Cell Stem Cell
Review

Neural Regeneration and Cell Replacement: A View from the Eye

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Neuronal degenerations in the retina are leading causes of blindness. Like most other areas of the CNS, the neurons of the mammalian retina are not replaced following degeneration. However, in nonmammalian vertebrates, endogenous repair processes restore neurons very efficiently, even after complete loss of the retina. We describe the phenomenon of retinal regeneration in nonmammalian vertebrates and attempts made in recent years to stimulate similar regenerative processes in the mammalian retina. In addition, we review the various strategies employed to replace lost neurons in the retina and the recent use of stem cell technologies to address problems of retinal repair.

Studies of eye regeneration have a long history, dating back to the 1700s when some of the first investigations of regeneration in animals were made. Soon after Abraham Trembley discovered the remarkable regenerative potential of hydra, his cousin, Charles Bonnet, discovered that newts could regenerate their eyes if all but a small part of it was surgically removed (Morgan, 1901). This led to further investigations and establishment of retinal and lens regeneration as key experimental systems for regeneration research in the first half of the 20th century (Stone, 1950). Building on the wealth of molecular information on eye development from the last 15 years, significant advances in our understanding of regeneration in this tissue have recently been made. In this review, we highlight some of the major developments in retinal regeneration and repair that have provided insights for regenerative biology and medicine. But first, we will describe the major retinal degenerations that could be addressed by retinal repair or replacement and some key features of retinal developmental biology that serve as a foundation for the studies of regeneration.

The Retina and Its Degenerations

The rod and cone photoreceptors of the eye that mediate our vision are part of a complex neural tissue known as the neural retina (Figure 1A). The photoreceptors respond to photons by changes in their membrane potential that are relayed by synaptic connections through a circuit of neurons to the brain. The rods are capable of responding to single photons and, as a consequence, are responsible for our night vision, while cones mediate high acuity color vision in the daylight. In humans, a high-density region of cones in the central retina is known as the fovea. The responses of the photoreceptors to changes in illumination lead to changes in their release of neurotransmitter at synapses with a group of retinal interneurons called bipolar cells. These cells in turn relay the signals to ganglion cells, either directly or through another class of retinal interneurons, called amacrine cells. The ganglion cells are the output neurons of the retina, connecting to visual centers in the brain via long axons that leave the eye through the optic nerve. The different types of retinal neurons are organized in three laminae: the nuclei of the rods and cones are in the outer nuclear layer (ONL), the nuclei of the bipolar cells and amacrine cells (and those of another type of interneuron—the horizontal cells) are in the inner nuclear layer (INL), and the innermost layer of the retina contains the ganglion cells and their axons. In addition to the neurons of the retina, there are several types of nonneuronal cells that are also critical for its function. The Müller cells are a type of glia that span the retinal epithelium and perform functions similar to astrocytes in other regions of the CNS. Adjacent to the photoreceptor layer is a layer of pigmented cells, called the retinal pigmented epithelium (RPE), which are essential for the maintenance of the rods and cones. Lastly, the inner surface of the retina contains a layer of astrocytes, similar to those in other regions of the CNS.

Like many other regions of the nervous system, the retina is subject to many different degenerative conditions. Macular degeneration (AMD) is a disease of the retina characterized by the degeneration of cells in the RPE and the photoreceptors in the central neural retina. AMD is the leading cause of blindness in the US, and because it occurs primarily in individuals over 65, its prevalence is increasing as the population ages. In addition to AMD, a number of other conditions lead to degeneration of retinal neurons. Glaucoma and diabetic retinopathy also lead to neuron death in the retina; the former results in loss of ganglion cells, while in the latter the degeneration encompasses other neuronal types as well. There are also a number of inherited retinal disorders that lead to either degeneration of retinal neurons: these include Usher syndrome, retinitis pigmentosa (RP), and Leber congenital amaurosis.

The various types of retinal degenerations impair vision by reducing the number of elements that process the visual scene. For example, degeneration of ganglion cells in glaucoma leads to fewer channels to communicate visual information to the brain. In addition, many of these degenerations begin in one cell type, only to later involve others. In AMD, for example, the pigmented epithelial cells in the RPE are the first to degenerate, but the cones die soon after. A similar codependency is observed for rods and cones in RP. Although most of the mutations that cause this disorder lead to degeneration of rod photoreceptors initially and a consequent loss in night vision, the cone

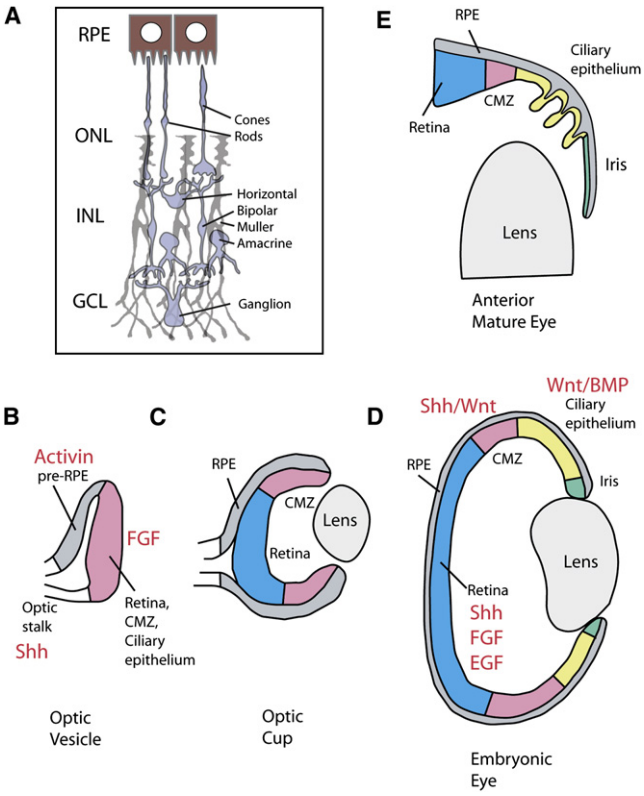

Figure 1. Development and Structure of the Retina

(A) The mature retina is made up of five types of neurons and one type of intrinsic glial cell—the Müller glia.

(B) The retina first becomes recognizable at the optic vesicle stage of development.

(C) The optic vesicles undergo a series of morphogenetic changes to form a two-layered optic cup. The inner layer develops into the neural retina (blue), while the outer layer acquires pigmentation (gray) and develops into the retinal pigmented epithelium (RPE).

