On the Road to iPS Cell Cardiovascular Applications

Timothy J. Kamp, Gary E. Lyons

The ability to generate induced pluripotent stem (iPS) cells from somatic cells by the overexpression of a limited number of stem cell–related genes has generated great excitement and interest in the biomedical research community including cardiovascular researchers. The pioneering study by Yamanaka and colleagues showing that overexpression of Oct3/4, Sox2, Klf4, and c-Myc could reprogram mouse fibroblasts to a pluripotent state similar to that of embryonic stem (ES) cells opened major new avenues of research. This epigenetic reprogramming was rapidly extrapolated to the human system using either the same combination of reprogramming factors or a slightly different combination of transgenes (Oct4, Nanog, Sox2, Lin28). Like embryonic stem (ES) cells, iPS cells can be used for basic developmental biology research and also as a cell source to generate theoretically unlimited quantities of desired cell types such as cardiomyocytes. Such differentiated cells types can be used in a wide range of basic research studies and potentially in clinical applications, which not only include cellular therapies but also drug discovery and safety testing.

One appealing aspect of human iPS cells compared to human ES cells is that they can be more readily generated without specialized expertise and access to human embryos, which also avoids the ethical challenges associated with human embryo research. Potentially the most powerful advantage of iPS cells over ES cells is that they can be generated from any patient to produce genetically identical pluripotent cells that can create human disease models or generate patient-specific cells for therapy. Already a number of iPS cell lines have been generated, and proof-of-principle iPS cellular therapies have been pioneered in mouse models.

Despite the speed at which the iPS cell field is racing forward, we are just at the beginning of a long road. Many major questions remain regarding iPS cells. Will they prove to be equivalent to ES cells in their properties? In other words, will iPS cells be equally as pluripotent as ES cells and readily generate all cell lineages represented in the 3 embryonic germ layers? Will different iPS cell lines show distinct differentiation profiles which may be an advantage or disadvantage for a given application? For example, will certain iPS cell lines be less able to differentiate into cardiovascular relevant cell types compared to other lines? Another critical question for therapeutic applications is whether reprogrammed iPS cell lines are prone to tumorigensis. Does the starting cell source matter? How does the technique of reprogramming impact iPS cell behavior? In this issue of Circulation Research, Martinez-Fernandez et al address some of these questions by carefully examining the cardiogenic potential of mouse iPS cells generated from murine fibroblasts using 5 reprogramming factors (Oct4, Klf4, and Sox2).

Most of the detailed understanding of the developmental potential of iPS cells comes from studies using mouse iPS cell lines generated with integrating retroviral vectors encoding 4 reprogramming factors (Oct3/4, Sox2, Klf4, and c-Myc), because this technology has been available the longest, for 3 full years. Some of these 4-factor iPS cells have been demonstrated to be competent to integrate into blastocyst stage embryos and generate chimeric mice which can exhibit germline transmission. This is strong evidence of pluripotency, but initial efforts at the most rigorous developmental test of pluripotency, what is called tetraploid complementation, failed. In tetraploid complementation, an embryo is made tetraploid by fusing cells at the 2-cell stage. The resulting tetraploid blastocyst can develop the cells that become placenta but cannot develop the embryo proper. Stem cells transplanted at the early blastocyst stage of the tetraploid embryo will give rise to a viable mouse strictly from the donor cells, if the cells are truly pluripotent. Very recently, 2 studies have demonstrated using tetraploid complementation that 4-factor iPS cell lines can support development of full term viable mice. Thus, some 4-factor iPS cell lines seem to be fully capable of developing all cell types present in the adult mouse, but the hint of caution is that in the limited data available, not all tested iPS cell lines proved successful in tetraploid complementation. This could be because of experimental limitations and inadequate testing or fundamental differences in the iPS cell lines. Likewise, in vitro studies of cardiac differentiation of 4-factor mouse iPS cell lines have produced mixed results, with some lines showing comparable cardiogenesis to ES cells and others showing delayed cardiogenesis.

Thus with the oldest of iPS cell technologies, some iPS cell lines seem to meet even the most stringent criteria for pluripotency, but it is unlikely that all 4-factor iPS cell lines will meet this standard.

It was quickly recognized that chimeric animals generated from 4-factor iPS cells were at increased risk for tumorigenesis attributable to reactivation of the c-Myc transgene, which clearly represents a safety concern for translation of this technology to humans. However, c-Myc was soon found not to be essential for generation of iPS cells, which could be generated using 3 virally encoded factors: Oct3/4, Sox2, and Klf4 (3F iPS cells). These 3F iPS cells expressed the
standard markers of pluripotency in culture and could generate chimeric offspring following blastocyst injection, providing the first developmental assay supporting pluripotency and, importantly, they did not display any evident increase in risk for tumorigenesis. However, at this stage, it remains to be determined whether these 3F iPS cells are fully pluripotent like ES cells and apparently like some 4-factor iPS cell lines.

