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Wnt5a and Wnt11 are essential for second heart field progenitor development

Ethan David Cohen[1,*], Mayumi F. Miller[2], Zichao Wang[1], Randall T. Moon[3] and Edward E. Morrisey[2,4,5,6,*]

SUMMARY

Wnt/β-catenin has a biphasic effect on cardiogenesis, promoting the induction of cardiac progenitors but later inhibiting their differentiation. Second heart field progenitors and expression of the second heart field transcription factor Islet1 are inhibited by the loss of β-catenin, indicating that Wnt/β-catenin signaling is necessary for second heart field development. However, expressing a constitutively active β-catenin with Islet1-Cre also inhibits endogenous Islet1 expression, reflecting the inhibitory effect of prolonged Wnt/β-catenin signaling on second heart field development. We show that two non-canonical Wnt ligands, Wnt5a and Wnt11, are co-required to regulate second heart field development in mice. Loss of Wnt5a and Wnt11 leads to a dramatic loss of second heart field progenitors in the developing heart. Importantly, this loss of Wnt5a and Wnt11 is accompanied by an increase in Wnt/β-catenin signaling, and ectopic Wnt5a/Wnt11 inhibits β-catenin signaling and promotes cardiac progenitor development in differentiating embryonic stem cells. These data show that Wnt5a and Wnt11 are essential regulators of the response of second heart field progenitors to Wnt/β-catenin signaling and that they act by restraining Wnt/β-catenin signaling during cardiac development.

KEY WORDS: Wnt, Cardiac development, Progenitor, Mouse

INTRODUCTION

The heart arises from two domains of lateral mesoderm that join at the anterior midline to form a crescent-shaped swathe of cardiogenic mesoderm under the head folds known as the cardiac crescent (Dyer and Kirby, 2009; Watanabe and Buckingham, 2010). The cardiac crescent contains two distinct fields of cardiac progenitors. The first heart field (FHF) occupies the medial region of the crescent and will coalesce at the ventral midline to form the initial linear heart tube, which is fated to become the myocardium of the left ventricle (LV). The second heart field (SHF) occupies more lateral regions of the crescent that are shifted dorsally into the pharyngeal mesenchyme as the heart tube fuses. SHF progenitors remain undifferentiated in the pharyngeal mesenchyme until later in development, when they migrate into the heart and contribute the majority of cardiomyocytes in the right ventricle (RV) and atria. How SHF progenitors and their derivatives differ from those of the FHF is poorly understood, but this knowledge will be crucial in our understanding of congenital heart defects and the potential differences in populations of cardiomyocytes derived from pluripotent stem cells.

Wnt proteins are secreted ligands that regulate diverse cellular behaviors and play pivotal roles in the development of cardiac progenitors (Tzahor, 2007; Cohen et al., 2008; Tian et al., 2010a). The canonical Wnt pathway stabilizes β-catenin and allows it to enter the nucleus, where it complexes with TCF family DNA-binding proteins to activate gene transcription (Molenaar et al., 1996; Brunner et al., 1997; Korinek et al., 1998; Young et al., 1998). Experiments performed on embryonic stem (ES) cell-derived embryoid bodies (EBs) and zebrafish embryos have revealed at least two temporally distinct roles for Wnt/β-catenin signaling during cardiogenesis (Naito et al., 2006; Ueno et al., 2007). The first phase promotes the induction of cardiac progenitors, whereas the second phase inhibits the maintenance of cardiac progenitor fate. SHF-specific β-catenin deletion reduces the numbers of SHF progenitors and their expression of the crucial SHF transcription factor Islet1 (Isl1) in mouse embryos, indicating that canonical Wnt signaling is required in SHF cells (Ai et al., 2007; Cohen et al., 2007; Klaus et al., 2007; Kwon et al., 2007). However, forced activation of β-catenin signaling in Isl1^{+} cells also disrupts SHF development, confirming that canonical Wnt signaling plays both positive and negative roles in Isl1^{+} SHF progenitors (Kwon et al., 2009).

Wnt proteins can also activate Rho family GTPases and intracellular Ca^{2+} release to affect cytoskeletal organization, adhesion and motility (Malbon, 2004; James et al., 2008; Schlessinger et al., 2009). This non-canonical Wnt signaling also inhibits Wnt/β-catenin signaling in many contexts and the balance between these activities may fine-tune the effects of Wnt proteins on responding cells (Torres et al., 1996; Ishitani et al., 1999; Ishitani et al., 2003; Mikels and Nusse, 2006; Yuan et al., 2011). Wnt5a and Wnt11 promote cardiogenesis in frog and chick embryos as well as in differentiating stem cells through the non-canonical Wnt pathways (Eisenberg and Eisenberg, 1999; Schneider and Mercola, 2001; Pandur et al., 2002; Terami et al., 2004; Koyanagi et al., 2005; Koyanagi et al., 2009). Although these data suggest that Wnt5a and Wnt11 act in early cardiac progenitors, *Wnt5a* and *Wnt11* mutations cause mild heart defects due to problems in cell-cell adhesion and cytoskeleton organization in differentiating cardiomyocytes (Schleiffarth et al., 2007; Zhou et al., 2007; Nagy et al., 2010). Here we show

[1]Department of Medicine, Division of Endocrinology, University of Rochester, Rochester, NY 14642, USA. [3]Howard Hughes Medical Institute, Institute for Stem Cell and Regenerative Medicine, Department of Pharmacology, University of Washington School of Medicine, Seattle, WA 98195, USA. [2]Department of Cell and Developmental Biology, [4]Department of Medicine, [5]Institute for Regenerative Medicine and [6]Cardiovascular Institute, University of Pennsylvania, Philadelphia, PA 19104, USA.

*Authors for correspondence (ethan_cohen@urmc.rochester.edu; emorrise@mail.med.upenn.edu)

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that Wnt5a and Wnt11 are co-required to restrain the inhibitory effects of prolonged canonical Wnt signaling on Isl1^{+} SHF progenitors.

MATERIALS AND METHODS

Generation of *Wnt5a; Wnt11* double-mutant mice

Mice carrying previously published alleles of *Wnt5a* and *Wnt11* (Yamaguchi et al., 1999; Majumdar et al., 2003) were intercrossed to produce double-mutant, single-mutant and wild-type embryos. To examine Wnt/β-catenin signaling in vivo, *Wnt5a*$^{-/+}$; *Wnt11*$^{-/+}$ mice were crossed into the BAT-GAL strain, which carries a TCF-responsive *lacZ* reporter (Maretto et al., 2003). Genotyping for *Wnt5a*, *Wnt11* and BAT-GAL was performed as described (Yamaguchi et al., 1999; Majumdar et al., 2003; Maretto et al., 2003). All protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Histology

Embryos were fixed in 4% PFA overnight before being embedded and sectioned. Staining with Isl1 (University of Iowa Hybridoma Bank, 40.2D6), Nkx2.5 (Santa Cruz, sc-8697) and Ki67 (DAKO, m7249) antibodies as well as TUNEL staining were performed as described (Cohen et al., 2007; Zhang et al., 2008) and examined with a confocal microscope. Labeling indexes for sections stained for Isl1, Nkx2.5, Ki67 and TUNEL were obtained by dividing the number of labeled cells by the total number of cells within the fame. Graphs represent the average value of indexes from images of three embryos of each genotype. Error bars represent s.d. $P<0.05$ by Student's *t*-test indicated statistical significance.

