

# 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 to 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- $\beta$  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  $\alpha$ MHC 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  $\mu$ M 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  $\alpha$ MHC 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  $\mu$ M) 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  $\sim$ 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.

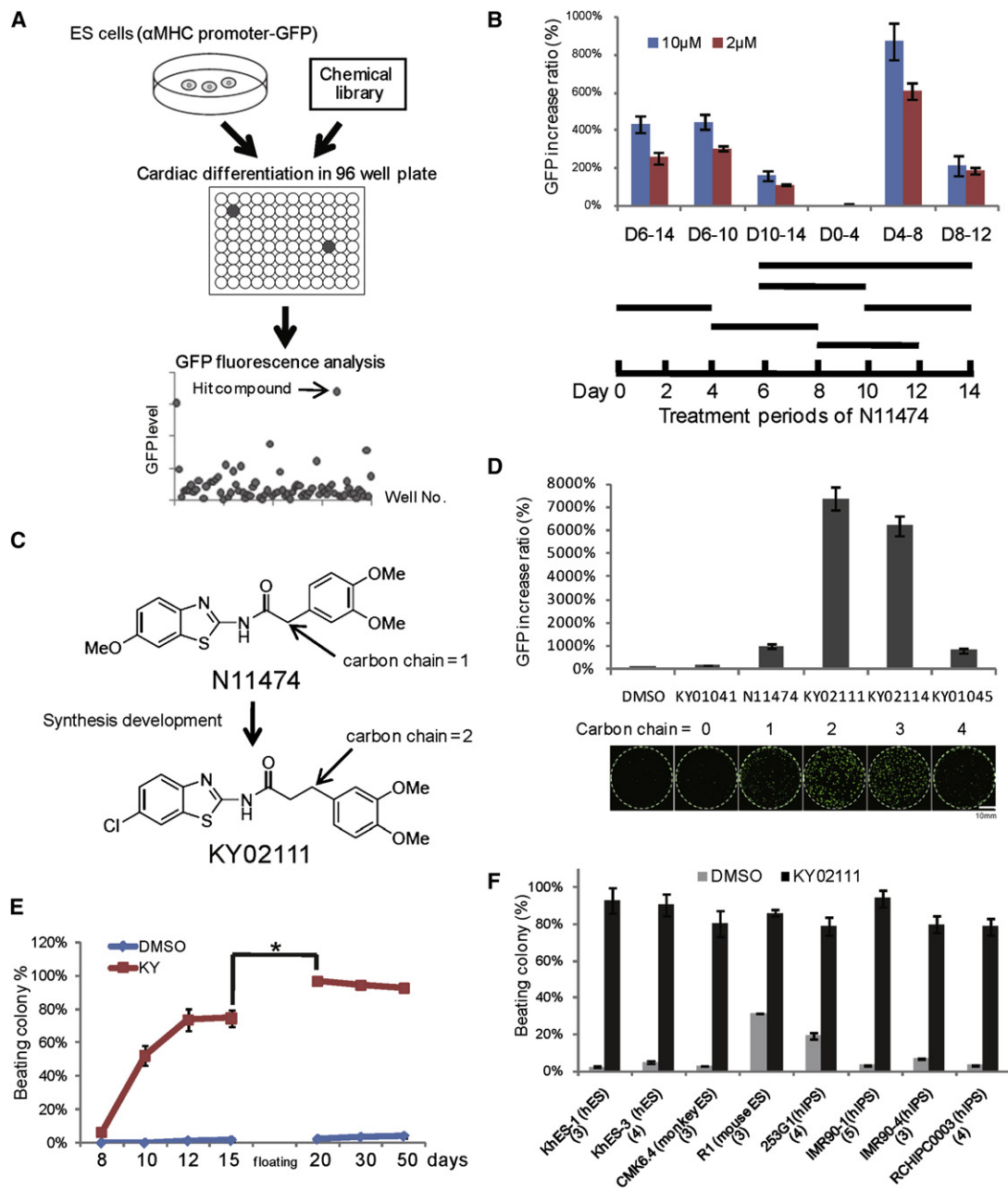
The general applicability of KY02111's activity was examined with a variety of primate and rodent PSCs (Figure 1F). KY02111 increased the ratio of beating cardiac colonies as much as 70%–94% in cell aggregates of two hESC lines (KhES-1 and KhES-3), four hiPSC lines (253G1, IMR90-1, IMR90-4, and RCHIPC0003), 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),  $\alpha$ Actinin, 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,  $\alpha$ MHC, NKX2.5, 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  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$ , 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  $37.0\% \pm 11.2\%$  for E4031 treatment, and  $42.1\% \pm 8.8\%$  for Chromanol293B treatment (Figure S2E). Moreover,



**Figure 1. Identification and Characterization of Small Molecules that Promote Cardiac Differentiation**

(A) Schematic of small molecule screening. One hit compound was identified from the chemical library of 9,600 compounds (Murakami et al., 2011), using human  $\alpha$ MHC promoter-driven EGFP transgenic monkey ESCs and a HCA system.

(B) Effective time window of hit compound, N11474. The expression level of the DMSO control is 100%. The experiment was independently performed three times ( $n = 3$ ). Mean  $\pm$  SEM. D, day.

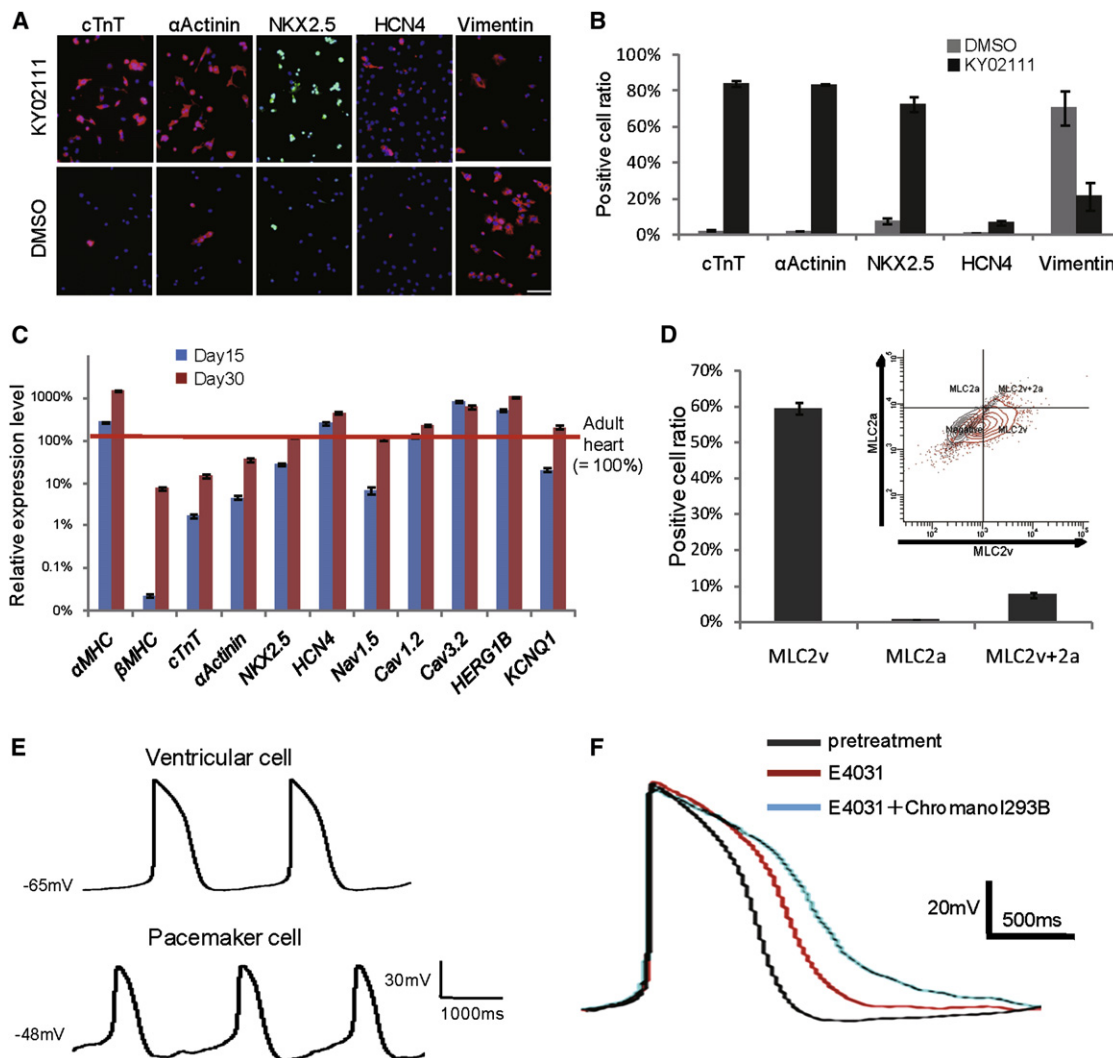
(C) Chemical structures of N11474 and KY02111.

