Zebrafish caudal fin regeneration: the effect of repeated amputations and ageing

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Regeneração da barbatana caudal do peixe zebra: o efeito de amputações repetidas e envelhecimento

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Abstract

Whilst all organisms developed schemes to respond to injury and illness, their capacity to recover from severe loss or damage of organs and appendages diverge quite a lot. A vertebrate organism that retained regenerative capacity is the zebrafish (*Danio rerio*). Its amenability to molecular and genetic manipulation turned it into a powerful regeneration model. In particular, zebrafish caudal fin regeneration has emerged as an ideal model to further study vertebrate regeneration due its accessibility and simple anatomical structure. The caudal fin is composed of several segmented bony rays. Each bony ray, with the exception of the most lateral, is bifurcated in the distal region of the fin.

Regarding the caudal fin regeneration process, it is commonly believed that regeneration efficiency is lost upon repeated amputations. The aim of my thesis was to characterize in detail whether there is a decrease in regeneration efficiency and to identify the signalling pathways that are altered, in response to repeated injuries. To this end, we designed a protocol of consecutive repeated amputations in which the same caudal fins were subjected to three consecutive amputations every month. This protocol was repeated 10 times and resulted in a total of 29 amputations in the end of the protocol.

Our results show that the size of the blastema, which is a structure comprised of progenitor cells that direct regeneration, and of the fully regenerated fin remains unchanged. Thus, consecutive repeated amputations of the zebrafish caudal fin do not reduce its regeneration capacity and do not compromise any of the successive regeneration steps: wound healing, blastema formation and regenerative outgrowth.

The inhibition of Wnt/β-catenin signalling using heat-shock-mediated overexpression of Dickkopf1 (Dkk1) completely blocks fin regeneration. We overexpressed *dkk1-gfp* twice daily starting shortly before fin amputation and until 4 days-post-amputation (dpa) to completely inhibit fin regeneration.

Once these fish were relieved from the heat-shock treatment, spontaneous regeneration did not occur. However, when fins were re-amputated at the non-inhibitory temperature, the caudal fin regenerated and reached its original length. To further challenge the regenerative capacity we performed repeated cycles of amputation, inhibition of Wnt/β-catenin signalling, recovery and second amputation. Remarkably, repeated blockage of blastema formation and fin regeneration after inhibition of Wnt/β-catenin signalling, did not diminish the regenerative capacity after a new amputation stimulus. We conclude that, blastema formation and regenerative outgrowth do not depend on a biological process that is permanently disrupted or depleted by loss of Wnt/β-catenin signalling.

In spite of this amazing capacity to regenerate, we observed that, while the bone distal to the amputation plane (new bone) regenerated with a normal morphology, the bone proximal to the amputation plane (old bone) became progressively thickened with the repeated cycles of amputations. We suggest that this progressive bone thickening can be due to an inappropriate activation of osteoblasts that secrete matrix far away from the amputation plane or, alternatively, an unbalanced ratio of bone-forming and bone-degrading cells.

Moreover, we detected an alteration in the original pattern of pigment cells and a distal shift in the position of the bony ray bifurcations in the regenerated caudal fins.

We wanted to further investigate how the positional information is established during fin regeneration and whether it is altered by repeated amputations at different proximo-distal (PD) places along the fin. Our results show that upon a first amputation at 4 segments of the bony ray from the base of the fin (proximal amputation), the bifurcation position was immediately distalized when compared to its previous position in the uncut fin. Following the second, third and fourth amputation, the bifurcation position was maintained in the regenerated fin. On the other hand, the bifurcation position was progressively distalized when the amputations were done at 1

segment proximal to the bifurcation (near bifurcation – distal amputation). Thus, we show that while amputations performed at a long distance from the bifurcation do not change its PD position in the regenerated fin (after a first amputation), consecutive distal amputations induce a positional reset and progressively shift its position distally. Therefore, it is possible that an amputation proximally near the bifurcation will inhibit the signal responsible to initiate the formation of a bifurcation and consequently delay this process.

We aimed to determine the signals involved in the control of the bifurcation position by the amputation place. To this end, we analyzed in detail the role of Sonic hedgehog (Shh), since previous reports propose that, preceding the formation of a bony ray bifurcation, *shh* duplicates its single domain. However, in contrast, our analysis shows that the dynamics of *shh* expression does not change in response to different amputation places, being always two domains of expression throughout the regeneration process. Thus, Shh does not seem to be the factor that modulates the bifurcation position during fin regeneration.

Given the fact that it has been proposed that Shh might play a role in the osteoblasts patterning and/or differentiation during fin regeneration we analyzed Zns5 expression, an osteoblast marker in a *shh-gfp* transgenic reporter line. We observed that soon after the detection of *shh* expression, the bone alters its growing tip, and the forming osteoblasts start to be aligned close to the basal layer of the epidermis next to *shh* expressing cells. This leads to the hypothesis that *shh* expression in two separate domains might be important to align and direct the growth of the regenerating bone.

Finally, we analyzed the implication of Fibroblast growth factor (Fgf) signalling in the modulation of the bifurcation position by the amputation place, since it was previously reported that the levels of Fgf signalling activation vary according to the PD place of amputation. This reveals the existence of positional memory in the regenerating fin that can be mediated or act through Fgf signalling. In order to investigate whether Fgf signalling would determine the PD position of the bifurcation in the regenerated fin, we

made use of the *hsp70:dn-fgfr1* zebrafish transgenic. This transgenic contains a dominant-negative *fgfr1-egfp* fusion gene (*dn-fgfr1*) driven by a heat-inducible zebrafish *hsp70* promoter and efficiently attenuates Fgf signalling during fin regeneration in a dose dependent manner. However, Fgf signalling attenuation did not alter the position of the bony ray bifurcation, when compared to the controls, indicating that Fgf signalling may not be the trigger signal for the formation of a bifurcation in zebrafish fin regeneration.

The establishment of positional memory during vertebrate regeneration has been mainly investigated in the amphibian limb. Nevertheless, the signals involved in the maintenance of positional memory remain poorly understood. The better understanding of this process in model organisms will be of great importance in the regenerative medicine field, namely to achieve the proper tridimensional structure for a successful and functional integration of the *in vitro* generated organs into patients.

Additionally, we believe that better understanding of the cellular mechanisms underlying the virtually unlimited regenerative capacity of fish caudal fin regeneration will be informative for efforts to improve repair in humans.

Resumo

Apesar de todos os organismos terem desenvolvido mecanismos de resposta a um ferimento ou doença, a sua capacidade de recuperar de uma perda ou dano de órgãos ou apêndices é muito variada. Um organismo vertebrado que mantém a capacidade regenerativa é o peixe zebra (*Danio rerio*). A facilidade de manipulação molecular e genética, tornou este organismo num poderoso modelo de estudo da regeneração. Em particular, a barbatana caudal do peixe zebra devido à sua acessibilidade e a uma estrutura anatómica simples, tornou-se um modelo ideal para aprofundar o estudo de regeneração em vertebrados. A barbatana caudal é constituída por vários ossos segmentados. Cada osso, com a excepção dos ossos mais laterais, é bifurcado na parte distal da barbatana.

Relativamente ao processo de regeneração da barbatana caudal é, na generalidade aceite, que haja uma perda de eficiência de regeneração após amputações repetidas. O objectivo da minha tese foi caracterizar em detalhe a hipótese de amputações repetidas provocarem uma diminuição da eficiência de regeneração e identificar as vias de sinalização envolvidas nessa resposta. Para isso, estabelecemos um protocolo de amputações repetidas, no qual as mesmas barbatanas caudais foram submetidas a três amputações consecutivas todos os meses. Este protocolo foi repetido 10 vezes, resultando num total de 29 amputações no final do protocolo.

Os nossos resultados mostram que o tamanho do blastema, estrutura constituída por células progenitoras essenciais no processo de regeneração, e o tamanho final da barbatana caudal completamente regenerada, não são alterados. Desta forma, amputações consecutivas repetidas da barbatana caudal do peixe zebra não diminuem a sua capacidade de regeneração e não afectam qualquer um dos passos sucessivos de regeneração: cicatrização, formação do blastema e crescimento regenerativo.

A inibição da via de sinalização Wnt/β-catenin através da sobre-expressão de Dickkopf1 (Dkk1) por método de choque térmico causa um bloqueio

completo da regeneração da barbatana. Iniciámos a sobre-expressão de *dkk1-gfp* imediatamente antes da amputação da barbatana, duas vezes por dia até aos 4 dias-após-amputação (dpa), para inibir completamente a regeneração da barbatana. Uma vez não sendo mais expostos ao tratamento de choque térmico verificou-se que não ocorreu regeneração espontânea nestes peixes. Contudo, quando as suas barbatanas foram novamente amputadas a uma temperatura não inibitória, a barbatana caudal regenerou e atingiu o seu tamanho original. A fim de colocar ainda mais à prova a capacidade de regeneração realizámos ciclos repetidos de amputação, inibição da sinalização Wnt/β-catenin, recuperação e segunda amputação. Notavelmente, o bloqueio repetido da formação do blastema e da regeneração da barbatana após inibição da via de sinalização Wnt/β-catenin não diminuiu a capacidade regenerativa após o estímulo de uma nova amputação. Estes resultados permitem-nos concluir que a formação do blastema e o crescimento regenerativo não dependem de um processo biológico que é destruído permanentemente ou esgotado pela perda da via de sinalização Wnt/β-catenin.

Apesar desta surpreendente capacidade de regenerar, observámos que, enquanto o osso distal em relação ao plano de amputação (osso novo) regenerou com a morfologia normal, o osso proximal em relação ao plano de amputação (osso velho) ficou progressivamente mais espesso com os ciclos repetidos de amputações. Sugerimos que este espessamento progressivo do osso possa ser devido a uma activação inapropriada de osteoblastos que secretaram matriz longe do plano de amputação ou, alternativamente, a um desequilíbrio no rácio de células que formam e degradam osso.

Além disso, detectámos uma alteração no padrão original de células de pigmento e uma distalização na posição das bifurcações dos ossos das barbatanas caudais regeneradas.

De seguida, investigámos como é estabelecida a informação posicional durante a regeneração da barbatana caudal e se é alterada por amputações repetidas a diferentes níveis proximo-distais (PD) ao longo da barbatana. Os

nossos resultados revelam que após uma primeira amputação a 4 segmentos da base da cauda (amputação proximal) a bifurcação é imediatamente distalizada quando comparada com a sua posição prévia na barbatana não amputada. Após a segunda, terceira e quarta amputação, a posição da bifurcação foi mantida na barbatana regenerada. Por outro lado, a posição da bifurcação foi progressivamente distalizada quando as amputações foram efectuadas a 1 segmento proximal da bifurcação (perto da bifurcação – amputação distal). Deste modo, mostramos que, enquanto amputações efectuadas a uma grande distância da bifurcação não alteram a sua posição PD (após uma primeira amputação), amputações distais consecutivas induzem um "reset" posicional e alteram a sua posição para progressivamente mais distal. Assim, é possível que uma amputação perto da bifurcação iniba o sinal responsável por iniciar a formação da bifurcação e consequentemente atrase esse processo.

Procurámos determinar os sinais envolvidos no controlo da posição da bifurcação pelo plano de amputação. Para este fim, analisámos em detalhe o papel de Sonic hedgehog (Shh) uma vez que, estudos anteriores propõem que, antes da formação de uma bifurcação de um osso, *shh* duplica o seu único domínio de expressão. Contudo, a nossa análise mostra que a dinâmica de expressão de *shh* não é alterada em resposta aos diferentes planos de amputação, estando sempre em dois domínios de expressão durante todo o processo de regeneração.

Dado que foi proposto que Shh poderá ter um papel na padronização ou diferenciação de osteoblastos durante a regeneração da barbatana, procedemos à análise da expressão de Zns5, um marcador de osteoblastos, numa linha reporter transgénica *shh-gfp*. Observámos que logo depois da detecção da expressão de *shh*, o osso altera a forma da sua extremidade de crescimento e os pré-osteoblastos começam a alinhar-se perto da camada basal da epiderme junto às células que expressam *shh*. Isto conduz à hipótese de que a expressão de *shh* em dois domínios separados poderá ser importante para alinhar e direccionar o crescimento do osso a regenerar.

Por fim, analisámos o envolvimento da via de sinalização Fibroblast growth factor (Fgf) na regulação da posição da bifurcação pelo plano de amputação, uma vez que já foi demonstrado que os níveis de activação da sinalização Fgf variam de acordo com o nível PD da amputação. Este dado revela a existência de memória posicional na barbatana durante a regeneração que pode ser mediada ou actuar através da via de sinalização Fgf. Com o intuito de investigar se a sinalização Fgf determina a posição PD da bifurcação na barbatana regenerada, utilizámos a linha transgénica de peixe zebra *hsp70:dn-fgfr1*. Este transgénico contém uma fusão genética *fgfr1-gfp* dominante-negativa (*dn-fgfr1*) sob influência do promotor induzido por choque térmico *hsp70* de peixe zebra e atenua com uma eficácia dose-dependente a via de sinalização Fgf durante a regeneração da barbatana. Contudo, a atenuação da sinalização Fgf não afectou a posição da bifurcação do osso quando comparada com os controlos, indicando que a sinalização Fgf parece não ser o sinal activador para a formação da bifurcação na regeneração da barbatana caudal do peixe zebra.

O estabelecimento de memória posicional durante a regeneração em vertebrados tem sido maioritariamente investigada no membro do anfíbio. Porém, os sinais envolvidos na manutenção da memória posicional continuam mal compreendidos. Uma melhor compreensão deste processo em organismos modelo terá uma grande importância na área da medicina regenerativa, nomeadamente para obter a estrutura tridimensional correcta dos orgãos criados *in vitro*,.de modo a assegurar com sucesso a integração funcional nos pacientes

Adicionalmente, acreditamos que uma maior compreensão dos mecanismos celulares que suportam a capacidade regenerativa virtualmente ilimitada da barbatana caudal do peixe zebra será informativa para as tentativas de aumento da capacidade de reparação de tecidos em humanos.

ABBREVIATIONS

AEC Apical epidermal cap
AER Apical ectodermal ridge
Bmp Bone morphogenetic protein
Dkk1 Dikkopf1
Dpa Days-post-amputation
Fgf Fibroblast growth factor
Fgfr1 Fibroblast growth factor receptor 1
Hh Hedgehog
Hpa Hours-post-amputation
Hsp Heat-shock protein
Igf Insulin-like growth factor
PCP Planar cell polarity
PD Proximo-distal
Ptc1 Patched1
qPCR Quantitative polymerase chain reaction
RAR Retinoic acid receptor
RXR Retinoic X receptor
Shh Sonic Hedgehog
Smo Smoothened
Tgf-β Transforming growth factor beta
Wpa Weeks-post-amputation

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CHAPTER I

Introduction

I.1. Regeneration

I.1.1 The importance of studying the mechanisms of regeneration

Regeneration is the ability to completely restore tissue architecture and function after injury and is one of the most elaborate processes that occur during adult life. Regeneration happens in organisms from distant phyla and with different levels of biological complexity, can be triggered by a variety of insults, can take place at different developmental stages and can proceed through a variety of cellular and molecular processes that are activated upon injury. Humans have only a limited capacity to regenerate their tissues and organs. In contrast, some other vertebrates present an amazing capacity to fully regenerate complex structures and organs as the limbs, the eye, the spinal cord or even the heart. These organisms are excellent models to understand the cellular and molecular mechanisms that could be used to develop regenerative strategies in humans and push forward the field of regenerative medicine. The ultimate goal is to have the knowledge to be able to restore cells, tissues and structures that are lost or damaged after injury, disease or as a consequence of aging. The field of regenerative medicine has brought hope with key achievements: the identification of stem and progenitor cells in most human organs holds promise for a tissue specific activation to induce regeneration; *in vitro* culturing of stem and progenitor cells and their differentiation into specific cell types suitable for implanting into patients; and *in vitro* growing of organs and tissues for transplantation into patients (Jopling et al., 2011; Poss, 2010; Stoick-Cooper et al., 2007a). However, in spite of these major achievements, there are still many limitations to overcome before we are able to successfully replace an organ. Some of these limitations have been related to the difficulty of efficiently control differentiation of stem cells into the target cell type and the isolation of the differentiated cells to obtain a pure population, in order to avoid the formation of teratomas upon transplantation into the host. In addition, it has been a major issue, to successfully and functionally integrate the *in vitro* generated

organ/differentiated cells into the patients' tissues (Koh and Atala, 2004). Therefore, even though the current strategies are promising, they will certainly benefit from continued regeneration studies in the different model organisms.

I.1.2. Regeneration Vs Repair Vs Homeostasis

The recovery of the damaged tissue upon injury can be viewed as a process of regeneration or repair. Regeneration refers to the complete restitution of lost or damaged tissues or organs, such as the re-growth of an amputated limb in amphibians. Conversely, repair leads to a partial recovery of the original tissues or organs and involves collagen deposition and the formation of scar tissue, which invariably results in impaired organ function (an example of this is seen in the mammalian cardiac muscle). Homeostasis is another form of tissue regeneration, which is transversal to all tissues and common to all animals. It occurs in a physiologic manner, regularly replacing cells lost by apoptosis and aging, through the activity of self-renewing stem cells. Examples of this type of regulation are observed in tissues like the mammalian skin, gastrointestinal epithelium and hematopoietic tissues. However, as opposed to the other forms of regeneration, it does not need to be activated by a stimulus like an injury (Krafts, 2010).

Even though the outcome of a regenerative response may be similar between species, the mechanisms used to accomplish such response can vary among them. Therefore, regeneration complexity as been classically divided into two main categories: morphallactic and epimorphic. As defined by Thomas Hunt Morgan in 1901, morphallactic regeneration takes place when the repair of lost or damaged structures does not dependent on cellular proliferation and relies on remodelling of the remaining tissues. This is the case of hydra head regeneration since, upon amputation, a new head will form from the existing tissue. Once the regeneration program is completed, the regenerated organism will be smaller and will grow to reach the original size through a proliferation-dependent mechanism. In contrast, epimorphic regeneration depends on

cellular proliferation and on the formation of a regeneration-specific structure named blastema, which comprises proliferative cells that will differentiate and lead to the complete recovery of the lost body structures (as seen for example, in the amphibian limb, tail and even spinal cord) (Galliot and Ghila, 2010). One could see these distinct mechanisms of regeneration as two opposing categorizations with several intermediate levels of contributions of each of them in the different species. This could be the reason why it has been difficult to describe a global mechanism including the different species-specific response (Galliot and Ghila, 2010).

Repair is the most frequent type of healing in mammals. Indeed, mammals have a limited capacity to regenerate whole organs and complex tissues after injury being the term regeneration applied usually to processes such as liver growth after partial resection, a process that consists of compensatory growth rather than true regeneration. In most cases the repair mechanism consists of a combination of two processes: replacement of the damaged tissue by new cells (often viewed as a true regeneration mechanism) and deposition of collagen. The contribution of each process depends on the rate of the tissue-specific cell turnover and on the extent of injury. Therefore, the repair of a damaged tissue with a high turnover rate will consist on a greater regeneration contribution, whereas a larger wound will result in a more extensive collagen deposition (Krafts, 2010).

I.1.3.The ability to regenerate declined during evolution

Key questions regarding the evolution of regeneration have been debated for more than a century. However, it is still not understood why the ability to replace lost body parts varies widely among animals. Examples that reflect this amazing variation are cnidarians and flatworms that can regenerate an entire individual from a small body fragment, whereas birds and mammals are largely or completely incapable of regenerating any structure (Figure 1.1). Even though it has been an old aspiration to identify the cause for regeneration

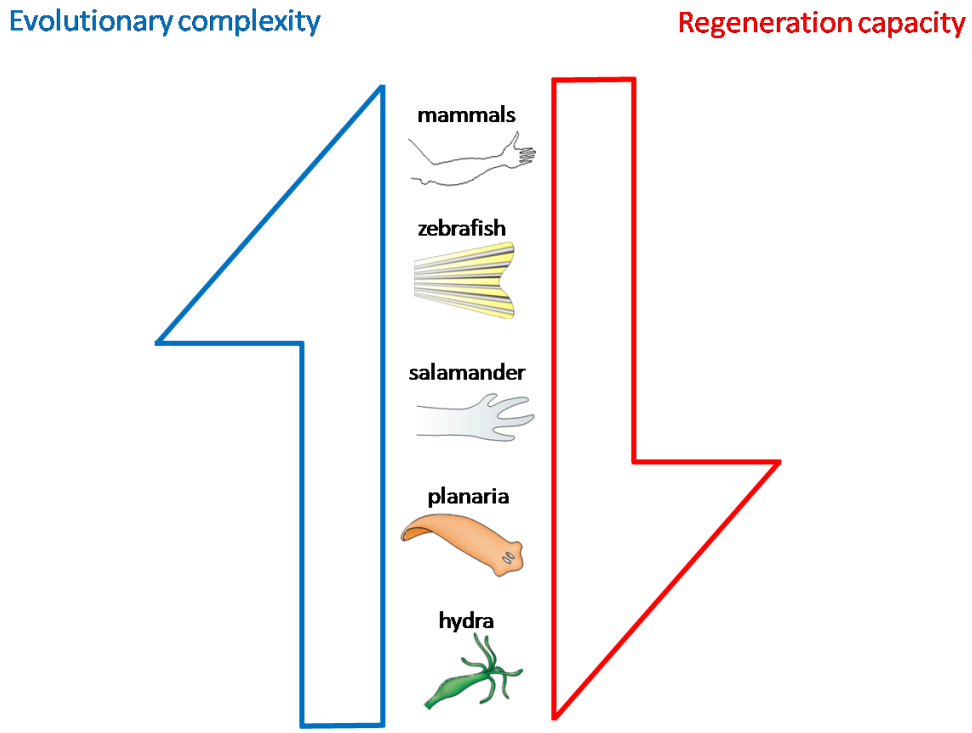

Figure 1.1. Inverse correlation between the evolutionary complexity and regeneration capacity. Whereas mammals have only a limited capacity to regenerate their tissues and organs, lower vertebrates, such as certain urodeles (salamander) and teleosts (zebrafish), present an elevated regenerative spectrum being able to regenerate complex structures and organs like the brain, spinal cord, retina and heart. Additionally, the invertebrates hydra and planarian can even regenerate an entire individual from a small body fragment. Salamander, hydra e planarian images were taken fom Poss, 2010.

variation, it has become increasingly evident that regeneration is shaped by a diversity of ecological and evolutionary factors.

Based on the phylogenetic distribution of regeneration, it seems likely that regeneration first arose in primordial animals, possibly coincident with the origin of multicellularity. Once regeneration ability evolved, it could be maintained by mechanisms other than those responsible for its origin and most likely associated with the ecological context. Certain species experience high frequencies of structure loss in nature. When a structure that is frequently lost results in a decreased fitness, it indicates that regeneration of this structure is important for the ecology of the organism (namely limb regeneration in urodeles or the lizard's tail). It also falls in this hypothesis, species that lose and regenerate a structure that is unimportant at the time of loss but that becomes important in a later stage of development (for example the anuran limb regeneration as larvae). Importantly, the benefits of replacing the structure should compensate the cost of its regeneration (Reichman, 1984).

Other theories considered to explain the retention of regeneration are the pleiotropy and phylogenetic inertia. The pleiotropy theory, considers that the ability to regenerate a structure was retained because it is tightly coupled with a related phenomenon, such as asexual reproduction or embryogenesis. In other words, the ability to regenerate a particular structure would not be part of an adaptation to a certain biological context, since it would take advantage of a shared developmental process. According to this theory, the high regenerative capacity of cnidarians could have been maintained due to the overlap of the cellular and molecular mechanisms used in regeneration and normal growth (Bely and Nyberg, 2010). On the other hand, the phylogenetic inertia hypothesis suggests that regeneration in certain species is an ancestral trait that is neither important for the ecology of the animal nor retained by pleiotropy. In this case, regeneration ability has simply not been eliminated but can still be in the future (Bely and Nyberg, 2010).

The hypotheses described above attempt to explain the maintenance of regeneration. However, the opposite, restriction or loss of regeneration ability has been a common feature across animal phylogeny. Why would species lose such an apparent beneficial trait? One possibility could be that regeneration becomes ecologically irrelevant due to an adaptive change in the species (namely, increased defence ability from predators) or a particular structure or body part could become essential for the immediate survival of the animal. Loosing such structure would lead to the organisms' death before it could be properly regenerated, resulting in a lower frequency of tissue loss (Bely and Nyberg, 2010). An example of this is the non-regenerating central nervous system (CNS) of higher vertebrates versus the regeneration of the rudimentary nervous system present in some invertebrates. Another additional difficulty common to birds and mammals is the fact that they are homeothermic. The maintenance of a constant body temperature increases the metabolic rate, which consequently increases the blood flow to the organs and the need of feeding. This will increase the chances of starving or bleeding to death upon a severe injury. Indeed, it has been suggested that throughout evolution these organisms have developed higher degrees of wound healing abilities to stop the life-threatening loss of blood. Importantly, the factors associated with wound healing in these organisms may inhibit regeneration (Reichman, 1984).

