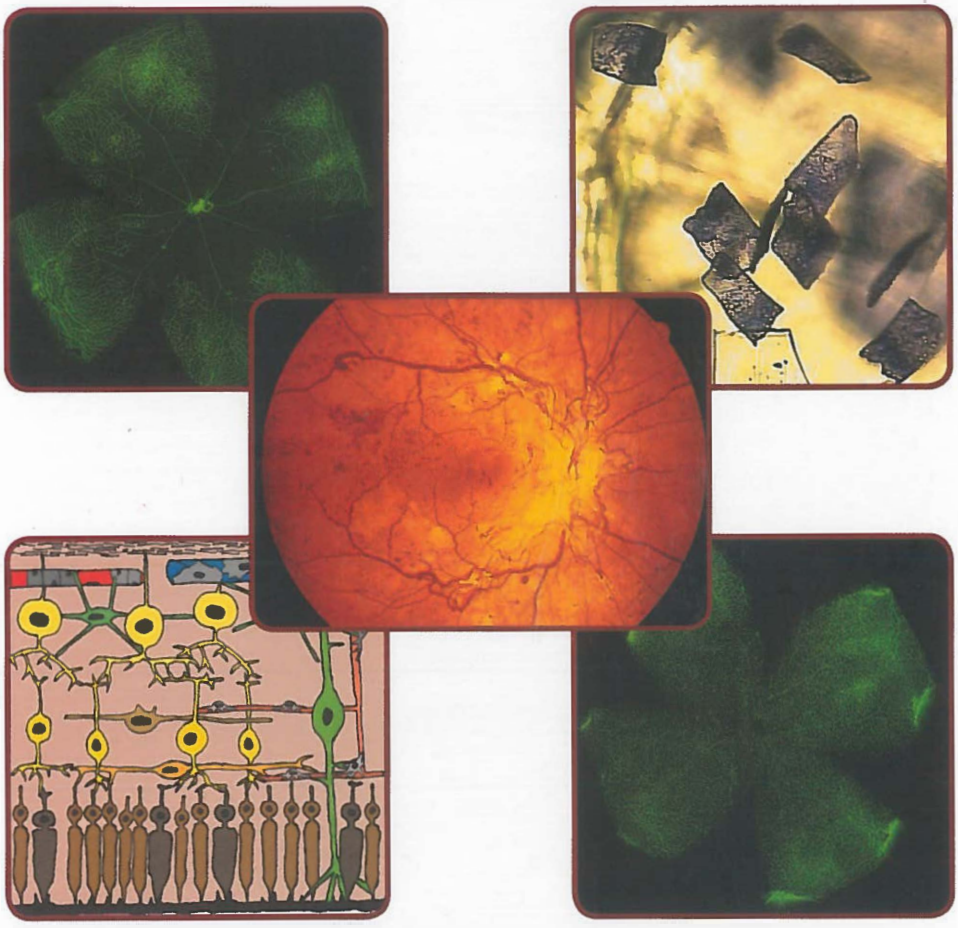
Experimental Models Of Retinal Angiogenesis:

the effect of Angiopoietin-2 and TNFα modulation



F. Gräfin vom Hagen

Experimental Models Of Retinal Angiogenesis: the effect of Angiopoietin-2 and TNFα modulation

Franziska M. Gräfin vom Hagen

Stellingen behorende bij het proefschrift
Theses pertaining to the dissertation

Experimental Models Of Retinal Angiogenesis: the effect of Angiopoietin-2 and TNFα modulation

1. Angiopietin-2 overexpression in the retina promotes postnatal vascular development and increases preretinal neovascularization in experimental proliferative retinopathy. (this thesis)

2. Angiopoietin-2 deficiency causes abnormal vascular patterning and outgrowth in the postnatal retina and causes persistent preretinal neovascularization via upregulation of VEGF. (this thesis)

3. Laser microdissection of retinal tissue prior to gene expression analysis elucidates spatial gene expression patterns and regulation in the physiologic and pathologic retina and allows zooming into the area of interest. (this thesis)

4. TNFα deficiency reduces experimental proliferative retinopathy, but does not influence postnatal retinal vascular development. (this thesis)

5. Intravitreal thalidomide reduces experimental proliferative retinopathy and is a therapeutic option for patients with proliferative retinopathies. (this thesis)

6. Research of physiological mechanisms should be funded to the same extent as research of pathological mechanisms as we can not understand one without the other.

7. A considerable amount of valuable scientific data can not be found in PubMed, as the editorial boards of journals do not facilitate publishing negative outcome studies.

8. There must be no barriers for freedom of inquiry. There is no place for dogma in science. The scientist is free and must be free to ask any

question, to doubt any assertion, to seek for any evidence, to correct any errors. (R. Oppenheimer)

9. An international graduate school provides opportunities to cross barriers in various scientific and non scientific aspects, if the members are open for it.

10. Armed with the scientific method, we can explore and understand nature to the limits of our intelligence. (J. W. Yewdell, Nat Rev Mol Cell Biol 9:413-416, 2008)

11. The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He, to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed. (Albert Einstein)

12. *Virtutum omnium pretium in ipsis est.* (Seneca, Epistulae morales ad Lucilium)

Franziska Gräfin vom Hagen

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Die Natur liebt es, sich zu verbergen.
Nature loves to hide.

Heraklit, Fragment 123

Paranimfen:	Elise Langenkamp
	Annelie Stöcker

Content

Content

Chapter I

Introduction

1.The Retina

Retina comes from the latin word rete, which means net or network or retinaculum, which means holder. The retina lines the posterior eye from the Ora serrata to the optic nerve entrance. Mammals have an inversed structured retina, meaning that the light has to pass the retina before it is detected by the photoreceptor cells. Three major components form the retina a. the neuronal network, b. the neuroglia and c. the retinal vasculature.

1.1 Retinal architecture

The retina is morphologically and functionally structured in several different layers (Fig. 1). The boundary of the retina towards the vitreous is the Inner Limiting Membrane (ILM), which is made of Mueller cell end processes, ganglion cell axons and astrocytic processes. While the axons of the ganglion cells form the nerve fibre layer (NFL), the cell bodies reside in the ganglion cell layer (GCL). The inner plexiform layer (IPL) contains the synapses and processes of bipolar cells, amacrine cells, horizontal cells and ganglion cells. Bipolar cells and Mueller cell bodies form the inner nuclear layer (INL). The following outer plexiform layer (OPL) contains the synaptic connections of the bipolar cells and the photoreceptors. Cell bodies of the photoreceptors are in the outer nuclear layer (ONL), then comes the outer limiting membrane (OLM) and after that the photoreceptor layer (PRL) with rods and cones. Finally, the retinal pigment epithelium layer (RPE) is the border to the choroidea.
The description of these layers is based on the architectural pattern of mainly the neuronal cells, which are actually represented by over 55 morphologically and probably also functionally different cell types (1, 2). Functionally, the light has to pass the retinal layers before it is detected by the photoreceptors. These take up the light photons and convert them first into biochemical and then into electrical action potentials. Basically, these are taken up by the bipolar cells, transferred to amacrine cells and/or horizontal cells and then go via the ganglion cells and the optic nerve to the optical center in the occipital lobe of the brain (3). To coordinate and process the multitude of photonic impulses a wide and complex neuronal network is necessary. During evolution this complex network developed in connection to the evolutionary older cones, whereas the later developed rods have to use the cone neuronal network via an indirect

postsynaptic connection.

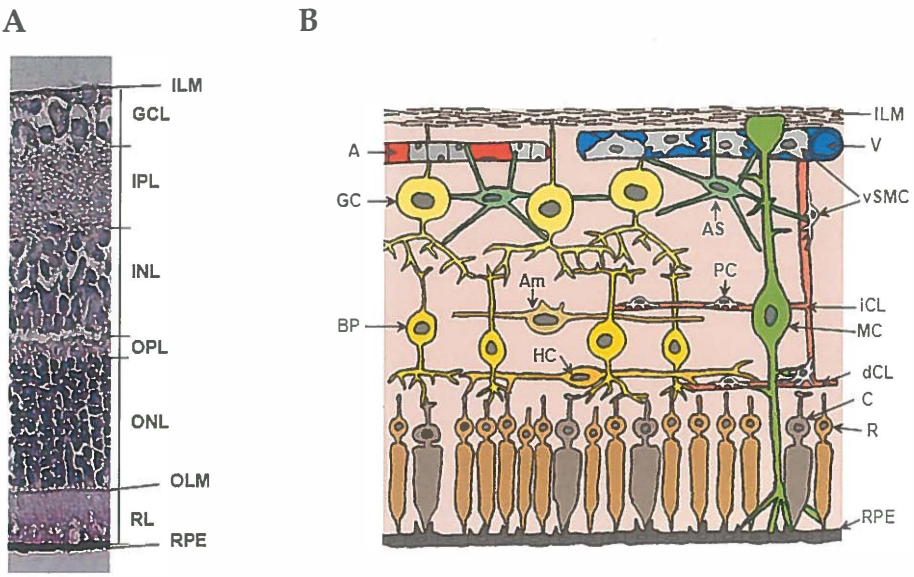

Figure 1: Retinal Architecture
A: The retinal layers are presented in a periodic acid- Schiff's reagent and hematoxylin stained paraffin section of the retina. B: A schematic drawing of the cellular components in the retina.
A=arteriole, Am=amacrine cell, AS=astrocyte, BP=bipolar cell, C=cone, GC=ganglion cell, HC=horizontal cell, MC=mueller cell, PC=pericyte, R=rod, RPE=retinal pigment epithelium, V=venule, vSMC=vascular smooth muscle cell, dCL=deep capillary layer, iCL=intermediate capillary layer, ILM=inner limiting membrane, GCL=ganglion cell layer, IPL=inner plexiform layer, INL=inner nuclear layer, OPL=outer plexiform layer, ONL=outer nuclear layer, OLM=outer limiting membrane, PRL=photoreceptor layer

Two cell types, the astrocytes and Mueller cells, belong to the glia, a further cellular component of the retina. The astrocytes reside in the NFL and GCL and cover with their long processes the superficial vascular plexus. The Mueller cells span the entire retina connecting to the ILM on one side and to the RPE on the other side with their processes (4). Their cell nuclei are part of the inner nuclear layer and their processes wrap around the deep vascular layer at the plane of the OPL. Functionally, the glia cells support the retinal neuronal cells. Their intimate contact to the retinal vasculature makes them part of the blood-retinal-barrier. A special function of the astrocytes is the formation of a growth template for the developing superficial retinal vascular plexus during postnatal retinal development

(5). The Mueller cell is a multitalent. In cooperation with neuronal cells they are involved in the production of lactate, glutamine and retinoic acid, which are basic components for neuronal signaling (6, 7). Furthermore, they produce vascular endothelial growth factor (VEGF) and Angiopoietin-2, both important angiogenic growth factors. As these cells are regulators of water homeostasis, pH and membrane potential, they function as a kind of lymphatic drainage system, which the retina is devoid of (7, 8). Recently, the Mueller cells have been shown to act like glass fibres allowing straight and proper light transmission through the retina (9).

The retinal vascular network is the third component of the retinal architecture, which develops postnatally in the mouse. The vascular network consists of three vascular plexi. The superficial retinal vascular plexus is found in the plane of the ILM to GCL and is formed by four to six arterioles and venules and their connecting capillary network. Arterioles and venules originate from the optic nerve vessels at the optic disc entrance and spread distally in a radial fashion in the mouse retina. The intermediate capillary plexus resides at the outer border of the IPL and is derived from the superficial vascular network. At the outer border of the INL the deep capillary plexus is found. Functionally, the retinal vasculature is essential for optimal distribution of oxygen and nutrients, especially to the upper or inner retinal layers facing the vitreous. The development, function and pathological reaction of the vasculature will be described in detail in the following chapters.

A further structure in the retina is the fovea. The fovea is a depression in the retina, in which the inner retinal layers and the retinal vasculature is absent. This area is only covered by cones and their axons run obliquely to connect to the bipolar cells, which are outside the fovea. While humans and some primates have a fovea, most mammals do not have a fovea. Instead they posess an area centralis, in which photoreceptor density is enhanced, but lack the foveal pit. In the clinic fovea and macula are often used synonymously. However, the macula defines a retinal region in which the density of xanthophyll pigment is highest and is termed macular pigment. The macula is larger than the fovea and includes the parafovea. The name macula lutea is derived from its lemon-yellow colour, which appears when the retina is removed from the eye. Its function is still poorly understood.

2. Angiogenesis

2.1 General concepts

Angiogenesis is the formation of new blood vessels by sprouting from preexisting vessels. In contrast, new vessel formation occurring from vascular progenitor cells is termed as vasculogenesis. Most angiogenesis concepts have been developed from animal models of tumor angiogenesis, and some of them have been transferred to retinal angiogenesis.

Angiogenesis starts with activation of endothelial cells of preexisting vessels. The major stimulus for endothelial activation in the retina is hypoxia. Hypoxia upregulates several angiogenesis associated genes, which then regulate the downstream signaling pathways. Due to these signaling pathways, which will be described in the following chapters a cascade of processes is initiated. Following endothelial activation, an increased vascular permeability occurs, which leads to the extravasation of matrix degrading molecules. Furthermore, inflammatory cells enter the tissue and release cytokines, which further drive the angiogenic process. Subsequently, endothelial cells start to migrate und proliferate according to the surrounding guidance and patterning cues (10). In some organs the detachment of pericytes, a hallmark of vessel destabilization is a prerequisite for the angiogenic process. After successful sprout and subsequent lumenized vessel formation the physiologic angiogenic process is followed by further vessel remodeling and maturation.

The molecular concept of angiogenesis started out with the identification of a vasoactive factor, later known as VEGF, and a description of its key role in angiogenesis (11). The number of molecules described to have angiogenic properties is still growing. Angiogenesis-related molecules encompass the group of angiogenic growth factors, anti-angiogenic factors, growth hormones, adhesion molecules, Ephrins, extracellular matrix (ECM) molecules, matrix-metallo-proteinases and cytokines, and the number of molecules with angiogenic properties is still growing. The role of these molecules in angiogenesis has been extensively reviewed (12-15) and some of these will be described in the subsequent chapters.

The molecular concepts of angiogenesis are mostly derived from animal models of tumor angiogenesis. Hypoxia is a trigger for the initiation of the angiogenic process (10). In the tumor hypoxia arises with the fast growth of the tumor tissue, which is followed by

the need to vascularize to get nutrients for further growth (16). The transcription of VEGF and other angiogenic factors is hypoxia sensitive (17, 18). Folkman et al. were the first to describe the balance of pro- to antiangiogenic factors to be the modulator of angiogenesis (19). The upregulation of proangiogenic factors by hypoxia switches the balance towards a proangiogenic environment (20). Whereas several studies investigated the role of VEGF and other proangiogenic factors in angiogenesis, less is known about the role of endogenous antiangiogenic factors. A difficulty of classification of angiogenesis-associated molecules arises as some molecules, such as the antiangiogenic thrombospondin-1 (Tsp-1) exerts also proangiogenic functions (21). Although the key players of angiogenesis seem to be identified, the modulation of the angiogenic process is still poorly understood, mainly due to its complex interactive network and the additional complexity induced by microenvironmental control of the processes involved (22). The current paradigm describes a role for the Angiopoietin-Tie2 system as an endogenous modulator of angiogenesis. Angiopoietin-1 (Ang1), the agonist for the tyrosine kinase receptor (Tie2) is essential for vascular survival, stability and integrity (23). In contrast, Angiopoietin-2 (Ang2) was originally described to be the natural antagonist to Ang1 blocking Tie-2 signaling, thereby destabilizing the vasculature (24). However, the action of Ang2 is more complex, as it can act vasoregressive in a VEGF low environment, and proangiogenic in a VEGF rich environment (Fig. 2) (12).

Inflammatory cells and molecules have been observed alongside each other in the angiogenic process. However, inflammation was associated more with pathological than with physiological angiogenesis (13). Recently, the contribution of inflammatory mediators to the angiogenic process came more into the focus of retinal angiogenesis research. Evidence for a close connection is for example presented by the cytokine tumor necrosis factor alpha (TNFα), which has been shown to have angiogenic properties (25). Vice versa, proinflammatory properties have been discovered for angiogenic molecules, such as VEGF and Ang2 (26-28).

Initially retinal vascular development has been described to be a vasculogenic process, but later on Fruttiger showed that it is an angiogenic process in the rodent retina (29). In the human retina the retinal vascular development is a combined vasculo- and angioge-

nic process.

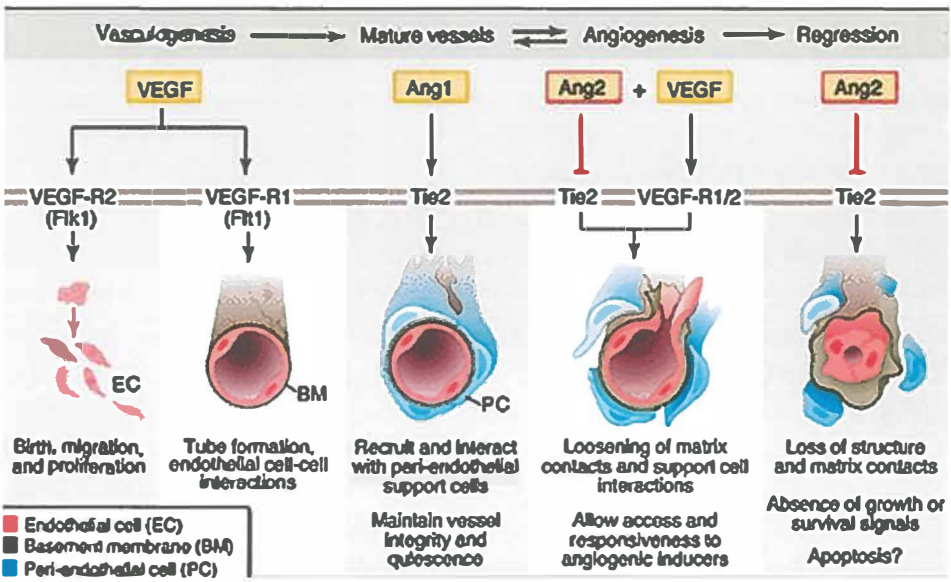

Figure 2: VEGF and angiopoietin signaling in vascular development
Regulation of vascular morphogenesis, maintenance, and remodeling by RTKs and their ligands. A model for regulation of the vascular endothelium manifested by the prototypical angiogenesis factor VEGF and a new class of angiogenic regulators, Ang1 and Ang2. All three ligands bind to RTKs that have similar cytoplasmic signaling domains. Yet their downstream signals elicit distinctive cellular responses. Only VEGF binding to the VEGF-R2 sends a classical proliferative signal. When first activated in embryogenesis, this interaction induces the birth and proliferation of endothelial cells. In contrast, VEGF binding to VEGF-R1 elicits endothelial cell-cell interactions and capillary tube formation, a process that follows closely proliferation and migration of endothelial cells. Ang1 binding to the Tie2 RTK recruits and likely maintains association of peri-endothelial support cells (pericytes, smooth muscle cells, myocardiocytes), thus solidifying and stabilizing a newly formed blood vessel. The newly discovered Ang2, although highly homologous to Ang1, does not activate the Tie2 RTK; rather, it binds and blocks kinase activation in endothelial cells. The Ang2 negative signal causes vessel structures to become loosened, reducing endothelial cell contacts with matrix and disassociating peri-endothelial support cells. This loosening appears to render the endothelial cells more accessible and responsive toward the angiogenic inducer VEGF (and likely to other inducers). Finally, Ang2 is expressed at uniformly high levels in vascular regression in nonproductive ovarian follicles; the lack of VEGF coexpression suggests that loosening of cell-matrix interactions in the absence of a growth or survival signal elicits endothelial cell death, likely by apoptosis. No doubt additional factors play into these distinctive states, including the emerging class of angiogenesis inhibitors that directly block endothelial cell proliferation and migration. Illustration: K. Sutliff

Ang=angiopoietin, RTK= receptor tyrosine kinase, Tie2=tyrosine kinase receptor for angiopoietins, VEGF=vascular endothelial growth factor, VEGF-R= VEGF receptor
From Hanahan et al., SCIENCE 227:48-50 (1997). Reprinted with permission from AAAS.

2.2 Retinal angiogenesis

2.2.1 Physiological retinal vascular development

Retinal vascular development

Before the retinal vasculature is described two other important vascular systems of the eye should be briefly mentioned. One constantly supplies the outer retinal layers with nutrients, while the other supplies the retina only during embryonic and early postnatal retinal development. The choroidal vascular system supplies the retina with sufficient oxygen and nutrients via diffusion during embryonic development. In adulthood the choroidea still supplies the outer human retina including the photoreceptor layer, the outer nuclear, and the outer plexiform layer into a depth of 130μm (30). Approximately 65 to 85% of the adult retina is thereby supplied by the choroid (31). The other system is the hyaloid vasculature. This is an arterial network, which is only temporarily formed during ocular development to provide blood supply for the developing lens. Hyaloid arteries anastomose via the tunica vasculosa lentis with the venous ringvessels of the choroid. The hyaloid vasculature regresses postnatally, when intraretinal vasularization is developing (Fig. 3) (32).

As long as the retinal thickness is below 90μm the choroidal supply of nutrients and oxygen is sufficient. The guinea pig, which has a thin retina even in adulthood, therefore does not develop a retinal vasculature. The further growing retinal thickness during retinal development attenuates the choroidal oxygen and nutrient supply. Subsequently, a hypoxic environment arises, which is thought to be the starting point for the formation of the superficial retinal vascular plexus (33). With the further growing retina another hypoxic period occurs, which leads to the formation of the intermediate and deep vascular plexus. The source of the high retinal demand for oxygen and nutrients are primarily the photoreceptors. These require high and constant delivery of oxygen to produce the amount of energy that is necessary for the conversion of photon energy into electrical action potentials. During retinal development the energy demand

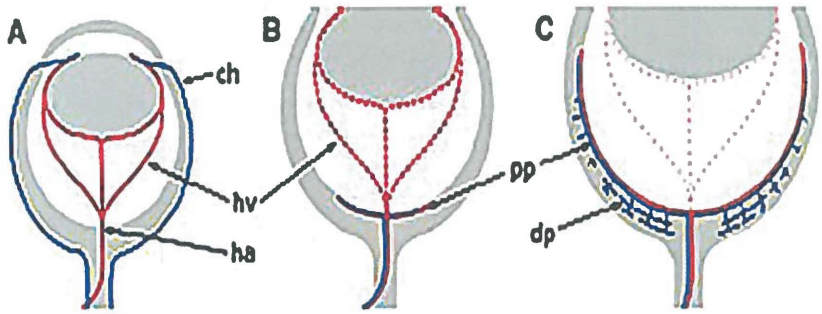

Figure 3: Development of the hyaloid and retinal vasculature
The vascular network inside the eye is remodelled during development. A: The hyaloid vasculature (hv) is supplied by the hyaloid artery (ha) and drained into the venous, choroidal net (ch) on the outside of the eye (the choroidal net is not shown in B and C). B: the hyaloid vasculature regresses as the primary plexus (pp) of the retinal vasculature grows into the retina. The primary plexus consists of arteries and veins. C: The deeper plexus (dp) of the retinal vasculature develops from veins in the primary plexus.
With kind permission from Springer Science+Business Media: Fruttiger M, Development of the retinal vasculature, Angiogenesis 2007, 10:77-88, Figure 1.

is increasing and with the opening of the eyes the full demand has to be granted by proper blood supply. Besides the demand of neuronal cells, the glia also demands high nutrient supply for proper function.
The human retina is vascularised by the retinal arteries and veins, which enter at the optic disc (Fig. 4 A). The central retinal artery divides into 4 main branches, which supply three layers of capillary networks. These are connected to venules, which fuse to the central retinal vein, leaving the retina via the optic disc. The vasculature covers the entire retina, except the avascular fovea centralis, which consists mostly of cones and is responsible for sharp central vision. Besides the lack of a fovea, which most mammalian species do not have, the rodent retina is similarly vascularised as the human retina. The superficial vascular plexus in the mouse retina is formed by 4-6 retinal arterioles and 4-6 retinal venules, which grow centrifugally towards the retinal periphery and are distributed in a radial fashion (Fig. 4 B). Arterioles and venules are connected via the superficial capillary plexus and the intermediate and deep vascular plexi are true capillary networks.

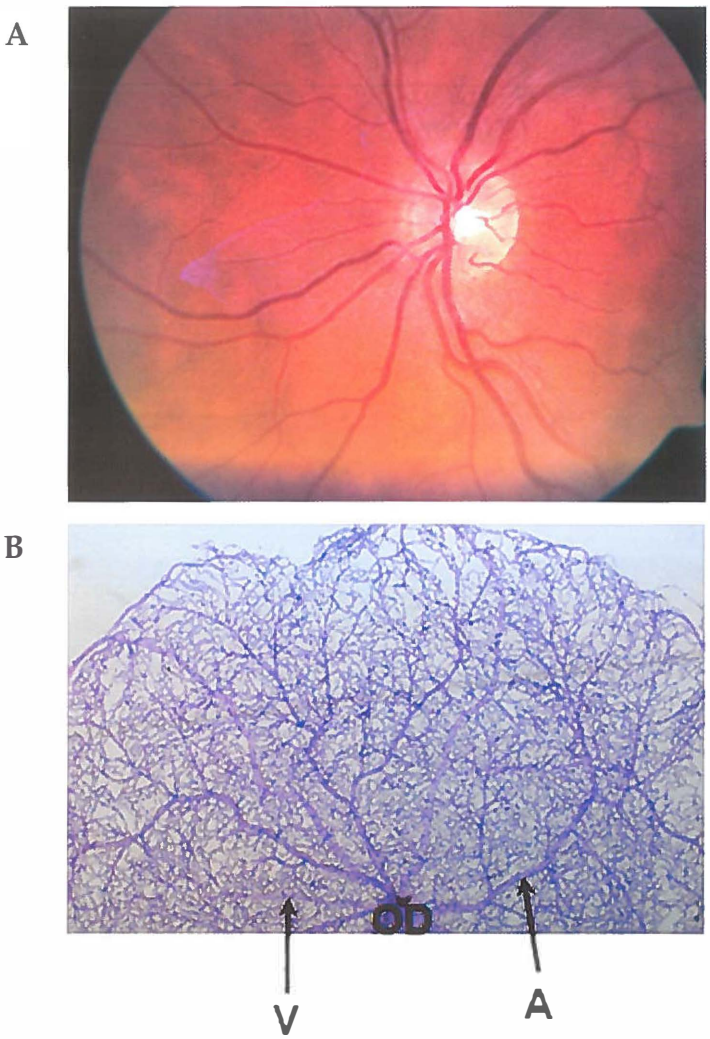

Figure 4: Human versus mouse retinal vasculature
A: Fundus image of a healthy human retina. B: Retinal digest preparation displaying the retinal vasculature of an adult mouse, which is stained with periodic acid-Schiff's reagent and hematoxylin. The radial fashion of smaller diameter arterioles and larger diameter venules, as well as the capillary network is discernible.
OD: vessels entering the retina at the optic disc, A: arteriole, V: venule

Time frame of development

The formation of retinal vasculature starts in the human retina already prenatally by vascular sprouting of the hyaloid vessels at the optic disc (8). In the rodent retina this process starts postnatally, in the mouse at postnatal day 0 (p0) and in the rat at p1-2.

The superficial plexus arises from hyaloid vasculature sprouts at the optic disc. The vessels sprout centrifugally following a preformed astrocyte template (5). The initial stimulus for this process is thought to be the hypoxic environment in the retina, as described before. The superficial plexus develops mainly between p0 and p7

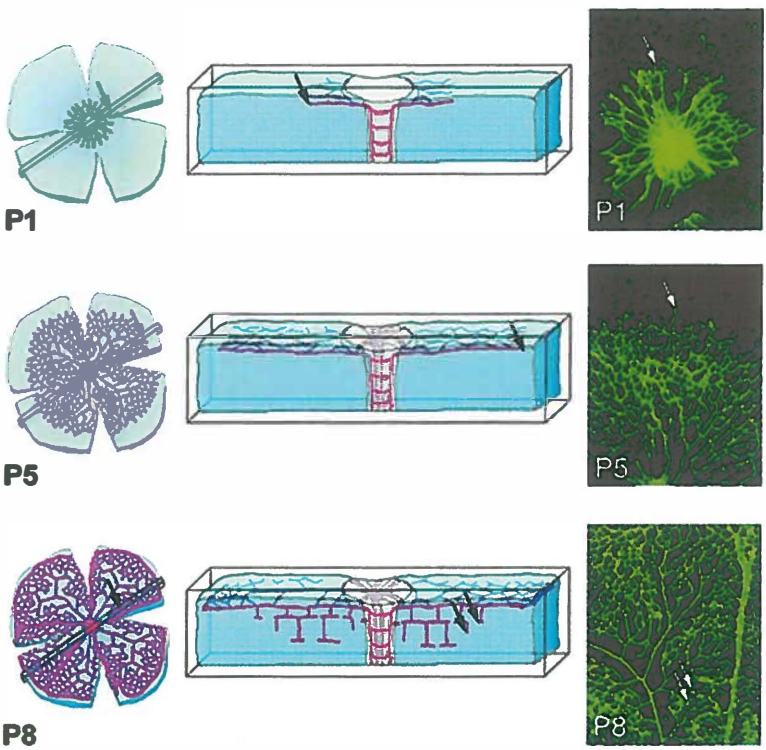

Figure 5: Retinal vascular development

Schematic presentation of retina development as a model system for investigation of angiogenic sprouting in the CNS. Corresponding top view micrographs of whole mount isolectin- labeled specimen are shown to the right. The top view displays the primary plexus in the fiber layer of the retina. Sprouting occurs toward the periphery in the primary plexus (P1 and P5, arrows) and subsequently into deeper layers (P8, arrows), where again branching and fusion leads to plexus formation.

(Fig. 5) (34). From p7 vascular sprouts are formed from the superficial vessels, predominantly from the venous site (33) and orientate into the deeper retinal layers. The deep plexus is then formed until p12. The intermediate plexus forms a bit later from p12 onwards and is finished around p20. The majority of the retinal vasculature is fully developed as the rodents open their eyes at p14 and remodeling occurs until p20.

Cellular interaction
Much more is known about the development of the superficial vascular plexus than of the deep plexus. The formation of the superficial plexus requires a tightly controlled and orchestrated cellular and molecular interaction. A preformed astrocytic template guides the growth of the sprouting vessels to the periphery and determines their patterning. There is a close interaction between astrocytes, endothelial cells (tip and stalk cells) and pericytes. Little is known about the contribution of the neuronal cells in the development of the superficial plexus. Sprouts from the vessels of the superficial layer, first arising from the venous site, form the intermediate and deep plexus (32, 33). Probably, the endothelial sprouts grow into the deeper layers along the Mueller cells, but the precise cellular and molecular guidance and patterning cues in this process are largely unknown (32).

2.2.2. Pathological retinal angiogenesis

Human retinopathies
Proliferative diabetic retinopathy
According to conservative epidemiological calculations the number of diabetic patients will increase to about 340 million worldwide by the year 2030 (35). In the developing countries the number of patients in the age group of 45-65 will double, while in the age group >65 it will triple until 2030. This will have a huge economical and social impact.
Until now, diabetic retinopathy is the leading cause for blindness in working age people in the western world. Despite the long time presence of this disease since its first description in 1870 (36), there is still insufficient knowledge about the molecular and cellular mechanisms of this disease and there is still no curative therapy available. Especially the proliferative form of diabetic retinopathy

is poorly understood. The reason lies in the fact that the human retina can only be studied in a limited manner, such as by fundus imaging and angiography. Patient material is not easily available and rarely represents the disease stages important for studying the initial process of the disease.

Proliferative diabetic retinopathy develops in 50% of Type-1 and 10% or more of Type-2 diabetic patients after fifteen years of diabetes duration (37, 38). The proliferative form of diabetic retinopathy is characterized by increased formation of new blood vessels (31), which arise from the superficial vascular plexus and grow towards the vitreous, breaking through the inner limiting membrane (Fig. 6). These preretinal neovascularization are weak vessels, which tend to rupture easily and thereby cause intravitreal hemorrhage. Later complications are the formation of preretinal fibrovascular membranes and their traction forces causing retinal detachment (39). The up to date treatment option is laser photocoagulation, which stops the process of preretinal neovascularization, but is destructive to

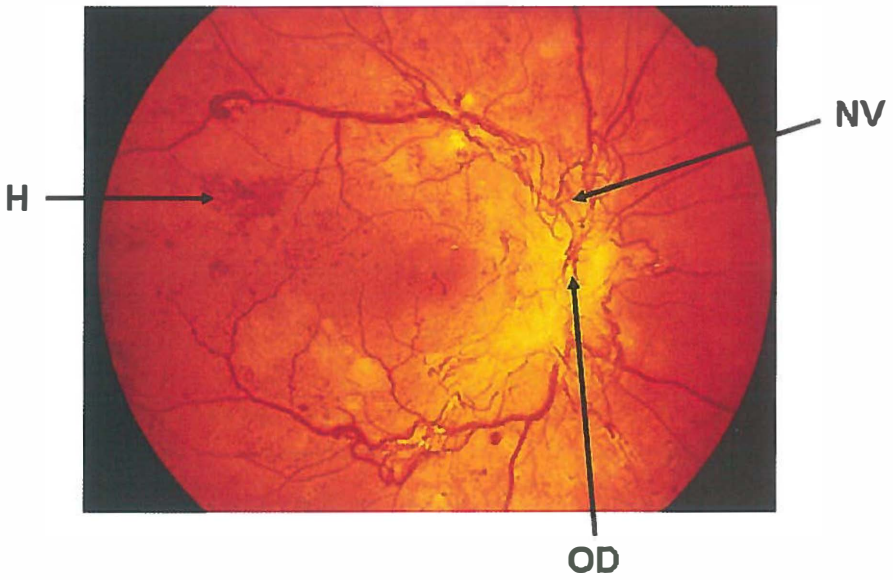

Figure 6: Fundus Image of a patient with proliferative diabetic retinopathy
The fundus image of a patient with proliferative diabetic retinopathy displays the charcateristics of the disease, such as hemorrage (H), and neovascular iaztion (NV) around the optic disc (OD).
Image cordially provided by J.B. Jonas, Department of Ophthalmology, Medical Faculty Mannheim, University of Heidelberg

the retina underneath (40, 41).
Preretinal neovascularizations are thought to be a consequence of hyperglycemia induced vasoregression, which causes retinal hypoxia (39). Hypoxia upregulates proangiogenic growth factors, which induce pathological sprouting of retinal vessels (42). The disease seems to be mediated by an imbalance of pro- and antiangiogenic factors, and probably has also an inflammatory aspect. Patient studies show increased levels of VEGF (43, 44), angiopoietins (45), intercellular adhesion molecule 1 (46), vascular cell adhesion molecule 1 (47), insulin like growth factor I (IGF-I) (48, 49), tumor necrosis factor α (TNFα) (50) and tumor necrosis factor receptors (TNFR) (51) in plasma, vitreous fluid and some in fibrovascular retinal membrane samples of diabetic retinopathy patients. The presence of these molecules in the vitreous fluid shows a disturbed blood-retinal barrier, but also increased retinal production and secretion. However, the presence of these factors in the vitreal fluid is not sufficient to explain the initiation and pathogenesis of preretinal neovascularization.
A successful new treatment option has to prevent or reduce preretinal neovascularization, while enhancing intraretinal neovascularization into areas of retinal vasoregression and protecting the existent intraretinal vasculature from vasoregressive and neovascular signals.

Retinopathy of prematurity
Retinopathy of prematurity (ROP) and its preretinal neovascularizations were first described by Terry in 1942 (52). Despite improved intensive care of preterm born infants 27-40% of babies born with a weight below 1500g develop a retinopathy of prematurity (53). The formation of retinal vasculature in the human retina starts in the 14th embryonic week with vasculogenesis and is followed by angiogenesis from the 15th embryonic week. The retinal vasculature is totally formed by the time of birth (53). Preterm born babies therefore have a still developing retinal vasculature, which is highly sensitive to oxygen concentrations. The incubation in high oxygen concentrations is used to allow proper lung development. At the same time, the retina senses the higher blood oxygenation and hypoxia regulated growth factors, especially VEGF, decrease. Furthermore, tissue oxygenation is higher after birth than in utero.

Moreover, IGF-I is a key factor in postnatal vascular development and its levels are reduced in preterm born babies. This results in a halt of retinal vasculature development and in an avascular retinal periphery during the first phase of ROP (54). Due to vasoregression and the return to normoxic condition after incubation the retina becomes hypoxic and upregulates proangiogenic growth factors, such as VEGF in the second phase of ROP (55). Consequently, preretinal neovascularization occurs predominantly at the growth front of the vascular plexus towards the avascular periphery (Fig. 7) (54).

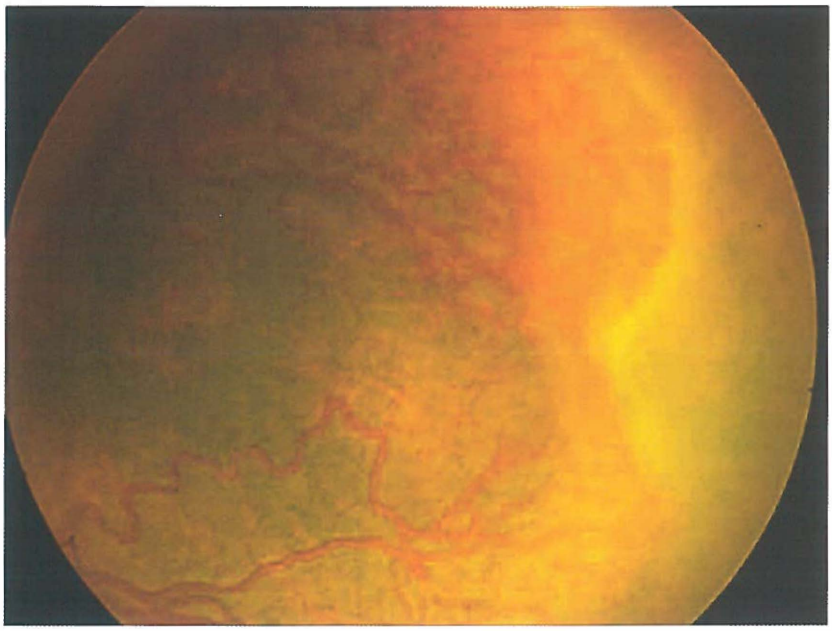

Figure 7: Fundus Image of a patient with retinopathy of prematurity
Retinopathy of prematurity (Stage 3 Zone I with plus disease) and flat neovascularization, which may have a broad appearance. The absence of a line or ridge makes it difficult to discern the interface between vascular and avascular retina. The arterioles are tortuous (black arrow) and at the vascularized ridge neovascularizations are present. The optic disc would be to the left side of the picture.
With kind permission from Wolters Kluwer/Lippincott, Williams&Wilkins: Quiram P.A., Capone A, Jr., Current understanding and management of retinopathy of prematurity, Curr Opin Ophthalmol 2007, 18:228-234

2.2.3. Mouse models of retinal angiogenesis

The earliest findings of retinal vascular damage were described in the human retina (56-59) and experimental studies investigating vascular changes in the diabetic retina were performed in the canine retina (60). Nowadays, the rodent is the most commonly used animal in retinal angiogenesis models.

Experimental diabetic retinopathy

Experimental diabetes in the rodent model is induced by streptozotocin, which leads to pancreatic β-cell apoptosis. The loss of insulin producing β-cells leads to a fast onset of hyperglycemia, which is stable four to five days after streptozotocin application. Another option is the use of the transgenic Ins2Akita mouse, in which the insulin gene is mutated (61). This leads to hyperglycemia between four and six weeks of age in male mice.

The rodent model of diabetic retinopathy only shows the features of early diabetic retinopathy. Early diabetic retinopathy is characterised by increased retinal vascular permeability, loss of pericytes/vascular smooth muscle cells and acellular capillary formation (39). Pericyte loss becomes quantifiable in the diabetic rat retina after three months of diabetes and is increasing afterwards. Longer lasting hyperglycemia leads to damage of capillaries, which occlude and are termed acellular capillaries in retinal research (59). Acellular capillary formation develops later and is in the rat retina established after 6 months of diabetes. Our own data shows that in the diabetic mouse retina pericyte loss is not as strong as in the rat retina. Acellular capillary formation is quantifiable already after three months of diabetes and progresses to six months of diabetes. The disadvantage of the rodent model of diabetic retinopathy is the lack of preretinal neovascularization development. The reason for the lack of preretinal neovascularization under hyperglycemia is still obscure.

Experimental proliferative retinopathy

The model of proliferative retinopathy has been first described by Smith et al. (62). The model induces preretinal neovascularization in the postnatal mouse retina by an exposure to hyperoxia, which is followed by relative hypoxia. The postnatal retina as described already in the earlier chapters is very sensitive to a change in oxygen

concentration. The incubation of seven day old mice in 75% oxygen for five days leads to a vasoregressive stimulus in the retina. The transfer from 75% to normoxia induces a relative hypoxia, which upregulates proangiogenic growth factors, such as VEGF. Consequently, preretinal neovascularization start to appear from p 14 onwards with a maximum at p17. At p25 the preretinal neovascularization is resolved. Smith et al. named the model retinopathy of prematurity model, as preretinal neovascularization is induced in a ROP like manner (62). The model was and is widely used to study proliferative diabetic retinopathy and retinopathy of prematurity as the mechanism of preretinal neovascularization appears similar. Later on some people started using the term hypoxia or oxygen induced retinopathy (OIR) model, but the term ROP model is the most commonly used. The advantage of the model is the inducibility of preretinal neovascularization in the mouse retina. A further advantage is the appearance of the morphological vascular changes in a relatively short time frame and in a precisely timed manner. Therefore, modulation of disease relevant genes can be easily studied at different time points of disease. Another advantage is the short time read out for systemic or local application of antiangiogenic therapeutics. Interestingly, preretinal neovascularization in the rat retina needs an undulating sort of oxygen modulation to induce preretinal neovascularization. Independent of the species, different mouse and rat strains have been described to differ in their angiogenic response and regulation of angiogenic growth factors in this model (63, 64). This makes the comparison of published data rather difficult.

2.2.4. Angiogenic molecules involved in retinal angiogenesis

VEGF/VEGFR signaling pathways

VEGF is known in six forms, VEGFA through F. VEGFA is the key molecule in vascular development and function and exists in various splicing variants named after their amino acid sequence length $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$, which are all one amino acid shorter in rodents. The variants are furthermore characterized by their difference in heparin binding affinity. In the rodent retina VEGF165 is the most abundant isoform (34). Furthermore, each of the isoforms have a proximal and distal splicing variant a

and b, which have distinct functional effects (65). While VEGF signals via the receptors VEGFR1 (flt-1) and VEGFR2 (Kdr or flk-1), $VEGF_{164}$ can signal also via Neuropilin 1. VEGFR1 has a stronger binding affinity for VEGF than VEGFR2, but a lower tyrosine kinase activity than VEGFR2. Furthermore, VEGFR1 exists as the full length form that is membrane associated and a soluble form, which is the extracellular domain.
In the retina VEGF is expressed in astrocytes, in mueller cells, pericytes, vascular smooth muscle cells. VEGFR1 and VEGFR2 are expressed in endothelial cells. While VEGFR1 is additionally expressed in cells of the macrophage lineage, hematopoietic precursor cells and in retinal neuronal cells, VEGFR2 is expressed on endothelial progenitor cells, as well as in neuronal cells in the early postnatal retina (66). During embryogenesis VEGFR2 is highly expressed in the developing vasculature and is essential for its proper development (67). In the adult vasculature VEGFR2 is expressed at low level, but is upregulated in neovascularization (68). VEGFA exerts its proangiogenic functions, such as induction of permeability, migration, proliferation and survival of endothelial cells mainly through VEGFR2. VEGFR2 signalling is complex. VEGFR2 binds phospholipase Cγ (PLCγ) and activates via protein kinase C (PKC) (69, 70) the p42/p44 mitogen activated protein kinase (MAPK) pathway, which mediates proliferation of endothelial cells. Binding of the adaptor molecule Shb, activation of phosphatidylinositol 3-kinase (PI3K) and the serine/threonine kinase AKT/protein kinase B pathway, leads to EC survival and further downstream via eNOS to the induction of vascular permeability (71). Vascular permeability is also mediated via activation of p38 MAPK. Several factors have been associated with VEGFR2 mediated cell migration: p38MAPK activation and heat shock protein 27, or via PI3K activation and Rac, or via focal adhesion kinase and paxillin, or via T-cell specific adaptor and tyrosine kinase Src (71). In retinal sprouting angiogenesis the VEGF system is highly fine tuned to allow proper vascular development. Gerhardt et al. described the existence of specialized endothelial cells at the sprouting tip, i.e. a tip cell, which migrates, and a stalk cell that proliferates and is sitting behind the tip cell (34). Both endothelial cell types respond differently to VEGFA. The migrating tip cell follows an astrocytic VEGFA distribution pattern in a VEGFR2 dependent process, while the proliferation of the stalk cell

depends on the local VEGFA concentrations, and is also VEGFR2 mediated (34).
VEGF is not only a key regulator of physiological, but also of pathological retinal angiogenesis. The major role of VEGF in retinal vascular pathology, especially in retinal hyperpermeability, has led to the successful development and approval of anti-VEGF therapies for patients with diabetic macular edema and neovascular age-related macular degeneration (72, 73). At the moment clinical trials are ongoing, which investigate the efficacy of these drugs to inhibit or prevent preretinal neovascularization. Recent trials in patients with diabetic macular edema and neovascular age-related macular degeneration showed that not all patients respond to these therapies, and that the effect is of much shorter duration than originally assumed based on experimental in vitro and in vivo studies. Regarding the complexity of retinal VEGF action, these clinical trial results are not surprising. Due to context dependent spatio-temporary controlled expression, VEGF can act differently on cellular subtypes. This has been shown, e.g. in physiological retinal angiogenesis for the endothelial tip versus stalk cells (34), as described above. It is easy to envision that this is also true in pathological retinal angiogenesis, yet despite this known local action of VEGF, most studies investigate total retinal VEGF levels instead of localized VEGF levels. Hypoxia upregulates retinal VEGFA via HIF1α and this induces preretinal neovascularization in the mouse model of retinopathy (17). As described above systemic or local application of anti-VEGF drugs can inhibit this effect. VEGF action occurs in a balance with other molecules, such as the angiopoietins (see Angiopoietin/Tie chapter). Moreover, various inflammatory molecules, such as TNFα and TGFβ can regulate VEGF expression as shown in in vitro experiments (74), thereby directly linking neovascularization with inflammatory signals. The $VEGF_{164}$ variant facilitates leukostasis and blood-retinal-barrier breakdown in the rat model of diabetic retinopathy and thus exerts also a proinflammatory function (26). In addition this study demonstrated that $VEGF_{164}$ is upregulated by hypoxia and that inhibition with an aptamer reduced preretinal neovascularization in the ROP model, indicating that angiogenesis and inflammation are linked.

Angiopoietin/Tie signaling pathways

The Tie receptors, Tie1 and Tie2 are tyrosine kinase receptors with Ig and EGF homology domains (Tie). Whereas the ligand for Tie1 is not known, the ligands for Tie2 are Ang1 to Ang4. Both Tie1 and Tie2 were originally described to be exclusively expressed by the vascular endothelium. It is now well accepted that Tie2 is also expressed in nonendothelial cells, including mural cells/ pericytes, neutrophils, monocytes, neuronal and glial cells. Ang1 is expressed in mural cells/pericytes, fibroblasts and in several non-vascular cells (23). Ang2 is primarily expressed in endothelial cells, but also in vascular smooth muscle cells, horizontal cells and retinal Mueller cells (24, 75). Ang1 and Ang2 were originally thought to be the agonist and the antagonist of Tie2, respectively (24). Ang1 binding to Tie2 induces receptor phosphorylation and downstream signaling via PI3-kinase/Akt (76), which promotes endothelial survival. Ang2 has been shown in vitro to bind to Tie2 without receptor phosphorylation (24), thereby reducing Ang1 binding and therefore acting as a competitive inhibitor of Ang1. Recent in vitro evidence suggests that Ang2 is capable to induce Tie2 phosphorylation in endothelial progenitor cells and in human umbilical vein endothelial cells under certain conditions (77), but in vivo evidence is lacking. Furthermore, inhibition of the PI3K/AKT pathway activates forkhead transcription factor 1, which results in increased endothelial Ang2 production (78). Some studies suggest a Tie2 independent signaling pathway via integrins for Ang1.

For embryonic vascular development Tie2 expression is essential as Tie2 k.o. mice are embryonically lethal (79, 80). The Tie2 deficient vascular phenotype resembles that of Ang1 deficient mice. In contrast, Ang2 overexpressing mice display a similar embryonic vascular development defect as Ang1 or Tie2 deficient mice (24). In adulthood, Ang1 supports endothelial survival and vascular maturation, and induces or maintains endothelial integrity by protection from vascular leakage (81, 82). Ang2 was originally thought to only destabilize the vasculature by inhibiting Ang1/Tie2 signalling, but its action is more complex. Figure 8 depicts the most recent view on Ang/Tie signaling in the vasculature (23).

