

## Cardiac Cell Cycle Activation as a Strategy to Improve iPSC-Derived Cardiomyocyte Therapy

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Over the past 2 decades, scientists and clinicians have strived to leverage stem cell therapy as a treatment for heart failure. However, a myriad of clinical trials investigating the potential of adult stem cells to restore damaged myocardium showed inconsistent effectiveness.<sup>1</sup> A subsequent paradigm shift emerged with evidence that the benefits of such treatment, if any, were derived from cardioprotective paracrine factors released by stem cells as opposed to direct myocardial regeneration. As a result, researchers have attempted to regenerate the lost myocardium by delivering cardiac cells to the sites of myocardial damage.

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With recent advances in induced pluripotent stem cell (iPSC) technology, cardiomyocytes can be efficiently and reproducibly generated from iPSCs.<sup>2</sup> The use of iPSCs in cell therapy is particularly attractive for the following reasons. First, iPSCs have extensive self-renewal and differentiation potential, which enables generation of a large number of iPSC-derived cardiomyocytes (iPSC-CMs) required for successful cell therapy. Second, this self-renewal property of iPSCs makes them amenable to desired genetic modification before differentiation.<sup>3</sup> Third, iPSC-CMs exhibit properties of beating cardiomyocytes and have been shown to engraft in the host myocardium and electromechanically couple to neighboring native cardiomyocytes.<sup>4</sup> Finally, because iPSCs are derived from patients' own somatic cells, the use of autologous iPSC-CMs may help circumvent potential immunogenicity of the transplanted cells.<sup>5</sup> Despite these beneficial characteristics of iPSCs, poor survival and engraftment of the transplanted cells remain major obstacles for efficient myocardial regeneration.<sup>1</sup>

In this issue of *Circulation Research*, Zhu et al<sup>6</sup> sought to test whether the use of genetically engineered, cell cycle-activated iPSC-CMs could improve cardiac cell therapy. Recognizing that only a small fraction of transplanted iPSC-CMs survive and engraft into the myocardium, they hypothesized that if iPSC-CMs could retain proliferative capacity,

then even a small initial population of engrafted cells might proliferate and significantly restore myocardium. To investigate this hypothesis, Zhu et al used a lentivirus to overexpress cyclin D2 (CCND2), an important cell cycle regulator of the G1-to-S phase transition, in iPSCs under the regulation of the  $\alpha$ -myosin heavy chain promoter. After differentiation, significantly more CCND2-overexpressing iPSC-CMs (iPSC-CCND2<sup>OE</sup>CMs) exhibited proliferative markers compared with wild-type CCND2 iPSC-CMs (iPSC-CCND2<sup>WT</sup>CMs). In a mouse myocardial infarction model, Zhu et al observed significantly improved left ventricular systolic function and decreased scar size after the treatment of iPSC-CCND2<sup>OE</sup>CMs compared with iPSC-CCND2<sup>WT</sup>CMs and sham injury groups. Consistent with this observation, bioluminescence imaging of the hearts showed increased engraftment of the cells in the iPSC-CCND2<sup>OE</sup>CM treatment group. Immunostaining of the heart sections with human specific troponin T and human nuclear antigen verified that the majority of these engrafted cells ( $\approx$ 96%) were cardiomyocytes.

