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Wnt/β-catenin signaling directs the regional expansion of first and second heart field-derived ventricular cardiomyocytes

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SUMMARY

In mammals, cardiac development proceeds from the formation of the linear heart tube, through complex looping and septation, all the while increasing in mass to provide the oxygen delivery demands of embryonic growth. The developing heart must orchestrate regional differences in cardiomyocyte proliferation to control cardiac morphogenesis. During ventricular wall formation, the compact myocardium proliferates more vigorously than the trabecular myocardium, but the mechanisms controlling such regional differences among cardiomyocyte populations are not understood. Control of definitive cardiomyocyte proliferation is of great importance for application to regenerative cell-based therapies. We have used murine and human pluripotent stem cell systems to demonstrate that, during *in vitro* cellular differentiation, early ventricular cardiac myocytes display a robust proliferative response to β-catenin-mediated signaling and conversely accelerate differentiation in response to inhibition of this pathway. Using gain- and loss-of-function murine genetic models, we show that β-catenin controls ventricular myocyte proliferation during development and the perinatal period. We further demonstrate that the differential activation of the Wnt/β-catenin signaling pathway accounts for the observed differences in the proliferation rates of the compact versus the trabecular myocardium during normal cardiac development. Collectively, these results provide a mechanistic explanation for the differences in localized proliferation rates of cardiac myocytes and point to a practical method for the generation of the large numbers of stem cell-derived cardiac myocytes necessary for clinical applications.

KEY WORDS: Cardiac development, Cardiomyocyte differentiation, Cardiomyocyte proliferation, First heart field, Gsk3 inhibitor, Wnt/β-catenin, Mouse

INTRODUCTION

Advanced heart failure represents a leading cause of morbidity and mortality in the developed world. A loss of viable and fully functional myocardial tissue results in a mismatch in myocardial oxygen demand and supply (Deedwania and Nelson, 1990; Ford et al., 2007; Levy et al., 2002; Lloyd-Jones et al., 2006; Quyyumi et al., 1984). Designing new approaches to augment the number of functioning human cardiac muscle cells serves as the foundation of modern regenerative cardiovascular medicine (van der Pol et al., 2012). Untangling the molecular pathways that control early cardiomyocyte (CM) proliferation during differentiation will not only lead to a better understanding of normal cardiac development, but will also allow advances in stem cell biology to be adapted for clinical end points.

The early mammalian heart arises from two regions of multipotent progenitor cells in the splanchnic mesoderm, described as the first (FHF) and second heart field (SHF) located posteriorly and medially to the cardiac crescent, and anteriorly to the pharyngeal mesoderm. The FHF progenitors of the cardiac crescent coalesce along the midline and give rise to the primitive linear heart tube and ultimately the majority of the cells of the left ventricle (LV) and inflow tract (IFT). Cells from the SHF contribute to the growth of the developing heart and eventually give rise to the right ventricle (RV), outflow tract (OFT) and parts of the IFT (Buckingham et al., 2005; Laugwitz et al., 2008; Martin-Puig et al., 2008). Significantly, this complex developmental program requires the coordinated expansion of the different populations of cardiac progenitors and early CMs to allow for a greater than 300-fold increase in muscle mass (von Gise et al., 2012). This increase of mass has to be tightly linked to complex morphological changes (Ishiwata et al., 2003; von Gise et al., 2012). Work from a number of laboratories over several decades has shown that cardiomyocytes in the compact myocardium have a significantly higher proliferation rate than cardiomyocytes in the trabecular myocardium during normal cardiac development. However, the precise mechanism that controls this difference in cardiac myocyte proliferation remains poorly understood (Jeter and Cameron, 1971; Luxán et al., 2013).

The Wnt/β-catenin signaling pathway has previously been shown to be involved in early cardiac development and growth. In the absence of Wnt ligands, a complex of axin, adenomatous polyposis coli (APC), casein kinase 1 (Ck1) and glycogen synthase kinase 3 (Gsk3), mediates phosphorylation, ubiquitylation and degradation of β-catenin. When present, receptor-bound ligands inactivate the phosphorylation complex and allow active β-catenin molecules to enter the nucleus. Nuclear β-catenin acts as a transcriptional co-activator of T-cell factor (Tcf) and leukemia enhancer factor (Lef) transcription factors to activate Wnt target genes (Bejsovec, 2005; Clevers, 2006; Rubinfeld et al., 1996; Tzahor, 2007).

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In murine and human embryonic development, Wnt has highly stage-specific effects on mesoderm and cardiac progenitor specification and expansion. Wnt signaling is required for primitive streak formation, and disruption of Wnt/β-catenin signaling leads to the absence of primitive streak, mesoderm and node formation (Barrow et al., 2007; Haegel et al., 1995; Liu et al., 1999; Rivera-Pérez and Magnuson, 2005). *In vitro*, the addition of Wnt3a to differentiating embryonic stem cells (ESCs) enhances mesoderm induction and is therefore necessary for cardiac differentiation. By contrast, repression of endogenous Wnt signaling diminishes brachyury (T) expression and blocks early mesoderm cell differentiation (Kattman et al., 2011; Kouskoff et al., 2005; Naito et al., 2006; Paige et al., 2010).

Interestingly, later during development, Wnt inhibition is necessary for the differentiation of mesodermal progenitors into cardiac precursors. Wnt antagonists such as crescent and dickkopf 1 (Dkk1) are expressed in the anterior endoderm and exert an inductive influence on the adjacent developing heart and promote the differentiation of precardiac mesodermal progenitors into cardiac progenitors (Rivera-Pérez and Magnuson, 2005; Schneider and Mercola, 2001). Similarly, during *in vitro* differentiation ESCs, repression of Wnt signaling promotes mesoderm progenitors to differentiate along the cardiac lineage (Naito et al., 2006; Paige et al., 2010; Wang et al., 2011; Willems et al., 2011).

Work from a number of laboratories has previously shown that canonical Wnt signaling promotes the *in vitro* expansion of multipotent cardiac progenitors that can subsequently differentiate into smooth muscle, endothelial and cardiomyogenic lineages. *In vivo*, inactivation of β-catenin results in a partial loss of multipotent Isl1 progenitor cells that contribute to the RV and OFT, during early embryonic development (E7.0-E9.5). Conversely, constitutive expression of β-catenin in these multipotent progenitors results in the expansion of the cells contributing to the right ventricle and outflow tract (Ai et al., 2007; Bu et al., 2009; Cohen et al., 2007; Kwon et al., 2007; Kwon et al., 2009; Lin et al., 2007; Qyang et al., 2007).

Herein, we examine the role of Wnt signaling in the development and cellular differentiation of early ventricular CMs from the SHF and the FHF, which contribute to the majority of the cells in the right and left ventricles, respectively. We exploit recent advances that have allowed the isolation of highly purified FHF and SHF transgenic marked early ventricular CMs from developing embryos and differentiating ESCs. These early CMs have an intrinsically limited proliferative capacity before they further differentiate (Domian et al., 2009). We demonstrate that during murine and human *in vitro* cellular differentiation, these early cardiac myocytes display a robust proliferative response to β-catenin-mediated signaling and conversely accelerated differentiation in response to inhibition of this pathway. During *in vivo* murine development, the differential activation of β-catenin signaling promotes the preferential expansion of compact versus trabecular myocardium. Taken together, our results provide a mechanistic explanation for the differences in localized proliferation rates of cardiac myocytes and point to a practical method for the generation of the large numbers of stem cell-derived cardiac myocytes necessary for clinical and translational applications.

MATERIAL AND METHODS

Generation of double-fluorescent reporter iPSC lines

Tail-tip fibroblasts (TTFs) from homozygous Nkx2.5-eGFP and anterior-heart-field-Mef2C-DsRed mice (Domian et al., 2009) were expanded and reprogrammed by transfection with four factors (Oct3/4, Sox2, Klf4, Myc) into iPSCs in LIF-containing media. In total ~30 lines were screened for their potential to differentiate into cardiac progeny *in vitro*. For the three lines used in this work, iPSCs were injected in E3.5 blastocysts and re-implanted in pseudo-pregnant foster mothers. At E9.5, chimerical contribution to the embryonic heart of the double transgenic cardiac reporter system was visualized under whole-mount fluorescence microscopy.

Mouse ESC and iPSC culture

Murine Nkx2.5-eGFP and anterior-heart-field-Mef2C-DsRed double transgenic embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) were cultured and differentiated as previously described (Domian et al., 2009). On day 6, EBs were trypsinized into single cell suspension and GFP+/DsRed– (FHF), GFP+/DsRed+ (SHF) or GFP–/DsRed– (NEG) cells were FACS purified. Purified cells were plated in 0.1% gelatin-coated 384-well plates in differentiation media. To study the effect of canonical Wnt signaling, compounds were added (at the following final concentrations) to the culture media 1 day after re-plating in 10× of final concentration to make a total volume of ~84 μl per well of a 384-well plate: DMSO carrier at 0.01%, 6-bromoindirubin-3′-oxime (BIO) at 0.5-2 μM, 1-azakenpaullone (AZA) at 2-8 μM (Sigma Aldrich), CHIR-99021 (CHI) (Sigma Aldrich) at 1-4 μM, Wnt3a (R&D) at 50-200 ng/ml, inhibitor of Wnt response 1 (IWR) (Sigma Aldrich) at 4-16 μM or PNU-74654 (PNU) (Sigma Aldrich) at 4-16 μM. Media were changed every 3rd day and compounds were subsequently added as described above. At 6 additional days of culture, cells were fixed in 4% paraformaldehyde for 10 minutes and stained and quantified for Troponin T and Ki67 expression.

Quantitative RT-PCR

Standard RT-PCR was performed on an Eppendorf Realplex 4. Prior to Tryzol (Invitrogen) RNA isolation, cells were treated with various small molecules in equal volumes of DMSO. Primer sequences are available in supplementary material Table S1.

Tissue engineering

Anisotropic fibronectin patterns were generated on PDMS substrates through microcontact printing. PDMS stamps with patterns consisting of 20 μm wide and 2 μm tall ridges, separated by 20 μm were generated using a micropatterned master. PDMS elastomer was mixed at a 10:1 base to curing agent ratio and cured against the master to obtain microtextured PDMS stamps. Micropatterned substrates were passivated with 1% pluronic acid for 10 minutes before cell seeding (Feinberg et al., 2007).

