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Gene expression differences during regeneration in model organisms

Studium podobnosti a odlišnosti genové exprese během regenerace u modelových organismů

Bachelor's thesis

Supervisor: Mgr. Radek Šindelka, Ph.D.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 30. 4. 2021

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Abstract

With new progressive methods allowing us to study natural regeneration in model organisms, we have an opportunity to gain important insights into the very essence of this process. These insights might help us radically improve the current state of therapeutic approaches based on tissue replacement. Many different animal models display an incredible ability to restore various body parts, allowing them to escape predators and avoid premature death. While invertebrate models give us a chance to investigate the fundamental elements of regeneration, vertebrates represent systems often more resembling human biology. This thesis outlines the variability of regeneration in frequently studied model organisms with a special emphasis on the impact of gene expression.

Keywords: regeneration, model organism, gene expression, injury

Abstrakt

Díky novým pokrokovým metodám, které nám umožňují studovat regeneraci v modelových organismech, máme příležitost získat důležité poznatky týkající se samotné podstaty tohoto procesu. Tyto poznatky nám mohou pomoci zásadně zlepšit momentální terapeutické přístupy. Mnoho různých živočichů disponuje schopností nahradit chybějící části svého těla. Tato schopnost jim často umožňuje uniknout predátorům a vyhnout se tak předčasnému úmrtí. Zatímco bezobratlí nám dávají příležitost prozkoumat regeneraci na té nejjednodušší úrovni, obratlovci reprezentují biologické systémy podobné tomu lidskému. Tato práce se pokouší nastínit variabilitu regenerace v běžně studovaných modelových organismech se speciálním důrazem na rozdíly v genové expresi.

Klíčová slova: regenerace, modelový organismus, genová exprese, poranění

Glossary

BMP-Bone morphogenic protein

CT-Connective tissue

ESC-Embryonic stem cell

FGF-Fibroblast growth factor

iPSC-Induced pluripotent stem cell

PCG-Position control gene

RA-Retinoic acid

ROC-Regeneration-organizing cell

SHH-Sonic hedgehog

Summary

Introduction

Regeneration, the ability to restore missing tissue or body part, has always posed a fascinating subject for scientists to study. Over the years, researchers examined a diverse pool of animals, and they found out that regeneration is undoubtedly a highly complex process that differs from one animal to another. Even before sophisticated methods of molecular biology became a standard tool in the research, it was clear that some of the animals can regrow an entire limb while others create just a fibrotic scar at the site of injury. We humans, as members of the mammalian class, belong predominantly to the second group. That is probably the strongest motivation for us to strive towards a better understanding of animals with the capacity to regenerate. Nowadays, using methods like single-cell imaging or confocal fluorescent microscopy, we can finally identify some of the essential elements in the process of regeneration.

This thesis aims to look for the variability of regeneration mechanisms in animals and to describe the differences and similarities between them. Since this is by itself a very ambitious goal, I have chosen four models (planarians, *Danio rerio* - zebrafish, *Ambystoma mexicanum* - axolotl, and *Xenopus laevis* - African-clawed frog) to illustrate what we know about regeneration so far. There are several criteria that animal need to fulfill to become an established experimental model. It must be available (cheap, available through academic and commercial providers) for a broad community and easy to keep and breed. The experimental protocols should be standardized so studies from other laboratories can be compared. Recently, progress in genome, transcriptome, and proteome analyses allowed high-throughput and precise quantification of various biomolecules. Experimental models should also have available sequences and tools shared by the scientific community. Since not all organisms meet those requirements, our efforts to understand the variability of regeneration in nature remain limited.

In 1744, Genevan naturalist Abraham Trembley published his findings on regeneration in *Hydra*. He observed that cutting a Hydra into halves resulted in the regeneration of complete animals from both fragments (Lenhoff et al., 1986). Such findings aroused curiosity among Trembley's colleagues, and they soon observed a similar situation in the earthworm, garden snail, and salamander (reviewed by (Tsonis & Fox, 2009)). Those pioneers of regeneration research were also the first to highlight the uneven distribution of regenerative capacity not only among different species but also between various body parts of the same animal. We nowadays distinguish multiple levels of body organization on which regeneration can occur (Fig.1). The regeneration itself can be triggered by different types of damage and is sometimes limited only to a certain part of a life cycle. All those variables make each regeneration mechanism unique and fully accustomed to a specific lifestyle of the animal. That is possibly the main reason why we mostly fail to find appropriate treatment for human patients with conditions such as limb

loss or heart attack. However, the possibility to analyse and compare gene expression among various model organisms put us closer to a scenario where we can help people with such diagnoses.

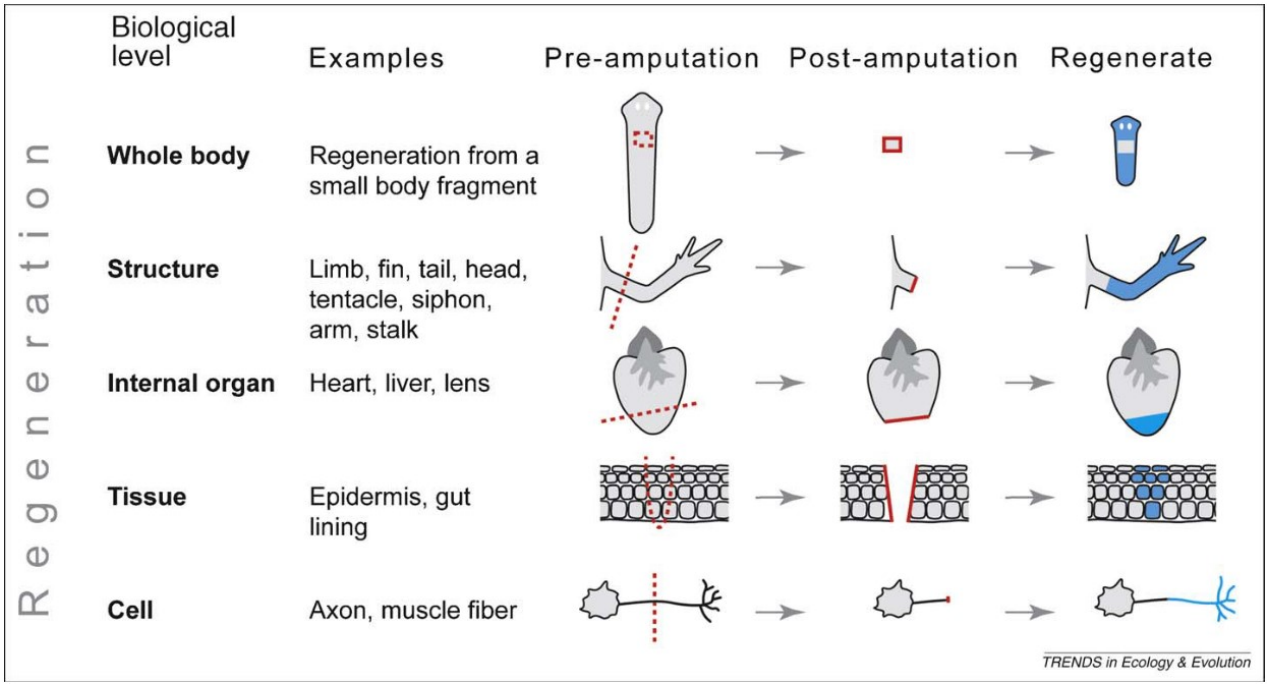

Figure 1 Biological levels of regeneration. Adopted from (Bely & Nyberg, 2010)

