Regeneration Biology
Rajesh Ramachandran
Department of Biological Sciences
IISER Mohali
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W6L26_Cellular, Molecular and Genetic factors involved in retina regeneration

Hello everyone, welcome back to another class on regenerative biology, and in today's class, we will learn about the cellular, molecular, and genetic factors that are involved in the regeneration of mainly retinal tissue. We will see different model systems, and we will also cover some of the reviews and the pictures represented there. So if you look at this picture, you can see this is a diagrammatic representation of the retinal architecture, which is taken from one of our own lab's reviews. Those who are interested can read it. So retinal architecture is very similar to that of humans in zebrafish. So except that they have a slightly, you know, spherical lens.

But when it comes to the retina, there is not too much of a difference among the vertebrate retinas. So you have the retinal pigment epithelium, which is usually black in color, and soon after that, there is an outer nuclear layer where you have rods and cones, and then you have the inner nuclear layer where you have horizontal cells, bipolar cells, amacrine cells, etc. And then you have the ganglion cell layer where the photoreceptors integrate the light signal and pass it on to various neuronal tracts, collecting at the ganglion cell layer, and each ganglion cell gives rise to one optic axon, which will merge thousands and millions of ganglion cells or ganglion axons, merging to give rise to the optic nerve. So remember, although it's a part of the central nervous system, the retina takes the signal from the environment in the form of light, creates action potentials, and goes into the brain.

So, unlike the other cranial nerves or the motor neurons where the brain is commanding, the retinal neuron is kind of reversed. So broadly, you can classify them into the outer nuclear layer, the inner nuclear layer, and the ganglion cell layer, and between the outer and inner nuclear layers, there is something called the outer plexiform layer, which is not a cellular layer but rather has more of cellular processes, like the extension of cytoplasm, and of course, in those places, you can have some. Microglial cells, etc., which will also migrate to macrophages, and then you have an inner plexiform layer (IPL) between the inner nuclear layer and the ganglion cell layer, and in the inner nuclear layer resides the most important cell type, which is the Müller glia, which is not a neuron but rather a glia that can reprogram upon a cellular insult. So they can give rise to other retinal neurons and also to the Müller glia itself.

So this is the kind of thing we have discussed. But in today's class, this is needed. That's why I'm bringing it back. So this is a pictorial representation of different methods of retinal damage. Whenever you want to study retina regeneration, you need to have different injury methods.

So people use either a chemical method or a photobleaching method, where you expose the animal to intense light, or you can even do some physical damage, like surgery or needle poking, etc. Different parts of the eye are shown along the retina. Three primary mechanisms of inducing damage in various retinal types are present, and needless to say, there is also a genetic ablation mechanism. People use either the thymidine kinase ganciclovir system or the nitroreductase metronidazole system. Different methods are available, but the more prevalent ones are the easier surgical, mechanical, light-induced, or photobleaching methods.

So in any case, whenever there is damage, it gives rise to a reprogramming or de-differentiation of the Müller glia, which gives rise to retinal progenitors that are capable of giving rise to all the retinal cell types. The three modes of physical injury involved are with a 30-gauge needle, which is used in our lab, and also a lot of mechanical injury methods in labs that follow the mechanical injury methods. So the removal of a small part of retinal tissue has also been done, which is more prevalent in amphibians, where you take a surgical approach, removing a small portion, somewhat like that of a one millimeter square area or a 1.5 millimeter square area, something like that. You can surgically remove it.

A patch of retinal tissue can be removed with a micro knife and subretinal injection to induce a retinal detachment; that's another method that has been used. What you are seeing is basically a physical, chemical, and light-induced method, and the damage occurs in the form of cell death. The progenitors are formed because of the Müller glia, and the lost damaged vision is restored; that's what you are seeing. Panel A and Panel B are what I mentioned about the physical damage that can be a needle poke or the removal of a patch of tissue, or you can induce a retinal detachment by causing the retina to peel off from the pigment epithelial layer, which is retinal detachment. And then C is done by damage to the different lateral layers caused by intravitreal injection of various concentrations of chemical reagents.