(D) Structure-activity relationships of N11474 and related compounds. Transgenic monkey ESCs were treated with 10  $\mu$ M of chemical compounds. Top graph shows GFP increase ratio. Green dots in the whole-well images (bottom photos) indicated colonies with GFP signals. Scale bar, 10 mm. The length of carbon chain that connects the benzothiazole ring to the dimethoxyphenyl ring was important for inducing GFP expression ( $n = 3$ ). Mean  $\pm$  SEM.

(E) Time course analysis of cardiac differentiation induced by KY02111. IMR90-1 was treated with 10  $\mu$ M KY02111. Cardiac colonies were transferred to floating culture on day 15 ( $n = 3$ ). Mean  $\pm$  SEM.

(F) Generality of KY02111 effect on PSC lines. hESC lines (KhES-1 and KhES-3), monkey ESC line (CMK6.4), mouse ESC line (R1), and hiPSC lines (IMR90-1, IMR90-4, 253G1, and RCHIPC0003) were differentiated into cardiomyocytes by treatment with 10  $\mu$ M KY02111. The ratio of beating colonies in floating culture was examined for all PSC lines on day 30 except mouse ESC. Mean  $\pm$  SEM. n, the number in parentheses.

See also Figure S1.



### Figure 2. Characterization of Cardiomyocytes Derived from hPSCs Treated with KY02111

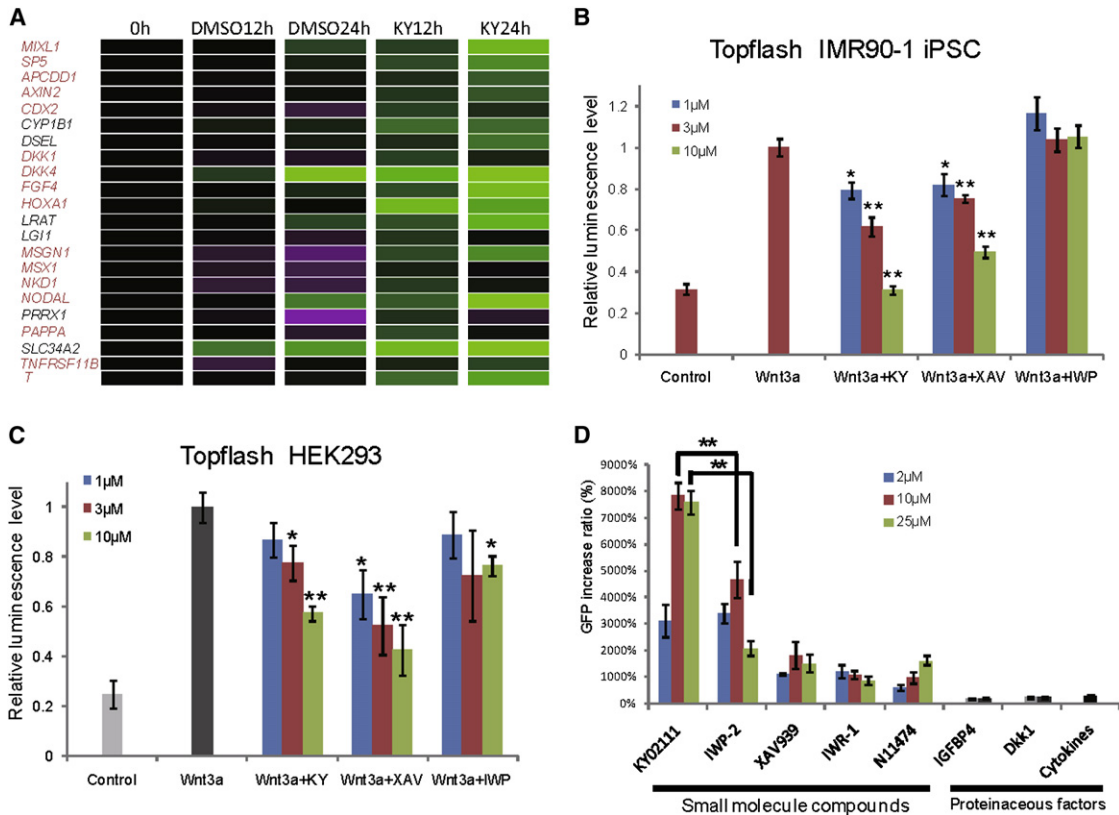
(A and B) KY02111 promoted the expression of cardiac markers. IMR90-1 hiPSCs were treated with 10  $\mu$ M KY02111, and then cardiac markers (cTnT,  $\alpha$ Actinin, and NKX2.5), pacemaker marker, HCN4, and fibroblast marker, Vimentin, were examined using floating cardiac colonies. (A) The upper and lower images are cells treated with KY02111 or DMSO, respectively. Scale bar, 100  $\mu$ m. (B) Immunopositive cell ratio in cells treated with KY02111 (n = 3). Mean  $\pm$  SEM. (C) qPCR gene expression analysis of KY-CMs. Total RNA was extracted from IMR90-1-derived KY-CMs on days 15 and 30 of cardiac differentiation using 10  $\mu$ M KY02111. Cardiac marker genes ( $\alpha$ MHC,  $\beta$ MHC, cTnT,  $\alpha$ Actinin, and NKX2.5) and channel genes (HCN4, Nav1.5, Cav1.2, Cav3.2, HERG1b, and KCNQ1) were highly expressed in KY-CMs. Almost all genes were upregulated at day 30, and their gene expression levels were nearly equal to the gene expression levels of adult heart tissue, which was considered to be 100% (mean  $\pm$  SEM; n = 3). All primers used are shown in Table S2. (D) FACS analysis of KY-CMs. A total of 30,000 cells of IMR90-1-derived KY-CMs were measured in each sample by FACSCanto II. Mean  $\pm$  SEM; n = 3. The inset shows representative flow cytometry data. Red indicates MLC2v and/or MLC2a-positive cells. Gray indicates negative controls without the primary antibody. (E and F) Functionality of KY-CMs. (E) Spontaneous ventricular-like or pacemaker-like action potential (AP) in patch-clamp recordings from day 30 KY-CMs. (F) Prolongation of APD induced by 100 nM E4031 and 4  $\mu$ M Chromanol293B. See also Figures S2 and S3.

voltage-dependent  $K^+$  currents were suppressed by treatment with E4031 and Chromanol293B (Figures S2D and S2E). These results indicated that the KY-CMs expressed functional functional HERG and KCNQ1 channels. A drug-induced QT prolongation test, performed using a microelectrode array (MEA), showed that ECG-like waves were emitted from KY-CM colonies (Figures S3A and S3B). Treatment with astemizole, which causes clinical QT prolongation (Suessbrich et al., 1996), prolonged the  $Na^+$ - $K^+$

interval of the ECG-like waves in a dose-dependent manner (Figure S3C). Overall, these results indicated that KY-CMs are electrically and pharmacologically functional.