Planarians

The first and the most primitive organisms discussed in this thesis would be planarians. These bilaterally symmetrical metazoans inhabiting marine, freshwater, and terrestrial ecosystems all around the world are known for their ability to replace large regions of missing tissue in a process taking only days or weeks. This quality made them a very attractive model for researchers studying stem cells and tissue renewal. In comparison to other experimental animals covered in the thesis, planarians represent regeneration in invertebrates. Their simple body structure (Fig.2) and low tissue complexity allow them to survive injuries that would be considered fatal for most vertebrates. Planarians completely lack the circulatory and respiratory systems. Exchange of oxygen and carbon dioxide is ensured by simple diffusion. Brain is extended to two parallel nerve chords stretched along the central body axis, creating the characteristic ladder shape. Food is being ingested by the muscular pharynx connected to a branched intestine. Pharynx serves both as the mouth and anus of the animal. Planarians also possess a unique protonephridial excretory system that removes unwanted liquid from the body and was found to have interesting homology with the human kidney (Thi-Kim Vu et al., 2015).

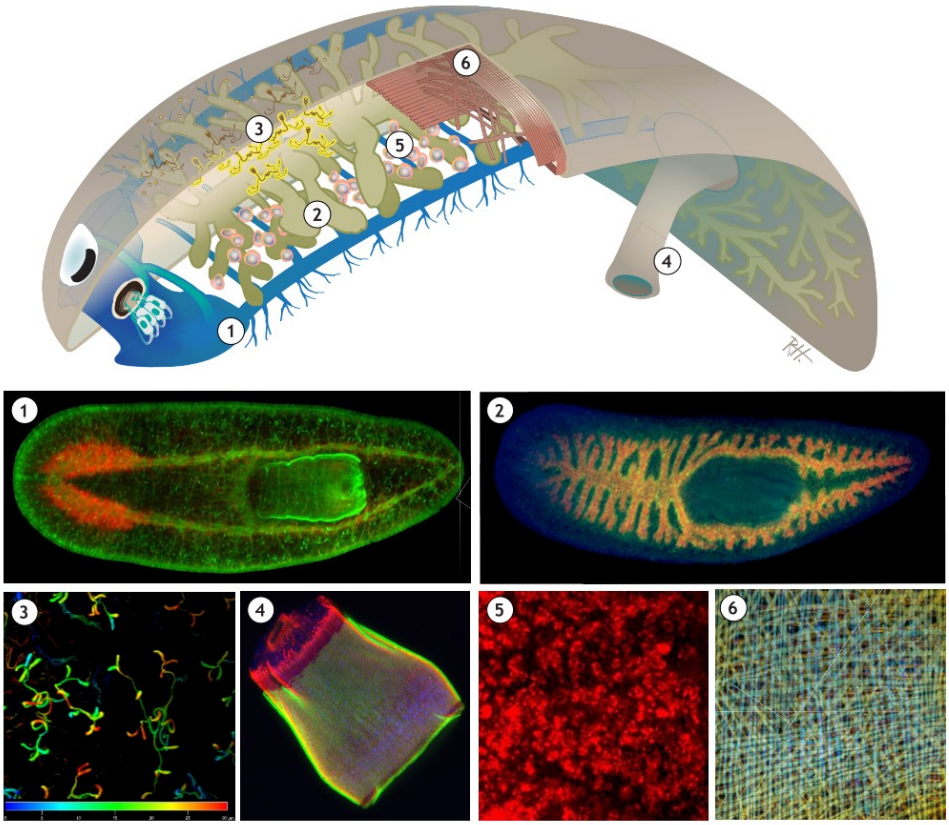

Figure 2 Planarian anatomy-schematic display (top), microscopical images (bottom). (1) Brain-red, nervous system and pharynx-green. (2) Intestine-orange on blue counterstaining. (3) Protonephridial units-green. (4) Pharynx-red and green. (5) Neoblasts-red. (6) Body wall musculature. Adopted from (Ivankovic et al., 2019)

Whole-body regeneration

As mentioned earlier, planarians are a diverse group of animals. In regards to that, their ability to regenerate significantly varies from one species to another. Some planarians such as *Dendrocoelum lacteum* or *Bdelloura candida* have only limited or nearly absent regenerative capacity, while species like *Dugesia japonica* and *Schmidtea mediterranea* can mount a robust whole-body regeneration (reviewed in (Ivankovic et al., 2019)). As a consequence, *Dugesia* and *Schmidtea* are nowadays the most prevalent planarians in regeneration research. The experimentation with planarians have a long history since the first observations of regenerating planarian required only a sharp razor and magnifying glass. Planarian can be chopped into several pieces, and each piece will regenerate into a new complete animal. This piece of tissue can be as small as 10 000 cells (Montgomery & Coward, 1974), and the incision can be made almost anywhere on the body from head to tail and also along the midline (medio-lateral

axis). The only exceptions to this rule are the areas in front of the photoreceptors and also the pharynx (Newmark & Sánchez Alvarado, 2000). Those pieces will consequently die if detached from the rest of the body.

The injury triggers a series of responses that should minimize tissue loss and prevent the animal from dying. As an immediate response to amputation, strong muscular contractions at the injury site start to close the wound (Chandebois, 1980). If the head is removed from the rest of the body, it will continue to move to escape possible predator while trunk fragments remain relatively stationary (Reddien & Sánchez Alvarado, 2004). Epithelial cells around the wound start to spread and cover the wound in the next 30 minutes creating a cellular layer called wound epidermis (Alvarado & Newmark, 1998; Chandebois, 1980). As much as this barrier is vital for isolation of the wound from the surrounding environment, the main driving force of planarian regeneration is an unusual adult stem cell called neoblast. Neoblasts are small round cells with a relatively large nucleus estimated to account for 20-30% of all cells (Baguñá & Romero, 1981). Underneath the wound epidermis, neoblasts create a differentiating cellular mass termed the regeneration blastema. Thanks to a high level of local neoblast proliferation, the blastema slowly grows to substitute for the missing tissue (Newmark & Sánchez Alvarado, 2000).

