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EXPERIMENTAL STUDIES OF CORNEAL NEOVASCULARISATION

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ABSTRACT

The cornea contains no vessels, which is a prerequisite for the maintenance of its transparency. Vessel ingrowth leads to opacification resulting in visual impairment, sometimes complete blindness. This important clinical problem affects patients of all ages and may be due to chronic inflammation, hypoxia, chemical injury, stem cell deficiency, infection, dry eyes, a loss of sensation or transplant rejection. Corneal neovascularisation (NV) may be inhibited by anti-inflammatory treatment but not completely reversed or prevented. Although angiogenic research has translated into the development of new promising drugs, a deeper knowledge of angiogesis is needed to define optimal treatment options and save vision.

The present studies aimed to broaden the understanding of corneal angiogenesis on a molecular, cellular and tissue level. All three studies were based on an experimental mouse model were a transcorneal silk suture causes inflammation and NV. Genetically modified mice lacking functional genes (knockout mice) for matrix metalloproteinase-2 (MMP-2) or interleukin-10 (IL-10) were used in the first two studies. In the third study the cornea model was an *in vivo*-part of an experiment cluster including the chick chorioallantoic membrane model (CAM), cell cultures, *in-vitro* gel-contraction and peptide-inhibitor experiments. The vessel visualization was performed with dye/ink perfusions and immunohystochemical stainings. NV was studied and quantified with a digital microscope with image analysis software, assessing spatiotemporal aspects. Studies of the inflammatory reaction were performed with immunohistochemistry for cellular markers while *in-situ* hybridization and real time-Polymerase Chain Reaction (rt-PCR) were used for identification of mRNA for relevant target molecules.

MMP-2, a member of an enzyme family essential for degradation of extracellular matrix (ECM) and the vascular basement membrane, acted as an angiogenic regulator in this model. NV correlated with increased expression of MMP-2 mRNA and protein, both mainly found in activated keratocytes. In the second study, the anti-inflammatory and angio-regulatory cytokine IL-10 was found to act pro-angiogenically which could be explained neither by its anti-inflammatory effects nor by an apparent cross-talk with other potent angiogenic and immuno-modulatory factors.
The third study revealed that tissue tension generated by activated myofibroblasts during wound contraction was able to mediate and direct the initial vascular invasion of the cornea and CAM-matrix gels through mainly vessel enlargement and elongation, independently of endothelial sprouting or proliferation. Neutralizing antibodies to vascular endothelial growth factor receptor -2 (VEGFR-2) could not prevent vessel ingrowth, while inhibition of gel contractibility did so. The results from this study may contribute to the already established theories on angiogenesis and this novel explanation model has been tentatively named “looping angiogenesis”.

Taken together, these studies show that corneal neovascularisation is dependent on both chemical signals and mechanical factors. Further investigations of the interaction between these mechanisms will hopefully broaden the understanding of the complex and vital biological process that angiongesis represents.

LIST OF PUBLICATIONS

The thesis is based on following original publications that will be referred to by their Roman numerals in the text.

I. Samolov B, Steen B, Seregard S, van der Ploeg I, Montan P, Kvanta A. Delayed inflammation-associated neovascularization in MMP-2-deficient mice. Exp Eye Res. 2005 Feb; 80 (2): 159-166

II. Samolov B, Kvanta A, van der Ploeg I. Delayed neovascularization in inflammation induced corneal neovascularization in IL-10-deficient mice. Acta Ophthalmol. 2008 Nov 25 [Epub ahead of print]

III. Kilarski WW, Samolov B, Petersson L, Kvanta A, Gerwins P. Biomechanical regulation of blood vessel growth during tissue vascularization. Nat Med. 2009 Jun; 15 (6): 657-64

CONTENTS

LIST OF ABBREVIATIONS

ACAID	Anterior chamber associated immune deviation
Ang	Angiopoietin
ANOVA	Analysis of variance, statistics
APC	Antingen-presenting cell
BM	Basement membrane
CAM	Chick chorioallantoic membrane model
C57BL/6J	Laboratory mouse; the most widely used inbred strain, with sequenced genome
cDNA	Complementary or copy DNA (deoxyribonucleic acid)
cRNA	Complementary RNA (ribonucleic acid)
EC	Endothelial cell
ECM	Extracellular matrix
EPC	Endothelial progenitor / precursor cells, angioblasts
bFGF	(basic)Fibroblast growth factor = FGF2
FITC	Fluorescein isothiocyanate, tracing dye used in microscopy
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase, common enzyme
GF	Growth factor
HASMC	Human aortic smooth muscle cells
HMVEC	Human microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
IFN-γ	Interferon gamma, cytokine
IgG	Immunoglobulin G
IL-10	Interleukin 10, cytokine
KO	Knockout mouse, genetically engineered mouse
LYVE-1	Lymphatic endothelial hyaluronan receptor 1
MMP	Matrix metalloproteinases, enzyme family
MT-MMP	Membrane type metalloproteinases, a subgroup of MMP
mRNA	Messenger RNA
NV	Neovascularisation; angiogenesis
PDGF	Platelet derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule 1
PEDF	Pigment epithelium derived factor
PlGF	Placental growth factor
rt-PCR	Real time- polymerase chain reaction
αSMA	Alpha smooth muscle actine
SMC	Smooth muscle cell
Tie (TIE)	Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains, receptors for angiopoietins
TIMPs	Tissue inhibitors of metalloproteinases
VEGF	Vascular endothelial growth factor
VEGFR	Receptor for VEGF
VPF	Vascular permeability factor
WHO	World Health Organisation
WT	Wild type mouse, normal genome mouse

1 INTRODUCTION

1.1 CORNEA

The cornea acts as a positive lens of about 43 diopters constituting the main refractive element of the eye. It can fulfill its chief function, to provide optimal visual acuity and quality, only if it remains transparent.
Anatomically, it is composed of three distinct layers: epithelium, stroma and endothelium. They are separated by two different membranes: the epithelial basement membrane, Bowman's layer, between the epithelium and the stroma and Descemet's membrane between the stroma and the endothelium. This compartment organization is considered to be essential for corneal physiology. Defect regeneration of respective limiting membranes following injury have been demonstrated to be associated with pathological wound healing resulting in a loss of transparency and vision (Matsubara et al 1991, Fini et al. 1992, Stramer et al 2003). The innermost layer, the endothelium, is responsible for the deturgescence, i.e. the relative dehydration, and hence for the transparency of the cornea, but acts also as an immunologic barrier to the aqueous humor in the anterior chamber of the eye.
While richly supplied with sensory nerves, the cornea is devoid of blood vessels. The oxygen is instead supplied by the tear film and the aqueous humor while the glucose is derived from the aqueous humor and the limbal vessels.
The limbus is the border between the transparent cornea and the opaque sclera. It contains the niche for corneal epithelial stem cells, the collector veins which are the pathway for aqueous humor flow and a vascular plexus derived from the ciliary arteries, branches of the ophthalmic artery.

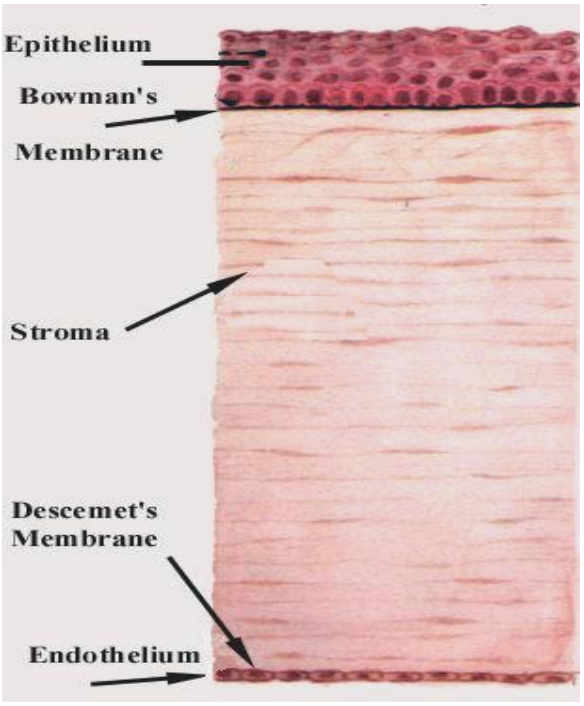

Figure 1. Corneal layers (hematoxylin-eosin staining of cornea section) (reproduced with permission from Dr. T.Caceci, Virginia, USA)

1.2 VASCULAR DEVELOPMENT

All cells are dependent on oxygen and nutrient supply. Due to its limited diffusion capacity, oxygen can reach only 150-200 μm into tissues, a distance that determines vessel density. Tissues also need a transport system for cells with immunological or repairing properties as well as for waste products and dead microorganisms. Blood and lymph vessels serve all these purposes. Lymphatics, though, transport larger molecules and cells than blood capillaries (i.e. dead bacteria and large proteins).
Accordingly, the vessels are needed in the entire body, from embryogenesis, enabling fetal growth and later throughout life. The only parts of the human body lacking vessels are the cornea and the crystalline lens of the eye, cartilage, nails and hair. They are avascular.
The efferent blood vessels come from the hearth. The arteries branch to smaller size units, becoming arterioles and finally capillaries. At the location of the blood-tissue exchange, capillaries build anastomoses with the smallest units of the afferent vessel-tree; the venous component of the capillary network. Venules lead the blood through larger veins back to the heart. The blood vessel phenotype differs with its location in the circulation network. The wall of the vessels is lined by endothelial cells (ECs) and supported by pericytes and smooth muscle cells (SMCs). The walls of veins are fenestrated and thinner, especially the smooth muscle layer, and less elastic than arteries. The walls of tiny capillaries are only one cell thick. The vessel phenotype also varies with the degree of maturation. The pericytes and tight junctions between ECs are more numerous in the mature vessels and the basement membrane is multilayered (Cursiefen et al.2003). The vessel wall properties also change in disordered conditions like inflammation when vascular permeability greatly increases.
Lymph vessels lie adjacent to the blood vessels. Lymphatic capillaries merge into larger truncs and ducts that empty into the venous system. Lymphatic capillaries are like blood capillaries lined by single layer of ECs, but have a poorly developed or even absent BM and lack the sheath of pericytes and SMCs. Larger, collecting lymphatic vessels do have a BM and are supported by SMCs, though (Leak et al. 1976).
Blood and lymph vessels are in a close interaction with the immune system. The blood circulation brings the immune cells to the place of injury or disease while lymphatics provide the access of antigens and antigen-presenting cells (APCs) to regional lymph nodes where they can trigger B- and T-cell activation.

1.2.1 Vasculogenesis

The cardiovascular system is the first organ system to develop during vertebrate embryogenesis. The formation of the first blood capillary plexus during embryogenesis, where no pre-existing vessels are present occurs initially through so called vasculogenesis. This *de novo* vessel formation emerges from endothelial precursor cells (EPCs), also called angioblasts, which differentiate to ECs and form primitive vascular networks. The process is characterized by a balance between pro- and anti-angiogenic factors. These pioneer vessels subsequently remodel, prune and expand by sprouting and intussusceptive angiogenesis (reviewed by Makanya et al. 2005). For a long time it was presumed that vasculogenesis occurred only prenataly. Now we know that vasculogenesis also takes place in adult life, e.g. in tumor growth and hypoxia.
Different pro-angiogenic factors, like vascular endothelial growth factor (VEGF) or

angiopoietin (Ang), can trigger recruitment and differentiation of endothelial progenitor cells, mesoangioblasts and multipotent adult progenitor cells to ECs that will take part in vessel formation at neovascularisation sites (Asahara et al. 1997, Takahashi et al. 1999, Ribatti et al. 2001, Gao et al 2009).

1.2.2 Angiogenesis (sprouting hemangiogenesis)

Angiogenesis is defined as formation of new blood vessels from pre-existing ones (Folkman 1971) and occurs in direct response to tissues metabolic demands (Rise, 1997). Physiological angiogenesis is involved in wound healing and in the menstruation cycle where monthly rebuilt vessels line the uterus. Angiogenesis is also seen in many pathologic conditions including tumor growth, atherosclerosis, rheumatoid arthritis, proliferative retinopathies and corneal neovascularisation. Basically, the same steps are involved in pathologic angiogenesis as in the physiologic one. However, vessels formed in pathologic angiogenesis are often functionally abnormal presenting leakage and defect perfusion, possibly due to an imbalance in the angiogenic process. Multiple factors contribute to angiogenesis, including growth and differentiating factors, extracellular matrix components, membrane-bound receptors and intracellular signal molecules. Several steps are involved; the angiogenic switch, stimulation of ECs through angiogenic growth factor receptors, proteolysis of the basement membrane (BM), proliferation and migration of vascular endothelial cells, proteolysis and invasion of extracellular matrix (ECM) by newly built vessel sprouts, elongation, vessel stabilization, recruitment of pericytes and smooth muscle cells and closing of arteriovenous loops (**Fig. 2**).

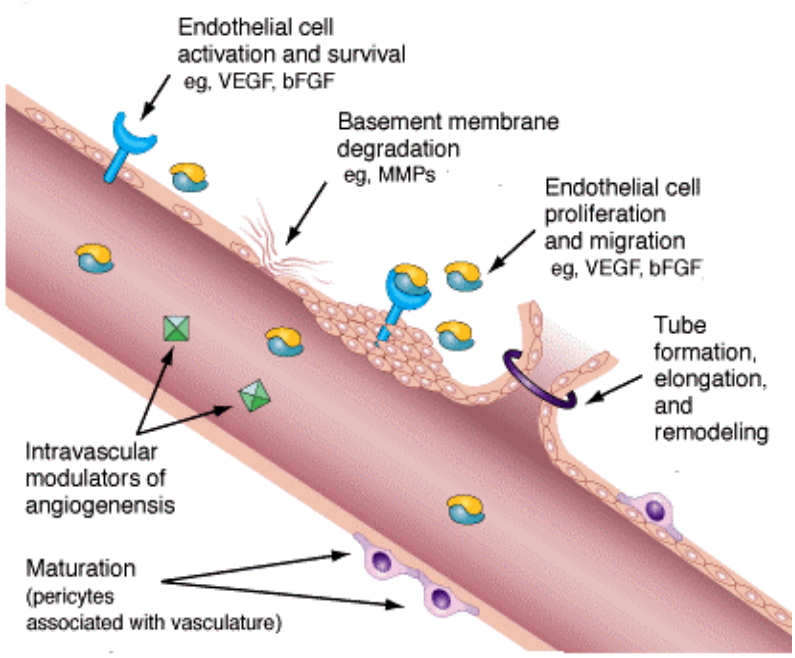

Figure 2. Sprouting angiogenesis, cascade of events

1.2.2.1 The angiogenic switch

Despite the presence of pro-angiogenic factors, EC turnover is normally very low. The maintenance of this endothelial quiescence is thought to be due to the endogenous negative regulators, anti-angiogenic factors. But, if the pro-angiogenic factors dominate, triggered by rising metabolic demands, such as in inflammation or growing tumors, the balance may be shifted and the endothelium activated. This event is called the angiogenic switch and represents the starting point for angiogenesis.

