Regeneration Biology
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W7L31_Different cellular signaling during regeneration-Overview

Hello everyone, welcome back to another session of regeneration biology. In today's class, we will learn about different signaling events that are induced soon after injury, with some reference to limb regeneration in axolotls. So there are different signaling events that contribute to a regenerative response, but what is more important is the initial phases of signaling and the other signaling events that contribute to later events. We will address them one by one, but we will try to learn. What are the contributions of the initial signaling in the regeneration event? So we are focusing on one tissue that is limb regeneration, but more or less in other tissues there will be more weight given to a given signaling than to the other. That is very likely.

Limb amputation in salamanders, which are a well-studied model, leads to a wound response that ultimately replaces the missing part, and we usually call it epimorphic regeneration. This is very unique among tetrapods and involves the migration and recruitment of multiple cell types because, in a limb, there are so many different types of tissues: bone, muscle, epithelium, connective tissues, blood vessels, etc. and also various other associated tissues, neurons, etc. comes into the picture.

And this includes immune cells, epithelial cells, axonal growth cones, and connective tissue cells. And these all contribute to the formation of the blastema, which contains the proliferating group of stem cells in the blastema. And we can also call them progenitor cells because they give rise to a specific tissue type to rebuild the exact lost part of the limb. So a number of signaling events and cell biological processes have been defined to bring about this remarkable phenomenon, so we should also understand the intimate coordination; this is very important. It's not just coordination; it's the intimate coordination.

Of physical events such as osmotic pressure, cell migration, cell communication, and can also bring changes in cell identity, such as de-differentiation and morphing into embryonic cell-like characteristics, we normally call these blastema cells mesenchymal cells because they are derived from adherent attached cells. Now they are becoming less adherent. They are more migratory. The formation of the blastema takes a prolonged period of around one to three weeks. Although the formation of blastema happens

immediately, it's a normal assumption of a tissue type, etc.

It can take up to around one to three weeks, depending upon the size and shape of the organ, as well as the age of the animal and the species of salamander you are working with. Whether it is in the larval stage or in a metamorphosed stage, everything determines whether limb regeneration can happen fast or slow. During this process, multiple signaling hubs, such as the wound epithelium, like we already discussed in the case of the zebrafish fin, will have an epithelium wound epithelium that has to cover the blood clot or wherever there is a wound. Soon after the blood clot is formed, the wound epithelium has to cover it. And the injured nerves, as well as the immune cells, come into the picture.

They signal each other, and it also contributes to the connective tissue cells. So immune cells, connective tissue cells, and the damaged nerve ending cells are included. That can help in the migration of all these cells, which are the mesenchymal cells, into the amputation site, which eventually leads to the formation of the blastema. But remember, everything is happening under the wound epithelium; the wound epithelium can be seen like a blanket. What you put on top of you when you are sleeping.

In salamanders. Responding keratinocytes. From the adjacent intact skin. They delaminate from the epithelium. Keratinocytes are present.

In the damaged limb. They can. Detach or delaminate; that means it loses its connection to the basal lamina, delaminates from the epithelium, presumably with the help of a transforming growth factor beta (TGF-beta) in short form, dependent on epithelial to mesenchymal transition because TGF-beta signaling is a pivotal signaling pathway that plays a role in the epithelial to mesenchymal transition, as I already told you. Epithelium is an adherent property of the tissue, but it has to become mesenchyme; only then can the blastema form, and this is helped by TGF-beta signaling, which in turn is assisted by factors that contribute to the induction of genes such as Snail and Slug. So, TGF beta signaling leads to the induction of snail and slug genes, and they are enriched in that wound region, which is necessary for the formation of the so-called blastema, or the mesenchymal-epithelial to mesenchymal transition, leading to blastema formation.

