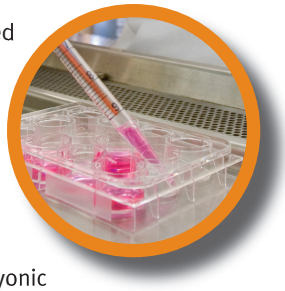
STEM CELLS:
BEYOND SOMATIC CELL NUCLEAR TRANSFER

Despite the heated ethical and moral debate, research on embryonic stem cells has been forging ahead at breakneck speed. But before these cells make a dent in the clinic or in drug discovery, researchers must learn how to create cell lines from diseased individuals, so they can, for instance, learn how to stymie disease progression in others. Here we examine some of the recent scientific advances and look at where the technology pack leaders may take us in the future.

By Jeffrey M. Perkel

It's been 25 years since Martin Evans and Matthew Kaufman at the University of Cambridge and Gail Martin at the University of California, San Francisco independently launched the stem cell revolution with their establishment of murine embryonic stem cell lines, and the impact on biomedical research is undeniable.



As a research tool, embryonic stem cells – pluripotent progenitors, harvested from the inner cell mass of developing embryos, that can differentiate into any cell type in the body – "have revolutionized the way we do biomedical research," says George Daley, associate professor at **Children's Hospital Boston** and at the **Harvard Stem Cell Institute**. "Think of all the mouse models of cancer, of neurodegenerative and cardiovascular diseases that have really come about because of our ability to manipulate genes in murine embryonic stem cells."

But more than that, Daley says, researchers' ability to differentiate embryonic stem cells in a culture dish has made them interesting objects of study in their own right. "Those in vitro systems allow us to ask questions about tissue differentiation in a manner that previously was inaccessible," he says. Plus, he adds, the resulting elucidation of cellular lineages and how they emerge will provide a biological organizing principle akin to that gained from the sequencing of the human genome or chemistry's establishment of the periodic table of elements.

Yet many technical challenges remain, especially in humans. Precious little is known about the markers that distinguish one developmental lineage from another; about which cellular signals can induce differentiation in a controlled, robust, and predictable manner; and perhaps most important, about how to make embryonic stem cell lines from diseased individuals – a critical step for many therapeutic and drug-development applications.

Blast into the Past

Last August Shinya Yamanaka, professor of stem cell biology at the **Institute for Frontier Medical Sciences at Kyoto University**, demonstrated it was possible to deprogram murine somatic cells—specifically fibroblasts—into embryonic-like stem cells by expressing four genes: *c-myc*, *oct3/4*, *sox2*, and *klf4*.

According to Yamanaka, the proteins encoded by these genes—identified from a pool of 24 candidates—each serve a specific role: c-Myc opens tightly compacted chromatin, enabling the transcription factors oct3/4 and sox2 to bind to gene regulatory regions in the genome and restore pluripotency. klf4, he says, appears to fulfill two roles, both as a cofactor to oct3/4 and sox2, and as an apoptotic inhibitor.

Peter Mountford, president and CEO of **Stem Cell Sciences**, a stem cell research and development company in Edinburgh, UK, describes the findings as continued ›

> "Embryonic stem cells have revolutionized the way we do biomedical research."

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"In terms of cell therapy in general, it's likely some diseases will be treated by embryonic stem cells, and others by adult stem cells."

"potentially one of the most important breakthroughs and certainly one of the most extraordinary experiments in recent times."

Yamanaka says work is ongoing in his lab to migrate these findings to humans. Preliminary evidence suggests additional factors may be required, he says, but perhaps the biggest challenge to using this technique to make therapeutic patient-matched embryonic stem cells involves the gene-delivery technique itself.

"So far we can generate these embryonic stem cell–like cells only by using a retroviral system," Yamanaka explains. But retroviral vectors have been linked to leukemia in some gene therapy trials – a problem that is all the more acute because *c-myc*, at least, is a known oncogene. "Because of that, I think using the retroviral system is very, in a sense, dangerous," Yamanaka says.

Not implicated in Yamanaka's study was *nanog*, which like oct3/4 and sox2 is also a key transcriptional regulator in pluripotent cells. Kiminobu Sugaya, director of the **Stem Cell Laboratory at the University of Central Florida**, has presented data at meetings showing that adult mesenchymal stem cells—bone marrow–derived cells that are predestined to form connective tissues like bone and cartilage—can be dedifferentiated into embryonic-like stem cells by expression of *nanog*.