The irreplaceable role of neoblasts in planarian regeneration is not only in their abundance but mainly in their ability to differentiate into almost any cell type (Fig. 3). Researchers have shown that even after transplantation of a single neoblast into a stem cell-depleted host, all the missing tissue will be gradually replaced (Wagner et al., 2011). Neoblasts are essential not only for regeneration but for the maintenance of homeostasis in general. They are possibly the only somatic cell type capable of division, which means that neoblasts are also the single source of new cells in planaria (Forsthoefel et al., 2011; Newmark & Sánchez Alvarado, 2000). Since neoblasts have multiple roles, we distinguish between several functional classes of them. While some classes are ready to proliferate in response to injury, others play a more prominent role in tissue maintenance (Van Wolfswinkel et al., 2014).

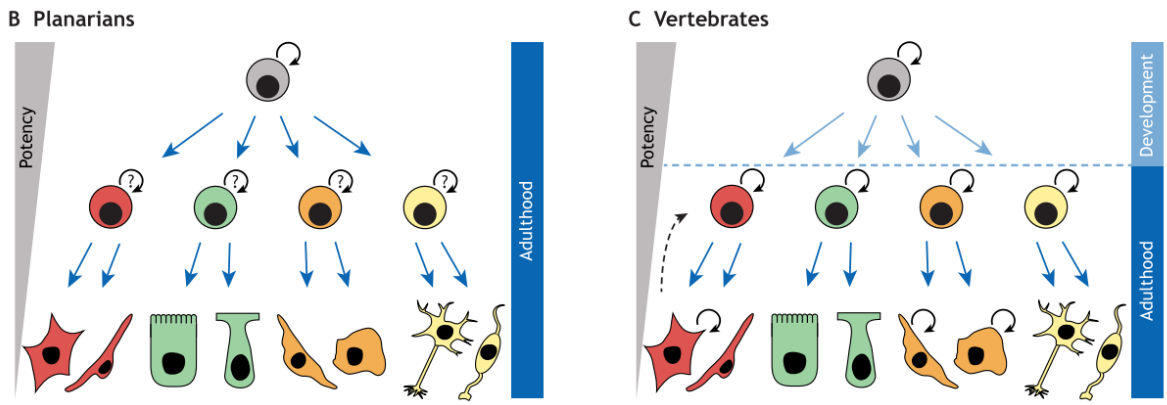

Figure 3 Planarian and vertebrate stem cell system. (B) Adult planarians possess pluripotent neoblast giving rise to lineage-committed progenitors with unknown self-renewal capacity that ultimately differentiate into postmitotic cell types. (C) In vertebrates, pluripotency occurs only in the early stages of development. In this case, multipotent stem cells persist in adults terminally differentiating into various cell types with occasional mitotic activity. Adopted from (Ivankovic et al., 2019)

It would seem that regeneration in planarians is only a matter of stem cells. However, the regenerating animal is usually unable to ingest food, so the blastema cannot rebuild the body to its original size. Instead, some structures like the pharynx can be restored by remodeling already existing tissues (Agata et al., 2007). Since one neoblast can potentially give rise to hundreds of different adult cell types, there must also be some mechanism to determine which cell type matches the situation. Single-cell RNA sequencing studies revealed that neoblast specification is a hierarchical process (Fig.3) that involves the initial differentiation into a lineage-restricted subclass of progenitors (Fincher et al., 2018; Molinaro & Pearson, 2016; Zeng et al., 2018). Those progenitors then migrate to their target organ, where they differentiate into specific postmitotic cell types (Wurtzel et al., 2017).

To a certain extent, this whole process resembles development. And as in development, every cell needs guidance in this "differentiation tree." The correct navigation is ensured by the existence of position control genes (PCGs) expressed by muscle cells (Witchley et al., 2013). PCGs show specific and regionalized expression along one or more body axis, and as a consequence, their protein products are important components of body patterning. Interference with those patterning components usually results in severe body transformations. For example, inhibition of canonical *Wnt* signaling pathway in regenerating animals causes reprogramming of the tail blastema into head development (Gurley et al., 2008; Petersen & Reddien, 2008). On the contrary, inhibition of *Wnt* antagonists will ultimately produce a tail at the anterior wound (Gurley et al., 2008). Those bizarre-looking two-headed and two-tailed phenotypes imply that the presence but also absence of the *Wnt* signaling pathway defines the central anterior-posterior body axis. Components of the bone morphogenic protein (BMP) signaling pathway have a similar impact on the re-specification of the dorsoventral body axis. Silencing some of the BMP components like *Smed-BMP* and *Smed-Smad* resulted in abnormal blastema and ventralization

of dorsal tissues (Molina et al., 2007). Since other components of the BMP pathway are necessary for the correct re-establishment of the mediolateral axis (Reddien et al., 2007), we can assume that each cell in the planarian body is provided with unique positional information.

The combination of pluripotent neoblasts and the gradient of signaling molecules makes regeneration in planarians outstanding both from a quantitative and qualitative perspective. Planarians nowadays represent one of the few invertebrate regeneration models, and despite being our distant evolutionary relatives, they might help us answer important questions regarding regeneration in general.

Xenopus laevis

The African clawed frog (*Xenopus laevis*) is an aquatic amphibian of the Pipidae family originally from African Sub-Saharan states like Sudan or Uganda (Tinsley, 1981). *X. laevis* spend its whole life in the water, nowadays inhabiting not only African ponds and rivers but also aquarium tanks worldwide. All species of *Xenopus* frogs have flattened bodies and very strong legs with a set of sharp keratinous claws on the tips of each foot. In order to become adult frogs, *Xenopus* tadpoles must undergo metamorphosis. This process is hormonally controlled like many other events in a frog's life. And it was the sensitivity to hormones that established *X. laevis* as a useful endocrinology model as well as one of the first bioassays for pregnancy diagnosis in the early 20th century (reviewed by (Gurdon & Hopwood, 2000)). These days *X. laevis* is one of the most common biological models used extensively in developmental biology and as a model for regeneration studies. Researchers mainly take advantage of its relatively short life cycle and of the possibility of stimulating egg production all year round.

X. laevis can regenerate multiple types of tissues such as the tail, spinal cord, lens, or brain (Endo et al., 2007; Gargioli et al., 2008; Gargioli & Slack, 2004; Gibbs et al., 2011). However, this ability is usually limited only to pre-metamorphic stages of tadpoles or progressively lost throughout animal's lifetime. In the previous chapter, we saw the robust regeneration in planarians. From this perspective, the regenerative capacity of *X. laevis* might not appear that impressive, but we must keep in mind the fundamental difference in the tissue complexity of vertebrates and invertebrates. Nevertheless, even among amphibians, *X. laevis* has a decent competition. Some laboratories use preferably closely related *Xenopus tropicalis*, an animal with a noticeably smaller genome. Even with tools like next-generation sequencing on the table, *X. tropicalis* is usually the "frog of choice" for genetic manipulations or epigenomic studies (reviewed by (Kakebeen & Wills, 2019)). Another amphibian,

Mexican axolotl (*Ambystoma mexicanum*), is a popular model organism intensely studied for its ability to reconstitute a fully functional limb (Kragl et al., 2009). Recent work on regeneration in *Ambystoma* will be discussed in the following chapters.

