Paul Schied

John Clardy and David Liu

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Taking the Next Step in Stem Cells: Small Molecule Reprogramming

Scientific progress is often a long plod, replete with detours and potholes. In the realm of stem cell research the pace seems to resemble more of a dead sprint. There are pitfalls and challenges to be sure, but the achievements of the past few years have generated exciting prospects for the future of stem cell research. The emergence of induced pluripotent stem (iPS) cells presents an alternative to the embryonic stem (ES) cells that originally had the scientific community buzzing. The topic of the day has become how to improve the efficacy and therapeutic potential of iPS cells. This is the next step in stem cells, and researchers are already in the process of making that stride. The path of iPS cell research is an intriguing one, with a fascinating recent history and a promising future. The scientific and socio-political questions that it raises are important ones, and the answers may well be dictated by the frenetic pace of progress and the immense promise of the field.

The starter's pistol that sent stem cell researchers around the globe off on a race to develop therapy-ready iPS cells was Shinya Yamanaka's 2006 breakthrough paper on the reprogramming of mouse adult somatic cells into pluripotent stem cells. By introducing a mere four factors— the genes *Oct3/4*, *Sox2*, *cMyc*, and *Klf4*—Yamanaka succeeded in getting his cells to exhibit "morphology and growth properties of ES cells."[1] The discovery was revolutionary because it amounted to the ability to produce cells with all of the utility of ES cells, but without

[1] Shinya Yamanaka, Kazutaka Takahashi, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell* **126**, 663 (Aug 10, 2006).

the use of an embryo. Yamanaka's team at Kyoto University in Japan introduced the four genes by infecting the cells with four retroviruses carrying a gene apiece.[2] This process produced novel results, but it also has several important flaws that have inspired the drive for improvements in iPS cell production methodology.

The retroviral reprogramming technique employed by Yamanaka is hampered by legitimate concerns about the efficiency of the reprogramming process and even more pressing worries about the long-term clinical potential for iPS cells developed in this way. The inherent problem in retroviral reprogramming is that the viruses that carry the four reprogramming genes work the same way that many viruses do: by inserting their genome into that of the host cell. This increases the risk of tumorigenicity; the tissues produced from the iPS cells are more likely to become cancerous.[3] This characteristic is an obvious deal breaker for clinical use, and severely limits the therapeutic potential of virally reprogrammed iPS cells. In addition to the oncogenic tendencies of the iPS cells, the problem of efficiency also plagues reprogramming. The reprogramming process was terribly inefficient, with less than .1 percent of cells infected with the four viruses developing into pluripotent stem cells. It is the combination of this inefficiency and the cancer risk—20 percent of Yamanaka's mice died of cancer—that has tempered the excitement created by the breakthrough.[4] The characteristics of iPS cells have been shown to be nearly identical to those of ES cells, but the crippling factor of the tumor formation necessitates a different method of reprogramming. The ideal method would be to eliminate the need for gene introduction entirely, and instead employ small molecules that could enter the

[2] Shinya Yamanaka, *et al.*, "Generation of Mouse Induced Pluripotent Stem Cells Without Viral Vectors," *Science* **322**, 949 (Nov 7, 2008).
[3] Yamanaka, "Generation of Mouse Induced Pluripotent Stem Cells Without Viral Vectors".
[4] C. B., "Mouse method turns skin cells to stem cells," *Science News* **172**, 29 (Jul 14, 2007).

nuclei of cells and enact the reprogramming.[5] This crucial next step is being taken by a lab at Harvard.

The need to reprogram without using viruses presented an interesting problem to the scientific community, and to the lab of Kevin Eggan and Doug Melton. The goal— to replace some or all of *Sox2, Oct3/4, cMyc* and *Klf4* in the reprogramming process—required finding a small molecule that would achieve the desired results, even though the exact method by which it would do so was unknown. The situation was strikingly similar to those facing drug designers, and the eventual approach taken resembled a modern drug discovery plan. Rational small molecule design was not an especially promising route to take because it was unclear if a small molecule replacement for one of the virally introduced genes would act directly on the genes of the cell or modify the cell through some alternate method. The prevailing wisdom in drug design is that when established knowledge cannot inform the discovery process, the best approach is to assay a large quantity of potential drug candidates in the hopes of finding a small molecule with the desired effect.[6] In the case of small molecule reprogramming, this same logic encouraged Eggan and colleagues to develop a "chemical screen" to obtain a replacement for *Sox2*- the gene on which they had decided to focus. Instead of using a random assortment of molecules, they chose a library of molecules with known bioactivity. The targets of the molecules were diverse, ranging from kinases to extracellular receptors, but the fact that they were selected from a "well annotated" database meant that, should any work, the mechanism through which they worked would be suggested. According to the paper "A Small Molecule Inhibitor of Tgf-βSignaling Replaces *Sox2* in Reprogramming by Inducing *Nanog*," this approach was favored especially

