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Review

Regeneration: The ultimate example of wound healing

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ABSTRACT

The outcome of wound repair in mammals is often characterized by fibrotic scaring. Vertebrates such as zebrafish, frogs, and salamanders not only heal scarlessly, but also can regenerate lost appendages. Decades of study on the process of animal regeneration has produced key insights into the mechanisms of how complex tissue is restored. By examining our current knowledge of regeneration, we can draw parallels with mammalian wound healing to identify the molecular determinants that produce such differing outcomes.

Contents

1. Introduction

Every animal in existence has evolved mechanisms of repair with the goal to restore tissue homeostasis and architecture after insult. Ultimately, this repair should recapitulate the original in both form and function, but for most mammals, such as humans, this is not the case. Instead, the normal outcome of both cutaneous and subcutaneous wounds is a fibrotic scar that disrupts the normal tissue organization with acellular deposits of extracellular matrix (ECM) [1,2]. A primary culprit are fibroblasts within the wound bed that fail to remodel and remove excessively deposited ECM that was used in early wound repair to restore tissue tension and structure and to provide tracks into the wound for other cell types. However, all the mechanisms that lead to the final outcome of fibrosis are far from clear. Wound repair is a multi-tissue process that involves epithelia, dermal and mesenchymal tissues, blood vessels, nerves, and immune cells that all respond during wound healing within a complex network of signals and behaviors necessary to resolve wounds. Changing any one parameter of wound healing can initiate a cascade of consequent alterations in the response of surrounding tissues, exacerbating or alleviating the final fibrotic outcome.

Abbreviations: ECM, extracellular matrix; AER, apical ectodermal ridge; AEC, apical ectodermal cap; MMP, matrix metalloproteinase; KGF, keratinocyte growth factor; ALM, accessory limb model; FGF, fibroblast growth factor; nAG, newt anterior gradient; TGFβ, transforming growth factor β; TN, tenascin; HA, hyaluronic acid; FN, fibronectin; FAK, focal adhesion kinase; RA, Retinoic acid; IGF2b, Insulin-like growth factor 2b; HB-EGF, Heparin-binding EGF-like growth factor; HGF, Hepatocyte growth factor; MHC, Major histocompatibility complex; Prod1, Newt ortholog of CD59.

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In spite of the daunting prospect of improving a complex, imperfect wound repair process, the promise of perfect wound healing does exist. Mammalian embryos retain the ability to heal epidermal wounds without scars through early stages of development, and certain epithelial surfaces such as the palm or buccal mucosa inside the mouth heal without the presence of a scar. In addition to enhanced healing, human and mouse neonates also maintain the ability to regenerate their digit tips [3,4].

One way to expand our thinking about wound healing is to consider situations where wound healing occurs flawlessly throughout the lifetime of an organism. Regenerating vertebrates such as zebrafish and salamanders offer a unique example where wounds are not only resolved without the formation of a fibrotic scar, but the wound tissue executes tissue patterning analogous to the original process of embryonic development to restore lost tissue. Salamanders can heal wounds and regenerate their jaws, limbs, tails, gills, lens (newt), and heart without scars throughout their lifetime, recapitulating the original tissue in form and function.

The primary characteristic of vertebrate regeneration is the formation of a regenerative structure called the blastema (Fig. 1). The blastema is formed after the migration of epidermis over the amputated appendage. The underlying mesenchyme that are in direct contact with the epidermis, begin to dedifferentiate into lineage-restricted progenitors and increase their proliferation. Signals from severed nerves and the covering epidermis, that has transitioned into a secretory epithelium termed the apical ectodermal cap (AEC), are essential components of the blastema. These two signaling centers maintain undifferentiated progenitors in the blastema and pattern differentiating cells to form a fully articulated limb.

What can we learn by looking at wound repair and regeneration in regenerating amphibians? Firstly, regeneration, as studied in the limb, involves most of the same tissues that are generally thought to participate in mammalian wound healing, sans specialized structures such as the hair follicle. The participating tissues also include nerve that has been somewhat understudied in mammalian wound healing and which plays a major role in limb regeneration. Secondly, because the process of regeneration mirrors developmental events, there is a wealth of knowledge about morphogenetic signaling pathways that are crucial for regeneration. There is some evidence that many of these same pathways are involved in amphibian wound healing, supporting a link between regeneration and perfect tissue repair. Still to be determined is if intrinsic properties allow regenerating cells to respond to morphogenetic signals or if it is a unique property of the surrounding tissue to produce such coordinated, morphogenetic signals. Similar questions relating to the intrinsic/extrinsic properties of cells and tissues are especially poignant in studying wound healing, underpinning the importance of comparing the two processes.

