Bmp signaling represses Vegfa to promote outflow tract cushion development

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SUMMARY
Congenital heart disease (CHD) is a devastating anomaly that affects ~1% of live births. Defects of the outflow tract (OFT) make up a large percentage of human CHD. We investigated Bmp signaling in mouse OFT development by conditionally deleting both Bmp4 and Bmp7 in the second heart field (SHF). SHF Bmp4/7 deficiency resulted in defective epithelial to mesenchymal transition (EMT) and reduced cardiac neural crest ingress, with resultant persistent truncus arteriosus. Using a candidate gene approach, we found that Vegfa was upregulated in the Bmp4/7 mutant hearts. To determine if Vegfa is a downstream Bmp effector during EMT, we examined whether Vegfa is transcriptionally regulated by the Bmp receptor-regulated Smad. Our findings indicate that Smad directly binds to Vegfa chromatin and represses Vegfa transcriptional activity. We also found that Vegfa is a direct target for the miR-17-92 cluster, which is also regulated by Bmp signaling in the SHF. Deletion of miR-17-92 reveals similar phenotypes to Bmp4/7 SHF deletion. To directly address the function of Vegfa repression in Bmp-mediated EMT, we performed ex vivo explant cultures from Bmp4/7 and miR-17-92 mutant hearts. EMT was defective in explants from the Bmp4/7 double conditional knockout (dCKO; Mef2c-Cre;Bmp4/7fl/fl) and miR-17-92 null. By antagonizing Vegfa activity in explants, EMT was rescued in Bmp4/7 dCKO and miR-17-92 null culture. Moreover, overexpression of miR-17-92 partially suppressed the EMT defect in Bmp4/7 mutant embryos. Our study reveals that Vegfa levels in the OFT are tightly controlled by Smad- and microRNA-dependent pathways to modulate OFT development.

KEY WORDS: Bone morphogenetic protein, microRNA, Vascular endothelial growth factor A, Epithelial to mesenchymal transition, Outflow tract, Mouse

INTRODUCTION
Congenital heart disease (CHD) occurs in nearly 1% of all live births and is the leading cause of infant mortality and morbidity in the western world. Persistent truncus arteriosus (PTA) is the most severe phenotype of outflow tract (OFT) defects, with an unfavorable prognosis as surgical repair is not always possible (Williams et al., 1999). Although discovering the genetic causes would provide insight into the pathogenesis of CHD, the etiology of most human CHD, including OFT defects, remains unknown because of the multifactorial nature of the diseases, indicating the need for more study (Bruneau, 2008; Olson, 2006; Yamagishi et al., 2009; Zhao and Srivastava, 2007).

The OFT is derived from the second heart field (SHF), with a major contribution from cardiac neural crest (CNC) (Diman et al., 2011). The OFT, which first forms as a single tube, separates into the aorta and pulmonary trunk through both epithelial to mesenchymal transition (EMT) proximally and CNC influx into distal OFT. Endocardial cells in the proximal OFT undergo EMT and contribute to the proximal OFT cushions and to a subset of cells within semilunar valves. Defects in EMT and CNC influx are associated with several CHDs. During OFT development, endocardial cells sense and respond to inductive signals from overlying myocardium and initiate the process of EMT to contribute to conotruncal cushions. Several genes have been shown to regulate EMT, including Twist1, Snai1 and Slug (Snai2) (Ma et al., 2005; Niessen et al., 2008; Yang et al., 2004).

Other studies show that Bmp signals are required for OFT morphogenesis (Liu et al., 2004; Ma et al., 2005; McCulley et al., 2008). Bmp2 is involved in EMT during atrioventricular (AV) cushion development (Ma et al., 2005), whereas Bmp4 is crucial for OFT separation and cushion development (Liu et al., 2004; McCulley et al., 2008). Bmp4 mutant embryos show variability in phenotype with incomplete penetrance, possibly owing to the functional redundancy of Bmp ligands. Although ablation of Bmp7 has no obvious phenotypic consequences in the heart (Solloway and Robertson, 1999), loss of Bmp4 causes upregulation of Bmp7 in OFT myocardium and compound mutants have a more severe phenotype (Liu et al., 2004). The overlapping expression of Bmp ligands in the OFT and their redundancy in function makes functional analysis of Bmp ligands challenging.

