Canonical Wnt signaling functions in second heart field to promote right ventricular growth

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The second heart field (SHF), progenitor cells that are initially sequestered outside the heart, migrates into the heart and gives rise to endocardium, myocardium, and smooth muscle. Because of its distinct developmental history, the SHF is likely subjected to different signals from that of the first heart field. Previous experiments revealed that canonical Wnt signaling negatively regulated first heart field specification. We inactivated the obligate canonical Wnt effector β-catenin using a *β-catenin* conditional null allele and the *Mef2c AHF cre* driver that directs cre activity specifically in SHF. We also expressed a stabilized form of β-catenin to model continuous Wnt signaling in SHF. Our data indicate that Wnt signaling acts in a positive fashion to promote right ventricular and interventricular myocardial expansion. *Cyclin D2* and *Tgfβ2* expression was drastically reduced in *β-catenin* loss-of-function mutants, indicating that Wnt signaling is required for patterning and expansion of SHF derivatives. Our findings reveal that Wnt signaling plays a major positive role in promoting growth and diversification of SHF precursors into right ventricular and interventricular myocardium.

conditional genetics | cardiac progenitor | mouse | development

Previous work using retrospective clonal analysis in mouse embryos indicated that the heart developed from two populations of cardiac progenitors. Cells within the first heart field (FHF) uniquely contribute to the left ventricular myocardium whereas the second heart field (SHF) contributes to outflow tract (OFT) and right ventricular (RV) myocardium, endocardium, and smooth muscle of the great vessels (1–5). Each progenitor population expresses distinct and overlapping molecular markers: the *Hand1* and *Tbx5* transcriptional regulators mark the FHF whereas *Fgf10* and the Lim-homeobox gene *Isl1* mark the SHF. Recent work indicates that *Isl1* may also be transiently expressed in the FHF (6). The *Nkx2.5* homeobox gene is expressed in both lineages (7).

Genetic experiments performed in mice revealed that *Isl1* had a critical function in SHF. Importantly, although *Isl1* is expressed in SHF cardiac progenitors, *Isl1* expression is extinguished in the majority of the heart with the exception of a subpopulation of cardiac progenitors that persist in the adult (4, 8). Other experiments, revealing that the Mef2c transcription factor is a direct Isl1 target, uncovered a SHF transcriptional hierarchy (9).

The SHF has been proposed to be a source of adult progenitor cells. Recent work has led to the proposal that *Isl1*-expressing progenitors provide a source of resident progenitor cells, or cardioblasts, that have the capability to differentiate into mature cardiomyocytes (8). Experiments performed in embryonic stem cells revealed that the *Isl1*-positive lineage gives rise to endothelial cells, smooth muscle cells, and cardiomyocytes, suggesting the intriguing possibility, as yet unproven, that the adult heart may harbor a stem cell niche (10–13).

Despite the importance of the SHF in cardiac development, a clear picture of the regulatory pathways controlling SHF development and diversification is lacking. An enhancer trap into the *Fgf10* locus provided early evidence for the existence of the SHF in mice and indicates, along with conditional loss-of-function experiments, the involvement of Fgf signaling in SHF formation (14–16). Likewise, Bmp signaling has been implicated in SHF development based on data from chick embryos and mouse conditional deletion studies (6, 17, 18).

The *Wnt* family of secreted glycoproteins has been implicated in numerous events in development and disease (19). *β-Catenin*, an obligate effector of the canonical Wnt pathway, is stabilized in the presence of *Wnt* signaling and enters the nucleus, where it interacts with TCF factors, such as *Lef1*, to regulate gene expression. In the absence of Wnt signaling, *β-catenin* is targeted for destruction by the *APC*, *Axin*, *Gsk3b* complex that phosphorylates *β-catenin* and directs it to a destruction pathway (19). *β-Catenin* also complexes with the cytoplasmic domain of *E-cadherin* where it plays a fundamental role in promoting cell adhesion.

Previous findings indicated that Wnt signaling from the neural tube inhibits FHF specification (20–22). It is unclear whether Wnt signaling influences the SHF in a similar fashion as the FHF. Because Wnt signaling is known to promote development of trunk skeletal muscle, it was conceivable that Wnt signaling could promote SHF development from splanchnic mesoderm (23).

In this work we investigated *β-catenin*-dependent Wnt signaling in the SHF. We inactivated *β-catenin* in the SHF using the *Mef2c AHF cre* (hereafter referred to as *Mef2c*^{cre}) that directs cre activity specifically in SHF (9, 24) (kindly provided by B. Black and M. Verzi, University of California, San Francisco, CA). Our findings indicate that *β-catenin* function is required for expansion of the RV and interventricular septum, as well as patterning of OFT cushions.