In the retina, the angiopoietins have a crucial role in the postnatal formation of the vascular plexus. Formation of the deep vascular plexus, a critical step in retinal vascular maturation, is dependent

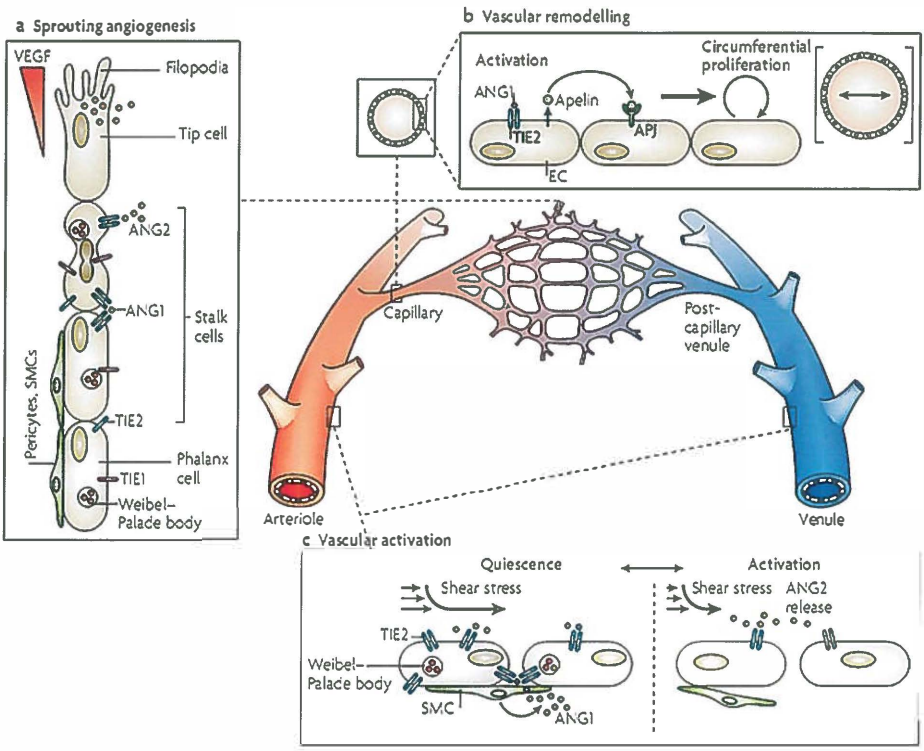

Figure 8: Physiological vascular effects of the angiopoietin–Tie system.
a: Sprouting angiogenesis is initiated in response to vascular endothelial growth factor (VEGF) gradients by invading endothelial cells (ECs), known as tip cells, that have numerous filopodia. These are followed by a zone of proliferating and differentiating ECs, known as stalk cells, that either lack or are only loosely covered by pericytes and smooth muscle cells (SMCs). Below these stalk cells, phalanx cells are in intimate contact with pericytes and SMCs, which keep them in a protected, quiescent state. Tie receptors are expressed by stalk cells and phalanx cells. Angiopoietin 2 (ANG2; also known as ANGPT2) is abundantly expressed by angiogenic ECs, but the precise positional expression pattern of ANG2 is not well defined. Stalk cells and phalanx cells might store ANG2 in Weibel–Palade bodies. Paracrine-acting ANG1 (also known as ANGPT1) might cluster TIE2 (also known as TEK) receptors of contacting ECs in trans. b: The overexpression of ANG1 induces vascular remodelling that leads to the formation of vessels with a wider diameter. TIE2-mediated EC activation controls the expression of endothelial apelin, which in turn acts in an autocrine manner on EC-expressed G-protein-coupled APJ receptors, the downstream signalling of which contributes to the control of vessel diameter. c: The quiescent EC phenotype is maintained by constitutive ANG1–TIE2 signalling. ANG1 clusters TIE2 junctionally at inter-endothelial cell junctions in trans to transduce survival signals. Differences in arterio-venous shear stress also control Ang–Tie signalling. During the transition from the quiescent to the activated phenotype, ECs liberate their endogenously

stored pools of ANG2, and this antagonizes ANG1–TIE2 signalling to facilitate EC responsiveness to exogenous cytokines. As such, the absence or presence of stored ANG2 contributes to the control of the adaptive plasticity of the vascular endothelium.
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on angiopoietins and PDGFB (83). Partial Ang2 deficiency disturbs retinal vascular development and total Ang2 deficiency leads to defective formation of the superficial vascular plexus in the retinal periphery, and the deeper capillary plexi are not formed until p18 (84). Moreover, Ang2 deficient mice have a persistent hyaloid vasculature (85). Interestingly, increased expression of Ang2 affects developing retinal vessels, but not healthy mature vessels (86).
In adulthood, the Ang/Tie system is involved in pathological angiogenesis. VEGF, hypoxia and inflammatory molecules, such as TNFα, modulate Ang1 and Ang2 gene expression (23). In the retina, the Ang/Tie2 system appears to be a modulator in pathological angiogenesis. As mentioned the angiopoietins act in balance with VEGF. The balance of VEGF and Ang2 determines, whether retinal vessel become angiogenic or regress. High Ang2 levels promote in a VEGF rich environment neovascularization (87), while high Ang2 levels in a VEGF low environment induce vascular regression (86). In the retina, Ang2 expression was correlated with the increase in preretinal neovascularization and reaches its highest level together with the maximum of preretinal neovascularization at p17 (88). Concordantly, Ang2 partially deficient mice showed reduced preretinal neovascularization in the ROP model (85). In contrast, Ang1 mRNA is upregulated early between p13 and p15 at the onset of preretinal neovascularization in the ROP model. A study using transgenic overexpression of Ang1 demonstrated that it effectively blocks the initiation and progression of neovascularization induced by VEGF, if upregulated at the same time as VEGF, but cannot reverse established neovascularization (89).
Tie2 regulation is mediated by receptor internalization and degradation, or induced shedding. Recently, a possible role of shedded sTie2 as neutralizer of Ang1/Ang2 signalling and its induction by VEGF has been indicated (90).
Interestingly, angiopoietins have inflammatory properties. While

Ang1 has anti-inflammatory propensities (91), Ang2 exerts proinflammatory function (28). In the retina Ang1 reduces VEGF induced retinal vascular permeability and VEGF mediated leukocyte adhesion via reduction of endothelial cell adhesion molecule expression (92). The proinflammatory action of Ang2 has been demonstrated in a peritonitis and tumor models, in which Ang2 sensitized endothelial cells to a TNFα stimulus (28).

Further molecules involved in retinal angiogenesis
Next to VEGF and the angiopoietin signaling pathway, other molecules that have been associated with retinal neovascularization include platelet derived growth factor B (PDGFB), insulin-like growth factor 1 (IGF-1), bFGF, erythropoietin and matrix-metallo-proteinases (55, 93). Furthermore, factors with antiangiogenic functions, including TGFß, PEDF and thrombospondin 1 have been suggested to play a role in retinal neovascularization as well (39, 94). In addition adhesion molecules are a major component of angiogenesis. Loosening of cell-cell interactions is a prerequisite for endothelial migration and for sprouting angiogenesis. Blocking pathological adhesion molecule regulation, especially integrins, has been successful to reduce preretinal neovascularization in animal models (95, 96). Furthermore, an intact blood-retinal barrier depends on proper function of the adhesion molecules of the cells forming the barrier. Finally, adhesion molecules play an essential role in the interaction of inflammatory cells with the endothelium and are the gate keepers for tissue invasion of these cells.

2.2.5. Contribution of inflammatory molecules to retinal angiogenesis

TNFα/TNFR signaling pathways
TNFα has been first described by Carswell in 1975 (97) and is situated on chromosome 6 in humans and chromosome 17 in mice. Originally described as cachexin or cachectin, the molecule was then named TNFα or TNF and belongs now to a superfamily which consists of over 20 homologues.
All ligands of the TNFα superfamily (98) form non-covalent homotrimers, which can be shed as soluble forms. TNFα is present as a transmembrane bound form of 26kD (mTNF) (99, 100), which is

cleaved by ADAM17, a desintegrin metallo-proteinase, to its soluble form (TNF) of 17kD, forming a trimer of 51kD upon release (101). While the membrane bound form of TNFα seems to mediate predominantly physiological signaling, the soluble form represents a cytokine. Both forms are ligands to TNFR1 and TNFR2. Soluble TNFα binds with high affinity to TNFR1 and with low affinity to TNFR2. In contrast, mTNF preferably binds and activates TNFR2 (102, 103). Activation of TNFR1 by mTNF goes via a ligand-passing mechanism (101, 104). The function of mTNF signaling is poorly understood. TNFR1, but not TNFR2, contains a death domain, which is involved in caspase mediated apoptosis signalling. Ligand binding to TNFR1 and its activation leads to a cascade of adapter molecule activation via kinase activity, which downstream activates a number of transcription factors, including NFκB. NFκB regulates genes which are responsible for cell growth, apoptosis, oncogenesis, immunity, inflammation and stress response (105).

The first description of the proangiogenic potential of TNFα was described in a cornea mouse model using a the TNFα inhibiting drug thalidomide by the group of Folkman (106). However, the role of TNFα in formation of the retinal vascular plexi is not known. Gardiner et al. have shown that the genetic deletion of TNFα does not affect intraretinal angiogenesis after exposure to the ROP model, which they termed physiological angiogenesis (107). A study of physiological retinal angiogenesis in TNFR1 and TNFR2 k.o. mice demonstrated that TNFR1 deletion does not affect retinal vascular development, while TNFR2 deletion reduced vascular outgrowth at p14 (108). However, vascular outgrowth is nearly completed at this late timepoint, and detailed analyses of early postnatal angiogenesis are missing.

As mentioned earlier, inflammatory molecules and inflammatory cells are thought to be contributors or even initiators of pathological angiogenesis (13, 93). The demarcation of angiogenic versus inflammatory becomes difficult as recent research has revealed that traditional angiogenic molecules have inflammatory properties, and vice versa. Genetic deletion of TNFα reduced preretinal neovascularization and enhanced revascularization of the vasoregressed areas in the mouse ROP model (107). These data indicate that TNFα might play an essential role in preretinal neovascularization and intraretinal vascular regrowth. With regard to the TNFα receptors,

TNFR1 k.o., but not TNFR2 k.o. mice showed an intraretinal revascularization defect in the ROP model at p20, when preretinal neovascularization start to regress (108). In this study, preretinal neovascularization was described, but not quantified, as more prominent in the TNFR1 k.o. than in TNFR2 k.o. and wildtype retina at p20. The impaired revascularization observed in the TNFR1 k.o. in this study was explained by a failure to upregulate VEGF and Ang1 after ROP exposure and a higher Ang2 expression at p14. This would hint to a more prominent role of TNFR1 than TNFR2 in retinal angiogenesis. However, these data are controversial as depletion of the ligand TNFα induces the opposite effect of depletion of TNFR1. Furthermore, physiological retinal vascular development has not been thoroughly studied in these transgenic mice. An alteration of physiological angiogenesis before the onset of the ROP model influences the extent of preretinal neovascularization, as we have previously demonstrated (83).

The use of thalidomide, a strong TNFα inhibiting drug, in the cornea angiogenesis model revealed its antiangiogenic potential for the first time (106). Thalidomide or the inhibition of TNFα resulted in a strong antiangiogenic effect in this bFGF stimulated model. The data explained the inhibitory action towards proper vascular limb bud formation in infants of thalidomide using pregnant mothers, who took the drug in the early 1960s to prevent morning sickness or as a sleeping pill. This usage resulted in defective limb formation of the developing infants, and was only in 1994 linked to its antiangiogenic potential. Lately the angiogenic action of TNFα and its interaction with physiological, as well as pathological angiogenic signaling is getting more into the focus of research. An in vivo study showed the ability of TNFα to induce a tip cell phenotype in endothelial cells via VEGFR2 modulation (25). Furthermore, Ang2 sensitizes endothelial cells to a proinflammatory stimulus of TNFα in mouse models of peritonitis and tumor angiogenesis (28). Despite these recent studies, the precise effects of TNFα and its interaction with angiogenic growth factors involved in pathologic retinal neovascularization remain to be investigated.

Further inflammatory signals involved in retinal angiogenesis

Inflammatory cells are tightly associated with angiogenic processes, both in health and disease. The presence of macrophages in epiretinal membranes of proliferative retinopathy patients has been shown already in a patient study in 1981 (109). In the ROP model, the number of macrophages is increased in the retina at p17, especially in close proximity and attached to preretinal neovascularization and within the retina at the border of the avascular to the vascularized area (110). In the same study depletion of macrophages reduced preretinal neovascularization and enhanced revascularization of the avascular areas. Myeloid progenitor cells have in addition been shown to migrate into the avascular retinal areas after exposure to ROP, to differentiate into microglia and mediate HIF1α dependent revascularization (111).

Furthermore, adhesion molecules are essential in mediating endothelial-inflammatory cell interaction and are gate keepers for transmigration of inflammatory cell into the tissue. The process of leukocyte-endothelial interaction and subsequent transmigration is in part mediated by TNFα. Several adhesion molecules mediate the process of leukocyte rolling, adhesion and transmigration through the endothelium (112). In diabetic retinopathy increased leukocyte adhesion to the retinal vasculature has been speculated to result in capillary obstruction leading to the formation of acellular capillaries (113). In experimental diabetic retinopathy the genetic deletion of ICAM-1 or CD18, both important mediators of leukocyte-endothelial interaction, resulted in reduced leukocyte adhesion and endothelial cell death (114). The concept of capillary obstruction by inflammatory cells is however debated as leukocytes trapped in retinal capillaries of diabetic retinae have never been observed. Despite the debate, studies investigating, whether the inhibition of enhanced leukocyte adhesion in the diabetic retinal vasculature is beneficial are performed in experimental models.

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Chapter I

Aims

In the previous overview, we introduced that proliferative retinopathies are common, sight-threatening diseases and are a major cause for blindness in the western world. These diseases, such as proliferative diabetic retinopathy and retinopathy of prematurity are characterized by the intravitreal growth of newly formed vessels (neovascularization). These vessels originate from the intraretinal superficial vascular plexus. It is known that vascular endothelial growth factor plays a key role in the retinal angiogenic process and drugs targeting VEGF have found their way into the clinic. The spatial distribution of molecules within the complex retinal structure, as well as the precise molecular mechanisms of preretinal neovascularization, especially at the neovascularization incipience is poorly understood. Several angiogenesis associated factors, such as the angiopoietins have been proposed to play a role in preretinal neovascularization. Recent evidence suggests also a role for inflammatory factors, such as TNFα in the pathogenesis of preretinal neovascularization.

The general aim of this research thesis was to further explore the role of angiogenesis associated factors, such as angiopoietin-2, and inflammatory factors, such as TNFα in the process of physiological retinal vascularization and pathological preretinal neovascularization in experimental mouse models. These experiments were performed with the aim that a further understanding of the molecular process of preretinal neovascularization would eventually optimize strategies to prevent/treat proliferative retinopathies. Furthermore, we tested, whether a therapeutic approach interfering with VEGF and TNFα signaling would effectively reduce experimental preretinal neovascularization and would be an example for future treatment strategies of proliferative retinopathy.

In **chapter 2** and **chapter 3** we aimed to investigate the effects of Ang2, a molecule with context dependent function on the vasculature, in physiological and pathological retinal angiogenesis. Therefore, we investigated whether the influence of Ang2 overexpression affects physiological retinal vascularization, and whether it influences the vascular response to hypoxia in the model of proliferative retinopathy. Furthermore, we studied the influence of Ang2 deficiency in physiological retinal vascularization.

Investigations to unravel the molecular mechanisms of preretinal neovascularization are hampered as quantitative analyses of con-

tributing factors in whole retinal lysates, the standard procedure so far, ignored the spatial distribution and localized regulation of molecules. In **chapter 4**, we therefore aimed at establishing a method that uncovers spatial inflammatory- and angiogenesis-associated gene expression patterns and reveals their hypoxic regulation in the area of neovascularization incipience in the retina.
In **chapter 5** we studied the effect of TNFα deficiency on physiological and pathological retinal neovascularization. Based on the results of chapter 4, we aimed at uncovering the molecular mechanism behind its effects by studying the influence of TNFα deficiency on spatial inflammatory and angiogenesis related gene expression and its influence on hypoxic regulation of these genes in the area of neovascularization incipience.
Understanding the importance of VEGF in the pathogenesis of preretinal neovascularization and based on the results of our studies and those of others, which demonstrate that inflammatory factors contribute to preretinal neovascularization, we hypothesized that a drug, which targets both, VEGF and TNFα, and inhibits inflammatory signaling would reduce experimental pathologic retinal neovascularization. We chose thalidomide, which is a potent TNFα inhibiting drug. Furthermore, thalidomide is antiangiogenic by inhibiting VEGF signaling, and besides blocking TNFα, it has further anti-inflammatory functions. Therefore, in **chapter 6**, we investigated the effects of intravitreal application of thalidomide on the retinal vasculature and the neuroretina in experimental proliferative retinopathy.
In **chapter 7** the results of the studies performed in the previous chapters are summarized and in **chapter 8** these results are discussed in a broader context and the future perspectives of physiological retinal angiogenesis as well as proliferative retinopathy research are delineated.

Chapter II

Impaired pericyte recruitment and abnormal retinal angiogenesis as a result of Angiopoietin-2 overexpression

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Abstract

Angiopoietin-2 (Ang2) is among the relevant growth factors induced by hypoxia and plays an important role in the initiation of retinal neovascularizations. Ang2 is also involved in incipient diabetic retinopathy, as it may cause pericyte loss. To investigate the impact of Ang2 on developmental and hypoxia-induced angiogenesis, we used a transgenic mouse line overexpressing human Ang2 in the mouse retina. Transgenic mice displayed a reduced coverage of capillaries with pericytes (-14 %; $p<0.01$) and a 46% increase of vascular density of the capillary network at postnatal day 10 compared to wild type mice. In the model of oxygen-induced retinopathy (OIR), Ang2 overexpression resulted in enhanced preretinal (+103%) and intraretinal neovascularization (+29%). Newly formed intraretinal vessels in OIR were also pericyte-deficient (-26 %; $p<0.01$). The total expression of Ang2 in transgenic mice was 7 fold, compared with wild type controls. Ang2 modulated expression of genes encoding VEGF (+65%) and Ang1 (+79%) in transgenic animals. These data suggest that Ang2 is involved in pericyte recruitment, and modulates intraretinal, and preretinal vessel formation in the eye under physiological and pathological conditions.

Introduction

Retinal neovascularization is a major cause of severe vision loss in several eye diseases (1-3). It has been established that hypoxia-induced VEGF plays a critical role in retinal neovascularization (4-6). VEGF is overexpressed in several cell types of the retina under hypoxia, such as endothelial cells, pericytes, ganglion cells and Müller cells (5, 7-9). Inhibition of VEGF is an effective intervention (10, 11). Angiopoietins and their cognate receptor, Tie2, an endothelial-specific tyrosine kinase receptor, play an important role in physiological vascular growth and in pathological angiogenesis such as diabetic retinopathy (12-15). Ang1 acts via phosphorylation of the Tie-2 receptor, stabilizing the endothelium and promoting periendothelial cell recruitment to the newly formed vessels. Ang1 deficient mice die during the formation of embryonic blood vessels because of absence of periendothelial cells, immediately after the induction of the primary capillary plexus by VEGF (16-18). Ang2 is originally identified as a natural antagonist of Ang1, inhibiting Ang1 induced Tie-2 phosphorylation and its subsequent effects on endothelial cells. Transgenic mice which overexpress Ang2 simulate the phenotype of the Ang1 deficient mouse (12). Similar to VEGF, Ang2 is induced by hypoxia (19-20). The effect of Ang2 is VEGF-dependent as Ang2 in combination with high VEGF levels induces sprouting angiogenesis, whereas Ang2 in the absence of VEGF results in capillary regression (14, 21-24). In the normal retina, Ang2 expression is found in endothelial cells, in vascular smooth muscle cells, in horizontal cells, in Mueller cells and in some undefined cells in the ganglion cell layer (25-30).

Pericytes in retinal capillaries protect the endothelial cells from regressive signals. In diabetic rodent retina, Ang2 is markedly increased before pericytes drop out, and intravitreal injection of Ang2 into a normal rat induces pericyte dropout. Ang2LacZ mice in which the reporter gene LacZ is controlled by a mouse Ang2 promoter, Ang2 is increased throughout the retina after 6 weeks of diabetes. Heterozygous Ang2 deficiency protects from pericyte loss and reduces subsequent formation of acellular capillaries in diabetic mice (15).

Together, these data suggest that Ang2 is a context-dependent molecule regulating vessel formation by affecting the crosstalk between pericytes and endothelial cells. Recently, it has been reported that the inducible expression of Ang2 in the retina results in increased

vascular density at p11 and in a significant increase of retinal neovascularization in mice with ischemic retinopathy, indicating the sensitivity of new forming vessels to the ambient concentration of Ang2 (31). Unfortunately, this study did not address the Ang2 effect on pericyte behaviors.
Therefore, our study investigated the role of Ang2 overexpression in pericyte recruitment and vessel formation under physiological and OIR conditions.

Materials and methods

Animals

A fragment encoding human angiopoietin-2 protein was cloned into a plasmid which contains a mouse opsin promoter upstream and a polyadenylation signal of SV40 downstream of the multiple cloning site. The transgenic mice were created by injecting the purified DNA into the pronuclei of fertilized oocytes. Genotyping of mOpsinhAng2 mouse was performed by PCR using the specific human Ang2 primers: forward 5'-GGA AGA GCA TGG ACA GCA TAG GA-3', reverse 5'-GCC ATT TGT GGT GTG TCC TGA TT-3'. The tail cuts were digested overnight at 55°C in the buffer containing 0.4μg/μl proteinase K, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3). The transgenic mice revealed an 821bp product.
The care and experimental use of all animals in the studies were in accordance with institutional guidelines and in compliance with the Association for Research in Vision and Opthamology (ARVO) statement. Nontransgenic littermates (C57BL/6J) served as controls. For pericyte coverage studies, the heterozygous mOpsinhAng2 mice were bred with homozygous XLacZ mice, which express the reporter gene LacZ specifically in smooth muscle cells and pericytes (32).

Retinal neovascularization

To study the effect of Ang2 overexpression on the proliferative retinopathy newborn mice were subjected to the model of Oxygen-Induced Retinopathy (OIR model)(4). Briefly, the mice at postnatal day 7 were exposed to 75% oxygen for 5 days with their nursing mother, mice were returned to room air at day 12. At day 17, eyes were enucleated under deep anesthesia and fixed in 4% buffered formalin, and vertical paraffin sections (6 μm) were prepared. The

quantification was performed as described previously (33). The numbers of nuclei in newly formed vessels at the vitreous side of the inner limiting membrane (ILM) were counted on the sections stained with Periodic Acid-Schiff (PAS), and the intraretinal capillary profiles were analyzed on the sections stained with TRITC-labeled isolectin B4 from Bandeiraea simplicifolia (Sigma). There are three capillary layers in the mouse retina, superficial capillary layer in the ganglion cell layer, intermediate capillary layer at the inner border of inner nuclear layer and deep capillary layer at the outer border of inner nuclear layer. Capillary profiles of the second and third layer excluding the influence of large vessels in the superficial layer were counted on the 200x photographs in a total length of 425μm retina in the areas not affected by hyperoxia-induced regression. The quantitation was conducted in a blinded fashion.

Immunofluorescence analysis

To exhibit the vasculature, whole mount retinae isolated from postnatal day 10 (p10) and OIR model at p17 were permeabilized and unspecific binding was blocked in 1% BSA and 0.5% Triton-100 at room temperature for 1 hour. After incubating with TRITC-labeled isolectin B4 (Sigma, 1:50) overnight at 4°C, retinae were washed in PBS. For pericyte coverage analysis, the primary antibody rabbit anti-beta gal (Promega, 1:500) for staining of the LacZ reporter gene positive cells was diluted in the blocking solution and incubated with retina overnight at 4°C followed by incubation with swine anti rabbit secondary antibody coupled to FITC (DAKOCytomation GmbH, Hamburg, Germany, dilution 1:20). The retinae were flat mounted in 50% glycerol and photomicropgraphs were obtained with a fluorescence microscope (Leica BMR, Bensheim, Germany) or a confocal microscope (Leica TCS SP2 Confocal Microscope, Leica, Wetzlar, Germany). The quantification of avascular zones and of filopodial length was performed as described previously (34, 35).
To determine the mitotic activity in the vasculature of the retinae of OIR model at p17, cryosections fixed with acetone were subjected to immunofluorescence staining. The primary antibodies used were rabbit-anti-mouse Ki67 (Abcom, Cambridge, UK, 1:500) and rat anti mouse PECAM-1 (BD Pharmingen, Heidelberg, Germany, 1:100), and the secondary antibodies were swine anti rabbit coupled with FITC (DAKOCytomation GmbH, Hamburg, Germany, 1:20)

and goat anti rat Alexa 594 (Invitrogen GmbH, Karlsruhe, Germany, 1:200), respectively.

Confocal microscopic evaluation

We evaluated the pericyte coverage on the capillaries within a circular area of 1200μm apart from optic disc by counting the ß gal positive cells in the heterozygous mOpsinhAng2 mice cross-bred with homozygous XLacZ mice. The analysis was performed using confocal microscopy of lectin-LacZ-stained retinae as described above. Photomicrographs were digitized in serial sections by scanning the deep capillary layer and pericyte numbers were quantitated with the modified method for quantitative retinal morphometry as described previously (15, 33).

RT-PCR and Real-Time PCR

Total RNA was extracted from retinae using Trizol (Invotrogen, Germany) according to the manufacturer's protocol. DNA contamination was removed by treating the isolated RNA with RNase-free DNase I (Amplification grade, Invitrogen, Germany). RNA was reverse transcribed using Superscript II transcriptase (Invitrogen, Germany) with Oligo(dT) and random primers. Subsequently, the expression of human Ang2 was assessed by PCR amplified with genotyping primers. RNA treated with RNase-free DNase I without RT and retinal RNA from wild type mice served as negative controls.

For quantitative analysis of transcriptional expression, cDNA was subjected to real-time quantitative PCR. Real-time PCR was performed using QuantiTectTM SYBR® Green PCR kit (Qiagen) in a 20μl reaction volume with HotStartTagTM DNA Polymerase according to the protocol provided by manufacturer. The condition for each gene of interest in real-time PCR was optimized. For generating the standard curve, the gene specific PCR product amplified with the specific primers was purified with QIAquick® PCR purification kit (Qiagen, Germany) and quantified by measuring the absorption at 260nm in a spectrophotometer and its integrity was controlled by 2% agarose gel electrophoresis. The concentrations of the standard were measured in series at intervals of 10-fold copies from 1pg/μl to 100ag/μl. The gene specific amplificants were confirmed by analyzing the melting curve and digesting the PCR products with

HpaI, and subsequent agarose gel electrophoresis. The gene expression levels were calculated by ratios of genes of interest to house keeping gene S16. The primers used in real-time PCR were mouse Ang1 forwards 5'-ACA ACA CCA ACG CTC TGC AA-3', reverse 5'-TGG CCG TGT GGT TTT GAA C-3'; mouse VEGF forwards 5'-TTA CTG CTG TAC CTC CAC C-3', reverse 5'-ACA GGA CGG CTT GAA GAT G-3'; S16 forwards 5'-CAC TGC AAA CGG GGA AAT GG-3', reverse 5'-TGA GAT GGA CTG TCG GAT GG-3'; mouse and human Ang2 forwards 5'-AAA CCA CCT TCA GAG ACT GTG C-3', reverse 5'-CAT GTC ACA GTA GGC CTT GAT CT-3'.

Statistics

Quantitative data are given as means ± SD, unless stated otherwise. Student T-test was used to make comparison between groups. A value of $p<0.05$ was considered as statistically significant.

Results

Human Ang2 is expressed in the transgenic retina at postnatal day 7.

To determine the transcriptional magnitude of the Ang2 overexpression, we assessed the human retinal Ang2 transcript in the transgenic mOpsinhAng2 mice at postnatal day 7. The Ang2 mRNA was analysed by RT-PCR using the genotyping primers for human Ang2. The presence of human Ang2 was demonstrated at p7, while amplificants were neither detected in the non-transgenic controls nor in the transgenic mice with RNA treated with DNase I, but without reverse transcription (Fig. 1).

Ang2 overexpression increases vascular density in the forming retinal network

To determine the effect of Ang2 overexpression on the physiological angiogenesis, we focused on the deep capillary layer which is adjacent to the site of Ang2 overexpression. Density of the deep capillary network in transgenic mice was significantly higher (46%) than in wild type mice at p10 (23.3±4.9 in mOpsinhAng2 mice vs. 16.0±3.9 in wild type mice, n=6; $p<0.05$).

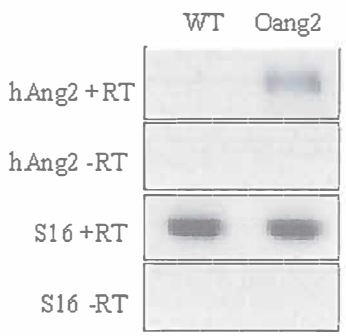


Figure 1:
Representative example for RT-PCR analysis of human Ang2 and S16 expression in the retina of transgenic mOpsin-Ang2 mouse and its wild type littermate at p7. Total retinal RNA isolated from mOpsinhAng2 mouse or wild type littermates, treated with DNase I to avoid DNA contamination, with or without reverse transcription, was subjected to PCR amplification with human Ang2 primer for genotyping. mOpsinhAng2 mouse expresses human Ang2 at postnatal day p7. n=10.
WT, wild type; Oang2, mOpsinhAng2 transgenic; RT, reverse transcription.

Ang2 overexpression reduces capillary pericyte coverage in the forming retinal network

In order to assess whether pericyte coverage in the deep capillary network is changed due to Ang2 overexpression during development, we assessed pericyte numbers in mice at p10 using the LacZ reporter gene in mOpsinhAng2-XLacZ mice. Pericyte numbers in the deep capillary layer in mOpsinhAng2 mice were 3398±319/mm^2 retinal capillary area, while control mice had 3928±133/mm^2 retinal capillary area ($p<0.01$ mOpsinhAng2 vs. control mice). Thus, overexpression of Ang2 in the photoreceptor layer changed capillary pericyte recruitment.

Ang2 promotes preretinal neovascularization and intraretinal angiogenesis in the OIR model

In order to determine the effect of Ang2 overexpression to retinal neovascularization, we quantitated preretinal neovascularizations in the OIR model. The transgenic mOpsinhAng2 mice showed a 103% increased number of neovascular nuclei on the vitreal side of the inner limiting membrane (79 ± 10 neovascular nuclei/section) in comparison with their wild type littermates (39 ± 4 neovascular nuclei/section) ($p<0.01$, Fig. 2A-2B). We also examined the neovascular response of intraretinal capillaries. The profiles of intraretinal capillaries were quantitated on the paraffin sections stained with lectin. A 29 % increase of these capillary profiles in mOpsinhAng2 mice was observed compared to the littermates (mOpsinhAng2 20.4 ± 1.0 vs wild type 15.8 ± 0.9 vascular segments/section, $p<0.01$, Fig.

2C-2D). These data revealed that increased Ang2 expression in the retina promotes a stronger preretinal and intraretinal angiogenic response of capillaries.

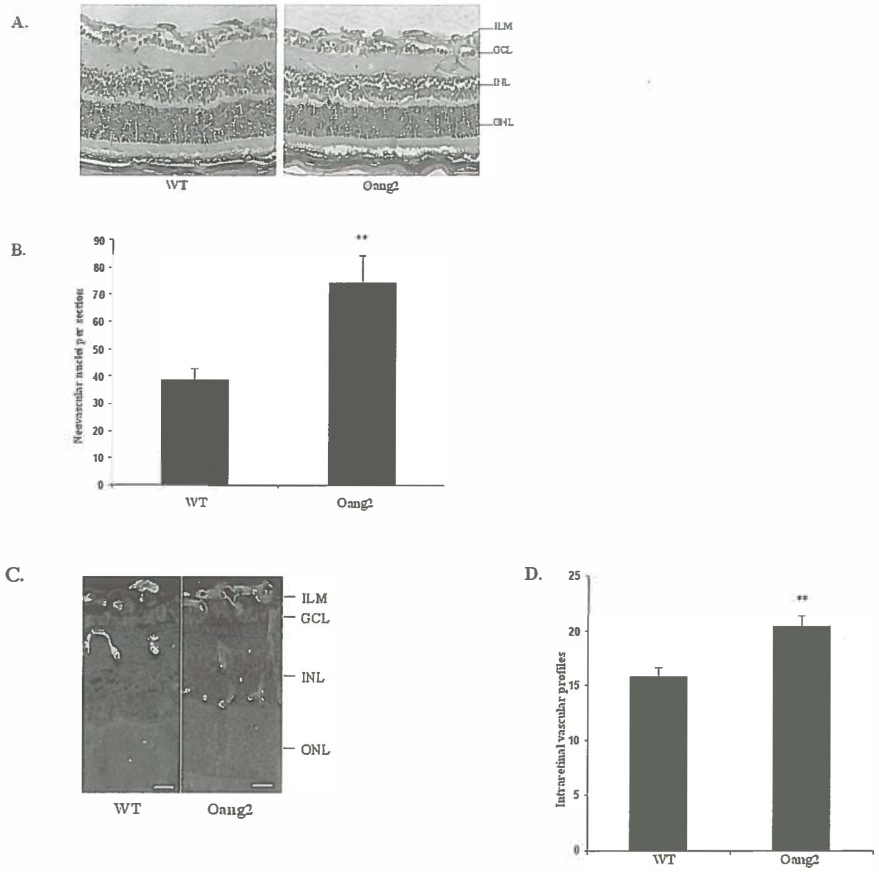


Figure 2:
Preretinal neovascularization and intraretinal vascularization of mOpsinhAng2 mice in the OIR model at p17. (A) representative example are shown for preretinal neovascularization analysis on six-µm paraffin sections of the retina stained with PAS from OIR model in mOpsinhAng2 mouse and its littermates. (B) Nuclei of neovascularization on the vitreal side of ILM are shown graphically. An increased preretinal neovascularization is observed in mOpsinhAng2 transgenic mice compared to its wild type littermates. (C) shows an example of capillary profiles counted on Lectin stained six-µm paraffin sections of retina in mOpsinhAng2 mouse and its wild type littermates. (D) Quantification of intraretinal blood vessel profiles displayed as a graphic. mOpsinhAng2 mice demonstrate significantly increased intraretinal neovascularization. Values are means ± SEM. n is 12 per group. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. WT, wild type; Oang2, mOpsinhAng2. ** $p<0.01$ compared to control WT group. Scale bar: A: 20 µm. C: 40 µm.

The examination of mitotic activity by double immunostaining with Ki67 and PECAM (Fig. 3A-3F) showed that mitotic cells were detectable both in the pre-retinal neovascularization and in the intra-retinal vascularization, confirming that new vessels in the eye in the OIR model are formed by angiogenesis.

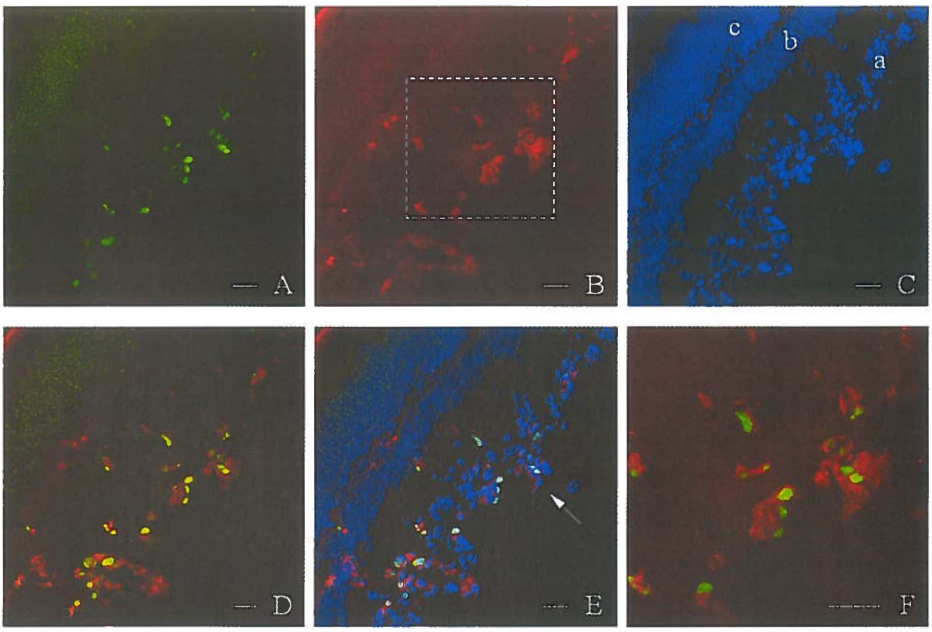


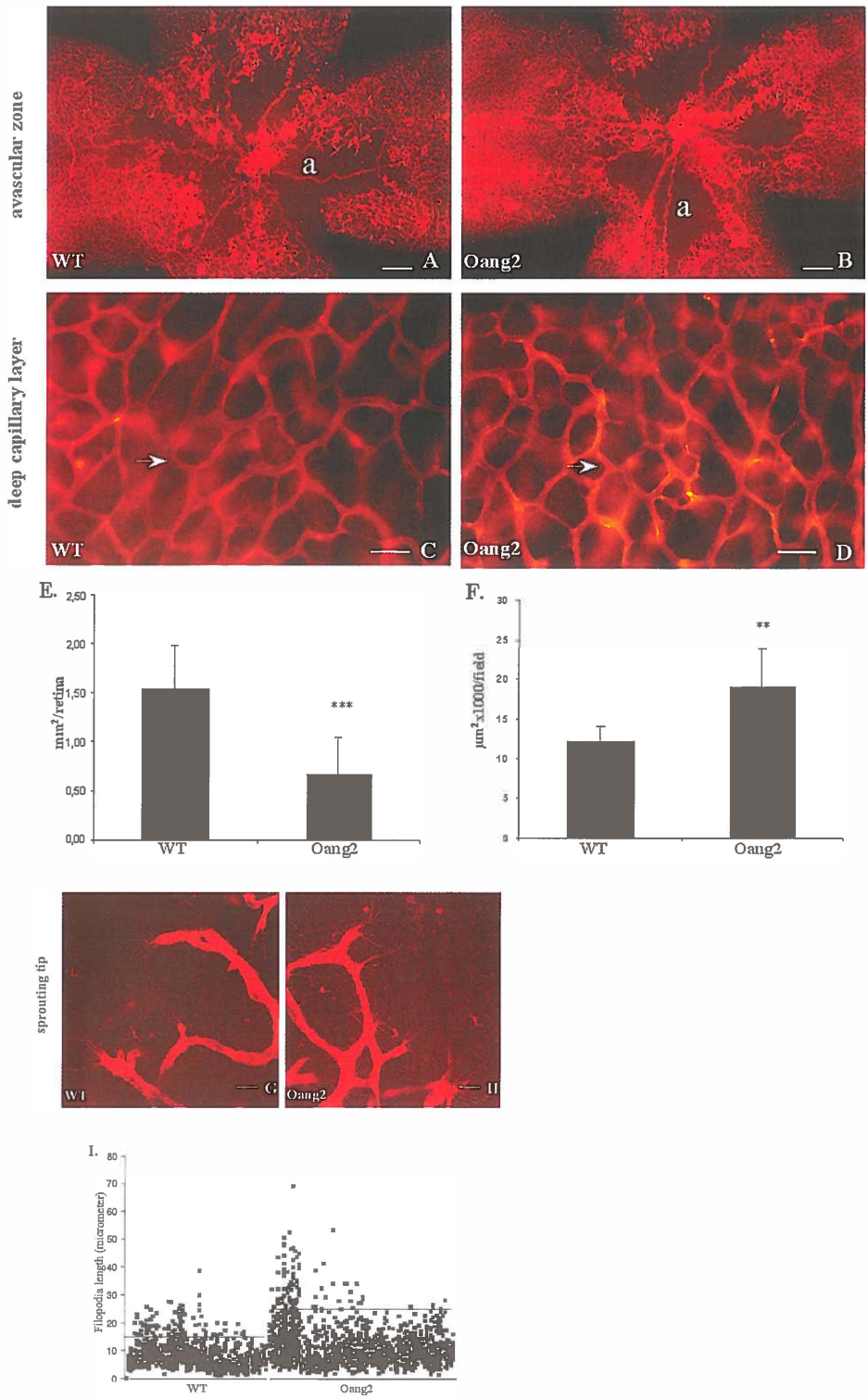
Figure 3:
Colocalization of Ki67 and PECAM-1 in the retina of OIR p17 mouse. A. Ki67 (green); B. PECAM (red); C. DAPI (blue); D. merged image of A and B, E. merged image of A, B and C; F. enlarged area of B in frame (Ki67 in green and PECAM in red). Arrow in E indicates preretinal neovascularization.
a. GCL; b. INL; c. ONL. Scale bar: 40 μm.

Using whole mount lectin staining we examined the response of the forming vessels to hypoxia. There was a consistently smaller central avascular zone in the mOpsinhAng2 retinae compared to wild type controls, confirming the observation that intraretinal vessels were more extensively formed (Fig. 4A, 4B, 4E). Of note, there was no difference between avascular zones of wild type (4.6±0.5 mm^2/retina) and mOpsinhAng2 mice (4.7±0.9 mm^2/retina) at p12, indicating the growth advantage under Ang2 overexpressing conditions.
We then examined the density of the deep capillary layers within a circular area of 1200 μm apart from the optic disc at p17. These

areas form only from p12 after the hyperoxic period, because the deep layer is still absent when the animals start hyperoxic exposure at p7 and does not form during p7-p12 because of lacking stimulus. Transgenic mOpsinhAng2 mice displayed an increase of capillary density in these areas compared to the wild type littermates (Fig. 4C, 4D). Quantitative analysis of the capillary density demonstrated a 58% higher vessel density (Fig 4F).
Vascular sprouts extending filopodia into the inner nuclear layer support orientation of the developing capillary network. As depicted in Fig 4G, 4H and 4I, filopodial extensions of the sprouting tips of vessels diving into the deep retinal layer in mOpsinhAng2 mice were significantly longer than the filopodia of wild type mice ($P<0.001$). Together with increased vascular densities in these mice, these data suggest that vessel formation may be more active under the influence of Ang2.

Increased retinal angiogenesis in the deep capillary layer in the OIR model is associated with less pericyte coverage
Angiogenesis is closely associated with pericyte coverage both in the physiological and pathological angiogenesis. Next, analysis of pericyte coverage was performed in double transgenic mice (mOpsinhAng2-XlacZ mice) in the OIR model at p17. The pericyte numbers were significantly decreased (26%) in the mOpsinhAng2 transgenic mice (Fig. 5A-5B).

VEGF and Ang1 levels are elevated in the mOpsinhAng2 mice in the OIR model
To investigate the molecular cross-talk of Ang2 overexpression with other growth factors such as VEGF and Ang1 in mOpsinhAng2 mice in the OIR model at day p17, we first designed a pair of primers recognizing a 113 base pair PCR product in both of mouse and human Ang2, in which human Ang2 sequences can be digested by restriction enzyme HpaI into 67bp and 46bp, but not mouse Ang2 (Fig. 6B). The melting curve analysis in real-time PCR confirmed the specific amplification of both human and mouse Ang2, which was further verified by digestion with restriction enzyme HpaI (Fig. 6A). The expression of total Ang2 in the mOpsinhAng2 mice was studied by real time PCR using this primer pair. A marked increase of Ang2 mRNA level was detected in the mOpsinhAng2 transgenic


avascular zone
a
WT
A
a
Oang2
B
deep capillary layer
WT
C
Oang2
D
E.
mm²/retina
2,50
2,00
1,50
1,00
0,50
0,00
***
WT
Oang2
F.
µm²x1000/field
30
25
20
15
10
5
0
**
WT
Oang2
sprouting tip
WT
G
Oang2
H
I.
Filopodia length (micrometer)
80
70
60
50
40
30
20
10
0
WT
Oang2

previous page

Figure 4

Representation of the influence of overexpressed retinal Ang2 on the capillary network of mOpsinhAng2 mice in OIR model at p17, confirming the accelerated retinal neovascularization. The whole mount retinae were stained with TRITC-labeled Lectin to demonstrate the vessel formation. (A, B) Examples show the narrowed avascular zones in mOpsinhAng2 mice. The area with a shows the central avascular zone. (C, D) The deep capillary layer is indicated by arrows within a circular area of 1200μm apart from the optic disc in whole mount retina. E and F illustrate quantification of avascular zones and deep capillary networks density, respectively. G and H display the sprouting tips in the deep capillary layer. I is the quantification of filopodia length in the deep capillary layer. Each dot indicates the length of one filopodium and the lines indicate the average of the longest filopodium. *** $p<0.001$ in E, ** $p<0.01$ in F, and $p<0.001$ in I compared to control group. n=8 for E and F. Data for I are collected from at least three individual retinae each group. WT, wild type; Oang2, mOpsinhAng2 transgenic. Scale bar: A and B: 200 μm, C and D: 40 μm. G and H: 20 μm.

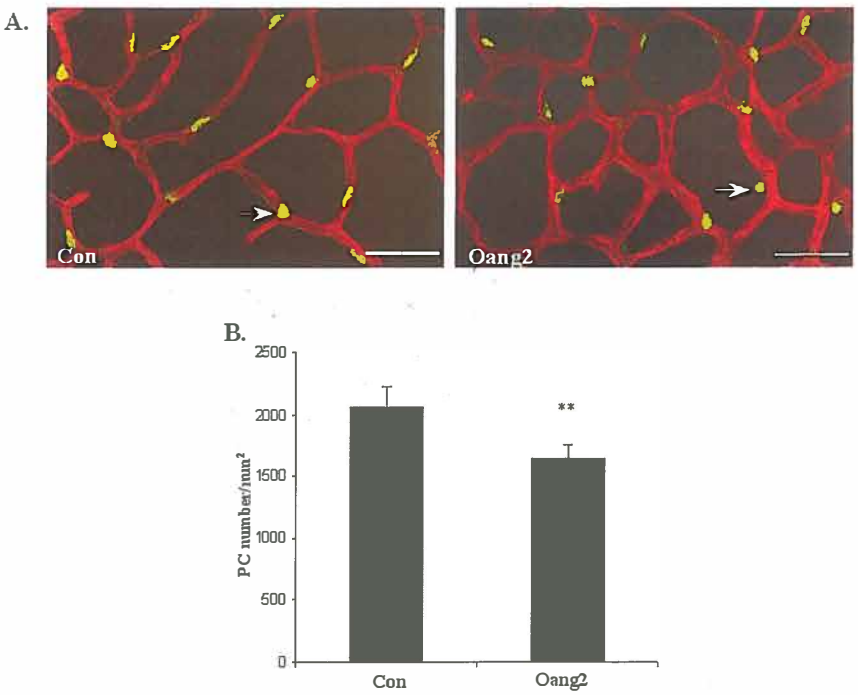


Figure 5:

Decreased pericyte coverage in overexpressed retinal Ang2 of mOpsinhAng2-XLacZ mice in OIR model at p17. (A) An example of a region for pericyte (arrowhead) coverage analysis in the deep capillary layer within a circular area of 1200μm apart from the optic disc. Pericyte coverage analysis (B) of mOpsinhAng2-XLacZ transgenic and XLacZ littermates. N is 5 for each group. ** $p<0.01$ compared to control group. Con, XLacZ transgenic; Oang2, mOpsinhAng2-XLacZ transgenic. Scale bar: 40 μm.

mice as compared with their littermates, suggesting overexpression of Ang2 (Fig. 6C).
Next, we examined whether the overexpression of Ang2 had any impact on the expression of growth factors implicated in the angiogenic response of the hypoxic retina. We therefore assessed the mRNA expression of VEGF and Ang1 in the OIR model. We determined a 79% increase of Ang1 and a 65% increase of VEGF mRNA expression (Fig. 6D, 6E). The expression levels of Tie2 and VEGFR2 were not changed in the retina of mOpsinhAng2 transgenic mice compared to the wild type mice subjected to the OIR model (data not shown).

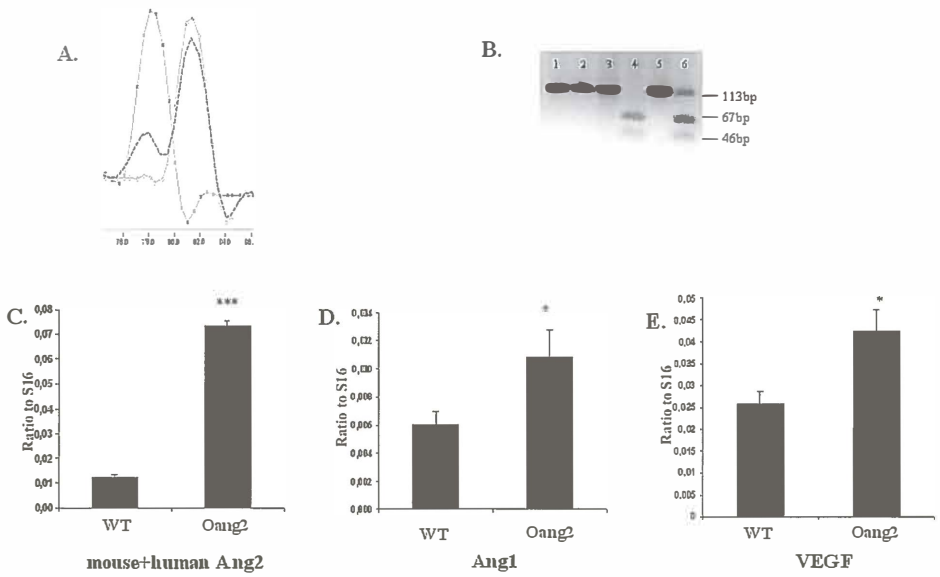


Figure 6:
Transcriptional analysis of angiogenic factors in retinae of mOpsinhAng2 and its wild type littermates from OIR model at p17. The specific amplification with mouse+human Ang2 primers was confirmed in Light cycler (A) by detecting the mouse (line with open diamonds) or human (line with black squares), mouse and human Ang2 (broken line) and on 2.5% agarose gel (B) after digestion by restriction enzyme HpaI which only cuts human 113bp Ang2 into 67bp and 46bp, but not murine. 1, 2 mouse Ang2; 3, 4 human Ang2; 5, 6 mouse+human Ang2; 1, 3, 5 without HpaI digestion; 2, 4, 6 with HpaI digestion. mRNA expression of mouse and human retinal Ang2 (C), Ang1 (D) and VEGF (E) was assessed by real-time PCR. Values are means ± SEM, n is 6 per group * $p<0.05$, *** $p<0.001$ compared to WT. WT, wild type; Oang2, mOpsinhAng2 transgenic.

Discussion

This study shows that transgenic overexpression of Ang2 in the photoreceptor layer of the retina reduces pericyte coverage of retinal capillaries of the deep layers and increases retinal neovascularization in the OIR mouse model. As novel observation, more preretinal vessels and a denser deep capillary vessel network were found. In addition, elevated retinal expressions of VEGF and Ang-1 were also found with Ang2 overexpression in the OIR model.

The dual functional role of the Ang-Tie system in pericyte recruitment has evolved from data showing that Ang1 signaling via Tie2 determines vascular (capillary) maturation, reduced leakiness, and resistance to growth promoting cytokines/survival factors (13, 18). In turn, hypoxic upregulation of the Ang1-inhibiting natural antagonist Ang2 was found to disrupt endothelial pericyte crosstalk (36). Our study provides evidence that during physiological development, pericyte coverage of capillaries in the vicinity of Ang2 overexpression is lowered. A shift in the balance between Ang1 and Ang2 with a resulting predominance of Ang2 can be expected as the underlying cause of inhibited pericyte recruitment. However, the exact mechanism by which Ang2 inhibits signaling leading to impaired pericyte recruitment remains undetermined. In vitro, the stabilizing and endothelial survival promoting effect of Ang1 is likely attributable to the inhibition of the forkhead transcription factor Foxo-1 (37). Several groups of genes involved in important vascular functions such as matrix remodeling and angiogenesis are dependent on Foxo-1 transcription, including Ang2 (38, 39). The current hypothesis is that the effect of Ang2 is due to its ability to block stabilizing signals provided by Ang1. Multiple other systems may be affected as a result of the shift in the Ang1/Ang2 balance such as the PDGF-B/receptor ß pair, and the expression of heparin-binding epidermal growth factor (40, 41).

