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A Recipe for T-tubules in Human iPS Cell-derived Cardiomyocytes

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Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) provide a powerful tool for investigating human cardiac biology, drug screening, and potentially therapeutic applications; however, these applications are limited by the relative immaturity of hPSC-CMs. In other words, the *in vitro* differentiated hPSC-CMs are more akin to early fetal cardiomyocytes than adult cardiomyocytes, which means they are structurally and functionally distinct in certain properties. One feature notably absent from immature hPSC-CMs relative to adult cardiomyocytes are t-tubules. The t-tubule system forms around the time of birth and is a complex network of interconnecting tubules and membranes contiguous with the extracellular space referred to as t-tubules for the predominant transverse components aligned with the z lines. Because ventricular cardiomyocytes are remarkably large cells, the t-tubule network enables the rapid spread of the electrical impulse throughout the cardiomyocyte. This is critical for the synchronized onset of contraction given that key components of excitation-contraction coupling are present in dyads where L-type Ca^{2+} channels in the t-tubules provided the triggering influx of Ca^{2+} during the action potential leading to intracellular Ca^{2+} release from the closely apposed ryanodine receptors of the junctional sarcoplasmic reticulum which together produce the Ca^{2+} transient activating contraction of the myofilaments. Thus, to investigate properties of adult cardiac excitation-contraction coupling and alterations in disease such as inherited cardiomyopathies or Ca^{2+} triggered arrhythmias, an hPSC-CM model with more mature features including a functional t-tubule network and adult-like Ca^{2+} cycling is desirable. Furthermore, the improved contractile performance and reduced spontaneous automaticity of

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mature ventricular-like hPSC-CMs may benefit cell therapy applications by improving the functional effect of integrated cells as well as reducing the risk of arrhythmias.

The quest for generating more mature hPSC-CMs has been shared by multiple laboratories using a range of approaches.[1] Simply maintaining the cells in culture for prolonged periods (3 months or more) can promote some degree of maturation,[2] but this approach is time consuming and resource intensive. Alternatively, investigators have looked for interventions to hasten the maturation in culture. For example, overexpression of the let-7 family miRNA, which is typically expressed late in cardiac development, has been demonstrated to accelerate maturation.[3] Another example is overexpression of *KCNJ2* which encodes the Kir2.1 inward rectifier potassium channel which is expressed later in development and in adult cardiomyocytes providing a more hyperpolarized resting membrane potential that results in more adult-like action potentials.[4] A five factor cocktail including insulin, dexamethasone, 3-isobutul-1-methilxanthine, rosiglitazone and indomethacin promoted metabolic maturation and unmasked the pathological signature of arrhythmogenic right ventricular cardiomyopathy in disease iPSC-CMs.[5] Others have tried different combinations of hormones and growth factors important in cardiac development including thyroid hormone (tri-iodo-l-thyronine, T3), the glucocorticoid dexamethasone (Dex) and IGF-1 to promote multiple features of mature cardiomyocytes, and this intervention enabled a hypertrophic cardiomyopathy *MYBPC3* mutation to be characterized.[6] In addition to soluble signaling molecules, the extracellular matrix composition and associated substrate stiffness have been found to be important variables in promoting maturation.[7, 8] However, these previous studies and multiple other related studies have not observed formation of t-tubules in hPSC-CMs or they have not looked specifically for this feature. Two recent studies did provide evidence that strategies for maturation could promote some t-tubule formation using either prolonged culture on nanopatterned surfaces or engineered substrates of optimized shape and stiffness.[9, 10] However, the extent of t-tubules detected in the hPSC-CMs in these studies was not clear nor was any functional impact of the t-tubules demonstrated.

In this issue of Circulation Research, Parikh and colleagues break the t-tubule barrier by discovering the appropriate combination of matrix and hormones to produce hPSC-CMs with a functional network of t-tubule producing more adult-like Ca^{2+} cycling.[11] The authors found that combining the Knollman lab's previously published Matrigel mattress technique[7] with T3 and Dex resulted in hPSC-CMs exhibiting abundant T-tubules with largely synchronized Ca^{2+} release throughout the myocytes similar to adult cardiomyocytes. This contrasted the untreated hPSC-CM that exhibited a delayed Ca^{2+} release present in the center of the cells. Furthermore, the gain of EC coupling, or the amount of intracellular Ca^{2+} released per unit of inward L-type Ca^{2+} current increased in the matured hPSC-CM along with more organized ryanodine receptors, both consistent with functional t-tubules involved in excitation-contraction coupling. The results provide the clearest evidence that hPSC-CMs can be coaxed in culture to behave as more mature cardiomyocytes regarding excitation-contraction coupling, and because this has been accomplished with single cells in culture, this is ideal for experimental approaches requiring single cells such as electrophysiological voltage clamp studies or single cell contractility characterization.

The study by Parikh et al. demonstrating t-tubules in the hPSC-CMs is a step forward, but we have not reached the promise of adult-like hPSC-CMs in a dish. The t-tubule network, while functional and throughout the myocytes, lacks the abundance and detailed organization found in adult ventricular cardiomyocytes. Furthermore, the kinetics of Ca^{2+} cycling appear relatively slow in these hPSC-CMs, in part because the studies were done at room temperature at a very slow rate of stimulation (0.2 Hz) that makes comparison to studies at physiological temperature and rate challenging. Furthermore, although hPSC-CMs treated with T3 and Dex on the Matrigel mattress were larger cells, they still fall far short of the size of adult cardiomyocytes which exhibit cell volumes closer to 30 pL relative to the 8 pL found in the hPSC-CMs in Parikih et al. In addition, it is unclear from the presented data whether other features of more mature cardiomyocytes result from this treatment, such as a change in metabolism to fatty acid oxidation, adult-like action potentials manifesting hyperpolarized diastolic potentials with faster action potential upstrokes, and developmental changes in myofilament protein isoforms. The protocol also has practical limitations as it requires careful pipetting of drops of concentrated Matrigel, an undefined basement membrane preparation commercially made from a mouse sarcoma line with hundreds of different proteins, which can vary from lot-to-lot. To determine the essential matrix components and the optimal substrate stiffness to promote t-tubule formation will require further study. Detailed mechanistic understanding of this intervention is limited in part because the mechanisms responsible for perinatal native cardiomyocyte development are understudied including the pathways governing the formation and dynamics of t-tubules. Nevertheless, this work strongly suggests that both the extracellular matrix and soluble signaling cues are essential to optimize this feature of maturation. Undoubtedly a multiplicity of signaling pathways and alterations in gene expression are activated by the broad cellular effects of thyroid and glucocorticoid hormones combined with extracellular matrix-based signaling. Future studies characterizing perinatal cardiac development along with studies using hPSC-CMs are needed to define the critical features of t-tubuologenesis to produce more adult-like hPSC-CMs as well as to gain insight into disease such as heart failure in which t-tubule remodeling is a key pathological feature.[12, 13]

Overall, a Matrigel mattress with a pinch of T3 and Dex provide a potent recipe to accelerate hPSC-CMs along the developmental path and form functional t-tubules, but questions remain aabout the properties of these matured hPSC-CMs and how closely they reflect adult human cardiomyocytes. Nevertheless, the study by Parikh and colleagues provides evidence for an ever improving cell system to model human heart disease and generate therapeutic products.

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