Regeneration Biology
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W8L38_Nerve-dependent regeneration-Axolotl and Newt

Hello everyone, welcome back to another class on regenerative biology. Today we will learn about nerve-dependent regeneration in axolotls. In the previous class, we saw how nAG contributes to the formation of the proper limb, even if the nerve is removed. So we will see a more detailed view of nerve-dependent regeneration in the axolotl model. So, the molecular basis of nerve-dependent limb regeneration in an adult vertebrate is quite interesting. So regeneration occurs only if there is simultaneous regeneration of the severed nerve.

Means when you cut the limb, you also cut the nerve, and the nerve also grows naturally through regeneration, but the nerve also secretes the signal that is not just for nerve regeneration but for the whole limb regeneration. We usually think of the nervous system as a means of carrying information between the nerve cells and their sensory and motor targets. Nerves are also essential for tissue regeneration because they are not just meant for their own regeneration but also for tissue regeneration. When a salamander's limb is amputated at any position from the shoulder to the fingertips, you can start from here, here, here, here, here, and you can go up to here.

Fingertips form a blastema at the stump, whichever part you cut; whether you cut the stump or the remaining portion of the organ, it forms a blastema, a mound of stem cells from which regeneration begins. So if the nerves are cut at the base of the limb, deeper than the regenerative tissue not it that cut the nerves are cut at the base of the limb deeper than the regenerative tissue that means here is the cut nerve is cut from here inside okay here is the cut so that is what you should know the limb stump is permanently denervated the axons cannot regenerate the limb regeneration fails because you have cut only nerve from inside limb is cut from here so this nerve will not grow up to here and allow the growth of the limb doesn't happen so after amputation the severed axons retract it shrinks if the axon is cut here it becomes short it retracts within the stump and then grow back along the nerve sheath first if it is cut here nerve is also cut but the nerve now shrink back and then it bounce back And you can see in the following figure. But as of now, we are only showing the message. The Schwann cells that make up the nerve sheath in this region express the so-called nAG. nAG comes from the Schwann cells of the newt.

The new anterior gradient protein is called nAG. As the first blastomere cells start dividing, later what happens is that the nAG appears within gland cells in the specialized wound epidermis at the end of the limb. This epidermis is critical for sustaining later cell proliferation in the blastema. So the initiation and the perpetuation are what is important. So the appearance of nAG in both the swan cells and the wound epidermis is abrogated by denervation.

If the nerve is removed, nAG cannot be formed because Schwann cells will not remain independent of the nerve; hence, Schwann cells will not secrete nAG, and you will not achieve proper limb formation. Isolated cells cultured from the blastema promote the proliferation in the denervated blastema treated with nAG. And the wound epidermis is reestablished. As you can see here, it's a new limbic cut and you can have the normal nAG protein that has been cultured and normal situation what happens is the amputated limb, you have the nerve, the black color nerve, it shrinks back and then it bounce back and then it is allowing the blastema formation that can start having the nAG expression and the limb is formed. But if the retracted nerve does not grow back, it means the nerve is amputated, much upstream denervation has happened; that is why the nerve is not there.

Then, if you supplement nAG, you can end up getting the regenerated limb, so this nAG protein can be made from nAG-producing cells, even in culture. So the regeneration of salamander limbs requires the concomitant regeneration of the severed nerves, which a single protein, nAG, can substitute for in regenerating nerve cells. So nAG is one of the early molecules that a wound epidermis is expecting to be released only by the Schwann cells. But this doesn't mean that nAG is good enough. nAG is good enough to complement the absence of the severed nerve or to compensate for or supplement the loss of nerve.

Neurogluin signaling is essential for nerve-dependent axolotl limb regeneration. Those who are interested can read this article. The Mexican axolotl, Ambystoma mexicanum, is capable of fully regenerating amputated limbs. Similar to nAG, axolotls also use a molecule called Neuregluin. So the molecular basis behind this phenomenon of limb regeneration remains poorly understood.

