Focus Article

Limb regeneration

András Simon[1]* and Elly M. Tanaka[2]*

Limb regeneration is observed in certain members of the animal phyla. Some animals keep this ability during their entire life while others lose it at some time during development. How do animals regenerate limbs? Is it possible to find unifying, conserved mechanisms of limb regeneration or have different species evolved distinct means of replacing a lost limb? How is limb regeneration similar or different to limb development? Studies on many organisms, including echinoderms, arthropods, and chordates have provided significant knowledge about limb regeneration. In this focus article, we concentrate on tetrapod limb regeneration as studied in three model amphibians: newts, axolotls, and frogs. We review recent progress on tissue interactions during limb regeneration, and place those findings into an evolutionary context.

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INTRODUCTION

Limb regeneration is defined as the phenomenon by which amputation of the limb anywhere along the proximal to distal axis leads to the reformation of a functional limb. This process involves the re-elaboration of the appropriate limb segments along the proximal–distal, the anterior–posterior, and dorsal–ventral axes.

This trait was first extensively documented for salamanders by Spallanzani in 1768[1] and has since fascinated generations of developmental biologists.

Differences in Limb Regeneration among Tetrapods: Conceptual and Experimental Considerations

Amphibian tetrapods display limb regeneration to differing degrees. The frog *Xenopus laevis*, an anuran amphibian (amphibians with no tails), completely regenerates limbs prior to metamorphosis up to stage 51 when the developing hindlimb is still paddle-shaped[2] (Figure 1). At these stages the limb has not yet terminated development. After stage 51, limb regeneration capacity is progressively lost. Post-metamorphic froglets only produce an outgrowth—a 'spike' lacking anterior/posterior patterning and digit elaboration[3] (Figure 1). In contrast, caudate (tailed) amphibians, commonly called salamanders, retain their regeneration abilities throughout life[4] (Figure 2). The most often used salamanders—newts (*Notophthalmus viridescens* and *Cynops pyrrogbaster*) and axolotls (*Ambystoma mexicanum*)—represent subtypes of salamanders with differing life cycles and display some distinctions in regeneration. Newts normally undergo metamorphosis, and become partially land dwelling while axolotls are a neotenic, aquatic species that fully grow limbs and achieve sexual maturity while retaining larval features like the gills. Full metamorphosis can be induced with thyroid hormone administration and the limb can generally regenerate post-thyroid hormone treatment.[5] Regeneration of body structures such as tail, brain tissue, and limb, have been extensively documented in both types of salamanders.[6] The best-known regeneration difference between newt and axolotl regeneration is regeneration of the lens, which occurs in newts but not in axolotls.[7]

The distinct properties of each animal model will provide a means to address the evolution of regeneration as the basic cellular and molecular processes underlying regeneration in the three models become delineated. For example, it is not yet known if the progenitors for limb regeneration in the larval *Xenopus* are the same or distinct from adult cell pools involved in salamander limb regeneration. Might limb regeneration in larval *Xenopus laevis* represent

*Correspondence to: elly.tanaka@crt-dresden.de; Andras.Simon@ki.se

[1]Department of Cell and Molecular Biology, Karolinska Institute, Stockholm, Sweden

[2]DFG Research Center for Regenerative Therapies Dresden, Technische Universität Dresden, Dresden, Germany