(D) At a slightly later stage of development, the iris (green) and ciliary epithelium (yellow) become distinct, and there is a region of undifferentiated, mitotically active progenitor cells that is located between the ciliary epithelium and the developing neural retina (red) that will ultimately contain the stem cells of the CMZ.

(E) The morphological arrangement of these domains is shown in the mature eye section.

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photoreceptors are frequently involved as well, and individuals can progress to total blindness. It has more recently been appreciated that loss of photoreceptors also leads to dramatic changes in retinal organization.

Like most regions of the CNS of mammals, loss of neurons in the retina does not lead to their replacement naturally. However, in many vertebrates, there are elegant repair processes that restore the normal number and types of neurons very efficiently, even after complete loss of the retina. In this review, we outline the current understanding of these endogenous repair processes and describe attempts that have been made in recent years to probe the limits to these regenerative processes in the mammalian retina. In addition, we describe the various strategies that have been employed to replace lost neurons in this tissue and the recent use of stem cell technologies to address problems of retinal repair. However, before discussing repair of the retina, we need to describe the basics of eye development.

Retinal Developmental Biology

The presumptive retina can be identified as early as neural plate stages in amphibians, classically through experimental embryology, and more recently through its distinct pattern of gene expression (Zuber et al., 2003). As the neural plate rolls into a tube, the lateral aspects of the anterior neural tube evaginate to form paired optic vesicles (Figure 1B). These vesicles undergo a series of morphogenetic changes to form two-layered optic cups (Figure 1C). The inner layer develops into the neural retina and the nonpigmented layer of the ciliary epithelium, while the outer layer acquires pigmentation and develops into the RPE and the pigmented layer of the ciliary epithelium. The patterning of these various domains involves several signaling pathways, and the details are not thoroughly understood. Current evidence suggests that at least three signaling pathways are required; FGF signaling is necessary for the development of the neural retina (Pittack et al., 1997; Vinothkumar et al., 2008), while activation of the activin signaling pathway promotes RPE development (Fuhrmann et al., 2000; Sakami et al., 2008). BMP and Wnt signaling promote ciliary epithelium development (Figure 1D) (Cho and Cepko, 2006; Liu et al., 2007). During this early stage of eye development, Shh promotes both RPE and optic stalk development. Though later in eye development, Shh is an important mitogen for retinal progenitors (Yu et al., 2006).

Once the optic cup has formed, the cells within this structure are known as retinal progenitor cells. Clonal analysis of the lineages of these cells using retroviral infection or cell-impermeant dye injection established that they have the potential to generate all the different types of neurons and Müller glia (Turner and Cepko, 1987). It has been known since the early 1960s that there is a defined sequence in which the neurons of the retina are generated that is largely conserved across vertebrates: ganglion cells, cone photoreceptors, horizontal cells, and most amacrine cells are generated during early stages of development (prenatally in mice), and most rod photoreceptors, bipolar cells, and Müller glia are generated in the latter half of the period of retinogenesis (in the first postnatal week in mice, Sidman, 1961). The retinal progenitors are similar to neural progenitors found elsewhere in the central nervous system, and they respond to mitogenic factors similar to those that stimulate proliferation of neural stem/progenitors elsewhere in the CNS, notably EGF, FGF, and Shh (see Nelson et al., 2007 for review). Also, retinal progenitors express and require many of the same genes as the neural stem/progenitor cells elsewhere in the CNS. Proneural *bHLH* transcription factors, such as *Ascl1*, *Neurog2*, *NeuroD1*, and *Math5*, are required for the production of one or more of the different retinal neurons, as they are for neurons in other areas of the CNS (Akagi et al., 2004). Components of the Notch pathway are also critical for the maintenance of the retinal progenitor pool (see Nelson et al., 2007 for review).

The CMZ: A Zone of Persistent Progenitors

During the normal development of the retina, there is a distinct central-to-peripheral gradient in histogenesis. In amphibians and fish, the central-most part of the retina is generated in a relatively short period of embryogenesis; however, most of the

retina in the mature animal is generated from a small zone of mitotically active cells at the peripheral margin of the retina. This zone has been called a variety of names in the literature, but we will use the term ciliary marginal zone (CMZ), due to its location at the junction between the ciliary epithelium and the retina (Figure 1E). The CMZ is analogous to other neural stem cell zones in the vertebrate CNS in that it can persist throughout the lifetime of the animal and can generate all the types of neurons and glia normally present in the retina (Hollyfield, 1968; Reh, 1987) The cells in the zone proliferate and generate new neurons at the junction with the retina, which subsequently differentiate and are incorporated into the retinal circuitry, essentially producing a spatial representation of the stages of retinal development (Perron et al., 1998; Reh and Nagy, 1989). The CMZ cells resemble retinal progenitors in their pattern of gene expression (Perron et al., 1998; Raymond et al., 2006) and respond to at least some of the same mitogenic factors as the embryonic retinal progenitors (Boucher and Hitchcock, 1998; Fischer and Reh, 2000; Moshiri et al., 2005; Otteson et al., 2002).

In amphibians, the CMZ responds to retinal damage by upregulating its production of new neurons (Reh, 1987). Early studies of retinal regeneration in amphibians demonstrated that this zone makes a contribution to regenerated retina, though the RPE is the primary source (see below). Studies of cell-specific retinal damage in fish and amphibians also show that the CMZ has some specificity in the response to injury, producing the specific types of neurons that were destroyed (Negishi et al., 1982; Reh, 1987; Reh and Tully, 1986). However, the extent of regeneration that occurs from the CMZ is limited due to the fact that the eye of mature vertebrates is a fairly large structure and the migration of neurons is limited to a few 100 microns (Johns, 1982; Reh and Tully, 1986; Reh, 1987).