According to Kevin Eggan, assistant professor of molecular and cellular biology at **Harvard University**, others have shown that nuclear reprogramming during cell fusion can likewise be made more efficient by overexpression of *nanog* in mouse embryonic stem cells. "But that doesn't directly show that *nanog* is directly required for reprogramming in the sense that Yamanaka did," he cautions.

Sugaya's goal is to develop neural stem cell–based therapies for conditions like stroke and Alzheimer's disease. The process can work using fetal tissue-derived neural cells, he notes, "but the availability of these cells isn't so great." And in any event, such cells could provoke an immune response – hence, Sugaya's interest in autologous, adult stem cells.

Before they can be converted into neural tissue, however, the cells must relearn how to be embryonic. "We have to erase the memory, the commitment of the cells," Sugaya says. "Then we have to teach them what to become."

The Essence of ESC

When Sugaya talks of cellular memory, he is referring essentially to the epigenetic changes that occur as embryonic stem cells differentiate. All cells contain the same DNA, but embryonic stem cells express different genes to the more developmentally committed lineages. That change arises in part due to changes in gene methylation, changes which must be reset if the cells are to be restored to pluripotency.

A cell is called pluripotent if it can develop into any of the three germ layers of the body: endoderm, mesoderm, and ectoderm. Ectoderm gives rise to the skin, epidermis, and the nervous system; from mesoderm arises heart tissue, kidney, gonads, continued ›

Selected Embryonic Stem Cell Tools

Embryonic stem cells present a number of unique challenges that distinguish them from run-of-the-mill cultured cells. For one thing, embryonic stem cells traditionally are cultured either on a murine embryonic fibroblast feeder layer, or in media that have been conditioned by such cells. In addition, like many cultured cells, embryonic stem cells require "black-box" additives, such as fetal bovine serum.

Both of these components pose not only a potential contamination risk, but also a batch-to-batch consistency problem. Sera, for instance, can vary based on the animal's age, diet, sex, health, and reproductive status, among other factors, says Peter Mountford, president and CEO of Stem Cell Sciences. "You really have to get rid of any noncharacterized component. It must be like a chemical formula, so that every time you make it, it is exactly the same."

Mountford's company has developed media formulations, available through Millipore, for murine embryonic stem cell culture that require neither feeder cells nor serum; human media formulations that no longer require serum, but still require feeder cells, are also available.

Invitrogen also is developing so-called fully defined media formulations, part of the company's cradle-to-grave strategy for embryonic stem cell work, according to Joydeep Goswami, Invitrogen's vice president of stem cell and regenerative medicine division. Invitrogen plans to release in the third quarter of 2007 "the first feeder-free and serum-free defined culture media for human embryonic stem cells," Goswami says.

Cellartis is looking to modify culture procedures for industrial applications. Embryonic stem cells traditionally are passaged mechanically, by physically transferring undifferentiated colonies into fresh culture dishes. Such a process can be neither scaled nor automated easily. The company has developed a single-cell, enzyme-based dissociation method that can be used to maintain cells in culture flasks rather than in dishes, without enriching for chromosomal abnormalities.

"That means you can get more cells, more easily, and in an undifferentiated state," says Johan Hyllner, Cellartis's chief scientific officer. The enzyme at the heart of this approach is TrypLE, a recombinant trypsin from Invitrogen. Used to passage cells, trypsin typically is of porcine origin, but Invitrogen's reagent removes that animal-based component from the workflow.

BD Biosciences, a segment of Becton, Dickinson and Company, focuses on monitoring stem cell differentiation, or lack thereof. Embryonic stem cells can form any of the body's 220 or so different cell types, and each one expresses a unique, and largely undefined, set of intracellular and surface markers. The company offers several thousand antibodies for research use, but according to Robert Balderas, vice president of research and development, life science research reagents, there currently is no "perfect" marker or biosignature that defines the pluripotent stem cell, let alone its myriad descendents.

"There is a continual need for the identification of new proteins on these subsets of cells so that applications like fluorescence activated cell sorting can be used to better define, characterize, and isolate these cells," Balderas says.

blood cells and vessels, muscle, and connective tissue; and from endoderm comes the gut tube, plus all the principal organs that bud off of that, including the liver, pancreas, and lungs.

Adult stem cells, such as hematopoietic or neural stem cells, are not pluripotent, because they can yield only a limited number of different cell types: hematopoietic stem cells can create blood cells, for instance, but not skin.

According to Leonard Zon, the Grousbeck Professor of Pediatrics at **Harvard Medical School**, "We need to get better at turning embryonic stem cells into tissues more robustly, and that involves understanding how normal embryos make tissues and adapting those gene programs to the embryonic stem cells in culture." Researchers at **Novocell**, a stem cell engineering firm in San Diego, used precisely that approach in 2005 to coax embryonic stem cells to form definitive endoderm.