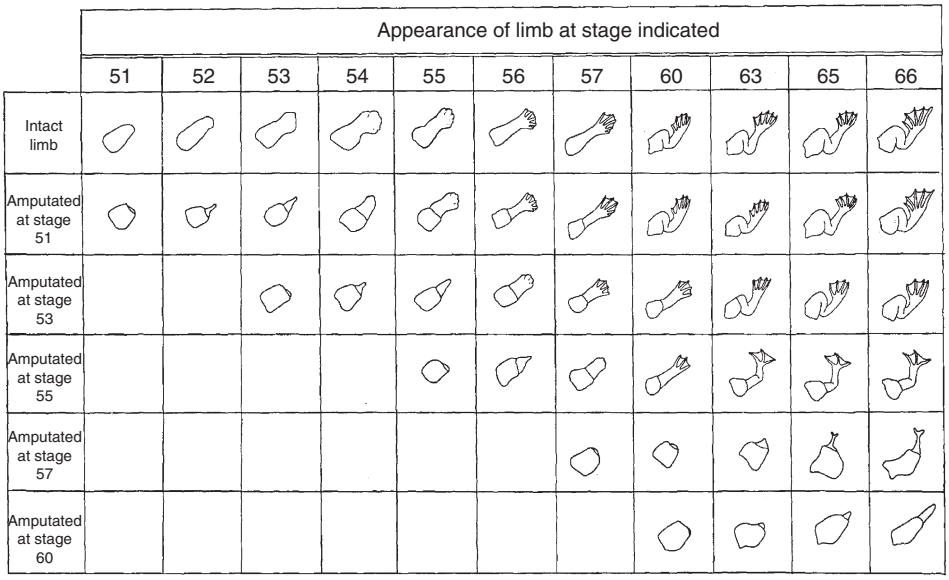

FIGURE 1 | Stage dependence of hindlimb regeneration in the frog, *Xenopus laevis*. (Reprinted with permission from Ref 3. Copyright 1962 John Wiley & Sons, Inc.)

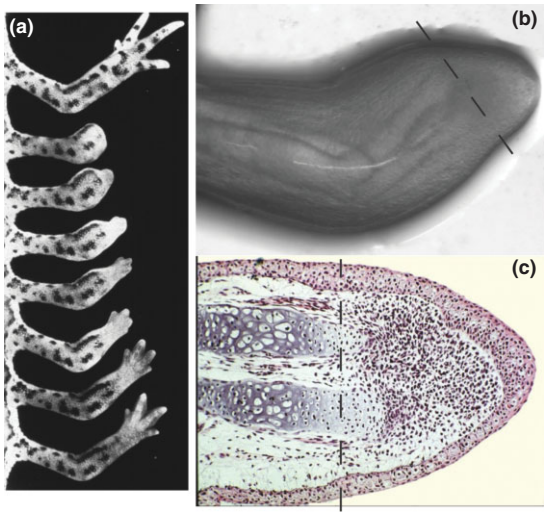

FIGURE 2 | Limb regeneration in salamanders. (a) Time course of forelimb regeneration over 10 weeks in the newt, *Notophthalmus viridescens*. (Reprinted with permission from Ref 8. Copyright 1969 Academic Press) (b) Brightfield, wholemount image of the regenerating axolotl limb at mid-bud blastema stage. Dashed line, amputation plane (Knapp and Tanaka, unpublished). (c) Hematoxylin/eosin stained longitudinal section of the regenerating limb blastema (b) (Meersburg, Knapp, and Tanaka, unpublished). Blastema cells appear homogeneous and are encased in epidermis. Dashed line, amputation plane.

'embryonic regulation' in the sense that remaining embryonic progenitor pools respond to tissue removal to make up the missing part, whereas salamander limb regeneration may rely on a different set of adult cells as the source of limb regeneration? Skeletal muscle provides an exemplar — muscle tissue harbors a stem cell population, the satellite cells that are distinguishable from the embryonic myoblasts that give rise to muscle during embryonic development.[9] In limb regeneration of frogs, newts, or axolotls, the precursor cell for muscle regeneration has not yet be definitively determined.

Another facet of limb regeneration, the dependence on nerve supply, also shows distinctions in the various regeneration contexts. Nerve dependence has been extensively characterized during limb regeneration of both newt and axolotl in all examined stages[10]—a topic that will be further discussed later in this article. Interestingly, innervation does not appear to be required during *Xenopus* tadpole limb bud regeneration, but is apparently required for blastema growth in post-metamorphic froglets.[11,12] Therefore, *Xenopus* presents a valuable opportunity to study stage dependence of regeneration mechanisms.

