Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 278 (2005) 35–48

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Frzb modulates Wnt-9a-mediated β-catenin signaling during avian atrioventricular cardiac cushion development

Anthony D. Person[a,*], Robert J. Garriock[a], Paul A. Krieg[a], Raymond B. Runyan[a], Scott E. Klewer[a,b]

[a]*Department of Cell Biology and Anatomy, University Medical Center, University of Arizona School of Medicine, 1501 N. Campbell Avenue, PO Box 245044, Tucson, AZ 85724, USA*

[b]*Department of Pediatrics, University of Arizona School of Medicine, Tucson, AZ 85724, USA*

Received for publication 9 June 2004, revised 8 October 2004, accepted 21 October 2004
Available online 13 November 2004

Abstract

Normal development of the cardiac atrioventricular (AV) endocardial cushions is essential for proper ventricular septation and morphogenesis of the mature mitral and tricuspid valves. In this study, we demonstrate spatially restricted expression of both Wnt-9a (formerly Wnt-14) and the secreted Wnt antagonist Frzb in AV endocardial cushions of the developing chicken heart. Wnt-9a expression is detected only in AV canal endocardial cells, while Frzb expression is detected in both endocardial and transformed mesenchymal cells of the developing AV cardiac cushions. We present evidence that Wnt-9a promotes cell proliferation in the AV canal and overexpression of Wnt-9a in ovo results in enlarged endocardial cushions and AV inlet obstruction. Wnt-9a stimulates β-catenin-responsive transcription in AV canal cells, duplicates the embryonic axis upon ventral injections in *Xenopus* embryos and appears to regulate cell proliferation by activating a Wnt/β-catenin signaling pathway. Additional functional studies reveal that Frzb inhibits Wnt-9a-mediated cell proliferation in cardiac cushions. Together, these data argue that Wnt-9a and Frzb regulate mesenchymal cell proliferation leading to proper AV canal cushion outgrowth and remodeling in the developing avian heart.

Keywords: Wnt-9a; Wnt-14; Frzb; Endocardial cushions; Cell proliferation; Apoptosis; β-catenin; Epithelial–mesenchymal transition (EMT); Heart; Cardiac

Introduction

The primitive heart consists of an outer myocardial cell layer and an inner endocardial cell layer separated by an acellular extracellular matrix (ECM) termed cardiac jelly (Kitten et al., 1987). Following initiation of cardiac looping localized increased extracellular matrix (ECM) secretion from the myocardium at the junctions between the atrium and ventricle (atrioventricular canal) and along the outflow tract creates thickened luminal projections called endocardial cushions (Markwald et al., 1975, 1977). During cardiac cushion epithelial–mesenchymal cell transition (EMT), a subpopulation of endocardial cells within the AV and outflow tract cushions proliferate and transform into migratory mesenchymal cells that populate the cardiac jelly between the endocardium and myocardium (Markwald et al., 1975, 1977). Lineage tracing experiments demonstrate that AV canal cushion mesenchymal cells contribute to the mature mitral and tricuspid valve leaflets as well as the interatrial and interventricular septa (de la Cruz et al., 1977, 1983; Lamers et al., 1995).

The majority of studies investigating cardiac cushion morphogenesis have focused on the process of epithelial–mesenchymal cell transition (EMT) (Akiyama et al., 2004; Brown et al., 1996, 1999; Camenisch et al., 2000; Galvin et al., 2000; Kim et al., 2001; Kitamura et al., 1999; Krug et al., 1985, 1987; Nakajima et al., 1997a,b; Potts and Runyan, 1989; Potts et al., 1991; Runyan and Markwald, 1983).

* Corresponding author. Fax: +1 520 626 6571.
E-mail address: aperson@email.arizona.edu (A.D. Person).

0012-1606/$ - see front matter © 2004 Published by Elsevier Inc.
doi:10.1016/j.ydbio.2004.10.013

However, the formation of mesenchymal cells in cardiac cushions is one of many developmental events necessary for proper heart valve morphogenesis. While restricted cardiac cushion expression of BMP-4 and NFATc is observed during EMT, genetic null models suggest direct roles for these candidate genes in cell proliferation, rather than EMT, during cushion morphogenesis (de la Pompa et al., 1998; Jiao et al., 2003; Ranger et al., 1998). In addition, although FGF-4 is expressed in developing chicken endocardial cushions during EMT, FGF-4 appears to affect cell proliferation important for later cushion remodeling and does not play a role in cushion EMT (Sugi et al., 2003).

Within the embryonic cardiac cushions, endocardial cells and newly formed mesenchymal cells display increased BRDU incorporation (De la Cruz and Markwald, 1998) and a gradient of proliferating cell nuclear antigen (PCNA) staining near the leading edge of the cushion projections (Abdelwahid et al., 2002). This gradient of cell proliferation has been proposed to promote directional outgrowth of cushions ultimately leading to mature valve leaflet formation (De la Cruz and Markwald, 1998). Apoptosis also seems to be involved in cardiac cushion remodeling. Apoptotic cells are found at low levels throughout the cushions with increased programmed cell death observed in cushion mesenchyme adjacent to the myocardium (Abdelwahid et al., 2002). Focal apoptosis within cardiac cushions may play a role in shaping early cushions into elongated mature valve leaflets. Thus, the coordinated balance of cell proliferation and apoptosis appears necessary for normal endocardial cushion outgrowth and remodeling.

Canonical Wnt glycoproteins (Wnt-1, Wnt-3a, Wnt-8) bind to Frizzled transmembrane receptors initiating a signaling cascade resulting in β-catenin nuclear translocation and activation of β-catenin responsive genes (reviewed by Miller, 2002). Many examples exist of Wnt/β-catenin signaling modulating cell proliferation throughout development and cancer (reviewed by Smalley and Dale, 1999). β-catenin activity affects cell proliferation with molecular studies revealing activating mutations of canonical Wnt signaling pathway components in 90% of colorectal cancers (reviewed in Giles et al., 2003). Wnt/β-catenin signaling also influences cell proliferation during embryogenesis. Wnt-1 and Wnt-3a stimulate cell proliferation in the developing neural tube and somites (Galli et al., 2004; Megason and McMahon, 2002). In addition, β-catenin activity regulates cell survival and proliferation in the developing mammary gland with activated β-catenin resulting in mammary gland neoplasia (Gallagher et al., 2002; Imbert et al., 2001; Rowlands et al., 2003). Wnt/β-catenin regulation of the transcription factor Pitx2 leads to a cascade of cell proliferation signals necessary for neural crest cell migration (Kioussi et al., 2002).

Recent reports using transgenic zebrafish and mouse embryos expressing β-catenin responsive reporter genes reveal an important role for β-catenin signaling in endocardial cushion morphogenesis (Gitler et al., 2003; Hurlstone et al., 2003). The hearts of zebrafish embryos with mutations in APC or Axin, both negative regulators of β-catenin signaling, exhibit ectopic β-catenin activity, expanded cardiac cushions, and increased cell proliferation (Hurlstone et al., 2003). Although downstream components of Wnt/β-catenin signaling are clearly involved in cardiac cushion development, the specific Wnt directing β-catenin-mediated cell proliferation in the cardiac cushions is not currently known. Here we show that Wnt-9a signals through β-catenin in the developing avian AV canal cushions. Wnt-9a overexpression increases AV cushion mesenchymal cell proliferation while a Wnt-9a truncation mutant, Wnt-9aΔ_{288}, increases mesenchymal cell apoptosis. We also present evidence that Frzb inhibits Wnt-9a-induced cell proliferation in avian AV canal cardiac cushions.

Materials and methods

Whole-mount and section in situ hybridizations

Specific cDNAs for chicken Wnt-9a (bp 231–800; GenBank no. AF031168) and chicken Frzb (bp 423–918; GenBank no. AF218057) were isolated by RT-PCR from HH stage 16 AV canals and cloned into pSTBlue-1 (Novagen). Whole-mount in situ hybridization protocols and Digoxigenin (DIG)-labeled riboprobe synthesis for chicken Wnt-9a and Frzb probes were as described (Nieto et al., 1996). Images of whole-mount embryos were captured on a dissecting microscope (Lieca model MZ125) with a digital camera (Nikon Cool Pix 995).

For in situ hybridization on sections, dissected embryos were fixed overnight in a 6:3:1 solution of ethanol/formalin/acetic acid prior to paraffin embedding and sectioning. Slides were briefly treated with proteinase K (20 ug/ml) for 7 min and post-fixed in 4% paraformaldehyde prior to overnight incubation in hybridization buffer containing DIG-labeled riboprobe (250 ng/ml) at 65°C. Slides were washed in SSC/50% formamide/0.1% Tween20 before overnight incubation at 4°C with an anti-DIG antibody (Roche) diluted 1/2000 in MABT (100 mM maleic acid pH 7.5, 150 mM NaCl, 1%Tween 20) with 20% goat serum and 2% blocking reagent (Roche). Slides were washed in MABT prior to color detection with NBT (Roche) and BCIP (Roche) in NTMT buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ and 1% Tween 20). Images were captured on an inverted microscope (Leica) equipped with Normarski Optics.

RCAS production and characterization

A RCASBPA proviral construct expressing full-length Wnt-9a was generously provided by Dr. Cliff Tabin (Hartmann and Tabin, 2001). Based upon published reports of truncated Wnts that function in a dominant-negative manner (Hoppler et al., 1996), a C-terminal truncated Wnt-

9a mutant (Wnt-9aΔ_{288}) was produced by PCR using Expand High Fidelity DNA Polymerase (Roche) using RCASBPA Wnt-9a proviral plasmid as a template with primers (5′CCATCGATTCTAGACCACTGTGG 3′ and 5′CCATCGATTCACAAAAGCTTGGGGAA 3′). *Cla*1 sites engineered into the PCR primers were used to ligate the Wnt-9a C-terminal truncation mutant (Wnt-9aΔ_{288}) into a RCASBPA proviral plasmid. Proviral constructs were transfected with Lipofectamine (Invitrogen) into avian DF-1 fibroblasts and concentrated virus (5×10^8 to 8×10^8 IU/ml) was collected as described (Morgan and Fekete, 1996). Western blot analysis of infected DF-1 cell lysates was performed using an affinity-purified polyclonal Wnt-9a anti-peptide antibody produced in rabbits injected with the following peptide (VCDRLKLEKKQRRMCR).