You know a lot, but you do not know as much as to Create a limb in a mammal. But previous studies have suggested that nerves support regeneration via the secretion of essential growth-promoting factors. A neuronally secreted protein, known as Neuregulin, in short form called NRG1, fulfills all these criteria in the axolotl. Immunohistochemistry and in situ hybridization of NRG1 and its active receptor ERBB2, which is the receptor, revealed that they are expressed in the regenerating blastema but are lost upon

denervation. You may remember that when we studied epidermal growth factors, we discussed ERBB receptors; different receptors are present.

In the previous class, we discussed ERBB1, ERBB2, ERBB3, and ERBB4, and how they form homodimers and heterodimers based on the ligand. At that time, we also discussed neuroglin, but that was related to regeneration. Some other tissues, such as the intestinal epithelium, etc., but here we are talking about limb regeneration, so NRG1 was localized to the wound epithelium prior to blastema formation, and it was later strongly expressed in the proliferating blastemal cells. Supplementation of or the implantation of NRG-soaked beads rescued regeneration to digits in the denervated limb, similar to that of nAG in the nude.

You can also see in axolotls that NRG1 contributes or can supplement the absence of nerves. Connection to the wound site. Pharmacological inhibition of NRG1 signaling reduced cell proliferation, blocked blastema formation, and induced aberrant collagen deposition. That is nothing but scar formation in fully innervated limbs. So, innervation is there.

You did not denervate it. Innervation is present. Amputation is present. But you blocked NRG1. So the nerve may be secreting some other factors as well.

The absence of NRG1 is powerful enough not to allow proper lymph to form. That is indicated by aberrant collagen deposition. The nerve-dependent NRG1-ERBB2 signaling promotes blastemal proliferation in the regenerating limb and may play an essential role in blastema formation. Thus, providing insight into the longstanding question of why nerves are required for axolotl limb regeneration. Nerves are needed, but we did not know why, and these experiments clarified that NRG1 and ERBB2 are expressed in the PNS and the regenerating blastema.

So how people explored it, we will get into the different details of this experiment. This is NRG1 in situ and of all types, and here are NRG1 type 1 and type 2. And also, you can see different locations in the blastema. This is a growing blastema. And you know it is an in situ hybridization.

It stands for in situ hybridization. Which means you are detecting the RNA of NRG1 isoforms and receptors expressed in the blastema 14 days post-amputation. Insets show sense of control.

Sense control means... Anti-sense is usually used as a probe. Sense control means you are using the same gene as a probe. So the same gene will not hybridize with the same

gene. You need an antisense of that gene for hybridization. So sense control means there will not be any signal.

RT-PCR analysis showed that the ER, EGF-like proteins, NRG1, and the receptor ERBB2 are also induced. And they have been confirmed by statistical analysis. So, in a nutshell, what do you understand? Both the ligand and the receptor are induced in the blastema so that when the nerves secrete the NRG1, it will act on the ERBB2 receptor and do what it is supposed to do. That is the blastema's formation. NRG1 and ERBB2 are expressed, as I told you, in the peripheral nervous system.

And this picture shows how different phases, like the G to K panel, demonstrate that NRG1 and ERBB2 are expressed in the mesenchyme of the regenerating blastema but are lost upon denervation. It's a normal scenario if a nerve is present, an amputee's severed nerve is present, NRG1 and the ERBB2 receptor, and everything happens smoothly. But if the nerve is not there, it is denervated; then you will not have blastema formation at all. You will have aberrant deposition of collagen. Green and orange fluorescence are due to autofluorescent cellular debris.

