Regeneration Biology
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W5L23_Zebrafish heart regeneration-in a brief

Hello everyone, welcome back to another lecture on regenerative biology. So today we will learn about zebrafish heart regeneration briefly. And zebrafish, as we discussed in the previous class, have enormous regenerative capability. But not every organ is equal in regeneration. As we know, the liver can be regenerated in every animal. But when it comes to the central nervous system, such as the brain and spinal cord, the regeneration ability is compromised in many species.

But when it comes to the heart, because it's a very unique tissue made of cardiomyocytes or cardiac muscles, which are different from skeletal muscle, every animal that undergoes the so-called reduced supply of blood to the heart muscle, which we usually call myocardial infarction, has damaged tissue that responds by scar formation, which can debilitate. The contractile ability of the ventricle, if the auricles are damaged, means the heart will continue to function; but if the ventricular muscles are damaged, the contraction is maximal and more powerful when it comes to the ventricles, and if that is damaged, the animal will not be able to supply blood to the rest of the body, and that animal may succumb to death. So animal models to study cardiac regeneration have been one of the interesting aspects or topics in regenerative medicine because everybody wants to understand why and how myocardial infarction takes the life of an organism, so a lot of research is going on to understand if we can avoid it. The formation of a scar or fibrosis after a myocardial infarction.

So animal models to study cardiac regeneration have been explored for various questions. Permanent fibrosis and chronic deterioration of heart function in patients after myocardial infarction, referred to in short as MI, present a major healthcare burden worldwide. And in contrast to the restricted potential for cellular and functional regeneration of the adult mammalian heart, a robust capacity for cardiac regeneration is seen during the neonatal period in mammals. Neonatal means soon after birth, the very early stage of the organism's lifespan. That is neonatal in the adults of many fish and amphibian species.

Neonatal mammals and adult fish and adult amphibians are able to cope with a damaged heart or an injured heart, but in adult mammals, the damage does not result in a

regenerative response, so that is the time when you experience some cardiac trouble, not during your neonatal phase; hence, the knowledge or understanding or ability to. Intervening to cause a proper regenerative response in adults is very important when a mammalian species is concerned. However, we lack a complete understanding of why cardiac regeneration occurs more efficiently in some species than in others. So this is something that baffles the researchers. The adult heart of some animals can regenerate after damage, whereas the adult face of some animals responds to cardiac injury with scar formation.

Why is that? This is a baffling question for the researchers. The capacity of the heart to regenerate after injury is controlled by a complex network of cellular and molecular mechanisms that form a regulatory landscape. either permitting or restricting regeneration. So in animals that can regenerate, the gene expression landscape allows for regeneration. The gene expression events allow for regeneration.

Whereas in the same tissue, in another species at the adult stage, the same landscape causes a set of gene expressions that do not allow, which means we call it a non-permissive environment, hence regeneration does not happen. So this is a dendrogram; you can see where mammals are grouped here, amphibians are grouped here, and fishes are grouped here. You can see the gray color box indicates low regenerative ability, while the red color indicates high regenerative capability, and it has been classified into three categories: fetal, neonatal, and adult. Fetal is shown as a triangle, neonatal is shown as a circle, and adult is shown as a square. So if you have a blue square, it means low regenerative ability; a red square means high regenerative ability.

So in mammals, you can see that fetal studies done in sheep do not mean that other fetal studies are missing; it means that they have not been explored. When it comes to neonatal studies, you can see that neonatal heart regeneration is excellent in rats because it is red in color and has a high muscle mass; it is also high in rabbits and humans. Of course, nobody injures humans and tests it, okay? So you should understand that any damage or surgery that has to be done heals without any scar formation; that is what we know. Also, in the neonatal stage of pigs, it is excellent, whereas in all the species that have excellent regenerative capability in the adult phase, you see a blue mark, which means they do not have low regenerative ability. They do not have excellent regenerative ability; rather, they have very minimal or nil regenerative ability when it comes to adults.

One exception is the spiny mouse. What you can see here is that the neonatal stage has not been tried; rather, you don't have to try the neonatal spiny mouse because the adult... The heart has excellent regenerative ability.

