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Specification of the mouse cardiac conduction system in the absence of Endothelin signaling

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ABSTRACT

Coordinated contraction of the heart is essential for survival and is regulated by the cardiac conduction system. Contraction of ventricular myocytes is controlled by the terminal part of the conduction system known as the Purkinje fiber network. Lineage analyses in chickens and mice have established that the Purkinje fibers of the peripheral ventricular conduction system arise from working myocytes during cardiac development. It has been proposed, based primarily on gain-of-function studies, that Endothelin signaling is responsible for myocyte-to-Purkinje fiber transdifferentiation during avian heart development. However, the role of Endothelin signaling in mammalian conduction system development is less clear, and the development of the cardiac conduction system in mice lacking Endothelin signaling has not been previously addressed. Here, we assessed the specification of the cardiac conduction system in mouse embryos lacking all Endothelin signaling. We found that mouse embryos that were homozygous null for both *ednra* and *ednrb*, the genes encoding the two Endothelin receptors in mice, were born at predicted Mendelian frequency and had normal specification of the cardiac conduction system and apparently normal electrocardiograms with normal QRS intervals. In addition, we found that *ednra* expression within the heart was restricted to the myocardium while *ednrb* expression in the heart was restricted to the endocardium and coronary endothelium. By establishing that *ednra* and *ednrb* are expressed in distinct compartments within the developing mammalian heart and that Endothelin signaling is dispensable for specification and function of the cardiac conduction system, this work has important implications for our understanding of mammalian cardiac development.

Introduction

The cardiac conduction system (CCS) is a specialized, electrically active tissue within the heart that carries electrical impulses to coordinate atrial and ventricular contraction in a rhythmic fashion (Mikawa and Hurtado, 2007). The major components of the CCS include the sinoatrial node (SAN), the atrioventricular node (AVN), the right and left bundle branches, and the peripheral ventricular conduction system. The SAN is the primary pacemaker of the heart and generates the initial electrical impulse that rapidly spreads through the atria (Bakker et al., 2010; Mikawa and Hurtado, 2007). The electrical impulse slows as it enters the AVN and then is propagated rapidly through the bundle of His, the right and left bundle branches, and the peripheral ventricular conduction system (Bakker et al., 2010; Mikawa and Hurtado, 2007). The peripheral ventricular conduction system consists of the Purkinje fiber network, which coordinates ventricular contraction beginning at the apex and propagating to the base, resulting in efficient emptying of the ventricles (Bakker et al., 2010; Mikawa and Hurtado, 2007).

Retroviral lineage labeling studies performed in chicken embryos and fate mapping studies in mouse embryos have established that the cells of the peripheral conduction system are derived from working myocytes (Mikawa et al., 2003; Miquerol et al., 2011; Munshi, 2012). Purkinje fiber differentiation occurs around areas of high blood flow and enhanced shear stress adjacent to the endocardium and near coronary arteries (Gourdie et al., 1995, 1999; Pennisi et al., 2002). In addition, based on work performed in the chick system, it has been proposed that

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myocyte-to-Purkinje fiber transdifferentiation occurs via activation of Endothelin signaling (Gourdie et al., 1998; Kanzawa et al., 2002; Takebayashi-Suzuki et al., 2000). Endothelin peptides are potent vasoactive peptides that control numerous aspects of normal physiological homeostasis, most notably regulating vascular tone (Barton and Yanagisawa, 2008). There are three Endothelin peptides (ET-1, ET-2, and ET-3) that induce signaling by binding to Endothelin receptors, which are seven-pass transmembrane G protein-coupled receptors (Barton and Yanagisawa, 2008; Kedzierski and Yanagisawa, 2001). Mammals have two Endothelin receptors, Endothelin receptor A (ET_A, encoded by the *ednra* gene) and Endothelin receptor B (ET_B, encoded by the *ednrb* gene) (Kedzierski and Yanagisawa, 2001). In addition to ET_A and ET_B, birds encode a third Endothelin receptor ET_{B2}, which is not found in mice (Kanzawa et al., 2002; Lecoin et al., 1998). Mature Endothelin peptides are 21 amino acids long but are synthesized as longer proteins that are subject to multiple steps of proteolytic processing (Barton and Yanagisawa, 2008; Kedzierski and Yanagisawa, 2001). Furin proteases digest preproendothelins into inactive intermediates, referred to as Big Endothelins; Big Endothelins are further processed to the mature peptides in a highly specific proteolytic event by one of two Endothelin-specific proteases, known as Endothelin-converting enzyme-1 (Ece-1) and -2 (Ece-2) (Barton and Yanagisawa, 2008; Kedzierski and Yanagisawa, 2001).

In the developing chick embryo, ET_A and ET_B are reported to be expressed in cardiomyocytes, while ET_{B2} is expressed in the developing valve leaflets (Kanzawa et al., 2002). Endothelin signaling in cardiac myocytes is sufficient for induction of chick cardiomyocyte transdifferentiation into peripheral Purkinje fibers (Kanzawa et al., 2002). The model for Endothelin induction of Purkinje fiber differentiation suggests that shear stress from blood flow induces expression of *Ece1* in the endocardium and coronary endothelium, and Ece-1 in turn processes Big Endothelin-1 expressed in endothelial cells into the active ET-1 peptide, which then allows endothelial-to-myocardial Endothelin signaling to occur (Hall et al., 2004; Takebayashi-Suzuki et al., 2000).

Loss-of-function mutations for Endothelin receptor genes in mice have established an essential role for Endothelin signaling in neural crest development (Clouthier et al., 1998; Hosoda et al., 1994; Yanagisawa et al., 1998). Inactivation of *ednra* results in neonatal lethality due to cranial neural crest-derived craniofacial and cardiac defects (Clouthier et al., 1998). Inactivation of *ednrb* results in pigmentation defects and megacolon due to defects in derivatives of trunk neural crest, leading to lethality at weaning (Hosoda et al., 1994). Double knockout of both *ednra* and *ednrb* in mice, resulting in complete loss of Endothelin signaling, was briefly reported to result in embryonic lethality (Yanagisawa et al., 1998), but a detailed analysis of those mice has not been reported. Additionally, conduction system development in the absence of Endothelin signaling has not been described.

