Stem Cell Reports, Volume *10*

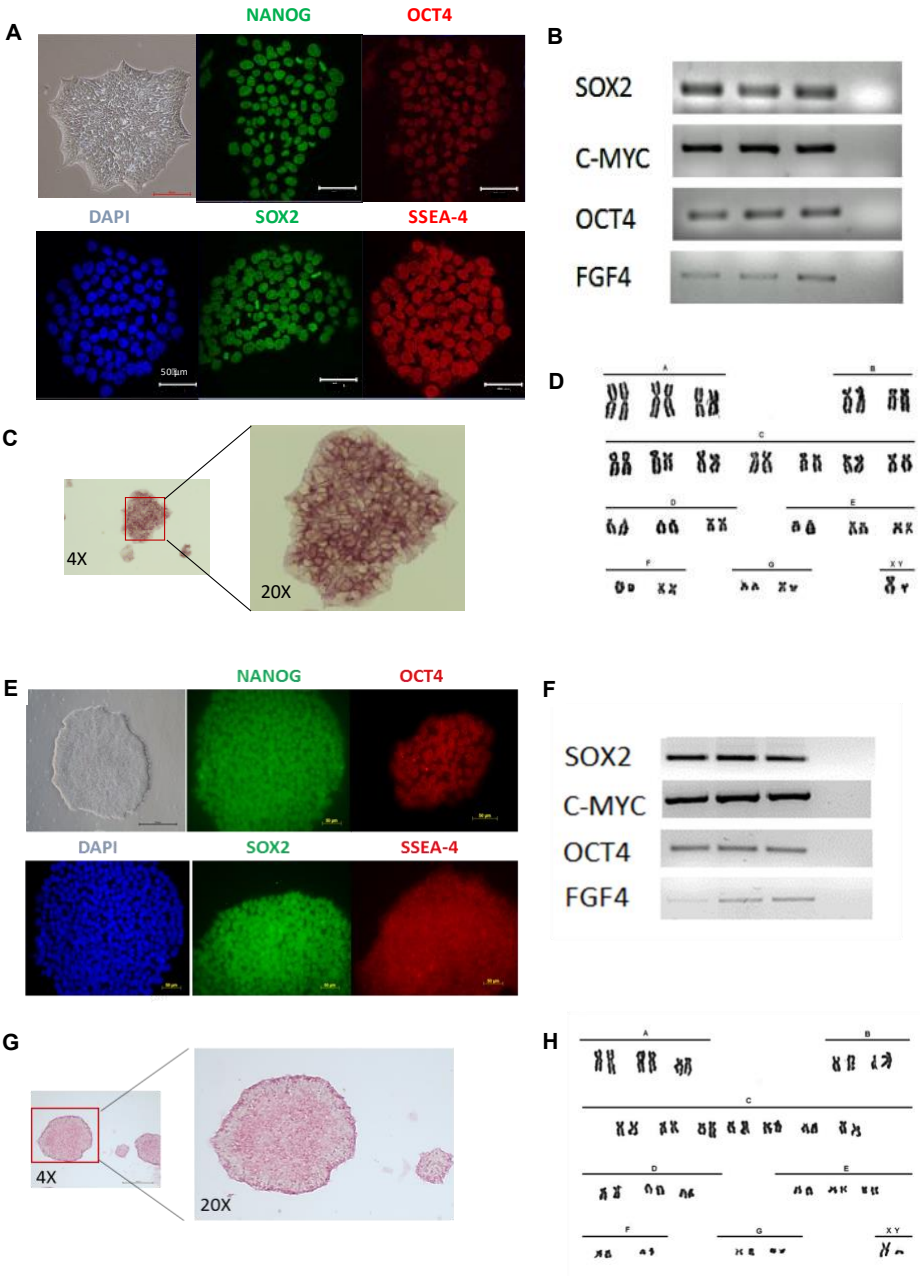
Supplemental Information

Electrophysiologic Characterization of Calcium Handling in Human Induced Pluripotent Stem Cell-Derived Atrial Cardiomyocytes