What about birds and mammals? The bird and mammalian retina also show a distinct central-to-peripheral gradient in histogenesis. In birds, nearly the entire retina is generated in a relatively short period of embryogenesis, but a small number of neurons/glia are added at the retinal margin in posthatch chicks (Fischer and Reh, 2000). The proliferation of cells in the normal posthatch chicken CMZ is relatively modest, but can be increased as much as 10-fold by intraocular delivery of growth factors (Fischer and Reh, 2003a). Unlike the CMZ of fish and amphibians, the cells at the margin of the chick retina only generate bipolar and amacrine cells (Fischer and Reh, 2000) in the undamaged retina, suggesting that the progenitors in the CMZ may be restricted to producing particular neuronal cell types. But the combination of insulin and FGF2 stimulate production of ganglion cells, suggesting that the types of cells produced in the avian CMZ are limited by local microenvironment rather than cell-intrinsic constraints (Fischer and Reh, 2003a). The mammalian retina is even more limited than the bird with respect to the potential for regeneration from the CMZ. Mammalian retina, including human, rats and mice, have no significant persistent neurogenic sources; in mice, neurogenesis is completed within the first 10 postnatal days and prolonged administration of BrdU in adults fails to label cells at the retinal margin (Moshiri and Reh, 2004). Analysis of primate retina has also failed to find convincing evidence of a zone of proliferation at the retinal margin analogous to that of nonmammalian vertebrates (Fischer et al., 2001). These results indicate that there has been a gradual loss of this neurogenic zone from fish to mammals. Nevertheless, the eyes of mammals grow quite extensively after the completion of neurogenesis, but this growth results in a thinning of the retina, rather than a production of new neurons as occurs in cold-blooded vertebrates.

What factors are responsible for the CMZ formation and maintenance? Some clues have come from the understanding of the patterning of ocular domains. Wnt signaling promotes ciliary epithelium development (Cho and Cepko, 2006); Wnt2b expressed in the developing iris may provide this signal (Kubo et al., 2003). The same signal may also be critical for the CMZ to form by maintaining the cells in a stem cell state (Kubo et al., 2003). Since Bmp4 also promotes ciliary epithelial formation (Zhao et al., 2002a), Noggin might also play a role in maintaining this niche, as it does for the SVZ (Lim et al., 2000). Shh, an important mitogenic factor for retinal progenitors during embryonic stages, is also expressed in the CMZ (Moshiri et al., 2005). Intraocular injections of Shh promote proliferation in this zone in birds (Moshiri et al., 2005) and mice with a mutation in the *Ptc* gene that leads to overactivation of the Shh signaling pathway have a zone of persistent proliferation at the retinal margin reminiscent of the CMZ in nonmammalian vertebrates (Moshiri and Reh, 2004).

Transdifferentiation I: Amphibians and the Pigment Epithelium

Urodele amphibians have remarkable regenerative abilities. It has been known for many years that the newt retina can regenerate nearly perfectly after its removal. The source of the new retina has been shown to be the RPE (Figure 2). If the retina is experimentally removed, the RPE proliferates and generates two new epithelial layers: a pigmented layer and a nonpigmented layer. The latter activates a retinal progenitor pattern of gene expression and undergoes extensive proliferation to generate the correct types and numbers of retinal cells (Moshiri et al., 2004). The process of regeneration appears to involve a dedifferentiation step and then a second step, which is essentially a recapitulation of normal development. The regeneration of retina from the RPE was one of the first demonstrations of ''transdifferentiation'' (Okada, 1980), in which a differentiated cell undergoes a change in fate to form a functionally distinct cell type. A similar phenomenon of RPE transdifferentiation also occurs in the embryonic eye of chicks (Coulombre and Coulombre, 1965; Park and Hollenberg, 1989; Pittack et al., 1991). If the neural retina is removed from the chick embryo eye within the first 3 to 4 days of incubation, the RPE dedifferentiates to form a layer of retinal progenitors, which then proliferate and differentiate to form a new retina, a process very similar to that which occurs in the newt. In the chick embryo, this process is stimulated by FGF, which also stimulates retinal regeneration in amphibians. However, while some amphibians have the ability to regenerate their retinas from the RPE throughout life, the chick embryo loses this ability after the first 4 days of incubation. The molecular mechanisms that regulate the regeneration of retina from the RPE are only beginning to be understood. As noted above, FGF and downstream signaling kinase pathway components stimulate regeneration (Spence et al., 2007), while activin signaling inhibits it (Sakami et al., 2008). Recent evidence indicates that activin signaling may be part of the mechanism that limits the competence of the

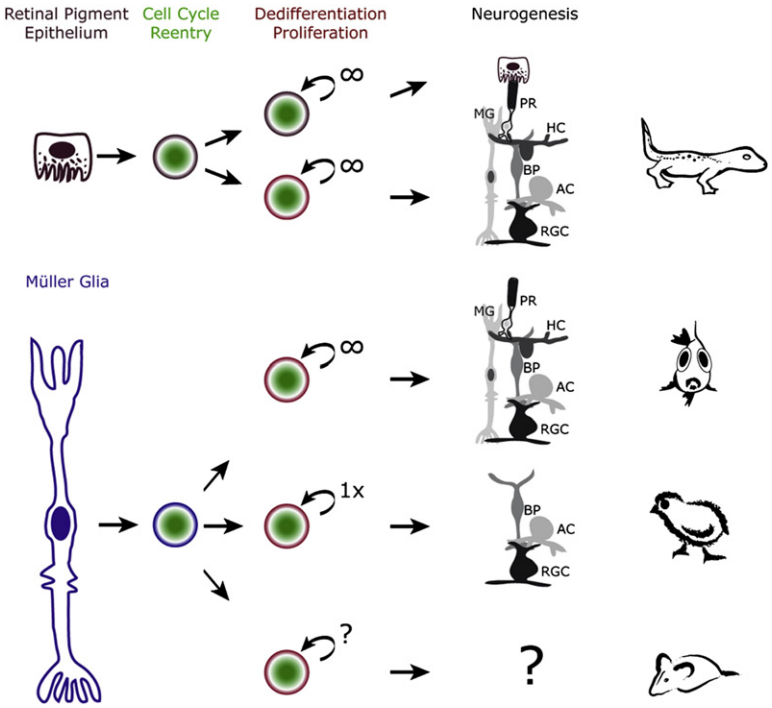

Figure 2. Regeneration in Various Vertebrates

Regeneration in the retina primarily relies on the dedifferentiation of support cells. The pigmented epithelium (RPE) is the source for retinal regeneration in amphibians. If the retina is removed in these animals, the RPE cells re-enter the cell cycle, lose their pigmentation, and re-express genes present in embryonic retinal progenitor cells. An entire retina, including new RPE cells, is generated. In fish and birds, Müller glia spontaneously re-enter the cell cycle after retinal damage, and they re-express embryonic retinal progenitor genes. In the fish, the progenitors generate all types of retinal neurons and a functional circuit; however, in birds, the regeneration is more limited and only a few types of neurons are replaced. In the mammalian retina, Müller cells do not spontaneously re-enter the cell cycle, but can be induced to do so by intraocular injections of growth factors.

