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The Stem Cell "Tunnel" Effect

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In this issue, Kane *et al.* suggest that induction of pluripotency from terminally differentiated cells may not always require reprogramming factors but might be prompted by integration of an empty retroviral vector alone.[1] These findings have potential implications for the safety of induced pluripotent stem (iPS) cells as well as for our understanding of the fundamental molecular principles underlying pluripotency.

It almost sounds like a control experiment gone wrong: iPS cells surface in a petri dish treated with empty vector retrovirus. And yet, as unlikely as this may appear, Kane and co-workers report just that. The result they describe is reminiscent of the "tunnel effect" in quantum physics, whereby a particle can "tunnel" through any barrier, but with a very low probability. According to this new report, somatic cells apparently can, on rare occasions, tunnel through the epigenetic barrier that normally prevents dedifferentiation into the primitive blank state of pluripotentiality.

Ironically, shortly after the first wave of reprogramming articles,[2–4] there was some discussion of the possibility that, in addition to the direct reprogramming activity of the four reprogramming factors Oct4, Sox2, Klf4, and c-Myc, positional mutagenesis due to retroviral integration could play at least a supportive role in the reprogramming process. Lending additional credence to this possibility was the fact that the early studies, which all used retrovirus-mediated gene transfer to deliver the reprogramming factors, commonly reported a relatively high number of integration sites.[3] Although subsequent careful and systematic mapping of the retroviral landing spots did not reveal any elite integration sites,[5,6] this issue remained a subject of debate.[7]

Kane and colleagues now report that a relatively high dose of retrovirus alone—i.e., without reprogramming factors—provoked the emergence of iPS-like cells in three of six human fibroblast cell lines tested.[1] Follow-up experiments revealed that the cell lines obtained under such circumstances not only resembled human iPS and embryonic stem cells morphologically but were apparently capable of differentiating into the three principal germ layers both *in vitro*, after embryoid body formation, and *in vivo*, after injection into immunocompromised mice, in which they formed teratocarcinomas at the injection site. These properties are the hallmarks of pluripotentiality and satisfy the criteria typically applied experimentally. Although such experiments are critical, additional follow-up studies will be needed to fully gauge the pluripotency of these cells. These should include an assessment of the DNA methylation patterns in the promoter regions of the *Oct4* and *Nanog* genes, tests to establish that clonally obtained cell lines retain the potential to differentiate into the three principal germ layers, determination of telomerase activity, and confirmation that physiologically functional cell types (e.g., beating cardiac myocytes and neurons with characteristic action potentials) can be obtained. Finally, future studies would need to carefully and rigorously compare the differentiation potential of these new iPS-like cells to other well-established "conventional" iPS and human embryonic stem cells.

Could the retroviral integration process play a functional role in this unorthodox reprogramming process? The authors argue "yes," citing their identification of certain genes previously associated with pluripotency near the viral genomic integration sites. However, this observation cannot distinguish between a possible epiphenomenon and a truly mechanistic association. As it currently stands, this study provokes a number of questions. First, did the lentivirus indeed play a causal role? The authors argue that published studies using nonintegrative means to deliver reprogramming factors are plagued by extremely low efficiencies; however, a recent study reported extremely high and efficient reprogramming using direct delivery of reprogramming factors via messenger RNA.[8] Second, was the retrovirus needed at all, or would a spontaneous conversion have occurred even in untreated cells, albeit at a much lower rate? Could viral infection potentially activate an extremely small subset of fibroblasts that already expressed some or all of the reprogramming factors? Studies suggest that reprogramming efficiency increases if some of the reprogramming factors are already present in the cell types to be reprogrammed.[9] It will also be important to assess the significance of the massive chromosomal abnormalities observed. Are these mere by-products, or are they necessary contributors to the reprogramming process? Does retroviral mutagenesis provoke such aberrations? A recent report describing an alarmingly high frequency of chromosome abnormalities in iPS cells suggests that it does.[10]

