Supplemental Information: Wnt Activation and Reduced Cell-Cell Contact Synergistically Induce Massive Expansion of Functional Human iPSC-derived Cardiomyocytes

Supplementary Figure 1	**Relates to Figure 1 and 2**
Supplementary Figure 2	**Relates to Figure 3**
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Supplemental Figure Legend

Figure S1: Phenotypic analysis of hiPSC-CM proliferation upon GSK-3β inhibition. Related to Figure 1,2. (A) Pluripotent, cardiac mesoderm, cardiac progenitor and cardiomyocyte transcription factor expression during hiPSC-CM differentiation. Dashed lines indicated gene expression in samples treated with 2.0 μM CHIR99021 (CHIR) or DMSO (CTR) from day 12 to 14. Immunofluorescence images and graphs displaying (**B**) Islet-1 (ISL1) (red) or (**C**) Caspase 3 (red) and cardiac troponin T (TnT) positive cells at day 14 as in A. (**D)** Graph displaying the fold increase in cell numbers of freshly differentiated or cryopreserved hiPSC-CMs sparsely cultured with CHIR for multiple passages (P). (**E**) Expression of ki67, a cell-cycle index, in TnT+ cells after treatment with CHIR or DMSO (CTR). (**F**) hiPSC-CMs after treatment with DMSO (CTR) or CHIR. CMs were identified by staining for ACTN2 (white) and cycling CMs based on Anillin-eGFP (turquoise) expression. Anillin-eGFP localizations indicative for cell division, such as midbodies (arrow) and contractile rings (white box) were preferentially visible in CHIR treated CMs. Inset is a close-up of the boxed CM with an anillin-eGFP+ midbody (green). Nuclei are stained by DAPI (blue). Bars: 10 μm, inset: 5 μm. (**G**) Quantification of hiPS-derived eGFP-anillin^{+} CMs treated with either vehicle (CTR) or CHIR. CHIR treatment significantly increased the number of eGFP-anillin^{+} CMs at day (D) 10. Averaged values are expressed as mean ±SEM; n=1; * = $p<0.05$. Quantification of (**H**) bi-nucleation and (**I**) Ki67 expression (i.e. cell cycle index) as a percentage of total cells. hiPSC-CMs were cultured for 9 days with DMSO (CTR), CHIR or 6 days with CHIR followed by 3 days of Wnt inhibitor C59 (4.0 μM). Data shown represent mean ± SD, unless indicated else. *$p<0.05$. Supplementary Table 1 specifies the replicates per experiment.

Figure S2: Single-cell gene expression analysis of hiPSC-CMs following CHIR or C59 treatment. Related to Figure 3. (**A**) Quality control (QC) graphs featuring RNA content of all captured cells that have been treated with DMSO (1:2500) (CTR- gray), CHIR (4.0 μM) (CHIR-yellow), or C59 (4.0 μM) (C59-black) for 24 hours starting at day 12. (**B**) Post QC gating and unsupervised clustering of cells. (**C**) Heat map of genes for identity assignment validation of 5 populations characterized as ventricular CMs in G1-phase (vCM (G1))(red), ventricular CMs in S/G2-M-phase (vCM (S/G2M))(yellow), atrial CMs in G1-phase (aCM (G1))(green), atrial CMs in S/G2-M-phase (aCM (S/G2M))(blue) and fibroblast (Fb)(pink). (**D**) UMAPS displaying th e5 characterized cell populations for the indicated treatments. (**E**) Heat map displaying top 10 highest expressed genes in the vCM (S/G2M), vCM (G1), aCM (G1) and aCM (S/G2M) populations. UMAP plots for (**F**) cardiac progenitor genes and (**G**) cardiac transcription factors for the indicated treatments.

Figure S3: **Confirmation of Wnt signaling at the receptor level to induce immature hiPSC-CM proliferation and mature hiPSC-CM cell cycle activity. Related to Figure 5.** (**A**) Experimental diagram of immature day 12 (D12) hiPSC-CMs treated with various Wnt ligands and CHIR for 6 days. (**B**) Representative immunofluorescence images of immature hiPSC-CMs treated with R-Spondin 1 (RSPO) (25nM), Wnt3A (100 ng/mL), scFv-DKK1c (200 nM), scFV-DKK1c (200nM) + RSPO (25 nM), CHIR (2.0 μM), or media alone (CTR). (**C**) Quantification of the TnT positive cell numbers after treatment with the indicated Wnt ligands and CHIR. (**D**) Immunohistochemistry for Ki67 and troponin T (TnT) for the indicated treatments. (**E**) Plots displaying the percentages of Ki67+ /TnT+ cells. (**F**) Immunohistomistry for mitosis marker pHH3 in hiPSC-CMs treated with various Wnt activators. (**G**) Plots displaying the percentages of pHH3+/TnT+ cells. Scale bars represent 100μm. Dot plots represent biological replicates and mean. **$p<0.005$ by unpaired t-test. Supplementary Table 1 specifies the replicates per experiment.

Figure S4: Kinase expression in hiPSC-CM treated with CHIR. Related to Figure 5. (A) Graph displaying the expression of 43 kinases in hiPSC-CMs at day 12 after 0 (T0), 10 (T10) and 100 minutes of stimulation with 4.0 μM of CHIR99021 (CHIR). **(B)** Multiple immunofluorescence images of pAKT T308 and TnT in hiPSC-CMs treated with CHIR for 3 passages.