In situ hybridization was performed as described (Cohen et al., 2007; Zhang et al., 2008). Probes were amplified from *Wnt5a* and *Wnt11* cDNAs (Open Biosystems) using the primers listed in supplementary material Table S1.

Generation and culture of EBs

TL1 mouse ES cells were grown on inactivated mouse embryonic fibroblasts in DMEM with 15% FBS (Hyclone) and LIF (Millipore ESGRO). To initiate differentiation, cells were dissociated, suspended at 5×10^4 cells/ml in LIF-free media containing 100 μg/ml ascorbic acid and used to make 20 ml droplets on Petri dish lids. Cells were cultured as hanging drops for 2 days to form EBs, which were then cultured in poly-HEMA (Sigma-Aldrich)-treated dishes for another 2 days. On day 4, EBs were transferred to gelatin (Sigma-Aldrich)-coated dishes containing control media, media with 200 ng/ml mouse Wnt5a and/or human WNT11 (R&D Systems), or media with 200 ng/ml human WNT3A (R&D Systems). EBs were cultured with meBIO (50 nM, Millipore), BIO (50 nM, Millipore) or XAV-939 (25 nM, Cayman Chemical) between days 4 and 6 of differentiation. The phosphorylation mutant form of active β-catenin (pCMV-act-β-catenin) has been reported previously (Provost et al., 2003). Additional groups of EBs were lysed on days 8, 10 and 12 for RNA isolation to detect Wnt and cardiac gene expression. ES cells were transfected with either pCMV-lacZ or pCMV-act-β-catenin using Effectine (Qiagen) as previously described (Ko et al., 2009; Liou et al., 2010).

Measuring TCF activity in EBs

EBs were made from ES cells transduced with pBarls, a TCF-responsive firefly luciferase virus (Fuerer and Nusse, 2010), and with a constitutive Renilla luciferase virus. On day 4, EBs were placed in gelatin-coated dishes containing either control media or media with 250 ng/ml Wnt5a and/or WNT11. After 24 hours, luciferase activity was assayed with the Dual Luciferase Assay System (Promega). Renilla luciferase activity was used to normalize for transduction efficiency. Reported values are the mean ± s.e.m. for the normalized pBarls activity from at least three assays. $P<0.05$ by two-tailed Student's *t*-test indicated statistical significance.

Quantitative PCR (Q-PCR) and western blotting

Relative gene expression was calculated by the ΔCT method using *Gapdh* as an endogenous control. For Q-PCR analysis of embryonic hearts, graphs represent the average results from three independent pools of RNA isolated from the heart-containing regions, defined as the first pharyngeal arch to the septum transversum, of at least five embryos. For Q-PCR analysis of EBs, graphs represent the average values from three pools of RNA isolated from independent experiments. RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and Q-PCR performed on an ABI 7900 Cycler with SYBR Green (Applied Biosystems) and the primers listed in supplementary material Table S1. The ΔCT values of all samples were normalized to the average ΔCT of control samples, thus allowing the standard deviation to be calculated from the ΔCT values of individual controls. Error bars represent s.d. $P<0.05$ by Student's *t*-test indicated statistical significance.

For western blotting, cells were collected 48 hours following transfection, lysed, and nuclear and cytoplasmic fractions were generated as described (Wang et al., 2005). Proteins were resolved on SDS-PAGE gels and transferred to PVDF membranes. Antibodies used in western blotting include: β-catenin (BD Transduction Laboratories), Gapdh (Abcam) and hnRNP (Santa Cruz).

RESULTS

Wnt5a and *Wnt11* are co-expressed at the anterior pole of the heart tube

In situ hybridization was performed to determine whether *Wnt5a* and *Wnt11* expression domains overlap in the embryonic heart, which could suggest a shared role in early cardiac development. We performed in situ hybridization for *Wnt5a* and *Wnt11* on sections of E7.5 embryos. Expression of *Wnt5a* was observed in the allantois, as has previously been reported, but expression of *Wnt5a* or *Wnt11* was not detected in the cardiac mesoderm at this stage (Fig. 1A,E) (Yamaguchi et al., 1999). By E8.5, *Wnt5a* and *Wnt11* were both expressed in the presumptive outflow tract (OFT) and RV and at lower levels in the LV and atria, similar to what has been reported previously (Fig. 1B-D,F-H) (Kispert et al., 1996; Yamaguchi et al., 1999; Terami et al., 2004). Co-expression of *Wnt5a* and *Wnt11* was maintained at the anterior pole of the heart until E10.5, at which time the patterns of *Wnt5a* and *Wnt11* expression began to diverge. *Wnt5a* became enriched in the mesoderm surrounding the branchial arch arteries and the laryngeal-tracheal grove as well as the medial layer of the OFT wall (Fig. 1I). *Wnt5a* expression continued in the compact myocardium of the RV, LV and atria (Fig. 1J). By contrast, *Wnt11* was expressed in both the inner and outer layers of the OFT wall and inner lining of the OFT cushion (Fig. 1K). Within the developing RV, LV and atria, *Wnt11* was expressed in the epicardium and endocardium, with the highest levels of expression observed in the epicardial cells surrounding the atria and inner lining of the endocardial cushions (Fig. 1L). *Wnt11* was also expressed in the endothelium of the arch arteries (Fig. 1K, arrowhead) and in the mesenchyme surrounding the trachea (Fig. 1L, asterisk).

Wnt5a and Wnt11 are co-required for cardiac development

The overlap between Wnt5a and Wnt11 expression in the OFT and RV of the embryonic heart suggested that these ligands play redundant roles in cardiac development. To test this hypothesis, mice carrying null alleles of *Wnt5a* and *Wnt11* (Yamaguchi et al., 1999; Majumdar et al., 2003) were intercrossed to generate *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos, as well as wild-type and single-mutant controls. Intriguingly, whereas *Wnt5a*$^{-/-}$ and *Wnt11*$^{-/-}$ embryos survived until birth, no *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos were found after E10.5. *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos were found at the expected Mendelian ratios in litters harvested at E9.5 and had beating hearts, indicating that *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos were viable at this stage.