In this study, we examined the expression of *ednra* and *ednrb* genes in the developing mouse heart, and we assessed the formation and function of the cardiac conduction system in mice lacking Endothelin signaling. We found that *ednra* expression within the heart was restricted to the myocardium and was not apparent in the endocardium. In contrast, we found that *ednrb* expression in the heart was largely restricted to the endocardium and coronary endothelium. We also found that *ednra*$^{-/-}$; *ednrb*$^{-/-}$ knockout embryos, which have no Endothelin signaling, were born at predicted Mendelian frequency on an outbred background. Importantly, we observed no alterations in the temporal or spatial expression pattern of the cardiac conduction system marker transgene CCS-*lacZ* or in the expression of *Gja1* or *Gja5*, markers of conducting tissue, in the developing heart in *ednra*$^{-/-}$; *ednrb*$^{-/-}$ knockout embryos when compared to wild type embryos. Similarly, fetuses lacking Endothelin signaling showed no obvious changes in the cardiac conduction system function compared to wild type control fetuses, including no change in PR interval or in the morphology or duration of the QRS complex, as measured by fetal electrocardiogram. These data demonstrate that Endothelin signaling is not required for conduction system marker gene expression or for basic cardiac conduction system function, including the function of the peripheral ventricular conduction system, and thus strongly suggest that Endothelin signaling is not required for cardiac conduction system specification in the mouse. This work has important implications for our understanding of conduction system development in mammals.

Materials and methods

Genetically modified mice and mouse embryo electrocardiography

CCS-*lacZ* transgenic and *ednra* and *ednrb* knockout mice have been described previously (Clouthier et al., 1998; Hosoda et al., 1994; Rentschler et al., 2001). To generate *ednra*$^{-/-}$; *ednrb*$^{-/-}$ embryos, we intercrossed *ednra*$^{+/-}$; *ednrb*$^{+/-}$ double heterozygous mice. CCS-*lacZ*$^{Tg/0}$; *ednra*$^{-/-}$; *ednrb*$^{-/-}$ mice were generated by crossing CCS-*lacZ*$^{Tg/0}$; *ednra*$^{+/-}$; *ednrb*$^{+/-}$ to *ednra*$^{+/-}$; *ednrb*$^{+/-}$ double heterozygotes.

For embryonic electrocardiography, pregnant mice were anesthetized with isoflurane when embryos were at embryonic day (E) 18.5, the peritoneal cavity was opened, and the uterus was exposed without disrupting its anatomical attachments or blood supply. Under direct visualization, 2 needle electrodes were placed through the uterus and yolk sac near the attachment of the upper limbs and thorax of each embryo. A single lead ECG recording was obtained in this manner for several seconds per embryo, with subsequent removal of embryos for genotyping. Signals were filtered with a signal conditioner (Animal BioAmp, AD Instruments, Colorado Springs, CO) and sampled at 10 kHz, using a PowerLab analog-to-digital converter and the Chart5Pro software package (v 5.4.2, AD Instruments). ECG analyses were performed with Chart5-Pro by an investigator blinded to fetus genotype. Several seconds of data for each embryo were averaged using automated R-wave detection, and intervals were measured with electronic calipers from averaged data. The QRS interval was measured from the onset of the sharp deflection in the Q wave to the nadir of the S wave. Data for each genotype were pooled, and statistical analyses were performed using a two-tailed Student's *t*-test.

Genotyping was performed by PCR or Southern blot on genomic DNA isolated from yolk sacs or tail biopsies. All experiments using animals were reviewed and approved by the UCSF Institutional Animal Care and Use Committee and complied with all institutional and federal guidelines.

X-gal staining and in situ hybridization

To visualize the cardiac conduction system, CCS-*lacZ*$^{Tg/0}$ hearts isolated from mouse embryos at E11.5 and E14.5 were stained with X-gal to detect β-galactosidase activity as previously described (Anderson et al., 2004). Following staining, embryonic hearts were dehydrated in ethanol and then either cleared in a 1:1 solution of benzyl benzoate:benzyl alcohol for whole mount visualization or sectioned at a thickness of 10 μm and counterstained with Nuclear Fast Red as previously described (Anderson et al., 2004).

In situ hybridization was performed as described previously (Morikawa et al., 2009). The *Gja1* (connexin 43), *Gja5* (connexin 40), and *Tnni3* in situ probe plasmids have been previously described (Koibuchi and Chin, 2007; Ruangvoravat and Lo, 1992;

Soufan et al., 2004). The *Gja1* probe was made by linearizing the in situ plasmid with BamHI and transcribing with T7 polymerase. The *Gja5* probe was made by linearizing the in situ plasmid with SpeI and transcribing with T7 polymerase. The *Tnni3* probe was made by linearizing with BamHI and transcribing with T3 polymerase. The *Flk1* in situ probe plasmid was made by amplifying a 712 bp region of the mouse *Flk1* cDNA extending from nucleotide 383 to nucleotide 1094 of the 5924 bp *Flk1* cDNA and cloning the resulting product into plasmid pCR2.1 (Life Technologies). Antisense *Flk1* probe was made by linearizing plasmid pCR2.1-*Flk1* with NotI and transcribing with T7 polymerase. The *ednra* and *ednrb* in situ plasmids were generated by amplifying regions of the *ednra* and *ednrb* genes by PCR from mouse embryonic cDNA by using primers *ednra*-F, 5′-ttcatggcccatgactaca-3′ and *ednra*-R, 5′-agttggggacaggatgga and *ednrb*-F, 5′-agaaaagacagcctgcga-3′ and *ednrb*-R, 5′-ggttaaacaaagatgttaggact-3′, and cloning the products into pBluescriptSKII(+). The *ednra* and *ednrb* probes were made by linearizing the in situ plasmids with NotI and HindIII, respectively, and transcribing with T7 polymerase.

