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RESEARCH ARTICLE

Review: Limb regeneration in humans: Dream or reality?

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ABSTRACT

Appendage regeneration occurs by a sequence of events resembling those that take place during development in the embryo. This requires embryonic conditions such as hydration and hyaluronate content where Wnt and other signaling pathways, together with non- coding RNAs, can be re-expressed. These conditions among vertebrates are fully met only in amputated limbs of amphibians, likely because they are neotenic and maintain larval characteristics, including immaturity of their immune system and permanence of numerous stem cells. Although some key genes orchestrating limb regeneration are also present in amniotes, including humans, these genes are not expressed after injury. In amniotes a key problem for regeneration derives from the efficient immune system, largely deficient in anamniotes. As a consequence, wounds and appendage loss tend to scar instead of regenerating. Efforts of regenerative medicine in the attempt to induce the regrowth of limbs in humans must produce outgrowths with high hydration and hyaluronate content in order to create the immune-suppressed conditions similar to those present during development. The induced blastema must be manipulated for long periods of time in order to maintain the same regions present during limb development, an apical epidermal ridge and a polarizing region that forms gradients of expression of Wnt, Shh, FGF, BMP and Hox-genes. Pharmacological treatments to direct the regenerating limb into normal growth without risk of inducing abnormal or tumorigenic growth must be monitored during the course of the regeneration process — a medical treatment lasting years to fully regain the size of the lost appendage.

1. Amniotes lack organ regeneration plasticity

Regeneration is a fundamental property of living matter. It occurs from biochemical molecules to cell organelles, in cells to the tissue level in most multicellular organisms, but only in some animal groups is the regeneration of functional organs possible (Goss, 1969; Reichman, 1984; Brokes and Kumar, 2008; Bely and Nyberg, 2009). Here we deal with a special case of organ regeneration in vertebrates, in an attempt to understand why limb regeneration only occurs in few vertebrates. It is also considered what might be expected from future attempts to induce limb regeneration in vertebrates which do not regenerate limbs — in particular humans. A basic biological principle to regenerate an organ, including limbs, is that the function of the lost organ is important but its loss is, however, compatible with the survival of the organism (Goss, 1969; Reichman, 1984). Since humans can survive after digit or limb loss, we have reasonable hopes that using biotechnology (regenerative medicine treatments) or bioengineering (artificial prosthesis) we can induce the regeneration of new limbs or limb-equivalent after limb loss.

Among vertebrates, the remarkable ability to regenerate diverse inner and external organs is present only in numerous urodele amphibians and tadpoles of frogs. This capability is unmatched in any other vertebrates (Mescher, 1996; Stocum, 2004; Carlson, 2007; Tanaka, 2016). The amazing regenerative power of limbs in urodele amphibians has made these lower vertebrates the most important model for analyzing the regenerative processes, with the assumption that what we learn from them can eventually be applied to make human limb regeneration possible. However, the study of biology of modern amphibians (lissamphibians) indicates that these are special types of adults, very different from most adults of amniotes. Therefore, we should carefully evaluate the "adult status" for extant amphibians (Vitt and Caldwell, 2009; Alibardi, 2010). Aquatic and more terrestrial "adult" urodeles are vertebrates still resembling their tadpole condition in various degrees. They have followed along a line of evolution that has generated neotenic or pedogenic forms capable to reproduce — a property that qualifies them as adults (Vitt and Caldwell, 2009; Grigoryan, 2016). Therefore, what we call "adults" in amphibians

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and some fish is a different "adult condition" from that of amniotes (Alibardi, 2010). In the latter, the adult is very different from the embryo, including the fact that adult tissues are over 20% drier and contain much less hyaluronate than their embryos. Besides, anamniotes in general, fish and amphibians, and also reptiles among amniotes, grow for most of their life, conserving active growing centers and stem cell niches throughout most of their lifetime (Vitt and Caldwell, 2009; McCusker et al., 2015; Grigoryan, 2016). Therefore, amphibians such as the axolotl or various aquatic newts are a larval type of adults, which conserve numerous embryonic-larval traits. In addition to mature gonads, they include aqueous tissues rich in hyaluronate, the glycosaminoglycan initially formed during development (Toole and Goss, 1971; Toole, 1997). Furthermore, their limbs have a relatively small size and an aqueous consistency compared to the large sizes and more solid skin and tissue present in limbs of most amniotes (Alibardi, 2017a). As we will discuss later, the limb size is important in relation to its capability and timing of regeneration, especially under dry terrestrial conditions, as is indicated from the absence of limb regeneration in large and more terrestrial-adapted amphibians (Scadding, 1977, 1981).

The complexity of the body, organization of the nervous circuits, and the immune system are lower in efficiency in amphibians than in amniotes, starting with reptiles (Zimmerman et al., 2010). The physiological mechanisms that control the inner milieu are so complex and refined in endotherm amniotes (birds and mammals) that the adult condition, once reached, cannot tolerate extensive injuries. In other words, amniotes have almost entirely lost their developmental plasticity. In particular, a major injury can only be repaired at the wound margin, in an attempt to close the wound as rapidly as possible (Ferguson and O'Kane, 2004). The evolution of the body of amniotes, especially endotherm as birds and mammals, has perfected their bodies at the expense of plasticity: they are efficient biological machines made to avoid injury. As part of the evolution of body complexity in multicellular organisms, the immune system is a basic indicator of stress or morphological and physiological alterations of the inner milieu. It has evolved different capabilities to respond to stress during evolution, in particular avoidance of non-self antigens (Danilova, 2006).

