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Authors

Liang, Wenbin
Han, Pengcheng
Kim, Elizabeth H
et al.

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Canonical Wnt signaling promotes pacemaker cell specification of cardiac mesodermal cells derived from mouse and human embryonic stem cells

Wenbin Liang[#1], **Pengcheng Han**[#2], **Elizabeth H. Kim**[2], **Jordan Mak**[2], **Rui Zhang**[3], **Angelo G. Torrente**[3], **Joshua I. Goldhaber**[3], **Eduardo Marbán**[3], **Hee Cheol Cho**[2,4]

[1]University of Ottawa Heart Institute and Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada

[2]Department of Pediatrics, Emory University, Atlanta, Georgia

[3]Cedars-Sinai Heart Institute, Los Angeles, California

[4]Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia

[#] These authors contributed equally to this work.

Abstract

Cardiac differentiation of embryonic stem cells (ESCs) can give rise to de novo chamber cardiomyocytes and nodal pacemaker cells. Compared with our understanding of direct differentiation toward atrial and ventricular myocytes, the mechanisms for nodal pacemaker cell commitment are not well understood. Taking a cue from the prominence of canonical Wnt signaling during cardiac pacemaker tissue development in chick embryos, we asked if modulations of Wnt signaling influence cardiac progenitors to bifurcate to either chamber cardiomyocytes or pacemaker cells. Omitting an exogenous Wnt inhibitor, which is routinely added to maximize cardiac myocyte yield during differentiation of mouse and human ESCs, led to increased yield of spontaneously beating cardiomyocytes with action potential properties similar to those of native sinoatrial node pacemaker cells. The pacemaker phenotype was accompanied by enhanced expression of genes and gene products that mark nodal pacemaker cells such as *Hcn4, Tbx18, Tbx3*, and *Shox2*. Addition of exogenous Wnt3a ligand, which activates canonical Wnt/β-catenin signaling, increased the yield of pacemaker-like myocytes while reducing cTNT-positive pan-cardiac differentiation. Conversely, addition of inhibitors of Wnt/β-catenin signaling led to

Correspondence: Wenbin Liang, MD, PhD, Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ontario, K1H 8M5, Canada. wliang@ottawaheart.ca; Hee Cheol Cho, PhD, Departments of Biomedical Engineering and Pediatrics, Emory University, Atlanta, GA 30332-0535. heecheol.cho@emory.edu.

AUTHOR CONTRIBUTIONS

W.L., H.C.C.: conceived the study, designed experiments, wrote the manuscript, performed experiments; W.L., P.H., E.H.K., J.M., R.Z., A.G.T.: performed experiments; E.M., J.I.G.: provided conceptual input.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

increased chamber myocyte lineage development at the expense of pacemaker cell specification. The positive impact of canonical Wnt signaling on nodal pacemaker cell differentiation was evidenced in direct differentiation of two human ESC lines and human induced pluripotent stem cells. Our data identify the Wnt/β-catenin pathway as a critical determinant of cardiac myocyte subtype commitment during ESC differentiation: endogenous Wnt signaling favors the pacemaker lineage, whereas its suppression promotes the chamber cardiomyocyte lineage.

Keywords

cardiac; cell culture; cell biology; cellular therapy; stem cells

1 | INTRODUCTION

Cardiogenesis gives rise to multiple excitable cardiac cell subtypes, including the working chamber cardiomyocytes of the atria and ventricles as well as the sinoatrial node (SAN) and the ventricular conduction system.[1] During mouse embryonic development, the SAN originates from a subset of progenitor cells that are positive for *Shox2, Tbx18*, and/or *Tbx3*. Differentiation of *Tbx18*-positive progenitor cells into SAN cells requires *Shox2*, a homeodomain transcription factor that suppresses chamber-lineage gene expression.[2,3] Chamber cardiomyocytes develop from *Nkx2.5*-positive progenitor cells, which constitute the first and second heart fields.[4–6] Thus, the developing SAN has been referred to as the tertiary heart field,[7] which is distinct from and mutually exclusive to the first and second heart fields.[8] The SAN appears to be distinct from the ventricular conduction system as well, which co-expresses *Nkx2.5* and *Tbx3*.[9,10]

Embryonic stem cells (ESCs) can give rise to multiple cardiac lineages, including chamber myocytes and pacemaker cells.[11] Recent advances have greatly improved the yield of cardiomyocytes from ESCs,[12–14] which are predominantly of ventricular phenotype.[15] Similarly, enrichment of atrial myocytes from pluripotent stem cells has been reported.[16,17] Enrichment of cardiac pacemaker cells during ESC differentiation could be achieved by suramin treatment,[18] via viral overexpression of *TBX3*[19] or *SHOX2*,[20] by CD166 cell isolation,[21] or by isolation of a NKX2.5-negative population.[22] Still, compared with our understanding of myocardial chamber specification, key mechanisms for nodal pacemaker commitment remain poorly understood.

Wnt signaling is one of the dominating pathways for proper cardiogenesis during development and cardiac differentiation of ESCs.[14,23] Specifically, the canonical Wnt/β-catenin signaling plays a biphasic role in cardiac differentiation[14,24,25]: it promotes cardiac mesoderm induction early on,[26,27] but inhibits the subsequent differentiation of cardiac progenitor cells into cardiomyocytes.[24,25] The non-canonical Wnt signaling that proceeds through Wnt/JNK and Wnt/Ca^{2+} pathways enhances cardiac development at both early and late stages.[28–30] Recent studies suggested a role for Wnt signaling in maintaining the *Tbx18*-positive progenitor pool in mouse embryos[31] and in cardiac SAN development in chick embryos.[7] Here, we tested the hypothesis that Wnt signaling differentially modulates the specification of pacemaker cells from the chamber myocyte lineages.

Using direct differentiation of mouse and human pluripotent stem cells as a platform, we controlled the degree of Wnt activation and analyzed for changes in the expression of genes and gene products that are enriched in the developing SAN. As a functional measure, we characterized the single-cell electrophysiology of the de novo cardiac myocytes over 2 months to phenotype their spontaneous electrical activity and action potential (AP) morphology in a longitudinal manner. Automaticity of the native SAN pacemaker cells is driven by a coupled mechanism involving two clocks: one driven by the spontaneous depolarization at the sarcolemma initiated by Hcn4 ion channels and the other driven by rhythmic releases of intracellular Ca^{2+} from the sarco/endoplasmic reticulum.[32,33] Examining the relative weight of these two clocks revealed that spontaneously beating myocytes derived with uninhibited Wnt signaling leveraged both clocks, whereas the spontaneously beating myocytes from control condition relied heavily on the Ca^{2+} clock.

2 | MATERIALS AND METHODS

2.1 | Maintenance and cardiac differentiation of mouse ESCs

R1 mouse embryonic stem cells (mESCs) were obtained from ATCC and cultured on feeder layers of mitomycin C-inactivated mouse embryonic fibroblasts (EDM Millipore) in knock-out DMEM (Life Technologies) supplemented with 15% knock-out serum replacement, 1% MEM nonessential amino acid, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1000 U/mL leukemia inhibitory factor (EMD Millipore). Culture medium was changed daily, and cells were passaged every other day at a 1:5 ratio.

Cardiac differentiation of mESCs was performed as previously described.[13] To obtain $Flk1^+$/PdgfR-α^+ cells from R1 mESCs, we optimized a protocol previously established for T-GFP mESC line[13]: treatment with 8 ng/mL Activin A and 0.5 ng/mL BMP4 for 40 hours led to >75% $Flk1^+$/PdgfR-α^+ cells from R1 mESCs (Figure S1B–D). Specifically, mESCs were trypsinized (0.05% trypsin/EDTA, Life Technologies) into single cells and seeded (D0) at 100,000 cells/mL in serum-free differentiation (SFD) medium containing 75% IMDM-GlutaMAX, 25% F12 nutrient, 1× B27 (vitamin A-free) supplement, 1× N2 supplement, 1% penicillin/streptomycin, and 4×10^{-4} M monothioglycerol (Sigma), in ultra-low attachment dishes (Corning) for 48 hours. At day 2, ESC aggregates were trypsinized (with 0.25% trypsin/EDTA for 3 minutes) into single cells and seeded in SFD medium supplemented with 5 ng/mL human VEGF, together with human Activin A and human BMP4 proteins at concentrations optimized for the R1 cell line (see above). At day 4, cell aggregates were trypsinized and $Flk1^+$/PdgfR-α^+ cells were FACS-sorted and seeded on a gelatin-coated glass surface at a density of 2×10^5 cells/cm^2 in StemPro-34 medium (Life Technologies) supplemented with 2 mM L-glutamine, 1 mM ascorbic acid, 1% penicillin/streptomycin, 5 ng/mL human VEGF, 10 ng/mL human basic FGF and 10 ng/mL human FGF10. For treatment groups, recombinant protein of Dkk1 (150 ng/mL), Sfrp1 (1 μg/mL), Sfrp5 (1 μg/mL), Wnt3a (100 ng/mL), or Wnt11 (1 μg/mL) was added from day 4 to day 8. Cell cultures were replenished daily with fresh medium with growth factors. To meet the high metabolism need of late-stage differentiated cells, a subset of spontaneously beating cardiac-differentiated cell cultures was maintained in IMDM + GlutMAX (Life Technologies) supplemented with 15% fetal bovine serum, 1% MEM nonessential amino acid, 0.1 mM β-

mercaptoethanol, and 0.5% penicillin/streptomycin. Human basic FGF was from Invitrogen and all other protein factors were purchased from R&D systems.

2.2 | Maintenance and cardiac differentiation of human pluripotent stem cells

The H9 human embryonic stem cells (hESCs), NKX2.5-GFP transgenic hESCs,[34] and a human inducible pluripotent stem cell line (hiPSCs, Exp13) were cultured in E8 medium (Stem cell Technologies) with daily media change. hESCs and hiPSCs were passaged every 5-6 days.

