Müller Glia, Vision-Guided Ocular Growth, Retinal Stem Cells, and a Little Serendipity

The Cogan Lecture

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Hypothesis-driven science is expected to result in a continuum of studies and findings along a discrete path. By comparison, serendipity can lead to new directions that branch into different paths. Herein, I describe a diverse series of findings that were motivated by hypotheses, but driven by serendipity. I summarize how investigations into vision-guided ocular growth in the chick eye led to the identification of glucagonergic amacrine cells as key regulators of ocular elongation. Studies designed to assess the impact of the ablation of different types of neurons on vision-guided ocular growth led to the finding of numerous proliferating cells within damaged retinas. These proliferating cells were Müller glia-derived retinal progenitors with a capacity to produce new neurons. Studies designed to investigate Müller glia-derived progenitors led to the identification of a domain of neural stem cells that form a circumferential marginal zone (CMZ) that lines the periphery of the retina. Accelerated ocular growth, caused by visual deprivation, stimulated the proliferation of CMZ progenitors. We formulated a hypothesis that growth-regulating glucagonergic cells may regulate both overall eye size (scleral growth) and the growth of the retina (proliferation of CMZ cells). Subsequent studies identified unusual types of glucagonergic neurons with terminals that ramify within the CMZ; these cells use visual cues to control equatorial ocular growth and the proliferation of CMZ cells. Finally, while studying the signaling pathways that stimulate CMZ and Müller glia-derived progenitors, serendipity led to the discovery of a novel type of glial cell that is scattered across the inner retinal layers. (*Invest Ophthalmol Vis Sci.* 2011;52:7705-7710) DOI:10.1167/iovs.11-8330

Losses in vision can occur from many different disorders of the eye, ranging from irreversible retinal degeneration to easily treated refractive errors. The most common vision disorder is myopia, or near-sightedness; an estimated 33% of adults in the United States are myopic.¹ Myopia is primarily caused by ocular elongation resulting from increases in vitreous chamber depth, which is determined by the growth of the sclera, the connective-tissue sheath of the eye. In myopia, images of distant objects are focused in front of the retina because of excessive ocular elongation. Eye growth is a complex, well-regulated process with an endpoint of minimal to no refractive error at maturity. This process, known as emmetropization, has been observed in many different animal models including chickens,

tree shrews, marmosets, mice, and monkeys (reviewed by Wallman and Winawer²). During emmetropization, the refractive elements of the eye—the lens and cornea—grow in a coordinated manner with the sclera, to achieve an axial length where light from distant objects converges to a focal point on the retina. At birth, most animals have some refractive error.³ During adolescence, the eye grows, and the refractive components of the eye change to eliminate this initial refractive error. Clearly, myopia occurs when the process of emmetropization fails to sufficiently slow rates of ocular growth and limit ocular elongation.

MYOPIA AND VISION-GUIDED OCULAR GROWTH

The process of emmetropization requires visual experience; clear images must be projected onto the retina. In animal models, attenuation of clear vision causes excessive ocular growth and myopic refractive errors; this process is known as form-deprivation myopia (FDM). The requirement for clear vision in achieving emmetropia has been documented in many different vertebrate species, including chicks, marmosets, tree shrews, mice, monkeys, and rabbits.^{2,4-9} Interestingly, lens-imposed defocus can influence rates of ocular growth. For example, the application of divergent (minus) lenses, which produces hyperopic defocus, increases rates of ocular growth and cause ocular elongation (Fig. 1).¹⁰ Conversely, the application of the convergent (plus) lenses that produce myopic defocus slows rates of ocular growth and prevents ocular elongation (Fig. 1).¹⁰ The identity of the retinal cell types that detect defocus and effect changes in the rates of eye growth have remained elusive for many years.

