Regeneration Biology
Rajesh Ramachandran
Department of Biological Sciences
IISER Mohali
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W11L56_The basics of regeneration put into practice in vitro

Hello, everyone. Welcome back to another class on regenerative biology. In today's class, we will learn about the basics of regeneration put into practice in vitro. So what developments have we made? What are the pros and cons we encounter when addressing regeneration in an in vitro laboratory setting? So we know we have many animal models of regeneration; we have been practicing or understanding their regenerative approaches extensively, and it is a very fascinating biological phenomenon that has continued to intrigue human beings and humanity for centuries. Many researchers have started working on it, but you cannot call it research. You decapitate the snail's head and the head will grow back or cut the planaria into pieces.

The lost head will come. Those kinds of research have intrigued people. By many researchers, laypeople, and others, it has also been observed that the lizard's tail is growing, but no one can comprehend, even today, its exact depth. If you understood each and every step of it, then you would be able to simulate the same thing in humans or any other mammal.

We haven't reached that stage yet, although the knowledge is piling up and being built. The study of regeneration promises to inform how adult tissue heals and rebuilds itself so that this process may someday be stimulated in a clinical setting. That is the overall idea and goal of studying so-called regenerative biology. Although mammals are limited in their ability to regenerate, both closely and distantly related species can perform astonishing regenerative feats. There are many examples, although mammals cannot be found among them.

Some examples, such as spiny mice, can regenerate their skin naturally, and many regeneration studies are also conducted. in that model as well. So many animals representing almost all phyla harness an innate ability to rebuild the adult structures lost due to injury. Replacing the missing structure can be a perfect substitute for running the show, like, you know, a stopgap arrangement. You can see it both ways.

However, it is unclear which aspects of regeneration are conserved and which are unique to a given context. For example, if you think zebrafish can regenerate but Japanese

medaka cannot, you are mistaken, as Japanese medaka is also a bony fish and an aquatic animal. So we cannot assume that if it is a fish, it can regenerate. In the same way, the mammal Mus musculus cannot, but Acomys species, spiny mice, can regenerate to a reasonable extent. So it varies from individual to individual and among all individuals.

What I mean is individual species in all other scenarios. Every organism can regenerate its liver, and it can regenerate its RBCs because blood cells and WBCs are all limited in their capacities. Their lifespan is limited. So, if you cannot regenerate your blood, you are dead. Or, if you cannot regenerate your damaged liver, you will die.

So, every organism has RBCs. a reasonable regenerative capacity, but it varies. Not every organism has a lifespan of 120 days for its RBCs. In humans, it is 120 days; whereas in other animals, it varies. So one aspect of regeneration that appears to be shared is the use of stem and progenitor cells to replace missing tissues.

No animal can directly make differentiated tissue from pre-existing differentiated tissue. Say one RBC cannot be formed from another RBC. You need to have your hematopoietic stem cells, or the so-called progenitor cells. While many animals regenerate, we limit our discussion mainly to amphibians, zebrafish, and planarians because they are well-studied bilaterally symmetrical organisms that demonstrate bilateral symmetry. And they invoke the so-called cell proliferation in response to injury.

Say Hydra has a morphallaxis that rearranges its cells. And form a miniature hydra, which then gives rise to it; it will continue growing by cell proliferation. Whereas other animals form the so-called blastema, which is created by cell proliferation and gives rise to the damaged tissue, humans do not. Except for planarians, the cellular source of regeneration remains mysterious in the sense that it regenerates; we know that it forms from the reprogramming of existing cells, but there is also the possibility, as we discussed, that stem cells from other tissues are moving to different tissues and then fixing them. These are all possibilities, but in a well-regenerating organism such as the zebrafish, this remains unclear.

The differentiated tissues, such as heart tissue and cardiomyocytes, reprogram and give rise to stem cells; in the retina, the Müller glia reprogram and give rise to stem cells. Similarly, in planaria, each tissue present in its limb or tail is reprogramming and giving rise to each type of cell. We know that, but Planarians have a dedicated system in which around 30 percent of their body is capable of regenerating constantly, and the reason we discussed is that every organ in planaria is short-lived, somewhat like our blood. Our blood is constantly revamped because none of the blood cells present in your body are more than 120 days old; the oldest blood in your body is 120 days old and will die today

or tomorrow. And it will start forming new cells soon.

So we should understand that none of the organs present in planaria are old. It keeps revamping now and then. Are stem cells that rebuild missing tissues present before the injury, or are they generated during the injury response? One question one can ask is this: If they are generated de novo, where do they come from? Are they coming from the site of the injury, or are they coming from somewhere else? Another question one can ask is. Can regenerative stem cells give rise to all missing cell types, or are multiple lineage-restricted stem cells required? This is another question that we can ask. And the current studies on animal regeneration are intensely focused on answering these central questions.

