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Regulation of the neurovascular patterning by growth factors and cytokines during anterior ocular development

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Abstract

Regulation of the neurovascular patterning by growth factors and cytokines during anterior ocular development

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Ana Ojeda

The cornea is a transparent, avascular, and one of the most innervated tissues of the body. Corneal diseases including injuries, neovascularization, congenital eye defects and degenerations, represent a major public health burden. Although, studies have been focused on understanding the basis of transparency, innervation, and neovascularization of the adult cornea, little is known about the molecular mechanisms that lead to this specialized structure results in a highly innervated but avascular tissue during embryogenesis.

The purpose of this work was to identify molecular regulators of the neurovascular patterning during cornea development. First, Sema3A, a well-known chemorepulser of axons, was identified as a key modulator in the establishment of cornea avascularity in both, avian and murine models. Moreover, I demonstrated that chemokines, initially described for their function in controlling immune cell migration, also play an important role in axon guidance and vasculogenesis during ocular development. Examination of the expression of the chemokine *CXCL14* by *in situ* hybridization and immunohistochemistry revealed novel patterns of localization in the corneal stroma, iris, lens epithelium, retina and trigeminal ganglion. Comparison in the expression of *CXCL14* and *CXCL12* shows that they are expressed in complementary patterns in most tissues during ocular development, suggesting an interactive regulation of these chemokines.
Immunohistochemical analysis of ocular nerves in CXCL14 knockdown chick embryos indicated exacerbated projection of sensory nerves into the corneal stroma, corneal epithelium and iris, which subsequently elevated nerve density in these tissues. *In vitro* analyses revealed that CXCL14 has an inhibitory effect on CXCL12-induced axon growth of trigeminal ganglion sensory neurons. Furthermore, Knockdown of CXCL14 in Tg(tie1:H2B:eYFP) transgenic Japanese quail embryos resulted in ectopic migration of YFP fluorescently labeled angioblasts into the cornea and exogenous CXCL14 inhibits VEGF- and CXCL12-induced angioblast migration into the cornea. This is the first time that CXCL14 has been shown to have a critical function during embryogenesis that may be mediated through inhibition of CXCL12 signaling.

Collectively, these results demonstrate that neurovascular patterning of the anterior eye during development depends on an intricate process and fine balance of growth factors and cytokines. These findings will contribute to a better understanding of the molecular mechanisms involved in pathological conditions such as cornea neovascularization, anterior segment ocular dysgeneses and wound healing, where angiogenesis and nerve regeneration are critically compromised.
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<tr>
<td>AREs:</td>
<td>AU-rich sequence element</td>
</tr>
<tr>
<td>ASLV:</td>
<td>Avian sarcoma-leukosis Virus</td>
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<tr>
<td>BDNF:</td>
<td>brain derived neurotrophic factor</td>
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<tr>
<td>BCIP:</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
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<td>BM:</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BMP:</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA:</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>ca:</td>
<td>ciliary artery</td>
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<td>cb:</td>
<td>ciliary body</td>
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<td>DMEM:</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>dpw:</td>
<td>days post-wound</td>
</tr>
<tr>
<td>E(#):</td>
<td>embryonic (day of gestation)</td>
</tr>
<tr>
<td>ECM:</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF:</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>en:</td>
<td>endothelium</td>
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</tr>
<tr>
<td>ey:</td>
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</tr>
<tr>
<td>FGF:</td>
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</tr>
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</tr>
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<td>GAPDH:</td>
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</tr>
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<td>GFP:</td>
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<td>GDNF:</td>
<td>glial cell-derived neurotrophic factor</td>
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<td>H2B:</td>
<td>histone-2B</td>
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<td>heparin sulfate proteoglycan</td>
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<td>IGF:</td>
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<td>IL8:</td>
<td>interleukin 8</td>
</tr>
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<td>INL:</td>
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<td>KSPG:</td>
<td>keratin sulfate proteoglycan</td>
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<td>L:</td>
<td>lens</td>
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<td>Definition</td>
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<td>LASIK</td>
<td>laser-assisted in situ keratomileusis</td>
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<tr>
<td>lef</td>
<td>lens fibers</td>
</tr>
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<td>lep</td>
<td>lens epithelium</td>
</tr>
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<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>LP</td>
<td>lens placode</td>
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<td>lv</td>
<td>limbal vasculature</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
</tr>
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<td>NBT</td>
<td>nitroblue tetrazolium</td>
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<td>NCCs</td>
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<tr>
<td>NGF</td>
<td>nerve growth factor</td>
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<td>NT-3</td>
<td>neurotrophin-3</td>
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</tr>
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<td>Nrp</td>
<td>neuropilin</td>
</tr>
<tr>
<td>nr</td>
<td>neuroretina</td>
</tr>
<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBT</td>
<td>PBS+ Triton X-100</td>
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<tr>
<td>PEDF</td>
<td>pigmented epithelium derived factor</td>
</tr>
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<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
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<td>Pen/Strep</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>pm</td>
<td>periocular mesenchyme</td>
</tr>
<tr>
<td>PRRs</td>
<td>pattern-recognition receptors</td>
</tr>
<tr>
<td>RCAS</td>
<td>replication competent avian sarcoma-leukosis virus</td>
</tr>
<tr>
<td>RCASBP</td>
<td>RCAS with Bryan polymerase</td>
</tr>
<tr>
<td>Robo</td>
<td>roundabout</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigmented epithelium</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-PCR</td>
</tr>
<tr>
<td>Sc</td>
<td>sclera</td>
</tr>
<tr>
<td>Scr</td>
<td>scrambled</td>
</tr>
<tr>
<td>SDF</td>
<td>stromal cell-derived factor</td>
</tr>
<tr>
<td>Sema3A</td>
<td>semaphorin 3A</td>
</tr>
<tr>
<td>sFlt</td>
<td>soluble feline McDonough sarcoma (fms)-like</td>
</tr>
<tr>
<td>SO</td>
<td>scleral ossicles</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>st</td>
<td>stroma</td>
</tr>
<tr>
<td>tca</td>
<td>temporal ciliary artery</td>
</tr>
<tr>
<td>Tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>TG</td>
<td>trigeminal ganglion</td>
</tr>
<tr>
<td>TNF α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TUJ1</td>
<td>neuron-specific class III beta-tubulin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>VEGF:</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR:</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>Wnt:</td>
<td>wingless-type MMTV integration site family</td>
</tr>
<tr>
<td>YFP:</td>
<td>Yellow fluorescent protein</td>
</tr>
<tr>
<td>WT:</td>
<td>wild type</td>
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Chapter 1: Literature Review

1.1 Structure, Function, and Dysfunctions of the Anterior Eye

The eye is an incredibly complex and unique organ whose function is to provide the sense of vision. In vertebrates, it is composed of several structures that are crucial for the transfer and processing of light. There is a general consensus that the anterior segment of the eye is composed by the cornea, iris, ciliary body, and lens (Figure 1.1A).

![Figure 1.1. Anatomy of the eye and histology of the cornea. (A) Schematic diagram showing cross section view of the eye. (B) Cross-section of the cornea stained with hematoxylin-eosin showing the epithelial, Bowman’s, Stromal, Descemet’s and endothelial layers. Figure adapted from Meeney and Mudhar, 2013.](image)

The cornea

The cornea is an avascular and highly innervated tissue located at the most anterior part of the eye. Due to its unique composition and transparency, the cornea permits transmission and refraction of the light that is essential for proper vision.
(Quantock and Young, 2008). In fact, the cornea is responsible for more than 60% of the total refractive power of the eye (Qazi et al., 2011).

Morphologically, the cornea is made up of five layers: Epithelium, Bowman’s Membrane, Stroma, Descemet’s membrane, and Endothelium (Figure 1.1 B) (Lwigale et al., 2005; Hassell and Birk, 2010). At the outermost part of the cornea, the epithelium is a stratified layer that acts as a barrier for foreign agents that might otherwise have contact with the eye. The corneal epithelial cells also secrete mucins that are critical for maintaining the corneal hydration (Gipson, 2004). Beneath the corneal epithelium, the Bowman’s layer is a specialized acellular matrix that separates the epithelium from the stroma and whose function is to provide substrates for the attachment of epithelial cells (Linsenmayer et al., 1998, Tisdale et al., 1988). Lying below the Bowman’s layer, the corneal stroma is one of the most organized matrices that occupy around 90% of the cornea thickness. It is composed primarily of collagen and proteoglycans that are secreted by specialized cells known as keratocytes (Linsenmayer et al., 1998; Leonard and Meek, 1997). The arrangement of precise, uniform and equally spaced collagen throughout the stroma allows, in large part, cornea transparency. Below the stroma, the Descemet’s layer is the posterior basement membrane comprised mainly of collagen VIII whose function is to provide adhesive substrates essential for endothelial cells attachment (Kapoor et al., 1986; Sawada et al., 1990; Linsenmayer et al., 1998). Finally, as the innermost cellular layer, the endothelium is a monolayer that acts as a physical barrier, but also permits active diffusion of nutrients such as glucose from the aqueous humor to the avascular cornea (Bourne, 2003).
The iris and ciliary body

Below the cornea, the iris is a disc-shaped pigmented contractile structure of the eye that functions to control the entry of light to the retina. It is made up of three cell types: the iris pigmented epithelium, the iridial muscles, and the iris stroma (Davis-Silberman and Ashery-Padan, 2008). The function of the iris is mediated by iris muscles that receive innervation from parasympathetic, sympathetic and sensory trigeminal axons (Neuhuber and Schödl, 2011). Continuous with the iris, the ciliary body consists of ocular muscles, a vascularized layer, and thin epithelial layer that connects to the retinal pigmented epithelium (RPE) and neural retina (Graw, 2003). The ciliary body is responsible for the production of the aqueous humor that provides oxygen and nutrients to the lens and the cornea (Davis-Silberman and Ashery-Padan, 2008).

The ocular lens

As the most posterior structure of the anterior segment of the eye, the ocular lens is a biconvex avascular, transparent and non-innervated tissue whose function, along with the cornea, is to focus light rays on the retina. Lens cells derive from the ectoderm that forms the lens placode (thickening of the ectoderm). As development progresses the lens placode invaginates to form the lens vesicle that differentiates into two cell types: lens fibers and lens epithelium (McAvoy et al., 1999; Graw, 2003). The lens fibers are elongated and transparent cells that form the body of the lens. On the other hand, the lens epithelium provides support to lens fiber cells, and also contains mitotically active stem/progenitor-like cells that are required for cell renewal (Cvekl and Ashery-Padan, 2014).
Congenital dysfunctions of the anterior eye

Congenital eye defects are a major health threat. It has been estimated that more than 20 million children present eye abnormalities worldwide (Graw, 2003). Anterior eye defects, collectively called anterior segment ocular dysgenesis (ASOD) (Sowden, 2007) include a wide variety of developmental conditions that affect the anatomy and function of the cornea, iris and lens. The most common syndromes associated with anterior eye defects are Peter’s anomaly and Axenfeld-Rieger syndrome, the first characterized by incomplete separation of the cornea from the iris or the lens that lead to corneal opacity, and the latter by abnormal angle of the iris (corectopia) that can cause glaucoma (Sowden, 2007; Reis and Semina, 2011). Although mutations in transcription factors such as PAX6, PITX2, FOXC1, FOXC2, and SOX2 have identified these genes as regulators of eye development, their mechanism of actions and interactions with other genes need further studies.

1.2 Cornea Development
1.2.1 Cornea Morphogenesis in Avians

During cornea morphogenesis, the three layers of the cornea originate from two different precursor cell populations (Hay et al., 1979, Hay, 1980). The cornea epithelium forms directly from the cranial ectoderm, while the endothelium and stroma are both derived from a highly migratory multipotent cell population known as neural crest cells (NCCs) (Figure 1.2 A) (Hay et al, 1979). In chick embryos, shortly after embryonic day (E)3, the presumptive cornea epithelium synthesizes extracellular matrix known as the
primary stroma onto which NCCs-derived mesenchyme that is going to form the endothelium migrates at about E4.5 (Figure 1.2 B-D) (Hendrix et al., 1982; Lwigale et al., 2005). By E6, a second wave of neural crest-derived mesenchyme cells migrate between the ectoderm and endothelial layer, and differentiate into keratocytes that synthesize extracellular matrix as indicated by the expression of keratin sulfate proteoglycan (KSPG) and COL-I keratocyte markers of the stroma (Figure 1.2 E and F) (Funderburgh et al., 1986; Linsenmayer et al., 1998; Lwigale et al., 2005).

Figure 1.2 Schematic diagram (A) illustrating the migratory neural crest cells (NCCs) delaminating from the dorsal neural tube (B-F) showing the resultant contribution of NCCs (in red) to the cornea at various developmental stages in chick embryos. At E3 (B) NCCs are located in the periocular region surrounding the primitive lens. At E4.5 (C), NCCs have begun migrating between the lens and epithelium such that by E5 (D), they have formed the inner layer called the endothelium. By E6 (E), NCCs are migrating between the epithelium and endothelium and by E7 (F), all layers of the cornea are formed with the endothelium and stroma being of neural crest descent. L, lens; Ep, epithelium; St, stroma; Cb, ciliary body; En, endothelium. Adapted from Lwigale et al., 2005.
From E7 to E12, the cornea stroma undergoes cell proliferation and extracellular matrix (ECM) components synthesis (Hay, 1980). At subsequent stages, the cornea stroma starts to dehydrate and the collagen fibers compact to have a complete transparent cornea by the time of hatching (Siegler and Quantock, 2002; Connon et al., 2004).

1.2.2 Cornea Morphogenesis in Murines

Although the origin of the cornea and its morphology are well conserved in most of the vertebrates, the processes of migration and differentiation of NCCs are slightly different in mouse compared to chick embryos. During mouse cornea development, between E12.5 – E13.5, NCCs-derived mesenchyme cells migrate between the lens and ectoderm to form first the presumptive stroma (Figure 1.3 A and B) (Hay, 1980; Cintron et al., 1983; Zieske, 2004). Between E14.5-15.5, cells of the posterior stroma become more connected to form a monolayer that corresponds to the corneal endothelium (Figure 1.3 C). Thus, by E16.5 all layers of the cornea are formed (Figure 1.3 D) (Cvekl and Tamm, 2004). Unlike chick cornea development, numerous events such proliferation and changes in gene expression occur postnatally before the opening of the eyelid in the mouse (Hay, 1980; Zieske, 2004). In spite of these differences, the innervation and avascularization of the cornea are features that are shared by the majority of vertebrate embryos.
Figure 1.3. Schematic diagram of ocular mesenchyme development in the mouse eye between embryonic days (E) 12.5-19.5. (A) at E12.5-13.5, the lens vesicle has detached from the surface epithelium (SE) and has become invaginated into the optic cup. Mesenchymal cells (ME) start to migrate into the space between the anterior epithelium of the lens vesicle and the surface of the ectoderm. The inner layer of the optic cup forms the neural retina (Re) and the outer layer the retinal pigmented epithelium (RPE). (B) At E13.5-14.5, the mesenchyme cells condense to form several flat layers that are separated from each other by a loose fibrillar extracellular matrix. In the lens (Le), the primary lens fibers elongate to close the lumen of the lens vesicle. (C) At E14.5-15.5, the posterior mesenchyme cells closest to the lens flatten, become connected by apicolateral contacts and form an endothelial monolayer. At the end of this process, all layers of the cornea have been defined. (B) After E15.5 mesenchyme cells migrate along the epithelial layers of the iris and ciliary body to differentiate into stroma of the iris (Sir) and ciliary body (SCB). Adapted from Cvekli and Tamm, 2004.
1.3 Corneal Innervation

Corneal nerves play a very important role in corneal transparency and health. Indeed, the presence of nerves is essential to protect the cornea from external agents and physical trauma as they are responsible for sensations of touch and pain (Belmonte et al., 2004). Moreover, it has been well documented that corneal nerves secrete trophic factors that stimulate growth and survival of corneal epithelial cells (Baker et al., 1993, Müller et al., 2003). In this section, I will review how corneal innervation occurs in chick embryos, the factors and mechanisms that are involved in maintaining the proper innervation during embryogenesis, and pathologies associated with corneal nerve dysfunction.

![Figure 1.4](image)

Figure 1.4. Progressive outgrowth of the trigeminal sensory axons during cornea development in the chick embryo. (A-C) Sensory axons initially avoid the cornea and extend dorsally (d) and ventrally (v), subsequently joining in the nasal region (n) to form a nerve ring. A subset of axons in the ventral stream forma plexus (asterisk) in the choroid fissure that later provides sensory innervation in the iris. (D-F) upon completion of the nerve ring, sensory axons begin to radially extend into the cornea, primarily innervating its periphery (D and E) then the entire surface (F). d, dorsal outgrowth; v, ventral outgrowth; n, nasal region of the cornea; (*), choroid fissure and ventral plexus. From Lwigale and Bronner-Fraser, 2007.
1.3.1 Trigeminal Ganglion Anatomy and Function

During early embryonic development, cells migrate long distances to condense into various ganglia. Among 10 cranial ganglia, the trigeminal ganglion (TG) is the largest cranial sensory ganglion that innervates the majority of the facial tissues, including the corneas, eyelids and mouth (Lwigale, 2001; Kirstein and Fariñas, 2002). It is composed of two different cell types, NCCs-derived and placode-derived neurons, that migrate to cluster between the eye and ear (Davies, 1988). Morphologically, the TG is divided into three branches: the ophthalmic branch, the mandibular branch and the maxillary branch. Tissues such as eyelid, iris and cornea are innervated by the ophthalmic branch of the TG (D'Amico-Martel and Noden, 1980; D'Amico-Martel and Noden, 1983; Lwigale, 2001, Lwigale et al., 2004). Although how the TG is assembled during development has been extensively studied, the molecular regulators involved in the precise patterning of cranial innervation, particularly in the ocular tissue, are still not well understood.

1.3.2 Development of Avian Corneal Innervation

Previous studies in the chick embryo have shown that there is a precise spatial and temporal pattern of innervation. The innervation of the cornea can be classified into three phases: the nerve ring formation phase, the corneal stromal innervation phase, and the corneal epithelium innervation phase. During the nerve ring formation, sensory nerves from the TG arrive at the ventro-temporal region of the developing cornea to form a ring around it that is complete by E8 (Figure 1.4 A-C) (Bee et al., 1986, Lwigale and Bronner-Fraser, 2007). In the second phase, by E9, the cornea becomes permissive to the sensory nerves as nerve bundles start growing and being attracted into the corneal
stroma (Figure 1.4 D and E). Subsequently, in the late phase, the nerve bundles from the stroma reach the center of the cornea and nerve fibers start to innervate the corneal epithelium, a process that is complete by E15 (Figure 1.4 F) (Lwigale and Bronner-Fraser, 2007; Schwend et al., 2012).

### 1.3.3 Regulation of Sensory Nerve Growth

Perhaps, one of the most intrinsic features of the sensory system is the ability of axons to grow and move long distances to reach their final tissue. Target areas are thought to regulate sensory innervation by producing or restricting the amount of neurotrophic factors. Among all the neurotrophic factors, the neurotrophins: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), neurophin-3 (NT-3) and NT-4/5 are known to be present in the cornea during development and also in the adults. They all activate tyrosine kinase receptors; NGF binds to TrkA, BDNF and NT4/5 bind to TrkB, NT-3 is the ligand for TrkC receptor, while GDNF activates RET receptor (You et al., 2000; Kirstein and Fariñas, 2002).

During corneal innervation NGF is a critical factor for corneal nerve survival, axonal branching and nerve regeneration (Müller et al., 2003). BDNF, similar to NGF, is expressed in human corneal epithelium and anterior stroma. *In vitro* and *in vivo* studies have shown that BDNF not only promotes axon growth and regeneration but also stimulates the expression of other growth factors such as NT-4/5 (Kobayashi et al., 1997; Shaheen et al., 2013). GDNF, expressed by stromal keratocytes, has been reported to promote nerve elongation and neurite branching of sensory neurons (Madduri et al., 2009; Simpson et al., 2012). Although expressed in developing and adult corneas, the
functions of NT-3 and NT-4 are not understood with regards to corneal innervation and regeneration

Semaphorin (Sema) family is a class of secreted and membrane bound proteins that play an important role as guidance molecules. One of the most extensively studied proteins from this family is Sema3A. Sema3A is a secreted protein that signals through neuropilin (Nrp) 1 receptor to act as a negative regulator of axon growth (Renzi et al., 2000). During embryonic development, Sema3A is strongly expressed by the lens vesicle and corneal epithelium and its function is required for proper innervation of the cornea. In fact, disruption of Sema3A/Npn1 signaling leads to aberrant axon projection in the initial phase of corneal innervation during development (Lwigale et al., 2007, Kubilus and Linsenmayer, 2010).

It is well documented that VEGF is a potent stimulator of blood vessel formation. Interestingly, it has also been implicated in nerve growth. VEGF is slightly present and upregulated in healthy and injured corneas, respectively. In fact, it has been shown that VEGF promotes neurite outgrowth and nerve regeneration in the cornea, and that its abrogation delays nerve growth, demonstrating the role of VEGF as a mediator of corneal nerve repair (Yu et al., 2008; Kim et al., 2010; Pan et al., 2013).

Slit/Robo signaling also plays an important role in axon guidance during development. Recent studies have suggested slit/Robo signaling has dual functions during cornea innervation: negative regulator of axon outgrowth at early stage of innervation, and positive regulator of corneal epithelial innervation later (Kubilus and Linsenmayer, 2010a; Schwend et al., 2012).
Cytokines, as inflammatory mediators, have been implicated in pathologic corneal inflammation and nerve regeneration (Sugaya et al., 2011). However, the understanding of cytokines functions on corneal innervation during embryogenesis remains poor.

1.3.4 Pathologies Associated with Corneal Nerve Dysfunction

Corneal fiber nerves play a crucial function in the maintenance of a healthy ocular surface. They are responsible for sensation of touch, pain, and tear production. Numerous studies have shown that corneal nerve dysfunction is a frequent pathology. Recently, it has been estimated that from approximately 285 million people worldwide that suffer impaired vision, 2.85 million have cornea opacity with nerve dysfunction (Pascolini and Mariotti, 2012).

There are several pathological conditions that can cause corneal diseases. For instance, virus, bacterial, and fungal infections in the eye negatively affect corneal innervation. This is characterized by a reduction in the number of nerve bundles and branches that affect corneal sensitivity (Müller et al., 2003; Chuair-Elliot et al., 2015). Diseases such as aneurysm and ischemia, and traumas that cause damage of the TG also lead to corneal innervation alterations (Kurbanyan et al., 2012; Shaheen et al., 2013). Even keratorefractive procedures such laser-assisted in situ keratomileusis (LASIK), commonly used to restore the refractive error causes, in some extent, damage of the corneal nerves (Wilson and Ambrósio, 2001). Despite the relatively high prevalence of corneal nerve dysfunction, treatments that promote nerve regeneration with appropriate return of function are still much needed.
1.4 Corneal Avascularity

Despite of being surrounded by highly vascularized periocular tissue, the cornea remains as an avascular tissue. This section reviews how the cornea avascularity is established during development, molecular mechanisms that are or might be involved in this process, and the pathological conditions associated with corneal neovascularization.

