Transgenic Mouse Models of Corneal Neovascularization: New Perspectives for Angiogenesis Research

Jakob Nikolas Kather and Jens Kroll

Department of Vascular Biology and Tumor Angiogenesis, Center for Biomedicine and Medical Technology Mannheim (CBTM), Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany and Division of Vascular Oncology and Metastasis, German Cancer Research Center (DKFZ-ZMBH Alliance), Heidelberg, Germany

Corneal neovascularization (NV) refers to the growth of blood vessels and/or lymphatics into the physiologically avascular cornea, which occurs in several pathological processes. In mouse models, corneal NV can be artificially induced to investigate mechanisms of corneal pathologies. However, mouse models of corneal NV are not restricted to cornea-specific research, but also are widely used to investigate general mechanisms of angiogenesis. Because the cornea is transparent and easily accessible, corneal NV models are among the most useful in vivo models in angiogenesis research.

The three different approaches that are used to study corneal NV in mice are based on direct application of proangiogenic or antiangiogenic transmitters, external injury to the cornea, or genetically engineered mice, which spontaneously develop corneal NV. The aim of this review is to compare the scope and limitations of the different approaches for corneal NV in mice. Our main focus is to highlight the potential of transgenic spontaneous models of corneal NV. Transgenic models do not require any experimental interference and make it possible to investigate different interconnected proangiogenic signaling cascades. As a result, transgenic models are highly useful for disease-centered angiogenesis research.

In summary, transgenic models of corneal NV will complement and advance existing ocular NV assays, and help to discover new angiogenesis-related treatment strategies for ocular and extraocular diseases.

Keywords: cornea, neovascularization, angiogenesis, transgenic mice, animal model
FIGURE 1. Regulation of physiological avascularity, induction of corneal NV and therapeutic intervention in the cornea. Whenever the balance of pro- and antiangiogenic transmitters in the cornea is disturbed, corneal NV occurs. In this Figure, the progression from physiological avascularity to complete vascularization is shown. Blue represents DAPI-positive epithelial cell nuclei, red lines represent lymphatics, green lines represent blood vessels. I, epithelium; II, Bowman’s layer; III, stroma; IV, Descemet’s membrane; V, endothelium. (A) A number of mediators (orange) actively maintain corneal avascularity. Green margin indicates the inhibition of blood vessel growth, red margin indicates the inhibition of lymphatic vessel growth. (B) The most relevant proangiogenic factors are shown in yellow. Various studies have shown that if the concentration of any one of these...
have been shown to be indispensable for maintaining the corneal lymphangiogenic privilege in physiological settings.\(^3\) Recent advances in investigating corneal avascularity include a novel antiangiogenic system that is orchestrated by the transcription factor FoxC1.\(^5\) Also, it has been shown recently that crosstalk between developing nerve fibers and blood vessels inhibits blood vessel sprouting into the cornea (Fig. 1A).\(^6\)

Another aspect of corneal avascularity is closely linked to integrity of the corneal epithelium. Unlike the corneal endothelium, the corneal epithelium has an extensive capacity of regeneration upon injury. However, this regeneration relies on functional limbal stem cells (LSCs), which express ATP-binding cassette, subfamily B, member 5 (Abcb5).\(^17\) Differenci-ation of these cells into corneal epithelial cells is orchestrated by WNT7A–PAX6 signaling.\(^19\) If this differentiation of LSCs into corneal epithelial cells is disturbed, the corneal epithelium becomes metaplastic and displays several skin-like characteristics, for example keratinization, loss of transparency, and subepithelial corneal NV.\(^19\) Thus, a functionally intact epithelium is another prerequisite of corneal avascularity.

**Proangiogenic Signaling in the Cornea**

Physiologically, antiangiogenic factors outmatch proangiogenic factors in the cornea. In several pathological processes, this equilibrium is disturbed and proangiogenic factors dominate the corneal signaling environment (Figs. 1A, 1B).\(^3,5,20\) In experimental models of corneal NV, this process is being used to induce HA or LA.

Classical proangiogenic factors in the cornea are VEGF and fibroblast growth factor (FGF) isoforms.\(^5\) Also, platelet-derived growth factor (PDGF) isoforms,\(^21\) (placental growth factor) PLGF,\(^22\) angiopoietin-1 (Ang-1),\(^23,24\) matrix metalloproteinases (MMPs),\(^30\) and CCR2 ligands\(^25\) have proangiogenic effects in the cornea in vivo. Ellenberg et al.\(^5\) have reviewed further angiogenesis regulating mediators. For VEGF isoforms, TGF-\(\beta\), IL-1, TNF-\(\alpha\), MMPs, IL-6, and IL-8, we know that they are secreted VEGF-C and VEGF-D, and induce LA (Fig. 1B).\(^5,5\) Furthermore, it has even been suggested that macrophages themselves can transdifferentiate and integrate into the nascent lymphatic vasculature.\(^5\)

Corneal HA and LA is regulated by an intricate interplay between many different signaling pathways. Although many components of these pathways have been identified, their interaction and pathophysiological involvement must be further clarified by in vivo approaches.

**Regression of Blood Vessels**

Regression of blood vessels, also called pruning, is an essential process during vessel maturation in embryonic and postnatal development leading to a functional and hierarchically organized vascular system.\(^3\) Hyaloid vessels of the eye are a prime example for a vessel type that is temporarily formed during embryonic development, but regresses at later stages of development. Although it still is not fully clear whether vessel regression is regulated by an active genetic program or whether it is simply due to withdrawal of prosurvival angiogenic factors that usually maintain the vasculature, pruning is associated with apoptotic endothelial cells.\(^5,35\) In addition, reduced flow in blood vessels\(^36\) induces pruning and several molecular mediators, including Rac1,\(^56\) FGD5,\(^37\) and the Wnt secretion factor Evi\(^58\) have been identified in this process. In the cornea, molecular mechanisms of vessel regression are so far not well understood. Corneal blood vessels can regress after photodynamic\(^59\) or antiangiogenic therapy,\(^60\) whereas corneal lymphatic vessels form and regress spontaneously within the first weeks after birth.\(^41\) Likewise, lymphatic vessels in adult mice can regress upon corneal recovery after an inflammatory response, which may partially be mediated by MMP-10 and TGF-\(\beta\).\(^42\) Yet, also in the cornea it remains unclear whether blood and lymphatic vessel regression can be induced by a specific genetic program.

