Regeneration Biology
Rajesh Ramachandran
Department of Biological Sciences
IISER Mohali
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W6L27_Detailed mechanisms of fin regeneration

Hello, everyone. Welcome back to another class on regenerative biology. In today's class, we will learn about a detailed mechanism of regeneration that pertains to the fin in the zebrafish model. So we have seen how efficiently zebrafish are able to regenerate various organs. And coming to different organs, such as the heart, the brain, the retina, the spinal cord, and the fin, they adopt a different strategy. So if you look further into retina regeneration, you know zebrafish exhibit epimorphic regeneration in which non-differentiated cell mass formed after amputation is able to fully regenerate the damaged part, which can be, say, limb, heart, muscle, etc.

The backbone of epimorphic regeneration is that it is capable of restoring the morphology as it was before the injury. In 2003, scientists from the Sanger Institute compared around 26,000 zebrafish genes to that of the human genome, and they reported that Almost 70% of the human genes have orthologs in zebrafish. That is very interesting and exciting news simply because although zebrafish and humans don't look the same, both are vertebrates, and at the genetic level, there is a lot of genomic homology and synteny, which is an exciting feature zebrafish possess when it comes to regeneration studies. So Aristotle, a very old Greek philosopher, in his articles dedicated to natural history, mentioned that animal embryos regenerate more efficiently than adults.

So he has observed. One interesting thing about regeneration biology is that it is one of the ancient branches of biology because everybody knew about planaria regeneration some 250 years ago. And still, it is an evolving area of research that seems like a conundrum. But that is a fact. Although the key genes responsible for limb regeneration and inducing the proliferation and differentiation of somatic and stem cells are similar in amniotes and anamniotes, which are the animals that have amnion and those without amnion, in mammals these genes are not activated after amputation, so we should understand that some animals, despite having the ability to regenerate, do not exhibit this capability.

And the genes responsible for regeneration exist. Sometimes they are not turned on in time, which can account for your lack of regeneration. The process of regeneration is complete in around two to three weeks, or around 14 to 28 days. It is completed

depending on the water quality, temperature, diet, pathogenic organisms, and infection at the wound site. And also the stocking density, how many fish you are putting in one tank.

So, fin regeneration, when you look closely at zebrafish, has five stages. The first stage is the formation of the wound epidermis, which is created not by proliferation but by the migration of cells from the surrounding area, along with mesenchymal disorganization, blastema formation, and regenerative growth. And the final stage is termination. So bleeding is stopped soon after an injury amputation. The first danger signal that is initiated by reactive oxygen species (ROS) attracts immune cells at the site of injury.

So ROS is capable of bringing in the cells, especially the immune cells that are necessary for regeneration to kick-start; neutrophils arrive within minutes of injury, and it causes blood clotting, removes antigen debris, etc. Wound epithelium formation is the next stage that occurs, and what happens is the migration of the cells that come from the rest of the body into the. The apical epidermal cap is one of the initial stages and a necessary stage required for regeneration to occur, and one of the pro-inflammatory cytokines, interleukin 1b, is induced at the site of injury, which causes inflammation. This credit also goes to the M1-like macrophages that are capable of, as you can see, different types of cells: epithelial cells, neutrophils, M1-like macrophages, and ROS; they are all present here in this injured area. Additionally, you can see the osteoblasts in this injured area; there is a lot of congregation of these cells, but the pro-inflammatory environment is one of the initial stages that is necessary.

So, with macrophage depletion, people have usually done different ways of analyzing and understanding fin regeneration. So how it works is that the macrophage depletion has resulted in a significant decrease in the caudal fin outgrowth. So we know M1 macrophages are necessary for the pro-inflammatory cytokine, but a significant decrease in caudal fin outgrowth occurs because of macrophage depletion. And it also causes changes in the fin morphology, including areas of aberrant tissue growth, defects in bone array patterning, and heterogenic bone calcification. So what do you understand is that if you deplete the macrophages, the pro-inflammatory environment will not be sufficient for the morphology and epimorphic regeneration to be established.

