Putting Regeneration into Regenerative Medicine

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The prevalence of blinding diseases such as age-related macular degeneration (AMD), glaucoma and diabetic retinopathy have significantly increased in the United States over the last decade.[1] In response to the higher incidence of retinal degenerative diseases, the National Eye Institute (NEI) of the US National Institutes of Health (NIH) has recently proposed the audacious goal of regenerating neurons and neural connections in the eye and visual system.[2]

To date, regenerative medicine has to a large extent focused on determining how to reprogram stem cells into retinal neurons that can be used to restore vision. Recent surprising findings, however, suggest that mammals may possess limited *in vivo* regenerative potential. The challenge now lies in understanding why the retina in other vertebrates regenerates, while mammals lack this ability. In this review, we summarize current knowledge on endogenous mechanisms of regeneration in frogs and how these findings might provide essential information to stimulate regeneration in the human retina.

ORGANIZATION OF THE RETINA AND CELL TYPES AFFECTED BY DAMAGE AND DISEASE

The retina is the light sensing tissue at the back of the eye that converts light into electrical signals that are interpreted as a visual image by the brain (Figure 1A). The vertebrate retina consists of seven major cell types: ganglion cells, horizontal cells, cone photoreceptors, rod photoreceptors, amacrine cells, bipolar cells and Müller glial cells. The retinal pigmented epithelium (RPE) is the pigmented cell layer outside the neural retina that supports photoreceptor function. The overall organization and major cell types present in the human retina are also present in other mammals as well as non-mammalian vertebrates.

Retinal diseases that cause blindness, either as a result of an inherited condition or age, often first affect a single cell type. The common denominator in these diseases is the initial death of a specific retinal cell type that results in subsequent loss of other retinal neurons and progressive retinal degeneration.[3] Rod photoreceptors are the first cell type affected in some types of Stargardt's disease, while both rods and cones are initially affected in some types of retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA).[4,5] The RPE is the initial cell target in AMD and Best's disease.[6-11] In glaucoma, ganglion cells are the first cells to be compromised in the retina.[12] Accidental traumatic retinal injury due to head injuries or direct damage to the eye often result in photoreceptor cell death.[9] Since the retina in humans and animal models of regeneration have the same retinal cell types and organization, these models can be used to understand how to regenerate the initial cell types lost during disease.

REGENERATION IN THE MAMMALIAN RETINA

Regeneration is the process by which lost or injured body parts are replaced.[13] Mammals, including humans, cannot regenerate damaged neural tissues. The retina in mammals does not regenerate spontaneously once injured. Instead, injury results in cell death and permanent loss of the affected neurons, often causing blindness.

Regeneration in non-mammalian vertebrates occurs by transdifferentiation of mature retinal/RPE cells or from an intrinsic pool of proliferating