**Therapeutic Inhibition of Corneal NV**

The VEGF-A was among the first proangiogenic transmitters to be isolated and is widely viewed as the principal proangiogenic transmitters in pathological NV. In the clinic, this has led to the introduction of VEGF-A–inhibiting drugs used to inhibit tumor angiogenesis or ocular NV.\(^1,20\) However, initial euphoria about this new therapeutic approach soon wore off as it turned out that VEGF inhibition alone is not enough to effectively inhibit tumor angiogenesis.\(^1\) In the treatment of corneal NV, VEGF-targeting therapy has been shown experimentally to induce a reduction of pathological corneal NV. Approaches for anti-VEGF therapy of corneal NV have been subject of two recent in-depth reviews by Bock et al.\(^3\) and Chang et al.\(^20\) Some limitations to anti-VEGF therapy have been described. For example, some studies report only a partial efficacy of experimental anti-VEGF therapy and others report neurotoxicity as a limiting factor of these agents.\(^3,20\) Another therapeutic compound is agaunins, an antisense oligonucleotide targeting insulin receptor substrate-1, which has very recently demonstrated safety and efficacy in a phase III study (Fig. 1C).\(^45\)

**Experimentally-Induced Angiogenesis in the Eye**

For several decades, experimentally-induced angiogenesis in the eye has provided a framework for standardized assessment...
of angiogenesis in model. As early as 1974, the rabbit cornea has been
used as an experimental model to test proangiogenic
substances in a corneal pocket assay. In this procedure, a
micropocket is cut into the cornea and a pellet containing a
proangiogenic factor is implanted into this pocket. This
substance then is slowly liberated over the course of several
days and induces vessel sprouting from the limbus. The
classical corneal pocket assay remains a cornerstone of in vivo
angiogenesis research (Table 1). It has been supplemented by
two different types of ocular angiogenesis models, namely
models of retinal angiogenesis and choroidal NV. A strength of
retinal models is that physiological angiogenesis during
development can be assessed in detail in this context. Much
of the knowledge we have of the complicated interplay
between tip cells and stalk cells in sprouting angiogenesis
has been obtained in retinal models.46 Moreover, transgenic models have long been used in retinal angiogenesis
research, but have only recently been introduced in the
corneal setting. We propose that with the advent of new
models of corneal NV, the mouse cornea can efficiently
complement other ocular angiogenesis models to assess
angiogenesis in the eye, accelerating in vivo angiogenesis
research.

Two aspects form the main rationale to add new corneal
models to the available ocular angiogenesis models. First,
corneal models are the basis to investigate pathological
angiogenesis in a previously avascular tissue, as opposed to a
multitude of studies investigating physiological angiogenesis
in the retina. Studies that investigate pathological hypervascula-
ration in the retina do not start off with a previously avascular
scaffold, like the cornea. In the cornea, de novo vessel
sprouting can be directly studied, while in the retina, the
change in a preexisting vascular network is the focus of
investigation of pathological angiogenesis.

Second, angiogenesis-mediating transmitters have been
shown to exert different functions in different ocular tissues.
Consequently, different context-dependent actions of proan-
giogenic transmitters can be assessed in different ocular
angiogenesis assays. An example is the widely discussed role
of angiopoietin-1 in angiogenesis. Angiopoietin-1 has been
described as a stabilizing factor for the quiescent vasculature
and as an angiogenesis-inducing factor.48,49 It is solidly
established that administration of angiopoietin-1 causes NV.
However, in the eye, opposite roles have been demonstrated
for angiopoietin-1: For the choroid, there is evidence for a
proangiogenic and an antiangiogenic role of angiopoietin-

### Table 1. Recent Corneal Pocket Assay Studies

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Intervention</th>
<th>Reference</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of HA by depletion of natural killer cells; reduced expression of VEGF-A, VEGF-C, and VEGFR3</td>
<td>Anti-NK.1 antibody</td>
<td>56</td>
<td>bFGF pellet</td>
</tr>
<tr>
<td>Inhibition of HA in FMOD−/− mice</td>
<td>FMOD−/− mice</td>
<td>109</td>
<td>bFGF pellet</td>
</tr>
<tr>
<td>Increased VEGF induced HA in Tie2-CYP2C8-Tr mice</td>
<td>Tie2-CYP2C8-Tr mice</td>
<td>110</td>
<td>VEGF pellet</td>
</tr>
<tr>
<td>Inhibition of VEGF induced HA</td>
<td>H-RN derived peptide from hepatocyte growth factor kringle 1 domain (HGF K1)</td>
<td>58</td>
<td>VEGF pellet</td>
</tr>
<tr>
<td>Lcn2 enhances VEGF induced HA</td>
<td>Lipocalin 2 (Lcn2) protein administration</td>
<td>111</td>
<td>VEGF pellet</td>
</tr>
<tr>
<td>Semaphorin 7A, inhibition of HA</td>
<td>PTK7 siRNA injection</td>
<td>112</td>
<td>VEGF pellet</td>
</tr>
<tr>
<td>Protein tyrosine kinase 7 (PTK7) forms complex with VEGFR-1, inhibition of HA after PTK7 silencing</td>
<td>Sem7A expression vector</td>
<td>113</td>
<td>bFGF pellet</td>
</tr>
</tbody>
</table>

A selection of recent studies are listed that made use of corneal pocket assays. We distinguished between mice developing only HA, or only LA, or both.

In recent years, this multitude of ocular experimental
angiogenesis assays has been complemented by two different
assays in the mouse cornea. Over time, the number of studies
investigating corneal NV in mouse models has rapidly
increased (Fig. 2). The corneal NV models that fueled this
increase are corneal injury models (Table 2) and, most recently,
spontaneous, transgenic models of corneal NV (Table 3).