That is what you see in panel C: chemical damage, intravitreal injection, and disruption of ion channels. You can give ovabin or superactivation of neurotransmitters such as NMDA, which means glutamate. Receptors get activated and can cause damage, and that is basically retinal layer-specific damage; you can choose the retinal layer and cause

damage, so that is what you see in C Panel. The chemical agents vary; people have been trying different types of reagents, but one thing we should keep in mind is that chemical methods, even when you use them, always come with some baggage, such as inadvertent toxic effects on the fish itself, so one has to be wary about that. The damage to the photoreceptor cells was induced after keeping the fish in a dark chamber for a long time, followed by short-term exposure to intense bright light; this is the photobleaching method.

Light exposure causes cellular damage through chemical, mechanical, and thermal mechanisms, so even when you damage retinal tissue, the extent of the damage or the mechanism through which the damage occurs can vary; for example, light-induced methods can have different effects. Chemical, mechanical, and thermal effects occur because of the cascading effect; when you put intense light, it can have other cascading effects, so retina regeneration, if you look at a glance, is basically referring to the mechanical injury. This is a normal retina. And soon after injury, you can see the Müller glia cells respond to the injury by reacting to the chemicals that are released and also by the phagocytic action of the Müller glia cells. And they de-differentiate and start expanding in number by cellular proliferation.

And this proliferated Müller glia-derived progenitor can migrate and give rise to normalcy. This picture we also discussed earlier. And if you look closely, what you see is that these cells can be tagged with some genetic tags, such as green fluorescent protein, and that's what you are seeing here in the retina. The neighboring cells you see here are all normal retina, which is not responding to the injury. The response to the injury is the one that is now tagged with the GFP, which has the proliferating marker BrdU, and this is the reason why this picture has been shown here; because of the GFP fluorescence, you can sort them using a cell sorter.

By making use of this green fluorescent protein, you can pick these individual cells and demarcate them with the. Other cell types, so that is what you can see here; that is what has been done here: that the progenitor cells can be purified using a cell sorter, and this is a transgenic line where you have got the marker GFP expressed in every Müller glia. It has GFP expression controlled by a Müller glia-specific marker called GFAP. And you can see this is the cell sorting window in which you can see plenty of cells that are present in this R3 window, which is nothing but this GFP. That means of the total retina in this window, what you are seeing? There are no GFP fluorescent cells, whereas in this window you have a GFP fluorescent cell from the same fish.

You can use it in an uninjured condition, and here you are seeing another transgenic line, which is a 2-bar GFP line, in which the uninjured retina does not have any GFP

expression. You can see there are plenty of non-fluorescing cells here, but there are no fluorescing cells in this R3 window coming from this. Uninjured fish from the same transgenic line, tuba gfp fish, when injured, show expression of gfp-positive Müller glial-derived progenitor cells, which are marked by gfp and can be sorted, and there are gfp-positive cells. You may wonder why the cell number is low; it is simply because. You are giving a mechanical poke, and in the retina, around 40% of the cells are Müller glia, and that is why, in this first window, the entire Müller glia is represented, which has GFP because it's a different transgenic line; whereas here, you get around 2 to 3% of the cells only, even if you give 10 to 12 pokes per eye; that is why the number is less.

And these cells can be picked up, and you can study the gene expression events; you can look into the transcriptome, you can look into the proteome, you can subject them to RNA sequencing methods, etc. You can also do qPCR. One such approach is what you are seeing here: you have the expression of both Thompson and Yamanaka factors in these cells. What you are seeing is that these cells have Thompson and Yamanaka factors. which is having KLF4, OCT4, cMYC, ASCL1A, LIN28, SOX2, and NANOG.

So this indicates that these retina-derived progenitor cells have the potential of a pluripotent cell or IPS cell because these genes are induced naturally. And to make things further, these... Cells you pick up sort them out, and if you deliver them into an embryo, what happens is they can participate in embryogenesis at around 4 dpa.

