

Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors

Chulan Kwon*[†], Joshua Arnold*[†], Edward C. Hsiao*[‡], Makoto M. Taketo[§], Bruce R. Conklin*^{†¶}, and Deepak Srivastava*^{†||**}

*Gladstone Institute of Cardiovascular Disease and Departments of [†]Pediatrics, [‡]Medicine, [¶]Cellular and Molecular Pharmacology, and ^{||}Biochemistry and Biophysics, University of California, 1650 Owens Street, San Francisco, CA 94158; and [§]Department of Pharmacology, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo Kyoto 606-8501, Japan

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Guiding multipotent cells into distinct lineages and controlling their expansion remain fundamental challenges in developmental and stem cell biology. Members of the Wnt pathway control many pivotal embryonic events, often promoting self-renewal or expansion of progenitor cells. In contrast, canonical Wnt ligands are thought to negatively regulate cardiomyogenesis in several species. However, the cell-autonomous role of canonical Wnt signaling within precardiac mesoderm, through its obligatory transcriptional mediator, β -catenin, is unknown. Using tissue-specific *in vivo* genetic manipulation, we found that β -catenin is required for development of cardiac progenitors and is a positive regulator of proliferative expansion of such progenitor cells. At discrete windows of development in embryonic stem cells, activation of canonical Wnt signaling promoted expansion of cardiac progenitors after initial commitment and was required for cardiac differentiation. Together, these data provide *in vivo* and *in vitro* evidence that canonical Wnt signaling promotes the expansion of cardiac progenitors and differentiation of cardiomyocytes.

cardiac development | embryonic stem cells | β -catenin | second heart field

Signaling and transcriptional networks that control early cardiac commitment, expansion, and differentiation are relevant for congenital heart malformations and attempts to guide stem cells into the cardiac lineage for therapeutic purposes. Soon after gastrulation begins, vertebrate heart formation begins in the anterior mesoderm, stimulated by the interplay of inductive and repressive signals (1, 2). Responding to signals from neighboring endoderm and ectoderm, two domains of mesoderm progenitors, the first heart field and second heart field (SHF), adopt a cardiac fate, each contributing to distinct cardiac regions (3–7). Unlike the first heart field, SHF cells remain undifferentiated until they migrate into the forming heart.

Despite evolutionary conservation of most cardiac developmental pathways, conflicting roles for Wnt signaling in cardiogenesis have been reported. Canonical Wnt signaling is initiated when Wnt ligands bind to Frizzled transmembrane receptors, stabilizing protein levels of β -catenin, a transcriptional coactivator that interacts with T cell factor (TCF/LEF) transcription factors to activate Wnt target genes (8, 9). In many contexts, canonical Wnt signaling regulates proliferation or cell fate decisions during embryonic development (10–12). In flies, Wingless, the founding member of the canonical Wnt family, is required for cardiac lineage determination (13, 14). However, in frogs and chicks, overexpression of canonical Wnts in dorsal mesoderm explants inhibits cardiomyocyte commitment or differentiation, likely by affecting endoderm (15, 16). A zebrafish mutant that globally decreases transcriptional activity of the β -catenin/TCF complex caused hyperproliferation of cardiomyocytes, suggesting β -catenin negatively regulates cardiac differentiation or expansion (17). Recent evidence suggests that canonical Wnt signaling may have temporal-specific effects on pluripotent cells (18). Non-canonical Wnt signaling mediated by alterations in intracel-

lular calcium dependent on G protein-coupled receptors may promote cardiogenesis (19), but the cell-autonomous role of either Wnt pathway within precardiac mesodermal progenitors is unknown.

Here, we demonstrate that β -catenin activity within precardiac mesoderm is required for development of the SHF and promotes proliferation of precardiac mesoderm cells *in vivo*, with up-regulation of one of its target genes, cyclin D2. Furthermore, after initial precardiac mesoderm differentiation in ES cells (ESC), canonical Wnt signals were required for further differentiation and proliferation of cardiac cells. These results indicate that canonical Wnt signaling can be a positive regulator of cardiogenesis during discrete windows of mammalian development.

