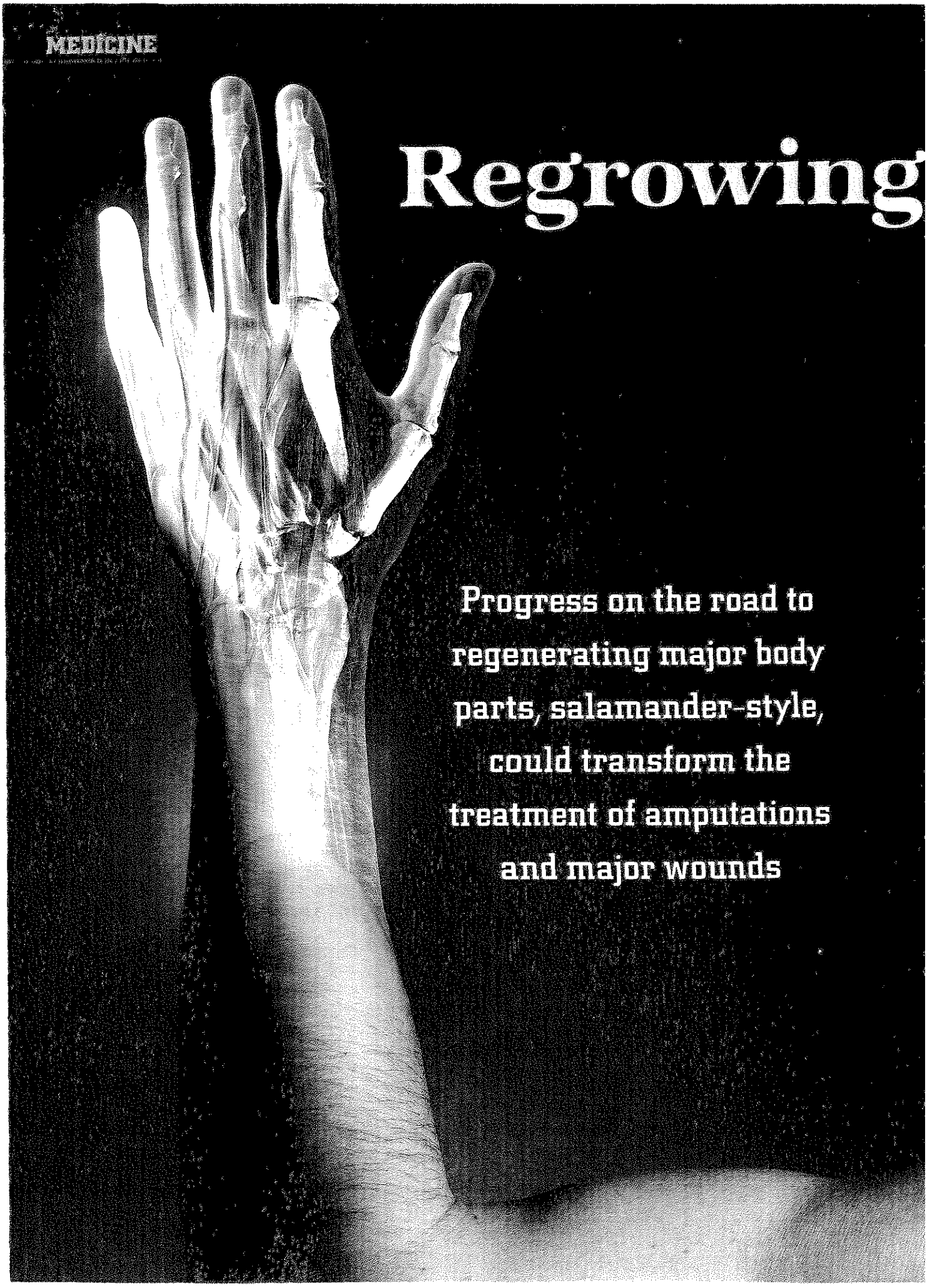


Regrowing

Progress on the road to regenerating major body parts, salamander-style, could transform the treatment of amputations and major wounds

Human Limbs

By Ken Muneoka, Manjong Han and David M. Gardiner

A salamander's limbs are smaller and a bit slimier than those of most people, but otherwise they are not that different from their human counterparts. The salamander limb is encased in skin, and inside it is composed of a bony skeleton, muscles, ligaments, tendons, nerves and blood vessels. A loose arrangement of cells called fibroblasts holds all these internal tissues together and gives the limb its shape.

Yet a salamander's limb is unique in the world of vertebrates in that it can regrow from a stump after an amputation. An adult salamander can regenerate a lost arm or leg this way over and over again, regardless of how many times the part is amputated. Frogs can rebuild a limb during tadpole stages when their limbs are first growing out, but they lose this ability in adulthood. Even mammalian embryos have some ability to replace developing limb buds, but that capacity also disappears well before birth. Indeed, this trend toward declining regenerative capacity over the course of an organism's development is mirrored in the evolution of higher animal forms, leaving the lowly salamander as the only vertebrate still able to regrow complex body parts throughout its lifetime.

