEGF-A, -B, -C, -D, -E and -PLGF), which bind with different affinities to three structural and functional disparate tyrosine kinase receptors, VEGFR1, VEGFR2, and VEGFR3 (Shibuya, 2011, Jeltsch et al., 2013), visualized in Fig. 6 A. This results in a receptor dimerization and autophosphorylization (Nilsson & Heymach, 2006, Mac Gabhann & Popel, 2007). The interplay between VEGF ligands, corresponding receptors, and other angiogenic and angio-inhibitory factors provides the opportunity to generate complex vascular networks (Carmeliet, 2005, Feeney et al., 2003, Sarlos et al., 2003).

More than 60 years ago researchers mentioned a molecule *Factor X* which controls retinal vascular development (Arroyo, 2019, Stone & Maslim, 1997) and is highly oxygen-sensitive (Shweiki et al., 1992). Today this factor is called VEGF-A and it is well understood that VEGF-A signaling through VEGFR2 is involved the key regulator of growing vessels (Senger et al., 1993, Lawson et al., 2002, Mukouyama et al., 2002, Stalmans et al., 2002). In fact, it is the strongest known promoter of both, vasculogenesis - the assembly of angioblasts in the primary vascular plexuses (Shalaby et al., 1995, Carmeliet et al., 1996, Ferrara et al., 1996, Abhinand et al., 2016), as well as sprouting angiogenesis (Ferrara et al., 1996). The final vessel morphogenesis is also defined by VEGF-A signaling (Coultas et al., 2005, Kowanetz, 2006, Li et al., 2008, Gerhardt, 2008). Thus, it is not surprising, that VEGF-A signaling became the major target of medical treatments in diseases with dysfunctional angiogenesis (tumors, diabetic retinopathies) and thereby one of the flagships of vascular biology (Simons et al., 2016, Apte et al., 2019).

In the developing mouse retina the majority of VEGF-A ligand is released by a preexisting network of GFAP-positive astrocytes, which maintains a gradient of VEGF-A with higher levels in the direction of sprouting (Gerhardt et al., 2003, Ruhrberg et al., 2002, Fruttiger, 2002, Pierce et al., 1995a, Provis et al., 1997, Stone & Maslim, 1997), visualization in Figures 5 and 6. Genetic targeting of retinal astrocytes affects VEGF-A signaling and ECs with the same proportions (discussed below). Deletion of a single allele of *Vegf-a* is not viable, pubs die at day E10-12 (Carmeliet et al., 1996, Ferrara et al., 1996). In contrast, genetic deletions of the other *Vegf* ligands show only subtle or no vascular phenotypes (Autiero et al., 2003, Reichelt et al., 2003).

Once secreted, VEGF-A binds with its C-terminal retention motif to heparan sulfate (HS) structures in the extracellular matrix in order to be presented at the right location (Stalmans et al., 2002). This implicates, that not only the VEGF-A quantity is crucial for physiological blood vessel sprouting, but also the right location of ligand presentation and quality of ECM interaction which is ensured by HS binding in the ECM. It is not surprising, that genetic overexpression of *Vegf-a*, as well as injection of VEGF-A in the eye leads to massive hypersprouting (Gerhardt et al., 2003), induces filopodia formation and Tip Cell gene expression is increased (Hellström et al., 2007).

Each of the three receptors fulfills specific functions and stands in clear connection to angiogenesis as single homozygous deletions of any of the *Vegfr1-3* lead to vascular abnormalities and embryonic death at around E10 (Dumont et al., 1998, Fong et al., 1995, Shalaby et al., 1995, Carmeliet et al., 1996). The VEGF-A isoforms signal principally through the two remaining receptors (VEGFR1 and 2), which are both expressed on the surface of sprouting ECs in a temporally and spatially distinct fashion, but have reverse effects on sprouting angiogenesis (Shih et al., 2003, Gariano et al., 2006, Gariano, 2003, Ferrara, 1999, Fruttiger, 2002), see Fig. 6 A. In the postnatal mouse retina, VEGFR2 (gene: *Flk1=Kdr1*) is the main mediator of sprouting angiogenesis and is involved in the majority of catalytic downstream cascades of VEGF-A (Takahashi & Shibuya, 2005, Simmons et al., 2018). Highest levels are expressed in Tip Cells and endothelial filopodia formations (Gerhardt et al., 2003), compare Fig. 6).
The second important receptor for VEGF-A is VEGFR1 (gene: *Flt-1*), which is also strongly expressed in ECs of blood vessels with highest levels in Stalk Cells and lower levels in Tip Cells (Chappell et al., 2019, Jakobsson et al., 2010). VEGFR1 has only weak kinase activity and acts as a decoy to trap VEGF-A, keeping it unavailable for the other VEGF-receptors, a process of negative regulation (Park et al., 1994).
VEGFR3 is present in all endothelia during development, but in adults becomes restricted to the lymphatic endothelium. It is of particular importance for the VEGF-C and VEGF-D ligands (Varney & Singh, 2015, Jacob et al., 2019, Zhang et al., 2018). However, VEGFR-3 is upregulated in the blood microvasculature of tumours and wounds. Tammela et al. (2008) demonstrates a key role of VEGFR3 for blood vascular sprouting and blocking VEGFR3 results in decreased sprouting, vascular density, vessel branching and endothelial cell proliferation in mouse angiogenesis models.

1.2.7 Tip cell selection and Endothelial Cell plasticity: the fine interplay of Notch and VEGF-A

VEGF ligands and their corresponding receptors are the major players in sprouting angiogenesis, but in addition to them, several mouse retina studies illustrate the importance of Notch signaling components including Dll4, Jagged-1 and Notch1 in EC specification during formation of a functional vascular network (Blanco & Gerhardt, 2013), compare Fig. 6. Thereby the actual detonator for sprouting angiogenesis is a VEGF-A gradient, in the retina originated by a network of astrocytes (discussed above). Every EC in the preexisting vessel network is expressing the corresponding *Vegfrs*, but with differential amounts resulting in an individual response proportion to VEGF-A. Neighboring ECs allocate themselves by communication through DLL4- and its corresponding Notch1-Receptor in two distinct categories: migratory Tip Cells, which lead the sprouting front, and proliferative Stalk Cells behind, giving stability and build the future lumen of the nascent vessel (Blanco & Gerhardt, 2013).

The selection of Tip Cells is a highly competitive and dynamic process, in which chosen Tip Cells assert their position by presenting DLL4 to neighboring ECs, a process of lateral inhibition (Fig. 6 B). The presentation of DLL4 increases Notch1-

downstream-signaling in the neighboring cells resulting in a decreased expression of *Vegfr2* and *Nrp1*, but an upregulation of *Vegfr1*, thereby trimming the sensitivity to the VEGF-A gradient. The affected cells are pushed to the Stalk position and the *Dll4* expressing cell takes the lead (Blanco & Gerhardt, 2013). In contrast, Stalk Cells of the second row present Jagged1, another cell surface-ligand, to the migratory Tip Cells thereby reducing Notch1-downstream signaling in the leading Tip Cells resulting in an increased VEGF-A sensitivity. This understanding and several confirming studies suggest that VEGF-A acts downstream of Notch signaling (Lawson et al., 2002, Weinstein & Lawson, 2002, Patel et al., 2005, Ridgway et al., 2006, Lobov et al., 2007), compare Fig. 6 B.

In fact, besides a VEGF-A-VEGFR2 regulation by DLL4-Jagged/Notch1 signaling, a modulation also occurs vice versa: ECs experiencing a stronger influence of VEGF-A-VEGFR2 signaling, e.g. Tip ECs, express increased levels of *Dll4* (Patel et al., 2005, Williams et al., 2006, Lobov et al., 2007). This effect is strengthening the mechanism described above making Notch and VEGF-A interact in a mutual regulation loop (Fig. 6 B). This fundamental principle is believed to represent the key mechanism of spacing vascular sprouts during normal development.

Many other signal cascades are described to interact, promote or to block Notch signaling in order to influence angiogenesis (Wüstehube et al., 2010). For the sake of clarity, I will not mention them at this point and refer to appropriate reviews (Andersson et al., 2011, Siekmann & Lawson, 2007, Kofler et al., 2011).

1.2.8 Retinal disease models in the mouse

The most commonly used model to mimic human ischemic retinal diseases like ROP and DR is the Oxygen-induced Retinopathy Mouse Model (Fig. 7 and 9). The key advantage of this model is not only the close phylogenetic similarity between mice and human, but once more the fact, that the murine retinal blood vasculature develops within several days after birth in a highly reliable manner. This development is not only rapid, but also predictable, and tightly regulated. It makes the OIR protocol in the mouse retina an optimal model to study the human diseases. Genetic and drug interventions are possible and well-established (Sharif & Klimko, 2007). There is a strong similarity between diseased human retinas and retinas from OIR mice. Importantly, ROP progresses in two phases. Initially, vaso-obliteration with subsequent vessel depletion can be observed. In a second step, pathological neovascularization occurs during the repair phase as a response to a relative hypoxia (Compare Fig. 7). These two hallmarks are closely phenocopied in OIR models. Similar finding have been observed in patients suffering from the wet form of AMD (Gammons & Bates, 2016, Zhou et al., 2019).
There are other models established to mimic ischemic retinopathies, e.g. streptozotocin-diabetic animals. Interested readers should look for appropriate protocols elsewhere.

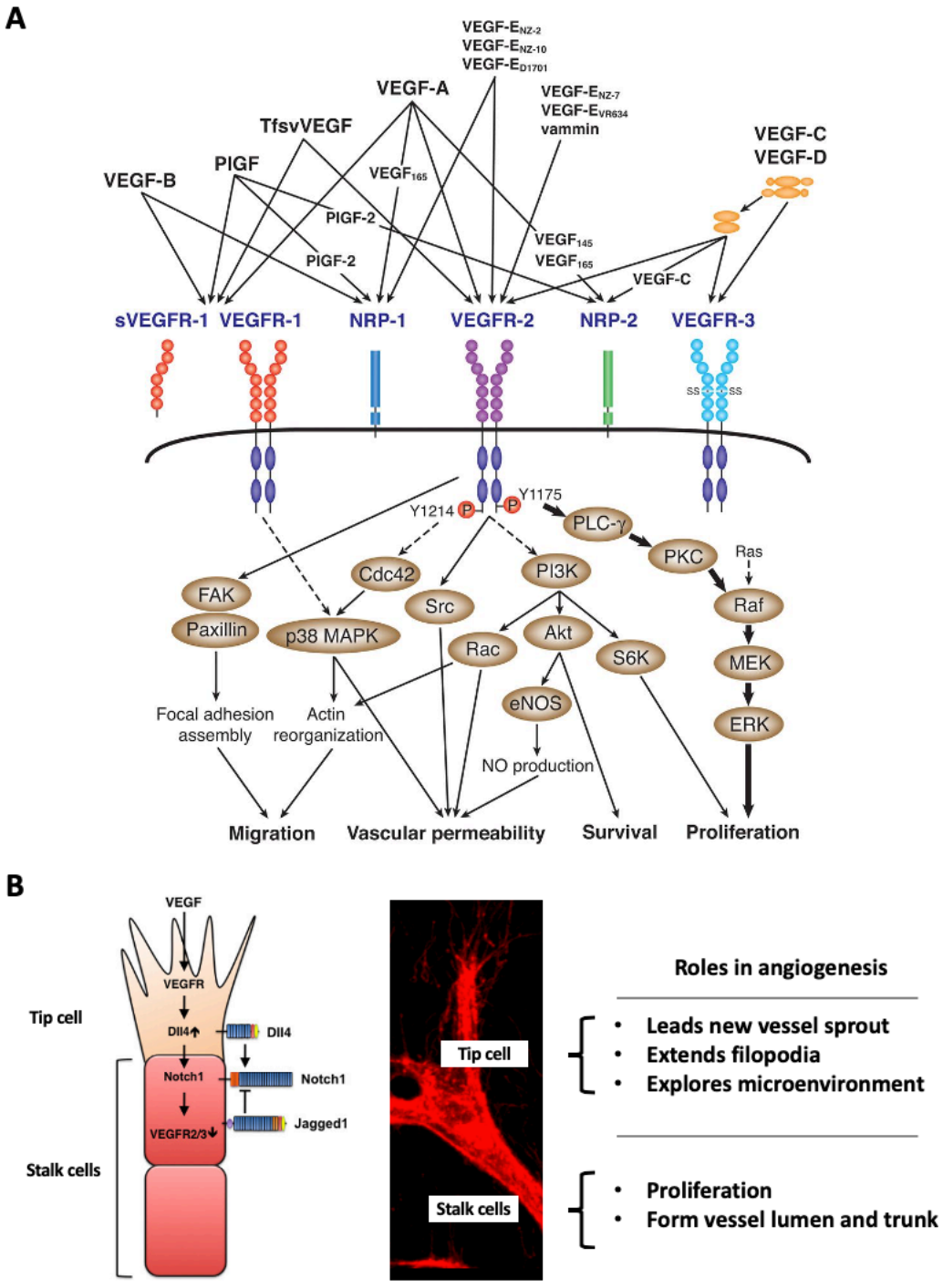


Figure 6: **VEGF signaling and current concepts of Tip and Stalk Cell selection.**
A VEGF ligands, receptors, downstream signaling cascades, and corresponding cell behavior.
B Tip and Stalk cell selection. Left: Schematic of the mutual regulation of VEGF-A and Notch signaling. Center: High manification of Tip and Stalk Cells, IB4 staining. Right: Overview of different roles of Tip and Stalk cells during angiogenesis.

References:
A: from Takahashi & Shibuya (2005) B: from Suchting & Eichmann (2009), Kume (2009)

	Health	ROP	Prol. DR	AMD	OIR
Neovascularization	1	11	21	31	41
Vessel obliteration	2	12	22	32	42
Microaneurysms	3	13	23	33	43
Edema	4	14	24	34	44
Hemorrhage	5	15	25	35	45
Exudates	6	16	26	36	46
Atrophy	7	17	27	37	47
Druses	(8)	18	28	38	48
Retina Detachment	9	19	29	39	49
Neovascular Tufts	10	20	30	40	50

Figure 7: **Vascular characteristics of the OIR model and their appearance in human retinal diseases.**
Grey highlighting: Corresponding feature is not regularly found in indicated conditions. Blue highlighting: Corresponding feature is regularly found in indicated conditions. *Digits 1-50 refer to references.*
As displayed the OIR model is mimicking the human diseases ROP, proliferative DR, and Wet AMD to a high degree. Every disease has individual features, such as pathological characteristics, degenerations, and malformations.

References:
1-10: Mehrle (2010); 8: Klein et al. (1992); 11,12: Sapieha et al. (2010); 13: Nudleman & Capone (2017); 14: Vinekar et al. (2015); 15: Jensen et al. (2011); 16: Ittiara et al. (2013); 17: Fishburne et al. (1997); 18: Nudleman & Capone (2017); 19: Kalina (1991); 20: Wallace et al. (1998); 21: Cho et al. (2013); 22: Shin et al. (2014); 23: Hammes et al. (2002); 24: Cohen & Gardner (2016); 25: Murugesan et al. (2015); 26: Esmann et al. (2009); 27: Park (2016); 29: Li et al. (2017); 30: Ishibazawa et al. (2016); 31: Chappelow & Kaiser (2008); 32-40: Holz et al. (2004); 38: Rudolf et al. (2008); 41-42, 45, 50: Dubrac and Künzel et. al. (2018); 43-49: Kim et al. (2016), Hartnett (2014);

1.3 Pericytes

Almost one hundred-fifty years ago, Eberth and shortly after Charles-Marie Rouget described Pericytes (PCs) for the first time. Rouget was the one who dared to give a definition of the then novel cell type as he described them as a population of contractile cells surrounding the ECs of small blood vessels. Zimmermann stated in 1923 that all transitional Mural Cell phenotypes expressing smooth muscle fibers are to be included. Since these early definitions several generations of scientists tried to define this interesting cell type. Studies focused on marker expression profiles, spatial distribution, contractility, surrounding vessel, single cell gene expressions, and other features. Today, scientists agree that not one, but different cell types cover the ECs from the abluminal side. However, the correct and sharp identification of MCs and particularly PCs is still a challenge in the field. I intend to make my contribution via this dissertation.

Pericytes are the mural cells (MCs) of blood microvessels and appear ubiquitously in the human body (Armulik et al., 2011). They cover the vascular endothelium from the abluminal side and are embedded within the vascular basement membrane (BM) in close proximity to ECs (Thomsen et al., 2017). Communication to neighboring ECs is maintained by numerous cell-cell-interactions, as well as through major paracrine signaling cascades, such as PDGF-B- and Tie2- signaling (Bjarnegard et al., 2004, Zonneville et al., 2018, Sweeney & Foldes, 2018). In this position, they fulfill several crucial functions, such as giving vascular stability, assembling the BM, and thereby guaranteeing barrier functions within the Blood-Brain Barrier (BBB) and the Blood-Retina Barrier (BRB), as stated in Park et al. (2017) and Armulik et al. (2010). Furthermore, PCs have long been proposed to play crucial roles in development and diseases, such as cancer, fibrosis, and retinopathies (Chen et al., 2016, Greenhalgh et al., 2013, Beltramo & Porta, 2013). Thus, a rising interest in PCs has led to a detailed understanding of PC biology. Nevertheless, detailed functions and characteristics of PCs are not fully understood today. Since PCs have just recently gained more and more interest in potential clinical interventions, a lot of aspects are still the subject of current studies. Today, scientists abandoned an EC-centered understanding of sprouting angiogenesis to a more complex model with more cells involved, including PCs and other MCs.

1.3.1 Identification

PCs belong to the family of MCs, which is not a singular fixed cell type, but an umbrella term, as it really refers to a number of cell types that have just recently been further distinguished (Vanlandewijck et al., 2018a). In general, MCs comprise PCs and SMCs. PCs appear omnipresent in the human body and consequently bear very different functions and demands depending on their specific location (Vanlandewijck et al., 2018b, Holm et al., 2018, Augustin & Koh, 2017). Thus, PCs display a highly organ-specific profile with distinct morphological characteristics and marker expression profiles (Armulik et al., 2011). It is challenging to identify the different subgroups of MCs and PCs, specifically at their location and tissue. What makes PC

identification even more difficult is the high plasticity of MCs (Herrmann et al., 2016). They potentially change their characteristic profile during developmental states (Armulik et al., 2011), in pathological conditions (Cathery et al., 2018) and during in vitro culture experiments (Cantoni et al., 2015).

PC identification can be achieved by using molecular marker expression profiles. The challenging character of this method is that PCs, SMCs, and other perivascular cell types express very similar and overlapping expression patterns (Vanlandewijck et al., 2018a), see also table 3.2. Many potential markers are proposed to be consulted in terms of PC identification, but only very few are well-studied for reliable conclusions (Schlingemann et al., 1991, Smyth et al., 2018). PCs are positive for unspecific MC markers, such as PDGFRβ, NG2, CD13, and Desmin (Armulik et al., 2011). While these are particularly powerful to distinguish MCs from other cell types, such as ECs or neurons, none of them is specific for one single subgroup of MCs, and there is no specific marker established to label PCs (Armulik et al., 2011). To overcome this hurdle, it is conducive that certain markers specifically identify SMCs, including α-SMA and MYH11 (Kumar et al., 2017, Allahverdian et al., 2018, Armulik et al., 2011, Vanlandewijck et al., 2018a). Thus, PCs should be defined by exclusion, and it is appropriate to use a combination of different markers for a sharp tissue-specific MC and PC identification. At this point, experts of the field adopted the view, that a PC can still be best descirbed by being PDGFRβ positive and α-SMA negative.

The rising importance of Single Cell Sequencing techniques opened new doors concerning the identification of certain MC subgroups, i.e. the advent of massive parallel sequencing now allows molecular profiling of PCs, as performed by Vanlandewijck et al. (2018a) and others. A much more accurate picture of brain MC gene expression could be observed. The studies confirm that there is no specific PC marker that can be used in every condition of health and disease to clearly distinguish PCs from other cell groups. Some novel markers have been identified but should undergo further examination in terms of PC specificity and reliability (compare also Table 3.2).

As a matter of fact, under certain circumstances, such as pathological conditions or development, a hard line cannot be drawn using only the expression marker profile. Thus, it is appropriate to not only distinguish PCs from SMCs and other cell types by their marker expression levels, but by consulting a combination of several criteria (Armulik et al., 2011). These can include: the cell morphology by high-resolution microscopy (Yamazaki & Mukouyama, 2018, Dore-Duffy & Cleary, 2011), the specific location and surrounded vessel (Trost et al., 2016, Armulik et al., 2011), cell function (Attwell et al., 2016), and other characteristics, which depend on the scientific question. Furthermore, PCs tend to strongly differentiate and change their appearance when it comes to disease conditions, developmental stages, or in vitro experiments (Schor et al., 1995). In this case, more elaborate protocols have to be applied, such as fate-mapping experiments (Cathery et al., 2018).

Marker molecule	*Gene name*	Expressing cell-types (others than PCs)	Important functions
Neural/Glial Antigen 2, Chondroitin Sulfate Proteoglycan (NG2)	*Cspg4*	Developing cartilage; bone; muscle; early postnatal skin; adult skin stem cells; adipocytes; vSMCs; neuronal progenitors; oligodendrocyte progenitors	Crucial for cell proliferation and migration, inhibitor of neurite outgrowth and growth cone collapse during axon regeneration, cell surface receptor for collagen alpha 2, more functions described.
Plateled-derived Growth Factor Receptor β (PDGFRβ)	*Pdgfrβ*	Interstitial mesenchymal cells during development; smooth muscle; in the CNS; certain neurons and neuronal progenitors; myofibroblasts; mesenchymal stem cells	Tyrosine-protein kinase that acts as cell-surface receptor for homodimeric PDGFB and PDGFD and for heterodimers formed by PDGFA and PDGFB, essential role in blood vessel development by promoting proliferation, migration and recruitment of PCs and SMCs to ECs, more functions described.
α-SMA (α-Smooth Muscle Actin)	*Acta2*	Smooth muscle; myofibroblasts; myoepithlium; all other eukaryotic cells	Cell motlity, constituent of thin filaments, Up-regulated in response to certain infections.
Myosin Heavy Chain 11 (MYH11)	*Myh11*	Muscle cells; almost every eukaryotic cell	Muscle contraction.
Desmin	*Des*	Skeletal, cardiac, smooth muscle cells	Intermediate filament essential for muscular structure and function. Crucial for sarcolemmal cytoskeleton, but also for nucleus and mitochondria. Important in cardiac cells.
Transgelin (SM22 α)	*Tagln*	SMCs; fibroblasts; many eukaryotic cell types	Actin cross-linking/ gelling protein. Involved in Ca interactions and contractile properties of the cell.
Regulator of G Protein Signaling 5 (RGS5)	*Rgs5*	vSMCs; heart muscle; lung cells; skeletal muscle; small intestine and many more tissues	Inhibitor of signal transduction cascades.
Leptin Receptor (LEPR)	*Lepr*	Hypothalamus cells; skeletal muscle; ECs	Receptor for hormone LEP.
Nestin	*Nes*	Neural stem cells; neural cells; progenitors; muscle cells; pancreatic cells	Intermediate filament protein, Brain and eye development, crucial for neural progenitor cells, many more functions described.
Forkhead Box D1 (Forkhead-related Transcription Factor 4)	*Foxd1*	SMC; fibroblasts; much lower levels in EC	Transcription factor, particular important for developing retina, optic chiasm, kidney and neuralization of ectodermal cells, many more functions described.
Glioma-Associated Oncogene Homolog 1 (GLI1 Family Zinc Finger 1)	*Gli1*	Muscle cells; nervous cells; different stem cells and progenitors	Transcription factor especially important during development of the CNS and the GI tract, interaction with SHH transduction cascade, regulator of stem cell proliferation.
ADAM12 Metallopeptidase Domain 12 (Disintegrin And Metalloproteinase Domain-Containin Protein 12, Meltrin-α, MLTN)	*Adam12*	Skeletal muscle cells; cardiac muscle cells; vSMC; osteoblast; osteoclast and many more	Disinegrin and metalloprotease domains, involved in skeletal msucle regeneration and bone cell formation.
anpep (CD13, Amino-peptidase N)	*Anpep*	vSMCs; inflamed and tumor endothelium; myeloid cells; epithelial cells in the kidney; gut	Final digestion of peptides, processing of peptide hormones like angiotensin III and IV, neuropeptides, chemokines, bound to MHC complex class II molecules, important role in angiogenesis and cholesterol crystallization.
delta-like 1 homolog, Delta Like Non-Canonical Notch Ligand 1, Protein δ Homolog 1 (DLK1)	*Dlk1*	vSMCs; hepatoblasts in the developing liver; adipocyte progenitors	Transmembrane protein containing multiple epidermal growth factor repeats, cell growth regulator.
ABCC9, ATP-binding cassette, subfamily C (SUR2)	*Abcc9*	Skeletal, cardiac, smooth muscle; renal tubular epithelium	Subunit of ATP-sensitive potassium channels (KATP).
Kir6.1 (KCNJ8, potassium inwardly rectifying channel, subfamily J)	*Kcnj8*	vSMCs; heart muscle cells	Potassium channel controled by G-proteins.
Endosialin (Tem1, CD248)	*Cd248*	vSMCs; myofibroblasts; fibroblasts; T-cells	Unclear; important in tumor angiogenesis.

Table 2: **Marker molecules for Pericyte identification.**
Notably, there is no single molecule that stands out as a specific and established PC marker. The table does not make a claim to completeness, but is instead intended to give an overview of proposed and commonly used markers.

References: Compilation from Armulik et al. (2011) and Vanlandewijck et al. (2018a).

1.3.2 Function

The role of PCs is complex, and many functions are still subject to research. Experts of the field adopted the view that PCs are crucial for the developing and mature vasculature (Bergers & Song, 2005). Thereby they guarantee vessel stability, integrity, barrier functions, and potentially regulate blood flow (Armulik et al., 2011).

An explanation for the importance of PCs for the vasculature can be found in the proximity between PCs and adjacent ECs (Choi et al., 2018). This close neighborhood supports the idea of an anchoring and stability function. PCs are also an essential part of the Basement Membrane (BM) and assemble proteins for it (Stalmans et al., 2002). The BM guarantees stability functions, acts as a mechanical barrier and plays a vital role in EC differentiation during angiogenesis (Thomsen et al., 2017).

Furthermore, and as discussed in more detail below, PCs are crucial for production, processing, and releasing of different growth factors and paracrine ligands to communicate with neighboring cells, especially ECs (Armulik et al., 2011). The outstanding importance of PCs for a functional vasculature was confirmed with experiments using transgenic mice in which PCs were impaired or depleted. The affected animals suffer from severe vascular abnormalities, including significant stability defects, widely enlarged vessels, leakage, and bleeding (Uemura et al., 2002, Enge, 2002).

SMCs predominantly cover larger caliber arteries where they provide strength and elasticity. They can potentially act as powerful ring-shaped sphincters that contract and relax to regulate blood flow to the brain and other organs (Xie & Chen, 2011). Whether or not PCs are capable of dynamically regulating the vessel diameter by contracting themselves is a point where expert opinions differ. Even if the mechanism is not revealed in detail, recent studies indicate that PCs are capable of controlling the blood flow (Armulik et al., 2011, Hamilton et al., 2010, Kelley et al., 1987).

