Regeneration Biology
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W4L18_Adult stem cells: Natural and induced and their roles in regeneration-zebrafish

Hello, everyone. Welcome back to another class on regenerative biology. So today we will learn about adult stem cells, both natural and induced, and their roles during regeneration, with a special focus on zebrafish. OK, so adult stem cells, we have seen from the very beginning that every tissue present in an organism should have some amount of stem cells. However, their load is going to dwindle as you progress. As the organism ages, the levels of these adult stem cells start dwindling, and this dwindling eventually leads to signs of old age, such as wrinkles in the skin and the tissue starting to show signs of compromised functioning.

And eventually, the organ will not be repaired or restored as it should have been or as it is being done during youth. So this is what happens when the stock of stem cells or adult stem cells dwindle. And adult stem cells are indeed present. Like you get a new nail that has grown.

If your nail gets damaged, you grow a new hair. If your hair gets damaged. Fallen off, and you get new skin that is formed after a minor cut or a small burn injury, etc. All credit goes to your stem cells, and every organ has some representation of stem cells, which we call adult stem cells. In the intestine, we have also seen and discussed this; those who have forgotten can refer back to the old lectures, where you can see that.

So adult stem cells, one of the well-studied groups, are in hematopoiesis. Stem cells in various tissues are essential for tissue turnover and homeostasis. And we know our bone marrow, inside the bone, that is called bone marrow, has dedicated stem cells that are meant for producing mainly WBCs and RBCs. Of course, it has calcium-producing cells and fat-storing cells as well. Aging has both quantitative and qualitative effects on stem cells; considering the balance, the qualitative changes of stem cells are more important because they determine whether stem cells can produce all cell types as they should.

For example, a given stem cell producing four types of cells in a ratio of 25, 25, 25, and 25 percent is optimal. Say that in old age, one of them goes higher, and one of them goes lower; then the balance is affected. So it can lead to some complications, which do occur. So, not only do you have the ability to produce, but you should also have the ability to

produce adequately or sufficiently. Say you are cooking food.

Normally, you take one cup of rice, and if you are eating with some curd or something, you will put in a pinch of salt. What if you make it the other way around? One pinch of rice and one cup of salt. You are putting in the same amount of curd. Will it taste good? Definitely not. So the combination is also important.

Not that mere presence. Sometimes if the combination has to be 1 to 1 to 1 to 1, it should be maintained. You cannot go that 2 to 1 to 1. Or 2 is to 1 is to 1 is to 0. It can't happen that way.

So this balance is very important for the normal functioning of many cell types. So again, the balance also depends on the availability of stem cells. What is the load of stem cells that is present? So the self-renewal potential of the adult stem cell is also very important, and it has developmental potential. That means the ability to give rise to a particular cell type from this stem cell and its interaction with extrinsic signals. For example, hematopoiesis is generally maintained at normal and reduced performance when old stem cells are subjected to stress.

If the stem cells are also getting equally old as the individual, and if you apply some stress—whether it be chemical stress, physical stress, or any other type of stress—then the cell will not produce as it should. The balance will be tilted. Let us see stem cell turnover in mouse tissues. You can see hematopoietic cells that are in a dormant state, and this includes hematocytes and epidermis, which is ear epidermis, hematopoietic cells when they are active, male germ cells, and small intestine epidermis. The epidermis first appears in the tail, then in the palm, and then in the colon and esophagus.

You know these tissues do have their stem cells, but when you talk about turnover, you see the time on the x-axis, which is mentioned in the number of days: 20, 40, 60, 80, 100, 120, 140, 160, and so on. As you grow older, even the oldest mice, around 140 or 160 days old, are considered really old mice. The turnover is present in hematopoietic cells, whereas around 100 days, the hepatocyte turnover is compromised by about 20-30 days; the epidermis, which means one month old, shows a decreasing turnover, and you can see some of them in the esophagus, colon, etc. In the very early stage, the average cycle of stem cells is getting shorter, so you can say that as the organism grows older, the turnover of the cells is not holding up as it was in the beginning, at around the zero stage or maybe the first few days. This is the first five days; the turnover is more or less equal for all cell types, meaning all are very vigorous and quite vigilant.

