Notch1b and neuregulin are required for specification of central cardiac conduction tissue

David J. Milan1,*, Andrea C. Giokas1, Fabrizio C. Serluca2, Randall T. Peterson1 and Calum A. MacRae1

Normal heart function is critically dependent on the timing and coordination provided by a complex network of specialized cells: the cardiac conduction system. We have employed functional assays in zebrafish to explore early steps in the patterning of the conduction system that previously have been inaccessible. We demonstrate that a ring of atrioventricular conduction tissue develops at 40 hours post-fertilization in the zebrafish heart. Analysis of the mutant cloche reveals a requirement for endocardial signals in the formation of this tissue. The differentiation of these specialized cells, unlike that of adjacent endocardial cushions and valves, is not dependent on blood flow or cardiac contraction. Finally, both neuregulin and notch1b are necessary for the development of atrioventricular conduction tissue. These results are the first demonstration of the endocardial signals required for patterning central ‘slow’ conduction tissue, and they reveal the operation of distinct local endocardial-myocardial interactions within the developing heart tube.

Key words: Notch, Neuregulin, Cardiac conduction system, Development, Zebrafish

INTRODUCTION

There is an increasing realization of the importance of the cardiac conduction system in human disease (Moorman and Christoffels, 2003). Failure of any single component of this complex network has immediate adverse effects, including heart failure and sudden cardiac death (Jongbloed et al., 2004; Kleber and Rudy, 2004). Cardiac electrical impulses arise in the sinoatrial node and pass rapidly through the atria to the atrioventricular (AV) node. Within the AV node, the impulse is significantly delayed prior to entering the His-Purkinje network or the distal conduction system. The AV node is one of the most complex structures in the cardiac conduction system and slows impulse propagation between atrium and ventricle, as well as setting an upper limit on the frequency of conducted impulses. The anatomy and physiology of the AV conduction tissue have been well described (Anderson and Ho, 1998; Kleber and Rudy, 2004), but the factors regulating its development are largely unknown (Gourdie et al., 1999; Moorman and Christoffels, 2003; Pennisi et al., 2002). The AV node is thought to arise from tissue that emerges at the boundary between atrium and ventricle. Evidence of such tissue has been observed in chick, manifest as an impulse delay between the atrium and ventricle in the tubular heart (de Jong et al., 1992; Sedmera et al., 2004), but study of the formation of the central conduction system has proven difficult, as it arises so early in cardiac development.

Lineage analyses have demonstrated that the entire cardiac conduction system arises from cardiomyocyte progenitors (Gourdie et al., 1995; Mikawa and Fischman, 1996). Conduction tissues can be classified, on the basis of function, into two broad divisions: the central conduction system, characterized by slow impulse propagation and prolonged refractory periods; and the peripheral conduction system, with rapid impulse propagation. Some of the molecular signals involved in the induction of the peripheral conduction system have been identified. Peripheral conduction fiber markers can be induced in cultured embryonic myocytes by treatment with exogenous endothelin 1 (Gourdie et al., 1998; Patel and Kos, 2005). Likewise, addition of exogenous neuregulin in whole mouse embryo culture causes expansion of conduction system marker expression and changes in the ventricular electrical activation pattern consistent with a recruitment of cells to the conduction system (Rentschler et al., 2002). The study of the effects of inactivation of these same pathways on the conduction system has proven less straightforward: endothelin 1-null mice display ventriculo-septal defects in 50% of pups, but there are no data on conduction system function (Kurihara et al., 1995). neuregulin knockout mice die around day 8.5 of generalized myocardial failure, but prior to a time when electrophysiological analysis is feasible (Kramer et al., 1996; Liu et al., 1998; Meyer and Birchmeier, 1995). By contrast, little is known of the signals required for the differentiation of the proximal conduction system (Gourdie et al., 2003; Moorman and Christoffels, 2003; Pennisi et al., 2002). An ongoing requirement for nKc2.5 expression has been established for the persistence of the central conduction system through adulthood (Pashmforoush et al., 2004), and haploinsufficiency of tbx5 causes defects in the postnatal maturation of this tissue (Moskowitz et al., 2004). However, the earliest steps in AV conduction system formation remain unclear, largely because these stages of development are inaccessible in most models.

