Chemically Defined Differentiation

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Stepwise Chemically Induced Cardiomyocyte Specification of Human Embryonic Stem Cells**

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Because human embryonic stem cells (hESCs) can differentiate into all cardiac lineages, including atrial, ventricular and pacemaker cells,[1a–c] they are a potential source of cardiomyocytes for cell-based therapy, as well as a useful tool for studies of early embryonic cardiac development. Previous studies generated cardiomyocytes from hESCs by first forming three-dimensional embryoid bodies which were then treated with a combinantion of Wnt, Activin, and BMP proteins in an attempt to recapitulate the key events that regulate cardiogensis.[1a,c,2a–c] However, the efficiency of these methods is poor and the signaling pathway(s) involved in the specification of cardiomyocytes from hESCs is not well defined. Cell permeable small molecules that modulate signal transduction pathways or gene expression have been used to selectively modulate ESC self-renewal or differentiation under defined monolayer cell culture conditions.[3] We therefore attempted to identify molecules that selectively and efficiently induce the differentiation of hESCs to cardiomyocytes in a stepwise process by first generating primitive streak cells and then differentiating them into cardiomyocytes.

Previous reports have shown that activation of the canonical Wnt signaling pathway promotes the generation of primitive streak cells from both mouse and human ESCs.[4a,b] Based on this notion, we sought to establish chemically defined culture conditions for primitive streak formation using small-molecule activators of Wnt signaling. To this end, H9 and H1 human ES cell lines were cultured in serum-free media without mitogenic factors or feeder cells (SFM; DMEM/F12 1X N2/B27) and treated with various concentrations of CHIR99021 (Figure 1a), a selective inhibitor of glycogen synthase kinase 3β (GSK3β) which activates the canonical Wnt signaling pathway.[5] After 2 days, cells were immunostained for Brachyury (T), a marker for primitive streak,[6] and analyzed with an Opera high-content confocal imaging system. Treatment of hESCs with 10 μM CHIR99021 led to greater than 90% Brachyury-positive cells (Figure 1b–f). To further characterize the population of Brachyury-positive cells derived from hESCs treated with CHIR99021, we performed microarray analysis 48 h after compound treatment. Primitive streak cell associated genes

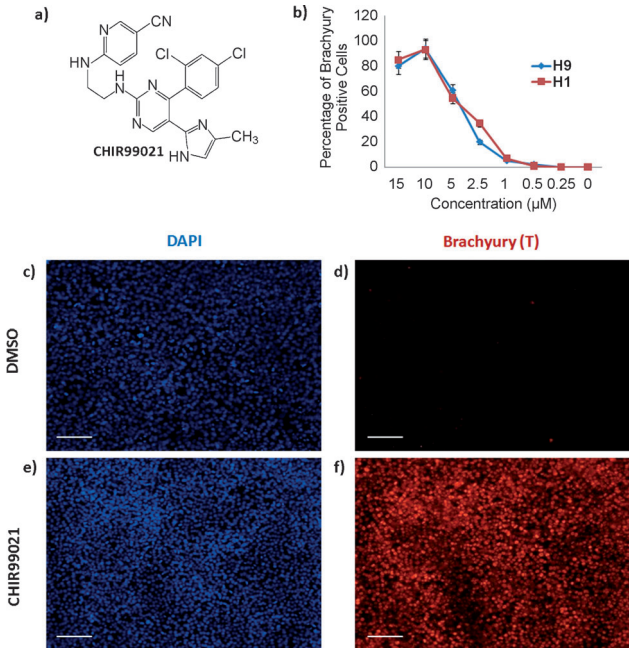

Figure 1. a) Structure of CHIR99021. b) HESC-H9 and H1 cells respond dose-dependently to CHIR99021 treatement. 48 h after treatment, the cells were fixed and immunostained with an anti-Brachyury antibody and analyzed with an Opera high-content confocal imaging system to quantify the percentage of Brachyury-positive cells. c–f) Immunofluorescent staining of H9 cells treated with 10 μM CHIR99021 or DMSO (vehicle control, 0.1%) for 48 h. After 48 h of treatment, the cells were fixed, stained, and analyzed as above (red). Cell nuclei were stained with DAPI (blue). Values are the mean ± s.d. for three measurements. (Scale bar, 100 μm.)

