Limb Regeneration in Humans: Micromanaging a Plastic Environment

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A popular topic as of late, stem cell research has intrigued many with its remarkable potential in the realm of human health. Multipotent stem cells have been found throughout the entire human body, indicating that adult tissues may be able to repair and regenerate themselves. Although organs such as the heart, liver, or lungs do not currently regenerate in humans, the presence of these somatic stem cells indicates that we possess the necessary structures to do so. By studying the axolotl, a species of salamander known to effectively regenerate limbs and organs, scientists have come closer to understanding human regeneration. After various studies performed on the axolotl, results have indicated that limb regeneration occurs in a step-wise fashion, in which certain qualifications must be met to progress to the next step. This is groundbreaking because the lack of human regeneration can be attributed to the absence of a correct preliminary environment, not in our sheer inability to do so. If we can determine the factors that influence each individual step of limb regeneration, we could theoretically micromanage regenerating environments to provide the exact outcome we desire.

In the last century, stem cell research has sparked a widespread interest in modern science. Stem cells are remarkable in their ability to develop into many different types of cells throughout development, indicating they potentially provide benefits for human health. As Elly M. Tanaka (2003) accurately puts it, "[t]he discovery of somatic stem cells in many locations suggests that adult tissues may have the latent capacity to regenerate" (p. 1). Although the goal of regenerating a functionally complex organ is still far-off and controversial, these multipotent cells have shown an extraordinary potential to help many people. Stem cells may eventually have the ability to create organs that will not be rejected by donors, or grow "replacement cells" for tissues in patients who suffer from Alzheimer's, Parkinson's, stroke, diabetes, and infinitely more diseases. Due to the powerful implications these cells have, scientists have become interested in studying classical models of regeneration. Surprisingly, these models are best understood by examining salamanders, who are known to effectively regenerate limbs, tails, eyes, jaws and even hearts. Overall, by examining the processes behind tissue repair and limb regeneration in salamanders, scientists grow one step closer to understanding human ability to regenerate cells, and maybe one day, complex organs.

Although much of the scientific research behind regeneration is rather dense, studies performed on the axolotl (a species of Mexican salamander with regenerative ability) provide basic understanding of how the process of regeneration occurs. In 2004, scientists Endo, Bryant, and Gardiner published an article titled, "A stepwise model system for limb regeneration", which highlights the important and necessary steps in initiating regeneration. However, instead of examining "normal" regeneration, the study induced ectopic (out of place) limbs to study growth. Instead of amputating axolotl limbs to monitor regeneration, the researchers made incisions in their upper arms in an attempt to regenerate an extra limb in this abnormal position. This procedure allowed them to analyze each step of regeneration and to determine which phases were essential to its success. In addition, ectopic "bumps" or blastemas were produced instead of full arms/limbs in certain cases. Unique to regenerating tissues, blastemas are large areas of undifferentiated cells formed in response to injury. Thus, the induction of blastema bumps provides a sufficient measure of whether or not regeneration will occur because they only appear in tissues that can regenerate. In order to examine the necessary elements for regeneration, Endo *et al*. generated various circumstances to test whether a bump could form. The results were as follows:

Table 1. Frequency of bump formation

(bump = blastema)

	N	No bump	Bump
Wound	3	3 (100%)	0 (0%)
Wound + Nerve	40	1 (2%)	39 (98%)
Wound + Nerve + Sham skin graft	4	1 (25%)	3 (75%)
Wound + Sham nerve + Skin graft	3	3 (100%)	0 (0%)

Group one consisted of salamanders that only had a simple skin wound, and none of the wounds formed blastemas. The second group had simple skin wounds and

a functional nerve deviated, or diverted into the wound. This was clearly the best way to induce regeneration, as 98% of salamanders formed blastemas. Thirdly, Endo *et al*. prepared a group with a wound, deviated nerve, and a sham skin graft (a fake square of skin); the regeneration was high in this group too. The last group also had a skin wound, but non-functioning nerves and a real piece of skin; no blastemas were formed in this group. Overall, this part of the experiment identified the role nerves play in creating proliferating regions – a deviated nerve was necessary to form a bump in all four groups. This was confirmed later on by denervating bumps in an early stage – they stopped growing within 2 days and completely disappeared in 4 days. In conclusion, nerves alone are sufficient to turn a healing wound into a regenerating blastema.

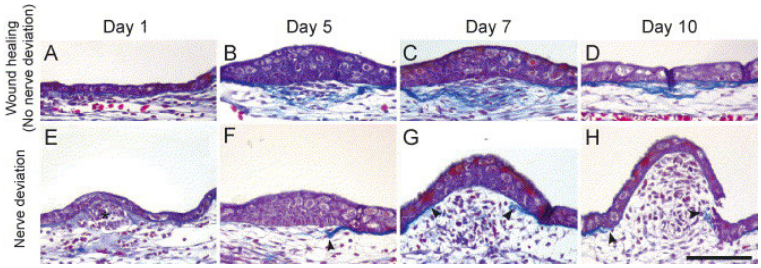

Figure 1. Comparison of bump formation during the healing process between normal wounds (A-D) and wounds with deviated nerves (E-H). Note that the bump continues to grow in the presence of a nerve, but without it begins to recede.