RNA isolation and quantitative real-time reverse transcriptase PCR (qPCR)

RNA was isolated from individual embryonic hearts collected at E11.5 and prepared using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. RNA was treated with DNaseI at 37°C for 1 h, and then cDNA synthesis using the Omniscript RT kit (Qiagen) was performed. 10 ng of cDNA was used for each qPCR using the MAXIMA SYBR Green kit (Fermentas). The following primers were used to amplify *Gja5*: 5′-agggctgagcttgcttctta-3′ and 5′-ttagtgccagtgtcgggaat-3′ and *Gja1*: 5′-ggtgatgaacagtctgccttt cg-3′ and 5′-gtgagccaagtacaggagtgtg-3′. Expression data were normalized to *gapdh* expression as described previously (Schachterle et al., 2012).

Results

Viability of ednra; ednrb double knockout mice

Previously, it was reported that *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout mice displayed 100% lethality at E13.5 when maintained on an inbred 129SvEv background (Yanagisawa et al., 1998). In contrast, we maintained *ednra*$^{+/-}$; *ednrb*$^{+/-}$ double heterozygous mice on an outbred, mixed background and found that intercrosses of double heterozygotes resulted in *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout mice born at normal Mendelian ratios (Table 1, χ^2 test=0.65). Importantly, the *ednra* and *ednrb* mutant alleles used here have each been shown previously to completely abolish responsiveness to Endothelin ligands, establishing that the mutations are true null alleles (Clouthier et al., 1998; Hosoda et al., 1994). We found that double knockout mice on an outbred background died shortly after birth with evidence of severe cyanosis and profound craniofacial closure defects (data not shown), consistent with the defects reported previously for *ednra*$^{-/-}$ single knockout mice (Clouthier et al., 1998). The survival of *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout mice to birth when maintained on an outbred background is consistent with the incompletely penetrant embryonic lethality of *Ece1*-null mice when maintained on a C57BL/6-129SvEv mixed background (Yanagisawa et al., 1998) and supports the strong possibility that additional genes are likely involved in the embryonic lethality observed in the absence of Endothelin signaling on an isogenic 129SvEv background.

Table 1
Viability of outbred *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout mice at E9.5, E13.5, and P0. Mice of all genotypes were recovered at normal Mendelian frequencies at P0, including *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockouts, χ^2 test=0.65. All *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockouts were cyanotic at birth and displayed craniofacial defects, consistent with the defects reported previously for *ednra*-null mice (Clouthier et al., 1998).

Gestational age	*ednra*$^{-/-}$; *ednrb*$^{-/-}$ mice recovered: actual (expected)	Total # of mice recovered: (all genotypes)	Percentage of *ednra*$^{-/-}$; *ednrb*$^{-/-}$ embryos recovered: actual (expected)
E9.5	4 (3)	53	7.5% (6.25%)
E13.5	5 (4)	62	8.1% (6.25%)
Postnatal day 0	4 (4)	63	6.3% (6.25%)

Distinct expression patterns for ednra and ednrb in the developing mouse heart

Previous studies of the developing chick heart have reported that ET_A and ET_B are expressed in cardiomyocytes and ET_{B2} is expressed in the developing valve leaflets (Kanzawa et al., 2002). By comparison, the expression of the genes encoding the Endothelin receptors has been less well described in the developing mouse heart (Asai et al., 2010; Clouthier et al., 1998; Lee et al., 2003). Therefore, to examine *ednra* and *ednrb* expression in the developing mouse heart from early development and throughout cardiogenesis, we performed in situ hybridization analyses of sectioned wild type mouse embryonic hearts from E8.5 to E14.5 (Fig. 1).

At E8.5, *ednra* was weakly expressed within cardiomyocytes of the early common ventricular chamber (Fig. 1A). Expression of *ednra* continued in cardiomyocytes at E9.5 and E10.5 (data not shown), as previously described (Asai et al., 2010; Clouthier et al., 1998). At E11.5, *ednra* was expressed in cardiac myocytes in both the trabecular and compact zones (Fig. 1C). Comparison of *ednra* expression to *Tnni3* expression on adjacent sections confirmed that the expression of *ednra* was restricted to the myocardium at E11.5 although expression in the myocardium was not as strong or as uniform as a structural gene like *Tnni3* (Fig. 1C and E). Expression of *ednra* in compact and trabecular myocardium continued at E14.5 (Fig. 1G and I). Again, expression of *ednra* was compared to the expression of *Tnni3* on adjacent sections, which showed that *ednra* expression was confined to the myocardium in a pattern overlapped by the strong expression of the cardiac specific *Tnni3* gene (Fig. 1I and K).

Transcripts for *ednrb* were not detected in the heart at E8.5; expression was first observed in the endocardium at E9.5 (Fig. 1B and data not shown). By E11.5, *ednrb* became robustly expressed in the endocardium associated with chamber myocardium (Fig. 1D). Expression of *ednrb* was not observed in the endothelial cells of the inflow or outflow tracts, nor was it observed in the endothelial cells of the aortic arch arteries (data not shown). *ednrb* expression was also observed within the endothelial cells of the coronary vasculature at E11.5 (Fig. 1D). Comparison of *ednrb* expression at E11.5 to the expression of the endothelial marker *Flk1* from an adjacent serial section (Fig. 1F) showed essentially identical patterns of expression of the two genes (Fig. 1D and F). Expression of *ednrb* remained largely restricted to the coronary endothelial cells and to the endocardium at E14.5 (Fig. 1H and J) in a pattern that was overlapping with the pattern of the endothelial marker *Flk1* on an adjacent serial section (Fig. 1L). Importantly, these data show that throughout cardiogenesis, *ednrb* was either not detected or only very weakly observed in the myocardium, at least at the level of detection by in situ hybridization. The restricted expression of *ednrb* to the endothelium and endocardium in the embryonic mouse heart is in contrast to the expression in the