Actually, these have been taken from the retina, and you have delivered them into the eye, and you can see them. This simply shows that the expression of pluripotency factors does have some relevance in embryogenesis as well. So now, if you look further into the overview of different retina regeneration mechanisms, you can see that the retina adopts a different strategy based on which species you are talking about. Say, the mammals do not have the ability to regenerate. The chick has a limited ability to regenerate.

So accordingly, every animal, if it has a damage, will try to fix it. So this is something you should keep in mind. In panel A, what you are discussing is transdifferentiation. The transdifferentiation concept we discussed in one of the earlier classes basically means you are going backward from a differentiated status to a dedifferentiated status. Or, in other words, to the stem cells from which this cell type has formed.

Now, instead of becoming this cell from which it transdifferentiated or dedifferentiated, it will become some other cell. In simplistic terms, I can tell you, with a very crude example, say your liver cell de-differentiated and became a stem cell, and now it is not giving rise to liver cells; it is giving rise to kidney cells or some other cells. So this is the idea you should keep: the transdifferentiation of RPE (retinal pigment epithelium) in the

embryonic stages of amphibians and chicks after whole retina removal is what has been shown in panel A. The RPE regenerates the whole retina by losing its pigmentation because the RPE has pigmentation and the retina does not want pigmentation, so it has to lose its pigmentation; it has to transdifferentiate, changing the morphology and proliferating to differentiate into different neuronal retinal cell types. This is what happens in amphibians, especially if you surgically remove the retina.

There are different categories of amphibians. We don't have to get into the details. The RPE needs the presence of a choroid. Choroid refers to the outermost layer of the retinal architecture that is farthest outside or the part of the optic cup. as support to form the whole retina.

So pigment epithelium cannot give rise to the retina when it is in the atmosphere or when it does not have any support structure. So the pigment epithelium needs the choroid structure in order to gain the ability to form the whole retina. In contrast, a portion of the neural retinal tissue ensures retinal regeneration in chick embryos. You can remove a lot of retina, but you need a small part of the retina; if you remove the entire retina, the chick embryo fails. Still, it is of reverse polarity; it will make the other way around: the ganglion photoreceptor layer will be facing towards the vitreous humor, and the ganglion cell will be facing towards the outer layer, that is, the choroid layer.

So there will be a polarity issue in chick embryos, and in panel B, what you are seeing here is the post-retinal damage that is responding. happening by Muller glial reprogramming. That means, as I told you in the earlier picture, that it expressed the Yamanaka and Thomson factors, and that chicks and mice involve sensing the damage by the glial cells. That is what happens. But they are not powerful enough to cause effective reprogrammed retinal cells, which then de-differentiate to form multipotent stem cells.

The de-differentiated Müller glia divide and migrate to different retinal layers through interkinetic migration, where they differentiate to form lost retinal cell types and restore division. This is what happens in the case of zebrafish. The regeneration by Müller-Glia reprogramming is inherently successful and complete in zebrafish without any external involvement. While in chicks, not all proliferated Muller-Glia form the neuronal cell types. Mice Muller-Glier is reluctant to reprogram.

But in some studies, it has been shown that genetic manipulation, such as the overexpression of ACL1A and inhibition of HDACs, has facilitated the production of more retinal neurons than usual. So what it indicates is that if you genetically program or genetically tweak the retina, it is now more vulnerable to regeneration. So you can see the different methods of reprogramming that are mentioned here. Then comes the

differentiation. The ciliary marginal zone, which is usually the edge of the retina and constantly keeps proliferating, containing constantly proliferating cells at the retinal periphery, contributes to retinal growth in adult zebrafish and amphibians throughout their lives.

So, in other words, the old fish has the bigger eye. A young fish has a smaller eye. Same with amphibians as well. The bigger frog has got a bigger eye. Means the old frog is the big frog, and the big frog has got the big eye.

So because their eye also keep growing just like the animal. They don't have a growth arrest like that of mammals after a certain age. In the chick retina, the CMC contributes to neuronal cell types by producing only amacrine and bipolar cells for up to one month post-hatching. CMC is also known to contribute to adult mice retinal growth by forming only ganglion cells. In response to light damage, the rod progenitors with their origin either in the outer nuclear layer or from the progenitors in the inner nuclear layer can contribute to the restoration of lost photoreceptors in the zebrafish retina; this has been shown schematically in this panel, so this is a cartoon way of depicting the facts we discussed.

