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Wnt signaling in heart valve development and osteogenic gene induction

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Abstract

Wnt signaling mediated by beta-catenin has been implicated in early endocardial cushion development, but its roles in later stages of heart valve maturation and homeostasis have not been identified. Multiple Wnt ligands and pathway genes are differentially expressed during heart valve development. At E12.5, *Wnt2* is expressed in cushion mesenchyme, whereas *Wnt4* and *Wnt9b* are predominant in overlying endothelial cells. At E17.5, both *Wnt3a* and *Wnt7b* are expressed in the remodeling atrioventricular (AV) and semilunar valves. In addition, the TOPGAL Wnt reporter transgene is active throughout the developing AV and semilunar valves at E16.5, with more localized expression in the stratified valve leaflets after birth. In chicken embryo aortic valves, genes characteristic of osteogenic cell lineages including *periostin*, *osteonectin*, and *Id2* are expressed specifically in the collagen-rich fibrosa layer at E14. Treatment of E14 aortic valve interstitial cells (VIC) in culture with osteogenic media results in increased expression of multiple genes associated with bone formation. Treatment of VIC with Wnt3a leads to nuclear localization of beta-catenin and induction of *periostin* and *matrix gla-protein*, but does not induce genes associated with later stages of osteogenesis. Together, these studies provide evidence for Wnt signaling as a regulator of endocardial cushion maturation as well as valve leaflet stratification, homeostasis and pathogenesis.

Keywords

Heart; valve; embryo; Wnt signaling; osteogenesis

Introduction

Developmental defects in atrioventricular and semilunar valves are among the most common congenital heart malformations, and aortic valve replacement is the second most common cardiac surgery in the United States (Freeman and Otto, 2005; Hoffman and Kaplan, 2002; Pierpont et al., 2007). The majority of the aortic valves that are replaced also have congenital malformations, establishing a link between abnormal valve development and valve disease later in life (Roberts and Ko, 2005). During vertebrate embryonic development, the cardiac valves initially arise from endocardial cushions that form in the atrioventricular (AV) canal and outflow tract (OFT) regions of the primitive heart tube (Armstrong and Bischoff, 2004; Person et al., 2005b). The endocardial cushions contribute to valve primordia that subsequently

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elongate into the mature leaflets of the AV and semilunar valves (Combs and Yutzey, 2009a). The mature valve leaflets are stratified into fibrosa, spongiosa and atrialis/ventricularis layers characterized by differential localization of extracellular matrix (ECM) proteins with distinct mechanical properties (Aikawa et al., 2006; Hinton et al., 2006). Heart valve disease is characterized by disorganization of ECM proteins as well as cellular abnormalities that contribute to dysfunction (Hinton et al., 2006; Rabkin et al., 2001; Rabkin-Aikawa et al., 2004). There is increasing evidence that signaling pathways and transcription factors critical for valve development also contribute to heart valve disease.

Wnt signaling has multiple functions at different stages of heart development with demonstrated functions at the earliest stages of cardiac myocyte lineage development during embryogenesis in vivo, as well as in embryonic stem cells (Cohen et al., 2008; Kwon et al., 2008; Tzahor, 2007). Manipulation of β-catenin expression in mice has demonstrated a further requirement for Wnt signaling in cell proliferation in the secondary heart field and in epicardial development (Grigoryan et al., 2008; Zamora et al., 2007). In the developing valves, Wnt signaling has been implicated in the regulation of endothelial-to-mesenchymal transition (EMT) and cell proliferation during the initial stages of endocardial cushion formation in multiple animal models (Hurlstone et al., 2003; Liebner et al., 2004; Person et al., 2005a). Later functions for Wnt signaling in valve maturation and homeostasis have not been reported. In adults, increased Wnt pathway gene expression is associated with calcific aortic valve disease (Caira et al., 2006; Rajamannan et al., 2005). However, the functions of Wnt signaling in valve development, homeostasis, and pathogenesis are not well understood.

The regulatory mechanisms that control heart valve formation share many molecular features with cartilage, tendon, and bone development (Lincoln et al., 2006b). Previous studies have shown extensive similarities in the molecular regulation of the valve leaflet patterning and differentiation with regulatory hierarchies that control cartilage and tendon development in both chicken and mouse embryos (Levay et al., 2008; Lincoln et al., 2006a; Lincoln et al., 2007). Less is known of the regulatory mechanisms that control maturation of the fibrosa layer, which has molecular and cellular characteristics similar to undifferentiated osteoblasts and other fibroblast lineages (Chakraborty et al., 2008). Wnt signaling functions at multiple stages during osteogenesis and also promotes fibrosis in multiple organ systems, consistent with a role in fibrous connective tissue development (He et al., 2009; Leucht et al., 2008; Morrisey, 2003). In adult human aortic valve sclerosis, the fibrosa layer is the primary site of valve calcification, further supporting parallels in valve development and disease mechanisms with osteogenesis (Freeman and Otto, 2005).

The expression and function of Wnt pathway genes and signaling activation in valvulogenesis was examined in chicken and mouse embryonic systems. Multiple Wnt pathway ligands, receptors, and modifying proteins are differentially expressed in the endocardial cushions and remodeling valves in both species. In addition, the TOPGAL Wnt reporter is active throughout remodeling atrioventricular (AV) and semilunar valves with restricted expression in the stratified valves after birth in mice. The function of Wnt signaling in activation of genes expressed in the stratified valves and pre-osteoblasts was examined in primary cultures of chicken embryonic (E14) aortic valve interstitial cells (VIC) that express fibrosa layer markers. Treatment of VIC with osteogenic media results in increased expression of multiple genes associated with the early stages of osteogenic differentiation. Treatment of VIC with Wnt3a leads to induction of *periostin (POSTN)* and *matrix-gla protein* (*MGP*) gene expression, and Wnt signaling inhibition blocks induction of these genes by osteogenic media. Together, these studies provide evidence for canonical Wnt signaling as a regulator of heart valve lineage maturation and pathogenesis.

Materials and Methods

Mouse and chicken embryo collection

Wild-type FVBN mouse embryos were isolated at E12.5 or E17.5 after timed matings, with evidence of a copulation plug considered E0.5. Whole E12.5 embryos or isolated E17.5 hearts were fixed in 4% paraformaldehyde prior to in situ hybridization as previously described (Chakraborty et al., 2008; Lange and Yutzey, 2006). Alternatively, E12.5 AV cushions or E17.5 AV valves were dissected, and RNA isolated as previously described (Chakraborty et al., 2008). Hearts from transgenic TOPGAL Wnt reporter mice (DasGupta and Fuchs, 1999) were assayed for β-gal expression by X-gal staining in histological sections as previously described (Lincoln et al., 2004). Fertilized white leghorn chicken eggs (Charles River Laboratories) were incubated at 38°C under high humidity. Embryos were collected at 14d of incubation and hearts isolated in 1X phosphate buffered saline (PBS) for in situ hybridization or valve leaflet dissection as previously described (Shelton and Yutzey, 2007; Shelton and Yutzey, 2008). All animal procedures were carried out using protocols approved by Cincinnati Children's Medical Center Biohazard Safety Committee and Institutional Animal Care and Use Committee.