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Results

Manipulation of β-Catenin Function in Second Cardiac Lineage. To investigate Wnt signaling in second cardiac lineage, we generated mouse embryos with loss and gain of *β-catenin* function specifically in SHF. The *Mef2c*^{cre} transgene directs cre activity specifically in SHF [supporting information (SI) Fig. 7*A*]. Lineage tracing with *Mef2c*^{cre} and *Rosa26Reporter* (R26R) allele labels cells in the dorsal medial aspect of the crescent at 7.5 days postcoitum (dpc) (SI Fig. 7 *B* and *C*). As cardiac development progresses, the *Mef2c*^{cre} lineage contributes to OFT myocardium, pharyngeal mesenchyme, and OFT endocardium (SI Fig. 7 *D*–*I*). Activity of the *Mef2c*^{cre} transgene is excluded from the pharyn-

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Abbreviations: FHF, first heart field; SHF, second heart field; OFT, outflow tract; RV, right ventricle; CNC, cardiac neural crest; dpc, days postcoitum.

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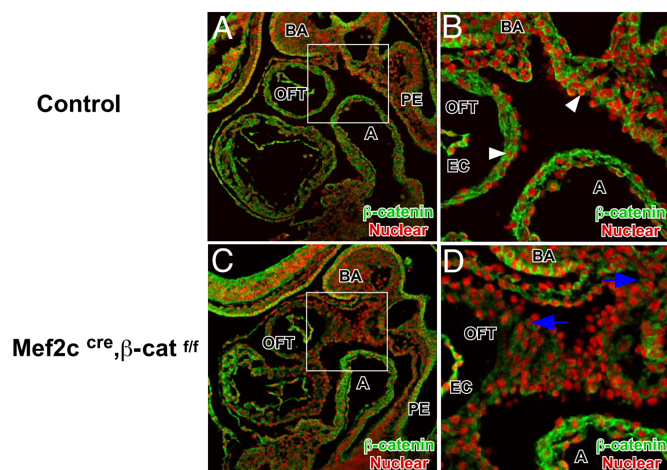

Fig. 1. *Mef2c^cre* induced β-catenin deletion in SHF. Immunofluorescent staining of β-catenin in 9.5 dpc sagittal sections of control (*A* and *B*) and *Mef2c^cre*; *β-catenin^flox* mutant (*C* and *D*) embryos revealing membrane-localized β-catenin staining in ectoderm and endoderm as well as nuclear β-catenin in OFT myocardium, posterior pericardial mesothelium, and pharyngeal mesenchyme (denoted by white arrowheads in *B*). In the *Mef2c^cre*; *β-catenin^flox* mutant there is reduced β-catenin expression in pharyngeal mesenchyme and OFT myocardium, but not endocardium, due to mosaic activity of *Mef2c^cre* in OFT endocardium (decreased β-catenin denoted by blue arrows). A, atrium; BA, branchial arch; EC, endocardium; PE, pharyngeal endoderm.

geal endoderm, making this an ideal cre driver to investigate gene function in the specified SHF (SI Fig. 7 *E*, *G*, and *I*) (24).

The *β-catenin^flox* allele contains LoxP sites flanking exons 2–6 and has been shown to be a conditional null allele (SI Fig. 7*J*) (25). We used the *Mef2c^cre* transgenic line to delete *β-catenin*, an obligatory downstream canonical Wnt effector. *β-Catenin* deletion in *Mef2c^cre*-expressing cells would render SHF resistant to Wnt signaling. As a complementary experiment, we expressed a stabilized form of *β-catenin* in the *Mef2c^cre*-expressing SHF to model gain of function or continuous Wnt signaling (26). The *β-catenin^ex3flox* allele is a mutant *β-catenin* allele containing a LoxP-flanked exon 3 that encodes residues that are required for negative regulation of β-catenin (SI Fig. 7*K*) (26). When *β-catenin* exon 3 is removed by cre-mediated recombination, the resulting mutant allele encodes a stabilized form of *β-catenin* that mimics sustained Wnt signaling.