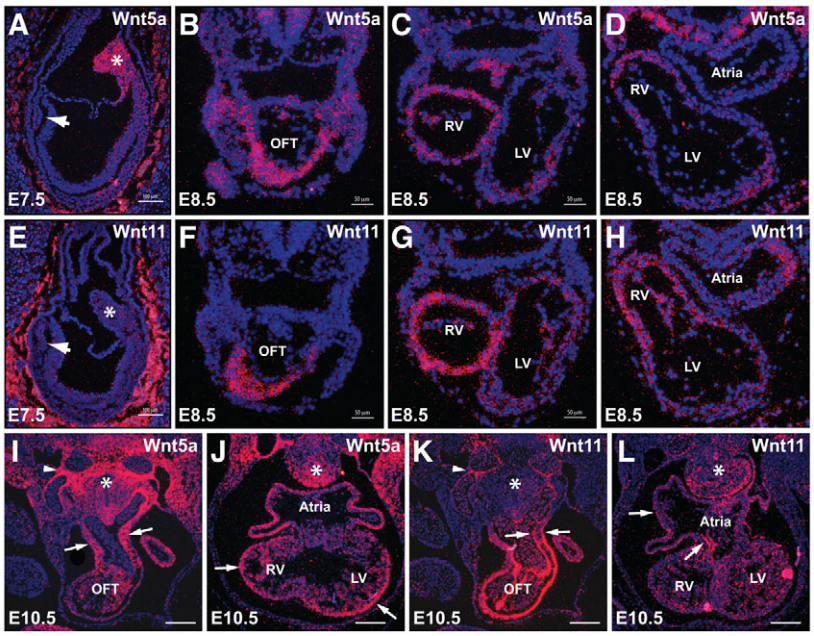

Fig. 1. Expression of *Wnt5a* and *Wnt11* during mouse early cardiac development. (**A**) In situ hybridization showing *Wnt5a* expression in the allantois (asterisk) at E7.5 but not in the precardiac mesoderm (arrow). (**B-D**) Expression of *Wnt5a* is observed in the developing OFT and right ventricle myocardium at E8.5. (**E-H**) Expression of *Wnt11* is not observed in the early precardiac mesoderm at E7.5 (arrow) and only at low levels in the allantois. Expression of *Wnt11* is observed in the developing OFT and right ventricle myocardium at E8.5. (**I**,**J**) *Wnt5a* expression is observed in OFT mesenchyme (I, arrows) and the branchial arch region at E10.5 (I, arrowhead) and also in the ventricular and atrial myocardium (J, arrows) at E10.5. (**K**,**L**) *Wnt11* expression is observed in the myocardium of the OFT (K, arrows) and at lower levels in the atrioventricular region and atrial myocardium (L, arrows) at E10.5. Expression of *Wnt11* is observed diffusely and at low levels in ventricular myocardium at E10.5 (L). OFT, outflow tract; LV, left ventricle; RV, right ventricle. Scale bars: 50 μm in A-H; 100 μm in I-L.

To assess the extent of disruption to cardiac development by loss of Wnt5a and Wnt11 expression, wild-type, *Wnt5a*$^{-/-}$, *Wnt11*$^{-/-}$ and *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ double-mutant embryos were harvested at E9.5 and processed for histological sectioning. Examining sections from anterior to posterior reveals severe cardiovascular defects in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ double-mutant embryos. *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos had a centrally located OFT leading to a single sac-like heart chamber that connects directly to the dorsal mesocardium at the posterior pole of the heart (Fig. 2Da-e). Although morphological abnormalities were apparent in *Wnt5a*$^{-/-}$ and *Wnt11*$^{-/-}$ hearts, these single-mutant hearts displayed evidence of chamber septation and development similar to wild-type hearts (Fig. 2Aa-d,Ba-e,Ca-e). By contrast, no chamber septation was apparent in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ hearts (Fig. 2Da-e). The OFT and RV of *Wnt5a*$^{-/-}$ hearts appeared dilated and dysmorphic relative to those of wild-type hearts (Fig. 2Ba-c), whereas the OFT and RV of *Wnt11*$^{-/-}$ hearts were small relative to those of controls (Fig. 2Ca-d). Although atrioventricular (AV) canal development was apparent in *Wnt5a*$^{-/-}$ and *Wnt11*$^{-/-}$ single mutants (Fig. 2Bc,d,Cc,d), no obvious AV canal development occurred in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ hearts (Fig. 2Da-e).

Development of SHF cardiac progenitors requires Wnt5a and Wnt11

Wnt5a and Wnt11 are co-expressed in the presumptive OFT and LV, and the single-chambered phenotype of *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ hearts was highly reminiscent of the cardiac defects found in *Isl1* mutants, suggesting that Wnt5a and Wnt11 act together to promote the development of SHF cardiac progenitors (Cai et al., 2003). To determine whether the numbers of Isl1^{+} SHF progenitors were reduced in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos, sections of E9.5 double-mutant, single-mutant and wild-type embryos were stained for Isl1 protein expression. Although nearly 40% of cells in the OFT and pharyngeal mesenchyme of wild-type and single-mutant control embryos were Isl1^{+}, less than 10% of these cells stained for Isl1 in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos (Fig. 3A-D,I). Furthermore, the Isl1^{+} cells in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos stained much less intensely than those in wild-type and single-mutant controls (Fig. 3A-D). By contrast, staining for Nkx2.5, which marks both FHF and SHF cardiac progenitors, was similar in all genotypes tested (Fig. 3E-I).

To further quantify FHF and SHF gene expression, Q-PCR for *Isl1* and *Nkx2.5* mRNA was performed on cardiac tissue dissected from E9.5 embryos. Consistent with the results for Isl1 and Nkx2.5 immunostaining, *Isl1* mRNA levels were more than 80% lower in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos than in wild-type and single-mutant controls, whereas the levels of *Nkx2.5* mRNA were unaffected (Fig. 3J). Like Isl1, the basic helix-loop-helix transcription factor Hand2 is expressed in the SHF and is crucial for its development (Tsuchihashi et al., 2011), whereas expression of the highly related Hand1 is restricted to the FHF and its derivatives (Riley et al., 1998; Thomas et al., 1998). Consistent with the loss of Isl1 expression in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos, *Hand2* expression was significantly reduced in double-mutant embryos, whereas *Hand1* expression was unaffected (Fig. 3J). These data indicate that Wnt5a and Wnt11 are required for the development of SHF cardiac progenitors.

Cardiac development was also assessed at E8.5 to determine whether there were any early defects associated with cardiac progenitor development, proliferation or apoptosis in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos. Although the *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos were smaller than single-mutant or wild-type controls at E9.5, *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos collected at E8.5 were similar in size and gross morphology to their wild-type and single-mutant littermates (Fig. 3K-N). *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ hearts at E8.5 had a centrally aligned OFT that connected to a single cardiac chamber (Fig. 3M,N). Moreover, there was little AV canal development in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos at E8.5 (Fig. 3M,N). Similar to the E9.5 embryos, Isl1 expression was reduced but Nkx2.5 expression was unaffected in E8.5 *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos, consistent with abnormal OFT and ventricular development observed at this stage (Fig. 3O-R,W).

