Regeneration Biology
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W8L37_Accessory limb model of regeneration: lessons from Axolotl and newt

Hello, everyone. Welcome back to another class on regenerative biology. In today's class, we will learn about a new topic that is the accessory limb model of regeneration. What we can learn from the axolotl and newt. We know that when it comes to limb regeneration, the best-studied, best-explored model is the axolotl. And what we learn from them has a lot of depth and many similarities to that of zebrafish fin regeneration.

But we have to accept the fact that a fin is not a limb. Hence, the research on limb regeneration focuses more on the axolotl. So this is a picture of an axolotl. If you look carefully, you can notice something different.

The hind limb is proper, but in the forelimb, you can see there is an extra limb in this organism. That means we will see how we are bringing in an extra limb while retaining one proper normal limb. In the previous class, we also discussed that if you have an injury and express a cocktail of factors, you can get an accessory limb. So studies of ectopic limb formation tell us that all you need is specialized epidermis. That means specialized epidermis refers to an epidermis that is wounded.

And a nerve connection, nerve and fibroblast-derived blastema cells. If you have these things, or if you take blastema cells from another individual and put them in an injury spot, you may end up getting an extra limb in that injury spot. So that is what people have been doing. This is mainly to avoid creating a monstrous organism. That is not the goal.

People want to understand how flexible the system is, how maneuverable the system is. So if an organism is capable of having multiple limbs that are originated, why can't mammals make at least the last part? So this is what the researchers have been baffled by: researchers have been unable to come up with an answer as to why a mammal does not regenerate. So let us see how to make an ectopic blastema. We have seen so far how blastema formed yesterday in previous classes; we have seen it, but now we are thinking about how an accessory limb or how an ectopic limb is created. First, what you need to do is make a wound; naturally, wound signaling is a must to deviate a nerve.

To the wound site to stimulate the formation of an ectopic blastema. So, what you

understand from here, you can see in this picture: there are, uh, this is a limb, and this "a" stands for the anterior side, while "p" stands for the posterior side. Anterior means this hand. Like in my hand, this is the anterior side and the back is the posterior side, so that is what the concept you should have. My limb is like this; this is anterior, that is facing front, and posterior is facing back.

In another picture, I can tell you, this is the anterior side and the backside is the posterior side. Here, this is the anterior side and this is the posterior side. Okay. So if you cause an injury and take a nerve, such as the hand nerve or sciatic nerve, and you deviate it and attach it near or bring it close to the injured area, you end up getting an accessory blastema that forms on top of an existing limb. It's almost like I started getting a new limb formed from here.

My limb has no issues. It is perfectly fine. So this is the picture of the ectopic blastema. So what has been done is that this is the nerve you see inside, and it is cut, and you placed this nerve near the wound site in a cartoonish manner. You can see it here.

So, how do you make an ectopic arm? Graft a piece of skin that includes the epidermis and dermis from the opposite side of the limb to the wound side. This is another approach that people have taken. So this is the right, and this is the left. And what people have done is take a small tissue from the back, which is the posterior side, and put it into the anterior side of the other limb of the same organism. So you took a tissue from here and put it in there.

And you put in a deviated nerve. And you end up getting it. So now it has an identity. It is my right hand. This is my left hand.

So by putting a tissue graft from here, And putting it into the anterior side. Along with the injury and a nerve that has been injured. You end up getting my right hand that is formed in my left hand because this tissue also holds the identity. The formation of the injury, everything, and the deviation of the nerve are needed. But all they have done is put a small tissue from the other side to the opposite arm in the front; as you can see here, you end up getting this limp that is forming on my left side as an accessory limp.

So regeneration is a stepwise process, and this picture, this cartoon, or this flowchart may look very complicated, but they are all congregating together. Signals can come from fibroblasts, signals can come from the nerves, and also soon after injury. As you can see here, the blood clot is formed, and this is the timeline you are seeing here. Regeneration is a very complex phenomenon, and it has an integrated sequence of events. Say you are sitting in one room; your brain knows you are sitting on the chair, you are

sitting in a room, and that room is in such and such a building, and that building is in such and such a city.

