

## Short Note

# Method Overview of Cardiomyocytes Generation from Pluripotent Stem Cells

Lin Jiang, Jialiang Liang, Wei Huang, Wenfeng Cai, Christian Paul, and Yigang Wang\*

Department of Pathology and Lab of Medicine, University of Cincinnati Medical Center, USA

## \*Corresponding author

Yigang Wang, Department of Pathology and Lab of Medicine, University of Cincinnati Medical Center, 231 Albert Sabin Way, USA, Tel: 513-558-5798; Fax: 513-558-2141; Email: yi-gang.wang@uc.edu

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**Abstract**

Pioneering preclinical studies have demonstrated that cardiomyocytes generated from pluripotent stem cells (PSC-CMs) hold tremendous potential for the treatment of tissue loss and insufficiency (such as HF and MI) that result from heart disease. PSCs stand out as the one of the most promising candidates due to their ability to differentiate into CMs. However, methods for inducing differentiation have lagged behind and are still considered controversial because of the poor capacity for CM differentiation. Here, we introduce alternative methods in various disciplines that promote cardiac differentiation and review the current efforts underway to refine techniques for regenerative purposes. Growth factors, small chemical molecules, electrical stimulation, miRs, and Nanopatterned (NP) engineering techniques are emerging as efficient and reproducible options for fine-tuning of desired cardiac lineage differentiation properties and therapeutic treatments for regeneration of tissue lost as a consequence of heart disease.

**INTRODUCTION**

Ischemic heart disease (IHD) is the major cause of morbidity and mortality worldwide [1]. IHD patients could benefit from therapies that accelerate the natural processes of postnatal cardiomyocytes (CM) regeneration and collateral vessel formation [2]. Although the treatment for acute myocardial infarction (MI) has improved over the past decades, there are no effective therapies for the associated problem of IHD [3]. It has been well established that adult mammalian heart tissue has limited regenerative capabilities post-infarction [4]. Most regenerative medicine techniques depend on the use of stem cell transplantation, which has emerged as a promising strategy to repair or replace damaged myocardium [5]. However, these adult stem cells do not possess sufficient plasticity to generate functional CM. PSCs, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), can undergo differentiation to generate derivatives of the three germ layers (all cell types present in the body) under defined conditions. However, inefficiency and immaturity of PSC-derived CMs (PSC-CMs) limits their potential clinical applications for cardiac regeneration [6]. Therefore, improvement and refinement of differentiation methods to generate PSC-CMs is a critical component of creating a reservoir of functional CMs for cardiac disease modeling and tissue engineering.

The approaches currently used to generate PSC-CM have been established through *in vitro* mimicking of the embryonic heart environment. Studies of heart development have demonstrated that embryonic signals (including growth factor signals, soluble factors, electrical fields, microRNAs, and NP techniques) determine the cardiac lineage commitment [7]. When PSCs undergo spontaneous differentiation into new CMs, the natural physiological cues of the environment become essential to maintain this process. Here we present a mini-review that briefly introduces the current methods and techniques used for differentiation of PSCs into CMs.

**Growth factors' controlling**

Activation of physiological signaling pathways related to heart development is important for generation of PSC-CMs under *in vitro* conditions. A growing body of evidence also indicates that CM differentiation fate is influenced by environmental signaling. Three prime growth factor families (WNT proteins, BMP/TGF- $\beta$  family, and bFGF) control cardiac development and CM formation [8]. WNT protein signaling has been implicated in cardiac differentiation, morphogenesis, and specification through inhibition of GSK3, leading to nuclear accumulation of  $\beta$ -catenin that is associated with Tcf/Lef, which activates cardiac gene transcription. Cardiogenic programming can be activated when *in vitro* cultured PSCs exposed to WNT's agonists