Let us see a few more initial mechanisms in various investigations of the substrate requirements that revealed the rapid migration. Whenever you study regeneration biology or work on stem cells, as we also discussed, when you are growing embryonic stem cells, you need a feeder layer, leukemia inhibitory factor, etc. This acts as an attachment and a substratum. And also, the cells need the so-called extracellular matrix. Various proteins contribute to the extracellular matrix, which includes fibronectin, collagen, and fibrinogen.

So this substrate specificity of fibrinogen is a clot component. Fibrinogen is present in the blood clots. Clot component that forms immediately. At the wound site. Presumably, it allows.

The rapid epithelial crawling is occurring. Because there is Fibrinogen is present in the blood clot. Which is a friendly ECM? For the cells to crawl. Rapid epithelial crawling.

Over the cloud. So, like I told you. How wound epithelium is formed. The blood clot has this so-called ECM called fibrinogen allows the cells to migrate over it. And salamanders, rather than being under the clot, turn over the clot and the granulation tissue. It normally happens over the blood clot, as seen in mammals.

Also, it attempts to do so, but this is not powerful enough to create a proper regenerative response. So, crosstalk between macrophages and the epithelium has been established to mediate epithelial migration in other animal models. So much research has happened. If you bring about an epithelium, can you achieve regeneration? Can the blastema form, etc.? However, what has been seen is that the depletion of macrophages from Axolotls does not inhibit epidermal cell migration over the stump, so macrophages are the initial phagocytic cells.

However, if you deplete them, we have seen how the depletion of macrophages has contributed to fin regeneration; it doesn't regenerate, and they are meant for interleukin-1 beta production in the case of zebrafish, but if you supplement interleukin-1 beta... Even after macrophages are depleted, you get more apoptosis, so macrophages have a much bigger role, not just producing interleukin 1 beta. In the same way, macrophage depletion has been shown not to inhibit epidermal cell migration over the stump that happens normally, but importantly, macrophage depletion reduced early wound epithelium-associated gene expression, such as MMP9 (matrix metalloproteinase 9).

And the wound epithelial marker, WE3. Wound epithelial marker WE3 gene. These are necessary for clearing this junk. That is the blood clot, etc.

The matrix clearance, etc. It is needed for the new cells to migrate. So even if the macrophages are lacking, The hydrogen peroxide is H2O2. That which is released in response to the wound is sufficient to attract the leukocytes, and this will trigger a normal regenerative response. So, all we need is the initial attraction of the immune cells. So hydrogen peroxide can do this.

So early events and amputation can mediate wound epidermis formation. What we have

seen now, we are seeing in pictorial form. So, first thing, the first part is that the osmotic changes sensed by the epithelium cause a release of ATP, and it will also promote hydrogen peroxide gradient formation, as you can see in this picture. Whenever there is damage, hydrogen peroxide will be created. The epithelium secretes migratory chemokines, which attract cells, especially immune cells, to act; so wherever chemokine production occurs, there will be a gradient leading to distant places, and based on this gradient, cells will come to that site.

The third phase is the recruitment of immune cells that respond to the injury signals, which will create TGF-beta and epidermal growth factor; immune cells also contribute to the TGF-beta signaling. As I told you, snail and slug production will happen, which is needed for the epithelial-mesenchymal transition. Step four: TGF-beta and ATP promote EMT (epithelial to mesenchymal transition) and epithelial sheet migration at the wound site. And then comes the step five movement of these cells to the wound region where the actual wound is located because the migration starts from the non-wound area and then moves into the wound area, and at step six, the cell migration completes, leading to a continuous wound epidermis formation that occurs, with these actions happening one by one in an orchestrated manner in response to injury. Then you only end up getting the proper wound epidermis.

This is normally done by the migration of connective tissue or epithelial tissue, which is in turn the result of all this collective effort by various signaling events. And if you look further at the early events after amputation, what you know is that there is another step of events that is happening: the peripheral cells respond to the injury by water intake. The environment in which the animal is living cannot attract the water molecules, and it will promote hypertrophy, which means the cells will become larger in size. Adjacent, as you can see here in step one of this panel. They are becoming a little bigger because the peripheral cells respond to the injury with water intake, causing a swelling; this is why these brown cells are a little bigger, and the epithelial cells are then pushed towards the damaged area.

