

Regeneration Biology
Rajesh Ramachandran
Department of Biological Sciences
IISER Mohali
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W7L33_Different cellular signaling during regeneration -Egf, and Hippo signaling

Hello everyone, welcome back to another session of regeneration biology. In today's class, we will learn about the importance of two signaling pathways, that is, EGF and Hippo signaling, and will give more priority to Hippo signaling. Although EGF signaling is well characterized and well studied, Hippo signaling is relatively new and quite interesting. So first, let us see what the role and importance of EGF, that is, epidermal growth factor, is in the intestinal epithelium. We know the intestinal epithelium is one of the constantly regenerating tissues in our body because that is the most important. Robust tissue that constantly undergoes erosion has to be replaced.

We have learned about the intestine since the very first class, and it has an availability of stem cells that give rise to all cell types of the intestine. Epidermal growth factor has long been known for its role in promoting the proliferation of intestinal epithelial cells. As I already mentioned, I will not focus on the importance of a given signaling event in every regenerating tissue type because that would be too cumbersome and beyond the scope of this class. Take individual tissues, some of which, like the intestinal epithelium, regenerate in every animal, irrespective of whether it is a mammal or a zebrafish, whereas some tissue regeneration is unique to, say, zebrafish or axolotls.

Therefore, we will focus on those animals and those tissues, as the intestinal epithelium regenerates in every animal with an intestine. EGF is produced by the epithelial niche cells at the base of the crypts in vivo and is routinely added to the culture medium to support the growth of the intestinal organoids if you are doing them ex vivo. So it has been well characterized that the importance of EGF is essential when it comes to regeneration. There is some recent identification of diverse stromal cell populations that reside underneath the intestinal crypts. The actual name is Crypts of Liberkuhn.

That is the name. Crypts that have enabled the characterization of key growth factor cues supplied by these cells. So the presence of cells is triggered mainly by the release of growth factors from the underlying cells, whatever is present. So, the nature of these signals and how they are derived, how they are delivered to drive the intestinal epithelial cell development right from embryonic development and also daily homeostasis, and how tissue regeneration following injury is being actively investigated by many research

groups, may lead you to wonder, "Oh, intestine, what is so special about the intestine?" After all, it is one of the most important organs in your body, and its cell division is faster than even cancerous cells in the intestinal epithelium. It is clear that apart from FGF, other ligands of the family, such as Neuroglin 1, EGF, and Neuroglin, have distinct roles in supporting the function of intestinal stem cells through the ERBB pathway.

ERBBs are receptors. So we can quickly look into the members of the EGF family of ligands and their respective receptors. In this picture, you can see that different EGFR receptors are present, where EGF is binding, and then there are ERBB2, ERBB4, and ERBB3 receptors, while EGF, TGF alpha, AREG, and EPGN are the ligands for the EGFR. And whereas HB-EGF, the other accessory ligands are HB-EGF, ER-EG, and BTC. These proteins can bind to EGFR as well as to ERBB4.

And then there are specific Neuroglins. Neuroglin 3 and Neuroglin 4 can bind to ERBB4. And Neuroglin 1 and Neuroglin 2 can bind to ERBB3 and ERBB4. So, you should also understand that they are vulnerable to receptor dimerization. ERBB4 and EGFR can form a heterodimer, and based on which ligand it is binding, it can decide.

And this also ERBB2, ERBB4 can also form a heterodimer. And we will not go into detail. In general, they can trigger the PI3, AKT pathway, MAP kinase, and JAK-STAT signaling. We have addressed JAK stat signaling in the previous class. So this is what you should understand.

These molecules have similar common EGF-like motifs, and due to their membrane-anchored nature, they can act in a juxtacrine manner between two neighboring cells. Juxtacrine means A and B cells that are touching each other. They can influence others. And in this way, they can also act in an autocrine-paracrine fashion via the proteolytic cleavage of the external EGF-like domain. Many of these proteins have an EGF-like domain in common, which results in the release of the extracellular compartment.