Cardiac differentiation of hESCs and hiPSCs was performed according to a reported protocol.[16] Briefly, undifferentiated hESCs and hiPSCs were seeded on matrigel (Corning)-coated plates at a density of 2.5×10^5 cells/cm^2 and cultured in E8 medium with 10 μM ROCK inhibitor Y27632 (Tocris) for 1-2 days until >90% confluence. To initiate cardiac differentiation, the medium was changed to differentiation medium RPMI1640 (Thermo Scientific) plus B27 (Thermo Scientific). At D0, the cells were treated with 10 ng/mL ActivinA, 10 ng/mL bFGF, and 10 ng/mL BMP4. At D3, the differentiated medium were changed to RPMI1640 plus B27 (Thermo Scientific) with or without 2 μM Wnt-C59 (Tocris). For NKX2.5-GFP transgenic hESCs, At D0 the cells were treated with 10 ng/mL ActivinA, 10 ng/mL bFGF, and 10 ng/mL BMP4. At D3, the differentiated medium were changed to RPMI1640 plus B27 (Thermo Scientific) with Noggin 100 ng/mL (R&D systems), At D5, the differentiated medium were changed to RPMI1640 plus B27 (Thermo Scientific) with or without 2 μM Wnt-C59 (Tocris). The medium was refreshed every 3 days. For cardiac differentiation of hiPSCs, to initiate cardiac differentiation, the medium was changed to differentiation medium RPMI1640 (Thermo Scientific) plus B27 without insulin (Thermo Scientific). At D0, the cells were treated with 6uM CHIR99021 (Tocris); at D2, the differentiated medium was changed to RPMI1640 plus B27 without insulin (Thermo Scientific). At D3, the cells were treated with gradient Wnt-C59, starting from 0 to 2 μM. The medium was refreshed every 3 days. The cardiomyocytes were collected on D14 or D20 for further analysis.

2.3 | Flow cytometry

D4mESC aggregates were trypsinized (0.25% trypsin/EDTA for 5 minutes) into single cells and incubated with phycoerythrin-conjugated anti-mouse Flk1 antibody and allophycocyanin-conjugated anti-mouse PdgfR-α antibody (eBiosciences, San Diego, California) at 4°C for 50 minutes. Cells were washed twice with 1.5% bovine serum albumin/phosphate-buffered saline (BSA/PBS) and sorted on a MoFlow cell sorter (Dako Cytomation, Carpinteria, California). Data were analyzed with FlowJo software (version 7.6.5; Tree Star, Inc, Ashland, Oregon). For quantification of cTnT-positive cells at day 8 of differentiation, cardiomyocyte monolayers were dissociated into single cells with collagenase B, fixed with 4% paraformaldehyde at 4°C for 20 minutes, and permeabilized in 0.1% Triton X-100 /1.5%BSA/PBS at room temperature for 10 minutes. Mouse monoclonal anti-cTnT antibody (Thermo Scientific) and a mouse IgG_1 isotype control antibody (Cell Signaling) were conjugated with a Zenon Alexa Fluor 405 mouse IgG1 labeling kit (Life Technologies) according to manufacturer's instruction. Cells were incubated with either the conjugated anti-cTnT antibody or the conjugated isotype control antibody for at 4°C for 50

minutes, washed twice, and cTnT-positive cells were quantified with a CyAn ADP flow cytometer (Dako) and the Summit 4.1 software package (Dako).

2.4 | Real-time quantitative PCR and gene arrays

Total RNA was isolated from differentiating cells with an RNeasy mini kit (Qiagen), and cDNA was synthesized with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California) according to manufacturer's instructions. In some studies, a Cells-to-Ct kit (Ambion) was used for RNA sample preparation and cDNA synthesis. Real-time quantitative PCR was performed with Taqman Gene Expression Assays (Life Technologies, Assay IDs are listed in Table S2) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Transcript level of target genes was normalized to that of 18S rRNA in the same sample and presented as $\times 10^7$ fold. A set of Wnt-related genes were examined with RT.^{2} Profiler PCR Arrays (PAMM-043Z, Qiagen).

2.5 | Immunostaining, blotting, and enzyme-linked immunosorbent assay

Differentiating cells cultured on gelatin-coated 8-chamber culture slides (BD Biosciences) were fixed with 100% methanol at −20°C for 10 minutes. Cells were washed with PBS for three times and permeabilized in 1% Triton X-100/PBS at room temperature for 35 minutes, followed by blocking in 0.1% Triton X-100/2% BSA/PBS for 1 hour. Cells were then incubated with primary antibodies at 4°C for overnight, followed by Alexa Fluor-conjugated secondary antibody incubation the next day. Mouse monoclonal anti-α-sarcomeric actinin (anti-α-SA; Sigma, 1:400), rabbit anti-Hcn4 (Alomone Labs, 1:100), and rabbit anti-Tbx3 (Abcam, 1:200) were used for primary antibodies. Secondary antibodies were donkey anti-mouse (Alexa Fluor-568, 1:600) and donkey anti-rabbit (Alexa Fluor-488, 1:600) immunoglobulin G.

For immunoblotting, cell lysates in RIPA buffer were run on a 4%-12% sodium dodecyl sulfate-polyacrylamide gel (10 μg per lane) and transferred onto a polyvinylidene difluoride membrane. The transferred membrane was incubated with a primary antibody overnight at 4°C, followed by a 2-hour incubation with a peroxidase-conjugated secondary antibody (1:2000). Primary antibodies used were rabbit anti-Nkx2.5 (Abcam, 1:1000), rabbit anti-Hcn4 (Abcam, 1:1000), mouse anti-cardiac troponin T (Abcam, 1:2000), mouse anti-Tbx18 (R&D system, 1:1000), rabbit anti-phospholamban phospho Serine-16 (EMD Millipore), and mouse anti-total phospholamban (Thermo Scientific). For loading control, HRP-conjugated mouse anti-β-actin (Sigma, 1:50,000) was used.

For enzyme-linked immunosorbent assay (ELISA), conditioned media from differentiating Flk1$^+$/PdgfR-α$^+$ cells were collected at days 5-8. Media were centrifuged at 1000g for 1 minute to remove cell debris, added with 1× Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) and stored at −80°C. Endogenous Dkk1 protein level in conditioned media was determined with a mouse Dkk1 Quantikine ELISA kit (R&D Systems) according to manufacturer's instruction.

2.6 | Intracellular calcium transient recording

For measurements of intracellular Ca^{2+} oscillations, cells were dissociated and seeded at 65,000 cells/cm^2 on 35-mm glass-bottom dishes (MatTek Cultureware). Cells were loaded with Cal-520™ (10 μM, AAT Bioquest Inc, Sunnyvale, California) for 1 hour at room temperature. Live-cell Ca^{2+} imaging was performed on a Nikon Eclipse 2000 inverted microscope equipped with a spinning disk confocal system (PerkinElmer Life Sciences) in normal Tyrode's solution at 30°C ± 2°C. Cardiomyocytes were field-stimulated at 1 or 2 Hz with a pair of platinum wires connected to an isolated pulse stimulator (Model 2100; A-M Systems, Carlsborg, Waltham). Spontaneous calcium transients of cells were recorded in the presence of a gap junction uncoupler, palmitoleic acid (Sigma, 10 μM). Offline analysis was performed using ImageJ.

2.7 | Isolation of myocytes from the SAN and neonatal rat ventricles

Single SAN myocytes were isolated from adult mouse hearts (C57BL/6, 8-12 weeks, Charles River Laboratories) as described previously.[35] The SAN was dissected and digested by collagenase type II (Worthington Biochemical), protease type XIV (Sigma-Aldrich), and elastase (Worthington Biochemical) for 30-35 minutes at 37°C in a modified Tyrode's solution containing (in mM): NaCl 140, KCl 5.4, KH_2PO_4 1.2, HEPES 5, taurine 50, glucose 18.5, $CaCl_2$ 0.066, and 1 mg/mL BSA; pH adjusted to 6.9 with NaOH. The SAN tissue was then transferred to a Kraft-Brühe (KB) solution containing (in mM): K-glutamate 100, K-aspartate 10, KCl 25, KH_2PO_4 10, $MgSO_4$ 2, taurine 20, creatinine 5, EGTA 0.5, glucose 20, HEPES 5, and 1.0% BSA; pH adjusted to 7.2 with KOH at 37°C. Cells were dispersed by gentle pipetting with a wide-mouth glass pipette. Ca^{2+} was gradually reintroduced to a final concentration of 1.4 mM, and cells were kept at room temperature for up to 6 hours for electrophysiology experiment. Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day-old neonatal rats and cultured on coverslips for 2 days before electrophysiology experiments. For pacing studies, NRVMs were plated in 24-well plates, and quiescent wells were selected for studies. Cell aggregates from Wnt-uninhibited medium (WM) condition were generated by a hanging-drop method described for mESC differentiation[20] and plated on top of NRVM monolayers. Beating of the cell aggregates and NRVMs were examined 3 to 4 days later.

2.8 | Electrophysiology

Monolayers of mESC-derived cardiomyocytes were dispersed into single cells with a protocol modified from a previous report.[36] Cell monolayers were digested with a nominally calcium-free solution (in mM: NaCl 140, KCl 5.4, $MgCl_2$ 0.5, $KH_2PO_4$1.2, HEPES 5, taurine 50, glucose 5.5, pH = 6.9 with NaOH) containing collagenase B (Roche, 0.5 mg/mL) at 37°C for 50 minutes followed by digestion with Liberase (Roche, 75 μg/mL) at 35°C for 20 minutes. Whole-cell electrophysiology recordings were performed using nystatin-perforated, whole-cell patch-clamp technique with an Axopatch 200B amplifier (Axon instruments) with a sampling rate of 20 kHz, and low-pass Bessel-filtered at 5 kHz. Experiments were performed at 36°C ± 1°C with cells perfused with a normal Tyrode's solution containing (mM): NaCl 138, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.5, glucose 10, and HEPES 10, pH = 7.4 with NaOH. Microelectrodes had tip resistances of 2 to 4 MΩ when filled with

the internal pipette solution, containing (mM): K-glutamate 98, KCl 50, $MgCl_2$ 1.0, HEPES 10, and 200 μg/mL nysatin, pH = 7.2 with KOH. Spontaneous APs were recorded in an $I = 0$ mode. Data were corrected for the estimated liquid junction potentials (−12.5 mV). Diastolic depolarization rate was determined from the slope of a 20-millisecond segment after the maximum diastolic potential. Purkinje cell APs were identified by their characteristic fast phase 1 repolarization with a large spike[37] and frequent early after depolarizations. Pacemaker cells were defined as cells with an action potential duration (APD_{90}) <92 milliseconds (the highest value observed in mouse SAN cells) and a beating rate >180 bpm. Myocytes with an APD_{90} >100 milliseconds and a spontaneous beating rate <180 bpm were categorized as atrial or ventricular myocytes. Myocytes that do not fall into any of the above groups were considered an intermediate type.[19] Funny current (I_f) was recorded in Tyrode solution containing 1mM $BaCl_2$ with a holding potential of −35 mV and a family of voltage steps from −25 to −135 mV for 1.5 seconds with 10 mV increment. I_f density was measured as ivabradine-sensitive current (Sigma, 10 μM).

2.9 | Statistical analysis

Data are expressed as mean ± SEM with $P < .05$ considered significant. Differences between two means were evaluated by Student's *t*-test. Differences among multiple means were assessed by one-way analysis of variance (ANOVA). When significance was detected by ANOVA, differences among individual means were evaluated post hoc by Bonferroni's test.

