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Limb Regeneration in Axolotl and Salamanders

Introduction

Axolotl's are an indigenous salamander species found in the lakes and canals of Xochimilco, Mexico. Though they can be found in aquariums and labs, they are only found in that area. They are an endangered species, but specifically studied for their unique ability to regenerate their limbs. Axolotl's are one of the only types of salamanders that can fully regenerate all their limbs, jaw, spinal cord, and skin. Why is this? They have progenitor cells that activate and makeup blastema which is the bases for the cells regeneration. They also grow back their limbs in perfect condition no matter of sex, size, or age. In most salamanders, regeneration happens in the tail and is considered autonomous, this leads to the questions of what promotes regeneration in Axolotl and what is different in them compared to other salamanders?

Ancestral evolution of regeneration

The relationship between regeneration in modern salamanders and their evolution has not been widely studied, but recently it has been brought back to the attention of scientists, due to interests with human regeneration. It is known that a 300-million-year-old fossil of the amphibian *Micromelerpeton* was supposedly capable of limb regeneration found in Fig 1 (Fröbish et al. 2014). It is possible that regeneration evolved locally in salamanders or is

ancestral or primordial property of metazoan (Garza-Garcia et al. 2010). Salamanders maintained limb regeneration but lost in tetrapod vertebrates, thus salamanders are the current vertebrates with regeneration abilities versus other.

Cell response activation in Axolotl

The regenerating process begins in the cells. Once the limb has been amputated or detached, within the first few hours a thin layer of epidermal cells form. The epidermal cells collect at the site and proliferate to then form a wound epidermis to induce the regenerating process (Haas & Whited, 2017). The wound encloses fast and rapidly to be completed in a few hours (Bryant et al. 2002). The progenitor cells are then activated in the surrounding tissues and tip of the stump to make up the blastemal which is formative material from which cells are developed. A pool of blastemas will form and when size is reached the bulbous structure flattens and cartilage cells condense and coalesce (Haas & Whited, 2017). Differentiation of the tissue takes place and a new limb is developed and formed perfectly from regeneration found in Fig 2 and Table 1.

Johnson et al. (2017) wanted to determine the types of cells in contralateral limbs and if they become activated and enter the cell cycle in response to amputation of a limb. They found that after amputation there was a significance in activation of skeletal cells identified at 5 dpa and remained until 28 dpa, days post amputation (Johnson et al. 2017). They also used anit-Pax7 to stain the tissue to mark muscle satellite cells from animals with a contralateral amputation that was given Edu, a type of time course experiment that labels cells that are actively synthesizing DNA throughout the regenerating process (Johnson et al. 2017). All these

tests were coordinated to find if a limb amputation leads to distant cellular activation elsewhere and they found that it indicated that many tissues have the capacity to respond by activation resident cells to re-enter the cell cycle (Johnson et al. 2017).

Grafts were performed for many years on Axolotl. In 1986 Muneoka did experiments on Axolotl using the grafting method. Using sibling Axolotl, the skin was removed from the upper arm and transplanted to the other and sutured in place (Muneoka et al. 1986). Such grafts with skin were used for skeleton grafts. The grafts then when attached to the skin or nerves then produced a new limb. Flowers et al (2017) using tissue grafting of single cells during digit tip could proliferative or differentiated chondrocytes do not contribute much to regeneration and that these cells are refilled by dermal fibroblasts. This is an interesting theory since proliferation is the foundation of regeneration and something that most scientists should think about.

Cell response activation in salamanders

Unlike the Axolotl, only a small percentage of salamander species can regenerate a limited number of body parts. The regeneration in a newt's limb proceeds by the closure of the wound and is dependent on the formation of a multipotent mesenchymal growth zone, the blastemal (Morrison et al. 2006). Through data Morrison found that mature tissue in the stump responds to amputation by histolysis, increased cellular proliferation, and disorganization or known as dedifferentiation (Morrison et al. 2006).

Connective tissue contains cells called fibroblasts that produce collagens and other fibers that interact and regulate growth and pattern formation (Gardiner et al. 2002). Grafting studies are used to show the function of connective tissue fibroblasts and how it induces patterns by cell contribution studies (Gardiner et al. 2002). The dermal fibroblasts progenies are

19% and 78% of early blastema cells, though less than 20% of those cells are of the stump (Gardiner et al. 2002). It can be suggested that the expansion in the blastema was populated by fibroblasts. According to Gardiner, because dermal fibroblasts account for half of all the fibroblasts in the limb, he wonders if it's possible that the early blastemal cells derive from fibroblasts.

Morrison used a monoclonal antibody against Pax7 to test whether the skeletal muscle in the limb of a newt contains satellite cell population. Pax7 cells are present in Newt limb skeletal muscle shown in fig. 3 (Morrison et al, 2006). They also tested whether the Pax7 cells would reenter the cell cycle after the amputation of the limb using immunostained limb sections including antibodys against phosphorylated histone 3 (H3P) (Morrsion et al. 2006). They also saw Pax7 outside the skeletal muscle tissue after amputation and within the blastema. This shows that the activation of satellite cells is a response to the removal of the limb and that satellite cells incorporate into the blastema by leaving their niche (Morrison et al. 2006).

Conclusion and Future Directions

When comparing Axolotl's and Salamanders to their regeneration, there have not been enough studies that show any similarities or differences between them. Most of the read articles have been about the overall aspect of the Axolotl's regenerating limbs. Therefore because of this conclusion, my thesis could not be directly answered.