This much information your brain gathers. If this connection is gone all of a sudden, if you lose that connection, you will not have an idea. That is where you are right now; you will not have an idea while traveling. Also, that is why if you are traveling, there are many people who have done a lot of research on that. You can see a lot of YouTube videos where people have gone inside one room for 72 hours.

No clock, nothing; there is always persistent light. After around 36 to 40 hours, you completely lose your circadian rhythm. You have no idea what time it is—Is it morning or evening? You have no idea at all; it gets reset. So, while an injury occurred to a limb.

.. We are seeing just the injury, but lots of other things come into the picture. That is what has been shown in this flowchart. So this is a timeline of up to 60 days, and a blood clot is formed within one to two days. The blood clot is stably formed, and by around five to seven days, wound closure and fibroblast migration occur, followed by fibroblast and collagen synthesis and wound contraction around 10 to 15 days later, culminating in scar formation around 20 to 30 days. But while this is happening, you should understand there is a wound closure occurring right from the injury site, and the proliferation and fibroblast migration happen; these are all influenced by various signals from the nerves, and there is also a signal occurring from the blastema cells, which will enhance the proliferation and differentiation.

And growth and pattern formation are important. Blastema, although we say it is a group of cells, has to have identity. And accordingly, you can see in a meagerly regenerating organism, scars form, with no regeneration. Whereas in regenerating organisms, whether it is skin or whether it has to form a proper limb, if it doesn't have signals coming from the nerves, then it will regenerate the skin. But if the signals are coming from the nerves in that damaged area as well, you end up getting a proper limb or a potential accessory limb that can develop.

So signals from the nerves are essential at an injury site of the skin. That the blastema formation is persistent, and the proliferation, ECM synthesis, and chondrogenesis have to come down. And if it comes down, you will end up in the so-called blast regress. So you don't want this ECM and chondrogenesis to decrease. So how is it done? It is done by strong signals that are coming from fibroblasts.

Which is also favored by the persistent nerve signaling; that is why nerve signaling can persistently occur in a wound site. Only by derouting a nerve, by attaching a nerve, you

won't get that continuity, so then growth and pattern formation happen, and you end up getting a properly regenerated limb. So you can see multiple stages where scar forms in a non-regeneration, skin regeneration happens, and blast regression occurs because it did not receive the persistent signal in spite of having the nerve signal. But the persistent nerve signal allows limb regeneration to happen properly. You can see here, understanding the pattern formation, that it is basically the blueprint function of the orientation of the blastema cells; that means blastema is a mass of cells, but it has an identity which is anterior, which is posterior, which is dorsal, which is ventral, etc.

So, if you tweak it, you end up getting limbs like this that almost look like branches of a tree; you have got three limbs coming from one stack. So, despite having a lot of emergent properties, the blueprint of the organ can be distorted by tweaking the orientation of the blastema cells. Or like I told you, take skin from here and put in here after cutting. So this can also have lots of implications. You can also see that the connective tissue fibroblasts are the progenitors of early blastema cells, which means the fibroblasts have positional information.

Although in this picture you are seeing fibroblasts, this fibroblast knows where it belongs, and this fibroblast knows where it belongs, and this fibroblast also knows where it belongs, and it will act accordingly. So positional interactions between fibroblasts also play a major role. Although fibroblasts are present, blastema cells are also present. And each of these cells, the congregation of both these mesenchymal cells, which have the potential to restore a damaged part, and also the fibroblast cell. And there is also an interaction between them.

And so the positional interaction between fibroblasts creates the blueprint of limb regeneration. If you distort it, as you can see in this picture, say this is a blastema: anterior, posterior. Anterior means this; posterior means this. Anterior, posterior. At the newly formed blastema, you twist it.

