Regeneration and the underlying mechanisms

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Abstract

Regeneration is generally defined as tissue replacement after pathological insult such as injury or disease. It has captured the attention of many biologists, clinicians and biomedical engineers, because understanding how regeneration is regulated will accelerate the development of regenerative medicine. Although many animals, including mammals, are able to regenerate damaged tissues, the degree to which this is possible varies considerably among species, as well as among their different body parts and tissues/organs. Consequently, various model organisms and systems, each offering specific strengths and weaknesses, are used to study regeneration in all its complexity. At the cellular level, the regenerative strategies, for example the source of cells regenerating a structure, are shown to differ widely between species and systems. In contrast, the molecular regulation seems less variable, as the same signaling pathways are commonly found to play crucial roles. The most important are Transforming growth factor β (TGF β), Fibroblast growth factor (FGF) and Wnt/β-Catenin signaling, directing all stages of the regenerative process across species and structures. Further, signaling through the growth factors IGF, VEGF, EGF and HGF, as well as MEIS- and Homeobox-factors is repeatedly found to be required for successful regeneration. Moreover, experimental interference with specific signals was shown to have the power to augment or even trigger the regenerative process in certain contexts, which allowed increasing our understanding of how regeneration is regulated. Here, I will give an overview of the different cellular aspects of regeneration and discuss the underlying molecular regulations. Differences and similarities between species and structures will be put forward and will furthermore be set into an evolutionary context. In addition, important future directions for regeneration research will be pointed out.

Key words:

Regeneration
Stem cells
Evolution
Molecular mechanisms
Metazoan

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Introduction

Regeneration is the process of tissue replacement triggered by pathological causes such as injury or disease, and is characterized by cell division and differentiation. Although both regeneration and homeostatic cell turnover represent a replacement of cells, they are two different processes. Physiological cell turnover takes place continuously and specific cells are deleted by genetic programs. In contrast, regeneration is initiated in case of an exogenous stimulus that caused acute damage and the removal of multiple tissue types at once [1].

Most animals are able to regenerate parts of their body, but the extent to which this is possible varies (Fig. 1). Humans replace lost blood through the activity of hematopoietic stem cells [2], while newts are able to re-grow a fully functional, normal limb after amputation [3]. Invertebrates like *Hydra* and planarians even bear the capacity to regenerate whole animals from tiny body pieces [4-6].

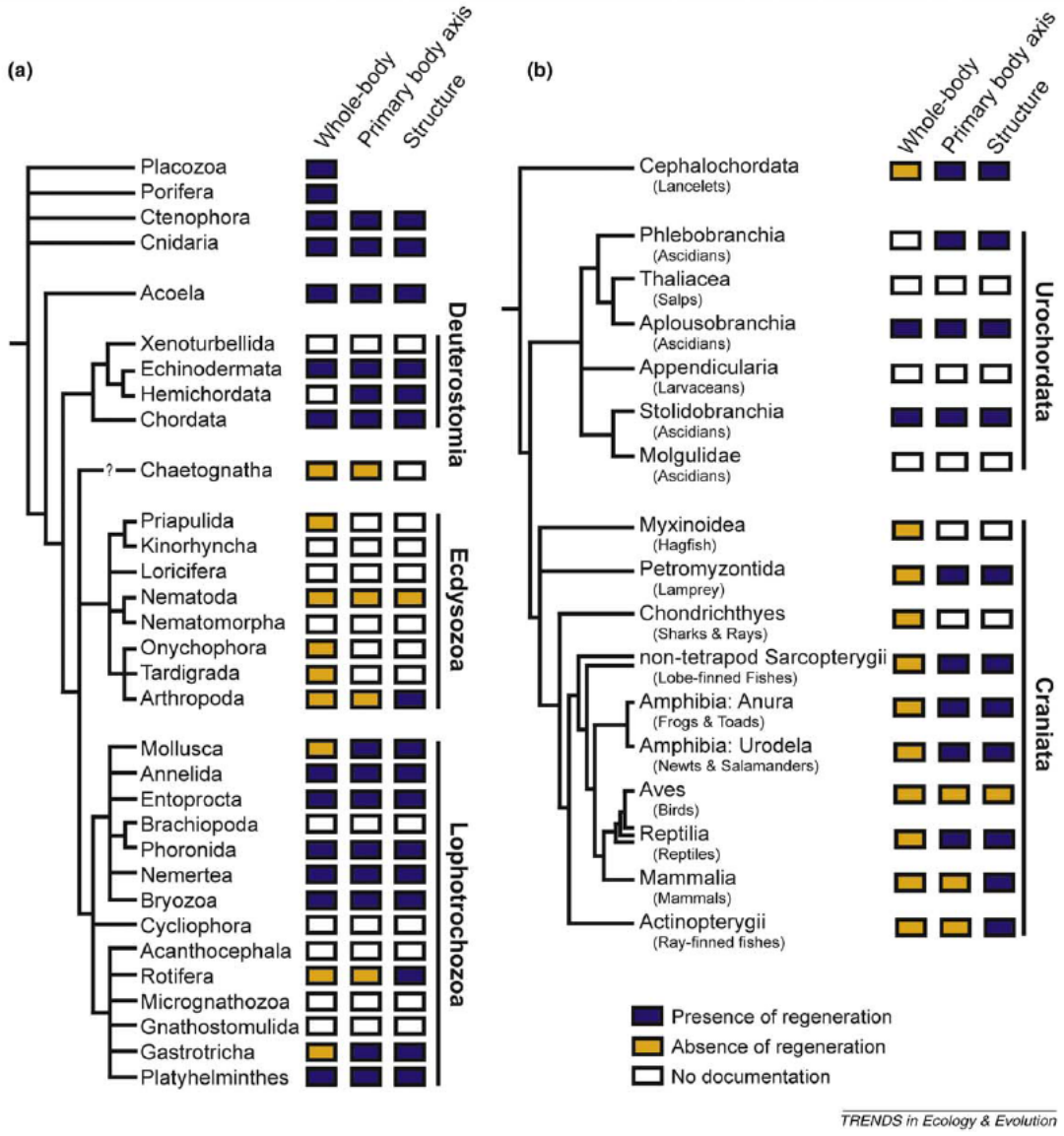

Figure 1: Phylogenetic distribution of regeneration across the Metazoa (a) and the Chordata (b). "Whole-body regeneration" is defined as the potential to regenerate every part of the body, but not necessarily from a tiny fragment or simultaneously. The category "presence of regeneration" indicates that at least one report of regeneration exists in that taxon, but does not imply that all species in that taxon are able to regenerate. The category "absence of regeneration", on the contrary, indicates that there is at least one report of the lack of regeneration and that the presence of regeneration has not been reported for that taxon. Adapted from Bely at al. [7]

Regeneration is a fascinating phenomenon from a biological point of view. But the potential to replace damaged tissues also caused biomedical interest ever since. However, until now, biomedical studies have mainly focused on stem cell biology *in vitro*, whereas *in vivo* research is needed to gain a full understanding of the regeneration process. Elucidation of the complex interactions among and within cells and tissues will contribute to a complete understanding of regeneration. In order to investigate such *in vivo* processes, the use of model organisms is essential and only here the necessary knowledge to eventually control and manipulate regenerative properties can be provided [8]. Moreover, it will be advantageous for

biomedicine if we understand the mechanisms and modes that are involved in the regenerative process in the different model organisms. Especially comparing the molecular mechanisms, the pathways and gene networks driving regeneration in different organisms will help to understand regeneration as a whole.

For over 200 years, biologists have studied regeneration in invertebrates [9]. The cnidarian *Hydra vulgaris* is a diploblast with a radial symmetry around an oral-aboral axis. It is composed of three distinguishable anatomical parts: the foot, the body column and the head region. The two germ layers – ectoderm and endoderm – are separated by the mesoglea, an extracellular matrix in which interstitial stem cells reside [10, 11]. *Hydra* is the first animal in which regeneration has been described [9, 12] and it is able to re-grow all essential body parts. Due to constant replacement of cells that are lost during physiological cell turnover, *Hydra* is considered to be negligibly senescent [13].

Another highly regenerative class of invertebrates that has been a classic model of animal regeneration are the free-living freshwater planarians. The flatworms that have received most attention are *Dugesia japonica* and *Schmidtea mediterranea* [4, 14]. In addition, *Macrostomum lignano* has emerged as a new model organism [15]. The only somatic mitotically active cells in planarians are the so-called neoblasts - small, round or ovoid undifferentiated cells with very little cytoplasm - which are essential for homeostatic cell turnover as well as regeneration [16-19]. According to T.H. Morgan, a fragment 1/279th the size of the original worm is able to regenerate a new, complete animal [20].

Among the vertebrates, the highest regenerative capacity is found in amphibian species, especially newts and salamanders (urodeles) [8]. An adult newt is able to regenerate many organs, limbs, the tail, the lens and retina, hair cells, the brain and spinal cord, the jaws and the heart. The neotenous (which retain traits that are usually seen only in juveniles) salamander axolotl has emerged as a model due to its amenability to routine breeding and because it can regenerate the spinal cord, the tail and limbs [21]. Anurans, which consist of frogs and toads, have a more limited regenerative capacity. The anurans *Xenopus laevis* and *Xenopus tropicalis* can regenerate fewer structures than salamanders or newts and only during certain pre-metamorphic stages [22].

Furthermore, fish also have good regenerative capabilities. An excellent model organism to study regeneration in lower vertebrates is the zebrafish *Danio rerio*. Its developmental

period is short, it can easily be reared in the laboratory and genetic screens have produced many mutants. Especially, fins and the heart are highly regenerative and research in these structures has produced high-quality results [23].

Compared with other vertebrates, the regenerative capacity of mammals is very low. Skeletal muscle and the liver were shown to regenerate. Additionally, the digit tips of mice do regenerate under certain conditions and research of these three structures is producing highly significant results, which will be elucidated below in detail [8].

Research on regeneration can be broken down to a few questions: How does injury-induced regeneration canalize the formation of new anatomically precise and functionally integrated tissues? Why do some organisms regenerate while others do not? What are the molecular mechanisms driving regeneration and what are the differences and similarities between these mechanisms in the diverse species? The aim of this work is to give an overview of the current knowledge about regeneration across animal phyla. Specifically, the underlying molecular mechanisms will be discussed. This will allow to point out common pathways and gene networks, but also to reveal differences and trends.