1.4.1 Development and Establishment of Cornea Avascularity

The mature cornea possesses the “angiogenic privilege”, a term that implies the absence of blood vessels. This process is actively maintained by a fine balance between pro-angiogenic and anti-angiogenic factors expressed in the cornea or the surrounding tissue (Ellenberg et al., 2010). In normal corneas, expression of anti-angiogenic factors are upregulated compared to the expression of pro-angiogenic factors. However, after corneal injury, trauma, or any disorder that involves the presence of blood vessels in the cornea there is an increased expression of pro-angiogenic factors that tilts the balance toward cornea neovascularization. Although many studies focus on understanding the processes that maintain cornea as an avascular tissue in the adults, little is known about the establishment of cornea avascularity. Recent work has shown that cornea avascularity is a process that takes place very early during development (Kwiatkowski et al., 2013). By using Tg (tie1:H2B:eYFP) transgenic quail embryos (Sato et al., 2010) which express YFP in angioblasts and endothelial cells, it was observed that by E3, angioblasts located in the periocular mesenchyme already avoid the presumptive cornea (Figure 1.5 A), a condition that is maintained throughout the entire process of cornea development and in adults under normal conditions (Figure 1.5 B, C, and data not
shown). Cross sections of developing corneas show how angioblasts are located in the periocular mesenchyme while NCCs start migrating between the lens and presumptive corneal epithelium to form the corneal endothelium and stroma (Figure 1.5 D-F).

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Figure 1.5. Patterning of the limbal vasculature in the anterior eye during avian corneal development. A–C: Expression of H2B-eYFP by angioblasts and vasculature in whole mount Tg(tie1:H2B:eYFP) quail eyes at E3, E5, and E7. Remodeling of the temporal ciliary artery forms a vascular plexus (arrows) while the nasal ciliary artery regresses (asterisk). D–F: Cross sections through E3, E5, and E7 Tg(tie1:H2B:eYFP) quail eyes showing localization of angioblasts and vasculature in the periocular region. Sections were counterstained with DAPI. Abbreviations: C, cornea; vr, vascular ring; tca, temporal ciliary artery; nca, nasal ciliary artery; ir, iridial ring artery; L, lens; OC, optic cup; pm, periocular mesenchyme; ep, corneal epithelium; en, corneal endothelium; st, corneal stroma. Scale bars in (A–C) = 500µm; (D–F) = 50µm. From Kwiatkowski et al., 2013.

Strikingly, both, pro- and anti-angiogenic factors are expressed in the anterior eye during development. For instance, expression of VEGF and FGF2 proangiogenic factors have been detected in the anterior segment of the eye along with anti-angiogenic factors such as sFlt1, Sema3A and Netrin1 (Kwiatkowski et al., 2013). Therefore, during anterior eye
development both pro-angiogenic and anti-angiogenic factors orchestrate the process of periocular vascularization and establishment of corneal avascularity.

1.4.2 Molecular Regulators of Cornea Avascularity

Several mechanisms contribute to the maintenance of the “corneal angiogenic privilege”. In fact, the regulation of angiogenesis results from a complex interaction of growth factors, extracellular matrix (ECM), vascular endothelial cells, and cytokines (Kvanta, 2006).

After injury, infection or trauma, cornea wound healing can occur in absence or presence of blood vessels. In the first situation, anti-angiogenic factors such as angiostatin, PEDF and endotastin are upregulated while factors such as VEGF and FGF are downregulated. On the other hand, if wound healing progresses into cornea vascularization pro-angiogenic factors such as VEGF-A, IGF, and PDGF are upregulated. In this case, there is a shift in factors that regulate angiogenesis towards vessels progression. Table 1 shows the factors that have been reported to regulate corneal angiogenesis (Ellenberg et al., 2010).

ECM remodeling proteins have also been implicated in regulation of corneal angiogenesis. It has been shown that matrix metalloproteinases (MMP), a group of proteolytic enzymes, not only participate in ECM remodeling but also in regulation of corneal angiogenesis. They do so by activating pro- and anti-angiogenic molecules (e.g., TNF-α and IGF), changing the architecture of matrix protein (cleavage of E-cadherin, perlecan and proteoglycans), and chemoattraction (formation of growth factors and chemokine gradients). Expression of MMP-2, MMP-9, and MMP14 have clearly been
shown to induce corneal angiogenesis, while MMP-7 maintains corneal avascularity through cleavage of collagen XVIII to generate a fragment with a potent anti-angiogenic activity (Kato et al., 2001; Ellenberg et al., 2010).

Among the secreted proteins of the ECM, PEDF glycoprotein, which is expressed during development and adult ocular tissues, has been reported as a factor with a potent anti-angiogenic activity (Karakousis et al., 2001; Kwiatkowski et al., 2013).

1.4.3 Disorders Associated with Corneal Neovascularization

According to the World Health Organization, approximately 4.9 million people worldwide are bilaterally corneal blind which account for 12% of total blindness (Kim and Hara, 2015; Avadhanam et al., 2015). Under certain pathological conditions, blood vessels coming from the limbal vasculature grow excessively into the cornea causing corneal neovascularization (CNV) that might lead to visual impairment and ultimately blindness. Although the estimated prevalence of CNV worldwide is still unknown, it represents a major public health burden considering that in the U.S. alone 4% of the population suffers vision impairment due to cornea neovascularization (Lee et al., 1998, Chan et al., 2001). Several corneal opacities due to congenital defects in the anterior eye as well as a variety of insults such as infectious keratitis, corneal graft rejection, contact lenses-related keratitis and hypoxia, alkali burns, and limbal stem cell deficiency are all associated with CNV (Ellenberg et al., 2010).
Table 1. Factors involved in the regulation of corneal angiogenesis. From Ellenberg et al., 2010.

<table>
<thead>
<tr>
<th>Pro-angiogenic factors (GF) (Cao et al., 2004; Chang et al., 2004)</th>
<th>Anti-angiogenic factors</th>
<th>Pro-/Anti-angiogenic factors</th>
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<tr>
<td>Fibroblast growth factor (Fgf) (Lai et al., 2007)</td>
<td>Endostatin</td>
<td>Transforming growth factor-β (TGF-β) (Friling et al., 1996; Sakamoto et al., 2000)</td>
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<td>Angiostatin (Cheng et al., 2007)</td>
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<td>Prolactin (Duenas et al., 1999; Ueda et al., 2005)</td>
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<td>Thrombospondin (Panigrahy et al., 2008; Simontov et al., 2005)</td>
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<td>Transforming growth factor-α (TGF-α) (Curtsiefen et al., 2000; Yamamoto et al., 1994)</td>
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<td>Insulin-like growth factor (IGF) (Yamada et al., 2006)</td>
<td>Arresten (Mundel and Kalluri, 2007; Nyberg et al., 2008)</td>
<td>Matrix metalloproteinases (MMPs) (Azar, 2006; Kure et al., 2003; Ma et al., 2006)</td>
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<td>Leptin (Park et al., 2001)</td>
<td>Canstatin (Mignon et al., 2007; Mundel and Kalluri, 2007)</td>
<td>Tumor necrosis factor-α (TNF-α) (Chen et al., 2004; Saika, 2007; Ueda et al., 1998)</td>
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<td>Integrins (Muehler et al., 2007)</td>
<td>Tumstatin (Goto et al., 2008; Mundel and Kalluri, 2007)</td>
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<td>Platelet-derived growth factors (PDGF) (Abdiu and Van Setten, 2008)</td>
<td>Pigment epithelium-derived factor (PEDF) (Dell et al., 2006)</td>
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<td>Angiogenin (Crabtree et al., 2007)</td>
<td>Fibuline (Xie et al., 2008)</td>
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<td>Hepatocyte growth factor/scatter factor (HGF/SF) (Grierson et al., 2000)</td>
<td>Endorepellin (Woodali et al., 2008)</td>
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<td>Connective tissue growth factor (CTGF) (Babic et al., 1999)</td>
<td>Antithrombin (Schedin-Weiss et al., 2008)</td>
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<td>Platelet activating factor (PAF) (Ma et al., 2004)</td>
<td>Vasostatin (Wu et al., 2005)</td>
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<td>Activin-A (Foulaki et al., 2004)</td>
<td>Neostatin-7 (Kojima et al., 2008)</td>
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<td>Thrombin (Hu et al., 2008)</td>
<td>IFN-γ (Kommineni et al., 2008)</td>
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Herpes simplex virus (HSV) stromal keratitis is one of the most common causes of CNV. HSV is a member of the herpesvirus family that infects and establishes latent infection in the TG. By anterograde transport, the virus from the TG nerves can reach the cornea and cause herpetic keratitis. Its incidence and prevalence is very significant in developed countries, being one of the most frequent causes of blindness in the US (Lee et al., 1998; Abdelfattah et al., 2015).

Contact lenses-related keratitis is also an important cause of CNV. It has been estimated that around 11-23% of contact lens users are linked to corneal angiogenesis (Ellenberg et al., 2010). Contact lenses-associated neovascularization is more likely to occur with soft-hydrogel lenses, probably due to the extended covered area of the cornea that stimulate hypoxia and limbal inflammation (Abdelfattah et al., 2015). Since the use of contact lenses has increased significantly during the past years, it is not surprising that contact lens use leads as one of the common causes of acquired CNV.

Corneal transplantation is the most common and successful restoring procedure as transplantation can be done without histocompatibility matching. However, it is not always amenable to treat all corneal diseases. It’s has been estimated that the success rate of corneal transplantation depends largely on the absence of vasculature and inflammatory response, otherwise it decreases to less than 50% if the cornea is inflamed or vascularized (Cursiefen et al., 2003). In this context, recent studies show that chemokines play an important role in inflammation and graft rejection. For instance, high risk vascularized beds express CXCL1 chemokine that is crucial for the induction of CXCL9 and CXC10 to attract T-cells with subsequent graft rejection (Amescua et al.,
2008). Thus, corneal rejection is directly related to the extent of corneal vascularization and inflammation prior to surgery.

1.5  **CXCL14: A Member of a Large Chemokine Family with Multiple Functions.**

1.5.1  **Chemokines Classification and Regulation**

Chemokines are a large family of small chemotactic heparin-binding cytokines (Kiefer and Siekmann, 2011). To date, it has been reported that in human the chemokine network involves approximately 20 receptors and 50 ligands (Allen et al., 2007). With the exception of the non-signaling scavenger receptors, chemokines exert their biological effects through seven-transmembrane-domain G-protein-coupled receptors leading the dissociation of G-protein complex into the Gα and Gβγ subunits. These subunits activate secondary messengers that regulate cell proliferation, migration, adhesion and differentiation (Rostene et al., 2007; Allen et al., 2007; Comerford and McColl, 2011; Allegrati et al., 2012).

Based on their function, chemokines can be classified as inflammatory or homeostatic. Inflammatory chemokines are expressed after injury, stress response or infection. Their functions consist of recruiting leukocytes after an inflammatory response. On the other hand, homeostatic chemokines are constitutively expressed by tissues and they can function independent of the immune system (Allen et al., 2007; Mortier et al., 2012).
In general, chemokines contain 4 well-conserved cysteine residues. Based on the number and location of the first two cysteine residues, they can be divided into CC, CXC, CX3 and C groups (Mehrad et al., 2007). Chemokines that have two cysteine residues next to each other are grouped as CC, whereas chemokines with the presence of one or three random aminoacids between the first cysteine residues are called CXC and CX3 respectively. A small group containing only one conserved cysteine residue in the amino-terminus corresponds to C chemokines (Figure 1.6) (Barbieri et al., 2010).

Moreover, depending on the presence or absence of three conserved amino acids (Glu-Leu-Arg; ELR) in the amino terminal region, CXCL chemokines can be sub-classified into ELR (+) with angiogenic activity or ELR (-) which are considered to be angiostatic factors (Table 2) (Mehrad et al., 2007; Kiefer and Siekmann, 2011). An exception of this classification is the ELR- chemokine CXCL12, which has been well

Figure 1.6. Chemokine subfamilies classification. The first cysteine (C) in the sequence forms a covalent bond with the third, the second and the fourth cysteines also form a disulfide bond to create the tertiary structure characteristic of chemokines. In the CC subfamily the first two cysteines are adjacent to each other, in the CXC group there is one amino acid between the first two cysteines, and in the CX3C group there are three amino acids between cysteines. From Barbieri et al., 2010.
documented to induce angiogenesis via binding to CXCR4 receptor (Balestrieri et al., 2008; Cheng et al., 2014).

There are several mechanisms that regulate the activity and availability of chemokines. Most of the inflammatory chemokines express the pathogen-associated molecular patterns (PAMPs) that are recognized by pattern-recognition receptors (PRRs) expressed by leukocytes to induce chemokine expression. Moreover, the mRNA of several chemokines presents an AU-rich sequence element (AREs) in the 3’ untranslated region where proteins interact to increase or reduce the mRNA stability. In relation to activity regulation, glycosaminoglycans (GAGs) play an important role. In fact, it has been reported that chemokines also bind to proteoglycans as a mechanism to concentrate and form a gradient necessary for their function (Proudfoot et al., 2003). So far, six classes of GAGs the chemokines bind to have been documented: heparin, heparin sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid (Mortier et al, 2012)

Increasing evidence demonstrates that chemokines act in physiological and pathological processes such as regulation of the central nervous system development, embryogenesis, organogenesis, angiogenesis, and cancer (Rostene et al., 2007; Olesnicky et. al., 2009; Banisadr et al., 2011; Kiefer and Siekmann, 2011).
1.5.2 CXCL14 Expression and Functions

CXCL14, also known as BRAK, Scyba, MIP-2γ or BMAC, is one of the most ancient chemokine whose aminoacid constitution is well conserved among vertebrates. It belongs to the CXCL ELR(-) subfamily whose receptor is still unknown (Table 2).

In human, CXCL14 is constitutively expressed in normal epithelial tissues such as skin, breast, kidney, brain, muscles, and lungs but absent or down regulated in various malignant cells (Hromas et al., 1999; Long et al., 2000; Meuter and Moser, 2008; Chen et. al., 2010; Hara and Tanegashima, 2012). Recent studies have shown that CXCL14 is a chemokine with pleotropic functions. In this section I will describe the function of CXCL14 in the nervous system and its role in angiogenesis and cancer.
**CXCL14 in the Nervous System**

In the nervous system, *CXCL14* is broadly expressed in the brain (Hromas et al., 1999; García-Andres and Torres, 2010). *In situ* hybridization analysis has shown expression of *CXCL14* in a subset group of microglia cells and γ-aminobutiric acid (GABA)-ergic neurons (Banisadr et al., 2011). Postnatally, *CXCL14* is expressed in the hippocampus, cortex, basal ganglia, septum, and also in the dorsal root ganglia in mice. Particularly, in the dentate gyrus (DG) of the hippocampus, Banisadr and coworkers (2011) reported that CXCL14 inhibits GABAergic transmission. Interestingly, GABAergic transmission is stimulated by CXCL12, suggesting an opposite function of these two chemokine in the DG. The role of CXCL14 in the developing nervous system is very limited. Park and coworkers (2012) reported that CXCL14 is strongly and transiently expressed in the developing cerebellum by Purkinje cells. Using *In vitro* studies they showed that CXCL14 stimulates granule cell migration which suggests a role of CXCL14 in the maturation of the cerebellum.

**CXCL14 in Angiogenesis and Cancer**

Pioneer studies have shown that *CXCL14* expression is repressed in several cancer samples and tumor cell lines (Hromas et al., 1999; Izukuri et al., 2010). Since CXCL14 is a potent chemoattractant of immature dendritic cells and natural killer cells, it has been suggested that its down regulation by malignant cells may contribute to their immunologic escape (Shellenberger et al., 2004). In addition, CXCL14 acts as an anti-angiogenic factor that inhibits endothelial cell chemotaxis *in vitro*, and blood vessel formation *in vivo*. *In vitro* chemotaxis assays studies have reported that CXCL14 inhibits
endothelial cell migration in a dose-dependent manner (Figure 1.7 A and B). Moreover, in vivo studies using the adult rat cornea micropocket assay have shown that CXCL14 abrogates induced angiogenesis of the well-known pro-angiogenic factors IL8, VEGF, and bFGF (Figure 1.7C). Since neovascularization is essential for tumor growth, it would explain why this chemokine is greatly suppressed. In fact, a recent study showed that CXCL14 transgenic mice that suppress growth of tumor cells presented not only significantly smaller tumors than those observed in wild type (WT) mice, but also difference in vasculature structure and size, with lower number of CD-31 positive endothelial cells (Izukuri et al., 2010).

Interestingly, in several cancer cell lines CXCL14 is regulated at the protein level by proteosomal degradation. In fact, a recent study revealed the presence of a unique aminoacid insertion between strands β2 and β3 that is responsible for its degradation via ubiquitin-mediated proteolysis. This study elucidated that CXCL14 not only is negatively regulated transcriptionally, but also post-transcriptionally in several malignant cells, highlighting the importance of new mechanisms by which transformed cells operate (Peterson et al. 2006).
Expression of CXCL14 has been reported in zebrafish, frog, chick and mouse embryos. In zebrafish embryos, CXCL14 is expressed in the vestibular-acoustic system, mid-hindbrain, diencephalon and cerebellum (Long et al., 2000). In Xenopus laevis, it has been reported that CXCL14 is expressed in the dorsal aspect of the retina, the
embryonic ectoderm and its derivatives, being regulated by both BMP and Wnt signaling (Park et. al., 2009). In chicken and mouse development, CXCL14 is widely expressed in the ectoderm, cranial ganglia, mesonephros, neural tubes, brain and limbs (García-Andres and Torres, 2010; Gordon et al., 2011). Interestingly, a complementary expression of CXCL14 and CXCL12 were found in several developing tissues, suggesting that these two chemokines might act cooperatively or antagonistically during embryogenesis. Along with CXCL12, CXCL14 is one of the most ancient chemokines with a high degree of conservation among vertebrates (Huising et al., 2004). Although the understanding of CXCL12 functions during development is vast, very little is known about the role of CXCL14. Thus, one of the main purposes of this project was to characterize the expression of CXCL14 and determine its role during ocular development.
Chapter 2: Sema3A Maintains Corneal Avascularity during Development by Inhibiting VEGF Induced Angioblast Migration


2.1 Introduction

Semaphorin3A (Sema3A) is a secreted molecule that belongs to the Semaphorin family of genes, well known for their roles as guidance cues for cell migration and neuron extension (Luo et al., 1993; Kolodkin, 1998; Raper, 2000). Sema3A signals by binding to neuropilin1 (Nrp1) receptor, which forms a complex with plexins (Bagri et al., 2009) that transmit the signal to the intracellular milieu, where actin polymerization is prevented and cell motility is reduced (Fan et al., 1993; Fournier et al., 2000). Sema3A is strongly expressed in the lens vesicle during ocular development (Luo et al., 1993; Chilton and Guthrie, 2003). Due to the proximity of the lens and presumptive cornea, the lens-derived Sema3A plays a crucial role during cornea development by regulating neural crest cell (NCC) migration (Lwigale and Bronner-Fraser, 2009) and its innervation (Lwigale and Bronner-Fraser, 2007; Kubilus and Linsenmayer, 2010; Schwend et al., 2012). Migration of the periocular NCCs and the extent of trigeminal sensory innervation of the cornea both depend on the expression of Nrp1 receptor (Lwigale and Bronner-Fraser, 2009; McKenna et al., 2012). Interestingly, Nrp1 is a co-receptor with binding domains for Sema3A and VEGF-A, also known as isoform 164 or 165. Sema3A binds to the a1a2 domain and VEGF binds to the b1b2 domains of Nrp1 (Olsson et al., 2006).
However, an overlap of Sema binding to the b1 domain has been reported (Giger et al., 1998; Miao et al., 1999; Gu et al., 2002).

VEGF is the most prominent pro-angiogenic factor involved in vasculogenesis (the formation of blood vessels de novo from angioblasts) and angiogenesis (sprouting of blood vessels from pre-existing vasculature) in embryonic and adult tissues. VEGF is a secreted protein that binds to a complex of Nrp1 and the receptor tyrosine kinase VEGFR2 (KDR/flk1) to stimulate proliferation, migration, survival, and differentiation of endothelial cells (Leung et al., 1989; Carmeliet et al., 1996; Ferrara et al., 2003). In mouse embryos, knockout of VEGF, Nrp1, or VEGFR2 are embryonic lethal due to cardiac and vascular defects (Carmeliet et al., 1996; Ferrara et al., 1996; Kitsukawa et al., 1997). Unlike VEGF, Sema3A knockout mice are viable but show defects in morphogenesis of the major blood vessels (Serini et al., 2003). Interestingly, mutation of the Sema binding domain of Nrp1 (Nrp1\textsuperscript{sema-}) did not result in cardiovascular defects and the embryos were viable, but at a reduced ratio (Gu et al., 2003). However, \textit{in vitro} studies showed that Sema3A inhibits VEGF-induced endothelial cell migration (Miao et al., 1999). Combined, these findings raise questions about the role of Sema3A in endothelial cell migration during vascular development. Both Sema3A (Shepherd et al., 1996; Chilton and Guthrie, 2003; Lwigale and Bronner-Fraser, 2007, 2009) and VEGF (Ash and Overbeek, 2000; Shui et al., 2003; Garcia et al., 2009; Saint-Geniez et al., 2009; Kwiatkowski et al., 2013) are expressed in the lens and secreted into the adjacent cornea, but their roles during ocular vasculogenesis remain unclear. In this study, we report that angioblasts and newly formed periocular vasculature express Nrp1 that bind to AP-Sema3A. The lens expresses significantly more Sema3A mRNA transcripts than
VEGF. Inhibition of Sema3A/Nrp1 signaling causes ectopic migration and aberrant vascularization of the embryonic cornea. We also show that exogenous Sema3A inhibits VEGF-induced angioblast migration into the cornea. In agreement with these findings, loss of Sema/Nrp1 signaling in Nrp1<sup>sema−</sup> mutant mice results in ectopic angioblast migration and vascularization of the embryonic corneas. Altogether, these data reveal an essential role of Sema3A signaling in the establishment of corneal avascularity during avian and mouse embryonic development.

