

HUMAN EMBRYO five to six days after fertilization, called a blastocyst, is opened to retrieve the inner cell mass (*red bulge*) that produces embryonic stem cells.

Stem cells raise the prospect of regenerating failing

body parts and curing diseases that have so far defied drug-based treatment. Patients are buoyed by reports of the cells' near-miraculous properties, but many of the most publicized scientific studies have subsequently been refuted, and other data have been distorted in debates over the propriety of deriving some of these cells from human embryos.

Provocative and conflicting claims have left the public (and most scientists) confused as to whether stem cell treatments are even medically feasible. If legal and funding restrictions in the U.S. and other countries were lifted immediately, could doctors start treating patients with stem cells the next day? Probably not. Many technical obstacles must be overcome and unanswered questions resolved before stem cells can safely fulfill their promise.

For instance, just identifying a true stem cell can be tricky. For scientists to be able to share results and gauge the success of techniques for controlling stem cell behavior, we must first know that the cells we are studying actually possess the ability to serve as the source, or "stem," of a variety of cell types while themselves remaining in a generic state of potential. But for all the intensive scrutiny of stem cells, they cannot be distinguished by appearance. They are defined by their behavior.

Most versatile are embryonic stem (ES) cells, first isolated in mice more than 20 years ago. ES cells come from the portion of a very early-stage embryo that would normally go on to form three distinctive germ layers within a later embryo [see illustration on page 95] and ultimately all the different tissues of the body. ES cells retain this potential ability to produce any cell type in the body, making them pluripotent.

Most of the existing human ES cell lines in the world were derived from unused embryos created for couples seeking in vitro fertilization (IVF). Researchers working with these cells have found that they usually recover after freezing and thawing and can differentiate into assorted cell types in a culture dish. But it is becoming clear that not all human ES cell lines are the same.

The Stem Cell Challenge

What hurdles stand between the promise of human stem cell therapies and real treatments in the clinic?

By Robert Lanza and Nadia Rosenthal

Seeking Stemness

SOME LINES WILL differentiate into only certain cell types; others grow sluggishly in culture. To ensure that these cells are pluripotent before using them in research, two possible tests, already common in nonhuman ES cell studies, have been proposed by a group of American and Canadian biologists hoping to set standards for experimentation with human ES cells. One would involve injecting the ES cells into an animal's body tissue. If they form a teratoma—a distinctive tumor containing cell types from all three embryonic layers—their pluripotency is proved. Another way to test putative ES cells is to mark them, then inject them into a developing animal embryo. When the animal is born, if the marked cells turn up in all its tissues, the cell line is deemed pluripotent. But testing human embryonic stem cells in this manner would create a chimeric animal with human DNA throughout its body, a prospect many find ethically troubling. What is more, passing the latter test does not always guarantee that the cells will differentiate in the lab.

The need to find more reliable markers that distinguish truly pluripotent ES cells is driving widespread attempts to catalogue the genes that are turned on or off at various times in cultured ES cells. Having such a gene expression profile would not only provide a way of identifying pluripotent ES cells, it would also offer tremendous insight into the properties that confer their "stemness." Unfortunately, to date, gene expression profiles of ES cells have yielded conflicting results, and the search for a clear ES cell signature continues.

Of course, the goal of stem cell research is to replace or regenerate failing body parts, such as pancreatic insulin-producing cells in diabetics or dopamine-producing neurons in people with Parkinson's disease. But techniques for coaxing ES cells to differentiate into desired cell types are far from perfected.

Left to their own devices in a culture dish, ES cells will spontaneously differentiate into a hodgepodge of tissue types. With timed administration of chemicals, we can often direct them to become one cell type or another. But they seem to prefer to become certain tissues—readily proliferating into patches of beating heart cells, for example—whereas other tissues are far more difficult to derive.

Putting Cells to Work

BECAUSE WE STILL do not understand the signals that normally instruct these cells to choose a particular pathway during embryonic development, many researchers are studying the natural embryonic "niche" to understand possible environmental cues. Other scientists are trying to profile embryonic cells' gene expression patterns as they differentiate in order to find genes that could be turned on or off to direct the cells toward a particular tissue type.