[5] Constance Holden, Gretchen Vogel, "A Seismic Shift for Stem Cell Research," *Science* **319**, 560 (Feb 1, 2008).
[6] David Liu, "Drug discovery and personalized medicine" (lecture, Harvard University, Cambridge, MA, Nov 19, 2009).

"because it was unbiased with respect to the mechanism by which a given chemical functioned . . . [it] would not only deliver chemical compounds with translational utility but would also provide novel insights into the pathways and mechanism controlling reprogramming."[7] The roulette approach of trying a multitude of molecules was academic on more than one level. Not only would a hit present a new way of reprogramming, it would also suggest in greater detail the mechanism through which this new way of reprogramming worked. This in turn could suggest further improvements to the process. The approach was sound, but what made the experiment noteworthy was its success.

In discovering a small molecule replacement for *Sox2*, the Eggan lab showed that it was possible to create iPS cells by means other than gene insertion via viruses. There were still viruses involved in this new reprogramming procedure, to be sure, but this early success suggests that the remaining viruses might be eliminated from the process by discovering small molecule replacements in similar ways. The actual process by which the molecules were screened was quite simple. *Oct4*, *Klf4*, and *cMyc* were introduced to cells, followed by the introduction of a small molecule. If reprogramming proceeded in the absence of *Sox2*, the small molecule was a potential replacement for *Sox2*. It is safe to say that a certain amount of luck was involved, as is true with any novel discovery, and three hits were initially found; however, two of the hits were found to be unable to successfully cause reprogramming without valproic acid, which had been used in the initial screening.[8] This small molecule was renamed RepSox, for its ability to replace *Sox2* in the reprogramming process. Since *cMyc* has been found to be unnecessary for reprogramming, despite increasing efficiency, it was proven that reprogramming is now possible with the introduction of only two retroviruses and a small molecule. If small molecule

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[7] Kevin Eggan, *et al.*, "A Small Molecule Inhibitor of Tgf-ßSignaling Replaces *Sox2* in Reprogramming by Inducing *Nanog*," *Cell Stem Cell* 491 (Oct 8 2009).
[8] Eggan.

replacements could be found for the remaining virally introduced genes—*Oct4* and *Klf4*—the risk of cancer in tissues created with iPS cells would be greatly decreased or eliminated, and the clinical utility of iPS cells would be immense.

The mechanism through which RepSox works in reprogramming was determined through it's known status as a Transforming Growth Factor-ß Receptor 1 kinase inhibitor and further experiments by the Eggan group. Unlike the genes inserted into the cell genome by the retroviruses, RepSox works indirectly by inhibiting Tgf-ß signaling. This inhibition induces the transcription of *Nanog*, a gene important to maintaining pluripotency, effectively bypassing the need for *Sox2*. This mechanism was determined in experiments to measure the expression of *Nanog*, which was observed to increase by 1000% within 48 hours of RepSox treatment.[9] This realization added to the success of finding a small molecule replacement for *Sox2* by outlining the process by which that small molecule is able to do so. Not only did they find something that worked, but they also found out why it worked. If the metaphorical stock price of iPS cells was high when they entered the market in 2006, it has risen still higher in 2009 with the RepSox breakthrough. As with any game-changing technological development, the ramifications of iPS cells have extended outside the science world, in this case impacting the moral and political issues inherent in the stem cell conversation.