### Figure 2. An exponentially increasing number of studies investigates corneal NV. In this plot, the cumulative number of new published studies on corneal NV is shown for the last 50 years. The cornea is emerging as a widely-used model to study angiogenesis and the number of new studies has increased strongly over time. Cumulative data, plotted for each year, not smoothed. Source: PubMed (National Center for Biotechnology Information [NCBI], National Institutes of Health [NIH], Bethesda MD, USA), search query “cornea AND (neovascularization OR angiogenesis)”.

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Transgenic Mouse Models of Corneal Neovascularization
TABLE 2. Non-VEGF–Based Strategies to Inhibit Corneal NV in Injury Models

<table>
<thead>
<tr>
<th>Mechanism/Molecules</th>
<th>HA</th>
<th>LA</th>
<th>Intervention</th>
<th>Reference</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of VEGF, bFGF, TGF-β1, and EGF; upregulation of Tsp-1, Tsp-2, and ADAMTS-1 CXCR4, CXCR7, and others</td>
<td></td>
<td></td>
<td>Largazole (class I HDAC inhibitor)</td>
<td>114</td>
<td>Alkali</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC14012 (CXCR4 antagonist and CXCR7 agonist)</td>
<td>115</td>
<td>Alkali</td>
</tr>
<tr>
<td>Inhibition of HA and LA in angiopoietin-2+/− mice</td>
<td></td>
<td></td>
<td>Ang-2+/− mice</td>
<td>116</td>
<td>Suture</td>
</tr>
<tr>
<td>C57BL/6 and FVB mice</td>
<td></td>
<td></td>
<td>Anti-TNF-α antibody</td>
<td>63</td>
<td>Alkali and suture</td>
</tr>
<tr>
<td>TNR-2</td>
<td></td>
<td></td>
<td>Neutralizing anti-SDF-1α antibody</td>
<td>120</td>
<td>Alkali</td>
</tr>
<tr>
<td>Negative regulation of HA, CXCL1, and CCL2</td>
<td></td>
<td></td>
<td>MMP12 KO mice</td>
<td>117</td>
<td>Alkali</td>
</tr>
<tr>
<td>Reduced TGF-β and IL6 expression</td>
<td></td>
<td></td>
<td>Rapamycin</td>
<td>65</td>
<td>Alkali</td>
</tr>
<tr>
<td>Angiopoietin-like protein 2; regulation of F4/80 and IL-1β expression</td>
<td></td>
<td></td>
<td>Angptl2+/− mice and K14-Angptl2 mice, subconjunctival Angptl2 siRNA injection</td>
<td>118</td>
<td>Suture</td>
</tr>
<tr>
<td>Intermediate-conductance Ca(2+)-activated K(+) channel (KCa3.1), inhibition of EGF-induced HA</td>
<td></td>
<td></td>
<td>TRAM-34</td>
<td>119</td>
<td>Alkali</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neutralizing anti-SDF-1α antibody</td>
<td>120</td>
<td>Alkali</td>
</tr>
<tr>
<td>Galectin-3 increases phosphorylation of VEGFR-2, positive regulation of HA</td>
<td></td>
<td></td>
<td>L1-10, an Ang-2-specific inhibitor</td>
<td>121</td>
<td>Suture</td>
</tr>
<tr>
<td>IRS-1, inhibition of HA and LA</td>
<td></td>
<td></td>
<td>GS-101 (Aganirsen), an antisense oligonucleotide against IRS-1</td>
<td>123</td>
<td>Suture</td>
</tr>
<tr>
<td>Increased expression of Robo4 upon HSV infection; Robo4 ligand Slit2 inhibits HA</td>
<td></td>
<td></td>
<td>Injection of Slit protein</td>
<td>74</td>
<td>HSV-infection</td>
</tr>
<tr>
<td>Inhibition of inflammation</td>
<td></td>
<td></td>
<td>Corticosteroids</td>
<td>124</td>
<td>Suture</td>
</tr>
<tr>
<td>Programmed death ligand-1 (PD-L1), negative regulator of HA</td>
<td></td>
<td></td>
<td>PD-L1+/− mice</td>
<td>67</td>
<td>Suture</td>
</tr>
<tr>
<td>Toll-like receptor 4 (TLR4), activation of HA by TLR4 ligand HMGB1</td>
<td></td>
<td></td>
<td>TLR4+/− and TLR2+/− mice, topical application of HMGB1</td>
<td>125</td>
<td>Alkali</td>
</tr>
<tr>
<td>Very late antigen-1 (VLA-1)</td>
<td></td>
<td></td>
<td>VLA-1 neutralizing antibody</td>
<td>126</td>
<td>Suture</td>
</tr>
<tr>
<td>Neureilin 2 (NRP2)</td>
<td></td>
<td></td>
<td>NRP2 artificial miRNA</td>
<td>127</td>
<td>Suture</td>
</tr>
</tbody>
</table>

Selected recent studies are listed investigating non-VEGF-related inhibition of NV in corneal injury models. We distinguished between mice developing only HA, or only LA, or both. √, reported by study; ×, not reported by study.

For the cornea, there is no such debate. At least four studies describe proangiogenic functions of angiopoietin-1 and/or its receptor Tie2 in the cornea.23,24,53,54 This example shows that well-characterized angiogenesis-related transmitters still may have different roles in different ocular tissues. Consequently, it is worth the effort to broaden the choice of available tools to investigate experimental angiogenesis in the eye.

We now will describe and compare the three different approaches to study angiogenesis in the cornea. These approaches are summarized in Figure 3.

CORNEAL POCKET ASSAYS AND INJURY MODELS

Corneal Pocket Assay

The classical angiogenesis assay in the mouse cornea is the corneal (micro)pocket assay in which a pellet is implanted into the cornea (Table 1). The pellet contains one or more proangiogenic factors, which are gradually set free over the course of several days. As a result, corneal limbal blood vessels are activated and start to grow toward the pellet.55,56 To assess hemangiogenesis and lymphangiogenesis, VEGF-A or bFGF pellets are used, while to assess lymphangiogenesis only, VEGF-C pellets can be used.45,57 The appropriate experimental protocols have been reviewed extensively.45,55,57 The extent of corneal NV typically is assessed 5 to 7 days after pellet implantation.45,56,58 Even though the experimental conditions in corneal pocket assays are highly standardized, there is considerable variation in angiogenic and lymphangiogenic response depending on the genetic background of the mouse strain.59–61 This shows that while corneal pocket assays are a good method for initial screening of proangiogenic substances, care should be taken when quantitatively comparing the results.