1.2.2.2 Pro- and anti-angiogenic factors

Since the most potent pro-angiogenic factor; vascular endothelial growth factor (VEGF) was discovered, initially named vascular permeability factor (VPF) (Senger et al.1983, Ferara et al. 1989), numerous pro- and anti-angiogenic molecules have been identified. Several of them showed to have the potential to act as both stimulators and inhibitors depending on receptor types, different ligands and the angiogenic context. They can also up- and downregulate each other, on gen, molecular or cellular levels. Many other molecules, primarily engaged in the regulation of other biological pathways, have also the capacity to induce neovascularisation, like various cytokines, e.g. Interleukin-10 (IL-10), more discussed later on, or IL-8 (Li et al. 2003) and hormones, e.g. thyroxine (Berg et al. 2005).

VEGF, also designated as VEGF-A, is the founding member of a family of homodymeric glycoproteins structurally related to another potent proangiogenic growth factor; platelet-derived growth factor (PDGF). This family also includes VEGF-B, -C, -D, -E and placenta growth factor (PlGF). They bind with overlapping specificity to cell-surface receptor tyrosine kinases; **VEGFR**-1, -2 and -3 and co-receptors neuropilins (Nrp). VEGFR-1 and -2 play a crucial role in vasculo- and angiogenesis. VEGF-A binds to both receptors; VEGF-B and PlGF bind to VEGFR-1, while VEGF-E and –C bind to VEGFR-2. VEGFR-3, on the other hand is important in lymphangiogenesis, being a ligand for VEGF-C and –D.
VEGFR-2 seems to mediate most of the VEGF-effects, while VEGFR-1 probably has a modifying function, mostly acting as decoy receptor (Shalaby et al. 1995, Hiratsuka et al 1998). VEGF stimulates migration and proliferation of EPCs (Shalaby et al. 1997, Bautch et al. 2000) as well as proliferation and migration of ECs, capillary tube formation and vascular permeability, all summarized by Klagsbrun et D´Amore 1996. There are six different isoforms of VEGF in humans, generated by alternative splicing, with $VEGF_{165}$ being the predominant one. Their interaction with neuropilins might explain their different potencies despite equal affinity to VEGF-receptors. The outcome of VEGF-VEGFR signaling is also dose dependent.

Another important receptor signaling system, beside VEGF-VEGFR, is the angiopoietin –Tie (tyrosine kinase with immunoglobulin (Ig)-like and epidermal growth factor (EGF) homology domains) -receptor system (**Ang-Tie**). Ang-Tie signaling regulates blood vessel development and also lymphangiogenesis. Its immuno-modulatory ability makes it a link between angiogenesis and inflammation. Ang-Tie is essential in embryonic vessel development and maturation (Dumont et al. 1992, Sato et al. 1993) and has a key function in the regulation of adult vascular homeostasis. It

controls sprouting angiogenesis, vascular remodeling and transformation of ECs from quiescent to an active state. The Tie receptors, TIE-1 and-2, are single transmembrane molecules almost exclusively expressed on ECs and haematopoietic cells. Their ligands are the paracrine angiopoietin1 (Ang-1) and autokrin angiopoietin 2 (Ang-2). Ang-1 acts as stimulator, agonist on TIE-2, while ANG-2 mediates both agonistic and antagonistic effects on TIE-2 (Daly et al. 2006, Fiedler et al. 2006, Reis et al. 2007). On the other hand, in lymphangiogenesis they act redundantly. Ang-1 has anti-inflammatory properties. It inhibits VEGF-induced expression of inflammatory EC-adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) (Kim et al. 2001, Gamble et al. 2000). Ang-2 acts pro-inflammatory, regulating the responsiveness of ECs to inflammation through their sensitization toward tumor necrosis factor alpha (TNF-α) (Fiedler et al. 2006). The role of TIE-1 is still rather unraveled.

Fibroblast growth factor 2 (**FGF-2**), also called basic FGF (bFGF), is another highly pro-angiogenic factor and as such frequently used in experimental studies of angiogenesis, e.g. in cornea models (Kenyon et al. 1996). It is a ubiquitous and pleiotrophic growth factor and a highly potent inducer of DNA-synthesis. As such it also plays an important role during embryogenesis. It is present in the subendothelial basement membrane of blood vessels in nearly all organs (Cordon-Cardo et al. 1990). During wound healing and tumor growth it becomes active and upregulated. FGF-2 binds to cell surface associated heparan-sulfate proteoglycans and its signal transduction is mediated through binding to tyrosine kinase FGF-receptors (Bikfavi et al 1989). Many studies indicate an intimate cross-talk between FGF-2 and VEGF during both vasculo-/angiogenesis and lymphangiogenesis. Pro-angiogenic events in ECs triggered by FGFs, particularly FGF-2, are basal lamina degradation, proliferation, morphogenesis and vessel maturation (reviewed by Presta et al, 2005).

1.2.2.3 Proteolysis of basement membrane and extracellular matrix

Proteolytic breakdown of vascular basement membrane and the surrounding ECM are essential events prior to outgrowth of a new vessel branch. Matrix metalloproteinases, MMPs, a family of zinc-depended endopeptidases have a crucial role in this enzymatic activity. MMP-2, -9, both represented in cornea, and MT1-MMP (se below) have been particularly implicated.
MMPs include 28 presently known members, structurally divided into two subgroups; secreted MMPs and membrane-type MMPs (MT-MMPs). Secreted MMPs are divided in to collagenases; gelatinases, such as gelatinase A also called MMP-2 and gelatinase B, or MMP-9; stromelysins and elastases. Most of them are synthetized as inactive precursors, zymogens, requiring processing in order to become active, often by proteolysis. ECs are able to synthesize a majority of MMPs and it has been shown that also EPCs express several members, e.g. MMP-2 and -9 (Yoon et al. 2005). Angiogenic growth factors and inflammatory cytokines can induce MMPs and at the same time MMPs are able to activate pro-angiogenic molecules such as VEGF by releasing them from proteoglycans of the ECM (Houch et al. 1992, Hashimoto et al. 2002).

MMPs can have ambiguous functions in angiogenesis. For example, the otherwise proangiogenic MMP-2 can also release anti-angiogenic fragments (Brooks PC et al. 1998) and allow production of potent angiostatic factors (O´Rilley et al. 1999). There is a strong redundancy between the MMP family members. Their activity is regulated and balanced by the endogenous inhibitors; tissue inhibitors of matrix metalloproteinases (TIMPs), produced e.g. by pericytes and ECs. The balance between proteolysis and proteinase inhibition on the one hand and between pro- and antiangiogenic factors on the other hand is a key element in the regulation of angiogenesis.

1.2.3 Intussusception

Intussusception, also known as splitting angiogenesis is a mode of blood vessel formation where a pre-existing vessel is divided into two new vessels by invagination of the vessel walls, resulting in a transluminal tissue bridge, called “pillar”. The pillar is composed of two new vessel wall segments that will enclose two new lumina. (Burri et Tarek 1990, Caduff et al 1986). Pericytes and myofibroblasts invade the pillars providing collagen for the ECM that supports new vessel walls (Burri et Djonov 2003). Intussusception has been described during organogenesis and in pathologic conditions in adult life, such as in tumours (Caduff et al. 1986, Djonov et al. 2001, Hlushchuk et al. 2008, Makanyan et al. 2005, 2007 and 2009). This reorganisation of existing vascular cells allows a vast increase in the number of capillaries without a corresponding increase in the number of ECs. In comparison to sprouting angiogenesis, it results in faster neovascularisation at less metabolic expense (Djonov et al.2000). Regulatory mechanisms of intussusception are not fully understood, but it is has been suggested that shear stress might be the initial stimulus (van Royen et al.2001) leading to uppregulated expression of adhesion molecules and proangiogenic factors by ECs (Gimbrone et al.1997).

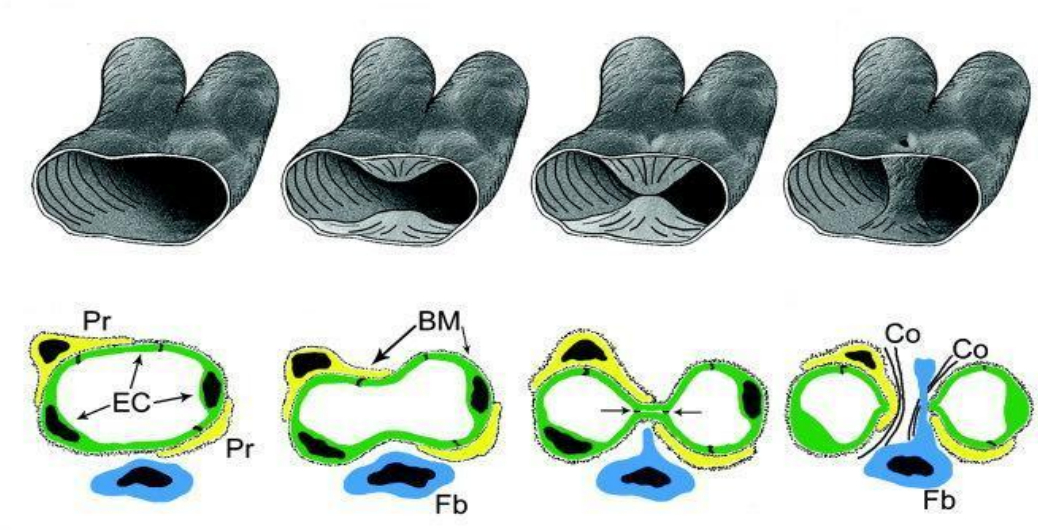

Figure 3. Intussusception, schematic illustration of the events. EC= endothelial cell, BM= basement membrane, Co= collagen, Fb= fibroblast, Pr= pericyte (reproduced from Kurz et al. 2003, with permission)

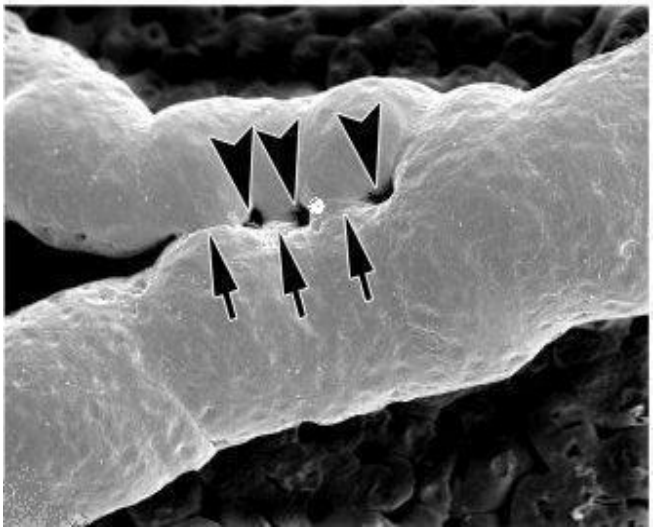

Figure 4. A scanning electron micrograph of intussusception in a CAM-vessel. Transcapillary tissue pillars in a row (arrow heads) and their fusion resulting in walls of two new vessels (arrows) (reproduced from Burri et al. 2004, with permission)

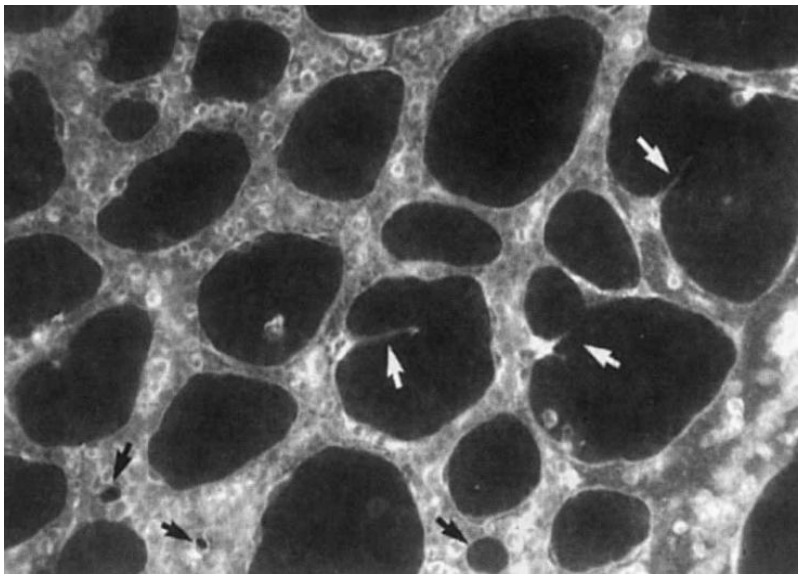

Figure 5. Sprouting and intussuseptive angiogenesis (white arrows: sprouts, black arrows: transcapillary pillars) (reproduced from Risau 1997, with permission)

1.2.4 Lymphangiogenesis

Similarly to blood vessels, the lymphatic vasculature in adults is quiescent but can be activated in wound healing, tumor growth and inflammation. Lymphangiogenesis is a process where new lymphatic vessels grow from pre-existing ones, primarily by sprouting. In addition, during pathological lymphangiongenesis hematopoietic cell-derived circulating endothelial progenitor cells and trans-differentiated macrophages can also serve as a source of lymphatic endothelial cells (LECs) (Maruyama et al. 2005, Religa et al. 2005). Two prime lymphangiogenic factors are VEGF-C and –D, acting

through VEGFR-3 (Kaipainen et al.1995, Achen et al. 1998, Joukov et al.1996, Mäkinen et al.2001). VEGF-C also binds to Nrp-2. Its importance in lymphangiogenesis was highlighted in a study where Nrp-2-defficient mice presented lymphatic hypoplasia (Yuan et al. 2002). VEGF-C is essential in embryonal lymphangiogenesis as homozygous deletion of this gene leads to a complete absence of lymphatics in a mouse embryo (Karkkainen et al. 2004). Cursiefen et al. have shown that even VEGF-A can promote lymphangiogenesis in a mouse model of inflammatory corneal neovascularisation (Cursiefen et al. 2004). Other lymphangiogenic factors known are fibroblast growth factor 2 (FGF-2), platelet-derived growth factor B and hepatocyte growth factor (Kubo et al. 2002, Cao R. et al. 2004, Kajiya et al. 2005). On the gene level, more regulatory factors have proved to be of importance, like Prox1 which is expressed on EC of the jugular vein during embryology (Wigle et al. 1999). These ECs sprout and migrate to form lymphatic vessels while deletion of Prox1 leads to a complete absence of lymphatic vasculature (Wigle et al. 2002).