In each section of this review, we will examine several tissues that play a role in both regeneration and wound repair and our current knowledge about how they function during these processes in regenerating organisms, specifically in amphibians such as the axolotl, newt, and frog.

2. The wound epidermis

In order to orchestrate a process such as limb regeneration, urodele amphibians (axolotl, newt) have to establish morphogenetic signaling centers that mirror those found during limb development in order to reestablish progenitor populations from mature tissue, increase cell proliferation, and coordinate new patterning events [5]. The epithelia that covers the amputated limb represents a major component of such a signaling center.

During regeneration, the wound epidermis has two important functions. At early time points, the epidermis recapitulates many of the same injury-related behaviors as any lateral wound, with the primary goal of barricading the wound site off from the extracellular environment. In later stages, however, the wound epidermis converts to a specialized secretory epithelia, referred to as the apical ectodermal cap (AEC), that is necessary to organize the blastema. Through this conversion, the regenerative epidermis becomes similar to the developing limb epidermis, or apical ectodermal ridge (AER). Both the AEC and AER are required for regeneration and limb development, respectively, and share a similar morphology and express similar genes. Despite their similarities, the wound secretory epithelium is created via events and signals following injury. This implies that the regenerative AEC is a somewhat hybrid structure: it is initiated by an injury response, but later utilizes developmental pathways to achieve regeneration.

2.1. Re-epithelialization

Re-establishing epithelial integrity is an essential first step to maintain the regenerative or wound healing response. Once a wound or amputation has occurred, blood coagulation creates a fibrinous clot to prevent fluid loss and act as a temporary covering. The first step to reestablish normal tissue architecture is to construct a more permanent epidermal layer to resist further insults and create a wound bed environment that is conducive to healing. Morphologically, this process is identical between regenerative

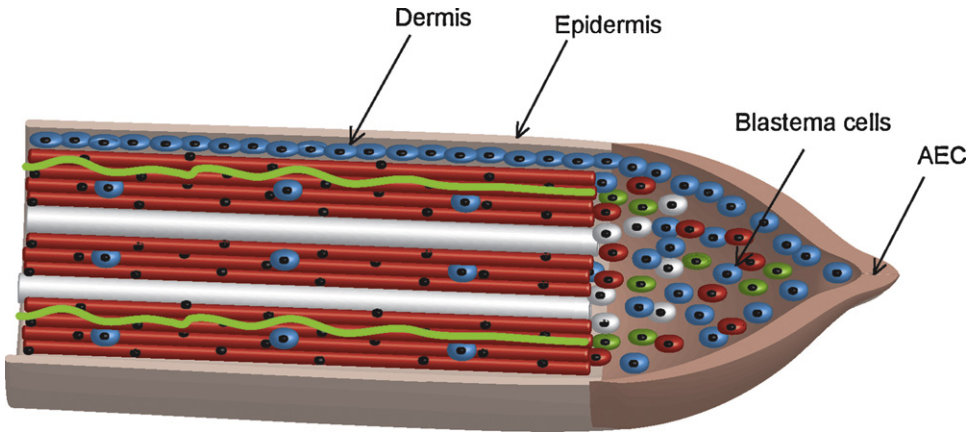

Fig. 1. Mid stage blastema of regenerating salamander limb. Upon limb amputation, epidermis migrates over the cut end and gives rise to a thickened layer of epidermis, known as the apical ectodermal cap (AEC). The AEC acts as a signaling center and promotes migration of the underlying mesenchyme to form an undifferentiated mass of cells known as blastema, which is a pool of lineage-restricted progenitors that will reconstruct the internal tissue of the limb. Bone (white), connective tissue/dermis (blue), muscle (red) and nerves (green) each contribute their own progenitors during blastema formation and limb regeneration.

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and mammalian wound healing, where keratinocytes at the wound edge extend lamellipodia and migrate as a continuous front to close the wound [6,7]. This requires leading edge cells to undergo large changes in gene expression to dissolve tight junctions, reorganize cytoskeletal machinery for migration, and upregulate specific integrins, matrix metalloproteases (MMPs), and ECM components necessary to enclose the wound or amputation.

