Nephronectin regulates atrioventricular canal differentiation via Bmp4-Has2 signaling in zebrafish

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SUMMARY
The extracellular matrix is crucial for organogenesis. It is a complex and dynamic component that regulates cell behavior by modulating the activity, bioavailability and presentation of growth factors to cell surface receptors. Here, we determined the role of the extracellular matrix protein Nephronectin (Npnt) in heart development using the zebrafish model system. The vertebrate heart is formed as a simple linear tube in which myocardium and endocardium are separated by a cell-free layer of extracellular matrix termed the cardiac jelly. During heart development, the cardiac jelly swells at the atrioventricular (AV) canal, which precedes valve formation. Here, we show that Npnt expression correlates with this process. Morpholino-mediated knockdown of Npnt prevents proper valve leaflet formation and trabeculation and results in greater than 85% lethality at 7 days post-fertilization. The earliest observed phenotype is an extended tube-like structure at the AV boundary. In addition, the expression of myocardial genes involved in cardiac valve formation (cspg2, fibulin 1, tbx2b, bmp4) is expanded and endocardial cells along the extended tube-like structure exhibit characteristics of AV cells (has2, notch1b and Alcam expression, cuboidal cell shape). Inhibition of has2 in npnt morphants rescues the endocardial, but not the myocardial, expansion. By contrast, reduction of BMP signaling in npnt morphants reduces the ectopic expression of myocardial and endocardial AV markers. Taken together, our results identify Npnt as a novel upstream regulator of Bmp4-Has2 signaling that plays a crucial role in AV canal differentiation.

KEY WORDS: Nephronectin, Atrioventricular canal, Bmp4, Zebrafish

INTRODUCTION
The vertebrate heart is formed as a simple linear tube in which myocardium and endocardium are separated by a cell-free layer of extracellular matrix (ECM) termed the cardiac jelly. Shortly after the onset of heart looping, local differentiation pathways are activated, initiating the formation of the atrial and ventricular chambers that are separated by a discrete domain known as the atrioventricular (AV) canal. The signaling pathways that underlie these processes are highly conserved across species ranging from zebrafish to human (Stainier, 2001; Beis et al., 2005; Srivastava, 2006). However, the mechanism by which the differentiation of the AV segment in the primary heart tube is controlled remains largely unknown, although some molecular circuits of the regulatory hierarchy, such as Notch and Tbx2/Bmp2, have been implicated in previous studies.

In zebrafish, heart looping starts at ~36 hours post-fertilization (hpf). The chambers express distinct myosin genes [vmhc, amhc (myh6 – Zebrafish Information Network) (Yelon, 2001; Berdugo et al., 2003)] whereas the expression of myocardial genes [e.g. bmp4, cspg2 (wvana – Zebrafish Information Network) (Walsh and Stainier, 2001), fibulin 1 (Zang et al., 1997)] and endocardial genes [e.g. notch1b (Walsh and Stainier, 2001), has2 (Smith et al., 2009)] becomes restricted to the AV boundary. In addition, AV endocardial cells differentiate into cuboidal cells that express Alcam (Alcama, DM-GRASP) (Beis et al., 2005). The differentiated AV endocardial and myocardial cells produce increased amounts of ECM components resulting in cardiac jelly swelling, which initiates valve formation (Moorman and Christoffels, 2003).

Defects in cardiac valves are the most common type of congenital malformation (Loffredo, 2000). Thus, it is important to identify novel regulators of cardiac valve development. Utilizing a microarray-based temporal RNA expression data set describing heart development, we identified the gene nephronectin (npnt) as transiently expressed in the heart at the time of valve initiation and formation. Npnt has previously been identified as a ligand of integrin αβ1 (Brandenberger et al., 2001; Sato et al., 2009). It contains an N-terminal signal peptide followed by EGF-like repeats, an RGD sequence and a C-terminal MAM domain, but it does not contain a transmembrane domain. Thus, Npnt has been classified as an ECM protein (Brandenberger et al., 2001). Recent data have shown that Npnt is required for the invasion of the metanephric mesenchyme by the ureteric bud during kidney development (Linton et al., 2007). Furthermore, it has been associated with malignant melanoma. Npnt overexpression in melanoma cell lines increased cell adhesion and decreased cell migration (Kuphal et al., 2008). In addition, overexpression of Npnt in mouse MC3T3-E1 cells promoted their differentiation into osteoblasts, an effect mediated via the EGF-like repeats (Fang et al., 2010; Kahai et al., 2010). However, to our knowledge, Npnt expression has not thus far been associated with heart development and its function remains poorly characterized.