Bmp signaling is transduced through a canonical pathway involving phosphorylation of Smad1/5/8 (R-Smad) from the ligand-receptor complex. Phosphorylated R-Smads then form a complex with Smad4 (Co-Smad) and this complex shuttles from cytoplasm to nucleus. Since Smads bind DNA motifs with low affinity and selectivity (Itoh et al., 2000; Karaulanov et al., 2004; Shi and Massagué, 2003; Zwijsen et al., 2003) they associate with various transcription factors to bind DNA and promote or repress transcription (Kawabata et al., 1998; Zwijsen et al., 2003).

Recent studies have shown that Bmp signaling also regulates downstream genes through microRNA (miRNA) maturation. In vascular smooth muscle, TGFβ- and Bmp-specific Smads are recruited to the p68 (Ddx5) RNA helicase-containing complex. The Smad-p68 complex interacts with the miRNA processing enzyme Drosha in the nucleus to regulate the biogenesis of a subset of...
miRNAs, including miR-21 (Davis et al., 2008). During cardiac development, Bmp2/4 regulate OFT myocardial differentiation by promoting Smad-mediated transcription of the mir-17-92 cluster (Wang et al., 2010). The mir-17-92 cluster, which encodes miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1, is oncogenic and also functions in the development of the heart, lungs and immune system (Koralov et al., 2008; Ventura et al., 2008; Vincentz et al., 2008; Xiao et al., 2008).

In this study, we discovered that loss of Bmp4/7 in the SHF resulted in PTA with upregulation of Vegfa, repression of which is required in the atrioventricular canal (AVC) to promote EMT (Chang et al., 2004). Our in vitro studies showed that Vegfa is repressed by both Smad and miR-17-20a. Moreover, in vivo and ex vivo studies showed that overexpression of mir-17-92 or repression of Vegfa can partially suppress the Bmp loss-of-function phenotype. Our study reveals that in the OFT, EMT and CMC influx are regulated by Bmp via a combination of Smad- and miRNA-dependent Vegfa repression.

MATERIALS AND METHODS

Mouse lines

The Bmp4 flox, mir-17-92 null and mir-17-92+/flox lines have been described previously (Liu et al., 2004; Ventura et al., 2008; Verzilli et al., 2005; Xiao et al., 2008). To generate the Bmp7 flox allele, a targeting vector was constructed that introduced one loxP site upstream of Bmp7 exon 4 followed by an FRT-flanked PGKneo cassette, while another loxP site was introduced downstream of Bmp7 exon 4 (see supplementary material Fig. S1).

β-galactosidase staining, histology and immunostaining

Embryos were fixed overnight in 4% paraformaldehyde or buffered formalin, dehydrated through a graded ethanol series, and paraffin embedded. Sections were cut at 7 μm and used for Hematoxylin and Eosin (H&E) staining or immunostaining. Detection of lacZ expression on whole embryos was performed as previously described (Lu et al., 1999). For Twist1 immunodetection, sections were deparaffinized and hydrated. For antigen retrieval, 10 mM citrate buffer was used. Monoclonal anti-Twist1 (Abcam, ab50887; 1:200) was used as primary antibody. HRP-conjugated anti-mouse secondary antibody (Bio-Rad) was visualized using TSA Plus Fluorescence Systems (PerkinElmer). Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen).

Quantitative real-time RT-PCR

Total RNA from OFT (E10.5 embryos) was isolated using the RNeasy Micro Kit (Qiagen). SuperScript II reverse transcriptase (Invitrogen) was used for reverse transcription (RT)-PCR with 20–100 ng mRNA and SYBR Green JumpStart Taq ReadyMix (Sigma) in triplicate reactions on a StepOnePlus Real-Time PCR System (ABI); Gapdh was used as internal control. Vegfa was detected by TaqMan probe (IDT, Mm.PT.47.7135538.g) with 18s rRNA (IDT) as internal control. For miRNAs, TaqMan MicroRNA Assay Kits (Applied Biosystems) were used according to the manufacturer’s guidelines. Sno-202 was used as internal control. For all qRT-PCR experiments, at least four mutant and four control embryos were analyzed. All error bars represent s.e.m. Primer sequences are available in supplementary material Table S1.