such as Brachyury (T), eomesodermin homolog (EOMES), Mix1 homeobox-like 1(MIXL), and mesoderm posterior 1 homolog (MESP-1) were upregulated greater than 4-fold (Figure 2a).[6,7a–c]

We also analyzed the expression of various primitive streak markers in CHIR99021-treated H9 cells using immunofluorescence and fluorescence-activated cell sorting (FACS). FACS analysis indicated that ≥90% of the 10 μM CHIR99021-treated H9 cells expressed apelin receptor (APLNR), platelet-derived growth factor receptor alpha (PDGFRα), fibronectin leucine rich transmembrane protein 3 (FLRT3), EPH receptor A4 (EPHA4), cadherin 11, type 2, OB-cadherin (CDH11) and the adhesion molecule with Ig-like domain 2 (AMIGO2) (Figure 2b). Immunofluorescent analysis indicated that H9 or H1 cells treated with CHIR99021 (10 μM) for 48 h were >90% positive for T, MIXL1, MESP-1, EOMES and SP5 (Figure 2g–j). Western

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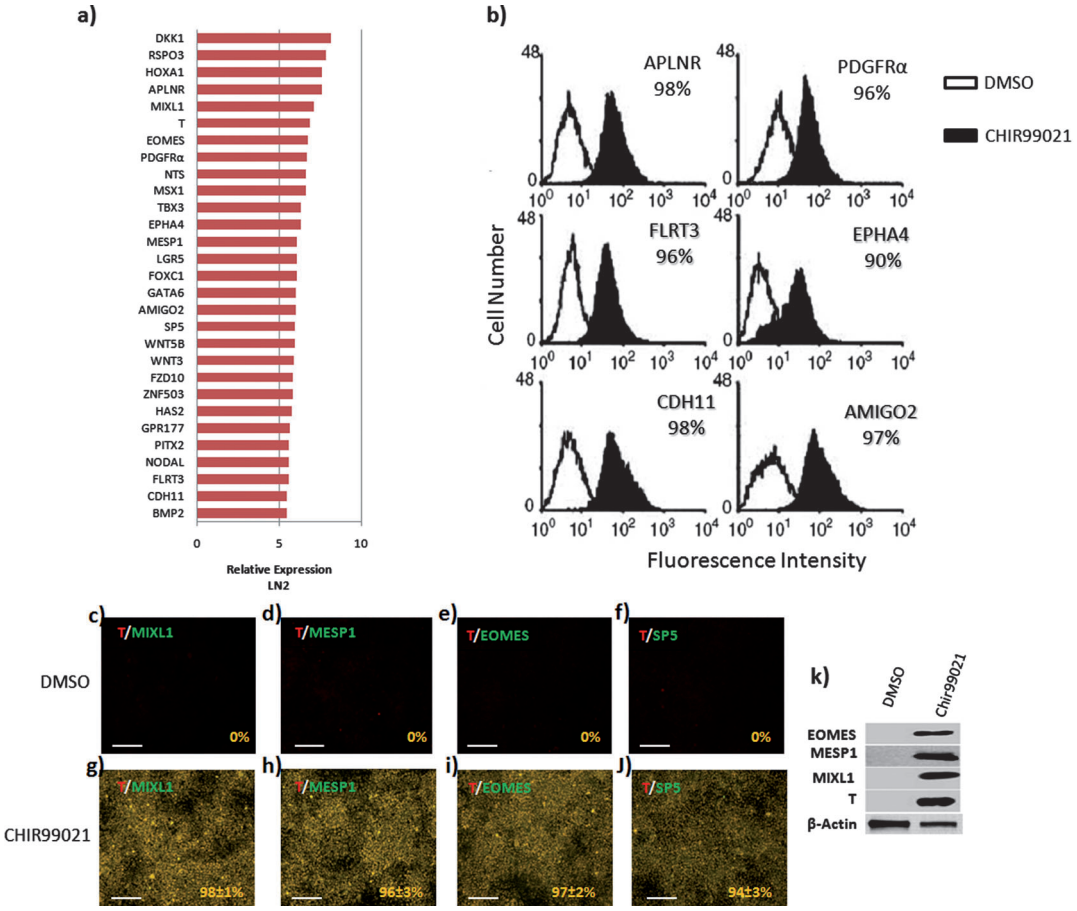

