TOP: © JOSE MANUEL GELPI DIAZ MIDDLE: JOEL SARTORE BOTTOM: © DK LIMITED/CORBIS

THE REGENERATION RECIPE

Can natural regenerators such as the newt teach scientists about the ingredients needed to grow new limbs and organs? Even in, say, mammals?

By Megan Scudellari

The first cut is too small. With gloved hands, Nobuyasu Maki slices the cornea again, this time with more pressure. The anesthetized amphibian doesn't twitch beneath the spotlights focused on its speckled yellow head, no bigger than a large Tic Tac. A timer beeps somewhere in the lab, like a heart monitor in a hospital room. Switching instruments, Maki raises a pair of miniature tweezers vertically above the newt's head, then plunges the point down into the eye. The alarm, unrelated to this experiment, stops. Maki pulls the tweezers up, extracting a small, clear orb, no larger than a pinhead. He lays down the instrument and picks up the motionless newt, placing it in inside a plastic carrier. "It'll wake up in an hour," he says, stripping the latex gloves from his hands.

But long before the animal stirs, macrophages will flood the injury site and mediate the removal of pigment from the dark iris epithelium. Four days later, the iris will begin to decondense as the epithelial cells elongate. In 10 days, a lens vesicle will form and start to express genes for lens development: fibroblast growth factors, *pax-6* and *prox-1*. Then, an event more familiar to science fiction fans than scientists: In less than a month, the newt (*Notophthalmus viridescens*) will regenerate a fully functional lens, indistinguishable from the original glistening sphere once stuck to the tip of Maki's tweezers.

Two floors above the University of Dayton, Ohio, lab where Maki is a second-year postdoc, Panagiotis Tsonis sits up and points to a sequence of four images on his computer screen. Four stained sections of a newt eye depict the regeneration of a lens from dorsal iris cells, the growing lens like a glob of ink falling from a dropper in stop motion. The newt lens is a classical case of regeneration by transdifferentiation, in which adult cells revert to a stem cell–like state, then redifferentiate into another type of adult cell. The first person to identify transdifferentiation in the newt eye was Tsonis's PhD advisor in Japan, Goro Eguchi, in the early 1970s.[1] When the newt lens is injured or removed, dark iris epithelium cells revert to a dedifferentiated state, proliferate, then slowly transform into clear lens fiber cells. "This is a terminally differentiated cell. It's an adult cell. It's not supposed to be doing that, but it does!" exclaims Tsonis. "So we're very interested to know why the newt does this and *how* it does it." If they can figure that out, of course, perhaps they can figure out how to induce other species—even, alluringly, humans—to do the same. "Can we do what the newt does?"

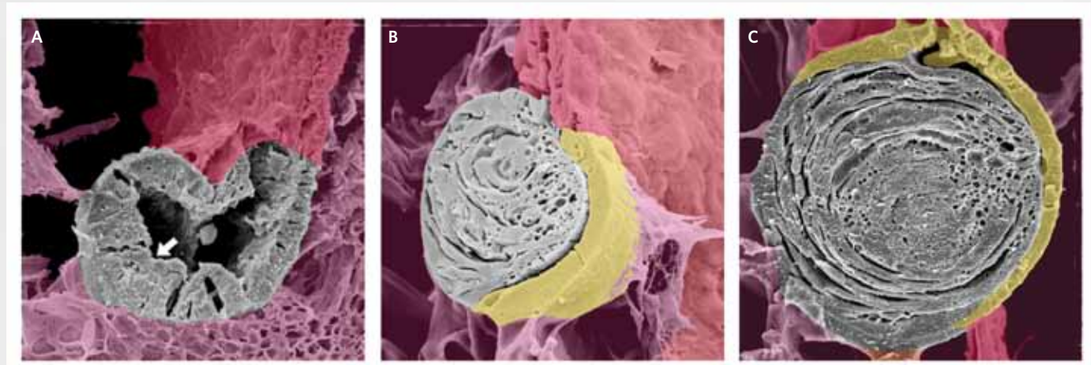
Eight years ago, Tsonis made the first move toward answering that question. With his wife and collaborator, Katia Del Rio-Tsonis, now a professor at Miami University in Ohio, the eager biologist embarked on what would be a 4-year attempt to induce regeneration in a nonregenerating tissue. And he had the perfect system in which to try: In the newt eye, dorsal pigmented epithelium cells (PEC) can regenerate the lens after injury, but the ventral PEC never do, despite being the same type of cells. The eye has a built-in negative control, and the married duo set out to use it as their testing ground. "We had found a slew of things required for regeneration," says Del Rio-Tsonis: *Pax-6*, a known regulator of mouse eye development was highly expressed during lens regeneration; fibroblast growth factor seemed to play a key role in PEC differentiation into fiber cells and lens morphogenesis; and sonic hedgehog regulated the overall process—without it, no regeneration occurred. "But the question was," Del Rio-Tsonis recalls, "what was sufficient?" ▶

