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RESEARCH HIGHLIGHT

Beating in a dish: new hopes for cardiomyocyte regeneration

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Functional human cardiomyocytes hold great promise in cell transplantation-based therapy to treat many heart diseases. To meet this devastating and clinical need, researchers are infatuated with developing novel technologies and methodologies to efficiently generate cardiomyocytes through either stem cell differentiation or cell lineage transdifferentiation. Though exciting progress has been made, challenges remain to be addressed before the translation from bench side to bed side can be fulfilled.

As a leading cause of death worldwide, heart failure represents a severe medical condition which renders both a challenge and opportunity to the field of regenerative medicine. One major cause of heart failure is the massive loss or dysfunction of cardiomyocytes after myocardial infarction. In addition, chronic cardiac disorders, such as hypertension and aging, also lead to progressive cardiomyocyte loss, and eventually to the development of heart failure. Unlike some other vertebrates, who are able to regenerate their hearts after injury, the adult mammalian heart is believed to have very limited innate regenerative capacity, making it virtually impossible for the injured heart to cure itself. Therefore, generation of functional cardiomyocytes to treat or prevent heart failure has become a much needed demand towards cardiac repair. Accompanied with the great advances in stem cell biology, developmental biology and tissue engineering, the field of cardiac regenerative medicine has developed rapidly over the last decade. Many researchers have developed novel strategies to generate cardiomyocytes by differentiation of pluripotent stem cells (PSCs) or transdifferentiation of adult somatic cells. These cellular platforms offer great opportunities to better understand normal cardiac development, to identify disease-related mechanisms, and to advance clinical therapies.

Human and mouse PSCs are able to differentiate into cardiac lineages spontaneously through embryoid body formation *in vitro*, but at a relatively low efficiency (~1% - 10% cardiomyocytes). To enhance specificity and efficiency of differentiation, cardiac lineage inducing cytokines and signaling modulating small compounds identified from developmental studies have been extensively tested in the *in vitro* culture systems. In 2007, Laflamme *et al*. [1] found that addition of Activin A and BMP4 enhanced cardiac differentiation from human embryonic stem cells (hESCs) in a monolayer culture system, with cardiomyocytes efficiencies up to 30%. Besides the closely resembled morphological features and gene expressions to their primary counterparts, these *in vitro*-derived cardiomyocytes were capable of rescuing heart failure, though partially, after transplantation into infarcted rat hearts [1]. Subsequently, a novel approach established by Yang *et al*. [2] firstly induced hESCs to primitive streak-like cells and cardiac mesoderm by Activin A, BMP4, and bFGF, and subsequently promoted cardiomyocyte differentiation by canonical Wnt inhibitor DKK1, and VEGF. This protocol further increased the cardiogenesis efficiency to around 50%. In a recent study by Lian *et al.* [3], a more robust cardiomyocyte differentiation was induced from both hESCs and hiPSCs via temporal modulation of Wnt signaling through genetic modification or small molecule inhibition. By using GSK3-specific inhibitor on the starting day of differentiation, followed by Wnt inhibitor IWP2 or IWP4 on day 3, a purity of greater than 82% cTnT-positive cells could be achieved in all six hESCs and hiPSCs lines tested. Thus, the use of small molecules alone provides a more reproducible and inexpensive protocol for cardiac regeneration. Another small molecule, ITD-1, a potent TGF-β inhibitor, was recently identified in a cardiogenesis screening performed by Willems *et al*. [4]. Early addition of ITD-1 inhibited mesoderm formation, whereas later addition selectively promoted the uncommitted mesoderm towards cardiomyocyte differentiation (~60% cardiomyocytes), but not to endothelial cells or vascular smooth muscle cells. Given that all three of the lineages are derived from the same cardiovascular progenitors, this study indicates that TGF-β plays a biphasic role in cardiogenesis by first promoting mesoderm induction and specifically repressing cardiomyocyte fate at a later stage. While most of these cardiac differentiation protocols focused on the modulation of cytokine pathways that are known to play important roles in cardiac development, other approaches like the recent study by Zhang *et al*. [5] has found that the extracellular matrix is also necessarily involved in cardiomyocyte differentiation from hESCs and hiPSCs. By culturing human PSCs as a monolayer on Matrigel, and subsequently overlaying with Matrigel again one

or two days before induction to form a matrix sandwich, cardiomyocytes with a high purity (up to 98%) were generated in combination with cytokine treatments (Activin A, BMP4 and bFGF).

The robust cardiomyocyte differentiation achieved from both mouse and human PSCs provides researchers with a good model to study the mechanisms underlying normal cardiac development. Using these platforms, in combination with genome-wide RNA-seq and ChIP-seq, two recent studies have analyzed the dynamic epigenetic regulation patterns during cardiac development [6, 7]. Wamstad *et al*. [6] discovered a new pre-activated pattern of chromatin modification that associates with cardiac-related genes, and identified stage-specific enhancer elements that predict the transcription networks regulating cardiac differentiation in mouse. Meanwhile, Paige *et al*. [7] identified a temporal histone modification "signature" which distinguishes key cardiac factors from other genes, and used this set of chromatin signatures to discover novel regulators of human cardiac development.