The primary difference between regenerative and non-regenerative wound closure is the timing required for the initial re-epithelialization. In the axolotl, complete coverage of an amputated axolotl limb takes between 10 and 24 h, depending on animal size. In contrast, the re-epithelialization of even a small lateral wound in mammals can take between 48 and 72 h. This difference in timing seems to occur at the initial lag phase, as keratinocytes prepare to migrate into the wound site (Fig. 2). In lateral wounds in mammals, the time between injury and first migration of epithelia can be more than 24 h. In explants of axolotl full thickness skin, which close wounds in vitro at a similar pace as in vivo, the initial migration of keratinocytes occurs at 1–2 h post injury [6]. While it is not known what allows axolotl and newt epithelia to so rapidly undergo this change in behavior, we do know several molecules required for the efficient migration of epithelia in the axolotl. keratinocyte growth factor (KGF), MMP-9 [6], and fibronectin/fibrinogen [8] (discussed in more detail later) are necessary components for epithelial migration as pharmacological inhibitors or blocking antibodies prevent wound epidermis formation in vivo and ex vivo and consequently block regeneration. Other molecules such as Heparin-binding EGF-like growth factor (HB-EGF) [9] and hepatocyte growth factor (HGF) [10], which have been implicated in mammalian re-epithelialization, have not yet been examined in amphibian regeneration or wound healing.

Although it is not clear if rapid epithelial migration would significantly impact scar formation in non-regenerating organisms, it is tempting to speculate that the lag between injury and epidermal coverage could prevent the epidermis from providing important instructional cues to the underlying mesenchyme as it does during regeneration.

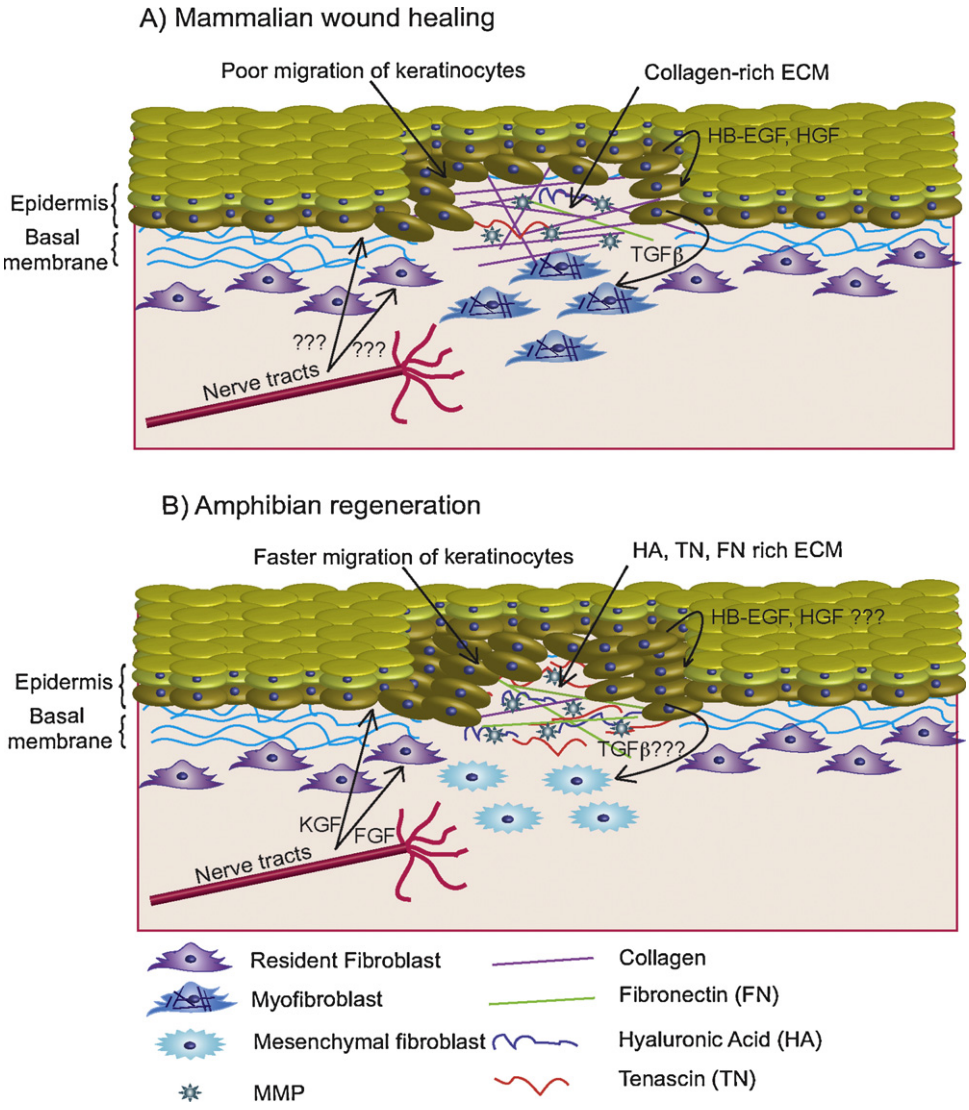

Fig. 2. Differences of wound healing between (A) mammals and (B) amphibians. (A) Mammals display a slower migration of keratinocytes in response to wounding as compared to amphibians. TGF β signaling is responsible for myofibroblast formation. Myoblasts produce collagen-rich ECM. The role of nerves in wound healing is understudied in mammals. (B) In amphibians, nerves are responsible for KGF, nAG, and FGF secretion. Roles for HB-EGF and HGF, that are known to promote keratinocyte migration in mammals, have not been explored in regenerating animals. TGF β signaling is necessary for regeneration, but it is not clear how resident fibroblasts escape the myofibroblast lineage. Mesenchymal cells accumulate under the epithelium and produce TN, HA, FN-rich ECM, which promotes scarless wound healing.