Human ESC culture

Human embryonic stem cells (ESCs) (HES7) were maintained on feeder-free Matrigel-coated (BD Biosciences) polystyrene plates with MEF-conditioned media. Conditioned medium [DMEM knockout, 10% knockout serum, 10% plasmanate, 1× NEAA, L-glutamine (1%), bFGF (5 ng/ml) and 2-mercaptoethanol (2-ME) (1:120,000)] was harvested from cultured iMEF. Human ESCs were passaged every 5-6 days using dispase 1 mg/ml (STEMCELL Technologies). Cardiac differentiation was induced with an earlier described two-dimensional directed differentiation approach (Kattman et al., 2011; Laflamme et al., 2007; Paige et al., 2010).

At day 21, human ESC-derived cardiomyocytes were dissociated into single cell suspension using collagenase A and B (1 mg/ml) in serum-containing media (DMEM, 20% serum) and subsequently trypsin 0.25% for 5 minutes. Dissociated cells were pelleted, re-suspended in human cardiac differentiation media, re-plated on fibronectin-coated (0.005%) 384-well plates and treated with compounds.

Animal breeding and constitutive activation of β-catenin in the myocardium

Conditional loss and gain-of-function studies were performed with the previously described $Myl2^{cre/+}$ mouse strain (Chen et al., 1998a). For the ventricular-specific β-catenin loss-of-function studies, the $Ctnnb1^{tm2Kem/tm2Kem}$ mice strain was used (Brault et al., 2001). For the gain of function of β-catenin, $Ctnnb1^{tm1Mmt/tm1Mmt}$ (Harada et al., 1999) mice were used. All animal work was performed within the institutional animal guidelines.

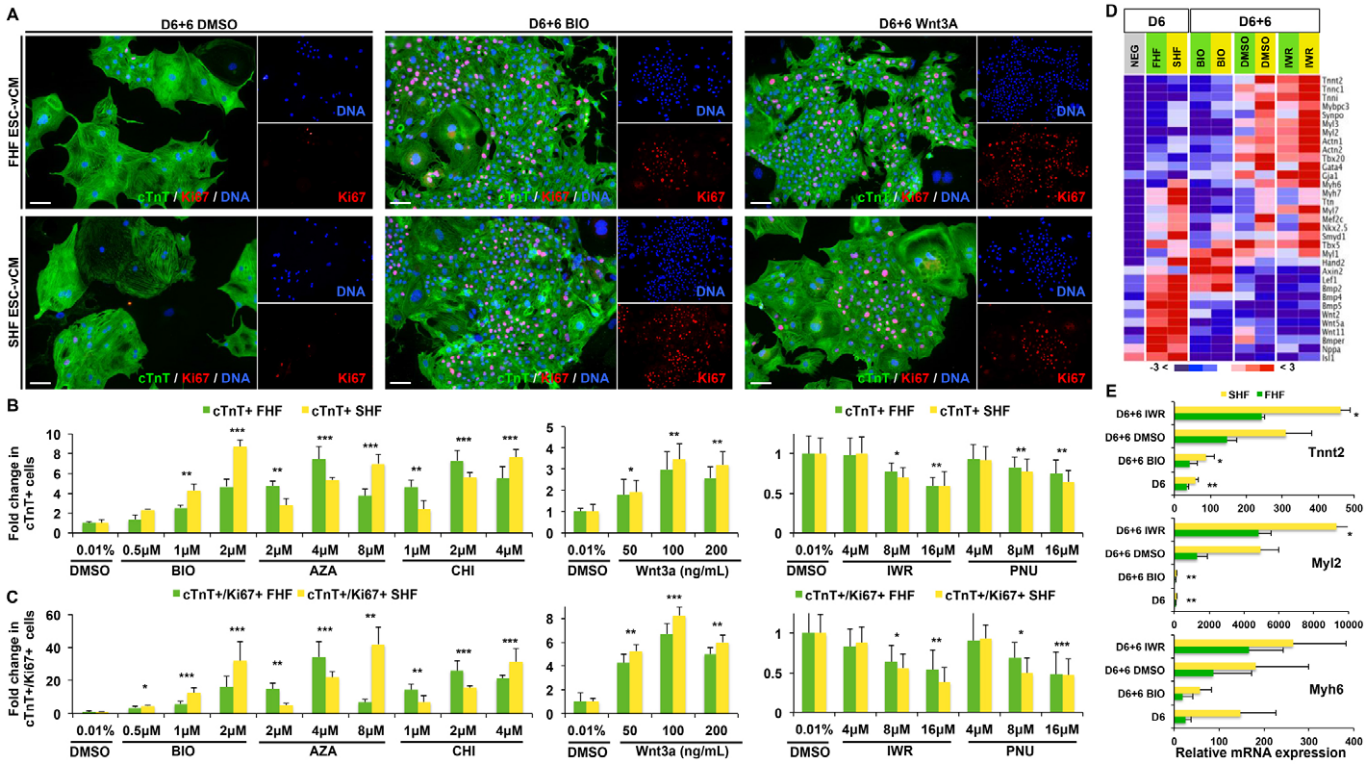

Fig. 1. Identification and characterization of Wnt activators that promote the expansion of early ventricular myocytes from the first and second heart fields. (**A**) Representative images of FHF- and SHF-marked ventricular cardiomyocytes (vCM) cultured for 6 days in (a) DMSO, 6-bromoindirubin-3′-oxime (BIO) or Wnt3a. Cardiomyocytes identified with cardiac Troponin T (cTnT) (green) staining, actively cycling cells with Ki67 (red) and nuclei with DAPI (blue). Scale bars: 50 μm. (**B**) Quantification of total FHF (green) and SHF (yellow) troponin (cTnT+)-positive cells as fold increase over DMSO control for three different GSK3 inhibitors [BIO, 1-azakenpaullone (AZA) and CHIR-99021 (CHI)], Wnt3a purified protein and Wnt signaling inhibitors [inhibitor of Wnt response 1 (IWR) and PNU 74654 (PNU)]. (**C**) Quantification of proliferating ventricular cardiomyocytes (vCMs) (cTnT+/Ki67+ cells) in BIO, AZA, CHI and Wnt3a. Error bars indicate s.d. (n>3). (**D**) Heatmap displaying the relative RNA expression pattern of ~35 selected genes for FHF- and SHF-marked cells at D6, D6+6 with BIO, D6+6 with DMSO or D6+6 with IWR. Gene expression in all groups was row normalized to the not transgenic-marked (NEG D6) population (n=3). Low expression is in blue; high-expression is in red. (**E**) Bar graphs show re-validation of three structural cardiac genes in FHF- and SHF-marked cells at D6, D6+6 with BIO, D6+6 with DMSO or D6+6 with IWR relative to D6-negative cells (n=3). *P<0.05, **P<0.01 and ***P<0.001.

For *ex vivo* cardiac cultures, hearts from Rosa26$^{lox(stop)YFP/+}$/Myl2$^{cre/+}$ or wild-type mice were digested for 1 hour in collagenase A and B (1 mg/ml) to obtain single-cell suspension. After FACS isolation, YFP+ cells were re-plated in mouse differentiation media.

Statistical analysis

Statistical analysis was performed using Student's *t*-test. Differences were considered significant if P was lower than 0.05 (P<0.05). Standard deviation is represented by error bars for each mean value mentioned in this manuscript.

RESULTS

Wnt signaling promotes the expansion of ESC-derived FHF- and SHF-marked ventricular CMs

We have previously reported the development of an *in vivo* multicolor reporter system in embryos and corresponding ESC lines that allowed for the purification of distinct subsets of heart-field progenitors and early proliferating cardiac myocytes. Specifically, we generated a double-transgenic mouse line with a red florescent protein (dsRed) under the control of an Isl1-dependent anterior heart field-specific enhancer of the transcription factor Mef2c (Dodou et al., 2003; Qyang et al., 2007) and with the enhanced green fluorescent protein (eGFP) under the control of a cardiac-specific Nkx2.5 enhancer (Lien et al., 1999; Wu et al., 2006). We were thereby able to isolate fluorescently marked FHF (eGFP+/DsRed–) early myocytes and SHF (eGFP+/DsRed+) early ventricular myocytes from embryos and corresponding differentiating ESC lines (Domian et al., 2009). At day 6 (D6) of ESC differentiation via embryoid bodies (EBs), FHF and SHF transgenic marked cells were isolated from beating EBs (supplementary material Fig. S1, Movie 1) by fluorescence-activated cell sorting (FACS) and re-plated in 384-well plates. FACS-purified cells were then cultured for an additional 6 days (D6+6) in the presence of 6-bromoindirubin-3′-oxime (BIO) [a reversible nonspecific Gsk3α and Gsk3β inhibitor (Meijer et al., 2003; Sato et al., 2004)], Wnt3a or carrier controls. Wnt3a or carrier controls. Cultured cells were then stained for cardiac troponin T (cTnT) and Ki67, a marker for actively cycling cells. As shown in Fig. 1A-C and supplementary material Fig. S2, induction of β-catenin in FHF and SHF early CMs with 1.0 and 2.0 μM BIO resulted in a four- to eightfold increase in total cTnT+ CMs, and a 10- to 20-fold increase in cTnT+/Ki67+ CMs.

To demonstrate that the robust expansion obtained with BIO treatment was due to Gsk3 inhibition and not due to off-target effects, we repeated a similar series of experiments using two other known analogous Gsk3 inhibitors: 1-azakenpaullone (AZA) (Kunick et al., 2004; Qyang et al., 2007) and CHIR-99021 (CHI) (Sineva and Pospelov, 2010; Ying et al., 2008). AZA and CHI were shown to selectively inhibit the β-isoform of cytoplasmic Gsk3. For