Tail regeneration

As mentioned earlier, *Xenopus laevis* tadpoles are capable of tail regeneration. Tadpoles develop in four days after fertilization, and their transparent tail can be easily accessible. Synchronous development and nearly perfectly same tail size of sibling tadpoles make them ideal models for regeneration after amputation. The developing tail is defined as the region posterior to the animal's proctodeum (anal opening). It is a complex tissue representing a continuation of the central body axis consisting of the same axial structures (spinal cord, notochord, and somites) as the trunk.

The tail can fully regenerate after amputation within 10-20 days, depending on the developmental stage (Slack et al., 2004). This occurs throughout the *X. laevis* development until the metamorphosis, when the tail is lost. There is a short period between stages 45 and 47 (refractory) when tadpoles lose the ability to regrow their tails. During the refractory period, a new tail fails to replace the lost tissue, and instead, a thick epidermis is produced covering the site of the injury (Beck et al., 2003). If these tadpoles are kept into later stages and re-amputated, they can successfully regrow the tail back.

Immediately after the amputation, the wound is covered with a layer of epidermal cells. The cut at the end of the spinal cord closes, and over the next few days, it forms a "blind-ended" tube with an enlarged distal lumen called the neural ampulla (Stefanelli, 1951). The notochord tip develops into a "bullet-shaped" cell mass. Myofibers around the neural ampulla and notochord tip show massive degeneration creating extracellular proteinaceous debris (Slack et al., 2004). Aside from these structures, there is a population of mesenchymal-like cells often referred to as regeneration blastema. We have already come across the concept of regeneration blastema before. In planarians, blastema was the foundation for the newly emerging body growing by neoblast division. In *X. laevis*, the blastema does not have such a crucial role since there are no pluripotent stem cells present. Researchers instead emphasize the reality that each component of the regenerating tail has a different rate of cell proliferation. In fact, the structures with the highest proliferation rate are the spinal cord and notochord (Beck et al., 2006). For this reason, the term regeneration bud is often used to describe the situation in regenerating tail of tadpoles (Fig. 4).

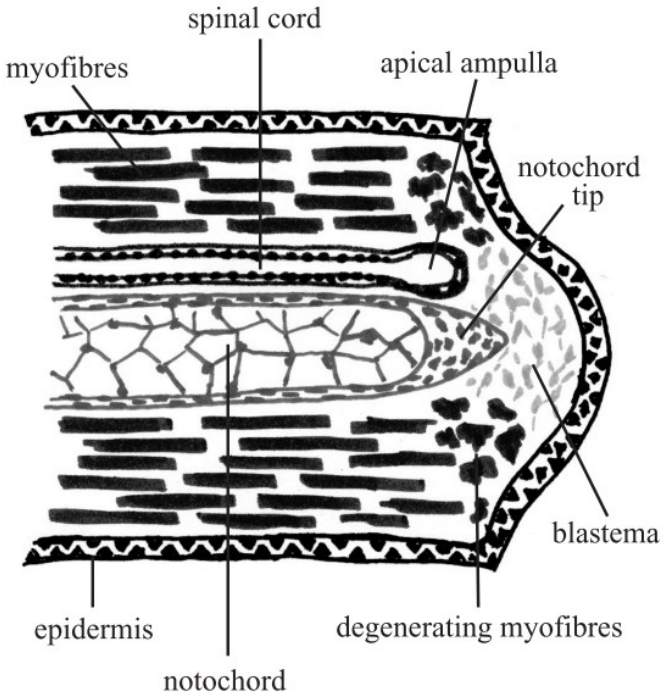

Figure 4 Schematic diagram of Xenopus laevis regeneration bud. Adopted from (Slack et al., 2004)

While the contribution of blastema to tail regeneration remains questionable, recent studies confirmed that wound epidermis plays a vital role in the process. Researchers identified a unique cell type they term the regeneration-organizing cell (ROC), which contributes to the formation of wound epidermis and ultimately successful regeneration (C. Aztekin et al., 2019). ROCs are generally present in the epidermis of tadpoles, and after the amputation, they relocalize to the wound site. Here they express ligands of signaling pathways such as Fibroblast growth factor (FGF), Bone morphogenic protein (BMP), or Wnt, promoting cell proliferation in the components of regeneration bud. That is also the explanation for the absence of stem cells in the wound. We will later observe similar signaling patterns in zebrafish (*Danio rerio*) caudal fin regeneration, also characterized by cell dedifferentiation (S. Stewart & Stankunas, 2012).

Another noteworthy phenomenon recently highlighted in scientific literature is the relationship between regeneration competence and immune system modulations. Animals with limited regeneration capacity, such as mammals, have a prolonged inflammatory phase after the injury, which inevitably leads to impairment of extracellular matrix remodeling and scar formation due to collagen deposition. On the contrary, suppression of inflammation shown improvements in injury repair and regeneration (reviewed in (Godwin, 2014)). Recent findings suggest that the presence of inflammatory myeloid cells characterizes regeneration incompetency in *X. laevis* tadpoles. Moreover, reparative myeloid cells are

required for several crucial events related to successful regeneration, such as tissue remodeling, ROC mobilization, and regulation of apoptosis (Can Aztekin et al., 2020).

Of course, the ultimate question is whether the regenerated *X. laevis* tail is identical to the original. And the answer is: No, it is not. The newly formed tail seems to be very similar at first. However, there are some critical differences in muscle segmentation and neuronal connection formation (Slack et al., 2008). Nevertheless, it seems that even without the original tail structure, *X. laevis* tadpoles can swim and survive. That leads us to an important conclusion: Regeneration is more than often an imperfect process, but in nature, many organisms can still benefit from it greatly.

Lens regeneration

In this short section, we will take a closer look at the regeneration of lens in *Xenopus laevis*. This process is very different from the tail situation or any other example mentioned in this thesis because it is driven by cell transdifferentiation. It means that after the lens is surgically removed, another already differentiated cell type will act as a source for the new emerging lens.