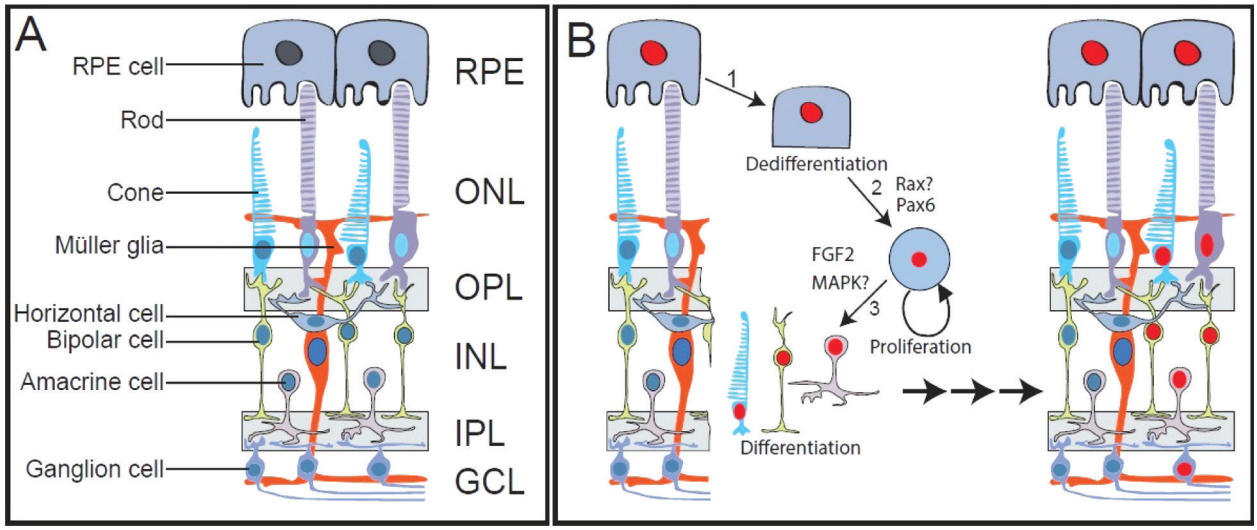

Figure 1. (A) Organization of the vertebrate retina. The vertebrate retina consists of seven retinal cell types and the RPE cells. Each cell type is labeled. The different retinal layers formed by the different cell types are indicated on the left. (B) Retinal regeneration in frogs. The frog retina regenerates by transdifferentiation of the RPE. After injury, RPE cells dedifferentiate (1), proliferate (2) to generate new retinal progenitors that differentiate (3) to restore all retinal cell types in the damaged retina. The cell progeny of the transdifferentiating RPE cell is indicated with red nuclei. The known factors involved in each step are shown. MAPK and Rax are likely to function at the steps indicated, however these steps have not yet been explicitly demonstrated.
RPE: retinal pigmented epithelium; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer, GCL: ganglion cell layer; FGF2: fibroblast growth factor 2; MAPK: mitogen activated protein kinase, Pax6: paired box protein 6; Rax: retinal homeobox

retinal progenitors.[14,15] Transdifferentiation occurs when a mature cell dedifferentiates and proliferates to generate new cells. Intrinsic progenitors are a population of cells that proliferate constantly throughout the life of the animal to produce new cells in response to growth or injury. In the mammalian retina, a source of intrinsic progenitors was discovered in the pigmented ciliary epithelium (PCE) of the ciliary body.[16-20] When cultured, PCE cells spontaneously formed clonogenic spheres, and when differentiated, generated progeny that expressed typical markers of retinal neurons.[21] More recently, it was determined that cultured PCE spheres lacked the ability to differentiate into photoreceptors and maintained characteristics of PCE cells.[22,23] Due to their therapeutic potential, the search for proliferating progenitors in the mammalian retina is ongoing.

RPE transdifferentiation in mammals has been explored as a possible source of new retinal cells. *In vitro*, rat and mouse RPE transdifferentiate into retinal neurons when treated with fibroblast growth factor 2 (FGF2) and when activin signaling is blocked.[21,24] *In vivo*, RPE transdifferentiation was observed only during embryogenesis after elimination of the RPE specific fate transcription factor Mitf or the Wnt signaling effector beta-catenin.[25,26] These findings suggest that the mammalian RPE loses its latent capacity to generate retinal cells early in development.

Müller glia can also transdifferentiate and produce new cells after injury. Growth factor treatment demonstrated the potential of Müller glia to proliferate in response to injury.[27-29] Chemical injury of the retina combined with growth factor treatment cause Müller glia to transdifferentiate and generate a limited number of neurons including amacrine, bipolar and rod photoreceptor cells.[27,30-35] Similar to RPE, the ability of Müller glia to transdifferentiate is restricted by various signaling systems. In mice, transforming growth factor beta (TGFß) signaling restricts Müller glia proliferation, an essential step required during transdifferentiation.[27] Misexpression of the proneural gene *Ascl1* in Müller glia results in reduction of Müller cell markers, an increase in neural markers and re-entry into the cell cycle.[36] Together, these studies

demonstrate that the mammalian retina has the potential to initiate a program of regeneration under favorable conditions. Further studies will be required to understand the barriers that prevent regeneration in the mammalian retina.

