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

Jan Willem Buikema¹,², Ahmed S. Mady¹,², Nikhil V. Mittal¹,², Ayhan Atmanli¹, Leslie Caron⁴, Pieter A. Doevendans⁵, Joost P. G. Sluijter³ and Ibrahim J. Domian¹,²,⁵,*

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; Lauwitz 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 et al., 2005; Clevers, 2006; Rubinfeld et al., 1996; Tzahor, 2007).

¹Cardiovascular Research Center, Massachusetts General Hospital, Charles River Plaza/CPSN 3200, 185 Cambridge Street, Boston, MA 02114-2790, USA. ²Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115, USA. ³Department of Cardiology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands. ⁴Genea/FSHD, 321 Kent Street, Sydney NSW 2000, Australia. ⁵Harvard Stem Cell Institute, 1350 Massachusetts Avenue, Cambridge, MA 02138, USA.

*Author for correspondence (idomian@partners.org)

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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 (TFFs) 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 reimplanted 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− (FFH), 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-bromindinurine-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 Appendol Realplex 4. Prior to Tryzol (Invitrogen) RNA isolation, cells were treated with various small molecules in equal volumes of DMEM. 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% placmanate, 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 Myl2cre+ mouse strain (Chen et al., 1998a). For the ventricular-specific β-catenin loss-of-function studies, the Ctnnb1tm1Mmt/tm1Mmt (Harada et al., 1999) mice strain was used (Braut et al., 2001). For the gain of function of β-catenin, Ctnnb1tm1Mmt/tm1Mmt (Harada et al., 1999) mice were used. All animal work was performed within the institutional animal guidelines.
For *ex vivo* cardiac cultures, hearts from Rosa26lox(stop)YFP/+/Myl2cre/+ 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 fluorescent 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+) and early ventricular 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.

**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.
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 DMSO, 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
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<sup>loxp(stop)YFP/+<sup>/Myl2cre/+ E12.5 embryos using FACS (Fig. 4A,B). YFP<sup>+</sup> ventricular myocytes were then cultured in a three-dimensional cardiac microsphere 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
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/\(\beta\)-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/\(\beta\)-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 \(\beta\)-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 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
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). 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 β-catenin loss-of-function mutants by crossing Ctnnb1tm1Mmt/tm1Mmt mice with Myl2Cre/− animals. The β-catenin molecule (Brault et al., 2001). Cardiac-specific β-catenin gain-of-function mutants were generated by crossing Ctnnb1tm1Mmt/tm1Mmt mice with Myl2Cre/+ 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 Ctnnb1tm1Mmt 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 Ctnnb1tm2Kem/tm2Kem mice with Myl2Cre/+ mice breeding with E12.5 embryos born from Ctnnb1tm2Kem/tm2Kem mice breeding with Myl2Cre/+ 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. 6H-L). Crucially, this reduction in the proliferation rate was observed to a similar extent in the compact and trabecular myocardium of both the LV and the RV (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...
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, Ctnnb1tm1Mmt/+Myl2cre/+ gain-of-function animals, expressing a constitutively active β-catenin mutant protein in ventricular myocytes, were live born in the predicated Mendelian ratio. Gain-of-function Ctnnb1tm1Mmt/+Myl2cre/+ 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.

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**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 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. (KL) 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.
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 Ctnnb1tm1Mmt/+/Myl2cre/+ 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 iPS 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 reduction 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.

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