Upon ligand-induced conformational change, ERBB receptors form homodimers or heterodimers, depending on the situation, which activates the intrinsic kinase domain and also causes the phosphorylation of specific tyrosine residues within the cytoplasmic side or cytoplasmic tails of these receptors. So we should understand that there are different ligands available, as you can list here, and it will undergo ligand-mediated dimerization, and accordingly, there will be a signaling event that is kicking in. Inside the cytoplasm. The EGF-like protein drives cellular signal transduction through the ERBB subclass of receptors, a tyrosine kinase superfamily, which consists of members such as the EGFR receptor, also known as ERBB1, to align with the ERBB nomenclature, including ERBB2, ERBB3, ERBB4, etc. The EGF family of ligands can be classified into four

subgroups mainly based on the distinct receptor binding qualities or properties; that is, the ligands that recognize ErbB1 only, such as EGF and TGF- α , are examples, as we saw in the earlier picture.

Additionally, the ligands that can bind both ErbB1 and ErbB4, such as HBGF, EREG1, and BTC, are also included. And the ligands that are specific for both ERBB3 and ERBB4 are mainly Neuroglin 1 and Neuroglin 2. And also the ligands that activate the ERBB4 are only NRG3 and NRG4. So these are all the main family members. It is also important to note that no ligands have been identified for ERBB2 to date in mammals.

All ERBB receptors contain an extracellular ligand-binding site, a single membrane-spanning region, and a cytoplasmic tyrosine kinase-containing domain that performs these phosphorylation activities. And this is a schematic diagram of the intestinal epithelium that deciphers the expression of ligands and receptors within the different cellular compartments of the intestinal crypt during normal homeostasis and following regeneration. You should understand that when we say homeostasis, there is also erosion of cell regeneration, which means it involves some real damage; otherwise, there is also a homeostatic condition, and there is constant regeneration going on because of the cellular erosion in mice and humans. Paneth cells are localized within the epithelium. Adjacent to the intestinal stem cells at the base of the crypts, as you can see here, different cell types are marked.

You can also see the distribution of various receptors that have been shown, and you should understand the expression level: if it is red in color, that means the levels are high; if it is white in color, the levels are low for different receptors. And also, the ligands' expression is being shown in different cell types. What is being shown here? Intestinal stem cells, Paneth cells, etc. And also, even macrophages are being studied because they will be migrating from the bloodstream into the intestine. And this is a normal crypt, and this is the regeneration following damage.

So, a normal crypt versus regeneration following damage; you can see some genes are constantly expressed as they are, whereas some genes, like you can see here, some gene which was white has now turned red. So, you can easily identify that in some cases the levels have already gone up; it was up, and it has gone up further after injury, and in some cases. The genes are absent; they were not induced, but they started appearing. One good example is what you have mentioned: NRG1 (Neuregulin 1). You can see here that it is in CD34 and PDGFRA in these stromal cells, and also you can see their levels are pretty absent in homeostasis, but after injury, the levels are going very high.

So, we should understand that when the pancreatic cells are depleted in vivo, there are

different methods by which you can deplete them or kill them. Other cellular sources can compensate for this loss, resulting in the maintenance of an intact epithelium. Also, you should note that the mesenchymally secreted molecules, which include NRG1, NRG2, AREG1, and AREG2, act on the epithelial cells. Their site of action is epithelial cells because their respective receptors are present in those cells. And they act via a paracrine mechanism because they are released from just the neighborhood, and the epithelial-produced ligands such as EGF, BTC, HBGF, and TGF-alpha can regulate cellular function in an autocrine manner, so in crypts, different cells can either.

