Regeneration Biology
Rajesh Ramachandran
Department of Biological Sciences
IISER Mohali
Week: 5
Lecture: 24

W5L24_Zebrafish retina regeneration-in a brief

Hello everyone, welcome back to another class on regenerative biology. We have been learning about different tissues and organ regeneration, and now we are picking different organs from a highly regenerative species, which is the zebrafish. So initially, we will see a brief outline, and then we will... Go deep in depth on various angles and aspects.

Okay, so this class will provide a brief outline about the regeneration in zebrafish. We know zebrafish is a well-studied or well-used organism whose genetics is well studied. It can reproduce year-round, has external fertilization, and has transparent embryos. The embryos can be injected with DNA or RNA, and they can also express proteins.

Zebrafish are amenable to large-scale genetic screens. Forward and reverse genetic screens and zebrafish share extensive genomic homology and synteny with that of humans; synteny is the order in which genes are arranged in a chromosome, and homology is the sequence similarity that means the functional similarity is attributed to the sequence similarity, so homology and synteny are hallmarks of deciding. whether or not an organism is suitable to study human physiology or genetics. So zebrafish can regenerate central nervous system neurons following damage, which makes them a more attractive model organism when regeneration biology is concerned. If you look closely at the zebrafish retina structure, it is very similar to that of a human; it has a lens, a cornea, and a retina.

As you can see here, it also has an optic nerve that is connected to the brain, and the image processing is very similar to that in mammals, except that we have a flat lens, whereas fish have a spherical lens. The cell types are also pretty much the same: rods and cones, which are the photoreceptors, and horizontal, amacrine, and bipolar cells, as well as the ganglion cells. Whereas the only glial cell type, the predominant glial cell type of the retina, is called a Müller glia, as you can see here, yellow in color. And broadly, the layers are classified into the outer nuclear layer, where photoreceptors are located, and the inner nuclear layer, where various neurons and glia are present. And then comes the glial cell layer where the ganglion cells reside.

There are different ways in which you can create damage in the eye, and the injury

model can vary, but regeneration has to happen in the retina no matter what method you use to injure it. In our lab, as well as in many other labs, we use something called mechanical injury, which is achieved through a needle poke. We use a 30-gauge needle from the back of the eye, which creates a hole in the retina. We usually give one poke per quadrant, and this is called the needle poke method of injury. It's a focal injury, meaning the entire eye's tissue is not affected, and the animal will not go completely blind.

Then the neighboring region and damaged retinal part will act as a controlled region, so it will provide better control than the other eye or another fish's eye. To study the regeneration, we have to use different models, different model organisms, and also different transgenic organisms of the same model; one such transgenic. A model or transgenic organism we use in zebrafish is the Tuba1 GFP fish. What is Tuba 1? Tuba 1 is part of the cytoskeleton. Whose cytoskeleton is it? Neuronal cytoskeleton.

But we haven't used the full-length tuba. We used a fragment of the Tuba 1 promoter. So, what do you understand from this? If the promoter is truncated, its expression will not be as it happens in the native gene. Say, for example, if a gene's promoter is 10,000 bases and if the entire 10,000 bases are there, the gene will be expressed as it should in the native condition. But if I cut down the 10,000 base into 1,000 base, I got rid of 9,000 bases.

Still, it is a promoter, but that promoter comes with a lot of limitations. It will not work in every cell type. So that is what you should understand about a truncated promoter. So, in the truncated promoter of tuba1a: GFP, what you see is that instead of expressing it in the entire neurons, it is expressed in the central nervous system of embryonic zebrafish, not adult embryonic zebrafish, as you can see in the brain and the spinal cord; it is there, and also it is expressed in the eye. You can see it is expressed in the eye, but whereas if you see it, it is there in the retina also, but in the adult retina, it is not expressed; in the adult brain, very little is expressed, and in the adult spinal cord, there is no expression.

But the interesting thing is that the same adult retina, uninjured retina, is not expressed. But in an injured retina, you can see the expression. See, this is the result of a focal injury. But here, in the surrounding area, there is no GFP expression. Look at this area.

