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Article

The Renewal and Differentiation of *Isl1*$^+$ Cardiovascular Progenitors Are Controlled by a Wnt/β-Catenin Pathway

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SUMMARY

***Isl1*$^+$ cardiovascular progenitors and their downstream progeny play a pivotal role in cardiogenesis and lineage diversification of the heart. The mechanisms that control their renewal and differentiation are largely unknown. Herein, we show that the Wnt/β-catenin pathway is a major component by which cardiac mesenchymal cells modulate the prespecification, renewal, and differentiation of *isl1*$^+$ cardiovascular progenitors. This microenvironment can be reconstituted by a Wnt3a-secreting feeder layer with ES cell-derived, embryonic, and postnatal *isl1*$^+$ cardiovascular progenitors. In vivo activation of β-catenin signaling in *isl1*$^+$ progenitors of the secondary heart field leads to their massive accumulation, inhibition of differentiation, and outflow tract (OFT) morphogenic defects. In addition, the mitosis rate in OFT myocytes is significantly reduced following β-catenin deletion in *isl1*$^+$ precursors. Agents that manipulate Wnt signals can markedly expand *isl1*$^+$ progenitors from human neonatal hearts, a key advance toward the cloning of human *isl1*$^+$ heart progenitors.**

INTRODUCTION

Cardiogenesis requires the formation of a diverse spectrum of muscle and nonmuscle cell lineages in specific tissue compartments in the heart. Understanding how embryonic precursor cells generate and control the formation of distinct endothelial, pacemaker, atrial, ventricular, and vascular smooth muscle lineages, as well as how these cells become positioned to form the specific chambers, aorta, coronary arteries, and conduction system in the heart, is of fundamental importance in unraveling the developmental logic and molecular cues that underlie both cardiovascular development and disease (for review, see Chien and Karsenty [2005]).

Toward this end, recent studies from our laboratory have identified multipotent *isl1*$^+$ cardiovascular progenitors (MICPs), marked by the transcriptional signature of *isl1/nkx2.5/flk-1*, which can generate the three major cell types in the heart: cardiac, smooth muscle, and endothelial cells (Moretti et al., 2006). MICPs have been cloned from both mouse ES (embryonic stem) cells and mouse embryos and can make this decision at a single cell level, suggesting a hematopoietic paradigm for how the diversity of cardiovascular lineages can be generated (Moretti et al., 2006). Supporting this concept, a hierarchy of distinct *isl1*$^+$ cardiovascular progenitors have been uncovered (Laugwitz et al., 2005; Moretti et al., 2006), including a rare subset of *isl1*$^+$ cardioblasts that persist until birth

and can develop into fully mature cardiac and smooth muscle cells. At the same time, independent studies from other laboratories also point to the existence of other multipotent and bipotent cardiovascular precursors (Kattman et al., 2006; Wu et al., 2006), which may also arise from the early heart field. Moreover, in vivo lineage tracing of the descendants of the *isl1*$^+$ cardiovascular progenitors documents their contribution to over two-thirds of all the cells in the embryonic heart, and to these three major cell types in all of the cardiovascular compartments, with the exception of the free left ventricular wall (Cai et al., 2003; Laugwitz et al., 2005). Uncovering the molecular pathways that control their formation, renewal, and differentiation into specific mature cellular progeny will be critical in unlocking the potential of stem cell therapy for a myriad of cardiovascular degenerative diseases.

In many model systems, microenvironmental cues are known to play a pivotal role in the control of stem cell formation, renewal, and differentiation (for review, see Scadden [2006]). While it is known that a single hematopoietic stem cell can regenerate the entire spectrum of blood cell lineages (Akashi et al., 2000), there is an absolute requirement for the in vivo bone marrow niche (for review, see Yin and Li [2006]). Accordingly, it is difficult to renew and expand hematopoietic stem cells ex vivo, a major inherent limitation to the system. In the case of the embryonic (Moretti et al., 2006) and postnatal (Laugwitz et al., 2005) *isl1*$^+$ cardiovascular progenitors, we have previously documented a role for the cardiac mesenchymal cells (CMC) in the expansion, inhibition of differentiation, and maintenance of their multipotency. In addition, the CMC play a role in the prespecification of MICPs, having an inductive effect on the transition of mesodermal precursors into the hierarchy of *isl1*$^+$ heart progenitors (Moretti et al., 2006). Uncovering the molecular basis for these effects of the CMC would be a major step forward in allowing the unlimited expansion of *isl*$^+$ cardiovascular progenitors while maintaining their multipotency. In addition, documenting the translation of these pathways to human *isl1*$^+$ progenitors would represent another important step in the critical pathway that might eventually allow these human cells to be harnessed as models of human cardiovascular disease, assay systems for cardiovascular drug development, and to be exploited for their potential therapeutic applications. We herein report that a Wnt/β-catenin pathway is a major component of the CMC microenvironment that controls the prespecification, renewal, and the subsequent differentiation of a hierarchy of *isl1*$^+$ cardiovascular progenitors. The inhibition of glycogen synthase kinase-3 (GSK-3) by chemical agents leads to a marked increase in the expansion of human *isl1*$^+$ cardiovascular progenitors. In addition, the in vivo constitutive activation of β-catenin pathways within *isl1*$^+$ progenitors in the secondary heart field results in their massive accumulation, inhibition of myocytic differentiation, and severe outflow tract (OFT) defects. Finally, the mitosis rate in OFT myocytes is significantly reduced following β-catenin deletion in *isl1*$^+$ precursors. Taken together, these findings are an important advance in understanding the control of the microenvironmental pathways by which the CMC can regulate the cell fate of *isl1*$^+$ cardiovascular progenitors during cardiogenesis, and how defects in their renewal and differentiation can lead to OFT abnormalities that are a major form of congenital heart disease. In addition, the studies represent an important step toward the isolation of clones of human *isl1*$^+$ cardiovascular progenitors via the manipulation of Wnt signals in CMC.

RESULTS

High-Throughput Screening Identifies Chemical Probes that Enhance CMC Cues for Expansion of *Isl1*$^+$ Cardiac Progenitors

To find CMC-derived environmental cues involved in the renewal of *isl1*$^+$ progenitors, we developed a high-throughput chemical screening system, based on the coculture of CMC with postnatal *isl1*$^+$ progenitors (Figure S1A). To genetically mark *isl1*$^+$ progenitors in the postnatal heart, we crossed *isl1-mER-Cre-mER* (*MCM*) mice with the conditional *Cre* reporter strain *R26R* (Laugwitz et al., 2005; Soriano, 1999). Recently, several synthetic small molecules from a combinatorial library of heterocyclic compounds were identified that regulate stem cell fate (Ding et al., 2003; Wu et al., 2004). We used this library to screen for small molecules that would expand the rare population of postnatal *isl1*$^+$ progenitors.

CMC from *isl1-MCM/R26R* mouse hearts were isolated as previously described (Laugwitz et al., 2005), expanded for 7 days, and treated with a DMSO control or small molecules for an additional 4 days. As seen in Figure S1B, β-galactosidase (β-gal) activity was directly proportional to the starting amount of CMC. The screening of over 15,000 independent compounds in four separate experiments identified 25 candidates that were able to significantly upregulate β-gal activity. Although there was only a small increase over the control, the effect of these compounds was highly reproducible and statistically significant ($p < 0.05$, 0.01, or 0.001, Figure S1C). A more sensitive assay of isl1 immunostaining was performed, and three candidates were noted to substantially increase the number of *isl1*$^+$ progenitors (Figure 1C and data not shown). Two of these compounds were unknown (compound A and compound B), and the third was 6-bromoindirubin-3′-oxime (BIO), previously shown to be an inhibitor of GSK-3 (Meijer et al., 2003). BIO has been recently shown to promote self-renewal of both human and mouse ES cells through activation of the Wnt/β-catenin pathway in combination with other signaling inputs (Sato et al., 2004). We therefore decided to explore the role of BIO on the renewal of *isl1*$^+$ progenitors.

