**Regeneration Biology**
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**Week: 7**
**Lecture: 34**

W7L34_Roles of Delta-Notch signaling during regeneration

Hello everyone, welcome back to another class on delta node signaling during regeneration. We have mentioned delta notch signaling multiple times in the previous classes because it is one of the most important signaling events that govern regeneration as well as embryonic development. And in different tissues, it plays different roles; say, for example, in some tissues, it is important to retain homeostasis. That means the day-to-day affairs of the tissue have to be taken care of by delta-notch signaling, whereas in other cases, they are important for transforming the stem cells into differentiated cell types, their expression is somewhat referred to as the salt and pepper way of induction; it means you take black pepper powder and sprinkle some salt. What pattern do you get? It's something like that in their expression. So let us quickly visit the delta node signaling pathway in an overview.

The picture seems a little complex, but don't worry, we can lighten it up. It's a schematic diagram of the node signaling pathway following the ligand-receptor translocation that is part of the NICD domain. And there are different proteins that come into the picture. ADAM stands for disintegrin and metalloproteases.

ANK is the one that is anchoring repeats. COR, which means co-repressor. CSL means a bunch of genes, CBF1. SU(H), LAG DSL means Delta Serrate LAG2, MAML means mastermind, NICD means notch intracellular domain, NLS means nuclear localization signal, NRR means negative regulatory region, and PEST stands for fluorine, glutamic acid, serine, and threonine; these are all well-accepted terminology, and TMD stands for transmembrane domain, etc. So you can see here there is an EGF-like repeat bearing delta molecule; this is one cell, and it is present, and this is another cell that has also got EGF-like repeats, but it is called a notch.

So the delta must interact with the notch for any activity to occur. There is also a step in the maturation of the notch ligand for which the ADAM family enzymes come into play, and what we should understand is that because of the deltas binding onto the notch, you end up getting a cleavage of this notch intracellular domain, which is favored by gamma secretase. There is a S2, S3, and S4. That means different sites of cleavage that occur. But the final action is done by gamma-secretase, and that triggers the release of the Notch

intracellular domain.

That is when this portion will be released. This portion will be released, and that will go into the nucleus NICD, which can turn on a bunch of genes; that is, it can be as many genes as there are, and this is the typical way of functioning of NICD or the Notch signaling events. So the diagrams that show how delta notch signaling contributes to the endocardium are the signaling events that happen after a myocardial infarction. If there is an ischemic heart injury, the blood supply to the heart muscles is stopped; how the endocardium or myocardium comes into the picture and contributes to retaining homeostasis. And different injury and stress models so we can see one situation that is (a) after ventricle ablation and the (b) after ventricle cryo injury.

So what we are seeing here is notch activation results in the non-cell autonomous initiation of myocardial Erbb2. Remember we saw in the previous class about the Erbb2, whose ligand is not known, but it is important in the EGF signaling, ERBB2, and BMP signaling are responsible for cardiomyocyte reprogramming and proliferation. So, as you can see here, upon insult in panel B, Notch signaling is significantly activated in the endocardial cells. So, the endocardium is the wall of the heart muscle that is facing the blood, which is the lumen of the heart; then comes the myocardium, followed by the epicardium, and above the epicardium is the pericardium. Endocardial cells restrict the expression of inflammatory factors and macrophages.

Recruitment coordinates with a gene or a protein called Serpene 1 to control endocardium maturation and cardiomyocyte proliferation. This is its normal route. So what we can see in this picture is that this is the cardiac lumen, and there are a lot of cells that are exposed to this cardiac lumen, and the notch is one of them. When the notch gets activated, you end up getting the NICD, which in turn will facilitate the production of more and more notch into the cell. Various signaling events can trigger an anti- or negative role on the migration of NICD to the nucleus, which is possible, but in general, this can trigger an ERBB2-mediated NRG1 and ERBB2, which we are discussing in a regenerating model.