### Inhibition of Canonical WNT Signaling Pathway by KY02111

To gain insight into how KY02111 promotes cardiac differentiation of hPSCs, gene expression profiles of IMR90-1 hiPSCs



**Figure 3. KY02111 Is a Canonical WNT Signaling Inhibitor**

(A) Heatmap of downregulated genes. IMR90-1 hiPSCs were treated with 10  $\mu$ M KY02111 or DMSO for 12 or 24 hr (KY12hr, KY24hr, DMSO12hr, or DMSO24hr), and 26 genes were identified. A total of 22 downregulated genes were used to generate the heatmap. Four upregulated genes are shown in Table S1. Red gene symbols indicate WNT-signaling target genes (see also Table S1).

(B and C) TCF reporter assay using IMR90-1 hiPSCs or HEK293 cells. The effect of KY02111 (KY), XAV939 (XAV), IWP-2 (IWP), or DMSO (Control) was examined. Wnt3a (60 ng/ml) was added to activate WNT signaling and TCF promoter activity. IWP-2 did not inhibit TCF promoter activities, probably because IWP-2 is an inhibitor of WNT ligand secretion.  $n = 3$  (B) and  $n = 3$  (C); mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  by Student's  $t$  test.

(D) Comparison of cardiac differentiation promoting activity among chemical and proteinaceous WNT inhibitors. The activity was measured using monkey ESCs with EGFP gene driven by human  $\alpha$ MHC promoter on day 14. The activity level of the DMSO control treatment was considered to be 100%. The small molecule WNT inhibitors were KY02111, IWP-2, XAV939, and IWR-1. The proteinaceous factors were IGFBP4 (0.2 and 1  $\mu$ g/ml), Dkk1 (0.1 and 0.3  $\mu$ g/ml), and a mixture of bFGF, BMP4, VEGF, DKK1, and Activin A (cytokines) (Yang et al., 2008). Mean  $\pm$  SEM;  $n = 4$ . \*\* $p < 0.01$  by Student's  $t$  test.

See also Figure S4.

treated with KY02111 were analyzed using the microarray technique. KY02111 or DMSO was added to the culture on day 3 of cardiac differentiation, and the cell population was harvested after 12 or 24 hr. A total of 22 downregulated genes and four upregulated genes were identified at both 12 and 24 hr after KY02111 treatment (Figure 3A; Table S1). Although only a few genes were extracted by the microarray analysis, perhaps due to high heterogeneity of the cell population, the microarray data were validated by qPCR (Figure S4A). When the 22 downregulated genes were examined for common transcription factor-binding sites, using DiRE (Gotea and Ovcharenko, 2008), which can predict distant regulatory elements, TCF4 was predicted as a common transcription factor (Figure S4B). Of the 22 downregulated genes, 16 (72.7%) were known target genes of canonical WNT signaling (Table S1). Furthermore, the effect of KY02111 on WNT target gene expression was very similar to that of other WNT inhibitors, such as XAV939 and

IWP-2, but not of BIO, a WNT activator (Figure S4C). These results suggested that KY02111 inhibits canonical WNT signaling in hPSCs.

The TOPflash assay, a reporter system generally used for measuring activities of canonical WNT signaling, was used to confirm that KY02111 is a WNT signaling inhibitor. IMR90-1 cells or HEK293 cells were transfected with TCF reporter plasmids and then were treated with Wnt3a along with 1, 3, or 10  $\mu$ M of KY02111, XAV939, or IWP-2. Treatment with KY02111 or XAV939 clearly reduced luciferase activities in both cell lines in a dose-dependent manner (Figures 3B and 3C). TOPflash results substantiated that KY02111 is an inhibitor of canonical WNT signaling.

We next compared the promotion of cardiac differentiation in transgenic monkey ESCs by KY02111, several chemical or proteinaceous WNT inhibitors (IWP-2, IWR-1, XAV939, DKK1, and IGFBP4; Zhu et al., 2008), and a combination of several

cytokines. All of them increased the intensity of the GFP signal driven by  $\alpha$ MHC promoter (Figure 3D). Of them, KY02111 was most potent at an effective concentration of 10–25  $\mu$ M. Treatment with KY02111 increased differentiation about 80-fold compared to the control and did not show toxicity to cells even at high concentration.

### Mechanism of WNT Signaling Inhibition by KY02111

To explore how KY02111 inhibits WNT signaling, we compared its effect with two WNT inhibitors, IWP-2 and XAV939, which inhibit O-acyltransferase and tankyrase, respectively (Chen et al., 2009; Wang et al., 2011). The other known inhibitor, IWR-1, was not used because the target of IWR-1 is the same as that of XAV939 (Narwal et al., 2012). The compounds were used at a concentration of 10  $\mu$ M, which was sufficient to inhibit the TCF reporter activity in the TOPflash assay and also to promote cardiac differentiation (Figures 3B–3D). SW480, a human colon adenocarcinoma cell, has mutation of adenomatous polyposis coli (APC) gene that stabilizes  $\beta$ -catenin (Morin et al., 1997). Hence, the basal luciferase activity of SW480 is very high even without Wnt3a treatment in the TOPflash assay (Figure 4A). Treatment of KY02111 significantly reduced luciferase activity in SW480 cells, whereas XAV939 and IWP-2 did not. Furthermore, additional assays were performed using IMR90-1 cells and HEK293 cells treated by a GSK3 $\beta$  inhibitor BIO, which prevents  $\beta$ -catenin degradation. Treatment of BIO along with KY02111 dramatically reduced luciferase activity induced by BIO, compared to XAV939 and IWP-2 (Figures 4B and 4C). In addition, we confirmed the dose-dependent effects of KY02111 across all the TOPflash assays performed (Figures S5A–S5C).

To evaluate the role of WNT signaling inhibition in cardiac differentiation, monkey ESCs and hiPSCs were induced to differentiate into cardiomyocytes in the presence of BIO. When BIO was added along with IWP-2 or XAV939, cardiac differentiation of monkey ESCs and hiPSCs was completely inhibited, whereas cardiac differentiation promoted by KY02111 remained unaffected (Figures 4D and 4E). KY02111 overcame the effect of BIO in a dose-dependent manner (Figures S5D and S5E). Together with the TOPflash results, these data suggest that the mechanism of WNT inhibition by KY02111 is different from that of IWP-2 or XAV939. It appears that KY02111 might be acting downstream of APC and GSK3 $\beta$  in the canonical WNT signaling pathway.