Follow an autocrine manner or a paracrine manner depending upon the availability of the receptor; ligand is free for all, no matter who is releasing it, but the availability of the receptor will decide whether it should act in an autocrine or paracrine manner. In spite of several reported observations, such as the stimulation of the proliferation of intestinal cells, there is little change in the expression of EGF during the regenerative response following injury in the intestinal epithelium; naturally, it is always the receptor that decides whether or the density of the receptor that decides to what extent it should respond to the ligand. In contrast, NRG1 is robustly upregulated following injury in macrophages, endothelial cells, and PDGF alpha plus trauma cells; NRG1 is induced upon injury. The effect of NRG1 on the epithelium induces both a strong proliferative response and the induction of stem cell characteristics in regenerating crypts. So NRG1 plays a very major role in the regeneration of the intestinal epithelium.

So the expression of ERBB3 is upregulated during regeneration. That is another unique feature you see compared to homeostasis. Uh, yeah, during regeneration or a post-injury scenario, ErbB3 is also upregulated. Loss of epithelial ErbB2 or ErbB3 decreases the ability of the intestine to regenerate efficiently following injury, although we don't know what the bona fide receptor of ErbB is or a bona fide ligand of the ErbB2 receptor; we don't know. But still, its level is important; probably it is contributing to dimerization, although it does not bind independently to a given ligand.

ErbB2 is a must receptor, although we don't know its ligand. Now we will get into hippo signaling. Hippo signaling is an important pathway that governs various tissue regenerations. The hippo pathway is regulated by the integration of a range of upstream stimuli. This includes mechanotransductive elements such as caveolae and piezo signaling.

These are some force-mediated, mechanical stress-mediated signaling events. Metabolism, extracellular matrix, and integrin signaling also contribute to the transduction of extracellular stimuli via mitogenic growth factor signaling and GPCRs, and cell polarity and cell-cell contacts can influence the HIPPO signaling. The activation

of the HIPPO signaling pathway triggers a phosphorylation cascade that leads to the phosphorylation of the HIPPO pathway effectors YAP and TAZ. YAP stands for Yes-associated protein. It is not important to go into the details of those genes.

YAP-TAS is acting as the effector of the HIPPO signaling. And the phosphorylation of YAP-TAS redistributes YAP and TAS to the cytoplasm, blocking TED-mediated gene expression. TED-mediated gene expression is occurring in the nucleus. HIPPO pathway inactivation. When the hypo signaling is inactivated, it prevents the apt as phosphorylation and allows their nuclear translocation; hence, the TED-mediated gene expression occurs.

So, the YAP now goes and pairs with the TED, binds, and turns on various gene expressions. One more thing to keep in mind is that MST1 and MST2 are mammalian. In mammals, this is called STE-20-like kinase 1 and 2. That is one nomenclature difference encoded by STK4 and TAZ by WWR1, so in mammals, these genes are encoded by other genes, but functionally they are the same. You can see here that different inductions, like various mechanical stimuli, can trigger responses, and when the Hippo pathway is inactive, the effectors do not function.

When the hippo pathway is active, the effectors do not function. When the hippo pathway is inactive, the TED and YAP go into the nucleus and turn off a bunch of genes. That is what you should understand here: active, non-functional, inactive, functional. So in this panel, we will look into how zebrafish heart regeneration depends on the Hippo signaling. Zebrafish's pretty much every tissue's regeneration involves Hippo signaling, but we will focus on heart regeneration.

The injury at the ventricle apex induces collagen and fibronectin deposition; injury can be inflicted in various ways, such as applying dry ice, pinching the heart, or cutting the heart, etc., and it will lead to scar formation. The deposition of collagen and fibronectin creates scar formation, and yes, one CTGFA and cave. CAV, CAV1 promote the appropriate and transient scar formation and that you can be seen in the panel B.

And now you move on to see what happens. The epicardium undergoes EMT, epithelial to mesenchymal transition, and inflammatory cells, which have been marked in blue, infiltrate into the scar. First, a scar is formed, and now these cells infiltrate the scar. And remember, fibronectin can act as a walking tool or walking surface for the cells. YAP1 and CTGF-A inhibit inflammatory cell infiltration. They can also counter this inflammatory cell infiltration, as you can see here in part C.