NRG1 can bind to ERBB2 in the case of regeneration and regenerative animals, such as zebrafish, that you should keep in mind. The BMP signaling triggers the differentiation and transdifferentiation of myocardial cells (MCS) or cardiomyocyte cells, and what happens if you have given a cryo-injury is that the serpentine molecules come into the picture for easy maturation and easy proliferation. In the neighboring cell, what happens is that it can also inhibit myocardial cell proliferation; the progenitors have to be formed for regeneration to occur, but we are talking about the epicardium and myocardium. The epicardial cells, not epicardial, sorry, endocardial cells, and also the myocardium interact

with each other; they are the ones triggering this delta node signaling for their intercellular communication. NOTCH triggers NICD production, helps in the release of various factors, and can negatively influence the inflammatory factors and macrophages.

Like we saw in the case of HIPPO-YAP signaling, in the heart you need to have a negatively influenced inflammatory response; too much of it is not welcome. They have to block the inflammatory factors, which is also favored by delta NOTCH signaling. This is the diagram that shows the functions of endocardial NOTCH signaling and various heart homeostasis, as well as stress injury models. Panel C is after ventricle amputation. So the inhibition of the endocardial notch signaling could cause a reduced expression of Wnt antagonist.

We are now talking about Wnt signaling. What are Wnt antagonists? They are WIF-1 and NOTAM-1B. NOTAM, you may remember, we have also studied invertebrate regeneration. Thus, the Wnt activity was increased and could inhibit cardiomyocyte proliferation and heart regeneration. This regenerative defect could be partially rescued after treatment with the Wnt inhibitor IWR, so in panel D, what you are seeing is that the notch signaling could control the balance between fibrotic and regenerative repair in the adult heart.

Interestingly, what we should note is that the notch 1 knockout—if you knock out notch 1 in cardiomyocytes—does not affect the jagged one induced. Anti-hypertrophic and anti-fibrotic responses occur because naturally various other signaling pathways, like the Yap signaling we have seen, come into the picture; they can also contribute to the cell types responsible for the beneficial functions of Notch1 overexpression, yet to be identified, and the endocardium stands out as a good candidate for this proposal. So you can see here the lack of signaling; once you kick-start the NICD, they can release the Wnt inhibitors so that in the myocardium, the Wnt signaling will not act very effectively because the Wnt signaling is prevented. Hence, they will not proliferate. You can also see in the case of jagged overexpression that if you do it, you are forcefully allowing the delta notch signaling to go up in the endocardial cells.

Then what you end up getting is that there will be... A lot of influence that is occurring in the myocardial precursor expansion will be favored, and also the myocardial proliferation will be favored, and you also end up getting a lot of fibrosis that is due to the fibroblast cells and the fibrosis that is coming into the scenario, so one signaling can do either job; at a minimum, it does one job, and at a maximum, it does another job. So this is what you should keep in mind: the same signaling will be able to perform different tasks.

So the role of the no signaling pathway in the recovery of cardiac function after

myocardial infarction. So myocardial infarction, we all know it is the inability of the heart muscles to perform. It normally occurs because of a lack of blood supply. That is typically known as ischemic heart injury. And it's a pathological process.

evidencing a massive death of cardiomyocytes associated with hypoxic and oxidative stress. The formation of areas of fibrosis that lead to heart failure. We know that there are some mechanisms that contribute to the functional repair of the heart, and some mechanisms may not restore the myocardium, but they can prevent the onset of excessive inflammation in most mammals, including humans. The Notch signaling pathway has a cardioprotective effect; it is involved in the formation of the heart during embryogenesis and in the restoration of cardiac function in myocardial infarction. Steps that are: step number one, reducing oxidative stress; step number two, prevention of apoptosis; step number three, regulation of inflammation; step number four, containment of fibrosis and hypertrophy of the cardiomyocytes; step number five, tissue revascularization; and step number six, regulation of proliferation and differentiation of the cardiomyocytes.