We have so far highlighted the differences between the frog versus salamander systems. It is additionally not yet clear if limb regeneration occurs by identical mechanisms in incompletely metamorphosed axolotl compared to the fully adult newt. The newt and axolotl do display differences in regeneration of eye tissues.[7] It is not known if this represents some underlying generic difference between larval/neotenic versus adult regeneration, or is merely a sporadic species difference specific to eye tissue regeneration.

The lifestyle and other features of the animal models also present different experimental advantages for regeneration studies outlined in Table 1. For example, live imaging of regeneration in larvae is

TABLE 1 | Comparison of Limb Regeneration Features Between Tetrapods

	Xenopus	Axolotl	Newt (*Notophthalmus viridescens* and *Cynops pyrroghaster* sp.)
Larval regeneration	Regeneration of developing limb bud	Regeneration of fully formed but still growing limb Advantage: regeneration is rapid and limb bud is optically clear	Regeneration of fully adult limb
Adult regeneration	Partial—cartilage spike forms	Yes, but animal is considered neotenic	Yes
Nerve dependence	Tadpole no Froglet spike yes	Yes	Yes
Tissue tracking/cell lineages	Not yet determined	Yes	Not yet determined
Live cell imaging	Yes, tadpoles are delicate	Yes	Probably but not yet described
Transgenesis	Yes	Yes	Yes, but breeding and egg number are limited (see text)
Cell culture	Yes, but not commonly used	Yes, but cell expansion requires some attention	Yes, multiple cell lines established
Genome sequence	Yes	No	No
ESTs/microarray	Yes	Yes	Moderate

most developed in the axolotl but has been reported also in *Xenopus* tadpoles.[13–16] The axolotl survives the conditions for live imaging more easily than *Xenopus* tadpoles that are quite delicate. Transgenesis is available for all organisms, and is most accessible in *Xenopus* and axolotl. Transgenesis in newt is possible and will be important facet of future regeneration research but the small number of eggs laid and the life cycle of the newt make the process more arduous.[17–20] Cell lines have been derived from all species, although anecdotally, it may be easier to derive continuous primary cultures from the newt compared to the axolotl (Tanaka, unpublished observations).[21]

Limb Regeneration Proceeds via Blastema Formation

The progenitor cells for limb regeneration are derived locally from the stump and form a coherent growth zone, called the blastema in which mesenchymal cells are covered by a simple epithelium (Figure 2).[22] By the mid-bud stage, the limb blastema, after transplantation to new sites, autonomously forms the limb structures corresponding to its origin.[23] Thus blastema cells, on one hand, display plasticity in that they convert from a cell type within resting adult tissue to a highly proliferative cell that can form new limb segments. On the other hand, blastema cells retain enough memory of positional and tissue origin, to appropriately elaborate only the missing portion of the limb. How are these dichotomous features of blastema cells reconciled? To understand this remarkable but apparently limited plasticity at the cellular and molecular level, we need to understand the origin of the blastema and its interplay with other tissues, such as the wound epidermis and nerves.

CELL SOURCES FOR REGENERATION

Mechanism of Blastema Cell Formation

Traditionally, blastema formation has been strongly associated with the term 'dedifferentiation'. It is, however, important to distinguish between the tissue-level definition of dedifferentiation and a cell level definition of dedifferentiation. There is no doubt that tissue-level dedifferentiation occurs, characterized by histolysis, disorganization of differentiated tissue architecture followed by the appearance of a morphologically homogenous cell population in the blastema.[24] Cellular dedifferentiation, defined as a post-mitotic, differentiated cell returning to the cell cycle and losing its differentiated character to form an undifferentiated blastema cell has been proposed and investigated.[25] At present, however, the quantitative input from dedifferentiating cells versus activation of resident stem and progenitor cells to blastema formation is not known.

