Regeneration Biology
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W8L39_Roles of Histone deacetylases in regeneration of vertebrates like fishes and amphibians

Hello everyone, welcome back to another class on regeneration biology. In today's class, we will try to learn some data generated in my lab, and I will explain the roles of histone deacetylases in the regeneration of vertebrates like fish and amphibians. We will mainly touch upon fish fins and amphibians. Tail and limb, so there are also several papers that have been published in this field from our lab and from other labs. In general, there is a discrepancy between the regenerative abilities of mammals and fishes. When it comes to histone deacetylases, in a simple sense, I can tell you that if histone deacetylases are inhibited in zebrafish, it does not favor regeneration.

Whereas in mammals, inhibiting it favors regeneration. So there is a significant difference between day and night. But we shall see. But the mechanism is not fully understood.

All we know is that if you do it, you get the result. But let us try to learn what the mechanism probably acting behind it is through these results. In this picture, you are seeing stages in zebrafish fin and axolotl limb regeneration, which you can find in several textbooks. The procedure is simple: take a zebrafish fin, amputate it, and you will have a wound epidermis. You will start seeing the blastema that is being formed in around two days, and the regenerative outgrowth happens completely.

and same with the limb also you cut it you have a wound epidermis formation just like fin and the innervation is necessary we saw in previous classes already and you have an apical ectodermal cap being formed and underneath there is a blastema that is formed and the new limb patterning happens and the limb gets regenerated fully as you can see in this picture simple but what is the take of or what is the role of Epigenome modifiers or epigenetic modifiers and how they intercalate into the mechanisms of regeneration. We will see them. So after amputation of the zebrafish fin, you dip the fish in tricostatin A, which is a histone deacetylase inhibitor. All families of HDACs will be blocked with tricostatin A. You do not get regeneration.

We know that as long as you keep the fish in TSA, you will not get fin regeneration at

all. But what we did was slightly different. What we did was take a normal fish exposed to trichostatin A. You blocked HDACs and then amputated and did not put TSA or block the HDAC after amputation. We wanted to see what the role is.

One of our earlier studies showed that if you withdraw it in the retina, what we have shown is that if you block the HDACs with the TSA, and after that you withdraw it, the regeneration continues; after, say, if the regeneration should have completed in two weeks, it takes three or four weeks, but regeneration happens. However, what we have seen is that if you block three days prior to amputation, as you can see, that is written as minus three. Minus 3, minus 2, minus 1, and 0 days post-amputation, you amputate it and then put the fish in just water; after collecting at 4 days post-amputation, you compare it to the control in a 4-day post-amputation scenario. This much blastema is supposed to be seen with tricostatin at 0.1 micromolar, 0.

25 micromolar, and 0.5 micromolar. There is an increasing dose concentration, and at the highest dose compared to the control, you get minimal blastema. So what this tells you is that even if you block the HDAC much before the amputation, you do not get an efficient regenerative response. In the same scenario, you get it for axolotl tail and limb; both of them you see it, so the procedure is the same, minus three days to zero days, and then put HFR solution, which is a normal solution in which the axolotl is kept.

So this is the pre-cut scenario, and this is zero DPA, but in this pre-cut scenario, the animal is exposed to TSA. Here it is DMSO, whereas here it is TSA that you should keep in mind, and after 0 DPA to 7 DPA, you are supposed to have this much blastema formed in DMSO, whereas at a 2 micromolar concentration, you do not get proper tail blastema. Same with limb; also, the axolotl procedure is the same: you amputate it, and you are supposed to get this smooth blastema in the stump that is formed. But here in TSA-treated, you get a sharp rudimentary blastema, which is not powerful enough to cause an adequate regenerative response in a given amount of time compared to that of the control. So if you look, we followed it up to 56 days in a normal pre-treatment scenario.