In addition to this, the no signaling pathway interacts with various other signaling events, as we saw how it influences Wnt signaling by producing Wnt antagonists. And it can also get involved in several pathogenic events of myocardial infarction and subsequent cardiac repair. So even in mammals, even though they cannot regenerate, they have a cardio protective role. And if you look into the tissue and cellular events of myocardial infarction, they have multiple phases. Ischemia, hypoxia, inflammation, termination.

This is the broad phase. After ischemic exposure, an increase in the level of ROS and a lack of ATP lead to cell death and the release of inflammatory mediators. During the inflammation stage, the following processes occur: let us see what they are. Phagocytosis of the dead cells must happen, and the debris should also be cleared. Additionally, new blood capillaries should form so that new cells can survive. At the termination stage, some conditions restore the integrity of the myocardium by replacing the dead cardiomyocytes (CMCs) with fibroblasts and hypertrophy of the surviving CMCs.

Hypertrophy means an expansion of size so that the damaged portion can be restored. This is a schematic representation we have already seen; this is a quick revisit of the delta notch signaling because delta notch signals, depending upon species, can have delta ligand, delta-like ligand (DLL), and jagged (JAG); all these things can come into the picture. And eventually, the ligand, no matter what it is, has to activate or bind the notch, and the notch receptor can be notch 1 to 4. The Notch intracellular domain is cleaved and activated when the Notch is bound to its ligand, and the activated NICD enters the nucleus. Where it interacts with the intracellular regulatory complexes, after which the transcription of the Notch target genes takes place.

As you can see here, Jagged is there, DLL is there; they are all acting on the Notch, and the Adam and the gamma secretase can cleave here, and the gamma secretase cleaves here, and this NICD is freed. So, Adam is the first cleavage, and gamma secretase is the second cleavage. And if NACD goes into the nucleus, it can work in a canonical way, and it can also engage in several non-canonical pathways. Many genes are turned on, such as HES, HEY, NKX, etc., and a lot of this cleavage of the notch occurs during maturation; there are multiple cleavage phases that take place, including furin cleavage, Adam cleavage, and gamma secretase cleavage, etc.

So that's how a notch matures. See the role of the notch signaling pathway in reducing oxidative stress in cardiomyocyte cells. So we can see that when the notch is turned on, it can favor NACD and LKB1, and it can trigger AMP kinase and also ATP production. And it can also negatively affect TNF alpha, which is a pro-inflammatory cytokine and in turn inhibits MFN2. So technically, Notch is activating MNF2, which is helping in mitophagy. And notch can also activate AKT, which is responsible for the production of several antioxidant enzymes.

So launching the notch signaling pathway enhances the synthesis of the MFN2 protein responsible for mitophagy. The notch 1 receptor can bind to tumor necrosis factor alpha and block its action. The production of antioxidant enzymes is controlled through the Akt cascade, and the energy conservation in the cells is achieved by activating the MAP kinase cascade, so this many roles the NO signaling can trigger. If you look into the significance of the NO signaling pathway in preventing cardiomyocyte apoptosis through the transmission of the NO signal, a decrease in the expression of Pro-apoptotic BACs and an increase in the expression of anti-apoptotic BCL-2. The decrease of pro-apoptotic BAX and the increase of anti-apoptotic BCL2 are achieved.

Additionally, the prevention of apoptosis can be achieved by increasing the expression of genes associated with autophagy. Inhibition of the pro-apoptotic tumor necrosis factor alpha, which is also a pro-inflammatory molecule, helped to prevent apoptosis. As you can see here, Notch activates Beclin 1, LC3I, and can also trigger autophagy. Additionally, it upregulates Bcl-2, which is anti-apoptotic, and downregulates Bax, thereby safeguarding the cells from apoptosis. It blocks NF-alpha, which is a facilitator of apoptosis, so if you block it, apoptosis will be prevented.