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Here, we identify Npnt as a novel regulator of heart development. Our data show that Npnt is an upstream regulator of Bmp4-Has2 signaling and that it is critically involved in AV differentiation during zebrafish heart development.

**MATERIALS AND METHODS**

**Zebrafish maintenance**
Wild-type AB and transgenic Tg(lymy17:EGFP)-Has2RASp3883 (D’Amico et al., 2007), Tg(kdr:EGFP)Z1436 (Jin et al., 2005), Tg(-5.1myl7:nDsRed2)j2 (Mably et al., 2003) and Tg(TOP:GFP)25 (Dorsky et al., 2002) zebrafish (Danio rerio) were maintained at 28°C as described (Westerfield, 1993).

**Microarray data**
Total RNA of rat cardiac ventricles from different developmental stages [embryonic day (E) 11 to postnatal day (P) 10.5] at 12-hour intervals was isolated using Trizol (Invitrogen). For each time point, the RNA was extracted from 6-12 pooled hearts after removal of the atria. In addition, three independent RNA samples were generated for E11, E15, E19 and P2.

Expression analysis was performed using the Affymetrix GeneChip RAT 230 Expression Set. Preprocessing of the data, including background subtraction, normalization and probe set summarization, were performed according to the robust multi-array average (RMA) procedure.

**Reverse transcriptase PCR (RT-PCR)**
RNA was extracted from 30 or more zebrafish embryos at the indicated developmental stages, adult zebrafish tail fin or rat heart ventricles (E11 to E20, n=10; P5, P10 and adult, n=3) using Trizol (Invitrogen). RT-PCR was performed following standard protocols. Primers (5′ to 3′) for rat were: Gapdh, CAGAAGACTGTGGATGCCC and AGTGATGCGCAG-GATGCCCT; Npnt total, CACAGTGCAAACAGGAGAG and GCTACGACGATGTACCGTTG; Npnt variants, CTGGGACAGTGTCAACCTT and GCTACGACGATGTACCGTTG. Primers for zebrafish were: gapdh, TGGGTGTCACCATGAGAAA and AACCTGGT-GCTCCCGCTGAT; Npnt F1, CATTGGGAGCTTCAAGTGT; F2, ATGGATCATAAATGGTAATG; F3, CATGATGCTCTGCGTAG; R, CTGAGGATGGAAGGCTCAT; R2, TGCTCATATGGG-GTATGTGGTA; and R3, TCATCCACTCAGCCTGTTG.

**In situ hybridization**
At 24 hpf, 0.2 mM 1-phenyl-2-thiourea was used to prevent pigmentation. Embryos were fixed in 4% paraformaldehyde (PFA) in PBS for 2 hours at room temperature (RT), washed twice (0.1% Tween 20 in PBS, 4°C), dehydrated and processed for in situ hybridization utilizing digoxigenin-labeled RNA probes against: vmhc (Yelon, 2001), amhc (Berdougo et al., 2003), csq2, notch1b, bmp4 (Walsh and Stainier, 2001), fibulin I (Zang et al., 1997), bta2b (Smith et al., 2009), has2 (Bakkers et al., 2004), gfp, npnt and zgc:172265 (NM_001114911.1), which is named in this study itga8 owing to its homology to itga8 (Dorsky et al., 2002) zebrafish (NM_001114911.1), which is named in this study itga8 owing to its homology to itga8 (Dorsky et al., 2002) and 5′-GACGGACCTGATGCAAACAAAGGCGGCTTAC-3′ and 5′-CTGAGGATGGAAGGCTCAT-3′ was used to inhibit BMP signaling (Yu et al., 2008) and was added at 10 μM at 25 hpf in E3 medium.

**Western blot**
Protein was isolated from 60 embryos at 2 days post-fertilization (dpf). Yolk was removed by shearing (pipetting), shaking twice for 3 minutes each at 1100 rpm in deyolking buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM NaHCO₃) and washing in 110 mM NaCl, 3.5 mM KCl, 2.7 mM CaCl₂, 10 mM Tris-HCl (pH 8.5). Embryos were pelleted (300 g, 1 minute, 4°C) and lysed by sonication (four pulses of 5 seconds each) in lysis buffer [Cell Signaling buffer plus protease inhibitor mix (Roche) and 1 mM PMSF (Sigma)]. After centrifugation (17,000 g, 5 minutes, 4°C) supernatants were resolved in NuPAGE Novex Bis-Tris Gels (Invitrogen), blotted and analyzed with anti-Npnt (1:300, Cosmo Bio), anti-p-Smad1/5/8 (1:1000, Cell Signaling), anti-acetylated tubulin (1:1000, Sigma) or anti-pan-Actin (Cell Signaling, 1:1000).