RPE to dedifferentiate into retinal progenitors, since inhibition of this pathway extends the competence period (Sakami et al., 2008). One key transcription factor, regulated by the activin and FGF signaling pathways is *Pax6*, and overexpression of *Pax6* is sufficient to stimulate the production of neurons from the RPE (Azuma et al., 2005).

Is the RPE capable of generating retinal progenitors in the mammalian retina? Several reports have shown that pigmented cells isolated from the ciliary epithelium of several mammals, including mice, pig, and humans, can give rise to neurospheres in vitro (Ahmad et al., 2000; Asami et al., 2007; Coles et al., 2004; Kohno et al., 2006; MacNeil et al., 2007; Sun et al., 2006; Tropepe et al., 2000). These have been termed retinal stem cells, and may resemble the transdifferentiation process that underlies regeneration in the amphibian and embryonic chick. Although FGF is not specifically required to initiate this process, other genes involved in retinal progenitor cells, such as *pax6*, are required for these cells to generate neurospheres (Xu et al., 2007). The cells that can be cultured from the ciliary epithelium express a variety of neural progenitor markers and can undergo extensive proliferation. Recently, attempts have been made to use the cultured cells in cell transplantation experiments (see below).

Transdifferentiation II: Fish and Birds—The Müller Glia Generate Neurons

The regenerative ability of urodeles is truly remarkable; however, teleost fish also retain the ability to regenerate new retinal neurons following neurotoxin damage or surgical lesions throughout life (Hitchcock et al., 2004; Raymond et al., 2006). All types of neurons can be regenerated in all regions of the retina, through a blastema-like population of proliferating cells that arises adjacent to the site of injury. As described above, the fish retina contains a CMZ at the retinal margin, and the proliferation in this zone is increased after damage to the retina. However, this zone is not thought to contribute to regeneration of neurons in central retina. Instead, the fish retina contains other cells that are mitotically active: the rod progenitors and the Müller glia (Figure 2). Under normal conditions, the mitotically active cells in the central retina generate only rod photoreceptors; they are therefore known as rod progenitors (Johns, 1982). In uninjured fish retina, it appears that very slowly dividing Müller glia generate the rod progenitor cells (Bernardos et al., 2007). For many years, it was thought that the rod progenitor was the primary source of regenerated neurons following damage (Hitchcock and Raymond, 1992); however, recently, the use of transgenic fish with GFP expressed in Müller glia has enabled several labs to demonstrate that these glial cells are the primary source of regenerated neurons in the fish retina. These cells are normally mitotically quiescent, but following damage to retinal neurons, they re-enter the cell cycle and dedifferentiate toward a progenitor-like phenotype (Bernardos et al., 2007; Faillace et al., 2002; Fausett and Goldman, 2006; Fimbel et al., 2007; Kassen et al., 2007; Raymond et al., 2006; Thummel et al., 2008; Vihtelic et al., 2006; Wu et al., 2001; Yurco and Cameron, 2005). Work in multiple labs has shown that fish retinal regeneration leads to almost full architectural, functional (electroretinogram), and simple behavioral recovery in as few as 28 days depending on the type of retinal damage (Fimbel et al., 2007; Vihtelic and Hyde, 2000). Recent studies have focused on the molecular pathways that regulate regeneration in this system (Cameron et al., 2005; Kassen et al., 2007; Raymond et al., 2006; Yurco and Cameron, 2007). These studies are beginning to reveal some of the critical genes that are required for Müller dedifferentiation to the retinal progenitor state. For example, Fausett et al. (2008) have found that *Ascl1*, a gene expressed in retinal progenitors, but not normally in Müller glia, is rapidly induced upon retinal damage (Fausett et al., 2008). Morpholino inhibition of *Ascl1* blocks Müller glial dedifferentiation and re-entry into the mitotic cycle. This result is interesting in that it demonstrates that Müller dedifferentiation is necessary prior to cell cycle re-entry, but also that *Ascl1* is necessary for regeneration of all retinal cell types, whereas in development, it is only required for bipolar cell development (Akagi et al., 2004). The implication is that regeneration and development may use the same

molecular pathways, but there may be differences in the molecular redundancy in the two processes.

The chick retina displays a limited regenerative capacity (Fischer and Reh, 2001a). Regeneration in this system was first demonstrated by eliciting neurotoxic damage of inner retinal neurons with N-methyl-D-aspartate (NMDA) in posthatch chicks (Fischer and Reh, 2001a). Two days after the NMDA induced damage, Müller glia re-enter the cell cycle and dedifferentiate into retinal progenitors (Fischer et al., 2002; Fischer and Reh, 2001a, 2003b; Hayes et al., 2007). Some of the progeny (<10%) of the Müller glial-derived progenitors differentiate into amacrine cells and bipolar cells. If the proliferating Müller glia are treated with Insulin and FGF, their progeny can also express markers of ganglion cells, including *Brn3* and *Neurofilament* (Fischer and Reh, 2003b). A striking feature of regeneration in the chick retina is that most (80%) of the progeny of the Müller glia continue to express the progenitor markers *Pax6* and *Vsx2* for up to two weeks after damage. Another interesting feature of regeneration in this system is that the regenerative response occurs in the central retina in birds less than 1 week of age but continues only at the periphery after day 7, up to at least 1 month of age (Fischer and Reh, 2003a).

Mammalian Müller glia, by contrast, do not spontaneously re-enter the mitotic cycle after retina damage (Chang et al., 2007; Close et al., 2006; Dyer and Cepko, 2000; Sahel et al., 1990; Zhao et al., 2005), though they can be stimulated to do so with growth factor treatments combined with damage. Some studies have proposed that mammalian Müller glia could also serve as a source of retinal regeneration in the adult rat retina. Ooto and colleagues used NMDA to induce damage in the adult rat retina (Ooto et al., 2004) and found a small number of Müller glia incorporated BrdU. Although these cells were limited in number, the authors report that some BrdU+ cells expressed markers of bipolar cells and photoreceptors. The number of regenerated neurons was increased by treatment with retinoic acid or the misexpression of basic helix-loop-helix and homeobox genes *Math3*, *NeuroD1* and *Pax6*, or *Crx* and *NeuroD1* to produce bipolar, amacrine cells or photoreceptors, respectively. The possibility that limited regeneration can occur in the mammalian retina is further supported by other recent publications that have also reported BrdU+ photoreceptors following either retinal damage or growth factor treatment in adult rodents (Close et al., 2006; Das et al., 2006; Osakada et al., 2007; Wan et al., 2007, 2008). A major caveat of in most of these experiments is that cytoplasmic markers were used to identify the cells, without confocal microscopic analysis; similar studies of adult neurogenesis in other regions of the nervous system have been disputed when more careful analyses were carried out (Dayer et al., 2005; Breunig et al., 2007). In addition, to date, there have been no studies of functional regeneration in either the chick or the mammalian retina, and so it is still not known whether Müller glial-derived cells that express neuronal markers are functionally integrated into the retinal circuit.