2.2 Materials and Methods

2.2.1 Embryos

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Rice University. Fertilized Tg(<i>tie1</i>:H2B:eYFP) transgenic Japanese quail eggs, whose embryos naturally express enhanced yellow fluorescent protein (eYFP) in nuclei of all endothelial cells (Sato et al., 2010), were obtained from Ozark Egg Company (Stover, MO). Fertilized White Leghorn chick eggs were obtained from Texas A&M Poultry Center (College Station, TX). Eggs were incubated at 38°C in a humidified incubator until the desired embryonic stage. Mutant Nrp1<sup>sema−</sup> mouse embryos that express normal levels of a modified Npn1 protein that binds to VEGF but is completely disrupted in its interaction with Semaphorin (Gu et al., 2003) were examined for vascular defects during cornea development. The Nrp1<sup>sema−</sup> mouse lines were maintained through heterozygous crosses and mutant embryos were identified by PCR genotyping. Embryos were staged with the morning of virginal plug formation counted as embryonic day (E)0.5.
2.2.2 In situ hybridization

Freshly isolated chick or mouse eyes were fixed overnight at 4°C in modified Carnoy's fixative (60% ethanol, 30% formaldehyde, and 10% glacial acetic acid) and embedded in paraffin. In situ hybridization was performed on 10–12 µm sections as described (Etchevers, et al., 2001). Digoxigenin-labeled riboprobes for chick Sema3A, VEGF, and Nrp1 were used as previously described (Lwigale and Bronner-Fraser, 2009), and riboprobes for mouse Nrp1 was made and used as described in McKenna et al., 2012.

2.2.3 Isolation of endothelial cells and cell culture

Temporal ciliary arteries or umbilical vessels were dissected from E5–E7 Tg(tie1:H2B:eYFP) embryos. Surrounding mesenchyme was carefully removed from the vascular tissue following 5 minutes treatment in dispase (Worthington) at 37°C. Blood vessels were treated with trypsin (Cellgro), rinsed in Ringer's solution, triturated in DMEM (Cellgro). The isolated endothelial cells were allowed to attach to a gelatin coated slide at 37°C for 6–8 h before replacing the culture media with control or AP-Sema3A conditioned media.

2.2.4 AP-Sema3A fusion protein binding

Analysis of AP-Sema3A fusion protein was conducted as previously described (Feiner et al., 1997). Briefly, media was harvested from HEK293T cells transiently transfected with AP-Sema3A construct and stored at 4°C until needed. Tg(tie1:H2B:eYFP)-positive endothelial cells were fixed in 4% paraformaldehyde (PFA), rinsed in phosphate buffered saline (PBS) containing 0.1% Triton-X (PBT), blocked in PBT containing 10% FBS, then
incubated overnight in AP-Sema3A conditioned media or control media from untransfected HEK293T cells. Endogenous alkaline phosphatase activity was inactivated by incubation of the endothelial cells at 65°C. Tissue-bound heat-stable AP-Sema3A activity was detected as an insoluble reaction product after treatment with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche).

2.2.5 Immunostaining

Embryos were fixed overnight at 4°C in 4% PFA. Immunostaining of eyes was performed following standard procedures with minor modifications. For sections, samples were embedded in gelatin, cryo-sectioned at 8–10 µm, then the gelatin was melted in 42°C phosphate buffered saline (PBS). Whole mount anterior eye tissue or sections were washed in PBS containing 0.1% Triton-X (PBT) and blocked in PBT containing 0.1% BSA and 5% heat-inactivated sheep serum. Samples were incubated overnight at 4°C in antibody solution containing rabbit anti-Sema3A (IgG, Abcam) diluted 1:50, mouse anti-VEGF (IgG1, Abcam) diluted 1:200, rabbit anti-GFP diluted 1:500 (IgG, Invitrogen), or rat anti-endomucin (IgG2a, eBioscience) diluted 1:500 in blocking solution. After extensive washes in PBT, samples were blocked for one hour then incubated overnight at 4°C in secondary antibody (Alexa 488 goat anti-rabbit IgG and Alexa488 goat anti-mouse IgG1, Invitrogen, Calsbad, CA; FITC goat anti-rat IgG2a, Bethyl, Montgomery, TX) diluted 1:200 in blocking solution. Samples were washed in PBS. Some sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to label all nuclei. All samples were mounted in PBS and imaged with a Zeiss AxioImager 2 fluorescence microscope with ApoTome and Axiocam (Carl Zeiss AG).
2.2.6 Quantitative real-time PCR

Chick lenses (E3, E5 and E7) or mouse lenses and corneas (E13.5 and E16.5) were dissected and pooled for each time point. Total RNA was isolated using Trizol Reagent (Invitrogen), genomic DNA was digested with TURBO DNA-free (Ambion), and cDNA was reverse transcribed using qScript cDNA SuperMix (Quanta BioSciences). Quantitative real-time PCR (qRT-PCR) was conducted on a CFX96 Real-time instrument/C1000 Thermal Cycler (Biorad) using Perfecta SYBR Green SuperMix (Quanta BioSciences). Gapdh was used as an internal control. Primers used for chick VEGF were as follows; FWD: 5’-CAGACCTGTAAATGTCTGC-3’; REV: 5’-CCTTCTTTCGCTGCTC-3’. Primers for GAPDH and Sema3A amplification were previously described (Kubilus and Linsenmayer, 2010; Spurlin and Lwigale, 2013). Primers used for mouse GAPDH FWD: 5’-GCATGGCCTTCCGTGTTCCA-3’; GAPDH REV: 5’-GCCTGCTTCACCACCTTCTGA-3’; Sema3A FWD: 5’-TGGAATTGCCTGTCTTTT-3’; Sema3A REV: 5’-GGCCAAGCCATTAAAGTGA-3’; VEGF FWD 5’-CTGCTGTAACGATGAAGCCCTG-3’; VEGF Reverse 5’-GCTGTAGGAAGCTCATCTCCTCC-3’. The fold change was calculated as the ΔΔCt relative to VEGF expression at each time point.

2.2.7 Lens removal

Lens removal was performed as previously described (Lwigale and Bronner-Fraser, 2007). Briefly, after 3-day incubation, quail eggs were windowed, and then a small incision was made in the presumptive cornea epithelium. The lens was gently removed from the right eye without disturbing the periocular region. Sham operated or unoperated
left eyes served as a control. Embryos were reincubated for 1–2 days and then collected for imaging.

2.2.8 Peptide injection

Peptide injections were performed as described (Lwigale and Bronner-Fraser, 2007). Briefly, a Sema3A-Ig peptide that binds to and blocks Sema3A interaction with Nrp1 (N-Ac-HAVEHGFMQTLLKVTLE-NH2) (Williams et al., 2005) or control scrambled peptide (N-Ac-AHLELLTQTGKVEVFHM-NH2) were pressure injected into the right lens of E3 quail embryos at 10 mM concentration. Embryos were analyzed after 2 days incubation.

2.2.9 Bead implantation

Beads were prepared as previously described (Bates et al., 2003) with minor modifications. Briefly, Cibacron blue 3 GA beads (Sigma-Aldrich) were rinsed in Ringer's solution then soaked overnight at 4°C in either 5 μl of 90 μg/ml recombinant mouse Sema3A (R&D Systems), 10 μg/ml recombinant mouse VEGF164 (R&D Systems), a 1:1 (vol:vol) combination of Sema3A and VEGF 164 proteins, or in Ringer's solution. Beads were implanted between the presumptive cornea epithelium and lens vesicle of E4 quail embryos. Vascularization of corneas was analyzed after 1–2 days incubation.

2.2.10 Quantification of anterior eye vasculature following bead implantation

Only the eyes with beads located within 300–600 μm of the edge of the vascular ring were selected for further analysis. To quantify the region covered by endothelial cells in the vascular ring and the cornea, a delineating line was drawn around the outside and
inside edges of the vascular ring and any of its extensions (dashed red line Fig. 3A, C, F, and H). The total area (within the outside boundary) and the area covered by endothelial cells (between the outside boundary and inside boundary) were determined using Image J program (Abramoff et al., 2004). Percent area covered by endothelial cells was calculated as the ratio between the areas covered by endothelial cells divided by the total area. The values reported are averages of each group. Statistical analysis was performed using unpaired Student’s t-test with a two-tailed P value.

2.3 Results

2.3.1 Angioblasts and Primitive Blood Vessels Colonize the Periocular region but Avoid the Developing Cornea

To determine the pattern of avascularity during development, we examined the eyes of Tg(tie1:H2B:eYFP) transgenic quail embryos between E3-E6. This time period corresponds with the formation of the three cellular layers of the cornea (Hay, 1980; Lwigale et al., 2005; Creuzet et al., 2005). By E3, numerous fluorescently labeled angioblasts were present in the periocular region, but they appeared to avoid the region of the presumptive cornea (Figure 2.1 A and 2.1A’). Cross sections through the eye at this time revealed that the angioblasts were localized adjacent to the optic cup (Figure 2.1D; arrowheads). By E5, angioblasts aggregated to form the primitive vasculature comprised of the temporal and nasal ciliary arteries, and the vascular ring that surrounds the cornea (Figure 2.1B and 2.1B’). Even though NCCs had migrated between the lens and ectoderm to form the cornea endothelium, the angioblasts remained adjacent to the optic cup in the periocular region and presumptive iris (Figure 2.1E). By E7, the nasal
ciliary artery had regressed from the pericorneal region (Figure 2.1C; asterisk) and only the temporal ciliary artery remained, thus supplying the vascular network of the anterior eye (Figure 2.1C and 2.1C'). Cross sections through the E7 eye show that all the cellular layers of the cornea are formed but it remains avascular while blood vessels form in the periocular region and iris (Figure 2.1F). This data agree with the results of Kwiatkowski and coworkers (2013) and demonstrate that during avian ocular development, angioblasts migrate into the anterior region of the eye to be located in the periocular mesenchyme, but avoid the cornea.

2.3.2 Expression of VEGF, Sema3A, and Nrp1 Correlate with Angioblast Migration and Vascular Patterning in the Anterior Eye Region.

Based on our observation that angioblasts migrate into the periocular region but appear to be inhibited from the presumptive cornea, we investigated the expression of VEGF and Sema3A during ocular development. Strong expression of VEGF was detected in the presumptive retinal pigment epithelium layer of the optic cup and in the lens epithelium at E3 (Figure 2.2A). Expression of VEGF persists in the lens epithelium through E7 (data not shown and Kwiatkowski et al., 2013). Sema3A is vividly expressed in the lens epithelium at E3 (Figure 2.2B) and its expression persists during subsequent stages of cornea development (data not shown and Lwigale and Bronner-Fraser, 2009).
Figure 2.1. Cornea avascularity is established early during avian ocular development. (A-C) Schematic view of E3, E5, E7 quail embryos showing vasculogenesis in the anterior eye and corneal avascularity. (A’-C’) Wholemount images of Tg(tie1:H2B:eYFP) quail embryos showing the localization and aggregation of fluorescently labeled nuclei of angioblasts in the boxed areas in A-C. (A’) Angioblasts migrate into the periocular region of the eye by E3, but they avoid the presumptive cornea. (B’) At E5, angioblasts coalesce to form primitive vasculature in the anterior eye region but not in the presumptive cornea. (C’) At E7, a primitive vascular network is established in the anterior eye region while the cornea remains avascular. (D-F) Transverse sections through the anterior eyes of E3, E5, E7 Tg(tie1:H2B:eYFP) quail embryos showing the localization of angioblasts in the periocular region during neural crest migration between the lens and ectoderm to form the endothelial and stromal layers of the cornea. Abbreviations: c, presumptive cornea; tca, temporal ciliary artery; nca, nasal ciliary artery; vr, vascular ring; pr, periocular region; ir, iris; ec, ectoderm; en, endothelium; ep, epithelium; L, lens; oc; optic cup; ret, retina. Asterisk indicates regression of the nasal ciliary artery. Scale bars: A’,C’ 200 µm; B’,C,D,E,F 100 µm.
Given that both Sema3A and VEGF were localized in the developing lens, we quantified their mRNA transcript levels using qRT-PCR. Sema3A was expressed at relatively higher levels than VEGF at all developmental stages analyzed (Figure 2.2C). The biggest fold difference between Sema3A and VEGF expression levels was at E3 (16.39 ± 6.97; \( P = 0.02 \)). At E5 and E7, Sema3A transcripts were (5.32 ± 3.24; \( P = 0.08 \)) and (5.61 ± 1.44; \( P = 0.005 \)) fold higher than VEGF, respectively. These results show that the expression of VEGF and Sema3A overlap in the lens during vasculogenesis of the anterior eye. The higher levels of Sema3A compared to VEGF transcript expression raise the possibility that Sema3A is more abundant than VEGF in the lens.

Next, we determined the expression of Nrp1 by angioblasts and forming vascular in the periocular region. Since Nrp1 is expressed by periocular neural crest cells (Chilton and Guthrie, 2003; Lwigale and Bronner-Fraser, 2009), we performed section in situ hybridization on eyes from Tg(tie1:H2B:eYFP) quail embryos to show specific expression in the vasculature. Our results show that Nrp1 colocalizes with the GFP-positive angioblasts and primitive vasculature in the periocular region and presumptive iris at E5 and E6 (Figure 2.2D and E; and insets).

To verify that Sema3A binds to angioblasts, we performed an AP-Sema3A binding assay on endothelial cells isolated from the primitive vasculature of the eye or the umbilical vein of Tg(tie1:H2B:eYFP) quail embryos. Endothelial cells incubated overnight with AP-Sema3A stained positive for alkaline phosphatase (Figure 2.2G), in contrast to endothelial cells grown in control media (Figure 2.2F). Thus, VEGF signaling from the optic cup and lens attracts Nrp1-positive angioblasts into the anterior eye region. Intriguingly, our results also raise the possibility that Sema3A signaling from the lens
utilizes the Nrp1 receptor on angioblasts to prevent their migration into the presumptive cornea region.

Figure 2.2. Expression of VEGF, Sema3A, and Nrp1 during vasculogenesis of the avian anterior eye. Transverse sections through the anterior eye at E3 showing the localization of (A) VEGF in the lens and optic cup, and (B) Sema3A in the lens. (C) Quantification of Sema3A and VEGF mRNA levels in the lens at E3, E5, and E7 by RT-QPCR. Each time point is an average of triplicate reactions and expressed as fold change relative to VEGF. Student's t-test, *P < 0.05 and **P < 0.01; error bars indicate s.d. (D and E) GFP-positive angioblasts and primitive blood vessels in the periorcular region express Nrp1. Insets in D and E are higher magnifications of outlined regions. (F and G) Binding of Sema3A with Tg(tie1:H2B:eYFP) endothelial cells in vitro. Cells cultured (G) with media containing AP-Sema3A show a dark precipitate indicating binding of Sema3A, which is absent in (F) cells cultured in control media. Abbreviations: ec, ectoderm; en, endothelium; ep, epithelium; L, lens; oc, optic cup; ret, retina; vr, vascular ring; ir, iris. Scale bars: A,B 100 µm; D,E 100 µm; D,E (insets) 50 µm F,G 200 µm.
2.3.3 Lens-derived Sema3A Signaling Prevents Angioblast Migration into the Developing Cornea

To identify the role of Sema3A during the formation of the avascular cornea, we inhibited Sema3A signaling by performing lens removal or by injecting Sema3A-Ig peptide (Williams et al., 2003) into the lens to block of lens-derived Sema3A. Previous studies have showed that following lens removal or injection of Sema3A-Ig peptide into the lens vesicle at E3, Nrp1-positive trigeminal nerves and periocular neural crest cells migrated precociously into the presumptive cornea (Lwigale and Bronner-Fraser, 2007; Lwigale and Bronner-Fraser, 2009). In the current study we analyzed Tg(tie1:H2B:eYFP) quail embryos at E5 to determine how angioblasts respond to Sema3A inhibition. We chose E5 because it corresponds to the time of rapid vasculogenesis in the periocular region as well as neural crest migration during the formation of the corneal endothelial layer. Controls for these experiments included sham operations and injection of scrambled peptide, which were all similar to unperturbed eyes (Figure 2.3A, B; and data not shown). However, lensless eyes revealed ectopic angioblast migration and aggregation in the presumptive cornea (Figure 2.3C; arrow). Histological analysis of lensless eyes revealed massive cell migration into the presumptive cornea, including GFP-positive angioblasts (Figure 2.3D; arrowheads). Similarly, E5 Sema3A-Ig peptide injected eyes showed ectopic angioblast migration and vascularization of the cornea (Figure 2.3E, F; arrowheads). As previously shown, periocular neural crest cells also aberrantly migrated into the cornea (Lwigale and Bronner-Fraser, 2009). These results demonstrate that lens-derived Sema3A inhibits angioblast migration into the presumptive
cornea, indicating that Sema3A prevents corneal vascularization during ocular development.

Figure 2.3 Removal of lens-derived Sema3A causes ectopic angioblast migration into the cornea. Whole mount (A,C,E) and transverse sections (B,D,F) of E5 Tg(tie1:H2B:eYFP) quail corneas showing localization of GFP-positive angioblasts. (A and B) Controls showing absence of angioblasts in the presumptive cornea. (C and D) Lens ablated, and (E and F) Sema3A-Ig peptide injected eyes showing ectopic angioblasts (arrows and arrowheads) in the presumptive cornea. Asterisk in D indicates absence of lens. Abbreviations: c, presumptive cornea; en, endothelium; ep, epithelium; L, lens; oc; optic cup. Scale bars: A,C,E, 200µm; B,D,F, 100 µm.
2.3.4 Exogenous Sema3A Prevents VEGF-induced Angioblast Migration into the Cornea

Based on the expression of VEGF and Sema3A, and the Sema3A inhibition results, we hypothesized that the high levels of lens-derived Sema3A counteract the pro-angiogenic effects of lens-derived VEGF to prevent angioblast migration into the cornea during development. To test this hypothesis we implanted beads soaked in VEGF, Sema3A, or a combination of VEGF and Sema3A, between the lens and overlaying ectoderm of E3 Tg(tie1:H2B:eYFP) quail embryos. Beads soaked in Ringers solution were used as control.

E5 eyes implanted with beads soaked in Ringer's solution (Figure 2.4A, B) revealed no ectopic angioblasts in the presumptive cornea after 2 days incubation. Similarly, there were no ectopic angioblasts in the corneas of eyes containing Sema3A soaked beads (Figure 2.4C, D), but there was a reduction in the thickness of the primitive vasculature including the vascular ring (Figure 2.4C). Analysis of the area covered by angioblasts revealed a marked decrease in the thickness of the vascular ring in the eyes implanted with Sema3A soaked beads compared with the controls (Figure 2.4E; $P = 0.04$). In contrast, eyes implanted with VEGF soaked beads showed expansion of the vascular ring as a result of massive angioblast migration into the presumptive cornea (Figure 2.4F, G). However, similar angioblast migration into the presumptive cornea was abolished in eyes implanted with beads soaked in a combination of VEGF and Sema3A. In a few cases a limited number of angioblasts migrated towards the beads (Figure 2.4H, arrowhead), but they migrated through the anterior chamber and did not enter the cornea (Figure 2.4I, arrowhead). This result suggests that in the presence
of both proteins, angioblasts remained responsive to VEGF but Sema3A prevented their massive invasion of the presumptive cornea. Statistical analysis of the area covered by angioblasts revealed a significant decrease ($P = 0.013$) of angioblast migration into the presumptive cornea region when beads soaked in a combination of VEGF and Sema3A were used versus VEGF alone (Figure 2.4J). These data demonstrate that elevated VEGF signaling induces aberrant migration of periocular angioblasts into the presumptive cornea, and that adding Sema3A can prevent the VEGF-induced angioblast migration to maintain corneal avascularity.

2.3.5 Ocular Vasculogenesis and Expression of Sema3A, and VEGF during Mouse Eye Development

Our results show that during avian ocular development, angioblasts do not migrate past the tip of the optic cup (Figure 2.1). However, in mice angioblasts migrate into the optic cup to form the hyaloid vasculature and also vascularize the lens until later stages of development (Mitchell et al., 1998; Saint-Geniez and D’Amore, 2004). To determine whether Sema3A/Nrp1 signaling is involved in angioblast migration during mouse cornea development, we first established the location of angioblasts at different time points of cornea development. By E10.5, endomucin-positive angioblasts were observed primarily adjacent to the optic cup as the lens placode and optic vesicle invaginated (Figure 2.5A). At E13.5, angioblasts were localized inside the optic cup and alongside the presumptive retinal pigment layer (Figure 2.5B).
Figure 2.4 Sema3A inhibits VEGF-induced angioblast migration into the cornea. Whole mount and transverse sections of (A-D) E5, and (E-F) E6 Tg(tie1:H2B:eYFP) eyes showing GFP-positive angioblasts. No ectopic angioblasts were observed in corneas after implantation of beads soaked in (A and B) Ringer’s solution or (C and D) Sema3A. Implantation of beads soaked in VEGF induced angioblast migration into the cornea (F and G), which was inhibited when beads were soaked in VEGF + Sema3A (H and I). Dotted white circles indicate location of the beads. (E and J) quantification of the area covered by angioblasts demarcated with red dotted lines: (E) Control (black bar, n = 4) against Sema3A (white bar, n = 4), and (J) VEGF (grey bar, n = 4) against VEGF + Sema3A (striped bar, n = 4). Student’s t-test, *P < 0.05 and **P < 0.01; error bars indicate s.d. Abbreviations: vr, vascular ring. Scale bars: ACFH, 100 µm; BDGI, 100 µm.
Although the lens vesicle was surrounded by angioblasts, they did not migrate into the mesenchyme of the presumptive cornea (Figure 2.5B), which remained avascular at E16.5 (Figure 2.5C). Nrp1 mRNA and protein are expressed in the regions (Erskine et al., 2011; McKenna et al., 2012) that also stain positive for endomucin, suggesting that the angioblasts and primitive vasculature have potential to respond to both Sema3A and VEGF signaling.

Similar to chick, expression of Sema3A is conserved in the mouse lens during cornea development (McKenna et al., 2012). To examine the expression of Sema3A and VEGF during cornea development, we immunostained sections through E13.5 mouse eyes. Sema3A staining was observed in the lens, ocular ectoderm, cornea mesenchyme, optic cup, and weakly in the periocular region (Figure 2.5D). The strong expression of Sema3A in the lens epithelium (Figure 2.5E, arrow) corresponded with the relatively fewer number of angioblasts in the papillary membrane on the lens surface compared the posterior region (Figure 2.5B and 5E). VEGF staining was observed in the lens, ocular ectoderm, cornea mesenchyme, hyaloid vasculature, and along the periphery of the optic cup and optic stalk (Figure 2.5F). Endomucin and VEGF staining were localized in similar regions except for the cornea and ocular ectoderm (compare Figure 2.5B and 5F). Vivid VEGF staining in the posterior lens (Figure 5G, arrow) was consistent with the large number of angioblasts that contributed to the hyaloid vasculature. Taken together, these expression results raise the possibility that both VEGF and Sema3A signaling play an essential role in vascular patterning during mouse ocular development.

Quantification of mRNA transcripts revealed significantly higher levels of Sema3A than VEGF in the lens at E13.5 (2.7 ± 0.35 fold, P = 0.006) and E16.5 (4.6 ± 0.657 fold,
Similarly, *Sema3A* was expressed at higher levels than *VEGF* in the presumptive cornea at E13.5 (4.4 ± 1.2 fold, *P* = 0.01). However, at E16.5 the level of *Sema3A* decreased to 1.5 ± 0.8 fold that of *VEGF* (Figure 2.5 H, *P* = 0.45). Therefore, the robust expression patterns of *Sema3A* in the lens and cornea in conjunction with the relatively low expression of *VEGF* in these tissues suggest that the absence of angioblasts in the developing mouse cornea and its avascularity may be due to combined *Sema3A* signaling from the lens and presumptive cornea.