To assess proliferation in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ hearts at E8.5, Ki67 immunostaining was performed. These data show increased proliferation in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos in areas corresponding to the SHF (Fig. 3S,T,W). By contrast, TUNEL staining did not reveal any changes in apoptosis in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos at

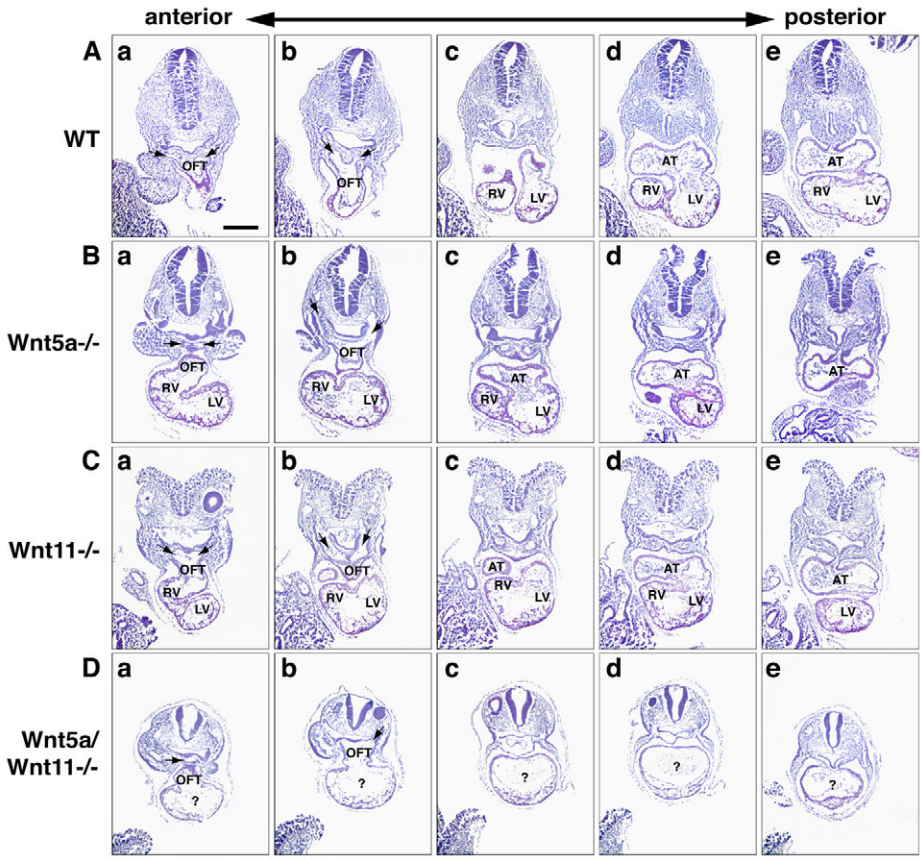

Fig. 2. *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos develop a single-chamber heart. Hematoxylin and Eosin (H+E) staining of histological sections at E9.5 of (**Aa-e**) *Wnt5a*$^{+/+}$; *Wnt11*$^{+/+}$, (**Ba-e**) *Wnt5a*$^{-/-}$; *Wnt11*$^{+/+}$, (**Ca-e**) *Wnt5a*$^{+/+}$; *Wnt11*$^{-/-}$ and (**Da-e**) *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ mouse embryos. Wild type as well as *Wnt5a*$^{-/-}$ and *Wnt11*$^{-/-}$ mutants display proper chamber septation. However, *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ mutants lack chamber septation and form a single-chamber heart (question mark in D). Arrows indicate where branchial arch arteries flow into the OFT of the heart. AT, atrium. Scale bar: 150 μm.

E8.5 (Fig. 3U-W). Taken together, these data suggested that the loss of Isl1^{+} cells in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos reflected a co-requirement of Wnt5a/Wnt11 signaling in SHF development and is not the result of either a broad developmental delay or changes in the survival of cells within the SHF.

Since Isl1^{+} progenitors contribute to all of the major cell types within the heart, markers of myocardial and non-myocardial cell populations within the heart were examined to determine whether these cell types are affected in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos. Immunostaining for the endothelial marker Flk1 (Kdr – Mouse Genome Informatics) was unaffected in E8.5 *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos relative to controls (Fig. 3X,Y). Q-PCR revealed that mRNA levels for the endothelial markers *Flk1* and *Cd31* (*Pecam1* – Mouse Genome Informatics) and smooth muscle markers smooth muscle α-actin (*Acta2*) and *Sm22a* (*Tagln* – Mouse Genome Informatics) were unaffected in the hearts of *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos (Fig. 3Z). By contrast, expression of the myocardial-specific markers *cTnI* (*Tnni3* – Mouse Genome Informatics) and *Mlc2v* (*Myl2* – Mouse Genome Informatics) was significantly reduced in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos (Fig. 3Z).

Taken together, these data suggest that the myocardial differentiation of Isl1^{+} SHF progenitors is specifically affected by the combined loss of Wnt5a and Wnt11.

Wnt5a and Wnt11 are co-required to induce cardiac progenitor gene expression in differentiating ES cells

To examine the temporal relationship between Wnt5a and Wnt11 expression and cardiac differentiation, EBs were formed from mouse ES cells using the hanging drop method and used to assess expression of various Wnt proteins. Expression of *Wnt3a* and *Wnt5a*, which are both expressed in the primitive streak, first began to rise on day 4 of EB differentiation and increased steadily, hitting a peak on days 8 and 10, respectively, and declining thereafter (Fig. 4A,B) (Yamaguchi et al., 1999; Baranski et al., 2000). By contrast, *Wnt11* expression increased slowly between days 0 and 8 before it increased abruptly on day 10 and remained high through day 14 of EB differentiation (Fig. 4C). To compare the timing of *Wnt5a* and *Wnt11* expression with the appearance of cardiac progenitor gene expression, the percentage maximum expression for *Wnt5a*, *Wnt11*, *Isl1*, *Nkx2.5* and the precardiac mesoderm marker *Mesp1* was plotted over the course of EB differentiation (Fig. 4D). This comparison shows that expression of *Wnt5a* roughly coincided with markers of early cardiac mesoderm including *Isl1*, *Nkx2.5* and *Mesp1*, whereas *Wnt11* expression increased somewhat later.

