Editorial

Persistent Integration of Reprogramming Factors Impairs the In Vitro Cardiogenic Potential of Induced Pluripotent Stem Cells

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Induced pluripotent cells (iPSCs) were first generated by the laboratory of Takahashi and Yamanaka in 2006 by reprogramming somatic cells into pluripotent stem cells capable of differentiating into any cell type. In a landmark series of experiments, the Yamanaka laboratory transduced mouse fibroblasts with retroviruses coding 4 transcription factors, the so-called reprogramming factors, Oct4, Sox2, c-Myc, and Klf4. Germline transmission of murine iPSC cells was reported in the following year. Shortly thereafter, human iPSCs were derived, opening new avenues for regenerative medicine with autologous patient-specific cells.

These early approaches of iPS cell production uniformly involved the introduction of reprogramming genes with retroviral or lentiviral vectors. Accordingly, the reprogramming genes irreversibly integrated into the host genome at multiple poorly defined sites. Interestingly, the expression of these genes was largely silenced after iPS induction, thereby allowing differentiation in vitro and in vivo. Nonetheless, it was known right from the second round of mouse iPS articles in 2007 that there is low residual expression of transgenes in reprogrammed cells. Because of this concern, inducible expression systems were developed with reduced likelihood of aberrant reprogramming gene expression. In all of these early approaches, however, the integrated reprogramming vectors remained in the host genome and could in principle become reactivated over time. Indeed, the laboratory of Yamanaka demonstrated that tumors arise in mice generated from iPSC cells, and that the c-myc transgene (a classical oncogene) is reactivated in the tumor cells. Subsequent work demonstrated that reprogramming could be accomplished with fewer pluripotency genes and, specifically, without the use of c-myc. Nonetheless, disruption of genomic DNA at the locus of integration (ie, the region where the transgenes integrate) could result in altered expression of normal host-cell genes. Collectively these results represented persuasive arguments that persistent viral integration precludes the use of this promising cell source in human clinical applications.

Given the potential of iPSC as an autologous cell source for human in vivo applications, several groups pursued novel methodologies for the induction of pluripotency with no or minimal genetic alteration. In 2008, 2 studies demonstrated that iPS cells can be produced by repeat transduction of adenoviral or plasmid-based constructs without any permanent modification to the host-cell genome. The efficiency of these approaches was low, however, and to date they have not been adapted for the reprogramming of human cells. These studies were followed by the use of the piggyBac transposon for the elimination of exogenous reprogramming factors after iPSC induction. This methodology allowed for the relatively efficient reprogramming of human cells from embryonic fibroblasts, while minimizing genomic modification and eliminating reprogramming factors. Subsequent efforts allowed for the reprogramming of somatic cells with purified protein factors or modified coding RNAs.

Despite this progress, significant barriers limit the use of nonintegrative iPSC generation strategies. Although DNA transfection-based methods in principle avoid altering the host genome, they do involve some risk of insertional mutagenesis. The piggyBac system can leave small genomic alterations that could result in altered host gene expression. Protein and modified RNA–based strategies require the use of reagents that are expensive and difficult to generate and purify in the quantities required for reprogramming. Furthermore, methodologies that rely on the repeated administration of reprogramming reagents typically have relatively low iPSC derivation efficiencies when compared with traditional methods.

As a result of these considerations and the difficulties associated with nonintegrating or minimally integrating reprogramming approaches, a large majority of iPS cells used for in vitro studies still rely on the earliest reprogramming strategies (namely retroviral or inducible lentiviral constructs). Indeed, except for iPSC specifically derived for clinical use in humans, there has been seemingly little imperative for the wide spread use of next generation nonintegrating methodologies.