In a second experiment, the researchers wondered under which circumstances full limbs were formed instead of bumps. These were the results:

Table 2. Frequency of limb formation

	N	Bump	Limb
Wound + Nerve	19	18 (95%)	1 (5%)
Wound + Nerve + Skin graft	15	4 (27%)	11 (73%)

To summarize, only one of the salamanders with a wound and deviated nerve formed a full limb, yet around 73% of salamanders with the same conditions, in addition to a skin graft, formed a limb. The skin graft was obtained from the opposite side of the contralateral limb (i.e.; a posterior skin graft for an anterior wound). The researchers also noted that if the skin graft was obtained from the same side of the arm as the wound, an ectopic limb did not form. Thus, it appeared that a skin graft from the opposite side of the limb (in addition to a nerve) is necessary for the blastema to develop into an ectopic limb. In fact, unless bumps receive a contralateral skin implant, they will eventually stop growing and recede. Thus, there appears to be communication between certain molecules in these opposing sides that causes a bump to become a regenerating limb. Although the specifics of signaling were not a focus of this study, it attributed this front/back skin communication to cells called dermal fibroblasts in the original and grafted skin. A fibroblast is a type of cell that secretes proteins to build and support the structure of connective tissue, including the extracellular matrix. Results from this study suggested that less extracellular matrix was observed in regenerating bumps than in healing bumps.

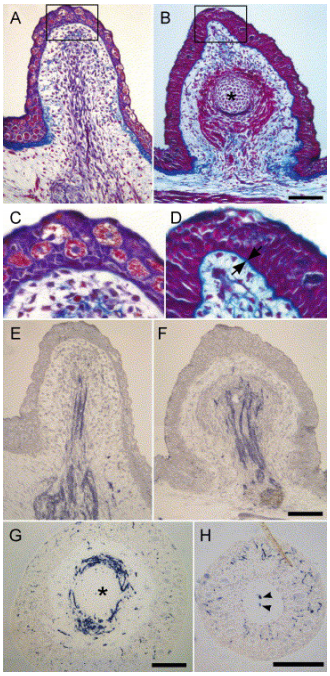

Figure 2. Differences in tissue between growing and regressing bumps. Growing bumps are pictured in (A) and (E), regressing bumps in (B) and (F). (C) and (D) are higher magnified areas of (A) and (B), respectively. (G) and (H) are transverse images of the tip of the regressing bump. Note the arrow in (D), points to the new collagen layer (extracellular matrix) forming in the regressing bump.

Thus, perhaps fibroblasts between opposing hosts and grafts communicate in a way to slow certain production of proteins they would otherwise individually produce to heal a wound. In conclusion, the step-by-step process for limb regeneration can be simplified as follows:

1) An injury occurs, the wound begins to close, cell division. Continue to 2a or 2b.

2a) NO SIGNAL FROM NERVES – Cells stop dividing, scar begins to form, ***skin regeneration***.

OR

2b) SIGNAL FROM NERVES – Cells continue to divide and gain ability to become a variety of cells, a bump/blastema is formed. Continue to 3a or 3b.

3a) NO SIGNAL FROM FIBROBLASTS – Decrease in cell division, increase in skin structure and stability, ***bump regression***.

OR

3b) SIGNAL FROM FIBROBLASTS – Bump continues to grow, patterns form in bump tissue, ***limb regeneration*.**

Overall, this study is interesting and applicable to mammalian regeneration because these results indicate that the "decision" to regenerate or not regenerate occurs very early on. This implies that perhaps the lack regeneration in humans does not stem from our inability to regenerate, but rather our failure to initiate the necessary preliminary steps.

Although Endo *et al*. provide a clear model for these preliminary steps, it merely brushes the surface of the type of complex regeneration needed to form bones and complex tissues that mammals, including us, could find useful. Interestingly enough, humans already do regenerate entire tissues on their own. Bone and cartilage are constantly growing and transforming throughout childhood and old age. We also have an intrinsic ability to completely regenerate broken bones over time, which many people have witnessed first hand. However, despite this inherent ability to regenerate skeletal tissue, if *too* much bone is lost, it will not be regenerated. This creates what Satoh *et al*. refer to as a "critical-size defect", or bone loss that has exceeded a threshold level at which it can no longer be regenerated. The ability to regenerate a critical-size defect is precisely what humans are interested in – large amounts of bone loss, replacement hearts and livers – these are all above our threshold ability at the present time. What Satoh *et al*. recognize as a key process in initiating critical-size defect limb development is "dedifferentiation".