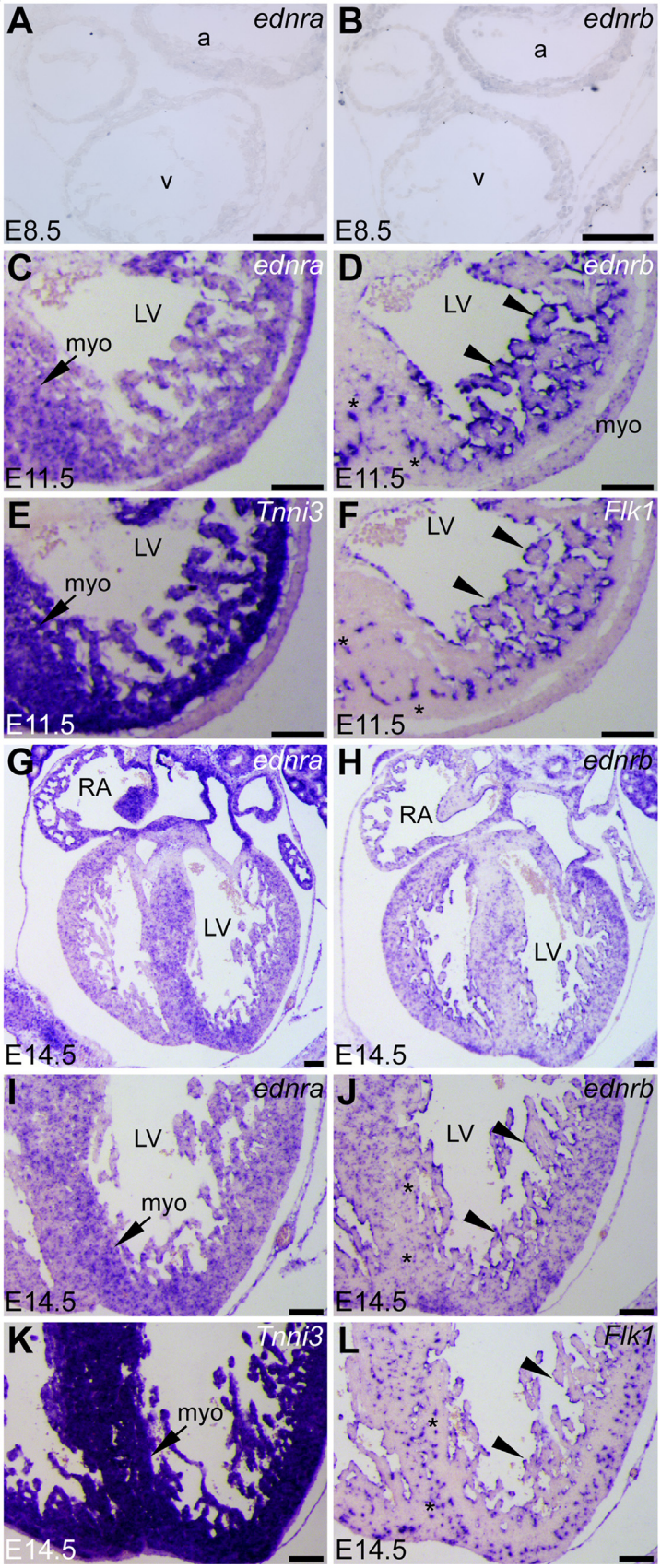

Fig. 1. Expression patterns of *ednra* and *ednrb* in the developing mouse heart. Expression of *ednra* (A, C, G, and I) and *ednrb* (B, D, H, and J) was determined by in situ hybridization and compared on serial transverse sections to the expression of the myocardial marker *Tnni3* (E and K) and the endothelial marker *Flk1* (F and L) from mouse embryos collected at E8.5 (A and B), E11.5 (C–F), and E14.5 (G–L). *ednra* expression was restricted to cardiomyocytes (arrows) in a pattern that was completely overlapped by, but more punctate than, the expression of *Tnni3*. *ednrb* expression was largely restricted to the endocardial cells (arrowheads) lining the atrial and ventricular chambers and to endothelial cells of the coronary vasculature (asterisks) in a pattern very similar to the pattern of expression of *Flk1*. a, atrium; LV, left ventricle; myo, myocardium; RA, right atrium; v, ventricle. The bar equals 100 μm in all panels.

chick heart, where *ednrb* expression has been reported in cardiomyocytes of the atria and left ventricle (Kanzawa et al., 2002; Takebayashi-Suzuki et al., 2000). Taken together, our data demonstrate that *ednra* and *ednrb* are expressed in different cell types within the developing mouse heart.

Cardiac conduction system markers exhibit a normal pattern of expression in the absence of Endothelin signaling

A difficulty in the study of the peripheral cardiac conduction system is a lack of highly specific molecular markers of the lineage during development (Christoffels and Moorman, 2009). In this regard, an important tool for the study of cardiac conduction system development is the cardiac conduction system (CCS)-*lacZ* transgenic mouse line, which expresses β-galactosidase in the cardiac conduction system, including the ventricular peripheral Purkinje fiber network (Rentschler et al., 2001). In addition, the presence of β-galactosidase-positive cells in the right bundle branch in CCS-*lacZ* transgenic mice has been directly correlated with overlapping functional maps of electrical propagation, supporting the notion that CCS-*lacZ*-expressing cells are functional components of the nascent cardiac conduction system (Rentschler et al., 2001). Therefore, to examine the specification of the cardiac conduction system in the mouse in the presence and absence of Endothelin signaling, we crossed the CCS-*lacZ* transgene into *ednra*$^{-/-}$, *ednrb*$^{-/-}$, and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ knockout backgrounds (Figs. 2 and 3) and examined the pattern of β-galactosidase activity as an indicator of conduction system specification and patterning. At E11.5, the pattern of CCS-*lacZ* expression showed minor variation among individual embryos, but no substantial differences were observed between wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout hearts (Fig. 2A and B). CCS-*lacZ* marked the major components of the cardiac conduction system, including the venous valve adjacent to the sinus node, the nascent atrioventricular node and the atrioventricular bundle (Fig. 2A and B), suggesting that these conduction system structures were specified in the absence of Endothelin signaling.

