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Strategies for Improving the Maturity of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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Over the past decade, induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) have emerged as one of the most important tools in the field of cardiac research because they hold the potential for numerous translational applications. Human iPSC-CMs, sharing identical genomic make-up as their donors, are employed for modeling inherited cardiac diseases such as hypertrophic cardiomyopathy (HCM)[1] and Brugada syndrome,[2] allowing us to investigate their pathology and discover novel therapeutic reagents.[3] In addition, iPSC-CMs offer a personalized platform for predicting cardiotoxicity of drugs such as the widely used chemotherapy reagents doxorubicin[4] and tyrosine kinase inhibitors.[5] Such prediction capability was nearly impossible before the advent of iPSC technology. However, while there is no doubt about the advantages of iPSC-CMs, successful translation of these cells into clinical settings requires them to recapitulate the physiology and pathology of adult cells. Unfortunately, iPSC-CMs generated using current methods are often structurally and functionally immature, resembling fetal CMs. This immature phenotype of iPSC-CMs may affect their response to drugs being tested, leading to less accurate predictions of their *in vivo* effects.[6] Therefore, there is an urgent need for methods that allow us to rapidly and consistently mature iPSC-CMs.

During natural cardiac maturation, CMs undergo physiological hypertrophy along with metabolic and structural changes such as increased oxidative metabolism and more aligned sarcomeres. These changes of CMs allow them to generate stronger contractions in a more efficient manner so that they can meet the growing workload demand. At the same time, subjecting CMs to increasing workload can actively drive cardiac maturation. Specifically,

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two recent work demonstrated the viability of this methodology via modulation of tissue stress[7] and increasing beating frequency,[8] respectively.

The work by Abilez et al.[7] showed that passive stretching of engineered heart muscle (EHM) improves its maturity. The EHMs were generated by mixing human iPSC-CMs or human embryonic stem cell-derived cardiomyocytes (ESC-CMs) with human IMR-90 fibroblasts in collagen hydrogels.[7] By growing the EHMs between two polydimethylsiloxane (PDMS) posts of varying distances (5 mm, 7 mm, 9 mm, or 0 mm with only one post), the investigators were able to stretch the EHMs to various lengths, resulting in distinct stress distributions across the EHMs as predicted by computational modeling. Interestingly, EHMs displayed significant variations in calcium handling under different stretching conditions. For instance, moderate tension (7 mm) led to increased calcium amplitude, slower beating rate, and longer duration compared to low tension (5 mm).[7] These results suggest modulation of tissue stress has a direct impact on its functional performance and may be optimized for improving cardiac maturation. To evaluate maturation on a molecular level, the authors performed qPCR analysis of a panel of genes associated with maturation. Overall, EHMs exhibited higher expression of all the tested genes than cells grown as monolayers. In particular, caveolin-3 (CAV), a T-tubule protein, was considerably upregulated in EHMs.[7] This work opens a new window for rational designs of EHMs with varying stress distributions. Depending on the downstream applications, this platform may be optimized for promoting cardiac maturation as well as for simulating pathological conditions such as hypertrophic cardiomyopathy.[7]

In addition to mechanical stress, electrical stimulation is also an effective approach for improving the maturity of iPSC-CMs. Recently, Ronaldson-Bouchard et al.[8] generated adult-like cardiac tissue from human iPSCs using a high-intensity training regime. Specifically, cardiac tissues assembled from early-stage CMs (isolated on day 12 of differentiation) were subjected to electrical stimulation with gradually increasing frequency, from 2 Hz to 6 Hz within 2 weeks, followed by another week of stimulation at 2 Hz. The resulting cardiac tissues exhibited remarkable maturity by many metrics.[8] On a transcriptional level, these intensity-trained tissues had comparable gene expression profile to adult heart tissue, such as the upregulation of *ITPR3*, *KCNH2*, *MYH7*, *CAV3*, and *RYR2* and the downregulation of *MYH6* and *HCN4*.[8] Structurally, transmission electron microscopy revealed well-developed ultrastructures that are characteristic of adult CMs: orderly aligned sarcomeres with clear I-bands, A-bands, Z lines, and M-lines and a high density of mitochondria (30%). Notably, T-tubules, a structure required for efficient excitation-contraction coupling and often missing in immature CMs, were also extensively found in these cells.[8] Consistent with structural maturation, their calcium handling also resembles the performance of mature CMs. Specifically, contraction force increased significantly in response to increasing concentrations of calcium, indicating a robust calcium-induced calcium response (CICR).[8] The authors also observed a positive force-frequency relationship (FFR), a hallmark of maturation that was not reported in previous iPSC-CM models. Together, these results indicate an advanced level of maturity, both structurally and functionally, in these intensity-trained cardiac tissues.

