

Exploring Future Cardiovascular Medicine: Heart Precursors Directed from Human Embryonic Stem Cells for Myocardium Regeneration

Xuejun H Parsons^{1,2*}

¹San Diego Regenerative Medicine Institute, San Diego, CA 92109, USA

²Xcelthera, San Diego Regenerative Medicine Institute, San Diego, CA 92109, USA

Given the limited capacity of the heart muscle for self-repair after birth, transplantation of cardiomyocyte stem/precursor/progenitor cells holds enormous potential in cell replacement therapy for cardiac repair. However, the lack of a clinically-suitable human cardiomyocyte stem/precursor/progenitor cell source with adequate myocardium regenerative potential has been the major setback in regenerating the damaged human heart, either by endogenous cells or by cell-based transplantation or cardiac tissue engineering [1,2]. Pluripotent human embryonic stem cells (hESCs) have the unconstrained capacity for long-term stable undifferentiated growth in culture and the intrinsic potential for differentiation into all somatic cell types in the human body, holding tremendous potential for restoring tissue and organ function [1]. Due to the prevalence of heart disease worldwide and acute shortage of donor organs or adequate human myocardial grafts, there is intense interest in developing hESC-based therapy for heart disease and failure [1,2].

However, realizing the developmental and therapeutic potential of hESC derivatives has been hindered by the inefficiency and instability of generating clinically-relevant functional cells from pluripotent cells through conventional uncontrollable and incomplete multi-lineage differentiation [1,2]. Conventional approaches rely on multi-lineage inclination of pluripotent cells through spontaneous germ layer differentiation, resulting in inefficient, incomplete, and uncontrollable lineage-commitment that is often followed by phenotypic heterogeneity and instability, hence, a high risk of tumorigenicity [1]. In addition, undefined foreign or animal biological supplements and/or feeders that have typically been used for the isolation, expansion, and differentiation of hESCs may make direct use of such cell-specialized grafts in patients problematic [1]. In hESC-differentiating multi-lineage aggregates or embryoid bodies, only a very small fraction of cells spontaneously differentiate into immature cardiomyocytes [1]. Grafts generated by such isolated or enriched hESC-derived immature cardiomyocytes have been small, insufficient to restore heart function [1,2]. Functional enhancement in experimental animal models has been limited to mid-term at most, equivalent to perhaps a few months in humans, which is not related to regeneration from the grafts [1,2]. For pluripotent cell-derived grafts to be used safely and effectively in cardiac repair, it requires the development of more practical, efficient, and controllable differentiation strategy to generate a large supply of human cardiomyocyte committed engraftable cells for myocardium regeneration.

Maintaining undifferentiated hESCs in a defined biologics-free culture system that allows faithful expansion and controllable direct differentiation is one of the keys to their therapeutic utility and potential [1]. Recent technology breakthroughs in hESC research have overcome some major obstacles in bringing hESC therapy derivatives towards clinical applications, including establishing defined culture systems for derivation and maintenance of clinical-grade pluripotent hESCs and lineage-specific differentiation of pluripotent hESCs by small molecule induction [1-10]. Such milestone advances and medical innovations in hESC research enable direct conversion of pluripotent hESCs into

a large supply of homogeneous populations of clinical-grade hESC neuronal and heart cell therapy products for developing safe and effective stem cell therapies [1-10]. Currently, these hESC neuronal and cardiomyocyte therapy derivatives are the only available human cell sources with adequate capacity to regenerate neurons and contractile heart muscles, vital for CNS and heart repair in the clinical setting. The availability of human stem/progenitor/precursor cells in high purity and large quantity with adequate myocardium regenerative potential will greatly facilitate developing safe and effective cell-based therapies against cardiovascular disease. Transforming pluripotent hESCs into fate-restricted therapy derivatives dramatically increases the clinical efficacy of graft-dependent repair and safety of hESC-derived cellular products, bringing regenerative medicine to a turning point.