Because targets differ among WNT inhibitors, we examined whether the use of a combination of WNT inhibitors might enhance differentiation of hPSCs to cardiomyocytes. KY02111 alone produced approximately 80% cTnT-positive cells; KY02111 in combination with other WNT inhibitors did not significantly increase differentiation efficiency (Figure S5F). However, treatment with a combination of WNT inhibitors did significantly enhance total cell numbers and beating colony numbers compared to treatment with KY02111 alone and the combination of XAV939 and IWP-2 (XAV+IWP) (Figures 4F and S5G). The number of cardiomyocytes produced by day 30 was 80-fold higher in the KY02111 and 130- to 180-fold higher in the combined treatments (KY+IWP and KY+XAV) than in the control.

### Defined, Cytokine-free, and Xeno-free Cardiac Differentiation

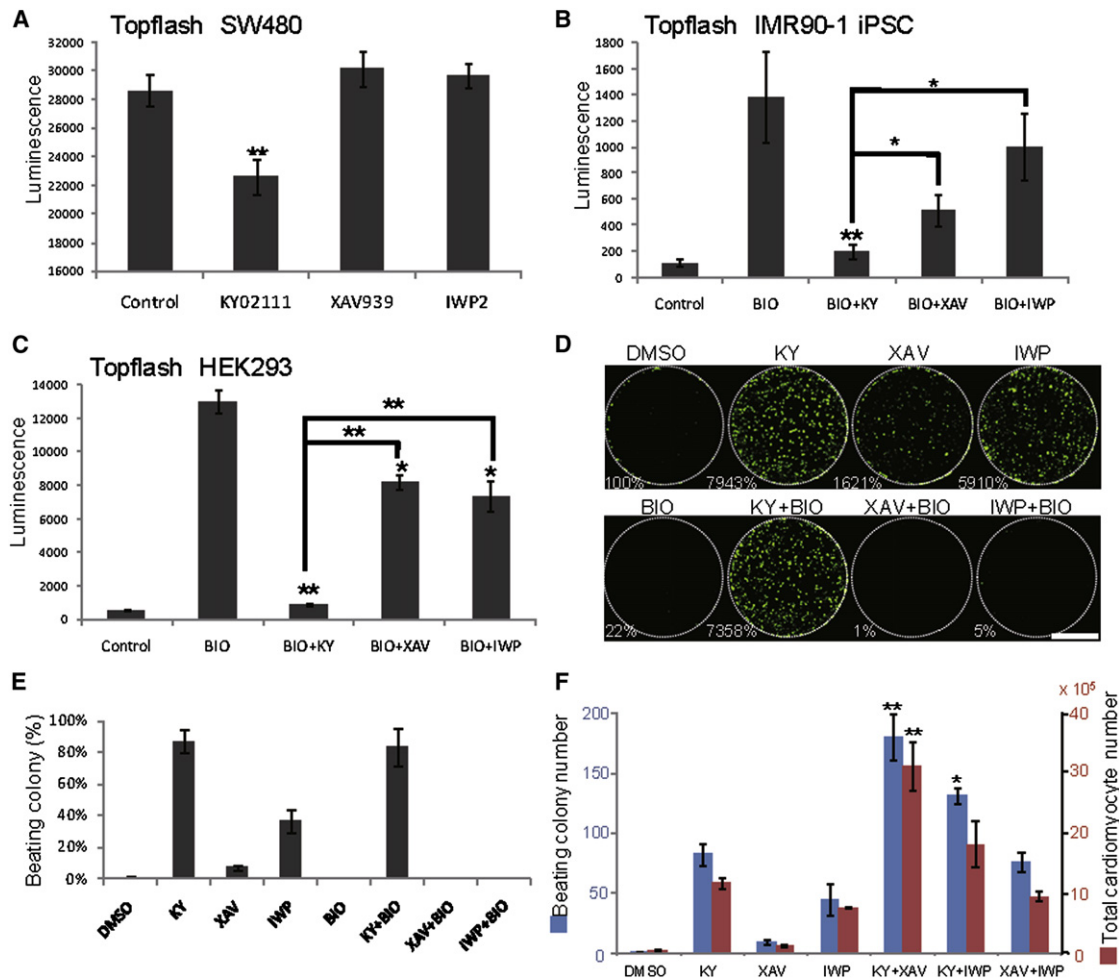
When cardiac differentiation in serum-free medium was examined, two requirements emerged: surface coating with gelatin or human laminin211, and an addition of 0.4% human serum albumin or 1%–2% BSA. Nonattached cells were minimally differentiated into cardiomyocytes, and cell death occurred in serum-free medium without albumin (data not shown).

Activation of WNT signaling is required for the early phase of cardiac differentiation in hPSCs. Therefore, the effect of WNT activators, BIO and CHIR99021 (CHIR), was examined in the early stage of differentiation, in the absence of BMP4 (Figure 5A). Although treatment with 2–4  $\mu$ M CHIR alone increased the number of cardiac beating colonies, the combined use of 1–2  $\mu$ M BIO and 4  $\mu$ M CHIR resulted in more efficient cardiac differentiation (Figure 5B). Treatment with the optimal concentrations of BIO and CHIR on days 0–3 led to an upregulation (4- to 14-fold) of mesodermal marker genes (*MSGN1*, *T*, *MIXL1*, and *NODAL*) in IMR90-1 hiPSCs (Figure 5C). Following treatment with KY02111 alone or in combination with XAV939 (KY+XAV), beating colonies emerged on days 8–10 (Movie S1). The treatment with these WNT signaling modulators resulted in 84%–98% cTnT-positive cell ratio of floating cardiac colonies derived from IMR90-1 hiPSCs under both serum-containing and serum-free, albumin-containing medium culture conditions (Figures 5D and 5E). These results suggest that small molecule-mediated activation of WNT signaling and endogenous signaling factors are sufficient to induce mesodermal cells during cardiac differentiation.

Treatment with KY+XAV did not result in significantly different cTnT-positive cell ratio of floating cardiac colonies compared to treatment with KY02111 alone (Figure 5D). However, FACS analysis showed that, under cytokine- and serum-free conditions, treatment with KY+XAV had slightly higher ratio (98.1%) than treatment with KY02111 alone (90.2%) (Figure 5F). Immunostaining analysis showed, furthermore, that treatment with KY+XAV produced a higher proportion of cTnT-positive cells ( $97.7\% \pm 0.8\%$ ) than KY02111 alone ( $93.3\% \pm 4.4\%$ ) (Figures 5D and 5E). Thus, the combined use of KY+XAV appears to result in highly efficient cardiac differentiation of hPSCs.

Treatment with KY02111 in the presence of serum gave a lower cTnT-positive cell ratio of floating cardiac colonies ( $83.7\% \pm 8.0\%$ ) compared to the treatment with KY02111 in the presence of albumin ( $93.3\% \pm 4.4\%$ ) (Figure 5D). The number of beating colonies and cardiomyocytes was about three times higher in serum-free KY02111 treatment compared to treatment under serum-containing conditions (Figure 5G). Similarly, treatment with KY+XAV without serum enhanced these numbers approximately 1.5-fold over treatment with serum. These results suggest that FBS might contain factor(s) that inhibits cardiac induction and/or proliferation of cardiac progenitors.