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2.2. Secretory epithelia formation

After a complete wound epidermis has formed over an amputated salamander limb, it undergoes a round of proliferation to thicken the epidermal layer and concomitantly begins to transition into a signaling secretory epithelium. There are several factors that likely contribute to this transition. The mixing and contact of tissue from different regions along the circumference of the limb likely plays a role in converting the wound epidermis into a signaling center. This requirement was shown in experiments that rotated the orientation of the blastema to create multiple boundaries of axis discontinuity, which resulted in supernumerary limb formation [11,12].

Another important input is nerve-dependent signals such as FGF2 [13,14], keratinocyte growth factor (KGF) [15], and nAG [16] (discussed below). Of particular note, KGF seems to have divergent functions in axolotl versus mammals in both the timing of its expression and the tissue source. In mammals, KGF appears to influence the rate of wound re-epithelialization and is secreted by fibroblasts in the wound bed [17]. In contrast, nerve-secreted KGF upregulates *sp9*, a marker of embryonic epidermis, in the regenerative AEC [15].

The importance of nerve and intermingling of cells from different circumferential positions was most recently demonstrated by Endo et al. who developed the accessory limb model (ALM) [18]. In this assay, nerve-deviation toward an anterior lateral site on the limb initiates a blastema-like bump of progenitors laterally, but this bump regresses unless a piece of posterior full thickness skin is grafted at the site of innervation. It is not yet known at which point the molecules associated with full AEC function become expressed in this model. Because of the close interaction of dermis and epidermis in the skin graft, the exact sequence of signals and their source that leads to the epidermis to AEC transformation remains to be determined in assays such as the ALM.

The underlying mesenchyme that gives rise to the regenerative blastema structure is also likely to influence the specialized identity of the AEC. Unlike differentiated tissue, the wound epidermis lacks a basement membrane and is in direct contact with the progenitor mesenchyme in the blastema. In a similar mechanism to limb development, a positive feedback loop of FGF-10 and FGF-8 between the blastema mesenchyme and wound epidermis, respectively, likely reinforces signaling from the wound epidermis [19–21]. In fact, supplying exogenous FGF-10 to normally non-regenerating adult Xenopus amputations can induce FGF-8 in the wound epidermis and produce an imperfect, albeit fully articulated, limb [22]. Wnt signaling also seems to display a feedback expression such that inhibition of Wnt signaling not only influences the proliferation of blastema cells, but also affects the morphology of the AEC [23]. Additionally, in zebrafish, IGF2b secreted from the blastema mesenchyme to the AEC is necessary for regeneration [24].

2.3. Function of the wound epidermis during regeneration

What, then, is the function of the secretory wound epidermis during axolotl limb regeneration? The wound epidermis is vital for maintaining a pool of proliferating progenitors within the blastema since replacing the wound epidermis with an unwounded skin graft halts regeneration [25]. The wound epidermis supplies a number of extracellular factors such as Wnt5 (in a positive feedback loop with the blastema mesenchyme) [23,26,27] and several FGFs (2, 4, and 8) [20,21] that putatively promote proliferation and keep blastema cells in an undifferentiated state. Interestingly, some of these same factors are important in maintaining mammalian hair follicle stem cells and are subsequently upregulated during mammalian wound healing [28–30].

In addition to a permissive role in maintaining the blastema, the wound epidermis also secretes several factors that likely influence the patterning of the regenerating limb. Retinoic acid (RA) is one such factor that is known to play a key role in determining proximal tissue fates during limb formation [31–33]. It is important to note that although comparisons are often made between the function of the limb bud AER and regenerative AEC, on the molecular level, molecules such as RA may have a more complex and confounding function during regeneration. During embryonic limb formation, RA is known to emanate from the somites as a proximal to distal gradient that influences proximal fate by upregulating the proximal positional marker Meis 1/2 [34]. Similarly to development, treating blastemas with exogenous RA is able to proximalize cells and leads to aberrant limb formation [33,35,36]. However it is not clear if RA normally functions in this capacity during regeneration. In contrast to embryonic limb formation, RA seems to be secreted by the wound epidermis, in an opposite orientation [32]. One possibility is that the levels of RA expressed by the AEC may play a secondary role to regulating proximal positional markers. RA has been shown to negatively regulate growth of cells in culture and completely inhibit limb development and regeneration, implying that it might act to maintain the proper rate of cell division as well as provide patterning and differentiation signals in the blastema [37,38].