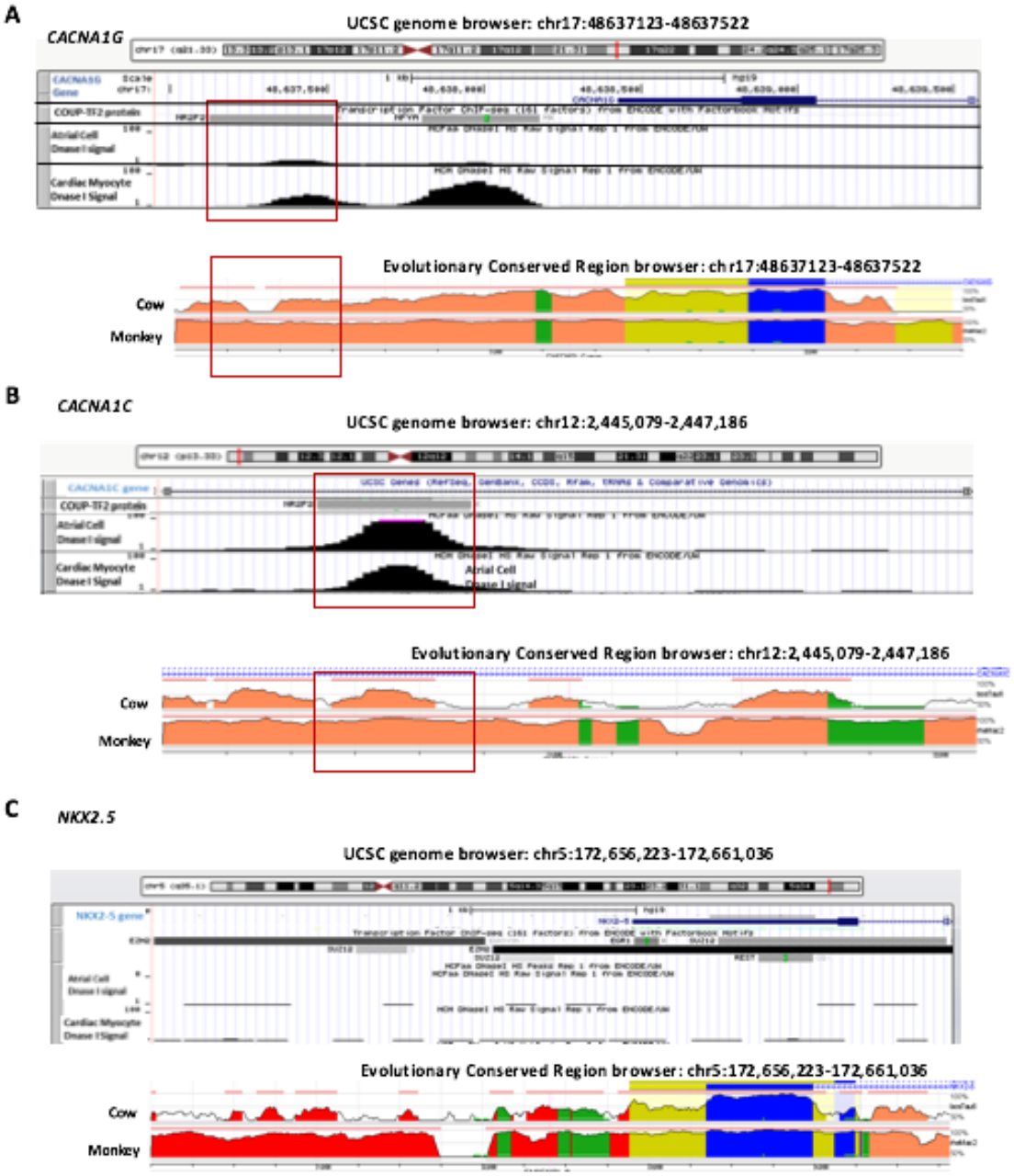
Mariana Argenziano, Erin Lambers, Liang Hong, Arvind Sridhar, Meihong Zhang, Brandon Chalazan, Ambili Menon, Eleonora Savio-Galimberti, Joseph C. Wu, Jalees Rehman, and Dawood Darbar