There is no GFP expression. Look at the southern areas. This is the place through which the needle has passed. It is a microscopic image; that is why it looks broader. Normally, the needle is a very small 30-gauge needle.

And for zebrafish eyes, when you take an image under the microscope, it will look like a larger area. And this vertical group of cells is nothing but a proliferating group of cells, as

you can see here. And they are regenerating cells. And if you see the merge, the green and the blue are merging, so what do you understand from here? In the tuba fish embryonic central nervous system, including the retina, it expresses; in adults, there is no expression, especially in the retina after injury. It expresses, and it expresses GFP in the proliferating cell, so this transgenic line is extensively used.

And these images were taken in 4 days. Why are 4 days so special? Because 4 days is the time for a needle poke injury, you can see that the maximum proliferation happens in 4 days. Roughly around here. And by around seven days, the proliferation is reduced to a bare minimum. So this is what you should keep in mind.

Now, which are these cells that made these cells proliferate? In the earlier picture, we saw that there is a Müller glia cell. The Müller glia cell is the assistant cell of a neuron, just like an officer has a personal secretary or a stenographer as a helper. Like that, the Müller glia are the helpers of neurons; they have some hallmark gene expressions that neurons don't have. One such gene is called glutamine synthetase. As you can see here, the red color is in this picture.

And this GFP is the same GFP that you saw here. And when you merge it, you see a lot of yellow-colored cells. So the GS-positive, glutamine synthetase-positive cell has GFP expression, and they are merged together, resulting in a strong expression of GFP. Glutamate synthetase is indicative of the Müller glia, and GFP expression indicates that it is now responding to the injury. This response is not expressed in every Müller glia; it is expressed in those Müller glia in which GFP is expressed, specifically in those Müller glia that have entered the cell cycle.

That is what you should keep in mind when looking at this picture. Now, if we can generalize what is happening. This is a normal retina. You have the nuclear layer, the inner nuclear layer, and the ganglion cell layer. After injury what happens there will be a response.

It can be anything. Responses can be anything. It can be cytokines or chemokines; it can be any cellular damage that will create some unrest in the environment. This unrest will be sensed by some cells, such as Muller glia. Muller glia have phagocytic properties, and phagocytosis means engulfing this damaged portion. When it is engulfing this damaged portion, it will sense there is an issue, that there is damage, which makes them reprogram.

We have already discussed what reprogramming, de-differentiation, etc. are. Once again, I will tell you that reprogramming means that if you are studying for a bachelor's degree, you are pushed into kindergarten. That is reprogramming. Once reprogramming happens,

they undergo proliferation and are called Müller glia-derived progenitor cells.

Because of this proliferation, they will increase in number and migrate into different layers; when they migrate to the outer nuclear, inner nuclear, and ganglion cell layers, they will give rise to retinal neurons and Müller glia, which are present in the eye. So whatever lost cell type will be restored, and the net result is that you will restore normalcy; it roughly takes around two to three weeks for the whole process to be done with a 30-gauge needle poke. What you should understand from here is that because of the injury, they are restoring normalcy in around three weeks, which involves a lot of. Factors raise lots of questions about what genetic factors control the Müller glia's de-differentiation or reprogramming and its proliferation, and its re-differentiation; it should come back, just like when you are studying in your bachelor's, you become a kindergarten member. From that phase, you are now coming back into bachelor's.

Not the same, but one fellow became a doctor, one fellow became an engineer, another fellow became an advocate, and another fellow became a chartered accountant. This is called redifferentiation. So, in other words, what you can see here is that the Muller glia is capable of giving rise to Muller glia-derived progenitor cells, as you can see here. But for this process, there have to be lots of signaling events such as Wnt signaling, FGF signaling, insulin IGF-PI3K pathway, HB-EGF MAP kinase pathway, and EGFR pathway.

These all contribute. These are all stimulating the reprogramming, but some pathways, such as delta no signaling, are not stimulating; rather, they are restricting. That means restricting basically means just like you are tying your dog to a leash. If you have a dog and you unleash it, it will run around, but by tying it to a thread, you are not. Completely stopping its movement, but you are restricting the dog so it can move as long as the length of the leash allows; if the leash length is one meter, the dog can move around a one square meter area; if it is five meters, it can go to a five square meter area. The same logic applies: delta notch signaling is not inhibiting reprogramming, but it restricts the zone in which the reprogramming or differentiation can happen.