But deriving what appear to be cells of the desired kind is just half the battle. ES cells will easily produce dishes full of neurons, for instance, but these are only useful if they can be placed in a living brain, make connections and "talk" with surrounding neurons. In 2001 stem cell researchers thought they had a major breakthrough when Ronald McKay of the National Institutes of Health report-

ed having generated insulin-producing cells—a coveted goal in stem cell research—from mouse ES cells. Last year, though, Douglas A. Melton of Harvard University reproduced McKay's experiment and found that the cells had absorbed insulin from their culture medium rather than producing it themselves. Discovering markers to identify truly functional cells is another urgent task for the stem cell research community.

It would be ideal if we could simply inject ES cells into the part of the body we wish to regenerate and let them take their cues from the surrounding environment. ES cells' pluripotency, however, makes this far too dangerous an approach for human therapy. The cells might form a teratoma or could differentiate into an undesirable tissue type, or both. In animal experiments, teratomas containing fully formed teeth have been reported.

Rather than risk creating a tumor or a tooth in a patient's brain or heart with direct ES cell injections or struggling to produce specific functional tissues, many ES cell researchers are now striving for a middle ground. By coaxing ES cells into a more stable, yet still flexible, progenitor-cell stage before administering them, we can avoid uncontrolled differentiation while still taking advantage of environmental cues to generate the desired cell types.

Even though these progenitor cells can take to their environment and initiate the generation of new tissue, they would still be subject to attack by the patient's own body. ES cells and their derivatives carry the same likelihood of immune rejection as a transplanted organ because, like all cells, they carry the surface proteins, or antigens, by which the immune system recognizes invaders. Hundreds of combinations of different types of antigens are possible, meaning that hundreds of thousands of ES cell lines might be needed to establish a bank of cells with immune matches for most potential patients. Creating that many lines could require millions of discarded embryos from IVF clinics.

Some researchers have speculated that such an extensive bank might not be necessary, that patients can be desensitized to ES cell derivatives or that the

Overview/*Stem Cell Prospects*

- The possibility of replacing or regenerating failing body parts with new tissues derived from stem cells has provoked hope, controversy and conflicting scientific claims.
- Embryonic stem cells offer primordial potential, but scientists are still struggling to understand and control them. Stem cells in the adult body may be easier to marshal for some tasks, but their true origin and range of abilities are still unresolved.
- Many hurdles, both scientific and political, remain before stem cell treatments can be widely applied to patients.

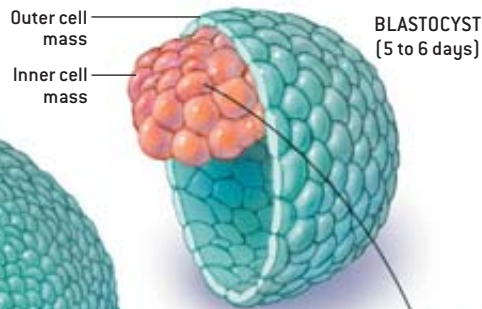
WHAT ARE EMBRYONIC STEM CELLS?

Embryonic stem (ES) cells are derived from the portion of a very early stage embryo that would eventually give rise to an entire

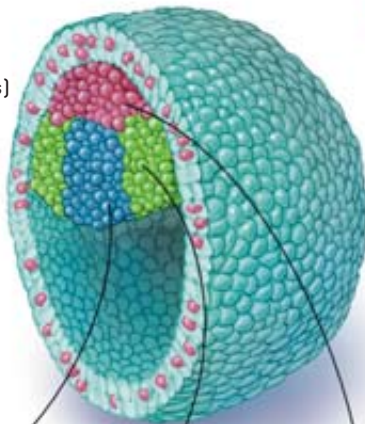
body. Because ES cells originate in this primordial stage, they retain the “pluripotent” ability to form any cell type in the body.

