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Review Paper

AXOLOTL - A SUPERMODEL FOR TISSUE REGENERATION

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Summary: The process of regeneration - the regrowth of tissue or body part, have been focus of human attention for many centuries. Among vertebrates, the ability to regenerate lost tissue or organs is best developed in urodele amphibians, such as axolotl (Ambystoma mexicanum), which can replace lost tissue throughout adulthood. Axolotl is neotenic amphibian that can regenerate wide range of organs including limbs, tails, gills, heart, spinal cord, jaws. Among the organs they can regenerate, the limb has been most widely studied so that it provides the basis of knowledge about the mechanisms regulating tissue regeneration. Limb regeneration progresses through a series of steps: wound healing, dedifferentiation and re-differentiation. A number of genes involved in the activation of regeneration have been already identified and cloned, but to this moment, exact molecular mechanisms of regeneration process are not yet known. With the new techniques of functional genomics that will help us to identify candidate genes and testing their functions, there are good prospects to discover the whole mystery of regeneration and achieve the ultimate goal - regeneration in mammals.

Key words: regeneration-genetics; cell differentiation; gene expression regulation, developmental; *Ambystoma mexicanum*

Introduction

Regeneration is the process of restoring a missing part of a tissue or an organ. Complete regeneration is present in most of the lower species but among vertebrates, only urodele amphibians are capable of complete regeneration of number of different tissues such as limb, tail and brain even as adults (1, 2). Among these organs, the limb has been most widely studied and therefore provides the basis of our knowledge about the mechanisms regulating tissue regeneration (3).

Limb regeneration is a process that goes through a series of steps. It starts with wound healing as reserved stem cells of epidermis migrate to cover the wound surface within few hours. Cells that lie underneath start to dedifferentiate and migrate under the WE (wound epidermis) to become mesenchymal-like stem cells that form blastema. Blastema then gradually begins to proliferate. Cells re-differentiate (by the process similar to the processes during embryonic development) to replace a missing limb (3-5).

A number of important conclusions came from studies of the urodele limb regeneration. We now know that complete regeneration is indeed possible, and number of key regulators has been identified. But even though the regeneration of salamander limb was first reported in eighteen century by Spallazani (6), the exact physiological and molecular mechanisms are still not clear. Resent advances in a field of functional genomics and cell biology are raising new hopes. With the high throughput techniques for screening of genes such as DNA microarrays, techniques for functional analysis such as transgenic salamanders, more efficient use of in vitro cell cultures and possibility of genetic manipulation (5, 7, 8), there is a better opportunity now to identify cellular and molecular events responsible for regeneration that will hopefully in the future give us opportunity to induce regeneration in other vertebrates and will help us to achieve long-lasting goal of human regeneration (9).

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Mexican axolotl *(Ambstoma mexicanum)*

Mexican axolotl (Ambystoma mexicanum) is a urodele amphibian (Figure 1). They are neotenic, so they remain in its aquatic larval form even as sexually mature adults and are not undergoing metamorphosis into a terrestrial form. Metamorphosis is very rare and occurs as a response to an extreme habitat or as a result of iodine or hormone thyroxine injections. Most attempts at inducing metamorphosis lead to death, although some were successful but animal lifespan was shorten drastically. Axolotls live usually more than 12 years. A fully grown axolotl ranges in length from 15-45 cm. Their natural habitat is Lake Xochimilco in Mexico, but as laboratory animals, they are bred all over the world.

They have distinctive "fern-like" gills that are not covered, usually three stalks on each side of the head; the colour of the fern-like part varies although the usual colour is red. Axolotls also breathe through the skin and possess lungs. They have tiny teeth that are hardly noticeable and are used to grip food rather than to tear and chew it. They are carnivores consuming small prey such as worms, insects, and small fish. Axolotls live in salty water (the optimal mixture is called Hoeltfreter's solution and is a mixture of different salts) with optimum temperature around 18°C. Temperature above 23°C can lead to stress and increased appetite (10).