As shown in Figures 1A–1E, BIO increased the number of *isl1*$^+$ progenitors in a dose-dependent manner, and a maximal effect was seen at 2.5 μM with an ~7-fold increase versus control. Immunostaining of cleaved caspase-3 showed no appreciable apoptosis in both BIO- and DMSO-treated CMC (data not shown), suggesting that the expansion of *isl1*$^+$ progenitors by BIO does not occur through repressing apoptosis. To further validate

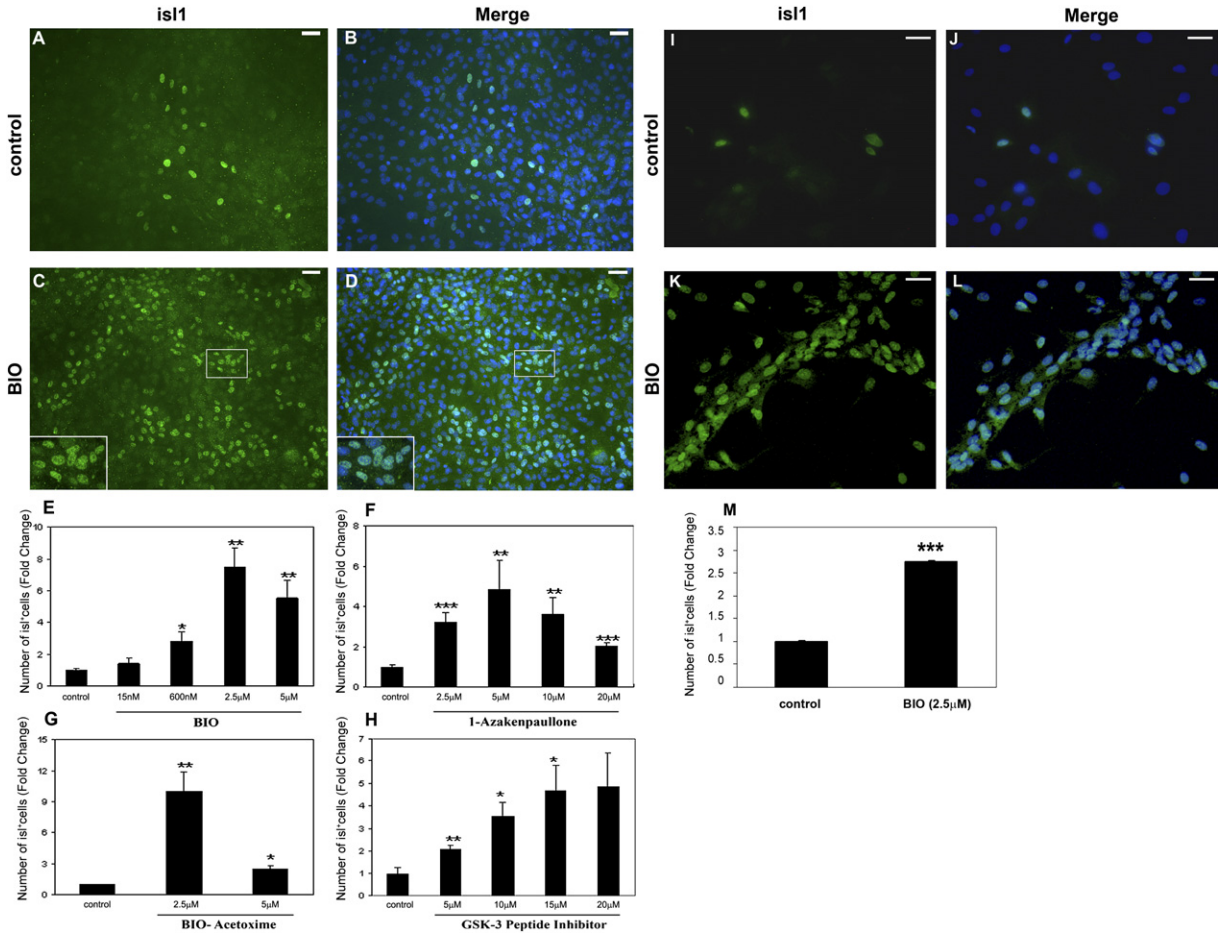


Figure 1. Identification and Characterization of a Chemical Probe that Augments the Expansion of Postnatal *Isl1*$^+$ Cardiovascular Progenitors from a High-Throughput Chemical Screening

Representative views of *isl1*$^+$ cells in the control (A and B) and in the BIO-treated sample (C and D). Insets show a magnification of *isl1*$^+$ cells. Nuclei were detected with Hoechst dye (B and D). Scale bar, 50 μm. Quantification of the effect of BIO (E), 1-Azakenpaullone (F), an acetoxime analog of BIO (G), or GSK-3 peptide inhibitor (H) treatment at different doses on the expansion of postnatal *isl1*$^+$ cardiovascular progenitors. Mean ± SEM, n ≥ 3. Note quantification of each treatment represents the total number of *isl*$^+$ cells per culture. (I–M) Human neonatal cardiac tissue-derived cells were cultured in the presence of either DMSO (control [I and J]) or BIO (K and L) and stained for isl1. Quantification of the effect of BIO on the expansion of postnatal human *isl1*$^+$ cardiovascular progenitors (M), mean ± SEM, n = 6. Scale bar, 25 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

the specificity of BIO function, we tested two other ATP-competitive GSK-3-specific inhibitors: an acetoxime analog of BIO and 1-Azakenpaullone (Kunick et al., 2004; Meijer et al., 2003). Both of these compounds substantially increased *isl1*$^+$ progenitor cell number versus control (Figures 1F and 1G). More importantly, a cell-permeable and substrate-competitive GSK-3 peptide inhibitor, which has negligible inhibitory effect on other protein kinases (Plotkin et al., 2003), was able to significantly expand *isl1*$^+$ progenitor cells (Figure 1H). Taken together, these results suggest that BIO promotes the expansion of *isl1*$^+$ progenitors by inhibiting GSK-3 activity.

In order to test whether BIO was capable of expanding human neonatal *isl1*$^+$ progenitors, human neonatal CMC were isolated from the biopsies of patients with congenital heart defects into single cells and cultured for 4 days in the presence or absence of BIO. Interestingly, BIO treatment markedly increased the number of human *isl1*$^+$ progenitors, detected by immunostaining (Figures 1I–1M), suggesting that Wnt/β-catenin pathways have an evolutionarily conserved role in expanding *isl1*$^+$ cardiovascular progenitors.

Wnt/β-Catenin Pathway Plays a Pivotal Role in the Control of *Isl1*$^+$ Progenitor Expansion

The above results suggested that CMC-derived cues promote the expansion of *isl1*$^+$ progenitors through the inhibition of GSK-3 activity. This prompted us to investigate the roles of signaling molecules in the GSK-3 pathway (Dominguez and Green, 2001). The Wnt/β-catenin pathway has previously been shown to be involved in the renewal of a variety of stem cell/progenitors (Reya et al., 2003; Sato et al., 2004). Thus, we examined whether Wnt3a, a well-established ligand in the Wnt/β-catenin

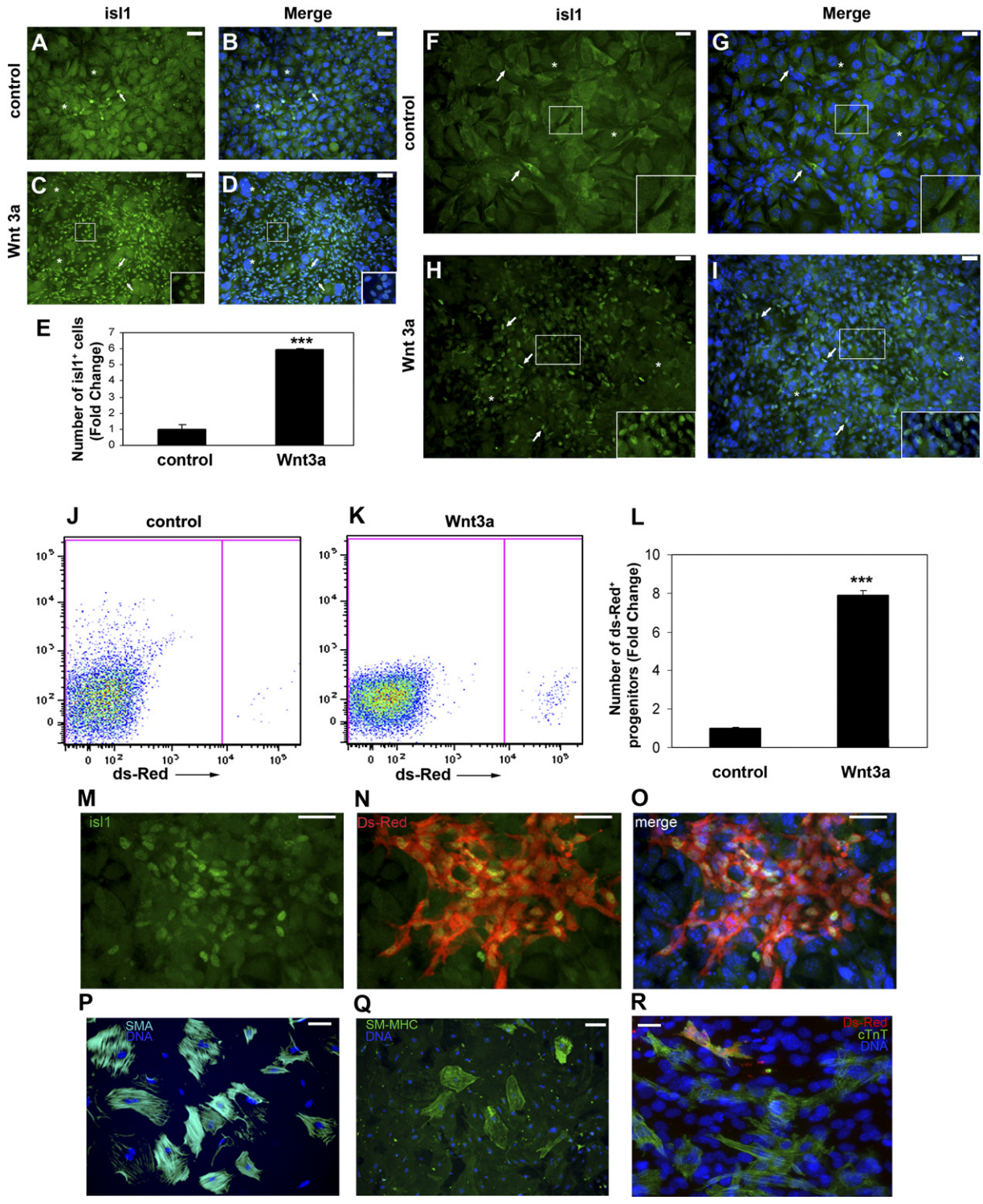


Figure 2. Wnt/β-Catenin Pathway Plays a Pivotal Role in the Control of the Expansion of *Isl1*$^+$ Cardiovascular Progenitors