These are all the roles played by Notch signaling in influencing apoptosis. Now, if you look further, the Notch pathway has a significant role in regulating the inflammatory response. Like in the previous class, we discussed a lot about M1 and M2 macrophages. We discussed that M1 macrophages create a more pro-inflammatory environment,

whereas M2 macrophages create more of an anti-inflammatory environment.

So, let us see how it is contributing. So the notch signaling promotes the acquisition of a pro-inflammatory M1 macrophage phenotype that secretes inflammatory mediators. So if you have active notch signaling, what happens is the production of M1 macrophages takes place, which can secrete IL-6, MCP-1, TNF-alpha, and many other pro-inflammatory cytokines. And if there is DAMP signaling that is triggered, then you end up getting more M2 macrophages. M2 macrophages, if present, are anti-inflammatory. But remember, for regeneration to occur, you need to have pro-inflammatory events triggered soon after injury.

And these come later: IL-10 and IL-RA; these are all derived from the M2 macrophages. So the no signaling pathway very selectively influences the upbringing of M1 macrophages. We can also see the myocardial remodeling in the fibroblasts, as well as the mechanism of inhibition of myocardial remodeling in fibroblasts. We know that fibroblasts contribute to fibrosis and are involved in scar formation after a myocardial infarction. The intracellular domain of the Notch receptor NICD can inhibit signal transmission along the TGF-beta 1 SMAD3 pathway due to competition for the alpha-SMA promoter.

Let us see, this is TGF-beta, and when SMAD3 is activated, it normally brings in alpha-SMA; the gene will be turned on and expressed. However, if the same cell has NICD, notch signaling is turned on; it allows alpha-SMA expression, but it does not allow SMAD3 to bind to it. So in this process, it will alleviate the action of TGF-beta signaling on the production of alpha-SMA; the gene expression is the same, but based on whether TGF-beta signaling is turned on or delta notch signaling is turned on, you end up getting alpha-SMA gene expression. However, you can say that NICD has a higher affinity for the alpha-SMA promoter than pSMAD3, so it can compete out and also trigger a more robust regenerative response. The differences between normal and hypertrophied CMCs indicate that when an injury occurs, fibrosis can happen in the heart, which can be cleared in the case of regenerating zebrafish; however, in the case of mammals, the fibrosis remains, which can debilitate the contraction of the heart muscle.

Now, even in animals that do not regenerate, even though there is fibrosis, the existing cardiomyocytes have the potential to undergo something called hypertrophy. That means, just like you know how a balloon looks: you take a balloon, it is very flaccid, but if you blow air into it, it will become bigger and bigger and bigger. So we can call it hypertrophy; the balloon got hypertrophy, but the number of balloons does not change; the number of balloons remains one. But earlier, the balloon was something you could happily put in your pocket, but once you blow the balloon up, you cannot put it in your

pocket, meaning it can occupy more space. More space, so this is the principle of hypertrophy; the muscle remains muscle, but now it can cater to a larger area.

That is a principle of hypertrophy. Normal cardiomyocytes respond to the action potential; soon after, potassium channels that are open will result in an action potential. Whereas in hypertrophied cardiomyocytes the action potential is occurring, it does not come to the resting stage very fast; it will stay for a longer time, so the hypertrophied cardiomyocytes differ from normal CMCs in terms of electrochemical features and they have a longer period. Of vulnerability to extra contractions. That means it contracted. If it comes down immediately, that is called repolarization.

This is depolarization and then repolarization. If it does not come back to normalcy, it will not contract again. They have a refractory period before they can respond to the injury. In a simple sense, if I say I gave you breakfast at nine o'clock and you did not finish it until one o'clock—four hours later—you are having breakfast now. How will you have lunch at twelve o'clock? You will not be able to, so you have to have a gap for digestion, and then you can go for a second round of food. The same logic applies; hypertrophied muscles will not respond because their relaxation takes a longer time and a longer period of vulnerability to extra contractions.

