Regeneration Biology
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W6L28_Retina regeneration

Hello everyone, welcome back to another class on regenerative biology. In this class, we will learn more in detail about retina regeneration with special reference to sonic hedgehog signaling and also the involvement of some epigenetic factors, such as Histone deacetylases' involvement in regeneration. And I will be presenting data published from my lab. So one of the articles is published where we studied the involvement of hedgehog signaling and how let-7 micro RNA influences it. So those who are interested can read this paper. The injury model we have discussed is done mainly through a needle poke from the back of the eye, and it's a focal injury that is very easy to perform, allowing you to analyze the neighboring tissue.

If you look at the cartoon of regeneration, you can see that soon after the injury, cytokines will be released from the retina, which will be sensed by the Müller glia, causing proliferation and restoring normalcy. And in this picture, we have also seen how the Müller glia reprogram into Müller glia-derived progenitor cells and how various signaling pathways, such as Wnt signaling, FGFR signaling, insulin signaling, and HBGF signaling, stimulate the Müller glia to transition into progenitor cells. And also, the delta notch signaling restricts the zone of proliferation and various other factors, such as INSM1A, which is a negative regulator in the sense that it's a transcriptional repressor. It is necessary to push the progenitor from exiting the cell cycle and to push the cell into exiting the cell cycle.

Lin28 is a negative regulator of RNA; let's say when micro RNA, it's an RNA-binding protein, and many various factors like ASCL1A contribute to the regenerative response. Let's say when micro RNA is a great deterrent to this Muller glia becoming the progenitor because. The let-7 microRNA retains the integrity of a neuron, which means it will keep it in a differentiated state. If you want it to move into a progenitor state, let-7 should be downregulated. So this is a retinal injury and the in situ expression of Lin28.

And you can also see that normally in a published paper, you will see a block area. So whenever you see a square, you should understand it's a part of the retina, and this is the injury spot that is highlighted. And you can see that Lin28 is a major blocker of the let-7 microRNA. And Lin28 is one of the pluripotency factors expressed in embryonic stem

cells and cancer cells. Lin28 is found both in the nucleus and cytoplasm and is capable of regulating the levels of the let-7 microRNAs.

One option is that let-7 is present in various parts of your genome. It is also embedded in the introns of other genes. So let's have a gene; when it is produced, it is produced in the form of pri-let-7 (primary let-7), which is acted upon by Drosha and DGCR8 to make it into pre-let-7 by chopping off this region. It will become pre-let-7, which is exported into the cytoplasm from the nucleus, and it is processed by Dicer to make mature let-7. So this pri-let7 bound drosha and DGCR8 will be blocked by lin28, one option.

And also, let-7 microRNA will be actively degraded by Lin28. So Lin28 acts in two phases. One is to prevent the formation of the pre-let-7, and it will also prevent the stability of the mature let-7 RNA. And the downregulation of Let-7 is necessary for regeneration because previous publications have shown that various regeneration-associated genes, even though they are induced, will not be translated into proteins if let-7 microRNA is present. So you can see ASCL1, HSPD1, LIN28, and PAX6B.

KL4, cMYC A, and cMYC B (OCT4) are all genes that are negatively regulated by let-7 microRNA. If, let's say, when microRNA is missing, then this mRNA can get translated in a given cell. So now, if you look into hedgehog signaling, because today's class is about hedgehog signaling, we'll come back to let-7 in a short while. So hedgehog signaling can be both inactive and active. What happens when the Hedgehog signaling pathway is inactive and not present? There is a receptor called a patch that will block the SMO from doing any function.

SMO is the major player in hedgehog signaling. When the patch is occupied by the SHH ligand, it is no longer able to block the SMO. Hence, the SMO will be able to inhibit a negative regulator called SUFU, because of which the GLI will be able to enter the nucleus and turn on various genes. But usually when SUFU is present, it blocks the Gli from going into the nucleus during active hedgehog signaling. This is active hedgehog signaling.