In vitro RCAS studies

Concentrated RCAS viruses (100 nl) containing full-length Wnt-9a, Wnt-9aΔ_{288}, or no insert control were injected directly into the heart lumen of Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951) stage 11 avian embryos explanted onto egg agar plates (Darnell and Schoenwolf, 2000) using a micromanipulator (Leica). Prior to injection, 1 μl of 0.1% fast green dye was added to 20 μl of each virus for visualization. Embryos were incubated at 37°C to stage 15 prior to AV canal isolation and cultured on hydrated collagen I gels as described (Romano and Runyan, 1999). After 24 h of culture, the number of AV canal mesenchymal cells observed within the collagen gel were manually counted by two independent observers. For each experiment, a minimum of 16 AV canal explants were examined.

Real-time PCR

Total RNA (250 ng) isolated from AV canal explants infected with either RCAS Wnt-9a, RCAS Wnt-9aΔ_{288}, or RCAS control was used in real time RT-PCR reactions with primers for Wnt-9a (F = 5′-CTCAAAGTGGGCAGCAC-TAC-3′; R = 5′-TGGGGAATCGTCAATGTAGA-3′) using the Quantitect SYBR RT-PCR Kit (Qiagen). These Wnt-9a PCR primers hybridize to both endogenous and exogenous Wnt-9a cDNAs. Real-time reactions were each done in triplicate on a Rotogene 3000 Real-Time PCR machine. Wnt-9a amplification was normalized to β-actin amplification for each experimental treatment.

β-catenin TOPFLASH reporter assays

Stage 15 AV canals where isolated from RCAS infected embryos and explanted onto collagen I gels as described above. For these studies, 40 AV canal explants were cultured on a single collagen I gel. TOPFLASH or FOPFLASH (6 μg) (Upstate Biotech) and 0.3 μg of pCMV-RL (Promega) were transfected into AV canal tissues with 5 μl of Lipofectamine (Invitrogen) in 150 μl of Media 199 (Invitrogen) for 2 h at 37°C. AV canal cultures where incubated for an additional 24 h in Media 199 with 1% chick serum and Pen/Strep antibiotics (Invitrogen). Firefly luciferase and Renilla luciferase activities were determined with a Luminomitor (TD-20/20, Turner Designs) using the Dual Luciferase System (Promega).

Xenopus injections

Full-length Wnt-9a and Wnt-9aΔ_{288} were directionally cloned into the T7TS RNA expression vector (Cleaver et al., 1996). Capped mRNAs were synthesized in vitro from *Bam*HI linearized pT7TS-Wnt-9a and pT7TS-Wnt-9aΔ_{288} using the T7 Message Machine in vitro transcription kit (Ambion). XWnt-8 in pSP64T (Christian et al., 1991) and XWnt-5a in pSP64T (Moon et al., 1993) were linearized with *Bam*HI and *Xba*1, respectively, and capped mRNA was synthesized using the SP6 Message Machine in vitro transcription kit (Ambion). Wnt-9a, Wnt-9aΔ_{288}, XWnt-8, or XWnt-5a mRNA were injected into a ventral vegetal blastomere at the four-cell stage. Wnt-9a- and XWnt-8-injected *Xenopus* embryos were examined at stage 27 for duplicated axes. XWnt-5a-injected embryos were scored at stage 15 for gastrulation defects. In situ hybridizations were performed as described (Harland, 1991) on stage 11 embryos using an XNot-1 DIG-labeled riboprobe to mark the dorsal mesoderm.

AV canal cell proliferation and apoptosis in vitro

Stage 15 AV canals infected with RCAS were isolated and explanted onto collagen I gels as described above. After incubation for 48 h, 100 μM of BRDU (BD Biosciences) was added for 5 h before fixation in 4% paraformaldehyde. BRDU incorporation was detected with an anti-BRDU antibody (BD Biosciences) diluted 1:200 and a donkey anti-mouse Texas Red conjugated secondary antibody (Jackson Laboratories) diluted 1:500. For apoptosis determinations, TdT-mediated dUTP Nick-End Labeling (TUNEL) staining was performed using the DeadEnd Fluorometric TUNEL System (Promega) on infected AV canal explants. Nuclei were counterstained with DAPI (10 μg/ml). Collagen gels were mounted on slides and 0.2-μm optical sections were captured using a deconvolution microscope (Deltavision). The percentage of BRDU-positive cells/DAPI-positive cells and TUNEL-positive cells/DAPI-positive cells were counted in 20 explants for each treatment.

Frzb studies

Recombinant Frizzled Related Protein-3 (FRP-3) (R&D Systems), the mouse orthologue of chicken Frzb, was used to treat stage 15 avian AV canals cultured in vitro. AV canals were isolated from either naïve embryos or embryos infected with RCAS Wnt-9a at stage 11 as described above. Mouse

FRP-3 was added to explants at concentrations of 1, 10 or 30 ng/ml in Media 199 with 1% chicken serum and Pen/Strep antibiotic. After 24 h in culture, mesenchymal cells beneath the gel surface were counted as described above.

To specifically target Frzb, antisense oligodeoxynucleotide experiments were performed as described (Romano and Runyan, 1999). Unmodified 16-mer oligodeoxynucleotides (Integrated DNA Technologies) were designed from the chicken Frzb sequence (GenBank no. AF218057) as follows: asFrzb (731–746) ACAGAGGCAGCCGGAG; asFrzb (801–816) AGAGTAACCTGGAGCG; and a reverse compliment control asFrzb (731–746 control) TGTCTCCGTCGGCCTC. Antisense treatments of explanted AV canals were performed as described (Romano and Runyan, 1999; Runyan et al., 1999). After 24 h in culture, mesenchymal cells were counted by two independent observers.

In vivo studies

To assess the effects of Wnt overexpression on AV canal morphogenesis in vivo, 100 nl of RCAS virus was injected into the heart lumen of stage 15 embryos explanted on egg agar. The embryos were then incubated at 37°C for 42 h prior to fixation, embedding, and sectioning. Cell proliferation and viral infection were determined using a monoclonal anti-PCNA (Zymed) 1:200 and a polyclonal anti-retroviral GAG antibody (p27) (Spafas) 1:200, respectively. TUNEL staining was also performed as described above. Goat anti-rabbit (Cy5) (Sigma) and donkey anti-mouse (TR) (Jackson Laboratories) secondary antibodies were used for double-channel immunofluorescence imaging. The percentage of TUNEL-positive mesenchymal cells and viral infected mesenchymal cells showing PCNA staining within the AV endocardial cushions was determined in serial optical sections for Wnt-9a, Wnt-9aΔ_{288}, and control infected embryos on a deconvolution microscope (Deltavision).

To define the long-term consequences of Wnt-9a overexpression on valve development, RCASBPA constructs were injected in ovo into the sinus venosus of windowed eggs at HH stage 11. This approach was required because avian embryos explanted onto egg agar do not develop beyond stage 20 (Darnell and Schoenwolf, 2000). After injections, the eggs were sealed with parafilm and incubated at 37°C until stage 26 or stage 33. Hearts were isolated and gross heart morphology was imaged. Dissected hearts were fixed in 4% paraformaldehyde, embedded and sectioned. Sections were immunostained with a monoclonal anti-GAG antibody (AMV-3C2, Developmental Studies Hybridoma Bank, University of Iowa) diluted 1:5 in PBS blocking buffer (0.1% Triton X-100/10% horse serum). Secondary detection was performed with the Peroxidase MIgG Vectastain Elite ABC kit (Vector Laboratories) and visualized with DAB staining (Vector Laboratories).

Results

Wnt-9a expression is restricted to AV canal endocardial cells during early valve development

Wnt-9a expression is initially detected at Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951) stage 16 in the endocardial cell population of the AV canal cushions (Figs. 1A, D). At stage 20, intense staining for Wnt-9a transcripts is observed in the AV canal endocardium (Figs. 1B, E). Restricted endocardial AV canal expression of Wnt-

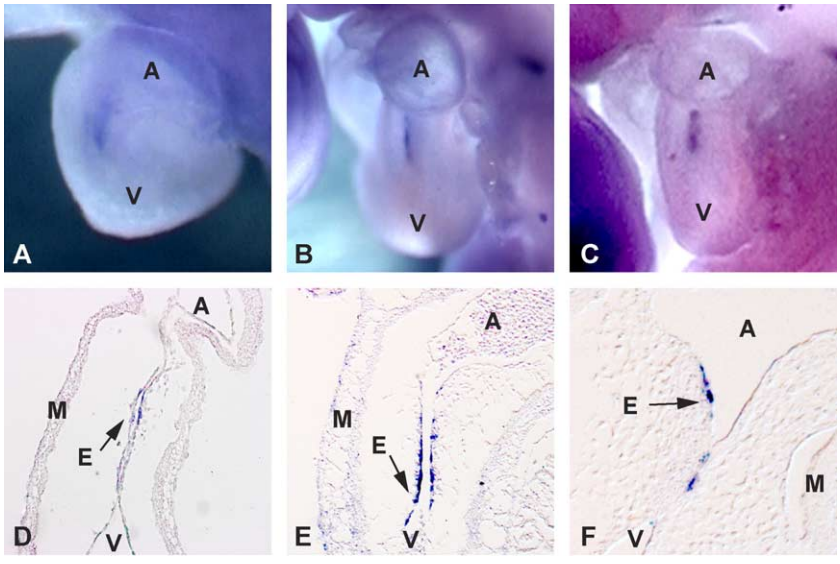

Fig. 1. Expression of Wnt-9a in the AV canal cushion endocardium. Whole-mount in situ hybridizations demonstrating Wnt-9a expression in the AV canal cardiac cushions of chicken hearts at stage 16 (A), stage 20 (B), and stage 24 (C). Section in situ hybridizations of stage 16 (D), stage 20 (E), and stage 24 (F) show Wnt-9a expression localized to the endocardial cell layer (arrows) of the AV canal cardiac cushions. Abbreviations: A = atrium; V = ventricle; M = myocardium; E = endocardium.