In a study published in this issue of Circulation Cardiovascular Genetics, however, Martinez-Fernandez et al demonstrate that even the in vitro differentiation potential of iPSC is altered by persistent vector integration. Specifically, the authors demonstrate that the cardiac differentiation of iPSCs is hindered by the sustained integration of reprogramming transgenes. The authors make use of the Tet-inducible expression system to regulate the
expression of reprogramming genes. This expression system is coupled to the piggyBac transposon/transposases system that allows the removal of the reprogramming transgenes from the host-cell genome. This approach allows for the direct comparison of the differentiation potential of iPSCs still harboring the silenced reprogramming transgenes with iPSCs from which the transgene has been excised. Because the excised and nonexcised iPSC lines share identical genetic programs, it is possible to compare the effect of sustained integration of the reprogramming vectors directly. Interestingly, transgene-free iPSC differentiated into the cardiac lineage earlier and more efficiently than their transgene-containing counterparts.

This reduction in cardiogenic potential of reprogrammed cells was correlated with the level of basal expression of the reprogramming factors in uninduced iPSC, suggesting leaky expression of pluripotency factors even in the absence of doxycycline treatment. Permanent excision of the integrated transgenes facilitated the optimum execution of cardiogenic programs. Importantly, this effect was observed across multiple independent iPSC lines, suggesting that the observed reduction in cardiac differentiation is independent of the locus of integration. Thus, it is likely that the reduction in cardiac differentiation is because of the residual transgene expression and not alterations in the reprogrammed cell genome. Indeed, the authors demonstrate a statistically significant increase in differentiative capacity of iPSCs and not in nonexcised versus excised cells. The doxycycline-inducible system was then used to vary the level of pluripotency markers in nonexcised versus excised cells. The doxycycline treatment. Permanent excision of the integrated transgenes facilitated the optimum execution of cardiogenic programs. Importantly, this effect was observed across multiple independent iPSC lines, suggesting that the observed reduction in cardiac differentiation is independent of the locus of integration. Thus, it is likely that the reduction in cardiac differentiation is because of the residual transgene expression and not alterations in the reprogrammed cell genome. Indeed, the authors demonstrate a statistically significant increase in differentiative capacity of iPSCs and not in nonexcised versus excised cells. The doxycycline-inducible system was then used to vary the level of pluripotency markers in nonexcised versus excised cells. The doxycycline-induced system was then used to vary the amount of residual reprogramming gene expression in nonexcised iPSC. As may be expected, there was an inverse correlation between the level of transgene expression and cardiac lineage commitment and cardiac-specific gene expression.

The authors also suggest that there may be important functional differences in the derived cardiomyocytes. Transgene-free iPSC-derived cardiomyocytes but not the transgene-containing controls seem to be responsive to isoproterenol stimulation. This observation is contrary to what has been reported by multiple laboratories that used other integrating vectors to reprogram fibroblasts.16–18 Additional studies are required to delineate whether the apparent reduced responsiveness of transgene-containing iPSC-derived cardiomyocytes to β-adrenergic stimulation is specific to the cell lines examined, is restricted to iPSC derived with the piggyBac system or is widely occurring but underappreciated in iPSC-derived cardiomyocytes.

Interestingly, although the excision of transgenes improved the cardiogenic potential of iPSC, endoderm- and ectoderm-related genes displayed similar patterns of expression in transgene-containing and transgene-free cells. These findings suggest that cardiac lineage commitment may be particularly susceptible to alterations in the equilibrium between the pluripotency and the differentiation transcriptional programs. It would be of great interest to elucidate the precise mechanism whereby the cardiac differentiation potential is specifically hindered by the sustained integration of pluripotency factors. Additional studies should also delineate the effect of persistent integration of reprogramming vectors on other noncardiac lineages.

Overall, the data represent persuasive arguments on the need to move toward transgene-free methodologies for the next generation of iPSCs. These considerations are of greater importance in the modeling of human disease because it is critical to identify potentially subtle phenotypic abnormalities. With rapid advances in reprogramming technologies, it will be increasingly feasible to transition current and future in vitro studies to transgene-free iPSC.

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Disclosures

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References


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