Regeneration and the underlying mechanisms

In order to regenerate successfully, several key problems need to be faced. Firstly, cells need to identify which body part is missing and thus has to be regenerated. Secondly, after a wound response, proliferation has to be induced either in stem cell pools, which are located in the residing tissue, or in intermediary cells during trans- or dedifferentiation of formerly post-mitotic cells. Thirdly, the new tissue needs to be properly patterned and cells need to differentiate to build a structure that is similar to the amputated structure in form and function. The axes (anterior-posterior, dorso-vetral, left-right, proximo-distal) have to be correctly established. Lastly, regeneration needs to be terminated after correct re-growth. Every one of these different steps during regeneration is tightly controlled by gene networks and molecular mechanisms. In the following sections, each step will be analyzed individually. Cellular processes of regeneration in different systems and species will be described and the underlying molecular mechanisms will be depicted. Further, the cellular and molecular mechanisms will be put into evolutionary context. Unfortunately, many questions about the molecular networks driving regeneration are still unanswered. For this reason, insights from selected model systems and organisms will be pooled and compared, in order to give an overview of the current state of knowledge.

1. Initiation of regeneration – insights from different model systems

The first step towards successful regeneration is the initiation of the regeneration process. Naturally, there are differences between different organs and animal models in terms of overall regeneration, including its initiation. Activation of the innate immune system following injury is common in all vertebrates. Signals regulating the immune response are likely to be involved in triggering the regenerative process. In this section, the initiation of regeneration - and the underlying molecular mechanisms - in selected organs and structures of vertebrates, and also invertebrates, will be discussed.

1.1 Initiation of regeneration in vertebrates

1.1.1 Liver regeneration

The liver represents the only mammalian organ with high regenerative capacity. After amputation/resection of up to 70% of liver mass, the original size will be restored within 7-10 days in rats and 3-6 months in humans [24, 25]. The earliest signals important to trigger regeneration of the liver are part of the innate immune system. Members of the cascade C3a

and C5a activate the Kupffer cells, the macrophages that are present in the liver, which in turn release cytokines that then act on hepatocytes and other cell types [26, 27]. The cytokines, mainly tumor necrosis factor (TNF) and Interleukin-6 (IL-6), are thought to make hepatocytes competent for cell division. IL-6 activates Stat3 and mitogen-activated kinase (MAPK) signaling in hepatocytes, resulting in modulation of transcription of many target genes [28]. Another TNF-related ligand, TWEAK, appears to be a specific regulator of progenitor cell-mediated liver regeneration, since, unlike other cytokines, it has no effects on differentiated hepatocytes [29]. After priming with cytokines, growth factors drive cell cycle progression of differentiated liver cells during regeneration. Hepatocyte growth factor (HGF) and ligands of the epidermal growth factor receptor (EGFR), namely EGF, Transforming growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin (AR) can induce proliferation of primed hepatocytes *in vitro* and *in vivo* [30-34]. Another effect of cytokines seems to be the activation of matrix metalloproteinases (MMPs), which degrade the extracellular components and as a result allow proliferation of hepatocytes [35]. Fibroblast growth factor (FGF) and Wnt/β-catenin signaling seem to be of significance for liver regeneration, as well [36-38].

1.1.2 Skeletal muscle regeneration

Skeletal muscle tissue also has a high regenerative capacity: the formation of muscle fibers from resident progenitor cells, the so-called 'satellite cells' (SCs), can repair local damage of muscle [39]. As part of the immunological response neutrophils and macrophages invade the muscle rapidly after injury, evidently attracted by several chemoattractants such as MCP-1, MDC, FKN, VEGF, μPAR and μPA [40]. There is evidence that macrophages, in contrast to neutrophils, have a beneficial role for muscle regeneration [41-44]. Upon injury, macrophages are attracted and it was shown that they directly affect SCs [45, 46]. Secretion of soluble factors, and also direct cell-cell contact, were shown to be mechanisms of interaction between SCs and macrophages [47]. Further, myogenic precursor cell proliferation is induced by pro-inflammatory macrophages, while differentiation and fusion of myogenic precursor cells is induced by anti-inflammatory macrophages [48]. As during liver regeneration, cytokines, secreted by macrophages, appear to take a part: deficiency of the TWEAK receptor *Fn14* leads to delayed regeneration of the muscle [49]. Moreover, MMPs have been shown to be crucial for regeneration to occur [50]. Two other matrix-specific molecules, Fibronectin (FN) and Tenascin-C (TN), have been suggested to be involved in cell proliferation [51].

The apical and basal attachment sides of SCs were shown to be essential for signal transduction [52, 53]. Attachment to the adjacent myofiber on the apical side is accomplished through M-cadherin [54]. Integrin α7βI links the cytoskeleton on the basal side with Laminin in the basal membrane to allow the transduction of mechanical forces to chemical signals, which is involved in regulating myogenesis [55]. Recently, it has been reported that Integrin α7βI is required for migration of SCs, where a crucial role in guidance is played by HGF [56]. HGF can bind to Heparan sulfate proteoglycans (HSPGs) either in the basal membrane, by which the signaling is negatively regulated [57, 58], or on the surface of SCs, causing their activation. Importantly, the HSPGs on SCs, e.g. Syndecan, are different in terms of their extracellular domains and they are part of the signal transduction [59, 60]. Activation of SCs leads to HSPG up-regulation and it was shown that they are required for proper FGF and HGF signaling [60, 61].

The role of FGF signaling during muscle regeneration is not clear, but Insulin-like growth factors (IGFs) have been shown to stimulate proliferation and eventually also differentiation. This happens via IGF receptor I-induced MAPK (Mitogen-activated protein kinase) signaling during proliferation, while phosphatidylinositol 3-kinase (PI3K) signaling leads to differentiation (for review see [62]).

1.1.3 Limb regeneration

During limb regeneration, the first step is healing of the wound. It is known that, in mammals, a large wound takes days to close after an initial inflammatory response, resulting in the formation of scar tissue due to accumulation of collagen bundles [63, 64]. In highly regenerative non-mammal vertebrates like salamanders the healing of the wound occurs differently. In urodeles or larval anurans, the wound is rapidly covered by epidermal cells forming a so-called “wound epidermis” (WE). Within a matter of hours this WE is formed through migration of epidermal cells from the edge of the amputation surface [65]. The formation of such WE was shown to be required for regeneration to occur [66]. An upregulation of MMPs, which are suggested to play a role in matrix degradation, contributes to the WE formation and is required for regeneration [50, 65]. In axolotls and frogs it was shown that Wnt/β-catenin signaling is required for structural maturation of the WE [67]. The WE then becomes a specialized structure, often referred to as apical epithelial cap (AEC), which is distinct from the normal epithelium in terms of morphology, gene expression and most of all its robust secretory activity [64, 65, 68]. This structure shares many biochemical and gene expression similarities with the apical ectodermal ridge (AER), which directs and patterns limb

outgrowth during amniote development. The main difference between these two structures is that the AEC regenerates after amputation, whereas the AER does not regenerate and neither does the limb [69, 70]. In this work, as in the existing literature, the terms WE and AEC will be used interchangeably.

The next step during limb regeneration is the formation of the regeneration blastema, induced by the WE. The blastema is comprised of progenitor cells which proliferate and direct regeneration [71, 72]. Dedifferentiated cells of various tissues in the urodele limb have been hypothesized to give rise to the blastema [73, 74]. The blood-clotting protease Thrombin is thought to act as extracellular signal to induce this process, as it can indirectly induce S-phase re-entry in cultured newt myotubes [75]. Additionally, intracellular phosphorylation of Retinoblastoma (Rb) protein and expression of *msx1*, a homeobox protein and transcriptional repressor expressed in many regenerating systems, is required for myotube cell cycle re-entry in vitro [76, 77]. The WE in both urodeles and larval anurans begins to express *fgf8*, which is known to be the only FGF individually necessary for normal limb development, during blastema formation [78-80]. Bone morphogenetic protein (BMP) signaling was shown to promote *msx1* and *fgf8* expression and is consequently required for blastema formation and cell proliferation [81]. Wnt/β-catenin signaling proved to be required for the blastema formation and cell proliferation, shown through loss-of-function studies where regeneration is completely inhibited [67, 82].

1.1.4 Tail regeneration

The general cellular processes in urodeles and larval anurans driving limb and tail regeneration appear to be very similar. Nevertheless, there are a few differences. It has been shown that during the first 24 hours post amputation (hpa) in *Xenopus*, apoptosis is required for successful regeneration. It has been hypothesized that endogenous inhibitory cells must be destroyed by Caspase-3 activity for regeneration to occur [83].

1.1.5 Fin regeneration

Similar to previously discussed limb regeneration (see section **1.1.3**), a thin epithelial layer covering the wound, the WE, is formed during the first 1-3 hpa of the fin. Proliferation and blood supply are not necessary for this process to occur [84-88]. The molecular signals for this process are unknown. Important for a correct formation of the WE is signaling through Fgf20a and Wnt10a, both up-regulated during the first 6 hpa [89, 90]. The blastema is then formed until 6 hpa, induced by signals from the WE. FGF and Wnt/β-catenin signaling

pathways continue to be required for the formation of the blastema [90, 91]. Expression of the *heat-shock protein 60* (*hsp60*) is increased during formation of the blastema cells and dysfunction was shown to lead to mitochondrial defects and apoptosis in these cells, indicating that Hsp60 is required for formation and maintenance of the blastema [92]. The TGFβ-related ligand Activin-βA was also found to be highly up-regulated during the first 6 hpa and is important for the progression of regeneration. Specifically, it is necessary for cell migration during wound healing and blastemal formation [93]. Furthermore, IGF signaling in the blastema and WE was demonstrated to be of importance for successful initiation and progression of fin regeneration [94].

1.2 Initiation of regeneration in invertebrates

1.2.1 Planarians

Adult planarians contain a number of somatic mitotically active cells, known as neoblasts, which can be seen as stem cell-like [95]. Except for the region of the photoreceptors and the pharynx, the neoblasts are distributed throughout the body and can, by formation of a blastema at the site of amputation within 2-3 days post amputation (dpa), give rise to new body parts [4]. It has been described that two distinct maxima in mitotic numbers, one 4-12 hpa and the other 2-4 dpa, occur after injury [96]. Recently, evidence was provided that distinct signaling events control these mitotic peaks in *S. mediterranea*. It was suggested that the first mitotic peak is a systemic response to all types of wounding and that, compared to this, the second peak is a local response to missing tissue, in other words where regeneration needs to occur. During the phase of decline in mitotic number between the two peaks (by 18 hpa), neoblasts accumulate at the wound site, suggesting a signal triggering neoblasts migration [97]. To date, the signals initiating the mitotic peaks and neoblast migration are unknown. However, Wnt/β-Catenin signaling was shown to be required for head formation[98-100], while BMP signaling is required for proper regeneration in general [101-103]. Furthermore, RNA interference experiments in *S. mediterranea* pointed out that signaling through EGF receptors (EGFRs) is involved in the control of cell proliferation, differentiation and morphogenesis not only during regeneration, but also in homeostasis [104].