Another important factor to consider is the level of amputation. Generally, during evolution, more proximal amputations became less likely to regenerate (Reichman, 1984). While hydra and planarian regenerate upon an amputation at any level, zebrafish regenerates the fins until a certain proximal limit, and mammals are only able to regenerate the distal digit tip. Therefore, with increased complexity a more proximal injury is more likely to trigger a severe lesion, leading to death before regeneration can occur.

In the case of redundant structures, these might not be important enough to worth the cost of a regeneration process. An example of this is the loss of a leg that does not result in a detectable impairment or reproductive cost in

some arachnids, possibly because of the functional redundancy that results from having many legs (Bely and Nyberg 2010; Reichman, 1984).

Finally, loss of regenerative capacity could also occur if pleiotropic interactions between regeneration and other developmental processes dissociated during evolution (Bely and Nyberg, 2010).

I.1.4. Evolutionary loss of regenerative capacity and its relation to cancer

In mammals, the ability to restore complex structures such as limbs is lost towards the end of embryonic development. The capacity of complete regeneration persists during adulthood in rare cases such as the deer antlers, the cartilage of the rabbit ear, the membrane of bat wings, or the human and mouse digit tip distal to the terminal phalangeal joint. However, before aiming to enhance this limited regenerative capacity in mammals, one should fully understand the stem cell system involved, since regeneration usually relies on a large accumulation of proliferating cells sharing potentially dangerous similarities with cancer. Like in regeneration, cancer develops from an initial injury (physical, chemical or biological) that leads to a permanent inflammatory response. In a regenerative process, an injury is followed by controlled cell migration, proliferation and functional integration within the pre-existing tissue, while in cancer, the proliferation and migration events are abnormal, resulting in the formation of a tumour (Oviedo and Beane, 2009). Importantly, the molecular pathways involved in cell migration and proliferation are the same during regeneration and carcinogenesis.

Mammals require an extended period of time to develop a complex body, exposing proliferating cells to an increased risk of damage. Moreover, during adulthood, tissues with a high cell turnover are supplied by a larger pool of activated stem cells, which increases the risk of malignant transformation. This might explain the overall higher incidence of cancer in the digestive, respiratory, genital and urinary systems (Meng and Riordan, 2006). Thus, as evolutionary complexity increased, it is likely that more regulatory checkpoints

were introduced to control pluripotency in development, homeostasis and repair. However, in addition to preventing the excessive proliferation that can lead to tumours, the increased number of regulatory checkpoints might have contributed to a progressive loss of the regenerative ability (Beachy et al., 2004; Egger, 2008; Gardiner, 2005; Sanchez Alvarado, 2000).

Urodeles are a remarkable example of a model organism that is able to regenerate and is also resistant to cancer. In these animals, not only spontaneous tumours are not found, but also carcinogen application in the regeneration-competent tissues results in normal morphogenesis and differentiation (Oviedo and Beane, 2009; Tsonis, 2000). In the near future, examples like this will require further investigation to better understand the (most likely small) differences between regeneration and cancer and to hopefully use this knowledge to treat cancer as a naturally healing wound.

I.1.5. Different model organisms used to study regeneration

In this section, I will discuss the classic regeneration model organisms: from the amazing invertebrate regenerators, hydra and planarian, to the poorly regenerating mammals. Anuran amphibians, urodele amphibians and zebrafish are also briefly described as powerful vertebrate models to use in regeneration studies. The mechanisms of zebrafish regeneration are further characterized, since it was the model organism used for the work presented in this thesis.

I.1.5.1. Invertebrates

Hydra and planarian regeneration has been explored for over a century. Initially, surgical manipulations and cellular observations were the methods used to study the regeneration of these organisms. However, more recently, the development of new tools such as reverse genetics through RNAi or, in the case of hydra, the sequenced genome and the possibility of producing transgenics, has allowed molecular and genetic studies. This has helped to uncover the cellular and molecular mechanisms of regeneration in these

organisms (Bosch, 2007; Reddien and Sanchez Alvarado, 2004).The advantages of using invertebrates such as hydra and planarian as models for morphological and molecular studies of regeneration include: optical transparency facilitating *in vivo* tracking of cells within the intact animal; rapid growth rate and mass culturing of clonally derived animals (Bosch, 2007).

I.1.5.1.1. Hydra

Hydras live as freshwater polyps with a body axis containing two poles separated by a body column: in one side the head with tentacles and on the opposite side a foot. These metazoans from the phylum Cnidaria possess two cell layers, the ectoderm and the endoderm, separated by an extracellular matrix, the mesoglea. Hydra presents an incredible capacity to regenerate and was the first animal model used in regeneration experiments. A whole organism can regenerate from a fragment with only a few hundred cells and even dissociated hydra cells can re-aggregate and produce a new animal. This ability is connected to the continuous tissue renewal and pluripotency that involves the contribution of stem cells present in the ectodermal, endodermal and interstitial tissue layers (Bosch, 2007; Bosch et al., 2010; Tanaka and Reddien, 2011).

A regenerating hydra fragment is polarized, which is likely based on gradients of molecules that provide positional information in a regenerating fragment, determining the formation of a head in the apical end and of a foot at the basal end (Bosch, 2007).

So far, a few pathways have been identified in the regulation of hydra regeneration. Wnt signalling is among those factors, previously shown to be necessary in hydra head regeneration. Curiously, its contribution varies according to the level of amputation. Upon head amputation, Wnt3 is strongly upregulated in interstitial epithelial cells driving morphollaxis-type of regeneration. On the other hand, after an amputation at mid-gastric level Wnt3 is first detected and released from a subset of apoptotic interstitial cells leading to the synchronous division of cycling interstitial cells. The latter mechanism of

Wnt signalling is required for this epimorphic-like response, which is specifically triggered in hydra head regeneration upon amputation at mid-gastric level (Chera et al., 2009; Galliot and Ghila, 2010).

Other pathways that have been identified in hydra regeneration are the mitogen activated protein pathway (MAPK), which plays a role in head regeneration (Bosch, 2006) and Bmp, demonstrated to be implicated in axial patterning and tentacle regeneration (Galliot and Chera, 2010; Reinhardt et al., 2004).

I.1.5.1.2. Planarian

Planarians are bilaterally symmetrical metazoans of the phylum Platyhelminthes. Its internal anatomy includes a nervous system, musculature, excretory system, epidermis, eyes, and intestine (Reddien and Sanchez Alvarado, 2004). Planarians are known for their capacity to produce all the organ systems and cell types in the adult as they can regenerate complete individuals from very small body parts. In a transverse amputation, muscle cells, nerve tracts, intestine and mesenchymal cells are usually affected. This extraordinary ability has been proposed to depend on a population of adult somatic stem cells called neoblasts. These cells are distributed throughout the planarian body in the parenchyma, which is beneath the basement membrane and body wall musculature, and surrounds the intestine and nervous system. The population of neoblasts constitutes ~25-30% of all the cells and are thought to be able to replace all the different tissues that constitute an adult planarian as they are the only mitotically active cells. Therefore, they are involved in the replacement of cells lost in homeostatic events and also give rise to the regeneration blastema in amputated animals. Evidence for the role of neoblasts in the formation of the regeneration blastema came from irradiation experiments, which lead to neoblast degeneration and blocked regeneration. Regeneration capacity was rescued after transplanting normal tissue into irradiated hosts. In addition, BrdU-labelling experiments demonstrate that dividing cells with undifferentiated morphology contribute to

blastema formation. However, in spite of these results strongly pointing to neoblasts as a crucial source for regeneration, the possibility of the contribution of processes such as dedifferentiation or transdifferentiation cannot be excluded (Reddien and Sanchez Alvarado, 2004; Tanaka and Reddien, 2011).

After wounding there is a strong muscular contraction to reduce the surface area of the wound and a protective mucus with possible immunological functions is released by specialized cells. Within 30 minutes a thin layer of epithelial cells covers the wound, a process that relies on cell migration and does not require cell proliferation. The blastema is originated from neoblasts that can migrate from long distances to the wound site, where they are induced to proliferate and differentiate to give rise to the new tissues (Reddien and Sanchez Alvarado, 2004; Tanaka and Reddien, 2011).

Regardless of whether there is an amputation of the head, removal of the head and midbody or even a greater body part, there is an identical outcome of the regenerative response, which is the formation of a new head. This means that the blastema tissue is not always able to fully recover the lost body parts. Thus, when more than the head is amputated the proportion of width/length of the regenerated animal is greater than the original. This is in most of the cases compensated by the lengthening and thinning of the pre-existing tissues (morphollaxis) (Reddien and Sanchez Alvarado, 2004).

Several players of signalling pathways, such as Bone morphogenetic protein (Bmp), Hedgehog (Hh) and Wnt, have been shown to be conserved in planarians and, more importantly, implicated in the establishment and maintenance of planarian axial polarity during the regeneration process.

Wnt/b-catenin signalling pathway determines where head and tail will form after an amputation. While low levels of Wnt signalling will lead to the formation of a head, the upregulation of this signalling pathway will result in tail formation (Adell et al., 2010; Tanaka and Weidinger, 2008). This differential anterior-posterior expression of *wnt* was recently shown to be

controlled by Hh signalling. Similarly to Wnt, reduced Hh signalling is required for head formation and elevated Hh signalling is required for tail formation (Rink et al., 2009).

On the other hand, Bmp signalling has been shown to be necessary for the establishment of a correct dorso-ventral axis, promoting dorsal and inhibiting ventral tissue regeneration (Adell et al., 2010; Reddien, 2011).

I.1.5.2.Vertebrates

I.1.5.2.1. Zebrafish

Zebrafish (*Danio rerio*) has emerged as a powerful model organism to study the process of regeneration. This teleost fish has the ability to regenerate various tissues and organs like the heart, the spinal cord, the retina and the fins. Due to its accessibility, its fast and robust regeneration and its simple architecture, the zebrafish caudal fin is currently one of the most powerful models for regenerative studies. The advantage of using the zebrafish is that, in contrast to what happens in amphibians, it is amenable for standard molecular and genetic manipulations. Other advantages of this model organism include a short generation time, the ability to raise and maintain a large number of animals and the availability of reagents and technology generated by zebrafish embryologists (Poss et al., 2003).

I.1.5.2.2. Anuran amphibians (frogs, toads)

Due to their permeable skin, anuran amphibians can be found in semi-aquatic or humid regions, but move easily on land and are able to regenerate limbs, tails and lens only as tadpoles. This ability declines during differentiation and metamorphosis, such that tadpoles can only regenerate complex structures while they are going through a period of morphological change. This suggests that regeneration in anuran amphibians may depend on the presence of undifferentiated cells, which are no longer present once differentiation has set in. This stage-dependent regenerative ability enables the gain and loss of

function studies to better understand the progressive loss of regeneration capacity.

Important tools, such as transgenic overexpression, were developed in the field of development biology in the frog and currently allow a detailed molecular understanding of the regeneration process in this model organism (Beck et al., 2009).

I.1.5.2.3. Urodele amphibians (salamanders, newts, axolotl)

Urodele amphibians can be fully aquatic, both terrestrial and aquatic or even entirely terrestrial. Among vertebrates, they are the true champions of regeneration. When injured, these animals regenerate several body parts anytime during their life cycle, including the upper and lower jaw, lens, retina, limb, tail, spinal cord, and intestine. In fact, limb regeneration in salamander, represents one of the best examples of complex vertebrate regeneration. Regeneration is a local response of the cells of the stump and results in a perfect replacement of the original structure (Brockes and Kumar, 2005; Han et al., 2005). The greater disadvantages of using urodele amphibians in regeneration studies, when compared to some of the previous model organisms described, is the lack of a sequenced genome and well-developed molecular and genetic tools (Poss, 2010; Poss et al., 2003). This becomes a major limitation in the dissection of the cellular and molecular mechanisms of vertebrate regeneration.

I.1.5.2.4. Mammals

In mammals, throughout adult life, the only part of the mature limb that is able to regenerate is the digit tips. Thus, digit tip regeneration has been the main model system used to study mammalian regeneration. It was found in humans as a result of fingertip amputation being a common injury, treated simply by preventing infection of the wound and allowing it to heal without suturing (Gardiner, 2005). However, the successful regeneration is dependent on the level of amputation and it is only observed when the digit is amputated

through the distal phalanx. Interestingly, while regeneration of bone is common following fracture, its regeneration from a free surface, such as the amputated distal phalanx, is a unique regenerative response in mammals (Han et al., 2005).

I.1.6. The different phases of zebrafish caudal fin regeneration

The caudal fin is composed of several segmented bony rays and inter-ray mesenchymal tissue. Each bony ray consists of 2 concave hemirays that define an inner space filled with intra-ray mesenchymal cells and, with the exception of the most lateral rays, is bifurcated in a distal position within the fin (Poss et al., 2003) (Figure 1.2). These bifurcations are responsible for generating the characteristic shape of the caudal fin and ultimately for increasing swimming efficiency. Blood vessels and nerve axons are found in both intra- and inter-ray tissues (Poss et al., 2003). Bony rays are produced and maintained by the osteoblasts, skeletogenic cells that secrete bone matrix (Hall, 2005). When a caudal fin is amputated, a regenerative program with stereotypic successive steps is activated and it takes approximately 2 weeks to fully regenerate all the tissues and structures that compose a functional fin. These steps include the closure of the wound by the epidermis to form the regeneration epidermis and the migration of the stump cells distally to form the blastema, which is a structure comprised of proliferating cells. The blastema cells proliferate, go through morphogenesis, pattern formation, and differentiation (Figure 1.3). During the regeneration process, important interactions take place between the blastema mesenchymal cells and the regeneration epidermis.

I.1.6.1. Wound healing

Upon amputation of the zebrafish caudal fin, there is little bleeding or inflammation and within the first 1-3 hours-post-amputation (hpa) the epithelial cells migrate to cover and close the wound. In the next 12 to 18 hours, the wound epidermis matures and accumulates additional layers, commonly referred as apical epidermal cap (AEC), which is thought to be

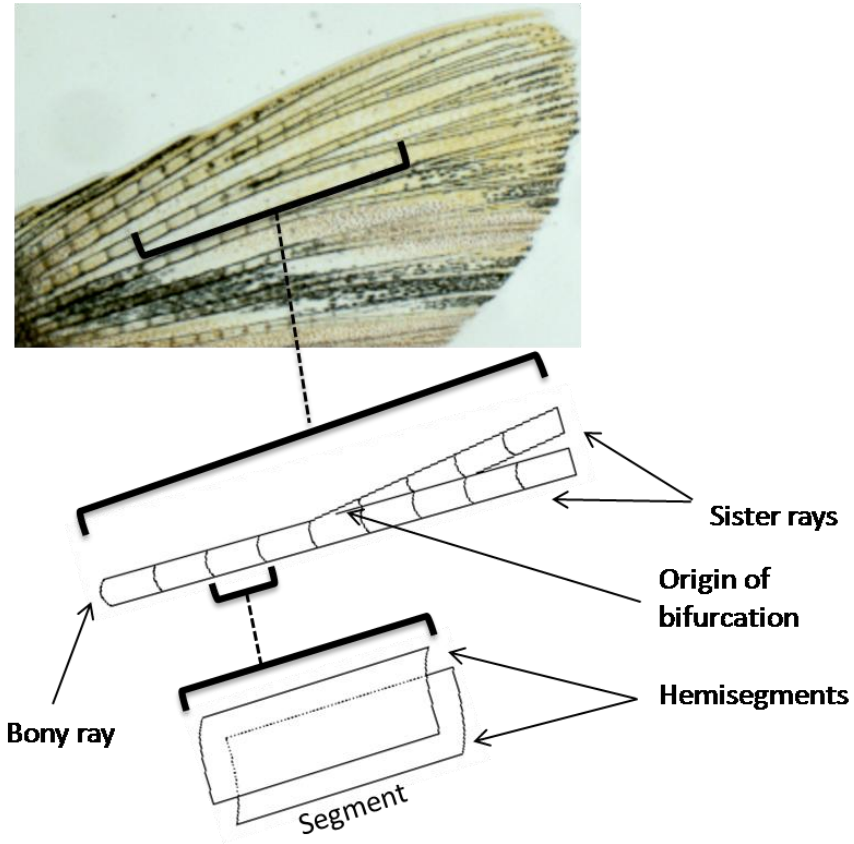

Figure 1.2. Zebrafish caudal fin architecture. The caudal fin is composed of segmented bony fin rays. Each ray is comprised of concave, facing hemirays (consisting of several hemisegments) and is bifurcated in the distal part of the fin (with the exception of the most lateral rays) originating the sister rays. (Adapted from Quint et al., 2002)

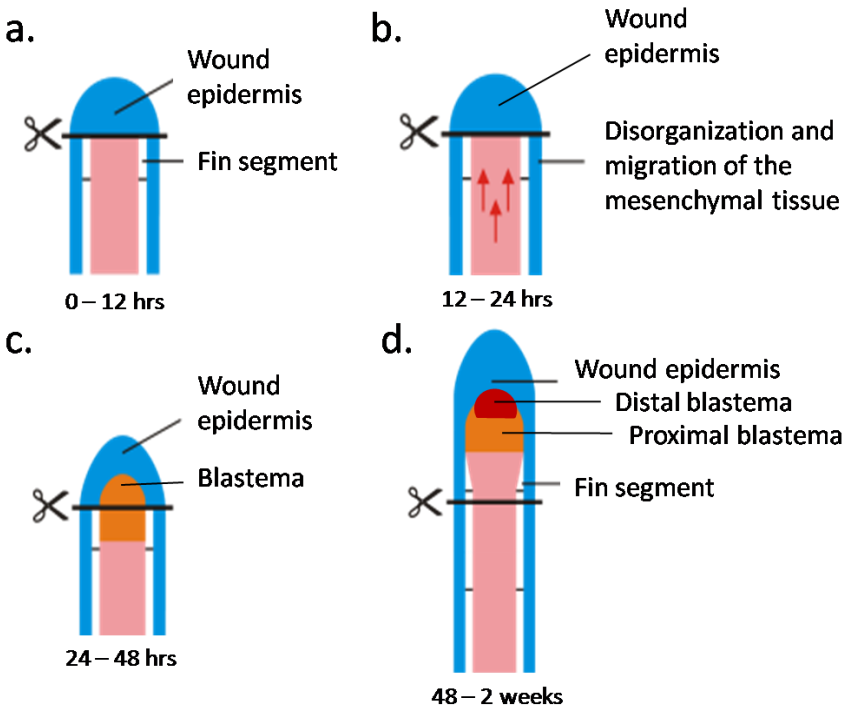

Figure 1.3. Zebrafish caudal fin regeneration steps represented in longitudinal sections. a. Wound healing. During the first 12 hr-post-amputation (hpa) epidermal cells migrate to cover the wound. **b.** Blastema formation. In the next 12 hpa, the wound epidermis thickens while the tissue proximal to the amputation plane disorganizes and cells migrate distally. **c.** Blastema formation. The blastema, a mass of proliferative cells, is formed distal to the amputation plane. **d**. Regenerative outgrowth. During this stage, blastema cells proliferate and differentiate to replace the missing structures. (Adapted from Poss, 2000b)

similar in function to the apical ectodermal ridge (AER) that forms in the limb bud during embryonic development.These processes are only dependent on migration events and do not involve cell proliferation. Around 18 – 24 hpa, when the blastema starts being formed, there is the arrangement of an epidermal basal layer of cells adjacent to the forming blastemal tissue. This basal epidermal layer of cells expresses several important markers throughout regeneration and is thought to interact with the blastema playing a key role in the fin growth and pattern formation (Poss et al., 2003).

Little is still known about the signals that trigger the formation of the AEC. The signalling pathways already identified to be important in this phase of regeneration are the Wnt, Activin βA, Insulin-like growth factor (IGF) and Retinoic acid (RA) signalling.

I.1.6.2. Blastema formation

The second regeneration step starts between 18 - 24 hpa when a mass of proliferative cells, accumulates underneath the AEC via migration to form a structure, at the top of each injured bony ray, called the blastema. The blastema cells are the cellular source for the replacement of the lost structures. The epidermis adjacent to the blastema cells is thought to influence position, size and mitotic activity of the blastema. Indeed, it has been known for a long time in newts, the importance of the wound epidermis in blastema formation. Once the wound epidermis is removed from a regenerating limb, regeneration is blocked until a new wound epidermis is formed. It is likely that the wound epidermis plays the same role in the zebrafish fin. It has also been demonstrated in newt that, blastema formation is dependent on innervation. In teleosts, data has similarly, provided evidence for the existence of nerve-derived factors that simulate blastema proliferation. However, similar evidences are still missing in zebrafish (Poss et al., 2003).

The formation of the blastema is a hallmark of epimorphic regeneration, an event that distinguishes regeneration from embryogenesis, even though

it displays embryonic characteristics and shares many of the developmentally signalling pathways including the Wnt, Activin βA, IGF, RA and Fibroblast growth factor (Fgf).

I.1.6.3. Regenerative Outgrowth

The transition to the regenerative outgrowth phase occurs by 48 hpa. At this time-point, the proximal regenerate starts to present differentiated tissue, namely osteoblasts, and the length of the cell cycle becomes shorter than during blastema formation. The blastema cells segregate into two morphologically indistinct compartments: a slowly proliferating distal blastema and a rapidly proliferating proximal blastema. The distal blastema seems to contain a pool of progenitors, contributing with daughter cells to the proximal blastema, which is a population of cells that migrate to new positions and differentiate to replace the lost tissues. At the molecular level, the transition from blastema formation to the regenerative outgrowth involves changes in the expression pattern of certain genes as well as upregulation of new genes. An example of this is the change in the pattern of expression of the blastema marker *msxb*. It starts by presenting a diffused mesenchymal expression during blastema formation that becomes limited to the distal blastema (in the slow proliferative cells) in the regenerative outgrowth (Poss et al., 2003).

Throughout outgrowth, the temporal and spatial regulation of epidermal signals, are crucial to regenerate the correct pattern and function. In fact, it has been demonstrated that the basal layer of the epidermis contains two spatially and functionally distinct cellular subtypes. While the distal domain expresses *wnt5b* and *pea3*, the proximal domain expresses *lef1* and *sonic hedgehog* (*shh*). Wnt and Fgf signallings are likely involved in the activation and maintenance of the markers of the two distinct cell populations within the basal epidermal layer. Wnt5b inhibits distal *shh* and *lef1,* restricting their expression to proximal domains while Fgf signalling induces the distal expression *wnt5b.* Thus, Fgf signalling inhibits distal *shh* and *lef1* expression through Wnt5b and, additionally, induces proximal *shh* and *lef1* expression

through a Wnt5b independent mechanism. These different epidermal compartments are important to signal throughout regenerative outgrowth to the adjacent blastema tissue (Lee et al., 2009).

Different signalling centers are necessary for the regenerative outgrowth phase, including Wnt, Activin βA, IGF, RA, Fgf, Bmp and Hedgehog (Hh) (Figure 1.4 and Figure 1.5).

I.1.7.Signalling centers involved in caudal fin regeneration

I.1.7.1. Wnt/β-catenin signalling regulates fin regeneration

An extracellular Wnt signal activates transduction pathway cascades in the cell, which includes the canonical or Wnt/β-catenin dependent pathway and non-canonical or β-catenin independent pathways. The non-canonical pathway can be divided into the Planar Cell Polarity pathway (PCP) and the Wnt/Ca^{2+} pathway (Komiya and Habas, 2008)(Figure 1.6). The Wnt ligands signal through binding to cell-surface receptors of the Frizzled (Fz) family and activate Dishevelled (Dsh). In the canonical Wnt pathway, Dsh activation will result in the accumulation and translocation of β-catenin to the nucleus where it complexes to the Lef/Tcf family members to mediate transcriptional induction of target genes (Figure 1.6a). On the other hand, Dsh recruitment in the non-canonical PCP pathway activates a downstream cascade that ultimately results in the remodeling of the cytoskeleton (Figure 1.6b) while in the non-canonical Wnt/Ca^{2+} pathway it modulates the intracellular calcium levels (Figure 1.6c). Through these pathways, Wnt signalling plays a determinant role during embryonic development, in cell differentiation and polarity (Komiya and Habas, 2008).