One molecule that has a more evident function to pattern the proximal–distal axis of the developing and regenerating salamander limb is Prod1/nAG. Prod1 is cell surface receptor that is expressed in a gradient along the limb proximal–distal axis (P > D) [39]. The ligand of Prod1, newt Anterior Gradient (nAG), is first secreted by severed nerves shortly after amputation [16]. After the secretory epithelium is formed, nAG expression is upregulated in the epidermis. The initial pulse of nerve-delivered nAG is essential for its expression in the wound epidermis, as denervated limbs lack nAG wound epidermis expression and do not regenerate. Regeneration can be rescued, however, by exogenous expression of nAG in the epidermis via electroporation. Limbs that developed without nerve innervation by removal of the neural tube retain high levels of nAG even after development is finished, ensuring that the epidermis can initiate nAG signaling even in the absence of nerve [40]. Currently it is not clear whether Prod1/nAG are found in mammalian wounds or have a similar expression or function in mammals.

3. Dermal biology

During regeneration a major target of signaling from the epithelium are underlying mesenchymal cells deriving from dermis and other connective tissues. Dermal tissues are one of the most intriguing cell types during wound healing due to their ability to produce various growth factors, ECM molecules, MMPs, and their role in healing wounds with or without scars. During mammalian wound healing, dermal fibroblasts are attracted to the wound site by various growth factors and cytokines and differentiate into myofibroblasts (Fig. 2a). These myofibroblasts are the major cell type in newly formed granulation tissue generated after injury. Myofibroblasts can be characterized by expression of α-smooth muscle actin (SMA) and actin stress fibers and are responsible for collagen-I production at the injury site [41,42]. Additionally, one of the major reasons for the scarring phenotype in adult mammals versus the scarless phenotype of fetal mammals is attributed to the high ratio of collagen-I/collagen-III produced by myofibroblasts [43]. Moreover, myofibroblasts align along the contraction axis in the granulation tissue and are responsible for wound contraction with the help of collagen fibers [41,44]. This collagen deposition in adult

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mammals undergoes very little remodeling over time and thus leaves the tissue injury site with a fibrotic scar.

In contrast, fibroblasts in axolotl wound healing and blastema formation do not display a myofibroblast phenotype and produce little collagen in comparison to adult mammalian fibroblasts (Fig. 2b) [14,45]. Additionally, during axolotl limb regeneration, dermis-derived cells not only regenerate the dermis but are also able to contribute to the regenerating skeletal elements, suggesting that limb amputation signals promote a mesenchymal stem cell phenotype—either through dedifferentiation of fibroblasts, or through selection of a resident stem cell [46,47]. Altogether, this raises two possibilities about fibroblasts that may contribute to the regenerative phenotype in axolotl: (1) either the fibroblast population in regenerating systems such as the axolotl have different intrinsic properties from their counterpart in mammals or (2) the fibroblasts of regenerating systems receive different signals from the neighboring tissue. While the answer to this question is not yet resolved, a number of molecular players associated with a regenerative phenotype have been studied in the axolotl.

3.1. Phenotype of dermis-derived cells during axolotl limb regeneration and scarless wound healing

During limb regeneration in amphibians, dermis derived cells re-express mesenchymal genes that are associated with limb development. This mesenchymal marker expression occurs not only after limb amputation, but also in simple lateral limb wounds. For example, Prrx1, which is expressed in the mesenchyme during limb bud stages in axolotl, frog and mice is re-expressed both in the blastema and lateral wounds of axolotl and frog which heal without fibrosis [7,14]. Conversely, Prrx1 is not re-expressed in mouse forearm wounds, suggesting that fibroblasts in amphibians during scarless lateral wound healing and blastema formation share many similarities whereas they might differ with their counterpart in adult mammals [7].

3.2. Signals that are responsible for inducing a mesenchymal phenotype in axolotl fibroblasts

On a molecular level, pathways that can lead to the upregulation of Prrx1 have started to emerge in the last two decades (Fig. 3). It has been shown that upon salamander limb regeneration, FGF family members are upregulated in dorsal root ganglia (DRG) [20,21]. These secreted FGF proteins bind to the FGFR1 receptor on fibroblasts and keratinocytes to promote the expression of various matrix metalloproteinases (MMPs) [14,48]. Interestingly, in an in vitro model of mouse fibroblasts and smooth muscle cells, it was shown that MMP activity, in particular collagenase, was sufficient to activate the expression of Prrx1 in a FAK and β3 integrin-dependent manner [49,50]. In support of this hypothesis, a recent study by Satoh et al. in axolotl suggests that insertion of beads coated with FGF2 and FGF8 at the wound site are able to reduce the deposition of collagen, promote phosphorylation of FAK, and increase the expression of β3 integrin and Prrx1 in resident fibroblasts [14]. Moreover, another study by the same group also showed that the degree of Prrx1 induction in axolotl wounds depends upon the depth of the wound and nerve injury, again suggesting that FGF signaling by nerve cells might promote a mesenchymal progenitor phenotype in dermis-derived cells [51].