Skeletal muscle is the classic system where cellular dedifferentiation versus stem cell activation has been most intensively investigated in tetrapod limb regeneration. Skeletal muscle in all vertebrates mounts

a regeneration response after injury. This response is mediated by a resident stem cell called the satellite cell that lies dormant between the muscle fiber and its surrounding basement membrane until tissue injury. After injury satellite cells proliferate, and differentiate as they fuse into existing muscle fibers.[9,26] Newt and axolotl limbs harbor a $Pax7^+$ satellite cell population and proliferative $Pax7^+$ cells are found during limb regeneration.[27–29] However, it is not resolved whether $Pax7^+$ satellite cells or other sources such as dedifferentiating muscle fibers are the major source of muscle progenitors in the blastema due to current lack of lineage tracing studies. The possibility of skeletal muscle dedifferentiation whereby the resident syncytial muscle fibers bud off mononucleate cells into the blastema has long been discussed as a potential salamander-specific process.[30,31] Several groups have reported uptake of thymidine analogs in skeletal muscle nuclei, which could be consistent with reentry by myonuclei into S-phase although such static observations do not rule out the possibility that proliferative myoblasts were labeled that had differentiated and fused into syncytial fibers during the labeling experiment.[32–34] Support for skeletal muscle dedifferentiation also comes from cell tracking studies where lineage labeled newt myotubes formed in culture were implanted into the blastema, resulting later in the appearance of mononucleate cells.[34,35] Although these data suggest skeletal muscle dedifferentiation can occur they do not resolve whether dedifferentiation of skeletal muscle makes a major contribution to the limb blastema. These transplantation-based experimental assays may reflect a generally higher degree of cellular plasticity of salamander cells compared to mammalian cells. The possibility that muscle dedifferentiation itself may be dispensable for limb regeneration and myogenic contribution is executed solely by satellite cells cannot at present be excluded. Therefore, genetic lineage tracking of fully differentiated, endogenous skeletal muscle cells versus satellite cells is required to definitively determine the relative contribution of muscle dedifferentiation versus satellite cell activation during newt and axolotl limb regeneration. Clearly, resolving the relative contribution by different cell populations is not trivial. For example, two different lineage tracing approaches prompted opposing interpretations on the origin of new cardiomyocytes during heart regeneration in zebrafish.[36,37,38] In addition, dedifferentiation can result in graded outcomes. Dedifferentiation of pigmented epithelial cells after lentectomy in the newt eye is followed by the transdifferentiation of the progeny into lens cells. While cell tracking studies in the zebrafish fin have also suggested that osteoblasts lose differentiation properties after fin amputation, their progeny redifferentiated into cartilage during regeneration and not into other cell types.[39,40] Hence, dedifferentiated cells may or may not retain their tissue identity.

Cell Lineage in the Blastema

Morphologically, cells of the blastema resemble each other, and therefore, blastema cells were often discussed as a single cell type, which implies that each individual cell had the potential to form all cells of the different limb lineages such as muscle, bone, and Schwann cells. Whether cells that entered into the blastema from different tissues were restricted to specific lineages or reverted toward a multipotent/pluripotent state was historically much discussed (for review see Ref 41). Recently, transgenic labeling of different tissue layers via embryonic grafting in axolotl established that most blastema cells retain their tissue identity and do not cross lineages during limb regeneration.[28] The data showed that despite its homogenous morphology, the blastema has a mosaic organization and is largely composed by lineage-restricted cells (Figure 3(a)). The connective tissue showed the most plasticity, contributing to skeletal elements during regeneration. This suggests that during regeneration connective tissue cells produce a blastema cell resembling a lateral plate mesoderm-derived limb progenitor found in the early developing limb bud. In contrast, a different technical approach used in the newt, involving implanting derivatives of clonally cultured muscle stem cells into the regenerating limb indicated that skeletal muscle-derived cells may contribute to multiple tissues, including cartilage[42] (Figure 3(b)). The diverging conclusions between the axolotl and newt studies may be rationalized in several ways. The underlying reason could simply be the different methodologies that were employed: transplantation of fluorescent tissues from one animal to the other in the case of the axolotl studies, and injection of *in vitro* expanded, virally labeled cells in the case of the newt studies. Alternatively, despite their close phylogenetic position, newts and axolotls may simply use different mechanisms to generate a blastema. Interestingly, lineage shifting occurs in newts during lens regeneration, which depends on the dedifferentiation and subsequent transdifferentiation of pigmented epithelial cells of the dorsal iris into lens. In contrast to newts, axolotls cannot regenerate the lens.[7] It remains to be seen whether the lack of lens regeneration in axolotl is due to axolotl cells being generally more refractory to lineage shifting in multiple regeneration contexts or whether this deficiency stems from a highly specific difference of a yet unknown parameter in the eye.