Efficient β-*Catenin* Deletion by *Mef2c^cre*. To remove *β-catenin* function from specified SHF, we crossed the *β-catenin^flox* allele to the *Mef2c^cre* transgenic line (24, 25). We used immunofluorescent microscopy to determine the efficiency of *β-catenin* deletion in SHF. At 9.5 dpc we detected β-catenin in the pharyngeal mesenchyme and OFT myocardium of control embryos (Fig. 1 *A* and *B*). We noted membrane-localized β-catenin in epithelial tissues such as branchial arch ectoderm and pharyngeal endoderm. Moreover, we detected nuclear-localized β-catenin, a hallmark of active Wnt signaling, in the pharyngeal mesenchyme and OFT myocardium (Fig. 1*B*). In *Mef2c^cre*; *β-catenin^flox* conditional mutants, we found a strong reduction in β-catenin immunostaining in the SHF-derived OFT myocardium and pharyngeal mesenchyme (Fig. 1 *C* and *D*), indicating that *Mef2c^cre* efficiently deleted β-catenin in SHF.

***Mef2c^cre*; *β-Catenin^flox* Conditional Mutants Have a Reduced Right Ventricle.** Examination of *Mef2c^cre*; *β-catenin* conditional null mutant embryos at multiple stages revealed that all *β-catenin* loss-of-function mutants had defective OFT and RV development. In particular, the RV was drastically reduced when compared with control embryos (Fig. 2 *A* and *A′*). Histologic analysis also indicated that, at 12.5 dpc, *β-catenin* conditional null mutants had defective separation of the distal OFT (Fig. 2 *B* and *B′*). More proximal sections revealed that, in the proximal OFT, the pulmonary trunk and aorta were separated (Fig. 2 *C* and *C′*). Sections through the RV indicated that the RV was reduced in size with a thin myocardium (Fig. 2 *D* and *D′*). The OFT length was also reduced in *β-catenin* conditional null mutants when compared with control (Fig. 2 *E* and *E′*). Together, the phenotypes detected in *β-catenin* conditional null mutants indicated a major deficiency in SHF derivatives.

Deficiency of SHF Derivatives in β-*Catenin* Conditional Mutants. We studied SHF markers to investigate the *β-catenin* conditional null mutant phenotype in more depth. Expression of *Isl1* was intact in the *β-catenin* conditional null mutants as would be expected because *Mef2c* is a downstream target of *Isl1* in SHF (9) (Fig. 3 *A* and *A′*). Moreover, expression of cardiac neural crest (CNC) markers indicated that CNC was specified correctly in *β-catenin* mutants (Fig. 3 *B*, *B′*, *C*, and *C′*). Lineage tracing with R26R, to follow the developmental progression of the *Mef2c^cre* lineage, indicated that in *β-catenin* mutant embryos the SHF was severely defective (Fig. 3 *E*, *E′*, *F*, and *F′*). Whole-mount LacZ staining showed the reduced size of the RV and defective distal OFT

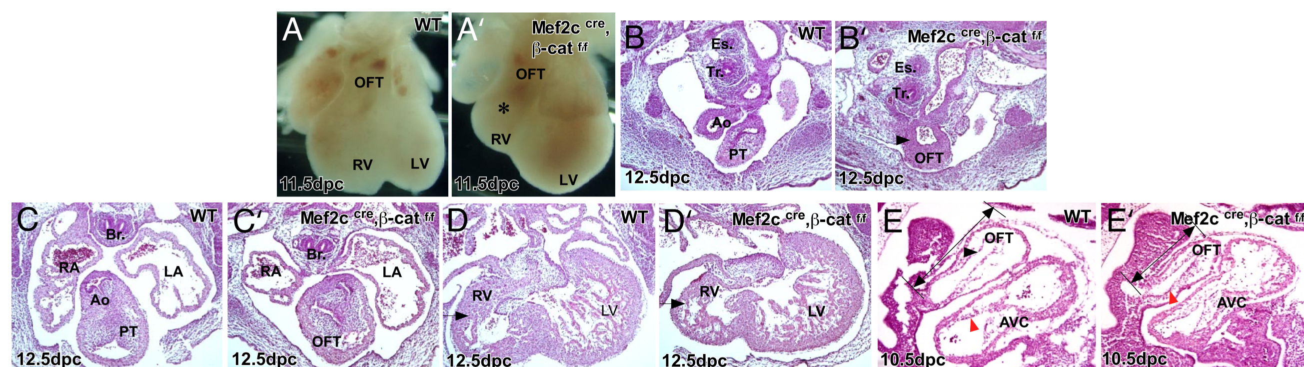

Fig. 2. Phenotype of *Mef2c^cre*; *β-catenin* mutant embryos. Shown are morphology and histology of 10.5–12.5 dpc *β-catenin* loss-of-function mutant hearts showing severe RV hypoplasia (*) (*A′*) compared with control littermate (*A*). Transverse section of 12.5 dpc (*B*, *B′*, *C*, *C′*, *D*, and *D′*) and sagittal section of 10.5 dpc (*E* and *E′*) control and *β-catenin* loss-of-function mutant hearts reveal severe cardiac abnormalities: OFT septation defect in the distal OFT (*B* and *B′*, arrowheads) but not in more proximal OFT (*C* and *C′*), and hypoplasia of RV myocardium (*D* and *D′*, arrow) and short OFT (*E* and *E′*, double-headed arrows). Endocardium is denoted by arrowheads. PT, pulmonary trunk; LV, left ventricle; RV, right ventricle; RA, right atrium; LA, left atrium; Es, esophagus, Tr, Trachea, Br, bronchi.