Figure 2.5. Ocular vasculogenesis during mouse eye development and the expression of *VEGF* and *Sema3A*. Cross sections through E10.5 (A), E13.5 (B), and E16.5 eyes immunostained with endomucin (green) to show the localization of angioblasts and primitive vasculature. Arrowheads in C indicate the papillary membrane. Immunolocalization of *Sema3A* (D and E) and *VEGF* (F and G) at E13.5. Arrow in (E) indicates high level of *Sema3A* expression in anterior lens and in (G) high level of *Vegf* expression in the posterior lens. (H) Quantification of *Sema3A* and *VEGF* mRNA levels in the mouse lens at E13.5 and E16.5 by RT-QPCR. Each time point is an average of triplicate reactions and expressed as fold change relative to *VEGF*. Student’s t-test, *P* < 0.01 and **P** < 0.001; error bars indicate s.d. Abbreviations: lpt, lens pit; oc, optic cup; c, presumptive cornea; L, lens; hv, hyaloid vasculature; ep, epithelium; st, stroma. Scale bars: AEG, 100 μm; BDF, 100 μm; C, 50 μm.
2.3.6 Disruption of Sema3A/Nrp1 Signaling Results in Ectopic Angioblast Migration and Vascularization of the Developing Mouse Cornea

To confirm that Sema3A/Nrp1 signaling plays a role in establishing mouse cornea avascularity during development, we immunostained eyes from Nrp1<sup>sema-</sup> mutants (Gu et al., 2003) and wild type embryos for endomucin and analyzed the whole-mounts and sections for vascular defects. In comparison to the avascular control corneas at E13.5 (Figure 2.6A), numerous angioblasts accumulated in the region of the presumptive cornea in Nrp1<sup>sema-</sup> eyes (Figure 2.6B; arrows) and they did not appear to organize into vessels at this time. Cross sections through E13.5 Nrp1<sup>sema-</sup> eyes revealed the presence of ectopic angioblasts in the cornea mesenchyme (Figure 2.6C; arrowheads) and expansion of the vessels in the pupillary membrane (Figure 2.6C’, arrows; also compare with Figure 2.5E and 5G). By E16.5, several blood vessels were visible in Nrp1<sup>sema-</sup> corneas (Figure 2.6E; arrows) but absent in the wild type control corneas (Figure 2.6D). In addition, cross sections through E16.5 Nrp1<sup>sema-</sup> eyes showed ectopic vasculature in the cornea stroma (Figure 2.6F; arrowheads), and the pupillary membrane was visibly thicker than wild type (Figure 2.6F, arrows; compare with Figure 2.5C, arrowheads). Together these results indicate that Sema/Nrp1 signaling plays a role in preventing angioblast migration and vascularization of the developing mouse cornea, and that it may also play a similar role by regulating the robustness of the transient lenticular vasculature.
2.4 Discussion

The cornea develops concurrently with the formation of the dense vascular network in the contiguous periocular region, making it an excellent model to study molecular signals that regulate vascular development and patterning. Our observation that angioblasts and primary vasculature express Nrp1, combined with the expression patterns of VEGF and Sema3A in the anterior eye region suggest their potential role in establishing the boundary between the avascular cornea and the highly vascularized periocular. Using various strategies to disrupt Sema3A/Nrp1 signaling in avian and mouse eyes, we provide compelling evidence that Sema3A signaling plays an essential role.
role in inhibiting angioblast migration into the cornea thus preventing its vascularization during development.

Analysis of ocular development in Tg(tie1:H2B:eYFP) quail or endomucin stained mouse tissue showed that angioblasts migrate into the periocular region, but unlike the periocular neural crest cells, which contribute to the cornea, angioblasts do not migrate into the cornea. Despite differences in angioblast migration and vascular patterning between quail and mouse, the corneas in both species maintained their avascular state during development. Together these results suggest that the vascular network in the anterior eye region emerges from vasculogenesis, and that angioblast migration and vascularization of the cornea are prevented early during development.

The cornea develops in close proximity with the lens, which provides signals that are crucial to neural crest cell migration and differentiation (Genis-Galvez, 1966; Beebe and Coats, 2000; Yamamoto and Jeffery, 2000; Lwigale and Bronner-Fraser, 2009), and the projection of trigeminal sensory nerves (Lwigale and Bronner-Fraser, 2007; Schwend et al., 2012). The lens vesicle expresses Sema3A during development (Chilton and Guthrie, 2003; Lwigale and Bronner-Fraser, 2009) and Sema3A secreted by the lens repels trigeminal and retinal ganglion axons in vitro (Ohta et al., 1999; Lwigale and Bronner-Fraser, 2007). Interestingly, the lens also expresses VEGF during development (Gerber et al., 1999; Shui et al., 2003; Saint-Geniez et al., 2006), which is also localized in regions occupied by migratory angioblasts and ocular blood vessels. Our results show that the expression patterns of VEGF and Sema3A in the mouse lens coordinate with the level of vascular formation. In support of this observation, conditional mouse mutants lacking VEGF expression in the lens show defects in the anterior papillary membrane,
but the tunica vasculosa lentis located at the posterior lens is not affected (Garcia et al., 2009).

Periocular neural crest cells and presumptive corneal nerves express Nrp1 where it plays a role in regulating their migration into the developing cornea (Lwigale and Bronner-Fraser, 2007, 2009; McKenna et al., 2012). Given that angioblasts and ocular blood vessels express Nrp1, and that Sema3A is expressed at higher levels than VEGF in the lens, we rigorously tested the role of Sema3A on periocular angioblast migration and development of corneal avascularity. Our data form Tg(tie1:H2B:eYFP) quail embryos show ectopic angioblast migration into the presumptive cornea in the absence of the lens. Similar observations were made in lens ablated chick eyes (Beebe and Coats, 2000), however the genes involved in the precocious vascularization of the cornea were not investigated. We also showed that pharmacological inhibition of lens-derived Sema3A recapitulated the lens ablation results, suggesting that lens-derived Sema3A plays an anti-angiogenic role that prevents vascularization of the avian cornea during development.

The absence of vasculature in the developing cornea may be a consequence of higher levels of Sema3A than VEGF. We found that VEGF-soaked beads induced robust angioblast migration into the presumptive cornea. This result was not surprising given that pellets containing VEGF induce neovascularization of adult corneas in micropocket assays (Amano et al., 1999; Rogers et al., 2007; Poche et al., 2009). But to our knowledge, the question of whether Sema3A can attenuate VEGF-induced vascularization of the cornea has not been addressed in vivo. We show that Sema3A inhibits VEGF-induced angioblast migration into the presumptive cornea. These data
support our inference that periocular angioblasts can respond to both VEGF and Sema3A, but the presence of higher levels of Sema3A in the presumptive cornea inhibits their ingression.

Similarly our studies in mice showed that loss of Sema3/Nrp1 signaling in Nrp1sema- mutant mice resulted in aberrant angioblast migration and vascularization of the developing cornea. Nonetheless, the adult mutant corneas were not vascularized (data not shown) suggesting that even under conditions of no Sema3/Nrp1 signaling, the adult cornea environment does not support stable vasculature. One possibility is that the soluble form of VEGFR1 (sflt), which plays an important role in preserving adult corneal avascularity (Ambati et al., 2006), is not present in the embryonic corneas where Sema3A plays a similar role. Despite the loss of ectopic vasculature in adult Nrp1sema- corneas, we cannot rule out the possibility of their increased susceptibility to pathological neovascularization. Given that Nrp1sema- mutant corneas are precociously innervated by Nrp1-positive trigeminal sensory axons (McKenna et al., 2012), we can further speculate that Sema3A/Nrp1 signaling plays a dual role in regulating neurovascular patterning during mouse ocular development.

Although earlier studies also reported neuropatterning defects in Nrp1sema- mutants, normal vascular development was observed in different regions of the developing embryo including the limbs, heart, and lungs (Vieira et al., 2007; Bhisitkul, 2006; Joza et al., 2013). In concert with the relatively high survival of Nrp1sema- mutants (Gu et al., 2003) compared with VEGF−/− and Nrp1−/− mutants that are embryonic lethal (Carmeliet et al., 1996; Ferrara et al., 1996; Kitsukawa et al., 1997; Takashima et al., 2002), it was suggested that Sema3A/Nrp1 signaling was not required for vascular
development in mice. However, these studies did not take into account the complexity of the signals in the environment to which the angioblasts and forming vasculature respond. Given that Sema3A inhibits VEGF-induced endothelial cell migration under defined culture conditions (Miao et al., 1999), the absence of vascular phenotype in some tissues in Nrp1\textsuperscript{sema-} null mutants could be interpreted in different ways. First, extrapolating from the current study, it is possible that Sema3A may be present at low levels in tissues where its absence does not result in apparent vascular defects as a result of aberrant angioblast migration. Second, the absence of Sema3A signaling in mice may be compensated for by other anti-angiogenic factors. For example, Sema3A co-localizes with Sema3E in most of the regions analyzed for vascular defects in the Nrp1\textsuperscript{sema-} null mutants (Behar et al., 1996; Meadows et al., 2012), whereas Sema3E\textsuperscript{−/−} null mutant mice exhibit cardiac and vascular patterning defects (Gitler et al., 2004; Gu et al., 2005). Third, as observed in the Nrp1\textsuperscript{sema-} null corneas, the aberrant vasculature in other tissues may not be stable in the ectopic environments resulting in their loss after vascular pruning and/or remodeling.

In summary, the current study presents cornea development as an excellent model to study the signals involved in vascular patterning. Results obtained from manipulation of avian eyes and analysis of vascular defects in Nrp1\textsuperscript{sema-} mutant mice corneas, provide direct evidence suggesting that Sema3A/Nrp1 signaling plays a crucial role in preventing angioblast migration and subsequent vascularization of the developing cornea. The schematic in Fig. 7 explains our current model of how Sema3A and VEGF regulate ocular vasculogenesis via signaling through Nrp1. VEGF from the optic cup is involved in the migration of Nrp1-positive angioblasts into the periocular region and the
establishment of primitive vasculature. However, despite the presence of VEGF in the presumptive cornea, the relatively higher levels of Sema3A accumulation in this region prevent angioblast migration into the cornea and maintain its avascularity during development. We can infer from this and previous studies that angioblast migration, a crucial step in vascular development, is dictated by the relative concentrations of Sema3A and VEGF in the embryonic environment. Interestingly, both Sema3A and VEGF are strongly expressed in the adult corneal epithelium (Morishige et al., 2010; Phillip et al., 2000; Ambati et al., 2006), where they may be required for maintaining normal physiology, but Sema3A also contributes to corneal avascularity. Identification of Sema3A as a key player in preventing vascularization of the developing cornea may provide insights into new strategies for treatment of neoangiogenesis in adult corneas and in pathological neovascularization such as cancer.

Figure 2.7. Model depicting the regulation of angioblast migration in the anterior eye by Sema3A and VEGF via Nrp1. Schematic of a cross section through the anterior eye showing the localization of Sema3A and VEGF in the anterior eye and the response to these cues by Nrp1-positive angioblasts. Angioblasts migrate to the periocular region in response to VEGF signaling from the optic cup. The lens secretes a higher concentration of Sema3A than VEGF into the region of the developing cornea. Therefore angioblasts encounter strong Sema3A repulsion at the interface between the cornea and periocular region that prevents their migration into the presumptive cornea. These unequal concentrations of Sema3A and VEGF signaling in the anterior eye allow for the formation of the limbal vasculature in the periocular region while maintaining avascularity in the presumptive cornea.
Chapter 3: Expression of CXCL12 and CXCL14 Chemokines During Eye Development in Chick and Mouse

A substantial portion of this chapter is from Ojeda AF, Munjaal RP, Lwigale PY. Expression of CXCL12 and CXCL14 during eye development in chick and mouse. Gene Expression Patterns. 13 (8) 303-10. 2013.

3.1 Introduction

Chemokines are a large family of small-secreted chemotactic cytokines that function in many physiological and pathological processes. Although initially identified as inducers of leukocyte migration during inflammatory response (Wong and Fish, 2003; Kiefer and Siekmann, 2011), recent studies have shown that chemokines are involved in tumor development, neurodegenerative diseases, angiogenesis, and embryogenesis (Rostene et al., 2007; Olesnicky et. al., 2009; Banisadr et al., 2011; Kiefer and Siekmann, 2011). Despite increasing evidence of the involvement of CXCL12 (stromal cell-derived factor-1, SDF-1) and CXCL14 (BRAK, Scyba, or MIP-2γ) in non-immune processes such as cell migration, proliferation, and differentiation, very little is known about their involvement in eye development.

CXCL12 signaling mediated by the receptors CXCR4 and/or CXCR7 plays an important role during hematopoiesis, angiogenesis, and in the migration of germ cells, neural crest cells, neural precursors, and limb progenitor cells (Lazarini et al., 2003; Stebler et al., 2004; Yusuf et al, 2006; Theveneau et al., 2010; Belmadani et al., 2009). During embryonic development, CXCL12 is expressed in the mesenchyme of the head, limbs, sclerotome, and in regions surrounding major blood vessels, gonads, and mesonephros (Vasyutina et al., 2005; Rehimi et al., 2008; García-Andres and Torres,
Mutations of CXCL12 or CXCR4 in mice are lethal between embryonic day (E)15 and birth, and the embryos have defective neurogenesis, cardiovascular development, and impaired hematopoiesis (Nagasawa et al., 1996; Ma et al., 1998; Zou et al., 1998). Similar defects are observed in CXCR7 deficient mice, although hematopoiesis seems not to be affected (Sierro et al., 2007).

Until recent, very little was known about the involvement of CXCL14 in embryonic development and the receptor(s) that mediate its function remain to be discovered. In adults, CXCL14 is constitutively expressed in normal epithelial tissues such as the breast, kidney, brain, and lungs (Hromas et al., 1999; Meuter and Moser, 2008). During early embryogenesis in chick and mouse, CXCL14 is expressed in the ectoderm, central nervous system, paraxial mesoderm, limbs, and in the trigeminal and dorsal root ganglia (Park et al., 2012; Gordon et al., 2011; Garcia-Andres and Torres, 2010). Other patterns of CXCL14 expression are observed in Xenopus and zebrafish embryos during development of the cement gland, otic vesicle, dorsal retina, and in migratory cranial neural crest cells and neuromasts of the lateral line (Park et al., 2009; Long et al., 2000). Although CXCL14 is a chemoattractant for immune cells (Kurth et al., 2001; Shellenberger et al., 2004; Tanegashima et al., 2010), CXCL14 deficient mice do not show severe defects in the immune system (Meuter et al., 2007). However, these mice show altered feeding behavior and glucose metabolism that is associated with improved insulin sensitivity (Nara et al., 2007; Tanegashima et al., 2010). CXCL14 inhibits endothelial cell migration in vitro and prevents neovascularization of the cornea in micropocket assays involving various pro-angiogenic factors (Shellenberger et al., 2004). Recent studies have reported that CXCL14 plays a role in differentiation of Langerhans
cells in the skin and Schwann cells (Schaefer et al., 2005; Barbaria et al., 2009; Hara and Tanegashima, 2012). Also CXCL14 is upregulated through calcium–calmodulin signaling in a cell density-dependent manner, accompanied by the upregulation of several keratinocyte differentiation markers of epithelial cells (Ikoma et al., 2012).

Vertebrate eye development is a multistep process that involves interactions between cells and tissues from different embryonic origins that result in the formation of a functional organ. At the rudimentary stage, the eye is comprised of the lens vesicle and overlaying ectoderm, the optic cup, and the periorcular mesenchyme comprised of neural crest cells and cranial mesoderm. The following events occur during subsequent development of the eye: (1) The lens vesicle forms the lens epithelium and crystalline cells (Cvekl and Mitton, 2010) (2) The periorcular mesenchyme combines with the overlying ectoderm to form the cornea and the eyelids (Johnston et al., 1979; Hay, 1980; Creuzet et al., 2005; Lwigale et al., 2005); (3) Angioblasts in the periorcular region undergo vasculogenesis to form the complex network of limbal blood vessels (Kwiatkowski et al, 2013); (4) The optic cup differentiates into the retinal pigment epithelium and the neuroretina (Venters et al., 2011; Fuhrmann, 2010); and (5) The trigeminal, ciliary, and oculomotor nerves project into the eye to provide sensory and sympathetic innervation (Narayanan and Narayanan, 1978). Signaling between the ocular tissues is essential for cell migration, proliferation, and differentiation that enable the proper formation of a functional eye. Although CXCL12 and CXCL14 have been associated with such cellular events, these chemokines have not been studied during eye development. To determine the potential roles of CXCL12 and CXCL14 during eye development, we performed a detailed examination of their expression patterns during
the formation of ocular tissues. Due to differences between avian (Johnston et al., 1979) and murine (Pei and Rhodin, 1970) eye development, we compared the expression of CXCL12 and CXCL14 between chick and mouse embryos. Our results show that CXCL12 and CXCL14 are expressed in complementary patterns in most tissues during ocular development. Expression of CXCL12 is conserved between chick and mouse in most ocular tissues, whereas CXCL14 expression is only conserved in the upper eyelid ectoderm. The expression patterns of CXCL12 and CXCL14 indicate their potential interaction and involvement in ocular development.

3.2 Material and Methods

3.2.1 Embryos

All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Rice University. Fertilized White Leghorn chicken eggs (Gallus gallus domesticus) were obtained from a commercial supplier. Eggs were incubated at 38°C under humidified conditions. Chick embryos were collected between embryonic day (E)3 and E17. Wild-type C57/B6 mouse embryos were collected between E11.5 and E16.5, with the first appearance of a virginal plug considered as E0.5. Mouse eyes were also collected at postnatal day (P)0 and from adults. All tissues were collected in Ringer’s solution, fixed overnight in modified Carnoy’s fixative (60% ethanol, 30% formadehyde, and 10% glacial acetic acid) at 4°C. Tissues were dehydrated in ethanol series, cleared in Histosol and embedded in paraffin, then sectioned between 10 and 12 µm.
3.2.2 Synthesis of mRNA riboprobes

Fragments of the genes of interest were obtained by amplification of the cDNA. The PCR products were cloned into pCRII-TOPO vector with dual promoters (Invitrogen) following manufacture’s protocol, and the gene inserts were confirmed by sequencing. Plasmids were linearized and digoxigenin-labeled riboprobes were generated using T7 or SP6 polymerases. Primers were used as follows: *Gallus gallus* CXCL14, 5’GATTCTCTAACGTACGGAAGC (forward) and 5’CCAGCATCACTCATGTACCTCT (reverse); *Gallus gallus* CXCL12, 5’TGGCTCTGCTCGCCTTTGC (forward) and 5’AAGTATCTGTGCTGGGGTCC (reverse); *Mus musculus* CXCL14, 5’ACTGCGAGGAGAAGATGGTTAT (forward) and 5’ GTAGAAGATGCTTCTGAGGCATC (reverse); *Mus musculus* CXCL12, 5’ GCCGCACTTTCACTCTCG (forward) and 5’GGTCATGCTAGGGTTGCCA (reverse).

3.2.3 In situ hybridization

*In situ* hybridization was performed on sections as described previously (Etchevers et al., 2001). Sense probes were used as control, and showed no signal.

3.2.4 Microscopy and imaging

Images of stained sections were acquired using AxioImager 2 fluorescence microscope with ApoTome and AxioCam (Carl Zeiss AG, Oberkochen, Germany).
3.3 Results and Discussion

3.3.1 Expression of CXCL12 and CXCL14 during Development of the Cornea and Iris

During eye development, periocular neural crest cells migrate between the lens vesicle and the ectoderm to form the cornea. In chick this is a well-coordinated process involving an initial wave of neural crest cell migration from the periocular mesenchyme to the region between the lens and ectoderm (presumptive cornea epithelium) to form the inner most layer of the cornea (the cornea endothelium). This is followed by a second wave of neural crest cell migration between the endothelium and ectoderm to form the cornea stroma (Johnston et al., 1979; Hay, 1980; Creuzet et al., 2005; Lwigale et al., 2005). In mouse, neural crest cells migrate as a single mass between the lens vesicle and ectoderm, then the cells adjacent to the lens differentiate into the cornea endothelium while the rest form the stroma (Pei and Rhodin, 1970; Gage et. al., 2005). Shortly after cornea formation, the periocular neural crest cells and neuroepithelium at the tip of the optic cup coalesce to form the iris.

Expression of CXCL12 in the chick eye is first detected in the periocular mesenchyme at E3 (appendix and Garcia-Andres and Torres, 2010). By E5, expression of CXCL12 persists in the periocular mesenchyme, and it is also expressed some extent in the newly formed cornea endothelium (Figure 3.1A, arrowhead), and blood vessels (Figure 3.1A, A', asterisk). At E7 and E12, expression of CXCL12 in the anterior eye is restricted to the periocular region in cells located adjacent to the retinal pigment epithelium, and it remains strong in the iris stroma and blood vessels (Figure 3.1B and C). Similarly, in the mouse eye, CXCL12 expression is initially robust in the periocular
mesenchyme at E11.5 (Figure 3.1G'), but it becomes restricted to the cells adjacent to the retinal pigment epithelium, iris stroma (Fig. 1G-I, arrows) and ocular blood vessels (Figure 3.1H, asterisk). Since CXCL12 is involved in migration of neural crest cells (Olesnicky Killian et al., 2009; Kasemeier-Kulesa et al., 2010; Theveneau et al., 2010) and other embryonic cell types (Doitsidou et al., 2002; Vasyutina et al., 2005; Chen et al., 2007; Li and Ransohoff, 2008), its expression by periocular neural crest cells suggests that it may play a similar role during cornea development. Expression of CXCL12 in the forming ocular blood vessels is consistent with its proangiogenic role during vascular development (Yamaguchi et al., 2003; Hiasa et al., 2004). Since CXCL12 is expressed in the limb during myogenesis (Garcia-Andres and Torres, 2010; Hunger et al., 2012), its presence in the iris stroma indicates a possible role during neural crest cell differentiation into iris muscles.

In contrast to CXCL12 expression, CXCL14 is not expressed in the anterior region of the chick eye by E3 and E5 (Figure 3.1D and data not shown). However, by E7 CXCL14 expression is prominent in the cornea stroma (Figure 3.1E and E') and coincides with neural crest differentiation into stromal keratocytes (Hay et al., 1979; Hay, 1980; Funderburgh et al., 1986). CXCL14 is also expressed at low levels in the lens epithelium (Figure 3.1E, arrowhead). During subsequent development, expression of CXCL14 is restricted to the keratocytes in anterior region of the corneal stroma at E12 (Figure 3.1F). By E17, CXCL14 expression is absent in the stroma, but it becomes expressed by cells on the surface and tip of the iris, where it persists through E19 (Figure 3.1F' and data not shown). Unlike most of the cranial and limb ectoderm where intense expression of CXCL14 is observed (Garcia-Andres and Torres, 2010; Gordon et
al., 2011), it is not expressed in the ectoderm of the presumptive cornea epithelium in chick (Figure 3.1D-F).