CELL FATE

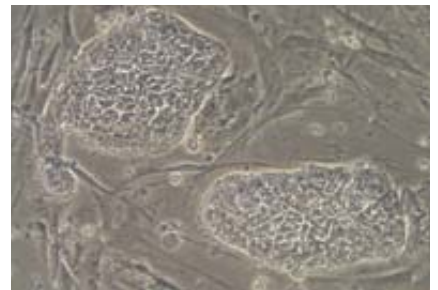
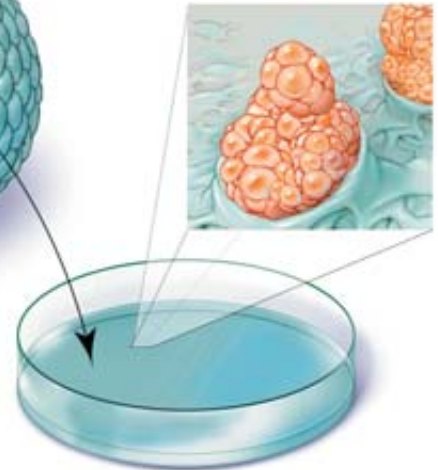
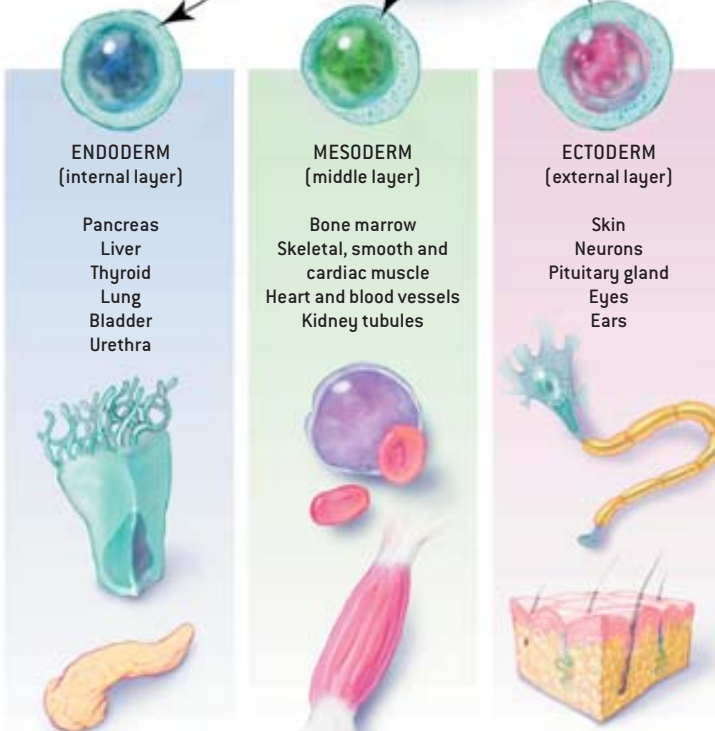
Less than a week after a human egg is fertilized, the developing embryo contains about 100 to 150 cells that have yet to differentiate. The embryo is a hollow ball, called a blastocyst, consisting only of an outer cell mass, which in a pregnancy would later form the placenta, and an inner cell mass (ICM), which would become the fetus. Inside a womb, these cells would continue multiplying, beginning to specialize by the third week. The embryo, called a gastrula at this stage, would contain three distinctive germ layers whose descendants would ultimately form hundreds of different tissue types in the human body.



GASTRULA (14 to 16 days)

