

REVIEW ARTICLE

MEDICAL PROGRESS

Prometheus's Vulture
and the Stem-Cell Promise

Nadia Rosenthal, Ph.D.

WHEN PROMETHEUS TRANSGRESSED THE LAW OF THE ANCIENT GODS and stole fire for humankind, to teach them civilization and the arts, his punishment was typically brutal. Jupiter had the great Titan chained to the side of Mount Caucasus, where a vulture preyed daily on his liver, which was renewed as quickly as it was devoured.

We mere mortals do not possess livers with quite so vigorous a regenerative capacity, but the legend captures well the remarkable potential of the body to rebuild itself. Throughout our lives we sustain less gruesome injuries from which we recover spontaneously, often without realizing we were hurt. Wound healing involves the recruitment and proliferation of cells capable of restoring tissues and even organs to their original form and function. These cells must retain a collective memory of the complex developmental process by which the tissue was first constructed. Fortunately for Prometheus, whose name means forethought, his liver was well prepared for its daily renewal, since it is one of the most highly regenerative organs of the human body.

What determines the healing potential of an injured tissue? If Prometheus's vulture had chosen a different organ on which to dine — the heart, for instance — the hero might not have survived his ordeal. Until recently, it was presumed that aboriginal populations of undifferentiated, self-renewing progenitor cells contributed exclusively to the regeneration of the organ in which they resided. This restriction of progenitor-cell activity neatly explained why some organs could not regenerate as well as others: they simply did not maintain a sufficiently robust population of progenitor cells.

This concept of organ-specific regeneration has been challenged by discoveries that multipotent cells can be isolated from many tissues of the body, even from some, such as the nervous system, that have historically been considered incapable of regeneration. Loosely referred to as stem cells, these cells in the adult body resemble pluripotent cell populations derived from the early embryo, which can contribute to virtually any type of tissue under appropriate experimental conditions. In the past two decades, embryonic stem cells have been the focus of intense study. It is important to note that embryonic stem cells originate from undetermined early embryos, with no possible history of differentiation, whereas the provenance of adult stem cells found in mature tissues is far less well understood. Nevertheless, our imaginations have been captivated by the possibility that our bodies have retained a population of reserve stem cells, perhaps set aside during gestation, that might be coerced into renewed regenerative service later in life. And if we cannot become Titans using our own stem-cell resources, perhaps we can resist the onslaught of time's vulture by transplanting pluripotent cells derived from early embryos to supplement our own waning supplies of stem cells.

EMBRYONIC STEM CELLS EXPLAINED

The flurry of recent studies touting the near-miraculous properties of stem cells underscores the need for more rigorous definitions. A true stem cell must satisfy several

From the European Molecular Biology Laboratory Mouse Biology Programme, Rome. Address reprint requests to Dr. Rosenthal at Mouse Biology Programme, the European Molecular Biology Laboratory, Via Ramarini 32, 00016 Monterotondo, (Rome), Italy, or at rosenthal@embl-monterotondo.it.

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operational criteria. First, it must be clonogenic, capable of unlimited self-renewal by symmetric division. Second, it must be able to divide asymmetrically, one daughter resembling its mother, the other daughter giving rise to multiple types of differentiated cells representing all three primitive embryonic germ layers (the ectoderm, mesoderm, and endoderm). Third, it must originate from an embryonic or adult stem-cell source. Thus, stem-cell reservoirs in the hematopoietic compartment can contribute to other tissue lineages, such as the pancreas or liver.^{1,2}

A culture of embryonic stem cells derived from the inner cell mass, or embryoblast, of an embryonic blastocyst satisfies all three criteria (Fig. 1). These differentiated derivatives of embryonic stem cells must retain a normal chromosomal complement and must maintain their functional properties when placed *in vivo*. Candidate embryonic stem cells can be tested in mice by using markers on embryonic stem cells used to form chimeric embryos and determining the contribution of these identifiable cells to the different types of tissue in the resulting mouse pups.^{3,4} In another test, embryonic stem cells are injected into immunodeficient mice to produce teratomas, which are themselves pluripotent in cell culture and which contain differentiated de-

rivatives of all three germ layers if left in the host.⁵ A third approach, only successfully achieved with mouse, primate, and human embryonic stem cells, involves inducing the cells to differentiate in culture to form embryoid bodies containing multiple types of cells.⁶⁻⁸