or antagonists. In addition, WNT/PKC-Ca<sup>2+</sup>/CamKII and WNT/JNK pathways are involved in the activation of cardiogenic gene programming [9,10]. BMP/TGF- $\beta$  is a cytokine super family that can efficiently promote the differentiation of cardiac mesoderm cells by upregulation of the downstream gene, such as SLUG and MSX2 via binding of phosphorylated Smad1/5/8 [11]. Previous studies found that even small changes in amount of BMP4 protein can dramatically alter the cardiac mesoderm formation in PSCs with an unbalanced of proportion of Flk-1/KDR and Pdgfr- $\alpha$ . FGF family is also composed of multifunctional proteins that regulate cardiogenesis. For instance, the absence of FGFR-2 or FGF10 expression led to abnormal heart positioning, while addition of FGF-2 induced cardiac specificity in epicardial cell lineages [12]. As a result, the addition of defined growth factors (including DKK-1, BMP4, bFGF and active A) can be applied as a 'cocktail' method for further enhancement of CM differentiation in PSCs.

### Small chemical molecules

Supported by the activated signaling pathways, sequential growth factor (GF) cocktail strategies have been widely used in PSC-CM generation. However, GFs are costly, degrade rapidly, and exhibit swift variation in their bioactivity. Hence, the application of chemically synthesized small molecules that potentially substitute GFs involved in CM differentiation is being pursued. Recently, GSK inhibitors (including CHIR99021, BIO, SB415286 etc.) and WNT signaling inhibitors (including IWP1, IWP2 and IWP4) were considered to be the standard protocol for generating CMs from PSCs [13]. The former inhibitors have been shown to promote cardiogenesis in mesoderm formation, and the latter is applied in the post-mesoderm process of cardiac differentiation. Addition of ALK inhibitors (through TGF- $\beta$ /Smad signaling, such as SB431542) also promoted cardiogenesis after mesoderm specification. JAK inhibitor (inhibitor of tyrosine kinases, such as JI-1) and VEGFR/FGFR/PDGFR/EGFR inhibitor (another kind of tyrosine kinase, such as SU5402) also have critical roles in the formation of cardiac progenitor cells [14]. The different targets or functions of small molecules should be clearly documented, and the timing of application according to the process of cardiac differentiation is extremely important.

Additionally, DNA methylation and histone modifications play a key role in the self-regulation of the epigenetic state of PSC-derived cardiac cells. Several chemical molecules targeting or modulating the epigenetic mechanisms that underlie cardiac differentiation have been discovered and utilized in PSC-CM generation approaches. Moreover, several chemical modifiers targeting histones such as TSA (HDAC inhibitor) can induce acetylation of histone H4 in cardiac genes, activating gene transcription [15]. These epigenetic modifiers can potentially influence the global chromatic structure and facilitate the accessibility of transcriptional activators to cardiac genes, although molecules with specific targeting effects on cardiac gene loci remain unavailable. Significantly, a combination of nine chemical compounds has been reported that activates cardiac lineage reprogramming, suggesting the promise of chemical approaches for CM generation without any viral manipulation [16]. PSC-CMs generated from chemical approaches will provide a safe, reproducible, and GMP-grade of resource CMs for the future clinical application.

### Electrical stimulation

The heart is an electro-mechanical pump with CMs exposed to regular electrical stimuli during the early cardiac lineage development. Bioelectrical pulses may determine the emergence of spatial patterns and aid in heart tissue morphogenesis. Therefore, in addition to the adjustment of soluble growth factors, research techniques that employ external electrical stimulation (EleS) have been designed to deliver electric signals mimicking the bioelectrical environment of native heart to facilitate the PSC-CM generation. Cardiac-mimetic EleS has therefore been applied as a conditioning treatment during the *in vitro* culture of CMs, particularly in myocardial tissue engineering. EleS approach also can promote the cardiac differentiation potential of stem cells such as cardiac progenitor cells and ESCs, and have been shown to enhance the therapeutic efficacy of cardiac stem cells in infarcted heart [17,18].