In the mouse, CXCL14 is initially expressed at low levels in the periocular mesenchyme, optic cup, and lens at E14.5 (Figure 3.1J). Expression of CXCL14 in the eyelid epithelium is strong at this time and during subsequent development. At E16.5 and P0, expression of CXCL14 is restricted to the cornea epithelium, optic cup, neuroepithelium of the iris, and in the lens epithelium (Figure 3.1K and 1L). Expression of CXCL14 in the mouse cornea coincides with the differentiation of the cornea epithelium as indicated by the expression of the epithelial keratin markers, K12 and K14 (Kurpakus et al., 1994; Tanifuji-Terai et al., 2006). Although expression of CXCL14 in the cornea is not conserved between chick and mouse, its presence in the chick corneal stroma and mouse corneal epithelium may indicate a possible role during keratocyte and epithelial cell differentiation, respectively.
Figure 3.1. Expression of CXCL12 and CXCL14 during development of the cornea and iris in chick and mouse. In situ hybridization was performed on paraffin sections through the anterior eye of chick and mouse embryos. (A-F) During chick eye development, CXCL12 is strongly expressed in the periocular mesenchyme and its derivatives between E5 and E12 (A-C). CXCL14 expression is not detected in the periocular region and presumptive cornea at E5 (D), but it is later expressed in the cornea, lens, and iris between E7-E17 (E-F). (G-L) During mouse eye development, CXCL12 is expressed in the periocular mesenchyme and its derivatives between E11.5 and P0 (G-I). CXCL14 is initially expressed at low levels at E14.5 (J), and then it becomes restricted to the cornea epithelium, optic cup, and lens epithelium between E16.5 and P0 (K-L). Asterisks in A-D and H indicate ocular blood vessels; c, cornea; en, cornea endothelium; ep, cornea epithelium; ey, eyelid; ir, iris; L, lens; oc, optic cup; pi, presumptive iris; pm, periocular mesenchyme; rpe, retinal pigmented epithelium; st, cornea stroma. Scale bar represents 100 μm in B, C, E, and F and 50 μm in A, D, G-L.
3.3.2 Expression of *CXCL14* in the Anterior Ocular Epithelium during Development

At E7 and during subsequent development of the chick eye, expression of *CXCL12* is limited to the blood vessels and periocular mesenchyme adjacent to the retinal pigment epithelium (Figure 3.1B and data not shown). The ectoderm spanning the anterior eye region of the chick does not express *CXCL14* during early stages of development (Figure 3.1D-1F and data not shown). However, by E7 expression of *CXCL14* is vivid in the region of the ectoderm (Figure 3.2A and 2A') corresponding to the location of the scleral papillae (Coulombre and Coulombre, 1962). Scleral papillae are thickenings in the conjunctival epithelium (Franz-Odendaal and Vickaryous, 2006), that induce the underlying neural crest mesenchyme to form skeletogenic condensations known as scleral ossicles (Coulombre and Coulombre, 1962; Pinto and Hall, 1991). Expression of *CXCL14* in the conjunctival epithelium coincides with the time when the mesenchyme becomes competent to induce the formation of scleral papillae in the overlying conjunctival epithelium (Wedlock et al., 1969; Hall, 1981; Duench and Franz-Odendaal, 2012). Section through a scleral papilla at more advanced stage of formation shows expression of *CXCL14* in the thickened ectoderm, but it is absent in the superficial cells above the thickest central region (Figure 3.2B, asterisk). *CXCL14* is expressed at low levels as the scleral papillae increase in size to form a visible outgrowth above the epithelium (Figure 3.2C, asterisk). By E11, expression of *CXCL14* is absent in this region of the conjunctival epithelium and coincides with the degeneration of the scleral papillae (Murray, 1943; Franz-Odendaal, 2008). Our results show *CXCL14* expression as a novel
early marker for scleral papillae and suggest that it may play a role in establishing the transient placodal phase of the conjunctival epithelium.

By E12, expression of CXCL14 is strong in another group of conjunctival epithelial cells and adjacent stroma located in the limbus region of the cornea (Fig. 2D and 2D'). By E17, cells expressing CXCL14 are located in the basal cell layer of limbal epithelium near the margin of the cornea epithelium (Figure 3.2E and 2E'). Unlike chick, expression of CXCL14 in the mouse is maintained at modest levels in the cornea and conjunctival epithelium during development (Figure 3.1K and 1L). In adult mouse corneas, CXCL14 is expressed in the basal and wing layers of the epithelium (Figure 3.2F and 2G). In the limbal epithelium, CXCL14 expression is maintained at a slightly higher level in comparison to the corneal epithelium (Figure 3.2H). In adult eyes, the basal layer of the limbal epithelium contains a reservoir of stem cells that replenish the cornea epithelium (Tseng, 1989; Schlötzer-Schrehardt et al., 2005), but their origin and when they are established is not known. Based on our results, it is possible that CXCL14 plays a role during the formation of the limbal stem cells. Similarly, previous studies have shown that CXCL14 is expressed in regions where stem cells reside in other tissues including the dentate gyrus of the hippocampus (Banisadr et al., 2011), skin epithelial cells (Schauerli et al., 2005; Frick et al., 2011), and at the base of the intestinal crypts (Meuter and Moser, 2008).
3.3.3 Expression of CXCL12 and CXCL14 in the Eyelids

The conjunctival epithelium also covers the inner eyelid and the nictitating membrane. The nictitating membrane is a transparent tissue that protects and lubricates the anterior surface of the eye. It exists in reptiles, birds, amphibians, and some mammals, but absent in primates and mice (Stibbe, 1928). The eyelids first appear as mesenchyme filled bumps in the ectoderm at about E7 in chick (Hamburger and
Hamilton, 1951) and between E13 and E14 in mouse (Pei and Rhodin, 1970). In chick, the nictitating membrane also appears at about E7 as an additional mesenchyme filled bump adjacent to the eyelid.

Expression of \textit{CXCL12} is not detected in the forming chick eyelid at E7 (Figure 3.3A). By E8, \textit{CXCL12} expression is prominent in the mesenchyme of the eyelid and low in the nictitating membrane (Figure 3.3B). At E12, expression of \textit{CXCL12} persists in the mid-region of the eyelid mesenchyme (Figure 3.3C) that later differentiates into the eyelid muscles (Noden, 1986; Creuzet et al., 2005). At this time only a few cells express \textit{CXCL12} in the mid-region of the nictitating membrane (data not shown). \textit{CXCL12} is also expressed in the mesenchyme surrounding the feather buds on the eyelids (Figure 3.3C’).

\textit{CXCL14} is strongly expressed in the eyelid ectoderm at E7 (Figure 3.3D). This pattern of \textit{CXCL14} expression persists in the ectoderm of the eyelid and nictitating membrane at E8 and E12 (Figure 3.3E and 3F). Expression of \textit{CXCL14} is also observed in a few cells in the mid-region of the feather buds and in the surrounding surface ectoderm (Fig. 3F’).

In the mouse, \textit{CXCL12} is strongly expressed in the mesenchyme located in the mid-region of the eyelid by E14.5 and its intensity decreases towards the edges (Figure 3.3G). This pattern of \textit{CXCL12} expression persists in the eyelid mesenchyme at E16.5 (Figure 3.3H) and during later stages of development (data not shown). In contrast, at E14.5 \textit{CXCL14} is expressed in the ectoderm of the outer eyelid, but absent in the inner eyelid. \textit{CXCL14} is also expressed in the mesenchyme at the anterior region of the eyelid at E14.5 (Figure 3.3J), and it persists at low levels in this region at E16.5 (Figure 3.3K).
Given that feather buds form at the posterior region of the eyelids in chick but hair follicles are at a relatively early stage of development in the mouse eyelids between E11.5-E16.5, we examined the expression patterns of CXCL12 and CXCL14 in the hair follicles of the whisker pad at E16.5. Although CXCL12 is expressed at low levels in the developing hair follicles (Figure 3.3I, dotted lines), it is strong in the surrounding mesenchyme. In contrast, CXCL14 is strongly expressed in the hair follicles (Figure 3.3L). Its expression is continuous with the basal layer of the skin and encompasses the outer root sheath, hair bulb (Figure 3.3L, arrow) and bulge (Figure 3.3L, arrowhead) regions of the hair follicle (Figure 3.3L). Expression of CXCL14 in the bulge region of the follicle where hair stem cells reside (Spradling et al., 2001; Wang et al., 2012) further indicates its potential role in stem cell development. Our results show that CXCL12 and CXCL14 are expressed in complementary patterns during eyelid development. Expression of CXCL12 in the eyelids is conserved between the chick and mouse at early stages of development but it is restricted in the posterior eyelid mesenchyme of the mouse by E16.5. Therefore CXCL12 may play similar role that occurs at different time in chick and mouse eyelid development. Expression of CXCL14 in the eyelids also appears to be conserved between chick and mouse, with the exception of the inner eyelid epithelium in mouse where it is absent.
Figure 3.3. Expression of CXCL12 and CXCL14 during eyelid development in chick and mouse. (A-F) Expression of CXCL12 (A-C) and CXCL14 (D-F) during chick eyelid development. CXCL12 is initially not expressed in the eyelid at E7 (A), but it becomes prominent between E8 and E12 (B-C). Expression of CXCL12 is absent in the feather buds (C'). Expression of CXCL14 is robust in the ectoderm of the eyelids and nictitating membrane between E7-E12 (D-F), but not continuous in the feather buds where it is expressed in the mesenchyme (F'). (G-L) Expression of CXCL12 (G-I) and CXCL14 (J-L) during eyelid and hair follicle development in the mouse. CXCL12 is strongly expressed in the eyelid mesenchyme between E14.5- E16.5 (G and H). CXCL12 is also expressed in the mesenchyme surrounding the hair follicles (I, dotted outline). CXCL14 is expressed in the eyelid ectoderm and mesenchyme between E14.5 and E16.5 (J and K), and in the hair follicles (L). ey, eyelid; nm, nictitating membrane; fb, feather buds; hf, hair follicle; in, inner eyelid epithelium; out, outer eyelid epithelium. Scale bar represents 100 μm in B, C, E, F, I, and L, and 50 μm in A, D, G, H, J, and K.
3.3.4 Expression of CXCL12 and CXCL14 in the Retina

Following the formation of the optic cup, the outer layer becomes the retinal pigmented epithelium (RPE) and an inner layer gives rise to the neural retina (Pei and Rhodin, 1970, Agathocleous and Harris, 2009). After successive proliferation and differentiation, the neural retina is divided into three cellular layers: the outer nuclear layer (ONL) that contains rod and cone photoreceptors; the inner nuclear layer (INL) consisting of horizontal, amacrine, Muller, and bipolar cells; and the ganglion cell layer (GCL) located near the inner surface and comprised of retinal ganglion cells (Chow and Lang, 2001; Bassett and Wallace, 2012).

Expression of CXCL12 in the posterior eye region of chick (Figure 3.4A and 4B) and mouse (Figure 3.4E and 4F) is restricted to the connective tissue and choroidal blood vessels adjacent to the RPE. A few cells in the anterior most region of the chick INL express CXCL12 at E12 (Figure 3.4B) but no expression of CXCL12 was observed in the mouse neuroretina by P0 (Figure 3.4F). Interestingly, a different pattern of CXCL12 expression was observed in the internal limiting membrane (ILM) of the human retina at 2 weeks of gestation and believed to function in vessel assembly (Hasegawa et al., 2008). In addition to its function in angiogenesis, CXCL12 may also contribute to retina development since it promotes the survival of embryonic retinal ganglion cells that express CXCR4 receptor (Chalasani et al., 2003).

Expression of CXCL14 is sparse and diffuse in the chick neural retina at E7 (Figure 3.4C). By E12, its expression is restricted to the INL in regions where amacrine and bipolar cells reside, and by a few cells in the GCL layers (Figure 3.4C). In the mouse, CXCL14 is broadly expressed in the neural retina at E16.5 (Figure 3.4G). By P0,
vivid expression of CXCL14 is maintained in the neuroretina (Figure 3.4H), but it is absent in the developing inner plexiform layer (IPL). All cell layers are formed in young adult mouse retinas and CXCL14 is expressed in the inner boundary of the INL populated by amacrine cells (Doh et al., 2010; Bassett and Wallace, 2012), and in the GCL (Figure 3.4 H'). Our results show that retina expression of CXCL14 is conserved between chick and mouse. The asymmetric distribution of CXCL14 in developing and adult retinas suggests a potential role in positioning, differentiation and/or maintenance of retinal cells in specific layers.

Figure 3.4. Expression of CXCL12 and CXCL14 during retina development in chick and mouse. (A-D) Expression of CXCL12 (A and B) and CXCL14 (C and D) in the chick retina at E7 and E12. (E-F) Expression of CXCL12 (E and F) and CXCL14 (G and H) in the mouse retina at E16.5, P0, and young adult (H'). gcl, ganglion cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; nr, neural retina; rpe, retinal pigmented epithelium. Scale bar represents 50 μm.
3.4 Conclusions

Our results revealed novel expression patterns of $CXCL12$ and $CXCL14$ and suggest that these two chemokines may play essential roles during ocular development in chick and mouse. Expression of $CXCL12$ in the ocular mesenchyme and blood vessels was conserved between chick and mouse suggesting similar function during neural crest cell differentiation into muscles of the eyelid and iris, and during ocular vasculogenesis in both species. Chick-specific expression of $CXCL12$ in the amacrine cells of the retina indicates potential role in cell proliferation or differentiation. In contrast, $CXCL14$ was expressed in the ocular ectoderm and corresponded with the formation of the scleral papillae and hair follicles, and it was localized in the basal layer of the limbal epithelium where stem cells reside. The observed expression patterns indicate potential involvement of $CXCL14$ in cell differentiation and proliferation in the ocular epithelium. $CXCL14$ expression in the eyelid mesenchyme and cornea suggests a possible function in neural crest differentiation into muscles or stromal keratocytes. $CXCL14$ may play an anti-angiogenic role (Shellenberger et al., 2004) by preventing $CXCL12$-expressing angioblasts and ocular blood vessels enter from the limbal region to the developing cornea. Complementary expression of $CXCL12$ and $CXCL14$ in the anterior eye and hair follicles suggests that interaction of these genes might be essential during development.
Chapter 4: RCAS System as an Effective Tool to Knockdown CXCL14 during Chick Ocular Development

4.1 Introduction

The RCAS system is a family of replication–competent retroviral vectors derived from the Rous sarcoma virus (RSV) (Hughes, 2004; Bromberg-White et al., 2004). In fact, the name RCAS stands for Replication-Competent Avian sarcoma-leukosis Virus (ASLV) long terminal repeat (LTR) with a Splice acceptor (Loftus et al., 2001). As all retroviruses, RCAS life cycle starts with the entry of the virus by the interaction of the viral envelope glycoprotein with its cognate receptor from the host cell. In the cytoplasm, viral RNA is replicated into DNA to be randomly inserted in the host genome. Once integrated, the viral genome is transcribed and translated into viral proteins necessary to form infectious viruses. In RCAS virus, Pol encodes the reverse transcriptase, gag the structural proteins of the virus (capsid) and env encodes the envelope. It also contains the oncogene src that can be removed and replaced by a gene or sequence of interest via a spliced message (Hughes, 2004). RCAS virus is an ideal method for gene expression in the avian system, considering that it spreads vertically to daughter cells, and horizontally to neighboring cells, resulting in a global, stable, and long lasting expression of the gene or sequence of interest. A series of RCAS vectors have been constructed that differ in the type of envelope protein that they express and the introduction of more efficient polymerase to increase the viral titer and infectivity (Gordon et al., 2009). The most commonly used RCAS vectors encode the Env A and B glycoproteins, designated as RCAS(A) and RCAS(B) respectively (Hunter, 1997,
Hughes, 2004). Both give a broad infection in the chick embryos, however, it has been reported that only RCAS(A) can infect quails (Holmen et al., 2001; Creuzet et al., 2002; Rainey et al., 2003). Another important modification of the original RCAS vector is the introduction of “Bryan polymerase”, a polymerase known to have higher efficiency that results in a significant increase of the titer viral stock. The resulting vectors are called RCASBP (Boerkoel et al., 1993; Hughes, 2004; von Werder et al., 2012).

Loss-of-function approaches using RCAS system have also been developed to efficiently knock down gene function in vitro and in vivo through short hairpin RNAs (shRNA) (Harpavat and Cepko, 2006). In this case, a U6 promoter that drives a shRNA sequence is inserted in the RCAS via ClaI site. In order to perform loss-of-function experiments using the RCAS virus, design of shRNA sequence is critical. There are several rules that can be followed: a) Avoid sequences rich in GC content. b) Avoid 4 or more nucleotide repeats. c) Avoid sequences with high homology to other related or unrelated genes. d) Start with a guanine residue to favor the transcription of Polymerase. e) Contain a hairpin loop sequence such as TTCAAGAGA. f) Have overhanging sequences corresponding to restriction endonuclease sites that are necessary for cloning. g) Include a RNA Pol III terminator sequence (poly T nucleotides) (Hannon, 2002; Mello and Conte, 2004).

The overall goal of this study was to knockdown CXCL14 in the ocular tissue at late stages of chick embryos by using the retrovirus-mediated shRNA delivery. Following the rules mentioned above I designed CXCL14-shRNA sequences that were integrated into RCAS vector and show that RCAS-CXCL14-shRNA virus could effectively knock down CXCL14 in the ocular tissues. Furthermore, I show that CXCL14 loss-of-function
embryos exhibit a distinct morphological phenotype that suggests CXCL14 plays a critical role in the development of ocular tissues during embryogenesis.

4.2 Material and Methods

Animals

Animals were processed as stated in material and methods of chapter 2. Chicken embryos were collected between E7 and E12, and processed for *in situ* hybridization, histology and immunostaining. Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Rice University.

4.2.2 Construction of RCAS-CXCL14-shRNA vector

Construction of RCAS-CXCL14-shRNA is described in detail in the result section of this chapter. Briefly, four CXCL14-shRNA sequences were designed using a free website tool (Dharmacon). The sense and antisense CXCL14-shRNA oligomers were annealed, phosphorylated and incorporated into the shuttle vector that contains a cU6 promoter (pSLAX-cU6). PSLAX-cU6-CXCL14-shRNA constructs were ClaI digested and inserted into the ClaI site of the avian retrovirus vector RCASBP(B) (Hughes, 2004) to produce RCASBP(B)-CXCL14-shRNA vectors. Primers RCAS forward: 5' ACGCTTTTGTCTGTGCTGC 3' and RCAS reverse: 5' ATCTCTGCAATGCGGAATTCAGTG 3' (Morgan and Fakete, 1996) were used to confirm the insertion of cU6-CXCL14-shRNA fragment into RCAS by PCR and DNA sequencing. Additionally, the cU6 forward primer 5' GAATTGTGGGACGGCGGAAG 3' that is located inside the cU6 reported was used to sequence the generated shRNA
fragments. For simplicity, RCASBP(B) is subsequently referred as RCAS(B). RCAS having the green fluorescent protein (GFP) reporter (RCASBP(B)-GFP) was kindly provided by Deneen lab to be used as control.

4.2.3 **In Ovo injection**

Eggs were incubated at 38°C in a humidified incubator until the desired embryonic stage. *In ovo* injections were carried out by injecting high-titer of RCAS-CXCL14-shRNA or RCAS-GFP control viruses resuspended in Dulbecco’s Modified Eagle Medium (DMEM) containing a small amount of fast green dye for visualization, into the lumen of the neural tube at stages Hamburger Hamilton (HH) 5-9 to target migratory NCCs of the cranial region (Hamburger and Hamilton, 1951). A pressure-based microinjection system (Picospritzer III, Parker Hannifin) connected to glass needles pulled from capillary tubes (VWR) was used to perform the injection. Windowed eggs were sealed after injections, reincubated until desired time of collection, and examined for ocular defects.

4.2.4 **Histology and immunostaining**

*Hematoxylin-Eosin Staining*

Hematoxylin-Eosin staining (H&E) were performed from E7 to E12 chick embryos. Eyes were fixed in modified Carnoy’s, paraffin embedded and sectioned. Sections were deparaffinized and rehydrated and stained with hematoxylin for 2 minutes, rinsed in water and destained with acid ethanol. After several washes in water, samples were
stained with eosin for 30-60 seconds and dehydrated in 95% and 100% ethanol. Stained sections were mounted in xylene based solution (Permount, Fisher Scientific).

**Immunohistochemistry in cell culture**

DF-1 cells were cultured on coverslip in 12 well-plates (MIDSCI) or 8-well chamber slides (Labtek, Nalge Nunc). 3-5 days post incubation cells were rinsed in PBS and fixed for 20 minutes in 4% PFA at room temperature. Washes were performed in 0.1% PBT and blocked for 1 hour in blocking solution containing 0.1% Triton X-100, 0.1% BSA and 5% heat-inactivated goat serum. Rabbit anti-GFP monoclonal (1:500, IgG, life technologies), or mouse anti-3C2 (1:1000, IgG1, DSHB) primary antibody was added and incubated for 2 hours at room temperature or overnight at 4°C. After several washes, Alexa 488 goat anti-rabbit IgG, Alexa 594 goat anti-mouse IgG1, or Alexa 488 goat anti-mouse IgG1 secondary antibodies (Invitrogen) were added and incubated at room temperature 1 hour. Coverslips or chambers were washed in PBS, stained with DAPI and mounted in aqueous based mounting media, Perma Fluor (Thermo Scientific).

**Section in situ hybridization and immunostaining.**

RNA *in situ* hybridization was performed in sectioned tissue as described in material and methods of chapter 2. After *in situ* color reaction, sections were post-fixed in 4% PFA for 30 minutes, blocked in blocking solution containing 0.2% Triton X-100, 0.2% BSA, and 5% heat-inactivated goat serum, and incubated with mouse anti-3C2 gag viral protein (1:500, IgG1, DSHB) primary antibody for two days at room temperature. Secondary
antibody, Alexa 488 goat anti-mouse IgG1 (Invitrogen) was used at a concentration of 1:200 and incubated for four hours at room temperature. Slides were cover-slipped with water based mounting solution Perma Flour (Thermo Scientific) and images were captured a Zeiss Axiocam mounted on a Zeiss Axioscope 2 microscope with ApoTome (Carl Zeiss, Germany).