In our experiments, Ang2 overexpression starts around birth, and according to our data, is active at least at p7. The effect on pericyte recruitment is measurable as early as p10. The level of pericyte loss is similar to the magnitude obtained when Ang2 is injected intravitreally into juvenile rats (15). In both cases, Ang2 acts distant from its site of administration/overproduction, suggesting that although Ang2 action is particularly dependent to three-dimensional interaction with the receptor, it can be spatially active over a considerable

distance.
When pericytes are not recruited to the developing capillaries, the underlying endothelial cells may be more susceptible to vasoregression. This had been observed in mice with a heterozygous deletion of PDGF-B, in which the number of acellular capillaries was 50 % higher than in wildtypes, and pericyte numbers recruited to the capillaries were reduced to a similar level as in the Ang2 overexpressing mice in this study (42). Oshima et al used transgenic mice with inducible expression of Ang2 and demonstrated that these mice had normal retinal circulations illustrating that Ang2 overexpression did not necessarily induce vessel regression (31). Not only do pericytes protect endothelial cells from regression, but also had earlier data suggested that Ang2 in the absence of high VEGF levels would induce vasogression in a variety of different vascular beds (14, 21-23). However, according to the present study, and the one by Oshima et al, analyses did not show vascular regression in the retinae of transgenic mice. Although levels of VEGF may be low in the developing retina, their distinct dose-dependent effect on endothelial cell survival may be sufficient to prevent developing vessels from regression. Furthermore, other growth factors, such as PDGF-B could work as backup, in particular when pericyte numbers are low and autocrine sources are therefore limited.
Mice with a reduction in PDGF-B have less pericytes, and less protection against vessel regression than wild type mice. The transient formation of a denser deep capillary network in Ang2 overexpressing mice, both in our experiments, and also reported by Oshima et al., suggest that sensitivity against Ang2 changes during retinal vessel development. The concept that multiple, partially unknown survival signals may explain the elusive effect of Ang2 on the developing vessels is supported by the notion that, with time, the agonist Ang1 increases rendering the vessel again insensitive to regressive signals, but also inhibiting the effect of Ang2 (in cooperation with VEGF) on vascular network formation. Ang1 is upregulated when the deep layer forms (43), and it may have taken some time until the primary effect of Ang2 had been compensated. In addition, we observed that the superficial layer was unaffected both by pericyte loss and by increased vessel formation in the development. This is consistent with a spatially limited mode of action of Ang2 in this specific environment.

The cooperation of Ang2 and VEGF in angiogenesis, and in particular in proliferative retinopathy has been well established. As a novel observation, we found that not only vessels from the remaining superficial network penetrated into the vitreous, but also more vessels traversing the inner plexiform layer were present, suggesting intraretinal vessel formation. We measured sevenfold higher expression of Ang2 compared with WT hypoxic retinae in our transgenic animals. From in-vitro studies it was noted that high concentrations of Ang2 can mimic Ang1 in enhancing endothelial survival through activation of Tie2 (44). Thus, since the deep capillary layer was absent before the exposure to hyperoxia, and was denser after a period of hypoxic stimulus, we conclude that Ang1 was somehow involved in de novo formation of intraretinal vessels under hypoxic stress in combination with Ang2.
As possible hint for improved intraretinal vascular repair, we observed longer filopodia in Ang2 overexpressing retinae. Filopodia extend from tip cells, and express VEGFR2 to guide sprouting angiogenesis (35). VEGF concentrations determine the extension of neovascularization while the VEGF gradient accounts for migratory activity of tip cells. It is thus conceivable that the higher level of VEGF together with improved intraretinal guidance may account for intraretinal vessel regrowth as a specific repair phenotype. This is a crucial demand for clinical cases in which intraretinal vessels have been permanently destroyed.

Acknowledgements

We thank Nadine Dietrich, Anja Rippert, and Petra Bugert for excellent technical assistance and animal care. This study was supported by grants from the German Diabetes Association and the Deutsche Forschungsgemeinschaft, SPP 1069 (HA1755/7-2) for YF, GRK 880 for FvH.

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Chapter III

The absence of Angiopoietin-2 leads to abnormal vascular maturation and persistent proliferative retinopathy

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Abstract

Angiopoietin-2 (Ang-2) antagonizes the maturing effect of angiopoietin-1 (Ang-1) on blood vessels, and cooperates with VEGF to induce neovascularization. In knockout mice, Ang-2 displayed a specific role in postnatal angiogenic remodeling. Here, we demonstrate that mice deficient in Ang-2 fail to form a proper spatial retinal vascular network. The retinal vasculature was characterized by reduced large vessel numbers and defects forming the superficial periphery mostly on the arteriolar site, and the secondary and tertiary deep capillary network. Hypoxia in the retinal periphery induced a fourfold VEGF upregulation and active endothelial proliferation for up to sixty days. Concomitantly, retinal digest preparations showed increased arteriolar (+33%) and capillary diameters (+90%), and fluorescein angiograms revealed leakiness of neovascular fronds. At one year of age, persistent preretinal vessels were non-leaky in accordance with a relative increase in the ratio of Ang-1 to VEGF. Taken together, the data suggest that Ang-2 has an important function in the spatial configuration of the three-dimensional retinal vasculature. Secondarily, prolonged VEGF activity results in a model of persistent proliferative retinopathy.

Introduction

Neovascularization is a key component of eye diseases such as proliferative diabetic retinopathy and age-related macular degeneration (1, 2). Several factors are central to specific steps during vascular development while others play additive or modulating roles (3). Many of the events during formation of the vasculature in the embryo are recapitulated during pathological neovascularization in the adult. Vascular endothelial growth factor (VEGF) is necessary to form a primitive vascular plexus during embryogenesis. The upregulation of angiopoietin-1 (Ang-1) marks the transition from an immature to a stable network and is associated with matrix formation and pericyte recruitment through signaling of the receptor tyrosine kinase Tie-2 (4). In contrast, angiopoietin-2 (Ang-2), the natural antagonist of angiopoietin-1 (Ang-1), inhibits phosphorylation of Tie-2 induced by Ang-1. Although there are exceptions, Ang-2 upregulation is a crucial step in postnatal vessel destabilization, for example in female reproduction and tumor vessel formation (5). Subsequent vascular responses are context-dependent. In collaboration with VEGF, Ang-2 is thought to be indispensable for sprouting angiogenesis. In the absence of VEGF, Ang-2 promotes vessel regression. Vessel regression induced by chronic hyperglycemia is a premise for hypoxia which induces new vessel formation in the retina of longstanding diabetic patients. Both, VEGF and Ang-2 are hypoxia-inducible, suggesting an essential role in the pathogenesis of proliferative diabetic retinopathy (6, 7). Interestingly, both factors are also inducible by specific diabetes-induced biochemical abnormalities, such as hyperglycemia, reactive oxygen species, and advanced glycation end products (8-12). While the upregulation of VEGF in a diabetic animal indicates increased need for support of endothelial survival, Ang-2 upregulation precedes diabetic pericyte loss, an early hallmark of incipient retinopathy (9). Intravitreal injection of recombinant Ang-2, and intraocular overexpression of Ang-2, both, induce pericyte loss in non-diabetic rats and mice, and aggravate pericyte loss in diabetic mice (9, 13). In turn, diabetes induced pericyte loss is inhibited in diabetic heterozygous Ang2LacZ mice which are partially Ang2 deficient. Since pericyte loss is considered as an early step of destabilization in the diabetic retina, Ang-2 upregulation opposing the maturing effect of Ang-1 during developmental retinal angiogenesis, is a key element of diabetic vessel re-

modeling. At later stages of diabetic eye disease, Ang-2 and VEGF cooperate to induce new retinal vessels, as indicated by increased levels of VEGF and Ang-2 in the vitreous of patients with proliferative diabetic retinopathy (7, 14, 15).
Since Ang-2, in contrast to VEGF and Ang-1, is dispensable for embryonic development, its function in postnatal vascular remodeling can be studied. Using homozygous Ang-2 knockout mice (Ang2-/-), Gale et al. demonstrated a rudimentary superficial vascular plexus restricted to the central areas of the retina (16). Peripheral absence of vessels was compensated by persistence of the hyaloid vessel system, which is usually regressed. Ang-2 expression, as assessed by a LacZ reporter, was observed in the central artery, in the vessel wall of large arteries, in vascular sprouts, and in the persistent hyaloid vessels. This observation is consistent with a dual role of Ang-2 in context-dependent sprouting angiogenesis and vascular regression, and a possible role in arterial function. Inside the retina, where VEGF levels are high in the presence of hypoxia, sprouting angiogenesis was promoted, while in the hyaloid vessels, in which VEGF is progressively turned off, Ang-2 deficiency led to the failure of vasoregression. Consistent with the dual function of Ang-2 in this early model, Ang-2 expression was found in vascular sprouts forming the superficial retinal vascular plexus, and in persistent hyaloid vessels. However, the role of Ang-2 as a complex regulator of vascular functions could not be studied because only very few animals survived beyond postnatal day 14. With continuous backcrossing of these animals to the C57BL/6J background, Ang2-/- mice became available which survived for as long as one year. Using these animals, we studied the function of Ang-2 in retinal vascular development with special emphasis on microvascular patterning and remodeling over a prolonged period.

Materials and methods

Animals

All experiments in this study were performed according to the guidelines of the statement of animal experimentation issued by the association for Research in Vision and Ophthalmology and were approved by the Institutional Animal Care and Use Committee.
Mice with a mixed background (129/J and C57BL/6J) carrying the LacZ reporter gene under control of mouse Ang-2 promoter exhi-

biting disrupted Ang-2 transcription were kindly provided by Regeneron Pharmaceuticals, Inc.. After breeding heterozygous Ang2LacZ mice to C57BL/6J mice for 10 generations, heterozygous Ang2LacZ mice were used to generate Ang2-/- and wildtype littermates for this study. Genotyping was performed with specific primers as has been described previously (17). Eyes were obtained from postnatal day 10 (p10), p20, p60 and one-year old animals under deep anaesthesia. The eyes were enucleated and fixed in 4% formalin for histological analysis or frozen immediately at –80°C for other analysis.

Retinopathy of prematurity (ROP) mouse model
Wildtype mice (C57BL/6J) were investigated for morphological comparison of preretinal neovascularization using the established mouse ROP model (18). Briefly, newborn pups at the age of 7 days were exposed to high oxygen for 5 days and returned to room air. Eyes were obtained at p17 for qualitative analysis of neovascularization using periodic-acid Schiff reagent (PAS) (6) stained vertical paraffin sections. Analysis of retinal histology and quantification of retinal neovascularization were performed on paraffin sections stained with PAS as described previously (18).

Whole mount immunofluorescence staining
Retinas for whole mount immunofluorescence staining were permeabilized in PBS with 1% BSA and 0.5% Triton X-100 at 4°C and incubated with FITC-conjugated lectin of Bandeiraea simplicifolia (1:50, Sigma, Munich, Germany) at 4°C. For double staining, the retinas were incubated with Cy3-conjugated smooth muscle actin (SMA, 1:100, Sigma, Munich, Germany) or rabbit-anti mouse Ki67 (1:100, Abcam, Cambridge, UK) overnight. Swine-anti rabbit IgG labeled with TRITC (1:20, DAKO, Hamburg, Germany) was used as secondary antibody to Ki67. Retinas were flat mounted in 50% glycerol and photographs were taken with a Leica DMRBE microscope connected to a video camera (Leica, Wetzlar, Germany) and where appropriate, with a confocal microscope (Leica TCS SP2 Confocal Microscope, Leica, Wetzlar, Germany).

Retinal digest preparation and quantitative vessel analysis
Eyes were obtained from p60 Ang2-/- mice and their wildtype litter-

mates (n = 5-7 for each group). Retinal vascular preparations were performed using a trypsin digestion technique as previously described (9). Briefly, the retinas stained with or without LacZ (see below) were incubated in 3% trypsin solution resolved in 0.2 mol/L Tris buffer (pH 7.4) for 120 min. For analysis of intraretinal vascular morphometry, the vessels above the inner limiting membrane were carefully removed. Subsequently, the retinal digest preparations were carefully washed with aqua bidest and flat mounted on slides. Finally, the samples were stained with PAS. Analysis of retinal vascular phenotype in Ang2-/- mice was performed on retinal digest preparations stained with PAS. The numbers of arterioles and venules were counted in PAS stained retinal digest preparations of 2 month old wildtype (n=5) and Ang-2 deficient mice (n=7). The diameters of retinal arterioles and venules were measured 200 micrometer distant from the retinal center using quantitative retinal image analysis (Analysis Pro System; Olympus Opticals, Hamburg, Germany). Capillary diameters were quantified 800 micrometer distant from the retinal center in the capillary area adjacent to venules and to arterioles (n=5). In Ang2-/- mice, capillary diameters were measured at the same distance from the retinal center in the area adjacent to the venules (Ang2v) and within the defective arteriolar tree (Ang2a) (each n=6).

LacZ staining

LacZ staining of whole-mount retinas was performed as described previously (9). Frozen eyes of mice at p60 were fixed in phosphate buffer (100 mM, pH 7.3) containing 1.5% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde for 20 min at room temperature. Subsequently, eyes were washed three times for 15 min in washing buffer [2 mM $MgCl_2$, 0.01% (w/v) sodium deoxycholate, 0.02% (v/v) Nonidet P-40 in 100 mM phosphate buffer, pH 7.3]. For preparation of the staining solution 0.1% X-gal (Roche Diagnostics GmbH, Mannheim, Germany), 5 mM $K_4Fe(CN)_6$, and 5 mM $K_3Fe(CN)_6$ were dissolved in washing buffer. The eyes were incubated in the staining solution over night at 37°C. On the next day, the staining solution was changed against washing buffer alone and retinas were dissected.

SLO Angiography

To visualize the vascular changes in the eyes of Ang2-/- in situ, we used indocyanine green (ICG; infrared laser, 795 nm; barrier, 800 nm) and fluorescein (FL; argon blue laser, 488 nm; barrier, 500 nm) Scanning Laser Ophthalmoscope (SLO) angiography (A) (Heidelberg Retina Angiograph; Heidelberg Engineering GmbH, Dossenheim, Germany). FLA followed subcutaneous injection of 75 mg/kg body weight fluorescein-Na (University Pharmacy; University of Tuebingen, Germany), and ICGA followed subcutaneous injection of 50 mg/kg body weight ICG (ICGPulsion; Pulsion Medical Systems AG, Munich, Germany) (19).

Determination of Retinal Hypoxia

To identify whether the abnormal retinas in Ang2-/- mice were suffering from hypoxic environment, pimonidazole as hypoxia marker from the Hypoxyprobe-1-Plus® kit for the detection of tissue hypoxia (Chemicon International, USA) was dissolved in 0.9% NaCl (20). A dose of 60mg/kg body weight was injected i.p. in p10 wildtype and Ang2-/- mice. After circulation for 4 hours, mouse eyes were dissected and fixed immediately in 4% formalin. Staining was performed on 6µm paraffin sections according to the manufacturer's protocol. For colocalization with vessels in p10 retinas, slices were subsequently stained with TRITC-conjugated lectin of Bandeiraea simplicifolia (1:50, Sigma, Munich, Germany) and DAPI.

RNA isolation and quantitative PCR

Retinal RNA was isolated from individual retinas homogenized in 1ml Trizol reagent (Invitrogen, Karlsruhe, Germany) at 4°C according to the manufacturer's instructions. RNA was reverse transcribed with QuantiTect Reverse Transcription kit (Qiagen GmbH, Germany) and subjected to Taqman analysis using the Taqman 2xPCR master Mix (Applied Biosystems, Weiterstadt, Germany). The expression of genes was analysed by the $2^{-\Delta\Delta CT}$ method using 18s ribosomal RNA as housekeeping control. Additionally, the expression of CD 31 was used as control to correct for the possible impact of reduced vessel (endothelial cell) numbers in Ang2-/- mice. All primers were designed according to the specific sequences in the Genbank and purchased from IBA (Göttingen, Germany) and primers and FAM-labeled Taqman TAMRA probes used were: S18: forward

5'-ACC ACA TCC AAG GAA GGC AG-3' reverse 5'-TTT TCG TCA CTA CCT CCC C-3', probe 5'-AGG CGC GCA AAT TAC CCA CTC CC-3'; VEGF: forward 5'-CGA GAT AGA GTA CAT CTT CAA GCC G-3' reverse 5'-TCA TCG TTA CAG CAG CCT GC-3' probe 5'-CTG TGT GCC GCT GAT GCG CTG-3'; Ang-1: forward 5'-CAA CAA CAG CAT CCT GCA GAA-3', reverse 5'-CTT TAG TGC AAA GGC TGA TAA GGT T-3', probe 5'-CAA CAA CTG GAG CTC ATG GAC ACA GTT CA-3'; VEGF164: forward 5'- TGA ATG CAG ACC AAA GAA AGA CAG-3', reverse 5'-GGA TCT TGG ACA AAC AAA TGC TT -3', probe 5'-AGA AAA TCA CTG TGA GCC TTG TTC AGA GCG -3'; VEGF188: forward 5'-GCG CAA GAA ATC CCG GT -3', reverse 5'-TGC TTT CTC CGC TCT GAA CA -3', probe 5'-AAA TCC TGG AGC GTT CAC TGT GAG CC -3'.

Statistical analysis

Data are expressed as mean ± SD unless otherwise stated. Unpaired T-test for quantification of intraretinal vessel parameters and for quantitative RT-PCR data was used. A value of $p < 0.05$ was considered statistically significant.

Results

Mice develop proliferative retinopathy in the absence of Ang-2 at postnatal day 10

The retinal vascular network in mice develops exclusively after birth with a progressive and continuous formation of a superficial and deep vascular system and a concomittant regression of the hyaloid vessel system. First, we assessed the development of the retinal vascular system of Ang2-/- mice backcrossed to the C57BL/6J background. Compared with wildtype retina at p10 and p17 (Fig 1 A and B), vertical paraffin-embedded PAS-stained sections at p10 revealed vascular profiles protruding from vessels of the superficial network through the inner limiting membrane and invading into the vitreous (Figure 1 C). Quantitatively, preretinal neovascularizations were significantly increased in Ang2-/- retinas at p10 compared with wildtype retinas (14.60 ± 1.30 in Ang2-/-, n=5; 0.35 ± 0.11 neovascular nuclei/per section in wildtype mice, n=6, mean ± SEM, $p<0.001$). Qualitatively, the preretinal vessels were identical to the neovascularization observed in the mouse ROP model (Figure 1 D).

The hyaloid vessels persisted at postnatal day 10 in Ang2-/- mice on the C57BL/6J background, while the hyaloid vessel system had largely regressed in wildtype retina (data not shown).
To further analyse spatial retinal vascularization, we used whole mount lectin staining and compared Ang2-/- with normal wildtype and ROP mouse retinas. Preparations of wildtype mice at p10 and p17 revealed proper extension of the superficial vasculature over the entire retina with regular vessels protruding perpendicularly into the deeper layers to form a dense deep capillary network in the inner nuclear layer (Fig. 1 E, F, I, J, M, N). Outgrowth of the superficial retinal vessels of Ang2-/- mice was impaired with vessels covering 30% of the entire retina (Figure 1 G). In contrast to wildtype mice, a deep capillary plexus had not formed and the network of small vessels showed irregular patterning and substantial larger diameters (Fig. 1 K and 1 O) than wildtype capillaries (Fig. 1 I, 1 J and 1 M, 1 N). Compared with Ang2-/- mice, retinal capillaries in the superficial layer of ROP mice at p17, i.e. during strongest neovascular response, were smaller in diameter and less tortuous (Fig. 1 L). Preretinal neovascular tufts of Ang2-/- mice originated from the venular site of the superficial vasculature, which corresponds to the origination of neovascular tufts in ROP mice which were similar in size and numbers (Fig. 1 O and P). However, while neovascular tufts of wildtype ROP mice form at the border to the hyperoxia-induced avascular zone (Fig 1 H, white arrows), neovascular tufts in the Ang2-/- mice grow in the intermediate third of the vascularized part of the retina (white arrow in Fig. 1 G).
Co-immunostaining with lectin and smooth muscle actin (SMA) was used to confirm that neovascular tufts of Ang2-/- mice formed in the vicinity of intraretinal venules. As depicted in Fig. 1 R, arterioles of wildtype retina at p10 were strong SMA-positive while capillaries and venules were negative. Arterioles of the p10 Ang2-/- retinas were positive for SMA as was the adjacent area of irregularly patterned capillaries (marked by the asterisk in Fig. 1 T), while the network adjacent to venules in which neovascular tufts were discernible was weak SMA-positive (marked by # in Fig. 1 T).

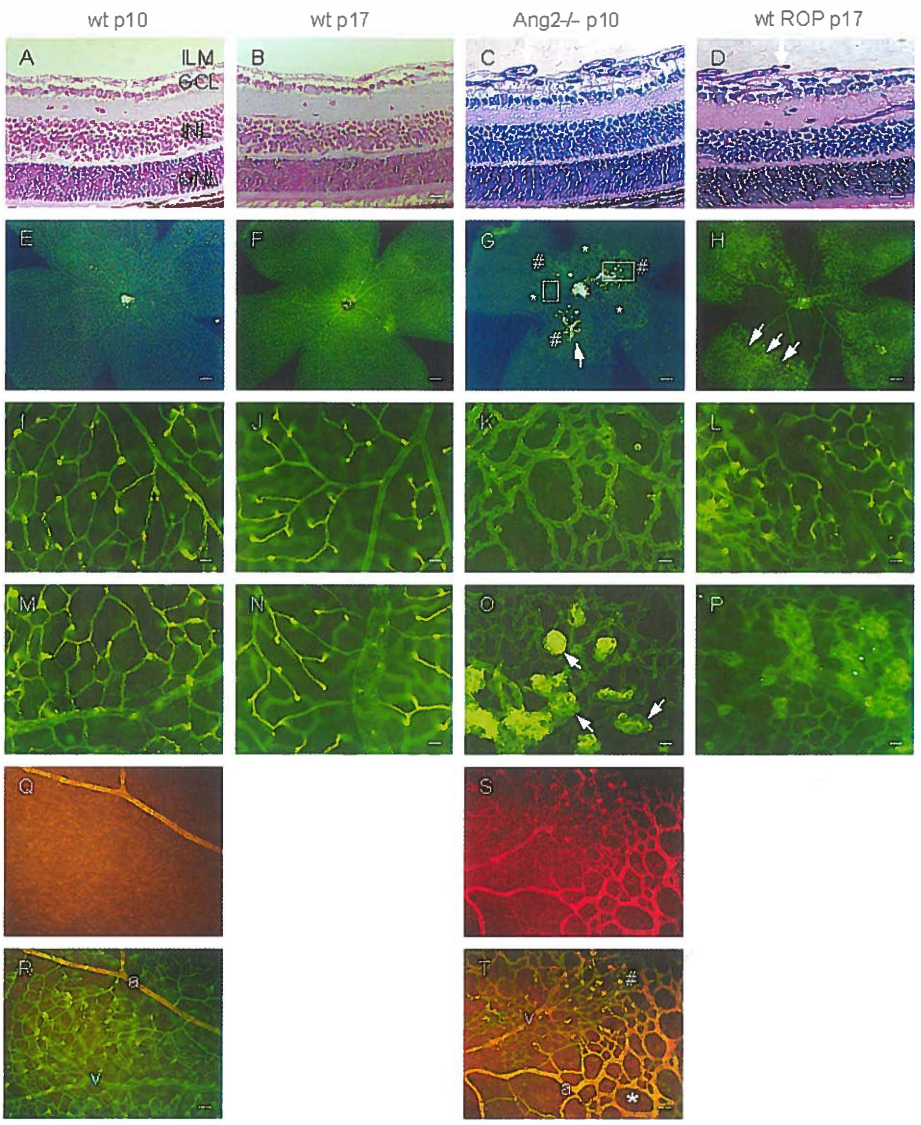

Figure 1: Proliferative retinopathy develops spontaneously in the absence of Ang-2 at p10.
Representative retinal PAS-stained paraffin sections of control wildtype (wt), ROP-wildtype and Ang2-/- mice (A-D) were used to determine histological changes and preretinal neovascularization at p10 (C) and ROP exposed wildtype mice at p17 (D). Both retinas show preretinal neovascular tufts of equal appearance, as indicated by arrows in C and D. A and B: non-exposed wt controls at p10 (A) and p17 (B); absence of preretinal neovascularization. E-H: Overview of retinal whole mount preparations of wt control (p10 E; p17 F), Ang2-/- (p10, G) and ROP-wt

mice (p17, H) stained with lectin to visualize the retinal vasculature. In contrast to regular vascular patterning in wt controls (E, F), three areas were discernible in Ang2-/- mice with increased (#) or reduced (*) capillary densities (G). Preretinal neovascular tufts developed from the areas of increased capillary density (arrow in G). Neovascular tufts in wt ROP developed at the border to the hyperoxia-induced avascular zones (arrows in H). I-P: Higher magnification of arteriolar (I-L) and venular (M-P) site of wt mice (p10 I+M, p17 J+N), Ang2-/- (K+O) and wt ROP mice (L+P). White box close to * in G marks the area of reduced capillary density of Ang2-/- mice magnified in K; white box close to # marks the corresponding area magnified in O indicating increased vascular density and neovascular tufts (arrows in O). Neovascular tufts of wt ROP (H, P) and Ang2-/- (G, O) were phenotypically comparable. Q-T: Co-staining of SMA (red) and lectin (green) was used to distinguish arterioles from venules in wt (Q: SMA; R: SMA+lectin) and Ang2-/- mice (S: SMA; T: SMA+lectin) at p10. "a" marks an arteriole and "v" a venule. * and # in T mark corresponding areas in G. ILM: inner limiting membrane; GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Scale bars: 25 μm (A-D, I-P); 200 μm (E-H). 100 μm (Q-T)

Proliferative retinopathy at p10 is induced by hypoxia in Ang2-/- mice

To evaluate the contribution of hypoxia to the phenotype of proliferative retinopathy in the Ang2-/- mouse, we analyzed the spatial pattern of hypoxia at p10. As shown in Figures 2 A, C, E, and G, hypoxia is absent in p10 wildtype control mice. In lectin-stained vertical sections (Fig. 2 B) the outgrowth of the retinal vascular plexus covered only the central part of the retina, while the periphery was avascular. Hypoxyprobe staining was strong in the avascular periphery and in the innermost layers of the retina from the inner limiting membrane to the outer part of the inner nuclear layer (Fig. 2 D). These data suggest that the stimulus to induce preretinal vessel outgrowth resides in the retinal periphery towards which vessels were insufficiently growing in the absence of Ang-2.

Preretinal neovascularization persist and progress through p20

The increased survival of the Ang2-/- mice enabled us to study the retinal vascular phenotype from p10 onward with a focus on the development of the deep capillary layers and the neovascularization. At p20, the extent of neovascularization and the affected areas were increasing. Apart from the persistent hyaloid vessel system, two distinct layers of intravitreal vessel networks (i.e. neovascularization) were discernible after p10, one directly on the surface of

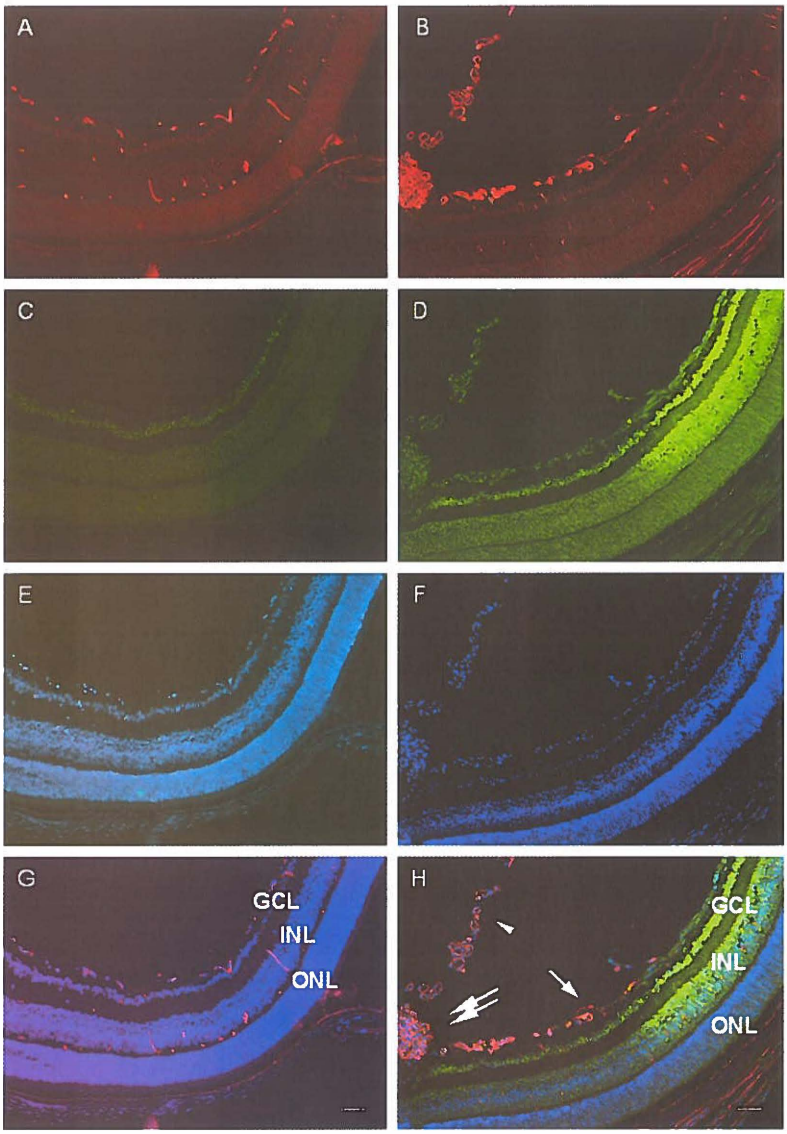

Figure 2: Persistent vascular outgrowth defect despite peripheral hypoxia. Retinal paraffin sections stained with lectin (A, B), hypoxyprobe (C, D) and DAPI (E, F) of wildtype (A, C, E, G) and Ang2-/- mice (B, D, F, H) at p10. G, H: merged images. Intense positive hypoxyprobe staining (D) throughout the peripheral inner retinal layers (from the inner limiting membrane towards the outer part of the inner nuclear layer) of Ang2-/- mice, which is devoid of vessels as shown in B. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer; H: Arrow: superficial retinal vessels; double arrow: vessel penetrating into the vitreous from the optic nerve area; arrowhead: persistent hyaloid vessels. Scale bars: 50 μm.

the retina in proximity to the inner limiting membrane, and one in intermediate distance between the retinal surface and the hyaloid vessel system (Figure 3 B). Figure 3 A shows an age-matched wild-type retina as control demonstrating the complete regression of the hyaloid vessels at this point.
Comparison of lectin-stained whole mount preparations of wild-type (Fig. 3 C) and Ang2-/- mice (Fig. 3 D) demonstrates the impairment of peripheral vascular outgrowth by the absence of Ang2. Retinal areas of increased vascular density alternated with areas of decreased density. Retinal arterioles appeared tortuous and occasionally anastomosed with neovascular loops arising from the adjacent venular sites (Fig. 3 D and K and supplementary Fig 1, videos). Preretinal neovascularization predominated on the venular sites of the network (# in Fig. 3 D and supplementary Fig 1, video2). Ki67-positive cells were found in lectin-positive areas at p20, and p60 in Ang2-/- mice suggesting active endothelial proliferation (supplementary Fig. 2).
Figures taken by confocal microscopy of lectin stained whole mounts 3 E-H depict the developmental status of retinal vessels in a wildtype mouse at p20 in the superficial layer (E, G) on the venular (E) and the arteriolar (G) site and the corresponding areas of the deep capillary layers beneath the venules (F) and the arterioles (H). Figures 3 I-L show the corresponding areas of Ang2-/- mice. On the venular site, neovascularization was present (marked by the white circle close to the diamond in Figure 3 D; higher magnification in Fig. 3 I). Underneath this area an incomplete deep capillary network had formed, however, with a normal appearing pattern (Fig. 3 J). On the arteriolar site (marked by the white circle in Fig. 3 D close to the asterisk) the development of the intraretinal deep capillary layer was substantially affected by the lack of Ang-2. The deep capillary network failed to form underneath the peripheral irregular tortuous and ramified arterioles. Multiple blind-ending sprouts were present at the growing edge indicating persistent neovascular activity.
These data are consistent with a possible functional role of Ang-2 in peripheral vascular outgrowth, three-dimensional vascular patterning and capillarisation of the arteriolar site.

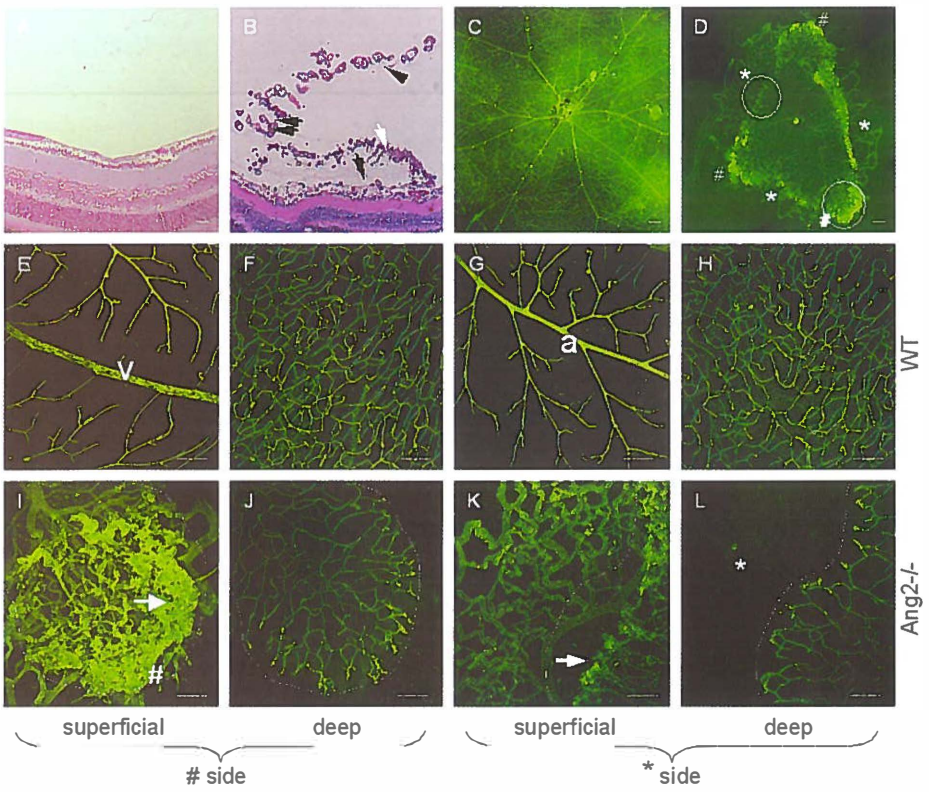

Figure 3: Persistent neovascularization at p20.
A, B: Vertical PAS-stained paraffin sections of wt control (A) and Ang-/- retinas (B) at p20.
A: no preretinal vessels are discernible in controls. B: Three layers of intravitreal vessels are discernible: persistent hyaloid vessels (black arrowhead), one intermediate layer distant from the retina (white arrow), and preretinal neovascularization above the inner limiting membrane (black arrow). Double arrow: points towards the entry of the optic nerve. C, D: Lectin stained retinal whole mounts of wt control (C) and Ang2-/- mice (D) at p20. C: Regular patterning of arterioles, venules and the capillary network. D: Areas of preretinal neovascularization develop at the venular edges of the sprouting superficial intraretinal vasculature. # and * mark areas of increased (#) and reduced (*) densities. E-L: confocal images of lectin-stained whole mount preparations of wt control (E-H) and Ang2-/- retinas (I-L) showing venolar and arteriolar sites and adjacent capillary areas in the developing superficial and deep layers. In contrast to wildtype controls, a capillary network has formed in the deep layer (J) underneath the venular plexi (I), whereas no vessels are detectable (* in L) underneath the underdeveloped arteriolar plexus (K). Dotted white line in I and K indicate the vascular edge, and are indicated in the deep layers for orientation. White arrows in I and K: preretinal vessels. v in E: venule; a in G: arteriole. Scale bars: 50μm (A,B), 200μm (C, D), 100μm (E-L).

Abnormal intraretinal vessels in p60 Ang2-/- mice

We studied remodeling and maturation of the intraretinal vasculature in mice at p60 using retinal digest preparations. Figures 4 A-C depict the vascular patterning around arterioles (a in Fig. 4 A and C), and venules (v in Fig. 4 A and B), and the capillary networks of the superficial and deep layers of wildtype mice. Compared with wildtype retinas (Fig. 4 A), Ang2-/- mice exhibited a sustained failure of vascular development, particularly on the arteriolar site. Arteriolar outgrowth was only 30-40% of the full distance of wildtype counterparts (Fig. 4 D). Arterioles were hypercellular, tortuous, exhibited focal dilatations and microaneurysms (Fig. 4 F), and were surrounded by large avascular zones. A severely distorted capillary network with multiple empty basement membrane sheets formed predominantly around arterioles (Fig. 4 F). Arteriovenous shunts were occasionally observed. Venules attained approximately 50 % of the outgrowth of the wildtype counterparts, and the capillary plexus surrounding venules was less dense compared to wildtype mice.

Quantitative analysis of the retinal vasculature revealed a significantly reduced number of arterioles (WT 4.78 ± 0.97 vs Ang2-/- 3.00 ± 0.58; $p<0.001$ Fig. 4 G) and venules (WT 4.67 ± 0.87 vs Ang2-/- 2.86 ± 0.69; $p<0.001$; Fig. 4 G). Diameters of arterioles yielded a 30 % dilatation compared to wildtype mice (Fig. 4 H), while venular diameters did not differ between groups (Fig. 4 H). Perivenular capillary diameters were 20 % larger than the corresponding capillaries of wildtype animals ($p<0.01$), while the periarteriolar vessels were twice as large as capillaries of wildtype mice (Fig. 4 I, $p <0.001$).

Thus, peripheral vascular outgrowth, vascular patterning and capillarisation of the arteriolar site continued to be affected by the lack of Ang-2.

Ang-2 is highly transcribed in endothelial cells

Next, we asked whether the phenotypic changes described above were accompagnied by Ang-2 expression in the affected regions. We monitored LacZ staining representing Ang-2 expression in retinal digest preparations and found distinct Ang-2 expression at three locations. As shown in Figure 5 A, strong LacZ staining was detected in preretinal neovascularization, which persisted throughout 2 months. In mice heterozygous for Ang-2, no clear LacZ expression

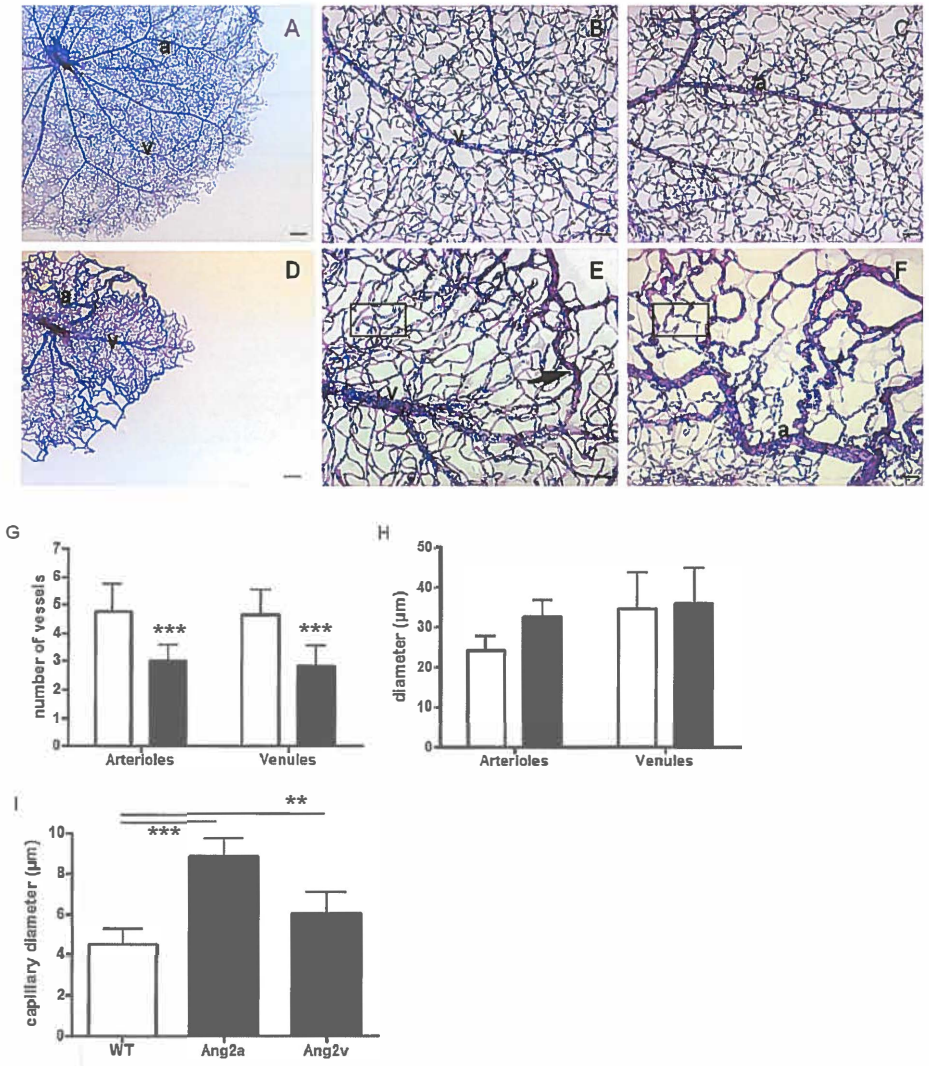

Figure 4: Abnormalities of intraretinal vessels at p60.
PAS-stained retinal digest preparations were used for analysis of intraretinal vasculature at p60. Preretinal vessels were removed. Substantial impairment of retinal outgrowth at the arteriolar (a) but also at the venular (v) sites (D - F) of Ang2-/- compared to wildtype mice (A - C). The retinas yielded a rarefied network at the venular site (E), and arteriolar tortuousity, aneurysms and a chaotic capillary network at the arteriolar sites (F). B and E are magnified images at venular site of A and D (v), C and F are magnified images at arteriolar site of A and D (a), respectively. Numbers of arterioles and venules (G) were significantly decreased in Ang2-/- compared to wildtype mice ($p<0.001$). Diameters of retinal arterioles (H; $p<0.05$) and retinal capillaries at the arteriolar site (Ang2a in I) were significantly increased (I; $p<0.001$ vs wt). Diameters of retinal venules (H) were

unchanged compared with wt controls. Capillaries on the venular site (I; Ang2v) were smaller than those of the arteriolar site, but still different from wt controls. Scale bars: 200µm (A, D), 50µm (B-C, E-F). *** p <0.001; ** p<0.01; * p<0.05. Black bars: Ang2-/- mice; white bars: wildtype mice. Squares in E and F indicate areas of analysis for data in I. Arrow in E marks an AV shunt.

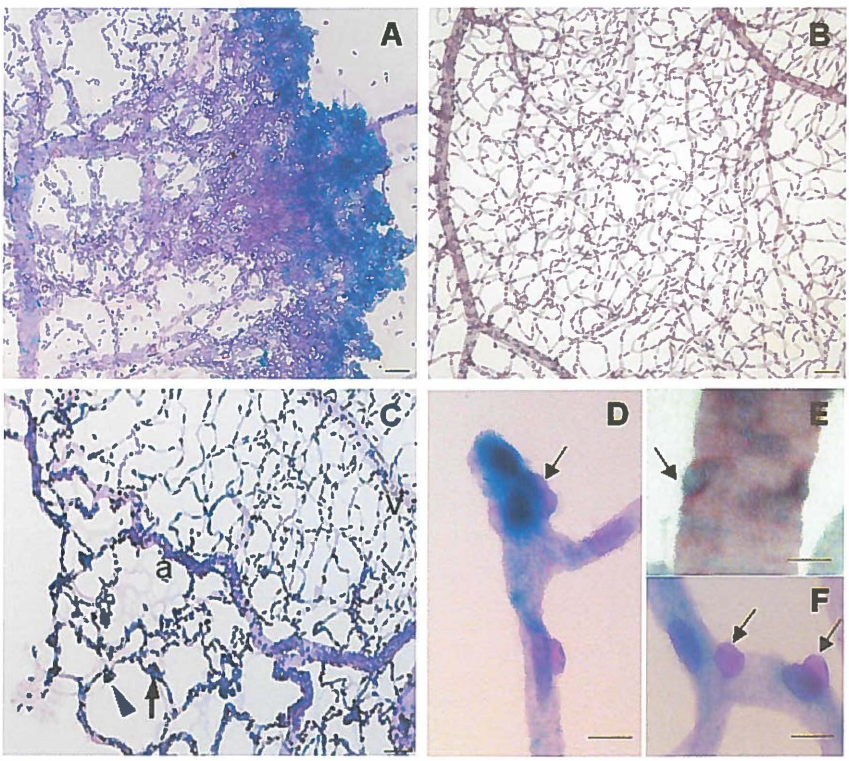

Figure 5: Ang-2 expression using LacZ as reporter at p 60.
Retinal digests were performed using p60 eyes of Ang2-/- (A) and Ang2+/- (B) mice stained with LacZ as reporter for Ang-2. (A) Ang-2 is strongly expressed at neovascular fronts of Ang2-/- mice (A) while LacZ staining was virtually absent in intraretinal vessels of heterozygous Ang2LacZ mice (B). In intraretinal vessels, LacZ positive cells were localized in tortuous arterioles ("a" in C), in aneurysms (arrow in C), and in the capillaries adjacent to the arterioles (C). Ang-2 is expressed in endothelial cells within sprouts (D) and in endothelial cells of straight capillaries (F), but not in pericytes (arrows in D and F). Notably, LacZ staining was also moderately positive in SMCs of arterioles (arrow in E). Scale bars: 50µm (A-C), 100µm (C-E). v in B: venule.

was discernible (Fig. 5 B). A moderate LacZ expression was seen in peripheral retinal arterioles (Fig. 5 C) which were characterized by irregular shape, tortuosity and hypercellularity, indicating remodeling defects and proliferative activity. At the capillary level, LacZ positive cells were found in microaneurysms (arrowhead in Fig. 5 C) and on sprouts (Fig. 5 D), but not in pericytes in the vicinity of sprouts or in capillaries (Fig. 5 F). Interestingly, LacZ positive cells of putative SMC positions were localized in arterioles Fig. 5 E).

Preretinal neovascularization are leaky in Ang-2 deficient mice
We studied the permeability of the irregular retinal vessels in Ang2-/- mice at p60 mice using indocyanin green and fluorescein angiography and scanning laser ophthalmoscopy. As depicted in Figure 6 A, C, and E, retinal vessels in homozygous Ang2-/- mice were perfused by ICG indicating patent blood flow. The neovascularization at the sprouting front, however, displayed severe leakiness as indicated by substantial extravasation of fluorescein (Fig. 6 B, D, and F).

Persistent preretinal neovascularization during ageing
With the availability of Ang2-/- animals surviving for up to one year on the C57BL/6J background, we examined the retinal vasculature for persistence of neovascularization and vessel patency. In wildtype mice, vertical sections confirmed the absence of preretinal vessels at 60 days and one year (Fig. 7 A and B). Preretinal vessels were still present at one year of age, but decreased in magnitude compared to p60 Ang2-/- retinas (Fig. 7 C and D, respectively). Interestingly, SLO in Ang2-/- mice at one year showed irregular preretinal vessels (Fig. 7 E), but these vessels were no longer leaky (Fig. 7 F). The hyaloid vessel system had grossly disappeared. Thus, the irregular preretinal vessels underwent remodeling towards maturation in the absence of Ang-2.

Pro-angiogenic imbalance of growth-/survival factors in Ang-2 deficient mice
Hypoxia induces expression of VEGF in human and in the murine ROP model. We investigated the expression time course of VEGF-A and Ang-1 by quantitative real-time RT-PCR. VEGF-A was upregulated 3.8 fold at p10 in Ang2-/- mice compared to wildtype mice. Until p60, VEGF expression remained significantly elevated,

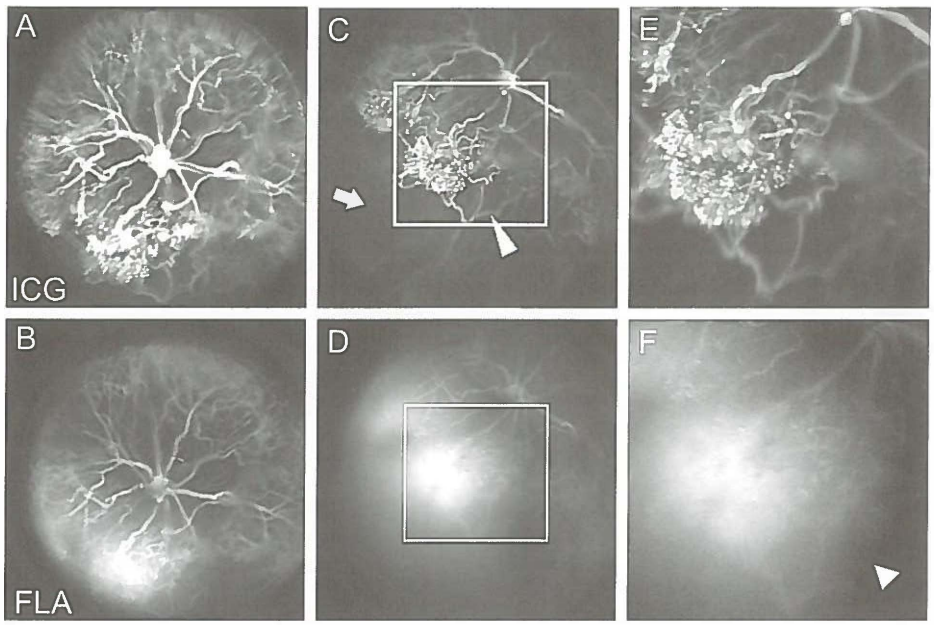

Figure 6: Preretinal neovascular vessels are leaky in Ang2 deficient mice. Scanning laser ophthalmoscopy was used to evaluate the permeability of preretinal neovessels. Vessels were visualized by indocyanine green (ICG) and leakage was detected by small fluorescein (FLA). Retinal vessels were incompletely matured with peripheral areas of leaky vessel convolutes (A and B). C. Neovascular areas were adjacent to large avascular areas (arrowhead) and the non-vascularized peripheral retina (arrow). D. Identical area as in C demonstrating strong leakage of the neovascularization. E and F: higher magnifications of the areas in white squares in C and D, respectively. Arrowhead in F marks the avascular periphery.

although the relative magnitude declined compared to wildtype retinas (Fig. 8 A). There was no major difference between the expressions of the VEGF isoforms 164 and 188 over time suggesting that both isoforms are regulated by hypoxia in the Ang2-/- mice (supplementary Fig. 3). We also assessed the expression of VEGFR2 and found a transient trend of increased expression in Ang2-/- mice at p10 (supplementary Fig. 3).

The expression of Ang-1, an important growth factor that stabilizes the endothelium by recruiting mural cells to established retinal vessels, was quantitated. The ratio of Ang-1 to VEGF was reduced by 60-80 % in Ang2-/- mice up to p60 (Fig. 8 B) and was normalized at one year of age.

