Solving the puzzle of pluripotent stem cell-derived cardiomyocyte maturation: piece by piece

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Comment on: Ruan JL, Tulloch NL, Razumova MV, *et al*. Mechanical Stress Conditioning and Electrical Stimulation Promote Contractility and Force Maturation of Induced Pluripotent Stem Cell-Derived Human Cardiac Tissue. Circulation 2016;134:1557-67.

Abstract: There is a growing need for *in vitro* models which can serve as platforms for drug screening and basic research. Human adult cardiomyocytes cannot be readily obtained or cultured, and so pluripotent stem cell-derived cardiomyocytes appear to be an attractive option. Unfortunately, these cells are structurally and functionally immature—more comparable to foetal cardiomyocytes than adult. A recent study by Ruan *et al*., provides new insights into accelerating the maturation process and takes us a step closer to solving the puzzle of pluripotent stem cell-derived cardiomyocyte maturation.

Keywords: Stem cells; cardiomyocyte; differentiation; maturation

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Cardiovascular disease is the leading cause of mortality in developed countries, responsible for more deaths than cancers, respiratory diseases and automobile accidents combined (1). Given that terminally-differentiated adult cardiomyocytes proliferate very poorly (2), there is an enormous need for new therapies to regenerate the heart following injury, whether in the form of small molecule drugs, protein growth factors, cell therapy or tissue engineering.

To develop new therapies, scientists utilise a variety of animal models—primarily surgical occlusion of the coronary artery in rodents, pigs and non-human primates. However, these animal models do not always accurately recapitulate the pathophysiology of myocardial infarction, nor do they mimic human pharmacokinetics, dynamics and toxicity after therapeutic intervention (3). Such models are also not financially or ethically viable for large-scale drug candidate screening, and *in vitro* methods would be preferable for early-stage screening. Human adult cardiomyocytes are difficult to obtain, highly heterogeneous, cannot be reliably maintained in culture, and thus cannot serve as a stable platform on the scales needed for commercial drug research and development. As an alternative, many drug companies still rely on human embryonic kidney or Chinese hamster ovary cell lines, engineered during the 1980's to express cardiomyocyte ion channels such as the hERG potassium channel, as their standard model to assess cardiotoxicity (4,5). These models clearly leave a lot to be desired and likely lead to the exclusion of many good candidate drugs for perceived toxicity which would not actually manifest in patients. It is also known that drugs with long-term cardiotoxicity have slipped through the safety net and entered onto the general market due to inadequate screening during drug development. Indeed, previously undetected cardiotoxicity is a major cause of drug attrition and withdrawal, and is not limited to drugs targeting cardiovascular diseases (4).

Human pluripotent stem cell-derived cardiomyocytes

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(PSC-CMs) are, on paper, an ideal solution to these challenges. Induced pluripotent stem cells (iPSCs) can be readily generated from a small blood sample, can be banked, cultured and maintained indefinitely (6), and reliable methods for differentiating and obtaining pure cultures of PSC-CMs have now been developed (7). These models have already been used to generate patient-specific PSC-CMs and to elucidate mechanisms underlying cardiovascular diseases (8,9). However, even after complete differentiation and expression of signature cardiomyocyte markers, these PSC-CMs retain an "immature" physiology, presenting a phenotype more akin to mid-term foetal cardiomyocytes rather than adult cells. This immaturity affects almost all aspects of PSC-CM behaviour, such as cell morphology, metabolism, electrophysiology, contractility and ion channel expression, all of which are potential sources of drug-induced cardiotoxicity, or potential therapeutic targets (10). Considerable efforts are currently being taken to develop robust PSC-CM platforms for drug development, as well as disease modelling and patient-specific drug screening. An excellent in-depth discussion of these efforts, as well as a summary of cardiomyocyte maturation is provided by Denning and colleagues (11).

This immature status of PSC-CMs is also a significant limiting factor in the development of cell transplantation therapy, where cardiomyocytes must successfully integrate and synchronise into a contracting heart with its own electrophysiological signalling. Indeed, two recent landmark studies found a degree of arrhythmia resulting from PSC-SM cell transplantation in non-human primates (12,13). This is unsurprising, given that one struggles to imagine how small, rounded cells with disorganised myofibrils, weak contraction force and spontaneous action potentials would integrate with large, aligned, rod-shaped cells in the native myocardium. To the same end, there are questions about whether these cells can suitably serve as accurate *in vitro* models when they utilise different metabolic pathways (glycolysis rather than fatty acid oxidation), have atypical electrophysiology and are totally absent of essential cardiomyocyte structures such as transverse tubules.

