Scg3 as a Novel Disease-Related Factor for Diabetic Retinopathy Therapy

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UNIVERSITY OF MIAMI

SCG3 AS A NOVEL DISEASE-RELATED FACTOR FOR DIABETIC RETINOPATHY THERAPY

By

Michelle Elizabeth LeBlanc

A DISSERTATION

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SCG3 AS A NOVEL DISEASE-RELATED FACTOR FOR DIABETIC
RETINOPATHY THERAPY

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Diabetic retinopathy (DR) is one of the most common diabetic vascular complications. Angiogenic factors, such as VEGF (vascular endothelial growth factor), play important roles in the pathogenesis of vision-threatening DR. A major breakthrough is the recent approval of anti-VEGF therapy for diabetic eye disease however, its limited therapeutic efficacy implicates the involvement of other angiogenic factors in the pathogenesis. Endothelial factors with preferential binding to the diseased vasculature may represent novel targets for alternative ligand-based therapies. In this thesis, I applied a new technology of “comparative ligandomics” to diabetic and healthy mice for the differential mapping of disease-related ligands. Secretogranin III (Scg3) displayed minimal binding to healthy vessels but ~1,731-fold increase in binding to the diabetic endothelium. In contrast, VEGF showed comparable binding to both the diabetic and healthy vasculature. These findings were independently verified using a cornea angiogenesis assay in which Scg3, but not VEGF, preferentially promoted angiogenesis in diabetic mice. Accordingly, Scg3 and VEGF activated distinct receptor signaling pathways. However, both factors were elevated in the diabetic vitreous fluid, suggesting that similar to VEGF, Scg3 may also play a role in DR pathogenesis. The role of Scg3 as a therapeutic target was confirmed using a neutralizing polyclonal antibody
(pAb) in a mouse model of diabetic retinal leakage and ocular neovascularization. To demonstrate how the identification of a disease-related factor by comparative ligandomics can impact ligand discovery, we generated a Scg3-neutralizing monoclonal antibody (mAb) and verified its therapeutic efficacy to reduce vascular leakage and angiogenesis \textit{in vivo}. These findings established Scg3 as a disease-related target for anti-angiogenic therapy and provide a foundation for future mAb humanization and clinical trials. Characterization of Scg3 supports the use of comparative ligandomics for identification of disease-associated ligands.
Dedication page

This work is dedicated to my parents Joseph and Margaret LeBlanc and my fiancé Dr. Jonathan Chinea, all of whom have supported me throughout the years. To my parents who instilled in me the belief that I could accomplish anything I set my mind to. I want to thank them for their continual love and guidance. They have gone above and beyond to help me achieve my goals. To Jonathan, I would like to thank him for his continual support over the past 4 years, his countless readings of this manuscript and perpetual encouragement. His faith in me has never dwindled and I could not imagine a better partner for this journey. I would also like to thank Barbara Chinea-Cobo, who always reminds me to stop and enjoy the simple pleasures in life. Finally, I would like to acknowledge my grandmother, Margaret Wich, who suffered from wet AMD and was one of the inspirations for my research interests in the eye. Thank you to all of you, I could not have done it without each and every one of you.
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Liu, Dr. Abigail Hackam and my external examiner Dr. Xin-Hai Pei. A special thanks to Dr. Liu for his intellectual discussion and helping to establish *in vitro* endothelial cultures. Thanks to Dr. Webster for allowing our lab to borrow his antibodies and for his intellectual discussions, particularly regarding *in vivo* therapy. Thank you to Dr. Hackam for her critical review of our manuscripts and for answering my many questions throughout the dissertation writing process.
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List of Abbreviations

AMD: Age-related macular degeneration
APP: Amyloid precursor protein
BRB: Blood retinal barrier
BSA: Bovine serum albumin
CgA: Chromogranin A
CgB: Chromogranin B
CNV: Choroid neovascularization
CSPG: Chondroitin sulfate proteoglycan
DFC: Dual functional cloning
Dil: 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate
DME: Diabetic macular edema
DR: Diabetic retinopathy
EC: Endothelial cell
ERK: Extracellular signal-regulated kinase
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate
GFP: Green fluorescent protein
Glut2: Glucose transporter 2 receptor
HDGF: Hepatoma-derived growth factor
HRMVEC: Human retinal microvascular endothelial cell
HRP-3/Hdgfrp3: hepatoma-derived growth factor-related protein 3
HSPG: Heparan sulfate proteoglycan
HUVEC: Human umbilical vein endothelial cell

IgG: Immunoglobulin G

JAK: Janus kinase

mAb: Monoclonal antibody

MAPK: Mitogen-activated protein kinase

MEK: Mitogen-activated protein/ extracellular signal-regulated kinase

NGS: Next generation DNA sequencing

NPDR: Non proliferative diabetic retinopathy

NV: Neovascularization

OPD: Open reading frame phage display

ORF: Open reading frame

pAb: Polyclonal antibody

PBS: Phosphate buffered saline

PDGF: Platelet-derived growth factor

PDR: Proliferative diabetic retinopathy

PFC: Phagocytosis-based functional cloning

PIGF: Placental growth factor

PS: Phosphatidylserine

RAGE: Receptor for advanced glycation end products

Rcn1: Reticulocalbin 1

RPE: Retinal pigment epithelium

Scg2: Secretogranin 2

Scg3: Secretogranin 3
SN: Secretoneurin
Src: Proto-oncogene c-Src
STAT: Signal transducer and activator of transcription
STZ: Streptozotocin
Tulp1: Tubby-like protein 1
VEGF: Vascular endothelial growth factor
VEGFR1: Vascular endothelial growth factor 1
VEGFR2: Vascular endothelial growth factor 2
Chapter 1: Introduction

1.1. The retina and its vascular system

The blood supply for the inner and outer retina arises from two distinct sources. The inner 2/3rd of the retina receives its blood supply from the central retinal artery, bringing in blood and nutrients through the optic nerve. This artery branches to form a dense connection of arteries and veins which together makes up the intermediate and deep capillary beds [1]. In contrast, the outer 1/3rd of the retina is devoid of blood vessels. This region receives its blood and oxygen from the choroidal vasculature. The choroid contains a dense network of blood vessels, known as the choroidal capillaries. This vascular bed has high blood flow and fenestrated endothelial cells (ECs) which allow nutrients to diffuse between the retina and the choroid [1].

In the retina, vascular EC tight junctions maintain the influx and efflux of plasma proteins. Together, these ECs form the inner blood retinal barrier (BRB), which confers immune privilege to this tissue [2]. Deregulation of essential growth factors, such as vascular endothelial growth factor (VEGF) and concurrent EC dysfunction can lead to the breakdown of the BRB under pathological conditions. This breakdown represents an early event in disease onset and contributes to the feedforward mechanism of vascular disease progression [1].

1.2. The functional role of VEGF in the vasculature

VEGF is an important growth factor which regulates endothelial function and helps maintain the integrity of the BRB [3]. This protein is part of a large family which includes placental growth factor (PIGF) and VEGFA-D. VEGFA can exist as six
different isoforms each with different biological availability depending on its location [4]. The isoforms for VEGFA are as follows: 121, 145, 165, 183, 189 and 206. VEGFA165 (hereafter referred to as VEGF) is the primary isoform implicated in endothelial angiogenesis and will be the focus for this thesis [5, 6].

In the eye, VEGF is secreted from the retinal pigment epithelium (RPE), ECs, pericytes, neurons, müller cells and astrocytes. VEGF was the first growth factor identified with dual roles as an angiogenic and pro-survival as well as a vascular permeability factor [7, 8]. Early studies using tumor models established this connection between EC permeability and angiogenesis [9]. Endothelial permeability may prime the adult vasculature to support new vessel growth. In the healthy vasculature, mature ECs are less dependent on VEGF stimulation which contributes to minimal new vessel formation [1]. Permeable vessels allow plasma proteins to deposit into the extracellular environment, altering the extracellular matrix composition and transforming it into a hospitable environment favoring pathogenic neovascularization (NV) [9]. In this manner, EC permeability may serve as a prerequisite for new vessel growth and define a link between vascular permeability and angiogenesis [9, 10].

VEGFA-induced vascular permeability and angiogenesis are mediated through interactions with tyrosine kinase receptors VEGF receptor 1 (VEGFR1, Flt-1) and VEGF receptor 2 (VEGFR2, Flk-1/KDR) expressed on the endothelial surface (Fig. 1.1). VEGFR2 binds VEGFA, VEGFB and VEGFC, and is the main promoter for VEGF-driven EC proliferation, migration, angiogenesis and survival [4, 11, 12]. VEGFR2−/− mice do not develop a functional vascular system and are
embryonically lethal (~embryonic day 8), highlighting the essential role of VEGFR2 in vessel development [13]. VEGFR1, binds VEGFA, PIGF and VEGFB. This receptor is believed to play an indirect role in angiogenesis possibly through EC migration [14-16]. VEGFR1 can exist as a membrane bound or soluble receptor. Soluble VEGFR1 arises from an alternative splice variant. Interestingly, VEGFA has a significantly higher binding affinity to VEGFR1 ($K_d \sim 5-20$ pM) than VEGFR2 ($K_d \sim 400-800$ pM) [17, 18] and therefore soluble or membrane bound VEGFR1 can act as an efficient decoy receptor, preventing VEGF from binding to VEGFR2 [19]. VEGFR1 shows weak auto-phosphorylation and kinase activation compared with VEGFR2. It contains an inhibitory domain which blocks PI3 kinase activation [20]. Mice lacking VEGFR1 die in utero and show pronounced defects in vascular organization and function, suggesting that like VEGFR2, VEGFR1 is essential for proper embryonic vasculature development [21]. In addition to its interactions with tyrosine kinase receptors, VEGF can bind endothelial surface molecules heparan sulfate proteoglycans (HSPGs) and neuropilin 1, and 2 (Fig. 1.2) [22]. In the absence of VEGF binding these co-receptors do not elicit a biological response [23]. However, in the presence of VEGF they enhance VEGF interactions with its tyrosine kinase receptors [4, 6]. This is the case for secretoneurin (SN), an angiogenic peptide derived from secretogranin 2 (Scg2), which binds HSPGs to effectively enhance VEGF binding to VEGFR2 [24]. In this manner, co-receptors can link previously unappreciated angiogenic factors with the VEGF signaling pathway [6].
Fig. 1.1. VEGF receptors and their functional roles

VEGFRs1-3 are expressed on the EC surface. VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR) are involved in EC angiogenesis. However VEGFR2 is the primary mediator of new vessel growth [1]. VEGF can also interact with cell surface molecules such as heparan-sulfate proteoglycans (HSPGs) and neuropilin 1, and 2 [22]. The role of these co-receptors are discussed in Fig. 1.2.

Fig. 1.2. HSPGs can facilitate VEGF binding to VEGFRs

VEGF can interact with endothelial surface molecules such as heparan-sulfate proteoglycans (HSPGs) and neuropilin 1, and 2. This binding event enhances VEGF interactions with tyrosine kinase receptors (VEGFR1-3) [4, 6, 22]. In this manner co-receptors serve to as a bridge to link angiogenic factors with VEGFRs for enhanced signaling [6].
VEGFR2 is the main tyrosine kinase receptor implicated in EC angiogenesis [1]. Through its molecular signaling, VEGF and VEGFR2 can activate the full range of angiogenic activities, including EC survival, proliferation, permeability and tube formation [25]. Survival and anti-apoptotic pathways are associated with phosphorylation of Akt kinase [26]. EC proliferation is mediated through activation of extracellular signal regulated kinase 1/2 (Erk1/2). The ability of VEGF to induce transient vascular permeability is modulated through Src protein kinase activation which induces dysregulation of endothelial tight junctions and adherens junctions [27]. EC migration and tube formation is attributed to activation on the P38/ mitogen-activated protein kinases (MAPK) pathways [28]. The Janus kinase/signal transducers and activators of transcription (Jak/Stat) pathway is one of the major signaling mechanisms activated by cytokines and growth factor receptors. Jak/Stat activation results in Stat dimerization and translocation into the nucleus, where this factor can up-or down-regulate gene transcription. Of importance to this thesis is the ability of Stat to upregulate gene expression of VEGF [29]. Furthermore, activation of Stat signaling exacerbates pathogenic vessel growth in a murine model of retinopathy of prematurity [30]. In RPE cells, Stat3 is involved in survival, inflammation and cytokine release. Stat3 is also associated with ocular diseases involving RPE and photoreceptor dysfunction, relevant to this thesis is its role in age-related macular degeneration [31]. It should be noted that the above-mentioned pathways represent only a handful of the protein kinases involved in EC angiogenesis. VEGF kinase activation has a
dynamic and complex profile. The summary in Fig. 1.3 highlights the angiogenic protein kinases that will be discussed in this thesis.

![Fig. 1.3. VEGF and VEGFR2 angiogenic signaling](image)

**Fig. 1.3. VEGF and VEGFR2 angiogenic signaling**

This schematic of VEGF signaling pathways shows only a handful of VEGFR2-mediated angiogenic kinases investigated in this thesis. VEGFR2 is the main tyrosine kinase receptor implicated in VEGF-mediated angiogenesis. In contrast, VEGFR1 is only indirectly involved in angiogenesis [1]. Through its molecular signaling, VEGF and VEGFR2 can activate a full range of angiogenic activities, including EC survival (Akt), proliferation (Erk1/2, Stat3), permeability (Src) and tube formation (Akt and Erk1/2) [25].

### 1.3. The role of VEGF in the diseased vasculature

VEGF dysregulation is associated with diseases marked by endothelial permeability and NV. Relevant examples include solid tumors, retinal vein occlusion and retinopathy of prematurity [19]. Relevant to this thesis is that VEGF in the vitreous of PDR patients correlates with disease progression [32, 33].

The role of VEGF in diabetic eye disease was independently validated using non-diabetic animal models. In rodents, an intravitreal injection of VEGF is
sufficient to trigger vascular changes seen in early stage diabetic eye disease, including retinal leakage and BRB breakdown. VEGF injected intravitreally into non-diabetic primates is sufficient to induce preretinal NV, hemorrhage, venous bleeding, microaneurysms, capillary closure and retinal permeability, all of which are vascular changes seen in late stage diabetic eye disease [34]. In diabetic animal models, neutralization of VEGF reduces pathogenic DR-associated vascular changes. These findings highlight the role of VEGF in early and late stage diabetic eye disease and the use of its inhibitors for disease therapy [35, 36].

1.4. Diabetes and diabetic retinopathy

It is estimated that ~25.6 million Americans over the age of 20 have diabetes [37] and 3 out of 5 diabetics will develop micro- or macrovascular complications. Diabetic complications include coronary heart disease, atherosclerosis, diabetic nephropathy, diabetic neuropathy and diabetic retinopathy (DR) [38]. Vision-threatening diabetic retinopathy (DR) is one of the most common diabetic complications affecting those with chronic diabetes [39]. It is also one of the leading causes of blindness in working-age Americans [11]. It is divided into two stages, the early non-proliferative stage (NPDR) and the later stage called proliferative DR (PDR). In either phase patients may develop vision-threatening diabetic macular edema (DME) [11, 40]. PDR and DME represent the most severe vision-threatening complications in diabetic eye disease [41]. The prevalence and severity of DR highlights the importance of developing new therapies for disease management [20, 38].
1.4.1. Non-proliferative diabetic retinopathy (NPDR)

Diabetic eye disease is progressive and results from endothelial dysfunction, characterized by the apoptosis of ECs and pericytes, vascular leakage and leukocyte adhesion [42]. These dysfunctions occur early in disease progression and may lead to acellular capillaries and microaneurysms, all of which contribute to the feedforward destruction of the BRB [3]. In NPDR patients do not present with the growth of abnormal blood vessels. This finding distinguishes NPDR from proliferative diabetic eye disease. Patients with mild or moderate NPDR can often be managed by controlling their blood glucose, blood pressure and lipid levels [1].

1.4.2. Diabetic macular edema (DME)

The etiology of DME is complex but begins with the breakdown of the BRB [43], similar to NPDR [42]. DME is defined as an increase in fluid retention, retinal thickness, exudate accumulation and lipid deposits present near or around the macula, the area of the retina responsible for central vision. Although DME can occur at any stage in DR, its prevalence and severity varies between type 1 and type 2 diabetics and correlates with disease progression [44]. Macular edema is not exclusive to diabetic eye disease and can also occur as a result of ocular surgery, retinal vein occlusion, intraocular inflammatory diseases or age-related macular degeneration (discussed in a later chapter) [43].

1.4.3. Proliferative diabetic retinopathy (PDR)

Advanced vision-threatening proliferative diabetic eye disease is marked by extensive capillary death, non-perfusion and hypoxia-induced angiogenesis and retinal vascular bleeding [37, 39, 45] (Fig. 1.4). In the retina, hypoxia markedly
induces VEGF expression, particularly in the pericytes, RPE, Müller cells, ganglion cells and glia [38]. This upregulation contributes to a feedforward mechanism of angiogenesis, marked by the growth of abnormal, fragile, leaky and unstable vessels [46]. If left untreated, pathogenic retinal NV can lead to hemorrhages, retinal detachment, neovascular glaucoma and significant vision loss [41].

Fig. 1.4. Cartoon illustration of a healthy retina versus late stage diabetic retinopathy

This cartoon illustrates the differences between a healthy retina (A) and a retina with PDR (B). In the healthy retina the vasculature is intact, and therefore there is negligible retinal leakage and minimal angiogenesis [1]. In contrast, PDR is marked by retinal leakage and NV, as the presence of NV distinguishes this phase from advanced NPDR. Owing to the retinal vasculature leakage, blood, protein and macromolecules can deposit into the eye [36, 38, 44]. Concurrently, abnormal NV can grow into the vitreous fluid and trigger retinal detachment which leads to severe vision loss [45].

1.5. Age-related macular degeneration (AMD)

Age-related macular degeneration (AMD) is one of the leading causes of vision loss in the elderly population. Of the 8 million Americans who have AMD, 1 million will go on to develop vision-threatening symptoms [47]. In advanced AMD, patients lose the ability to recognize faces, read and drive.

AMD is divided into early, intermediate and late stages. Only in the later stages, it is further classified into dry and wet AMD. AMD dysfunction occurs in four
functionally interdependent tissues: photoreceptors, RPE, Bruch’s membrane and choroidal capillaries. Dry AMD is marked by the degeneration of the outer retina, particularly the RPE and photoreceptors. This degeneration of the outer retina is known as geographic atrophy. Additionally, dry AMD patients may present with drusen deposits. Unlike dry AMD, the hallmark of wet AMD is the presence of choroidal NV (CNV). If left untreated, CNV can invade across Bruch’s membrane, through the RPE layers and into the retina. These vessels can leak blood in the subretinal space or vitreous fluid to cause visual impairment or blindness (Fig. 1.5). Advanced wet AMD can culminate in RPE detachment, hemorrhage, gliosis and calcification [47]. AMD is not static and patients can progress from dry to wet AMD, although the mechanism behind this transition is not entirely understood [48]. One possibility is that subretinal deposits of drusen in dry AMD contain angiogenic metabolites which are sufficient to trigger CNV [49].

![Fig. 1.5. Cartoon illustration of wet age-related macular degeneration](image)

This cartoon highlights some of the phenotypic changes seen in wet AMD. This illustration shows the growth of new vessels from the choroidal vasculature. These vessels are centered near the macula, the part of the retina responsible for central vision. If left untreated, these vessels will grow into the retina and cause bleeding with severe vision loss. Also illustrated in this image are drusen deposits, which are clinically correlated with the transition from dry to wet AMD [46].
1.6. Laser and steroid therapy for ocular disease

Focal or grid laser photocoagulation therapy can be beneficial for patients with diabetic eye disease and wet AMD. The primary goal of photocoagulation therapy is visual preservation not visual improvement [1]. Photocoagulation therapy for DME is effective in reducing fluid retention specifically macular swelling, and can help restore visual acuity. Laser therapy can also minimize retinal bleeding and prevent additional vessel growth [46]. However, photocoagulation is not without side effects and can lead to retinal scarring with possible secondary NV and peripheral blind spots [46].

In 2014, long-lasting slow-release corticosteroid implants, Dexamethasone and Fluocinolone, were approved for DME therapy due to their anti-inflammatory and anti-permeability properties [42, 50]. However, intraocular steroid use can lead to elevated intraocular pressure with the possibility of secondary corticosteroid-induced glaucoma [51]. Ocular steroid use also increases the chance of developing cataracts [52].

1.7. Anti-VEGF therapy for ocular disease

VEGF inhibitors are the only anti-angiogenic therapy approved for DME, PDR in patients with DME and wet AMD, highlighting anti-angiogenic therapy as a valuable strategy for ocular disease management [43, 53]. FDA-approved anti-VEGF agents include: bevacizumab (Avastin, Genetech/Roche), ranibizumab (Lucentis, Genetech), pegaptanib (Macugen, EyeTech) and aflibercept (Eylea, Regeneron) (Table 1.1) [54].
Bevacizumab (Genetech) is a recombinant humanized full-length 149 kDa monoclonal antibody (mAb), with high affinity for VEGFA. It was first approved for systemic administration in patients with colon cancer [54]. Although bevacizumab is not FDA-approved for diabetic eye disease or wet AMD, it is used as an off-label therapy [54].

Ranibizumab (Genetech) was developed for intraocular administration in patients with ocular disease. Ranibizumab is a soluble Fab fragment (~49kDa) generated from the full-length humanized anti-VEGF mAb. As an improvement over bevacizumab, ranibizumab shows enhanced potency, affinity, and efficacy for VEGFA [55]. Since this inhibitor is a Fab fragment, as opposed to a full-length antibody, it has a shorter half-life and a reduced chance of adverse systemic events. It is FDA-approved for DME, PDR with DME, and wet AMD [54].

Pegaptanib (Eye Tech) is a PEGylated aptamer of a modified oligonucleotide against VEGF165 [54]. Aptamers are oligonucleotides which are selected based on high binding affinity to select protein targets. Pegaptanib was approved for wet AMD in 2004 and for being the first aptamer approved for human therapy [56].

Aflibercept (Regeneron) is a soluble VEGF-trap containing the chimeric extracellular domains of VEGFR1 (the second domain) and VEGFR2 (the third domain) covalently fused to human Immunoglobulin G1 (IgG1) Fc domain [57]. Aflibercept binds and neutralizes VEGFA, PIGF and VEGFB across species with high affinity [54]. It exhibits higher binding affinity to VEGFA than either bevacizumab or ranibizumab, and is FDA-approved for DME and wet AMD [55].
1.7.1. Anti-VEGF resistance

Although VEGF inhibitors display moderate clinical efficacy, they are not without limitations. One obvious limitation is that all the above mentioned inhibitors target the VEGF pathway and thereby act through a similar molecular mechanism (Table 1.1). This is supported by the finding that VEGF inhibitors are only capable of restoring vision in 21-50% of patients with diabetic eye disease [38, 50]. Owing to the limited therapeutic options targeting molecules other than VEGF, these non-responders are frequently switched to a different VEGF inhibitor, often aflibercept [58], despite similar molecular mechanisms [55, 59]. This highlights the importance of identifying alternative ligand-based therapies with distinct molecular pathways, specifically for those who show a poor response to anti-VEGF therapy.