Microarray analysis

RNA isolated from mouse E12.5 AV cushions or E17.5 AV valves was amplified, biotin-labeled and hybridized in biological replicates to Affymetrix Mouse Genome 430 2.0 arrays as previously described (Chakraborty et al., 2008). The scanned gene expression data were subjected to Robust Multichip Average (RMA) analysis, and gene expression values for E12.5 AV cushions versus E17.5 AV valves were calculated and subjected to statistical analysis as previously described. A list of 3119 differentially expressed genes (>2-fold) was generated and submitted to the GEO database (http://www.ncbi.nlm.gov/geo/) with accession number **GSE11040** (Chakraborty et al., 2008). Genes encoding proteins in the canonical and noncanonical Wnt pathways were identified based on homology to characterized proteins (http://www.stanford.edu/~rnusse/wntwindow.html (Cohen et al., 2008)) and a heat map of relative expression levels generated as previously described (Chakraborty et al., 2008).

in situ hybridization

In situ hybridizations were performed on paraffin-embedded and sectioned (14 μm) E12.5 mouse embryos, isolated mouse E17.5 hearts or isolated chicken E14 hearts as previously described (Chakraborty et al., 2008; Shelton and Yutzey, 2008; Somi et al., 2004). Sequences corresponding to mouse *Wnt2*, *Wnt4*, *Wnt3a*, *Wnt7b*, *Wnt9b*, *Lef1*, *Fzd2*, as well as chicken *matrix gla protein* (*MGP*), *osteonectin*, *Id2*, *Wnt3a* and *LRP5* were amplified by RT-PCR from embryonic limb or heart RNA. Forward and reverse primers, annealing temperatures and amplified sequence length are shown in Table 1. For each transcript, the amplified DNA was subcloned into pGEM T-vector (Promega) and identity confirmed by sequencing. Chicken *POSTN* sequence was amplified and probe generated as described previously (Shelton and Yutzey, 2008). Digoxigenin-labeled antisense RNA probes were generated as previously described (Ehrman and Yutzey, 1999; Shelton and Yutzey, 2007). Probes for *mWnt3a*, *mWnt4*, *mWnt7b* were synthesized with Sp6 polymerase from plasmids linearized with Nco1. The probe for *mWnt2* was synthesized with Sp6 polymerase from plasmid linearized with SacII. Probes for *cLRP5* and *cMGP* were synthesized with T7 polymerase after linearization with Not1 or Nde1, respectively.

Valve cell culture

Primary aortic valve interstitial cells (VIC) were isolated from chicken embryos at E14 and cultured on collagen-coated chamber slides. Aortic valve leaflets were explanted, dissected away from the aorta with fine forceps and subjected to treatment with Collagenase II

(Worthington; 100U/ml) in Hank's balanced salt solution (Cellgro) at 37°C 3 × 7 minutes in a shaking water bath (Evans et al., 2003). Pooled supernatants from 4–6 valves were spun at 800rpm and the cell pellet resuspended in 3ml of complete M199 culture medium containing 10% fetal bovine serum (Hyclone) plus penicillin/streptomycin (Invitrogen). The cells were plated on collagen-coated 2 well permanox chamber slides (Nunc) and cultured for 2–5 days. For studies of osteogenic gene induction, the cells were treated with complete media containing β-glycerophosphate (10mM) and ascorbic acid (50 μg/ml) (β-GP/AA) (Gerstenfeld and Landis, 1991). Osteogenic media was added after 3 days in culture and replenished after an additional 2 days. Alternatively, osteogenesis was induced using the Millipore stem cell osteogenesis kit (complete media with 10mM β-glycerophosphate, 0.2μM ascorbic acid, 0.1μM dexamethasone) with similar results. For analysis of canonical Wnt signaling, isolated aortic VIC cultures were treated with recombinant mouse Wnt3a protein (150 ng/ml; R&D systems), the Wnt inhibitor mouse sFRP3 (150 ng/ml; R&D systems) or control vehicle BSA (150ng/ml), in the presence or absence of β-GP/AA osteogenic media. After an additional 2 days, RNA was isolated for qRT-PCR or cells were fixed for immunostaining as previously described (Shelton and Yutzey, 2007; Shelton and Yutzey, 2008).

Immunostaining and confocal analysis

Smooth muscle α-actin (Sigma) immunostaining of paraffin-embedded E12.5 embryo sections (7 μm) was performed as previously described (Combs and Yutzey, 2009b). Cultured valve cells were fixed in cold 70% methanol for 20 min and preblocked in PBS with 5% BSA for at least 1 hr. Antibodies used for immunostaining include mouse monoclonal antibodies to unphosphorylated β-catenin (Santa Cruz) [1:50]; smooth muscle α-actin (Sigma) [1:100]; collagen I (M38; Developmental Hybridoma Bank, Iowa) [1:100]; collagen VI (39; Developmental Hybridoma Bank, Iowa) [1:100]; and rabbit polyclonal antisera for periostin (Abcam) [1:200], fibronectin (Dako Cytomation) [1:200] and Id1/2 (Santa Cruz) [1:100]. Primary antibodies were incubated at indicated dilutions in PBS/blocking solution (1% BSA, 0.1% cold water fish skin gelatin, 0.1% Tween-20, 0.05% NaN_3) at 4°C overnight. Secondary antisera for immunofluorescence including Alexa-goat anti-mouse-488, Alexa-goat anti-rabbit-568 or Alexa goat anti-rabbit-488 (Invitrogen) were used at 1:200 in PBS for 1 hr. ToPro3 iodide (Invitrogen) was used at (1:1000) in PBS for nuclear labeling. Immunofluorescence was detected using a Zeiss LSM 510 confocal microscope and images were captured using LSM version 3.2 SP2 software.

Real time qRT-PCR

RNA was isolated from valve cell cultures or dissected embryo heart valves and used to generate cDNA as previously described (Shelton and Yutzey, 2007). Quantitative real-time PCR (qRT-PCR) was performed using primers in Table 1 with indicated annealing temperatures and expected product size or as reported previously (Shelton and Yutzey, 2007; Shelton and Yutzey, 2008). The identity of each of the amplified DNA fragments was confirmed by sequencing. 1μg cDNA was used in qRT-PCR reactions performed in triplicate as previously reported (Shelton and Yutzey, 2007; Shelton and Yutzey, 2008). Gene expression levels were determined based on a threshold cycle of detection for each amplified product relative to a standard curve of calibration for each primer pair. Standard curves were generated with cDNA from E8 chicken heart or E14 chicken wing and all values were normalized to GAPDH expression. Fold changes of gene expression were calculated in triplicate for at least three independent experiments. Statistical significance of observed differences was calculated using a student's t-test ($p<0.05–0.01$)

Results

Differential expression of Wnt pathway genes in mouse heart valve development

Affymetrix microarray gene expression analysis was performed on RNA isolated from mouse E12.5 AV cushions and E17.5 AV valves in order to identify genes differentially expressed during early and late stages of valvulogenesis (Chakraborty et al., 2008). A list of 3119 differentially expressed genes (at least 2X difference) was generated that can be accessed in the GEO database (**GSE11040**). Several identified genes in the Wnt signaling pathway are among the differentially expressed genes based on published pathways (Cohen et al., 2008; Nusse, 2005). Differential expression of Wnt ligands, receptors, downstream transcription factors, and modulating proteins was observed (Figure 1; Supplemental Table 1). Wnt signaling pathway genes elevated in the E12.5 AV cushions relative to remodeling AV valves include *Wnt2*, *Wnt4*, *Wnt5b*, *Wnt9b*, *Lef1*, *Dvl2*, *Dkk1* and *Fzd2*. At later stages of valvulogenesis, increased expression of Wnt inhibitors *Wisp1* and *Wif1* was detected. Together these expression data provide evidence for dynamic regulation of multiple components and regulators of Wnt signaling at early and late stages of valvulogenesis.

The localized expression of selected Wnt pathway genes elevated in mouse E12.5 AV cushions relative to E17.5 AV valves was examined by in situ hybridization (Figure 2). *Wnt2* is expressed in the AV and OFT cushion mesenchyme and endothelial cells, but not in the adjacent OFT myocardium (Figure 2A, B, data not shown). Expression of the Wnt receptor *Frizzled 2* (*Fzd2*) and the Wnt-responsive transcription factor *Lef1* also is apparent in the AV and OFT endocardial cushion mesenchyme at E12.5, with little or no expression in the endocardial endothelial cells (Figure 2C–F). *Lef1* expression is specific to the endocardial cushions, whereas *Frzd2* expression also is apparent in myocardium and smooth muscle. In contrast, *Wnt4* and *Wnt9b* are expressed together specifically in endocardial endothelial cells overlying the AV and OFT cushions (Figure 2G–J). Interestingly, *Wnt4* and *Wnt9b* are not expressed in the endocardium of the atria or ventricles, suggesting a specific role in endocardial endothelial cushion development. Microarray expression analysis indicated that each of these genes is downregulated in E17.5 remodeling AV valves (Figure 1), and loss of expression in E17.5 AV and OFT valves was confirmed by in situ hybridization (data not shown). Together, these studies support the presence of Wnt pathway activity through specific ligands in endothelial and mesenchymal cells of the developing AV and OFT endocardial cushions after initiation of EMT.