At E11.5, the Purkinje fibers of the peripheral conduction system have not fully differentiated, but unorganized conduction tissue is present, and a contraction sequence has begun to be established (Rentschler et al., 2001; Sankova et al., 2012); these early conducting cells in the ventricles express *Gja1* and *Gja5*, which encode connexin 43 and connexin 40, respectively (Bakker et al., 2010; Delorme et al., 1995; Myers and Fishman, 2003; Pfenniger et al., 2011; Ruangvoravat and Lo, 1992). *Gja1* and *Gja5* are expressed in the myocardium but become associated with and are enriched in the developing cardiac conduction system (Bakker et al., 2010; Delorme et al., 1995; Myers and Fishman, 2003; Ruangvoravat and Lo, 1992). At E11.5, *Gja5* (connexin 40) is expressed broadly in trabecular cardiomyocytes and in some compact zone cardiomyocytes, and then its expression gradually restricts to the ventricular conduction system concomitant with a down regulation in compact layer myocardium (Delorme et al., 1995). *Gja1* (connexin 43) is co-expressed with *Gja5* in trabecular myocardium and later is expressed in the Purkinje fibers in the trabecular myocardium (Giovannone et al., 2012; Gourdie et al., 1993). Therefore, to further examine the development of the ventricles and the ventricular conduction system, we also examined *Gja1* and *Gja5* expression in wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout hearts (Fig. 2C–F). Although some embryo to embryo variation was observed, the overall spatial expression patterns of *Gja1* (connexin 43; Fig. 2C and D) and *Gja5* (connexin 40; Fig. 2E and F) were unaffected by the combined loss of ET_A and ET_B. Previous studies reported down regulation of *Gja5* at E9.5 in *ednra*-null mice (Asai et al., 2010). In contrast, we readily detected *Gja5* expression in *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double null embryos at E11.5 (Fig. 2F).

Because of the previously reported down regulation of *Gja5* in *ednra*-null hearts, we examined *Gja5* expression quantitatively in wild type and *ednra* single null hearts by qPCR analysis of RNA isolated from whole embryonic hearts at E11.5. We did not observe a statistically significant difference in *Gja5* expression between wild type (mean expression = 1.00, SD = 0.49, $n = 4$) and *ednra*$^{-/-}$ (mean expression = 0.86, SD = 0.08, $n = 4$) hearts, $p = 0.598$. We extended these analyses by examining the expression of *Gja1* and *Gja5* specifically in the right and left ventricles of wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double null embryos (Fig. 2G). To do this, we removed hearts at E11.5 from wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout embryos, removed the atria, and then dissected the right and left ventricles from each other by pinching the hearts in half at the point of the interventricular sulcus. The dissected ventricles were then used for qPCR analyses to examine *Gja1* and *Gja5* expression levels quantitatively. Although there was substantial embryo to embryo variation in expression level in both wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double null embryos as detected by qPCR, importantly, there was no significant difference in the level of either marker in either the left ventricle or the right ventricle when comparing wild type and double knockout hearts (Fig. 2G). Taken together, these results further support the overall notion that Endothelin signaling in the mouse is not required for the expression level or pattern of markers of the ventricular conduction system.

We also examined the expression of CCS-*lacZ* at E14.5 in wild type, *ednra*$^{-/-}$ and *ednrb*$^{-/-}$ single knockout, and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout hearts (Fig. 3). At this stage, the conduction system is nearly fully differentiated and exhibits a mature conduction pattern (Rentschler et al., 2001; Sankova et al., 2012). CCS-*lacZ* expression was observed in the atrioventricular node, atrioventricular bundle, left and right bundle branches, and the Purkinje fiber network in wild type hearts at this stage (Fig. 3A and A′). Importantly, the pattern and level of β-galactosidase in hearts from all three mutant genotypes (Fig. 3B–D and B′–D′) were indistinguishable from the pattern and level of CCS-*lacZ* in wild type hearts (Fig. 3A and A′). These observations, taken together with apparently normal morphology and contraction of *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout hearts, strongly suggests that the conduction system develops and matures normally in the absence of Endothelin signaling in the mouse.

Intact conduction system function in the absence of Endothelin signaling

Because of perinatal lethality, it was not possible to assess peripheral conduction system function in postnatal *ednra*$^{-/-}$; *ednrb*$^{-/-}$ animals. We circumvented this problem by recording electrocardiograms (ECG) from E18.5 *ednra*$^{-/-}$; *ednrb*$^{-/-}$ fetuses in utero. Needle electrodes were inserted through the exposed uterus and through the yolk sac into the right and left upper limb–thorax junction of each embryo. ECG signals were recorded for several seconds sequentially from each embryo in a litter (Fig. 4). ECG data were measured from 7 wild type and 5 *ednra*$^{-/-}$; *ednrb*$^{-/-}$ fetuses and PR interval and QRS durations were measured. Although baseline noise was present and varied from fetus to fetus, both wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout fetuses were in sinus rhythm (Fig. 4A). Importantly, no gross differences in the ECG tracings were observed for wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ fetuses (Fig. 4A).