DMSO treated one; you can see pre-cut, cut 8 days, 12 days, 16 days, 20 days, 24 days, 28 days, 32 days, and 56 days. What you are seeing is that after 56 days, the limb is somewhat similar to that of the pre-cut scenario you end up getting. But when you put two different concentrations of TSA, one at one micromolar and another at two micromolar, you see that the blastema formed is very slow and eventually it forms. And the same with the two micromolar as well. Blastema forms slowly, and eventually it develops.

So if you look carefully, say here by around 24 days, the digit formation starts 24 days

post-amputation in the control scenario. Whereas in one micromolar, the same digit formation starts in 28 days. Because on day 24 you don't see anything the same way, at 2 micromolar you see there is still blastema present, and even on day 28 digits haven't formed; by day 32 the digit has formed. So there is a 24-day, 28-day, and 32-day equality that means there is a delay, similar to what you saw in the retina. There is a delay in that the experiment was different in which you injure the retina, put it in TSA, observe no regeneration, and then withdraw the drug.

Here we have done the TSA treatment beforehand, and there is a reason behind it. We wanted to know what changes in the homeostasis of the animal take place that prevent the blastema formation. We are more interested in knowing the role of HDAC inhibition prior to amputation. We want to de-link the role of HDAC in post-amputation scenarios. So that is why we were exposed in a pre-amputated scenario.

So eventually, in a delayed process, the limb can regenerate in around 32 days, but 56 days has been shown because we wanted to have a comparison with the rest of them. By 56 days, they all look the same, but at two micromolar concentration, the regeneration is a slow process now. We looked at what the possible enhancements were, so one of the candidates we considered was based on literature that indicated HDACs have good control over collagen gene expression. Thus, we examined the zebrafish caudal fin, axolotl tail, and axolotl limb for the levels of collagen 4 and also assessed the levels of histone 3 lysine 27 acetylation. Normally, histone deacetylases are supposed to remove the acetyl group from the histone proteins and also various other proteins, but we are interested in the histone proteins, especially the histone 3 lysine 27 residue.

If the acetyl group is there, the chromatin can be very loose and gene expression can occur; if the acetyl group is removed, that will make the lysine moiety vulnerable to methylation, and the chromatin can become very tight in any case. If you block the HDACs, then the acetyl group on this histone will remain; that is what you can see: the acetyl group is very strongly present compared to the DMSO. That is why the increasing concentration of TSA increases the H3K27 acetylation level, and GAPDH is the control. It is interesting to note that the collagen 4 levels are increasing because of TSA pretreatment. Remember, these are all non-amputated, no amputation.

See, this is the regime: minus 3 to 0 days. Although you amputated it, you are not waiting for any regenerative response, so technically, in an uninjured condition, just because of the TSA treatment, you have got increased levels of collagen 4 and increased levels of lysine H3K27. The same thing is seen with axolotl tails and axolotl limbs, so what you learn from here is that the pre-treatment of TSA, or the prior to amputation, if you block the histone deacetylases, you end up having too much collagen 4. We have

discussed that collagens are part of the ECM (extracellular matrix) protein, and these are super abundant. That is what you should understand: the matrix proteins are super abundant in the fin, tail, or limb because histone deacetylase is being blocked.

Thank you. The same thing is confirmed by immunofluorescence levels, not just western zebrafish caudal fin 0 dpa, axolotl tail 0 dpa, and axolotl limb 0 dpa, but they all have received TSA pre-treatment. You can see the green color; collagen 4 expression is very strong at 0.5 and 1 micromolar of TSA. The levels are high, and this is showing the DAPI, which is the nuclear stain, and this is the merge. You can also see in the axolotl tail that there are increased levels of collagen 4, and you can see that this is the DMSO level; this should have been the actual level, but because of the TSA treatment, you have an increase in level.