Figure S5: **Treatment of mice with CHIR results in increased myocardial cell-division in the late embryonic but not the postnatal heart. Related to Figure 6.** (**A**) Representative immunofluorescence for Troponin T (TnT), phospho Histone H3 (pHH3) and 4',6-diamidino-2-phenylindole (DAPI) of whole hearts and myocardial sections with magnified views from day 20.5 (E20.5) embryo's that were treated at

E16.5 for 4 consecutive days with a once-daily injection of 50mg/kg CHIR99021 (CHIR) (N = 3) or DMSO carrier control (CTR) (N = 3). (**B**) Relative number of mitotic cardiomyocytes (pHH3+/TnT+) in the myocardium of E20.5 embryo's treated with CHIR or CTR. (**C**) Percentages of mitotic cells in the heart of E20.5 embryo's treated with CHIR or CTR. (**D**) Representative immunofluorescence images of heart sections from embryo's treated with CHIR or CTR for wheat germ agglutin (WGA) and DAPI. (**E**) Quantification of the relative pixels/area indicating cell size for both groups. (**F**) Immunofluorescence in postnatal day 10 (P10) animals treated for 6 days with a once-daily injection of CHIR 50mg/kg or CTR. (**G**) percentages of mitotic cells (pHH3+) in the myocardium of P10 animals treated with CHIR or CTR. Scale bars represent 50μm. Dot plots represent biological replicates and mean. Data shown in bar graph represents mean±SD. **p<0.005 by unpaired t-test. Supplementary Table 1 specifies the replicates per experiment.

Supplementary Table 1: Data Transparency. Related to all figures.

Figure	Subpanel	Cell Line	Biological Replicate	Technical Replicate	Total Number of Cells Analyzed
1	B	CVI-111	4-8	2	N/A
1	D	CVI-111	6	3 (for dense (w/o PSG), 30 (for sparse) *	w/o PSG (6601), Dense (6056), Sparse (8573).
1	F	CVI-111	6	3 (for dense (w/o PSG), 30 (for sparse) *	w/o PSG (7688), Dense (6817), Sparse (6115).
1	I	CVI-111	4	2	Fresh (234), 1X C/M (231), 0.1X C/M (229), 0.01X C/M (260).
1	L	CVI-111	3	4-5 (for dense), 15-17 (for sparse) *	Dense (3058), Sparse (1109).
1	N	CVI-111	6	3-4 (for dense), 9-10 (for sparse) *	Dense (7327), Sparse (3071).
2	C	CVI-111, CVI-113, CVI-202, CVI-273	4	2	n/a
2	E	CVI-111, CVI-113, CVI-202, CVI-273	4	2	n/a
2	F	CVI-111, CVI-113, CVI-202, CVI-273	4	2	n/a
2	H	CVI-111, CVI-113, CVI-202, CVI-273	4	2	n/a
2	I-J	CVI-111, CVI-202, CVI-273	3	2	600000
2	L	CVI-111	2	2	175
3	D-E	CVI-111	20	1	60
3	G-H	CVI-111	12	1	n/a
3	I-K	CVI-111	3	2	n/a

3	L-P	CVI-273	1	1	8381
4	B	CVI-111	7	5 (for dense), 10-14 (for sparse) *	Dense (4814), Sparse (1812).
4	E	CVI-111	3	10	1 kPa (1416), 10 kPa (1778), 60 kPa (893), TCP (3397).
4	G	CVI-111	3	3	1 kPa (204), 10 kPa (166), 60 kPa (324), TCP (348).
4	I	CVI-111	3-4	3	Dense/DMSO(1681), Sparse/DMSO(708), Sparse/0.1uM(426), Sparse/1uM(858), Sparse/10uM(593).
4	K	CVI-111	6	3	Dense/DMSO(1793), Sparse/DMSO(657), Sparse/0.1uM(861), Sparse/1uM(1165), Sparse/10uM(1020).
5	A	CVI-111	6	1	n/a
5	B	CVI-111	4	2	996
5	C	CVI-111	2	2	n/a
5	D	CVI-111	1	2	n/a
5	F	CVI-111, CVI-273	2	1	n/a
5	H-I	CVI-111	4	2	1144
5	J	CVI-111	4	2	n/a
5	K	CVI-111	2	2	n/a
5	N	CVI-111	4	2	955
5	P	CVI-111	4	2	1368
6	B	CVI-111	3	2	n/a
6	F-G	CVI-111, CVI-273	12-20	1	n/a
S1	A	CVI-111	3	2	n/a
S1	B	CVI-111	2	2	263
S1	C	CVI-111	2	2	438
S1	D	CVI-111	2	2	n/a
S1	H-I	CVI-111	2	2	274
S2	A-G	CVI-273	1	1	8381
S3	C, E, G	CVI-111	4	2	5098
S4	A-B	CVI-111	1	2	n/a
S5	B-C	Mice	4	2	112162
S5	E	Mice	3	2	600
S5	G	Mice	3	4	157280

* to analyze the comparable number of single cells,
w/o = without, PSG = Passaging, n/a = not applicable