One of the complex technical issues surrounding the isolation and propagation of embryonic stem cells *in vitro* is the identification of the proper culture conditions, which can keep the cells in an undifferentiated state or induce their differentiation into a particular type of cell. In addition to soluble factors, plastic surfaces can affect cell morphology, cell density can influence interactions between cells, and the transfection of differentiation-inducing genes can help guide the pluripotent embryonic stem cell to a specific cell fate. This process is more an art than a science, and current studies rarely satisfy both of the operational criteria for embryonic stem cells. Yet with the use of these techniques, single mouse precursor cells cultured from the inner cell mass have been induced to generate multiple types of cells, including vascular,⁹ neuronal,¹⁰ and pancreatic¹¹ precursors and even haploid oocytes.¹²

A parallel set of studies has focused on human embryonic germ cells, isolated from the fetal gonadal ridge of 8-to-10-week-old embryos.¹³ Like embryonic stem cells, embryonic germ cells are highly proliferative, and when differentiated in culture, they express multiple differentiation-specific markers. Although to date embryonic germ cells have been shown to be viable for only 70 to 80 passages in culture, a potential advantage is that they do not form teratomas when injected into mice, and they may therefore represent a safer source of transplantable tissue.

Although similar studies involving human cells date only from 1998, when human embryonic stem cells were first successfully propagated,^{8,13} this milestone relied heavily on the gradual progress made in the previous 20 years,^{14,15} when mouse and primate embryonic stem cells were also being extensively characterized.¹⁶ Generating cultures of embryonic stem cells from early human embryos that were not used for *in vitro* fertilization procedures required certain adjustments, such as delaying the dissociation of the embryo until five days after conception, a time when normal human implantation would occur. The requirement for mouse feeder-cell layers in cultures of human embryonic stem cells was only recently overcome.¹⁷ Nevertheless, the past five years have witnessed an exponential increase in

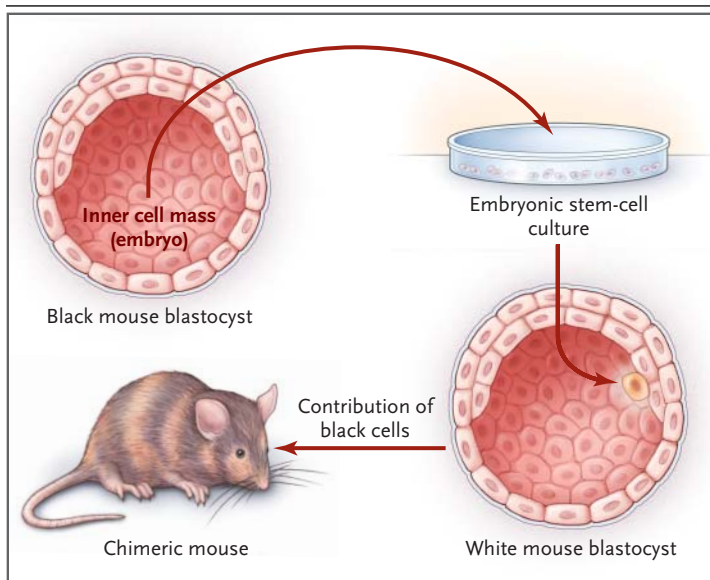


Figure 1. The Origin and Pluripotency of Embryonic Stem Cells.

The inner cell mass of a blastocyst from a black mouse is transferred to tissue culture, and the resulting embryonic stem cells are inserted into the blastocyst of a white mouse. This produces a chimeric offspring in which some of the cells in each tissue are derived from the cultured embryonic stem cells.

experiments aimed at generating human tissues for transplantation, such as pancreatic endocrine cells, skeletal myocytes, and neurons. In a recent test of the therapeutic potential of embryonic stem cells,¹⁸ dopamine-producing neurons were derived from embryonic stem cells expressing a transgenic neuronal determinant, and they established appropriate electrophysiologic connections when they were injected into the brains of rats with symptoms of Parkinson's disease. Remarkably, motor function was improved, with no associated tumor formation, removing yet another stumbling block to therapies based on embryonic stem cells.