Similarly, two concentrations of TSA have been tried in the limb, and you are having an increase in the levels of collagen 4 because of TSA treatment or the blocking of HDAC. Now we thought, if collagen deposition is the culprit, if collagen deposition is to be blamed, then what if you get rid of the excess collagen? So, one enzyme that we can use for this purpose is collagenase. So what we did was take these fissures, which are pre-treated. The pretreatment increased the collagen levels, so we also gave the collagenase enzyme in the blastema. Although the blastema forms very little, we gave collagenase enzyme in the blastema, and we could rescue the effect.

You can see that TSA treatment at 0.5 and 1 micromolar results in very low blastema levels, as indicated by the yellow dotted line. If you look, you can see that the blastema levels are very low; however, because of the collagenase treatment at 0.05, 0.5, and 5 milligrams per milliliter, which is the collagen concentration we used, we can see that it rescued the TSA-mediated inhibition.

This should have been the blastema levels, and you could observe this at the highest concentration. In the same way, one micromolar should have been the blastema level, but we could rescue it. An interesting thing to note is that in the normal DMSO1, where there is no collagen deposit, normal collagen is present, but there is no excessive deposit. When you remove the collagen, you see a reduced blastema, so what you understand from here is that collagen is necessary at a threshold level. Too high is also a problem, and too little is also a problem.

It's like, again, I am giving an example of the salt used in food. If you put no salt, food will not taste good. If you put too much salt, the food will not taste good. But there is a threshold, an adjacent level. If there is less salt in the food, adding salt will make it tasty.

If there is more salt in the food, getting rid of it by whatever mechanism will make the food tasty. You should keep the same logic in mind. It's not that collagenase is a favorable enzyme. If collagen is a troublesome molecule, then collagenase is a favorable enzyme. When collagenase is not a troublesome molecule, it will do harm.

So that is what you get from the DMSO treatment. Now we can obtain the same result through collagenase treatment that rescues the TSA phenotype in the limb and also in the tail of the axolotl. So you can see how the DMSO collagenase treatment is reducing the blastema in the DMSO, whereas in TSA, the reduced blastema, as you can see here, is present in the PBS treated without collagenase; you don't have any rescue now, so you get a reduced blastema, but that will bounce back because of collagenase treatment. The same thing we can see in the axolotl tail also. In the PBS, you have reduced blastema formation.

But if you give collagenase, you can rescue the blastema and it will bounce back. So, from this, what you understand is that because of the TSA treatment or the inhibition of histone deacetylases, the excessive collagen is to be blamed for the lack of or the delayed regeneration. So if you can prevent or reduce that excessive collagen by collagenase treatment, then the trouble is alleviated. So this is the information we have gained from here. We then performed an RNA sequencing analysis.

That is, we looked for all the ECM genes. Collagenase is one of the ECM proteins. So, if you look for what the general take of the ECM genes is because of histone deacetylase inhibition, we found that these genes are upregulated, and these are all the downregulated; this is a gene enrichment analysis that has been done, and this whole group is for the extracellular matrix genes. The procedure is the same, minus three to zero days, and on zero days, we have done two things: one is Zero-day RNA-seq and three-day RNA-seq. So what we found is that there is an upregulation of collagen or collagen family extracellular matrix genes because of the TSA treatment.

compared to the DMSO. TSA in general upregulates extracellular matrix genes, and the levels of downregulated genes are lower. Upregulated genes are more prevalent, which belong to the extracellular matrix family. But there are some ECM proteins, which show that if there are 100 ECM proteins in an animal, all 100 out of 100 are upregulated. Some are downregulated, but the majority are upregulated in 3DPA. So we have also done zero DPA, but due to time limitations, we cannot go and analyze all of them.

3 DPA has been used here. Then we created a heat map representation of the RNA-seq data, which shows the collagen and MMP genes. So we looked at what the role of this collagen is if collagen has to be increased. In the heat map, you can see the red color.

There are so many collagen family genes.

Collagen is not just collagen 4. There are also plenty of other collagens. You can see a bunch of collagen genes in 3DPA, which should have been downregulated because of.