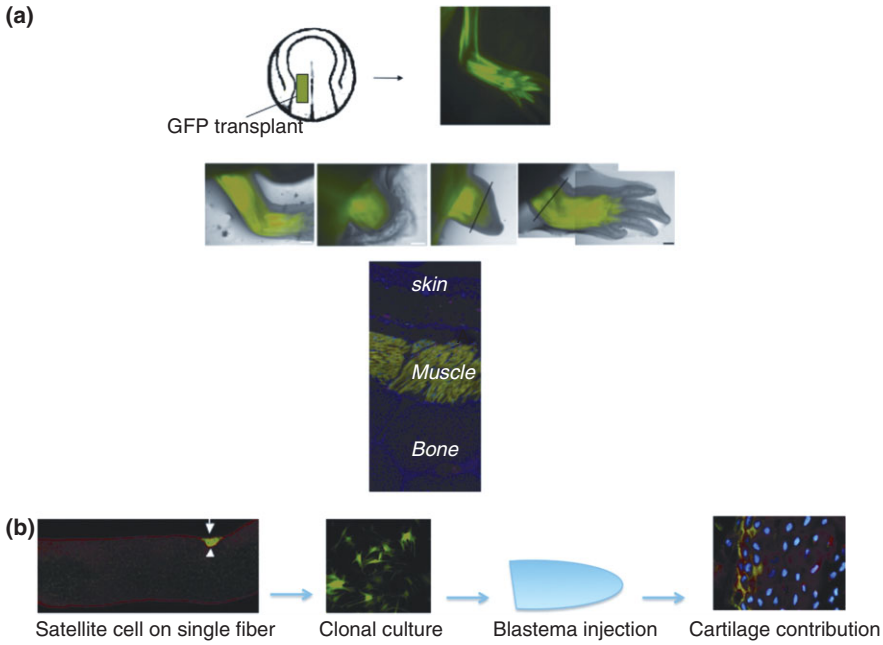

FIGURE 3 | Diverging conclusions on muscle-derived cell fate during axolotl and newt limb regeneration. (a) Cell tracking of fluorescently tagged muscle tissue results in muscle-specific labeling in the regenerated axolotl limb. Top row, muscle tissue was labeled by transplanting presomitic mesoderm from GFP-transgenic neurla stage embryos to normal hosts. Middle row, time course of limb regeneration in labeled animals. Bottom row, histological section of regenerated limb shows GFP is restricted to muscle tissue and not found in cartilage or epidermis. (Reprinted with permission from Ref 28. Copyright 2009 Macmillan Publishiing) (b) Cell tracking of virally infected, clonal muscle stem cell cultures during newt limb regeneration. Clonal cell cultures of muscle satellite cells were derived from *Notophthalmus viridescens*. Cells were infected with adenovirus, and implanted into the regenerating limb blastema. After regeneration, labeled cells were observed in cartilage. First panel: green, Pax7 (arrowheads); red, collagen IV (arrow). Fourth panel: green, GFP; red, collagen II. (Reprinted with permission from Ref 42. Copyright 2010 FASEB)

Beyond amphibians, the mouse digit has been shown to regenerate at all stages of the life cycle when cut within the most distal segment.[43–45] The fate of the main tissue layers, ectoderm, bone, and ligaments were followed using cre/loxP-based genetic fate mapping.[46,47] Again, lineage crossing was not observed, as ectodermal cells gave rise to ectoderm but not mesoderm, bone-derived cells gave rise exclusively to bone, and ligament cells produced more ligament. In these studies, it was not resolved whether soft connective tissue can give rise to bone as is found in the axolotl.