So the macrophages regulate tissue homeostasis, organ repair, and angiogenesis, migrate to the wound, and secrete growth factors and cytokines, mainly pro-inflammatory cytokines, that in turn activate the ECM, extracellular matrix production. ECM is a necessary component so that these newly formed blastema cells can migrate. So ECM is a must, as it presents all tissues and can regulate wound healing, providing a patch to protect the damaged area. So the ECM production by the immune response cells is important for establishing structural stability in the injured area. In contrast to zebrafish

and other fully regenerating species, mammals form scar tissue after amputation, which is nothing but an overproduction of ECM protein.

After amputation, the deposition of fibronectin and collagens, the major components of ECM leading to fibrosis, can hamper the proper migration of cells due to the overproduction of ECM, but the structural stability is retained on the injured side. In zebrafish, when bleeding following caudal fin amputation is stopped, the amounts of fibrin and fibronectin decrease. What makes, and what causes, to prevent the scarring. Otherwise, scarring can happen. Prevent scarring because when the wound is closed, proteolytic enzymes such as matrix metalloproteinases, or MMPs for short, remodel the matrix protein to protect the healing of the organ or tissue and allow migrating cells to replace the lost tissue.

So, in other words, you can tell that you want to occupy a house. for a room. But if that room has too much junk, unless you clean it, no one can get in. The room had 200 chairs. How will you enter the room? So you have to clean it.

So this cleaning help is provided by the MMPs. Blocking MMPs negatively influences fin regeneration. MMP enzymes positively influence the activation of chemokines. Chemokines are molecules that allow cell migration. They normally help provide directionality to the cells that stimulate the migration of immune cells to the injury site.

And it also helps in maintaining homeostasis and controlling cell migration during embryogenesis and regeneration. So these are all the important roles played by the MMPs. So, if you block MMPs, you don't get these features. Hence, you don't receive the regeneration. Another pro-inflammatory cytokine, such as interleukin 1B, is induced at the site of injury, triggering regeneration-inducing genes.

at that particular damaged area. However, in the absence of macrophages and with the overexpression of interleukin 1B, we have already seen that if you get rid of macrophages, you don't achieve proper regeneration. And we also know that the interleukins are produced by the macrophages. So you depleted the macrophages and overexpressed interleukin 1B. It causes the apoptosis of the regenerative tissue.

So what you understand from here is that the role of macrophages is not just to produce interleukins; their role is interleukin production plus many more functions. That is why, if you want normal epimorphic regeneration, macrophages and interleukins are a must; just supplementing interleukins is not a compensation for the absence of macrophages. So another pro-inflammatory cytokine is TNF-alpha. It was identified as a crucial component of caudal fin regeneration. This TNF-alpha is synthesized by M1-like

macrophages.

There are two types of macrophages: M1 and M2. M1 always produces pro-inflammatory cytokines. M2 macrophages always produce anti-inflammatory cytokines. And they get recruited to an injury region and exhibit dual action of enhancing the accumulation of macrophages and participating in blastema formation because they can produce more TNF-alpha. TNF-alpha means more M1 macrophages.

More M1 macrophages mean there are more pro-inflammatory cytokines. So it's a kind of positive feedback cycle. Another molecule, hyaluronic acid, has been shown to be highly abundant in the extracellular matrix (ECM) of the regenerating fin. The enzyme hyaluron synthase 3 is also known as HAS3. Gene is overexpressed within six hours after the caudal fin amputation and increases its maximum concentration within 24 hours, so what you understand is that hyaluron synthase and hyaluronic acid production are necessary in the first few hours of amputation.

Inhibition of hyaluronic acid synthesis during the first 24 hours post-amputation blocks it by whatever mechanism. You can block the HAS3 enzyme, which produces the suppression of blastema formation and regeneration. Whereas the inhibition at later stages, say post 24 hours, is being inhibited. It had no significant effects on the regeneration process. So the initial few steps, which are the production or synthesis of hyaluronic acid within the first 24 hours, are very crucial for the blastema formation to initiate.