"I thought that was a breakthrough," says Johan Hyllner, chief scientific officer at **Cellartis**, a Swedish/Scottish company that uses human embryonic stems to derive both hepatocyte- and cardiomyocyte-like cells. "Endoderm was one of the trickier germ layers to grow. Ectoderm and mesoderm are somewhat easier to get, but true endoderm has been a challenge until this paper came out."

As Emmanuel Baetge, Novocell's chief scientific officer, puts it, the trick was to heed the lessons of cell biology. The three germ layers are established during gastrulation, occurring in the second to third week of human development. "We said, let's see if we can make human embryonic stem cells undergo a process like gastrulation to make definitive endoderm," Baetge explains.

The key was to force the cells to differentiate by both removing serum and the growth factors—fibroblast growth factor and insulin—and by adding a protein called activin. Activin is a member of the transforming growth factor (TGF)-ß family of signaling molecules, another member of which (Nodal) helps define germ layer fate during gastrulation.

Baetge and his team published a follow-up paper last year describing how to push the development even further, to create in four discrete steps fetal pancreatic islets – the insulin-producing cells that one day could be used as transplants for type 1 diabetics.

As with the first study, the key to this latest advance lay in the developmental biology literature, by knowing which factors to add, and in what order, says Baetge.

"If you want to work with stem cells, you need to apply developmental biology principles," he says. "They can provide a framework in which you should try to operate with these cells if you want to make defined lineages."

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The Embryo with No Father

According to Eggan, Yamanaka's technique is one of three "ponies" in the race to develop patient-specific cell lines. The others are somatic cell nuclear transfer (SCNT), the technique used to clone Dolly the sheep, and cellular fusion, a technique in which a somatic cell is fused with an embryonic stem cell line, thereby sending the somatic cell back to its embryonic roots.

Each has pros and cons, says Eggan. Yamanaka's cells, for instance, "aren't really embryonic stem cells" – because they are neither completely reprogrammed nor express the full complement of embryonic genes. SCNT requires human eggs, which are hard to come by, while with cell fusion, "no one has figured out how to get rid of the embryonic chromosomes."

Daley at Harvard is working on yet another strategy: parthenogenesis. Literally meaning "virgin birth," parthenogenesis is a process in which an egg is tricked into thinking it has been fertilized and begins to develop into an embryo, without the contribution of a sperm. At the same time, the cell is prevented from completing the second round of meiosis that normally occurs, so that the cell ends up with a diploid chromosome content, all of which comes from the mother.

Ignoring the effects of crossing over, "it's like taking half a deck of cards and then photocopying them to give 52 cards," explains Michael West, president and CEO of **Advanced Cell Technology**, a stem cell company that also pursues parthenogenic stem cell lines.

According to Daley, parthenogenesis, which to date has only been achieved in mice, is more efficient than SCNT and requires fewer human eggs. In addition, the strategy could theoretically be used to create banks of HLA-matched cells for use in therapeutic applications.

That's because parthenogenic stem cell lines typically contain only half of the six HLA loci of a normal diploid cell. "It's a lot easier to match patients at three loci rather than six," says Daley.

There are, however, a few potential glitches in the system. First, parthenogenic embryos could have problems with imprinted genes. In addition, there exists a class of natural killer cells that recognize cells that don't express the full complement of an individual's MHC antigens. Daley suspects this will not be an issue for most tissue grafts, but could be a problem for blood tissue transplantation, which may require full HLA matches.

Despite these advances, all agree it could be a decade or more before embryonic stem cell–derived therapies arrive in the clinic. Those desiring more immediate benefits can look to the many adult stem cell–derived therapies winding their way through clinical trials right now.

In March, for instance, **Osiris Therapeutics** announced the results of a phase 3 clinical trial that used intravenous human mesenchymal stem cells for repair of cardiac tissue following heart attack. "In terms of cell therapy in general," says Harvard's Zon, "it's likely some diseases will be treated by embryonic stem cells, and others by adult stem cells. I think the science of each field informs each other, so it's important that both succeed."

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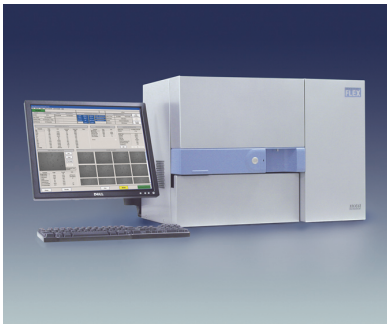

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