Traditional analyses of development have focused largely on molecular markers to categorize cell identity. However, the relationship between such markers and the ultimate cellular phenotype is not always straightforward (Cleaver and Melton, 2003; Morrishead, 2004; Parmacek and Epstein, 2005). As methods are developed for the study of embryonic physiology, the relationship between molecular markers and cell function will become more clearly defined. There are few known markers of the early conduction system, and their relationship to cellular function is undefined. In order to better understand central conduction tissue formation, we developed techniques to study the electrophysiological function of differentiating cardiomyocytes in vivo throughout development.

The external fertilization and development of the zebrafish enables the observation and manipulation of cardiac physiology at the earliest stages (Stainier et al., 1996; Warren and Fishman, 1998;
Yelon, 2001). By using three independent techniques, we have established that myocytes within the AV ring differentiate into slowly conducting cells with prolonged refractory periods at 40 hours post-fertilization (hpf) in zebrafish. These functional assays were employed as physiological reporters of cellular differentiation to explore endothelial-myocardial signaling in conduction tissue development. Using a combination of known mutants and antisense morpholino knockdown (Nasevicius and Ekker, 2000), we demonstrate that the formation of central ‘slow’ conduction tissue is not dependent on blood flow, but does require endocardial signals, including neuregulin and notch.

MATERIALS AND METHODS

Fish
All experiments were performed in wild-type zebrafish (Tübingen AB) or the mutant lines silent heart (tc3006) and cloche (m39), raised and maintained using standard methods. Embryos were staged according to morphological criteria (somite number) and by timing in hours post-fertilization.

Pacing
Tungsten 0.5 MΩ bipolar fork electrodes (WPI, Sarasota, FL) mounted on a Narishige micromanipulator were introduced into the pericardial space of zebrafish embryos anesthetized with propofol at 10 μg/ml in E3 medium at room temperature. Pacing was performed with a Medtronic 5328 stimulator (Medtronic, Minneapolis, MN) at the cycle lengths indicated and at outputs of twice diastolic threshold.

Drug treatment
Embryos were treated with a dose of terfenadine (12 μg/ml; Sigma Chemicals) empirically defined to cause 2:1 AV block in 100% of wild-type embryos at 48 hpf. Fish were exposed to drug at 36 hpf and observed at 48 hpf (or the indicated time) by video microscopy using a Nikon TE200 inverted microscope and an ORCA-ER CCD camera (Hamamatsu, Hamamatsu City, Japan). Images were captured and analyzed using commercially available image processing software (Metamorph, Universal Imaging Corporation, Downingtown, PA) (Milan et al., 2003).

Morpholino injections
Morpholinos (Gene Tools, Philomath, OR) were resuspended in sterile water to a concentration of 1 mM and diluted to 10-100 μM with 1× Danieau’s [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 0.5 mM Hepes, pH 7.6]. The morpholinos were injected at the single-cell stage in a volume of ~5 nl. Morpholino sequences were as follows:

- EDN-1, 5’-GTAGTATGCAAGTCCCGTATTCCAG-3’;
- Troponin T, 5’-CATGTTTGCTCTGATCTGACACGCA-3’;
- Neuregulin exon 6 acceptor, 5’-TGCTGGTGCTGCTGACAGAGGA-3’;
- Neuregulin exon 6 donor, 5’-ATGCCGATTTCGCCGCAATACCTGCA-3’;
- Notch 1b exon 27 donor, 5’-AATCTCAACTGACCTCAAACCGAC-3’;
- Notch 1b exon 27 acceptor, 5’-TGCTGGTGCTGCTGACAGAGGA-3’;

For each experiment, appropriate four-base mismatch morpholino controls were employed. The effects of morpholinos on mRNA were assayed independently using quantitative real-time RT-PCR from pooled, injected embryos.

Calcium imaging
Embryos at the single-cell stage were injected (into the body of the cell) with dextran-coupled calcium-green (Molecular Probes, Eugene, OR), of an average Mw of 3000, at a concentration of 3 mM. Embryos then were allowed to develop in a dark environment before imaging at the indicated time on an inverted fluorescence microscope with video capture using a CCD camera. In all cases, except for the silent heart mutant embryos, motion artifact was suppressed with an antisense morpholino targeted against the cardiac troponin T transcript (Sehnert et al., 2002). Images were analyzed off-line and isochronal maps were generated with Metamorph software (Universal Imaging Corporation, Downingtown, PA). Calcium wavefront velocities in the atrium were calculated by measuring the distance along the greater curvature of the atrium and then dividing by the elapsed time. To minimize the inherent inaccuracies imposed by foreshortening, we have reported the maximum velocity averaged over several beats. At the video frame rates used, we were unable to reliably measure the ventricular velocities in wild-type fish at 48 hpf; therefore we have reported the lower limit for these datapoints.

