Supporting Information

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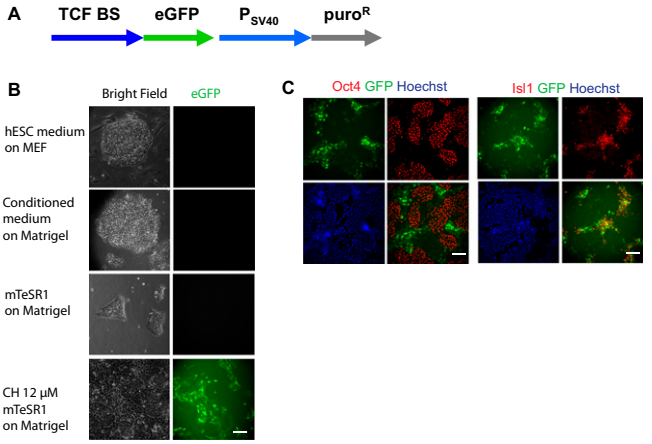


Fig. S1. Activation of Wnt/β-catenin signaling by glycogen synthase kinase 3 (Gsk3) inhibitors treatment induces differentiation in hPSCs. (*A*) Schematic of the 7TGP lentiviral promoter-reporter construct. "TCF BS" represents seven repeats of T-cell factor/lymphoid enhancer-binding factor consensus promoter binding sites. (*B*) H9 7TGP cells were cultured in hESC medium on MEF feeders or in conditioned medium, mTeSR1, or mTeSR1 plus 12 μM CHIR99021 (CH) on Matrigel. GFP expression was detected by immunofluorescent microscopy. (Scale bar, 50 μm.) (*C*) 19-9-11 7TGP cells were cultured in mTeSR1 plus 12 μM CH on Matrigel. GFP localization and immunostaining of Oct4 and Isl1 was performed 3 d after the addition of CH. (Scale bars, 50 μm.)