That means if this is anterior, then this is posterior. And the blastema formed as you twisted this way. This is cut, and you twist it, as you can see here. Anterior-posterior and then posterior-anterior; you fused it, as you can see here. Then you end up getting completely different limbs.

Instead of a limb formed like this, it will now form like that. So that is what you see in this picture. Anterior, posterior, and now anterior, posterior. You end up getting a multi-fingered limb. And people have done lots of such, you know, mix-and-match combinations.

The outcome of regeneration is dependent on the positional information. Now you can see here: anterior, anterior, posterior, anterior, anterior, anterior, posterior, anterior. This is the scenario, but what they have done is put anterior with anterior, so what they have done is taken the posterior and removed it, replacing it with an anterior. So whatever they have, the entire blastema has got only anterior information. And then, on posterior anterior, you are replacing with a posterior anterior that gives a normal limb because posterior anterior is now restored, fused with another posterior anterior.

Here, another anterior anterior was done with anterior posterior, and then you end up getting a multi-limbed structure. If posterior anterior didn't fuse with the anterior posterior, then you end up getting a multi-limbed structure where only the anterior part is kept. And it has only one toe that is somewhat equal to your thumb that is being formed; restore this one posterior anterior, which is fused with the posterior anterior. The same thing would have happened if the anterior posterior fused with another anterior posterior; you will still get a normal limb even though the blastema is from another source. The growth and pattern formations are controlled by local cell interactions.

This is the proof that this is a blastema and how you. Handle the blastema's positional information; you can tweak it across different positions within that blastema. You end up getting this kind of cells, this kind of limb structure. The creation of the amphibian regeneration blastema may also depend on maintaining ion currents through the stump. The stump means that the growth of the stump; if this electric field is suppressed, the regeneration blastema fails to form.

So we should understand that the electric current has a strong influence from neurons and also from the innate properties of the cells because we know the interior of the cell membrane is negatively charged and the outside is positively charged. Skin flaps inhibit both the current of injury at the amputation surface and the regeneration of that limb as shown in newts. This is a published paper. Such fields have shown that they are important for the regeneration of the tails of Xenopus laevis. So the same orientation identity is followed in tail regeneration as well.

In this frog, where VATPS, a gene, activates the proton pump within six hours after tail amputation, changing the membrane potential and establishing the flow of protons through the blastema, is the role of this VATPS. If this proton pump is inactivated, there are different ways in which you can inactivate it, either by mutation or by drugs; the depolarization of the blastema cells fails to occur, and there is no regeneration. Depolarization means the interior membranes are negatively charged; they become positively charged locally. That is depolarization; that is the principle behind every neurotransmission, like when you are seeing something. An action potential is created

due to depolarization.

So if this proton pump is inactivated, then it can be done by various methods and VATPS plays a major role for that. You will not get a regenerative response at all. So this has also been done in a published paper that shows H plus pump-dependent changes in the membrane voltage are an early mechanism necessary and sufficient to induce Xenopus tail regeneration. Those who are interested can read the article. So bone, dermis, and cartilage just beneath the site of amputation contribute to the regeneration blastema, as do the satellite cells from the nearby muscles.

So the bone dermis also plays a role. Unlike the flatworm, where the regeneration blastema tissue comes from the blueprint of stem cells, in the salamander, most of the regeneration blastema appears to arise from the differentiation of adult cells, followed by cell division and re-differentiation. Of those cells back into their original cell types, so this is how normal regeneration happens in an amputated organism. However, there is a lot of controversy about whether this is true: the differentiation of normally post-mitotic cells or whether much of the lymphoblastema. Is formed from the activation of uncommitted stem cells residing within the adult tissue means whether they are present in every tissue, like skin, which has skin stem cells; your retina has rod progenitor cells. Whether those kinds of cells are coming and expanding in number or they are formed.