Wnt signaling status was examined in the remodeling valve primordia of mouse embryos at E16.5 and in stratified valve leaflets one month after birth. Previous studies demonstrated active Wnt signaling throughout the endocardial cushions of the AV canal at E12.5 using TOPGAL transgenic mice that express β-galactosidase where canonical Wnt signaling is active (DasGupta and Fuchs, 1999; Gitler et al., 2003). TOPGAL Wnt reporter activity was assessed at later stages in the remodeling valve leaflets by X-gal staining in histological sections. At E16.5, TOPGAL reporter activity is predominant throughout the AV and OFT valve leaflet primordia, indicating widespread Wnt pathway activation in the remodeling valves (Figure 3A, B). At one month after birth, TOPGAL activity is apparent in a subset of cells in the stratified aortic and mitral valves, with predominant activity on the fibrosa side away from blood flow (Figure 3C, D). The widespread activation of the TOPGAL reporter gene in the prenatal valve primordia and restricted expression after birth are consistent with a role for canonical Wnt signaling in remodeling, stratification and homeostasis of the valve leaflets.

Expression of *Wnt3a* and *Wnt7b* was examined in the remodeling valves at E17.5. These ligands were chosen based on their developmental roles in fibroblast and osteogenic cell lineages that share molecular characteristics with the fibrosa cell layer of the remodeling AV and semilunar valves (Chakraborty et al., 2008; Derfoul et al., 2004; Hu et al., 2005). While

Wnt ligands with increased expression at E17.5 were not initially identified in the valve developmental expression screen, TOPGAL reporter gene activation indicates Wnt pathway activity in the remodeling valve leaflets (Figure 3). Expression of both *Wnt3a* and *Wnt7b* was detected in the AV and semilunar valve leaflets at E17.5 (Figure 4A–D) and also in osteoblasts of the developing bone (data not shown). In the developing mitral valve leaflets, *Wnt3a* is predominant at the tips of the valves (Figure 4A). *Wnt7b* is expressed more highly on the fibrosa surface, although there is some expression on the atrialis side of the mitral valve leaflets (Figure 4B). Expression of *Wnt3a* is apparent in the aortic valve leaflets preferentially on the fibrosa side (Figure 4B, arrows), whereas *Wnt7a* expression is detected throughout the leaflets (Figure 4D, arrows). *Wnt3a* and *Wnt7b* also are expressed in the AV canal adjacent to the mitral valve (Figure 4A, B asterisks) as well as in scattered cells present in the interventricular septum and ventricular myocardium. In the developing valves, expression of *Wnt3a* and *Wnt7b* ligands overlaps with regions where the Wnt pathway is active as indicated by TOPGAL reporter mice.

Localized expression of ECM, osteogenic and Wnt pathway genes in stratified E14 chicken aortic valves

The expression of genes associated with the valve fibrosa layer, preosteoblast/fibroblast cell types as well as Wnt signaling was examined in E14 chicken embryo aortic valves (Figure 5). The secreted proteins periostin (POSTN) and matrix-gla protein (MGP) are highly expressed in pre-osteoblast and fibroblast lineages with the potential to undergo ECM mineralization (Kruzynska-Frejtag et al., 2001;Murshed et al., 2004). Periostin has a role in fibrillar collagen biosynthesis, whereas MGP inhibits calcification of fibrous tissues and osteogenic progenitors (Norris et al., 2007;Yagami et al., 1999). *POSTN* and *MGP* expression is increased in the stratified valves relative to endocardial cushions in both avian and mouse embryos (data not shown (Chakraborty et al., 2008)). *POSTN* gene expression is predominant in the fibrosa layer of the E14 aortic valve cusp and annulus (Figure 5B). MGP expression is predominant at the base of the aortic valve and also on the fibrosa surface of the valve as well as in the aorta (Figure 5D). *Osteonection* and *Id2*, associated with multiple fibroblast and preosteoblast lineages, also are predominantly expressed in the fibrosa layer of the E14 aortic valve (Figure 5A, C). Wnt3a and its coreceptor LRP5 have previously been implicated in osteogenesis and vascular development (Kato et al., 2002;Zhou et al., 2008). In the aortic valve, *Wnt3a* is expressed at the base of the cusp and also on the fibrosa surface (Figure 5E). *LRP5* is predominantly expressed at the tips of the valves, and expression of both *Wnt3a* and *LRP5* is apparent in the aorta (Figure 5E, F). Expression of *Wnt3a* also was detected in the developing skeleton and in featherbuds as previously reported (Yue et al., 2006). Together, these expression analyses demonstrate fibrosa-restricted gene expression and the presence of Wnt pathway genes in the avian E14 aortic valve leaflets.

Induction of osteogenic genes in chicken aortic valve interstitial cell cultures is affected by Wnt signaling

A chicken embryonic valve cell culture system was devised in order to examine regulatory mechanisms of valve cell lineage maturation as well as pathogenic mechanisms. E14 chicken embryo aortic valves were used for isolation of VIC for cell culture. At this stage, the valve leaflets are stratified with *osteonectin*, *POSTN*, and *Id2* expression predominant in the fibrosa layer (Figure 5A–C). The expression of proteins associated with fibrosa layer was examined in isolated E14 aortic valve cells. After 5 days in culture, nearly all of the cells (~90%) express type 1 collagen (Hinton et al., 2006), Id1/2, POSTN and fibronectin (FN) (Kruithof et al., 2007), characteristic of the fibrosa layer (Figure 6). Widespread expression of smooth muscle α-actin (SMA) is indicative of an activated myofibroblast phenotype typical of cultured VIC (Liu et al., 2007). Decreased expression of the endothelial marker Tie2 was observed in cultured VIC relative to intact aortic valves as detected by RT-PCR (data not shown). Therefore the isolated E14 avian aortic VIC population after several days in culture consists predominantly

of cells with a fibrosa-like phenotype, as has been demonstrated for VIC isolated from adult large animal models (Liu et al., 2007).

The ability to manipulate canonical Wnt signaling was assessed by immunolocalization of β-catenin in avian E14 VIC in the presence of increased or decreased Wnt signaling. Under normal culture conditions, β-catenin protein expression is apparent in a subset of cells with localization predominantly at the cell membrane (Figure 7A). Treatment with recombinant Wnt3a protein resulted in widespread stabilization, as well as nuclear localization, of β-catenin protein apparent in 1hr (Figure 7B). Inhibition of Wnt signaling was achieved with treatment of cells with the Wnt pathway antagonist soluble frizzled-related protein-3 (sFRP-3). In the sFRP-3 treated cultures, decreased immunoreactivity for β-catenin protein was observed relative to cells in normal growth medium or in cells treated with Wnt3a ligand (Figure 7C). These studies demonstrate that Wnt signaling can be manipulated in avian E14 aortic valve interstitial cells with the predicted effects on β-catenin stability and subcellular localization.

The potential of heart valve interstitial cells to activate an osteogenic gene profile was examined in isolated primary cell cultures from chicken embryo day 14 aortic valve leaflets. For osteogenic gene induction, E14 VIC were treated with osteogenic medium containing β-glycerophosphate (10mM) and ascorbic acid (50μg/ml) (β-GP/AA), which induces calcification of human VIC and promotes bone mineralization in osteoblast cell cultures (Gerstenfeld and Landis, 1991; Mathieu et al., 2005). E14 VIC also were treated with osteogenic medium containing dexamethasone with similar results (data not shown). Several genes associated with fibrous connective tissues, osteogenesis and valve calcification were examined in treated VIC cultures. *POSTN* and *MGP* are expressed in preosteoblasts as well as in multiple types of fibroblasts and connective tissues capable of undergoing calcification (Kruzynska-Frejtag et al., 2001; Murshed et al., 2005; Takeshita et al., 1993). The transcription factor *Runx2* is expressed during the early stages of osteoblast differentiation and also in human calcified aortic valves (Karsenty and Wagner, 2002; O'Brien, 2006; Rajamannan et al., 2003). *Alkaline phosphatase* (ALP) and *osteocalcin* (OCN) are markers of bone differentiation and also are characteristic of human valve calcification (Karsenty and Wagner, 2002; Mathieu et al., 2005). Expression of genes associated with osteogenesis and human aortic valve disease was examined in chicken aortic VIC cultures by qRT-PCR using sequence-specific primers shown in Table 1. In these experiments, *POSTN* and *MGP* are strongly induced with β-GP/AA treatment. *Runx2*, *OCN* and *ALP* expression also is significantly increased (Figure 8A). These studies demonstrate that avian E14 aortic valve interstitial cells treated with osteogenic medium in culture have the potential to activate several genes associated with bone lineage development and/or pathological calcification of fibrous connective tissues.