The PR interval reflects transit time through the atrium, AV node, and AV bundle, while the QRS duration reflects the time course of ventricular activation and is therefore a direct assessment of conduction through the bundle branches and peripheral

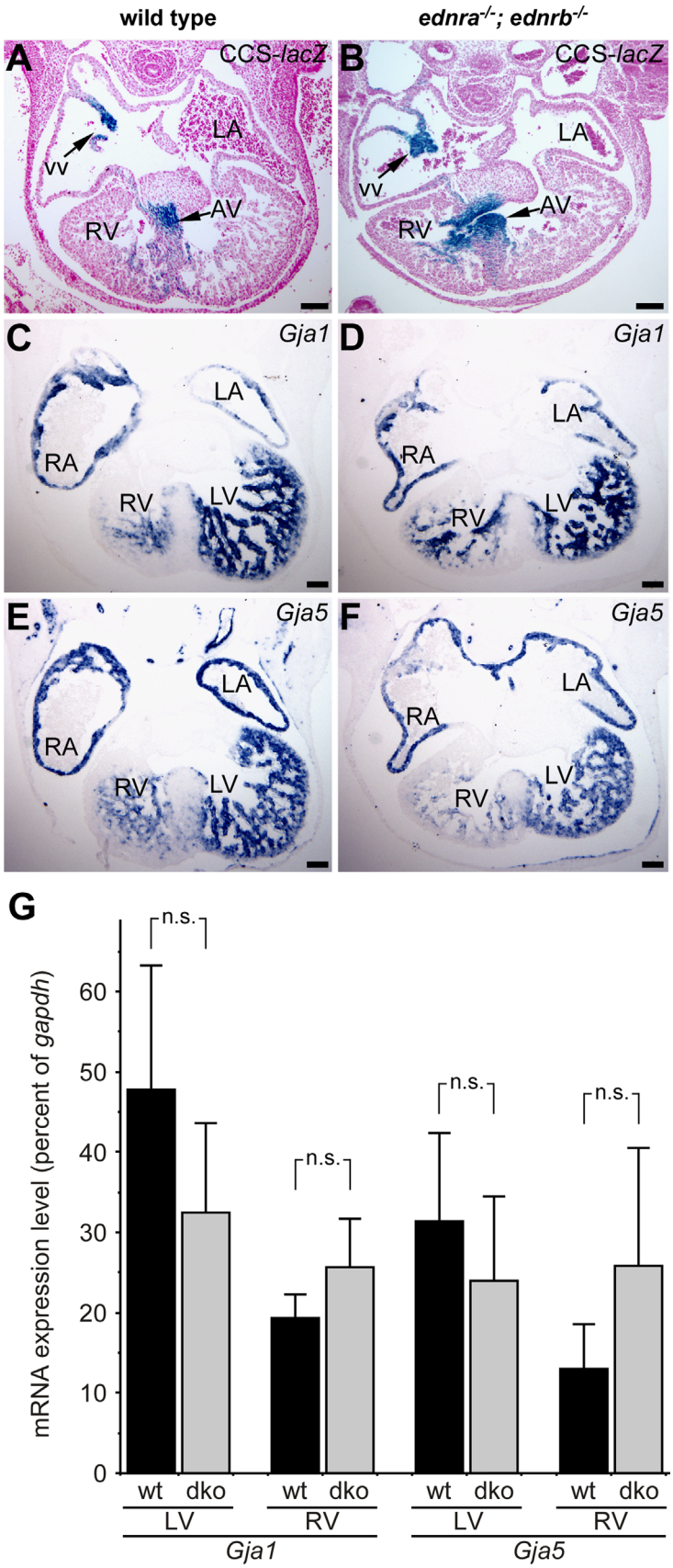

Fig. 2. Normal expression of cardiac conduction system markers in *ednra*$^{-/-}$; *ednrb*$^{-/-}$ knockout mouse hearts at E11.5. Hearts were isolated at E11.5 and transverse sections were cut to analyze CCS-*lacZ* (A and B), *Gja1* (connexin 43) (C and D), and *Gja5* (connexin 40) (E and F) expression in embryos with wild type Endothelin receptor genes (A, C, and E) or in *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout embryos (B, D, and F). X-gal staining for β-galactosidase in wild type CCS-*lacZ* (A) and CCS-*lacZ*; *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout (B) embryonic hearts appeared similar. Likewise, RNA in situ hybridization analyses of *Gja1* (C and D) and *Gja5* (E and F) appeared to be nearly identical in wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout embryos. AV, atrioventricular bundle; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; vv, ventricular valve. Representative images are shown. The bar equals 100 μm in all panels. (G) qPCR analyses of *Gja1* and *Gja5* expression from isolated right ventricles (RV) and left ventricles (LV) of wild type (wt) and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout (dko) embryos collected at E11.5 showed no significant (n.s.) differences in expression of either marker in either ventricle. Data are shown as the mean expression level as a percentage of *gapdh* expression ± SEM for 3 samples in each group.

