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A role for DNA methylation in regulation of *EphA5* receptor expression in the mouse retina

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ABSTRACT

Understanding the mechanisms regulating expression of retinal ganglion cell (RGC) specific and axon-guidance genes during development and in retinal stem cells will be critical for successful optic nerve regeneration. Müller glia have some characteristics of retinal stem cells but in mammals have demonstrated limited potential to differentiate into RGCs. Chromatin remodeling through histone deacetylation and DNA methylation are a potential mechanism for silencing genes necessary for neuronal differentiation of glial cells. We investigated DNA methylation as a mechanism for regulating expression of mouse *EphA5*, one member of a large family of ephrin receptor genes that regulate patterning of the topographic connections of RGCs during visual system development. We analyzed spatial and age-related patterns of *EphA5* promoter methylation by bisulfite sequencing and mRNA expression by quantitative RT-PCR in the mouse retina. The CpG island in the *EphA5* promoter was hypomethylated in the retina and showed no change in overall methylation with age, despite a decline in *EphA5* mRNA expression levels in the adult retina. In the nasal retina of post-natal day 0 mice, there was a modest, but statistically significant increase in methylation. Increased methylation corresponded with lower levels of receptor mRNA expression in the nasal retina. We cloned the *EphA5* promoter and found that site-specific differences in methylation could preferentially activate or repress promoter activity in transient transfections of rat retinal progenitor cells (R28) using luciferase assays. In sphere cultures generated by EGF/FGF2 stimulation of conditionally immortalized mouse Müller glia (ImM10), *EphA5* promoter was hypermethylated and *EphA5* mRNA was not detected. Demethylation using 5-azadeoxycytidine (AzadC) resulted in a significant decrease of methylation of the *EphA5* promoter and re-expression of the *EphA5* mRNA. The inverse relationship between *EphA5* promoter methylation and mRNA expression is consistent with a role for DNA methylation in modulating the spatial patterns of *EphA5* gene expression in the retina and in silencing *EphA5* expression in ImM10 cells. The robust up-regulation of *EphA5* in ImM10 cells following demethylation suggests that modulation of chromatin structure may be a useful approach for promoting expression of silenced developmental genes and increasing the neurogenic potential of Müller glia.

1. Introduction

Currently there are no cures for neurodegenerative diseases that affect the optic nerve. Even if progression can be stopped, there is a need to develop regenerative therapies to restore lost vision. In mammals, the retina and optic nerve do not regenerate. However, in teleost fish, Müller glia are the source of the retinal stem cells in the inner retina that generate rod photoreceptors during normal growth and can regenerate all classes of retinal neurons including RGCs following injury (Bernardos, Barthel, Meyers, & Raymond, 2007; Otteson, D'Costa, & Hitchcock, 2001). The robust neurogenic capacity of Müller glia in fish and, to a more limited extent, in birds (Fischer & Reh, 2003) has stimulated research to understand the stem cell properties and neurogenic potential of Müller glia in the mammalian retina both *in vivo* and *in vitro* (Bhatia, Singhal, Lawrence, Khaw, & Limb, 2009; Dyer & Cepko, 2000; Karl et al., 2008; Ooto et al., 2004; Wohl, Schmeer, Kretz, Witte, & Isenmann, 2009). The challenges for developing cell-replacement strategies to treat optic neuropathies will require not only identifying how to regenerate retinal ganglion cells (RGCs), but also how to promote

Abbreviations: qRT-PCR, quantitative real time reverse transcriptase PCR; CpG, cytosine–guanine dinucleotide; Ct, crossing threshold; RGC, retinal ganglion cell; gDNA, genomic DNA; AzadC, 5-azadeoxycytidine; LB, Luria broth; MTT, methylthiazolyldiphenyl-tetrazolium bromide; TSS, transcription start site; ImM10, Immortomouse Müller glia P10 cell line.