So it is logical to assume that neonates and fetuses will have much more efficient regenerative abilities. Unlike all the mammals tested, the adult spiny mouse heart has the ability to regenerate. When it comes to amphibians, nobody bothered to study fetal and neonatal stages because adults have excellent regenerative ability. You take any newt of your choice; you take it. And Xenopus, the African clawed frog, adults have minimal regenerative ability.

And whereas Xenopus tropicalis and Xenopus levis have less ability, Xenopus tropicalis has less. Adult faces also have regenerative ability. An axolotl has excellent regenerative ability as an adult. And when it comes to zebrafish, nobody bothers to study fetal and neonatal heart because adults regenerate excellently. The exception is the Mexican tetra.

It has two forms: surface-dwelling form and cave-dwelling form. Surface-dwelling forms, adults have good regenerative ability, whereas the cave-dwelling form has the least regenerative ability. Remember, both come under the same genus, that is, Astyanax mexicanus, which is the Mexican tetra; they are the same species and the same genus, but the surface-dwelling form has excellent heart regeneration ability, while the cave-dwelling form, which is basically blind, does not have the ability to regenerate. This is something worth knowing, and Japanese medaka do not have regenerative ability at all. It doesn't have the heart regeneration ability, whereas the killifish has excellent regenerative ability, and zebrafish, as you can see here, have excellent regenerative ability.

Additionally, there is another species called the giant danio that also has regenerative ability. Goldfish have excellent regenerative ability as well. Tetra fish, which is the surface-dwelling form, can regenerate, while the cave-dwelling form cannot, and medaka cannot regenerate; so this picture lets you know that regeneration is not a blessing, but it can often cause trouble for some organisms' survival. If it has the ability to regenerate, then it will make a better living going forward, whereas in mammals you may wonder. It doesn't have the regenerative ability; then how is the species existing? So we cannot connect the regenerative ability of the heart to the survival of a species.

Like you have seen, some fish cannot regenerate, so it has to be understood that whether or not a species is regenerating, the ability to regenerate gives an added advantage to that particular species. So, when you look into heart regeneration, there is no significant regeneration of adult mammalian cardiac muscle after experimental injury paradigms. This efficiency is highly relevant to the human disease that we refer to as myocardial infarction. And the scarring is the primary cause of morbidity and mortality. Like I told you some time ago, the formation of the scar renders the cardiac muscle less contracted.

So this is not a friendly thing to happen. So you want the heart muscle to contract, but

the scar will come in between. It is just like you are eating some food and in the middle, I am putting in a stone and you are biting through the stone. How horrible a feeling that will be. Same logic applies when the heart is contracting; the scar will be an impeding, intervening obstacle.

So there are currently several injury models that stimulate heart regeneration in zebrafish, including surgical resection of the ventricular apex because zebrafish have two-chambered hearts and the ventricle has a conical edge. It's almost like an inverted pyramid. So that con is very easy to cut. You can either touch or handle everything. Without affecting the normal functioning of the heart, you can amputate it, or you can use dry ice, which is similar to that of a myocardial infarction, and that is called cryo-injury.

People also damage cells using genetic ablation; for example, you can express a gene called nitroreductase, which is from bacteria, and then you can use metronidazole. The nitroreductase acts on the metronidazole and kills the cells. So, this is a genetic ablation. There are different methods. People also use the thymidine kinase-ganciclovir system to damage the cells.

We don't have to go into the details, but the most prominently used ones are either surgical amputation or treating cryo injury and dry ice. So, because dry ice mediated is something similar to that of myocardial infarction. Because of a myocardial infarction, nobody comes and cuts your heart; rather, the blood supply is blocked. Whereas cryoinjury mimics aspects of MI, that is myocardial infarction, genetic ablation produces massive injuries that remove up to 60% or more of the cardiomyocytes. That is why it is a very alarming situation, and it is not very logical or close to that of myocardial infarction that occurs in humans.