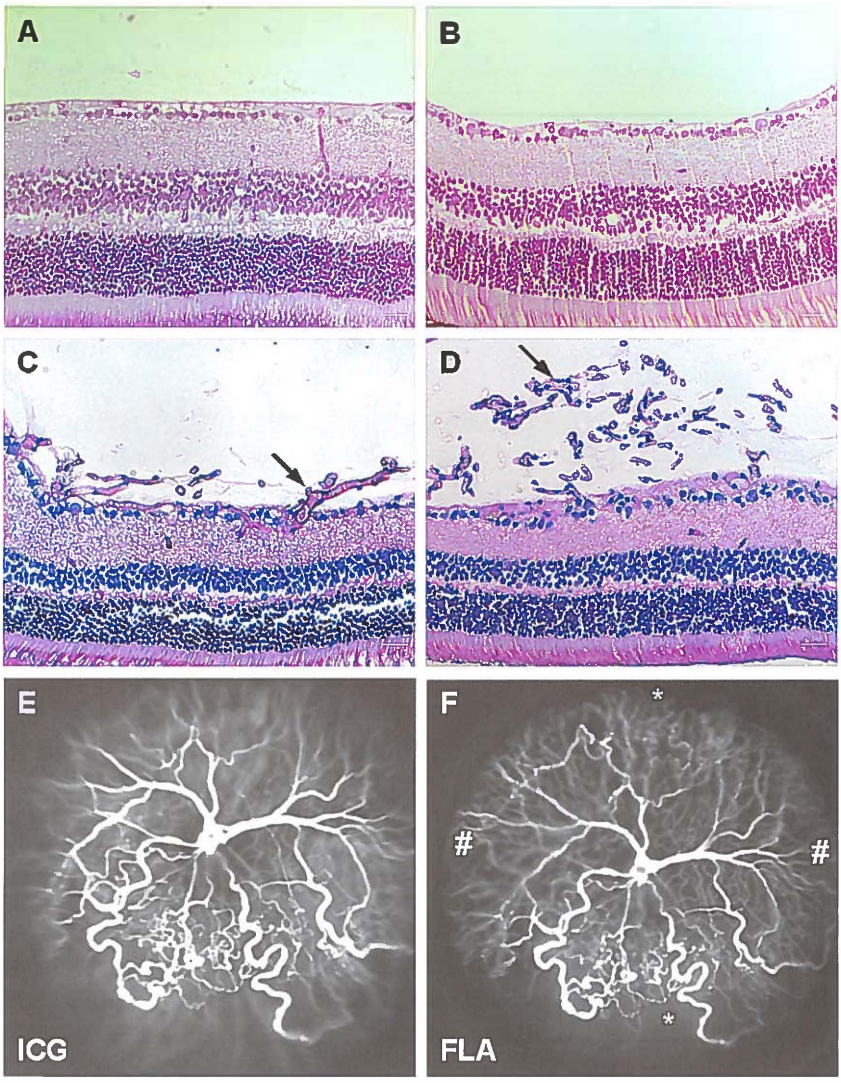

Figure 7: Persistence of preretinal vessels for up to one year in Ang2-/- mice. Vertical PAS-stained paraffin sections of 12 month-old (A, C) and and 60 days old (B, D) wt controls (A,B) and Ang2-/- (C, D) retinas, showing persistent neovascularization in Ang2-/- mice. The extent of neovascularization was reduced at one year compared to p60 (C vs D). Preretinal vessels visualized by ICG (E) were not leaky at one year of age in Ang2-/- mice (F: FLA). v: venular site; a: arteriolar site. Scale bars: 25μm (A-D)

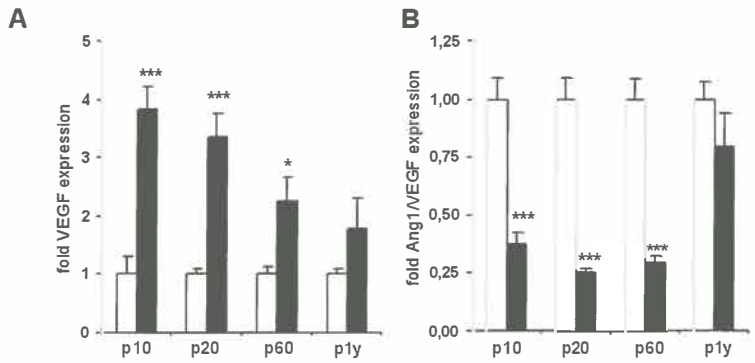

Figure 8: Time course of VEGF and Ang-1/VEGF expression.
VEGF (A) and Ang-1/VEGF ratio (B) mRNA expression was measured by quantitative RT-PCR in Ang2-/- (■) and wt retinas (□) at p10, p20, p60 and at one year of age (p1y). VEGF was upregulated fourfold at p10 and remained upregulated up to p60. The ratio of Ang-1 relative to VEGF was significantly lower in the Ang2-/- mice during the first month of age and returned to normal at one year of age. Bars show mean ± SEM. *** $p < 0.001$; *$p < 0.05$ all Ang2-/- versus wt at same age.

Discussion

Our studies in retinal vascular development after postnatal day 10 revealed a major role for Ang-2 in sprouting angiogenesis and adult vascular patterning. Without Ang-2, the arborization of the retinal vascular tree remains defective, and the promotion, and completion of the peripheral and deep capillary networks fails. However, spontaneous proliferative retinopathy develops centrally distant from tissue hypoxia. Furthermore, the progressive decline of VEGF-A and the concomitant increment of Ang-1 appear to determine successive vascular impermeability irrespective of Ang-2.

Previous studies have focused on the early postnatal retinal defects of Ang-2 knockout mice. Gale et al. concluded that Ang-2 is absolutely required for coupled vascular regression and sprouting (16). From their work, it was established that a. Ang-2 is not essential for embryonic development as the mice were viable; b. Ang-2 is essential for hyaloid vessel regression, and c. Ang-2 is preferentially expressed in vascular sprouts, and in periendothelial cells of large arteries. They used Ang2-/- mice on a genetic mouse background, which limited their survival to a short postnatal period (appr. 2 weeks with only few animals living longer). They found a rudimentary superficial intraretinal vascular plexus, and a hyaloid vessel

system that persisted for up to 25 days while normally regressing shortly after birth. Our data are consistent with their observations, but contrast in some important aspects. We noted that a. preretinal neovascularization develop in the absence of Ang-2, b. Ang-2 is expressed in capillary endothelial cells, and c. preretinal proliferations rather than hyaloid vessels invade the peripheral retina. With increased life-span, persistent Ang-2 deficiency affects predominantly the remodeling at the arteriolar site of the vascular tree (exemplified in Figs. 4 D and F, 5 C), the peripheral (Figs. 1 G, 2 B and D, and 3 D) and the deep vasculature (Figs 2 B and 3 L). Thus, Ang-2 and VEGF are important partners in the coordination of three steps: a. correct arterial development and patterning, b. the formation of the deep capillary layers, and c. the appropriate formation of the vascular periphery. The latter two defects in early postnatal retinal development were already noted by Hackett et al. using Ang2LacZ mice (however of unknown strain assignment) and studying Ang-2 expression and vessel patterning for up to 18 days, and in the ROP model (21). Consistent with our data, there was a profound impairment of the formation of the peripheral and the outer retinal layers in Ang2-/- mice. However, the predominance of the impairment at the arteriolar site, and the ability to form new vessels in the absence of Ang-2 was not noted. An explanation for the differences may lie in the different mouse strains used. It is known that mice vary in their propensities to form new vessels in response to hypoxia (22). It is conceivable that proliferative retinopathy is the result of the absolute VEGF-levels, and that Ang-2 may play a modifier role. However, the findings in our study challenge the paradigm of the cooperation of VEGF and Ang-2 in causing pathological neovascularization. Further experiments, in which Ang-2 is reconstituted during later periods, would assist in discrimination between Ang-2 specific and secondary effects on the retinal phenotype.

The altered numbers of large vessels is reminiscent of an altered ratio of the different VEGF isoforms. According to Stalmans et al., the numbers of retinal arterioles is determined by the predominance of the appropriate VEGF-isotype (23). When $VEGF_{164}$ prevails, the number of arterioles at p9 does not differ from wildtype animals. However, with the exclusive expression of $VEGF_{188}$ and $VEGF_{120}$, respectively, the numbers of arterioles (2.8 ± 1.5 and 3.1 ± 0.3) are comparable with Ang2-/- mice in the present study. Arterial specifi-

cation depends on shear-stress induced remodeling and the recruitment of specific vascular smooth muscle cells. Our data, which are consistent with previous findings, suggest that Ang-2 is expressed in vascular smooth muscle cells of retinal arterioles, contributing to the formation and patterning of the arteriolar site of the vascular tree. According to recent data, Ang-2 is silenced in endothelial cells upon high shear stress via an AMP-Kinase-Foxo-dependent pathway (24). The finding that Ang-2 expression was seen in tortuous enlarged arterioles piled with aneurysms suggests persistent impairment of blood flow in these parts of the retina.

According to our observations, Ang-2 deficiency caused the selective failure of the secondary intraretinal capillary network below the arteriolar site. This patterning defect is unprecedented. It is known that Müller cells express Ang-2 and VEGF, thus contributing growth, promoting cytokines for the correct spatial orientation of the forming vasculature (9, 25). When guidance cues are lacking, such as in Norrie k.o. mice, the intermediate and deep capillary layers fail to form completey with no areal preponderance (26). The failure induces the retinal overexpression of VEGF, Ang-2 and integrins, among others. Since the lack of Ang-2 is associated with defective angiogenesis on the arteriolar, but not on the venular site, a role of flow changes is, again, suggested. However, it remains unclear, whether molecules relevant for arterio-venous differentiation such as neuropilin-1, notch, delta-like 4, or signal mediators of the TGF-ß family are involved.

The reduction of large vessel numbers, the failure of a proper peripheral arborisation, and the occurrence of preretinal neovascularization suggest that VEGF is highly upregulated, as measured in our study. Gerhardt et al. suggested that the proper behaviour of the growing capillary sprouts depends on specific properties of VEGF (27). Guided migration of tip cells depends on the VEGF-A gradient and the proliferation of endothelial stalk cells depends on the concentration of VEGF-A. The patterning of vessels was similarly deranged as some of the retinal vessels in Ang2-/- mice, with chaotic branching, irregular vascular diameters and focal aneurysms. Mouse strains differ in their capacity to overexpress VEGF in the retina upon hypoxic stimulation (22). The relevance of the VEGF-dosage is further supported by data from Stalmans et al.. They found regular arborisation of the retinal vascular tree, when only (normal

levels of) VEGF164 was present. Together, these data suggest that a part of the retinal pathology is not explained exclusively by the lack of Ang-2, but by the concomitant VEGF overproduction and possibly by the abolition of a functional VEGF gradient. It is noteworthy that proliferative retinopathy can develop as the sole consequence of VEGF abundance.

Vascular permeability is tightly regulated, in particular in the retina. VEGF is the most potent inducer of increased vascular permeability as has been found in eye disease in which hyperpermeability was clinically evident (28). Angiopoietin-1 has been demonstrated to reduce VEGF dependent and -independent hyperpermeation in different vascular beds (29, 30). In our study, VEGF is upregulated 4 fold, and Ang-1 is downregulated by 60 %, resulting in a profound change in the VEGF/Ang-1 ratio. Ang-1 regulates permeability by thightening junctions, and consolidating cell-matrix interactions. Pericytes may have an additional impact. Overexpression of Ang-2 induces pericyte loss, but whether the opposite is true has not been demonstrated. At least, in heterozygous Ang2LacZ mice there is no evidence for increased recruitment of pericytes to the retinal capillary system. The normalization of leakage at one year of age corresponds with the marked shift in the VEGF/Ang-1 ratio. Whether VEGF in this setting is responsible for the persistence of neovascularization, and Ang-1 for the reduction in leakage, however, remains speculative.

Finally, the vascular phenotype of the Ang2-/- mouse producing spontaneous proliferative retinopathy, has large similarities with the mouse model of retinopathy of prematurity. In both cases, the retina is hypoxic, inducing high VEGF levels, with proliferative retinopathy, and leakiness of the vessels (25, 31). However, apart from the absence of Ang-2, there are also discrepancies. In the Ang2-/- mouse, the induction is spontaneous, neovascularization persist for at least one year, and the avascular zone is in the periphery. These characteristics render the model much closer to human proliferative retinopathies. In particular, one major criticism of the ROP model – the transience – does not apply.

In summary, we demonstrate that Ang-2 is an important determinant of retinal vascular patterning. With our findings, the concept that Ang-2 is highly context-dependent is supported. However, we also demonstrate that persistent neovascularization is conceivable

in the absence of Ang-2.

Acknowledgements

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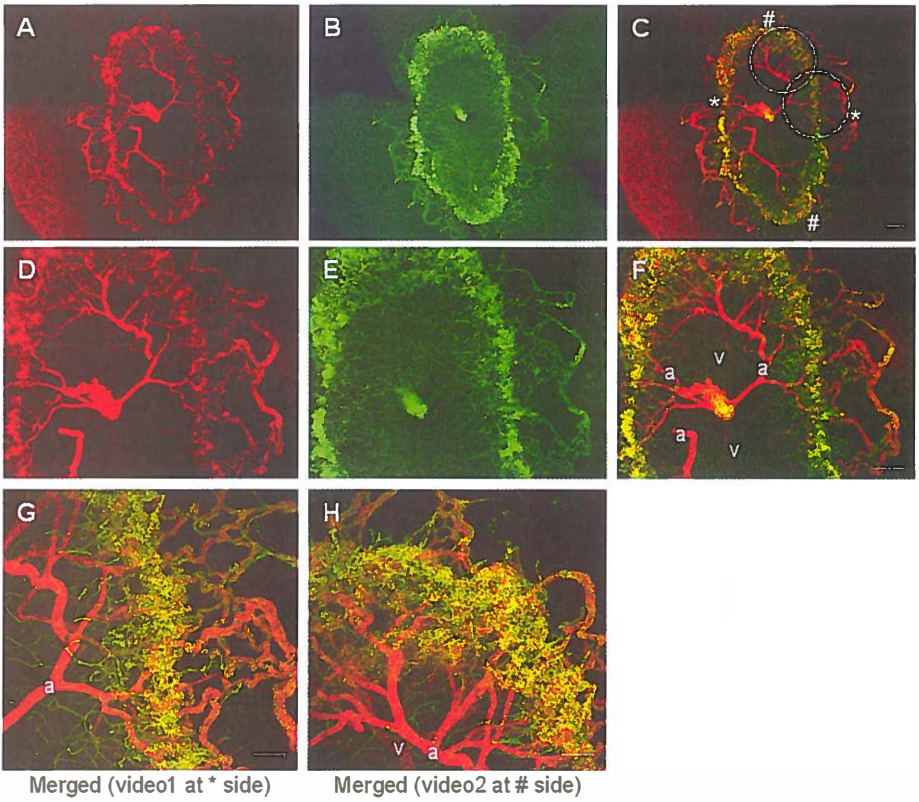

Supplemental Figure 1:
Epifluorescent image (A-F) and confocal images (G, H) of a p20 whole mount retina of an Ang2-/- mouse stained with lectin (green) and SMA (red). C: overview showing the defective branching of arterioles (strong SMA labeling) and of venules (weak SMA labeling) and the area of neovascularization (strong lectin labeling) in relation to the outgrowth of the intraretinal vessels. D-F: enlarged images. G: Confocal image of a higher magnification of * corresponding the encircled area marked by * in Figure 3 D and supplement 1 C, showing arterioles (SMA-labeled, red) and a preretinal neovascular network (strong lectin-labeled). H: depicts the corresponding area marked by the circle # of Figure 3 D and supplement 1 C. Scales: 25μm (A-F), 50μm (G, H).

Precise spatial orientation is provided by the concomitant confocal video 1 and 2.
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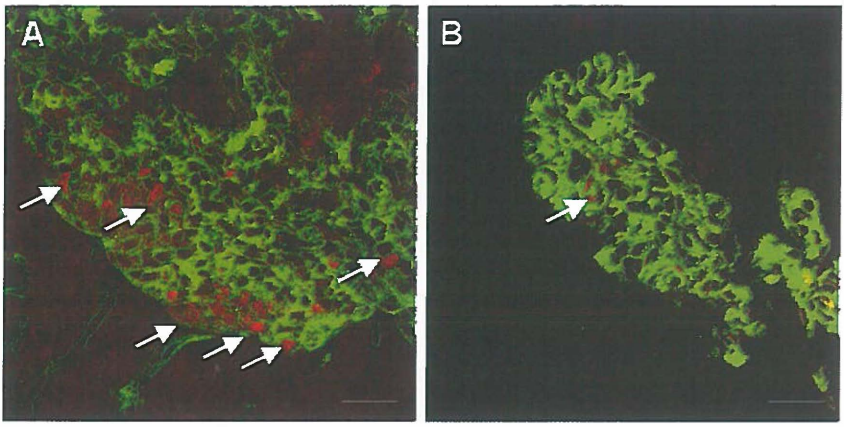
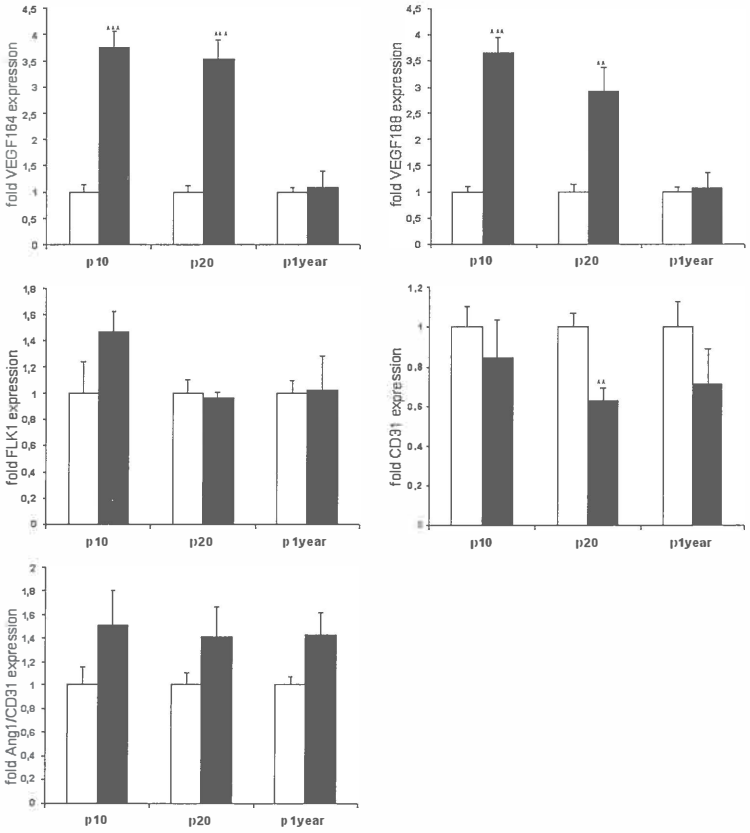
video 2: www.ma.uni-heidelberg.de/hidden/video2.avi



Supplemental Figure 2:
Confocal microscopic image of the co-staining of lectin (green) and Ki67 (red) of preretinal neovascularizations in Ang2-/- mice at p20 (A) and p60 (B) indicating persistent neovascularization activity. White arrows: proliferating endothelial cells in neovascular tufts. Scales: 50μm (A, B).



Supplemental Figure 3:
Quantitative real time PCR of VEGF isoforms 164 and 188, the VEGF receptor flk-1, the endothelial marker CD 31, and the Ang-1/CD31 ratio of wt (white bars) and Ang2-/- mice (black bars) at p10, p20, and p1year. Primers and probes of Flk1 (Mm01222431-m1) and CD31 (Mm00476702-m1) were purchased from Applied Biosystems. ** $p<0.01$, *** $p<0.001$.

Chapter IV

Spatial patterning of inflammation- and angiogenesis-associated genes in the retina and localized regulation in experimental proliferative retinopathy

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Abstract

Proliferative retinopathies form new vessels from the intraretinal superficial plexus. Inflammatory- and angiogenesis-associated (or angioflammatory) factors play a role in pathologic angiogenesis. However, little is known about the spatial patterning and their expression levels at the site and at the timepoint of angiogenesis incipience. C57BL/6J mice were exposed to the model of retinopathy of prematurity (ROP) or kept at room air condition (control). Eyes were obtained at postnatal day 13, the timepoint of neovascularization incipience. The expression of 46 angioflammatory genes was analysed in two vascularised retinal areas, selected by laser dissection microscopy, and in whole retinal lysates, using quantitative RT-PCR. Some factors were validated by immunohistochemistry. In control mice, 25 angioflammatory genes differed in their spatial distribution and 20 genes were predominantly expressed in the vascularised layers compared to the whole retina. In the ROP retina, 20 genes including angiopoietin-2 and tumor necrosis factor receptor 1, but also thrombospondin-1 were significantly upregulated in the area of neovascularization incipience compared to controls. Together, our data demonstrate that angioflammatory genes are differentially expressed in the layers of the retina, both in control and in ROP mice. Only a selected number of angioflammatory factors localized at the site of neovascularization incipience. This knowledge will further promote our understanding of the complexity of spatial expression and hypoxic regulation of angioflammatory genes in the retina.

Introduction

Proliferative retinopathies, such as proliferative diabetic retinopathy, retinopathy of prematurity, and neovascular age-related macular degeneration are characterized by retinal neovascularization. These diseases cause visual impairment and remain to be the most common cause for blindness (1, 2).

The retina consists mostly of neuronal and glia cells, which are termed the neuroretina (3). Different neuronal cell types are found in distinct locations in the retina and create a pattern of distinct retinal layers. While the astrocytes are present in the innermost retinal layers enwrapping the superficial vascular plexus, the glial Mueller cells span the entire retina and enwrap the deep vascular plexus. Postnatally the retinal vasculature develops within the neuroretina in three plexi, vascularising the inner retinal layers. The superficial vascular plexus is located in the nerve fiber layer, the intermediate plexus is in the inner plexiform layer close to the inner nuclear layer, and the deep capillary plexus is at the outer border of the inner nuclear layer (4). While the superficial vascular plexus is fully outgrown at postnatal day 8-10, the deep vascular plexus forms from postnatal day 7-8 onwards (5). In the mouse model of proliferative retinopathy (ROP), five days of incubation in 75% oxygen halts the postnatal development of the retinal vasculature and leads to vasoregression in the central retina around the optic disc (6). Therefore, in this model, the retinal vasculature as a vascular plexus is mainly present in the nerve fiber layer of the retinal periphery at p12. Retinal hypoxia develops with the drop in oxygen concentration when the animals are returned to room air condition, and leads to the upregulation of proangiogenic growth factors (7). Subsequently, neovascularization arises from the vessels of the superficial plexus of the retinal periphery, which grow preretinal and intravitreally, as in proliferative diabetic retinopathy and retinopathy of prematurity.

The key angiogenic molecule vascular endothelial growth factor (VEGF) plays an essential role in the development of retinal neovascularization (8). In experimental proliferative retinopathy relative hypoxia, which occurs after the hyperoxic incubation, induces VEGF upregulation. VEGF is produced by retinal glial cells, the Mueller cells, in response to hypoxia, as demonstrated in vitro (9). Downstream proangiogenic signaling of VEGF is mainly mediated via its endothelial receptor VEGFR2 (10). VEGF-VEGFR2 signaling

underlines the importance of the glial microenvironment for retinal vascular disease. However, VEGF is not the only player in the pathogenesis of retinal neovascularization. Other angiogenic growth factors, such as the angiopoietins (Ang), platelet derived growth factor beta (PDGFB) and their receptors as well as integrins, adhesion molecules and matrix-metallo-proteinases participate in the retinal angiogenic process (11).

Inflammation has been described in pathological retinal angiogenesis, but its precise role is unknown (12). It is debated, whether inflammation plays an essential role in the initiation or whether it occurs alongside the progression of angiogenesis. In experimental models, anti-inflammatory drugs reduced preretinal neovascularization. Furthermore, macrophages, which are increased in the hypoxic retina, may contribute to the formation of preretinal neovascularization. There is a link between inflammation and angiogenesis since angiogenic factors, such as VEGF and angiopoietin-2 (Ang2) have proinflammatory properties and vice versa, inflammatory factors, such as tumor necrosis factor α (TNFα) have angiogenic potential (13, 14).

Recently, an analysis of angiogenesis related genes has been performed in the timecourse of experimental proliferative retinopathy (15). The study showed hypoxia induced upregulation of VEGF, but for other angiogenic and inflammatory factors such as the angiopoietins a hypoxia induced regulation was not observed at early time points (15). Like this study, many others have analysed retinal gene expression in whole retinal lysates. We hypothesized that the knowledge created by total retinal gene expression analysis may be limited by the fact that spatial differences in gene expression can be masked in whole organ analysis. Furthermore, the spatial distribution and the origin of gene expression seem critical in retinal neovascularization as it happens in a small part of the organ. The cellular distribution of some genes has been revealed in studies using immunohistochemistry and in situ hybridization techniques, as well as tissue selection by laser dissection microscopy prior to gene expression analysis. However, these studies focused on a limited number of genes of interest in a specific context. Hence, comprehensive data about the spatial distribution of broader series of angiogenesis-associated gene expression in the retina, especially in the area of the peripheral superficial vascular plexus, are missing.

Furthermore, many studies focus on postnatal day 17 (p17), the timepoint of maximal neovascularization in the ROP model, while the first preretinal neovascularization is already present at p14. Given the time of growth from the intraretinal venous vessels, through the inner limiting membrane and a delay between transcription and translation of the factors needed for this process, p13 is supposedly the timepoint to study the factors that initiate retinal neovascularization. So far, little is known about gene expression at this early timepoint and most studies ignored spatial patterning by whole retinal analysis.
Understanding the spatial complexity of gene expression is essential for a rational design of therapeutic intervention. Therefore, we studied the spatial distribution of inflammatory- and angiogenesis related genes, termed angioflammatory, in the control postnatal retina and in the mouse model of proliferative retinopathy at the incipience of preretinal neovascularization. For this, we employed tissue selection using laser dissection microscopy prior to low density array real-time RT-PCR. This strategy provided us with means to obtain a representative view of the nature and complexity of spatial angioflammatory gene expression in the physiologic and pathologic angiogenic retina.

Material and Methods

Animals

All experiments in this study were performed according to the guidelines of the statement of animal experimentation issued by the Association for Research in Vision and Ophthalmology and were approved by the Institutional Animal Care and Use Committee (Regierungspräsidium Karlsruhe, Karlsruhe, Germany).
Wildtype (C57BL/6J) mice were used in this study. Breeding of the mice and exposure to the ROP model was performed at the laboratory animal facility of the Medical Faculty Mannheim (University of Heidelberg, Germany). Animals were kept at a 12 hours day-12 hours night light cycle and fed with standard chow and tap water.

Proliferative retinopathy mouse model

The mouse retinopathy of prematurity model was used as previously described (6). Briefly, newborn mouse litters were split and

either exposed from postnatal day 7 (p7) until p12 in an incubation chamber with 75% oxygen and then returned to room air (ROP), or were kept at room air the entire time (controls). Eyes obtained at p13 were stored at -80°C for gene expression analysis.

Laser Dissection Microscopy

Laser Dissection Microscopy (LDM) was performed on retinal serial cryosections (6μm), which were mounted on polyethylene-naphtalene membranes attached to glass slides (P.A.L.M. Microlaser Technology AG, Bernried, Germany). Before laser dissection, cryosections were briefly acetone fixed, stained with Mayer's haematoxylin solution and air dried. Areas for dissection were selected using P.A.L.M. software on a Laser Robot Microbeam System at 200x magnification (P.A.L.M. Microlaser Technology AG). The area from the inner limiting membrane to the outer plexiform layer (LDM_VL=laser dissected tissue of the vascularised retinal layers), which represents the vascularised retinal layers, was dissected and collected over the entire retina of each section (Fig.1 A). The area from the inner limiting membrane to the ganglion cell layer (LDM_VP=laser dissected tissue of the area of the peripheral superficial vascular plexus), which consists of the peripheral superficial vascular plexus, was dissected and collected from the outer 2/3 of each retinal section (Fig.1 B). For LDM_VP, the selection range was measured for each section separately before cutting. Both areas were cut from sections of the same eye. Approximately $3x10^6$ μm^2 tissue per retinal area of individual animals (n=5) was dissected.

mRNA gene expression analysis

Retina was isolated from frozen eyes of ROP exposed and control mice at p13. Retina was homogenized and total retinal RNA/per animal was isolated using the RNeasy Mini Plus kit (Qiagen, Leusden, The Netherlands). These samples were termed retinal lysates (RL) and represent the standard source of RNA that has been used in most studies published so far. Retinal lysates were retrieved from the eyes contralateral to the eyes used for laser dissection. The RNA of laser dissected tissue of the two retinal areas of ROP exposed and control eyes (n=5 retinae from individual animals) was individually isolated using the RNeasy Micro kit (Qiagen). The cDNA was synthesized from 10μg RNA using Superscript III, RNase OUT (In-

vitrogen, Carlsbad, CA, U.S.A) and random hexamers (Promega, Madison, WI, U.S.A). The cDNA of each retinal lysate and of each dissected area of individual animals (n=5) was loaded in duplicate according to the manufacturer's protocol on custom designed 48 gene low density array plates (LDA, Applied Biosystems, Foster City, CA, U.S.A). 46 key inflammatory- and angiogenesis associated genes were selected from the literature and GAPDH was used as housekeeping gene (see Table1). Quantitative real-time RT-PCR was performed with an ABI PRISM 7900 HT Sequence Detector (Applied Biosystems). Ct values ≥40 were considered below the detection limit. If more than four out of ten Ct values per group of samples were missing, the gene was considered as not detectable in this group. Data was analysed with the SDS 2.3 software (Applied Biosystems), mRNA expression was calculated relative to the housekeeping gene GAPDH using the $2^{-\Delta Ct}$ method (16) and values were multiplied by 10^5.

Table 1: List of the selected 47 angioflammatory genes analysed by real-time RT-PCR.

General name	Gene name	Gene Symbol	Assay ID
GAPDH	glycerinaldehyde 3-phosphate dehydrogenase	Gapdh	Mm99999915_g1
Ang1	angiopoietin-1	Angpt1	Mm00456503_m1
Ang2	angiopoietin-2	Angpt2	Mm00545822_m1
B2m	ß-2-microglobulin	B2m	Mm00437762_m1
Bcl2	B-cell lymphoma 2	Bcl2	Mm00477631_m1
MCP-1	chemokine ligand 2, monocyte chemotactic protein 1	Ccl2	Mm00441242_m1
VE-cadherin	vascular endothelium cadherin 5, type2	Cdh5	Mm00486938_m1
EphrinB2	ephrinB2	Efnb2	Mm00438670_m1
Endoglin	endoglin	Eng	Mm00468256_m1
EphB4	Eph receptor B4	Ephb4	Mm00438750_m1
EPO	eryhropoietin	Epo	Mm00433126_m1

General name	Gene name	Gene Symbol	Assay ID
EPOR	erythropoietin receptor	Epor	Mm00438760_m1
bFGF	basic fibroblast growth factor	Fgf2	Mm00433287_m1
FGFR1	fibroblast growth factor receptor 1	Fgfr1	Mm00438923_m1
FGFR2	fibroblast growth factor receptor 2	Fgfr2	Mm00438941_m1
VEGFR1	vascular endothelial growth factor receptor 1	Flt1	Mm00438980_m1
HIF1α	hypoxia inducible factor 1 α	Hif1a	Mm00468869_m1
ICAM-1	intercellular adhesion molecule	Icam1	Mm00516023_m1
Integrin αv	integrin αv	Itgav	Mm00434506_m1
Integrin ß3	integrin ß3	Itgb3	Mm00443980_m1
VEGFR2	vascular endothelial growth factor receptor 2	Kdr	Mm00440099_m1
MMP2	matrix metallopeptidase 2	Mmp2	Mm00439508_m1
MMP9	matrix metallopeptidase 9	Mmp9	Mm00442991_m1
iNOS	inducible nitric oxide synthase	Nos2	Mm00440485_m1
eNOS	endothelial nitric oxide synthase	Nos3	Mm00435204_m1
Nur77	nur77	Nr4a1	Mm00439358_m1
PDGFB	platelet derived growth factor B	Pdgfb	Mm00440678_m1
PDGFRB	platelet derived growth factor receptor B	Pdgfrb	Mm00435546_m1
PECAM-1	platelet/endothelial cell adhesion molecule	Pecam1	Mm00476702_m1
PLGF	placental growth factor	Pgf	Mm00435613_m1
tPA	tissue plasminogen activator	Plat	Mm00476931_m1
uPA	urokinase plasminogen activator	Plau	Mm00447054_m1
COX-2	cycloxygenase 2	Ptgs2	Mm00478374_m1
CD45	protein tyrosine phosphatase, CD 45	Ptprc	Mm00448463_m1
PEDF	pigment epithelium derived growth factor	Serpinf1	Mm00441270_m1

General name	Gene name	Gene Symbol	Assay ID
Tie2	endothelial tyrosine kinase	Tek	Mm00443242_m1
TGFβ1	transforming growth factor B 1	Tgfb1	Mm00441724_m1
TGFβ1R	transforming growth factor B 1 receptor	Tgfbr1	Mm00436964_m1
Tsp-1	thrombospondin-1	Thbs1	Mm00449022_m1
TIMP-1	metallopeptidase inhibitor 1	Timp1	Mm00441818_m1
TNFα	tumor necrosis factor α	Tnf	Mm00443258_m1
TNFR1	tumor necrosis factor receptor superfamily, member 1A	Tnfrsf1a	Mm00441875_m1
TNFR2	tumor necrosis factor receptor superfamily, member 1B	Tnfrsf1b	Mm00441889_m1
VCAM-1	vascular cell adhesion molecule 1	Vcam1	Mm00449197_m1
NG-2	versican, chondroitin sulfate proteoglycan 2	Vcan	Mm00490179_m1
VEGFA	vascular endothelial growth factor a	Vegfa	Mm00437304_m1
vWF	von Willebrand factor	Vwf	Mm00550376_m1

Immunohistochemistry

Serial retinal cryosections (8μm) of ROP exposed and control mice at p13 were fixed in acetone and endogenous peroxidase was blocked. After a washing step the sections were incubated with rat-anti-mouse PECAM-1 (1:100, BD Pharmingen, Heidelberg, Germany), rat-anti-mouse ICAM-1 (YN1/1.7.4 hybridoma supernatant, ATTC CRL-1878, U.S.A), and rat-anti-mouse CD45 (1:50, BD Pharmingen) at 4°C overnight. Another washing step followed and then the sections were incubated with the secondary HRP-conjugated antibody rabbit-anti-rat (1:40, DAKO, Hamburg, Germany) for 60min at room temperature. All primary antibodies were diluted in 1% Bovine Serum Albumine solution in PBS and the secondary antibody in 1% Bovine Serum Albumine (Sigma, Munich, Germany) and 2% Normal Mouse Serum (DAKO) in a PBS solution. For negative controls sections were incubated with the respective rat IgG2a or IgG2b isotype controls (1:50, Antigenix America Inc., NY, U.S.A) instead of the first antibody. Detection was performed with AEC solution

(Vector, Burlingame, U.S.A.). VCAM-1 and VE-cadherin detection was performed using the EnVision kit (DAKO). Briefly, retinal sections were stained with rat-anti-mouse VCAM-1 (1:10, M/K 2.7. hybridoma supernatant, ATTC CRL-1909, U.S.A) or rat IgG1 isotype control (1:50, Antigenix America) as negative control at 4°C overnight. Other sections were incubated with VE-cadherin antibody (1:100, kindly provided by E. Dejana) at 4°C overnight. After washing, the sections were incubated with the secondary antibody rabbit-anti-rat (1:300, Vector) diluted in 1% Bovine Serum Albumine (Sigma) and 1% Normal Mouse Serum (DAKO) for 60min at room temperature. Another washing step was followed by incubation with anti-rabbit HRP Polymer solution (EnVision kit, DAKO) for 30 min at RT, and detection was performed with AEC reagent from the EnVision kit according to the manufacturer's protocol. VE-cadherin staining was performed with HBSS (GIBCO, Invitrogen, Karlsruhe, Germany) solution instead of PBS in all steps.

Statistics

Real-time RT-PCR data were analyzed using the R software (R Development Core Team, R Foundation, Vienna, Austria) with a linear mixed effect model (LME). P values were adjusted for false discovery rate of multiple comparisons according to the Benjamini and Hochberg procedure (17). Adjusted P-values <0.05 were considered significant.

Results

Area selection and relative gene expression in the control retina at p13

We analysed the expression of 46 angioflammatory genes in three different retinal isolates of control and ROP mice at p13. First we analysed gene expression in retinal lysates (RL), in which spatial distribution cannot be assessed. The second isolate was made of the vascularised retinal tissue, which was selected by laser dissection microscopy between the inner limiting membrane and the outer plexiform layer (termed LDM_VL) (Fig.1 A). The third isolate consisted of the tissue between the inner limiting membrane and the ganglion cell layer, which was selected by laser dissection microscopy and was only retrieved from the outer two third of the re-

tina (termed LDM_VP), i.e. this area remains vascularised and from this area neovascularizations arise in ROP animals (Fig. 1 B). The expression levels of the angioflammatory genes differed widely in the control retina at p13 (Fig.1 C). Hypoxia-inducible factor 1 α, VEGFA, VEGF-receptor 2 and beta-2-microglobulin were the most abundantly expressed genes. The angiopoietins, intercellular adhesion molecule-1 and the leukocyte marker CD45 were expressed at low level. Chemokine ligand 2, cyclooxygenase-2, erythropoietin, -receptor and tumor necrosis factor α showed inconsistent detection. Furthermore, expression of matrix-metallo-proteinase 2 was not detectable in the samples examined.

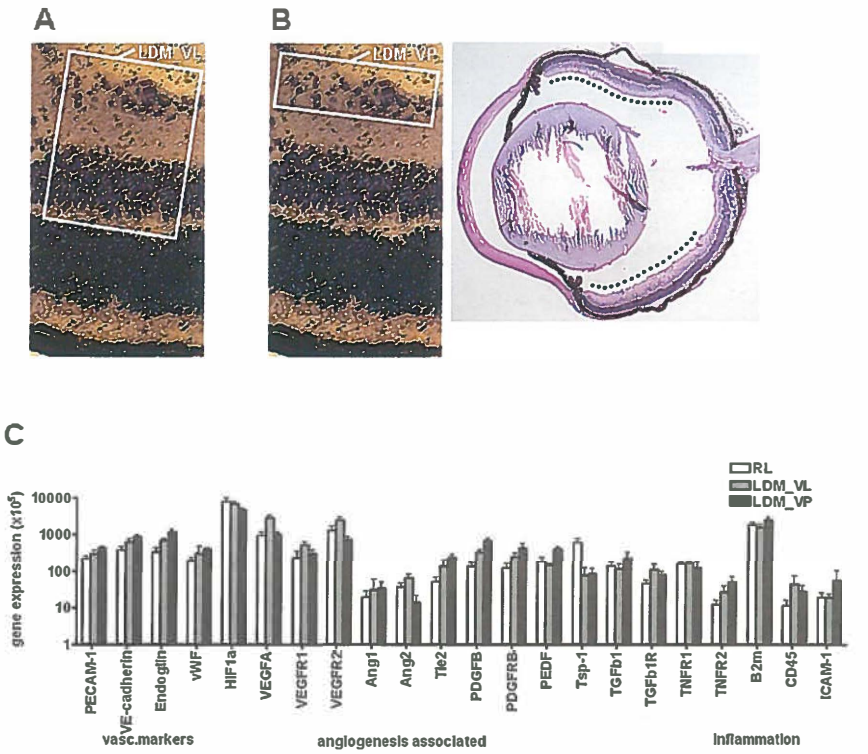


Figure 1: Selection of retinal areas by Laser Dissection Microscopy (LDM) and relative gene expression.

A: The area between the inner limiting membrane and the outer plexiform layer (LDM_VL) was dissected and collected from the retina. B: In the ROP retina at p13 the peripheral twothird (dotted black line) of the retina between inner limiting membrane and ganglion cell layer is vascularised. Therefore this area (LDM_VP) was dissected from each retinal section. C: Relative expression level of selected vascular marker-, angiogenesis-associated and inflammatory genes in control retina at p13. Gene expression is shown relative to GAPDH (x10^5) on a logarithmic scale.

Angioflammatory gene expression shows different spatial distribution patterns in the p13 control retina

Comparison of gene expression between the three data sets (see supplementary Table 1 for relative mRNA levels of all genes examined in control retina) revealed six significant spatial distribution patterns, termed pattern A through F, in the control retina at p13. Genes, such as Tsp-1, assigned to pattern A were predominantly expressed in the retinal lysates compared to the two vascularised areas, indicating that their expression was primarily localized in the outer avascular neuroretina (Fig.2 A). Pattern B genes, such as HIF1α (Fig.2 B), were equally expressed in retinal lysates and in LDM_VL, but significantly lower in the LDM_VP area, suggesting their predominant expression in the outer avascular neuroretina and in the vascularised intermediate retina. Expression pattern C describes genes, such as VEGFR2 (Fig.2 C) which were predominantly expressed in the LDM_VL area, indicative of abundant expression in the vascularised intermediate retina. Pattern D represents genes, such as PEDF (Fig.2 D) which were primarily expressed in the LDM_VP dissected area, thus present in the peripheral superficial vascular plexus area. Genes, which were significantly higher expressed in LDM_VL than in RL and significantly higher expressed in LDM_VP than in LDM_VL, were assigned to pattern E. These genes, such as PDGFB (Fig.2 E) were enriched in the vascularised intermediate retina and predominantly expressed in the area of the peripheral superficial vascular plexus. The last pattern F included genes, which were equally distributed in the two vascularised retinal areas and expressed at low level in retinal lysates. Hence, these genes were primarily expressed in the vascularised retina (iNOS, Fig.2 F). The 20 genes expressed in pattern C, D, E and F were primarily expressed in the vascularised retina from the inner limiting membrane to the outer plexiform layer, although with different spatial distribution therein. Vascular genes were either predominantly expressed in the superficial vascular plexus area, such as PECAM-1, or predominantly in the vascularised intermediate retina, such as VEGFR2 or evenly expressed in the entire retinal vasculature, such as PDGFRB.

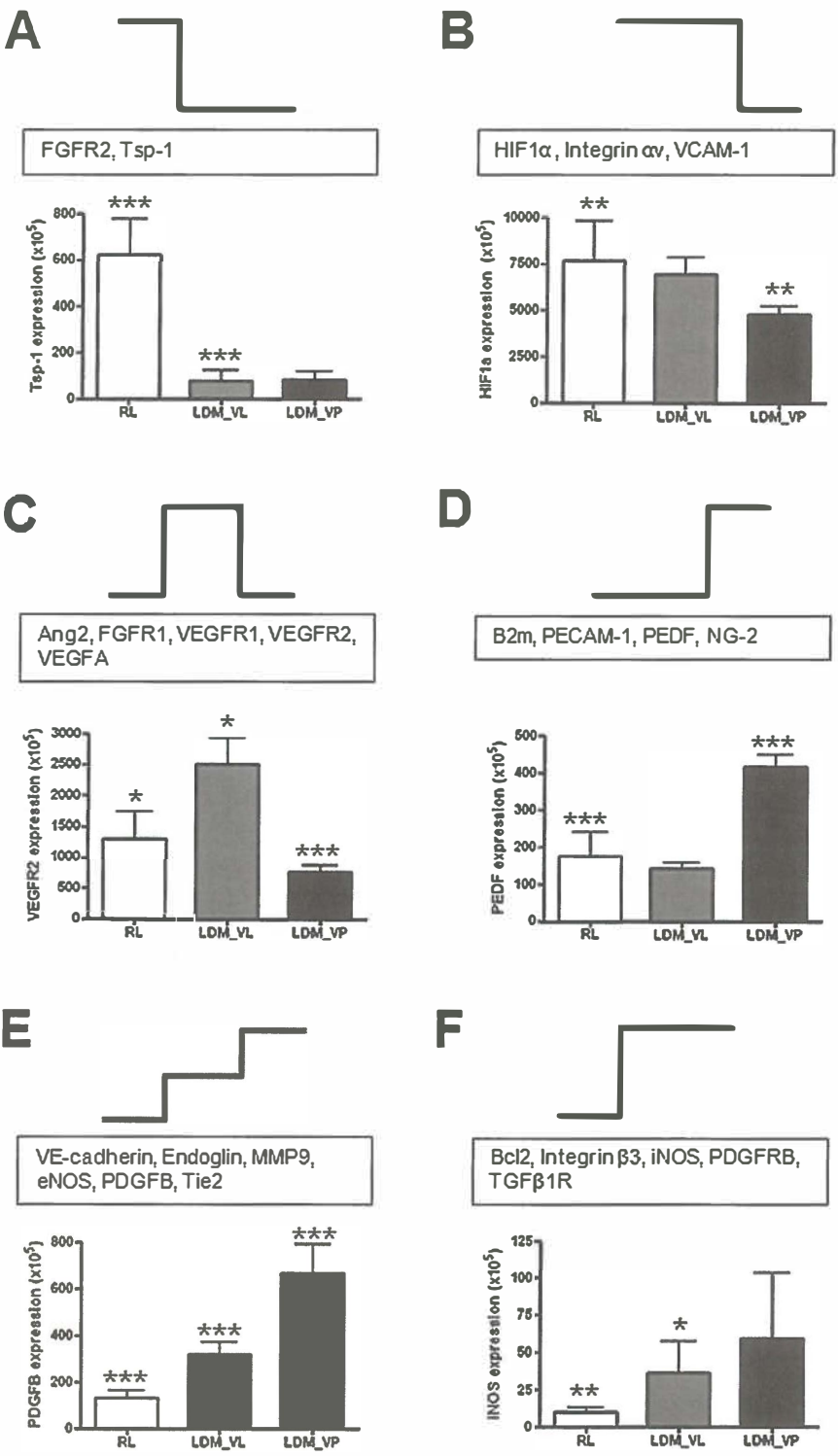

A
FGFR2, Tsp-1
Tsp-1 expression (x10⁵)
800
600
400
200
0
***
***
RL
LDM_VL
LDM_VP
B
HIF1α, Integrin αv, VCAM-1
HIF1a expression (x10⁵)
10000
7500
5000
2500
0
**
**
RL
LDM_VL
LDM_VP
C
Ang2, FGFR1, VEGFR1, VEGFR2, VEGFA
VEGFR2 expression (x10⁵)
3000
2500
2000
1500
1000
500
0
*
*
***
RL
LDM_VL
LDM_VP
D
B2m, PECAM-1, PEDF, NG-2
PEDF expression (x10⁵)
500
400
300
200
100
0
***
***
RL
LDM_VL
LDM_VP
E
VE-cadherin, Endoglin, MMP9, eNOS, PDGFB, Tie2
PDGFB expression (x10⁵)
800
600
400
200
0
***
***
***
RL
LDM_VL
LDM_VP
F
Bcl2, Integrin β3, iNOS, PDGFRB, TGFβ1R
iNOS expression (x10⁵)
125
100
75
50
25
0
**
*
RL
LDM_VL
LDM_VP

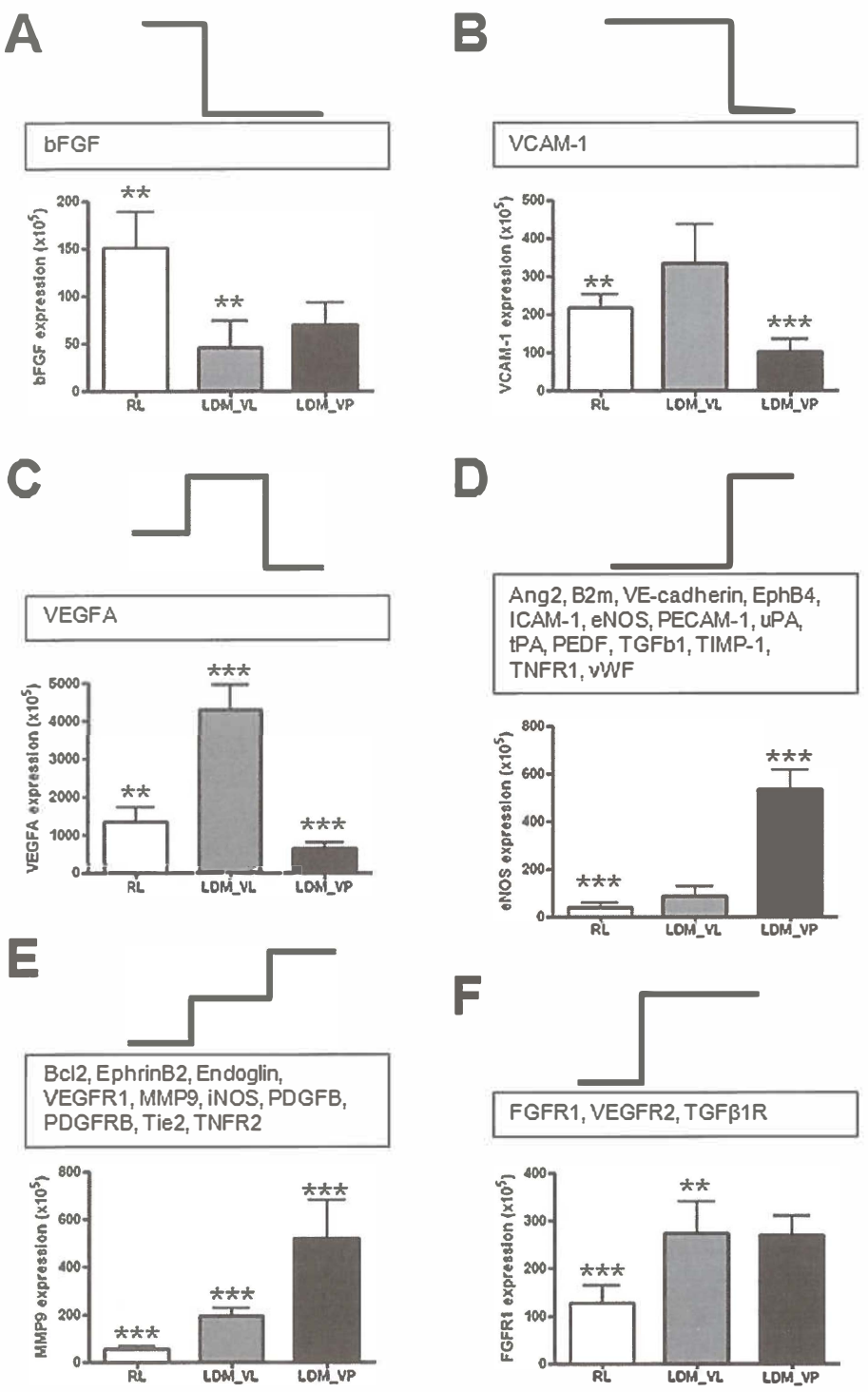
Figure 2: Angioflammatory genes show differed in their spatial expression patterns in control retina at p13.
Six significantly different spatial expression patterns termed A through F of retinal angioflammatory genes were present in control retina at p13. Each pattern is depicted by a schematic line drawing and the significant genes expressed in this pattern are listed in the boxes. For each pattern the data of one gene is shown as an example.
A: Pattern A genes were predominantly expressed in the outer avascular retina and are listed in the box. For example, Tsp-1 expression is shown: RL versus LDM_VL ($p<0.001$), and RL versus LDM_VP ($p<0.001$). B: Pattern B genes were predominantly expressed in the outer and intermediate retina. For example, HIF1α expression is depicted: LDM_VL versus LDM_VP ($p<0.01$), and RL versus LDM_VP ($p<0.01$). C: Pattern C genes were predominantly expressed in the intermediate retina. For example, VEGFR2 expression is displayed: RL versus LDM_VL ($p=0.01$), LDM_VL versus LDM_VP ($p<0.001$). Whereas for FGFR1, VEGFR1 and VEGFA expression in RL and in LDM_VP was comparable, it was significantly higher in RL than in LDM_VP for VEGFR2 ($p<0.05$) and Ang2 ($p<0.01$). D: Pattern D genes were predominantly expressed in the area of the superficial vascular plexus. For example, PEDF expression is depicted: RL versus LDM_VP ($p<0.001$), and LDM_VL versus LDM_VP ($p<0.001$). E: Pattern E genes were enhanced expressed in the intermediate retina and predominantly expressed in the area of the peripheral superficial vascular plexus. PDGFB expression is shown as example: RL versus LDM_VL ($p<0.001$), LDM_VL versus LDM_VP ($p<0.001$), and RL versus LDM_VP ($p<0.001$). F: Pattern F genes were predominantly expressed in the intermediate retina and in the area of the peripheral superficial vascular plexus. For example, iNOS expression is displayed: RL versus LDM_VL ($p=0.01$), and RL versus LDM_VP ($p<0.01$).