Figure 2. hESC-H9 cells were treated with 10 μM CHIR99021 or DMSO (vehicle control, 0.1%) in SFM medium for 48 h. a) The top 30 CHIR99021 upregulated mRNAs in hESCs after 48 h treatment relative to vehicle control treated cells as determined by microarray analysis. b) FACS analysis of apelin receptor (APLNR), platelet-derived growth factor receptor, alpha (PDGFRα), fibronectin leucine rich transmembrane protein 3 (FLRT3), EPH receptor A4 (EPHA4), cadherin 11, type 2, OB-cadherin (CDH11), and adhesion molecule with Ig-like domain 2 (AMIGO2) cell surface protein expression. c–f) Immunofluorescent staining of hESC-H1 cells that were grown in SFM medium and treated with 0.1% DMSO (c–f) or 10 μM CHIR99021 (g–j). After 48 h, cells were fixed and stained with anti-T, MIXL, EOMES, SP5 and MESP1 antibodies. Fluorescent images were analyzed with an Opera high-content confocal imaging system to quantitate the percentages of double positive cells (yellow). Values are the mean ±s.d. for three measurements. (Scale bar, 100 μm.) k) Western blot analysis of T, MIXL, EOMES, and MESP1 2 days after hESCs were treated with 0.1% DMSO or 10 μM CHIR99021 in SFM medium.

analysis also showed that 10 μM CHIR99021 and not 0.1% DMSO (dimethylsulfoxide; vehicle control) induced T, MIXL1, MESP-1 and EOMES expression in hESC-H9 cells that were treated in SFM medium for 48 h (Figure 2k). Collectively, these results demonstrate that the activation of Wnt signaling promotes the generation of primitve streak cells from hESCs. Furthermore, the application of CHIR99021 during a defined temporal window gives rise to

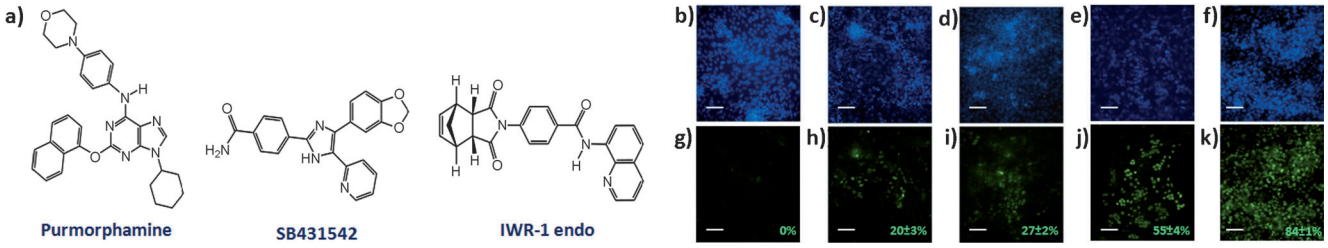

Figure 3. a) Structure of the three small molecules that efficiently induce Nkx2.5 expression from hESCs-derived primitive streak cells. b–k) Immunofluorescent images of hESC-H9-derived primitive streak cells that were treated with either 0.1% DMSO (b,g), SB431542(5 μM) (c,h), Purmorphamine (5 μM) (d,i), IWR-1endo (5 μM) (e,j), or a chemical cocktail consisting of IWR-1endo (5 μM), purmorphamine (5 μM), and SB431542 (5 μM) (f,k) in CDM. Fluorescent images were then analyzed with an Opera high-content confocal imaging system to quantitate the percentages of Nkx2.5-positive cells (green). Values are the mean ±s.d. for three measurements. (Scale bar, 100 μm.)

a highly homogenous population of primitive streak cells with >90% efficiency that can be used in downstream differentiation protocols.

