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Technical Note

PlexinD1 is required for proper patterning of the periocular vascular network and for the establishment of corneal avascularity during avian ocular development

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ABSTRACT

The anterior eye is comprised of an avascular cornea surrounded by a dense periocular vascular network and therefore serves as an excellent model for angiogenesis. Although signaling through PlexinD1 underlies various vascular patterning events during embryonic development, its role during the formation of the periocular vascular network is yet to be determined. Our recent study showed that *PlexinD1* mRNA is expressed by periocular angioblasts and blood vessels during ocular vasculogenesis in patterns that suggest its involvement with Sema3 ligands that are concurrently expressed in the anterior eye. In this study, we used *in vivo* knockdown experiments to determine the role of PlexinD1 during vascular patterning in the anterior eye of the developing avian embryos. Knockdown of PlexinD1 in the anterior eye caused mispatterning of the vascular network in the presumptive iris, which was accompanied by lose of vascular integrity and profuse hemorrhaging in the anterior chamber. We also observed ectopic vascularization of the cornea in PlexinD1 knockdown eyes, which coincided with the formation of the limbal vasculature in controls. Finally we show that Sema3E and Sema3C transcripts are expressed in ocular tissue that is devoid of vasculature. These results indicate that PlexinD1 plays a critical role during vascular patterning in the iris and limbus, and is essential for the establishment of corneal avascularity during development. We conclude that PlexinD1 is involved in vascular response to antiangiogenic Sema3 signaling that guides the formation of the iris and limbal blood vessels by inhibiting VEGF signaling.

1. Introduction

Corneal avascularity is required to maintain transparency and vision clarity. Avascularity is maintained throughout adult life, but it is first established concomitantly with both the formative morphological events of the embryonic cornea and the vascularization of the periocular region. This process occurs when neural crest cells migrate between the lens and overlying ectoderm to form the corneal endothelium and stroma (Creuzet et al., 2005; Hay, 1980; Lwigale et al., 2005), while migratory angioblasts undergo vasculogenesis in the periocular region. The angioblasts and nascent blood vessels do not enter the cornea, but instead form a dense vascular network in the periocular region (Hiruma, 1996; Kwiatkowski et al., 2013), which distributes oxygen and nutrients to the tissues in the anterior eye including the cornea.

The mechanism by which angioblasts and ocular blood vessels form the dense vascular network in the anterior eye as well as their exclusion from the adjacent embryonic cornea remains unclear. A debated hypothesis speculates that the limbus epithelium functions as a physical barrier against vascularization of the cornea (Ellenberg et al., 2010; Lim et al., 2009; Ma et al., 2004). However, substantial evidence also suggests that the cornea provides a chemical barrier by secreting antiangiogenic factors that restrict vascular invasion, also known as neovascularization. Much of the evidence supporting this hypothesis originates from studying the role of antiangiogenic factors in adult corneas. For example, the mammalian adult corneal epithelium expresses the proangiogenic vascular endothelial growth factor (VEGF), but neovascularization is prevented by the presence of soluble VEGF receptor-1 (sVEGFR-1, also known as sFlt1). Knockdown of sFlt1 increases the bioavailability of VEGF and results in corneal neovascularization (Ambati et al., 2006, 2007). Other endogenously expressed antiangiogenic factors that promote corneal avascularity include thrombospondin, pigment epithelium-derived factor (PEDF), Slit2, and Netrin1 (Aiello et al., 1995; Ellenberg et al., 2010; Han and Zhang, 2010; Jin et al., 2010).

The role of antiangiogenic factors during corneal development is supported by our recent study showing that lens-derived

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Semaphorin3A (Sema3A) inhibits VEGF-induced angioblast migration and vascularization of the cornea by signaling through a common Neuropilin1 (Nrp1) receptor (McKenna et al., 2014). Loss of Sema3A (in chick) or Nrp1/Sema signaling (in mouse) resulted in spontaneous vascularization in the embryonic cornea. Besides Sema3A, other antiangiogenic factors are expressed in the anterior eye including *Sema3E*, *Sema3F* (Chilton and Guthrie, 2003), *sFlt1*, *Netrin1*, *Netrin4* (Kwiatkowski et al., 2013), and *CXCL14* (Ojeda et al., 2013), however their putative function during the formation of the periocular vascular network and in establishing corneal avascularity alongside Sema3A have not been demonstrated. Pertinent to this study is the vivid expression of *Sema3E* in the optic cup and iridocorneal angle, and the concomitant expression of *PlexinD1* by angioblasts and the forming periocular vascular network (Kwiatkowski et al., 2013). Interestingly, *PlexinD1* expression coincides with *Nrp1* during the formation of the periocular vasculature, but it is not clear whether PlexinD1 functions independently or in conjunction with Nrp1 to regulate vascular patterning during ocular development.

PlexinD1 is a receptor for secreted Sema3 ligands. During embryonic development, it is expressed by endothelial cells, retinal blood vessels, and cardiac tissue (Fukushima et al., 2011; Gay et al., 2011; Oh and Gu, 2013a; Toyofuku et al., 2008). Endothelial PlexinD1 signaling inhibits angiogenesis by preventing endothelial migration, upregulating endothelial expression of sVEGFR-1, and antagonizing VEGF-induced relaxation of lateral inhibition (Kim et al., 2011, Torres-Vazquez et al., 2004; Zygmunt et al., 2011). This role has been exemplified in several contexts, including in the developing retina and in somites where knockout of PlexinD1 disrupts vascular patterning (Fukushima et al., 2011; Gay et al., 2011; Gu et al., 2005; Kim et al., 2011; Meadows et al., 2013; Oh and Gu, 2013b; Torres-Vázquez et al., 2004). In this study, we aim to identify a new role of PlexinD1 during the formation of the periocular vascular network and maintenance of corneal avascularity during ocular development. We utilized RCAS-mediated overexpression of PlexinD1-shRNA in chick and Tg(*tie1*:H2B:eYFP) quail embryos (Sato et al., 2010) to knockdown PlexinD1 in the anterior eye. Our results show that in the periocular region PlexinD1 is requisite for proper function of endothelial cells that is required for proper patterning and formation of the iridial and limbal vasculature, and for the maintenance of corneal avascularity during development. Thus, PlexinD1 plays a critical role in the vascular guidance cues that are essential for the formation and patterning of the intricate vasculature of the anterior eye.