In situ analyses of expression
Whole-mount in situ hybridization and immunohistochemistry were carried out according to standard procedures (Jowett and Lettice, 1994). Images were acquired using a color digital CCD camera and processed with Adobe Photoshop (Adobe Systems, San Jose, CA). To synthesize digoxigenin-labeled antisense RNA probes, a plasmid containing neuregulin cDNA (pCR-II:Neuregulin-full length) was amplified with M13 forward and reverse primers to yield a linear template, prior to transcription with T7 RNA polymerase (Promega). The plasmid for notch1b was a kind gift from Michael Lardelli (University of Adelaide, Australia). The plasmid for bmp4 was a kind gift from Dr Didier Stainier (Walsh and Stainier, 2001). The S46 antibody was obtained from the Developmental Studies Hybridoma Bank.

neuregulin cDNA cloning
Using the human, mouse and chick neuregulin cDNAs, degenerate primers were synthesized and a zebrafish XZap cDNA library was screened. A 2840 bp clone was identified and sequenced completely in both directions, revealing a 1797 bp open-reading frame encoding a 599 amino acid protein with high homology to known neuregulins (GenBank Accession Number DQ366108).

RESULTS

Slow conduction tissue develops in the atrioventricular ring at 40 hpf
The two defining characteristics of central conduction tissue are slow conduction and prolonged refractory periods. To assess these physiological parameters in the developing zebrafish heart we employed calcium-sensitive fluorophores. At present, signal-to-noise considerations prevent the use of voltage-sensitive dyes in the study of cardiac activation patterns in zebrafish embryos. Although they can become dissociated at high frequencies or during arrhythmias, calcium activation and membrane depolarization are tightly coupled under most physiological situations (Laurita and Singal, 2001). In the current studies we have employed calcium imaging to study the excitation pattern during normal baseline rhythm and therefore anticipate calcium excitation to closely parallel voltage activation. Injection of calcium green at the single-cell stage allows the visualization of cardiac excitation in vivo, as well as the analysis of evolving patterns of excitation during development. In Fig. 1A, the calcium activation sequence of the embryonic heart at 36 hpf is illustrated using isochronal lines superimposed on the maximum intensity projection from the complete stack of fluorescent images. These lines are spaced at 50 milliseconds intervals. The uniform spacing of isochronal lines in the atrium demonstrates smooth progression of the activation wave front, which proceeds to the ventricle without pause. Acceleration of the calcium transient in the ventricle is visible as increased spacing between the isochronal lines. The atrial average maximal wavefront velocity was 403±67 microns/second, whereas the ventricular average velocity was 912±109 microns/second. The activation pattern of a 48 hpf embryo is shown in Fig. 1B. In contrast to the 36 hpf heart, there is a significant slowing of the excitation wave front in the AV ring, indicated by the compressed spacing of the isochronal lines. At 48 hpf, the atrial average maximal wavefront velocity was 2853±580 microns/second, whereas the lower limit for the
ventricular average velocity was 2051±203 microns/second. These data demonstrate that, between 36 and 48 hours of development, myocytes in the AV ring differentiate to a more slowly conducting phenotype.

Refractory periods in any excitable tissue are revealed only when the input stimulus interval encroaches on the recovery time of the tissue. In the central conduction system this manifests as an AV block, when atrial impulses arrive at the AV conduction tissue while it is still refractory. Thus, a simple method to define the functional refractory period of AV tissue is to pace the atrium at incrementally shorter stimulus intervals until some of the impulses no longer conduct to the ventricle. We performed atrial pacing, using a bipolar needle electrode, in embryos at multiple time points over the first 48 hours of development. At 36 hpf, none of the embryos exhibited a pacing-induced 2:1 AV block (Fig. 1C), but, by 40 hpf, 80% of the embryos displayed 2:1 AV conduction. At 48 hpf, all of the embryos studied developed 2:1 heart block with incremental atrial pacing. Ten embryos were studied at each time point. The mean cycle length at which 2:1 AV block developed in the 40 hpf embryos was 754±46 milliseconds, and at 48 hpf was 777±72 milliseconds. The shortest cycle length required for 2:1 block in the 48 hpf embryos was 690 milliseconds. All other embryos were paced at incrementally faster rates until a loss of atrial capture was demonstrated. In all cases, this occurred at cycle lengths at or below 690 milliseconds.