Additional experiments confirmed that other hPSCs (KhES-3, H1, H9, RCHIPC0003, and 253G1) efficiently differentiated into cardiomyocytes in cytokine- and xeno-free, defined medium containing KY02111, BIO, CHIR, and XAV939. As observed in IMR90-1 hiPSCs, these treatments enhanced the proportions of beating colonies and cTnT-positive cells in all the cell lines tested (Figures 6A and 6B). Immunostaining of  $\alpha$ Actinin clearly



**Figure 4. KY02111 and Other WNT Inhibitors Cooperatively Promote Cardiac Differentiation through Distinct Manner of WNT Signaling Inhibition**

(A) TCF reporter assay using SW480 cells. The effect of 10  $\mu$ M KY02111 (KY), 10  $\mu$ M XAV939 (XAV), 10  $\mu$ M IWP-2 (IWP), or DMSO (Control) was examined. Mean  $\pm$  SEM; n = 4. \*\*p < 0.01 by Student's t test.

(B and C) TCF reporter assay using IMR90-1 hiPSCs and HEK293 cells. BIO (3  $\mu$ M) was added to activate WNT signaling. n = 3 (B) and n = 3 (C); mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01 by Student's t test.

(D and E) A GSK3 $\beta$  inhibitor did not abolish the effect of KY02111 on cardiac differentiation. Colonies with GFP signal driven by human  $\alpha$ MHC promoter were produced using 10  $\mu$ M KY, XAV, or IWP treatment, with or without 5  $\mu$ M BIO. (D) Representative images of the GFP signal (green dots) in whole wells. Images were captured at day 14. Numbers at bottom left of each image indicate the increase in GFP signal ratio (DMSO = 100%). (E) Proportions of day 30 beating colonies of IMR90-1 hiPSCs. Mean  $\pm$  SEM; n = 3.

(F) Cooperative effects of KY02111 with XAV939 or IWP-2. On day 0 of differentiation,  $6 \times 10^6$  IMR90-1 cells per well were added to a 6-well plate. Blue bars show the number of beating cardiac colonies induced by KY, XAV, IWP, KY+XAV, KY+IWP, and XAV+IWP. Each chemical concentration was 10  $\mu$ M. Red bars show the total number of cardiomyocytes, calculated by multiplying total cell numbers of all colonies by the ratio of cTnT-positive cells (see Figures S5F and S5G). Mean  $\pm$  SEM; n = 4. \*p < 0.05, \*\*p < 0.01 for Student's t test comparing each treatment to KY02111 alone.

See also Figure S5.

showed sarcomere structures in cardiomyocytes derived from IMR90-1 hiPSCs under cytokine- and xeno-free, defined condition (Figure 6C). Also, electron microscopy revealed the structural features of hPSC-derived cardiomyocytes, such as the presence of myofibrils, Z bands, mitochondria, intercalated disks with desmosomes, and sarcoplasmic reticula (Figures 6D and S6). Cardiac colonies, which were harvested on day 15, continued beating in floating culture (Movie S2). Further-

more, the gene expression levels of cardiac markers ( $\alpha$ MHC,  $\alpha$ Actinin, and NKX2.5) and all of ion channel genes examined were nearly equal to those of adult heart tissue on both day 15 and day 30 (Figure 6E). Overall, these results showed that the KY02111 differentiation method, using WNT signal-modulating small molecules and defined medium, effectively produced a high proportion of functional cardiomyocytes from hPSCs.

## DISCUSSION

The present study reports on a small molecule, KY02111 (original molecule, N11474), that promotes cardiac differentiation and acts as a WNT inhibitor in hPSCs. The effective window for treatment with KY02111 is during the middle phase of cardiac differentiation (days 3–8); treatment during the early phase (days 0–3) resulted in suppression of cardiac differentiation. This timing is consistent with previous reports that canonical WNT signaling is required for induction of cardiac differentiation or mesoderm induction of hPSCs, and not for later stages of differentiation (Laflamme and Murry, 2011; Mignone et al., 2010; Naito et al., 2006; Paige et al., 2010). Results presented in this study suggest that KY02111 is a WNT inhibitor and produces more efficient cardiac differentiation than other known WNT inhibitors. Efficient cardiac differentiation in all eight hPSC lines examined was achieved using a fixed concentration of KY02111, indicating that optimization of the differentiation procedure might not be required or might be relatively simple.

Treatment with KY02111 and other WNT inhibitors, such as XAV939 and IWP-2, had a cooperative effect, increasing the number of both cardiomyocytes and beating colonies. The combined use of BIO and CHIR in the early phase of cardiac differentiation, and use of KY+XAV in the middle phase, produced up to 98% cardiomyocytes in cytokine- and serum-free, xeno-free, defined medium. Thus, these treatments might be useful for producing cardiomyocytes for cell-based therapy because all components of defined media are relatively safe and inexpensive. However, cell attachment via gelatin or laminin211 is essential for efficient differentiation, suggesting that mechanotransduction or integrin signaling from interaction with these substrates might be important (Jacot et al., 2010).

Serum-free conditions produced greater numbers of cardiomyocytes in our cardiac differentiation protocol than serum-containing conditions. Although serum is essential for several other differentiation methods, it had an inhibitory effect in our method. Although no inhibitory factors in serum are known, serum contains insulin, which was reported to be an inhibitor of cardiac differentiation (Burrige et al., 2011). Our method requires only small molecules that regulate WNT signaling, and not exogenous BMP4, Activin A, or bFGF, which are often used to increase efficiency of cardiac differentiation (Burrige et al., 2011; Yang et al., 2008). Thus, two WNT activators, BIO and CHIR, appear to fully lead to cardiac mesoderm induction.

A very recently published paper reported that known WNT signaling modulators effectively induced cardiomyocytes in the defined medium with B27 supplement containing insulin, a few steroid hormones, and BSA (Lian et al., 2012). Interestingly, their method predominantly produced MLC2a-positive ventricular cardiomyocytes without any cardiac pacemaker cells, whereas our method mainly produced MLC2v-positive/MLC2a-negative ventricular cardiomyocytes and did include some cardiac pacemaker cells. This suggests that KY02111 itself, the combination of small molecules, or the absence of B27 supplement in our cytokine and xeno-free condition might be able to induce to generation of MLC2v-positive/MLC2a-negative cardiomyocytes and pacemaker cells from hPSCs.

Cardiomyocytes derived from hPSCs described in earlier reports (reviewed in Mummery et al., 2012) were thought to be immature and similar to human fetal cardiomyocytes because of the presence of many MLC2v/MLC2a double-positive ventricular cardiomyocytes with disorganized sarcomeres and weak HERG channel responses. However, our method mainly produced MLC2v-positive/MLC2a-negative cardiomyocytes with well-organized sarcomeres and strong HERG channel responses, indicating that our method might produce more mature cardiomyocytes, though complete maturation did not occur.

The method developed in the present study produced functional cardiomyocytes. These cells have potential for use in stem cell-based therapy. They also have potential pharmaceutical applications, e.g., safety screening for detecting side effects such as drug-inducible QT prolongation (Asai et al., 2010). Large numbers of cardiomyocytes are required for these applications. Large-scale KY+XAV-mediated differentiation could potentially produce a sufficient number of cardiomyocytes from hPSCs. The method described herein produced  $\sim 4.2 \times 10^6$  cardiomyocytes from approximately  $6.0 \times 10^6$  cells seeded initially in one well of a 6-well plate. Although there is room for improvement, our differentiation method produces safe, functional cardiomyocytes and may provide a practical procedure for cell-based therapy of heart diseases.

The direct target of KY02111 is still unknown, but results from this study indicate that KY02111 might function downstream from GSK3 $\beta$  and APC in  $\beta$ -catenin destruction complex and suggest that KY02111 may be a powerful tool for not only cardiac research using hPSCs but also WNT signaling studies. Future research should identify the direct target of KY02111.