Results

β -Catenin Regulation of SHF Progenitors. To determine whether canonical Wnt signaling might be active in the heart-forming region, we examined β -catenin protein expression in mouse embryos. At embryonic day (E)8.5, the myocardial layer was enriched in β -catenin, suggesting preferential stabilization of the protein in this region (Fig. 1A). β -Catenin protein was abundant in all chambers of the heart and in the SHF-derived outflow tract at E10.5. Of the canonical Wnt molecules expressed in the developing heart (*Wnt2*, *Wnt7a*, and *Wnt8a*) (20–22), *Wnt8a* was most specifically expressed in heart precursors at E8.5 (Fig. 1C and D). *Wnt8a* was expressed initially throughout the developing heart tube. By E10.5, protein was expressed in all chambers and in the outflow tract. *Wnt8a* persisted in the ventricular chambers at E12.5, including the compact and trabecular layers. β -Catenin and *Wnt8a* expression persisted at later stages of development (Fig. 1B, E, and F).

To examine the cell-autonomous role of canonical Wnt signaling in precardiac mesodermal progenitors or cardiomyocytes, we disrupted or stabilized β -catenin expression in cardiac mesodermal progenitors with *ctnnb1^{tm2Kem}* (23) or *β -catenin/loxP(ex3)* (24) mice, respectively. In *ctnnb1^{tm2Kem}* mice, Cre recombinase-mediated excision of exons 2–6 produced a β -catenin-null allele; in *β -catenin/loxP(ex3)* mice, Cre expression activated Wnt/ β -catenin signaling by generating stable β -catenin [supporting information (SI) Fig. 5A]. We deleted β -catenin in SHF progenitors before cardiac differentiation by crossing *ctnnb1^{tm2Kem}* mice with *Islet1-cre* mice, in which

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Abbreviations: SHF, second heart field; En, embryonic day *n*; RV, right ventricle; ESC, ES cell; EB, embryoid body; EB*n*, day *n* of EB differentiation; DM, differentiation medium.

**To whom correspondence should be addressed. E-mail: dsrivastava@gladstone.ucsf.edu.

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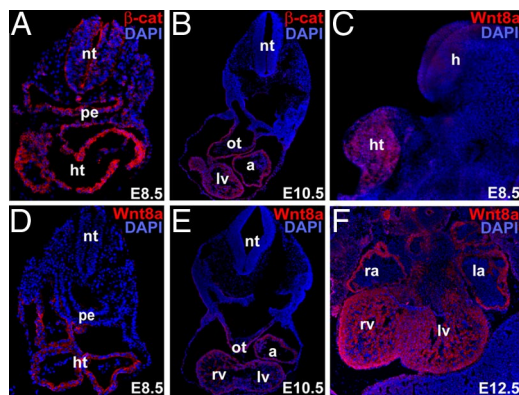


Fig. 1. Wnt/ β -catenin signaling is active in the developing heart. (A and B) Immunohistochemistry of mouse embryos for β -catenin protein (β -cat, red) at E8.5 (A) and E10.5 (B). (C) Lateral view of whole-mount immunostained E8.5 mouse embryo for Wnt8a (red) protein expression. (D–F) Immunohistochemistry with antibodies against Wnt8a (red) at E8.5 (D), E10.5 (E), and E12.5 (F). DAPI (blue) was used to counterstain the nuclei. All cryosections were transverse. a, atria; h, head; ht, heart tube; la, left atrium; lv, left ventricle; nt, neural tube; ot, outflow tract; pe, pharyngeal region of foregut endoderm; ra, right atrium; and rv, right ventricle.

Cre is expressed at E7.0–7.5 in undifferentiated mesodermal progenitors that give rise to the right ventricle (RV) and outflow tract (3). In *Islet1-cre ctnnb1^{tm2Kem}* homozygous embryos, the SHF failed to form the RV, but the left ventricle (LV) was relatively normal in size (Fig. 2 A–C and G–I). *In situ* hybridization with a probe recognizing the LV-specific transcript *Hand1* (25, 26), also expressed in the outflow tract, confirmed the identity of the residual ventricular chamber (Fig. 2 C and I). Consistent with the activity of *Islet1-Cre* in pharyngeal arch mesoderm, deletion of β -catenin led to hypoplasia of these structures (Fig. 2 B and H). It should be noted that this *Islet1-Cre* is not very active in the pharyngeal endoderm despite *Isl1* mRNA expression in the endoderm. Thus, SHF mesoderm progenitors may require cell-autonomous Wnt/ β -catenin signaling for specification, migration, or expansion.