**Immunohistochemistry and histological analysis**
Immunohistochemistry was performed as described (Dong et al., 2007). Samples were incubated with anti-Alcian (zm8) (1:10, DSHB) or 4G10 (1:10, DSHB) antibody, DsRed antibody (1:200, Clontech) and subsequently labeled with Alexa-conjugated secondary antibodies (Molecular Probes). Staining with anti-p-Smad1/5/8 antibody (1:200, Cell Signalling) was performed with minor modifications: fixation in 2% PFA in PBS for 1.5 hours at RT; blocking in 10% goat serum/0.3% Triton X-100 in PBS. For F-actin staining, samples were incubated with Rhodamine-labeled phallolidin (1.75, Invitrogen), blotted and analyzed with anti-Npnt (1:300, Cosmo Bio), anti-p-Smad1/5/8 (1:1000, Cell Signaling), anti-acetylated tubulin (1:1000, Sigma) or anti-pan-Actin (Cell Signaling, 1:1000).

**Morphological analysis, confocal and transmission electron microscopy (TEM)**
For Nomarski (Zeiss) and confocal microscopy, tricane-anesthetized embryos were mounted in 1% low-melting-point agarose in E3 medium. Confocal sections (1.3 μm) covering the entire heart were imaged on a Zeiss LSM710 and images were processed to obtain projections (LSM Image Browser, Zeiss). For TEM, embryos were fixed by immersion in 1.5% glutaraldehyde/1.5% paraformaldehyde in 0.15 M HEPES buffer (pH 7.3) for at least 24 hours. The specimens were subsequently osmicated, stained en bloc with uranyl acetate, dehydrated in an ascending ethanol series, stained en bloc with uranyl acetate, dehydrated in an ascending ethanol series and embedded in epoxy resin. Ultrathin sections from comparable regions of the heart were generated and analyzed using a Zeiss TEM 902.

**Live imaging**
Movies of embryos embedded in 1% low-melting-point agarose in E3 medium under a Leica DM6000 B microscope were recorded with a Sony HDR-SR12 camcorder and formatted (Wondershare or iSkysoft video converter).
Statistical analysis
Data are expressed as the mean ± s.e.m. of at least three independent experiments. Statistical significance of differences was evaluated by one-way ANOVA followed by Bonferroni’s post-hoc test (GraphPad Prism). *P*<0.05 was considered statistically significant.

RESULTS

**npnt** is transiently expressed during heart development

Based on a large-scale temporal RNA expression analysis we have identified nephronectin (**npnt**) as transiently expressed in the rat heart at the time of valve initiation and formation (Fig. 1A) (Okagawa et al., 1996). These results were confirmed by RT-PCR for both known variants of rat **npnt** using independent sets of mRNA (Fig. 1B). To determine the expression pattern of **npnt** in zebrafish, we performed whole-mount in situ hybridization analyses. At 24 hpf, **npnt** is markedly expressed at the tail bud, head, in the posterior part of the gut and in the pharyngeal endoderm (Fig. 1C,D). At 34 hpf, highest expression was observed in the pronephric region, with persistent expression in the head (Fig. 1E,F). Cardiac **npnt** expression was still observed at 48 hpf, but was undetectable at 53 hpf when **npnt** expression was still observed at 48 hpf but was undetectable at 53 hpf when **npnt** expression was most prominent in the pharynx, esophagus and the pharyngeal endoderm (Fig. 1K,L). Taken together, our data demonstrate that **npnt** is transiently expressed during heart development.

Npnt knockdown disrupts heart development and is lethal

To assess the role of Npnt in zebrafish heart development we applied antisense morpholino oligonucleotides. We designed one translation-inhibitory morpholino (MO1) and two splicing-inhibitory morpholinos that target the splice donor site of exon E5 (MO2) or exon E1 (MO3) (Fig. 2A, see Fig. S1A in the supplementary material). Our data indicate that both MO2 and MO3 were effective in disrupting correct splicing of npnt pre-mRNA, resulting in a marked decrease of mature **npnt** mRNA (Fig. 2B,C, see Fig. S1B in the supplementary material). RT-PCR analyses demonstrated that MO2 injection resulted in intron insertion (Fig. 2B) and exon E5 deletion, which was confirmed by sequencing (Fig. 2C). Western blot analysis demonstrated that injection of MO1 (3.5 pmol), MO2 (1.4 pmol) and MO3 (2.4 pmol) resulted in a marked decrease in the Npnt protein level (Fig. 2D, see Fig. S1C in the supplementary material). Therefore, all subsequent studies were performed using these amounts of morpholinos. Collectively, our results demonstrate that injection of MO1, MO2 and MO3 efficiently knocked down the expression of Npnt on the mRNA and/or protein level.