During zebrafish fin regeneration, Wnt signalling was shown to be activated and to play an essential role. Upon caudal fin amputation there is a rapid upregulation of β-catenin (Poss et al., 2003) . β-catenin expression is

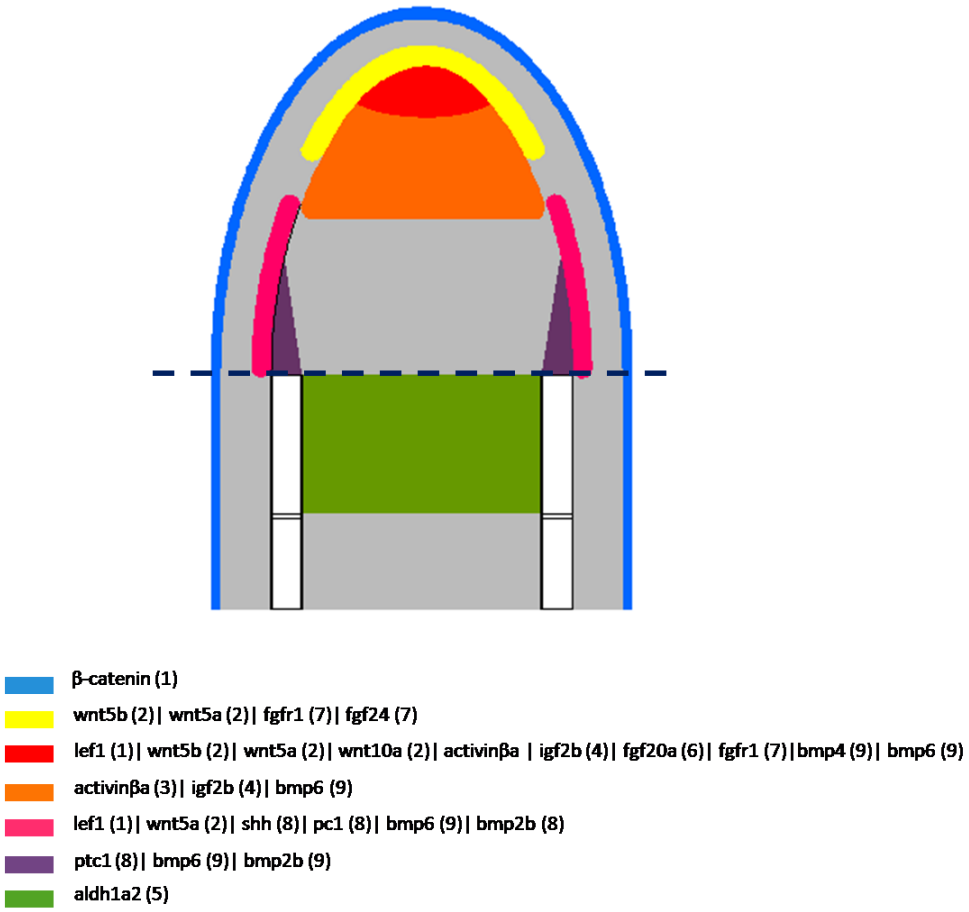

Figure 1.4. Signalling centers present during the regenerative outgrowth phase represented in a longitudinal section of the caudal fin. The tissue of expression is color-coded to match the corresponding color of the different players grouped according to their expression domains. The dashed line represents the amputation plane. References: (1) Poss et al., 2000a; (2) Stoick-Cooper et al., 2007; (3) Jazwinska et al., 2007; (4) Chablais and Jazwinska, 2010; (5) Blum and Begemann, 2012; (6) Whitehead et al., 2005; (7) Poss et al., 2000b; (8) Laforest et al., 1998; (9) Smith et al., 2006.

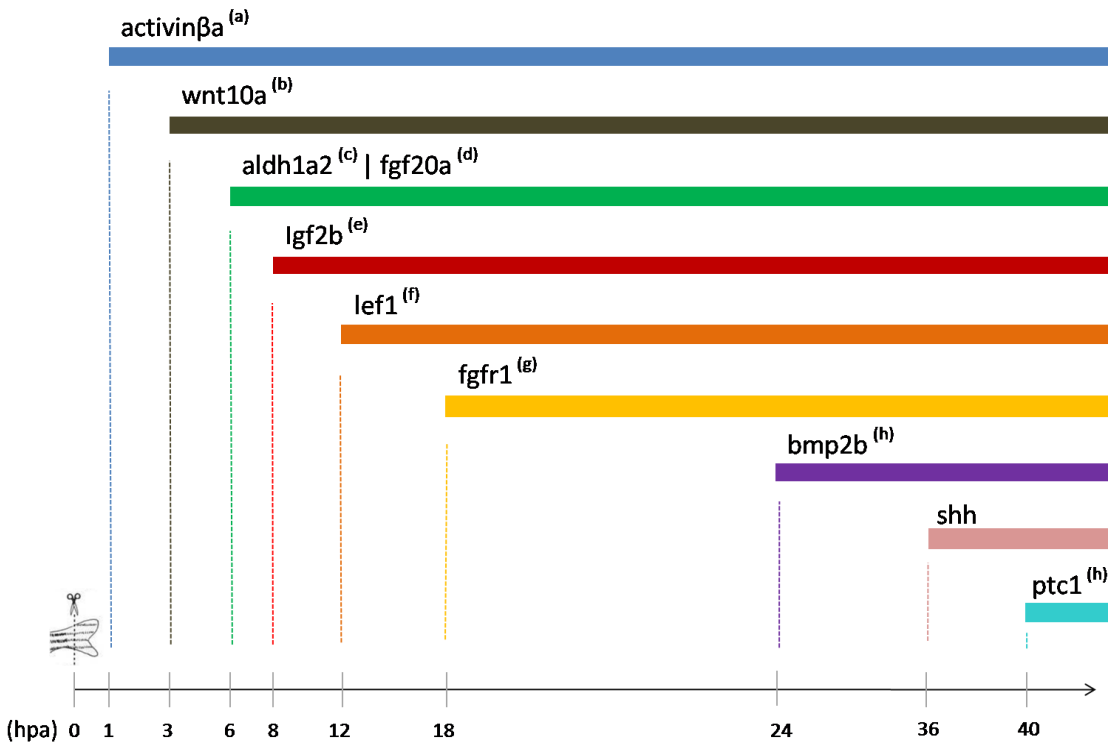

Figure 1.5. Timeline of activation of different players during zebrafish caudal fin regeneration.

Please note that the time of initiation of these players has been based on the available data (except for *shh*, which was based on our own data). It does not mean that it is the absolute initiation time. References: (a) Jazwinska et al., 2007; (b) Stoick-Cooper et al., 2007; (c) Blum and Begemann, 2012; (d) Whitehead et al., 2005; (e) Chablais and Jazwinska, 2010; (f) Poss K et al., 2000a; (g) Poss et al., 2000b; (h) Laforest et al., 1998

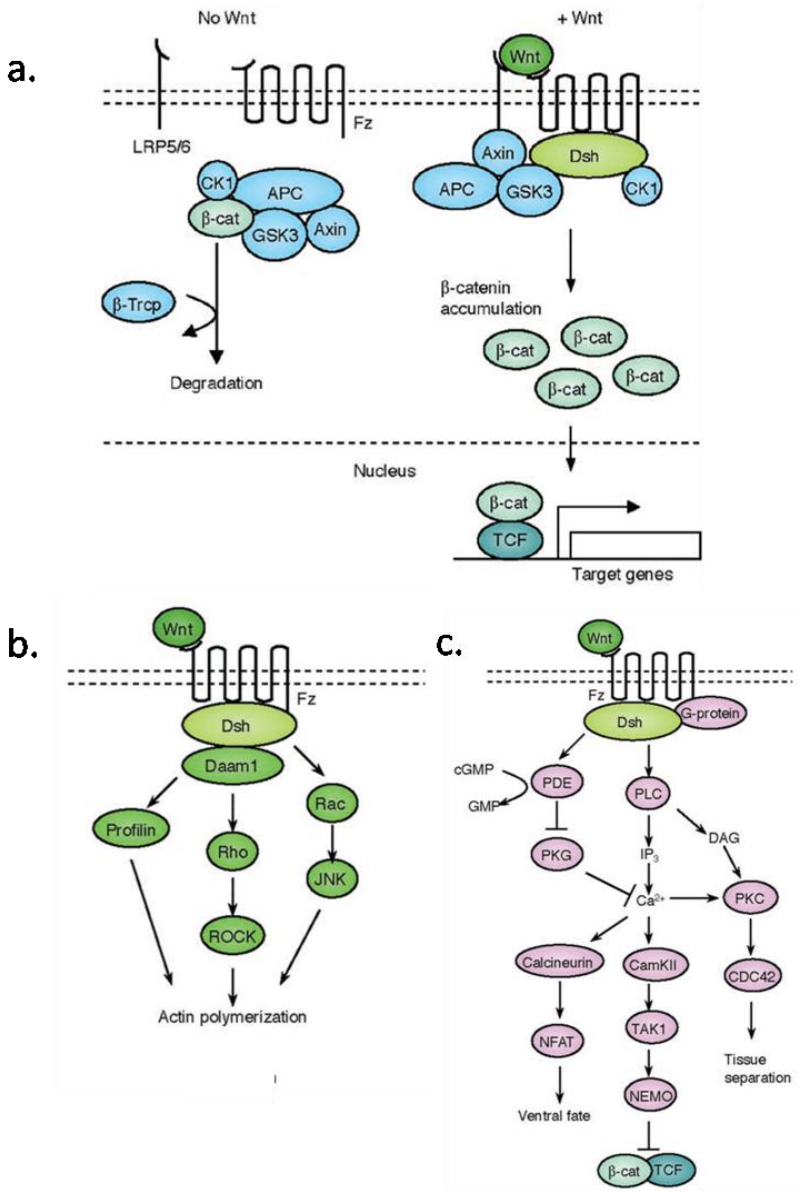

Figure 1.6. Canonical Wnt signalling pathway (a), Planar Cell Polarity transduction cascade (b) and Wnt/Ca $^{2+}$ signal transduction cascade (c). (a) Upon Wnt stimulation, stabilization of β-catenin is induced. β-catenin translocates into the nucleus where it mediates the transcriptional induction of targets. **(b)** Wnt signalling transduction leads to the regulation of the cytoskeleton through c-Jun N-terminal kinases (Jnk), Profilin and Rho kinase (ROCK). **(c)** Wnt signaling transduction through the modulation of Ca^{2+} levels can inhibit β-catenin/TCF function and regulate ventral cell fates, tissue separation and cell movements. Adapted from Komiya and Habas, 2008.

induced in the external-most layers of the regeneration epidermis and also in the epidermal regions several segments proximal to the amputation plane. This expression pattern is maintained throughout regeneration (Poss et al., 2000a) and could be important to maintain cell-cell interactions and facilitate migration (Poss et al., 2003).

wnt10a is the earliest Wnt ligand detectable already at 3 hpa by quantitative PCR (qPCR), possibly playing a role in the early activation of the β-catenin pathway (Stoick-Cooper et al., 2007b) (Figure 1.5). At 12 hpa *lef1* starts to be expressed in wound epidermal cells just distal to the amputation plane, before the formation of the epidermal basal layer. During these early stages, Lef1 might be involved in the formation of the basal epidermal layer and/or in blastema induction. Later, during blastema formation, *lef1* marks the basal epidermal layer surrounding the forming blastema and in the regenerative outgrowth phase, *lef1* expression is localized in the proximal region of the basal epidermal layer and in the distal blastema (Poss et al., 2000a) (Figure 1.4). Both *wnt5a* and *wnt5b* are expressed in the basal epidermal layer of the epidermis and in the distal blastema, with *wnt5a* extending further proximally in the basal epidermal layer (Stoick-Cooper et al., 2007b) (Figure 1.4).

Blocking Wnt signalling shortly before amputation, using a heat-shock inducible transgenic for Dickkopf1 (Dkk1), an inhibitor of the Wnt/β catenin signalling pathway, reveals that cells are still able to successfully migrate and cover the wound. However, *lef1* expression is lost, indicating that the basal layer of the wound epidermis is not specified correctly (Stoick-Cooper et al., 2007b). Moreover inhibition of Wnt/β-catenin signaling pathway severely impairs formation of the regeneration blastema and its subsequent proliferation in the outgrowth phase. On the other hand, it is also possible to enhance Wnt/β-catenin signalling during fin regeneration using a transgenic zebrafish line that overexpresses Wnt8 after heat shock. Wnt8 overexpression increases

the expression of the Wnt target *axin2*, proliferation of the blastema mesenchyme and overlying epithelium 6 hours after induction of the transgene. In spite of presenting an increased proliferation, the regenerated fin length is unaffected even after repeated pulses of activation of the transgene. However, an increase in the fin length after 10 days of regeneration is observed in a zebrafish mutant that has a mutation in one copy of *axin1*, an inhibitor of the Wnt/β-catenin signalling pathway. The faster regeneration in the *axin1*$^{+/-}$ zebrafish could be explained due to a more prolonged and consistent activation of the pathway (Stoick-Cooper et al., 2007b).

On the other hand, the activation of the β-catenin independent pathway using a transgenic line carrying a heat-shock inducible *wnt5b-gfp*, causes defects similar to the inhibition of Wnt/β-catenin signaling pathway through Dkk1 overexpresion and blocks regeneration. In fact, Wnt5b overexpression leads to a reduced proliferation of the blastema mesenchyme and overlying epithelium 6 hours after induction. Conversely, the homozygous *wnt5b* (*pipetail*) mutant zebrafish had longer regenerates than wild-type siblings at 4 and 7 dpa, showing that *wnt5b* mutant fins regenerate faster, without presenting any patterning defects or inappropriate growth (Stoick-Cooper et al., 2007b).

I.1.7.2 Activin βA signalling is required during the three phases of fin regeneration

Activin βA is a secreted ligand that belongs to the Tgf-β protein superfamily and signals through serine/threonine kinase cell surface transmembrane receptors, regulating a large variety of genes during embryogenesis as well as in mature tissues (Shi and Massague, 2003) (Figure 1.7).

In the zebrafish caudal fin regeneration *activin-βA* is detected as early as 1 hpa by qPCR (Figure 1.5) and at 6 to 12 hpa by *in situ* hybridization, in mesenchymal cells at the wound margin of the interrays. At 24 hpa, *activin-βA* is additionally induced in the mesenchyme underlying the wound epidermis of

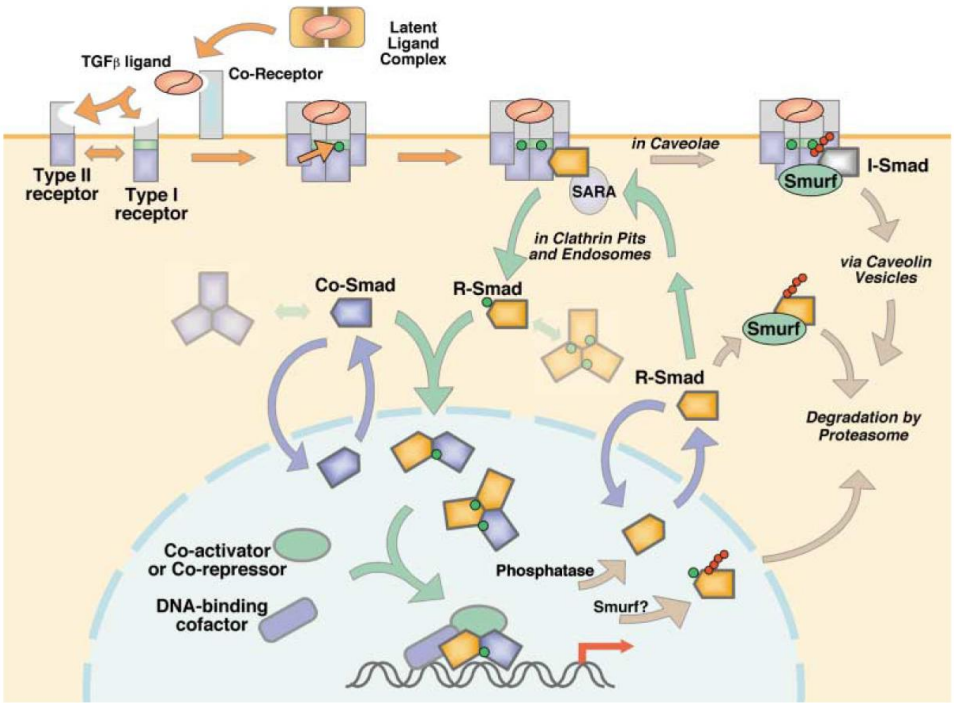

Figure 1.7. Tgf-β signalling pathway. A Tgf-β ligand initiates signalling by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface. This allows receptor II to phosphorylate the receptor I kinase domain, which then propagates the signal through phosphorylation of the Smad proteins. The activated Smad complexes translocate to the nucleus and, together with other nuclear cofactors, regulate the transcription of target genes. Adapted from Shi and Massague, 2003.

the rays, where the blastema is formed and at 72 hpa the expression is strongly detected in the blastema (Jazwinska et al., 2007) (Figure 1.4).

Activin-βA signalling is required in the three regeneration phases: wound healing, blastema formation and regenerative outgrowth. Its pharmacological inhibition during wound healing results in retraction of the interrays from the amputation plane. During blastema formation, pharmacological inhibition of Activin-βA signalling reveals its involvement in mesenchymal remodelling, mesenchymal proliferation and specification of blastema cells. In the regenerative outgrowth phase, the pharmacological blockage of this signalling demonstrates its requirement for the maintenance of the blastema proliferative potential and, in addition, the MO-mediated knockdown of *activin-βA* and of its receptor *alk4* impairs normally initiated regeneration (Jazwinska et al., 2007).

I.1.7.3. IGF signalling is activated and necessary during fin regeneration

The IGF signalling consists of two cell surface receptors (Igf1r and Igf2r), two ligands (Igf-1 and Igf-2), a family of six high-affinity Igf-binding proteins (Igfbp), as well as a range of Igfbp degrading proteases (Edmondson et al., 2003) (Figure 1.8).

IGF signalling has been considered required for mammalian skin homeostasis and wound healing (Edmondson et al., 2003; Semenova et al., 2008; Werner and Grose, 2003). However, only recently the contribution of Igf signalling in fin regeneration was addressed (Chablais and Jazwinska, 2010).

When the zebrafish caudal fin is amputated, *igf2b* expression starts to be detected during the wound healing phase, at 8 hpa by qPCR, and progressively increases its expression levels in the subsequent phases of regeneration (Figure 1.5). By *in situ* hybridization, *igf2b* is detected in the the blastema at 24, 48 and 72 hpa (Figure 1.4). In addition, *igf1* receptors expression is ubiquitous in the uncut and regenerating fin and the phosphorylated form of Igf1r is induced at the wound margin upon amputation, indicating the activation of this signalling during the regeneration process (Chablais and Jazwinska, 2010).

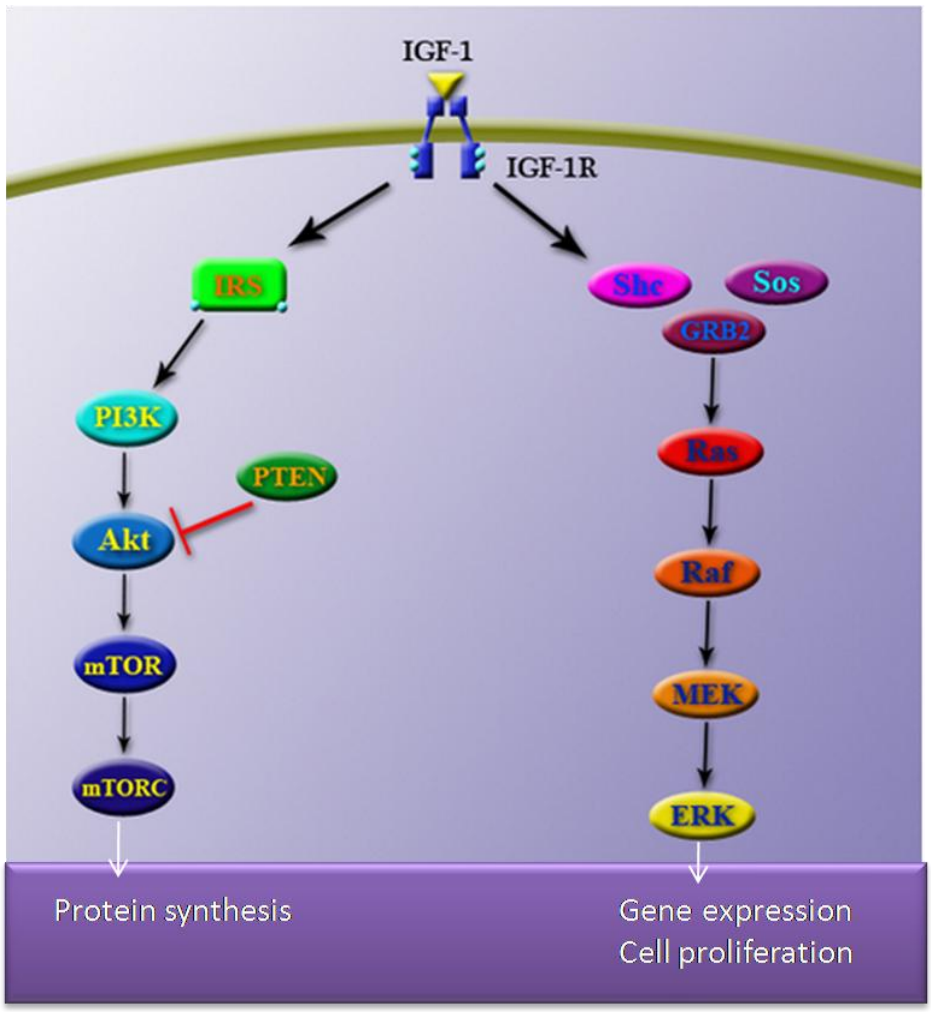

Figure 1.8. Igf1r signalling pathway. Igf1 or Igf2 binding to Igfr1 results in the phosphorylation of the insulin receptor substrate (IRS), initiating a cascade of events that will ultimately lead to protein synthesis. Signalling through the Igf1r also activates the adaptor proteins Shc and Grb2, leading to the activation of MAPK, which will interfere with gene expression and result in cell proliferation. Adapted from Scartozzi et al., 2011.

Importantly, using a pharmacological approach, it was addressed the requirement of IGF signalling in all phases of fin regeneration: wound healing, blastema formation and blastema function and maintenance during the regenerative outgrowth phase. During the wound healing phase it acts as a survival factor, is implicated in the formation of a well-structured wound epidermis and in the maintenance of intrinsic molecular properties of the basal epidermal layer (Chablais and Jazwinska, 2010). During blastema formation, IGF signalling has a mitogenic role and regulates the expression of the blastema markers *msxb* and *fgf20a*. The pharmacological inhibition at the beginning of the outgrowth phase, affects the expression of molecular markers and proliferation of the blastema cells resulting in the impairment of fin regeneration (Chablais and Jazwinska, 2010).

I.1.7.4. RA signalling is essential throughout the different regeneration phases

RA is the biologically active form of vitamin A and is an important molecule during growth and development. RA signalling is mediated by the retinoic acid receptors (RAR) and retinoid X receptors (RXR). Binding of RA ligand to receptors alters the conformation of the receptor, which affects the binding of other proteins that either induce or repress transcription (Vilhais-Neto and Pourquie, 2008) (Figure 1.9).

Only recently it was shown the importance of RA in fin regeneration (Blum and Begemann, 2012). Following amputation, the RA-synthesizing enzyme *aldh1a2* expression is detected at 6 hpa by qPCR (Figure 1.5). At 18 hpa, by in situ hybridization, aldh1a2 expression is observed within one segment proximal do the amputation plane in the ray and inter-ray mesenchyme (Figure 1.4).