3 | RESULTS

3.1 | Flk1$^+$/PdgfR-α$^+$ progenitors give rise to pacemaker cell precursors as well as chamber cardiomyocytes

A population of cardiovascular progenitor cells has been identified by their cell surface expression of Flk1 and PdgfR-α proteins.[13] These progenitors are multipotent, capable of differentiating into cardiomyocytes and vascular lineages.[13,38] To obtain Flk1$^+$/PdgfR-α$^+$ cells, R1 mouse ESCs were treated with optimized concentrations of Activin A and BMP 4 (Figure 1A, Figure S1). The high expression levels of *Brachyury* (*T*), a pan-mesodermal marker and *Mesp1*, the earliest cardiac progenitor marker, in Flk1$^+$/PdgfR-α$^+$ cells at day 4 (Figure 1B) suggest that they are cardiac mesodermal progenitor cells. Accordingly, when Flk1$^+$/PdgfR-α$^+$ cells were FACS-purified (Figure 1A, day 4) and cultured as monolayers in differentiation medium, α-SA-positive cardiomyocytes were routinely observed (Figure 1A, day 8).

The SAN pacemaker is derived from mesenchymal progenitors in the sinus venosus region that expresses *Tbx18* and *Shox2*,[2,8] whereas atrial/ventricular myocytes are developed from *Nkx2.5*$^+$ first heart field and *Nkx2.5*$^+$/*Isl1*$^+$ second heart field progenitors. We set out to examine if Flk1$^+$/PdgfR-α$^+$ cardiac mesodermal cells have the potential to differentiate to the three different progenitor pools. Examination of heart field marker genes in the differentiating progenitor cells revealed that their sequence of appearance (ie, *Isl1, Nkx2.5, Shox2*, and *Tbx18* from early to late, Figure 1C, D) mirrored their expression pattern in the developing mouse heart: *Isl1* (E7),[5] *Nkx2.5* (E7.5),[4] *Shox2* (E8.5),[3] and *Tbx18* (E9-9.5).[8,39] Specifically, *Isl1* and *Nkx2.5* were first detected at days 5 and 6, respectively (Figure 1C).

Hcn4, a marker transiently expressed in the first heart field[40,41] peaked at day 6 (Figure 1C). In contrast, *Shox2* and *Tbx18*, two marker genes for the SAN pacemaker lineage, were not expressed until day 7 of differentiation (Figure 1D). The deferred expression of pacemaker lineage markers during mESC differentiation is consistent with embryonic heart development in which pacemaker cell lineage development is preceded by the first and second heart field lineages.[42] These results indicate that $Flk1^+$/PdgfR-α^+ cells are capable of differentiating into all three different cardiac progenitor pools, validating their use as a model to study chamber myocyte/pacemaker subtype specification.

3.2 | Canonical Wnt signaling promotes pacemaker lineage specification from mESCs

Canonical Wnt signaling is thought to inhibit late-phase cardiogenesis.[23] Dickkopf-1 (Dkk1) is a secreted inhibitor of the canonical Wnt pathway and is endogenously expressed in differentiating $Flk1^+$/PdgfR-α^+ cells at ~3 ng/mL (Figure S2A). Saturating levels of exogenous Dkk1 is often added to culture media to enhance overall cardiac differentiation.[13] We asked if active Wnt signaling positively regulates cardiac pacemaker lineage specification by culturing the $Flk1^+$/PdgfR-α^+ cells either in the differentiation medium containing 150 ng/mL recombinant Dkk1 from day 4 to day 8 (cardiac differentiation medium, CDM) or in the differentiation medium without exogenous Dkk1 protein (WM). Consistent with an inhibitory role of Wnt in cardiogenesis,[23] WM group exhibited a reduced level of cardiac troponin T (cTnT) proteins (Figure 1E) and a less number of cTnT-positive cells (Figure S2B) compared with CDM group. This corresponded to an output of three cTnT-positive cardiomyocytes per single $Flk1^+$/PdgfR-α^+ progenitor cell in WM group and four cardiomyocytes for CDM group (Figure S2C). The ratio of cardiac TnI (cTnI, *Tnni3*) to slow skeletal TnI (ssTnI, *Tnni1*) levels, a marker for cardiomyocyte maturity,[43] is not significantly different ($P = .2$) between WM (0.23 ± 0.01) and CDM (0.26 ± 0.02) groups (Figure S2D), suggesting similar degree of maturity.

The expression levels of SAN pacemaker lineage marker genes, *Shox2* and *Tbx18*, were higher in WM than in CDM and the atrial/ventricular myocyte lineage marker, *Nkx2.5*, was lower in WM than in CDM ($P<0.01$, Figure 1C, D). In addition, genes enriched in pacemaker myocytes *Tbx3, Cx45*, and *Cx30.2*[8] were expressed at a higher level in WM, whereas genes that are highly expressed in atrial and ventricular myocytes, *Nppa*[44,45] and *Scn5a* (encoding $Na_v1.5$),[46] respectively, were lower ($P < .01$) in WM. *Cacna1c* (encoding $Ca_v1.2$) was similar ($P = .93$) between CDM and WM groups when normalized to cTnT levels (Figure 1F), consistent with the fact that the $Ca_v1.2$ channel (mediating cardiac L-type Ca^{2+} current) is important for both the upstroke of pacemaker APs[47] and the plateau phase of atrial/ventricular myocyte APs. Accordingly, Nkx2.5 protein level was lower, whereas Tbx18 protein level was higher in the WM group (Figure 1G). *Hcn4* is transiently expressed in the first heart field but becomes restricted to the nodal tissues at a late stage.[40,41] *Hcn4* level peaked at day 6 in CDM and returned to a baseline in 2 days, a time frame in which expression of a first heart field marker, *Nkx2.5*, started to rise (Figure 1C). In contrast, *Hcn4* expression sustained and reached a plateau at day 7 in WM at the same time when *Shox2* and *Tbx18* expression spiked (Figure 1D). CD166 has been shown to be selectively expressed in $Hcn4^+$ cells at day 8 of serum-driven mESC differentiation but becomes nonspecifically expressed thereafter.[21] Under our experimental conditions, CD166

expression was higher in WM at day 7, but not at day 6 or day 8 (Figure S2E). As expected, expression of a pluripotency gene, Oct4, decreased dramatically after day 4 (Figure S2F). Taken together, our data indicate that canonical Wnt signaling increases cardiac pacemaker cell gene expression at the expense of chamber cardiomyocyte specification.

We asked if endogenously secreted Dkk1 in WM could still suppress Wnt activation. We treated WM cells with a neutralizing anti-Dkk1 antibody, which reduced free Dkk1 protein level by 90.4% (0.2 ± 0.1 ng/mL vs. 2.5 ± 0.2 ng/mL, with or without anti-Dkk1 antibody, respectively, Figure S2G). Inhibition of endogenous Dkk1 further enhanced *Tbx18 and Hcn4* expression beyond the levels observed in WM (Figure S2H), indicating that endogenously secreted Dkk1 could still suppress Wnt signaling and the subsequent cardiac pacemaker cell specification. Interestingly, addition of Dkk1 antibody in WM did not affect *Nkx2.5* expression but increased *Isl1* and *cTnT* levels (Figure S2I). Previous studies have shown that *Isl1*, although being a second heart field marker, is also expressed in a subset of SAN cells during mouse development at E11.5,[48] E14.5,[49] and E16.5,[41] as well as in zebrafish.[50] Immunostaining indicated that the proportion of $Hcn4^+$ cardiomyocytes over α-SA^+ total cardiomyocytes (Figure 2A, Figure S3A) was higher in WM than in CDM (34% ± 3%, n = 10 fields of view vs.12% ± 4%, n = 7, $P < .01$). Similarly, a higher percentage of $Tbx3^+$ cardiomyocytes[44,45] was found in WM than in CDM (29% ± 2% vs. 12% ± 3%, n = 5 fields of view, $P < .01$, Figure 2B, Figure S3B). Furthermore, a higher ratio of Hcn4-to-cTnT proteins was observed in WM compared with CDM (Figure 2C). Together, the data demonstrate increased pacemaker cell specification when endogenous Wnt signaling was uninhibited.

3.3 | Canonical Wnt signaling promotes SAN-like pacemaker cell generation from mESCs

Compared with Chamber myocytes, SAN-like pacemaker cells beat faster and have a relatively positive maximum diastolic potential, a small phase 0 upstroke velocity and prominent phase-4 depolarization maintaining SAN pacemaker dominance,[51] which means higher If density. During cardiac differentiation, spontaneously beating regions were first observed at day 6.5 in both CDM and WM groups. Beating rates in the CDM group rose to 125 ± 6 bpm at day 10 and declined thereafter, leveling off at 41 ± 4 bpm at day 17 (n = 12, Figure 3A); this is consistent with stage-dependent slowing of the beating rates observed in mouse and human ESC-derived embryoid bodies.[52–54] In contrast, beating rates in the WM group continued to increase, reaching 154 ± 13 bpm at day 23 (n = 10, Figure 3A). To assess the number and frequency of autonomously beating myocytes, gap junctional communication between individual cardiomyocytes from 3- to 4-week monolayers were uncoupled with 10 μM palmitoleic acid.[55] More myocytes exhibited spontaneous Ca^{2+} oscillations in WM than in CDM (22% ± 3% vs. 8% ± 3%, $P < .01$, n = 5 monolayers, Figure 3B, Figure S4A). The rate of spontaneous Ca^{2+} oscillations was also higher in WM (44 ± 3 bpm, n = 27 cells vs. 20 ± 2 bpm in CDM, n = 13 cells, $P < .01$, Figure S4B). Index of automaticity, defined as the product of the rate and percentage of spontaneously beating cells,[56] was 6.2-fold higher in WM than in CDM (Figure S4C).