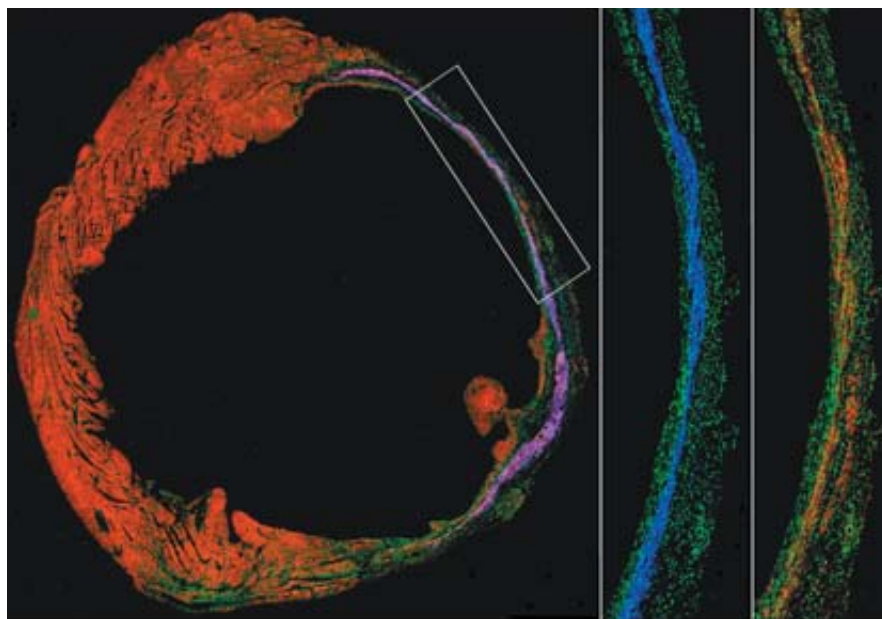


EMBRYONIC GERM LAYERS AND SOME OF THE TISSUES IN THEIR LINEAGES



MAKING EMBRYONIC STEM CELLS

To create ES cell lines, scientists remove the inner cell mass from a blastocyst created in the laboratory, usually left over from an attempt at in vitro fertilization. The ICM is placed on a plate containing feeder cells, to which it soon attaches. In a few days, new cells grow out of the ICM and form colonies (above). These cells are formally called embryonic stem cells only if they meet two criteria: they display markers known to characterize ES cells, and they undergo several generations of cell division, or passages, demonstrating that they constitute a stable, or immortalized, cell line.



MOUSE HEART in cross section shows the left ventricle, where 38 percent of tissue destroyed by myocardial infarction [*first frame, rectangle*] was repaired by cloned stem cells within a month. Close-ups of the damaged area display cloned cells (*blue*) and new heart cells (*red*).

antigenic properties of the cells themselves can be reduced. But those feats have yet to be conclusively demonstrated. At present, the only sure way to circumvent the problem of immune rejection would be to create an ES cell line using a patient's own genetic material through nuclear transfer or cloning. This technique has inspired considerable controversy and has its own practical hurdles to overcome, but it has also produced encouraging results in animal experiments for regenerating failing tissues.

Turning Back the Clock

CLONING CAN BE VIEWED as a way to restore embryonic potential to a patient's old cells. The human body is made of more than 200 kinds of cells, and in mammals, once a cell is committed to a particular type, there is normally no turn-

ing back. It is said to be "terminally differentiated." An exception to this rule is when the nucleus containing an unfertilized egg's genetic material is extracted and the nucleus of a somatic (body) cell is placed into the egg instead. The egg is tricked into behaving as though it has been fertilized and begins dividing like a normal embryo. The ES cells derived from this embryo will contain the donor somatic cell's DNA. But the somatic cell will have been reprogrammed—reset to a state of stemness, capable of generating any tissue type.

One of us (Lanza) recently showed that partially differentiated stem cells from a cloned mouse embryo could be injected into the donor mouse's heart, where they homed in on the site of injury from a heart attack, replacing 38 percent of the scar with healthy heart tissue within a month [*see illustration above*]. And this

year, for the first time, somatic cell nuclear transfer (SCNT) yielded a human ES cell line. A few in the scientific community had started to wonder whether the nuclear-transfer technique would work with primate physiology to produce therapeutic stem cells. But Woo Suk Hwang of Seoul National University and his colleagues proved that it could be done. The Korean team announced this past February that they had created a human embryo through SCNT, grew it into a blastocyst and derived a pluripotent ES cell line. Their accomplishment represents a major milestone. It also demonstrates how many unknowns we still face.

Because Hwang's group had 242 donated eggs to work with, they were able to experiment with techniques, timing and conditions at every step. Even so, from hundreds of eggs the effort yielded only a single ES cell line, and the researchers have said that they are not certain which of their methods was responsible for that success. Much remains to be learned about the mysterious mechanism of reprogramming within the egg and all that could go wrong while creating and culturing a nuclear-transfer embryo.