Figure 6. Corneal neovascularisation, starting from limbal vessels at the bottom of the picture. CD31staining representing blood vessels (blue), LYVE-1staining representing lymphatic vessels (red) (author's picture)

1.3 ANGIOGENIC AND IMMUNOLOGIC PRIVILEGE OF THE CORNEA

Maintenance of corneal avascularity is an active process where the production of anti-aniogenic factors counterbalances the pro-angiogenic factors that are upregulated i.e. in wound healing (Azar, 2006, Cursiefen et al. 2006 and 2007, Ambati et al. 2006, Dana 2006). There are numerous anti-angiogenic factors that block corneal NV:
a) endogenous inhibitors of angiogenesis, like trombospondin 1 (TSP-1) (Cursiefen et al. 2004b), pigment epithelium derived factor (PEDF) (Dawson et al. 1999, Karakousis et al. 2001), angiostatin (Shin et al. 2000) and endotstatin (Vazquez et al.1999),
b) receptor decoys like soluble VEGFR-1 (sFlt-1) found in the aqueous humor and corneal epithelium (Lai et al. 2001, Ambati et al. 2006), ectopically expressed VEGFR-

3 in corneal epithelium (Cursiefen et al. 2006), and heparan sulfat binding to fibroblast growth factor (FGF) (Fannon et al. 2003). The cornea also expresses inhibitory PAS domain protein (IPAS), an inhibitor of hypoxia-driven VEGF-upregulation (Makino et al. 2001). Corneal avascularity due to this equilibrium between different angioregulatory factors has been termed "angiogenic privilege".

The absence of blood and lymphatic vessels in the cornea also contributes to its immunologic privilege, explaining the relatively low rejection rate of cornea allografts. Other mechanisms also play a role, like the reduced level of antigen-presenting cells (APCs) and the anterior chamber associated immune deviation (ACAID), which is the theorised capacity to actively suppress induction of delayed-type hypersensitivity to intracameral antigens (Medawar, 1940, Dana and Streilein 1996). Accordingly, vessel ingrowth in the cornea threatens to abrogate its immunologic privilege, and conversely, disturbed immunologic privilege jeopardizes the angiogenic privilege of the cornea. In other words these privileges are not absolute and they are interdependent. Reflecting these phenomena, many angiogenic factors have an immunomodulatory role and vice versa. For example. VEGF-A can act as a chemoattractant for macrophages through its VEGFR-1 (Cursiefen et al. 2004a) and VEGF-C can recruit dendritic cells through VEGFR-3 (Chen et al. 2004), interleukin-8 (IL-8) can act pro-angiogenically (Li et al. 2003a, Mizukami et al. 2005) and anti-inflammatory interleukin-10 (IL-10) can both stimulate (Apte et al. 2006, Samolov et al. 2008) and inhibit angiogenesis (Silvestre et al. 2000 and 2001, Cole et al. 2003). Macrophages are an important source of VEGF-A, -C and –D, and their local depletion can prevent hem- and lymphangiogenesis (Cursiefen et al. 2004a). Blocking antibodies to VEGFR-3 can mediate dendritic cell migration to regional lymph nodes and improve corneal graft survival (Chen et al. 2004). The interaction between these two systems is complex and many important factors show redundancy, explaining why there still is not a therapy that completely prevents or abolishes neovascularisation in the cornea.

1.4 CORNEAL NEOVASCULARISATION – CLINICAL ASPECTS

Transparency of the cornea, the prerequisite for good vision is threatened by three major pathological events; 1. dysfunction of corneal endothelial cells and their pump function, resulting in oedema, 2. inflammation with subsequent scarring, 3. neovascularisation with leakage of inflammatory cells, erythrocytes and fluid into the tissue (Cursiefen et al. 2006, Chang et al. 2001). Endothelial failure is usually due to degeneration. It might represent a complication to ocular surgery, but it can also be caused by inflammation and neovascularisation. Endothelial dysfunction is not therapeutically reversible but the damaged endothelium can be successfully replaced with a healthy transplant restoring vision. Chronic inflammation and neovascularisation may lead to severely impaired vision and represents a therapeutic challenge in everyday clinical work.

Trachoma, corneal neovascularisation and scarring due to chronic conjunctival infection with Chlamydia trachomatis is the leading cause of preventable blindness in the world. In 2003 World Health Organization (WHO) estimated that 84 million people had active, sight threatening trachoma, and that at least 7,6 million of these suffer from

severe visual loss. This deleterious infection is rare in the developed part of the world where herpetic disease is the leading cause of infectious keratitis.
The angiogenic stimuli in the cornea can be divided into inflammatory, infectious, degenerative, hereditary, traumatic disorders and hypoxia (**Table 1**). They usually overlap and more than one stimulus is often responsible for majority of the given clinical conditions below. Corneal neovascularisation is a particularly serious problem in corneal grafts as transplantation often represents the last treatment option for an otherwise severely diseased cornea.

Table 1. List of angiogenic stimuli in cornea, with examples

INFLAMMATORY DISORDERS
Atopic and vernal keratoconjunctivitis
Rosacea
Ocular pemphigoid
Stevens Johnson syndrome
Graft versus host disease (GVHD)
Corneal graft rejection
INFECTIOUS KERATITIS
Virus (Herpes simplex and zoster)
Bacteria (Pseudomonas aeruginosa, Chlamydia trachomatis, Syphilis)
Fungi (Candida, Fusarium, Aspergillus)
Parasites (Acanthamoeba, Onchocerciasis)
DEGENERATIVE DISORDERS
Pterygium
Terrien marginal degeneration
HEREDITARY DISORDERS AND STEM CELL DEFICIENCY
Aniridia
Meretoja´s syndrome
Chemical burns
TRAUMA, EXPOSURE AND HYPOXIA
Traumatic ulceration
Lagophtalmus (endocrine ophthalmopathy, n.VII-palsy)
Contact lens wear

Vessels invade the cornea in three different patterns: as vascular pannus, e.g. pterygium, as superficial neovascularisation and as deep stromal neovascularisation. Superficial vessels grow just beneath the corneal epithelium and are usually the result of mild chemical injury, trauma, inflammation or infection. Deep vessels grow beneath Bowman layer at any stromal depth. This kind of neovascularisation is usually inflicted by severe chemical burns and injuries or pronounced inflammation and infection (e.g. alkali burns, Stevens-Johnson syndrome, stromal herpes) and it usually results in pronounced visual disability.

Topical steroids have been and still remain the main treatment against corneal neovascularisation. Cortisone is able to suppress corneal neovessels to some extent, but cannot completely reverse or prevent them. The risk for sight threatening side effects (glaucoma, cataract) is also to be taken into account with this treatment. Cyclosporine

A is an alternative option, but not as effective and with an intense burning sensation accompanying administration. Case reports with laser induced occlusion of corneal neovessels, e.g. argon laser, photodynamic therapy (PDT) with Verteporfin, neodymium-doped yttrium aluminium garnet (Nd:YAG), indicate a role for these methods in the armamentarium, but their true effect in the long run remains to be demonstrated. In recent years, we have seen a growing number of case reports and small clinical studies demonstrating the benefit of off-label use of anti-angiogenic drugs in corneal neovascularisation. Bevacizumab, a monoclonal antibody against VEGF, initially approved for the treatment of colon cancer, has shown promising antiangiogenic effects also in corneal neovascularisation, when administered either topically as drops (Boch et al. 2008, Uy et al. 2008, Dastjerdi et al. 2009) or as a subconjunctival injection (Bahar et al. 2008, Oh et al. 2009, Zaki et al. 2009). The use of antisense oligonucleotide eye drops against the proangiogenic insulin receptor 1 (IRS-1) is tested in a European multicenter study.
Prospective randomized studies with more patients and longer follow-up time are necessary to define indications, contraindications, efficacy and safety of these and other emerging drugs.

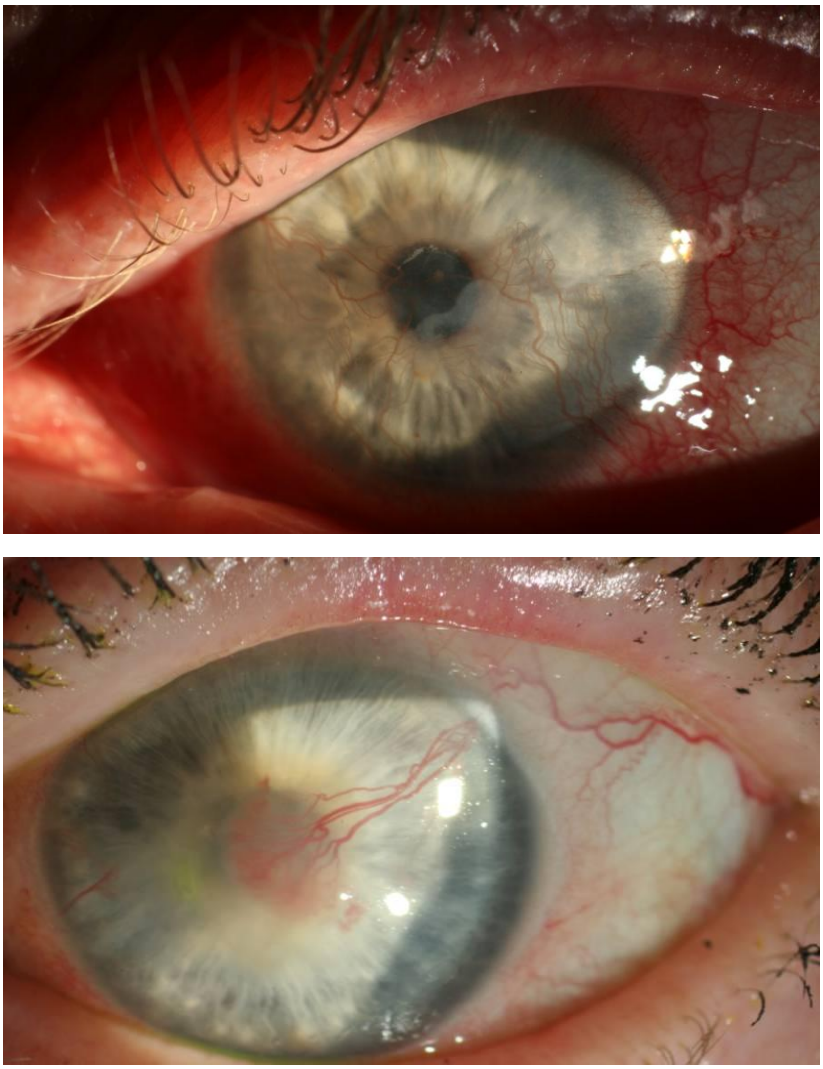

Figure 7. Corneal neovascularisation in atopic keratokonjuncitivits after secondary Candida infection (above) and in stromal keratitis caused by Herpes simplex virus (below) (author's pictures)

1.5 WOUND HEALING

Tissue repair, or wound healing, starts directly after the loss of tissue integrity and continuity that can be caused by chemical or physical tissue damage, infection, cancer, ischemia or an immunologic response. The process involves a complex pathway of both physical and chemical events, consisting of four overlapping phases: haemostasis, inflammation, proliferation and remodelling (Diegelman et Evans 2004).
As injury interrupts vascular bed the platelets come into contact with exposed collagen and other elements of the ECM. This contact triggers the platelets to release coagulation factors, growth factors and cytokines including platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-ß). Following formation of a fibrin clot haemostasis is achieved. The neutrophils then enter the wound and phagocyte damaged tissue and foreign materials, such as bacteria. Macorphages also take part in this inflammatory phase, continuing the process of phagocytosis as well as releasing more PDGF and TGFß. Once the wound site is cleaned, fibroblasts migrate in to begin the proliferative phase and deposit new extracellular matrix. During the final, remodelling phase the new collagen matrix becomes cross-linked and organized and the wound contracts (summarized by Martin 1997, Diegelman et Evans, 2004, Shaw et Martin 2009). This well-orchestrated cascade of events is an efficient repair process, apparently controlled by numerous molecular and cell-signaling steps.

Activated and proliferating fibroblasts, (proto)myofibroblasts, are one of the key cells in wound healing. They are induced by tensile stress and contract the wound matrix through a stress fiber-rich contractile apparatus (Tomasek et al. 2002). In response to tensile stress, extra domain A (ED-A) fibronectin and macrophage-derived growth factors, such as TGFß, cause protomyofibroblasts to differentiate into highly contractile myofibroblasts that express α smooth muscle actin (αSMA)(Serini et al. 1998). Interconnected by gap junctions, myofibroblasts both secrete ECM-components and contract the wound by transmitting tension across intracellular actin stress fibers connected to the ECM (Singer et al. 1999, Gabbiani et al. 1976).

Restoring blood flow to the wound area is a prerequisite for a successful repair response. Angiogenesis is therefore an essential component of wound healing. Neovessels are necessary for delivery of oxygen, nutrients and inflammatory cells to the site of injury. They also assist in debris removal and are an integral part of the granulation tissue that closes the wound. Angiogenesis, during wound healing, is initiated immediately after injury and peaks during the proliferating phase. Vessel formation in wound healing has traditionally been explained by sprouting (reviewed by Tonnesen et al. 2000, Li et al. 2003b) or intussusceptiv angiogenesis (Patan et al. 2001, Pettersson et al. 2000, Burri et Djonov 2002). Additionally, several studies describe the contributing role of EPCs (Asahara et al. 1997, Takahashi et al. 1999, Rafii et al. 2002, Majka et al. 2003), implying the role of vasculogenesis in wound healing. The role of myofibroblasts and the effect of mechanical forces on wound closure have been thoroughly described but their implication in the (neo)vascularisation of the wound has not been proposed until now (*Paper III*).

2 AIMS OF THIS PROJECT

To develop an experimental model of inflammation-associated corneal neovascularisation that is reproducible and clinically relevant

To study different ways of visualization of corneal neovessels

To study the expression and role of the MMP-2 in this model

To study the role of the anti-inflammatory cytokine IL-10 in this model

To study the mode of vascular development and the role of biomechanical forces in this model

To study if VEGF is essential for corneal neovascularisation in this model

3 MATERIAL AND METHODS

3.1 LABORATORY MICE, KNOCKOUT MICE

Paper I: MMP-2-deficient, or knockout (KO) mice: the primary strain of this mouse was generously provided by Professor Shigeyoshi Itohara, RIKEN Brain Science Institutet, Saitama, Japan.
The generation of MMP-2 KO mice has previously been described (Itoh et al. 1998). Briefly, 8-9 weeks old homozygous wild type (WT) and MMP-2 KO mice of both genders, with a mixed genetic background of 97% C57BL/6J and 3% 129 -strain, were used throughout this study. To examine the genotype of each mouse, polymerase chain reaction analysis (PCR) was performed on deoxyribonucleic acid (DNA) extracted from the tail, using oligonucleotide primers designed to detect the Neomycin cassette and the MMP-2 locus.

Paper II: IL-10 KO mice: homozygous IL-10-deficient mice with a C57BL/6/ and129/Ola background, backcrossed to C57BL/6J for 10 generations were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) with authorization from Werner Müller, Institut für Genetik der Universität zu Köln, Cologne, Germany. Control, C57BL/6J-mice (WT) were obtained from Charles River (Sulzfeldt, Germany).
The generation of IL-10 KO mice has previously been described (Kühn et al. 1993). 8-9 weeks old homozygous IL-10 KO mice and WT of either sex were used throughout the study. Genotyping with genomic tail DNA was performed using the PCR with primers and protocol recommended by The Jackson Laboratory.

Paper III: 8-9 weeks old C57BL/6J mice of both genders were used throughout the study.

All the animals were treated in accordance with the Association for Research in Vision and Ophthalmology's (ARVO`s) Statement for the Use of Animals in Ophthalmic and Vision Research.

