International Journal of Cardiology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Making human cardiomyocytes up to date: Derivation, maturation state and perspectives

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ARTICLE INFO

Article history:
Received 3 January 2017
Received in revised form 24 February 2017
Accepted 21 March 2017
Available online xxxx

Keywords:
Stem cells
Induced pluripotent stem cells
Cardiomyocyte derivation protocols
Cardiomyocyte maturation
In vitro derived-cardiomyocyte applications

ABSTRACT

In vitro generation of cardiomyocytes (CMs) from human cells opens the possibility to develop patient-specific therapies to various cardiomyopathies. By establishing the *in vitro* reprograming methods that produce human CMs, we learn about what is involved in the development of specific CM subtypes. In this review, we summarize the latest achievements in CM generation technologies, emphasizing the differentiation methods of specific CM subtypes. We also relate the biological properties and functions of the *in vitro*-generated CMs to those of their *in vivo* counterparts. Furthermore, we describe the main problem of current CM derivation methods – maturation of CMs. We subsequently discuss biochemical and physical stimuli that are used to overcome the maturation problems of *in vitro*-derived CMs. As a result, a more holistic approach with controllable environment and timing of specific stimuli for creation of more mature engineered heart tissues is described as well. Finally, we propose a novel approach in which enhancing energy transfer mechanisms in the immature CMs might help to overcome the current hurdle of incomplete *in vitro* differentiation.
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1. Introduction

Cardiomyocytes (CMs) are responsible for the contractile force of heart muscle. Most cardiac disorders are due to reduced activity of CMs. Currently, therapeutic strategies are palliative and have limited success in improving morbidity and reducing mortality. To develop new strategies for improving CM performance, several laboratories are working to manufacture CMs *in vitro* and then use them for several clinically related purposes. To produce fully functional CMs in culture, detailed knowledge about CM biology and differentiation is indispensable.

In recent years, milestones to manufacturing CMs *in vitro* have been reached. One is the production of ventricular-, atrial- and pacemaker-like CMs from pluripotent stem cells (PSCs) [1–4]. The second milestone encompasses the transdifferentiation of non-myocytes into CMs [5]. The *in vitro* generation of CMs provides opportunities to model cardiac diseases and target other related problems. However, the derivation and transdifferentiation methods used so far still produce CMs with immature phenotypes. Therefore, complete differentiation requires identifying the missing factors that govern maturation. A number of factors have been investigated *e.g.* biochemical stimuli, mechanical load or electrical activity, and integrated into approaches for generation of engineered heart tissue (EHT). However, existing differentiation (2D and 3D) methods often focus on single factors instead of combining them to more holistic approaches.

This review provides an overview of current advancements in *in vitro*-derived CM maturation by covering three major points:

Abbreviations: AM, Atrial cardiomyocyte; AP, Action potential; APA, Action potential amplitude; APD, Action potential duration; CICR, Ca^{2+}-induced Ca^{2+} release; CK, Creatine kinase; CM, Cardiomyocyte; cTNI, Troponin I, cardiac type; cTnT, Cardiac troponin T; C × 40, Connexin 40; C × 43, Connexin 43; C × 45, Connexin 45; ECM, Extracellular matrix; EHT, Engineered heart tissue; ELF-PEMF, Extremely-low-frequency pulsed electromagnetic field; FGF, Fibroblast growth factor; Gata4, GATA binding protein 4; Hand2, Heart and neural crest derivatives expressed 2; HCN 1 (or 2, 4), Hyperpolarization activated cyclic nucleotide gated potassium channel 1 (or 2, 4); hESCs, Human embryonic stem cells; Hey1, Hairy/enhancer-of-split related with YRPW motif 1; hiPSC-CMs, Human induced pluripotent stem cell-derived cardiomyocytes; hiPSCs, Human induced pluripotent stem cells; iCMs, Induced cardiomyocytes; I_f, Funny current, cardiac pacemaker current; IGF-1, Insulin growth factor 1; $I_{K,ATP}$, ATP-sensitive potassium current; I_{K1}, Inward rectifier potassium current; I_{Na}, Sodium current; iPSCs, Induced pluripotent stem cells; Irx4, Iroquois homeobox 4; JNK, C-Jun amino-terminal kinase; Mef2c, Myocyte enhancer factor 2C; Mlc2a, Myosin light chain 2, atrial; Mlc2v, Myosin light chain 2, ventricular; MYH6, Myosin heavy chain 6; MYH7, Myosin heavy chain 7; N2B, Titin isoform N2B (shorter, stiffer); N2BA, Titin isoform N2BA (longer, more compliant); NCX, Na^+/Ca^{2+} exchanger; Nk × 2.5, NK2 homeobox 5; NRVMs, Neonatal rat ventricular myocytes; Ox-Phos, Oxidative phosphorylation; PSCs, Pluripotent stem cells; RMP, Resting membrane potential; RyR2, Ryanodine receptor 2; SAN, Sino-atrial node; SERCA, Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SR, Sarcoplasmic reticulum; ssTNI, Troponin, slow skeletal type; T3, Triiodothyronine; Tbx5, T-box 5; TGF-β, Transforming growth factor beta; VEGF, Vascular endothelial growth factor; VM, Ventricular cardiomyocyte; V_{max}, Maximal upstroke velocity; VPA, Valproic acid.