Isl1 immunofluorescence on a control (A and B) or Wnt3a-producing (C and D) feeder layer. Arrows point to *isl1*$^+$ cells. Asterisks indicate feeder layer cells. Scale bar, 25 μm. (E) Quantification of the number of *isl1*$^+$ cells detected by immunostaining on a Wnt3a feeder layer compared with control. Mean values ± SEM, n = 6, ***p < 0.001. Immunofluorescence analysis of embryonic E8.5 *isl1*$^+$ progenitors on a control (F and G) or Wnt3a-producing (H and I) feeder layer. Scale bar, 50 μm. (J and K) Flow cytometry profile of E8.5 cells from AHF enriched tissue of double transgenic *isl1-IRES-Cre; Z/RED* embryos after expansion on a control or Wnt3a feeder layer for 7 days. (L) Quantification of the number of dsRed$^+$ progenitors on Wnt3a versus control feeder. Mean values ± SEM, n = 3, ***p < 0.001. (M–O) dsRed signal (N) correlates highly with isl1 expression (M) in cells from double transgenic

pathway (Logan and Nusse, 2004), was able to expand postnatal *isl1*$^+$ progenitors. Treatment with Wnt3a-conditioned medium resulted in an ~2-fold increase of *isl1*$^+$ progenitors compared with the control (Figures S2A–S2E). We further cocultured CMC with a feeder layer stably secreting Wnt3a, hence providing a higher sustained level of Wnt3a activity, and observed a nearly 6-fold increase of *isl1*$^+$ progenitors versus control (Figures 2A–2E).

To test whether Wnt signaling is required for the expansion of postnatal *isl1*$^+$ progenitors, we utilized a potent extracellular inhibitor of Wnt signaling, Dickkopf-1 (Dkk1) (Logan and Nusse, 2004). CMC incubated with Dkk1-conditioned medium showed a nearly 40% reduction in the number of *isl1*$^+$ progenitor cells versus the control treatment (Figures S2F–S2J).

Given the fact that Wnt3a can enhance the expansion of postnatal *isl1*$^+$ progenitors, we tested whether this ligand also had a similar effect on the *isl1*$^+$ embryonic progenitor subset. Single cell preparations from the secondary heart field region of approximately E8.5 embryos were generated as previously described (Moretti et al., 2006) and plated at a low density on a feeder layer consisting of a cell line stably secreting Wnt3a or its control. As shown in Figures 2H and 2I, the Wnt3a-secreting feeder layer triggered a marked expansion of embryonic *isl1*$^+$ progenitors, while the control feeder essentially failed to maintain the expression of *isl1* (Figures 2F and 2G).

To investigate the differentiation potential of these expanded embryonic *isl1*$^+$ progenitors, we genetically marked *isl1*-expresssing cells by crossing *isl1-IRES-Cre* mice (Laugwitz et al., 2005) into the *Cre* reporter strain *Z/RED* (Vintersten et al., 2004), thereby enabling us to purify the *isl1*$^+$ cells by flow cytometry. After coculture on feeder layers for 7 days, dsRed-expressing cells were isolated as a distinct population by FACS analysis (Figures 2J and 2K). As seen in Figure 2L, there was a significant expansion of dsRed$^+$ cells on the Wnt3a feeder compared to the control. These dsRed$^+$ cells were highly enriched for isl1 as confirmed by colocalization of isl1 and dsRed double immunostaining (Figures 2M–2O). In addition, dsRed positive cells showed essentially no expression of the cardiac marker troponin T (cTnT) or smooth muscle cell markers (α-smooth muscle actin [SMA] and smooth muscle myosin heavy chain [SM-MHC]) (data not shown). These results suggest that dsRed-expressing cells are in an undifferentiated progenitor state after expansion on Wnt3a feeder layers. When cultured in the absence of feeder layers after FACS purification, a significant proportion of dsRed$^+$ progenitors differentiated either into smooth muscle cells (4.5 ± 0.3%) or into cardiomyocytes (5.8 ± 0.4%) (Figures 2P–2R), showing that these Wnt3a-expanded embryonic *isl1*$^+$ progenitors maintain their capacity for directed differentiation.

We next performed, in *isl1*$^+$ progenitors, immunostaining analysis of activated β-catenin. Previous studies have established a reliable Wnt signaling indicator mouse strain, TOPGAL, which expresses β-gal under the control of a LEF/TCF- and β-catenin-inducible promoter (Figure S3A, DasGupta and Fuchs, 1999). Immunostaining revealed that a significant population of *isl1*$^+$ progenitors was positive for β-gal expression in the OFT (Figures S3B–S3E) and/or left atrial region (Figures S3B–S3H) of an E10.5 TOPGAL heart, suggesting that *isl1*$^+$ progenitors possess active nuclear β-catenin transcriptional activity in vivo. Taken together, these data strongly suggest that the Wnt/β-catenin pathway plays a pivotal role in the expansion of *isl1*$^+$ progenitors.

Canonical Wnt Ligands Lead to a Marked Expansion of *Isl1*$^+$ Anterior Heart Field Lineage Cells

In order to further study the effect of Wnt/β-catenin on the renewal and differentiation of *isl1*$^+$ progenitors, we decided to establish an ES cell system to provide a reliable source of purified cardiac progenitor cells. We initially generated an ES cell line in which eGFP was targeted to the genomic *isl1* locus, but this system proved suboptimal as the GFP signal was not strong enough for FACS detection.

Mef2c is a direct downstream target of isl1, and an enhancer/promoter of this gene has been recently shown to be specifically expressed within the isl1 domain of the anterior heart field (AHF) (Dodou et al., 2004). Within the minimally essential region of this enhancer/promoter two isl1 binding sites were identified (Figure 3A), and point mutations in these sites completely abrogated its expression, showing the requirement of *isl1* expression for this enhancer/promoter to function (Dodou et al., 2004).

The AHF enhancer/promoter (kindly provided by Dr. Brian Black, UCSF) was used to generate a transgenic mouse line that showed a GFP expression pattern that was completely restricted to the AHF and its derivatives, identical to that previously described (Figure 3A and Dodou et al., 2004). ES cell lines were derived from these transgenic mice. Following differentiation, these ES cell lines showed areas of strong GFP expression by embryoid body (EB) day 5 to 6, and by EB day 10, the majority of GFP$^+$ areas were beating. Figure 3B shows the FACS profile of EB day 6 differentiated ES cells. When the GFP$^+$ cells were sorted and plated onto fibronectin-coated slides, they demonstrated the ability to spontaneously differentiate into cardiomyocytes and smooth muscle cells (Figures 3C and 3D). To confirm the AHF identity of the GFP$^+$ cells, we measured *isl1* and *mef2c* expression in freshly sorted GFP$^+$ cells from EB day 6. As seen in Figure 3E, there was a significant enrichment

embryos expanded on Wnt3a feeder layer. Scale bar, 25 μm. Spontaneous differentiation of dsRed$^+$ progenitors into smooth muscle cells, revealed by expression of SMA (P) and SM-MHC (Q). Differentiation of ds-Red$^+$ progenitors driven by coculture with neonatal murine cardiomyocytes (R). Scale bar: 25, 50, and 25 μm, respectively.