SUPPLEMENTAL FIGURES AND TEXT

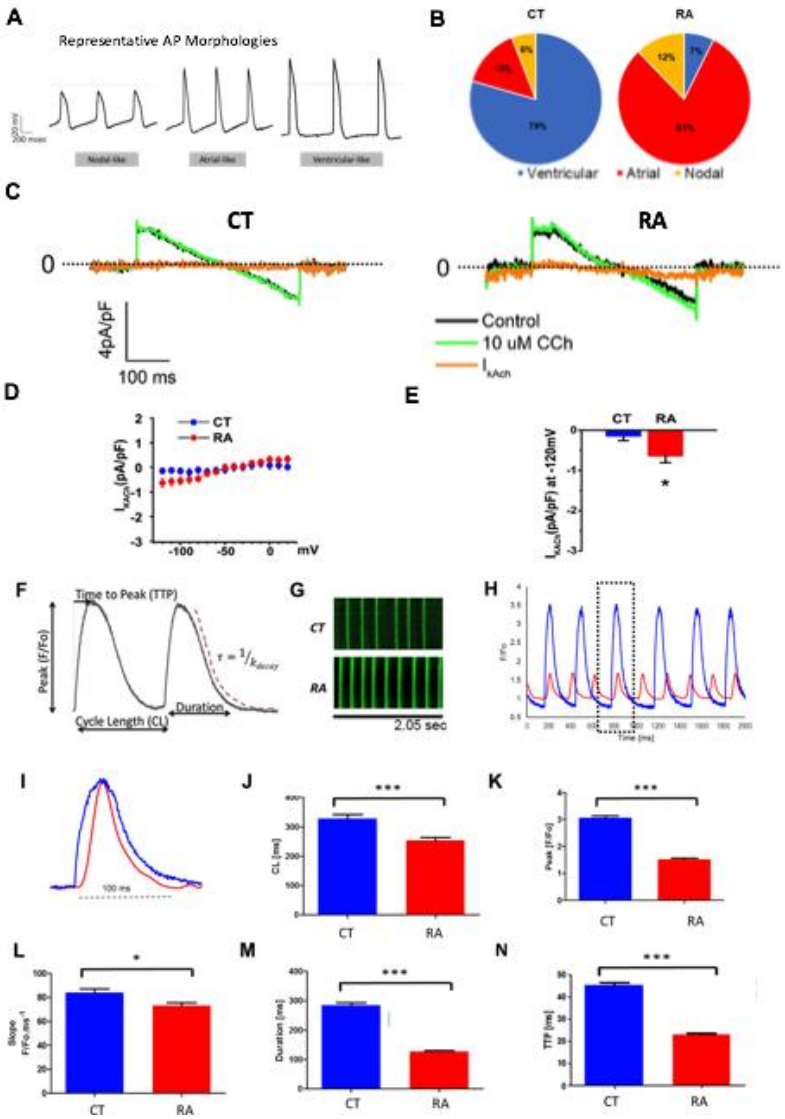
Supplemental Figure 1. Characterization of hiPSCs demonstrate pluripotent properties and normal karyotype in two independently derived hiPSC lines. A: Bright field and fluorescent immunostaining images of pluripotency markers NANOG, OCT4, SOX2 and SSEA4 in hiPSC-L1 at passage 20; **B**: RT-PCR analysis showing the expression of pluripotency markers *SOX2*, *C-MYC*, *OCT4* and *FGF4* for 3 independent hiPSC-L1 clones; **C**: Alkaline phosphatase activity assay. Undifferentiated (pluripotent) hiPSC-L1 cells appear red or purple, whereas differentiated cells appear colorless; **D**: Karyotype obtained from hiPSC-L1 cells at passage 20. **E**: Bright field and fluorescent immunostaining images of pluripotency markers NANOG, OCT4, SOX2 and SSEA4 in hiPSC-L2 at passage 20; **F**: RT-PCR analysis showing the expression of pluripotency markers *SOX2*, *C-MYC*, *OCT4* and *FGF4* for 3 independent hiPSC-L2 clones; **G**: Alkaline phosphatase activity assay. Undifferentiated (pluripotent) hiPSC-L2 cells appear red or purple, whereas differentiated cells appear colorless; **H**: Karyotype obtained from hiPSC-L2 cells at passage 20



Supplemental Figure 2. COUP-TFII is associated with calcium channel genes known to regulate atrial-like and pacemaker-like cellular functions. UCSC Encode ChIP-seq data showing enrichment of human **A:** *CACNA1G* and **B:** *CACNA1C* loci associated with COUP-TFII (*NR2F2*) as viewed on the UCSC Human Genome Browser (http://genome.ucsc.edu). Red boxes indicate genomic loci that are both associated with COUP-TFII protein and hypersensitive to cleavage by DNase 1 indicating open chromatin accessibility in atrial cells and cardiomyocytes to transcriptional regulation. COUP-TFII binding regions, indicated by red boxes, are located in highly conserved regions as shown by the evolutionary conserve region browser (ecrbrowser.dcode.org). Yellow represents 5' and 3'-UTRs; blue represents coding regions; salmon represents Introns; green represents transposons and simple repeats. **C:** *NKX2.5* loci is not associated with COUPTF-II.



