Regeneration Biology
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W5L22_Zebrafish fin regeneration-in a brief

Hello everyone, welcome back to another class on regenerative biology. In today's class, we will learn a brief outline about different tissue regenerations, starting with fin regeneration. The word "fin" itself indicates that we are talking about a fish, specifically the zebrafish. And we will come back to the regeneration of fins in detail in a later class. But first we need to introduce the species and also the basic things we need to know about regeneration. So this is a zebrafish.

Many of you would have seen it in the pet shop, which is very prevalent in Indian aquarium pet shops. It is also native to India. Zebrafish are a very interesting species of fish; they belong to the genus Danio and come under the family Cyprinidae. That is a bonnie fish group.

It's also called teleost fish. So, zebrafish are native to the streams of the southeastern Himalayan region and are found in parts of India, Pakistan, Bangladesh, Nepal, and Burma. And the species arose in the Ganges, the river Ganges, and in eastern India. And this commonly inhabits streams, canals, ditches, ponds, and it can also live in stagnant water bodies and even in rice paddy fields, etc. Means its ability to grow in a diverse water habitat; it’s known as a freshwater fish, and hence it can be easily maintained in laboratory conditions.

The zebrafish is named for five uniform pigmented horizontal blue stripes on its body, which are reminiscent of the animal zebra. and which extends to the end of the caudal fin. It walks into the caudal fin from the part of the body, shaping its fusiform and laterally compressed form, with its mouth slightly directed upwards. So male zebrafish are torpedo-shaped with golden stripes, slight yellowish-golden stripes between the blue stripes, whereas the females have larger whitish bellies, which is due to the presence of the ovaries and the yolk. A whitish belly and silver stripes instead of gold allow for easy identification; there is a slight color variation between blue and yellow, and the body shape will also differ.

In the case of females, adult females exhibit a small genital papilla in front of the anal fin origin. So if you use a dissection microscope, you can identify the female by its genital

papilla. So there are ways to easily identify the male or female gender in zebrafish. It's an excellent regeneration model due to various features and factors associated with these animals. Two features make the teleosteophage zebrafish a powerful complementary model system to study organ regeneration.

And we all know that to study organ regeneration, we have to get into gene expression or different types of genes, genomes, etc. And zebrafish share almost 70% homology with that of humans. And quite a few of the genes have extensive genomic synteny. That means the order in which the genes are arranged is important. So this is quite beneficial because the order of genes also plays a major role in their expression pattern.

First, they are highly regenerative, equipped to regrow amputated fins, injured retinae, transected optic nerves, spinal cords, resected heart muscles, scales, etc. Second, unlike the salamanders, which are amphibians, they are responsive to both forward and reverse genetic approaches. Salamanders, these forward and reverse genetic approaches are limited because they cannot provide as many eggs, and they may not breed throughout the year as efficiently as zebrafish; additionally, the genomes of many salamanders have only recently been identified, and some of them have been sequenced recently. So the forward and genetic screen approaches also depend on the size of their eggs, whether they are amenable to microinjection or maneuverability. Some of them may not be very transparent in their embryonic development.

Zebrafish have excellent transparency in their embryonic development. Hence, these all make it a very easy model organism to monitor. And when it comes to regeneration, it is as good as that of amphibians. As is customary with the genetic model system, various community resources are available for gene discovery and molecular characterization of zebrafish. This is an important feature; if you are studying a species, the availability of resources or reagents across the research groups or research fraternity is very important.

If you are starting a new species and no resources are available, you have to... Identify and discover those features, and then study; it's almost like you have to make the road and ride on it. That can be very cumbersome when it comes to doing effective or easier research, including mutagenesis screens, transgenesis, microarrays, developmental markers, and genome sequencing.