Hyaluronic acid molecules activate signaling cascades required for blastema establishment through the induction of specific blastema markers. This is one of the assigned functions of hyaluronic acid. Within one to three hours post-amputation, HPA means hours post-amputation, the site is covered by a multilayer wound epidermis that is migrating from the surrounding side tissue of the fin formed by non-proliferating epithelial cells distally migrating into the site and creating an apical epidermal cap. So these cells migrate into the wound site and create a cap called the apical epidermal cap. Which works as a physical barrier between the body and the environment.

Of course, it will protect against getting an infection. And around this is happening within 1 to 3 hours only. At around 18 to 24 hours, the mesenchymal cells underlining the wound epidermis, the apical epidermal cap below that, mainly osteoblasts and fibroblasts lose their organization and turn into a highly proliferative whitish mass of cells called blastema; that is formed by around 24 hours. You can see under the microscope that can be detected under the apical cap, which is the apical ectodermal cap. Beneath that, you will find a blastema cell that is formed.

Blastema has two main functions. One is to support the outgrowth and form specific structural elements essential for fin restoration. And it's mainly regulated by Wnt beta-catenin signaling, sonic hedgehog signaling, fibroblast growth factor signaling, bone morphogenetic protein signaling, active insulin-like growth factor, and notch and retinoic acid pathways, which contribute to the activation and maintenance of the blastema. So many signaling events undertake the establishment of the blastema and the migration at later stages. If you look closely, the TGF-beta signaling is very interesting; it is a graphical illustration of TGF-beta signaling, which has two phases: one is canonical, and the other is non-canonical. The left side is canonical, and the right side is non-canonical.

In canonical TGF-beta signaling, the TGF-beta ligand binds to TGFBR1 and TGFBR2. There are two receptors, TGF beta receptor 1 and receptor 2. Receptors result in their activation to form a heterotetrameric complex. That means four receptors will come together to form a heterotetrameric complex. So the intracellular events that include SMAD2-3 are next activated by phosphorylation due to the TGF ligand binding to the receptor; as you can see here, it formed a heterotetrameric complex.

This leads to the activation of SMAD2-3, which are activated by phosphorylation initiated by TGFBR1 of this heterotetrameric complex. PSMAD2-3 form a complex with SMAD4. Another free-moving molecule, SMAD2-3, when activated, will recruit SMAD4 together with certain transcription factors. They activate specific target genes that contain SMAD-binding elements.

In short, it is called SBE. So in the DNA of those gene regulatory regions, if there is an SMAD binding region, this complex, SMAD2-3, can go and bind to them. And they... Usually, those genes that are implicated in growth inhibition, apoptosis, ECM synthesis, and immune response have.

Whereas in the non-canonical pathway, what we have seen is a canonical pathway where these MAD genes bind to the SBA and cause gene expression. In a non-canonical pathway, TGF beta induces the expression of a molecule called TRAF6 and ubiquitinase, and it ubiquitinates that particular TRAF6 molecule in a lysine 63-dependent manner, promoting its catalytic activity. What happens to the TRAF6 then activates another molecule called TASE and PSEN1, and these two molecules get activated, resulting in the proteolytic cleavage of the TGF beta R1, the same receptor that forms a hetero-tetrameric receptor. TGFB R1 generates a soluble TGF beta R1 intracellular domain. So you end up getting a free-moving domain of TGF-beta R1.

And this is, in short form, called a TGFB R1 ICD. ICD stands for intracellular domain.

The endosomal adapter proteins called APPL1 and APPL2, along with various microtubules, are required for the translocation of the TGFB R1 intracellular domain to the nucleus. So the function of this intracellular domain is within the nucleus. where it contributes to the activation of specific target genes, whereas this MAD is not involved here.

TGF beta R1 is directly involved, and other modes of non-canonical TGF beta signaling pathways are also discussed and described. So that is what you are seeing here in A; on the left side, you are seeing the canonical, whereas here you are seeing the On the right side, you are seeing the non-canonical TGF beta signaling. And there is another TGF-beta signaling. They can also induce EMT, epithelial-to-mesenchymal transition, through the activation of the non-SMAD pathway. This is also TGF beta signaling, but it's a non-SMAD pathway where it involves something called Rho-GTPase via phosphorylation as a polarity protein, also known as PAR6, leading to cytoskeletal rearrangement.