RETINAL REGENERATION IN FROGS

Frogs are among the non-mammalian vertebrates that regenerate the retina. An established developmental model currently used for regeneration studies is the African clawed frog *Xenopus laevis*. Females of this species can be induced to lay thousands of eggs for experiments by injection of human chorionic gonadotropin. Embryos are fertilized *in vitro* and develop externally, allowing easy experimental manipulation and observation. Also, molecular tools are available to alter gene expression and investigate the function of genes during development. Justification for the use of *X. laevis* in regeneration studies stems from the fact that this species regenerates the retina at both larval and adult stages.

The frog retina regenerates following complete or partial surgical removal, devascularization, chemical damage or genetic ablation.[37-43] The time required for regeneration is dependent on the injury. Partial or complete surgical removal of tadpole or adult retina results in a fully regenerated retina by one month post-injury.[40,43,44] Injury by devascularization caused severe retinal degeneration, after which a new retina was observed two months later.[42] Specific ablation of rod photoreceptors lead to complete regeneration by 30 days, although this injury method also causes retinal degeneration.[37] Thus, in *X. laevis* complete regeneration is observed regardless of the injury paradigm.

Most retinal regeneration in frogs occurs via RPE transdifferentiation with relatively minor contribution from ciliary marginal zone (CMZ) progenitors.[14] RPE transdifferentiation was observed in tadpoles of *Rana catesbienna* and *X. laevis* adults.[42,44] The source of regenerated retina in *X. laevis* tadpoles has not been conclusively determined, but it is likely the cells are derived from the same tissues as in the adult. Retinal regeneration was still observed when the CMZ was removed following retinectomy, suggesting that RPE transdifferentiation might be the source of regenerating cells in tadpoles.[43] The CMZ is thought to contribute some new cells during regeneration. CMZ-derived cells were detected close to the CMZ following chemical injury.[45]

The regenerated retina in frogs has the same architecture of the normal retina and appears to contain all retinal cell types. Following retinectomy or partial resection, immune labeling with antibodies specific for different cell types detected the presence of ganglion, amacrine, bipolar, horizontal, photoreceptor and Müller glial cells.[40,43] Furthermore, regenerated ganglion cells formed an optic nerve.[43] It is unknown if the regenerated frog retina is functional.

The molecules and signaling pathways involved in *X. laevis* regeneration are now being uncovered (Figure 1B). FGF2 promotes RPE transdifferentiation *in vitro* and *in vivo*.[43,46,47] After retinectomy, FGF2 activates the mitogen activated protein kinase (MAPK) pathway; blocking this pathway decreases the amount of regenerated retina.[43] FGF2 functions to promote differentiation of RPE-derived proliferating retinal progenitors into new retinal cells.[46] Transcription factors involved in the specification of retinal progenitors may also be necessary during regeneration. For example, *pax6* is up-regulated in dedifferentiated RPE and its expression is required during the initial steps of RPE transdifferentiation.[46] Also, *rax* expression is up-regulated in dedifferentiating RPE cells during transdifferentiation and *rax* knock-down impairs retinal regeneration in tadpoles.[40,48,49] It is not known if rax functions at the same step of transdifferentiation as *pax6*.

REGENERATIVE MEDICINE IN THE RETINA: DRIVING STEM CELLS TO RETINAL CELLS

The goal of producing retinal cells from stem cells *in vitro* is to restore visual function by transplanting these cells into the damaged retina. Accomplishing this goal requires three steps: (1) generation of cell types necessary for transplantation (2) integration of transplanted cells into the retina (3) functionality of new cells

integrated into the retina.