Newly de novo formation, so many such debates are also going on, but it is well accepted that it is most likely the existing tissue type that dedifferentiates. But do we have some evidence? Can we show it? So a major question is, do the blastema cells keep a memory of what they have been? We say that blastema is formed by the de-differentiation reprogramming of the cells present at that injury site. But in that case, do those cells carry some memory? Do we have any proof? Kragel and colleagues in 2009 found that the blastema is not simply a collection of homogeneous, fully dedifferentiated cells. Rather, in the regenerating limb of a salamander, muscle cells arise from the previous muscle cells. So, previous muscle cells reprogram, become blastema, and then give rise to new muscle cells.

Dermal cells form only from the old dermal cells. And the cartilage cells can arise only from pre-existing old cartilage cells or old dermal cells. Thus, the blastema is not simply a collection of unspecified multipotent progenitor cells. It's like if you have a limb, you have muscle, nerve, skin, blood, bone, cartilage, and all those stem cells. They are derived from those tissues.

No tissues are going back. Bone is making its own contributions. Muscle is making its own contributions. Skin is making its own contribution. Now they all look like a

undifferentiated or de-differentiated stem cell like butt.

They still have the identity from where I have come. Just like if you go to America or London, you still know what your village name is and where you belong. Even you may tell your kids, oh, this is our hometown. So such an identity exists. Thus, the blastema is not simply a connection of unspecified cells. Rather, the cells retain their specification, and the blastma is a heterogeneous assortment of restricted progenitor cells.

They are all progenitors, but they have identities. So, blastema cells retain their specification even though they are capable of differentiating. So, what is done is they have a cartilage, a GFP-expressing limb, and they took a bone cartilage or cartilage tissue and transplanted it into a wild-type cartilage. So GFP expressing one small limb is taken and put it into a wild type limb of a recipient and then they cut it. Here, they created a cut and then saw that these GFP are the newly formed blastema cells that have the GFP, and those GFP-bearing blastema cells are giving rise only to the cartilage. See, you can see that the grafting has been done, and the amputation is done right at the site of the graft so that the GFP transplant, or the transplanted GFP-bearing cartilage, is injured.

And now the blastema is formed from there, and that GFP is now getting into the only bone; it doesn't give muscle, doesn't give rise to skin, and gives rise to regeneration. The newly formed limb cartilage had this GFP, so cartilage from the specific portion of the limb of a salamander expressing GFP is transplanted into an equivalent position in the cartilage of a salamander limb that does not express GFP. Thus, the grafted tissue integrates within the host tissue. Naturally, salamanders or axolotls do not.

Throw away or reject tissues. Amputation is performed in the middle of the grafted tissue. That means it has GFP. That grafted tissue is injured. The blastema forms contain GFP-expressing cells. The regenerated limb is studied to see if the GFP-negative or GFP-expressing cells are present only in the regenerated cartilage or other tissues.

What did they find? It is present only in the regenerated cartilage tissue, not in other tissues. So, this is another article published in Nature. Cells retain memory of their tissue origin during axolotl limb regeneration. Those who are interested can read this article because it is a good discovery that the blastema retained the memory of where it originated. Longitudinal section of the regenerating limb 30 days after amputation: muscle cells are stained in red, nuclei are stained in blue, and the majority of the GFP-positive cells, shown in green, are seen in the regenerated cartilage only because cartilage had the GFP from the donor and not in the other regenerated tissue, such as muscle.

So the proliferation of blastema cells is the requirement of the nerves and apical

ectodermal cells. So we saw at the beginning of the class that the nerves are important. Let us see how nerves can contribute. So the AEC epical ectodermal cap stimulates the growth of the blastema by secreting a factor called FGF8, just like the AER epical ectodermal ridge does during normal limb development, but the effect of the AEC is only possible when the nerves are present, even though an epical ectodermal cap is present.