To determine the effect of Npnt knockdown on heart development we injected morpholinos into the embryos of **Tg(myl7:EGFP-HsHRAS)** transgenic zebrafish (n=200, four independent experiments), in which GFP is localized at the cardiomyocyte plasma membrane, facilitating live imaging of heart morphology. MO1, MO2 or MO3 injection did not show any obvious effect on zebrafish development until 40 hpf. However, Npnt knockdown resulted in 89±7.9% (mean ± s.e.m.) lethality at
The first obvious phenotype was pericardial edema at 75 hpf, indicating a cardiac defect (Fig. 2F, see Fig. S1D in the supplementary material). A closer analysis of morphant hearts revealed that valve formation and trabeculation at 110 hpf were perturbed in 86±3.9%, with more than 30% of those lacking valve leaflets as well as trabeculation (Fig. 2G-I). The morphants are also characterized by cardiac jelly swelling throughout the heart (Fig. 2I). Live imaging revealed no obvious differences between wild type and morphants in cardiac function and circulation at 36 hpf and 52 hpf (see Movies 1-4 in the supplementary material). Taken together, our data indicate that npnt is essential for cardiac development in zebrafish.

npnt morphant hearts have an extended AV canal

To determine the earliest heart phenotype we utilized transgenic zebrafish lines Tg(myl7:EGFP-HsHRAS)s883 and Tg(-5.1myl7:nDsRed2)f2, which express RFP in cardiomyocyte nuclei. Npnt depletion induced formation of an extended tube-like...
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Fig. 3. Npnt knockdown causes extension of the AV canal. (A-D) Projections of confocal images of hearts from control (A,C) and MO2-injected (B,D) embryos from transgenic Tg(myl7:EGFP-HsHRAS)s883 (A,B) and Tg(f5.1myl7:nDsRed2)I2 (C,D) zebrafish at 52 hpf, suggesting an extension of the AV canal (brackets) in npnt morphants. (E) Representative images of hearts from control, MO2-injected and MO2 + npnt mRNA-injected embryos from transgenic Tg(myl7:EGFP-HsHRAS)s883 zebrafish at 52 hpf. Brackets indicate the AV boundary. (F) Quantitative analysis (n>160 from three independent experiments; mean ± s.e.m.). Note that injection of npnt mRNA rescued the MO2-mediated AV canal extension. (G) Quantitative analysis scoring of type I (mild extension of the AV canal), type II (obvious extension of the AV canal) and type III (straight heart) AV canal defects (mean ± s.e.m.). A, atrium; V, ventricle. Scale bars: 50 μm.

structure at the AV boundary at 52 hpf, as compared with control hearts from non-injected or Phenol Red-injected embryos (n>200, four independent experiments) (Fig. 3A-D, see Fig. S1E in the supplementary material). Based on the severity of the cardiac defect at 52 hpf, morphants were categorized into three classes: type I, mild AV canal extension; type II, obvious AV canal extension; type III, straight heart (see Fig. S2A in the supplementary material). MO1 injection caused heart defects in 71±4.0% of embryos (type I, 29±2.6%; type II, 54±5.3%; type III, 17±8.3%) and MO2 injection in 86±4.4% of embryos (type I, 21±3.2%; type II, 56±4.0%; type III, 23±3.9%) (see Fig. S2B,C in the supplementary material). All three morpholinos resulted in NPNT knockdown and in similar phenotypes, suggesting that the observed phenotypes are a functional consequence of NPNT depletion.

To prove that the observed AV canal phenotype is specific to NPNT depletion we performed rescue experiments by co-injecting capped npnt mRNA and MO2. Note that MO2, as a splice-inhibitory morpholino, does not affect the translation of injected npnt mRNA. Sequencing indicated that all cloned npnt cDNAs lacked exon E3 (51 bp) and a part of exon E13 and contained an additional exon E14, as compared with the npnt sequence published by NCBI (see Fig. S3 in the supplementary material). RT-PCR analyses of cDNA derived from zebrafish embryos at different developmental stages confirmed that this is the major splice variant (see Fig. S4A,B in the supplementary material). In adult zebrafish tail fin tissue only, we detected a second variant (see Fig. S4B in the supplementary material). Sequencing analysis demonstrated that this variant contains exon E3 but lacks part of exon E13. A MotifScan analysis revealed no change in the overall domain structure of the proteins encoded by the two variants (see Fig. S4C,D in the supplementary material).