Regeneration of lens has been extensively studied in newts where the restored lens is formed from epithelial cells of the iris (reviewed by (Tsonis et al., 2004)). Whereas newt can regenerate lens even as an adult animal, *X. laevis* do so only in the stage of a pre-metamorphic tadpole. The regenerated lens in *X. laevis* transdifferentiates from the cornea (Freeman, 1963), more specifically from the inner layer of the outer cornea (Reeve & Wild, 1981). The lens and inner cornea normally form a physical barrier between the outer cornea and the rest of the eye. After lentectomy (surgical removal of the lens), this barrier is impaired (Fig. 5), and the contact of the outer cornea with a factor present in the vitreous induces transdifferentiation (Cioni et al., 1982; Reeve & Wild, 1981). Tadpole's ability to regenerate the lens is slowly decreasing from stage 50 (Freeman, 1963). As the animal matures, the inner cornea heals faster, and the contact of the vitreous factor with the outer cornea is limited. In *X. tropicalis*, the rapid healing of the inner cornea occurs even sooner in the development, which means that successful lens regeneration in this species typically occurs at very low frequency (Henry & Elkins, 2001).

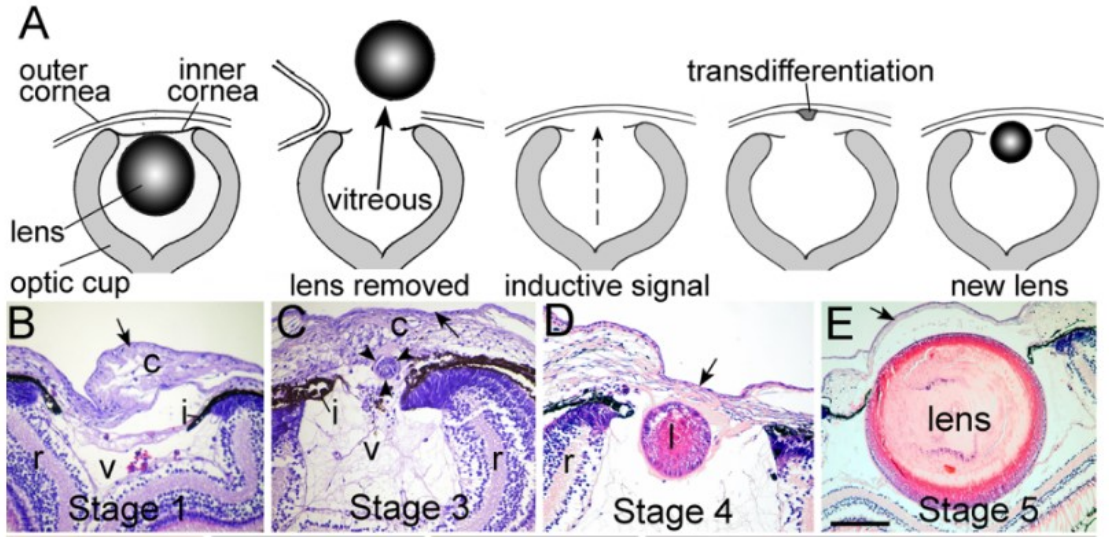

Figure 5 Lens regeneration in *Xenopus laevis*. (A) Schematic display of lentectomy and consequent regeneration of lens. Both outer and inner cornea are damaged during the extraction. (B-E) Histological sections of the eye (B=24hr, C=2 days, D=3days, E=8 days) after lentectomy. Descriptions (c-cornea, r-retina, i-iris, v-vitreous, l-lentoid), arrows mark the outermost layer of the cornea. Scale bar: 50 µm. Stages according to (Freeman, 1963). Adopted from (Beck et al., 2009)

In terms of gene expression, the lens regeneration in *X. laevis* resembles the development to a certain extent. Genes that encode transcription factors involved in embryogenesis (*pax6, otx2, sox3, or prox1*) are also expressed during transdifferentiation of the cornea (Schaefer et al., 1999). Specifically, *pax6* seems to have an essential role in the re-activation of lens differentiation pathways (Gargioli et al., 2008). In the past decade, the discoveries concerning lens regeneration in *X. laevis* have become less common. One of the exceptions would be an experiment with the role of Fibroblast growth factor (FGF) as a key signaling molecule in the process (Fukui & Henry, 2011). This way, many questions such as those dealing with the nature of the vitreous factor remain unanswered.

Danio rerio

At this point, it is fair to ask a simple question: Why do we have so many model organisms to study one phenomenon? Well, there are probably several reasons. As we could see in the previous two examples, regeneration is a highly variable process. Each organism possesses a unique way to restore damaged body parts and organs. But an even more important point is that regeneration is just much more complicated at the cellular and molecular levels than was previously anticipated. New progressive methods have been introduced during recent years, allowing researchers to design new and

often very complicated experiments. And still, the more information about regeneration we collect, the more distant from the understanding we are. Each of the models offers the opportunity to look at the process from a different perspective, introduce new approaches, and make an important reference point for later comparisons.

Zebrafish (*Danio rerio*) is a freshwater fish originally from south Asian rivers. It was first introduced as a laboratory model by George Streisinger used mainly for vertebrae genetic analysis (Streisinger et al., 1981, 1986). Over the years, *Danio* has become a prevalent and universal laboratory animal used in developmental biology, pharmacology, and as a model of many human diseases. That is not surprising since *Danio* has a short generation time, large clutches of externally developing eggs, and a relatively small genome (about 1.7 Gbp). However, the main feature I want to emphasize here is *Danio*'s regenerative ability because adult fish can regenerate multiple tissues, including the caudal fin, heart, kidney, liver, and structures linked to the central nervous system (Burkhardt-Holm et al., 1999; Kroehne et al., 2011; Nechiporuk & Keating, 2002; Poss et al., 2002; Reimschuessel, 2001). Similar to *Xenopus*, each tissue has its unique properties and mechanism of how to regrow after injury.

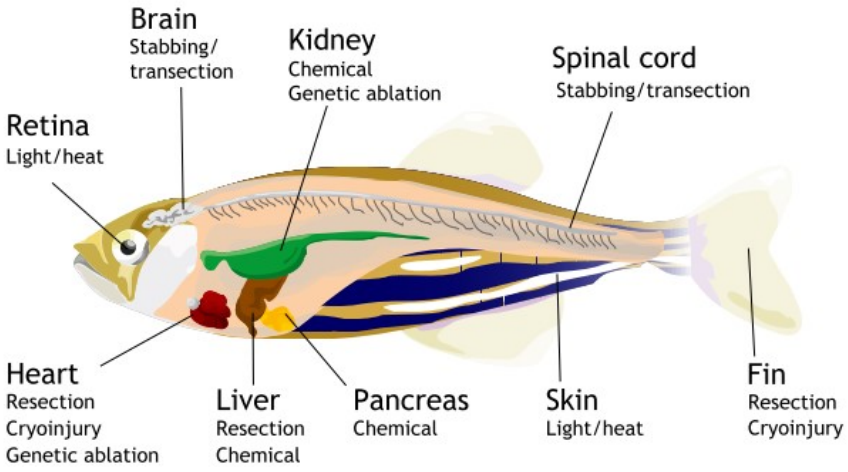

Figure 6 Schematic display of regeneration in Danio rerio. Each organ is annotated with a preferred injury model. Adopted from (Marques et al., 2019)

Caudal fin regeneration

The first regeneration observed in *Danio* was the amputated caudal fin (Broussonet, 1789). That is not surprising since fins are external body parts that can be easily manipulated, and later observations do not require any special equipment. Besides the caudal fin, zebrafish has a dorsal fin, anal fin, pelvic fin, and pectoral fin. The caudal fin of *Danio* has a bi-lobed shape stabilized by rays of segmented dermal

bone interconnected by soft tissue. This morphology of the tail is excellent for analysis of growth rate. When we compare it to usually complex and irregularly shaped structures such as an amphibian limb, it is not so challenging to quantify the regeneration in time. It is also important to mention that in contrast to amphibian limbs, *Danio's* fins maintain the capacity of increasing their size throughout the entire lifespan (Goldsmith et al., 2006).