Normally, in tissues, you will get unwanted fluorescence based on the tissue you are studying, but that can be ignored. In the L to O panel, what you are seeing is that NRG1 and ERBB2 are expressed in the dorsal root ganglia and peripheral nerves. And this P and P dash, what you are seeing is that NRG1 is expressed in the wound epithelium, that is WE. The mesenchyme wound epithelium is underneath the mesenchyme cells of six days post-amputated limbs, along with the proliferating BrdU-positive cells, and in Q and Q dash, what you are seeing is extensive energy one expression and co-localization with the BrdU. In 16 DPA blastema, co-localization with the BrdU indicates energy is expressed in the proliferating cells.

ROs indicate the co-labeled cells, both BrdU and NRG1. NRG1 and BrdU co-localize along with peripheral nerves in the regenerating limb at 16 dpa. De-nervation significantly decreases the percentage of BrdU and NRG1 localization in panel S. At 16 dpa, because you waited until 16 dpa, you still don't have the blastema formation due to the denervation in panel T. The western blot of the nrg1 at 16 dpa shows a band at the expected size of 47 kilodaltons and a greater band intensity in the blastemal tissue relative to the denervated tissue.

So what you are seeing is that in denervated tissue, you do not get proper NRG1 expression. So what you learn from here is that these researchers show that NRG1 depends on the presence of a severed nerve. So, supplementation with NRG1 rescues regeneration in denervated limbs. So you can see here the limbs are amputated and

wound healing happens, kick starts and the beads are implanted that is at the denervated limbs which are soaked with NRG1 and the blastema starts forming and limbs are regenerated. Reformed or they are formed again; they act as if they are reinnervated.

That feeling comes because the soaked beads are exposed, and the blastema formation and limb formation take place. So, panel A is the timeline of the energy supplementation experiment, and it's supplemented with energy-soaked beads. Up to this point, they have gone up to around 20 to 21 days post-amputation. Arrows indicate the plane of amputation in panel E. From 6 to 20 days post-amputation, NRG1 supplemented up to seven individuals, and they have regenerated significantly more tissues than a denervated animal.

As you can see here, in the innervation normal blastema, with denervation you don't get a blastema. When you have denervation and you don't have any supplementation with NRG1, you only get a stump. So it is somewhat closer to an innervated scenario, but definitely drastically different from that of a denervated scenario, because in the innervated scenario, you don't have any blastema that is formed. So NRG1-supplemented limbs regenerated in the absence of innervation or in the presence of denervation. So supplementation with NRG can rescue the denervated limbs, and in this panel, what you are seeing is the timeline of late energy supplementation.

Instead of giving at an early stage, if you give at a late stage, what happens? Implantation of NRG1 socked beads into the denervated limb rescues regeneration to the point of digit formation at 36 dPa. Until the digit formation time you waited, it could work. Arrows indicate the plane of amputation, and the dotted lines outline the regenerating tissue, as you can see in this picture. So, panel M, what you are seeing here is that NRG1-supplemented limbs regenerated significantly more tissue than the innervated ones, and it can lead to the formation of the proper limb itself. Significantly less tissue than innervated means that supplementation is not as good as having a proper nerve present, but it does the job.

The N to O panel shows L-CN blue staining, indicating digit formation in control and NRG1-treated limbs. So NRG1 induced growth and digit formation in fully denervated limbs. That is the message we have to convey. So, inhibition of ERBB2—now you are going into the receptor. So far, we are playing with the ligand ERBB2 receptor; if you block it, it blocks regeneration, inhibits proliferation, and induces aberrant collagen deposition, which is as good as blocking or as good as a denervated scenario, even though the nerve is present.

So, a to c panel inhibition of Erbb2 with the 500 nanomole mubritinib blocks the

blastema formation. It's a drug at 13 dpa. The picture is taken at 13 dpa because that is a time when you can have a proper limb bud that is being formed, as you can see in the innervated scenario. When you treat with mubritinib, it is as good as a dinner-weighted scenario because the stump is not growing. The stump is not growing, but the stump from here has grown up to here.