Assuming that the cloned cDNA represents the npnt mRNA at 52 hpf, we used linearized pCS2+-Fnpnt plasmid as template to generate capped npnt mRNA. Injection of 50 pg capped npnt mRNA rescued the AV extension in a subset of MO2-injected embryos (Fig. 3E,F) and had only a minor effect on cardiac development when injected alone (88±1.5% normal hearts; Fig. 3F). Importantly, 40±2.3% of embryos had normal hearts after co-injection of npnt mRNA and MO2 as compared with 11±2.0% after MO2 injection alone (Fig. 3F). In addition, injection of 50 pg capped npnt mRNA markedly reduced the severity of the AV canal defect in the remaining embryos, with defective hearts showing an increase in type I defects (from 23.6±1.2% to 48.7±2.4%) and a decrease in type II defects (from 60±1.1% to 26.5±2.6%) (Fig. 3G). The slight increase in the type III phenotype (from 16.4±2.4% to 24.8±2.4%) might be due to npnt overexpression. In summary, the rescue experiments confirm that the MO-mediated phenotypes are due to NPNT depletion.

To characterize the extended tube-like structure at the AV boundary we performed Alcam staining of embryos from the transgenic zebrafish line Tg(kdrl:EGFP)1243, in which GFP is expressed in all endothelial cells. At 52 hpf, all cardiomyocytes and endocardial cells at the AV boundary express Alcam. The AV canal in wild-type animals is 5-6 tiers long at the superior AV boundary (S AV) and 3-4 tiers long at the inferior AV boundary (I AV) of Alcam-positive endocardial cells (Fig. 4A). In npnt morphants, the AV canal was extended by 84.4±27.6% (8-12 tiers, S AV) and 85.0±22.5% (5-7 tiers, I AV) (Fig. 4B,C). In accordance with these data, endocardial expression of notch1b was also expanded in npnt morphant hearts at the AV boundary as compared with control hearts (Fig. 4D). These data suggest that NPNT knockdown causes an increase in the number of AV endocardial cells and thus an expansion of endocardial AV specification.

In contrast to the AV endocardium, there is no AV myocardium-specific marker available. Therefore, we used genes as markers whose expression becomes restricted during development to the AV myocardium: cspg2 (Versican), fibulin 1 and bmp4 (Zang et al., 1997; Walsh and Stainier, 2001); bmp4 is also expressed at the outflow and inflow tract (Fig. 4E-G) (Walsh and Stainier, 2001). Compared with control hearts, expression of cspg2, fibulin 1 and
bmp4 was expanded in npnt morphant hearts at 52 hpf (Fig. 4E-G). In addition, ectopic expression of cspg2 was detected at the inflow tract (Fig. 4E). By contrast, chamber-specific expression of the cardiac marker genes vmhc and amhc (Yelon et al., 1999) appeared normal (Fig. 4H). Our data suggest that the extended tube-like structure represents, on a molecular level, an extended AV canal. However, owing to the lack of an AV myocardium-specific marker it remains unclear whether Npnt regulates myocardial in addition to endocardial AV specification.

To determine the origin of the additional myocardial AV canal-like cells we performed cell count experiments with double-transgenic animals $[Tg(–5.1myl7:nDsRed2)f2/H11003]$ $Tg(myl7:EGFP-HsHRAS)s883]$. The overall number of cardiomyocytes (chambers + AV boundary) was not significantly different between control and morphant hearts (Fig. 4I,J). These data support the assumption that the AV boundary extension is not due to an increase in cardiomyocyte number, but rather to differentiation into AV canal-like cells at the expense of chamber cells.

Npnt knockdown causes cardiac jelly expansion and increases has2 expression

One characteristic of AV cells is increased expression of ECM components at the onset of looping. Signaling between myocardium and endocardium is essential for heart development, including valve formation (Armstrong and Bischoff, 2004; Hsieh et al., 2006; Holtzman et al., 2007). In addition, it has been suggested that an increase in cardiac jelly can interfere with this signaling and perturb heart development (Shirai et al., 2009). To determine whether the extended AV canal produces normal amounts of cardiac jelly we used $Tg(kdrl:EGFP)s843$ embryos, counterstained with Rhodamine-phalloidin (which detects F-actin) to visualize the myocardium. Analysis of confocal sections of hearts from control and npnt morphant embryos at 52 hpf showed that the space between endocardium and myocardium is markedly increased in morphants (Fig. 5A,B), suggesting that Npnt knockdown causes cardiac jelly expansion.