The whole process of caudal fin regeneration takes approximately three weeks (Fig 7). Almost immediately after the cut is made, the connective tissue of the fin contracts while epidermal cells cover the wound. This wound epidermis then thickens, and the surrounding connective tissue is disorganized (Nechiporuk & Keating, 2002). At this point, mesenchymal cells start to express tissue remodeling proteins and migrate to create a regeneration blastema (Jaźwińska et al., 2007). We know that blastema is also present in previously mentioned organisms (i.e., planarian and frog), and so it is even more important to mention that interaction between epidermal and mesenchymal cells seems to be very much essential for proper regeneration. The wound epidermis is not only a physical barrier but acts as an effective organizer of the later blastema growth. By secreting growth factors such as Sonic hedgehog (SHH), Fibroblast growth factor (FGF), or Bone morphogenic protein (BMP), it provides the blastemal cells with important positional information (Lee et al., 2009; Poss et al., 2000; Quint et al., 2002). On the other hand, some growth factors and chemokines secreted by blastema are essential for the wound epithelium formation (Blum & Begemann, 2012; Bouzaffour et al., 2009; Dufourcq & Vriz, 2006; Whitehead et al., 2005). This signaling relationship between the epidermis and mesenchyme is of great importance. Without its proper function, chances of successful regeneration decrease rapidly.

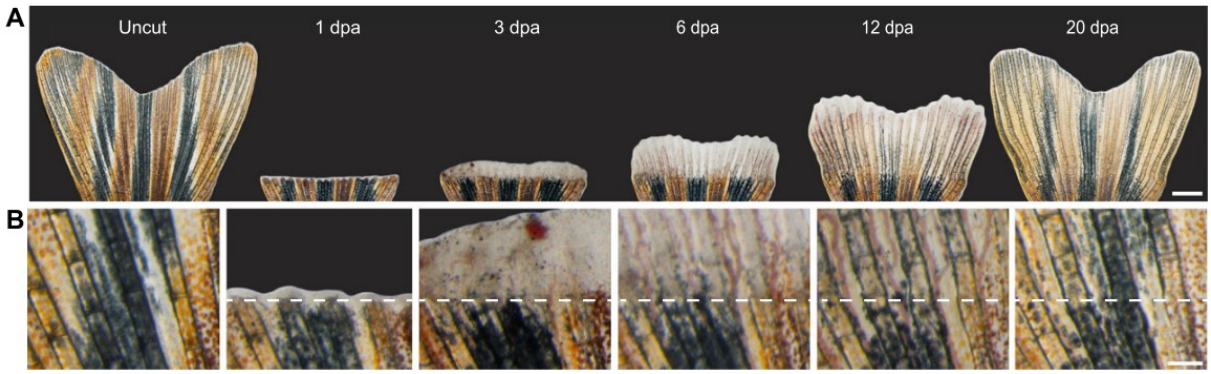

Figure 7 Time-lapse of caudal fin regeneration in *Danio rerio*. Scale bars: (A) 1000 μm; (B) 200 μm. Adopted from (Pfefferli & Jaźwińska, 2015)

Nevertheless, we are also interested in a more detailed description of what is exactly happening in the blastema. Because when we talk about blastema, we tend to define it as a mass of proliferative cells. However, the blastema organizes itself over time into distinct compartments. At this point, we

distinguish between four compartments, each having a unique gene expression profile and proliferation rate (Haase et al., 2014). The idea is that while the apical part of blastema has mainly an organizational and regulatory role, the proximal compartment is highly proliferative. This balance seems to be maintained mainly by the Notch signaling pathway (Grotek et al., 2013; Münch et al., 2013).

Since the establishment of single-cell transcriptional profiling (Zheng et al., 2017), we can monitor transitions between cellular states, which is by itself a very useful piece of information. The results can be later used for cell clustering analysis showing us cell types contributing to the blastema at specific time points after injury (Hou et al., 2020). This recent study confirmed what was anticipated before. In *Danio's* regeneration blastema, there are no multipotent progenitors present. It rather seems that in response to the amputation, cells localized near the wound undergo transcriptional reprogramming. This reprogramming allows them to detach from their original location, migrate towards the wound site and re-enter the cell cycle. Comparison between preinjury and post-injury samples revealed that even though there are some differences, the major cell composition in the tissue stays the same both before and after the injury (Hou et al., 2020).

Heart regeneration

I have already mentioned before that *Danio* is very well established as an experimental model organism for multiple reasons. While amphibians are leading models in the study of limb and tail regeneration, *Danio* has this precedence in the field of heart regeneration. Despite being our distant evolutionary relative, there are numerous similarities between *Danio's* heart and its mammalian equivalent in terms of development and cellular composition (reviewed in (Staudt & Stainier, 2012)). Nowadays, heart disease is still one of the leading causes of death worldwide (Benjamin et al., 2017). So it is no surprise that researchers and physicians are eager to understand the properties of the healing process in animals capable of regeneration.

At the beginning of the last century, doctors were not sure if the human heart is a regenerative organ or not. Some believed that there is convincing evidence for cardiomyocyte (cardiac muscle cell) division happening in children's hearts (MacMahon, 1937). However, the original idea that cardiac growth is due to an increase in cardiomyocyte size without cell division (Karsner et al., 1925) was ultimately considered to be the correct one. This inability of cardiomyocytes to divide is generally accepted as the main reason mammals are not capable of heart regeneration. More recent studies showed a slight cardiomyocyte renewal even in the adult human heart (Bergmann et al., 2009; Senyo et al., 2013). Nevertheless, this rate is clearly insufficient to compensate for myocardium loss due to a sudden event such as a heart attack.