To assess whether β -catenin was required for specification or maintenance of the initial *Isl1*-positive cells, we examined *Isl1* expression in the *Islet1-Cre/ β -catenin* mutant mice. *Isl1* was expressed at similar levels and in a comparable domain in mutants and wild-type embryos (Fig. 2 E and K), suggesting that the initial formation and maintenance of SHF progenitors was intact. Migration also appeared intact as *Fgf10*, marking SHF cells that migrate into the cardiac outflow tract (3, 5, 27), was also expressed appropriately in the β -catenin mutants (Fig. 2 F and L). Finally, we observed no evidence of SHF progenitor cell death (not shown), indicating that the primary defect may lie in failure of SHF cells to differentiate and/or expand in number after specification.

To directly test this idea, we used the *Islet1-Cre* and β -catenin/*loxP(ex3)* mice to stabilize β -catenin in the *Isl1*-positive SHF progenitors. Gain-of-function of β -catenin in these progenitors resulted in a greatly enlarged RV segment from E8.5–9.5 compared with the LV, which was marked by *Hand1* expression (Fig. 2 M–O and S–U). In some, the RV segment was so large at an early stage that protrusion of the heart tube was apparent with cardiac dysfunction resulting in developmental delay (Fig. 2 Y, Z, and A'). Most strikingly, the SHF progenitor pool dorsal to the developing heart tube was greatly expanded (Fig. 2 P, Q, V, and W) and contained an increased percentage of phosphohistone H3 (PH3)-positive cells (Fig. 2 R and X), indicating that β -catenin stabilization resulted in marked proliferation of SHF progenitors.

β -Catenin Promotes Proliferation and Differentiation of Cardiomyocytes *in Vivo*. To determine whether Wnt/ β -catenin signaling could also affect proliferation of cardiac cells even after they

have begun to differentiate, we used a transgenic mouse line in which *cre* expression is under control of a ventricular-specific enhancer of the cardiac regulatory gene, *Nkx2.5*. Unlike the endogenous *Nkx2.5* gene, which is expressed in the SHF, this *Nkx2.5* enhancer is not active in undifferentiated SHF cells (28). The *Cre*-mediated excision event begins at E8.0–8.5 in the cells that have already arrived into the heart and have begun differentiation (28); excision of β -catenin was confirmed by western analysis as nearly complete (SI Fig. 5B).

Nkx2.5-cre, ctnnb1^{tm2Kem} homozygous embryos died around E12.5 from cardiac dysfunction (Fig. 3 A and G), with significant reductions in ventricular size, particularly the RV (Fig. 3 B–F and H–L) and a thinner compact layer in the ventricular wall (Fig. 3 F and L). To identify the source of the myocardial alterations, we analyzed hearts by immunostaining with anti-PH3 antibody to detect alterations in cell-cycle control or by TUNEL assay to detect apoptosis. The percentage of PH3-positive cells was lower ($P < 0.01$) in *Nkx2.5-cre, ctnnb1^{tm2Kem}* hearts and greater ($P < 0.01$) in *Nkx2.5-cre, β -catenin/loxP(ex3)* hearts than in wild-type hearts (Fig. 3M). Similarly, the percentage of cyclin D2-positive cells, a direct target of the β -catenin/TCF complex that promotes cell cycling (29), was increased in *Nkx2.5-cre, β -catenin/loxP(ex3)* hearts and decreased in *Nkx2.5-cre, ctnnb1^{tm2Kem}* hearts (Fig. 3N Upper, $P < 0.01$). Total protein levels of cyclin D2 were correspondingly regulated in heart lysates (Fig. 3N Lower). Apoptosis was unaffected (not shown). Expression of numerous regulators of cardiogenesis, including *BMP4*, *Hand2*, *Hrt2*, *Mef2c*, and *Sall4*, was down-regulated in *Nkx2.5-cre, ctnnb1^{tm2Kem}* hearts, and *BMP4* and *Mef2c* expression was up-regulated in *Nkx2.5-cre, β -catenin/loxP(ex3)* hearts (Fig. 3O). Thus, Wnt/ β -catenin activity within cardiac mesoderm appears to positively regulate proliferation and full differentiation of ventricular cardiomyocytes even after early commitment steps have occurred.

Canonical Wnt Signaling Promotes Expansion and Differentiation of Mesodermal Progenitors into Cardiomyocytes in the ESC System. To assess the earlier function of Wnt signals in cells already committed to the mesodermal but not yet to the cardiac fate and to more precisely control the timing of Wnt stimulation or disruption, we turned to the ESC system. ESCs grown in hanging drops form clusters of cells called embryoid bodies (EBs) that randomly differentiate into derivatives of the three germ layers and can form beating cardiomyocytes, although with low frequency. The frequency is even lower when EBs are grown in suspension, which is technically much easier and would allow higher throughput generation of cardiomyocytes from ESCs.