Furthermore, findings have suggested that exogenous EleS exerts complex effects during cardiogenesis and subsequent maturation, resulting in a higher percentage of spontaneously beating PSC-CMs and closer contraction frequency when compare to adult human heart [17]. Thus, EleS is a potent inducer for cardiac differentiation of PSCs. Our recent study shows EleS as an efficacious and timesaving approach for CM generation from human iPSCs (Ma et al., manuscript submitted). The global RNA profiling in this study demonstrated that EleS accelerated the cardiac differentiation of human iPSCs through activation of multiple pathways, such as calcium signaling and MAPK (Data unpublished). EleS was also shown to enhance the efficiency of cardiac differentiation in human iPSCs and promote CM maturation.

### MicroRNAs

Specific microRNAs (miRs) involved in cardiac differentiation have been revealed in the results of expression profiling of PSCs-CMs. For instance, the expression level of miR-1, miR-133, and miR-499 was shown to significantly change during PSC differentiation [19]. MiR-1 can promote cardiomyogenesis and CM differentiation by targeting HDAC4 (histone deacetylase 4), FZD7 (frizzeld family receptor 7), and FRS2 (fibroblast growth factor receptor substrate 2) or repressing WNT and FGF signaling pathway [20]. In contrast to miR-1, over expression of miR-133 suppresses cardiac differentiation of PSCs and decreases CM proliferation [21]. Consistent with these findings, expression of miR-499 resulted in upregulation of cardiac markers such as Nkx2.5 and Gata4 [22]. However, inhibition of miR-499 in PSCs inhibits cardiomyocyte differentiation [23]. This suggests that miRNAs are of key importance in the specialization of cells towards a CM fate. Since miRNAs are powerful regulators of gene expression, they represent an efficient strategy to modulate commitment of PSCs to a cardiovascular lineage under *in vitro* conditions. Importantly, the technically feasible miR supplementation or antisense oligonucleotides potentially facilitate the CM differentiation approaches of PSCs.

### Nanopatterned (NP) engineering techniques

A variety of methods have been used to improve cardiomyocyte induction, including mechanical stretching,

electrical stimulation, and miRNA regulation. However, heart tissue exhibits complex organization on multiple scales, and the effects of different-scaled molecules (or patterns) on cardiac cell and tissue function are not clearly understood. Previous studies have demonstrated that NP cues could help to create a cardiac stem cell niche and promote regeneration [24]. Recently, marked improvements at the subcellular and nanoscale level have been reported that show the potential importance for CM's maturity and function [25]. Macadangang used NP silicon masters and fabricated polyurethane acrylate (PUA) molds to perform capillary force lithography (CFL). Using CFL nanofabrication methods, hESC-CMs helped to align  $\alpha$ -SMA and F-actin fibers (mimicking native cardiac ECM fiber alignment), while the control group had only randomly oriented fibers [26]. With the addition of an *in vivo*-like extracellular matrix (ECM) nano-cue as a layer, it presents a simple technique for creating biomimetic nanotopographic cell culture substrates for hiPSC-CMs culturing, which has the potential to enhance the structure and morphology of CMs [27]. A second technique employed by Kim uses biocompatible polyethylene glycol (PEG) hydrogel arrays to form a nanofabricated substratum, which closely imitates myocardial ECM [28]. Clearly, NP engineering techniques are attractive as they afford the possibility for improved cardiac tissue construction and CM function for regenerative purposes.

In summary, recent advancements in the methods of inducing differentiation including utilization of growth factors, small chemical molecules, electrical stimulation, miRs, and NP engineering have been well established for the acquisition of PSC-CMs. Studies have unveiled that the determinants of cardiogenesis can facilitate the development of robust and reliable methodologies to generate CMs from PSCs. Understanding the methods employed for cardiac lineage differentiation of PSCs may enable the development of safer and more efficient cell resources to regenerate the infarcted heart, but it is necessary to globally screen the master regulators controlling cardiac lineage commitments in order to design specific, powerful, and effective tools to further improve the CM differentiation and maturation. The optimization and applied timing of CM growth factors, molecules, and electrophysiological conditions will benefit large-scale generation of CMs in cardiac tissue engineering for the repair of heart tissue after infarction.

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