Many more organ-specific functions are proposed and have to undergo further scientific evaluation. They are involved in scar formation (Gautam & Yao, 2018), and potentially regulate inflammation (Rudziak et al., 2019). Interestingly, some PCs even act as mesenchymal stem or progenitor cells and can give rise to tissues from a broad range of background, even heart and bone tissue (Armulik et al., 2011). On top of that, the phagocytic activity of PCs following ischemia and injury of the central nervous system has been described (Winkler et al., 2014, Ueta et al., 2011). Both the activity as a progenitor, as well as their phagocytic function need further investigation.

1.3.3 Location and abundance

MCs are ubiquitously present in the vertebral body, with SMCs wrapping around larger vessels and forming a dense layer around arteries, arterioles and precapillary arterioles (Bacakova et al., 2018, Xie & Chen, 2011). PCs on the other hand are located on veins, venules and capillaries with an entirely different morphology. They have round bodies and form extending branching processes - often described as stellate in electro-microscopic studies. With this, one PC can have contact with numerous ECs. Thus, it is reasonable to think about vessels as a complex, teamplay model (Armulik et al., 2011). Observations about PC frequency in different tissues reveal that their

number in relation to the number of ECs varies between 1:1 to 1:50 or even less, depending on their distinct location. Studies in muscle tissue, for example, reveal a very low number of PCs, while in the brain the number of PCs is approximately equal to the number of ECs. In the brain, about 30 percent of the abluminal EC surface is covered by PCs with numerous interactions between ECs and PCs. A possible explanation for this higher number of PCs in the CNS is their crucial contribution to the BBB and BRB (Park et al., 2017). Nevertheless, even in the brain the number of PCs in relation to the number of ECs is highly flexible with the highest density in the retinal vasculature (Sims, 2000, Armulik et al., 2011).

Interestingly, while PCs can be found throughout all vessels in the human body, they do not cover lymphatic vessels. An exception to this are pathological conditions in which ectopic PCs migrate to lymphatic vessels, e.g. in lymphedema (Petrova et al., 2004, Ohtani & Ohtani, 2012, Armulik et al., 2005).

1.3.4 Origin

The origin of MCs varies depending of the cell type and the tissue. It has been proposed that MCs originate from mesenchymal cells, which adhere at the abluminal side of the vessel. However, recent observations disproved this hypothesis and uncovered that there is a higher level of complexity with more than one origin of PCs. Indeed, blood vessels are covered by MCs from several origins, depending on the exact location. For example, SMCs from the aorta grow out of at least four origins: secondary heart field, neural crest, somites, and splanchnic mesoderm (Armulik et al., 2011). This creates a mosaic at one single vessel. The majority of MCs of the forebrain and especially the PCs of retinal microvessels are arising from the cranial neural crest. Nevertheless, the origin of PCs remains a current research topic (Armulik et al., 2011, Yamazaki & Mukouyama, 2018).

1.3.5 Pericyte and Endothelial Cell communication

ECs and MCs commonly produce extracellular matrix proteins and share the BM (Eble and Niland, 2009). The proximity between the two cell types guarantees a powerful communication between ECs and MCs (Fig. 8). Even one PC can have direct contact with numerous ECs (discussed above). Although the proximity suggests a significant mechanical interaction, this introduction will focus on juxtacrine and paracrine signaling cascades between the two cell types (Fig. 8).

PDGF-B-PDGFRβ The importance of Platelet-derived growth-factor B (PDGF-B) for MCs gained momentum, when Russel Ross shared his observations about the growth factor and its critical role as a mediator of MC accumulation in wounded arteries in 1974 (Ross et al., 1974). Through the years more and more transgenic and interventional mouse studies focused on PDGF-B signaling through its corresponding receptor, PDGFRβ (Kohler & Lipton, 1974). Today it is well-understood, that PDGF-B is a prominent molecule of MC biology as it mediates MC migration, survival, proliferation, and differentiation in this group of cells (Armulik et al., 2011), compare Fig. 8.

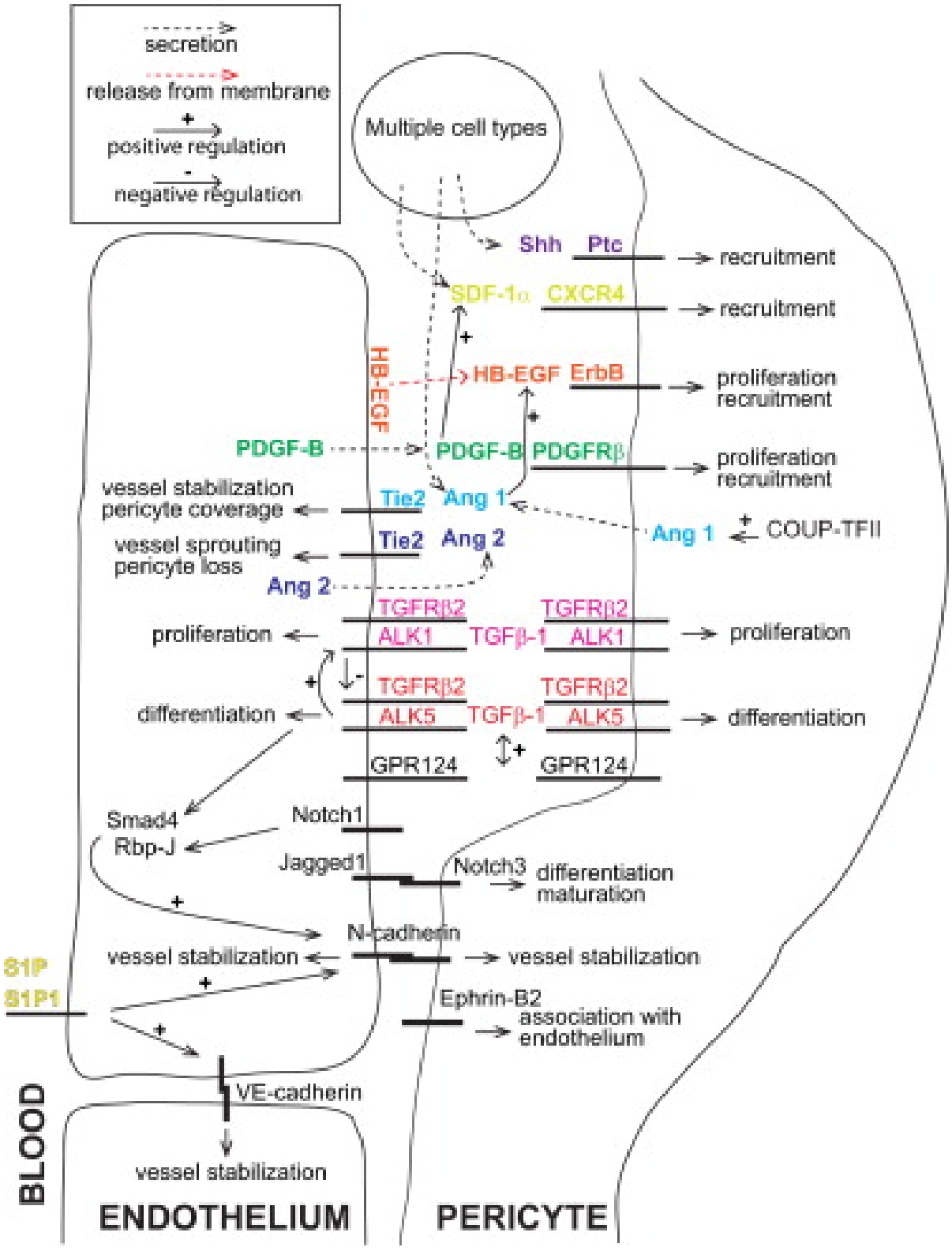


Figure 8: **Signaling pathways between PCs and ECs.**
Multiple ligand-receptor complexes are known to mediate PC recruitment, differentiation, sprouting angiogenesis, and vessel stabilization. These include PDGF-B-PDGFRβ, Ang1/Tie-2, and many more. A ligand-receptor pair is indicated by the same color. N-cadherin and Notch-mediated vessel stabilization requires direct contact between PCs and ECs. Note, that some signaling pathways are proposed to be relevant for pathological angiogenesis.

from Armulik et al. (2011)

The platelet-derived growth factor family includes four members (PDGF-A, -B, -C, and -D). All of them function as secreted, disulfide-linked homodimers composed of two chains, e.g., two -A (-AA) or two -B (-BB) chains (Chen et al., 2013, Andrae et al., 2008). Only PDGF-A and -B are capable of forming functional heterodimers (-AB). Although as the name suggests - PDGF is synthesized, stored, and released by platelets upon activation, it is also produced by a plethora of other cells. These include smooth muscle cells, activated macrophages and - most importantly for this dissertation - by ECs (Bjarnegard et al. (2004). Fig. 8 which is modified from Armulik et al. (2011) underlines the fact, that PDGFb is a major ligand in the communication between ECs and PCs. The PDGF receptor, PDGFR, is classified as a transmembrane receptor tyrosine kinase and cell surface receptor. Two types of PDGFRs are crucial for MC biology: α-type and β-type (Gronwald et al., 1988, Hart et al., 1988, Heldin et al., 1988, Matsui et al., 1989). The different PDGF ligands have distinct affinities for the receptor isoforms, and the receptor isoforms may variably form hetero- or homodimers. Upon activation by PDGF, the receptors dimerize and are "switched on" by auto-phosphorylation of several sites on their cytosolic domains. These sites determine a specificity of downstream signaling with a broad range of cascades involved (Kazlauskas & Cooper, 1989, Loukinova et al., 2002).

Both MC types, SMCs and PCs, express the β type *Pdgfr* which binds with high affinity to PDGF-BB and PDGF-AB (Hart et al., 1988, Heldin et al., 1988). During the process of retinal sprouting angiogenesis, ECs express *Pdgf-b (-bb)* with the highest levels in arteries and endothelial Tip Cells (Lindahl et al., 1997, Lindblom et al., 2003, Gerhardt et al., 2003). Thereby MCs are attracted to follow the endothelial sprout in a close range and to cover the newly formed vessel from the abluminal side. This one-direction communication is crucial, as the transgenic, global KO of the ligand or its receptor is not viable in mice (Hellström et al., 2001, Bjarnegard et al., 2004, Lindahl et al., 1997, Levéen et al., 1994, Suri et al., 1996).

Constitutive EC-specific deletion of *Pdgf-b* is viable, but mutant mice display a broad loss of MCs, and enlarged and unstable naked vessels. This observation confirms that ECs are the primary source of PDGF-B in the developing mouse retina (Bjarnegard et al., 2004, Enge, 2002). Interestingly, the incidence of MCs correlates to the number of receptors expressed and the number of activated signal transduction pathways in MCs (Tallquist et al., 2003).

In similarity with other prominent vascular growth factor families, like VEGF or TGF, the PDGF ligands express a C-terminal retention motif. This motif binds to the ECM anchor Heparan Sulfate (HS) to keep the ligand in the right position and to present it to target cell types expressing the appropriate receptors. Interestingly, mice with a genetically inactivated C-terminal retention motif, but a fully functional remaining sequence for PDGF-B, lose their regular investment of MCs which detach from ECs (Lindblom et al., 2003, Nystrom et al., 2006).

The important role of a stable MC recruitment for a proper vasculature is confirmed by a bunch of studies, confirming that whenever PCs are depleted or massively impaired, the retinal vessel wall and the plexus is retarded and sprouting appears only very sparsely (Lindblom et al., 2003, Uemura et al., 2002). These findings underline the crucial importance of PCs for a proper vascular development.

Although this ligand-receptor interaction has gained increasing scientific interest, some key questions are still not answered. This dissertation scrutinizes for the first time the postnatally induced deletion of EC-specific *Pdgf-b*. Furthermore, I will focus on downstream signaling cascades since none of them can be related to a specific PC behavior at this point.

TGFβ Another important signaling cascade in the biology of PCs is the Transforming Growth Factor β (TGFβ) ligand and its corresponding Activine Receptor-like Kinase (ALK) receptors (Fig. 8). It is described to be involved in MC differentiation from early mesenchyme, in survival and proliferation (Yamazaki et al., 2017, Rustenhoven et al., 2016, Walshe et al., 2009, Armulik et al., 2011). *TGFβ* and the correspondent receptors are expressed on ECs and PCs and studies suggest that an interplay between both cell types is required for the activation of the signaling cascade (Sato & Rifkin, 1989). Thus, TGFβ is another example for the teamwork character of PC-EC interactions (Fig. 8).

The ligand TGFβ is binding to several distinct ALK-receptors. *Alk1* and *Alk5* are expressed on ECs and MCs (Vanlandewijck et al., 2018a). Thereby ALK5 is mediating downstream signaling via phosphorylation of the intracellular signal transducers and transcriptional modulators SMAD2 (Mothers against Decapentaplegic 2) and SMAD3; ALK1 via SMAD1 and SMAD5, respectively (Moustakas et al., 2001). Although the two receptors are binding the same ligands, they have partially opposing effects on expressing cells. Thereby ALK5 is an important factor for MC differentiation, while ALK1 is opposing differentiation and is leading to cell proliferation and migration . Thus, ALK5 is a promoter of vessel maturation, while ALK1 has opposing effects. Notably, *Alk1* and *Alk5* differ in expression strength during different stages of development, with one receptor dominating at certain time points (Heldin & Moustakas, 2016, Goumans et al., 2002, 2003b,a, Moustakas et al., 2001).

The outstanding importance of TGFβ signaling for an appropriate vascularization is underlined by the fact, that mice with genetically deleted ligands, receptors or even downstream SMAD proteins die in utero with major vascular defects (Weiss & Attisano, 2013, Roman & Hinck, 2017). Even EC-specific KOs of *SMAD4* result in vascular dilation, arteriovenous malformations, reduced MC coverage in brain vessels and lethality (Ola et al., 2018, Li et al., 2011). Interestingly the KO of distinct related genes impairs TGFβ signaling and leads to vascular defects particularly affecting MCs. These include connexins, which are important for gap junctions, integrins and others

(Hirschi et al., 2003, Cambier et al., 2005, Krüger et al., 2000). A close interaction of TGFβ signaling is described with Notch, N-Cadherin regulation and other important players of vascular biology (Li et al., 2011).

The human disease Hereditary Hemorrhagic Telangiectasia (HHT) is a rare autosomal-dominant genetic disorder that leads to abnormal blood vessel formation in the skin, mucous membranes, and often in organs such as the lungs, liver, and brain. Five genetic loci are recognized to cause HHT. 80% of patients have a mutated allele for *Endoglin* or *ALK1*, both important players in TGFβ signaling. Another gene associated with HHT is *SMAD4*, a downstream mediator for the receptors for Tgfβ ligands. Homozygosity appears to be fatal in utero (Govani & Shovlin, 2009, McDonald & Pyeritz, 1993).

Thus, TGFβ signaling is crucial in vascular development and disease (Hirschi et al., 1998, ten Dijke & Arthur, 2007), but its role in PCs and in PC-mediated non-cell autonomous regulation of ECs remains elusive. *ALK1* is not expressed in PCs of the CNS (Van Geest et al., 2010, Vanlandewijck et al., 2018a). Dave et al. were the first to show that embryonic mice with a deletion of Alk5 PCs develop a markedly severe cerebral phenotype in the germinal matrix (Dave et al., 2018). Furthermore, it could be shown that ALK5 signaling in PCs is a key regulator of EC morphogenesis during BBB development (Dave et al., 2018).

Ang1-Tie2 The ANG ligands and corresponding TIE receptors act as another paracrine signaling communication system between MCs and ECs. The ligand *Angiopoietin-1 (Ang-1)* is mainly expressed by perivascular cells, such as PCs and SMCs, while TIE-2, the primary receptor for ANG-1 is predominantly found in ECs (Eklund et al., 2017, La Porta et al., 2018, Vanlandewijck et al., 2018a). Once ANG-1 binds to TiE-2, it is promoting EC maturation, stabilization, and differentiation, thereby reducing vascular leakage (Thomas et al., 2010, Fukuhara et al., 2010, Teichert et al., 2017). Thus, it is mainly reciprocal to PDGF-B-signaling (compare Fig. 8). KO animals for the ligand or receptor die in utero with significant vascular abnormalities suggesting a crucial role of ANG-1-TIE-2 in vascular biology (Jeansson et al., 2011, Milam & Parikh, 2015). ANG-2 is another key regulator of angiogenesis which also binds to TIE2 thereby acting as a negative regulator of ANG-1/TIE2 signaling. However, its role in angiogenesis is context-depending as under certain conditions, such as tumors, ANG-2 can also promote angiogenesis (Felcht et al., 2012).

Of particular importance for this dissertation is the finding, that genetically impaired mice for ANG-1-TIE-2 display a lack of MCs (Suri et al., 1996, Patan, 1998). On the other hand, *Ang2* overexpression in tumor and rat models correlates inversely with PC coverage (Hammes et al., 2004, Zhang et al., 2003, Stratman & Davis, 2012). These findings indicate for the first time that *Tie2* is potentially also expressed in MCs. Thus, ANG1-TIE2 signaling is not a mono-directional signal cascade as it has been supposed to be. Augustin et al. (2016) could finally show, that *Tie2* is also expressed in PCs and

that the Ang-1-Tie2-cascade in PCs controls angiogenesis and maturation in mice. Interestingly, PC-specific KO of Tie2 results in a pro-migratory phenotype and is thereby an important regulator of sprouting angiogenesis. Although Ang-Tie signaling is not in the centre of my work, it is a highly promising signal cascade with recent novel insights into blood vessel and lymphatic angiogenesis (Savant et al., 2015, Zheng et al., 2014, Kim et al., 2019a,b), tumour growth (Nasarre et al., 2009), glaucoma (Kim et al., 2017), and sepsis (Ziegler et al., 2013).

others There are more signaling cascades described regulating PC development, morphology and behavior (see Fig. 8). Only very few of them are fully understood today. These include Heparin-Binding Epidermal Growth Factor (HB-EGF) signaling through EGF receptors (ErbBs) in connection with ADAM17 protease (Yu et al., 2012, Lin et al., 2011), the stromal-derived factor 1-a (SDF-1α)/CXCR4 pathway (Hamdan et al., 2014), sonic hedgehog (SHH) (Bohannon et al., 2019), sphingosine-1-phosphate (Nitta et al., 2014), Notch signaling (Kofler et al., 2015), and Ephrin-Eph receptor cascades (Teichert et al., 2017, Malik & Di Benedetto, 2018). Interested readers should look out for reviews (Armulik et al., 2011, Malik & Di Benedetto, 2018).

1.3.6 Pericytes as part of the Blood-Brain Barrier

The vasculature of the CNS is characterized by extreme tightness and an impermeability to the passive transport of cells, proteins, and bioactive compounds. This is guaranteed by a multicomponent system which is summarised under the term Blood-Brain Barrier (BBB) and underlies cellular and molecular complexity. Key cell components are PCs, ECs, Astrocytes, and even Neurons and Microglia. The term "neurovascular unit" describes the CNS vasculature and its structural and functional connection to the neural tissue (Abbott et al., 2010, Zlokovic, 2008).
Several studies confirm, that PCs play a crucial role in the maturation and maintance of the BBB (Armulik et al., 2010, 2011, Bell et al., 2010, Daneman & Prat, 2015). In fact, PCs of the CNS show a distinct profile in comparison to PCs from other tissues concerning shape and marker expression (Bondjers et al., 2006, Armulik et al., 2010). Furthermore, the CNS vasculature shows the highest PC coverage among all organs analyzed so far (discussed above).

The BBB is of great relevance concerning its role in pathologic conditions. An abnormal increase in vascular permeability is associated with sepsis, lung edema, and allergic reactions (Wang & Dudek, 2009, Armulik et al., 2011). However, the detailed role of PCs in the majority of these pathological conditions has not been finally clarified, yet. In vitro studies indicate that PCs regulate the BBB at the level of endothelial junctions by increasing transendothelial electrical resistance of EC monolayers (Cardoso et al., 2010, Lai & Kuo, 2005, Abbott et al., 2010). Two in vivo studies challenge this in vitro finding (Armulik et al., 2010, Daneman & Prat, 2015). Both of them target the PDGFB-PDGFRβ signaling cascade thereby depleting PCs. The studies conclude independently that PC deficiency causes increased brain vessel permeability. Interestingly, the extent of permeability correlates directly with the density of brain PCs.

The detailed mechanism and role of PC contribution to the healthy and diseased BBB requires further studies. Current findings suggest, that ECs of the CNS in PC-depleted animals display a characteristic upregulation of *Vegfa, Ang2*, and *Adrenomedullin*, and a downregulation of *Ang1* expression. All of these genes have been proposed to regulate endothelial permeability by mediating endothelial cell-cell junctions; the VEGFA ligand has been described to regulate transendothelial permeability by inducing vesiculo-tubular organelles (Daneman & Prat, 2015, Daneman et al., 2010, Mehta & Malik, 2006, Temmesfeld-Wollbrück et al., 2007). This is in accordance with the finding, that early BBB damage is associated with increased transcytosis, whereas the loss of endothelial junctional integrity takes place later (Nag et al., 2011).
PC-EC interaction is not the only affected microsystem in PC-depleted animals. Interestingly, Astrocyte endfeet lack polarization in transgenic animals (Armulik et al., 2010). This indicates that the neurovascular unit is affected on different levels.
Moreover, PCs have been described to leave the vessel wall during several pathological conditions, such as tumors, trauma, sepsis, diabetic microangiopathy, and fibrosis (Benjamin et al., 1998, 1999). The pathogenic significance of this PC loss is currently unknown. Interestingly, PC loss and an increase in vessel permeability in the CNS have been proposed to result in neurodegeneration in aging animals (Bell et al., 2010).

1.3.7 Role of Pericytes in diseases

PC loss, differentiation, proliferation, and migration are described to be involved in different human diseases and pathologic conditions from a broad range of background. Although a detailed understanding could only be gained in very few diseases, PCs are proposed as promising targets in a full range of conditions. These include tumors (Lu & Shenoy, 2017), stroke (Yang et al., 2017), Alzheimers disease (Salmina et al., 2019), fibrosis (Greenhalgh et al., 2013), traumatic brain injury (Dore-Duffy et al., 2000), and retinopathies (Beltramo & Porta, 2013, Christian et al., 2008). On top of that, they are potential stromal targets for cancer therapy (Raza et al., 2010).

Recently, scientists working in ischemic retinopathies drew more and more attention to PCs and left the established disease understanding that focused exclusively on ECs. This shift of perception is based on observations of PC morphology and microcapillary coverage (Trost et al., 2016, Armulik et al., 2011, Beltramo & Porta, 2013, Arboleda-Velasquez et al., 2015). Furthermore, VEGF-A is not the only upregulated growth factor in neoproliferative diseases. Notably, superphysiological levels of PDGF-B, TGFβ, and others can be detected (discussed above). BM thickening, as well as an impaired BRB in ischemic retinal diseases, underline the importance of PCs in ischemic retinopathies (Roy et al., 2010, Antonetti et al., 2008, Cunha-Vaz, 2012). The increased permeability regularly leads to macular edema, which, in turn, precedes vascular proliferation (Ockrim & Yorston, 2010, Cohen & Gardner, 2016). Furthermore, PC free capillaries, as well as pockets in the vessel wall, can be observed (Beltramo & Porta, 2013, Armulik et al., 2011). These so-called PC ghosts are commonly understood as the morphological correlate of PC death. The observed vascular

changes result in severe blood flow changes, edema, leakage, and finally, even bleeding (Barber et al., 2011, Kern et al., 2010).

The exact role of PCs during this process is not fully understood today. It is not clear whether PCs play a disease-triggering role, have a preventing character, or are only affected in terms of collateral damages. This is one of the superordinate research questions of my work and will be further discussed during my dissertation.

1.4 Aims of the present study

Current disease-related and translational studies have prompted a strong scientific interest in PCs and other microvascular MCs (Ophthotech Company, 2016, Birbrair, 2018). It is well understood that PC detachment and loss of BRB integrity lead to pathological permeability and edema in ischemic retinopathies (described above). Thus, PCs have already been implicated in the initiation and the progression of retinal diseases. However, antagonizing PDGF-B did not show an improvement in visual acuity in combination with established anti-VEGF-A treatments in patients suffering from AMD (Ophthotech Company, 2016). Does this study drive a nail in the coffin of PCs in vascular diseases of the retina and the CNS?
My current work claims the opposite. The observed findings suggest that PCs remain promising targets for future medical therapies of ischemic retinopathies, but also underline the urgent need to further investigate PC biology in order to achieve a potential clinical impact. Furthermore, I try to provide a better understanding of this interesting cell type, its interaction with ECs during sprouting angiogenesis and function in health and disease.

(1) One current obstacle in PC biology is the lack of a specific PC marker (Armulik et al., 2011). This is of particular importance since PCs can be found ubiquitously in the human body, but in a highly organo- and tissue-specific manner. Furthermore, the unique identification of PCs is even more difficult since a second MC type SMCs appears in a very similar manner also at the abluminal side of the ECs. Thus, the first part of my work focuses on the comparison of different MC markers in order to find a sharp expression pattern guaranteeing unique marker profiles of MCs in the mouse retina. Thereby, I consulted a set of well-established PC markers and evaluated them based on their specific expression pattern located on microvascular arteries, veins and capillaries. This expression pattern was contrasted with the spatial appearance of PCs and SMCs to create a clear assignment of markers and cell types.
(2) It is well-known, that MCs can potentially change gene expression profiles when it comes to pathological differentiation processes. Thus, the provided marker expression pattern is challenged in disease models, such as OIR experiments. To stick with a sharp identification strategy of disease-involved cell types, much effort of the current study is put in fatemapping experiments. Thereby, utilization of several, newly-crossed transgenic mouselines and elaborated protocols guarantee a precise logging of MC fate in diseased retinas. This protocol is not only important for the results of my work, but aims to provide a novel technique to unambiguously identify the fate of different cell types.
(3) After the identification as important disease-drivers of ischemic retinopathies, the next step is to specifically impair PCs, thereby potentially improving diseased retinas. PDGF-B from ECs is the strongest known regulator of PC migration, proliferation, and survival - and thus, a potential target in retinopathies (Armulik et al., 2011). In the current study I test the importance of endothelial Pdgf-b by utilizing transgenic mouselines. Much is already known about deletion of *Pdgf-b* for sprouting angio-

genesis, e.g. constitutional deletion of EC-specific *Pdgf-b* and different molecular trap models. However, this is the first study to create a model in which EC-specific *Pdgf-b* is genetically deleted postnatally during development. This strategy provides important insights into the roles of PCs for sprouting angiogenesis and supports the current understanding, that not only ECs are crucial for sprouting vessels. Furthermore, utilizing the transgenic mice is particularly powerful for my research question, since *Pdgf-b* of ECs can be deleted precisely during the period of pathological hypersprouting.
(4) The results of this experiment (see results) partially confirm the failure of current clinical studies, in which PDGF-B signaling was blocked in addition to established VEGF-A antagonization. This leads me to the hypothesis that a more precise target than the complete block of PDGF-B signaling has to be found. Nck1/2 are intracellular adaptor protein which have already been described to interact with the fibroblast and MC characteristic Pdgfrβ in mice. Thus, it is natural to test the role of Nck1/2 in PCs. In the current study, I provide a precise characterization of the function of NCK1/2 proteins in HBVPCs with a particular focus on PDGFRβ downstream signaling pathways. These are involved in PC recruitment, attachment, and survival. By performing signaling experiments, I scrutinize the expression and activation of a set of already described and well-established signaling mediators in presence and absence of NCK1/2. Of particular interest are the tyrosine phosphosites of the intracellular domain of the PDGFRβ. Furthermore, since NCK1/2 is already described to connect the intracellular domain to downstream signaling cascades, I have a closer look on those downstream molecules. These include p21-activated kinase (PAK) family of serine/threonine kinases and their upstream activators, RAC1/CDC42 and others.
Concerning these in vitro experiments, it is important to mention, that established protocols to culture PCs in vitro are not fulfilling important criteria of PC biology and processes of differentiation are frequently observed. Thus, it is an important aim of this study to establish and utilize a novel in vitro protocol that closely fits the PC biology in living organisms.
(5) However, in vitro experiments underlie certain limitations. Thus, it is of critical relevance whether the in vitro findings can be confirmed in vivo. In order to achieve this, I created inducible MC specific KO mice for Nck1/2 by crossing available transgenic mouse lines. These mice are tested for a phenotypic change of the vasculature and MCs in vivo. Thereby, I focus on development and disease models to provide a detailed characterization of the role of Nck1/2 in MCs during sprouting angiogenesis. The experiments try to answer the superordinate research question whether deleting *Nck1/2* is a sufficient, but more subtle way to impair the contribution of PC in pathological sprouting. As described above, the current knowledge of NCK1/2 as an adaptor between PDGFRβ and intracellular cascades was gained in fibroblast experiments. Thus, this study is the first one to provide a detailed characterization of NCK1/2 in PCs.