The effects of increased or decreased Wnt signaling on osteogenic gene induction were examined in cultured E14 aortic VIC (Figure 8B). Treatment with Wnt3a alone results in increased expression of *POSTN* and *MGP*, both of which are expressed in preosteoblast lineages and multiple types of fibrous connective tissues as well as the developing valves. In contrast, expression of *Runx2*, *OCN* and *ALP* markers of osteoblasts and differentiating bone was not induced by Wnt3a treatment. Treatment with Wnt3a together with osteogenic medium enhances induction of *POSTN* and *MGP*, with strong synergistic induction of *MGP* (35-fold). However, addition of Wnt3a did not augment the induction of *Runx2*, *OCN* or *ALP* expression under osteogenic conditions. Treatment of aortic VIC with osteogenic medium in the presence of the Wnt antagonist sFRP-3 blunted the induction of *POSTN* and *MGP*, but had no apparent effect on the osteoblast lineage markers *Runx2*, *OCN* and *ALP*. Overall these studies demonstrate that Wnt signaling is necessary and sufficient for induction of *POSTN* and *MGP*, which are normally present in the stratifying aortic valve and other fibrous connective tissues. However increased Wnt signaling alone is not sufficient to promote the complete osteogenic gene program in cultured aortic VIC.

Discussion

There is emerging evidence that Wnt signaling has multiple complex roles in vertebrate heart valve formation, homeostasis and disease. In this study we report differential expression of Wnt ligands and pathway genes at early and late stages of valvulogenesis. In the E12.5 endocardial cushions, *Wnt2* is predominant in the mesenchyme whereas *Wnt4* and *Wnt9b* are coexpressed in endothelial cells. The Wnt signaling pathway is active in the late fetal as well as post-natal valve leaflets, and expression of *Wnt3a* and *Wnt7b* is apparent at E17.5. In adult aortic valves, canonical Wnt signaling has been implicated in pathologic calcification that could be related to its function in osteoblast lineage development (Caira et al., 2006; Leucht et al., 2008; Rajamannan et al., 2005). Chicken E14 aortic VIC express multiple proteins that characterize the fibrosa layer of the stratified valves and also have the potential to activate genes associated with early and late stages of osteogenesis. Increased Wnt signaling in aortic VIC cultures leads to increased expression of *POSTN* and *MGP*, which also are expressed pre-osteoblasts and other connective tissues with the potential to calcify. However Wnt signaling alone is not sufficient to induce genes associated with osteoblast differentiation, consistent with the reported role of Wnt signaling in osteoblast cell lineage determination and progenitor expansion (Leucht et al., 2008). Together these results support the involvement of Wnt signaling through specific distinct ligands at multiple stages of valve development as well as contributing to valve homeostasis and pathogenesis.

Expression of multiple Wnt pathway genes in the endocardial cushion mesenchyme and endothelial cells is consistent with functions in EMT and valve progenitor cell proliferation. Endothelial cell-specific loss of β-catenin in mice demonstrates that Wnt signaling is required for EMT during endocardial cushion formation (Liebner et al., 2004). Manipulation of the Wnt receptor Frzb and Wnt9a in avian embryos demonstrated a role for Wnt signaling in promoting mesenchymal cell proliferation in the AV cushions (Person et al., 2005a). Likewise, activation of canonical Wnt signaling throughout AV cushion mesenchyme is apparent in TOPGAL Wnt reporter gene expression at E12.5 in mice (Gitler et al., 2003). Here we report similar mesenchymal expression of additional Wnt pathway genes *Wnt2* (*Wnt2a*), *LEF-1* and *Fzd2* in mouse AV as well as OFT cushions consistent with a role in mesenchymal cell proliferation. Wnt2 also is required for cardiac lineage differentiation in embryonic stem cells, but defects in heart development have not been reported for mice lacking Wnt2 that exhibit neonatal lethality with placental defects (Monkley et al., 1996; Wang et al., 2007). It has been proposed that Wnt2b may compensate for Wnt2a in regulation of cardiac development (Cohen et al., 2008), but further studies are necessary to determine the specific functions of Wnt2/2a in cardiac lineage development and endocardial cushion maturation.

The localized expression of *Wnt9b* and *Wnt4* in the endothelial cells specifically overlying the endocardial cushions has not previously been reported. In the developing kidney, these ligands also are coexpressed in the ureteric bud and pretubular aggregates, where Wnt9b regulates Wnt4 to promote mesenchymal to epithelial transitions (Carroll et al., 2005). Wnt9b also has been implicated in epicardial cell activation, consistent with emerging evidence for common regulatory mechanisms in endocardial cushion maturation and epicardial-derived cell lineage maturation (Merki et al., 2005; Shelton and Yutzey, 2008). Unlike the developing kidney, both endocardial cushion formation and epicardial cell activation require an epithelial to mesenchymal transition, and the roles of Wnt9b or Wnt4 in these cardiac structures are unknown. Mice lacking Wnt9b die within 24 hr of birth, presumably due to kidney agenesis, and mice lacking Wnt4 also are born with defective kidney and gonadal development (Carroll et al., 2005; Stark et al., 1994; Vainio et al., 1999). It is possible that mice lacking either Wnt9b or Wnt4 have undetected cardiac defects. However, based on their expression together in the endocardial cushion endothelial cells, loss of both may be necessary to reveal their functions

in the early stages of valve development. Mice lacking both Wnt4 and Wnt9b have not yet been described.

The expression of *Wnt3a* and *Wnt7b* ligands in the remodeling valves is consistent with a role in valve stratification. In developing osteoblasts, which share many characteristics with the valve fibrosa layer, Wnt3a promotes osteoblast lineage commitment that can be inhibited by sFRP1 in culture (Zhou et al., 2008). Likewise, Wnt7b has been identified as a potential endogenous ligand important in osteoblast lineage development, and it also functions in vascular smooth muscle development (Hu et al., 2005; Shu et al., 2002). Wnt pathway activation, as indicated by TOPGAL reporter activity, occurs throughout the valve primordia, but then is localized in the valve leaflets with predominant expression on the fibrosa surface at one month after birth. The expression of several fibrosa layer markers along with the TOPGAL reporter throughout the valve primordia, followed by restriction to the fibrosa layer at later stages of stratification, is consistent with a mechanism whereby the fibrosa phenotype is a default cell fate that is repressed on the flow side of the valve leaflet during stratification. It is likely that hemodynamics and shear stress contribute to the localization of Wnt pathway activation and valve stratification, but the regulatory hierarchies involved have not yet been fully defined.

The hallmark of the valve fibrosa layer is extensive highly organized type I collagen fibers, and additional ECM proteins characteristic of preosteoblasts and fibroblasts, including fibronectin, osteonectin and collagen III, also are preferentially localized to the fibrosa layer of the stratified valves (Hinton et al., 2006; Kruithof et al., 2007). These proteins are also coexpressed in cultured osteoblasts and other connective tissues with the potential to mineralize (Murshed et al., 2005). In cultured aortic VIC, Wnt signaling is necessary and sufficient for induction of *POSTN* and *MGP* gene expression characteristic of fibrous connective tissue. Periostin was initially described as an osteoblast-specific protein; but it is dynamically regulated in the developing heart valves, as well as other collagen-rich connective tissues, and has a putative role in collagen fibrillogenesis (Kruzynska-Frejtag et al., 2001; Norris et al., 2007; Snider et al., 2008). MGP also is expressed in undifferentiated osteoblasts and is an inhibitor of vascular and cartilage calcification (Barone et al., 1991; Luo et al., 1997). MGP null mice exhibit spontaneous calcification of the arteries, but no valve abnormalities were reported (Luo et al., 1997). MGP expression in the E14 avian aortic valve is predominant in proteoglycan-rich spongiosa region, which may be related to its function in cartilaginous structures. However, a protective role for MGP in preventing aortic valve calcification has not yet been demonstrated.