9a persists in later stages of heart development when the cushions are extensively populated with mesenchymal cells at stage 24 (Figs. 1C, F). In all stages examined, Wnt-9a expression is not detected in transformed mesenchymal cells of AV canal cardiac cushions or in cardiac myocytes (Figs. 1D–F).

Wnt-9a stimulates cell proliferation in AV cardiac cushions in vitro

To investigate the role of Wnt-9a in AV canal cushion morphogenesis, we used an avian replication competent retrovirus (RCAS) to overexpress Wnt-9a in the heart. Wnt-9aΔ_{288} was generated by truncating the C-terminus of the coding sequence, after the SPSFC motif, similar to reported dominant-negative mWnt-1 and XWnt-8 constructs (Hoppler et al., 1996) (Fig. 2A). Western blot analysis of avian DF-1 fibroblasts infected with RCAS containing either the Wnt-9a or Wnt-9aΔ_{288} proviral constructs confirm expression of a proteins consistent with the predicted sizes of ~45 and ~38 kDa, respectively (Fig. 2B).

To directly test the role of Wnt-9a in AV canal development, RCAS Wnt-9a and RCAS Wnt-9aΔ_{288} were used to infect AV canals. As studies using RCAS demonstrate viral protein detection 18 h after infection (Morgan and Fekete, 1996), virus was injected into the lumen of stage 11 hearts 24 h prior to AV canal EMT. In situ hybridization detected extensive infection of RCAS

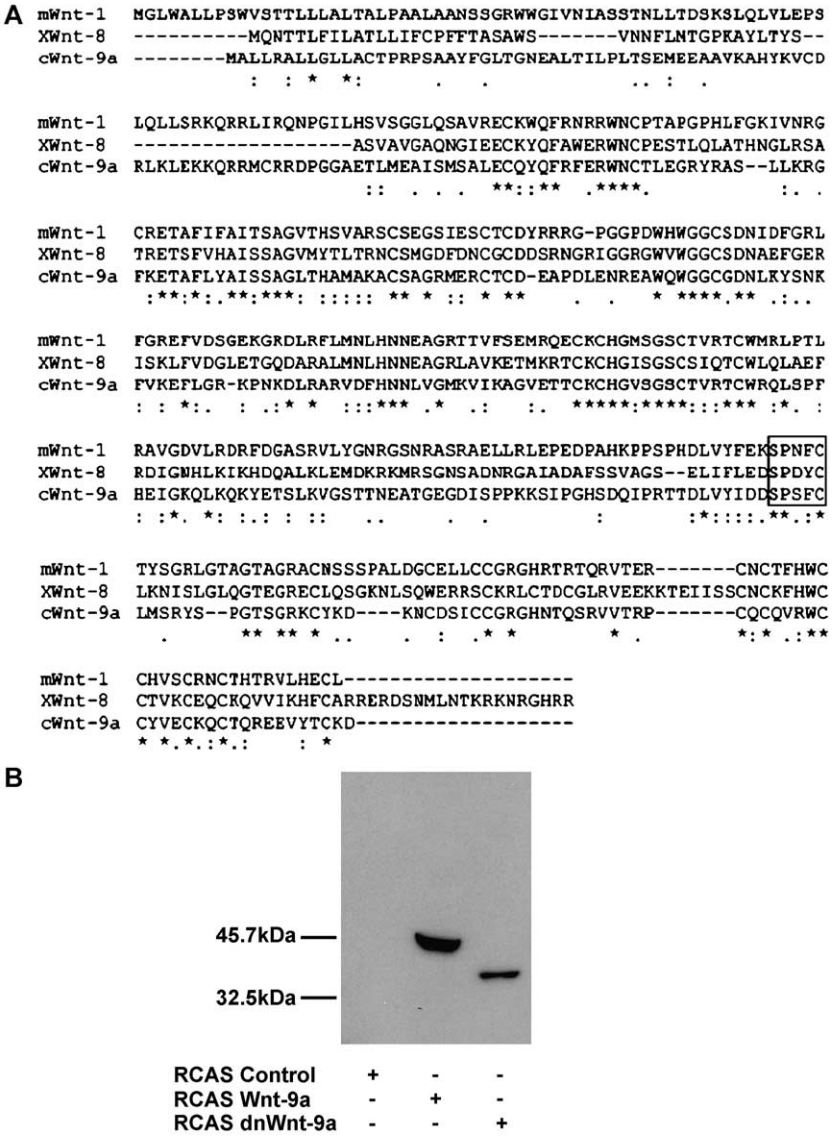

Fig. 2. Production and characterization of RCAS Wnt-9a and Wnt-9aΔ_{288}. (A) Alignment of mWnt-1 and XWnt-8 with chicken Wnt-9a nucleotide sequences. C-terminal truncations of both mWnt-1 and XWnt-8 after the SPXXC motif (box) create Wnts that function as dominant-negative inhibitors (Hoppler et al., 1996). A similar 288 amino acid avian Wnt-9a construct (Wnt-9aΔ_{288}) was generated by truncating the C-terminal 66 amino acids after the SPSFC domain. (B) Western blot analysis of DF-1 cell lysates infected with RCAS Wnt-9a and Wnt-9aΔ_{288} viruses detect proteins of ~45 and ~38 kDa, respectively. Wnt-9a is not detected in DF-1 cells infected with RCAS control virus.

virus throughout the heart at stage 15 (data not shown). At stage 15, the AV canals of infected embryos were explanted onto collagen I gels to assay for mesenchymal cell formation as described (Romano and Runyan, 1999). AV canal explants from RCAS Wnt-9a-injected hearts displayed >100% increase in mesenchymal cell number compared to controls (Fig. 3A). Infection of AV canals with RCAS Wnt-9aΔ_{288} attenuated mesenchymal cell formation resulting in a 73% reduction in migratory mesenchymal cells compared to controls (Fig. 3A). This approach for RCAS delivery of Wnt constructs resulted in >6-fold transgene expression above endogenous AV canal Wnt-9a detected in RCAS control infected AV canals as assayed by real-time PCR (Fig. 3B).

Several studies report that Wnts promote cell proliferation during development (Kioussi et al., 2002; Reya et al., 2000; Shu et al., 2002). AV canals infected with RCAS Wnt-9a displayed a significant increase in BRDU incorporation compared to control AV canals (Fig. 3C). In contrast, Wnt-9aΔ_{288} did not alter BRDU incorporation (Fig. 3C). Thus, increased cushion mesenchymal cells observed in Wnt-9a overexpression studies are due, at least in part, to increased cell proliferation. Studies in tumor cells have shown that Wnts can promote cell survival (Chen et al., 2001; Longo et al., 2002; You et al., 2002) and inhibiting Wnt signaling leads to programmed cell death (He et al., 2004). In AV canal cultures, Wnt-9aΔ_{288} overexpression results in a significant increase in TUNEL-positive cells compared to control or Wnt-9a-infected AV canals (Fig. 3D). Wnt-9a-infected AV canals show a reduction in TUNEL-positive cells compared to controls (Fig. 3D).

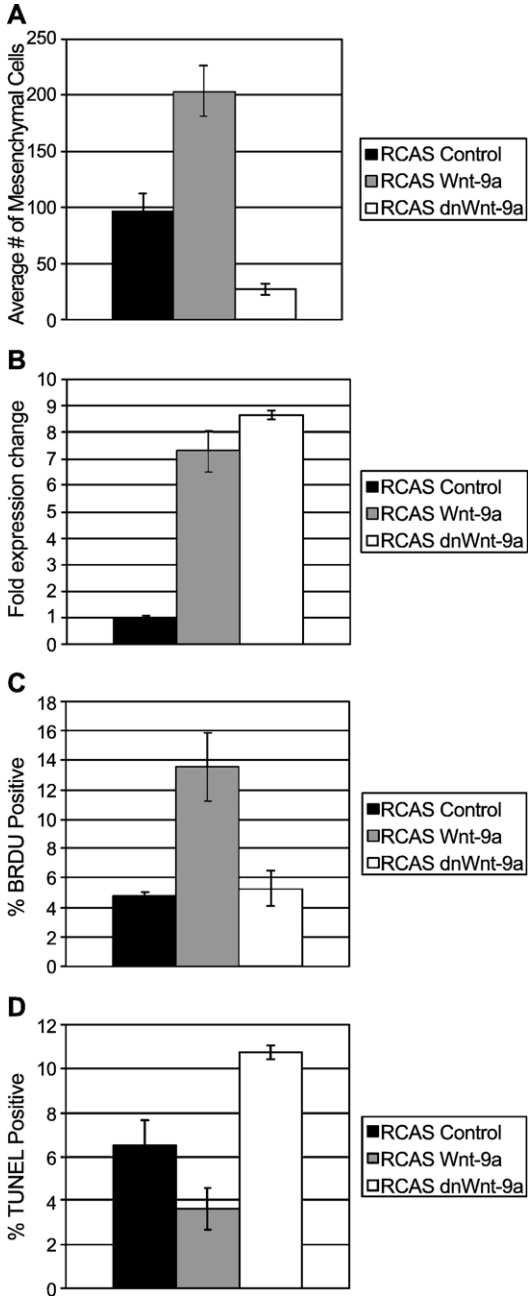

Fig. 3. Wnt-9a and Wnt-9aΔ_{288} affect cell proliferation and apoptosis in AV canal cultures. (A) Infections of AV canal cultures with RCAS Wnt-9a results in a significant increase in migratory mesenchymal cells compared to RCAS control infected AV canal cultures. Infection of AV canal cultures with RCAS Wnt-9aΔ_{288} results in a decrease in migratory mesenchymal cells compared to RCAS control infected AV canal cultures. (B) Real-time PCR data showing infections of AV canal cultures with RCAS Wnt-9a and RCAS Wnt-9aΔ_{288} resulting in a 6.3- and 7.7-fold expression increase respectively over endogenous Wnt-9a in RCAS controls. (C) AV canal cultures infected with RCAS Wnt-9a show an increase in BRDU incorporation compared to RCAS control infected AV canal cultures. RCAS Wnt-9aΔ_{288}-infected cultures did not show differences in BRDU incorporation compared to controls. (D) Wnt-9a overexpression in AV canal explants resulted in decreased TUNEL staining compared to RCAS controls. Overexpression of Wnt-9aΔ_{288} results in an increase in TUNEL staining compared to controls (D). All experiments consisted of at least 16 AV canal explants and were performed in triplicate.