Dedifferentiation is the creation of a blastema from multipotent cells by reverting back to an embryonic-like state. Thus, as Endo *et al*. touched on in their paper, dedifferentiation causes cells to act as if they are merely developing the first limb, not regenerating a lost one. One cell type that acts in this manner is the fibroblast, whose signals ultimately induce limb/skeletal regeneration by reverting back to a blastema. Therefore, in order to regenerate a critical-size defect, Satoh *et al*. attempted to identify the signals that induce fibroblast dedifferentiation and cause regeneration of bone in salamanders, and could potentially in humans.

Satoh *et al*. began by removing a 2 mm piece of bone from the axolotl forearm. This served as a critical-size defect, because bone regeneration did not occur on its own. As before, a nerve was also deviated to the center of the defect. Although a blastema did not form, the wound environment still provided signals that could maintain blastema cells. For example, when foreign blastemas were planted in the wound, the cells grew just as if the host had created them. Thus, the wound environment provided adequate signals to maintain blastema cells, even though the signals to create the blastema cells themselves were not present. These results help answer the paradox of axolotl regeneration: how can an entire arm be regenerated in the case of amputation, but a tiny deletion cannot? Clearly, the key is the formation of a blastema, which occurs during amputation but not in a forearm excision.

However, what signals are responsible for these intricate jobs? The researchers initially looked at bone morphogenetic proteins (BMPs), one of the most important groups of molecules in regulating bone formation and healing. They were interested in whether or not BMP signaling was activated under critical-size defect circumstances, due to their key role in development. In fact, inserting beads soaked in BMP into the defect lead to closure of the defect. In other words, by artificially increasing BMP signaling, the critical-size defect was eliminated and the forearm was regenerated. Satoh *et al*. also examined the signaling between wound tissue and the deviated nerve to see if their effects were the same as in limb amputation. When both the tissue and nerve were present, around half of the defects were regenerated. Signaling between these two structures created blastemas exactly like those created in amputation. This data indicates that signals from the wound/nerve environment are specifically responsible for fibroblast dedifferentiation into blastema cells. So, without these signals, more differentiated fibroblast cells are unable to revert back or dedifferentiate into the blastema cells that are necessary for regeneration.

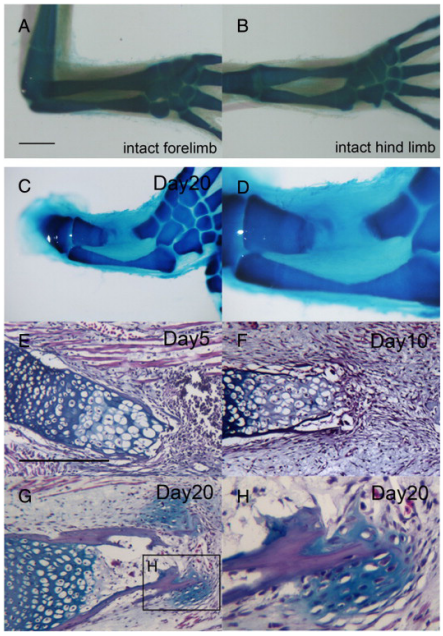

Figure 3. Normal anatomy of axolotl forelimb (A) and hind limb (B). After 2mm excision in forelimb (C), higher magnification (D). At day 5 post surgery, decreased extracellular matrix (E). At day 10, radius begins to expand (F). At day 20, callus begins to form (G), higher magnification (H).

The authors of this study ultimately conclude that regeneration is not simply dependent on causing the differentiation of a missing cell type, but that the environment must communicate and instruct the cells along the way. In fact, even when foreign blastema cells are placed into a blastema-free environment, the communication and instruction appears to still be present. Ultimately, a wide variety of regenerative responses can occur if the appropriate wound signals are provided. By this manner, regeneration can be viewed as a multistep process. In theory, if each step could be modified and perfected, a precise regenerative response could be produced. The key to human regeneration would then lie in our ability to direct development, or set up our own "blueprint" for regeneration in the host environment.

In conclusion, regeneration is not a far-off fiction of sci-fi movies. With regard to human regeneration, our failure thus far lies in the absence of a correct environment to initiate the necessary preliminary steps. Although a major setback in our ability to regenerate, the human wound environment is truly more useful for our everyday lives. When presented with a paper cut or skinned knee, we can rely on our bodies to heal the wound – keeping out infection and preventing illness. Growth of the extracellular matrix and scar formation are essential to successful healing, but in turn prevent regeneration. Thus, in an environment where we are much more prone to scrapes and scuffles than losing a limb, it only makes sense that we revert to healing instead of regeneration in response to injury. Although we are not theoretically far off from beginning to understand human regeneration, it still remains a science of the future due to ethical limitations. These studies can only reap major benefits for humans by experimenting with human embryos – an extremely controversial and debated topic. For now, we can learn all that is possible from the axolotl and other animals, understanding the minutiae of each step during development. By scrutinizing each of these steps, we might eventually have the power to micromanage a regenerating environment and change the developmental fate of certain cells. By such mechanisms, we would have the power to create the exact result we want: a new tissue, bone, or maybe even a functioning organ.

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