And D16, this is D0 treatment, and D16, you can also see that you are able to see a proper limb not formed as well as in the denervated scenario, whereas in the innervated scenario, it started forming the digits. D2F, the Mubritinib application after 16 DPA, much later, blocks lymph proliferation but not patterning and appears phenotypically similar to 16-day denervation. 16-day denervation, what do you get from it? An aberrant lymph will be formed. It looks like you blocked at a much later stage. Daughtered lines outline the regenerating tissues.

Blocking means that in this whole panel, you are blocking only the ERBB2 receptor. Picro's serious staining showed at 23 days of submersion in 10 micromolar. A little higher concentration. Earlier, they had done 500 nanomolar.

Now you are doing higher concentration. 10 micromolar Mubritinib results in the contraction of the epidermis. Very severe blocking of the ERBB2 receptor takes place. That aberrant collagen deposition is seen. In the mesenchyme, the contrast to the minimal fibrotic deposition is seen in control blastema. So you can see a huge contrast compared to the 10 micromolar Mubritinib-treated limbs.

And the dotted lines indicate the boundary of the epidermis. So now, to continue further, the Masson's trichome staining of control and mubritinib-treated limbs at 12 days post-amputation shows a lack of blastomere accumulation in the drug-treated limbs. Drug means ERBB2 is blocked. Treatment that you can see in panels I and J and panels K, L, and N shows that mebritinib does not reduce innervation but significantly decreases the proliferative index of the amputated limbs. Dotted lines indicate the plane of amputation in this picture, also at 14 dpa.

M panel 14 DPA mubritinib-treated limbs had regenerated significantly less area than the control, but not as low as denervated limbs. This means the presence of the nerve helps in some way, but not adequately enough to cause regeneration if the ErbB2 receptor is blocked, so that energy neuregulin has to act on them. Panel O, limbs that were either denervated or treated with Mubritinib at 16 dPa, regenerated significantly less tissue than the control limbs. That is what has been plotted here. You can see different enervated, denervated, and enervated plus Mubritinib.

Arrows indicate the plane of amputation there as well. So, EGFR inhibition now refers to another receptor that belongs to the same family, the epidermal growth factor receptor, because they also act through ERBB1, ERBB2, and ERBB3, unlike the different ERBB4. You can refer back to our earlier class where we discussed that. EGFR inhibition inhibits wound closure and is phenotypically distinct from ERBB2 inhibition. One may wonder that because of the blocking of the ERBB2 receptor, you blocked epidermal growth factor signaling, but no.

It can specifically block the neuregulin function. That is what has been shown here. In the A to C panel, what you are seeing is that the proliferating cells are localized to the mesenchyme in the AG1478-treated limbs. That is the EGFR blocker and the epidermis in the Mubritinib-treated limbs at 6 dPa. So two experiments: one where EGFR is blocked and another. The ERBB2 receptor is blocked, so EGFR is meant for all EGF signaling, whereas this drug, AG1478, is used; Mibritinib blocks ERBB2, so keep that in mind.

The arrowheads indicate autofluorescent cells; you can ignore the debris and rod cells. Dotted lines indicate the boundary between the wound epidermis and the mesenchyme. So keep in mind that in the beta-tubulin 3, BrdU, and DAPI staining shown here, you can see that the proliferating cells are localized in the mesenchyme. Although the nuclei are present and the green color of BrdU indicates the actively proliferating cells, when you have EGF blocked, you do not see much of the proliferation that is occurring in the blastema cells. Whereas in the same way, the Mubritinib treatment also shows a significant decline.

There are some stumps of cells that are being formed. They do not properly form the proliferative blastema, even though the nerve is present. So what you learn from here is that ERBP2 receptor blockade affected the blastema formation in spite of having the neuron. It is not through EGFR signaling but through NRG1 signaling that neuregulin signaling occurs, so EGFR inhibition inhibits wound closure and is phenotypically distinct from ERBB2 inhibition; this is further evidence that EGFR is needed for EGFR signaling on the EGFR receptor. Wound closure to happen, so if you block the EGFR receptor, wound closure gets affected; that is what is being shown here.