Consistent with previous studies,[19] we could identify APs of all major cardiac subtypes when freshly isolated, single myocytes from cultures of days 20-40 were recorded by

nystatin-perforated whole-cell patch-clamp (Figure 3C). Of note, 73% of the WM cells exhibited spontaneous APs (Figure 3C, F), which resembled the AP profile recorded from native mouse SAN pacemaker cells (Figure 3D). Specifically, the nodal-type cells in WM exhibited faster beating rate (292 ± 14 bpm), gradual phase 4 depolarization (113 ± 11 mV/s), short AP duration at 90% repolarization (APD_{90}, 58 ± 3 milliseconds), and a small AP amplitude (78 ± 3 mV) (Figure 3G, Table S1). WM myocytes at a later stage (40-60 days, Figure S4D, E) maintained the pacemaker phonotype with fast automaticity and short AP duration, suggesting that the nodal-type myocytes are pacemaker cells rather than immature cardiomyocytes. Conversely, when the $Flk1^+$/PdgfR-α^+ cells were cultured in CDM, 39% of the cells showed APs characteristic of the chamber (atrial/ventricular) myocytes with a slow beating rate of 81 ± 12 bpm, longer APD_{90} of 229 ± 41 milliseconds, and a large amplitude of 94 ± 4 mV (Figure 3C, F, Table S2). Spontaneous APs were measured from neonatal rat ventricular myocytes as a comparative measure of spontaneous electrical activity from immature chamber cardiomyocytes (Figure 3E). Only 13% of the CDM myocytes displayed nodal-like APs (Figure 3F, Table S2). Of note, 10% of the CDM cells showed Purkinje-like APs with a large upstroke amplitude (132 ± 9 mV) and fast phase 1 repolarization (APD_{40}, 3 ± 2 milliseconds) with frequent early after depolarizations (Figure 3C, F, Table S2). Chamber cardiomyocytes of the atria and the ventricles exhibit hyperpolarized and stable resting membrane potential during diastole due to strong inward rectifier K^+ current, I_{K1}.[57–59] In contrast, pacemaker cells in the SAN exhibit spontaneously depolarizing diastolic potential driven by “funny current,” (I_f, and lack of I_{K1}.[51] We measured (I_f density from the two groups of myocytes to understand their comparative ability to spontaneously depolarize. WM myocytes exhibited a much higher I_f density than CDM myocytes did (−6 ± 1 vs. −2 ± 0.5 pA/pF at −105 mV, $P < .01$; Figure 4A, B). Still, the I_f density in WM cells was relatively smaller than that reported for adult mouse SAN cells (eg, −15 pA/pF, −90 mV),[60] suggesting that the de novo pacemaker cells maintained in culture may be less developed than adult SAN pacemaker cells.

3.4 | WM pacemaker cells from mESCs are driven by both membrane and Ca^{2+} clock mechanisms

The automaticity of native SAN pacemaker cells are driven by ion channels on the sarcolemma (the “membrane-clock”) and the spontaneous Ca^{2+}-release events from the sarcoplasmic reticulum (“Ca^{2+} clock”) mechanisms.[32] In order to understand relative contributions of membrane-clock or Ca^{2+}-clock mechanisms[32,33] of automaticity in WM and CDM cells, an I_f blocker ivabradine was applied to minimize the membrane clock, whereas caffeine and thapsigargin were applied to debilitate the Ca^{2+}-clock. Ivabradine reduced the rate of single-cell automaticity in the WM group by 57% ± 7% (n = 12), whereas its effect was smaller in the CDM group (32% ± 6%, n = 6, $P < .05$, Figure 4C, E). Co-application of caffeine and thapsigargin slowed down the rate of spontaneous AP oscillations in the WM group by 58% ± 15% (n = 7), which is consistent with previous studies in rabbit SAN myocytes showing that inhibition of the Ca^{2+} clock with ryanodine slowed, but did not stop, spontaneous AP oscillations.[61] In contrast, the effect of suppressing calcium cycling with caffeine and thapsigargin dramatically reduced spontaneous AP oscillations in CDM myocytes (89% ± 6%, n = 9, $P < .05$, Figure 4D, E). Continuous recording of the oscillating APs illustrated that CDM myocytes gradually ceased to fire APs

within 5 minutes of washing in caffeine and thapsigargin (Figure S5, top). In contrast, WM myocytes appeared to lose the automaticity upon washing in of caffeine and thapsigargin but then recovered the rhythmic APs albeit at a slower rate (Figure S5, bottom). The ratio of rate reduction induced by ivabradine to that by caffeine/thapsigargin illustrates a balanced contribution of membrane-clock and Ca^{2+}-clock mechanisms in the WM group (0.9 ± 0.2, n = 5); in contrast, Ca^{2+} cycling is the major driver of spontaneous activity in the CDM group (0.4 ± 0.06, n = 5, $P < .05$, Figure 4E). These results indicate that pacemaker cells in WM utilize both the membrane-clock and the Ca^{2+}-clock mechanisms for pacemaking, similar to native SAN pacemaker cells,[32] whereas the spontaneous activity in CDM relies on oscillations of Ca^{2+} release from intracellular stores. Native pacemaker cells exhibit a higher basal cAMP/PKA activity than atrial/ventricular myocytes,[62] which is critical for their pacemaker activity by coupling the Ca^{2+} and membrane clocks. WM group had a higher phosphorylation level of phospholamban (Figure S6A), indicating enhanced cAMP/PKA activities in these pacemaker cells. Taken together, the data demonstrate that active Wnt signaling during ESC differentiation promotes generation of genuine cardiac pacemaker cells.

Enhanced cAMP/PKA signaling would lead to higher autonomic response in WM pacemaker cells. Indeed, WM pacemaker cells accelerated and decelerated significantly more in response to β-adrenergic stimulation with isoproterenol and cholinergic suppression with acetylcholine, respectively (Figure S6B, C). We asked if the superior automaticity of pacemaker cells in WM would allow them to pace a monolayer of ventricular myocytes with nominal spontaneous activity. Small aggregates of WM myocytes were formed by hanging drop and then plated on top of NRVM monolayers. Three days later, the co-culture of WM myocyte aggregates and NRVM monolayer illustrated synchronous contractions, suggesting electrical coupling between the two cell types (Video S4). The spontaneous beating rate of co-cultured WM myocytes and NRVMs was faster than that of monolayers of NRVMs without WM myocytes (212 ± 11 bpm, n = 4, vs. 51 ± 25 bpm, n = 9, $P < .01$). Noting that the spontaneous beating rate of the co-cultures was similar to that of WM myocytes alone (Figure S6C), these data suggest that WM myocytes are able to pace the neighboring ventricular myocytes.

3.5 | Canonical Wnt signaling promotes pacemaker lineage specification from mESCs

Gene array indicated that three canonical Wnt ligands (*Wnt3, Wnt2*, and *Wnt2b*) as well as two non-canonical Wnt ligands (*Wnt5b* and *Wnt11*) were highly expressed at the cardiac mesodermal stage (day 4, Figure S7A, left). By day 6, the WM group expressed a higher level of *Wnt3* but a lower level of *Wnt11* than the CDM group (Figure S7A, right). Consistent with a higher activity of Wnt/β-catenin pathway in WM, Wnt/β-catenin target genes, *Axin*[63], *Lef1*[64], and *Pitx2*[65] were increased in WM (Figure S7A, right).

Secreted frizzled-related proteins (Sfrp) bind to Wnt ligands preventing Wnt ligand-receptor interaction.[66] A 4-fold higher *Sfrp1* and a 12-fold increase in *Sfrp5* mRNA levels were observed at day 8 in WM compared with CDM (Figure S7B). This is in line with earlier studies indicating that activation of the Wnt/β-catenin pathway is followed by upregulated expression of secreted Wnt inhibitors as a negative feedback mechanism.[67,68] To investigate

if Sfrp proteins affect cardiac lineage differentiation, recombinant Sfrp1/5 proteins were added to the WM culture media. A high dose of Sfrp 1/5 (1 μg/mL), but not a low dose (0.1-0.3 μg/mL), significantly reduced expression of *Shox2* and *Tbx18* (Figure S8A and B). The reduced expression of *Shox2* and *Tbx18*, but increased expression of *Nkx2.5* (Figure S8A, right panel), by Sfrp1/5 are consistent with similar changes effected by Dkk1 (Figure 1).

Treating the $Flk1^+$/PdgfR-α^+ cardiac progenitor cells with Wnt3a (a canonical Wnt/β-catenin pathway ligand) increased expression of the pacemaker lineage marker genes *Shox2* and *Tbx18*, while reducing expression of a chamber cardiomyocyte gene *Nkx2.5* (Figure 5A). Conversely, treatment with Wnt11 (a non-canonical Wnt pathway ligand) decreased *Shox2* expression while increasing *Nkx2.5* expression (Figure 5A). Specific degradation of β-catenin with a small-molecule blocker, IWR-1,[69] reduced *Shox2* and *Tbx18* expression but increased *Nkx2.5* and *Isl1* expression (Figure 5A). Furthermore, Wnt3a-treated WM myocytes expressed a higher level of Hcn4 protein but a lower level of cTnT protein (Figure 5B). Interestingly, Wnt3a-treatment led to formation of numerous independent, spontaneously beating foci in WM monolayers. In contrast, monolayers of CDM myocytes as well as monolayers of WM myocytes without Wnt3a exhibited syncytial contractions (Videos S1–S3). The spontaneously beating colonies in Wnt3a-treated WM myocytes were strongly positive for Hcn4 and Tbx3 (Figure 5C) and showed faster automaticity compared with monolayers grown in CDM or WM without Wnt3a (Figure 5D). Taken together, the data demonstrate that the canonical Wnt pathway promotes pacemaker lineage specification.

3.6 | Wnt signaling specifies SAN-like pacemaker cells in human ESCs and human iPSCs

We tested if Wnt-induced cardiac pacemaker cell specification could be generalized to human pluripotent stem cells. Cardiac differentiation of hESCs was performed according to a Wnt inhibition protocol[16,70] in which mesoderm progenitors are directed to cardiac mesoderm with a small molecule Wnt inhibitor (Wnt-C59) during D5-D8 of differentiation (Fig. 6A). In the following experiments with hESCs, CDM is defined as the control condition with Wnt-C59, whereas WM is defined as the condition without Wnt-C59. Cardiac differentiation of H9 hESCs in WM led to significantly higher expression levels of cardiac pacemaker-enriched genes such as *SHOX2, TBX18*, and *TBX3* compared with the differentiated cardiomyocytes in CDM (Figure 6B). Similar to the mouse ESC data, the total yield of $CTNT^+$ cardiomyocytes was ~50% lower in WM compared with that in CDM (Figure 6B, right panel). We examined a second hESC line which harbors a GFP reporter in exon 1 of the *NKX2.5* locus in one of the two alleles.[34] This transgenic NKX2.5-GFP line of H3 hESCs enables real-time monitoring of developing chamber cardiomyocytes from $NKX2.5^+$ progenitors[34] as well as nodal-like myocytes from $NKX2.5^-$ progenitors.[71] Cardiac differentiation of H3 NKX2.5-GFP hESCs led to a significantly higher proportion of $NKX2.5^-$ $CTNT^+$ cardiomyocytes over total $CTNT^+$ cardiomyocytes in WM compared with in CDM (Figure 6C, D). Again, the total cardiomyocyte pool over all cells was ~50% lower in WM compared with in CDM (Figure 6D, right panel). Similar to the data from H9 hESC line, expression levels of nodal pacemaker-enriched genes such as *SHOX2, TBX18, TBX3*, and *HCN4* were higher in WM than in CDM (Figure 6E). To probe nodal gene expression at the level of single myocytes, the de novo cardiomyocytes were immunostained