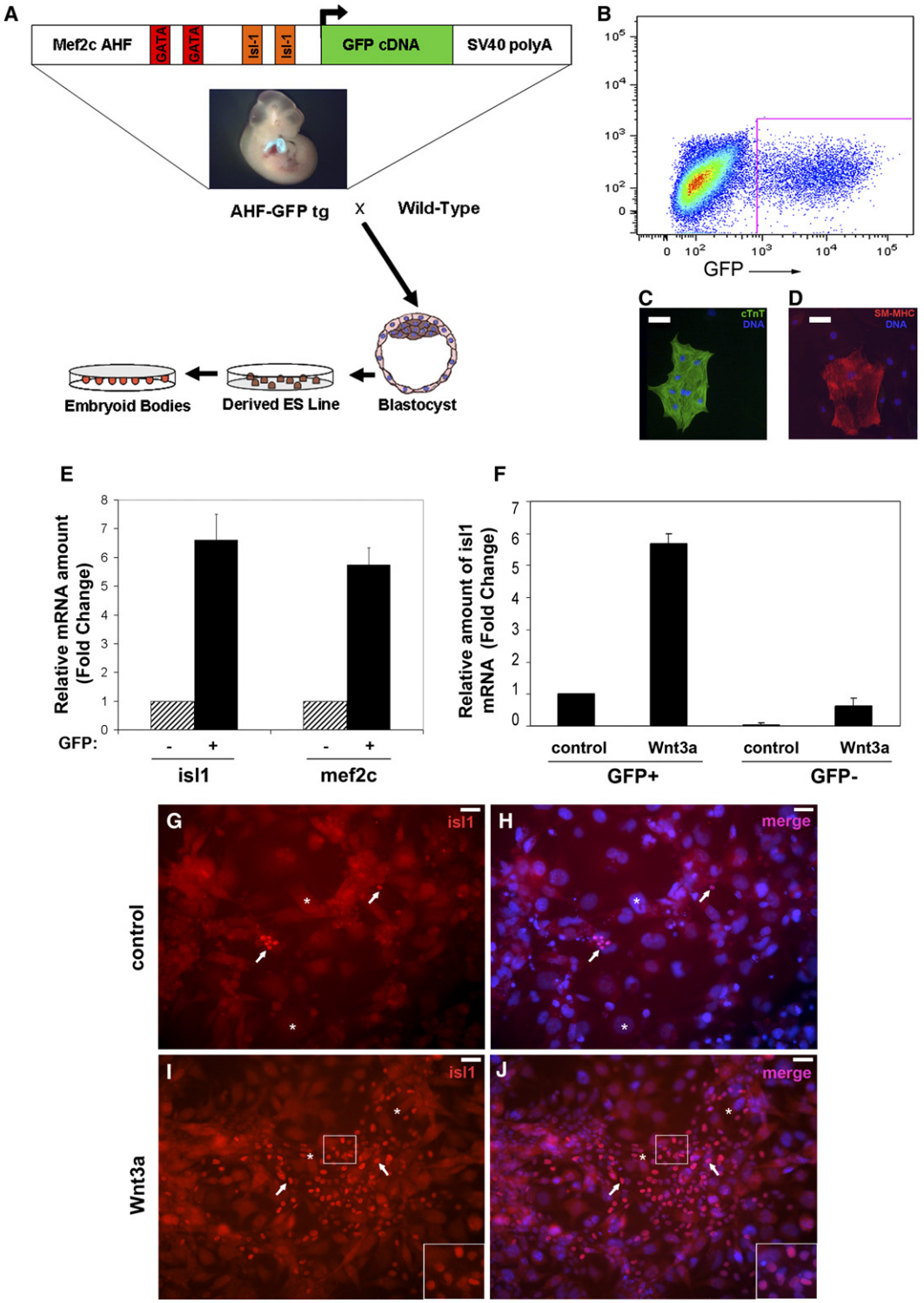


Figure 3. Expansion of *Isl1*$^{+}$ AHF Cells by a Wnt3a Feeder Layer

(A) Schematic diagram of the AHF construct, AHF-GFP transgenic mouse, and embryonic stem cell derivation strategy.

(B) FACS profile of EB day 6 differentiated AHF-GFP ES cells.

(C and D) cTnT and SM-MHC staining of sorted EB day 6 GFP^{+} cells. Scale bar, 25 μm.

(E) Quantitative PCR analysis showing the *isl1* and *mef2c* expression levels normalized by GAPDH in freshly sorted GFP^{+} and GFP^{-} cells from EB day 6 differentiated AHF-GFP ES cells. Mean ± SD, n = 3.

of isl1 and mef2c message in the GFP$^+$ compared to the GFP$^-$ population.

To test the ability of Wnt/β-catenin signals to stimulate the expansion of the ES-derived cardiac progenitors, freshly sorted AHF-GFP$^+$ cells were directly plated onto control cells or cells stably secreting Wnt3a for 7 days. As seen in Figure 3F, there was a significant enrichment of *isl1* expression in GFP$^+$ cells plated on the Wnt3a feeder layer compared with GFP$^+$ cells plated on the control layer. This observation was further confirmed by isl1 immunostaining (Figures 3G–3J).

We next performed studies on *isl1*$^+$ AHF lineage cells to investigate their ability to differentiate into cardiomyocytes and smooth muscle cells following their expansion by Wnt3a or BIO. As seen in Figures S4A–S4D, Wnt3a- or BIO-expanded progenitor cells had similar ability to differentiate into both cell lineages as control treated cells.

The Wnt/β-Catenin Pathway Regulates the Prespecification, Expansion, and Differentiation of *Isl1*$^+$ Cardiovascular Progenitors

Because the CMC-derived cues lead to the prespecification of *isl1*$^+$ cardiovascular progenitors from mesodermal precursors (Moretti et al., 2006) and activation of Wnt/β-catenin signaling enhances cardiac commitment during early phase of EB formation (Naito et al., 2006), we next examined whether Wnt signals are capable of augmenting the initial number of ES cell-derived *isl1*$^+$ clones. In order to do this, we used a previously described *isl1-nlacZ* knockin ES cell line (Moretti et al., 2006). After 4.5 days of differentiation, EBs were dissociated into single cells and plated at low density on a CMC feeder layer. To score the effect of the CMC feeder on the prespecification of mesodermal precursors toward MICPs, we quantified the single β-gal$^+$ cells 24 hr after treatment with various reagents (Figure 4A). Interestingly, the addition of Wnt3a-conditioned medium resulted in a marked inhibition in the formation of MICPs (Figures 4B–4D), raising the possibility that canonical Wnt ligands from CMC have an inhibitory effect on this step. In order to investigate whether inhibition of the Wnt signal leads to a higher rate of prespecification, we tested the effect of Dkk1-conditioned medium finding a significant increase of single β-gal$^+$ cells (Figures 4E–4G).

These results suggest that the CMC feeder layer utilizes a Wnt/β-catenin pathway to carefully titrate the number of MICPs via a negative regulatory pathway that inhibits prespecification, a result that is consistent with previous studies in other systems that have demonstrated that the Wnt/β-catenin pathway can markedly inhibit cardiogenesis (Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001).

Given the fact that the Wnt/β-catenin pathway can expand a hierarchy of *isl1*$^+$ progenitors, we further hypothesized that once mesodermal precursors have been committed to MICPs, the CMC-derived Wnt cues may promote the expansion of these prespecified cardiovascular progenitors. Thus, we cocultured mesodermal precursors arising from *isl1-nlacZ* knockin ES cells with the CMC feeder layers for 3 days, during which the feeder cells presumably prespecified a substantial number of mesodermal precursors toward MICPs. We then added either control- or Wnt3a-conditioned media and allowed the coculture to proceed for another 3 days. We scored the effect on promoting the expansion of these prespecified MICPs by comparing the size and homogeneity of β-gal$^+$ colonies. Interestingly, we frequently observed that the addition of Wnt3a-conditioned medium resulted in the formation of markedly expanded and relatively homogeneous β-gal$^+$ colonies (Figure 4I). In contrast, treatment with control-conditioned medium produced colonies that generally had a significantly sparser distribution of β-gal$^+$ cells (Figure 4H). Figure 4J shows the quantitative effect of Wnt3a treatment versus control. To test whether the canonical Wnt signal is required for the expansion of prespecified MICPs, we partially blocked the Wnt pathway with Dkk1-conditioned media. While the control-conditioned medium allowed a basal level of expansion of MICPs (Figure 4K), Dkk1 caused a marked reduction of the expansion of the committed MICPs with primarily single β-gal$^+$ cells distributed within the colony (Figures 4L and 4M).

We next examined whether the Wnt/β-catenin pathway regulates the differentiation of *isl1*$^+$ cardiovascular progenitors. In order to obtain a purified population of cardiac progenitors to perform these studies, we used freshly sorted AHF-GFP$^+$ cells from day 6 EBs as described in the previous section (Figure 3A). These cells were directly plated onto fibronectin-coated slides and allowed to undergo spontaneous differentiation. The presence of Wnt3a-conditioned media resulted in a significant decrease of differentiated cardiomyocytes, when compared with control media (Figures 4N–4P), even though the total cell number in both samples was comparable (data not shown). Consistent with this observation, when AHF-GFP$^+$ cells were cocultured on a Wnt3a-secreting feeder layer, cardiomyocyte differentiation was completely abrogated compared to that on the control feeder (Figures 4Q and 4R).