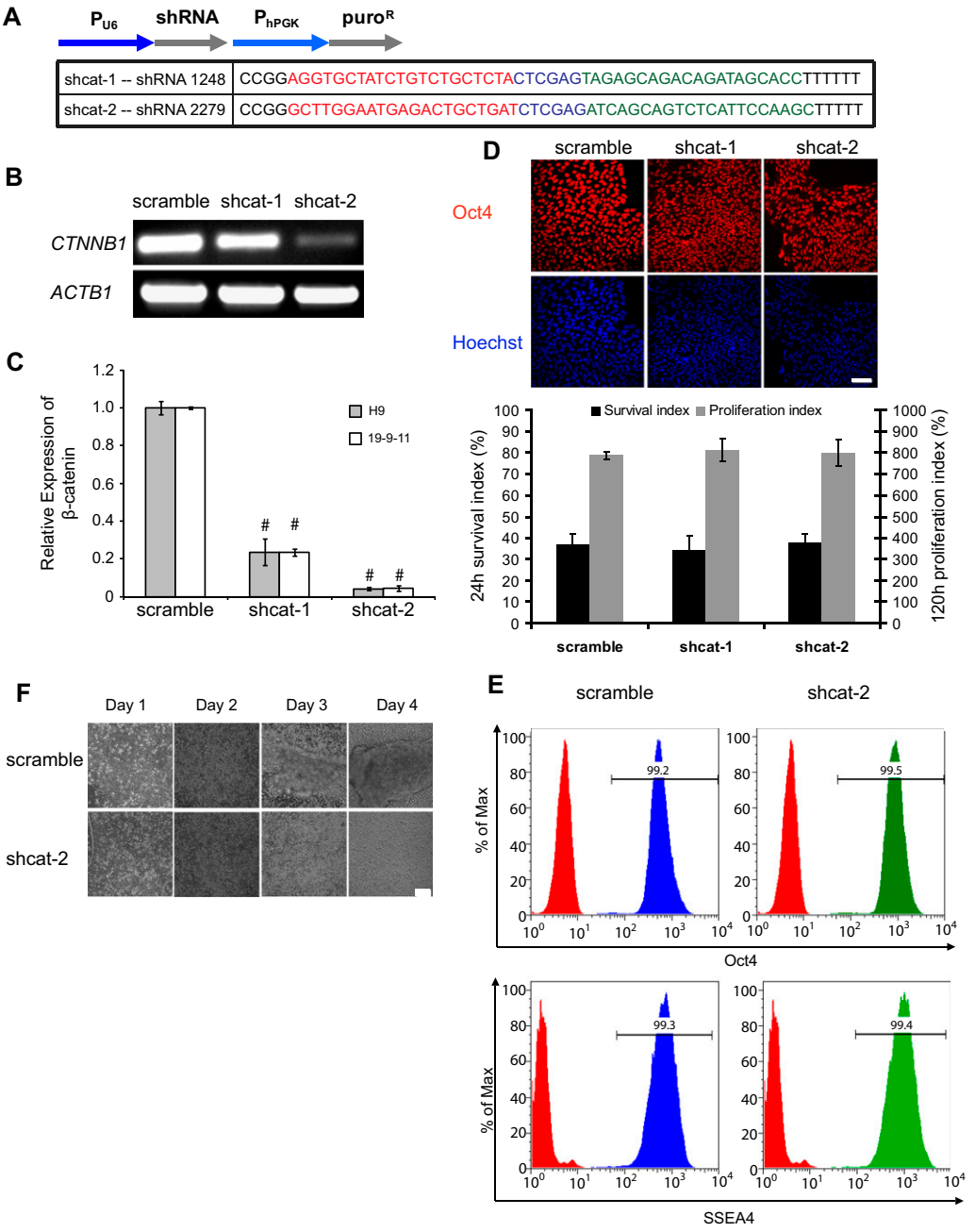


Fig. S2. Differentiation induced by treatment with Gsk3 inhibitors in hPSCs is β-catenin dependent. (*A*) Schematic of construct for constitutive knockdown of β-catenin expression and shRNA sequences targeting β-catenin. P_{U6} and P_{hPGK} are human U6 and human phosphoglycerate kinase-1 promoters, respectively. Red and green sequences are forward and reverse shRNA sequences of β-catenin, respectively; the blue sequence represents the loop sequence. (*B*) H9 β-catenin knockdown and scramble cell lines were cultured in mTeSR1, and expression of β-catenin was analyzed by RT-PCR. (*C*) Quantitative RT-PCR gene-expression analysis of β-catenin and β-actin for scramble and β-catenin knockdown lines in H9 and 19-9-11 cells. $^{\#}P < 0.005$, shcat-1 versus scramble and shcat-2 versus scramble (Student's *t* test). (*D*) (*Upper*) H9 scramble, shcat-1, and shcat-2 cells were cultured in mTeSR1 on Matrigel for 3 d and immunostained for Oct4. (Scale bar, 50 μm.) (*Lower*) Three hundred thousand H9 scramble, shcat-1, and shcat-2 cells were seeded in one well of a six-well plate coated with Matrigel, cultured with mTeSR1 medium, and counted 120 h later. The survival index and proliferation index are defined as the cell numbers at a 24 h and 120 h, respectively, normalized to the number of input cells. (*E*) 19-9-11 shcat-2 and scramble lines were cultured in mTeSR1 on Matrigel for 3 d, and flow cytometry for Oct4 and SSEA4 was performed. The blue (scramble) and green (shcat-2) histograms represent Oct4 or SSEA4 expression, and the red histogram is an isotype control. (*F*) 19-9-11 shcat-2 and scramble cells were cultured on Matrigel in mTeSR1 containing 12 μM CH for 4 d, and cell morphology was assessed by phase-contrast imaging. (Scale bar, 50 μm.)