CELL–CELL INTERACTIONS AND MOLECULAR SIGNALING IN THE BLASTEMA

Blastema and Wound Epidermis

The first step of limb regeneration is the formation of the wound epidermis, which forms by rapid migration of keratinocytes over the cut limb. Subsequently, cell proliferation leads to the multilayered wound epidermis.[48] The wound epidermis does not produce the typical basement membrane between skin and underlying cells and this direct contact between wound epidermis and the underlying cells is thought to serve an essential signaling function during regeneration. The wound epidermis is absolutely required for limb regeneration, and determines the orientation of the outgrowing limb.[49] It also expresses many of the growth factors, such as FGF and WNT family members, known from embryological studies to be essential for limb outgrowth (for review see, Ref 50). The molecular role of several such signaling pathways has been tested in *Xenopus* limb regeneration by production of transgenic animals overexpressing molecules that block a given signaling pathway under control of the heat-shock promoter. In *Xenopus* and axolotl, inhibition of the canonical WNT pathway via overexpression of DKK1 early after limb bud amputation blocked tadpole limb regeneration.[51] Molecularly, the onset of FGF8 expression was inhibited.[52] Interestingly, froglet spike regeneration was refractory, unless the limbs were deprived of some innervation.[53] In addition, blocking of BMP via overexpression of noggin also blocked regeneration, but this pathway seemed to be required throughout the process of regeneration.[54] A number of other signaling pathways have been investigated during salamander and

Xenopus limb regeneration, with many factors having potent inhibitory effects on regeneration.[55–57]

How exactly the epidermal/mesenchymal interaction is established is unclear but members of the matrix metalloprotease (MMP) family could play important functions in histolysis and blastema formation.[58,59] Increased expression level of MMPs and their regulators was observed in the wound epidermis, which correlated with blastema formation, when regeneration competent and regeneration deficient amphibian limbs were compared.[60] Accordingly, inhibition of MMPs results in arrested limb regeneration.

Blastema, Peripheral Nerves, and Wound Epidermis

In salamanders and post-metamorphic frogs, limb regeneration via a blastema depends on signals from peripheral nerves. Several loss of function experiments demonstrated that if the nerves are absent, the blastema fails to develop into a new limb. Studies characterizing the nerve supply indicate that the nerve effect is independent of nerve type, and only depends on the total quantity of nerves growing into the blastema.[61] Furthermore, this signaling may not depend on electrical conduction.[62,63] In addition, gain of function studies by the deviation of nerves to ectopic sites demonstrated the ability of nerves to induce a blastema-like structure after wounding.[64]

A number of factors have been proposed to represent nerve-derived signals that promote blastema growth. Most recently, one set of studies in axolotls has focused on the role of KGF (FGF7). KGF is expressed in the dorsal root ganglia and presumably transported along the axons to induce *Sp9* expression in the wound epidermis.[65] Other studies in the newt identified a function for the secreted molecule named anterior gradient (AG) protein. When the newt limb is denervated, limb regeneration can be rescued by the expression of AG.[66] During limb development, AG is expressed in the embryonic limb epidermis, until ingrowth of nerves causes its down regulation as the limb matures.[67] During normal limb regeneration, Schwann cells in the injured nerve sheaths are first to express AG. Later gland cells associated with the wound epidermis start to express AG in response to the AG expression initiated by cut nerves.[66,68] AG binds a cell surface protein, Prod1, a molecule that interestingly has the capacity to proximalize cells during regeneration.[13] It is unclear whether binding of AG to Prod1 is indeed involved in the regulation of cellular positioning in the limb but *in vitro* data showed that an antibody against Prod1 inhibits AG stimulation of blastema cell proliferation.[66] Given that Prod1 is expressed as a gradient along the P/D axis,[69] it is possible that stimulation of cell division by AG through Prod1 is related to intercalation, which is a process that can be described by cells filling the missing gap between their assigned discontinuous positional values after amputation.[23]

Currently, it is not known if the nerve-supplied FGFs seen in axolotl, and the involvement of AG/Prod1 system in newt, are both universal features of nerve dependence of limb regeneration that operate in both species. So far complete rescue of full limb regeneration after denervation has only been demonstrated by AG overexpression in the newt. The axolotl and newt Prod1, show distinct structural and chemical properties although both molecules are competent to activate the same downstream signaling events.[70] Loss of function approaches for AG and Prod1 in the two salamander types could further clarify this issue.