2. Methods

2.1. Embryos

Fertilized White Leghorn chicken eggs were obtained from the Texas A&M University Poultry Center (College Station, TX). Fertilized Tg(*tie1*:H2B:eYFP) transgenic quail eggs were obtained from Ozark Egg Company (Stover, MO). All experiments involving embryos were approved by the Institutional Animal Care and Use Committee (IACUC) at Rice University.

2.2. Immunohistochemistry

Tg(*tie1*:H2B:eYFP) quail and chick eyes were collected at embryonic day (E)12, fixed overnight at 4 °C in 4% PFA, and washed in PBS containing 0.1% Triton. Some eyes were embedded in gelatin and cryosectioned at 12 μm. Whole-mount and sectioned eyes were immunostained using standard protocols. Primary antibodies used were: mouse anti-GFP monoclonal antibody (1:2000, IgG1, Covance), rabbit anti-GFP polyclonal primary antibody (1:500, IgG, Invitrogen), rabbit anti-mCherry polyclonal antibody (1:500, IgG, Abcam), and mouse anti-Claudin-5 (1:250, IgG1, Fisher). The secondary antibodies (Alexa 488 sheep anti-mouse, Alexa 488 anti-rabbit antibody, and Alexa 594 sheep anti-rabbit, Invitrogen) were used at a concentration of 1:200.

2.3. Section in situ hybridization and immunostaining for GFP

Tg(*tie1*:H2B:eYFP) quail eggs were incubated at 37 °C and embryos were collected at E7, E10, and E12. Eyes were enucleated and fixed overnight at 4 °C in modified Carnoy's fixative (60% ethanol, 30% formaldehyde, and 10% glacial acetic acid). Fixed eye balls were dehydrated through ethanol series, embedded in paraffin, and sectioned at 12 μm. Riboprobes for *PlexinD1*, *Sema3C*, and *Sema3E* were generated as previously described (Kwiatkowski et al., 2013). RNA *in situ* hybridization was performed in sectioned tissue as described (Etchevers et al., 2001). After color reaction, sections were post-fixed in 4% paraformaldehyde (PFA) then immunostained for GFP as described above to fluorescently label the endothelial cells in Tg(*tie1*:H2B:eYFP) embryos.

2.4. Generation of RCAS-shRNA constructs

Using free online tools (Invitrogen, Dharmacon, Genscript), PlexinD1-specific sequences having 21 base pairs, GC content ranging between 30% and 50%, and beginning with a guanine residue were selected as PlexinD1-shRNA sequences. A scrambled (SCR) sequence (Ferrario et al., 2012) was also selected to serve as a negative control shRNA. The PlexinD1-specific and scrambled sequences were incorporated into complementary pairs of oligomers (Sigma) comprising a stem-loop sequence and restriction endonuclease sites to facilitate future cloning steps (Fig. S1). pSLAX-mCherry-cU6-(PlexinD1-shRNA) and pSLAX-(mCherry)-cU6-(SCR-shRNA) were generated from a pSLAX-GFP-cU6-(Sox9-shRNA) shuttle vector (Deneen et al., 2006), a gift from David Anderson (Caltech), and pFlk1:myr-mCherry vector (Larina et al., 2009), a gift from Mary Dickinson (Baylor College of Medicine). mCherry-cU6-(PlexinD1-shRNA) and mCherry-cU6-(SCR-shRNA) fragments were cloned into the ClaI restriction site of RCASBP (A) or RCASBP(B), referred to herein as RCASA or RCASB, using PCR-based cloning kits (*InFusion*, Clontech or *CloneEZ*, Genscript).

2.5. Cell culture infection and production of RCAS-shRNA viral stocks

A DF-1 chicken fibroblast cell line (ATCC) was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen). DF-1 cells were seeded into 12-well plates (MidSci) and incubated at 37 °C and 5% CO_2 for two days until ~50% confluent. Cultures were transfected using Lipofectamine (Invitrogen) according to manufacturer's protocol then reincubated until they reached 95% confluence. At least 90% of the cells were infected by RCAS virus, as determined by expression of mCherry. After screening for fluorescence, cell cultures were collected to isolate total RNA and generate cDNA using superscript III reverse transcriptase (Invitrogen). Transcription of *PlexinD1* was analyzed by semi-quantitative reverse transcription PCR (RT-PCR) using MyTaq hotstart PCR mastermix (Bioline) and gel electrophoresis. Transcription of *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control.

For production of virus, DF-1 cells transfected with shRNA-expressing RCASA and RCASB vectors were expanded in T75 flasks (MidSci) for up to a week until the cultures reached 80% confluence. Media was replaced with fresh DMEM without FBS. Cultures were reincubated for four days and the medium was harvested, pooled, and filtered through 0.45 μm syringe filters (Millipore) to remove cell debris. Medium was ultracentrifuged