Like all teleost fishes, *Danio* has a heart consisting of two chambers, atrium and ventricle, interconnected by an atrio-ventricular valve. It is usually the thick ventricular myocardium that is removed or damaged during the manipulation. In one of the first experiments, it was shown that as much as 20% of the ventricle could be cut off without long-term consequences for the animal (Poss et al., 2002). After the resection, heavy bleeding occurs, stopped after several seconds by a fibrin clot. This clot is over the next 4-8 weeks replaced by new fully functional muscle. This unexpected discovery motivated researchers to focus on some still unanswered questions. And for several years, surgical resection was the only technique used to study heart regeneration in *Danio*. This method is based on simple tissue removal, and as a result, there is no dead necrotic tissue left. To simulate human ischemic myocardial infarction with necrotic tissue presence, researchers employed methods such as cryoinjury or genetic ablation. Cryoinjured heart ultimately fully regenerates (Chablais et al., 2011; González-Rosa et al., 2011; Schnabel et al., 2011); however, during the process, a temporal fibrotic scar is formed.

Heart regeneration in *Danio* is a very dynamic process driven mainly by cardiomyocyte proliferation (Jopling et al., 2010; Kikuchi et al., 2010). Already existing cardiomyocytes re-enter the cell cycle and proliferate to create new myocardium. However, cardiomyocyte activation is dependent on several precedent events. Regardless of the method causing the injury, inflammation is always part of the first response. If the inflammation is suppressed, the cardiomyocyte division is impaired, and heart regeneration fails (Huang et al., 2013). A similar impact of inflammation was found in the case of *Danio's* brain regeneration, where it provides an important stimulus for neural progenitors to divide (Kyritsis et al., 2012). Accompanying the inflammatory response, endocardial cells start to re-express some embryonic markers such as retinoic acid (RA) synthesizing enzyme raldh2 (Kikuchi et al., 2011). Both inflammatory cytokines and RA activate subsequent signaling pathways necessary for myocardial regeneration. Please note that the role of inflammation in *Danio's* heart regeneration seems to be very much different from what was observed in the regeneration of *Xenopus laevis.*

However, without immediate revascularization of the damaged area, the proliferation rate of cardiomyocytes can be significantly reduced or blocked permanently (Marín-Juez et al., 2016). In the case of cryoinjury fibrotic scar is formed during the weeks following the procedure (González-Rosa et al., 2011). Because of its stiffness, the scar might pose a risk of heart failure. *Danio* expresses matrix metalloproteinases *mmp2* and *mmp14a* with collagenase activity which degrade the area of the scar and allow proper myocardial regeneration (Gamba et al., 2017).

Ambystoma mexicanum

The axolotl (*Ambystoma mexicanum*) is a urodele amphibian inhabiting freshwater lakes in Mexico and also the last model organism used for the regeneration studies mentioned in this thesis. Despite being popular as a model organism and as a pet, *Ambystoma* is considered a critically endangered species due to water pollution and ecological imbalance.

But, what is so unique about this animal that it attracts researchers studying it? *Ambystoma* is, like other salamanders, able to restore very complex body parts such as a limb or spinal cord (Kragl et al., 2009; Mchedlishvili et al., 2007). It also has a relatively short generation time-about one year, and there are multiple tools available for editing *Ambystoma's* genome (reviewed in (Haas & Whited, 2017)). This chapter covers *Ambystoma's* capacity to regenerate an amputated limb.

Limb regeneration

As we saw in the previous chapters, regeneration is sometimes limited only to a specific time frame in an animal's life. Anuran amphibians (e.g., frogs) lose this ability after the metamorphosis, but what about *Ambystoma*? In this case, the animal does not undergo the process of metamorphosis and instead remains in a larval stage with tail and external gills for the rest of its life. That is why we sometimes say that *Ambystoma* is so-called neotenic. One might consider this to be the main reason for *Ambystoma's* regenerative capacity. The possibility to experimentally induce metamorphosis (Page & Voss, 2009) allowed researchers to test this hypothesis. It appears that after the animal undergoes the induced metamorphosis, its ability to regenerate a limb persists. However, regeneration in postmetamorphic animals slows down, and newly formed limb shows defects in patterning and growth (Monaghan et al., 2014).

The sequence of events happening after the amputation has been well-described in the literature (Campbell & Crews, 2008). Immediately after the cut is made, epidermal cells start to migrate towards it, and over the next few days, they proliferate to create a wound epidermis. Following epidermal cell migration, resident progenitor cells re-enter the cell cycle and accumulate at the tip of the stump just beneath the newly formed wound epidermis. They make up a highly proliferative tissue we call the blastema. It is important to note that each progenitor contributing to the blastema is to a certain extend tissue-specific and so has restricted differentiation potential (Kragl et al., 2009). There are multiple cell types present in the blastema, but the most abundant contributing lineage is the connective tissue (CT) progenitor. This CT progenitor cell is considered the key to successful limb regeneration since it

provides the scaffold that guides the renewal of other tissue types such as muscle (Gerber et al., 2018). The heterogeneous pool of cells grows until it reaches a critical size. At this point, the structure flattens into a "palette" (Fig. 8A), and progenitors start to differentiate into the definitive cell types creating new fully functional limb.