Year after year went by and every combination of growth factors, chemical stimulants, and signaling molecules failed to induce regeneration in the ventral PEC. For each attempt, the researchers removed the dorsal and ventral iris from the eye, transfected the ventral cells in culture with the factors of the day, then aggregated the cells back in the newt eye. "It was a nightmare," recalls Tsonis. Not only are newt eyes tiny, but the animals are unhappy lab guests, difficult to breed and unable to generate knockouts. Finally, it happened. They stumbled on not only one but two recipes for regeneration. Retinoic acid and *six-3*, a gene known to induce the growth of lenses during embryogenesis, were sufficient to cajole lens cells from the ventral iris PEC. In addition, simply inhibiting the bone morphogenetic protein (BMP) pathway, an important patterning pathway in embryogenesis, gave the same result. In 2005, the couple published their results in *Nature*: They had grown a lens where none had grown before.[2]

Tsonis and his colleagues are not the only ones who have regenerated a complex tissue from a nonregenerating source—one group, for instance, performed the same feat with *Xenopus* tails.[3] These classical regeneration researchers, who study the organisms that regenerate naturally, eventually presented their data to scientists working in regenerative medicine, a field tasked with finding methods to repair or replace damaged organs or tissues and often focusing on stem cells and bioengineering. "Evolution has solved the problem," says Peter Reddien, a planarian researcher at the Massachusetts Institute of Technology. "These are organisms that can do exactly what one would want to see applied in regenerative medicine."

But they met silence from the community most likely to use the findings. "A lot of [scientists studying regenerative medicine] don't realize the relevance of amphibian limb regeneration to mammalian repair and regeneration. You'll hear comments like 'What do amphibians have to do with humans?'" says David Stocum, director of the Center for Regenerative Biology and Medicine at Indiana University–Purdue University Indianapolis, who has studied regeneration in the axolotl, a type of tiger salamander, for almost half a century.

But some researchers predict that if scientists studying regeneration in amphibians and other animals joined forces with stem cell biologists and bioengineers, the repair or replacement of human limbs and organs would be possible within years. "I think it took some time for the amazing and interesting information from these classical regenerating organisms to be able to be used in a way that can be transferred to these mammalian or tissue engineering contexts, but I think that's coming," says Elly Tanaka, at the Center for Regenerative Therapies at the University of Technology in Dresden, Germany. And a growing arm of evidence is starting to agree.



▲ (A) Regenerating lens 15 days post-lentectomy. Note that the cells in the posterior part of the vesicle (arrow) elongate as they differentiate to lens fibers. (B) Regenerating lens 20 days post-lentectomy. Note the definite formation of the lens epithelium (yellow), the differentiation of lens fibers at the equator. (C) Regenerating lens 25 days post-lentectomy, depicting the more advanced stage of growth.