And there are other factors such as INSM1A, which are inhibitors, whereas ACL1A, Lin28a, are activators of reprogramming. So there are many transcription factors that will favor the formation of the Müller Glia-derived progenitor cells.

But there are other factors that... Do not favor it that much. That means something like the brake and accelerator in a car. You cannot say, "I want a car that has only an accelerator." Because you can't stop it. You can't crash every time and stop the car.

So brakes are also equally important as those of the car. So, transcriptional activators are important. But when should we end the show? When should we stop the show? That has to be decided by inhibitors such as INSM1A. One principal inhibitor is Let7 microRNA. Let-7 is a microRNA that is expressed in every differentiated neuron to maintain homeostasis.

What is homeostasis? Neurons do not want to divide. Neurons want to stay in a fixed state of excitation. They will do their metabolic work, their physiology; every job will be done, but they do not proliferate. What if there are some pro-proliferative genes that got turned on? Then the neurons will be compelled to proliferate, or the neurons will get into some unrest situation.

So you don't want that to happen. Because of that, the Let7 microRNA ensures that even if these pro-proliferative genes are turned on, they will never get translated. RNA is not causing any harm. So the lead sevens are the guardians. They are the guardians of maintaining the differentiated status. So we can say when a differentiated Muller glia, which has a high level of lead seven, has to become differentiated.

reprogrammed or de-differentiated into progenitor cells, you want to get rid of let-7. If let-7s are present, let-7 microRNA is present, then the so-called proliferative phase or the next phase will not happen. So we can call let-7 a principal inhibitor of glia's migration into the progenitor path. So what you can see is that there are different ways of dealing with a different way of analyzing the regeneration event.

So this is from a review recently published. It's a timeline of Müller glia reprogramming and retinal regeneration in zebrafish. So you can see that there are different phases, and you can also notice that there are different timelines. 1, 2, 3, 4, it's mentioned, but 1 DPI, some events happen. 2 DPI, some events happen.

4 DPI, some events happen. And in 7 to 21 days, some other events happen. So every process is initiated from a Muller glia reprogramming event. And it's also important to note that every Müller glia, if the retina has 1000 Müller glia, in that injury spot—I'm not talking about the whole retina—near the injury spot, if there are 1000 Müller glia, every Müller glia will not reprogram. Only a selected number, say 100 Muller glia, will reprogram. What stopped other Müller glia from reprogramming? Of course, we can justify why they do not.

If a job can be done by one person, we don't want 50% of the position to be filled. That is understood. But who chose that one person? This is the question you should keep in mind. So in the uninjured retina, Müller glia are embedded in a sea of neurons. Retinal

injury results in neuronal damage, and this stimulates a partial reprogramming event at the very beginning in the uninjured retina.

So any insult that happens, like I told you earlier, there will be release of some factors which will be sensed by the mullerglia. You can see that these abbreviations are there. DPI means days post-injury.

MG for Mullerglia. V for vascular endothelium. VEGF stands for vascular endothelial growth factor. And here you can see different phases, like you mentioned, number one and number two, right? Number one in MG that is characterized by ascl1a and lin28 expression remembers ascl1a can upregulate lin28; also, lin28 can be upregulated by various other pathways, but ascl1a is one of the members that can upregulate lin28 around one dpi (one day post injury). Immune cells, mostly from the microglia, accumulate near the injury site. Microglia are another wandering type of glial cells that come into the retina. They accumulate near the injury site and appear to stimulate a second reprogramming event.

First, Muller glia will sense these damaged particles, and they are vulnerable to reprogramming. But the presence of microglia in the injury spot creates a second reprogramming event, as you can see here. Uh, neuronal damage occurs in one event, and then comes the second reprogramming event, which allows for Müller glia division. The second reprogramming event stimulates a division response, meaning it has to divide and increase in number.