As you grow old, around every 100 to 140 days, the hepatocytes disappear; this means

that the turnover is mainly for the hematopoietic stem cells. As the organism grows older, the representation of stem cells, which is reflected in the form of turnover, gets compromised, so this is a fact. The diminished functional capacity of hematopoietic stem cells during aging is a fact. Now let us see this picture in cartoon form. The red color represents young active stem cells, and the purple color represents quiescent stem cells.

And this one circle with a cut across is an apoptotic stem cell. So in a normal scenario, at a young age, you can see that four of them are young and active. Only two of them are quiescent, and only one is an apoptotic cell. And they are differentiating. This blue color one is becoming either lymphoid identity or myeloid identity if it is a hematopoietic stem cell.

As you grow old, you see that this apoptotic cell has doubled, and quite a few of them have already undergone old active stem cell. That number is increasingly active, but they are very old. And you can also see that the number of quiescent stem cells remains more or less the same. And the differentiation is happening now. But when you give stress, what happens is that the apoptotic stem cell increases.

The number one, which was young, has now become four. That means four times increase. And although the other type of cells is maintained, the stress in old age causes a dramatic change in the survival of these young animals to middle age and old age, which, combined with the stress, creates a lot of havoc. And you can also see the restriction in the developmental potency of stem cells during the aging process. Now restriction basically means that during the developmental stage, what happens to the potency of the stem cell? What affects the stem cells? Of course, we all know that the inner cell mass of the blastula that gives rise to an embryonic stem cell, or at the very beginning stage, the zygote, is nothing but a totipotent cell; it can give rise to an entire organism.

As you can see here, embryonic stem cell lines derived from the inner cell mass retain totipotency and can be maintained in culture, but if one individual cell is placed in the uterus in the right environment, it can give rise to a An entire organism so that we call it a totipotent cell, and this ground cell that you are growing coexists to give rise to other cell types, let's say hepatocyte, skeletal, endothelial, neuronal, and whatnot; you can give rise to any cell type, and hematopoietic stem cells can also give rise to their routine lymphoid and myeloid lineages, but this can be. Tweaked by some given conditions, you can also use what is called a trans-differentiation hematopoietic stem cell that should give rise to lymphoid or myeloid lineages, but now we can twist the arm or force this stem cell to give rise to hepatocytes, skeletal, endothelial, or neuronal cell types as well. However, if it grows old, like you can see here in this graph, this sharp end shows it is the youngest, and as you grow wider, it is the oldest. As the aging continues, what happens is the

hematopoietic stem cells are differentiating, but you may see this is a thin arrow; this is a thick arrow. What does it mean? Hematopoietic stem cells usually.

.. Used to give rise to lymphoid and myeloid lineages equally. Now the lymphoid lineage is minimized and the myeloid lineage is more pronounced. As I mentioned at the beginning of the lecture, 1 is to 1, or equal, or the proportion that is affected. Same hematopoietic stem cell; it was giving equal lymphoid and equal myeloid, but now it is becoming less lymphoid and more myeloid. And the most interesting thing is that they are not vulnerable to transdifferentiation, which means their plasticity is reduced.

Same hematopoietic stem cell, just because the cell was older. It is not able to transdifferentiate; whatever you do, that means in young age, these stem cells do not retain the potency to produce a new individual, but they can give rise to any new cell type. In young age, however, differentiation of the hematopoietic lineage is skewed. Toward differentiation at the expense of lymphopoiesis means that it is forcing more differentiation in old age because it does not want to maintain a sturdy population of itself, does not make a copy of itself, and is now waiting for a chance to just finish off the job. For example, small kids, when they are working, will do so very meticulously; they will not do.

Like they enjoy doing it. But once it became a routine thing, one mundane thing, you just wanted to see the end point. You just want to finish it. That is what these cells do as well. They just want to differentiate and finish off the job. And do not explore this trans-differentiation strategy, even if you pick them out and try to culture and force them.