Although human embryonic stem cells are as pluripotent *in vitro* as those isolated directly from embryos¹⁹⁻²² it is obviously not possible to test them as rigorously as animal models, since reimplantation into a human embryo is not ethical. In addition, cultures of embryonic stem cells may include rare "cancer" stem cells, which may then proliferate.²³ Many other uncertainties remain. First, mammalian embryonic stem cells derive from the inner cell mass of the preimplantation embryo, yet it is not known whether these cells, if left in place, would go on to exhibit the features they display in culture. Second, since most of our knowledge comes from experiments in mice, we cannot be sure that human stem cells possess the same potential. Third, human embryonic stem cells, like mouse embryonic stem cells, appear capable of unlimited proliferation *in vitro*, but it is still a matter of debate whether prolonged culturing affects their pluripotency.

ADULT STEM CELLS UNBOUND

The astonishing capacity of embryonic stem cells to give rise to virtually any type of tissue has intensified the search for similar cell lineages in the adult that may contribute to self-renewal. Rigorous criteria are required to distinguish a stem cell in the adult milieu from partially committed precursor cells that have more limited potential. Like embryonic stem cells, adult stem cells must be clonogenic and self-renewing during the lifetime of an organism through asymmetric division, with one daughter cell remaining multipotent to maintain a stem-cell lineage and the other daughter cell free to mature into a specialized type of cell with typical morphology and function. The task facing the committed daughter cell is daunting: it must take up residence in the right place, attain the necessary shape, set up the correct contacts, and perform the necessary

functions for multiple adult tissues, behaving appropriately in a variety of different cellular environments. The criteria for defining stem cells in the adult are still difficult to satisfy experimentally. There is no predictable location for stem cells in most adult tissues, and we still possess only limited tools for identifying them.

Searches for adult stem cells have relied on information derived primarily from studies of stem cells in the bone marrow, which must renew themselves daily to maintain the body's blood supply. In mouse bone marrow, stem cells are as rare as 1 in 10,000 cells, and they may be even less common in humans, yet they proliferate constantly to replace circulating blood cells that die.²⁴ They give rise to intermediate precursor- or progenitor-cell populations that partially differentiate and commit to various blood-cell lineages. Stem-cell-containing populations can be highly purified as a side population by means of fluorometric cytometry.²⁵ Hematopoietic stem cells have additional characteristic morphologic appearances and cell-surface markers²⁶⁻²⁸ that allow them to be labeled and tracked in the bloodstream and target tissues or to be isolated and cultured *in vitro*.

An understanding of the plasticity of adult stem cells initially grew from observations that donor cells were found in nonhematopoietic tissues in the recipients of bone marrow transplants. Accounts of the repopulation of adult organs by stem cells derived from bone marrow have since flooded the literature, suggesting that under the right conditions, these rare cells can contribute to virtually any type of tissue. As proof of principle, a lone hematopoietic stem cell, genetically marked and mixed with unmarked bone marrow, was injected into a mouse that had received a lethal dose of radiation. Several weeks later, the marked descendants of that stem cell were found in multiple tissues, attesting to the plasticity of bone marrow precursors.²⁹

These dramatic reports of the versatility of stem cells must be tempered with a note of caution. A separate analysis involving the use of a single marked cell to reconstitute lethally irradiated mouse bone marrow failed to detect appreciable numbers of descendants of the stem cell in non-hematopoietic tissues.³⁰ Two other studies^{31,32} challenged the intrinsic plasticity of tissue stem cells on the basis of the observation that the spontaneous fusion of embryonic stem cells with cocultured brain cells resulted in tetraploid hybrids that retained full pluripotency, including their multilineage contribution to chi-

meras when they were injected into mouse blastocysts. More recently, two reports have verified that cell fusion is the most prevalent source of bone marrow–derived hepatocytes.^{33,34} These findings offer an alternative explanation for the presumed transdifferentiation of hematopoietic stem cells in new environments, but they also carry the broader, more interesting implication that fusion may be just another facet of stem-cell biology.