Previously, we observed that treatment with drugs that inhibit the repolarizing potassium current IKr results in 2:1 AV block in zebrafish embryos at 48 hpf but not at 24 hpf (Milan et al., 2003). In order to better understand the relationship between this drug response and the onset of physiological AV delay, we defined the timing of the onset of the drug-mediated 2:1 block using video microscopy (Milan et al., 2003). We monitored embryos exposed to the IKr blocker terfenadine (12 μg/ml) every hour after the initiation of a heartbeat. The embryos developed heart block over a narrow time window, so that, by 40 hpf, 90% of the embryos exhibit 2:1 AV block (n=40; Fig. 1C). Taken together, these results demonstrate that, between 37 and 41 hpf, myocytes within the AV ring develop the key electrophysiological properties of central conduction tissue: slowed conduction and prolonged refractory periods.

In addition to the physiological evidence for specialized conduction tissue at this point in development, the AV ring exhibits a unique pattern of gene expression. We examined the in situ expression of the genes notch1b and bmp4 in wild-type embryos (Walsh and Stainier, 2001; Westin and Lardelli, 1997). The expression of notch1b is highest in the central nervous system, but is also evident in the heart, where it is accentuated in the endocardium of the AV ring (Fig. 1D) (Walsh and Stainier, 2001). Likewise, bmp4 is expressed in the myocardium of the AV ring (Fig. 1E). These restricted patterns of expression further support uniquely specified populations of endocardial and myocardial cells at the boundary between atrium and ventricle.

It has been proposed that early AV conduction delay is a result of a source-sink mismatch due to differing tissue architecture between the thin-walled atrium and thicker-walled ventricle (Markhasin et al., 2003; Rohr et al., 1999). If this were the case, it would be expected that impulses arising in the ventricle and conducting retrogradely would not exhibit conduction slowing at the AV interface. During calcium imaging, we observed spontaneous ventricular premature beats that conducted to the atrium. These retrograde impulses displayed conduction slowing in the AV ring (Fig. 2A,B; see also Movie 1 in the supplementary material). This demonstration of both anterograde and retrograde conduction slowing effectively excludes source-sink mismatch as the operative mechanism of AV delay.

---

**Fig. 1. Normal development of AV conduction tissue.**

(A,B) Calcium activation maps from a single cardiac cycle in wild-type zebrafish at 36 hpf (A) and 48 hpf (B). Isochronal lines (50 mseconds) obtained by fluorescence microscopy are superimposed on maximum intensity projection images. These data demonstrate smooth conduction throughout the heart with ventricular acceleration at 36 hpf (A). Marked slowing at the AV junction can be seen at 48 hpf (B). Scale bars: 25 μm in A; 50 μm in B. (C) Time course of onset of 2:1 or higher grade AV block in response to atrial pacing (bars) or terfenadine (line) expressed as a percentage of the embryos studied. (D,E) In situ expression patterns of notch1b (D) and bmp4 (E) in wild-type embryos at 48 hpf. Arrowheads indicate expression in the AV ring a dotted line outlines the cardiac silhouette in D.

---

**Fig. 2. Characteristics of retrograde conduction in the zebrafish heart at 48 hpf.** (A,B) Calcium activation maps for normal anterograde cardiac cycle (A) and spontaneous premature ventricular beat (B). Both anterograde and retrograde AV delay are evident as compressed spacing of isochronal lines (100 mseconds intervals). Scale bars: 50 μm. (C) Contemporary recordings of atrial and ventricular conduction using intensity-time plots from regions over the respective chambers, during ventricular pacing at 48 hpf. The ladder diagram depicts the resulting Wenckebach-type retrograde ventriculo-atrial block.
Similarly, it is possible that the conduction block seen with atrial pacing is a result of a prolonged refractory period, not in the AV ring, but in the ventricular myocardium. To discriminate between these two possibilities, we performed incremental ventricular pacing in wild-type embryos at 48 hpf. We observed retrograde ventriculo-atrial conduction block of a Wenckebach type (characterized by a prolongation of ventriculo-atrial conduction times prior to the failure of impulse propagation; Fig. 2C). The combination of anterograde and retrograde block can only be explained by the presence of tissue in the AV ring with a refractory period longer than that of the chambers on either side. These results confirm the existence of specialized conduction tissue in the AV junction.