External Injury and Corneal Transplantation

The corneal epithelium has a pronounced capacity for regeneration after injury. Therefore, in mice, superficial corneal abrasion that leaves the corneal limbus intact, completely heals within 7 to 14 days.62 However, more severe external injury or prolonged epithelial defects (e.g., by infection) can disturb corneal integrity in such a way that stromal NV occurs. This effect is used in injury models for NV in murine corneas, which we will explain in detail in the following section.

Most routinely used injury models rely on suture injury or alkali burn (Table 2).65 Here, we briefly present the most widely used injury approaches. In alkali burn models, a specified amount of NaOH (mostly 0.1 N or 1 N) is applied for a specified time (typically 10–30 seconds).64–66 In suture models, one or more nylon sutures are placed eccentrically in the corneal stroma.67,68 These nylon threads remain in the cornea and induce inflammation and NV (HA and LA). Another mechanical injury approach makes use of extensive removal of the corneal epithelium, including the limbus.69 This epithelial
### Table 3. Mouse Models Showing Spontaneous Corneal NV

<table>
<thead>
<tr>
<th>Mouse Model</th>
<th>Mechanism</th>
<th>HA</th>
<th>LA</th>
<th>Time Course</th>
<th>Intervention</th>
<th>Special Features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Destrin⁻/⁻ mice, corn1</td>
<td>Pro-angiogenic VEGF-3 signaling, deficient in sVEGFR-1</td>
<td>✓</td>
<td>✓</td>
<td>Starts at 20 days</td>
<td>Rescue by VEGFR-3 neutralization or application of sVEGFR-1</td>
<td>Spontaneous corneal HA and LA, differentially regulated by genetic background, different time kinetics of HA and LA</td>
<td>10, 77, 78, 94, 96, 97</td>
</tr>
<tr>
<td>TSP-1⁻/⁻ mice</td>
<td>Loss of antiangiogenic signaling of CD36+ macrophages</td>
<td>×</td>
<td>✓</td>
<td>Starts at 6 mo</td>
<td>Rescued by wildtype bone marrow cells</td>
<td>TSP-1 inhibits VEGF-C release and LA by binding to its receptor CD36 on monocytes</td>
<td>13</td>
</tr>
<tr>
<td>CD36⁻/⁻ mice</td>
<td>Loss of TSP-1 – CD36 interaction</td>
<td>✓</td>
<td>✓</td>
<td>Late onset</td>
<td>None</td>
<td>Uregulation of VEGF-A is observed when CD36 is knocked out</td>
<td>13, 84</td>
</tr>
<tr>
<td>KLEH/KLHL20⁻/⁻ mice</td>
<td>Proangiogenic miR-204 – Ang-1 pathway</td>
<td>✓</td>
<td>✓</td>
<td>Median time 8 wk</td>
<td>Anti-VEGF therapy not effective</td>
<td>Includes macrophage infiltration and spontaneous corneal NV; accelerated by superficial abrasion</td>
<td>24, 62</td>
</tr>
<tr>
<td>LRIG1⁻/⁻ mice</td>
<td>LRIG1 acts as a negative regulator of STAT3</td>
<td>✓</td>
<td>×</td>
<td>Median time 18 mo</td>
<td>STAT3 inhibition by STA21 or wildtype bone marrow cells rescue phenotype</td>
<td>Spontaneous, can be induced by injury</td>
<td>85</td>
</tr>
<tr>
<td>K5.Stat3C mice</td>
<td>Proinflammatory STAT3 signaling</td>
<td>✓</td>
<td>×</td>
<td>Similar to LRIG1⁻/⁻</td>
<td>None</td>
<td>Similar phenotype to LRIG1 deficient mice</td>
<td>85, 86, 87</td>
</tr>
<tr>
<td>JamA⁻/⁻ mice</td>
<td>VEGF expression and TGF-β activation</td>
<td>✓</td>
<td>×</td>
<td>Starting early but low prevalence</td>
<td>Inhibition of HA by DC101 (anti VEGFR-2 antibody)</td>
<td>Spontaneous, can be accelerated by injury, approximately 50% prevalence at 6–12 mo</td>
<td>80% prevalence in homozygous mice (only in C57BL/6 strain). Null mice are not viable</td>
</tr>
<tr>
<td>ADAMTS9⁻/⁻ mice</td>
<td>ADAMTS9 is an antiangiogenic metalloprotease</td>
<td>✓</td>
<td>×</td>
<td>Median time</td>
<td>None</td>
<td>High penetration</td>
<td>129</td>
</tr>
<tr>
<td>LeCre;Cited2loxP/loxP;MxCre;Rbp4⁻/⁻ mice</td>
<td>Presumably via Pax6 and Klf4</td>
<td>✓</td>
<td>×</td>
<td>8 mo</td>
<td>None</td>
<td>Spontaneous NV affects various ocular and extraocular tissues (cornea, retina, liver, lung)</td>
<td>130</td>
</tr>
<tr>
<td>Pax6 overexpression using the Aldh3a1 promoter</td>
<td>Chi3h4, Flt-1, Wif1</td>
<td>✓</td>
<td>×</td>
<td>Approximately 6–12 wk</td>
<td>None</td>
<td>Up to 60% prevalence, associated with corneal inflammation</td>
<td>88</td>
</tr>
<tr>
<td>Pax6⁻/⁻ mice</td>
<td>Presumably due to epithelial defects; sVEGFR-1 deficiency</td>
<td>✓</td>
<td>×</td>
<td>Early phenotype starting at 2 weeks</td>
<td>Rescued by application of sVEGFR-1</td>
<td>Incomplete prevalence (different prevalence reports), several anterior eye defects including corneal NV</td>
<td>10, 89, 90</td>
</tr>
<tr>
<td>Global FoxC1⁻/⁻ mice and neural-crest specific FoxC1⁻/⁻</td>
<td>Via VEGF – VEGFR-2 signaling</td>
<td>✓</td>
<td>✓</td>
<td>Detectable at 15 days after birth</td>
<td>Inhibition of HA by blocking VEGF-2 signaling</td>
<td>Linked to disrupted epithelial architecture; FoxC1⁻/⁻ mice show spontaneous corneal NV, FoxC1⁻/⁻ mice show increased corneal NV upon injury</td>
<td>15</td>
</tr>
<tr>
<td>LeCre;VEGFR-2loxP/loxP;MxCre;Rbp4⁻/⁻ mice</td>
<td>Lack of sVEGFR-2</td>
<td>×</td>
<td>✓</td>
<td>At birth</td>
<td>Inhibition of LA by blocking VEGF-3 signaling</td>
<td>100% prevalence in N = 30 mice</td>
<td>14</td>
</tr>
<tr>
<td>pCre;VEGFR-1loxP/loxP;MxCre;Rbp4⁻/⁻ mice</td>
<td>Lack of sVEGFR-1</td>
<td>✓</td>
<td>×</td>
<td>Starting 2 d after pCre injection</td>
<td>None</td>
<td>Fully vascularized at 14 days, high prevalence in n = 10 mice</td>
<td>10</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td>Unknown</td>
<td>×</td>
<td>×</td>
<td>P6–P14</td>
<td>None</td>
<td>During development, the mouse cornea presents lymphatic vessel sprouting and regression</td>
<td>41</td>
</tr>
<tr>
<td>Nude mice, nu/nu, and hairless mutant mouse strain, SKH1/hr/hr</td>
<td>Proangiogenic factors probably coming from the epithelium</td>
<td>✓</td>
<td>✓</td>
<td>6–12 wk old mice</td>
<td>None</td>
<td>Spontaneous corneal NV associated with absence of hair in 2 different genetic mouse models</td>
<td>76, 92</td>
</tr>
<tr>
<td>Heme oxygenase (HO)-2</td>
<td>MMP-2</td>
<td>✓</td>
<td>×</td>
<td>Not applicable</td>
<td>shRNA injection</td>
<td>Local knockdown, not transgenic. Negative regulation of HA</td>
<td>131</td>
</tr>
</tbody>
</table>