Fig. 3. Marker analysis of SHF and CNC and *Mef2c*^{cre} lineage tracing in *Mef2c*^{cre}; *β-catenin*^{flox} mutants. *In situ* hybridizations of *Isl1* (*A* and *A′*), *Sox10* (*B* and *B′*), and *Ap2* (*C* and *C′*) at 9.5 dpc indicated that SHF and CNC were correctly specified in *Mef2c*^{cre}; *β-catenin* conditional mutant embryos (yellow arrowheads denote hybridization signal). *Mef2c*^{cre} lineage tracing (*D* and *D′*) and transverse section (*E*, *E′*, *F*, and *F′*) in wild type and *β-catenin* mutant revealed a severe deficiency in the *Mef2c* lineage in *Mef2c*^{cre}; *β-catenin* conditional mutant embryos. LV, left ventricle; RV, right ventricle; RA, right atrium; LA, left atrium; AO, aorta; PT, pulmonary trunk; Ba, branchial arch.

separation in *β-catenin* mutant embryos (Fig. 3 *D* and *D′*). Sections revealed a major deficiency of SHF-derived cells in the RV and interventricular septum (Fig. 3 *E*, *E′*, *F*, and *F′*). Dysmorphology of the OFT cushions was also evident in these sections, suggesting that a β-catenin target is a signaling molecule that functions to pattern ingressing CNC (Fig. 3 *E* and *E′*). These findings indicate that, in contrast to its inhibitory influence on the FHF, Wnt signaling promotes SHF development.

Defective Expression of Myocardial Markers in *β-Catenin* Conditional Mutants. To address the genetic pathways regulated by Wnt signaling in SHF, we examined molecular markers of the SHF and its derivatives. *Cyclin D2*, a direct target of β-catenin-dependent Wnt signaling, is expressed in pharyngeal mesenchyme and in proximal OFT (27). At both 9.5 dpc and 10.5 dpc, *CyclinD2* was absent in the OFT of *β-catenin* mutants validating loss of competence to respond to Wnt signaling (Fig. 4 *A–D*). To determine whether there was a defect in regionalization of the *β-catenin* mutant OFT, we examined *Tgfβ2* expression that marks the most distal OFT myocardium. In *β-catenin* mutants, *Tgfβ2* was severely diminished, indicating that both distal and proximal OFT myocardium was defective in *β-catenin* mutants (Fig. 4 *E* and *F*). In contrast, expression of *Smarcd3*, which encodes a component of the BAF chromatin remodeling complex and a proposed Wnt target in the heart, was unchanged (Fig. 4 *G* and *H*) (28). Absence of *Cyclin D2* expression in *β-catenin* mutants suggested a defect in proliferation. Expression of phospho-Histone H3, which marks mitotic cells, was greatly reduced in *β-catenin* mutants, supporting the idea that canonical Wnt signaling was required for normal proliferation of OFT and RV myocardium (Fig. 4 *I* and *J*). Taken together, these data indicate that patterning and expansion of SHF-derived myocardium are severely disrupted in *β-catenin* mutant embryos.

Expression of Stabilized β-Catenin Causes Defective Right Ventricle Development. We used the *β-catenin*^{ex3flox} allele to express stabilized *β-catenin* in the SHF. This gain-of-function experiment models the effect of continuous, deregulated Wnt signaling on SHF progenitor cells. The RV was severely reduced in *Mef2c*^{cre}; *β-catenin*^{ex3flox} embryos (Fig. 5 *A* and *B*). Sectioning indicated that OFT and RV myocardium was thickened and hypercellular in the *Mef2c*^{cre}; *β-catenin*^{ex3flox} embryos (Fig. 5 *C–F*). We also noted that a region of the left ventricular myocardium was thickened (Fig. 5*F*, arrowhead). This likely reflects the contribution of the SHF to the left ventricular region near the interventricular septum (24). Taken together, these data indicate that both loss and gain of β-catenin function resulted in defective RV development. It is notable that in other systems, such as tooth development, excess and reduced Wnt signaling results in a similar tooth-loss phenotype (29, 30).