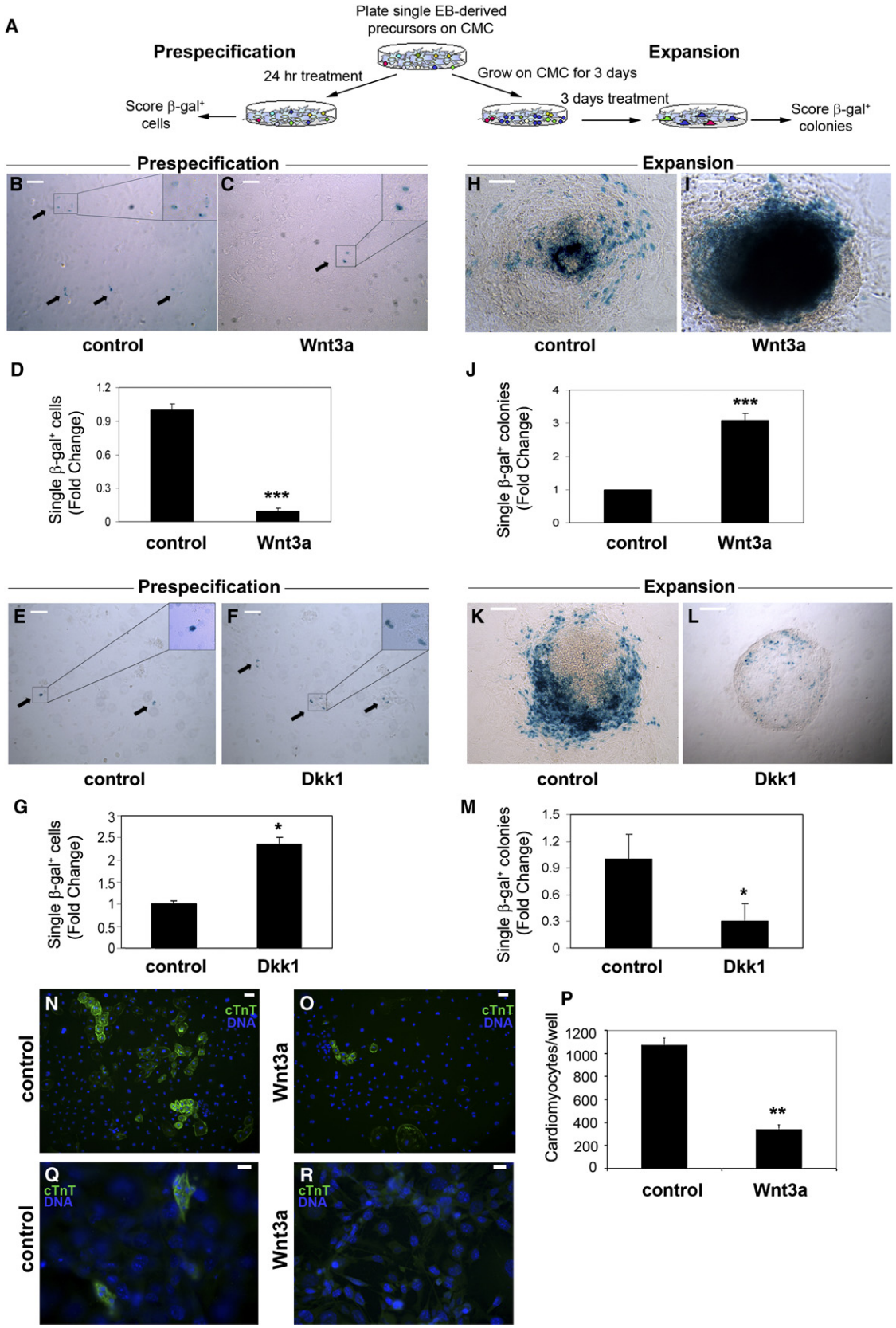
Taken together, these studies have uncovered a triphasic Wnt/β-catenin paradigm that represents a major component of the molecular mechanism by which each specific step—prespecification, renewal, and subsequent differentiation—is differentially regulated during cardiogenesis.

Expression of a Stabilized Form of β-Catenin in AHF Lineage Cells In Vivo Leads to a Markedly Expanded *Isl1*$^+$ Second Heart Field and Negatively Regulates the Differentiation of *Isl1*$^+$ Progenitors in OFT

To unravel the effects of Wnt/β-catenin on the renewal and differentiation of *isl1*$^+$ cardiovascular progenitors in vivo,

(F) Quantitative PCR analysis of *isl1* expression in GFP$^+$ and GFP$^-$ cells plated on cells stably expressing Wnt3a or control cells for 7 days. Mean ± SD, n = 3.

(G–J) Isl1 immunostaining of GFP$^+$ cells plated on control (G and H) or Wnt3a secreting feeder layer (I and J). Arrows point to *isl1*$^+$ cells. Asterisks indicate feeder cells. Scale bar, 25 μm.


A
Plate single EB-derived precursors on CMC
Prespecification
Expansion
24 hr treatment
Grow on CMC for 3 days
3 days treatment
Score β-gal+ cells
Score β-gal+ colonies
Prespecification
Expansion
B
C
H
I
control
Wnt3a
control
Wnt3a
D
J
Single β-gal+ cells (Fold Change)
Single β-gal+ colonies (Fold Change)
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Cardiomyocytes/well
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control
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cTnT
DNA
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we examined the consequences of constitutively activating β-catenin in the *isl1*$^+$ progenitors and their derivatives in the AHF lineage cells. Previous studies have established that various serine/threonine residues located in the exon3 of *β-catenin* are the targets of phosphorylation of GSK-3 and deletion of exon3 prevents this phosphorylation and subsequent degradation of β-catenin, thereby generating a stabilized form (Logan and Nusse, 2004). A mouse strain in which exon3 of *β-catenin* is flanked by *loxP* sites was generated previously (*Catnb*$^{+/lox(ex3)}$, Harada et al., 1999).

We decided to utilize a transgenic *mef2c-AHF-Cre* mouse line, in which the Cre expression is controlled by an enhancer/promoter region in the *mef2c* gene that exclusively directs expression to the AHF and its derivatives, and is dependent on isl1 for its expression (Verzi et al., 2005; Dodou et al., 2004). *Catnb*$^{+/lox(ex3)}$ mice were crossed with *mef2c-AHF-Cre* line to generate double heterozygous *mef2c-AHF-Cre; Catnb*$^{+/lox(ex3)}$ embryos (hereafter referred to as *β-cat[ex3]*$_{AHF}$), in which Cre-mediated removal of exon3 in the *β-catenin* gene results in the production of a stabilized and constitutively active molecule specifically in the AHF. We chose to analyze E9.5 embryos, because the AHF and its derivatives give rise to recognizable cardiac structures at this time. As shown in Figures 5A–5C′, while the primary atrium and left ventricle looked essentially normal in *β-cat(ex3)*$_{AHF}$ embryos, the OFT appeared to have a morphogenic defect characterized by a marked dilation, with a larger cross-sectional diameter, and truncated length when compared with somite-matched controls, a defect that appeared with complete penetrance (4/4). In addition, the mutants failed to exhibit a distinct right ventricular structure, which was readily appreciable in the control embryos. The rest of the embryonic structures appear normal in the mutants compared to controls (data not shown).

To further study the OFT abnormalities in *β-cat(ex3)*$_{AHF}$ embryos, we performed coimmunostaining on sections with antibodies for isl1 and SMA, a marker for embryonic myocardium (Xu et al., 2004; Sun et al., 2007). Consistent with the morphological defects observed in whole mount embryos (Figures 5A–5C′), sections of the mutants showed a relatively larger OFT with a discontinuous immunoreaction for SMA across the myocardial layer of the OFT, while the control sections maintained uninterrupted signals (Figures 5D–5F′ and Figures S5A–S5F′). Interestingly, although all the isl1-expressing cells in the myocardial layer of control OFT coexpressed SMA (Figure 5F and Figures S5C and S5F), in agreement with a previous study (Sun et al., 2007), there are a considerable number of isl1-expressing cells negative for SMA in the mutant OFT "myocardial" layer (Figure 5F′ and Figures S5C′ and S5F′). Given that cardiac progenitor cells from the AHF express cardiomyocytic markers once they migrate into the OFT (Waldo et al., 2001), lack of SMA expression in the isl1-expressing cells in the mutant raises an intriguing possibility that gain of function of β-catenin in the *isl1*$^+$ AHF progenitors inhibits their differentiation in the OFT. This would be in full agreement with our in vitro results showing inhibition of the differentiation of *isl1*$^+$ cardiac progenitors by canonical Wnt signals (Figures 4N–4R).