.. which favors the regenerative response. But you have seen in the TSA-treated sample that, because of the HDAC inhibition, a large number of collagen family members have gone up in red in color. And concomitantly, we can also consider why collagen genes will increase. Or even if collagen genes go up, we have also shown by Western and immunofluorescence that collagen 4 levels are high. So we thought there is probably a possibility that the degrading molecule, like matrix metalloproteases, may have an opposite trend. So we saw that because of the collagenase, the HDAC inhibition, along with the collagen protein levels going up, their transcription going up, and protein going up, some of the metalloprotease levels are going down.

That means the transcription of collagen is already high, translation is happening, but its degradation is also now prevented because many of the MMP proteins are downregulated. When we looked further into the RNA-seq data to understand another angle, we found that the immunofluorescence data of collagen gave us some idea similar to the excessive deposits of collagen that are creating a huge matrix of extracellular deposition. This may interfere with some cell migration into that site, which may be delayed.

It is not stopping. It is delayed. It's as if you have more hurdles. Then we thought, can this interfere somehow with some immune response because of this TSA treatment or because of HDAC inhibition? To our surprise, what we saw compared to the actual scenario where there is no treatment is a decrease in the immune response genes at that injured site. So, if you pre-treat the animal with TSA, the immune response genes are downregulated. As you can see in this gene expression or gene ontology analysis, this red color indicates a defense response. Response to biotic stimuli and a lot of these highlighted red color genes are downregulated, which occur because of TSA treatment.

We also found something significant, and this has been done both on day 0 and at 3 DPA, so in both scenarios due to TSA treatment or post-injury. We have seen that some of the interesting immune response genes are downregulated; we looked further to see if we can make an extra plot looking for candidate genes. So far, in the previous class, we have learned that pro-inflammatory cytokines are needed soon after injury, which should be taken over by anti-inflammatory mechanisms. Both are needed. So we looked for the levels of pro-inflammatory and anti-inflammatory cytokines.

What we saw because of the TSA treatment in both 0 DPA and 3 DPA is a decrease in the anti-inflammatory. That means the environment remains pro-inflammatory; then we looked at what about pro-inflammatory because of the TSA treatment. We saw there is an increase in the pro-inflammatory gene expression events, as you can see here, so the pro-inflammatory scenario remains for a longer time, and the anti-inflammatory scenario doesn't kick in.

So, it's just like... You are earning less money, but the income increased; also, something like that is what is happening here: anti-inflammatory cytokine levels go down, and pro-inflammatory cytokine levels go up. These together probably could have contributed to the delayed regenerative response. We have also discussed in the previous classes that in mice, in many scenarios where pro-inflammation is favored or facilitated, regeneration is not welcomed or cannot happen effectively. Now we looked into another mechanism that is specifically looked at for the regulatory T cells.

Regulatory T cells are important. One of the next classes we will be discussing is regulatory T cells. But understand that regulatory T cells are one of the earlier granulocytes, which are CD4-positive cells. And they're important; they are very much important to have a normal regenerative response, so we looked at the caudal fin of the zebrafish and examined the level of CD4-positive cells that are FOXP3 positive. FOXP3 positive is an indicator of regulatory T cells; in short form, they are known as Tregs. We saw that in both 0 HPA and 3 HPA, our post-amputation caudal fin levels in TSA treatment, the Tregs, that is, regulatory T cells, are decreasing.

And probably that could account for the lack of or the delayed regeneration because of TSA treatment. And what we looked at further was, of course, we all know that soon after an injury, there has to be a free radical mechanism. There is a reactive oxygen species involved, which is necessary for the attraction of immune cells. So we looked: can we mimic that? Can we mimic this scenario by treating either with TSA or, simply put, LPS? What is LPS? Bacterial lipopolysaccharide. When Bacteria Lipopolysaccharide is deposited at the injury site, pro-inflammation will persist for a longer time because it will mimic an infection.