Scientists are still not sure whether reprogramming itself or other aspects of handling these embryos might introduce gene mutations that could predispose the resulting ES cells to senescence or cancer, and more research is needed to detect these potential problems. Inherited gene mutations, such as those that cause hemophilia or muscular dystrophy, would have to be corrected as well before using a patient's own cells to create ES cells. But techniques for gene-specific modifications routinely performed in mouse ES cells have been successfully applied to human ES cells, providing a means of safely correcting mutations before administering cells to patients.

The overall health of ES cells derived from clone embryos has also been questioned because efforts to produce live animals through cloning have met with an unusually high rate of deformities and mortality. When a cloned ES cell line's potential is tested by injecting the cells into a developing animal blastocyst, though, the resulting animals seem to be

THE AUTHORS

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POLITICS: THE BIGGEST OBSTACLE OF ALL

Research involving stem cells from the adult body is uncontroversial and unrestricted. But the versatility of adult stem cells is also the least proved. Many scientists believe that embryonic stem (ES) cells will provide more powerful treatments but that the greatest obstacle to assessing and harnessing the potential of ES cells is a lack of freedom and funding to do the work.

In the U.K., Singapore, South Korea, China, Japan and a handful of other nations, research on ES cells enjoys generous government support. The European Parliament, however, has been struggling to agree on a policy, leaving member countries to decide their own rules for now. A United Nations effort to draft a global convention has been deadlocked for two years.

U.S. scientists have been laboring under a partial ban decreed three years ago by President George W. Bush. Any researcher receiving government funding—the vast majority in both academia and industry do receive some kind of government grant money—may work only with embryonic stem cell lines created before the policy was announced in August 2001. The grantees can get federal support, but less than \$20 million of the National Institutes of Health's \$27-billion budget for 2003 actually went to fund studies using those so-called presidential cell lines.

The situation might as well be a total ban, according to many scientists. At present, only about 15 of the presidential lines are even available to researchers. Some of those are sickly and difficult to cultivate; others have started displaying genetic abnormalities. And all have spent time on a culture medium containing mouse cells, creating a possibility of contamination by nonhuman viruses. The U.S. Food and Drug Administration is now considering whether to allow clinical trials with these cells.

Since 2001, techniques for keeping ES cells alive have improved considerably, and scientists and their supporters in Congress have been clamoring for permission to produce new healthy lines. Some have not waited. Douglas A. Melton of Harvard University, whose two children have type 1 diabetes, is an outspoken critic of the current policy, and in February he announced that he had created 17 brand-new ES cell lines with private funds. He is making the lines freely available to researchers, but most investigators in the U.S. cannot afford to

follow government regulations as Melton did by setting up a separate lab for his ES cell work, without so much as a federally funded pipette in it.

A trend toward private funding of ES cell research may make it possible for more U.S. scientists to participate. Andrew S. Grove, founder of Intel, gave \$5 million to the University of California at San Francisco to make new ES cell lines. Stanford University started an institute to study cancer using ES cells with a \$12-million anonymous grant. The Howard Hughes Medical Institute and the Juvenile Diabetes Foundation funded Melton, and the Michael J. Fox Foundation for Parkinson's Research has given more than \$5 million to institutions and individual researchers. But the political climate has driven many

scientists away from the field entirely and has dampened investor enthusiasm, leaving some biotechnology firms struggling, too.

A few states are trying to turn the tide. Recognizing the potential windfall if ES cell research pays off, California was the first state to endorse stem cell studies officially, in 2002, and will hold a referendum in November seeking \$3 billion in state funding for scientists. New Jersey added its endorsement last year and has promised \$50 million over five years for the state's researchers.

—Christine Soares



DOUGLAS A. MELTON of Harvard University created 17 ES cell lines from donated IVF embryos in a basement laboratory separate from his government-funded research. He will co-direct a new stem cell institute for which Harvard is seeking to raise \$100 million.

perfectly normal. This outcome suggests that although reproductive cloning is clearly too unpredictable to consider for humans, ES cells derived by nuclear transfer, at least for therapeutic purposes, are equivalent to regular ES cells.