This is due to a change in the amount and conductive properties of the potential-dependent potassium channels; because of this hypertrophy, the potassium channel distribution can be affected; hence, they will not enter the refractory period very quickly, meaning they will have to wait for a longer time. And if you look, there is no signaling in angiogenesis. We all know angiogenesis is a necessary step in every regeneration because angiogenesis is nothing but the formation of new blood vessels, for which the endothelium comes into the picture. Endothelial cells have to proliferate and then assemble into blood vessels; this is how blood vessels are formed. Vegef is an important angiogenic factor; the vascular epithelial growth factor vegef triggers the expression of DLL4.

DLL4 is the ligand for Delta-Notch signaling, dealing with ligands in the leading tip of the cell, triggering Notch in the adjacent stalk cells. So when you have this ligand, which is vegef, present... And it will trigger the expression of another ligand, DLL4, in the tip of the cell, and that can influence or tell the target cell or the adjacent cell that has a Notch signaling pathway, so the direction of migration of the tip cells is determined by the CXCR4 chemokine receptor and chemokines.

We know chemokines attract the cells when chemokines are released; it's almost like bait when you go fishing. You have a rod, a hook, and bait that you put on it. Ultimately, if a

fish is on your hook, it will come to you. You can tell that the gradient, wherever the hook goes, doesn't matter. When the fish are there, you can bring the fish back to you.

The same logic applies. The chemokines bring a cell to where they want it to go. It will attract. Just like you put out a light, the insects come to the light at night, right? Something like this is logical. Same logic. So, you should understand that there are veins and that there are arteries.

Both require, you know, angiogenesis. Angiogenesis means not just veins, not just arteries. Both are needed when it comes to angiogenesis. So, in these cells, when they are growing, this blood vessel is actually growing, okay. When it is growing, the tip cells have VEGF present.

And when VEGF is acting, it has its own receptor. They produce VEGF. Based on the CXCR4, which is a chemokine that allows the migration of cells in a given direction, these stalk cells have sharp or pointed edges and express DLL4, which binds to neighboring cells that have notch signaling, triggering a notch signaling event. And hence, they will continue to proliferate, so that not signaling cells will produce genes that will allow the progenitors to proliferate; hence, you will have more endothelial cells and more blood vessels that will be triggered, and it is decided by the VEGF. The DLL4 expression is determined by the wedge of availability, and DLL4, in turn, allows the node signaling to kickstart. So this is how the migration or growth of the blood vessels takes place.

So, if you derive the conclusion, the Notch signaling pathway mediates cardioprotective effects at various stages of myocardial infarction. Ischemic damage to the cardiomyocytes occurs at the reactive stage and also during the scarring stage. In the early stages of myocardial infarction, Notch causes a decrease in oxidative stress by regulating the number of mitochondria and activating the MFN protein. Notch also activates the Akt pathway, which in turn is involved in producing antioxidant enzymes in the mitochondria, so it has to safeguard the cell; you don't want the damage to go further after a myocardial infarction. Finally, Notch signaling is involved in the activation of a pathway associated with AMP-activated protein kinase.

This is also a role played by the notch signaling, which promotes energy reservation. That means cells are starving now because of the ischemia, and they should learn to conserve the existing energy. So the prevention of apoptosis is achieved by stimulating the production of survival factors such as PI3 kinase, phosphatidylinositol 3 kinase, the AKT pathway, and anti-apoptotic proteins. So that notch stimulates the survival of cardiomyocyte cells, thereby reducing the damage caused by myocardial infarction.

Otherwise, the whole heart can suffer, but it will be localized. Notch 1 is able to reduce myocardial fibrosis by inhibiting the transmission of TGF-beta-1 SMAD3 signals and reducing the production of ECM in the myofibroblasts. We will study more about regeneration in the next class. Thank you.