They are capable to regenerate wide range of organs including limbs, tails, gills, heart, spinal cord and jaws throughout their whole life (11). The regeneration is very rapid (usually a couple of weeks) and the animals are easy to handle, so they provide excellent model organisms for studying mechanisms of regeneration in vertebrates. Among organs that can regenerate, limb has been most extensively studied.

Limb regeneration

Regeneration of axolotl limbs occurs by the process of dedifferentiation. It is completed within a month. The timing of limb regeneration depends on axolotl size and age and it is very consistent. Usually older axolotls regenerate longer. There is no limit in the number of amputation of the same limb; each time the limb will regenerate completely. Regeneration progresses through a series of steps. Minimum requirements for limb regeneration are: a skin wound, adequate innervations and positional diverse blastema. This requirements separate the process into three distinct phases (Figure 2) (1, 2, 4). The first phase is wound healing, phase II is re-differentiation and phase III is dedifferentiation. The most unique is a second phase of re-differentiation while the dedifferentiation is a recapitulation of developmental phase with some differences (4). Summary of regeneration events is presented in figure 3.

Phase I: Wound healing

Within the first hour the epithelial cells from basal lamina begin to migrate to close the wound. Wound closure is incredibly fast; in young axolotls it is closed in 4 hours. Compared to mammals where it takes days, the speed is extraordinary. Outcomes of rapid wound healing include minimizing damage, infection and inflammation (12, 13). The wound is healed without a scar. Wound epidermis (WE) is formed which is crucial for the onset of regeneration and is essential for triggering expression of particular genes. If it is removed, the regeneration does not proceed. Covering the amputation wound surface with mature skin completely inhibits the regeneration response. The WE is crucial for epithelial/mesenchymal interaction (4, 8, 11). Extracellular matrix restructuring is initiated with MMP (matrix metalloproteinases) MMP-2, MMP-9 and MMP-13. Msx-2 gene is also expressed prior to wound closure, so it does not depend on WE as does expression of all other genes (11, 14). Recently, overexpression of Msx-1 was found to be important for inhibition of differentiation, suggesting that this gene may play a role in maintaining cells in a undifferentiated state (13, 15, 16), and it might be expressed at the same time as Msx-2. WE than begins to thicken and cells dedifferentiate to form AEC (apical ectodermal epithelium) that promotes limb regeneration. This stage is not dependent on nerves (4, 17). It is similar to wound healing in other vertebrates with some crucial exceptions; the inflammation response in axolotl is very mild and results in low cytokine synthesis whereas in mammals, there is apparently high concentration of interleukins 1 and 6 (IL-2 and IL-6) and some other cytokines that activate scaring and consequentially inhibit regeneration. Another difference is the presence of proteoglycan tenascin-C, which apparently helps to maintain differentiated state of cells (13).

Phase II: Dedifferentiation

This phase is unique to the process of regeneration. The phase II in regenerating limb is the period of limb regeneration when cells in the mature tissue start to dedifferentiate, losing their specialized functions and become migratory. Some days after amputation (day 2-3) cells begin to migrate under the WE and accumulate at the distal tip of the stump. At the same time as they dedifferentiate, they lose their specific function and than progress to proliferation. The final result is the genesis of population of undifferentiated, proliferated blastema cells (2, 4, 17) . Although overlying epidermis does not contribute to the blastema, it does influence the location at which blastema cells accumulate. Blastema arise from cells located 1-2 mm of the amputation plane. There is some evidence that all tissue participates in formation of blastema, several experiments demonstrated that the majority of cells that are essential for growth control and pattern formation in blastema are fibroblast of the connective tissue from amputated stump (2). The first divergence of phase II regeneration to normal skin regeneration is expression of Hoxa-9 and Hoxa-13 genes in the distal part of the stump 12-24h after amputation. Hox genes in general regulate morphogenetic events during regeneration. HoxA gene is responsible for specification of proximal-distal limb axis and may be the first transitional event on the limb regeneration pathway (18). Also HoxD (HoxD8, HoxD10) genes are expressed that play a role in re-expression of positional memory. Specification of the distal-most region of the pattern (autopod) is a consequence of co-expression of both 3' and 5' members of HoxA and HoxD, which is in contrast to the events during limb development (19). The early establishment of the distal tip of the limb ensures that the regenerate will always be a exact replacement of the amputated portion of the limb (4, 11, 17).