Humans have long wondered how the salamander pulls off this feat. How does the regrowing part of the limb "know" how much limb is missing and needs to be replaced? Why doesn't the skin at the stump form a scar to seal off the wound as it would in humans? How can adult salamander tissue retain the embryonic potential to build an entire limb from scratch multiple times? Biologists are closing in on the answers to those questions. And if we can understand how the regeneration process works in nature, we hope to be able to trigger it in people to regenerate amputated limbs, for example, and transform the healing of other major wounds.

The human body's initial responses to such a serious injury are not that different from those of a salamander, but soon afterward the human and amphibian wound-healing strategies diverge. Ours results in a scar and amounts to a failed regeneration response, but several signs indicate that humans do have the potential to rebuild complex parts. The key to making that happen will be tapping into our latent abilities so that our own wound healing becomes more salamanderlike. For this reason, our research first focused on the experts to learn how it is done.

Lessons from the Salamander

When the tiny salamander limb is amputated, blood vessels in the remaining stump contract quickly, so bleeding is limited, and a layer of skin cells rapidly covers the surface of the amputation site. During the first few days after injury, this so-called wound epidermis transforms into a layer of signaling cells called the apical epithelial cap (AEC), which is indispensable for successful regeneration. In the meantime, fibroblasts break free from the connective tissue meshwork and migrate across the amputation surface to meet at the center of the wound. There they proliferate to form a blastema—an aggregation of stemlike cells that will serve as progenitors for the new limb [*see box on next two pages*].

Many years ago studies in the laboratory of our colleague Susan V. Bryant at the University of California, Irvine, demonstrated that the cells in the blastema are equivalent to the cells in the developing limb bud of the salamander embryo. This discovery suggested that the construction of a limb by the blastema is essentially a recapitulation of the limb formation that took place during the animal's original development. An important implication of this insight was that the same genetic program is involved in both situations, and because humans make limbs as embryos, in principle we should already have the necessary programming to regenerate them as adults, too. It seemed, therefore, that all scientists needed to do was figure out how to

KEY CONCEPTS

- The gold standard for limb regeneration is the salamander, which can grow perfect replacements for lost body parts throughout its lifetime. Understanding how can provide a road map for human limb regeneration.
- The early responses of tissues at an amputation site are not that different in salamanders and in humans, but eventually human tissues form a scar, whereas the salamander's reactivate an embryonic development program to build a new limb.
- Learning to control the human wound environment to trigger salamanderlike healing could make it possible to regenerate large body parts.

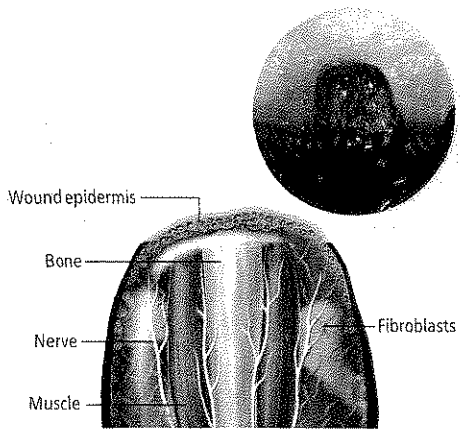
—The Editors

AARON GOODMAN

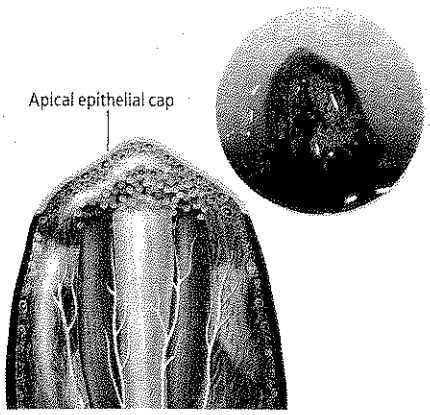
[THE GOLD STANDARD]

PERFECT REGENERATION

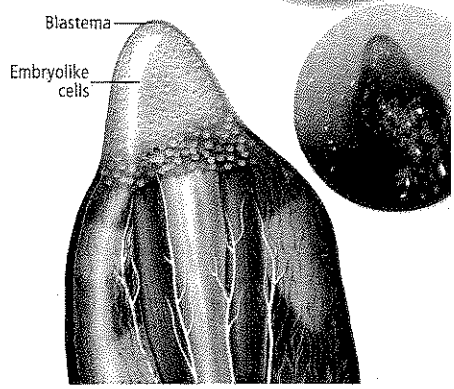
Salamanders are the only vertebrates able to regrow lost limbs, as well as many other body parts, throughout their lifetimes—and they can do it repeatedly. Studies of how a limb forms on the salamander have revealed that the process begins with rapid wound closure and a rush of cells from stump tissues to the amputation site. The next stages involve reversion of those cells to an embryonic state and their building of a new limb following the same steps as in embryonic development.