Increased canonical Wnt signaling, evident in expression of the Wnt receptor *Lrp5* and stabilized β-catenin has been associated with adult calcific valve disease (Caira et al., 2006; Rajamannan et al., 2005). In human aortic valve disease and mouse models of valve calcification, increased expression of *Runx2* (Cbfa-1), *osteopontin*, *osteocalcin* and alkaline phosphatase, characteristic of mineralized bone, is observed (Aikawa et al., 2007; Caira et al., 2006; Rajamannan et al., 2003). In this study, expression of each of these genes is increased in isolated embryonic VIC treated with osteogenic medium. However, increased Wnt signaling is not sufficient to induce a full osteogenic response, which is consistent with Wnt pathway activation in developing valves and postnatal valves that do not normally calcify. Similarly, osteogenic media induces multiple markers of bone differentiation and calcification in primary calvarial cultures and fibroblast cell lines; whereas increased Wnt signaling promotes early osteoblast progenitor lineage commitment and expansion, but has an inhibitory role in osteocyte differentiation (Leucht et al., 2008; Shi et al., 2007; Zhou et al., 2008). Wnt signaling also has been implicated in pathological fibrosis of the lung and kidney (He et al., 2009; Morrisey, 2003), which could be related to a role in valve pathogenesis. Therefore our data provide initial evidence that Wnt signaling could contribute to valve pathogenesis through

alterations in the ECM that affects its susceptibility to calcification. However, the full calcification response likely requires multiple stimuli that together contribute to heart valve dysfunction and insufficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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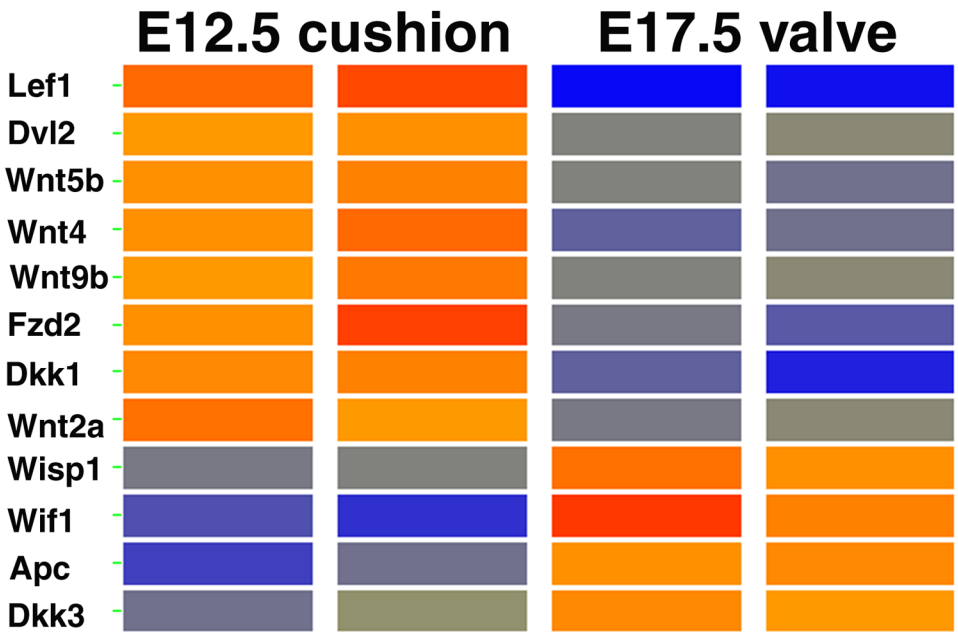

Figure 1. Wnt pathway genes are differentially expressed in the developing AV valves at E12.5 and E17.5

An Affymetrix microarray gene expression analysis was performed on duplicate samples of RNA isolated from E12.5 AV cushions or E17.5 AV valves. The presence of Wnt signaling pathway genes was determined among genes with >2.0 fold statistically significant change at E12.5 versus E17.5 (Chakraborty et al., 2008). A heat map was generated of relative gene expression levels based on raw intensity values. Red indicates increased expression, blue indicates decreased expression, and yellow indicates no change in expression.

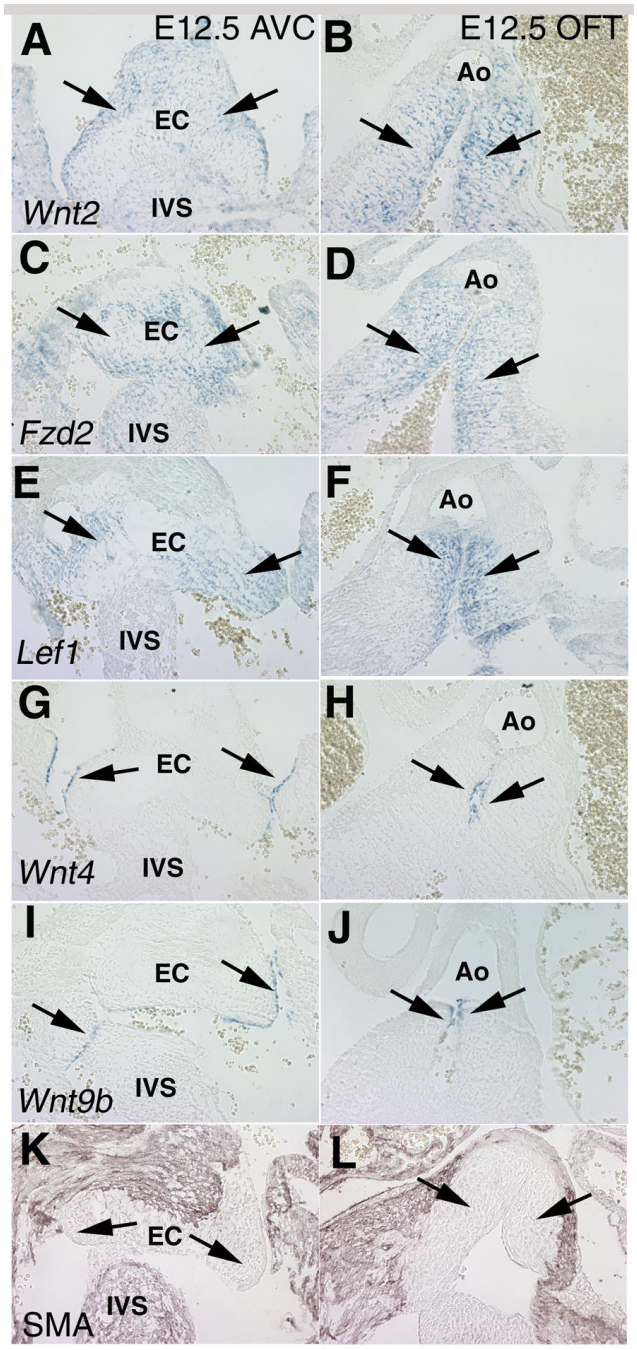

Figure 2. Wnt pathway genes are differentially expressed in mesenchymal and endothelial cells of mouse E12.5 endocardial cushions

In situ hybridizations of sectioned hearts show Wnt pathway gene expression in both atrioventricular canal (AVC; left panels) and outflow tract (OFT; right panels) cushions at E12.5. The ligand *Wnt2* (A, B), receptor frizzled2 (*Fzd2*; C,D) and downstream transcription factor *LEF1* (E,F) are expressed in cushion mesenchyme (indicated by arrows). In contrast, the ligands *Wnt4* (G,H) and *Wnt9b* (I,J) are expressed specifically in the cushion endothelial layer (indicated by arrows). Antibody staining for smooth muscle α-actin (SMA) indicates muscle cells (K,L). Endocardial cushions (EC), interventricular septum (IVS) and Aorta (Ao) are indicated.