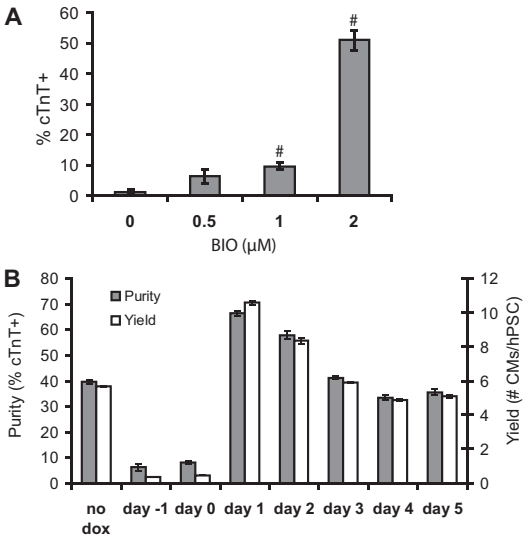


Fig. S3. Temporal regulation of Wnt/β-catenin signaling synergistically enhances cardiac differentiation with other signaling growth factors. (*A*) IMR90C4 cells were cultured in mTeSR1 containing different concentrations of 6-bromoindirubin-3′-oxime (BIO). Differentiation was induced by 100 ng/mL activin A at day 0 and 5 ng/mL bone morphogenic factor 4 (BMP4) at day 1 in RPMI/B27-insulin medium. Fifteen days after addition of activin A, the percentage of cells expressing cardiac troponin T (cTnT) was quantified by flow cytometry. $^{\#}P < 0.005$, each point versus no BIO (Student's *t* test). (*B*) 19-9-11 ishcat-2 cells were cultured in mTeSR1 and treated with BIO before being exposed to 100 ng/mL activin A at day 0 and 5 ng/mL BMP4 at day 1, with the addition of 2 μg/mL doxycycline (dox) at the indicated times. Fifteen days after the initiation of differentiation, cells were counted and analyzed for cTnT expression by flow cytometry. Error bars represent SEM of three independent experiments. $^{\#}P < 0.005$, each time point versus no dox (Student's *t* test).