3.2 EXPERIMENTAL MODEL OF CORNEAL ANGIOGENESIS

Paper I, II, III: This experimental model for corneal neovascularisation in mice is a modification of suture models previously described by others (Suzuki et al. 2000, Stuart et al. 2003). A single, penetrating 8-0 silk suture was put through the centre of the mouse cornea resulting in inflammatory reaction and vessel ingrowth. The first signs of neovascularisation in this model, i.e. dilatation and looping of limbal vessels, emptying of limbal capillary bed, sprouting, appear 2-3 days after suturing and the cornea becomes fully vascularised by day 12-14.

3.3 VISUALISATION AND QUANTIFICATION OF CORNEAL NEOVESSELS

The vessels were visualized with immunohistochemistry (*Paper I, II, III*) (see separate chapter), or by intracardiac injection of: a) fluorescein-isothiocyanate (FITC) -dextran (*Paper I, II*); b) FITC -labeled Lectin I (*Paper III*); c) ink.
After sacrifice, the corneas were flat-mounted in order to quantify perfusion-stained neovessels [a), b)], or in order to immunostain them. Alternatively, ink-perfused whole bulbs were fixated in zinc (Zn)-fixative and then treated with a diaminobenzidin (DAB)-solution that permanently stains erythrocytes giving them a red/brown colour that persists during tissue- clarifying treatment with benzyl benzoate/benzyl alcohol (BBBA) [c)]. This staining protocol allowed discrimination of actively perfused vessels, black vessels, from those with low or no perfusion, red/brown vessels, in preserved whole eye bulbs.

Quantification of the neovascularization was done by a) calculation of the neovascularized area (*Paper I, II, III*) and b) measuring the length of the neovessels (*Paper III*), using a digital microscope and image analysis software. The neovascularised area was defined as the percentage of the total corneal flat mount area that was covered with vessels. The innermost limbal vessel was used as the outer border.

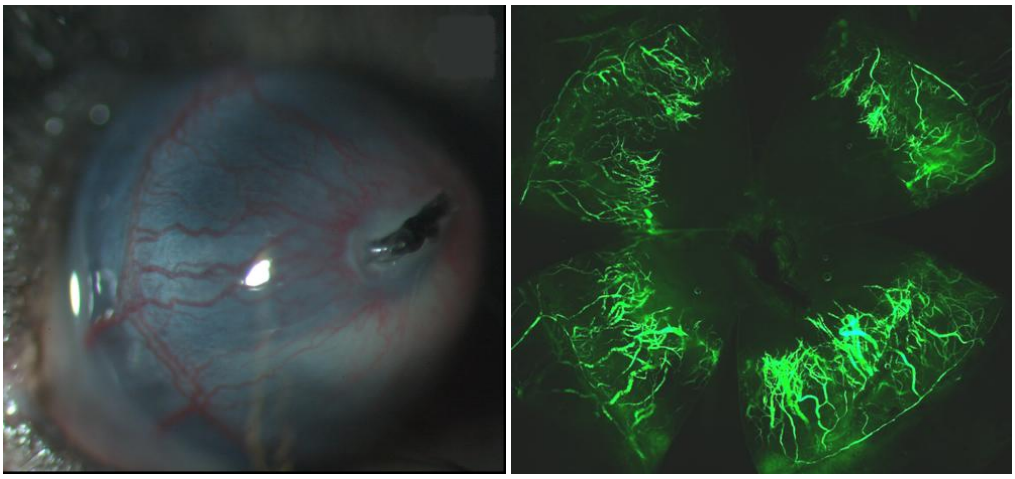

Figure 8. Eye of a mouse with a centrally placed silk suture and neovessels (to the left). Corneal flat-mount with fluorescein perfused neovessels (to the right) (author's picture)

3.4 NEUTRALIZING ANTIBODIES

Paper III: A monoclonal rat anti-mouse VEGFR-2 antibody; DC101, was administered intra-peritonealy (i.p.) at the dose of 1 mg/mouse, which is a dose that has previously been shown to normalize vessels and have antitumor effects in vivo. The control group received 1 mg i.p. of an irrelevant rat IgG1 control antibody against the Horseradish Peroxidase (HRPN). Antibodies were given before corneal suturing (day 0) and repeated every second day (day 2 and 4).

3.5 *IN-SITU* HYBRIDISATION

Paper I: In situ hybridization was performed on 4μm thick serial sagital sections adjacent to the corneal suture. Analyses were done on day 0 (the day of suturing), 3, 6 and 12 days after suturing. The sections were formaldehyde-fixed and T3 and T7 ribonucleic acid (RNA) polymerase-derived sense and anti-sense complementary RNA (cRNA) probes [generated from a rat complementary deoxyribonucleic acid (cDNA) probe to MMP-2], labeled with sulfur-35 –uridine triphosphate (^{35}S-UTP) were used, as previously described (Kvanta et al. 2000). Briefly, the sections were placed on poly-L-lysine coated slides, dewaxed in xylene and after rehydration, incubated in HCl and then treated with proteinase K and glycine. After washing in phosphate buffered saline (PBS), sections were fixed in 4% paraformaldehyde in PBS, acetylated in acetic anhydride with triethanolamine and dehydrated in graded ethanol. Antisense and sense riboprobes were then added to the hybridization buffer. The mixture was heated to 65ºC for 5 minutes and 50 μl was added to each section, whereafter the sections were incubated in a humidified chamber at 50ºC for 16 hours. Sections were then washed four times at 20ºC in saline-sodium citrate (SSC) and dithiothreitol (DTT), rinsed in RNAse buffer and then repeatedly washed in SSC and DTT. After a final wash at 20ºC in SSC plus DTT, the sections were dehydrated in graded ethanol, air dried, dipped in photo- emulsion and exposed for 7 weeks at 4ºC. After development, the sections were counterstained with Mayer's hematoxylin and studied with light and dark-field microscopy.

3.6 REAL TIME- POLYMERASE CHAIN REACTION (RT-PCR)

Paper II: Excised corneas were immediately submerged in RNAlater and stored for a maximum of 4 weeks at 4°C. Total RNA was extracted from each individual specimen with the RNeasy mini kit, essentially according to the manufacturer's instructions for tissue with abundance of collagen. DTT was used instead of β-mercaptoethanol and disruption was performed by grinding with mortar and pestle, followed by homogenization with syringe and needle. The RNA quality in each cornea was determined by measuring the 260 nm/280 nm ratios in the samples. Equal amounts of RNA were reversely transcribed to cDNA with the iScript cDNA synthesis kit after Amplification Grade DNase I treatment of the RNA samples. rt-PCR was performed using the My iQ single-colour rt-PCR detection system using the Bio-Rad iQ SYBR Green method. Primers were used both for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and for the genes of interest; MMP-2, MMP-9, VEGF, Ang-1 and Ang-2 (the primers were previously described by Kato et al. 2001; Luo et al. 2004; Brafman et al. 2004; Pomyje et al. 2001). Melting curves were performed after initial optimization of PCR experiments and PCR products were checked on agarose gels. The relative ratios between the genes of interest and GAPDH messenger RNA (mRNA) were determined at each time-point of the rt-PCR experiments. To optimize the specificity of the rt-PCR for IL-10, a fluorescence resonance energy transfer (FRET)-probe for mouse IL-10 cDNA was used.

3.7 IMMUNOHISTOCHEMISTRY

Paper I: Cryosections were used for staining of vascular ECs with monoclonal anti-mouse antibody against CD31 [platelet endothelial cell adhesion molecule-1 (PECAM-1)]. Formaldehyde fixed paraffin embedded serial sections were stained with antibodies against neutrophils (rat anti-mouse neutrophil, clone 7/4), macrophages (monoclonal rat anti-mouse macrophage, F4/80), activated keratocytes (rabbit polyclonal anti-S100A4), MMP-2 (a sheep monoclonal antibody against human MMP-2)(Hipps et al. 1991) and MT1-MMP (two different polyclonal goat antisera against the N-or C-terminal of human MT1-MMP). The sections were subsequently incubated with biotinylated secondary antibody, and then with an avidin-horseradish peroxidase complex. Peroxidase activity was visualized using 3-amino-9 ethyl carbazole as a red chormogen. Finally the sections were counterstained with haematoxylin and mounted with glycerol-gelatine. All procedures were performed at room temperature.

Paper II: Serial, "optimal cutting temperature compound" (OCT) -embedded, 7 μm thick cryosections were stained with antibodies against neutrophils, macrophages, activated keratocytes (antibodies as specified above for *Paper I*) and T-lymphocytes (Rabbit anti-CD3). Endogenous tissue peroxidase was blocked prior to the staining by incubation in H_2O_2. 1% normal serum, obtained from the animal species in which the secondary antibody was produced, was used as the blocking agent. Biotinylated secondary antibodies and VECTASTAIN Elite ABC kit was used as detection system. The colour was developed with the AEC-substrate kit according to the manufacturer's instructions. The sections were counterstained with Mayer's haematoxylin and mounted with glycerol–gelatine. The number of stained cells was counted manually in masked sections by one of the investigators at a magnification of × 400 and statistically analysed.

Paper III: a) Enucleated eyes were fixed in Zn-fixative, dehydrated and embedded in paraffin. For Bromodeoxyuridine (BrdU) and "Proliferating cell nuclear antigen" (PCNA) stainings, slides were treated for 30 min with HCl. Five μm thick sections were deparaffinized and blocked in NEL blocking buffer. Slides were probed with antibodies raised against CD31, αSMA, von Willebrand factor (vWf), α-laminin, cytokeratin (specific for 1, 5, 10 and 14 keratins), mouse CD45, laminin, BrdU (where indicated mice received 150 μl of 1mg/ml BrdU i.p. 2 hours before they were sacrificed) and PCNA or with biotinylated Sambucus nigra (SN-1) lectin. This was followed by incubation with appropriate biotinylated secondary antibodies and a streptavidin-peroxidase complex. Immunocomplexes were detected after a 5 min incubation with ammonium nickel sulphate, DAB, H_2O_2 in potassium acetate. Masson trichrome and hematoxylin-eosin stainings were performed according to standard protocols. PCNA positive nuclei associated with luminal structures were counted at a 40x magnification. The chick chorioallantoic membrane model (CAM)-implants were stained using the same procedure.
b) Whole-mount staining of cornea. The lumens of perfused vessels were stained by an intracardiac injection of FITC-labeled Lectin I that circulated for 3-5 min before the eyes were enucleated and then immediately fixed in 4% buffered formalin overnight. Flat mounts of corneas were prepared and washed in tris buffered saline (TBS)/Tween followed by successive overnight incubations with antibodies against vascular ECs (primary rat anti-mouse CD31), lymphatic ECs [rabbit anti-mouse lymphatic endothelial hyaluronan receptor 1 (LYVE1)] or pericytes [(rabbit anti-mouse

nerve/glial antigen 2 (NG2)]. Samples were washed in TBS/Tween and then incubated with secondary antibodies together with the nuclear stain Hoechst 33342 in NEL blocking buffer in TBS.

3.8 CHICK CHORIOALLANTOIC MEMBRANE MODEL (CAM) OF MATRIX VASCULARISATION

Paper III: The assay was performed essentially as described by Kilarski et al. 2003. A window in the shell of a white Leghorn egg was cut on day 0 of embryo incubation and eggs were then incubated in a humidified atmosphere at 38°C. A 0.5 cm high plastic tube was glued on a nylon mesh. A provisional matrix was formed by adding 100 µl of a solution composed of fibrinogen and rat tail collagen I. After 10 minutes of collagen polymerization at 37°C, thrombin dissolved in clotting buffer ($CaCl_2$ and $MgCl_2$), amphotericin B (Fungizone)/ penicillin/ streptomycin (FPEST), bovine serum albumin (BSA), FGF-2, aprotinin and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was overlaid on the collagen/fibrinogen gel. The solution was immediately covered with plant oil and allowed to polymerize overnight at 37°C in a humidified atmosphere. A single construct was placed on the CAM at day 12 of embryo development and eggs were incubated for another 6 days before the entire egg was fixed in 2×concentrated Zn-fixative. Constructs were then cut out from the CAM and the plastic tube removed leaving the fibrin/collagen gel, ingrown tissue with vessels, and underlying pre-existing CAM attached to the nylon mesh.
To visualize all blood-containing structures the tissues were treated with a DAB-solution that permanently stains erythrocytes and gives them a red/brown colour that persists during treatment with BBBA that is used to clarify tissues. This staining protocol allowed discrimination between actively perfused vessels (black vessels) and those with low or no perfusion (red/brown vessels).

3.9 GEL MATRICES WITH REDUCED CELL- DEPENDENT CONTRACTIBILITY AND MYOFIBROBLAST POPULATED SPONGES

Paper III: For Vitrogen based gels, rat-tail collagen was replaced with Vitrogen (pepsin-solubized collagen type I) or PureCol collagen. Borosilicate glass fibre (GF) reinforced matrices were made by adding GFs to the fibrin/collagen solution immediately before the gel solutions were poured into the constructs. GFs were prepared by rubbing isolation glass wool through a nylon grid. GFs were then washed in ethanol and water, sterilized in 70% ethanol and dried.
Chemically crosslinked collagen–fibrin matrices were prepared as previously described (Lee et al. 2001). Briefly, immediately prior to use, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) were dissolved in 2-(N-morpholino)ethanesulfonic acid (MES) buffer. Constructs with pre-polymerized gels were incubated for 2 hours in this reaction buffer at room temperature. Control gels were incubated in MES buffer without EDAC and NHS. The gels were then washed in PBS, rinsed with sterile water and covered with new clotting buffer with FGF-2 and plant oil.
To differentiate the provisional matrix from the ingrown tissue on the CAM a non-diffusible ink-stain was added to the fibrin-collagen solution of the implants. Ingrown

vascular tissue with the remaining ink-containing gel was then stained with the DAB-solution and clarified in BBBA as previously described.

Primary chicken myofibroblasts were isolated as previously described (Kilarski et al. 2005). Cylindrical sterile polyvinyl alcohol sponges were cut into cylinders and placed on the grid at the bottom of empty sterile gel chambers in cell dishes. Cultured myofibroblasts were trypsinized, spun down and diluted in fetal bovine serum (FBS) with addition of gelatine. The cell suspension was added to each chamber and incubated at 37°C overnight covered with Ham-F12 medium with FBS and FPEST. Before application of constructs to the CAM, serum with FGF-2 was added to each chamber. Control sponges were covered with FBS only.

3.10 INHIBITION OF A-SMA BY CELL PENETRATING PEPTIDES

The NH2-terminal sequence of αSMA (Ac-EEED) was delivered to the implanted matrix on the CAM in the form of fusion peptides with cell penetrating sequences from antennapedia (Ant) or the human immunodeficiency virus (HIV) transactivator of transcription (Tat), as previously described (Hinz et al. 2002, Lindgren et al. 2000). The corresponding sequence Ac-DEDE from skeletal muscle actin (SKA) was used as a control. The following peptides were used: Tat-SMA (Ac-EEEDRKKRRQRRR-NH2, Tat-SKA (Ac-DEDERKKRRQRRR-NH2), Ant-SMA (Ac-EEEDRQIKIWFQNRRMKWKK-NH2) and Ant-SKA (Ac-DEDERQIKIWFQNRRMKWKK-NH2). Peptides were dissolved in sterile H_2O and added to FGF-2 stimulated constructs. TAT-SKA/TAT-SMA and Ant-SKA/Ant-SMA were added daily during 6 days of incubation.