To target canonical Wnt signaling only after mesoderm commitment, we first profiled mesoderm and cardiac gene expression during ESC differentiation in suspension, given that sensitivity to Wnt effects occurs in narrow windows of time. The early mesoderm marker *Brachyury* (*Bry*) was strongly induced on day 3 of EB differentiation (EB3) but was down-regulated afterward, suggesting mesoderm commitment by EB3 (SI Fig. 6). Other early cardiac mesoderm markers, such as the transcription factors *Nkx2.5*, *Tbx5*, *Gata4*, and *Mesp1*, were highly induced at EB4–6. Late cardiac markers, such as the sarcomeric genes *Myh6*, *Myh7*, *Mlc2a*, and *Mlc2v*, were induced by EB7–9 (SI Fig. 7).

Canonical Wnt signaling is required for general mesoderm formation *in vivo* (30, 31) and in EBs (32). We therefore initially targeted canonical Wnt signaling after mesoderm commitment (EB3) but before induction of early cardiac genes (EB4–5). Wnt3a, the soluble canonical Wnt ligand available, and Dickkopf1 (*Dkk1*), an inhibitor of canonical Wnts, were used to promote or disrupt Wnt/ β -catenin signaling, respectively. *Bry* expression was unaltered by addition of these factors at EB4–6 (Fig. 4A), suggesting that stimulating or inhibiting canonical Wnt signaling after EB3 did not alter the number of mesodermal progenitors. In contrast, stimulation of Wnt/ β -catenin between

