Landmark Approach to Generating Human Stem Cells

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Building on the breakthrough discovery that differentiated cells can be returned to a pluripotent state, a team of researchers led by Dr. Derrick Rossi of Harvard Medical School have developed a powerful new technique for cellular reprogramming that could have a broad range of applications in both clinical and research settings.

The First Steps

Ongoing ethical concerns about the study of embryonic stem cells for the treatment of disease propelled researchers to search for methods to generate pluripotent human stem cells in the laboratory. The first milestone was reported in 2006 by a team of investigators led by Shinya Yamanaka of Kyoto University. Their research focused on 24 select genes believed to contribute to the regulation of embryonic stem cell properties. After screening each of the candidate genes in cultures of mouse embryonic and adult cells, they showed that only four were sufficient to induce pluripotency. The genes encode transcription factors identified as Klf4, c-Myc, Oct4, and Sox2, and the reprogrammed cells were termed induced pluripotent stem (iPS) cells.1

In a subsequent investigation, Yamanaka and colleagues studied the effects of the same four genes in fully differentiated, adult human dermal fibroblasts. By using retroviruses to incorporate the genes into the fibroblast DNA, the researchers forced the coexpression of the four transcription factors and reverted the mature cells back to a pluripotent state. The result was the first iPS cells made from mature, differentiated human cells.2

Yamanaka’s research marked a major turning point in cellular reprogramming; however, his technique was not without important limitations. First, it was inefficient, with an approximate yield of only 1 iPS colony from 50,000 transduced human fibroblasts. In addition, the first colonies resembling human embryonic cells did not appear for about 25 days, and the integration of retroviral DNA into the genome of the host cell brought with it the risk of insertional mutagenesis and tumor formation.2,3

Still, Yamanaka’s achievements were remarkable and launched an intense search for more efficient approaches to return adult human cells to a pluripotent state without altering the genome. A variety of methods were investigated, such as the use of excisable viral vectors or transient plasmid vectors.3 DNA-free techniques were also studied by a number of researchers, including Rossi, who considered genomic integration, “a hurdle for clinical translation because the gene therapy trials of the 1980s and 1990s taught us that you can’t leave viruses in genomes because often they activate proto-oncogenes and deregulate genes that live in the neighborhood where they integrate.”

Rossi and his colleagues focused on an RNA vector, specifically messenger RNA (mRNA). According to Rossi, “RNA does not integrate into DNA, so it doesn’t compromise genomic integrity.” Further, the instructions for the manufacture of every protein in the human body are encoded by a unique gene transcribed on a complementary mRNA template. The distinct sequence of ribonucleotides on this single-stranded molecule is the framework used by the cell to construct the protein. It was the harnessing of this fundamental cellular process of protein synthesis that led to the next major advance in cellular reprogramming.

A New Stride

Rossi’s objective was to induce mature human fibroblasts to manufacture the four transcription factors used in the Yamanaka studies by supplying the cells with the mRNA templates for each.

The researchers synthesized complementary mRNA molecules for each factor. Because all exogenous single-stranded RNA can activate host antiviral defenses, investigators used two strategies to minimize the immunogenic profile of their synthetic mRNA. First, they treated the mRNA with a phosphatase to reduce their 5’ triphosphates, which are recognized as foreign by RIG-I, a primary sensor of single-stranded RNA in eukaryotic cells. Next, they modified the ribonucleoside bases by substituting cytidine and/or uridine with 5-methylcytidine or pseudouridine, respectively. As was later observed, these alterations effectively reduced the immunogenicity of the synthetic mRNA, allowing for increased expression of the encoded proteins. The researchers called their creation synthetic modified RNA.

To further reduce innate immune responses to exogenous RNA, a decoy receptor was added to the culture media to inhibit receptors for type 1 interferons, known to mediate potent immune responses against foreign RNA.3

The fibroblasts were then exposed to daily transfection with the modified synthetic mRNAs. The cells responded by synthesizing the transcription factors in sufficient levels to reprogram the cells to pluripotency. Approximately 1% to 4% of the treated cells were reprogrammed within 2 weeks, a conversion efficiency that was ~36-fold higher than that achieved with the viral method. Furthermore, functional and molecular assays showed that the genes expressed by the reprogrammed cells exhibited characteristic similar to those of true embryonic stem cells. Rossi and his team termed the cells RNA-induced pluripotent stem cells (RiPSCs) (Figure 1).3
Although the markedly increased efficiency over Yamanaka’s method may one day prove beneficial in patients with limited cells available for reprogramming, it is not the most critical finding in this study, according to Rossi: “The important thing is that we have done it without compromising genomic integrity.”

The team then set out to determine if the fate of the RiPSCs could be directed toward a specific cell type. They used a synthetic modified RNA to express MYOD, a transcription factor that regulates skeletal myogenesis. The RiPSCs were transfected with the MYOD synthetic modified RNA, and upon analysis, the researchers observed a high percentage (40% of cells) of myogenic markers.