Wnt-9a activates β-catenin signaling

The TOPFLASH/FOPFLASH β-catenin reporter system was used to address whether the Wnt-9a-mediated increase in cell proliferation during AV canal cushion morphogenesis is due to β-catenin activation. This TOPFLASH/FOPFLASH reporter system has been used extensively to assay for β-catenin activity in a variety of systems (Liu et al., 2003; Nakamura et al., 2003; van de Wetering et al., 1997). The TOPFLASH reporter contains three copies of the optimal T-Cell Factor (TCF) transcription factor binding motif upstream of a minimal c-Fos promoter driving luciferase expression. The FOPFLASH reporter has mutated TCF binding sites and does not respond to β-catenin activity (van de Wetering et al., 1997). AV canal explants infected with RCAS Wnt-9a show a 4.6-fold activation of the TOPFLASH reporter compared to the negative control FOPFLASH reporter with mutated TCF binding sites (Fig. 4A).

Injection of Wnt mRNA into a ventral vegetal blastomere of *Xenopus* embryos at the 4-cell stage is an established method to classify Wnts as canonical or noncanonical. In this assay, early ventral injections of Wnts that increase β-catenin stability cause axis duplications (Sokol et al., 1991). Wnt-9a

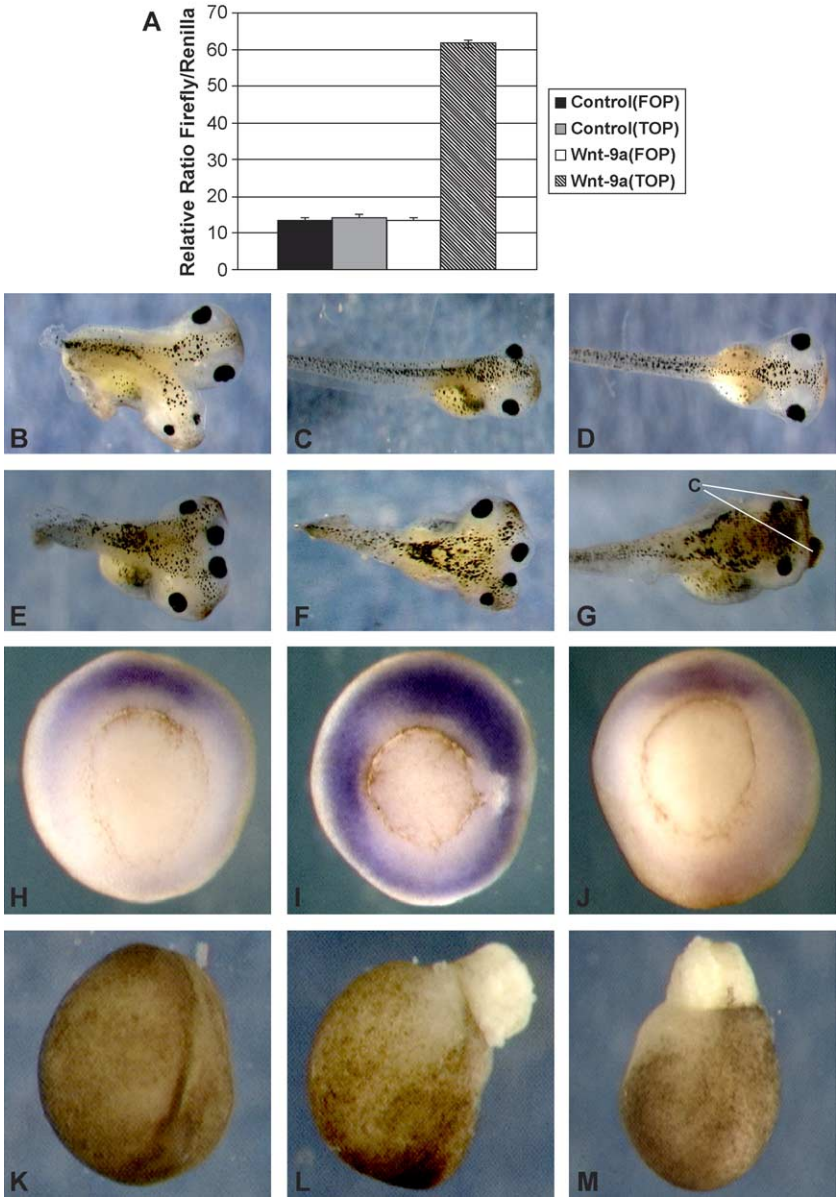

Fig. 4. Wnt-9a activates β-catenin in AV canals and causes axis duplications in *Xenopus* embryos. (A) AV canal explants infected with RCAS Wnt-9a activate the β-catenin responsive TOPFLASH reporter but not the FOPFLASH reporter containing mutated TCF binding sites. Infection of AV canal explants with RCAS control virus does not activate the TOPFLASH or FOPFLASH reporter. (B) Wnt-9a mRNA (50 pg) injected into a ventral blastomere of four-cell *Xenopus* embryos causes duplication of the embryonic axis. Co-injecting 50 pg of Wnt-9a with 200 pg (C) or 500 pg (D) of Wnt-9aΔ_{288} rescues axis duplications. Injection of 50 pg of XWnt-8 also results in axis duplications (E) that are not rescued with co-injection with 200 pg of Wnt-9aΔ_{288} (F). Co-injection of Xwnt-8 with 500 pg of Wnt-9aΔ_{288} partially rescues XWnt-8-induced twinning as two cement glands are still evident (G). Uninjected embryos showing Xnot-1 expression in the mesoderm overlying the dorsal blastopore lip (H). Stage 11 embryos injected with 50 pg of Wnt-9a mRNA show ectopic Xnot-1 expression consistent with secondary axis formation (I). This ectopic Xnot-1 expression is no longer detected in embryos co-injected with Wnt-9a (50 pg) and Wnt-9aΔ_{288} (500 pg) (J). Uninjected control embryos at stage 15 undergoing normal gastrulation (K). Ventral injection of XWnt-5a inhibits convergent extension creating gastrulation defects (L). These gastrulation defects caused by XWnt-5a injections are not rescued by co-injection with 500 pg of Wnt-9aΔ_{288} (M). Abbreviation: C = cement gland.

mRNA injection of 50 pg results in axis duplications in a significant number of embryos (Fig. 4B and Table 1). Co-injection of 200 pg of Wnt-9aΔ_{288} with 50 pg of Wnt-9a reduces duplicated axes to near background levels (Fig. 4C and Table 1). This low level of axis duplication is not further reduced by co-injecting 500 pg of Wnt-9aΔ_{288} (Fig. 4D and Table 1).

The dorsal mesoderm marker Xnot-1 is normally expressed in uninjected embryos adjacent to the blastopore lip of gastrulating stage 11 embryos (Fig. 4H). Xnot-1 is expressed ectopically in Wnt-9a-injected embryos consistent with double-axis formation (Fig. 4I). In contrast, localized Xnot-1 expression is only detected directly above the blastopore lip in Wnt-9a/Wnt-9aΔ_{288}

Table 1
Percentage of *Xenopus* embryos with duplicated axes or defective gastrulation produced by ventral injection of various Wnts in the presence or absence of Wnt-9aΔ_{288}

	Ventral injection of Wnt mRNA	Wnt-9aΔ_{288} co-injection	
		(200 pg)	(500 pg)
Wnt-9a (50 pg)[a]	40.3% (n = 109)	2.7% (n = 37)	2.8% (n = 107)
XWnt-8 (50 pg)[a,b]	69.2% (n = 39)	61.7% (n = 47)	29.7% (n = 37)
Uninjected[a]	0.0% (n = 52)	0.0% (n = 49)[c]	0.0% (n = 40)[d]
XWnt-5a (50 pg)[e,f]	70.0% (n = 30)	85.0% (n = 48)	81.0% (n = 32)
Uninjected[e]	0.0% (n = 35)		

[a] Embryos scored for duplicated axes at stage 27.
[b] Canonical Wnt previously demonstrated to cause axis duplications (Christian et al., 1991).
[c] 200 pg of Wnt-9aΔ_{288} injected alone.
[d] 500 pg of Wnt-9aΔ_{288} injected alone.
[e] Embryos scored for defective gastrulation at stage 15.
[f] Noncanonical Wnt previously demonstrated to inhibit convergent extension (Moon et al., 1993).

co-injected embryos (Fig. 4J). These results argue that Wnt-9a activates a β-catenin signaling pathway and that Wnt-9aΔ_{288} functions as a dominant-negative inhibitor of Wnt-9a.

The specificity of Wnt-9aΔ_{288} for Wnt-9a was further examined by co-injection with XWnt-8 or XWnt-5a. XWnt-8 efficiently produces duplicated axes when injected ventrally in *Xenopus* embryos (Christian et al., 1991). Injections of 50 pg of XWnt-8 mRNA results in a majority of embryos with duplicated axes (Fig. 4E and Table 1). Co-injection of 50 pg of XWnt-8 with 200 pg of Wnt-9aΔ_{288} mildly reduces the number of axis duplications, and co-injection with 500 pg of Wnt-9aΔ_{288} reduces axis duplications in half (Figs. 4F, G and Table 1). Therefore, Wnt-9aΔ_{288} can inhibit XWnt-8 function but not as efficiently as it inhibits Wnt-9a function. Ventral injection of XWnt-5a does not result in axis duplication (Moon et al., 1993), but inhibits convergent extension resulting in gastrulation defects (Du et al., 1995; Moon et al., 1993). XWnt-5a (50 pg) injected into a ventral blastomere of 4-cell *Xenopus* embryos results in severe gastrulation defects in 70% of embryos (Fig. 4L and Table 1). Co-injection of either 200 or 500 pg of Wnt-9aΔ_{288} with 50 pg of XWnt-5a does not inhibit XWnt-5a-induced gastulation defects (Fig. 4M and Table 1). We conclude that Wnt-9aΔ_{288} inhibits Wnt-9a and to a lesser extent XWnt-8, but does not inhibit non-canonical XWnt-5a signaling.