Physically exercising cardiac cells, via either mechanical or electrical methods, has arisen to be a promising maturation strategy in recent years.[9, 10] However, our knowledge of the underlying mechanisms is lacking. Though previous work suggested the potential role of reactive oxygen species (ROS) in mediating physical stimulations,[10] the downstream signaling pathways involved in maturation are still largely elusive. A recent work by Hodgkinson et al.[11] may offer us some valuable insights on this front. This study found that activation of nuclear factor kappa B (NFκB) by toll-like receptor 3 (TLR3) is required for the formation of mature CMs during cardiac reprogramming. Specifically, the authors performed reprogramming of mouse neonatal cardiac fibroblasts into CMs using a known combination of miRNAs. Pharmacological inhibition or siRNA knockdown of TLR3 during the reprogramming effectively abolished CM maturation, as evidenced by the lack of sarcomere structure and the low expression of sarcomere genes such as *TNNI3* and *MYH6*. Interestingly, inhibition of TLR3 did not affect the expression of cardiac precursor genes such as *GATA4*, *MEF2C,* and *TBX5,*[11] indicating that TLR3 signaling is required only for the maturation stage but not the earlier initiation stage. To further identify the downstream player responsible for these effects of TLR3, the investigators examined two candidates, NFκB and AP-1, which are well-known transcriptional factors regulated by TLR3. Inhibition of NFκB, but not AP1, prevented the formation of mature CMs. Similarly, siRNA knockdown of ikbkb, an activator of NFkB signaling, attained the same results. These two experiments confirmed that NFκB, but not AP1, mediated the effects of TLR3 in cardiac maturation. Subsequently, the authors successfully identified RelA, a main member of the NFκB family, as the key player. Specifically, knockdown of RelA, just like the inhibition of TLR3, prevented the upregulation of sarcomere genes in response to cardiac repgrogramming.[11]

The next important question is how RelA regulates cardiac maturation. By performing a CHIP assay, the authors found RelA was significantly enriched in the promoter regions of several key cardiac sarcomere genes such as *MYH6, MYPN, TNNI3,* and *ACTN2*. In particular, among these genes, *MYH6* (for mouse) and *TNNI3* are known markers for cardiac maturation as their expression increases along with maturation. In summary, this work uncovered a novel role of TLR3-NFκB (RelA) pathway in cardiac maturation by regulating the expression of cardiac sarcomere genes, including those indicative of maturation.[11] It should be noted that this work was performed with cardiac reprogramming rather than iPSC cardiac differentiation, and functional maturity was not evaluated. Nevertheless, it offers us valuable insights for future investigations into the molecular mechanisms of cardiac maturation. Moreover, it is also worth examining how the maturation methods, mechanical stretching[7] and intensity training[8] discussed above affect the activity of NFkB, specifically RelA, in iPSC-CMs.

Recent years have witnessed exciting progress in developing maturation strategies for iPSC-CMs. These methods range widely, including both physical stimulations (e.g., electrical stimulation,[8] mechanical stress,[7] and substrate stiffness[12]) and biochemical stimulations (e.g., T3 hormone treatment,[13] microRNA overexpression,[14] and metabolic manipulation[15]) (Figure 1), with various degrees of success. However, we still have a long way to go in pursuit of an ideal maturation strategy. To date, even with the most advanced techniques exemplified by the work of Ronaldson-Bouchard et al.,[8] there is still room for improvement.