What are the differences among the retinas of fish, birds, and mammals that have led to a progressive reduction in the ability of the Müller glia in these tissues to regenerate lost neurons? The process of regeneration in this system involves at least the following four steps: (1) Müller glia re-enter the mitotic cycle, (2) dedifferentiate into retinal progenitors, and (3) generate neurons and glia that (4) integrate stably into functional circuits. In fish, all these steps occur relatively seamlessly. In birds, the first step occurs, though proliferation appears to cease after a single mitotic cycle. Nevertheless, a considerable amount of dedifferentiation occurs, such that many progenitor genes are re-expressed in at least some of the progeny of the Müller glia. The later steps in the process, however, are much less robust in birds than in fish, and few of the progeny stably express neuronal markers for weeks after the damage (Hayes et al., 2007; Moshiri et al., 2004). In the mammalian retina, none of these steps seem to occur spontaneously following damage to the extent that they occur in fish. Although Müller glial are postmitotic in the mature retina, proliferation can be stimulated with mitogens, but it is still not clear to what extent the progeny proliferate and dedifferentiate, or whether any of the progeny can differentiate as neurons that are stably incorporated into existing retinal circuits.

The lack of proliferation in the mammalian retina following retinal damage suggests that some mechanisms are in place to limit proliferation in these cells. Close et al. (2005) investigated this question by analyzing the factors that signal the termination of neurogenesis in the developing retina. They found TGFβ2, produced by retinal neurons, activates the TGFβ-receptor in progenitors, and then later in Müller glia, to promote mitotic quiescence. The mechanism of cytostasis in this system involves TGFβ-dependent expression of the cyclin dependent kinase inhibitor, $p27^{kip}$. TGFβ also acts as a cytostatic factor on adult neurogenesis in the brain (Aigner and Bogdahn, 2008; Buckwalter et al., 2006; Wachs et al., 2006), and the mechanisms appear to be similar to those described for the retina. In addition to the inhibition of proliferation mediated by TGFβ2, Müller glia show a marked decline in their expression of EGF receptor as they mature (Close et al., 2006). The combination of active inhibition and loss of sensitivity to mitogens leads to a tight regulation of proliferation that appears to be absent in both fish and posthatch chick Müller glia.

Another major difference between fish, chick, and mammalian retinal regeneration is the survival of dedifferentiated Müller glia and newly generated neurons. In the fish retina, no apoptosis of Müller glia has been observed during the process of regeneration; however, PCNA knockdown after damage led to significant apoptosis and Müller glia loss (Thummel et al., 2008). In retinal regeneration of the fish, 50% of the Müller glial-derived progenitors or their progeny undergo apoptosis during the first week (Fausett and Goldman, 2006), but regenerated neurons survive up to 180 days after injury. A similar phenomenon is observed in the chick retina; less than half of the cells generated by the Müller glia are still alive after 2 weeks (Hayes et al., 2007). The failure of most neurons to be maintained after their genesis in the adult CNS has also been reported for the olfactory bulb and the hippocampal dentate gyrus; it appears that many are called but few are chosen, even in those parts of the CNS that display exemplary properties of endogenous repair.

Despite the progressive decline in regenerative potential during vertebrate evolution, there are reasons for optimism in the possibility that endogenous repair mechanisms can be stimulated in this system in higher vertebrates. First, it was long thought that the source of regeneration in the fish was either the rod precursor (a cell that is not present in the mammalian retina) or a cryptic endogenous stem cell (a cell that has not been

found in the mammalian retina). However, recent studies have shown that the Müller glia, a cell found in all vertebrate retinas, is the primary source of new neurons after damage, and therefore, the cellular basis for regeneration is present in mammalian retinas. Second, the process of dedifferentiation in the fish and avian retinas are remarkably similar. The Müller glial cells respond to the damage of surrounding neurons by re-expressing key components of the neurogenic mechanism, such as *Notch1*, *Pax6*, and *Ascl1*. Although it is not yet known whether mammalian Müller glia re-express the components of this molecular program after injury, it is clear that at least in fish and birds, *Ascl1* and *Notch1* are necessary for Müller glial dedifferentiation. Therefore, it should be possible to determine whether the bottleneck in mammalian regeneration occurs prior to or following this point. Third, the retina has served as a model to understand the mechanisms of neuronal development for over 20 years, and there is a wealth of information on the regulation of retinal progenitors to draw on in attempts to stimulate endogenous repair.

Cell Replacement Strategies for Retinal Repair

The lack of an endogenous repair mechanism in the mammalian retina has stimulated a number of groups to explore the possibility of neuronal replacement to restore retinal function following degeneration. The first report of retinal transplantation in mammals was in 1946 by Katharine Tansley, who transplanted embryonic rat eyes into the brains of 2-day-old rats (Tansley, 1946). Subsequently, a number of intra-cranial transplantation studies were carried out by Raymond Lund's lab in 1980s and 1990s to address developmental questions of axon pathfinding and topographic map formation in mammals. They found that the axons of the transplanted cells managed to make contacts along the visual pathway (Lund and Hankin, 1995; McLoon et al., 1981; McLoon and Lund, 1980) and develop light responses (Klassen and Lund, 1987). The rats could be trained to respond to certain behavioral tests using just the visual information from the graft site (Coffey et al., 1989; Klassen and Lund, 1990). This early work showed that the mature nervous system retained the potential to make functional connections with transplanted neural tissue. More direct transplantation of cells into the eye also has a long history. Royo and Quay (1959) transplanted rat fetal neural retinas into the anterior chamber of adult eyes and saw long-term survival of these grafts. These studies were followed up by del Cerro and colleagues to determine whether the eye was immune-privileged enough to allow survival of the graft (del Cerro et al., 1985, 1987). The grafts survived and differentiated well, but there was no clear migration of the transplanted cells into the posterior chamber of the eye and their subsequent integration into host retina.