In short, the following research questions have been addressed:
1. Which MC markers can be consulted in order to uniquely identify PCs in the mouse

retina, with a particular interest in the distinction to SMCs?
2. Which is the original phylogenetic cell type of activated α-SMA positive PCs in diseased retinas?
3. Is PDGF-B a promising target in ischemic retinopathies? Which role does endothelial PDGF-B play during development and disease in vivo?
4. What is the exact role of the intracellular, scaffold protein NCK1/2 in PC biology? Is it responsible for a specific PC behavior and through which signaling cascades is it mediated?
5. To which degree does NCK1/2 in PCs contribute to physiological and pathophysiological vascular sprouting? Is NCK1/2 in PCs a potential target in ischemic retinopathies?

2 Material and Methods

2.1 In vivo

2.1.1 Procedures

Mouse keeping and breeding

All mice were maintained in the Animal Research Center of Yale University. Experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee of Yale University. Well-being and physicial health of mice was strictly monitored by experts of the animal facility and Yale University. Every experiment underwent critically questioning in terms of the harm-benefit ratio.
Rita Webber and Nicole Copeland from the animal facility were critical in maintaining and breeding animals.

Crossing of transgenic mouselines

For inducible *Cre*-mediated recombination, *Nck1-/-;Nck2lox/lox* mice, *Pdgf-blox/lox* mice and *mTmG reporter* mice were bred with *Cdh5-CreERT2* mice, *Pdgfrβ-CreERT2* mice, *α-Sma-CreERT2* mice and *Myh11-CreERT2* mice. All transgenic mouse lines were available at independent research institutes such as the Jackson Laboratory or at collaborating laboratories as indicated in Dubrac and Künzel et. al. (2018). I would like to express my sincere thanks to Prof. Ralf Adams (MPI Münster) in this matter.
To avoid misunderstanding: In this dissertation and the original publication *lox/lox* is indicated by *l/l* as for example in *Pdgf-b l/l* animals. *iEC* or *iPC* indicates "deleted in PCs", or "deleted in ECs", respectively.

In terms of animal crossing, I used protocols and expertise of local animal facility and researchers, combined with protocols from Jackson Laboratory. The following premises were guaranteed at all times.
Female animals were not older than 16 weeks, and not younger than 8 weeks, in order to increase the rate of successful pregnancies. I used group-housing (with a maximum of 4 females per cage) prior to mating. This promotes a longer, more irregular estrous stage of the reproductive cycle (in which ovulation occurs). The phenomenon is called Lee-Boot effect. Estrous stage was determined by visual inspection of the female external genitalia. Females that have mated during the night were monitored and checked for vaginal plugs early next morning. Since no experiments were performed on embrionic stage, I dispensed close monitoring for weight (in order to detect verry early pregnancis), but isolated pregnant females early.
Male animals were kept individually for about 10 days prior to mating. It is described that individual housing of male animals maximizes pregnancy chances by increasing the number of sperm counts. Older mates were preferred over younger animals.
Mice were from mixed backgrounds.

Genotyping

Genotyping of all animals and mouselines was performed by PCR of genomic DNA.

This was gained from mouse ears in adults, and toes of pubs, respectively. Genotyping was performed in my own hands. Samples were friendly provided by Rita Webber. Pubs were genotyped after birth, and - as a control - a second time after euthanasia before and parallel to the experiment. All samples were stored at -20 °C for safety and regenotyping.
Samples were prepared and lysed in 0,1ml l DirectPCR Lysis Reagent containing 10 microgram proteinase K at 60 ° Celsius and 300 rpm overnight. Proteinase K activity was stopped by incubation (95 °C for 20 minutes). After centrifugation, the supernatant was directly used for genotyping.
For genotyping I used: 10x Coral load PCR buffer, dNTPs, MgCl2, the oligonnucleotide primers and Taq polymerase. Genotyping revealed individual bands (e.g. Cre at 100bp). A positive and negative CT was used. The system for exact thermocycling was an Applied Biosystems Thermocycler. I used a 2 percent agarose gelelectrophorersis gel.

Tamoxifen application
Intraperitoneal injections with 50 μg tamoxifen (Sigma, T5648; 1 mg/ml) of pups at P0, P1, and P2 in developmental experiments, at day P6 and P7 in OIR cell tracking experiments, and at day P12, P13, and P14 in the pathology experiments, respectively, were used for gene deletion. Mice were sacrificed at day P5, P8, or P17 depending on the experiment. The corresponding protocol is indicated in the figures. *Cre*-negative littermates were also treated with tamoxifen and subsequently used as control mice.

Euthanasia
In order to examine mouse eyes at different developmental stages, and following our experimental strategies, mice were sacrificed at different timepoints as indicated in figures. Thereby, animals were decapitated after anaesthesia with fluran following animal protocols.

Eye isolation and prefixation
The eyes of sacrificed mice were taken out after euthanasia at the indicated day (see corresponding Figures). Therefore, eyeballs were carefullly isolated and removed with a specialized, bended forceps, that was positioned behind the eyeball on both sides of the optic nerve and optic vasculature. The eyeball was then disconnected by carefully pulling out the eyeball. Subsequently, the eyeballs were prefixed in 4 % paraformaldehyde for 20 minutes at room temperature.

Retinal isolation
The next step was the dissection under a conventional low-magnification microscope. During this process, scleral structures, as well as vitreous body and lens were carefully removed. In some cases, the optic nerve was still present, and was removed. Subsequent blocking during 30 minutes at room temperature in blocking buffer (BB: 1 % FBS, 3 % BSA, 0,5 % Triton X-100, 0.01 % Na-deoxycholate, 0.02 % Na-azide in PBS at pH 7.4).

Retinal antibody staining

I used whole mount retina staining following existing protocols. In order to do so, retinas were already blocked (described in previous step). The retinas were then incubated with the first antibodies in Blocking Buffer over one night, or - stained with TRITC-conjugated Lectin (1:150). In the latter case, no secondary antibody was needed. I did not experience problems of staining at this point. The retinas were washed afterwards and subsequently incubated the corresponding secondary antibody for 2h at room temperature. In contrast to other known protocols, I used only one washing step, and no secondary blocking.

Mounting of retinal tissue

The final step was the mounting procedure in fluorescent mounting medium, acquired from Dako (Carpinteria, CA, USA), again under a conventional low-magnification microscope. Thereby, retinal layers were flattened by adding four radial incisions and opening the ball like structure in the shape of a four-leave clover. The retina was then covered by a thin glass.

Image acquisition and analysis

I used a Leica SP5 confocal microscope with a Leica spectral detection system (Leica 15 SP detector) and the Leica application suite advanced fluorescence (LAS-AF) software to acquire high-resolution images. The microscope is equipped with four diode lasers (405, 488, 555, 639), allowing parallel acquisition of different stainings at once. Images were taken as stacks with differing numbers of stacks per image. This allowed to precisely visualize vessels and NVT formations.
Images were analyzed with the ImageJ software. α-SMA, Desmin, NG2, and PDGFRβ coverage was determined by quantifying the α-SMA positive, Desmin positive, NG2 positive or PDGFRβ positive area, respectively, normalized to IB4 positive areas of the vasculature in 63x images. Special techniques were utilized to quantify the RBC leakage and the Fibrinogen deposition, as I counted the number of extravascular TER119 positive cells normalized to the total number of all TER119 positive cells in the 63x image and normalized Fibrinogen staining to IB4 positive area in 20x images. The pups used for Fibrinogen staining were not perfused.

2.1.2 Experimental strategies

Fatemapping experiments

In order to describe which detailed celltypes contributed to NVT formations, I utilized fate mapping experiments. Fate mapping is a method for tracing cell lineages and a fundamental tool of developmental biology and embryology. Thereby, I utilized the advantage of genetic deletion, and the fact, that a deleted gene is deleted constantly - in contrast to drugs or antibody, that might only work temporary. Thereby, I deleted the corresponding genes during the OIR experiment before the neovascular sprouting

occcurs (deletion is indicated in the figure, P6/7). This deletion enabled me, to tag a given cell by an expressed gene (e.g. PDGFRβ), and visualize later. Confirmation of gene deletion occured in a highly reliable manner and was confirmed by staining of littermates shortly after injection (P8). This strategy was performed on three different mouselines individually and allowed precise identification of PCs as tuft covering cells.

Oxygen-induced Retinopathy Model

The Oxygen-induced Retinopathy model (OIR model) is a common model to study ischemic retinopathies, due to its ability to mimic corresponding diseases (discussed above). The protocol is well-established and highly reproducible (Fig. 9).

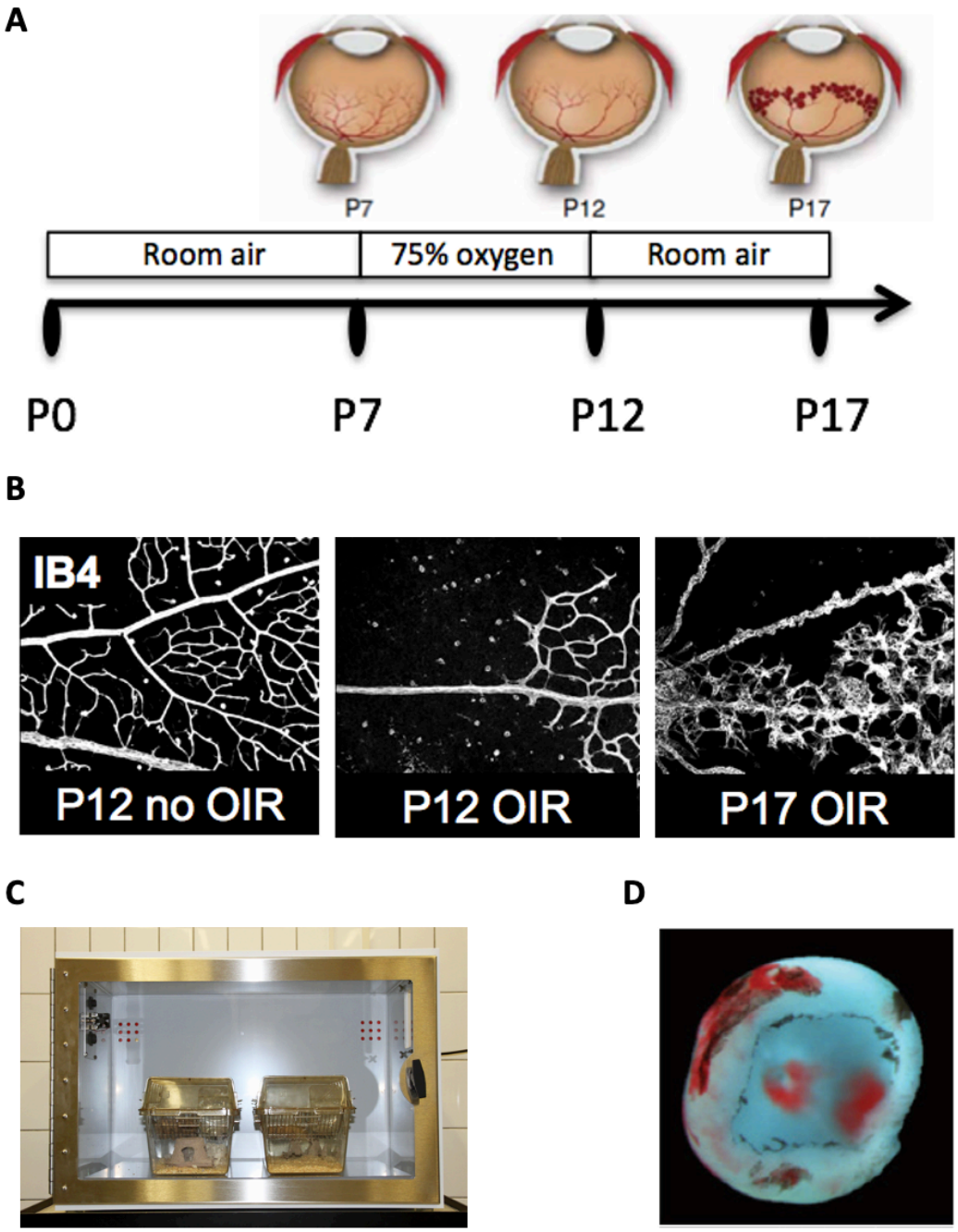


Figure 9: **OIR protocol.**
A Experimental strategy of the OIR protocol and illustration of observed phenotypes. **B** IB4 staining of retinal vasculature at different timepoints following physiological development and the OIR protocol. Note, that after five days of hyperoxia (P7-P12) vessel depletion and obliteration occurs (compare left: P12 no OIR and central: P12 OIR). At day P17, massive hypersprouting and NVTs can be observed. *Kindly provided by Alexandre Dubrac, PhD, 2019.*
C Hyperoxia chamber. *Photography by Zürich Integrative Rodent Physiology (ZIRP), 2019.*
D Photography of a mouse retina after prefixation, but before dissection and before any staining or manipulation. Red areas represent bleeding. *Kindly provided by Alexandre Dubrac, PhD, 2019.*

2.1.3 Antibodies

Different antibodies from a broad range of companies were utilized in vivo and in vitro. Antibodies used were as follows: anti-NCK1 (1/200, ab168940, Abcam), anti-NCK2 (1/200, ab109239, Abcam), anti-PDGF-B (1/50, AB23914, Abcam), anti-PDGFRβ (1/100, AF1042, RD Systems), anti-NG2 (1/200, AB5320, Millipore), anti-MYH11 (1/200, AB53219, Abcam), anti-Desmin (1/200, AF3844, RD Systems), anti-TER119 eFluor 450 (1/50, 48-5921-82, eBioscience-ThermoFisher Scientific), anti-fibronectin (1/300, F3648, Sigma-Aldrich), anti-laminin (1/300, AB11575, Abcam), anti-collagen IV (1/300, AB769, Millipore), anti-GFP (1/300, G10362, Life Technologies), anti-ERG1/2/3 (1/100, SC353, Santa Cruz), PDGF-BB (220-BB, RD Systems), anti-phospho-histone 3 (1/100, 06-570, Millipore), anti-α-smooth muscle actin CY3 (1/200, α-SMA, C6198, Sigma), anti-PDGFRβ 751 (1/1000, 4549, Cell Signaling), anti-PDGFRα 1009 (1/1000, 3124, Cell Signaling), anti-PDGFRα 1021 (1/1000, 2227, Cell Signaling), anti-PDGFRα (1/1000, 3169, Cell Signaling), anti-p44/42 MAP kinase (1/1000, phospho-ERK, 9106, Cell Signaling), anti-44/42 MAP kinase (1/1000, total ERK, 9102, Cell Signaling), anti-pAKT (1/1000, 4060, Cell Signaling), and anti-panAKT (1/1000, total AKT, 4685, Cell Signaling). Appropriate secondary antibodies were conjugated to horseradish peroxidase (Vector Laboratories) or fluorescently labeled (Life Technologies). IB4 was purchased from Life Technologies.

2.2 In vitro

2.2.1 Cell culture

HBVPCs and HBMECs can be obtained from ScienCell. The HBVPCs were cultured in 0.1 % gelatin-coated plates in EGM2 medium (Lonza). I established these conditions for HBVPCs to promote PC marker expression and inhibit SMC marker expression (discussed in results). HBVSMCs can also be obtained from ScienCell and were cultured on 0.1 % gelatin-covered plates in Dulbeccos modified Eagles medium (DMEM), which could be obtained from Gibco by Life Technologies. I supplemented this medium with 10 % FBS and 1 % of penicillin/streptomycin solution. Maximum number of passage for HBVSMCs was 4; in average substantially less. For HBVPCs and HBMECs maximum passage was 5 and 4, respectively; in average again less.

2.2.2 siRNA transfection

For siRNA transfection, the HBVPCs were starved overnight in EBM2 supplemented with 2 % FBS only (instead of EGM2). I treated them with PDGF-B (Time and amount indicated in the corresponding figure). siRNA (Flexitube siRNA) can be obtained from Dharmacon.
The used *siRNA* are:
- NCK1 (SMARTpool: ON-TARGETplus *NCK1 siRNA* L-006354-00-0005),
- NCK2 (SMARTpool: ON- TARGETplus *NCK2 siRNA* L-019547-00-0005),
- PDGFRβ (SMARTpool: ON- TARGETplus *PDGFRβ siRNA* L-003163-00-0005)
- negative control (ON-TARGETplus *Non-targeting Pool siRNA* D-001810-10-05).

During the transfection, the cells were cultured in Opti-MEM I Reduced Serum Medium (Cat. No. 31985-062). The cells were cultured in 6-well plates. To each well 25 pmol of *siRNA* and 2 μl RNAiMax (Invitrogen) were added. Medium change after 4 hours.

2.2.3 Scratch assay

I performed scratch assays with HBVPCs, which I grew in 6-well plates. *siRNA* transfection was performed in these plates prior to any scratch. 48 hours later, a straight wound was created using a sterile 200 μl pipette tip. After scratching, the cells were washed with EBM2 at 37 ^0C and incubated in EGM2 under different conditions, e.g. with PDGF-B (25 ng/ml). Incubation for 6 h at 37 ^0C. Wound closure was evaluated using images just before the scratch and 6 h after. Measurements were performed with the ImageJ software.

2.2.4 Proliferation assay

Proliferation of HBVPCs under different knockdown treatments was evaluated every 5h for 48h by xCELLigence RTCA DP analyzer. Cells were transfected with *siRNA* for *PDGFRβ* and *NCK1/2*. 10.0/200 cells per well and stimulation with PDGF-B -(100 ng/ml).

2.2.5 Western blotting

Before westernblotting, cells were starved overnight in EBM2 supplemented with 2 % FBS. Stimulation occurred with PDGF-B (20 ng/ml) for different time periods. Times are indicated in the corresponding figure. Cell lysation was performed with Laemmli buffer including phosphatase and protease inhbitors (Thermo Scientific, 78420, 1862209). I used 15 μg of cell proteins and separated them on 4-15 % Criterion precast geld (Bio-Rad, 567-1084). Subsequently, proteins were transferred in nitrocellulose membranes (Bio-Rad). For development of Western Blots a Luminescent image analyzer, ImageQuant LAS 4000 mini was used (GE Healthcare).

2.2.6 Quantitative realtime qPCR

RNeasy Kit (Qiagen) was used to purify *RNAs* from HBVPCs or from retina. 1 μg of *RNA* was reversely transcribed with SuperScript III (Invitrogen). Quantitative PCR were assayed using corresponding primers (Qiagen). *HsActb*, *MmActb*, and *HsGapdh* were used for normalization. More details about used primers can be found in Dubrac and Künzel et. al. (2018).

2.3 Data

2.3.1 Statistical analysis

No statistical analysis to determine sample size before experiments, no blinding, and no randomization were used. Control and intervention groups were determined by corresponding genotypes. *Cre* negative littermates were used as controls. All data are

shown as mean + - standard error of the mean (SEM). In samples with equal variances Mann-Whitney U test or two-tailed Students t-test were used between groups. P value 0.05 was considered to be statistically significant. For all analysis Prism 6.0 (GraphPad) was used.

2.3.2 Data availability

All data that support findings of this dissertation can be found in this script or in Dubrac and Künzel et. al. (2018).

3 Results

3.1 Identification of Mural Cell markers in the developing mouse retina

Despite its peripheral location, the retina which is the neural and vascular portion of the eye, is morphologically considered a part of the CNS; and is actually brain tissue. Thus, vessels of the retina are considered as brain vasculature with PCs and SMCs as a subgroup of brain MCs. As stated above, PCs appear with high similarity to SMCs; and clear distinction of PCs and SMCs is still a hurdle in the field. Many different PC markers have been proposed as promising measures for brain PC identification (Fig. 3.2). However, the majority of the suggested markers are weak, unspecific, or not helpful for scientific questions of the brain and retinal vasculature. In fact, literature research provides contradictory statements concerning MC expression markers (Armulik et al., 2011). Today, no specific PC marker is established in the field (discussed above).

In this study, I test several of the proposed MC markers to build the foundation for the ongoing experiments. The tested markers include PDGFRβ, NG2, Desmin, α-SMA and MYH11; and were chosen based on literature research and key questions of this dissertation. P5 retinas of healthy WT mice were immunostained for the particular markers. Location of marker staining was then superimpositioned with the known location of SMCs (predominantly on arteries) and PCs (capillaries, veins). This allows to assign the single markers to PCs and SMCs, in order to find sharp marker expression profiles.

Staining for PDGFRβ, NG2, and Desmin could be found on all three vessel types: arteries, capillaries, and veins (Fig. 10 + 12). This implies, that they can not distinguish between PCs and SMCs. In contrast, α-SMA and MYH11 coverage was sharply limited to arteries (Fig. 10+11+12). Thus, α-SMA and MYH11 are reliable markers for SMCs of the retinal arteries during development. Notably, no α-SMA or MYH11 and consequently no SMCs could be detected on capillaries or veins during physiological development. This underlines that PCs are not expressing *α-Sma* or *Myh11* in developmental states; and can be identified by exclusion from SMCs. This expression profile applies to the vascular plexus in the center of the retina, as well as the sprouting front at the outer edge (Fig. 10+11+12).

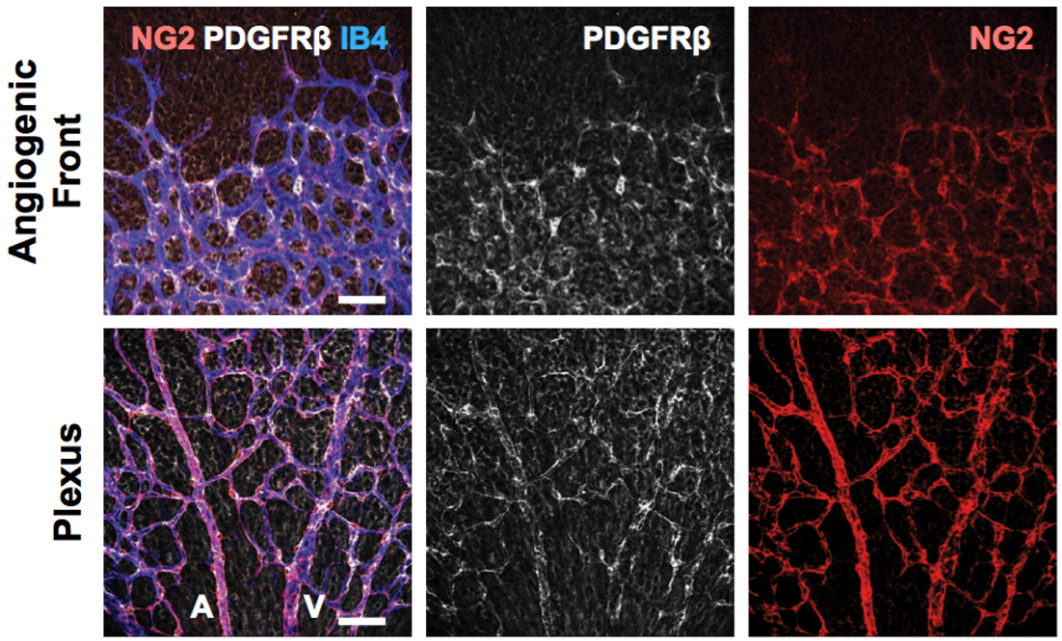


Figure 10: **MC markers of the mouse retinal vasculature. (1)**

Left: Triple-staining for NG2, PDGFRβ and IB4; Center: Staining for PDGFRβ; Right: Staining for NG2.
Top row images represent the vascular front. Bottom row images represent vessels near the vascular plexus. Artery, vein, and capillaries, can be compared in bottom row images. Corresponding vessel type is indicated by white letter. Scale bars: 100 μm
V=vein, A=artery.

From: Dubrac and Künzel et. al. (2018)

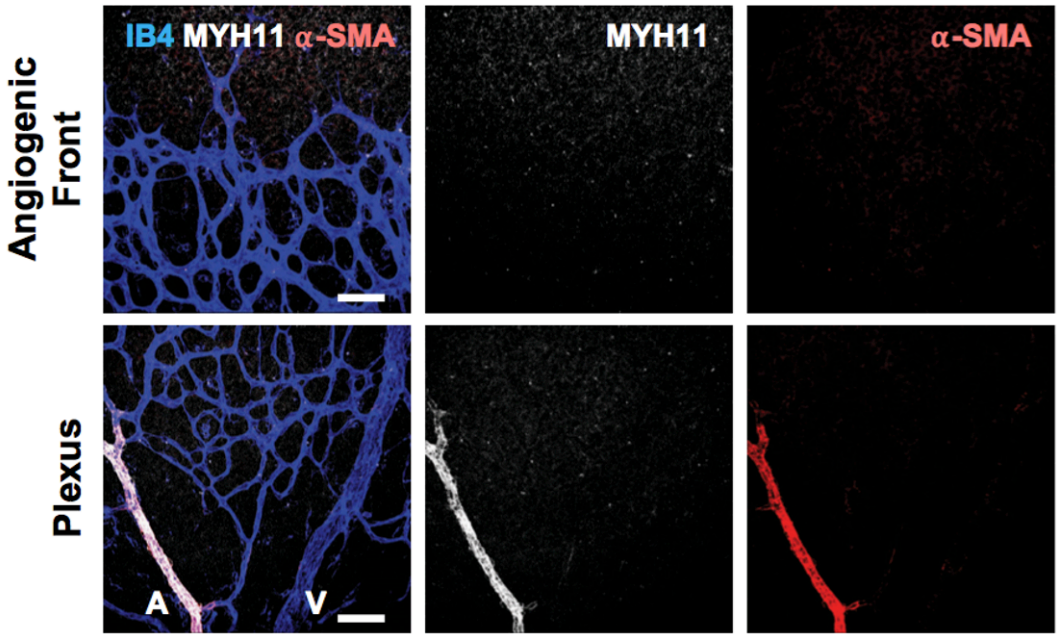


Figure 11: **MC markers of the mouse retinal vasculature. (2)**

Left: Triple-staining for MYH11, α-SMA and IB4; Center: Staining for MYH11; Right: Staining for α-SMA.
Top row images represent the vascular front. Bottom row images represent vessels near the vascular plexus. Artery, vein, and capillaries, can be compared in bottom row images. Corresponding vessel type is indicated by white letter. Scale bars: 100 μm.
V=vein, A=artery.