A major constituent of cardiac jelly is glycosaminoglycan hyaluronan (HA), which is synthesized by the product of hyaluronan synthase 2 (has2), a downstream target of Tbx2 (Shirai et al., 2009). Both has2 and tbx2 are regulated by BMP signaling (Camenisch et al., 2000; Gaussen et al., 2002; Shirai et al., 2009). In wild-type zebrafish, the expression of both genes is restricted to the AV boundary at 2 dpf (Walsh and Stainier, 2001; Hurlstone et al., 2003; Chi et al., 2008) (Fig. 5C,E). By contrast, expression of has2 and tbx2b was expanded in npnt morphants (Fig. 5D,F). Importantly, our data demonstrate that the phenotype was also rescued on a molecular (bmp4, cspg2) and cellular (Alcam) (see Fig. S5 in the supplementary material) level in morphologically rescued npnt morphants.
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The specificity of HA staining was tested using hyaluronidase (see Fig. 5 in the supplementary material). Npnt knockdown increased the amount of HA in the cardiac jelly compared with wild-type embryos (Fig. 5G,H). Has2 knockdown caused a marked reduction of HA in the cardiac jelly, both in npnt morphants (Fig. 5I) and in wild-type embryos (Fig. 5J). In addition, TEM analyses revealed that the density of cardiac jelly in wild type and npnt morphants is comparable (Fig. 5K,L) and that the junctional complexes (comprising adherens and tight junctions) in the endocardium in npnt morphants are still intact, suggesting that the expanded cardiac jelly is not due to increased permeability of the endocardium (Fig. 5M,N). These results show that Npnt is an inhibitory upstream regulator of bmp4, tbx2b and has2 expression and thus regulates the composition of the cardiac jelly. At 40 hpf, before bmp4 expression, bmp4 expression was restricted to the AV canal in both wild-type embryos and npnt morphants (see Fig. S7A in the supplementary material). This indicates that Npnt is required to maintain the restricted expression of AV canal markers.

**Has2 knockdown rescues the npnt morphant endocardial phenotype**

Has2 plays an essential role during valve formation (Camenisch et al., 2000). An effect on the number of AV canal cells has not been reported. To determine whether expanded expression of has2 caused the increased number of AV endocardial cells we performed Has2 knockdown experiments. One-cell stage embryos were injected with has2 and npnt morpholinos and analyzed for AV endocardial (Alcam and notch1b) and myocardial (cspg2, bmp4) specific gene expression at 52 hpf (Fig. 6). Has2 knockdown in npnt morphants reduced the number of Alcam-positive AV endocardial cells from 180.8±21.9% (8-11 tiers, SAV) or 188.2±33.5% (5-8 tiers, IAV) to 30.8±10.5% (1-2 tiers, SAV) or 11.0±16.1% (0-1 tier, IAV) compared with wild-type embryos (Fig. 6A,B). Has2 knockdown in wild-type embryos resulted in a reduction from 100±8.6% (5-6 tiers, SAV) or 100±16.1% (3-4 tiers, IAV) to 25.1±16.1% (0-2 tiers, SAV) or 5.9±13.2% (0-1 tier, IAV) (Fig. 6A,B). In addition, Has2 knockdown rescued the expansion of notch1b (Fig. 6C). These data demonstrate that has2 is required for prevallvalvar endocardial cell differentiation. Taken together, our data show that the increased number of AV endocardial cells in npnt morphants is due to the expanded expression of has2.

To assess whether expanded expression of has2 caused the myocardial phenotype, we determined the expression of cspg2 and bmp4 after Has2 knockdown in control and npnt morphant embryos. The expression of these genes in npnt morphants as well as in control embryos either remained unchanged or was upregulated after Has2 knockdown (Fig. 6C). These data indicate that ectopic endocardial cell differentiation, but not the myocardial phenotype, in npnt morphants is due to ectopic expression of has2.