Sequence analysis and miRNA target prediction

For sequence analysis and alignment, NCBI (http://www.ncbi.nlm.nih.gov/), Ensemble (http://uswest.ensemble.org/index.html), rVista 2.0 (http://rvista.dcode.org/) and TFSEARCH (http://mbs.cbrj.isrpm舠h) were used. For miRNA target prediction, miranda (http://www.microrna.org), TargetScan (http://www.targetscan.org) and miRDB (http://miadb.org/miRDB) were used.

Generation of constructs

To generate the Vegfa promoter luciferase reporter plasmid, a 1563 bp fragment of the mouse Vegfa promoter was amplified using a high-fidelity PCR system (Roche) and subcloned into the pGL3-Basic vector (Promega). To generate the Vegfa 3’UTR luciferase reporter plasmid, 1881 bp of Vegfa 3’UTR genomic sequence was amplified using the high-fidelity PCR system from a cDNA clone (#6816435, Open Biosystems) and subcloned into the pCMV-REPORT Lucifase miRNA Expression Reporter Vector (Ambion). Site-directed mutagenesis of the seed sites in the Vegfa 3’UTR and Vegfa promoter was performed with the QuickChange II Site-Directed Mutagenesis Kit (Stratagene). Primers are listed in supplementary material Table S1.

Luciferase activity assay

P19 cells were transfected with the Vegfa promoter and 3’UTR reporter plasmids described above using Lipofectamine 2000 (Invitrogen). Luciferase activity assays were performed using the Dual-Luciferase Reporter Assay System (Promega). The Renilla luciferase-encoding plasmid pRL-TK was used as the normalization control. Ca-Alk3 (Wang et al., 2010) was used to activate the Smad pathway. miRNA mimics were purchased from Thermo Scientific.

Ex vivo OFT explant culture

A solution (1.5 mg/ml) of rat tail collagen type I (BD Biosciences) was dispensed into 24-well microculture dishes and allowed to solidify in a 37°C: 5% CO2 incubator. Collagen gels were washed several times with DMEM containing 10% fetal bovine serum (FBS), 0.1% insulin-transferrin selenium (ITS, Gibco, Invitrogen) and 100 unit/ml penicillin, 100 μg/ml streptomycin (1:100, penicillin-streptomycin solution, Hyclone, Thermo Scientific). OFTs were harvested in sterile Tyrode’s Salt Solution (Sigma-Aldrich) from E10.5 embryos. OFTs were carefully dissected, and endocardium was exposed to the collagen gel, with the myocardium overlaying. Four hours after attachment, medium (100 μl/well) was added and explants cultured for up to 3 days. Explants were fixed and stained with anti-alpha-SMA-Cyan3 (1:100; Sigma-Aldrich) to detect mesenchymal cells and phallolidin-FITC (1:100; Sigma-Aldrich) to reveal the actin cytoskeleton as described (Luna-Zurita et al., 2010). TO-PRO-3 iodide (TOP3; Invitrogen) was used for nuclei staining. For explant treatments, the medium was supplemented with sFlt (10 ng/ml; R&D Systems, 471-F1). Medium was replaced every 24 hours.

In vivo chromatin immunoprecipitation

About 80 OFTs of E10.5 wild-type embryos were dissected and subjected to chromatin immunoprecipitation (ChIP) analysis. Soluble chromatin was prepared after formaldehyde crosslinking and sonication. Smad1/5/8 antibody (sc-6031 X, Santa Cruz) was used to immunoprecipitate protein-bound DNA fragments; rabbit IgG was used as control. The PCR products for the SBE1, SBE2 and SBE3 fragments are 280 bp, 402 bp and 204 bp, respectively. Primers that amplified sequence irrelevant for Smad binding were used as a negative control. SYBR Green was used for ChIP-qPCR, and results were analyzed by the percentage input method (Invitrogen). Primer sequences are listed in supplementary material Table S1.