In this way, the neighboring cells will be pushed, just like when you are sitting in a bus that can only seat two people, and a third person, a larger individual, comes and sits in the middle. What will happen? The left fellow will go to the left side, and the right fellow will go to the right side. So this swelling creates a pushing of the cells, and this will trigger the movement of the wound epidermis. Remember, these cells are moved not because of proliferation, but rather because of the migration of the cells. And this, in turn, while this is happening, will be triggered by all this hydrogen peroxide formation and attraction of the immune cells, etc.

happen side by side. So if you look further, you will find the pharmacological inhibition of the canonical and non-canonical TGF beta signaling. We know TGF beta signaling is important for the epithelial to mesenchymal transition, which is triggered by immune cells, etc. But what if you block it either by using some drug or by some genetic method? And it was shown to delay wound closure. And after the limb amputation, naturally, because EMT is not happening. So where is the blastema? So wound closure can take its own time.

However, it is not clear whether this delay is sufficient to adversely affect the regenerative outcomes, as the prolonged TGF beta inhibition results in its inability to form the blastema, which might also affect other target cell populations. So if you make an immediate blockade, it will cause a delay, but a prolonged blockade will cause no regeneration at all. So in parallel, what happens is a wound response occurs in the underlying fibroblasts, and possibly other cell types also get affected in the injured limb stump. So the application of some molecules, such as MARCKS-like proteins, was shown to be sufficient to induce the cell cycle in uninjured axolotls. Injured axolotls, we know, normally respond.

Uninjured axolotls, if you supply some of these MARCKS-like proteins, are normally more of a membrane-bound protein. Tissue is likely secreted by injured nerve tracks as well as the earliest wound epidermis. So MARCKS-like proteins are one of the early induced genes that are needed for the normal wound-induced expression and the subsequent regeneration. So, the comparison of gene expression between limbs that are amputated and those that were wounded laterally without amputation means one thing: you can do amputation, and another is that you can wound it in the same place, but you did not amputate it.

Just like this is my finger. I cut it off his amputation, but I took another animal and just made an injury here. I did not cut it. I just sustained an injury. That's a wound, but not an amputation. So that showed that early regulators of the limb development program, including fibroblast growth factor 10 (FGF10) and bone morphogenetic protein 2 (BMP2), are highly induced upon wounding.

And it acts as an injury-responsive factor. Even if you do not amputate it. Just because you created a wound, these genes are induced to help attract the mesenchyme. However, just because a wound does not mean a new limb will be formed. We will learn about that in future classes. But you understand the purpose of the induction of these genes, not just for healing the wound.

It is for a regenerative response. However, it is not forming a tumor. New limb on an

existing limb from a wound is because it further has to be supported and triggered by the amputated nerve itself. Here, you created a wound. You did not amputate the nerve. And the blastema formation is basically a group of mesenchymal cells that will undergo an extended period of cell renewal and proliferative divisions before undertaking limb morphogenesis and differentiation.

Like I told you, in a wound, a simple wound, although genes such as FGF10 and BMP2 are induced, they are not going to form a blastema. Hence, they are not going to give rise to new limbs. So nerve-dependent switch of wound epithelium to the development of an apical ectodermal cap. Like I told you, the apical ectodermal cap is the place where the blastema congregates, and it can create lots of signaling molecules from this apical ectodermal cap, allowing the blastema cells to become different tissues. And eventually you end up getting a full-fledged organ.

So nerve, the amputation; every organ has a nerve. So when you cut the limb, you also cut the nerve. So nerves also play an important role. Around three days post-amputation, the wound epithelium undergoes an important nerve-dependent phenotypic change into an apical ectodermal cap called AEC, which can be considered analogous to the apical ectodermal ridge in embryonic development. That is called AER. That is found in amniotic limb buds during embryonic development, thus considered to be characterized by differentiation.