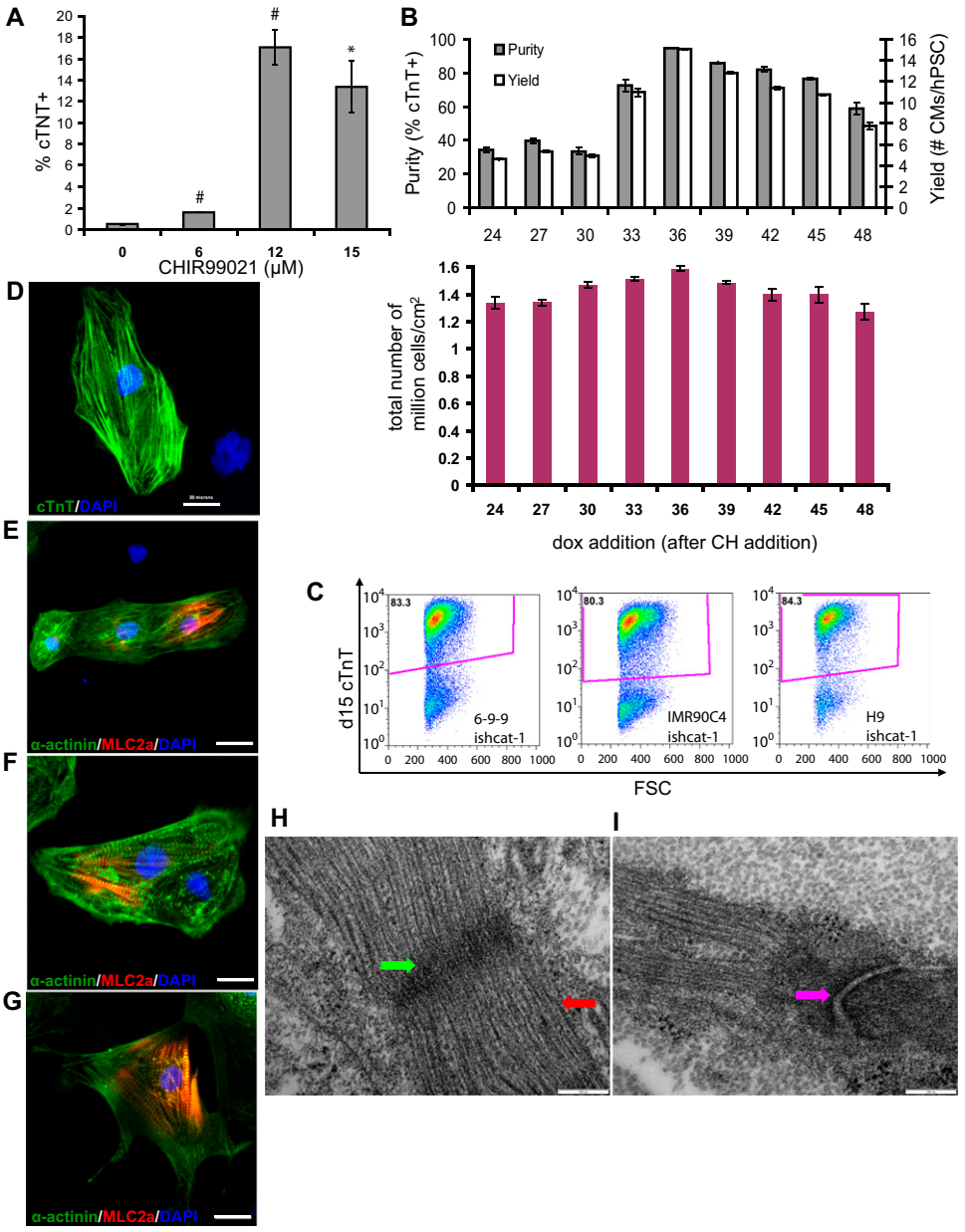


Fig. S4. Modulation of regulatory elements in Wnt signaling is sufficient to induce robust cardiac differentiation in hPSCs. (*A*) 19-9-11 ishcat-1 cells were treated with different concentrations of CH in RPMI/B27-insulin for 24 h, and then the medium was changed to RPMI/B27-insulin at day 1. Starting from day 7, cells were cultured in RPMI/B27. Flow cytometry of cTnT was performed at day 15 after the addition of CH. Error bars represent the SEM of three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.005$, each point versus no CH (Student's *t* test). (*B*) 19-9-11 ishcat-1 cells were cultured in mTeSR1 before exposure to 12 μM CH in RPMI/B27-insulin for 24 h. Dox (2 μg/mL) was added at different time points following CH addition. Cell counting and flow cytometry of cTnT were performed at day 15 after the addition of CH. Error bars represent the SEM of three independent experiments. (*C*) Three additional hPSC lines (IMR90C4, 6–9-9, and H9) transduced with inducible β-catenin shRNA construct ishcat-1 cells were cultured in mTeSR1 and treated with 12 μM CH followed by the addition of 2 μg/mL dox 36 h later. Flow cytometry of cells expressing cTnT was performed 15 d following CH addition. (*D*–*G*) Immunostaining of day 30 cardiomyocytes generated from (*D*) 19-9-11 ishcat-1, (*E*) IMR90C4 ishcat-1, (*F*) 6–9-9 ishcat-1, and (*G*) H9 ishcat-1 cells cultured in mTeSR1 and treated with the addition of 12 μM CH and 2 μg/mL dox 36 h later. Cells were immunostained for cTnT, α-actinin, and MLC2a to show sarcomere structure. (Scale bars, 20 μm.) (*H* and *I*) Transmission electron microscopic images of beating clusters derived from 19-9-11 ishcat-1 cells following culture in mTeSR1 and treated with the addition of 12 μM CH and 2 μg/mL dox 36 h later. (*H*) Myofibrils (red arrow) with Z-bands (green arrow). (*I*) Intercalated disks with desmosomes (pink arrowhead). (Scale bars, 200 nm.)