So this is also non-canonical TGF-beta signaling, but it's a non-SMAD pathway involving the PAR6 protein. And this leads to the rearrangement of the cytoskeletal proteins, often causing a breakdown of epithelial cell junctions. Because we should understand that for the blastematiform epithelium to migrate, etc., you cannot have cells attached to each other. You need to break that junction so that the cells can move across.

So the TGF-beta signaling plays some unique roles in defining the regenerative capacity of the fin. Another signaling pathway, which can be called a sibling signaling pathway, is called BMP signaling because both BMP and the TGF-beta signaling make use of SMAD proteins. Let us see how BMP signaling contributes. You may remember that BMP signaling is also important in determining the dorsal area or the dorsalization of the planar embryos. You may recollect that in the previous classes we studied.

So, the complex regulation of bone morphogenetic protein signaling is quite interesting when it comes to the regeneration of fin tissue. BMPs are processed by a pro-protein peptidase to generate mature dimers that then bind to two copies of type 1 and type 2 BMP receptors. Two types of receptors are type 1 and type 2. Generating a heterohexameric complex.

In the case of TGF-beta, we saw heterotetrameric. Here we have six copies of the hexameric complex. Binding of BMP homodimers to their cognate receptors leads to phosphorylation of the type 1 receptor and also by the type 2 receptor in the GS domain. This is what happens; it is a domain present in these receptors. Activated BMP receptors then phosphorylate a different kind of SMAD.

They are SMAD1, SMAD5, and SMAD8. Earlier in TGF beta, we saw SMAD 2, 3, and 4. Here are 1, 5, and 8 proteins that dimerize with SMAD 4. SMAD 4 is common for both TGF beta and BMP signaling because you can say they will compete for SMAD 4. and accumulate in the nucleus, where they mediate changes in BMP-regulated gene expression. So BMP has its own target genes; unlike TGF-beta and BMP, they act on separate sets of genes.

Regulation of this pathway occurs extracellularly via the binding of extracellular antagonists such as Gremlin, or GREM1 for short. NOGGIN, these are antagonists of BMP signaling, NOGGIN, or in the plasma membrane via the action of pseudo receptors such as BMP and actin membrane-bound inhibitor; in short form, it is called BAMBI. In addition, inhibitory constraints or receptor-mediated SMAD1,5,8 phosphorylation can occur via FK binding protein. These are all amino acid residues: phenylalanine and lysine.

FK means FK binding proteins 1 and 2. FKBP12 is the name of that protein binding and inhibitory SMAD6 binding. This will be triggered in. which is relieved by the action of a methyltransferase known as PRMT1. Additional regulation of BMP signaling occurs via the cytoplasmic phosphatase and ubiquitin ligases such as SMURF, as well as through micro RNA and methylation-mediated pathways, which can control BMP-mediated gene expression. That means there are checks and balances occurring throughout, so there will be a push and there will be a drag, and again there will be a push and there will be a drag.

So this is the mechanism through which various pathways work. Whatever we discussed is listed here, like BMP; it gets activated and forms a dimer, and it can be influenced by its antagonists. If they are present, they will not get a chance to bind to the receptor. If the antagonist doesn't trigger, then they will bind, and it will lead to activation. Either the receptor can be activated and trigger Co-SMAD4 and R-SMAD. They can work on gene expression even in the nucleus, or they can also get involved in other pathways where R-SMAD can be influenced by other complexes such as SMAD7, SMARF, and phosphatases.

They can influence the availability of RSMAD1,5,8. Another mechanism is that once the BMP is bound to its receptor, it can influence the expression of SMAD6, which will also tweak the BMP signaling events. So what you should understand is that the BMP comprises a large subgroup of ligands from the transforming growth factor beta family. Upon ligand binding, the BMP receptor 1 dimer phosphorylates the BMP receptor 2 dimer and initiates its kinase activity, stimulating the action of various downstream mediators. The main targets of BMP receptors and phosphorylation are mainly the SMAD proteins, which are regulators of fibrosis and translocate from the cytoplasm to

the nucleus where they bind to the DNA at SMAD binding motifs of various DNA sequences of regulatory elements of various genes, causing tissue-specific gene expression. Different homologs of type 1 BMP receptors are expressed in almost all regenerating fin tissue types because BMP signaling is one of the pivotal signaling pathways for fin regeneration.