So this is another diagram that depicts a set of molecular events. So far we have been talking about what the structural features are or what the species-specific qualities are that are needed. Now let us think about what gene expression events are needed. There are several studies that have picked up the retinal progenitors and used them for transcriptome studies, microarray analyses, etc.

And it is available in the public database. But the most important thing is: can we use these gene expression events in a non-regenerating species such as mice or humans? Then the study becomes more important. So here you can see resting Müller glia and activated Müller glia. What happens? One of the major transcription factors that is in the Linton diet gets upregulated, and the cytokines get upregulated. And because of the upregulation of the linton diet, the levels of seven micro RNAs go down. Any retinal damage induces the activation of the Müller glia from the resting state to an activated state.

A number of factors lead to the de-differentiation of activated Müller glia attributed mainly to the increase in the Linton diet, which is roughly induced by around 100-fold. Soon after injury, it can increase expression, which degrades the lead 7 microRNA. This series of events is accompanied by a hike in pro-inflammatory cytokines. Pro-inflammatory cytokines are triggered mainly by your immune system in the retina, and pluripotency-inducing factors increase in the Müller-Glia-derived progenitor cells, which

leads to the activation of many signaling pathways; this can trigger a burst of proliferative events that are needed for the restoration of the neuronal cell type. A decline follows soon after the proliferative phase; the decline of this pro-inflammatory cytokine and the decline of pluripotency factors also occur, as the expression of PIF stands for pluripotency-inducing factors.

And simultaneously increase the expression of neuronal markers, that is what you say, culminating in the regeneration of different retinal cell types and Müller glia itself. So once it is de-differentiated, PIFs and the signaling pathways go up, proliferation occurs. Then PIFs cannot stay high; they have to come down, and the neuronal markers will start expressing so that there are rod cells, common cells, amacrine cells, bipolar cells, etc. So this is a diagram that eventually gives rise to all retinal cell types after differentiation from the proliferating molecularly derived retinal progenitors. So if you look at retinal regeneration at a glance, in a comparative picture, what are the things we should keep in mind? The potential of Muller glia to become neuronal progenitor cells is seen in the avian retina, a species that has regenerative capacity around one month after its birth or hatching.

After that, it loses its ability, but it still has potential. There are differences in the proliferative and neurogenic capacity of the Muller glia among fish compared to that of avian or mammalian rodent models. So although the rodent and the avian models have the Muller glia, they have the ability to reprogram. Somehow, it is incomplete. The limited capacity of Müller glia to reprogram is seen in the avian and rodent retinas.

The findings suggest that various research findings indicate that the secreted factors, signaling pathways, and cell intrinsic factors are implicated in the formation of Müller-Weir derived progenitors. So it is not that all genes are present or all signaling pathways are present, but whether they are turned on effectively in the time that is required. You are going to take an exam. A question arose. You know the answer, but it came to your mind after the exam.

It is useless. You will not get it. It is as good as if you have no knowledge about the answer. But actually, you know, you could not produce it. The same logic applies to the mammalian and chick retina. So there exist several key similarities and differences between fish, rodents, and chick model systems.

Highlight several key transcription factors. That means which factors turn down at what time and at what intensity. And the signaling pathways that regulate the formation of Müller-glia-derived progenitor cells. Here you can see a schematic diagram that depicts factors influencing retinal regeneration. It may look a little crowded, but we will go one

by one.

The Wnts are one of the primary signaling events. The Wnt ligands are one of the... One of the major factors is that we clearly do not know where they are coming from. Some studies say that it is the damaged cells that have the stock of Wnt because of the damage they release.

So there are several theories, and some studies say that some neighboring cells actively secrete because Wnt is a secreted molecule. In any case, there is production of Wnt at the sources. And it is usually blocked. The ability of the Wnts to act on a target cell is blocked by DKK.