WOUND CLOSURE
Within hours of a leg amputation, epidermal skin cells migrate across the wound to seal it, forming a wound epidermis.



HEALING SIGNALS
Epidermal cells form a ridge known as an apical epithelial cap, which generates critical signals that guide the behavior of other cells. Fibroblasts and muscle cells start migrating toward the wound site.



RETURN TO THE WOMB
Cells that migrated to the wound revert to a less specialized embryonic state and begin dividing to populate the bud of a new limb, called a blastema.

induce an amputated limb to form a blastema.

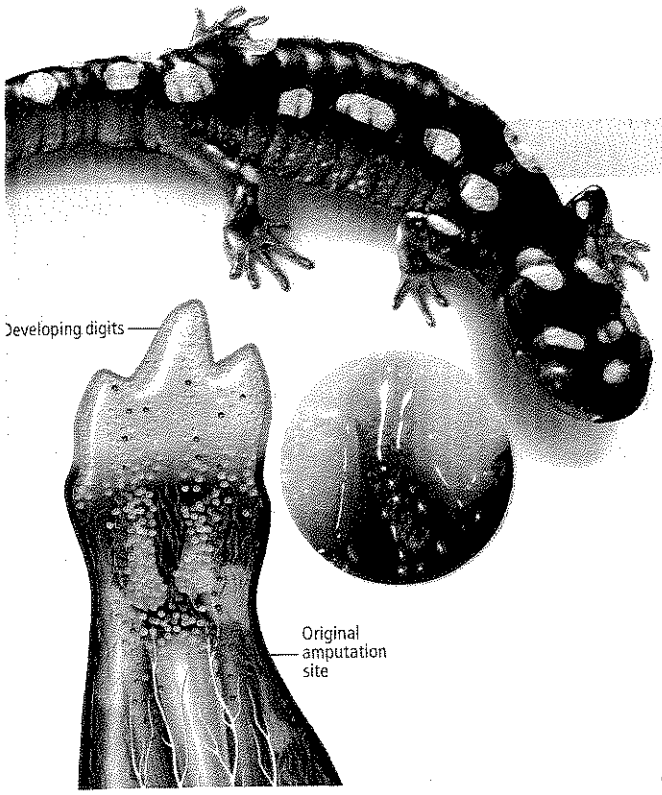
One of us (Gardiner)—working with Tetsuya Endo of U.C. Irvine a few years ago—took a minimalist approach to answering the basic question of how to make a blastema. Instead of studying amputation sites on the salamander, where a blastema would naturally form, we looked at simple wounds on the side of a salamander limb, which would normally heal just by regenerating the skin. Our idea was that such wounds are similar to the site of an amputated mammalian limb that fails to generate a new limb. If we could get an entire limb to grow where a simple wound-healing response would typically occur, then we could further dissect the regeneration process.

After we made a small incision in the salamander leg, epidermal cells migrated to cover and seal the wound, as they would at an amputation site, and fibroblasts from the dermis layer of the skin also moved in to replace the missing skin. But if we carefully deviated a nerve to the wound site, we could induce those fibroblasts to form a blastema instead. Marcus Singer of Case Western Reserve University had already demonstrated more than half a century ago that innervation was required for a regeneration response, but our experiments clarified that unknown factors provided by the nerve were influencing regeneration by altering the behavior of resident fibroblasts.

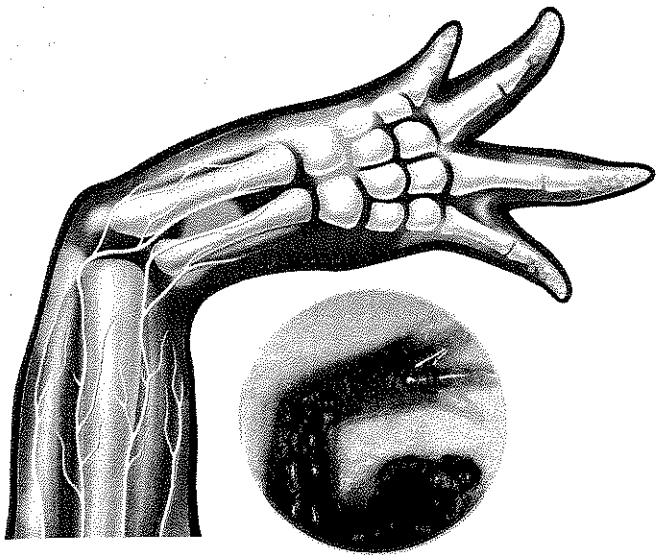
These induced blastemas never progressed to the later stages of regeneration to form a new limb, however. One more ingredient was needed. The key to inducing a blastema that produced a new limb was to graft a piece of skin from the opposite side of the limb to the wound site, which allowed fibroblasts from opposite regions of the limb to participate in the healing response. The resulting accessory limb was, of course, growing out at an abnormal location, but it was anatomically normal [*see box on pages 62 and 63*]. So the basic recipe for making a blastema seemed relatively simple: you need a wound epidermis, nerves and fibroblasts from opposite sides of the limb. With this minimal view of limb regeneration in mind, we began to focus on understanding the roles of the individual ingredients.