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http://dx.doi.org/10.1016/j.ijcard.2017.03.099
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(1) recent achievements in CM derivation technology, (2) the remaining differences in properties and functions between *in vitro* PSC-derived and adult CMs, and (3) the developed approaches that further the maturation of derived CMs. In addition, we propose energy regulation as a mechanism that might push *in vitro*-derived CMs towards a more mature phenotype and better mimic adult CM physiology.

2. Derivation of CMs *in vitro*

Three main approaches for obtaining highly enriched CM cultures (up to 95%) from human cells have been established (Fig. 1): (1) generation of induced PSCs (iPSCs) and subsequent differentiation of iPSCs into CMs, (2) direct reprogramming of somatic cells into induced CMs (iCMs) without going through a pluripotent state, and (3) reprogramming somatic cells into CMs *via* a transient acquisition of pluripotency and subsequent differentiation. Although the iPSC differentiation protocol seems to be the most efficient and potent one, the other two methods offer increased clinical safety and maturation.

2.1. CMs derived from iPSCs

Human embryonic stem cells (hESCs) and iPSCs (hiPSCs) are valued as potential sources for *in vitro*-production of tissues, due to their differentiation potential. Compared to hESCs, hiPSCs have several advantages. First, their use is not associated with ethical problems, since no embryo tissues are used. Second, hiPSCs are derived from adult somatic cells, allowing autologous or at least HLA-matched transplantations. Third, their ease derivation *in vitro* leads to higher availability of hiPSCs for research and clinical use.

Most of the currently available methods for CM differentiation generate a heterogeneous mix of several subtypes, including ventricular-, atrial- and pacemaker-like CMs. This heterogeneity may limit their applications because mixed populations consist of cells with different action potential (AP) properties [6,7], and can result in life-threatening arrhythmogenecity when introduced into the heart. Many attempts have been undertaken to produce pure populations of each CM subtype. Several physiological and molecular markers are used to discriminate between CM subtypes, including AP properties, the expression and switch of specific gene isoforms, ion channels and transcription factor profiles [1,3,4,6–9].

Ventricular cardiomyocytes (VMs) are derived from PSCs (PSC-VMs) in an efficient manner using protocols with three steps: early brachyury-expressing (>90%) mesendoderm derivation mediated by GSK3 inhibitor, Nk × 2.5^+ cardiac mesoderm (>50%) induction by Wnt signaling inhibitor and cardiac troponin T-positive ($cTnT^+$) CM (>80%) differentiation [4]. Additionally, culturing in physiological normoxia (5% O_2) leads to generation of PSC-VMs [10]. PSC-VMs typically show increased expression of ventricle-specific genes (such as transcription factor *Hey2* or myosin light chain 2, ventricular isoform, *Mlc2v*), Ca^{2+} handling properties and membrane excitability. Human PSC-VMs develop ventricle-specific AP profile, but the AP duration (APD), the maximal upstroke velocity (V_{max}) and the AP amplitude (APA) are still significantly different from their mature counterparts, the adult VMs [2,4,7,10].

Protocols for atrial cardiomyocyte (AM) differentiation are currently less efficient than those for VMs [1,4,10,11]. Treatment of hESCs with retinoic acid and noggin leads to 50% cardiac differentiation efficiency, but 94% of CMs showing AM-like APs [1]. Moreover, BMP antagonist Gremlin2 significantly increases the differentiation efficiency of AMs from hESCs by inducing JNK signal transduction, which upregulates atrial-specific genes encoding ion channels (*Kcnj3*, *Kcnj5*, and *Cacna1d*) and transcription factors (CoupTFII and Hey1) and downregulates the expression of atrial-fate repressors *Irx4* and *Hey2* [11].