Stromal cells from adult bone marrow are a richer, if less well characterized, source of stem cells that can be induced to undergo differentiation in a variety of adult tissues.³⁵ These cells may be identical to mesenchymal stem cells.³⁶ The ready proliferation of cultured mesenchymal stem cells makes them attractive candidates for therapy.³⁷ In one instance, purified mesenchymal stem cells from adult human bone marrow engrafted into the heart of an immunodeficient mouse persisted in the myocardium, taking on the characteristics of cardiomyocytes.³⁸ Mesenchymal stem cells are likely to be a heterogeneous population, in which only a percentage of cells maintain true pluripotency. With the use of appropriate conditions, it may be possible to culture this subpopulation. This point was recently evidenced by the isolation of unusually versatile multipotent adult progenitor cells derived from culturing rat, mouse, and human bone marrow, which readily differentiated into multiple types of cell in culture.³⁹ When reintroduced into mouse blastocysts, multipotent adult progenitor cells could contribute all three embryonic germ layers and appeared as blood and epithelia when they were injected intravenously into adult mice, providing new promise for the therapeutic use of adult stem cells.

One uncontested concept emerging from the flurry of recent articles is that injury is a prerequisite for circulating-cell participation in differentiated tissue structure and function. Although the reported low frequencies at which adult stem cells repopulate injured tissues preclude their use in the regeneration of whole organs, small numbers of stem cells may be all that is necessary to induce endogenous regenerative programs. Mice or rats with experimentally induced myocardial infarcts had surprising recoveries after bone-marrow–derived stem cells were administered either directly into the heart⁴⁰ or through the circulation.⁴¹ Although in both studies the number of foreign stem cells lodged in the injured myocardium was far too low to replace necrotic tissues, the cells were more concentrated in the infarcted area than elsewhere in the heart,

suggesting that stem-cell repopulation of certain tissues may actually be triggered by injury. Increasing evidence suggests that stem cells, like metastatic tumor cells, use common chemoattractive mechanisms to home to damaged tissues (Fig. 2).⁴² Furthermore, one of the studies reported the induction of neovascularization of surrounding host tissues in the border zone,⁴¹ which presumably contributed to the improvement in recovery. If stem cells can home to sites of injury and promote healing in damaged tissues, we may not need as many of them as we think.

ONE STEM CELL FIXES ALL?

The remarkable plasticity and pluripotency of bone marrow cells raise the exciting possibility of a universal stem cell that can roam throughout the body, taking up residence wherever it is needed to promote regeneration. Recent evidence suggests that the plasticity of stem cells residing locally in adult tissues may be similar to that of bone marrow cells.^{43,44} The option of using a nonhematopoietic stem cell for clinical applications would be particularly relevant in the treatment of circulating tumors, since such cells could provide a source of healthy autologous cells to repopulate irradiated bone marrow (Table 1).

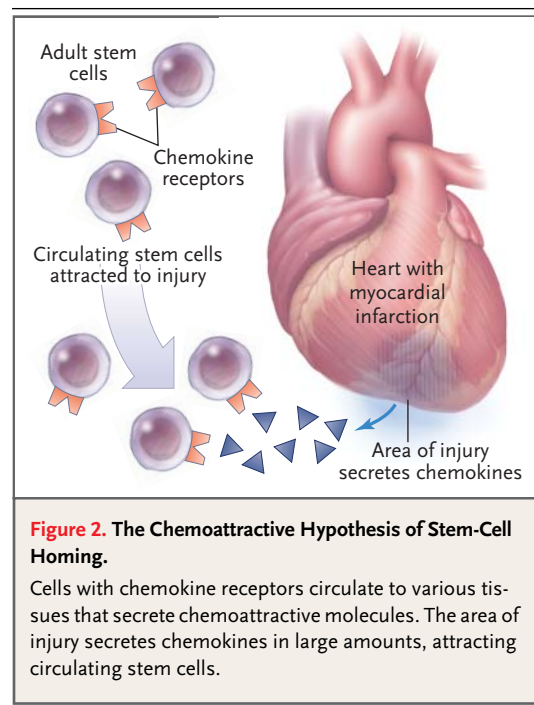


Figure 2. The Chemoattractive Hypothesis of Stem-Cell Homing.