Since these early studies, most of the work in this field has concentrated on replacement of cells within the retina, by transplantation to either the vitreous or the subretinal space. Following intravitreal transplantation, it has been observed that migration of the transplanted cells into the retina is usually limited to developing retinas of newborn rodents and is almost nonexistent in adults (Takahashi et al., 1998). However, degenerating retinas appear to stimulate migration and integration of intravitreal transplantation. Cell migration into the outer nuclear layer is more effective in normal adult retina if a subretinal approach is used instead of the intravitreal injection. However, even the most successful transplantation results in only approximately 1% of all transplanted cells to migrate into the retina (Lu et al., 2002; MacLaren et al., 2006).

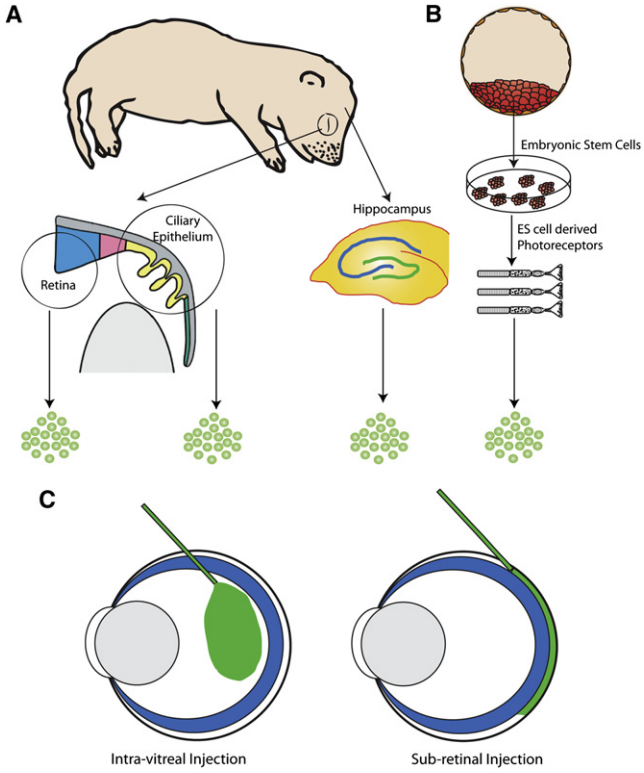

Figure 3. Sources and Routes of Transplantation into the Retina
(A) Cells from either the eye or the brain of embryonic or newborn mice have been most successfully used for transplantation. The two main sources of cells from the eye are retinal cells or stem cells isolated from the ciliary epithelium. Brain-derived neural progenitors have been isolated from the Hippocampus, the site of constant neurogenesis.
(B) A potential alternate renewable source is embryonic stem cells obtained from inner cell mass of the blastocyst. These pluripotent cells can be induced to a photoreceptor fate and then used for repair.
(C) The two main routes of transplantation into the eye are intravitreal, i.e., injecting cells into the vitreal cavity, the space between the lens and the retina, or subretinal i.e., injecting into the potential space between the retina and the pigment epithelium.

In addition to testing different sites for transplantation, several potential sources for replacement neurons and photoreceptor cells have been analyzed (Figure 3). These include (1) intact sheets of embryonic retina, (2) dissociated retinal cells, (3) multipotent retinal progenitor cells, (4) neural progenitors derived from the hippocampal progenitor zone, (5) human embryonic stem cells, and (6) cells derived from the pigmented ciliary epithelium. A thorough discussion of the results obtained from each of these sources is beyond the scope of this review; however, some general principles have emerged from a comparison of these very different cell preparations.

The most common source of cells for transplantation is the developing retina. At either embryonic or neonatal stages in rodents, the retina contains a mix of retinal progenitors and differentiating neurons and photoreceptor cells. Developing retinal cells have been transplanted as intact retinal sheets (Seiler and

Aramant, 1998), small pieces containing tens to hundreds of retinal cells, i.e., microaggregates (del Cerro et al., 1991; Kwan et al., 1999), or as dissociated cell suspensions (Chacko et al., 2000; Qiu et al., 2005). Although retinal sheets and microaggregates survive and differentiate well in the subretinal space, the cells in these grafts typically develop into rosette-like structures, which results in poor migration of cells out of the grafts, severely restricting the integration of the transplanted cells with the host tissue. Enzymatically dissociating the developing retinas and transplanting retinal cell suspensions circumvents this problem somewhat (depending on the age of the donor cells) and results in better integration of the transplanted cells (Qiu et al., 2005).

The most successful use of this approach was in a recent study by MacLaren et al. (2006); the authors transplanted a suspension of retinal cells obtained from GFP-expressing transgenic mice into the subretinal space of wild-type mice of different ages. They found that GFP-expressing cells migrated into the outer nuclear layer, developed the morphology of differentiated photoreceptors, including organized outer segments, and made synaptic connections in the outer plexiform layer with the host bipolar cells. The transplanted cells expressed rod phototransduction pathway proteins, rhodopsin and peripherin, and rod-specific glutamate receptor mGluR8. Interestingly, they found that the successful integration of the dissociated cells that they transplanted was not dependent on the age of the host that received the transplant, but rather was critically dependent on the age of the donor cells. As expected, cells derived from mature retinas did not survive or integrate following transplantation. Surprisingly, the most immature donor cells, from embryonic stages, also were not effective for transplantation; the cells from embryonic retinas did not integrate into the host retina, but rather formed rosettes in the subretinal space. By contrast, those donor cells most effective at integration into host retinas after subretinal transplantation were derived from retinas of animals in their first postnatal week. This corresponds to the stage of retinal development when the majority of rod photoreceptors are generated, and the authors suggest that newly postmitotic rod photoreceptors, rather than progenitor cells, have the greatest potential for successful integration into the host retinal circuit. This conclusion was confirmed by selectively transplanting newly born rod photoreceptors from transgenic mice, which express GFP in all postmitotic rods under the *Nrl* promoter (Mears et al., 2001). On transplantation, the *Nrl*-GFP+ rod photoreceptors integrated as effectively as the unpurified cell suspension.