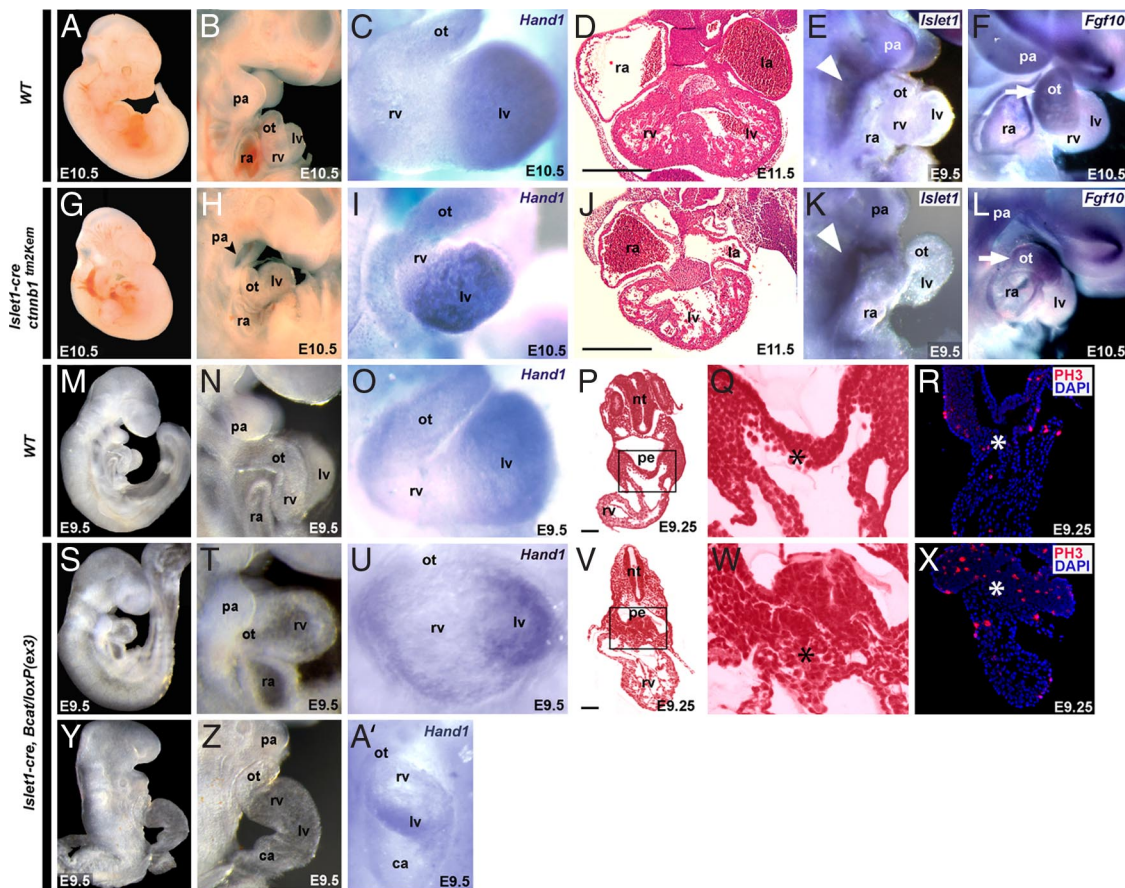


Fig. 2. β -Catenin is required for expansion of SHF cardiac progenitors. (A–F and M–R) WT embryos. (G–L) *Islet1-cre, ctnnb1^{tm2Kem}* homozygous embryos. (S–A') *Islet1-cre, β -catenin^{loxP(ex3)}* heterozygous embryos. (A and G) Lateral views of E10.5 embryos. (B and H) Lateral view focused on cardiac and pharyngeal arch (pa) regions showing hypoplasia of pharyngeal arches (pa) (arrowhead) and RV (rv). (C and I) Frontal view of E10.5 hearts hybridized with *Hand1* riboprobe marking left ventricle (lv) and outflow tract (ot). (D and J) Transverse sections through the heart. (Scale bars, 250 μ m.) (E, F, K, and L) Lateral views of embryos hybridized with SHF markers, *Islet1* (R and K, E9.5) and *Fgf10* (F and L, E10.5) riboprobes. Arrowheads indicate SHF; arrows indicate *Fgf10* in ot. (M, S, and Y) Lateral views of E9.5 embryos. (N, T, and Z) Lateral view focused on cardiac and pa regions showing significantly enlarged rv region. (O, U, and A') Frontal view of E9.0 hearts hybridized with *Hand1* riboprobe showing expanded rv. (P, Q, V, and W) Transverse sections of E9.25 embryos (P and V) with enlargement of boxed areas (Q and W) showing hyperplasia of SHF cardiac progenitors (asterisk) ventral to the pharyngeal region of foregut endoderm (pe). (Scale bars, 50 μ m.) (R and X) PH3 immunostaining (red) of transverse sections through the ot region. DAPI was used to counterstain the nuclei. ca, common atrium; la, left atrium; nt, neural tube; ra, right atrium.

EB4–6 with soluble Wnt3a increased the number of beating EBs at EB12 from 10% to >50% when cells were grown in suspension (Fig. 4B Left, $P < 0.01$). Inhibiting canonical Wnt signaling with Dkk-1 between EB4–6 resulted in a complete absence of beating EBs (Fig. 4B Left), suggesting that canonical Wnt signaling during this window not only promotes but also is required for cardiomyocyte formation in the ESC differentiation system.

The presence of beating EBs is useful for gross analyses but does not address the number of progenitors or differentiated cardiomyocytes. To more rigorously assess this aspect, we used an ESC line containing GFP under control of the endogenous *Nkx2.5* regulatory elements to mark and sort early cardiac mesoderm (B.R.C. and E.C.H., unpublished results). We found that stimulation or inhibition of Wnt signaling between EB4 and EB6 did not affect the initial number of progenitors in day 6 EBs. However, flow cytometry revealed a progressive expansion of the *Nkx2.5*-positive pool upon Wnt3a stimulation and a decrease in the progenitor pool upon Wnt inhibition as further differentiation proceeded in EBs (Fig. 4C). The numbers of cells positive for Tropomyosin, a late cardiac sarcomeric marker, were similarly increased by the Wnt agonist by EB12 (Fig. 4D). Notably, no Tropomyosin⁺ cells were observed in EBs treated with the

Wnt antagonist after EB4, consistent with the absence of beating EBs (Fig. 4B Left).

In agreement with a positive role for Wnt in the expansion and possibly early differentiation of the cardiac lineage, expression levels of the early cardiac genes *Nkx2.5* and *Tbx5* was up-regulated by exposure to Wnt3a from EB4–6 and down-regulated by similar exposure to Dkk-1 (Fig. 4E Upper). The SHF marker, *Islet1*, and the RV marker, *Hand2* (26), showed similar responses to Wnt3a and Dkk-1, as did cardiac sarcomeric genes (Fig. 4E Upper).