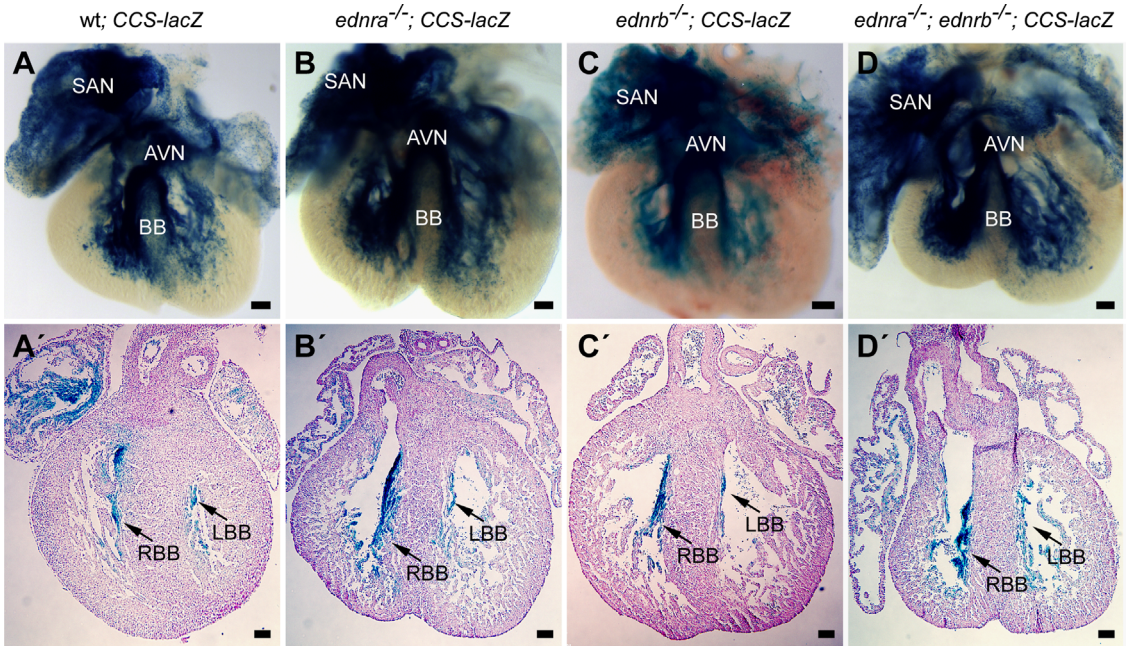

Fig. 3. Normal heart morphology and expression of CCS-*lacZ* in the absence of Endothelin signaling in mouse embryos at E14.5. Hearts were excised at E14.5 and stained with X-gal. Whole mount, ventral view images are shown in (A–D). Coronal sections, counterstained with Nuclear Fast Red, are shown in A′–D′. Morphology and β-galactosidase activity were comparable in wild type CCS-*lacZ* (A and A′), CCS-*lacZ*; *ednra*$^{-/-}$ (B and B′), CCS-*lacZ*; *ednrb*$^{-/-}$ (C and C′), and CCS-*lacZ*; *ednra*$^{-/-}$; *ednrb*$^{-/-}$ (D and D′) transgenic hearts and all major components of the cardiac conduction system appeared to be present. AVN, atrioventricular node; BB, bundle branches; LBB, left bundle branch; RBB, right bundle branch; SAN, sinoatrial node. A minimum of three embryos for each genotype was examined; representative images are shown. The bar equals 100 μm in all panels.

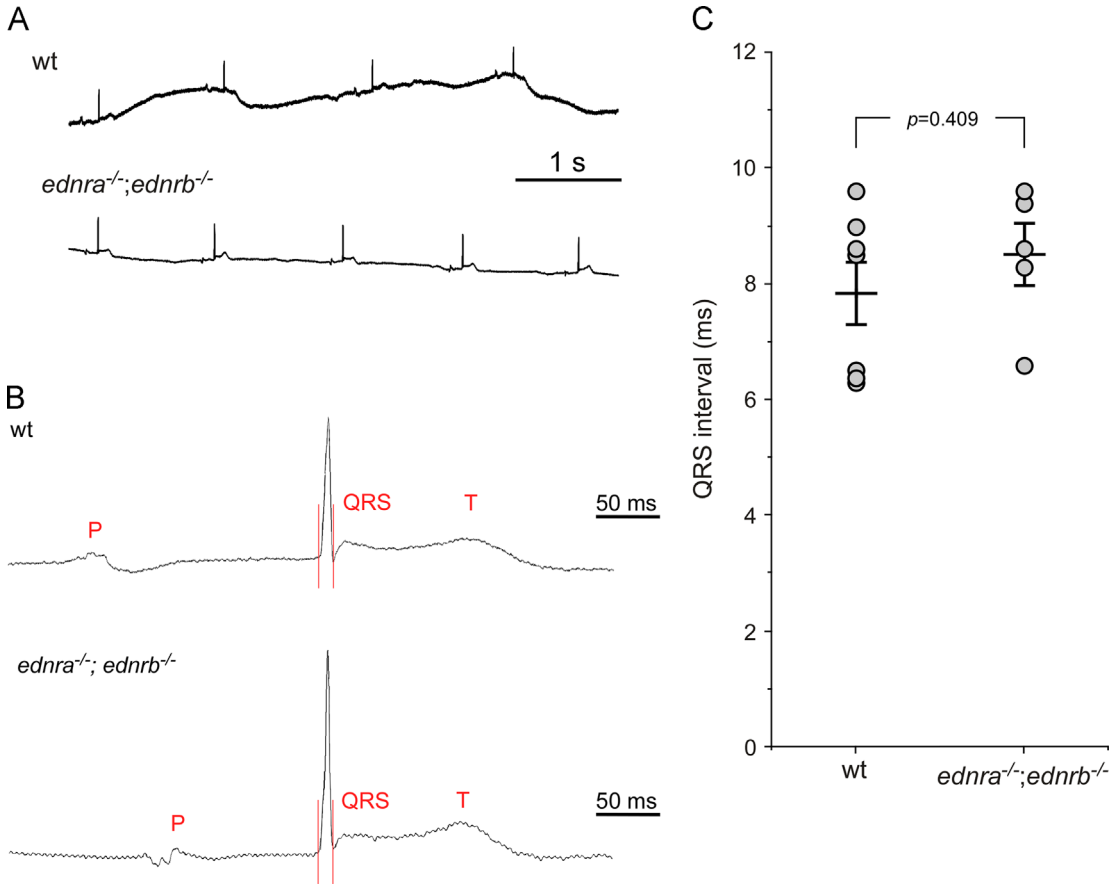

Fig. 4. Apparently normal conduction system function in the absence of Endothelin signaling. (A) Continuous tracings of single lead fetal electrocardiograms (ECG) from representative wild type (wt) (top) and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ (bottom) fetuses showing normal sinus rhythm in both fetuses. Seven wild type and five *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockouts were examined. Tracings from representative fetuses are shown; scale bar, 1 s. (B) Averaged tracings of a representative wt (top) and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout fetus with P, QRS, and T waves shown. Both wt and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout fetuses exhibited similar ECG morphology. 50 ms scale bars are shown for both recordings. (C) Mean QRS intervals ± standard deviation for seven wt and 5 *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout fetuses are shown. Notably, no significant difference ($p=0.409$) was observed between wt and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ fetuses, suggesting similar conduction properties through the ventricular conduction system.

ventricular conduction system. The mean PR interval was not significantly different between wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ fetuses (wild type, 108.6 ± 19.25 ms, $n=6$; *ednra*$^{-/-}$; *ednrb*$^{-/-}$, 96.7 ± 13.24 ms, $n=5$; $p=0.638$, not significant). Likewise, there were no morphological differences between wild type and double mutant QRS complexes (Fig. 4B), nor was there any significant difference in QRS duration between wild type and *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double mutant fetuses (Fig. 4C). These data strongly support the notion that the ventricular cardiac conduction system is specified and functional in the absence of Endothelin signaling.