Disrupted *Bmp4* Expression in *β-Catenin* Conditional Mutants. We compared expression of SHF markers in the *β-catenin* loss- and gain-of-function embryos. *Nkx2.5*, which plays an early role in SHF specification, was expressed in the pharyngeal mesenchyme and SHF derivatives of both loss- and gain-of-function embryos, supporting the idea that SHF is correctly specified in *Mef2c*^{cre}; *β-catenin*^{flox} mutant embryos (Fig. 6 *A–C*) (6). In contrast, *Bmp4* was greatly reduced in the pharyngeal mesenchyme and OFT myocardium of *β-catenin* loss-of-function embryos (Fig. 6 *D* and *E*). *β-Catenin* gain-of-function embryos expressed *Bmp4* in both pharyngeal mesenchyme and OFT myocardium (Fig. 6 *D* and *F*). *Fgf10* expression in pharyngeal mesenchyme was unchanged in *β-catenin* loss-of-function embryos and had a considerably reduced expression domain in *β-catenin* gain-of-function embryos (Fig. 6 *G–I*). We also noted low levels of *Fgf10* in the OFT that was diminished in both *β-catenin* loss- and gain-of-function embryos. *MLC2a*, a marker of differentiated myocardium, was reduced in *β-catenin* gain-of-function embryos, indicating a differentiation defect. In *β-catenin* loss-of-function mutants, expression of *MLC2a* was similar to control embryos (Fig. 6 *J–L*).

Discussion

Previous experiments revealed an inhibitory role for canonical Wnt signaling in FHF precursors (22, 31, 32). In contrast, canonical Wnt signaling has been shown to promote trunk skeletal muscle development (33, 34). Our data indicate that canonical Wnt signals promote development of SHF progenitors into RV and interventricular myocardium. These findings un-

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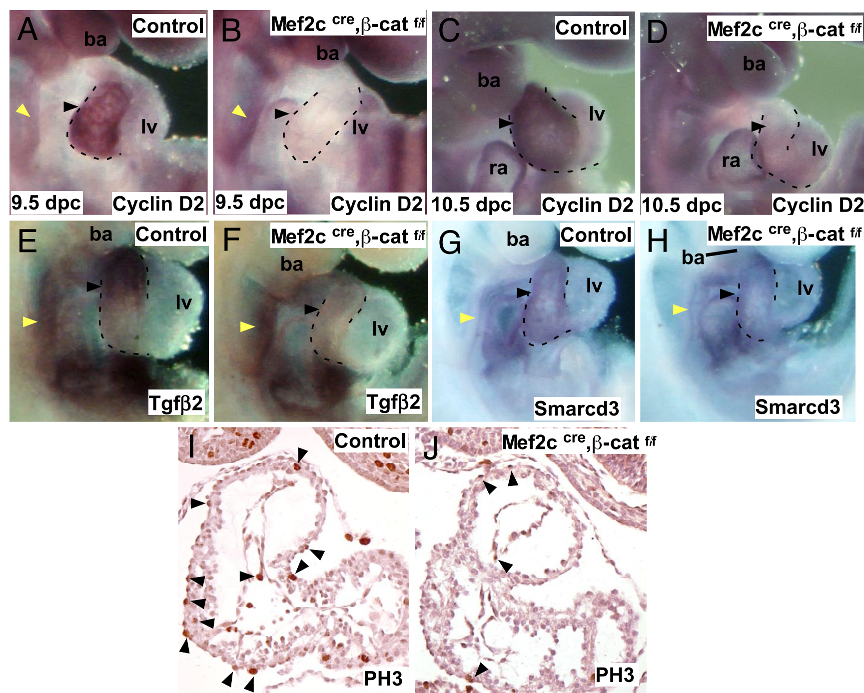

Fig. 4. Marker analysis of OFT myocardium in *Mef2c*^{cre}; *β-catenin*^{flox} mutants. *In situ* hybridization with *Cyclin D2* (*A–D*), *Tgfβ2* (*E* and *F*), and *Smarcd3* (*G* and *H*) in control and *Mef2c*^{cre}; *β-catenin*^{flox} mutants at 9.5 dpc. Pharyngeal mesenchyme is denoted by yellow arrowheads, and OFT is denoted by black arrowheads. (*I* and *J*) Sagittal sections of phospho-histone H3 immunostaining at 9.5 dpc. Arrowheads denote PH3 signal. lv, left ventricle; ba, brachial arch.

cover a striking difference between the two cardiac progenitor populations in the use of Wnt signaling to promote or repress formation of the mature cardiomyocyte phenotype.