We next examined the effect of cell-autonomous changes of the canonical Wnt pathway in the *isl1*$^+$ AHF in E9.5 *β-cat(ex3)*$_{AHF}$ embryos. Previous studies have established that a substantial portion of the AHF is composed of the pharyngeal mesoderm between the OFT and the inflow tract (IFT) of the early embryonic heart and that isl1-expressing cells mark a substantial amount of AHF lineage (Waldo et al., 2001; Cai et al., 2003). Immunostaining on sagittal sections of E9.5 embryos revealed that the *isl1*$^+$ pharyngeal mesodermal cells, as outlined by the orange dashed line in Figures 5G–5H′, appeared to be markedly expanded in the *β-cat(ex3)*$_{AHF}$ embryo compared to that in the somite-matched litter-mate control in both medial (Figures 5G and 5G′) and lateral (Figures 5H and 5H′) regions. 3D reconstruction from serial sections was next performed to better appreciate the effect of the gain of function of β-catenin on the expansion of the *isl1*$^+$ AHF. Consistent with the results from the representative lateral and medial sections, the isl1$^+$ pharyngeal mesoderm between the OFT and the IFT was significantly enlarged in the mutant compared to the control (Figures 5I and 5I′). To test whether the expansion of the AHF in the mutant was associated with an increased proliferation of isl1-expressing cells, we counted cells double stained for isl1 and the mitotic marker, phosphorylated histone H3 (pi-H3). The proportion of pi-H3 and isl1 double positive cells in the AHF from the average of two E9.5 mutant embryos was 15.8%, which was significantly higher than that seen in control embryos (9.0%,

Figure 4. Regulation of the Prespecification, Expansion, and Differentiation of *Isl1*$^+$ Cardiovascular Progenitors by the Wnt/β-Catenin Pathway

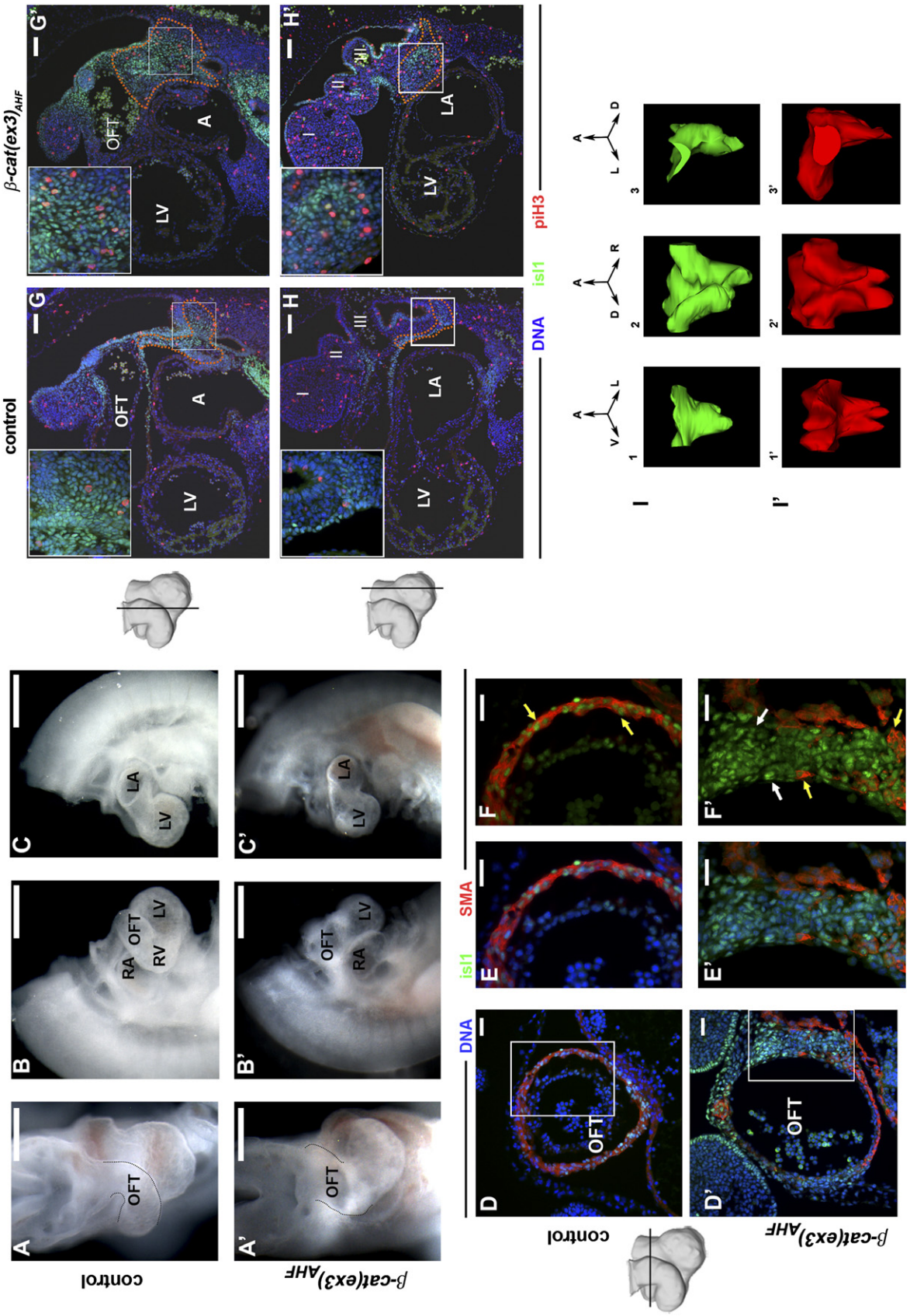
(A) Schematic representation of the experimental strategy.

(B–D) Wnt3a treatment markedly inhibits the formation of MICPs. Control (B)- or Wnt3a-conditioned medium (C) was added to the coculture for 24 hr, and single β-gal$^+$ cells were scored after X-gal staining. Bar graph (D) represents mean values ± SEM, n = 5, ***p < 0.001.

(E–G) Dkk1 treatment significantly promotes the formation of MICPs. Control (E)- or Dkk1-conditioned medium (F) was added to the coculture for 24 hr, and single β-gal$^+$ cells were scored after X-gal staining. Bar graph (G) represents mean values ± SEM, n = 3, *p < 0.05. Scale bar, 50 μm.

(H–M) Effects of the Wnt/β-catenin pathway on the expansion of prespecified MICPs. Single EB-derived precursors were plated on CMC and allowed to grow for 3 days. Wnt3a (I) and Dkk1 (L) conditional media or their respective controls (H and K) were then added to the coculture for an additional 3 days prior to the assessment of β-gal$^+$ colonies. (J and M) Bar graphs represent mean values ± SEM, n = 3. *p < 0.05, ***p < 0.001. Scale bars, 100 μm.

(N–R) Wnt3a inhibits cardiomyocyte differentiation of *isl1*$^+$ AHF cells. AHF-GFP$^+$ cells sorted on EB day 6 were plated on fibronectin-coated slides in the presence of Wnt3a (O)- or control (N)-conditioned media. Following fixation and cTnT immunostaining, the total number of *cTnT*$^+$ cells per well was scored (P). AHF-GFP ES cells were sorted on EB day 6, and GFP$^+$ cells were plated on control feeder layers (Q) or cells stably transfected with Wnt3a (R) followed by immunostaining.


control
β-cat(ex3)AHF
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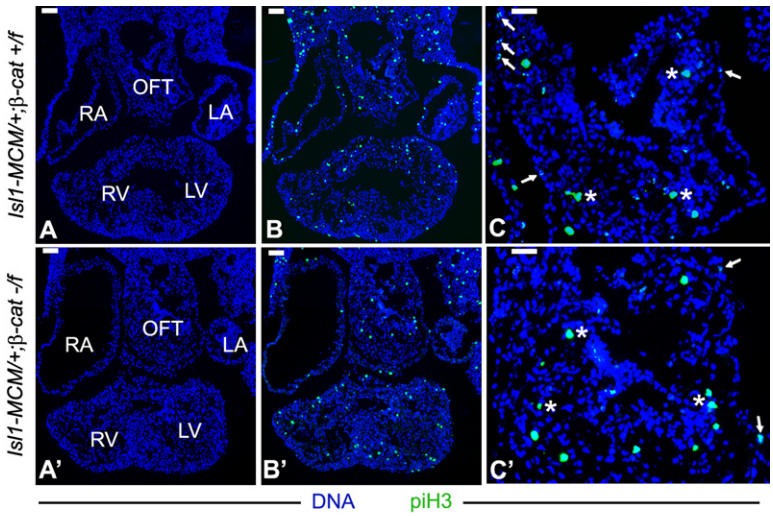

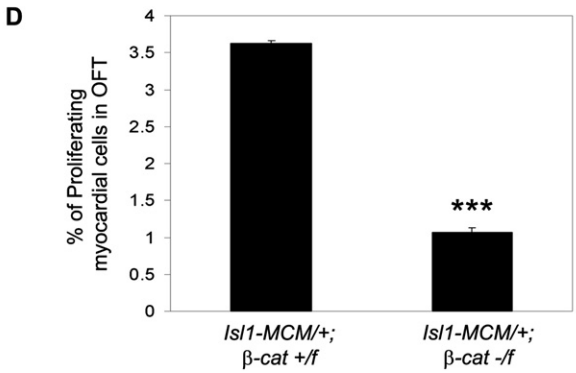

Figure 6. Decreased Proliferation of the OFT Myocardial Cells in Murine Embryos with a Temporally Controlled Loss of Function of β-Catenin

(A–C′) Representative transverse sections of a control (*Isl1-MCM*$^{/+}$; *β-cat*$^{+/f}$ [A–C]) and a mutant (*Isl1-MCM*$^{/+}$; *β-cat*$^{-/f}$ [A′–C′]) embryo (E11.5) immunostained for pi-H3. (C) and (C′) are the magnified views of the OFT for (B) and (B′), respectively. Tamoxifen was injected to pregnant females at E9.5, and embryos were harvested at E11.5. Nuclei were marked by DAPI. White arrows indicate proliferating myocardial cells in OFT. Asterisks mark proliferating endocardial cells in OFT.