They are highly expressed in the Müller glia. They are blocked. And there is another signaling ligand. They are called BMPs. That is bone morphogenetic protein. From the inner retinal neurons likely to act to keep the Müller glia post-mitotic. Post-mitotic means it has completed mitosis and does not want to continue in the cell cycle.

So the legend has different color codings in this picture. Orange arrow indicates the proliferation or transdifferentiation. Green line indicates activate. Red line indicates inhibit or suppress. And the different codes C for chick, M for mammal, and F for fish are all some of the abbreviations used in this; so you can see here normal retina and different cell types are marked, color-coded: Müller glia, microglia, and NRG cell, and chick, mammal, and fish.

Here you can see there are many factors transitioning. SOX2, SOX9, NKX2.2, OLIG2, etc. These are all seen in chick. And you can also see the similar ways in other factors that are induced, which are CD45 and LMG.

These factors are induced. And you can also see in the Muller Glia that there are several factors such as HES, CHX10, PAX6, PAX2, and Six3; this continues. Plenty of these genes influence, but make sure that the DKK does not allow the Wnts to act in the Müller glia, usually. So the DKK also has to be addressed and manipulated effectively. And there are several glia-specific genes, such as GFAP and glutamine synthetase.

are present in a steady state level. When they reprogram, the levels of these housekeeping genes also go down. So you can see there are many membrane glycoproteins and many cytoskeletal proteins, such as GFAP (glial fibrillary acidic protein). And there are also cellular and retinaldehyde binding proteins. And glutamate, aspartate, transporter protein, delta-like ligand, patched smooth end, etc.

also comes into the picture when the Muller glia has to reprogram. We will study more about sonic hedgehog signaling and delta-notch signaling in the coming classes. So we will discuss that at a later stage. And here in this picture, what you are seeing is another schematic of diagrams that summarizes the signaling transcription factors and the interactions between the glial cells when the Müller glia have been stimulated to become progenitor-like. In a steady state level, the Müller glia do not reprogram.

So it needs a cellular insult. It can be a hypoxic insult, a chemical insult, or a mechanical insult—anything. The same code applies: C4 for chick, M4 for mammal, F4 for fish. What you are seeing here, and this activation inhibition signal, has also been mentioned, and proliferation indicates a curved arrow. So, several structural proteins also have to come into the picture, but what is important is.

.. How the genes, these genes that are induced, not all genes that are induced favor the proliferation. So this is what you should understand: if a gene X is induced, it need not necessarily favor proliferation. Just because it is proliferating, we cannot say, "Oh, this gene is facilitating proliferation.

" Some genes will be present. They are induced to control proliferation. In the zebrafish retina, the delta node signaling limits the zone of proliferation. It does not inhibit proliferation, but it limits the zone. So if you block the delta node signaling, it expands the zone, which means the proliferation happens in those places much beyond the scope of the actual injury. So in this legend, you can see the orange arrow that represents proliferation or transdifferentiation; the green line basically indicates activation, while the red line represents inhibition or suppression. You can see that the factors involved are FGF2, one of the preliminary factors that induce fibroblast growth factor, EGF (epidermal growth factor), and IGF (insulin-like growth factor).

Insulin signaling is very pivotal in homeostasis and proliferation. And IGF contributes to it, CNTF (ciliary neurotrophic factor), ASCL1 (Achaete-Scute homolog like 1A), MAP kinase (mitogen-activated protein kinase delta like ligand), lysosomal membrane glycoprotein, glial fibrillary acidic protein, retinaldehyde binding protein, and also the glutamate-aspartate transport. What you can see here, the ASCL1A has the ability to favor the Müller glia to reprogram and proliferate. You can also see that the notch signaling and FGF and Wnt signaling contribute to the gene expression events, allowing the Müller glia to proliferate. So many of these factors contribute, and they interplay. So among themselves, not that the signaling events are independent, signaling events also interact with each other, and they contribute to a proper regeneration mechanism in the Müller-Clea-derived retinal progenitor formation. We will study more about regenerative biology in the next class. Thank you.