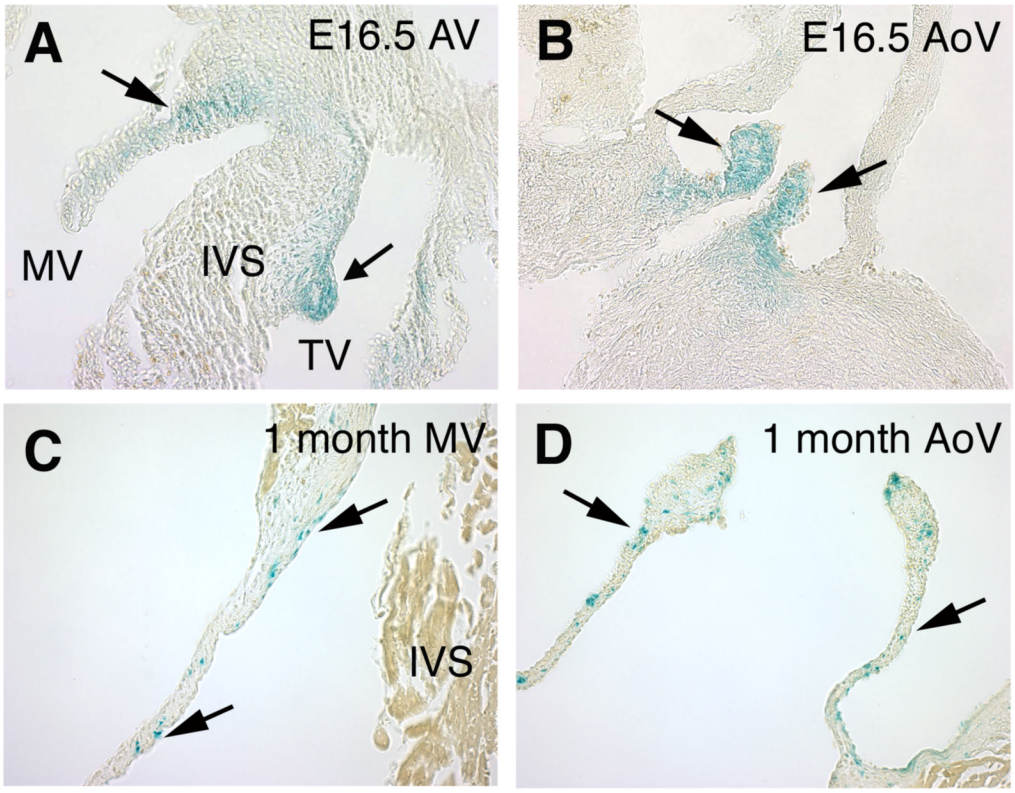

Figure 3. The Wnt pathway is active in mouse mitral and aortic valves as indicated by TOPGAL reporter expression

(A–D) Analysis of TOPGAL Wnt pathway reporter mice demonstrates pathway activation in the mitral valve (MV) and tricuspid valve (TV) primordia as well as the aortic valve (AoV) at E16.5 (A, B), as indicated by X-gal staining. Localized TOPGAL activity also is apparent in MV and AoV leaflets 1 month after birth (C,D), predominantly on the fibrosa side (indicated by arrows). The interventricular septum (IVS) is indicated.

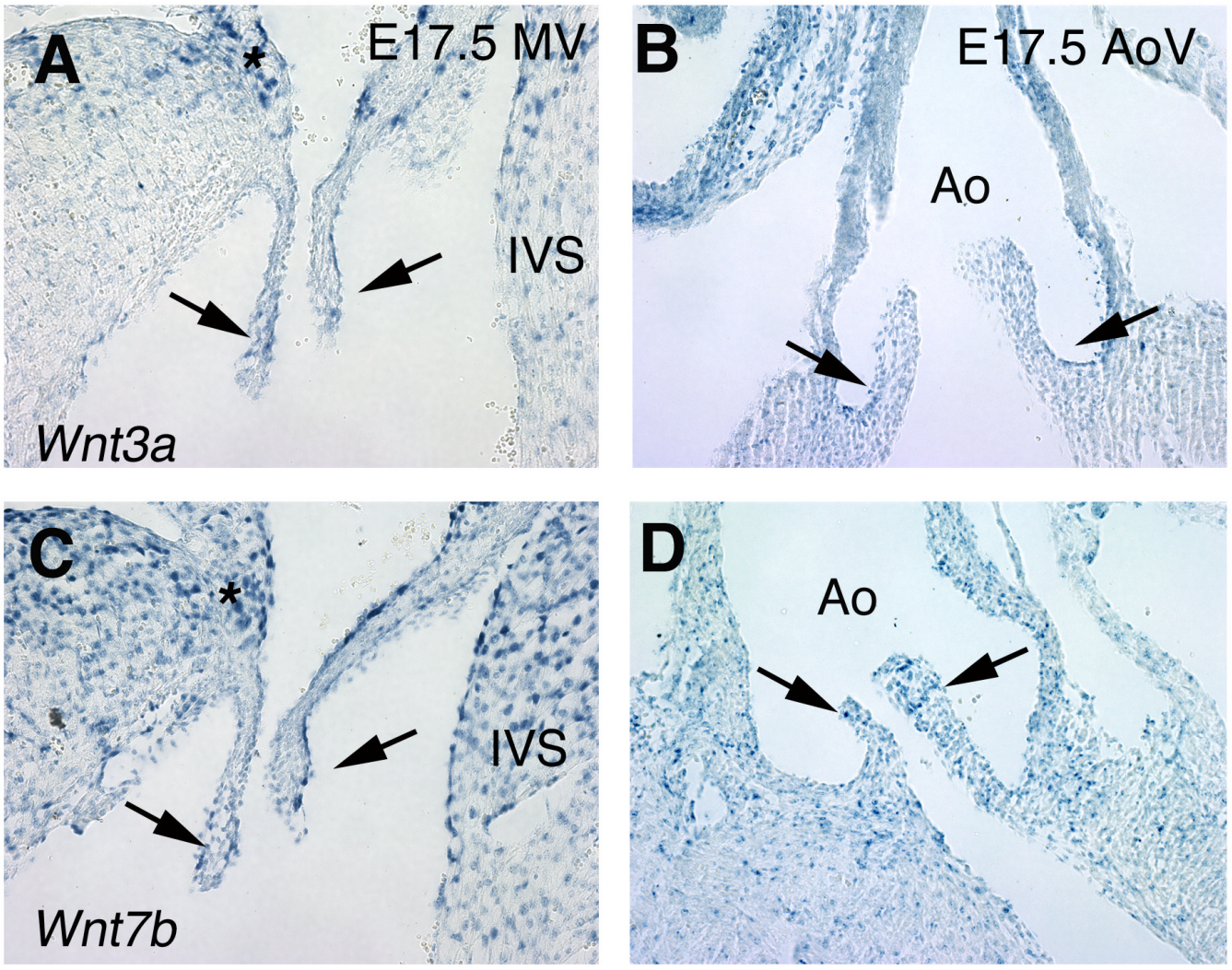

Figure 4. *Wnt3a* and *Wnt7b* are expressed in mouse mitral and aortic valves at E17.5

(A–D) In situ hybridization of sectioned E17.5 mouse hearts demonstrates that *Wnt3a* (A,B) and *Wnt7b* (C,D) are expressed in the remodeling mitral (MV) and aortic (AoV) valves. *Wnt3a* and *Wnt7b* expression in the AV valves is apparent at the tips of the leaflets (indicated by arrows) and in the AV canal (indicated by asterisks). *Wnt3a* is expressed preferentially on the fibrosa side the aortic valve leaflets (arrows, B). *Wnt7b* expression is apparent throughout the AoV leaflets (arrows, D). The interventricular septum (IVS) and aorta (Ao) are indicated.