In an effort to establish chemically defined conditions to further differentiate hESCs into cardiomyocytes, we used CHIR99021-treated hESCs to screen a kinase and phosphatase inhibitor collection for inducers of the expression of Nkx2.5, a transcription factor expressed in cardiomyocytes downstream of several cardiomyogenic transcriptional factors.[8] H9-hESCs were first treated with 10 μM CHIR99021 in SFM for 48 h to generate primitive streak cells; medium was then replaced with a chemically defined medium (CDM; RPMI + 1X B27) and the primitive streak cells were treated with a in-house collection of 300 known signal transduction modulators at a final concentration of 5 μM for 7 days. Treated cells were grown for an additional 21 days in CDM without compound, with media changes every two days and then stained with Nkx2.5 antibodies and analyzed. We found that IWR-1endo (Figure 3a), a reported Wnt antagonist, yielded the highest percentage (≥50%) of Nkx2.5-positive cells (Figure 3f,k). Additionally, lower percentages (≤20–30%) of Nkx2.5-positive cells were observed in wells treated with purmorphamine, a Sonic Hedgehog (Shh) signaling agonist (Figure 3d,i), and SB431542, a TGFβ/Nodal receptor kinase antagonist (Figure 3c,h). Wnt, Nodal, and Shh signaling are all known to participate in metazoan cardiogenesis.[9a–d] For example, the protein Dkk-1, an inhibitor of the Wnt pathway, induces the formation of ectopic heart tissue in vertebrates.[9a,b] Similarly, both cerberus, an antagonist of Wnt, BMP, and nodal signaling,[10a,b] and truncated Cer-S protein, which has nodal inhibitory activity,[10b] can initiate ectopic cardiogenesis in *Xenopus* embryos.[10b] In addition, it has been previously reported that the Hedgehog signaling pathway plays an import role in cardiovascular progenitor specification.[9d] Thus the mechanism of action of the ES cell differentiation-inducing small-molecule inhibitors identified herein appears to recapitulate the activity of these signaling pathways observed in organismal development.

We next determined if a combination of IWR-1, purmorphamine and/or SB431542 would yield a higher percentage of Nkx2.5-positive cells than individual compound treatment. Indeed, we found that a chemical cocktail consisting of IWR-1 (5 μM), SB431542 (5 μM) and purmorphamine (5 μM) (ISP) produced the highest percentage (70–80%) of Nkx2.5-positive cells from hESC-derived primitive streak cells 28 days after initial treatment (25 days post ISP treatment) (Figure 3g,l). RT-PCR and Western blot analyses indicated that ISP-treated primitive streak cells expressed several cardiac lineage-associated genes, including Nkx2.5, alpha-myosin heavy chain (α-MHC), α-actinin, and cardiac troponin T (cTNT) (Figure 4a,h). Immunofluorescent staining followed by Opera high-content confocal image analysis revealed that ≥80% of the cells were positive for Nkx2.5,