Embryonic stem cells have been persistently embroiled in controversy for obvious reasons. An embryo can develop into a human being, and this simple fact has led critics to decry playing around with what could be construed as a human life. On the other side of the coin, stem cell proponents see the vast potential of stem cell therapies as a way to *save* human lives, and

[9] Eggan.

posit that many of the embryos used to procure ES cells would be destroyed anyway. Even within the scientific community, the ambiguous status of the human embryo has caused hesitation, or at least contemplation. Yamanaka himself was first inspired to look for ways of inducing the formation of pluripotent stem cells by an image of an embryo that reminded him of his young children.[10] Into the firestorm of the ES cell debate was thrown the new development of somatic cell reprogramming, followed shortly by the first small molecule replacement. Some analysts have claimed that Yamanaka, Eggan, and company have sounded the death knell for ES cells. "The embryonic stem cell debate is over," Charles Krauthammer, who formerly served on the President's Council on Bioethics, declared. "Scientific reasons alone will now incline even the most willful researchers to leave the human embryo alone."[11] What amounted to a way to avoid using human embryos should have settled the debate in theory, but the realities of iPS cells have complicated the issue. The current necessity of two retroviruses represents the biggest roadblock on the road to efficacious iPS cells. Even if other small molecule replacements are found that can eliminate the need for viruses entirely, the future is uncertain, and it is unclear if iPS cells will ever have the utility of ES cells. Some go so far as to claim that even virus free reprogramming won't be able to produce cells as valuable as ES cells. iPS cells "can't possibly be used for therapies," asserts Thomas Okarma, President of stem cell company Geron Corp. Somatic cells could be damaged by age or toxins, as opposed to "pure crystal-clear" ES cells, says Okarma.[12] These claims may be slightly overblown, but they underline the uncertainty about the future of iPS cells, an uncertainty that complicates the juxtaposition of iPS cells and ES

[10] Dennis Normile, "Shinya Yamanaka: Modest Researcher, Results to Brag About," *Science* **319**, 562 (Feb 1 2008).
[11] Holden, Vogel.
[12] Holden, Vogel.

cells. Bioethical concerns aside, the potential of iPS cells has an impact on funding for all stem cell research, an issue of great practical importance as researchers look to move forward.

Despite being out of the headlines with the economic crisis taking center stage, the debate over stem cell research funding is by no means over. President Obama did reverse former President Bush's executive order banning federal funding for ES cell research, but the issue of receiving and effectively utilizing that funding remains. The first human ES cell lines since Bush's ban were approved on December 3, 2009.[13] It is easy to assume that the issue of stem cell funding is over with a liberal administration in Washington, but it is important to remember that the growth of the body of stem cell knowledge depends on more than the major research institutions of the United States. The race for effective stem cell therapies is an international one, with scientists in the United Kingdom and Japan playing an especially vital role. While the UK is typically open to stem cell research, Japan has historically been stringent when it comes to human ES cell restrictions. The emergence of iPS cells has changed the face of stem cell research funding almost overnight. Yamanaka, who once considered moving his research to California so that he would have freer access to ES cells, has said he now feels obligated to stay in Japan by the sums of money the government is investing in iPS research.[14] Likewise, the US Congress has shown strong bipartisan support for iPS cell research that ES cell research has never enjoyed. Everyone seems ready to invest in a procedure that has the potential to provide all of the benefits with none—or at least less—of the bioethical concerns, and scientists are certainly not going to turn down the money. "I would welcome any infusion of resources," said George Daley of Harvard Medical School, "as long as it's not used as an excuse to further delay funding for the methodology we know works today. You move ahead on all fronts. Scientists

[13] Rob Stein, "U.S. set to fund more stem cell study," *Washington Post*, December 3, 2009.
[14] Normile.

will in the end use what works best."[15] The question becomes: will iPS cells kill ES cell research? The answer is probably not. As long as scientists view ES cells as a worthwhile area of study, enough politicians will defer to the men and women in the laboratory to keep ES cell research alive. iPS cells present enormous potential and will inevitably be pursued by scientists as a novel tool of regenerative medicine and a solution to the constant bioethical and political headaches of ES cell research. Funding for iPS cells should be sought vigorously, but ES cell research should be pursued as well. As Daley suggests, the best and most likely scenario is to proceed on all fronts. iPS cells may be the exciting future of stem cell research, but ES cells are the equally exciting, if somewhat more controversial, present.

In reality, the political and social questions related to stem cells might well be rendered moot as the science progresses rapidly in new directions. Nevertheless, the onus is on scientists and policy makers alike to make sure that stem cell research proceeds as responsibly and rapidly as possible. The stakes are too high and the potential benefits too great not to continue moving forward. There is a long road ahead for stem cells, but the pace of progress is swift and showing no signs of slowing down. The next steps are already being taken.

[15] Gretchen Vogel, "Embryo-Free Techniques Gain Momentum," *Science* **309**, 240 (July 8 2005).

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