So BMP is active in the distal blastema, wound epidermis, osteoblasts, and blood vessels of the regenerating tissues. Initially, BMPs are essential to remodel the plexus into blood vessels. Naturally, the newly formed blastema needs nutrition, which comes from angiogenesis, that is, blood vessel formation. BMP promotes the deposition of collagen fibers into the basement membrane while closing the wound, which induces the actinotrichia formation, a unique structural protein of the fins. So if you look further, what are the early steps of fin regeneration? After amputation, what happens? The wound is closed within 12 hours post-amputation by migrating epithelial cells, which form a multi-layered epithelium called the wound epidermis.

Next, the blastema is shown in orange. Accumulation happens by proliferative undifferentiated cells, which we often call blastema cells. Under each fin ray, we have seen the structure of the fin. It has about 16 to 18 fin rays per fin, which are made of hemi rays and look like a bracket for structural stability. And next, the blastema, which is shown in orange, causes an accumulation that results in a congregation of proliferative masses of cells and differentiated cells.

They are formed atop the amputated site below the epidermis. Each ray keeps allowing because the epidermis is covering the whole wound, and the blastema is forming mainly on top of individual rays and between the rays, the so-called interrays, where you have mainly fibroblast tissue. They are not true blastema tissues. They are digitally migrating cells during regeneration; the blastema cells proliferate and differentiate to replace the missing tissue. As you can see here, msxb is one gene that serves as a blastema marker. You can see a blue color right below the individual rays, but between the two blue areas, there is a white area where there is no msxb expression.

There, there are fibroblasts; they are not. True blastema, but it is just a mass of cells. So if we look further, there are a few more molecular events. Inhibition of SHH signaling in the injured tissue by cyclopamine leads to the suppression of Wnt beta-catenin, FGF, and retinoic acid genes and loss of regeneration. However, the activation of the Wnt pathway results in the restoration of the regenerative processes. At the level of blastema establishment, the canonical Wnt/beta-catenin signaling pathway regulating stem cell pluripotency is critical to blastema formation, which in turn is essential for fin ray development.

So the Wnt beta-catenin pathway during the regeneration process is to establish the organizing centers of the blastema to indirectly regulate the activity of other signaling pathways such as FGF and BMP signaling, which control cellular proliferation and differentiation, which we have already seen. During the buildup of the cell mass and the superexpression of the Wnt beta-catenin inhibitor, DKK1, which suppresses tumorigenesis and cell proliferation in a normal scenario, it causes the cessation of blastema formation and regeneration. Therefore, as a negative regulator of Wnt signaling, if you overexpress it, you don't achieve normal regeneration. Knocking out the DKK1 gene results in impaired limb and brain development during embryogenesis in mice. Thus, the regular Wnt beta-catenin DKK1 interaction is required to successfully initiate the regeneration.

And if you look further at the downstream events, what you can see is that FGF is expressed at the early stages of fin regeneration, which controls blastema formation, osteoblast de-differentiation, proliferation, actinotrichia, and fin restoration. Errors in regulating the FGF pathway result in severe abnormalities, such as tumors, that are often seen. Fibroblast growth factor receptors on which the fibroblast growth factor acts significantly contribute to effective regeneration. FGF is essential to maintaining a subpopulation of epidermal and blastemal cells to initiate blastema proliferation.

A mutant zebrafish with the FGF20a gene was temporarily knocked down. Epithelial cells covered the injury site, but the site did not completely regenerate. Wild type fish do not bring about blastema cell formation, but we do not replace them. The wound healed entirely without restoring the missing fin. So wounds can get healed, but regeneration will not happen if FGF20a is deleted. It was found that the notch is activated in response to caudal fin amputation, but is not involved in cell migration or de-differentiation after the blastema has formed.

And you also, one more thing I want to tell you: if you cut the blastema into two different pieces, one proximal and one distal. So the proximal cut will have more regenerative ability than the distal cut. So the site specificity is also influenced by genes like LDH2A and FGF signaling. So we will study more about regenerative biology in the next class. Thank you.