Many secreted peptides and neurotransmitters have been implicated as growth regulators in animal models of myopia (reviewed by Wallman and Winawer²). To date, one of the most promising candidates for a retina-derived signal that regulates ocular growth is glucagon peptide. We found that the immediate early gene *Egr1* is differentially expressed by amacrine cells in response to growth-slowing and growth-accelerating visual stimuli. *Egr1* is also known as ZENK, zif268, NGF-induced 1a, and Krox24. Interestingly, *Egr1* was differentially regulated by a minor population of amacrine cells, those that express glucagon.¹¹ The glucagon-expressing amacrine cells (GACs) respond to growth-slowing visual stimuli (plus defocus and recovery from FDM) by upregulating *Egr1* (Fig. 1).¹¹ By contrast, the glucagon-expressing amacrine cells respond to growth-accelerating visual cues (minus defocus and form deprivation) by downregulating *Egr1* (Fig. 1).¹¹ Subsequent reports confirmed our findings and indicated that glucagon and glucagon receptor antagonists influence rates of ocular growth.¹²⁻¹⁴ The expression of *Egr1* by glucagonergic retinal neurons is believed to influence glucagon synthesis and release.^{11-13,15,16} However, definitive evidence of the direct transcriptional regulation of glucagon by *Egr1* is lacking. Further-

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FIGURE 1. Summary of the effects of imposed defocus on Egr1 expression in glucagonergic amacrine cells, levels of glucagon synthesis, and scleral growth.

more, the downstream targets of retinal glucagon that regulate ocular growth remain uncertain, in part because the expression of glucagon receptors appears to be nearly ubiquitous across ocular tissues.^{16–18}

Hints that the glucagon-expressing amacrine cells are key regulators of ocular growth were first exposed by studies involving the ablation of different populations of retinal neurons. Studies have indicated that the different retinal toxins that ablate different types of retinal interneurons cause ocular enlargement,^{19–22} consistent with the notion that retinal interneurons normally provide growth-slowing signals. A serendipitous observation, revealed by Ian Morgan,²³ while studying colchicine-mediated ablation of ganglion cells, suggested that treated eyes appeared significantly larger than untreated eyes. An in-depth study confirmed that colchicine treatment results in excessive growth and significant ocular enlargement.²⁴ Interestingly, in addition to the loss of ganglion cells, we found that there was a discrete loss of two different populations of large-field amacrine cells, including those that use the neurotransmitters dopamine or glucagon. These findings are consistent with the notion that glucagon and the GACs are essential to slow rates of ocular growth.

PROLIFERATING PROGENITORS IN DAMAGED RETINAS

While studying the effects of different retinal toxins on ocular growth and the destruction of retinal neurons, we noted the accumulation of numerous proliferating cells in the inner nuclear layer (INL) (Fischer and Stell, unpublished observations, 1998). Originally, the observation of proliferating cells was an unexpected outcome that was peripheral to the focus of our myopia-related research. Accordingly, these proliferating cells were ignored until these data were shared with Thomas Reh

and investigations were pursued in his laboratory. We found that the proliferating cells in damaged retinas were retinal progenitors, but these cells were derived from mature Müller glia.²⁵ The proliferating Müller glia appear to become progenitor-like with the upregulation of many different markers of embryonic retinal progenitors including Pax6, Chx10, Six3, transitin (nestin), Notch1, Hes1, Hes5, and ascl1a.^{25–30} Further, normal and proliferating Müller glia express the transcription factors Sox2 and Sox9, which are associated with retinal stem cells.^{26,30–33} Interestingly, the transcriptome of Müller glia, unlike the transcriptome of other types of retinal cells, overlaps significantly with that of retinal progenitors.^{34,35}

The ability of Müller glia-derived progenitors to produce new neurons is most likely restricted by cell-intrinsic factors and environmental cues. In vivo, the Müller glia-derived progenitors go through only one round of cell division, but are capable of multiple rounds of division if dissociated and grown in culture.²⁵ This finding suggests that the intact retina provides signals that suppress the proliferation of Müller glia-derived progenitors. A small percentage (<5%) of the Müller glia-derived progenitors in damaged retinas go on to form new neurons, whereas most Müller glia-derived progenitors appear to remain undifferentiated.^{25,36} This finding suggests that the proliferating progenitors that are derived from Müller glia have a limited capacity to produce new neurons. Alternatively, the cues that support neuronal differentiation are absent or differentiation is inhibited within the mature, damaged retina. Collectively, these findings support the possibility that Müller glia could be stimulated, by activating or inhibiting cell-intrinsic factors and environmental cues, to form proliferating progenitors and neuronal differentiation of glia-derived progenitors enhanced to drive retinal regeneration. Further, these findings provide the possibility that the neurogenic potential of cells