So one could use those genetic ablation studies for academic purposes but may not have clinical relevance. Inducing signs of end-stage heart failure. Unlike severe heart failure in humans, these signs regress within weeks, and the animals typically make a full recovery concomitant with muscle regeneration. In those animals we saw in the previous chart that are capable of regenerating the heart, they will completely restore, which doesn't happen in human heart regeneration, involves two fundamental components: one component is the proliferation of existing cardiomyocytes. The heart is made of cardiomyocytes, and the cells of cardiac muscle are called cardiomyocytes.

As the primary cellular source, they are proliferative and can proliferate to some extent; however, that is not the only thing in an environment that stimulates muscle regeneration from this source. Cardiomyocytes are proliferating, but are you able to make? Muscles, the contractile muscles, are what is important. You have the raw material, but are we

making the end product? So both are needed. Raw materials have to be formed. Cardiomycetes, if they are not able to proliferate adequately, undergo reprogramming, become stem cells, proliferate in number, and then differentiate into cardiomyocytes and cardiac muscle.

So heart regeneration is something people have done experiments called genetic fate mapping, which basically means if I give a simple example, you will understand: you lit an agarbati or you created smoke in your house, and you can see where all the smoke is going. But normal air is also moving like that. But you can't see. Normal air is not colored. So what you did was put a tracer in the air in the form of smoke.

So if you light an Agarbathi that gives off smoke, you can see where all this smoke is going for a certain distance. You cannot keep it; you put an Agarbathi on Monday, and you cannot trace that smoke on Wednesday. That is not possible because it diffuses. The law of diffusion applies. Similarly, if you have a cell, the origin of the cell, and you mark it with something, there is a—oh, today, if this cell proliferates, I am marking it with the GFP or some traceable protein.

So it will become a permanent marker. Like you may have seen in some people who go to, like, you know, some temple like Tirupati or maybe to a large pilgrimage spot, the law authority will give you. Some stickers on your hand will indicate that there is a barcode showing when you entered and how long it is valid, etc. So wherever you go, you have a tag that signifies you are a bona fide, authenticated visitor, and anyone can scan it to know, "Oh, this person entered at this time and will leave this premise at this time," similar to genetic fate mapping. Allows researchers to understand how the cell is formed and where the cell is going to go eventually, just like how you will know if a kindergarten child will become a doctor, a lawyer, or an engineer; you have no way of knowing, but if you have some tag at that stage and if he becomes an engineer, you will know this tag as a kindergarten tag. If he is having this tag now and he became an engineer, that means you are detecting it.

That is what we will do. Fate mapping experiments in zebrafish have made it clear that the regenerative ability of the zebrafish heart relies mainly on... exclusively on the proliferation of the existing cardiomyocytes.

The heart has cardiomyocytes. They have to enter the cell cycle. Normally, they don't proliferate. They have to enter the cell cycle from G0 to G1. It has to migrate. These source cardiomyocytes show characteristics of dedifferentiation, including a reduction in the contractile structure.

Cardiomyocytes, which are basically the cells of the cardiac muscle, have the ability to contract. But when they get into a de-differentiation mode, they do not contract because if they keep contracting, they cannot proliferate effectively. So they reduce their contractile properties, have more of their proliferative properties, and increase in number. And then they become these contractile cardiac muscles. Cardiomyocyte proliferation occurs at a low rate in the adult zebrafish heart, regardless of whether there is an injury or not, but it is sharply increased after an injury is made or because of an injury response or tissue damage; this proliferative rate of the cardiomyocytes increases tremendously.

And because of this, you are able to create a mass, a mass of cells, something similar to that of the bleastema, which we have been discussing in planaria, zebrafish fins, etc. So, injury to the zebrafish heart initiates an organ-wide reaction detectable as the induced expression of a gene called RALDH2. What is RALDH2? A retinoic acid-synthesizing enzyme. In other words, it is called retinaldehyde dehydrogenase. So, what is retinoic acid? Vitamin A was also observed in the fin regeneration.

So, RALDH, when it is induced, tells that there is an abundant supply of vitamin A at that spot because it is needed. You need to have retinoic acid. It is as early as one hour after the injury. Within one hour of injury, you end up getting a severe induction or a huge increase of RALDH.