3.3. Signals that specify a scarring versus regenerative phenotype

For many years, the transforming growth factor β (TGFβ) signaling pathway has been implicated in promoting the differentiation of fibroblasts to myofibroblasts in non-regenerating systems and is considered to promote scarring during adult wound-healing [42,52]. Several independent studies have shown that inhibition of the TGFβ pathway reduces scar formation in non-regenerating systems [53,54]. Surprisingly in axolotl, TGFβ is upregulated during blastema formation and lateral wound injury and inhibition of TGFβ signaling using a specific inhibitor to TGFβ-type 1 receptor impairs limb regeneration in axolotl [45,55]. This contradictory behavior of TGFβ in regenerating and non-regenerating systems may be due to a difference in the duration of expression, since TGFβ is upregulated only transiently in the axolotl as compared to mammals where it persists for many days [45]. These results raise the question as to whether prolonged or higher TGFβ signaling in the axolotl would inhibit regeneration and lead to scarring.

4. ECM environment of the regenerative blastema

The extracellular matrix (ECM) of the blastema and adult mammalian wounds differs significantly in its composition. Compared to the collagen-rich granulation tissue in mammalian wounds, the blastema and lateral wound of the axolotl are rich in tenascin (TN), hyaluronic acid (HA) and fibronectin (FN) [56–58]. This TN, FN and HA-rich ECM of blastema is often called "regeneration specific ECM" and lacks the stiffness of the collagen-rich granulation tissue found in adult mammals [59].

In addition, extracellular matrix is a known modulator of various cellular processes such as cell migration, proliferation and differentiation and even during regeneration, ECM components play crucial

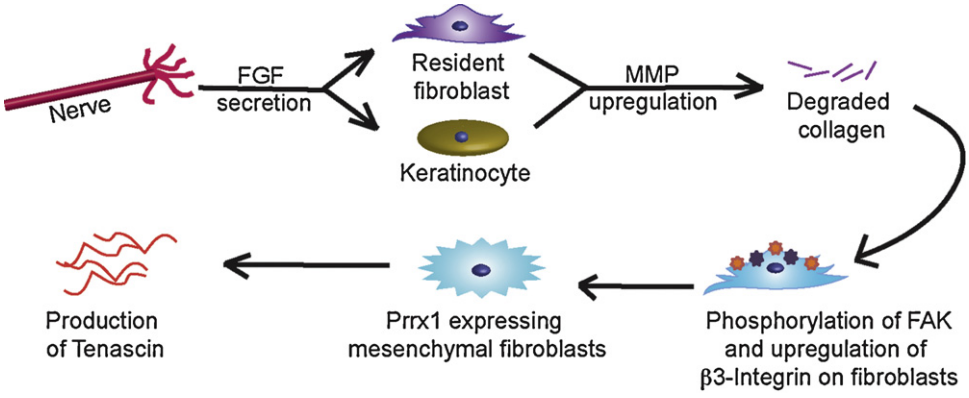

Fig. 3. Possible pathway that leads to the activation of mesenchymal fibroblasts and synthesis of "regeneration specific ECM" in regenerating animals. Upon injury, nerves secret FGF molecules, which leads to the expression of MMPs from keratinocytes and fibroblasts. MMPs degrade collagen, which leads to the upregulation of β3 integrin and phosphorylation of FAK (focal adhesion kinase) through an intracellular signaling cascade. This in turn promotes Prrx1 activity, which is a characteristic of mesenchymal fibroblasts that produce tenascin-rich ECM.

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Table 1
Effect of different ECM molecules on cell properties of newt myoblasts and myotubes.