They just have a laid-back attitude toward those cells. Under certain experimental and possibly physiological conditions, stem cells in young bone marrow have expanded developmental potential. That's what has been mentioned here: to produce several other cell types. Although it is not the purpose of this hematopoietic stem cell, they have vulnerability or potential. So it has been measured in old age that vulnerability or maneuverability is lost.

And also do not give rise to lymphoid or myeloid lineage. Properly, and now if you see further what the stem cell response to stress is, you can see in this picture that it's an intact tissue and a wounded tissue, and how it is responding. We have seen how wound healing, etc., happens and how it is responding to the wound. You can see wound repair in the esophageal epithelium; that is what you are seeing here following wounding the progenitor cells next to the wound's immediate neighborhood.

Exit the cell cycle that is marked as W; here you can see the exit of the cell cycle and the

migration towards the defect in the yellow area, as shown in this picture. Behind this migrating front, cycling progenitors undergo divisions heavily biased towards self-duplication; they make a copy of themselves because you need raw material to fill the gap. Cycling progenitors undergo divisions heavily. Biased towards self-duplication, which is shown in the green areas you can see here, and expanding the progenitor compartment to generate the excess cells required to repair the epithelium. This is what has been shown in cartoon form in this picture.

Then, what happens in the B wound repair epidermis? How does it follow? As you can see here, the thickness has increased in this epidermis, and in the epidermis, the progenitor cells change their behavior as Shown in the esophagus, the wound repair is supported by the flux of cells into the epidermis from hair follicles, which are the neighboring cells indicated by the purple arrows. This has been demonstrated in the case of skin epidermis and the tail skin. The mobilization of quiescent cells in the epidermis and the migration explains the appearance of radial clones, which means they'll come in a circular fashion around a healed wound. Lineage tracing is an experiment where people mark a location and then observe where it ends up; this is called a lineage tracing experiment in the simplistic sense. For example, if you put an agarbatti in the house, the smoke spreads, and wherever that smoke goes, you can detect its fragrance.

While smoke is opaque, you can observe its movement, but... If that smoke were not there, you would not be able to watch.

So that smoke is now acting as a tracer. So this is a simplistic example to show how cells are moving across in the animal. And the same logic applies if you have any ablation and repopulation experiments that have been done in various tissue types. Following ablation, stem cells in the bulge, as you can see here, are projected where you have done the ablation wherever you like. This is an ablated area, and now the stem cells have to come from here, migrate, colonize, and fill the gap. That is what the restoration of normalcy has happened, but the contribution of individual cells from the neighborhood is very important.

If these cells are stressed, then this orchestration or organization is affected badly. Those who are interested can read the article that is cited here. So now we will move on to another very interesting organism called the zebrafish. Zebrafish is extensively used to study as a model organism because it has several advantages. One reason is that it has external fertilization.

Transparent embryos can be injected with RNA, DNA, protein, etc. And it is amenable for large-scale genetic screening, forward genetic screening, and reverse genetic

screening. You can do it, and you can study the mutants. And the humans and zebrafish share extensive genomic homology and synteny. Synteny is the order in which the genes are arranged on a chromosome.

Genes are arranged in the chromosome, and zebrafish can regenerate very complex organs, such as the central nervous system, which makes them a very attractive model. This fish is a typical aquarium fish present in many pet shops. If you look at the brief structure of the zebrafish retina, you can see the zebrafish eye in this cartoon. You can see that this yellow color is in the retina.

This blue color is bluish. The blue color is the lens. And this purple color in the front is the cornea. You can see there are multiple layers: the outer nuclear layer, the inner nuclear layer, and the ganglion cell layer that are present. You can see rods and cones, which are basically the photoreceptor layer, and behind the photoreceptor layer, you have the pigment epithelium, which is black in color and attracts light. In the middle, the most important cell that contributes to retinal function and regeneration is the Müller glial cell, which you can see here; it wraps around every neuron and provides structural and functional support.