Due to the limited supply of embryonic or fetal tissue for ultimate clinical application, several groups have attempted to use retinal cells that have been expanded in vitro. It has been known for some time that retinal progenitors from both rodents and humans can be expanded in dissociated cell cultures and will retain their potential to differentiate into various types of retinal neurons, including rod and cone photoreceptors (Anchan et al., 1991; Kelley et al., 1995). Various protocols have been used to propagate retinal progenitors prior to transplantation. In general these studies have shown that progenitors can survive in either the vitreous or subretinal space following transplantation and that, like freshly dissociated cells, can integrate into the various retinal layers (Klassen et al., 2004). The transplanted cells also express proteins consistent with neuronal differentiation, like MAP-2. However, unlike the studies of freshly dissociated retinal cells described above, the cells derived from the progenitor cultures do not develop the morphology or expression profile of rod or cone photoreceptors.

Another potential source of neural progenitor cells that can be expanded in vitro are hippocampal progenitor cells (Takahashi et al., 1998; Young et al., 2000). These progenitors are obtained from the dentate gyrus of the hippocampus, a site of active neurogenesis even in adult mice. Hippocampal-derived neural progenitors exhibit a high degree of migration into all layers of the retina following intravitreal injection in either newborn mice or adult mice with retinal degeneration. Despite their hippocampal origin, they develop morphologies highly reminiscent of several types of retinal neurons; however, they did not show an ability to differentiate into photoreceptors, i.e., none of the cells expressed opsin.

As mentioned above, several laboratories have reported that neurospheres can be generated from adult pigmented iris and ciliary epithelium of rodents, humans, and pigs (Ahmad et al., 2000; Asami et al., 2007; Coles et al., 2004; Kohno et al., 2006; MacNeil et al., 2007; Sun et al., 2006; Tropepe et al., 2000). Since the presence of sphere-generating cells in the CNS has been associated with neural stem properties (Reynolds et al., 1992), the fact that pigmented cells from the iris and ciliary epithelium can generate spheres raises the possibility that they are latent retinal stem cells (Tropepe et al., 2000). However, it is also possible that these cells arise from a transdifferentiation process similar to that present in the RPE of lower vertebrates. The presence of a central-to-peripheral gradient in development in the eye means that cells of the peripheral ocular tissues are less mature than those located more centrally. Even in the adult eye, the most peripheral RPE and ciliary epithelial pigmented cells may, therefore, remain relatively undifferentiated (Asami et al., 2007; Fischer and Reh, 2001b). In all the above reports, the progeny of the iris or ciliary epithelial-derived spheres can differentiate into cells that express neuronal proteins and morphology consistent with neuronal differentiation. Interestingly, intraocular injections of growth factors can induce neuronal differentiation within the ciliary epithelium in vivo as well (Fischer and Reh, 2003a). The evidence for photoreceptor differentiation, however, is less consistent. Some groups have reported rhodopsin expression (Asami et al., 2007; Tropepe et al., 2000), though others have failed to find definitive photoreceptor markers (MacNeil et al., 2007). In vitro, many aspects of photoreceptor development do not occur; outer segments do not form, for example (Reh and Kljavin, 1989). Therefore, to promote further differentiation of photoreceptors, Coles et al. (2004) transplanted ciliary derived spheres into newborn mice using the intravitreal approach. They reported migration of the transplanted cells to the subretinal space and some integration into the outer nuclear layer. Some of the cells expressed the photoreceptor marker Rom1, though many also retained aspects of their pigment epithelial fate and continued to express bestrophin. Taken together, the evidence indicates that pigmented cells in the iris and ciliary epithelium of many species, including humans, can be maintained in vitro for extended periods of time and generate large numbers of progeny; however, it remains to be determined whether these cells can be used to generate photoreceptors that will be useful for cell replacement therapy.

Embryonic stem cells (ESCs) might also be a source for replacement retinal neurons since they are pluripotent with the

ability to differentiate into any cell type in the body. Their property of indefinite self-renewal makes them an ideal source for cellular replacement therapy. The challenge is to be able to efficiently coax them to develop as retinal progenitors or preferably postmitotic photoreceptors. A few groups have published reports of inducing a retinal fate in mouse embryonic stem cells either using growth factors or by transfecting retinal progenitor genes (Ikeda et al., 2005; Sugie et al., 2005; Tabata et al., 2004; Zhao et al., 2002b). The most efficient induction was shown by Ikeda and coworkers using extrinsic factors normally involved in neuroretinal development (Ikeda et al., 2005). Using lefty-A, Dkk1, and Activin A to induce differentiation, they found that 25%–30% of cells expressed *Pax6* and *Rx*, two important eye-field transcription factors. The cells, upon differentiation by coculture with adult retinas, formed rhodopsin- and recoverin-expressing photoreceptors.

Human ESCs can also be directed to a retinal cell fate using a combination of a BMP inhibitor, a Wnt inhibitor, and IGF-1 (Lamba et al., 2006) or a combination of Wnt and Nodal antagonists (Osakada et al., 2008). The Lamba protocol resulted in approximately 80% of all cells acquiring a retinal progenitor fate as assessed by expression of eye field transcription factors (Lamba et al., 2006). The cells differentiated under this protocol also expressed early markers of photoreceptor differentiation like Crx, Nrl, and recoverin. When these human ESC-derived retinal cells were cocultured with explants of adult retinas from wild-type and degenerated retinas, the donor cells integrated with the host explants and underwent enhanced photoreceptor differentiation; this suggests that microenvironmental cues may play a role in the final differentiation of these cells. More recently, another group has reported a similar protocol for directing human ESCs to a retinal fate. Though both protocols use related antagonists of Wnt and BMP/nodal pathways (Osakada et al., 2008), the Lamba protocol led to only limited photoreceptor differentiation, while the Okasada protocol was more efficient in this regard (although this took approximately 5 to 6 months). Recently, human ESC-like cells, known as induced pluripotent stem cells (iPSCs), have been derived by overexpressing a group of key pluripotency genes in adult fibroblasts (Takahashi et al., 2007; Yu et al., 2007). These cells have most characteristics of ESCs and could help curtail some of the ethical concerns associated with the use of embryonic stem cells and also help in creating nonimmunogenic cells for transplants. Thus, human ESCs could be an excellent source for retinal replacement therapy as they could provide the millions of cells needed for such procedures.