Selected spontaneous mouse models of corneal NV are summarized, most of which represent transgenic mice. We distinguished between mice developing only HA, or only LA, or both, ✓, reported by study; ×, not reported by study.
Debridement must be distinguished from superficial epithelial abrasion, which does not induce corneal NV in wild-type mice and completely heals within a few days.62 Another way to induce corneal NV by injury aims at damaging the LSCs, which are needed for corneal epithelial regeneration and contribute to corneal avascularity. In patients with LSC deficiency (LSCD), corneal NV is observed commonly.18 Three approaches to induce LSCD with corneal NV in mouse models use chemical injury by benzalkonium chloride,70 transplantation of corneas lacking Abcb5-positive LSCs,17 or UV-B irradiation.71 Other similar models use thermal cauterization.

Another very useful approach is the corneal transplantation model, which can be used to study corneal NV in a clinically highly relevant setting. After allograft transplantation, corneal inflammation and NV occur, both of which can be pharmacologically attenuated.72,73 It is not clear whether corneal NV in these models is primarily due to inflammation and/or due to mechanical injury. Therefore, we subsume this model in the category of external injury models.

Another very elegant, yet rarely applied approach is based on corneal infection, for example by external application of HSV-1 virus particles to the murine cornea.74,75 These injury-based models are well standardized and are used widely in different variations. Their major disadvantage is that the damage inflicted upon the cornea is so grave that not only angiogenesis is induced, but also a number of other processes, for example wound healing, epithelial proliferation, and inflammation. Still, some of these approaches are highly relevant models for clinically relevant scenarios. For example, the investigation of angiogenesis in the context of corneal transplantation can yield important insights into mechanisms of allograft failure in patients. Furthermore, the interplay between inflammation and angiogenesis can be studied efficiently in corneal injury and corneal infection models, which, therefore, are highly useful for translation of knowledge to the clinic.

Transgenic Mouse Models in Corneal NV

Research

Two decades ago, spontaneously occurring corneal NV has been demonstrated in two mouse models.76 Yet, this discovery has not stimulated the use of spontaneous models of corneal NV in angiogenesis research. Recently, however, several genetically engineered mice developing spontaneous corneal NV were presented. Here, relevant transgenic models of corneal NV are explained (see also Table 3 for a summary of these models).

Destrin\(^*/\) Mice (Corn1)

Destrin\(^*/\) model mice carry a homozygous mutation in the destrin gene and develop spontaneous corneal HA and LA.77,78 Both processes can be diminished by blocking VEGF-VEGFR-3 signaling: efficient inhibition of NV is achieved by external application of a blocking anti-VEGFR-3 antibody or by application of sVEGFR-1 (for which these corneas are deficient).10,78 Corneal HA reaches 100% prevalence at 4 weeks after birth and does not regress within 12 months, while prevalence of corneal LA is incomplete (reaches 60% at 3 months and decreases to approximately 15% at 12 months).78 Notably, the destrin\(^*/\) model shows a low-inflammatory form of angiogenesis. Inflammatory activity as measured by the number of CD45-positive cells in corneal sections of vascularized corn1 corneas is much lower than in vascularized corneas after allograft transplantation.79 Although corneal HA partially regresses after application of anti-VEGFR-3 antibody, some degree of pathological HA still is present.78 Yet, external application of sVEGFR-1 completely inhibits corneal NV in this model.10 These findings show that the destrin\(^*/\) model can be used to characterize antiangiogenic potential of pharmacological inhibitors. Because the destrin\(^*/\) phenotype has an early onset and a high penetrance, it is very well suited for angiogenesis research.

TSP-1\(^*/\) Mice and CD36\(^*/\) Mice

In 2004, it was reported that knockout mice for TSP-1 and/or TSP-2 do not show spontaneous hemangiogenesis.79 Interestingly, six years later, the same research group found out that TSP-1\(^*/\) mice do show spontaneously lymphatically vascularized corneas at the age of six months, while control corneas are devoid of lymphatics at that time point.13 Additionally, TSP-1\(^*/\) mice also show decreased intraocular pressure (IOP).80 However, it has not yet been investigated whether decreased IOP is linked to increased ocular lymphangiogenesis this model.