1.2.2 *Hydra*

Regeneration in *Hydra* can be carried out by epithelial cells only [105, 106] (the cellular source will be discussed in section **2.4**). Nevertheless, there are two additional requirements for regeneration to occur: the extracellular matrix (ECM) separating epithelium and

endothelium has to be intact [107-109] and a critical minimum tissue size of 300 epithelial cells needs to be provided [110]. The importance of the ECM is that its anchorage to the epithelial cells is necessary for their survival [111]. Cytokines have also been detected in hydra as initial signals for regeneration. One is HyBMP5-8b, a BMP5-8 orthologue, which is active in tentacle formation and patterning of the lower end of the body [112]. Additionally, src-type receptor tyrosine kinase (STK) activity is strongly increased 6 hpa and high levels of STK are correlated with head, but not foot, regeneration [113]. Serine/threonine protein kinases are also up-regulated during head regeneration [114] and the PI(3)K-PKB pathway was also found to participate during regeneration [115]. Moreover, the canonical Wnt pathway is suggested to play a role in the formation and maintenance of the head organizer region, since HyWnt is found right at the terminus of the body axis and β-Catenin and TCF were also found to be up-regulated in a broader region of the head [116]. Additionally, the activity of the serine-protease inhibitor Kazal1, which also prevents excessive autophagy and has a cytoprotective function against wounding stress, is needed for regeneration, during which the *kazal1* gene is highly up-regulated [117].

1.3 Conclusion

In general, activation of the innate immune system commonly is one of the first reactions to injury. Consequently, signals from this early immune response theoretically present good candidates for inducing regeneration. As was shown above, this has been proven to be correct: cytokines have been identified to trigger the regenerative processes in liver and muscle regeneration and also during general regeneration in *Hydra*. Surely the future will show whether they are involved in inducing regeneration of other organs, appendages and whole body-parts, as well. Furthermore, IGF and EGF signaling and the action of MMPs seem to be of significance for correct regeneration. Although these factors have not yet been proven to be required in every single system/species, there were no contradicting results. Requirement for FGF, TGFβ and Wnt/β-catenin signaling was shown in even more systems, pointing out their high importance for initiation of regeneration. Additionally, signals released from dying cells seem to bear capacity to induce regeneration, as described for the tail regeneration in tadpoles, for example [83]. These results indicate that the initiation process during regeneration is very similar across species. Nevertheless, this hypothesis has to be met with caution: although the same above-mentioned pathways are active, this does not necessarily mean that the exact same molecules are involved in the regeneration of every structure. Often, various ligands can bind and activate the same receptor-type. This means, in consequence, that detected activation

of, for example, the FGF-receptor can be achieved through a specific FGF-ligand in species A and through a different FGF-ligand in species B (i.e. FGF8 in urodele limb regeneration and FGF20 in zebrafish fin regeneration). More specific experiments are required to unravel the exact players in these pathways in order to determine whether the initiation of regeneration is as similar across structures and species as it seems to be from our present point of view.

2. Source of regenerating cells: Stem cells vs. trans-/dedifferentiation

In order to successfully regenerate, there needs to be a population of proliferating cells in order to replace the lost tissue. An important feature of most metazoan somatic cells is that all necessary genetic information to produce complete organisms is contained in their nuclei [118-120]. Animals must have acquired mechanisms to access such inherent totipotentiality since regeneration generally involves the formation of one or more pools of proliferating cell populations. In essence, there are two mechanisms by which the cellular source for regeneration can be provided: on the one side are stem cells or progenitor cells and opposed to this are cells within the tissue requiring transdifferentiation or dedifferentiation [121]. In the following, the term "dedifferentiation" will be used for post-mitotic cells losing differentiated character and acquiring proliferative capacity, irrespectively of what cell types they make later. "Transdifferentiation" will be defined as the conversion from one differentiated cell type to a different cell type, irrespective of whether there is or is not an obvious less differentiated intermediary cell type. This definition was chosen due to the occasional difficulty to determine the existence of an intermediate less-differentiated state (definitions were adopted from Antos & Tanaka [122]).

Progenitor cells – originating from either stem cells or dedifferentiation – have a different epigenetic status than fully differentiated cells on the one hand and quiescent, not activated stem cells on the other hand. Therefore, changes in epigenetic and gene transcription programs must be involved in the process leading to proliferation.

The focus of this section will be the comparison between the different cellular sources. Several case studies where a specific cellular source has been intensely investigated will be discussed and a summary of which cellular source is used for regeneration of which specific structure/species will be given. Moreover, the known molecular mechanisms involved in the regulation of such cells will be elucidated.

2.1 Eye regeneration - a model of transdifferentiation

2.1.1 Regeneration of the lens

One of the best studied cases of transdifferentiation in vertebrates is the regeneration of the amphibian eye. Removal of the lens in newts induces the dorsal iris pigment epithelium (dIPE) to lose its pigmentation, undergo proliferation and eventually transdifferentiate into lens [123]. While the IPE originates from the neurectoderm, the lens forms from the non-neural ectoderm, which illustrates that both structures come from different developmental lineages [124]. Experiments with clonal cultures of IPE from chick embryos showed that application of basic FGF and the de-pigmenting enzyme PTU is sufficient to achieve transdifferentiation from pigment epithelium to lens [125]. Similarly, FGF2 has been identified to stimulate dIPE to form ectopic lenses *in vitro* and also in intact eyes in the newt system [126, 127]. Interestingly, only the dorsal IPE transdifferentiates *in vivo*, while the ventral IPE re-enters the cell cycle after injury, but does not form a lens [127]. Retinoic acid, however, combined with over-expression of the transcription factor *sine oculis homeobox-3* (*six-3*) and the inhibition of BMP signaling induces ectopic regeneration of the lens by the ventral IPE. A key step to this accomplishment is the inhibition of BMP signaling, which can restore competence without Retinoic acid and Six-3 at a much lower frequency [128]. *Sox2*, *myc* and *klf4* expression, a fraction of the factors used to induce pluripotent stem cells (iPS) in mammalian research, have recently been shown to be up-regulated during regeneration of the lens [129]. The fact that *oct4* and *nanog* expression were not detected indicates that IPE cells do not become fully pluripotent [129].

2.1.2 Regeneration of the retina

Regeneration of the retina in amphibians occurs through de-lamination, followed by dedifferentiation and transdifferentiation of retinal pigment epithelium (RPE) to generate the neuronal cell types [130]. Evidence has been provided that transcriptional programs that are not taking part in retinal development are involved during this transdifferentiation: *Cellular retinal-binding protein* (*CRALBP*), which is else wise active in embryonic pigment epithelium and Müller glia cells, but cannot be found in embryonic retinal progenitor cells, is expressed by the dedifferentiated RPE cells [131, 132].

The protein bFGF can induce transdifferentiation in mammalian and avian embryonic RPE. However, the result is a complete conversion to retinal cells, while a second, de-laminated cell layer is not produced. As a consequence, the retinal pigment epithelium is

depleted to form a retinal layer [133, 134]. Additionally, the avian and mammalian RPE is only responsive to FGF-induced retina formation while the eye is developing [133, 135, 136]. Inhibition of Activin signaling, which promotes RPE differentiation, can delay this restriction [137]. In comparison to the induction by the embryonic chick RPE to regenerate retinal neurons, only Müller glia within the retina can generate new retina neurons in postnatal chicks [138].

Similar to the chick, regeneration of the retina in fish is generated by the Müller glia in the inner nuclear layer of the uninjured retina and can be induced by damage to the photoreceptor cells [139, 140]. Müller glia, thus, appear to act as progenitor cells during regeneration of the retina. In goldfish, regeneration is initiated after damage to rod cells, photoreceptor cells and dopaminergic neurons within the retina [141]. Surprisingly, whereas selective damage to dopaminergic neurons does not result in their restoration, selective damage to rod cells or cone cells is followed by restoration [142].

2.2 Dedifferentiation: the main mechanism of heart and limb regeneration

2.2.1 Heart regeneration

It has been shown that injured or resected ventricles can be regenerated in fish and amphibians, resulting in a fully functional, normal heart [143, 144]. Recent publications suggested that the major source of regenerating heart muscle are pre-existing, dedifferentiating cardiomyocytes [145, 146]. Genes involved during cardiac development, like *gata4*, *hand2* and *nkx2.5*, but also genes known to affect progenitor cells, like *notch1b*, *deltaC* and *msxb*, are evidently re-expressed by the cardiomyocytes in regenerating hearts [147, 148]. Although dedifferentiation is generally regarded as the main cellular source for cardiac regeneration, there is still an ongoing discussion. For instance, Jopling and colleagues were not able to confirm the upregulation of *hand2* and *nkx2.5*, arguing against extensive dedifferentiation of cardiomyocytes as a pre-requisite for their proliferation [146]. Since research on skeletal muscle regeneration has produced such a vast amount of data about satellite cell-mediated regeneration (see section **2.3.1**), it is tempting to generalize that the prevalent source for muscle regeneration are stem/progenitor cells. In this context, the reported *gata4* expression during heart regeneration could possibly mark a pool of progenitor cells [145]. Further, growth factors, in particular the Platelet-derived Growth Factor (PDGF), seem to be involved in cell cycle progression of cardiomyocytes during heart regeneration [149]. Despite the discussed contradicting results, the common opinion remains that cardiomyocyte dedifferentiation is the main cellular source for heart regeneration.

2.2.2 Limb regeneration

As described above (see section **1.1.3**), appendages, for example in salamanders, regenerate by formation of a blastema which contains progenitor cells. Evidence has been provided that the source of the blastema cells primarily originates from the first few millimeters of the plane of amputation and that these cells lose their differentiated morphology [73, 150, 151]. Recent publications suggested that the majority of myogenic progenitor cells contributing to the blastema are dedifferentiated cells from muscle fibers and myotubes [74, 152-154]. Only a small fraction of stem cells, like satellite cells for example (the muscle stem cells), is suggested to contribute to the blastema. In addition to muscle cells, other cell types like cartilage, fibroblasts, Schwann and connective tissue cells are also thought to contribute to the blastema, forming the regenerated appendage [73, 150, 151, 155-157]. The question whether the cells in the blastema are multipotent or only give rise to the tissue they originate from has recently been resolved: tissue-specific cell labeling in the axolotl revealed that each tissue produced restricted progenitor cells which in turn contributed to a very limited range of tissues [155] (Fig. 2).