To determine whether Wnt5a and Wnt11 could induce markers of early cardiac mesoderm progenitors, EBs were formed as hanging drops and allowed to develop until day 4, the point at which Mesp1^{+} cardiac competent mesoderm first appears (Bondue et al., 2008). EBs were then cultured in either control media, media containing recombinant Wnt5a and/or WNT11 proteins or media containing recombinant WNT3A protein and Q-PCR used to measure gene expression. Since Wnt/β-catenin signaling was previously shown to promote the formation of cardiac competent mesoderm, we first examined the expression of *Mesp1*, a helix-loop-helix transcription factor expressed in early precardiac mesoderm and the cardiac crescent but later extinguished as the heart tube develops (Saga et al., 1999; Kitajima et al., 2000; Saga et al., 2000; Lindsley et al., 2006;

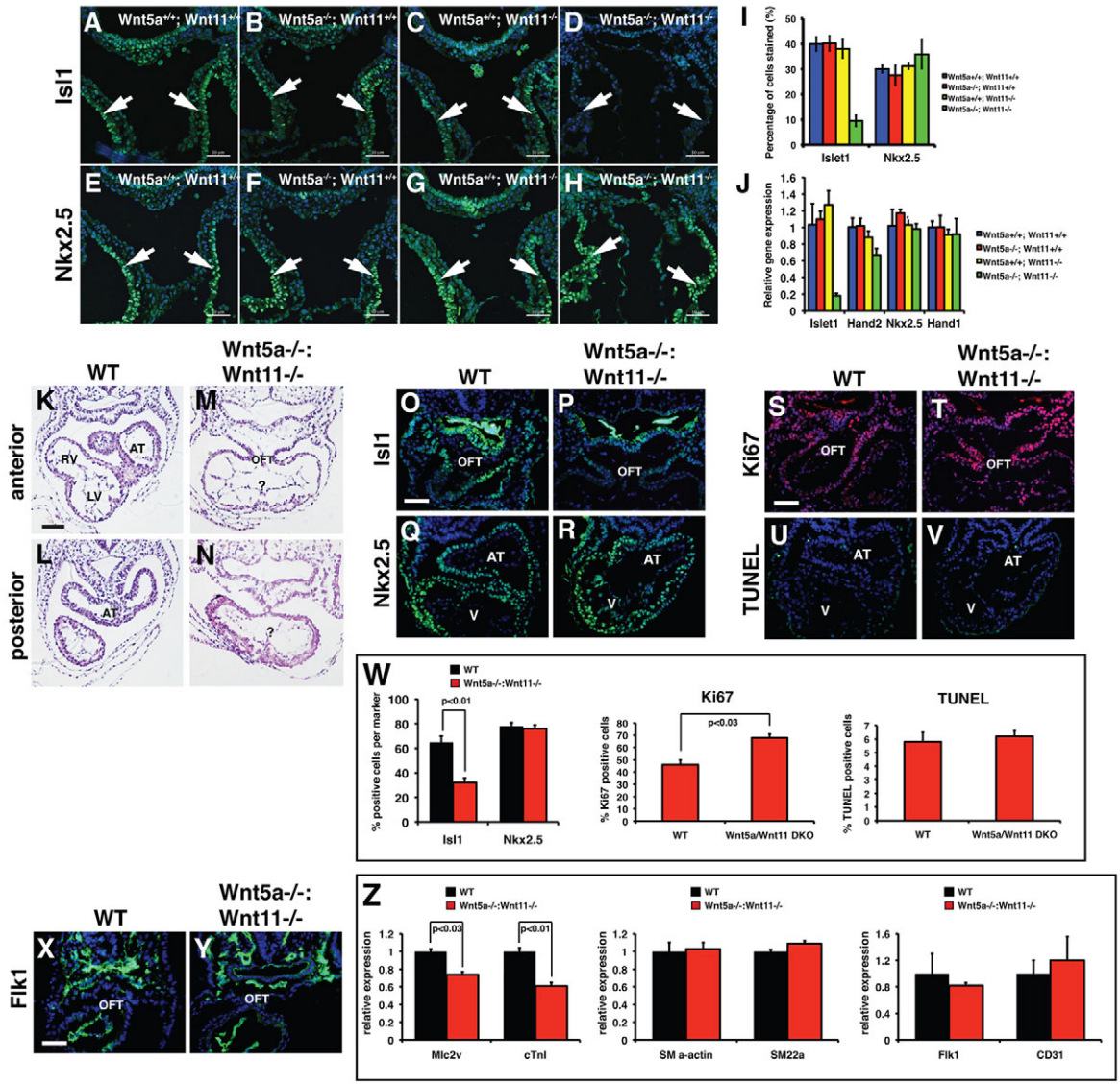

Fig. 3. Wnt5a and Wnt11 are necessary for SHF progenitor development and myocardial differentiation. (**A-D**) Immunostaining shows that Isl1 protein expression (arrows) is dramatically reduced in *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ mouse embryos compared with wild type or single mutants. (**E-H**) By contrast, Nkx2.5 protein expression (arrows) is not significantly altered in *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ mutants compared with wild type and single mutants. (**I**) Quantitation of Isl1^{+} cells in single and double mutants. (**J**) Q-PCR shows that expression of *Isl1* and *Hand2* is reduced in *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ mutants, whereas *Nkx2.5* and *Hand1* expression is not altered. (**K-N**) H+E-stained sections of wild type and *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ mutants at E8.5 showing lack of chamber septation in the *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ heart. Question mark indicates single-chamber heart. (**O-R**) Isl1 (O,P) and Nkx2.5 (Q,R) immunostaining at E8.5 in wild type and *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ mutants. (**S-V**) Ki67 (S,T) and Nkx2.5 (U,V) immunostaining at E8.5 in wild type and *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ mutants. (**W**) Quantitation of Isl1^{+}, Nkx2.5^{+}, Ki67^{+} and TUNEL^{+} cells in wild type and *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ mutants. (**X**,**Y**) Flk1 immunostaining in wild type (X) and *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ mutants (Y). (**Z**) Q-PCR for *Mlc2v*, *cTnI*, smooth muscle α-actin, *Flk1* and *Cd31* in wild type and *Wnt5a*$^{-/-}$*; Wnt11*$^{-/-}$ mutants. $P<0.05$ by Student's *t*-test indicates statistical significance. DKO, double knockout. Error bars indicate s.e.m. Scale bars: 50 μm in A-H; 150 μm in K-N; 100 μm in O-V,X,Y.

Lindsley et al., 2008). Treatment with Wnt5a, WNT11 or WNT3A individually caused a ~50% increase in peak *Mesp1* expression relative to controls, and treatment with Wnt5a and WNT11 together caused only a slight increase in *Mesp1* expression relative to treatment with either Wnt alone (Fig. 4E). By contrast, expression of *Tbx5*, which is expressed in the precardiac mesoderm and cardiac crescent before being restricted to the venous pole of the heart (Bruneau et al., 1999), is increased in EBs treated with Wnt5a and WNT11 together as compared with those treated with Wnt5a, WNT11 or WNT3A alone (Fig. 4F). Most importantly, combined Wnt5a WNT11 treatment also caused cooperative increases in the levels of both *Nkx2.5* and *Isl1* expression over the levels of expression found in either control EBs or those treated with Wnt5a, WNT11 or WNT3A alone (Fig. 4G,H). This increase in *Nkx2.5* could be the result of an expansion of early precardiac mesoderm by Wnt5a and WNT11 that leads to an overall increase in cardiogenesis.

Taken together, these data suggest that Wnt5a and Wnt11 are co-required to increase both FHF and SHF progenitor markers during EB differentiation.