FIGURE 2. Summary of the organization and markers expressed by retinal stem cells at the CMZ and in NPE of the ciliary body. PCNA, proliferating cell nuclear antigen; NCAM, neural cell adhesion molecule; IGF1, insulin-like growth factor 1; EGF, epidermal growth factor; Shh, sonic hedgehog; FGF2, fibroblast growth factor 2; NPE, non-pigmented epithelium; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; ILM, inner limiting membrane.

endogenous to the retina could be harnessed to restore vision to treat sight-threatening diseases of the retina.

It makes no sense to destroy neurons to activate the neurogenic potential of Müller glia-derived progenitors in retinas that are slowly degenerating. Thus, it is important to identify the mechanisms that stimulate neurogenesis from Müller glia-derived progenitors without damaging retinal neurons. We found that it is possible to stimulate the formation of Müller glia-derived progenitors without damaging the retina; consecutive daily injections of insulin and FGF2 stimulated numerous Müller glia to dedifferentiate, proliferate, become progenitor-like cells, and produce a few new neurons.³⁶ The insulin and FGF2 act synergistically and exposure must be sustained; injections of either factor alone or a single large dose is insufficient to activate the neurogenic potential of Müller glia.³⁶ Activation of FGF receptors and MAPK-signaling are necessary for Müller glia to become proliferating progenitor-like cells.^{27,30,33} MAPK-mediated activation of Müller glia-derived progenitors requires that baseline levels of Notch-signaling be maintained.²⁷ Notch-signaling appears to be sustained at low

levels in mature Müller glia, and this signaling likely maintains progenitor-like qualities and affects the neuroprotective functions of these glia.^{26,27} Notch-signaling is essential for Müller glia to re-enter the cell cycle in response to acute retinal damage; however, once Müller glia-derived progenitors are formed, Notch-signaling inhibits neuronal differentiation.^{26,27} Nevertheless, most Müller glia-derived progenitors remain undifferentiated whether Notch is inhibited,²⁶ proneural bHLH factors are misexpressed,³⁷ or prodifferentiation growth factors applied.³⁸ Clearly, the cell-signaling pathways and cell-intrinsic limitations that influence the proliferation and neurogenic potential of Müller glia are complicated and require additional study.

Studies have indicated that Müller glia are a source of retinal regeneration in vertebrate classes in addition to birds. Functional regeneration of the fish retina has been known for decades (reviewed by Hitchcock and Raymond³⁹). Only recently have the Müller glia been identified as the cellular source of the neural regeneration in the fish retina.^{40,41} Interestingly, Müller glia have also been identified as the source of rod progenitors that are scattered across the fish retina⁴⁰; these progenitors are known to produce rod photoreceptors to maintain the density of photoreceptors as the eye grows and as the retina stretches (reviewed by Hitchcock et al.⁴²). In addition, Müller glia have been identified as a source for neuronal regeneration in the mammalian retina.^{43,44} Similar to Müller glia-derived progenitors in the bird retina, the Müller glia-derived progenitors in the mammalian retina produce only a few new neurons—a regenerative response that is insufficient to restore vision. Collectively, these findings raise the question as to why Müller glia in the fish effectively produce retinal progenitors that regenerate numerous functional neurons to restore visual function, whereas Müller glia in the avian and mammalian retinas effectively produce retinal progenitors but fail to regenerate a significant number of functional neurons.