To assess effects of canonical Wnt signaling after induction of early cardiac mesoderm in ESCs, we treated EBs with Wnt3a or Dkk-1 between EB 7 and EB9. Nearly 50% of EBs treated with Wnt3a and grown in suspension were beating by EB12 (Fig. 4B Right, $P < 0.01$), and most early and late cardiac markers were up-regulated (Fig. 4E Lower), suggesting that Wnt had a positive effect on cardiomyocyte development even at this relatively late stage. This timing of Wnt manipulation is similar to the ventricular-restricted *Nkx2.5-cre* transgenic line used in the *in vivo* studies. Approximately 10% of EBs treated with Dkk-1 were beating (vs. 13% of control EBs; Fig. 4B Right, $P > 0.2$), and cardiac gene expression was not changed upon Wnt inhibition (Fig. 4E Lower). Thus, after cardiac mesoderm commitment,

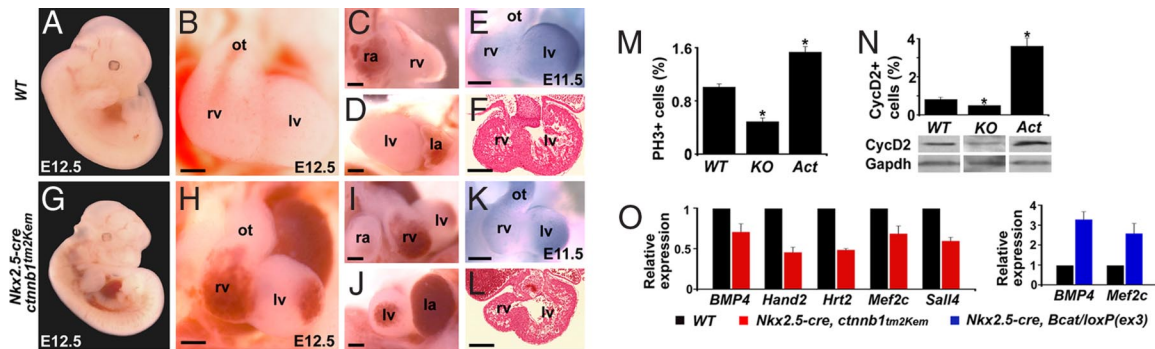


Fig. 3. β -Catenin regulates proliferation of ventricular cardiomyocytes. (A–F) WT embryos. (G–L) *Nkx2.5-cre, cttnnb1^{tm2Kem}* homozygous embryos. (A and G) Lateral embryonic view of E12.5 embryo. (B–D and H–J) Closeup frontal, right lateral, and left lateral views of the heart, respectively. (E and K) *In situ* hybridization with *Hand1* riboprobe. (F and L) Transverse section of E11.5 heart. (M) Percentage of PH3-positive cells in WT, *Nkx2.5-cre, cttnnb1^{tm2Kem}* homozygous (KO) and *Nkx2.5-cre, β -catenin/loxP(ex3)* heterozygous (Act) embryos at E11.5. (N) Percentage of Cyclin D2 (CycD2)-positive cells and Western blot of ventricles using CyclinD2 antibody in indicated mutants. GAPDH antibody was used as a control. (O) Quantitative real-time RT-PCR of indicated genes from ventricles of WT and mutant embryos at E12.5 (Left) or E13.5 (Right). *, $P < 0.01$. Error bars indicate standard deviations. la, left atrium; lv, left ventricle; ot, outflow tract; ra, right atrium; rv, right ventricle. (Scale bars, 250 μ m.)

canonical Wnt signals appear to be dispensable for full cardiac differentiation but may still recruit mesodermal precursors or expand cardiac progenitors. This result is consistent with our *in vivo* studies with the *Nkx2.5-cre* mouse described above.

Discussion

Published observations suggest that canonical Wnt signals likely play distinct roles during discrete developmental windows, first positively regulating mesoderm commitment and then possibly playing a negative role in the initial induction of cardiac progenitors (15, 16, 18, 30–32). The loss- and gain-of-function studies of canonical Wnt signaling in a spatiotemporally restricted manner described here provide compelling evidence that Wnt/ β -catenin signaling is required in a cell autonomous fashion for the expansion and development of precardiac mesoderm and cardiac mesoderm in mouse. Thus, narrow developmental windows may exist during which canonical Wnt signaling sequentially inhibits then promotes cardiac development. This may be similar to the function of Bmp signaling during cardiogenesis (33).

