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Small molecules and human cardiomyogenesis: Is there a bottleneck in current research?

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Abstract

Human pluripotent stem cell derived cardiomyocytes (hPSC-derived CMs) have a vast potential in drug discovery, disease modeling and regenerative medicine. In recent years various differentiation protocols for hPSC-derived CMs have been developed. Most of them utilize the modulation of human cardiomyogenesis via small-molecule compounds. However, setbacks to the large-scale application of hPSC-derived CMs still abound: insufficient insight into important signaling pathways for cardiac lineage-specific differentiation and identification of suitable small-molecule modulators; inconsistent results due to unstandardised culturing techniques; lack of effective maturation of hPSC-derived CMs in vitro. So is there a bottleneck in current research? This paper attempts to answer this question.

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Abbreviations: BMP – bone morphogenic protein;CM – cardiomyocyte; EGFP - enhanced greenfluorescent protein;hPSC – human pluripotent stem cell; hPSC-derived CMs - human pluripotent stem cell-derived cardiomyocytes;HCS - high-content screening; HTS - high-throughput screening; KDR– kinase insert domain receptor; mTOR –mammalian target of rapamycin;PDGFR-α–platelet-derived growth factor receptor; SAR - structure-activity relationship; TGF-β–tumor growth factor β.

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Human pluripotent stem cell-derived cardiomyocytes (hPSC-derived CMs) are currently an area of active interest to biomedical researchers due to the vast number of potential applications they have in drug discovery [1-3], disease modeling [2, 3] and regenerative medicine [2, 3]. This research enthusiasm, notwithstanding, a "revolution" in stem cell biology has being stirring up for some time. For over a decade now the number of reports regarding small-molecule compounds with cardiomyogenic potential and molecular pathways of hPSC differentiation into cardiomyocytes has increased exponentially [4-6]. As a result, novel small molecule-directed differentiation protocols for derivation of hPSC-derived CMs have been developed [2, 6]. In this regard Karakikes and co-workers [7] have reported a methodology for CM differentiation, based on modulation of the canonical Wnt pathway via the small molecule IWR-1, with an efficiency close to 100%. The paper, published in Stem Cell Translational Medicine provides a detailed analysis of the hPSC-derived CMs in terms of tissue-specific biomarkers and electrophysiology. Thus, it advances the use of an integrated systems biology approach in the research of human cardiomyogenesis, a notion advocated back in 2011 by Young et al. [8]. The work of Karakikes *et al.* also addresses some research limitations in this fields

formulated by Redig and Adler [9], specifically: the simplicity of the protocol, its efficiency in terms of percentage of differentiated cells out of total number of cells, as well as the reliability of identification of the newly derived cells as cells belonging to the cardiomyocyte lineage. However, it must be stated that the canonical Wnt pathway is one of the most extensively studied and commonly used pathways for inducing cardiomyogenesis in hPSCs via small molecules [2, 6, 10-12]. This implies a rather intriguing question: "Is the potential of hPSC-derived CMs in areas such as drug discovery, disease modeling and regenerative therapies still limited due to our current understanding of human biology?".

Human cardiomyogenesis is a complex sequential process which goes through different numerous developmental stages. The major phases in cardiac development are as follows: (1) establishment of mesoderm formation (from hPSCs); (2) patterning toward cardiac mesoderm; (3) cardiac mesoderm formation; (4) cardiomyocyte development and maturation [10-12]. Temporal regulation of metabolic and signaling pathways is key crucial for successful hPSCs differentiation to CMs, as demonstrated by previously published experimental data [2, 6, 7, 10, 11]. Each stage of this gradual transition between cell types can be monitored via the detection of temporally specific biomarkers like transcription factors (e.g. Mesp 1), cell-surface markers (e.g. VCAM-1) or tissue specific proteins (e.g. cTnT) [10-14]. In vitro, differentiation of pluripotent cells towards the cardiogenic lineage is, in principle, quite easy (at least when compared to other types of differentiated cells). In fact, as soon as the factors that maintain the undifferentiated state of the cells in culture are removed, spontaneous differentiation begins. One of the most commonly encountered cell types produced by spontaneous differentiation are cardiomyocyte-like cells. Targeted differentiation of pluripotent cells along the cardiogenic lineage is also feasible, and several key protocols have been developed [11, 12]. However, hPSC-derived CMs obtained via currently used differentiation protocols areusually within a heterogeneous cell population and may recapitulate features of foetal and adult human CMs [15]. In addition, not all pluripotent cell lines are equal with regards to their capacity to differentiate along the cardiogenic lineage. In this regard, Sepac and associates [16] have reported that there is a difference in the cardiomyogenic potential of commonly used hPSC lines like H1 and H9 (embryonic stem cell lines) in comparison to C2a and C6a (induced pluripotent cell lines). Additionally, they concluded that human embryonic stem cells have a greater potential for terminal differentiation to functional CMs than human induced pluripotent stem cells. This conclusion was supported by a recent report by Burridge et al. regarding the human induced pluripotent stem cell differentiation to CMs [17]. Also, pluripotent stem cell lines, despite their high proliferative potential, are often prone to aging. There is also the fact that in vitro cultured cells may, with increasing number of divisions, experience genomic rearrangements resembling the increased genomic flux typical of cancer cells. Understandably, both greatly decrease the potential use of pre-established stem cell lines. In this regard, some authors believe that characterization of the individual capacity for maintenance of genomic integrity is crucially important for pre-existing and newly established pluripotent stem cell lines alike, in order to screen out prospective cell lines that may have very limited potential for applications [18, 19]. There is also pressing need for establishment of new pluripotent stem cell lines that have not come into contact with substances of animal origin, in order to avoid transmission of zoonotic agents by preparations designed for potential use in humans; and to compensate for the inevitable loss of cell lines due to decreased replicative potential or risk of carcinogenic transformation [20].