So this is the information you should know. You should also understand that there are ways in which you can prevent the migration of SMO from the cytoplasm to the nucleus. So that will also prevent the hedgehog from signaling. So, understanding the SMO receptor is the actual player in the hedgehog signaling. Patched is basically a blocker for SMO.

from acting. So Smo, when it is free and relieved from the repression of patched receptor, will inhibit SUFU from its ability to block Gli. As a result, the Gli will go to the

nucleus and turn on various genes. So, hedgehog signaling is turned on at various time points post retinal injury. If you see, almost all of the genes are induced in the retina, and the expression pattern is not important for us as of now. And if you pick up a few of the hedgehog signaling components, such as Shh-a, SMO, and PATCH, you can see they are induced right from one day post-injury, two days it is high, four days it is high, and by seven days it comes down to a normal state.

This indicates that the qPCR analysis also confirms the gel picture data. So now, if you block the hedgehog signaling using a drug called cyclopamine, which is present in Californian lily, if it is eaten by a pregnant goat or any animal, then they will end up getting cyclopia because hedgehog signaling is a developmentally essential pathway. We checked in zebrafish if giving cyclopamine results in cyclopia, and the interest in this experiment is to determine what concentration of cyclopamine can be used for retina regeneration studies. We found that around 30 micromolar of cyclopamine leads to a fused eye, referred to as cyclopia, and you also end up getting a lot of.

.. Cell death or animal survival is compromised because of this cyclopia, so now, when cyclopamine is delivered into the eye, what you see is a drastic decline in the progenitor number, the retinal progenitor. This is the proliferating retinal progenitor that is Müller-glia-derived; their number is stained with a proliferation marker. Such as proliferating cell nuclear antigen, which is called PCNA, and their number is decreased. This is the quantification of it. So that indicates that in regeneration, for normal proliferation to occur, we need normal hedgehog signaling.

So another approach we use is that if you want to knock down a gene, you can take zebrafish anesthetized, deliver the morpholino into the vitreous, and then give an electric shock so that the morpholino will get into the retina, as you can see here, and then you can do the analysis. Once you do this electroporation and gene knockdown, you can give a BrdU pulse, and then you can perform sectioning and image for morpholino immunostaining, etc. That is the workflow of gene knockdown studies in the zebrafish retina. An image that shows if you knock down SHH using antisense morpholino indicates that you see a decline in progenitor proliferation; this is control morpholino that does not knock down any gene, and you can see normal proliferation. This is the merge, so the lisamine tag is the tag associated with the morpholino; wherever there is red color, it means it is because of the presence of the morpholino, as it has a fluorescent tag.

So Shh-a morpholino, you can see how much decline in the cell number is observed. And on the other hand, if you knock down SHH but supplement with the SHH protein, then you rescue the regeneration. So it indicates that the decline in proliferation was due to the absence of SHH protein. In the same way, the same knockdown condition was used

with the control morpholino, cyclopamine-treated, SHH morpholino, and Gli morpholino. You remember that Gli is the actual player of Shh-a signaling or sonic hedgehog signaling in the nucleus.

So you can see one of the important genes, ASCL1A; its level is quite good in the control morpholino, but with cyclopamine, its level goes down. With the Shh-a morpholino, the level goes down, and with the Gli morpholino, the level goes down. So not only is the proliferation going down, but the regeneration-associated genes are also going down. Additionally, we performed another double in situ using patch one and SHH signaling. Remember, the patch expression is regulated by hedgehog signaling itself.

So, if the hedgehog signaling is turned on, then the patch expression is positively regulated. So it's actually more patch in the cell than the SHH will be sequestered by these overexpressed patches. So it's the way. regulating the hedgehog signaling itself. So you can see the patch expression, the lin28 expression, and the BrdU expression; they are in the same cell.

So lin28 expression happens in the patch-expressing cell, which in turn is due to the hedgehog signaling. So we looked into the promoter of the LIN28 gene for various GLI binding sites where the actual transcription factor GLI can go and bind. We saw several binding sites that indeed bind with the GLI when you performed a GLI-specific antibody. We saw this in chromatin immunoprecipitation. So these sites, when it is site one or site two, when they are present, have the ability to bind, especially site one, which is closer to the ATG start site.

There is a strong binding of the Gli protein to the chromatin immunoprecipitants. What you do is break the DNA, fix the cells, fix the DNA, break the DNA, and pull down using the Glee antibody, and then simply do a PCR. If the unbound one is washed off, the bound one will be retained; this is a forward primer, a reverse primer, and that is what the gel has been done for. This is chromatin immunoprecipitation. And after proving that LIN28 is bound by the Gli protein and is indeed regulating the hedgehog signaling.