From: Dubrac and Künzel et. al. (2018)

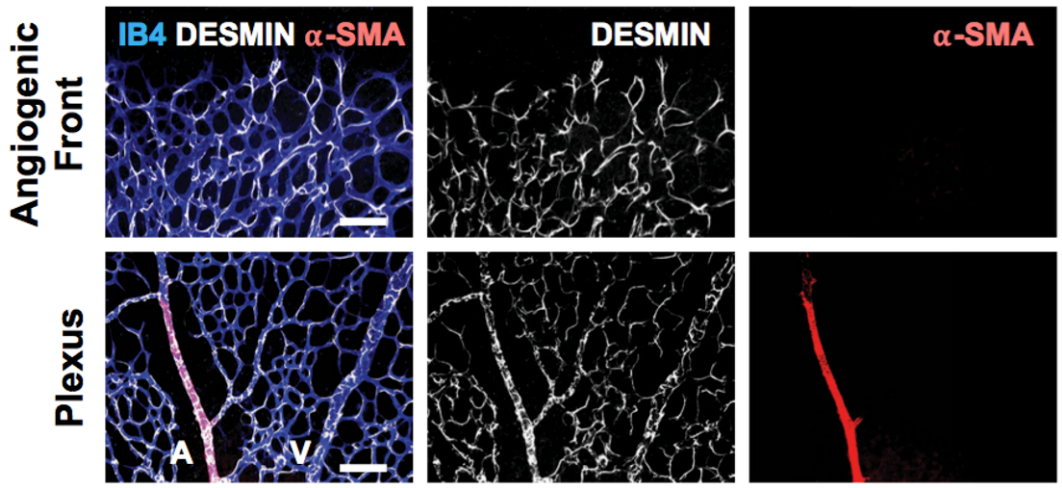


Figure 12: **MC markers of the mouse retinal vasculature. (3)**

Left: Triple-staining for Desmin, α-SMA and IB4; Center: Staining for Desmin; Right: Staining for α-SMA.
Top row images represent the vascular front. Bottom row images represent vessels near the vascular plexus. Artery, vein, and capillaries, can be compared in bottom row images. Corresponding vessel type is indicated by white letter. Scale bars: 200 μm.
V=vein, A=artery.

From: Dubrac and Künzel et. al. (2018)

3.2 A novel protocol for in vitro culturing of Pericytes

To develop a reductionist in vitro model to test the in vivo findings, I tested one established and several modified in vitro PC culturing protocols (the best one is shown) on commercially available HBVPCs, kept as indicated in Fig. 13 and specified in Material and Methods. Especially in vitro culturing of PCs is a challenging technique, in which substantial cell differentiation regularly appears; α-SMA is an important differentiation marker in this context (Armulik et al., 2011). The assessment criteria for the protocols were predominantly the expression profiles of the MC markers observed in the in vivo experiments (Fig. 10), quantified by qPCR analysis (Fig. 13 A).
Following one of the well-established protocols in the field, PCs were cultured in DMEM + 10% FBS. Surprisingly, this state-of-the-art approach did result in high expression levels of *α-SMA (ACTA2)* and *MYH11* in cultured HBVPCs (Fig. 13 A). Expression of *NG2 (CSPG4)* and *PDGFRβ* was satisfyingly high (Fig. 13). Nevertheless, the observed profile resembled the one of SMCs in vivo, and I concluded that the used protocol led to massive cell differentiation; the original PC character was lost (Compare Fig. and 13 A).
To develop a protocol that more accurately mimicked the in vivo gene expression profile of PCs, I varied the conditions. The most promising protocol is shown in Fig. 13. Thereby, I switched the culturing medium for HBVPCs to EGM2. The chapter Materials and Methods provides a detailed presentation of the novel culturing technique. Under this new condition, *α-SMA (ACTA2)* was almost not expressed in PCs, while a substantial decrease of *MYH11* could be observed. Furthermore, *NG2 (CSPG4)* and *PDGFRβ* expression levels were decreased in comparison to the established protocol, but not gone (Fig. 13 A). Thus, the novel protocol with EGM2 as culture medium guarantees a more "PC-like" phenotype of cultured HBVPCs than the previously established protocol.
I also compared HUVECs and SMCs to PCs in these culture conditions. As expected PCs did not express any EC marker, but *NG2 (CSPG4)* and *PDGFRβ*. As described above, *α-SMA (ACTA2)* was only slightly expressed in PCs, while *MYH11* expression was sharply decreased. In contrast, ECs did not express any of the MC markers mentioned above, but *CD31*. *CD31* is specific for ECs in this context, since neither PCs nor SMCs express this EC marker. Notably, SMCs which were exposed to the novel protocol did not lose their identifying expression of *α-SMA (ACTA2)* and *MYH11* (Fig. 13 B).
Thus, the novel protocol is a powerful and reliable technique to culture HBVPCs and HBVSMCs in vitro.

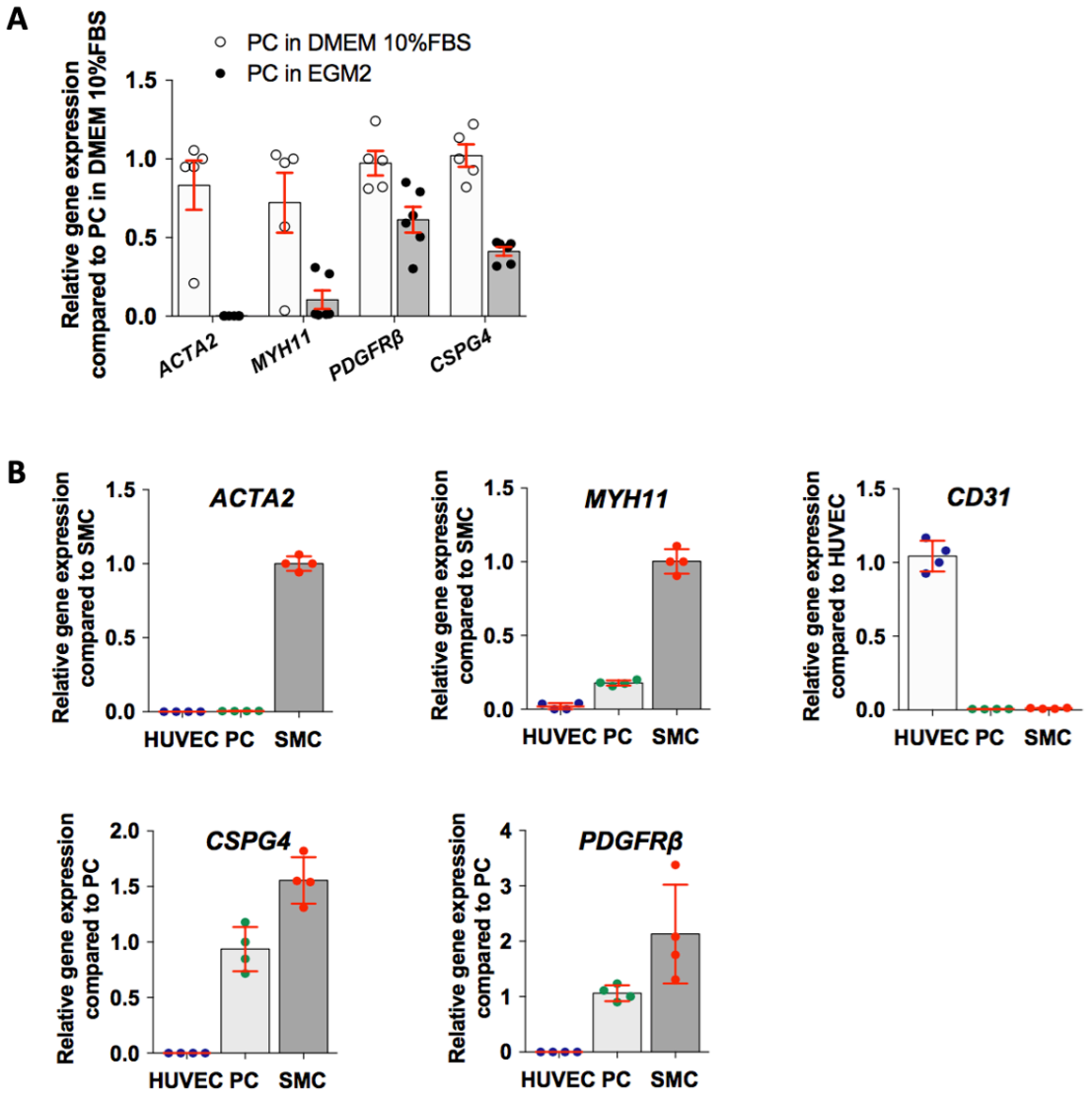


Figure 13: **EGM2 is an appropriate medium to keep PCs in vitro.**
A qPCR analysis for *α-SMA (ACTA2), MYH11, PDGFRβ*, and *NG2 (CSPG4)* expression of HBVPCs under different treatment conditions. The medium is indicated in the figure. *(n=5 and 6 independent experiments)*
B qPCR analysis of *CD31, α-SMA (ACTA2), MYH11, PDGFRβ* and *NG2 (CSPG4)* expression in HBPVCs in relation to SMCs and HUVECs (n = 4 independent experiments). All cells treated with EGM2.
*Error bars represent s.e.m. ** P<0.01, Students t-test.*

From: Dubrac and Künzel et. al. (2018)

3.3 Differentiated Mural Cells contribute to Neovascular Tuft Formations in the OIR mouse model

To test the hypothesis that MCs play a crucial role in ischemic retinopathies, WT mice were subjected to the OIR protocol to produce ischemic retinas. After taking the mice out of the hyperoxia chamber at P12 and leaving them at room air for five more days (see OIR protocol in Fig. 9 and Fig. 14 A), retinal gene expression of *Vegf-a* and *Pdgf-b* was measured by qPCR experiments. As expected and already well-described, a significant increase of *Vegf-a* gene expression could be detected. Interestingly, gene expression of *Pdgf-b* was also strongly elevated. This finding was the first indication that a pathological MC recruitment could contribute to ischemic retinopathies (Fig. 14 B).

Having a closer look at the blood vessel network in P17 OIR retinas, led to the observation of pathological hypersprouting, as indicated by numerous small vascular sprouts growing out of the veins (Fig. 14 C). Furthermore, a high number of NVTs can be detected (Fig. 14 C, white arrows). This hypersprouting could be observed in parallel appearance to large avascular areas of the retina (Fig. 14 C), a typical hallmark of ischemic retinopathies (compare Fig. 1). Immunostaining of the P17 OIR retinas for PDGF-B indicated high levels of the growth factor, spatially on those NVT areas. This is a strong indicator, that MCs were attracted to NVTs by PDGF-B.

The NVT areas could be strongly stained for NG2 and PDGFRβ in comparison to surrounding vessels (Fig. 14 C bottom row). Notably, almost every single tuft was covered by PDGFRβ and NG2 positive cells (Fig. 14 C and Fig. 15 B). This finding could be confirmed by antibody staining against Desmin (Fig. 17 A) where higher magnification showed a close wrapping of the Desmin filaments around the tuft-forming vessels. These observations indicate a strong MC coverage of NVTs by MCs. The coverage of NVTs with α-SMA and MYH11 did not clearly identify PCs or SMCs as NVT covering cells. In fact, more than 90 percent of the NVTs could be strongly stained for α-SMA. This observation indicates that the tuft-covering MCs were potentially SMCs. However, only a very small percentage of NVTs could be stained for MYH11, the second specific marker of SMCs (Fig. 15 A, B). This contradiction raised the critical scientific question whether PCs or SMCs contribute to NVT formations.

qPCR experiments were performed to quantify the expression of *α-Sma* and *Myh11* in the P17 OIR retinas. In accordance with the findings from the immunostaining images, the amount of expressed *α-Sma* was strongly increased while expressed *Myh11* in OIR retinas was reduced (Fig. 15 C).

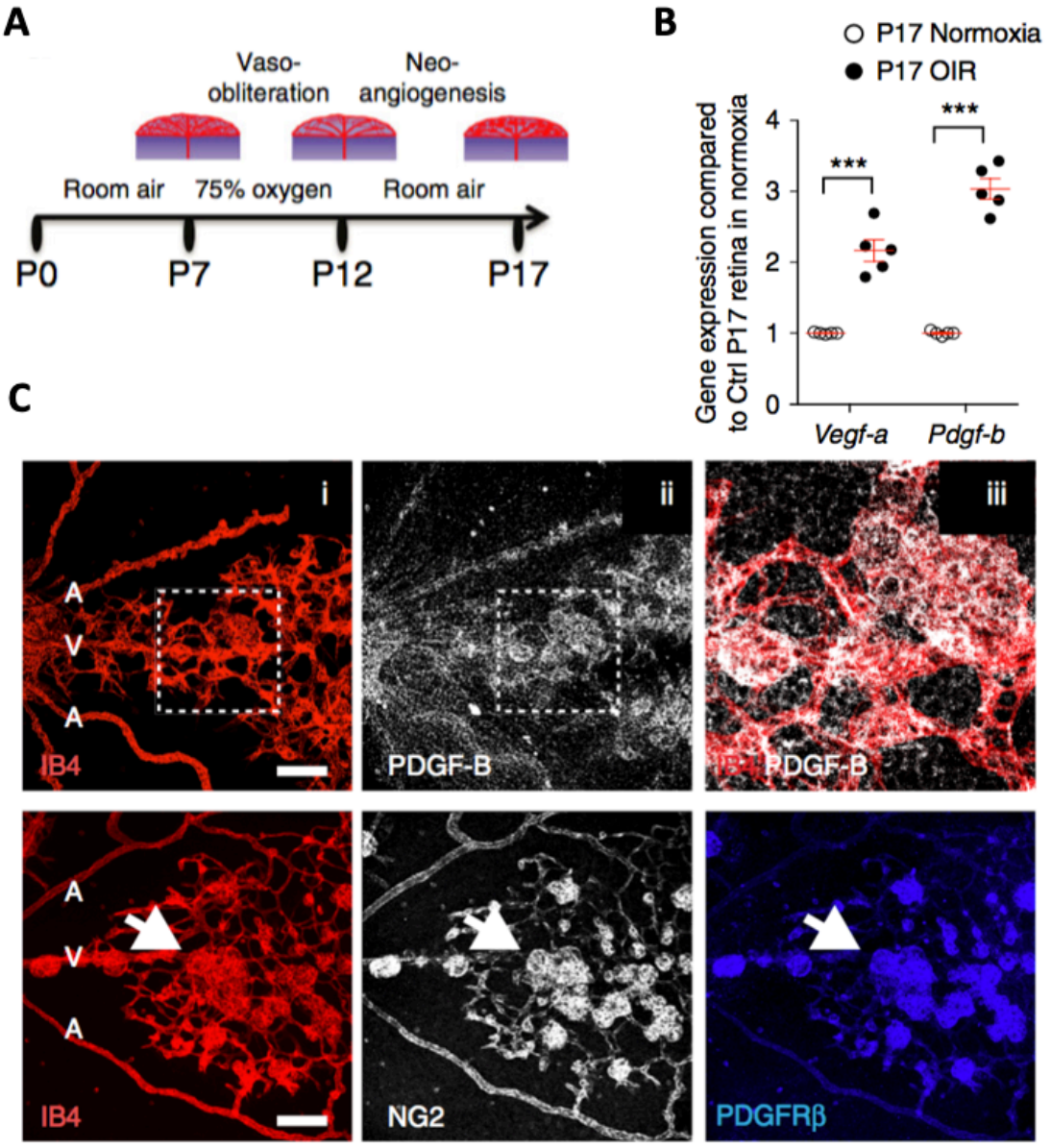


Figure 14: α**-SMA positive MCs cover NVTs. (1)**

A Study protocol of the OIR model. More information in Materials and Methods (Fig. 9).
B qPCR analysis of *Vegf-a* and *Pdgf-b* gene expression measured in P17 OIR retinas compared to P17 retinas in normoxia (n = 5 mice). *Error bars: s.e.m. *** P < 0.001, two-way ANOVA Sidaks multiple comparisons test.*
C Top: Immunostaining for IB4 (i), PDGF-B (ii) and doublestaining (iii). Bottom row: Immunostaining for IB4, NG2 and PDGFRβ. Retinas from P17 OIR mice. *Scale bars: 100 μm.*

From: Dubrac and Künzel et. al. (2018)

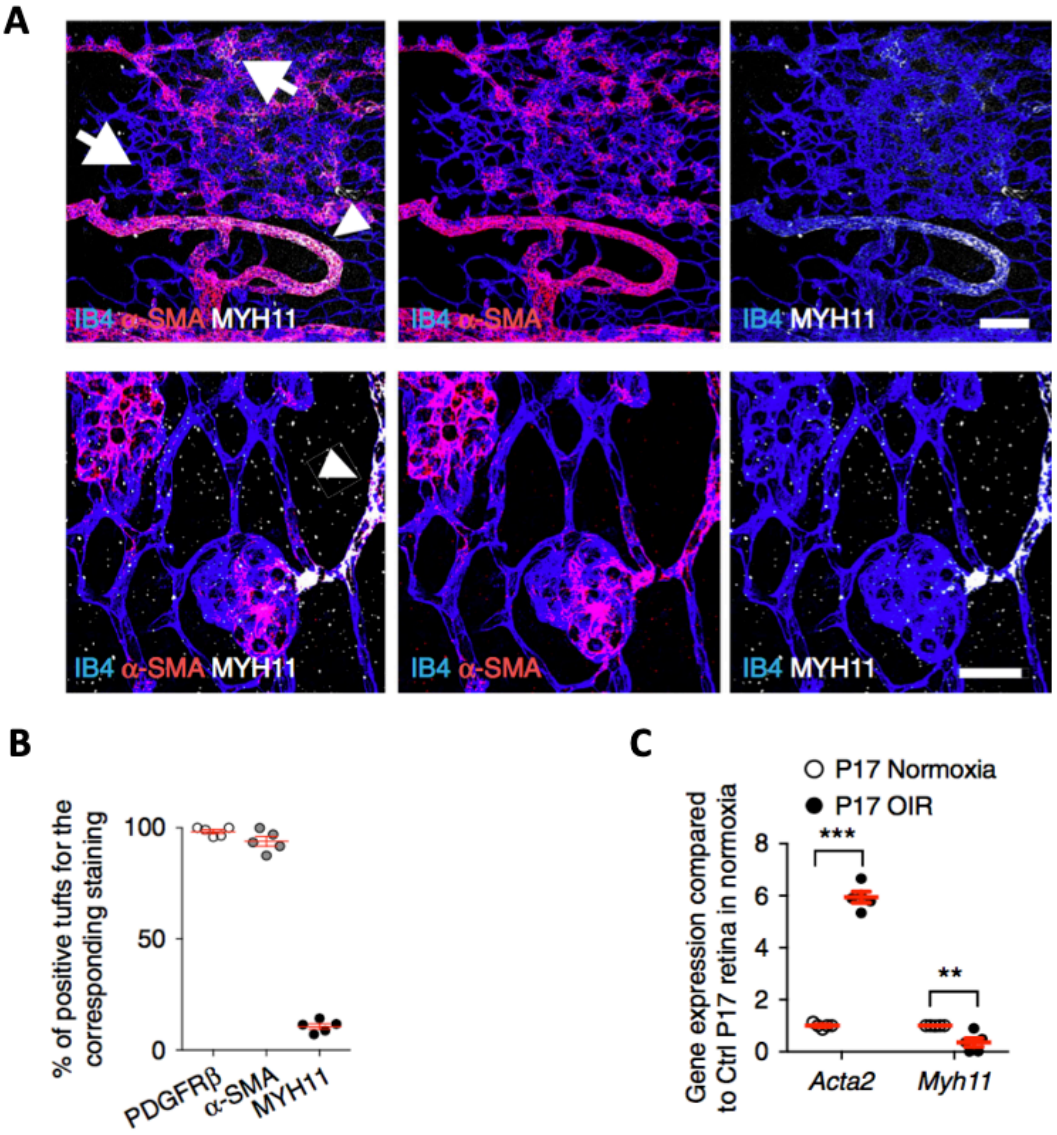


Figure 15: **α-SMA positive MCs cover NVTs. (2)**

A Triple staining for IB4 and the SMC markers α-SMA and MYH11. Staining indicated in corresponding images. Bottom images represent higher magnifications. White arrows on top image: NVTs covered by α-SMA positive cells. White arrowheads on bottom image: Artery covered by MYH11 positive cells. *Scale bars in top images: 100 µm; in bottom images: 30 µm.*
B Quantification of NVTs positive for indicated markers.
C qPCR quantification of *α-SMA* and *Myh11* expression of P17 OIR retinas in relation to normoxia retinas (n = 5 mice). *Error bars: s.e.m. $P < 0.001$, $P < 0.01$, two-way ANOVA Sidaks multiple comparisons test.*

From: Dubrac and Künzel et. al. (2018)

Staining for PDGFRβ and α-SMA was overlapping on NVTs (Fig. 16). This strongly indicates, that it is the same MC type covering the NVTs. Furthermore, I scrutinized the gene expression of different growth factors and MC markers at P15, a time point where hypersprouting and NVTs already occur, as seen in Fig. 17, A.

Similar to P17 retinas, not only *Vegf-a* but also *Pdgf-b* was increased at this time point. Second, while no significant change in *Pdgfrβ* expression could be observed, the SMC marker *α-Sma (Acta2)* was stronger expressed in OIR retinas. In contrast to this, no difference could be detected in *Myh11* expression (Fig. 17 B).
The increase in α-SMA covering cells could be confirmed by quantifying the percentage of the vasculature that was covered by α-SMA positive cells (Fig. 17 D). A strong increase could be detected, while the percentage of vasculature covered by any MC as indicated by NG2 did not significantly change (Fig. 17 C).

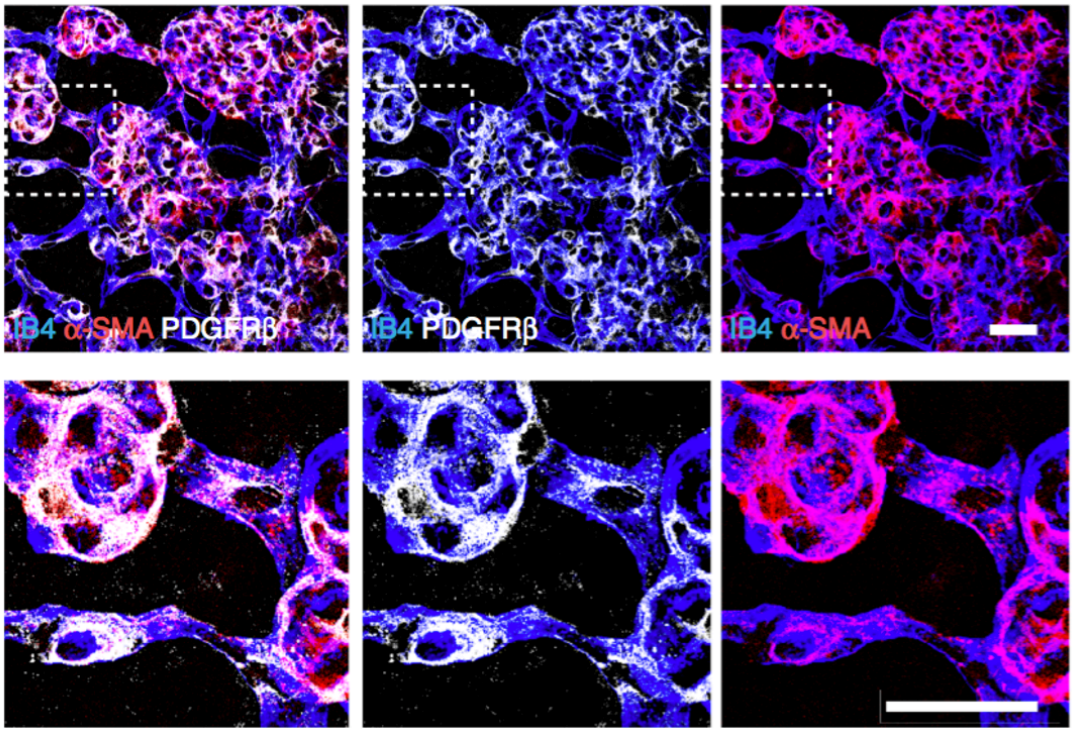


Figure 16: **α-SMA positive MCs cover NVTs. (3)**

IB4, α-SMA, and PDGFRβ triple staining of NVTs from P17 OIR retinas. Staining indicated in corresponding images. Bottom images are higher magnification of boxed areas *Scale bars: 30 µm.*

From Dubrac and Künzel et. al. (2018)

The observations of these experiments support the hypothesis of my dissertation that MCs are pathologically activated or affected in diseased retinas. *Pdgf-b* expression is strongly increased and NVTs are heavily covered by MC marker expressing cells. Surprisingly, *Myh11* expression decreases, and vessel coverage by *Myh11* expressing cells appears only scarcely. In contrast to this, and as a striking result, *α-Sma* expression and vessel coverage are strongly elevated in the NVT areas. These findings raised important questions for the understanding of MCs in diseased retinas. Of particular importance for this study is to answer which cell type contributes to the coverage of NVTs. While the increase of α-SMA promotes SMCs as potential candidates, the low levels of *Myh11* expression support the hypothesis, that PCs cover NVTs.