Frzb expression in the AV canal endocardial cushions

Expression of secreted Wnt antagonist, Frzb, was reported in the AV canal endocardium and transformed mesenchymal cells of stage 24 AV endocardial cushions (Ladher et al., 2000). To determine if Frzb is expressed during earlier stages of avian endocardial cushion morphogenesis, in situ hybridizations were performed from stages 16 through stage 24 (Fig. 5). Expression of Frzb at stage 16 is primarily seen in the AV canal endocardial cell population (Figs. 5A, D). Newly formed mesenchymal cells also show expression at stage 16 (Fig. 5D). By stage 20 (Figs. 5B, E) and stage 24 (Figs. 5C, F), expression of Frzb remains restricted to the endocardial cushions. However, in contrast to continued Wnt-9a expression limited to AV canal endocardial cells (Fig. 1), Frzb is expressed by endocardial and mesenchymal cells of the AV canal during early cushion development (Fig. 5).

Frzb inhibits Wnt-9a-induced AV cushion mesenchyme proliferation

The addition of recombinant Frizzled Related Protein-3 (FRP-3), the mouse orthologue of chicken Frzb, to AV canal explant cultures results in a reduction in mesenchymal cells (Fig. 6A). FRP-3 at 10 or 30 ng/ml results in a similar ~60% reduction in mesenchymal cell number arguing that doses of 10 ng/ml saturate Wnt activity in this culture system (Fig. 6A). To further investigate Frzb function, Frzb antisense oligodeoxynucleotides were used to perturb Frzb in AV canal explants. This approach has proven effective in the study of other factors in the AV canal explant system (Potts et al., 1991; Romano and Runyan, 1999). Consistent with Frzb functioning as a negative regulator of Wnt signaling, targeted degradation of Frzb mRNA with two independent antisense oligos resulted in amplified mesenchymal cell numbers in AV canal explants (Fig. 6B).

To test if Frzb inhibits Wnt-9a signaling during AV canal development, Wnt-9a-infected AV canals were treated with exogenous FRP-3. FRP-3 offset the Wnt-9a-mediated increase in cell proliferation in a dose-dependent manner (Fig. 6C). These data suggest that Frzb acts to modulate Wnt-9a-induced mesenchyme cell proliferation in the developing AV canal.

Overexpression of Wnt-9a and Wnt-9aΔ_{288} in vivo

Cell proliferation and apoptosis were examined in the developing AV endocardial cushions of avian embryos at stage 18 following injection with RCAS containing either full-length Wnt-9a or dominant-negative Wnt-9aΔ_{288} constructs. At stage 18, Wnt-9a overexpressing endocardial cushions are larger in size with more mesenchymal cells compared to controls (Fig. 7B). Wnt-9a-infected cushions contained 112 ± 11 cells compared to 46 ± 5 mesenchymal cells in control hearts. Stage 18 AV endocardial cushions infected with Wnt-9aΔ_{288} displayed 35 ± 5 cells. The percentage of viral-infected mesenchymal cells under-

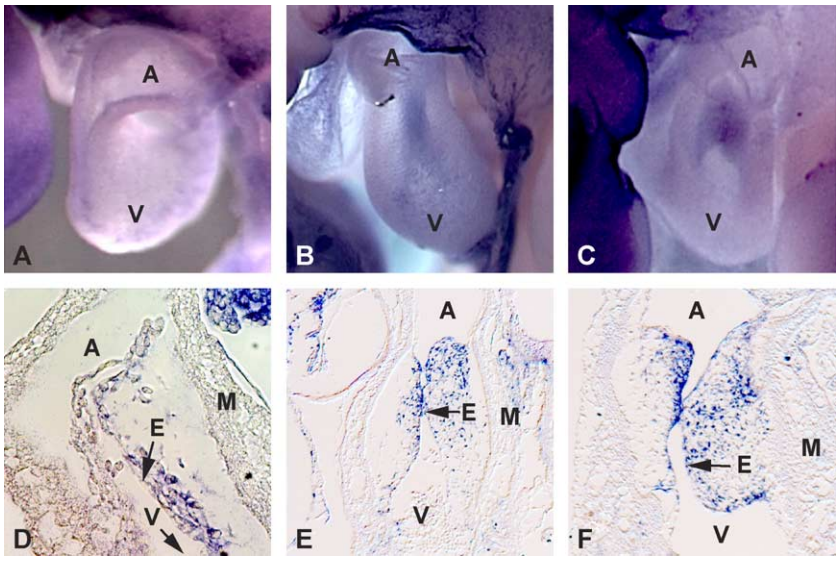

Fig. 5. Frzb is expressed in the endocardial and mesenchymal cells of the AV canal cushions. Whole-mount in situ hybridizations show Frzb expression in the AV canal cushions at HH stage 16 (A), stage 20 (B), and stage 24 (C). In situ hybridization analysis of sections reveals Frzb is expressed in the endocardium and transformed mesenchyme of AV canal cushions at stage 16 (D), stage 20 (E), and stage 24 (F). Frzb expression is absent from the myocardium at all stages examined. Abbreviations: A = atrium; V = ventricle; E = endocardium; M = myocardium.

going cell proliferation, identified by anti-viral and anti-PCNA co-immunolocalization, was 29.1% in RCAS Wnt-9a-infected hearts (n = 5), 16.9% in RCAS Wnt-9aΔ_{288}-infected hearts (n = 5), and 14.4% in controls (n = 5). PCNA immunostaining also appeared more pronounced in the endocardial cell layer of hearts infected with RCAS Wnt-9a (Fig. 7B). Consistent with AV canal explant results, in vivo TUNEL staining of infected cushions reveals increased TUNEL-positive cells in dominant-negative Wnt-9aΔ_{288}-infected cushions (9.8%) compared to controls (5.4%). Cushions from Wnt-9a overexpressing hearts displayed only (4.4%) TUNEL-positive mesenchymal cells (Figs. 7D–F).

RCAS injections were performed in ovo to determine the effects of Wnt-9a perturbation on later stages of cardiac valve development. Wnt-9a overexpression caused a reproducible phenotype with expanded, hyperproliferative cushions that created an elongated inlet between the atrium and ventricle at HH stage 26 (Figs. 7K, L). While control hearts display single endocardial cushion luminal projections at stage 26, Wnt-9a-infected cushions have multiple ectopic projections (Figs. 7H, L). Wnt-9a-infected hearts have massive atrial enlargement and atrial blood pooling, consistent with significant AV inlet stenosis (Fig. 7K). By stage 33, the AV canal normally forms separate mitral and tricuspid inlets (Figs. 7I, J). In contrast, hearts infected with dominant-negative Wnt-9aΔ_{288} exhibit arrested development and maintain a single AV junction at stage 33 (Fig. 7M). Dominant-negative Wnt-9aΔ_{288} hearts at this stage display hypocellular endocardial cushions, delayed cardiac looping, and severe cushion hypoplasia (Figs. 7M, N).

Discussion

Cardiac cushion morphogenesis encompasses several steps leading to formation of mature valvular and septal structures. After the bilateral heart primordia in the lateral plate mesoderm coalesce to form a tubular heart, regional increases in extracellular matrix secretion at the AV and outflow tract regions results in the formation of cardiac cushions. Endocardial cells within these cardiac cushions then receive inductive signals from the myocardium leading to the formation of migratory mesenchymal cells that populate the cardiac jelly (Runyan and Markwald, 1983). Subsequent morphogenic events include directional cushion elongation and eventual formation of mature valve leaflets as well as completion of chamber septation. The molecular mechanisms of later cushion outgrowth and remodeling are just beginning to be understood.

In this study, we demonstrate that Wnt-9a directs cell proliferation in the developing avian cardiac AV endocardial cushions. Viral-mediated overexpression of Wnt-9a in vitro and in vivo supports this. Wnt-9a overexpression increases cell proliferation and mesenchymal cell number in the AV canal explant system, and results in enlarged, hyperproliferative cushions in ovo (Fig. 7). Cushion formation is not detected outside the normal cushion forming regions, arguing that Wnt-9a overexpression is not sufficient to induce ectopic EMT. In addition, a dominant-negative Wnt-9a does not completely inhibit mesenchyme formation in cardiac cushions (Fig. 7); rather, Wnt-9aΔ_{288} increases apoptosis leading to a reduction in cushion mesenchyme (Fig. 7). These data suggest that Wnt-9a is not a critical stimulus for the change in endothelial to mesenchymal phenotype in the developing avian AV canal, but rather is an

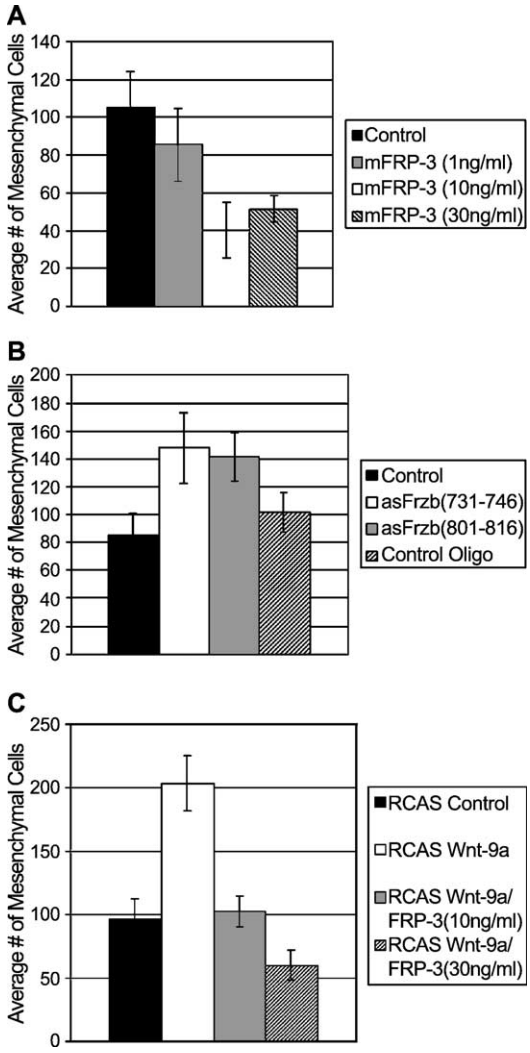

Fig. 6. Frzb is a dose-dependent negative regulator of Wnt-9a-mediated cell proliferation. (A) Addition of recombinant FRP-3, the mouse orthologue of chicken Frzb, results in a decrease in mesenchymal cell number in AV canal cultures. (B) Treatments of AV canal explants with two distinct Frzb antisense oligodeoxynucleotides result in an increase in mesenchymal cell number compared to controls (Lipofectamine alone and control oligo treatments). (C) Infection of AV canal cultures with RCAS Wnt-9a results in an increase in mesenchymal cell number while treating RCAS Wnt-9a-infected cultures with FRP-3 at 10 ng/ml reduces mesenchymal levels to those seen in RCAS control infected cultures. FRP-3 (30 ng/ml) further decreases mesenchymal cell number in RCAS Wnt-9a-infected cultures (C).

important regulator of the expansion of endocardial cushion endothelial and mesenchymal cell populations that are critical for appropriate growth and remodeling of the AV valves.