**Inhibition of BMP signaling reduces AV canal expansion**

Bmp4 is a major regulator for cardiac valve formation (Wessels and Markwald, 2000; Jiao et al., 2003). However, there is a debate as to whether Bmp4 is an upstream regulator of has2 expression. Using explant cultures it has been shown that inhibition of BMP signaling does not affect has2 expression (Klewer et al., 2006). By contrast, tbx2 overexpression studies suggested that has2 expression is controlled by BMP-Smad signaling during cushion formation (Shirai et al., 2009). To determine whether BMP signaling acts downstream of Npnt we inhibited BMP signaling in npnt morphants with dorsomorphin.
a chemical antagonist of BMP receptors (Yu et al., 2008), at 25 hpf (see Fig. S7B,C in the supplementary material), and analyzed the embryos at 52 hpf.

Dorsomorphin treatment in npnt morphants reduced the number of Alcam-positive AV endocardial cells from 163.6±18.2% (8-10 tiers, SA V) or 176.8±17.3% (5-8 tiers, IA V) to 127.3±12.9% (6-8 tiers, SA V) or 107.4±17.3% (3-4 tiers, IA V) compared with wild-type embryos (Fig. 7A,B) and rescued the expanded expression of notch1b (Fig. 7C). In wild-type embryos, inhibition of BMP signaling had no effect on the number of Alcam-positive cells (see Fig. S7D in the supplementary material) or upon notch1b expression (see Fig. S7E in the supplementary material).

To assess whether ectopic expression of has2 and cspg2 is dependent on BMP signaling, we performed in situ hybridization analyses on 52 hpf dorsomorphin-treated npnt morphants and wild-type embryos. Dorsomorphin partially rescued the expanded expression of has2 and cspg2 at the AV region in npnt morphants and abolished ectopic expression of cspg2 in the inflow tract (Fig. 7C). Expression in wild-type embryos was unaffected (see Fig. S7E in the supplementary material).

To better understand Bmp4-Smad1/5/8 signaling in the AV canal of zebrafish we analyzed the cellular origin of bmp4 expression and Smad1/5/8 phosphorylation at 52 hpf. Thin sections of wild-type and npnt morphant hearts demonstrated that bmp4 is expressed in the myocardium of the AV canal (see Fig. S7F in the supplementary material). Phosphorylated Smad1/5/8 (p-Smad1/5/8) could be detected in the myocardium and at very low levels in the endocardium (see Fig. S7G,H in the supplementary material). This suggests that myocardial Bmp4 can diffuse in a controlled manner to the AV endocardium, activating p-Smad1/5/8 signaling. By contrast, the level of p-Smad1/5/8 was increased in the expanded AV endocardium of npnt morphants to similar levels as in the myocardium (see Fig. S7G,H in the supplementary material).

Thus, our data indicate that knockdown of Npnt causes expanded expression of bmp4, which is mainly responsible for the phenotype of npnt morphants, and suggest that BMP signaling is an upstream regulator of has2 and cspg2 in zebrafish heart (Fig. 7D). Based on our data, we suggest two models. First, Npnt binds to a receptor that is expressed in the chamber myocardium and that this mediates the repression of bmp4 expression (Fig. 7D, model 1). Second, Npnt regulates the diffusion of Bmp4, preventing paracrine signaling towards the endocardium and the chambers (Fig. 7D, model 2).
DISCUSSION

Our data identify Npnt as a novel regulator of early heart development. Several lines of evidence support this conclusion. First, Npnt knockdown causes 89±7.9% lethality. Second, npnt morphant hearts were characterized by an expanded A V canal, increased cardiac jelly, impaired trabeculation and a failure of proper A V valve formation. Third, Has2 knockdown rescued the endocardial phenotype in npnt morphants. Fourth, chemical inhibition of BMP signaling rescued A V canal extension in npnt morphants. In summary, our data indicate that Npnt regulates the differentiation of the A V segment via Bmp4-Has2 signaling.

It has been shown that regionally specific interactions of myocardium and endocardium are required to initiate the formation of prevallvular mesenchyme (Krug et al., 1985; Mjaatvedt et al., 1987; Wagner and Siddiqui, 2007). Changes in the composition or amount of the ECM can interfere with valve formation. Knockdown of Cspg2 or Has2 results in reduced amounts of cardiac jelly (Mjaatvedt et al., 1998; Camenisch et al., 2000) and in a failure of endocardial cushion formation. By contrast, knockdown of the ECM protein Npnt caused an increase in cardiac jelly. Moreover, it resulted in an extended A V canal on the morphological, cellular and molecular level. This suggests that Npnt does not simply act as a structural protein but functions as a negative regulator of genes involved in A V canal differentiation.