STEM CELLS WITHIN THE RETINAL MARGIN AND NONPIGMENTED EPITHELIUM OF THE CILIARY BODY

While applying BrdU to label proliferating Müller glia, we noticed a cluster of proliferating cells at the far peripheral edge of control (nondamaged) retinas.⁴⁵ These proliferating cells are retinal stem cells that form a circumferential marginal zone (CMZ) at the transition of neural retina to non-pigmented epithelium (NPE) of the ciliary body (Fig. 2), reminiscent of the CMZ described at the retinal periphery in lower vertebrates (reviewed by Reh and Levine,⁴⁶ Hitchcock et al.,⁴² and Raymond and Hitchcock⁴⁷). In the chick eye, CMZ cells are similar to embryonic retinal stem cells, in that they express Pax6, Chx10, Six3, Notch1, Hes1, transitin, Egr1, Sox2, Sox9, and NCAM (Fig. 2).^{28,33,45,48,49} The CMZ cells appear to be maintained into adulthood.^{45,50} The proliferation of CMZ stem cells and the differentiation of the daughter cells can be influenced by exogenous growth factors, including insulin, IGF1, FGF2, and EGF (Fig. 2).^{45,49} In addition, we found that some of the NPE cells in the pars plana, anterior to the CMZ, can be stimulated by growth factors in vivo to proliferate and produce new neurons.⁵¹ Interestingly, we found that form deprivation stimulates the proliferation of CMZ stem cells and addition of new cells to the peripheral edge of the retina.⁴⁵ In other words, when overall eye growth is stimulated by form deprivation, the addition of new cells to the peripheral edge of the retina is enhanced, implying that scleral growth and retinal growth are coordinated by visual experience.

We next formulated a hypothesis based on a series of observations: (1) glucagon peptide released from amacrine

FIGURE 3. Bullwhip and minibullwhip cells are unique types of retinal neurons that are scattered across peripheral regions of the retina. Whole-mounts (**a**, **b**) and vertical sections (**d**) of the retina were labeled with antibodies to glucagon alone (**d**) or combined with substance P (**a**, **b**). Arrows: the somata of bullwhip and minibullwhip cells.

cells slows rates of ocular growth, (2) form deprivation, which accelerates rates of ocular growth, causes the glucagonergic amacrine cells to downregulate *Egr1* and glucagon synthesis, and (3) the increased rates of ocular growth, that result from form deprivation, increase the proliferation CMZ progenitors and addition of new cells to the peripheral edge of the retina. Thus, we hypothesized that glucagon peptide released from glucagonergic amacrine cells inhibits the proliferation of CMZ progenitors. We began by examining whether glucagon-containing neurites are present in close proximity to the CMZ. Indeed, there is a massive accumulation of glucagon-immunoreactive neurites clustered in the CMZ.¹⁷ Further, we found that intraocular injections of glucagon peptide inhibits the proliferation of CMZ progenitors, and inhibition of the glucagon receptor increases the proliferation of CMZ progenitors.¹⁷

The cells that produce the terminals that ramify in the CMZ were revealed to be unique types of cells that are distinct from the GACs. Within vertical sections of the retina, we occasionally observed unusual, glucagon-immunoreactive unipolar neurons with large somata in the INL, neurites in the distal inner plexiform layer (IPL), and axon-like structures that project toward the retinal periphery.^{17,52} The axons of the large glucagonergic cells project to and ramify within the CMZ.¹⁷ Cells similar to the large glucagon-expressing neurons were described more than two decades ago in a study that investigated the expression of substance P in ganglion cells; the unusual cells were described as having a morphology that is reminiscent of a bullwhip.⁵³ Glucagon and substance P are colocalized to large “bullwhip cells,” which are found only in ventral regions of the retina; smaller “minibullwhip cells,” which are found only in dorsal regions of the retina; and neurites, which are densely clustered in the CMZ (Fig. 3).⁵² There have been a

few reports briefly describing large glucagon-expressing neurons (putative bullwhip and minibullwhip cells) in the avian retina.^{54–56} Perhaps little attention has been paid to the large glucagon-positive neurons because these cells are rather rare. On average, there are only 240 bullwhip cells and 800 minibullwhip cells in the postnatal chicken retina.⁵²

One of the functions of the bullwhip and minibullwhip cells is to regulate equatorial ocular growth. If we lesion the bullwhip and minibullwhip cells by using a low dose of colchicine applied at postnatal day 7, when the ganglion cells and conventional glucagonergic amacrine cells are no longer sensitive to colchicine, the eye grows significantly larger around the equator, but not in axial length.¹⁶ The excessive ocular growth that results from colchicine is completely blocked by intraocular injections of glucagon.¹⁶ Similar to the GACs, the bullwhip and minibullwhip cells differentially express *Egr1* in response to growth-slowing and growth-accelerating visual stimuli.¹⁶ Collectively, these findings suggest that the bullwhip and minibullwhip cells respond to growth-guiding visual cues to regulate and coordinate equatorial growth of the sclera and the addition of new cells to the peripheral edge of the retina by influencing the proliferation of CMZ progenitors.