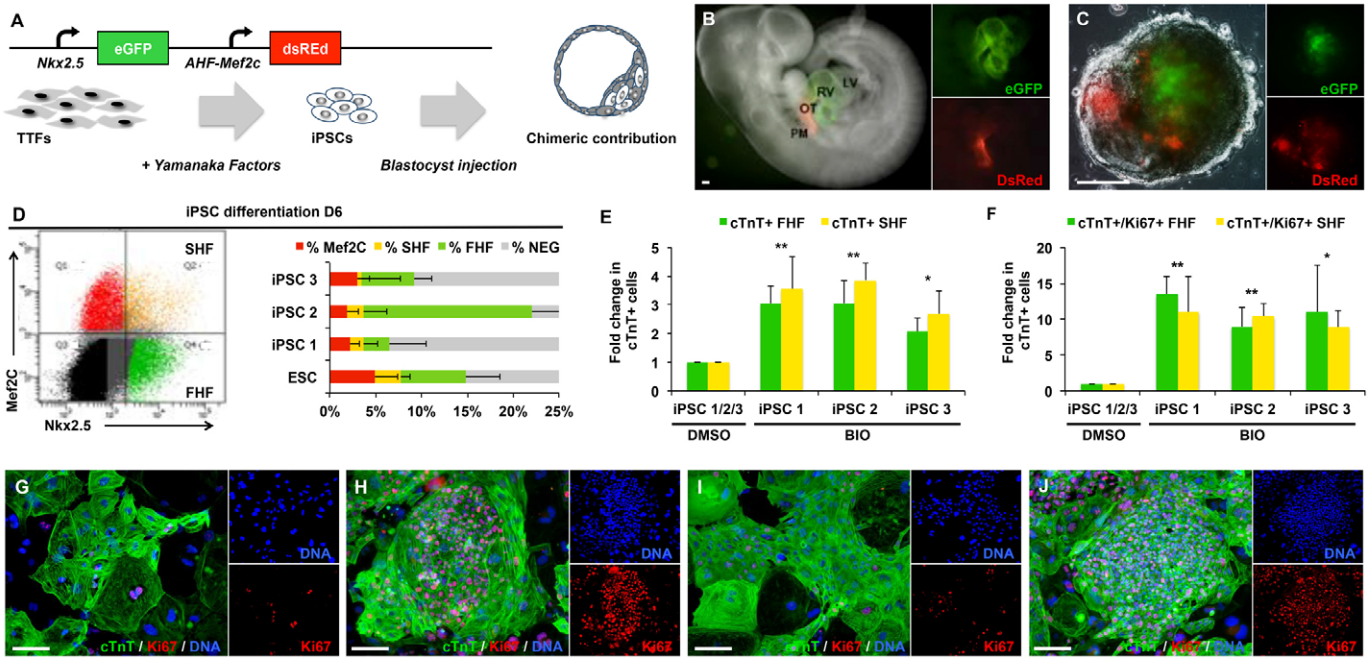

Fig. 2. Generation of double transgenic iPSC lines and expansion of iPSC-derived CMs. (**A**) Representation of tail-tip fibroblast (TTF) conversion into induced pluripotent stem cells (iPSCs) and validation via chimera formation. (**B**) Fluorescence image of an E9.5 chimera embryo resulting from iPSC injection into wild-type blastocysts. (**C**) Live cell image of an differentiated iPSC embryoid body. (**D**) FACS plot of cells isolated from dissociated embryoid bodies at day 6 (D6), showing the FHF and SHF transgenic marked populations. Graph represents mean percentages of colored cell populations obtained from iPSC lines 1, 2 and 3 compared with ESC-derived populations at D6. (**E**,**F**) Quantification of iPSC-derived FHF- (green) and SHF- (yellow) marked cTnT+ cells and (F) cTnT+/Ki67+ cells expanding in BIO at D6+6. Data are represented as mean fold increase over DMSO control. Error bars indicate s.d. (n>3). (**G-J**) Representative images of iPSC derived FHF-marked ventricular cardiomyocytes cultured for an additional 6 days (D6+6) with (G) DMSO or (H) BIO, and SHF-marked ventricular myocytes cultures with (I) DMSO or (J) BIO. Cardiomyocytes were identified with cardiac troponin T (cTnT) (green) staining, active cycling cells were identified with Ki67 (red) and nuclei were identified with DAPI (blue). Scale bars: 50 μm. *P<0.01, **P<0.001.

each analog, we found a four- to eightfold increase in cTnT+ and a 15- to 20-fold increase in cTnT+/Ki67+ cell number (Fig. 1B,C). To further determine whether canonical Wnt signaling and β-catenin activation can promote the proliferation of early ventricular CMs, we cultured cells with purified Wnt3a (Logan and Nusse, 2004). This resulted in an approximately threefold increase in cTnT positive CMs and sixfold increase in proliferating CMs compared with controls (Fig. 1B,C). Thus, activation of the canonical Wnt and β-catenin signaling pathway results in the robust expansion of FHF and SHF ventricular CMs. To verify that Troponin-positive CMs also expressed other markers of definitive functional myocardial cells, we stained expanded cells as well as their respective controls for α-sarcomeric actinin (α-SA) and myosin heavy chain (MF20) in combination with Ki67, or the ventricular-specific myosin light chain protein (Mlc2v) co-stained with cTnT (supplementary material Fig. S3). As demonstrated, expanding ventricular myocytes express multiple definitive markers of functional cardiac myocytes.

To determine whether suppression of β-catenin signaling promotes definitive cardiac differentiation, we then examined the effect of Wnt suppression on early CMs. FACS-purified early ventricular CMs were cultured in the presence or absence of the small molecule inhibitor of Wnt response 1 (IWR) or PNU 74654 (PNU). IWR has been previously shown to stabilize axin proteins and thereby facilitate β-catenin degradation (Chen et al., 2009; Lu et al., 2009); PNU has been previously shown to bind to β-catenin and inhibit the interaction between β-catenin and T cell factor 4 at the nuclear level (Trosset et al., 2006). FHF and SHF early ventricular CMs were therefore cultured in the presence of IWR, PNU or DMSO. After 3 days (D6+3) we found a reduced number of cTnT+ early CMs and a twofold reduction in the number of cTnT+/Ki67+ cells (Fig. 1B,C).

To evaluate the effect of Wnt signaling on differentiation, FHF and SHF early ventricular CMs were cultured in the presence of IWR, BIO or a carrier control. After culturing the cells for 6 days, the cells were lysed and gene expression was quantitatively analyzed by real-time polymerase chain reaction (RT-PCR) and plotted as a heatmap (Fig. 1D; supplementary material Fig. S4). As expected, D6 FHF and SHF early ventricular CMs expressed increased levels of axin 2 and Lef1, known targets of β-catenin. In the presence of DMSO or IWR, Wnt signaling decreased within 5 days of isolation, but was upregulated in the presence of BIO (Fig. 1D; supplementary material Fig. S5). Pan-cardiac transcription factors remained relatively stable in expanding and quiescent cultures. Interestingly, treatment of the CMs with the Wnt inhibitor IWR resulted in an increased expression level of cTnT, Myl2 and Myh6 when compared with treatment with the Wnt agonist BIO. The DMSO control exhibited an intermediate expression level (Fig. 1E). Remarkably, the expression of markers of functional cardiac myocytes is inversely correlated with the expression of Wnt target genes in FHF and SHF ventricular cells treated with DMSO, BIO or IWR (R^2=0.61 and R^2=0.71) (supplementary material Fig. S4).

Isolation and expansion of iPSC-derived FHF and SHF early ventricular CMs

Induced pluripotent stem cells (iPSCs) represent a potentially novel patient-specific source of ventricular CMs that can be used to

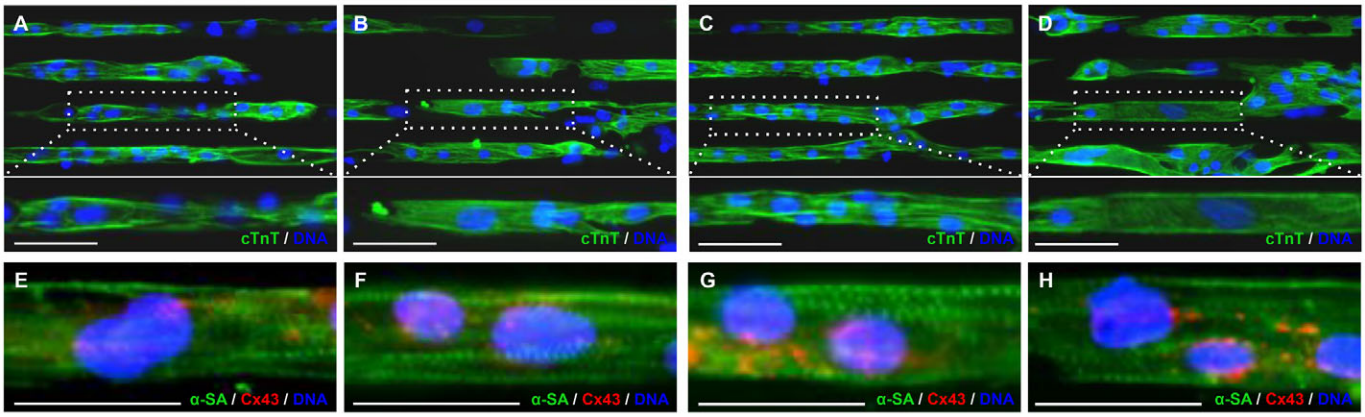

Fig. 3. Expanded early ventricular CMs are a useful source for tissue engineering and retain the potential to further differentiate. (**A**,**B**) Images illustrating the alignment of (A) control (D6+3) and (B) expanded (D6+5+3) ES-derived FHF early ventricular cardiomyocytes (vCMs) on micropatterned surfaces. (**C**,**D**) Alignment of (C) control and (D) expanded SHF marked early vCMs. All images show troponin T (cTnT) (green) stain with DAPI (DNA) (blue). Lower panels show further magnified images. (**E**,**F**) Immunofluorescence images of (E) control and (F) expanded FHF-marked early vCMs stained for connexin 43 (Cx43) (red) and α-sarcomeric actinin (α-SA) (green) with nuclear dye DAPI (DNA) (blue). (**G**,**H**) Immunofluorescence images of (G) control and (H) expanded SHF-marked cells.

generate functional cardiac tissue that may be exploited for clinically useful applications. To study the effect of Gsk3 inhibition and Wnt/β-catenin activation on iPSC-derived CM proliferation, we derived several iPSC lines. Tail-tip fibroblasts were isolated from the previously described Nkx2.5-eGFP/SHF-dsRed dual transgenic reporter mouse. Tail-tip fibroblasts were then cultured *in vitro* and reprogrammed into iPSCs with a lentiviral-based reprogramming strategy, using the Yamanaka factors (Takahashi and Yamanaka, 2006) (Fig. 2A). To ensure the pluripotency, as well as the fidelity, of fluorescent marker expression in these new double transgenic iPSC lines, we injected the iPSC lines into wild-type mouse blastocysts to generate ED9.5 mouse chimera. Fluorescence microscopy of these embryos revealed faithful recapitulation of marker expression (Fig. 2B; supplementary material Fig. S6), validating the *in vivo* accuracy of transgene expression of *in vitro* generated iPSCs. *In vitro* differentiation of these iPSC lines by embryoid body (EB) formation revealed discrete populations of FHF (Nkx2.5+/Mef2c–) and SHF (Nkx2.5+/Mef2c+) transgenic marked cells that were clearly evident by day 6 of EB differentiation as assessed by immunofluorescence microscopy (Fig. 2C). To isolate iPSC-derived FHF and SHF early ventricular cells, we dissociated day 6 EBs into single cell suspensions and FACS-purified distinct populations (supplementary material Fig. S6) of cardiac cells similar to those found during ESC differentiation. As previously described (Kaichi et al., 2010), we observed that the different iPSC lines had widely varying efficiencies of cardiac differentiation (Fig. 2D). Once purified, CMs were isolated; however, they all had similar differentiation potential and a similar robust response to Gsk3 inhibition and activated β-catenin signaling (Fig. 3E-J). Thus, although different iPSC lines differentiate into the myocardial lineage with variable efficiencies, small molecule-mediated Gsk3 inhibition is capable of enhancing FHF and SHF early ventricular CM proliferation.