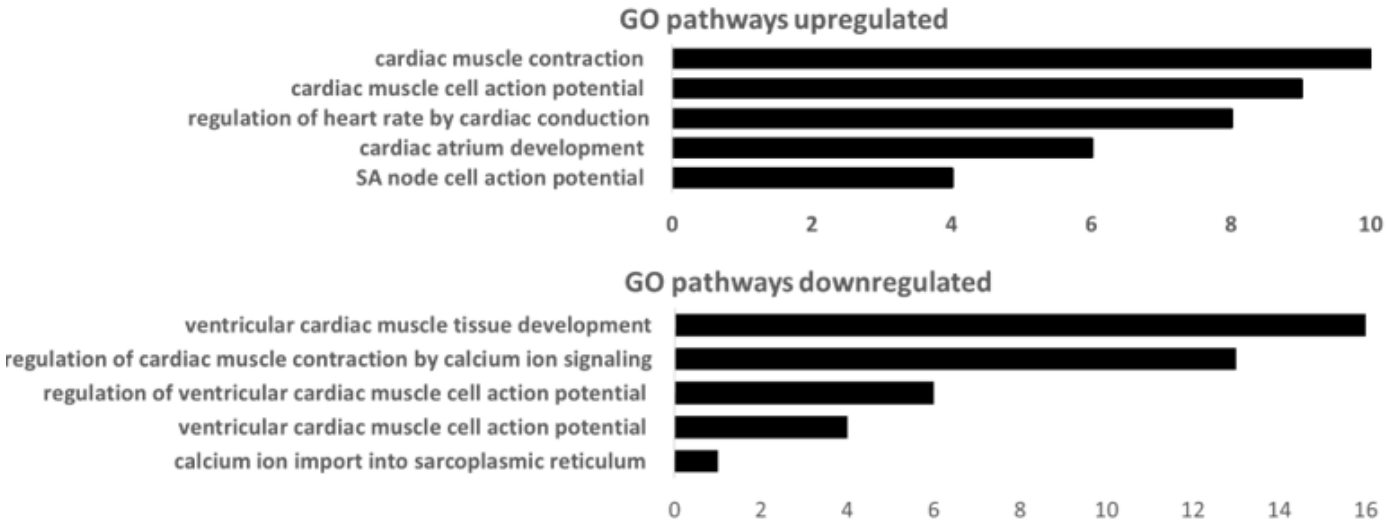
Supplemental Figure 3: RA Treatment increases atrial-like morphologies, atrial-specific currents, and calcium uptake and reléase in an independently derived cell line. A: Representative images of three major types of AP morphologies observed in hiPSC-L2-CMs: atrial-, nodal-, and ventricular-like. AP morphologies were recorded using intracellular sharp microelectrode recordings of single cells within monolayers. **B:** Pie graphs displaying the percentage of morphologies in observed in RA-treated (n=32 cells) and CT- hiPSC-L2-CMs (n=42 cells). **C:** Representative recordings of 10uM carbachol (Cch) sensitive current ($I_{K,Ach}$) in ventricular-like (CT) and atrial-like hiPSC-L2-CMs (RA); voltage protocol is shown in inset; n= 3 cells per group. **D:** Current-voltage relationship (I-V curve) for Cch sensitive $I_{K,Ach}$ densities in ventricular-like and atrial-like hiPSC-L2-CMs; n= 3 cells per group. **E**: Quantification of $I_{K,Ach}$ densities at -120mV in ventricular-like and atrial-like CMs; n=3 each group **F:** Space-averaged calcium transients illustrating the parameters to be analyzed. **G:** Representative line scans showing the spontaneous Ca release events (sCaRE) in CT and RA hiPSC-L1-CMs. **H:** Space-averaged calcium transients comparing spontaneous activity between CT (blue line) and RA (red line) hiPSC-CMs n=25 cells per group. **I:** Overlap of normalized calcium transients from CT and RA CMs showing differences in time to peak (TTP), duration and decay. Comparison of sCaRE parameters from CT- and RA-treated CMs. **J:** Cycle length (CL) n=25 cells per group **K:** Peak amplitude n=25 cells per group **L:** Slope n=25 cells per group **M:** Duration n=25 cells per group **N:** TTP, n=25 cells per group. Data shown in all panels represent independent biological experiments displayed as mean ± SD, * P<0.05, ** P<0.01, *** P<0.001



Supplemental Table 1: Differentially expressed GO pathway gene lists in RA treated vs CT hiPSC-L1-CMs related to cardiac development, conduction, and calcium handling.

Upregulated Gene Ontology Pathway	Gene Symbols
cardiac atrium development (GO:0003230)	CCM2L, GJA5, DAND5, NOG, BMP10, MESP1,
SA node cell action potential (GO:0086015)	GJA5, SCN3B, HCN4, CACNA1G
regulation of heart rate by cardiac conduction (GO:0086091)	KCNH6, KCNJ2, KCNJ3, GJA5, SCN3B, HCN4, CACNA1G, SCN4B,
cardiac muscle cell action potential (GO:0086001)	KCNIP2, SCN1A, KCNJ2, KCNJ3, GJA5, SCN3B, HCN4, CACNA1G, SCN4B
cardiac muscle contraction (GO:0060048)	SCN1A, MYK2, KCNJ2, TNNI1, KCNJ3, GJA5, SCN3B, CACNA1G, SCN4B

Downregulated Gene Ontology Pathway	Gene Symbols
ventricular cardiac muscle tissue development (GO:0003229)	TNNT2, MYBPC3, MYL3, OU4F1, TPM1, MYL2, HAND1, PKP2, TGFB2, NRG1, TNNC1, NKX2.5, MYH7, TGFBR1, MYH6
ventricular cardiac muscle cell action potential (GO:0086005)	KCNQ1, ANK2, PKP2, SNTA1
calcium ion import into sarcoplasmic reticulum (GO:1990036)	ANK2, TRDN, ATP2A2
regulation of ventricular cardiac muscle cell action potential (GO:0098911)	TRPM4, DSC3, JUP, CACNA1C, PKP2, CTNNA3
regulation of cardiac muscle contraction by calcium ion signaling (GO:0010882)	GST01, PLN, CACNA1C, CAMK2D, CASQ2, ANK2, PRKACA, ASPH, SLC8A1, TNNC1, HRC, ATP2A2, ATP1B1,