It is not yet clear, which are the factors responsible for activation of dedifferentiation and regeneration in generally but some of them definitely are connected to inervation. Phase II is dependent on nerves (4). If nerve supply is cut off, regeneration does not proceed. The nerves exert their effect through FGF-mediated pathway (11). Genes like fgf-1, fgf-2 and their receptors (fgfr-1, fgfr-2) are expressed in AEC. There is also a gene that is known to involve altered mechanisms of regulation in response to enervation. Dlx-3 (distal less) expression starts at early bud blastema and peaks at the transition from nerve dependency to independency of regeneration. Dlx-3 expression is up-regulated in blastemal stages of regeneration and is dependent on signals from nerves. Denervation inhibits Dlx-3 expression but addition of FGF sustains Dlx-3 expression and rescues regeneration (11, 20).

Phase III: Redevelopment

At the beginning of phase III, the large mass of undifferentiated cells (blastema) begins to differentiate and behave like a developing limb (4). Similar genes are expressed as in developing limb, but with some differences. Among expressed genes is sonic hedgehog (shh), which is expressed transiently in a small group of posterior cells and plays a crucial role in the establishment of the anterior-posterior limb pattern during both limb development and regeneration (21). Fgf-10 expressed in blastemal cells stimulate the expression of shh (22). In phase III genes that are characteristic of late stage regenerating limbs are expressed (HoxD11, Dlx-3). Expression of HoxC10 is specific for late phase blastema. Genes within HoxC complex are involved in specification of positional identity along the rostro-caudal axis. HoxC-10 is expressed in regenerating forelimbs and hind limbs, while in the development; it is expressed only in hind limbs, what indicates the presence of regeneration-specific signals (23). Cell differentiation that occurs in this phase is nerve-independent, but continued growth depends on nerves (17). In less than 6 weeks morphologically perfect limb is regenerated and from that moment on it only grows to reach its normal size.

Functional analysis

For many years studies concerning regeneration in urodels could not progress as wished due to the lack of tools for functional analysis of gene expression. In recent years, however, different approaches have been developed to achieve this goal. By far the most important approach in functional genomic analyses is knock-out and transgenic technology developed in mice. However, these technologies are very difficult to apply to axolotl models because of axolotl slow sexual maturity and lack of knowledge about their genome. Very recently, a big progress have been made in the development of transgenic salamander (7, 8). Transgenic axolotls were also generated by using I-Sce1 meganuclease method which is used to produce transgenic frogs and

fishes (24). Both methods resulted in the expression of the reporter green fluorescent protein in all tissues observed. In addition, tissue-specific promoters have already been shown to work in salamanders. By using this technology, one can study overexpression of cDNAs to increase protein expression or short hairpin RNAs to inhibit gene expression of a specific gene (8). There are other techniques for performing functional analysis such as overexpression of a gene introduced to a cell by electoporation, or shutting down a gene by antisense morpholinos (25). Furthermore, vaccine viruses have been used to introduce shh gene, that causes polydactyly (26). Different way to perform functional analyses is the pharmacological approach. Cyclopamine is a natural compound isolated from cabbage and is a potential inhibitor of hedgehog signalling. Treatment of axolotls with cyclopamine resulted in loss of a regeneration (27). All these tools will help us to answer questions pertaining to the functional roles of specific genes in different phases of regeneration.