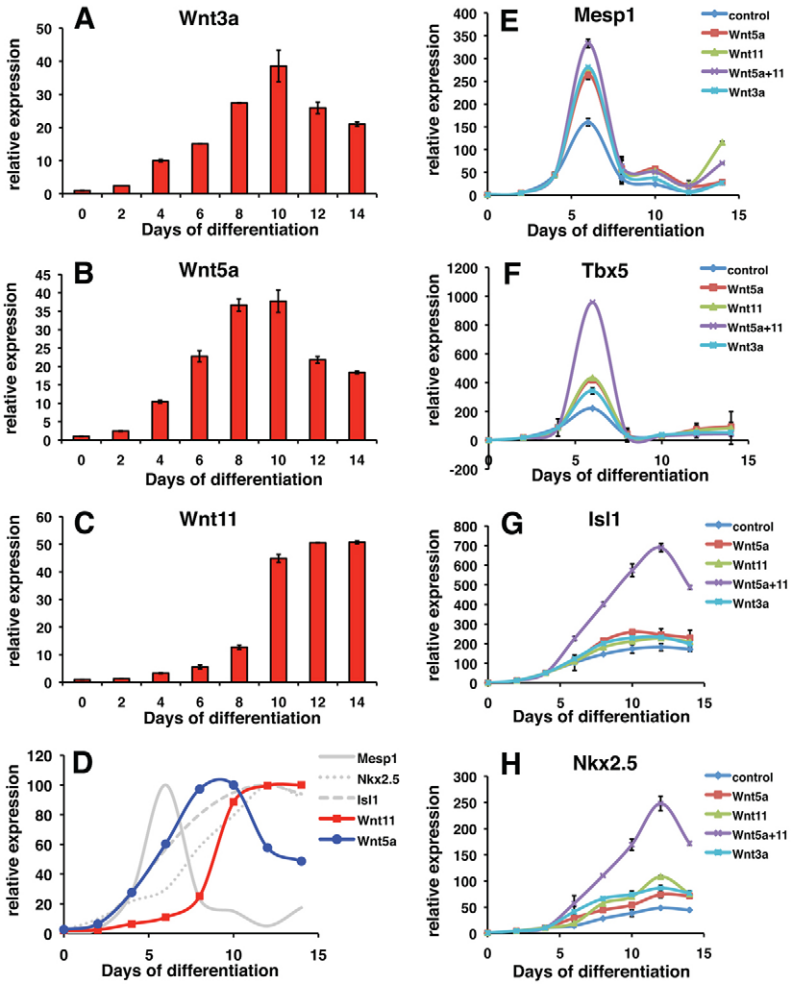

Fig. 4. Wnt5a and Wnt11 cooperatively promote early cardiac progenitor development in differentiating ES cells. (**A-D**) Temporal expression of *Wnt3a* (A), *Wnt5a* (B) and *Wnt11* (C) in mouse ES cell-derived embryoid body (EB) cultures at the days indicated. Overlay of temporal expression patterns of *Wnt5a*, *Wnt11*, *Mesp1*, *Isl1* and *Nkx2.5* (D). (**E-H**) Q-PCR analysis showing effects of recombinant WNT3A, Wnt5a, WNT11, or Wnt5a plus WNT11 on expression of *Mesp1* (E), *Tbx5* (F), *Isl1* (G) and *Nkx2.5* (H) in ES cell-derived EB cultures at the days indicated. Error bars indicate s.e.m.

Wnt5a and Wnt11 are co-required to repress canonical Wnt signaling in SHF progenitors

Although Wnt5a and Wnt11 are thought to signal via non-canonical pathways, they can also co-activate Wnt/β-catenin signaling during early *Xenopus* development in a manner that is reminiscent of their cooperative regulation of Isl1 and SHF development (Cha et al., 2008; Cha et al., 2009). To assess canonical Wnt signaling activity in the SHF of *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos, we crossed the BAT-GAL strain Wnt reporter line into the *Wnt5a*$^{+/-}$; *Wnt11*$^{+/-}$ mutants (Maretto et al., 2003). Sections through the hearts of E9.5 embryos stained with X-gal reveal that the number of stained cells within the walls of the OFT and surrounding mesenchyme are increased in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ mutants relative to controls, suggesting that Wnt5a and Wnt11 are co-required to repress canonical Wnt signaling in the SHF (Fig. 5A,B).

A previous study identified several genes, including *Bhlhb2* (*Bhlhe40*), *Ndrg1*, *Fgf11* and *Rora*, as being upregulated by β-catenin signaling in Isl1^{+} SHF progenitors (Kwon et al., 2009). To confirm that β-catenin signaling is increased in the SHF progenitors of *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos, the expression levels of these genes were examined by Q-PCR. These data show that levels of *Bhlhb2*, *Ndrg1*, *Fgf11* and *Rora* mRNA were all dramatically increased in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ hearts relative to either single-mutant or wild-type controls, further indicating that β-catenin signaling is upregulated in double-mutant SHF progenitors (Fig. 5C-F).

Forced activation of β-catenin signaling blocks the effects of Wnt5a and Wnt11 on cardiac differentiation

The increased BAT-GAL activity in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos suggested that Wnt5a and Wnt11 are normally co-required to attenuate canonical Wnt signaling in the SHF. To determine whether Wnt5a and Wnt11 are sufficient to inhibit canonical Wnt signaling, EBs were produced from ES cells transduced with the TCF-responsive luciferase reporter virus pBarls (Biechele et al., 2009). On day 4 of differentiation, these EBs were placed in control media as well as media containing recombinant Wnt5a, WNT11, or Wnt5a plus WNT11. After 24 hours, EBs were lysed and luciferase activities determined. Concordant with the increased levels of β-catenin signaling in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos, treating EBs with media containing both Wnt5a and WNT11 caused a significant decrease in pBarls activity (Fig. 6A).

To further test the effects of activated Wnt/β-catenin signaling on cardiac progenitor development in differentiating ES cells using the pBarls Wnt reporter, day-4 EBs were transferred to media containing combinations of the Gsk3β inhibitor BIO, its inactive form meBIO, and Wnt5a and/or WNT11 proteins, for 48 hours. The combined action of Wnt5a and WNT11 inhibited pBarls Wnt reporter activity as expected in the presence of the inactive Gsk3β inhibitor meBIO (Fig. 6B). However, In the presence of BIO, Wnt5a and WNT11 were unable to repress pBarls Wnt reporter activity (Fig. 6B). Q-PCR showed that activation of Wnt signaling