Some animals we know, such as zebrafish and axolotls, are known for their ability to form stem cells. But in other animals where we know of some regenerative abilities, we always wonder where these stem cells come from. Let us see a few facts about an adult animal to replace the missing structures with an exact copy of what is missing. It is clear that the developmental programs must be redeployed because organs are formed during development. So, if the same organ is to be formed, we must understand that it should rekindle the regenerative response.

It is only possible through the developmental program or the embryonic approach. However, the dynamics of cell communication and proliferation are vastly different, and as the cell types involved depend on which tissue or organ you are creating, they will change accordingly. To accomplish regeneration, adult animals may invoke the proliferation of differentiated cells, the activation of reserve stem cells, the formation of new stem cells with limited capacity for self-renewal, or a combination of these strategies. Apply any of these approaches. Let us see a few more facts.

Which cells in an adult animal divide and differentiate to replace the various cell types required during the regenerative response? So, what is that starting cell type? While this is a basic fundamental question formulated and reformulated through successive generations of biologists, its resiliency against experimental attacks has proven to be surprising and, in many cases, quite frustrating. Although we see something—regeneration—we are not able to come up with a concrete set of events that trigger this whole process. Nonetheless, we should understand that different tissues, both within the same organism and among the tissues of different organisms, use different strategies to achieve tissue repair and regeneration. Not that there is a Swiss Army knife approach. There are no standard operating procedures for each tissue type.

It varies from tissue to tissue and from organ to organ. For example, the vertebrate liver

undergoes compensatory regeneration after the removal of two liver lobes. whereby the remaining lobe proliferates to acquire the original tissue mass without replacing the missing lobes. So it is called compensatory regeneration. So regeneration can be compensatory in the case of the liver, tissue-specific in the case of the heart and skeletal muscle, and organ-specific in the case of the pancreas, lens, retina, etc.

Or it can rebuild complex structures containing multiple tissue types or organ types, such as limbs, fins, and tails. So species vary; organs vary. Accordingly, it can have a different way of approach for implementing the overall regenerative response. Now let us quickly examine the dedifferentiation process. So the term de-differentiation is often employed in the regeneration literature to describe the loss of differentiated characteristics of cells after amputation or injury and their concurrent acquisition of an undifferentiated morphology during blastema formation.

So, in a nutshell, an existing cell going back in time means it is taking a few steps back so that it can lose its existing differentiated character and become de-differentiated, allowing it not necessarily to return through the same route and give rise to the cell type from which the de-differentiated cell originated, but rather to give rise to another cell type. So the differentiation process could be the release of living cells from the confines of their previous organization, with the accompanying active mitosis of the cells; in a simple way, it can define how they move. a way moving in a different direction from this existing differentiated identity. However, the term de-differentiation is easily misinterpreted as implying that a cell has attained a multipotent, undifferentiated state. That is not always correct because there is currently insufficient evidence to suggest or rule out a major role for reserving stem or progenitor cells in urodial blastema formation.

The term de-differentiation, as originally coined, may therefore refer to a reversal of the differentiated state—just a reversal. Sometimes it may still have the differentiated cell-specific proteins, but the levels may be low. in the de-differentiated state. An activation of reser, stem, cell, or progenitor cell status, or a combination of both. It, as a differentiated cell, does not have any progenitor genes turned on.

It has many tissue-specific genes that are turned on. So the tissue-specific genes will be turned down, not off; turned down, and it will turn on a lot of progenitor-specific genes, and both of these gene types may stay together, and eventually, the progenitor-specific genes may pick up, and the differentiated cells may completely shut off. Then one can claim, "Oh, this progenitor has completely moved away from its differentiated status." What is the differentiation potential of the cells present in the blastema? Let us quickly look at this picture. The blastema may be composed of cells that are restricted to give rise to the same tissues from which it was derived.

The cells that were multipotent gave rise to different tissue types. A complex mix of cells with a variety of origins and potentials exists. These are all the possibilities. In the larval tail of the urodele, whether it is a salamander, axolotl, or anything else, fluorescently tagged glial cells from the spinal cord proliferate during regeneration and can give rise to tissues outside the spinal cord. Originating from the spinal cord, it has a tag.

Now it is not giving rise to just a spinal cord; it can give rise to others as well. Data currently indicate that, at least in larval tissues, the urodele blastema contains a complex mixture of lineage-restricted and multipotent cells. They are present. However, very little is known about the potential of blastema cells in adult urodeles. In anuran amphibians, some examples we have studied include the bones marked with GFP, etc.