If the nerve is not there, you will not get the regeneration. Singer, in 1954, demonstrated that a minimum number of nerve fibers must be present for regeneration to take place. Remember the year? It was done in 1954. The neurons are believed to secrete factors necessary for the proliferation of the blastema cells. Among the many candidates for nerve-derived blastema mitogens, the best-known example is the newt anterior gradient protein.

It is also known as nAG. Newt anterior gradient protein is secreted from the Schwann cells. Remember, not from the neurons. It is secreted from the Schwann cells, which are the glial supporting cells of the peripheral nervous system. Your limbs have a peripheral nervous system.

Only the brain and spinal cord are the central nervous system. So, the Schwann cells secrete this new anterior gradient protein known as NAG. This protein permits normal regeneration in limbs that have been denervated. So if the nerve is not there, amputation happens. But if you put nAG in that spot, then regeneration can happen.

Nerve is not present. If nerves are there, naturally the Schwann cells will be there. If the nerve is not there, Schwann cells will not be there. Hence, nAG will not be there. But if you supply nAG, you can end up getting limb regeneration.

So this protein permits normal regeneration in limbs that have been denervated. That means the nerve has been removed. Axolotls use something called a Neuregulin secretion from the neurons that are of the sciatic nerve. It is not produced by Schwann cells. It is produced by the sciatic nerve itself as a major factor contributing to the regeneration of the limb.

You can see it here. the right limb denervated you can see here in this picture it is denervation happened and then so right limb is denervated and both limbs are amputated and electroporation with nAG so what you can see here here the nerve is removed and it's amputated but nerve is not there whereas in left there is amputation but nerve also is there and now this is not supposed to regenerate but if you put nAG then, like this limb, this also will regenerate despite not having the nerve, so you can read about the molecular basis of neurodependent limb regeneration in adult vertebrates in an article published in

Science. It is a very interesting article; those who are interested in this field can read about it. Here, this is a picture from an article published in Developmental Biology, and this has also been discussed well in Current Opinion in Genetics and Development in 2008. So, this is a newt limb, which is normally present and has been cut, but what has happened here is that they have removed the sciatic nerve.

You can see the wound healed after cutting. The left limb is intact, and even if you cut it, it can regenerate since new nerves are removed right from where the star mark is. The wound heals; it doesn't give rise to anything. However, if you administer the nAG by electroporation, you end up getting the limb that is being formed. So, these are all indicators of new regulators of vertebrate appendage regeneration.

You may remember, despite all the odds, this complex picture we discussed. We should understand that each of them cannot be assumed to be good enough; neither nAG nor Neuragulin is sufficient. Other signaling, such as that from blastema, causes its own signaling, or the AEC (apical ectodermal cap) creates the FGF8 signaling. Additionally, the involvement of delta node signaling or Wnt signaling cannot be sidelined because they are all important contributors. Otherwise, what will happen is that you will end up with a scar formed, as you can see here in each of these regeneration approaches.

there will be formation of this scar in the first place. Later, this scar will have to be dissolved. And this happens because of the proper induction of a lot of metalloproteases and collagen-degrading enzymes so that this scar will be removed and the cells will be replaced. And these cells now differentiate and form the proper lymph structures. So we should understand that in the same animal, despite having nerves properly present, in this limb, even if you cut it, that nerve is not coming to help the adjacent limb.

Because if there is an endocrine factor, it is secreted into the bloodstream. Naturally, whatever receptors that are making use of nAG or Neuroglin must be present here also. So they are not available in the bloodstream. If they were available through the bloodstream, it would have been able to cause a proper regenerative response, so just like in mammals, after injury there is a wound healing response, and also a scar is formed, but the scar gets dissolved because the early deposition of the blood clot, which contains fibronectin, allows the migration of the cells. into that site and they can now also retain the positional identity so that the limb is formed forward not formed backward but you can make it from backward if you twist the anterior into the posterior side and posterior into the anterior side you can end up getting a limb grown this direction so that much plastic is the system So we will learn more about nerve-dependent limb regeneration in the subsequent classes. Thank you.