This is very important for the biology of the retina and its function. So, if you see the retina, you need to have a proper injury model. Like if you want to study regeneration, one should know how to injure the retina. So we use something called the needle poke method. What is the needle-poke method? You take a 30 gauge needle and poke from the back of the eye, as you can see here.

In the back of the eye, when you poke it, it creates a hole that uniformly injures the entire eye, making it easy to perform focal injury so the undamaged neighboring tissue can act as a control. Say you have damaged here, so you can see here, or here, or here, or here; anywhere you can see as a control. That means you do not have to study the eye of another fish or the eye of the same fish to study the regenerative response. You do not have to get another tissue.

That is the beauty of focal injuries. Although people use other methods such as chemical methods, photo bleaching, etc. But you don't have to go behind those methods because they are not uniformly damaging. Light is damaging more of the photoreceptor layers. Sometimes this chemical treatment damages more of the ganglion cell layer. Whereas the injury model damages all layers equally, So this is a retina regeneration cartoon in which you can see a normal retina, and you have a stab wound.

These Müller glial cells enter the cell cycle, migrate into various retinal layers, and

restore normalcy. So what are these spatial and temporal factors, and genetic and epigenetic factors controlling these events? That is a very interesting area of research; you can see here that this Müller glia de-differentiate and give rise to pluripotent progenitor cells, and they can give rise to the Müller glia itself, which gives rise to bipolar cells, horizontal cells, ganglion cells, rod cells, cone cells, and amacrine cells. So this is how regeneration is restored; there is no presence of dedicated stem cells in the retina, only the existing Müller glia. De-differentiate to give rise to all the other retinal neurons and the Müller glia itself. Now, the most interesting thing is how it is doing, and how it is able to do that.

This is something very exciting after so many years. Like you have seen here, you have got the Muller glia, and it gave rise to a de-differentiated population of cells. Now, who will tell you that you need to have only this many numbers? Say you have 1,000 Müller glia in the retina. How many de-differentiated cells are formed in the eyes? How many of them are capable of giving rise to a specific number? Who decides? Of course, we can identify some specific factors. Transcription factors are important. You would have also heard about iPSC-induced pluripotent stem cells.

However, in the retina, no one is there to transfect, nor is anyone there to deliver this protein. Incorporate these factors into the Müller glia so the induction has to happen ingeniously or indigenously within the cell, and it cannot go too far; if its expression is too high, then it can become overly hyperproliferative, leading to reactive gliosis or even tumor formation. This number is fixed around 30% that undergoes apoptosis at a later stage, but it never overproduces; it produces an adequate number and gives rise to the same set of retinal neurons. It won't happen that there is too much abundance of bipolar cells or too much abundance of rods.

The same proportion is maintained. And this happens because of the help of many pluripotency factors that are induced during retinal regeneration. This is the qPCR data of the retina, which was taken from one of my previous papers. Those who are interested can read this article. And this graph shows various times post-injury: 0 hours, 15 hours, 2 days, 4 days, 6 days, and 8 days.

At this time point, this last point is 7 days, not 8 days. And that is why the graph is ending here. You can see LIN28, cMYC, SOX2, NANOG, KLF4, and OCT4; these are pluripotency factors. Another important factor is ASCL1A. So there are six pluripotency factors, which include both Thomson and Yamanaka factors.

They are induced in this retina. At various times post-retinal injury. Some of them say you can see that the cMYC B level is very high within 15 hours, whereas some of them

are induced very high at around 2 DPA and 4 DPA, but the take-home message is that they are induced significantly in the eye. Now these are able to reprogram the Muller glia. Muller glia is losing its identity. It is now becoming spindle-shaped or spherical or spheroid-shaped cells that are stem cells.

So each of these pluripotency factors has the ability to reprogram the genome of the Muller glia, and it can give rise to a specific proliferating group of stem cells that are able to differentiate, but you should also understand their level has to come down. As you can see here, two days and four days are very important because, in a needle poke injury, four days is the peak of proliferation, which means The stem cells or this progenitor cell, retinal progenitor cells derived from the Muller glia, are maximally expressed at 4 dpa. But see, interestingly, by around 7 days, when the proliferation has come to a minimal level, the levels of these genes are decreasing and dwindling.