Another important question is whether retrovirus-mediated reprogramming poses additional poorly understood risks. And might it, in and of itself, affect cell fate and phenotype? The answers to these questions could turn out to be relevant not only for the nascent reprogramming field but also for other gene therapy studies in which retroviral gene transfer methods are utilized therapeutically. Although there appears to be a general consensus that integration-free reprogramming methods would be safer in a clinical setting, it has also been acknowledged that, at least in initial clinical trials that utilize iPS cell technology, the ability to specifically trace genetically marked, transplanted cells is extraordinarily useful for assessing safety and efficacy.[11]

The most worrisome aspect of the study by Kane *et al.*[1] is also the most intriguing: if rigorously confirmed, the described ability to obtain iPS cells without reprogramming factors severely challenges

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our understanding of the reprogramming process, compromising the current view that sequential stepwise activation of individual reprogramming factors fulfills very specific defined roles. Instead, it implies that stochasticity could play a much greater role than we currently assume.

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Liver Regeneration From Induced Pluripotent Stem Cells

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Are induced pluripotent stem (iPS) cells capable of regenerating an injured organ? A recent study by Espejel *et al.*[1] published in the *Journal of Clinical Investigation* indicates that the answer to this question is yes. In this study, hepatocytes derived from chimeric mice generated with iPS cells were analyzed functionally *in vivo*. IPS cell–derived hepatocytes were found to regenerate after a two-thirds hepatectomy in a manner similar to that of normal hepatocytes and displayed repopulation ability equivalent to that of normal hepatocytes in a competitive liver-reconstitution model. These findings indicate the full potency of iPS cell–derived hepatocytes in liver regeneration and function. Although exciting as a proof of principle, these studies utilized a chimeric mouse model to generate the hepatocytes, which is not a viable option for the generation of hepatocytes from human iPS cells and highlights the need to optimize methods for the *in vitro* generation of functional hepatocytes from iPS cells before clinical use of these cells can become a reality.

The advent of iPS cell technology in mid-2006 has made possible for the first time the generation of patient-specific pluripotent stem cells for cell-based therapy (reviewed by Nishikawa *et al.*[2]). These cells should circumvent the need for immune suppression and at the same time alleviate the ethical considerations of using human embryonic stem (ES) cells. The most convincing data for the developmental potential of iPS cells are in the mouse system, in which germline-competent chimeric mice can be generated,[3] and more recently it was shown that viable mice could be produced that were completely derived from iPS cells using the tetraploid complementation system.[4] However, other work suggests that iPS cells may have some limitations with regard to developmental potential and/or proliferative capacity of their differentiated progeny.[5–7] This is of concern if iPS cells are to be used to reconstitute the function of an entire organ, which would require both efficient differentiation and proliferative ability.

The work described by Espejel and colleagues addresses not only whether iPS cells could generate a liver but also whether the iPS-derived hepatocytes had the proliferative capacity to both regenerate a functional liver and reconstitute a genetic mouse model of liver damage. To generate chimeric mice such that the entire liver is derived from iPS cells, blastocysts from the fumarylacetoacetate hydrolase (FAH)–deficient mouse model were injected with wild-type iPS cells. FAH-mutant mice develop tyrosinemia and die from progressive liver failure[8] unless treated by the administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), a drug that mitigates hepatocyte toxicity in FAH-deficient animals. Therefore, the wild-type iPS cells have a competitive advantage and could achieve nearly 100% liver contribution in some chimeric mice after NTBC withdrawal. Consistent with these repopulation rates, blood analyses of liver function in the chimeric animals indicated completely restored liver function within a month after NTBC withdrawal. The liver function remained normal in these animals after 10 months of NTBC withdrawal, demonstrating that iPS cell–derived hepatocytes can repopulate the damaged liver efficiently and restore liver function over the long term.

Using chimeric mice whose livers were entirely repopulated by iPS cell–derived cells, Espejel *et al.* were then able to evaluate the proliferative capabilities of iPS cell–derived hepatocytes. Either by two-thirds hepatectomy or by transplanting iPS cell–derived hepatocytes into FAH-deficient adult mice, the authors found comparable proliferation rates of the iPS cell–derived liver cells as compared with those of normal hepatocytes. Finally, when performing a competitive reconstitution assay with

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