3.11 TIME-LAPS RECORDINGS

Paper III: The plastic tube was carefully removed from a CAM implant after 6 days of incubation. Pancuronium bromide was poured on the CAM to inhibit skeletal muscle-dependent embryo movements. The opening in the shell was covered with a glass cover slip and the selected area with ingrowing neovessels was photographed every 2 minutes for 26 hours through a microscope equipped with a digital camera.

3.12 MATRIX CONTRACTIBILITY ASSAY

Paper III: Modified matrices were prepared to determine the ability of different cells to adhere and spread on rigid, crosslinked and borosilicate glass fibre-containing gels. 24 well plates were seeded with primary cultures of human umbilical vein endothelial cells (HUVEC), human microvascular endothelial cells (HMVEC), human aortic smooth muscle cells (HASMC) or chick-derived myofibroblasts. Cells were photographed after 24 hours of culture at 37°C in Ham-F12 medium with FBS. Myofibroblasts were isolated from sprouting chicken embryo aortas as previously described (Kilarski et al. 2005), while HMVEC, HUVEC and HASMC were purchased. Rat-tail collagen or Vitrogen was neutralized with buffer (HEPES, NaCl, pH 8.6) at 4°C. Where indicated, 100 mg of glass fibres was mixed with 1 ml rat-tail collagen solution. Neutralized collagen solution was then mixed with cells (2nd-3rd

passage) and cell containing droplets were then immediately applied on the bottom of a cell-culture plate and incubated at 37°C. After 2 hours, polymerized gels were covered with Ham's F-12 medium with fetal calf serum (FCS) and cultured for 7 to 14 days. HUVEC and HASMC were mixed in varying ratios: 100% HASMC (0% HUVEC), 75%HASMC / 25%HUVEC, 50%HASMC / 50%HUVEC, 25%HASMC / 75%HUVEC, (0% HASMC) 100% HUVEC. Total gel number in each group was 37. Contraction of gels from each group was counted daily, for ten days, and the data statistically analyzed.

3.13 SPROUTING FROM SPHEROIDS IN FIBRIN GELS

Paper III: The assay is based on a method for studies of endothelial cell sprouting from spheroids previously described (Korf & Augustin 1999). HUVEC, HMVEC, HASMC and myofibroblasts were cultured for 3 days in Ham-F12 medium containing FCS and methylcellulose. Droplets with cells were placed on the internal part of a lid to a bacterial dish. Sterile PBS was poured into the dish and the dish was then covered with the lid carrying approximately 80 hanging droplets. Spheroids were harvested in serum free Ham-F12 medium and resuspended in a filtrated fibrinogen solution in Ham-F12. The fibrinogen solution was then poured on prepolymerized fibrin gels in a 24 well plate. After overnight polymerization of this second layer, Ham-F12 medium containing FCS supplemented with VEGF was added. Spheroids were cultured for 3 to 6 days and photographed.

3.14 STATISTICAL ANALYSES

Paper I: Quantitative comparison of the corneal neovascularisation area between KO and WT mice was performed using Student's t-test for unpaired data.

Paper II: The Mann–Whitney U-test was used for quantitative comparison of the corneal neovascularisation area as well as for the assessment of differences in the number of inflammatory cells and activated keratocytes/cornea between KO and WT mice. The calculation of neovascularised corneal area was based on the ratio between the neovascularised corneal area and the total corneal area, to avoid errors caused by differences in corneal size.

Paper III: The Student's t-test for independent variables with separate variance estimates was used to analyze differences between cellular sprouting and distances of vascular outgrowth as well as for the differences in number of sprouts and pericyte coverage of vessel loops in DC101 versus control antibody treated corneas. Change in sprout area was tested with one-way ANOVA. ANOVA and Tukey HSD for unequal "n" were used to assess differences in proliferation and vascular/lymphatic ingrowth between DC101 and control antibody groups. Data analyzed with ANOVA were checked for equal variance and normality. Implant vascularization on CAM was scored in binominal manner and p-values calculated using Fisher's exact test. Quantification of the matrix contractile ability was assessed by comparing multiple samples, Statistica Survival analysis.

In all papers a statistical difference was considered significant when the p-value was less than 0.05.

4 RESULTS

4.1 MMP-2 STUDY (*PAPER I*)

The neovascular response in this experimental model of corneal neovascularisation was highly reproducible. It was evident at 2-3 days after suturing and involved the entire cornea after about 12 days. Neovascularisation was delayed in MMP-2-KO mice as compared to WT mice. The difference was apparent at 6 days after suturing and statistically significant at day 9. No apparent morphological differences of the neovessels were seen between KO and WT mice. Histological analysis revealed neovessels both superficially and deeply in the corneal stroma accompanied by a pronounced inflammatory response, seen as neutrophil invasion in the anterior chamber and in corneal stroma.

In the WT mice, MMP-2 mRNA was conspicuously upregulated in the limbal region and throughout the corneal stroma, spatiotemporally preceding the ingrowth of neovessels. Immunostaining for MMP-2 protein was positive in the corneal stroma corresponding to the distribution of cells staining positive for S100A4, i.e. activated keratocytes. MMP-2 protein was also constitutively expressed in the corneal epithelium. It did not appear to increase after suturing and, in fact, mRNA for MMP-2 was not detected at this site. Neither neutrophils nor the corneal endothelium expressed MMP-2mRNA or protein.

4.2 IL-10 STUDY (*PAPER II*)

Both KO and WT mice responded with corneal angiogenesis to the inflammatory stimulus induced by the silk suture, but the response was delayed in KO mice compared to WT mice. This tendency was observed already 3 days after suturing, reached statistical significance on day 6 when about half of the entire cornea was vascularised and maintained a 24% inhibition of neovascularisation at day 9 after suturing. New vessel formation involved the entire cornea including the subepithelial space and stroma. No apparent differences in morphology or vessel density were seen between KO and WT mice.

Immunohistochemistry revealed infiltration of inflammatory cells, especially neutrophils but also macrophages in both WT and KO mice corneas, and they were mainly localized to the anterior chamber and the limbal area. T-cells were scarce (maximally a few cells/cornea) in both groups. Activated keratocytes could be detected in both WT and KO corneas. No different patterns in the inflammatory response could be observed in the corneal stroma between the groups in terms of markers for neutrophils, macrophages and activated keratocytes.

The mRNA-expression of a panel of molecules known to interact with IL-10, including MMP-2, MMP-9, VEGF-A, Ang-1 and Ang-2 did not show any significant difference between KO and WT mice.

4.3 BIOMECHANICAL VESSEL GROWTH REGULATION (*PAPER III*)

Cornea. Insertion of a silk suture through the cornea caused a wound with a healing reaction characterised by tissue oedema, inflammation and a neovascular response. αSMA-positive myofibroblasts were found in and around the wound, as well as in outer parts of the corneal stroma starting from the limbus. Limbal vessels became enlarged and translocated in the configuration of vascular loops toward the wound, leaving the vasculature in the limbus depleted of capillaries. Perfusion stainings combined with stainings of vascular endothelial cells and pericytes showed that the neovessels were functionally connected to the limbal circulation. They were also supported by a basal lamina and covered with αSMA-positive mural cells. Buds and sprouts were located on the pericyte-free side of the vascular loops facing the wound, while the part of the vessels oriented toward the limbus was covered with pericytes. Many of the sprouts and blunt-ended buds were unperfused. Sprouts did not contain nuclei and hence probably represented extensions of cells. Whole-mount stainings of corneas 3, 4, 5 and 6 days after suturing showed a linear increase of the total vascularised area that correlated with a decreased number of vascular outgrowths and an increased pericyte coverage of the vessels (at day 6).
Mice treated with DC101, an antibody against VEGFR-2, exhibited no difference in the size of the vascularised area compared to controls on day 3. However, the number of outgrowing vascular buds and sprouts was 70% lower and the proliferation of vascular cells 58% less while pericyte coverage of vascular loops was 90% greater in DC101-treated mice. At day 6, there was still a 64% inhibition of proliferation in DC101-treated mice, which was paralleled by a 20% inhibition of neovascularisation. No proliferating vascular cells were detected in the limbal vasculature of uninjured eyes.
Hemangiogenesis was accompanied by lymphangiogenesis. We observed outgrowth of LYVE-1 positive lymphatic vessels from the limbus into the cornea. Treatment with DC101 reduced lymphatic outgrowth at day 6 by 21%, whereas there was no difference on day 3.
The migration distance after 6 days in the sprouting experiments *in vitro*, using HMVEC, HUVEC, HASMC and myofibroblasts was compared with the length of cornea vessels 6 days after suturing. The distance of cell sprouts showed to be significantly shorter than the distance of vessel outgrowth in the cornea.

CAM and matrix gels. In response to FGF-2, the gel gradually contracted, partly digested by a cell infiltrate consisting of αSMA-negative protomyofibroblasts, αSMA-positive myofibroblasts and leukocytes and got invaded by ingrowing vascularised tissue. The lumens in the dense capillary network underlying the CAM implant became first enlarged, and then the entire capillary network moved toward the implant. The vessels were embedded in connective CAM tissue. These, initially bud-shaped structures elongated in order to expand. A clear interface between the implanted matrix and the neovascular tissue was identifiable at all stages of vascular ingrowth. Perfusion stainings with intracardiac ink injection and vessel wall stainings with DAB, H_2O_2 and BBBA distinguished vessels with functional circulation (black vessels) from those filled with blood but with no or low circulation (red/brown vessels). Moreover, it was found that the vast majority of the neovessels was functionally connected with the rest of the circulation. Neovessels were supported by a basal lamina and smooth muscle cells, the characteristic elements of mature vessels. Proliferating endothelial and mural cells were found in capillaries as well as in larger vessels.

The gel contracted concentrically generating a tension force that was perpendicular to the CAM and highest in the periphery. Chemical alteration of gel matrix (Vitrogen), i.e. removal of cellular binding sites, as collagen telopeptides (Woodley et al.1991) inhibited the development of cell-mediated tension and impaired neovascular ingrowth. Impairment of Vitrogen contractibility had no effect on myofibroblast invasion or migration (αSMA-positive cells in the gel), but blocked their concentric orientation parallel to the gel surface, which is necessary for the myofibroblasts to induce contraction (Eastwood et al. 1998). Using contraction-resistant gels, i.e. borosilicate glass fibre enriched matrix, resulted in complete inhibition of myofibroblasts-mediated matrix contraction *in vitro* and in 45% inhibition of vascularisation *in vivo*, when placed on the CAM. Correspondingly, chemical cross-linking of gels with EDAC abrogated implant contraction as well as vascular ingrowth, without affecting the ability of the cells to invade or spread within the matrix.

Endothelial cells embedded in rat collagen were not able to develop tensional forces and contract the gel in contrast to the myofibroblasts. To rule out the possibility that (proto)myofibroblasts might create a chemical environment that induces and directs neovascularisation without the need of contraction we replaced the fibrin and collagen gel with polyvinyl alcohol sponges that cannot be contracted by cells. When sponges were preseeded with purified myofibroblasts no vascularisation was observed.

Time-lapse recordings of neovascuarisation showed that the entire vasculature moved toward the contracting implant, instead of single ingrowing vessels. During this translocation, individual vessels were remodelled by enlargement, elongation, pruning and thinning and were further accompanied by angiogenesis with vessel splitting and intussusception.

5 DISCUSSION

Modern angiogenic research started with Judah Folkman's publication in New England Journal of Medicine in 1971 where he hypothesized that prevention and inhibition of angiogenesis could be a new means of cure for cancer (Folkman 1971). Although Dr. Folkman continued his admirable research and contributed with many important insights, like the role of bFGF in angiogenesis, it was not until Ferrara and Henzel discovered VEGF in 1989 that a wide interest in this research field surged (Ferrara et Henzel 1989). With the publication of anti-VEGF treatment for colorectal carcinoma in 2004, Hurwitz and coworkers presented "proof of concept" and this scientific area became one of the most active ones in biomedicine (Hurwitz et al. 2004).
Since then, the anti-angiogenic treatment has also entered the field of ophthalmology, primarily in the management of neovascular age-related macular degeneration [including VEGF inhibitors such as bevacizumab (Avastin®), ranibizumab (Lucentis®), and pegaptanib (Macugen®)]. Treatment of corneal angiogenesis has also been addressed, although so far only by off-label drugs (primarily bevacizumab) (Erdurmus et al. 2007, Bahar et al. 2008, Bock et al. 2008a and 2008b, Uy et al. 2008, Dastjerdi et al. 2009, Oh et al. 2009, Zaki et Farid 2009). The reported clinical effects are promising, although the published number of patients is still low and no long term data are available.
Still we lack a substance that can completely prevent or abolish the neovascularisation, a fact that mirrors the complexity of this pathophysiological process. A 100% potent anti-angiogenic drug would, however, most likely raise a number of safety issues given an expected wide range of serious side effects. As an example, VEGF is a potent neurotropic growth factor (reviewed by Carmeliet et Storkebaum 2002) and a potent chemoattractant for inflammatory cells, like macrophages (Cursiefen et al. 2004a). Anti-VEGF-treatment in the cornea might therefore, in the long run, have negative effects on wound healing, which could be deleterious. A profound understanding of the angiogenic complexity is thus a prerequisite for a successful search of candidate medicines.

Due to its physiological lack of vessels combined with its ability to become vascularized, the cornea has been a prime experimental tissue for angiogenic research since the 70s (Gimbrone et al. 1974). Consequently, the majority of these studies, has not been designed with clinical aspects of corneal neovascularisation in mind. Most experimental neovascular models on the cornea are therefore well suited to answer questions about basic angiogenic principles but not necessarily clinically applicable. This is, to a large extent, still the case in angiogenic studies using corneal models.
The experimental neovascularisation model used in present studies is a suture model where corneal hem- and lymphangiogenesis are elicited by a silk suture that acts as an inflammatory stimulus. It thereby probably mimics clinically relevant situations better than experimental designs where a defined angiogenic substance is applied in excess to cause neovascular response, often without any significant inflammation, as in the widely used corneal micropocket assay (Kato et al. 2001, Kure et al. 2003), or the traumatizing model of limbal or chemical wounding (alkaline burn) where corneal stem cell damage is instrumental in the process. The model used in present studies proved also to be highly reproducible, which makes it suitable for quantitative studies of this kind.