With TSP-1\(^*/\) mice, a model to investigate mechanisms of spontaneous, isolated LA is available. Since the description of the TSP-1\(^*/\) model, several interesting facets of this ocular phenotype have been reported. For example, the corneal and lacrimal gland phenotype of the TSP-1\(^*/\) mouse resembles human Sjögren’s syndrome.81 Therefore, TSP-1\(^*/\) mice have been used to investigate pathophysiological mechanisms of Sjögren’s syndrome.81,82

In the TSP-1\(^*/\) model, prolymphangiogenic signaling has been shown to critically depend on the presence of CD36-positive monocytes/macrophages.13 This is consistent with the observation that maintenance of lymphatic vessels depends on corneal stromal macrophages in a corneal injury model.83

FIGURE 3. Common experimental approaches to induce corneal NV in mice. In this Figure, the four most frequently used approaches for NV induction in murine corneas are shown. (A) corneal pocket assay (depicted here is the growth of blood vessels and lymphatics from opposite sides as observed with bFGF pellets). (B) Suture injury model. (C) Chemical injury by alkali burn. (D) Spontaneous transgenic model. Red arrows represent lymphatics, green arrows represent blood vessels.
Additionally, spontaneous corneal LA in TSP-1−/− mice parallels spontaneous corneal LA in CD36−/− mice.84 However, corneal LA in CD36−/− mice takes approximately 1 year to develop.84

In short, two main features make the TSP-1−/− model well suited for angiogenesis research: First, its proximity to Sjögren’s syndrome qualifies it as a disease-oriented model (as opposed to corneal injury models and corneal pocket assays). Second, the complex interaction of macrophages and lymphatics in this model can be used to investigate further basic mechanisms of lymphangiogenesis.

**LRIG1+/− Mice and K5.Stat3C Transgenic Mice**

The protein leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) is a marker of corneal epithelial LSCs and ocular epithelial basal cells.85 Its knockout leads to spontaneous corneal epithelial dysplasia and stromal NV.85 The mechanism behind this phenotype is disinhibition of a proinflammatory Stat3 pathway after LRIG1 knockout. K5.Stat3C mice, which express a constitutively active form of Stat3, show a comparable phenotype. Consequently, LRIG1+/− mice and K5.Stat3C transgenic mice can serve as models to investigate mechanisms of spontaneous inflammatory angiogenesis.

**Inducible VEGFR-1 and VEGFR-2 Knockout Mice**

From two recent studies, we know that sVEGFR-1 and sVEGFR-2 are important mediators of corneal angiogenic or lymphangiogenic privilege.10,14 In these studies, inducible pCre,VEGFR-1loxP/loxP and LeCre,VEGFR-2loxP/loxP mice were created that lacked these soluble receptors and consequently showed spontaneous hem- or lymphangiogenesis in the cornea.

**Jam-A+/− Mice**

Knockout of junctional adhesion molecule A (Jam-A) results in spontaneous corneal NV, inflammation, and opacification. These changes are linked to activation of various proinflammatory and proangiogenic systems, among them the TGF-β pathway and the VEGF-A–VEGFR-2 pathway.86 Interestingly, in this model of corneal NV, a contribution of bFGF is unlikely because presence of Jam-A has been shown to be essential for bFGF-induced angiogenesis.87 However, a limitation for the practical use of these mice is the incomplete penetrance of this phenotype.86 Still, mechanistically, the Jam-A+/− model is a very attractive model for angiogenesis research.

**FoxC1+/− or NC-FoxC1−/−, and FoxC1+−/− or NC-FoxC1−/− Mice**

In 2011, Seo et al.15 identified the transcription factor FOXC1 as an essential mediator of corneal clarity and avascularity, and described the following knockout phenotype. Global knockout mice and neural crest (NC) specific knockout mice show spontaneous corneal HA and LA dependent on VEGFR-2 signaling. Interestingly, corneal HA starts during embryonic development and the cornea is completely covered by blood vessels at birth (P0). Also, lymphatic vessels reach deep into the cornea at P0. Moreover, heterozygous mice also show highly increased angiogenic sprout formation in the cornea, but do not become fully vascularized. For angiogenesis research, this model is highly attractive because it facilitates experimental procedures in two ways. First, in these mice, corneal NV is present at birth and second, heterozygous mice also can be used for research, which triples biological sample size per litter.

**C57BL/6 Mice**

Recently, Zhang et al.41 systematically investigated the murine corneal development in C57BL/6 mice. They discovered that during postnatal days P6 and P14, LYVE-1-positive lymphatic vessels reach deep into the corneal stroma. After P21, these vessels regress and the cornea remains devoid of blood vessels. This process of lymphangiogenesis and subsequent spontaneous regression is exceptional and offers wide-ranging research perspectives. For example, a full molecular characterization of signaling pathways involved in this process would possibly elucidate new basic mechanisms of lymphangiogenesis. Although this mouse model is not a transgenic model, we subsume it here because of the spontaneous vessel growth and vessel regression seen in these mice.

**Pax6 Overexpression or Heterozygocity**

Corneal overexpression of Pax6 using the aldehyde dehydrogenase family 3, subfamily A1 (Aldh3a1) promoter results in epithelial abnormalities and inflammatory stromal NV.88 This combination of epithelial and stromal phenotypes is consistent with the notion that the corneal epithelium helps to maintain corneal avascularity. Remarkably, some of these features (including stromal NV) also occur in Pax6+/− mice (for which one study reports partial prevalence89 and another study reports 100% prevalence90). A recent study shed light on this situation because it was shown that Pax6 is the single most important regulator of corneal epithelial cell identity.19 Apparently, an impairment of tightly regulated Pax6 signaling either way (partial loss of function or gain of function) compromises corneal epithelial quiescence, leading to secondary stromal NV. One mechanism of stromal NV in Pax6+/− mice is deficiency for sVEGFR-1 and external application of sVEGFR-1 rescues Pax6+/− phenotype.10 For practical use in angiogenesis research, Pax6+/− mice are partly suited: although phenotype onset occurs as early as 2 weeks after birth, penetrance is incomplete.89 In Pax6 overexpressing mice, prevalence of neovascular phenotype differs depending on the genetic background and is always below 70%.89 To establish these mice as standard models of corneal NV, breeding could optimize this prevalence.