### Cardiac Cell Cycle Activation

This work by Zhu et al<sup>6</sup> highlights the feasibility of resetting the regenerative potential of iPSC-CMs by inducing cell cycle activity to improve cell therapy. Human and animal studies have repeatedly shown that embryonic/fetal cardiomyocytes can proliferate. However, as they mature, they lose their ability to multiply and eventually undergo cell cycle arrest (post-mitosis).<sup>7</sup> Similarly, differentiated iPSC-CMs also become post-mitotic and lose their ability to replicate as they mature. A potential solution for cardiac regeneration is to alter the cell cycle of cardiomyocytes to remain in the active replication cycle (Figure). To this end, extensive research has been conducted toward understanding how the cardiomyocyte cell cycle is regulated during cardiac development. Previous research found that while embryonic cardiomyocytes have a high expression of positive cell cycle regulators such as cyclins, cyclin-dependent kinases, and proto-oncogenes, adult cardiomyocytes have a low expression of these proteins.<sup>7</sup> Thus, it has been postulated that direct overexpression of relevant cell cycle regulators may allow sustained myocardial proliferation. Among the regulators, D-type cyclins appear particularly promising in modulating myocardial proliferation. D-type cyclins have been shown to be highly expressed during embryogenesis, but their levels dramatically decrease after birth, reflecting corresponding cardiac cell cycle activity. Although mice lacking D-type cyclins had progressive heart defects during embryogenesis that led to their premature death,<sup>8</sup> CCND2 overexpression in mice resulted in significantly enhanced cell cycle activity with decreased infarct size after myocardial injury.<sup>9</sup> Motivated by these studies, Zhu et al<sup>6</sup> created CCND2-overexpressing iPSC-CMs and demonstrated increased and

The opinions expressed in this article are not necessarily those of the editors or of the American Heart Association.

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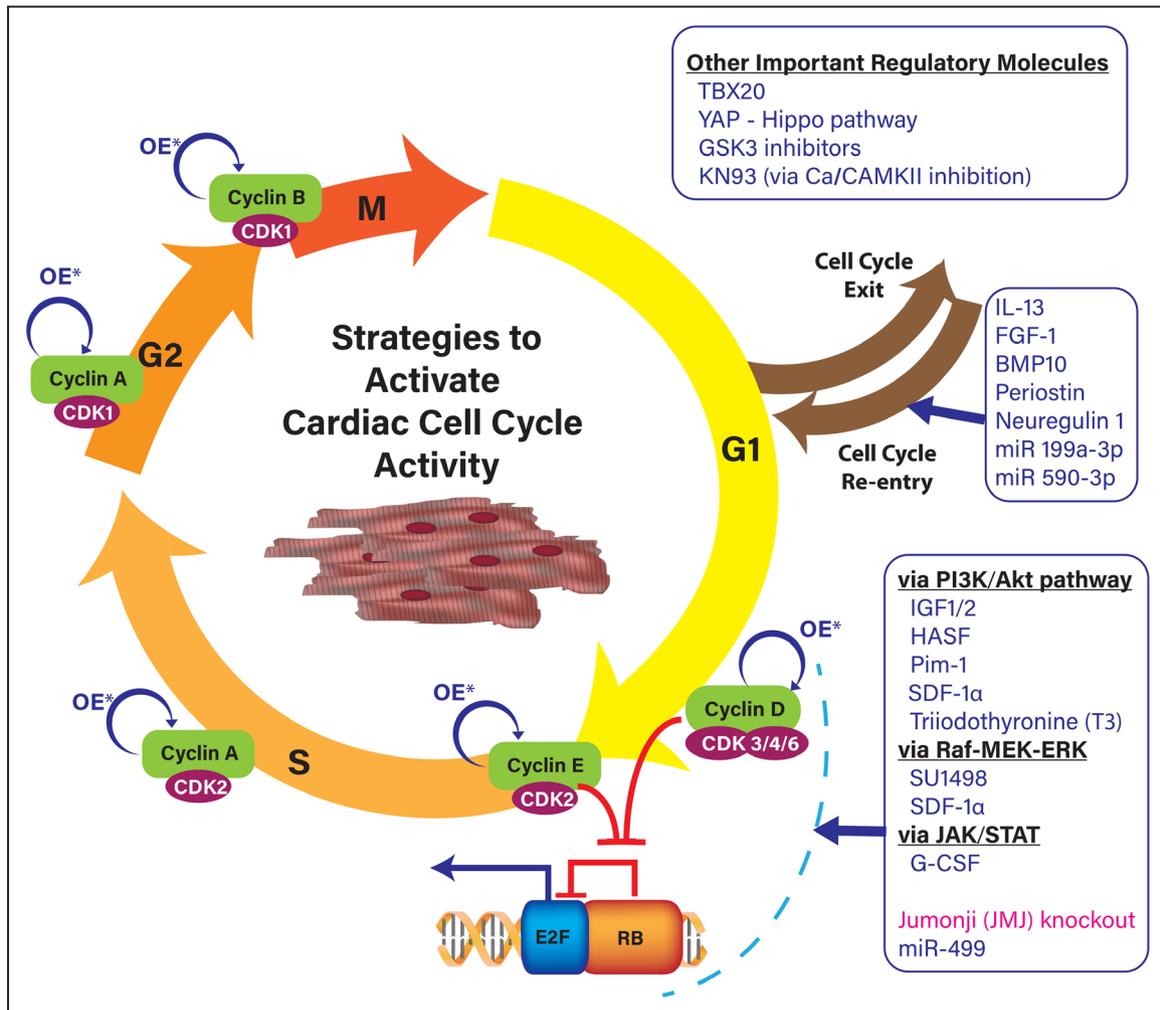
(*Circ Res.* 2018;122:14-16.