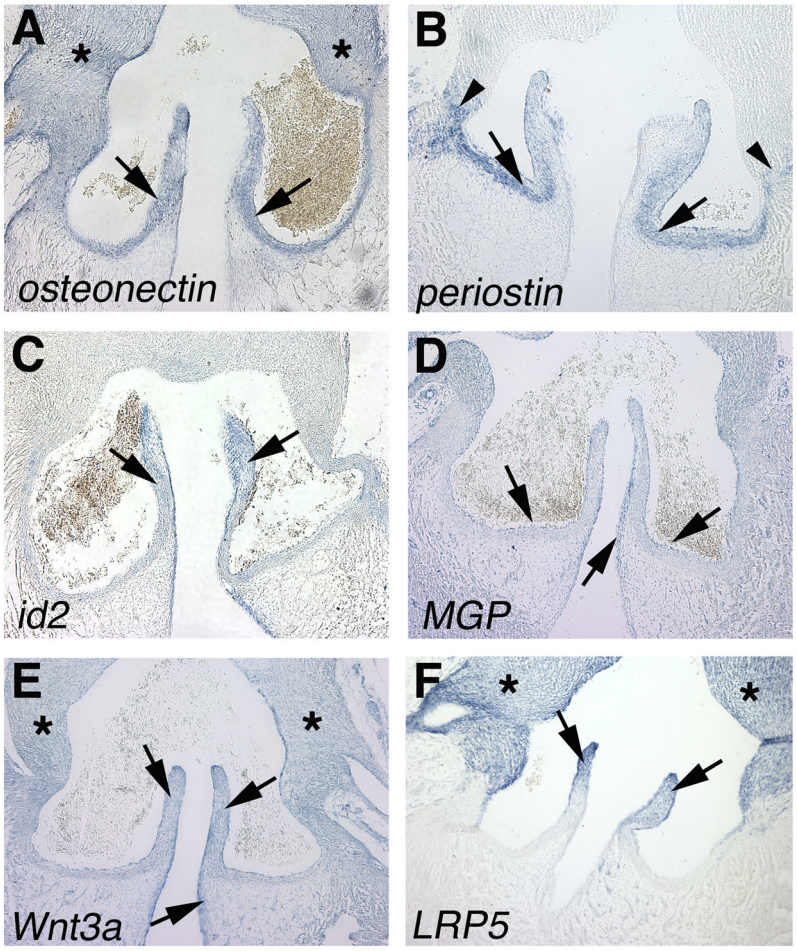

Figure 5. ECM, osteogenic and Wnt pathway genes are locally expressed during aortic valve stratification in chicken embryos

In situ hybridization of sectioned E14 chicken embryo hearts shows expression of (A) *osteonectin*, (B) *periostin*, (C) *id2*, (D) *matrix gla protein* (MGP), (E) *Wnt3a* and (F) *LRP5* in aortic valve cusps (arrows). Expression also is apparent in the aorta (asterisks). The aortic valve annulus is indicated by arrowheads in B.

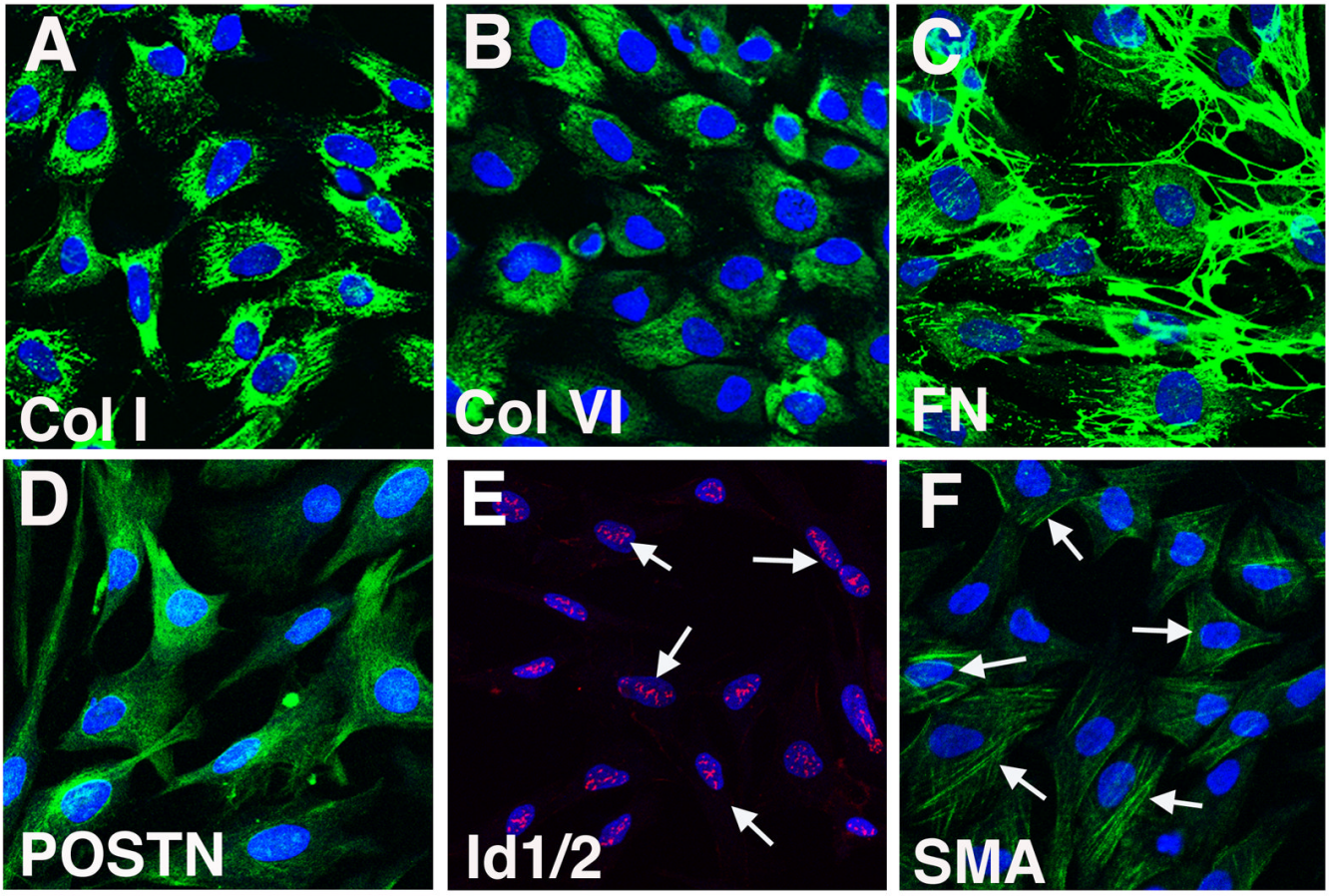

Figure 6. Cultured E14 chicken aortic valve cells have a fibrosa-like phenotype

Immunostaining and confocal analysis demonstrates expression of (A) Collagen I, (B) Collagen VI, (C) fibronectin (FN), (D) periostin (POSTN), (E) Id1/2 and (F) smooth muscle α-actin (SMA) in aortic valve cells cultured for 5d. Indicated proteins are shown in green with the exception of Id1/2, which is red. Nuclear staining of Id1/2 and distribution of SMA are indicated by arrows. Nuclei are stained blue with ToPro3.

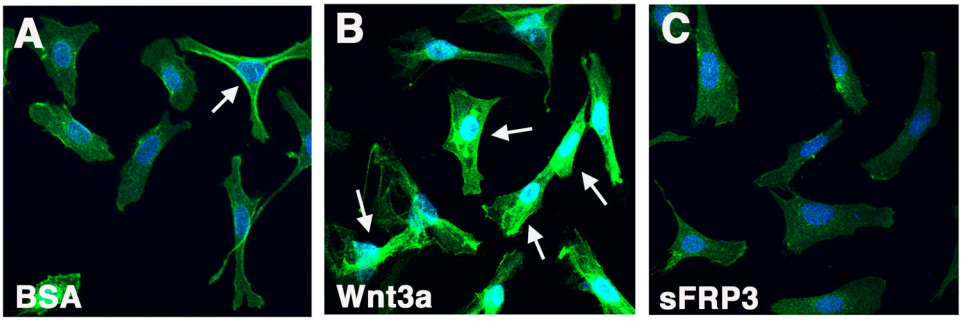

Figure 7. β-catenin protein expression is increased in E14 Aortic VIC treated with Wnt3a but not sFRP3

Primary E14 aortic VIC cultures were treated with (A) control vehicle BSA, (B) Wnt3a or (C) sFRP3 for 1hr. Increased nuclear and cytoplasmic β-catenin protein (green) was detected by immunofluorescence and confocal microscopy (arrows) in Wnt3a-treated relative to control or sFRP3-treated cells. Nuclei (blue) were visualized using ToPro3.