Some future direction that should be considered is, is there a way to use Salamanders, like Axolotl's, for humans to regenerate spinal cords, limbs, etc. According to Alvarado, most phyla possess one or many species that are capable of regenerating missing body parts or even

organisms (Alvarado, 2000). Are there different versions of regeneration in earlier metazoans? It is wondered if regeneration is an analogous trait or homologous trait (Alvarado, 2000). This could also be part of the question, were humans able to regenerate in earlier times, and why now can we regenerate our bone marrow and skin but not our limbs? In every article read there was always the correlation to, is there ever going to be a time that scientist find a way to inhibit cells that will then proliferate and regenerate in other species. Even though my thesis could not be answered, the aspect of future direction can be greatly considered with more research and detailed studies.

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Figure 1. Whole specimen of Micromelerpeton credneri. Specimen MB.Am.1210 showing the exceptional quality of preservation of fossil amphi- bians from the fossil lake deposits of Lake Odernheim. Note the preservation of 'skin shadow', external gills, retinal pigments and scalation patterns. Scale bar equals 1 cm.

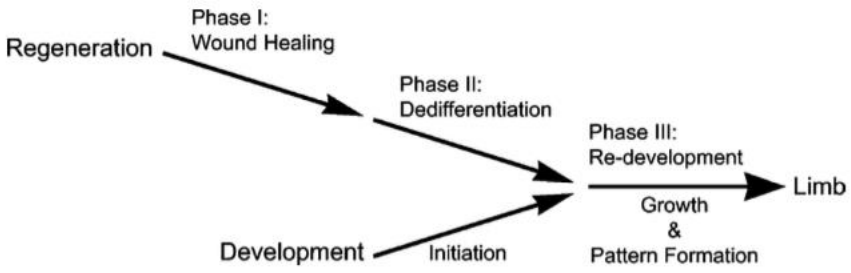

Figure 2. The converging pathways of limb regeneration and limb development (Gardiner et al. 2002).

TABLE 1

THE PHASES OF REGENERATION

PHASE I - WOUND HEALING	
Epidermal healing	Epidermal sheet migrates to cover the wound area within 1-2 hrs
Induction of gene expression	Genes common to wound healing and limb regeneration are expressed (e.g. *msx-2* and *MMP-9*)
Nerve dependency	Not dependent on nerves
PHASE II - DEDIFFERENTIATION	
Dedifferentiation	Cells in the stump tissues lose their specialized characteristics and become migratory
Blastema formation	Cells derived from fibroblasts migrate to form the blastema and begin to proliferate
Induction of gene expression	Re-expressed genes show spatial and/or temporal patterns that differ from development
Nerve dependency	No regeneration if nerve supply is interrupted
PHASE III - REDEVELOPMENT	
Growth and pattern formation	Classic responses to grafting are the same as in developing limbs; developing and regenerating limbs can cooperate to form a chimeric limb
Induction of gene expression	Genes show similar expression and function as in developing limbs
Nerve dependency	Continued growth depends on nerves, but differentiation is nerve-independent
Positional dependency	Requirement for a blastema consisting of cells that are positionally diverse in origin

(Bryant et al. 2002)

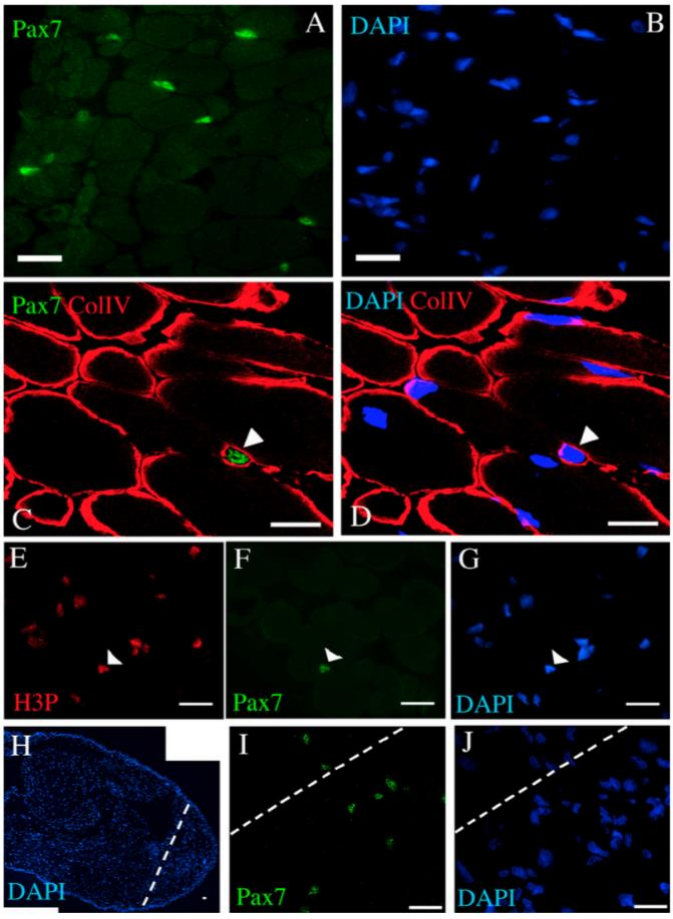

Figure 3. **Pax7 cells are present in newt limb skeletal muscle.** (A and B) Immunostaining of limb skeletal muscle identifies Pax7 cells within skeletal muscle tissue. DAPI staining shows the nuclei in the tissue section. (C and) Photomicrographs showing a typical Pax7 cell being surrounded by basement membrane. Arrowheads point to a Pax7 cell nucleus. (E-G) Photomicrographs showing a mitotic Pax7 cell 4 d after amputation. Arrowheads point to a Pax7/H3P double-positive cell. (H-J) Pax7 cells appear in an early bud stage blastema. Dotted line marks the level of amputation. Bars, 50 µm (Morrison et al. 2006)