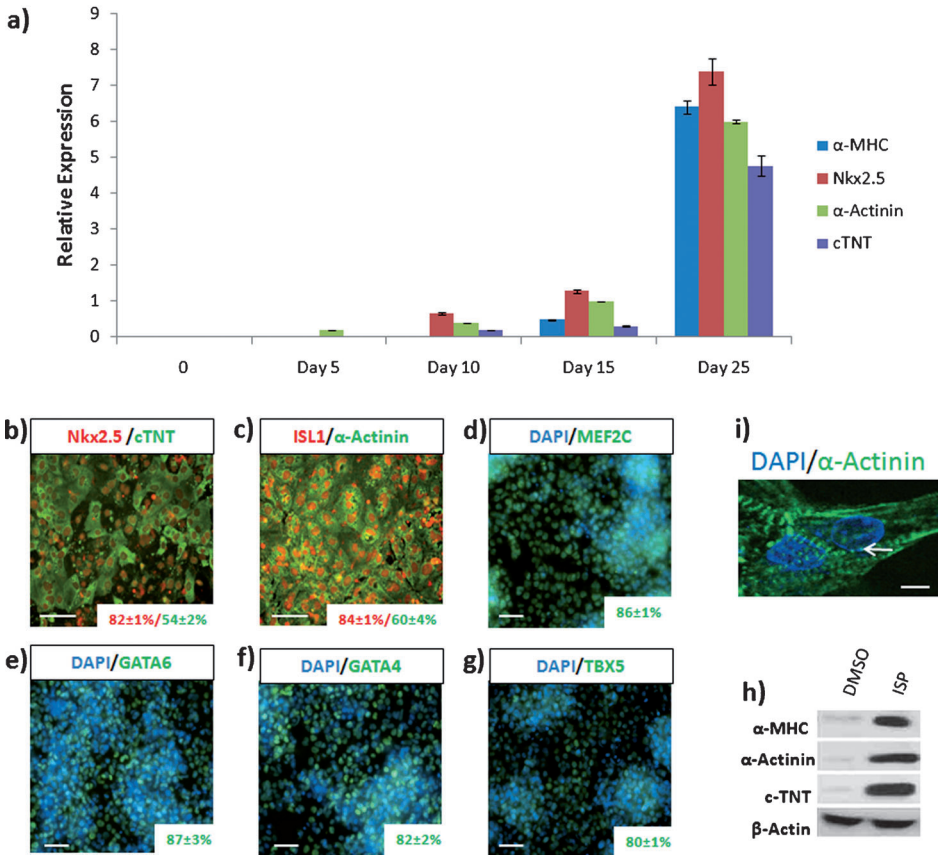

Figure 4. a) hESC-derived primitive streak cells were treated with a chemical cocktail containg IWR-1endo (5 μM), purmorphamine (5 μM), and SB431542 (5 μM) (ISP) in CDM medium (RPMI + 1X B27) for 25 days and then total RNA was isolated and used for RT-PCR analysis for cardiac lineage associated α-myosin heavy chain (α-MHC), Nkx2.5, cardiac α-actinin and cardiac troponin (cTNT) gene expression. b–g) Immunofluorescent images of hESC-H9-derived primitive streak cells that were treated with a chemical cocktail containing ISP in CDM medium for 25 days and then stained using antibodies against cardiac cell lineage associated proteins: b) Nkx2.5 and cTNT; c) ISL1 and cardiac α-actinin; d) MEF2C; e) GATA6; f) GATA4; g) TBX5 antibodies, and then analyzed with an Opera high-content confocal imaging system to determine the percentages of positive cells. Values are the mean ± s.d. for three measurements. Nuclei were stained with DAPI (blue). (Scale bar, 100 μm.) i) High-magnification immunofluorescent image of hESC-H9-derived primitive streak cells that were treated with a chemical cocktail containing ISP in CDM medium for 25 days and then stained using an antibody against cardiac α-actinin (green). White arrow: Z-bands with regular striated pattern that is consistent with labeling of the cardiac sarcomere. Nuclei were stain with DAPI (blue). (Scale bar, 4 μm.) h) Western blot analysis of cardiac cell lineage associated α-MHC, α-actinin, and cTNT protein expression 25 days after hESC-derived primitive streak cells were treated with 0.1% DMSO or 10 μM CHIR99021 in SFM medium.

GATA4, GATA6, ISL-1, and TBX5 (Figure 4b–i). Compared to a previous "standard" embryoid body-based system, which uses serum to induce differentiation (typically yielding $<10\%$ cTNT-positive cardiomyocytes),[2b] the ISP monolayer chemically defined protocol consistently yielded $\geq 50\%$ cTNT and α-Actinin positive cardiomyocytes (Figure 4b,f) and produced cardiac clusters that beat rhythmically (see movie in the Supporting Information). Collectively, these results indicate that hESC-derived primitive streak cells can be efficiently specified into the cardiomyocyte lineage using the ISP chemical cocktail.

In summary, we have developed a stepwise strategy to identify a chemically defined method to generate cardiomyocytes from hESCs in monolayer culture with good efficiency and homogeneity. From our screens, we identified IWR-1endo, a Wnt antagonist, purmorphamine, a Shh signaling agonist, and SB431542, a TGFβ/nodal receptor kinase antagonist, as small molecules that promote cardiomyocyte differentiation from hESC-derived primitive streak cells. It will be essential to explore the interactions between these various pathways to further optimize cardiac differentiation from hESCs. This will greatly enhance our ability to produce cardiomyocytes for future studies and possibly cardiac repair.