In the previous lectures, you could revisit them. GFP-positive grafts into GFP-negative hosts illustrate that the cells of the regenerating spinal cord and notochord are derived from the cells of those very same tissues in adults. Bone also gives rise to only bone-derived stem cells that generate new bone; it doesn't give rise to other cell types. However, in larvae, it can be different; larvae can give rise to a mix of other tissues. Also, spinal cord-derived cells can give rise to other cell types.

Therefore, The progenitor cells during anurian tadpole regeneration appear to be restricted in their potential, as you can see here with blastema cells; once they are marked, as shown in this picture, you can see that they specifically give rise to that particular cell type from which they originated, but in panels B and C, we can see in the next panel. So, B is a multinucleated myofiber. How do myofibers react? In panel B, the injection of dye into multinucleated muscle fibers prior to amputation illustrates that once clipped or injured, some of these fibers can fragment into proliferating mononuclear cells that contribute to blastema formation. The long-term fate of these cells is still under active investigation. We can see here that they have clipped it and mononucleated the cells because they are already tagged, and you can see how they are migrating.

And in C, the muscle fibers in adult nudes contain satellite-like cells that express PAX7 genes, which are seen in green. And they are separated from the rest of the cell by the basement membrane, which is shown in red. And right in the middle here, you can see that both are present. When these cells are isolated and cultured, they are already tagged red and implanted into the regenerating new limbs. They contribute to the blastema and give rise to unrelated tissue types, including cartilage and epidermis.

This is also one that has to be kept in mind in adults; although it is restricted in a given

tissue, it can be restricted in some other tissues, and it can also move to another lineage through some kind of transdifferentiation. Now let us see the mitral study. Do we have any examples? Muscle cell culture experiments focus on newt A1 and mouse C2C12 cells. Okay, there are two different cell lines. A1 cells from the newt and C2C12 cells from the mouse.

Both mononucleated cells, they can be induced to fuse and form myotubes during differentiation. They are individual cells. They will fuse and form myotubes if you provide the right cues. In culture, upon serum deprivation, one approach is to use serum-free conditions.

They will form muscle fibers. These experiments uncovered a key difference between the newt and mammalian in vitro-derived myotubes. Both are myotubes. Both are cells. Although the names are different, both are biologically muscle precursors. What is the difference? Newt A1 myotubes will enter the S phase when exposed to high levels of serum.

S phase refers to DNA replication and lower levels of thrombin-treated serum. A1 that is from the newt, the frog, or the salamander. At the same time, the mouse, which is a mammal, shows that C2C12 cells do not respond, indicating the presence of an active signal in animal serum that leads to the proliferation of A1 myotube nuclei. S phase means proliferation, and whereas C12 cells don't respond, newts can respond to something that is present in the serum, and they enter proliferation.

However, let us note this further. It should be noted that the extent of differentiation and, therefore, the relative developmental starting points for the two cell types remain unclear. What made them different? Although functionally, they eventually become myotubes. For example, newt A1 cells or these precursor cells of the myotubes do not exhibit striation or peripheral alignment of the nucleus, as when the syncytial nucleus is formed in myofibers or myotubes. In addition, in vivo urodial amphibian muscles contain satellite-like cells that express PAX7, which is a marker, and are separated from the myofibers by a basement membrane.

So this is what you see in vitro and in vivo. Let us see further. Forced expression of another gene, MSX1, a homeodomain protein that is a non-repressor and has repressor function in C2C12 myotubes, causes a small fraction, around 5% of the cells, to fragment into proliferating mononuclear cells. This has been seen. Under proper culture conditions, these cells can differentiate into other cell types, such as adipocytes, chondrocytes, myocytes, and osteocytes. They give rise to different types of tissues, such as fat, cartilage, muscle, and bone.

Because the C2C12 cells are multipotent, these results should be treated with caution. Nonetheless, this was a key demonstration that mammalian myofibers can be induced to reverse their differentiated state. That is what the whole point of it is. Complementary studies were also carried out in nudes in which the primary larval lymph myofibers normally fragment upon dissociation, and they are inhibited from doing so through MSX knockdown. If you don't allow MSX to be there, then the blastema formation will be jeopardized.

Given the caveats mentioned above for cultured cells, the data argue that MSX1 may be necessary and sufficient for differentiated muscle to fragment into proliferating mononuclear cells, which are necessary for blastema formation. To the contrary, in vivo morpholino-mediated knockdown of MSX1 in individual larval axolotl tail muscle cells has had no negative effects on the ability of these animals to regenerate. This shows an opposite result. That means that even if MSX is missing, some other genes are able to contribute to this fragmentation process. This discrepancy has several potential explanations, including but not limited to the differences that likely exist between in vitro and in vivo conditions, because in vitro petri dish conditions are different from live animal conditions, and the muscle cells of the limb and tail could exhibit a differential requirement for MSX1 expression during fragmentation.