	Myoblast EDU incorporation	Migration of newt myoblast	Myotube fragmentation	Myoblast fusion (differentiation)
TN	Yes	Yes	Yes	No
HA	No	Yes	Yes	No
FN	Yes	No	No	Yes
Collagen type-1	Yes	No	No	Yes (poor)

role. Evidence for this came from a recent in vitro study on newt myoblasts and myotubes grown on different matrices by Calve et al. [59,60]. Their results show that primary newt myoblast cells, when grown on FN, TN, and collagen show more cell proliferation compared to cells grown on HA. In contrast, these myoblast cells showed faster migration on HA and TN compared to FN and collagen. Moreover, their study also implicates ECM in key cellular events like myotube fragmentation (dedifferentiation) and fusion (differentiation). In summary, these results showed that TN and HA, but not FN and collagen, promote myotube fragmentation whereas fusion of myoblasts, an important event for myotube formation, is increased when cells were grown on FN and collagen, but not for cells grown on TN and HA (Table 1). Taken together, it seems that the stoichiometry of regeneration-specific ECM components is an important factor in blastema formation and scarless wound healing.

Often, the inability of adult mammals to remodel a scar has been attributed to limited amounts of matrix metalloproteases (MMPs) produced at the wound site [61]. Matrix metalloproteases are a class of zinc-dependent peptidases, which are secreted from fibroblasts and keratinocytes and are best known for their ability to degrade ECM components. In contrast, during blastema formation and wound healing in axolotl and newt, various matrix-remodeling proteases including collagenase (nCol and AxCol, MMP13), gelatinase (MMP9), and stromelysins (MMP3 and MMP10) are upregulated within hours of limb amputation by dermal fibroblasts and keratinocytes [14,62–64]. MMP activity is considered indispensable for regeneration since pharmacological inhibition of MMP activity blocks regeneration [63]. How do MMPs promote regeneration then? It seems that the activity of MMPs are necessary for the upregulation of Prrx1, and thus promote the mesenchymal nature of blastema cells, perhaps through the degradation of collagen as a substrate [14]. Interestingly, similar to axolotl, tenascin, HA, fibronectin, and various MMPs are upregulated during fetal wound healing in mammals and promote scarless wound healing [61,65]. This raises the possibility that dermis-derived cells in a regenerating system and in the mammalian fetus deploy the same route to heal wounds.

5. The immune response

A theory that has gained momentum in recent years is that scar formation is a result of an over-active inflammatory response [66,67]. Vertebrate immunity is a complex milieu of different cell types and signals that pleiotropically affects local tissues involved in wound healing. Below, we will briefly discuss the role of immunity in the context of embryonic wound healing and regeneration and the evidence that an attenuated immune reaction supports scar-free healing.

The primary source of evidence linking immunity and scar formation comes from comparisons of embryonic and adult wound healing in mammals, which has been reviewed extensively elsewhere [68–70]. Studies in numerous mammals and observations of human fetal surgeries have shown that fetal skin wound healing is predominantly a scarless process with the onset of scar-based healing occurring in late gestation, concomitant with an increased inflammatory response; roughly equivalent to the third trimester in human fetal development. Scar-based healing does occur in fetal skin, but only when highly necrotic cauterizations are induced, causing a large influx of inflammatory cells to the wound site [71]. Several studies have harnessed transgenic knockout mice lines to selectively remove populations of inflammatory cells such as neutrophils or macrophages [53,72]. Although often constrained by the deleterious effect of abrogating broad cell lineages, these studies have been able to shed light on the extensive role that immune cells play during wound repair [73]. Inflammatory cells are not only responsible for clearing the wound site of pathogens and dead cells, but also release cytokines and other signaling factors that influence the gene expression and activity of cells at the wound site. In particular, macrophages are the primary source at wound sites of TGFβ, which has been directly related to fibroblast behavior and the fibrotic outcome of wound healing (as discussed earlier). Interestingly, TGFβ is found at much lower levels in mammalian embryos than neonates or adults.

In general, fetal skin wound healing is an interesting parallel in mammals to epimorphic regeneration in amphibians. While attenuating the inflammatory response in mammals will likely not result in appendage regeneration, could it lead to a more perfect wound healing? In considering the role of the immune system it is vital to consider the potential of the wound tissue to recapitulate the events of fetal skin healing. In this context, does the immune response exert a neutral or positive influence or does it interfere with the embryonic potential of wound cells? We will describe a few examples that implicate immunomodulation during regeneration and how it may differ from mammalian wound healing.

5.1. Immunity in amphibians

While there is a large body of work regarding the components of immunity in urodele and anuran amphibians, our knowledge about how immunomodulation regulates regeneration is still in its nascent stages [67]. Amphibians have a similar adaptive and innate immune system as mammals with T cells, B cells, and antigen presenting cells (APCs) with MHC I and II receptors. Overall, the sensitivity of immunity among amphibians seems to correspond with the efficiency and extent of regeneration. Urodele amphibians such as the axolotl and newt are regarded to have weak immune responses [74], while anurans such as *Xenopus laevis* have a more robust immune response and less regenerative potential. In urodeles, this is thought to be the case due to a low diversity of MHC receptors and a simple immunoglobulin repertoire, with only a weak IgM immunity [74]. Consequently, it is thought that T cell activation, cytokine secretion, and subsequently, inflammation are attenuated in salamander wound healing and regeneration. Salamanders also show very little, if any, rejection of tissue grafts from mismatched donors, which has been a fortuitous attribute for researchers to uncover the mechanisms of regeneration using novel transplantation techniques [46,75]. Recent proteomic analysis of Xenopus limb blastemas revealed the expression of several immune-suppressing peptides, highlighting the use of immunomodulation during regeneration [76].