4.3 Results

4.3.1 Generation of RCAS CXCL14-shRNA Vectors

CXCL14-ShRNA sequences were designed using the Dharmacon siRNA design tool (www.dharmacon.com). Sequences were designed to be 21 base pairs in length, GC content from 30-50%, and beginning with a guanine. Four siRNA sequences were chosen referred to as 170, 213, 268 and 308 based on the initiation of the Gallus gallus CXCL14 mRNA sequence (NCBI reference sequence No. NM_204712.2) (Figure 4.1A). Complementary oligonucleotides containing a stem-loop sequence (TTCAAGAGA) and its antisense sequence were acquired (Sigma), phosphorylated with T4 polynucleotide kinase (NEB) at 37°C for one hour and then annealed at 95°C for three minutes and room temperature for one hour. A two-step cloning was performed for the RCAS-CXCL14 shRNA construction vector. First, the shRNA fragment was cloned into a shuttle vector (pSLAX) that contains the cU6 promoter and multiple cloning sites flanked by ClaI restriction sites. pSLAX- cU6 shuttle vector was linearized with EcoRI and Pmel, and ligated with CXCL14-shRNA fragment using T4 ligation (New England BioLabs). This results in pSLAX-cU6-CXCL14-shRNA vector (Figure 4.1B). Second, the insert is released from the shuttle vector and inserted into the RCAS(B) vector. Both, pSLAX-
cU6-CXCL14shRNA and RCAS(B) were linearized using Clal and ligated by T4 ligation (New England BioLabs). This results in RCAS(B)-CXCL14-shRNA driven by cU6 promoter (Figure 4.1C).

Figure 4.1. Generation of RCAS-CXCL14-shRNA vector. A) Four CXCL14-specific sequences were selected and named as 170, 213, 268 and 308 based on the initiation of Gallus gallus CXCL14 mRNA sequence. All sequences were located within the coding region of CXCL14. B) SLAX shuttle vector encoding cU6 promoter was linearized with Pmel and HindIII to insert CXCL14-specific shRNA. The modified shuttle vector resulted in SLAX-CXCL14-shRNA. C) Schematic representation of the RCASBP vector encoding the CXCL14-specific shRNA sequence. The fragment encoding the cU6 promoter and CXCL14-shRNA from the shuttle vector was linearized with Clal enzyme and inserted in the RCASBP vector. It results in RCASBP-CXCL14-shRNA driven by the cU6 promoter.
4.3.2 Production, Concentration and Infectivity of RCAS Vectors

For virus production, it is first necessary to transfect avian cells with the RCAS plasmid. The embryonic chicken fibroblast (DF-1) cells are widely used for this purpose, since they proliferate continuously and are free of endogenous proviruses closely related to RCAS (Schaefer-Klein et al., 1998).

To determine the production of large amounts of retrovirus, we transfected DF-1 cells with RCAS-GFP control, or RCAS-CXCL14-shRNA construct. We verified the successful transfection shown by cells that stained positive for GAG viral protein (3C2) in cells transfected with RCAS(B)-CXCL14-shRNA (Figure 4.2B) or GFP and 3C2 in cells transfected with RCAS(B)-GFP control vector (Figure 4.2A and insets). After two or three passages, and once confirmed that at least 95% of DF-1 cells were producing RCAS viruses, supernatant was collected, centrifuged to remove cell debris, and stored at -80°C until needed.

Figure 4.2. Transfection of avian DF-1 cells with RCAS plasmids. Cells were transfected with (A) RCAS-GFP control or (B) RCAS-CXCL14-shRNA. Five days post-transfection virtually all the cells are infected with RCAS viruses, shown by GFP (A) or 3C2 (anti GAG)-positive cells (A and B). Non-transfected virus-free DF-1 cells showed no staining with 3C2 which validates the specificity of the antibody (B, inset).
For in vivo studies, it is desirable to have a high titer of viral particles that allows high infection efficiency. For viral particle concentration, the harvested supernatant was thawed on ice for about 4 to 5 hours. Supernatant was filtered and centrifuged at 21000 rpm at 4°C for 1.5 hours, using the 45Ti rotor. The supernatant was then discarded and 500µl of DMEM was added to resuspend the viral particles pellet. The concentrated viral particles were aliquoted and stored at -80°C until use (Figure 4.3 A and B). Concentrated virus was tested at different dilutions for viral titration and infectivity in DF-1 cells. Three days post infection, about 95% of the cells were 3C2 or/and GFP positive (data not shown).

4.3.3 In ovo Injection of Concentrated Viral Particles

As CXCL14-shRNA fragment is incorporated into functional RCAS provirus, viral particles can be directly injected into chick embryos. This is convenient, considering that electroporation usually causes high rate of mortality. First, fertilized eggs are incubated in horizontal position at 37°C under humidifying conditions. After 24 hour of incubation, 3-4 mL of albumin is removed to facilitate the windowing and the access of the embryo. Once windowed, 3-4 drops of Ringer’s solution containing Penicillin (100 U/mL)/Streptomycin (10ug/ml) antibiotics (Gibco) is added on top of the embryo to prevent contamination. Then, concentrated viral particles can be directly injected into the neural tube of early stage chick embryos (HH6 to HH8+) to target migratory cranial NCCs that will form the corneal endothelium and stroma, among other cranial tissues.
Injected embryos are then sealed with transparent tape, reincubated for additional days, and collected at desired stage for further analysis (Figure 4.3 D).

4.3.4 Validation of RCAS-CXCL14shRNA by in situ Hybridization

The four generated RCAS-CXCL14-shRNA constructs were tested in vivo by CXCL14 in situ hybridization and 3C2 viral protein immunostaining. Embryos stages HH6-HH8+ were injected with RCAS(B)-CXCL14-shRNA constructs or RCAS(B)-GFP.
control and collected at E7, the time point where there is strong expression of CXCL14 in the NCCs-derived corneal stroma keratocytes (Figure 3.1 E, and E’). In situ hybridization revealed that embryos injected with RCAS-GFP control, 213 or 308 constructs do not attenuate the expression of CXCL14 in the stroma, being comparable to CXCL14 expression of non-injected control embryos (Figure 4.4A, B, D and F). On the other hand, from the four shRNA constructs that were tested, CXCL14 was greatly reduced in embryos injected with 170 and 268 constructs (Figure 4.4C and E, red arrows). To confirm the infectivity of RCAS in the cornea, 3C2 immunostaining after in situ hybridization was performed. Injected embryos showed strong 3C2 fluorescence in the cornea and surrounding tissues, demonstrating the high efficiency of the virus to spread and infect avian cells of the ocular region (Figure 4.4G-I, and data not shown). Our results indicate that RCAS viruses can integrate and spread to neighboring dividing cells, and that constructs 170 and 268 are effective in knocking down CXCL14. Since viral titer and number of embryos that showed reduction of CXCL14 expression by in situ hybridization were greater when injected with 170 construct, I proceeded to use this construct to further analysis and referred to it as RCAS-CXCL14-shRNA.
4.3.5 CXCL14 Knockdown Causes Distinct Morphological Eye Defects during Development

Since the role of CXCL14 during ocular development is completely unknown, I first focused on the phenotypic characteristic of embryos that were injected with RCAS-CXCL14-shRNA. Visual examination of CXCL14 Knockdown embryos revealed defective eyelids and relatively smaller eyes compared to RCAS-GFP controls (Figure 4.5A, A’, B and B’). Various CXCL14 knockdown embryos also exhibited defects in beak formation,
especially a delayed development of the lower jaw (Figure 4.5 B and B’). Eye cross-sections of RCAS-CXCL14-shRNA injected embryos stained with H&E revealed smaller lenses with severe defects in lens fibers, denoted by the presence of vacuoles (Figure 4.5C and D). At this stage, we also observed that the iris appeared less elongated and misshapen compared to controls (Figure 4.5C’ and D’). Gross analysis of the cornea indicates there are not evident defects in thickness and morphology (Figure 4.5C and D, and data not shown). Interestingly, CXCL14 loss-of-function embryos exhibited morphological neuroretinal defects. Close examination of the retina at E12 stained with H&E shows distinct boundaries of cell layers in controls. The ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL) of the neuroretina are formed in a normal well-organized multi-layer pattern in RCAS-GFP injected embryos (Figure 4.5 E). Conversely, various CXCL14 loss-of-function embryos showed a chaotic pattern with undistinguishable INL and ONL layers (Figure 4.5 F, asterisk), and an apparent hyperplasia of the GCL layer (Figure 4.5 F, red arrows).

Altogether, these data indicate that the Knockdown of CXCL14 is having an effect on distinct tissues of the developing anterior eye and retina, suggesting that CXCL14 has crucial roles during ocular development.
Figure 4.5. Knockdown of CXCL14 causes morphological defects in chick embryos. (A,B) Gross anatomical features of RCAS-CXCL14-shRNA embryos (B) revealed slightly smaller eyes and defects in eyelid and beak formation compared to controls (A). (C-F) Histological analyses depicting defects in lens (D), iris morphology (D’), and neuroretina development (F) of CXCL14 knockdown embryos compared to controls (C, C’, E). co, cornea; Ey, eyelid; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ir, iris; If, lens fibers; lep, lens epithelium; nr, neuroretina; ONL, outer nuclear layer; OPL; outer plexiform layer; rpe, retinal pigmented epithelium. Scale bars: C and D, 200µm; E and F, 100µm.
4.4 Discussion

CXCL14, one of the most ancient chemokines in vertebrates, is widely expressed during embryonic development (García-Andrés and Torres, 2010). It is one of the few chemokines whose cognate receptor(s) remains unknown; as a result, very little is known about its signals and physiological roles during embryogenesis and/or organogenesis. Particularly in the ocular tissue, CXCL14 was found to be strongly expressed in the stroma of the cornea from E7 to E12 (Figure 3.1E and F). As critical events such as stromal keratocyte differentiation, stroma proliferation, corneal innervation and corneal transparency occur during this period, it is possible that CXCL14 plays an important role during these processes. Thus, the overall goal of this study was to determine the potential role of CXCL14 during ocular development.

To perform functional studies, I first developed the RCAS-CXCL14-shRNA system to knock down CXCL14 in avians. I showed that RCAS-system to deliver shRNA is an efficient tool to knockdown CXCL14 in vitro an in vivo. Certainly, the replication-competent retrovirus mediated loss-of-function method offers distinct advantages compared to electroporation techniques. For instance, because the viral vector integrates into the genome of the host cells, virus particles not only infect neighboring cells but also transmit it to daughter cells making this model and ideal system for loss of function experiments at late stages during embryonic development due to its more stable delivery method (Yang, 2002). Increased survival is also observed when viral particles are injected compared to electroporation techniques where the rate is reduced presumably due to tissue damage caused by the electric field. However, there are also
several considerations that should be taken into account when interpreting the biological phenotypes. First, compared to loss of function electroporated-mediated experiments, RCAS-mediated gene expression is more stable but with a delayed onset. Second, since infected cells can spread the viruses to neighboring cells, distinct cell types are targeted at a different time point of infection that can interfere with the interpretation of biological phenotypes. Thus, embryos infected with RCAS-GFP or RCAS-Scramble-shRNA controls are essential for the analysis of potential phenotypes.

As the main purpose of this study was to determine whether CXCL14 loss-of-function causes any ocular abnormalities during chick development, embryos were initially examined for ocular defects. Gross anatomy analysis revealed that CXCL14 knockdown embryos presented smaller body size compared to RCAS-GFP injected controls. Previous works have reported that CXCL14 knockout mice exhibit lower body weight and reduced birth rate compared to heterozygous and wild type littermates (Meuter et al., 2007; Tanegashima at., 2010). This suggests that CXCL14 may be an important metabolic regulator. We also noticed morphological defects in eyelid and beak formation when CXCL14 is being knocked down. In situ hybridization expression pattern characterized by us and others (García-Andres and Torres, 2010; Gordon et al., 2011; Ojeda et al., 2013) revealed that CXCL14 is strongly expressed in the anterior side of Rathke’s pouch, pharyngeal arches and presumptive eyelid epithelium, that give rise to the pituitary, oral cavity and eyelid, respectively (Figure 3.3D-F, and data not shown). These data suggest that CXCL14 might command processes such cell migration, proliferation and differentiation that are crucial for the proper development of these tissues. The majority of the CXCL14-loss-of-function embryos exhibited lens defects
with severe vacuolization of lens fibers that correlated with the degree of infection. Since mRNA expression of CXCL14 was also detected in the lens epithelium (Figure 3.1 E) it is reasonable to infer that CXCL14 may act as a paracrine signaling necessary for the differentiation and elongation of fiber cells. It was also observed that the developing retina of various CXCL14 loss-of-function embryos presented serious defects in the organization of the retinal layers, frequently accompanied by abnormal misfolding. In situ hybridization analysis shows diffuse expression of CXCL14 at early stage of retinal cell differentiation, but it becomes restricted to the INL and GCL once distinct and definitive cell morphologies are observed (Ojeda et al, 2013). During retinal development, intense cell proliferation, cell migration, and cell differentiation take place from E7 to E14 (Doh et al., 2010). Based on the morphological and histological analysis, it is plausible that CXCL14 may be a critical regulator in one of these processes. Further studies are necessary to determine the role of CXCL14 in retina development.

Interestingly, corneas of CXCL14 knockdown embryos did not exhibit any apparent phenotype compared to controls. The three cellular layers of the cornea were well-formed with no obvious difference in thickness and length. Thus, it is unlikely that CXCL14 is involved in migration, survival and/or proliferation of NNCs derived mesenchymal cells and keratocytes to establish the formation of the three corneal cellular layers (Lwigale et al., 2005).

Taken together, by using the RCAS system to deliver hairpins, we showed that CXCL14 can be knocked down in vivo and that its deficiency causes distinct morphological ocular defects.
Chapter 5: The Chemokine CXCL14 is Required for Proper Neurovascular Patterning during Cornea Development

5.1 Introduction

The cornea is one of the most innervated tissues of the body, yet deprived of blood vessels. However, under certain pathological conditions such as infectious keratitis, corneal graft rejection, dry eye syndrome, diabetes, and limbal stem cell deficiency, corneal avascularity and innervation can be seriously compromised (Müller et al., 2003; Ellenberg et al., 2010). It has been documented that corneal avascularity and innervation are both processes that are established during embryonic development and that are regulated by a fine balance of growth factors and secreted guidance molecules (Schwend et al., 2012; Kwiatkowski et al., 2013). For instance, well-known guidance molecules such as Semaphorins (Sema), Slits, Netrins, and Nephrins that play important functions in innervation, also regulate vasculogenesis or angiogenesis (Tam and Watts, 2010; McKenna et al., 2014). In this regard, we have shown that the cell guidance chemorepellent Sema3A expressed by the lens epithelium is not only a critical modulator of the trigeminal sensory innervation of the cornea but also essential to maintain cornea avascularity at early stages of development (Lwigale and Bronner-Fraser, 2007; McKenna et al., 2014). However, Sema3A chemorepulsive function is blocked during later stages of cornea development, presumably by the establishment of physical barriers such as the endothelial cell layer, corneal basement membranes and ECM components that prevent the flow of Sema3A from the lens to the cornea. Hence, it is reasonable to infer that other secreted molecules by stromal keratocytes might be
involved in maintaining cornea avascularity and proper innervation at later stages of development.

CXCL14 is a small chemotactic cytokine that is expressed in several tissues during embryogenesis. Particularly in ocular development, we have shown that CXCL14 is expressed in the corneal stroma, periorcular region, iris, trigeminal ganglion and retina (Ojeda et al., 2013). Although the receptor by which CXCL14 performs its chemotactic function remains to be discovered (Kiefer and Siekmann, 2011), several studies have shown that CXCL14 is a chemokine with pleiotropic functions. For instance, in the CNS CXCL14 acts as a negative regulator of neurotransmission while in various malignant cancer cells, tumor suppressive functions have been attributed, in part, by its ability to inhibit angiogenesis (Shellenberger et al., 2004; Banisadr et al., 2011). Based on the expression pattern of CXCL14 in ocular development and what is known about its functions, I aimed to investigate whether or not CXCL14 plays a role in the establishment of corneal innervation and avascularity during ocular development.

Here, I reported that knockdown of CXCL14 in the anterior eye resulted in increased sensory innervation in the corneal stroma as well as precocious innervation of corneal epithelium. Moreover, I also showed that knockdown of CXCL14 resulted in exacerbated iridial innervation. In vitro studies suggest that CXCL14 may act as a modulator of corneal innervation by inhibiting CXCL12-mediated axon growth of sensory neurons. Furthermore, knockdown of CXCL14 in Tg(tie1: H2B:eYFP) transgenic Japanese quail resulted in ectopic migration of YFP fluorescently labeled angioblasts into the cornea, and exogenous CXCL14 prevents VEGF- and CXCL12-induced vasculogenesis in this tissue. Altogether, these results indicate that CXCL14 chemokine is a key factor for the
precise patterning of sensory innervation and cornea avascularity, and that it might function through inhibition of CXCL12 signaling.

5.2 Material and Methods

5.2.1 Animals

Fertilized White Leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from Texas A&M (College Station, TX) and Tg(tie1:H2B:eYFP) transgenic quail eggs were obtained from Ozark Egg Company (Stover, MO). Transgenic embryos were screened for green fluorescence of angioblasts and blood vessels before processing. Eggs were incubated at 38°C under humidifying conditions and embryos were collected between E7 and E17. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Rice University.

5.2.2 Immunohistochemistry on whole-mount

Whole-mount immunostaining of tissues followed standard protocol as previously described (Lwigale, 2001). Eyes were fixed in 4%PFA and washed thoroughly in PBS. The cornea and iris were dissected, washed in PBT (PBS, 0.3% Triton X-100) and then blocked in buffer solution for one hour (PBS, 5% goat serum, 0.2% BSA, 0.3% Triton X-100). Samples were incubated two days at 4°C in mouse anti-mammalian neuron-specific-tubulin-TUJ1 (1:500; IgG, Covance) rabbit anti-GFP (1:500; IgG, Invitrogen) or mouse anti-avian myoblastosis virus-3C2 (1:500; IgG1, DSHB) primary antibodies diluted in buffer solution. Slides were washed 3 times in PBT for one hour followed by incubation with anti-rabbit Alexa Fluor 488, anti-mouse IgG1 488, or anti-mouse IgG2a
(1:200, Invitrogen) secondary antibodies. Tissues were rinsed and mounted in PBS for imaging. Some Immunostained tissues were embedded in gelatin, cryosectioned at 12µm and counterstained with 4,6-diamidino-2-phenylindole (DAPI) when needed.

5.2.3 Immunohistochemistry on cross-sections

Anterior eyes were dissected and fixed as described above. After washes in PBS, samples were equilibrated in 5% and 15% sucrose in PBS, overnight at 4°C. Samples were then embedded in gelatin (7.4%) and cryosectioned at 10-12µm. Gelatin was removed from the sections by incubation at 42°C in PBS for 20 min. Sections were blocked for 1 hour in blocking solution (PBS, 5% goat serum, 0.2% BSA, 0.2% Triton X-100) and rabbit anti chicken-CXCL14 (1:100, IgG, GenScript), mouse anti-mammalian neuron-specific-tubulin-TUJ1(1:500, IgG2a, Covance) or rabbit anti-GFP (1:500, IgG, Invitrogen) primary antibodies were added in blocking solution and incubated overnight at 4°C. Signals were detected by using anti-rabbit Alexa Fluor 488, anti-mouse IgG1 488, or anti-mouse IgG2a (1:200, Invitrogen) secondary antibodies. Sections were counterstained using DAPI to label the nuclei.

5.2.4 RNA isolation and semi-quantitative reverse transcription PCR

Reverse transcription (RT)-PCR was used to examine the expression of CXCL14 in vitro. Total RNA was isolated from DF-1 cells by using Trizol (Life technologies) following the manufacturer’s protocol. cDNA was synthesized with reverse transcriptase (SuperScript III RT First-Strand Synthesis Systems, Invitrogen). The PCR primers used for each gene are: *gallus gallus* CXCL14 5'GCTTAGCCAGTGACGAGGA (forward) and
5′CACTGTGTTCCTGGCGTTTGG (reverse); and GAPDH *gallus gallus*
5′GATTCTACACACGGACACTT (forward) and 5′TCTGCCATTTGATGTTGCT (reverse). The PCR product was analyzed by using 2% agarose gel.

5.2.5 **Primary Culture of Dissociated Trigeminal Ganglion neurons for axon outgrowth**

TG sensory neurons from E10-E12 chick embryos were dissected and the ganglia were cut into small pieces with a tungsten needle. Cells were incubated in 0.25% trypsin/EDTA (Gibco) and mechanically dissociated. Following dissociation, cells were resuspended in DMEM supplemented with 10% of FBS (Invitrogen) and penicillin/streptomycin (Gibco) referred as complete media. For neurite outgrowth, 8-well chamber slides (Labtek Nalge Nunc) were coated with 100ug/ml of poly-lysine (Sigma) at room temperature for 1 hour and subsequently with 2ug/mL of laminin (Sigma) at 37°C for 2-3 hours. Cells were seeded at a final density of 5,000 cells/well and incubated in complete medium. To determine the stimulatory or inhibitory effect of cytokines, the medium was replaced with complete medium containing 10ng/mL of NGF (Sigma) and 100ng/mL of human recombinant (hr) CXCL14 (R&D system), 100ng/mL of hrCXCL12 (R&D system), or combination of hrCXCL12 and hrCXCL14 for 24-48 hours.

For neurite growth quantification, digital images were processed in image J (Abramoff et al., 2004) and neurite extent was quantified using Neuron J (Meijering et al., 2004). The total neurite length was determined per neuron. In all cases, I measured between 30-50 neurons per condition and each experiment was performed at least in triplicate.
5.2.6 Generation of RCAS-based vectors

RCAS-GFP-CXCL14shRNA vector

The avian retroviral vector RCASBP(B) was used to knockdown chick CXCL14. From the four CXCL14-specific sequences that were designed (see material and methods chapter 4), I selected the CXCL14-shRNA sequence named as 170, as its efficiency to knockdown CXCL14 was already tested by *in situ* hybridization (Figure 4.4C). RCASBP(B) containing a scramble shRNA sequence (RCAS(B)-Scr-shRNA) (Ferrario et al., 2012) or RCAS(B)-GFP constructs were used as negative controls. For each of shRNA designed sequences, complementary strands comprising a stem-loop sequence and restriction endonuclease sites were synthesized (Sigma) to generate double stranded fragments suitable for cloning. pSLAX-GFP-cU6-(CXCL14-shRNA) and pSLAX-GFP-cU6-(Scr-shRNA) were generated from a pSLAX-GFP-cU6-(Sox9-shRNA) shuttle vector (Deneen et al., 2006), kindly provided by David Anderson (Caltech). In brief, the Sox9-shRNA fragment was replaced by CXCL14-shRNA or Scr-shRNA and the resulting GFP-cU6-(CXCL14-shRNA) or GFP-cU6-(SCR-shRNA) fragments were cloned into the ClaI restriction site of RCASBP(B) using PCR-based cloning kits (*InFusion*, Clontech or *CloneEZ*, Genscript). It basically consists of amplifying the insert by PCR, using primers that have about 15 base sequences homologous to the insertion site at the 5’-end of the linearized vector. The kits contain enzymes with exonuclease activity that creates a 5` overhangs on the PCR and the linearized RCAS vector to allow recombination. New clones resulted in RCASBP (B)-GFP-CXCL14-shRNA and RCASBP(B)-GFP-SCR-shRNA.
RCAS-CXCL14 overexpression vector

RCAS(A)-CXCL14 viral construct was generated by cloning a 296-bp cDNA fragment containing the coding region of gallus gallus CXCL14 (NCBI accession number: NM_204712.2). Briefly, pSLAX-GFP-cU6-(Sox9-shRNA) shuttle vector (Deneen et al., 2006), was linearized with XbaI and HindIII to remove the GFP-cU6-(Sox9-shRNA) fragment and insert the gallus gallus CXCL14 fragment. CXCL14 was then cloned into the ClaI restriction site of RCASBP(A) using PCR-based cloning kits (InFusion, Clontech or CloneEZ, Genscript) that resulted in RCASBP(A)-CXCL14 vector. DNA vector was isolated and sequenced (GeneSriipt) to confirm the presence of CXCL14 fragment.