The role of MMP-2 in the initial steps on angiogenesis, by proteolysis of ECM and basement membrane, is comprehensively described in a number of studies. *Paper I*

corroborates the role of MMP-2 in angiogenesis in general and in inflammation associated angiogenesis of the cornea in particular. The lack of MMP-2 did not abolish neovascularisation, but it suppressed and delayed it. In this model, the target of the proteolytic activity of MMP-2 seemed primarily to be ECM, since there was an obvious spatiotemporal discrepancy between MMP-2 mRNA-expression and the neovascular zone (WT animals). Thus, MMP-2 mRNA was expressed throughout the cornea while the neovessels reached barely half of it. The lack of observation of any morphological differences in neovessels between the KO and WT animals, e.g. vessel shape and size or density of the sprouts and neovessel, suggests that the proteolysis of basement membrane was not strongly affected. Additionally a hypothetical preservation of the basement membrane proteolysis due to the lack of MMP-2 in this case, might have resulted in more conspicuous inhibition of neovascularisation than it was the case in this study. Other studies have shown that inflammation-associated angiogenesis in the eye is less dependent on MMP-2 than in models where angiogenesis is induced by growth factors solely [Kato et al. 2001, Berglin et al. 2003, Kure et al. (MMP-7) 2003]. This emphasizes the importance of analyzing the experimental results in their biological context. The expression of different members of vast MMP family and the extent of their redundancy may also differ between the tissues or types of angiogenic stimuli.

Inflammation is a major cause of angiogenesis and formation of corneal neovessels is almost always related to an inflammatory process. As previously described, it is known that many cells and molecules instrumental in immunologic responses can have an angioregullatory function as well. Also vascular cells and molecules essential in angiogenesis, e.g. VEGF, can act as immunomodulators. The multifunctional cytokine IL-10 is mostly known for its anti-inflammatory and regulatory effects on the immune response (Moore et al. 2001), but several studies have shown its regulatory effects on angiogenesis as well. Interestingly, in some studies it showed proangiogenic activity (Hatanaka et al. 2001, Garcia-Hernandez et al. 2002, Apte et al. 2006), while it in others played an anti-angiogenic role (Huang et al. 1999, Stearns et al. 1999, Silvestre et al. 2000 and 2001, Cole et al. 2003). In our model, *Paper II*, IL-10 acted proangiogenically, which could neither be explained by its anti-inflammatory effects nor by an apparent cross-talk with some of the most potent angiogenic factors that also are known to interfere with inflammatory responses, such as VEGF, MMP-2, MMP-9, Ang-1, Ang-2. Still, the results in this study further confirm the presumed link between inflammation and angiogenesis and also illustrate the complexity of this interaction. The proangiogenic role of IL-10 in this corneal model is in good agreement with results from other studies of neovascularisation in the eye, including choroidal and retinal neovascularisation (Apte et al. 2006, Dace et al. 2008). IL-10 seems to act as a proangiogenic factor in ocular angiogenesis regardless of site.

The extensive angiogenic research has mapped out many intricate molecular regulatory pathways. It is still an enigma, though, how an avascular tissue, like the void of the wound, can gain a fully functioning circulation within a few days. Many steps in angiogenesis have been described in detail, such as the role of tip- and stalk cells in sprouting (Gerhardt et al. 2003). In contrast, the final steps of neovascularisation when *de novo* formed branches coalesce into functional anastomoses are yet to be fully outlined. Neither is there a complete understanding of the redirection of the functional circulation from ancestor vessels towards the new vascular plexa. These poorly explained phenomena as well as the difficulty to grasp a wider picture of angiogenesis from intricate patterns of redundant and diversely acting angioregulatory factors became a point of departure for our third study.

The results in *Paper III* suggest that translocation of the pre-existing vasculature is responsible for the initial rapid formation of functional vessels in granulation tissue. Our results revitalize the concept of biomechanical regulation as a contributor to molecular guidance of neovascularisation. The concept in fact originates from the 90's (Chen et al.1999, Ingberg 2002). In addition, recent studies of Mammoto and Thodeti and coworkers elegantly described evidence of conversion of physical factors into chemical signals in angiogenesis (Mammoto et al. 2009, Thodeti et al. 2009). The main actors in the process of vessel translocation in our study (*Paper III*) seemed to be proto- and myofibroblasts. They generated tensional forces resulting in contraction of the provisional matrix and the corneal tissue with subsequent vessel ingrowth. The gel matrices contracted concentrically generating a tension force that was perpendicular to the CAM and strongest in the periphery. The myofibroblastic phenotype is induced by mechanical tension (Hinz et al. 2001). This probably explains why myofibroblasts appeared preferentially in the periphery of the gel implant, a pattern that also was followed by the ingrowing vascularised tissue. The extent of neovascularisation correlated with the contractile ability of different collagen preparation, while, conversely, the prevention of matrix contraction by reinforcing its rigidity reduced neovascularisation, without affecting the ingrowth and spreading of the cells. This indicated that the tissue contraction was a prerequisite for neovascularisation. To rule out the possibility that invading cells released factors mediating vessel ingrowth we tested if there was any vessel invasion in non-contractile sponges seeded with myofibroblasts. The results were negative which strengthened our hypothesis on tissue contractibility as an essential initial step in angiogenesis.

In the cornea model, the number of sprouts and vascular buds decreased, while the total vascularised area increased in a linear fashion during the observation time. Also the percentage of pericyte-covered mature vessels increased with time. If sprouting angiogenesis was the only mechanism of neovascularisation, one would expect the number of sprouts to increase proportionally with the growth of the neovascular area. It would also be anticipated that the percentage of immature, pericyte-free, vessels would increase, which was not the case. Furthermore the sprouts lacked nuclei, suggesting that the majority represented cellular extensions, rather than endothelial tip cells that would be expected in pure sprouting angiogenesis (Gerhardt et al. 2003). Inhibition of VEGFR-2 markedly reduced formation of sprouts and increased pericyte-coverage, normalizing vessel morphology, but only partially inhibiting the early steps of vascularisation. Proliferating endothelial cells were found not only in capillaries but also in larger vessels and thus represented vascular remodeling as well as sprouting. This proliferative activity was markedly reduced by inhibition of VEGFR-2 without affecting the degree of neovascularisation, as would be expected if only sprouting was driving the neovascularisation. Taken together, this indicates that sprouting was not critical for the initial neovascularisation in this model. These findings are in agreement with the conclusions in a previous study of Sholly et al, which showed that migration and redistribution of existing endothelial cells from the limbal vessels enable vascular sprouting and elongation without cellular proliferation (Sholley et al. 1984). Our findings are also in agreement with the study of Scroggs et al. where total body irradiation could not significantly suppress corneal neovascularisation in rats with chemically injured corneas (Scroggs et al 1991). This indicates that endothelial cell migration plays a more important role than cell replication in pathogenesis of corneal angiogenesis. This in turn can be further compared with studies of angiogenesis in muscles in which intussusceptive, non-sprouting, angiogenesis was sufficient to expand the capillary network under certain circumstances (Egginton et al. 2001). Still, contrary to our results concerning the initial steps of neovascularisation, at later stages we

observed decreased cell proliferation that later during the observation time was associated with reduced neovascularisation, implicating that cellular proliferation is required to a higher degree over longer distances. Also time-laps recordings of CAM implants revealed angiogenic activity, mostly via intussusception. Still vascular cell cultures showed significantly lower sprout capacity; shorter sprout distance compared to average vessel outgrowth *in vivo*, during the same time period of 6 days, indicating that other mechanisms beside mitotic cell activity are responsible for the observed neovascularisation.
These results propose that mechanical tension is both necessary and sufficient to mediate and direct the early phases of vascular tissue expansion in tissue healing, whereas sprouting and intussusceptiv angiogenesis is needed to fine-tune and further develop the newly formed vasculature.

Future studies on mechanisms regulating the interplay between physical factors and chemical signals in angiogenesis are necessary for a genuine understanding of this complex process. The characteristics of neovascularisation and its regulatory mechanisms appear to differ depending on tissue type and primary progangiogenic stimulus. This is important to take in to account when designing new pharmaceuticals interfering with angiogenesis - therapeutic means that undoubtedly will be vital for public health in the future.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

As most of the studies on angiogenesis use quantitative analysis of neovessels their optimal visualization is essential, but also far from easy or straightforward. This is due to several reasons. Visualisation techniques usually take place *post mortem* which implies low blood pressure. Since capillaries and the smallest venules have thin walls lacking reinforcement of smooth muscle cells and elastic fibres their lumina collapse when the blood and injected visualisation stains move to larger vessels. This is due to the pressure of surrounding interstitial fluid. Such microvesells can be misinterpreted as blunt vascular structures in sprouting or vascular remodelling. Pronounced leakage and defect perfusion of neovessels can make the visualisation with intravasal stains a challenge. Confounding lymphatic vessels for blood vessels is also one reason for unreliable results as well as imperfect detection of three-dimensional vessel extension by two-dimensional assessment owing to deficient measurement systems. Additionally almost all techniques provide "frozen" moments of a highly dynamic process which is actually the prime interest. Even if repeated images taken during the observation period will compensate for this to some extent, the realization of continuous monitoring should be more revealing. Accordingly, there is a need for tools improving visualization of vessels and their morphogenesis, for providing more precise quantification. Several adjacent areas of interest regarding vessel type and state of function during neovascularisation remain unanswered. Important questions are: How are haemodynamics preserved in short isolated vessel protrusions that still did not fuse into anastomoses with other vessel branches? How are the vessels that do not sustain the flow eliminated? Is the phenotype of neovessels determined by physical factors or chemical signals, or perhaps both?

Presented studies confirmed the role of two important angio-regulatory factors, MMP-2 and IL-10, in inflammation associated corneal angiogenesis. The results, however, elucidate the complexity of this process that, in part, is due to manifest redundancy of critical angiogenic factors and intricate interactions between the factors. Moreover, there seems to be a modulatory role of local tissue changes on chemical signals regulating angiogenesis, which was one of the conclusions of our third study. The same factor can evidently act both pro- and anti-angiogenically due to the type of tissue or due to the primary angiogenic stimulus. So can, as previously described and referred to, MMP-2 play a much more pronounced pro-angiogenic role in the cornea micropocket assay, than in our inflammation-associated suture model. Likewise, IL-10 acts anti-angiogenically in the ischemic limb and pro-angiongenically in our inflammatory cornea model. Hence, the analysis of the angiogenic context seems to be as necessary as the properties of single regulatory factor.
The results from our third study support the concept that the local changes in the tissue structure and mechanics co-operate with chemical signals in the regulation of angiogenesis. This should prompt further exploration of the interaction between biomechanical and molecular regulation of vessel growth and morphogenesis. As these regulatory pathways are tightly linked during embryogenesis, there may be a genetic program for interpretation of biomechanical and morphological to chemical signals and vice versa? Which key molecules respond to cellular stress in different cell types and tissues and how is this translated to a change of phenotype?

Could an increased rigidity of a corneal graft delay or even prevent vessel ingrowth and rejection? If so, would locally increased concentrations of proangiogenic factors with persisting pro-angiogenic stimuli result in tissue proteolysis and neovascularisation in adjacent tissues, similar to neovascularisation of the iris secondary to retinal vein occlusion?

The interplay between lymph- and hemangiogenesis, especially in the case of corneal graft rejection merits further study. Can the inhibition of lymphangiogenesis prevent graft rejection if the blood neovessels already are present, as in the event of re-grafting? What happens with the lymphatic neovessels after therapeutic inhibition of hemangiongenesis?

There are several levels of regulation of vessel growth; genetic, molecular, cellular, tissue/organ and organism. These levels interact in a complex manner and regulatory molecules involved can play different roles depending on the circumstances, like in health versus disease or ischemia versus inflammation. This complex situation may require integrative interdisciplinary studies with other theoretic sciences, as mathematical and computational probability models, more then acknowledged hitherto (Chaplain et al. 2006, Peirce 2008, Qutub et Popel 2009).

New anti-angiogenic drugs have made a great change in the treatment of sight-threatening age-related macular degeneration. So far, this might be the most positive clinical effect of these new drugs. The same drugs are used in corneal neovascularisation, but so far only off label. There is a need for prospective randomized studies with a long follow-up time recruiting a sufficient number of patients sub-grouped according to the neovascular condition. The primary endpoint would not only include a regress of vessels but also the clinical benefit for the patient, expressed as an improved best corrected visual acuity sustained over time. A multi-center study would be best suited for this goal.

The past decades have taught us much about one of the most basic and essential processes in a living organism; the vessel formation. During this time the cornea has been an important and widely used tissue for angiogenic research making contribution to insights that have led to new revolutionary drugs. Further angiogenic research will hopefully lead to even better treatment solutions, none the less for sight threatening corneal angiogenesis.

7 SVENSK SAMMANFATTNING

Utan kärl inget liv. För att få näring och syre får en cells avstånd till närmaste kärl inte överskrida 0,15-0,2 mm. Utan förmågan att bilda nya kärl skulle inte ett foster kunna utvecklas och bli till ett barn och inga sår skulle kunna läkas. Idag vet vi dessutom att kärlnybildning är en del av sjukdomsprocessen i några av de vanligaste och farligaste folksjukdomarna såsom ateroskleros, hjärtinfarkt, cancer, stroke, reumatism, diabetes och osteoporos, för att nämna de viktigaste. Peter Carmeliet, en auktoritet inom kärlnybildning, postulerade 2005 i en översiktsartikel i den ansedda tidskriften *Nature* att mer än en halv miljard människor världen över skulle kunna dra nytta av mediciner som kunde reglera kärlnybildning, angiogenes, antingen genom att hämma eller stimulera processen. Ögat, kan precis som andra delar av kroppen drabbas av kärlnybildning och tillståndet orsakar många gånger grav synnedsättning eller i värsta fall blindhet. Av detta lider flera hundra millioner människor världen över.

Ögats fönster, hornhinnan, är en av kroppens få vävnader som saknar kärl. Avsaknad av kärl är förutsättning för dess genomskinlighet, vilket i sin tur är helt avgörande för en god synskärpa. Denna egenskap har också gjort hornhinnan till en vida använd experimentell modell för studier av kärlnybildning, eftersom det inet finns någon risk för förväxling mellan gamla och nybildade kärl. Kärl kan växa in i hornhinna vid en rad olika tillstånd; t. ex. vid syrebrist, inflammation, infektion, kemisk skada, stamcellskada, ögontorrhet, nedsatt känslighet eller vid avstötning av hornhinnetransplantat. Det sistnämnda är extra vanskligt då just transplantation ofta utgör det sista behandlingsalternativet för svårt sjuka hornhinnor. Kärlnybildning i hornhinnan är ett viktigt kliniskt problem som drabbar patienter i alla åldrar. Effektiv behandling saknas alltjämt. Anti-inflammatoriska mediciner kan i viss mån hämma kärlinväxten, men utan att för den skull helt kunna förebygga eller upphäva dent. I och med den intensiva forskningen kring kärlnybildning har nya mediciner börjat nå kliniken, men mycket mer kunskap om angiogenesens basala principer behövs för att få fram nya adekvata läkemedel.