“First, we turned fibroblasts into RiPSCs, and then, we turned RiPSCs into terminally differentiated myogenic cells,” said Rossi. “All of a sudden, here is a new technology for actually directing cell fate, not just for reprogramming, but for reestablishing differentiated cell fates.”

Other investigators in stem cell research applaud this innovative technology. “The approach is scientifically very sound, and its achievability hinges on the ingenious steps that were taken to overcome cells’ antiviral responses to exogenous single-stranded RNA and ensure sustained protein expression,” states Professor Michael D. Schneider, MD FMedSci, British Heart Foundation Simon Marks Chair of Regenerative Cardiology and Director of the British Heart Foundation Centre of Research Excellence at Imperial College in London. A notable advantage of this technique, according to Schneider, is the very high efficiency for successful reprogramming and evidence from microarray studies that demonstrates that the RiPSCs are more similar to embryonic stem cells than are iPS cells manufactured using viral vectors.

Yamanaka is optimistic, as well, noting, “I think that the method to generate iPS cells, described in the paper, seems promising in inducing clinical-grade iPS cells and would like to have someone in my lab try the protocol.”

**Potential Cardiovascular Applications**

According to Rossi, the RNA-based technique is an important first step in generating patient-specific cells for the treatment of disease without concerns of immune rejection. However, he believes that the initial utility of RiPSCs will be in research rather than cell-based therapies. For example, this new technology of introducing RNA into cells can be used to create models of diseases to study the underlying pathophysiology and screen potential drug therapies “first in a dish,” rather than in humans or animals.

Stem cell scientist Kenneth R. Chien agrees. Chien is the Director of the Massachusetts General Hospital Cardiovascular Research Center and the Charles Addison and Elizabeth Ann Sanders Professor in the Department of Stem Cell and Regenerative Biology at Harvard University. Chien, Rossi, and Robert Langer of Massachusetts Institute of Technology are cofounders of a company called ModeRNA Therapeutics. “What this technology has allowed us to do is to generate model systems to study human cardiogenesis, not only from

![RNA-based method of cellular reprogramming](https://example.com/illustration.png)

**Figure.** RNA-based method of cellular reprogramming. (Illustration credit: Cosmocyte/Robert Thornevale)
normal patients, but also from patients that have developmental abnormalities, like congenital heart disease,” said Chien.

That the conventional iPS systems have the potential to serve as human models of disease has been demonstrated in a study by Karl-Ludwig Laugwitz published in The New England Journal of Medicine in October. Retroviral vectors were used to introduce the four transcription factors into dermal fibroblasts acquired from two related patients and two healthy controls. After the reprogrammed cells were induced to differentiate into functional cardiac myocytes, those derived from the patients with long-QT syndrome type I retained biophysical characteristics resembling features of the disease.4

According to Chien, a challenge of such standard reprogramming methods is the high degree of variability that can occur between cell lines generated from the skin fibroblasts of an individual patient. The mechanisms underlying the variability are likely attributable to the differential integration of the four key genes and other factors that can vary from line to line. Because Rossi’s technology is not expected to alter the genome of the host cell, Chien believes it has the potential for more uniform generation of iPS cell lines from patients with developmental defects.

This approach may also be used to study normal cardiac development. The relatively high efficiency of protein expression in Rossi’s stem cell model system may facilitate the identification of genes that contribute to cardiogenesis, including those that drive the differentiation of cardiac cells, explains Chien. He theorizes that the forced expression of genes in cardiac and progenitor cells can be facilitated with modified RNA, allowing researchers to “quickly define the precise role of individual genes in mammalian cardiogenesis in ways that we would never have been able to approach before.”

The same methodology when applied to stem cell development could eventually uncover the genetic program that induces the differentiation of cells toward a very specific cardiac fate, such as a pacemaker or ventricular cell, Chien said.

He also expects that modified RNA will have a future role in identifying therapeutic targets for human cardiovascular disease. Expressing exogenous genes in human cells allows scientists to observe the functional effects of the protein products in the development of disease with the goal of developing highly specific therapies directed at the underlying biochemical defects. To date, this has been accomplished with other methods, including the use of viral vectors to incorporate specific genes into the cells. However, according to Chien, this new RNA-based approach may one day prove to be a very rapid and efficient method of expressing foreign proteins in heart muscle cells. “The efficiency of expression will be the key here,” he notes.

The next step may be to determine the cardiovascular effects of modified RNA in vivo. This technology may be particularly useful for structural forms of cardiac muscle disease in which the primary therapeutic targets may be intracellular and less responsive to drug therapy, states Chien, and may lead to investigations of modified RNA in protein replacement therapy for diseases with cardiovascular endpoints.

Although both Rossi and Chien believe that the new technology will have a major impact on the study of cardiovascular development and disease, more studies are needed on the safety and efficacy of this new type of induced pluripotent cell. Furthermore, Yamanka notes, “The quality of the iPS cells generated by this method should be carefully examined because their characteristics vary depending on the induction methods and the origin of the resulting cells.”

While much research lies ahead, Rossi and his team may have provided investigators around the world with a powerful new method for expressing select proteins in cells without altering the genome, with potential applications across the biomedical spectrum.

References
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