## EXPERIMENTAL PROCEDURES

### Culture of hESC, hiPSC, and Monkey ESC

All PSC lines were maintained as described (Suemori and Nakatsuji, 2006). hESC lines used were KhES-1, KhES-3, H1, and H9 (Suemori et al., 2006; Thomson et al., 1998). hiPSC lines used were 253G1, IMR90-1, IMR90-4 (Takahashi et al., 2007; Yu et al., 2007), and RCHIPC0003. RCHIPC0003 was established from human fibroblasts by ReproCELL. The monkey ESC line was CMK6.4 (Suemori and Nakatsuji, 2006). The hESC lines were used in conformity with the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

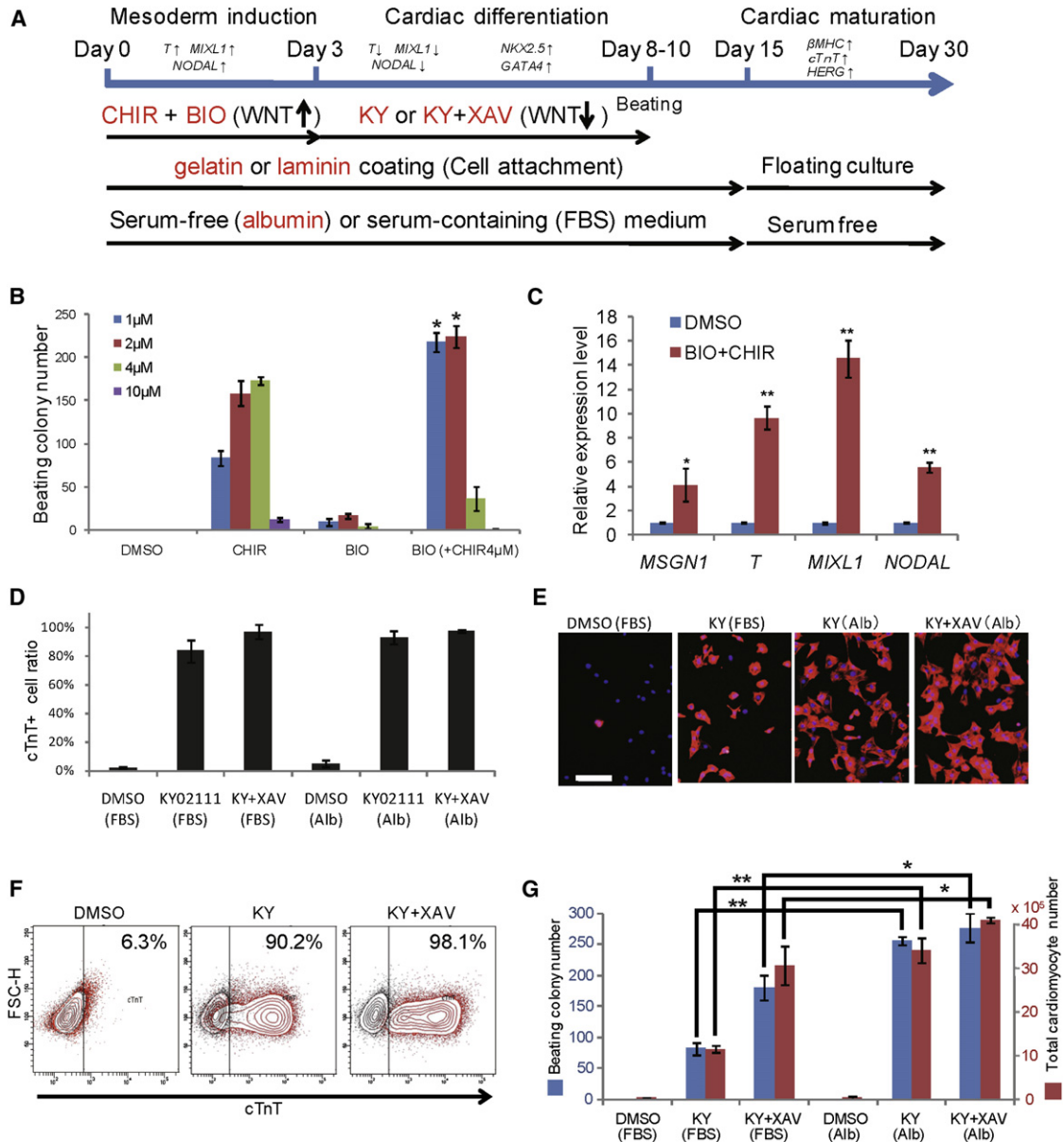
### Transgenic Monkey ESC Line

The human  $\alpha$ MHC promoter-driven EGFP-expressing vector was constructed in the previous study (Otsuji et al., 2010). The linearized vector was introduced into CMK6.4 monkey ESCs by electroporation. Transgenic ESC clones were selectively grown with G418 (Sigma-Aldrich). The expression of EGFP was confirmed in beating colonies.

### HCA Screening of Small Molecules for Promotion of Cardiac Differentiation

The  $\alpha$ MHC-GFP transgenic monkey ESCs were used for HCA screening. During days 6–14 of cardiac differentiation, 9,600 compounds from the chemical library were screened. On day 14, GFP fluorescence of ESCs was measured by whole-plate scanning, using the MetaMorph imaging system (Molecular Devices). See the Extended Experimental Procedures for detailed procedures.





**Figure 5. Cardiac Differentiation in Serum-free Defined Medium or in Serum-Containing Medium Using Small Molecules that Regulate WNT Signaling**

(A) A scheme of cardiac differentiation using WNT signaling regulators. In the early phase of differentiation (days 0–3), precultured hPSC aggregates were cultured in serum-free IMDM-based medium or serum-containing medium, including 0.4%–2% albumin and GSK3β inhibitors (2 μM BIO and 4 μM CHIR) on gelatin or laminin211-coated dishes. In the middle phase (days 3–9), cells were cultured in medium with 10 μM KY02111 (KY), or 10 μM KY and 10 μM XAV939 (KY+XAV). Beating cardiac colonies usually emerged between day 8 and day 10. In the late phase (days 15–30), cardiac colonies were cultured in defined medium without WNT signaling regulators as floating colonies.

(B) The effects of CHIR and BIO on IMR90-1 iPSC-derived cardiac differentiation under serum/cytokine-free condition. DMSO, CHIR, BIO, or BIO along with 4 μM CHIR (BIO+CHIR4 μM) were added in early phase, and 10 μM KY in middle phase. The number of beating colonies was examined on day 30. Mean ± SEM; n = 3. \*p < 0.05 for Student's t test comparing to treatment of 4 μM CHIR alone.

(C) qPCR gene expression analysis of IMR90-1 hiPSCs treated with CHIR and BIO under serum-free condition. Total RNA was extracted from differentiated cells on day 3 of cardiac differentiation after 4 μM CHIR+2 μM BIO or DMSO treatment. Mean ± SEM; n = 3. \*p < 0.05, \*\*p < 0.01 for Student's t test.

(D) Proportion of cTnT-positive cells under serum-free and serum-containing conditions. Cardiac differentiation was carried out according to (A), using medium with 20% serum (FBS) or 1%–2% BSA (Alb). The proportion of cTnT-positive cells was measured by MetaMorph software. Mean ± SEM; n = 3.

(E) cTnT immunostaining of differentiated cells under serum-containing (FBS) and serum-free (Alb) conditions. Red indicates cTnT staining; blue indicates DAPI staining. Scale bar, 100 μm.