Spatial patterning of angioflammatory gene expression is altered in the ROP retina

In the ROP retina at p13, gene expression was analysed in the three retinal isolates as described for the control retina. This method provided an opportunity to analyse the impact of hypoxia on the spatial distribution of the angioflammatory genes (see supplementary Table 2 for relative mRNA levels of all genes examined in the ROP retina).
The same significant gene expression patterns A through F as described in detail in the control retina were also present in the ROP retina. Additionally, another pattern was found, which was termed pattern G. Pattern G genes were predominantly expressed in the RL and in the LDM_VP area, indicating predominant expression in the outer avascular retina and in the area of the superficial vascular plexus. For some genes the expression pattern was altered by hy-

poxia. bFGF was only in the ROP retina predominantly expressed in the outer avascular area (Fig. 3 A). While HIF1a and Integrin αv lost their predominant expression in the outer avascular and in the intermediate ROP retina, VCAM-1 expression was not altered by hypoxia (Fig. 3 B). Hypoxia altered the expression pattern of Ang2, Fgfr1, VEGFR1 and VEGFR2, but not of VEGFA which remained predominantly expressed in the intermediate retina (Fig. 3 C). Ang2, VE-cadherin and eNOS expression was shifted by hypoxia to predominant expression in the area of the superficial vascular plexus, where hypoxia also increased the amount of genes expressed in this pattern (eNOS, Fig. 3 D). While the expression of Bcl2, iNOS, PDGFRB and VEGFR1 was altered by hypoxia to enhanced expression in the intermediate and predominant expression in the peripheral superficial vascular plexus area, the expression of Endoglin, MMP9, PDGFB and Tie2 remained in this pattern (MMP9, Fig. 3 E). Furthermore, only in the ROP retina EphrinB2 and TNFR2 were expressed in this pattern. While TGFβ1R remained predominantly expressed both vascularised retinal areas, FGFR1 and VEGFR2 were shifted by hypoxia into this expression pattern (FGFR1, Fig. 3F). Tsp-1 expression was changed by hypoxia from predominant expression in the outer avascular retina to additional predominant expression in the area of the superficial vascular plexus, where also Nur77 was predominantly expressed in the ROP retina (Tsp-1, Fig. 3 G).

In summary, 24 genes were mainly present in the area of the peripheral superficial vascular plexus, where the main vasculature is located in the ROP retina and from which neovascularization arise at this timepoint. Interestingly, the inflammatory genes TNFR1, TNFR2 and ICAM-1, as well as the antiangiogenic Tsp-1, PEDF and TIMP-1 were enriched in this area.


A
bFGF
bFGF expression (x10⁵)
200
150
100
50
0
**
**
RL
LDM_VL
LDM_VP
B
VCAM-1
VCAM-1 expression (x10⁵)
500
400
300
200
100
0
**
***
RL
LDM_VL
LDM_VP
C
VEGFA
VEGFA expression (x10⁵)
5000
4000
3000
2000
1000
0
**
***
***
RL
LDM_VL
LDM_VP
D
Ang2, B2m, VE-cadherin, EphB4,
ICAM-1, eNOS, PECAM-1, uPA,
tPA, PEDF, TGFb1, TIMP-1,
TNFR1, vWF
eNOS expression (x10⁵)
800
600
400
200
0
***
***
RL
LDM_VL
LDM_VP
E
Bcl2, EphrinB2, Endoglin,
VEGFR1, MMP9, iNOS, PDGFB,
PDGFRB, Tie2, TNFR2
MMP9 expression (x10⁵)
800
600
400
200
0
***
***
***
RL
LDM_VL
LDM_VP
F
FGFR1, VEGFR2, TGFβ1R
FGFR1 expression (x10⁵)
400
300
200
100
0
***
**
RL
LDM_VL
LDM_VP

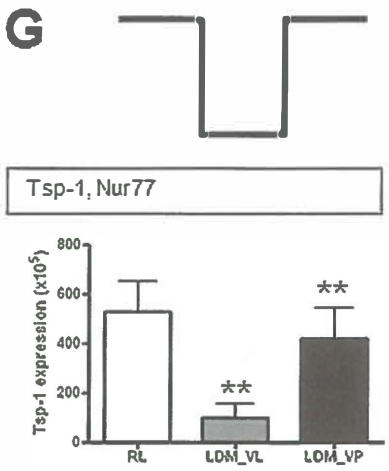


Figure 3: Hypoxia altered the assignment of genes to the described spatial expression patterns.

The same expression patterns A through F, as described for the control retina and one additional pattern, termed G, were found in the ROP retina at p13. The assignment of genes to the patterns was altered by hypoxia. As in Figure 2 each pattern is depicted by a schematic line drawing, the genes that were significantly expressed in this pattern are listed in the boxes and for each pattern the data of one gene is shown as example.

A: FGF2 expression was altered by hypoxia from even expression in the control retina to predominant expression in the outer avascular retina. RL versus LDM_VL ($p<0.01$), and RL versus LDM_VP ($p<0.01$). B: VCAM-1 was the only gene, which remained to be predominantly expressed in the outer avascular and in the intermediate area of the ROP retina. LDM_VL versus LDM_VP ($p<0.001$), and RL versus LDM_VP ($p=0.001$). C: Similarly, the VEGFA expression pattern was not altered by hypoxia and it was predominantly expressed in the intermediate area of the ROP retina. RL versus LDM_VL ($p<0.001$), LDM_VL versus LDM_VP ($p<0.001$), RL versus LDM_VP ($p<0.01$). D: Hypoxia increased the amount of genes which were predominantly expressed in the area of the peripheral superficial vascular plexus. For example, eNOS expression is depicted, which is also altered by hypoxia: RL versus LDM_VP ($p<0.001$), LDM_VL versus LDM_VP ($p<0.001$). E: While hypoxia altered VEGFR1 expression to enhanced expression in the intermediate and predominant expression in the superficial vascular plexus area, MMP9 expression pattern was not altered. RL versus LDM_VL ($p<0.001$), LDM_VL versus LDM_VP ($p<0.001$), and RL versus LDM_VP ($p<0.001$). F: Hypoxia shifted FGFR1 expression from predominant expression in the intermediate area of control retina to additional predominance in the peripheral superficial vascular plexus area in the ROP retina. RL versus LDM_VL ($p<0.01$), and RL versus LDM_VP ($p<0.001$). G: While Tsp-1 was predominantly expressed in the outer retina and in the area of the peripheral superficial vascular plexus in the ROP retina, it was only predominantly expressed in the outer avascular area of the control retina. RL versus LDM_VL ($p<0.01$), and LDM_VL versus LDM_VP ($p<0.01$).

Hypoxia induced gene expression especially in the area of the peripheral superficial vascular plexus and differentially regulated expression at distinct localizations

To investigate the hypoxia induced changes in angioflammatory gene expression, we compared the expression in the retinal lysates and in the two selected areas between ROP and control retina. Only three of the 46 angioflammatory genes, i.e. bFGF, uPA and TIMP-1, were significantly upregulated in the retinal lysates by hypoxia compared to the controls (Fig. 4 A). In contrast, zooming into the retina revealed that 20 genes were upregulated in the area of the peripheral superficial vascular plexus. VEGFA was the only gene identified to be upregulated in the intermediate retina, demonstrating the added value of unmasking spatial gene expression changes by area selection using laser dissection microscopy prior to gene expression analysis.

In the area of the peripheral superficial vascular plexus, from which the first sprouts arise, 20 genes were upregulated in the ROP retina. The strongest regulated genes of this area were Ang2 (15-fold versus control, p=0.00007), uPA (6-fold versus control, p=0.0002), TGFβ1 and Tsp-1 (6-fold, p=0.0006 and p=0.0005, respectively), Endoglin and TNFR1 (4-fold, p=0.000005 and p=0.003, respectively). Ang2, uPA, TGFβ1, TNFR1 and to a lesser extent tPA, eNOS and TIMP-1 were the strongest hypoxia regulated genes in the peripheral superficial vascular plexus area and were only in the ROP retina predominantly expressed in this area.

As the retinal vasculature at ROP p13 mostly consists of the peripheral superficial vascular plexus and neovascular sprouts arise from this plexus, we expected to find genes of vascular origin and angiogenesis-associated genes primarily expressed and upregulated by hypoxia in this area compared to the control retina. Indeed some vascular genes, such as VE-cadherin, eNOS, PECAM-1, PDGFB, PDGFRB and Tie2 were predominantly expressed in the superficial vascular plexus and were upregulated by hypoxia. For example the endothelial PECAM-1 was upregulated threefold by hypoxia in the peripheral superficial vascular plexus (p=0.00007, Fig.4 B). B2m, iNOS, ICAM-1, Endoglin and MMP9, which indicate angiogenic/inflammatory activated endothelium, followed the PECAM-1 expression pattern and regulation, and appeared to be primarily expressed in the superficial vascular plexus. The VEGF

receptor genes VEGFR1 and VEGFR2 were mainly expressed in the vascularised intermediate part of the control retina and were upregulated in the peripheral superficial vascular plexus area under hypoxic conditions. The spatial expression pattern of the vascular genes EphB4, iNOS and vWF was altered by hypoxia from predominant expression in both vascularised parts of the control retina, which indicates expression in the entire retinal vasculature, to a primary expression and upregulation in the area of the peripheral superficial vascular plexus (Fig. 3 D+E, and Fig. 4 A). Their expression patterns thus followed the ROP induced altered localization of the retinal vasculature. In summary, the genes of vascular origin differed in their spatial expression in the vascularised area of the control retina and became predominantly expressed and upregulated in the peripheral superficial vascular plexus under the influence of ROP.

The expression of VEGFA was differentially altered in distinct areas by hypoxia. While VEGFA was upregulated 2-fold in the intermediate retina ($p=0.03$), where it was predominantly expressed and where vasoregression occurs, it was downregulated 2-fold ($p=0.048$) in the area of the peripheral superficial vascular plexus (Fig.4 C). Hypoxia furthermore altered the Ang2 expression from primary expression in the vascularised intermediate area of the control retina to predominant expression in the peripheral superficial vascular plexus area of the ROP retina, where it was upregulated 15-fold ($p=0.00005$, Fig.4 E). These data imply that while in the control retina Ang2 is expressed by cells of the deep and intermediate vascular plexus area, it is induced in cells of the peripheral superficial vascular plexus area in the angiogenic retina. The genes PGF, TGFβ1 and TNFR1 were evenly expressed in the control retina and 3-fold, 5-fold and 4-fold upregulated by hypoxia in the area of the peripheral superficial vascular plexus ($p=0.048$, $p=0.0006$, $p=0.003$), respectively. Surprisingly, Tsp-1, which was predominantly expressed in the outer avascular control retina, was fivefold upregulated in the peripheral superficial vascular plexus area by hypoxia ($p=0.0005$, see Fig. 2A and Fig.3 G). The expression of uPA was upregulated in two distinct areas by hypoxia, 3-fold in the avascular outer retina and 6-fold in the area of the peripheral superficial vascular plexus ($p=0.03$, $p=0.0002$, respectively) (Fig.4 D). Similarly, TIMP-1 was 6-fold upregulated in the avascular outer retina ($p=0.002$) and was

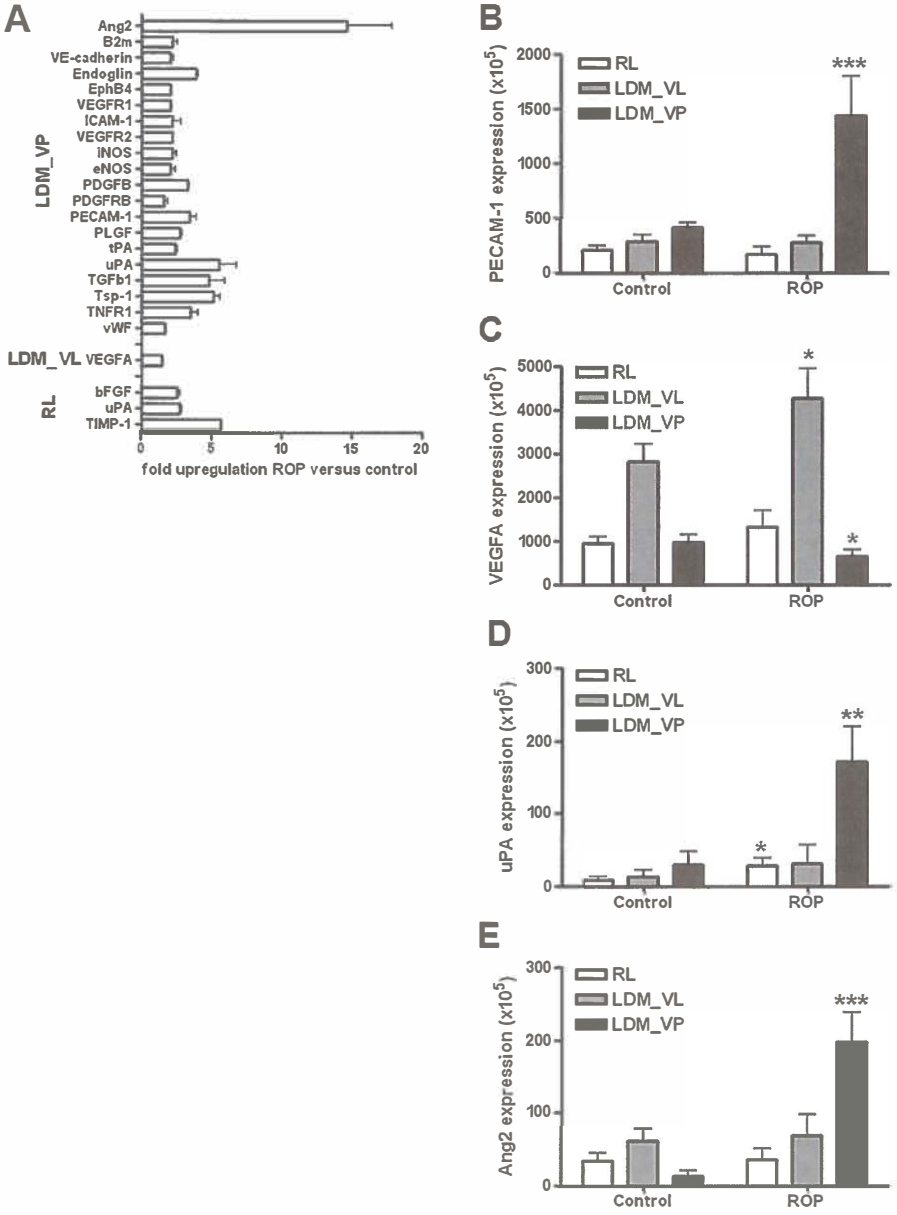


Figure 4: Retinal angioflammatory genes were upregulated by hypoxia, especially in the area of the peripheral superficial vascular plexus.
A: Significantly ($p<0.05$) upregulated genes are listed in the respective area with their fold expression versus control retina. B: PECAM-1 is displayed as example for hypoxia induced genes, which are primarily expressed in the peripheral inner retinal layers in control and ROP retina. PECAM-1 is upregulated threefold by hypoxia ($p<0.001$) in the area of the peripheral superficial vascular plexus. C: VEGFA is twofold upregulated ($p<0.05$) in the LDM_VL and twofold downregu-

lated in LDM_VP area ($p<0.05$) by hypoxia. D: uPA is displayed for genes, which are upregulated in more than one area by hypoxia. uPA is threefold upregulated ($p<0.05$) in the retinal lysates and sixfold ($p<0.01$) in the peripheral superficial vascular plexus area compared to the respective areas of control retina. E: Hypoxia induced a shift of Ang2 expression to primary expression in the area of the peripheral superficial vascular plexus, where it was 15-fold upregulated ($p<0.001$).

detectable in the peripheral superficial vascular plexus area of the ROP in contrast to the control retina (see supplementary Table 1 and 2). Conclusively, Tsp-1, uPA and TIMP-1, which appeared to be predominantly expressed in cells of the outer avascular area in control retina, were induced by hypoxia in a different sort of cells in the peripheral superficial vascular plexus area.

Downregulation by hypoxia of angioflammatory genes

Figure 5A depicts those genes that were downregulated by hypoxia in different retinal areas. In the retinal lysates two genes, VCAM-1 and vWF were downregulated by hypoxia. Moreover, in the vasoregressed intermediate retina HIF1α was almost 2-fold downregulated by hypoxia at p13 ($p<0.01$, Fig. 5 B). Thus, hypoxia altered HIF1α expression pattern from being predominant in the outer avascular area and in the vascular intermediate area of the control retina to even expression throughout the ROP retina. The expression of VCAM-1 was twofold downregulated in the outer avascular retina ($p=0.001$) and its expression was most prominent in the vasoregressed intermediate area in the ROP retina. Hypoxia differentially altered vWF expression at two distinct localizations. While vWF was downregulated twofold in the outer avascular retina ($p<0.01$), it was upregulated twofold in the area of the peripheral superficial vascular plexus ($p<0.01$, Fig.5 C). As mentioned, VEGFA expression was downregulated selectively in the area of the peripheral superficial vascular plexus.

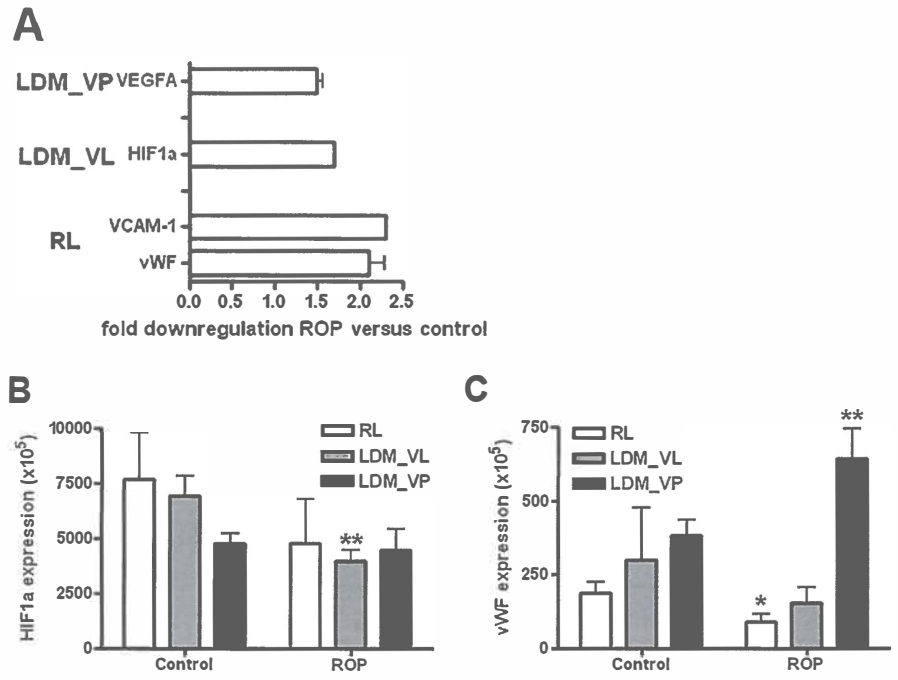


Figure 5: Hypoxia downregulated angioflammatory gene expressions in distinct retinal areas.
A: Four genes were significantly downregulated by hypoxia in distinct areas in the ROP retina. B: HIF1α expression displayed is an example for downregulation by hypoxia in a single area. HIF1α was downregulated twofold in the LDM_VL area (p=0.004). C: vWF was downregulated twofold in RL (p=0.03) and twofold upregulated (p=0.003) in the peripheral superficial vascular plexus area by hypoxia.

Retinal protein expression correlated with gene expression patterns for vascular markers, not for the inflammatory molecules ICAM-1 and CD45

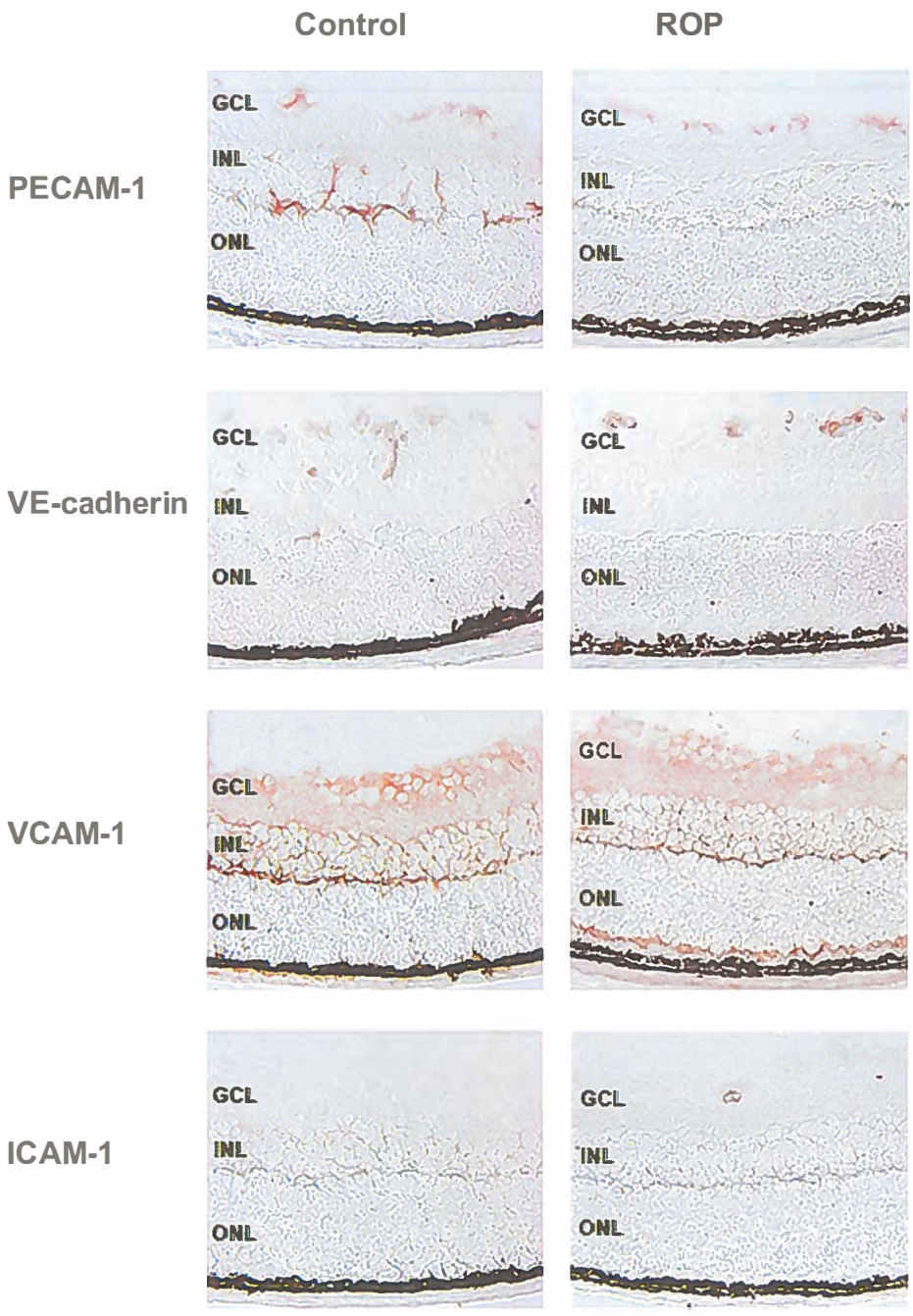
A selection of vascular and inflammatory molecules was analysed by immunohistochemistry to correlate localization of gene expression with protein expression (Figure 6). The endothelial marker gene PECAM-1 stained in the retinal vascular plexi at the outer border, within and at the inner border of the inner nuclear layer, as well as in the nerve fiber layer in the control retina at p13. In the ROP retina, PECAM-1 staining was mostly present in the peripheral nerve fiber layer in the peripheral superficial vascular plexus, corroborating the spatial gene expression pattern. VE-cadherin staining pat-

tern correlated with the PECAM-1 staining pattern in both control and ROP retina and correlated with the spatial gene expression pattern. In contrast to PECAM-1 and VE-cadherin, VCAM-1 staining did not show a vascular pattern, which was supported by the RNA expression pattern (see Fig.2 B and Fig.3 B). In both control and ROP retina, its expression was strongly detectable from the border of the photoreceptor layer to the retinal pigment epithelium, in the outer plexiform layer, and from the inner plexiform layer to the inner limiting membrane. Furthermore, the staining intensity increased from the retinal periphery towards the central retina. While VCAM-1 protein was present in the inner retinal layers, its mRNA was expressed at low level in this area. In contrast to the mRNA expression patterning, the staining for the inflammatory factor ICAM-1 was negative in the control retina and only single positive cells were occasionally found in the nerve fiber layer in the ROP retina at p13. Moreover, the induction of ICAM-1 gene expression in the area of the peripheral superficial vascular plexus by hypoxia was not consistently detectable at the protein level. Although CD45 mRNA was detectable in the retina, staining was neither found in control, nor in ROP retina (data not shown).

next page

Figure 6: Retinal protein patterns of vascular markers and inflammatory molecules in control and ROP retina.

Top row: PECAM-1 staining was detected at the broders and within the inner nuclear layer and in the nerve fiber layer in control retina at p13. In the ROP retina PECAM-1 positive cell were mainly found in the nerve fiber layer. Second row: While in control retina VE-cadherin weekly stained at the site of the three vascular plexi, it was stronger in the peripheral superficial vascular plexus area in the ROP retina. Third row: Unexpectedly, VCAM-1 staining was abundant at the outer border of the photoreceptor layer, the outer plexiform layer, throughout the inner plexiform layer and in the nerve fiber layer and did not show a vascular pattern. Fourth row: ICAM-1 staining was negative in control and only single ICAM-1 positive cells were occasionally found in the ROP retina at p13.


Control
ROP
PECAM-1
VE-cadherin
VCAM-1
ICAM-1
GCL
INL
ONL

Discussion

The current study demonstrates that angioflammatory genes differ in their spatial distribution in the retina and that their spatial expression patterns and their localised expression levels change under influence of hypoxia in the ROP model. Furthermore, several angiogenesis associated genes could be assigned to the area of the peripheral superficial vascular plexus at the onset of neovascularization, which has not been reported before. Some of these genes, including Ang2, uPA, TGFβ1 and TNFR1, were only in the ROP retina predominantly expressed in this area and were the most strongly regulated genes by hypoxia, which suggests an important role in angiogenesis incipience.

These new insights in complex spatial gene expression control could not have been created without the selection of distinct retinal areas by laser microdissection prior to gene expression analysis. The analyses revealed for the majority of genes distinct spatial distribution patterns in the postnatal retina and differential spatial expression and localized regulation in the ROP retina. Hypoxia enhanced angioflammatory gene expression especially in the area of the peripheral superficial vascular plexus, from which neovascularization arise at p13. While uPA, TGFβ1 and TNFR1 were evenly expressed in the control retina, they were strongly induced and predominantly expressed at the site of angiogenesis in the ROP retina. All three genes have been associated with retinopathy, but were previously not conceived as important players of the early phase of neovascularization. Vitreal samples of patients with proliferative diabetic retinopathy have detectable levels of the urokinase plasminogen activator (18) and the genetic deletion of uPA reduces preretinal neovascularization in experimental proliferative retinopathy by 60% (Hammes and Carmeliet, unpublished). These and our experimental data assign a proangiogenic function to uPA in the incipience of retinal neovascularization. In line with this, the intravitreal application of recombinant plasminogen activator inhibitor effectively reduced proliferative retinopathy in a rat model (19). Studies suggest glia cells, endothelial cells and macrophages as sources of uPA (20, 21), also in experimental proliferative retinopathy (Hammes et al., unpublished).

The functions of TGFβ1 are diverse and context-dependent. A patient study showed that vitreal levels of active TGFβ1 were decre-

ased in active proliferative diabetic retinopathy, while increased in quiescent proliferative diabetic retinopathy (22). Application of exogenous activated TGFβ1 rescued vessels from experimental hyperoxia-induced vasoregression by inducing endothelial VEGFR1 expression (23). In addition, TGFβ1 synergizes with VEGF to induce migration of the sprouting tip cell, but inhibits proliferation of the stalk cells in sprouting angiogenesis in the neonatal retina (24). In the light of these data, upregulation in the area of the superficial vascular plexus might reflect the need of the promigratory function of TGFβ1 at the incipience of neovascularization, but could also indicate intraretinal vessel stabilization as a counterbalance to hyperoxia induced intraretinal vasoregression. At p13, the intraretinal vasculature of the ROP retina is at the transition between hyperoxia induced vasoregression, hypoxia induced intraretinal regrowth and preretinal neovascularization. The cellular origins of TGFβ1 are retinal pericytes (23) and glia cells, especially under in vitro hypoxic condition (25). Our finding of predominant TGFβ1 expression in the peripheral superficial vascular plexus area might indicate expression by pericytes.

As we aimed to link inflammatory activity with neovascularization activity in the retina, it was of interest to note that TNFR1, a receptor involved in proinflammatory and in apoptotic signaling, was predominantly expressed and upregulated at the site of angiogenesis incipience in the ROP retina. Our observation would support the current concept that inflammatory factors participate or even initiate retinal neovascularization. In line with this concept, elevated vitreal levels of soluble TNFR1 are found in patients with proliferative diabetic retinopathy (26) and in TNFR1 knockout mice vascular regrowth is limited in the ROP model at p20 (27). Furthermore, other inflammatory molecules, such as ICAM-1 and B2m were upregulated by hypoxia at the angiogenic site. In contrast, staining of ICAM-1 was only occasionally found in single cells in the ROP retina at p13, which may indicate that we analysed ICAM-1 protein expression during the time window, when the translation from RNA to protein was not yet complete. Similar to ICAM-1, we did not detect CD45 on protein level neither in the control, nor in the ROP retina, although it was expressed at RNA level. The reason for these discrepancies is at present unknown, but justifies further investigations.

Surprisingly, we found hypoxia induced upregulation of Tsp-1 in the area of the peripheral superficial vascular plexus and predominant PEDF expression in the superficial vascular plexus of both the control and ROP retina. These genes are known to exercise antiangiogenic functions, as exemplified in studies in which human recombinant pigment epithelium derived factor intravitreally injected inhibited preretinal neovascularization in the mouse ROP model (28). In addition, physiological retinal vascularization was enhanced in PEDF knockout mice (29). PEDF is thought to be mainly expressed in the retinal pigment epithelium, and in vitro it is downregulated by hypoxia in Mueller cells (30), which is contradictory to the RNA profile we found. In the ROP model, Tsp-1 overexpression reduces vascular density, while absence of Tsp-1 enhances vascular density and maturation of retinal vascularization (31, 32). Furthermore, increasing PEDF and Tsp-1 RNA levels are associated with less preretinal neovascularization in certain mouse strains exposed to the ROP model (33). These and similar data in the rat support the concept of an antiangiogenic function of PEDF and Tsp-1 in the retina. Furthermore, based on these data the concept was proposed that the balance between pro- and antiangiogenic factors analysed in the entire retina is the determinant for retinal neovascularization. This concept was derived from tumor models in which the term angiogenic switch has been assigned to the observed overexpression of proangiogenic factors over antiangiogenic factors at the onset of angiogenesis (34). Recently, this switch was associated with transcriptional upregulation of Tsp-1 in dormant tumors versus its proangiogenic counterparts (35). In contrast, Suzuma et al. observed upregulation by hypoxia of Tsp-1 in retinal lysates at ROP p13 and the protein expression was enhanced in the neovascular tufts of the ROP p17 retina. Concordant with this study we observed fivefold induction of Tsp-1 in the area of the peripheral superficial vascular plexus, but we did not find altered expression in retinal lysates of the ROP p13 retina. The overexpression of antiangiogenic factors at the incipience of neovascularization appears paradox, but indicates that zooming into distinct retinal areas can reveal data, which seem to contrast current concepts. In some cancer patients and in experimental models Tsp-1 can also exert a proangiogenic function (36), which might indicate that Tsp-1 has a dual role in this context, however this needs further investigation.

These data indicate that retinal neovascularization is initiated despite the expression of a number of antiangiogenic genes and possibly, induced Ang2 expression is instrumental in shifting the balance to a proangiogenic stimulus. This underlies the challenging hypothesis that retinal neovascularization is mediated by local changes in expression levels and by local context dependent function of angiogenesis-associated factors. It is well accepted that high levels of angiopietin-2 in a VEGF rich environment induce neovascularization and our data show a 15-fold upregulation by hypoxia of Ang2 expression in the area of the peripheral superficial vascular plexus at the timepoint of neovascularization incipience. VEGFA is upregulated by hypoxia in the intermediate retina, where the Mueller cells are located, and these cells are a major production site for VEGF (10). In the area of the peripheral superficial vascular plexus VEGFA was downregulated by hypoxia, although it has been demonstrated to be expressed by astrocytes in front of the sprouting edge of the superficial vascular plexus in developmental retinal vascularization (37). It appears that in the ROP retina VEGFA mRNA production is reduced in the astrocytes and occurs predominantly in the Mueller cells. At the same time VEGFR2, which is key to downstream angiogenic signaling induced by VEGF, is expressed at higher level in this area in the ROP retina. Our data probably indicate that Mueller cell derived VEGFA signals via the endothelial VEGFR2 (38) in the area of neovascularization incipience, which supports the importance of endothelial-glial interaction in this process. Based on the current concepts of angiogenesis induction and our data, we put forward that the local constellation of strong hypoxic upregulation of Ang2 in the presence of VEGF, and together with upregulation of VEGFR2 in the area of neovascularization incipience is key in initiating neovascularization in this model. Furthermore, the loss of probably astrocytic VEGFA in the peripheral superficial vascular plexus area might indicate that the stimulus to preretinal growth of neovascular sprouts is due to the loss of VEGF patterning and disruption of VEGF gradients in this area, as previously indicated in a study of Gerhardt et al. in the early postnatal retina (37).
We expected to find genes of vascular origin enriched in the vascularised areas of the control retina. Indeed, we found that most of the 20 genes predominantly expressed in the vascularised retina and most of the 10 genes with predominant expression in the area of the

peripheral superficial vascular plexus were of vascular origin. The vascular restricted genes showed a differential spatial expression pattern within the vascularised control retina. Some genes, such as PECAM-1 were primarily expressed in the area of the superficial vascular plexus, while others were predominantly expressed in the area of the intermediate and deep vascular layer, such as the VEGFR2. Other vascular genes, such as the pericyte marker gene PDGFRB were evenly expressed in the vascularised layers. In the ROP retina the vasculature of the intermediate and deep vascular layer and around the optic nerve entrance in the superficial vascular layer mostly regresses during hyperoxia. At ROP p13 the retinal vasculature mostly consists of the peripheral superficial vascular plexus. Vascular genes were predominantly expressed in the area of the superficial vascular plexus in the ROP retina and thus followed the altered localization pattern of the retinal vasculature. Our staining of the endothelial markers PECAM-1 and VE-cadherin corroborated the altered localization of the vasculature in the ROP retina and was concordant with previously published data (37, 39).

Area selection by laser dissection microscopy prior to gene expression analysis uncovered spatial gene expression patterns and revealed hypoxic regulation of more angiogenic genes than analysis of retinal lysates. In our study of the 46 angioflammatory genes analysed, only three genes were detected as upregulated by hypoxia and two genes were downregulated at p13. All classical angiogenesis-associated genes, such as VEGFA were not regulated in whole retinal lysates, a result that is in line with previously published data (15). However, some differences existed, i.e. we did not find upregulation of HIF1α and TNFα, nor downregulation of PDGFRB, as previously reported in the same model (15). This discrepancy may be due to the difference in genetic background of the mice used. In the ROP model gene expression and angiogenic response is highly determined by the genetic background (33).

In summary, we unmasked the nature of spatial angioflammatory gene expression and its alterations induced by hypoxia in the retinae of mice subjected to the ROP model. Concordant with current concepts we found strong induction of Ang2 and VEGFR2 in the area of neovascularization incipience. Probably, Mueller cell derived VEGFA supported neovascularization incipience, while the loss of VEGFA expression in the area of neovascularization inci-

pience might attenuate VEGFA patterning. This is possibly causal for the preretinal growth of the neovascularization. Moreover, we revealed predominant expression of the antiangiogenic PEDF and strong induction of Tsp-1 in the same area. These data imply functions for these molecules, which at first sight cannot be explained by current paradigms. However, they will eventually contribute to the understanding of the molecular basis in the initiation of neovascularization in experimental proliferative retinopathy. Finally, we demonstrate that area selection by laser dissection microscopy prior to expression analysis is a valuable/necessary tool to understand the local molecular complexity of pathologic retinal angiogenesis.

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Supplementary Table 1: Angioflammatory gene expression in retinal lysates (RL), the laser dissected vascularised retina (LDM_VL) and the laser dissected peripheral superficial vascular plexus area (LDM_VP) of wildtype mice raised in normoxic condition at p13.

The table lists the relative gene expression levels relative to GAPDH (x10^5). Data is presented as mean ± standard deviation (SD). Genes below the detection level or with inconsistent detection are marked not detectable (N.D.).

	RL		LDM_VL		LDM_VP	
Gene	mean	SD	mean	SD	mean	SD
Ang1	19	8	30	26	33	14
Ang2	35	10	62	17	13	7
B2m	1884	260	1669	273	2640	321
Bcl2	43	21	107	41	90	38
MCP-1	N.D.		N.D.		N.D.	
VE-cadherin	366	90	616	142	855	95
EphrinB2	357	115	706	331	900	63
Endoglin	322	118	697	63	1200	160
EphB4	87	22	131	92	170	44
EPO	10	3	33	20	N.D.	
EPOR	6	3	N.D.		28	25
bFGF	58	21	41	18	56	28
FGFR1	145	42	359	59	212	58
FGFR2	34	7	6	4	N.D.	
VEGFR1	226	118	513	115	299	81
HIF1α	7675	2098	6902	888	4739	467
ICAM-1	18	6	19	3	53	45
Integrin αv	678	178	814	269	399	59
Integrin ß3	13	3	44	17	75	57
VEGFR2	1299	442	2501	422	762	109
MMP9	87	27	287	49	596	114
iNOS	10	3	36	21	59	44
eNOS	44	4	92	22	250	87
Nur77	327	73	365	142	441	183
PDGFB	133	31	319	51	668	124
PDGFRB	118	39	253	60	430	127
PECAM-1	211	38	293	62	425	35
PLGF	38	11	56	16	53	32
tPA	107	44	156	36	123	38

	RL		LDM_VL		LDM_VP	
Gene	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
uPA	10	4	14	9	31	18
COX-2	1	0	N.D.		N.D.	
CD45	11	5	44	26	27	13
PEDF	175	65	141	16	416	30
Tie2	50	16	131	62	232	49
TGFβ1	133	35	112	38	223	97
TGFβ1R	46	7	109	39	77	17
Tsp-1	622	155	75	45	81	33
TIMP-1	5	2	N.D.		N.D.	
TNFα	N.D.		N.D.		N.D.	
TNFR1	154	19	163	16	117	57
TNFR2	12	4	26	12	50	19
VCAM-1	497	75	543	74	83	50
NG-2	29	20	47	16	146	53
VEGFA	960	155	2820	409	976	168
vWF	188	39	299	177	384	52

Supplementary Table 2: Angioflammatory gene expression in retinal lysates (RL), the laser dissected vascularised retina (LDM_VL) and the laser dissected peripheral superficial vascular plexus area (LDM_VP) of wildtype mice subjected to the model of proliferative retinopathy at p13.
The table lists the relative gene expression levels relative to GAPDH (x10^5). Data is presented as mean ± standard deviation (SD). Genes below the detection level or with inconsistent detection are marked not detectable (N.D.).

	RL		LDM_VL		LDM_VP	
Gene	mean	SD	mean	SD	mean	SD
Ang1	23	15	26	4	43	10
Ang2	36	15	70	28	197	40
B2m	1853	573	1708	474	5864	1813
Bcl2	35	13	64	6	118	39
MCP-1	11	7	N.D.		22	22
VE-cadherin	239	107	337	116	1830	421
EphrinB2	227	62	638	176	1071	232
Endoglin	354	187	749	254	4733	454
EphB4	85	28	108	60	358	96
EPO	18	3	31	12	N.D.	
EPOR	3	1	N.D.		36	13
bFGF	151	38	45	29	70	22
FGFR1	128	35	274	65	270	39
FGFR2	36	12	6	1	26	19
VEGFR1	151	60	363	112	620	165
HIF1α	4779	1959	3983	487	4478	948
ICAM-1	29	5	29	21	118	41
Integrin αv	538	79	634	106	514	49
Integrin ß3	19	7	45	29	87	27
VEGFR2	733	198	1596	453	1652	246
MMP9	54	11	194	35	520	157
iNOS	11	3	30	17	129	66
eNOS	39	18	86	41	536	80
Nur77	375	47	198	64	503	87
PDGFB	147	55	470	95	2175	399
PDGFRB	65	26	145	43	705	57
PECAM-1	167	70	279	64	1445	359
PLGF	49	10	56	31	150	103

	RL		LDM_VL		LDM_VP	
Gene	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
tPA	111	49	108	25	295	69
uPA	28	11	32	25	172	47
COX-2	1	0	N.D.		N.D.	
CD45	13	6	32	11	36	15
PEDF	202	128	111	41	453	78
Tie2	31	11	72	32	234	53
TGFβ1	153	43	186	93	1059	90
TGFβ1R	35	8	66	28	81	21
Tsp-1	528	123	100	56	420	123
TIMP-1	29	11	18	9	70	16
TNFα	7	3	N.D.		N.D.	
TNFR1	177	47	153	22	409	110
TNFR2	10	6	32	10	76	27
VCAM-1	218	34	335	103	103	32
NG-2	53	45	40	16	99	39
VEGFA	1340	376	4288	658	658	148
vWF	90	29	153	53	641	103

Chapter V

Deletion of TNFα reduces preretinal neovascularization without affecting developmental vascularization, and alters spatial inflammation- and angiogenesis-associated gene expression and regulation in experimental proliferative retinopathy

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Abstract

Preretinal neovascularization arising from intraretinal vessels characterize proliferative retinopathies. As the contribution of inflammation in this process is poorly understood, we investigated the effect of tumor necrosis factor α (TNFα) on retinal vascularization and on spatial expression of inflammatory- and angiogenesis-associated (termed angioflammatory) factors in experimental proliferative retinopathy. C57BL/6J and TNF-/- mice were kept in room air or subjected to the model of retinopathy of prematurity (ROP). Retinal vasculature was analysed at postnatal day 7 (p7), p12 and p17. At p13 the expression of 46 angioflammatory genes was analysed in vascularised retinal layers selected by laser dissection microscopy, part of which were followed up for protein expression. TNFα deficiency neither influenced vascularization at p7, nor vascular response to the hyperoxic period at p12, but reduced preretinal neovascularization by 54% at p17. In TNF-/- mice 28 of the 46 angioflammatory genes differed in their spatial distribution pattern in the control, and for some genes, e.g. angiopoietin-2 the pattern was altered in the ROP retina. Although the expression of angioflammatory genes/area were comparable, the spatial expression patterns differed for, e.g. basic fibroblast growth factor between TNF-/- and wildtype mice. Expression of angioflammatory genes, e.g. thrombospondin-1 and urokinase plasminogen activator were differently induced by hypoxia in the area of neovascularization incipience in TNF-/- mice. Hypoxia induced upregulation of VEGF protein was reduced in TNF-/- mice at p13. TNFα deficiency reduced preretinal neovascularization at p17 without affecting physiological vascularization and vascular response to the hyperoxic period. The reduction in preretinal neovascularization is likely due to reduced VEGF levels in the TNF-/- mice. Zooming into the vascularised retina revealed differences in angioflammatory gene expression between wildtype and TNF-/- mice, which need further investigation.

Introduction

Retinal neovascularization is the hallmark of proliferative retinopathies, such as proliferative diabetic retinopathy and retinopathy of prematurity (ROP) (1, 2). Intravitreal growth of neovascularization causes visual impairment and can ultimately lead to blindness.

A key player in the pathogenesis of preretinal neovascularization is vascular endothelial growth factor (VEGF) (3). VEGF is expressed in the retinal glia cells, which are the Mueller cells and the astrocytes (4). In developmental retinal angiogenesis VEGF produced by astrocytes signals via vascular endothelial growth factor receptor 2 (VEGFR2), which is expressed by endothelial cells and guides angiogenic sprouting in a complex manner (5). VEGF is regulated by hypoxia, which arises during retinal vascular development, due to diabetes induced or hyperoxia induced vasoregression in the clinical proliferative retinal diseases (6). Based on the essential role of VEGF in pathologic retinal angiogenesis anti-VEGF drugs have been developed, which either target directly VEGF or its receptor (7). The interaction of glial VEGF on its endothelial receptor indicates the importance of the neuroglial microenvironment in the development of retinal neovascularization. However, VEGF is not the only player in the pathogenesis of retinal neovascularization. Several other angiogenesis-associated factors have been studied in experimental proliferative retinopathy. These studies demonstrated that the angiopoietins (Ang), insulin like growth factor 1, pigment epithelium derived growth factor (PEDF) as well as integrins, adhesion molecules and matrix molecules participate in the retinal angiogenic process (8).

The angiogenic process is accompanied by signs of inflammation (9), such as increased vascular permeability, the upregulation of inflammatory factors and the occurrence of inflammatory cells at the site of angiogenesis. In patients with proliferative retinopathies increased vascular permeability and the occurrence of inflammatory factors in vitreal fluids can be observed (10, 11). Furthermore, macrophages are increased in the hypoxic retina and seem to play a role in the formation of preretinal neovascularization (12). Anti-inflammatory drugs, such as nepafenac reduced preretinal neovascularization in experimental proliferative retinopathy (13). However, it is still unknown, whether inflammation plays an essential role in the initiation of angiogenesis or whether it occurs alongside

the angiogenic process. Tumor necrosis factor α (TNFα) is a key player in inflammatory signaling. Recently, a study indicated that Ang2 sensitizes endothelial cells to a TNFα stimulus, which links inflammation and angiogenesis (14). In the model of proliferative retinopathy, genetic deletion of TNFα and its inhibition by semapimod reduced preretinal neovascularization. Despite these studies it remains unknown, whether the effect of TNFα deletion on preretinal neovascularization is brought about by altered developmental angiogenesis, or by modified responses to hyperoxia induced vasoregression. These data are important, as we have previously shown that maturation of the retinal vasculature at the onset of the mouse model of proliferative retinopathy reduces the preretinal neovascularization response (15). Furthermore, the molecular mechanisms of the TNFα effect is obscure.

Preretinal neovascularization arise from the intraretinal vessels of the peripheral superficial vascular plexus from postnatal day 13 (p13) onwards. So far, gene expression in this area is poorly understood as gene expression analyses are usually performed in whole retinal lysates, which do not disclose spatial expression. We suggested that zooming into this area by tissue selection using laser microdissection prior to gene expression analysis at this time point of neovascularization incipience would uncover the molecular mechanism behind the TNFα mediated effect on preretinal neovascularization.

First, we studied the effect of TNFα deletion on developmental retinal angiogenesis prior to the exposure to the ROP model and its effect on hyperoxia induced vasoregression immediately after the hyperoxic exposure. We, furthermore investigated the effects of TNFα deletion on spatial distribution and localized regulation of inflammatory- and angiogenesis-associated gene expression (termed angioflammatory genes) in the postnatal and in the incipient angiogenic retina. Moreover, a selection of genes was analysed on protein level to corroborate mRNA levels.

Material and Methods

Animals

All experiments in this study were performed according to the guidelines of the statement of animal experimentation issued by the Association for Research in Vision and Ophthalmology and were approved by the Institutional Animal Care and Use Committee.
Wildtype (C57BL/6J) and TNF knockout (TNF-/-) (16) mice were used in this study. Breeding of the mice and exposure to the ROP model was performed at the laboratory animal facility of the Medical Faculty Mannheim (University of Heidelberg, Germany). Animals were kept at a 12 hours day-12 hours night light cycle and fed with standard chow and tap water.

Physiological angiogenesis

To assess the influence of TNFα deletion on physiological angiogenesis wildtype and TNF-/- mice, which were kept in room air condition, were sacrificed at postnatal day 7 (p7). The eyes were obtained and immediately frozen in liquid nitrogen and stored at -80°C.

Proliferative retinopathy mouse model

The mouse retinopathy of prematurity model was used as previously described (17). Briefly, newborn mouse litters were split and either exposed from p7 until p12 in an incubation chamber with 75% oxygen and then returned to room air (ROP), or were kept at room air the entire time (controls). Eyes obtained at p12, p13 and p17 were immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

Retinal whole mount staining

Whole eyes were fixed in 4% formalin (Merck, Darmstadt, Germany) for 2 hours and then washed in phosphate buffered saline. The retina was isolated and washed again. After incubation in a 0.5% Triton X-100 (Sigma, Munich, Germany) and 1% bovine serum albumine (Sigma) solution for 30 minutes at room temperature, the retinae were incubated with a TRITC labelled lectin of Bandeiraea simplicifolia (1:50, Sigma) at 4°C overnight. The next day the retina was incised at four positions in the retinal periphery and flat mounted on glass slides in 50% glycerole (Sigma) solution.

Quantification of vascularised and avascular area, capillary diameter and capillary density

Lectin stained retinal whole mount preparations of wildtype and TNF-/- mice at p7 were analysed for vascularised area, capillary diameter and capillary density. For quantification of the vascularised area images were taken at 100x magnification at a DMRBE microscope (Leica, Bensheim, Germany) for each retinal leaf. Using the drawing tool of the Qwin (Leica) software the area of each retinal leaf was measured. The area covered by the retinal vasculature was measured the same way. The data was calculated for each leaf separately and the mean was taken for each animal (n=4-5). Capillary diameter was measured with the QWin (Leica) software in images taken at 200x magnification in the capillary area of the superficial vascular plexus (n=4-5). Capillary density was measured in the same images converted into grayscale images and the area covered by capillaries was calculated by the AnalySIS software (Olympus, Hamburg, Germany).