DOI: 10.1161/CIRCRESAHA.117.312287.)

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*Circulation Research* is available at <http://circres.ahajournals.org>

DOI: 10.1161/CIRCRESAHA.117.312287



**Figure.** Strategies to activate cardiac cell cycle activity for myocardial regeneration. Positive cell cycle regulators such as cyclin-cyclin-dependent kinase (CDK) complexes facilitate cell cycle entry and progression. Overexpression (OE\*) of cyclins have been shown to promote cell cycle activity. Treatments with various hormones, growth factors, microRNAs (miRs), or small molecules have also been used to (1) induce reentry into the cell cycle or (2) modulate activities of direct cell cycle regulators and their molecular correlates (eg, tumor suppressor retinoblastoma [RB]) along the various points of the cell cycle. BMP, bone morphogenetic protein; FGF, fibroblast growth factors; G-CSF, granulocyte colony-stimulating factor; GSK3, glycogen synthase kinase 3; HASF, hypoxia-regulated Akt-mediated stem cell factor; IGF, insulin-like growth factor; SDF, stromal cell-derived factor; and T3, triiodothyronine.

sustained proliferative activity of the transplanted iPSC-CMs resulting in extensive myocardial regeneration in vivo.

Another approach to promote cell cycle activity is by treating cardiac cells with hormones, growth factors, microRNAs, or small molecules to enhance cell cycle signaling or to induce re-entry of differentiated myocytes (Figure).<sup>10</sup> For example, treatment of embryonic stem cell-derived CMs with insulin-like growth factor was shown to enhance their proliferation by activating the phosphoinositide 3-kinase/Akt signaling pathway.<sup>11</sup> Via a different pathway of JAK/STAT (janus tyrosine kinase-signal transducer and activator of transcription), treatment of iPSC-CMs and embryonic stem cell-derived CMs with granulocyte colony-stimulating factor resulted in significant proliferation of the derived cardiomyocytes.<sup>12</sup> Many microRNAs were also reported to enhance cardiac cell cycle activity when they were overexpressed or inhibited/knocked down in cardiomyocytes in vivo.<sup>10</sup> More recently, using a high-throughput combinatorial screening approach, several small molecules enhancing iPSC-CM proliferation have also been identified.<sup>13</sup>