**Endocardial signaling is required for the development of AV conduction tissue**

The zebrafish mutant cloche fails to develop endothelium and, therefore, provided a unique opportunity to study the development of specialized AV conduction tissue in the absence of any endocardium (Stainier et al., 1995). Calcium activation patterns in 52 hpf mutant embryos showed no evidence of AV delay, but rather exhibited virtually constant calcium transient velocities throughout the heart (Fig. 3A). Similarly, incremental atrial pacing in cloche embryos failed to elicit 2:1 AV block at cycle lengths consistently lower than those resulting in a conduction block in all wild-type embryos (Fig. 3B). In addition, treatment with terfenadine over a broad range of doses failed to cause AV block (Fig. 3B). As expected, notch1b, one of the potential endocardial signals for myocyte differentiation in the AV ring, is absent from the hearts of cloche mutants (Fig. 3C). Cloche embryos also display abnormal localization of the marker bmp4, which is absent from the AV ring, and which instead shows more prominent expression in myocytes in the inflow tract of the heart (Fig. 3D).

**Blood flow and endothelin 1 are not required for AV conduction development tissue**

Given the requirements for blood flow in the development of the peripheral conduction system, we determined the role of flow in the development of central AV conduction tissue (Gourdie et al., 1998; Hall et al., 2004; Hyer et al., 1999; Kanzawa et al., 2002). Owing to a missense substitution in the cardiac troponin T gene, the zebrafish mutant silent heart never exhibits cardiac contraction (Sehnert et al., 2002). Calcium imaging of silent heart mutant embryos at 48 hpf revealed normal conduction slowing in the AV ring (Fig. 4B). Furthermore, treatment of these embryos with terfenadine resulted in 2:1 AV block that could be visualized by calcium imaging (see Movie 2 in the supplementary material). The atrial and ventricular rates from a representative fish are seen in Fig. 4C, confirming 2:1 AV block. Similar results were obtained using 2,3-butanedione 2-monoxime to prevent cardiac contraction for the first 48 hours post-fertilization (data not shown). These findings confirm the formation of normal AV conduction tissue in hearts that have never contracted nor been exposed to physiological blood flow.

Endothelin 1 is a known endocardial signaling molecule that has been implicated in the flow-mediated induction of the peripheral conduction system phenotype (Gourdie et al., 1998; Hyer et al., 1999; Patel and Kos, 2005). We therefore sought to determine the role of endothelin 1 in the development of AV conduction tissue. Using a previously described antisense morpholino, we knocked down endothelin 1 mRNA translation (Miller and Kimmel, 2001). The resulting embryos exhibited the characteristic pharyngeal arch compression spacing of isochronal lines (50 mseconds) in the AV ring (Fig. 4B). Furthermore, treatment of these embryos with terfenadine resulted in 2:1 AV block that could be visualized by calcium imaging (see Movie 2 in the supplementary material). The atrial and ventricular rates from a representative fish are seen in Fig. 4C, confirming 2:1 AV block. Similar results were obtained using 2,3-butanedione 2-monoxime to prevent cardiac contraction for the first 48 hours post-fertilization (data not shown). These findings confirm the formation of normal AV conduction tissue in hearts that have never contracted nor been exposed to physiological blood flow.

**Fig. 3. Endocardial signaling is required for the development of AV conduction tissue.** (A) Calcium activation map of a single representative cardiac cycle in cloche mutant embryos at 48 hpf. Isochronal lines (black) obtained by fluorescence microscopy are superimposed on a maximum intensity projection of the same heart and demonstrate the failure of AV conduction tissue development in the absence of endocardium. (B) Lack of AV conduction block in cloche embryos compared with wild-type siblings is demonstrated by atrial pacing (white bars) and terfenadine exposure (gray bars). Inset of contemporaneous recordings of atrial and ventricular contraction using intensity-time plots from regions over the respective chambers in a terfenadine treated cloche embryo demonstrating 1:1 AV conduction. (C,D). In situ expression patterns of notch1b (C) and bmp4 (D) in cloche embryos at 48 hpf. Despite overstaining in the brain there is no evidence of notch1b signal in the heart (C). In D, the black arrowhead indicates the location of the AV ring; the white arrowhead denotes intense expression of bmp4 at the sinus venosus.