### Table. 1.1. VEGF inhibitors

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Brand name</th>
<th>Company</th>
<th>Ligands Bound</th>
<th>Type of neutralization</th>
<th>FDA-approved for ocular disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevacizumab</td>
<td>Avastin</td>
<td>Genetech/ Roche</td>
<td>VEGFA</td>
<td>full-length humanized mAb</td>
<td>No * Off label</td>
</tr>
<tr>
<td>Ranibizumab</td>
<td>Lucentis</td>
<td>Genetech</td>
<td>VEGFA</td>
<td>Fab fragment (49 kDa)</td>
<td>DME, PDR with DME and wet AMD</td>
</tr>
<tr>
<td>Pegaptanib</td>
<td>Macugen</td>
<td>EyeTech</td>
<td>VEGFA165</td>
<td>PEGylated aptamer</td>
<td>wet AMD</td>
</tr>
<tr>
<td>Aflibercept</td>
<td>Eylea</td>
<td>Regeneron</td>
<td>VEGFA, PIGF, VEGFB</td>
<td>VEGF-trap</td>
<td>DME, PDR with DME and wet AMD</td>
</tr>
</tbody>
</table>

* approved for colon cancer

1.7.2. Safety limitations of anti-VEGF therapy

Due to potential systemic side effects, VEGF inhibitors are only approved for ocular therapy via intravitreal administration [60]. This is based on the finding that systemic delivery of bevacizumab for colon cancer showed an increased risk of
severe or fatal side effects including, gastrointestinal perforation, delayed wound healing, hemorrhage, thromboembolic events, hypertension and proteinuria [60]. Despite targeted intraocular delivery, anti-VEGF may still enter systemic circulation, albeit at a relatively low level, and lead to unwanted side effects [59, 61, 62].

Adverse effects associated with VEGF inhibitors can be attributed to the essential role of this factor in ECs, neurons and RPE cells [63, 64]. Accordingly, mice with the deletion of a single VEGF allele result in embryonic lethality [65]. This is further supported by in vitro data in which ECs treated with high doses of bevacizumab and ranibizumab showed an increase in apoptosis and reduced proliferation [66, 67]. Using in vivo models, repetitive injections of VEGF inhibitors at high doses reduced organ vessel density [68]. These findings illustrate the safety concerns associated with VEGF inhibitors and the importance of developing additional therapeutics against alternative targets with minimal side effects.

1.8. Cellular ligands as target for disease therapy

Cellular ligands extrinsically regulate a broad spectrum of physiological and pathological functions. They can also be harnessed for ligand-based therapy by either overexpressing beneficial factors (e.g., insulin for diabetes) or blocking detrimental factors (e.g., anti-VEGF therapy) [69-71]. In regards to ocular diseases, this is best illustrated by the discovery of anti-VEGF for the management of patients with diabetic eye disease and wet AMD. The identification of disease-related factors may delineate novel targets for ligand-based therapy. However, the challenge is how to systematically identify EC ligands with therapeutic potentials.
1.9. Open reading frame phage display (OPD) for ligand identification

All endothelial ligands, including angiogenic factors, are traditionally identified on a case-by-case basis with technical challenges. This knowledge gap in turn hinders the capacity to develop additional ligand-based therapies. Conventional phage display with an antibody or random peptide library has been used to identify antibodies or small peptides for drug targeting [72] or vascular imaging [73]. However, these identified targets are not VEGF-like endogenous ligands and cannot help understand the extrinsic regulation of cellular functions. In other applications, conventional phage display using cDNA libraries has been used to identify protein binding partners [74]. The pitfall to this conventional system is the high rate of out-of-frame unnatural short peptides, as opposed to endogenous ligands. For example, ~80-94% of phage clones enriched from conventional cDNA libraries were non-open reading frames (non-ORFs), encoding unnatural short peptides without biological implications [74, 75].

To tackle these limitations, my group previously developed a new technology of open reading frame phage display (OPD) to systemically identify cellular proteins with specific binding or functional activities, including cellular ligands. The main distinction between OPD and conventional phage display is its ability to identify real endogenous ligands with a high percentage of enriched clones in open reading frames (ORFs) [76]. This approach markedly improves the probability of identifying endogenous binding proteins or cellular ligands with biological activity.
1.9.1. OPD as a tool to identify biologically relevant ligands

OPD was developed as a new dimension of functional proteomics with versatile applications to identify cellular proteins with specific binding or functional activities. We first demonstrated that OPD can efficiently enrich phage clone binding to immobilized protein bait (e.g., tubby) and rapidly identify protein-protein interactions [77, 78]. The majority of identified proteins were independently verified. We then applied OPD to phosphatidylserine (PS) immobilized on ELISA plates as a non-protein bait and successfully identified PS-binding proteins [74]. Furthermore, my group showed that OPD can efficiently identify substrates cleavable by a protease [79]. These findings support OPD technology as a versatile, convenient and efficient tool to identify cellular proteins with specific binding or functional activities.

Based on the above successes, my lab expanded OPD to cellular ligand research. We designed the phage selection strategy of phagocytosis-based functional cloning (PFC) to enrich OPD clones displaying phagocytosis ligands. Tubby-like protein 1 (Tulp1) was identified as a novel phagocytosis ligand [77]. A subsequent study verified Tulp1 as a new ligand for phagocytosis receptor MerTK [80]. My group then developed a selection strategy of dual functional cloning (DFC) by combining PFC with receptor-based binding selection to enrich receptor-specific phagocytosis ligands. This new approach discovered galactin-3 (Gal-3) as a novel phagocytosis ligand for MerTK receptor [81]. These studies suggest that OPD technology is capable of efficiently identifying cellular ligands or receptor-specific ligands [82].
1.9.2. OPD-NGS as the first paradigm of ligandomics

The above mentioned accomplishments from my group were significant in that it demonstrated the versatility of OPD to identify endogenous ligands with biological activity. However, all previously identified ligands were selected by manually picking and analyzing enriched phage clones. To convert this low throughput approach into high throughput profiling, we combined OPD with next generation DNA sequencing (NGS). This shifted OPD technology from a labor intensive procedure to a systematic screening technology with high efficiency. My group applied OPD-NGS to phagocytes to globally identify all enriched phagocytosis ligands in the absence of receptor information. When applied to RPE cells, a phagocyte in the retina, we demonstrated that OPD-NGS can efficiently identify Abcf1 as a novel phagocytosis ligand [83]. In a separate study, we showed that OPD-NGS successfully identified Reticulocalbin 1 (Rcn1) as a new microglial phagocytosis ligand [83, 84].

Phagocytosis ligands are a special type of cellular ligands that can be separated from non-phagocytosis ligands by PFC selection for OPD-NGS analysis [77]. However, most cell surface ligands are not phagocytosis ligands. An important question is whether OPD-NGS is broadly applicable to these cell surface-binding, non-phagocytosis ligands. To address this question, my co-worker performed three rounds of OPD by in vivo binding selection in live mice to enrich endothelial ligands (Fig. 1.6), as previously described [85, 86]. Following this in vivo selection, I preformed NGS analysis to identify all enriched ligands. Through this process I identified and independently verified hepatoma-derived
growth factor-related protein 3 (Hdgfrp3 or HRP-3) as a novel endothelial ligand with angiogenic activity [85]. Additionally, I further identified hepatoma-derived growth factor (HDGF) from OPD-NGS data analyses and characterized this protein as an angiogenic factor in the eye [86].

**Fig.1.6. OPD-based in vivo binding selection**

OPD cDNA libraries were purified and intravenously injected into mice. After circulating for 20 min, unbound phages were removed by intracardial perfusion. Retinas were isolated and homogenized to release endothelium-bound phages, which were amplified in bacteria and used as input for the next round of selection. After 3 rounds of selection, cDNA inserts of enriched phage clones were amplified and identified by NGS with simultaneous quantification of the cDNA inserts for individual clones [84, 85].

Given the capacity for OPD-NGS to globally identify cell-wide ligands, either phagocytosis or non-phagocytosis ligands, we termed this high-throughput approach as “ligandomics” [85]. To my knowledge, prior to this study ligandomics had not been possible for any cell type. Thus, OPD-NGS is the first paradigm of
ligandomics that is capable of identifying all enriched ligands in the absence of receptor information. We predict that ligandomics will drastically improve the technical capability to identify unknown endothelial factors for novel ligand-based therapies, as highlighted by the discovery of secretogranin III (Scg3). In this thesis, characterization of Scg3 as a novel EC factor with potential for ligand-based therapy will in turn support the validity of this technology.

1.10. The granin family

Scg3 belongs to the granin family which includes chromogranin A (CgA), chromogranin B (CgB) and Secretogranin (Scg) II-VII. All granin family members share a calcium-binding domain and an acidic isoelectric point. Many granin proteins are precursors for biologically active peptides. This is consistent with the fact that granin proteins contain repeating basic amino acid sequences, which serve as enzymatic cleavage sites [87, 88]. Granins are predominantly localized to endocrine and neuroendocrine cells, nervous tissue and peptidergic neurons, where they play key roles in the regulated secretory pathway [89]. Regulated secretion, unlike constitutive protein secretion, is tightly managed for the controlled release of peptides, hormones, neurotransmitters and growth factors. Prior to secretion, these factors must be sorted and packaged into secretory vesicles. This mechanism for vesicle biogenesis, packaging and protein sorting is dependent on granin proteins [90]. Granins as well as the prohormones they sort are targeted to this pathway through interactions with specific receptors. Some granin proteins themselves can function as sorting receptors [90, 91].
CgA was the first granin family member to be isolated and characterized for its role in the regulated secretory pathway. Like other family members, CgA promotes granule biogenesis and peptide hormone sorting [89]. CgB and Scg2 also play important roles in the secretory pathway where they function as chaperones for proteins destined for secretory vesicles. However, some granin proteins, such as CgA and Scg2, forgo their roles in regulating peptide hormone secretion and are themselves processed into biologically active peptides [90]. These peptides are released along with other hormones in a regulated manner. Granin-derived biologically active peptides are involved in pain, inflammation, metabolism, mood disorders, blood pressure and angiogenesis [90].

1.10.1. The granin family in angiogenesis

CgA and Scg2 are angiogenic peptide precursors [92-95]. Due to a lack of knowledge regarding the functional role of Scg3, we have looked to these granin prohormones and their peptides as models to help elucidate the putative angiogenic role of Scg3.

Scg2 is the precursor for the angiogenic peptide secretoneurin (SN) [95]. SN promotes neurite outgrowth, attracts monocytes and induces EC proliferation [96]. It is a potent angiogenic factor which promotes cornea angiogenesis in vivo and tube formation in vitro [97]. As a hypoxia-driven factor, SN has been investigated for its role in coronary artery disease, myocardial infarction and DR [95, 96]. Interestingly, in some tissue-specific endothelial cells SN promotes its angiogenic activity through an indirect activation of known growth factors, such as VEGF [98].
Another well-studied granin family member implicated in angiogenesis is CgA. Full-length CgA itself can function as an angiogenic inhibitor. Additionally, CgA can also be cleaved to produce two peptides: vasostatin-1 (CgA\textsubscript{1-76}) and cestatin (CgA\textsubscript{352-372}). Although both are implicated in angiogenesis, these two peptides play opposing roles. Vasotatin-1 is a potent angiogenic inhibitor \cite{92, 93}, whereas cestatin promotes endothelial chemotaxis, proliferation, angiogenesis and vascularization \cite{94}. The identification of Scg2 and CgA as angiogenic precursors was significant in that it defined a new and previously unappreciated functional role for granin family members in this pathway.

1.10.2. The functional role of Scg3

Like other granin family members, Scg3 is present in endocrine and neuroendocrine cells where it plays an important intracellular role in the regulated secretory pathway \cite{99, 100}. Prohormones and granin proteins are targeted to the regulated secretory pathway through interactions with sorting receptors and scaffolds such as cholesterol. Scg3 itself is sorted through binding to cholesterol-rich domains in secretory vesicles, and in this manner it facilitates its own recruitment. A unique property of Scg3 is that when anchored to cholesterol, it can bind and recruit CgA and Scg2 to these vesicles \cite{90, 91}. In this manner, Scg3 plays an integral role as an intracellular protein involved in recruitment of itself, other granin proteins and peptide hormones.

The role of Scg3 as an extracellular protein is less clear. Scg3 undergoes post translational modifications as a chondroitin sulfate proteoglycan (CSPG). CSPGs signal through hormonal, autocrine or paracrine mechanisms and are
implicated in angiogenesis and morphogenesis [101-103]. Although full-length Scg3 is secreted from endocrine and neuroendocrine cells [91], its secretion is elevated from activated platelets [104] and dysfunctional β-cells [105]. This implicates a role for Scg3 in the diseased vasculature (i.e. atherosclerosis and diabetic vascular complications). However, the functional role of Scg3 remains the least characterized of all granin family members. To date, Scg3 has no known receptor and prior to this thesis its role as a cellular ligand or angiogenic factor had not been defined.

1.11. Research objective and hypothesis

The objective for this thesis is to validate ligandomics as a tool for the identification of disease-related factors with therapeutic potential. To accomplish this objective, we performed a ligandomics analysis in a mouse model of DR to uncover Scg3 as a novel DR-high ligand with preferential binding to the diabetic vasculature. The hypothesis is that Scg3 functions as a unique disease-associated angiogenic and exudative factor, with potential for ligand-based therapy (Fig. 1.7). Characterization of Scg3 in this thesis will in turn validate ligandomics as an efficient tool for discovery and rapid development of disease-related ligand-based therapies. Although initially identified in a model of vascular permeability, Scg3 may also function as an angiogenic factor, similar to VEGF, albeit with enhanced activity in diabetic ECs. This preferential activity will be independently verified using a cornea angiogenesis assay in diabetic and healthy mice. This work is significant in that it is the first study to investigate the extracellular functions of Scg3 in the vasculature.
The ability of anti-Scg3 therapy to reduce diabetic retinal leakage and NV was evaluated using a neutralizing polyclonal antibody (pAb). To further illustrate how the discovery of a novel disease-associated angiogenic factor by comparative ligandomics can impact ligand-based therapy, we developed a Scg3-neutralizing monoclonal antibody (mAb) and characterize its potential for the therapy of DME (retinal leakage), PDR (retinal angiogenesis) and wet AMD (choroidal angiogenesis). This will provide the basis for humanization and future clinical trials using Scg3 mAb. Data from this thesis will in turn facilitate the translation of anti-Scg3 from bench to bedside.
Fig. 1.7. Hypothesis cartoon

Using comparative ligandomics, Scg3 was identified as a DR-high ligand with preferential binding towards the diabetic vasculature, possibly due to an upregulation of its receptor on the diseased vasculature. Based on family homology, Scg3 is predicted to function as a vascular permeability and angiogenic factor. Accordingly, Scg3 inhibition may represent a novel therapeutic target for a reduction in pathogenic leakage and NV, observed in DR, PDR and wet AMD. Therefore, the hypothesis is that Scg3 functions as a unique disease-associated angiogenic and exudative factor, with potential for ligand-based therapy.
Chapter 2: Materials and methods

2.1. Reagents

Reagents and their sources were as follows: C57BL/6 mice (~6 weeks old, Cat. #664) and C57BL/6-Ins2 Akita/J mice (Cat. #003548) were from the Jackson Laboratory. HRMVECs (#ACBRI 181) and complete classic medium kit with serum and CultureBoost (#4Z0-500) were from Cell Systems. HUVECs (#C2519) and EGM-2 bulletkit (#CC-3162), including EBM-2 medium (#CC3156), were from Lonza. 293 SFM II medium (#11686-029) was from Life Technologies. Human Scg3 (#16012-H08H) and mouse Scg3 (#51561-M08H) were from Sino Biological. Human VEGF165 (#293-VE-010/CF), VEGFR1-Fc (#321-FL-050/FC) and VEGFR2-Fc (#357-KD-050/FC) were from R&D Systems. Amicon centrifugal filter units (#UFC501008) were from Millipore. Affinity-purified anti-Scg3 pAb (#10954-1-AP), anti-Plekha1 pAb (#10238-1-AP) and anti-VEGFR2 (#26415-1-AP) were from Proteintech. Aflibercept (Eylea) (NDC 61755-0005-02) was from Regeneron Pharmaceuticals. Anti-EKR1/2 (#9102), anti-phosphorylated EKR1/2 (#4377), anti-Akt (#4691), anti-phosphorylated Akt T308 (#13038), anti-phosphorylated MEK (#23385), anti-MEK (#9126), anti-phosphorylated Stat3 S727 (#9145), anti-Stat3 (#12640), anti-Src (#2109), anti-phosphorylated Src Y416 (#2101) anti-phosphorylated VEGFR2 (Y1175, #2478s) and anti-β-actin (#4967) were from Cell Signaling. Alexa Fluor 488-conjugated isoelectin B4 (#I21411) and Alexa Fluor 488-conjugated anti-rabbit antibody (#A11008) were from Life Technologies. STZ (#S0130), FITC-dextran (#FD4), methycellulose (# M0512), fibrinogen (#F8630), aprotinin (#A1153) and thrombin (#T4648) were from Sigma. Whatman filter paper
(Grade 3) (#1003-090) was from GE Healthcare Life Sciences. Evans blue (#2129), Dil (#42364) and protein G-beads (#P7700) were from Sigma. High concentration Growth Factor Reduced (GFR) Matrigel (#354263) was from Corning Life Sciences. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (#I6758), polyethylene glycol 8000 (#1546605), DNase I (#D5025) and CsCl (#289329) were from Sigma. 9E10 hybridoma was from Developmental Studies Hybridoma Bank. Serum-free hybridoma medium was from Thermo Fisher Scientific (#12045-084). Optimal cutting temperature compound (OCT) (#4583) was obtained from Sakura Finetek USA Inc. A 1-ml syringe (#329461) was purchased from B&D Biosciences. A 33-gauge Hamilton needle was obtained from Hamilton Company (#65457-01). Isoflurane in oxygen from a precision vaporizer was purchased from Primal Healthcare. A topical antibiotic containing neomycin and polymyxin B sulfates and dexamethasone ophthalmic ointment was purchased from Bausch & Lomb. Phenylephrine hydrochloride ophthalmic solution (2.5%) was purchased from Akorn. Balanced salt solution (BSS) was purchased from Thermo Fischer Scientific. Leica SP5 confocal microscope was used for image analyses. Volocity software was acquired from PerkinElmer. Cell sorting was accomplished using FACS Aria-II. NanoVue Plus was purchased from GE Healthcare. HRP-3 protein was expressed and purified from E.Coli as previously described [85]. All proteins were run on SDS-PAGE gels and confirmed using a Western blot prior to any experiments.
2.2. Antibody purification

Anti-Scg3 mAb was raised against the full-length human Scg3 through a subcontract service with Creative Biolabs and purified from serum-free hybridoma-conditioned medium using protein G columns [106]. Hybridoma cells were further sorted into in 96-well plates with one cell per well. After cells were expanded, ELISA analyses were used to identify hybridoma clones with a high yield of anti-Scg3 mAb. Anti-c-Myc mAb was purified from the conditioned medium of 9E10 hybridoma and used as mouse control IgG1. Control rabbit IgG was purified from rabbit serum.

Briefly, Protein G agarose columns were used to purify mAbs from hybridoma conditioned media. The column was washed using (phosphate buffered saline) PBS. The conditioned media was collected, filtered through a 0.2-µl filter unit, and passed through the Protein G column. After washing with phosphate-buffered saline (PBS) and then with phosphate buffer (10 mM, pH 7.4), bound mAbs were eluted using low pH buffer (0.1 M glycine, pH 3.0, 0.5 ml/fraction) and collected into tubes containing 25 µl NaCl (3 M) and 17 µl Tris HCl (1 M, pH 9.0). The purified mAb was then exchanged to PBS and concentrated in an Amicon centrifugal filter unit. The ability of Scg3 mAbs to recognize Scg3 recombinant protein was validated using a Dot blot and ELISA assay. A cell proliferation assay and Western blot were used to determine is Scg3 mAbs can neutralize Scg3-induced functional activity. See Methods section 2.9 for details on in vitro validation assays.
2.3. Animals

All animal procedures in this study were approved by the Institutional Animal Care and use Committee at the University of Miami (Protocol #13–229) and complied with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH).

2.3.1. Anesthesia

Mice were anaesthetized using the cocktail of ketamine (1.5 mg/0.1 ml) and xylazine (0.3 mg/0.1 ml) delivered through intraperitoneal injection (100 ml/Kg body weight).

2.4. Animal models of diabetes

2.4.1. Streptozotocin (STZ)-induced diabetic mouse model

C57BL/6 mice (6 weeks old, male) were induced for type 1 diabetes with streptozotocin (STZ) in citrate buffer (100 mM, pH 4.5) or mock control with citrate buffer alone. Only male mice were used for STZ induction [107] (for discussion please see Chapter 7).

Mice were starved for 4 h then received STZ (40 µg/g body weight) via intraperitoneal injection for 5 consecutive days. STZ was dissolved in citrate buffer (7.5 mg/ml) immediately before the injection. Mice were monitored for blood glucose biweekly and considered diabetic when blood glucose was ≥ 350 mg/dL, usually starting at 2 weeks post STZ treatment. Mice were aged for 4 months post STZ to develop retinal leakage.
2.4.2. Ins2<sup>Akita</sup> diabetic mouse model

The Ins2<sup>Akita</sup> is a genetic mouse model of type I diabetes, in which a single amino acid mutation results in the misfolding of the insulin protein. This model was used as a non-pharmacological DR model. Heterozygous Ins2<sup>Akita</sup> mice were bred to C57BL/6J mice, and male offspring were monitored for hyperglycemia, beginning at 4.5 weeks of age. Only male mice were used for experiments [107] (for discussion please see Chapter 7). Mice were aged for 6 months to develop retinal leakage. Previous groups have shown that these mice exhibit retinal leakage between 4 to 36 weeks of age [108, 109].

2.5. In vivo experiments in diabetic and healthy mice

Healthy and diabetic mice were used for in vivo experiments. Diabetic mice were generated as described above.

2.5.1. Intravitreal injection

Mice were anesthetized as described above and the eyes were locally anesthetized using 1 drop of Proparacaine Hydrochloride Ophthalmic Solution (0.5%). A small incision was made in the pars plana, located behind the limbal vessel, and a 1.5 cm 33-gauge Hamilton needle was inserted at a 45° angle, taking care to avoid hitting the lens. A topical antibiotic of neomycin and polymyxin B sulfates and dexamethasone ophthalmic ointment was applied liberally on the eye.