Supplemental Table 2. Electrophysiological parameters of spontaneously beating hiPSC-L2-CMs and paced hiPSC-L1-CMs and hiPSC-L2-CMs (2Hz) treated with RA versus CT. AP, action potential; APA, AP amplitude; APD50, AP duration at 50% repolarization; APD90, AP duration at 90% repolarization; CT, control (DMSO) * P<0.05, ** P<0.01, *** P<0.001

hiPSC-L2-CMs - Spontaneous	AP Parameters	CT	RA	P-value
	n (%)	34	41	
	RMP [mV]	-45.1±1.4	-48.8±2.0	ns
	APA [mV]	63.0±2.3	56.0±2.0	p<0.05
	APD50 [msec]	231.6±10.8	75.5±3.5	p<0.01
	APD90 [msec]	294.1±13.0	109.1±7.0	P<0.001
	APD90/APD50 [RO]	1.5±0.03	1.3±0.01	P<0.01
	APD30-40/APD70-80	1.0±0.07	0.49±0.05	P<0.01
	Upstroke Velocity [V/sec]	13.0±0.7	13.2±0.9	ns
hiPSC-L2-CMs – 2Hz	**AP**	**CT**	**RA**	**P-value**
	n (%)	9	9	
	RMP [mV]	-52.7±4.7	-48.7±2.3	ns
	APA [mV]	71.1±7.8	48.2±4.9	p<0.05
	APD50 [msec]	215.9±18.8	86.2±4.7	p<0.001
	APD90 [msec]	269.8±22.1	138.7±13.8	p<0.001
	APD90/APD50 [RO]	1.4±0.05	1.28±0.02	p<0.05
	APD30-40/APD70-80	0.80±0.08	0.46±0.05	p<0.05
	Upstroke Velocity [V/sec]	12.8±1.7	8.9±2.0	ns
hiPSC-L1-CMs - 2Hz	**AP Parameter**	**CT**	**RA**	**P-value**
	n (%)	12	9	
	RMP [mV]	-51.3±3.2	-44.8±3.5	ns
	APA [mV]	72.2±4.8	46.7±3.6	p<0.01
	APD50 [msec]	124.3±12.8	63.0±5.9	p<0.001
	APD90 [msec]	204.0±18.2	90.8±6.8	p<0.001
	APD90/APD50 [RO]	1.7±0.11	1.45±0.04	p<0.05
	APD30-40/APD70-80	0.99±0.07	0.54±0.06	p<0.05
	Upstroke Velocity [V/sec]	34.4±6.5	14.4±1.2	p<0.05

Supplemental Table 3: RNA-seq dataset with comprehensive list of upregulated and downregulated genes in RA-treated vs CT-treated hiPSC-L1-CMs at day 30 of differentiation

Supplemental Movie 1: Spontaneous beating of retinoic acid (CT)-treated hiPSC-L1-CMs at day 30 of differentiation.

Supplemental Movie 2: Spontaneous beating of control (RA) hiPSC-L1-CMs at day 30 of differentiation.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

1. Cell Culture

HiPSC culture: HiPSC-L1-CMs and hiPSC-L2-CMs were generated , as previously described (Jia et al., 2010) by the Stanford iPSC Biobank and LIMR iPSC core respectively. Briefly, three independent clones of each cell line were derived from peripheral blood by lentivirus based reprogramming. HiPSCs were maintained on human recombinant vitronectin (hrVTN) coated plates (1:100 dilution, DPBS) in Essential 8 Flex medium (Gibco). Cells were passaged every 4 days using 0.5 mmol/L EDTA (Life Technologies) in D-PBS without $CaCl_2$ or $MgCl_2$ (Life Technologies). Cells were maintained at 37 ºC, with 5% CO_2.

Cardiac differentiation: iPSC were seeded at an initial density of 500,000 cells per well on 12-well plates coated with hrVTN. Cells were cultured in Essential 8 Flex (Gibco) media with daily media exchange until 80-90% confluent (day 0). They were then differentiated using medium A of conditioned cardiomyocyte differentiation kit (Gibco) for two days. At day 3, medium is replaced for cardiomyocyte differentiation medium B for two days. To guide iPSC-derived CMs into atrial maturation, at day 5, we used an adaptation of the method previously described by Devala *et al* whereby hiPSC-CMs are treated with 1 µmol/l all-*trans* retinoic acid (RA) (Devalla et al., 2015). We treated cells with RA during five days with media changes every other day. Control cells were treated with equivalent concentrations of DMSO. Cells are maintained in standard RPMI 1640 media with B27 complete until spontaneous contraction is observed, routinely around days 9-10. At day 10, a low-glucose metabolic selection step is used to enrich cardiomyocyte culture (Sharma et al., 2015). Cells were harvested at days 10, 15 and 30 to analyze gene expression and Ca dynamics.

Replating of hiPSC-CMs: HiPSC-CMs were rinsed with D-PBS without $CaCl_2$ or $MgCl_2$ (Gibco) and incubated with TrypLE Express (Gibco) for 10 minutes at 37ºC to dissociate cells, at day 20-25 post differentiation. They were then filtered (100 µm) (Falcon) and plated at low density (40,000 cells) on hrVTN.