A NOVEL TYPE OF GLIAL CELL

More than a decade ago, we observed an accumulation of numerous *Nkx2.2*-positive cells in the IPL of NMDA (*N*-methyl-D-aspartate)-damaged retinas (Fig. 4; Fischer and Reh, unpublished observations, 2001). This finding was unexpected, and the identity of these cells was uncertain. Thus, we shelved these data without having a reasonable interpretation. These

FIGURE 4. Nkx2.2-expressing cells accumulate within the IPL of NMDA-damaged retinas. The eyes were injected with saline (control; **a**) or 2 picomoles NMDA (**b**) at P7, and the retinas were harvested at P10. *Arrows*: the nuclei of Nkx2.2-positive cells. Vertical sections of the retina were labeled with antibodies to Nkx2.2.

cells were again observed several years later, while we were investigating the effects of insulin and IGF1 on cell-signaling and Müller glia, and we decided to pay more attention to their accumulation within the IPL. We observed accumulations of Nkx2.2/transitin-positive cells scattered across the IPL of treated retinas.^{32,33} Based on their morphology and position within the retina, we believed that these cells were microglial-like cells. However, the cells were negative for the microglial markers CD45, RCA1, and lysosomal membrane glycoprotein.³² Based on the phenotype and location within the retina, we termed these cells nonastrocytic inner retinal glial (NIRG) cells.³² NIRG cells are distinctly different from Müller glia and oligodendrocytes; these cells were positive for Sox2, Sox9, Nkx2.2, vimentin and transitin, but were negative for other well-established glial markers, including glutamine synthetase, Pax2, GFAP, S100 β , 2M6 (Top_{AP}), and transferin-binding protein.³² We propose that the NIRG cells are not a type of astrocyte, because they are negative for both GFAP and Pax2, they are not associated with blood vessels (unlike most astrocytes in mammalian retinas), and they do not upregulate GFAP in response to damage.^{32,57} In all species examined, retinal astrocytes express GFAP and Pax2, and GFAP expression is dramatically increased in damaged tissues.^{57,58} Although NIRG-like cells were not observed in the retinas of mice or guinea pigs, they have been found in the retinas of dogs and monkeys,⁵⁹ suggesting the presence of similar cells in the eyes of humans.

The NIRG cells that we identified are likely to be the same cells that were recently described as astrocytes and diacytes by Rompani and Cepko.⁶⁰ In this study, the authors identified an optic nerve–derived glial progenitor that gives rise to different types of retinal glia: oligodendrocytes, astrocytes, and a novel cell type that they termed “diacytes.” The authors describe presumptive astrocytes in the IPL and diacytes in the ganglion cell layer (GCL) based on morphology. The diacytes and presumptive astrocytes express Olig2 (similar to oligodendrocytes), whereas these cells do not express GFAP and other well-established markers of oligodendrocytes.⁶⁰

Intraocular injections of IGF1 stimulated the NIRG cells to upregulate transitin and to proliferate and accumulate within the IPL.³² Although IGF1-mediated stimulation of NIRG cells is detrimental to the survival of retinal neurons and Müller glia,³² nothing is known about the functions of the NIRG cells in normal, healthy retinas. Further studies are needed to identify the functions of the NIRG cells in normal retinas and thereby to better ascribe an identity to these cells.

CONCLUDING THOUGHTS

Empiric hypothesis-driven investigation is, without doubt, the preferred mode of scientific exploration. Nevertheless, serendipity can be a powerful scientific tool, as suggested by the tales of investigation described herein. Thus, I offer the follow-

ing advice: Do not always stare straight at the expected hypothesis-driven outcomes; try to look at all the data, even those that do not support the hypothesis and those that are unrelated to the hypothesis. Do not ignore or disregard unexpected outcomes; carefully assess whether something new and interesting lies in the unexpected.

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