We knew that the epidermis is derived from one of three layers of primitive cells within an early developing embryo, the ectoderm, which is also well known to provide signals that control the outgrowth of limbs from limb buds on the embryo. Ectoderm cells gather in the bud to form an apical ectodermal ridge (AER), which transiently produces chemical signals that guide

DAVID M. GARDINER (*photographs*); ALICE Y. CHEN (*limb illustrations*); LISA APFELBACHER (*salamander*)



TAKING SHAPE
As the blastema grows, it begins to form the outline of the new limb, including the tip that will become the foot. The embryonic cells give rise to new tissues by proliferating and differentiating into bone, muscle, fibroblasts, and so on.



FLESHING OUT
As its internal anatomy and outline become more mature, the limb lengthens to fill in the missing segment between the original amputation plane and the toes.

the migration and proliferation of the underlying limb bud cells.

Although some of the critical signals from the epidermis have not yet been identified, members of the family of fibroblast growth factors (FGFs) are involved. The AER produces a number of FGFs that stimulate the underlying cells of the limb bud to produce other FGFs, fueling a feedback circuit of signaling between the AER and limb bud cells that is essential for the outgrowth of a limb. A similar feedback circuit spurred by the AEC is thought to function in the same way during limb regeneration, and Hiroyuki Ide of Tohoku University in Japan discovered that the progressive loss of regenerative ability in frog tadpoles is associated with a failure to activate the FGF circuit. By treating older nonregenerating tadpole limbs with FGF10, he was able to jump-start this signaling circuit and stimulate partial regeneration of amputated limbs.

The excitement this result inspired was tempered, however, by the fact that the induced regenerates were abnormal, consisting of irregularly placed limb parts, which raises the important issue of how regeneration is controlled so that all the appropriate anatomical structures that are lost when the limb is amputated are accurately replaced. It turns out that the other primary cellular players, the fibroblasts, carry out this function.

Location, Location, Location

Recall from the minimalist accessory-limb experiments that the presence of fibroblasts *per se* was not sufficient for regeneration because fibroblasts are present at the simple wound site that does not make a new limb. It was the fibroblasts from the opposite side of the limb that proved essential. That discovery illustrates the importance of cellular position in triggering a regeneration response. In an embryo, the sequence of events in limb development always begins with formation of the base of the limb (the shoulder or hip) and is followed by progressive building of more distal structures until the process terminates with the making of fingers or toes. In salamander regeneration, on the other hand (or foot), the site of amputation can be anywhere along the length of the limb and regardless of where the wound is located, only those parts of the limb that were amputated regrow.

This variable response indicates that cells at the amputation wound edge must "know" where they are in relation to the entire limb. Such positional information is what controls the cellular and molecular processes leading to

[THE AUTHORS]

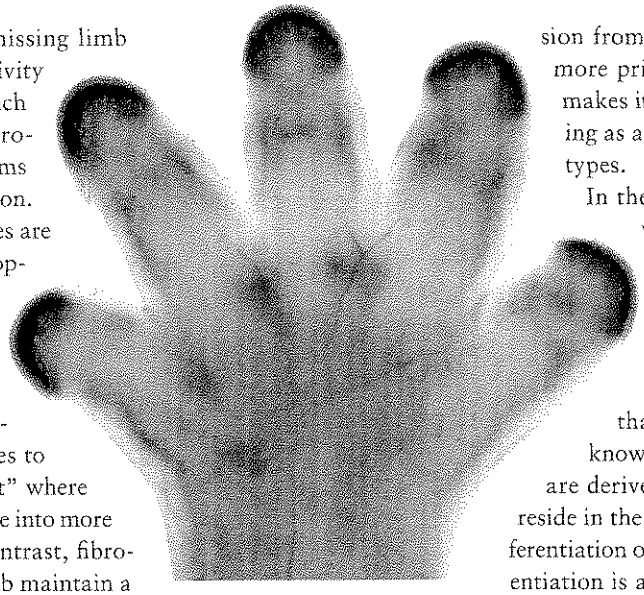
Ken Muneoka, Manjong Han and David M. Gardiner are part of a multi-institution research team working toward regenerating a mammalian limb. Their group, led by Muneoka, is one of only two to have received a multimillion-dollar grant from the Defense Advanced Research Projects Agency to pursue human limb regeneration. Muneoka is a professor and Han an assistant research professor in the department of cell and molecular biology at Tulane University. Gardiner is a research biologist in the department of cell and molecular biology at the University of California, Irvine, where Muneoka also completed a postdoctoral fellowship. Muneoka unexpectedly found himself working there once again after Hurricane Katrina forced him to relocate his family and his Tulane research staff to Irvine for five months.