2. Immunofluorescence and Alkaline Phospatase Staining

HiPSCs and hiPSC-CMs were plated in Matek(R) glass bottom dishes coated with hrVTN and were allowed to grow for 2 days. Cells were fixed for 5 minutes with ice cold methanol, permeabilized with PBS + 0.1% Triton x-100 for 10 minutes at room temperature and blocked with 1% BSA, 22.52 mg/ml glycine in PBST for 30 minutes at room temperature. Staining was performed for 1 hour at room temperature using primary antibodies diluted in 1% BSA in PBS for SOX2 (Santa Cruz, sc-17320), OCT4 (Santa Cruz, sc-5279), NANOG (Santa Cruz, 33759), SSEA-4 (Santa Cruz, 21704), NKX2-5 (Molecular Probes, A25974),and troponin-T (TNNT; Molecular Probes, A25969). Cells were washed three times with cold PBS and incubated at room temperature for 1 hour with secondary antibodies, diluted in 1% BSA in PBS, Alexa Fluor 594 donkey anti-rabbit IgG (Molecular Probes, A25970), Alexa Fluor 488 donkey anti mouse IgG (Molecular Probes, A25972), FITC goat anti-rabbit IgG (Santa Cruz, sc-3839), PE goat anti-mouse IgG (Santa Cruz, sc-3798) and FITC donkey anti-goat IgG (Santa Cruz, sc-3853). Cells were washed three times with PBS, co-stained with 1µg/ml of DAPI (Thermo Scientific) and mounted with SlowFade Diamond Antifade Mountant (Molecular Probes). Samples were imaged using an LSM710 Meta Confocal Microscope (Zeiss). Detection of alkaline phosphatase was performed using the alkaline phosphatase staining kit (Stemgent) following manufacturer's instructions. Samples were imaged with an EVOS FL cell imaging system (Thermofisher Scientific).

3. Flow Cytometry

HiPSC-CMs were rinsed with D-PBS without $CaCl_2$ or $MgCl_2$ (Gibco) and incubated with TrypLE Express (Gibco) for 10 minutes at 37ºC to dissociate the cells. Cells were washed and stained with a fixable Live/dead stain (Invitrogen, MP34955) for 30 minutes to distinguish between live and dead populations. Cells were then fixed with 4% formaldehyde for 10 minutes and permeabilized with 0.5% triton X. Cells were incubated with primary antibodies MLC2v (Abcam ab79935) and KCN1.5 (Santa Cruz sc-377110) according to manufacturer instructions: 1uL/ 10,000,00 cells and 1:50 volume dilutions respectively. Undifferentiated hiPSC-L1 at Day 0 served as a negative gating

control for assessing markers of ventricular and atrial differentiation in RA-treated and CT hiPSC-L1-CMs at Day 30.

4. Karyotyping

For karyotyping, iPSCs were incubated with 0.1 µg/ml of colcemid for 3 hours and harvested with 0.5 mM EDTA. Cells were centrifuged for 5 minutes at 122g and after discarding the supernatant, we added 5 ml of 75 mM KCl and incubated for 15 minutes at 37ºC. Cells were centrifuged 5 minutes at 122g, the supernatant was replaced by 3 ml of fixative solution (methanol/glacial acetic acid 3:1) and incubated at 4ºC overnight. On the next day, cells are centrifuged 5 minutes at 122g to replace the fixative solution for fresh solution. After last centrifugation, the supernatant was discarded and cells were resuspended in 200 µl of fixative solution. We added 30 µl to a clean slide and let it dry overnight at 55ºC. Slides were stained with Gurrs Giemsa (3 ml Giemsa + 48 ml Gurrs Buffer 6.8 + 3 drops 2.5% Trypsin, Gibco) and washed 3 times with Gurrs buffer. Once dried, slides were mounted with toluene based mounting media and incubated overnight at 55ºC. Images were obtained using an Olympus BX51 microscope.

5. PCR and RNA-seq

RNA isolation: Cells were harvested using 0.5 ml of Trizol reagent (Invitrogen), incubated for 5 minutes at room temperature and 0.2 ml of chloroform per ml of Trizol was added. After 2–3 minutes of incubation, samples were centrifuged at 12000g for 15 minutes at 4°C. The clear phase was transferred to a new microcentrifuge tube where 0.5 ml of isopropanol were added and samples were centrifuged at 12000g for 10 minutes at 4°C. After centrifugation, the supernatant was removed and 1 ml of 75% ethanol per ml of Trizol was added previous to centrifugation at 7500g for 5 minutes at 4°C. After the supernatant was removed, samples were dried for 30 minutes and the pellet dissolved in 30 µl of RNAse-free water.

Reverse transcription: RNA was quantified by using the Nanodrop 1000 (Thermofisher Scientific). For the retrotranscription, 2 µg of RNA were mixed with 1 µl of Random hexamers (200 ng/µl, Invitrogen), 1 µl of dNTPs (10 mM each, Invitrogen) and DEPC treated water up to 13 µl. After

incubating for 5 minutes at 65°C, 7.5 µl of a MIX solution containing 4 µl of 5X reaction buffer, 0.5 µl of RNAseOUT (40 U/µl), 1 µl DTT (100 mM), and 1 µl of SuperScript IV Reverse Transcriptase (200 U/ul, Invitrogen) was added before the thermal cycles began according to manufacturer's instructions.