2. Organ regeneration in vertebrates has to deal with the immune system

While tissue regeneration is limited in amniotes, in both mammals and sauropsids (reptiles and birds), the regeneration of inner organs, (with some exceptions like the liver) and of body appendages are completely blocked in these vertebrates (Tsonis, 2002; Han et al., 2005; Carlson, 2007). In contrast, in tissues and some organs of numerous fish and amphibians (anamniotes), regeneration is frequent and in some urodele amphibians also body appendages, such as the tail and limbs, can regenerate anatomically and functionally (Tsonis, 1996; Stocum, 2004; McCusker et al., 2015).

Morphological, biochemical and cell biology studies, recently integrated by transcriptome analysis, have identified the cell processes and the key genes implicated in appendage regeneration in urodeles (Wu et al., 2013; Kochegarov et al., 2015; Bryant et al., 2017) and lizards (Alibardi, 2014a, 2014b, 2016; Hutchins et al., 2014, 2016; Liu et al., 2015; Vitulo et al., 2017a, 2017b). The latter study on genes activated in the blastema of the lizard tail versus those activated in the scarring blastema of the limb of the same lizard has shown that organ regeneration requires the re-activation of an embryonic program dominated by the Wnt pathway, and the activation of several small non-coding RNAs (Vitulo et al., 2017a; Alibardi, 2017b). This program determines the formation of a regenerative blastema, an outgrowth of proliferating cells with embryonic characteristics. This is an obligatory passage needed to regenerate a limb or a tail (Mescher, 1996; Nye et al., 2003; Alibardi, 2010, 2014a; McCusker et al., 2015; Tanaka, 2016). In the best regenerators among vertebrates, the newts, the amputated limb regenerates following a similar process present during the original development of this organ. Particularly the formation of an embryonic-like mass of mesenchymal and epidermal cells occurs. The regenerative blastema is similar to the embryonic limb bud. However, while the limb bud is integrated into the remaining organs of the developing embryo, the regenerative blastema is a new and growing embryonic organ connected to an adult body that is vascularized by blood and circulating immune cells.

Though it has been known for many years that the efficiency of the immune system of anamniotes and amniotes differs, the correlation between immune systems and the loss of organ regeneration in amniotes has only become clear in recent years (Harty et al., 2003; Goodwin and Brookes, 2006; Mescher and Neff, 2006; Han et al., 2005; King et al., 2012; Goodwin and Rosenthal, 2014; Mescher et al., 2016; Alibardi, 2017b, 2017e). This hypothesis states that a possible pitfall of the adaptive immune system, related to regeneration, is the timing of acquisition of the full immune tolerance towards the organism. This process, which requires time and maturation, is only fully reached by the end of development, or even after birth (Simon et al., 2015). We hypothesize that numerous antigens formed during the early stages of development, have disappeared at later stages as part of the differentiation process. These resemble non-self antigens for mature immune cells, since the latter have never encountered and learned to tolerate these embryonic antigens. Therefore, the re-expression of these antigens following a wound or appendage loss in adult vertebrates is an obligatory passage in order to form the embryonic-like cells of the regenerative blastema. Thus, these embryonic antigens are exposed to an immune system incapable of recognizing them as self. The presence of embryonic cells and their antigens in blastema (embryonic-like) cells, likewise in cancer cells (also presenting embryonic characteristics), is detected as non-self cells from the definitive immune cells of the adult body. As a result, these embryonic-like cells are attacked and rejected.

Based on the above consideration, to permit the permanence of a regenerative blastema, the immune system must be kept under control, either by its low efficiency (anamniotes) or by evolving a process of temporary immune-suppression or immune-evasion (amniotes), or by combining these two processes. The best example of the acquisition of immune-competence in conjunction with the loss of regenerative capability is observed during the transition from a larval stage in amphibian anurans (frogs), adapted to a life in water, to the adult form, adapted to terrestrial life. This is accomplished through the process of metamorphosis (Harty et al., 2003; Mescher and Neff, 2006; Han et al., 2005; King et al., 2012; Mescher et al., 2016; Alibardi, 2017e). In fact, the water to land transition has potentiated the immune system, as terrestrial conditions require a more effective immune system. It is therefore important to realize that what we learn from amphibians cannot be directly transfer ed into organ regeneration for mammals, since a very different biology occurs between anamniotes and amniotes. In particular, the presence of a more efficient immune system occurs in the latter. In order to remain connected to the rest of the body and avoiding rejection by the immune system, the cells of the regenerating blastema of amphibians and lizards likely have immune-evasive capability, like it similarly occurs in some tumor cells (Gabrilovich et al., 2012). Recent studies have begun to address the problem of immuno-evasion in lizard and amphibian blastemas.