the perfect replacement of the missing limb parts, and it is encoded in the activity of various genes. Examining which genes are at work during these processes helps to reveal the mechanisms controlling this stage of regeneration.

Although a large number of genes are involved during embryonic development in educating cells about their position in the limb, the activity of a gene family called *Hox* is critical. In most animals, cells in the developing limb bud use the positional code provided by *Hox* genes to form a limb, but then they "forget" where they came from as they differentiate into more specialized tissues later on. In contrast, fibroblasts in the adult salamander limb maintain a memory of this information system and can reaccess the positional *Hox* code in the process of limb regeneration.

During regeneration the fibroblasts bring this information with them as they migrate across the wound to initiate blastema formation, and once in the blastema, cells are able to "talk" to one another to assess the extent of the injury. The content of this crosstalk is still largely a mystery, but we do know that one outcome of the conversation is that the regenerating limb first establishes its boundaries, including the outline of the hand or foot, so that cells can use their positional information to fill in the missing parts between the amputation plane and the fingers or toes.

Because muscle and bone make up the bulk of a limb, we are also interested in understanding where the raw material for those tissues originates and what mechanisms control their formation. When the regenerative response is initiated, one of the key early events involves a poorly understood process called dedifferentiation. The term is typically used to describe a cell's reversion from a mature specialized state to a more primitive, embryonic state, which makes it capable of multiplying and serving as a progenitor of one or more tissue types.



MOUSE PAW produces a growth factor called BMP4 (*purple stain*) during fetal digit development that is also essential for natural digit-tip regeneration in mice.

In the field of regeneration, the word was first used by early scientists who observed under the microscope that the salamander stump tissues, particularly the muscle, appeared to break down and give rise to proliferative cells that formed the blastema. We now know that those muscle-associated cells are derived from stem cells that normally reside in the muscle tissue and not from dedifferentiation of muscle. Whether or not dedifferentiation is actually happening in the case of every tissue type within a regenerating limb has yet to be proved, although it is clear that a variation of this theme does occur during regeneration. Fibroblasts that enter the blastema and become primitive blastemal cells have the ability to differentiate into skeletal tissues (bone and cartilage) as well as to redifferentiate into the fibroblasts that will form the interstitial meshwork of the new limb, for instance.

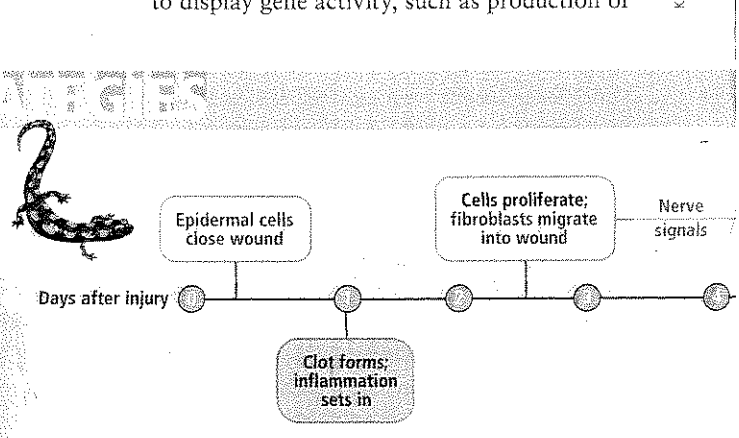
Returning to another of the central cellular players in blastema formation, the epidermal cells, we can also pinpoint moments in the regeneration process when it seems these cells are making a transition to a more embryonic state. A number of genes active in the embryonic ectoderm are critical for limb development, including *Fgf8* and *Wnt7a,* but as the ectoderm of the embryo differentiates to form the multilayered epidermis of the adult, these genes are turned off. During regeneration in the adult, the epidermal cells that migrate across the amputation wound and establish a wound epidermis initially begin to display gene activity, such as production of

KEN MUNEOKA (*photograph*); ALICE Y. CHEN (*illustrations*)

[THE HUMAN CHALLENGE]

EPIDERMIS HEALING STRATEGIES

In mammals and salamanders, cell responses to a severe injury such as an amputation are similar in some ways (*red type*), although the pace of healing is much slower in mammals and environmental signals that promote regeneration are not present. Salamanders close a wound within hours but do not form scars. Instead cells respond to signals from one another and from the wound environment to begin a rebuilding process within days. Mammalian wound healing aims to just seal off the damaged area, first with a scab and then with a scar. By the time human epidermal cells close a wound, the salamander is already forming a blastema in preparation for regeneration.



wound-healing keratin proteins, that is not specifically related to regeneration. Later the wound epidermal cells activate *Fgf8* and *Wnt7a*, the two important developmental genes. For practical purposes, then, the essential definition of dedifferentiation—as it pertains to the epidermis and other cell types—is the specific reactivation of essential developmental genes.