Målet med de tre studier som presenteras i denna avhandling var att nå djupare kunskap om angiogenes generellt och angiogenes i hornhinna i synnerhet, på molekylär, cellulär och vävnadsnivå. Alla tre studier baseras på en experimentell musmodell där en silkessutur som sätts genom hornhinnan framkallar inflammation och kärlinväxt. De två första studierna använder sig av genetiskt manipulerade möss med en defekt, icke fungerande gen för matrix metalloproteinas-2 (MMP-2) respektive interleukin-10 (IL-10), så kallade knockout möss (KO). Den tredje studien utgörs av en rad experiment där hornhinneförsöken är en *in-vivo*-modell, vid sidan av den så kallade chick chorioallantoic membrane model (CAM), cellkulturer, *in-vitro* gel-kontraktioner och experiment med peptid-inhibition. De nybildade kärlen har visualiserats med hjälp av perfusionsfärgningar samt immunohistokemi. Kärlnybildningen har studerats och kvantifierats med hjälp av mikroskop försett med bildbehandlarprogram, utifrån aspekter av tid och lokalisation/utbredning. Den inflammatoriska reaktionen har analyserats med hjälp av immunohistokemi. In-situ hybridisering och *real time*-

polymerase chain reaction (rt-PCR) har använts för detektering av genuttryck (mRNA) av relevanta molekyler.

Matrix metalloproteinaser (MMP) är en stor grupp enzymer som spjälkar vävnad mellan cellerna och kärlväggens basalmembran. Därför anses de ligga bakom de inledande stegen vid kärlnybildning. I den första studien bekräftas betydelsen av MMP-2 som mediator av kärlnybildning i denna hornhinnemodell. Kärlnybildningen korrelerade med ökat uttryck för MMP-2, på både gen- och proteinnivå, vilket framför allt hittades i de aktiverade keratocyterna. I den andra studien stimulerade den anti-inflammatoriska cytokinen IL-10 till kärlnybildning, vilket dock inte kunde förklaras av dess anti-inflammatoriska egenskaper (undersökt med histologiska metoder) eller av dess påverkan på någon av de mest potenta proangiogena faktorerna (visat med rt-PCR).
I den tredje studien konstaterades att aktiverade myofibroblaster vid sårläkning orsakar spänningar och kontraktioner i vävnaden som i sin tur drar de ursprungliga kärlen till de ställen där nya kärl behövs (experiment med hornhinnor och CAM). Studien beskriver biomekaniska processer som hittills inte har varit accepterade som regleringsmekanism vid kärlnybildning. I de inledande stegen av kärlnybildning vidgades de ursprungliga kärlen och tänjdes ut utan att endotelcellsavknoppningar till nya kärlgrenar, så kallad sprouting, var nödvändig. Neutraliserande antikroppar DC101 mot vascular endothelial growth factor receptor-2 (VEGFR-2) som styr kärlnybildning genom sprouting, kunde därför inte förhindra kärlnybildningen. När man däremot förhindrade sammandragningar i den experimentella vävnaden hämmades kärlnybildningen. Likaså uteblev kärlnybildningen om myofibroblasternas förmåga att dra och spänna vävnaden inhiberades. Denna studie bidrar med ny kunskap till dem redan etablerade teorierna om kärlnybildning och ett namnförslag till den nya förklaringsmodellen, ”looping angiogenesis”, har lanserats (News and Views. Bennet AV, Augustin HG. Nat Med. 2009;15(6):08-610)

Kärlnybildningen kan gestalta sig olika beroende på var i kroppen den sker och under vilka förutsättningar. Tillsammans visar de presenterade experimentella studierna att kärlnybildning i hornhinnan styrs av både kemiska signaler och mekaniska faktorer. Fortsatta studier av mekanismer som styr samspelet mellan dessa är oundgängliga för att nå fullödig kunskap om den komplexa och vitala biologiska process som kärlnybildning representerar. Bättre förståelse kommer förhoppningsvis att resultera i nya läkemedel som kan få avgörande betydelse för många sjukdomar, inte minst för synhotande hornhinnesjukdomar.

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9 REFERENCES

Achen MG, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 1998;95**:**548–553.

Ambati BK, Nozaki M, Singh N, Takda A, Jani PD, Suthar T, Albuquerque RJ, Richter E, Skurai E, Newcomb MT, Kleinman ME, Caldwell , Lin Q, Ogura Y, Orecchia A, Samuelson DA, Agnew DW, St Leger J, Green WR, Mahasreshti PJ, Curiel DT, Kwan D, Marsh H, Iked S, Leiper LJ, Collinson JM, Bogdanovich S, Khurana TS, Shibuya M, Baldwin ME, Ferrara N, Gerber HP, De Falco S, Witta J, Baffi JZ, Raisler BJ, Ambati J. Corneal avascularity is due to soluble VEGF receptor-1. Nature 2006;443:993-997.

Apte RS, Richter J, Herndon J, Ferguson TA. Macrophages inhibit neovascularization in a murine model of age-related macular degeneration. PLoS Med. 2006;3(8):e310.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964–967.

Bahar I, Kaiserman I, McAllum P, Rootman D, Slomovic A. Subconjunctival bevacizumab injection for corneal neovascularization. Cornea 2008;27:142–147.

Bautch VL, Redick SD, Scalia A, Harmaty M, Carmeliet P, Rapoport R. Characterization of the vasculogenic block in the absence of vascular endothelial growth factor-A. Blood 2000;95(6):1979-1987.

Bergh JJ, Lin HY, Lansing L, Mohamed SN, Davis FB, Mousa S, Davis PJ. Integrin αvβ3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. Endocrinology 2005;146:2864–2871.

Berglin L, Sarman S, van der Ploeg I, Steén B, Ming Y, Itohara S, Seregard S, Kvanta A. Reduced choroidal neovascular membrane formation in matrix metalloproteinase-2-deficient mice. Invest Ophthalmol Vis Sci. 2003;44:403-408.

Bikfalvi A, Dupuy E, Inyang AL, Fayein N, Leseche G, Courtois Y, Tobelem G. Binding, internalization, and degradation of basic fibroblast growth factor in human microvascular endothelial cells. Exp Cell Res.1989;181**:**75–84.

Bock F, Konig Y, Kruse F, Baier M, Cursiefen C. Bevacizumab (Avastin) eye drops inhibit corneal neovascularization. Graefes Arch Clin Exp Ophthalmol. 2008a;246: 281–284.

Bock F, Onderka J, Dietrich T, Bachmann B, Pytowski B, Cursiefen C. Blockade of VEGFR3-signalling specifically inhibits lymphangiogenesis in inflammatory corneal neovascularisation.Graefes Arch Clin Exp Ophthalmol. 2008b;246:115–119.

Brafman A, Mett I, Shafir M, Gottlieb H, Damari G, Gozlan-Kelner S, Vishnevskia-Dai V, Skaliter R, Einat P, Faerman A, Feinstein E, Shoshani T. Inhibition of oxygen-induced retinopathy in RTP801-deficient mice. Invest Ophthalmol Vis Sci. 2004;45: 3796–3805.

Brooks PC, Silletti S, von Schalscha TL, Friedlander M, Cheresh DA. Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. Cell 1998, 92: 391–400.

Burri PH, Tarrek MR. A novel mechanism of capillary growth in the rat pulmonary microcirculation. Anat Rec.1990;228(1):35-45.

Burri PH, Djonov V. Intussusceptive angiogenesis—the alternative to capillary sprouting. Mol Asp Med. 2002;23(6, Suppl. 1):1-27.

Burri PH, Hlushchuk R, Djonov V. Intussusceptive angiogenesis: Its emergence, its characteristics, and its significance. Developm Dyn. 2004;231(3):474-488.

Cadff SH, Fischer LC, Burri PH. Scanning electron microscope study of the developing microvasculature in the postnatal rat lung. Anat Rec.1986;216(2):154-164.

Cao R, Björndahl MA, Religa P, Clasper S, Garvin S, Galter D, Meister B, Ikomi F, Tritsaris K, Dissing S, Ohhashi T, Jackson DG, Cao Y. PDGF-BB induces intratumoral lymphangiogenesis and promotes tumour metastasis. Cancer Cell 2004**;**6(4)**:**333–345.

Carmeliet P, Storkebaum E. Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. Semin Cell Dev Biol.2002;13:39–53.

Chang JH, Gabison EE, Kato T, Azar DT. Corneal neovascularization. Curr Opin Ophthalmol. 2001;12:241-249.

Chaplain MA, McDougall SR, Anderson AR. Mathematical modeling of tumor-induced angiogenesis. Annu Rev Biomed Eng. 2006;8:233-257.

Chen KD, Li S, Kim M, Yuan S, Chien S, Shyy JY. Mechanotransduction in response to shear stress. Role of receptor tyrosine kinases, integrins and Shc. J Biol Chem. 1999; 274:18393–18400

Chen L, Hamrah P, Cursiefen C, Jackson D, Streilein JW, Dana MR. Vascular endothelial growth factor receptor-3 (VEGFR-3) mediates dendritic cell migration to lymph nodes and induction of immunity to corneal transplants. Nat Med. 2004;10:813–815.

Cole N, Krockenberger M, Stapleton F, Khan S, Hume E, Husband AJ, Willcox M. Experimental Pseudomonas aeruginosa kertitits in interleukin-10 gene knock out mice. Infect Immun. 2003;71:1328–1336.

Cordon-Cardo C, Vlodavsky I, Haimovitz-Friedman A, Hicklin D, Fuks Z. Expression of basic fibroblast growth factor. Lab Invest. 1990;63:832-840.

Cursiefen C, Chen L, Borges L, Jackson D, Cao J, Radziejewski C, D'Amore PA, Dana MR,Wiegand SJ, Streilein JW. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. J Clin Invest. 2004a;113:1040–1050.

Cursiefen C, Masli S, Ng TF, Dana MR, Bornstein P, Lawler J, Streilein JW. Roles of thrombospondin 1 and 2 in regulating spontaneous and induced angiogenesis in the cornea and iris. Invest Ophthalmol Vis Sci. 2004b;45:1117–11124.

Cursiefen C, Chen L, SaintGeniez M, Hamrah P, Jin Y, Rashid S, Pytowski B, Persaud K, Wu Y, Streilein JW, DanaR. Nonvascular VEGF receptor 3 expression by corneal epithelium maintains avascularity and vision. Proc Natl Acad Sci USA 2006;103:11405-11410.

Dace DS, Khan AA, Kelly J, Apte RS. Interleukin-10 promotes pathological angiogenesis by regulating macrophage response to hypoxia during development. PLoS One. 2008;3(10):e3381.

Daly C, Pasnikowski E, Burova E, Wong V, Aldrich TH, Griffiths J, Ioffe E, Daly TJ, Fandl JP, Papadopoulos N, McDonald DM, Thurston G, Yancopoulos GD, Rudge JS. Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. Proc Natl Acad Sci USA 2006;103(42):15491-15496.

Dana MR, Streilein JW. Coreal neovascularization and ocular immune privilege. Invest Ophthalmol Vis Sci. 1996;37:2485-2494.

Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W, Bouck NP. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 1999, 285:245–248.

Dastjerdi MH, Al-Arfaj KM, Nallasamy N, Hamrah P, Jurkunas UV, Pineda R 2nd, Pavan-Langston D, Dana R.Topical bevacizumab in the treatment of corneal neovascularization: results of a prospective, open-label, noncomparative study. Arch Ophthalmol. 2009;127(4):381-389.

Diegelman RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci. 2004;9:283-289.

Djonov V, Schmid M, Tschamz SA, Burri P. Intussusceptiv angiogenesis. Its role in embryonic vascular network formation. Circ Res. 2000;86:286-292

Djonov V, Andres AC, Ziemiecki A.Vascular remodelling during the normal and malignant life cycle of the mammary gland. Microsci Res Technol. 2001;52**:**182–189

Djonov V, Baum O, Burri PH. Vascular remodeling by intussusceptive angiogenesis. Cell Tissue Res. 2003;314(1):107-117.

Dumont DJ, Yamaguchi TP, Conlon RA., Rossant J, Breitman, ML. tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. Oncogene 1992;7(8):1471-1480.

Eastwood M, Mudera VC, McGrouther DA, Brown RA. Effects of precise mechanical loading on fibroblast populated collagen lattices: morphological changes. Cell Motil Cytoskeleton 1998;40:13-21.

Egginton S, Zhou AL, Brown MD, Hudlická O. Unorthodox angiogenesis in skeletal muscle. Cardiovasc Res. 2001;49(3):634-646.

Erdurmus M, Totan Y. Subconjunctival bevacizumab for corneal neovascularization. Graefes Arch Clin Exp Ophthalmol. 2007;245:1577–1579.

Fannon M, Forsten-Willimas K, Dowd CJ, Freedmann DA, Folkman J, Nugent MA. Binding inhibition of angiogenic factors by heparan sulfate proteoglycans in aqueous humor: potential mechanism for maintenance of an avascular environment. FASEB J. 2003;17:902–904.

Fernando NH, Hurwitz HI. Targeted therapy of colorectal cancer: clinical experience with bevacizumab. Oncologist 2004;9 Suppl 1:11-18.

Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun.1989; 161(2):851-858.

Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzenrath M, Rosseau S, Suttorp N, Sobke A, Herrmann M, Preissner KT, Vajkoczy P, Augustin HG. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med. 2006;12(2):235-239.

Fini ME, Girard MT, Matsubara M. Collagenolytic/gelatinolytic enzymes in corneal wound healing. Acta Ophthalmol Suppl. 1992;70:26–33.

Folkman J et al. Tumor angiogenesis: Therapeutica implications. N Eng J Med. 1971;285:1182-1186.

Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR, Majno G. Granulation tissue as a contractile organ. A study of structure and function. J Exp Med. 1972;135(4):719-34.

Gamble JR, Drew J, Trezise L, Underwood A, Parsons M, Kasminkas L, Rudge J, Yancopoulos G, Vadas MA. Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. Circ Res. 2000;87(7):603-607.

Gao D, Nolan D, McDonnell K, Vahdat L, Benezra R, Altorki N, Mittal V. Bone marrow-derived endothelial progenitor cells contribute to the angiogenic switch in tumor growth and metastatic progression. Biochim Biophys Acta. 2009;1796(1):33-40.

Garcia-Hernandez ML. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. Immunology 2002;105: 231–243.

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol. 2003;161(6):1163-1177.

Gimbrone Jr MA, Cotran RS, Folkman J. Tumor growth neovascularization: an experimental model using rabbit cornea. J Natl Canc Inst. 1974;52:413–427.

Gimbrone MA Jr., Nagel T, Topper JN. Biomechanical activation: an emerging paradigm in endothelial adhesion biology. J Clin Invest. 1997;99:1809–1813.

Hatanaka H. Significant correlation between interleukin 10 expression and vascularization through angiopoietin/TIE2 networks in non-small cell lung cancer. Clin Cancer Res. 2001;7:1287–1292.

Hinz B, Mastrangelo D, Iselin CE, Chaponnier C, Gabbiani G. Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. Am J Pathol. 2001;159:1009–1020.

Hinz B, Gabbiani G, Chaponnier C. The NH2-terminal peptide of alpha-smooth muscle actin inhibits force generation by the myofibroblast in vitro and in vivo. J Cell Biol. 2002;157:657-663.

Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci USA 1998;95(16):9349-9354.