Previous studies have not clearly resolved the mechanism of Wnt-9a signal transduction. Wnt-9a (formerly Wnt-14) functions prominently to initiate synovial joint formation, but overexpression of activated versions of β-catenin, RhoA, CamKII, or a dominant negative Dishevelled fail to phenocopy Wnt-9a overexpression during limb formation. These data caused the authors to speculate that Wnt-9a may transduce intracellular signaling by a novel mechanism (Hartmann and Tabin, 2001). We provide direct evidence that Wnt-9a activates β-catenin responsive transcription in AV canal cells using a β-catenin reporter assay (Fig. 4). In addition, Wnt-9a induces axis duplications in *Xenopus* embryos further arguing that Wnt-9a signals via a Wnt/β-catenin pathway. Taken together, our data suggest that Wnt-9a mediates β-catenin activity in the endocardial cell layer of the avian AV canal cushions, but we cannot exclude a role for additional related Wnts. In a survey of Wnt isoform expression in the AV canal, Wnt-9a is the predominant Wnt isoform expressed during cushion formation, but Wnt-6 is expressed in a subset of myocardial cells in the AV canal (Person et al., in preparation).

The novel C-terminal truncation Wnt-9a mutant used in this study proved useful to perturb Wnt-9a function during avian endocardial cushion morphogenesis. The Wnt-9a truncation after the SPXXC motif is similar to XWnt-8 and mouse Wnt-1 dominant negatives used in a previously published report (Hoppler et al., 1996). The Wnt-9a truncation effectively inhibits Wnt-9a-induced twinning in *Xenopus* embryos and Wnt-9a function during avian valve development in an apparent dominant negative manner. While the Wnt-9aΔ_{288} construct abolished Wnt-9a effects on axis duplication in *Xenopus* embryos, results with XWnt-8 and Wnt-9aΔ_{288} co-injection suggest this truncation mutant has some potential to affect other Wnt/β-catenin pathways.

Our results in an embryonic chicken model are consistent with reports in zebrafish mutants lacking the intracellular β-catenin antagonist APC. Zebrafish APC mutants display expanded expression domains of cushion markers (Has-2, Versican, BMP-2 and Notch-1b), consistent with enlarged cardiac cushions (Hurlstone et al., 2003). However, cardiac mesenchymal cells are not obvious in areas outside the normal cushion forming regions in zebrafish APC mutants (Hurlstone et al., 2003), suggesting that activated β-catenin is necessary for cell proliferation but is likely not sufficient to induce ectopic EMT. A similar increase in cell proliferation rather than cell transformation per se is observed in human melanoma cell lines upon reducing APC expression levels (Worm et al., 2004).

Frzb is the founding member of a larger group of secreted frizzled related proteins (sFRPs) that share sequence homology with frizzled transmembrane receptors. FRPs and Wnts are often expressed in overlapping regions and FRPs are thought to delineate Wnt signaling boundaries during development (Jones and Jomary, 2002). The restricted AV canal expression of Frzb and the expansion of transformed mesenchymal cells following Frzb antisense oligo targeting in AV canal explants suggests Frzb is a negative regulator of avian endocardial cushion morphogenesis. Although inhibition of Wnt-9a-mediated cell proliferation in avian AV canal explants supplemented with the Frzb orthologue FRP-3 suggests a direct interaction between these factors, FRP-3 inhibition of other endocardial cushion Wnts, including Wnt-6 (Schubert et al., 2002), cannot be excluded.

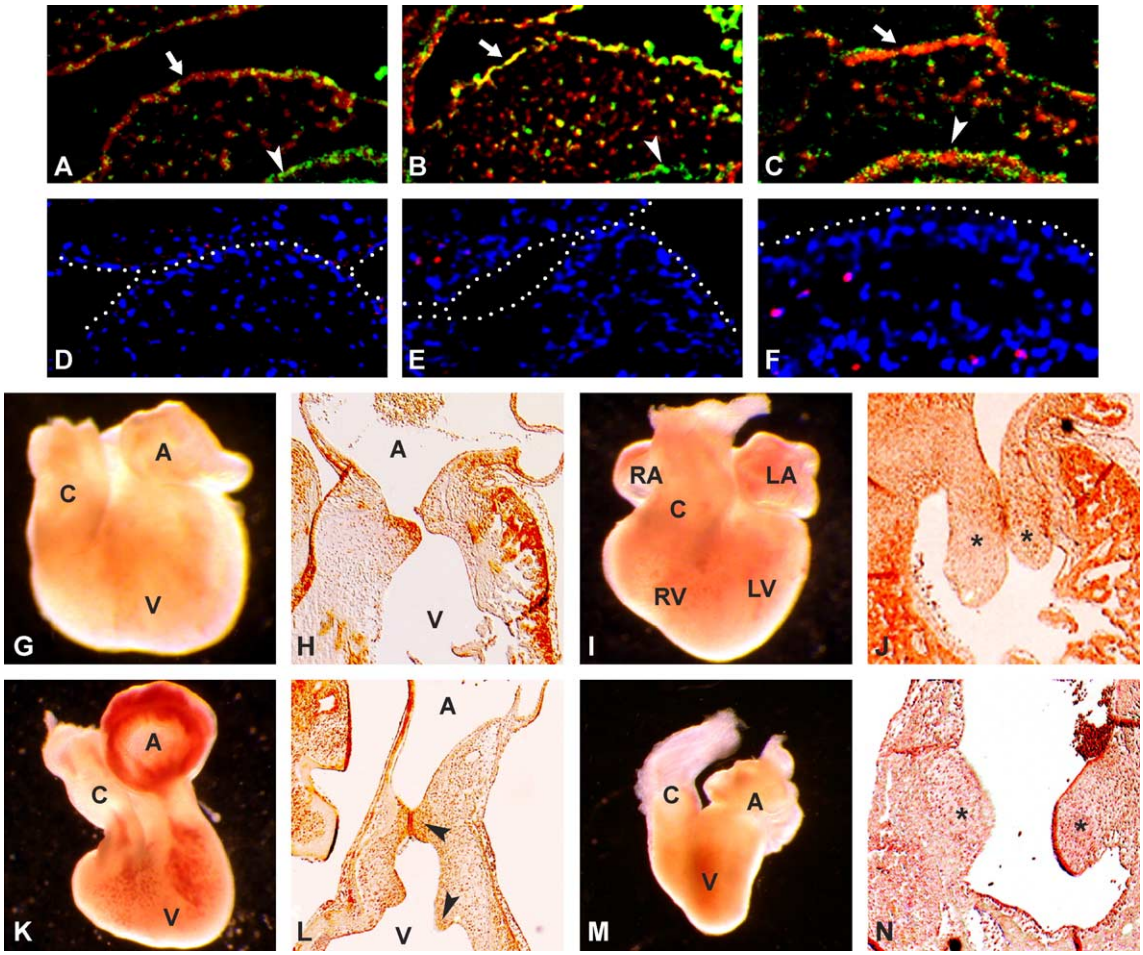

Fig. 7. Wnt-9a and Wnt-9aΔ_{288} affect cell proliferation, apoptosis, and cushion morphology in vivo. (A–F) Analysis of stage 18 AV endocardial cushions following Wnt-9a perturbations. (A–C) RCAS infection (anti-p27) is shown in red and PCNA immunostaining is shown in green. Co-expressing cells are yellow. Wnt-9a-infected AV cushions show a pronounced increase in PCNA staining in both infected endocardial and mesenchymal cells compared to control and Wnt-9aΔ_{288}-infected hearts. Arrows indicate the endocardium while arrowheads point to the myocardium of the AV canal junction. (D–F) TUNEL staining (seen as red) is increased in cushion mesenchymal cells of Wnt-9aΔ_{288}-infected hearts compared to control and Wnt-9a-infected hearts. The endocardial cell layer is marked by a white dotted line. (G–L) Heart valve remodeling defects following Wnt-9a overexpression. (K) Wnt-9a-infected hearts at stage 26 display an elongated AV inlet and an enlarged atrium with blood pooling. (L) Wnt-9a-treated AV cushions are enlarged, hyperplastic, and display multiple AV cushion projections into the lumen (arrowheads). RCAS Wnt-9aΔ_{288}-infected hearts, at stage 33, display looping abnormalities and a single AV inlet with hypoplastic AV cushions (M, N). Viral infection is detected with anti-GAG immunostaining shown in red (H, J, L, N). Abbreviations: A = atrium; V = ventricle; C = conus arteriosus; RA = right ventricle; LA = left ventricle; RA = right atrium; LA = left atrium.