Ventricular CMs expanded by β-catenin activation maintain the capacity to differentiate into functional ventricular muscle

A key consideration is that expanded CMs preserve the functional properties, cardiac gene expression pattern, protein organization and cellular alignment of the unexpanded native CMs. We and others have previously shown that neonatal CMs (Feinberg et al., 2007) and ESC-derived CMs (Domian et al., 2009) have the capacity to functionally align on micro-patterned substrates and form two-dimensional anisotropic functional myocardial tissue. In order to determine whether expanded early ventricular CMs retain this capacity, we directly compared expanded and non-expanded CMs. FHF (Fig. 3A,B) and SHF (Fig. 3C,D) early CMs were FACS purified from ESCs differentiated *in vitro* and expanded for an additional 6 days in the presence of the Gsk3 inhibitor BIO. Cells were dissociated into a single cell suspension and plated on micro-patterned substrates as shown in Fig. 3B,D. Alternately, early ventricular CMs were cultured for 3 days (without prior expansion), dissociated into a single cell suspension and again plated on micro-patterned substrates (Fig. 3A,C). As shown in Fig. 3E-H, FHF and SHF CMs maintained their capacity to generate anisotropic two-dimensional myocardial tissue even after being expanded for 6 days by Gsk3 inhibition. Anisotropic organized ESC-derived CMs contracted synchronously (supplementary material Movie 2) and maintained a typical cardio-myogenic transcriptional profile (supplementary material Fig. S7) regardless of whether or not they had been previously expanded *in vitro*.

Wnt/β-catenin signaling is necessary for the maintenance of proliferation of embryonic ventricular CMs but is not sufficient for the induction of proliferation in quiescent CMs

We then sought to determine the role of Wnt/β-catenin signaling in the expansion of fetal cardiomyocytes. We isolated yellow fluorescent protein (YFP)-positive ventricular myocytes from $Rosa26^{lox(stop)YFP/+}$/$Myl2^{cre/+}$ E12.5 embryos using FACS (Fig. 4A,B). YFP+ ventricular myocytes were then cultured in a three-dimensional cardiac microspheres in the presence of Gsk3β inhibitor CHI or DMSO control. Cardiac microspheres cultured in the presence of CHI had an increased diameter compared with DMSO ($P<0.05$) within only 3 days (E12.5+3) of *ex vivo* culture, indicating an increased cell number (Fig. 4C,D). We then obtained unsorted wild-type ventricular myocytes from E12.5 hearts. Ventricular CMs were cultured in the presence or absence Wnt

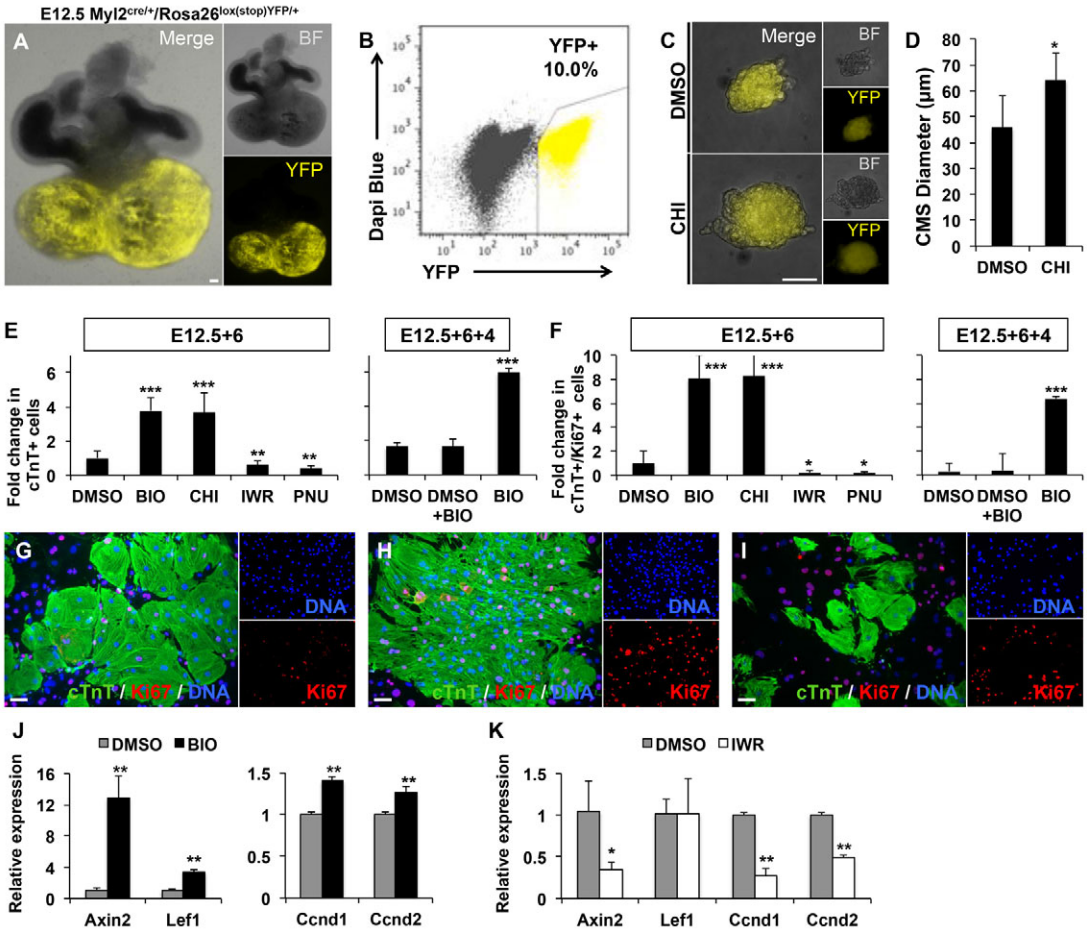

Fig. 4. Wnt/β-catenin signaling is necessary for the maintenance of proliferation of embryonic ventricular CMs. (**A**) Bright-field (BF) and immunofluorescence images of the Myl2 ventricular-specific YFP (yellow) expression in E12.5 hearts. (**B**) FACS isolation of Myl2+ (yellow) ventricular myocytes. (**C**) Live-cell YFP immunofluorescence (yellow) and BF images of beating cardiac microspheres at E12.5+3 made from Myl2+/YFP+ cells cultured with DMSO or GSK3β inhibitor CHIR-99021 (CHI). (**D**) Quantification of cardiac microsphere (CMS) diameter (in μm) after 3 days of 0.01% DMSO or 4.0 μM CHI treatment. (**E**,**F**) Quantification of (E) cTnT+ and (F) cTnT+/Ki67+ cells in whole ventricle cultures treated with 0.01% DMSO, 2.0 μM 6-bromoindirubin-3′-oxime (BIO), 4.0 μM CHIR-99021 (CHI), 8.0 μM inhibitor of Wnt response 1 (IWR) or 8.0 μM PNU 74654 (PNU) at E12.5+6 (left panels) and E12.5+6+4. (**G-I**) Images of *ex vivo* cells treated with (G) DMSO, (H) BIO or (I) IWR, and stained for troponin T (cTnT) (green), Ki67 (red) and DAPI (DNA) (blue). (**J**,**K**) Relative RNA expression pattern of Wnt target genes *Axin2* and *Lef1* and cell cycling genes *Ccnd1* and *Ccnd2* in *ex vivo* ventricular cells treated with (J) BIO or (K) IWR. (n=3). Scale bars: 50 μm. *P<0.05, **P<0.01, ***P<0.001.

agonists BIO (2.0 μM) and/or CHI (4.0 μM), the Wnt inhibitors IWR (8.0 μM) and PNU (8.0 μM), or a DMSO carrier control for 6 days (E12.5+6). At all described concentrations, we observed beating clusters of cardiac myocytes. At E12.5+6, we found a significant approximately fourfold (P<0.01) increase in cTnT+ and approximately eightfold (P<0.01) increase in cTnT+/Ki67+ ventricular cells treated with BIO or CHI. Conversely, we found a 30% (P<0.05) reduction in cTnT+ cells and a 60% (P<0.01) reduction in cTnT+/Ki67+ cells when ventricular myocytes were cultured in the presence of Wnt inhibitors IWR or PNU (Fig. 4E-I). Next, we hypothesized that Wnt/β-catenin signaling promotes proliferation through the inhibition of differentiation. To test this, we cultured ventricular myocytes for 6 days (E12.5+6) in DMSO and then added BIO for an additional 4 days (E12.5+6+4). As shown in Fig. 4E,F (right graphs), activation of Wnt/β-catenin signaling through Gsk3 inhibition was not sufficient to promote the proliferation of ventricular myocytes that have exited the cell cycle. Furthermore, RT-PCR analysis for downstream targets of β-catenin revealed that mRNA levels of axin 2, Lef1 and cell cycle genes such as Ccnd1 and Ccnd2 were significantly increased in ventricular myocytes treated with the Gsk3 inhibitor BIO (Fig. 4J) and decreased in myocytes treated with IWR when compared with DMSO control (Fig. 4K). We then performed microdissection of E12.5 hearts to isolate LV and RV ventricular cells and found no statistical differences in the two populations of ventricular myocytes in response to modulating Wnt signaling (supplementary material Fig. S8).