2.5.2. Retinal vascular leakage assay

Retinal vascular leakage assay using Evans blue dye was performed as previously described [85, 86, 110]. Evans blue (0.15 mg/g body weight) was intravenously injected and allowed to circulate for 2.5 h prior to sacrifice. Mice were euthanatized
using CO₂ inhalation. Blood was collected via a cardiac punch, and mice were intracardially perfused with pre-warmed (37°C) sodium citrate buffer (100 mM, pH 4.5). After perfusion, retinas were isolated and incubated in formamide (50 µl/retina) overnight at 70°C to extract Evans blue dye from the retina. The solution was centrifuged at 180,000 g at 4°C for 45 min. Evans blue dye in the supernatant was quantified spectometrically at 620 nm and 740 nm (background) and compared to a standard curve of Evans blue dye. The blood samples were centrifuged at 3,550 x g for 15 min at 25°C, diluted and quantified at the same wavelengths. Evans blue dye was calculated using the following formula: [leaked Evans blue concentration (mg/ml)/retinal weight (mg)]/[blood Evans blue concentration (mg/ml) x circulation time (h)]. All data were normalized to the PBS-injected contralateral control eye to account for individual differences in baseline leakage.

For Evans blue experiments in healthy mice, Scg3 (0.25 µg/1 µl/eye), VEGF (0.1 µg/1 µl/eye) or BSA (0.25 µg/1 µl/eye) was injected via intravitreal injection, as described. PBS was injected into fellow eyes as a negative control.

For retinal leakage therapy in diabetic mice, anti-Scg3 pAb (0.36 µg/eye), mAb (0.36 µg/eye), mock affinity-purified antibody (0.36 µg/eye), control IgG (0.36 µg/1 µl/eye) or aflibercept (2 µg/eye) was intravitreally injected into one eye. The contralateral eye received a PBS injection.

### 2.5.3. Cornea angiogenesis assay

The assay was performed as described [85, 86]. Briefly, sterilized Whatman filter paper was cut into small pieces (~0.125 mm²). The papers were soaked in HRP-3
(1 µg/ml), VEGF (0.1 µg/ml), Scg3 (0.25 µg/ml), or PBS for 2 h at 4°C, and implanted into corneal pockets of anesthetized C57BL/6 mice (8–10 weeks old, male; 1 paper/cornea; 2 pockets/mouse). After 6 days, angiogenesis in each eye was evaluated using a slit-lamp microscope and photographed. The number of new sprouting vessels and their branching points were quantified. We modified a scoring system to provide a semi-quantitative analysis for the number, density and length of visible corneal blood vessels. Mice were euthanized by CO₂ inhalation, followed by immediate intracardial perfusion with a lipophilic fluorescent 1,1'-Dioctadecyl-3,3',3',3'-Tetramethylindocarbocyanine Perchlorate dye (Dil). Eyes were removed and fixed in 10% formalin for 24 h at 4°C. Corneas were dissected at the limbal vessel, flat-mounted in 50% glycerol/PBS, and imaged by confocal microscopy to detect Dil-labeled vessels.

2.5.4. Immunohistochemistry

STZ-induced diabetic and healthy mice (C57BL/6, 8-10 weeks old) were euthanatized by CO₂ inhalation and intracardially perfused with PBS followed by 10% formalin. After euthanasia, eyes were isolated and fixed overnight at 4°C. After removal of the cornea and lens, eye cups were incubated in a sucrose gradient solution (10% and 20% for 3 h each; 30% for overnight) at 4°C, followed by 3 rounds of freeze-thaw and OCT embedding. Frozen tissue sections in 10-µm thickness were permeabilized in acetone at 4°C for 15 min. Endogenous peroxidase was blocked using 3% H₂O₂ for 10 min at room temperature. Sections were blocked in 5% fetal bovine serum (FBS) in 0.3 M glycine in PBS for 1 h at room temperature. Sections were incubated with rabbit anti-Scg3 antibody
overnight at 4°C. The following day sections were incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG antibody. Coverslips were applied in the presence of 10% glycerol diluted in PBS containing 4',6-diamidino-2-phenylindole (DAPI, 0.1 µg/ml) to counterstain for cell nuclei. The signals were analyzed by confocal microscopy.

Control slides which omitted the primary antibody incubation step were used to verify the specificity of the antibodies. Microscope settings, including fluorescence exposure times, were kept constant between the antibody and control sections for comparison and analyses.

2.6. Oxygen-induced retinopathy mouse model

Oxygen-induced retinopathy (OIR) mice were generated as described [111, 112]. Briefly, C57BL/6 mice at postnatal day 7 (P7) and their nursing mother were exposed to 75% oxygen in a regulated chamber for 5 days. Mice received an intravitreal injection of anti-angiogenic drug or control on P12. Briefly, mice were anesthetized using isoflurane in oxygen from a precision vaporizer (1-3% for maintenance; up to 5% for induction). The mouse eye lid was surgically opened using scissors prior to intravitreal injection. Mice received an intravitreal injection of anti-Scg3 mAb (0.36 µg/1 µl/eye), anti-Scg3 pAb (0.36 µg/1 µl/eye), aflibercept (2 µg/1 µl/eye), IgG (0.36 µg/1 µl/eye) or PBS. Topical antibiotic neomycin and polymyxin B sulfates and dexamethasone ophthalmic ointment was applied liberally on the eye. Non-injected eyes were used as controls. Pups and their mother were returned to ambient oxygen after injection.
At P17 mice were euthanized by CO$_2$ inhalation, followed by cervical dislocation. Eyes were enucleated and incubated in 4% paraformaldehyde diluted in PBS for 45 min at room temperature. Retinas were isolated and incubated in 1% Triton X-100 rocking overnight at 4°C in a 48-well culture dish. Retinas were washed twice and incubated with Alexa Fluor 488-isolectin B4 (10 μg/ml, 0.5 ml/retina) while rocking overnight at room temperature. The following day, retinas were washed 4 times in PBS and flat mounted with 50% glycerol in PBS. Images were acquired (10x magnification) using tiling and z-stack confocal software.

NV has a higher fluorescence intensity than non-NV regions and was quantified using the magic wand tool in Adobe PhotoShop CSS Extended software, as previously described [112]. For each retina, results were normalized to the total retinal pixel intensity. All data was normalized to non-injection controls. Total number of NV tufts were manually counted for each retina using the counting tool in Photoshop. To determine the number of branching points two squares of a predefined size (200 x 200 pixels) were drawn in Photoshop 100 pixels from the most peripheral region of the retina (1 μm= 1 pixel). Within each square the number of branching points were counted using the counting tool in PhotoShop CSS Extended software. The number of branching points were averaged between the 2 squares to obtain the average per retina. Healthy age-matched retinas, not exposed to high oxygen treatment, served as the control for this analysis.

2.7. Animal models of wet AMD

Wet AMD was generated using either a laser-induced CNV [113-115] or Matrigel-induced CNV [116, 117] mouse model, as described below.
2.7.1. Laser-induced choroidal neovascularization

Mice (C57BL/6 mice, 8 weeks old, male or female) were anesthetized. Eyes were dilated using phenylephrine hydrochloride ophthalmic solution (2.5%). Mice were subjected to laser photocoagulation (Argon laser, 532 nm, 100 mW, 100 ms, 100 µm, 4 spots/retina around the optic disc) on Day 0. Lesions were confirmed by subretinal bubbles which developed immediately after laser treatment.

Laser-induced CNV mice received an intravitreal injection of anti-Scg3 mAb (0.36 µg/1 µl/eye), anti-Scg3 pAb (0.36 µg/1 µl/eye), aflibercept (2 µg/1 µl/eye) or IgG control (0.36 µg/1 µl/eye) on Day 3. PBS was injected into the fellow eye, and all data were normalized to this control eye. CNV was quantified using fluorescein angiography on Day 7 and choroid flat analysis on Day 8.

2.7.2. Subretinal Matrigel-induced choroidal neovascularization

High concentration growth factor-reduced Matrigel was diluted 1:4 (vol/vol) with PBS and 0.8 µl/ eye was injected into the subretinal space of C57BL/6 mice (6-7 weeks old, 0.8 µl/retina) on Day 0. Briefly, mice were anesthetized and a small incision was made in the conjunctiva and sclera of the eye exposing the subretinal space and a 1.5 cm 33-gauge Hamilton needle was inserted between the RPE and retina. Topical antibiotic neomycin and polymyxin B sulfates and dexamethasone ophthalmic ointment was applied liberally on the eye. After injection the needle was held in place for 1 min to allow the Matrigel to solidify.

Mice with Matrigel-induced CNV received a subcutaneous injection of anti-Scg3 pAb (25 µg/Kg body weight), mAb (25 µg/Kg), aflibercept (250 µg/Kg) or IgG (25 µg/Kg). The first injection was carried out prior to the Matrigel injection on Day
0, and again on Day 2 and 4. All antibodies were injected via a subcutaneous injection in a final volume of 100 µl diluted with PBS. All data were normalized to Matrigel only controls. Mice were analyzed by Fluorescein angiography on Day 7.

2.7.3. Fluorescein angiography

Mice were anesthetized, and eyes were dilated using phenylephrine hydrochloride ophthalmic solution (2.5%). Eyes were kept moist with BSS. Fluorescein sodium (2.5%, 0.1 mL) was injected into the intraperitoneal cavity. Mice were imaged 6 min post injection using Heidelberg Engineering Multiline HRA SN 2884 imaging system.

2.7.4. Fluorescein angiography quantification

CNV lesions were imaged by fluorescein angiography, as described above. The area of each CNV lesion was quantified for fluorescence intensity using Adobe PhotoShop CSS Extended software. Lesions with choroidal hemorrhage and linear or fused lesions were excluded as described [118].

2.7.5. Choroidal vessel quantification

Mice were euthanized by CO₂ inhalation 24 h post fluorescein angiography to allow the clearance of the fluorescence dye. Eyes were enucleated and fixed in 4% paraformaldehyde for 45 min at room temperature. The eyecups of retinal pigment epithelium-choroid-complex were prepared and incubated in PBS containing 0.5% Triton X-100 at 4°C for 24 h and stained with Alexa Fluor 488-isolectin B4 (10 µg/ml, 0.5 ml/retina) overnight at room temperature. The following day, eyecups were washed 4 times in PBS for 45 min each. Complexes were flat mounted, and each CNV lesion was analyzed by z-stack using Volocity software. To calculate
CNV area of each lesion, a single z-stack image with the largest diameter was measured to determine the area using ImageJ Software (NIH). Relative lesion pixel intensity was measured from the same section as an indicator of vessel density. Pixel intensity was quantified by measuring the threshold intensity of each CNV lesion using the Magic Wand Tool in Adobe Photoshop CSS Extended software.

2.8. Cell Culture

Cells were cultured according to manufacturers’ instruction, as described [85, 86].

2.8.1. Human retinal microvascular endothelial cells

Human retinal microvascular endothelial cells (HRMVECs) were purchased from Cell Systems. Flasks were coated with attachment factor. Experiments using HRMVECs were conducted at low passages (P3 - P8). Cell cultures were maintained using complete media in 5% CO₂ at 37°C. Media was changed every 2 days.

2.8.2. Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. Flasks were coated with 1% gelatin. Experiments using HUVECs were conducted at low passages (P3-P8). Cell cultures were maintained using EBM-2 complete media and maintained in 5% CO₂ at 37°C. Media was changed every 2 days.

2.8.3. Cell Dissociation with trypsin

Culture media were removed using aspiration, and cells were washed with PBS followed by aspiration. Trypsin (0.025%) was added to completely cover the bottom of the flask, and cells were placed in the 37°C incubator for approximately 2 min, or until detached by gentle tapping. Cell detachment was confirmed using
an inverted microscope. FBS (10%) was added to neutralize the trypsin. Cells were resuspended and centrifuged at 200 x g for 5 min.

2.8.4. Hybridoma culture and adaptation

Anti-Scg3 hybridomas were cultured in complete media (RPMI + 10% FBS + 1% penicillin Streptomycin (P/S, 10,000 U/ml) + 1 X Glutamax). Hybridomas were subcloned in 96-well plates (one cell/well) using FACS Aria II cell sorter. The conditioned medium of all individual hybridoma clones were analyzed for the presence of anti-Scg3 mAbs by ELISA. Clone ML49.3 anti-Scg3 mAb was used for this study.

ML49.3 hybridoma was adapted into serum-free hybridoma medium as follows. Cells were first cultured in 75% complete media and 25% serum-free hybridoma media supplemented with 1% P/S and 1 X Glutamax. Cells were then gradually adapted to 50% and then 25% complete media with increased percentage of serum-free hybridoma media. Eventually, cells were cultured in 100% serum-free hybridoma media.

Conditioned serum-free hybridoma media were collected when cells reached to high density when the media began to appear yellowish. Cells were spun down at 200 x g for 5 min. The supernatant containing the secreted anti-Scg3 mAbs were collected and stored at -20°C until antibody purification.

2.9. In vitro functional assays using endothelial cells

2.9.1. Protein neutralization

Scg3 (1 µg/ml) was preincubated with anti-Scg3 mAb (2 µg/ml), anti-Scg3 pAb (2 µg/ml), aflibercept (2 µg/ml), or IgG (2 µg/ml) in 20 µl of PBS rotating for 1 h at 4°C.
As a control, VEGF (100 ng/ml) was preincubated with aflibercept (2 µg/ml) in 20 µl of PBS for 1 h at 4°C.

2.9.2. Cell proliferation assay

A cell proliferation assay was performed as previously described [85, 86, 119]. HRMVECs (1x10^4 cell / well) were seeded in 48-well plates coated with attachment factor and grown in complete media. HUVEC (1x10^4 cell / well) were seeded in 48-well plates pre-coated with 1% gelatin. Fresh medium and growth factors were added every 24 h. After 24 or 48 h media was removed and the wells were washed once with PBS. Cells were collected by trypsin digestion, followed by neutralization in 10% FBS in PBS. Viable cells were quantified with 1 mM trypan blue using a hemocytometer.

2.9.3. Migration assay

A wound healing assay was performed as previously described [85, 86, 120]. HRMVECs were cultured in 12-well plates coated with Cell Attachment Factor until ~90–100% confluent. Cells were starved for 3 h in serum free medium supplemented with 2% FBS. A scratch was made with a sterile tip down the center of each well, approximate 1 mm in width, to mimic the wound region. Dislodged cells were removed by rinsing and the remaining cells were cultured in basal media supplemented with 2% FBS in the presence of Scg3 (1 µg/ml), VEGF (50 ng/ml) or media only. Cell migration into the denuded area was monitored at 0 and 20 h as follows. At 0 and 20 h, at least six images per well were acquired with phase-contrast microscopy. At 0 h the average width of the original scratch was
calculated. At 20 h, the percentage of the denuded area covered by migrated cells was calculated using ImageJ software.

2.9.4. Tube formation assay

A tube formation assay was performed as previously described [85, 86, 121]. High concentration Matrigel (growth factor-reduced) was diluted 1:4 (vol/vol) in EBM-2 medium, plated in 96-well plates (50 μl/well) and allowed to solidify at 37°C for 30 min. HUVECs were starved overnight in serum-free EBM-2 medium and plated on Matrigel-coated wells (15,000 cells/well) in the presence of Scg3 (300 ng/ml), VEGF (50 ng/ml) or media only. Bright field images were obtained after 3 h of incubation at 37°C. Total tube length, the number of tubes and the number of branching points per viewing field were quantified as follows. To quantify total tube length, the distance (μm) of each tube was measured by ImageJ software to calculate the total tube length per viewing field [122]. The number of tubes, irrespective of tube distance, was also counted for each viewing field. The total number of branching points was calculated per viewing field [123].

2.9.5. Spheroid sprouting assay

A spheroid sprouting assay was performed as previously described [85, 86, 124]. Methocel solution was prepared by dissolving methycellulose in serum-free medium at 1.2% and centrifuged at 5,000 x g for 2 h at 4°C to clear debris. HUVECs or HRMVECs at 80% confluence were harvested, counted, resuspended in medium containing 20% methocel and 10% FBS, seeded at 750 cells/well in non-adhesive 96-well round-bottomed plates and cultured for 24 h. The spheroids were harvested, resuspended in medium containing fibrinogen (2.5 mg/ml) and
aprotinin (0.05 mg/ml), and seeded in 24-well plates (~50 spheroids/ml/well). Clotting was induced by adding thrombin (12 units/ml) to each well. The spheroid-embedded fibrin gel was allowed to clot for 5 min at room temperature followed by 20 min at 37°C. The fibrin gel was equilibrated with 1 ml of medium containing aprotinin (0.05 mg/ml) in the presence of Scg3 (1 µg/ml), VEGF (50 ng/ml) or control, and incubated for 48 h at 37°C. Photographs were taken at 48 h using a phase contrast microscope and the average sprout length per spheroid was quantified using ImageJ. One spheroid was equivalent to n=1.

2.9.6. Endothelial permeability assay

An endothelial permeability assay was performed as previously described [85, 86, 125]. HUVECs or HRMVECs (1x10^5 cell/ well) were plated on gelatin-coated transwell inserts with 3-µm pores in a 24-well dish, in the presence of 1 ml in the lower chamber and 200 µl in the upper chamber of complete media. I demonstrated that at this density cells formed a confluent monolayer after 24 h. Dextran-FITC (3 kDa) in the presence of Scg3 (200 ng/ml), VEGF (100 ng/ml) or PBS was added to the lower chamber. When Dextran-FITC was placed into the lower chamber of the transwell insert, it freely diffused into the upper chamber when ECs were not present. However, in the presence of a confluent EC monolayer, the diffusion was blocked. Increased diffusion of dextran-FITC across the EC monolayer into the upper chamber indicated an increase in endothelial permeability. After 24 h, media from the upper chamber was collected, and FITC fluorescence was quantified read using a plate reader (excitation 488 nm, emission
525 nm). Total FITC (µg) was calculated for each condition against a FITC standard curve.

2.9.7. Western blot analyses

HRMVECs or HUVECs grown in 6-well plates were lysed in RIPA buffer (50 µl/well) containing proteinase inhibitors. A phosphatase inhibitor cocktail (1 tablet/ 10 ml) was also included to prevent protein dephosphorylation. Total protein concentration was measured at 280 nm (A280) using a NanoVue Plus.

Anesthetized mice were sacrificed by CO₂ inhalation, followed by cervical dislocation. Retinas were removed and lysed (50 µl/retina) as described above. Retinas were homogenized with a 26-gauge needle and a 1-ml syringe to further dissociate retinal tissue. Samples were centrifuged at 5,000 x g for 5 min at 4°C, and total protein concentration was measured using a NanoVue Plus. For vitreous samples, mice were anesthetized and the vitreous fluid was removed using a 1.5 cm 33-gauge Hamilton needle. Protein concentration was directly measured using a NanoVue Plus.

Protein lysate was loaded onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 70 V for 60 minutes. Membranes were blocked for 1 h in PBS with 5% blotto and probed using the appropriate primary antibody overnight at 4°C. The next day membranes were washed three times in PBST (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4) followed by incubation in anti-rabbit, mouse or goat secondary antibodies conjugated with horse radish peroxidase (HRP) enzyme
diluted in 5% blotto in PBS. Secondary antibody incubation lasted for 1 h at room temperature. The protein bands were visualized through chemiluminescence using either SuperSignal West Femto maximum sensitivity substrate kit or substrate kit and imaged using a Fujifilm LAS 4000 imaging system. Protein band density was quantified using ImageJ software and normalized to total protein or β-actin density to correct for loading differences.

2.9.8. Protein kinase activation

HUVECs were seeded in 6-well plates precoated with 1% gelatin or Cell Attachment Factor, respectively. All cells were cultured to ~90% confluence. Cells were incubated 3 times serum-free media for 15 min each at 37°C to reduce background kinase activation. The following day cells were incubated with Scg3 (1 µg/ml), VEGF (0.1 µg/ml) or media only in serum-free media for 10 or 30 min in 37°C. After washing, cells were lysed in RIPA buffer in the presence of a phosphatase inhibitor cocktail and protease inhibitors. The lysates were analyzed by Western blot using an antibody against phosphorylated Erk1/2, Mek, Akt, Src or Stat3. Membranes were also probed for total Erk1/2, Mek, Akt, Src or Stat3. β-actin was used as a loading control followed by HRP-labeled secondary antibody for chemiluminescence to detect signals. VEGF was included as a positive control.

2.9.9. Protein pull down assay

To detect Scg3 interactions with VEGFRs-Fc, recombinant protein Scg3 (0.1 µg/ml), VEGF (0.1 µg/ml) or BSA (0.1 µg/ml) was incubated with indicated VEGFR1-Fc (0.1 µg/ml), VEGFR2-Fc (0.1 µg/ml) or aflibercept (0.1 µg/ml) for 1 h at 4°C in a final volume of 100 µl of PBS containing 0.1% BSA. Protein A beads
(10 μl) were pre-blocked in 100 μl of 0.1% BSA in PBS for 1 h rotating at 4°C, added to the samples and incubated for additional 30 min at 4°C. After centrifugation at 3,000 g for 5 min, beads were washed 4 times with PBS, treated with SDS-PAGE loading buffer, boiled and analyzed by Western blot using anti-Scg3 mAb or anti-VEGF.

To detect Scg3 interactions with VEGF, Scg3 (0.1 μg/ml) was incubated with VEGF (0.1 μg/ml) for 1 h at 4°C in a final volume of 100 μl of PBS with 0.1% BSA. After incubation anti-Scg3 pAb was added and samples were incubated for an additional 30 min rotating at 4°C. Protein A beads were pre-blocked and incubated with the samples for protein pull down assay as above.

2.9.10. Dot Blot
Scg3 (0.5 μg/in 1 μl) was added to PVDF membranes and allowed to dry for 15 min at room temperature. Membranes were incubated with hybridoma-conditioned media (25% vol/vol in PBS) overnight at 4°C. Anti-Scg3 pAb was included as a positive control. The following day membranes were washed and probed with anti-mouse or anti-rabbit HRP-conjugated secondary antibodies. Membranes were washed 3 x in PBST. The protein was visualized through chemiluminescence using either SuperSignal West Femto maximum sensitivity substrate kit or substrate kit and imaged using a Fujifilm LAS 4000 imaging system.

2.9.11. ELISA
ELISA plates were coated with BSA (1 μg/ml), hScg3 (1 μg/ml), mScg3 (1 μg/ml) or VEGF (1 μg/ml) overnight at 4°C. After washing, wells were blocked with 1% polyvinyl alcohol in PBS for 1 h with gentle shaking at room temperature. Wells
were washed in PBS and incubated with either hybridoma-conditioned media (25% vol/vol in PBS), BSA, aflibercept, hVEGFR1-Fc or hVEGFR2-Fc (1 µg/ml each) for 1 h at room temperature. Wells were then washed 3 x in PBST and detected using the appropriate HRP-labeled anti-human IgG secondary antibodies for 1 h at room temperature. Bound HRP-antibodies were detected by incubating with HRP substrate (ophenylenediamine dihydrochloride, citric acid, hydrogen peroxide) for 15 min in the dark at room temperature, as described [67]. The reaction was quenched with in 1 M sulfuric acid (H₂SO₄) and read at OD 490 nm using a fluorescence plate reader.

To detect Scg3 binding to VEGF, ELISA plates were coated with BSA (1 µg/ml) or VEGF (1 µg/ml). hScg3 recombinant protein (1 µg/ml) was added followed by incubation with anti-Scg3 pAb (1 µg/ml each) for 1 h at room temperature. Bound antibody was detected by colorimetric assay using HRP-labeled anti-rabbit IgG secondary antibodies, as described above.