Cells with chemokine receptors circulate to various tissues that secrete chemoattractive molecules. The area of injury secretes chemokines in large amounts, attracting circulating stem cells.

The pluripotency of adult stem cells from non-hematopoietic sources has been demonstrated most convincingly by studies in which cells extracted from various adult tissues were transplanted into irradiated animals and took up residence in the bone marrow, reconstituting the entire hematopoietic system. Even tissues that have historically been considered terminally differentiated, with low potential for regeneration, contain enough stem cells to qualify. Stem cells harvested from the central nervous system have now been coaxed to reconstitute bone marrow⁴⁶ or skeletal muscle⁴⁷ in adult animals. These results lend further support to the concept of the plasticity of neural cells, which was previously suggested by studies demonstrating increased neurogenesis in the adult brain after injury. Although contradictory reports question how frequent a phenomenon neural stem-cell repopulation might be,⁴⁸ differing protocols may be responsible for the variable rates of success in demonstrating engraftment and differentiation of these progenitors.⁴⁹

Stem cells isolated from skeletal muscle were also capable of repopulating the bone marrow after transplantation into hosts that had received lethal doses of irradiation.^{26,50} It is not yet known whether these cells are the progenitors of muscle satellite cells, a population of precursor cells capable of regenerating muscle and demonstrating self-renewal but presumably committed to the myogenic lineage. It is equally probable that the regenerative potential of the tissues in these studies derived from circulating hematopoietic stem cells, as suggested by a recent report documenting the hematopoietic origin of muscle-derived stem cells.²⁸ Further studies are needed to determine whether heterogeneous populations of stem cells emanating from the bone marrow and lodging in peripheral postmitotic tissues may constitute the true developmental origin of most peripheral stem cells.⁵¹

Ultimately, a naturally rejuvenating tissue such as the skin would be a more likely source of stem cells. In an initial test of this possibility, genetically marked skin-derived stem cells were obtained from neonatal mice and introduced into early mouse embryos, where their descendants integrated into multiple cell lineages and could later be found in various tissues of the adult progeny.⁵² It remains to be seen whether the demonstrated plasticity of epidermal stem cells would be maintained beyond the permissive environment of the early embryo, but if they are truly pluripotent, the skin could provide a readi-

Table 1. Potential Plasticity of Stem Cells.*

Location of Stem Cell	Type of Cells Generated
Brain	Neurons, oligodendrites, skeletal muscle, blood cells
Bone marrow	Endothelial cells, blood cells, cartilage, bone, adipocytes, cardiac muscle, skeletal muscle, neuronal cells, skin, oval cells, gastrointestinal tract cells, thymus, pulmonary epithelial cells
Skeletal muscle	Skeletal muscle, bone, cartilage, fat, smooth muscle
Myocardium	Myocytes, endothelial cells
Skin	Keratinocytes
Liver	Liver cells
Testis and ovaries	Gonads
Pancreatic ducts	Islet cells
Fatty tissue	Fat, muscle, cartilage, bone

* Data are adapted from Kuehnle and Goodell⁴⁴ and Tsonis.⁴⁵

ly accessible source of stem cells for use in the clinic.

These examples highlight the necessity for a more extensive battery of markers for stem cells of different origins to confirm their provenance. In general, the process of documenting that a stem cell from one tissue generates differentiated types of cells in another must be particularly rigorous, in order to rule out the possible contribution of cell fusion or of circulating hematopoietic stem cells. However, research to date suggests that stem-cell populations in adult mammals are not fixed entities and that after exposure to a new cellular environment, they may be able to contribute to the regeneration of multiple types of tissue.