Furthermore, lectin stained retinal whole mount preparations of wildtype and TNF-/- exposed to the ROP model at p12 (n=4-5), immediately after return to normoxia, were quantified for the size of the vascularised area, the avascular (vasoregression) area, for capillary diameter and capillary density. For area measurement images were taken at 100x magnification at a DMRBE microscope (Leica). As described above, the drawing tool of the QWin software (Leica) was used to measure the area of each retinal leaf, the vascularised area and the central asvacular area. Furthermore, capillary diameter and capillary density were measured as described above.

Quantification of preretinal neovascularization

At p17 eyes were obtained from ROP exposed wildtype and TNF-/- mice for quantitative analysis of preretinal neovascularization (pNV). Serial paraffin sections (6μm) of the eye were stained with periodic-acid Schiff's reagent (Sigma) and hematoxylin (Merck; PAS-H). Preretinal neovascularization nuclei were counted as described previously (18). Briefly, nuclei in lumenized profiles at the vitreal side of the inner limiting membrane were counted using a DMLS2 microscope (Leica) at 400x magnification. 10 retinal sections, each 12μm apart were quantified per animal (n=7).

Laser Dissection Microscopy
Laser Dissection Microscopy (LDM) was performed on retinal serial cryosections (6μm), which were mounted on polyethylene-naphtalene membranes attached to glass slides (P.A.L.M. Microlaser Technology AG, Bernried, Germany). Before laser dissection cryosections were briefly acetone fixed, stained with Mayer's haematoxylin solution and air dried. Areas for dissection were selected using P.A.L.M. software on a Laser Robot Microbeam System at 200x magnification (P.A.L.M. Microlaser Technology AG). The area from the inner limiting membrane to the outer plexiform layer (LDM_VL), which consists of the vascularised retinal layers, was cut and collected over the entire retina of each section. The area from the inner limiting membrane to the ganglion cell layer (LDM_VP), which consists of the superficial vascular plexus), was cut and collected from the outer 2/3 of each retinal section. For LDM_VP the selection range was calculated for each section separately before cutting. Both areas were ideally cut from the sections of the same eye, or one area was obtained from another eye, if the tissue was not sufficient. Approximately $3x10^6$ μm^2 retinal tissue per area and per retina of individual animals (n=5) was dissected.

mRNA gene expression analysis
Retinae were isolated from frozen eyes of ROP exposed and control mice at p13. Retinae were homogenized and total retinal RNA/per retina/per animal was isolated using the RNeasy Mini Plus kit (Qiagen, Leusden, The Netherlands). These samples were termed retinal lysates (RL) and consisted of the eyes contralateral from the ones used for laser dissection. Total RNA of laser dissection material of ROP exposed and control eyes of two retinal areas (n=5) was individually isolated using the RNeasy Micro kit (Qiagen). The cDNA was synthesized from 10μg RNA using Superscript III, RNase OUT (Invitrogen, Carlsbad, CA, U.S.A) and random hexamers (Promega, Madison, WI, U.S.A). The cDNA of each retinal lysate and of each dissected area of individual animals (n=5) was loaded according to the manufacturer's protocol in duplicate on custom designed 48 gene low density array plates (LDA, Applied Biosystems, Foster City, CA, U.S.A). Key inflammatory- and angiogenesis associated genes were selected from the literature (see Table1). Quantitative real-time RT-PCR was performed with an ABI PRISM 7900

HT Sequence Detector (Applied Biosystems). Ct values ≥40 were considered as undetermined. If more than four out of ten values per group/area were missing the gene was considered as undetermined in this group. Data was analysed with the SDS 2.3. software (Applied Biosystems) and mRNA expression was calculated relative to the housekeeping gene GAPDH using the $2^{-\Delta Ct}$ method (19).

Detection of retinal VEGF protein levels

The retinae were isolated from frozen eyes of control and ROP exposed wildtype and TNF-/- mice at p13 and p17 (n=5 per group and time point). The retinae were homogenized through needles using a RIPA lysis buffer (PBS [pH 7.4], 0.25% Sodiumdesoxycholat, 15 NP-40, 150mM NaCl, 1mM EDTA, 0.1%SDS, 1mM PMSF, 1mM Na_3VO_4, 1mM NaF) with freshly added protease inhibitors (Complete mini, Roche, Mannheim, Germany). Homogenates were centrifuged at 15,000rpm for 15min at 4°C. Protein concentration was measured in the supernatants using RC/DC protein assay (Bio-Rad, Munich, Germany). A mouse VEGF ELISA (R&D, Mannheim, Germany) was performed according to the manufacturer's protocol using 10µg retinal protein. Colorimetric change was measured with a microplate reader (SLT Labinstruments GmbH, Crailsheim, Germany). All samples were assayed in duplicate.

Immunohistochemistry

Serial retinal cryosections (8µm) of ROP exposed and control mice at p13 were fixed in acetone and then blocked for endogeneous peroxidase. After a washing step the sections were incubated with rat-a-mouse PECAM-1 (1:100, BD Pharmingen, Heidelberg, Germany), rat-a-mouse ICAM-1 (YN1/1.7.4 hybridoma supernatant, ATTC CRL-1878, U.S.A), and rat-a-mouse CD45 (1:50, BD Pharmingen) at 4°C overnight. Another washing step followed and then the sections were incubated with the secondary antibody rabbit-a-rat (1:40, DAKO, Hamburg, Germany) for 60min at room temperature. All primary antibodies were diluted in 1% bovine serum albumine solution in PBS and the secondary antibody in 1% bovine serum albumine (Sigma, Munich, Germany) and 2% normal mouse serum (DAKO) in a PBS solution. As negative controls sections were incubated with the respective rat IgG2b or IgG2a isotype controls (1:50, Antigenix America Inc., NY, U.S.A) instead of the first antibody.

The detection was performed with AEC solution (Vector, Burlingame, U.S.A.). Detection of VCAM-1 and VE-cadherin was performed using the EnVision kit (DAKO). Briefly, retinal sections were stained with rat-anti-mouse VCAM-1 (1:10, M/K-2.7. hybridoma supernatant, ATTC CRL-1909, U.S.A) or rat IgG1 isotype control (1:50, Antigenix America) as negative control at 4°C overnight. Other sections were incubated with VE-cadherin antibody (1:100, kindly provided by E. Dejana) at 4°C overnight. After washing the sections were incubated with the secondary antibody rabbit-a-rat (1:300, Vector) diluted in 1% bovine serum albumine (Sigma) and 1% normal mouse serum (DAKO) in PBS solution for 60min at room temperature. Another washing step was followed by incubation with a-rabbit HRP Polymer solution (EnVision kit, DAKO) for 30 min at RT and detection was performed with AEC reagent from the EnVision kit according to the manufacturer's protocol. VE-cadherin staining was performed with HBSS (GIBCO, Invitrogen, Karlsruhe, Germany) solution instead of PBS in all steps.

Statistics

Data of two groups was analysed by unpaired t-test. One way analysis of variance (ANOVA) and Tukey's posttest for multiple comparisons was used for data comparison of more than two groups. Quantitative real-time RT-PCR data was analyzed using the R software (R Development Core Team, R Foundation, Vienna, Austria) with a linear mixed effect model (LME). The gene expression in the three data sets and in the control versus the ROP groups of the TNF-/- mice was statistically compared. Furthermore, we compared spatial gene expression and regulation by hypoxia between the TNF-/- data sets and the wildtype data sets, which we generated in another study (vom Hagen et al., manuscript submitted). P values were adjusted for false discovery rate of multiple comparisons according to the Benjamini and Hochberg procedure (20). Adjusted p-values <0.05 were considered significant.

Table 1: List of the selected 47 angioflammatory genes analysed by real-time RT-PCR.

General name	Gene name	Gene Symbol	Assay ID
GAPDH	glycerinaldehyde 3-phosphate dehydrogenase	Gapdh	Mm99999915_g1
Ang1	angiopoietin-1	Angpt1	Mm00456503_m1
Ang2	angiopoietin-2	Angpt2	Mm00545822_m1
B2m	ß-2-microglobulin	B2m	Mm00437762_m1
Bcl2	B-cell lymphoma 2	Bcl2	Mm00477631_m1
MCP-1	chemokine ligand 2, monocyte chemotactic protein 1	Ccl2	Mm00441242_m1
VE-cadherin	vascular endothelium cadherin 5, type2	Cdh5	Mm00486938_m1
EphrinB2	ephrinB2	Efnb2	Mm00438670_m1
Endoglin	endoglin	Eng	Mm00468256_m1
EphB4	Eph receptor B4	Ephb4	Mm00438750_m1
EPO	eryhropoietin	Epo	Mm00433126_m1
EPOR	erythropoietin receptor	Epor	Mm00438760_m1
bFGF	basic fibroblast growth factor	Fgf2	Mm00433287_m1
FGFR1	fibroblast growth factor receptor 1	Fgfr1	Mm00438923_m1
FGFR2	fibroblast growth factor receptor 2	Fgfr2	Mm00438941_m1
VEGFR1	vascular endothelial growth factor receptor 1	Flt1	Mm00438980_m1
HIF1α	hypoxia inducible factor 1 α	Hif1a	Mm00468869_m1
ICAM-1	intercellular adhesion molecule	Icam1	Mm00516023_m1
Integrin αv	integrin αv	Itgav	Mm00434506_m1
Integrin ß3	integrin ß3	Itgb3	Mm00443980_m1
VEGFR2	vascular endothelial growth factor receptor 2	Kdr	Mm00440099_m1
MMP2	matrix metallopeptidase 2	Mmp2	Mm00439508_m1
MMP9	matrix metallopeptidase 9	Mmp9	Mm00442991_m1

General name	Gene name	Gene Symbol	Assay ID
iNOS	inducible nitric oxide synthase	Nos2	Mm00440485_m1
eNOS	endothelial nitric oxide synthase	Nos3	Mm00435204_m1
Nur77	nur77	Nr4a1	Mm00439358_m1
PDGFB	platelet derived growth factor B	Pdgfb	Mm00440678_m1
PDGFRB	platelet derived growth factor receptor B	Pdgfrb	Mm00435546_m1
PECAM-1	platelet/endothelial cell adhesion molecule	Pecam1	Mm00476702_m1
PLGF	placental growth factor	Pgf	Mm00435613_m1
tPA	tissue plasminogen activator	Plat	Mm00476931_m1
uPA	urokinase plasminogen activator	Plau	Mm00447054_m1
COX-2	cycloxygenase 2	Ptgs2	Mm00478374_m1
CD45	protein tyrosine phosphatase, CD 45	Ptprc	Mm00448463_m1
PEDF	pigment epithelium derived growth factor	Serpinf1	Mm00441270_m1
Tie2	endothelial tyrosine kinase	Tek	Mm00443242_m1
TGFβ1	transforming growth factor B 1	Tgfb1	Mm00441724_m1
TGFβ1R	transforming growth factor B 1 receptor	Tgfbr1	Mm00436964_m1
Tsp-1	thrombospondin-1	Thbs1	Mm00449022_m1
TIMP-1	metallopeptidase inhibitor 1	Timp1	Mm00441818_m1
TNFα	tumor necrosis factor α	Tnf	Mm00443258_m1
TNFR1	tumor necrosis factor receptor superfamily, member 1A	Tnfrsf1a	Mm00441875_m1
TNFR2	tumor necrosis factor receptor superfamily, member 1B	Tnfrsf1b	Mm00441889_m1
VCAM-1	vascular cell adhesion molecule 1	Vcam1	Mm00449197_m1
NG-2	versican, chondroitin sulfate proteoglycan 2	Vcan	Mm00490179_m1
VEGFA	vascular endothelial growth factor a	Vegfa	Mm00437304_m1
vWF	von Willebrand factor	Vwf	Mm00550376_m1

Results

Absence of TNFα does not alter physiological angiogenesis at p7

Prior to subjecting the TNF-/- mice to the ROP model, we assessed the effect of TNFα deletion on physiological angiogenesis at p7, the time point at which the animals become exposed to hyperoxia. At p7 the vascularised retinal area and capillary density in retinae of TNF-/- mice was comparable to wildtypes (Fig.1 A and B, respectively). Capillary diameter was increased in the TNF-/- mice compared to wildtype mice (TNF-/-: 8.6±0.4 μm versus control (Ctrl): 8.0±0.6 μm, $p<0.001$, Fig.1 C).

Depletion of TNFα reduces preretinal neovascularization at p17, but does not influence retinal vascularization at p12 after ROP

First, we assessed hyperoxia induced vasoregression at ROP p12 immediately after return to normoxia in the TNFα deficient mice, as an influence here alters preretinal neovascularization. The size of the vascularised retinal area (TNF-/-: 64±13% and Ctrl 64±9 %) and the size of the vascular area (TNF-/-: 27±11% and Ctrl: 27±8%) was comparable between TNF-/- and wildtype retinae (Fig. 2 A). Similarly, capillary density and capillary diameter were not altered in the retinae of TNFα and wildtype mice (Fig. 2 B+C). Thus, absence of TNFα did not influence hyperoxia induced vasoregression.

The preretinal neovascularization count was significantly reduced by 54% in TNF-/- mice (26±15 nuclei/section) compared to wildtypes (57±17 nuclei/section) subjected to ROP at p17 ($p=0.01$, Fig.2 D).

Angioflammatory gene expression in selected vascularised retinal areas of TNF-/- mice

The expression of 46 inflammatory- and angiogenesis- associated genes (Table 1) was analysed in retinal lysates (RL) of control and ROP exposed TNF-/- mice at p13. Furthermore, two retinal vascularised areas were selected using laser dissection microscopy prior to gene expression analysis (Fig. 3). One area (LDM_VL) was dissected from the outer plexiform layer to the inner limiting membrane of control and ROP retina. This area consists of the neuroglia and the three retinal vascular plexi in the control retina. In the ROP retina at p13, the retinal vasculature is mainly present as the superficial vascular plexus in the retinal periphery due to hyperoxia induced vasoregression. The second area (LDM_VP) was selected from the

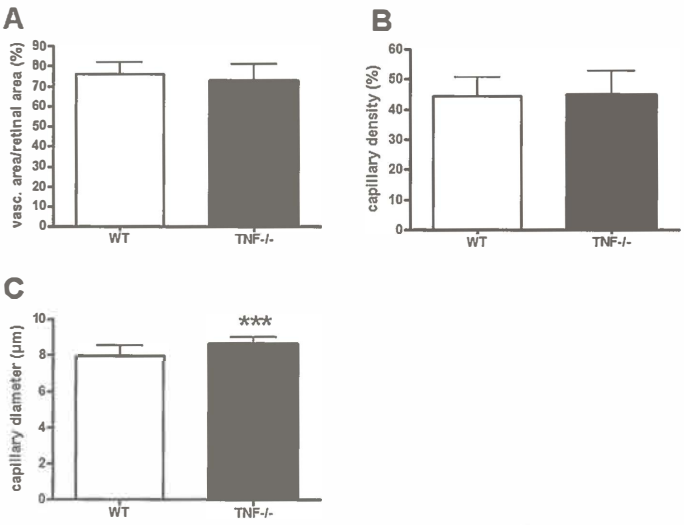


Figure 1: TNFα deficiency did not alter retinal vascularisation at p7.
A: The retinal area covered by the outgrowing retinal vasculature is comparable between wildtypes (□) and TNF-/- mice (■). While capillary density (B) was comparable in wildtype and TNF-/- mice, capillary diameter (C) was increased in TNF-/- mice at p7 (p=0.0005). Data is expressed as mean ± SD.

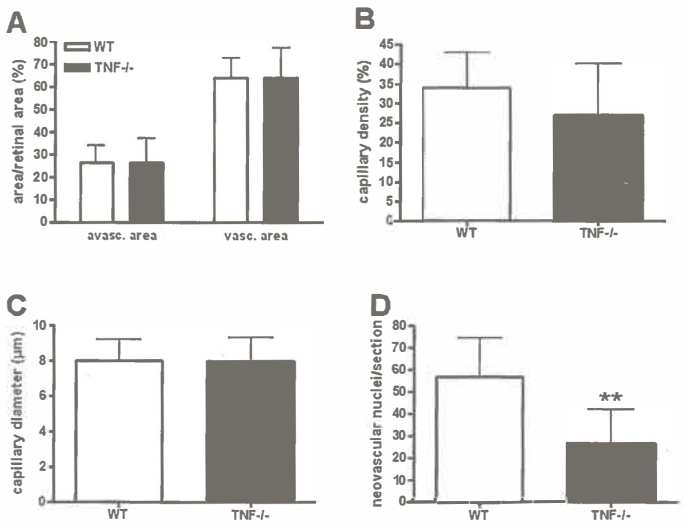


Figure 2: TNFα deficiency reduced preretinal neovascularization at p17, but did not alter response to hyperoxia-induced vasoregression at p12
A: Preretinal neovascularization was significantly reduced by 54% in TNF-/- mice compared to wildtypes after exposure to ROP at p17 (p=0.01). B: Avascular area (avasc.area) and vascular area (vasc.area) were of similar size in TNF-/- (■) and wildtype mice (□) at ROP p12. Capillary density (C) and diameter (D) were not altered in TNF-/- mice at ROP p12. Data is expressed as mean ± SD.

inner limiting membrane to the ganglion cell layer of the retinal periphery and contains the peripheral superficial vascular plexus and its surrounding neuroglia. In these two areas of control (supplementary Table 1) and ROP retina (supplementary Table 2) the expression of 46 angioflammatory genes was analysed by real-time RT-PCR. 41 of the 46 genes were fully detectable in the retinal lysates and in the selected vascularised retinal areas. The expression of erythropoietin, cyclooxygenase-2 and tissue-inhibitor of matrix-metallo-proteinase-1 was in some areas inconsistently detectable, and chemokine ligand-2 was only detectable in the ROP retina. The expression of matrix-metallo-proteinase 2 and TNFα was not detectable in all groups in the retina of TNF-/- mice.

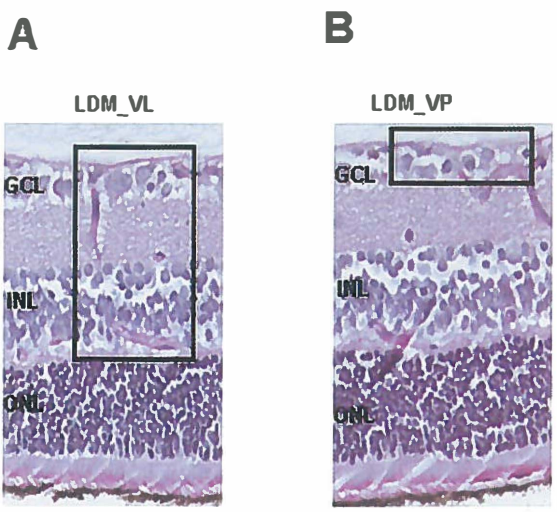


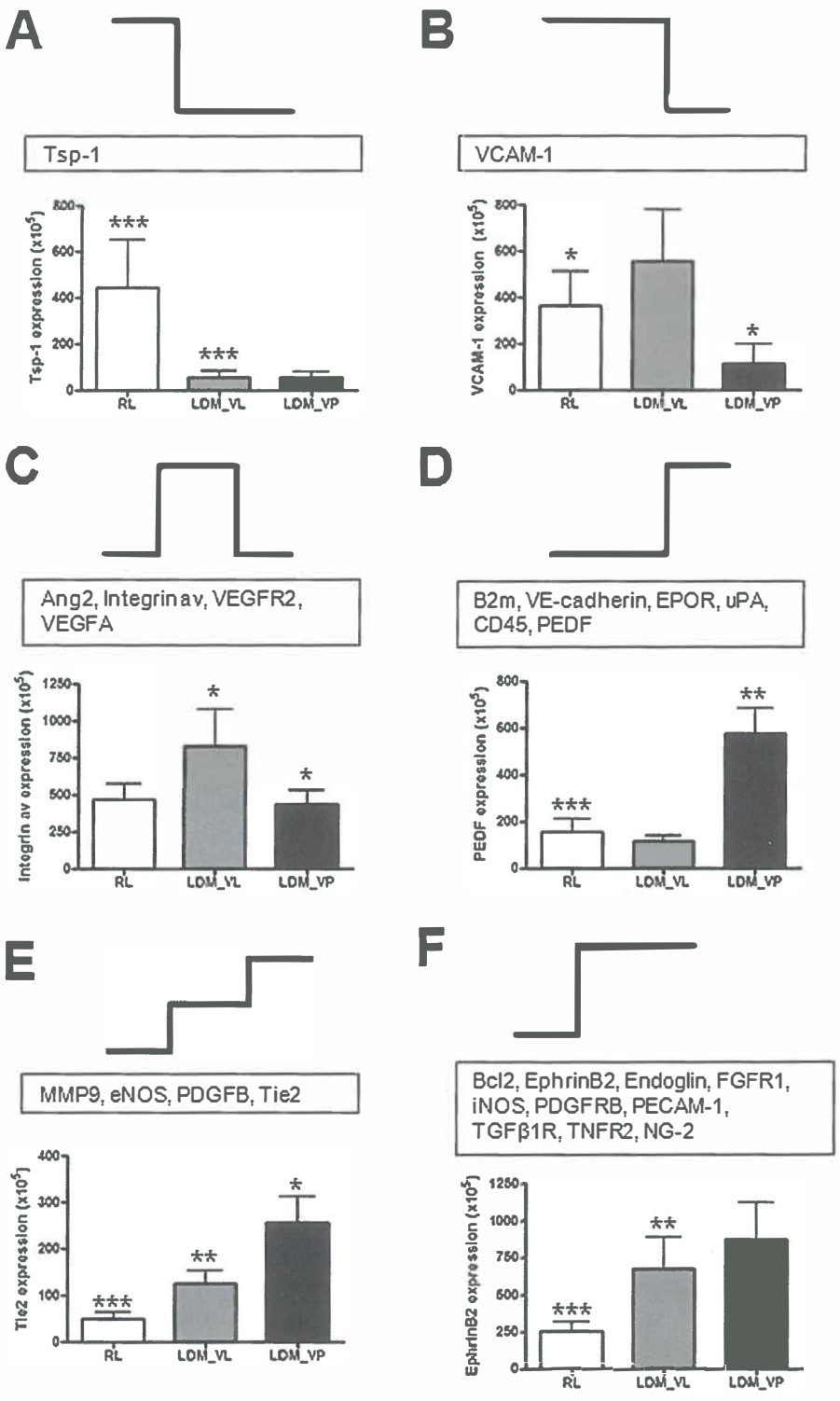
Figure 3: Two vascularised retinal areas were selected by laser dissection microscopy prior to gene expression analysis.
A: The area between the inner limiting membrane and outer plexiform layer (LDM_VL), in which the three plexis of the retinal vasculature are present, was selected by laser dissection microscopy from the retina. B: The area between the inner limiting membrane and the ganglion cell layer, in which the superficial vascular plexus is present, was selected by laser dissection microscopy from the retinal periphery (LDM_VP). In the ROP retina preretinal neovascularization arise from the peripheral superficial vascular plexus.

TNFα depletion affects spatial distribution of angioflammatory genes in the control retina at p13

In the control retina, we compared the gene expression of 46 angioflammatory genes in the retinal lysates to expression in the LDM_VL area and in the LDM_VP area. Furthermore, we compared expression between the LDM_VL and LDM_VP area. By these comparisons, we found seven different spatial expression patterns, which we termed pattern A through G, in the control retina of TNF-/- mice at p13.

Pattern A genes were significantly higher expressed in the retinal lysates compared to the two selected vascularised retinal areas, indicating predominant expression of these genes in the outer avascular retina. Tsp-1 was the only gene representing pattern A (Fig. 4 A). VCAM-1 was the only gene expressed in pattern B (Fig. 4 B) and showed predominant expression in the RL and in the LDM_VL, indicating predominant expression in the outer avascular and in the vascularised intermediate retina between outer plexiform and inner plexiform layer. Genes of pattern C, such as Integrin αv (Fig. 4 C) were predominantly expressed in the intermediate retina as their expression was higher in the LDM_VL area compared to RL and the LDM_VP area. Predominant gene expression in the LDM_VP area, which represents the area of the peripheral superficial vascular plexus, was termed pattern D. Genes, such as PEDF belonged to the pattern D (Fig. 4 D). Pattern E genes, such as Tie2 (Fig. 4 E) showed enhanced expression in the LDM_VL compared to RL and predominant expression in LDM_VP. These genes were enhanced expressed in the vascularised intermediate retina and predominant expression in the area of the superficial vascular plexus. Genes that were predominantly expressed in the LDM_VL and in the LDM_VP area belonged to pattern F. EphrinB2 belonged to pattern F and showed predominant expression in the vascularised area of the retina (Fig. 4 F). FGFR2 was the only gene expressed according to the pattern described in G, and was predominantly expressed in the RL and in the LDM_VP area, which represents predominant expression in the outer avascular and in the area of the superficial vascular plexus (Fig. 4 G).

Taken together genes expressed in pattern C, D, E and F, 25 out of 46 analysed genes were predominantly expressed in the vascularised retinal layers in the control retina of TNF-/- mice.


A
Tsp-1
Tsp-1 expression (x10⁵)
***
***
RL
LDM_VL
LDM_VP
B
VCAM-1
VCAM-1 expression (x10⁵)
*
*
RL
LDM_VL
LDM_VP
C
Ang2, Integrin av, VEGFR2, VEGFA
Integrin av expression (x10⁵)
*
*
RL
LDM_VL
LDM_VP
D
B2m, VE-cadherin, EPOR, uPA, CD45, PEDF
PEDF expression (x10⁵)
***
**
RL
LDM_VL
LDM_VP
E
MMP9, eNOS, PDGFB, Tie2
Tie2 expression (x10⁵)
***
**
*
RL
LDM_VL
LDM_VP
F
Bcl2, EphrinB2, Endoglin, FGFR1, iNOS, PDGFRB, PECAM-1, TGFβ1R, TNFR2, NG-2
EphrinB2 expression (x10⁵)
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RL
LDM_VL
LDM_VP

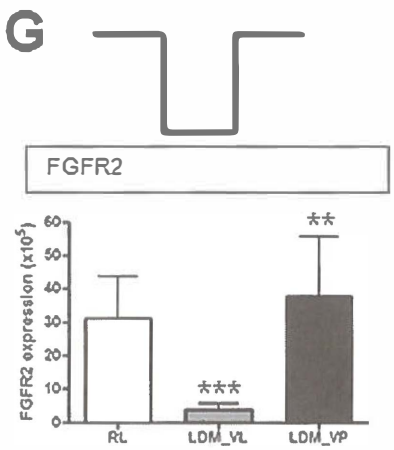


Figure 4: Angioflammatory genes were spatially distributed in seven expression patterns in the control retina of TNF-/- mice
Each pattern, A through G, is depicted by a schematic line drawing, and the genes that significantly follow these expression patterns are listed in the boxes. For each pattern the data of one gene is shown as example.
A: Pattern A genes were significantly higher expressed in RL than in the LDM_VL and LDM_VP area, indicating predominant expression of these genes in the outer avascular retina. For example, Tsp-1 expression is depicted: RL versus LDM_VL ($p<0.001$), RL versus LDM_VP ($p<0.001$). B: Pattern B genes were significantly lower expressed in the LDM_VP area compared to the RL and the LDM_VL area, indicating predominant expression in the outer avascular and in the intermediate retina. For example, VCAM-1 expression is shown: RL versus LDM_VP ($p<0.05$), and LDM_VL versus LDM_VP ($p<0.05$). C: Pattern C genes were significantly higher expressed in the LDM_VL area compared to RL and the LDM_VP area, indicating predominant expression in the intermediate retina. For example, Integrin αv expression: RL versus LDM_VL ($p<0.05$), and RL versus LDM_VP area ($p=0.01$). D: Pattern D genes were predominantly expressed in the LDM_VP area, which represents the area of the peripheral superficial vascular plexus. For example, PEDF expression is displayed: RL versus LDM_VP ($p<0.001$), and RL versus LDM_VL ($p<0.001$). E: Pattern E genes, was significantly higher expressed in the LDM_VL area than in the RL, and predominantly expressed in the LDM_VP area. For example, Tie2 expression is depicted: RL versus LDM_VL ($p=0.001$), and LDM_VL versus LDM_VP ($p=0.046$), and RL versus LDM_VP ($p<0.001$). F: Genes of pattern F were significantly lower expressed in RL than in the LDM_VL and LDM_VP areas, representing predominant expression in the vascularised retinal areas. For example, EphrinB2 expression is displayed: RL versus LDM_VL area ($p<0.01$), and RL versus LDM_VP ($p<0.001$). G: Pattern G genes were significantly lower expressed in the LDM_VL area compared to RL and the LDM_VP area, indicating predominant expression in the outer avascular and in the area of the peripheral superficial vascular plexus. For example, FGFR2 expression is shown: RL versus LDM_VL ($p<0.001$), and LDM_VL versus LDM_VP area ($p<0.01$).

Spatial expression patterns of angioflammatory genes were altered in TNF-/- mice at ROP p13

In the ROP retina of the TNF-/- mice we made the same comparisons as described for the control retina. We found six gene expression patterns, which were identical to pattern B through G, as described for the control retina. However, hypoxia shifted genes to other expression patterns, as described below.

None of the 46 angioflammatory genes was predominantly expressed in the outer avascular layers of the ROP retina (Fig. 5 A). The expression pattern of VCAM-1 was not altered by hypoxia and it was predominantly expressed in the outer avascular and intermediate retina (Fig. 5 B). While the expression pattern of Ang2, Integrin αv and VEGFR2 was altered in the ROP retina, VEGFA expression remained predominantly expressed in the intermediate retina (Fig. 5 C). Hypoxia increased the number of genes with preferential expression in the area of the superficial vascular plexus. In addition to B2m, VE-cadherin, EPOR, uPA and PEDF, the genes Ang1, MCP-1, ICAM-1, Integrin β3, PLGF, TIMP-1, TGFβ1 and TNFR1 were expressed in this pattern (ICAM-1, Fig. 5 D). Hypoxia shifted Ang2 from predominant expression in the intermediate retina, and Bcl2, Endoglin, TNFR2, NG-2, iNOS and PDGFRB from predominant expression in both vascularised retinal layers to enhanced expression in the intermediate and predominant expression in the area of the peripheral superficial vascular plexus. In addition, VEGFR1 and tPA were expressed in this pattern only in the ROP retina (VEGFR1, Fig. 5 E). While EphrinB2, FGFR1and TGFß1R expression was not changed in the ROP retina, VEGFR2 and CD45 were shifted by hypoxia to predominant expression in the intermediate retina and in the area of the superficial vascular plexus (VEGFR2, Fig. 5 F). Hypoxia shifted the expression of Tsp-1 from predominant expression in the outer avascular area of the control retina to predominant expression in the outer avascular and in the peripheral superficial vascular plexus area (Fig. 5 G).

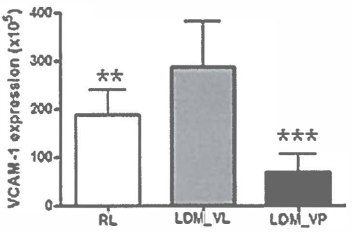
In summary, 34 of the 46 genes analysed were predominantly expressed in the area of the peripheral superficial vascular plexus in the ROP retina of TNF-/- mice.

A

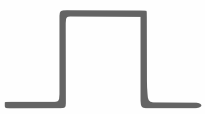
Not existent

B

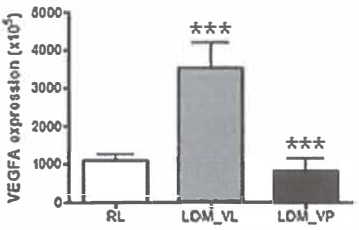
VCAM-1



C

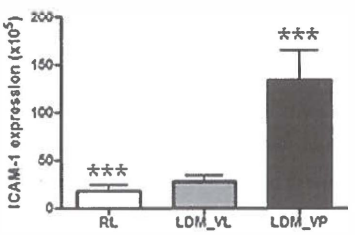


VEGFA

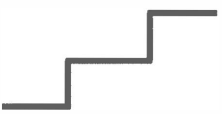


D

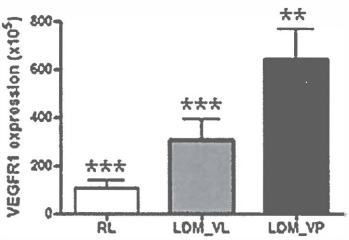
Ang1, B2m, MCP-1, VE-cadherin, EPOR, ICAM-1, Integrinβ3, PEDF, PLGF, uPA, TIMP-1, TGFβ1, TNFR1



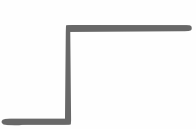
E



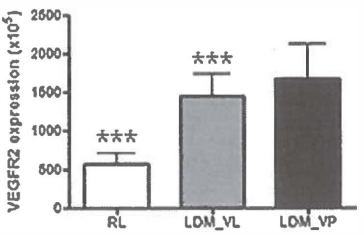
Ang2, Bcl2, Endoglin, EphB4, VEGFR1, MMP9, iNOS, eNOS, PDGFB, PDGFRB, PECAM-1, tPA, Tie2, TNFR2, NG-2, vWF



F



EphrinB2, FGFR1, VEGFR2, CD45, TGFβ1R



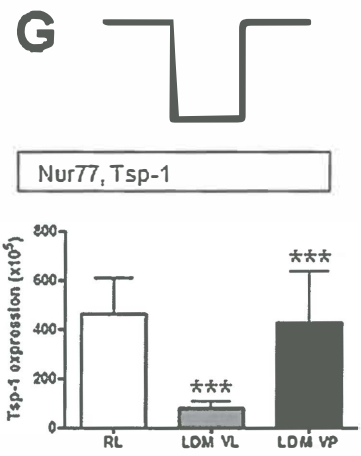


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Figure 5: Spatial distribution patterns of angioflammatory genes were altered in the ROP retina of TNF-/- mice.

Six of the seven gene expression patterns, A through G, described in the control retina were found in the ROP retina of TNF-/- mice. Each pattern is depicted by a schematic line drawing, and the genes that significantly follow these expression patterns are listed in the boxes. For each pattern the data of one gene is shown as an example.

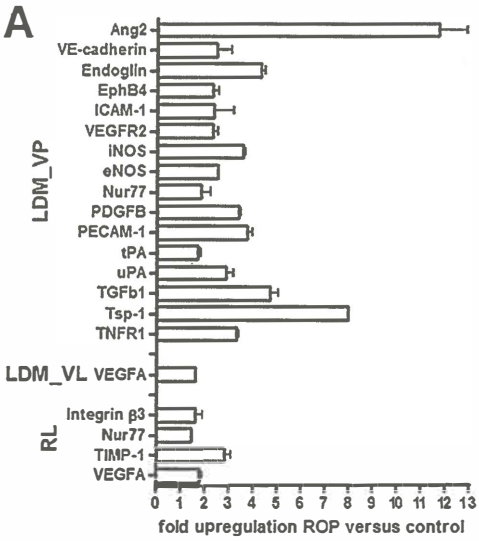
A: No genes with a pattern A expression were found in the analysis. B: Pattern B expression is exemplified by VCAM-1 expression, which was predominantly expressed in the outer avascular and in the intermediate retina: RL versus LDM_VP ($p<0.01$), and LDM_VL versus LDM_VP ($p<0.001$) area. C: Pattern C gene expression is exemplified by VEGFA expression, which was preferentially expressed in the intermediate retina: RL versus LDM_VL ($p<0.001$), and LDM_VL versus LDM_VP ($p<0.001$). D: ICAM-1 represents pattern D gene expression and was primarily expressed in the area of the peripheral superficial vascular plexus: RL versus LDM_VP area ($p<0.001$), LDM_VL versus LDM_VP ($p<0.001$). E: Genes expressed in pattern E, exemplified by VEGFR1 expression, were enriched in the intermediate retina and predominantly expressed in the area of the peripheral superficial vascular plexus: RL versus LDM_VL area ($p=0.001$), and LDM_VL versus LDM_VP ($p=0.001$), and RL versus LDM_VP ($p<0.001$) F: Pattern F expression is exemplified by VEGFR2, which was primarily expressed in both by laser microdissection selected areas: RL versus LDM_VL ($p<0.001$), and RL versus LDM_VP ($p<0.001$). G: Genes of pattern G, exemplified by Tsp-1 were predominantly expressed in the outer avascular and in the area of the peripheral superficial vascular plexus: RL versus LDM_VL ($p=0.001$), and LDM_VL versus LDM_VP ($p<0.001$).

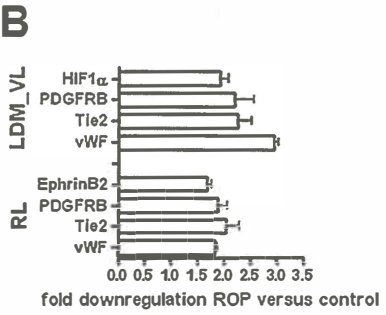
Hypoxia altered gene expression, especially in the area of neovascularization incipience in the TNF-/- retina

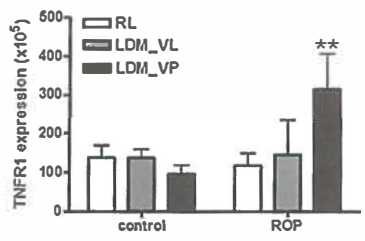
Comparison of angioflammatory gene expression in retinal lysates of the ROP and the control retina of TNF-/- mice revealed that 4 out of 46 analysed genes, e.g. TIMP-1 were upregulated (Fig. 6 A) and 4 genes, e.g. as Tie2 were downregulated by hypoxia (Fig. 6 B).

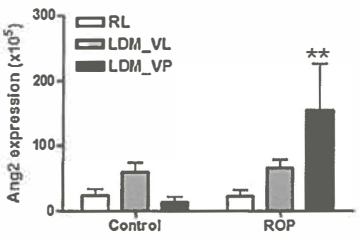
In contrast to the retinal lysates, laser dissection of retinal areas prior to gene expression analysis revealed that 16 angioflammatory genes were upregulated in the area of the peripheral superficial vascular plexus, from which the neovascular sprouts arise. The strongest hypoxia regulated genes in this area were Ang2 and Tsp-1 with 12- and 8- fold induction versus control ($p<0.01$, $p<0.001$, respectively). Interestingly, TNFR1 (Fig. 6 C) and Nur77, which were evenly expressed in the control retina were 3-fold and 2-fold ($p<0.01$, $p<0.01$, respectively) upregulated in the area of the peripheral superficial vascular plexus. While Ang2 (Fig. 6 D), VEGFR2 and Tsp-1 were predominantly expressed outside the peripheral superficial vascular plexus area in the control retina, they were significantly induced in the peripheral superficial vascular plexus area by hypoxia. VE-cadherin, EphB4, eNOS, PECAM-1 and PDGFB were upregulated by hypoxia in the superficial vascular plexus area. VEGFA was upregulated 2-fold by hypoxia in the intermediate retina, where it was predominantly expressed in control and ROP retina ($p=0.01$, Fig. 6 E). Furthermore, in retinal lysates of ROP retinae VEGFA was upregulated 2-fold ($p<0.05$).

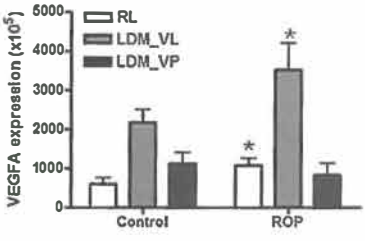
The vascular genes PDGFRB and Tie2 were downregulated 2-fold ($p=0.01$, $p=0.01$), and vWF was downregulated 3-fold ($p=0.01$, Fig. 5 F) by hypoxia in the intermediate retina, which is characterized by vasoregression. The expression pattern of these genes followed the altered localization of the retinal vasculature in the ROP compared to control retina. In the intermediate area HIF1α was 2-fold downregulated by hypoxia ($p<0.01$), resulting in even expression levels throughout the retina.

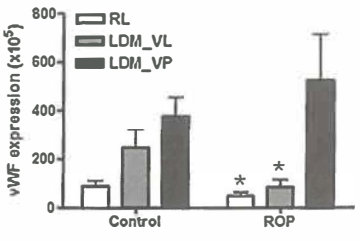

A
Ang2
VE-cadherin
Endoglin
EphB4
ICAM-1
VEGFR2
iNOS
eNOS
Nur77
PDGFB
PECAM-1
tPA
uPA
TGFb1
Tsp-1
TNFR1
LDM_VP
LDM_VL VEGFA
Integrin β3
Nur77
TIMP-1
VEGFA
RL
0 1 2 3 4 5 6 7 8 9 10 11 12 13
fold upregulation ROP versus control


B
LDM_VL
HIF1α
PDGFRB
Tie2
vWF
RL
EphrinB2
PDGFRB
Tie2
vWF
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
fold downregulation ROP versus control


C
TNFR1 expression (x10⁵)
RL
LDM_VL
LDM_VP
**
control
ROP


D
Ang2 expression (x10⁵)
RL
LDM_VL
LDM_VP
**
Control
ROP


E
VEGFA expression (x10⁵)
RL
LDM_VL
LDM_VP
*
*
Control
ROP


F
vWF expression (x10⁵)
RL
LDM_VL
LDM_VP
*
*
Control
ROP

previous page
Figure 6: Hypoxia differentially up- or downregulated localized angioflammatory gene expression in the TNFα depleted retina.
A: Comparison of angioflammatory gene expression revealed that 16 genes were upregulated by hypoxia in the peripheral superficial vascular plexus area, while in retinal lysates only 4 genes were upregulated by hypoxia. B: While four genes were downregulated by hypoxia in the retinal lysates, three of them were also downregulated in the LDM_VL area. C: TNFR1 represents genes that were evenly expressed in the control retina and exclusively upregulated by hypoxia in the peripheral superficial vascular plexus area. D: Expression of genes, such as Ang2 was induced in the superficial vascular plexus area, although they were predominantly expressed outside this area in the control retina. E: VEGFA expression is shown as example for induced expression by hypoxia in two retinal areas. F: Hypoxia downregulated the vascular genes, such as vWF in the retinal lysates and in the LDM_VL area.

Spatial patterns and hypoxic regulation of angioflammatory gene expression- a comparison between TNF-/- and wildtype mice

A comparison of the relative angioflammatory gene expression levels in control retina of wildtype and TNF-/- mice at p13 revealed no significant difference. However, the spatial expression pattern was changed for some genes in the control retina of TNF-/- mice compared to wildtypes. FGFR2 was predominantly expressed in the outer avascular retina in both strains, but additionally enhanced in the area of the peripheral superficial vascular plexus in the TNFα depleted mice (Fig. 7 A). Furthermore, genes with predominant expression in the outer avascular retina and in the area of the peripheral superficial vascular plexus were present in the TNF-/-, but missing in the wildtype control retina. VE-cadherin, Endoglin, PECAM-1 and NG-2 showed altered expression patterns in the TNF-/- mice compared to the wildtypes. Interestingly, EPOR was consistently detectable in all areas in the TNFα depleted mice, and showed predominant expression in the area of the superficial vascular plexus, while inconsistently detectable in wildtypes.
Next, we compared the relative angioflammatory gene expression levels in the ROP retina of wildtype and TNF-/- mice at p13, and this revealed no significant difference. However, like in the control retina TNFα deficiency influenced spatial gene expression patterns in the ROP retina. While in the ROP retina of wildtypes expression pattern A through G were present, pattern A was missing in the TNFα depleted mice. bFGF was not enhanced expressed in the ou-

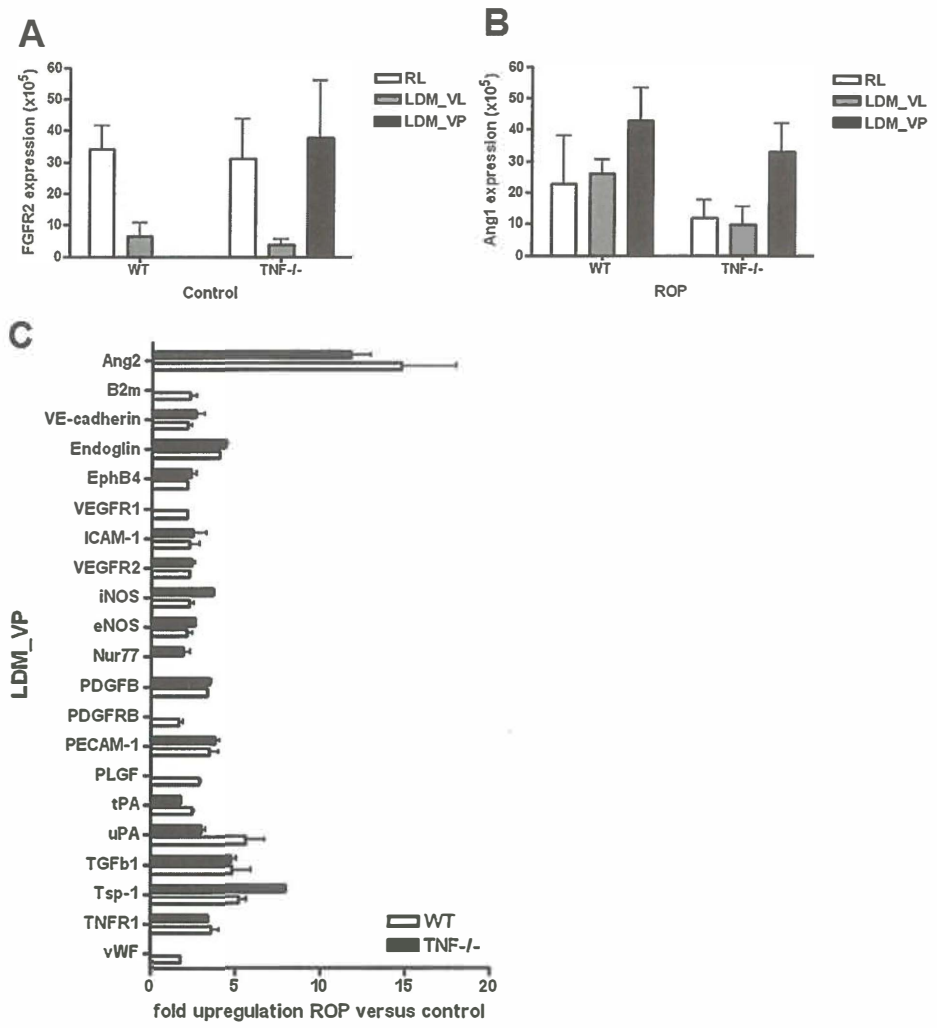


Figure 7: Differences observed in spatial expression patterns and hypoxia induced regulation of angioflammatory genes between TNF-/- and wildtype mice.

A: FGFR2 expression is depicted as example for altered spatial gene expression pattern in the control retina of TNF-/- mice at p13. B: Ang1 expression is displayed as example for altered spatial gene expression pattern in the ROP retina of TNF-/- mice at p13. C: All significantly upregulated genes in the area of the superficial vascular plexus, from which neovascularization arises, are depicted. Differences and similarities between TNF-/- and wildtype mice are shown with regard to hypoxia induced changes in angioflammatory gene expression in the area of the superficial vascular plexus.

ter avascular area in the TNF-/- retina. For pattern B to G the assignment of genes to each pattern was similar for genes, such as VCAM-1, VEGFA, VE-cadherin, PDGFB, VEGFR2 and Tsp-1. Of note, Ang1 and MCP-1 were enhanced expressed in the peripheral superficial vascular plexus area only in the TNF-/- ROP retina (Ang1, Fig. 7 B). Some vascular marker genes, i.e. VE-cadherin, PECAM-1 and EphB4 were enhanced expressed in the total vascularised retinal area in the TNFα depleted mice subjected to ROP, while only predominantly expressed in the peripheral superficial vascular plexus in the wildtype ROP retina. These genes did not follow the hyperoxia induced alteration of vascular localization, which was observed in the wildtype ROP retina.
Furthermore, we compared the hypoxic regulation effect on angioflammatory gene expression in ROP versus control retina in both breeds. This comparison revealed that more genes, i.e. Integrin β3, Nur77, EphrinB2, PDGFRB and Tie2 were regulated in retinal lysates of TNFα deficient mice compared to wildtypes. In the selected vascularised retinal areas some genes, such as VE-cadherin and VEGFA were upregulated by hypoxia or down regulated, such as Hif1a. In the TNF-/- mice B2m, VEGFR1, PLGF and vWF were not induced by hypoxia in the peripheral superficial vascular plexus area (Fig. 7 C). While VEGFA was regulated by hypoxia in the retinal lysates in TNFα depleted mice, its regulation was not detectable in wildtype retinal lysates. In the LDM_VL area VEGFA upregulation by hypoxia was similar in both breeds, but its downregulation in the LDM_VP area was only found in the wildtype retina. Furthermore, the fold regulation by hypoxia of the genes at their respective localization was altered by the absence of TNFα. iNOS and Tsp-1 were much stronger induced by hypoxia in the superficial vascular plexus area of TNF-/- mice (4-fold and 8-fold, respectively) compared to wildtypes (2-fold and 5-fold, respectively). Vice versa, in the same area uPA and Ang2 (although not significant) were much stronger induced by hypoxia in the wildtypes than in the TNF-/- mice (6- and 15-fold versus 3-fold and 12-fold, respectively).

Expression of vascular, but not inflammatory proteins was enhanced in TNF-/- mice at p13

The vascular markers PECAM-1 and VE-cadherin were expressed in the three vascular plexi of the control retina of TNF-/- mice (Fig. 8). In the ROP retina of TNF-/- mice their expression was present in the peripheral superficial vascular plexus area, which correlated with the altered localization of the retinal vasculature in the ROP retina. The staining pattern of PECAM-1 and VE-cadherin followed the spatial mRNA expression pattern, and VE-cadherin staining was mildly enhanced in the TNF-/- mice compared to wildtypes (wildtype data not shown). VCAM-1 staining showed a nonvascular expression pattern in the control retina and in the ROP retina of TNFα depleted mice. The staining was found in both plexiform layers and from the ganglion cell layer up to the inner limiting membrane. The intensity increased from the retinal periphery to the optic nerve entrance of the retina, but was not altered by hypoxia in the TNF-/- and wildtype mice (wildtype data not shown). The staining pattern of VCAM-1 did not follow the predominant VCAM-1 expression in the intermediate area of the retina that we observed on RNA level in the control and the ROP retina. Staining of the inflammatory factors ICAM-1 and CD45 (not shown) was negative in both control and ROP retina of TNFα depleted mice. ICAM-1 staining contrasted the observed predominant ICAM-1 mRNA expression in the superficial vascular plexus area in control and ROP retina. Similarly, CD45 staining did not follow the by hypoxia enhanced CD45 mRNA expression in the peripheral superficial vascular plexus area.

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Figure 8: Retinal staining patterns of vascular markers and inflammatory molecules in control and ROP retina of TNF-/- mice at p13.