2.10. Ligandomic data analysis

NGS data were aligned against NCBI CCDS database to identify enriched ligands. The copy numbers of cDNA inserts identified represents the relative binding activities of their cognate ligands to the retinal endothelium. These results were quantitatively compared using a Chi-square test to identify DR-related endothelial ligands. To calculate the binding activity ratios, the copy numbers of individual ligands were normalized with the following formula. Normalized readout of individual ligands in control retina = (individual ligand read in control retina) x (total NGS read for diabetic retina) ÷ (total NGS read for control retina). In this study,
total NGS reads for control and diabetic retinal were 473,965 and 489,126, respectively. Thus, the formula was simplified as follows: normalized readout of individual ligands in the control retina = 1.03 x (individual ligand reads in control retina). The adjustment is negligible. After data normalization, the activity ratio = (DR +1)/(Control +1) as in Table 3.1.

CCDS ID of NGS data were batch-converted to Uniprot accession numbers and analyzed by PANTHER (http://www.pantherdb.org/) and DAVID (http://david.abcc.ncifcrf.gov/summary.jsp). Identified ligands were categorized based on gene ontology (GO) terms “Cellular Component” and “Biological Process”.

2.11. Statistical analyses
Student’s t-test or analysis of variance (ANOVA) test with appropriate post-hoc analysis was used for statistical analysis. P values less than 0.05 were regarded as significant. Regression and correlation analysis was conducted and reported as a Pearson correlation coefficient.
Chapter 3: Comparative ligandomics data analysis to identify disease-associated endothelial ligands

The objective for this chapter is to develop the first paradigm of “comparative ligandomics” to systematically identify disease-related cellular ligands. This innovative approach is centered around my lab’s recent applications of ligandomics in vitro, in which we identified biologically relevant phagocytosis ligands based on their functional activity [77, 78, 83, 84]. We further expanded upon this technology to identify endothelial ligands using in vivo binding selection, in the absence of receptor information [85]. Based on this success of ligandomics analysis, we applied comparative ligandomics data analysis to systematically delineate factors with preferential binding activity to the diseased vasculature for the discovery of novel pathogenic ligands with therapeutic potential.

3.1. Ligandomics to globally identify EC ligands

For ligandomics analysis, other team members, Dr. Nora Caberoy and Ms. Gabriela Alvarado, performed three rounds of in vivo binding selection to enrich for endothelial ligands with binding activity to the retinal vasculature in healthy mice [85]. To demonstrate the validity of this technology, I analyzed the entire ligandome data set for healthy mice, with the goal of identifying novel EC ligands.

To verify the ability of this technology to identify endogenous ligands with biological relevance, all identified factors were classified according to their known functional roles. For example, ligands were identified with roles in endothelial cellular processes including, angiogenesis, apoptosis, cell migration and adhesion (Fig. 3.1). Ligands without known roles in the vasculature could not be classified and required further independent functional validation.
Ligandomics analysis identified HRP-3 and HDGF as putative retinal endothelial ligands. While HDGF is a known angiogenic factor [126], family member HRP-3 had not been reported as an endothelial ligand or growth factor. As part of a separate project, I characterized the novel role of both ligands as retinal angiogenic factors using *in vitro* and *in vivo* models. The results of these studies were published in two recent papers [85, 86]. Independent characterization of HDGF demonstrated the validity of ligandomics to identify endogenous ligands. More significant is the characterization of HRP-3 as a novel endothelial ligand, highlights the capacity of ligandomics to identify factors with previously unappreciated roles in the vasculature.

![Fig. 3.1. Protein classification of identified ligands](image)

Protein classified based on their biological process. All identified ligands were categorized based on gene ontology (GO) terms “Cellular Component” and “Biological Process”. Identified roles include: apoptosis, cell migration, biological adhesion, blood vessel development, cellular compartment biogenesis, biological regulation, metabolic processes, and immune regulation.
3.2. Quantitative ligandomics

The next objective was to establish the feasibility of binding activity quantification using ligandomics. We predict that the relative copy numbers of cDNA inserts identified by NGS analyses correlates to the binding activity of its cognate ligand. To investigate this prediction, other lab members performed high-throughput ligandomics screenings in healthy and diabetic mice with an internal positive and negative control as follows.

Diabetic mice were generated with a STZ injection into the intraperitoneal cavity to destroy the β-islet cells, as previously described [107]. Mice were monitored for blood glucose weekly and developed hyperglycemia 2 weeks post STZ injection (Fig. 3.2A-B). Hyperglycemic mice were aged for 4 months to develop diabetes-induced retinal leakage. I performed Evans blue assay to confirm that diabetic mice aged 4-months developed significant retinal vascular leakage, a major symptom of DR (Fig. 3.2C).

![Fig. 3.2. A mouse model of diabetes induced by STZ exhibits retinal leakage](image)

Characterization of the STZ diabetic phenotype. (A, B) STZ-induced mice are hyperglycemic. Blood glucose (A) and body weight (B) were monitored biweekly. Mice with blood glucose ≥ 350 mg/dL were considered diabetic (Mean ± SD, n=5, p<0.001). (C) STZ-induced mice show enhanced retinal permeability. Retinal leakage was confirmed in STZ mice aged 4-months using Evans blue assay (n=8, p<0.01). All data are mean ± SEM, Student’s t-test.
To establish the feasibility of the binding activity quantification by ligandomics, two clonal phages were constructed and spiked into the OPD mouse libraries at the equal titer (1:1,000) before in vivo binding selection. One clonal phage displayed human VEGF (hVEGF-Phage) with artificial codons (Fig. 3.3). The other displayed green fluorescent protein (GFP-Phage). After three rounds of in vivo binding selection (Fig. 3.4A), the spiked-in positive and negative control phages with non-mouse codons were simultaneously identified within the enriched mouse library by NGS (Fig. 3.4B).

**Fig. 3.3. VEGF-phage displays wild-type human VEGF protein with artificial codons**

(A) The coding sequence with altered codons is 65.8% and 66.4% identical to human and mouse VEGF. (B) The encoded protein is 100% identical to human VEGF \(1-110\) (Accession # NM_001171623). Altered codons were optimized for bacteria expression and phage display.
My analysis of these two ligandome data sets showed that hVEGF-Phage was identified with 2,420 and 408 copies in normal and diabetic mice, whereas GFP-Phage was identified with 10 copies in both mouse models (Fig. 3.4D). The depletion of GFP-Phage and relative enrichment of hVEGF-Phage by NSG quantification (GFP-Phage versus. VEGF-Phage) after three rounds of selection confirmed that phage copy numbers correlates to differential ligand binding activities in vivo. These results established GFP-Phage as a baseline for non-specific binding. Establishing this binding activity quantification using ligandomics was critical to ensure the reliability of comparative ligandomics data analyses for the systematic identification of disease-associated ligands in the next section.

3.3 Comparative ligandomic profiling

Ligandomics analysis in diabetic and healthy mice identified a total of 489,126 and 473,965 valid sequence reads that aligned to 1,548 (diabetic retina) and 844 (healthy retina) proteins in the NCBI CCDS database (Table 3.1). This included a total of 1,772 non-redundant proteins identified within both groups (healthy and diabetic) (Fig. 3.5C). By setting GFP-Phage as the background, quantitative ligandomics analysis revealed that 817/1,548 and 417/844 isolated ligands specifically bound above this background baseline to the diabetic and healthy retinas, respectively (Fig. 3.5, spectrum A versus B).

Analysis of the entire ligandome data set revealed the overall patterns for diabtotic and healthy retinas were relatively similar (Fig. 3.5, spectrum A versus B). However, a more detailed comparison identified subtle distinctions between individual ligands in these two groups. To tease out these individual differences,
we performed a quantitative comparison of the entire ligandome profile for diabetic versus control retinas by Pearson correlation analysis. Results showed that the global binding activity patterns were markedly different between diabetic and healthy mice (Pearson correlation coefficient $r=0.498$ for all identified ligands, Fig. 3.4E). Further analyses identified 1,114 disease-associated endothelial ligands ($p < 0.05$) by Chi-square ($\chi^2$) test (Fig. 3.5D).

Two categories of ligands were further defined based on increased (DR-high) or decreased (DR-low) binding to diabetic retinal ECs using a binding activity plot (Fig. 3.3E). However, a plot of the Chi-square value versus the binding activity ratio for diabetes:control uncovered many ligands with minimal, albeit statistically significant, changes in binding activity between the two conditions (Fig. 3.4E). To improve the reliability of identifying disease-associated ligands, we further defined DR-high or DR-low ligands with the following more stringent, arbitrary criteria: $p < 0.001$; diabetes:control binding activity ratio $> 10$ or $< 0.1$; copy number in DR or control $\geq 30$ (Fig. 3.5D). Based on this filter we identified 353 DR-high and 105 DR-low ligands (Table 3.1, Fig. 3.5D). We predict that DR-high ligands play more prominent roles in DR pathogenesis than DR-low and non-DR-specific ligands.

The validity of comparative ligandomics was confirmed by the identification of several known ligands, including amyloid β and subunits of the C1q complement factor, with increased binding to diabetic endothelium (Table 3.1). Amyloid β is a product of amyloid precursor protein (APP), which is a known ligand for the RAGE (receptor for advanced glycation end products). This is significant in that RAGE is upregulated on the diabetic endothelium [127]. C1qb is the β subunit of C1q
complement factor that interacts with at least two endothelial receptors, cC1qR and gC1qR/p33 [128]. C1q is present in significant quantities at the site of atherosclerotic lesions [129], which are one of the diabetic vascular complications [45]. Identification of these proteins as DR-high ligands suggests that comparative ligandomics is a reliable tool to globally map disease-associated factors.

3.4. Identification of Scg3 as a DR-high ligand by comparative ligandomics

Scg3 and HRP-3 were identified by comparative ligandomics as DR-high and DR-low ligands, respectively (Table 3.1). Scg3 with no detectable binding to normal vessels was found with 1,731 copies to the diabetic retinal endothelium by NGS (Fig. 3.4D). This preferential binding activity suggests that the receptor for Scg3 is upregulated in the diabetic but not healthy vasculature. In contrast, HRP-3 showed reduced binding to the diabetic vasculature by ~227-fold, suggesting that its receptor is downregulated in the diabetic vasculature (Fig. 3.4D). This is consistent with my results defining HRP-3 as a potent angiogenic factor in the healthy mouse retina [85]. By comparison, VEGF copy number was marginally altered by ~5-fold between the diabetic versus healthy vasculature (Fig. 3.4D). Based on the above criteria, VEGF is a non-DR-associated ligand. These results suggest that the expression of VEGFRs are not markedly altered in the 4-month diabetic mouse vasculature compared to healthy controls.
A Multi-round in vivo binding selection by open reading frame phage display (OPD) to enrich for retinal endothelial ligands in diabetic and healthy mice. (B) Global identification of all enriched ligands. After 3 rounds of selection, the cDNA inserts of enriched ligands were identified by NGS with simultaneous binding activity quantification for all identified ligands. (C) Quantitative comparison of the entire ligandome profiles for diabetic versus control retina was performed to systematically identify DR-associated endothelial ligands. (D) Enrichment of Scg3 and reduction of HRP-3 in the diabetic vasculature. VEGF and GFP served as internal positive and negative controls, respectively. (E) Binding activity plot. DR-associated ligands with increased or decreased binding to the diabetic endothelium are classified as DR-high or DR-low, respectively. Background and non-DR-associated ligands showed comparable binding in both conditions. DR-high Scg3, DR-low HRP-3 and GFP are indicated. Pearson correlation coefficient r=0.489.
Table 3.1. DR-associated endothelial ligands identified by comparative ligandomics

<table>
<thead>
<tr>
<th>CCDS ID</th>
<th>Protein</th>
<th>DR</th>
<th>Control</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCDS23347</td>
<td>Scg3*</td>
<td>1,731</td>
<td>0</td>
<td>1,732</td>
</tr>
<tr>
<td>CCDS18810</td>
<td>C1qb*</td>
<td>837</td>
<td>0</td>
<td>838</td>
</tr>
<tr>
<td>CCDS15031</td>
<td>Fn1*</td>
<td>419</td>
<td>0</td>
<td>420</td>
</tr>
<tr>
<td>CCDS35631</td>
<td>Col4a3*</td>
<td>409</td>
<td>2</td>
<td>137</td>
</tr>
<tr>
<td>CCDS28285</td>
<td>APP*</td>
<td>206</td>
<td>1</td>
<td>104</td>
</tr>
<tr>
<td>CCDS22638</td>
<td>Cdh1*</td>
<td>132</td>
<td>0</td>
<td>133</td>
</tr>
</tbody>
</table>

DR-high ligands with increased binding to diabetic ECs

| CCDS40011   | HRP-3*  | 48        | 11,140    | 0.0044         |
| CCDS17457   | HDGF*   | 0         | 83        | 0.0119         |

Internal positive and negative controls

| VEGF-Phage  | 408     | 2,420     | 0.1689    |
| GFP-Phage   | 10      | 10        | 1.0       |

Total identified sequences: 489,126
Total identified ligands: 1,548
Diabetes-related ligands*: 353↑ 105↓

*P<0.001, DR versus control, χ² test. Activity ratio = (DR +1)/(Control +1).

Table 3.1. DR-related endothelial ligands identified by OPD-NGS

A total of 489,126 and 473,965 valid sequence reads were identified by NGS for diabetic and control retina and matched to 1,548 and 844 proteins in NCBI CCDS database, respectively. The copy numbers of cDNA inserts for enriched clones identified by NGS are the equivalent of the EC-binding activity of their cognate ligands. Quantitative comparison of the entire ligand profiles for diabetic versus healthy retina systematically identified 353 ligands with increased binding to diabetic ECs and 105 with decreased binding (p<0.0001, χ² test). HRP-3 showed reduced binding to diabetic retinal ECs, whereas Scg3 exhibited increased binding to the diabetic endothelium. In contrast, VEGF bound to retinal ECs with ~5-fold less activity in diabetic than in healthy mice.
Fig. 3.5. Quantitative and comparative ligandomic analyses

(A, B) Quantitative and comparative ligandomic profiling. A total of 844 and 1548 putative ligands were identified by ligandomics for control (A) and diabetic (B) retinas. VEGF-Phage and GFP-Phage were internal positive and negative controls indicated by red columns. DR-high Scg3 and DR-low HRP-3 are indicated by yellow columns. Scg3 was not detected in healthy retina. GFP is used as the baseline of non-specific binding to distinguish 417 and 817 ligands in control and diabetic retina with binding activity higher than background (seen in A versus B). (C) Activity ratio analyses to summarize the distribution patterns of binding (left Y axis for vertical bars) and activity ratios (right Y axis for the orange curve) for all 1772 non-redundant ligands identified in the two conditions. (D) Chi-square value distribution. The reliability of Chi-square ($\chi^2$) test is analyzed by plotting $\chi^2$ value against binding activity ratio. The results indicate that many ligands within the red triangle area have minimal binding activity changes but high $\chi^2$ values, suggesting that Chi-square test alone may result in a large number of false positives. Based on this analysis, stringent criteria described in main text were used to identify 353 DR-high ligands and 105 DR-low ligands.
3.5. Conclusions and significance

We recently developed the first paradigm of ligandomics to identify cell-wide ligands, in the absence of receptor information. My analysis of the ligandomics data set identified HRP-3 and HDGF as novel retinal angiogenic ligands [85, 86]. Identification and subsequent validation of these factors and controls established the capacity of ligandomics for global binding activity quantification.

For comparative ligandomics, I implemented an alternative data analysis strategy to identify disease-associated endothelial ligands. This technology allows for the identification of previously unappreciated EC ligands in the healthy and diabetic vasculature, based on their distinct binding activities. Comparative ligandomics led to the discovery of Scg3 as a DR-high ligand and HRP-3 as a DR-low ligand. Disease-high ligands with minimal binding to the healthy vasculature could be easily missed using traditional ligand identification techniques. Although ligandomics cannot exhaustively identify all endogenous ligands it is the only available approach to systemically delineate disease-related factors. To my knowledge this is the first application of comparative ligandomics for the identification of such ligands. In this thesis, validation of Scg3 as a DR-high ligand and a therapeutic target will in turn support the validity of comparative ligandomics to identify endogenous factors with disease relevance.
Chapter 4: Scg3 as a vascular permeability factor *in vitro* and *in vivo*

The working hypothesis is that Scg3 functions as a vascular permeability factor *in vitro* and *in vivo*. The rationale for this hypothesis is based on comparative ligandomics data, in which Scg3 preferentially bound to the diabetic vasculature of STZ mice. Since STZ-induced diabetic mice only exhibit retinal leakage and do not progress towards angiogenesis, Scg3 was predicted to promote vascular permeability.

To verify Scg3 as a permeability factor, an *in vitro* transwell insert system and an Evans blue *in vivo* assay were implemented. Although these experiments were carried out using healthy ECs, we predicted that the functional activity of Scg3 would be the same, albeit with reduced activity compared to the diabetic vasculature.

**4.1. Scg3 facilitates endothelial permeability *in vitro***

The transwell insert endothelial permeability assay is illustrated in Fig. 4.1A [85, 86, 125]. Briefly, ECs were plated on gelatin-coated inserts. After attachment, ECs formed a confluent monolayer. At confluency, fluorescein isothiocyanate (FITC)-dextran (3 kDa, 1 µg/ml) in the presence of Scg3, VEGF or media only was added into the lower chamber. FITC diffusion from the lower chamber into the upper chamber will be detected only if the EC monolayer becomes permeable. Therefore, an increase in FITC in the upper chamber is indicative of increase in growth factor-induced endothelial permeability [125].
Results showed that Scg3 at 200 ng/ml increased HUVEC permeability after 24-h treatment (P<0.05). As a positive control, VEGF at 100 ng/ml also enhanced HUVEC permeability (P<0.05, Fig. 4.1B). These findings were independently verified using HRMVECs (Fig. 4.1C).

**Fig. 4.1. Scg3 facilitates endothelial permeability**

(A) Cartoon illustration of the transwell insert assay. HUVECs or HRMVECs were plated in a confluent monolayer in the presence of FITC-Dextran (3 kDa) and Scg3 (200 ng/ml), VEGF (100 ng/ml) or media only for 24 h. An increase in FITC in the upper chamber is indicative of an increase in EC permeability. (B) HUVEC permeability. Scg3 promoted HUVEC permeability (p<0.05). (C) HRMVEC permeability. Scg3 promoted HRMVEC permeability (p<0.05). n=3. All data are mean ± SEM, Student’s t-test.

### 4.2. Scg3 promotes retinal vascular leakage *in vivo*

In animal models, an intravitreal injection of VEGF triggers vascular changes seen in the early stages of DR, most notably retinal leakage [36]. Therefore, the objective of this experiment was to investigate if Scg3, like VEGF, could induce retinal permeability following an intravitreal injection into healthy mice.

Retinal vascular leakage was assessed using Evans blue dye 4 h post intravitreal injection, as previously described [85, 86]. A cartoon illustration is shown in Fig. 4.2A. Evans blue dye has a high binding affinity to albumin, which is
leaked from the retina vessels after the breakdown of the BRB and can be quantified to determine leakage [110, 131-133]. Scg3, VEGF, BSA or PBS was intravitreally injected into healthy mice. All data was normalized to the PBS-injected eye.

Results showed that intravitreal Scg3 (0.25 µg/1 µl/ eye) promoted retinal permeability in healthy mice (P<0.001). As the positive control, VEGF (0.1 µg/1 µl/ eye) induced comparable leakage (P<0.01). In contrast, BSA (0.25 µg/1 µl/ eye) had no effect (Fig. 4.2B).

![Fig. 4.2. Scg3 promotes retinal vascular permeability](image)

**Fig. 4.2. Scg3 promotes retinal vascular permeability**

**(A)** Cartoon illustration of Evans blue assay. Mice received an intravitreal injection of Scg3 (0.25 µg/1 µl/ eye), VEGF (0.1 µg/1 µl/ eye), BSA (0.25 µg/1 µl/ eye) or PBS. Evans blue was assessed after 4 h. **(B)** Quantification. Scg3 promoted retinal vascular permeability in healthy mice (P<0.001). VEGF also induced EC permeability (P<0.01). BSA did not induce retinal leakage. Data was normalized to PBS. n=5. All data are mean ± SEM, Student's t-test.

### 4.3. Conclusions and significance

These results are significant in that this is the first study to define the role of Scg3 as a vascular permeability factor using *in vitro* and *in vivo* models. These results suggest that like VEGF, Scg3 may play a role in diabetic retinal leakage. This notion will be investigated in a later chapter. Delineating this functional role of Scg3
is the first step in defining its pathogenic role in DR and its utility for ligand-based DME therapy. Furthermore, these results characterize Scg3 as an exudative factor in both retinal and non-retinal ECs, suggesting a role for this factor beyond the retina. Results from this chapter support the validity of comparative ligandomics to identify genuine EC ligands with biological activity.

For the in vivo leakage analysis I chose to use an Evans blue assay. An alternative method is Western blot analysis to directly measure leaked albumin in perfused retinas using an anti-albumin antibody [134]. Although both methods have been established [86], my experience indicates that the Evans blue assay is a far more reliable, quantitative and sensitive tool to detect retinal vascular leakage. Similarly, fluorescein angiography is excellent to detect retinal vascular leakage with focal lesions [110] however, it is not ideal for accurate quantification of diffused retinal leakage induced by intravitreal VEGF or Scg3. This method is also insensitive to detect and quantify disseminated retinal leakage in DR. For in vitro assays, I chose to use commercially available primary HUVECs and HRMVECs. Using commercially available ECs avoids the technical complications of isolating primary mouse or rat retinal ECs, contamination of other cells and low yield of rodent retinal ECs.
Chapter 5: Scg3 as an angiogenic factor *in vitro*

The working hypothesis of this chapter is that Scg3 plays a dual role as a vascular permeability and angiogenic factor. The rationale for this hypothesis is modeled after VEGF, which functions as both an exudative and angiogenic factor [135]. In Chapter 4, Scg3 was characterized as a vascular permeability factor *in vitro* and *in vivo*. This chapter expands upon this knowledge by defining the role of Scg3 in EC angiogenesis. Interestingly, prior to this thesis Scg3 had never been reported as an angiogenic ligand, despite the fact that several family members and their precursors are involved in angiogenesis [93, 96].

To interrogate the angiogenic role of Scg3 in healthy ECs, I utilized an *in vitro* cell proliferation, migration, tube formation and spheroid sprouting assay [124]. Similar to the *in vitro* permeability assays, I predicted that Scg3 functional activity would not be different in healthy ECs, but rather show less robust effects compared to diabetic ECs. *In vivo* data will be presented in a following chapter.