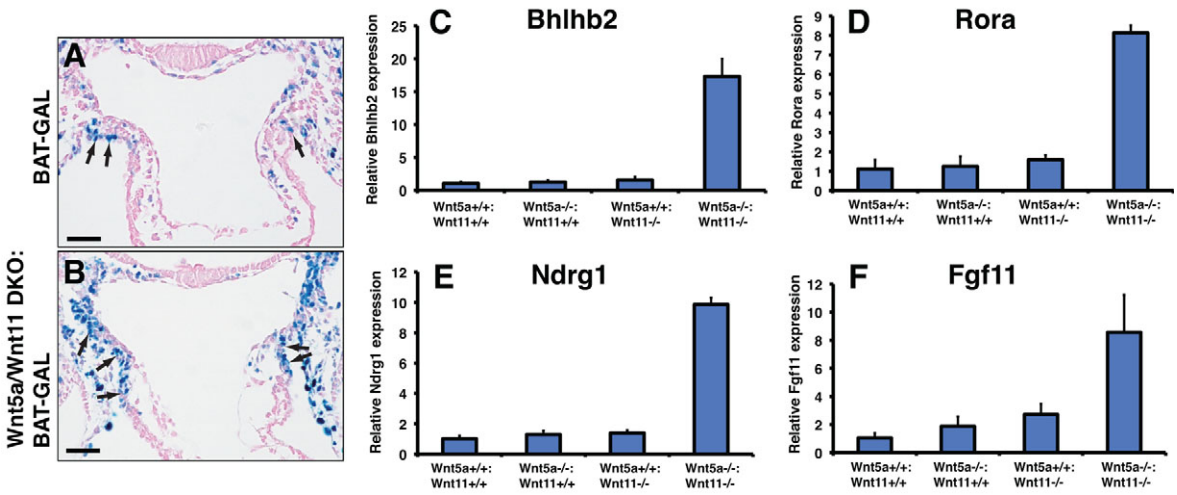

Fig. 5. Combined loss of Wnt5a and Wnt11 during mouse development increases canonical Wnt/β-catenin signaling activity and SHF target gene expression. (**A**,**B**) BAT-GAL *lacZ* expression (arrows) is increased in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ mutants compared with wild-type controls. (**C-F**) Expression of *Bhlhb2* (C), *Rora* (D), *Ndrg1* (E) and *Fgf11* (F) is increased in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ hearts but not single-mutant hearts. For all four genes, only the increased expression in the *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ mutants was considered significant (P<0.01). Error bars indicate s.e.m. Scale bars: 50 μm.

by BIO inhibited the increase of *Isl1* and *Nkx2.5* gene expression caused by treatment of differentiating EBs with Wnt5a and WNT11 (Fig. 6C,D). Moreover, expression of an activated form of β-catenin containing mutations in the Gsk3β phosphorylation sites during EB differentiation also inhibited the increase in *Isl1* and *Nkx2.5* cardiac gene expression in comparison to control *lacZ* expression (Fig. 6E,F).

To assess whether the combined action of Wnt5a and WNT11 affected additional readouts of canonical Wnt signaling, we performed western blots to assess the levels of nuclear β-catenin upon treatment of differentiating EBs. These data show that the combination of Wnt5a and WNT11 causes a decrease in nuclear β-catenin levels (Fig. 6G). Moreover, this decrease in β-catenin was not observed in the presence of BIO, consistent with the observed effects on the pBarls Wnt reporter above (Fig. 6H). Finally, to assess whether direct inhibition of Wnt/β-catenin signaling could affect cardiac gene expression, we treated differentiating EBs with the tankyrase inhibitor XAV-939, which promotes axin-Gsk3β interactions and thereby represses canonical Wnt signaling (Huang et al., 2009). Using this method, we observed a significant increase in *Isl1* and *Nkx2.5* gene expression (Fig. 6I). Thus, the ability of Wnt5a and Wnt11 to cooperatively repress β-catenin signaling in differentiating EBs is essential for their activation of cardiac gene expression and promotion of cardiac progenitor development.

DISCUSSION

The data presented here support a novel role for Wnt5a and Wnt11 in SHF development that is mediated by blocking the later inhibitory effect of Wnt/β-catenin signaling on Isl1^{+} SHF progenitors. We show that *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos die early in embryogenesis and have single-chamber sac-like hearts reminiscent of those found in *Isl1*$^{-/-}$ embryos. Moreover, expression of the SHF markers Isl1 and Hand2 is significantly reduced in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos, whereas Nkx2.5, which is expressed in both FHF and SHF, is unaffected. Interestingly, treating EBs with Wnt5a and Wnt11 causes a strong cooperative increase in the expression of Isl1, as well as other early cardiac progenitor markers including Nkx2.5, that far exceeds those caused by either Wnt alone, and this requires inhibition of β-catenin signaling. These data suggest that Wnt5a and Wnt11 are co-required to promote cardiogenesis, in part, through inhibition of β-catenin signaling.

Experiments in ES cells and zebrafish embryos indicate that canonical Wnt signaling plays two temporally distinct roles in cardiogenesis, first promoting the appearance of early precardiac mesoderm and then later inhibiting cardiac differentiation. Consistent with these data, the canonical Wnt ligand Wnt3a is sufficient to induce Mesp1 expression in differentiating ES cells, but overexpressing Mesp1 in ES cells promotes cardiac differentiation only if canonical Wnt signaling is simultaneously inhibited (Bondue et al., 2008). We were unable to detect either Wnt5a or Wnt11 in the cardiogenic mesoderm of E7.5 head-fold stage mouse embryos, which is consistent with the results of previous studies indicating that expression of Wnt11 in the heart begins at E8.0 (Kispert et al., 1996; Terami et al., 2004). These data suggest that reports of Wnt11 expression in the cardiac crescent of the chick embryo might reflect species-specific differences between avian and mammalian development or that Wnt11 expression is present in early precardiac mesoderm prior to heart tube formation but is dramatically upregulated upon fusion of the lateral cardiac primordia (Eisenberg and Eisenberg, 1999; Hardy et al., 2008). Our expression studies on Wnt5a and Wnt11 in E8.5 embryos revealed a previously unappreciated degree of overlap between the expression of these Wnt ligands in the presumptive OFT and RV at the anterior pole of the heart tube. Furthermore, although Wnt5a and Wnt11 expression no longer overlap in the mouse heart at E10.5, they are expressed in a complementary pattern that allows them to signal cooperatively in a paracrine fashion at this stage. Thus, the in vivo developmental role of Wnt5a and Wnt11 appears to be restricted to regulation of SHF progenitor development.

How Wnt5a and Wnt11 co-regulate early cardiac development is still unclear. Our data show that the combination of Wnt5a and Wnt11 mediates a decrease in nuclear β-catenin levels, which corresponds to a decrease in canonical Wnt activity. This function of Wnt5a and Wnt11 appears to act after the time period when Wnt3a induction of canonical Wnt signaling occurs in cardiogenesis in differentiating EBs. Previous studies have shown that Wnt5a and Wnt11 can dimerize to promote canonical Wnt