Information is a must if you want to study a species; zebrafish fulfills all these criteria. Zebrafish fin regeneration is historically important because it is quite compelling to know that almost 250 years ago, people knew that fish can regenerate, as we have seen in planaria; when we studied planaria, many scientists reported the regenerative capacity of planaria, similar to the regenerative capacity of zebrafish, which people also knew. It is a

sad fact that despite having knowledge about the regenerative ability, which is one of the earliest research areas or topics that have emerged for scientists, it still remains incomplete. That means, although we know a lot about the regeneration mechanism, we are far from eliciting these regenerative tactics or skills of lower vertebrates in higher vertebrates, such as mammals. So that is an area that needs to be fulfilled.

The ability of teleosts to regenerate amputated fins was first reported in 1786. In the pectoral fins of goldfish by Bussonet, the scientist's name. Although Thomas Hunt Morgan, the famous scientist who had done a lot of work on mutagenesis and mutations, was fascinated by fin regeneration, at the turn of the 20th century, fin regeneration took nearly an additional century to reach the genetic era. At that time, people didn't know much about genes, genotypes, genomes, etc. They know that it regenerates.

That's it. Even cell biology was in its infancy then. In 1995, Johnson and Western described a screen for mutations that disrupt the regeneration of tail fins in adult zebrafish. That was some of the almost 30 years ago. Later, a handful of mutations that inhibited fin regeneration could be localized to specific molecular defects by positional cloning. So how people do forward genetic screening is to identify a defect in the organism.

And you don't know why you can take an animal, do some expose them to some mutagen or this is you create some mutations in the genome and then you see whether it is able to regenerate or not. And if it is not able to regenerate, what we know is that I damaged some part of the genome or that some gene is defective now, which is why it is not able to regenerate. But who will tell you what that gene is? So you have to identify, and that is called forward genetic screening. You have to do positional cloning; I will not go into the details of positional cloning. You need to have a lot of genome markers, etc.

, but you can eventually narrow down and identify which chromosome and which part of the genome. DNA, or the part of the genome that contains this defective gene, is the reason the animal is not regenerating. The reverse genetic screen is the other way around: you have a gene in hand and you don't know what its function is. You disrupt it, knock it down, knock it out, or do something similar, and then see what it is doing. That approach is called reverse genetic screening.

So a lot of such research done in mutagenic studies has shown that regeneration can be compromised in zebrafish due to defects in or due to induced defects in some genes. New advances in high-throughput genome, exome, and transcriptome sequencing are likely to reboot the forward genetic approaches to study regeneration. Since we know the genome of the entire animal, even the sequencing of the genome of a mutant that you have in your

hand is rather easy these days. Hence, identifying a defect is much easier. You don't typically have to go for the positional cloning.

So, this is a very interesting picture. That is the time-lapse imaging of the fin after amputation. That is done at around 27 degrees. That is the temperature at which the zebrafish is housed. Normally, the water temperature is around 27 degrees Celsius.

This is an uncut fin, and this is one day after amputation. So this is the plane where it is cut, and it is called a DPA. 1 DPA means one day post-amputation. And this is 3 DPA.

This is 6 DPA. This is 12 DPA, and this is 20 DPA. 20 DPA and uncut look more or less the same. There is no difference. So here, the whitish cell mass that you are seeing is called Blastema. And here there is barely any blastema.

Here little bit Blastemma. There are more blastemata. Here it is: Blastemma is there, but the fin is already formed. Here there is no Blastemma. The fin is completely formed, and it is also restoring the same stripe pattern.

As you had. There is a slight difference here, but it is more or less the same in three weeks' time. And the same thing at a higher magnified level, what you are seeing in the B panel, the blastema at a higher magnification, you can see here. And that you look at this panel, see what is happening. So, this is homeostasis, which is a steady state level in an uncut condition. This is amputation at zero days, and the wound closure will happen within a few hours.