RESULTS

Bmp2, Bmp4 and Bmp7 regulate EMT and have redundant functions

Bmp2, Bmp4 and Bmp7 show overlapping expression patterns and functional redundancy (Bonilla-Claudio et al., 2012; Dudley and Robertson, 1997; Furuta et al., 1997; Wang et al., 2010). We compared their expression patterns using lacZ knock-in mouse lines for Bmp2 and Bmp4 and in situ hybridization of Bmp7. Bmp2 and Bmp4 were expressed in the OFT at E9.5, whereas Bmp7 was broadly expressed throughout the heart tube (supplementary material Fig. S2A,B,G,H,M,N). Bmp4 expression was found in the inflow tract at E9.5, but was limited to the OFT at E10.5. Analysis of the sections revealed that Bmp2, Bmp4 and Bmp7 were exclusively co-expressed in OFT myocardium (supplementary material Fig. S2E,I,L,O,R). At E10.5, expression of Bmp2 in the OFT was decreased (supplementary material Fig. S2G,J), whereas Bmp4 and Bmp7 expression was maintained at similar levels (supplementary material Fig. S2A,D,M,P).
To examine functional redundancy, we deleted Bmp2, Bmp4 and Bmp7 in different combinations in the OFT using Mef2c-Cre (Fig. 1A-H). Proximal OFT mesenchymal cells were absent from Bmp2/4, Bmp4/7 and Bmp2/7 double conditional knockout (dCKO) embryos (Fig. 1C-F). Bmp4/7 deletion resulted in OFT cushion defects in CNC-derived distal OFT, as well as proximal OFT (Fig. 1D). The distal OFT defects were not observed upon deleting Bmp2 in a Bmp4 homozygous or heterozygous background, suggesting that Bmp2 has limited function in the OFT proper (Fig. 1C,F).

Consistent with the defective CNC influx and proximal EMT observed at E10.5, Bmp4/7 mutants had severe OFT defects at E14.5, with failure of OFT separation resulting in PTA (Table 1, Fig. 1I-L). One Bmp4/7 dCKO embryo had double outlet right ventricle (DORV), with both aortic and pulmonary arteries draining the right ventricle (Table 1). In addition to defective great vessel separation, histological sections revealed hypoplastic semilunar valves and an interventricular septal defect (VSD; data not shown) in the Bmp4/7 dCKO embryos at E10.5. RT-PCR analysis was performed using SYBR Green for Id1/2, Snai1 and Slug and TaqMan for Vegfa. Control, n=5; mutant, n=4. Error bars represent s.e.m. *P<0.004, **P<0.03, ***P<0.04 (two-tailed t-test). Scale bars: 100 μm in A-I; 500 μm in J,L; 50 μm in M,N.

Table 1. OFT defects in Bmp2/4/7 conditional knockout embryos at E14.5

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<th>DORV</th>
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<tr>
<td>Mef2c-Cre;Bmp2f/+Bmp4f/f</td>
<td>5/5</td>
<td>n/a</td>
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<tr>
<td>Mef2c-Cre;Bmp2f/+Bmp4f/f</td>
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The frequency of persistent truncus arteriosus (PTA) and double outlet right ventricle (DORV) among total embryos examined is shown for each genotype. n/a, not applicable.

Fig. 1. Inactivation of Bmp2, Bmp4 and Bmp7 in the SHF. (A-H) Hematoxylin and Eosin-stained sagittal sections of E10.5 mouse embryos with different combinations of Bmp gene deletion. The Bmp genes were knocked out conditionally in the second heart field (SHF) using Mef2c-Cre. Black arrowheads indicate proximal outflow tract (OFT) and white arrowheads distal OFT. For A-H, n=6, 5, 6, 3, 2, 5, 1-34, 1-5 (I-L) Whole-mount (I,K) and sections (J,L) of control and Bmp4/7 dCKO hearts at E14.5. The arrowhead in K indicates persistent truncus arteriosus (PTA); ba, branchial arch; m, myocardium; mc, mesenchymal cushion; e, epithelium; ao, aorta; pa, pulmonary artery; la, left atrium; ra, right atrium; lv, left ventricle; rv, right ventricle; av, aorta valve; sv, semilunar valve; pta, persistent truncus arteriosus; ivs, interventricular septum. (M,N) Immunostaining of Twist1 in control and Bmp4/7 dCKO embryos at E10.5. Arrowheads point to Twist1-positive cells. (O) The expression of EMT-related genes was examined in the OFT of control and Bmp4/7 dCKO mice at E10.5. *P<0.004, **P<0.03, ***P<0.04 (two-tailed t-test). Scale bars: 100 μm in A-I; 500 μm in J,L; 50 μm in M,N.