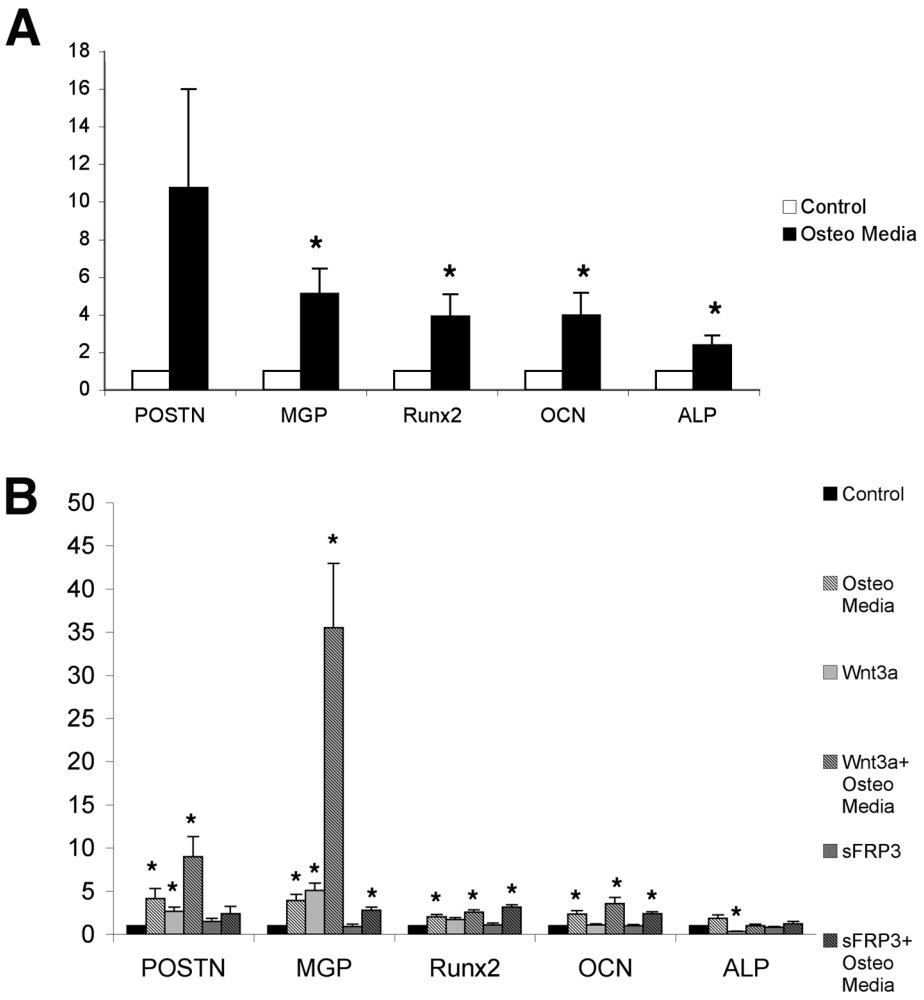

Figure 8. Induction of osteoblast-associated genes in avian E14 aortic VIC cultures treated with β-glycerophosphate/ascorbic acid is enhanced by increased Wnt signaling

(A) E14 embryonic aortic valve cells were cultured in the presence or absence of osteogenic (osteo) media containing β-glycerophosphate (10mM) and ascorbic acid (50μg/ml). Expression levels of *periostin (POSTN)*, *matrix-gla protein (MGP)*, *Runx2*, *osteocalcin (OCN)* and *alkaline phosphatase (ALP)* transcripts were determined by qRT-PCR in untreated (white bars) and treated (black bars) VIC cultures. Control values were set to 1 and fold differences are shown with error bars representing s.e.m. Statistically significant increases in gene expression in induced cells ($p<0.05$) are indicated by asterisks. Baseline levels of expression are *POSTN* (19.26), *MGP* (3710.23), *Runx2* (957.22), *OCN* (10.90), *ALP* (100.37) relative fluorescence units. (B) Primary chicken embryo (E14) aortic valve cells were cultured as in (A) with the addition of groups treated with Wnt3a or the Wnt antagonist sFRP3. Expression levels of *POSTN*, *MGP*, *Runx2*, *OCN* and *ALP* transcripts were determined by qRT-PCR for all groups. Control values were set to 1, and fold differences are shown for a representative experiment. Note induction of *MGP* and *POSTN* in the presence of osteogenic media is enhanced by Wnt3a and is inhibited by sFRP3 treatments.

Table 1

Oligonucleotide sequences of primers for PCR

A. Primers for generation of anti-sense RNA probes			
Gene	**Product size**	**Annealing temp °C**	**Sequence**
Mouse			
Wnt2	831	62	5′-CTGGCTCTGGCTCCCTCTGCTCTT-3′
NM_023653.4			5′-GCCTGCCTCTCGGTCCCTGATACA-3′
Wnt3a	795	63	5′-AGCCACCCGGGAGTCAGCCTTTGT-3′
NM_009522.1			5′-GGAACCTTGCCCCTCGGAATGAAC-3′
Wnt4	776	62	5′-CGGAACCGGCGCTGGAACT-3′
NM_009523.1			5′-GCACTGCCGGCACTTGACGAA-3′
Wnt7b	844	63	5′-CAGCGGTGCAGGTGGAGGTGGTG-3′
NM_009528.2			5′-AGGGAGGGGCCGGGAGGAGTGAG-3′
Wnt9b	758	61	5′-AGGACTTGGGGCCGGAGAACAGAC-3′
NM_011719			5′-TGAGAGCAGGGGGTGGAAGCAGAC-3′
Lef1	639	62	5′-GCAGCGGAGCGGAGATTACACAGC-3′
NM_010703			5′-CCAACGCCGCCCGGAGACAGG-3′
Fzd2	878	64	5′-CGTCACGGCGCGGAGCAGAT-3′
NM_020510.2			5′-AAGCCGGCCAGCAGGAAGGATGTA-3′
Chicken			
Osteonectin	608	58	5′-GCTGCAAGATGAGAACCTGGAT-3′
NM_204410.1			5′-GGCGCTTCTCATTCTCGTGGAT-3′
Id2	891	58	5′-ACTCCAAGCTGAAGGAGCTG-3′
NM_205002.1			5′-AAACGTCCATTTCTGCCACT-3′
MGP	615	60	5′-TACAGGCTGGCTGGGAAG-3′
NM_205044.1			5′-CAAACACCCACATCCTGAAC-3′
Wnt3a	782	60	5′-AAGTCGTTCTGCAGCGAAGT-3′
NM_204675			5′-AGCGCTGTCGTATTTGTCCT-3′
LRP5	820	60	5′-TTGACGCGCATCTGAGGTGTAA-3′
NM_001012897			5′-CCACACCTCGCAGCCAATACATG-3′
B. Primers for qRT-PCR			
Chicken			
Periostin	271	65	5′-GTGCTGTCCTGGCTACATGA-3′
NM_001030541			5′-TGTGGTGGTGGAGAGCATTA-3′
MGP	615	60	5′-TACAGGCTGGCTGGGAAG-3′
NM_205044.1			5′-CAAACACCCACATCCTGAAC-3′
Runx2	211	65	5′-CCCCGGCTCCTCCCAAAACCAA-3′
NM_204128			5′-CCGCCTCCACACCGTCACTCTG-3′
Osteocalcin	175	60	5′-AGTGGAGCTGCACCATGAA-3′
NM_205387.1			5′-GCTGTCCTGGGCGTAGTG-3′
ALP	210	60	5′-GCGGCTGCAGCACCTCAACC-3′
XM_423297			5′-CGCGCTGTCGGGCACCTGT-3′