Gsk3 inhibition results in the expansion of human stem cell-derived cardiomyocytes

A key requirement for the translation of our findings to clinically relevant applications is their extension to human stem cell-derived CMs. Accordingly, we generated human ESC-derived CMs using using a previously described directed differentiation protocol (Paige et al., 2010). After 3 weeks (D21) of *in vitro* differentiation, cardiac myocytes were dissociated into a single cell suspension and plated

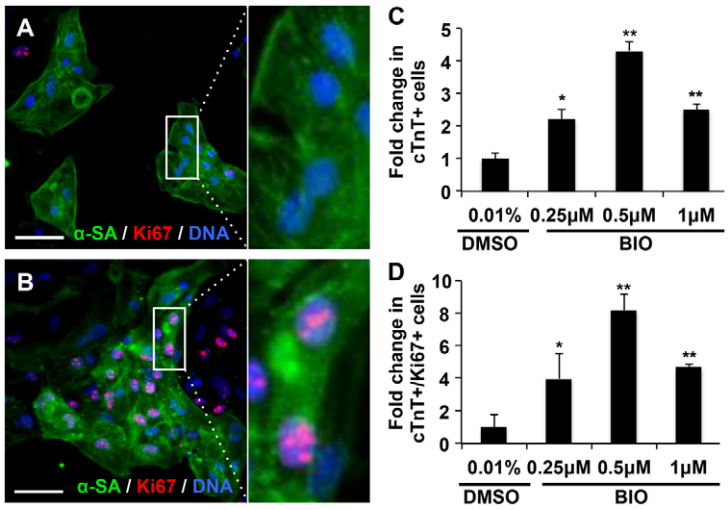

Fig. 5. Canonical Wnt signaling promotes proliferation of human ESC-derived cardiomyocytes. (**A**,**B**) Representative images of human CMs cultured for 6 days in the (A) absence or (B) presence of the GSK3 inhibitor BIO stained for Ki67 (red). The fraction of CMs is visualized with α-sarcomeric actinin (α-SA) (green), proliferating cells are visualized with Ki67 (red) and nuclei are stained with DAPI (blue). Scale bars: 50 μm. (**C**,**D**) Quantification of total (C) cTnT+ cells and (D) cTnT+/Ki67+ cells as fold increase over DMSO control for various concentrations of BIO. Error bars indicate s.d. (n>3). *P<0.01, **P<0.001.

into individual wells of a 384-well tissue culture plate. Cells were then cultured in the presence of the Gsk3 inhibitor BIO or carrier controls (Fig. 5A-D; supplementary material Movie 3). After an additional 6 days of *in vitro* culture, cells were fixed and co-stained for cTnT or α-SA and the proliferation marker Ki67. We found that the number of total troponin-positive cells was increased approximately fourfold (P<0.01), while the number proliferating (Ki67-positive) troponin-positive cells was increased ninefold (P<0.001) in BIO-treated cells compared with carrier control (Fig. 5C,D). RT-PCR analysis revealed increased expression of axin 2 and Lef1 (supplementary material Fig. S5), and a trend for decreased expression of structural cardiac genes (data not shown). Thus, in humans, as in mice, the inhibition of Gsk3 and the subsequent activation of β-catenin signaling promotes the robust expansion of stem cell-derived cardiac myocytes.

Selective activation of β-catenin controls the differential rate of proliferation in compact versus trabecular cardiomyocytes

Work from multiple laboratories has previously demonstrated that compact myocardial cells have a significantly higher proliferation rate than trabecular cardiomyocytes. To examine the role of β-catenin signaling during normal cardiac development, we stained E12.5 embryos for the active nuclear form β-catenin, as well as Ki67. Approximately 65% of the LV and RV compact ventricular myocytes were β-catenin+ compared with only ~30% of trabecular cardiac myocytes (Fig. 6A,B). Furthermore, the majority (greater than 70%) of β-catenin+ myocytes were Ki67 positive (Fig. 6C; supplementary material Fig. S9) and that this correlation was independent of whether or not the cardiac myocytes were trabecular or compact. Conversely, the majority of β-catenin– cells were quiescent. These findings suggest that canonical Wnt/β-catenin signaling may control the differential proliferation rates of these two myocardial sub-compartments.

To more closely examine this possibility, we generated cardiac specific gain and loss of function β-catenin mutants. Cardiac specific β-catenin loss-of-function mutants were generated by crossing $Ctnnb1^{tm2Kem/tm2Kem}$ mice (Brault et al., 2001) with $Myl2^{Cre/+}$/$Ctnnb1^{tm2Kem/+}$ mice, in which the Cre recombinase is under the transcriptional control of the ventricular myocyte-specific Myl2 promoter (Chen et al., 1998a; Chen et al., 1998b). The $Ctnnb1^{tm2Kem}$ allele carries loxP sites flanking exon 2-6 encoding the N-terminal domain and the Armadillo repeats 1-4 of the Ctnnb1 gene. Cre recombinase-mediated excision results in a short inactive β-catenin molecule (Brault et al., 2001). Cardiac-specific β-catenin gain-of-function mutants were generated by crossing $Ctnnb1^{tm1Mmt/tm1Mmt}$ mice with $Myl2^{Cre/+}$ animals. The serine/threonine residues of the β-catenin protein, encoded in exon 3 of the gene, are the phosphorylation targets of Gsk3β (Sparks et al., 1998) and deletion of exon 3 results in the constitutive activation of the β-catenin protein (Iwao et al., 1998). In the previously described $Ctnnb1^{tm1Mmt}$ allele, the loxP sequences flank exon 3 of the β-catenin gene and Cre-mediated deletion results in a truncated constitutively active mutant protein (Harada et al., 1999).

β-Catenin was essential for normal cardiac development and embryonic viability with no cardiac β-catenin loss-of-function embryos born from $Ctnnb1^{tm2Kem/tm2Kem}$ mice breeding with $Myl2^{Cre/+}$/$Ctnnb1^{tm2Kem/+}$ mice (n=26 live born animals). Hearts of loss-of-function embryos at E12.5 have a thin compact layer of both the LV and RV wall compared with littermate controls (Fig. 4D-G). Furthermore, the proliferation rate of cardiac myocytes (cTnT+/Ki67+) was markedly decreased to ~5% (P<0.001) compared with ~45% and ~25% cTnT+/Ki67+ in the compact and trabecular myocardium of control hearts (Fig. 6J-L). Crucially, this reduction in the proliferation rate was observed to a similar extent in the compact and trabecular myocardium of both the RV and the LV (Fig. 6K,L). This suggests that activated β-catenin is necessary for normal cardiac myocyte proliferation and that its normal preferential activation in compact myocardium is necessary to maintain the higher proliferation rate of compact compared with trabecular myocardium.

By contrast, cardiac-specific β-catenin gain-of-function pups were born at the predicted Mendelian ratios. Furthermore, the proliferation rate in the trabecular myocardium of gain-of-function E12.5 pups was increased from ~25% to ~40% (P<0.001) in both ventricles, while the proliferation rate of the compact myocardium was not significantly changed from control hearts (P=0.76) (Fig. 6H-L). In addition, the LV and RV compact myocardial thickness in loss-of-function embryonic hearts was decreased from ~40 μm to ~15 μm. By contrast, the compact myocardial wall thickness was similar in the gain-of-function embryos compared with controls (Fig. 6J). Thus, during fetal development β-catenin is essential for the normal proliferation of LV and RV myocardial cells, and that the differential activation of β-catenin plays a key role in

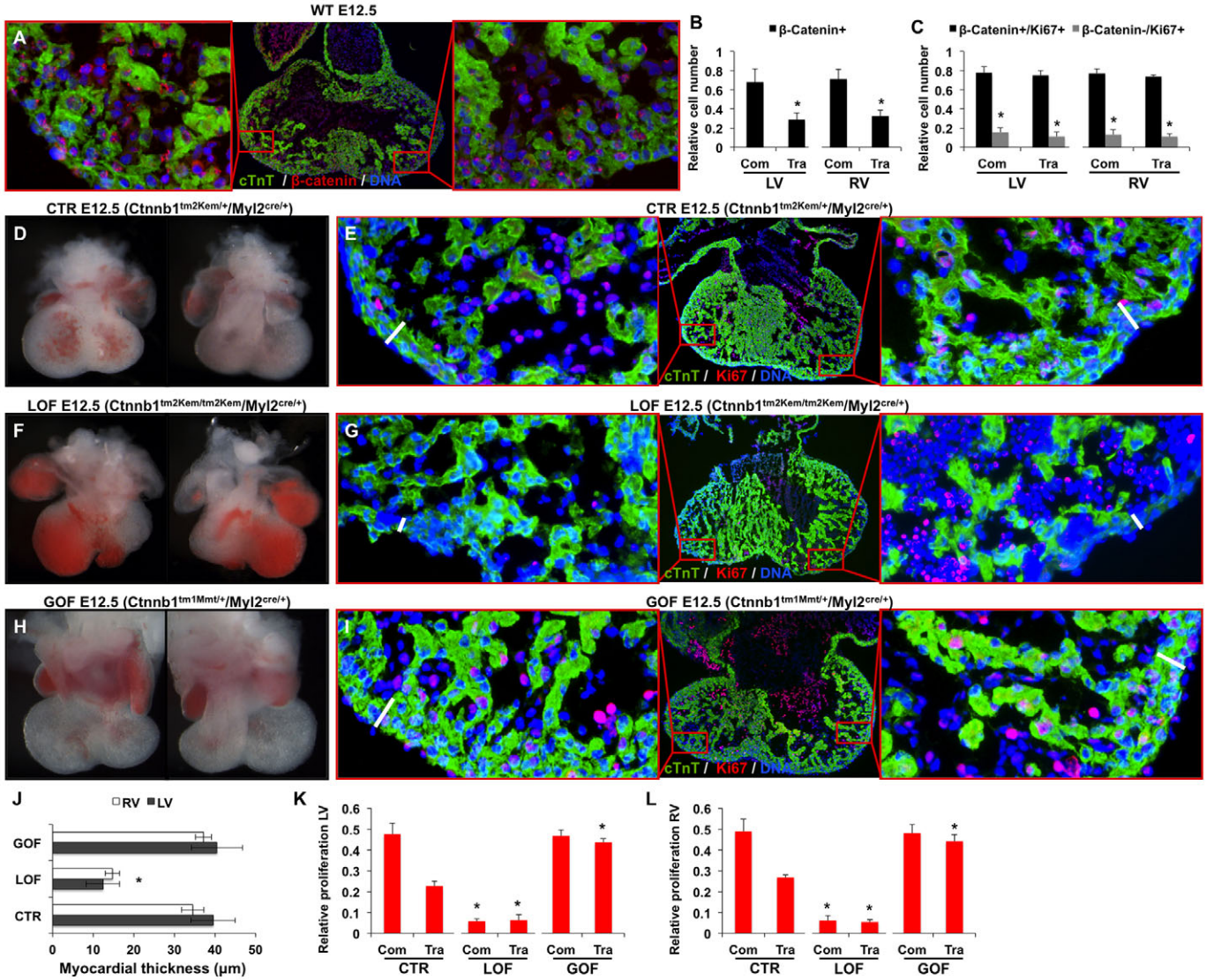

Fig. 6. β-Catenin plays a pivotal role in growth of compact myocardium. (**A**) Representative images of sections of E12.5 wild-type (WT) hearts stained for cTnT (green), active β-catenin (red) and DAPI (blue). (**B**) Percentage of ventricular myocytes (cTnT+) staining positive for active β-catenin in the compact or trabecular myocardium. (**C**) Percentage of proliferating ventricular myocytes staining positive for active β-catenin (black) or negative (gray). (*n*=3). (**D-I**) Representative color illustrations and images of stained of sections of E12.5 (D,E) control (CTR) ventricles, (F,G) β-catenin loss-of-function (LOF) ventricles and (H,I) β-catenin gain-of-function (GOF) ventricles stained for troponin T (cTnT) (green), Ki67 (red) and DAPI (DNA) (blue). Scale bars: 50 μm. (**J**) Thickness (μm) of the compact layer of LV and RV myocardium in CTR, loss-of-function and gain-of-function animals. (**K**,**L**) Percentage of active cycling myocytes (cTnT+/Ki67+) in the compact (Com) and trabecular (Tra) myocardium of the (K) left ventricle (LV) and (L) right ventricle (RV) of CTR, LOF and GOF hearts (*n*=2 or 3). *P<0.001.

controlling the differential proliferation rates of cardiac myocytes in the compact and trabecular myocardium.