**Fig. 4. Flow is not required for the development of AV conduction tissue.** (A) Time line of the experiments with silent heart embryos. (B) Calcium imaging of silent heart embryo at 48 hpf. Compressed spacing of isochronal lines (50 mseconds) in the AV ring demonstrates physiological conduction delay. Scale bar: 50 μm. (C) silent heart mutant embryos develop 2:1 block at 48 hpf in response to terfenadine treatment, as demonstrated in this plot of atrial and ventricular fluorescence intensity.
defects (Fig. 5A) seen in the genetic mutant sucker, which is null at the endothelin 1 locus (Miller et al., 2000). Despite phenocopy of the sucker mutant, the morphants exhibited normal AV conduction physiology (Fig. 5B,C), suggesting that, in contrast to observations in the peripheral conduction system, endothelin 1 is not required for the specification of AV conduction tissue.

**Neuregulin signaling is necessary for development of AV conduction tissue**

We cloned the zebrafish neuregulin cDNA and characterized its expression pattern in 48 hpf embryos. Zebrafish neuregulin expression is strongest in neural tissue, but can also be seen in the heart with accentuated expression in the AV ring (Fig. 6A) that is restricted to the endocardium (Fig. 6A, inset). We designed a splice-acceptor morpholino to target exon 6 of the neuregulin gene (Falls, 2003). This exon contains the EGF-like domain that is crucial for the function of all neuregulin splice forms (Falls, 2003). In neuregulin morphant fish, calcium activation mapping revealed a generally slowed conduction throughout the heart, and a loss of physiological AV delay, as demonstrated in the typical isochronal map seen in Fig. 6B. In these morphants, the average maximal atrial wavefront velocity was 434±14 microns/second, whereas the average ventricular wavefront velocity was 386±10 microns/second. The morphants also failed to develop 2:1 AV block with terfenadine (Fig. 6C). The morpholino effect was dose dependent, with 25% of embryos (n=20) lacking functional AV conduction tissue following a 50 µM morpholino injection, and 100% failing to develop 2:1 AV block at 100 µM. Progressive knockdown of neuregulin mRNA in these embryos was confirmed by quantitative RT-PCR (Fig. 6D). Finally, we designed a second non-overlapping antisense morpholino targeting the donor splice site of exon 6, which reproduced the failure of AV delay phenotype in calcium imaging experiments (5 out of 10 fish). A four-basepair mismatch morpholino had no effect on the development of AV delay (data not shown). All control-injected fish developed 2:1 AV block at 40 hpf. Taken together, these data define an early requirement for neuregulin in the differentiation of the AV conduction tissue, while also suggesting a more general requirement for neuregulin in the electrical maturation of the chambers (Liu et al., 1998; Meyer and Birchmeier, 1995; Rentschler et al., 2002; Zhao et al., 1998).

**Notch signaling is required for the normal formation of AV conduction tissue**

In light of the importance of notch signaling in cell fate determination, and of the localization of notch1b to AV endocardium, we investigated the role of this isoform in the development of AV conduction tissue. We targeted the splice donor
of exon 27, which contains the intracellular signaling domain. Calcium imaging in notch1b morphant embryos revealed a loss of AV conduction slowing, as shown in Fig. 7A. At doses that knocked down 69% of processed mRNA, 28% of embryos (30/108) failed to develop 2:1 AV block on treatment with terfenadine (Fig. 7B). Higher doses of morpholino were uniformly lethal, causing developmental arrest in the first 24 hours post-fertilization. In order to confirm the specificity of these results, a second non-overlapping morpholino targeting the splice acceptor of exon 27 was designed. This morpholino resulted in the failure of AV delay development in injected embryos, both by calcium imaging (6 out of 10 injected embryos) and by terfenadine treatment (25% of injected embryos failed to develop 2:1 AV block, n=28; Fig. 7B). Failure of AV delay development was never seen in any of the control embryos (n=50). These results confirm a requirement for notch1b in the formation of central conduction tissue. Because notch signaling is an important determinant of cell fate, we considered whether its inhibition might have more global effects on cardiac chamber specification. We therefore examined known atrial and ventricular specific markers, which demonstrated normal patterns of expression and excluded any major effects on chamber fate (see Fig. S1 in the supplementary material).