Top row: PECAM-1 vascular staining was detected in the outer plexiform layer, the inner border of the inner nuclear layer and in the nerve fiber layer in control retina at p13. In the ROP retina PECAM-1 positive cells were mainly found in the nerve fiber layer in the superficial vascular plexus. TNFα deficiency did not alter the staining pattern of PECAM-1 compared to wildtypes (data not shown). Second row: VE-cadherin staining followed the pattern of the PECAM-1 staining, staining the three vascular plexi in the control and the superficial vascular plexus in the ROP retina. The staining pattern of VE-cadherin was not altered in the TNF-/- mice compared to wildtypes (data not shown). Third row: VCAM-1 staining of the neuroretina was abundant at the outer border of the photoreceptor layer, the outer plexiform layer, throughout the inner plexiform layer and in the nerve fiber layer and was not altered by hypoxia. The staining intensity increased

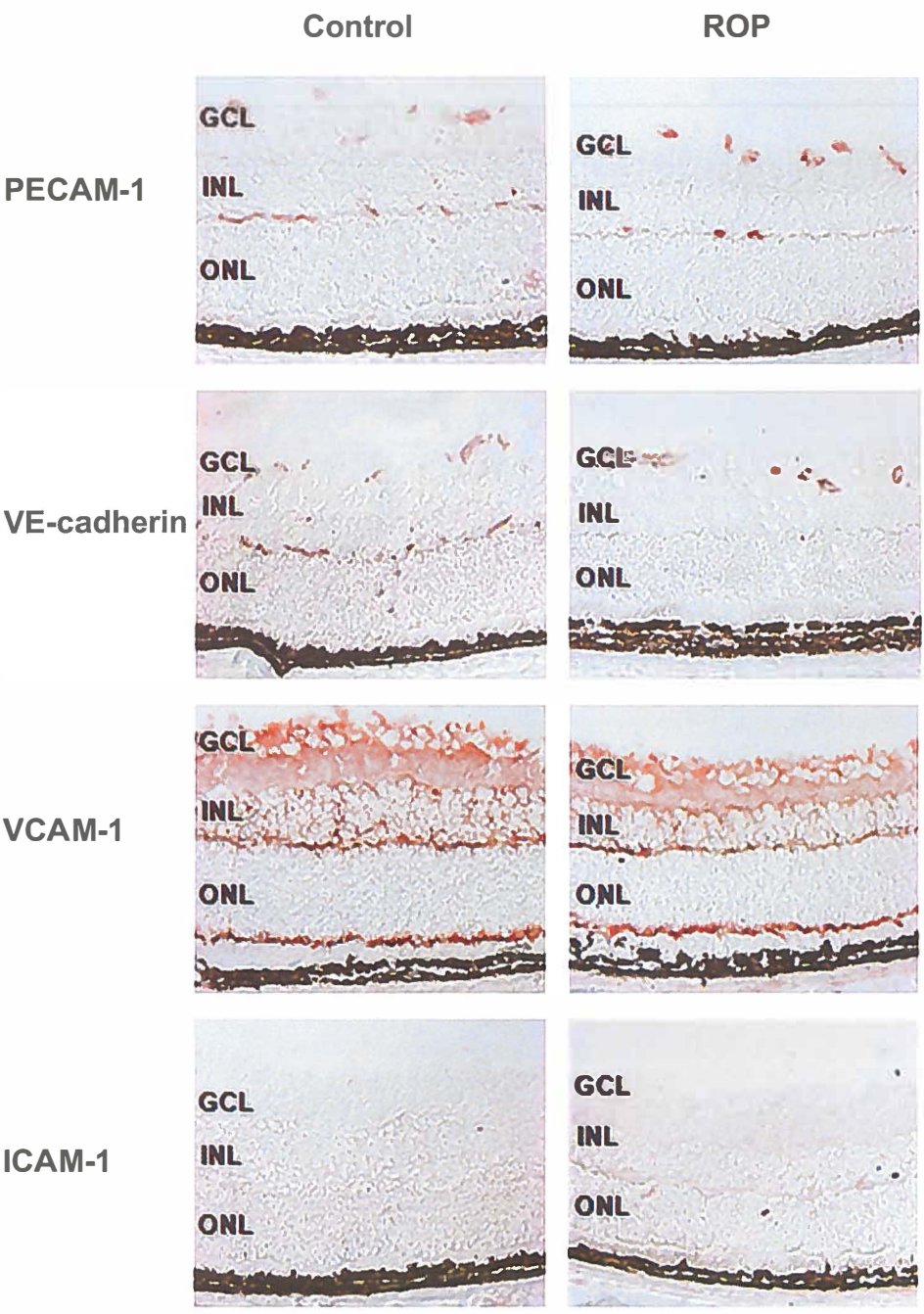

Figure 8 continued
from the retinal periphery towards the optic nerve entrance. TNFα deficiency did not influence the VCAM-1 staining pattern. Fourth row: ICAM-1 staining was negative in the control and ROP retina of TNF-/- mice.

Genetic deletion of TNFα significantly reduces retinal VEGF levels at p13, but not at p17 in the ROP model

At p13 and p17 the influence of TNFα deficiency on retinal VEGF protein expression was analysed. The expression was measured in retinal lysates of wildtype and TNF-/- mice, which were either room air raised controls or subjected to the ROP (Fig. 9 A). VEGF protein expression was significantly elevated in the retinae of wildtype mice subjected to the ROP model (504±60 pg/ml) compared to control littermates (117±10 pg/ml, $p<0.001$) at p13. At p17, VEGF protein was still elevated in the wildtype ROP mice (248±60 pg/ml versus control 71±11 pg/ml, $p<0.001$), but to a lesser extent. In the TNF-/- ROP retina VEGF protein expression was increased by hypoxia (393±59 pg/ml) compared to control littermates (107±14 pg/ml, $p<0.001$) at p13, but was significantly reduced compared to the levels of wildtype ROP retinae at p13 ($p<0.05$). At p17 VEGF protein levels were still increased in ROP TNF-/- retinae compared to the controls ($p<0.05$), but to a lesser extent as at p13. Furthermore, VEGF protein expression was comparable in both breeds in the control and in the ROP retina at p17.

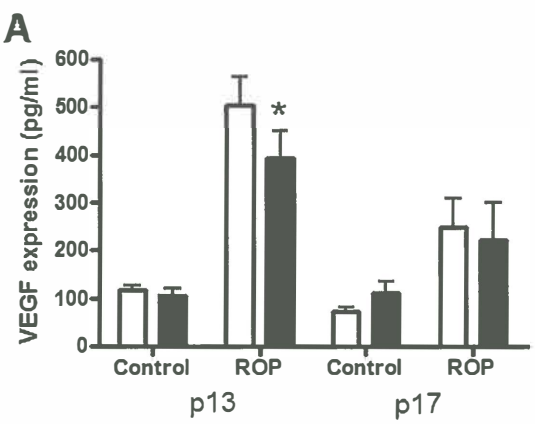

Figure 9: TNFα deficiency reduces retinal VEGF protein expression at p13 after ROP exposure.

A: VEGF protein expression was 4-fold upregulated in the wildtype (□) ROP retina at p13 compared to control ($p<0.001$). TNFα deficiency (■) reduced the hypoxia-induced upregulation (versus control: $p<0.001$) of VEGF compared to wildtypes in the ROP retina at p13 (versus wildtype: $p<0.05$). In wildtype and TNF-/- mice the VEGF expression was comparable at p17 in both conditions and was 3- and 2-fold upregulated in the ROP retina compared to control ($p<0.001$, $p<0.05$, respectively). Data is expressed as mean ± SD.

Discussion

Our experiments revealed that the reduction of preretinal neovascularization mediated by the absence of TNFα in the ROP model is not due to the influence of TNFα on developmental angiogenesis or vascular response to hyperoxia induced vasoregression. Zooming into the ROP retina revealed that genes, such as Tsp-1 and uPA showed differential regulation by hypoxia in the area of the peripheral superficial vascular plexus in TNF-/- mice. Furthermore, we observed reduced retinal VEGF protein levels in TNFα depleted mice subjected to the ROP model at p13. These data indicate that the reduction of preretinal neovascularization in TNFα deficiency is most likely modulated by altered levels of VEGF, Ang2, Ang1, Tsp-1 and uPA.

Our data demonstrate that TNFα depletion affects preretinal neovascularization. This result agrees with the study of Gardiner et al., which showed a similar effect in the same model (21). Furthermore, we show that TNFα neither alters physiological retinal vascularization before the onset of hyperoxia, nor changes the vascular response to hyperoxia induced vasoregression in the model of proliferative retinopathy. This is an important aspect as a more mature retinal vasculature and formation of the deep capillary plexus at the onset of hyperoxia reduces pretinal neovascularization in this model (15). VEGF is a key factor in preretinal neovascularization and is regulated by hypoxia. Accordingly, we observed hypoxia induced upregulation of VEGFA in the total ROP retinae of TNF-/- mice, as described in a study by Sato et al. for wildtypes (22). In the intermediate retina, where VEGFA is predominantly expressed, we found similar hypoxia induced VEGF mRNA upregulation in wildtype and TNF-/- mice. On the protein level, the hypoxic induction of VEGFA was much stronger than on RNA level, and was significantly lower in the TNF-/- mice. Reduction of VEGF by several strategies in experimental models of proliferative retinopathy (23-25) and by intravitreal bevacizumab in patients with proliferative retinopathy reduced neovascularization (7). These data most likely indicate that the decreased preretinal neovascularization in the TNF-/- mice is VEGF mediated.

Our data did not reveal any significant differences in relative angioflammatory gene expression levels in their respective area between TNF-/- and wildtype mice. However, we observed differences

in hypoxic regulation of gene expression in the area of neovascularization incipience between TNF-/- and wildtype mice. The antiangiogenic Tsp-1 was stronger induced by hypoxia in the area of the peripheral superficial vascular plexus in the TNF-/- than in the wildtype mice. This observation is concordant with the antiangiogenic action of Tsp-1, which has been demonstrated in experimental tumor-, wound healing- and ocular angiogenesis models27. Furthermore, high Tsp-1 levels are correlated with better prognosis in patients with invasive cervical cancer (26). The antiangiogenic action of Tsp-1 is mediated by two domains, the type-1 repeats and the procollagen domain. The endothelial receptor CD36 of Tsp-1 is relevant for most of its antiangiogenic actions (27). An upregulation of Tsp-1 in the area and at the timepoint of neovascularization incipience might indicate that Tsp-1 participates in the reduction of neovascularization, which was observed in TNF-/- mice.

The proangiogenic action of uPA has been shown in several in vivo studies and is mediated by its matrix degrading effects. Furthermore, uPA releases matrix bound proangiogenic growth factors, such as bFGF (28). In an experimental model of proliferative retinopathy uPA-/- mice show a 60% reduction in neovascularization (Hammes and Carmeliet, unpublished observation) and intravitreal application of plasminogen activator inhibitor 1 (PAI-1) inhibits pretinal neovascularization in a similar ROP rat model. Most likely, uPA is produced by endothelial cells, glial cells and invading macrophages in the retina (Hammeset al., not published). In wildtype mice we observed a strong induction of Plau in the area of the peripheral superficial vascular plexus at the incipience of neovascularization (vom Hagen et al, manuscript submitted). In contrast, hypoxic induction of uPA was less prominent in the same area of TNF-/- mice and could therefore contribute to the antiangiogenic effect observed in the TNF-/- mice.

While VEGF mediates vascular stabilization together with Ang1 (29), it induces retinal angiogenesis together with high levels of Ang2 (30). In the model of proliferative retinopathy overexpression of Ang2 enhanced preretinal neovascularization (31) and partial deficiency of Ang2 reduced it (Feng et al., in press). In our study, Ang2 showed a trend to less strong hypoxia induced upregulation in the area of neovascularization incipience in the TNFα depleted mice. This would further support the observed reduction of pre-

retinal neovascularization. Furthermore, the enhanced presence of Ang1 in the area of the superficial vascular plexus in the ROP retina of TNFα depleted mice might assist in shifting the balance towards vascular stability.

Our observations are not in line with data showing that thrombospondin-1 is not only antiangiogenic, but also exerts proangiogenic functions via its heparin binding domain, which signals to integrin receptors or syndecan-4 (32). The stronger induction by hypoxia, which we observed in the peripheral superficial vascular plexus area of TNF-/- mice, if it indicates the proangiogenic function of Tsp-1, would be in contrast to the observed reduction in preretinal neovascularization. Furthermore, endothelial NO production and its downstream signaling via cGMP promotes angiogenesis. Along this line, deficiency of eNOS, which is a generator of endothelial NO, decreased neovascularization in experimental proliferative retinopathy32. Our observation that eNOS is more strongly induced by hypoxia in the area of the peripheral superficial vascular plexus in TNF-/- mice, is in contrast to the morphologically observed reduced preretinal neovascularization in these mice. However, Tsp-1 can block eNOS signaling via its endothelial receptor CD47 (33). Therefore, the precise role of Tsp-1 and eNOS remain to be further elucidated in the pathogenesis of neovascularization.

Inflammatory cells, such as leukocytes participate in the angiogenic process, especially during vascular remodeling. A study by Ishida et al. demonstrated that leucocytes prune the retinal vasculature from p5 onwards in the postnatal retina (34). In this study, this effect was mediated by ICAM-1/CD18 dependent leukocyte adhesion to the endothelium and was followed by FasL induced endothelial apoptosis. Moreover, TNFα not only mediates FasL induced apoptosis through TNFR1, which associates with the FasL binding domain FADD (35), but also influences retinal leukocyte–endothelial adhesion and ICAM-1 expression (36). Therefore, the slight increase in capillary diameter in the TNF-/- mice at p7 could indicate that TNFα depletion influences the pruning effect of leucocytes in the postnatal retina. Apparently, this altered pruning did not affect the vascular response to hyperoxia induced vasoregression in the TNF-/- mice.

In the TNF-/- mice we observed a difference in spatial distribution of angioflammatory gene expression in the ROP compared to con-

trol retina. In the ROP retina, more genes were enhanced expressed in the area of the peripheral superficial vascular plexus, from which neovascularization arise. In the same area 16 of the 46 analysed angioflammatory genes were induced by hypoxia. This observation is in line with our previous data generated in wildtype mice (vom Hagen et al., manuscript submitted). Like in the wildtypes, TGFβ and TNFR1 were exclusively upregulated by hypoxia in the area of the superficial vascular plexus, indicating their possible participation in the initiation of neovascularization.

Recently, an analysis of angiogenesis-associated genes has been performed in the timecourse of experimental proliferative retinopathy (22). This study demonstrated hypoxia induced upregulation of TNFα and upregulation of VEGF expression by analysis of retinal lysates. However, this method did not reveal hypoxia induced regulation for several other angiogenic and inflammatory factors, such as the angiopoietins at the time point of neovascularization incipience in the ROP model (22). By tissue selection using laser dissection microscopy prior to gene expression analysis we have unmasked the spatial distribution of angioflammatory gene expression and their alterations in the ROP model of TNF-/- mice and revealed differences in comparison to wildtypes.

In summary, we revealed that reduction of preretinal neovascularization in TNFα deficient mice is not mediated by affecting developmental vascularization or vascular response to hyperoxia. The antiangiogenic effect of TNFα deficiency is likely mediated by the reduction of VEGF protein levels. Tissue selection by laser dissection microscopy prior to gene expression analysis revealed differences in spatial gene expression and in localized induction by hypoxia between TNF-/- and wildtype mice. Probably, altered Tsp-1, uPA and Ang2 expression in the area of neovascularization incipience is involved in the antiangiogenic effect observed in the TNF-/- mice. Further studies have to show the detailed mechanisms and interaction of these molecules in the process of preretinal neovascularization. These data enhance our knowledge about the influence of TNFα on spatial expression and local regulation of angioflammatory genes in preretinal neovascularization. As such they contribute to the understanding of the molecular mechanism of proliferative retinopathy.

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Supplementary Table 1: Angioflammatory gene expression in retinal lysates (RL), the laser dissected vascularised retina (LDM_VL) and the laser dissected retinal peripheral superficial vascular plexus area (LDM_VP) of TNF-/- mice raised in room air (control) at p13.

The table lists the relative gene expression levels relative to GAPDH (x105). Data is presented as mean ± standard deviation (SD). Genes below the detection level or with inconsistent detection are marked not detectable (N.D.).

	RL		LDM_VL		LDM_VP	
Gene	mean	SD	mean	SD	mean	SD
Ang1	19	11	18	10	88	76
Ang2	24	8	60	14	13	8
B2m	1877	347	1518	322	3169	561
Bcl2	34	6	95	36	116	45
MCP-1	N.D.		N.D.		N.D.	
VE-cadherin	319	126	442	130	860	106
Ephrin B2	255	62	674	210	870	248
Endoglin	251	62	756	126	1055	259
EphB4	78	28	116	42	177	83
EPO	8	6	15	10	N.D.	
EPOR	5	3	13	8	47	19
bFGF	40	21	52	13	61	38
FGFR1	119	24	298	73	237	40
FGFR2	31	13	4	2	38	18
VEGFR1	164	24	500	156	304	132
HIF1α	5200	1512	6692	680	5685	592
ICAM-1	17	8	29	7	56	47
Integrin αv	468	108	832	248	433	89
Integrin β3	9	3	23	22	61	38
VEGFR2	928	205	2438	1086	720	287
MMP9	78	27	241	68	777	224
iNOS	7	4	34	11	43	20
eNOS	36	12	92	27	245	99
Nur77	231	45	302	128	267	50
PDGFB	93	20	327	67	665	224
PDGFRB	80	7	285	49	435	154
PECAM-1	162	36	262	65	403	99
PLGF	28	7	64	54	100	27

	RL		LDM_VL		LDM_VP	
Gene	mean	SD	mean	SD	mean	SD
uPA	68	22	106	34	136	36
tPA	10	6	18	12	53	23
COX-2	1	0	N.D.		N.D.	
CD45	9	3	13	8	69	23
PEDF	157	55	115	23	577	106
Tie2	50	14	125	28	255	56
TGFβ1	115	45	131	28	195	40
TGFb1R	34	14	70	27	92	49
Tsp-1	443	208	57	27	54	26
TIMP-1	5	2	12	7	N.D.	
TNF	N.D.		N.D.		N.D.	
TNFR1	139	29	137	20	96	23
TNFR2	12	4	32	14	68	20
VCAM-1	363	149	560	223	110	84
NG-2	21	9	67	27	118	45
VEGFA	608	155	2191	315	1122	277
vWF	87	23	246	72	378	75

Supplementary Table 2: Angioflammatory gene expression in retinal lysates (RL), the laser dissected vascularised retina (LDM_VL) and the laser dissected retinal peripheral superficial vascular plexus area (LDM_VP) of TNF-/- mice subjected to the model of proliferative retinopathy at p13.
The table lists the relative gene expression levels relative to GAPDH (x105). Data is presented as mean ± standard deviation (SD). Genes below the detection level or with inconsistent detection are marked not detectable (N.D.).

	RL		LDM_VL		LDM_VP	
Gene	mean	SD	mean	SD	mean	SD
Ang1	12	6	10	6	33	9
Ang2	22	9	66	12	155	70
B2m	1514	289	1285	331	3796	1104
Bcl2	26	5	51	15	112	36
MCP-1	5	6	9	6	34	17
VE-cadherin	174	50	263	55	2148	825
Ephrin B2	152	29	575	253	908	191
Endoglin	249	57	676	186	4573	913
EphB4	52	17	112	20	406	114
EPO	17	4	33	21	N.D.	
EPOR	4	2	7	5	27	11
bFGF	97	55	43	18	60	31
FGFR1	82	20	248	43	250	96
FGFR2	19	5	5	5	14	4
VEGFR1	109	30	309	82	644	121
HIF1α	3794	468	3501	718	4759	1593
ICAM-1	18	6	28	6	134	30
Integrin αv	397	83	611	152	494	127
Integrin β3	14	2	19	7	138	20
VEGFR2	574	139	1447	292	1671	458
MMP9	45	11	160	38	570	216
iNOS	8	4	24	8	154	67
eNOS	28	14	89	18	621	230
Nur77	335	50	198	52	493	216
PDGFB	124	32	392	90	2259	662
PDGFRB	43	9	129	52	594	172
PECAM-1	128	23	199	39	1509	491
PLGF	35	13	53	33	130	17
uPA	64	20	94	13	234	45

	RL		LDM_VL		LDM_VP	
Gene	mean	SD	mean	SD	mean	SD
tPA	17	7	24	8	155	44
COX-2	1	0	N.D.		N.D.	
CD45	5	1	20	9	40	15
PEDF	130	65	78	29	417	86
Tie2	24	3	56	21	268	78
TGFβ1	125	23	159	62	920	264
TGFb1R	25	5	72	23	94	46
Tsp-1	462	145	77	29	426	208
TIMP-1	14	5	16	11	70	38
TNF	N.D.		N.D.		N.D.	
TNFR1	118	30	144	88	317	88
TNFR2	8	2	23	9	93	44
VCAM-1	188	53	288	94	70	35
NG-2	15	7	44	21	81	15
VEGFA	1088	163	3540	657	818	318
vWF	48	11	84	27	526	185

Chapter VI

Intravitreal thalidomide reduces experimental preretinal neovascularization without induction of retinal toxicity

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Abstract

Background/aims:

Proliferative retinopathies have remained the most common causes of blindness. Retinal neovascularization is induced by hypoxic up-regulation of angiogenic growth factors, such as vascular endothelial growth factor (VEGF). Thalidomide has been shown to be anti-angiogenic via the reduction of VEGF. We investigated the effect of intravitreal application of thalidomide on neovascularization and retinal toxicity in a mouse model of proliferative retinopathy.

Methods:

C57BL/6J mice were exposed to 75% oxygen from postnatal day 7 (p7) to 12. Immediately after transfer to room air at p12, mice received an intravitreal injection of 150μg/μl thalidomide or control solution. Preretinal neovascularization was quantified at p17. VEGF levels were assessed in whole retinal lysates at p13 and p17. Retinal toxicity was assessed by measuring retinal layer thickness and by analysing caspase-3 activity and apoptotic cell counts in retinal layers to examine retinal apoptosis.

Results:

Intravitreal application of thalidomide significantly reduced preretinal neovascularization by 62% compared to control treated contralateral eyes (p=0.01). Interestingly, this effect was established without a change in retinal VEGF levels. Intravitreal thalidomide was not toxic as retinal layer thickness and neither retinal caspase-3 activity nor apoptotic cell counts were altered.

Conclusion:

These data indicate that intravitreal application of thalidomide can be an effective and safe way to treat retinal neovascularization.

Introduction

Proliferative retinopathies, such as proliferative diabetic retinopathy and neovascular age-related macular degeneration are the most common causes for blindness (1, 2). New vessels arise from preexisting intraretinal vessels and grow intravitreally (preretinal neovascularization).

The pathological angiogenic process is triggered by retinal hypoxia, induced by hyperglycemic capillary vasoregression in proliferative diabetic retinopathy (3). In retinopathy of prematurity (ROP) oxygen incubation of neonates halts the retinal vasculature development and thus generates retinal hypoxia, which induces neovascularization (4). The transcription of angiogenic growth factors, especially of vascular endothelial growth factor (VEGF), is induced by hypoxia. VEGF plays a key role in the induction and modulation of the neovascularization process (5). Besides VEGF other angiogenic growth factors, integrins, adhesion molecules, and matrix molecules participate in the formation of new vessels. Furthermore, several data indicate that inflammatory molecules and inflammatory cells play a role in pathological retinal angiogenesis (6).

Laser photocoagulation has been the standard treatment of neovascularization in proliferative diabetic retinopathy, but irreversibly damages the retinal tissue (7). Increasing knowledge of angiogenesis and its aetiology have led to the development of drugs that target the VEGF pathway, such as bevacizumab, ranibizumab and pegaptanib. These drugs limited for the first time progression of the disease or even improved visual acuity in patients with exsudative age related macular degeneration due to their anti-edematous effect. Several case studies showed that ranibizumab or bevacizumab, intraocularly applied, have a pronounced anti-angiogenic effect in patients with retinal neovascularisation (8). However, in the case of neovascular age-related macular degeneration the anti-angiogenic effect of these drugs has been limited, so that re-injections are necessary and quite often a complete standstill could not be achieved. The complexity of the angiogenic process might require a more diverse intervention like, for example, the combination of a VEGF inhibitor combined with a vascular endothelial cadherin mediated adhesion blocker, which reduces experimental preretinal neovascularisation (9).

Thalidomide was developed in 1958 as an oral sedative, but was la-

ter banned due to its teratogenicity. The comeback was established as thalidomide's efficacy in cancer patients with relapsed or refractive multiple myeloma led to the approval by the Food and Drug Administration. Besides the strong teratogenic potential, thalidomide has a low scale side-effect profile as clinical studies showed (10). The anti-angiogenic action of thalidomide was first described in the rabbit corneal neovascularization model (11). In further studies using the same model in other species it blocked VEGF- and basic fibroblast growth factor-induced neovascularisation (12, 13). In vitro, thalidomide inhibited endothelial cell migration and proliferation (14, 15), which are both essential processes for neovascularization and are VEGF dependent. Moreover, thalidomide has further properties influencing angiogenic pathways. Thalidomide downregulates the expression of integrins (16) and could therefore reduce experimental preretinal neovascularization via this pathway, which has been proven by the experimental application of other integrin antagonists (17, 18). Furthermore, thalidomide is anti-inflammatory and thus affects inflammatory pathways involved in pathological angiogenesis. For example, tumor necrosis factor α (TNFα) inhibition decreases experimental preretinal neovascularisation (19), and thalidomide is a potent TNFα inhibitor as it enhances TNFα mRNA degradation (20). Moreover, thalidomide inhibits secretion of proinflammatoy cytokines from activated macrophages (21), a cell population that is involved in the pathogenesis of preretinal neovascularisation (22).

Recently, the systemic application of thalidomide was not effective in reducing preretinal neovascularization in a mouse model of proliferative retinopathy (23). Based on the pioneering works by Machemer, Tano and others, the ocular vitreous cavity has increasingly been used to treat retinal diseases locally, e.g. by an injection into the vitreous body (24). We hypothesized that an intravitreal injection of thalidomide could create high concentrations directly at the location of the disease without causing side-effects as already been shown with triamcinolone (25).

Therefore, we studied the pharmacological effects of intravitreal thalidomide on neovascularization in a mouse model of proliferative retinopathy.

Material and methods

Animals

All experiments were performed according to the guidelines of the statement of animal experimentation issued by the Association for Research in Vision and Ophthalmology and were approved by the Institutional Animal Care and Use Committee. Wild type mice (C57BL/6J) used in this study were kept at a 12 h light-dark cycle, had free access to standard chow and tap water.

Retinopathy of prematurity (ROP) mouse model

Preretinal neovascularization was investigated in C57BL/6J mice in the established mouse ROP model (26). Briefly, newborn pups at postnatal day 7 (p7) were exposed to 75% oxygen in an incubation chamber for 5 days and then returned to room air. Eyes obtained at p13 and p17 were stored in 4% formalin for histological analysis and at -80°C for protein analysis.

Intravitreal application of thalidomide

Thalidomide was purchased from Fagron (Barsbüttel, Germany) and dissolved in 60% propyleneglycole solution at 150µg/µl. At p12 upon return to normoxic condition the newborns were anesthetised with diethylether and intravitreally injected with 1µl thalidomide into the right eye and with the control solution (1µl 60% propyleneglycole, later referred to as control) into the left eye. Intravitreal injection was performed with a Hamilton syringe (Bonaduz, Switzerland) and a 30G needle penetrating the eye through the sclera just posterior to the limbus at the 12 o'clock position. Thalidomide was injected at p12 to interfere with hypoxia induced up regulation of angiogenic growth factors and signalling pathways involved in initiation of pathological angiogenesis.

Quantification of preretinal neovascularization

At p17 eyes were obtained for quantitative analysis of preretinal neovascularization (pNV) using periodic-acid Schiff's reagent and hematoxylin (PAS-H) stained serial retinal paraffin sections (6µm). Preretinal neovascularization nuclei were counted as described previously (18). Briefly, nuclei at the vitreal side of the inner limiting membrane in lumenized profiles were counted using a microscope (Leica DMLS2, Bensheim, Germany) at 400x magnification. 10 reti-

nal sections, each 12μm apart were quantified per animal. Animals of which only one eye could be analysed were excluded from the analysis. Both eyes of 8 animals of two litters (4 for each litter) were analysed (n=8).

Quantification of retinal layer thickness

Retinal layer thickness was measured in the same PAS-H stained sections as used for quantification of pNV (n=8 animals). Thickness of photoreceptor- (PRL), outer nuclear-(ONL), outer plexiform-(OPL), inner nuclear-(INL), inner plexiform-(IPL) and ganglion cell layer (GCL) were measured 400μm distant from the Ora serrata in the retinal periphery. Measurements were taken in 10 retinal sections per animal at the exact position, and in both directions 50μm apart. Thickness was measured using Leica QWin software on a Leica DMRBE microscope (Leica, Bensheim, Germany) at 200x magnification.

Retinal VEGF levels

The retinae were isolated from frozen thalidomide/control treated eyes of ROP exposed mice at p13 and p17 (n=6-7 per group and time point). The retinae were homogenized through needles using a RIPA lysis buffer (PBS [pH 7.4], 0.25% Sodiumdesoxycholat, 15 NP-40, 150mM NaCl, 1mM EDTA, 0.1%SDS, 1mM PMSF, 1mM Na_3VO_4, 1mM NaF) with freshly added protease inhibitors (Complete mini, Roche, Mannheim, Germany). Homogenates were centrifuged at 15,000rpm for 15min at 4°C. Protein concentration was measured in the supernatants using RC/DC protein assay (BioRad, Munich, Germany). A mouse VEGF ELISA (R&D, Mannheim, Germany) was performed according to the manufacturer's protocol using 10μg retinal protein. Colorimetric change was measured with a microplate reader (SLT Labinstruments GmbH, Crailsheim, Germany). All samples were assayed in duplicate and the assay was repeated.

Retinal Apoptosis – TdT-mediated dUTP-biotin nick end labeling (TUNEL) Staining

Serial retinal paraffin sections were fluorescently stained using the TUNEL kit (Promega, Mannheim, Germany) according to the manufacturer's protocol. DAPI (Sigma, Munich, Germany) was

used as counterstain for cell nuclei. TUNEL positive apoptotic nuclei were counted in retinal sections of Thalidomide and control treated eyes of ROP exposed mice at p17 in the ganglion cell-, the inner nuclear- and outer nuclear layer (n=4 animals per group). Images were taken at 200x magnification at a Leica DMRBE microscope (Leica, Bensheim, Germany).

Retinal Apoptosis – Caspase-3 activity

Retinal caspase-3 activity was assessed in ROP exposed mice at p13 and p17, which were either intravitreally injected with thalidomide or with the control solution at p12 (n=5-7 per group and time point). Furthermore, adult mice were anesthetized and intravitreally injected with thalidomide or with the control solution as described above (n=4 per group). Eyes were obtained one day after injection and frozen at -80°C. The retinae were isolated from all frozen eyes and retinal protein was extracted using a hypotonic lysis buffer (25mM HEPES [pH 7.5], 5mM $MgCl_2$, 5mM EDTA, 5mM DTT, 1% NP-40, 2mM PMSF, 10µg/ml Leupeptin, 10µg/ml Pepstatin A). The retinae were homogenized through needles and centrifuged at 16,000xg for 20 min at 4°C. The protein concentration was measured in the supernatants using the RC/DC protein assay (BioRad, Munich, Germany). Caspase-3 activity was analysed using the fluorometric CaspACE Assay system (Promega, Mannheim, Germany). 20µg retinal protein per sample was assayed in duplicate and in presence/absence of the caspase-3 inhibitor (Ac-DEVD-CHO) according to the manufacturer's protocol with incubations at 30°C in a 96-well plate format. As positive controls mouse 3T3 fibroblasts were cultured and treated with 500nM hydroxyurea to induce apoptosis. Cells were harvested with a cell scraper 16 hours after addition of hydroxyurea. Cells were lysed by four freeze-thawing cycles in the same buffer as tissue and handled the same as the tissue. Fluorescence was measured with an Infinite M200 microplate reader (TECAN, Crailsheim, Germany). Caspase-3 activity was measured as pM AMC release directly and 5 Minutes after stop of the final incubation. The data was calculated with the mean of the two measurements according to the manufacturer's recommendations.

Statistics

Data with two groups were analysed by unpaired t-test and data

with more than two groups were analysed by one-way ANOVA with Dunn's post-test for multiple comparison (Prism, Graph Pad, San Diego, USA). Data is expressed as mean ± standard deviation (SD), for preretinal neovascularization count the data is presented as mean ± standard error of the mean (SEM). A p-value <0.05 was considered statistically significant.

Results

Intravitreally applied thalidomide reduced preretinal neovascularization

The neovascularization count in the eyes intravitreally injected with thalidomide was 23±24 nuclei/section and in the contralateral control injected eyes 60±27 nuclei/section. Intravitreal thalidomide significantly reduced preretinal neovascularization by 62% compared to the contralateral control injected eyes (p=0.01; Fig. 1).

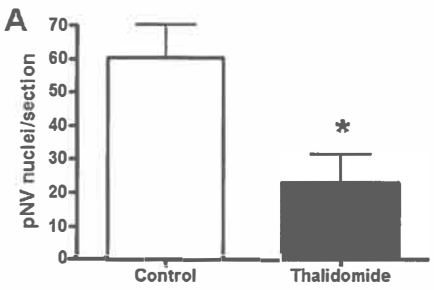

Figure 1:
Preretinal neovascularization (pNV) was significantly reduced in intravitreally thalidomide treated (■) versus control treated eyes (□) in ROP exposed mice at p17 (A). Data is expressed as mean ± SEM.

Retinal VEGF levels were not affected by intravitreal thalidomide

The intravitreal treatment with thalidomide at p12 did not change VEGF protein expression in retinal lysates of ROP exposed animals at p13 (Fig.2: thalidomide eyes: 15±4pg VEGF/μg retinal protein versus control eyes: 22±9pgVEGF/μg retinal protein). At p17 VEGF protein levels were significantly lower compared to p13, yet thalidomide treated eyes contained similar VEGF protein levels as control treated eyes at p17 (3±1pgVEGF/μg retinal protein versus 3±1pgVEGF/μg retinal protein; Fig. 2).

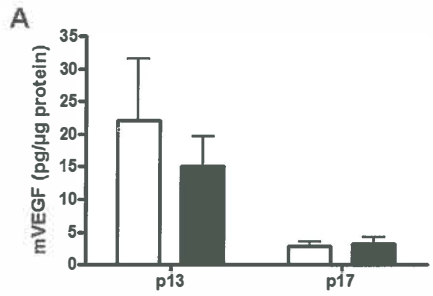

Figure 2
Intravitreal application of thalidomide at p12 did not influence total retinal VEGF protein levels. VEGF levels were comparable in Thalidomide treated (■) and control treated (□) mice at p13 and p17 after ROP exposure (A).

The thickness of retinal layers was not altered in thalidomide treated eyes

To assess possible retinal toxic side-effects of intravitreally applied thalidomide the retinal layer thickness was measured. Thalidomide did not influence the thickness of the ganglion cell layer (22.6±5.4µm versus control 24.7±1.9µm), the inner plexiform layer (34.0±6.7µm versus control 34.5±3.6µm), the inner nuclear layer (44.0±6.2µm versus control 43.1±5.5µm), the outer plexiform layer (9.9±1.6µm versus control 10.6±1.6µm), the outer nuclear layer (61.7±5.6µm versus control 62.1±10.2µm) and the photoreceptor layer (22.6±3.4µm versus control 22.8±3.4µm) in mice at p17 (Fig.3).

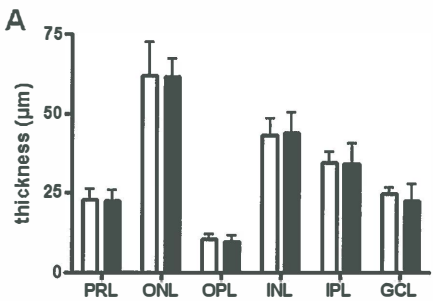

Figure 3
Thickness of retinal layers was similar in intravitreally thalidomide (■) treated and control (□) eyes of ROP exposed mice at p17. PRL: Photoreceptor layer, ONL: Outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner nuclear layer, GCL: ganglion cell layer

Intravitreal application of thalidomide did not induce retinal apoptosis

The retinal apoptosis was determined by counting TUNEL positive cell nuclei in retinal sections of thalidomide and control treated eyes of ROP mice at p17 (Fig.4 A-C). The apoptotic cell count was comparable in both groups for the ganglion cell layer (3±2 cells/section in thalidomide eyes and 4±4 cells/section in control eyes), the inner nuclear layer (7±6 cells/section in thalidomide eyes and 7±4 cells/section in control eyes), and the outer nuclear layer (33±18 cells/section in thalidomide and 18±9 cells/section in control eyes) (Fig.4 D).

To substantiate the data from the TUNEL stain, caspase-3 activity was measured in retinal lysates of ROP exposed and adult mice, which were intravitreally injected with thalidomide or with the control solution. Caspase-3 activity was neither altered by thalidomide in the ROP retina at p13 (3.6±2.1 pM AMC versus 3.2±1.8 pM AMC in control), nor in the ROP retina at p17 (1.0±0.6 pM AMC versus 1.4±1.4 pM AMC in control, Fig.4E). In the adult retina one day after the intravitreal injection of thalidomide the caspase-3 activity was comparable to control. In the control eyes at ROP p13 caspase-3 activity was about threefold higher than in the control treated adult retina and in the ROP retina at p17. Mouse 3T3 fibroblasts treated with hydroxyurea, used as an internal control, revealed significantly elevated caspase-3 activity compared to untreated cells, and caspase-3 activity could be reduced to no AMC release by the application of the caspase-3 inhibitor, which was also used as a control in all retinal samples to proof specificity of the assay.

Discussion

In this study we aimed to determine the pharmacological effects of intravitreal application of thalidomide on preretinal neovascularization in a mouse model of ROP. Our data demonstrate that the local application of the drug effectively reduced neovascularization. Toxic side-effects, such as apoptosis induction or structural retinal damage, were not observed. Interestingly, the effect was not brought about by inhibition of retinal VEGF production during the pathogenesis.

The anti-angiogenic and anti-inflammatory potential of thalidomi-

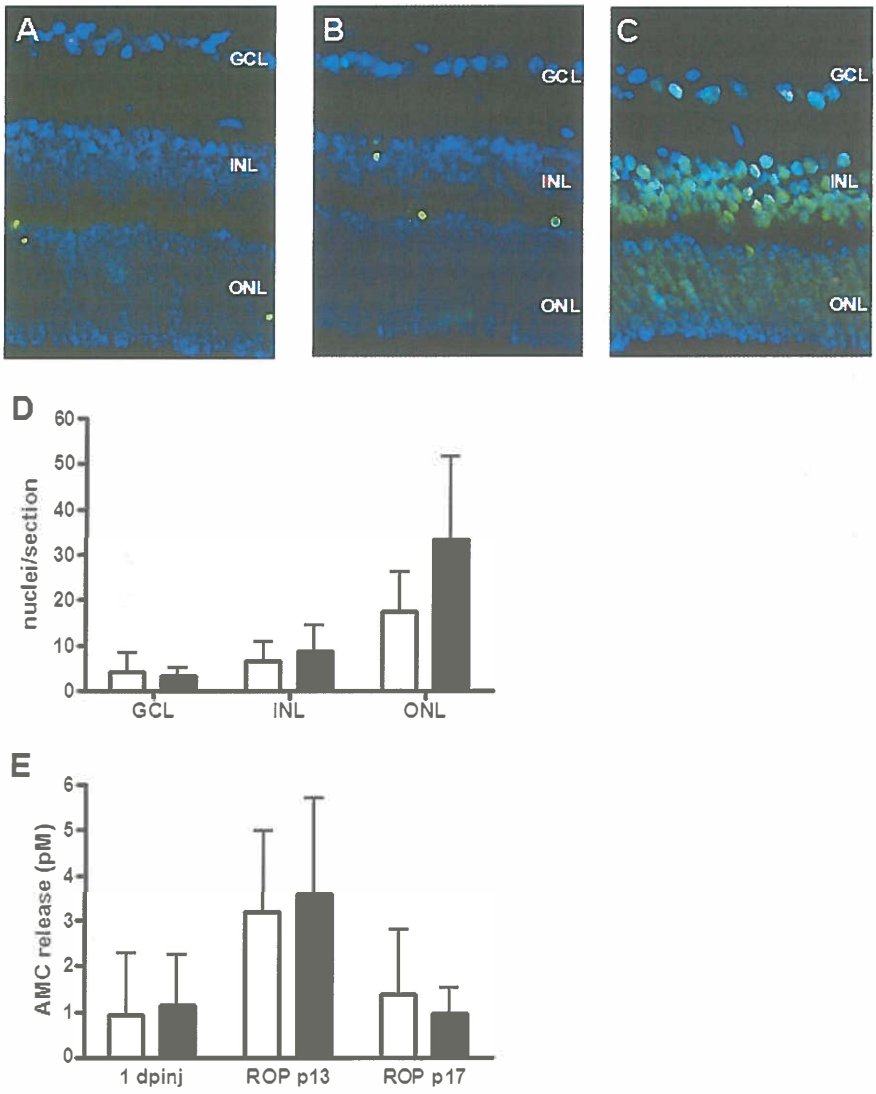

Figure 4
A-C: TUNEL (green) and DAPI (blue) stained retinal paraffin sections of control treated (A) and thalidomide (B) treated eyes of ROP exposed animals at p17. C shows a positive control stain for TUNEL at 200x magnification. D: Thalidomide treated eyes (■) showed similar apoptotic cell counts in the respective retinal nuclear layers compared to control treated eyes (□) of ROP exposed mice at p17. E: Caspase-3 activity was not influenced by thalidomide (■) compared to control (□) intravitreal injection in all groups. Caspase-3 activity was enhanced in ROP exposed retina at p13. GCL: ganglion cell layer, INL. Inner nuclear layer, ONL: outer nuclear layer

de suggested it to be a novel and potent drug to reduce preretinal neovascularization in proliferative retinopathies. Intravitreal application of thalidomide reduced preretinal neovascularization in the model of experimental proliferative retinopathy. The reduction in the neovascularization count by 60% suggests that the anti-angiogenic potency of thalidomide is stronger than the anti-angiogenic potency of a systemic application of a VEGF inhibitor alone, or of the genetic deletion of TNFα, or of the systemic inhibition of TNFα in this model (9, 19). The anti-angiogenic potency of thalidomide is comparable to the systemic application of a VEGF inhibitor combined with an integrin antagonist or a VEGF inhibitor combined with a VE-cadherin mediated adhesion blocking agent in the mouse ROP model (9).

In the study of Rabinowitz et al., the daily intraperitoneal injection of 200mg/kg thalidomide did not affect neovascularization in the same model (23). An explanation for the difference in the results of the studies may be the difference in the application route. The intravitreal application delivers the drug in close proximity to the localization of pathology and the vitreous serves as a drug reservoir, which keeps the drug longer at the site. A further reason for the discrepancies between both studies is the difference in the dosages used.

In the cornea micropocket model thalidomide was able to reduce VEGF induced corneal vascularization in the mouse by intraperitoneal and in the rabbit by oral application (11-13). In tumor patients, in vivo tumor models, and in vitro models thalidomide reduces VEGF expression and secretion (27-29). In our study thalidomide did not reduce total retinal VEGF protein levels. Likely, a local action of thalidomide on VEGF production at the site of pathology could be masked by total retinal VEGF analysis or the anti-angiogenic effect of thalidomide is not primarily mediated via VEGF.

Thalidomide's modulation of angiogenesis can be due to its anti-inflammatory properties as it is a potent inhibitor of TNFα, and inhibition of TNFα has been related to reduced preretinal neovascularization in the mouse model of proliferative retinopathy (19). Moreover, thalidomide blocks pro-inflammatory cytokine release from activated macrophages, and macrophages are localised adjacent to preretinal neovascularization (22).

Apart from its teratogenicity thalidomide has a low scale side-effect

profile and is approved in many countries worldwide. Possible toxic side effects for the retina like apoptosis induction and structural damage of the retina could not be observed in our study. We observed an increase of caspase-3 activity in the ROP exposed retina at p13. This is in line with published data of increased apoptosis in the avascular area at p14 compared to p17 in the ROP exposed mouse retina by TUNEL staining (30).
In conclusion the intravitreal administration of the "old" drug Thalidomide is a novel and retina safe option to potently reduce experimental proliferative retinopathy. Further studies may elucidate the mode of action in this model and examine whether thalidomide can be a therapeutic option for patients with proliferative retinopathies, such as proliferative diabetic retinopathy and exsudative age-related macular degeneration.

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Chapter VII

Summary

Summary

Proliferative retinopathies, such as proliferative diabetic retinopathy and retinopathy of prematurity are common causes of blindness. Epidemiologic studies propose an increase in diabetic patients over the coming years and thus the amount of people affected by proliferative retinopathy will also increase. Vascular endothelial growth factor (VEGF) has been identified as a key molecular target in this disease and anti-VEGF drugs have found their way into the clinic, yet. The experiments presented in this thesis were performed to further investigate the molecular mechanisms of physiological and pathological retinal angiogenesis and to increase understanding of the pathogenesis of pathological neovascularization. Based on studies demonstrating the importance of the angiopoietin-2 (Ang2)/VEGF balance in angiogenesis, one part of the studies was focused on the effects of Ang2 overexpression and deficiency in experimental mouse models of retinal angiogenesis. In the next part, we aimed at establishing a method to analyse spatial inflammatory- and angiogenesis associated gene expression and their localized regulation by hypoxia in the retina. Furthermore, we investigated the contribution of inflammatory factors, i.e. tumor necrosis factor α (TNFα) in the process of retinal neovascularization. Finally, we tested the influence of intravitreally adminstered thalidomide, which is a drug that inhibits TNFα signalling and interferes with VEGF signalling, on preretinal neovascularization.

The **first chapter** introduces the cellular and vascular architecture of the retina. The current knowledge of the timeframe of postnatal vascular development, the molecular control, as well as the cellular interactions is summarized for physiological retinal angiogenesis. Pathological retinal angiogenesis is introduced by two common clinical diseases, i.e. proliferative diabetic retinopathy and retinopathy of prematurity, which are characterized by preretinal neovascularization. Furthermore, it informs about the experimental models of retinal angiogenesis and summarizes the current concepts about the molecular mechanisms of pathologic retinal neovascularization, in which VEGF signaling is a key pathway. The angiopoietin/Tie2 system is introduced as a modulator physiologic and pathologic angiogenesis. Moreover, the new concepts of inflammatory contribution to retinal neovascularization are discussed. Based on

the current knowledge and on the new concepts, we designed the following chapters of the thesis.

Ang2 can act dependent on the VEGF environment either proangiogenic or vasoregressive, but its precise role in physiological and pathological retinal angiogenesis is not known. The experiments of the **second chapter** were designed to investigate the effect of transgenic overexpression of human Ang2 in the photoreceptor layer, on postnatal retinal vascular development and on the vascular response in the model of proliferative retinopathy (ROP). We observed that during physiological angiogenesis retinal vascular outgrowth and capillary density is enhanced, while pericyte coverage of the retinal capillaries was reduced in the Ang2 overexpressing mice. Our data extended previous data, which demonstrated that Ang2 is an important modulator of pericyte-endothelial association not only during pathological, but also developmental retinal angiogenesis. Exposure of Ang2 overexpressing mice to hyperoxia followed by relative hypoxia in the ROP model resulted in enhanced intraretinal regrowth and enhanced preretinal neovascularization at postnatal day 17 (p17). In the ROP retina, transgenic mice showed 7-fold higher Ang2 and increased VEGF expression compared to the wildtypes. As high levels of Ang2 in the presence of abundant VEGF induce angiogenesis, we concluded that this effect might be the reason for enhanced preretinal neovascularization. The increased intraretinal regrowth could be partly due to increased angiopoietin-1 (Ang1) levels, which we observed in the transgenic ROP retinae.
The results obtained from studying the role of Ang2 in a gain-of-function model in the second chapter were the basis for the design of the experiments in an Ang2 loss-of-function model. The study in the **third chapter** tested the retinal vascular response to the lack of Ang2 in the postnatal and adult retina. Crossing heterozygous Ang-2LacZ mice back on the C57bl/6J background enhanced the lifespan of the animals, and crossing heterozygous Ang2LacZ mice resulted in homozygous offspring. In these homozygous Ang2LacZ mice, in which the LacZ reporter gene is placed behind the Ang2 promoter resulting in disrupted Ang2 transcription, we studied the effect of Ang2 deficiency from p10 onwards, during maturation of the retinal vasculature, and up to one year of age in the adult vasculature. The

Ang2 deficient mice showed highly abnormal vascularization in the postnatal retina. The arterioles were reduced in number, of larger diameter and tortuous indicating a remodelling defect. Furthermore, the outgrowth of the vasculature towards the retinal periphery mostly on the arteriolar site was defective, and formation of the deep capillary layers was reduced. The defective formation of the retinal vasculature resulted in hypoxia, which upregulated VEGFA. Unexpectedly, these animals demonstrated massive preretinal neovascularization, originating from intraretinal venules. These neovascularizations were characterized by strong LacZ expression representing Ang2, endothelial proliferation and leakiness in the early phase. In one year old animals the preretinal neovascularization was still present while the leakiness resolved over time, which was likely due to the increase of Ang1 during the timecourse studied. We concluded that Ang2 has an important function in appropriate outgrowth and patterning of the vascular periphery, especially on the arteriolar site and the formation of the deeper capillary layers. Furthermore, prolonged VEGF activity results in persistent proliferative retinopathy.
In summary, our data derived from the studies in **chapter 2** and **chapter 3** demonstrate that Ang2 is necessary for proper retinal vascular development and vascular remodelling, and that the coordination of Ang2 and VEGF levels needs to be tightly controlled to prevent abnormal retinal vascular development. These data suggest a prominent role of Ang2 in postnatal retinal vascular remodeling and in proliferative retinopathy.

Understanding the molecular mechanism of retinal vascular disease requires knowledge of the molecular makeup of the neovascular area and of the molecular regulation during the neovascularization process. Retinal molecular studies have been limited as gene expression analysis and quantitative protein analysis were only feasible in whole retinal lysates. These analyses ignore the spatial distribution and local regulation of molecules. Studies addressing the cellular origin of molecules and the local regulation were also limited due to the small amount of available tissue. Furthermore, these studies investigated only small numbers of molecules by in situ hybridization, immunohistochemistry and recently by laser dissection microscopy prior to gene expression analysis. In **chapter 4**, we therefore

established a method to analyse spatial distribution of the expression of 46 inflammatory- and angiogenesis associated (angioflammatory) genes. These genes were analysed in selected vascularised retinal areas of normoxic controls and of animals subjected to the model of proliferative retinopathy. The areas, one consisting of the vascularised retinal layers and the other of the peripheral superficial vascular plexus area, which is the site of neovascularization incipience, were selected by laser dissection microscopy prior to gene expression analysis. We focused on the timepoint of neovascularization incipience, which is p13 in the ROP model and we compared the patterns obtained in the retinal areas with the expression in the whole retina. Our study revealed that angioflammatory genes differ in their spatial expression patterns with most genes predominantly expressed in the vascularised retinal areas of the control retina. Hypoxia induced angioflammatory gene expression, especially in the area of neovascularization incipience. In the peripheral superficial vascular plexus area, we observed strong upregulation by hypoxia of Ang2 corroborating our conclusions derived from chapter 2 and 3 that Ang2 is involved in preretinal neovascularization. Furthermore, we demonstrated that tumor necrosis factor receptor 1 (TNFR1), urokinase plasminogen activator (uPA) and transforming growth factor beta 1 (TGFβ1) were strongly upregulated by hypoxia in the area of neovascularization incipience, which is a new finding. Challenging current concepts, we found hypoxic upregulation of the antiangiogenic thrombospondin-1 (Tsp-1) in the same area and the antiangiogenic pigment epithelium derived factor (PEDF) was expressed to a higher extent in the same area of the control and ROP retina. In summary, zooming into different retinal layers allows a more detailed view about spatial patterning and local regulation of genes, especially at the site of pathology.