The CMs from the sino-atrial node (SAN) are still difficult to produce. The *in vivo* SAN consists of a heterogeneous population of CMs that vary in morphology, ion currents and connexin (C×) expression. So far, the most effective protocol to generate SAN cells from hESCs *in vitro* is the inhibition of neregulin1β/ErbB signaling, which leads to 3-fold increase of CMs with nodal-like APs (from 20% to around 60%) and the upregulation of SAN-CM-specific genes (*Tbx3*, *Hcn4*, and *Cacna1g*) [3]. However, their purification from highly mixed CM populations is problematic due to the lack of selective and specific markers for SAN-CMs (for more details, please see review [12]).

In addition to the difficulties in obtaining pure populations of specific CM subtypes *in vitro*, it takes at least 3–6 months for the generation of

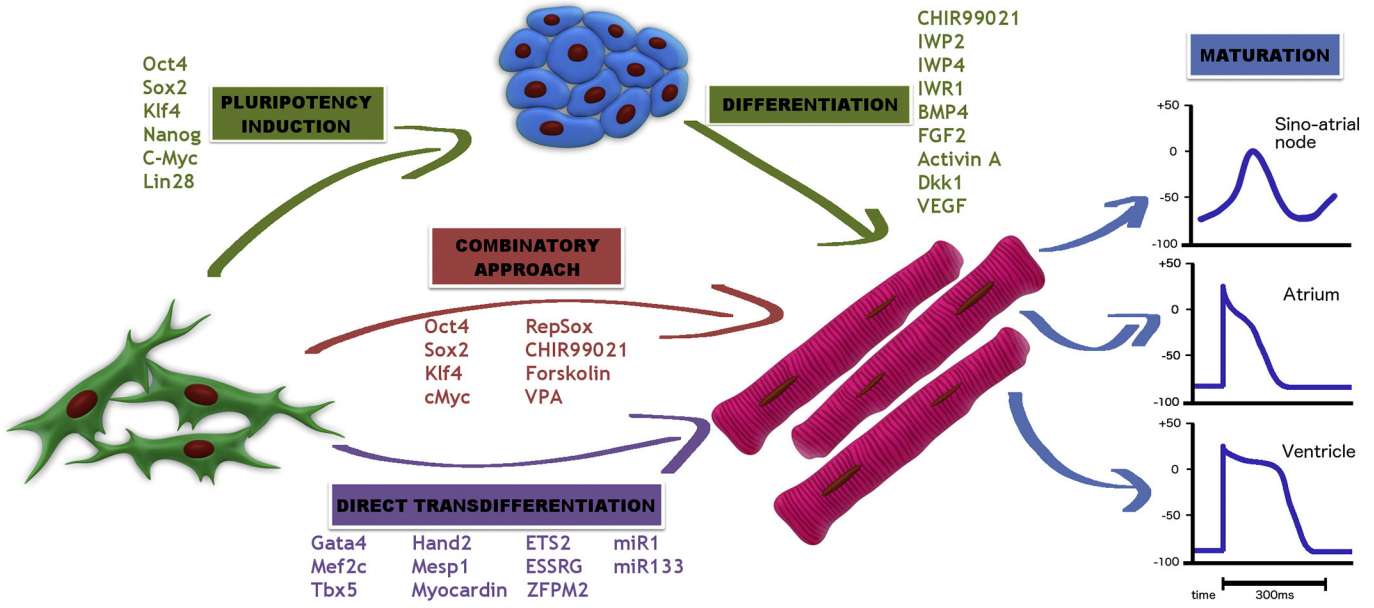

Fig. 1. Derivation of cardiomyocytes (CMs) from somatic cells by *in vitro* culture. CMs can be obtained from somatic cells using at least three different approaches: (1) induction of pluripotency by establishing iPSCs and subsequent differentiation to CMs (green), (2) direct reprogramming (violet), or manipulating these two pathways and performing (3) combinatory approach (dark red). In each approach, significant changes could be evoked by a set of transcription factors, small molecules or miRNAs, and some reported factors were mentioned. The second stage is maturation of derived CMs towards chamber-specific cells including ventricular-, atrial- and sino-atrial nodal-like cells (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article as: T.J. Kolanowski, et al., Making human cardiomyocytes up to date: Derivation, maturation state and perspectives, Int J Cardiol (2017), http://dx.doi.org/10.1016/j.ijcard.2017.03.099

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