And then you have another situation that is a new coronary in panel D. New coronary

vessels will contribute to the revascularization of the injury site because the newly formed cells need nutrition. So that's why angiogenesis is also an important part. And in panel E, mature cardiomyocytes are shown in pink, and they de-differentiate into progenitor cells, which are shown in yellow. So the pink cell that is present on the site is now becoming a yellow cell.

These are all diagrammatic. Don't think that the cell color will change. Their property will change. That is what we show in this cartoon as a yellow color. Progenitors proliferate to create a progenitor cell pool that is necessary for fixing the damaged area, which matures back into cardiomyocytes (CMs) to reform the heart muscle.

CTGFA and CAV-1 promote cell proliferation. So that is what you are seeing in different cartoons in each of these stages. The individual molecule that is most important is YAP1, and CTGFA blocks too much inflammation happening at that site. So the importance of this hippo signaling is governed through the effector, such as YAP. And now, if you look into spinal cord regeneration, you will find another tissue where regeneration happens effectively in zebrafish. This is a structure of the uninjured spinal cord and the ependymal radial glial cell, which is ERG, shown in green color, living in the central canal of the motor neurons of the spinal cord.

And then panel B shows the spinal cord transection. That means you have damaged the spinal cord and disrupted the neuronal process. There is a discontinuity. In panel C, the ERGs, which are the ependymal radial glial cells, undergo EMT (epithelial-mesenchymal transition) to form ERG progenitors, which are colored blue, and they migrate to the site of the injury. The YAP1 promotes the EMT of the ERGs, and the epithelial to mesenchymal transition is favored or facilitated by the YAP1.

Additionally, the YAP1 and CTGF promote progenitor cell proliferation, as seen in the case of heart and ERG1. Progenitors extend the processes across the injury site to form a glial bridge; as you can see here, they are slowly starting to attach to each other, which is shown in the gray-colored glial bridge, and Yap1 and Ctgfa promote the formation of a glial bridge. Their expression, as well as Yap1 and Ctgfa, is important for the promotion of this glial bridge. The neuronal processes that extend across the injury site bring in communication, guided by the glial bridge, to promote the remodeling and deformation of the spinal cord, eventually establishing continuity and resulting in the formation of ependymal radial glial cells after regeneration. Now, if you look into the overview of zebrafish tail fin regeneration, you can see how this works.

This is a normal caudal fin, and it has different bony structures. It also has bony ray segments, as you can see here in the cartoon of the cross-section. You have created a cut

here, as you can see in panel A, along the cut line. In the cut line, what happens is that the wound covering occurs because of the wound epithelium formation, and now comes the YAP; the blastema formation via cell de-differentiation and proliferation normally has to happen effectively, and YAP, when present, does not allow this reprogramming to happen effectively, but when YAP is present.

.. it favor this reprogramming. What happens is it allows the stem cells to return to differentiated cell's regenerative outgrowth and maturation, and the patterning is favored. You can see here that amputation of the tail fin disrupts the bone array fragment, as shown in this picture. In uninjured tissue, it is present, and that is the goal we are trying to achieve after an amputation. In panel B, you can see the osteoblasts getting affected soon after an amputation, as you have shown in this picture. Osteoblasts and other mature cells de-differentiate and proliferate at the wound tip only in the site where the actual injury has occurred.

And the progenitors are now given a green color. And YAP1 inhibits osteoblast dedifferentiation. And BMP4, bone morphogenetic protein 4, enhances blastema cell proliferation. This is the equation between YAP and BMP4 when it comes to progenitor proliferation. But the bony fragment segments extend, but the blastema doesn't help in forming the blastema; it doesn't form the fin. The blastema cells have to differentiate back into the bony segment, so the bony ray fragment extends through the maturation of the progenitor cell, and progenitors are there outside the bony fragment.