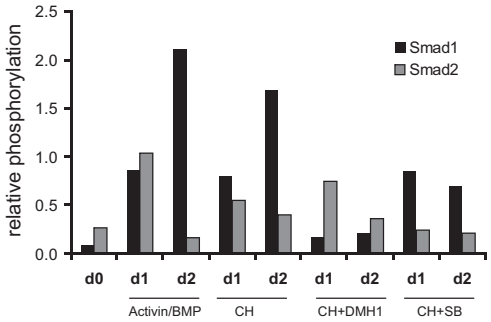


Fig. S5. Induction of TGF-β superfamily signaling by Gsk3 inhibitors. 19-9-11 ishcat-1 cells cultured in mTeSR1 were treated with 12 μM CH, 12 μM CH and 0.5–4 μM SB431542, or 12 μM CH and 0.2–1 μM DMH1 for 24 h in RPMI/B27-insulin. Expression and phosphorylation of Smad proteins were assessed by Western blotting at different time points following CH treatment. The plot shows densitometry measurements of pSmad1/5 protein bands relative to total Smad1 and pSmad2 protein bands relative to total Smad2.

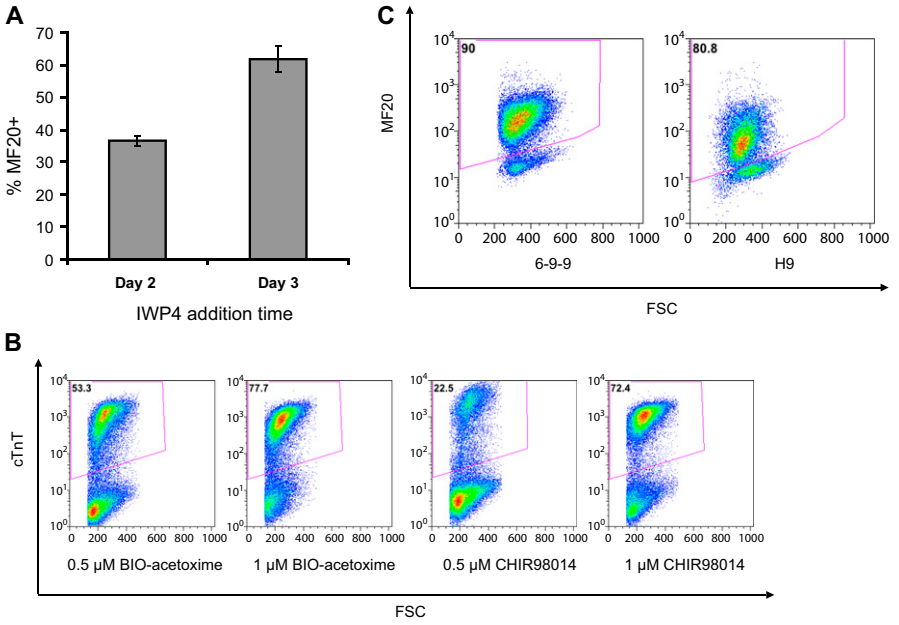


Fig. S6. Differentiation of hPSCs to cardiomyocytes in fully defined conditions with small molecules. (*A*) 19-9-11 cells were cultured in mTeSR1 on Matrigel and treated with 12 μM CH followed by the addition of 1 μM Inducer of Wnt production (IWP4) 2 or 3 d later. Flow cytometry for MF20 was performed at day 15. Error bars represent the SEM of three independent experiments. (*B*) 19-9-11 cells were cultured in mTeSR1 on Matrigel for 5 d before exposure to indicated concentrations of CH98014 or BIO-acetoxime (Tocris) at day 0 for 24 h, and IWP4 added at day 3, in RPMI/B27-insulin. At day 15, cTnT expression was assessed by flow cytometry. (*C*) 6–9-9 and H9 cells were cultured in mTeSR1 on Synthemax surface (Corning) and treated with 12 μM CH followed by the addition of 5 μM IWP4 3 d later. Flow cytometry for MF20 versus forward scatter (FSC) was performed at day 15.