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axon growth and connectivity. For functional vision, the spatial relationship between neighboring retinal ganglion cells (RGC) in the retina must be preserved in the topographic organization of their synaptic connections in the visual centers of the brain. In the visual system, forward and reverse signaling through Ephrin receptors (*Eph*) and ephrin ligands functions as chemoaffinity signals that regulate retinotopic mapping.

Eph receptors constitute a family of tyrosine kinase receptors that regulate multiple aspects of development and disease including vascular development (Zhang & Hughes, 2006) and synaptogenesis (Murai & Pasquale, 2004; Otal, Burgaya, Frisen, Soriano, & Martinez, 2006). More recently, disregulation of EPH/ephrin signaling has also been implicated in oncogenesis and tumor progression (Merlos-Suarez & Batlle, 2008). However, one of their most well known functions is in patterning the formation of sensory maps in the central nervous system during development (Scicolone, Ortalli, & Carri, 2009). In the visual system, gradients of *Eph* receptors along the nasal/temporal and dorsal/ventral axes of the retina respond to corresponding gradients of ephrin ligands in target regions within the brain to create the positional information that guides the initial spatial organization of RGC connectivity (Feldheim et al., 2004; Flanagan, 2006; Scicolone et al., 2009). In the mouse retina, *EphA5* is expressed in an increasing nasal to temporal gradient and functions in patterning the location of retinal axon termination along the anterior–posterior axis in the visual centers in the brain. In the mouse retina, *EphA5* expression is initiated during the optic vesicle stage. By E11, when the first retinal ganglion cells begin to differentiate, *EphA5* expression in the neuroblastic layer and nascent RGCs extends in a temporal (high) to nasal (low) gradient across the optic cup (Cooper, Crockett, Nowakowski, Gale, & Zhou, 2009). Axon outgrowth begins at E12 and by E17, RGCs have projected through the optic tract and have begun to reach targets and arborize in the ventral lateral geniculate nucleus and superior colliculus (Bovolenta & Mason, 1987; Godement, Salaun, & Imbert, 1984). Graded *EphA5* expression is prominent in the inner retina and RGC layer beginning at E12, and by E17.5, when all RGCs have been generated, the gradient of *EphA5* is robust in the RGC layer (Cooper et al., 2009). By P0, the retinal ganglion cells fibers have established their terminal connections, but have not yet entered the subsequent period of connection refinement. Although the gradient of *EphA5* expression persists in the ganglion cell layer in the adult retina, there is a gradual decline in overall expression and a flattening of the gradient with age (Rodger et al., 2001). Loss of *EphA5* receptor leads to mapping abnormalities along the anterior/posterior axis of the mouse superior colliculus (Feldheim et al., 2004) confirming the importance of the receptor in the positional termination of retinal axons.

While there is extensive literature showing direct regulation of *EphB* receptors by transcription factors (reviewed by (Petros, Rebsam, & Mason, 2008), little is known about transcriptional mechanisms regulating *EphA5* expression in the retina. *Hmx* family transcription factors, *SOHO* and *GH6*, have been implicated in regulation of *EphA3* expression in chick (Schulte & Cepko, 2000) and, in the developing mouse retina, *Hmx1* is expressed in a nasal high/temporal low gradient (Wang, Lo, Frasch, & Lufkin, 2000). However, the role of *Hmx1* in regulating *EphA5* expression in the mouse retina has not been determined. *FoxD1* and *FoxG1* are expressed in reciprocal gradients in the developing mouse retina (Hatini, Tao, & Lai, 1994) and regulate formation of the contralateral and ipsilateral pathways and optic chiasm in the mouse (Herrera et al., 2004; Pratt, Tian, Simpson, Mason, & Price, 2004; Tian, Pratt, & Price, 2008). However, only *EphB1* has been proposed as a downstream target of FOX transcription factors in the mouse retina and the specific transcription factors that regulate *EphA5* expression in the retina have not been determined.