Using a transgenic line that allows heat shock-inducible degradation of endogenous RA, it was demonstrated the requirement of RA signalling in the three regeneration phases. RA is involved in the formation of a well-structured and specified wound epidermis, controls cell cycle entry during blastema formation and also subsequent proliferation in the regenerative outgrowth phase. Importantly, RA regulates Fgf, Wnt and Igf signallings in the fin stump

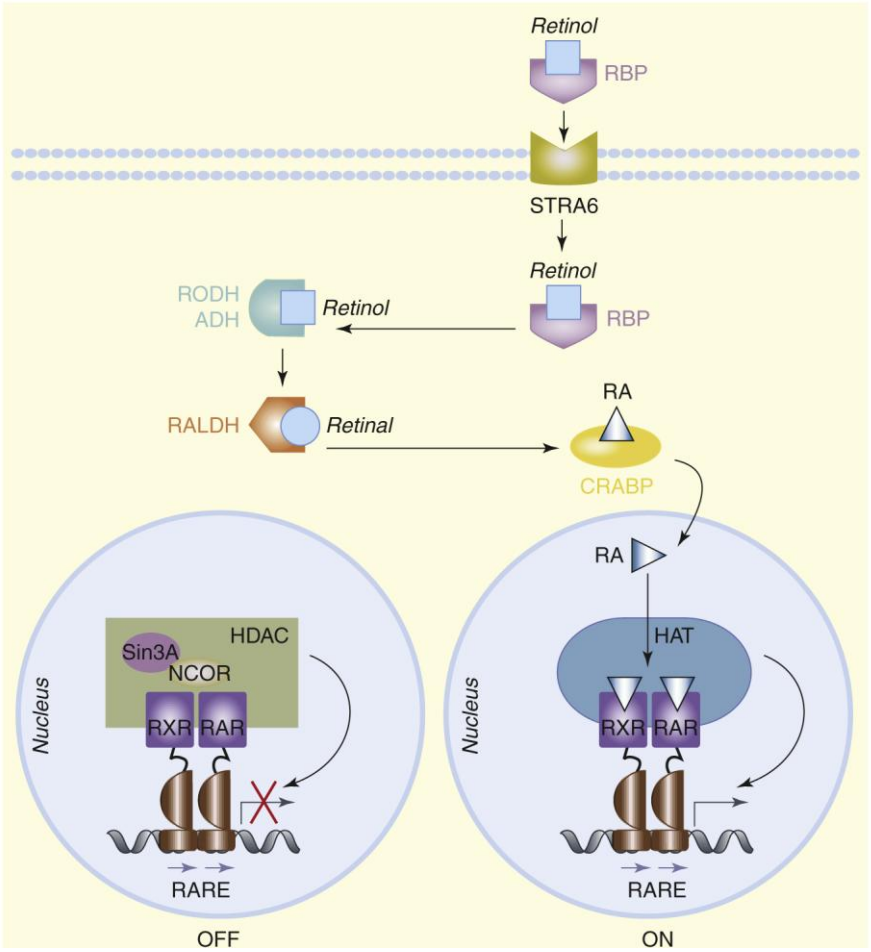

Figure 1.9. Overview of the RA function in the cell. In the absence of RA, RAR/RXR heterodimers recruit the co-repressor complex NCOR/Sin3A/HDAC (left nucleus). Upon retinoic acid binding to the RAR/RXR heterodimers, co-activator complex HAT is recruited and transcription is initiated in the DNA regions called retinoic acid response elements (RAREs) (right nucleus). Adapted from Vilhais-Neto and Pourquie, 2008.

and mediates a pro-survival mechanism in the blastema cells through upregulation of *bcl2* expression (Blum and Begemann, 2012).

I.1.7.5. Fgf signalling plays a key role in blastema formation and regenerative outgrowth

Fgfs are key regulators of several developmental processes in which cell fate and differentiation to various tissue lineages are determined. The Fgf ligands signal via a family of tyrosine kinase receptors and, depending on the cell type or stage of maturation, produce diverse biological responses that include proliferation, growth arrest, differentiation or apoptosis (Ornitz and Itoh, 2001) (Figure 1.10).

A few studies have addressed the role of Fgf signalling in fin regeneration. Soon after amputation, at 6 hpa, *fgf20a* is detected (Figure 1.5) in mesenchymal cells adjacent to the epidermis. During blastema formation, *fgf20a* expression is observed in the blastema cells, where it colocalizes with *msxb*. This overlap is maintained in the regenerative outgrowth phase in a distal subset of *msxb* expressing cells (Whitehead et al., 2005) (Figure 1.4).

The *fgf receptor 1* (*fgfr1*) expression is detected at 18 hpa in cells that seem to be in the process of forming a blastema (Figure 1.5). The onset and pattern of expression of *fgfr1* is coincident with the blastema markers *msxb* and *msxc*, expressed in the cycling cells during blastema formation At 48 hpa *fgfr1* is expressed in the mesenchymal cells of distal blastema and bilaterally in the basal layer of the epidermis (Poss et al., 2000b) (Figure 1.4).

At the onset of regenerative outgrowth, 48 hpa, *fgf24* (Figure 1.5) starts to be expressed in the distal regeneration epidermis overlying the distal blastema where *fgfr1* and *msxb/c* are expressed (Poss et al., 2000b) (Figure 1.4).

The absence of Fgf20a, in the Fgf20a zebrafish mutant, does not lead to primary defects in the wound closure, but results in an abnormally structured basal epidermal layer and the lack of expression of basal epithelium markers (*lef1* and *sparc1*). In addition, the mesenchymal disorganization and the

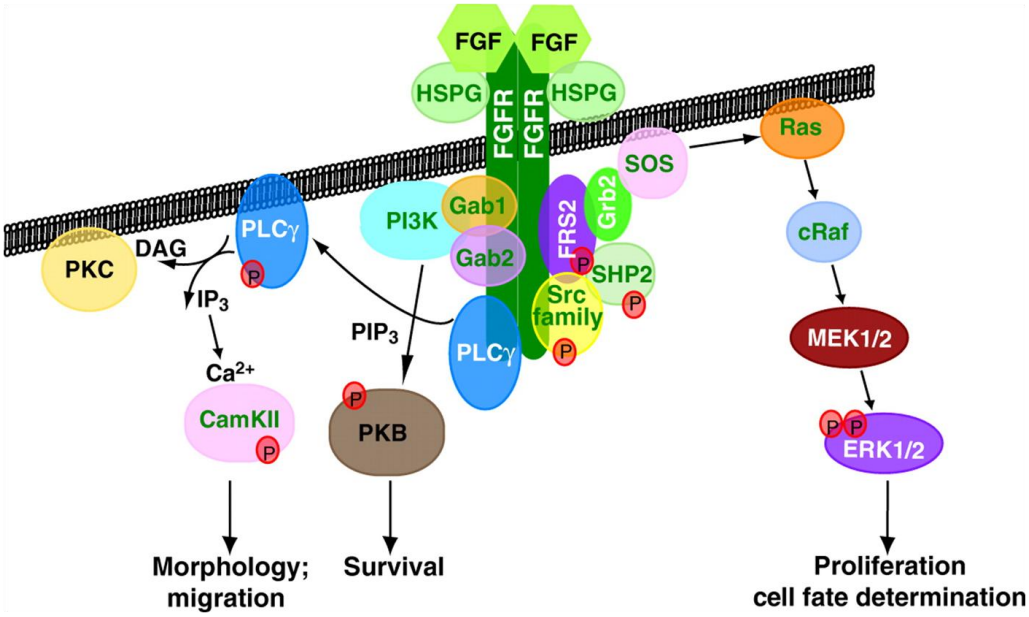

Figure 1.10. Fgf signalling pathway. Ligand binding leads to receptor dimerization, which results in a conformational shift in the receptor structure activating the intracellular kinase domain. This is followed by the activation of several intracellular signalling pathways that ultimately result in a cellular response to regulate morphology, migration, survival, proliferation and cell fate determination. Adapted from Dorey and Amaya, 2010.

subsequent blastema formation are impaired (Whitehead et al., 2005). Likewise, specifically inhibiting Fgfr1 does not affect formation of the wound epidermis. However, treatment with a specific Fgfr1 inhibitor, decreases *msxb*/*c* expression levels, impairs blastema formation and consequently blocks the regeneration process. The reduction of *msx* genes expression, suggests that *msxb* and *msxc* might be downstream targets of Fgf signalling pathway in the induction of blastema formation. This hypothesis suggests a molecular and cellular mechanism for the contribution of this pathway to the process of blastema formation and outgrowth (Poss et al., 2000b). Furthermore, treatment with an Fgfr1 inhibitor decreases the expression of the patterning gene *shh*. Thus, in addition to its essential role in proliferation, Fgf might directly or indirectly regulate *shh* transcription. Furthermore, the levels of *fgf20a* transcripts are suppressed already at 3 hpa in Dkk1 overexpressing fins suggesting a direct regulation of *fgf20a* expression by Wnt/β-catenin signalling pathway (Stoick-Cooper et al., 2007b).

In addition, depletion of Fgf signalling during regenerative outgrowth, using a heat-shock inducible transgenic for the dominant negative form of Fgfr1, affects blastema proliferation. Importantly, the decrease in cell proliferation is only observed in the distal blastema, the region flanked by the epidermal expression of Fgf target genes (Lee et al., 2005).

I.1.7.6. Hh signalling is necessary for the fin outgrowth

The Hh signalling pathway is one of the key regulators of animal development and is present in all bilaterians. The Hh ligand signals through the binding to Patched-1 (Ptc1) receptor. Ptc1 inhibits Smoothened (Smo), a downstream protein in the pathway, in the absence of ligand. Thus, binding of Hh will relieve Smo inhibition, leading to activation of Gli transcription factors, which then accumulate in the nucleus and regulate the transcription of Hh target genes (Huangfu and Anderson, 2006) (Figure 1.11). During fin regeneration, *shh* starts to be expressed around 36 hpa (Figure 1.5) in a subset of cells

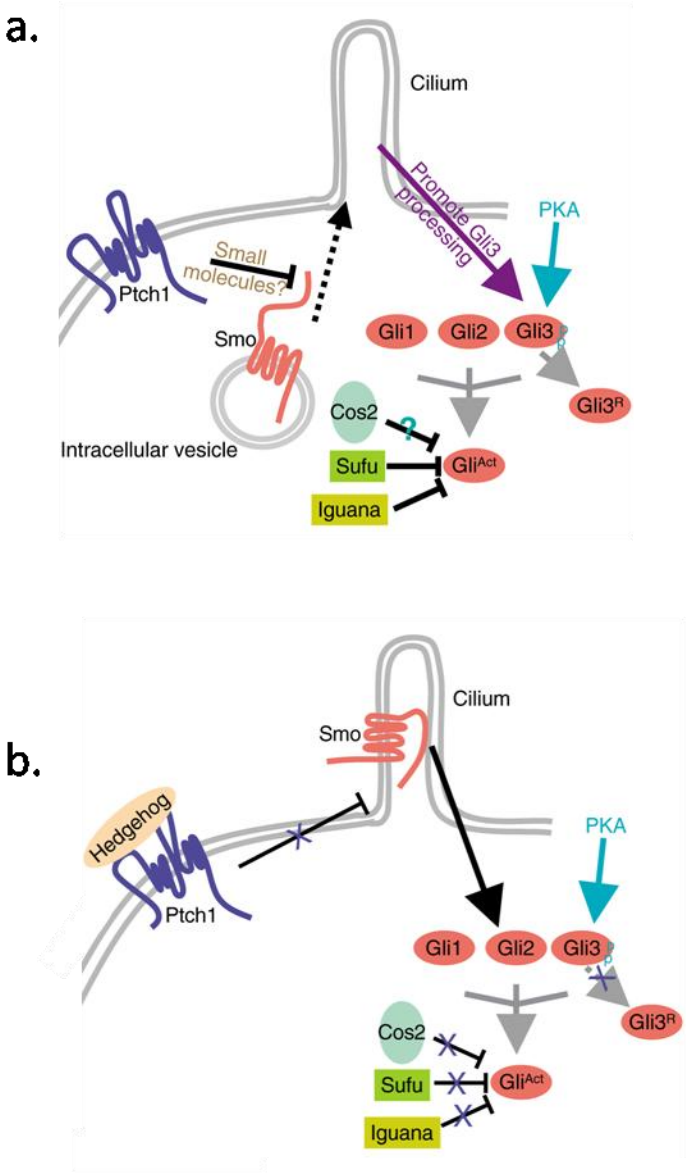

Figure 1.11. Hh signalling pathway. (a) In the absence of ligand, Ptc1 inhibits Smo, preventing Smo accumulation in cilia and the downstream events of the pathway. **(b)** In the presence of Hedgehoh, Smo inhibition is relieved and Smo is targeted to cilia, activating Gli proteins in a cilia-dependent manner. Adapted from Huangfu and Anderson, 2006.

on the proximal compartment of the basal layer of the epidermis, adjacent to the newly formed and aligned osteoblasts (Figure 1.4). The Hh membrane receptor *ptc1* starts to be expressed around 40 hpa (Laforest et al., 1998) (Figure 1.5) in the basal epidermal layer and in adjacent newly formed osteoblasts at a similar proximo-distal (PD) position along the fin ray as *shh* (Figure 1.4). However, while *shh* expression is observed in two groups of cells, ptc1 transcripts occupy the whole width of the fin ray (Laforest et al., 1998).

Disruption of Hh signalling by inhibiting its receptor Smo, after treatment with cyclopamine, causes a decrease in cell proliferation and cessation of fin outgrowth. Conversely, the ectopic expression of the ligand Shh leads to additional bone deposition, suggesting a role in proliferation and differentiation of osteoblasts. Interestingly, this bone deposition is inhibited by coinjection with chordin, an inhibitor of Bmp signalling, indicating that Bmp signalling pathway is required for the bone formation induced by Shh. On the other hand, cyclopamine treatments do not arrest bone matrix deposition by already differentiated osteoblasts, suggesting that Shh has no effect on bone matrix synthesis and release (Quint et al., 2002).

Treatment with SU5402, an inhibitor of Fgf signalling, declines *shh* expression. Conversely, *fgfr1* expression decreases after cyclopamine treatment. This suggests the existence of a relationship between Fgf and Hh signalling pathways which requires further investigation (Akimenko et al., 2003). Moreover, RA treatment downregulates *shh* expression after 1 hour of treatment and delays deposition of bone matrix after 24 hours of treatment. The rapid downregulation of *shh* expression suggests that RA may directly regulate this gene (Laforest et al., 1998). In fact, the zebrafish *shh* promoter contains a RA response element, which was already shown to be regulated by RA receptors in HeLa cells (Chang et al., 1997).

I.1.7.7. Bmp signalling is induced and required in the outgrowth phase

The Bmps are soluble proteins belonging to the Tgf-β superfamily. Bmp ligands signal through binding to a complex of specific receptors on the cell surface consisting of the Bmp receptor type I and Bmp receptor type II. This leads to the phosphorylation of the receptor type I that subsequently phosphorylates the Bmp-specific Smads, which will translocate to the nucleus to act as transcriptional enhancers (Figure 1.7). The Bmp signalling is essential during embryonic development, patterning and early skeletal formation (Bleuming et al., 2007).

In the zebrafish fin regeneration, Bmp signalling was already shown to play a role during the regenerative outgrowth phase. *bmp6* is expressed in the differentiating osteoblasts, basal layer of the epidermis and proliferating blastema, *bmp4* is expressed in the distal blastema and *bmp2b* expression is detected at 24 hpa (Figure 1.5) in the differentiating osteoblasts (Smith et al., 2006), as well as in the adjacent cells of the basal epidermis where it overlaps with *shh* (Laforest et al., 1998) (Figure 1.4). Importantly, ectopic expression of Bmp2b in the inter-ray tissue induces bone matrix deposition leading to the fusion of the bony rays (Quint et al., 2002). This suggests that Bmp2b might play a role in the differentiation of osteoblasts or in the correct patterning of the bone, possibly through interactions with the Hedgehog signalling pathway. On the other hand, ectopic expression of chordin, a Bmp inhibitor, induces a transient arrest of fin outgrowth, decreasing *msxb* expression and cell proliferation, possibly through the inhibition of Bmp4 and/or Bmp6 signalling in the distal blastema. In addition, ectopic expression of chordin also downregulates *runx2a* and *runx2b* expression in the osteoblasts ultimately resulting in a delayed bone matrix deposition. This phenotype is likely related to the inhibition of Bmp2b and/or Bmp6 signalling in the differentiating osteoblasts (Smith et al., 2006).

I.1.8. Cellular sources of regeneration

Until recently, little was known about the source that supplies new cells for the regeneration process. This has been an intriguing question that has for long raised interest in the field of regenerative medicine. Additionally, another major question has been to uncover whether all cells of the blastema are equally potent or lineage restricted. Uncovering these cellular and molecular mechanisms is an important step towards the development of regenerative strategies in humans.

The main mechanisms providing the cellular sources for regeneration have been generally classified as relying in stem/progenitor cells or in cell dedifferentiation/transdifferentiation (Jopling et al., 2011; Poss, 2010; Tanaka and Reddien, 2011).

I.1.8.1. Stem/progenitor-cell based regeneration

The stem/progenitor-cell-based regeneration requires the maintenance of a population of undifferentiated cell types which is used to regenerate tissue after injury. The identification of such population has often been limited due to the absence of undifferentiated cell markers and lack of tools for lineage-tracing studies (Poss, 2010; Tanaka and Reddien, 2011).

Well-understood examples of model organisms which have been shown to present a stem-cell-based regeneration are the invertebrates hydra and planarian, as previously described. In hydra, there is the contribution of three stem cell types (ectodermal and endodermal epithelial cells, and interstitial stem cells) while in planarian, a population of adult dividing cells, called neoblasts, is responsible for new tissue formation during regeneration (Tanaka and Reddien, 2011).

In vertebrates, many tissues maintain a stem cell population, including blood, skin, brain, lung, gut epithelium and skeletal muscle (Poss, 2010). The adult stem cells present in these tissues are known to be mainly involved in homeostasis and repair. Notably, it has so far been unknown whether these

cells participate in the regeneration of complex vertebrate tissues and organs in classic model organisms such as salamanders, frogs or zebrafish.

I.1.8.2. Dedifferentiation and transdifferentiation based regeneration

Dedifferentiation or transdifferentiation based regeneration occurs through mechanisms that do not require a population of multipotent stem cell or undifferentiated progenitors. Dedifferentiation refers to a reduction in the molecular and functional properties of a differentiated cell type and might lead to a multipotent state. On the other hand, transdifferentiation is the conversion from one cell type to another, sometimes through an undifferentiated intermediate (Jopling et al., 2011; Poss, 2010).

Recent studies in zebrafish suggest that a dedifferentiation mechanism is present in heart regeneration. Using Cre/loxP-based genetic labeling to track cardiomyocytes, these studies show that cardiomyocyte dedifferentiation and proliferation is the primary source for heart regeneration (Jopling et al., 2010; Kikuchi et al., 2010). In another study, it was also demonstrated that epicardial cell lineage do not contribute to cardiomyocyte formation during heart regeneration, demonstrating the existence of lineage restriction (Kikuchi et al., 2011).

Similar fate mapping studies in the zebrafish fin regeneration show that mature osteoblasts dedifferentiate to form part of the blastema (Knopf et al., 2011; Sousa et al., 2011). Osteoblast-derived blastema cells remain lineage restricted and give rise only to osteoblasts in the regenerating fin (Knopf et al., 2011).

Altogether, the heart and fin regeneration studies in zebrafish provide strong evidence for mature cells as the source for vertebrate organ and tissue regeneration.

The regeneration of the salamander limb represents one of the most complex vertebrate regeneration examples. For this reason, it has been one of the most extensively studied models over the last century. However, the various experiments performed since 1961, led to many possible

interpretations about the cellular sources of the limb blastema. Indeed, it is still lacking in *vivo* evidence for the contribution of mature differentiated cells to limb regeneration based on molecular markers of cellular differentiation status and genetic lineage tracing (Poss, 2010; Tanaka and Reddien, 2011).

Importantly, previous work has shown that the different cell lineages retain their fate when they go through a regenerative process. This was demonstrated for vessel/artery, osteoblast, fibroblast, glial, melanophore/xanthophore, iridiphore, epidermis and lateral line cell lineages in the zebrafish fin (Tu and Johnson, 2011) and Schwann cells, muscle and cartilage/connective tissue in the salamander limb (Kragl et al., 2009). More recently, a similar fate restriction was documented in neonatal (Lehoczky et al., 2011) and adult (Rinkevich et al., 2011) mouse digit tip regeneration. Thus, the mechanism of cellular transdifferentiation does not seem to be involved in the regeneration process in these models.

A well studied, and possibly the only reported example of a transdifferentiation mechanism in a regeneration model organism, is the newts lens regeneration. Upon removal of the lens, pigmented epithelial cells from the dorsal iris undergo transdifferentiation events and regenerate a new functional lens (Jopling et al., 2011; Poss, 2010).

I.2. Aims and outline of the thesis

The aims of my PhD work were to address the regenerative capacity limit of the zebrafish caudal fin with a detailed characterization of the morphology, molecular markers and positional information.

In **Chapter 1**, I review the literature in the regeneration field. The reviewed topics include: classic model organisms used in the regeneration studies, the several hypothesis to explain the loss of regenerative ability during evolution, the main signaling pathways involved in the successive steps of zebrafish caudal fin regeneration and the cellular sources of regeneration in several contexts/animal models.

In **Chapter 2**, I present the data of the paper Azevedo et al., 2011 published in Plos One. We show that consecutive repeated amputations of zebrafish caudal fin do not reduce its regeneration capacity and do not compromise any of the successive regeneration steps: wound healing, blastema formation and regenerative outgrowth. Even after inhibition of regeneration caused by the loss of Wnt/β−catenin signalling, a new amputation resets the regeneration capacity within the caudal fin, suggesting that blastema formation does not depend on a pool of stem/progenitor cells that require Wnt/β-catenin signalling for their survival.

In **Chapter 3**, in an unpublished manuscript format, we demonstrate that positional information of the bony ray bifurcation is affected with repeated amputations at different levels. We show that there is a progressive distalization of the position of this structure in the regenerated fin when the repeated amputations are done proximally near the bifurcation. On the other hand, its position is maintained with repeated amputations at a more proximal level. By using a transgenic containing a dominant-negative *fgfr1-egfp* fusion gene and a transgenic line expressing GFP under the control of *shh* promoter, we have analyzed the role of Fgf and Shh in the determination of the

bifurcation position. Using these tools we could observe that they do not seem to be the instructive signals.

In **Chapter 4**, I summarize the main findings of the 3 year work presented in the thesis, discussing and integrating them with the literature. I also propose the follow up experiments to go further in the understanding of the main unresolved questions.

CHAPTER II

The regenerative capacity of the zebrafish caudal fin is not affected by repeated amputations

The work presented was published in Azevedo, A. S., Grotek, B., Jacinto, A., Weidinger, G. and Saude, L. (2011). "The regenerative capacity of the zebrafish caudal fin is not affected by repeated amputations." *PLoS One* **6**(7): e22820.

The regenerative capacity of the zebrafish caudal fin is not affected by repeated amputations

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Abstract

Background

The zebrafish has the capacity to regenerate many tissues and organs. The caudal fin is one of the most convenient tissues to approach experimentally due to its accessibility, simple structure and fast regeneration. In this work we investigate how the regenerative capacity is affected by recurrent fin amputations and by experimental manipulations that block regeneration.

Methodology/Principal Findings

We show that consecutive repeated amputations of zebrafish caudal fin do not reduce its regeneration capacity and do not compromise any of the successive regeneration steps: wound healing, blastema formation and regenerative outgrowth. Interfering with Wnt/ß-catenin signalling using heat-shock-mediated overexpression of Dickkopf1 completely blocks fin regeneration. Notably, if these fins were re-amputated at the non-inhibitory temperature, the regenerated caudal fin reached the original length, even after several rounds of consecutive Wnt/ß-catenin signalling inhibition and re-amputation.

Conclusions/Significance

We show that the caudal fin has an almost unlimited capacity to regenerate. Even after inhibition of regeneration caused by the loss of Wnt/ß-catenin signalling, a new amputation resets the regeneration capacity within the caudal fin, suggesting that blastema formation does not depend on a pool of stem/progenitor cells that require Wnt/ß-catenin signalling for their survival.

Introduction

In contrast to humans, some organisms retain the extraordinary capacity to regenerate throughout adult life. One of such organisms is the zebrafish, a vertebrate that is able to regenerate fins, scales, retina, spinal cord and heart among other internal organs [1].

Due to its accessibility, its fast and robust regeneration and its simple architecture, the zebrafish caudal fin is one of the most powerful models for regenerative studies. The caudal fin is composed of several segmented bony rays and inter-ray mesenchymal tissue, all enclosed by an epidermis. Each bony ray consists of 2 concave hemirays that define an inner space filled with intra-ray mesenchymal cells. Blood vessels and nerve axons are found in both intra- and inter-ray tissues [2]. Bony rays are produced and maintained by osteoblasts (also called scleroblasts), skeletogenic cells that secrete bone matrix [3].

When a caudal fin is amputated, a regenerative program with stereotypic successive steps is activated and it takes approximately 2 weeks to fully regenerate all the tissues and structures that compose a functional fin. Within 1-3 hours-post-amputation (hpa), epithelial cells migrate to cover and close the wound. By 18-24 hpa, an apical epidermal cap (AEC) is formed and a mass of undifferentiated mesenchymal cells called the blastema accumulates underneath the AEC [2]. At 24 hpa the blastema cells segregate into two morphologically indistinct compartments: a slowly proliferating distal blastema and a rapidly proliferating proximal blastema. The distal blastema contributes with daughter cells to the proximal blastema, which is a population of cells that migrate to new positions and differentiate to replace the lost tissues. After 48 hpa the regeneration program is installed and the regenerative outgrowth continues until the original tissue architecture is reconstituted [4].