Specifically, both the contractile force and the conduction velocity of their engineered cardiac tissues were significantly lower than those of adult myocardium, indicating suboptimal functional maturation. Furthermore, in contrast to our detailed knowledge of cardiac differentiation regarding its developmental stages and the associated signaling pathways, our understanding of the maturation process is limited. For instance, it is unclear whether or not cardiac maturation can be dissected into well-defined stages, similar to cardiac differentiation. In addition, as mentioned earlier, the mechanism by which physical stimulations promote maturation has yet to be elucidated. These critical gaps in knowledge prevent us from developing a systematic approach to optimize the maturation protocol. Therefore, in future studies, our efforts should focus not only on developing novel maturation methods, but also on understanding the molecular mechanisms governing the course of cardiac maturation. Together, these efforts may ultimately help us achieve a comprehensive maturation of iPSC-CMs and unlock their full potential in disease modeling and drug testing.

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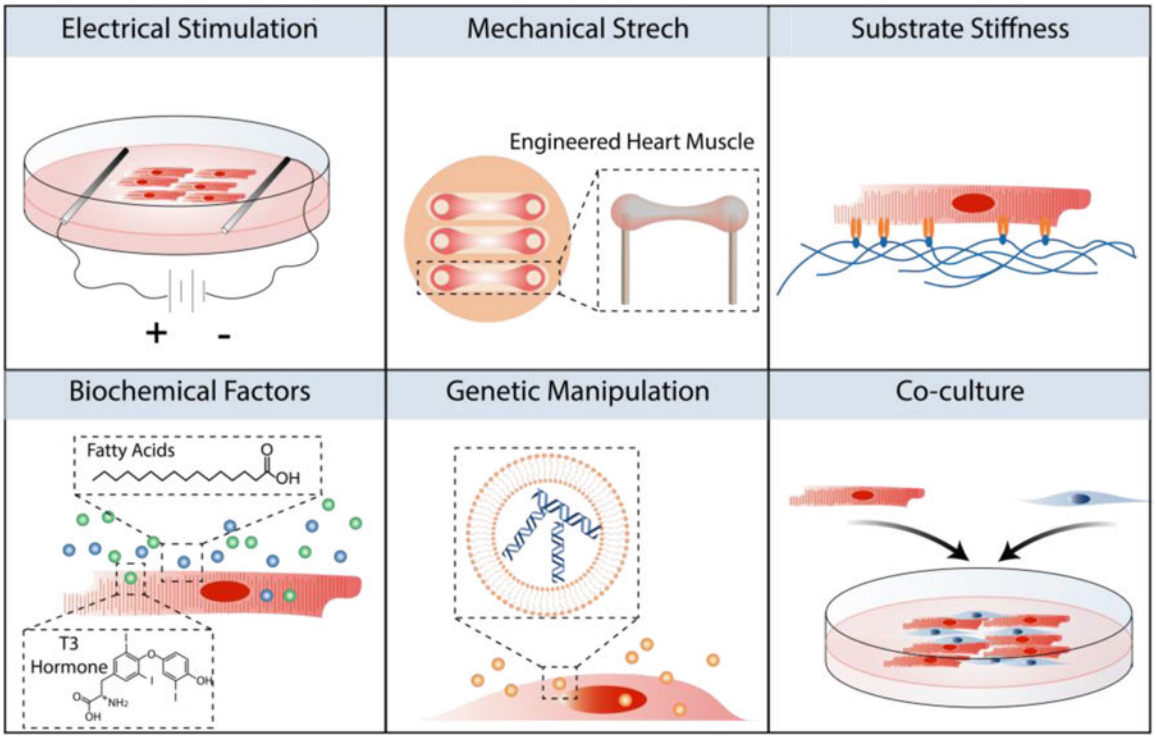

Figure 1.

Schematic illustration of existing in vitro maturation strategies for stem cell-derived CMs. Biophysical methods (upper panel) include electrical stimulations (e.g., field stimulation and biowire), mechanical stretch (e.g., cyclic stretch and static stretch), and substrate modulation (e.g., stiffness and ligands). Biochemical methods (lower panel) include metabolic modulation (e.g., fatty acids and hormones), genetic manipulation (e.g., miRNA overexpression), and co-culture with other cell types (e.g., fibroblasts and endothelial cells).