Experimental Section

HESC cell culture and plating into 96-well plates. hESC lines H9 (WA-09) and H1 (WA-01) were used for this study. hESCs were cultured on mitotically inactivated MEFs as described previously[11] followed by feeder-free growth on Matrigel in MEF-CM[12] prior to 96-well plating. For plating, cells were harvested following Accutase dissociation for 20 min at 37 °C. At this stage, single-cell suspensions could be obtained without further mechanical dissociation, and dissociated cells displayed high levels of viability (>95% based on trypan exclusion). hESCs were plated at 16000 cells per well in SFM medium (Dulbecco's modified Eagle's medium/F-12 + 1X N2/B27 Supplement [Invitrogen]). Medium was replaced every two days.

Immunocytochemistry. Cultures were fixed with 4% paraformaldehyde and blocked in 1 × PBS containing 0.2% Triton X-100 and 2% BSA (bovine serum albumin). The cells were incubated with the primary antibody in 0.1% Triton X-100 in PBS (phosphate-buffered saline) at 4 °C overnight. After washing with 1 × PBS containing 0.1% Triton X-100, secondary antibody labeled with Alexa 488 or 594 (Invitrogen) was then added and incubated at room temperature for 45 min. After staining with DAPI (4′,6-diamidino-2-phenylindole), cells were visualized with a fluorescence microscope. The following primary antibodies were used for the immunofluorescence analysis: POU51/OCT4, Nkx2.5, SOX2, GATA4, GATA6 (Santa Cruz Biotechnology), mouse anti-a-actinin (Sigma–Aldrich), cTNT, MEF2C, ISL1, and TBX5 (Abcam). AlexaFluor 488-, 546-, 555-, or 647-conjugated secondary antibodies were purchased from Invitrogen. For the quantification of Nkx2.5, cTNT, GATA4, GATA6, MEF2C, α-actinin, cTNT, and TBX5 expressing cells, at least ten images for each sample were taken using an Opera high-content confocal imaging system. Quantification for each treatment was done for three independent experiments. Data represent mean values ± s.d.

FACS analysis. Cells were dissociated using 0.05% trypsin/0.5 mM EDTA (ethylendiaminetetraacetic acid; Invitrogen) at 37 °C for 3 min followed by neutralization in DMEM/F12 with 10% fetal bovine serum (FBS). After washing three times in Staining Buffer (BD Biosciences) 1.25×10^5 cells were aliquoted for each antibody staining. Cells were resuspended in 200 μL of the same buffer and first Fc-blocked by treatment with 50 μL human serum supplement (Irvine Scientific) for 15 min on ice. 1.25×10^5 pelleted cells were fixed in 100 μL of 4% paraformaldehyde (BD Biosciences) PBS solution at 4 °C for 15 min. Cells were washed three times in Staining Buffer (BD Biosciences). The Fc-blocked cells were then incubated with primary antibodies: apelin receptor (APLNR), platelet-derived growth factor receptor alpha (PDGFRα), fibronectin leucine rich transmembrane protein 3 (FLRT3), EPH receptor A4 (EPHA4), cadherin 11, type 2, OB-cadherin (CDH11), and the adhesion molecule with Ig-like domain 2 (AMIGO2) (R&D Systems) for 30 min on ice. After incubation with the primary antibodies, cells were washed three times in Staining Buffer (BD Biosciences) and then incubated with PE-labeled secondary antibody (Invitrogen) for 30 min on ice.

Quantitative RT-PCR analysis. Total RNA was extracted using the RNeasy Micro Kit (QIAGEN, Valencia, VA, USA) and reverse-transcribed using the Omniscript RT Kit (QIAGEN) according to the manufacturer's instructions. Semi-quantitative RT-PCR was carried out using Ex-Taq Polymerase (Takara Biotechnology, Tokyo, Japan). Quantitative RT-PCR was carried out using SYBR Green RT-PCR reagents (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocols. The relative amount of each gene was normalized against GAPDH using the comparative threshold cycle (CT) method. The data represent mean values ± s.d. from three independent experiments. The expression level of each gene in (0.1%) DMSO treated cells was defined as 1.0.

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