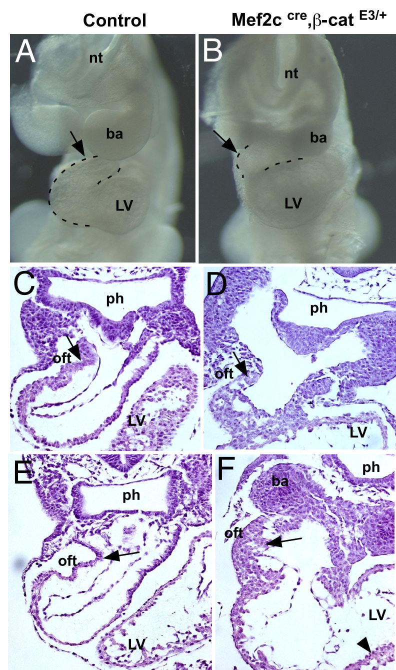

Fig. 5. Phenotype of *Mef2c*^{cre}; *β-catenin*^{Ex3flox} mutant embryos. Morphology and serial, transverse sections of 9.5 dpc wild-type (*A*, *C*, and *E*) and *Mef2c*^{cre}; *β-catenin*^{Ex3flox} mutant embryos (*B*, *D*, and *F*) showing short but more dense and hypercellular OFT myocardium (denoted by black arrows). nt, neural tube; ba, branchial arch; LV, left ventricle; ph, pharynx.

Wnt Signaling Is Critical for Development of the Right Ventricle. We deleted β-catenin using the *Mef2c*^{cre} driver that is active after *Isl1* expression has been initiated in SHF. Therefore, we removed competence to respond to Wnt signaling after SHF specification. Although *in situ* data indicate that patterning of the proximal and distal OFT is disrupted, the most severely affected cells are the RV and interventricular progenitors. Our findings are consistent with the previous observation that RV and interventricular myocardium derives from SHF (35). One explanation for the stronger RV and interventricular myocardial phenotype may be that the OFT myocardial lineage is specified or expands earlier than RV and interventricular septum. This notion is analogous to data from ES cells in which Nkx2.5 GFP-expressing cells are more restricted than Brachury GFP-expressing cells in their potential for lineage diversification or expansion (36). It is conceivable that other signals, such as Bmp signaling, have a role in specification or expansion of OFT myocardium and endocardial lineages (see below).

Previous work revealed that noncanonical Wnt signaling plays a critical role in cardiac development. The noncanonical Wnt signaling pathway is β-catenin-independent but utilizes Dsh and Rho (37). *Wnt11* has been shown to regulate cardiac development through a noncanonical pathway in *Xenopus* and zebrafish embryos (38, 39). Moreover, *Loop-tail* (*Lp*), a naturally occurring mouse mutant that was initially identified based on severe defects in neural tube closure, has cardiac OFT defects. The gene mutated in *Lp* is *Vangl2*, a homolog of the *Drosophila* planar cell polarity gene *Strabismus*. Recent work has shown that *Vangl2* functions in the OFT myocardium to regulate OFT septation (40).

Our work provides insight into the role of Wnt signaling in SHF by showing that the canonical Wnt pathway promotes SHF development. It is notable that *Wnt11*, highly expressed in OFT myocardium, has also been implicated in canonical Wnt signaling in *Xenopus* embryos (41). Therefore, it is conceivable that *Wnt11* activates the canonical pathway in SHF derivatives, but further experiments will be required to investigate this.