The capacity to make and organize a blastema is a shared feature of all organisms that are able to efficiently regenerate upon appendage

amputation. Although the active cell proliferation of the blastema is required for the progression of regeneration, little is known about the origin and fate of the blastema cells in the fish fin. Regarding the origin of blastema cells, we could consider two hypotheses. One possibility is that stem/progenitor cells become activated upon amputation and migrate distally to form the blastema. While stem cells are the source of regenerating tissues in invertebrates such as planarians and annelids among others [5], little evidence for the contribution of resident stem cells to the formation of the blastema has been obtained in vertebrate appendage regeneration, with the exception of a potential role of muscle satellite cells in salamander limb regeneration [6]. Another possibility that has been proposed to occur in urodele amphibians is that blastema cells originate from a process of dedifferentiation of adult differentiated cells [7]. Lineage tracing analysis using injection of dyes has suggested that muscle fibers disintegrate and that cells containing the dye are found in the forming blastema in regenerating urodele limbs [8,9]. However, whether muscle-derived cells contribute to the forming regenerate has not been shown. Thus, *in vivo* evidence for the contribution of mature differentiated cells to appendage regeneration based on molecular markers of the cellular differentiation status and genetic lineage tracing is lacking for the salamander. We have recently used such tools to address the cellular mechanism of bone regeneration in the zebrafish caudal fin [10]. Interestingly, we found that mature osteoblasts dedifferentiate to form part of the appendage blastema. Osteoblast-derived blastema cells remain lineage restricted and give rise only to osteoblasts in the regenerating fin. Thus, strong evidence for mature cells as the source of regenerating vertebrate appendages is starting to accumulate. Other recent studies have shown that other cell lineages also retain their fate when they go through a regenerative process in the zebrafish fin [11] and in the salamander limb [12]. Therefore, transdifferentiation from one lineage into another does not occur during vertebrate appendage regeneration and blastema cells, whether they form

by dedifferentiation or from progenitor cells, do not appear to be multipotent.

Regeneration of a complex organ must involve a number of signalling pathways to coordinate blastema formation, cell proliferation, differentiation and patterning events. Although we are beginning to understand the molecular mechanisms of regeneration, it is becoming clear that signalling pathways such as Hedgehog (Hh), Fibroblast growth factor (Fgf) and Wnt among other molecules are activated upon amputation and control different aspects of caudal fin regeneration in zebrafish [1,13]. Fin regeneration is impaired due to a reduction in cell proliferation when Hh signalling is disrupted by inhibiting its receptor Smoothened using cyclopamine. Conversely, the ectopic overexpression of *sonic hedgehog* (*shh*) leads to excessive bone deposition in regenerating fins, suggesting a role in proliferation and differentiation of bone-secreting cells [14]. The formation of the blastema is impaired in *fgf20a* mutants, when Fgfr1 is pharmacologically inhibited and in a transgenic line expressing a dominant-negative Fgfr1, [15,16,17]. The Wnt signalling pathway also plays a role during appendage regeneration in zebrafish. Increasing canonical Wnt/ß-catenin signalling, either by overactivating *wnt8* or in *axin1* heterozygous mutants, is sufficient to augment regeneration while inhibition of Wnt/ß-catenin signalling by overactivating the specific inhibitor Dkk1 leads to failure to form the blastema and to a block in regeneration [13]. In contrast, overexpression of non-canonical *wnt5b* inhibits fin regeneration, possibly by interfering with Wnt/ß-catenin signalling. In agreement, fin regeneration is accelerated in *wnt5b* homozygous mutants [13]. Therefore, a balance between canonical and non-canonical Wnt signalling seems to be required for successful fin regeneration. A big challenge now is to understand the interplay between these signalling pathways and to uncover the ways by which they are modulated during regeneration.

In this study, we have evaluated the robustness of the regenerative capacity of zebrafish caudal fins. We show that consecutive repeated amputations over a long period of time do not compromise blastema formation and outgrowth. This reveals an almost unlimited capacity to reconstitute a complex structure, possibly only limited by the life span of the fish. In addition, we challenged the regenerative capacity even further, by asking whether fin regeneration could occur normally after it has been repeatedly blocked with cycles of amputation and inhibition of Wnt/ß-catenin signalling. Once again we found that even in this extreme situation, the permanent block of regeneration caused by overexpression of Dkk1 can be relieved by a subsequent re-amputation, which then leads to normal regeneration.

Results

II.1. The caudal fin maintains its original size after consecutive repeated amputations

We designed a consecutive repeated amputation experiment to evaluate whether caudal fin regeneration is limited (Figure 2.1). The caudal fin of initially 24 adult zebrafish siblings was subjected to three amputations every month. During the first 6 months the first amputation (1st amp) was done one bone segment below the most proximal bony ray bifurcation. In the following months, the first amputation (1st amp) was done 6 segments distally to the base of the fin. After 8 hours (8hpa), a second amputation (2nd amp) was performed to collect the regenerate portion (RP) together with stump tissue of one bone segment in length (the non-regenerate portion, NRP). After 72 hours (72 hpa), a third amputation (3rd amp) was performed to collect separately the RP and the NRP to evaluate the effect of consecutive repeated amputations on regenerative outgrowth. Thereafter, we allowed the caudal fin

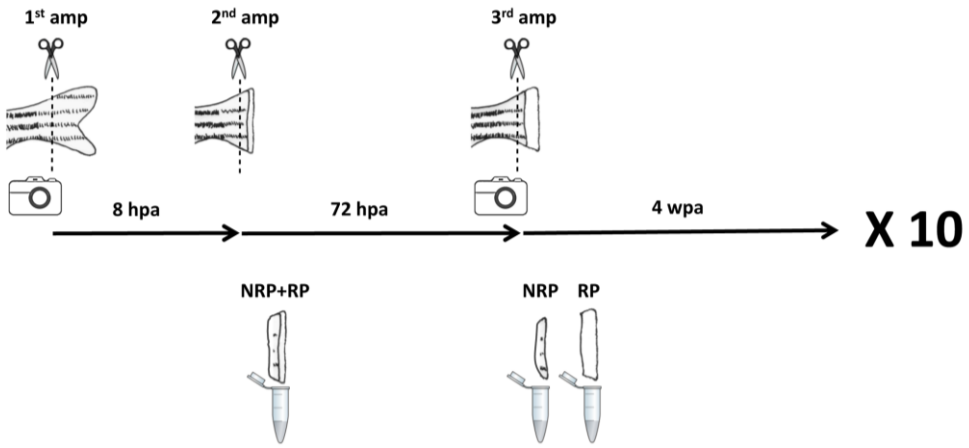

Figure 2.1. Outline of the consecutive repeated caudal fin amputations performed every month over an 11-month period. Each month, the fully regenerated caudal fin was photographed and amputated. After 8 hpa, it was subjected to a second amputation and the amputated tissue was collected. After 72 hpa, the caudal fin was photographed again, a third amputation was performed and the amputated tissues were collected. After 4 wpa, the procedure was repeated. The entire procedure was done 10 times. AMP: amputation; NRP: non-regenerate portion; RP: regenerate portion

to regenerate for 4 weeks (4 wpa) to ensure a complete regeneration. This amputation protocol was repeated 10 times spanning a period of approximately 11 months. To evaluate the regenerative outgrowth state following consecutive repeated amputations, we measured every month the 4 wpa full caudal fin area of each fish. As a control, we also measured the uncut caudal fin area of each fish just before initiating the consecutive repeated amputation experiment. The area of the 4 wpa full caudal fin did not change when we compared the uncut caudal fin area (n=24) with the one obtained after 27 cuts (n=14) (Figure 2.2A, B). To control for possible influence of fish age, we also measured the caudal fin area of zebrafish siblings (n=10) that were never amputated but were maintained over the experimental period in the exact same conditions. Again, we found no differences in the caudal fin area of these age-matched zebrafish siblings (Figure 2.2C). These results show that the regenerative outgrowth of the zebrafish caudal fin does not decline with repeated amputations.

II.2. Blastema formation is not impaired after consecutive repeated amputations

We next asked whether early events after amputation, in particular wound healing and blastema formation, might be affected by repeated amputations. To this end, we measured the size of the regenerate (RP) at 72 hpa. When we correct these values for the overall individual caudal fin size by dividing the RP area by the 4 wpa full caudal fin area on each month, we found that the relative area of the 72 hpa RP did not decrease significantly even when we compared the 72 hpa RP obtained after 2 cuts (n=24) with the one obtained after 29 cuts (n=14) (Figure 2.3A, B). To complement this data with a molecular analysis, we quantified the expression levels of the wound healing marker, *mmp9* [18] and the blastema cell marker, *msxb* [4]. Although the level of *mmp9* expression in 8 hpa NRP+RP showed a decrease after 14 cuts, this level was maintained in subsequent amputations (Figure 2.3C). The levels of *msxb* also slightly decreased, even though not significantly, with increasing

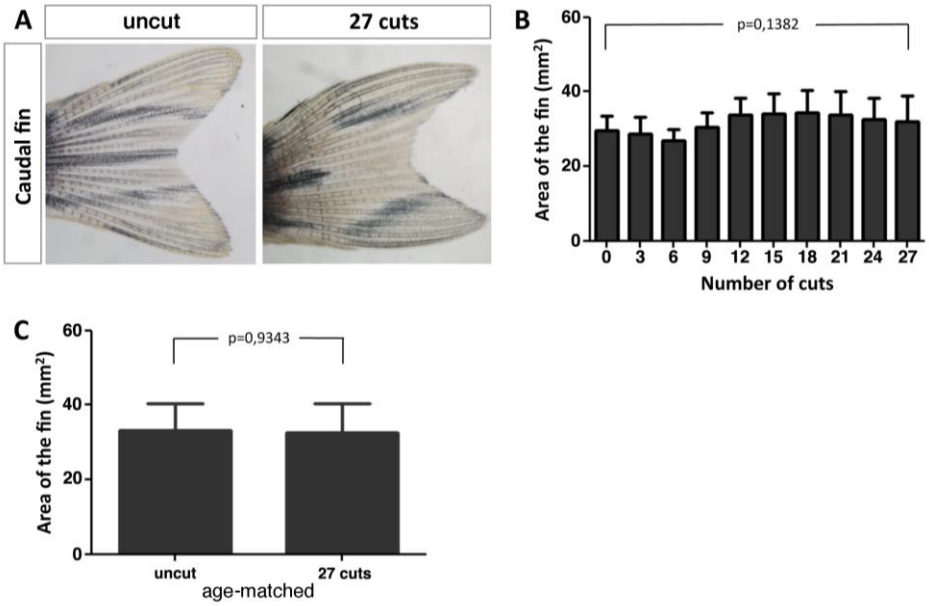

Figure 2.2. Consecutive repeated amputations maintain the original size of the fully regenerated caudal fin. (A) The same caudal fin before any amputation (0 cuts) and 4 wpa after 27 consecutive cuts. (B) Area of the 4 wpa regenerated caudal fin with increasing number of cuts. (C) Comparison of the caudal fin area of zebrafish siblings that were amputated 27 consecutive times with age matched siblings that were never amputated.

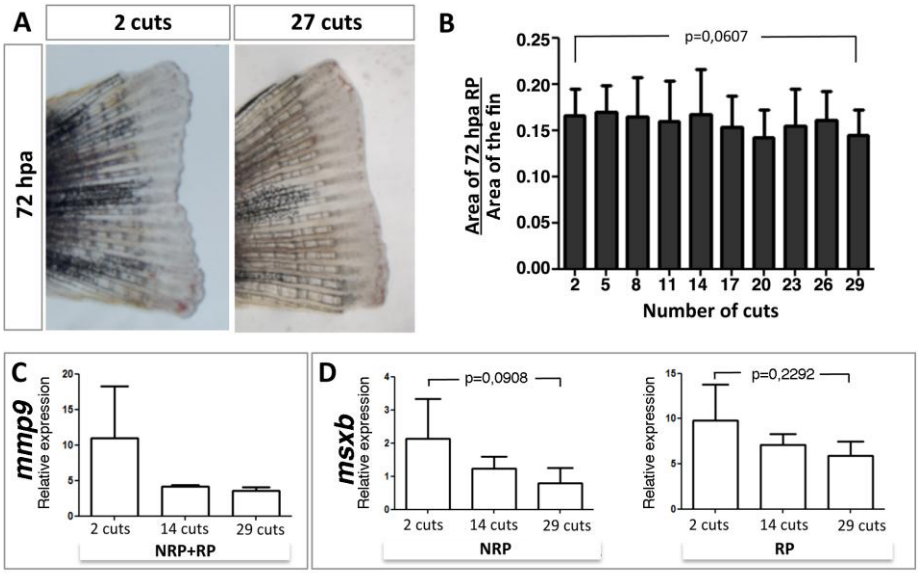

Figure 2.3. The 72 hpa regenerate size of the caudal fin is maintained with consecutive repeated amputations over an 11-month period. (A) A 72 hpa caudal fin obtained after the second consecutive amputation and after the twenty-seventh consecutive amputation. (B) Area of the 72 hpa regenerate over the area of the fully regenerated caudal fin immediately before the amputation measured with increasing number of cuts. (C) *mmp9* expression levels at 8 hpa with increasing number of cuts. (D) *msxb* expression levels at 72 hpa in both non-regenerate portions (NRP) and regenerate portions (RP) with increasing number of cuts.

number of amputations (Figure 2.3D). Since *msxb* is a blastema marker, it is not surprising that the levels of expression were higher in the 72 hpa RP when compared with the 72 hpa NRP (Figure 2.3D). These results reveal that, even if the expression of these markers slightly decreases with repeated amputations, these changes do not result in a decline of the fin's ability to successfully accomplish wound healing and blastema formation.

II.3. Consecutive repeated amputations affect the non-regenerated bone

A closer look at the bony rays present in caudal fins obtained after 27 consecutive amputations revealed a clear difference between the bone segments located proximal to the amputation plane (bone that was never amputated or old bone) and bone segments located distally to the amputation plane (regenerated or new bone). Overall, old bony rays got wider and bone segment boundaries became less defined along the entire proximal-distal axis (Figure 2.4B). This phenotype is not age dependent since the bony rays of uncut age-matched siblings did not change bone width and segment boundaries definition with time (Figure 2.4A).

To be able to characterize and quantify the bone phenotype, we performed an independent consecutive repeated amputation experiment where two amputations were performed every other week. The first amputation of the week was always done 6 segments distally to the base of the fin and the second amputation was always done one segment below the previous one. We observed that the old bone got progressively thicker after an increased number of amputations and a clear difference between the old and the new bone was already visible after 7 cuts (Figure 2.4C-E). Histological longitudinal sections of bony rays stained with Masson's trichrome expose the collagen content. This staining showed that the amount of collagen was increased in old bone (Figure 2.4G) when compared with new bone regenerated after 14 cuts (Figure 2.4H). Interestingly, the new bone showed

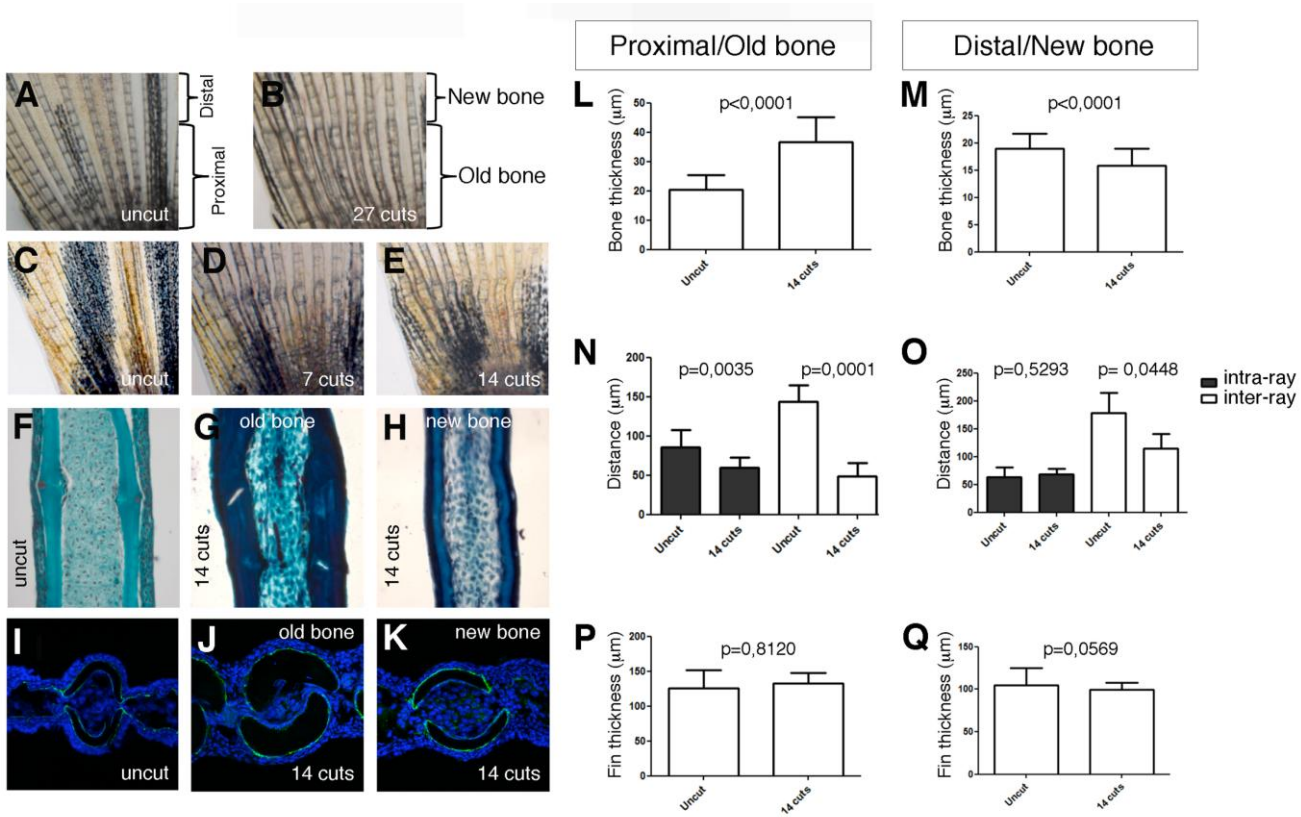

Figure 2.4. Consecutive repeated amputations affect the structure of the bone proximal to the amputation plane. Picture of the dorsal lobe of an uncut caudal fin (A) and its age-matched sibling after 27 cuts (B). Picture of the dorsal lobe of an uncut caudal fin (C) and a caudal fin after 7 (D) and 14 cuts (E). Masson´s trichrome staining of longitudinal sections of an uncut bony ray (F) and of an old (G) and regenerated (H) regions of a bony ray after 14 cuts. Confocal images of transverse sections of a Zns5 immunostained proximal region of an uncut caudal fin (I) and of the old (J) and new (K) tissue of a caudal fin after 14 cuts. Quantification of the bone thickness, inter- and intra-ray tissue and fin thickness in the old (L, N, P) and new (M, O, Q).

a similar amount of collagen when compared to the one present in the control uncut caudal fin (compare Figure 2.4H with Figure 2.4F). To determine if the increase in collagen content was accompanied by an increase in the number of osteoblasts, we analysed transverse sections of caudal fins immunostained with Zns5 by confocal microscopy. A single layer of Zns5^{+} cells was found to line the bone matrix in uncut controls and in old and new bone of fins after 14 cuts (Figure 2.4I-K), indicating that the number of osteoblasts lining the hemirays did not increase with repeated amputations. Quantification of the bone thickness, the space between the hemirays (intra-ray) and the space between rays (inter-ray) showed that the thickness of old bone increased significantly after 14 cuts, while the intra- and inter-ray space decreased concomitantly (Figure 2.4I,J,L,N). In contrast, the regenerated new tissue presented a slight decrease in the bone thickness and a mild reduction of the inter-ray space, while the amount of intra-ray tissue is slightly increased although not significantly when compared to the uncut caudal fins (Figure 2.4I,K,M,O). However the overall fin thickness, which is the sum of the bone thickness and the intra-ray space, was not affected proximally (old tissue) or distally (regenerated tissue) after 14 cuts. (Figure 2.4P,Q). We conclude that repeated amputations result in abnormal remodelling of the bone and mesenchymal tissue proximal to the amputation plane.

II.4. Regenerative capacity is not affected after repeated inhibition of caudal fin regeneration following Wnt/ß-catenin signalling perturbation

When Wnt/ß-catenin signalling is inhibited immediately after fin amputation, a wound epidermis forms, but blastema formation does not occur and regeneration is completely blocked [13,19,20]. We analyzed whether fin regeneration could occur normally after it has been previously perturbed.

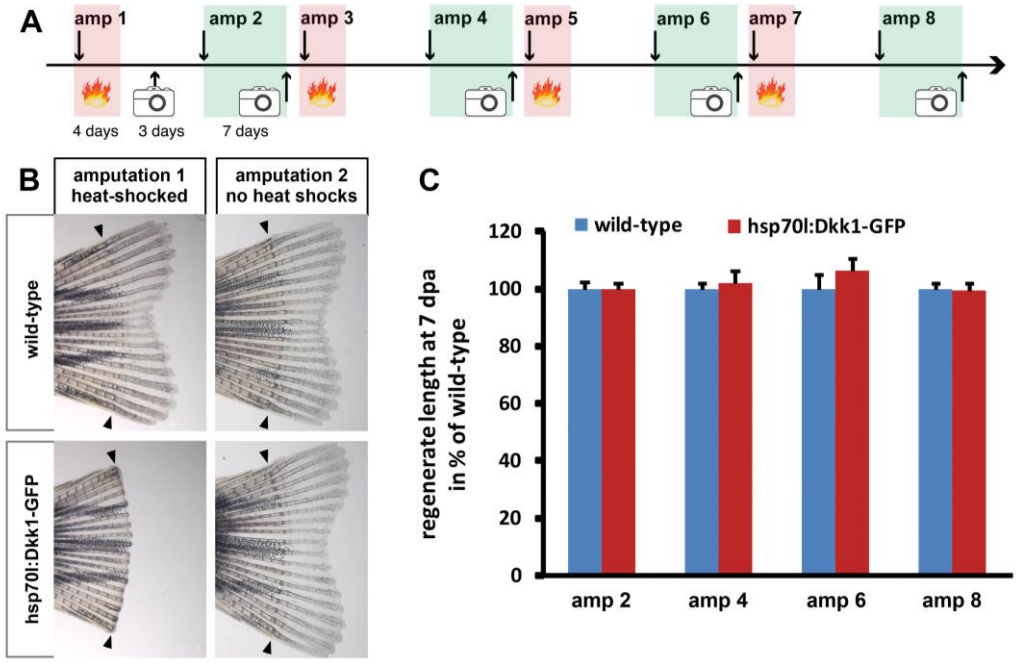

Figure 2.5. Repeated inhibition of fin regeneration by interference with Wnt/b-catenin signalling does not diminish regenerative capacity. (A) Schematic illustration of the experimental scheme. Red shaded areas indicate periods in which fish were heat-shocked twice daily, green areas indicate periods in which fish were allowed to regenerate in the absence of heat-shock. amp = amputation, phot = photo of the tail fin. (B) Wild-type and *hsp70l:dkk1-gfp* transgenic tail fins heat-shocked until 4 dpa and photographed 7 days after amputation 1 (left column) and photographed after amputation 2 without heat-shocks (right column). Note that heat-shocked wild-type fins regenerated, while *dkk1-gfp* expressing fins did not, yet both fins regenerated in the absence of heat-shocks in response to amputation 2. (C) The average regenerate length 7 days post amputation number 2, 4, 6, and 8 were normalized to the length of wild-type fish. Note that there are no significant differences in regenerate length between wild-type and *hsp70l:dkk1-gfp* fish.

To inhibit fin regeneration, we overexpressed the Wnt pathway inhibitor Dkk1 using heat-shock inducible transgenic *hsp70l:dkk1-gfp* fish. Overexpression of *dkk1-gfp* twice daily starting shortly before fin amputation and continuing until 4 days-post-amputation (dpa) was sufficient to completely inhibit fin regeneration (amputation 1 in Figure 2.5B) [13]. When fish were relieved from the heat-shock treatment, spontaneous regeneration did not occur. In contrast, when these fins that did not regenerate were re-amputated and fish were kept at non-inducing standard temperatures, fins completely regenerated (amputation 2 in Figure 2.5B). Thus, the ability to regenerate after Wnt signalling inhibition requires a novel amputation stimulus. Importantly, this also shows that inhibition of Wnt/ß-catenin signalling does not permanently block the regenerative capacity of the zebrafish caudal fin. To test whether repeated cycles of regenerative inhibition caused by blockage of Wnt signalling can diminish the regenerative capacity, we repeated the cycle of amputation, heat-shocking, recovery and second amputation 4 times (Figure 2.5A). We measured the length of the regenerate formed after every other amputation (in the absence of heat-shock) and plotted the length of the *hsp70l:dkk1-gfp* transgenic regenerates normalized to the one of their wild-type siblings. As shown in Figure 2.5C, no significant difference between the two groups could be detected. Thus, repeated blockage of blastema formation and fin regeneration by interference of Wnt/ß-catenin signalling did not diminish the regenerative capacity after a new amputation stimulus. We conclude that blastema formation and regenerative outgrowth do not depend on a biological process that is permanently disrupted or depleted by loss of Wnt/ß-catenin signalling.