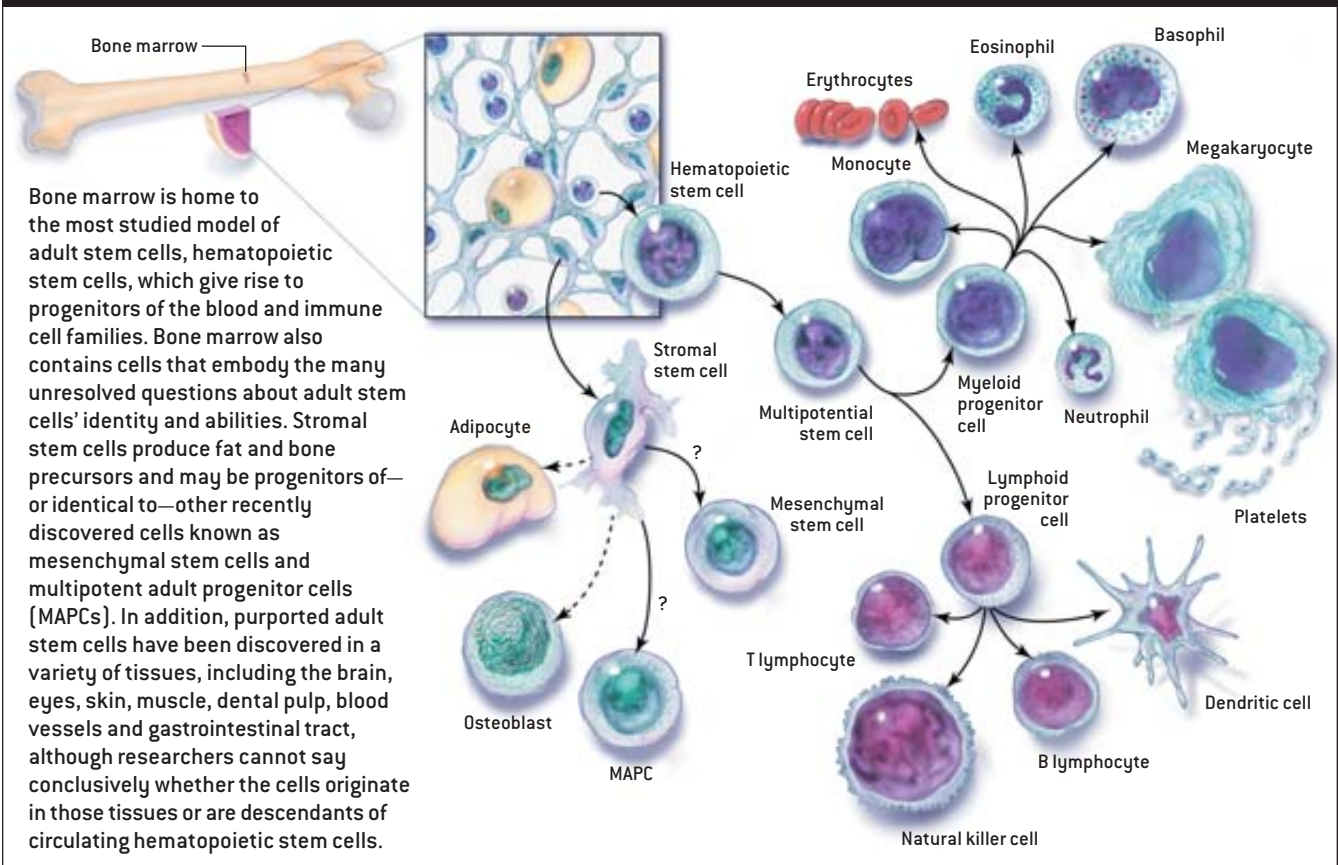
Similar safety questions must also be resolved for a different technique that produces ES cells without nuclear-transfer or IVF embryos. In a process called parthenogenesis (from Greek for “virgin birth”),

an unfertilized egg can be chemically tricked into beginning cell division as though it has been fertilized. These pseudo-embryos, or parthenotes, are considerably easier to grow than nuclear-transfer embryos. In animal studies, parthenotes have yielded ES cells able to differentiate into multiple tissue types in culture and to pass the teratoma test, forming cells from all three embryonic germ layers.