Our Frzb results are consistent with inhibition of cardiac cushion formation following injections of Dkk-1 mRNA in zebrafish embryos (Hurlstone et al., 2003). Although expression of Dkk-1 and Dkk-3 are reported in the AV canal endocardial cushions of mouse hearts at E12.5 p.c. (Monaghan et al., 1999), it is not clear if Dkk family members are expressed in the AV canal during the earlier stages of cushion morphogenesis investigated in the current study. Similar to Frzb, Dkk-1 antagonizes canonical Wnt signaling in several developmental contexts (Fedi et al., 1999; Glinka et al., 1998; Semenov et al., 2001). It is possible that both Frzb and Dkk-1 regulate Wnt-9a-mediated cell proliferation in developing avian AV cushions. Further investigations are needed to investigate potential interplay between Wnt-9a, Frzb, Dkk-1, and other Wnt signaling components during cardiac cushion development.

The endocardial cushion extracellular environment is likely critical for directing cell proliferation and later valve leaflet remodeling. Our data are consistent with a model whereby Wnt-9a secreted from AV endocardial cells creates a Wnt-9a concentration gradient supporting cell proliferation in proximity to the endocardium. The expression of Frzb by mesenchymal cells in the cardiac cushions likely plays a role in localizing cell proliferation to the proximity of the endocardial cell layer promoting directional luminal cushion outgrowth. Invasive cushion mesenchymal cells are likely exposed to decreasing Wnt-9a stimulation in the setting of persistent Frzb modulation as they migrate into cardiac cushions. Frzb inhibition of Wnt signaling within the cardiac cushions may promote differentiation of mesenchymal cells already populating the cardiac jelly leading to mature valve leaflet formation.

There is a growing appreciation for molecular and morphologic differences in heart valve formation between chickens and mice (Camenisch et al., 2002). To explore a potential role for Wnt-9a and Frzb in mouse AV endocardial cushion morphogenesis, the spatial expression patterns for these factors were examined by in situ hybridization. Consistent with published data demonstrating no significant expression of Frzb in the developing mouse heart (Borello et al., 1999), the embryonic mouse cardiac cushions do not express significant Frzb or Wnt-9a (data not shown). Thus, Wnt-9a/Frzb regulation of AV endocardial cushion cell proliferation does not appear to be functioning during mouse development. Other Wnts and Wnt inhibitors (Dkks) are likely responsible for regulating cell proliferation in expanding endocardial cushions of mammals.

It is becoming clear that endocardial cushion morphogenesis is dependent on multiple molecular cascades (Akiyama et al., 2004). Our data strongly suggest that Wnt-9a activates β-catenin signaling within AV canal cushions, and that Wnt-9a activity is regulated my Frzb. Important next steps in understanding Wnt-9a and Frzb function during endocardial cushion morphogenesis will include identification of Frizzled receptors that partner with Wnt-9a along with defining the influences of Wnts expressed in other cell types within the developing heart. In addition, defining downstream effectors of Wnt-9a within AV canal cardiac cushions will be necessary to integrate Wnt signaling with the rapidly expanding number of genes regulating heart valve development (Barnett and Desgrosellier, 2003).

In summary, we demonstrate interrelated functional roles for Wnt-9a and the secreted Wnt antagonist Frzb during avian AV canal cushion morphogenesis. Wnt-9a overexpression in ovo results in hyperplastic cushions and AV canal obstruction, while dominant-negative Wnt-9a causes hypoplastic AV canal cushions and arrested cardiac development. These data suggest an important role for Wnt-9a and Frzb in the proper remodeling of AV canal cushions into mature septal and valvular structures.

Acknowledgments

We would like to thank Dr. Ian N. Jongewaard for his invaluable training and mentoring of young scientists. We would also like to thank Dr. Cliff Tabin who generously provided the RCASBPA Wnt-9a proviral construct and Dr. Stephen Ekker who provided the XWnt-8 and XWnt-5a constructs. We also thank Drs. Robert Erickson, Todd Camenisch and Simon Conway for providing us with mouse embryos for in situ comparisons. This work was supported by American Heart Association Predoctoral Fellowship # 0215182Z and projects #1 and #3 of a National Institutes of Health Program Project Grant # 5P01HL063926-04.

References

Abdelwahid, E., Pelliniemi, L.J., Jokinen, E., 2002. Cell death and differentiation in the development of the endocardial cushion of the embryonic heart. Microsc. Res. Tech. 58, 395–403.

Akiyama, H., Chaboissier, M.C., Behringer, R.R., Rowitch, D.H., Schedl, A., Epstein, J.A., De Crombrugghe, B., 2004. Essential role of Sox9 in the pathway that controls formation of cardiac valves and septa. Proc. Natl. Acad. Sci. U. S. A. 101, 6502–6507.

Barnett, J.V., Desgrosellier, J.S., 2003. Early events in valvulogenesis: a signaling perspective. Birth Defects Res. Part C, Embryo Today 69, 58–72.

Borello, U., Coletta, M., Tajbakhsh, S., Leyns, L., De Robertis, E.M., Buckingham, M., Cossu, G., 1999. Transplacental delivery of the Wnt antagonist Frzb1 inhibits development of caudal paraxial mesoderm and skeletal myogenesis in mouse embryos. Development 126, 4247–4255.

Brown, C.B., Boyer, A.S., Runyan, R.B., Barnett, J.V., 1996. Antibodies to the Type II TGFbeta receptor block cell activation and migration during atrioventricular cushion transformation in the heart. Dev. Biol. 174, 248–257.

Brown, C.B., Boyer, A.S., Runyan, R.B., Barnett, J.V., 1999. Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. Science 283, 2080–2082.

Camenisch, T.D., Spicer, A.P., Brehm-Gibson, T., Biesterfeldt, J., Augustine, M.L., Calabro Jr., A., Kubalak, S., Klewer, S.E., McDonald, J.A., 2000. Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme [see comment]. J. Clin. Invest. 106, 349–360.

Camenisch, T.D., Molin, D.G., Person, A., Runyan, R.B., Gittenberger-de Groot, A.C., McDonald, J.A., Klewer, S.E., 2002. Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. Dev. Biol. 248, 170–181.

Chen, S., Guttridge, D.C., You, Z., Zhang, Z., Fribley, A., Mayo, M.W., Kitajewski, J., Wang, C.Y., 2001. Wnt-1 signaling inhibits apoptosis by activating beta-catenin/T cell factor-mediated transcription. J. Cell Biol. 152, 87–96.

Christian, J.L., McMahon, J.A., McMahon, A.P., Moon, R.T., 1991. Xwnt-8, a *Xenopus* Wnt-1/int-1-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. Development 111, 1045–1055.

Cleaver, O.B., Patterson, K.D., Krieg, P.A., 1996. Overexpression of the tinman-related genes XNkx-2.5 and XNkx-2.3 in *Xenopus* embryos results in myocardial hyperplasia. Development 122, 3549–3556.

Darnell, D.K., Schoenwolf, G.C., 2000. Culture of avian embryos. Methods Mol. Biol. 135, 31–38.

De la Cruz, M.V., Markwald, R.R., 1998. Living Morphogenesis of the Heart. Birkhäuser, Boston.

De la Cruz, M.V., Sanchez Gomez, C., Arteaga, M.M., Arguello, C., 1977. Experimental study of the development of the truncus and the conus in the chick embryo. J. Anat. 123, 661–686.

De la Cruz, M.V., Gimenez-Ribotta, M., Saravalli, O., Cayre, R., 1983. The contribution of the inferior endocardial cushion of the atrioventricular canal to cardiac septation and to the development of the atrioventricular valves: study in the chick embryo. Am. J. Anat. 166, 63–72.

de la Pompa, J.L., Timmerman, L.A., Takimoto, H., Yoshida, H., Elia, A.J., Samper, E., Potter, J., Wakeham, A., Marengere, L., Langille, B.L., Crabtree, G.R., Mak, T.W., 1998. Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. Nature 392, 182–186.

Du, S.J., Purcell, S.M., Christian, J.L., McGrew, L.L., Moon, R.T., 1995. Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in *Xenopus* embryos. Mol. Cell. Biol. 15, 2625–2634.

Fedi, P., Bafico, A., Nieto Soria, A., Burgess, W.H., Miki, T., Bottaro, D.P., Kraus, M.H., Aaronson, S.A., 1999. Isolation and biochemical characterization of the human Dkk-1 homologue, a novel