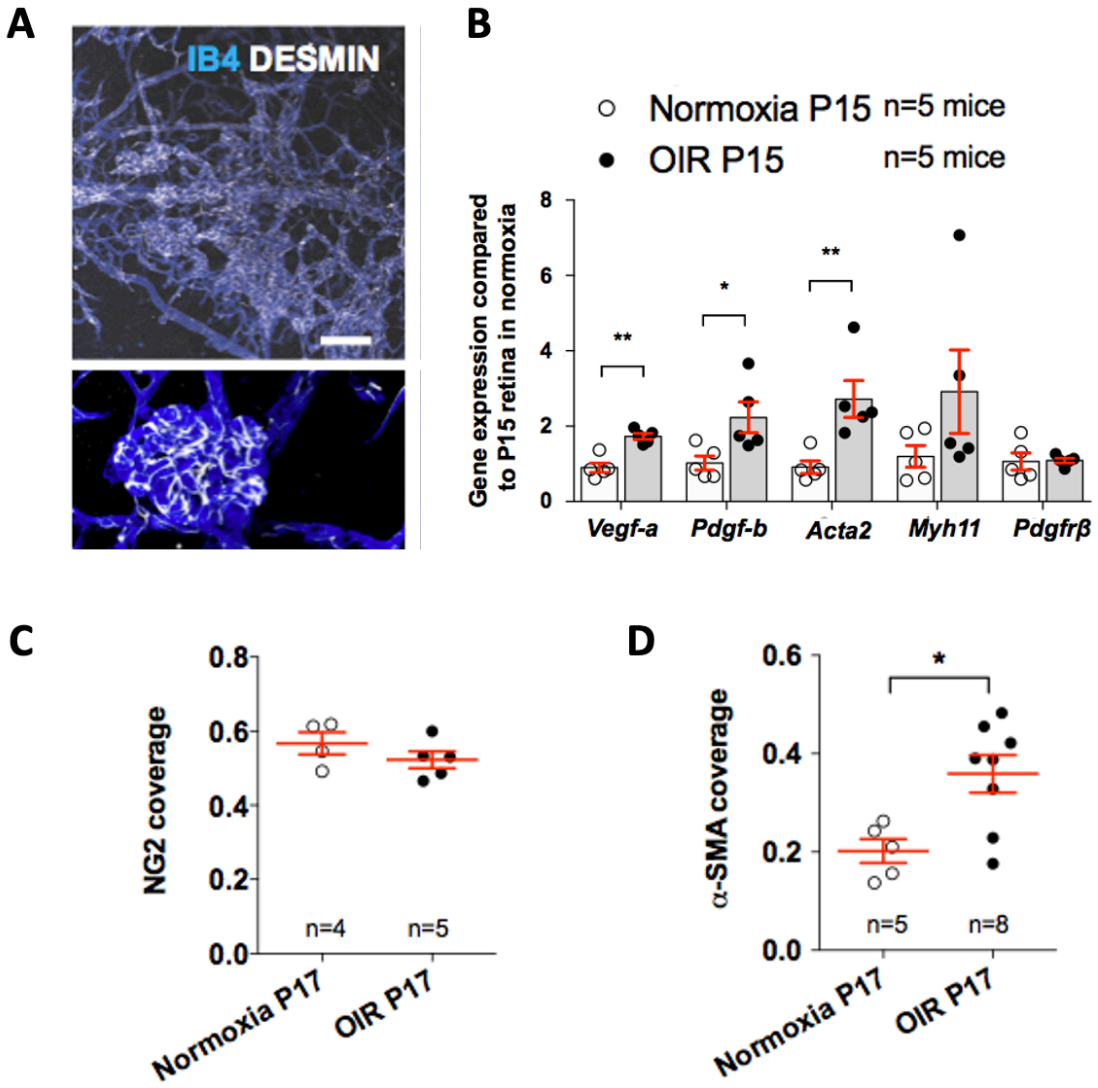


Figure 17: **α-SMA positive MCs cover NVTs. (4)**

A IB4 and Desmin double staining of NVTs from P15 OIR retinas. Bottom: Higher magnification of single NVT. *Scale bars: Top: 100 µm; Bottom: 10 µm*
B qPCR analysis of *Vegf-a, Pdgf-b, α-Sma (Acta2), Myh11* and *Pdgfrβ* expression in P15 OIR retinas compared to P15 retinas in normoxia. The number of animals used for the experiment is indicated. *Error bars: s.e.m. * P<0.05, MannWhitney U test; ** P<0.01, MannWhitney U test.*
C, D Quantification of NG2 and α-SMA coverage of P17 retina in normoxia and OIR. Number of retinas used for quantification is indicated. *Error bars represent s.e.m. * P<0.05, Mann Whitney U test.*

From Dubrac and Künzel et. al. (2018)

3.4 Pericytes and not Smooth Muscle Cells differentiate and cover Neovascular Tuft Formations

NVTs are covered by α-SMA positive, but MYH11 negative MCs (Fig. 16+17). Thus, it is unclear which cell type is covering these tufts with three possibilities. First, PCs which differentiate and start to express *α-Sma* could cover NVTs. Second, SMCs which lose their typical expression of *Myh11*. Third, another cell type could be attracted to the NVTs. Lineage tracing experiments were used to answer that question, thereby crossing *Pdgfrβ-CreERT2, Myh11-CreERT2,* and *α-Sma-CreERT2* mice with *Rosa26;mTmG* reporter mice to genetically label the MCs and their descendants. TXF injection at day P6 and P7 guarantees, that corresponding cells are labeled by expressing *Gfp* before the NVT formation occurs (Fig. 18 A). Mice were sacrificed at day P17 following the normal OIR protocol (Fig. 9 and Fig. 18 A). Notably, gene deletion and subsequent *Gfp* expression occurs with a very high efficiency as images taken one day after TXF injection show almost completely green labeled cells (Fig. 18 C and Fig. 19 A, B, P8 pictures in top row). Notably, as *Pdgfrβ-CreERT2* is labeling all MCs, the complete vasculature is green, while *α-Sma-CreERT2* and *Myh11-CreERT2* are selectively affecting SMCs which predominantly cover arteries.
Importantly, in P17 retina more than 95% of the α-SMA positive tufts are GFP labeled in the *Pdgfrβ-CreERT2* mice, which indicates that the α-SMA positive NVT covering cells derive from originally PDGFRβ positive cells (Fig. 18 B, C) and not from ECs or cells from another origin. This is a key finding in this dissertation, since it undoubtedly demonstrates a critical MC contribution to NVT formations, though not distinguishing between PCs and SMCs.
Notably, less than 10% of NVTs are labeled with GFP in *α-Sma-CreERT2* or *Myh11CreERT2* P17 retinas (Fig. 18 B and Fig. 19 B). Thus, originally α-SMA positive and MYH11 positive SMCs are not the origin of the α-SMA positive NVT covering cells, but PDGFRβ positive, α-SMA negative, MYH11 negative PCs are the major origin of NVT covering MC.

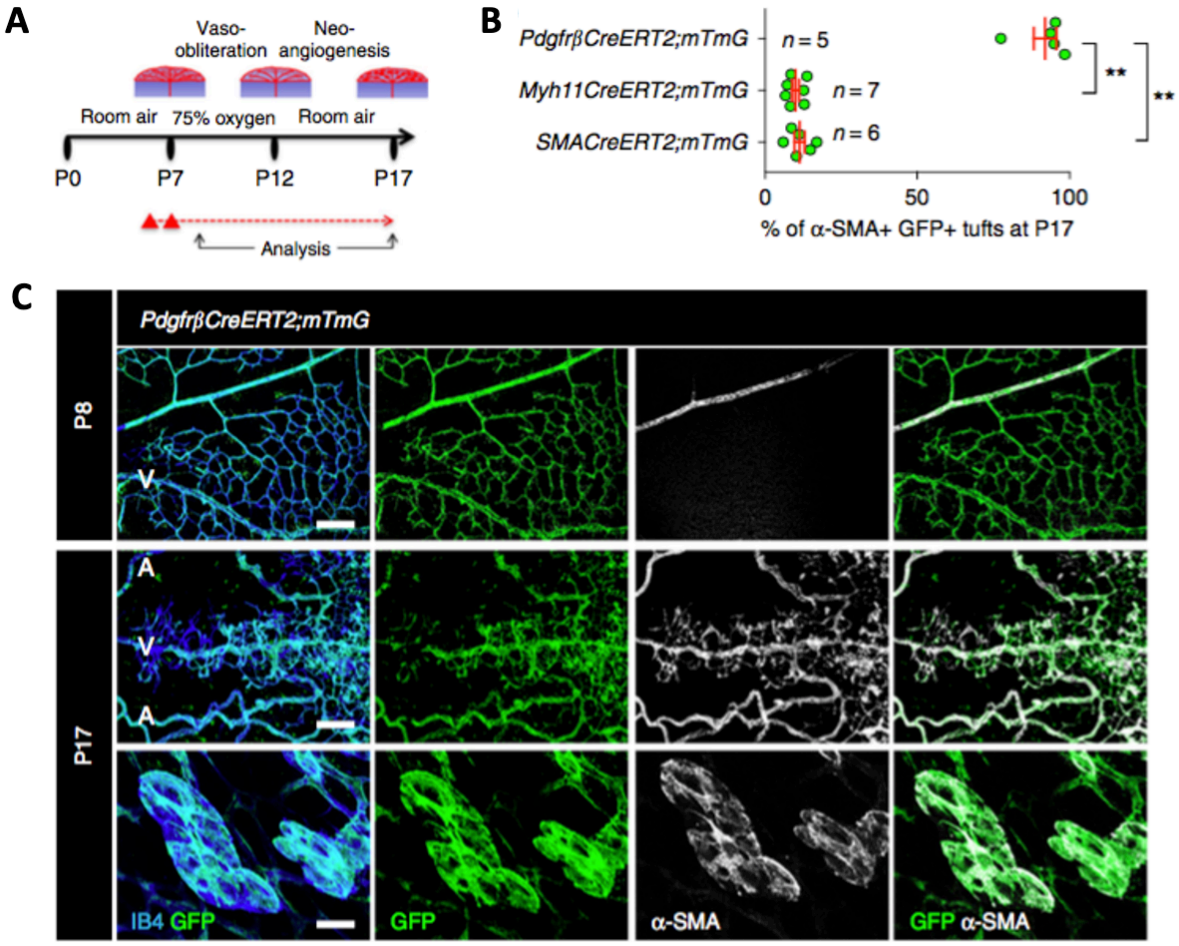


Figure 18: **NVTs are covered by differentiated α-SMA positive Pericytes. (1)**
A Schematic of lineage-tracing experiment: *CreERT2;mTmG* mice were exposed to the OIR protocol. TXF treatment is indicated by red triangles.
B Quantification of α-SMA positive NVTs labeled with GFP in different mouse lines. All pictures from OIR P17 retinas. The number of retinas used for quantification is indicated. *Error bars: s.e.m. P < 0.01, MannWhitney U test.*
C IB4, α-SMA, and GFP triple staining of retinas from P8 and P17 *Pdgfrβ-CreERT2;mTmG* mice.
Scale bars: 100 µm (top and middle) and 60 µm (bottom).

From Dubrac and Künzel et. al. (2018)

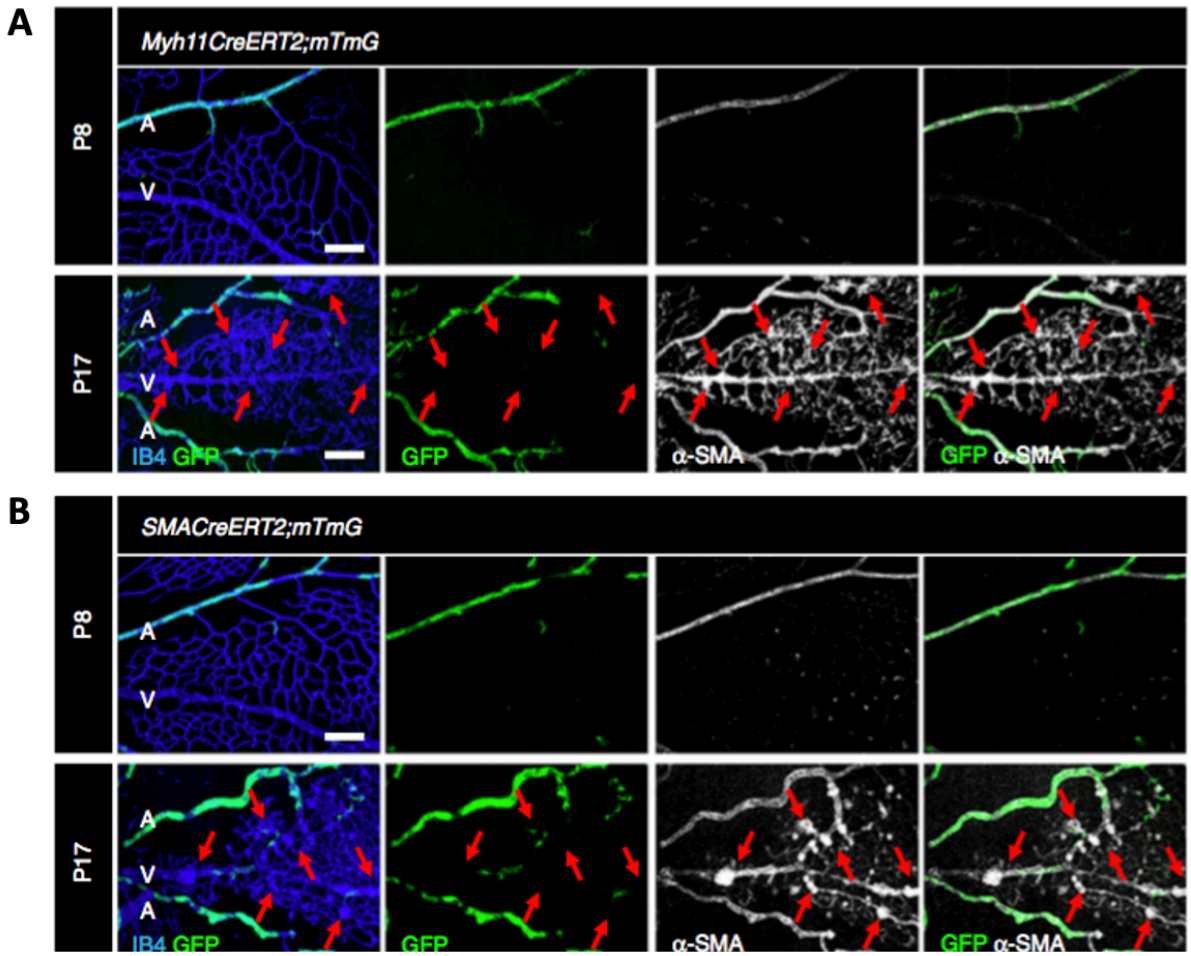


Figure 19: **NVTs are covered by differentiated α-SMA positive Pericytes. (2)**
A IB4, α-SMA, and GFP triple staining of retinas from P8 and P17 *Myh11-CreERT2;mTmG* mice.
B IB4, α-SMA, and GFP triple staining of retinas from P8 and P17 *α-Sma-CreERT2;mTmG* mice.
Red arrows show α-SMA positive GFP negative NVTs. *Scale bars: 100 µm.*

From Dubrac and Künzel et. al. (2018)

3.5 Endothelial *Pdgf-b* expression is crucial for a proper Mural Cell recruitment

After the observation of increased levels of *Pdgf-b* expression in diseased retinas; and identification of differentiated PCs as NVT covering cells, I evaluated the role of endothelial PDGF-B in the developing mouse retina. Thus, transgenic *Pdgf-b lox* (*Pdgf-bl/l*) mice were interbred with inducible *Cdh5-CreERT2* mice. *Cdh5* is predominantly expressed in ECs in tis model, and hence PDGF-B was targetted in ECs. Gene deletion was then induced by TXF injection starting on the first days of life (P0-2), to elucidate the role of PDGF-B during early retinal blood vessel development after birth. Mice were sacrificed at P5 when the retinal vasculature was still on a developmental stage (Fig. 20 A). To confirm an efficient gene deletion, *Pdgf-b* levels in the retina were determined by qPCR of the whole retina. Notably, *Pdgf-b* expression was strongly decreased, but could still be detected (Fig. 20 B).
Interestingly, in P5 mutant retinas, PC expression markers such as *Pdgfrβ* were also significantly decreased and almost not detectable. Very similar to this, *Myh11* and *α-Sma (Acta2)* were detected at reduced levels in mutant animals (Fig. 20 C). Immunostained P5 retinas could confirm these qPCR results. While the retinal vasculature of WT mice was fully covered by NG2 positive MCs, mutant mice displayed almost naked vessels with no MC gene expressing cells. This finding was particularly true for veins and capillaries since not a single one of them showed any MC coverage. Furthermore, this strong phenotype was not depending on the particular location in the vasculature. Capillaries of the vascular front of WT mice were fully covered by MCs, while capillaries at the same location of mutant mice were not covered at all. Arteries were also strongly affected, but not as strong as veins or capillaries. While MCs were completely depleted on veins and capillaries, SMC coverage on arteries was only strongly decreased, but not fully gone. A coverage of NG2 and α-SMA positive SMCs could still be detected (Fig. 20 D). In addition to this finding of naked vessels, retinas of mutant mice frequently displayed enlarged and dilated vessels, as well as microaneurysms (Fig. 20 D; white arrows). These vessel abnormalities underlined the crucial functions of a proper MC recruitment for vessel stability.
Furthermore, the absence of PCs massively impaired vascular outgrowth, branching and radial expansion. The number of newly formed sprouts is another indicator of effective vascular sprouting (Fig. 21 A, C). Quantification of these revealed a substantial decrease and thus, impaired sprouting. In the developing mouse retina, EC proliferation only occurs at the vascular front and on veins. I evaluated EC proliferation by measuring ERG123 positive ECs . Interestingly, when PCs were depleted, much less ERG123 positive ECs could be counted on veins (Fig. 20 B, D). Thus, PCs play a crucial function in sprouting angiogenesis, EC proliferation (discussed below), and development of an appropriate retinal vasculature.
Another important parameter for vessel stability is the evaluation of vessel regression. Vascular regression can be evaluated by the appearance of vessel sleeves. These could be observed by identifying IB4 negative and Collagen4 positive structures, or IB4 negative and Fibronectin positive structures, respectively (Fig. 21 F and not shown).

Comparing WT and mutant animals, PC depleted retinas demonstrated a strong increase in those vessel sleeves. Thus, the loss of PCs led to less vessel stability and more regression (Fig. 21 F, G). In summary, the data suggest that endothelial PDGF-B is essential for proper PC recruitment during sprouting angiogenesis. The loss of PCs resulted in impaired sprouting, less proliferative ECs and unstable, dilated vessels.

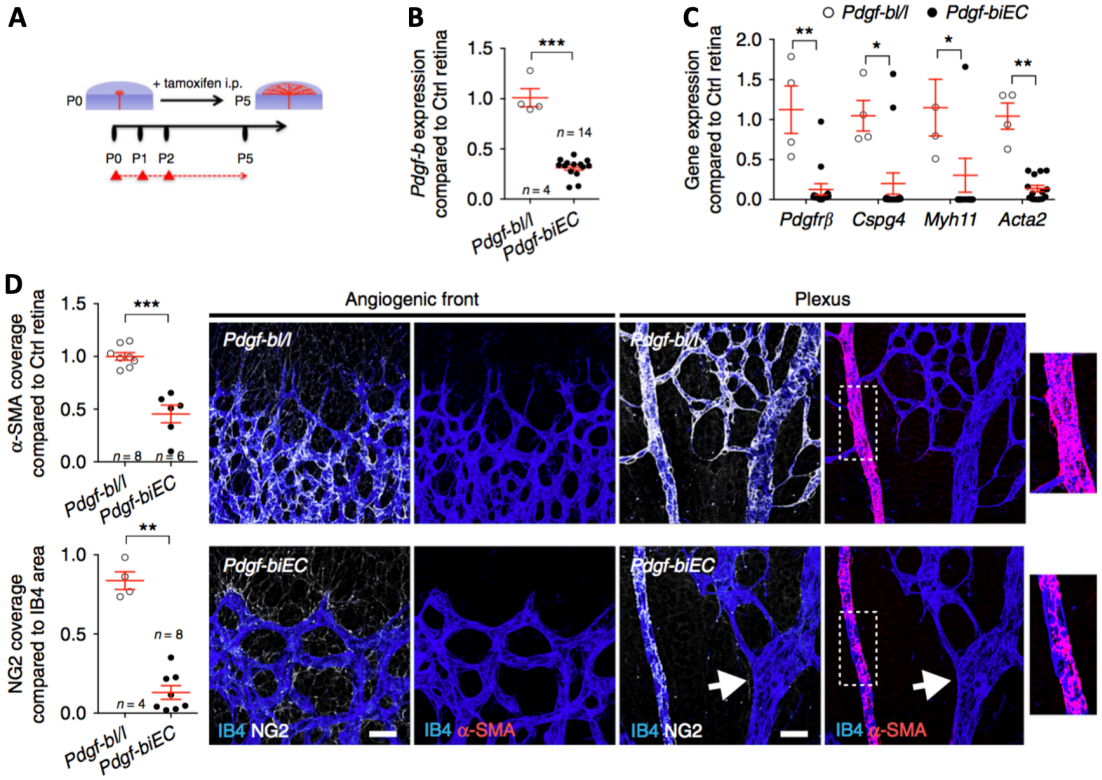


Figure 20: **Postnatally induced deletion of endothelial *Pdgf-b* results in "naked", enlarged vessels and impaired sprouting. (1)**

A Concept of the experiment. Red triangles mark TXF injections at day P0-P2. Mice were sacrificed at P5.
B, C Realtime qPCR results of *Pdgf-b, Pdgfrβ, Cspg4 (NG2), Myh11,* and *α-SMA (Acta2)* in whole P5 retinas: mutant vs control. *N = 4 Pdgf-bl/l and N = 14 Pdgf-biEC retinas. Error bars represent s.e.m. P < 0.05; P < 0.01, two-way ANOVA Sidaks multiple comparisons test.*
D Left, top: Quantification of α-SMA coverage: mutant vs control. Left, bottom: Quantification of NG2 coverage in relation to IB4 stained vessels. All retinas from P5 mice. *Number of retinas used for quantification is indicated. Error bars represent s.e.m. P < 0.01; P < 0.001, MannWhitney U test.* Right: Staining for IB4, NG2, and α-SMA of the angiogenic front and the retinal plexus of P5 retina. White arrows point on microaneurysm formation. Higher magnification of α-SMA stained arteries in boxed areas on the right. *Scale bars 30 µm.*

From Dubrac and Künzel et. al. (2018)

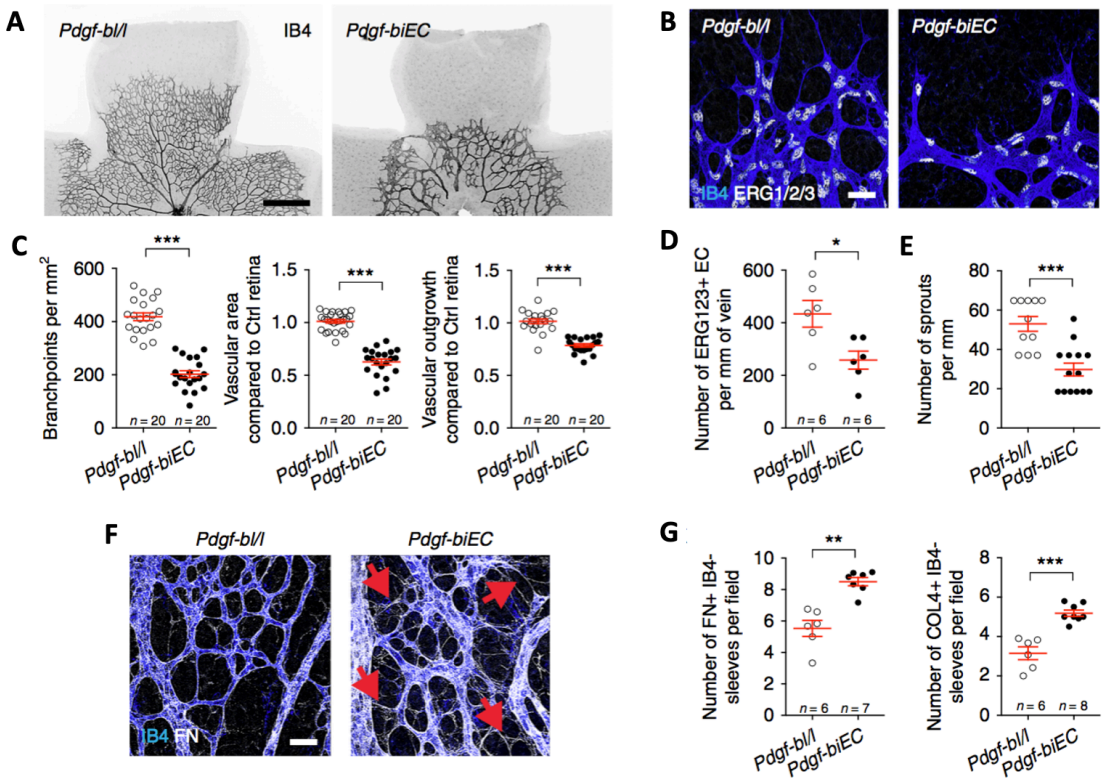


Figure 21: **Postnatally induced deletion of endothelial *Pdgf-b* results in "naked", enlarged vessels and impaired sprouting. (2)**

A IB4 staining of the retinal vasculature at P5 (negative images of the fluorescent signal). *Scale bars 500 µm.*
B IB4 and ERG1/2/3 double staining of the angiogenic front of P5 retina. *Scale bars 30 µm.*
C Quantification of branchpoints, vascular area, and vascular outgrowth. *Error bars represent s.e.m. $P < 0.001$, MannWhitney U test.*
D Quantification of *Erg1/2/3* positive cells in the vessels of P5 retina. *Number of retinas used for quantification is indicated. Error bars represent s.e.m. $P < 0.05$, MannWhitney U test.*
E Quantification of sprouts in the angiogenic front. *$n = 11$ imaging fields for Pdgf-bl/l from four retinas and $n = 14$ for Pdgf-biEC from four retinas. Error bars represent s.e.m. $P < 0.001$, MannWhitney U test.*
F IB4 and Fibronectin staining of P5 retina. Empty BM sleeves of retracting vessels are indicated by red arrows. *Scale bars 30 µm.*
G Quantification of FN positive IB4 negative and COL4 positive IB4 negative sleeves per 63x images. *Number of retinas used for quantification is indicated. Error bars represent s.e.m. $P < 0.01$, $P < 0.001$, MannWhitney U test.*

From Dubrac and Künzel et. al. (2018)

3.6 Deleting endothelial *Pdgf-b* in the OIR experiment reduces Neovascular Tuft formation, but impairs retinal revascularization

The next aim was to scrutinize the distribution of the endothelial PDGF-b-dependent PC recruitment to NVTs and pathological sprouting in ischemic retinopathies. To further evaluate the role of PCs, I subjected the transgenic *Pdgf-bl/l-Cdh5-CreERT2* mice, as well as *Cre* negative littermates to the established OIR protocol. The gene deletion was induced by TXF injections for three days, beginning at P12 when animals were taken out of the hyperoxia chambers back to room air (Compare Fig. 22, A). The protocol was performed this way, to delete *Pdgf-b* precisely during the time, when the high pathological levels of the vascular growth factor were detected (Compare Fig. 14). Measurement of retinal *Pdgf-b* expression by qPCR revealed a decrease of only 60 % indicating potential other cells expressing the growth factor in the given model and in the ischemic retina (not shown in this dissertation, but in Dubrac and Künzel et. al. (2018)). Nevertheless, a promising finding in the mutant P17 OIR retinas was a substantial reduction of NVT area in mutant mice. The decline of the measured NVT area of about 50 % was strongly significant (Fig. 22 B + Fig. 23 B). Despite the incomplete loss of *Pdgf-b* expression, the coverage of IB4 positive tufts by PDGFRβ and α-SMA positive cells was strongly decreased (Fig. 22 B, C). Furthermore, when having a closer look at the vessels, the number of sprouts growing out of larger vessels was lower in mutants (Fig. 23 A). Since NVTs, as well as new sprouts, are an indicator for pathological vascular sprouting, deletion of *Pdgf-b* was a promising target, when consulting these parameters for evaluation.
However, another critical parameter in the OIR protocol is the size of the avascular area. Experts of the field adopted the view that a large avascular area is a correlate of impaired revascularization. The mutant mice of this experiment displayed a larger avascular area, and thus, a decreased revascularization (Fig. 23 A, B).
Another common hallmark of ischemic retinopathies is retinal bleeding into the eye. In this experiment, I evaluated bleeding by antibody staining for TER119, which is a marker of myeloid cells. As red blood cells are positive for TER119, immunostaining against TER119 is a powerful tool to evaluate retinal bleeding. As expected, the WT mice in the OIR model suffered from severe bleeding with red blood cells outside of the vessels (Fig. 23 B, C). Interestingly, the bleeding in the eyes of mutant mice was strongly increased by a factor larger than 20. Images of retinal flat mounts, in which red blood cells infiltrated the whole retinal tissue, could be observed frequently (Fig. 23 B, C).
Thus, deletion of endothelial *Pdgf-b* during the OIR protocol is promising, since the NVT area, as well as neovascular sprouting, was decreased. On the other hand, the mutant mice displayed several critical characteristics: the avascular area as an indicator of impaired revascularization was increased in mutant mice. Furthermore, an extraordinary amount of red blood cells in the retinal tissue could be observed.