Significant research efforts have been made to discover genetically modifiable or druggable targets to enhance cardiac cell cycle activity. Although promising, we still know relatively little about the mechanism underlying cardiac cell cycle regulation. Because of the complex molecular circuits involved in this process, it is difficult to fully understand how the treatment of the proproliferative molecules affects overall cell cycle activity and function. Rather than targeting upstream signaling pathways, overexpression of direct cell cycle regulators may provide a more reliable and consistent method to activate the cell cycle. In particular, with advances in genome-editing technologies such as CRISPR/Cas9, genetic engineering of iPSCs has become highly efficient, precise, and convenient.<sup>14</sup> This ability to genetically modify iPSCs is particularly attractive because it does not require temporal and spatial regulation of drug treatments. The continued myocardial regeneration of iPSC-CMs observed by Zhu et al<sup>6</sup> also supports the durable effectiveness of genetically engineered cell cycle activation. Although more research is needed to

understand the long-term effects of CCND2 overexpression in iPSC-CMs, the approach by Zhu et al to genetically reset cardiac cell cycle activity seems to be an attractive strategy for efficient myocardial regeneration.

### Potential Hurdles for Safe and Effective Clinical Application

Before the use of cell cycle-activated iPSC-CMs for safe and effective clinical translation, multiple hurdles need to be addressed. First, the generation of iPSCs takes time (6–12 weeks) and remains costly, especially if any genetic modifications are needed. Second, the iPSC-CM therapy may increase the risk of developing potentially life-threatening arrhythmias. Shiba et al<sup>4</sup> recently reported allogeneic transplantation of iPSC-CMs in a nonhuman primate model using cynomolgus monkey (*Macaca fascicularis*). Although they observed successful engraftment of the transplanted iPSC-CMs, all 5 recipient monkeys developed transient episodes of ventricular tachycardia. Currently, it is unknown whether the proliferation of cell cycle-activated iPSC-CMs increases the risk of arrhythmia because small animal models do not accurately recapitulate the underlying arrhythmia risk. Thus, further investigation of their arrhythmogenicity is essential before commencing safe clinical translation. Finally, transplantation of iPSC-CMs may carry a risk of tumorigenicity.<sup>15</sup> This risk increases with the extent of incompletely differentiated cells remaining in culture before transplantation. With advances in our differentiation and maturation strategies, we are now able to generate >90% pure, functional iPSC-CMs.<sup>2</sup> Yet, whether cell cycle-activated iPSC-CMs have increased oncogenic potential remains unknown and requires further studies. Additionally, tumorigenicity may be increased in the presence of culture-acquired mutations,<sup>16</sup> especially when iPSCs undergo genetic modification such as with lentivirus-mediated transduction. Although it is encouraging that Zhu et al did not observe tumor formation at 4 weeks after transplantation, this does not rule out potential long-term tumorigenic risk in humans. Thus, extended monitoring in preclinical stage will be crucial to demonstrate safety.

### Conclusions

The study by Zhu et al<sup>6</sup> is a valuable addition to the growing body of literature investigating cell cycle activation as a strategy to improve iPSC-CM regeneration. By genetically engineering iPSCs to overexpress an important cell cycle regulator CCND2, Zhu et al showed significantly increased and sustained proliferation of the differentiated iPSC-CMs. Transplantation of these cell cycle-activated iPSC-CMs resulted in extensive myocardial regeneration and subsequent improved cardiac function after myocardial injury in vivo. Although more studies are warranted to address potential risks of arrhythmogenicity and tumorigenicity, CCND2-overexpressing iPSC-CMs seem to be a promising solution to improve our current state of cardiac cell therapy.

### Acknowledgments

This publication was supported in part by research grants from the National Institutes of Health (NIH) F32 HL134221-01 (J.-W. Rhee), R01 HL113006, NIH R01 HL126527, NIH R01 HL133272, American Heart Association (AHA) 17MERIT33610009, and California Institute of Regenerative Medicine (CIRM) DR2A-05394 and CIRM RT3-07798 (J.C. Wu).

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KEY WORDS: Editorials ■ cell cycle ■ cell transplantation ■ heart failure ■ myocyte, cardiac ■ pluripotent stem cell

# Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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*Circ Res.* 2018;122:14-16

doi: 10.1161/CIRCRESAHA.117.312287

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

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