COURTESY OF PANAGIOTIS A. TSONIS / THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY

▼ **LENS REGENERATION: Outline of events and pathways likely to be involved in lens regeneration. Upon lens removal or injury, PECs must re-enter the cell cycle and thrombin is suspected to play a role in this. Following proliferation, the PECs dedifferentiate and then redifferentiate. BMP antagonists have been shown to upregulate TFs such as *Six-3*.**

Redrawn with permission from Fig. 1 / Seminars in Cell & Developmental Biology 17 (2006) 753–758

The genes and molecules involved in regeneration in even the most primitive organisms, like the planarian *S. mediterranea*, are often conserved in humans. And in higher-order animals such as amphibians, the similarities to mammals are even more numerous.

With a small piece of rolled-up paper towel, Kazu Kikuchi dabs the fish's belly, soaking up the excess blood. As it wakes from anesthesia, he scoops up the zebrafish with a plastic spoon and drops it into a plastic tank where two previous surgical patients are swimming around. Except for the small red dots on each of their abdomens, it's hard to tell that each fish just had a chunk of its heart removed, part of an experiment at Duke University to tease out the molecular steps behind the species' ability to regenerate its heart.

The headliners of regeneration have abilities that regenerative medicine can only dream about. The newt is the champion of regeneration, able to reconstruct almost any body part, including the brain, spinal cord, heart, and limbs. Planarians, a high-school laboratory favorite, can be sliced to bits and each piece will regenerate a new individual, complete with nervous, muscular, and intestinal tissues. The hydra, a simple freshwater predator the size of a staple, can be experimentally dissociated into single cells, then recombined into clumps that will naturally self-organize and in 2 days form a normal, fully intact animal. Zebrafish, in addition to regenerating the heart, are such experts at fin regeneration that even those who study the fish can't tell the difference between original and regenerated fins.

In humans or zebrafish, a heart injury causes the same initial event: fibrinogen accumulates at the site of the injury and is converted by thrombin into fibrin, a clotting factor that forms a mesh over the wound. In humans, fibrin is replaced by scar tissue over several weeks, forming an irreversible and nonfunctional patch at the site of injury. But in a zebrafish, new cardiomyocytes supplant the fibrin, and in a month the fish boasts a new, functional wall of muscle. But the zebrafish heart can scar, as Kenneth Poss and colleagues at Children's Hospital in Boston described in a 2002 *Science* paper. Zebrafish with a mutation in *mps1*, the gene for a critical cell cycle kinase, do not regenerate their heart. Instead, they form scars, suggesting that regeneration and scarring are competing events in the vertebrate heart.[4] "Regeneration of heart muscle is perhaps ▶

The MRL Mouse

The experiment was ruined, thought Ellen Heber-Katz, staring at a cage of white mice at the Wistar Institute in Philadelphia in the late 1990s. Frustrated, she left the animal facility and went to find the postdoc who was supposed to have punched identification holes in the ears of the mice, half of whom were being treated with a new drug to combat an autoimmune response. The postdoc swore she did. Heber-Katz ordered more of the Murphy Roths Large (MRL) mice, a model for autoimmune disease, and punched their ears again. Over a matter of weeks, Heber-Katz watched with amazement as the holes closed, growing new cartilage and even hair follicles.

Excited, Heber-Katz and colleagues at Wistar and the Hahnemann Medical School, now part of Drexel University College of Medicine, began studying the genetics of the MRL mouse and identified loci controlling the wound healing process. They sent their results to *Nature* and *Nature Genetics*, but in both instances the papers were returned without review. "We had a terrible time getting it published," recalls Heber-Katz. Finally, at the 1998 AAAS meeting in Philadelphia, Heber-Katz was invited to present her work. *Science News* hailed the "super-healing mouse, a rare example of a mammal that regenerates complex, scar-free tissue." But the scientific community remained skeptical. "I remember when she first presented those results, people were saying it's not regeneration," says David Stocum, an axolotl researcher at Indiana University–Purdue University Indianapolis.