We looked at the expression of Shh protein, and we saw good expression of Shh protein in the proliferating cells. Interestingly, these proliferating cells are the ones that have downregulated let-7. As I told you, downregulation of let-7 happens because of the Lin28 expression. As you can see here, these proliferating cells have a reduced expression of let-7 microRNA. And this Let-7 microRNA, what we then did next was analyze whether the Let-7 microRNA binding site is present in the component genes of the hedgehog signaling.

We looked for SHH-A, SHH-B, PATCH, and SMO. We found several Let-7 binding sites in these genes, and we made a construct where the GFP is fused with the Let-7 binding regions of various SHHA, AB, etc. and then overexpressed in a cell line along with the let-7 microRNA. So this is the workflow. CMV promoter, GFP, and the microRNA binding sequence of various genes were transfected.

And also, cells will be lysed and a Western blotting assay will be performed. What we found was that Shh a b is more and Patched as the Let-7 micro RNA concentration is increasing; you see a decreased proliferation. So, what you know is that the activation of Hedgehog signaling must. It is accompanied by lin28 and the downregulation of let-7 because it is needed for the proper translation of this protein. So if the SHH signaling has to be sustained, then Lin28 becomes very important because you have to get rid of the let-7 microRNA.

So in summary, we can say that the SMO migration, if blocked with cyclopamine, can inhibit hedgehog signaling. But the usual mechanism is SHHA, patch1 , Smo, and Gli, and this is the actual player. The Gli can turn on lin28; it can also influence the ascl1a production. Ascl1 can induce the lin28 production, and lin28 negatively regulates let-7, while let-7 can negatively inhibit lin28 and also Ascl1 itself. So this is basically a cycle that occurs, and let-7 can negatively regulate SHHA, Patch, and SMO as well.

So let-7 must go off from the progenitor if the hedgehog signaling is to turn on. That is the story from that paper. But how is SHHA being actively secreted, and who produces it? At first, it is an interesting question. So these are the other two papers published from our lab. One is talking about how Lin28 and MYC interplay in governing the Muller glia-derived progenitor proliferation at different stages, and another is about how histone deacetylase comes into the picture, as well as how the HER4 and Lin28 axis comes into the picture.

So these two stories, those who are interested can read them, and I will give a gist of them. So HDAC is histone deacetylase, and the enzyme's main role is to remove the acetyl group from the histone protein. It can also deacetylate various other proteins. But when histones are acetylated, the chromatin becomes loose.

Gene expression can occur. When it is deacetylated, it will make a compact chromatin. The histone will bind strongly to the DNA, and the chromatin will be condensed. So if acetylation occurs in the histone, gene expression can occur effectively. So this is the epigenetic process.

The role played by HDACs. Different HDACs have been looked into, and our main

focus was on HDAC1 because HDAC1 is a primary histone deacetylase. So if you look into the expression pattern of HDAC, what we see is that this is a proliferating PCNA marker and this is a BrdU marker, and you can see the expression of HDAC is neighboring the proliferating cell. In the immunostain of the HDAC, you can see that the BrdU-positive cells do not have HDAC expression, whereas the other cells do. So what it tells you is that HDAC is secluded from the actively proliferating cell. That means you don't want deacetylation to happen actively in the proliferating cell.

That is why HDAC is missing. If HDAC is present, it will deacetylate it, and chromatin will be compact. So from this observation, this is what you can get. However, there is a catch. If you look at HDAC, which is excluded from the proliferating cell, but if it is associated with another protein called MycB, MycB can influence the lin28 expression in both ways.

It can either be an activator or a repressor. Let us see how it goes. MycB normally binds to the E-box binding region, and around 25% of the total polymerase II-transcribed genes have MycB binding sites. So MycB activating lint on diet is just a normal thing. And in the paper, we have also confirmed it by chromatin immunoprecipitation. So the Myc-b is present in the Lin28-expressing cell, as you can see here.