**KLEIP+/− Mice (KLHL20+/−)**

Homozygous mice deficient for the intracellular protein KLEIP/KLHL20 show a progressive corneal dystrophy with spontaneous stromal HA and LA, reaching 100% prevalence at 4 months after birth.52,91 Spontaneous corneal NV in this model is promoted by angiopoietin-1, which in turn is upregulated because its inhibition by miR-204 is lost in this model.24 It can, therefore, serve as a starting point to evaluate non-VEGF targeting therapy approaches for pathological angiogenesis. Corneal NV in KLEIP+/− mice is associated with presence of macrophages in the dystrophic cornea, but it is presently unknown whether these macrophages are required for HA and/or LA in this model.24,62 In practice, KLEIP+/− corneal NV is well suited for angiogenesis research because it has a high penetrance and the time course of phenotype progression is well characterized.

**Nude Mice and Hairless SKH1;hr/hr Mice**

Lastly, we want to point out an almost 25-year-old finding by Niederkorn et al.76 They reported that immunodeficient nude (nu/nu) mice show subepithelial corneal NV. This phenotype is not due to immunodeficiency because severe combined immunodeficient (SCID) mice do not show any corneal blood
vessels. Rather, the phenotype seems to be related to the lack of hair because another hairless mutant strain (SKH1/hr/hr) displayed a similar phenotype. Later, this phenotype was roughly linked to epithelium-derived proangiogenic factors.

To our knowledge, the exact molecular mechanisms of corneal NV in hairless mice have not been investigated by today’s state of the art methods. This example shows that spontaneous corneal NV in transgenic mice offers a significant and currently unused potential for angiogenesis research.

**Advantages and Limitations of Transgenic Models of Corneal NV**

**Similar Etiologic Factors in Different Transgenic Models of Corneal NV**

In transgenic mouse models, corneal stromal NV often is linked to a disturbed corneal epithelial architecture and the transformation of corneal epithelium toward skin-like epithelium. For example, this is the case in LRIG1⁻/⁻, KLEIP⁻/⁻, Pax6⁻/⁻, destrin⁻/⁻/corne1, and Notch1⁻/⁻ mice (Notch1⁻/⁻ only after corneal injury). A comparable change is characteristic of some human corneal diseases, for example xeropthalmia. Two etiologic factors contributing to these changes of corneal epithelial architecture in mouse models are LSC deficiency and disturbed control of epithelial cell proliferation.

The LSC deficiency, corneal epithelial dysplasia, and stromal NV are observed in mice deficient for LRIG1, a moderately specific LSC marker and regulator of inflammation. Interestingly, the corneal phenotype of LRIG1⁻/⁻ and KLEIP⁻/⁻ mice is similar morphologically and on an expression level. Our unpublished data show that LRIG1 expression is reduced 2.5 fold in late-stage KLEIP⁻/⁻ vascularized corneas (P = 0.0018, N = 3 ± 3 mice, see the methods of Kather et al.) suggesting an involvement of LSCs in KLEIP⁻/⁻ corneal phenotype development.

Furthermore, control of corneal epithelial cell proliferations requires functional actin cytoskeleton dynamics. For example, corneal epithelial dystrophy in destrin⁻/⁻/corne1 is due to compromised intracellular actin cytoskeleton remodeling. Similarly, the KLEIP protein has been linked to regulation of actin cytoskeleton assembly so that also in KLEIP⁻/⁻ corneal NV development, corneal epithelial actin polymerization might be an etiologic factor.

These findings show that different transgenic mouse models of corneal NV may share mechanisms of phenotype development. This emphasizes the usefulness of spontaneous corneal NV models in angiogenesis research, because these models make it possible to identify and investigate several intertwined signaling pathways that ultimately lead to corneal NV.

**Comparison of Transgenic Models to Corneal Pocket Assays**

In corneal pocket assays, one or more proangiogenic transmitters are directly implanted into the cornea, and stimulate the growth of blood vessels and/or lymphatics toward a gradient. However, in basic and translational angiogenesis research, the reductionist approaches that centered on single-transmitter mechanisms of angiogenesis are replaced by a more comprehensive view entailing interconnected regulatory mechanisms of angiogenesis. In transgenic corneal NV models, proangiogenic signaling is partly due to canonical VEGF signaling (like in destrin⁻/⁻/corne1 model) or to VEGF-independent signaling (like in late stage KLEIP⁻/⁻ corneal NV). In several transgenic corneal NV models, an involvement of several different proangiogenic mechanisms is conceivable. Thereby, transgenic models offer a possibility to investigate these intertwined mechanisms in a well-characterized and experimentally accessible setting.

It should be noted that, as mentioned above, the extent of the angiogenic and lymphangiogenic response in corneal pocket assays depends on the genetic background of the strain. This also has been reported with transgenic models of corneal NV, for example in destrin⁻/⁻ (corn1) mice. In this model, the corneal phenotype is more pronounced in A.BY than in C57Bl/6 mice. In contrast, in transgenic models of corneal NV, high-inflammatory (such as TSP-1⁻/⁻) and low-inflammatory (such as destrin⁻/⁻/corn1) models are available. Also, in other transgenic models of corneal NV, like the KLEIP⁻/⁻ model, inflammation is present but the mechanistic link to angiogenesis has not been investigated yet. Therefore, transgenic models of corneal NV offer a convenient framework to investigate new mechanisms that link inflammation to angiogenesis in the cornea.

Still, transgenic corneal NV models will complement, but not replace, existing corneal NV models.