So you are going back in time to your early embryonic developmental stage. So, the terminology is different. One is the apical ectodermal ridge, which occurs during development, and the other is the apical ectodermal cap in the case of a limb. The AEC express a number of developmentally associated factors such as the transcription factor SP9 and the extracellular factors Wnt5 and anterior gradient (AG), nerve-derived factor FGF, and signaling that appears to be important for the induction. This early signaling from the AEC is important for the induction of SP9 and one important factor known as Neuregulin, which is released from the neuron.

We will learn about Neuregulin and how nerves are important in the subsequent class. But now we can learn that Neuregulin is a nerve-derived factor that is contributing to regeneration. A recently identified molecule is Medkine, which is also important in retina regeneration. We should also understand that Medkine is a cytokine that has a strong connection with the circadian rhythm. So like if you amputate an animal and you keep it in extreme bright or extreme dark and you know delineate its light dark cycle then the medkine levels are affected hence the regeneration can be negatively influenced no matter whether it is zebrafish or axolotl so medkine is an important factor small 13-kDa cytokine and it's a factor expressed in the aec apical ectodermal cap And also in the distal

mesenchyme.

So Blastema has two parts. One is closer to the animal that is proximal and farther away from the animal that is distal. So there that is, medkine is expressed in the distal mesenchyme, and that is essential for the maintenance of the apical ectodermal cap. So the medkine decides how long, how far, or how strong the AEC should be maintained. So although AEC is important for the signaling events, the maintenance of AEC is also important; it is just like a father in the house who is the income generator, but the maintenance of the father is also important; the father is not invincible. So in the same way, AEC is formed, but it has to be maintained.

Only then can it continue to give the signaling. So Medkine plays an important role in that. And AEC also helps in leukocyte recruitment because a lot of granulocytes are involved in the early immune response, and it contributes; the AEC contributes to the establishment of limb patterning, which is limb regeneration or the proper so-called organogenesis. Pharmacological or CRISPR-Cas-based mediated genetic interference with the medkine has resulted in the inhibition of regeneration characterized by a lack of AEC morphogenesis. That means AEC is an important signaling place similar to that of AER in the embryonic development of the limb. But Medkine, if you disturb it, you will not get a proper AEC formed.

Now you forget about the signaling emanating from the AEC. So this is what you should keep in mind. Another signature of the Medkine repression was the sustained inflammatory signaling. Remember what happens in a diabetic ulcer: sustained inflammatory signaling. and the presence of monocytes points to a potential role of WE wound epithelial specificity and AEC in immunomodulation. So WE is a gene that is also W3; we saw it, and W is a short form of the wound epithelium.

The wound epithelium leads to the formation of the apical ectodermal cap, which is a signaling event that, in turn, is a place of induction for various signaling events and AEC. Formation depends on the Medkine, and the absence of Medkine keeps a pro-inflammatory environment. The environment of the injured area remains inflammatory, which is not welcome. Initial inflammation is necessary. Then it should shift to anti-inflammatory cytokines that have to come into the picture.

Hence, Medkine is a very important contributing molecule to the establishment of the AEC itself. So, connective tissue de-differentiation and stemness are established by paracrine signals and special localization. So, whatever we have seen, any tissue that is especially the blood vasculature tissue has to develop into the blastema. Then only will they get the food, glucose, and oxygen. So they play a major role, and their de-

differentiation, the connective tissue de-differentiation, is a must.

So you can see it here step by step. First thing, the cues originating from the severed nerve, which means amputated nerve, the apical ectodermal cap, and immune cells that are contributing to the connective tissue's de-differentiation. The nerve and the Schwann cells are also there. Every neuron, whenever you talk about a nerve or neuron, also has associated cells called glial cells, specifically Schwann cells in the peripheral nervous system. And these CT cells, what you are seeing here, which are engulfed in the ECM, are helping in the de-differentiation for which the involvement of damaged ECM proteins and also MMPs contributes to the so-called reprogramming. And CT cells respond to the signals by dedifferentiating to a progenitor-like state.