for TBX3. The majority of $CTNT^+$ cardiomyocytes were $TBX3^+$ in WM, whereas they were mostly $TBX3^-$ in CDM (Figure 7A, B, 83% ± 7% vs.17% ± 4%, n = 3, $P < .05$). We sought to gain more quantitative measure of nodal cell yield by flow cytometry. Unfortunately, none of the commercially available antibodies that we tested against TBX18, TBX3, and SHOX2 were able to discern cells over isotype controls for flow cytometry applications (data not shown, please refer to Methods for antibody information). Finally, we used hiPSCs derived from cord blood of a healthy newborn. Cardiac differentiation of hiPSC lines often require optimizing the concentrations of growth factors and/or small molecules,[71] and we determined a small molecule-based protocol is superior to a growth factor-based protocol for maximizing cardiac mesoderm specification (Figure 7C). We first optimized the cardiac differentiation protocol by performing a dose-response experiment by varying the concentration of a small molecule Wnt inhibitor, Wnt-C59. Unlike the two hESC lines examined above, the hiPSC line failed to yield $cTNT^+$ cardiomyocytes when Wnt-C59 was completely withdrawn and reached maximal cardiomyocyte yield at concentrations above 0.1 μM (Figure 7D). Expression levels of cardiac pacemaker-enriched genes such as *SHOX2, TBX18, TBX3*, and *HCN4* were the highest at the lowest dose (0.01 μM) of Wnt-C59 (Figure 7E). An exception was *TBX18*, which was expressed the most when Wnt-C59 was omitted (Figure 7E, top right). Noting that TBX18 is expressed not only in the developing SAN but also in the proepicardium,[72] this observation could be due to increased derivation of epicardial lineage cells in the absence of Wnt inhibition. Taken together, these data demonstrate that canonical Wnt pathway promotes pacemaker lineage specification from human pluripotent stem cells.

4 | DISCUSSION

In this study, we report a bifurcating pathway dictated by Wnt signaling which strongly influences pluripotent stem cell-derived, $Flk1^+$/PdgfR-α^+ cardiac mesodermal cells to differentiate into either SAN-like pacemaker cells or chamber cardiomyocytes. Withdrawal of a natural Wnt inhibitor, Dkk1, increased transcript levels of *Shox2, Tbx18*, and *Hcn4* as well as the number of Tbx3 and Hcn4-positive cardiomyocytes compared with control at 3 weeks after the start of differentiation (Figure 1D). Supplementing WM with Wnt3a, an activator of the β-catenin-mediated Wnt pathway, further potentiated expression of pacemaker cell lineage markers (Figure 5A–C). Canonical Wnt signaling antagonized cardiac myocyte generation, decreasing the expression of genes characteristic of the first and second heart fields (*Nkx2.5, Isl1, Scn5a*, Figures 1C, E, and 5A), resulting in lower yield of total cardiomyocytes in WM or WM+Wnt3a (Figures 2A and 5C). This is consistent with reports that early-stage Wnt/β-catenin signaling during ESC differentiation reduces *Nkx2.5* expression and cTnT-positive cells.[24,25]

The percentage of pacemaker-like myocytes (Figure 3F) and their beating rates (Figure 5D) were significantly higher in WM than in CDM. These findings are in line with an earlier work in which Wnt3 increased the rate of diastolic depolarization in beating cardiac tissues.[7] Time-dependent cardiomyocyte maturation process (from days 20 to 60) did not appear to make a significant impact on the molecular determinants of automaticity and the shape of the WM pacemaker-like myocytes' APs (Figure 3G). This does not mean that the de novo cardiomyocytes did not advance in their maturation state during the 2 months of culture.

This notion warrants further investigation. The automaticity of genuine SAN pacemaker cells involves both membrane-clock and Ca^{2+}-clock mechanisms[32,33]; in contrast, the spontaneous activity of immature cardiomyocytes originates primarily from Ca^{2+} cycling.[73,74] Nodal pacemaker-like cardiomyocytes in WM exhibited a balanced dependence on membrane- and Ca^{2+}-clock mechanisms, similar to genuine SAN cells, whereas spontaneously beating cardiomyocytes in CDM relied heavily on intracellular Ca^{2+} cycling mechanism.

When cultured in CDM, $Flk1^{+}$/PdgfR-α^{+} progenitor cells expressed >2-fold higher Wnt11 compared with those cultured in WM (Figure S7A, right panel; Figure S7C). Addition of exogenous Wnt11 upregulated *Nkx2.5*, while downregulating *Shox2* (WM+Wnt11, Figure 5A). Since Wnt11 is known to promote cardiomyogenesis in an intracellular Ca^{2+}-dependent pathway,[28,30,75] our data indicate that suppression of Wnt/β-catenin pathway by Dkk1 not only downregulates nodal cell specification but also promotes chamber cardiomyogenesis.

Given the apparent importance of Wnt/β-catenin signaling in ESC-derived nodal pacemaker cell specification, it is unexpected that conditional knockout of the β-catenin gene, *Ctnnb1*, via a *Tbx18* promoter-driven Cre did not appreciably affect SAN formation.[31] Tbx18 is a transcription factor known to be imperative for proper SAN development.[8] A potential explanation is that Wnt/β-catenin signaling may be an upstream regulator of *Tbx18*-mediated pacemaker tissue specification, a notion that warrants further investigation.

Our data indicate that $Flk1^{+}$/PdgfR-α^{+} progenitor cells express both canonical and non-canonical Wnt ligands (Figure S7A), suggesting that other temporal and/or spatial cues are in play to direct the cardiac mesodermal progenitors to either the nodal or chamber sublineages. We conclude that canonical Wnt ligands promote nodal pacemaker myocyte specification at the expense of chamber cardiomyocyte generation, reciprocating the effects of non-canonical Wnt ligands (Figure 7F). Such opposing roles of β-catenin-dependent vs. -independent Wnt signaling are common design principles in multiple biological circuits, including heart, kidney, and lymphoid development[23,76,77] as well as zebrafish fin regeneration.[78] Manipulation of the Wnt pathway represents a new strategy to enrich either the pacemaker cell population for creating biological pacemakers,[79,80] or, conversely, atrial/ventricular myocytes for cell replacement therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance statement

Despite recent advances in cardiac chamber-specific differentiation of pluripotent stem cells, molecular drivers that dictate cardiac pacemaker cell differentiation are not well understood. The present study demonstrates that Canonical Wnt signaling promotes cardiac pacemaker lineage from $Flk1^{+}$/PdgfR-α^{+} cardiac progenitors. The de novo pacemaker cells exhibit hallmark features of native cardiac pacemaker cells, attesting to their therapeutic potential for cardiac rhythm-related diseases.

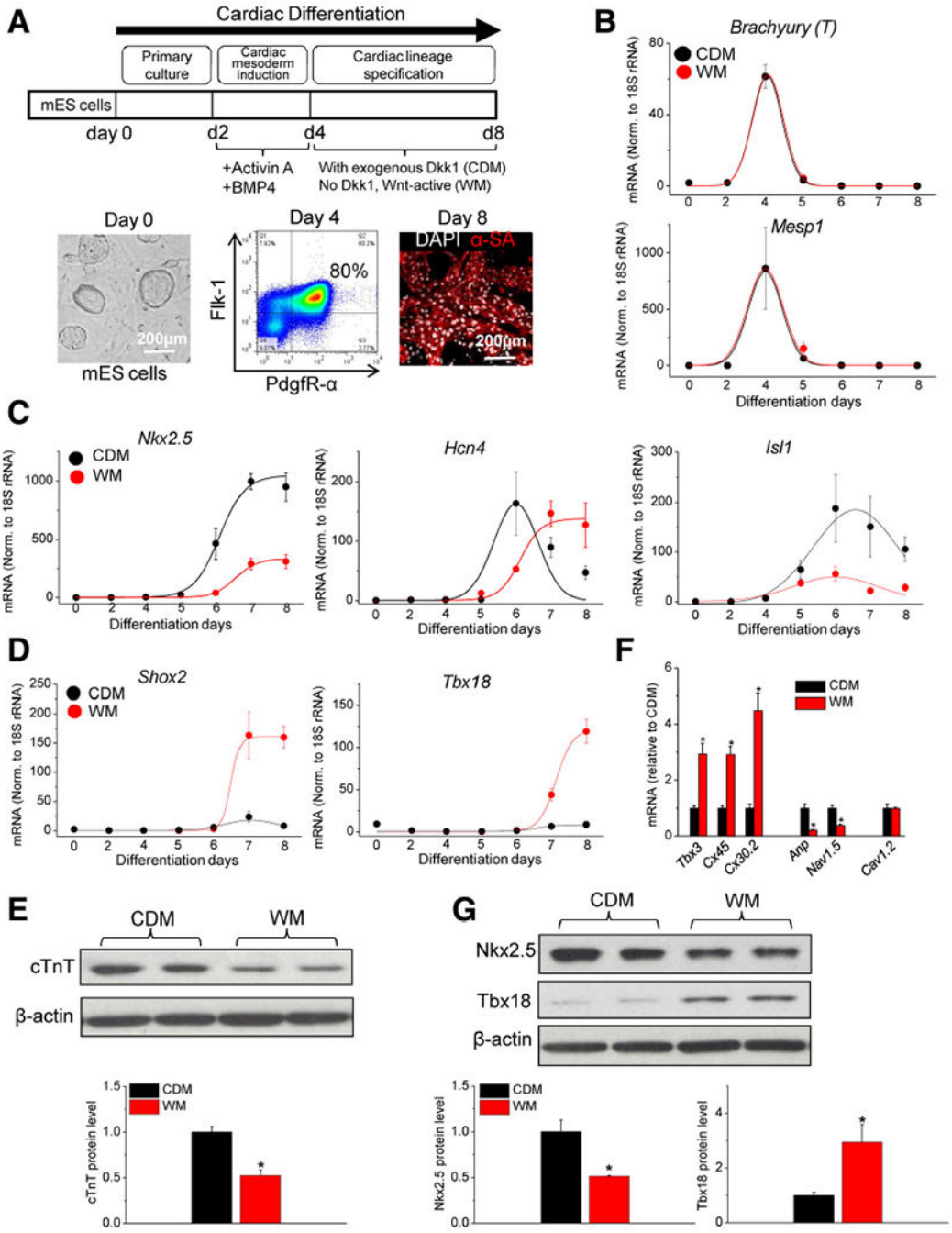

FIGURE 1.