Many approaches have been taken to improve on the maturation of PSC-CMs, aiming to accelerate a complex natural process which occurs over several years in the human body. Indeed, long-term culture of PSC-CMs does improve their maturity over the course of several months, albeit with obvious limitations for large-scale use (14). Other approaches typically revolve around mimicking the *in vivo* maturation environment during development, including delivering hormones, growth factors, microRNAs (15), manipulating extracellular substrate characteristics (16), providing electrical stimulation (17), or co-culturing PSC-CMs with relevant cells (18). A recent paper from our lab showed that co-culture of human PSC-derived cardiomyocytes with endothelial cells significantly improved many aspects of maturity through the action of miRNAs (19).

A recent study published in Circulation by Ruan *et al*., demonstrated incremental improvements in iPSC-CM maturation by combining several of the above-mentioned approaches. The authors used collagen Type I, the dominant load-bearing protein in the developing heart, seeded with immature iPSC-CMs to create cardiac constructs. These constructs were then subjected to a combination of mechanical and electrical stimulation to enhance their maturity over 2 weeks. The creation of a cardiac construct is certainly a more representative model than PSC-CMs cultured on standard 2D plasticware, and the authors showed a good survival rate of cells in the construct. Ruan and colleagues found that constructs subjected to static stretch conditioning displayed improved morphology, sarcomere alignment and formed stiffer constructs overall, all of which are signs of improved maturation. Interestingly, the addition of electrical pacing did not further alter cell morphology (size, alignment etc.) or calcium handling above and beyond static stretch conditioning alone, but did further improve contractility. This supports the widely-held hypothesis that maturation results from the combined effect of multiple stimuli. The data presented in this paper support the idea that morphological maturation may be primarily influenced by physical/mechanical cues, whereas contractile maturation may be more affected by electrical stimulation. The authors also demonstrated a few other aspects of improved maturation in the constructs including primitive intercalated disk formation, increased SERCA2 expression and moderate improvements in the force-frequency relationship following mechanical and electrical stimulation. This is certainly useful information for future research in the field.

Although encouraging, the study is not without limitations and caveats. Perhaps the largest limitation is that the maturity of the obtained PSC-CMs appears no better than those already achievable by simple 2D culture methods and culture media supplementation, and the overall maturity reached still pales in comparison to endogenous adult cardiomyocytes. For example, the authors revealed an average contraction force of 1.5 mN/mm^2 after mechanical and electrical stimulation, far lower than native

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adult human cardiomyocytes which contract in the range of 40–80 mN/mm^2. However, this is typical of most papers published in the field, and not a specific criticism of this maturation protocol. It also would have been beneficial to examine other crucial aspects of maturation, such as the metabolic switch, resting membrane potential, or t-tubule formation, to build a more complete picture of the effect of each treatment on the constructs.

We are surprised that the addition of electrical pacing showed only limited improvements, given the successful use of electrical pacing by other authors (17). As the authors mentioned, it is likely that the parameters could be adjusted to further improve PSC-CM development. We also believe that integrating cardiac endothelial cells into the engineered constructs, or the addition of key growth factors could further enhance the results—albeit adding a significant degree of complexity to the study.

The authors also, in our opinion, missed an opportunity to clearly demonstrate that these matured constructs were more useful and predictive for drug screening, or as a model to investigate developmental cues, compared to their immature counterparts. They did show that the engineered PSC-CM constructs were responsive to external stimuli such as the beta-adrenergic agonist isoproterenol and extracellular calcium levels, but unfortunately they did not explore this further. Another weakness is that the authors did not clearly separate the effects of electrical stimulation alone. It would have been useful to see whether electrical stimulation alone could still enhance contractility in the absence of mechanical stimulation. We look forward to subsequent publications where the authors endeavour to further untangle these cues.

Clearly, endogenous cardiomyocyte maturation is a complex, lengthy process and it would be naïve to believe that any single "magic bullet" solution exists which can easily provide a homogenous population of adult-like PSC-CMs *in vitro*. We believe that the authors, and other research groups in the field, are all on the right track, aiming to piece together multiple aspects of the puzzle to improve PSC-CM maturation. For example, in 2007 differentiated PSC-CMs were typically obtained with an efficiency of <5%, but this has risen to >95% in 2016 thanks to piece-by-piece improvements in protocols and biological understanding (20). Similarly, it is inevitable that some time-course of factors will be found that can produce differentiated PSC-CMs with high similarity to adult human cardiomyocytes. An ideal solution would reproduce most important aspects of adult cells, but also needs to be accessible and scalable to the levels demanded by industry, and provided at a feasible cost-benefit ratio. This is a significant challenge, and the large-scale adoption of PSC-CMs by pharmaceutical companies will likely take many more years of basic research, as well as development of new culture methods, assays and equipment. However, the benefits will be too large to be ignored.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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