We and others have found that preretinal neovascularization is reduced in TNFα depleted mice in the ROP model, but the mechanism involved is still unknown. In **chapter 5**, we therefore used the new method established in the previous chapter to uncover the molecular mechanism(s) behind the TNFα effect. Before employing the new method, we tested whether TNFα depletion altered postnatal retinal vascularization or changed the response to hyperoxia induced vasoregression in the ROP model. An alteration of the va-

sculature prior to hyperoxic exposure in the model, and/or during the hyperoxic phase influences the extent of preretinal neovascularization, as we have previously demonstrated. Furthermore, we investigated the effect of TNFα deficiency on spatial distribution of angioflammatory gene expression and its effect on local gene regulation by hypoxia, especially in the area of angiogenesis incipience. Like in the previous study in chapter 4, we focused on p13 as the timepoint of neovascularization incipience. Similar to the wildtypes, we observed strong hypoxic induction of TNFR1 and TGFβ1 expression in the area of neovascularization incipience, indicating their participation in the initial phase of the neovascularization process. Although we did not find an obvious explanation for the TNFα mediated effect, we observed local TNFα modulated molecular trends, which may play a role in this process. In the area of neovascularization incipience, we observed less strong induction by hypoxia of the proangiogenic Ang2 and uPA, and a stronger upregulation of the antiangiogenic Tsp-1 compared to wildtypes. The observed shift towards antiangiogenic factors in TNFα deficient mice may cause the reduction in neovascularization. We can, however, not exclude that effects of TNFα deficiency on preretinal neovascularization occur later in the timecourse of neovascularization, or that the genes involved in this modulation as key regulators were not part of the set of genes in our study. Furthermore, we only selectively studied the protein level of some factors, such as the endothelial PECAM-1 and VE-cadherin. The protein expression pattern of these factors followed the RNA expression pattern. VEGF RNA and protein expression were upregulated by hypoxia at p13, but only on protein level VEGF was reduced in the TNF-/- mice at ROP p13.

New hope arose for patients suffering from proliferative retinopathies from the development of anti-VEGF drugs, but as described above clinical studies showed that their use is in many ways restricted. Recently, it has been the matter of debate whether a multitargeted approach might be more beneficial in this disease. Based on the knowledge of the role of VEGF in pathologic retinal neovascularization and our experimentally observed effect of TNFα deficiency on this, we hypothesized in **chapter 6** that the "old" drug thalidomide could be beneficial in this disease. Thalidomide is first of all

a potent inhibitor of TNFα, and its antiangiogenic effects are long known. The antiangiogenic effects have been first described to be due to VEGF inhibition, but could also be mediated via TNFα inhibition. Furthermore, thalidomide has antiinflammatory properties that could support its effect on preretinal neovascularization inhibition. We therefore tested the effect of intravitreally applied thalidomide on preretinal neovascularization in the model of proliferative retinopathy. As we expected, thalidomide effectively reduced preretinal neovascularization. Moreover, thalidomide did not cause any negative side effects on the retina. We concluded that thalidomide might be a promising drug to treat proliferative retinopathy.

Our studies demonstrate that Ang2 plays an essential role in physiological retinal vascular patterning and maturation, as well as in pathological retinal neovascularization. In contrast the inflammatory factor TNFα neither modulates physiological, nor hyperoxia-induced vasoregression, but promotes pathologic retinal neovascularization. Furthermore, the new method of comprehensive retinal gene expression analysis in selected areas, which we established, allows zooming into the area of retinal pathology. Although this method revealed data that challenge current concepts, they will eventually contribute to a further understanding of the molecular mechanism of preretinal neovascularization. Overall, our data show that detailed knowledge of spatial and local molecular regulations are necessary to develop therapeutic strategies that show their added value not only in experimental models, but more importantly also in the clinic. The intravitreal application of drugs is a good way to locally treat retinal disease.

Chapter VIII

Discussion and Future Perspectives

In the previous chapter we summarized the aims and results of our studies performed in this thesis and discussed the results obtained. In this chapter, we discuss the results in a broader context of retinal research, and based on our results we delineate future perspectives in this field.

Angiopoietin-2 in physiological and pathological retinal angiogenesis

Our findings in chapter 3 indicate that angiopoietins-2 (Ang2) is essential for proper formation and patterning of the arteriolar tree, the formation of the deep capillary plexus and the development of the vasculature in the retinal periphery during physiological retinal angiogenesis. Furthermore, Ang2 is involved in regression of the hyaloid vascular plexus. The combination of impaired hyaloid vessel regression and impaired formation of a deep vascular plexus we observed in the Ang2LacZ mice was also observed in other transgenic animals, such as Norrin and Frizzled-4 knockout mice (1, 2). It is likely, that the formation of the deep vascular plexus and hyaloid vessel regression are linked or share signalling cues.

Unexpectedly, we found that preretinal neovascularization develop and are persistent in the absence of Ang2. This challenges the current concept that both, vascular endothelial growth factor (VEGF) and Ang2 are necessary for pathological angiogenesis. However, in chapter 4 we observed that of the 46 angioflammatory genes we investigated, Ang2 is the strongest upregulated gene in the area of neovascularization incipience in the model of proliferative retinopathy (ROP). In line with this, Ang2 overexpression resulted in enhanced preretinal neovascularization. These data indicate that Ang2 modulates, but might not be essential in the pathogenesis of preretinal neovascularization. Further studies have to unravel the precise mechanism of the Ang/Tie2 system in this process.

Inflammation in physiological and pathological angiogenesis

Inflammation has been associated with the pathogenesis of proliferative retinopathies. We could show in chapter 4 that inflammatory genes, such as tumor necrosis factor receptor 1 (TNFR1) are locally upregulated already at the incipience of neovascularization. The retinal vascularization is altered in TNFR1 knockout mice exposed to the ROP model (3), but its effect on preretinal neovascularization

remained unclear. In tumor necrosis factor α (TNFα) deficient mice preretinal neovascularization is reduced, as we observed in chapter 5. The moderate reduction in VEGF protein expression might be an explanation for the observed effect. Spatial expression analysis of inflammatory and angiogenesis-associated genes did not reveal strong regulatory effects. Only hypoxia induced regulation of some genes, e.g. urokinase plasminogen activator (uPA) and thrombospondin-1 was altered in the area of neovascularization incipience. This might indicate participation of these factors in preretinal neovascularization, but needs further investigation. Furthermore, identifying the TNFα mediated effect probably requires the analysis of a broader set of genes, and/or more time points are necessary.

Other inflammatory molecules, such as intercellular adhesion molecule 1 (ICAM-1) and CD18 have been studied in hyperglycemia induced vascular damage in the model of early diabetic retinopathy (4), but not in the ROP model. Their role in preretinal neovascularization thus remains undetermined. In the diabetic retina, the formation of advanced glycation end products (AGE) (5, 6) is increased and signal via their receptor of advanced glycation end products (RAGE) (7), inducing vasoregression in experimental models of early diabetic retinopathy (8). As an important mediator of inflammation signalling, RAGE could play a role in the pathogenesis of preretinal neovascularization. However, we found that preretinal neovascularization is not altered by the administration of sRAGE, nor in RAGE-/- mice in the ROP model (vom Hagen et al., unpublished observations). These recent data imply that RAGE is not involved in preretinal neovascularization.

Another aspect is the contribution of inflammatory cells to preretinal neovascularization. Macrophages are a major source for inflammatory cytokines and matrix degrading factors (9), such as uPA, which we found distinctively regulated in our studies in chapter 4 and 5. Macrophages can be present in the vitreous as cells recruited from the systemic circulation or in the retina as resident microglia, which become activated. During postnatal retinal vascular development, macrophages are tightly associated with the growing vascular plexus and in front of the plexus (10). The depletion of macrophages has been experimentally induced by intravitreal application of clodronate liposomes, which reduced the number of resident retinal microglia and reduced postnatal vascular de-

velopment in the rat (10). Furthermore, they are essential for the regression of the hyaloid vascular plexus, as transgenic macrophage ablation inhibits this process (11). Endothelial apoptosis during hyaloid vascular plexus regression depends on the interaction of Ang2 and macrophage derived Wnt7b signalling (12). In the ROP model macrophages are continuously increased in proximity to the inner limiting membrane from postnatal day 12 (p12) to p17 and around the neovascular tufts (13, 14), but reduced in the hyperoxia induced vasoregressed areas of the ROP retina (10). The increase of macrophages in the area of preretinal neovascularization is correlated with an increase in monocyte chemoattractant protein 1 (MCP-1) expression in retinal lysates (14). Interestingly, MCP-1 deficiency did not alter the formation of preretinal neovascularization, but delayed the regression thereof in the ROP model (15). This could indicate that macrophages are involved in the regression of neovascular tufts rather than in their formation. It would be interesting to investigate, whether the intraretinal and preretinal numbers of macrophages are altered in the TNF-/- mice in the time course of the ROP model.

Angiogenesis and inflammation are more tightly associated as originally thought. Angiogenic molecules, such as VEGFA can execute proinflammatory functions (16) and inflammatory factors, such as TNFα can execute proangiogenic functions (17) in pathologic retinal neovascularization, as described in more detail in chapter 1. Ang2 and TNFα are linked by a study demonstrating that Ang2 sensitizes endothelial cells to a TNFα stimulus and that it promotes inflammation (18). In our studies in chapter 4 and 5, we found that Ang2 is selectively upregulated in the area of neovascularization incipience and this regulation is influenced in TNFα deficient mice. Tie2 is predominantly expressed in the same area as Ang2, but is not upregulated in the ROP retina compared to control. Both TNF receptors, TNFR1 and TNFR2 are mainly expressed in the area of neovascularization incipience as well, but only TNFR1 is induced by hypoxia in this area. Based on these results a further investigation of the interaction of TNFα and Ang2 and their downstream signaling pathways in retinal neovascularization could include expression analysis of TNFα and its receptors in the vascularised retina in the Ang2 deficient and Ang2 overexpressing mice. This experiment might indicate, whether TNFα is involved in the vascular response

to Ang2 in the pathogenesis of retinal neovascularization. While for the Ang/Tie2 system and the TNFα/TNFR system the ligands and receptors are mainly expressed in the area of neovascularization incipience, we observed induced VEGFA expression distant from induced VEGFR2 expression and distant from VEGFR1 expression in the ROP retina. The localization of the VEGF receptors correlated with the expression of the TNFα/TNFR and the Ang/Tie2 system, but VEGF was predominantly expressed in the intermediate retina. It would be therefore interesting to study how VEGF is distributed to act on its receptors, and whether this distribution is altered in the diseased retina.

Methods to unravel the molecular complexity of physiological and pathological retinal angiogenesis

In chapter 4 we isolated vascularised retinal areas by laser dissection microscopy prior to gene expression analysis to create new knowledge regarding the spatial patterning. Of special interest were the gene expression changes at the site where angiogenesis ensues in the model of proliferative retinopathy. The results demonstrate that this method is an exquisite tool to unravel the spatial expression and regulation of genes in the retina, and would be useful also in other animal models, e.g. in models of diabetic retinopathy. The selection of retinal areas prior to gene expression analysis would also be a valuable tool to study the local molecular changes in the time course of the ROP model. Thereby, not only the molecular basis of neovascularization incipience, but also of intraretinal regrowth and regression of neovascularization could be further elucidated. Using this method, we observed localized molecular regulations, which, next to VEGF, might be involved in the reduction of neovascularization in TNF-/- mice. However, we did not unravel key mechanisms involved in the TNFα effect on retinal neovascularization. We cannot exclude that more prominent molecular changes mediated by TNFα deficiency occur at other time points during neovascularization.

In diabetic retinopathy and in retinopathy of prematurity, impairments of the intraretinal vascular network, either by regression or by incomplete formation precedes preretinal neovascularization (19, 20). A future therapy would at best enhance intraretinal regrowth and prevent or reduce preretinal neovascularization. The

ROP model is suitable to investigate cellular and molecular mechanisms that mediate preretinal neovascularization and intraretinal regrowth. Our new method can assist in these studies, and in those which investigate the effects of potential therapeutics for patients with proliferative retinopathies. Since the key failure leading to preretinal neovascularization is the absence of a proper functioning intraretinal vasculature, it would be interesting to perform a comprehensive gene expression study during the distinctive steps of developmental retinal angiogenesis, i.e. during the formation of the deep capillary layers. A profound knowledge of the molecular mechanisms of physiological angiogenesis probably will help to elucidate key regulators of disease. With the aim to identify new genes that are involved in these processes one could investigate this by microarray analysis of retinal tissue selected by laser dissection microscopy. Buckanovich et al. successfully used this method on endothelial cells from tumors (21), and it seems also feasible with the amount of material that we collected. The amplification of mRNA, however, is still necessary, and by this mRNA signals of some low abundant expressed genes can get lost (22). Another aspect is that we selected material from large retinal areas. The new types of laser dissection microscopes allow collecting material from much smaller retinal areas or even certain cell types. The dissection of single cells is also feasible, but the RNA retrieved is low and would require amplification, which has its limitations.

The use of reporter genes in transgenic animals is a further possibility to enhance our understanding of the molecular mechanism of preretinal neovascularization. In chapter 3 we employed the reporter gene LacZ to reveal the colocalization of Ang2 expression in or next to the pathologic vasculature. However, all these kind of studies cover only gene expression patterns, and not the exact regulation thereof nor their downstream effectors. A next step is to elucidate the protein expression and protein functions of relevant factors. The success of these studies will depend on the sensitivity and specificity of the available methods. A future option would be a proteomic comparison of tissue selected by laser dissection microscopy of the relevant areas of disease or physiological angiogenic processes. So far, the amount of retinal tissue selected by laser dissection microscopy is not sufficient for these methods, if the tissue is not pooled from several retinae. Moreover, the results that would

be obtained from protein studies would have to be further corroborated by experiments demonstrating the local functional activities of the proteins, which is so far only feasible in whole retinal lysates.

Animal models of physiological and pathological retinal angiogenesis

A general problem in animal research is that researchers tend to see and treat their animals, especially transgenic animals as tools. However, reliable results are dependent on the well being of the animals. Furthermore, the nature of species and/or the differences in strain characteristics have to be considered (23). The models can deliver reliable results, if the factors that influence the animals in their response to the models are considered. Often, even trivial factors are neglected. Despite these possible caveats, animal models are the best method to investigate the molecular mechanism of retinal neovascularization in health and disease. Furthermore, they are valuable to evaluate and re-evaluate drugs and might assist in unravelling new targets to treat proliferative retinopathies.

The postnatal retina is a useful model to investigate cellular and molecular mechanisms of vascular development (24, 25). In a limited amount of time the complexity of a three dimensional vascular plexus formation can be studied in many aspects, such as cellular interaction, molecular guidance or patterning cues, and in different phases of the process, such as initiation, formation and maturation. In postnatal retinal angiogenesis and in the model of proliferative retinopathy drugs can be applied and depending on the postnatal age even intravitreally, to study their effects on the structure/mechanism of interest in the retina. A further possibility to investigate the outcome of molecular modulation in retinal angiogenesis is the use of genetically manipulated animals, such as knock-outs, knock-ins, inducible cell specific gain or loss of function, and/or transgenics. A possible disadvantage is the occurrence of compensatory functions resulting from the genetic manipulation in these animals.

The ROP mouse model (26) is well characterized and is the gold-standard model used to study proliferative retinopathy. Although it does not model all aspects of human proliferative retinopathy, it is suitable to investigate cellular functions in certain aspects of disease. With regard to retinopathy of prematurity the model displays well the common characteristics of hyperoxia induced vasoregression

followed by hypoxia induced neovascularization (20). A discrepancy is the defective peripheral outgrowth of the retinal vasculature, as well as the occurrence of neovascularization at the peripheral growth edge of the retinal vasculature in the human disease (20). In proliferative diabetic retinopathy preretinal neovascularization is initiated by hypoxia, which results from hyperglycemia induced capillary damage and subsequent vasoregression (19). The animal ROP model mimicks some characteristics of vasoregression prior to hypoxia induced neovascularization in proliferative diabetic retinopathy. Some have criticized that the model relies on the vascular plasticity in newborn mice, while proliferative diabetic retinopathy is a disease of the adult, often aged retina, and that the impact of hyperglycemia on the neovascularization process is missing.
Data derived from the described model of proliferative retinopathy is often abused to also apply in patients with neovascular age-related macular degeneration, and vice versa. In animal models, choroidal neovascularization (CNV) is induced by laser burns in the fundus, and its detrimental effect depends on the destruction of Bruch's membrane (27). Bruch's membrane looses its barrier function during aging in the human and in the model by laser destruction, and choroidal neovascularization can grow into the subretinal space or retinal vessels of the deep vascular plexus grow into the subretinal space (28). The laser induced CNV model has its limitations as the holes burned into the fundus induce a wound healing response. Furthermore, the human disease occurs predominantly in the macula, a structure that most mammals, except humans and some primates do not have. Some transgenic models, such as mice lacking MCP-1 develop several signs of age-related macular degeneration including CNV (29, 30).

Limitations of research related to proliferative retinopathies
Research in human retinal diseases is limited by the fact that retinal tissue can not be spared. While in other organs, biopsy can help elucidating the molecular mechanisms of disease, this is not feasible in the retina. Although pathological processes in the retinal vasculature are visible non-invasively, i.e. by ophthalmoscopy, optical coherence tomography and angiography, they represent some point of vascular response to injury. Despite their importance to understand the progress of a disease, these methods can only assist

in elucidating the causal mechanism of disease. The cellular and molecular complexity of the retina and its interactions limit the value of cell culture studies. Retinal angiogenesis is a highly complex mechanism, which requires intimate interaction of several cellular components. Moreover, the retina has unique characteristics, such as the blood-retinal-barrier and being an immune privileged site. Cell culture research of retinal cells is not always representative, as many cell types loose their specific phenotypic and functional characteristics in culture. Furthermore, the cellular crosstalk of the tissue is not mimicked in vitro. Animal models are a valuable tool to study retinal angiogenesis in physiology and disease, but they also have their limitations as models of human disease. Another limitation is the amount of material that is derived from experimental animal studies. Only recently, the sensitivity improved so that comprehensive gene expression studies in small amounts of retinal samples became feasible. On the protein level, however, the sensitivity of quantitative molecular methods is still insufficient for comprehensive analyses, but the development of these methods is rapid.

Therapeutic Strategies for proliferative retinopathies

There are several human diseases, which are characterized by ocular neovascularization. While in proliferative diabetic retinopathy and retinopathy of prematurity neovascularization preretinal growth of intraretinal vessels occurs (31, 32), in neovascular age-related macular degeneration neovascularization arises from the choroidal vessels, which grow into the subretinal space (28). In the studies presented in chapter 2, 4, 5, 6 and 7, we focused on the pathogenesis of neovascularization of intraretinal vessels. Caution has to be taken to translate these results to the pathogenesis of choroidal neovascularization, and vice versa, i.e. to apply concepts derived from experimental models of choroidal neovascularization to preretinal neovascularization.

For patients with diabetes the perfect therapy is the prevention of the disease itself. Meanwhile, tight blood sugar and blood pressure control in diabetic patients are potent means to delay the onset of diabetic retinopathy and to prevent/delay proliferative retinopathy (33-35). The standard therapy of proliferative retinopathies is laser photocoagulation of the neovascular areas to stop preretinal

growth. This is effective in halting the disease process, but destroys healthy tissue surrounding the laser treated area. The therapy of retinopathies has been somewhat revolutionized during past years by the use of anti-VEGF drugs. Pegaptanib and ranibizumab have been approved for their beneficial effect in reducing macular edema in diabetic patients (36). The efficacy of anti-VEGF drugs to reduce preretinal neovascularization in diabetic patients is currently investigated in clinical trials. So far, anti-VEGF drugs appear more potent in the resolution of vascular leakage than in reduction of preretinal neovascularization. Some case reports have demonstrated that the application of bevacizumab alone or in combination with laser therapy reduced preretinal neovascularization in patients with proliferative diabetic retinopathy (37-39). Besides their not yet proven efficacy in patients with proliferative retinopathies, limitations of these drugs arise by their systemic side effects. These side effects are relevant in the multimorbid diabetic patients and in the multimorbid old patients with neovascular age-related macular degeneration. Ranibizumab slightly increased the incidence of stroke and myocardial infarction in the MARINA and ANCHOR trials and it was recommended to limit its use in patients at higher risk for these events (40-42). Further limitations arise from the high costs of these drugs, which can often not be afforded by patients and is not covered by the insurance. Thus, the off-label use of bevacizumab is common. Caution is required in the long term application of anti-VEGF drugs in patients with retinal vascular disease, as the precise role of VEGF function with regard to maintenance and survival not only of the vasculature in the adult retina is poorly understood (43, 44). It appears that VEGF is essential in this process.

Generally, our data in chapter 4 demonstrate that a local highly complex modulation of molecular expression is the basis of preretinal neovascularization. These local complex modulations are poorly understood. Recently, new therapeutic strategies have been proposed based on multiple targets, such as receptor tyrosine kinases. However, broad kinase inhibition has to show efficacy and it could lead to even more severe side-effects than specific anti-VEGF strategies. Other approaches to target integrins and VEGF seem to be promising in experimental models (45) and blocking of integrins previously has been shown to be a potent method to inhibit experimental preretinal neovascularization (46). The first anti-TNFα and

anti-inflammatory drugs are currently investigated in clinical trials for their effects in patients with macular edema. Based on our studies in chapter 5, TNFα inhibition could be beneficial also in reducing preretinal neovascularization. Anti-inflammatory drugs must be used cautiously in patients with diabetes, as their systemic side effects, especially in diabetic nephropathy, would be detrimental.
Our observation in chapter 6 that thalidomide reduces preretinal neovascularization, indicates that a multitargeted approach combining anti-angiogenic and anti-inflammatory effects might be a future therapeutic strategy. Furthermore, it shows that long known drugs should be re-evaluated for their potential use in other diseases. This would limit the time of drug development from bench to bedside. Thalidomide is approved for patients with multiple myeloma and systemic erythema nodosum leprosum and its beneficial effects have been described for several immunological disorders, such as systemic lupus erythematosus, Behcet's disease and rheumatic diseases. Immunological disorders are often associated with retinal vascular disease. Retinopathy in patients with, e.g. lupus erythematosus is characterised by microangiopathic changes, such as hemorrhage or less frequently retinal vaso-occlusive disease (47, 48). Data about the effects of thalidomide on the retinal vascular damage in these patients, if existent, would be valuable for considering its use in proliferative retinopathy patients. It might be that diabetic patients are already treated sytemically with thalidomide for another indication. If that is the case, a study could show, if the treatment influenced/influences their retinal disease stage. Before considering the use of thalidomide in patients with proliferative retinopathy, its formulation has to become transparent to avoid visual impairment by its opacity. Furthermore, a phase II clinical trial has to reveal the optimal dose that is going to be used for intravitreal application.
Increasing knowledge about the hyperoxia induced effects on retinal vascular development in preterm born babies have improved the critical care measurements (32). Great care is now taken that the incubators do not increase the blood oxygen saturation above physiological levels to prevent retinal vascular responses. However despite these efforts, the occurrence of retinopathy of prematurity is not prevented, which indicates that other factors, such as the degree of prematurity and birth weight also contribute to the formation

of retinopathy of prematurity (32). The incidence of retinopathy of prematurity even increases as due to medical advances more immature infants are saved after preterm birth (49). Clinical trials evaluating the efficacy and safety of anti-VEGF drugs in these patients are currently planned. These trials also have to demonstrate that these drugs do not interfere with the developmental angiogenesis and vascular maturation in these premature infants.

Next to anti-VEGF, strategies to interfere with growth hormone and insulin-like growth factor 1 (IGF-1) signalling were suggested to have potential in limiting preretinal neovascularization in patients with proliferative retinopathies (50). The hypothesis was derived from observations that retinopathy in growth hormone deficient diabetic patients is rare and that pituitary ablation leads to neovascular regression (51-53). In patients with proliferative diabetic retinopathy therapeutic strategies aim to inhibit IGF-1 signalling, e.g. by application of somatostatin analogues (54). The somatostatin analogue octreotide, which interferes with VEGF and IGF-1 signalling showed efficacy in small case studies (55) and was therefore investigated in two large phase III trials in type 1 and type 2 diabetic retinopathy patients (54). In retinopathy of prematurity IGF-1 is of interest as preterm born infants have reduced IGF-1 and IGF binding protein 3 (IGFBP-3) levels, which are probably the reason for the defective postnatal retinal neuronal and vascular development (56-58). Clinical trials are in the planning phase to supplement IGF-1 or IGF binding protein 3 to promote retinal development and thus to prevent or reduce the severity of ROP, which was successful in the mouse ROP model (59).

Recently, the ocular vitreous has been assigned the function of a drug reservoir for the treatment of ocular/retinal diseases. The advantage is that higher drug concentrations can be achieved at the site of pathology. Furthermore, as indication of the high acceptance rate of repeated intravitreal injections, the drop-out of patients in studies was low. We used this approach to administer thalidomide and could demonstrate an effect that was not observed by others using a systemic approach (chapter 6). However, systemic resorption of some anti-VEGF drugs is still large enough to cause toxic side effects, as described for ranibizumab above. Furthermore, intravitreal injections can cause intraocular side-effects, such as endophthalmitis, retinal detachment and cataract. Clincial studies, such as

the VISION trial have shown that the occurrence of these ocular side-effects is low (42). A new approach considers the application of anti-angiogenic drugs in an eye drop formulation (60). Recently, two experimental studies using a multiple target approach in an eye drop formulation demonstrated reduction of preretinal neovascularization (61, 62). This strategy would ease repeated application of anti-angiogenic drugs in patients, and would promote outpatient care. This application route and these drugs have to be carefully tested for their possible side-effects on other ocular structures, such as the cornea and the iris vasculature.

In summary, the research of this thesis could further elucidate the effects of Ang2 in developmental retinal angiogenesis. Furthermore, we demonstrated that retinal angioflammatory genes differ in their spatial expression patterns and these are influenced in the ROP model. We put forward that laser dissection of retinal areas prior to gene expression analyses is a valuable tool in retinal research. The aim to discover the molecular mechanism of TNFα deficiency effects on preretinal neovascularization was not reached, but the study revealed interesting molecules for further investigations, as delineated above. The reason for our failure to uncover the molecular effects of TNFα deficiency on preretinal neovascularization could be due to the limitations described. Overall our studies are a small piece in the big puzzle of the molecular mechanisms of retinal angiogenesis.

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Appendix

Nederlandse Samenvatting

Proliferatieve retinopathieën, zoals proliferatieve diabetische retinopathie en retinopathie van prematuriteit (retinopathy of prematurity; ROP) worden gekarakteriseerd door overmatige nieuwvorming van bloedvaten (neovascularisatie) in de retina. Deze nieuwgevormde bloedvaten komen voort uit het netwerk van bloedvaten dat zich in de retina bevindt en groeien in de richting van het glasachtig lichaam, in het zogenaamde preretinale gebied aan de voorzijde van de retina. Groei van deze veelal lekkende bloedvaten in het preretinale gebied heeft ernstige consequenties voor het gezichtsvermogen, en kan uiteindelijk leiden tot blindheid. Proliferatieve retinopathieën zijn daarmee de meest voorkomende oorzaak van blindheid onder de werkende populatie in de Westerse wereld. Voor de komende jaren voorspellen epidemiologische studies een sterke toename in het aantal patiënten met diabetes, en het aantal mensen dat met proliferatieve retinopathie te maken krijgt, zal derhalve ook stijgen.
Een van de belangrijkste groeifactoren die aanzetten tot (overmatige) neovascularisatie is 'vascular endothelial growth factor' (VEGF), en inmiddels hebben geneesmiddelen die de functie van VEGF remmen hun weg naar de kliniek gevonden. De experimenten beschreven in dit proefschrift zijn uitgevoerd om de moleculaire mechanismen die ten grondslag liggen aan de pathogenese van pathologische neovascularisatie verder te onderzoeken. Gebaseerd op studies die het belang van de balans in de productie van de moleculen Angiopoietine-2 (Ang2) en VEGF voor angiogenese laten zien, hebben we ervoor gekozen om in het eerste deel van onze studies middels overexpressie en knock-out de precieze moleculaire effecten van Ang2 op angiogenese in de retina te evalueren. In het hiernavolgende deel was ons doel een methode te ontwikkelen om de ruimtelijke verdeling van de expressie van moleculen te analyseren die betrokken zijn bij de regulatie van angiogenese en inflammatoire processen. Tevens hebben we onderzocht hoe de locale regulatie van deze genen verandert onder invloed van hypoxie, een belangrijke factor die een primaire aanleiding geeft tot initiatie van overmatige vaatvorming in het model voor ROP. Bovendien hebben we de bijdrage van inflammatoire genen, zoals Tumor Necrosis Factor α (TNFα) aan het proces van neovascularisatie in de retina

geanalyseerd. Ten slotte hebben we de effecten van thalidomide, een geneesmiddel dat de functie van TNFα remt en interfereert met VEGF signaaltransductie, op preretinale neovascularisatie onderzocht.

Het **eerste hoofdstuk** introduceert de cellulaire en vasculaire architectuur van de retina. Het geeft een samenvatting van de hedendaagse kennis omtrent het tijdsverloop van de postnatale vasculaire ontwikkeling, de regulatie hiervan op moleculair niveau en de cellulaire interacties binnen het proces van fysiologische angiogenese in de retina. Pathologische angiogenese wordt geïntroduceerd door twee veelvoorkomende klinische symptomen, namelijk proliferatieve diabetische retinopathie en retinopathie van prematuriteit, ziektes die beide gekarakteriseerd worden door overmatige neovascularisatie in het preretinale gebied. Verder beschrijft dit hoofdstuk de experimentele modellen die voor onderzoek naar (afwijkingen van) angiogenese in de retina gebruikt worden, en geeft het een samenvatting van de huidige concepten omtrent de moleculaire mechanismen achter pathologische neovascularisatie in de retina, een proces waarin VEGF wordt gezien als een van de sleutelrolspelers. Daarnaast wordt het angiopoietine-Tie2 systeem geïntroduceerd als een modulator van fysiologische en pathologische angiogenese. Bovendien worden in dit hoofdstuk de nieuwe concepten aangaande de bijdragen van een inflammatoire component aan neovascularisatie in de retina bediscussieerd. Gebaseerd op de bestaande kennis, en op deze nieuwe concepten, hebben we de hoofdstukken van dit proefschrift vormgegeven zoals hieronder beschreven.

Afhankelijk van de aanwezigheid van VEGF, heeft Ang2 een tweeledige functie op het bloedvatstelsel, namelijk pro-angiogeen of juist vasoregressie-inducerend. Echter, de precieze rol van Ang2 in fysiologische en pathologische angiogenese in de retina is onbekend. De experimenten in het **tweede hoofdstuk** zijn ontworpen om de effecten van transgene overexpressie van Ang2 in de laag met fotoreceptoren op postnatale ontwikkeling van het vaatstelsel van een zich normaal ontwikkelende retina te onderzoeken. Daarnaast hebben we ook gekeken naar de consequenties van Ang2 overexpressie voor pathologische vascularisatie in de retina in het model voor proliferatieve retinopathie. Onze observaties brachten aan het licht

dat gedurende fysiologische angiogenese de uitgroei van bloedvaten, en daarmee de microvasculaire dichtheid, toeneemt onder invloed van verhoogde Ang2 expressie, terwijl het aantal steuncellen, pericyten, dat de bloedvaten omgeeft, is afgenomen in verhoogde Ang2 condities. Onze data bevestigden eerder gegenereerde data die lieten zien dat Ang2 een belangrijke modulator is van interacties tussen pericyten en endotheelcellen, niet alleen gedurende pathologische, maar ook tijdens fysiologische angiogenese in de zich ontwikkelende retina. Blootstelling van muizen die Angiopoietine-2 tot overexpressie brengen aan hyperoxie, gevolgd door een periode van relatieve hypoxie in het ROP model resulteerde in een versterkte hergroei van vaten binnen de retina, en ook in toegenomen neovascularisatie in het gebied voor de retina op postnatale dag 17 (p17). Binnen het ROP model lieten de transgene muizen een zevenvoudig hogere expressie van Ang2, en tevens verhoogde VEGF niveaus zien, in vergelijking tot wildtype muizen. Omdat bekend is dat hoge Ang2 productie in de aanwezigheid van overvloedig VEGF leidt tot de initiatie van angiogenese, hebben we geconcludeerd dat dit effect de reden voor de versterkte preretinale neovascularisatie zou kunnen zijn.
De resultaten omtrent de rol van Ang2 in angiogenese in de retina, verkregen zoals hierboven beschreven door verhoogde productie van Ang2 in een gain-of-function model, vormden de basis voor het ontwerp van een studie waarin werd gekeken naar de effecten van afwezigheid van Ang2 in een loss-of-function model. In de studie omschreven in het **derde hoofdstuk** maakten we gebruik van Ang2LacZ muizen om de respons van het retinale vaatstelsel op de afwezigheid van Ang2, in de postnatale en in de volwassen retina te onderzoeken. Ang2LacZ muizen worden gekenmerkt door verstoorde Ang2-expressie, doordat in hun DNA het LacZ-reporter gen direct achter de Ang2-promotor is geplaatst en daarmee de transcriptie van Ang2 verstoort. In deze muizen hebben we de effecten van Ang2-deficiëntie op de vasculaire ontwikkeling in de retina onderzocht, vanaf postnatale dag 10, tijdens maturatie van het bloedvatstelsel, tot aan de uiteindelijke ontwikkeling van een volwassen vaatstelsel in de retina op de leeftijd van een jaar. Ang2-deficiënte muizen ontwikkelen een sterk abnormaal vascularisatiepatroon in de postnatale retina. Het aantal arteriolen was afgenomen, terwijl een toename in vaatdiameter gepaard ging met verhoogde tortuo-

siteit, observaties die duiden op een defect in remodelling van het bloedvatenstelsel. Bovendien was de uitgroei van bloedvaten in de richting van de retinale periferie, vooral aan de kant van de arteriolen, onvolledig, en de vorming van de diepere capillaire lagen was verminderd. Deze onvolkomen ontwikkeling van het retinale bloedvatstelsel resulteerde in hypoxie, en daarmee in een opregulatie van expressie van VEGF. Tegen onze verwachting in bleek in deze dieren een aanzienlijke neovascularisatie in het preretinale gebied op te treden, welke ontsprongen vanuit de intraretinale venules. Deze nieuwgevormde vaten werden gekarakteriseerd door sterke LacZ expressie, wat duidt op activiteit van de Ang2-promotor, door proliferatie van het endotheel en door permeabiliteit van de vaten in twee maanden oude muizen. Op de leeftijd van een jaar waren de nieuwgevormde vaten in het preretinale gebied nog steeds aanwezig, terwijl de permeabiliteit van deze vaten afgenomen was. Dit laatste is waarschijnlijk te wijten aan een verhoging van Ang1 expressieniveaus, welke wij gedurende het tijdsbestek van de studie gemeten hebben. Dit bracht ons tot de conclusie dat Ang2 een belangrijke rol speelt in het reguleren van de vorming van een adequate architectuur van de vasculaire periferie, met name aan de arteriole zijde en voor de vorming van de diepere capillaire lagen. Bovendien resulteert langdurige activiteit van VEGF in aanhoudende proliferatieve retinopathie.
Samengevat laten onze data verkregen in de studies uit **hoofdstuk 2 en hoofdstuk 3** zien dat Ang2 noodzakelijk is voor een adequate vaatontwikkeling in de retina, en dat de coördinatie van Ang2 en VEGF productie strikt gereguleerd moet worden om abnormale ontwikkeling van bloedvaten in de retina te voorkomen. Deze data suggereren een prominente rol voor Ang2 in postnatale vasculaire remodelling in de normale retina, en ook tijdens proliferatieve retinopathie.

Begrip van de moleculaire mechanismen die verantwoordelijk zijn voor vaatziekten in de retina vereist kennis van de moleculaire opmaak van de nieuwgevormde vaten en het omliggende gebied, en ook van de moleculaire regulatie van het proces van neovascularisatie. Tot nu toe zijn dergelijke moleculaire studies in de retina erg beperkt, omdat kwantitatieve analyses van gen- en eiwitexpressie alleen mogelijk waren in monsters van de gehele retina. Daarbij gaat

belangrijke informatie omtrent de ruimtelijke verdeling van deze moleculen binnen het weefsel, en de locale regulatie daarvan, verloren. Ook is de cellulaire oorsprong van deze moleculen slechts in beperkte mate bestudeerd vanwege de beperkte hoeveelheid beschikbaar weefsel, en deze studies limiteerden zich veelal tot de analyse van een klein aantal moleculen in in situ hybridisaties, immunohistochemie en recentelijk ook door toepassing van laserdissectiemicroscopie voorafgaand aan genexpressie-profilering. In **hoofdstuk 4** hebben we daarom een methode opgezet om de ruimtelijke expressiepatronen van 46 angiogenese gerelateerde en inflammatoire genen (gezamenlijk angioflammatoir genoemd) binnen de retina te onderzoeken. We hebben de expressie van deze genen geanalyseerd in geselecteerde vaatgebieden in de retina van muizen die zijn blootgesteld aan het model voor proliferatieve retinopathie, en in die van controle muizen opgegroeid in normoxische omstandigheden. Deze gebieden, waarvan de een bestond uit de gevasculariseerde lagen van de retina en de andere uit de perifere superficiële vasculaire plexus, het gebied waar het neovascularisatieproces in gang wordt gezet, zijn geïsoleerd middels laserdissectiemicroscopie en onderworpen aan genexpressie-analyse. Onze focus lag op het tijdspunt waarop het angiogeneseproces begint, namelijk p13 in het ROP model, en we hebben expressiepatronen van angioflammatoire genen in de verschillende vascularisatie-gebieden vergeleken met expressiepatronen in de retina als geheel. Dit bracht naar voren dat angioflammatoire genen variëren in hun ruimtelijk expressiepatroon. Voor de meeste genen gold een overwegende expressie in de gevasculariseerde laag van de retina van controle muizen. Hypoxie induceerde angioflammatoire genexpressie voornamelijk in het superficiële plexus gebied, van waaruit neovascularisatie van start gaat. In dit gebied zagen we sterke opregulatie van Ang2 door hypoxie, een gegeven dat onze conclusies uit hoofdstuk 2 en 3 aangaande de rol van Ang2 in preretinale neovascularisatie bevestigt. Bovendien hebben we laten zien dat hypoxie in het gebied van neovascularisatie-initiatie ook leidt tot opregulatie van genen zoals tumor necrosis factor receptor 1 (TNFR1), urokinase plasminogen activator (uPA) en transforming growth factor beta 1 (TGFβ1), iets wat nog niet eerder is aangetoond. In tegenspraak met de huidige concepten, hebben we bewezen dat ook het anti-angiogene molecuul thrombospondin 1 (Tsp-1) is opgereguleerd onder invloed van

hypoxie in de superficiële vasculaire plexus. Erg interessant is het feit dat we verhoogde expressie van het anti-angiogene pigment epithelium-derived factor (PEDF) hebben gedetecteerd in de vasculaire plexus van zowel normale als pro-angiogene ROP retinae, terwijl tot nu toe aangenomen werd dat dit molecuul alleen door het pigment epitheel wordt geproduceerd. Samengevat geeft inzoomen op de verschillende (gevascularisseerde) lagen in de retina door lasermicrodissectie ons de mogelijkheid om, vooral op die locatie waar pathologie ontstaat, een meer gedetailleerd beeld van de ruimtelijke patronen van genexpressie, en locale regulatie hiervan, te creëren.

Wij, en ook anderen, hebben bewezen dat preretinale neovascularisatie in het ROP model is verminderd in muizen die het gen voor TNFα missen. Echter, het mechanisme hierachter is nog steeds onbekend. In **hoofdstuk 5** hebben we de nieuw ontworpen methode zoals beschreven in het vorige hoofdstuk ingezet om de moleculaire mechanismen achter dit TNFα-effect bloot te leggen. Op vergelijkbare wijze als in **hoofdstuk 4** hebben we de effecten van TNFα-deficiëntie op de ruimtelijke distributiepatronen van expressie van angioflammatoire genen in de retina, en op de locale regulatie hiervan in hypoxische omstandigheden, onderzocht. Weer kozen we ervoor onze studie te richten op de moleculaire veranderingen die optreden op postnatale dag 13, de dag waarop neovascularisatie begint. Net als in de wildtype muizen zagen we ook hier een sterke inductie van TNFR1 en TGFβ1 expressie in het gebied van neovascularisatie-inductie onder invloed van hypoxie, een observatie die hun rol in de beginfase van angiogenese bevestigt. Hoewel we geen duidelijke verklaring voor het TNFα-gemedieerde effect op neovascularisatie konden vinden, zagen we wel locale door TNFα gemoduleerde moleculaire veranderingen, trends die een rol in het TNFα-effect zouden kunnen spelen. In vergelijking tot wildtype muizen, vertoonde het gebied waar neovascularisatie begint in TNFα-deficiënte muizen een minder sterke hypoxie-gedreven opregulatie van de pro-angiogene moleculen Ang2 en uPA, en een sterkere inductie van het anti-angiogene Tsp-1. Deze verschuiving naar de productie van minder pro-angiogene en meer anti-angiogene factoren in muizen die het gen voor TNFα missen zou de vermindering in de mate van neovascularisatie kunnen verklaren. We

kunnen echter niet uitsluiten dat de effecten van TNFα-deficiëntie op preretinale neovascularisatie pas op een later tijdspunt in het neovascularisatieverloop optreden. Ook is het mogelijk dat juist die genen die een sleutelrol in het TNFα-effect spelen geen onderdeel uitmaakten van de selecte groep onderzochte genen in onze studieopzet. Bovendien hebben we van slechts een selectie van deze genen ook expressie op eiwitniveau onderzocht. Eiwitexpressie van PECAM-1 en VE-cadherine volgde het RNA-patroon. Zowel VEGF eiwit als RNA werden opgereguleerd door hypoxie op p13, maar in vergelijking tot wildtype muizen vertoonde alleen VEGF eiwit een minder sterke opregulatie onder invloed van hypoxie in de TNF α-deficiënte muizen.

De ontwikkeling van VEGF-remmende geneesmiddelen gaf patiënten die lijden aan proliferatieve retinopathieën nieuwe hoop, maar de mogelijkheden om de effectiviteit van dergelijke geneesmiddelen in klinische studies te onderzoeken zijn, zoals al eerder genoemd, sterk beperkt. Recentelijk is er veel discussie ontstaan over de vraag of deze patiënten wellicht meer baat hebben bij een multitarget benadering, een strategie die zich richt op de remming van meer dan één pro-angiogeen molecuul. Gebaseerd op de kennis betreffende de rol van VEGF in pathologische neovascularisatie in de retina, en op onze waarnemingen rondom het anti-angiogene effect van TNFα-deficiëntie hierop, kwamen we in **hoofdstuk 6** tot de hypothese dat het 'oude' geneesmiddel Thalidomide een goede kandidaat voor de behandeling van proliferatieve retinopathieën kan zijn. Thalidomide is niet alleen een potente remmer van TNFα, ook de anti-angiogene effecten van dit middel zijn al sinds lange tijd bekend. Aanvankelijk werd gedacht dat dit anti-angiogene effect gemedieerd werd door remming van de angiogenese-inducerende functie van VEGF, maar dit zou ook te wijten kunnen zijn aan de inhibitie van TNFα. Bovendien heeft Thalidomide anti-inflammatoire eigenschappen die het remmende effect op preretinale neovascularisatie kunnen versterken. Daarom hebben we het effect van Thalidomide, dat direct werd toegediend in het glasachtig lichaam van het oog, op pretinale neovascularisatie onderzocht in ons model voor proliferatieve retinopathie. Zoals verwacht, liet Thalidomide een effectieve remming van neovascularisatie in het preretinale gebied zien. Bovendien had het geen negatieve bijwerkingen op de

retina. Thalidomide zou daarom een veelbelovend geneesmiddel voor de behandeling van proliferatieve retinopathie kunnen zijn.

Samengevat laten de studies beschreven in dit proefschrift zien dat Ang2 een essentiële rol speelt in zowel fysiologische vasculaire ontwikkeling, als in pathologische neovascularisatie in de retina. Daarentegen speelt de pro-inflammatoire factor TNFα alleen een versterkende rol in pathologische neovascularisatie, en laat fysiologische neovascularisatie ongemoeid. Daarnaast hebben we een methode ontwikkelt om, op hypothesegedreven en uitgebreide wijze, genexpressie in bepaalde van tevoren geselecteerde gebieden in de retina te onderzoeken, een methode die ons de mogelijkheid biedt om in te zoomen in het gebied waar pathologie ontstaat. Hoewel deze methode data heeft opgeleverd die huidige concepten ter discussie stellen, zal het uiteindelijk bijdragen aan een completer begrip van de moleculaire mechanismen van neovascularisatie in het preretinale gebied. Bij elkaar genomen laten onze data zien dat gedetailleerde kennis van ruimtelijke en locale moleculaire regulaties van angiogenese nodig is om therapeutische strategieën te kunnen ontwerpen die een toegevoegde waarde hebben, niet alleen in experimentele modellen, maar, belangrijker, ook in de kliniek. Toediening van geneesmiddelen direct in het glasachtig lichaam van het oog is een goede manier om locaal ziektes van de retina te kunnen behandelen.

List of Abbreviations

Angioflammatory	angiogenic, inflammatory and angiogenesis associated genes
Ang1	angiopoietin-1, Ang-1, gene symbol Angpt1
Ang2	angiopoietin-2, Ang-2, gene symbol Angpt2
Ang2LacZ	transgenic mouse that expresses LacZ instead of Ang2, Ang2 deficient mouse
Angpt1	gene symbol for angiopoietin-1
Angpt2	gene symbol for angiopoietin-2
B2m	beta-2-microglobulin
Bcl2	B-cell-lymphoma 2
bFGF	basic fibroblast growth factor, gene symbol Fgf2
BSA	bovine serum albumine
Ccl2	chemokine ligand 2, gene symbol for MCP-1
Cdh5	gene symbol for VE-cadherin
CNV	choroidal neovascularization
Efnb2	ephrin B2
Eng	endoglin
EphB4	Eph-receptor B4
EPO	erythropoietin, gene symbol Epo
EPOR	erythropoietin receptor, gene symbol Epor
Fgf2	gene symbol for basic fibroblast growth factor, bFGF
FGFR1	fibroblast growth factor receptor 1, gene symbol Fgfr1
FGFR2	fibroblast growth factor receptor 2, gene symbol Fgfr2
GAPDH	glycerinaldehyde 3-phosphate dehydrogenase, gene symbol Gapdh
Flt1	gene symbol for VEGFR1
GCL	ganglion cell layer
HIF1α	hypoxia inducible factor 1 α, gene symbol Hif1a

ICAM-1	intercellular adhesion molecule 1, gene symbol Icam1
Integrin αv	integrin α v, gene symbol Itgav
Integrin β3	integrin β3, gene symbol Itgb3
ILM	inner limiting membrane
INL	inner nuclear layer
eNOS	endothelial nitric oxide synthase, gene symbol Nos3
iNOS	inducible nitric oxide synthase, gene symbol Nos2
IPL	inner plexiform layer
Kdr	gene symbol for VEGFR2
LDA	low density array
LDM	laser dissection microscopy
LDM_VL	laser dissected tissue of the vascularised retinal layers
LDM_VP	laser dissected tissue of the peripheral superficial vascular plexus area
mOpsinhAng2	transgenic mouse that overexpresses human Ang2 in the photoreceptors via mouse Opsin promotor
MCP-1	monocyte chemotactic protein 1, gene symbol Ccl2
MMP2	matrix metalloproteinase 2, gene symbol Mmp2
MMP9	matrix metallopeptidase 9, gene symbol Mmp9
NFL	nerve fibre layer
NG-2	chondroitin sulfate proteoglycan, gene symbol Vcan
Nos2	gene symbol for iNOS
Nos3	gene symbol for eNOS
Nur77	nur77, gene symbol Nr4a1
Nr4a1	gene symbol for nur77
OIR	oxygen induced retinopathy, identical with ROP model
OPL	outer plexiform layer
p	postnatal day
PAS-H	periodic acid-Schiff's reagent and he-

	matoxylin staining
PBS	phosphate buffered saline
PDGFB	platelet derived growth factor B, gene symbol Pdgfb
PDGFRB	platelet derived growth factor receptor B, gene symbol Pdgfrb
PEDF	pigment epithelium derived factor, gene symbol serpinf1
PECAM-1	platelet/endothelial cell adhesion molecule, gene symbol Pecam-1
Pgf	gene symbol PLGF
PLGF	placental growth factor, gene symbol Pgf
Plat	gene symbol for tPA
Plau	gene symbol for uPA
pNV	preretinal neovascularization
RPE	retinal pigment epithelium
PRL	photoreceptor layer
Ptgs2	gene symbol for cyclooxygenase2, COX-2
Ptprc	gene symbol for protein tyrosine phosphatase, CD45
RL	retinal lysates
ROP	retinopathy of prematurity
SD	standard deviation
S.E.M	standard error of the mean
Serpinf1	gene symbol for pigment epithelium derived growth factor, PEDF
Tek	gene symbol for endothelial tyrosine kinase, Tie2
TGFβ1	transforming growth factor β1, gene symbol Tgfb1
TGFβ1R	transforming growth factor β1, gene symbol Tgfb1r
Thbs1	thrombospondin-1, gene symbol Thbs1
Tie2	endothelial tyrosine kinase, Tie-2, gene symbol Tek
TIMP-1	tissue inhibitor of metalloproteinases 1, gene symbol Timp1

TNFα	tumor necrosis factor α, TNF, gene symbol Tnf
TNF-/-	TNFα knockout mouse
TNFR1	tumor necrosis factor receptor superfamily, member 1A
Tnfrsf1a	gene symbol for TNFR1
TNFR2	tumor necrosis factor receptor superfamily, member 1B
Tnfrsf1b	gene symbol for TNFR2
tPA	tissue plasminogen activator, gene symbol Plat
Tsp-1	thrombospondin-1, gene symbol Thbs1
uPA	urokinase plasminogen activator, gene symbol Plau
VCAM-1	vascular cell adhesion molecule 1, gene symbol Vcam1
Vcan	gene symbol for NG-2
VE-cadherin	vascular endothelium cadherin 5, type 2
VEGFA	vascular endothelial growth factor A, VEGF-A, gene symbol Vegfa
vWF	von Willebrand factor, gene symbol Vwf

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as it is called "re-search".

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Further publications by the author

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Awards

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