Wnt signaling regulates cardiac subtype myocyte lineage specification. A, Schematic outlining the cardiac differentiation protocol. R1 mouse embryonic stem cells were treated with Activin A and BMP 4 from day 2 to day 4; Flk-1^{+}/PdgfR-α^{+} cells were FACS-purified at day 4 and cultured as a monolayer in the cardiac differentiation medium (CDM, containing 150 ng/mL recombinant Dkk1 which is a Wnt inhibitor) or in a modified CDM with no added Dkk1 (Wnt-uninhibited medium, WM) from day 4 to day 8. B-D, Expressions of marker genes for pan-mesoderm (*Brachyury*) and cardiac mesoderm (*Mesp1*, panel B),

markers for the atrial/ventricular lineages (first and second heart fields, *Nkx2.5, Hcn4*, and *Isl1*, panel C), and markers for the third heart field (*Shox2* and *Tbx18*, panel D) in CDM (black dots) and WM (red dots) groups during ESC differentiation. **P* < .01 versus corresponding values in CDM group, n = 3-8 biological replicates per group. E, Western blotting analyses of cTnT of CDM (black bars) and WM (red bars) groups at day 8 of differentiation. Bar graphs summarize protein levels normalized to β-action. **P* < .05 versus corresponding values in CDM, n = 3-4 biological replicates per group. F, Expression of pacemaker lineage markers (*Tbx3, Cx45*, and *Cx30.2*), atrial/ventricular markers (*Anp* and *Nav1.5* α), and *Cav1.2* α, normalized to *cTnT* level, in CDM (black bars) and WM (red bars) at day 8. **P* < .05 versus corresponding values in CDM, n = 4-9 biological replicates per group. G, Western blotting analyses of Nkx2.5, Tbx18 of CDM (black bars) and WM (red bars) groups at day 8 of differentiation. Bar graphs summarize protein levels normalized to β-action. **P* < .05 versus corresponding values in CDM, n = 3-4 biological replicates per group

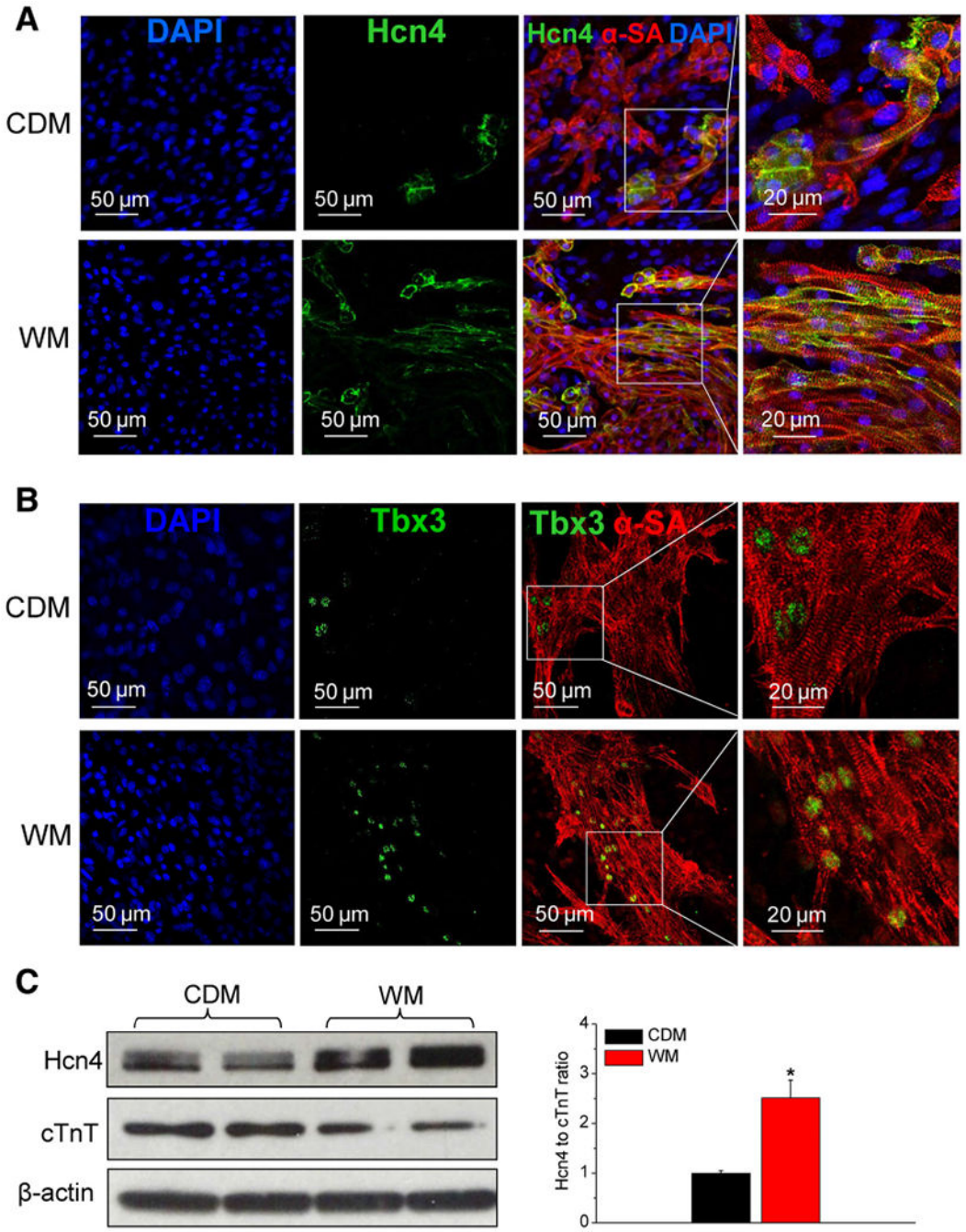

FIGURE 2.

Endogenous Wnt signaling promotes Hcn4-positive and Tbx3-positive cardiomyocytes from mouse embryonic stem cells. A, Representative confocal images of 3-week-old differentiated cells of the CDM group (top row) and WM group (bottom row) stained with antibodies against α-sarcomeric actinin (α-SA, red) and Hcn4 (green). Nuclei were stained with DAPI (blue). B, Confocal images of the CDM group (top row) and WM group (bottom row) cells stained with antibodies against α-SA (red) and Tbx3 (green). C, Western blotting analyses of Hcn4, cTnT, and β-actin of CDM (black bar) and WM (red bar) groups. *$P < .01$

versus corresponding values in CDM, n = 4 biological replicates per group. CDM, cardiac differentiation medium; WM, Wnt-uninhibited medium

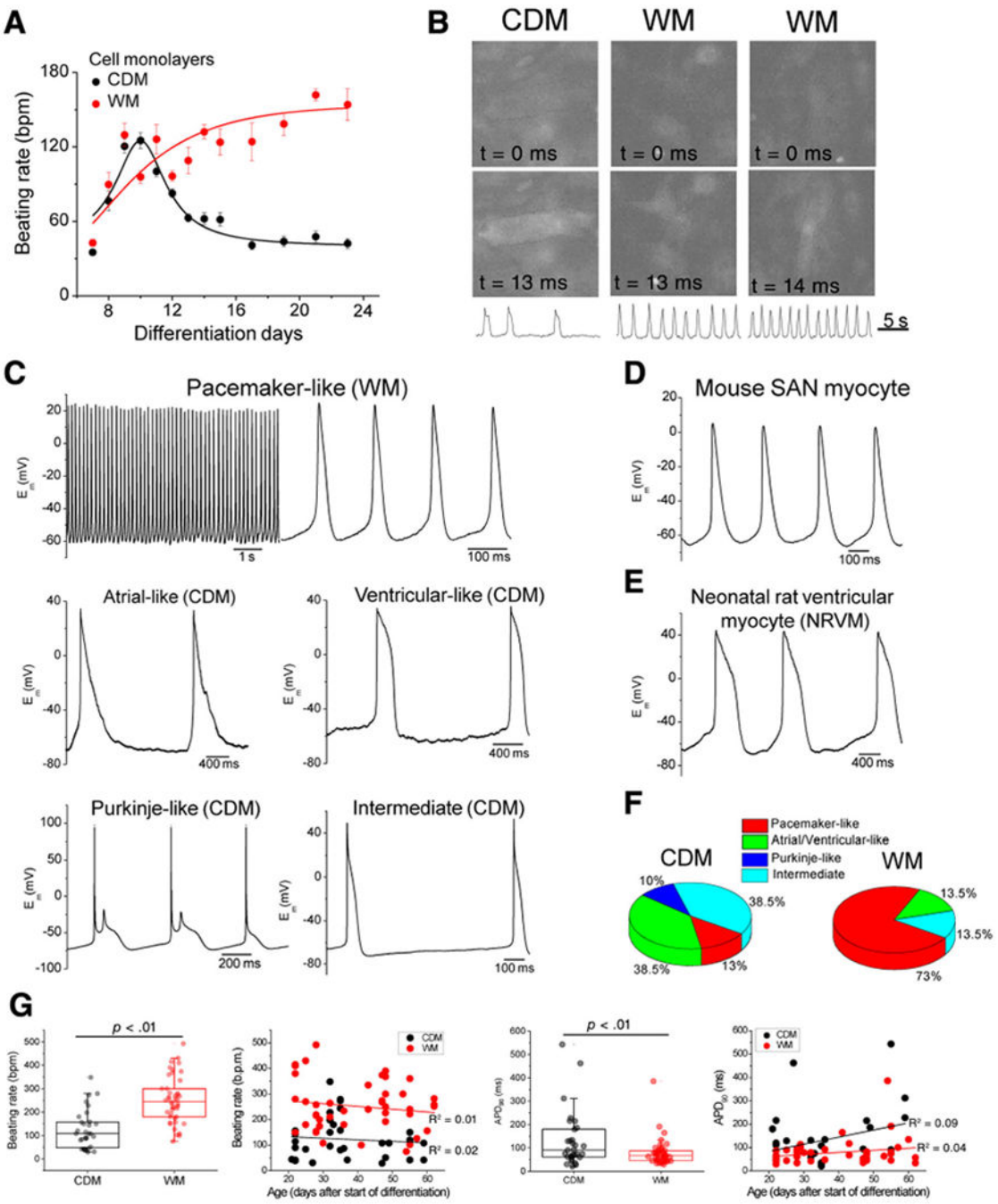

FIGURE 3.