5.1. Scg3 promotes endothelial proliferation

EC proliferation is required for new vessel growth. An *in vitro* endothelial proliferation assay was used to evaluate Scg3 as a potential angiogenic factor [85, 86, 119]. For this assay, HUVECs were plated and incubated with increasing concentrations of Scg3 to determine the optimal dose. Results showed that Scg3 induced HUVEC proliferation only at 1 µg/ml but not at lower concentrations (P<0.001, Fig. 5.1A). In contrast, VEGF promoted HUVEC proliferation at 0.05 µg/ml (P<0.001, Fig. 5.1B). Similar results were obtained using HRMVECs (Fig. 5.1C).
5.2. Scg3 stimulates endothelial tube formation

A tube formation assay on Matrigel-coated wells was used to assess the angiogenic activity of Scg3. Matrigel contains a mixture of extracellular matrix proteins which facilitate EC attachment. When plated on Matrigel-coated wells, ECs form tube networks in the presence of an angiogenic factor [85, 86]. In an effort to reduce background we selected growth factor-reduced (GFR) Matrigel [121, 124]. Cells were incubated in the presence of Scg3, VEGF or media alone and imaged after 4 h (Fig. 5.2A).

Results showed that Scg3 at 300 ng/ml significantly promoted HUVEC tube formation as measured by total tube length (P<0.05, Fig. 5.2B), number of tubes (P<0.05, Fig. 5.2C) and number of branching points (P<0.01, Fig. 5.2D). As the
positive control, VEGF (50 ng/ml) increased the total tube length (P<0.01, Fig. 5.2B), number of tubes (P<0.05, Fig. 5.2C) and number of branching points (P<0.01, Fig. 5.2D).

**Fig. 5.2. Scg3 stimulates endothelial tube formation**

(A) Representative images after 4 h. (B) Quantification of total tube length. Scg3 increased total tube length at 300 ng/ml (P<0.05). As the positive control, VEGF also increased tube length at 50 ng/ml (P<0.01). (C) Quantification of the total number of tubes. Scg3 (P<0.05) and VEGF (P<0.01) increased the total number of tubes. (D) Quantification of the number of branching points. Scg3 (P<0.01) and VEGF (P<0.01) increased the number of branching points. n=4. All data are mean +/- SEM, Student’s t-test.

5.3. **Scg3 promotes endothelial spheroid sprouting**

Angiogenesis is the growth of new blood vessels from pre-existing vasculature. During this process, vessels breakdown their extracellular matrix and migrate towards a stimulus. This process can be mimicked *in vitro* using a tube formation assay as previously described (Fig. 5.2). However, this assay has its limitations since the tube formation assay does not accurately reflect the *in vivo* 3D microenvironment. To compensate for these limitations, a 3D spheroid sprouting assay was developed [124, 136]. In this assay, a defined number of ECs form a spheroid which was then embedded in a fibrin gel. In the presence of an angiogenic factor these spheroids develop sprouts of different lengths. This design ensures
that ECs sprout from pre-existing tubes (i.e. the spheres) and migrate through the extracellular matrix [124]. This assay was performed as previously described by my lab [85, 86].

Representative images of HRMVEC sprouts after 48 h incubation with Scg3, VEGF or media only are shown (Fig. 5.3A). The average sprout length per viewing field was quantified. Results demonstrated that Scg3 (1 µg/ml) stimulated HRMVEC sprouting (P<0.001). As the positive control, VEGF (50 ng/ml) induced HRMVEC sprouting (P<0.001). Minimal sprouting was seen when spheroids were incubated in media alone (Fig. 5.3B). Similar results were obtained using HUVECs (Fig. 5.3C).

![Figure 5.3](image.png)

**Fig. 5.3. Scg3 promotes endothelial sprouting**

(A) Representative images after 48 h. Yellow arrows indicate spheroid sprouts. (B) HRMVEC sprout length. Average sprout length per viewing field was quantified. Scg3 (P<0.001) and VEGF (P<0.001) significantly promoted HRMVEC spheroid sprouting. (C) HUVEC sprout length. Scg3 (P<0.001) and VEGF (P<0.001) promoted HUVEC sprouting (C). n=8. All data are mean ± SEM, Student’s t-test.
5.4. Sdg3 induces endothelial migration

Endothelial migration is an essential step in the angiogenic process. This phenomenon can be mimicked in vitro using a wound healing assay, also known as a migration assay [85, 86, 120]. HRMVECs were cultured to confluency and a scratch was made down the center of each well to mimic the wound region. Cells were incubated in the presence of Sdg3, VEGF or media alone. Wells were imaged after 0 and 20 h (Fig. 5.4A), and the percentage of area covered by the migrated cells was quantified. Results showed Sdg3 (1 µg/ml) promoted an increase in the percentage of migrated cells into the denuded region after 20 h (P<0.05). As the positive control, VEGF (50 ng/ml) also induced EC migration (P<0.01, Fig. 5.4B).

![Fig. 5.4. Sdg3 induces endothelial migration (wound healing)](image)

(A) Representative images are shown at 0 and 20 h. Boundaries of the scratch are indicated by the black bars. (B) Quantification of migrated cells. Sdg3 increased the percentage of migrated cells at 20 h (P<0.05). VEGF also promoted cell migration at this time (P<0.01). n=3. All data are mean +/- SEM, Student’s t-test.

5.5. Conclusions and significance

This chapter provides a substantial conceptual advance beyond the existing literature for Sdg3. Prior to this study, only CgA and Sdg2 were implicated in angiogenesis, as either full-length proteins or precursors for angiogenic peptides.
[93, 96]. Although Scg3 secretion was upregulated in activated platelets [104] and dysfunctional β-cells [105], suggestive of a role in the diseased vascular, its functional role was not known. In this chapter, the angiogenic properties of Scg3 were independently verified using in vitro models of various stages in the angiogenic cascade such as proliferation (Fig. 5.1), tube formation (Fig. 5.2), spheroid sprouting (Fig. 5.3) and migration (Fig. 5.4) albeit at a much higher concentration than VEGF. This may suggest that Scg3 could have relatively low functional activity in normal vessels, consistent with the ligandomics finding that Scg3 minimally binds to normal vessels, supporting its predicted role as a DR-high ligand.

In summary, this chapter in combination with Chapter 4, defines a dual role for full-length Scg3 as both an exudative and angiogenic factor, similar to VEGF [97, 137]. Defining these functional roles of Scg3 is essential for us to evaluate its preferential activity in the diabetic versus healthy vasculature.
Chapter 6: Independent validation of Scg3 as a disease-associated angiogenic factor in vivo

The working hypothesis of this chapter is that Scg3 stimulates angiogenesis preferentially in the diabetic vasculature. The rationale of this hypothesis is based on comparative ligandomics data, in which Scg3 bound preferentially to diabetic ECs (Fig. 3.4), possibly due to the upregulation of its receptor. Scg3 as a disease-related ligand is further supported by the high concentration of Scg3 required for in vitro angiogenesis (Chapter 5). If Scg3 is a genuine DR-high angiogenic factor, this factor may preferentially induce new vessel growth in diabetic but not healthy mice at a comparable concentration. We chose a cornea angiogenesis assay to evaluate Scg3 as a DR-high ligand. An assay in which Scg3 evoked a significant response in healthy mice, such as Evans blue, would not be ideal to detect this difference.

6.1. Scg3 functional activity is upregulated in the diabetic vasculature

The cornea is a valuable organ to study angiogenesis due to its avascular nature and convenient visualization in live mice. A modified cornea angiogenesis assay was used to assess the growth-promoting properties of Scg3 in vivo, as described in Fig. 6.1A [85, 86, 138]. This assay was performed in 4-month STZ diabetic and age-matched healthy control mice. The 4-month time point was used to ensure that Scg3 binding was elevated in the diabetic vasculature, consistent with the ligandomics analyses (Fig. 3.4).

Briefly, filter paper was saturated with Scg3 (250 ng/ml), HRP-3 (1,000 µg/ml), VEGF (100 ng/ml) or PBS, and implanted into the cornea of anesthetized mice. VEGF was included as a positive control based on its identification as a non-
diseased-associated ligand. In contrast, HRP-3 was identified as a DR-low ligand and was included as an opposing control to DR-high Scg3 (Fig. 3.4). PBS was used for the contralateral cornea as a negative control. After 6 days, corneal angiogenesis was quantified by several criteria using slit lamp microscopy: number of new vessels, number of branching points and a total vessel score. The total vessel score was calculated by taking into account the number of quadrants with vessels, length and the number of branching points, as described in Table 6.1 [85, 86, 138]. Representative images of slit lamp microscopy are shown in Fig. 6.1B. After quantification mice were perfused with 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) to label blood vessel. Representative images of DiI stained corneas are shown in Fig. 6.1C.

Results demonstrated that Scg3 at 250 ng/ml significantly increased the number of corneal vessels (P<0.0001, Fig. 6.1D), number of branching points (P<0.01, Fig. 6.1E) and the total angiogenesis score (P<0.0001, Fig. 6.1F) in diabetic versus healthy mice. In contrast, HRP-3 (1,000 µg/ml) preferentially increased the number of corneal vessels (P<0.001, Fig. 10D), number of branching points (P<0.01, Fig. 10E) and total angiogenesis score (P<0.05, Fig. 10F) in healthy but not diabetic mice. VEGF (100 ng/ml) induced comparable vessel growth and number of branching points in both diabetic and healthy mice (Fig. 10D,E). However, VEGF did promote a marginal, albeit statistically significant increase in the total new vessel score of diabetic mice (P<0.05, Fig. 10F). As the negative control, PBS promoted negligible vessel growth after 6 days.
Fig. 6.1. Scg3 functional activity is upregulated in the diabetic vasculature

DR-high Scg3 selectively stimulated corneal angiogenesis in diabetic but not control mice. In contrast, DR-low HRP-3 induced angiogenesis in healthy but not diabetic mice. VEGF promoted angiogenesis in both healthy and diabetic mice. (A) A schematic of the cornea angiogenesis assay is shown. Briefly, Scg3 (250 ng/ml), HRP-3 (1,000 µg/ml), VEGF (100 ng/ml) or PBS was implanted into the anesthetized mouse cornea. After 6 days angiogenesis was quantified. (B) Representative photographic images of corneal angiogenesis in diabetic and healthy mice. (C) Dil staining of corneal blood vessels. * in B and C indicates the position of corneal implant. (D-F) Quantification of corneal angiogenesis. (D) Total number of corneal vessels (Scg3, P<0.0001; HRP-3, P<0.001; VEGF, n/s for not significant). (E) Number of branching points (Scg3, P<0.01; HRP-3, P<0.01; VEGF, n/s). (F) Total angiogenesis score (Scg3, P<0.0001; HRP-3, P<0.05; VEGF, P<0.05). Sample sizes (# of cornea) are indicated at the bottom. All data were normalized against PBS. All data are mean +/- SEM, Two-way ANOVA test.
6.2. Conclusions and significance

This chapter independently verified the comparative ligandomics data and supported Scg3 as a DR-high ligand. Results showed that at matched concentrations, Scg3 promoted angiogenesis preferentially in diabetic ECs. The opposing activity patterns for DR-high Scg3 and DR-low HRP-3 in the diabetic and healthy vasculature suggests that these results are not due to chance. In contrast to Scg3, VEGF as a non-disease-associated ligand promoted equal activity in both the diabetic and healthy vasculature (Fig. 6.1D,E). Although, VEGF did promote a marginal, albeit statistically significant increase in the total cornea angiogenesis score in diabetic versus healthy mice (Fig. 6.1F), we believe this can be explained by the high sensitivity of this quantification method. The opposing activity patterns observed for Scg3 and VEGF suggests that these ligands may regulate NV through distinct pathways and will be investigated in Chapter 9.

The dichotomy between these results and Scg3-induced angiogenesis in healthy ECs in Chapter 5, can be explained by the relatively high concentration of

<table>
<thead>
<tr>
<th>Score</th>
<th>Number of Quadrants with corneal neovascularization</th>
<th>Number of cornea vessels / quadrant</th>
<th>Length of longest cornea vessel between limbus and cornea center / quadrant</th>
<th>Number of branch points / quadrant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1-5</td>
<td>0.05-0.25</td>
<td>1-5</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>6-10</td>
<td>0.26-0.50</td>
<td>8-10</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>11-15</td>
<td>0.51-0.75</td>
<td>11-15</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>&gt;15</td>
<td>&gt;0.75</td>
<td>&gt;15</td>
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Table. 6.1. Total angiogenesis score: Criteria for grading the severity of corneal neovascularization

Cornea neovascularization was assessed using a modified scoring system [138]. A score of 0-4 was assigned for number, length and branch points for new cornea vessels. The total NV score for each mouse was used as an indicator of corneal angiogenesis.
Scg3 required for its in vitro activities. In contrast, Scg3 and VEGF concentrations were matched for the cornea angiogenesis assay. If Scg3 concentrations were elevated in this assay, as in the in vitro angiogenesis experiments, we predict Scg3 may induce modest vessel growth. However, the purpose of this chapter was to evaluate the differential functional activity of Scg3 and VEGF in diabetic and healthy mice at matched concentrations.

I evaluated Scg3 functional activity, in lieu of quantifying its receptor expression in the diabetic versus healthy vasculature. This was largely based on the fact that the receptor for Scg3 is unknown. However, the preferential activity of Scg3 in the diabetic vasculature may be due to an upregulation of its cognate receptor in diseased ECs. Therefore, future work should identify the receptor for Scg3 and quantify its expression in diseased vasculature.

The cornea was selected as a surrogate angiogenic model due to the fact that diabetic mice do not develop retinal NV. The ability of Scg3 to induce corneal NV in diabetic mice demonstrates that its functional activity is not exclusive to the retina and highlights this factor as a valuable disease-related target applicable for other tissues. I chose to modify the traditional cornea angiogenesis protocol which utilized a slow-releasing pellet of a growth factor [139]. This pellet was formed by mixing the growth factor with hydron and sucralfate on a mesh bedding. It was mentioned in the original protocol that the growth factor used, such as VEGF, must be free of any carrier (e.g., BSA) to avoid unstable pellets [139]. Although Scg3 was carrier-free, we were not able to generate stable intact pellets and therefore could not be inserted into the mouse cornea. This could be due to the chemical
property of Scg3, which may be more closely related to BSA, rather than VEGF, in terms of its stability for the formation of slow-releasing pellets. The use of filter paper as the growth factor carrier proved to be a valuable alternative to assess angiogenic activity in vivo [85, 86, 140]. Using this technique, I obtained intact pellets with highly reproducible data while minimizing time-consuming preparation.

In summary, these results independently corroborate the use of comparative ligandomics for identification of the DR-high and DR-low ligands. To my knowledge, such distinct disease-related angiogenic activity patterns have not been reported. Although the preferential activity of Scg3 was defined in the context of a diabetic mouse model, I predict that this upregulation is not exclusive to high glucose conditions. The role of Scg3 as a “disease-associated” or “disease-related”, but not a “disease-specific”, ligand and its involvement in other diseased tissues will be addressed in upcoming chapters.
Chapter 7: Anti-Scg3 therapy for diabetic retinal leakage

The working hypothesis is that Scg3 inhibition using a neutralizing antibody can reduce retinal EC leakage, associated with DME. This rationale is based on the results from Chapter 4, in which Scg3 was characterized as a vascular permeability factor \textit{in vitro} and \textit{in vivo}. Comparative ligandomics results identified Scg3 as a DR-high ligand with its receptor upregulated on the diabetic retinal vasculature (Fig. 3.4). Accordingly, Scg3 displayed selective functional activity in diabetic corneal ECs \textit{in vivo} (Fig. 6.1). Taken together, these results suggest that Scg3 is involved in diabetic retinal leakage and raises the question whether Scg3 expression is elevated in the retina or vitreous fluid of diabetic mice.

This working hypothesis was tested using two mouse models of DR, in which extracellular Scg3 was blocked using an affinity-purified Scg3-neutralizing pAb. However, pAbs may non-specifically recognize other proteins and are not appropriate for clinical translation due to possible off-target effects. To overcome this issue, we generated a Scg3-neutralizing mAb.

7.1. Mouse models of diabetic retinopathy

Diabetic mouse models develop early stages of DR which are characterized by an increase in retinal vascular permeability, but do not progress towards angiogenesis. When selecting a type 1 diabetic model, I chose the STZ and Ins2\textsuperscript{Akita} mouse models for my experiments.

STZ enters the pancreatic β cells through the glucose transporter 2 receptor (Glut2) and induces type I diabetes through the chemical destruction of these cells [141]. STZ-induced diabetic mice show increased vascular permeability ~4 months
post STZ induction [142]. STZ is more effective in males than females possibly due to the protective effect of estradiol on pancreatic β cells [107]. Although this is a cost efficient, quick and low maintenance mouse model of type 1 diabetes, it is not without its limitations.

Since STZ enters via the Glut2 receptor, it is not specific for pancreatic β cells and may also affect the liver and kidneys [107]. This model induces diabetes through a chemical destruction of the β cells and therefore does not reflect the natural onset of disease. The ability of STZ to induce type I diabetes is largely dependent on the mouse strain, with C57BL/6 mice considered high responders [107]. Nonetheless, variations in the severity of diabetes still exist within the C57BL/6 strain. Given these limitations, I evaluated the effectiveness of anti-Scg3 therapy using an additional type 1 diabetic mouse model to ensure that the therapeutic effect was not due to this pharmacological model.

The Ins2Akita is a genetically-induced spontaneous type 1 non-pharmacological diabetic mouse model. These mice contain an autosomal dominant single amino acid mutation in the insulin 2 gene, which results in the misfolding of the insulin protein [108, 109]. Mice lose β cell function and exhibit increased hyperglycemia beginning ~4.5 weeks of age [109], however retinal leakage and degenerating capillaries do not appear until ~4 to 36 weeks of age [108, 142]. Similar to STZ mice, males show an enhanced diabetic phenotype compared to females.

In these studies, STZ and Ins2Akita mice with hyperglycemia were aged for 4 and 6 months to ensure the development of retinal leakage by mimicking the
chronic process similar to human DR. Only male STZ and Ins2<sup>Akita</sup> mice were used for the experiments. Results showed that STZ (P<0.01) and Ins2<sup>Akita</sup> (P<0.05) exhibited a ~3-fold increase in retinal leakage compared to healthy controls, using an Evans blue assay (Fig. 7.1).

![Fig. 7.1. Retinal leakage in STZ and Ins2<sup>Akita</sup>](image)

Retinal leakage was confirmed in STZ mice (P<0.01) aged 4-months and Ins2<sup>Akita</sup> mice (P<0.05) aged 6-months using Evans blue assay. n=8. All data are mean +/- SEM, Student’s t-test.

### 7.2. Scg3 expression in diabetic and healthy mice

Scg3 contains a signal peptide and is secreted from endocrine and neuroendocrine cells [91]. Scg3 secretion is elevated from activated platelets [104] and dysfunctional β-cells [105], suggesting that its expression may be altered in the diabetic vasculature. In patients with PDR, VEGF is increased in the vitreous fluid, highlighting the importance of this factor in DR pathogenesis [32, 33]. Therefore, Scg3 upregulation under similar conditions may suggest a role for this factor in DR pathogenesis and illustrate its potential as a target for ligand-based therapy, similar to VEGF.
To determine if Scg3 is elevated in diabetes, the vitreous fluid was collected from healthy and diabetic mice. A Western blot showed that Scg3 expression was increased in the vitreous fluid of diabetic mice aged 4 and 12 months (Fig. 7.2A,B). Quantification of 4-month diabetic mouse vitreous fluid showed a significant elevation in its expression at this time (P<0.05, Fig. 7.2C). Due to the limited number of aged 12-month diabetic mice, Scg3 expression was not quantified in the vitreous fluid of diabetic mice at this time.

β-actin was used as a loading control for the Western blot, as previously described for healthy human and mouse samples [143]. Accordingly, β-actin can be detected in the vitreous fluid of healthy and diabetic mice using proteomics analyses [143-145]. However, the means through which β-actin enters the vitreous is not known. Due to the fact that this fluid is predominantly acellular it is possible β-actin is leaked out of ocular cells. However, my group did not detect a pronounced difference in expression between diabetic and healthy mice for this protein in mouse vitreous fluid.

Fig. 7.2. Scg3 is increased in the vitreous fluid of diabetic mice

(A-B) Representative images of Scg3 expression in the vitreous fluid of STZ-induced diabetic 4-month (seen in A) and 12-month (seen in B) mice, with age-matched controls. (C) Scg3 was significantly upregulated in the vitreous of 4-month diabetic mice (P<0.05). n=4 (healthy), 6 (diabetic). All data are mean +/- SEM, Student’s t-test.
The next question was to determine which retinal cells express Scg3. Endogenous Scg3 expression was compared between healthy and diabetic retinas using immunohistochemistry and Western blot. Results showed that Scg3 was expressed in multiple layers of the diabetic and healthy mouse retinas by immunohistochemistry (Fig. 7.3). Scg3 was detected in the retinal ganglion cells, inner and outer plexiform layers, photoreceptor inner segments and RPE, all of which contain secretory vesicles. Few Scg3 signals were detected in the inner and outer nuclear layers as well as the photoreceptor outer segments, which contain no secretory vesicles. The signal specificity was confirmed by the absence of Scg3 signals in the control panels (left column in Fig. 7.3), in which anti-Scg3 primary antibody was omitted.

**Fig. 7.3. Scg3 is expressed in the diabetic and healthy retina**

Scg3 is expressed in the mouse retina, particularly in the retinal ganglion cell layer (RGC), inner plexiform layer, outer plexiform layer, photoreceptor inner segments and RPE cells. Few Scg3 signals were detected in the inner and outer nuclear layer, and photoreceptor outer segments. Frozen tissue sections were incubated with rabbit anti-Scg3, followed by Alexa Fluor 488-labeled goat anti-rabbit IgG antibody. The nuclei were visualized with DAPI. The signals were analyzed by confocal microscopy (Scale bar = 50 µm). The left column (Control Merged) was analyzed without the primary anti-Scg3 pAb. Scg3: Green, DAPI, Blue.
The next objective was to determine if Scg3 expression was upregulated in 4-month diabetic versus aged-matched control retinas. A representative image of the Western blot is shown in Fig. 7.4A. Western blots are more sensitive to detect subtle differences in protein expression, compared to immunohistochemistry (Fig. 7.3). Western blot results showed that Scg3 expression was not significantly upregulated in the diabetic versus healthy mouse retinas (Fig. 7.4B).

![Image of Western blot results showing Scg3 expression in diabetic and healthy retinas.]

Fig. 7.4. Scg3 is not elevated in the diabetic retina

(A) Representative image of Scg3 expression in the retina of diabetic and healthy mice. (B) Scg3 was not upregulated in the 4-month diabetic retina versus control. n=4 (healthy), 6 (diabetic). All data are mean +/- SEM, Student's t-test.

7.3. Anti-Scg3 pAb reduces diabetic retinal leakage in STZ mice

A commercially available anti-Scg3 pAb was obtained and its ability to neutralize Scg3 was verified in vitro using endothelial proliferation assay in HRMVECs. Results showed that anti-Scg3 pAb (2 µg/ml) blocked Scg3-induced (1 µg/ml) proliferation (P<0.001), but had no effect on VEGF-induced (100 ng/ml) proliferation. As the control, aflibercept (2 µg/ml) prevented VEGF-induced proliferation (P<0.005). IgG (2 µg/ml) and anti-Scg3 pAb alone did not affect EC proliferation. All results were normalized to media only control (Fig. 7.5).
Once its neutralizing capacity was verified in vitro, the ability of anti-Scg3 pAb to reduce diabetic retinal leakage was evaluated in STZ mice. To determine the optimal therapeutic dose, anti-Scg3 pAb (0.1 µg - 0.36 µg/ 1 µl/ eye) or IgG (0.36 µg/ 1 µl/ eye) was injected intravitreally. Retinal leakage was assessed using an Evans blue assay. Results showed that anti-Scg3 pAb at 0.36 µg/ 1 µl/ eye significantly reduced retinal leakage (P<0.005). Anti-Scg3 pAb at 0.1 µg/ eye did not alleviate DR leakage (Fig. 7.6A).