Previous studies in other species suggested that canonical Wnt signaling inhibited early cardiac commitment in a noncell autonomous fashion, likely by acting on adjacent endoderm. Recent studies implicate activation of Sox17 as a potential mediator of this endodermal effect (34). The multiple hearts that arise with endoderm-specific deletion of β -catenin in mice is consistent with the negative effects of an endoderm-derived factor (35). In contrast, β -catenin within the mesoderm after initial cardiac commitment is clearly involved in both cardiac proliferation and differentiation, particularly in the SHF. Because *Hand2* plays a critical role in SHF expansion (28, 36) and an upstream genomic region is reportedly bound by the β -catenin/TCF complex (37), it is possible that its down-regulation in part mediates the effects of β -catenin in regulating cardiac mesoderm.

The striking increase in proliferating SHF cells upon stabilization of Wnt/ β -catenin suggests an instructive role in cardiac cell proliferation. This is similar to the hyperplasia of cardiac progenitors in *Drosophila* upon overexpression of Wg (13). This effect may be mediated through up-regulation of cyclin D2, a direct transcriptional target of β -catenin/TCF (29). These observations were supported by findings in ES-derived cells, in which Wnt signaling was necessary for cardiac differentiation from mesodermal precursors and induced expansion of cardiac precursors after their initial induction. Thus, we have shown that Wnt/ β -catenin signaling is essential for development of heart progenitors in mammals and that canonical Wnt signaling can be

manipulated to regulate expansion and differentiation of cardiac progenitor cells in ESCs. It will be interesting to determine whether similar Wnt signaling regulates self-renewal or expansion of cardiac progenitors in the postnatal heart.

Materials and Methods

Mouse Lines and Genetics. The *Nkx2.5-cre, cttnnb1^{tm2Kem}* or *Islet1-cre, cttnnb1^{tm2Kem}* homozygous embryos were obtained by crossing *Nkx2.5-cre, cttnnb1^{tm2Kem} flox/+* or *Islet1-cre, cttnnb1^{tm2Kem} flox/+* lines with *cttnnb1^{tm2Kem} flox/flox* lines, respectively. *Nkx2.5-cre, β -catenin/loxP(ex3)*, or *Islet1-cre, β -catenin/loxP(ex3)* heterozygous embryos were obtained by crossing *Nkx2.5-cre* or *Islet1-cre* lines with *β -catenin/loxP(ex3) flox/+* lines, respectively. Wild-type and mutant embryos were identified as described (23, 24).

Immunohistochemistry, Western Blotting, and *In Situ* Hybridization.

Whole or cryosectioned embryos and EBs were stained with the following antibodies: mouse anti-Islet1 (1:200) and anti-Tropomyosin (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA), goat anti-Wnt8a (15 μ g/ml, R&D Systems, Minneapolis, MN), goat anti- β -catenin (1:50), and rat anti-cyclin D2 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-histone H3 (1:100; Upstate Biotechnology, Charlottesville, VA), and horseradish peroxidase-conjugated (with TSA plus Fluorescent Systems; PerkinElmer, Waltham, MA) secondary antibodies (The Jackson Laboratory, Bar Harbor, ME). For Western blotting, lysates from E12.5 ventricles were analyzed by using antibodies against β -catenin, cyclin D2, and GAPDH (1:50, 1:150, and 1:100, respectively; Santa Cruz Biotechnology). mRNA *in situ* hybridization was performed with designated antisense probes as described (3, 5, 27).

ESC Culture, Flow Cytometry, and Gene Expression Analysis.

Murine ESCs were propagated undifferentiated in maintenance medium of Glasgow MEM (Sigma, St. Louis, MO; G5154) supplemented with 10% FBS (HyClone, Logan, UT; SH30071.03), 1 mM 2-mercaptoethanol (Sigma M7522), 2 mM L-glutamine (Gibco-BRL, Carlsbad, CA; 25030-081), 1 mM sodium pyruvate (GIBCO-BRL 11360-070), 0.1 mM minimum essential medium containing nonessential amino acids, and leukemia inhibitory factor (LIF)-conditioned medium (1:1,000). EBs were formed by culturing ESCs (6×10^5 per well) for 3 days in ultra-low-attachment six-well plates (Corning, Lowell, MA; 07200601) in differentiation medium (DM), which contained the same components as maintenance medium but had 20% FBS and no LIF. For early treatment, the medium was replaced with DM con-