5.2.7 Virus production

Avian DF-1 cells (ATCC) were transfected with RCAS plasmid using lipofectamine following manufacture’s protocol. 1 ug of plasmid DNA was mixed with lipofectamine reagents diluted in Opti-MEM medium (Life Technologies), and added to 60-70% confluency of DF-1 cells grown in complete media. Virus-containing supernatants were collected 5 to 10 days after transfection where at least 90% of the cells were infected by RCAS virus, as determined by GFP or 3C2 immunostaining. Viral particles were concentrated by ultracentrifugation for 1.5 hours at 21000rpm in a 45Ti rotor (Beckman Coulter), resuspended in DMEM with no supplements, aliquoted and stored at -80°C until use. Viral titer was estimated by adding serial dilutions to non-infected DF-1 cells and tested for GFP fluorescence.
5.2.8 *In ovo* infection

In *ovo* infections were achieved by injecting high-titer virus with small amount of fast green color for visualization, into the neural tube of Hamburger-Hamilton (HH) stage 7-9 embryos to target cranial NCCs (Hamburger and Hamilton, 1951). Eggs were sealed and reincubated at 37°C for additional 6-16 days. Eyeballs were harvested and fixed overnight at 4°C in 4% PFA or modified Carnoy’s for immunostaining and/or *in situ* hybridization respectively.

5.2.9 Quantification of corneal innervation

To determine the extent of corneal sensory nerves at E10, three concentric circles named as ring1, ring2 and ring3 were superimposed over the TUJ1- stained whole mount cornea captured images. Cornea nerves that reached the concentric rings in RCAS-CXCL14 shRNA and control injected embryos were quantified and graphed (Figure 5.4G).

For quantification of corneal epithelial innervation, optical cross-sectioning on corneas stained for nerves in whole mount was generated using the apotome mode on the Zeiss Axioskop 2 microscope (Figure 5.1A and B). 20X magnification was used to observe epithelial innervation at high resolution where terminal nerves that invade the basement membrane and the corneal epithelium can be detected in the X/Z, Y/Z and X/Y displayed images (Figure 5.1C, blue arrowheads).
For stromal nerve density and stromal nerve occupancy, cross-sectioned E12 corneas stained with TUJ-1 images were obtained and processed using image J. Stromal nerve density was calculated by using brightness values which basically counts and measure objects (in this case nerves) in binary or threshold images. For stromal nerve positions, the total area and the area covered by nerves were calculated to be expressed as percentage of stromal area covered by nerves.
5.2.10 Bead Implantation

Cibacron blue 3GA beads (Sigma-Aldrich) were rinsed in sterile PBS solution and soaked overnight at 4°C in 10ul of 5µg/ml hrVEGF, 5µg/ml hrCXCL14, 5µg hrCXCL12, or a combination of hrVEGF and hrCXCL14, or hrCXCL12 and hrCXCL14. Beads were implanted between the presumptive cornea and the lens of E3.5 transgenic quail embryos and reincubated for additional two days. To standardize vasculature quantification, only corneas where beads were localized within 200-400µm from the edge of the iridial ring artery were considered in the analysis. Quantification was performed as described in material and methods section in chapter 2.

5.2.11 Quantification of corneal vascularization

The area of corneal vascularization was quantified in whole mount corneas of E12 Tg(tie1:H2B:eYFP) CXCL14 knockdown and controls embryos using ImageJ. Briefly, delineating lines were drawn around the outside edge of the limbal vasculature and blood vessels that were located in the inner edge of the limbus and cornea. The area covered by angioblasts were divided by the total area (outside edge) to calculate the percent area of corneal vascularization (Figure 5.2 A-D).
5.2.12 Imaging and statistical analyses

Images of whole-mount and stained sections were acquired using a Zeiss Axiocam mounted on an AxioImager2 fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany). Comparisons among data sets were conducted by Student t-test. P<0.05 was considered statistically significant.

Figure 5.2. Quantification of corneal neovascularization. (A,C) A line was drawn to delimit and measure the total area of the cornea in control (A) and CXCL14 knockdown eyes (C). (B,D) An internal line is drawn to measure the non-vascular area in control (B) and CXCL14 knockdown eyes (D). The internal area was subtracted from the outside area to express it as percent of vascularized area.
5.3 Results

5.3.1 CXCL14 Protein Expression and RCAS-GFP-CXCL14-shRNA Validation during Chick Eye Development

We previously showed that \textit{CXCL14} is strongly expressed in the corneal stroma from E7 to E12. As we recently obtained a specific chick CXCL14 antibody (GeneScript), I next aimed to determine the expression of CXCL14 at the protein level by immunostainig. Interestingly, CXCL14 protein was not detected in the stroma by E7, however strong expression was observed in the periocular epithelium that corresponds to the scleral papillae as also observed by \textit{in situ} hybridization (Figure 5.3 A, and data not shown). CXCL14 protein was detected in the stroma from E10 to E15, being the strongest expression at E12 (Figure 5.3B, B inset, and data not shown). By E17 expression of CXCL14 in the stroma was greatly diminished, however strong fluorescence was observed in the retina and iris (Figure 5.3C and data not shown). Although a delay is observed in the expression of CXCL14 protein in the corneal stroma compared to the mRNA expression, a gradient expression pattern from anterior to posterior stroma is in line with our findings by \textit{in situ} hybridization. Therefore, CXCL14 protein is transiently present in the anterior stroma during a critical time where processes such as innervation and corneal transparency occur. These findings prompted us to determine the role of CXCL14 in corneal innervation and establishment of cornea avascularity by performing loss-of-function studies.

I previously generated and validated the RCAS-CXCL14-shRNA construct by \textit{in situ} hybridization and 3C2 staining (see Figure4.4 C and I). However, screening of the
injected embryos was difficult since this method is limited by the uncertainty in predicting the efficiency of the shRNA and site of injection. To facilitate the screening and determine the degree of RCAS-CXCL14-shRNA infection in vivo, I generated RCAS-GFP-CXCL14-shRNA, having GFP under the viral promoter and CXCL14-shRNA driven by the cU6 promoter (Figure 5.3 D). GFP and CXCL14-shRNA were co-expressed in the same cells and tissue, and screening of the embryos was based on the expression of GFP. As shown in Figure 5.3 E, at E7 strong GFP fluorescence is observed not only in the eye but in the entire cranial region. Cross-sections of the cornea at E10 show that the infection by RCAS-CXCL14-shRNA is robust and distributed in the three cellular layers (Figure 5.3 F).

To further validate the new CXCL14 knockdown RCAS retrovirus-mediated construct, I performed in vitro and in vivo studies. In vitro validation was tested in DF-1 cells. DF-1 cells do not express CXCL14 endogenously, thus, CXCL14 was artificially overexpressed in this cell line. Taking the advantage that RCAS(A) and RCAS(B) bind to different receptors, I overexpressed full length CXCL14 in RCAS(A) while CXCL14-shRNA fragment was cloned in RCAS(B). RT-PCR analysis showed that in the DF-1 cells that were infected with RCAS(A)-CXCL14 and RCAS(B)-GFP control, there was a strong expression of CXCL14, demonstrating that the co-infection with RCAS-GFP does not affect the expression of this chemokine. On the other hand, in cells that were co-infected with RCAS(A)-CXCL14 and RCAS(B)-GFP-CXCL14-shRNA, the expression of CXCL14 was greatly decreased (Figure 5.3G). This analysis confirmed that the new shRNA construct can efficiently knockdown CXCL14 in vitro.
Validation of CXCL14-shRNA construct at the protein level was conducted by immunohistochemistry in cornea cross-sections. As shown in figure 5.3H, at E12 expression of CXCL14 in the stroma was remarkably reduced in CXCL14 knockdown embryos compared to control embryos where the gradient pattern in the anterior stroma was clearly present (Figure 5.3I). These results further validate the efficiency of the CXCL14-shRNA virus to spread and knockdown CXCL14 at the mRNA and protein level.

Figure 5.3. Knockdown of CXCL14 using RCAS-GFP-CXCL14-shRNA construct. (A-C) Expression of CXCL14 protein in the cornea. CXCL14 was not detected in the corneal stroma by E7 (A), however strong expression of CXCL14 was observed in the anterior stroma from at E10 (B, inset) and E12 (B). Minimal expression of CXCL14 is detected in the stroma by E17 (C). (D) Plasmid map for RCAS-CXCL14-shRNA vector. (E-F) Screening of embryo (E) and cross-section of E10 cornea (F) showing robust expression of GFP which represents the degree of RCAS infectivity. G) in vitro validation of RCAS-CXCL14-shRNA in DF-1 embryonic avian cells. Note that only cells infected with RCAS-CXCL14-shRNA show remarkably reduction of CXCL14 as seen in RT-PCR. (H,I) Validation of RCAS-CXCL14-shRNA at the protein level was performed by CXCL14 immunostaining on E12 corneas. Only corneas infected with RCAS-CXCL14shRNA revealed reduction in the expression of CXL14 (H) compared to controls (I). Scale bar in C represents 100µm for A,B and C and scale bar in I represents 200µm for H and I.
5.3.2 Corneal Stromal Innervation is Accelerated in CXCL14 Knockdown Embryos

Chemokines are known to have a function in the development of the central and peripheral nervous systems (Li and Ransohoff, 2009; Kasemeier-Kulesa et al., 2010). For instance, it has been reported that one of the most studied chemokines, CXCL12, not only regulates migration of precursor neurons in brain development but also participates as an axon cue in peripheral processes (Li and Ransohoff, 2008; Lerner et al., 2009; Ferrairo et al., 2012).

Corneal innervation in chick begins at about E9.5 when trigeminal afferents branch from the pericorneal nerve ring penetrate and radially innervate the periphery of the stroma (Bee, 1982; Lwigale and Bronner-Fraser, 2007). This is a critical time point where a temporal modulation of key factors that allow corneal innervation takes place. Based on spatial-temporal expression of CXCL14 in the stroma, I hypothesized that CXCL14 regulates corneal innervation during embryonic development. Whole-mount immunostaining of E10 corneas showed that sensory nerves projected further towards the center of the cornea from embryos injected with CXCL14-shRNA (Figure 5.4B) compared with control embryos injected with RCAS-GFP (Figure 5.4A). TUJ-1 immunostaining from cross-sectioned corneas corroborated the accelerated projection of nerve bundles into the center of the cornea in CXCL14 knockdown embryos as opposed to injected controls that showed absolute absence of corneal nerves in the central corneal stroma (Figure 5.4C and D, red arrows). The robust expression of GFP in control and CXCL14 knockdown corneas confirmed the high rate of RCAS infection in this tissue (Figure 5.4E and F). To quantitate corneal innervation, concentric rings template was
drawn and superimposed on each corneal innervation image, and the numbers of nerves reaching each ring were counted (Figure 5.4G) These data show that a significantly large number of nerves reached each concentric ring in CXCL14 knockdown corneas compared to controls (Figure 5.4H), with the highest number towards the corneal periphery (ring 1) and subsequent reduction towards the center (ring 2 and ring 3). These results indicate that knockdown of CXCL14 exacerbates sensory nerve projection into the cornea during development.
Figure 5.4. Precocious innervation of the corneal stroma in CXCL14 knockdown embryos. (A,B) E10 whole mount corneas immunostained with TUJ-1 revealed accelerated projection of sensory axons from the periphery to the center of the cornea in CXCL14 knockdown embryos (B) compared to controls where nerves are located more peripherally (A). (C-F) Cross-sections from whole mount corneas confirmed the location of nerves in the stroma (C,D) and the broad corneal infection by RCAS-GFP control (C) and RCAS-CXCL14-shRNA (F). (G) Schematic showing concentric rings template to quantify innervation. (H) Quantification of nerve extensions from periphery to the center of the cornea. Student’s t-test, **P<0.01, ***P<0.001.
5.3.3 Precocious Innervation of Corneal Epithelium in CXCL14 Knockdown

**Embryos**

Because CXCL14 is strongly expressed in the anterior corneal stroma at E12 (Figure 5.3B), I next asked whether it affects the innervation process at this time point. As expected (Lwigale and Bronner-Fraser, 2007), in RCAS-GFP injected control corneas, nerve bundles innervate the entire corneal stroma from the periphery to the center (Figure 5.5B). A similar pattern of stromal innervation was observed in RCAS-CXCL14-shRNA injected embryos (Figure 5.5F). Since corneal epithelial innervation in chick begins from the corneal periphery at E12 (Bee, 1982), I examined this process by taking z-stack images at the peripheral and central cornea regions. In CXCL14 knockdown corneas, innervation of both the stroma and epithelium was observed throughout the entire cornea (Figure 5.5G and H). Control corneas also showed complete stromal innervation, but only the peripheral epithelium was innervated (Figure 5.5C and D). Consistent with this observation, there was no significant difference between peripheral epithelial innervation between CXCL14 knockdown and control corneas (Figure 5.5I), as opposed to the central epithelial innervation, which showed a significant difference (Figure 5.5J). Therefore, the absence of CXCL14 in the cornea causes rapid innervation of the epithelium.
Figure 5.5. Corneal epithelium is precociously innervated in CXCL14 loss-of-function embryos. (A,B,E and F) Whole-mount staining for GFP and TUJ-1 of E12 corneas showing extent of RCAS infection and nerve projections in control (A,B) and CXCL14 knockdown embryos (E,F). (C,D,G,H) Z-stack higher magnification images were obtained from whole mounts to show focal points that represents corneal epithelial innervation. Note that CXCL14 knockdown eyes show increased epithelial innervation of the corneal center (H, red arrow) but no the periphery (G) compared to controls (C,D). (I,J) Quantification of epithelial innervation in corneal periphery (I) and corneal center (J). White lines in C, D, G and H mark the limit of the z-section of the corneal epithelium and part of the stroma. Student’s test, n.s non-significant; **P< 0.01. en, endothelium; ep, epithelium; st, stroma.
During development, stromal and epithelial nerves occupy the anterior third of the cornea (Riley et al., 2001; Müller et al., 2003; McKenna and Lwigale, 2011). Since CXCL14 expression corresponds with the region of stromal innervation, I examined the distribution and density of stromal nerves in cross-sections through E12 corneas. Analysis of CXCL14 knockdown corneas revealed significant expansion of nerves into the mid- and posterior stroma (Figure 5.6B, bracket; Figure 5.6 C), compared to the anterior occupation in control corneas (Figure 5.6 A and C). Further analysis also revealed significant increase in nerve density in the stroma of CXCL14 knockdown corneas (Figure 5.6D). Taken together, these data indicate that expression of CXCL14 during chick corneal innervation limits and slows down sensory nerve projections into the stroma and their subsequent innervation of their final target, the corneal epithelium.

![Figure 5.6](image_url)

Figure 5.6. Knockdown of CXCL14 results in misdirected projection and increased innervation of the corneal stroma. (A, B) Cross sections of the cornea at E12 showing TUJ-1 immunostaining for nerves. Note the higher density and abnormal distribution of nerves in the stroma of CXCL14 knockdown corneas (B) compared to controls (A). (C,D). Quantification of stromal nerve occupancy and nerve density in controls and CXCL14 Knockdown corneas. Red brackets in A and B indicate thickness of stromal nerve plexus. Student’s t-test, ***P<0.001.
5.3.4 CXCL14 Knockdown Exacerbates Nerve Thickness in the Iris

The iris is a muscular structure localized behind the cornea and adjacent to the ciliary body whose function is to control the size of the pupil. It is comprised of three layers: the anterior layer, the stroma, and posterior epithelial layer. Since the iris muscles contract and expand to regulate the amount of light reaching the retina, the iris is majorly innervated by autonomic nerves, although sensory innervation is also found (Neuhuber and Schödl, 2011). When anterior eye whole mount TUJ-1 immunostaining was performed from E10 to E12 some embryos injected with RCAS-CXCL14-shRNA revealed extensive nerve fascicles coming from choroid fissure to the iris (Figure 5.7D and E) compared to GFP injected controls (Figure 5.7A and B). Cross sections of CXCL14-shRNA injected embryos confirmed an abnormal iris innervation with remarkably larger axons emerging from the ciliary body towards the iris (Figure 5.7F) compared to control (Figure 5.7C). These results further suggest that CXCL14 plays an important role in regulating innervation by limiting the axon projection to its target tissue. Strikingly, only 15% of RCAS-CXCL14-shRNA injected embryos showing robust expression of GFP in the cornea and periocular mesenchyme exhibited this phenotype. This suggests that this defect might be due to a secondary effect of CXCL14 being knocked down in a distinct tissue besides the cornea that regulates innervation of the iris. Further analysis is necessary to determine whether sensory, parasympathetic or sympathetic innervations are affected.
5.3.5 CXCL14 Inhibits CXCL12-mediated TG Sensory Axon Growth In Vitro

Previous studies propose that CXCL14 acts as a natural inhibitor of CXCL12 signaling in various cell types (Tanegashima et al., 2013). For example, opposite functions have been documented in the central nervous system of adult mice, where CXCL12 was found to activate GABAergic neural transmission while CXCL14 treatment represses it (Banisadr et al., 2011). During development, CXCL12 and CXCL14 present a complementary pattern expression in various tissues (García-Andrés and Torres, 2010). In fact, we reported that both chemokines are expressed in a non-overlapping pattern during anterior eye development (Ojeda et al., 2013). Extensive studies on CXCL12 have shown that this chemokine acts as an important cue in the development
and establishment of the sensory nervous system (Knaut et al., 2005). Previous reports have also shown that CXCL12 promotes axon neurite growth in presence of chemorepulsive signals (Chalasani et al., Opatz et al. 2009; Opatz et al., 2009). Thus, I hypothesized that CXCL14 is a negative regulator of axon growth induced by CXCL12.

In vitro analysis of dissociated sensory neurons showed that CXCL12 is not an inducer of neurite growth by itself but significantly potentiates axon growth of embryonic sensory neurons when combined with NGF (Figure 5.8B, E and G). On the other hand, CXCL14 does not have an effect on neurite growth when added to the media alone or in combination with NGF (Figure 5.8C, and data not shown). However, when TG sensory neurons were cultured with equal concentration of CXCL12 and CXCL14, the presence of CXCL14 significantly reduced neurite growth induced by CXCL12 (Figure 5.8F and G). Hence, these results support the hypothesis that CXCL14 regulates sensory innervation of the cornea by inhibiting CXCL12-induced TG sensory axon growth during embryonic development.
Figure 5.8. Culture of Trigeminal Sensory Neurons. Trigeminal neurons show negligible growth when cultured in (A) Neurobasal medium alone, (B) supplemented with 100 ng/ml CXCL12, or (C) supplemented with 100 ng/ml CXCL14. (D,E) Cooperative effect on neurite outgrowth is observed when medium is supplemented with 10 ng/ml NGF+100 ng/ml CXCL12 (E) compared to NGF alone (D). (F) The effect on axon growth of CXCL12 is inhibited by CXCL14. (E) Quantification of total neurite growth under different conditions. Student’s t test, *P<0.005; ***P<0.001.
5.3.6 CXCL14 Knockdown Causes Ectopic Migration of Angioblasts into the Cornea

The fact that CXCL14 has been described as a potent anti-angiogenic factor and that it is expressed in a critical time during the development of cornea avascularity, conducted me to hypothesize that CXCL14 also plays a role in maintenance of corneal avascularization. To test this hypothesis, I used transgenic Tg(tie1:H2B:eYFP) quail embryos. As it has been previously reported that RCAS(B) does not infect quails (Holmen et al., 2001), I generated RCAS(A)-CXCL14-shRNA. Tg embryos were injected with RCAS(A)-CXCL14-shRNA or RCAS(A)-Scr-shRNA virus at stages HH-5 to HH-9 and analyzed for corneal vascularization. GFP whole mount immunostaining at E12 revealed that corneas from CXCL14 knockdown embryos exhibited GFP+ angioblasts and blood vessels growing from limbal vasculature to the cornea (Figure 5.9B). Conversely, injected control embryos showed that GFP+ angioblasts and blood vessels were located in the limbus but the cornea remained avascular (Figure 5.9A).

Figure 5.9. Tg(tie1:H2B:eYFP) quail eyes showing vascularization of the anterior eye. (A and C) Control eye showing limbal vasculature (lv) and corneal avascularity. (B and D) CXCL14-Knockdown cornea showing ectopic vasculature in the cornea (arrows). (E) Quantification of the extent of corneal neovascularization. Dotted lines delineate the corneal periphery from the adjacent limbus. Student’s test, **P<0.01. Abbreviations: co, cornea; lv, limbal vasculature.
Cross sections of control and CXCL14-shRNA injected corneas showed that endothelial cells that migrated from the limbal vasculature are located in the anterior corneal stroma of CXCL14 loss-of-function embryos, but in controls they resided in the periocular mesenchyme (Figure 5.9C and D). Quantification of cornea neovascularization from whole-mount, showed that in CXCL14 knockdown eyes, about 40% of the cornea was invaded by ectopic migration of endothelial cells from the limbal vasculature. In contrast, very few endothelial cells were localized at the corneal margin of non-injected or SCR-shRNA injected embryos (Figure 5.9E). Hence, knockdown of CXCL14 permits corneal neovascularization which indicates CXCL14 is required for maintaining corneal avascularity during development.

5.3.7 CXCL14 Prevents VEGF- and CXCL12-Induced Angiogenesis

Based on the effect of on proangiogenic factors, I hypothesized that CXCL14 counteracts the pro-angiogenic effect of VEGF and CXCL12 to prevent endothelial cell migration into the cornea during development. To test this hypothesis, I first implanted beads soaked in VEGF, CXCL14 or a combination of VEGF and CXCL14 between the lens and overlaying ectoderm of E3.5 transgenic quail embryos (tie1:H2B:eYFP). As previously reported (McKenna et al., 2014), VEGF-soaked beads induced massive ectopic migration of angioblasts into the cornea (Figure 5.10A). In contrast, eyes that were implanted with beads soaked in a combination of VEGF and CXCL14, angioblasts migration into the cornea was significantly reduced (Figure 5.10 B and E). No ectopic angioblasts were observed in eyes implanted with beads soaked in Ringer’s solution or CXCL14 (Figure 5.1 D and data not shown). We next sought to determine whether the
pro-angiogenic chemokine CXCL12 is also able to induce angiogenesis in the developing corneas and whether CXCL14 acts as a negative regulator of CXCL12-induced vascularization. Thus, we implanted beads soaked in CXCL12 or a combination of CXCL12 and CXCL14. Although angioblasts migration was induced by CXCL12, it was to lesser extent compared to VEGF, so its effect was limited by the region where the beads were placed. In other words, only CXCL12-soaked beads located near the temporal ciliary artery (TCA) induced ectopic migration of angioblast into the presumptive cornea (Figure 5.11A), while in those where beads were located at the center of the cornea or toward the nasal ciliary artery, ectopic migration was not observed (data not shown). Contrarily, none of the eyes implanted in similar locations with CXCL12+CXCL14 soaked beads showed angioblast migration into the cornea (Figure 5.11B).