Then, what happens is a disorganization and de-differentiation before 1 dpa. At 1 dpa, you have mesenchymal proliferation and the formation of a blastema, a gentle or slight quantity of blastema that you can see at 1 dpa. You need high magnification, and then you have a blastema outgrowth. There will be an expansion of the blastema and also the formation of actinotrichia between one and three days. By around 5 to 10 days, what you will see is that the apical blastema maintenance at the extreme end will still need to have the blastema.

Between 5 and 10 days, the rapid growth of the fin occurs, and behind that, between the blastema and the cut region, there is a formation or differentiation of the fin, followed by progressive re-differentiation, which means the blastema gives rise to the fin tissue. And then it has to have the morphogenesis that morphologically restores the old structure, and by around 20 to 25 days, there is a termination of regeneration, which means you don't need blastema anymore, and homeostasis is restored as found in the uninjured condition. Those who are interested can read the paper cited here. So if you look closely, in

zebrafish fin regeneration, the amputated fin ray is covered within the first several hours by the epidermis, and the regeneration blastema forms within one to two days. So soon after the injury, a blood clot will form, and then there will be a migration of the cells from the surroundings.

Adjacent side of the fin. If I can show you here, if there is a cut here, the layer will be formed here by the movement of cells from here. So that it's acting almost like a blanket. And if this epidermal layer covering is not formed for some reason, you cannot get any blastema that is being formed. Or in other words, this epithelial layer formed from the migration of cells from the surrounding tissue is essential for the blastema to kickstart.

So blastemas take around one to two days. The blastema is a proliferative mass of morphologically similar cells formed through the disorganization and distal migration of fibroblasts and osteoblasts or scleroblasts proximal to the amputation plane. So two terms you should know are proximal and distal. So, proximal means something closer to the body. Distal means something that is away from the body. This is what you should get if you have a fin.

If I can show the previous picture once again, it is because the animal is below this; this is a proximal fin, and this is the distal fin. So, as with fin blastema in other classical regenerating systems such as the salamander's limb and the planarian's head, the fin ray blastema is the major source of new structures seen in salamander limb regeneration; similarly, in planarians' head regeneration, the formation of blastema occurs here in fin regeneration as well. The blastema is a must, and it performs more or less a similar job or function. So if you look closely, the anatomy of the blastema is very important in multiple ways. The first thing is that anatomy tells you what the tissue types are that need to be formed.

Say if the fin is made of muscle, neurons, and bones. After regeneration, you cannot have only a group of fibroblasts. You need to have these structures formed precisely in the right place. It should be an extension of the existing body parts. You don't want a completely different pattern that is being formed.

So, understanding the anatomy is very important. If you look here, you can see there are 16 to 18 rays, and this is the proximal fin. This is the distal fin. And here is the amputation plane. And if you see, the blastema that is formed is basically an extension of the 16 to 18 rays you can see when it is being formed. And the higher the magnification when you look at it, the better structures you can see.

This brown-colored one is the ray. And then this slight yellowish color one is the newly

formed rays in the blastema, and it is proximal and distal; the distal-most blastema does not have the ray because it is still growing. It is slowly getting into that zone or walking into it. If you look here, what you are seeing is that you can do sections in multiple places. You can have a proximal, distal, and distal-most section. This is a cross-section from the proximal, and then you have the distal and the distal-most sections.

As I told you, the distal-most does not have any fin rays, so this is a cross-section. So when you look at this cross-section, you can see that this proximal one has something called hemi rays. And these rays are something like a bracket. It is not a cylinder.

So it's like a bracket that allows for some flexing. So that is what you see here. You have got hemi rays that are located. And what is in this green color is the actinotrichia.

And the osteoblasts are... Slightly brownish in color, the bone matrix is deep brownish in color, and the epidermis, which is the skin, is gray in color, while the red circular areas are the blood vessels. So you can see in the proximal area, you have a very thick bony matrix of the fin rays, but still, the hemi rays are seen nicely. So, rays between rays are inter-rays, and each ray is made of two bony plates; that is, each half plate is called hemi-rays. The proximal has a much thicker bony plate, and the distal one has the rays, but it is not that much thicker. The distal most has only blastema, but it has actinotrichia; the epidermis is there.