Discussion

Repeated amputation experiments are fundamental to uncover the regenerative capacity limit of lower vertebrates. Some reports reveal a progressive increase of defects in the regenerated limb with an increasing number of amputations in both larval *Bufo regularis* and adult *Notophthalmus viridescens* newts [21,22]. In contrast, regeneration is successfully accomplished with only minor defects after 16 tail amputations in adult *Triturus carnifex* newts [23,24]. This led the authors to propose that regeneration of the spinal cord in *Triturus carnifex* relies on differentiated cells present in the stump that dedifferentiate contributing to the regenerate. Whether the difference in capacity to repeatedly regenerate these structures completely without defects is due to differences between newt species or whether tails have a higher capacity to regenerate than limbs is unsolved.

Only very recently, the regeneration limit of the zebrafish caudal fin was investigated [25]. In this report, it was shown that the regenerative capacity of the zebrafish caudal fin does not decline when amputated up to 9 times. This conclusion was based on the amount of regenerated tissue at 7 dpa and on analysis of expression of *msxb* and *fgf20a* at 48 hpa. In our study, we extended these results by showing that repeated amputations up to 29 times over a period of 11 months do not alter regenerative capacity. However, in contrast to this recent report, we observed a slight decrease of expression levels of the wound healing marker *mmp9* and the blastema marker *msxb* with repeated cycles of regeneration (Figure 2.3C,D). Nonetheless, these levels are still enough to accomplish a successful regeneration since the size of the 72 hpa regenerate and 4 wpa full caudal fin did not significantly change (Figure 2.2). Altogether, these data show that wound healing, blastema formation and regenerative outgrowth are not affected when the caudal fin is challenged with repeated amputations. Interestingly, it was recently demonstrated that telomere length is not maintained upon 3 repeated amputations in fish older than 3 months [26]. In this scenario, one could

speculate that consecutive amputations could lead to cell senescence. However, our results demonstrate the amazing regenerative potential of the zebrafish caudal fin even when challenged with a severe protocol of repeated amputations in older fish. Therefore, cell senescence can not be a limiting factor.

This almost unlimited capacity to regenerate that we have uncovered in our study could be due to either the presence of stem cells, dedifferentiation of mature cells or the contribution of both. In principle, each amputation could activate the pool of putative stem cells that might be present in different fin tissues, leading to the differentiation of all the missing structures. Importantly, the decision between self-renewal and the initiation of differentiation is controlled by signals provided by the tissue microenvironment, or niche, where stem cells are believed to reside. The Wnt signalling pathway plays a fundamental role in the control of maintenance and proliferation initiation of adult stem cells reservoirs in the intestine [27] and skin [28]. We made use of the heat-shock inducible transgenic *hsp70l:dkk1-gfp* fish, to efficiently and in a time-controlled manner inhibit Wnt signalling. Inhibition of Wnt signalling twice daily shortly before fin amputation and until 4 dpa completely impaired fin regeneration. However, if the fins that did not regenerate were re-amputated and allowed to have an intact Wnt signalling by keeping them at a non-inducing temperature, fins regenerated completely (Figure 2.5). This reveals that there is a time window for the initiation of regeneration that is triggered soon after each amputation and that is absolutely dependent on Wnt/ß-catenin signalling. Importantly, these experiments also indicate that blastema formation does not depend on a pool of progenitor cells that requires Wnt for its maintenance. While these data do not completely rule out a contribution of progenitor cells, it is more compatible with the alternative model of regeneration based on dedifferentiation. In fact, this model is now supported by recent findings showing that mature osteoblasts dedifferentiate to form part of the blastema and regenerate bone in the zebrafish caudal fin [10]. According to these

findings, Wnt signalling could be required for dedifferentiation and/or expansion of the dedifferentiated cells to form a blastema.

In spite of this amazing capacity to regenerate, the bone proximally to the amputation plane becomes thickened with repeated cycles of amputations (Figure 2.4). Interestingly, we could not detect a clear difference in Zns5 staining, indicating that the number of osteoblasts did not change with increased amputations (Figure 2.4I-K). Progressive bone thickening might be a consequence of inappropriate activation of osteoblasts to secrete matrix far away from the amputation plane. In fact there is strong evidence that osteoblasts enter the cell cycle following amputation [10,29] and that differentiated cells can be induced to proliferate even far from the amputation plane [10,30]. Thus, while some dedifferentiated osteoblasts migrate distally to form the blastema, it is unlikely that newly formed osteoblasts that far from the amputation plane would participate in blastema formation. Rather, they likely represent a source of cells replacing those moving into the blastema. It is possible that activation of proliferation also causes these cells to re-activate matrix secretion, which after repeated cycles results in bone thickening. Alternatively, the increase in bone matrix could be caused by an unbalanced ratio of bone-forming and bone-degrading cells. Due to the thickening of the bone, it seems that the inter- and intra-ray tissues became compacted and therefore reduced in size. Interestingly, the newly regenerated tissue of the fin exhibits a decreased bone thickness and inter-ray space probably because these are recently formed tissues that are still being remodelled.

A better understanding of the cellular mechanisms underlying the virtually unlimited regenerative capacity of fish appendage regeneration will be informative for efforts to improve repair, in particular of bone, in humans.

Materials and methods

Ethics Statement

All experiments involving animals were approved by the Animal User and Ethical Committees at Instituto de Medicina Molecular, according with directives from Direcção Geral Veterinária (PORT 1005/92). All animal experiments at the Biotechnology Center of the TU Dresden were performed in accordance with the guidelines of the state of Saxony and have been approved by the Regierungspräsidium Dresden, permit number 24D-9168.11-1/2008-1.

Zebrafish lines, maintenance and surgery

48 AB WT zebrafish were purchased from ZIRC. The repeated amputations protocol was initiated when fish were 1 year of age. 24 experimental animals were maintained at 30°C in separate tanks (one individual per tank) during the time of the experiment (approximately 11 months). 24 control uncut animals were kept together in a large tank, at the same temperature. To perform the amputations, fish were anesthetized in 0.6 mM Tricaine and amputated using a razor blade.

Repeated inhibition of regeneration

hsp70l:*dkk1-gfp*^{w32} transgenic fish, carrying one copy of the transgene and their wild-type siblings were used. To induce heat-shocks, fish were kept in an automated waterbath at 28°C, and twice daily heated to 37°C within 10 minutes, followed by sustained incubation at 37°C for 1 hour, and active cooling to 28°C within 15 minutes. To ensure complete block of fin regeneration in *dkk1-gfp* expressing fish, the first heat-shock was applied 6 hours prior to fin amputation. To document regenerative capacity after inhibition, fish were heat-shocked twice daily for 4 days without feeding, then allowed to recover for 1 week at 28°C with feeding, followed by re-amputation of the fin in wild-types or the non-regenerated fin stump in *hsp70l:dkk1-*

gfp^{w32} transgenic fish. For re-amputation, the fin was cut 1 bone segment proximal to the initial amputation plane. Fish were allowed to regenerate with feeding at 28°C for 1 week, after which the fin was photographed.

Quantification of regenerate area and length and caudal fin area

The 4 wpa full caudal fin and the 72 hpa regenerate area were measured each month using Image J software (NIH). Since zebrafish are very heterogeneous regarding its size, the 72 hpa regenerate area was corrected to the size of the fin by dividing its value in each month by the 4 wpa full caudal fin area in the corresponding month. The 7 dpa regenerate length of *hsp70l:dkk1-gfp* fish was normalized to the average regenerate length of wild-type sibling fish. For this quantification, the length of the 2nd, 3rd, 4th and 5th dorsal fin rays was measured from the amputation plane to the distal tip of the ray using Image J software and the average length calculated for each fish.

Quantitative RT-PCR

RP and NRP tissues were collected and preserved at -20°C in RNA Later solution (Ambion) during the time of the experiment. Total RNA was extracted from fin regenerates using TRIZOL (Invitrogen) according to the manufacturer's protocol. 8 regenerates were used to extract RNA for the 8 hpa time-point and 4 RP or NRP were used to extract RNA for the 72 hpa time-point. 1 µg of RNA from each sample was reverse transcribed with the Revertaid^{TM} H minus first strand cDNA synthesis kit (Fermentas) using random hexamer primers. Primers for quantitative RT-PCR of *mmp9* were 5-CTGGGCACCTGCTCGTTG-3 and 5-ATTGGAGATGACCGCCTGC-3 and for *msxb* were 5-AGGAACAGAGCACTTGGTCAAACT-3 and 5-TGAGGTTGAGGGAGTTGGAGAAC-3. Quantitative PCR was performed using Corbet Rotorgene 6000 and the SYBR Green labelling system. *mmp9* and *msxb* levels were normalized to the housekeeping gene *ef1a* (primers 5-ACGCCCTCCTGGCTTTCACCC-3 and 5-TGGGACGAAGGCAACACTGGC-3).

Quantification of the relative expression was performed using the $2^{-\Delta CT}$ method and normalized against the relative expression obtained for the uncut caudal fin. Data were analyzed using Student's t test.

Tissue sectioning and histology

Fins were embedded in gelatin and sectioned at 12 µm using a cryostat. For the Masson's trichrome staining, gelatin was washed in PBS at 37°C for approximately 30 minutes and sections were stained with Weigert's hematoxilin for 10 minutes, washed in warm running tap water for 5 minutes and rinsed in distilled water. After this washing, sections were stained with Biebrich scarlet-acid fuchsin for 5-10 minutes. The excess of this solution was removed by rinsing with distilled water and the unspecific staining was cleared with phosphomolybdic acid 1% for 10 minutes. Collagen was stained with light green at 2% for 1 minute. Finally, sections were dehydrated in ethanol 95% 30 seconds, ethanol 100% 30 seconds, cleared in xylol for 5-10 minutes and slides were mounted in Entellan.

Immunohistochemistry

The fins were fixed in a solution with 80% MeOH/20% DMSO (Sigma) and were rehydrated in a MeOH/PBS series, permeabilized with acetone at -20°C for 20 minutes, followed by two washes in PBS. An additional permeabilization was done with PBST 0.5% solution (PBS with 0.5% Triton X-100) during 30 minutes. Followed by several washes with PBS, fins were blocked in PBS with 10% Fetal Bovine Serum (FBS) and incubated with 1:250 primary antibody Zns5 (ZIRC 011604) overnight at 4°C. Fins were washed several times in PBS and the incubation with the secondary antibody and DAPI (D9564 Sigma) was done overnight at 4°C. Immunostained caudal fins were post-fixed for 20 minutes in 4% PFA (paraformaldehyde), washed in PBS and passed through a 30% sucrose/PBS solution for cryoprotection. Transverse sections of 12 µm of immunostained fins of 2 uncut controls and 2 caudal fins subjected to 14 amputations were obtained by cryosectioning and analysed by confocal

microscopy. In each of the controls and experimental fins the following measurements were performed using Image J software: proximal and distal bone thickness of dorsal and ventral hemi-rays of 5 - 9 bony rays was measured; the amount of 3 inter-ray tissues at a proximal and distal level was quantified by measuring the distance between two bony rays; the proximal and distal intra-ray tissue was quantified by measuring the length between two hemi-rays in 5 – 9 bony rays. Data were analyzed using Student's t-test.

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CHAPTER III

An amputation resets positional memory to a proximal identity in the regenerating zebrafish caudal fin blastema

An amputation resets positional information to a proximal identity in the regenerating zebrafish caudal fin blastema

Abstract

The questions of how the original size, pattern and replacement of only those structures removed by amputation is achieved, are among the most interesting aspects of regeneration. However, how the relative position of the different tissues and structures that compose the lost appendage is maintained upon amputation remains unknown. Zebrafish has emerged as a powerful model organism to study the process of regeneration. This teleost fish has the ability to regenerate various tissues and organs like the heart, the spinal cord, the retina and fins. In this study, we took advantage of the existence of an excellent morphological reference in the zebrafish caudal fin, the bony ray bifurcations, as a model to study positional information upon amputation. We investigated how the positional information is established during fin regeneration and whether it is altered by repeated amputations at different proximo-distal (PD) places along the fin. We show for the first time that, while amputations performed at a long distance from the bifurcation do not change its proximal/distal position in the regenerated fin (after a first amputation), consecutive distal amputations induce a positional reset and progressively shift its position distally. In contrast to what was previously believed, these findings reveal that, depending on the place of amputation, positional memory is not maintained in the regenerating fin.

Introduction

Tissue regeneration in humans can occur in a limited extent in structures like the skin, gut, skeletal muscle, bone, digit tips, liver and blood. However, other vertebrate species have the extraordinary capacity to regenerate lost tissues and organs throughout adult life. One of such organisms is the zebrafish, a well-established model to study general mechanisms of regeneration, since it is able to regenerate fins, scales, retina, spinal cord and heart among other internal organs (Iovine, 2007).

Due to its accessibility, caudal fin regeneration is an example of a powerful and efficient model for regenerative studies. The zebrafish caudal fin is composed of several segmented bony rays, mesenchymal tissue, blood vessels and nerve axons. Each bony ray is made of two concave hemirays and, with the exception of the most lateral rays, is bifurcated in a distal position within the fin (Poss et al., 2003). These bifurcations are responsible for generating the characteristic shape of the caudal fin and ultimately for increasing swimming efficiency.

In the zebrafish caudal fin, an amputation triggers a regenerative program that occurs in three phases: wound healing, blastema formation and regenerative outgrowth. Within the first 12 hours-post-amputation (hpa), the injury is healed through migration of epidermal cells that cover and close the wound (Poss et al., 2003). In the next 12-48 hpa, the wound epithelium thickens forming an apical epidermal cap (AEC) and the tissue proximal to the amputation plane disorganizes, begins to proliferate and migrates distally to form the blastema, which is a mass of proliferating cells (Poss et al., 2003). The onset of regenerative outgrowth is at 48 hpa, and at this stage the blastema becomes subdivided into a distal region comprising slow proliferative cells and an intensely proliferative proximal region (Nechiporuk and Keating, 2002). Within 2 weeks after amputation, the blastema reconstitutes the original architecture of the caudal fin with all its different tissues and structures (Nechiporuk and Keating, 2002).

Although we are beginning to understand the molecular mechanisms of regeneration, it is becoming clear that distinct pathways are activated upon amputation. Fibroblast growth factor (Fgf) signalling seems to be required for blastema formation (Whitehead et al., 2005), canonical Wnt/β-catenin signalling enhances proliferation of progenitors cells while non-canonical Wnt/Planar cell polarity (PCP) pathway seem to promote the opposite (Stoick-Cooper et al., 2007b) and Hedgehog (Hh) signalling seems to play a latter role by controlling bone differentiation (Quint et al., 2002). It is true that a tight control of cell proliferation and differentiation is critical to regenerate a fully functional caudal fin. Nonetheless, equally important is to be able to reconstitute the relative arrangement of the different regenerating tissues and structures, which means that during fin regeneration there must be ways of keeping positional memory. This is a fascinating question in the regeneration field for which we know very little.

In the present study, we took advantage of the zebrafish caudal fin as a model to study positional information upon amputation, since the stereotypic PD position of the bony ray bifurcations provides an excellent morphological reference. We tested how positional information of bony ray bifurcations is affected by repeated amputations performed at different levels along the PD axis of the fin. We show that there is a progressive distalization of the position of the bifurcations in the regenerated fin, when the repeated amputations were done proximally near the bifurcation (distal amputations). On the other hand, after a first amputation, its position is maintained in subsequent amputations done near the base of the fin, therefore away from the bifurcation (proximal amputations). Thus, we show for the first time that the positional memory of the bifurcation is maintained in proximal but not in distal amputations. Furthermore, we analysed the role of Fgf and Hh signalling and concluded that they do not seem to be the instructive signals that determine the bifurcation position.

Results

III.1. Repeated amputations progressively shift the bifurcation position distally

In Chapter II, I describe an amputation protocol that allowed us to conclude that the regenerative capacity of the zebrafish caudal fin is not affected by repeated amputations or ageing. In this protocol, the caudal fin was subjected to three amputations every month. This protocol was repeated 10 times. During the first 6 months (corresponding to the first 15 cuts) the third consecutive amputation (the last before allowing the fin to completely regenerate) was done three bone segments below the most proximal bony ray bifurcation (near the bifurcation). In the following 4 months (corresponding to the next 12 cuts), the third consecutive amputation was done 4 segments distally from the base of the fin (distant from the bifurcation) (Azevedo et al., 2011). Although the regenerative capacity was not affected, we detected an alteration in the original pattern of pigment cells and a distal shift in the position of the bony ray bifurcations in the regenerated caudal fins (Figure 3.1a,b).

We quantified the number of segments formed between the base of the fin and the 3rd dorsal ray bifurcation in the regenerated fin in order to determine the PD position of the bifurcation after each set of consecutive amputations. We observed that, during the first 6 months, there was an increase in the number of segments formed between the base of the fin and the 3rd dorsal ray bifurcation. This reveals that the position of the bifurcations was progressively shifted distally when compared to its position before amputation (Figure 3.1c - near bifurcation). In the following 4 months, the number of segments formed between the base of the fin and the 3rd dorsal ray bifurcation was maintained, showing that the PD position of the bifurcations was unaltered (Figure 3.1c – 4 segments). The overall number of segments within the regenerated caudal fin was unchanged (data not shown)

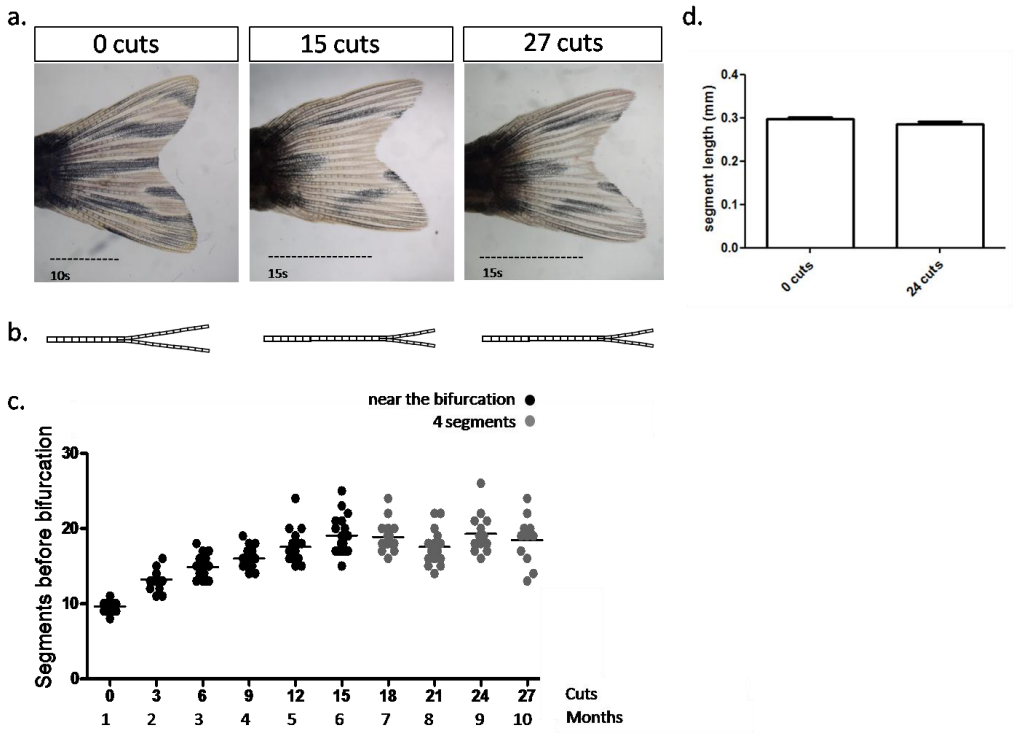

Figure 3.1. The bifurcation position is distalized with repeated amputations. (a) The same caudal fin before amputation and after 15 and 27 amputations. The dashed line in each panel marks the number of segments from the base of the fin until the bifurcation in the 3rd dorsal ray. **(b)** Schematic representation of the bifurcation distalization with the repeated amputations. **(c)** Number of segments formed in the 3rd dorsal ray between the base of the fin and the bifurcation after consecutive amputations. In the first 6 months, the third consecutive amputation was performed three segments below the bifurcation (near the bifurcation) and in the following 4 months, the third amputation was done at 4 segments from the base of the fin (distant from the bifurcation). **(d)** 3rd dorsal ray segment length before any amputation and after 24 amputations.

during the repeated rounds of amputations as well as the segment length (Figure 3.1d).These results suggest that the bifurcation position is distalized when the amputations are performed proximally near the bifurcation, but remains unaltered when the amputations are done near the base of the fin (distant from the bifurcation).

III.2. The bifurcation position is only shifted distally when the amputations are performed repeatedly near the bifurcation

One possibility to explain the maintenance of the proximal-distal position of the bifurcation observed in the last 4 months of our experimental setting could be that the distalization of the bifurcation reached its maximum limit after 6 months of consecutive amputations. Another possibility could be that the increased amputation distance to the bifurcation place, performed in the last 4 months, would decrease the possible influence of an amputation in the PD bifurcation position.

To distinguish between these two possibilities, we designed a more controlled amputation protocol (Figure 3.2a). We performed a first amputation at 4 segments from the base of the fin (distant from the bifurcation) in 20 adult zebrafish and allowed the fin to completely regenerate. The second, third and fourth amputations were performed at 4 segments from the base of the fin in 10 of the animals and, in the remaining 10, the second, third and fourth amputations were performed at 1 segment below the most proximal bifurcation (near the bifurcation).

Upon a first amputation at 4 segments from the base of the fin, the bifurcation was immediately distalized when compared to its position in the uncut fin (Figure 3.2b). Following the second, third and fourth amputations, the bifurcation position was maintained in the regenerated fin when the amputations were done at 4 segments from the base of the fin (Figure 3.2b') but it was progressively distalized when the amputations were done 1 segment proximal to the bifurcation (near bifurcation) (Figure 3.2b''). These data show that while amputations performed at a long distance from the

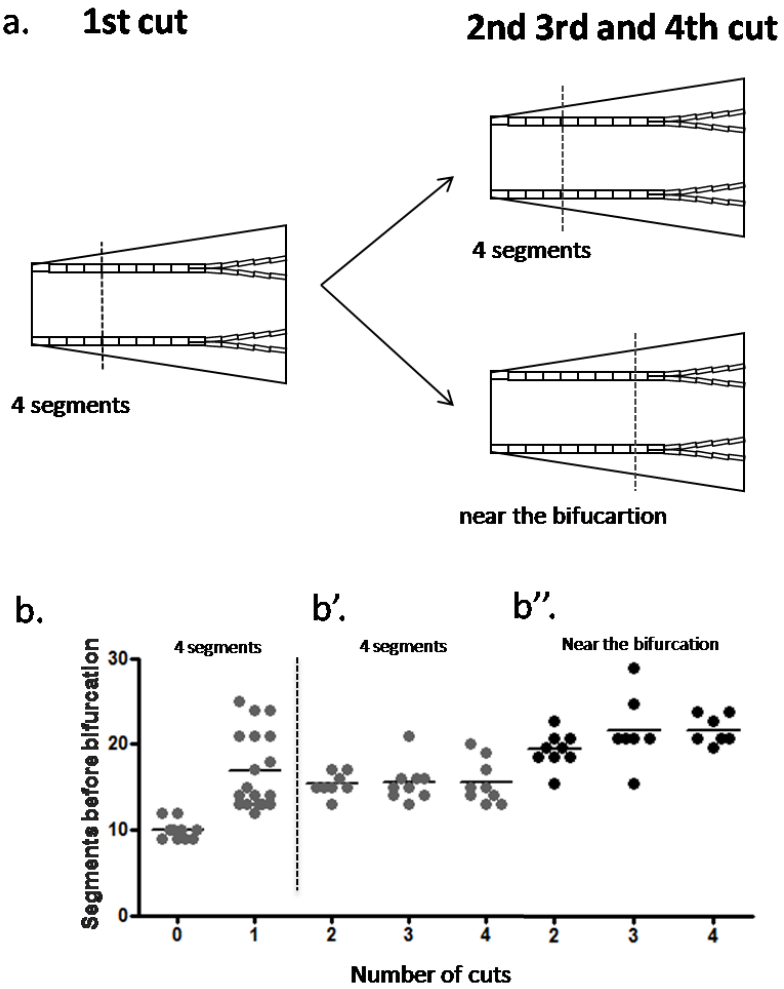

Figure 3.2. The distalization of the bifurcation is dependent on the PD level of amputation. (a) After a first amputation performed at 4 segments from the base of the fin, the fish were divided into two groups. One group was amputated a second, third and fourth time at 4 segments from the base of the fin and the second group was amputated one segment below the bifurcation (near the bifurcation). **(b)** Number of segments formed in the third dorsal ray between the base of the fin and the bifurcation after consecutive amputations performed always at 4 segments from the base of the fin **(b')** and after a first amputation performed at 4 segments from the base of the fin followed by a second, third and fourth amputations near the bifurcation **(b'')**.

bifurcation do not change its PD position in the regenerated fin, consecutive amputations near the bifurcation induce a positional reset and progressively shift its position distally.