Despite the resistance, Heber-Katz and her team quickly extended their studies to other organs and in 2001 published results that an MRL mouse heart heals from cryoinjury in 2 months with ▶

minimal scarring and restored function. "Cardiologists went crazy," says Heber-Katz: no less than six different labs have since published evidence refuting its results. In experiments using cryoinjury and other models of heart damage, researchers have repeatedly found that the MRL mouse heart does not regenerate, does not recover function, and scars.

"It's controversial," says Kenneth Poss, who studies zebrafish heart regeneration at Duke University. "With different groups using slightly different ways of injuring the animals, it can be hard to tell what's going on."

"I'm not 100% sure the MRL mouse is a good model for regeneration," says Panagiotis Tsonis, who studies newt regeneration at the University of Dayton in Ohio. But on this point, the research community remains divided. "I trust her science," says Stocum. "What is regeneration? It's the reproduction of the original tissue architecture, and that's exactly what happens in her ear punch holes. I don't know why people argue about this."

The response to the 2001 heart regeneration paper was so "nasty," says Heber-Katz, that the lab stopped heart research. They are now investigating global mechanisms that might explain the animal's unique abilities, and recently published evidence that adult MRL mice widely utilize aerobic glycolysis, a type of metabolism normally used by embryos, tumors, and stem cells. As most mammals mature, they switch to oxidative phosphorylation, but MRLs stick with aerobic glycolysis. Maintenance of an embryonic-like state through adulthood may be the source of the mouse's regenerative abilities, says Heber-Katz. "Everything I have seen in this mouse is unusual." ■

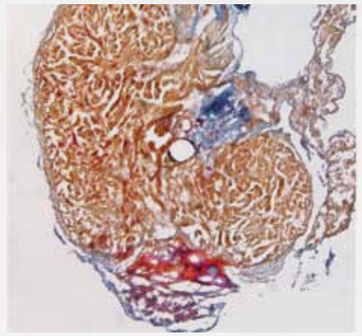
out-competing the process of scar formation in fish," says Poss. "It's reasonable to think that in mammals, if we could tip the balance in the same way and provoke regenerative mechanisms, we might be able to slow or prevent scar formation in human hearts."

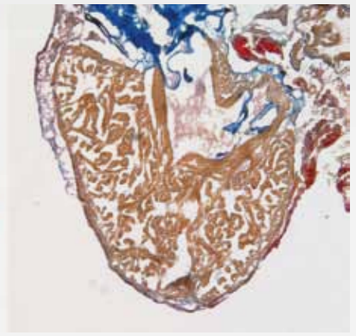
Poss and others have good reason to believe they might one day impose the abilities of regenerating animals onto humans: The genes and molecules involved in regeneration in even the most primitive organisms, like the planarian *S. mediterranea*, are often conserved in humans. And in higher-order animals such as amphibians, the similarities to mammals are even more numerous. For instance, thrombin, a human coagulation protein, is vital for amphibian limb regeneration, as are proteins like glial growth factor and fibroblast growth factor. This widespread overlap, scientists say, suggests they could one day induce these genes to perform the same regenerative functions in humans.

When scientists did an RNA interference screen of more than a thousand planarian genes, by feeding the animals bacteria expressing small, silencing RNAs, they identified 240 that were involved in planarian regeneration, a process driven by neoblasts, pools of adult stem cells that normally replace aging cells in the animal. Of the 240 genes, 85% can be found elsewhere in the animal kingdom and between 60–80% have homologs in humans, says Reddien, first author on the 2005 study.[5] "I'm not surprised a lot of the genes impacting regeneration are conserved because there are a lot of fundamental processes involved in regeneration," he says. "Neoblasts are stem cells, and because stem cells appeared long ago in evolution, we would anticipate that genes involved in controlling the neoblasts are performing similar stem-cell–like functions in humans."