All it is lacking is the blood vessels. Thick ray, the calcium, or the bony structures are missing, and if you look here, you can also see the lateral view of the distal-most blastema, the distal blastema, and the proximal blastema, and this is the stump. Stump basically means the remaining part of the body. So the regenerating stump of the caudal zebrafish fin three days post-amputation is what has been shown here. This blastema formed is almost three days post-amputation, and you can recognize it by this dashed line; the rest of them are very easily understandable when you take a section and look under the microscope, and you can stain them so that you can get a better view of this whole structure. So organ regeneration basically means the restoration of cellular and physiological normalcy of an organ.

As you can see here, if there is damage that occurs like this, this is the structure that is normally present. And this and this are developing, and this extreme end is the growing cone or the growing blastema cells; they are trying to mimic that of this stump. So this is what you should keep in your mind, and this light brownish color is retaining this original structure, but it is not complete yet because it will take around 21 days. So that is what this organ regeneration is: the restoration of cellular and physiological normalcy of an organism.

Just having a similar structure is not going to help. If you lost your nail, you don't want another structure. You want only the nail, which should look exactly like the nail you had before the damage. So, if you look at fin regeneration closely, zebrafish fins are complex appendages that quickly and reliably regenerate after amputation, restoring both size and shape. So the key regenerative units are the many rays of the dermal bone, which are segmented and lined by osteoblasts. The word osteo, when you hear it, should make you know that it refers to a bone structure.

Rays are cylindrical and hollow. As I told you, they are like this. They are cylindrical, but they are not touching each other. It's like two brackets in the cross section. You can see it like that. The two concave hemirays surround an inner mesenchymal tissue that is innervated, vascularized, and primarily comprised of fibroblasts.

Fibroblasts, we know, are sticky tissues and are present. They perform the blanketing function. And there is an innervation because the bony structures should also be mobile, and they need to have a muscular structure; all these structures together give rise to a functional fin. An amputated fin ray is covered within the first several hours by the epidermis, as I already told you. And within one or two days, a regeneration blastema has to form. So either you block the epidermis formation, or you block the blastema formation.

Just forget about getting proper regeneration. So the blastema is a proliferative mass of morphologically similar cells. We can compare them to that of the blastomere cells or the stem cells that are present in a developing embryo, something similar although they are not identical, because it can give rise to all cell types present in a normal fin. Although the blastema is a congregation of different types of cells, it can differentiate in the right place. That is the beauty of this regeneration. And they are morphologically similar cells formed through the disorganization and distal migration of fibroblasts and osteoblasts or scleroblasts proximal to the amputation plane.

So blastema formation is only one step in zebrafish fin regeneration. That means it is looking in the right direction. Just because there is a blastema, we cannot be sure that a fin will be formed. That is the beginning step. For the first successful step, the fins must then grow to their appropriate size, and the regenerative outgrowth occurs through two major processes. One is the maintenance of a proliferative compartment at the distal end; that means in the previous picture we saw there is a stump: the proximal, distal, and the distal-most part should have.

Blastema, as it is from the very beginning, whatever blastema is formed should be there

in the distal most plane, and the differentiation of the more proximal cells keeps occurring. If I can quickly show this once again, when the blastema is being formed, I will show here that the blastema is being formed in this area. This is the proximal part, and it will be differentiating.

As you can see here, there is... Even pigment cells, etc. They are formed, whereas here there is no pigment, and they still look whitish. So it is not waiting for the full fin to form. Even before this is reached, even the cells that are proximal to this boundary stump and the proximal fin are now differentiating, and they are just walking and walking into the extreme tip. So the distal end of the fin retains this blastema, and the differentiation happens in the more proximal ends. So if you look at regeneration, there are so many growth factors that come into the picture.