III.3. *shh* expression pattern is independent of the place of amputation

We next asked what factors determine the bifurcation position and how they are influenced by the amputation place.

Sonic hedgehog (Shh) is a strong candidate due to its previously described dynamic expression, correlated with the formation of a bifurcation during fin regeneration. It was shown that at 2 days-post-amputation (dpa), a strong single domain of *shh* expression is detected at the level of amputation on the top of each hemiray. By 4 dpa, this *shh* single domain starts to split into two groups of cells located laterally in the proximal region of the basal wound epidermal layer. This change in *shh* expression from one to two domains was proposed to be the trigger for bifurcation formation (Laforest et al., 1998).

Thus, we wanted to determine how this dynamic expression pattern of *shh* is modulated by the amputation place and whether Shh would be the instructive factor to form the bifurcation or a downstream factor in this process.

To this end we performed two rounds of amputations at different places, at 1 segment proximal to the bifurcation (near the bifurcation) or at 4 segments from the base of the fin (distant from the bifurcation) and analysed by *in situ* hybridization the expression of *shh* at 3 and 4 dpa. We observed that, independently of the number or places of amputations, *shh* was consistently expressed in two separate cellular domains already at 3 dpa (Figure 3.3a-h). These results show that *shh* expression is not modulated by the amputation place. Moreover, at 4 dpa, in a caudal fin that does not have any bifurcations due to being subjected to several distal amputations, *shh* expression was localized in two groups of cells located laterally in the proximal region of the basal wound epidermal layer (Figure 3.3i, j). This

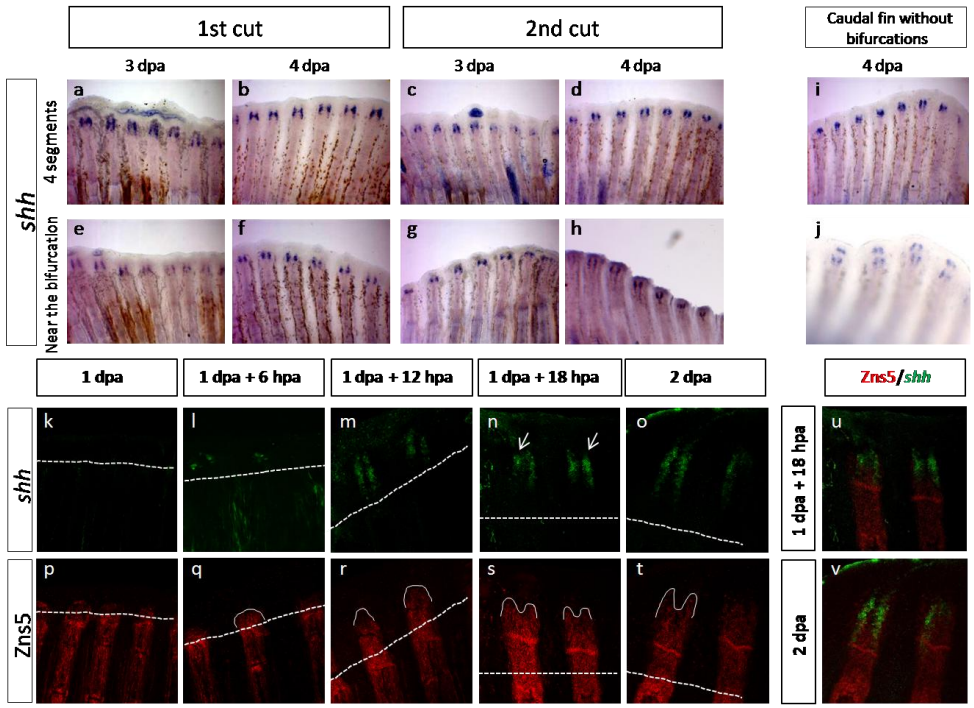

Figure 3.3. The expression pattern of *shh* during regeneration does not change with the PD level or the number of amputations. **(a-d)** Caudal fins were amputated at 4 segments from the base of the fin once or twice and *shh* expression was determined at 3 and 4 days following the amputation. **(e-h)** Caudal fins were amputated near the bifurcation once or twice and *shh* expression was determined at 3 and 4 days following the amputation. **(i)** Caudal fin with no bifurcations was amputated at 4 segments from the base of the fin and *shh* expression was examined at 4 days following the amputation. **(J)** Top view of the caudal fin shown in I. **(k-v)** Caudal fins of 2.2*shh*:*gfp:*ABC#15 transgenic fish were amputated at 1 segment proximal to the bifurcation (near the bifurcation) and analyzed at different time-points after amputation by a double immunostaining with anti-GFP **(k-o)** and anti-Zns5 **(p-t)** antibodies. **(u,v)** merge of 1 dpa+18 hpa **(u)** and 2 dpa **(v)**.Arrows indicate irregular domains of expression of *shh*. Dashed line represents the amputation plane.

strongly suggests that Shh is not sufficient to trigger the formation of bifurcations.

To analyse with increased cellular resolution the dynamics of *shh* expression in the zebrafish regenerating fin, we made use of a transgenic line expressing GFP under the control of *shh* promoter (2.2*shh*:*gfp:*ABC#15) (Shkumatava et al., 2004). Using this transgenic zebrafish line, we performed one amputation, at 1 segment proximal to the bifurcation (near bifurcation) and analysed the expression of *shh,* every 6 hours from 1 to 2 dpa. The time course analysis revealed that the establishment of *shh* expression pattern during regeneration is around 1 dpa + 12 hpa. However, in a few cases, *shh* expression could be detected at earlier time points, in a small number of cells, in only one or both sides of the regenerating hemiray (Figure 3.3i). From its onset of expression (at 1 dpa + 12 hpa) until 2 dpa, *shh* was always present with the same pattern of expression, namely in two separate groups of cells located laterally in the proximal region of the basal epidermal layer (Figure 3.3m-o). Since we have never observed a transition in *shh* expression from one to two domains during fin regeneration, these results provide additional support to the conclusion that Shh may not be the instructor to form the bifurcation.

In addition, it has also been proposed that Shh plays a role in the patterning and/or differentiation of osteoblasts within the blastema during fin regeneration (Quint et al., 2002). In order to determine whether there is a correlation between the restriction of *shh* expression in two epidermal domains and the dynamics of bone formation during regeneration, we performed a Zns5 (osteoblast marker) immunostaining time-course analysis (every 6 hours from 1 to 2 dpa) in the 2.2*shh*:*gfp:*ABC#15 transgenic fish. Interestingly, we observed that soon after the onset of *shh* expression, the growing bone alters the shape of its tip from a cone to a "V" shape (Figure 3.3s). This suggests that, Zns5+ cells cease to accumulate in the middle of the blastema and are aligned close to the basal layer of the epidermis where *shh* mRNA is produced (Figure 3.3n-t,u,v). Interestingly, we have also

observed that *shh* expression domains can be irregular in the form and differs in the number of *shh* positive cells in each individual blastema of the same fin (Figure 3.3n - arrows). Consequently, the visibility of *shh* separation in two cellular domains depends on the regenerating ray and blastema shape. Similarly, irregularities in the shape are also visible in the spatial organization of Zns5+ cells in the regenerating tip of each ray (Figure 3.3p-t).

Altogether, these results suggest that *shh* expression in two separate domains in the basal layer of the epidermis is not determining the PD position of the bone bifurcation, but could have an important role in bone formation and growth through osteoblasts alignment by attracting them to the region where *shh* is being produced.

III.4. Fgf signalling does not play a role in the determination of the bony ray bifurcation position

It was already demonstrated that the levels of Fgf signalling activation vary according to the PD place of amputation. Upon amputation, the expression levels of Fgf downstream targets such as *mkp3*, *sef* and *spry4* are higher following a proximal amputation when compared to a distal amputation (Lee et al., 2005). This suggests the existence of positional memory that can be mediated or act through Fgf signalling.

In order to investigate whether Fgf signalling determines the PD position of the bifurcation in the regenerated fin, we made use of the *hsp70:dn-fgfr1* transgenic zebrafish (Lee et al., 2005). This transgenic contains a dominant-negative *fgfr1-egfp* fusion gene (*dn-fgfr1*) driven by a heat-inducible zebrafish *hsp70* promoter. It was previously demonstrated that this construct attenuates Fgf signalling during fin regeneration in a dose dependent manner. Upon heat-shock, the regeneration growth rate is affected. This phenotype is highly sensitive to 1ºC temperature increments (Lee et al., 2005).

The *hsp70:dn-fgfr1* transgenic zebrafish were amputated once, at 1 segment proximal to the bifurcation (near the bifurcation) and Fgf signalling was partially inhibited by heat-shocking at 35°C for 1 hour daily, starting at

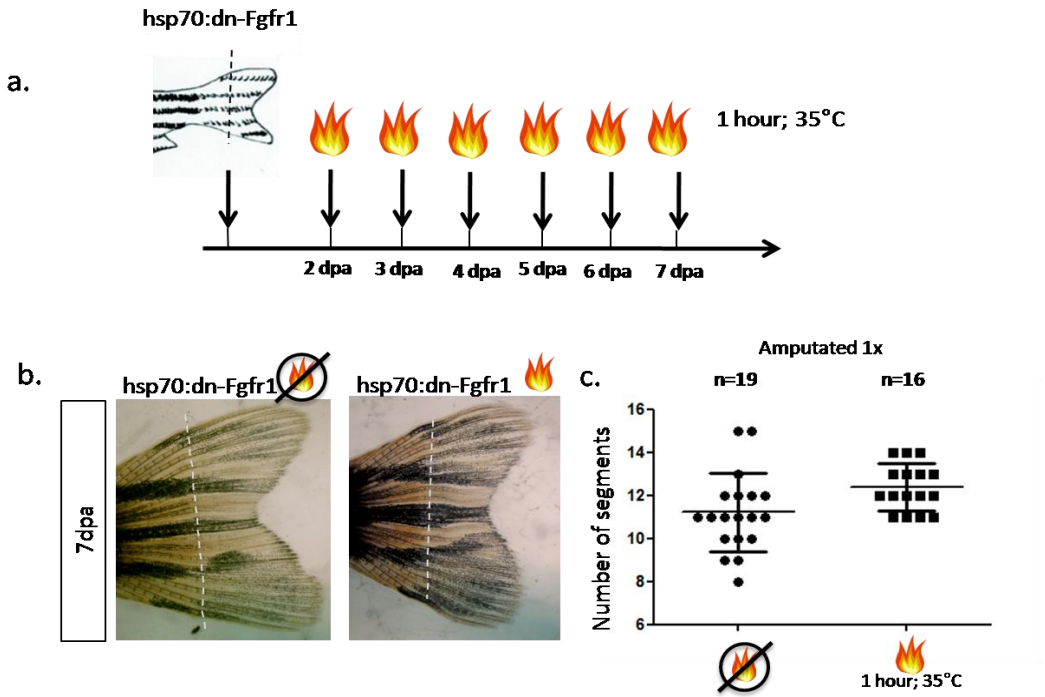

Figure 3.4. Fgf signalling does not seem to play a role in the determination of the PD position where the bifurcation is formed (a) Heat-shock protocol following an amputation. Transgenic hsp70:dn-fgfr1 fish were amputated at 1 segment proximal to the bifurcation and heat-shocked at 35°C for 1 hour, during 6 days, starting at day 2 post amputation. **(b)** Picture of a 7 days-post-amputation regenerated caudal fin of *hsp70:dn-fgfr1* transgenic fish with or without the heat-shock protocol. **(c)** Number of segments formed in the 3rd dorsal ray between the base of the fin and the bifurcation in heat-shocked and non-heat-shocked siblings.

day 2 until day 7 post amputation (Figure 3.4a). The time-window of this protocol was designed to target the regenerative outgrowth phase (when the bifurcations are signalled to form) at a temperature that does not block regeneration. The induction of *dn-fgfr1* upon heat-shock was confirmed by the detection of GFP in the regenerating fins (data not shown). The regenerated caudal fins after this protocol presented the bifurcation place in the same PD position as the amputated non heat-shocked siblings, as analysed by counting the number of segments formed between the base of the fin and the bifurcation in the 3rd dorsal ray (Figure 3.4b,c). Other protocols of attenuation of Fgf signalling were tested by heat-shocking at different temperatures, durations or time-points of regeneration. However, none of the protocols tested affected the bifurcation position (i.e. the number of segments formed between the base of the fin and the bone bifurcation in the regenerated caudal fin) (see Figure S3.5 in the supplementary data). These results suggest that Fgf signalling is not involved in the determination of the bony ray bifurcation position during caudal fin regeneration.

Discussion

Our results clearly demonstrate that the amputation place influences the bony ray bifurcation position and that repeated amputations performed proximally near the bifurcation will progressively induce a distal shift, changing the original position of the bifurcation and resetting its positional memory (Figure 3.1 and Figure 3.2). Thus, it is possible that the formation of a blastema after an amputation proximally near the bifurcation will inhibit the signal responsible to initiate a bifurcation and consequently delay its formation. This means that a certain number of segments will need to be formed/ differentiated before a bifurcation is signalled to form.

We wanted to investigate the mechanism controlling the position/formation of a bifurcation during caudal fin regeneration.

A previous report indicates that, in caudal fin regeneration, preceding the formation of a bony ray bifurcation, *shh* duplicates its single domain of expression in the basal layer of the epidermis (Laforest et al., 1998). This indicates that Shh would be a good candidate to signal the formation of a bifurcation (Laforest et al., 1998; Quint et al., 2002). However, we have observed that the dynamics of *shh* expression does not change, being always expressed in two separate groups of cells in the basal layer of the epidermis, after two consecutive amputations at different levels relatively to the bifurcation place: 4 segments distal from the base of the fin (proximal amputation) or near the bifurcation (distal amputation) (Figure 3.3a-h). Furthermore, *shh* expression in two separate domains was clear at 4 dpa even in a caudal fin that did not have any bifurcations due to being subjected to several distal amputations (Figure 3.3i,j).

In addition, we have precisely followed s*hh* expression using the 2.2*shh*:*gfp:*ABC#15 transgenic zebrafish line. We analyzed *shh* expression, every 6 hours from 1 to 2 dpa at 1 segment proximal to the bifurcation (near the bifurcation) and demonstrated that its expression is initiated at 1 dpa + 12 hpa when it is already detected in two separate domains, maintaining this expression in all subsequent time-points (Figure 3.3i-o). Altogether, these results suggest that Shh signalling does not seem to have an instructive role in settling the position of the bony ray bifurcation, even though it might be required for the formation of this structure.

To uncover the functional relevance for the expression of *shh* in two separate domains in the basal layer of the epidermis, we did a Zns5 expression time-course (osteoblast marker), since it has been proposed that Shh might play a role in the osteoblasts patterning and/or differentiation during fin regeneration (Quint et al., 2002). Interestingly, soon after the detection of *shh* expression, the bone alters its growing tip, as a cone shape, and the forming osteoblasts start to be aligned close to the basal layer of the epidermis in "V" shape, next to *shh* expressing cells (Figure 3.3s). This observation suggests that Shh might act has an attractor of bone progenitors

aligning them, directing the bone growth and possibly controlling the width of the bony ray in the regenerating fin.

Another possible candidate to control the bifurcation position is Fgf signalling pathway, which has been proposed as a possible pathway involved in the regulation of positional memory during regeneration (Lee et al., 2005). In order to address a potential role of Fgf signalling in the determination of the bifurcation position, we made use of the heat-shock inducible transgenic *hsp70:dn-fgfr1* to attenuate Fgf signalling in a time controlled manner. All the different protocols used to transiently attenuate Fgf signalling did not alter the position of the bony ray bifurcation when compared to the controls, with unaffected Fgf levels (Figure 3.4 and Figure S3.5). This indicates that Fgf signalling may not be the factor controlling the formation of a bony ray bifurcation in the zebrafish regenerating caudal fin.

In the regenerating zebrafish fin it has been reported that retinoic acid (RA) treatment distalizes the bifurcation point due to the fusion of fin rays (Geraudie et al., 1995; White et al., 1994). It is not clear though, whether this is caused by a proximalization of the regenerating tissue, by the downregulation of *shh* (Laforest et al., 1998) or even toxicity, perturbing proper bone formation/patterning (Quint et al., 2002) following RA treatment. In addition, previous work has demonstrated that the crosstalk between blastema, distal regenerating epidermis and inter-ray tissue is essential for signalling the formation of a bifurcation in the zebrafish fin (Murciano et al., 2002). Local interactions between the different cellular domains present in the regenerating fin seem to be key regulatory mechanisms in the patterning of a regenerating appendage. Nevertheless, the signalling(s) that gives positional information to the regenerating fin tissue remains to be discovered.

Supplementary data

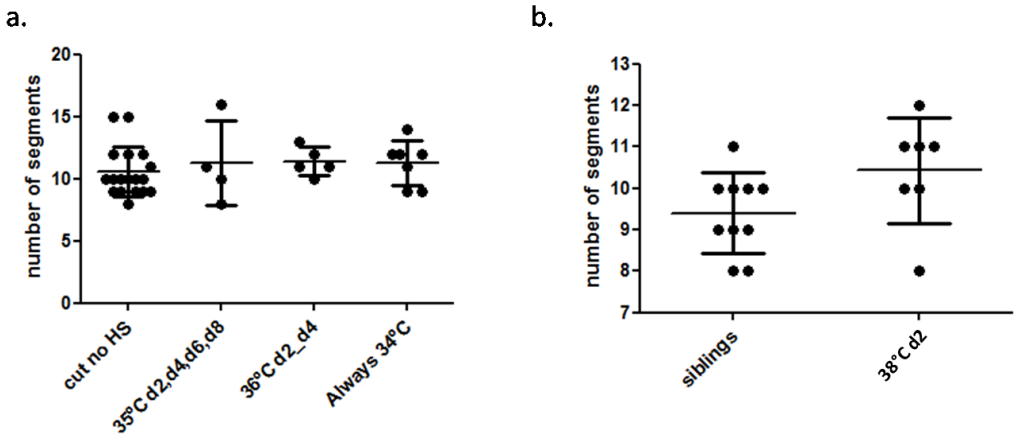

Figure S3.5. Fgf signalling does not seem to play a role in the determination of the PD position of the bifurcation. Transgenic *hsp70:dn-fgfr1* fish were amputated 1 at segment proximal to the bifurcation and heat-shocked at: 35ºC for 1 hour, every other day, from day 2 post amputation until day 8 post amputation; 36ºC for 1 hour daily, during 3 days, starting at day 2 post amputation; 34ºC permanently, from the time of amputation until the accomplishment of a complete regeneration; once at 38ºC for 1 hour at 2 dpa. The number of segments formed in the 3rd dorsal ray between the base of the fin and the bifurcation in the heat shocked zebrafish were counted and compared to the non-heat-shocked siblings **(a)** or to the heat-shocked siblings, negative for *hsp70:dn-fgfr1* insertion **(b)**.

Materials and methods

Ethics Statement

All experiments involving animals were approved by the Animal User and Ethical Committees at Instituto de Medicina Molecular, according with directives from Direcção Geral Veterinária (PORT 1005/92).

Zebrafish lines, maintenance and surgery

The following zebrafish strains were used in this study: wild-type AB strain (from ZIRC), *Tg*(*hsp70:dn-fgfr1*]^{pd1} strain (Poss 2005) and 2.2*shh*:*gfp:*ABC#15 (Shkumatava et al 2004) . Fish of 6-24 months of age were anaesthetized in 0.1% tricaine (Sigma- Aldrich), and caudal-fin amputations were performed with razor blades. Animals were allowed to regenerate for various times in water kept at 30-33°C, except the *Tg*(*hsp70:dn-fgfr1*]^{pd1} strain that was keep at 28.5°C.

Adult heat induction experiments

A heated incubator was used to maintain the water of breeding boxes warmed to the heat-shock temperature of 35°C or 34°C, 36°C and 38°C. To give the heat-shock, zebrafish were transferred from a temperature of 28,5°C to the breeding boxes with heated water in the incubator.

In situ Hybridization

The antisense *shh* RNA probe was synthesized with a digoxigenin labelling kit (Promega) and as previously described by Henrique et al. (1995). The plasmid containing *shh* cDNA was kindly provided by David Wilkinson's lab.

In situ hybridization of zebrafish fins was perfomed as follows. Fin regenerates were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and transferred to ethanol at room temperature (RT) and stored at -20°C, at least one overnight. Fins were rehydrated stepwise through ethanol in PBS-0,1% Triton (PBT) and washed in

two changes of PBT for 10 minutes. A solution of 6% of H_2O_2 in PBT was used during 30 minutes to inactivate endogenous peroxidases, followed by two washes for 5 minutes in PBT. Proteinase K (10 mg/ml) digestion was performed for 15 minutes and then stopped by washing with a glycine solution (2mg/ml in PBT). After two washes for 5 minutes in PBT, fins were refixed with 3.7% Formaldehyde solution, 0.2% Glutaraldehyde in PBT for 20 minutes followed by another two PBT brief washes. Pre-hybridization was allowed for ≥1 hour at 70°C, in hybridization solution (Hyb solution) containing: 60% formamide, 5x SSC (20x ph 6.0), 500mg/ml tRNA, 0,1% Tween20 (10%), 50mg/ml heparin, in miliQ H_2O. Fins were then hybridized in Hyb solution, containing 5ml/ml digoxigenin-labeled RNA probe, overnight at 70°C. Unhybridized probe was removed using washing solutions I and II (washing solution I: Formamide 50%, 1x SCC, 0.1% Tween 20; washing solution II: 50% Wash I, 50 % TBST) at 70°C (wash I: 2 x 15 minutes + 2 x 30 minutes; wash II: 2x 20 minutes). After this fins were washed with TRIS-buffered saline in 0.1% Tween 20 (TBST), incubated in a blocking solution (10% sheep serum in TBST) at RT for ≥1 hour and incubated with anti-digoxigenin antibody coupled to alkaline phosphatase Fab fragment (Roche), 1:2500 in blocking solution (10% goat serum in TBST), overnight at 4°C. The excess of anti- digoxigenin antibody was removed with at least four TBST washes for 15 minutes. For the alkaline phosphatase reaction, fins were first washed in reaction buffer NTMT (5M NaCl, 1M Tris HCl pH 9.5, 1M $MgCl_2$, Tween20, H_2O MQ) for 5 minutes followed by two washes for 10 minutes. The staining signal was developed with the staining reaction containing 2 µL/mL NBT and 3.5 µL/mL BCIP (Roche).

Immunohistochemistry

The fins were fixed in a solution of 80% methanol, 20% DMSO (Sigma) overnight at 4ºC, rehydrated in a methanol-PBS series, permeabilised with acetone at –20°C for 20 minutes, followed by two washes in PBS. An additional permeabilisation step was done with a PBST 0.5% solution (PBS

with 0.5% Triton X-100) for 30 minutes. Fins were then washed several times with PBS, blocked in PBS with 10% foetal bovine serum (FBS) and incubated with the primary monoclonal antibody anti-Zns5 antibody (dilution 1:250) (ZIRC 011604) to mark osteoblasts and anti-GFP antibody (dilution 1:100) (Abcam) overnight at 4°C. After several washes in PBS fins were incubated with the secondary antibody overnight at 4°C and then mounted for analysis.

Microscopy

Images of in situ hybridisation were obtained with a Leica Z6APO stereomicroscope equipped with a Leica DFC490 digital camera. Immunostaining of the -2.4*shh*:*gfp*ABC transgenic fish were obtained on a Zeiss LSM 510 META confocal microscope. Captured Z stacks were analysed using ImageJ software.

CHAPTER IV

Discussion

IV.1. The potential of the zebrafish caudal fin as regeneration model

"Regeneration is arguably among the most awe inspiring biological phenomena known to exist" (Gurley and Alvarado, 2008). Discovered centuries ago, regeneration continues to be a fascinating biological process.