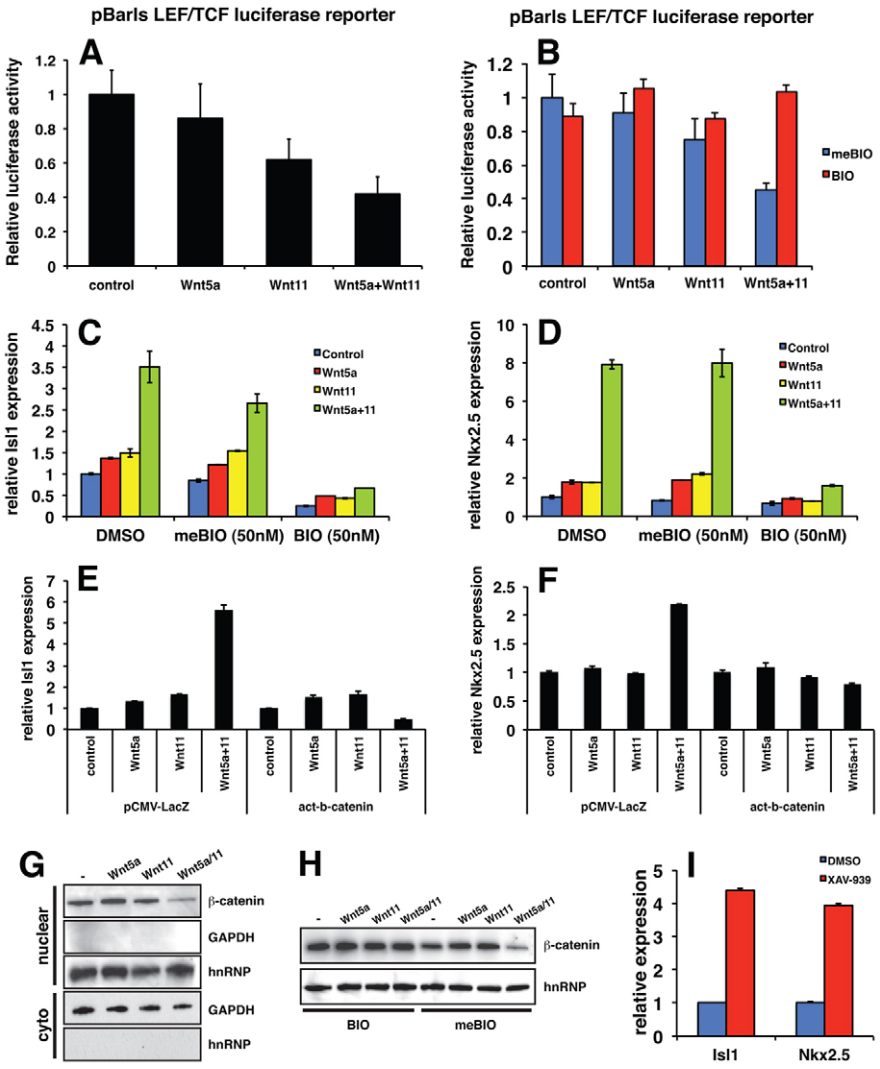

Fig. 6. Wnt5a and Wnt11 cooperatively inhibit Wnt/β-catenin signaling activity in differentiating ES cells. (**A**) Recombinant Wnt5a and WNT11 in combination can repress Wnt/β-catenin signaling in mouse EBs as shown using the Wnt/β-catenin reporter pBarls. (**B**) The Gsk3β inhibitor BIO inhibits the repressive effects on Wnt/β-catenin signaling caused by the combined addition of Wnt5a and WNT11 to EBs, whereas the inactive form meBIO does not. (**C**,**D**) Q-PCR analysis showing that addition of BIO inhibits the activation of *Isl1* (C) and *Nkx2.5* (D) caused by co-treatment with Wnt5a and WNT11, whereas DMSO and meBIO have little effect. (**E**,**F**) Expression of an activated form of β-catenin inhibits the activation of *Isl1* (E) and *Nkx2.5* (F) caused by co-treatment with Wnt5a and WNT11. (**G**) Western blot for expression of nuclear β-catenin showing that the combined action of Wnt5a and WNT11 decreases nuclear β-catenin levels in differentiating EBs. Sequestered protein expression of hnRNP and Gapdh show that the nuclear and cytoplasmic fractionation worked and that these fractions are relatively pure. (**H**) Western blot showing that nuclear levels of β-catenin could not be decreased by the combined action of Wnt5a and WNT11 in the presence of BIO. (**I**) Treatment of differentiating EBs with XAV-939 increases *Isl1* and *Nkx2.5* expression. Decreases in pBarls activity in A and B ($P<0.05$), increases in *Isl1* and *Nkx2.5* gene expression in C-F ($P<0.005$) and the increase in *Isl1* and *Nkx2.5* expression in I ($P<0.001$) are significant. Error bars indicate s.e.m.

signaling in *Xenopus* (Cha et al., 2008). In cardiac development and in cardiogenesis in differentiating EBs, we observe an inhibition of nuclear β-catenin levels and canonical Wnt signaling activity. This discrepancy might simply reflect differences in the activity of these Wnt ligands in different developmental contexts. Interestingly, previous reports have shown that Wnt11 can promote cardiogenesis through caspase 3-mediated degradation of β-catenin that appears to be independent of the cytoplasmic destruction complex (Abdul-Ghani et al., 2011). Although we did not observe decreased nuclear β-catenin with treatment of EBs with Wnt11 alone, such a mechanism could still be active during the cardiogenesis promoted by the combination of Wnt5a and Wnt11.

Although Isl1^{+} SHF progenitors are specifically affected in *Wnt5a*$^{-/-}$; *Wnt11*$^{-/-}$ embryos, levels of *Tbx5*, *Nkx2.5* and *Isl1* mRNAs are all dramatically increased in EBs treated with the combination of Wnt5a and Wnt11. Whereas Nkx2.5 is expressed in SHF progenitors, Tbx5 is not expressed in the SHF or its derivatives but rather in the LV and atria and is therefore considered a specific marker of the FHF (Bruneau et al., 1999). Given these data, the increased Tbx5 expression in EBs treated with Wnt5a and Wnt11 suggests that cooperative signaling by these Wnt proteins might be sufficient to promote both FHF and SHF fates if present during early precardiac mesoderm development. These data are consistent with the results of studies of heart development in *Xenopus*, which indicate that inhibition of canonical Wnt signaling is both necessary and sufficient for FHF formation (Schneider and Mercola, 2001). The ability of Wnt5a and Wnt11 to induce FHF markers including Mesp1 and Tbx5 occurs earlier than induction of SHF markers, suggesting that Wnt5a and Wnt11 might act via different mechanisms in a temporal-specific fashion during cardiogenesis. In the EB culture system, FHF and SHF progenitors are likely to co-develop at similar times and might be affected in the same fashion in gain-of-function experiments using exogenous Wnt ligands. Also, such differences are often observed between gain-of-function and loss-of-function experiments and might not be reflective of the physiological role for the specific Wnt ligands in question.

The endogenous factors responsible for inhibiting the negative effects of prolonged Wnt signaling during cardiac progenitor development have yet to be identified, and the mechanisms by which the activities of Wnt agonists and repressors are balanced in both FHF and SHF cardiac progenitors remain poorly understood. Additional research will be needed to determine how pathways that inhibit canonical signaling, such as the suppression of β-catenin signaling by Wnt5a/Wnt11, are balanced with the active promotion of Wnt/β-catenin signaling in the heart by ligands such as Wnt3a, Wnt8a and Wnt2 (Tian et al., 2010b).

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Competing interests statement
The authors declare no competing financial interests.

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