Thus, our studies of salamanders are revealing that the regeneration process can be divided into pivotal stages, beginning with the wound-healing response, followed by the formation of a blastema by cells that revert to some degree to an embryonic state, and finally, the initiation of a developmental program to build the new limb. As we move toward the challenge of inducing limb regeneration in humans, we rely on these insights to guide our efforts. Indeed, the hardest things to discover in science are those that do not already occur, and limb regeneration in humans fits snugly into this category, although that does not mean humans have no natural regenerative capacity.

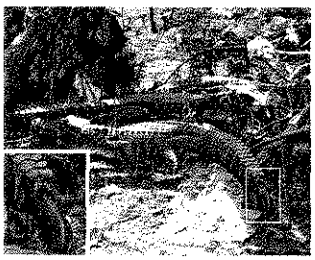
Potential at Our Fingertips

One of the most encouraging signs that human limb regeneration is a feasible goal is the fact that our fingertips already have an intrinsic ability to regenerate. This observation was made first in young children more than 30 years ago, but since then similar findings have been reported in teenagers and even adults. Fostering regeneration in a fingertip amputation injury is apparently as simple as cleaning the wound and covering it with a simple dressing. If allowed to heal naturally, the fingertip restores its contour, fingerprint and sensation and undergoes a varying degree of lengthening. The success of this conservative treatment of fingertip amputation injuries has been documented in medical journals thousands of times. Interestingly, the alternative protocol for such injuries typically included operating to suture a skin flap over the amputation wound, a "treatment" that we now know will inhibit regeneration even in the salamander because it interferes with formation of the wound epidermis. The profound message in these reports is that human beings have inherent regenerative capabilities that, sadly, have been suppressed by some of our own traditional medical practices.

It is not easy to study how natural human fingertip regeneration works because we cannot go around amputating fingers to do experiments, but the same response has been demonstrated in both juvenile and adult mice by several researchers. In recent years two of us (Muneoka and Han) have been studying the mouse digit-tip regeneration response in more detail. We have determined that a wound epidermis does form after digit-tip amputation, but it covers the regenerating wound much more slowly than occurs in the salamander. We have also shown that during digit-tip regeneration, important embryonic genes are active in a population of undifferentiated, proliferating cells at the wound site, indicating that they are blastema cells. And indirect evidence suggests that they are derived from fibroblasts residing in the interstitial connective tissues and in bone marrow.

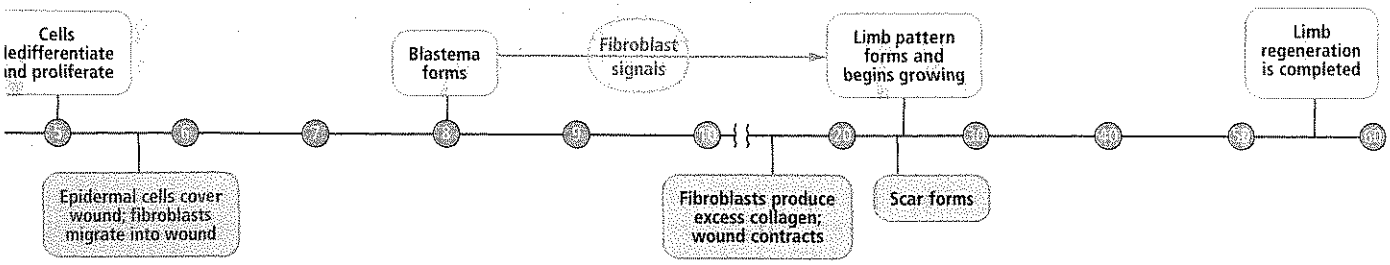
To explore the roles of specific genes and growth factors during the mouse-digit regeneration response, we developed a tissue culture that serves as a model for fetal mouse-digit regeneration. With it, we found that if we experimentally depleted a growth factor called bone morphogenetic protein 4 (BMP4) from the fetal amputation wound, we inhibited regeneration. In addition, we have shown that a mutant mouse lacking a gene called *Msx1* is unable to regenerate its digit tips. In the fetal digit tip, *Msx1* is critical to the production of BMP4, and we were able to restore the regener-

FAST FACTS



Young American alligator photographed in Louisiana is regenerating its amputated tail.