β-Catenin activation promotes limited late fetal and early neonatal ventricular CM expansion, and results in an increase in heart size

As described above, Ctnnb1$^{tm1Mmt/+}$/Myl2$^{cre/+}$ gain-of-function animals, expressing a constitutively active β-catenin mutant protein in ventricular myocytes, were live born in the predicated Mendelian ration. Gain-of-function Ctnnb1$^{tm1Mmt/+}$/Myl2$^{cre/+}$ day 1 (P1) pups have a significant increase in the number of replicating ventricular myocytes when compared with littermate controls (Fig. 7A-C) and the LV and RV walls, as well as the septum appeared to be thicker, especially the trabecular myocardium. No abnormalities of the outflow tract or atrioventricular valves were observed. To examine whether the increase in thickness of gain-of-function ventricles correlated with increased proliferation, we again performed immunohistochemistry for the proliferation marker Ki67, along with cTnT, for marking all cardiac myocytes. We found that in the LV and RV of β-catenin gain-of-function hearts ~13% and ~17% of cTnT+ cells, respectively, were Ki67+ versus ~6% and 8%, respectively, in littermate controls (P<0.001) (Fig. 7C). Interestingly, the approximately twofold increase in proliferation rate was equally distributed over the compact and trabecular myocardium, consistent with the finding that there is no significant difference in Wnt responsiveness between the different myocardial compartments. At

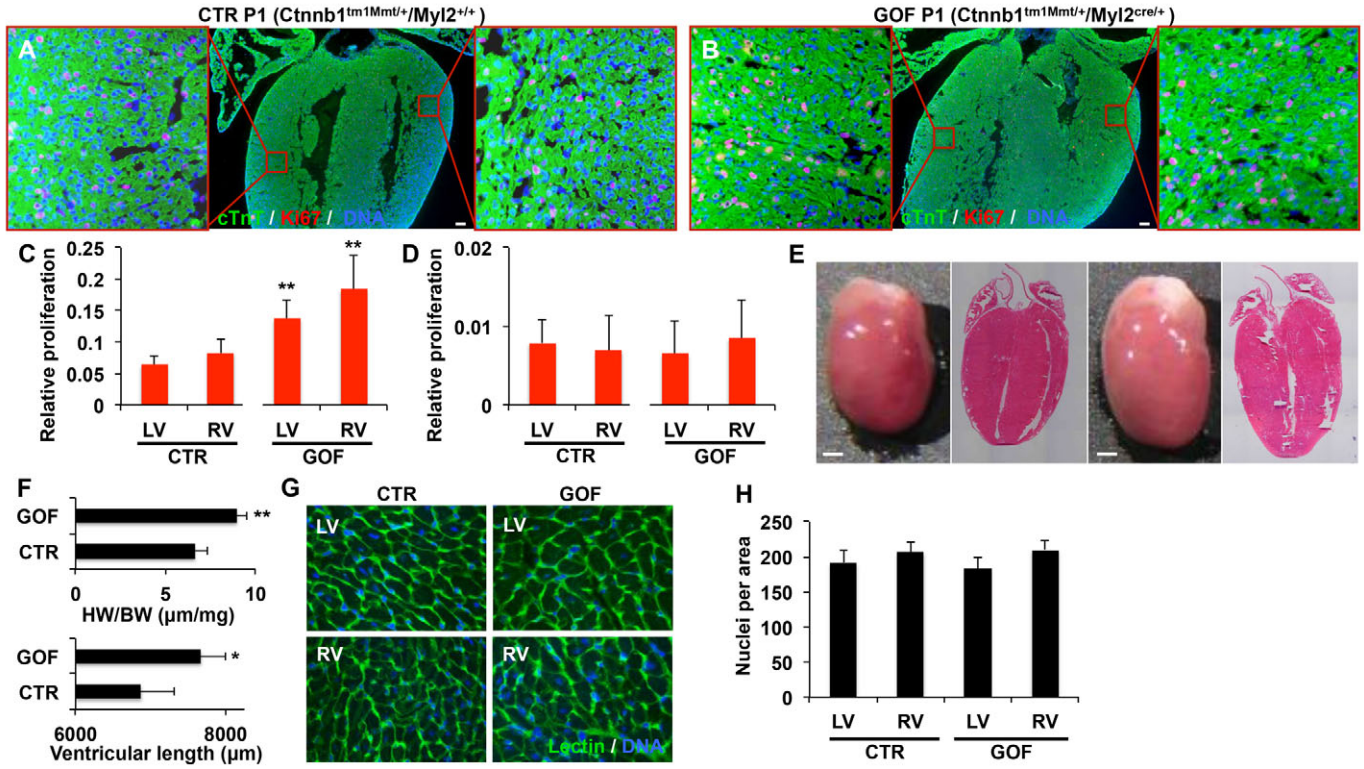

Fig. 7. Constitutive activation of β-catenin in the ventricular myogenic lineage. (**A**,**B**) Representative images of sections of (A) early neonatal (CTR) (P1) and (B) P1 β-catenin gain-of-function (GOF) ventricles stained for troponin T (cTnT) (green), Ki67 (red) and DAPI (DNA) (blue). Scale bars: 50 μm. (**C**) Percentage of cTnT+/Ki67+ cells in CTR and GOF left ventricle (LV) and right ventricle (RV) at P1 (n=3). (**D**) Percentage of cTnT+/Ki67+ cells in CTR and GOF left ventricle (LV) and right ventricle (RV) at P30 (n=3). (**E**) Color images, and Hematoxylin and Eosin staining of P30 CTR hearts (left panels) and β-catenin GOF hearts (right panels). Scale bars: 1 mm. (**F**) Heart-weight/body-weight (HW/BW) ratios (upper panel) of CTR and GOF animals, and ventricular length in μm (lower panel) at P30 (n=4-6 per group). (**G**) Image of ventricular cells stained for lectin (green) and DAPI (DNA) (blue) in CTR and GOF hearts. (**H**) Ventricular cell size measurements of CTR and GOF LV and RV cells (n=3, 25 replicates per ventricle). Number of nuclei per area of 40× picture (n=3). Error bars indicate s.d. *P<0.05, **P<0.01.

P1, RNA was isolated from the LV and RV of neonatal hearts. Expression analysis of Wnt target genes demonstrated that in the β-catenin gain-of-function ventricles, axin2, a known downstream target of β-catenin, was expressed at an 8- to 15-fold higher level than in littermate controls (supplementary material Fig. S5).

We next examined the ventricular size in response to constitutive activation of Wnt/β-catenin signaling in the myocardium. Morphologically, LV, RV and septum shape of Ctnnb1$^{tm1Mmt/+}$/Myl2$^{cre/+}$ adult mice was comparable with cre-negative littermate controls. Furthermore, there was no difference in the number of cTnT+/Ki67+ cells between groups at this stage (P=0.54 and P=0.64) (Fig. 7D; supplementary material Fig. S10, Table S2). At P30, we did observe an 11% increase in ventricular length/size (P<0.01) and an increased heart-weight/body-weight ratio in β-catenin gain-of-function hearts compared with littermate controls (Fig. 7E,F). The total number of nuclei per area in the left and right ventricle did not differ between gain-of-function and control ventricles (Fig. 7G,H). Collectively, these results indicate that Wnt signaling exerts a profound proliferative effect on early cardiac myocytes but not on mature myocytes.

DISCUSSION

A crucial step in cardiogenesis is the formation and expansion of the ventricular myocyte lineage necessary for normal cardiac contractile function. The finding that the proliferation of FHF and SHF early ventricular CMs from embryos and corresponding ESC lines are controlled by the β-catenin signaling pathway uncovers a mechanistic pathway for the control of *in vitro* cardiogenesis and *in vivo* adult heart size. From a developmental perspective, we showed that, in the developing myocardium, β-catenin is predominantly active in the compact myocardium and that the activated protein is expressed in the majority of proliferating cardiac myocytes. Furthermore, cardiac loss-of-function of β-catenin results in an abrogation of myocyte proliferation. Conversely, activation of β-catenin and canonical Wnt signaling drives the proliferation of early ventricular CMs. Work from a number of laboratories over several decades has shown that during mammalian development, the compact myocardium has a significantly higher rate of cardiomyocyte proliferation. Our studies begin to provide a mechanistic explanation of how this is achieved. Specifically, we show that the differential proliferation in the different cardiac sub-compartments requires the differential activation of β-catenin signaling. Cardiac-specific overexpression or deletion of β-catenin, such that it is equally expressed in both the compact and trabecular myocardium, abrogates the normally observed differences in their proliferation rates. This raises the possibility that inductive signals from the epicardium or repressive signals from the endocardium may control the differential activation of β-catenin and thereby direct the observed differences in proliferation rates in compact versus trabecular myocardium.

The organized assembly of various cell types is fundamental to organogenesis and organ regeneration. Complete regeneration of complex organs such as the adult heart appears to be largely restricted to lower vertebrates such as zebrafish (Jopling et al., 2010; Kikuchi et al., 2010; Poss et al., 2002). In mammals, the neonatal heart has been shown to regenerate from apical injury, but this potential fades shortly after birth (Porrello et al., 2011), and annual cardiac myocyte turnover decreases to less than 2-4% in adults (Bergmann et al., 2009; Porrello et al., 2011; Senyo et al., 2013). Our incomplete understanding of the molecular mechanisms driving cardiomyocyte expansion during cardiogenesis limits our ability to develop novel strategies for regenerative cardiovascular medicine. In that regard, the results described herein represent an important advance in defining pro-proliferative pathways in early FHF- and SHF-derived ventricular CMs.