Müller glia undergo self-renewing asymmetric cell division. Asymmetric cell division is very important because you want to have a differential distribution of delta and Notch, and many cells will have it. Both, like, let's say for example a rich father is distributing this, distributing, say, if he has got three restaurants or he has got four restaurants and four movie theaters. So if he is distributing, he will give restaurants to one son and the movie theaters to another son; he will never distribute. Okay, two restaurants and two movie theaters to one son and two restaurants and two movie theaters to the other son.

Why? Because then there will be competition among them. So the asymmetric division ensures that there is a difference between them, which allows them to have a different fate. One cell will differentiate; the other will proliferate. So, asymmetric division is a necessity. It's not happening because of a mistake. MG undergo cell-renewing asymmetric cell division, producing multipotent progenitors that transiently amplify before differentiating into one of the major retinal cell types.

Because the retina has six types of retinal neurons and one type of glia. So it can give

rise to any of them. But based on the demand and the need, you should keep in mind that this is a multi-step process involving retinal injury and Müller glia reprogramming. This picture shows a yellow-colored cell, which is a Müller glia, responding to the microglia present, as you can see here with immune cells. The color code is given for immune cells, damaged neurons, uninjured neurons are gray, damaged neurons are pink, and then Müller glia-derived progenitor spindle-shaped cells.

And the Müller glia undergo a division, and then the progenitor starts getting amplification. And in the final phase, 7 to 21 days, these progenitors migrate and differentiate into various neuronal cell types. And this two-step process of Mueller-Glia reprogramming in the injured retina is also allowing a controlled response. You don't want a pan-retinal response. So there has to be, it's just like, say you got one phone call from someone, some unknown number that you know there is some trouble with the so-and-so person.

You will not immediately panic. You will call someone more reliable and ask, "I heard so-and-so." Is that true? So this is a second level of confirmation. So your two level of reprogramming ensures that you don't want an alarming response, an unwanted alarming response. So in what happens in this picture, there are two steps given: one is molecular reprogramming by the damaged neurons; you can see here there is a neuron injury and there is an expression of some of the ligands of delta-notch signaling, DLL4 and DLLB. This will also trigger delta-notch signaling and facilitate molecular reprogramming, and you can also see what happens.

That some of the cells are the vascular endothelial cells because the newly formed cells also need a blood supply, so the endothelium has to assemble for the blood vessels to form; thus, the same neuronal injury can create a wedge of expression in the Müller glia. Vascular endothelial growth factor can inhibit the expression of DLL4, unlike the stimulation you see here; it can inhibit and favor the production of Notch, which can also favor Müller glia reprogramming. So you can understand, various neuronal signaling comes into the picture, like GABA and glutamate.

They all come into the picture. We will not dwell on those right now. So injured neurons initiate the first stimuli reprogramming event and involve not signaling inhibition via the suppression of DLL4 and DLB. What are the ligands of the Notch signaling? So notch signaling will be suppressed in the neurons. DLL4 expression will be favored in the VE cells. Those are vascular endothelial cells.

This is needed for the formation of the blood vessels. Suppression of quiescence-promoting factors such as TGF-beta and FGF8. TGF beta 3 and FGF8 by unknown

mechanism. And suppression of GABA signaling from HCs.

That is what you can see here. HC cells are mentioned. Horizontal cells are mentioned here. And they are present in the inner nuclear layer. These HC cells are done via reduced glutamate from the PRs. The PR basically refers to what is present in the photoreceptor layer, or the rods and cones, which are present in the photoreceptor layer. The secondary programming event is initiated by the microglia, which are distributed by the immune cells accumulating at the injury site.

The mechanism underlying this second event is unknown but may involve TNF signaling; TNF means tumor necrosis factor alpha (TNF-α), and it usually involves TNF signaling due to the presence of microglia, not the TNF-α from the injured. It has also been suggested that dying neurons stimulate the Muller-Glia reprogramming. So many cells store these factors when they are present in an inactive form. When they are broken or damaged, they will be released immediately outside.