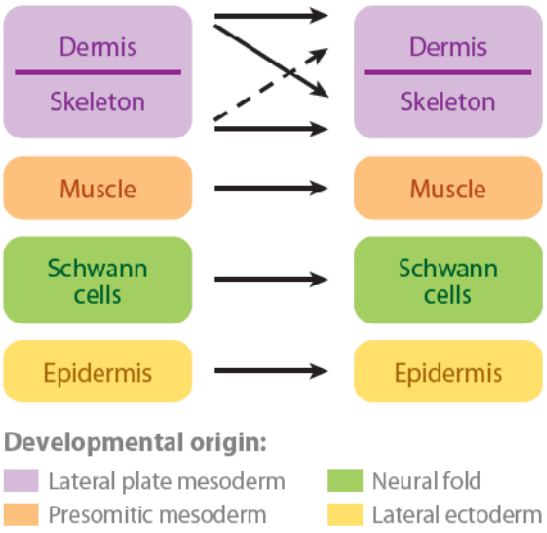

Figure 2: Schematic representation of cell fates during regeneration of the axolotl limb, as revealed by cell fate mapping [155]. Cell types contributing to the regenerate are strongly restricted to their own lineage. Figure adapted from Nacu et al. [158]

That cells are very strongly restricted to their lineage has also been confirmed by Rinkevich and colleagues (2011). Furthermore, they showed that, during regeneration of the digit tip in mice, several lineage-restricted stem cell/progenitor cell pools mostly contribute to the regenerate, since progenitor cell-specific GFP expression was sustained over a 3-month period [159]. The possibility that Kragl and colleagues also detected these lineage-restricted stem

cell/progenitor cell pools - instead of dedifferentiated cells that contribute to the blastema - cannot be excluded due to the limitation of the methods they used. Thus, in contrast to the common hypothesis of dedifferentiation, pools of progenitor cells might play a larger role than is commonly thought during regeneration of the vertebrate limb. Future experiments need to clarify whether these two processes act in parallel or whether one is superior.

2.3 Regeneration through stem cells

2.3.1 Skeletal muscle regeneration

The stem cells residing in skeletal muscle, called satellite cells, are located between the sarcolemma (plasma membrane) of the myofiber and the basal membrane [160, 161]. Satellite cells (SCs) are normally quiescent but after myotrauma they become activated and undergo proliferation, self-renewal and differentiation into new myofibers [162-165] (Fig. 3). The healing process generally consists of three phases: destruction, repair and remodeling. SCs can be characterized and distinguished by expression of specific markers during the different phases. Expression of *pax7* can be observed in quiescent SCs migrating to the site of injury, while they up-regulate *myoD* and *myf5*, two myogenic regulatory factors (MRFs), in the transition phase to become proliferative [52, 166-169]. From this point on, SCs are called myoblasts, characterized by down-regulation of *pax7* and up-regulation of *mrf4* and *myogenin*, which are also MRFs [52, 166, 167, 170, 171]. Eventually, the differentiated myoblasts either fuse to damaged myofibers or form new multinucleated myofibers [164, 172]. The current knowledge about the signals inducing SC activation was discussed above (see section **1.1.2**).

During skeletal muscle regeneration, the highly mitogenic IGF-I seems to be the main growth factor involved, although others like IGF-2, HGF, FGF-2 and -6, VEGF, PDGF-AA and –BB and SDF-I also play a role [164, 172-178]. *In vitro* studies showed that IGF-I, and later also IGF-2, can alter the expression of MRFs and additionally promote the proliferation of SC-derived myoblasts and later their differentiation [164, 179]. Further, IGF-I is essential for skeletal muscle growth in particular [180, 181], which was confirmed by over-expression studies leading to muscle hypertrophy [182].

The major inhibitory factors identified in skeletal muscle regeneration are members of the TGF-β superfamily, namely Myostatin, BMPs, TGF-α and –βI [183]. Myostatin is thought to maintain quiescence of SCs and induce $p21^{CIP}$, which leads to repression of self-renewal [165, 184]. The consequence of inhibition of TGF-β is prevention of muscle fibrosis and enhancement of skeletal muscle regeneration, pointing out the inhibitory effects of TGF-β [181].

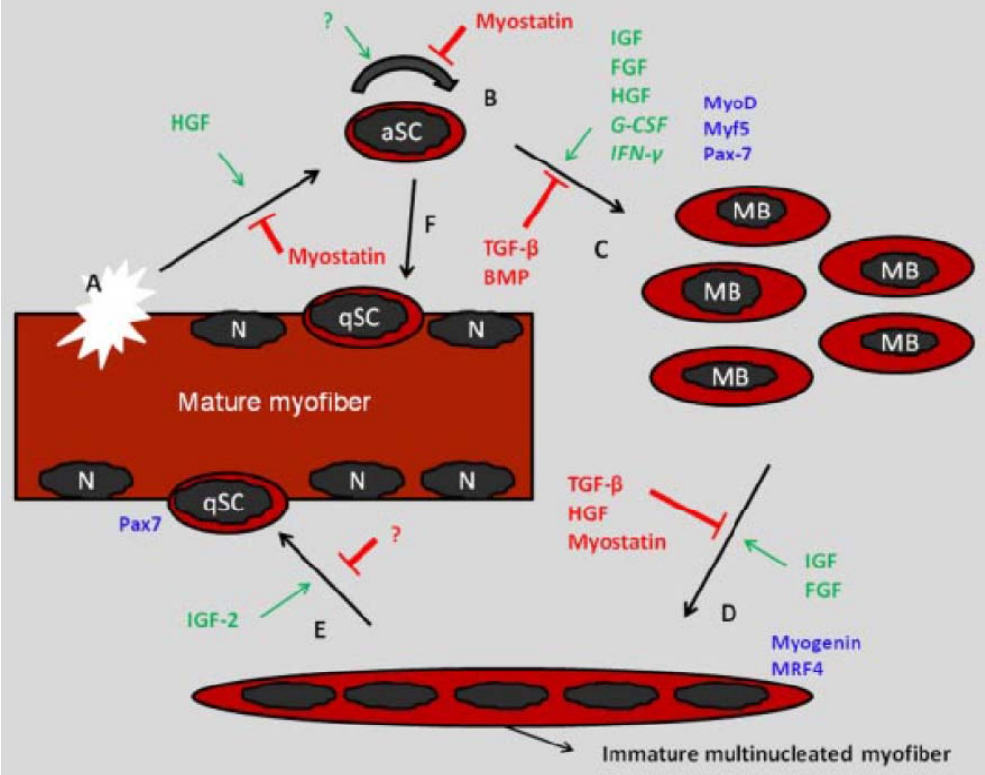

Figure 3: Regulation of the muscle stem cells, the satellite cells. Following injury of the muscle (myotrauma) (A), quiescent satellite cells are activated and self-renew (B) or proliferate (C). The differentiated myoblasts fuse to damaged myofibers (not shown) or form new immature multinucleated myofibers (D). Eventually, the central satellite cell nuclei migrate to a subsarcolemmal position in mature fibers (E). Additionally, a subset of satellite cells re-enters the quiescent state to replenish the satellite cell pool (F). Activating factors are shown in green with the arrow pointing at the step that they activate. Inhibiting factors are shown in red. Purple color indicates characteristic expression of a certain marker for a specific cell type. aSC: activated satellite cell; qSC: quiescent satellite cell; MB: myoblast; N: nucleus. Figure adapted from Ten Broek at al. [185]

Several other stem cells in addition to SCs show myogenic potential, hematopoietic stem cells (HSCs) being the most relevant ones after SCs in skeletal muscle regeneration [164, 186-188].

2.3.2 Planarian regeneration

The remarkable regenerative ability of planarians is strictly dependent on neoblasts [14, 95, 189-191], which can give rise to all cell types, somatic as well as germline cells [192-194]. Generally, neoblasts have a low cytosolic content and are characterized by *piwi* and *bruno-like* expression, while being the only somatic proliferating cells in the organism [195, 196]. Although the existence of pluripotent neoblasts has been shown, it is not known if all neoblasts are pluripotent or if the neoblasts-population is heterogeneous and also includes more restricted pools [4, 197]. Apart from germline precursors, it is unclear whether there are lineage-committed adult stem cells in flatworms [198].

2.4. Hydra uses diverse cellular sources for regeneration

During the first 12 hpa in *Hydra*, proliferation of cells at the site of cutting strongly decreases [199]. Further, there is no localized proliferation of endodermal epithelial cells at the tip of the regenerating tissue [200], demonstrating that the prevalent mechanism of regeneration in *Hydra* is morphallaxis, a type of regeneration in which the existing body parts or tissues transdifferentiate into newly organized structures in the absence of proliferation [6, 20]. Besides the above-mentioned mechanism, *Hydra* can also regenerate from stem cells under specific circumstances. Three stem cell types are known that enable new tissue production: ectodermal and endodermal epithelial cells and interstitial cells [6]. The interstitial cells are found within the epithelial cell layers. Following mid-gastric amputation, stimulation of these interstitial cells occurs: they become highly proliferative and contribute to regeneration [201, 202]. Experiments showed that interstitial cells are able to generate nematocytes, neurons, secretory cells and gametes, but are unable to form epithelial layers [203].

2.5. Conclusion

In summary, the origin of cells contributing to regeneration can either be a pool of sequestered stem/progenitor cells or cells in mature tissue that undergo cellular trans-/dedifferentiation. In invertebrates, the cellular source can be stem cell only (planarians) or stem cells combined with morphallaxis (*Hydra*). In vertebrates, most organs and tissues seem to regenerate on stem cell basis. Skeletal muscle regeneration represents one of the best studied examples for stem cell-mediated regeneration. Limbs and tails are thought to regenerate through dedifferentiation, but there has not been definite proof for this hypothesis, whereas recent studies indicate that lineage-restricted progenitor cell pools are the main cellular source for limb regeneration. Either way, genes expressed in progenitor cells of the different tissues/organisms during development are regularly found up-regulated in cells building the regenerate. These genes can be specific for the lineage, e.g. *myogenin* during muscle regeneration/development, but also general, for instance factors involved in FGF, TGF-β or Wnt/β-Catenin signaling pathways.

Taking everything into account, it is obvious that stem cells are widely used as the cellular source for regeneration, while only few cases are known to regenerate via trans- or dedifferentiation. Further, specific cellular sources are not restricted to one species or structure only. The question, why a specific cellular source is used for regeneration of a

specific structure remains unanswered; experiments to address this research question are needed.

3. Proliferation and patterning during regeneration

The mechanisms determining the growth, the correct scale and shape of the regenerating organ are one of the most fascinating aspects of regeneration. Similar to other examples of organogenesis, there is a requirement for mitogens and patterning signals and the correct scale and shape have to be reached before the regeneration process is terminated. Due to the obvious similarities with embryonic development, I will describe regeneration capacity as a function of developmental stage before going into detail about the molecular mechanisms that control proliferation and patterning.

