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A Small Molecule that Promotes Cardiac Differentiation of Human Pluripotent Stem Cells under Defined, Cytokine- and Xeno-free Conditions

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SUMMARY

Human pluripotent stem cells (hPSCs), including embryonic stem cells and induced pluripotent stem cells, are potentially useful in regenerative therapies for heart disease. For medical applications, clinical-grade cardiac cells must be produced from hPSCs in a defined, cost-effective manner. Cell-based screening led to the discovery of KY02111, a small molecule that promotes differentiation of hPSCs to cardiomyocytes. Although the direct target of KY02111 remains unknown, results of the present study suggest that KY02111 promotes differentiation by inhibiting WNT signaling in hPSCs but in a manner that is distinct from that of previously studied WNT inhibitors. Combined use of KY02111 and WNT signaling modulators produced robust cardiac differentiation of hPSCs in a xeno-free, defined medium, devoid of serum and any kind of recombinant cytokines and hormones, such as BMP4, Activin A, or insulin. The methodology has potential as a means for the practical production of human cardiomyocytes for regeneration therapies.

INTRODUCTION

Human pluripotent stem cells (hPSCs), including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), can proliferate indefinitely in an undifferentiated state and differentiate into many types of cells in human tissues, including the heart (Chien et al., 2004; Irion et al., 2008; Lutolf et al., 2009). Therefore, hPSCs are potentially useful in cell-based therapies for heart disease (Chien et al., 2008; Hansson et al., 2009; Laflamme and Murry, 2011; Menasché, 2009; Passier et al., 2008; Segers and Lee, 2008). Efficient production of functional cardiac cells from hPSCs is required for cell-based therapy. Multiple cardiac differentiation methods have been described, and these procedures need animal cells, fetal bovine serum (FBS), or various cytokines (Burridge et al., 2012; Laflamme and Murry, 2011; Rajala et al., 2011). However, an efficient single method cannot always be applied to all hPSC lines because of the differences in differentiation propensity among cell lines (Osafune et al., 2008). Recently, a universal cardiac differentiation method that is independent of hPSC lines was reported, but it requires FBS or human serum for highly efficient differentiation (Burridge et al., 2011). Another study using serum-free medium showed that optimal cytokine concentrations for cardiac induction differ among individual hPSC lines (Kattman et al., 2011). However, use of recombinant cytokines is not cost effective for large-scale production, and the use of serum needs be avoided for clinical use because of the potential risk for disease infection.

Small molecules have great potential as substitutes for recombinant cytokines and unknown factors in serum (Xu et al., 2008), and they are suitable for making defined media for large-scale culture. To date, small molecules have been used to activate or inhibit signaling pathways, such as WNT or TGF-β signaling (Chen et al., 2009; Ichida et al., 2009), or to regulate the expression of genes instead of transcription factors (Kamisuki et al., 2009; Sato et al., 2006). A number of small molecules have been examined or screened for promotion of differentiation: a BMP signaling inhibitor, a p38MAPK signaling inhibitor, a WNT signaling activator, and WNT signaling inhibitors were all reported to promote cardiac differentiation (Graichen et al., 2008; Hao et al., 2008; Naito et al., 2006; Qyang et al., 2007; Ren et al., 2011; Wang et al., 2011; Willems et al., 2011). However, these chemical treatments resulted in only 10%–60% differentiation to cardiomyocytes (Naito et al., 2006;

Qyang et al., 2007; Ren et al., 2011; Wang et al., 2011; Willems et al., 2011). Hence, a small molecule that produces more efficient differentiation is needed for clinical applications.

An enrichment of hPSC-derived cardiomyocytes as well as an increase in differentiation efficiency are important. Recently, enrichment procedures without using genetic modifications were reported by Dubois et al. (2011) and Hattori et al. (2010). These methods utilize fluorescence-activated cell sorting (FACS) technology based on mitochondria content or cell surface molecules. However, a FACS-based enrichment procedure would be time consuming for the preparation of a large amount of cells.

In this study, we report a small molecule promoting cardiac differentiation of hPSCs. By using this chemical, xeno-free and cytokine-free cardiac differentiation was achieved. Moreover, functional cardiomyocytes derived from hPSCs were enriched (up to 98%) by a simple floating culture protocol without FACS procedures.

RESULTS

Discovery and Characterization of KY02111

To identify small molecules that efficiently promote cardiomyocyte differentiation from hPSCs, we established a high-content analysis (HCA) system, using monkey ESCs that express EGFP driven by human *αMHC* promoter (Figure 1A). The chemical-screening protocol for the detection of an EGFP signal is described in Experimental Procedures. We identified one molecule, N11474, that significantly enhanced the values of HCA parameters compared to the control (Student's t test, $p = 0.015$).