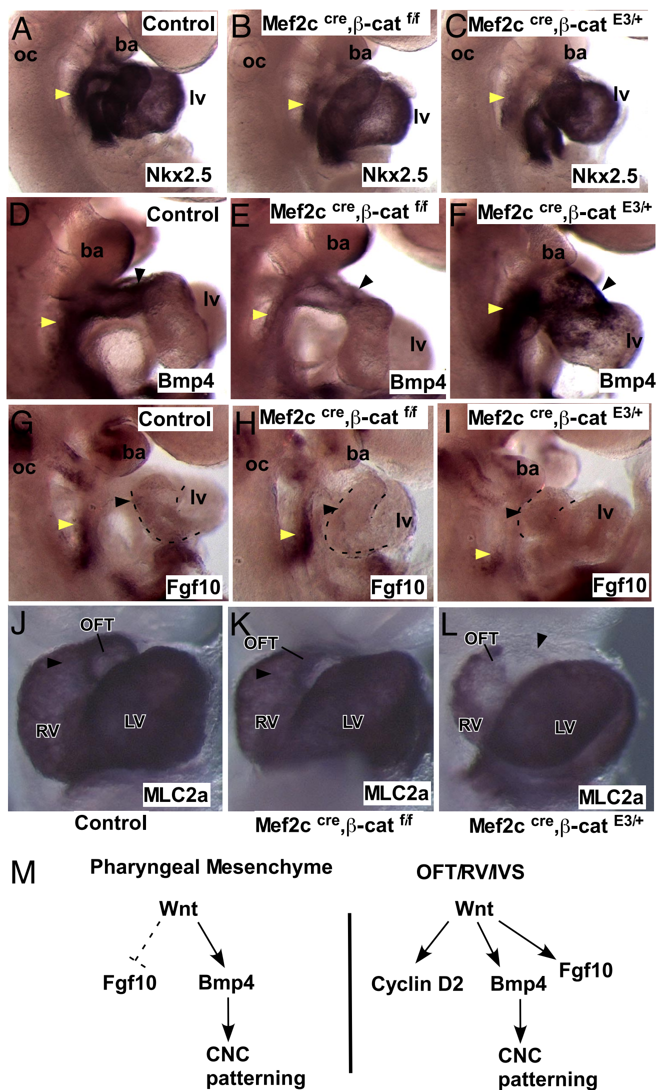

Fig. 6. Marker analysis in *β-catenin* loss-of-function and gain-of-function mutant embryos. (*A–L*) Analysis of markers for SHF and differentiated myocardium in 9.5-dpc embryos. Genotypes and markers are labeled. Pharyngeal mesenchyme is denoted by yellow arrowheads, and OFT is denoted by black arrowheads. OFT is outlined by a dotted line in *G–I* for clarity. (*M*) Model depicting the proposed role of canonical Wnt signaling in SHF. Based on loss- and gain-of-function experiments, canonical Wnt signaling promotes *Bmp4* expression while concurrently acting to restrict *Fgf10* in the SHF. The dotted line for *Fgf10* repression indicates that this may be a minor function for Wnt signaling in the SHF because the data supporting this inhibitory function were observed only in the gain-of-function experiment. In the SHF-derived myocardium of the OFT, RV, and interventricular septum (IVS), canonical Wnt signaling regulates *Bmp4* and promotes cell proliferation through regulation of *Cyclin D*. ba, branchial arch; RV, right ventricle; lv and LV, left ventricle; oc, otic capsule.

The Intersection of Wnt and Bmp Signaling in SHF and SHF Derivatives. Bmp signaling has been implicated in the addition of SHF progenitors to the OFT and has been considered to be a prodifferentiation pathway in the SHF. For example, Noggin-coated bead implantation in SHF explants resulted in increased cell proliferation (18). Conditional genetic experiments in mice support this interpretation because *Mesp*^{cre}; *Smad1*$^{f/f}$ mutants, which delete the Bmp effector *Smad1* widely in mesoderm, have elongated OFTs and elevated proliferation in SHF but not OFT myocardium (6). In addition, *Nkx2.5*^{cre}; *Bmp4*^{flox} mutants, which have a broad *Bmp4* deletion in SHF, SHF derivatives, and pharyngeal endoderm, show elevated proliferation in OFT myocardium (17). In contrast to what might have been predicted, our data indicate that *β*-catenin loss-of-function embryos with reduced *Bmp4* in pharyngeal mesenchyme and OFT myocardium have less myocardial proliferation.

We also noted that *β-catenin* loss-of-function embryos had defective patterning of OFT cushions that are partly derived from CNC. Because our strategy to inhibit Wnt signaling competence was limited to the SHF, the defect in CNC patterning indicates that there is a nonautonomous signal acting downstream of *β*-catenin. Published data reveal that inactivation of Bmp ligands in SHF, and type I Bmp receptors in CNC resulted in defective cardiac cushion development, supporting the notion that Bmp signals from SHF regulate development of the CNC (17, 42–44).

Previous work has uncovered dosage-dependent functions for Bmp signaling in mandibular development (45). This notion of distinct, dosage-dependent functions for Bmp signaling likely also applies to the SHF and SHF derivatives as suggested by the OFT phenotype of the *Bmpr2* hypomorph previously reported by Lyons and colleagues (46). Moreover, we have recently found that *Bmp2* and *Bmp4* function cooperatively in the SHF to regulate distinct events in the forming OFT (L. Ma and J.F.M., unpublished observations). Together our findings suggest that residual *Bmp4* expression in *β-catenin* loss-of-function embryos is sufficient to maintain the antiproliferative Bmp function but fails to support CNC patterning (Fig. 6*M*). The *β-catenin* loss-of-function embryos had reduced *Fgf10* expression in the OFT myocardium, which also likely contributes to diminished proliferation in the mutant OFT. Fgf signaling has been shown to be an important signal that promotes expansion of SHF derivatives (15, 16).