3.1 Relation to embryonic development

In *Xenopus* a correlation between regenerative capacity and developmental stage has been shown. Regeneration of amputated limbs can be observed in early stages - accompanied by an expression program including *fgf10* - but not in later stages in development [204]. *Xenopus* tails can regenerate robustly until metamorphosis, except for the stages 45-47 [22]. Suppression of this refractory period can be accomplished by manipulating H^+ ion flow or by experimentally increasing BMP or Msx1 signaling [22, 205]. A similar correlation between regenerative capacity and developmental stage has also been described in mammals. An amputated digit tip has been reported to regenerate in human children [206, 207]. Mice of all stages show the same phenomenon, provided that the amputation is distal to the first phalange [208]. The capacity for digit-tip regeneration is associated with *msx1* expression and BMP signaling [209]. Additionally, while there is no regeneration after cardiac muscle injury in adult mice, regeneration of cardiac muscle cells has been shown to occur in fetal mice [210]. A possible explanation for these observations might be that these juvenile or embryonic tissues are still in their growth phase and thus have easier access to the for regeneration necessary programs. Supporting this hypothesis is the fact that organisms with a high regenerative capacity continue to grow for most parts of their adult life [211]. In this context, studies have indicated that similar factors involved in development and regeneration are expressed at substantial levels in adult salamanders and zebrafish, presumably being used for either homeostatic maintenance or growth [212-214].

However, the growth of regenerating tissues should not be seen as a strict repeat of embryonic development. One of the key differences between regeneration and embryonic

development is that, during regeneration, only the injured organs respond to developmental cues that increase mass, whereas uninjured tissues retain their size. In contrast to this, organs develop and gain mass simultaneously during growth of the embryo. Another difference is the source of the dividing cells: as discussed above (see section **2**), pools of progenitor cells, generated by stem cells or dedifferentiated cells, form the new organ during most cases of regeneration. Either way, the cells have been maintained for months or years even, while tissues in the embryo develop from pools of progenitor cells, which origination from the fertilized egg has been only a short time ago.

3.2 Mechanisms of cellular positional memory, patterning and proliferation

Positional memory of cells is defined as the retained information about their proximodistal position in the mature limb. Based on results from transplantation experiments, which showed that in amputated appendages of salamanders only distal identities were regenerated compared to the positional memory of the cells at the amputation plane (Fig. 4), the 'rule of distal transformation' was formulated [215]. Interestingly, this rule only applies for vertebrates and not for insects, which do not exhibit restriction to distalization [216, 217]. Since, as discussed above (see section **2.2.2**), the blastema is a mixture of progenitor cells, it was not clear whether all cells contain a positional memory. So far, it has been shown that cartilage-derived cells obey the rule of distal transformation, while Schwann cell-derived cells do not [155].

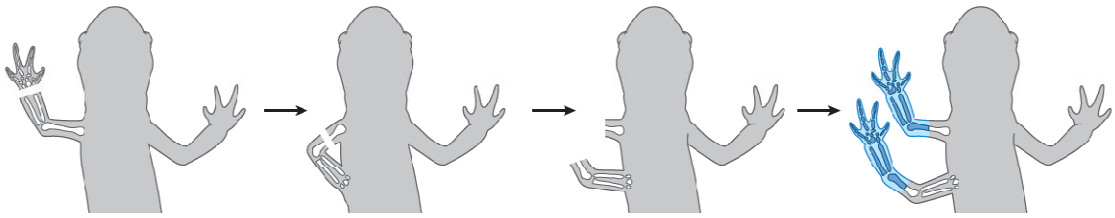

Figure 4: The rule of distal transformation, as formulated by Butler after amputation experiments [215]. A limb was amputated through the hand and inserted into the flank of the salamander. Afterwards, the limb is intersected through the upper arm. Only limb elements distal to the amputation plane (blue) regenerate in both cases. Figure adapted from Nacu et al. [158]

In order to assure the correct size of the regenerating organ, a coupling between cell proliferation and differentiation states by guidance mechanisms regulating the re-establishment of tissues must be present. In this section, I will give examples of such guidance mechanisms during regeneration.

3.2.1 Cadherin signaling

A signaling system controlling tissue growth has been identified which relies on graded differences of cell surface molecules: Two members of the cadherin family, *fat* and *dachsous*, both characterized in cricket leg intercalation and *Drosophila* wing disc cells, were shown to interact in a heterophilic way. An expression in opposing gradients of *dachsous* and *four jointed*, a regulator of *fat/dachsous* interaction, has been described [218, 219]. Further, differences in *dachsous* expression between neighboring cells promote proliferation whereas a flattening of the gradient acts as a cell proliferation inhibitor [220]. Interruption of *fat* signaling during cricket leg regeneration leads to decrease in size and abnormal shape of specific leg segments, while cell number or identity of distal segments were shown to be un-affected [216].

3.2.2 RA-Prod1-MEIS signaling

Retinoic acid (RA), which is provided by the WE during limb regeneration, was also shown to be a potent morphogen [221]. If regenerating or developing appendages were treated with distinct levels of RA, the proximo-distal patterning was adjusted so that blastemas that normally formed distal structures were reprogrammed to form proximal and distal structures [222-224]. The mediation of the proximalizing effect of RA is accomplished through binding to the RA receptor δ2 isoform (RARδ2), which is a nuclear hormone receptor [225]. By experimentally making use of the effect of RA, the extracellular glycosylphosphatidylinositol (GPI)-linked protein Prod1 was identified. *Prod1* is expressed at a higher level in proximal blastemas than in distal blastemas during normal regeneration [226]. Furthermore, a proximalization of the final location in distal blastema cells was induced by ectopic expression of *prod1* [227]. It was suggested that *prod1* is a downstream target of MEIS, due to two MEIS binding sites in upstream sequences of *prod1* [228]. Axolotl MEIS factors, a family of homeodomain transcription factors, were shown to be critical mediators of the RA-induced proximalizing effect. Transcripts of *meis* are increasingly found in upper limb compared to lower limb blastemas and MEIS protein is only nuclear-localized in upper arm blastemas, while it is localized in the cytoplasm in hand blastemas [155, 229].

3.2.3 HOX signaling

HOX genes are a topographically controlled set of genes and well-known regulators of morphogenesis. HOXA homeodomain transcription factors are known for their importance during limb development [230]. Surprisingly, the initial order of expression of *hoxa* genes was shown to be different in regeneration compared to development [231]: during limb development,

the expression domain of *hoxa13* is more distally restricted than that of *hoxa9*, which is also expressed earlier, pointing out that HoxA9 has a function in specifying more proximal limb structures, while HoxA13 specifies distal structures. During regeneration, on the other hand, *hoxa9* and *hoxa13* expression start simultaneously within 24 hpa and their expression domains within the blastema segregate to a proximo-distal distribution only in later stages of regeneration. These data suggest that during regeneration, unlike development, the appearance of distal structures is observed first, with subsequent intercalation of intermediate values [231].

Research on limb development revealed a gene network between *fgf8*, *RA*, *RALDH2* (the RA-synthesizing enzyme Retinaldehyde dehydrogenase 2), *MEIS1/2*, *prod1*, *bmp2* and *hoxa13/d13* [158] (Fig. 5). Since all these factors were also shown to play a role during limb regeneration, it can be hypothesized that they interact in a similar way and that the same gene network applies for regeneration as well.

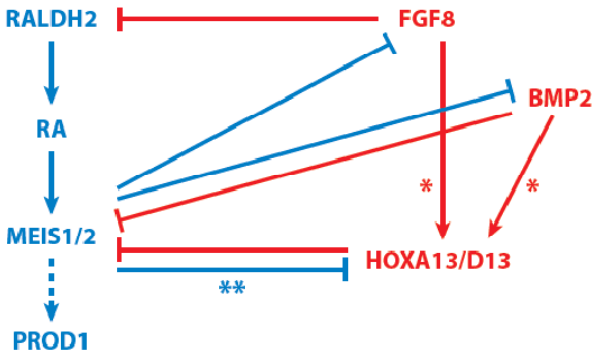

Figure 5: Interactions between distalizing (red) and proximalizing (blue) factors during limb development and seemingly also limb regeneration. Dashed arrows indicate potential activation, solid arrows indicate definite activation, and inhibition lines indicate repression. Single asterisk-marked pathways may function through direct activation of the MEIS pathway, while the pathway marked with a double asterisk may function through inhibition of upstream factors or direct inhibition. RALDH2: Retinaldehyde dehydrogenase 2 (RA-synthesizing enzyme); RA: Retinoic acid. Figure adapted from Nacu et al. [158]

3.2.4 Wnt signaling

As mentioned above (see section **1.1.3** and **1.1.5**), Wnt signaling was shown to be required for appendage regeneration in frogs, salamanders and fish [67, 82, 90]. The remarkable self-organization of aggregates made from dissociated cell suspensions in *Hydra* was shown to be at least partly mediated by canonical Wnt signaling. Expression of *wnt* and *tcf* was detected in the putative *Hydra* head organizer and they are, together with *β-catenin*, which is transcriptionally up-regulated during head regeneration, required for regeneration of the head region. Thus, Wnt/β-Catenin signaling is likely to be strongly involved in a molecular

network guiding axis induction [116]. Knock-down of β-Catenin in planarians consequences in ectopic head formation in all body regions, pointing at a possible gradient in canonical Wnt signaling [98, 100]. If the neural guidance molecule *slit* is knocked-down, on the other hand, structures from the nervous system form ectopically [232]. An active maintenance of patterning signals, like morphogen gradients, thus plays a role in setting up a positional memory in planarians.

3.2.5 Membrane voltage

It is known that biophysical signaling events, i.e. membrane depolarization, accompany early embryonic development. Recently, a similar biophysical signal was demonstrated to have clear effects on flatworm regeneration. A chemical genetic screen identified the H^+/K^+ ATPase as a regulator of anterior fate [233]. Additionally, it was shown that membrane voltage is regulated by H^+/K^+ ATPase during anterior regeneration, since chemical inhibition of H^+/K^+ ATPase activity in high K^+ medium, which depolarized the plasma membrane of regenerating flatworm fragments, led to restored head regeneration [234]. Further validation was obtained by the use of voltage-sensitive dyes: depolarization was detected in membranes of fragments undergoing anterior regeneration, while hyperpolarization was found in membranes of posterior regeneration [234]. Beane and colleagues also showed that anterior blastemas exhibit elevated levels of intracellular Ca^{2+}, relative to posterior blastemas, and that chemical inhibition of voltage-gated calcium channels resulted in anterior regeneration defects. These results support the hypothesis that H^+/K^+ ATPase-induced membrane depolarization might activate voltage-gated calcium channels [234]. The followed increase in intracellular Ca^{2+} could affect calcium-responsive transcription factors, such as CREB or NFAT [235]. In line with this hypothesis are results demonstrating that *in vivo* RNAi of voltage-operated Ca^{2+} channel (VOCC) β subunits ablated the bipolarizing effects of the drug PZQ [236]. An extension to the above-mentioned hypothesis can be added: since Ca^{2+} signals are associated with antagonistic effects on canonical Wnt signaling during patterning events in other systems [237], changes in Ca^{2+} influx could have an impact on the distribution and concentration of β-Catenin during regeneration and consequently on overall anterior-posterior patterning in flatworms [236].