During chemical screening, monkey ESCs were treated with 1–5 μM of small molecules for 8 days (days 6–14). To determine the period during which N11474 effectively promotes differentiation, we used several treatment patterns and measured the total signal intensity of *αMHC* promoter-driven EGFP on day 14 (Figure 1B). N11474 treatment on days 6–10 was similarly effective to the screening protocol, and treatment after day 8 (days 8–12 or days 10–14) was less effective. Treatment on days 4–8 maximized the increase in GFP expression, whereas treatment on days 0–4 completely repressed GFP expression. The treatment with a higher concentration (10 μM) revealed similar results and more efficacious increase of GFP intensity on days 4–8. These results suggested that in monkey ESCs, N11474 acts as an inhibitor in the early phase (days 0–4) and as a promoter in the middle phase (days 4–8) of cardiac differentiation.

We chemically synthesized analogs of N11474 (Figures S1A and S1B) and assayed their ability to promote cardiac differentiation of monkey ESCs. Structure-activity relationship studies revealed that substitution of the methoxy group at the benzothiazole ring with an electron-withdrawing group, and adjustment of the length of the methylene linker, greatly improved biological activity, resulting in the molecule KY02111 (Figures 1C, 1D, and S1C). This drug-like small molecule promoted cardiac differentiation ~73 times more effectively than the DMSO control and 7.4 times more effectively than N11474.

Time course experiments using IMR90-1 hiPSCs showed that beating colonies emerged on day 8 and increased in number until day 12 under adherent conditions (Figure 1E). When all the colonies were collected on day 15 as described in Experimental Procedures, the subsequent proportion of beating colonies evidently increased up to 90% in floating culture, probably due to reduced mechanical inhibition (Otsuji et al., 2010). This result suggested that a large proportion of cell colonies emerged in the cardiac differentiation protocol using KY02111 might be cardiac beating colonies. The cardiac colonies continued beating until at least day 50.

The general applicability of KY02111's activity was examined with a variety of primate and rodent PSCs (Figure 1F). KY02111 increased the ratio of beating cardiac colonies as much as 70%–94% in cell aggregates of two hESC lines (KhES-1 and KhES-3), four hiPSC lines (253G1, IMR90-1, IMR90-4, and RCHIPC0003), and a mouse ESC line (R1).

Characterization of Cardiomyocytes Produced by KY02111

Immunocytochemical analysis of day 30 cardiac colonies, which were switched to floating culture on day 15, showed that approximately 73%–85% of IMR90-1 hiPSCs treated with KY02111 expressed the cardiac markers, cardiac troponin T (cTnT), αActinin, or NKX2.5, whereas only a few DMSO-treated cells were positive for the markers (Figures 2A and 2B). The cardiac pacemaker marker, HCN4, was expressed in 16% of KY02111-treated cells, whereas the ratio of Vimentin-positive cells (fibroblasts) decreased 3.3-fold (Figure 2B). SMA, a marker of smooth muscle, was almost undetectable in KY02111-treated cells (data not shown). These results suggested that hPSC-derived cardiomyocytes can be enriched by simply harvesting KY02111-induced cell colonies, without resorting to cell-sorting procedures. Real-time PCR analysis on days 15 and 30 showed that KY02111-induced cardiomyocytes (KY-CMs) expressed the cardiac markers, *αMHC*, *NKH2.5*, and *HCN4*, and that all of the ion channel genes examined were expressed at levels similar to those of adult heart tissue (Figure 2C).

FACS analysis revealed that approximately 60% or 8% of day 30 KY-CMs were MLC2v-positive mature ventricular cardiomyocytes or MLC2v/MLC2a double-positive immature ventricular cardiomyocytes, respectively, and that few MLC2a-positive cells were detected (Figure 2D), suggesting that the majority of KY-CMs are ventricular cardiomyocytes.

Electrophysiological analysis by the whole-cell patch-clamp method was used to examine whether day 30 KY-CMs are functional cells. Action potential properties indicated that the population of KY-CMs included ventricular cells and pacemaker cells (Figure 2E). The properties of voltage-dependent Ca^{2+}, Na^{+}, and HCN channel currents were examined using the ion channel blockers, nifedipine, lidocaine, and zatebradine, respectively (Figures S2A–S2C). The current density-voltage relationships indicated that KY-CMs were electrophysiologically functional. Treatment of KY-CMs with HERG channel blocker, E4031, and KCNQ1 channel blocker, Chromanol293B, increased action potential duration (APD), which is equivalent to QT prolongation detected by an electrocardiogram (ECG) (Figure 2F). The increases in duration of APD90 (APD at 90% repolarization) were 37.0% ± 11.2% for E4031 treatment, and 42.1% ± 8.8% for Chromanol293B treatment (Figure S2E). Moreover,