In addition to the investigation of normal cardiac development, the above mentioned approaches of cardiac differentiation from hiPSCs are also employed to study the pathogenesis of cardiac disorders. In 2010, Moretti *et al*. [8] generated iPSCs from fibroblasts of patients with Long-QT syndrome type 1 and carrying the *KCNQ1* gene mutation, and further differentiated these iPSCs into functional cardiomyocytes. Compared to cardiomyocytes generated from iPSCs of healthy controls, the patient-derived cardiomyocytes showed significantly prolonged duration of action potential, altered activation and deactivation properties of the potassium channel encoded by *KCNQ1* gene, as well as abnormalities in response to catecholamine stimulation. Similarly, Itzhati *et al*. [9] and Yazawa *et al*. [10] simultaneously established disease models using cardiomyocytes derived from patient-specific iPSCs to study the molecular and cellular mechanisms of Long-QT syndrome. Taken together, all these research platforms have greatly enhanced our knowledge in different aspects of the field, including the development of *in vitro* disease models that recapitulate the pathological phenotypes of cardiac diseases, new mechanistic insights as well as novel tools for drug discovery.

In recent years, another exciting finding in the stem cell field is the development of methods to convert terminally differentiated cells towards different lineages. The notion that a functional cardiomyocyte can be obtained through a transdifferentiation strategy bypassing all the uncertainties associated with the pluripotent state has attracted attention and opened up a new avenue in the field. Recently, by local retroviral delivery of three cardiogenic transcription factors (Gata4, Mef2c, Tbx5) in the infarcted area of mouse adult hearts, Qian *et al*. [11] reported the successful reprogramming of cardiac fibroblasts into cardiomyocytes *in vivo*. These *in vivo* induced cardiomyocytes showed evidence of electrical coupling and maturation, decreased infarct size and attenuated cardiomyocyte dysfunction. Song *et al*. [12] also reported that forced expression of four transcription factors (Gata4, Hand2, Mef2c, Tbx5) in the injured mouse heart reprogrammed non-myocytes into functional cardiomyocytes. They also found that exogenous expression of these factors in infarcted heart reduced fibrosis and increased cardiac function.

Despite the significance of all these observations and approaches, there are still many hurdles to be overcome before they can be translated into the clinical setting. Primary concerns include issues regarding safety (in both previous transdifferentiation reports, viruses were utilized to ensure efficient delivery of transgenes) and scale (unlike ESCs or iPSCs, for which expansion potential is literally unlimited, transdifferentiation from a restrictively proliferative source would inevitably impinge on the final cell numbers). Additionally, for those heart disease patients bearing known pathogenic mutations, targeted correction of the inherited mutations by specific genome-editing tools in patient-specific iPSCs will be required [13, 14]. Moreover, a relatively pure population of human cardiomyocytes may significantly help to increase the safety for transplantation and accuracy for drug screening. In this concern, robust cardiac-specific surface markers need to be identified to enable efficient purification and enrichment. Notably, three cardiomyocyte-specific markers (EMILIN2 [15], SIRPA [16], VCAM1 [17]) have been recently identified to enable the isolation of live cardiomyocytes from other lineages or undifferentiated cells. Furthermore, particular clinical applications may need certain subtypes of cardiomyocytes and the current described protocols do not allow for a distinction between atrial, ventricular, and nodal-like cells. Therefore, novel methodologies need to be developed for the generation of subtype-specific cardiomyocyte populations. Potential hopes may reside in subtype directed differentiation such as a preliminary report from Zhang *et al*. [18] and improved cell isolation approaches which distinguish and enrich different cell subtypes.

Finally, and returning to the issues and concerns of safety and transplantation, several lines of research need to be enhanced: 1) even derived from autologous iPSCs, the immunogenicity of donor cardiomyocytes still has to be carefully investigated before transplantation, and a complete removal of contaminated iPSCs from transplantation materials should be thoroughly verified; 2) it is important to evaluate how well the cell engrafts can integrate with host cells structurally and functionally, while minimizing the damage to the host myocardium; 3) the epigenomic and genomic stability of iPSCs cardiac

derivatives needs to be carefully examined *in vitro* before transplantation to minimize the potential risk caused by the drifts of epigenetic status or the generation of harmful genetic variations; 4) long-term survival of engrafted cells needs to be studied in order to evaluate the pathological improvement of the transplantation to patients; 5) efficient methods of cardiomyocyte transplantation need to be developed. In addition to cell injection approaches, it is noteworthy to mention the development of a 3D reconstruction methodology that may help to improve the overall efficacy of cardiomyocyte transplantation. Kawamura *et al*. [19] have cultured hiPSC-derived cardiomyocytes in thermoresponsive dishes to generate cardiomyocyte sheets, enabling the transplantation of a large number of cells. These sheets, when transplanted into a porcine ischemic cardiomyopathy model, integrate structurally and electromechanically into host myocardium. This is one of the clearest examples of how tissue engineering may offer novel tissue substitutes which could increase the size and survival of cardiomyocyte grafts. While several of the approaches and methodologies described here may not end up being used in the clinic, certainly they constitute the basis on which to build and combine views and initiatives from different fields. Hopefully, not in the too distant future, all efforts made would move us forward to the final goal, to treat and ameliorate some of the most devastating human health problems, including heart disease.

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