Regeneration in mammals

Regeneration capabilities are very good in mammal embryos, but regeneration of body parts in adult mammals is much more limited. There is, however, continuous renewal of tissue in mammals as a part of the tissue homeostasis such as gametogenesis, skin-renewal, haematopoiesis, and in some other processes. Such homeostasis is achieved mostly by stimulation of stem cells and for this reason, most studies of regeneration focus on the role of stem cells (28). Some tissues in digestive system also have great potential for renewal. Liver and intestine are the most prominent organs. It is known that after partial hepatectomy (as much as one third of a liver), the remaining lobes of the liver enlarge to replace the missing tissue by the process of proliferation without any apparent dedifferentiaton or transdifferantiation (29).

There is also expanding evidence about olfactory-neuron regeneration and regeneration of new neurons in adult brain. Many attempts have been made to induce neuronal regeneration by applying FGF and integrins, but complete regeneration of part of CNS has yet to be achieved (30).

Many researchers have shown the plasticity and capability of adult and embryonic stem cells to repopulate tissues such as brain, heart, retina but so far no successful restoration of missing tissue or an organ has been documented (31). Apart from the focus on stem cells, the researchers should perhaps also focus their attentions more to one example of complete regeneration of tissue seen in mammals - regeneration of a digit tip in mice and in humans. The term “fingertip” refers to a part of the digit extending distally to interphalangeal joint (15). This phenomenon was first described for children but latter shown to extend to adults. If a wound after amputation is simply covered with sterile dressing and allowed to heal, it will result in perfect restoration of a portion of a finger after 5 weeks. While in mice, the process of digit tip regeneration goes through the formation of blastema, fibroblastic cells appear to be involved in the regeneration in humans. In mice, the capacity for digit-tip regeneration has been correlated to BMP (bone morphogenic factor) and Msx-1 expression, perhaps to maintain the dedifferentiation state of the blastema (28).

Prospects

It is a great interest of regeneration researchers to understand the difference between species that can regenerate lost tissue and those that can not. In case of humans and urodels, this is the matter of current interest in regenerative medicine. With the new techniques for identification of candidate genes and testing their functions, there are good prospects for enhancing regeneration in mammals, what is the ultimate goal of these studies. Axolotl model gave us a proof that regeneration is indeed possible. The fact that human embryos can regenerate more extensively and that some of those regeneration capabilities extend into an adult life, shows us that mechanisms are presents within us, we just have to find out how to activate and control them.

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AKSOLOTL – SUPERZVEZDA PRI REGENERACIJI TKIVA

M. Goršič

Povzetek: Razumevanje procesa regeneracije-ponovne rasti tkiva ali dela telesa ter njegova spodbuditev pri organizmih, ki tega sami niso sposobni, že več stoletij predstavlja izziv raziskovalcem. Med vretenčarji je sposobnost regeneracije tkiv in organov najbolje razvita pri repatih dvoživkah, kamor spada tudi aksolotl (*Ambystoma mexicanum*), ki regenerira tkiva skozi vse življenje. Aksolotl je neotenična dvoživka, sposobna regeneracije različnih organov, kot so okončine, rep, škrge, srce, hrbtenjača in čeljust. Med temi organi je najbolj proučena okončina, ki predstavlja osnovo znanj o regulatornih mehanizmih regeneracije. Proces regeneracije okončine poteka prek različnih stopenj: celjenje rane, dediferenciacija in rediferenciacija celic. Nakateri geni, vpleteni v aktivacijo regeneracije, so že znani in klonirani, natančni molekularni mehanizmi pa še niso jasni. S pomočjo novih tehnik funkcijske genomike in proteomike, ki nam bodo pomagale identificirati kandidatne gene in preveriti njihovo funkcijo, se odpirajo nove možnosti spoznavanja skrivnosti regeneracije in končno doseči najvišji cilj - regeneracijo pri sesalcih.

Ključne besede: regeneracija-genetika; celična diferenciacija; gensko izražanje, regulacija razvojna; *Ambystoma mexicanum*