Newly formed, but now these progenitors of the blastema should become the bony fragment; so wherever the bony fragment is, whether from the unamputated or the previous body part, it should now touch the progenitors, which should become the bony part. That is what is called the maturation of these progenitor cells back to their original cell type, and Yap promotes osteoprogenitor maturation. So that is the role of Yap in fin regeneration. If you want to conclude, From this, what you understand is the positive effect of YAP and TAZ signaling on the regeneration of the zebrafish, which appears to be latent in mammals.

This is what you should understand. Signaling is present in mammals as well, but during the regeneration event after an injury, two calls have to be made. Call number one, wound healing. Call number two is regeneration. Now does the YAP-TAZ pathway or YAP-TAS signaling kick in or not? And it appears to be latent in mammals. This suggests some therapeutic potential in promoting YAP-TAZ signaling to enhance mammalian regeneration.

However, it must be carefully noted that although many of the processes associated with

enhanced regeneration are linked with an increased risk of cancer, these processes, which facilitate progenitor proliferation and maintenance, can attract the risk of becoming cancerous, such as an elevated cell proliferation rate, which is necessary for every regeneration event, but they can also trigger a cancerous response. And cellular heterogeneity that has to be maintained; otherwise, it will be a simple wound healing process, filled by fibroblasts, and increased stemness. Cancer cells also have stem cell-like characteristics, so the stemness, when held back at the injury site for a prolonged time, can attract or trigger some trouble. So, dysregulation of the HIPPO pathway and, thereby, pathological hyperactivation of YAP and TAZ promote carcinogenesis in most, if not all, types of solid tumors. One important point I want to make about heart regeneration is that when collagen and fibronectin are present soon after injury, YAP and CTGF favor and facilitate scar formation; however, YAP and CTGF-A prevent inflammation.

Still, inflammation occurs in the scar tissue because it triggers the induction of many metalloproteases, which are required for clearing. Thus, the initial scar formed with fibronectin acts like a migratory platform for the cells and the inflammatory cells must be there, but the YAP and CTGF α make sure that inflammation does not go beyond a certain limit; it is held up to the necessary limit. Beyond that, it is problematic. So that is what you should keep in mind: the YAP and CTGF-A prevent overactivation of the inflammatory response.

And again lately, the cardiomyocyte dedifferentiation has come. The CTGF-A is a facilitator of the cardiomyocyte migration. The migration of these cardiomyocytes is facilitated and the proliferation of the progenitors is facilitated by CTGF and CAV1. So these molecules interact. So from this, what we are learning is that the Yap signaling or the Hippo/Yap pathway is contributing to the regeneration; however, it also depends on other factors. In mammals, if the Hippo signaling is turned off, to facilitate it, you know that Hippo signaling has to be turned off for it to get active, but It also accompanies, but if it is not aided by other signaling events, then you may not get the required regenerative response.

So this is what you should keep in mind: based on this, we can have some more questions. What are the interactions and feedback between Hippo signaling and the immune system in regeneration that result in the current contradictory observations? That is described, which means contradictory observations. What does it mean? The immune system has to come, but the signaling is blocking it, so that is why in the earlier class I also mentioned many times that when a signaling event is triggered, we should not think it is all favoring regeneration; sometimes, some signaling events are turned on to act as a contradictory mechanism. I don't want too fast regeneration, and I don't want too fast

proliferation. So, many times whenever it is present, you should always think it is meant for helping; sometimes, it is antagonizing.

So, does the Hippo pathway function differently in distinct immune processes? Do they have different roles in regulating your immune system? Enhanced Yap/Taz signaling is linked to an increased risk of tumorigenesis. Is there a way to activate Yap/Taz signaling spatio-temporally and precisely in specific cell types to promote regeneration without a cancer risk? So these are all some interesting questions that we should work on and think about. We'll study more about regenerative biology in another class. Thank you.