Similarly, *Xenopus* studies have revealed that V-ATPase H^+ pump activity is required for tail regeneration, but not tail development or wound healing [205]. Besides the failure to regenerate, Adams et al. also showed that a loss-of-function of the V-ATPase led to a drastic decrease in cell proliferation and a miss-patterning of neural components [205]. Comparable to

these results, ion transport through the voltage-gated sodium channel $Na_v1.2$ is necessary and sufficient to induce regeneration of *Xenopus* appendages [238]. A possible interaction between these two bioelectrical regulators can be hypothesized. Moreover, recent experiments showed that membrane voltage controls differentiation of human mesenchymal stem cells (MSCs) *in vitro*, suggesting a possible similar role during regeneration [239, 240].

3.2.6 TGFβ signaling and the Hippo pathway

TGFβ signaling was found to be of importance during the re-establishment of the bilateral symmetry in regeneration of irregularly cut planarians. Identified to regulate this process were three genes: *smelloid-1* (a *bmp1/tolloid* homologue), *smedsmad4-1* (a *smad4*-like gene) and *smedbmp4-1* (a homologue of *bmp2/4/dpp*). New *smedbmp4-1* expression prior to blastema formation was detected in asymmetric fragments lacking a midline. In contrast, a towards the wound expanded *smedbmp4-1* expression was displayed by asymmetric fragments containing the midline. These results give indication of how BMP activity resets the midline in injured animals that lack left-right symmetry [103].

TGFβ/BMP signaling-induced self-renewal in embryonic stem cells was shown to be regulated by YAP and TAZ, two core components in the Hippo pathway, during development [241, 242]. These results point towards a role of the Hippo pathway during regulation of self-renewal in stem cells and progenitor cells. It therefore is likely that components of the Hippo pathway have similar effects during regeneration. Indeed, loss of YAP severely impairs intestinal regeneration [243]. It remains to be determined, though, whether Hippo pathway activity is generally involved in regeneration.

3.2.7 Hedgehog signaling

Activity of Sonic hedgehog (Shh) is required in regenerating tissues in amphibians and fish. Inhibition of Shh signaling leads to severe reduction in the number of distal digits in salamanders and similarly blocks regenerative outgrowth of fish fins [244, 245]. So far, Shh appears to have the same role during regeneration and embryonic development [122].

3.2.8 FGF signaling

Experiments in fish have shown that the rate of outgrowth of the regenerating fin decreases as it reaches distal structures. Results showing that the expression of Fgf transcriptional downstream targets decreases as the growth rate decreases suggest that the positional information has to be coupled to the level of Fgf signaling [246]. Additionally, the

gene *fam53b/simplet* (*smp*) has been associated with the regulation of tissue patterning and cell proliferation in vertebrates, since experimental knock-down inhibited cell proliferation and thus reduced the regenerative outgrowth [247].

3.2.9 microRNA signaling

Moreover, microRNAs have been suggested to play an important role during tissue formation and patterning. For example, expression of the miRNA-133 is down-regulated in regeneration compared to the uninjured fin in zebrafish. Loss of miRNA-133 even has the capacity to compensate for lost Fgf signaling und consequently restore regeneration in fish fins [248]. Another microRNA shown to be important for regeneration is miR-196, which is also expressed in the blastema. Inhibition in salamanders leads to abnormally shortened tails with defects in the spinal cord [249].

3.2.10 Secreted peptides

Secreted peptides in *Hydra* have been shown to have an impact on foot and head regeneration. Shown to be a potent inducer of head formation and apical fate was the 12-amino-acid peptide HEADY [250]. Hym-301, another novel peptide, was identified as having direct effects on the epithelial cells that form the tentacles: if knocked-down, the formation of tentacles is reduced [251]. Furthermore, Hym-346 appears to have accelerating effects on foot regeneration [252]. The gene *anklet* seems to play a role in basal disk formation, since suppression of its expression during foot regeneration resulted in a significant decrease in basal disk size and a smaller foot [253].

3.3 Conclusion

Many concepts seen during embryonic development are re-used during regeneration. Expression of *hox* genes provides positional information as do gradients of morphogens of proteins involved in Wnt/β-Catenin and TGFβ signaling. Retinoic acid as well as Sonic Hedgehog play a role and microRNAs are also involved in patterning the growing organ. Additionally, a role of bioelectrical signaling events, as in early embryonic development, is suggested to affect the patterning process in both vertebrates and invertebrates. But still, there are many gaps in our current knowledge as we do not understand how the correct size and shape are established or how exactly regeneration is eventually terminated. So far, it seems that the proliferation and patterning processes in regeneration and embryonic development are very similar, as only few examples of differences have been reported. Does this reflect the reality or is it possible that scientists prematurely accepted the hypothesis that, aside from the

initiation of regeneration, mechanisms are basically a recapitulation of embryonic development?

4. Influence of aging on regeneration

4.1 The role of $p16^{INK4A}$

Similar to the differences of regenerative capacities between developmental stages (see section **3.1**), there is also a significant impact of aging on the regenerative capacity of organisms. It has been repeatedly stated that numbers and/or functionality of stem cells decrease during aging and, consequently, homeostasis and regeneration become disrupted [254-258]. Hematopoietic stem cells originating from older mice, for example, display a decreased capacity for long-term reconstitution in irrediated mice [259]. With age, a reduced islet cell proliferation coupled with lower regenerative capacity has been observed in the pancreas [260]. Less neurogenesis in the subventricular zone takes place in aged mice and, to name a last example, decreased satellite cell-mediated myogenesis in response to injury has been described for aged mice [261, 262]. The molecular mechanisms behind this inverse relationship between regenerative capacity and age are largely unknown. It has been described, however, that increasing $p16^{INK4a}$ levels negatively affect regeneration of aforementioned forebrain progenitors and islets in the pancreas, and also cause stem cell aging [259-261]. Moreover, an observed age-related decrease in regenerative capacity in mice is due to the niche rather than the stem cells themselves, as shown by parabiotic pairings of mice [262]. Recent studies pointed out that an age-induced increase of Wnt signalling in myogenic progenitor cells is responsible for alterations of satellite cell fate in muscles and an increase in fibrosis [263, 264].

4.2 Epigenetic causes

A comparison between young and old mice brought to light that the forebrain neural stem cells of senescent mice have a lower self-renewing capacity than their embryonic, fetal and early post-natal equivalents. A correlation was made to the High mobility group A2 (HMGA2) protein, which is involved in recruitment of transcription factors and chromatin structure. While HMGA2 was switched off in senescent mice, it was shown to be a promotor of self-renewal in young mice [265].

4.3 Rejuvenation and Regeneration

Repeated injury in annelids was shown to have rejuvinating effects on their soma [266]. Similarly, fission, starvation and regeneration are often claimed to have rejuvenating effects

in flatworms [14-16, 267-270]. This hypothesis is based on two important observations. Firstly, during these three processes flatworms transform to a physical condition resembling that of juveniles after which they grow again. In other words, a phase of reduction of tissue volume is followed by cell proliferation and renewal leading to regeneration and regrowth to the original sized worm with new, "younger" cells [267, 269]. Secondly, starved and regenerated flatworms have an increased life-span [15, 268-270]. However, as the observed life-span extension could also be accomplished by slowing down the aging process, it does not necessarely include rejuvenation [271]. Thus, the reversal of aging was not unambiguously proven yet and thorough experiments are needed to answer the question of rejuvenation in flatworms and annelids.

4.4 Conclusion

Taken together, aging clearly has an effect on the regenerative ability of organisms. On the other hand, regeneration also seems to affect aging by slowing it down or even reversing the aging process (rejuvenation). Unfortunately, very little is known to date about the molecular mechanisms underlying these phenomena and more research in this direction is needed. The main questions include: Are there differences between the age-related decrease of regenerative capacity between different species? Is aging actually reversed in repeatedly cut worms or just slowed down? What are the molecular causes of this relationship between aging and regeneration?

5. Regeneration in the light of evolution

The ability to regenerate lost parts of the body varies widely in metazoan phyla (Fig. 1). The regenerative capacity can even vary between closely related organisms [272-274]. In the following section, different theories about the evolutionary perspective of regeneration will be discussed.

5.1 Origin and loss of regeneration

Phylogenetic analysis leads to the critical hypothesis that regeneration is primordial and basic attribute of metazoans and not a mechanism that newly and independently evolved in a variety of contexts [275, 276]. Further, due to obvious similarities and strongly coupled occurrence across animal phyla, it has been hypothesized that regeneration has emerged out of asexual reproduction [275].

In the assumption of active selection of regenerative capacity during the course of evolution, several predictions should be met. A specific structure should be frequently lost, its

absence should significantly decrease the fitness of the animal, yet, the loss of the structure should not lead to death of the animal during regeneration and the costs of regeneration should not outweigh the benefits of replacement [7, 276]. More targeted experiments are needed, though, to assess the costs and benefits of tissue loss and regeneration.

Surprisingly, there is no record of high amputation frequencies in the wild for *Hydra* and planarians, some of the most highly regenerative animals. As a consequence, it is most likely that there are other mechanisms than direct selection maintaining regeneration [7]. The **pleiotrophy hypothesis** states that the regenerative capacity of a specific structure is retained because it is developmentally coupled with a different, related phenomenon, for example asexual reproduction. The high correlational incidence of regeneration and asexual reproduction in most animals may reflect the origin of regeneration, i.e. as a co-option of asexual reproductive mechanisms [275]. The **phylogenetic inertia hypothesis** suggests historical reasons for the retainability of regeneration that is currently neither advantageous nor retained by pleiotrophy [7]. Very similar to this is Morgan's **epiphenomenon hypothesis**. Taking into account that regeneration can be lost in closely related species and that the capacity was lost in numerous events across animal phyla, Morgan (1901) postulated that regeneration is an epiphenomenon - a by-product - that is neither selected for, nor against, its adaptive significance [20]. In this model, regeneration would not be under selective pressure and could be lost for a variety of reasons. Regarding the epiphenomenon hypothesis, it can further be hypothesized that neoblasts-mediated regeneration in *M. lignano* is a by-product of tissue homeostasis. Clearly, neoblasts are central players in homeostasis, regeneration, development and asexual reproduction, however, asexual reproduction is lost in *M. lignano* (and several other flatworms) and results have been published pointing out a special relation between homeostasis and regeneration. First, elimination of neoblasts through radiation inhibits both processes. Second, expression of *macpiwi* in neoblast subpopulations is required for regeneration and homeostasis, but not for early development [277]. These results suggest that regeneration could be a by-product of homeostasis. Nevertheless, the disrupting effect on cell proliferation of *macpiwi* RNAi can only be observed in *M. lignano* and not in other flatworms, whereas differentiation of stem cell progenitors is generally disrupted [195, 277]. Therefore, no evolutionary conservation of this special relation between regeneration and homeostasis has been shown and a straightforward answer regarding the homeostasis by-product hypothesis in flatworms cannot be given. Further investigations will need to clarify this matter.