These recent studies found that formulation of minimal essential defined conditions for undifferentiated hESCs rendered small molecule nicotinamide (NAM) sufficient to induce the specification of cardiomesoderm direct from the pluripotent state of hESCs by promoting the expression of the earliest cardiac-specific transcription factor *Csx/Nkx2.5* and triggering progression to cardiac precursors and beating cardiomyocytes with high efficiency [1-4]. Upon exposure of undifferentiated hESCs maintained in the defined culture to NAM, all the cells within the colony underwent morphology changes to large differentiated cells that down-regulated the expression of pluripotency-associated markers (e.g., *Oct-4*) and began expressing the earliest marker for heart precursor (e.g., *Nkx2.5*), but not markers associated with other lineages (Stage 1 --- Human Cardiomesodermal Cells) [1,3,4]. These differentiating hESCs then formed cardioblasts that uniformly expressed *Nkx2.5* in suspension (Stage 2 --- Human Cardiac Precursor Cells) [1,3,4]. After permitting the cardioblasts to attach and further treating them with NAM, beating cardiomyocytes began to appear after withdrawal of NAM with a drastic increase in efficiency when compared to similarly cultured cells derived from untreated embryoid bodies (Stage 3 --- Human Cardiomyocytes) [1,3,4]. Cells within the beating cardiospheres expressed markers characteristic of cardiomyocytes [1,3,4]. Electrical profiles of the cardiomyocytes confirmed their contractions to be strong rhythmic impulses reminiscent of the p-QRS-T-complexes seen from body surface electrodes in clinical electrocardiograms [3]. This technology breakthrough enables cardiac lineage-specific differentiation direct

*Corresponding author: Xuejun H Parsons, San Diego Regenerative Medicine Institute, San Diego, CA 92109, USA, Tel: 858-274-1621; Fax: 858-581-3213; E-mail: parsons@SDRMI.org

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from the pluripotent state of hESCs with small molecule induction, providing a large supply of clinical-grade human cardiomyocyte precursors and cardiomyocytes for myocardial tissue engineering and cell therapy. NAM appeared to trigger the activation of the class III NAD-dependent histone deacetylase SIRT1 that leads to global chromatin silencing yet selective activation of a subset of cardiac-specific genes, and subsequently cardiac fate determination of pluripotent hESCs [5]. Further unveiling the nucleoprotein complex regulation in hESC cardiomyocyte specification will provide critical understanding to the molecular mechanism underlying human embryonic cardiogenesis, thereby aid the development of more effective and safe stem cell-based therapeutic approaches in the heart field.

Large-scale profiling of developmental regulators and histone modifications by genome-wide approaches, including human microRNA (miRNA) expression microarrays and chromatin/nucleosome-immunoprecipitation-coupled DNA microarray analysis (ChIP/NuIP-chip), has been used to identify the developmental associated genetic and epigenetic regulators in high-resolution in hESCs and their derivatives [5,9,10]. In addition, recent advances in chromatin-immunoprecipitation-combined second-generation high-throughput sequencing (ChIP-seq) have provided powerful genome-wide, high-throughput, and high resolution techniques that lead to great advances in our understanding of the global phenomena of human developmental processes [11]. However, without a practical strategy to convert pluripotent cells direct into a specific lineage, previous studies are limited to profiling of hESCs differentiating multi-lineage aggregates, such as embryoid body that contain mixed cell types of endoderm, mesoderm, and ectoderm cells or a heterogeneous population of embryoid body-derived cardiac cells that contain mixed cell types of cardiomyocytes, smooth muscle cells, and endothelial cells [12-14]. Their findings have been limited to a small group of genes that have been identified previously in non-human systems, and thus, have not uncovered any new regulatory pathways unique to human development [12-14]. Although genome-wide mapping of histone modifications and chromatin-associated proteins have already begun to reveal the mechanisms in mouse ESC differentiation, similar studies in hESCs are currently lacking due to the difficulty of conventional multi-lineage differentiation approaches in obtaining the large number of purified cells, particularly cardiomyocytes, typically required for ChIP and ChIP-seq experiments [11,14,15]. Recent technology breakthrough in lineage-specific differentiation of pluripotent hESCs by small molecule direct induction allows generation of homogeneous populations of neural or cardiac cells direct from hESCs without going through the multi-lineage embryoid body stage [1-10]. This novel small molecule direct induction approach renders a cascade of neural or cardiac lineage-specific progression directly from the pluripotent state of hESCs, providing much-needed *in vitro* model systems for investigating the genetic and epigenetic programs governing the human embryonic CNS or heart formation. Such *in vitro* hESC model systems enable direct generation of large numbers of high purity hESC neuronal or cardiomyocyte derivatives required for miRNA and ChIP-seq profiling to reveal the mechanisms responsible for regulating the patterns of gene expression in hESC neuronal or cardiomyocyte

specification. It opens the door for further characterizing, identifying, and validating functional elements during human embryonic development in a comprehensive manner. Further using genome-wide approaches to study hESC models of human heart formation will not only provide missing knowledge regarding molecular cardiogenesis in human embryonic development, but also facilitate rapid progress on identification of molecular and genetic therapeutic targets for the prevention and treatment of cardiovascular disease.

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