Otherwise, you won't get it. So why RALDH? You need to have retinoic acid production. Where the endothelial lining of the lumen is, there is the wall of the heart that is facing the bloodstream; it is bathed in the blood. The endothelial lining of the lumen of the heart, within a day or two of the injury, shows an analogous organ-wide response of RALDH2. That means you may have made a cut in one area. So if the heart has 100 regions, you cut only in the 100th region, but the induction of RALDH will be from sector 1 to sector 100.

So every area, irrespective of where you caused the injury, the entire heart now produces the RALDH production. This doesn't mean that the whole heart is regenerating, but the response of the heart is pan-heart or it is panning the entire heart. Induction then causes the epicardial cells to proliferate and surround the regenerating muscle. Epicardium is the outermost layer. Okay, the epicardium, myocardium, and endocardium are the three layers present.

The next picture will have better images of that. where they release signals that facilitate cardiomyocytes proliferation. So we can see the epicardium is the one that is allowing their aldehyde expression, and the epicardial cells enter a proliferative phase and surround the regenerating muscle, where they release signals that can facilitate the

proliferation of cardiomyocytes. You can see heart regeneration in this picture. You can see that this is an uninjured heart. This is basically the SA node, AV node, and what you call the pacemaker region.

And you can see that soon after the injury, there will be blood, and blood will be present six hours post-amputation, six HPA. You have created a cut here in this picture, and the endocardial activation happens; later, epicardial activation occurs by 3 days post-amputation, which causes upregulation of retinoic acid because RALDH2 is activated, along with TGF beta, sonic hedgehog, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), and hypoxia factor. These are upregulated, and some microRNAs, such as MIR-133, are downregulated. By 7 dpa, there is a peak of proliferation in the amputation method, and by around 14 dpa, vascularization occurs, meaning that the blood supply or blood vessels are being formed, and muscle regeneration happens by around 14 dpa; then comes electrical coupling and the dissolution of the clot.

by around 30 dpa. Electrical coupling means the pacemaker should be able to give a signal to these newly formed cardiomyocytes so that they can contribute to contraction. So the blue color is the clot, as you can see here. And the brown color represents the blood vessels that you can see here. And RAL-DH2 mRNA is this sky-green color.

So you can see here its expression. Initially, you see 6 hours post-amputation; the entire heart is having RAL-DH. That's why it is blue. And the activated GATA4, which is a cardiac transcription factor regulatory sequence, you can see in green as you can see here. Here, it also responds up to this point, and then it is restricted to the site of injury; by around 30 dpi, you see a little bit of this GATA4 expression, which is a heart-specific transcription factor.

So this is the layers of the heart. What we have seen of the heart wall is the epicardium, and this is the myocardium, where the cardiomyocytes and the cardiac muscles are. There is the muscular middle layer, which is very thick, and the endocardium, the thinner layer. That is what we said about the lumen of the heart or the lining of the heart to the blood; that is the inside. So this is a small portion. This is not a zebrafish heart; this is a human heart, but it's a cartoon just for understanding.

The same layers are being shown. restored and retained in zebrafish also. So, the epicardium is the one that contributes to epicardial activation, which in turn contributes to these signaling events. Initial activation is the endocardial activation. That is what you see here in the very beginning. Heart regeneration, when you look closely, is a very interesting aspect.

What we can reveal is that if you tweak or prevent some signaling events, like you saw in this previous picture, there are so many: RA is there, TGF is there, SHH is there, PDGF is there, IGF is there, FGF is there. When this many are there, we think, oh, if I can get rid of one, what might happen? Oh, there are that many, no? It is not like you have an apple, an orange, a banana, and maybe other guavas; there are so many fruits. If I remove one fruit, you will not starve. But from this angle, they are not like a fruit or vegetable basket.

Each one has a root. So they are not expressed in a fashionable way. Each one. If you get rid of RA, you will find something. If you get rid of IGF, you will find something.

That means heart regeneration may occur. But it is... Think about a human baby that is being born; everything is fine, but the eyes are missing, so you wouldn't call it a human baby. It is not a perfect baby, which means the life of that particular baby will be compromised. If someone doesn't have ears, you may wonder, "Oh, it is still human." Yes, it is human, but it is not perfect. So if you get rid of any of the signaling events, sometimes it may not regenerate at all.