In order to determine the relationship between neuregulin and notch in AV myocyte differentiation, we characterized notch1b expression in neuregulin morphants. Cardiac notch1b expression is normal in these fish (Fig. 7C). Interestingly, neuregulin expression was also normal in notch 1b morphant embryos that failed to develop 2:1 AV block (Fig. 7D). The expression pattern of bmp4 was unperturbed in affected neuregulin and notch1b morphants (data not shown). These data suggest that there is no interaction between notch1b and neuregulin at the mRNA level, but do not exclude post-transcriptional cross-talk between these pathways.

**DISCUSSION**

Several elegant genetic screens designed to identify the crucial pathways in cardiogenesis have taken advantage of the zebrafish (Fishman and Olson, 1997; Sehnert and Stainier, 2002; Stainier et al., 1996). Recent efforts have extended into developmental physiology, where early work has suggested parallels between the zebrafish and higher vertebrates (Milan et al., 2003). In the work presented here, we have developed methods to study the formation of specialized AV conduction tissue in zebrafish, using physiological endpoints rather than expression analyses. We were able to use these techniques in combination with well-characterized zebrafish mutants and morpholino gene knockdown to explore the role of endocardial signals regulating the induction of AV conduction tissue.

**Functional imaging of myocyte differentiation**

By using three independent techniques, we have demonstrated that myocytes in the AV ring undergo differentiation into cells with slow conduction properties and prolonged refractory periods. These are the defining features of central ‘slow’ conduction tissue. The propagation of electrical activity in the embryonic heart is much slower than in the adult. It has been proposed that the entire embryonic heart is a ‘nodal’ structure and, that, as the chambers differentiate, they develop more rapid conduction, while AV ring conduction remains slow. In this model, the properties of the AV conduction tissue are the default pathway (de Jong et al., 1992). However, our results demonstrate that the development of conduction delay in the AV ring is an active process, rather than the persistence of a primitive myocardial phenotype.

**Flow-independent endocardial signals induce AV conduction tissue**

The zebrafish mutant cloche is a valuable tool with which to explore the role of endothelial tissue interactions during development, as it lacks all endocardial tissue and almost all endothelial cells (Stainier et al., 1995). We found that the cloche mutants failed to develop AV conduction tissue, highlighting an inductive role for the endocardium in this process. There is a growing recognition that endothelial cells participate in many developmental events (Cleaver and Melton, 2003). Endothelial signals and physiological blood flow are required for the formation of specialized vascular structures, including renal glomeruli (Majumdar and Drummond, 1999; Patel and Kos, 2005; Serluca et al., 2002; Stainier et al., 1995). Co-culture experiments, and in some cases in vivo studies, have demonstrated that endothelial cells induce the differentiation of many cell types, particularly neural and neuroendocrine fates (Lammert et al., 2001; Lammert et al., 2003). In many of these settings, there is evidence for bidirectional signaling between endothelial cells and surrounding tissues, and there are several examples where these signals are dependent on mechanical forces including physiological blood flow (Bartman et al., 2004; Hove et al., 2003), e.g. the development of the peripheral conduction system (Hyer et al., 1999). Here, it is thought that flow triggers endothelin 1 release, which in conjunction with other cues, causes surrounding myocytes to differentiate into Purkinje fibers (Gourdie et al., 1998; Hall et al., 2004; Kanzawa et al., 2002). In vivo data directly implicating blood flow as a signal for conduction system development have not been previously available.
Arresting blood flow during development is challenging in most experimental models, but the zebrafish allows the in vivo investigation of the role of blood flow, without the disruption of other contextual developmental signals (Stainier et al., 1996). Under conditions of circulatory arrest, we observed that AV conduction tissue develops normally. This is in contrast to the available data on the peripheral conduction system (Hall et al., 2004; Hyer et al., 1999), and suggests that a different set of cues is used to signal myocytes to become central ‘slow’ conduction tissue. Consistent with this interpretation, knockdown of *endothelin 1*, a signal implicated in hemodynamic induction of the peripheral conduction system, had no effect on the differentiation of AV conduction tissue (Gourdie et al., 1998; Hall et al., 2004). Additionally, these results demonstrate that the pathways regulating central conduction system development are distinct from those involved in valvulogenesis, where contraction and flow-mediated signaling are essential (Bartman et al., 2004; Hove et al., 2003).