5.2 Regeneration as a neutral trait

If regeneration indeed is a neutral trait, it could be lost for several reasons. The **frequency** of structure loss could be significantly decreased, making regeneration ecologically irrelevant. Either a change in the species itself could have occurred, e.g. increased defense ability or predator avoidance, or the type of damage caused by a predator could have changed, e.g. increased predator efficiency resulting in lethal damage rather than sub-lethal damage [7].

Further, a decrease or increase in the **functional importance** of the structure could also have an effect on the evolution of regeneration. According to the principle that regenerating structures must be important but not crucial [276, 278], a decrease in functional importance of a structure would make regeneration not worthwhile anymore, while an increase in functional importance would make the structure too important and the animal would die before being able to regenerate.

Also, a break in tight pleiotropic interaction could lead to an uncoupling of regenerative ability which could then be lost due to direct selection [7, 279]. Experiments revealed that limb development in amphibians is delayed relative to amniotes. It has been decoupled from interactions with a transient structure like the somites, and such interactions are no longer present at this late stage. Therefore, the limb seems to develop as a semi-independent module, enabling regeneration to occur in this background [279]. Finally, a change in energy allocation tradeoffs and non-energetic tradeoffs could play a role. In this context, the transition to warm-blooded vertebrates might be of particular interest, since these have much lower regenerative capacities [276, 280]. Sheer physical size could also be limiting regeneration in humans and other mammals. Embryonic limb development occurs when all the organ systems are small. Pattern formation and morphogenesis - e.g. cell migration, diffusion or cell-cell interactions - in a large structure may limit regeneration considering the diameter of a human arm, compared to the size of a human embryonic forelimb bud. Surprisingly, there is some evidence that tail regeneration can occur in alligators [64, 281], although there is no proof that full functionality of the regenerated tail is regained (Fig. 6). Still, it proofs the point that large structures can be regenerated as well.

Figure 6: Picture of four juvenile alligators (Alligator mississippiensis) of ca. 3-4 feet in length, Barataria Preserve of Jean Lafitte National Historical Park and Preserve, south of New Orleans. According to park rangers, one alligator was in the process of regeneration of an amputated tail (inset). Photographed in November 2004 by Carol A. Burdsal, adapted from Han et al. [64]

5.3 Increase of regulation during the evolution of regeneration

It was previously hypothesized by Sánchez Alvarado (2000) that with increasing morphological complexity an increase in regulation of cellular pluripotency is observed (Fig. 7). While wound healing always represents the first step, several regulatory steps are added as evolutionary complexity increases: in comparison to *Hydra*, additional blastema formation can be observed in planarians. The widely recognized hypothesis is that, in vertebrates, the blastema is formed by dedifferentiation of post-mitotic cells [275]. This hypothesis has to be met with caution, as discussed above (see section **2.2.2**), since no hard evidence has been published that undoubtedly proofs dedifferentiation as the main mechanism for vertebrate blastema formation. Further, in vertebrates, blastema formation is only required for regeneration of appendages, while other organs like the eye and the heart, for example, do not form a blastema during regeneration. This also emphasizes the restriction of Sanchez' hypothesis. On the other hand, a clear evolutionary link can be drawn: the formation of the blastema has not been observed in *Hydra*, while blastema formation is necessary for successful regeneration in planarians and is also required for appendage regeneration in vertebrates. A possible regulatory step from totipotent stem cells in planarians to strongly lineage-restricted progenitor cell pools in vertebrates can further be hypothesized. But, as mentioned before (see section **2.3.2**), it has not yet been shown whether all neoblasts - except for germline precursors - is totipotent [4, 197, 198]. Lineage analysis will be required to clarify the

population characteristics and dynamics of neoblasts. Similarly, regarding heart regeneration (see section **2.2.1**), it can be hypothesized that additional steps regulating progenitor cell activation have been introduced in higher vertebrates. This would explain why fish and salamanders are able to regenerate parts of their hearts while mammals cannot.

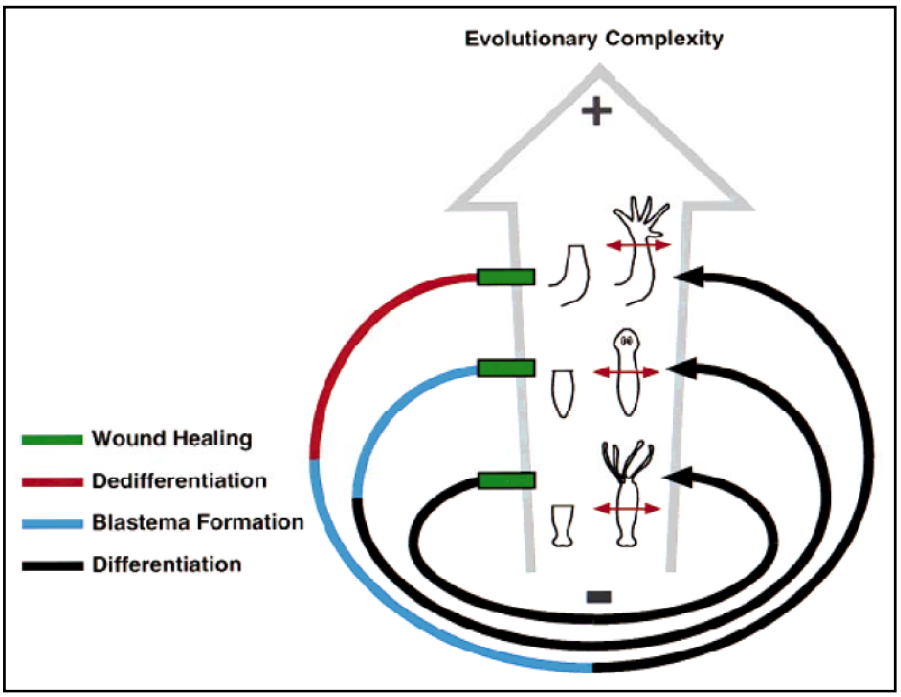

Figure 7: Increasing morphological complexity leads to increased regulation of cellular pluripotency. As evolutionary complexity increases, several regulatory steps are added: wound healing, blastema formation, dedifferentiation and differentiation. Three representative model organisms are included: *Hydra* (Cnidaria), planarian (Plathelmithes) and the salamander (Chordata) limb (from bottom to top, with increasing evolutionary complexity). Adapted from Sánchez Alvarado [275]

Nevertheless, there seems to be an obvious evolutionary trend towards higher lineage-restriction of the regenerating cells.

5.4 Regeneration and cancer

It further has been hypothesized that higher regenerative capacity in mammals could lead to a higher incidence of cancer. This is due to the obvious reason that regeneration involves a certain degree of plasticity, predisposing the organism to proliferative disorders [282, 283]. Contradicting this hypothesis are results showing that urodeles are remarkably resistant to carcinogenesis and that application of carcinogenic chemicals does not evoke tumors but rather supernumerary eyes [284]. There is no support for the initial hypothesis that regenerative capacity is coupled to a higher incidence of cancer [282].

5.5 Conclusion

In summary, regeneration seems to be an ancestral trait. This is confirmed by the high correlation between molecular mechanisms used in various organisms, as elucidated above and below. The frequent loss across animal phylogeny suggests that regeneration in general is an epiphenomenon rather than under selective pressure. Nevertheless, the ultimate causes of regeneration loss remain poorly understood and molecular data supporting one or the other hypothesis is not yet available. Further, during the course of evolution, more steps regulating regeneration have been introduced, i.e. higher lineage-restriction of the regenerating cells.

Conclusion and future perspective

In order to regenerate successfully, several key steps need to be taken. As described above, cells first need to identify which body part is missing and thus has to be regenerated. After a wound response, the lost tissue has to be replaced. Proliferation has to be induced either in stem cell pools or in intermediary cells during trans- or dedifferentiation of formerly post-mitotic cells. Thirdly, patterning and differentiation of the new tissue need to be properly executed in order to build a structure that is similar to the amputated structure in form and function. Lastly, after correct re-growth, regeneration needs to be terminated. Every one of these different steps during regeneration is tightly controlled by gene networks and molecular mechanisms; the current state of knowledge has been presented in the previous sections.

For a long time, research on regeneration has lagged behind other fields in reaching the molecular age. Further, the inverse proportional relationship between the regenerative capacity and the available molecular methods in models organisms of regeneration has restricted the progress in this field [8]. During the last years, though, great progress was made (Table 1). On the one hand, the genetic toolbox of the traditional models of regeneration has been extended. For example, RNAi knock-down and transgenesis are now available methods for research in flatworms [285, 286]. Genome and transcriptome sequence information is further increasing, as well [287-289]. On the other hand, genetically more amenable species have started to being used for studying regeneration. Mice, for example, offer a broad range of methods. Especially advanced tissue- and stage-specific tools, for instance under the control of the Cre recombinase, are of great use[290-292].

Table 1: Model organisms and systems and the available tools for regeneration studies. Adapted from Poss [211]

	Hydra	Planarians	Zebrafish	*Xenopus*	Axolotl	Newt	Mouse	*Drosophila*
Key tissues assessed in regeneration studies	Whole animal	Whole animal	Fins	Tadpole tail	Tail	Limbs	Blood	Midgut
		Germ cells	Heart	Tadpole limbs	Limbs	Lens	Skeletal muscle	Germ cells
		Nervous system	Retina	Retina	Spinal cord	Heart	Liver	
			Spinal cord			Tail	Pancreas	
			Hair cells			Spinal cord	Peripheral nerve	
						Retina	Skin	
							Gut epithelium	
							Germ cells	
Tools:								
Genome sequence finished or ongoing	√	√	√	√	-	-	√	√
Knockout by homologous recombination	-	-	-	-	-	-	√	√
RNAi	√	√	-	-	-	-	√	√
Transgenesis	√	-	√	√	√	-	√	√
Recombinase driven lineage tracing	-	-	√	-	-	-	√	√
Cell transplantation or tissue grafting	√	√	-	√	√	√	√	-
Forward genetics	-	-	√	-	-	-	√	√
Potential for real-time imaging of regeneration	√	√	√(larvae)	-	-	-	-	√
Stage- or age-dependent regenerative capacity	-	-	-	√	-	-	√	-

Shown in this table are the tissues assessed in regeneration studies of selected model organisms (top) as well as the currently available tools for research in these organisms, indicated by a check mark (bottom).