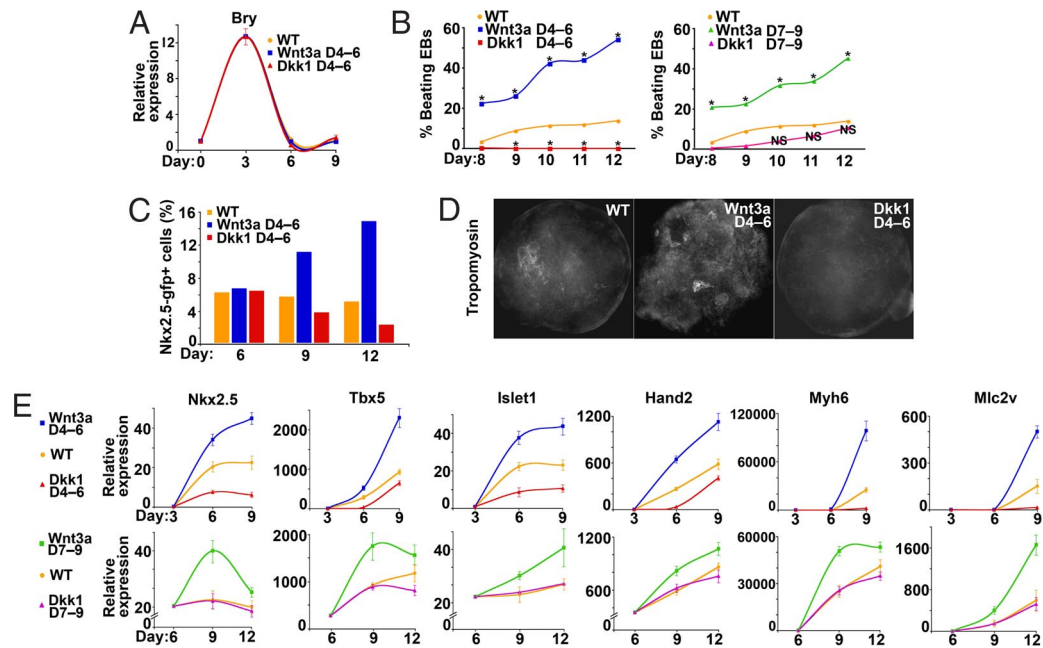


Fig. 4. Canonical Wnt signaling regulates cardiac expansion and differentiation in ESCs. (A) Brachyury expression profiles, determined by quantitative real-time PCR (qPCR), of undifferentiated ESCs on day 0 and EBs harvested on days 3, 6, and 9 after early Wnt3a or Dkk-1 treatment compared with control. (B) Beating EBs grown in suspension were counted from days 8 to 12. Percentage of beating EBs after early (days 4–6) or late (days 7–9) treatment with Wnt3a or Dkk-1. Asterisks indicate significant difference in beating percentage of treatment groups versus untreated EBs ($P < 0.01$). (C) Percentage of Nkx2.5-gfp⁺ cells at days 6, 9, and 12 in untreated EBs or those treated with Wnt3a or Dkk1 from days 4–6. (Bars represent percent of Nkx2.5-gfp⁺ cells from 4×10^4 cells analyzed). (D) Immunohistochemistry of day 12 EBs with anti-Tropomyosin. (E) Cardiac gene expression after early (days 4–6, *Upper*) or late (days 7–9, *Lower*) treatment with Wnt3a or Dkk-1. EBs were harvested on days 3, 6, and 9 (early treatment) or 6, 9, and 12 (late treatment). Fold change in expression of all indicated genes (y axis) in EBs with respect to undifferentiated ESCs was assessed by qPCR. NS, not significant. *, $P < 0.01$.

taining Wnt3a (150 ng/ml, R&D Systems) or Dkk1 (500 ng/ml, R&D Systems) at the start of day 4; on day 6, the medium was replaced with fresh DM. For late treatment, DM was replaced with Wnt3a- or Dkk1-containing DM at the start of day 7 and switched to fresh DM on day 9. Respective media were changed for early-, late-, and untreated EBs every 3 days. EBs were monitored for beating from day 8 to day 12 and collected between days 3 and 12 at regular intervals. At respective days of differentiation, EBs were dissociated via trypsin and passed through a nylon cell strainer. A Becton Dickinson (Franklin Lakes, NJ) FACS Diva flow cytometer and cell sorter was used for detecting Nkx2.5-GFP⁺ cells. For gene expression analysis, total RNA was isolated, and real-time PCR was performed with the ABI Prism system (7900HT, Applied Biosystems, Foster City, CA). TaqMan primers used in this study are listed in **SI Table 1**. All samples were run in triplicate. Real-time PCR data

were normalized and standardized with SDS2.2 software (Applied Biosystems).

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