DNA methylation is an epigenetic mechanism for regulating gene transcription in embryonic stem cells during differentiation and development (Yeo et al., 2007) and increased DNA methylation of CpG islands is associated with gene silencing (Bird, 2002). Except in oocytes and cancer, hypermethylation has typically been thought of as an irreversible chromatin modification. However, several studies have shown that demethylation of cell-type specific genes is dynamic (Frank et al., 1990; Meissner et al., 2008; Song et al., 2009). During development, 2% of the CpG islands that are methylated in embryonic stem cells become demethylated in neural progenitors (Meissner et al., 2008). All of the *Eph* receptor genes contain CpG islands within their proximal promoters and hypo- and hypermethylation can alter *Eph* receptor expression in cancer. Hypermethylation down-regulates *EphA3* in hematopoietic tumors (Dottori, Down, Huttmann, Fitzpatrick, & Boyd, 1999) and *EphA7* in colon cancer (Wang et al., 2005) and increased methylation of *EphA5* is correlated with decreased expression in primary breast cancer (Fu et al.). Conditional knockout of *Dnmt1*, the maintenance DNA methyltransferase, in mouse neural progenitors resulted in increased differentiation of astroglia in the mouse brain, indicating a role for DNA methylation in the switch from neurogenesis to gliogenesis.

Recent transcriptome analysis has revealed that Müller glia express many, but not all of the same genes expressed by undifferentiated retinal progenitor cells in the embryonic retina (Roesch et al., 2008). In the mature retina in mammals, some Müller glia proliferate in the context of retinal injury or disease *in vivo* and a fraction up-regulate genes characteristic of differentiated retinal neurons (Dyer & Cepko, 2000; Karl et al., 2008; Wohl, Schmeer, Kretz, Witte, & Isenmann, 2009). Cultured Müller glia from human (Lawrence et al., 2007), rat (Kubota, Nishida, Nakashima, & Tano, 2006) and mouse (Das et al., 2006; Otteson & Phillips, 2010; Phillips, Guirguis, Beach, Pillai, & Otteson, 2008) show stem cell characteristics, including expression of multiple retinal stem cell genes and formation of proliferating neurospheres in response to epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2). However, Müller-derived cells are quite limited in their neurogenic potential both *in vivo* and *in vitro*, and only a fraction acquire neuronal characteristics or up-regulate genes characteristic of differentiated retinal neurons. Single cell microarray analysis of gene expression showed that many key genes that regulate retinal ganglion cell development and differentiation, including *Atoh7*, *Pou4F2*, *Isl2* and *EphA5*, were either not expressed or expressed at low levels only in a subset of Müller glia cells examined (Roesch et al., 2008).

Understanding the mechanisms that regulate expression of key genes involved in RGC developmental and axon guidance in the retina and in Müller glia will contribute to the development of regenerative therapies to restore the optic nerve and vision in patients with glaucoma. We describe here the cloning of the mouse *EphA5* promoter and address the potential role of DNA methylation in regulating *EphA5* expression in the mouse retina *in vivo* and in conditionally immortalized Müller glia (ImM10 cell line) *in vitro*.

2. Materials and methods

2.1. Cell culture

The following cell lines were used: the R28 rat retinal progenitor cell line (Seigel, 1996) and the ImM10 conditionally immortalized Müller glia cell line (Otteson & Phillips, 2010). R28 cells were cultured in DMEM (Sigma; St. Louis, MO) supplemented with 1.125% sodium bicarbonate, 10% calf serum, 1% each MEM non-essential amino acids and MEM vitamins, 2 mM L-glutamine and 0.1 mg/ml gentamicin. ImM10 cells were cultured in growth medium (Neurobasal, 2% FBS, B27 supplement, 20 mM L-glutamine, 50 U/ml IFNγ, Pen/strep antibiotics) at 33 °C. To generate spheres,