During fragmentation, MSX dependency can vary depending on the species and the tissue being discussed. Let us see a cardiac example. In implantation experiments, cells isolated from the newt heart, cardiomyocytes, commonly called CMs, were tagged and implanted into unamputated or amputated limbs. I took cells from the heart and placed them in the limbs. While the cardiomyocytes implanted into unamputated limbs were stable and exhibited no special behavior, they were activated when implanted into day-5 regenerating limb blastemas, and 65% gave rise to skeletal myotubes.

Means from the heart; it became a skeletal muscle. At the same time, if you express the cartilage cell marker, this is clear evidence of the plasticity of the differentiated state in adult urodele amphibians. Still, the magnitude of the change in fate is unclear because the transplanted cardiomyocytes continue to express desmin, which is a marker found in many types of muscle cells. So, while these experiments do not rule out the role of reserve stem cell progenitor cells, they clearly illustrate that a large fraction of isolated newt cardiomyocytes can at least switch between muscle cell types. Heart to skeletal muscle may sound small, but it is very large when you think about it as a cell type. Proliferating cells derived from satellite-like precursors were isolated in culture from adult newt myofiber explants.

This is another set of experiments done on salamanders. When tagged and implanted into regenerating adult limbs, these cells contributed to the blastema, and many appeared to switch lineages to cartilage and even to epidermal cells. On the contrary, another group of researchers found that implanted primary myofibers from juvenile axolotls can fragment and proliferate without even the satellite cells being present. Implanted primary myofibers from juvenile axolotls can fragment and proliferate even without the inclusion of satellite cells. It is possible that this disparity or the variation in results can be explained by the differences in the species and the life cycle stage, whether they are juvenile or adult, or by the different criteria used to assess whether the satellite cells were present or not; that is the question that was asked.

It is also possible that it remains unanswered. It is to be observed that both myofiber fragmentation and satellite cell proliferation may contribute to the blastema in vivo. In reality, that is what is observed. And their relative contribution may be in an age-, species-, or tissue-specific manner. It can vary. Now, if you see an overview, in vitro animal regeneration involves studying and replicating regeneration processes outside of living organisms, typically in a laboratory setting.

That is why in vitro studies have to be monitored and validated in real life under in vivo conditions. This approach allows researchers to isolate specific factors and mechanisms involved in regeneration and to manipulate them under controlled conditions. The basics of animal regeneration, such as stem cell activation, de-differentiation, and trans-differentiation, can be explored in vitro much more effectively than in vivo using techniques like cell culture, organoid models, and tissue engineering. That is why the in vitro approach is very fascinating. To elaborate further, many animals utilize stem cells to regenerate damaged tissues through stem cell activation and differentiation.

In vitro, researchers can study how these stem cells are activated, proliferate, and differentiate into new cell types that are needed for regeneration. For example, they might investigate how certain growth factors or signaling molecules promote stem cell activation and differentiation in a dish so that these can be used in organ culture. De-differentiation and trans-differentiation. Some animal species can regenerate by inducing cells to lose their specialized characteristics; this process is called de-differentiation, and then they differentiate into new cell types through transdifferentiation. In vitro, researchers can examine the factors that trigger these processes, such as changes in gene expression and exposure to specific chemicals.

That is the beauty of in vitro studies. Tissue and organ regeneration. In vitro, researchers can also attempt to regenerate entire tissues or even small organs. This involves creating a 3D environment like an organoid that mimics tissue structures and provides the

necessary signals for cell proliferation and differentiation. For example, researchers might use some cells to build components such as a small intestine, lung, or muscle fiber. And in in vitro models and techniques, various in vitro methods are used to study and replicate the regeneration that occurs in the in vivo scenario. This includes cell culture that grows cells from an animal species in a dish to study their behavior and potential for regeneration, and organoid models that create 3D structures that mimic the architecture and function of an organ.

And that allows for more complex regeneration studies. And then comes tissue engineering itself, which uses scaffolds and cells to create new tissues or organs in a lab setting. So, if you look further, the applications include in vitro regeneration research, which has tremendous potential to lead to new regenerative medicines and therapies because of organoid culture, the scaffold, and stem cells on the scaffold, etc. is nothing but in vitro system. By understanding the mechanisms of regeneration in vitro, scientists can potentially develop treatments for injuries, diseases, and conditions.

That tissue gene regeneration is limited or absent. So the idea is that when you look into an in vitro system, you are trying to learn about how a given tissue interacts or how a given cell type needs help from another cell or another cytokine. Can we increase or decrease it? These are all very much possible in an in vitro study. This should be taken into a real scenario for in vivo help for a patient or an organism model that you are studying. So we will learn more in detail about the different aspects of regeneration in the next class. Thank you.