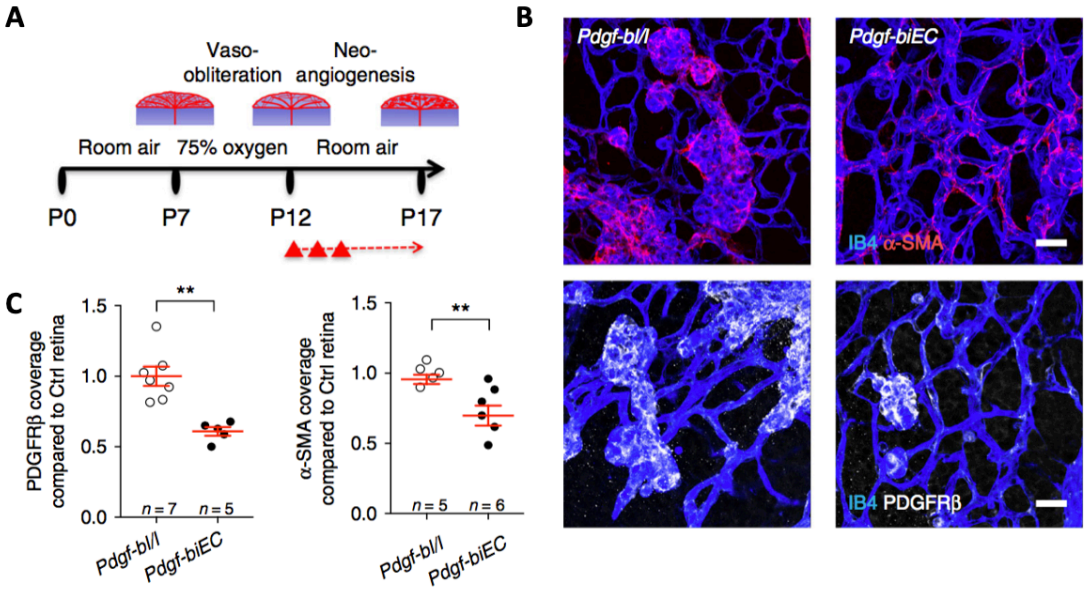


Figure 22: **PDGF-B is a driver of pathological neovascularization in the OIR experiment. (1)**

A Concept of the experiment. *Pdgf-bl/l-Cdh5-CreERT2* mice and corresponding control littermates were exposed to the OIR protocol. Red triangles indicate TXF injections at P12-P14.
B Double staining for IB4 and α-SM (top); and IB4 and PDGFRβ (bottom) of the NVTs in mutants (right) and controls (left). *Scale bars 30 µm.*
C Quantification of α-SMA and PDGFRβ coverage of IB4 positive neovascular tufts. *Number of retinas used for quantification is indicated. Error bars represent s.e.m. $P < 0.01$, MannWhitney U test*

From Dubrac and Künzel et. al. (2018)

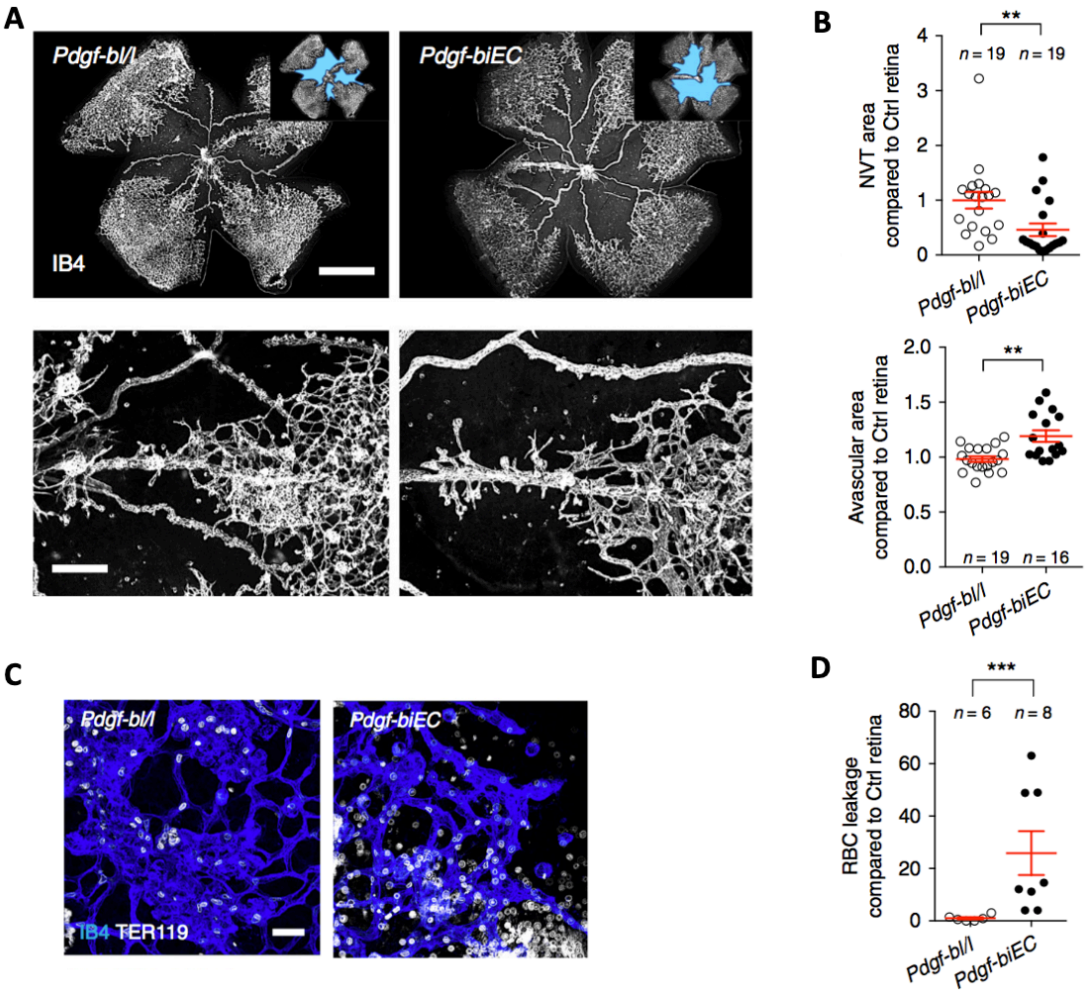


Figure 23: **PDGF-B is a driver of pathological neovascularization in the OIR experiment. (2)**

A IB4 staining of P17 OIR retinas. Smaller insets at top right corner demonstrate avascular area (indicated in blue). Lower images are in higher-magnification to show pathological NVTs. *Scale bars: 1 mm (Top). 100 µm (Bottom)*
B NVT (top) and avascular area (bottom) quantification. *Number of retinas used for quantification is indicated. Error bars represent s.e.m. $P < 0.01$, MannWhitney U test.*
C TER119 and IB4 double staining of P17 OIR retinas to quantify RBC leakage in Pdgf-biEC mice compared to control littermates. *Scale bars 30 µm.*
D Quantification of (F). Number of mice used for quantification is indicated. *Error bars represent s.e.m. $P < 0.001$, MannWhitney U test.*

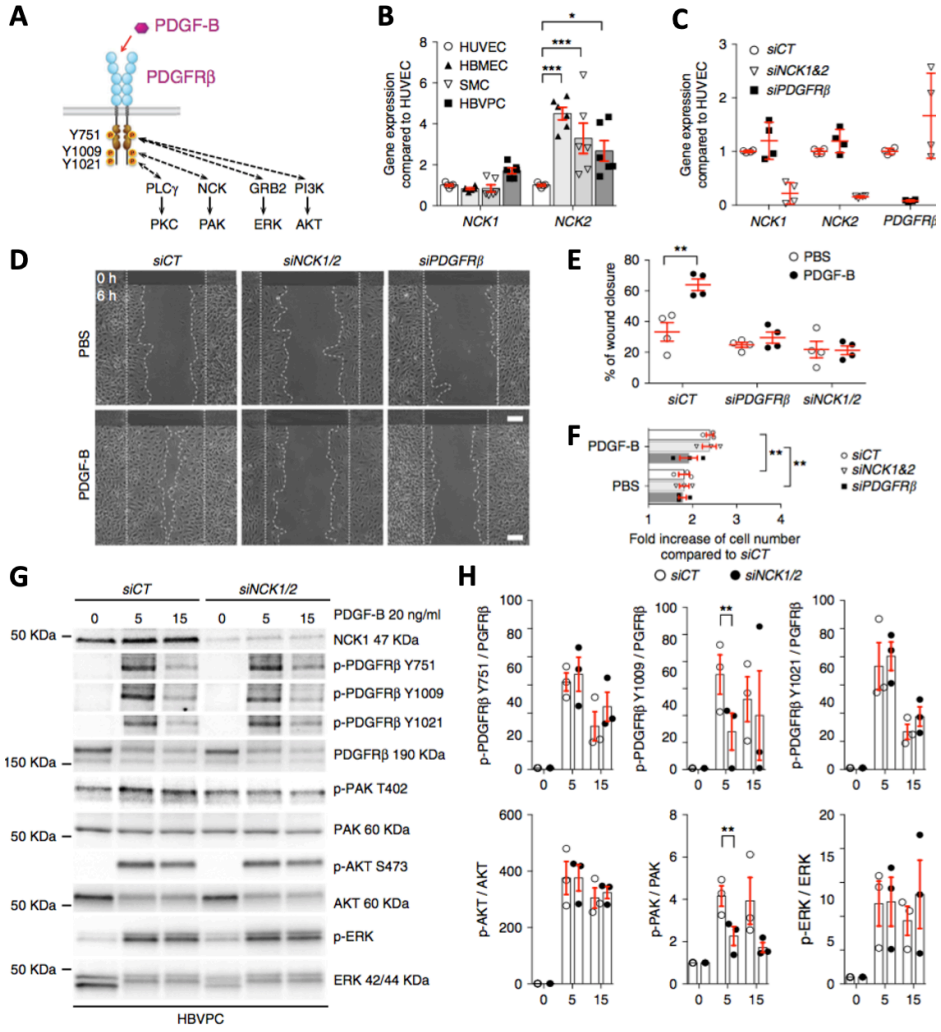
From Dubrac and Künzel et. al. (2018)

3.7 The intracellular adaptor proteins NCK1/2 promote specifically migration in Pericytes

Targeting PCs by their primary regulator PDGF-B reduced NVTs and pathological hypersprouting in vivo (Fig. 22). The complete deletion of *Pdgf-b* resulted in naked vessels with strongly reduced MC coverage and in major vascular defects in vivo in development as well as in disease (Fig. 20, 21, 22, 23).
Thus, I reasoned to target PDGF-B signaling in a more subtle and specific downstream cascade. The PDGFRβ is a receptor tyrosine kinase, with the intracellular domain consisting of several phosphosites (discussed above, Fig. 24 A). Each of them interacts with different intracellular adaptor proteins. As mentioned earlier and in other publications about fibroblasts which also express *Pdgfrβ*, the phosphosite Y1009 interacts with NCK1 and NCK2. NCKs are intracellular "scaffold" proteins. In mammals, *Nck1/22* have broad and overlapping expression patterns, and function redundantly. They act as adapters by linking receptor kinases to downstream signaling cascades. It is already shown, that NCK1/2 interact with p21-activated kinase (PAK) family of serine/threonine kinases and upstream activators, RAC1/CDC42, to regulate cytoskeletal dynamics. In fibroblasts, NCKs bind to phosphorylated Tyr-751 and Tyr-1009 of PDGFRβ (Bladt et al., 2003). Furthermore, Dubrac et al. (2016) could show, that NCKs are crucial for endothelial front rear polarity and vascular sprouting. However, the role of NCK1/2 in PCs is totally unknown at this point.

Before scrutinizing the role of NCKs in vivo, I tested them for a specific function in PC biology in vitro. First, I confirmed the expression of *Nck1* and *Nck2* in PCs by real-time qPCR of cultured HBVPCs and other cell types. I found out that they are strongly expressed in HBVPCs (Fig. 24 B). Interestingly, expression of *Nck1* and *Nck2* was much higher in HBVPCs than in ECs (Fig. 24 B, C). However, the in vitro observations indicated, that *Nck1* and *Nck2* were also expressed in SMCs (Fig. 24 B).
To further scrutinize the function of NCK1, NCK2 and to compare it to PDGFRβ, a protocol was established to specifically knockdown *Nck1, Nck2,* and *Pdgfrβ* by transfecting HBVPC with siRNA against the corresponding RNAs. Notably, knockdown of one of the three proteins did not significantly affect the expression of one of the other mentioned proteins, so that specific conclusions could be drawn (Fig. 24 C). As NCK1 and NCK2 share a very similar structure with reciprocal functions, a complete deletion of one of their genes could potentially be rescued by the other one (mentioned earlier). Thus, knockdown was always performed for both NCKs.
To evaluate PC migration under different conditions, I used the scratch assay, which is described in more detail in Materials and Methods. It was not surprising that HBVPCs did not migrate actively when stimulated without any growth factors, but only with PBS (Fig. 24 D top row, E). As expected, HBVPCs stimulated with PDGF-B and without knockdown of any gene did migrate and almost closed the wound after 6h (Fig. 24 D bottom row left, E). This effect could be prevented by knockdown of the corresponding receptor for PDGF-B (Fig. 24 D bottom row right, E). Interestingly, double knockdown of *Nck1* and *Nck2* phenocopied this observation very closely (Fig.

24 D bottom row center, E). This observation indicates that NCK1/2 are mediating PC migration through PDGF-B signaling in vitro .
It is well-established, that the growth factor PDGF-B is mediating other PC behaviors, such as proliferation and survival. To test whether NCK1 and NCK2 were also mediating proliferation, a quantification of the number of HBVPCs at different time points under different conditions was performed. Interestingly, only the knockdown of the *Pdgfrβ* could block PDGF-B-induced proliferation, but not the double knockdown of *Nck1* and *Nck2* (Fig. 24 F). This observation demonstrated that NCK1 and NCK2 are not mediating PC proliferation or survival.
Furthermore, previous studies revealed that NCK1 and NCK2 are potentially interacting with the PDGFRβ phosphosites Tyr-751 and Tyr-1009, but much less is known about NCKs in PCs and through which signal cascade NCK1 and NCK2 are mediating their functions. In this study, it could be shown by Western Blot signaling experiments, that NCK1 and NCK2 are mediating PDGF-B signaling through the p-PAK signaling cascade (Fig. 24 G, H).
Summarising the in vitro experiments on HBVPCs, NCK1 and NCK2 are specifically mediating PDGF-B induced PC migration through the phosphosite Y1009 of the PDGFRβ and PAK. PC proliferation and survival were not significantly affected.


A
PDGF-B
PDGFRβ
Y751
Y1009
Y1021
PLCγ
NCK
GRB2
PI3K
PKC
PAK
ERK
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B
HUVEC
HBMEC
SMC
HBVPC
Gene expression compared to HUVEC
NCK1
NCK2
C
siCT
siNCK1&2
siPDGFRβ
Gene expression compared to HUVEC
NCK1
NCK2
PDGFRβ
D
siCT
siNCK1/2
siPDGFRβ
0 h
6 h
PBS
PDGF-B
E
PBS
PDGF-B
% of wound closure
siCT
siPDGFRβ
siNCK1/2
F
PDGF-B
PBS
siCT
siNCK1&2
siPDGFRβ
Fold increase of cell number compared to siCT
G
siCT
siNCK1/2
PDGF-B 20 ng/ml
NCK1 47 KDa
p-PDGFRβ Y751
p-PDGFRβ Y1009
p-PDGFRβ Y1021
PDGFRβ 190 KDa
p-PAK T402
PAK 60 KDa
p-AKT S473
AKT 60 KDa
p-ERK
ERK 42/44 KDa
50 KDa
150 KDa
HBVPC
H
siCT
siNCK1/2
p-PDGFRβ Y751 / PGFRβ
p-PDGFRβ Y1009 / PGFRβ
p-PDGFRβ Y1021 / PGFRβ
p-AKT / AKT
p-PAK / PAK
p-ERK / ERK

Figure 24: **NCK1 and NCK2 specifically mediate PDGF-B-induced Pericyte migration in vitro.**
A Schematic of PDGFRβ with its phosphosites and selected downstream pathways. NCK1 and NCK2 interact with the phospho-Y1009 of the PDGFRβ receptor to regulate PDGF-B-induced PAK activation, while other effectors bind to different phosphosites.
B Gene expression of *Nck1* and *Nck2* in different celltypes. Measurements by qPCR in HBMECs (n = 6), human brain SMCs (n = 6), and HBVPCs (n = 6) compared to HUVECs (n = 5). *Error bars represent s.e.m. $P < 0.05$, $P < 0.001$, two-way ANOVA with Sidaks multiple comparisons test.*
C qPCR analysis of transfected HBVPCs. Knockdowns of *Nck1, Nck2,* and *Pdgfrβ*, compared to control *siRNA (siCT). n = 4 experiments. Error bars represent s.e.m.*
D Wound-closure assay on a plate coated with HBVPCs in different conditions. siRNA knockdowns against *Nck1, Nck2, Pdgfrβ* and a CT sequence, respectively. Cells were stimulated with recombinant PDGF-B or with PBS (CT). Top stripe images show wound at P0, large image after 6 h. Dashed lines mark wound migration edges at 0 h (straight lines) and 6 h. *Scale bars 150 µm.*
E Quantification of D (n = 4 exp.). *Error bars represent s.e.m. $P < 0.01$, Stud. t test.*
F Cell number in proliferation assay. HBVPCs transfected and stimulated as indicated (n = 3 experiments). *Error bars represent s.e.m. $P < 0.01$, Stud. t test.*
G Western Blots of HBVPC lysates probed with indicated antibodies.
H Quantifications of blots shown in **G**, measured values indicated in the figure (n = 3 experiments). *Error bars represent s.e.m. $P < 0.01$, Students t test.*

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3.8 NCK1/2 promote Pericyte migration and retinal revascularization in vivo

After having been able to show, that NCK1 and NCK2 are specific mediators of PC migration in vitro (compare Fig. 24), I tested whether this vital function of NCK1/2 for PC biology could be observed in vivo. As described above, NCKs are highly similar in structure and function. A complete loss of one of them could be fully rescued by the remaining partner protein. Constitutional, global KO of *Nck1* or *Nck2* in mice did not show vascular defects in previous in vivo studies (Bladt et al., 2003, Dubrac et al., 2016). Thus, complete KO mice for *Nck1* (*Nck1-/-*) were interbred with *Nck2 lox* and *Pdgfrβ-CreERT2* animals (Fig. 25 A). The *Pdgfrβ-CreERT2* recombinase is not fully specific for PCs, as the gene for *Pdgfrβ* is also expressed in SMCs (discussed above). Thus, the interbred animals were called *Nck1-/-Nck2l/liMC*, the corresponding *Cre* negative CT animals *Nck1-/-Nck2l/l*.
To figure out, whether the transgenic mice displayed a vascular phenotype during development, *Nck2* was deleted by TXF administration at days P0-P2 on top of the *Nck1* null background (*Nck1-/-*). Mice were then sacrificed at day P5 to examine the flat-mounted retinas (Fig. 25 A). Loss of NCK2 was confirmed by Western Blot. Importantly, deletion of *Nck2* in MCs did not affect the amount of PDGFRβ (Fig. 25 B). As this protocol did not allow to perform live-imaging in order to figure out whether PC migration was impaired in mutant mice, PC migration was evaluated by measuring the density of PCs at the vascular front, an area where PCs have had to migrate to. Specific identification of PCs was guaranteed by antibody staining for NG2 and Desmin. The density of the vascular front was then compared between the mutant and WT animals; and subsequently set in relation to the comparison of the density at the retinal plexus, where PCs did not have to migrate to. Strikingly, a massively decreased PC density at the vascular front in mutant mice could be observed, while the plexus showed no significant difference between mutant and WT animals (Fig. 25 C and Fig. 26 A). The explanation for the reduced number of PCs at the vascular front could be found in the impaired ability to migrate, which supported the in vitro findings (Compare Fig. 24). Interestingly, α-SMA coverage of arteries was not decreased. Thus, SMCs were not significantly affected in *Nck1/2* mutants, despite their expression of *Pdgfrβ* (Fig. 25 D and Fig. 26 A). Next, I wanted to evaluate whether PC migration also affected sprouting angiogenesis in the developing mouse retina. IB4 staining of the vasculature revealed that deleting *Nck1* and *Nck2* in MCs negatively affected sprouting angiogenesis (Fig. 26 C). Not only vascular outgrowth but also the number of sprouts, the total vascular area, and branchpoints were significantly decreased (Fig. 26 C, E). Interestingly no significant change in EC proliferation measured by ERG123 positive ECs at the vascular front could be observed, so that NCK1/2 in MCs did not affect ECs proliferation in this experiment (Fig. 26 D, F).

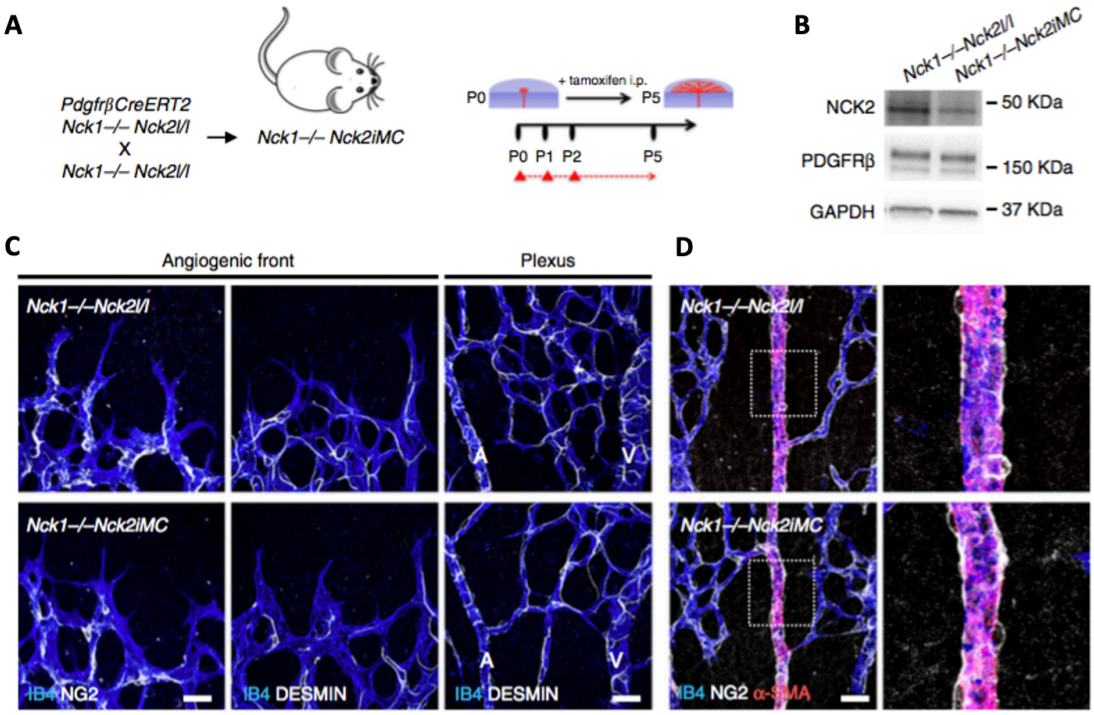

Figure 25: **NCK1/2 mediate PC migration in vivo and promote retinal vascularization. (1)**

A Left: Concept of mouse breeding for *Nck1/2*. Mice with a *Nck1* null background (*Nck1-/-*) were interbred with *Nck2 lox* mice (*Nck2l/l*) and with *Pdgfrβ-CreERT2* mice. The transgenic mouseline was then called *Nck1-/-Nck2l/liMC*; corresponding *Cre* negative CT animals *Nck1-/-Nck2l/l*. Right: Time schedule of the experiment. The red triangles indicate TXF injections at day P0-P2. Mice were sacrificed at day P5.
B Western Blot for indicated proteins using P5 total lung lysate from TXF-injected mice. Notably, deletion of *Nck2* in MCs did not affect the amount of PDGFRβ.
C Double staining for IB4 and NG2 (left), and IB4 and Desmin (center) of the angiogenic front in P5 retinas. Right: Doublestaining for IB4 and Desmin of the vascular plexus in P5 retinas. *Scale bars 30 µm.*
D (Left) Triple staining for IB4, NG2, and α-SMA of P5 retinal arteries. (Right) Higher magnification of the boxed areas. *Scale bars 30 µm.*

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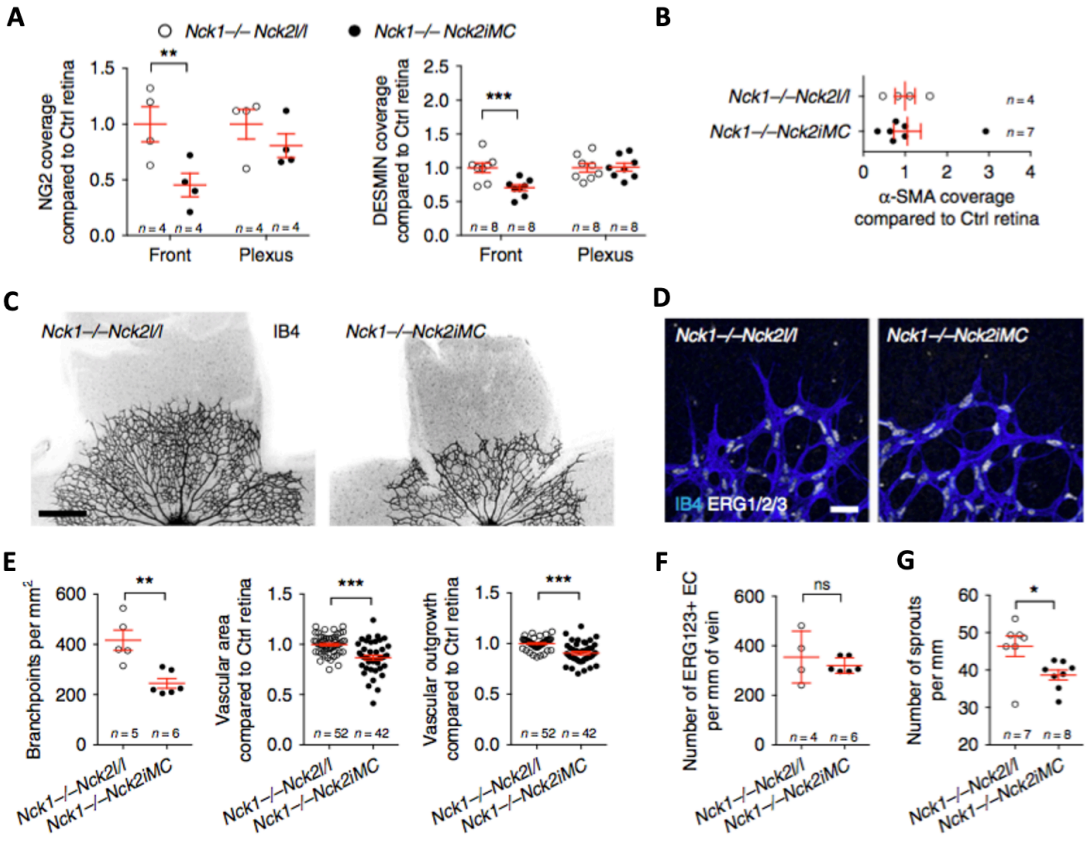

Figure 26: **NCK1/2 mediate PC migration in vivo and promote retinal vascularization. (2)**

A Quantification of NG2 (left) and Desmin (right) coverage of IB4 positive vessels of the angiogenic front and the plexus of P5 retinas to determine MC coverage. *Error bars represent s.e.m. P < 0.001, MannWhitney U test.*
B Quantification of α-SMA coverage of images in A. *Error bars represent s.e.m.*
C IB4 staining of the retinal vasculature (negative images of the fluorescent signal) of P5 retinas. *Scale bars 500 µm.*
D Double staining for IB4, ERG1/2/3 of the angiogenic front of P5 retinas. *Scale bars 30 µm..*
E Quantification of branchpoints, vascular area, and vascular outgrowth. *Error bars represent s.e.m. P < 0.01, P < 0.001, MannWhitney U test.*
F Quantification the ERG1/2/3-positive ECs. *Error bars represent s.e.m. NS: non-significant. MannWhitney U test.*
G Quantification of sprouts in the angiogenic front. *Error bars represent s.e.m. P < 0.01, MannWhitney U test.*

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3.9 Deletion of *Nck1/2* in MCs reduces pathological Neovascular Tuft formation and promotes revascularization

The next aim was to evaluate the role of NCK1 and NCK2 in PCs during ischemic retinopathies. In order to do so, I introduced the *Nck1-/-Nck2iMC* and corresponding CT mice to the OIR protocol. Gene deletion in MCs was performed by TMX injections at days P12-14 after taking the mice out of the hyperoxia chamber (Fig. 27 A). Notably, mutant mice showed a significant decrease in NVT area (Fig. 27 B, C). Furthermore, while no difference between NVT coverage of PDGFRβ positive cells could be observed, the tuft covering MCs were less activated as indicated by α-SMA coverage (Fig. 28 A). Thus, NVTs were not only decreased, but the remaining NVTs were less covered by α-SMA positive cells than in CT retinas.
The second important observation was the reduction of the avascular area in mutants (Fig. 27 B, C). This finding indicates, that the lack of NCK1/2 in MCs promotes revascularization of avascular areas. Interestingly, similar to the observations in the developmental experiments (Fig. 25 + 26), the sprouts at the vascular front in the OIR retinas were less covered by MCs in comparison to CT littermates as indicated by Desmin staining (Fig. 28 B).
Vascular leakage is another key problem in human ischemic retinopathies (discussed above). To evaluate the degree of leakage in the in vivo disease model, Fibrinogen was injected with subsequent measurement of leakage (Fig. 28 D). Strikingly, Fibrinogen leakage was less severe in mutant animals (Fig. 28 B). These data suggest that NCK1 and NCK2 in PCs are vital for pathological neovascularization in ischemic retinas. Thus, deletion of *Nck1* and *Nck2* is promising, as it resulted in fewer NVTs, less leakage, fewer α-SMA activated PC coverage of NVTs, and in improved revascularization.