- The tail has about the same diameter as a human limb, suggesting that the ability to regenerate an appendage is not limited by the size of the amputation wound surface.
- Regrowing an adult human limb also might not take as long as it took to grow the first time. In salamanders, a poorly understood phenomenon known as catch-up allows the regenerating limb to go through a phase of rapid growth, resulting in a final limb that is appropriately scaled to the rest of the animal.

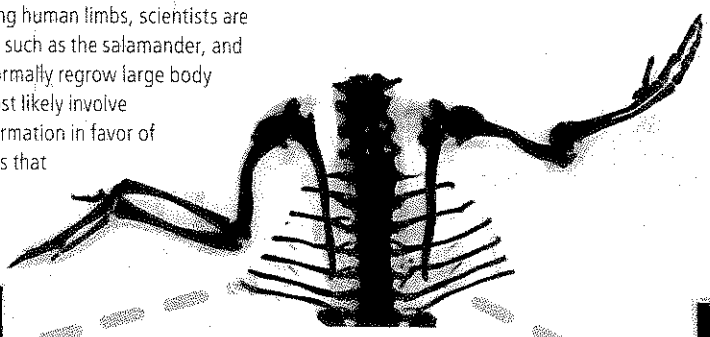


CAROL BURDSAL (*photograph*); FROM "LIMB REGENERATION IN HIGHER VERTEBRATES: DEVELOPING A ROADMAP" BY MANJONG HAN ET AL., IN *ANATOMICAL RECORD PART B: THE NEW ANATOMIST*, VOL. 287B, ISSUE 1, NOVEMBER 2005

THE ROAD TO REGENERATION

Taking a step-by-step approach toward the goal of regrowing human limbs, scientists are learning how to control the process in natural regenerators, such as the salamander, and how to trigger similar mechanisms in animals that do not normally regrow large body parts. Tapping the regenerative potential in humans will most likely involve redirecting our wound-healing responses away from scar formation in favor of a limb-building program similar to the biological instructions that first create our limbs during fetal development.

▲ **REDIRECTED WOUND HEALING**
Causing a new limb to grow from the site of an incision on front of the leg of an axolotl established the basic requirements in salamanders for triggering a limb-regeneration response where normally only simple wound healing would occur.



▲ **NONREGENERATING VERTEBRATE**
A normal ankle and foot grew from the "elbow" of a chicken embryo's wing (*above right*) after leg tissue was grafted into the wing bud earlier in the chick's development. Regrowth of the amputated leg segment in an animal that does not naturally regenerate shows that limb-building programs can be reactivated when the wound environment is permissive.

ation response by adding BMP4 to the wound in the *Msx1*-deficient mouse, confirming BMP4's necessity for regeneration.

Studies by Cory Abate-Shen and her colleagues at the Robert Wood Johnson Medical School have also demonstrated that the protein encoded by *Msx1* inhibits differentiation in a variety of cell types during embryonic development. That link to the control of differentiation suggests that the protein plays a role in the regeneration response by causing cells to dedifferentiate. Although *Msx1* is not active during the early dedifferentiation stages of salamander limb regeneration, its sister gene *Msx2* is one of the first genes reactivated during regeneration and very likely serves a similar function.

The Human Challenge

The idea of regenerating a human limb may still seem more like fantasy than a plausible possibility, but with insights such as those we have been describing, we can evaluate in a logical stepwise manner how it might happen. An amputated human limb results in a large and complex wound surface that transects a number of different tissues, including epidermis, dermis and interstitial connective tissue, adipose tissue, muscle, bone, nerve and vasculature. Looking at those different tissue types individually, we find that most of them are actually very capable of regenerating after a small-scale injury.

In fact, the one tissue type within a limb that lacks regenerative ability is the dermis, which is composed of a heterogeneous population of cells, many of which are fibroblasts—the same cells that play such a pivotal role in the salamander regeneration response. After an injury in humans and other mammals, these cells undergo a process called fibrosis that "heals" wounds by depositing an unorganized network of extracellular matrix material, which ultimately forms scar tissue. The most striking difference between regeneration in the salamander and regenerative failure in mammals is that mammalian fibroblasts form scars and salamander fibroblasts do not. That fibrotic response in mammals not only hampers regeneration but can be a very serious medical problem unto itself, one that permanently and progressively harms the functioning of many organs, such as the liver and heart, in the aftermath of injury or disease.