Urodele amphibians are the true champions of regeneration among vertebrates, being able to regenerate several body parts throughout adult life including the upper and lower jaw, lens, retina, limb, tail, spinal cord and intestine (Brockes and Kumar, 2005; Han et al., 2005). Therefore, for many years amphibians have been the model of choice to study vertebrate regeneration. However, the lack of a sequenced genome and well-developed molecular and genetic tools, have been a great limitation for the understanding of the cellular and molecular mechanisms of vertebrate regeneration (Poss, 2010; Poss et al., 2003).

In contrast, the teleost zebrafish is amenable for standard molecular and genetic manipulations and has the genome almost completely sequenced. In addition, similarly to amphibians, zebrafish has the amazing capacity to regenerate various tissues and organs like the heart, spinal cord, retina and fins throughout life. Other advantages of this model organism include a short generation time, the ability to raise and maintain a large number of animals and the availability of reagents and technology generated by the zebrafish community (Poss et al., 2003). For these reasons, zebrafish has recently emerged as a powerful model organism to study the process of regeneration. In particular, the zebrafish caudal fin, due to its accessibility, fast and robust regeneration and simple architecture, is currently one of the most convenient models for regenerative studies. It is composed of several segmented bony rays, mesenchymal tissue, blood vessels and nerve axons. The bony rays consist of 2 concave hemirays, and are bifurcated in the distal part of the fin (with the exception of the lateral rays) (Poss et al., 2003). These features combined with a well-established regenerative program composed of stereotypic successive steps activated upon injury, make the zebrafish caudal fin regeneration the ideal model to use in the work performed during my PhD

thesis. The caudal fin regeneration steps include the closure of the wound by the epidermis to form the regeneration epidermis and the migration of stump cells distally to form the blastema. In the outgrowth phase, the blastema cells proliferate, go through morphogenesis, pattern formation, and differentiation (Poss et al., 2003).

IV.2. Zebrafish caudal fin regeneration does not decline with consecutive repeated amputations and aging

Repeated amputation experiments are fundamental to uncover the regenerative capacity limit of lower vertebrates. A few studies have investigated this in different tissues and model organisms.

Two reports show a progressive accumulation of defects in the regenerated limb with an increasing number of amputations in both larval and adult *Notophthalmus viridescens* newts (Dearlove and Dresden, 1976; Abdel-Karim and Michael, 1993). In contrast, two other studies demonstrate that regeneration is successfully accomplished with only minor defects after 16 tail amputations in adult *Trituruscarnifex* newts (Margotta et al., 2002; Margotta, 2008). Also in the newt *Cynops pyrrhogaster*, another recent study, analyzed the regenerative capacity of the lens. In this study, structural and gene expression analysis revealed that regeneration efficiency is not compromised upon 18 amputations spanning 16 years (Eguchi et al., 2011). Whether the difference in the capacity to regenerate these structures completely without defects is due to differences between newt species or whether the newt tails and lens have a higher capacity to regenerate than limbs is unsolved.

Only very recently, the regeneration limit of the zebrafish caudal fin was investigated. In the first reported study, the gene expression analysis and the size of regenerated tissue at 7 dpa show that the regenerative capacity of the zebrafish caudal fin does not decline after 9 amputations (Shao et al., 2011). In Chapter II, we extended these results by showing that repeated amputations up to 29 times over a period of 11 months (Figure 2.1A) do not affect the regenerative capacity of the caudal fin. We show that the size of the 72 hours-post-amputation (hpa) (Figure 2.3A,B) regenerate and 4 weeks-

post-amputation (wpa) (Figure 2.2) fully regenerated caudal fin did not significantly change, even though there was a slight decrease in the gene expression markers analyzed (Figure 2.3C,D). Altogether, these data show that wound healing, blastema formation and regenerative outgrowth are not affected when the fin is challenged with 29 consecutive repeated amputations, demonstrating a virtually unlimited regenerative capacity of the zebrafish caudal fin.

However, in spite of this amazing capacity to regenerate, we observed that the bone proximal to the amputation plane (old bone), but not the regenerated bone (new bone), became progressively thickened with repeated cycles of amputations (Figure 2.4). Since we could not detect a difference in the number of osteoblasts (Figure 2.4I, J), the progressive bone thickening might be a consequence of inappropriate activation of osteoblasts that secrete matrix far away from the amputation plane. Indeed, there is now strong evidence that osteoblasts enter the cell cycle following amputation (Johnson and Bennett, 1999; Knopf et al., 2011; Sousa et al., 2011) and that differentiated cells can be induced to proliferate even far from the amputation plane (Knopf et al., 2011; Santos-Ruiz et al., 2002). Thus, while some dedifferentiated osteoblasts migrate distally to form the blastema, it is unlikely that newly formed osteoblasts distant from the amputation plane would participate in blastema formation. Rather, they likely represent a source of cells replacing those moving into the blastema. Therefore, it is possible that activation of proliferation causes these cells to re-activate matrix secretion, which after repeated cycles results in bone thickening. Alternatively, the increase in bone matrix could be caused by an unbalanced ratio of bone-forming (osteoblasts) and bone-degrading cells (osteoclasts) or to a decrease in the production/activation of the enzymes responsible for collagen degradation. This hypothesis could be further investigated by determining whether there is a progressive decrease in the number of osteoclasts with increased number of amputations, using the osteoclasts markers Calcitonin receptor (Hattersley and Chambers, 1989) and osteoclast-

associated receptor (OSCAR) (Kim et al., 2002). On the other hand, we could also analyse if repeated amputations result in a decrease or inactivation of the enzymes involved in bone resorption, such as the matrix metalloproteinases (Mmps) or Cathepsin K (Murphy and Lee, 2005). We could also determine if the overexpression of enzymes implicated in the process of bone resorption would rescue the thickened bone phenotype.

IV.3. Stem-cell niches maintained by Wnt signaling do not contribute to the robust regeneration capacity of the zebrafish caudal fin

The almost unlimited regenerative capacity of the zebrafish caudal fin that we have uncovered could be due to either the presence of stem cells, dedifferentiation of mature cells or the contribution of both. We hypothesized that each amputation could activate the pool of putative stem cells that might be present in different fin tissues, leading to the differentiation of all the missing structures. Importantly, the decision between self-renewal and the initiation of differentiation is controlled by signals provided by the tissue microenvironment, or niche, where stem cells are believed to reside. The Wnt signalling pathway plays a fundamental role in the control of maintenance and proliferation initiation of adult stem cells reservoirs (Korinek et al., 1998; Blanpain and Fuchs, 2006).

In Chapter II, we made use of the heat-shock inducible transgenic *hsp70l:dkk1-gfp* to block Wnt/β-catenin signalling (Figure 2.5A). Fin regeneration was impaired after Wnt signalling inhibition upon heat-shock and spontaneous regeneration did not occur when fish were relieved from the heat-shock treatment (Figure 2.5B). However, if the fins were re-amputated and allowed to have an intact Wnt signalling by keeping them at a non-inducing temperature, fins regenerated completely and reached the original length even after several rounds of consecutive Wnt/β-catenin signalling inhibition and re-amputation (Figure 2.5B,C). These results show that the ability to regenerate after Wnt signalling inhibition requires a novel

amputation stimulus and suggest that blastema formation does not depend on a pool of progenitor cells that requires Wnt for its maintenance. While these data do not completely rule out a contribution of progenitor cells, it is more compatible with the alternative model of regeneration based on dedifferentiation. This is supported by the recent finding that mature osteoblasts dedifferentiate to form part of the blastema (Knopf et al., 2011; Sousa et al., 2011) and regenerate bone (Knopf et al., 2011) in the zebrafish caudal fin. In this model, Wnt signalling could be involved in the mechanisms of dedifferentiation, migration and/or expansion of the dedifferentiated cells to form the blastema. To address whether Wnt signalling plays a role in these early processes of fin regeneration, one could take advantage of *hsp70I:dkk1-gfp* transgenic line and, by blocking Wnt signalling in a time-controlled manner, analyse its contribution to the early regenerative events that will lead to blastema formation.

IV.4. Dedifferentiation and implications for regenerative medicine

Our data in Chapter II suggests that zebrafish regeneration capacity does not depend on a stem cell niche controlled by Wnt signalling. This fits with the model proposed by others in the zebrafish fin (Knopf et al., 2011; Sousa et al., 2011) and heart regeneration (Jopling et al., 2010; Kikuchi et al., 2010) in which dedifferentiation might be the major mechanism contributing to the regeneration process. Thus, vertebrate regeneration does not seem to be related to a homeostatic event (that relies on a pool of stem cell to replace cells lost through apoptosis and aging). In contrast, an amputation will trigger an unknown signal required for cell dedifferentiation, proliferation and migration to the wound. Importantly, according to several recent studies in different regeneration models (namely in the zebrafish fin and heart, salamander limb and mouse digit tip), this dedifferentiation does not lead to a pluripotent cell state since these studies demonstrate that there is cell-lineage restriction during the regeneration process (Kikuchi et al., 2011; Knopf et al., 2011; Kragl et al., 2009; Lehoczky et al., 2011; Rinkevich et al., 2011).

These discoveries bring significant implications for the regenerative medicine field since it is now well established that this capacity of differentiated cells to go through a dedifferentiation mechanism is not specific to lower vertebrates. Indeed, a major accomplishment in the regenerative medicine field was achieved when it was reported the possibility to experimentally force differentiated fibroblasts cells back into a pluripotent stem cell state (*in vitro*), from which all cell lineages could be derived (Takahashi and Yamanaka, 2006). This ability to reverse the differentiated state of mammalian cells opens the possibility to induce *in vivo* regeneration upon injury or disease in mammals. To this end, further studies in regenerating model organisms will be essential.

A notable example of translation from research performed in newts to mammals is the case of the discovery of the factors required for mammalian muscle dedifferentiation. The effect of Retinoblastoma protein (RB) inactivation in mammalian muscle cell cycle re-entry was investigated after being reported that its inactivation is required for muscle proliferation during newt limb regeneration (Tanaka et al., 1997). In the case of mammals, it was found that RB inactivation alone is not sufficient to induce mammalian muscle cell cycle re-entry (Camarda et al., 2004). This is due to RB inactivation being compensated by the action of the tumour suppressor alternate reading frame (ARF), which by itself is sufficient to induce cell cycle arrest (Tago et al., 2005). However, inactivation of both RB and Arf could successfully induce mammalian muscle cells to dedifferentiate and proliferate (Pajcini et al., 2010).

It has been thought that organs which are only composed of differentiated cells are not able to self renew. Importantly, this old regenerative medicine concept is now starting to change. An example of this is the recent and major discovery that mammalian cardiac muscle cells are able to renew. In this study, elevated carbon 14 was found integrated in the

heart muscle DNA, from people born before 1955, when nuclear bomb testing during the Cold War generated high levels of radioactive in the Earth's atmosphere. This finding demonstrates that cardiac cells divided after birth. Moreover, with this analysis, it was also possible to estimate that a 20 year old person renews about 1% of heart muscle cells per year, having about 45 percent of the heart muscle cells renewed by the age of 50 (Bergmann et al., 2009). This relevant work in the mammalian heart, together with the identification of cardiomyocyte dedifferentiation as the main mechanism contributing to the heart regeneration in zebrafish (Jopling et al., 2010; Kikuchi et al., 2010) are essential starting points to future strategies in the induction of mammalian cardiac regeneration.

IV.5. Positional memory in regenerating appendages

The questions of how the original size, pattern and replacement of only those structures removed by amputation is achieved, are among the most interesting aspects of regeneration. However, how the relative position of the different tissues and structures that compose the lost appendage is maintained upon amputation remains unknown. The positional memory instructors should be present in a gradient or restricted pattern in the intact adult structure and their ectopic expression or downregulation should affect the pattern of the regenerated appendage. Studies from the past decades mainly in amphibian limb regeneration have attempted to identify differences between proximal and distal regenerates. Relevant work indicates a gradient of retinoic acid (RA) and of the cell surface protein CD59, with higher levels in more proximal blastemas when compared to the distal ones (da Silva et al., 2002; Scadding and Maden, 1994). In addition, treatment with RA proximalizes the regenerate in a concentration-dependent fashion (Crawford and Stocum, 1988; Maden, 2002) by increasing the levels of CD59 (da Silva et al., 2002). These data provide evidence for a model in which cell interactions take place locally between adjacent cells conferring different adhesion properties which enable to distinguish proximal from distal

regenerates (Crawford and Stocum, 1988; Maden, 2002). It was also shown that proximal amputations have a faster regeneration rate when compared with distal amputations. This was observed in the salamander limb, as well as in lower vertebrates and invertebrates suggesting an evolutionary conserved role that might be important for the setup of positional memory during a regeneration process (Morgan 1900, Lee et al., 2005).

Relevant work in planarian has identified graded or region-specific expression of certain signalling molecules which confer positional memory in the intact animal as well as in regeneration. Shh, Wnt and Bmp signalling pathways are implicated in the instruction and maintenance of planarian axial polarity (as described above in the invertebrates section of Chapter I). Misregulation of these pathways causes severe patterning defects during regeneration, as well as an abnormal body shape in the intact animal (Poss, 2010).

In the regenerating zebrafish fin it has been proposed that after an amputation, distal to the bifurcation, RA treatment leads to the fusion of the bifurcated sister rays and consequently, distalizes the bifurcation point (Geraudie et al., 1995; White et al., 1994). It is not clear though, whether this is directly caused by proximalization of the regenerating tissue or indirectly by the downregulation of *shh*, caused by the RA treatment (Laforest et al., 1998), which affects proper bone formation/patterning (Quint et al., 2002). On the other hand, a recent study hypothesizes that these patterning defects are a result of toxicity and secondary effects due to the high concentration of RA used in the earlier studies (Blum and Begemann, 2012).

It was also demonstrated that Fgf targets show higher expression in proximal regenerates when compared to distal ones. This correlates to an increased cell proliferation detected in proximal regenerates and to the possibility of an Fgf gradient in the regenerating fin, suggesting that Fgf signalling might be implicated in positional memory during fin regeneration (Lee et al., 2005). In agreement with a supposed role of Shh and Fgf in the positional memory of the caudal fin is their expression in the intact fin,

possibly maintaining the positional cues in the adult cells (Poss, 2010). However, since a gradient was never observed for these (or any) signalling molecules, their expression in the intact fin could just simply be required to the continuous growth of the caudal fin observed throughout the life of the animal. Nevertheless, the signalling(s) that give positional information to the regenerating fin tissue remains to be discovered.

VI.6. Positional memory of the caudal fin bifurcation is influenced by the amputation place

In Chapter III, we took advantage of the zebrafish caudal fin as a model to study positional information upon amputation, since the bony ray bifurcations provide an excellent morphological reference of the PD axis. We observed how positional information of the bony ray bifurcation is affected with repeated amputations at different levels. Our results show that there is a progressive distalization of the position of this structure in the regenerated fin, when the repeated amputations are done at 1 segment proximal to the bifurcation (near the bifurcation) (Figure 3.2). On the other hand, its position is maintained (after a first amputation) with repeated amputations at a more proximal level (4 segments distally from the base of the fin) (Figure 3.2). This indicates that while amputations proximally distant from the bifurcation do not affect its PD position in the regenerated fin, successive amputations proximally near the bifurcation induce a positional reset and will progressively shift its place distally. Thus, it is possible that the formation of a blastema during the regeneration process, after an amputation proximally near the bifurcation, will inhibit or delay its formation. This means that a certain number of segments will need to be formed/differentiated before a bifurcation is signalled to form.

IV.7. Shh is not the signal for the formation of a bony ray bifurcation

In Chapter III we investigated potential pathways involved in the control of the position/formation of a bifurcation during caudal fin regeneration. One of the investigated pathways was Shh, since a previous report indicates that, in caudal fin regeneration, preceding the formation of a bony ray bifurcation, *shh* duplicates its single mesenchymal domain of expression in the basal layer of the epidermis (Laforest et al., 1998). This would provide a good indication that Shh could be signalling the formation of a bifurcation during caudal fin regeneration (Laforest et al., 1998; Quint et al., 2002). According to this idea, an amputation proximally distant from the bifurcation would induce a delay in the duplication of the single domain of *shh* expression when compared to an amputation proximally near the bifurcation. However, we have observed that *shh* expression was not differently expressed after successive amputations at the two different levels relatively to the bifurcation place, being in both cases detected in two separate groups of cells in the basal layer of the epidermis (Figure 3.3a-h). Furthermore, *shh* expression in two separate domains was clear at 4 dpa even in a caudal fin that did not have any bifurcations due to being subjected to several distal amputations (Figure 3.3i,j).

We made use of the *shh*:*gfp* reporter transgenic zebrafish line to precisely follow s*hh* expression every 6 hours from 1 to 2 days post amputation at 1 segment proximal to the bifurcation. We observed that its expression is initiated at 1 dpa + 12 hpa when it is already detected in two separate domains in the basal layer of the epidermis. This expression was maintained in all subsequent time-points (Figure 3.3k-o).

Altogether, these results suggest that Shh signalling does not seem to have an instructive role in setting the position of the bony ray bifurcation, even though it might be required for the formation of this structure.

IV.8. *shh* expression in two separate epidermal domains might be required for bone alignment during regeneration

In Chapter III, to uncover the functional relevance of *shh* expression in two separate domains in the basal layer of the epidermis, we performed a Zns5 expression time-course (osteoblast marker), since it has been proposed that Shh might play a role in the osteoblasts patterning and/or differentiation during fin regeneration (Quint et al., 2002). Interestingly, soon after the detection of *shh* expression, the bone alters its growing tip, as a cone shape, and the forming osteoblasts start to be aligned close to the basal layer of the epidermis in "V" shape, next to *shh* expressing cells (Figure 3.3p-t). This observation suggests that Shh might act has an attractor of bone progenitors directing the bone growth and width in the regenerating fin. In order to test this hypothesis, an interesting experiment would be to implant Shh-coated beads in regenerating and intact fins, and observe if bone cells would migrate towards the bead.

IV.9. Fgf signalling does not seem to be involved in the determination of the bifurcation position

Another possible candidate to control the bifurcation position is Fgf signalling, since it has been implicated as a possible mediator of the positional memory in the regenerating fin (Lee et al., 2005). In order to address a potential role of Fgf signalling in instructing positional information and determining the bifurcation position, we made use of the heat-shock inducible transgenic *hsp70:dn-fgfr1* to attenuate Fgf signalling in a time controlled manner. All the different protocols used to transiently attenuate Fgf signalling did not alter the position of the bony ray bifurcation when compared to the controls, with unaffected Fgf levels (Figure 3.4 and Figure 3.5). This indicates that Fgf signalling may not be the factor which controls the formation of a bifurcation in the zebrafish regenerating caudal fin.

An interesting candidate signalling to investigate next in order to pursue this work, would be the RA signalling pathway, since it was previously demonstrated to play a role in the establishment of positional information in the regenerating amphibian limb (as described above, in this chapter) (Crawford and Stocum, 1988; da Silva et al., 2002; Maden, 2002; Scadding and Maden, 1994).

In addition, previous work has demonstrated that the crosstalk between blastema, distal regenerating epidermis and inter-ray tissue is essential for signalling the formation of a bifurcation in the zebrafish fin (Murciano et al., 2002). Therefore, local interactions between the different cellular domains present in the regenerating fin seem to be key regulatory mechanisms in the patterning of a regenerating appendage. Therefore, it would be interesting to further investigate the role of these interactions in the triggering of the formation of a bony ray bifurcation.

IV.10. Central questions in the field of regeneration

Intriguingly, the classic questions in regeneration research remain much as they were a long time ago possibly because the powerful genetic and molecular tools only very recently started to become available. This means that we are now able to begin to increase the knowledge in the understanding of the fundamental issues of this fascinating phenomenon that has for long been in the scientists' minds.

The question of what defines and controls regenerative potential has captured the imagination of scientists for centuries. The idea that regeneration capacity has been progressively lost during evolution is currently well accepted and several hypotheses have emerged to explain why some animals regenerate while others fail to do so (Reichman 1984, Bely et al 2009). Nevertheless, the ultimate answer to this question remains to be addressed.

The origin of the cellular sources of vertebrate regeneration has also intrigued researchers for a long time. Very recent discoveries in the zebrafish

heart (Jopling et al., 2010; Kikuchi et al., 2010) and fin (Knopf et al., 2011; Sousa et al., 2011) have finally shed some light on this subject. Notwithstanding, the cellular sources of regeneration are still poorly understood and these findings are only the beginning of the understanding of this fascinating question.

Another major unresolved issue that has relevant implications in the regenerative field is to discover the factors necessary to trigger regeneration. A few developmental genes including Fgf20a (Whitehead et al., 2005), Wnt ligands (Kawakami et al., 2006; Stoick-Cooper et al., 2007b) and Activin-βA (Jazwinska et al., 2007) were identified to be expressed early in amphibian and fish appendage regeneration. However, it remains unknown what is the mechanism responsible to induce their expression. Possibly cell stress and/or death are involved, as shown for Wnt3 induction in apoptotic cells in hydra head regeneration (Chera et al., 2009).

Finally, how the positional memory of the lost body part is maintained and how the re-growth is controlled are other unresolved mysteries. To date, a few developmental signalling pathways have been implicated in positional memory in planarian and hydra regeneration (Bosch, 2007; Chera et al., 2009; Galliot and Chera, 2010; Reinhardt et al., 2004). Conversely, in vertebrate appendage regeneration, it remains unknown which are the signals involved in the maintenance of positional memory, aside from RA and Prod1 in the amphibian limb (Crawford and Stocum, 1988; da Silva et al., 2002; Maden, 2002; Scadding and Maden, 1994).

IV.11. Future perspectives in the regenerative medicine field

Regenerative medicine aims to find new therapies for patients with severe injuries or chronic diseases, which do not naturally recover new functional tissues. Stem cells are the primary source used to repair, regenerate, and replace tissues and organs. These cells may be derived from embryonic, fetal or adult tissues. Moreover, they can be allogeneic or autologous, added

exogenously or recruited from the host, expanded and/or differentiated *in vitro* (Atala et al., 2010).

Cell-based therapies have hold promise for a variety of clinical problems and the goal of a successful treatment ultimately depends upon the ability of cells to respond to their environment and function in a clinically relevant manner. This represents one of the most simple, and yet most complex principles for cell-based therapies. Many factors contribute to decide which would be the most indicated cell source for the cell-based therapy in a given patient. The clinical condition and the type of damaged tissue are primary factors to consider (Atala et al., 2010).

The application of stem/progenitor cell therapy based on the expansion of adult stem cells is limited to tissues in which these cells are possible to isolate, culture/expand and re-differentiate *in vitro*. Moreover, since adult stem cells are often a very small percentage of the total cells isolated from a given tissue, generating a pure population is difficult (Koh and Atala, 2004). Bone marrow and blood-derived stem cells have been the most thoroughly investigated. However, since the yield of stem cell isolation from these tissues is low, this motivates efforts to find alternative adult stem cell sources, namely the umbilical cord and the fat tissue (Atala et al., 2010). The umbilical cord has been considered an exciting resource for regenerative medicine applications since it is a widely available source of stem cells with extensive expansion capabilities in vitro (Chiu et al., 2005). Likewise, the fat tissue, another abundant adult stem cell source, has also already been shown to have the potential to differentiate into multiple cell types (Ashjian et al., 2003; Huang et al., 2004; Zuk et al., 2002).

Overall, the proven differentiation potential of human adult stem cells is limited. Therefore, this cell replacement strategy will benefit from further translation from basic discoveries (namely in animal models of regeneration) regarding the identity and behavior of stem cells into applied therapies.

On the other hand, the high proliferation and pluripotency of embryonic stem cells are their major advantages and, at the same time, potential

limitations to the use of such cells for regenerative medicine. Indeed, the current main challenges for the clinical application of these cells are to efficiently direct their differentiation to a pure population of given cell type, without the presence of residual stem cells that can lead to the formation of tumors upon *in vivo* implantation (Odorico et al., 2001). These challenges will likely extend the timeline of usage of these cells in tissue engineering applications. In contrast, adult tissue-specific stem cells may provide a more direct route to clinical translation and it is likely that they are a safer cell source for clinical applications with or without prior differentiation.

Importantly, the understanding of the cellular interaction with extracellular matrices and biological factors has improved during the past years allowing significant progress in the *in vitro* generation of three-dimensional tissue-engineered skin, cartilage, and blood vessels. It was also discovered the importance of providing proper physical and biological context in order to elicit the desired cellular response. Understanding these interactions will continue to guide the future development of clinically useful engineered tissues or organs in the practice of regenerative medicine (Atala et al., 2010).

Stem cell technology shows potential in contributing to regenerative medicine. Nevertheless, many scientific obstacles will need to be overcome for each stem cell type before clinical use. Extensive ongoing research indicates the confidence of researchers in the ability to overcome these obstacles and in the potential of stem cells to have a positive impact on clinical applications. Progress in this field will hopefully help to treat many currently incurable diseases, face the lack of organs available for transplantation and will possibly allow customization of therapies for each patient.

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