(D) Quantification of proliferating myocardial cells in the OFT of control and mutant embryos. Mean ± SEM, n = 3, ***p < 0.001. Scale bars, 100 μm in (A)–(B′) and 50 μm in (C) and (C′).

p < 0.01, χ^2 test). In contrast, there was no appreciable difference in the proliferation rate of neuroepithelial cells between mutants (11.6%) and controls (10.6%, p = 0.42).

Decreased Proliferation of the OFT Myocardial Cells in Murine Embryos with a Temporally Controlled Loss of Function of β-Catenin

We next performed loss-of-function experiments as shown in Figure 6. We crossed double heterozygous *isl1-MCM*$^{+}$; *β-catenin*$^{+/-}$ mice with *β-catenin* floxed homozygous mice to obtain *isl1-MCM*$^{+/-}$; *β-cat*$^{-/f}$ mutants and *isl1-MCM*$^{+/-}$; *β-cat*$^{+/f}$ controls. Tamoxifen was injected into pregnant females at E9.5, and embryos were harvested at E11.5. Pi-H3 immunostaining showed a markedly decreased proliferation rate of myocardial cells in the OFT of the mutant when compared to control embryos (Figures 6A–6D). As myocardial cells in the OFT are primarily derived from *isl1*$^{+}$ secondary heart field progenitors (Cai et al., 2003), these results strongly suggest that β-catenin plays an important role in the proliferation of *isl1*$^{+}$ lineage cells.

Figure 5. Abnormal OFT Morphology, Disrupted OFT Myocardial Differentiation, and Marked Expansion of *Isl1*$^{+}$ Pharyngeal Mesodermal Progenitors in Murine Embryos that Harbor a Constitutive Activation of β-Catenin within AHF Lineages

(A–C′) Anatomical morphology of the heart in a control (A–C) and a mutant (*β-cat[ex3]*$_{AHF}$ [A′–C′]) E9.5 embryo. The head and pharyngeal arches 1 to 2 were removed to allow an optimal view of the heart components. LV, left ventricle; RA, right side of the primary atrium; LA, left side of the primary atrium. Scale bars, 500 μm.

(D–F′) Coronal sections through the OFT of a control (D–F) and a mutant (D′–F′) E9.5 embryo immunostained for isl1 and SMA. Boxed areas are magnified on the right of the row. The yellow arrows indicate *isl1*$^{+}$; *sma*$^{+}$ cells, and the white arrows indicate *isl1*$^{+}$; *sma*$^{-}$ cells. The cutting plane at the medial part of the OFT is indicated on the schematic heart image. Scale bars: 25 μm in (D) and (D′), 50 μm in (E)–(F′).

(G–H′) Sagittal sections of a control (G and H) and a mutant (*β-cat[ex3]*$_{AHF}$) (G′ and H′) E9.5 embryo immunostained for isl1 and pi-H3. The cutting planes at the medial (G and G′) and lateral (H and H′) part of the embryos are indicated on the schematic heart image. The *isl1*$^{+}$ cardiac progenitor population between the cardiac OFT and IFT is outlined with orange dashed lines. The boxed area in each panel is magnified on the top-left corner. I, II, III: first, second, and third pharyngeal arches; A, medial part of the primary atrium. Scale bars, 100 μm.

(I and I′) 3D reconstruction of *isl1*$^{+}$ pharyngeal mesoderm between the cardiac OFT and IFT from serial sections (represented by the areas outlined by orange dashed lines in [G]–[H′]). The control is represented in green and the mutant in red. Shown are ventral (1 and 1′), dorsal (2 and 2′), and left (3 and 3′) views of the reconstructed structures.

DISCUSSION

High-Throughput Chemical Screening and the Identification of Key Steps in Cardiovascular Cell Lineage Diversification

In the current study, we employed high-throughput screening to identify a series of compounds that can trigger renewal of the postnatal *isl1*$^+$ progenitors. The ability to reconstitute the CMC niche with FACS-purified *isl1*$^+$ cardiovascular progenitors derived from murine ES cells opens the possibility of developing new chemical screens to identify additional renewal signals for *isl1*$^+$ progenitors, and pathways that drive their differentiation into cardiac, smooth muscle, and endothelial cellular progeny. It will be of particular interest to identify specific chemical agents that might drive the directed differentiation of MICPs into coronary arterial, cardiac muscle, and pacemaker lineages, as this could ultimately lead to the large scale engineering of certain heart tissue components that have immediate clinical therapeutic value.

CMC and the Microenvironmental Cues for the Renewal of a Hierarchy of *Isl1*$^+$ Cardiovascular Lineages

As described previously, a key step in amplifying *isl1*$^+$ cardiovascular progenitors was the ability to expand the rare pool of these progenitors on CMC feeder layers derived from the neonatal and embryonic heart. This feeder layer allowed the renewal of *isl1*$^+$ cardiovascular progenitors with the maintenance of their multipotentiality. Because these cells are normally found in the embryonic and postnatal heart, the possibility exists that the CMC act as the in vivo microenvironment that serves to inhibit differentiation, activate their expansion, and maintain their multipotency. Consistent with this notion, we found that there was a preferential localization of clusters of *isl1*$^+$ cells in the neonatal mouse (Figures S6B and S6C) and human heart (Figures S6F and S6G) in an in vivo microenvironment of surrounding nonmyocytic CMC that most likely serve as an insulator from triggers of cellular differentiation. Because *isl1*$^+$ progenitors are largely localized in the secondary heart field and migrate into a region of differentiating cardiac cells in the primordial heart tube, it is possible that the *isl1*$^+$ cardiovascular progenitors first encounter this microenvironment early during the course of cardiogenesis, and that this plays a critical role in the maintenance of the multipotency of these precursors that are destined to form distinct cell lineages in discrete regions of the heart.

Canonical Wnt Signals Are a Major Component of the CMC Microenvironment that Controls the Renewal of a Hierarchy of *Isl1*$^+$ Cardiovascular Progenitors

Through the use of chemical screening and a panel of gain- and loss-of-function studies, we show that the effects of canonical Wnt ligands appear to be sufficient to renew the hierarchy of *isl1*$^+$ cardiovascular progenitors, as noted by studies on postnatal, embryonic, and ES cell systems. Taken together, these results represent the beginning of the molecular unraveling of the microenvironmental niche for the hierarchy of *isl1*$^+$ cardiovascular progenitors. While previous studies have established a role for canonical Wnt signals in cardiac specification in ES cells, there has been some controversy, as two studies proposed a positive role of Wnts in this function (Nakamura et al., 2003; Naito et al., 2006) while another suggested the opposite (Liu et al., 2007). This discrepancy may largely be due to the reliance of these previous studies on differentiation assays of intact beating EBs that are composed of a complex mosaic of embryonic cell types. (Nakamura et al., 2003; Naito et al., 2006; Singh et al., 2007; Liu et al., 2007). As such, it has proven difficult to precisely pinpoint the exact molecular mechanism by which Wnt ligands might exert control on the complex process of cardiogenesis. Utilizing FACS-purified embryonic and ES cell-derived cardiovascular progenitors, we have provided evidence that Wnt signals emanating from the CMC play a major role in cardiogenesis. The current studies attain a level of resolution of Wnt signaling on discrete cell fate steps on specific subsets of *isl1*$^+$ progenitor cell lineages (the mesodermal precursors that give rise to MICPs, where it is inhibitory, the MICP and bipotent precursors where it activates renewal, and the transitional isl1$^+$/sma$^+$ cells in the myocardium of the OFT, where it inhibits differentiation) (Figure 7). This underscores the complexity of Wnt signaling within cardiogenesis and reinforces the notion that the use of single cell systems, such as FACS-purified progenitors or ES cell-derived clonal assays, coupled with classic in vivo gain- and loss-of-function models, will be important to identify specific cues that control the fate of cardiac progenitors. In this regard, the in vivo constitutive activation of β-catenin pathways within *isl1*$^+$ AHF progenitors results in their massive accumulation, near complete inhibition of myocytic differentiation, and the onset of severe OFT defects. The requirement for Wnt/β-catenin signals is directly supported by the current findings of a decrease in the proliferative capacity of *isl1*$^+$ derivatives in the OFT of murine embryos that harbor a loss of β-catenin in isl1 lineage cells. Taken together, these data suggest that defects in Wnt/β-catenin pathways that control the renewal and differentiation of *isl1*$^+$ cardiovascular progenitors in the AHF may be related to the onset of severe OFT abnormalities, which constitute a major form of human congenital heart disease.