PCR reaction: For PCR reactions, 1 µl of each sample was mixed with 12.5 ul Platinum Hot Start PCR 2X Master Mix (Invitrogen), 0.5 µl 10 mM forward primer, 0.5 µl 10 mM reverse primer and water to 25 µl, thermal cycle proceeded as follows: initial denaturation at 94°C 2 minutes and 30 cycles consisting of 94°C 30 seconds, 60°C 30 seconds and 72°C 20 seconds. To visualize the bands, samples were run in 2% agarose gels with ethidium bromide and imaged using ChemiDoc Imaging Systems (BioRad).

The real time PCR reaction (qPCR) was carried out using FastStart Universal SYBR Green Master Mix (Applied Biosystems). The cycling program consisted of one cycle at 50°C for 2 min, one cycle at 95°C for 10 min (first denaturation) and 40 cycles consisting of: 95°C for 20 s (denaturation), 60°C for 1 min (annealing) and 72°C for 20 s (extension). Expression of mRNAs was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Data were analyzed using the Pfaffl method (Pfaffl, 2001). Primers were ordered from LifeTechnologies with the following catalog numbers: KCNA5 qPCR primer 4331182-HS00165693; MYL2 qPCR primer 4331182-Hs00166405_m1; MYH7 qPCR primer 4453320-Hs01110632_m1; NR2F2 qPCR primer 4331182-Hs00819630_m1; CACNA1G qPCR primer 4453320-Hs 00367969_mL; CACNA1C qPCR primer, 4453320-Hs 00167681_mL.

RNAseq: To obtain the whole transcriptome of the iPSC-CMs, we performed RNAseq using TempO-Seq (BioSpyder) from cell lysates following manufacturer's instructions. Briefly, cells lysates from three independent experiments are annealed to chimeric detector oligos. After annealing, excess oligos are removed by nuclease digestion, and ligated to form an amplifiable template. The ligated oligos are amplified with one pair of primers. The purified amplicon library is run on an Illumina next-generation sequencer to count the number of ligated detector oligo sequences per

sample. Read counts were analyzed for differential expression genes (DEGs) among samples. Genes with the mean absolute log2 fold change >2 were included in the analysis ($P<0.05$). Unsupervised hierarchical clustering of all detected genes was performed using the Euclidean distance and complete linkage method. Volcano plots were analyzed using R platform. The enriched biological processes were obtained using Gene Ontology (GO) (www.geneontology.org) and iPathways from Advaita Bioinformatics.

6. Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using a ChIP Assay kit (Millipore, Billerica, MA, http://www.millipore.com, Cat#17-295) per manufacturer's protocol. Immunoprecipitation was performed using a ChIP grade COUP-TFII antibody antibody to pulldown COUP-TFII: Abcam, Cambridge, U.K., http://www.abcam.com, Cat# ab41859 PCR detection was performed using Applied Biosystems Fast Real-time PCR 7500 system according to manufacturer's instructions with gene-specific primers spanning CACNA1G and CACNA1C binding site regions.

7. Ca Measurements

Fluo-4 AM (Molecular Probes) was used to measure changes in Ca concentration. The dye was dissolved in 45 µL DMSO (Molecular Probes) with 2.5% Pluronic F-127 (Molecular Probes) and added to 1 mL of 1 mM Ca Tyrode's solution (NaCl 134 mM, KCl 5.4 mM, $MgCl_2$ 1mM, HEPES 10 mM, Glucose 10 mM, $CaCl_2$ 1 mM or 2 mM). Cell were incubated in 5 µM of Fluo-4-AM during 15 minutes at room temperature and washed for 15 minutes with Tyrode's solution containing 2 mM Ca at room temperature before Ca measurements. All experiments were performed using 2 mM Ca concentration. Linescans were registered using an LSM710 Meta Confocal Microscope (Zeiss) using an oil 63X objective and analyzed using imageJ.

8. Action Potential Recordings

Sharp microelectrode technique with monolayer: HiPSC-CMs were differentiated on 35 mm petri dishes using the protocol described above. Between days 25-35, APs were obtained from iPSC-CMs monolayers using conventional sharp microelectrode techniques. Briefly, cells were perfused