Research in various model organisms and organs has provided the data at hand and in the following section the correlation between cellular and molecular mechanisms will be looked at. Also, I will discuss similarities and differences between the molecular mechanisms driving the various steps of regeneration across species. To end, future directions will be pointed out.

Cellular mechanisms of regeneration

As already mentioned above, there are, in terms of cellular mechanisms of regeneration, obvious differences between different organisms and also between different structures. The invertebrate *Hydra* mainly uses morphallaxis as regenerative response, while flatworms form a blastema at the site of injury, which directs the regenerative process. Similarly, appendage regeneration in vertebrates also requires the formation of a blastema. Regeneration of inner organs, such as skeletal muscle or the eye, are not directed by a blastema. Further, there are differences in the cellular sources driving regeneration. Morphallaxis in *Hydra* is mainly

driven by transdifferentiation, but *Hydra* can also regenerate through stem cells. Neoblasts, the flatworm stem cells, are responsible for regeneration in *S. mediterranea* and co. In vertebrates, the cellular source is different depending on the structure that is regenerating. Dedifferentiation of post-mitotic cells is the widely accepted mechanism hypothesized to drive heart regeneration, while the amphibian eye regenerates through transdifferentiation. Skeletal muscle regenerates by stem cell proliferation and differentiation. For the last years, dedifferentiation was hypothesized to drive vertebrate appendage regeneration, whereas recent results suggest that highly lineage-restricted progenitor cells, rather than dedifferentiated cells, mainly contribute to the re-growth. Whether the cellular source for limb regeneration are highly lineage-restricted dedifferentiated cells or highly lineage-restricted progenitor cell pools, in both cases a clear trend towards lower plasticity of the cells is observable in vertebrates compared to invertebrates. Stem cells in *Hydra* are pluripotent and can form any cell type. Whether flatworm neoblasts are exclusively (aside from germline precursors) pluripotent or whether there are some neoblasts-pools of more restricted potential is not clear to date. Regenerating cells in vertebrates on the other hand are evidently very lineage-restricted, whether we deal with progenitor cell pools or dedifferentiated cells in a blastema. In summary, growing complexity leads to lower plasticity of the cellular source of regeneration, which in most cases is a pool of stem or progenitor cells, representing an evolutionary trend of increased regulation.

Another form of increased regulation during the evolution of regeneration is the formation of the regeneration blastema, as hypothesized by Sánchez Alvarado [275]. Blastemas are not formed in *Hydra*, but appear in planarians and during limb regeneration of vertebrates. But if blastema formation represents an additional regulatory step, why is – in vertebrates – blastema formation not involved in the regeneration of other structures beside the appendages? Amputation of a leg, tail or fin leads to a large wound surface and cells from all three germ-layers need to be regenerated. It can be hypothesized that either a blastema is forming on (relatively) large external wounds or that a blastema is formed if complex structures including all three germ-layers need to be regenerated. At this point, neither hypothesis can be approved or rejected since there is no publication to date specifically investigating this matter.

Molecular mechanisms of regeneration

As elucidated above, the majority of the pathways and molecular mechanisms for regeneration are very similar across species and animal phyla. TGFβ, FGF and Wnt/β-Catenin signaling seems to be required for all steps of regeneration, in almost all structures and species covered in this thesis. As wound-response, cells from the immune system are attracted to the site of injury. Signaling through cytokines has been shown to be important in several systems/organisms. Commonly involved in initiation of regeneration are FGF, TGFβ, IGF and Wnt/β-Catenin signaling pathways. Often, Matrix metalloproteinases (MMPs) degrade the extracellular matrix. Additionally to the already mentioned growth factors, signaling through VEGF, HGF and EGF pathways further contributes to the proliferation, growth and patterning process, although this is not known yet for all above mentioned systems and organisms, i.e. in regeneration of appendages or in *Hydra*. Gene networks involving *retinoic acid*, *meis*- and *homeobox-factors*, *fgfs* and *bmps* are required for a correct patterning and positional memory in some cases like limb regeneration. Similarly, cadherin signaling, microRNAs and secreted small peptides were shown to play a role in limb, fin and *Hydra* regeneration, respectively. It has to be mentioned that there is no evidence for the hypothesis that these signaling cascades are generally used in regeneration of all systems and species. Nevertheless, no results contradicting this hypothesis have been reported. The fact that most of these signaling pathways are found to drive regeneration in several model organisms underlines the hypothesis that regeneration is a primordial, basic attribute (Table 2).

Table 2: Signaling pathways involved in regeneration of selected model organisms and systems.

	Hydra	Planarians	Salamander limb	Vertebrate skeletal muscle	Vertebrate liver
Cytokines	√			√	√
MMPs			√	√	√
FGF signaling		√	√	√	√
TGFβ signaling	√	√	√	√	
EGF signaling		√			√
Wnt/β-Catenin signaling	√	√	√		√

Shown in this table are the most important signaling pathways involved in regeneration of selected model organisms/systems, indicated by a check mark. Blank fields indicate that signaling through a certain pathway has not yet been proven and that further research needs to verify whether it is involved in the regeneration of this system/organism or not.

So far, the differences observed in the cellular mechanisms are not reflected by the molecular mechanisms. The main reason for this is probably our restricted knowledge about the molecular networks contributing to regeneration. First of all, we still do not understand the regenerative process as a whole and secondly we need to be able to compare specific processes in several structures/species in order to unravel the molecular differences that are coupled to the choice of the cellular source, for example, the formation of a blastema or the decision to regenerate or not to regenerate.

Further, most of the pathways mentioned in this report play a similar, if not identical, role during development. As most molecular mechanisms have already been established during development, the adaption of these pathways for regeneration seems economical. Considering this, it is not surprising to find the same factors fulfilling the same actions during development and regeneration.

But why have mammals such low regenerative capacities compared to other species of the lower vertebrates? And why can some invertebrate species regenerate from tiny body pieces if developmental pathways are just being recycled? Clearly, the processes of development and

regeneration are decoupled and additional regulators are required for regeneration. During evolution towards the higher vertebrates, as the complexity of the body is increasing, more regulatory steps must have been added [275], which identification will be a key to understanding regeneration as a whole. In my opinion, most of the processes involving differentiation and patterning are very similar to embryonic development and in regeneration only minor modifications need to be applied. The initiation of the regenerative process, on the other hand, needs much more control and the biggest differences to embryonic development will supposedly lie here.

Another striking question is why there is such a poor correlation between regenerative capacity and normal physiological cell turnover? Part of an answer to this question could be that cell turnover is accomplished through the deletion of specific cells by genetic programs. Regeneration, on the other hand, is initiated by an exogenous stimulus. As was shown above, cytokines, as part of the inflammatory response provoked by such acute damage, were shown to initiate the regenerative process in some systems. In summary, the initiating mechanisms are completely different [1].

Future directions

The role of developmental pathways during regeneration is being uncovered in more and more organisms, as well as some regeneration-specific modifications. In some cases research has already unraveled so many molecular pathways that we come close to actually understanding large parts of the regenerative process, especially in regeneration of the skeletal muscle [185]. But still, the most important step of regeneration is not understood: how are developmental programs re-accessed? Wound-healing occurs in all animals, but how do factors of the immune system initiate regeneration in the one species while another species does not react to (the same?) cues? Or are there other signals apart from the immune system that bear power to initiate regeneration? In order to identify the mechanisms which are responsible for the variation in regenerative capacity, the comparison of closely related species with a largely similar body plan but a strong variation in regeneration abilities is necessary. An excellent group to investigate regeneration abilities in a comparative context are the annelids [273]. The two species *Diopatra cuprea* and *Americonuphis magna* were previously described to be well-suited for this purpose [293].

Further, future studies will have to clarify which cellular source is used for the different organs. Experiments making use of cell-lineage tracing will hopefully lead to the answer of

this question soon. Especially interesting is also the question why a certain cellular source is preferably used in a specific case? Where are the differences to the regenerative process between progenitor cell-based proliferation compared to trans- or dedifferentiation?

Another interesting and promising line of research is the study of bioelectrical signals during regeneration. Recently, state-of-the-art work in development and other fields has begun to identify the genetic networks that shape the bioelectric signals, the responsible proteins and the mechanisms that transduce the bioelectric information [239]. Certainly, results obtained here will contribute to a better understanding of regenerative mechanisms as well.

Likewise, deep insights will be gained if the specific differences between wound healing in fish or amphibians and in mammals become unraveled. Investigation of the molecular basis of the apoptotic strategies removing damaged tissue in fish, compared to scaring in mammals, will hopefully lead the way to possible manipulations in mammals. Particularly interesting to regenerative medicine are the capabilities of fish to regenerate brain tissue and spinal cord [276].

Moreover, the identification of markers that allow the distinction between differentiated and non-differentiated cells will be of great use for regeneration studies, in particular by determining the role of stem cells in it. For example, it is already known that there are differences in chromatin organization between the genomes of differentiated cells and embryonic stem cells [294]. The protein Nucleostemin, which was discovered in rats, was found to induce such differences. Nucleostemin can be detected in embryonic and adult neuronal stem cells - where it resides in the nucleolus - and also in many cancer cell lines. On the contrary, it is absent from all differentiated cells tested to date. *Nucleostemin* expression becomes undetectable if stem cells undergo differentiation and similarly elimination of Nucleostemin in stem cells prevented their self-renewing capacity and drove them into differentiation [295]. In salamanders, Nucleostemin appeared in nucleoli of cells preceding dedifferentiation in the eye and also blastema formation in the limb, suggesting that Nucleostemin is not only necessary for the maintenance of stem cells, but is also associated with dedifferentiation [296]. Recent studies indicate that the growth-suppressive activity of p53, a protein known for its ability to regulate local and global modulation of chromatin modifications [297], can be inhibited by interaction with Nucleostemin [298]. The necessary chromatin organization required by toti-/pluripotent cells may be determined by such interaction. This example clearly highlights the importance of the identification of differentiation/non-differentiation markers. Not only can they be used for visualization

purposes, but they obviously also play an active role in maintenance of a specific state or the induction of changes.

Many human diseases are caused by quantitative or functional deficiency of particular cells. Examples include certain forms of liver and heart disease, neurodegenerative disorders, diabetes and some types of blindness and deafness. Regeneration research bears the power to reveal the molecular mechanisms that trigger regeneration in organisms with higher regenerative capacities and, as a consequence, can point out which interferences in signaling pathways bear good prospects to greatly improve the patients' health. The above mentioned examples thus represent the most promising targets of regenerative medicine in the future [299].

To sum up, with the current rate of progress in regeneration-research fundamental insights can soon be expected, which will eventually lead to new therapeutic possibilities in the field of biomedicine.

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