Table S1. Percent of cTnT$^+$ cardiomyocytes present at day 15 after differentiation via embryoid body (EB) generation, directed differentiation using a Gsk3 inhibitor and shRNA knockdown of β-catenin, or directed differentiation using a Gsk3 inhibitor and inhibitors of Wnt production (IWPs)

Cell line	EB methods (%)	Gsk3 inhibitor + shRNA (%)	Gsk3 inhibitor + IWPs (%)
H9	2.56 ± 0.97	85.03 ± 2.87	82.70 ± 2.33
H13	ND	ND	85.60 ± 1.78
H14	ND	ND	85.57 ± 4.17
19-9-11	0.019 ± 0.019	97.53 ± 0.50	95.20 ± 1.06
6-9-9	0.021 ± 0.01	86.30 ± 2.61	89.50 ± 2.10
IMR90C4	0.74 ± 0.29	83.83 ± 3.12	91.03 ± 3.45

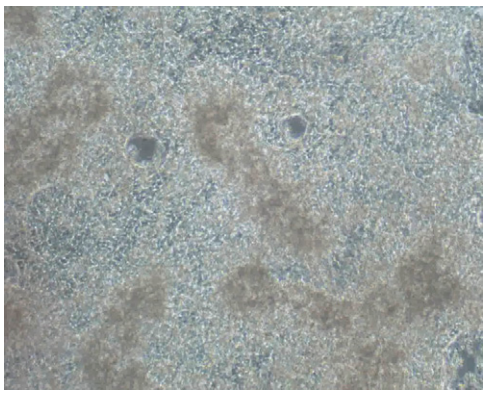
Data are presented as mean ± SD of three independent experiments. ND, not determined.