AG1478 is for blocking the EGFR, and this is the control. You can see here in both conditions, 3 DPA and 7 DPA, and also H and I is a later time point, as you can see here. H is for 10 days; much later, 10 days. So what we see is the AG 1478 treated limb showing aberrant wound closure over time compared with the control limb at 3DPA and 7DPA. Both scenarios, that is what you are seeing. In panel H, what you are seeing is a limb treated with AG1478 for 10 days, demonstrating aberrant development of

iridophores, which are needed for proper melanin pigmentation, etc.

That has to happen: the skin pigmentation. The I-panel is the control limb treated with DMSO for 10 days, showing a lack of iridiophores. So there is an aberrant migration of iridiophores seen in the EGF signaling-blocked scenario. Regeneration is compromised there as well. So why has EGFR signaling been selectively shown here, even if NRG signaling is normally occurring? The absence of EGFR can also interfere. So that's why I have told many classes that the absence of one signals that the regeneration is blocked.

That doesn't mean that signaling is the one and only signaling that is contributing. Other signaling is also just like cooking; if you forget the salt, the food will not be tasty at all. But you can claim, "Oh, I put only one; the rest has been put," but it will not be tasty food. So every component has its own role. The percentage of proliferating epidermal and mesenchymal cells is compared in the control in the J and K panel.

Mubritin-attributed and AG1478-treated limbs at 6 days post-amputation. It is done in approximately 5 to 6 biological replicates. So if you look closely at NRG1 signaling, what do we learn from it? A single nerve-derived protein known as Neuregulin 1 is capable of supporting blastemal growth and tissue regeneration up to the point of digit formation in the denervated axolotl limb. So keep in mind that Neuregulin signaling, if it is blocked until a certain stage of development, could be supplemented with the new regulin before digit formation; you can still rescue regeneration, as the system is very plastic and wants to regenerate. That is the idea you should gain from these studies: regeneration is very much a normalcy in this animal, whereas.

.. absence of some molecules such as neuroglans can completely shut it off. So it would be interesting to look at why these genes are not functioning in mammals, or can we learn a lot from them, etc. Nerve-dependent NRG1-ERBB2 signaling is crucial for blastemal proliferation and may also be an essential component of blastema formation and scar prevention.

program. Scar is also a bottleneck in the regeneration program. NRG1 is the first protein known to have been shown to be capable of rescuing regeneration in digits up to those in an axolotl limb. Newt anterior gradient protein (nAG) has also been shown to rescue regeneration in the denervated newt limbs. It may be interesting to see if nAG can do the job in an axolotl or if NRG1 can do a similar job in a newt. So such experiments will also tell how plastic the system can be or how it can talk to each other. Exploration of the relationship between these two signaling pathways is necessary in order to fully characterize the underlying cause of nerve dependency in axolotl limbs.

We know nerve is needed; now we know more about why nerve is needed in newts as well as in axolotls, given the conserved role, which means evolutionary conservation across diverse species, of NRG1 and ERBB2 signaling in the peripheral nerves, as well as its necessity. In other animal models of cardiac and peripheral nerve regeneration, elucidating the function and mechanism of this signaling pathway in axolotls. This pathway may have far-reaching impacts on the field of regenerative medicine. So we should understand or we should try to explore the energy one is contributing at what level, until what time it is continuing to produce, whether it is produced till the last digit is being formed, or if the level of energy is changing, or if this formed energy is still actively doing the ERBB2 receptor binding.

All these things one has to look into because it is very much possible. NRG released in the very beginning may not be released at the same level until the fag end of regeneration. So these are all the fine-tuning observations and fine-tuning analyses one has to perform in order to fully understand limb regeneration. We will learn more about limb regeneration in the next class. Thank you.