Using the pre-established dose for anti-Scg3 (0.36 µg/ 1 µl/ eye), diabetic mice received an intravitreal injection of either anti-Scg3 pAb, aflibercept, IgG, mock control or PBS. One group of mice received no injection in one eye and a PBS injection in the fellow eye. All data was normalized to the PBS-injected eye to account for differences in baseline retinal leakage.
Results showed anti-Scg3 pAb (0.36 µg/ 1 µl/ eye) significantly reduced retinal leakage after 4 h (P<0.001). These results were similar to those obtained using a clinically relevant dose of aflibercept (2 µg/ 1 µl/ eye), a commercial available anti-angiogenic drug (P<0.001). IgG and mock antibody control (0.36 µg/ 1 µl/ eye) did not show a significant reduction in retinal leakage. As expected, there was no difference in retinal leakage between non-injected control eyes and PBS injected eyes, confirming that the observed effects are not due to an injection-related event (Fig. 7.6B).

**Fig. 7.6. Anti-Scg3 pAb reduces retinal leakage in STZ mice**

Anti-Scg3 pAb, mAb, aflibercept or control was intravitreally injected into STZ mice. Evans blue assay in STZ mice. Anti-Scg3 pAb (0.36 µg/1 µl/eye) reduced retinal leakage after 4 h (P<0.01). Aflibercept (2 µg/1 µl/eye), as a positive control, also reduced retinal leakage (P<0.01). There was no effect seen using IgG, mock control antibody or non-injection control eyes. Data was normalized against PBS and expressed as a percentage of reduction in leakage after 4 h. n=4 eyes. All data are mean +/- SEM, Student’s t-test.
7.4. Generation and characterization of anti-Scg3 mAb

Although anti-Scg3 pAb effectively reduced diabetic vascular leakage, pAbs recognizing multiple epitopes may have off-target effects and are not appropriate for clinical therapy. To address this problem, we generated seven hybridoma clones against human Scg3. The presence of Scg3-specific mAbs in the hybridoma conditioned media was detected using ELISA assay. Plates were coated with Scg3 and incubated in the presence of the indicated anti-Scg3 hybridoma clone (Fig. 7.7A). These results were independently verified using a Dot blot assay (Fig. 7.7B). We next tested the ability these mAb clones to neutralize Scg3-induced functional activity using endothelial proliferation assay. For this assay, Scg3 (1 µg/ml) was incubated with HRMVECs in the presence of hybridoma-conditioned serum-free media (1:1 for conditioned medium:HRMVEC medium) for 24 h. The results showed that all hybridoma clones, except ML190, significantly reduced Scg3-induced cell proliferation.

We selected clone ML49 for further validation based on its consistent neutralization of Scg3-induced functional activity (P<0.01, Fig. 7.7B). ML49 hybridoma cells were further sorted into in 96-well plates with one cell per well. After cells were expanded, ELISA analysis identified ML49.3 hybridoma subcloned with a high yield of anti-Scg3 mAb (data not shown). ML49.3 mAb was then purified, column exchanged and used for all subsequent experiments. This clone was fully characterized including its full-length sequence for the heavy and light chains. Any reference to anti-Scg3 mAb in the remainder of this thesis is referencing Clone ML49.3.
Anti-Scg3 mAb, raised against human Scg3, recognized both mouse (mScg3, P<0.001) and human Scg3 (hScg3, P<0.001, Fig. 7.8) using an ELISA assay. This can be attributed to the high cross species homology between Scg3. The next step was to verify that affinity-purified anti-Scg3 mAb could neutralize Scg3-induced functional activity using a HRMVEC proliferation assay. Results show anti-Scg3 mAb (2 µg/ml) neutralized Scg3-induced (1 µg/ml) cell proliferation (P<0.001) after 24 h. IgG (2 µg/ml) had no effect on Scg3-induced proliferation. Both anti-Scg3 mAb and IgG alone had no effect on EC proliferation (Fig. 7.9).
To further verify its neutralizing capacity, anti-Scg3 mAb was tested for its ability to block Scg3-induced protein kinase phosphorylation. A representative image of the Western blot is shown (Fig. 7.10A). Results showed anti-Scg3 mAb (2 µg/ml) prevented Scg3-induced (1 µg/ml) phosphorylation of Src protein kinase.

Fig. 7.8. Anti-Scg3 mAb (ML49) recognizes mouse and human Scg3.

Results show anti-Scg3 mAb (ML49) recognized both mouse (P<0.001) and human Scg3 (P<0.001). n=3. All data are mean +/- SEM, Student’s t-test.

Fig. 7.9. Anti-Scg3 mAb prevents Scg3-induced endothelial proliferation

Anti-Scg3 mAb (2 µg/ml) significantly reduced Scg3-induced (1 µg/ml) cell proliferation in HRMVECs after 24 h (P<0.001). IgG had no effect on Scg3-induced cell proliferation. IgG or anti-Scg3 mAb alone did not affect proliferation. n=5. All data are mean +/- SEM, Student’s t-test.

To further verify its neutralizing capacity, anti-Scg3 mAb was tested for its ability to block Scg3-induced protein kinase phosphorylation. A representative image of the Western blot is shown (Fig. 7.10A). Results showed anti-Scg3 mAb (2 µg/ml) prevented Scg3-induced (1 µg/ml) phosphorylation of Src protein kinase.
in HRMVECs (Fig. 7.10B). These data in combination with the in vitro cell proliferation assay suggest that anti-Scg3 mAb is capable of neutralizing Scg3 functional activity.

**Fig. 7.10. Anti-Scg3 mAb prevents Scg3-induced Src activation**

(A) Representative image. (B) Quantification of p-Src. Signal intensity of p-Src was normalized against total Src. Results show that Scg3 alone stimulated Src phosphorylation in HRMVECs (P<0.05). This activation was blocked when Scg3 was pre-incubated with anti-Scg3 mAb (P<0.05). n=5. All data are mean +/- SEM, Student’s t-test.

### 7.5. Anti-Scg3 mAb reduces diabetic retinal leakage in STZ mice

Similar to anti-Scg3 pAb, anti-Scg3 mAb should be verified for its ability to reduce diabetic retinal leakage using the STZ mouse model. Diabetic mice were injected with either anti-Scg3 mAb, aflibercept, IgG, mock control or PBS. Anti-Scg3 mAb was injected at the same therapeutic dose as anti-Scg3 pAb (0.36 µg/ 1 µl/ eye). All data was normalized to the PBS injected eye to account for differences in baseline retinal leakage.

Results showed anti-Scg3 mAb (0.36 µg/ 1 µl/ eye) reduced diabetic retinal leakage using Evans blue assay (73% reduction, P<0.01). As the positive control, aflibercept (2 µg/ 1 µl/ eye) significantly reduced diabetic retinal leakage (73% reduction, P<0.01). Neither IgG (0.36 µg/ 1 µl/ eye), mock antibody control (0.36
µg/ 1 µl/ eye), nor PBS reduced retinal leakage at this time. There was no difference in retinal leakage detected between non-injected control and PBS-injected eyes (Fig. 7.11).

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**Fig. 7.11. Anti-Scg3 mAb reduces retinal leakage in STZ mice**

Anti-Scg3 mAb (0.36 µg/1 µl/eye) reduced retinal leakage in STZ mice (P<0.01). Aflibercept (2 µg/1 µl/eye), as a positive control, also reduced retinal leakage (P<0.01). There was no effect of IgG, mock control antibody or non-injection control. Data was normalized against PBS and expressed as a percentage of reduction in leakage after 4 h. n=5 eyes. All data are mean +/- SEM, Student’s t-test.

7.6. **Anti-Scg3 mAb reduces diabetic retinal leakage in Ins2<sup>Akita</sup> mice**

Ins2<sup>Akita</sup> mouse model was used to confirm the therapeutic effects of anti-Scg3 mAb on diabetic retinal leakage, to ensure that these results were not due to the model itself. Ins2<sup>Akita</sup> mice at the age of 6 months received an intravitreal injection of anti-Scg3 mAb, IgG, aflibercept or PBS. All data were normalized to PBS-injected control eyes.

Results showed anti-Scg3 mAb (0.36 µg/ 1 µl/ eye) reduced diabetic retinal leakage using Evans blue assay (64% reduction, P<0.01). As the positive control,
aflibercept (2 µg/ 1 µl/ eye) also reduced diabetic retinal leakage (75% reduction, P<0.01). IgG (0.36 µg/ 1 µl/eye) did not show an effect on retinal leakage (Fig. 12).

7.7. Conclusions and significance

This chapter expands upon the existing literature for Scg3 and further defines its functional role in the diseased vasculature. The therapeutic effect of anti-Scg3 for DR leakage supports the role of this ligand in pathogenic retinal leakage, with applications for ligand-based DME therapy. These results showed that Scg3 expression was increased in the vitreous fluid of diabetic mice (Fig. 7.2). The significance of this finding is reinforced by the fact that VEGF is elevated in the vitreous fluid of patients with PDR [32, 33]. Since Scg3 was expressed (Fig. 7.3) but not upregulated in the diabetic mouse retina (Fig. 7.4), its secretion may be tightly controlled, similar to the regulated release of cytotoxic T-lymphocyte-
associated protein 4 [147]. It is possible that Scg3 may only be released under pathogenic conditions, such as diabetes. Scg3 as a biomarker of disease should be investigated in future directions.

Scg3 inhibition in vitro was achieved using a neutralizing pAb (Fig. 7.5) and mAb (Figs. 7.9, 7.10). Intravitreal injection of Scg3-neutralizing pAb or mAb inhibited extracellular, but not intracellular Scg3. Accordingly, inhibition of Scg3 significantly reduced diabetic retinal leakage in both STZ (Fig. 7.6, 7.11) and Ins2Akita (Fig. 7.12) mouse models of DR. The use of two DR models ensured that the therapeutic effect observed was not model-specific. Unlike most pAbs purified using protein G columns from the serum of immunized animals, anti-Scg3 pAb was affinity purified to remove all non-Scg3-recognizing pAbs. Thus, this affinity-purified pAb should have markedly reduced non-specific recognition to other proteins. Nonetheless, pAbs are not appropriate for clinical trials due to possible off-target effects. To overcome this obstacle, we generated a Scg3-neutralizing mAb and characterized its therapeutic effects, similar to those of anti-Scg3 pAb. Taken together with the results using Scg3 pAb, these data provide additional confirmation of the pathogenic role of Scg3 in vascular leakage.

Characterization of anti-Scg3 mAb is one of the most significant accomplishments of this thesis. The full-length sequences of ML49.3 mAb, including its heavy and light chains, have been fully characterized. This is a necessary step to facilitate the humanization of this antibody for bench-to-bedside translation, similar to Lucentis and Avastin for anti-VEGF therapy. Future work will focus on humanization of this mAb for clinical trials.
In these experiments FDA-approved aflibercept was selected as the positive control for VEGF inhibition. Aflibercept binds and neutralizes VEGFA across species with high affinity [54], thus ensuring that it can successfully neutralize murine VEGF \textit{in vivo}. This is in contrast to anti-VEGF mAbs, ranibizumab and bevacizumab, whose ability to neutralize murine VEGF is controversial. Some groups, including my lab have shown that ranibizumab can neutralize murine VEGF in models of DR [148], whereas others report no therapeutic effect in murine models [149]. It should be noted that in addition to VEGFA neutralization, aflibercept can also block PIGF and VEGFB [54]. Future directions should compare anti-Scg3 mAb to murine specific anti-VEGF mAb.

Much of the \textit{in vivo} therapy data presented in this thesis serves as a proof of concept supporting the pathogenic role of Scg3, and although significant, it remains pilot data. For example, in these experiments two concentrations of anti-Scg3 pAb were tested for their ability to reduce diabetic retinal leakage (Fig. 7.6A). We selected the dose at which anti-Scg3 significantly reduced this leakage compared to IgG. I chose a clinically-relevant dose of aflibercept as the positive control. This concentration was selected based on its therapeutic dose in patients and adjusted accordingly, to account for the difference between mouse and human vitreous fluid volume. In the future a dose curve should be performed to determine the optimal therapeutic concentration of anti-Scg3 inhibit retinal leakage in DR mice and compared to matched concentrations of aflibercept. This will allow for a more accurate comparison of their maximal therapeutic efficacy ($E_{\text{max}}$) and potency ($EC_{50}$). Furthermore, in these experiments retinal leakage was evaluated 4h post
therapeutic injection. Pilot data showed that anti-Scg3 mAb was still effective in reducing diabetic retinal leakage after 24 h (data not shown). Although beyond the scope of this thesis future experiments should investigate the therapeutic duration of anti-Scg3. We also predict that humanized Fab fragment of anti-Scg3 mAb should exhibit similar pharmacokinetics to ranibizumab, which is also a humanized Fab antibody of an anti-VEGF mAb. Thus, anti-Scg3 with high efficacy and predictable pharmacokinetics should be promising therapy for clinical translation.
Chapter 8: Anti-Scg3 for ocular neovascularization

The working hypothesis is that Scg3 inhibition, using a neutralizing mAb or pAb, can reduce NV. The rationale for this working hypothesis is based on the ability of Scg3 to function as both an exudative and angiogenic factor in retinal and non-retinal ECs (Chapters 4 & 5). The ability of anti-Scg3 pAb and mAb to reduce DR-induced leakage was defined in the previous chapter. Accordingly, this chapter will expand upon this knowledge and investigate the effectiveness of Scg3 inhibition for ocular NV.

To test the working hypothesis, we implemented several non-diabetic surrogate mouse models to study the effect of anti-angiogenic therapy for retinal and choroidal NV. To evaluate Scg3 mAb for retinal NV, we used an OIR mouse model. The role of Scg3 mAb for choroidal NV was evaluated using laser- and Matrigel-induced CNV mouse models. Therefore, by extension, anti-Scg3 was evaluated for its use in PDR and wet AMD.

8.1. Anti-Scg3 mAb therapy for proliferative DR

Diabetic animal models do not progress towards late stage PDR, marked by NV. To overcome this limitation, a non-diabetic OIR mouse model was utilized as a surrogate model to study the effects of anti-angiogenic agents for PDR therapy. In this model, high oxygen induces retinal EC death. Subsequent exposure to ambient oxygen is perceived as a hypoxic insult to the developing retina and triggers the upregulation of angiogenic factors and retinal NV. Anti-angiogenic therapeutic intervention on postnatal day 12 (P12), such as anti-VEGF, prevents this pathological NV [111, 112].
The timeline for the OIR mouse model is illustrated in Fig. 8.1. For this experiment, mice received an intravitreal injection of anti-Scg3 mAb, pAb, aflibercept, IgG or PBS on P12. On P17, retinal angiogenesis was quantified by calculating the percentage of NV, number of tufts and branching points per retina. All data was normalized to non-injected high oxygen treated control eyes, as described [111, 112].

![Timeline for the OIR mouse model](image)

**Fig. 8.1. Oxygen-induced retinopathy (OIR) mouse model can be used to assess anti-angiogenic therapies**

Pups and their nursing mother were exposed to high oxygen (75%) from postnatal day 7-12. On day 12 pups were returned to ambient oxygen until day 17. Mice received an intravitreal injection of anti-Scg3 mAb (0.36 µg/ 1 µl/eye), pAb (0.36 µg/ 1 µl/eye), aflibercept (2 µg/ 1 µl/eye), IgG (0.36 µg/ 1 µl/eye) or PBS on P12. Mice were sacrificed and NV was assessed on P17.

Results showed anti-Scg3 pAb (0.36 µg/ 1 µl/eye) significantly prevented the percentage of NV (P<0.001, Fig. 8.2B), number of tufts (P<0.001, Fig. 8.2C) and the number of branching points (P<0.05, Fig. 8.2D). These findings were independently verified using anti-Scg3 mAb (0.36 µg/ 1 µl/eye). Anti-Scg3 mAb also reduced the percentage of NV (P<0.001, Fig. 8.2B), number of tufts (P<0.001,
Fig. 8.2C) and the number of branching points (P<0.001, Fig. 8.2D). As the positive control, aflibercept (2.0 µg/ 1 µl/eye) showed a significant reduction in NV across all conditions (Fig. 8.2B-D). In contrast, IgG (0.36 µg/ 1 µl/eye) and PBS did not have a therapeutic effect.

**Fig. 8.2. Anti-Scg3 prevents retinal neovascularization**

(A) OIR representative images. Arrowheads indicate NV and NV tufts. Doses are as described in Fig. 8.1. (B) NV quantification. Anti-Scg3 mAb, pAb and aflibercept significantly prevented retinal NV (mAb, P<0.001; pAb, P<0.001; aflibercept, P<0.001). (C) Tufts quantification. Anti-Scg3 mAb, pAb and aflibercept significantly prevented tuft formation (mAb, P<0.001; pAb, P<0.001; aflibercept, P<0.001). (D) Branching points. Anti-Scg3 mAb, pAb and aflibercept significantly reduced the number of branching points (mAb, P<0.001; pAb, P<0.001; aflibercept, P<0.001). All data were normalized to non-injected controls. n=13 (PBS), 11 (IgG),12 (aflibercept), 11 (pAb), 13 (mAb). All data are mean +/- SEM, Student’s t-test. P-value listed versus PBS.
8.2. Anti-Scg3 mAb for choroidal neovascularization

We implemented two mouse models of wet AMD to evaluate the ability of anti-Scg3 to prevent CNV. The first was a laser-induced CNV mouse model. The experimental timeline for this model is outlined in Fig. 8.3. Briefly, mice were subjected to laser photocoagulation on Day 0. Laser photocoagulation damages Bruch’s membrane and is sufficient to trigger choroidal angiogenesis [113-115].

Three days post laser photocoagulation, anti-Scg3 pAb, mAb, control IgG or aflibercept were intravitreally injected. On Day 7, Fluorescein angiography was performed to quantify CNV-associated leakage by measuring the pixel intensity of each lesion using PhotoShop Software. Mice were allowed to recover for 24 h to allow for the clearance of fluorescein and euthanized on Day 8 to analyze CNV 3D volume, lesion area size and vessel density.

**Fig. 8.3. Laser-induced choroidal neovascularization mouse model**

Mice were subjected to laser photocoagulation to induce CNV on Day 0. Three days post laser injury mice received an intravitreal injection of anti-Scg3 mAb (0.36 µg/ 1 µl/eye), pAb (0.36 µg/ 1 µl/eye), aflibercept (2 µg/ 1 µl/eye) or IgG (0.36 µg/ 1 µl/eye). Fluorescein angiography was performed on Day 7, and CNV vessels were imaged on Day 8.
Representative images are shown for each condition (Fig. 8.4A). Fluorescein angiography results showed that anti-Scg3 pAb (0.36 µg/1 µl/eye) reduced CNV-associated leakage (P<0.05). This finding was independently validated using anti-Scg3 mAb (0.36 µg/1 µl/eye), which showed a comparable reduction in CNV leakage (P<0.001). As the positive control, aflibercept (2.0 µg/1 µl/eye) also decreased CNV-associated leakage (P<0.05, Fig. 8.4B).

**Fig. 8.4. Anti-Scg3 mAb reduces laser-induced leakage**

On Day 7 mice were analyzed for retinal leakage by fluorescence angiography. Doses as described in Fig. 8.3. (A) Representative images of each condition. Yellow arrows indicate laser-induced lesions (B) Quantification. Anti-Scg3 pAb, mAb and aflibercept reduced leakage (anti-Scg3 pAb, P<0.05; mAb, P<0.001, aflibercept, p<0.05). All data was normalized to IgG. n=30 (anti-Scg3 pAb), 40 (mAb), 18 (aflibercept), 52 (IgG). All data are mean +/- SEM, Student’s t-test.

Following Fluorescein angiography, mice were allowed to recover, and choroidal angiogenesis was assessed the following day (Day 8). At this time, mice were sacrificed, choroids were removed and labeled using Alexa Fluor 488-
conjugated isolectin B4 to visualize new vessel growth. CNV lesions appear as circular regions of high pixel intensity (Fig. 8.5A). CNV was quantified using three criteria: intensity, area and volume. All choroids were imaged using z-stack confocal microscopy. Since fluorescence signal corresponds to labeled vasculature, high pixel intensity is indicative of increased vessel density. Although each lesion was imaged using z-stack confocal images, only the single largest image was quantified for each lesion. This usually corresponded to the center-most image. From this image, arbitrary pixel intensity was calculated using Adobe PhotoShop Software. Relative pixel intensity was normalized to IgG controls. Quantification of the lesion area was performed in a similar manner using the same z-stack image which was analyzed for pixel intensity. Area of each lesion was measured using ImageJ software. For the quantification of CNV 3D volume, all z-stack images were compiled using Volocity Software to calculate a total lesion volume. All three parameters were compiled to obtain a better assessment of the ability of anti-Scg3 to reduce CNV.

Representative images of CNV lesions are shown (Fig. 8.5A). Results showed that anti-Scg3 pAb (0.36 µg/ 1 µl/eye) reduced lesion CNV intensity (P<0.01, Fig. 8.5B), area (P<0.001, Fig. 8.5C) and 3D volume (P<0.01, Fig. 8.5D). This finding was independently verified using anti-Scg3 mAb (0.36 µg/ 1 µl/eye), which showed a similar reduction in CNV lesion intensity (P<0.001, Fig. 8.5B), area (P<0.01, Fig. 8.5C) and 3D volume (P<0.01, Fig. 8.5D). As the positive control, aflibercept (2.0 µg/ 1 µl/eye) also minimized CNV lesion intensity (P<0.01, Fig.
area (P<0.01, Fig. 8.5C) and 3D volume (P<0.05, Fig. 8.5D). IgG (0.36 µg/1 µl/eye) showed no therapeutic effect.

**Fig. 8.5. Anti-Scg3 mAb reduces laser-induced choroidal neovascularization**

Choroid flat mounts were analyzed for NV. Doses as described in Fig. 8.3. (A) Representative images from each condition. Yellow arrows indicate isolectin (488) stained CNV lesions (B) Fluorescence intensity quantification. Anti-Scg3 pAb, mAb and aflibercept reduced CNV lesion intensity (pAb, P<0.001; mAb, p<0.001; aflibercept, P<0.01). Data was normalized to IgG. (C) Lesion area quantification. Anti-Scg3 pAb, mAb and aflibercept reduced CNV area (pAb, P<0.01; mAb, p<0.01; aflibercept, P<0.01). (D) Volume quantification. Anti-Scg3 pAb, mAb and aflibercept reduced CNV lesion volume (pAb, P<0.01; mAb, p<0.01; aflibercept, P<0.05). n (# of laser spots) is indicated on the bottom. All data are mean +/- SEM, Student’s t-test.