But interestingly, one thing you should see is that in one cell where Myc-b expression is present, Lin28 is missing. But it is a proliferating cell. You can see that BrdU is there. So what made this cell not have Lin28? So, this made us believe that there is a possibility that Myc-b, although present, is not activating Lin28. So we later found in this study that MycB can interact with HDAC and bind to the lin28 promoter and cause repression of gene expression.

So HDAC1 can normally be seen as missing from the proliferating cell. So here proliferation is present in both the cells, but MycB is present while lin28 is missing. So this is because Myc-b can choose a partner, whether it should be HDAC, which can cause repression of the Lin28 promoter, or it can act as an activator. So, HDAC knockdown blocks retinal progenitor proliferation. You have used two different morpholinos, two different HDACs, one morpholino, and a second target; two targets we used.

And in a concentration-dependent manner, you see a decline in PCNA expression. Uh, like control morphol, you know there is a normal proliferation of HDAC; both the concentration of HDAC and the concentration show a decrease in the proliferation, as you can see here. So how this action happens is that histone acetyltransferases add an acetyl group onto the histone protein, whereas HDAC does the reverse. So if you inhibit HDAC, various chemicals are available; trichostatin is one, valproic acid is another, and

you can block HDAC to explore what happens to regeneration. So we found that the HDAC-mediated pathway, when you block HDAC, shows a decrease in Lin 28 expression.

As you can see here in the qPCR, there is a concentration-dependent decline in both TSA and valproic acid. There are two inhibitors of HDAC. We see a decrease in Lin28 expression. And you can also see the same decline in the RNA in situ hybridization LIN28 expression, which is decreased because of the SDAC inhibition; accordingly, proliferation is also decreased. Since the LIN28 level is decreased, there will be an increase in the let-7 expressions also.

So the let-7 levels do go up. And let-7, you should understand they don't allow the translation of various regeneration-associated genes. They don't allow the hedgehog signaling components either. Highlet-7 means that whatever it is, protein translation will not happen. So the increased let-7 could account for why there is a decrease in proliferation because of HDAC inhibition. So, HDAC inhibition-mediated reduction in proliferation is reversible.

Like in various regeneration studies, we have seen that. In fin regeneration, we have seen it as well. Like FGF20A, if you block it, the wound heals, but there is no blastema formation or regeneration. We have seen that. Follistatin knockdown in planarians. If you knock it down, you get a wound healing, but the planaria will not regenerate.

We have seen that. But the interesting thing here is in the retina, what we looked at, if you remove the blocker. So when you have a blocker, such as trichostatin or valproic acid, its presence prevents regeneration. So what we did was block up to four days. Four days is the peak of proliferation in an injury mechanism.

So we removed the VPA. and until four day, no regeneration. As you can see here, if you put it in, then you don't find any proliferative response. Then what we did was remove the VPA and wait up to eight days, meaning another four more days. Injury was sustained on the zero day and treated with medication for four days. On the fourth day, we put the fish in normal water and then allowed it to grow for up to eight days.

What we found was that the regeneration bounces back. So there is an increase in PCNA proliferation. And this is simply because the retina is so plastic that it knows how to come back, how to bounce back, and how to restore its damaged portions. So that was something very interesting that, unlike planaria or zebrafish fins, if you block HDACs, there is a chance that the animal will bounce back. And another interesting thing during this reentry, we also looked for lin28 expression. What we found is that once you

withdraw the drug, put it in for four days, and then stop it, that means you shouldn't inhibit the HDACs when you withdraw it.

You will see an increase in the expression of Lin28. And naturally you can expect that the let7 levels will go down, which we have also checked and is mentioned in the paper. So when the let7 levels go down, the regeneration-associated genes can happily get translated. So this is the increased level of lin28a, and this is the qPCR showing that the lin28 level goes up. So what you can understand from here is that there is an increase in how Lin28 and Let-7 are influencing and regulating; in this picture, what you can see here is SHH.

Follow this workflow: we have seen this SHH, a patch one Smo, and Gli. So patch 1 is a negative regulator of SMO, and SMO is a positive regulator of Gli because SMO's role is to get rid of the repression of Gli by SUFU. So the moment SMO is free, it will recruit the SUFU and free the Gli. That is how it goes to the nucleus, and Gli helps in the activation of LIN28. We are discussing only lin28 and its implications in let7, as well as its ability to activate ascl1a. In one of our earlier studies, we showed that ascl1a can activate lin28, and lin28 is the major negative regulator of let7 when let7 is present.