Unlike normal body cells, which con-

tain a set of chromosomes from each parent, parthenotes contain a doubled set of the egg donor's chromosomes. This duplication gives a parthenote a full complement of genes but prevents it from being viable if it were implanted in a woman's womb. Having a single “parent” also means that parthenote cells carry half the normal potential combinations of antigens, making them much easier to match to patients. A bank of fewer than

STEM CELL STOREHOUSE



1,000 parthenogenic ES cell lines could probably provide immunological matches for most of the U.S. population.

How long it will take for any ES cell therapies to be tested in humans will be determined as much by politics as by the remaining scientific questions [see box on preceding page]. Well-understood and easy-to-control cell types derived from ES cells, such as dopamine-producing neurons or the eyes' retinal pigment epithelium cells, could be ready for human trials in less than two years. In the meantime, the extraordinary regenerative potential of embryonic stem cells has intensified the search for similar cells that may be involved in normal healing in the adult body.

Hidden Potential?

SKIN BEGINS REPAIRING itself immediately after being injured. The human liver can regenerate up to 50 percent of its mass within weeks, just as a salamander regrows a severed tail. Our red blood cells are replaced at a rate of 350 million per minute. We know that prolific stem

cells must be at work in such rapidly regenerating tissues. But their very vigor raises questions about why other organs, such as the brain and heart, seem incapable of significant self-repair, especially when purported stem cells have also recently been discovered in those tissues.

The best-known stem cells in the adult body are the hematopoietic stem cells found in bone marrow, which are the source of more than half a dozen kinds of blood cells. Their ability to generate a variety of cell types, at least within a specific tissue family, is why hematopoietic stem cells have been described as multipotent.

There is great hope that similar multipotent stem cells found in other body tissues might be drafted into repairing damage without the need to involve embryos—or better still, that an adult stem cell with more versatility, approaching the pluripotency of embryonic cells, might be discovered.

But scientists are just beginning to investigate whether natural regeneration is

somehow blocked in tissues that do not repair themselves easily and, if so, whether unblocking their regenerative capacity will be possible. The very source, as well as the potential of various adult stem cells, is still disputed among researchers. We cannot say for sure whether tissue-specific adult stem cells originate within those tissues or are descendants of circulating hematopoietic stem cells. Nor do we know how far these cells can be pushed to differentiate into functional tissues outside their specific type or whether such transdifferentiation produced in the laboratory could be reproduced in a living organism.

The idea that certain adult stem cells might have greater potential first came from observations following human bone marrow transplants, when donor cells were subsequently found in a wide range of recipients' tissues. These accounts implied that under the right conditions, stem cells from the bone marrow could contribute to virtually any part of the body. (Similar claims have been made for the so-called fetal stem cells found in umbilical

cord blood, which resemble hematopoietic stem cells.)

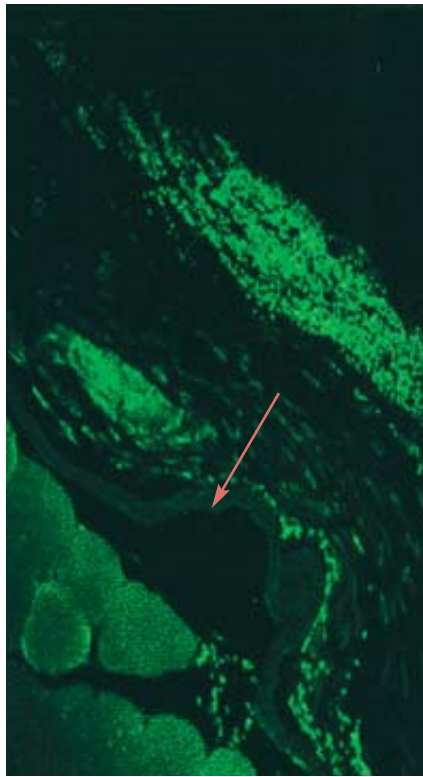
Attempts to directly test this theory in living organisms, however, have not found consistent evidence of such plasticity. In March separate reports from Leora Balsam and her colleagues at Stanford University and from a group led by Charles E. Murry of the University of Washington both described using powerful tracking methods to see if hematopoietic stem cells would incorporate into injured heart muscle, a nonhematopoietic tissue. Neither group detected contribution of new tissue by the stem cells.