inhibitor of mammalian Wnt signaling. J. Biol. Chem. 274, 19465–19472.
Gallagher, R.C., Hay, T., Meniel, V., Naughton, C., Anderson, T.J., Shibata, H., Ito, M., Clevers, H., Noda, T., Sansom, O.J., Mason, J.O., Clarke, A.R., 2002. Inactivation of Apc perturbs mammary development, but only directly results in acanthoma in the context of Tcf-1 deficiency. Oncogene 21, 6446–6457.
Galli, L.M., Willert, K., Nusse, R., Yablonka-Reuveni, Z., Nohno, T., Denetclaw, W., Burrus, L.W., 2004. A proliferative role for Wnt-3a in chick somites. Dev. Biol. 269, 489–504.
Galvin, K.M., Donovan, M.J., Lynch, C.A., Meyer, R.I., Paul, R.J., Lorenz, J.N., Fairchild-Huntress, V., Dixon, K.L., Dunmore, J.H., Gimbrone Jr., M.A., Falb, D., Huszar, D., 2000. A role for smad6 in development and homeostasis of the cardiovascular system. Nat. Genet. 24, 171–174.
Giles, R.H., van Es, J.H., Clevers, H., 2003. Caught up in a Wnt storm: Wnt signaling in cancer. Biochim. Biophys. Acta 1653, 1–24.
Gitler, A.D., Lu, M.M., Jiang, Y.Q., Epstein, J.A., Gruber, P.J., 2003. Molecular markers of cardiac endocardial cushion development. Dev. Dyn. 228, 643–650.
Glinka, A., Wu, W., Delius, H., Monaghan, A.P., Blumenstock, C., Niehrs, C., 1998. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391, 357–362.
Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49–92.
Harland, R.M., 1991. In situ hybridization: an improved whole-mount method for *Xenopus* embryos. Methods Cell Biol. 36, 685–695.
Hartmann, C., Tabin, C.J., 2001. Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. Cell 104, 341–351.
He, B., You, L., Uematsu, K., Xu, Z., Lee, A.Y., Matsangou, M., McCormick, F., Jablons, D.M., 2004. A monoclonal antibody against Wnt-1 induces apoptosis in human cancer cells. Neoplasia (New York) 6, 7–14.
Hoppler, S., Brown, J.D., Moon, R.T., 1996. Expression of a dominant-negative Wnt blocks induction of MyoD in *Xenopus* embryos. Genes Dev. 10, 2805–2817.
Hurlstone, A.F., Haramis, A.P., Wienholds, E., Begthel, H., Korving, J., Van Eeden, F., Cuppen, E., Zivkovic, D., Plasterk, R.H., Clevers, H., 2003. The Wnt/beta-catenin pathway regulates cardiac valve formation. Nature 425, 633–637.
Imbert, A., Eelkema, R., Jordan, S., Feiner, H., Cowin, P., 2001. Delta N89 beta-catenin induces precocious development, differentiation, and neoplasia in mammary gland. J. Cell Biol. 153, 555–568.
Jiao, K., Kulessa, H., Tompkins, K., Zhou, Y., Batts, L., Baldwin, H.S., Hogan, B.L., 2003. An essential role of Bmp4 in the atrioventricular septation of the mouse heart. Genes Dev. 17, 2362–2367.
Jones, S.E., Jomary, C., 2002. Secreted Frizzled-related proteins: searching for relationships and patterns. BioEssays 24, 811–820.
Kim, R.Y., Robertson, E.J., Solloway, M.J., 2001. Bmp6 and Bmp7 are required for cushion formation and septation in the developing mouse heart. Dev. Biol. 235, 449–466.
Kioussi, C., Briata, P., Baek, S.H., Rose, D.W., Hamblet, N.S., Herman, T., Ohgi, K.A., Lin, C.J., Gleiberman, A., Wang, J.B., Brault, V., Ruiz-Lozano, P., Nguyen, H.D., Kemler, R., Glass, C.K., Wynshaw-Boris, A., Rosenfeld, M.G., 2002. Identification of a Wnt/DVI/beta-catenin → Pitx2 pathway mediating cell-type-specific proliferation during development. Cell 111, 673–685.
Kitamura, K., Miura, H., Miyagawa-Tomita, S., Yanazawa, M., Katoh-Fukui, Y., Suzuki, R., Ohuchi, H., Suehiro, A., Motegi, Y., Nakahara, Y., Kondo, S., Yokoyama, M., 1999. Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. Development 126, 5749–5758.
Kitten, G.T., Markwald, R.R., Bolender, D.L., 1987. Distribution of basement membrane antigens in cryopreserved early embryonic hearts. Anat. Rec. 217, 379–390.
Krug, E.L., Runyan, R.B., Markwald, R.R., 1985. Protein extracts from early embryonic hearts initiate cardiac endothelial cytodifferentiation. Dev. Biol. 112, 414–426.
Krug, E.L., Mjaatvedt, C.H., Markwald, R.R., 1987. Extracellular matrix from embryonic myocardium elicits an early morphogenetic event in cardiac endothelial differentiation. Dev. Biol. 120, 348–355.
Ladher, R.K., Church, V.L., Allen, S., Robson, L., Abdelfattah, A., Brown, N.A., Hattersley, G., Rosen, V., Luyten, F.P., Dale, L., Francis-West, P.H., 2000. Cloning and expression of the Wnt antagonists Sfrp-2 and Frzb during chick development. Dev. Biol. 218, 183–198.
Lamers, W.H., Viragh, S., Wessels, A., Moorman, A.F., Anderson, R.H., 1995. Formation of the tricuspid valve in the human heart. Circulation 91, 111–121.
Liu, G., Bafico, A., Harris, V.K., Aaronson, S.A., 2003. A novel mechanism for Wnt activation of canonical signaling through the LRP6 receptor. Mol. Cell. Biol. 23, 5825–5835.
Longo, K.A., Kennell, J.A., Ochocinska, M.J., Ross, S.E., Wright, W.S., MacDougald, O.A., 2002. Wnt signaling protects 3T3-L1 preadipocytes from apoptosis through induction of insulin-like growth factors. J. Biol. Chem. 277, 38239–38244.
Markwald, R.R., Fitzharris, T.P., Smith, W.N., 1975. Sturctural analysis of endocardial cytodifferentiation. Dev. Biol. 42, 160–180.
Markwald, R.R., Fitzharris, T.P., Manasek, F.J., 1977. Structural development of endocardial cushions. Am. J. Anat. 148, 85–119.
Megason, S.G., McMahon, A.P., 2002. A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. Development 129, 2087–2098.
Miller, J.R., 2002. The Wnts. Genome Biol. 3, REVIEWS3001.
Monaghan, A.P., Kioschis, P., Wu, W., Zuniga, A., Bock, D., Poustka, A., Delius, H., Niehrs, C., 1999. Dickkopf genes are co-ordinately expressed in mesodermal lineages. Mech. Dev. 87, 45–56.
Moon, R.T., Campbell, R.M., Christian, J.L., McGrew, L.L., Shih, J., Fraser, S., 1993. Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of *Xenopus laevis*. Development 119, 97–111.
Morgan, B.A., Fekete, D.M., 1996. Manipulating gene expression with replication-competent retroviruses. Methods Cell Biol. 51, 185–218.
Nakajima, Y., Mironov, V., Yamagishi, T., Nakamura, H., Markwald, R.R., 1997a. Expression of smooth muscle alpha-actin in mesenchymal cells during formation of avian endocardial cushion tissue: a role for transforming growth factor beta3. Dev. Dyn. 209, 296–309.
Nakajima, Y., Miyazono, K., Kato, M., Takase, M., Yamagishi, T., Nakamura, H., 1997b. Extracellular fibrillar structure of latent TGF beta binding protein-1: role in TGF beta-dependent endothelial–mesenchymal transformation during endocardial cushion tissue formation in mouse embryonic heart. J. Cell Biol. 136, 193–204.
Nakamura, T., Sano, M., Songyang, Z., Schneider, M.D., 2003. A Wnt- and beta-catenin-dependent pathway for mammalian cardiac myogenesis. Proc. Natl. Acad. Sci. U. S. A. 100, 5834–5839.
Nieto, M.A., Patel, K., Wilkinson, D.G., 1996. In situ hybridization analysis of chick embryos in whole mount and tissue sections. Methods Cell Biol. 51, 219–235.
Potts, J.D., Runyan, R.B., 1989. Epithelial–mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor beta. Dev. Biol. 134, 392–401.
Potts, J.D., Dagle, J.M., Walder, J.A., Weeks, D.L., Runyan, R.B., 1991. Epithelial–mesenchymal transformation of embryonic cardiac endothelial cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor beta 3. Proc. Natl. Acad. Sci. U. S. A. 88, 1516–1520.
Ranger, A.M., Grusby, M.J., Hodge, M.R., Gravallese, E.M., de la Brousse, F.C., Hoey, T., Mickanin, C., Baldwin, H.S., Glimcher, L.H., 1998. The transcription factor NF-ATc is essential for cardiac valve formation. Nature 392, 186–190.
Reya, T., O'Riordan, M., Okamura, R., Devaney, E., Willert, K., Nusse, R., Grosschedl, R., 2000. Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. Immunity 13, 15–24.
Romano, L.A., Runyan, R.B., 1999. Slug is a mediator of epithelial–

mesenchymal cell transformation in the developing chicken heart. Dev. Biol. 212, 243–254.

Rowlands, T.M., Pechenkina, I.V., Hatsell, S.J., Pestell, R.G., Cowin, P., 2003. Dissecting the roles of beta-catenin and cyclin D1 during mammary development and neoplasia. Proc. Natl. Acad. Sci. U. S. A. 100, 11400–11405.

Runyan, R.B., Markwald, R.R., 1983. Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue. Dev. Biol. 95, 108–114.

Runyan, R.B., Wendler, C.C., Romano, L.A., Boyer, A.S., Dagle, J.M., Weeks, D.L., 1999. Utilization of antisense oligodeoxynucleotides with embryonic tissues in culture. Methods 18, 316–321.

Schubert, F.R., Mootoosamy, R.C., Walters, E.H., Graham, A., Tumiotto, L., Munsterberg, A.E., Lumsden, A., Dietrich, S., 2002. Wnt6 marks sites of epithelial transformations in the chick embryo. Mech. Dev. 114, 143–148.

Semenov, M.V., Tamai, K., Brott, B.K., Kuhl, M., Sokol, S., He, X., 2001. Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6 [see comment]. Curr. Biol. 11, 951–961.

Shu, W., Jiang, Y.Q., Lu, M.M., Morrisey, E.E., 2002. Wnt7b regulates mesenchymal proliferation and vascular development in the lung. Development 129, 4831–4842.

Smalley, M.J., Dale, T.C., 1999. Wnt signalling in mammalian development and cancer. Cancer Metastasis Rev. 18, 215–230.

Sokol, S., Christian, J.L., Moon, R.T., Melton, D.A., 1991. Injected Wnt RNA induces a complete body axis in *Xenopus* embryos. Cell 67, 741–752.

Sugi, Y., Ito, N., Szebenyi, G., Myers, K., Fallon, J.F., Mikawa, T., Markwald, R.R., 2003. Fibroblast growth factor (FGF)-4 can induce proliferation of cardiac cushion mesenchymal cells during early valve leaflet formation. Dev. Biol. 258, 252–263.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., Clevers, H., 1997. Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. Cell 88, 789–799.

Worm, J., Christensen, C., Gronbaek, K., Tulchinsky, E., Guldberg, P., 2004. Genetic and epigenetic alterations of the APC gene in malignant melanoma. Oncogene 23, 5215–5226.

You, Z., Saims, D., Chen, S., Zhang, Z., Guttridge, D.C., Guan, K.L., MacDougald, O.A., Brown, A.M., Evan, G., Kitajewski, J., Wang, C.Y., 2002. Wnt signaling promotes oncogenic transformation by inhibiting c-Myc-induced apoptosis. J. Cell Biol. 157, 429–440.