Smad directly represses Vegfa transcription
To investigate EMT in the Bmp4/7 dCKO, we performed phosphohistone H3 (pH3) analysis for cell proliferation and did not observe any obvious difference between the control and Bmp4/7 dCKO in the myocardium (data not shown). Immunostaining showed fewer Twist1-expressing mesenchymal cells in the OFT of the Bmp4/7 dCKO than the control (Fig. 1M,N), indicating that fewer cells progressed through EMT in the Bmp4/7 mutant. To determine the mechanism of EMT regulation by Bmp, the expression of genes involved in the Bmp pathway and EMT was examined by qRT-PCR. The Bmp-regulated EMT genes Snai1, Slug, Id1 and Id2 were significantly decreased in the Bmp4/7 dCKO OFT, whereas Vegfa was upregulated 1.8-fold (Fig. 1O).
These results indicate that Smad1/5/8 can directly bind Vegfa chromatin in the OFT.

To determine whether Smad directly regulates Vegfa transcription, we cloned a ~1500 bp region upstream of Vegfa and generated a luciferase reporter (Fig. 2D). To activate the Smad-dependent Bmp pathway, we co-transfected a constitutively active form of the type 1 Bmp receptor Alk3 (ca-Alk3) into the mouse P19 cell line (Wang et al., 2010) (Alk3 is also known as Bmpr1a – Mouse Genome Informatics). Luciferase assays demonstrated that Vegfa promoter activity was significantly diminished, by 35%, in the presence of ca-Alk3 (Fig. 2E). To determine whether Vegfa promoter repression was due to direct Smad binding, we introduced mutations into the predicted Smad binding sites (Fig. 2D).

Mutations in SBE1, SBE2 and/or SBE3 resulted in loss of promoter repression was due to direct Smad binding, we introduced mutations into the predicted Smad binding sites (Fig. 2D).

Alternative downstream Bmp effectors include miRNAs, which are involved in the refinement of gene expression. miR-17-92 is downstream of the Bmp pathway in cardiac development (Wang et al., 2010). Bioinformatic analysis predicted a highly conserved miR-17-92 binding site in the 3′ UTR of Vegfa (Fig. 3A). The levels of pri-miR-17-92 and mature miR-17/20a were decreased in the OFT downstream of Bmp4/7 dCKO embryos (Fig. 3B), showing that miR-17-20a are downstream of Bmp4/7.

To determine the role of miR-17-92 during OFT development, we analyzed miR-17-92 null (miR-17-92ΔN) embryos. At E10.5, miR-17-92ΔN embryos showed OPT defects, including lack of mesenchymal cushion formation (Fig. 3C,D). miR-17-92ΔN embryos developed no mesenchymal cells in either the proximal or distal OFT, resembling the phenotype of the Bmp4/7 dCKO. At E14.5, miR-17-92ΔN embryos exhibited the DORV phenotype, together with abnormal semilunar valve and VSD (Fig. 3E,F). Notably, loss of miR-17-92 resulted in a 1.7-fold increase in the level of Vegfa (Fig. 3G). This upregulation of Vegfa is similar to that observed in the Bmp4/7 dCKO (Fig. 1O). Taken together, our results suggest that miR-17-92 is downstream of Bmp signaling during EMT in the OFT.