Table S2. Primers for RT-PCR and quantitative PCR

Genes	Sequences (5′–3′)	Size (bp)/ temperature m °C/no. of cycles
Primers for RT-PCR		
OCT4	F: CAGTGCCCGAAACCCACAC	161/58/30
	R: GGAGACCCAGCAGCCTCAAA	
NANOG	F: CGAAGAATAGCAATGGTGTGACG	328/58/30
	R: TTCCAAAGCAGCCTCCAAGTC	
SOX2	F: CAAGATGCACAACTCGGAGA	300/58/30
	R: GTTCATGTGCGCGTAACTGT	
CTNNB1	F: GAATGAGACTGCTGATCTTGGAC	250/58/30
	R: CTGATTGCTGTCACCTGGAG	
GSC	F: CGAGGAGAAAGTGGAGGTCTGG	261/55/35
	R: GCAGCGCGTGTGCAAGAAA	
MIXL1	F: CAGAGTGGGAAATCCTTCCA	231/58/35
	R: TGAGTCCAGCTTTGAACCAA	
T	F: CTTCCCTGAGACCCAGTTCA	289/58/35
	R: CAGGGTTGGGTACCTGTCAC	
MSX1	F: CCGAGAGGACCCCGTGGATGC	280/58/35
	R: GCCTCTTGTAGTCTCTTTGCC	
ISL1	F: CACAAGCGTCTCGGGATT	202/58/40
	R: AGTGGCAAGTCTTCCGACA	
WNT3A	F: GCCCCACTCGGATACTTC T	189/58/40
	R: GGCATGATCTCCACGTAGT	
WNT8A	F: ACAGGTCCCAAGGCCTATCT	335/58/40
	R: ATCCTTTCCCCAAATTCCAC	
NKX2-5	F: GCGATTATGCAGCGTGCAATGAGT	220/58/35
	R: AACATAAATACGGGTGGGTGCGTG	
GATA4	F: TCCAAACCAGAAAACGGAAG	352/58/40
	R: AAGACCAGGCTGTTCCAAGA	
MEF2C	F: AGCCCTGAGTCTGAGGACAA	195/58/40
	R: GTGAGCCAGTGGCAATAGGT	
TBX5	F: GAAACCCAGCATAGGAGCTG	191/58/40
	R: CAGCCTCACATCTTACCCTGT	
TBX2	F: AGTGGATGGCTAAGCCTGTG	249/58/40
	R: ACGGGTTGTTGTCGATCTTC	
TNNI3	F: CTGCAGATTGCAAAGCAAGA	379/58/40
	R: CCTCCTTCTTCACCTGCTTG	
TNNT2	F: TTCACCAAAGATCTGCTCCTCGCT	165/58/40
	R: TTATTACTGGTGTGGAGTGGGTGTGG	
MYL7	F: GAGGAGAATGGCCAGCAGGAA	449/58/35
	R: GCGAACATCTGCTCCACCTCA	
MYL2	F: ACATCATCACCCACGGAGAAGAGA	164/58/40
	R: ATTGGAACATGGCCTCTGGATGGA	
PLN	F: ACAGCTGCCAAGGCTACCTA	191/58/40
	R: GCTTTTGACGTGCTTGTTGA	
CD31	F: GCTGACCCTTCTGCTCTGTT	238/55/35
	R: TGAGAGGTGGTGCTGACATC	
NODAL	F: CTTCCTGAGCCAACAAGAGG	197/58/40
	R: AGGTGACCTGGGACAAAGTG	
BMP2	F: TCAAGCCAAACACAAACAGC	197/58/40
	R: ACGTCTGAACAATGGCATGA	
BMP4	F: TGAGCCTTTCCAGCAAGTTT	180/58/40
	R: CTTCCCCGTCTCAGGTATCA	
NOGGIN	F: TCGAACACCCAGACCCTATC	298/58/40
	R: TGTAACTTCCTCCGCAGCTT	
GAPDH	F: CCCCTTCATTGACCTCAACTACA	342/58/30
	R: TTGCTGATGATCTTGAGGCTGT	
ACTB	F: CCTGAACCCTAAGGCCAACCG	400/58/30
	R: GCTCATAGCTCTTCTCCAGGG	
WT1	F: GGGCAGAGCAACCACAGCACA	469/58/35
	R: GCCACCGACAGCTGAAGGGC	
Primers for quantitative RT-PCR		
GAPDH	F: GTGGACCTGACCTGCCGTCT	152
	R: GGAGGAGTGGGTGTCGCTGT	
T	F: AAGAAGGAAATGCAGCCTCA	101
	R: TACTGCAGGTGTGAGCAAGG	
CTNNB1	F: CCCACTAATGTCCAGCGTTT	217
	R: AACGCATGATAGCGTGTCTG	

Table S3. Antibodies for immunostaining (IS), Western blotting (WB), and flow cytometry (FC)

Antibody	Source	Application
Cardiac troponin T	Lab Vision, mouse IgG1, Clone: 13–11 ms-295-p1	1:200 (FC)
MF20	Developmental Studies Hybridoma Bank Mouse IgG2b	1:20 (FC)
α-Actinin	Sigma, mouse IgG1, Clone: EA-53	1:500 (IS)
Brachyury	R&D, Polyclonal Ab, Goat IgG Clone: AF2085	1:100 (FC)
ISL1	DSHB, mouse IgG2b, Clone: 39.4D5-s	1:20 (IS)
MLC2a	Synaptic Systems, IgG2b Cat: 311011, Clone: 56F5	1:400 (FC)
MLC2v	ProteinTech Group Rabbit anti-MYL2, poly, PTG10906-1-AP	1:200 (FC)
Oct-3/4	Santa Cruz, Rabbit IgG, Clone: H-134, sc-9081	1:40 (FC)
Oct-3/4	Santa Cruz, Mouse IgG_{2b} Clone: C-10 sc-5279	1:100 (IS)
NKX2-5	Santa Cruz, Rabbit IgG, Clone: H-114, sc-14033	1:75 (IS)
Phospho-Smad1/5 (Ser463/465)	Cell Signaling Technology, Rabbit mAb, 41D10 Cat: 9516S	1:500(WB)
Phospho-Smad2 (Ser465/467)	Cell Signaling Technology, Rabbit mAb, 138D4 3108S	1:500(WB)
Smad1	Cell Signaling Technology, Rabbit mAb, D59D7, 6944P	1:1,000(WB)
Smad2/3	Cell Signaling Technology, Rabbit IgG, Cat: 5678S	1:1,000(WB)
BMP2/4	Santa Cruz, mouse IgG2a, Clone H-1, sc-137087	1:500(WB)
β-Actin	Cell Signaling Technology, Rabbit mAb (HRP Conjugate), 13E5, 5125S	1:1,000(WB)
Goat anti-mouse IgG-HRP	Santa Cruz, sc-2005	1:1,000(WB)