(F) Representative flow cytometry data for cTnT-positive cells induced by DMSO, KY, or KY+XAV in serum-free (Alb) medium. Red indicates cTnT-positive cardiomyocytes. Gray indicates negative controls without the primary antibody. A total of 30,000 cells were measured in each sample by FACSCanto II.

### Cardiac Differentiation in Serum-Containing Medium

Confluent hESCs and hiPSCs were enzymatically detached and transferred into Petri dishes (BD BioSciences) with ESC Culture Medium (ReproCELL, Japan). Cells were held in suspension culture for 8–24 hr to form aggregates (0.3–1 mm in diameter). Following suspension culture, cell aggregates were allowed to attach to culture dishes ( $3\text{--}10 \times 10^5$  cells/cm<sup>2</sup>) in cardiac differentiation medium: IMDM (Sigma-Aldrich) containing 20% FBS (GIBCO), 1% MEM nonessential amino acid solution (Sigma-Aldrich), 1% penicillin-streptomycin (GIBCO), 2 mM L-glutamine (Sigma-Aldrich), 0.001% 2-mercaptoethanol (GIBCO), and 0.005 N NaOH, with 10 ng/ml BMP4 (R&D Systems). The medium was changed to cardiac differentiation medium with KY02111 and/or other WNT inhibitors added on day 3 (hiPSC lines) or day 4 (human or monkey ESC lines): XAV939 (Wako, Japan); IWP-2 (Santa Cruz Biotechnology); IWR-1 (Merck4BioSciences); IGFBP4; Dkk1; and the mixture of bFGF, BMP4, VEGF, DKK1, and Activin A (Yang et al., 2008). The cytokines were obtained from R&D Systems. Medium was changed every 2–3 days; KY02111 and other WNT inhibitors were added until day 9 for hiPSC lines and the monkey ESC line or day 14 for hESC lines. On day 15, cardiac cell colonies on dishes were incubated with protease solution (0.1% collagenase type I, 0.25% trypsin, 1 U/ml DNase I, 116 mM NaCl, 20 mM HEPES, 12.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.6 mM glucose, 5.4 mM KCl, and 0.8 mM MgSO<sub>4</sub> [pH 7.35]) for 0.5–2 hr, until all cardiac colonies were detached. The detached cardiac colonies were transferred into 15 ml tubes with fresh cardiac differentiation medium and then the supernatant was removed by aspiration. The cardiac colonies were transferred into Ultra-low culture dishes (Corning) or 6-well plates (Corning) in cardiac differentiation medium without both serum and NaOH. The floating cardiac colonies were maintained for more than 1 month, and the media were changed every 5 days. A differentiation method of mouse ESC (R1 line) was carried out as previously described by Yuasa et al. (2005), and KY02111 was added during days 3–6. Beating colonies were counted on day 9.

### Cardiac Differentiation in Serum-free and Defined Medium

Human PSCs were precultured using serum-free cardiac differentiation medium (see below) without chemicals in Petri dishes as described above, and then cell aggregates were allowed to attach to culture dishes coated with gelatin (Sigma-Aldrich) or human laminin211 (BioLamina, Sweden) at  $3\text{--}10 \times 10^5$  cells/cm<sup>2</sup>, in serum-free cardiac differentiation medium: IMDM containing 1% MEM nonessential amino acid solution, 1% penicillin-streptomycin, 2 mM L-glutamine, 0.5 mM L-carnitine (Sigma-Aldrich), 0.001% 2-mercaptoethanol, and 1%–2% BSA (Wako) or 0.4% human serum albumin (Sigma-Aldrich), with 4  $\mu$ M CHIR (Axon) and 2  $\mu$ M BIO (Calbiochem). On days 3–9, 10  $\mu$ M KY02111 and/or other WNT inhibitors (XAV939 and/or IWP-2) were added to cell cultures, and the medium was changed every 2 days. On day 15, the cardiac colonies were incubated with the protease solution without collagenase for 5–10 min, until all colonies were detached. The cardiac colonies were transferred to Ultra-low culture 6-well plates with serum-free cardiac differentiation medium including 0.1% albumin. The floating cardiac colonies were maintained for more than 1 month, and the media were changed every 5 days.

### Immunostaining and Flow Cytometry

Cardiomyocytes derived from hiPSCs were fixed with 4% paraformaldehyde and then incubated with primary antibodies and secondary antibodies. Nuclei were visualized by DAPI. The number of immunopositive cells was counted from three random areas, using the MetaMorph imaging system. Approximately 1,000 DAPI-stained cells were used to calculate the proportion of immunopositive cells. For flow cytometry, cells treated with antibodies were analyzed using a FACSCanto II Flow Cytometer (BD Biosciences). Data were analyzed using FACSDiva software (BD Biosciences). See the [Extended](#)

[Experimental Procedures](#) for detailed procedures and antibodies used in this study.

### Microarray Experiments

IMR90-1 iPSCs were cultured in cardiac differentiation medium containing serum. On day 3, cells were treated with 10  $\mu$ M KY02111 or 0.1% DMSO for 0, 12, or 24 hr. Nontreated cells (0 hr), cells treated with KY02111 for 12 hr (KY12hr) or 24 hr (KY24hr), and cells treated with 0.1% DMSO for 12 hr (DMSO12hr) or 24 hr (DMSO24hr) were used for microarray analysis using the Human Gene 1.0 ST array (Affymetrix). For analysis of Distant Regulating Elements of co-regulated genes (DiRE analysis), all downregulated genes (based on the KY12hr/DMSO12hr ratio) were analyzed using the web-based program (<http://dire.dcode.org/>) (Gotea and Ovcharenko, 2008). See the [Extended Experimental Procedures](#) for detailed procedures.

### Quantitative RT-PCR

Total RNA (0.5  $\mu$ g) was subjected to cDNA synthesis using the SuperScript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed using SYBER Green PCR Master Mix on a 7500 Real-Time PCR System (Applied Biosystems). Total RNA from human adult heart tissue (BioChain) was used to evaluate the expression levels of cardiac marker genes for cardiomyocytes derived from hiPSCs. All values were normalized with respect to GAPDH expression level and expressed relative to the corresponding values in human adult hearts (100%). All of the primer sets are listed in [Table S2](#).

### TOPflash Assay

IMR90-1 hiPSCs were cultured in the serum-containing differentiation culture. HEK293 and SW480 cells were cultured in 10% FBS-DMEM and Leibovitz's L-15 medium (GIBCO), respectively. Cells were transfected with TOPflash plasmid (Millipore). After 24 hr, KY02111, XAV939, or IWP-2 was added to the medium, along with either 60 ng/ml of mouse Wnt3a (Wako) or 3  $\mu$ M BIO. Luciferase activities were measured 48 hr after transfection. See the [Extended Experimental Procedures](#) for detailed procedures.

### Patch-Clamp Recordings

Data from the whole-cell patch-clamp configuration were recorded from spontaneously beating cells, using a HEKA EPC10 amplifier (HEKA Instruments). In some experiments, cells were treated with 0.1  $\mu$ M E4031, 4  $\mu$ M Chromanol293B, 4  $\mu$ M zatebradine, 1  $\mu$ M TTX, or 4  $\mu$ M nifedipine for 5 min. The inhibitors were obtained from Sigma-Aldrich. See the [Extended Experimental Procedures](#) for detailed procedures.