To determine whether Vegfa can be regulated by miR-17 and miR-20a, we cloned 1874 bp of the native Vegfa 3′UTR and constructed a luciferase reporter vector (Fig. 3H). Co-transfection with miR-17/20a resulted in suppression of the Vegfa 3′UTR reporter (Fig. 3I), whereas miR-92a did not suppress the luciferase activity. Then we mutated the miR-17/20a binding site in the Vegfa 3′UTR (Fig. 3H). Suppression by both miR-17 and miR-20a was abolished in the mutated Vegfa reporter (Fig. 3I), showing that the putative miR-17/20a site was responsible for 3′UTR silencing of Vegfa transcription.
Vegfa. These results strongly suggest that miR-17/20a are downstream effectors of Bmp4/7 in repression of Vegfa.

**Repression of Vegfa regulates EMT during OFT development**

Vegfa is known to inhibit mesenchymal transformation in the AV cushion (Chang et al., 2004); however, the potential roles of Vegfa in EMT during Bmp-mediated OFT development have not been investigated. To determine if Bmp regulates EMT through Vegfa repression, we investigated whether reducing Vegfa can rescue the EMT defect caused by Bmp4/7 deletion. We performed ex vivo analysis by culturing the OFT from E10.5 embryos on collagen gel, with the endocardium exposed to the gel (Fig. 4A). In control explants, a halo of mesenchymal cells formed around the myocardium, with non-invasive mesenchymal cells on the surface of the gel and invasive mesenchymal cells invading the collagen depths (Fig. 4B,C). In the OFT of the Bmp4/7 dCKO, both invasive and non-invasive EMT were absent (Fig. 4D,E). Administration of sFlt (Flt1 – Mouse Genome Informatics), a soluble Vegfa antagonist, partially suppressed the mesenchymal transformation defect in the Bmp4/7 dCKO by rescuing non-invasive mesenchyme cell formation (Fig. 4F). Invasive EMT was not rescued by antagonizing Vegfa (Fig. 4G), suggesting that this process is regulated by other downstream Bmp targets, such as Snai1.

Next, to determine whether miR-17 and miR-20a regulate EMT through Vegfa, we performed explant culture from miR-17-92N/N OFT (Fig. 4H,I). The OFT from the miR-17-92 null lacked both invasive and non-invasive EMT (Fig. 4H,I), recapitulating the explant from the Bmp4/7 dCKO (Fig. 4D). After addition of the Vegfa antagonist sFlt to the explant, both the non-invasive and invasive EMT processes were rescued in the miR-17-92N/N explants (Fig. 4J,K), showing that Vegfa downregulation was sufficient to rescue the miR-17-92 loss-of-function phenotype. Since antagonizing Vegfa was not sufficient to rescue invasive EMT in the explant from the Bmp4/7 dCKO (Fig. 4G), regulation of Vegfa by miR-17-92 must be just one of multiple downstream effectors of Bmp signaling.

**miR-17-92 can rescue defects in EMT caused by loss of Bmp**

To address the hypothesis that miR-17/20a are direct downstream effectors of Bmp in the EMT process in vivo, we overexpressed miR-17-92 in the Bmp4/7 dCKO by crossing miR-17-92OE/+(Xiao et al., 2008) and Mef2c-Cre lines. qRT-PCR data showed that miR-17 and miR-20a were moderately downregulated in the OFT of Mef2c-Cre;miR-17-92OE/+(Fig. 5A). Vegfa levels were moderately downregulated in the OFT of Mef2c-Cre;miR-17-92OE/+(Fig. 5A), demonstrating that miR-17-92 represses Vegfa in vivo. To determine whether miR-17-92 can compensate for the loss of Bmp4/7, we generated Mef2c-Cre;Bmp4/7f/f;miR-17-92OE/+(Fig. 5A), and OFT septation was partially rescued in the distal part (data not shown). The level of Vegfa in Mef2c-Cre;Bmp4/7f/f;miR-17-92OE/+(Fig. 5A) was decreased compared with Mef2c-Cre;Bmp4/7f/f and comparable to that of control embryos (Fig. 5B), suggesting that suppression of Vegfa by Bmp was mediated by miR-17 and miR-20a. These results strongly suggest that miR-17 and miR-20a are downstream effectors of the Bmp signal during OFT development.
Although cushion formation was observed in Mef2c-Cre;Bmp4/7+/miR-17-92OE/+ embryos, there were fewer mesenchymal cushion-forming cells in the rescued embryos, indicating partial suppression (Fig. 5C-F). In addition, the extent of mesenchymal cushion formation was variable (Fig. 5C-F, Table 2). Together, these data support the hypothesis that Bmp signals are mediated by effector mechanisms in addition to miR-17.