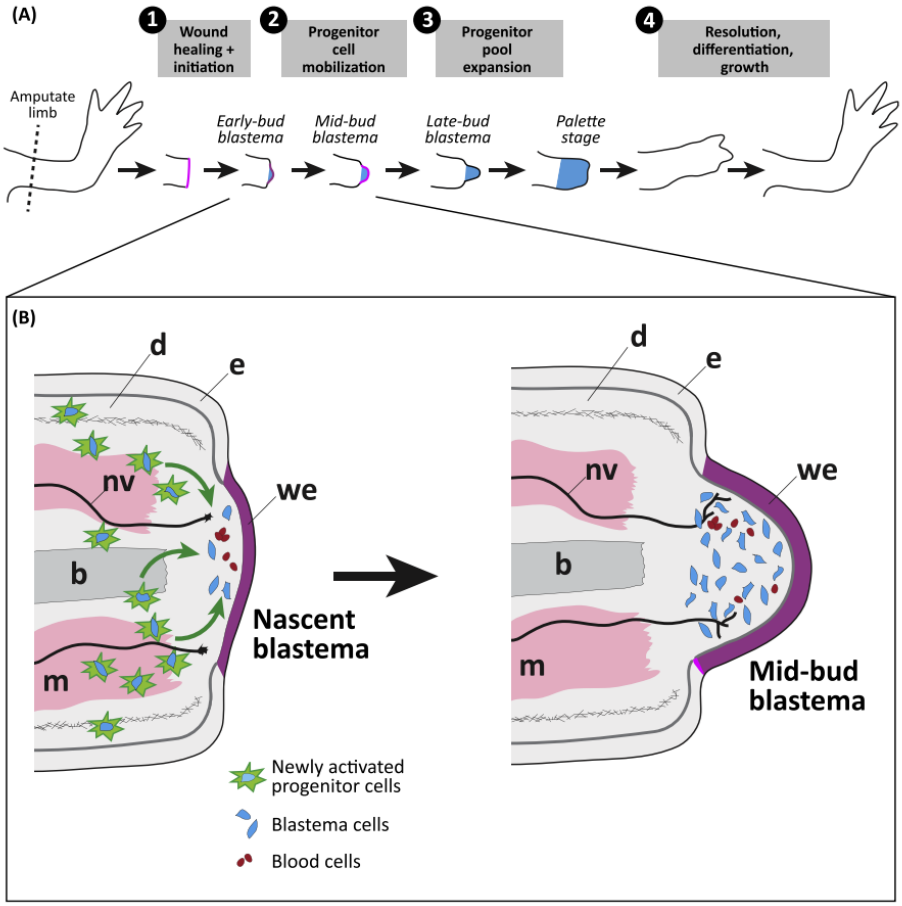

Figure 8 (A) Progression of regeneration after the amputation (B) Developing blastema in detail. Noted are: wound epidermis (we), muscle (m), nerves (nv), bone (b), dermis (d), and epidermis (e). Adopted from (Haas & Whited, 2017)

As we saw in the previous chapters, there are usually profound changes in gene expression during the regeneration process, and the *Ambystoma* is no exception here. In this context, I want to emphasize the role of genes with oncogenic activity. Various authors have shown that alterations in gene expression of oncogenes and tumor suppressors are vital for proper blastema formation (R. Stewart et al., 2013; Yun et al., 2013). We can see the close relationship between development, regeneration, and cancer not only because they are all intensely studied by researchers but because they involve similar patterns such as cell migration, proliferation, and signaling. Since *Ambystoma* is able to regenerate a relatively complicated structure such as its limb, researchers and medical professionals are eager to implement new findings in human therapy in the future. However, the slight difference between

therapeutic impact and damaging illness poses a great challenge to all of us. Another, maybe discouraging information was revealed during RNA sequencing of regenerating limb (Bryant et al., 2017). It seems that many blastema-enriched transcripts are unique to salamanders and might be lost by other tetrapods in evolution. That might be part of the explanation why mammals do not have the capacity to create the blastema or some similar structure after injury.

Discussion

For a while, it seemed that more than 250 years after Trembley's publication on the *Hydra*, we have finally reached the revolution of regenerative medicine. In 2006 Shinya Yamanaka and Kazutoshi Takahashi published an article on the induction of pluripotency in adult somatic cells (Takahashi & Yamanaka, 2006). By introducing carefully selected genes, they could wind back the developmental clock and transform a mouse fibroblast into something that looked and behaved as an embryonic stem cell (ESC). These reprogrammed cells later earned a special designation induced pluripotent stem cells or iPSCs.

In the chapter covering planarian regeneration, we witnessed the impact of stem cells on tissue maintenance and regeneration. So such discovery promised an incredible advancement in the therapy of various diseases. The patient's blood or skin might be taken to be reprogrammed into iPSCs and later used to grow new neurons, liver cells, or whatever cell type is needed for treatment. This personalized approach would evade the risk of rejection by the patient´s immune system and do not involve the ethically questionable use of embryos. Nevertheless, as the years passed, the development of a safe and effective therapy using iPSCs has proved very challenging, and several clinical trials were delayed or stopped. In the meantime, many laboratories worldwide started to use iPSCs as a source of human tissue for experiments, and in this way, Yamanaka and Takahashi indirectly contributed to many discoveries across multiple fields.

One of the objectives this thesis aimed for was to illustrate why it is so difficult to find a reliable and effective way to use regeneration as a therapeutic tool. The story of iPSCs is a great example of such effort. Although this method has enormous potential, there are just a few ongoing clinical trials involving iPSCs even after 15 years from their discovery (Deinsberger et al., 2020). And there is a reason for that. As I tried to illustrate in each chapter, natural regeneration requires the cooperation of many elements. Alterations in gene expression might allow one cell type to re-enter a cell cycle and another one to emit positional patterning signal into the surrounding tissue. Changing just one variable will not have the desired effect. Creating an artificial regeneration-permissive environment will require identifying all the missing variables and then imitating them outside the original animal tissue.

In this context, we are still at the beginning, and the variability of regeneration makes the whole process even more confusing (Tab. 1). One way to get new and relevant information would be to look for regeneration among animals with a similar evolutionary background. As much as vertebrate models like *Ambystoma* or *Danio* resemble human biology, they are still not mammals. The mammalian class members were always considered, with a few exceptions, to have very limited regeneration abilities. With an article describing regeneration in a spiny mouse (*Acomys*) (Seifert et al., 2012), the whole paradigm changed. This rodent has a similar appearance as the laboratory mouse (*Mus musculus*), but its reaction to injury is entirely different. *Acomys* is able to mount a non-fibrotic regenerative response in various internal and external organs such as skin, skeletal muscle, spinal cord, or heart (Maden et al., 2018; Qi et al., 2017; Seifert et al., 2012; Streeter et al., 2020). The main systemic differences between *Acomys* and other mammals are a controlled inflammatory response and the overall tissue composition (reviewed by (Sandoval & Maden, 2020)). Since the research on regeneration in *Acomys* is in its beginnings, the information we have at the moment is still incomplete. Nevertheless, *Acomys* might be the first key to unlocking the secrets of mammalian regeneration.

Regeneration	Developmental limit	Mechanism	Injury model
Planarian (whole body)	No	Neoblast proliferation	Incision
X. laevis (tail)	Tadpole stage	Dedifferentiation	Incision
X. laevis (lens)	Tadpole stage	Transdifferentiation	Incision
D. rerio (caudal fin)	No	Dedifferentiation	Incision
D. rerio (heart)	No	Dedifferentiation	Incision, cryoinjury, genetic ablation
A. mexicanum (limb)	No	Progenitor proliferation	Incision

Table 1 Summarizing table showing variability across model organisms used in regeneration research.

What might also accelerate the process of uncovering missing information is the development of new or already existing methods. The current toolkit allows us to analyze animal´s genome, proteome, or transcriptome for a reasonable amount of financial resources. Such analyses can be performed nowadays on the level of single cells, which provide us detailed information about cell activity and its gene expression profile at a specific time point (Zheng et al., 2017). This technology is widespread among researchers studying regeneration as well as the nuclease system CRISPR-Cas 9 used for genome editing (Jinek et al., 2012). Combination of these tools can speed up our effort to better understand regeneration mechanism at molecular and cellular levels.

One day there might be a way to regenerate human organs using cellular reprogramming or tissue engineering techniques. Overcoming the many constraints Mother nature has put on us will require integrating knowledge across multiple fields but mainly a tremendous amount of patience. And as much new technology can help us search for important details, it will always be the model organisms at the center of our attention like a beam of light showing us the way on this monumental journey.

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