A key consideration is that expanded CMs preserve their potential to further differentiate into functional CMs without significant epigenetic or phenotypic drift. We show that early FHF and SHF ventricular CMs, derived from murine and human ES and iPSC sources, have similar responsiveness to Wnt/β-catenin signaling. Quantitative gene expression analysis for common cardiac genes illustrated that expanding early ventricular CMs are in an intermediate differentiated state between freshly isolated and their differentiated progeny. Withdrawal of Gsk3 inhibition allows expanded CMs to further differentiate into functional CMs. Active forced stabilization of axin by IWR resulted in the further enhanced differentiation of expanded CMs. Remarkably, expanded FHF and SHF CMs aligned into functional myocardial tissue with similar potential to their unexpanded counterparts. Thus, expanded cardiac myocytes maintain the functional properties, cardiac gene expression pattern, protein organization and cellular alignment of the unexpanded native CMs. This underlines the potential of Gsk3 inhibition-driven expansion of early CMs for yielding large numbers of functional cardiomyocytes, as required for chemical screens and future therapeutic strategies.

Although we found an increase in proliferating ventricular myocardium of neonatal β-catenin gain-of-function mice, this effect was limited. By P30 we no longer found any statistical difference in the myocardial proliferation rate. In addition, we observed an increase in ventricular size between gain-of-function mice and littermate controls, with the number of nuclei per area remaining equal. However, we could not rule out earlier cell cycle withdrawal or increased apoptosis rates in hearts of gain-of-function animals compared with controls. During the transition from the neonatal to the adult period, a number of cellular changes occur that allow neonatal CMs to mature into their adult counterparts. Collectively, these changes result in cardiac myocytes that are no longer capable of proliferating in response to activated β-catenin signaling. As such, activation of a recently discovered Hippo/Yap1/Tead pathway that orchestrates heart size control, could be downregulating postnatal β-catenin-mediated CM cell cycling (Heallen et al., 2011; von Gise et al., 2012). Furthermore, TGF-β/BMP regulatory pathways, especially known to influence growth and differentiation early on in cardiac specification and differentiation (Klaus et al., 2012; Wang et al., 2010), may contribute to molecular changes causing cardiac maturation and/or redundancy of Wnt signals.

We have examined the role of the Wnt/β-catenin pathway in controlling the expansion and differentiation of early CMs from different sources by employing several validated small molecule inhibitors of Gsk3. Disruption of Gsk3-mediated degradation of β-catenin results in the activation of β-catenin and its nuclear translocation, subsequently resulting in the Lef1/Tcf-mediated activation of Wnt target genes (Jho et al., 2002; Lustig et al., 2002). Although Gsk3 is described as being part of the canonical Wnt cascade, it also forms the cornerstone for molecular crosstalk. Signaling pathways orchestrated by PI3K/Akt and Raf/Mek/Erk indirectly inhibit Gsk3 at the cytoplasmic level and thereby can act synergistically with activated Wnt signaling (Shiojima and Walsh, 2006; Singh et al., 2012). We demonstrate that Wnt signaling can promote early CM proliferation during development, but our results do not rule out the possibility that Akt signaling may also converge on the β-catenin signaling pathway.

Advanced heart failure is a major unmet clinical problem that arises from a loss of viable and/or fully functional cardiac muscle cells (Jessup and Brozena, 2003). Currently, a number of clinical trials have been designed to augment the function of cardiac muscle via bone marrow or mesenchymal stem cell transplantations. To date, although there have been encouraging early suggestions of a small therapeutic benefit, there has not been evidence for actual regeneration of heart muscle tissue (Bolli et al., 2011; Menasché, 2008; Pouly et al., 2008), underscoring the need for new approaches. A central challenge for cell-based therapy has been the isolation and expansion of sufficient numbers of cardiac myocytes necessary for tissue regeneration. In addition, it is crucial that the expanded cells retain their differentiation potential as well as their capacity to self-organize into functional myocardial tissue. Herein we demonstrate that early cardiac myocytes from a renewable cell source can be robustly expanded while maintaining their capacity to differentiate into functional myocardial tissue.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

J.W.B. performed experiments reported in in Figs 1-7 and wrote the manuscript; A.S.M. performed experiments reported in Fig. 7; N.V.M. performed experiments reported in Fig. 1; A.A. performed experiments reported in Fig. 3; L.C. performed experiments reported in Fig. 2; J.P.G.S. mentored colleagues and edited the manuscript; P.A.D. mentored colleagues and edited the manuscript; I.J.D. mentored colleagues and wrote the manuscript.

Supplementary material

Supplementary material available online at
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References

Ai, D., Fu, X., Wang, J., Lu, M. F., Chen, L., Baldini, A., Klein, W. H. and Martin, J. F. (2007). Canonical Wnt signaling functions in second heart field to promote right ventricular growth. *Proc. Natl. Acad. Sci. USA* **104**, 9319-9324.

Barrow, J. R., Howell, W. D., Rule, M., Hayashi, S., Thomas, K. R., Capecchi, M. R. and McMahon, A. P. (2007). Wnt3 signaling in the epiblast is required for proper orientation of the anteroposterior axis. *Dev. Biol.* **312**, 312-320.

Bejsovec, A. (2005). Wnt pathway activation: new relations and locations. *Cell* **120**, 11-14.

Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B. A., Druid, H. et al. (2009). Evidence for cardiomyocyte renewal in humans. *Science* **324**, 98-102.
Bolli, R., Chugh, A. R., D'Amario, D., Loughran, J. H., Stoddard, M. F., Ikram, S., Beache, G. M., Wagner, S. G., Leri, A., Hosoda, T. et al. (2011). Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. *Lancet* **378**, 1847-1857.
Brault, V., Moore, R., Kutsch, S., Ishibashi, M., Rowitch, D. H., McMahon, A. P., Sommer, L., Boussadia, O. and Kemler, R. (2001). Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* **128**, 1253-1264.
Bu, L., Jiang, X., Martin-Puig, S., Caron, L., Zhu, S., Shao, Y., Roberts, D. J., Huang, P. L., Domian, I. J. and Chien, K. R. (2009). Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature* **460**, 113-117.
Buckingham, M., Meilhac, S. and Zaffran, S. (2005). Building the mammalian heart from two sources of myocardial cells. *Nat. Rev. Genet.* **6**, 826-837.
Chen, J., Kubalak, S. W. and Chien, K. R. (1998a). Ventricular muscle-restricted targeting of the RXRalpha gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis. *Development* **125**, 1943-1949.
Chen, J., Kubalak, S. W., Minamisawa, S., Price, R. L., Becker, K. D., Hickey, R., Ross, J., Jr and Chien, K. R. (1998b). Selective requirement of myosin light chain 2v in embryonic heart function. *J. Biol. Chem.* **273**, 1252-1256.
Chen, B., Dodge, M. E., Tang, W., Lu, J., Ma, Z., Fan, C. W., Wei, S., Hao, W., Kilgore, J., Williams, N. S. et al. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat. Chem. Biol.* **5**, 100-107.
Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. *Cell* **127**, 469-480.
Cohen, E. D., Wang, Z., Lepore, J. J., Lu, M. M., Taketo, M. M., Epstein, D. J. and Morrisey, E. E. (2007). Wnt/beta-catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. *J. Clin. Invest.* **117**, 1794-1804.
Deedwania, P. C. and Nelson, J. R. (1990). Pathophysiology of silent myocardial ischemia during daily life. Hemodynamic evaluation by simultaneous electrocardiographic and blood pressure monitoring. *Circulation* **82**, 1296-1304.
Dodou, E., Xu, S. M. and Black, B. L. (2003). mef2c is activated directly by myogenic basic helix-loop-helix proteins during skeletal muscle development in vivo. *Mech. Dev.* **120**, 1021-1032.
Domian, I. J., Chiravuri, M., van der Meer, P., Feinberg, A. W., Shi, X., Shao, Y., Wu, S. M., Parker, K. K. and Chien, K. R. (2009). Generation of functional ventricular heart muscle from mouse ventricular progenitor cells. *Science* **326**, 426-429.
Feinberg, A. W., Feigel, A., Shevkoplyas, S. S., Sheehy, S., Whitesides, G. M. and Parker, K. K. (2007). Muscular thin films for building actuators and powering devices. *Science* **317**, 1366-1370.
Ford, E. S., Ajani, U. A., Croft, J. B., Critchley, J. A., Labarthe, D. R., Kottke, T. E., Giles, W. H. and Capewell, S. (2007). Explaining the decrease in U.S. deaths from coronary disease, 1980-2000. *N. Engl. J. Med.* **356**, 2388-2398.
Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K. and Kemler, R. (1995). Lack of beta-catenin affects mouse development at gastrulation. *Development* **121**, 3529-3537.
Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M. and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J.* **18**, 5931-5942.
Heallen, T., Zhang, M., Wang, J., Bonilla-Claudio, M., Klysik, E., Johnson, R. L. and Martin, J. F. (2011). Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science* **332**, 458-461.
Ishiwata, T., Nakazawa, M., Pu, W. T., Tevosian, S. G. and Izumo, S. (2003). Developmental changes in ventricular diastolic function correlate with changes in ventricular myoarchitecture in normal mouse embryos. *Circ. Res.* **93**, 857-865.
Iwao, K., Nakamori, S., Kameyama, M., Imaoka, S., Kinoshita, M., Fukui, T., Ishiguro, S., Nakamura, Y. and Miyoshi, Y. (1998). Activation of the beta-catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations. *Cancer Res.* **58**, 1021-1026.
Jessup, M. and Brozena, S. (2003). Heart failure. *N. Engl. J. Med.* **348**, 2007-2018.
Jeter, J. R., Jr and Cameron, I. L. (1971). Cell proliferation patterns during cytodifferentiation in embryonic chick tissues: liver, heart and erythrocytes. *J. Embryol. Exp. Morphol.* **25**, 405-422.
Jho, E. H., Zhang, T., Domon, C., Joo, C. K., Freund, J. N. and Costantini, F. (2002). Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol. Cell. Biol.* **22**, 1172-1183.
Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A. and Izpisúa Belmonte, J. C. (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**, 606-609.
Kaichi, S., Hasegawa, K., Takaya, T., Yokoo, N., Mima, T., Kawamura, T., Morimoto, T., Ono, K., Baba, S., Doi, H. et al. (2010). Cell line-dependent differentiation of induced pluripotent stem cells into cardiomyocytes in mice. *Cardiovasc. Res.* **88**, 314-323.
Kattman, S. J., Witty, A. D., Gagliardi, M., Dubois, N. C., Niapour, M., Hotta, A., Ellis, J. and Keller, G. (2011). Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* **8**, 228-240.
Kikuchi, K., Holdway, J. E., Werdich, A. A., Anderson, R. M., Fang, Y., Egnaczyk, G. F., Evans, T., Macrae, C. A., Stainier, D. Y. and Poss, K. D. (2010). Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature* **464**, 601-605.
Klaus, A., Müller, M., Schulz, H., Saga, Y., Martin, J. F. and Birchmeier, W. (2012). Wnt/β-catenin and Bmp signals control distinct sets of transcription factors in cardiac progenitor cells. *Proc. Natl. Acad. Sci. USA* **109**, 10921-10926.
Kouskoff, V., Lacaud, G., Schwantz, S., Fehling, H. J. and Keller, G. (2005). Sequential development of hematopoietic and cardiac mesoderm during embryonic stem cell differentiation. *Proc. Natl. Acad. Sci. USA* **102**, 13170-13175.
Kunick, C., Lauenroth, K., Leost, M., Meijer, L. and Lemcke, T. (2004). 1-Azakenpaullone is a selective inhibitor of glycogen synthase kinase-3 beta. *Bioorg. Med. Chem. Lett.* **14**, 413-416.
Kwon, C., Arnold, J., Hsiao, E. C., Taketo, M. M., Conklin, B. R. and Srivastava, D. (2007). Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. *Proc. Natl. Acad. Sci. USA* **104**, 10894-10899.
Kwon, C., Qian, L., Cheng, P., Nigam, V., Arnold, J. and Srivastava, D. (2009). A regulatory pathway involving Notch1/beta-catenin/Isl1 determines cardiac progenitor cell fate. *Nat. Cell Biol.* **11**, 951-957.
Laflamme, M. A., Chen, K. Y., Naumova, A. V., Muskheli, V., Fugate, J. A., Dupras, S. K., Reinecke, H., Xu, C., Hassanipour, M., Police, S. et al. (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat. Biotechnol.* **25**, 1015-1024.
Laugwitz, K. L., Moretti, A., Caron, L., Nakano, A. and Chien, K. R. (2008). Islet1 cardiovascular progenitors: a single source for heart lineages? *Development* **135**, 193-205.
Levy, D., Kenchaiah, S., Larson, M. G., Benjamin, E. J., Kupka, M. J., Ho, K. K., Murabito, J. M. and Vasan, R. S. (2002). Long-term trends in the incidence of and survival with heart failure. *N. Engl. J. Med.* **347**, 1397-1402.
Lien, C. L., Wu, C., Mercer, B., Webb, R., Richardson, J. A. and Olson, E. N. (1999). Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. *Development* **126**, 75-84.
Lin, L., Cui, L., Zhou, W., Dufort, D., Zhang, X., Cai, C. L., Bu, L., Yang, L., Martin, J., Kemler, R. et al. (2007). Beta-catenin directly regulates Islet1 expression in cardiovascular progenitors and is required for multiple aspects of cardiogenesis. *Proc. Natl. Acad. Sci. USA* **104**, 9313-9318.
Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R. and Bradley, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nat. Genet.* **22**, 361-365.
Lloyd-Jones, D. M., Leip, E. P., Larson, M. G., D'Agostino, R. B., Beiser, A., Wilson, P. W., Wolf, P. A. and Levy, D. (2006). Prediction of lifetime risk for cardiovascular disease by risk factor burden at 50 years of age. *Circulation* **113**, 791-798.
Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.
Lu, J., Ma, Z., Hsieh, J. C., Fan, C. W., Chen, B., Longgood, J. C., Williams, N. S., Amatruda, J. F., Lum, L. and Chen, C. (2009). Structure-activity relationship studies of small-molecule inhibitors of Wnt response. *Bioorg. Med. Chem. Lett.* **19**, 3825-3827.
Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P. M., Birchmeier, W. et al. (2002). Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol. Cell. Biol.* **22**, 1184-1193.
Luxán, G., Casanova, J. C., Martínez-Poveda, B., Prados, B., D'Amato, G., MacGrogan, D., Gonzalez-Rajal, A., Dobarro, D., Torroja, C., Martinez, F. et al. (2013). Mutations in the NOTCH pathway regulator MIB1 cause left ventricular noncompaction cardiomyopathy. *Nat. Med.* **19**, 193-201.
Martin-Puig, S., Wang, Z. and Chien, K. R. (2008). Lives of a heart cell: tracing the origins of cardiac progenitors. *Cell Stem Cell* **2**, 320-331.
Meijer, L., Skaltsounis, A. L., Magiatis, P., Polychronopoulos, P., Knockaert, M., Leost, M., Ryan, X. P., Vonica, C. A., Brivanlou, A., Dajani, R. et al. (2003). GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem. Biol.* **10**, 1255-1266.
Menasché, P. (2008). Current status and future prospects for cell transplantation to prevent congestive heart failure. *Semin. Thorac. Cardiovasc. Surg.* **20**, 131-137.
Naito, A. T., Shiojima, I., Akazawa, H., Hidaka, K., Morisaki, T., Kikuchi, A. and Komuro, I. (2006). Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc. Natl. Acad. Sci. USA* **103**, 19812-19817.
Paige, S. L., Osugi, T., Afanasiev, O. K., Pabon, L., Reinecke, H. and Murry, C. E. (2010). Endogenous Wnt/beta-catenin signaling is required for cardiac differentiation in human embryonic stem cells. *PLoS ONE* **5**, e11134.

Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N. and Sadek, H. A. (2011). Transient regenerative potential of the neonatal mouse heart. *Science* **331**, 1078-1080.
Poss, K. D., Wilson, L. G. and Keating, M. T. (2002). Heart regeneration in zebrafish. *Science* **298**, 2188-2190.
Pouly, J., Bruneval, P., Mandet, C., Proksch, S., Peyrard, S., Amrein, C., Bousseaux, V., Guillemain, R., Deloche, A., Fabiani, J. N. et al. (2008). Cardiac stem cells in the real world. *J. Thorac. Cardiovasc. Surg.* **135**, 673-678.
Quyyumi, A. A., Wright, C. A., Mockus, L. J. and Fox, K. M. (1984). Mechanisms of nocturnal angina pectoris: importance of increased myocardial oxygen demand in patients with severe coronary artery disease. *Lancet* **323**, 1207-1209.
Qyang, Y., Martin-Puig, S., Chiravuri, M., Chen, S., Xu, H., Bu, L., Jiang, X., Lin, L., Granger, A., Moretti, A. et al. (2007). The renewal and differentiation of Isl1+ cardiovascular progenitors are controlled by a Wnt/beta-catenin pathway. *Cell Stem Cell* **1**, 165-179.
Rivera-Pérez, J. A. and Magnuson, T. (2005). Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. *Dev. Biol.* **288**, 363-371.
Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. *Science* **272**, 1023-1026.
Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. and Brivanlou, A. H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat. Med.* **10**, 55-63.
Schneider, V. A. and Mercola, M. (2001). Wnt antagonism initiates cardiogenesis in Xenopus laevis. *Genes Dev.* **15**, 304-315.
Senyo, S. E., Steinhauser, M. L., Pizzimenti, C. L., Yang, V. K., Cai, L., Wang, M., Wu, T. D., Guerquin-Kern, J. L., Lechene, C. P. and Lee, R. T. (2013). Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* **493**, 433-436.
Shiojima, I. and Walsh, K. (2006). Regulation of cardiac growth and coronary angiogenesis by the Akt/PKB signaling pathway. *Genes Dev.* **20**, 3347-3365.
Sineva, G. S. and Pospelov, V. A. (2010). Inhibition of GSK3beta enhances both adhesive and signalling activities of beta-catenin in mouse embryonic stem cells. *Biol. Cell* **102**, 549-564.
Singh, A. M., Reynolds, D., Cliff, T., Ohtsuka, S., Mattheyses, A. L., Sun, Y., Menendez, L., Kulik, M. and Dalton, S. (2012). Signaling network crosstalk in human pluripotent cells: a Smad2/3-regulated switch that controls the balance between self-renewal and differentiation. *Cell Stem Cell* **10**, 312-326.
Sparks, A. B., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res.* **58**, 1130-1134.
Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676.
Trosset, J. Y., Dalvit, C., Knapp, S., Fasolini, M., Veronesi, M., Mantegani, S., Gianellini, L. M., Catana, C., Sundström, M., Stouten, P. F. et al. (2006). Inhibition of protein-protein interactions: the discovery of druglike beta-catenin inhibitors by combining virtual and biophysical screening. *Proteins* **64**, 60-67.
Tzahor, E. (2007). Wnt/beta-catenin signaling and cardiogenesis: timing does matter. *Dev. Cell* **13**, 10-13.
van der Pol, A., Domian, I. and van der Meer, P. (2012). Cardiac regeneration in left ventricular dysfunction: are we asking the right questions? *Eur. J. Heart Fail.* **14**, 1-4.
von Gise, A., Lin, Z., Schlegelmilch, K., Honor, L. B., Pan, G. M., Buck, J. N., Ma, Q., Ishiwata, T., Zhou, B., Camargo, F. D. et al. (2012). YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proc. Natl. Acad. Sci. USA* **109**, 2394-2399.
Wang, J., Greene, S. B., Bonilla-Claudio, M., Tao, Y., Zhang, J., Bai, Y., Huang, Z., Black, B. L., Wang, F. and Martin, J. F. (2010). Bmp signaling regulates myocardial differentiation from cardiac progenitors through a MicroRNA-mediated mechanism. *Dev. Cell* **19**, 903-912.
Wang, H., Hao, J. and Hong, C. C. (2011). Cardiac induction of embryonic stem cells by a small molecule inhibitor of Wnt/β-catenin signaling. *ACS Chem. Biol.* **6**, 192-197.
Willems, E., Spiering, S., Davidovics, H., Lanier, M., Xia, Z., Dawson, M., Cashman, J. and Mercola, M. (2011). Small-molecule inhibitors of the Wnt pathway potently promote cardiomyocytes from human embryonic stem cell-derived mesoderm. *Circ. Res.* **109**, 360-364.
Wu, S. M., Fujiwara, Y., Cibulsky, S. M., Clapham, D. E., Lien, C. L., Schultheiss, T. M. and Orkin, S. H. (2006). Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* **127**, 1137-1150.
Ying, Q. L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P. and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519-523.