Wnt/β-Catenin Pathways and Cardiovascular Regenerative Medicine

One of the major limitations in cardiovascular regenerative medicine relates to the difficulty of expanding clonal cardiovascular progenitor populations, from either intact human tissue, or ES cell-based systems. In particular, the feasibility of utilizing human ES cells as a source for differentiated cardiac myocytes has been limited largely due to the inability to markedly enhance in vitro cardiogenesis, as less than 1% of the differentiated progeny enter cardiac lineages. The current study suggests that the

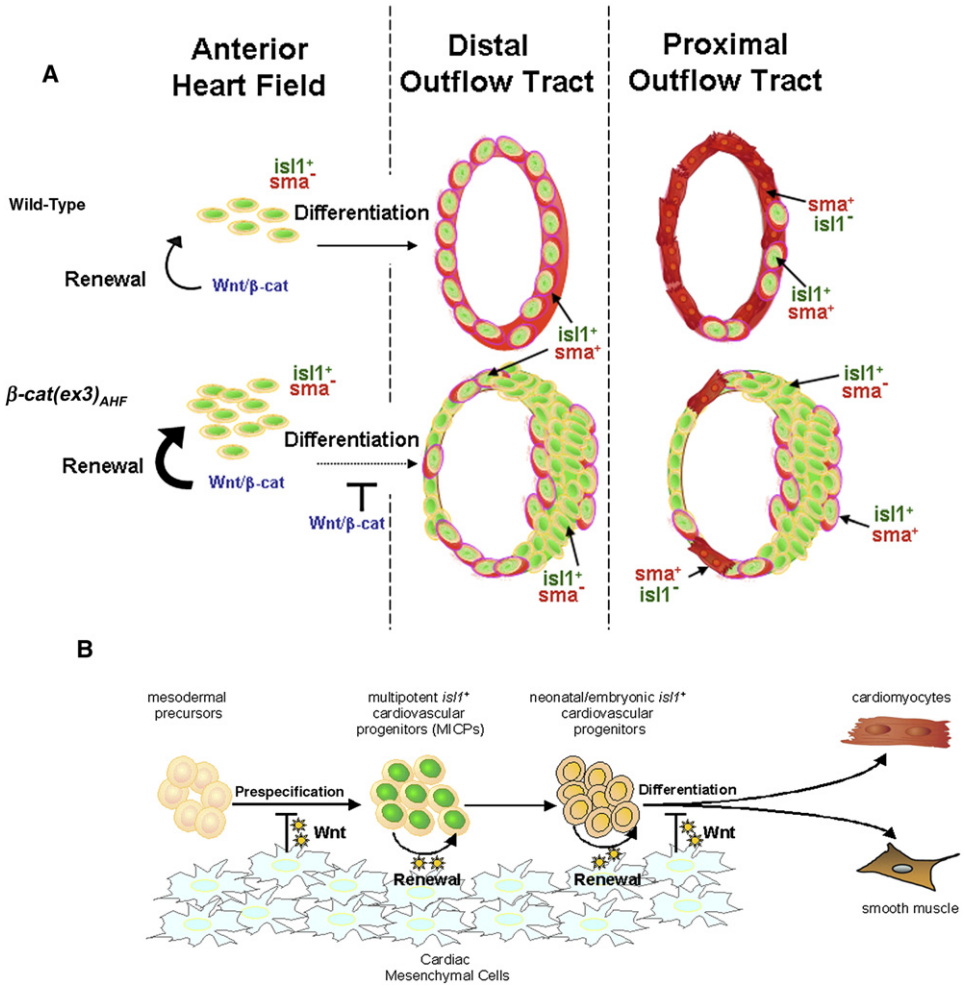

Figure 7. Models of the Effects of Wnt/β-Catenin Signaling on the Renewal and Differentiation of *Isl1*$^+$ Cardiac Progenitor Cells and Their Progenies

(A) In vivo model. In the AHF of wild-type embryos, Wnt/β-catenin signaling promotes the proliferation of *isl1*$^+$ cardiac progenitors, which are negative for *sma*. The progenitors migrate to the OFT myocardium and undergo stepwise differentiation. While the cells in the distal part of the OFT start to express sma, they remain positive for *isl1*. In contrast, in the proximal part of the OFT *isl1* expression is lost in a considerable portion of the cells. In the *β-cat(ex3)$_{AHF}$ mutant*, with augmented Wnt/β-catenin signaling in the AHF and its derivatives, there is an increased proliferation of the *isl1*$^+$ cardiac progenitors in the AHF but inhibited differentiation of the progenitors and their progenies after they migrate to the myocardial layer of the OFT. (B) A model of the roles of Wnt/β-catenin signals from CMC feeder on the prespecification, renewal, and differentiation of a hierarchy of *isl1*$^+$ cardiovascular progenitors.

manipulation of Wnt signals might have a direct effect on the isolation, cloning, and expansion of rare human *isl1*$^+$ cardiovascular progenitors from either ES cells or intact human heart tissue.

EXPERIMENTAL PROCEDURES

Isolation, Amplification, and Differentiation of Embryonic Cardiovascular Progenitor Cells

For isolation of embryonic cardiovascular progenitors, we crossed *isl1-IRES-Cre* mice (generously provided by Thomas M. Jessel) into the *Cre* reporter strain *Z/RED* (Vintersten et al., 2004). Approximately 80 E8.5 embryos were dissected and dissociated into single cells by treatment with a mix of 1 ml collagenase A&B (Roche) at 10 mg/ml during 1 hr at 37°C followed by treatment with trypsin 0.25% for 5–10 min. The dissociated cells were filtered through a 40 μm cell strainer (Falcon) and plated as single cells on the mitomycin-treated feeder layers, stably transfected with Wnt3a, at a density of 10,000 cells/cm^2 in DMEM/F12 complete media for 7 days. Smooth muscle spontaneous differentiation was performed as previously described (Moretti et al., 2006).

Isolation and Culturing of Human Postnatal Cardiac Progenitors

Biopsies were cut in small pieces and washed in solution A (10 mM HEPES, 35 mM NaCl, 10 mM glucose, 134 mM sucrose, 16 mM Na_2HPO_4, 25 mM $NaHCO_3$, 7.75 mM KCl, and 1.18 mM KH_2PO_4 [pH 7.4]), supplemented with 30 mM 2,3 butanedione 2-monoxime and 0.5 mM EGTA. The first digestion step was performed in solution A supplemented with 0.5% BSA, 200 U/ml of collagenase type II (Worthington), and 6 U/ml protease type XXIV (Sigma) for 20 min at 37°C to remove red blood cells and cell debris. This was followed by four

digestion steps performed in solution A supplemented with 400 U/ml collagenase type II for 20 min at 37°C and centrifuged at 30 × g for 1 min. The supernatant was neutralized from the collagenase by adding 1/5 volume of newborn calf serum (NCS) and then centrifuged at 1300 rpm for 3–5 min. The pellet was resuspended in DMEM supplemented with 10% NCS, 5% FBS, and pen/strep, and cells were seeded on chamber slides. BIO was added to the culture at different doses and cultured for 4 days prior to immunostaining for isl1.

Generation of AHF-GFP ES Cell Lines

Timed matings were performed between AHF-GFP transgenic males and C57Bl/6 females. On day 3.5 PC, the females were sacked and the blastocysts flushed from the uterine horns using M2 medium (Sigma-Aldrich, MO). After washing with M2 media, the zona pellucida was removed with acidic Tyrode's Solution (Sigma-Aldrich, MO) and the blastocysts were further washed three times in M2 media. The blastocysts were then adapted onto mouse embryonic feeder cells (MEF) with derivation media (DMEM with 15% KOSR, pen/strep, pyruvate, nonessential amino acids, and leukemia inhibitory factor [LIF] [Chemicon, CA]).

Production of Reagents for Wnt/β-Catenin Pathway

Wnt3a- or control-conditioned medium was produced as follows: a Wnt3a-secreting cell line (ATCC) was allowed to grow to confluency and subcultured at a 1:20 ratio prior to replenishment with fresh medium. Three batches of conditioned medium were harvested every 48 hr. Dkk1-conditioned medium was produced by transiently transfecting a Dkk1-expressing cDNA into the HEK293T cell line with FuGENE 6 (Roche). The supernatant was harvested 72 hr after transfection.

Supplemental Data

Supplemental Data include six figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/1/2/165/DC1/.

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