Generating the number of retinal cells required for successful transplantation requires a renewable supply of cells. A great deal of work has been done to generate neural and retinal precursors *in vitro* from embryonic stem cells (ESCs) of different species including humans.[50-62] Also, adult fibroblasts, RPE or blood T cells have been reprogrammed into pluripotent stem cells (induced pluripotent stem cells or iPSCs) and differentiated into photoreceptors, RPE, ganglion cells, or optic cup-like structures.[63-68]

Successful integration of ESC- or iPSC-derived cells into the retina has been observed. Transplanted ESC- or iPSC-derived cells partially integrate into the retina, express the appropriate markers but in most cases do not exhibit the morphology of differentiated neurons.[51,52,57-60,64,66,69,70] In other cases transplanted cells failed to differentiate, or worse, formed tumors.[64,71,72] Successful transplantation strategies were observed in experiments where isolated mouse rod precursors were transplanted into the diseased mouse retina.[73,74] The donor rod precursors matured into rod photoreceptors when transplanted into models lacking functional rods.[73-76]

More recently, evidence of functional retinal cells derived from ESC- or iPSC-derived retinal progenitors has been observed. Human ESC-derived retinal progenitor cells were shown to integrate into the retina of *Crx*$^{-/-}$ mice (a model of LCA), express cone and rod photoreceptor markers, and restore some visual function.[69] Using a protocol first developed by Sasai and colleagues to generate *in vitro* three-dimensional retinas, some visual function was restored using mouse ESC-derived rod photoreceptors transplanted into the retina of adult *Gnat1*$^{-/-}$ mice (a model of stationary night blindness).[77,78] Importantly, these cells were shown to integrate in multiple mouse retinal disease models.[75] Vision recovery has been incomplete in all reports thus far, and two major barriers to success have been the number of exogenous cells that integrated, and the extent of gliosis.[75] Further work is needed to determine how to generate multipotent retinal progenitors that generate other retinal cell types and address the problem of gliosis in order to allow more efficient integration and survival of functional retinal cells.

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Frogs and, to a very limited extent, mammals regenerate retina. Regeneration is achieved from either intrinsic stem cells or by transdifferentiation. The molecules and signaling pathways required to stimulate regeneration are only beginning to be elucidated. It is, however, becoming increasingly clear that loss of regenerative potential is tightly linked to inactivation of certain genes as the organism ages. Much remains to be learned about these molecular mechanisms to overcome barriers facing retinal regeneration in mammals.

Many questions pertaining to regeneration have not been sufficiently addressed such as similarities between regeneration and embryonic development. As a mature cell dedifferentiates and proliferates to produce retinal progenitors that generate new cells during regeneration, it returns to an embryonic-like (but not necessarily identical) progenitor state. Yet, injury-induced retinal progenitors in the postnatal retina of some species, like mammals, clearly lack the multipotency of true embryonic progenitors. What restricts the multipotency of progenitors generated during regeneration? If regenerating progenitors truly express the same genes as embryonic progenitors, why are they not capable of producing all retinal cell types?

Additional unanswered questions are linked to the relationship between degeneration and regeneration. Some regenerative species undergo a process of retinal degeneration before the retina can regenerate. How is it that these species can overcome the degenerative phase and still regenerate the retina? Conversely, some regenerative species appear only capable of regeneration; however, the retina does not progress beyond a certain critical point. What are the cellular and molecular events that define this critical point beyond which regeneration is no longer possible?

Basic developmental processes have been

proven repeatedly to be conserved throughout evolution. Regeneration is a blatant exception to this general rule. This is precisely why studying regeneration in model systems is important. By determining how "lower" vertebrates can regenerate retina, we will likely identify why mammals cannot. These discoveries may provide new approaches to delay or reverse degeneration of damaged or diseased mammalian retina, which currently always leads to blindness.

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Conflicts of Interest

None.

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