Recent advances in the development of novel or improved differentiation protocols for hPSC-derived CMs [7, 17] have only been made thanks to the knowledge gained by years of ongoing research focused on cardiomyogenic small-molecule compounds [4, 5, 11, 13, 15, 21]. Nevertheless, there are some researchers who point out several bottlenecks in current research [4, 8, 9, 13, 15, 22].

Bottleneck 1: Compound screening, available testbeds and druggable targets

In recent years there is an ongoing debate about how small-molecule compounds should be screened for cardiomyogenic properties [4, 8, 11, 12]. Small molecule screening techniques, application of various stem cell types as testbeds for identification of cardiomyogenic potential and validation of druggable targets are among

the most commonly discussed topics.

Compound screening. Over the years both synthetic and natural small-molecule compounds have been identified as inducers of cardyomyogenesis [4, 5, 22, 23]. A classical method to investigate the cardiomyogenic potential of discrete, well-characterized chemical entities in pluripotent stem cells is the high-throughput screening (HTS) [5, 23]. In brief, this methodology allows the analysis of large amounts of data collected in a parallel fashion. It is typically achieved with the aid of fluorescent, luminescent or color label/s that mark the molecule/s of interest. The signal is read out by a detector/s that may record and interpret many data points at once, e.g. a plate reader. In terms of cell-based HTS, labelled reporter proteins are commonly used. Usually, GFP is a staple label in stem cell research, and studies of differentiation into the cardiogenic lineage are no exception [4]. A variety of cardiomyogenic small molecules like ascorbic acid and classes of compounds like cardiogenols andsulfonyl-hydrazones have been discovered using HTS [5, 23]. However, despite the speed and simplicity of assay development [4] HTS is not particularly suitable for the identification of potent cardiomyogenesis-inducing molecules, as procardiogenic effects of such compounds could vary depending on culturing conditions [23]. Another caveat is the often inconsistent production of late-stage cardiac mesoderm progenitors needed for the development of reliable assays [13]. Therefore, the discovery of small molecules with procardiogenic potential via high-content screening (HCS) has been recently advocated [4, 13]. This methodology is based on the selection of cells which have a signal intensity corresponding to the content of a specific substance or metabolite measured by automated microscope imaging of reporter proteins [4, 13]. Advanced approaches to HCS also include flow cytometric analysis (e.g. counting differentiated versus undifferentiated cells) and, potentially, cell sorting, allowing for the identification of cellular subset sub-populations [4]. In comparison to HTS, HCS is a more complex type of assay. It is more time-consuming and may not allow the screening of many compounds simultaneously [4, 13]. On the other hand, imaging analysis inherent to HCS enables morphological analysis as well [4]. However, it is still to be decided among biomedical researchers whether one assay type should be looked upon more favorably than the other.

Available testbeds. Nowadays biomedical researchers have access to various stem cell lines which can be used to produce stem-cell-derived cardiomyocytes for applications like drug discovery and regenerative medicine or to probe the signaling pathways behind cardiomyogenesis. However, the need to choose one characterized cell line from a pool of available stem cell types (e.g. murine or human; embryonic or induced pluripotent) can be rather daunting and results obtained in animal cell lines may not be translatable to human cell lines. For example, the cardiomyogenic effect of ascorbic acid was first discovered in stably transfected EGFP-mouse embryonic stem cells back in 2003 [5], but in mouse induced pluripotent stem cells the yield of cardiomyocytes using ascorbic acid has recently been reported to be inconsistent [24]. Therefore, pre-existing data may indicate that ascorbic acid ought to promote cardiomyogenesis in mouse induced pluripotent stem cells, but that statement remains purely speculative, as the results apparently do not support this hypothesis. In this regard, multi-testbed screening of compounds can be a great time-saver as speculative assumptions will be quickly disproven by experimental data. Moreover, the use of both murine and human stem cell lines in the same experiment promises a faster generation of data about the stem cell biology of the two species. There have been reports that despite the apparent closeness of the two species, there are some essential differences on molecular level that show greatly in their capacity for maintenance of the undifferentiated state and targeting into differentiation into different cell types [25]. The report of Kattmanet *et al*. [26] about the stage-specific optimization of Activin/Nodal and BMP signaling promoting cardiac differentiation of mouse and human pluripotent stem cell lines is an excellent example of multi-testbed-based research. Their results show that the formation of cardiac mesoderm in both mouse and human pluripotent stem cells can be monitored by co-expression of KDR and PDGFR-α. Additionally, it was found that efficient cardiac differentiation was dependent on optimal levels of Activin/Nodal and BMP signaling in all tested stem cell lines. In a nutshell, translational research like that of Kattmanet *et al*. has the potential to "revolutionize" our understanding not only of just cardiomyogenesis, but of stem cell biology as a whole.