I agree that some of them are staying behind. cMYC levels stay high. ASCL1A levels still stay high. which is not a pluripotency factor, but it is a pro-neural gene that pushes the stem cell into the neuronal lineage and is also an important gene required for the formation of an organ called the pituitary. All of you have heard about this pituitary gland. And here, the Lin 28 level also stays high. In this paper, if you read it, you will know how ASCL1A regulates Lin 28, which is a pluripotency factor, and CMIC also remains high.

But there are others like Oct4, SOX2, or Nanog; their levels come down. So what you understand from here is that the induction of pluripotency factors is essential for the so-called de-differentiability of the Müller glial cell. And that is able to give rise to all the retinal neurons. Now, the question is: are they pluripotent? We are saying, yes, they are able to give rise to the retinal neurons and the glia, including Müller glia itself. But do we have any evidence for that? What we did was make use of a transgenic line.

What is the transgenic line? One line is a platelet. GFAP, which is a glial fibrillary acidic protein, is driving GFP. And another line is called a truncated tubulin promoter line. What is the difference between them? The GFAP is turned on in every Müller glia. So you can easily sort them. Every Müller glia will express this GFP under the control of glial fibrillary acidic protein, which is a cytoskeletal protein of the glial cell.

Whereas TUBA1 is also part of the cytoskeleton since it is truncated, it will not be expressed in every neuron or every glia. It rather expresses in those cells which have some proliferative capacity. So if you injure this tuba GFP line, you will end up getting proliferating GFP expression only in the proliferating cells; they will have BRDU also, but they will also have the GFP, so you can make use of these lines to sort them. As you

can see here, this is a cell sorting, fluorescent activated cell sorting done, and in this window, what you are seeing is Muller glia cells. In this window, the middle one is a 2 bar GFP, but no injury is given; hence, no cells are represented here.

Injury is given for DPI; you can see in this window the. A number of cells that you can see here; these cells can be picked, and you can do whatever experiment you want. You can do RNA sequencing, you can do a microarray, whatever experiment you want to do. But what we did—those who are interested can read my other paper, which was published a few years ago. You can read this paper to know more details about this. What we did was pick the cells and think, can we put them into some embryonic stages or a developing embryo? If they are able to give rise to only one retinal cell type, they will not get incorporated during development.

But if they are able to give rise to other non-retinal or non-eye-related genes, then they will be able to give rise to some other tissue type, or at least they can participate in embryogenesis. To test this, we took these cells and delivered them. The Muller glia-derived progenitors can participate in embryogenesis or not. That was the question. So what we did was take these cells, as you can see here in this group, in this window, this R3 window, not from here because these are quiescent Müller glia; that is, normal retinal Müller glia.

They are not activated; rather, they are not proliferating. They have GFP expression as a steady-state feature because GFAP is a housekeeping gene of the Müller glia. But these ones are activated. Mueller means they are proliferating, or rather, they have all pluripotency-inducing factors, as I saw in the previous graph. They are now being picked. That means from this window, these cells are picked and delivered into a normal embryo, which is a normal embryo.

Zebrafish embryos that are developing in the later stages, roughly around six to seven hours of development post-fertilization, were delivered when we allowed them to grow. We have seen that they are contributing, as you can see in this picture; they can give rise to... Every cell type—we haven't characterized them—but I just want to tell you these are muscles, as well as epidermal and various other cell types.

As you can see here, these green parts that you are seeing are nothing but these retinal progenitors. Muller glia-derived retinal progenitors from the retina are incorporated into the yolk, and this is the growing embryo. Incorporated, it was able to colonize, and this is a five-day, four-day-old embryo, four days post-fertilization; it's 96 hours after which you could see these green-expressing cells that are present, and they are contributing to embryogenesis. This is something interesting that shows from the retina you can get real

pluripotent stem cells. So we'll continue with regenerative biology in another class. Thank you.