**DISCUSSION**

Our data uncover a direct interaction between the Bmp and Vegfa signaling pathways that are important for normal OFT development. Moreover, our findings uncover a mechanism to tightly modulate Vegfa activity levels in the OFT. Our results also provide new insight into the mechanism for coordinated development of CNC and endocardial cells as they contribute to the conotruncal cushions. Lastly, our data reveal that miR-17-92 indirectly, via non-cell-autonomous mechanisms, regulates CNC development in addition to that of SHF-derived tissues.

**Coordinated regulation of cushion formation derived from multiple cell types**

The conotruncal cushions are derived from both CNC and the endocardium. Bmp signaling has been implicated previously in EMT within the AVC. We now show that Bmp signaling is also important for EMT within the OFT. Previous data in the AVC indicate that Bmp regulates genes such as Twist and Snai1 to promote EMT and, although we did not focus on that mechanism here, it is likely that similar mechanisms are at work in both the OFT and AVC. Our finding that Bmp signaling negatively regulates Vegfa in the OFT provides important insight into the coordinated regulation of the OFT cushions. In the OFT, in contrast to the AVC, there are three separate tissues developing in close proximity: myocardium, endocardium and CNC. We provide evidence that Vegfa repression mediated by Bmp–miR-17-92 is a crucial mechanism to ensure coordinated OFT development.

The importance of Vegf signaling in endocardium has recently been demonstrated. Deleting the Vegf receptor neuropilin 1 (Nrp1)
in endocardial cells disrupts OFT development, with abnormal cushion morphogenesis (Zhou et al., 2012). This indicates that endocardium is a target cell for Vegfa signaling. There are no previous findings implicating Vegfa signaling in CNC development. Our data suggest that elevated Vegfa inhibits CNC influx into the OFT; however, more experiments are required to confirm this.

A mechanism to tightly modulate Vegfa activity in the developing heart

Previous genetic studies have shown that the Vegfa dose must be maintained within a narrow window for normal embryonic development. Vegfa heterozygosity, as well as small gains in Vegfa activity, result in defective embryonic development. Loss of a single Vegfa allele is embryonic lethal at mid-gestation due to impaired angiogenesis and blood island formation (Ferrara et al., 1996). Moreover, there are cardiomyoblast differentiation defects in Vegfa heterozygotes. Haigh et al. found that heterozygotic inactivation of Vegfa using Col2a1-Cre caused lethality at E10.5, with defects in the myocardial and endocardial layers of the heart (Haigh et al., 2000). A different Vegfa allele, Vegfa<sup>−/−</sup>, results in 3-fold overexpression of Vegfa during development; heterozygotes for this allele are viable, whereas homozygous embryos die at E9.0 with dorsal aortae defects and severe abnormalities in yolk sac vasculature (Damer et al., 2002).

Our data indicate that Vegfa activity in the OFT is concurrently regulated by Smad transcriptional repression and post-transcriptional repression by miRNAs. Vegfa has previously been reported to be regulated by miRNAs, including the miR-17 family, in other contexts including cultured breast cancer cells and diabetes (Cascio et al., 2010; Long et al., 2010). However, our finding that miRNA regulation cooperates with Smad-mediated repression provides new insight into the mechanisms underlying tight Vegfa dosage regulation.

Bmp interacts with calcineurin-NFAT signaling

Previous work indicated that Nfatc1 mutants have cotrunical valve phenotypes similar to those of a Mef2c-Cre;Bmp2/4/7 triple mutant (Y.B. and J.F.M., unpublished). Moreover, similar to what we have discovered for Bmp signaling, Nfatc1 inhibits Vegfa expression in the E9.5 OFT to permit EMT (Chang et al., 2004; Stankunas et al., 2010). Nfatc1 expression is reduced in the AV cushion endocardium of Bmp2 conditional mutant embryos (Ma et al., 2005). There is evidence from pulmonary vascular cells that NFAT and Bmp are in a linear genetic pathway (Chan et al., 2011). Other intriguing data indicate that NFAT can promote chondrogenesis in vitro, a known function for Bmp signaling (Tomita et al., 2002). More work will be required to reveal potential interactions between calcineurin-NFAT and Bmp signaling in the developing OFT.