**Neuregulin is necessary for the induction of AV conduction tissue**

A role for neuregulin in promoting the expression of conduction system markers and in the development of rapid ventricular conduction has been defined in the mouse (Patel and Kos, 2005; Rentschler et al., 2002). However, mice null for *neuregulin* fail to develop normal trabecular myocardium, and die as a result of ‘myocardial failure’ at a stage too early to observe the effects on conduction tissue. We sought to define the effect of loss of neuregulin signaling on AV conduction tissue development.

We have cloned the zebrafish *neuregulin* cDNA and demonstrate that its expression is concentrated in AV ring endocardium at a time that is critical for the development of central conduction tissue. The knockdown of *neuregulin*, using morpholinos targeted to the exon encoding the crucial EGF-like domain, establishes an essential role for this signaling molecule in the induction of slowly conducting AV tissue. In *neuregulin* morphant fish we also observed that propagation velocities throughout the heart remained at basal levels similar to those seen in the 36 hpf embryo, suggesting that neuregulin may be required for the proper electrophysiological maturation of atrial and ventricular myocytes, in addition to the AV ring.

**Notch regulates the formation of AV conduction tissue**

We have demonstrated that zebrafish *notch1b* is expressed in the AV ring endocardium at a time point when AV conduction tissue is forming. Targeted knockdown of *notch1b* expression resulted in a failure of AV conduction tissue development in 25-28% of embryos. Higher doses of the morpholino cause substantial lethality, presumably because of requirements for *notch1b* at earlier stages of development. As is the case with the her genes in mice, there may be redundancy in AV ring notch signaling (Kokubo et al., 2005). Our data suggest that notch signaling not only affects endocardial cushion formation, but is also necessary for conduction tissue differentiation, and thus is likely to be active at an early step in AV ring specification. Expression analyses of *neuregulin* and *notch1b* in the respective morphant fish revealed no evidence of interactions between these pathways at the mRNA level.

Several endothelial-tissue inductive interactions, including those required for the differentiation of insulin-producing islet cells within the pancreas, are known to involve notch-delta pathway members (Clear and Melton, 2003; Lammert et al., 2000). Evidence for the role of notch signaling in endocardial cushion formation comes from several sources (Kokubo et al., 2005; Kokubo et al., 2004; Noseda et al., 2004; Timmerman et al., 2004). Recent work has identified rare dominant mutations in human Notch 1 that cause complex congenital heart disease and aortic stenosis. No conduction system disease was reported in these families, but it is conceivable that there may be selection in favor of milder phenotypes or the human mutations may act through a gain of function (Garg et al., 2005).

At a stage of development when there are 350 or fewer cells in the entire heart, there is already functional evidence of atrial, ventricular and AV conduction tissue characterized by distinct electrophysiological properties. The functional approach outlined here may enable further characterization of the notch-delta pathway members required for the patterning of the AV ring, as well as of cross-talk with other key developmental pathways, including Bmp and Wnt signaling.

**Conclusion**

We have employed the zebrafish to study the earliest steps in central conduction system development that previously have been inaccessible. Using physiological assays, we have demonstrated for the first time that a distinct population of slowly conducting cells arises in the AV ring of the developing heart. The formation of this specialized conduction tissue is a result of an active developmental process driven by flow-independent endocardial signals. Future application of these assays will enable an unbiased investigation of other signals involved in cardiac conduction tissue development and in the physiological patterning of the vertebrate heart.

The authors would like to thank Drs Patrick Ellinor, CharlesHong, Jordan Shin, Ashok Srinivasan and Jeremy Ruskin for their thoughtful comments and suggestions. This work was funded by NIH grants HL076361 (D.J.M.), GM075546 (C.A.M. and D.J.M.), HL065962 (C.A.M.) and HL079267 (R.T.P.), and by a grant from the Cardiovascular Research Foundation (D.J.M.).

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/6/1125/DC1

**References**