Endogenous Wnt signaling promotes pacemaker-like cell generation from mouse embryonic stem cells. A, Spontaneous beating rates of synchronized cell monolayers of CDM (black) and WM (red) groups. CDM cells were cultured in a medium with added Dkk1 from day 4 to day 8. n = 4-8 biological replicates for each time point. B, Representative confocal images of cells (outlined with black lines) with spontaneous calcium transients in CDM (left column) and WM (middle and right columns) group. Cells were loaded with Cal-520 and treated with 10 μM palmitoleic acid, a connexin inhibitor. Changes of fluorescence intensity

over time for each indicated cell were shown at the bottom. C, Representative pacemaker-like action potentials from WM group, atrial-like, ventricular-like, and purkinje-like action potentials from CDM group. D and E, Representative pacemaker-like and ventricular-like action potentials from freshly isolated tissue. F, Summary of different subtypes' proportion in CDM (n = 31) and WM (n = 44 cells). G, Summary of action potential parameters (beating rate and APD90) of spontaneously beating cells from CDM (n = 31 cells, black) and WM (n = 44 cells, red), $*P < .01$ versus CDM. CDM, cardiac differentiation medium; WM, Wnt-uninhibited medium

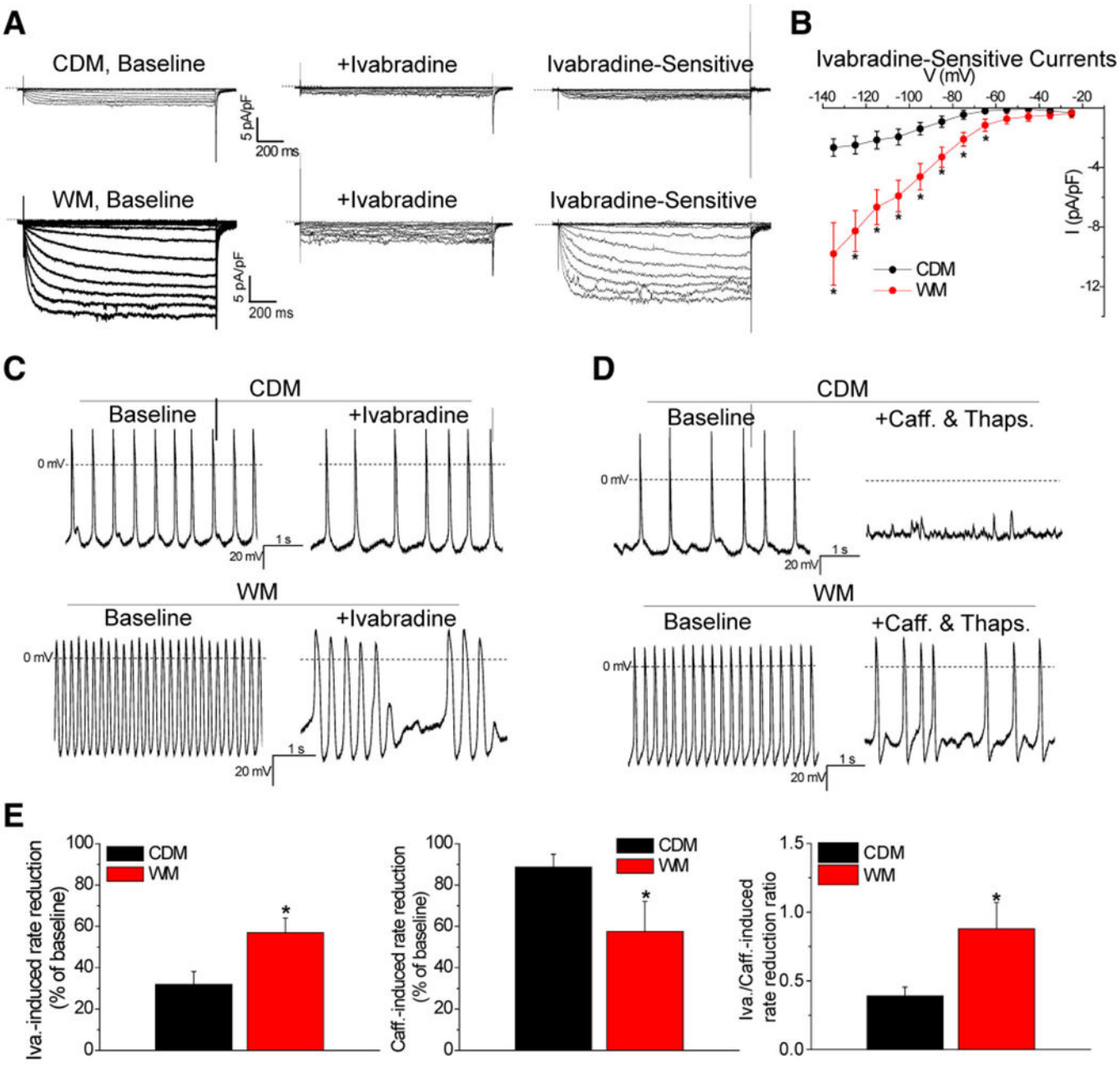

FIGURE 4.

Wnt signaling promotes pacemaker cells from mouse embryonic stem cells that use both membrane voltage clock and calcium clock mechanisms. A, Representative funny currents of spontaneously beating cells in CDM group (top panels) and WM group (bottom panels) before (left panels) and after (middle panels) ivabradine treatment (10 μM). Right panels show the difference of currents before and after ivabradine treatment. Currents were recorded in voltage-clamp mode with 1 mM Ba^{2+} in bath solution. Holding potential was set at −35 mV and voltage steps from −25 to −135 mV were applied to elicit the funny currents. Dashed lines indicate the position of zero current. B, Current-voltage relationships of ivabradine-sensitive currents in CDM (black circles, n = 11 cells) and WM (red circles, n = 9

cells) groups. Current density was normalized to cell capacitance. *$P < .05$ versus corresponding values in CDM. C and D, Representative action potential traces of spontaneously beating cells of CDM group (top rows) and WM group (bottom rows) before (left column) and after treatment with ivabradine (10 μM, a funny current inhibitor, panel C) or caffeine (10 mM)/thapsigargain (0.5 μM, inhibitors of calcium clock, panel D). Action potentials were recorded with patch-clamp technique in current clamp ($I = 0$) mode with nystatin-perforated method. E, Summary of percent beating rate reduction induced by ivabradine (left, n = 6-12 cells) or caffeine/thapsigargin (middle, n = 7-9 cells), and the ratio of ivabradine-induced versus caffeine/thapsigargin-induced rate reduction in the same cells (right, n = 5 cells) in CDM (black) and WM (red) groups. *$P < .05$ versus CDM. CDM, cardiac differentiation medium; WM, Wnt-uninhibited medium

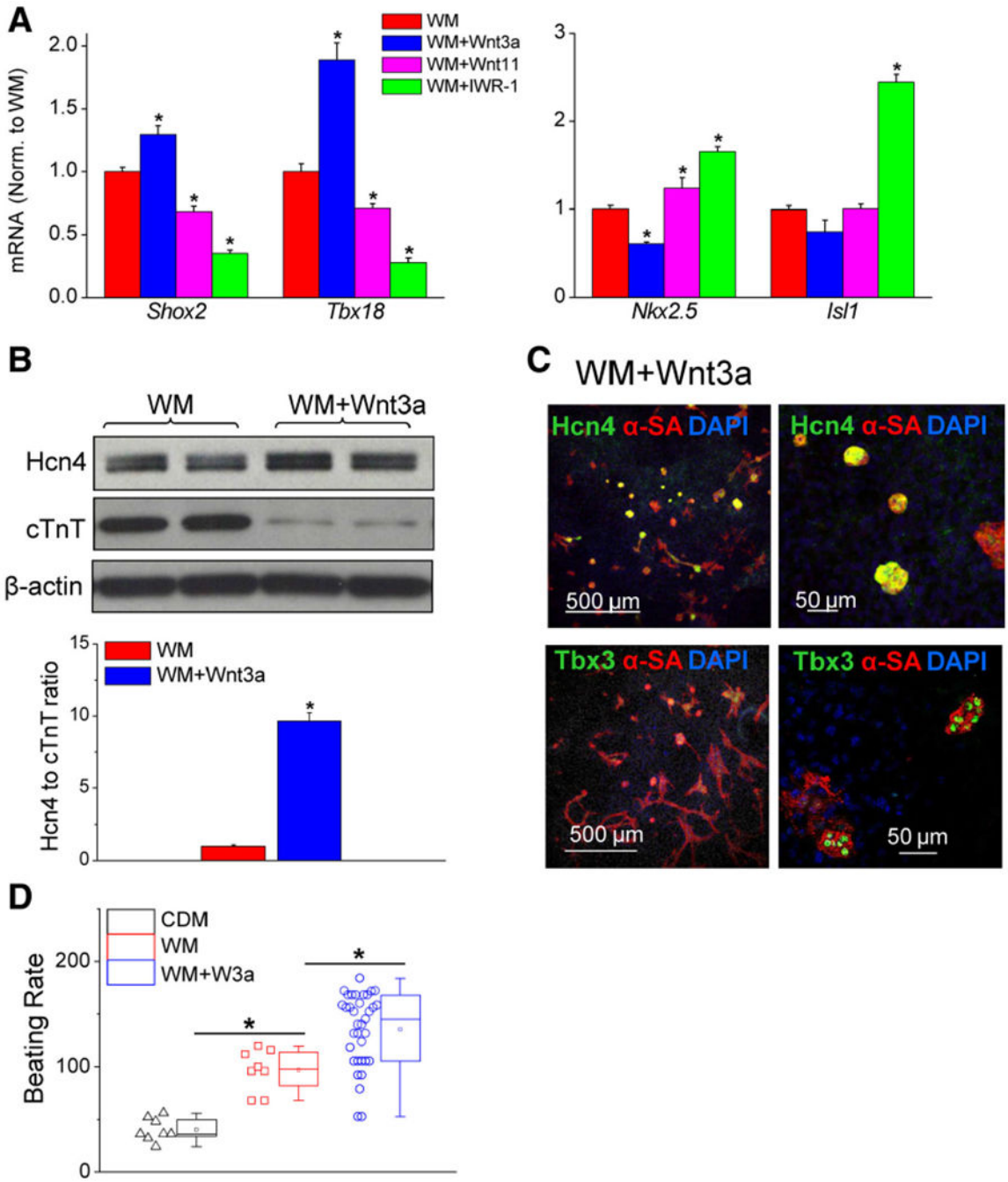

FIGURE 5.