To address this question with a focus on cardiac differentiation, in this issue of Circulation Research, Martinez-Fernandez and colleagues present an elegant study of 3F mouse iPS cells focused on their ability to undergo cardiogenesis. These authors began their studies by transducing mouse embryonic fibroblasts using lentiviral delivered human transgenes for SOX2, OCT4, and KLF4 to produce 3F iPS cells. The first test of the cardiogenic potential of the 3F iPS cells was performed by subjecting them to in vitro differentiation in embryoid bodies. The anticipated temporal change in expression of genes indicative of cardiac differentiation was observed, with an upregulation of cardiac transcription factors followed by myofilament proteins and the presence of contracting cardiomyocytes in the embryoid bodies. The cardiomyocytes exhibited organized myofibrils and were electrically coupled based on the presence of gap junction and synchronized multicellular contraction patterns. Furthermore, they demonstrated spontaneous cardiac-like action potentials and Ca\(^{2+}\)-transients. Initial voltage-clamp experiments showed that the iPS cell-derived cardiomyocytes exhibited some of the anticipated ionic currents expected in cardiomyocytes.

Although this in vitro characterization convincingly demonstrates the ability of the 3F iPS cells to generate functional cardiomyocytes, it is only a beginning study of these cardiomyocytes. How do these iPS cell cardiomyocytes compare to ES cell cardiomyocytes under the authors’ same experimental conditions? For example, is there evidence (from the action potentials or other markers) for different types of cardiomyocytes developing, eg, atrial, ventricular, and nodal-like? Likewise, the authors see primarily Ca\(^{2+}\) inward currents without evidence for Na\(^{+}\) current in these cardiomyocytes despite using a ramp protocol that should allow measurement of Na\(^{+}\) current. Mouse ES cell-derived cardiomyocytes exhibit properties typical of cardiac muscle such as robust intracellular Ca\(^{2+}\)-stores with Ca\(^{2+}\)-induced Ca\(^{2+}\)-release?

Despite these unresolved questions, the authors provide evidence that these 3F iPS cells can robustly undergo cardiogenesis based on their embryonic developmental studies. The authors take a slightly different route by aggregating the 3F iPS cells to morula stage embryos using a technique called noncoerced diploid aggregation. If the iPS cells are fully pluripotent, they would be anticipated to contribute to the generation of a chimeric blastocyst which develops into a chimeric mouse. Because the iPS cells include a lacZ transgene, they can be readily tracked, and the study demonstrates a mosaic distribution of lacZ-positive iPS cell progeny in the developing mouse embryos. The iPS cells were found to be clearly present in the heart at all stages of development studied, and live-born chimeric mice exhibited normal cardiac function. Although these observations do not quite reach the pluripotency-defining bar of tetraploid complementation, at least for the focused analysis on cardiac development, it seems likely that the 3F iPS cells generated by the Martinez-Fernandez can undergo normal cardiac development. Furthermore, the authors did not observe tumors in the chimeric offspring from the 3F iPS cells, but the number of mice studied was relatively small. Overall, the study of Martinez-Fernandez provides the most detailed analysis of cardiogenesis of 3F iPS cells to date, and the news is good thus far in that these cells are readily able to differentiate to form cardiac muscle.

However, we have not reached our desired destination with current 3F iPS cells because reprogramming without c-Myc reduces but does not eliminate the risk of tumorigenesis. A recent safety study of iPS cell lines examining their potential to generate teratomas from differentiated progeny revealed that secondary neurospheres differentiated from iPS cells could form teratomas when transplanted into mouse brains regardless of the presence or absence of the c-Myc transgene. Another clear risk for oncogenic transformation of iPS cells is related to the uncontrolled integration of the transgenes into the genomes of the reprogrammed cells. Unfortunately, the potential for insertional mutagenesis to activate protooncogenes has been demonstrated to be a real risk in the case of some gene therapy protocols. Hence, integrating lentiviral 3F iPS cells are only one more stop on the road of perfecting iPS cell technology.

The approaches to generate iPS cells are changing at a remarkable pace. Small molecules have been used to increase the efficiency of generating iPS cell lines and have enabled the use of only 2 transgenes to generate iPS cell lines. Mouse iPS cells have now been generated using adenovirus or plasmid-mediated transfections, which avoid the potential problems associated with viral integration of transgenes. Generation of mouse iPS cells using only recombinant proteins without transgenes has also been described. Furthermore, techniques using nonintegrating transgenes have succeeded in generating iPS cells from human cells. This exponential growth in technologies to produce iPS cells has, however, outpaced the ability to thoroughly investigate the properties of the resulting iPS cells. Careful studies like those of Martinez-Fernandez are needed for next generation iPS cells.

The groundbreaking studies done with mouse iPS cells have greatly advanced the field, but ultimately, for clinical applications, human iPS cells need the closest scrutiny. How can we demonstrate that human iPS cells are pluripotent without access to embryological studies? In vitro studies will
certainly play an important role in characterizing lineage potential development.28 What will be the best way to assess tumor risk in the human iPS cells? What human iPS cell technologies will provide cells optimal for repair of the injured myocardium? We are on the road to powerful cardiovascular applications of iPS cells, but this remains a largely unmapped route that requires us to proceed expeditiously but cautiously.

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References

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