Figure 5.10. CXCL14 inhibits VEGF-induced angiogenesis in the developing cornea. (A,B) rVEGF-coated bead were implanted alone (A) or in combination with rCXCL14 (B) in the cornea. Two days following implantation, rCXCL14 inhibited the angiogenic response of rVEGF (B). Non-implanted or rCXCL14-coated bead implantation alone did not show any defects (C and D). (E) Quantification of the vascularized area in implanted with either rVEGF alone or rVEGF+rCXCL14 coated beads. Student’s test, ***P<0.0001. co, cornea; tca, temporal ciliary artery.
Thus, these results show angioblast invasion induced by CXCL12 is prevented by CXCL14.

Altogether, these data demonstrate that CXCL14 is necessary to maintain cornea avascularity during development and that its anti-angiogenic effect to prevent corneal neovascularization may be through inhibition of VEGF and CXCL12 signaling.

5.4 Discussion

Formation of the vascular and peripheral nervous systems are both key events during embryonic development characterized by being highly branched structures. Indeed, they both have remarkable similarities at the anatomical, cellular and molecular level since growth cones of nerves and endothelial cells are regulated, in part, by common environmental cues or diffusible factors (Tam and Watts, 2010). Interestingly, the cornea is one of the most innervated tissues of the body yet completely deprived of blood vessels. Although the cornea has been the subject of many studies for years, to date the molecular mechanisms of how guidance molecules that regulate sensory

Figure 5.11. Inhibitory effect of CXCL14 on CXCL12-induced angiogenesis in the developing cornea. (A) Non-implanted control eyes display a completely avascular cornea and the formation of a vascular plexus in the tca. (B,C) rCXCL12-coated bead were implanted alone (A) or in combination with rCXCL14 (B) in the cornea. Two days following implantation near the tca plexus, rCXCL14 inhibited the angiogenic effect of rCXCL12 (C). co, cornea; tca, temporal ciliary artery.
innervation and/or promote cornea avascularity interact during development are not well-understood. Here, for the first time, I provided substantial evidence of the role of CXCL14 chemokine in regulating the neurovascular network that results in proper corneal innervation and avascularity during ocular development.

**CXCL14 as a key regulator of cornea and iris innervation**

The cornea is one of the most densely innervated tissues, yet little is known about the molecular mechanisms that are involved in this process. During development, NCCs-derived TG sensory nerve fascicles innervate the cornea in a series of spatiotemporal events. Initially, TG nerves approach the periphery of the cornea to form a pericorneal ring that surrounds the cornea but does not innervate it (Figure 5.12A and C). Subsequently, sensory nerves branches begin to enter the cornea to populate the anterior third of the stroma that becomes fully innervated by E12 (Figure 5.12D and F) (Schwend et al., 2012). At this time, nerves from the anterior stroma also project through the Bowman’s basement membrane to innervate the corneal epithelium (Riley et al., 2001). These innervation events raise the question of what converts the cornea from being non-permissive to permissive for axon growth. Previous studies have shown that sema3A is a critical regulator of the early phase of corneal innervation. In this regard, Lwigale and Bronner-Fraser (2007) showed that lens-derived sema3A regulates the formation of the pericorneal ring since its disruption caused aberrant and precocious innervation of the cornea at this phase. However, at later stages lens-derived sema3A is presumably blocked by the formation of a functional corneal endothelial layer and the
separation of the lens and cornea by the anterior chamber. This raises the possibility that other factors might account for the maintenance of proper corneal innervation.

Given that the spatiotemporal expression of CXCL14 corresponds with the period of corneal stromal and epithelial innervation, I performed loss-of-function experiments to determine its putative role in these processes. Examination of CXCL14 loss-of-function embryos for corneal innervation defects revealed exacerbated and misdirected axon projections in the cornea and iris. Although CXCL14 is one of the few chemokines whose receptor has not yet been identified, current studies have shown that CXCL14 acts as a natural inhibitor of CXCL12/CXCR4 signaling (Tanegashima et al., 2013). Numerous studies have reported that CXCL12 is essential in the development of the central and peripheral nervous system (Chen et al., 2014; Kasemeier-Kulesa et al., 2010). In particular, recent studies on guidance cue revealed that CXCL12 stimulates neurite outgrowth even in the presence of neurorepellent molecules such as sema3A and slit (Chalasani et al., 2003; Chalasani et al., 2007).

When I tested whether CXCL14 inhibits the effect of CXCL12-induced neurite growth in vitro, I found that CXCL14 had a negative effect on neurite outgrowth stimulated by CXCL12. However, CXCL14 inhibitory effect was not as drastic as expected compared to the in vivo observations when CXCL14 was knocked down in the cornea. Since TG sensory neurons are derived from two different populations (NCCs-derived and placode-derived neurons) (D'Amico-Martel and Noden, 1983), it is plausible that CXCL14 exerts its effect in a particular subtype of neurons. Furthermore, the placode and neural crest derived nociceptive neurons (neurons that sense noxious stimuli) that innervate the cornea and periocular tissues can be further sub-classified into peptidergic and non-
petidergic neurons depending on whether they produce neuropeptides such as calcitonin and substance P (Woolf and Ma, 2007). In this context, neurons that secrete different neuropeptides may be differentially affected by CXCL14. On the other hand, neurotrophins and axon guidance cues such as GDNF, VEGF and Sema3A, implicated in corneal innervation and nerve regeneration, are known to interact directly or indirectly with CXCL12 (Arthur et al., 2009; Ferrario et al., 2012; Pan et al., 2013). Thus, it is possible that CXCL14 also interacts with any of these growth factors which might account for its negative effects on neurite outgrowth observed in vivo.

Therefore, utilizing both in vivo and in vitro analysis I have demonstrated that CXCL14 is an important regulator of sensory innervation, whose function is to limit neurite growth in target tissues. Further experiments are required to determine whether CXCL14 acts as direct or indirect modulator of CXCL12 pathways in neurite outgrowth.
Requirement of CXCL14 to maintain avascularity of the cornea during development

Despite the vascular network that forms in the periorcular mesenchyme, angioblasts and blood vessels never enter into the developing cornea (Figure 5.12A, B, D and E) (Kwiatkowski et al., 2013). Substantial evidence suggests that ocular tissues provide chemical barriers by secreting factors that impede vascular invasion in the cornea but promote its innervation in a precise pattern and time during development (Kubilus and Linsenmayer, 2010; Schwend et al., 2012; McKenna et al., 2014). Establishment of corneal avascularity in embryogenesis is, indeed, an intricate process that depends on the interaction of angioblasts, NCCs-derived mesenchymal cells, and pro and anti-angiogenic factors. Our previous study showed that sema3A plays an important role in the establishment of cornea avascularity (McKenna et al., 2014). However, at later
stages barriers coming from the cornea limit the effect of Sema3A secreted by the lens. Since the cornea is an avascular tissue throughout all developmental stages, it raised the question of which other factors might account to maintain the cornea as an avascular tissue. In this work, I showed that CXCL14 is necessary and sufficient to prevent corneal neovascularization since its depletion caused ectopic migration of angioblasts from the limbal plexus to the stroma of the cornea, and addition of exogenous CXCL14 prevented VEGF-induced angioblast migration into the cornea.

CXC chemokines containing the ELR motif are known to display angiogenic activity, as opposed to chemokines that lack this motif which are potent anti-angiogenic factors (see table 2) (Kiefer and Siekmann, 2011). The expression pattern in the cornea as well as its known anti-angiogenic effect led me to hypothesize that CXCL14 regulates cornea avascularity. Certainly, by knocking down CXCL14, I found that it is required for maintaining corneal avascularity. Although the mode of action by which CXCL14 performs its anti-angiogenic function is not well-understood, it has been shown that CXCL14 inhibits the interaction of pro-angiogenic factors such as FGF, IL8 and VEGF to their respective receptors (Shellenberger et al., 2004; Rivera et al., 2015).

VEGF and CXCL12 are both proangiogenic factors whose mRNA transcripts are expressed in corneal epithelium and periocular mesenchyme, respectively (Van Setten, 1997; Ojeda et al., 2013). Under pathological conditions that result in cornea neovascularization, VEGF and CXCL12 are upregulated to promote angiogenesis (Philipp et al., 2000; Lima et al., 2007). Hence, I next sought to determine the effect of CXCL14 on preventing corneal neovascularization induced by VEGF and/or CXCL12 signaling during cornea development. By using bead implantation experiments, I found...
that CXCL14 abrogates ectopic corneal neovascularization driven by both VEGF and CXCL12, demonstrating thus the ability of CXCL14 to inhibit angiogenesis. The effect of CXCL14 on VEGF-induced angiogenesis is not surprising given that a previous work has shown that the efficacy of VEGF inhibitors commonly used as anti-angiogenic therapy in cancer depend on the induction of the angiostatic CXCL14 chemokine (Rivera et al., 2015). However, to my knowledge, this is the first time that the inhibitory effect of CXCL14 on CXCL12-induced angiogenesis has been shown. This is important, if we consider that endothelial progenitor and mature cells express the CXCL12 receptor, CXCR4, and that its expression is upregulated by pro-angiogenic factors such as VEGF and FGF (Salcedo et al., 1999).

Based on my results I propose a model (Figure 5.13) that during ocular development CXCL14 regulates neurovascular patterning by restricting CXCL12 signaling. CXCL12 expression in the iris and periocular mesenchyme is involved in sending signals to attract sensory nerves and angioblasts while CXCL14 expression in the cornea limits the amount of CXCL12 in these tissues. Therefore, if CXCL14 expression is reduced, axons and angioblasts respond to CXCL12 signal (that positively interacts with VEGF signaling) of the periocular region. This would explain why we observed rapid and increased innervation of the cornea as well as neovascularization when CXCL14 is knocked down.
Although these studies provide mechanistic evidence for regulation of neurovascular patterning by CXCL14, whether CXCL14 inhibits CXCL12 signaling by direct binding to CXCR4 or CXCR7 receptors or by the formation of heterodimers with CXCL12 is completely unknown. Further studies are necessary to elucidate the complete pathways involved in this process.

In conclusion, the findings presented in these studies identify CXCL14 as a novel and critical factor during the formation of the neurovascular network of the anterior eye. The fact that CXCL14 is essential for maintaining proper innervation and avascularization, not only contributes to a better understanding of neurovascular defects in the anterior eye during development, but also makes this chemokine suitable as a
potential therapeutic target of pathological neovascularization and aberrant innervation of ocular tissues in adults.
Chapter 6: Summary, Significance and Future Directions

6.1 Summary and Significance

Neurovascular patterning is a complex process that does not occur randomly. It is precisely controlled by environmental factors that act as guidance cues to form a specific neurovascular pattern. In peripheral tissues nerves often run along blood vessel reflecting their intimate association and interaction. Interestingly, the cornea is one of the tissues with the highest density of sensory innervation but avascular in its uninjured state. Both, the establishment of cornea avascularity and innervation occur during eye development. While blood vessels form a vascular plexus in the periocular region but exclude the developing cornea, the sensory nerves that initially form a nerve ring abundantly innervate the corneal stroma and epithelium. This suggests that there is a barrier during embryogenesis that regulates the interaction of blood vessels and nerves in this tissue.

This dissertation demonstrates the crucial roles of growth factors and cytokines in the establishment of the proper neurovascular patterning during cornea development. I showed that Sema3A, a secreted molecule well-known for its role as a guidance cue in the development of corneal innervation (Lwigale and Bronner-Fraser, 2007), has also an essential role for the establishment of corneal avascularity during avian and mouse embryonic development. We used various strategies to provide evidence about the role of Sema3A in preventing angioblast migration by inhibiting VEGF signaling. in situ hybridization and immunostaining revealed that Sema3A and VEGF are expressed by the lens, while Nrp1 receptor is expressed by angioblasts in the anterior eye during
corneal development. Blockade of Sema3A signaling via lens removal or injection of synthetic sema3A signaling causes ectopic migration of angioblasts into the cornea which results in corneal vascularization. Additionally, we demonstrated that exogenous sema3A protein inhibits VEGF-induced angiogenesis in the cornea. Furthermore, and in line with these results, loss of Sema/Nrp1 signaling in Nrp1^sema^-mutant mice resulted in cornea vascularization during embryogenesis. The contribution of Sema3A signaling in the establishment of cornea avascularity during development is a novel finding that provides additional insights into the mechanisms that are involved in cornea avascularity as well as new strategies for treatment of pathologies associated with neovascularization. However, based on the transient expression of Sema3A as well as the absence of vascular phenotype in the cornea of postnatal Nrp1^sema^-mice, it appears that lens-derived Sema3A is critical for a proper neurovascular patterning exclusively at early stages of cornea development. These results suggest that other molecules are involved in maintaining the proper neurovascular network at later stages.

CXCL14 is a chemotactic chemokine that has been described as one of the most ancient chemokine along with CXCL12 (Huising et al., 2004). It belongs to the subfamily of CXC chemokines that lack the amino terminal motif Glu-Leu-Arg (ELR) and are angiostatic (Strieter et al., 1995). Although the receptor through which CXCL14 acts remains unknown, current studies have shown that CXCL14 antagonizes CXCL12 signaling (Tanegashima et al., 2013). CXCL14 and CXCL12 are both widely expressed in the central and peripheral nervous system during development. However, in contrast to the numerous studies of CXCL12 function, very little is known about CXCL14 (Hara and Tanegashima, 2012). Microarray analysis conducted by our lab showed that
**CXCL14** was upregulated during corneal stroma formation. Considering that the cornea is avascular but highly innervated by sensory neurons, I hypothesized that CXCL14 plays an important role in the regulation of the neurovascular network during cornea development by restricting CXCL12 signaling. To support this hypothesis, I first determined the expression patterns of **CXCL14** and **CXCL12** during corneal development by RNA *in situ* hybridization. These experiments revealed that **CXCL14** was expressed by corneal stromal keratocytes from E7 to E12, while expression of **CXCL12** was mainly detected in the periocular mesenchyme and blood vessels. The pattern expression of both chemokines suggested that CXCL14 might prevent vascularization of the cornea as opposed to CXCL12 that might stimulate angiogenesis that is required for the formation of the vascular plexus in the periocular region.

My next goal was to identify the role of CXCL14 in the development of the neurovascular network in the cornea. To determine this function, RCAS shRNA-based strategy was designed to knockdown CXCL14 in the eye of chick embryos. RCAS system was utilized to insert CXCL14-shRNA fragment because it is a replication competent virus that infect exclusively avian cells. Knockdown of CXCL14 resulted in distinct phenotypes. First, eyes expressing RCAS-CXCL14-shRNA showed increased and precocious innervation of the cornea and iris, suggesting that CXCL14 acts as a negative regulator of sensory axon growth. Given that it is well-documented that sensory neurons respond to CXCL12 signaling to promote axon growth, I determined the effect of CXCL14 on CXCL12-induced neurite outgrowth. *In vitro* assays demonstrated that CXCL14 acts as an inhibitor of sensory axon growth that is induced by CXCL12. Hence, CXCL14 regulates sensory innervation by limiting the response of sensory axons to
CXCL12 signaling. Second, knockdown of CXCL14 caused corneal neovascularization originated from the limbal vasculature. Since the angiogenic effect of VEGF is amplified by CXCL12 signaling, I tested the effect of CXCL14 on VEGF- and CXCL12-induced angiogenesis in the cornea by using bead implantation experiments. Exogenous CXCL14 inhibited both, VEGF- and CXCL12-induced angioblast migration into the cornea. These results suggest that CXCL14 maintains corneal avascularity during development by inhibiting the function of pro-angiogenic factors.

Given that very little is known about CXCL14 during embryonic development, this study will significantly contribute to a better understanding of the role of this chemokine and its interaction with CXCL12 signaling in key developmental events as well as physiological and pathological conditions that involve angiogenesis and sensory innervation.

In conclusion, this work demonstrates that Sema3A signaling that is required for proper corneal innervation is also required for the establishment corneal avascularity at early stages of corneal development. At later stages, the function of maintaining the neurovascular pattern is provided by CXCL14 signaling. This is the first time that CXCL14 has been identified as a key regulation of innervation and angiogenesis during embryonic development which might lead to new avenues in finding effective treatments for cornea neovascularization and innervation.
6.2 Future Directions

In this dissertation I have determined the function of growth factors and cytokines in the establishment of the neurovascular pattern of the cornea during development. Particularly, this is the first time that CXCL14 has been identified to have a critical function in regulating innervation and cornea avascularity during development. Although I found that CXCL14 inhibits CXCL12 signaling in these processes, it is currently unknown the molecular mechanisms by which CXCL14 operates. It would be of interest to determine whether CXCL14 binds to CXCL12 receptors, CXCR4 and CXCR7 that are expressed by TG-derived sensory neurons and angioblasts during development, or whether CXCL14 binds directly CXCL12 to inhibit its function. It would be also interesting to determine whether CXCL14 interacts with other growth factors such as Semaphorin, Robo and VEGF that are expressed in the cornea and are also involved in neurovascular patterning. Elucidating molecular mechanisms of how CXCL14 interacts with other factors in the cornea would vastly expand the current understanding of corneal innervation and avascularity.

Detailed analysis of CXCL14 expression in mouse embryos revealed that although CXCL14 expression is not conserved compared to chick embryos, vivid expression of CXCL14 was detected in the corneal epithelium. Studies on CXCL14 knockout mice have shown that they are resistant to high fat diet and present lower body weight that is linked to a reduction of food intake (Tanegashima et al., 2010). However, CXCL14 knockout mice have not been examined for developmental or ocular defects. In this regards, it would be of interest to investigate whether CXCL14-null mice exhibit corneal neovascularization and/or aberrant innervation as seen in avian models.
Recent work conducted by our lab has shown that wounded embryonic corneas exhibit complete regeneration and innervation with no detectable scar formation (Spurlin and Lwigale, 2013). During regeneration, corneal nerves and inflammatory cells are determinant factors for wound repair (Wilson et al., 2001). Given that CXCL14 has been found to be a potent chemoattractant for monocytes and immature dendritic cells, and that I have identified CXCL14 as a critical regulator of corneal innervation, it would be interesting to investigate whether CXCL14 plays an essential role during wound healing. Preliminary results show that CXCL14 protein is differentially expressed during corneal regeneration (Figure 6.1). Cross-sections of regenerating corneas show that CXCL14 protein is localized in the wound, adjacent to the regenerating epithelium (Figure 6.1A). TUJ-1 immunostaining revealed that nerves avoid the stroma to project directly into the corneal epithelium (Figure 6.1B). Thus, it is plausible that CXCL14 might direct epithelial innervation during corneal regeneration.

These studies will not only expand our understanding of the development of corneal innervation and avascularity, but also will provide new insights on the role of CXCL14 in tissue regeneration and its potential as a therapeutic agent.
Figure 7.1. CXCL14 protein is expressed in the wound of regenerating embryonic cornea (A) Strong CXCL14 protein expression was located adjacent to the regenerating epithelium (yellow arrow). (B) Cross-section of 5dpw cornea identifies nerves migrating through the stroma to project into the corneal epithelium, avoiding the regenerating stroma (B, arrowheads). Abbreviations: dpw: days post-wound; en: endothelium; ep: epithelium; st: stroma. Figure B provided by Dr. James Spurlin.
Appendix

Expression of CXCR4 and CXCR7 during chick eye development

As described earlier, CXCL12 is a member of the CXC subfamily of chemokines that has been implicated in many physiological and pathological processes (Li and Ransohoff, 2008; Cheng et al., 2014). It performs its function by binding to their cognate receptors CXCR4 and CXCR7 (Bleul et al., 1996; Balabanian et al., 2005). CXCR4 receptor, which is highly conserved across species and whose only ligand is CXCL12, has been found to be expressed by a variety of cells that include immune cells, progenitor neurons and endothelial cells (Zhou et al., 1998; Li and Ransohoff, 2009; Bussmann et al., 2011). Mice deficient in CXCR4 exhibit defects in blood vessel formation of the gastrointestinal tract which suggests a crucial role of CXCL12/CXCR4 signaling in vascular development (Tachibana et al., 1998) CXCR7 is also widely expressed and has crucial roles during embryonic development (Sierro et al, 2007). In this regard, it has been reported that knockout of CXCR7 in mice results in severe cardiac and vascular defects (Li and Ransohoff, 2009). Although both receptors are expressed in a variety of tissues during organogenesis, their expression pattern had not been addressed during ocular development.

To determine the spatiotemporal expression of both receptors in the anterior eye I performed in situ hybridization. During chick eye development, CXCR4 was found to be strongly expressed by angioblasts throughout the embryo including the newly blood vessels that are forming in the pericocular region (Figure 7.1 A-C). On the other hand, CXCR7 was strongly expressed in the pericocular mesenchyme, optic cup, presumptive iris, corneal endothelium and lens epithelium (Figure 7.1 D-E).
Expression of *CXCR4* during eye development suggests that this gene may be required for the development of the vasculature in the periocular mesenchyme, while *CXCR7* might be involved in the migration of NCCs-derived periocular mesenchyme to form the cornea endothelium and stroma. Expression of *CXCR7* in the cornea endothelium from E7 and subsequent stages suggests that this gene might also mediate processes such as, proliferation, differentiation or survival of endothelial cells.

Figure 7.1. Expression of *CXCR4* and *CXCR7* receptors during anterior eye development. (A-C) Section through E3-E7 eyes showing *CXCR4* expression by angioblasts (A, arrow) and periocular blood vessels (B, B’, C, C’ arrow). (D-E) Expression of *CXCR7* from E3 to E7. At E3, strong expression of *CXCR7* is detected in the periocular migratory mesenchyme cells (D, red arrow) and optic cup (D, arrowhead). At E5, *CXCR7* expression is maintained in the periocular mesenchyme (E’ red arrow) and expression in the presumptive iris is apparent (E, E’, asterisk). Subsequently (at E7) *CXCR7* expression is still present in the presumptive iris and periocular mesenchyme adjacent to RPE (F, asterisk and red arrow), but vivid expression is also detected in the lens epithelium and corneal endothelium (F). en, endothelium; ep, epithelium, ir, presumptive iris; L, lens; lep, lens epithelium; oc, optic cup; rpe, retinal pigmented epithelium. Scale bar: 100µm.
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