So immune cells will go there. So what we saw is similar to what you saw in the TSA-treated one micromolar concentration; you see no blastema. In the same way, with LPS treatment, you also see a decrease in the blastema, so the blastema levels are on par with TSA treatment. TSA treatment also prevents the anti-inflammatory environment; it always favors the pro-inflammatory environment, and LPS also favors the pro-inflammatory environment. The same effect is seen with blastema formation, as you can see here at different times; when you go further, you can see the TSA treatment and how

the blastema is low, while the LPS treatment also keeps the blastema low. Here, you can see the same thing if we further add another compound called N-acetylcysteine, which is a free radical scavenger.

So, free radical scavengers basically, whenever there is tissue damage, there will be a lot of reactive oxygen species, and whenever there is LPS treatment, a lot of free radicals will be formed. But if you scavenge them, can the immune system be told not to respond? What we found is that if you prevent the reactive oxygen species from doing their job, Doing its job is what attracts the immune cells and makes the environment pro-inflammatory, so what we did was, as you can see here, NAC plus, minus, plus, minus, plus, minus; that means in each experiment control we have two scenarios: with NAC and without NAC, TSA also with NAC and without NAC, LPS also with NAC and without NAC, but with LPS and NAC, it rescues. In the same way, with TSA and NAC, it rescues, but without NAC, with TSA, and without NAC with LPS, the blastema formed is minimal, so what it tells you is that NAC is acting as a scavenger of free radicals. So it will sequester away the free radicals and allow less immune response at that site, so that the pro-inflammatory environment will not be as robust, which favors the blastema formation. You can also see the collagen deposit, and because of the NAC treatment, the collagen deposit is not going as high as it should have been, because the immune cells play a strong role in the deposition of collagen.

And this NAC rescues and restores the Treg levels as well. You can see DMSO and TSA here in the caudal fin. These are the regulatory T cell levels.

Not much. This is NS, non-significant. But TSA is with NAC. TSA reduces the Treg levels, which we know. But if you put NAC, That means the free radical scavenging happens, which means less immune response, then less collagen; also, the Tregs are more present in the injury site, but NAC alone doesn't do anything because it has to have TSA so that more of the collagen deposit and a more pro-inflammatory environment are present. Then NAC comes to the rescue. In other words, if you are not hungry, whatever food I give will not look tasty to you.

But if you are hungry, even my food is not as tasty. You will find it tasty. In the same way, TSA creates a pro-inflammatory environment that reduces the Treg population migration there, but NAC allows for more regulatory T cells, which restores not only Treg levels but also allows regeneration to continue properly. So in this proposed mechanism, what you are seeing here is the resting state, the unamputated state; you can see acetylation in certain residues. The histone acetylation is present, and what happens is when histones are acetylated, a set of genes will be turned on. A set of genes that are off will not have any significant role in maintaining homeostasis, but if you treat with

tricostatin for three days, acetylation levels will remain strong and high; no deacetylation is happening, and because of this, several pro-inflammatory cytokines and extracellular matrix genes are increasing, as acetylation favors gene expression, which is what you are seeing here.

If you have an uncut scenario, if you expose them to TSA, acetylation levels are high, along with ECM genes and pro-inflammatory cytokines, you have an abundant deposit of ECM proteins, as you can see in this film. Remember, it is uncut. and now you are cutting it when you are cutting it remember there is abundant collagen and other matrix proteins are already there in the fin and the cells are not able to migrate effectively and if you treat them with TSA and if you rescue it with NAC or collagenase then what will happen, you will get the MMP levels and other anti-inflammatory cytokines comes into normalcy and you will end up getting a proper anti-inflammatory response so that there will be a delayed regeneration that you can see it And in normal blastema formation, we can see it. This information tells us how plastic the system is, and we can learn more about this regeneration and the Tregs in the next class. Thank you.