Once it happens, you end up getting an epic ectodermal cap, as you can see here, which is a bunch of cells that act like a signaling center, like AER in embryonic development. And there are a lot of such molecules. PDGF is there, NRG is there, MLP is there, NAG is there, FGF is there, and MK, MLP; all these things, the to and fro. Some signaling facilitates the epical ectodermal cap, and some signaling coming from the epical ectodermal cap contributes to the connective tissue de-differentiation. After injury, different niches such as nerves, Schwann cells, AEC, blood vessels, and immune cells create a complex signaling network, as you can see in this picture.

And if you continue to see, you will see the CT-responsive cells; they migrate digitally and accumulate under the AEC because the AEC is a thick cap now. It is thicker than that of the epithelium. or wound epithelia. Early and mid CT cells will contribute to the multipotent progenitor population, and late migrating cells will contribute to soft connective tissue formation. These differentiated connective tissues migrate into the different layers.

That's what you are seeing here. Distance from the injury is required. And this is the maximum distance. And this is the injured spot. And this is closer to the animal. That is what the distribution you are seeing in a cartoonish manner; the cells migrate early in migration, and they enter multipotency. Those closer to the body have limited potency because they are closer to the initial location of the CT cells relative to the injury, which will influence the timing of migration and destination within the blastema, whether it will stay in the middle of the blastema, closer to the body, or far away.

From the body, those things will be decided, and this will determine the cellular stemness. What is far away from the body is actively proliferating, while what is closer to the body is now waiting for differentiation. In this scheme, the arrows you see in this picture represent non-interactions, and the dashed arrow represents possible but unknown

indirect or direct linkages. And the short forms you can read, like mk, med, kind, nrg, new, regulant, etc. So now what we know we need to know is that an early anti-inflammatory immune system plays a major role.

So far, we have seen that pro-inflammatory pro-inflammation is a must, but while pro-inflammation is necessary, you also need to have anti-inflammation. We kind of discussed m1 and m2 macrophages earlier, but this is the deciding factor. Pro-inflammation brings the immune cells, TGF beta, and other signaling events happen, and you don't want that pro-inflammation to continue. Just like when you go to a restaurant, you don't want to sit there permanently.

You have your food and come out of the restaurant. Same logic. Cytokine profiling and leukocyte characterization point to early activation and infiltration of immune-resolving macrophages. Remember, in the fin, we also saw that they are called M1 macrophages, immune-resolving macrophages, in the blastema in the case of axolotls. The same thing happens with the zebrafish fin as well. One gene called interleukin-8, IL-8, is a cytokine that is expressed in mesenchymal blastema and is necessary and sufficient to attract monocytes. So what we understand is that the IL-8 induction occurring at the injury site is sufficient to attract the monocytes, which in turn can create a pro-inflammatory environment.

In axolotls, annotation of the fibroblast molecular program showed an inflammatory response and the loss of differentiation markers, namely the ECM expression and the extracellular matrix itself. Not only did the fibroblast lose ECM expression, but they also actively expressed molecules such as MMPs that break down the ECM. Remember, when the blastema has to form, you need to have an epithelial-mesenchymal transition. If you want this mesenchyme to be successful, you need to have minimal obstructions in the form of ECM.

So the ECM is needed, but you don't want too much ECM. So too much ECM has to be degraded by metalloproteases such as MMPs. So these fibroblasts will become more migratory in nature, and they will have more MMPs expressed so that there is adequate ECM, but not an unwanted high quantity of ECM. Fibronectin expression is likely supported by the presence of Wnt and TGF-beta signaling, as fibronectin is a direct target of both pathways. So Wnt and TGF beta signaling have to come into the picture for the continuation of the fibrinoidin, which is part of the ECM. And this same ECM is used by the cells to migrate while it is not beyond a certain threshold level. So we will study more about other signaling events in the next class. Thank you.