Let7, when present in a tissue, simply will not allow the translation of various genes. You have seen various genes that are associated with regeneration, especially the hedgehog signaling. The same let-7; I will show that picture once again quickly so that you will remember. The same let-7 can negatively regulate lin28, and it can negatively regulate ascl1a. And here what we show is that the HDAC can combine with the Myc-b and negatively regulate the LIN28.

Myc-b alone positively regulates LIN28. That is one way; apart from ASL1 and ASCL2, My-b can also positively regulate LIN28. It can influence the HDAC-expressing cell. HDAC-expressing cells, even if Myc-b is expressed, will not allow activation of lin28. If lin28 is not activated, what will happen? The let-7 level will remain high.

And because of this, there will not be any translations. I'll quickly show that let-7 regulation. Here, this is an earlier paper that I published, where ASCL1 is negatively regulated by Let-7, and Lin28 is also negatively regulated by Let-7. So even though these are all regeneration-associated genes, they are essential. These are all pluripotency factors, and KLF and OCT4 are only marginally downregulated compared to the control.

But whereas these genes, ASCL1, HSPD, LIN28, etc., have binding sites. Various regeneration-associated genes may not have binding sites, but they are not good enough. Say there are 5 genes or 10 genes required for regeneration, but 6 of them have a let-7

binding site. The remaining four are not good enough; even though they are translated, they are not adequate or sufficient for causing a regenerative response. So this is the idea you should keep in mind. That is why in the last model, we showed that ASCL1A and lin28 negatively regulate each other.

And the cMyc itself, that's another thing we should keep in mind. The cMyc itself is negatively regulated by seven microRNAs. Now, if you go into it, another thing we have seen is this: this let-7 itself regulates, so this one is the let-7 which is going off from the proliferating cell, and credit goes to the lin28 expression. You can see that the neighboring cells have it; they don't proliferate, as you can see here. This is the merge, so this is the non-proliferating cell. They go off, whereas in the uninjured retina, you see the entire inner nuclear layer and the ganglion cell layer has got the let-7 expression.

This is the RNA in situ hybridization. Then comes how hedgehog signaling components such as A, B, Smad, and Patched are regulated. Here, this is basically an MRE mutant where the microRNA recognition element is mutated in this construct, where you have the normal protein. And whereas in these cases the protein levels are downregulated, that is why it becomes so foolproof that its levels are influenced exclusively by the abundance of a microRNA. Based on that, we have come up with this model. So that is why we can see here that the HDAC expression is present everywhere except in the proliferating cell.

Because if HDAC expression is present, even if MYC is present, it will not cause proliferation because Lin28 expression is negatively regulated. And in this model, you can see that this is what we have discussed here: that the Myc-b is negatively regulating the Lin28 expression. Then there's another story, but I will not go into the details. Those who are interested can read that we found another transcript, another tumor suppressor called PTEN that associates with various gene regulatory events that are essential for retina regeneration. So soon after the injury, just like HDAC is going off, the P10 also goes off from the proliferating progenitors.

And this is necessary for activating the AKT. So PTEN, when it is super repressed, like you got rid of a PTEN from various other cells which normally don't have to get rid of because it is not a responsive cell, then you get too much of a proliferative response. So those who are interested can read this article that is published in Glia. We also looked for the role of Tgf-beta signaling in mammals; Tgf-beta signaling is pro-proliferative and anti-proliferative, whereas in zebrafish, Tgf-beta signaling is pro-proliferative. This is something very interesting; those who are interested can read this article, which is cited here.

We also looked at how OCT4 is influencing. Which is a pluripotency factor and how it is

influencing the regenerative response at different phases, such as influencing the epithelial-mesenchymal transition or mesenchymal-to-epithelial transition. We are also looking for these articles that will be published soon, which involve yin yang protein, polycomb repressor protein CTCF, and long noncoding RNA such as MALAT1, how they influence regeneration, how the NURD complex influences regeneration, how type 2 diabetes influences regeneration, and how hippo signaling and Neuregulin KDM pathways influence regeneration. So these are all the angles from which regeneration can be studied. We'll study more about regeneration in the coming classes. Thank you.