Acknowledgements

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Table 2. OFT mesenchymal phenotype of the Bmp4/7 mutant

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The occurrence of mesenchymal cushion cells in the proximal and distal OFT among total embryos examined is shown for each genotype.


Fig. S1. Targeting scheme for the inactivation of Bmp7. (A) Targeting strategy for Bmp7 ablation. (B) Southern blot analysis of the Bmp7 flox allele. (C) qRT-PCR analysis of Bmp7 in wild-type and knockout samples. (D) Quantification of Bmp7 mRNA level.
Fig. S2. Expression of Bmp2/4/7 in the heart at E9.5 and E10.5. (A-F) Bmp4 is expressed in SHF, OFT, inflow tract and right ventricle at E9.5. Sections reveal that Bmp4 is exclusively expressed in myocardium and not in endocardium or cushion mesenchyme of the OFT. (G-L) Bmp2 is expressed in the OFT and AV cushion in E9.5 and E10.5 embryos. Its expression in the OFT is limited. (M-R) In situ hybridization shows that Bmp7 is broadly expressed throughout the entire heart tube. At E10.5, Bmp4 expression is restricted to the myocardium of the OFT; Bmp7 expression is similar to that at E9.5. Scale bars: 100 µm.
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<td>GGTGATCAAACCTACCTAAACCTCAAGAAGAAG</td>
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<tr>
<td>SBE1-mut</td>
<td>CAGGAGGAAACAGGGCCCTCTGAATCCAGCTGTCCTGCTTCAG</td>
<td>GAAGGAGGAGACCTGGAATTCAGAGGCCCTTGATTCCTC</td>
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<tr>
<td>SBE2-mut</td>
<td>CTGGCTCCAGGGCTCTGCTGAGACAGATGCACACGTC</td>
<td>CCACGTATGCACCTGTCTCGAGGCAAGAGGCCCTGAGAG</td>
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<tr>
<td>SBE3-mut</td>
<td>CGTGAAACTTTGGGGAGCAGGACTTTTCGTAGGGGAGGAGCCCTTGA</td>
<td>CACACGCGCCTCCCTCACGAATTTTCGGCTCGAGCACAG</td>
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<td>3’UTR-mut</td>
<td>GGAGACTCTTCGAGGACTCTCGAGGGTCGGGGAGGAGGGAG</td>
<td>GGAGACTCTTCGAGGACTCTCGAGGAGGTCCGGAGGAGGAGACTCCCG</td>
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<tr>
<td>Site1-ChIP</td>
<td>TGGGGAGGAGACAGATCACCCCTGA</td>
<td>CAGAGGCCTTTGTCTCCCTCAG</td>
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<tr>
<td>Site2-ChIP</td>
<td>GGCAACCCTGCGTTTCAGGTCC</td>
<td>TGCGTGGTTGACCTCGGAAAGC</td>
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<tr>
<td>Site3-ChIP</td>
<td>CCCCTTTCCAGACCCTGCACCCCTGA</td>
<td>ACGGGCTTTCTCCCTCAG</td>
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<tr>
<td>Id1</td>
<td>CGGCTCACAGCACCCTGACAG</td>
<td>CCGGGTGGGACGAGACAAC</td>
</tr>
<tr>
<td>Id2</td>
<td>TCCAGGAGCGCCGTGACC</td>
<td>CAAGCTCGTTTCTCCTGGAATGGC</td>
</tr>
<tr>
<td>Slug</td>
<td>GCACGGATGCCAGCTCAGT</td>
<td>CAGTGAAGGGCAAGAGGAAAG</td>
</tr>
<tr>
<td>Snail</td>
<td>AAACCCACTCGGATGTAAG</td>
<td>GAAGGAGTCCTGGCAGTSEG</td>
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Underlines indicate restriction sites in Vegfa primers and mutation sites in other primers.