It depends upon what the hierarchical position of that particular signaling event is. Is it sitting at the base of a branch of signaling events? Is it a master gene, or is it a player gene? But understand, every gene has a role. That is what we'll be seeing by blocking some pathway. What do we get? So you have done the amputation and what you are seeing here soon after there is a proliferation.

There is a key given here. Undifferentiated progenitor cells are present. And the green color differentiates cardiomyocytes because undifferentiated cells can differentiate back. And here you can see these newly differentiated cardiomyocytes. Undifferentiated and newly differentiated. Both are functionally the same but depend on their age. And new vasculature, which is shown in red, this vessel and cells derived from the epicardium are shown in blue here.

As you can see, there are one, two, three, four, five, six different stages. Stages five and six are important because they are somewhat towards the terminal phase of regeneration. What they have done is they just got rid of FGF signaling. Remember, FGF signaling is listed here; you can see that FGF signaling is there. And they got rid of the FGF signaling, and what did they end up getting? They end up with a scar. Zebrafish that are capable of regenerating showed that when you blocked the FGF signaling, wound healing occurred.

The heart is fine also, but there is the formation of a scar, which means contraction may not be perfect; although regeneration happened, contraction may not be perfect. This is why you got an unwanted scar formed in between, which can interfere with the proper contraction of the cardiac muscle. Cardiac muscle characteristics, if you look very closely, are something very interesting. Cardiac muscle characteristics are associated with different types of heart regeneration depending on which species you are talking about. What we have seen so far is that many animals vary based on their fetal, neonatal, or adult stages.

Mammals, the majority of which are neonatal and fetal, have the ability to regenerate, not the adults. Exception is for the spiny mouse, which is of the Acomys species. Whereas when it comes to fish and frogs, nobody has bothered to study the regeneration ability of fetal or neonatal forms. But adults can regenerate.

Exceptions are medaka or maybe some cave-dwelling form of fish, etc. Now, how are they responding at the tissue level: mass musculus, that is, house mouse versus spiny mouse, if you compare increased neovascularization in higher numbers of cells and the fibrotic wound tissue and the elevated number of diploids? Mononucleated cardiomyocytes are associated with increased cardiac regeneration potential in the spiny mouse compared to that of the house mouse. You can see here in this panel that this is Mus musculus, which is a comic species, and the ECM is very strongly formed. The cardiomyocytes that are formed have a huge energy consumption, and there is also the formation of an unhealed area, which means there is a scar being formed. Whereas in the case of the spiny mouse, you can see the cardiomyocytes that are formed and the fibroblasts formed are.

not contributing to the formation of the scar. The same thing applies to the medaka or Japanese rice fish or ICS Latipus versus zebrafish. Or, while ICS Latipus cannot regenerate, zebrafish can regenerate. An interesting thing you can see is that when you injure the medaka, you have a lot of neutrophils being formed, and macrophages are also being formed. You have some healing, but no complete regeneration. In contrast, in zebrafish, you have the same neutrophil cells and also macrophages, but in much greater quantity.

Here, the inflammation is very high, whereas in zebrafish, the inflammation is minimal, and the anti-inflammatory macrophages are very high, hence there is no scar, and complete normal regeneration happens. The restricted presence of neutrophils and the early recruitment of macrophages are associated with high cardiac regeneration potential in zebrafish compared to that of medaka. Now, let us come to the cave-dwelling fish. Very interesting. Cave-dwelling versus surface-dwelling forms of the Mexican tetra,

Astyanax mexicanus, elevated energy metabolism, restricted immune response, and reduced fibrosis are associated with high cardiac regeneration potential in the surface-dwelling form, not the cave-dwelling form.

Compared to the cave-dwelling form, you can see here their energy metabolism, and you can also see their immune cells. In the cave-dwelling form, the immune response is strong compared to that of the surface-dwelling form, and because of the low immune response, you are able to get proper regeneration. So, we will study another topic in the next class. Thank you.