So this is how TNF-alpha becomes available to the neighboring cell. So the potential roles of notch ligand, delta A, C, and D induction in the Muller-Glier-derived progenitor cells. So here you can see that notch ligands act on the neighboring progenitors to stimulate their proliferation because delta and notch are present. They do a juxtacrine signaling. It is just like two rooms in a hall.

Like in many hotels, you would have seen that rooms are arranged in a hall. So basically, if you make a hole in one room, you can enter the other room. That's how they are arranged. So that is what it's called. So you made a hole in the hotel wall and you're communicating with the neighbor.

So you can call this a juxtacrine signaling. So how it works is that every cell can have delta. Every cell can have a notch. So in a given cell, if Delta is present and binding to the notch of the neighboring cell, the notch-expressing cell will proliferate. But on the other hand, the same notch-expressing cell may also have Delta, and that will be binding onto the notch of the previous cell.

Then neither will proliferate. Although both have got delta, both will have notches. So here comes the asymmetric division that I mentioned earlier. Because of the asymmetric division, there will be a differential distribution. Because if a given cell has 100 delta and 100 notch, when it divides, it is not split into 50 delta and 50 notch. It will be split in such a way that one gets 60 delta and 40 notch.

The other gets 60 notches and 40 deltas. This way, this asymmetric distribution will have

the upper hand. So the delta-expressing cell will not proliferate. because it has fewer notches. And the other way around, the more notch-expressing cell will have fewer deltas.

Hence, it will proliferate. The more notch-expressing cells will proliferate. So this is an idea you should keep in mind. And the notch ligands act on the neighboring progenitor cells by juxtacrine signaling. Notch ligands act on neighboring progenitors to stimulate their differentiation.

So the notch-expressing cell will not differentiate. It will proliferate. But the notch-expressing cell tells the delta-expressing cell to differentiate. Don't proliferate. Differentiate into retinal neurons. And notch ligands act on their parent reprogrammed Müller glia to help return them to the basal state.

This also happens. As you can see in this stage 1 progenitor proliferation, stage 2 progenitor differentiation, and stage 3 return of Müller glia to the basal state. Because many times the reprogrammed Muller glia must return to its normalcy. So otherwise it will remain in a reprogrammed state, which is useless once the job is done. Like you may have seen, whenever there is a spiritual function going on, you will find a lot of local shops that are coming up.

Once the function is done and the festival is over, all the local shops will disappear. What is the point of having those local shops? So these Müller glia must return to their normalcy. And this is a typical in situ injury pattern in the retina. This is a lin28 gene expression because earlier I talked about a let 7, and the killer of let 7 is lin28. And you can take a small portion, as you can see in this box, and you can see it under the microscope.

So Lin28 is an inhibitor of let 7 microRNA. LIN28 is one of the pluripotency factors, like the iPS factor; you know, the Yamanaka and Thomson factors. Of those six factors, one of them is LIN28, which is expressed in embryonic stem cells and cancer cells. LIN28 is found both in the nucleus and the cytoplasm and regulates let-7 microRNA levels. Let seven is a very interesting molecule.

It has independent genes, and it can also be embedded in the introns of other genes. Okay. In both ways, it is present and produced as primary let-7, which is processed by Drosha and DGCR8 to become pre-let-7, the preliminary or precursor let-7 microRNA, because this portion is chopped off, and you end up getting it, which is exported to the cytoplasm and acted upon by Dicer here. It is acted upon and you end up getting a let7 microRNA and This will help maintaining the differentiated status. But lin28 can

degrade the pri-let7-bound Drosha DGCR8, and it can also cause the mature let7 to degrade by polyuridylation. So it ensures that when lin28 is present, there is no let-7 present in the cell.

And the downregulation of let-7 is necessary for regeneration. Why? Because many pluripotency factors are induced during retinal regeneration. But these let-7 microRNAs have binding sites in those RNAs: Cl1A, HSPD, Lin28 itself, FAC-6, KLA-4, CMYK-A, CMYK-B, AUK-4. So you can see there is an increased concentration of let7. You see reduced protein expression that is present.

Those who are interested can read this article, which is mentioned here. So this is how the absence of let7 allows the translation. The presence of let-7 prevents the translation of these genes. So we'll study regeneration biology in more detail in the next class. Thank you.