Druggable targets. The discovery of new druggable targets influencing cardiac differentiation is often

coupled with breakthroughs in small molecule HTS. However, the opposite may also be true [22]. Interestingly, the biological effect of several small molecules involves the inhibition rather than activation of specific signaling pathways [22, 27]. In this regard one of the most studied and well-known molecular pathways is Wnt signaling [11, 21, 28, 29]. Up-to-date several cardiomyogenic Wnt inhibitors have been discovered and are currently being used in differentiation protocols [6, 16]. The success story of Wnt inhibitor mediated cardiac differentiation [7, 29] has tempted biomedical researchers to overlook other druggable targets. However, in recent years the interest toward previously poorly studied signaling pathways like MAPK has increased [30, 31]. Other druggable targets under investigation include: microRNAs [32, 33]; mTOR signaling pathway [34]; TGF-β signaling pathway [35]; Neuregulin/ErbB signaling pathway [36]; retinoid signaling [37]. Additionally, the regulation of cardiac differentiation in stem cell via metabolic oxidation is also of key research interest [38, 39] as well as the role of mitochondria [40]. Furthermore, the interplay between small-molecule compounds and signaling molecules has been found to have a profound effect on cell type specification [6, 41]. Therefore the structure-activity relationship (SAR) and pharmacological properties (e.g. selectivity, solubility etc.) of a tested compound are key to its' effectiveness as an inducer of cardiac development in pluripotent stem cells. In this regard, molecular analogues of some previously tested small molecules have also been found to possess cardiomyogenic potential [42]. Moreover, some intriguing compounds like angiotensin II [43] and ghrelin (28-amino-acid peptide) [44] have recently been identified as Cardiomyogenic. All in all, druggable targets and small molecules are only parts of the puzzle. Only by applying our knowledge of both medicinal chemistry and stem cell biology can we solve the puzzle: a reliable method to obtain hPSC-derived CMs on a large scale. Something that is hardly possible in the present!

Bottleneck 2: Cardiomyocyte maturation in vitro

A common setback of most currently used differentiation protocols for hPSC-derived CMstoday is the insufficient cell culture maturation in vitro [11, 15-17]. In this regard Robertson et al. [15] propose that hPSC-derived CM cultures be defined as 'early-phase' or 'late-phase' based on specific a sets of biomarkers, e.g. membrane ion channels and sarcomeric organization. Early-phase hPSC-derived CM culture is defined by Robertson *et al*. as contractile cells possessing some proliferative capacity and electrophysiological properties similar to the properties of embryonic cells, whereas the late-phase culture is characterized by loss of proliferative capacity and electrophysiological properties similar to those of adult cells [15]. The group of Robertson advocates the need for development of novel culturing methods. However, despite the recent advancement in chemically defined culture media [17] or improvements in differentiation protocols [7], the researchers still face many challenges, such as: mainly obtaining adult-like cardiomyocyte cells with morphology and electrophysiology typical of mature cardiomyocytes [15]. There is also the problem of standardization. Without a robust universally accepted method for evaluation of the identity of cardiomyocyte-like cells the analysis of the pros and cons of different cardiomyogenesis protocols in terms of efficiency is difficult [9].

Concluding remarks

Presently we face more than one bottlenecks in development of potential applications of small molecule-mediated cardiomyogenesis in hPSCs for large scale production of human CMs for the purposes of drug discovery, safety pharmacology, disease modeling and regenerative medicine. Firstly, the spacial-temporal complexity of cardiomyogenesis in human stem cells significantly slows down the identification of differentiation pathways and differentiation modulating molecules, that may successfully be manipulated used in a large-scale production. In addition, the cost of technology transfer alone may prove to be prohibitive for many currently developed differentiation protocols. Secondly, without the methodology for reliable assessment of hPSC-derived CMs for adult-like morphological and electrophysiologyical properties, their potential use is questionable, especially in the field of regenerative and reparative medicine. It could be expected that those problems could be solved using an interdisciplinary approach to human cardiomyocyte differentiation complemented by tissue engineering.

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