with Tyrode's solution at 37ºC, APs were recorded using intracellular microelectrodes (DC resistance, 30 to 50 MOhm) filled with 2.7 mol/L KCl and connected to a high-input impedance amplifier. AP characteristics were obtained during spontaneous beating and also during pacing at the same rate above the spontaneous rate at 2 Hz with the values for AP corrected by frequency using the Bazzet formula. All amplified signals were digitized, stored on magnetic media and WORM-CD, and analyzed using Spike 2 (Cambridge Electronic Design). All AP duration (APD) measurements are reported at 90% repolarization (APD90) and 50% (APD50). Resting membrane potential (RMP) and AP amplitude (APA) are also evaluated. In all cases, Bazzet's correction was applied (APD/$\sqrt{CL}$). The following criteria were used to distinguish the AP phenotypes: atrial- and ventricular-like APs displayed more negative RMPs with higher dV/dt_{max} values and larger amplitudes than nodal-like APs. Ventricular-like APs had a distinct plateau phase (phase 2) after which repolarization accelerates (phase 3). Ventricular, atrial, and nodal APs were classified according to published criteria (Ma et al., 2011). Ventricular-like APs had a ratio (APD30–40/APD70–80) ~1 compared with the atrial-like ratio of ~0.5 and nodal-like ratio of ~0.2. Ventricular-like APs had longer duration APDs with slower rates of spontaneous beating, whereas atrial-like APs had shorter durations and higher spontaneous beating rates. Nodal-like APs had less negative RMPs and smaller APAs with lower dV/dtmax values (<10 V/sec).

Patch clamp technique with single CMs: Patch-clamp measurements were performed in whole-cell configuration configurations using an Axopatch 200B amplifier controlled by pClamp10 software through an Axon Digidata 1440A (Kim et al., 2014). For measurement of Ca currents, the extracellular solution contained 150mM Tris, 1mM $MgCl_2$, 10mM $CaCl_2$, and 10mM glucose, adjusted to pH 7.4 with methanesulfonic acid. The intracellular solution containing: 135mM CsCl, 1mM $MgCl_2$, 4mM MgATP, 10 mM EGTA, and 10mM HEPES adjusted to pH 7.3 with CsOH. Steady-state activation G-V for Ca currents were fitted by the Boltzmann equation described previously (Pathak et al., 2016): $G/G_{max}=1/(1+exp(V_{1/2}-V)/K)$, where G/G_{max} is the relative conductance normalized by the maximal conductance, $V_{1/2}$ is the potential of half activation, V is test pulse, and

k is the Boltzmann coefficient. Pipettes had 2-4 MΩ access resistance. Current densities were calculated by whole-cell current amplitude and capacitance value taken from readings of the amplifier after electronic subtraction of the capacitive transients.

For measurement of the I_{kAch} current, the extracellular solution contained 140mM NaCl, 4mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES and 10mM Glucose, adjusted to pH 7.4 with NaOH. The intracellular solution containing: 110mM KCl, 10mM NaCl, 10mM HEPES, 0.4mM $MgCl_2$, 5mM Glucose, 5mM K_2ATP dihydrate, 0.5mM GTP-Tris salt, 5mM BAPTA, adjusted to pH 7.4 with KOH.

9. Data Analysis

Data are presented as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD) as indicated. For datasets with normal distributions, statistical significance was determined by Student's t-test (two-tailed) for two groups or one-way ANOVA for multiple groups with post-hoc test Bonferroni.

References:

DEVALLA, H. D., SCHWACH, V., FORD, J. W., MILNES, J. T., EL-HAOU, S., JACKSON, C., GKATZIS, K., ELLIOTT, D. A., CHUVA DE SOUSA LOPES, S. M., MUMMERY, C. L., VERKERK, A. O. & PASSIER, R. 2015. Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology. *EMBO Mol Med,* 7**,** 394-410.

JIA, F., WILSON, K. D., SUN, N., GUPTA, D. M., HUANG, M., LI, Z., PANETTA, N. J., CHEN, Z. Y., ROBBINS, R. C., KAY, M. A., LONGAKER, M. T. & WU, J. C. 2010. A nonviral minicircle vector for deriving human iPS cells. *Nat Methods,* 7**,** 197-9.

KIM, I. H., HEVEZI, P., VARGA, C., PATHAK, M. M., HONG, L., TA, D., TRAN, C. T., ZLOTNIK, A., SOLTESZ, I. & TOMBOLA, F. 2014. Evidence for functional diversity between the voltage-gated proton channel Hv1 and its closest related protein HVRP1. *PLoS One,* 9**,** e105926.

MA, J., GUO, L., FIENE, S. J., ANSON, B. D., THOMSON, J. A., KAMP, T. J., KOLAJA, K. L., SWANSON, B. J. & JANUARY, C. T. 2011. High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents. *Am J Physiol Heart Circ Physiol,* 301**,** H2006-17.

PATHAK, M. M., TRAN, T., HONG, L., JOOS, B., MORRIS, C. E. & TOMBOLA, F. 2016. The Hv1 proton channel responds to mechanical stimuli. *J Gen Physiol,* 148**,** 405-418.

PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res,* 29**,** e45.

SHARMA, A., LI, G., RAJARAJAN, K., HAMAGUCHI, R., BURRIDGE, P. W. & WU, S. M. 2015. Derivation of highly purified cardiomyocytes from human induced pluripotent stem cells using small molecule-modulated differentiation and subsequent glucose starvation. *J Vis Exp*.