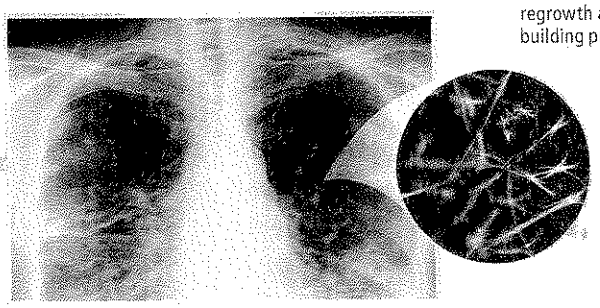
Studies of deep wounds have shown that at least two populations of fibroblasts invade an injury during healing. Some of these cells are fibroblasts that reside in the dermis, and the others are derived from circulating fibroblast-like stem cells. Both types are attracted to the wound by signals from immune cells that have also rushed to the scene. Once in the wound, the fibroblasts migrate and proliferate, eventually producing and modifying the extracellular matrix of the area. This early process is not that dissimilar to the regeneration response in a salamander wound, but the mammalian fibroblasts produce an excessive amount of matrix that becomes abnormally cross-linked as the scar tissue matures. In contrast, salamander fibroblasts stop producing matrix once the normal architecture has been restored.

An exception to this pattern in mammals does exist, however. Wounds in fetal skin heal without forming scars—yielding perfect skin

DAVID M. GARDINER (*axolotl*); KEN MUNEOKA (*chicken and mouse digit*)

◀ **HUMAN POTENTIAL**
Natural human regeneration of amputated fingertips has been well documented, including the recent case of Lee Spievak. His middle finger, about an inch of which was severed by a model airplane propeller, is shown after complete healing. The injury was treated with a protein powder that might have aided regeneration by acting as a scaffold for regrowing tissues.

▼ **REBUILDING LIMBS**
Most human tissues are individually able to regenerate, which suggests that regrowing complex body parts is a realistic goal. Regenerating whole limbs will require changing the signals cells receive in the wound environment so that brakes on regrowth are removed and our innate limb-building programs are reactivated.



◀ **MAMMALIAN DIGIT TIP**
New bone (*red stain*) growing from the site where a mouse's digit tip was amputated (*green stain*) illustrates the regenerative potential present in mammals. The authors have also shown that a blastema forms at the site where a mouse digit will regenerate.

▲ **BLOCKING FIBROSIS**
Fibroblast cells (*inset*) that form scar tissue at a wound site also cause organ-scarring diseases, such as pulmonary fibrosis, which constricts breathing. Learning to prevent scarring in amputation wounds as a prelude to regeneration should also yield treatments for unwanted fibrosis in other body tissues.

regeneration and indicating that the switch to a fibrotic response arises with the developmental maturation of the skin. Although this difference could reflect a change in the biology of the fibroblasts, it is more likely a result of altered signaling from the extracellular wound environment modulating the behavior of the fibroblasts, which in turn suggests that therapeutically modifying those signals could change the healing response. At the same time, the fact that limb amputations during fetal stages of development do not result in regeneration of the limb reminds us that scar-free wound healing is likely to be necessary but not sufficient for regeneration.

To advance our understanding of what it will take to induce limb regeneration in people, we are continuing our work with mice. Our research group has already described a natural blastema in a mouse amputation injury, and our goal within the next year is to induce a blastema where it would not normally occur. Like the accessory-limb experiments in salamanders, this achievement would establish the minimal requirements for blastema formation. We hope that this line of investigation will also reveal whether, as we suspect, the blastema itself provides critical signaling that prevents fibrosis in the wound site.

If we succeed in generating a blastema in a mammal, the next big hurdle for us would be coaxing the site of a digit amputation to regenerate the entire digit. The complexity of that task is many times greater than regenerating a simple digit tip because a whole digit includes joints, which are among the most complicated skeletal structures formed in the body during embryonic development. Developmental biologists are still trying to understand how joints are made naturally, so building a regenerated mouse digit, joints and all, would be a major milestone in the regeneration field. We hope to reach it in the next few years, and after that, the prospect of regenerating an entire mouse paw, and then an arm, will not seem so remote.

Indeed, when we consider all that we have learned about wound healing and regeneration from studies in various animal models, the surprising conclusion is that we may be only a decade or two away from a day when we can regenerate human body parts. The striking contrast between the behavior of fibroblasts in directing the regeneration response in salamanders versus the fibrotic response leading to scarring in mammals suggests that the road to successful regeneration is lined with these cells. Equally encouraging is the recent discovery by Howard Y. Chang and John L. Rinn of Stanford University that adult human fibroblasts, like salamander fibroblasts, retain a memory of the spatial coordinate system used to establish the body plan early in the embryo's development. Given that such positional information is required for regeneration in salamanders, its existence in human fibroblasts enhances the feasibility of tapping into and activating developmental programs necessary for regeneration.

Now, as we watch a salamander grow back an arm, we are no longer quite as mystified by how it happens. Soon humans might be able to harness this truly awesome ability ourselves, replacing damaged and diseased body parts at will, perhaps indefinitely. ■

MORE TO EXPLORE

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