For cell replacement therapy to be effective in retinal repair, the transplanted cells will have to integrate into the existing retinal circuit. While the retina is an ideal tissue to test for functional repair, due to the extensive literature on retinal structure function analysis, most of the studies to date using transplantation have not analyzed the effects of the new cells on the function of the retina. However, more recent studies have analyzed retinal function using electrophysiological methods and behavioral tests. For example, using pupillometry and extracellular field recording in $Nrl^{-/-}$ mice, MacLaren et al. (2006) showed that the transplanted cells both responded to light flashes as well as transferred the information to the downstream retinal circuit. These results provide evidence that transplanted cells can integrate into mature neuronal circuits, even in regions of the CNS where neuronal replacement is not normally observed. They also show that even a relatively small number of transplanted photoreceptors, a few thousand even in their best cases, can be enough to provide significant functional responses. This is remarkable given that the mouse retina contains millions of rods but is consistent with clinical reports that patients with very small numbers of photoreceptors retain some vision.

A consistent observation in transplantation studies in the retina is the very limited migration of transplanted cells from the subretinal space. This could be either due to formation of a barrier as the Müller glia mature and the astrocytes migrate into the retina (Zhang et al., 2003) or a change in extracellular matrix (ECM) composition of the mature retina. Some studies have shown that migration is enhanced if the retinal architecture is compromised, either following light damage in albino rats (del Cerro et al., 1988) or following transplantation in retinal dystrophic rats (Young et al., 2000). Breaking the glial barriers could be a way to allow more cells to move into the retina. This idea has been tested by a recent study that used mice lacking intermediate filament protein normally present in glia (Kinouchi et al., 2003). The group used the glial fibrillary acidic protein and vimentin double knockout mice and assessed migration of cells following subretinal transplantation of newborn retinal cells. The group found that there was a 3- to 4-fold increase in the number of cells that migrated into the various retinal layers. These results suggest that the astrocytes and Müller glia act as barriers to the movement of cells into the retina, and manipulating them using certain pharmacological agents could potentially help in improving the therapeutic response. As mentioned above, the maturation of the ECM in adult retinas may also serve to inhibit transplant cell migration. Altering the ECM by injection of matrix metalloprotease (MMP-2) resulted in enhanced migration of retinal progenitors in explant retinas (Suzuki et al., 2006).

A final approach in which cell transplants may prove useful for the treatment of retinal diseases is through the replacement of the RPE. As noted in the first section of this review, AMD, one of the most common retinal degenerations, leads first to the loss of the RPE cells, and then the loss of the cone photoreceptors in the fovea. If the dying RPE cells could be replaced prior to the degeneration of the cones, this could obviate the need for direct neural retinal repair. Toward this end, two groups have developed methods to derive RPE cells from human ESCs. Haruta et al. (2004) found that RPE cells, easily identified by their pigmentation, spontaneously developed in monkey ESC cultures when exposed to the PA6 stromal cell line. The cells survived transplantation to the subretinal space of a rat model of retinal degeneration, and even improved visually mediated behaviors. Similar results were obtained by the groups of Lund and Lanza (Lund et al., 2006) with human ESC-derived RPE cells; the RPE cells derived from human ESCs were from any of 18 different lines. These promising animal results will likely be translated soon into one of the first clinical trials of human ESC products sometime this year by Advanced Cell Technology.

Concluding Remarks

The regeneration of the vertebrate retina has been studied for many years, and although classic studies have characterized the basic phenomena, there remains a great deal to learn. Nevertheless, the results from experiments in the retina can inform

studies of other tissues. For example, there has been a debate as to whether regeneration of tissues arises from the dedifferentiation of existing cells or from existing stem or progenitor cells resident in the tissue. In the retina, both types of regenerations are observed: the stem cells of the CMZ in fish and amphibians responds to retinal damage by increasing the production of new neurons, whereas the differentiated cells of the RPE layer in urodeles can dedifferentiate into retinal progenitors that go on to replace the neurons and glia of the retina. Another phenomenon that is clear in the retina, and perhaps less well appreciated in other tissues, is that there is a progressive loss in regenerative potential with eye evolution. The same cell, the Müller glia, can be identified in all vertebrate retinas, but while this cell undergoes spontaneous dedifferentiation into a retinal progenitor in fish, its potential for dedifferentiation is more limited in birds and almost nonexistent in mammals. A similar phenomenon occurs in the loss of the RPE to contribute to regeneration. In the amphibian, this tissue is able to completely dedifferentiate into a cell with all the potential of retinal progenitors, while in birds and mammals, these cells have this capacity only during embryonic stages. The loss in dedifferentiation "potential" appears to occur through stages in development in birds and mammals, while newts and other urodelian amphibians retain the ability of their RPE to dedifferentiate throughout their lifetime. The progressive stabilization of phenotypes, either RPE or Müller glial, appears to occur through several stages and may be due to restrictions in gene activation as the cells mature. Changes in chromatin accessibility or expression of miRNAs directed to progenitor transcripts are among the possible mechanisms by which phenotype of these cells might be "solidified" over development and over evolutionary time.

In addition to the attention the eye has received in the regeneration field, the retina is perhaps the best understood part of the CNS in terms of its circuitry and function. As a result, there are good tests for functional repair in the retina, and this makes it an attractive tissue to study cell replacement in the CNS. The contributions of transplanted or regenerated cells to visually guided behavior make the retina an ideal place to test the capacity for neuronal regeneration and replacement in the CNS. Although the retina, like most regions of the CNS, does not have ongoing neurogenesis, transplant studies indicate that new neurons can be incorporated into the circuitry and contribute to visual mediated behaviors.

In summary, research in retinal regeneration provides a good example of how the concepts and methods of regenerative medicine can be brought to bear on a clinically significant group of degenerative disorders. Studies across nonmammalian vertebrates have shown there are many strategies for endogenous repair mechanisms to restore the retina after damage; however, none of these are spontaneously engaged in the mammal. Recent research has focused on gaining a better understanding of the molecular mechanisms that limit successful regeneration from mammalian Müller glia, but it has also recently become clear that functional cell replacement, potentially using retinal cells derived from human ESCs, is a feasible and promising alternative. Over 200 years after Bonnet, the renewed focus of regenerative medicine and stem cells on retinal repair has generated a new optimism in both the scientists in this field and the patients they hope to help.

WEB RESOURCES

Advanced Cell Technology (2008). Fact Sheet, http://www.advancedcell.com/fact-sheet

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