Stabilized *β*-Catenin Disrupts SHF Development. The phenotype we observed in *β-catenin* gain-of-function embryos, a short hypercellular OFT, likely represents a disruption of the normal balance between progenitor cell expansion and precisely timed differentiation. In support of this idea, we observed reduced *Fgf10* expression in the SHF of *β-catenin* gain-of-function embryos. As noted above, Fgf signaling in SHF is generally considered to promote proliferation; reduced Fgf signaling would likely result in a shorter OFT (15, 16, 18). In contrast, once SHF cells have moved into the OFT canonical Wnt signaling has a more direct role in promoting cellular proliferation and myocardial expansion (Fig. 6*M*). This would account for the myocardial hypercellularity and reduced *MLC2a* expression in the *β-catenin* gain-of-function embryos.

Moreover, these data suggest that elevated Wnt signaling has a negative influence on expansion of undifferentiated SHF in pharyngeal mesenchyme. These data support previous suggestions that Wnt signaling may have a role in delaying SHF from contributing to myocardium until looping stages.

Implications for Signaling in Cardiac Progenitor Cells and Right Ventricular Development. Our findings show that Wnt signaling plays a critical, positive role in promoting SHF expansion and diversification. This observation may have therapeutic implications because a number of laboratories have identified multipotent cardiac progenitor cells (10–12). An important goal would be to identify the signaling molecules that promote progenitor cell expansion. Our data indicate that Wnt signaling is an excellent candidate to promote growth of cardiac progenitors. Moreover, our findings indicate that Bmp signaling likely has a downstream function in this pathway (Fig. 6*M*). This is particularly intriguing considering the proposed role for *Bmp4* in self-renewal in embryonic stem cells (47).

Materials and Methods

Whole-Mount *in Situ* Hybridization. Whole-mount *in situ* hybridization was performed as previously described (48). Details about the *in situ* probes will be provided upon request. For all

experiments, at least three embryos were used for each probe at each time point examined.

LacZ Staining and Histology. For histology, embryos were fixed overnight in Bouin's fixative or buffered formalin, dehydrated through graded ethanol, and embedded in paraffin. Sections were cut at 7–10 μm and stained with H&E. Staining for β-galactosidase (LacZ) was as previously described (48).

Mouse Alleles and Transgenes. The *Mef2cAHF*^{cre} transgenic line has been previously described (9, 24). Briefly, the *Mef2cAHF*^{cre} transgenic line uses a 3,970-bp enhancer and promoter element from the *Mef2c* gene to direct *cre* recombinase expression in the SHF. The β-catenin conditional null allele has been described previously (25). For genotyping, DNA was extracted from yolk sacs or tails of embryos and adult mice, respectively. PCR was used to determine the genotype. The primers used were described (25). The β-catenin dominant stable allele has also been described previously (26). The genotyping primers used were 5′-AGCTGCTGTGACACCGCT-3′ and 5′-GCTACTTGCTCTTGCGTGAA-3′. The amplified product for the mutant allele is 700 bp.

Immunohistochemical Staining. Embryos were collected at desired stages and fixed in 4% paraformaldehyde for 30 min. Tissues were washed with PBS twice, dehydrated, and embedded in paraffin as previously described. Immunofluorescence staining was carried out on 5-μm sections as previously described (49). Briefly, sections were first blocked with 2% BSA in PBST and incubated with anti-β-catenin (1:600; Cell Signaling Technology, Beverly, MA) for 1 h. After three 10-min washings, Alexa Fluor 488-conjugated secondary anti-rabbit IgG (1:800; Molecular Probes, Eugene, OR) was added and incubated for 1 h. The secondary antibody was then washed off, and the sections were counterstained with propidium iodide and mounted with Aqua-Mount (Lerner Laboratories, Pittsburgh, PA). Images were captured by an Olympus FluoView confocal microscopy system.

For phospho-histone H3 (PH3) immunostaining antigen retrieval was performed by boiling slides in 10 mM sodium citrate (pH 6.0, 5 min) followed by treatment with 3% H_2O_2 to quench endogenous peroxidase. Specimens were blocked in 2% normal goat serum (NGS) for 1 h at room temperature and incubated overnight at 4°C with the anti-phospho-Histone H3 (Ser-10) antibody (Upstate Biotechnology, Lake Placid, NY) diluted 1:200 in 2% NGS, followed by incubation in biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in 2% NGS for 1 h at room temperature. Sections were processed by immunoperoxidase labeling using the Vectastain ABC Kit (Vector Laboratories) and visualized with a DAB kit (Vector Laboratories).

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