Zebrafish share not only common genes with humans, but common cells. Müller glial cells perform similar support functions in human and zebrafish eyes, yet the zebrafish variants have an added perk: They also act as retinal stem cells.[6] Even the hydra, a tiny, tubular predator with only two body layers, has a lot in common with us. Silencing an evolutionary conserved gene, *Kazal1*, leads to a similar phenotype in both humans and hydra: In hydra, lack of the gene causes autophagy in gland cells—they fill with vacuoles, being slowly degraded internally by digestive enzymes. The cells subsequently do not survive the stress of amputation.[7] A homologous enzyme mutated in humans has been linked to excessive autophagy in the pancreas, causing chronic pancreatitis. "The same gene is doing the same job in our pancreas and in hydra," says Brigitte Galliot, a researcher at the University of Geneva and senior author on the paper. When the gene is silenced, "hydra become like you and me. You cut them in two pieces, and they die."

Most regeneration researchers think that mammals do not lack the capacity to regenerate, but instead have brakes on the system. "The genes that comprise those pathways are there," says Stocum. "It's just a matter of turning them on." If researchers can discover differences in how the genes are regulated in regenerating and nonregenerating animals, than perhaps they can flip the switch. "That's the goal of what we do, to show how natural regeneration works in a nice model and for that to provide fruit for the field, especially those who are working in mammalian systems," says Poss.





Zebrafish hearts regenerate. Within 30 days after resection of the tip of the ventricle, the blood clot sealing the wound disappears and no scar remains.

KRISTIN SCHNABEL & GILBERT WEIDINGER / UNIVERSITY OF TECHNOLOGY DRESDEN.

But despite the numerous overlaps between mammals and classical regenerating models, the field has not yet become a major contributor to regenerative therapeutics, in part due to poor tools and funding. Not only do many of the animals have glacial generation times compared to fruitflies and mice (a newt reaches sexual maturity at 5 years old), researchers cannot make traditional knockouts using newts or zebrafish, as the organisms do not have the embryonic stem cells required for the experiment. And few of the organisms' genomes, including the newt's, have been sequenced. The tools needed to advance the field just haven't been available, says Del Rio-Tsonis. "For a very long time, we were limited to expression studies, so we are a little bit behind on being able to contribute more functional studies." Poss agrees: "It appears to be taking some time to catch up to other fields," he says, such as stem cells and bioengineering. "Truthfully, [the field] hasn't lit up as quickly as I would have predicted."

The egg cracks, and Katia Del Rio-Tsonis leans forward off her stool, only the tips of her toes touching the ground. Christian Gutierrez, a postdoc in her lab, peels off the thin shell, opening a small window to the chicken (*Gallus domesticus*) embryo floating below. "There, do you see that?" asks Del Rio-Tsonis. She points a finger toward a television screen at the magnified image of a round, dark embryonic chick eye. "That looks great." With miniature scissors, Gutierrez cuts through the retinal pigmented epithelium, a dark disc surrounded by pale pink tissue streaked with blood vessels. Lifting it back, he grasps some invisible structure with forceps, and tugs. The chick retina slips out of its membrane, and Gutierrez lifts it from the egg. It is barely visible—a clear sliver of gelatin, like a thick contact lens. Setting the retina aside, Guiterrez places a small bead into the eye, a condensed pellet that will slowly secrete fibroblast growth factor (FGF) to induce the regeneration of a new retina. This is the baseline experiment—the scientists will also manipulate other pathways, such as Shh, Wnt and BMP, to see how they affect regeneration with and without FGF present, and ultimately uncover the right recipe for retinal regeneration. "If we can induce regeneration at later stages," when the embryonic chick retina does not normally regenerate, says Del Rio-Tsonis, "that will give us the green light to try that mechanism in the mouse."