Our data indicate that Npnt is required to maintain the restricted expression of Bmp4 and that expanded bmp4 expression in npnt morphants causes increased expression of cspg2 and has2, which explains the increase in cardiac jelly. has2 plays an essential role in the formation of cardiac jelly and in the transformation of epithelium to mesenchyme (Camenisch et al., 2000). Has2 knockdown in npnt morphants rescued the endocardial phenotype and demonstrates that Npnt controls AV endocardial cell differentiation by regulating has2 expression. Has2 knockdown in npnt morphants, however, did not rescue the ectopic expression of bmp4 or cspg2. Thus, it appears that Bmp4 is an upstream regulator of has2 expression and that the endocardial phenotype is a consequence of ectopic myocardial bmp4 expression.
BMP signaling has been shown to regulate has2 expression (Shirai et al., 2009), although the data are controversial (Klewer et al., 2006). Inhibition of BMP signaling in npnt morphants fully rescued the endocardial phenotype, partially rescued the AV canal extension and reduced the expression of has2 and cspg2. In wild-type embryos, dorsomorphin did not affect the basal expression of AV canal marker genes. This might be due to the fact that dorsomorphin treatment resulted in only partial inhibition of BMP-vergence signaling and that complete inhibition could not be achieved even at higher dorsomorphin concentrations (see Fig. S7B,C in the supplementary material). Another possible explanation is that endogenous BMP signaling is redundant at the AV canal. Taken together, our data suggest that Npnt is an upstream regulator of BMP signaling. Moreover, these findings indicate that ectopic BMP signaling regulates has2 and cspg2 expression.

It has been suggested that AV endocardial differentiation is mediated through Wnt/β-catenin signaling. npnt morphants show many similarities to zebrafish apc mutants, in which the Wnt signaling pathway is constitutively activated (Hurlstone et al., 2003). Hearts of both appear normal at 36 hpf but subsequently form excessive cardiac jelly. In apc mutants, Wnt/β-catenin signaling is active throughout the heart, resulting in expanded has2 expression from the AV canal throughout the heart at 72 hpf (Hurlstone et al., 2003). However, our analyses indicate that Npnt knockdown does not result in ectopic activation of Wnt/β-catenin signaling at 52 hpf (see Fig. S8 in the supplementary material).

Our data have identified Npnt as a crucial regulator of AV canal differentiation and ECM composition by controlling Bmp4-Has2 signaling. It will be important in the future to determine how Npnt regulates BMP signaling. It is possible that chamber myocardium expresses a receptor for Npnt that represses Bmp4 signaling (Fig. 7D, model 1). It has been shown that Npnt acts during kidney development as a ligand of integrin α8β1, regulating migration (Brandenberger et al., 2001; Sato et al., 2009). However, during the time of npnt expression in the zebrafish heart we did not detect integrin α8 (tiga8) expression in the heart by in situ hybridization, whereas strong expression was detected in other tissues (see Fig. S9 in the supplementary material). This indicates that Npnt does not signal through integrin α8 during zebrafish heart development. Alternatively, Npnt might activate growth factor receptor signaling. It has been suggested that Npnt can induce signaling through its EGF-like repeats, which are required for Npnt-induced osteoblast differentiation (Kahai et al., 2010). Similarly, it has been suggested that the EGF-like repeats of Versican can bind and activate growth factor receptors (Wight, 2002). Thus, it will be important to determine whether Npnt signals through a receptor during heart development. Another possibility is that Npnt modulates the bioavailability of Bmp4 (Fig. 7D, model 2). Npnt might be required to establish an ECM in which Bmp4 can act only in an autocrine fashion. Knockdown of Npnt might therefore enable diffusion of Bmp4, and thus paracrine signaling, towards the endocardium as well as the chambers (Fig. 7D, model 2).

Previously, it has been shown that mouse embryos lacking a functional Npnt gene are born at the expected Mendelian frequency but frequently display kidney agenesis or hypoplasia (Linton et al., 2007). In addition, Npnt knockout mice exhibit a skin phenotype (Fujiwara et al., 2011). The heart has thus far not been investigated. The fact that the mouse Npnt knockout is not embryonic lethal suggests that the regulation of Bmp4 signaling is more stringent in mammals. This assumption is supported by the finding that the Npnt family member Efg16 is upregulated in Npnt knockout mice, partially compensating for Npnt (Fujiwara et al., 2011).

In conclusion, Npnt acts as an inhibitor of Bmp4-Has2 signaling to restrict AV canal differentiation and cardiac jelly swelling in zebrafish. Interference with this pathway results in an expanded AV canal, excessive cardiac jelly and a failure of valve formation.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Nephronectin regulates heart development

DEVELOPMENT

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