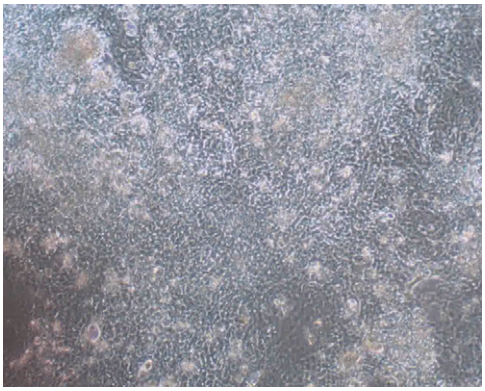


Movie S1. H9 cells were treated with 1 μM BIO for 3 d before exposure to 100 ng/mL activin A at day 0 and 5 ng/mL BMP4 at day 1. Movie S1 shows day 15 cardiomyocytes.

Movie S1

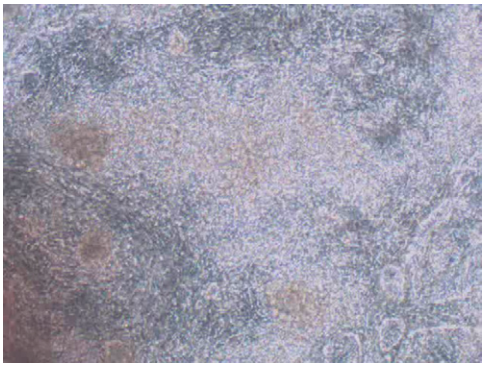
Movie S2. 19-9-11 ishcat-1 cells were treated with 12 μM CH at day 0 and 2 μg/mL dox at 36 h. Movie S2 shows relatively pure cardiomyocytes that contract as coordinated sheets in multiple independent wells (n = 9 wells) demonstrating consistency and reproducibility.

Movie S2



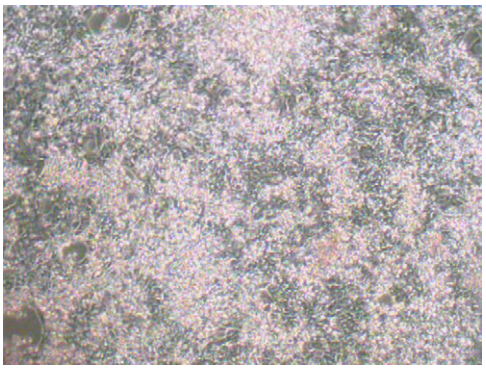
Movie S3. 19-9-11 ishcat-2 cells were differentiated as described in Movie S2. Cardiomyocytes maintained for 6 mo are shown.

Movie S3



Movie S4. 19-9-11 cells were differentiated with 12 μM CH at day 0 and 5 μM IWP4 at day 3 on a Synthemax plate. Movie S4 shows day 15 cardiomyocytes.

Movie S4



Movie S5. IMR90C4 cells were differentiated with 12 μM CH at day 0 and 5 μM IWP4 at day 3 on a Synthemax plate. Movie S5 shows day 15 cardiomyocytes at 10× magnification.

Movie S5