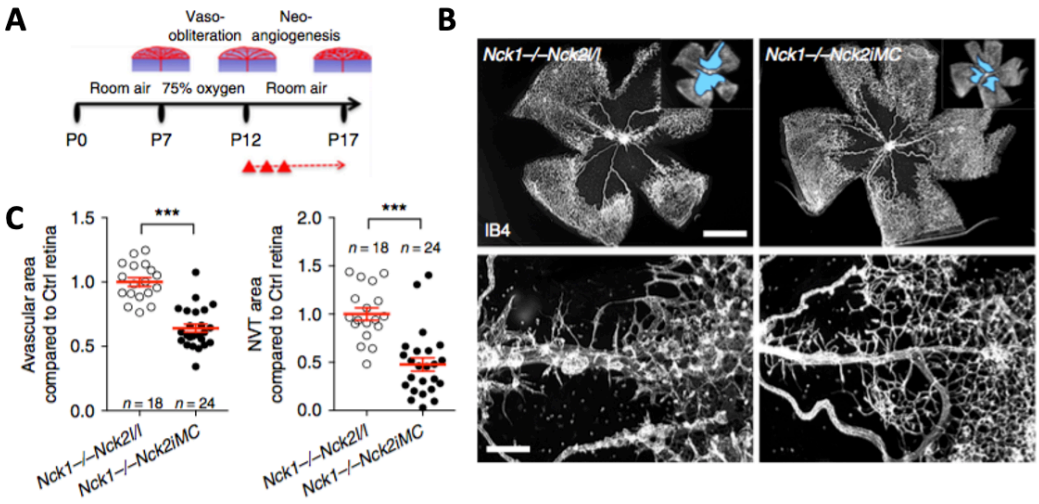

Figure 27: **Loss of NCK1/2 in Mural Cells results in less Neovascular Tuft Formations and an increase in revascularization. (1)**

A Concept of the experiment. *Nck1-/-Nck2l/liMC* and *Nck1-/-Nck2l/l* (CT) mice were subjected to the OIR protocol. Red triangles indicate TXF injections at P12-P14. Mice were sacrificed at day P17.
B Flat mounts of OIR retinas of *Nck1-/-Nck2l/liMC* mice and littermate controls. Blue area in top right corner images indicates measured avascular area. Bottom images show higher magnification of NVT areas surrounding retinal veins of OIR retinas to evaluate sprouting. *Scale bars top images 1 mm ; bottom images 100 µm.*
C Quantification of avascular area (left) and NVT area (right). *Error bars represent s.e.m. $P < 0.001$, MannWhitney U test.*

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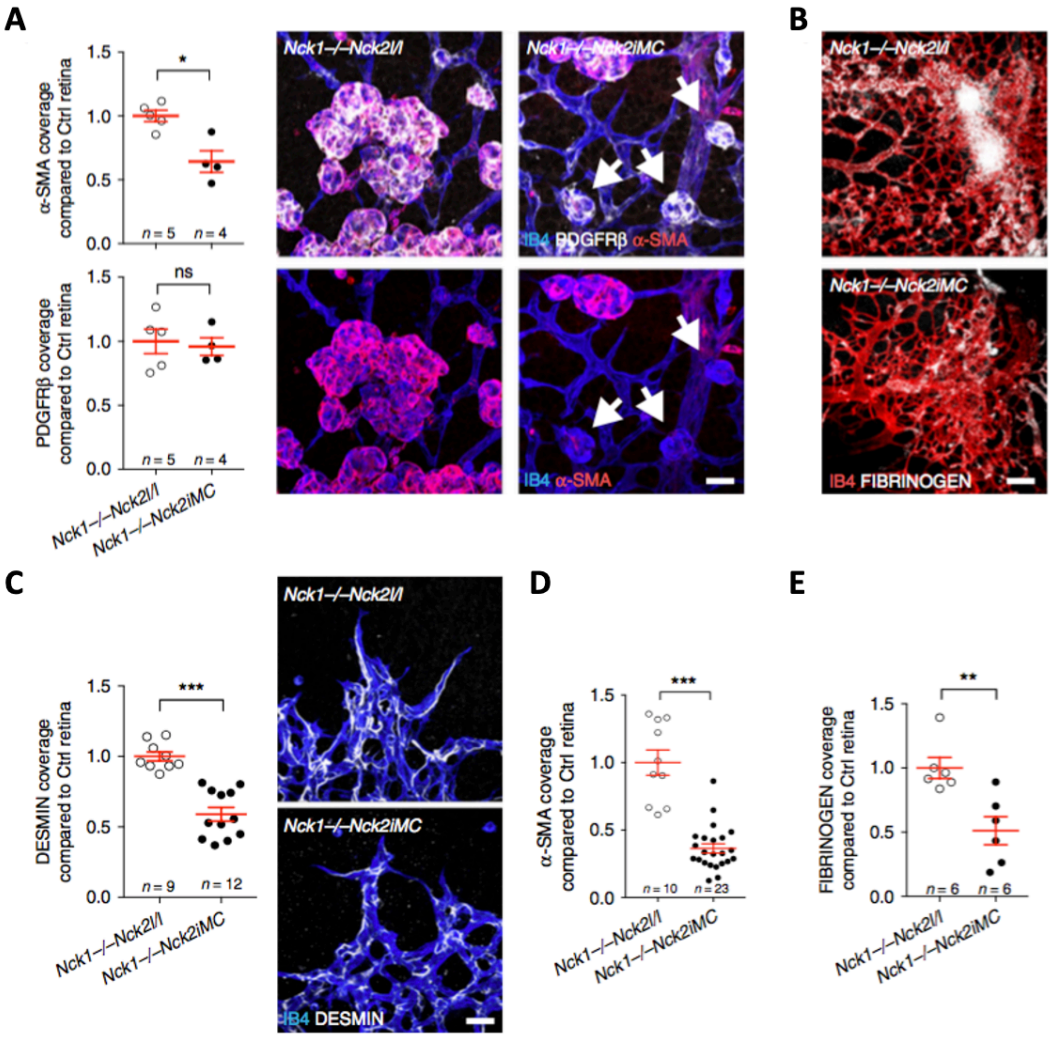

Figure 28: **Loss of NCK1/2 in Mural Cells results in less Neovascular Tuft Formations and an increase in revascularization. (2)**

A (Left) Quantification of activated MC coverage of NVT indicated by α-SMA (top) and PDGFRβ (bottom) staining in relation to IB4 positive tuft areas. *Error bars represent s.e.m. NS: non-significant. P < 0.05, MannWhitney U test.*
(Right) Triple staining for IB4, PDGFRβ, and α-SMA of the NVTs. The arrows point on tufts in *Nck1-/-Nck2l/liMC* mice. Notably, those tufts were smaller than in CT littermates. *Scale bars 30 µm.*
B (Left) Quantification of MC coverage of vascular sprouts in P17 OIR retinas (n = 9 imaging fields for control *Nck1-/-Nck2l/l* animals from 6 retinas and n = 12 for *Nck1-/-Nck2iMC* animals from 6 retinas).
(Right) IB4 and Desmin double staining of the angiogenic sprouts. *Error bars represent s.e.m. P < 0.001, MannWhitney U test. Scale bars 30 µm.*
C Quantification of α-SMA coverage of IB4 positive vasc. sprouts in P17 OIR retinas. *n = 10 imaging fields for control Nck1/Nck2l/l anim. from 2 ret. and n = 23 for Nck1-/-Nck2iMC anim. from 4 ret.). Error bars repr. s.e.m. P < 0.001, MannWhitney U test.*
D and E Images (D) and quantification (E) of Fibrinogen leakage in *Nck1/Nck2iMC* compared to CT littermate mice. *Error bars represent s.e.m. P < 0.01, MannWhitney U test. Scale bars 100 µm.*

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4 Discussion

The understanding of sprouting angiogenesis in retinal development has shifted from an EC-centered to a multi-player teamwork model, in which ECs actively interact with MCs and other perivascular cell types to build an efficient and stable blood vessel network. Disease models suggest that dysregulation of MCs is a significant driver of neovascular retinal diseases. Thus, microvascular MCs have gained increasing scientific importance due to their role in health and disease. Despite this keen interest, major aspects of MCs are only poorly understood today, and the biology of MCs is more complicated than our current understanding might suggest.

This study is about retinal PCs and their role in physiological and pathological sprouting angiogenesis of the retinal layer. Key findings are that a special subgroup of differentiated α-SMA positive PCs contributes to NVTs and to pathological sprouting in a model of ischemic retinopathies. Targeting these cells is potentially improving and normalizing retinal vascularization. Furthermore, this is the first report describing the role of the two intracellular scaffold proteins NCK1 and NCK2 in MCs. This gives the novel opportunity to specifically target one particular PC behavior - migration. Furthermore, this study provides novel information about the mechanisms of PDGF-B signaling, gives insights into PC biology, and supports the rising importance of PCs in the current understanding of sprouting angiogenesis. The study proves that vascular biologists have every right and reason to target perivascular cells in retinal disease models.
Several aspects, implications, and limitations of the present study should be subject of particular attention and are further discussed here. The central scientific idea of this study is staggered in seven sub-sections, which I will process separately.

(1) PCs underlie an organ-specific character. The focus of this study is on the retinal vasculature. Thus, the current study establishes a sharp expression marker profile of retinal MCs, which is important for the subsequent experiments and the field of vascular biology.

(2) It is already well-understood that PCs tend to change their specific gene expression marker profile during disease processes, development, in vitro culturing, and other environmental changes. Subjecting mice to the OIR protocol led to the observation of differentiated PCs which acquire the expression of the SMC marker α-SMA and contribute to pathological retinal angiogenesis. To answer the obvious question about the origin of these α-*Sma* expressing MCs, the current study establishes a novel concept for precise identification of MCs in disease conditions. Thereby the OIR model was combined with different transgenic *Cre* recombinase mouse lines. Notably, α-SMA plays a phenotype-defining role in this experiment. Even though a final mechanism of α-SMA activation is not established in the field so far, it is essential to discuss its role as an indicator for PC differentiation and activation.

(3) PDGF-B is well-described as a crucial ligand in MC biology. Much is already known about its role in MC biology. Nevertheless, this is the first study that scrutinizes the postnatally induced EC-specific deletion of *Pdgf-b* in transgenic mice. The stable phenotype with an almost complete PC loss and major vascular defects gives essential insights into MC biology. Furthermore, since current clinical studies focus on antibody treatments targeting PDGF-B signaling in retinopathy patients, I induced endothelial *Pdgf-b* deletion in disease models. Notably, the loss of PDGF-B in ECs reduced NVT formations but increased vascular leakage and bleeding. Thus, the mouse model gives important insights into the potential and the limitations of anti-PDGF-B treatments in ischemic retinopathies.

(4) In vitro experiments were used to decipher the mechanistic features behind the in vivo observations. As stated above, PCs tend to differentiate and lose their unique identity when cultured in vitro. The current study replaces existing culturing strategies with a novel protocol that phenocopies in vivo marker expression profiles to a much higher degree than established approaches.

(5) In vitro observations also led to the finding, that targeting specifically PC migration can be achieved by blocking the intracellular adaptor proteins NCK1/2. This observation could be confirmed in vivo by interbreeding different transgenic mice, thereby creating a new mouse line which allows deleting Nck1 globally and Nck2 specifically in MCs. The decrease of MC coverage at the retinal vascular front, but not at the plexus, is consistent with the in vitro findings of impaired PC migration. Thus, this is the first work describing particular and specific functions of NCK1/2 in PCs in vitro and in vivo.

(6) As a critical experiment for potential translational therapies, the mouse line which allows the inducible PC-specific migration block has been exposed to the OIR disease model. Observations indicate that targeting specifically PC migration is a promising approach for anti-angiogenic therapies in ischemic retinopathies.

(7) NCK1 and 2 in PCs as a potential target in retinopathies - where does the journey lead? My research aims to improve therapies of today for a better treatment of ischemic retinopathies and AMD of tomorrow. However, as *Nck12* are expressed in several cell types, and can be detected only on the inside of cells, it is particularly challenging to develop a specific and powerful medication or treatment option. Here, I will discuss challenges, limitations, as well as opportunities of the current findings.

4.1 Characterization of retinal Mural Cells in the developing retina - a continuum of Pericytes and Smooth Muscle Cells

In the current study, PCs that cover the NVT areas in pathological conditions, acquire α-SMA expression at some point during the disease process. This finding makes Zimmermanns definition from 1923 (all transitional Mural Cell phenotypes expressing smooth muscle fibers are to be included) quite accurate, and is consistent with other studies demonstrating a sensitivity of MCs when it comes to microenvironmental changes. Thus, PC identities do not only underlie organ- and vessel-specificity, but also plasticity when it comes to developmental stages, disease and in vitro culturing. This heterogenity and plasticity still causes confusion. PCs of the retina, for example, are very similar to SMCs concerning several important features and expression markers, and scientists even proposed a continuum with PCs on the one and SMCs on the the other end (discussed above). Thus, it has been crucial for this study to provide a sharp expression profile to identify PCs and distinguish them from SMCs. This organ-specific profile is the basis for observations of subsequent experiments.

However, the urgency to establish this expression profile underlines two important characteristics. First, as many studies in the field state, there is still no specific molecular marker known that can be used to unequivocally identify PCs and distinguish them from SMCs and other mesenchymal cells. The lack of a marker forms an obstacle in the field and remains a restricting challenge in PC research. Novel discoveries obtained by single-cell sequencing are just gaining ground, and there is legitimate reason that stable and specific markers or even a potential multi-genetic expression profile will set new standards for a sharp cell discrimination soon. Second, the lack of a specific PC marker and consequently of a transgenic mouse line for specifically targeting genes in PCs limits in vivo approaches. However, I used KO animals for *Nck1* and *Nck2*. Thus, cell specific deletion is indicated by iMC instead of iPC, since *Nck2* deletion appeared in all cells expressing the corresponding *Pdgfrβ*. These explicitly include SMCs and other cell types, potentially not even of the retina (since genes are deleted globally). However, recent studies from Nikolakopoulou et al. (2019) and others demonstrate, that *Pdgfrβ* Cre recombinase is predominantly deleting in PCs and to a much lower degree in SMCs, and other cells. Regardless of this lack of a specific PC marker and systemic deletions, the results are promising and phenotypes strong. Thus, the overarching idea of this study was not limited.

Are PCs and SMCs completely different cell types or from the same progeny only in different conditions? The tendency of PCs to differentiate at least partially to a SMC-like profile, and potentially vice versa will be on the scientific agenda for longer. PC and SMC heterogeneity and plasticity regarding protein expression, function and morphology remain a restricting challenge in MC research. Full genome analyses have only lately suggested a heterogeneity regarding protein expression of MCs and opened unprecedented opportunities to define PCs and SMCs. Possibly, it is time to adjust the definition of MC subgroups to a more precise description. Faced with latest

observations, scientists in the field cannot evade the question as to whether it is time to get past the immemorial definitions of PCs and SMCs and to adopt a more flexible nomenclature. This could involve more cell identification features, a bigger number of genes, and would potentially reveal numerous subgroups of MCs throughout the human body or in one single organ or even vessel, described by Vanlandewijck et al. (2018a) and others. In fact, current data gained by massive parallel sequencing suggest a continuum of cell phenotypes with SMCs at the one and PCs at the other end of the spectrum. Further efforts should be made to answer whether the field of vascular biology is still looking at two, one or even more distinct cell subtypes (Vanlandewijck et al., 2018a).

4.2 Finding the suspicious cell-type and the crucial role of α-SMA

One of the key observations of the current study is the finding of an intense coverage of NVTs by cells, that express MC genes. These genes include *Pdgfrβ, NG2, Desmin*, and the SMC marker *α-Sma*. A major surprise is the observation that the mentioned cells do not express *Myh11*, the second important SMC marker of this study. Again, the lack of a specific marker for an unambiguous PC identification combined with the described MC plasticity and tendency to differentiate is a challenge at this point (discussed above). The pivotal question about the provenance of the NVT covering MCs is of particular importance, since one of the aims of the current study is to find novel targets in ischemic retinopathies.
The suspicious cells express a strong MC marker profile and cover the NVTs from the outer side. Thus, it is likely, that they arise from PCs or SMCs. However, the possibility that a completely different cell type differentiates and adopts a MC-like marker expression profile is still feasible. A sophisticated protocol had to be established to ensure that PCs, and not SMCs or another cell types, start to express *α-Sma* and cover the NVTs. Thereby, four different transgenic mouse lines were subjected to the OIR protocol to test whether the NVT covering cells were originally expressing *Pdgfrβ, Cdh5*, and the SMC markers *α-Sma* and *Myh11*. Even though this is the first study to clearly identify PCs as NVT covering cells, several important questions are still subject of discussion and not fully answered, yet.

i. What is the role of α-SMA in PCs in ischemic retinas and how is it regulated?
α-SMA has a phenotype-defining role in this study, as it is one of the specific SMC markers, that distinguish SMCs from PCs. However, this role was challenged at different time points and the clear demarcation between PCs and SMCs was more and more softened in the OIR model and in vitro. Thus, α-SMA overtook a role as indicator for PC differentiation and disease, e.g. the novel established in vitro culturing protocol consulted expression levels of *α-SMA* to evaluate PC differentiation.
α-Sma is expressed in SMCs, myofibroblasts, the myoepithelium and hepatic perisinusoidal cells without notable expression levels in PCs of the CNS (Armulik et al., 2005, Vanlandewijck et al., 2018a). However, this work is by far not the first one that describes *α-Sma* expressing PCs of the retina or brain under certain disease conditions. In fact, studies linked *α-Sma* expression of PCs to the ability to contract and regulate blood flow (Alarcon-Martinez et al., 2018), others described it as a differentiation marker, indicator for inflammation, fibrosis and tumor growth (Nehls & Drenckhahn, 1993, Nisancioglu et al., 2010, Bergers & Song, 2005, Sennino et al., 2007). In the current study, when WT mice are subjected to the OIR protocol, *α-Sma* expressing PCs strongly cover NVTs and vascular sprouts. Similar observations could be obtained in PC in vitro culturing experiments. Thus, the obtained observations are in accordance with the literature, when claiming that α-SMA is an indicator for pathological conditions.
Notably, when *Pdgf-b* in ECs and *Nck1/2* in MCs are deleted, mice undergoing the OIR protocol show less tufts and less pathological sprouting. The relative number of NVTs covered by PCs that express *α-Sma* is also significantly decreased. A very sim-

ilar observation could be obtained for the pathological sprouts. This is an important result, since it raises three important hypothesizes, that are not fully answered at this point.

First, is *α-Sma* expression mechanistically regulated by PDGF-B or NCK1/2? Song proposed in 2005, that TGFβ, which is involved in SMC maturation, is a key driver of the expression of *α-Sma* in tumor PCs. Much less is known about the mechanism behind *α-Sma* expression in PCs of the CNS and the retina; especially as a responce to PDGF-B exposure. This study could not reveal an exact regulation process or signal cascade that drives *α-Sma* expression, but it is one of the first reports describing the influence of endothelial PDGF-B on α-SMA in MCs, and the very first one doing so for NCK1/2. Despite of this novelty, it is not clear at all, how and why PCs start to expresss *α-Sma*. Is it a response to the upregulation of PDGFB, e.g. a dose response? And if so, is there a critical threshold above which PCs become α-SMA positive? Which other framework conditions have to be given that this differentiation takes place? It might also be interesting to elucidate whether all PCs are sensitive to elevated levels of PDGF-B or whether this phenomenon is potentially limited to PCs of the CNS, to PCs of a specific vessel type (e.g. vein PCs), or to another subgroup of MCs. Putting current findings into context with previous observations of the developing and mature CNS vasculature suggests, that especially developing PCs are potentially vulnerable for microenvironmental changes. Massive parallel sequencing experiments could decipher a genomic signature of "young" PCs in contrast to mature PCs, or even a gradual development; and the mechanisms behind (Stapor et al., 2014, Harrell et al., 2018). Further research is needed to answer these questions and to determine whether PDGF-B and NCK1/2 are mediating *α-Sma* expression by themselves, or whether they interact with TGFβ signaling by crosstalk. A detailed understanding of the mechanisms could reveal potential therapeutic targets.

Second, the appearance of NVTs and pathological sprouts developed proportionally to the amount of coverage by *α-Sma* expressing PCs. This raises the pivotal question, whether α-SMA in PCs is only an indicator of pathological conditions or whether there is a causative relation between α-SMA in PCs and pathological sprouting. It is important to mention, that the study cannot show such a causative relation between α-SMA in PCs and pathological sprouting, but the proportional observation was stable through all flat-mounted, diseased mouse retinas. Since the overarching goal of biomedical science is to deliver clinical implications and potential targets, this question has to be cleared in the near future. Finding a causative relation would also facilitate the search for a potential underlying mechanism of α-SMA in PCs and sprouting angiogenesis. I hypothesize, that aberrant positioning around growing angiogenic Tip ECs, and modification of PCs contractile properties affecting Tip cell extention may play a role.
In this context it could also be valuable to think about the very few remaining α-SMA positive MCs on the NVTs of mice which bear an *Nck1/2* deletion in MCs. Do the remaining MCs still express *Nck1/2* due to a lack of deletion? Or is NCK1/2 not a key regulator of *α-Sma* expression? Again, novel methods like massive parallel sequencing

could shed light on these remaining cells in ongoing experiments.

Third, the in vitro data of the current study suggest, that PC activation is a reversible process. PCs, that were previously in an activated state (e.g. by keeping them in inappropriate in vitro conditions), can lose their expression of *α-Sma* and can come back to their previous identity when treated with the novel protocol. It is questionable whether this is possible in vivo. In fact, by deleting *Nck1/2* after taking the mice out of the hyperoxia chamber during the OIR model, less tufts show coverage of α-SMA positive MCs. This could be explained by less cells becoming α-SMA positive or by more cells coming back to their original identity. It might be promising to stop the OIR experiment at different timepoints, and to have a closer look on the cells that are capable of expressing *α-Sma* and losing the expression again. Are all MCs able to do so? If yes, how? If no, what are the (genomic) crucial prerequisites determining the MC fate in development and disease.

ii. What happens during retinal ischemia behind the curtain of technical limitations?

This study provides details about MCs during pathological sprouting. However, it is important to mention, that this elaborate protocol has certain limitations due to only two time points of observation at day P8 and P17, and to limited observed expression markers. While the flat-mounted retinas at P8 are important for the proof of the successful gene deletion, the main findings are obtained at a single time point at P17. Furthermore, the protocol only covers five expression markers and it is quite feasible that another, not considered cell type contributes to the NVT formations and pathological sprouts.

Thus, knowledge about the exact formation processes of the NVTs, as well as information about the timing of *α-SMA* expression, and of other genes remain elusive. It is not clear, whether PCs migrate to the NVTs over longer distances or whether they extensively cover NVTs by proliferation. It is difficult to conclude, whether all PCs of the retina are evenly attracted to the NVTs or whether it is only a certain subgroup of PCs that is involved in NVT formations.

Thus, it remains unclear, whether *α-Sma* expressing PCs all go back to one cell for every tuft, similar to a tumor, or whether they migrate from different locations to the tufts. In fact, proliferation markers are strongly expressed in MCs of NVTs. On the other hand, the current study shows, that by specifically targeting PC migration NVT area is decreased which would support the hypothesis, that the MCs on NVT migrate from local vessels to the NVT. A process of both, proliferation and migration, is also feasible.