Although the accumulating experimental evidence of the plasticity of adult stem cells is ever more convincing, there are several hurdles to overcome. The origin of stem cells in the bone marrow is well established, but the location of stem cells in other tissues remains elusive. What are the sources of these cells? Has the body had enough forethought to set aside pluripotent stem cells from its early moments as an embryo? If not, how do adult stem cells arise in nonhematopoietic tissues, and how do they remain in suspended animation until repair is necessary? Do they need a stimulus, such as traumatic injury, to expand the population for repair of the injured tissue? If adult stem cells are truly pluripotent, why is the response to injury to tissues such as the heart or spinal cord so inefficient under normal circumstances? Do adult stem cells carry surface markers that would make them immu-

nogenic in a foreign host, making embryonic stem cells better tolerated in the recipient? These questions may seem academic, but the future of adult stem-cell-mediated therapies depends on their resolution.

THE PROMETHEAN PROMISE PENDING

The prospect of improved regeneration is not the only promise held out by stem-cell research. Critical studies of the unique aspects of early human development are now within reach with the use of embryonic stem cells (Fig. 3). The origin and mechanistic basis of chromosomal abnormalities underlying many congenital defects can be elucidated, and the early childhood tumors resulting from these mutations could be analyzed in the culture dish. Embryonic stem cells also provide a potential source of material for the preclinical testing of candidate therapeutic drugs in multiple types of human tissue. And given the promising preclinical evidence,¹⁷ therapeutic trials of embryonic stem cells in neurodegenerative disease are probably imminent. The specter of human genetic engineering should not

preclude the exploration of therapeutic applications once our understanding of these extraordinary cells is broadened.

Even if results in animals are cleared by ethical committees for study in humans, formidable obstacles need to be overcome before the therapeutic utility of embryonic stem cells is tested in the clinic. To demonstrate the safety of embryonic stem cells, researchers need to rule out any chance that the administration of embryonic stem cells could cause tumor formation or the transmission of infectious agents. This may require screening cells for neoplastic potential or for signs of viral infection or delivering cells that have already been differentiated into the desired type of tissue. To be effective, cells derived from embryonic stem cells must integrate into host tissue in sufficient numbers and, once engrafted, take over or at least induce appropriate physiological functions. In a dramatic proof of principle, a genetic defect in the mouse hematopoietic system was successfully treated through the engraftment of genetically corrected autologous embryonic stem cells.⁵³ Preventing immunorejection under these circumstances in humans may require the use of immunosuppression unless the cultured cells are