**Comparison of Transgenic Models to Retinal and Choroidal Models**

In retinal and choroidal NV models, transgenic mice have long been incorporated into angiogenesis research. Physiological angiogenesis in these tissues has been used to elucidate basic mechanisms of angiogenesis, such as paracrine signaling in angiogenic sprouts. Also, retinal models of pathological hyper-vascularization have yielded insights into the pathogenesis of various clinically relevant diseases, such as diabetic retinopathy or retinopathy of prematurity. Thus, these models already have proven to be very useful, but transgenic models of corneal NV still offer different perspectives: Compared to retinal and choroidal models, corneal NV models start off with an avascular tissue but still represent pathological angiogenesis.

While the focus of retinal models of pathological angiogenesis is the transition of a healthy vasculature into an aberrant and harmful vascular network, corneal NV model allow to easily image the pathological de novo vascularization of a tissue.

Furthermore, transgenic mouse models of corneal NV differ from the widely established transgenic models of choroidal NV in one important aspect: Whereas in choroidal NV models, phenotype prevalence is incomplete and limits experimental feasibility, this is not the case with all transgenic corneal NV models. For example, LRIG1⁻/⁻ and KLEIP⁻/⁻ mice develop corneal NV in a well-characterized time course and with 100% prevalence. In FoxC1⁻/⁻ mice, corneal NV develops even
in heterozygous mice, which allows for an even higher yield of samples.\textsuperscript{15}

Therefore, transgenic models of corneal NV can complement retinal and choroidal NV models and help to clarify mechanisms of de novo angiogenesis. Because the extent of this form of angiogenesis can be easily quantified in the cornea, we will describe the most relevant imaging models in the following section.

**IMAGING AND QUANTIFYING CORNEAL NEOVESSELS**

As the cornea is a thin, easily accessible, and physiologically transparent tissue, imaging blood vessels and lymphatics is relatively easy in the cornea. Using confocal microscopy, the complete corneal vasculature can be imaged. Blood vessels and lymphatics inside the corneal stroma can be fully captured in detail (Fig. 4). Using two-photon microscopy, it is even possible to capture cellular migration in vivo in real time. For example, a corneal NV model made it possible to capture immune cell immigration into lymphatic vessels for the first time.\textsuperscript{100}

Ex vivo, corneal specimens usually are assessed quantitatively in a planar dimension (in whole mount samples, Fig. 5) or in an apical-basal dimension (in tissue sections, Fig. 6).

In a planar dimension, the most common parameter for NV is area covered by vessels, which can be measured either manually or semiautomatically. Bock et al.\textsuperscript{101} have introduced a semiautomatic, threshold-based method for morphometry of angiogenesis and lymphangiogenesis in corneal whole mounts. This method excels manual counting techniques in accuracy, objectivity, and reproducibility.\textsuperscript{101} Blacher et al. have extended this toolkit to measurement of morphological features, such as density of vessel extremities or density of branching points in the corneal neovascular plexus.\textsuperscript{102} A similar method has been used by Dastjerdi et al.\textsuperscript{103} to quantify corneal NV in slit-lamp images. Several other image analysis approaches for blood vessel segmentation have been implemented for images of the retinal vasculature, but not yet for the cornea.\textsuperscript{104}

Analysis of vessel coverage of the cornea has yielded surprising results. For example, it has been shown that vascularization in the nasal part of the cornea develops in a different way than vascularization in the temporal part.\textsuperscript{105} Also, lymphatic vessels in the nasal part of the cornea show more lymphatic valves than vessels in the temporal cornea.\textsuperscript{106} Additionally, the morphology of the growing vasculature differs depending on the etiologic factor.\textsuperscript{107} These findings highlight the benefit of accurately analyzing planar vascular growth patterns in mouse corneas.

**FIGURE 4.** Corneal blood vessels and lymphatics imaged with confocal microscopy. Compared to other mouse tissues, the cornea is an ideal organ to study experimentally induced angiogenesis, because it can easily be imaged in conventional and confocal fluorescence microscopy. Using confocal microscopy, the corneal tissue can be imaged as a whole, so that the complete corneal vasculature can be analyzed. In this Figure, CD31-positive blood and lymphatic vessels (A) and LYVE-1-positive lymphatics (B) in a KLEIP\textsuperscript{+/−} cornea are shown. (C) Shows the overlay of CD31 and LYVE-1 staining.

**FIGURE 5.** Manual quantification of NV in corneal whole mounts. In mouse models of corneal NV, such as the transgenic KLEIP\textsuperscript{+/−} model, vessels sprout from the vascular plexus at the limbus toward the corneal center. The extent of corneal NV can be quantified by measuring the remaining avascular area. This is shown in (A) for blood vessels and in (B) for lymphatics (area was delineated by hand in sample images). (C) A DAPI staining of a control cornea is shown. Measuring DAPI-positive area permits normalization of area measurements to total corneal area.
In an apical-basal direction, different patterns of corneal NV can be distinguished, for example subepithelial and deep stromal NV. These vascularization patterns can be distinguished by quantitative measurements in corneal sections. This parallels blood vessel growth in the mouse retina, where different apical-basal blood vessel distribution patterns have been described.46

CLINICAL PERSPECTIVES

In recent years, antiangiogenic therapy has been a widely-discussed issue in the context of corneal NV, retinal hypervascularization, and also in the context of extraocular diseases20,108. In the cornea, NV is associated with loss of visual acuity and allograft failure. In extraocular pathologies, antiangiogenic therapy offers strong therapeutic perspectives. However, until now, most antiangiogenic therapy approaches have been of limited efficacy. A possible reason for this discrepancy between the high expectations and the limited success of antiangiogenic therapy could be the focus on therapeutic options targeting only one transmitter. In vivo models, such as transgenic spontaneous corneal NV models, make it possible to investigate blood vessel growth that develops under real pathophysiological conditions, with minimal to no experimental intervention. Transgenic mouse models of corneal NV have been used to identify several NV-related signaling cascades, such as TSP-1 - CD36 - VEGF-C pathway, STAT3 signaling, miR-204–Ang-1 axis and others (Table 3). Yet, it still must be investigated how these transmitters affect corneal NV in humans.

In summary, transgenic models of corneal NV can be used to characterize the complex interaction between various proangiogenic pathways, thereby helping to overcome the limitations of current antiangiogenic therapy in the clinic.

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