Competence for Patterning

The impressive aspect of bonafide limb regeneration is the re-elaboration of a limb with its original pattern (for detailed review, see Ref 71). Many signaling pathways, including hedgehog, WNT, and FGF that are essential for development of a fully patterned limb, are also utilized during limb regeneration (see above and Ref 72). Interestingly, several developmental factors, such as sonic hedgehog are not present after amputation in *Xenopus* froglets. This lack of reactivation could provide a rationale for the hypomorphic regeneration in froglets. The stage and species-specific differences in re-expression of sonic hedgehog during regeneration inversely correlated with the degree of DNA methylation at a known limb enhancer, suggesting that epigenetic factors may influence the competence for forming a patterned limb.[73] The intrinsic nature of these mechanisms was emphasized by cross-stage transplantation experiments between *Xenopus* tadpoles and froglets, which showed that the froglet environment permits the tadpole blastema to form a complete limb. Conversely, transplantation of froglet cells on amputated tadpole limbs results in defective regeneration.[74]

It is clear that in regenerative tetrapods, the mature limb retains some positional identity both along the proximodistal and the anterior–posterior axes. For example, cartilage cells from amputation plane are normally restricted to only form limb elements more distal to their origin. The molecular basis for this restriction is largely not known. The outgrowth of a patterned limb depends on the interaction between cells with anterior and posterior limb identities. Grafts that generate a limb with double

anterior halves (destroying the posterior tissue) only regenerate a spike.[75] Furthermore, an ectopic limb can be formed on the lateral side of a limb when anterior limb tissue and posterior limb tissue are juxtaposed and the site is innervated.[64] An important question is whether maintenance of transcription factor codes that determine these identities during limb development underlies the positional memory system for limb regeneration in adult animals, and whether the same codes are used during larval and adult regeneration in species where both can occur. Interestingly, in the tail, the axolotl spinal cord maintains the homeodomain transcription factor code for dorsal/ventral progenitor cell identities that exists during mouse and chicken embryonic development but disappears during caudal neural tube development.[15] In the newt limb, the cell surface molecule, Prod1, that can proximalize limb blastema cells during regeneration is expressed as a shallow gradient in the adult limb, and therefore has been proposed to be part of the positional memory system.[13,69,76] The role of Prod1 during the development of the newt or other tetrapod limbs is currently unknown.

EVOLUTIONARY CONSIDERATIONS

Similar to the discussion on the origin and the composition of the blastema, the debate on the origin of limb regeneration capacity as such has recently gained new momentum. Regeneration could be regarded as a fundamental property of biological systems, i.e., the inherent architecture of regulatory circuits and cell–cell interactions involved in development/tissue formation predispose the system to the ability to regenerate. Reaccess to those developmental circuits from the adult state may have been blocked in some species but not others.[77] A radically different view is that regeneration is an adaptive trait and a result of natural selection. The former possibility was by far the most preferred one by a majority of regeneration biologists during the last decades. However, the recent finding that limb regeneration in newts involves a molecular program, in which one of the components, Prod1 is a salamander-specific variant of the Ly6 protein family brings up the possibility that local evolution of the genome is linked to salamanders' unique regeneration abilities.[78] The two opposing views are not necessarily irreconcilable. A plausible hypothesis is that a certain degree of cellular flexibility allowing appropriate regenerative responses after amputation is an ancient trait, possibly originating from asexual reproduction.[79,80] How different animals solved the particular 'task' of limb regeneration could, on the other hand, vary and it may have developed by specific adaptation mechanisms, which are superimposed on the general cellular flexibility. This composite view could rationalize the apparent variations among organ systems and animal species and also explain why most species have lost their regeneration capacity. Thus future research could, on one hand, target epigenetic mechanisms that allow cells to retain or regain access to *de novo* tissue formation, complemented by experiments dealing with the interactions between the key elements of the regulatory pathways.

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