In 1999, still in the midst of their lens regeneration work, Del Rio-Tsonis left Tsonis's lab for her own at Miami University in Ohio, an hour's ride from Dayton. There, she decided to tackle retinal regeneration, this time using a simpler animal model. "Retinal regeneration is challenging for the newt," she laughs—the amphibian takes between 45 and 65 days to complete the process. But in the self-contained embryonic chick, which is cheaper and easier to manipulate than a newt, the process takes only 7 days.

The embryonic chick regenerates its retina only during a short period of development and by two mechanisms. An embryonic chick can regenerate a retina by either the proliferation and differentiation of stem cells present in the ciliary margin, a small zone at the edge of the iris, or by transdifferentiation of the retinal pigmented epithelium (RPE), a layer of dark cells wrapped around the retina. Scientists have not determined which mechanism drives regeneration in amphibian and fish limbs and fins, respectively. But transdifferentiation, a pre- ▸

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viously obscure cellular process, has found recent fame thanks to research in a nonregenerating animal—the human.

"I had an undergraduate interested in working with me in the summer, and I gave her one of my reviews," recalls Del Rio-Tsonis. "She has just taken a stem cell course, and she was talking to me about what she read in the review. I looked down and saw she had written on the side of the paper 'natural iPS.'" Del Rio-Tsonis laughs. "She got the message."

In the summer of 2006, Shinya Yamanaka of Kyoto University in Japan showed that cultured human skin cells could be reverted to a pluripotent embryonic state by inserting four factors: Oct4, Sox2, c-Myc and Klf4. Reprogramming became a laboratory buzzword, but in some labs it had already been known by another name—transdifferentiation. "There's all this talk about iPS," says Stocum, "and here's the salamander, who knows how to do it naturally."

Last year, Tsonis and Del Rio-Tsonis's labs decided to compare the process of reprogramming in newt and human iPS cells. In recently published results, they demonstrated that three of the four famous iPS factors, Sox-2, c-myc, and Klf4, are expressed during limb and lens regeneration in the newt.[8] Tsonis is now testing the one factor that wasn't present, Oct4, to see what effect it might have on newt cells. Will Oct4, like the retinoic acid and *six-3*, be sufficient to induce lens regeneration from the ventral PEC? "It we succeed, this will be spectacular. It will unite stem cells and transdifferentiation," he says, thereby showing that a small number of factors have the same effect on both types of cells, becoming a keystone in the bridge between newts and mammals.

Slowly, there are signs that the stem cell community is recognizing such overlaps, such as infrequent collaborations. "I think the problem is that we haven't had the motivation—translate that into 'grant money'—to embark on an effort to talk to each other," says Jeanne Loring, a human embryonic stem cell researcher at the Scripps Research Institute in California. Recently, a colleague encouraged Loring to speak with regeneration researchers about a systems biology project to find common mechanisms between hES cells and those involved in regeneration. "It was like a date," she laughs. "We speak different languages, but we came up with common viewpoints." (The six researchers—from Caltech, UC Irvine, Scripps, and UC San Diego—did not receive funding from their first grant application, although they will apply again, says Loring.) Poss, in turn, works closely with Duke cardiologists to compare his models of zebrafish heart regeneration to mice. "I do think genetics, biochemistry, engineering, and stem cells are all blending into a regenerative medicine movement," he says.

Back in Ohio, Del Rio-Tsonis sits cross-legged on her stool, again chatting about what factors might induce retinal regeneration in chick embryos. Maybe FGF and Wnt together. Maybe activin, a retinal maintenance factor that showed success in another lab. "It's a balancing act," she says. Gutierrez listens as he sticks a piece of tape over the hole in the egg and places the embryo, sans retina, back in its Styrofoam incubator. Del Rio-Tsonis stands, and Gutierrez switches off the microscope. As they head toward the door, Del Rio-Tsonis looks back. "We have to find the right recipe," she says to herself. "We need to push the system." ■

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Have a comment? E-mail us at mail@the-scientist.com

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