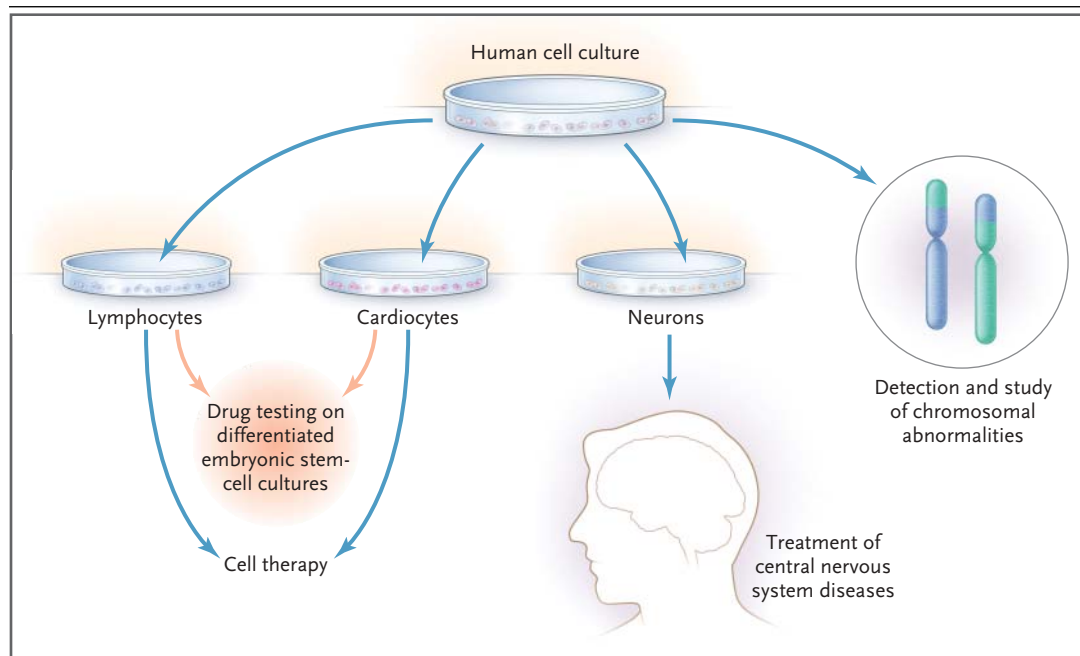


Figure 3. The Use of Human Embryonic Stem-Cell Cultures for Basic Research.

The uses include drug testing on differentiated tissue types, neuronal repopulation of brain tissue in Parkinson's disease, and the detection and study of chromosomal abnormalities in childhood tumors.

placed in an immunoprivileged tissue such as the brain or are genetically engineered to be tolerated by the host tissues.

Despite these caveats, the prospect of harnessing the prodigious powers of embryonic stem cells for clinical applications has impelled scientists to pursue the isolation of specific lineages derived from embryonic stem cells. Experimental schemes are being developed that will select for a particular characteristic in a culture of embryonic stem cells by exposure to specific mixtures of growth factors, by screening homogeneous populations of embryonic stem cells for certain surface markers, or by genetically engineering embryonic stem cells to generate differentiated phenotypes. We still do not have sufficient information to identify the stages of differentiation of embryonic stem cells that are optimal for transplantation or to ensure the survival and function of transplanted human embryonic stem cells as an integral part of the host tissue. Sorting through all these variables will require further research, and such research can only be carried out if the basic material is available for study. Once we gain a more complete understanding of the prodigious capacity of embryonic stem cells for self-renewal, we may then be able to apply this information to the manipulation of adult stem cells.

Perhaps the biggest hurdle to the clinical application of research on adult stem cells is the small number of cells that can be isolated from any adult tissue. The recent successful propagation of a multipotent adult stem cell³⁹ and the development of cytokine “cocktails” for optimizing the proliferation of adult stem cells⁵⁴ suggest that expansion in culture may be the answer. Yet it is still possible that extensive culture of human adult stem cells *ex vivo* may subtly change their intrinsic properties, rendering them unfit for restoring injured or diseased tissues in patients.

The Promethean promise notwithstanding, the low level of repopulation of exogenous stem cells in injured tissues argues against the possibility that

this approach can be used to rebuild entire organs. Other mechanisms at work in the natural process of regeneration may be more successfully harnessed to increase the efficiency of stem-cell-mediated regeneration. A lesson might be learned from the behavior of metastatic tumor cells, which, once dissociated from the primary neoplasm, roam the body much like a stem cell, drawn to their selective target organs through specific chemoattractive mechanisms. Remarkably, many of the surface chemoreceptors expressed by tumor cells are also found on stem cells,^{55,56} and it is tempting to speculate that metastasis is the dark side of regeneration, with tumor cells coopting similar guidance pathways for more nefarious purposes. If this is the case, specific chemoattractants might be identified and protocols might be developed to enhance the migration of healing stem cells into the lesions of specific types of tissue. Another avenue to be explored is the potential paracrine action in stem-cell-mediated regeneration. If this proves to be a general principle, stem cells might be engineered to express increased levels of these paracrine factors, acting as a targeted, readily administered system of delivery for therapeutic molecules.

In the process of pursuing the elusive stem cell and its promise of universal healing, we stand to gain important insight into the nature of human life itself. Along with our obvious advances, we have evolved into a species with remarkably restricted regenerative capacity. Our bodies have long lost the forethought of indefinite growth possessed by the sequoia or the carp. Unlike starfish or newts, we can no longer replace lost limbs. And as we grow older, our own aging populations of stem cells cannot keep up with our failing bodies. We have paid a heavy price for our high vantage point on the evolutionary tree. It remains to be seen whether a growing understanding of our own phylogenetic limitations will be sufficiently profound to overcome them. The Promethean prospect of eternal regeneration awaits us, while time's culture looks on.

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