To independently corroborate the above findings, a Matrigel-induced CNV mouse model was utilized [116, 117]. The timeline for this model is shown in Fig. 8.6. Briefly, Matrigel was injected into the subretinal space of healthy mice on Day 0. Injected Matrigel was initially located between the photoreceptors and RPE cells but gradually translocated into sub-RPE space to induce CNV by mimicking drusen...
as insoluble deposits [47]. Thus, this model is distinctly different from laser-induced CNV with tissue injury to Bruch’s membrane. Mice received a subcutaneous injection of either anti-Scg3 pAb, mAb, IgG, or aflibercept immediately after the Matrigel injection on Day 0. Treatments were repeated on Day 2 and 4. CNV-associated leakage was assessed using Fluorescein angiography on Day 7, as described for laser-induced CNV analyses. All data were normalized to Matrigel only controls.

Fig. 8.6. Matrigel-induced choroidal neovascularization

Mice received a subretinal injection of Matrigel to induce CNV by mimicking drusen deposits on Day 0. Mice received a subcutaneous injection of anti-Scg3 pAb (25 µg/Kg body weight), mAb (25 µg/Kg), aflibercept (250 µg/Kg) or IgG (25 µg/Kg) on Day 0, 2 and 4. On Day 7 fluorescein angiography was performed to analyze CNV-induced leakage.

Representative images are shown (Fig. 8.7A). Results showed that anti-Scg3 pAb (25 µg/Kg body weight) reduced CNV-associated leakage (P<0.0001). The role of Scg3 in CNV pathogenesis was independently verified using anti-Scg3 mAb (25 µg/Kg). Scg3 mAb significantly reduced CNV-associated leakage (P<0.001). As the positive control, aflibercept (250 µg/Kg) also showed a
significant reduction in CNV-associated leakage (P<0.0001). IgG (25 µg/Kg) did not have any effect on CNV (Fig. 8.7B).

**Fig. 8.7. Anti-Scg3 mAb reduces Matrigel-induced leakage**

On Day 7 mice were analyzed for leakage using fluorescence angiography. Doses are as described in Fig. 8.6. **(A)** Representative images of each condition. Yellow arrows indicated Matrigel-induced lesions **(B)** Quantification. Anti-Scg3 pAb, mAb and aflibercept reduced CNV-associated leakage (anti-Scg3 pAb, P<0.0001; mAb, P<0.001, aflibercept, P<0.0001). IgG injected mice showed no reduction in leakage. n=9 (anti-Scg3 pAb), 9 (mAb), 6 (aflibercept), 5 (IgG). All data were normalized to Matrigel only controls, mean +/- SEM, Student’s t-test.

### 8.3. Conclusions and significance

This chapter defines Scg3 as a target for anti-angiogenic therapy. Comparative ligandomics data initially identified Scg3 as a disease-associated factor in a mouse model of retinal leakage (Fig. 3.4). Results from Chapters 4 and 5, defined the ability of Scg3 to promote both EC permeability and angiogenesis. Accordingly, we proposed that anti-Scg3 was effective for dual inhibition of vascular leakage and angiogenesis. These results expanded on this concept and
further defined the anti-angiogenic potential of Scg3 inhibition beyond the diabetic retinal vasculature.

In this chapter anti-Scg3 therapy was evaluated exclusively in ocular NV disease. However, we predict that this is not the only application for this therapy. Anti-Scg3 could be applicable to non-diabetic diseases marked by pathological NV. This is supported by my data in which anti-Scg3 prevented retinal NV in the non-diabetic OIR mouse model (Fig. 8.2). These findings suggest that the upregulation of its receptor is not exclusive to diabetic retina. The ability of Scg3 to function outside the retina is supported by results from Chapters 4 and 5, in which Scg3 promoted vascular permeability and angiogenesis in retinal and non-retinal ECs. This is supported by my results showing that anti-Scg3 reduced choroidal angiogenesis (Figs. 8.7, 8.5, 8.4), suggestive of its role in wet AMD. Taken together, these results suggest that the preferential activity of Scg3 is not exclusive to the diabetic retina, and that its receptor upregulation may occur in diseases beyond the eye, such as cancer. Possible implications for this finding will be addressed in the Discussion section of this thesis.

Another novel application of anti-Scg3 is its use in preventative medicine. Feasibility of anti-Scg3 for preventative medicine is based on my OIR data (see OIR timeline, Fig. 8.1), in which an intravitreal injection of anti-Scg3 prevented retinal NV (Fig. 8.2). Additionally, anti-Scg3 mAb was effective in minimizing CNV lesions (Fig. 8.7) when administered before CNV onset in the Matrigel-induced CNV mouse model (see Matrigel-induced CNV timeline, Fig. 8.6). However, intravitreal injections are not ideal for preventative therapy because repetitive
injections may cause eye damage. Accordingly, my data showed that anti-Scg3 is effective when administered via systemic delivery, as seen in the Matrigel-induced CNV mouse model (Fig. 8.7). Systemic therapy of anti-Scg3 will not only treat the first eye with CNV but also provide the collateral benefit of preventing CNV onset in the fellow eyes, which are at high risk. The application of anti-Scg3 for systemic delivery in preventative medicine makes this disease-high angiogenic factor a unique therapeutic target. To my knowledge, this is the first study to demonstrate the application of anti-Scg3 for the prevention of NV in PDR and wet AMD.
Chapter 9: Molecular mechanisms of Scg3

The working hypothesis for this chapter is that Scg3 promotes angiogenesis through a distinct molecular pathway, independent of VEGF and VEGFR signaling cascades. The rationale behind this hypothesis is based on comparative ligandomics (Fig. 3.4) and cornea angiogenesis results (Fig. 6.1), in which Scg3 and VEGF showed differential binding and functional activity in the diabetic vasculature. In this chapter, the molecular mechanisms of Scg3 and VEGF were compared (Fig. 9.1). This was accomplished using an ELISA and protein pull down assay to confirm that Scg3 does not interact with VEGF and VEGFRs. A Western blot was used to determine if Scg3 upregulates VEGF expression or activates VEGF-mediated angiogenic signaling cascades.

Fig. 9.1. Scg3 molecular mechanisms

In this chapter, the molecular mechanisms of Scg3 were investigated and compared to VEGF. This cartoon summarizes known VEGF interactions and highlights possible Scg3 interactions. Known interactions are indicated by solid green lines. Putative interactions are indicted by dashed black lines.
9.1. Scg3 does not interact with VEGFRs

The receptor and binding partners required for Scg3-mediated angiogenesis are largely unknown. Therefore, it is possible that Scg3 binds to one of the VEGFRs expressed on the endothelial surface. As previously discussed, VEGFR2 is the main driver of VEGF-induced EC angiogenesis, while VEGFR1 plays an indirect role and VEGFR3 plays an exclusive role in lymph angiogenesis [1]. Therefore, in this experiment we evaluated Scg3 interactions with only VEGFR1 and VEGR2.

Pull down results showed that Scg3 did not interact with VEGFR1-Fc or VEGFR2-Fc. As the positive control, VEGF did pull down with both VEGFR1-Fc and VEGFR2-Fc (Fig. 9.2A,C). Similar results were obtained for the ELISA assay, in which Scg3 did not interact with VEGFR1-Fc or VEGFR2-Fc. As expected, VEGF interacted with VEGFR1-Fc (P<0.001) and VEGFR2-Fc (P<0.0001). As the negative control, neither Scg3 nor VEGF interacted with BSA (Fig. 9.2B,D).

Aflibercept is a recombinant fusion protein consisting of human VEGFR1 and VEGFR2 fused to the Fc portion of human IgG1. Results showed that Scg3 did not interact with aflibercept using a pull down assay. As the positive control, VEGF did show interact with aflibercept (Fig. 9.2E). Similar results were obtained using an ELISA assay. Scg3 did not interact with aflibercept, however VEGF as the positive control did show a positive interaction (P<0.0001, Fig. 9.2F).
Although Scg3 did not directly interact with VEGFR1 or VEGFR2 (Fig. 9.2), this did not eliminate the possibility that Scg3 might indirectly activate VEGFRs to promote its angiogenic activity. Indirect activation of tyrosine kinase receptors is observed for the SN peptide, which activates VEGFR2, fibroblast growth factor receptor-3 and insulin-like growth factor-1 receptor [98]. Therefore, the objective of this experiment was to determine if Scg3 activates VEGFRs in a similar manner. Due to the fact that VEGFR2 is the primary mediator of VEGF-induced EC angiogenesis [1], in this experiment we only investigated Scg3-induced...
phosphorylation of VEGFR2 (p-VEGFR2, Y1175). Results showed that Scg3 (0.25 µg/ml) did not phosphorylate VEGFR2. As the positive control, VEGF (0.1 µg/ml) activated VEGFR2 [150] (Fig. 9.3).

![Image of Western Blot](image.png)

**Fig. 9.3. Scg3 does not activate VEGFR2**

HRMVECs were stimulated with Scg3 (0.25 µg/ml), VEGF (0.1 µg/ml) or medium control for 24 h, harvested and probed with the indicated antibody. Results showed that VEGF but not Scg3 activated pVEGFR2 (Y1175) in HRMVECs. A representative image is shown n=1.

### 9.3. Scg3 does not interact with VEGF recombinant protein

The objective of this experiment is to determine if Scg3 interacts with VEGF recombinant protein. The rationale behind this experiment is based on the finding that VEGF contains a binding domain for HSPGs [22]. This interaction allows HSPGs to function as co-receptors to enhance VEGF and VEGFR interactions.

Since Scg3 is structurally akin to a HSPG [6], it was possible that VEGF recognizes Scg3 in a similar manner. If an interaction was detected between Scg3 and VEGF this would represent a potential mechanism of action for Scg3-induced angiogenesis. To investigate this possibility, Scg3 and VEGF interactions were detected using an ELISA (Methods 2.9.11) and pull down assay (Methods 2.9.9).

Results showed that Scg3 did not interact with VEGF recombinant protein (Fig. 9.4A). As the positive control, VEGF interacted with VEGFR2-Fc (P<0.005, Fig. 9.4B). Neither Scg3 nor VEGF interacted with BSA. Accordingly, Scg3 also did not
interact with VEGF using a pull down assay. However, as the positive control VEGF was pulled down with VEGFR2-Fc (Fig. 9.4C).

**Fig. 9.4. Scg3 does not bind VEGF**

(A, B) ELISA. Scg3 did not interact with VEGF (seen in A). VEGF showed a positive interaction with VEGFR2-Fc (P<0.005, seen in B). Neither Scg3 nor VEGF interacted with BSA, which served as the negative control. (C) Pull down assay. Scg3 did not pull down with VEGF. VEGF was pulled down in the presence of VEGFR2-Fc. n=3. All data are mean +/- SEM, Student’s t-test.

**9.4. Scg3 does not upregulate VEGF protein expression**

The objective of this experiment was to determine if Scg3 upregulates VEGF protein expression, and vice versa. This was based on the finding that angiogenic factors, such as platelet-derived growth factor, fibroblast growth factor 2, epidermal growth factor [150-152] and even VEGF [153] can upregulate VEGF expression. To test this possibility, HRMVECs were stimulated with either VEGF, Scg3 or control for 48 h, followed by Western blot analyses to detect the cellular expression of VEGF and Scg3 using anti-Scg3 or anti-VEGF antibodies.

Representative images are shown in Fig. 9.5A. Results showed that Scg3 (0.25 µg/ml) stimulation did not increase its own expression (Fig. 9.5B) or the expression of VEGF (Fig. 9.5C). VEGF (0.1 µg/ml) also failed to induce Scg3
upregulation (Fig. 9.5B), however it did significantly upregulate its own expression (P<0.05, Fig. 9.4C), as previously reported [153].

![Image](image.png)

**Fig. 9.5. Scg3 does not upregulate VEGF expression**

HRMVECs were stimulated with Scg3 (0.25 µg/ml), VEGF (0.1 µg/ml) or media only for 48 h, harvested and analyzed by Western blot with the indicated antibody. **(A)** Representative images are shown. **(B)** Quantification of Scg3 signal in (A). Scg3 did not upregulate its own expression. VEGF did not upregulate Scg3. **(C)** Quantification of VEGF signal in (A). Scg3 did not upregulate VEGF. As the positive control, VEGF did upregulate its own expression (P<0.05). n=5. All data are mean +/- SEM, Student’s t-test.

### 9.5. Scg3 activates angiogenic signaling pathways

The objective of this experiment was to compare protein kinase activation between Scg3 and VEGF. Since VEGFR2 is the main driver of VEGF-induced angiogenesis, we focused on VEGFR2 protein kinase activation [1]. VEGF activation of VEGFR2 can activate the full range of angiogenic cascades: EC survival (Akt), proliferation (Erk1/2, Stat3), permeability (Src) and tube formation (Akt and Erk1/2) [25]. A full review of these pathways is presented in Chapter 1.2. The ability of Scg3 to activate angiogenesis-related protein kinases was investigated using a Western blot with antibodies against total and phosphorylated Erk1/2, Mek, Akt, Src and Stat3.
Results showed that Scg3 (0.25 µg/ml) induced the phosphorylation of Erk1/2 and Mek protein kinases but not Akt or Stat3. In contrast, VEGF (0.1 µg/ml) activated Erk1/2, Mek, Akt and Stat3 (Fig. 9.6A-D). Results further showed that both Scg3 and VEGF phosphorylated Src protein kinase (Fig. 9.6E).

**Fig. 9.6. Scg3 activates angiogenic signaling pathways**

Scg3-induced activation of well-known protein kinases were investigated. VEGF served as the positive control. (A-E) Representative images are shown. Results show that Scg3 activated (A) Erk1/2, (B) Mek and (E) Src but not (C) Akt or (D) Stat3. In contrast, VEGF activated all protein kinases.

**9.6. Conclusions and significance**

In this thesis the molecular mechanism of Scg3 was evaluated in terms of its overlap with the VEGF angiogenic pathway. This choice was based on the fact that VEGF inhibitors are the only FDA-approved drugs for ocular NV diseases. By defining the molecular mechanism of Scg3, in terms of its overlap with VEGF signaling pathways, this thesis provides a foundation for understanding signaling
Scg3 molecular signaling and an opportunity for alternative or combination therapy with Scg3 and VEGF inhibitors.

The results of this chapter demonstrated that Scg3 and VEGF show distinct upstream molecular signaling pathways, particularly at the receptor level. This is supported by the results showing that Scg3 neither binds to VEGFR1 or 2 (Fig. 9.2). Scg3 and VEGF do not cross-regulate each other's protein expression in vitro (Fig. 9.5), suggesting that Scg3-induced angiogenesis is not dependent upon VEGF upregulation. My data suggest that although the receptor for Scg3 is unknown, it does not mediate its angiogenic activity through activation of VEGFR2 (Fig. 9.3), the predominant receptor of angiogenesis signaling [1]. At the protein kinase levels, VEGF but not Scg3 activated Akt protein kinase (Fig. 9.6C) and Stat3 transcription factor (Fig. 9.6D). Differential signaling pathways are further supported by opposing binding and functional activity patterns observed between Scg3 and VEGF in the ligandomics (Fig. 3.4) and cornea angiogenesis assays (Fig. 6.1), respectively.

Although both Scg3 and VEGF show distinct upstream signaling, these factors exhibit partial convergence upon shared protein kinases. This is supported by the finding that both Scg3 and VEGF activated Erk1/2 (Fig. 9.6A), Mek (Fig. 9.6B) and Src protein kinases (Fig. 9.6E). Taken together, these data present strong evidence that Scg3 promotes its activity through a distinct molecular, albeit with minimal overlap of VEGF downstream signaling.
Chapter 10: Discussion

10.1 Summary

In this thesis, I applied a new technology of “comparative ligandomics” to diabetic and healthy mice for the differential mapping of disease-related ligands. Scg3 displayed minimal binding to healthy vessels but ~1,731-fold increase in binding to the diabetic endothelium. In contrast, VEGF showed comparable binding to both the diabetic and healthy vasculature. These findings were independently verified using a cornea angiogenesis assay in which Scg3, but not VEGF, preferentially promoted angiogenesis in diabetic mice. Accordingly, Scg3 and VEGF activated distinct receptor signaling pathways. Both factors were elevated in the diabetic vitreous fluid, suggesting Scg3 may play a role in DR pathogenesis. The role of Scg3 as a therapeutic target was confirmed using a neutralizing pAb and mAb in two mouse models of diabetic retinal leakage and ocular neovascularization. These results support the use the anti-Scg3 therapy for non-proliferative and proliferative DR as well as wet AMD. These findings established Scg3 as a disease-related target for anti-angiogenic therapy and provide a foundation for future mAb humanization and clinical trials. Characterization of Scg3 supports the use of comparative ligandomics for identification of disease-associated ligands.

10.2. Advantages of comparative ligandomics

In this thesis, I played an integral role in validating OPD-NGS as the first paradigm of ligandomics. Ligandomics allows for global profiling of the entire ligandome by quantitatively mapping the binding activities of cell-wide factors. In contrast, conventional proteomics can only compare proteome expression profiles to assess
relative protein abundance, and cannot globally map cellular ligandomes to quantify their ligand binding activity [154]. In this regard, ligandomics is fundamentally distinct from conventional proteomics using mass spectrometry, and highlights the quantitative capacity of ligandomics for data analyses. The data presented in this thesis supports the use of quantitative ligandomics as a technique for global identification of disease-related cellular ligands with simultaneous binding activity quantification.

Identifying ligands with disease relevance represents a daunting challenge. The feasibility of quantitative ligandomics to distinguish disease-related ligands is highlighted by the identification of DR-high (Scg3), DR-low (HRP-3) and non-disease-related factors (VEGF) in a diabetic mouse model. DR-high ligands with their receptor(s) upregulated in the disease condition represent valuable targets for therapy, due to their preferential binding and functional activity in the diseased but not healthy vasculature. In contrast, DR-low ligands with downregulated binding to the diseased endothelium have a reduced capacity to modulate the functional activity of diabetic vessels. Therefore, characterization of DR-high ligands in the disease pathogenesis will have a significant impact on future ligand-based therapy. In addition to Scg3, this high-throughput screen identified over 350 putative disease-related factors. Additional identification of such factors will allow us to launch parallel investigations to identify additional Scg3-like factors. Characterization of Scg3 as a genuine DR-high factor with therapeutic potential in
turn supports comparative ligandomics as a valid tool to identify novel ligands with therapeutic applications in the absence of receptor information (Fig. 10.1).

**Fig. 10.1. Summary of comparative ligandomics**

Comparative ligandomics identified unique disease-related factors (i.e. DR-high ligands) whose binding activities were preferentially upregulated on the diabetic vasculature. These candidates may represent novel therapeutic targets. Characterization of Scg3 as a DR-high ligand with therapeutic potential supports the validity of this technology as a valuable tool to identify disease-related ligands.

Another unique advantage of ligandomics is its ability to longitudinally map changes in ligand binding activity to reveal age- or stage-specific factors throughout disease progression. Advanced age is a known risk factor for cardiovascular disease, cancer and neurodegeneration [155]. Yet despite this knowledge little is known about the identity and role of age-related ligands in geriatric diseases. Delineating age-related factors may represent novel targets for therapy by inhibiting detrimental ligands or overexpressing protective factors. Despite the fact that patients with uncontrolled or advanced diabetes are at an increased risk for vascular complications, little is known about the identity and role of stage-specific factors that may contribute to the onset and progression of these complications. Ligand-based therapies targeting these stage-specific factors may be useful for
early detection and intervention at different points in disease progression. Ligandomics is a versatile technology that is broadly applicable to any cells or diseases using *in vitro* or *in vivo* settings. Therefore, comparative ligandomics may be applied to healthy versus diseased, young versus aged, or receptor-expressing versus receptor-deficient/silenced cells to identify disease-related cellular ligands.

**10.3. Scg3 as a novel angiogenic and vascular permeability factor**

Prior to this thesis, Scg3 has never been reported as a cellular ligand or angiogenic factor, despite the fact that family members CgA and Scg2 are implicated in angiogenesis as either full-length proteins or cleaved peptides [94, 96]. Before this study, Scg3 was only known to play important a role in the regulated secretory pathway as an intracellular protein [99, 100]. In contrast, although it does contain a signal peptide, its role as a secreted extracellular protein was less defined. Scg3 secretion is elevated from activated platelets in atherosclerosis [104] and dysfunctional β-cells in diabetes [105]. These findings suggest that Scg3 may play a role in the diseased endothelium. My data expanded upon this limited knowledge and defined the biological function of Scg3 in the vasculature. Scg3 was detected in the vitreous fluid of healthy mice (Fig. 7.2), suggesting that this factor can be secreted and its release is upregulated in diabetic mice.

In this thesis, full-length Scg3 can function as an exudative (Chapter 4) and angiogenic factor (Chapter 5), in both retinal and non-retinal ECs. Although many granin proteins are cleaved to generate biologically active peptides, my data suggests that full-length Scg3 itself is functionally active. This is based on the fact that full-length Scg3 stimulated protein kinase activation after only 10 min in serum-
free media (Fig. 9.6), suggesting that it does not require proteolytic processing for its activity. Although processing is not essential for its biological activity, future work should be performed to determine if Scg3-derived peptides may function as angiogenic stimulators or inhibitors, similar to Scg2-derived SN or CgA-derived catestatin and vasostatin-1 [94, 96]. If alternative isoforms do exist, they may differ in regards to their biological activity. These peptides could play roles as either angiogenic or exudative stimulators (e.g., catestatin) and inhibitors (e.g., vasostain-1), with separate overlapping molecular mechanisms distinct from full-length Scg3.

10.4. Scg3 expression in non-endocrine cells

My data showed that Scg3 was expressed in multiple layers of the retina including the retinal ganglion cells, inner and outer plexiform layers, photoreceptor inner segments and RPE, all of which contain secretory vesicles (Fig. 7.3). This is consistent with the fact that Scg3 with a signal peptide is expressed in endocrine and neuroendocrine cells and is secreted in a manner similar to neurotransmitter release [88]. Accordingly, few Scg3 signals were detected in the inner and outer nuclear layers and photoreceptor outer segments, which contain no secretory vesicles (Fig. 7.3). Future directions should investigate Scg3 expression and secretion from non-endocrine or non-neuroendocrine cells. This knowledge will provide further insight into the regulation and bioavailability of Scg3.

10.5. The molecular mechanisms of SN versus Scg3

Scg2-derived SN is one of the most thoroughly studied angiogenic peptides of the granin family [96]. Much work has been done to characterize its mechanism of
action in ischemia-induced conditions such as peripheral and coronary artery diseases, DR and tumors [96, 98]. In a similar manner, Scg3 was also predicted to function as a growth factor with potential for anti-angiogenic therapy. Prior to this study, there was a lack of mechanistic knowledge regarding the signaling pathways for Scg3. To address this knowledge gap, I looked to SN for clues about the molecular mechanisms of Scg3.