What has increasingly been found is extensive fusion of bone marrow stem cells to cells in the heart, liver and brain, offering an alternative explanation for the presumed transdifferentiation. In future studies of adult stem cell potential, it will be crucial to rule out the possibility that stem cells are merely fusing to local cells rather than generating new ones.

Still, tissue-specific cells have already produced encouraging results. In the German TOPCARE-AMI study of patients with severe heart damage following myocardial infarction, the patients' own heart progenitor cells were infused directly into the infarcted artery. Four months later the size of the damaged tissue swath had decreased by nearly 36 percent, and the patients' heart function had increased by 10 percent.

The small number of stem cells that can be isolated from any adult tissue remains the biggest technical hurdle to applying this type of research more widely in the clinic. In mouse bone marrow, stem cells are as rare as one in 10,000, and the ratio may be even greater in humans. In most tissues, there is no predictable location for stem cells, and we possess only limited tools for identifying them using surface markers or gene expression signatures.

Once isolated, adult stem cells are also notoriously slow and labor-intensive to grow. As is true of embryonic cells, so little is understood about the factors that may control the adult stem cells' fate that we do not yet know whether extensive time spent in culture could harm their ability to restore tissues in patients.



BONE MARROW STEM CELLS marked with green fluorescence migrated to leg muscle that was producing the injury-alarm protein IGF-1 [insulinlike growth factor]. The stem cells homed toward the damaged area (arrow), where they initiated tissue regeneration.

Rather than hunting for a patient's stem cells to remove, cultivate and then replace them, we may be able to summon the body's hidden stores. Increasing evidence suggests that stem cells, like metastatic tumor cells, respond to common chemical signals leading them to sites of injury. One of us (Rosenthal) recently showed in mice that stem cells will travel great distances to reach an injury when summoned with the help of a protein called IGF-1 [see illustration above].

Marshaling the body's own ability to trigger tissue regeneration by stem cells will require a better grasp of the roles played by such chemical signals. Rosen-

thal and her collaborator Antonio Musarò have demonstrated that IGF-1 helps to beckon stem cells, but we suspect that this molecule may also take part in causing some of the injured cells to revert to a multipotent state and begin differentiating into the required tissue types. This phenomenon, known as epimorphic regeneration, underlies the ability of newts and zebra fish to regrow entire limbs and organs.

Regenerative medicine's ideal would be to find a means to cause such controlled dedifferentiation of adult tissue—in essence turning a terminally differentiated cell back into a stem cell. Many researchers are looking for the magic molecules that can produce this transformation, and some very preliminary successes have recently been reported. But therapeutic regeneration through dedifferentiation is a long way off and will most likely come from a much better understanding of stem cells themselves—both adult and embryonic.

Which Way Forward?

AS OFTEN HAPPENS in science, stem cell research has raised as many new questions as it has answered, but the field is advancing. Early tests of human adult stem cells in treating cardiovascular disease are encouraging and will certainly lead to more extensive trials in the near future. Given much promising experimental evidence in animals, therapeutic trials of human ES cell derivatives in neurodegenerative disease are probably imminent.

As the appropriate source of cells for both research and eventual therapeutic applications continues to be hotly debated, restrictions on this research are slowing progress. But we believe that generating replacement cells and regenerating organs are feasible and realistic goals. The remaining hurdles are difficult but not insurmountable. SA

MORE TO EXPLORE

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