Hipps DS, Hembry RM, Docherty AJ, Reynolds JJ, Murphy G. Purification and characterization of human 72-kDa gelatinase (type IV collagenase). Use of immunolocalisation to demonstrate the non-coordinate regulation of the 72-kDa and 95-kDa gelatinases by human fibroblasts. Biol Chem Hoppe Seyler.1991;3372(4):287-296.

Hlushchuk R, Riesterer O, Baum O, Wood J, Gruber G, Pruschy M, Djonov V. Tumor recovery by angiogenic switch from sprouting to intussusceptive angiogenesis after treatment with PTK787/ZK222584 or ionizing radiation. Am J Pathol. 2008;173(4):1173-1185.

Huang S. Regulation of tumor growth and metastasis by interleukin-10: the melanoma experience. J Interferon Cytokine Res. 1999;19:697–703.

Ingberg DE. Mechanical signaling and the cellular response to extracellular matrix in angiogenesis and cardiovascular physiology. Circ Res. 2002;91(10):877-887

ItohT, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S. Reduced angiongesis and tumor progression in gelatinase A-deficient mice. Cancer Res. 1998;58:1048-1051.

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J. 1996;15,290–298.

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K. Expression of the fms-like tyrosine kinase FLT4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 1995;92,3566–3570.

Kajiya K, Hirakawa S, Ma B, Drinnenberg I, Detmar M. Hepatocyte growth factor promotes lymphatic vessel formation and function. EMBO J. 2005;24**:**2885–2895.
Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006(6):392-401.

Karakousis PC, John SK, Behling KC, Surace EM, Smith JE, Hendrickson A, Tang WX, Bennett J, Milam AH. Localization of pigment epithelium derived factor (PEDF) in developing and adult human ocular tissues. Mol Vis. 2001;7:154-163.

Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K.Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nature Immunol. 2004;5**:**74–80.

Kato T, Kure T, Chang JH, Gabison EE, Itoh T, Itohara S, Azar DT. Diminished corneal angiogenesis in gelatinase A-deficient mice. FEBS Lett. 2001;508:187–190.

Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J, D'Amato RJ. A model of angiogenesis in the mouse cornea. Invest. Ophthalmol. Visual Sci. 1996;37**:**1625–1632.

Kilarski WW, Jura N, Gerwins P. Inactivation of Src family kinases inhibits angiogenesis in vivo: implications for a mechanism involving organization of the actin cytoskeleton. Exp Cell Res. 2003;291:70-82.

Kilarski WW, Jura N, Gerwins P. An ex vivo model for functional studies of myofibroblasts. Lab Invest. 2005;85:643-654.

Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. J Biol Chem. 2001;276(10):7614-7620.

Klagsbrun M, D'Amore PA. Vacular endothelial growth factor and its receptors. Cytokine Growth Factor Rev. 1996;7(3):259-270.

Korff T, Augustin H.G. Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. Journal of cell science 1999;112(Pt 19):3249-3258.

Kubo, H, Cao R, Brakenhielm E, Mäkinen T, Cao Y, Alitalo K. Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. Proc Natl Acad Sci USA 2002; 99**:**8868–8873.

KureT, Chang JH, Kato T, Hernandez-Quintela E, Ye H, Chung-Shien Lu P, Matrisian LM, Gatinel D, Shapiro S, Gosheh F, Azar DT. Corneal neovascularization after excimer keratectomy wounds in matrilysin-deficient mice. Invest Ophthalmol Vis Sci. 2003;44:137-144.

Kurz H, Burri PH, Djonov VG. Angiogenesis and vascular remodelling by intussusception: from form to function. News Physiol Sci. 2003;18:65-70.

Kühn R, Löhler J, Rennick D, Raywski K & Müllern W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 1993;75: 263–274.

Lai CM, Brankov M, Zaknich T, Lai YK, Shen WY, Constable IJ, Kovesdi I, Rakoczy PE. Inhibition of angiogenesis by adenovirus-mediated sFlt-1 expression in a rat model of corneal neovascularization. Hum Gene Ther. 2001;12:1299–1310.

Leak LV. The structure of lymphatic capillaries in lymph formation. Fed Proc. 1976;35:1863–1871.

Lee, CR, Grodzinsky AJ, Spector, M. The effects of cross-linking of collagenglycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomaterials 2001;22:3145-3154.

Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol. 2003a;170(6):3369-3376.

Li J, Zhang YP, Kirsner RS. Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. Microsc Res Tech 2003b;60**:**107–114.

Lindgren M, Hallbrink M, Prochiantz A, Langel U. Cell-penetrating peptides. Trends Pharmacol Sci. 2000;21:99-103.

Luo L, Li D-Q, Doshi A, Farley W, Corrales RM, Pflugfelder SC. Experimental dry eye stimulates production of inflammatory cytokines and MMP-9 and activates MAPK signaling pathways on the ocular surface. Invest Ophthalmol Vis Sci. 2004;45: 4293–4301.

Majka SM, Jackson KA, Kienstra KA, Majesky MW, Goodell MA, Hirschi KK. Distinct progenitor populations in skeletal muscle are bone marrow derived and exhibit different cell fates during vascular regeneration. J Clin Invest. 2003;111:71–79.

Makanya AN, Hlushchuk R, Djonov VG. Intussusceptive angiogenesis and its role in vascular morphogenesis, patterning, and remodeling. Angiogenesis 2009;12(2):113-23.
Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature 2001;414:550–554.

Makanya AN, Stauffer D, Ribatti D, Burri PH, Djonov V. Microvascular growth, development, and remodeling in the embryonic avian kidney: the interplay between sprouting and intussusceptive angiogenic mechanisms. Microsc Res Tech. 2005; 15;66(6):275-288.

Makanya AN, Hlushchuk R, Baum O, Velinov N, Ochs M, Djonov V. Microvascular endowment in the developing chicken embryo lung. Am J Physiol Lung Cell Mol Physiol. 2007;292(5):L1136-1146.

Mammoto A, Connor KM, Mammoto T, Yung CW, Huh D, Aderman CM, Mostoslavsky G, Smith LE, Ingber DE.A mechanosensitive transcriptional mechanism that controls angiogenesis. Nature 2009;457(7233):1103-1108.

Mariotti SP, Pascolini D, Rose-Nussbaumer J. Trachoma: global magnitude of a preventable cause of blindness. Br J Ophthalmol. 2009;93(5):563-568.
Martin P. Wound healing - aiming for perfect skin regeneration. Science 1997;276(5309):75–81.

Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, Van Rooijen N, Takenaka H, D'Amore PA, Stein-Streilein J, Losordo DW, Streilein JW. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin Invest. 2005;115**:**2363-2372.

Matsubara M, Zieske JD, Fini ME. Mechanism of basement membrane dissolution preceding corneal ulceration. Invest Ophthalmol Vis Sci. 1991;32:3221–3237.

Mizukami Y, Jo WS, Duerr EM, Gala M, Li J, Zhang X, Zimmer MA, Iliopoulos O, Zukerberg LR, Kohgo Y, Lynch MP, Rueda BR, Chung DC. Induction of interleukin-8 preserves the angiogenic response in HIF-1 deficient colon cancer cells. Nature Med. 2005;11,992–997.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683–765.

Mäkinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, Wise L, Mercer A, Kowalski H, Kerjaschki D, Stacker SA, Achen MG, Alitalo K. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. EMBO J. 2001;20**:**4762–4773.

Oh JY, Kim MK, Wee WR. Subconjunctival and Intracorneal Bevacizumab Injection for Corneal Neovascularization in Lipid Keratopathy. Cornea. 2009 Aug 29. [Epub ahead of print]

O'Reilly MS, Wiederschain D, Stetler-Stevenson WG, Folkman J, Moses MA. Regulation of angiostatin production by matrix metalloproteinase-2 in a model of concomitant resistance. J Biol Chem. 1999, 274:29568–29571.

Patan S, Munn LL, Tanda S, Roberge S, Jain RK, Jones RC. Vascular morphogenesis and remodeling in a model of tissue repair: blood vessel formation and growth in the ovarian pedicle after ovariectomy. Circ Res. 2001;89(8):723-731.

Peirce SM. Computational and mathematical modeling of angiogenesis. Microcirculation 2008;15(8):739-751.

Pettersson A, Nagy JA, Brown LF, Sundberg C, Morgan E, Jungles S, Carter R, Krieger JE, Manseau EJ, Harvey VS, Eckelhoefer IA, Feng D, Dvorak AM, Mulligan RC, Dvorak HF. Heterogeneity of the Angiogenic Response Induced in Different Normal Adult Tissues by Vascular Permeability Factor/Vascular Endothelial Growth Factor. Lab Invest. 2000;80(1):99-115.

Pomyje J, Zivný JH, Stopka T, Simák J, Vanková H, Necas E. Angiopoietin-1, angiopoietin-2 and Tie-2 in tumour and non-tumour tissues during growth of experimental melanoma. Melanoma Res. 2001;11:639–643.

Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M**.** Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. Cytokine Growth Factor Rev. 2005;16(2):159-178.

Qutub AA, Popel AS. Elongation, proliferation & migration differentiate endothelial cell phenotypes and determine capillary sprouting. BMC Syst Biol. 2009;3:13.

Rafii S, Meeus S, Dias S, Hattori K, Heissig B, Shmelkov S, Rafii D, Lyden D. Contribution of marrow-derived progenitors to vascular and cardiac regeneration. Semin Cell Dev Biol. 2002;13:61–67.

Reiss Y, Droste J, Heil M, Tribulova S, Schmidt MH, Schaper W, Dumont DJ, Plate KH. Angiopoietin-2 impairs revascularization after limb ischemia. Circ Res. 2007; 101,88–96.

Religa P, Cao R, Bjorndahl M, Zhou Z, Zhu Z, Cao Y. Presence of bone marrow-derived circulating progenitor endothelial cells in the newly formed lymphatic vessels. Blood 2005;106:4184–4190.

Ribatti D, Vacca A, Nico B, Roncali L, Dammacco F. Postnatal vasculogenesis. Mech Dev. 2001;100(2):157-163.

Rissau W. Mechanisms of angiogenesis. Nature 1997;386 (6626):671-674.

Sato TN, Quin Y, Kozak CA, Audus KL. Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc Natl Acad Sci USA 1993;90:9355-9358.

Scroggs MW, Proia AD, Smith CF, Halperin EC, Klintworth GK. The effect of total-body irradiation on corneal neovascularization in the Fischer 344 rat after chemical cauterization. Invest Ophthalmol Vis Sci. 1991;32(7):2105-2111.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983;219:983–985.

Serini G, Bochaton-Piallat ML, Ropraz P, Geinoz A, Borsi L, Zardi L, Gabbiani G. J The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1.Cell Biol. 1998;142(3):873-881.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995;376(6535):62-66.

Shalaby F, Ho J, Stanford WL, Fischer KD, Schuh AC, Schwartz L, Bernstein A, Rossant J. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell 1997;89(6):981-90.

Shaw TJ, Martin P. Wound repair at a glance. J Cell Sci. 2009;122(Pt 18):3209-3213.

Shin SH, Kim JC, Chang SI, Lee H, Chung SI. Recombinant kringle 1–3 of plasminogen inhibits rabbit corneal angiogenesis induced by angiogenin. Cornea 2000, 19:212–217.

Sholley MM, Ferguson GP, Seibel HR, Montuor JL, Wilson JD. Mechanisms of neovascularization. Vascular sprouting can occur without proliferation of endothelial cells. Lab Invest. 1984;51(6):624-34.

Silvestre JS, Mallat Z, Duriez M, Tamarat R, Bureau MF, Scherman D, Duverger N, Branellec D, Tedgui A, Levy BI. Antiangiogenic effect of interleukin-10 in ischemia-induced angiogenesis in mice hindlimb. Circ Res. 2000;87:448–452.

Silvestre JS, Mallat Z, Tamarat R, Duriez M, Tedgui A, Levy BI. Regulation of matrix metalloproteinase activity in ischemic tissue by interleukin-10: role in ischemia-induced angiogenesis. Circ Res. 2001;89:259–264.

Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med. 1999;341(10):738-746.

Stearns ME, Rhim J, Wang M. Interleukin 10 (IL-10) inhibition of primary human prostate cell-induced angiogenesis: IL-10 stimulation of metalloproteinase-1 and inhibition of matrix metallo-proteinase (MMP)-2/MMP-9 secretion. Clin Cancer Res. 1999;5:189–196.

Stramer BM, Zieske JD, Jung JC, Austin JS, Fini ME. Molecular mechanisms controlling the fibrotic repair phenotype in cornea: implications for surgical outcomes, Invest Ophthalmol Vis Sci. 2003;44:4237–4246.

Stuart PM, Pan F, Plambeck S, Ferguson TA. FasL-Fas interactions regulate neovascularization in the cornea. Invest Ophthalmol Vis Sci. 2003;44:93-98.

Suzuki T, Sano Y, Kinoshita S. Conjunctival inflammation induces Langerhans cell migration into the cornea. Curr Eye Res. 2000;21:550-553.

Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. Ischemiaand cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med. 1999;5:434–438.

Thodeti CK, Matthews B, Ravi A, Mammoto A, Ghosh K, Bracha AL, Ingber DE.TRPV4 channels mediate cyclic strain-induced endothelial cell reorientation through integrin-to-integrin signaling. Circ Res. 2009;104(9):1123-1130.

Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. J Investig Dermatol Symp Proc. 2000;5(1):40-46.

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissuer remodeling. Nat Rev Mol Cell Biol. 2002;3:349-363.

Uy HS, Chan PS, Ang RE. Topical bevacizumab and ocular surface neovascularization in patients with Stevens–Johnson syndrome. Cornea 2008;27:70–73.

van Royen N, Piek JJ, Buschmann I, Hoefer I, Voskuil M, Schaper W. Stimulation of arteriogenesis; a new concept for the treatment of arterial occlusive disease. Cardiovasc Res. 2001;49:543–553.

Vazquez F, Hastings G, Ortega MA, Lane TF, Oikemus S, Lombardo M, Iruela-Arispe ML. METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angio-inhibitory activity. J Biol Chem. 1999;274: 23349–23357.

Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. Cell 1999;98**:**769–778.

Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, Jackson DG, Oliver G.An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J. 2002;21**:**1505–1513.

Woodley DT, Yamauchi M, Wynn KC, Mechanic G, Briggaman RA. Collagen telopeptides (cross-linking sites) play a role in collagen gel lattice contraction. J. Invest. Dermatol.1991;97:580–585.

Yoon CH, Hur J, Park KW, Kim JH, Lee CS, Oh IY, Kim TY, Cho HJ, Kang HJ, Chae IH, Yang HK, Oh BH, Park YB, Kim HS. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. Circulation. 2005;112(11):1618-1627.

Yuan, L. Moyon D, Pardanaud L, Bréant C, Karkkainen MJ, Alitalo K, Eichmann A. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. Development 2002;129**:**4797–4806.

Zaki AA, Farid SF. Subconjunctival bevacizumab for corneal neovascularization. Acta Ophthalmol. 2009 Jun 10. [Epub ahead of print]

Zhou Z, Apte SS, Soninen R, cao R, Baaklini GY, Rause RW, Wang J, Cao Y, Tryggvson K. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metealloproteinase I. Proc Natl Acad Sci USA 2000;97:4052-4057.