SN-mediated angiogenic activity is dependent upon interactions with VEGF and HSPGs [24, 98]. HSPGs bind to VEGF and function as co-receptors to enhance VEGF binding to its tyrosine kinase receptors. This interaction is attributed to the fact that VEGF contains an HSPG binding site [6]. In dermal and coronary ECs, inhibition of HSPGs prevents SN-induced angiogenesis, highlighting the importance of these co-receptors for VEGF functional activity [24, 98]. Interestingly, SN does not directly bind to VEGF and HSPGs but rather mediates its effects through an indirect regulation of these factors [24]. Similarly, although Scg3 is structurally akin to a HSPG [103], it did not directly interact with VEGF (Fig. 9.4). Scg3 also did not upregulate VEGF protein expression (Fig. 9.5), suggesting that Scg3-mediated angiogenic activity is not through VEGF. However, experiments investigating the ability of Scg3 to promote angiogenesis in the presence of VEGF inhibition should also be investigated but should be carried out in diseased mice, such as diabetic mice, with upregulated Scg3 binding. Although not tested in this thesis, future experiments should investigate if HSPGs are required for Scg3-mediated angiogenesis.
Activation of VEGFR2 is essential for SN-mediated angiogenesis [98]. My data showed that Scg3 did not directly interact with VEGFR1 or VEGFR2 (Fig. 9.2). Accordingly, Scg3 stimulation was not sufficient to phosphorylate VEGFR2 (Fig. 9.3). This finding suggests that in contrast to SN, Scg3 functional activity is not mediated through interaction with or activation of VEGFR2, through either a direct or indirect mechanism. Taken together this data implies that, unlike SN, Scg3-induced angiogenesis is through a VEGF-independent pathway.

Differences between Scg3 and SN are further highlighted by the fact that Scg3 was identified as a disease-related factor, whereas SN had not been described as such a factor. Although in this thesis SN was not investigated directly, future work should examine SN as a potential disease-related ligand and perform a more in-depth comparison between Scg3 and SN.

10.6. The functional roles of Scg3 versus VEGF in disease pathogenesis

In this thesis, Scg3 and VEGF were evaluated and compared in terms of their functional activities, pathogenic roles and therapeutic potentials. Scg3 and VEGF share functional similarities in the healthy and diseased vasculature, namely their ability to promote vascular permeability and angiogenesis. Scg3, like VEGF [32, 33], was upregulated in the diabetic vitreous fluid (Fig. 7.2), implicating a similar role for this factor in disease progression. The ability of Scg3 and VEGF inhibitors to reduce pathogenic leakage was demonstrated in two mouse models of diabetic retinal leakage (Figs. 7.6, 7.11, 7.12). The ability of these inhibitors to prevent retinal and choroidal NV was demonstrated using mouse models of OIR (Fig. 8.2) and CNV (Figs. 8.4, 8.5, 8.7).
On the other hand, my data suggest that Scg3 and VEGF play fundamentally distinct roles in disease pathogenesis. Differences between these two factors are highlighted by comparative ligandomics data in which Scg3 was identified as a DR-high ligand, with preferential binding to the diabetic vasculature, while VEGF was identified as a non-disease-related factor with comparable binding to both vasculatures (Fig. 3.4). This notion of disease- versus non-disease-association was verified using a cornea angiogenesis assay, in which Scg3 preferentially induced vessel growth in the diabetic but not normal vasculature (Fig. 6.1). In contrast, VEGF promoted the angiogenesis of both diabetic and normal vessels (Fig. 6.1). Furthermore, although both Scg3 and VEGF promoted vascular permeability (Chapter 4) and angiogenesis (Chapter 5) in vitro, Scg3 was functionally active only at a substantially higher concentration compared to VEGF. This may be attributed to the role of Scg3 as a disease-related ligand. Accordingly, my data suggests that Scg3 and VEGF promote their functional activity through distinct molecular mechanisms (Chapter 9).

10.7. Obstacles for the identification of the Scg3 receptor

The receptor for Scg3 is still unknown, and its identification presents unique challenges due to its preferential upregulation in the diabetic vasculature. Accordingly, low receptor expression on healthy ECs is a major barrier. One option is to treat HUVEC or HRMVEC with high-glucose to facilitate receptor upregulation, although there is no guarantee that the receptor will be upregulated under these non-diabetic in vitro conditions. Due to this preferential upregulation and the difficulty in creating an appropriate diabetic milieu in vitro, only primary ECs
isolated from diabetic animals should be used for receptor identification. Nonetheless, isolation of diabetic ECs is challenging and does not guarantee that these ECs will maintain similar \textit{in vivo} receptor expression patterns without receptor degradation during EC isolation.

Future directions should focus on overcoming these obstacles, as receptor identification would provide several distinct advantages for this study. Quantification of Scg3 receptor expression in healthy versus diseased vessels would corroborate Scg3 as a DR-high ligand. Additionally, the identification of the Scg3 receptor is necessary to generate an aflibercept-like soluble decoy receptor as an alternative approach for anti-Scg3 mAb therapy.

\textbf{10.8. Scg3 as a potential biomarker for diabetic vascular disease}

Previous groups have shown that Scg3 is secreted from dysfunctional \(\beta\)-cells \cite{105} and activated platelets \cite{104}, suggestive of its role as a biomarker for diabetic vascular disease. This prediction is supported by my results in which Scg3 was upregulated in the vitreous fluid of diabetic mice (Fig. 7.2). The significance of this result is based on previous reports that VEGF, with well-known pathogenic roles in DR is also upregulated in the vitreous fluid of PDR patients \cite{32, 33}. Since Scg3 secretion (Fig. 7.2) but not its intracellular expression (Fig. 7.4) is preferentially upregulated in the diseased vasculature, this factor may play a role as a secreted biomarker in diabetes. Although not investigated in this thesis, Scg3 expression in the serum of type 1 and type 2 diabetic versus healthy patients should also be quantified to further define this role.
10.9. Scg3 and VEGF inhibitors for combination therapy

Combination therapy using Scg3 and VEGF inhibitors may provide higher efficacy than either inhibitor alone. Although several anti-VEGF drugs (Aflibercept, Ranibizumab & Bevacizumab) are available for ocular therapy, a limitation is that they all inhibit the same molecular pathway (i.e. VEGF, see Table 1.1) [55, 59]. Alternative angiogenic factors, with VEGF-independent molecular signaling pathways, are currently being investigated. Possible candidates include Fovista (targeting platelet-derived growth factor, PDGF), Luminate (ALG-1001, targeting integrin receptor), ASP8232 (targeting vascular adhesion protein-1) and AKB-9778 (targeting the angiopoietin receptor, Tie-2) which are now in clinical trials [156-158]. However, some of these conventional angiogenic factors with functional activity in normal vessels may interact with VEGF at various signaling pathways. For example, PDGF can upregulate VEGF expression [159].

Scg3 inhibitors are advantageous for combination therapy based on the role of Scg3 as a DR-high ligand. VEGF is not the only angiogenic factor involved in pathological NV, and other factors (e.g., PDGF) should be investigated for possible combination therapy with anti-Scg3.

The appeal of DR-high Scg3 as a target for combination therapy lies in its ability to promote angiogenesis through a distinct VEGF-independent molecular mechanism. Evidence supporting this differential signaling pathways is largely derived from the fact that Scg3 and VEGF activate distinct EC surface receptors and upstream molecular pathways. In this thesis, Scg3 did not bind to either VEGFR1 or VEGFR2 (Fig. 9.2). More importantly, Scg3 did not stimulate VEGFR2
activation through a direct or indirect mechanism (Fig. 9.3), suggesting that Scg3 and VEGF bind to and activate non-overlapping EC receptors. Scg3 also did not upregulate VEGF expression (Fig. 9.5), suggesting that its mechanism of action is not through activation of the VEGF pathway. Based on this distinct molecular signaling, I predict that Scg3 will have a synergistic effect when combined with VEGF inhibitors. If validated, this will be the first disease-related factor for ligand-based combination therapy.

The distinction between Scg3 and VEGF molecular mechanisms become less clear downstream, as they show partial convergence onto common protein kinases. For example, Scg3 and VEGF phosphorylated Erk1/2, Mek and Src protein kinase, but not Akt and Stat3 (Fig. 9.5). Downstream convergence on a common signaling pathway may be one possible explanation as to why inhibition of both factors show almost a complete phenotype reversal. Pathway convergence is further supported by the finding that both anti-Scg3 and aflibercept independently showed almost a complete reduction of in vivo leakage (Figs. 7.6, 7.11, 7.12) and NV (Figs. 8.2, 8.4, 8.5, 8.7). Complete inhibition by either therapy alone implies that both factors are essential for this phenomena. This notion of downstream convergence is further supported by the fact that Erk can be activated by both tyrosine kinase receptors (i.e. VEGFRs) and G protein-coupled receptors (GPCRs) [147]. It is possible that since Scg3 interacts with one of the GPCRs. Activation of Erk by both factors independently, through different upstream receptors, can markedly influence angiogenesis with similar efficacy. Nonetheless, the relative importance of the Erk pathway in Scg3 signaling is unknown. Future
work should identify the signaling pathway(s) essential to Scg3 functional activity and develop additional inhibitors to block the pathways as an alternative approach for anti-Scg3 therapy. Identification of Scg3 receptor(s) could also be helpful in understanding the molecular signaling pathways of Scg3. These results will more accurately define VEGF and Scg3 downstream converge onto common protein kinases.

Alternatively, Scg3 and VEGF could regulate two separate molecular steps, both of which may be important for angiogenesis. For example, VEGF could upregulate gene expression for angiogenesis by activating transcription factors, or Scg3 might regulate gene expression through protein synthesis, degradation, microRNA, etc. Thus, either anti-Scg3 or anti-VEGF could totally inhibit angiogenesis. An overview of Scg3 and VEGF molecular signaling pathways is shown in Fig. 10.2.
Advantages of disease-related ligands for therapy

Preventative therapy may be applicable for ocular diseases such as wet AMD and diabetic eye disease. For example, patients with wet AMD in one eye have a higher risk of bilateral involvement [160]. Additionally, those with advanced diabetic eye disease are also at an increased risk (~71%) to develop DME. When considering preventative therapy, it is important to evaluate the risk-to-benefit ratio. Repetitive intravitreal injections are not appropriate for preventative therapy due to the increased chance of ocular injection-related adverse effects, including endophthalmitis, retinal detachment, increased intraocular pressure, hemorrhage.
and cataract [59]. Therefore, the challenge for preventative medicine is how to develop an anti-angiogenic therapy suitable for systemic delivery with high efficacy and minimal side effects.

VEGF inhibitors administered systemically may trigger severe or fatal effects, including gastrointestinal perforation, delayed wound healing, hemorrhage, thromboembolic events, hypertension and proteinuria [60]. As a result, anti-VEGF is only approved for intravitreal administration in wet AMD and DME patients to minimize systemic adverse effects [60]. Despite this targeted delivery, VEGF inhibitors may still diffuse into the blood and cause systemic effects, albeit at relatively low rates [59, 61, 62]. Consequently, anti-VEGF is not appropriate for preventive therapy of wet AMD and DME in high-risk patients.

Given that Scg3 is a disease-associated ligand with minimal binding and angiogenic activity in healthy ECs, its inhibitors are predicted to exert negligible side effects on healthy vessels or cells. Preliminary data showed that systemic or intravitreal administration of anti-Scg3 mAb in high doses did not induce retinal or vascular toxicity, as detected through TUNEL assay and pattern electroretinography (data not shown). These findings are supported by a previous report that mice with a homozygous deletion of the Scg3 gene (1B1075 gene) displayed a normal phenotype [161]. In contrast, mice with the deletion of a single VEGF allele resulted in embryonic lethality [65]. Accordingly, inhibition of VEGF was associated with a reduction in vessel density in vivo [68], as well as retinal EC, RPE and neuronal toxicity in vitro [66, 67]. Although beyond the scope of this thesis, future directions should further investigate the safety of anti-Scg3 mAb. If
minimal side effects of anti-Scg3 mAb could be confirmed following treatment, this
would lead to alternative routes of delivery and opportunities for preventative
medicine. Feasibility of anti-Scg3 mAb for prevention of NV was demonstrated
using the Matrigel-induced CNV mouse model, in which systemic anti-Scg3
significantly prevented CNV (Fig. 8.7). Conceivably, systemic delivery of anti-Scg3
for the treatment of DME, PDR or wet AMD in one eye may offer a protective
advantage to the fellow eye.

10.11. Scg3 as a disease-related ligand beyond diabetic vascular disease
Scg3 with increased binding to diseased vessels is “associated” with multiple
diseases, but not “specific” to a single disease. In this regard, we prefer to call
Scg3 a “disease-associated” or “related” ligand, rather than a “disease-specific”
ligand. The concept of Scg3 as a disease-associated ligand is based on the ability
of anti-Scg3 mAb to prevent NV in non-diabetic mouse models of OIR (Fig. 8.2)
and wet AMD (Figs. 8.4, 8.5, 8.7). Based on these findings it is predicted that the
receptor for Scg3 may also be upregulated in the vessels of pathological NV seen
in PDR and wet AMD. However, future studies will be needed to confirm this
preferential binding and receptor upregulation in these non-diabetic conditions.

Accordingly, I predict that Scg3 as a disease-associated angiogenic factor
can be expanded beyond the retina and applied to cancer-associated vessel
growth. The rationale for this prediction is based on my data in which Scg3
promoted angiogenesis in both retinal and non-retinal ECs (Chapter 5). Due to
their ubiquitous expression and involvement in the regulated secretory pathway,
granin proteins have been widely characterized as cancer biomarkers [162].
Accordingly, Scg3 is upregulated in neuroendocrine tumors, small cell lung and hepatoma carcinoma [99, 163, 164]. In small cell lung carcinoma, it is a sensitive and specific marker for cancer progression and elevated levels are inversely correlated with a favorable chemotherapy response [164]. Scg3 indirectly promotes hepatocellular carcinoma proliferation, possibly through cytokine release [99], however its functional role in cancer is less defined [165].

Additional support for this prediction is based on the role of family member CgA as a biomarker and growth factor for cancer. CgA is one of the most well studied cancer biomarkers of the granin family. CgA plasma levels can be used as a diagnostic marker for early detection of endocrine neoplasms [166, 167]. In carcinoid tumors, a specific type of endocrine cancer, CgA can be increased over 1,000-fold [168]. In prostate cancer, its elevation may occur before a rise in prostate-specific markers [169]. In neuroblastoma, plasma CgA levels can be used to identify tumor burden and disease progression. Elevated CgA levels are inversely predictive of a positive therapeutic response. The functional role of CgA in cancer has been defined in the context of neuroblastoma, in which it regulates tumor angiogenesis, vascular permeability, endothelial barrier function, metastasis and epithelial to mesenchymal transition [170]. Based on family homology, I predict that Scg3 may also preform similar angiogenic roles in cancer. If Scg3 can be verified as an angiogenic factor with cancer association, this factor may represent an alternative target for anti-angiogenic cancer therapy, similar to the VEGF inhibitor (Bevacizumab). This would define a novel application of anti-Scg3 mAb therapy beyond the eye.
10.12. Limitations and future directions

10.12.1. Comparative ligandomics

One limitation of OPD is that the expression of mammalian proteins in bacterial systems may not contain proper folding or post-translational modifications. This obstacle could in turn limit the amount of endogenous ligands identified using this system. The critical issue to using this technology as a tool to identify ligands with biological activity is whether this obstacle will limit the applicability of phage display to only a small subset of cellular ligands. To address this concern, we randomly surveyed hundreds of biologically relevant extracellular soluble growth factors (VEGF, epidermal growth factor, fibroblast growth factor), cytokines and chemotactic proteins, commercially-available from Life Technologies and Abcam. About 80-88% of ligands could be successfully expressed in bacteria and did not lose their biological activity due to misfolding or loss of posttranslational modifications. Of interest is the finding that glycosylation is not required for VEGF angiogenic activity, but rather serves to increase secretion efficiency [171]. Furthermore, as a CSPG, Scg3 contains glycosaminoglycan chains. Despite these posttranslational modifications Scg3 was identified using ligandomics. This suggests that these glycosylation sites are not in the receptor-binding domain and are not required for its binding or functional activity. Therefore, despite missing a small percentage of ligands ligandomics should be applicable to identify many physiologically relevant cellular ligands.

Although OPD shows an improvement in endogenous ligand identification compared to traditional phage display, OPD can only identify ~66% of enriched
ligands as phage capsid fusion proteins in correct reading frames. This is compared to ~16% identified by conventional phage display. Despite the improvement, 1/3 of the proteins identified by OPD-NGS may still be out of frame and should be filtered using bioinformatics analyses. Although ligandomics cannot exhaustively identify all possible EC ligands, it should be applicable to identify a large number of biologically relevant ligands. Following ligand identification by ligandomics, it is crucial to verify the functional activity and disease relevance of individual ligands using a phage-independent platform. Independent validation will serve as a quality control checkpoint to minimize the identification of false positives.

Quantitative ligandomics can globally quantify the copy number of all identified ligands. As a cautionary note, this value is a reflection of the relative ligand binding activity and should not be interpreted as an absolute value. Furthermore, phage copy number is reflective of relative receptor expression and ligand-receptor binding affinity, and does not necessarily correlate with ligand abundance. This number may also be influenced by the total sequence reads identified by NGS. Therefore, failure to detect Scg3 binding to the healthy vasculature by ligandomics only suggests that Scg3 receptor is below the detection limit of this assay. This should not be interpreted as a lack of absolute binding, but rather as an indication of low receptor expression. Beyond the scope of this thesis, future work should be done to identify the receptor for each ligand and quantify its expression in the diseased and healthy vasculature, as an independent validation of comparative ligandomics technology.
10.12.2. Anti-Scg3 in vivo therapy

In this thesis, I demonstrated that Scg3 inhibition using a neutralizing mAb or pAb can reduce vascular leakage and NV. Since we are only interested in blocking extracellular Scg3, a neutralizing antibody was used as opposed to intracellular gene deletion. However, in future studies a genetic approach should be implemented to independently verify the role of Scg3 in vascular leakage and NV. Scg3 knockout mice (1B1075 gene) were characterized in 1990, before much was known about the role of Scg3 in ECs or DR [161]. Re-characterization of Scg3 knockout mice should be performed with an emphasis on the retina and its vasculature. If Scg3 plays an important role in DR, Scg3-deficient mice, treated with STZ to induce diabetes, should show reduced DR severity. By a similar token, aflibercept but not anti-Scg3 mAb should be therapeutically active in diabetic Scg3-null mice. This later experiment will also serve to independently validate Scg3 mAb for its specificity.

Future work should use a dose curve to establish the optimal therapeutic dose for anti-Scg3 therapy. A titrated dose curve is necessary to accurately compare the maximal efficacy ($E_{max}$) and potency ($EC_{50}$) of anti-Scg3 and aflibercept. In this thesis, the dose of aflibercept (2 mg/mouse eye) was equivalent to its clinical dose (2 mg/human eye) based on human and mouse vitreous volume. The dose for anti-Scg3 was selected based on a ~70% reduction in retinal leakage compared to IgG (Fig. 7.6). Future studies should compare the effect of anti-Scg3 mAb versus murine VEGF-neutralizing mAb in vitro and in vivo. This is based on the fact that aflibercept is a soluble VEGFR that may also neutralize both VEGF
and PIGF. Additional work is also needed to identify the epitope to which anti-Scg3 mAb binds. If this sequence is identified, neutralizing peptides could be generated to inhibit Scg3. This may provide an alternative therapeutic option for Scg3 inhibition in addition to the neutralizing mAb.

10.13. Conclusions and impact

A high-throughput technique identified Scg3 as a novel disease-associated ligand. This thesis expanded upon this limited knowledge regarding the function of Scg3 and defined its role in the endothelium (Chapters 4 and 5). My results characterized Scg3 as a unique exudative and angiogenic factor with enhanced binding (Chapter 3) and functional activity (Chapter 6) in the diabetic vasculature. Scg3 as a DR-high ligand was independently verified using a cornea angiogenesis assay, in which Scg3 promoted angiogenesis in the diabetic but not healthy mouse vasculature. In contrast, VEGF promoted comparable vessel growth in both diabetic and healthy vasculatures, supporting its role as a non-disease-related EC factor (Fig. 6.1). This differential binding and functional activity suggests that Scg3 and VEGF mediate angiogenesis through two independent pathways. Accordingly, I demonstrated that Scg3 and VEGF activated distinct angiogenic signaling pathways (Chapter 9). These findings are summarized in Fig. 10.2. The role of Scg3 in DR pathogenesis was supported by its upregulation in the diabetic vitreous fluid (Fig. 7.2). Scg3 inhibition using a neutralizing pAb significantly reduced diabetic retinal leakage (Fig. 7.6), retinal NV (Fig. 8.2) and choroidal NV (Figs. 8.4, 8.5, 8.7). To further illustrate how the discovery of a novel disease-associated angiogenic factor by comparative ligandomics can impact ligand-based therapy,
we generated a Scg3-neutralizing mAb. Similarly, this mAb markedly reduced diabetic leakage (Figs. 7.11, 7.12) and NV (Figs. 8.2, 8.4, 8.5, 8.7).

Owing to the limited efficacy of VEGF inhibitors, there is a need to identify alternative therapeutic targets and harness their potential for combination therapy. Identification of Scg3 as a target for vascular leakage, PDR and wet AMD provides an alternative therapeutic option beyond anti-VEGF for these diseases. Humanization of anti-Scg3 mAb, similar to ranibizumab [172], will facilitate bench-to-bedside translation for alternative or combination therapy.

Based on its disease-association Scg3 inhibitors may offer the advantages of high disease relevance, optimal high efficacy, minimal side effects and flexible administration through a VEGF-independent mechanism. A summary of Scg3 and its therapeutic potential can be seen in Fig. 10.3. Due to these advantages Scg3 mAb may be applicable for both treatment and prevention of DME, PDR and wet AMD. Although this thesis is restricted to anti-Scg3 for ocular NV diseases, my results suggest that this mAb may have a significant impact on NV disease beyond the eye. This highlights the uniqueness and diverse applications for anti-Scg3 therapy. The therapeutic potential of anti-Scg3 uncovered in this thesis in turn establishes comparative ligandomics as a powerful and valuable tool for systematic identification of disease-related factors and will impact the rapid development of ligand-based therapies. To my knowledge this is the first application of a disease-related factor for ligand-based therapy.
Fig. 10.3. Scg3 as a disease-related ligand with therapeutic potential

This thesis characterized Scg3 as a novel angiogenic and vascular permeability factor \textit{in vitro} and \textit{in vivo}. The role of Scg3 as a disease-related ligand with preferential activity in the diabetic vasculature was independently verified using a cornea angiogenesis assay. We predict that the upregulation of the Scg3 receptor contributes to its enhanced activity in diabetic ECs. In this thesis, I demonstrated that Scg3-neutralizing mAb and pAb are effective for anti-angiogenic therapy of DME, PDR and wet AMD. Neutralization of Scg3 resulted in a reduction in pathological retinal leakage and NV. Although this factor was first identified in a mouse model of diabetic retinal leakage, we predict its receptor is also upregulated in other disease conditions.
References


157. ClinicalTrial.gov, *A study to evaluate ASP8232 in reducing central retinal thickness in subjects with diabetic macular edema (DME) (ClinicalTrials.gov Identifier: NCT02302079).*

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