Visualizing PC migration and proliferation in vivo in real-time would be an important experiment in this context. However, live imaging of the retina is difficult and does not allow confocal microscopy, nor double or even triple staining for different markers. Thus, to achieve a better and precise understanding of the processes during ischemic retinopathies, retinas should additionally be observed at more timepoints (as discussed above).

iii. What is the actual role of PCs during the formation of NVTs: pathological

contribution, preventing character or collateral damage?
Since an understanding of ECs as the only important cell type during pathological blood vessel sprouting was abandoned, other cell types were moved to the center of interest. PCs have long been proposed to play a crucial role during pathological retinal sprouting (Armulik et al., 2011). However, experts in the field do not fully agree on the detailled role PCs play during pathological sprouting of the retina. The current study demonstrates that deletion of endothelial *Pdgf-b* which massively reduces the number of PCs partially improves retinal vascularization, but also bears the disadvantage of impaired revascularization and bleeding.
In fact, PC detachment, death, BM thickening and subsequent capillary leakage and edema are well-described in diabetic retinopathy models in mice Armulik et al. (2011). Even PC ghosts have been observed. This is to a certain degree contradictory to the findings of the current study, since antibody staining for established MC markers reveals a broad and intense coverage of NVTs by activated PCs, and not a loss of PCs. This suggests, that PC migration and proliferation plays a central role in disease development, instead of apoptosis or death (Bresnick et al., 1977, Kern et al., 2000, Barber et al., 2011).
However, other studies describe, that during the second phase of proliferation, a strong up-regulation of growth factors and a subsequent pathological sprouting appears. This also includes PCs which can be targeted by anti-PDGF-B therapies (Ophthotech Company, 2016). Observations from the last decades revealed that PDGF-B signaling through its corresponding receptor, as well as EC cell ablation or reduced levels of PDGF-B can potentially recapitulate the PC loss and aneurysms and some of the vascular changes that characterize retinopathies. This is in partial accordance with the observations of the current study (Enge, 2002, Hammes et al., 2002).
Another key attribute of DR is the loss of barrier functions of the vasculature and a disturbed BRB integrity which precedes vascular proliferation (Ockrim & Yorston, 2010). The current study could confirm this finding by the injection and detection of Fibrinogen in the retinal tissue.
However, many critical aspects of vascular defects and the participation of PCs on those remain unclear and cannot be solved with the current study. The question about the detailed role of PCs in ischemic retinopathies is of high relevance for the field, since even current clinical studies try to improve the life and vision of patients suffering from AMD with anti-PDGF-B therapies (Ophthotech Company, 2016).
Although, *α-Sma* expression and its potential role in PCs has been discussed in more detail above, it is quite important to think about α-SMA activation in PCs as an exacerbator of the disease, or in contrast, as a self-regulatory healing process. Even by identifying the detailed mechanism which underlies the activation of α-SMA it has to be elucidated whether *α-Sma* expression of PCs is a desirable condition or whether it should be inhibited to improve the pathological situation. The observations of this study strongly suggests that *α-Sma* expression correlates with retinal bleeding, more tufts and less revascularization. All of these three conditions have to be considered as negative conditions. Nevertheless, one should be careful to not run into danger to derive causality from the given correlation of this experiment.

Two different scenarios are also possible. First, as proposed above, *α-Sma* expression could occur in a way of self-healing process in diseased retinas. This would explain why more diseased retinas show stronger α-SMA coverage in tufts. Second, the high levels of α-SMA could underlie a confounding which means that α-SMA itself would probably not have a function in the disease, but could somehow come up as a by-product. This could also be true for the experiments of research groups worldwide in which *α-Sma* expression was strongly increased in PCs of pathological conditions. To address these questions and exclude the two possible scenarios it would be interesting to overexpress *α-Sma* in healthy, and to block *α-Sma* expression in diseased animals.

4.3 Endothelial *Pdgf-b* deletion results in Mural Cell free, dilated vessels during development and in less NVTs, but more bleeding in the OIR model

Much is already known about PDGF-B signaling and its importance for vascular biology. In fact, this study does not provide totally unexpected results in the developmental experiments using genetically targeted animals for *Pdgf-b* in ECs. It is already known, that the global KO of the ligand or the receptor is not viable and results in major defects of mural cells with dilated capillaries and subsequent haemorrhaging and edema. Mice with a heterozygote KO of the ligand are viable, but display vascular defects due to a reduction of MCs (discussed above). Furthermore, a constitutive endothelial-specific deletion of *PDGF-B* generates viable mice with decreased densities of PCs and microvascular abnormalities (Enge, 2002). Umuera was one of the first to show, that even postnatally induced blockage of the signal cascade by using an antibody against PDGFRβ results in MC-depleted vessels (Uemura et al., 2002). The current study is in accordance with all of the above-mentioned, and combines the EC-specifity of Enges experiments, with the inducible protocol of Uemura by utilizing a transgenic mouse line *Pdgf-biEC* that deletes *Pdgf-b* by TXF injection in ECs. Thus, this is the first study to reveal, that the postnatal endothelial-specific deletion of *Pdgf-b* leads to a massive loss of PCs and major vascular abnormalities (Fig. 20+21). This experiment was also crucial to justify the strategy of targeting PCs in the OIR protocol (Fig. 22+23).

Interestingly, while PCs were depleted to an almost complete degree, SMCs were only partially gone. Despite the fact, that SMCs are not in the center of the current study, it gives an important insight into the importance of PDGF-B for SMCs during development and disease, and whether *α-Sma* expression potentially downregulates the sensitivity for PDGF-B signaling. Another explanation would be, that SMC is a priori a different cell type, that is not sensitive to a (partial) loss of PDGF-B. If so, it would be important to elucidate key signal cascades of SMCs in the developing vasculature. Again, massive parallel sequencing could give important insights into genomic features and signatures of SMCs in comparison to other MCs.

Furthermore, it is well-understood in the field, that ECs are the main source of PDGF-B during retinal and CNS development. This understanding was just recently confirmed by massive parallel sequencing experiments. However, in the developmental experiment of the current study, PDGF-B was strongly decreased, but could still be detected. In the OIR model, injected *Pdgf-biEC* mice only showed a decrease of about 60 % of *Pdgf-b* expression compared to WT animals. These results indicate the potential presence of other sources of *Pdgf-b* during sprouting angiogenesis in development and disease. Although, there are more cell types described that potentially express *Pdgf-b*, such as Müller cells, Angioblasts, Mesangial Cells, photoreceptors and others, none of them has been observed to function during retinal sprouting, yet. Thus, it remains questionable where the remaining PDGF-B comes from. It is also not excluded, that gene deletion did not fully work due to technical limitations. However, observations of the study do not show evidence for this, since problems with gene deletion could not be observed in any other experiment. In vice versa, during developmental

experiments, the observed PC loss was almost complete. This confirms, that PDGF-B is the major player driving PC recruitment in this tissue.

Another interesting observation of the *Pdgf-biEC* mice in the OIR experiments is the massive leakage of Fibrinogen around α-SMA positive NVTs. PCs are described to contribute to a functional BRB and retinal vascular homeostasis. Similar to the developmental experiments, the *Pdgf-biEC* mice in the OIR experiment displayed a massive loss of PC coverage and vascular defects. These included miroaneurysms, impaired revascularization, and hemorrhage (compare Fig. 20+21). Interestingly, NVTs that were covered by α-SMA positive, activated PCs massively leaked Fibrinogen. This observation is a strong indicator, that activated PCs impair the BRB, and potentially the BBB, formation. A similar observation could be made in two recent study, which show, that the loss of *Foxc1* and *Foxc2* transcription factors increased brain PC density, but resulted in BBB defects (Siegenthaler et al., 2013). Thus, the current studies supports recent findings, that PCs play a crucial role in the BBB formation. As discussed above, an abnormal increase in vascular permeability is associated with sepsis, lung edema, allergic reactions and other pathologic conditions (Wang & Dudek, 2009, Armulik et al., 2011). Much less is known about PCs in these diseases. Thus, it is high time to draw attention to PCs in these conditions. Furthermore, in vitro studies indicate that PCs regulate the BBB at the level of endothelial junctions (discussed above). Considering the strong endothelial phenotype in PC depleted retinas of this study - inter alia delayed sprouting, enlarged vessels, less sprouts - justifies to have a closer look on PC-EC and the BBB/BRB in PC-affected animals of future experiments. Another ultimate goal of this line of thought would be the discovery of a key regulator of the BBB: a signal cascade or target cell type, that allows to spatially open and close the BBB/BRB for drug administration, local treatment, and control of leaking substances. The current work underlines, that there is no way around PCs concerning this aim.

Furthermore, the observation of less NVTs, less pathological sprouts and less revascularization of avascular areas underlines the anti-sprouting character of the mutant mice and the importance of PCs for sprouting angiogenesis (compare Fig. 22+23). However, the study does not reveal a mechanism of how PCs interact with ECs to promote sprouting. As discussed above, the anatomical relationship between PCs and ECs suggests a close interactions. These include paracrine und juxtracrine signaling (discussed above). However, my current work supports the understanding in the field, that sprouting is not a single player process of ECs, but has a teamplay character with more than one cell type involved.

4.4 In vitro culturing of Pericytes: the application of a new protocol

To find an accurate protocol of culturing PCs in vitro, the current study compares several PC culturing protocols and evaluates them by measuring different gene expression parameters to evaluate the state of PC differentiation. The observed genes are selected from the genes that could be determined in the in vivo experiments (Fig. 10+11). Notably, PCs were not purified by me, but obtained from *ScienCell TM*. One of the key challenges in PC in vitro experiments is their tendency to differentiate. While some researchers observe PCs which were more or less stable in their expression profiles for many passages (Capetandes & Gerritsen, 1990, Gitlin & D'Amore, 1983, Vanlandewijck et al., 2018a, Helmbold et al., 2001, Tigges et al., 2012), more recent studies describe culturing experiments in which PCs rapidly differentiate to other cell types (Armulik et al., 2005, Dellavalle et al., 2007, Sundberg et al., 2002) Thus, a strong focus of the current study is placed on PC differentiation, especially against the background of the new understanding of PC plasticity and a potential PC-SMC differentiation continuum.

In the past, the majority of PC in vitro experiments has been performed with DMEM (*Dulbeccos Modified Eagle Medium*) and high levels of fetal bovine serum (FBS). The present study tests this mixture to evaluate whether it is an adequate solution to culture PCs. Interestingly, all samples of PCs which were kept in DMEM expressed high levels of *α-Sma* (compare Fig. 13). This indicates a PC activation and differentiation to a SMC-like identity, and fits very well with the literature. Tigges et al. (2012) obtained similar results and identified DMEM as driver of PC differentiation by observing an increase of *α-Sma*, a decrease in growth rate, and cell enlargement of PCs in this medium. Unfortunately, the majority of PC experiments and in vitro discoveries have been obtained using DMEM. I strongly recommend to have the aspect of PC activation and differentiation in mind when working with PCs in vitro.

However, it remains questionable, whether PCs cultured in DMEM differentiate to full identity SMCs and whether DMEM is consequently a PC-to-SMC-converter. More studies and novel strategies should be performed to test this hypothesis.

Tigges was also one of the first to describe a novel technique proposing the medium ECGM (*Endothelial Cell Growth Medium*) as an adequate medium for culturing PCs and other mesenchymal cells. The current study also tests these media which were originally developed for ECs. Findings of the study suggest, that utilizing those is promising and superior to existing protocols. In particular, the levels of the SMC expression markers *α-Sma* and *Myh11* were low while unspecific MC markers were strongly expressed.

However, it is highly questionable, whether the established protocol is the last word on this subject. In fact, to evaluate and to compare the novel protocol, only very few gene expression patterns are utilized as parameters. There are much more features to a cell type than only expression markers: Cell shape, ability to migrate, contractility, proliferation, and many more. A better and more precise understanding of MCs - partially obtained by massive parallel sequencing - could help to find better protocols for "species-appropriate" culturing.

Having those limitations in mind, it is still unclear, whether the novel protocol is an almost perfect way to culture PCs in vitro. One argument for this is the fact that the majority of my in vitro results could be confirmed in vivo. This suggests, that the newly developed in vitro conditions are adequate enough to perform reliable experiments and to take important conclusions from them. The novel and currently best protocol is described in detail above, and I recommend to use the protocol to work with PCs in vitro, until we know more about this cell type.

4.5 NCK1/2 as specific mediators of Pericyte migration in vitro and in vivo

Another key novelty of the present study is the observation that the intracellular scaffold proteins NCK1 and NCK2 mediate specifically Pericyte migration induced by PDGF-B in vitro and in vivo. It is the first study to reveal the expression, the function and specific role in signaling cascades of NCK1/2 in PCs.
The idea to choose NCK1 and NCK2 as potential targets arose from the hypothesis whether it is possible to target one specific PC behavior, since the total block of endothelial PDGF-B resulted in major vascular defects limiting its use in ischemic retinopathies (Compare Fig. 20+21). The indication of testing NCK as specific mediator arose from previous studies revealing it as downstream mediator of PDGFRβ in fibroblasts which also express *Pdgfr* subtypes. The current data show a strong expression of *Nck1/2* in PCs, and could recently be confirmed by single-cell sequencing of brain tissue (Vanlandewijck et al., 2018a).
Transgenic mice are used to perform the in vivo experiments. *Nck1* KO animals (*Nck1-/-*) do not show a phenotype concerning the retinal vasculature which is most probably explained by a rescue of NCK2 which is very similar to NCK1 concerning structure and functions. Thus, *Nck1-/-* mice were crossed with *Nck2l/l* mice and with *Pdgfrβ-CreERT2* mice (discussed above). Notably, this recombinase deletes not only in PCs, but also in SMCs and other cells expressing the *Pdgfrβ-CreERT2*.
The potential deletion in SMCs rises two concerns, that are not addressed in the current study. First, it can not be excluded, that the deletion in SMCs contributes to the observed phenotype of the retinal vasculature. Second, it would be interesting to know, whether SMCs are also affected in their ability to migrate. The fact, that SMCs stick closely to arteries around the vascular plexus and cannot be observed at the sprouting front during physiological retinal vascularization limits the retina model to evaluate this question. The development of live imaging techniques could be an important tool at this point.
Interestingly, observations in mice at postnatal day P12 reveal that PCs can catch up and cover the complete retinal vasculature, even at the vascular front. Several explanations have to be considered to explain this. First, it is not clear, whether PC migration is blocked completely or only strongly delayed. Furthermore, an incomplete deletion and a migration advantage of not deleted PCs in the migration competition should be considered. However, this possibility can be excluded by intercrossing the *Nck1-/-Nck2l/liMC* with *mTmG* in order to genetically label deleted cells (not shown and still in progress). Another explanation is the possiblity that PCs move forward step by step being dragged by migrating ECs.
Experiments performed by A. Dubrac reveal that a lack of NCK1/2 in human and mouse PCs decreased PDGFRβ Y1009 phosphorylation, whereas the deletion has only much smaller effects on other phosphosites such as Y751. Similar observations have been made by A. Dubrac in another study about the role of NCK1/2 for VEGF-R2 signaling in ECs and the influence of this on EC migration. These observations suggest the existence of signaling mediators regulated by NCK1/2 that affect site-specific phosphorylation of the PDGFRβ. One candidate that should be considered is the ty-

rosine phosphatase SHP-2 that binds to the phosphosite PDGFRβ Y1009. SHP-2 is proposed to mediate cell migration (Noël et al., 2014). Not much is known about those mediators in PCs and efforts should be made to further investigate the role of Y1009 in vivo.

Since the current study is the first to show the expression of *Nck1/2* in PCs and there are more signaling pathways involved in PC migration than the one investigated in this study, more efforts have to be made to further identify the function of NCK1/2 in PCs. A crosstalk with other receptors and cascades should be considered. Furthermore, it cannot be ruled out, that NCK1/2 is involved in a bunch of other cell behaviors and not only PC migration. Thus, the present study should be understood as the kickoff to more studies about NCK1/2 in PCs.

4.6 Blocking Pericyte migration by blocking NCK1/2 is promising in ischemic retinopathies

Exposing *Nck1-/-Nck2iMC* mice to the OIR model results in the reduction of NVT formations, less activated PCs, but improved revascularization in comparison to CT animals. Notably, all consulted parameters to evaluate the health of ischemic retinas are improved in MT animals. Thus, this is a major new finding as it identifies a novel and never before described target in the treatment of ischemic retinopathies. However, several aspects of the observations should be discussed in this context.

First, NVT formations and pathological sprouting are processes, that are formed by ECs. Gene deletion is performed in MCs, but the phenotype of less sprouting is predominantly endothelial. How do PCs interact with ECs to determine how and when to migrate, proliferate and sprout? At this point, it is unclear whether this is happening through changes in the PC-EC paracrine communication, due to mechanistic push or pull, or whether it is due to structural changes of the vessel wall and at the vascular sprouts. It might be promising to have a closer look an ECs to test whether and to what degree their polarization and sprouting is impaired in MT and CT animals (discussed above).

Answering this question could also help to understand the contradictory character of NCK1/2 in terms of sprouting angiogenesis: In fact, deleted animals display less NVTs and less pathological sprouts, but an increased revascularization. Thus, deletion of *Nck1/2* in PCs leads to less sprouting on the one, and more sprouting on the other hand. This study does not provide an explanation to this.

Most importantly, the observations suggest that inhibiting PC migration by blocking NCK1/2 holds promise as a potential novel strategy to treat patients with retinopathies, and selective inhibitors were recently developed by Borroto et al. After the failure of anti-PDGF-B therapies in clinical trials, the next step should be to consider targeting NCK1/2. Interestingly, genetic deletion of *Nck1/2* in ECs led to an improved retinal vascularization in ischemic retinopathies (Dubrac et al., 2016). Thus, targetting NCK1/2 in ECs and PCs in a combinatorial manner should be considered.

4.7 NCK in ischemic retinopathies and AMD - quo vadis?

The key finding - blocking NCK12-mediated PC migration improves retinal revascularization and blocks pathological hypersprouting - is of particular importance for potential clinical interventions. However, targeting NCK12 is challenging, since the two proteins are intracellular adaptor proteins. Thus, a potential candidate molecule would need to meet the requirement of being able to cross the cell membrane. Small molecules of *800 g/mol* and smaller are a class of substances that comes to mind in terms of intracellular targeting of proteins (compare Bevacicumab *149,196.82 g/mol*). However, caution should be exercised, since a small percentage of small molecule drugs are capable of crossing the BBB, potentially causing neuronal damage and other side-effects (Pardridge, 2005), especially when administered in CNS tissues. The second challenge of targeting NCK12 in PCs is the fact, that the two *Nck* proteins are expressed in cells from a broad range of background in the CNS, including all major vascular cells, Fibroblasts, Oligodendrocytes, and to a much lower level Astrocytes and Microglia (compare the database from Vanlandewijck et al. (2018a), available online under http://betsholtzlab.org/VascularSingleCells/database.html, last visit: 14.02.2020). In fact, important roles of NCK are already discovered in some of these cells (Ngoenkam et al., 2014, Guan et al., 2019). Finding a small molecule that is capable of affecting only one particular cell type is - to my knowledge - extraordinarily difficult. However, it is questionable whether such a celltype-specific targeting is necessary. In fact, a similar study to mine examined the role of NCK12 in ECs. Interestingly, in ECs the two proteins are crucial for front-rear polarity, and genetic deletion of *Nck1* and *Nck2* in ECs results in an improvement concerning retinal vascularization in the OIR model (Dubrac et al., 2016), similar to the observations in this study when deleting in PCs. This is promising, since it might be sufficient to target NCK globally and not PC-specific. However, two important approaches have to be developed in order to target NCK12 globally. First, the roles of NCK12 in the remaining *Nck*-expressing retinal cells have to be elucidated, with a particular emphasis on disease models (e.g. OIR). Second, a global KO model has to be developed in order to elucidate the potential improvements, but also risks of a global targeting of NCK12.
Furthermore, the field of ophthalmology is on the cusp of utilizing novel treatment options. MacLaren & Pearson (2007) and others are trying to improve ophthalmological therapies by administering stem cells. First cells have already been administered and tested on safety (Edwards et al., 2018). Furthermore, gene therapies are finding their way from the lab to clinic, also in ophthalmology (Xue et al., 2018). Thus, the current understanding of the biologic function of single genes can be very important for genetic interventions in stem cells and gene therapies. Developing a drug, that targets specifically NCK, is not the only option to bring the current observations to clinic.

5 Summary

Today, anti-angiogenesis therapy for neovascular retinal diseases is limited to a small number of drugs that target VEGF-A, an important player of EC biology. Due to a changed understanding of retinal angiogenesis, MCs are increasingly of interest in ophthalmology. However, the role of MCs and especially PCs in ischemic retinopathies is only poorly understood.

My current work provides important tools, how to distinguish PCs from SMCs in the retina by establishing sharp marker profiles. These were subsequently used in an elaborate fatemapping experiment to show as a key finding, that a specialized subgroup of activated, α-SMA positive PCs cover NVT formations in diseased retinas. Furthermore, transgenic mouse lines were used to target specifically the postnatal expression of *Pdgf-b* in ECs. It is the first study showing, that this late deletion is sufficient to yield PC-free, "naked" vessels, that are extensively enlarged and unstable.

To answer the superordinate research question whether it is promising to target PCs in ischemic retina models, I subjected the transgenic mice to the OIR protocol and deleted *Pdgf-b* during the time, when hypersprouting occurs. Surprisingly, PCs were not gone in these retina, but NVT area and pathological sprouts were strongly decreased. Nevertheless, the retinas displayed large avascular areas and massive bleeding. The conclusion of this experiment was, that it is worth to target PCs, but the complete deletion of *Pdgf-b* is destabilizing retinal vasculature to a high degree.

In order to find novel targets that affect MCs in a more subtle way, I switched to in vitro experiments. Notably, all the established protocols of PC in vitro culturing are not satisfying, as they produce MCs that strongly express SMC markers, which is a sign of differentiation. Thus, I developed a novel technique, that guarantees conditions for PC culturing. I utilized this novel technique to show, that the intracellular adaptor proteins NCK1 and NCK2 interact with certain phosphosites of the PDGFRβ and are mediating specifically PC migration in vitro. To demonstrate this finding in vivo, I interbred different mouse lines in order to delete *Nck1/2* in PCs and could show, that PC migration is massively impaired in developmental experiments. This is the first study demonstrating, that it is possible to target specifically one PC behavior - migration.

Exposure of these mice to the OIR protocol revealed less NVTs, less hypersprouting, but increased revascularization. Thus, targeting specifically PC migration by blocking NCK1/2 in PCs is a promising approach and a potential target for future therapies.

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7 Publications

- *Dubrac A*, **Künzel SE*,** Künzel SH, Li J, Chandran RR, Martin K, Greif DM, Adams RH, Eichmann A.* NCK-dependent pericyte migration promotes pathological neovascularization in ischemic retinopathy. **Nat Commun.** 2018 Aug 27;9(1):3463. ***equal contribution**

- *Zhang F, Zarkada G, Han J, Li J, Dubrac A, Ola R, Genet G, Boye K, Michon P, **Künzel SE,** CamporezJP, Singh AK, Fong GH, Simons M, Fernández-Hernando C, Sessa WC, Shulman GI, Eichmann A.*Lacteal junction zippering protects against diet-induced obesity. **Science.** 2018 Aug 10;361(6402):599-603.

- *Genet G, Boyé K, Mathivet T, Ola R, Zhang F, Dubrac A, Li J, Genet N, Henrique Geraldo L, Benedetti L, **Künzel SE,** Pibouin-Fragner L, Thomas JL, Eichmann A.*Endophilin-A2 dependent VEGFR2 endocytosis promotes sprouting angiogenesis. **Nat Commun.** 2019 May 28;10(1):2350.

STEFFEN E. KÜNZEL | physician

Simrockstraße 16 – 53619 Rheinbreitbach – RLP, Germany
+49 (1633) 779266 • +49 (2224) 70368 • +49 (30 450) 7554900
steffen-emil.kuenzel@charite.de

Education

Charité Universitätsmedizin — **Berlin**
Residency in Ophthalmology — *October 2020 – today*

Rheinische Friedrich-Wilhelms-Universität — **Bonn**
Medical School — *October 2012 – June 2020*

Yale University, Yale Cardiovascular Research Center — **New Haven**
Medical Dissertation, Laboratory of Prof. Anne Eichmann — *May 2016 – May 2017*
Focus on pericyte biology and neovascular diseases

Siebengebirgsgymnasium — **Bad Honnef**
High school — *October 2003 – August 2012*
final grade: 1,1 ("very good"), core courses: Mathematics, Geography
highest-ranked student

Publications

- *Dubrac A*,* ***Künzel SE*,*** *Künzel SH, Li J, Chandran RR, Martin K, Greif DM, Adams RH, Eichmann A.* NCK-dependent pericyte migration promotes pathological neovascularization in ischemic retinopathy. **Nat Commun.** 2018 Aug 27;9(1):3463. ***equal contribution**
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Key Clinical Experiences

Massachusetts Eye and Ear, Harvard Medical School — **Boston**
Elective in surgery — *October–November 2018*

Children's Hospital Los Angeles, CHLA — **Los Angeles**
Medical Traineeship — *September – October 2017*
Rotation in Pediatric-Oncology

Univ. of Oxford, Radcliffe Department of Medicine — **Oxford**
Translational Research Stay — *March – May 2016*
Cardiology, Methodical research on cardiomyopathies

Nepal Kathmandu Model Hospital — **Kathmandu**
Clinical Trainee-ship — *February – March 2016*
Six-week assistance intervention after Nepal's 2015 earthquake disaster

Awards, Scholarships, Invitations

- *October 2020* **1st prize of Springer Charity Award 2020**
  Award for outstanding social commitment. Endowed with 30.000 Euro in Cash, as well as 50.000 Euro in Media Budget.
- *January 2013 – present:* **Scholarship of the German National Academic Association:**
  Scholarship covering tuition, travel, living expenses, language courses, and conferences
- *December 2017 – present:* Founder and CEO of **Get Vaccinated (Verein für Impfaufklärung für Deutschland e.V.)**
  Student Association promoting vaccination in Germany. We provide high school education programs, and programs aimed at improving refugees access to vaccination. We also advocate for online access to the Vaccination Bureaucracy (eHealth). For more information visit `get-vaccinated.com/impf-dich.org`.
- *2016-today:* **Invited Speaker at:**
  GRC Vascular Cell Biology Ventura 2017 and 2019, NAVBO Helsinki 2018, GRS Vascular Medicine 2017 and 2019, NAVBO Developmental Vascular Biology and Genetics Monterey 2017
- *February – March 2018:* Key organizer of a **research and educational expedition through oman**
- *September 2017 – present:* **Young Titans Scholarship**
  'For students with an outstanding personality who are part of the best 0.1 percent of their peer group'
- *January 2019 – present:* Scholar of **London School of Economics, German Symposium**
  Conference speaker and participant
- *October 2014 – 2015:* Co-founder of the regional group **Education against Tobacco:**
  Voluntary educational work for minors by medical students to reduce the incidence of smoking in society.
- *January 2014 – June 2015:* Participant in **PreScimed:**
  4 week research preparation training for selected MD students
- *Since 2017:* member of **NAVBO**
  Conference speaker and participant at scientific meetings worldwide
- *October 2012:* Winner of **Judith-Ernst-Prize:**
  For academic achievements in Mathematics
- *August 2010 – present:* Member of **MENSA Club Germany:**
  Club for creative, technologically and scientifically gifted people, psychological report
- *2008 – 2012:* Participant in numerous **High school math competitions:**
  Team leader, mentor, advisor at high school maths competitions, Federal round of the Mathematical Olympiad in North Rhine-Westphalia, qualification tournament for the European competition
- *October 2009 – June 2010:* Team leader at **Business@School:**
  *European Business Competence Licence EBC*L, Team business competitions:*
  1st round: first place, 2nd round: first place, 3rd round: second place
- *2008 – 2011:* Program for gifted students at **Research Center CAESAR:**
  dynamical systems in physics, introduction to quantum physics, mathematical encryption technologies, frontiers of neurobiology, HPCL-measurements to detect caffeine

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