The proliferative compartment is maintained by signaling interactions between the mesenchyme and the distal epidermis. Mesenchyme, like I discussed in the previous classes, is also involved in the mesenchymal-to-epithelial transition. The mesenchyme is something that has the ability to migrate. This is what you should keep in mind. A normal epithelial characteristic, despite having all the abilities, is not free to move.

So, the mesenchyme has the ability to move. So if you want the blastema to form after an amputation, the formation of mesenchymal cells is a must. The proliferative compartment is maintained by signaling interactions between the mesenchyme and the basal epidermis. So these two have to cross-talk. Then only will you know how much proliferating cell has to be accompanied or how many in the blastema and how many cells have to be differentiated. In addition to regulating blastema formation, there is one molecule called retinoic acid.

Retinoic acid; all of you would be familiar with retinoic acid. Another common name for it is vitamin A. Fibroblast growth factor FGF-2 plays a major role, whereas other FGFs also contribute. Canonical Wnt signaling positively regulates blast ml proliferation and outgrowth. These signaling events—retinoic acid, FGF, and Wnt signaling—contribute to positive outward growth. The Wnt signaling pathways are a group of signal transduction pathways made of proteins that pass signals from outside the cell through the cell surface receptor to inside the cell.

So the ligand of Wnt signaling is called Wnt. And the receptor of Wnt signaling is called the free cell. The moment Wnt binds to the free cell, it will create an environment inside the cell, because of which beta-catenin will be stabilized. Beta-catenin is normally degraded.

Every cell has beta-catenin. But the excess beta-catenin will be degraded. But Wnt signaling ensures that beta-catenin is not degraded. If beta-catenin is not degraded, it will go into the nucleus and create a lot of pro-proliferative gene expression events, which are needed for blastema formation. So, that is the typical scenario.

Canonical Wnt signaling. Three Wnt-signaling pathways have been characterized. Three different types of actions. One is the canonical Wnt signaling pathway, the non-canonical planar polarity pathway, and the non-canonical Wnt calcium pathway. So the Wnt ligand can make the vicinity respond in three different angles. All three Wnt signaling pathways are activated by the binding of a Wnt protein ligand to the Frizzled family receptor, which passes the biological signal to the protein disheveled inside the cell.

Disheveled is part of a destruction complex that normally degrades beta-catenin. Once the disheveled is hijacked by this Frizzled, the degradation of beta-catenin is prevented. Hence, it will go to the nucleus in a typical canonical Wnt signaling event. So you can see here, the canonical Wnt pathway leads to the regulation of gene transcription. Like I told you, many genes, including MYC, are present, and many cyclins are there.

These are all turned on because of the Wnt signaling. Remember, Wnt signaling is important. We have seen in planaria regeneration that Wnt signaling is essential for many biological pathways. And even if you have a scratch or wound, even if you have a mosquito or ant bite, the healing involves Wnt signaling. And many times the cancer also formed because of Wnt signals.

The non-canonical planar cell polarity pathway regulates the cytoskeleton. So the canonical Wnt signaling pathway activates gene expression events. Whereas another non-canonical planar cell polarity pathway of Wnt signaling regulates the cytoskeleton, which is responsible for the shape of the cell. The newly formed cells must have a particular shape. The mesenchyme should have a spindle shape so that it can migrate, but once it has lost its mesenchymal property as a part of differentiation, it retains its epithelial property.

Then it will settle down, and the morphology changes. So the regulation of the cytoskeleton is important because it decides the shape of the cell, which is governed by this Wnt signaling and the non-canonical Wnt calcium pathway that can regulate the calcium inside the cell. So, which is also important for various signaling events. So you can see here, this is one day after amputation: the control, and seven days after amputation, a normal fin is formed; whereas, when you inhibit Wnt signaling, you don't get it. Even after seven days, it looked just like one day itself.

That means if you block the Wnt signaling, you do not get normal regeneration. We will study more about fin in a later class, and we will learn about different aspects of regeneration in the next class. Thank you.