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Xenopus laevis has been the standard amphibian model to study the role of immunity in regeneration since metamorphosis is a developmentally programmed event that produces a profound change not only in regenerative potential, but also in immunity. Postmetamorphic Xenopus show a larger complement of immune cells compared to salamanders and larval frog stages with both MHC I and II expression [77]. In addition, the adult immune system is sufficiently different and/or alien from that of larvae such that larval antigens and grafts are often attacked or rejected by the adult immune system [78,79].

Xenopus larvae are able to regenerate arms and tails and scarlessly heal lateral wounds [7,80]. Beginning at prometamorphosis, this regenerative potential dramatically declines, such that adults heal wounds with scarring and amputated arms form a "pseudoblastema" that arrests as a spike of differentiated connective tissue [81]. The formation of these heteromorphic spikes is thought to be due to the lack of dedifferentiation by certain cell lineages, an absence of correct patterning, and an overproliferation of connective tissue cells [80]. Regeneration can be partially rescued by exogenous growth factors [22], demonstrating that a regenerative capacity is not completely lost, but merely misdirected or lacking initial key steps. Considering how the immune system instructs mesenchyme behavior via cytokine and TGF-β expression, it is tempting to speculate that the immune system creates overriding signals that push mesenchyme toward scarring rather than regeneration.

The most convincing evidence that immunomodulation influences regeneration comes from studies of tadpoles during the "refractory period", a developmental vignette where regeneration is briefly inhibited [82]. Comparative expression profiles revealed that genes related to immune function where upregulated in tadpoles within the refractory period. Immune suppression by pharmocological inhibition or morpholinos against PU.1, which is important for neutrophil development, significantly increased the tail regeneration of refractory stage tadpoles. The authors postulated that increases in regulatory T cells, which are known to exert immunosuppressant activities, restore the regenerative potential of post-refractory tadpoles. It will be important in the future to apply these results to postmetamorphic frogs or perhaps impose a loss of regeneration in salamanders or post-refractory tadpoles by heightening their immune response.

6. Discussion

Here we have discussed several features that distinguish regenerative healing in the salamander versus scar-associated healing in adult mammals. First, the timing and phenotype of the epithelium including its direct interaction with the underlying mesenchymal cells are significantly different in the two contexts. Whether the very early re-epithelialization in the salamander plays an important role or reflects an important characteristic of regenerative epithelium is an important question to be pursued. The maturation of the epithelium to a secretory epithelium, acting as a signaling center for morphogenesis is without doubt an important aspect of promoting regeneration. Here, the input of nerve, supplying factors such as FGF and nAG, has emerged as a key event associated with regeneration that is understudied in mammals. Could the supplementation of mammalian wounds with such factors promote a pro-regenerative phenotype? One main target tissue of signaling from the wound epithelium are the underlying dermis-derived cells that in salamanders take on an embryonic mesenchymal phenotype. These cells appear to produce a spectrum of extracellular matrix molecules distinct from the collagen I that is typically associated with myofibroblasts that populate a mammalian wound. How the timing, magnitude, or interpretation of common wound-associated signals such as TGFβ is modulated toward a regenerative phenotype is an important future direction to ameliorate scarring. In summary, the increased molecular understanding of regeneration in the salamander now provides a rich source of inspiration for novel modes of promoting scar-free healing in mammals.

After the submission of this manuscript, two studies have highlighted new aspects of wound healing and regeneration. One report focused on full thickness wound healing along the axolotl dorsal trunk, a region far removed from regenerating structures that were historically used such as the limb and tail [83]. They found that wound healing remains a flawless process for both larval axolotls as well as animals that have undergone induced metamorphosis, although the process of repair is not the same between larval and adult states. Another study from the same group has also uncovered an extraordinary example of regenerative wound repair in mammals [84]. As an adaptation to escape predators, the skin of the African spiny mouse (genus Acomys*) tears under very low tension, but is able to fully regenerate the entire skin including hair follicles and glands. This tissue restoration also extended to ear punch wounds, that heal by a blastema-like structure at the wound edge. Overall, this suggests that the regenerative repertoire of mammals may not be as limited as previously thought.*

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