Canonical Wnt signaling promotes cardiac pacemaker lineage specification from mouse embryonic stem cells. A, Expression of pacemaker lineage-specific genes (*Shox2* and *Tbx18*, left) and atrial/ventricular lineage-specific genes (*Nkx2.5* and *Isl1*, right) in differentiating cells at day 8 after treatment from day 4 to day 8 with Wnt3a (blue bars, n = 8 biological replicates), Wnt11 (purple bars, n = 4 biological replicates), or with the small molecule β-catenin inhibitor IWR-1 (green bars, n = 4 biological replicates). **P* < .01 versus WM. B, Western blotting analyses of Hcn4, cTnT, and β-actin of WM group and WM plus

Wnt3a protein treatment group at day 8 of differentiation. *P < .01 versus WM, n = 4 biological replicates per group. C, Representative confocal images of Wnt3a protein-treated cells stained with an antibody against α-sarcomeric actinin (α-SA, red), as well as an antibody against Hcn4 (green, top) or Tbx3 (green, bottom). Nuclei were stained with DAPI (blue). D, Spontaneous beating rates of synchronized cell monolayer at 3-week cultured in CDM (black, n = 8 technical replicates) or WM (red, n = 8 technical replicates) medium, or rates of the isolated beating colonies of Wnt3a-treated cells (blue, n = 35 beating foci). *P < .01 versus CDM or WM. CDM, cardiac differentiation medium; WM, Wnt-uninhibited medium

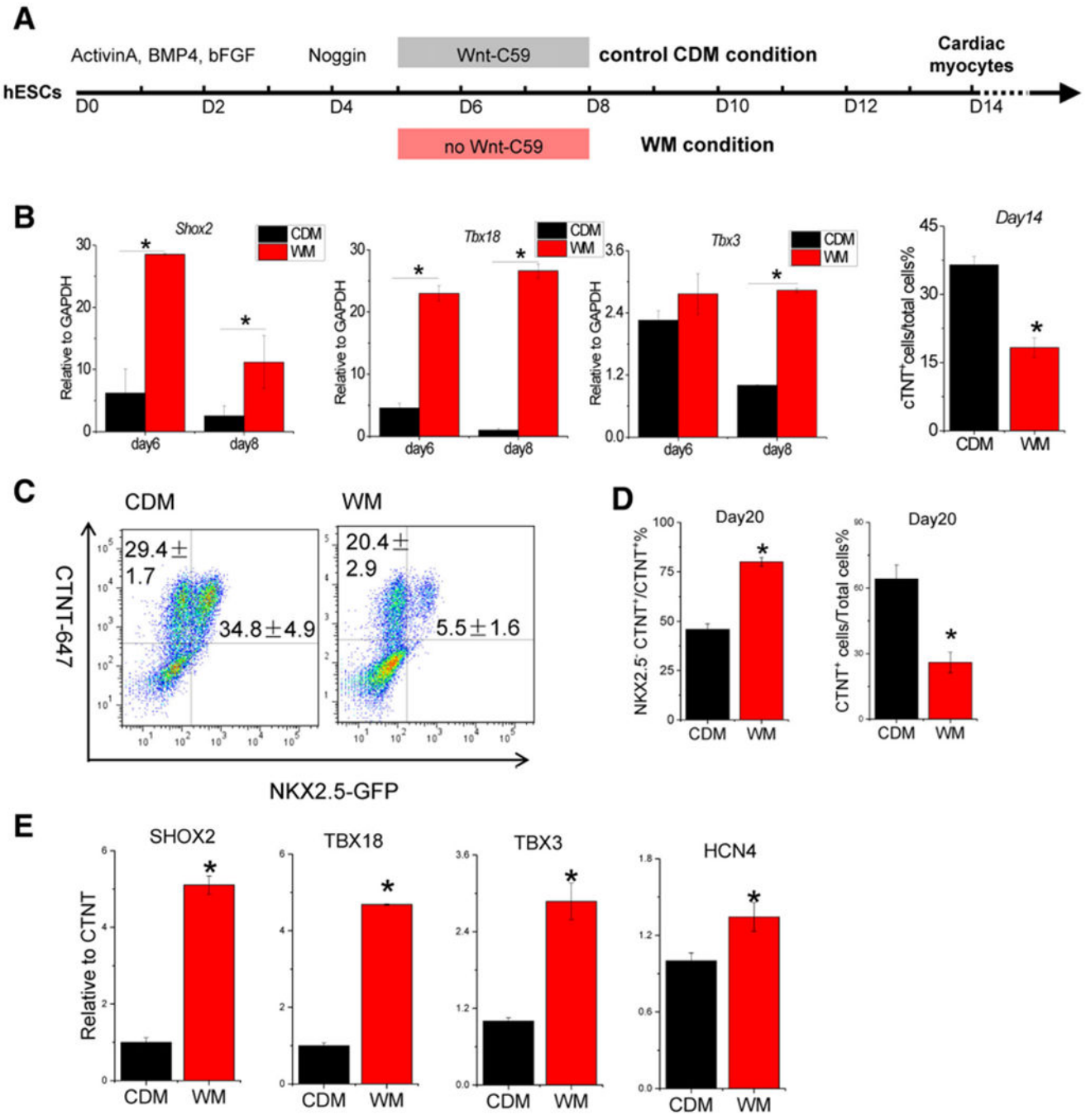

FIGURE 6.

Wnt signaling promotes pacemaker cells from hESCs. A, Schematic outlining the cardiac differentiation protocol of hESCs in CDM and WM condition. B, Relative expression of human pacemaker lineage-specific genes (Shox2, Tbx18, and Tbx3) in differentiating cells from hESCs at day 8 with CDM (black) or without WM (red) treatment of Wnt inhibitor Wnt-C59 groups, $*P < .05$ versus CDM. Flow cytometry analysis of cTnT-positive cells in CDM (black) and WM (red) groups at day 14. $*P < .05$, n = 3. C and D, Flow cytometry analysis of cTnT-positive cells derived from NKX2.5-GFP hESCs in CDM (black) and WM

(red) groups at day 20. *$P < .05$, n = 3. E, Expression of human pacemaker lineage-specific genes (*Shox2, Tbx18, Tbx3*, and *HCN4*) in differentiating cells from NKX2.5-GFP hESCs at day 20 with CDM (black) or without WM (red) treatment of Wnt inhibitor Wnt-C59 groups, *$P < .05$ versus CDM. CDM, cardiac differentiation medium; hESCs, human embryonic stem cells; WM, Wnt-uninhibited medium

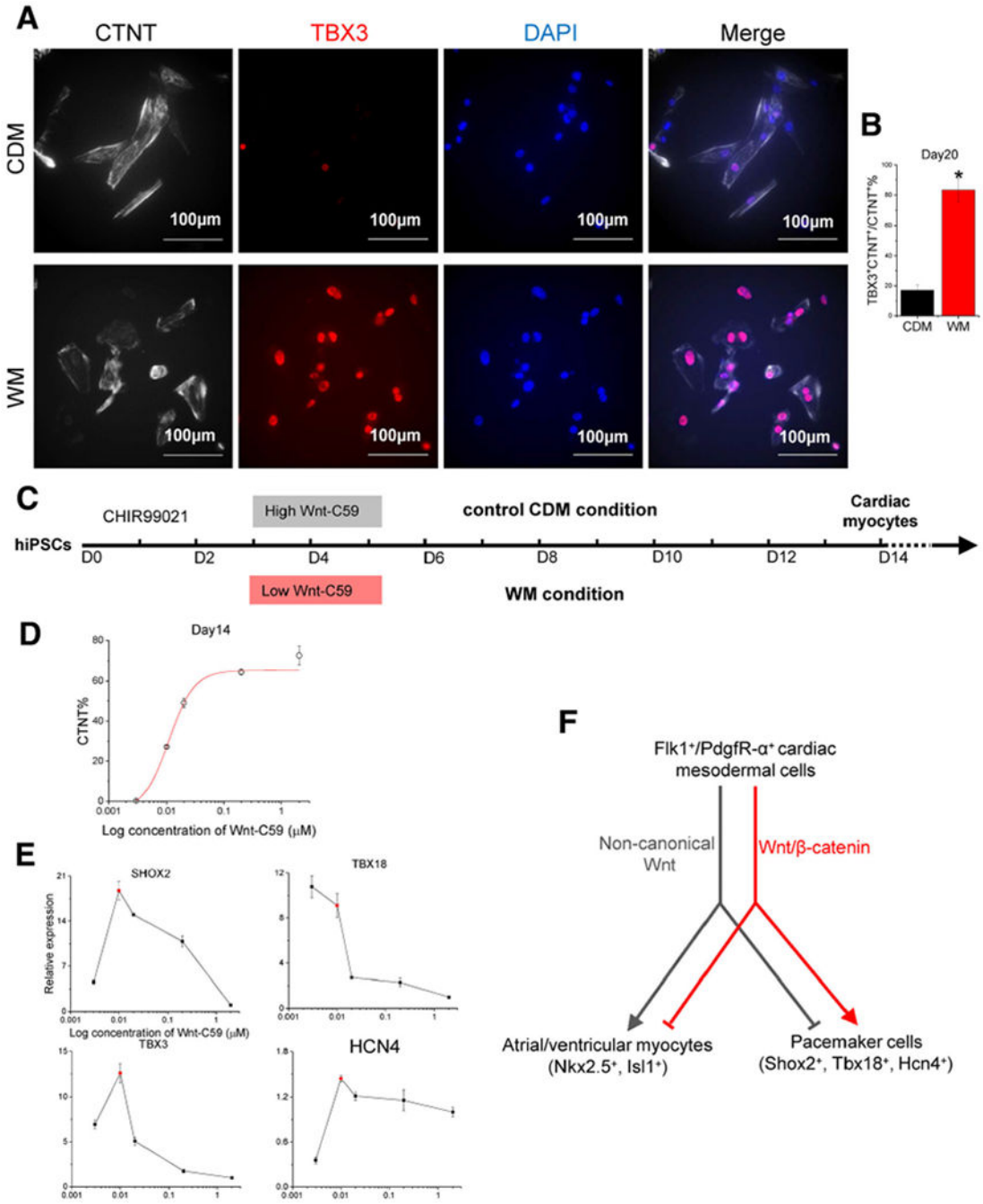

FIGURE 7.

Wnt signaling promotes pacemaker cells from hiPSCs. A and B, Immunostaining of D20 old differentiated cells of the CDM group (top row) and WM group (bottom row) stained with antibodies against CTNT (white) and TBX3 (red). Nuclei were stained with DAPI (blue). **P* < .05, n = 3. Scale bars = 100 μm. C, Schematic outlining the cardiac differentiation protocol of hiPSCs in CDM and WM condition. D, Flow cytometry analysis of cTnT-positive cells derived from hiPSCs at indicated dosages of Wnt inhibitor Wnt-C59 groups on D14, **P* < .05 versus minus wnt-C59 group. E, Relative expression of human pacemaker lineage-

specific genes (*Shox2, Tbx18, Tbx3*, and *HCN4*) in differentiating cells from hiPSCs at indicated dosages of Wnt inhibitor Wnt-C59 groups on D14.F, Schematic summary of the study. The Wnt/β-catenin signaling promotes the differentiation of cardiac progenitor cells towards the pacemaker lineage, whereas non-canonical Wnt signaling promotes atrial/ventricular myocyte lineage development. CDM, cardiac differentiation medium; hiPSCs, human inducible pluripotent stem cells; WM, Wnt-uninhibited medium