### QT Interval Prolongation Assay

ECG-like waves of KY-CMs were recorded using MEA dishes and an MEA amplifier (Multi Channel Systems, Germany). Beating rate, Na<sup>+</sup> amplitude, K<sup>+</sup> amplitude, and Na<sup>+</sup>-K<sup>+</sup> interval were analyzed by LabChart software v7 (ADInstruments, Australia). See the [Extended Experimental Procedures](#) for detailed procedures.

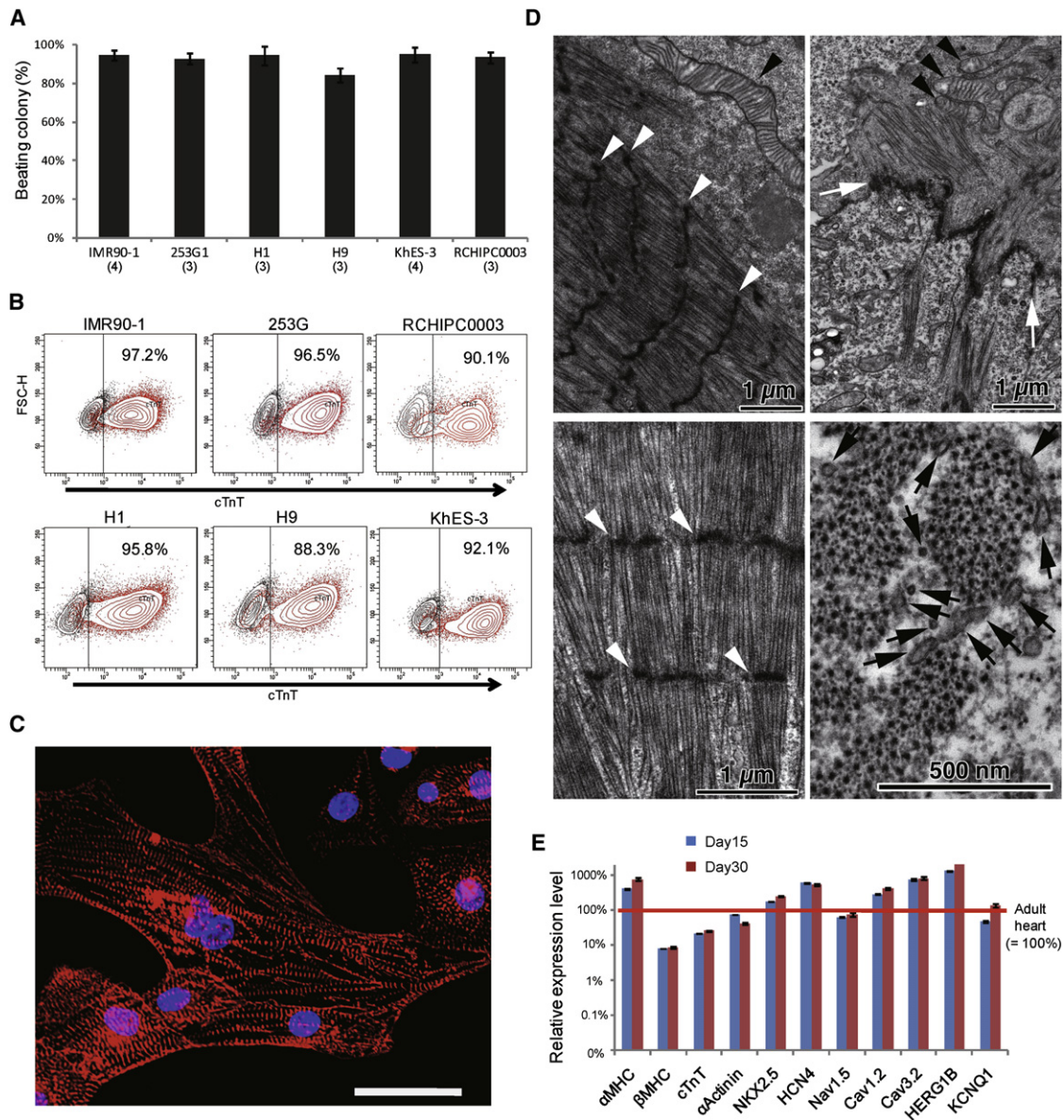
### Electron Microscopy

The hiPSC-derived cardiomyocytes were fixed and then embedded in Araldite 502 resin (Polysciences, Warrington, PA, USA). The ultrathin sections (~60 nm) were observed by conventional TEM (JEOL JEM1400, Japan). See the [Extended Experimental Procedures](#) for detailed procedures.

### Statistical Analysis

Each experiment was independently performed three or more times ( $n \geq 3$ ). All results were expressed as mean  $\pm$  SEM. Unpaired two-tailed Student's *t* tests were used to compare mean values of measurements from different treatments. Differences were considered significant at  $p < 0.05$ .

(G) The number of beating colonies and total number of cardiomyocytes in serum-free and defined culture conditions. On day 0 of differentiation,  $6 \times 10^6$  IMR90-1 cells per well were added to a 6-well plate. Red bars show cardiomyocyte numbers in each well, calculated by multiplying total cell numbers of all colonies by the ratio of cTnT-positive cells. Both the number of beating colonies and the total number of cardiomyocytes induced by KY or KY+XAV increased more in defined medium (Alb) than in serum-containing medium (FBS). Mean  $\pm$  SEM;  $n = 4$ . \* $p < 0.05$ , \*\* $p < 0.01$  for Student's *t* test. See also [Movie S1](#).



**Figure 6. Cardiac Differentiation of Several hPSC Lines in Cytokine-free, Serum-free, and Xeno-free Defined Condition**

(A) hESC lines (H1, H9, and KhES-3) and hiPSC lines (IMR90-1, RCHIPC0003, and 253G1) were highly efficiently differentiated into cardiomyocytes under xeno-free and cytokine-free condition as Figure 5A except XAV939 concentration (2  $\mu$ M instead of 10  $\mu$ M). Mean  $\pm$  SEM. n, the number in parentheses.

(B) Representative flow cytometry data of cTnT-positive cells generated from each hPSC line. Red indicates cTnT-positive cardiomyocytes. Gray indicates negative control without the primary antibody. A total of 30,000 cells were measured in each sample by FACSCanto II.

(C) The immunostaining of  $\alpha$ Actinin (red) in IMR90-1 hiPSC-derived cardiomyocytes. Images were deconvolved using the MetaMorph software. Scale bar, 30  $\mu$ m. DAPI is in blue.

(D) Transmission electron microscopic images of 253G1 hiPSC-derived cardiomyocytes. In parallel sections (left and upper-right images), mitochondria (black arrowheads), myofibrils, with Z bands (white arrowheads), and candidate structures of intercalated disks with desmosomes (white arrows) were observed. In the cross section (bottom right image), sarcoplasmic reticula around myofibrils represented by thin actin and thick myosin filaments were found (black arrows).

(E) Gene expression analysis of IMR90-1 hiPSC-derived cardiomyocytes. Total RNA was extracted from cells on days 15 and 30 of cardiac differentiation. The expression levels of cardiac marker genes ( $\alpha$ MHC,  $\alpha$ Actinin, and NKX2.5) and channel genes (HCN4, Nav1.5, Cav1.2, Cav3.2, HERG1b, and KCNQ1) were nearly equal to the gene expression levels of adult heart tissue, which was considered to be 100% (mean  $\pm$  SEM, n = 3).

See also Figure S6 and Movie S2.

## ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the microarray data reported in this paper is GSE33622.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, two tables, and two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2012.09.015>.

## LICENSING INFORMATION

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