Discussion

Components of the cardiac conduction system, including the peripheral Purkinje fiber network originate from the transdifferentiation of cardiomyocytes (Mikawa et al., 2003; Miquerol et al., 2011; Munshi, 2012). The formation of the peripheral conduction system has been shown to occur in areas of high hemodynamic flow, and it has been proposed, based primarily on work performed in the chick system, that myocyte-to-Purkinje fiber transdifferentiation occurs in response to shear stress (Gourdie et al., 1995, 1999; Pennisi et al., 2002). Chick embryos exposed to gadolinium, an antagonist for stretch activated channels, exhibit reduced expression of *Gja5* in the ventricles, and gadolinium-injected hearts fail to develop a mature conduction activation sequence, supporting the notion that hemodynamic flow plays an essential role in Purkinje fiber induction and patterning (Hall et al., 2004).

Hemodynamic flow has been linked to the activation of Endothelin signaling in multiple contexts (Barton and Yanagisawa, 2008; Hall et al., 2004; Morita et al., 1993; Yoshizumi et al., 1989). The connection of hemodynamic flow to both peripheral conduction system development and Endothelin signaling suggests a model whereby hemodynamic flow induces Endothelin signaling, which in turn, induces Purkinje fiber transdifferentiation (Mikawa and Hurtado, 2007; Pennisi et al., 2002). This model has been strongly supported by work performed in the chick system using gain-of-function approaches. Treatment of embryonic chicken hearts or myocytes with ET-1 induces Purkinje fiber differentiation (Gourdie et al., 1998; Takebayashi-Suzuki et al., 2000). Similarly, co-expression of preproendothelin-1 and Ece-1 was also shown to be sufficient to induce ectopic Purkinje fiber formation in developing chick hearts (Takebayashi-Suzuki et al., 2000). Continued expression of ET_A using retroviral overexpression extends the period of time for which chicken cardiomyocytes are permissive to ET-1-induced differentiation of myocytes into Purkinje fibers (Kanzawa et al., 2002).

Gain-of-function studies in mammalian systems have been less conclusive about the role of Endothelin signaling in conduction system development. ET-1 treatment of cultured cardiomyocytes isolated from embryonic mice or Nkx2-5^{+} cardiac progenitor cells isolated from embryonic rats induced expression of cardiac conduction system markers (Patel and Kos, 2005; Zhang et al., 2012). Similarly, pacemaker cells with distinct morphology and fast beating rate could be induced by treatment of embryonic stem cells differentiated into cardiac myocytes with ET-1 (Gassanov et al., 2004). On the other hand, CCS-*lacZ* hearts cultured ex vivo showed little or no ectopic β-galactosidase expression when treated with ET-1 (Rentschler et al., 2002). Thus, gain-of-function experiments in mice have not resolved the sufficiency of Endothelin signaling for Purkinje fiber induction. Surprisingly, an analysis of conduction system development has not been reported in previous mouse genetic studies in which Endothelin signaling had been abolished in the heart (Asai et al., 2010; Clouthier et al., 1998; Yanagisawa et al., 1998, 2000). Here, we found that complete abrogation of Endothelin signaling did not affect the initial specification or patterning of the conduction system as highlighted by the expression of CCS-*lacZ* and expression of *Gja1* and *Gja5*, nor did loss of Endothelin signaling disrupt conduction system function in Endothelin signaling-null fetuses, which exhibited PR intervals and QRS durations indistinguishable from wild type fetuses.

We also found that complete loss of Endothelin signaling on an outbred background did not result in embryonic demise and that *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout mice survived to birth at predicted Mendelian frequency (Table 1). Previous studies briefly reported embryonic demise at approximately E13.5 in the absence of Endothelin signaling (Yanagisawa et al., 1998). The survival of fetuses on an outbred background supports the idea that additional genes are involved in the observed embryonic lethality when Endothelin signaling is abrogated on an inbred background. Alternatively, it is possible that other genes contribute to survival of *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout fetuses on an outbred background, but if this were the case, one might expect partially penetrant embryonic lethality or underrepresentation of *ednra*$^{-/-}$; *ednrb*$^{-/-}$ double knockout fetuses when maintained on a mixed, outbred background. However, underrepresentation of double knockouts was not observed in our studies (Table 1).

The studies presented here support the idea that birds and mammals may have different requirements for Endothelin signaling in cardiac conduction system development. Our data demonstrate that Endothelin signaling is dispensable for cardiac conduction system specification in the mouse. On the other hand, gain-of-function experiments strongly suggest that Endothelin signaling is important for the development of the avian conduction system (Gourdie et al., 1998; Hall et al., 2004; Kanzawa et al., 2002; Takebayashi-Suzuki et al., 2000). The apparent difference in the role of Endothelin signaling in conduction system development between mammals and birds may simply reflect the differences between gain-of-function and loss-of function approaches. Although it is clear that Endothelin signaling is capable of inducing myocyte-to-Purkinje fiber transdifferentiation in the chick, it may not be required for that process, and loss-of-function studies have not been performed in an avian system to rigorously test the requirement for Endothelin signaling in development of the peripheral conduction system. On the other hand, it is possible that birds and mammals may have evolved distinct signaling mechanisms for the specification of the peripheral ventricular conduction system. In this regard, hemodynamic forces may play different roles in peripheral conduction system development in birds and mammals. Ventricular conduction system development in birds occurs in association with hemodynamic forces (Gourdie et al., 1995, 1999; Pennisi et al., 2002). In contrast, Purkinje fiber differentiation in the mouse appears to occur prior to the onset of high-pressure circulation (Rentschler et al., 2002), and blood flow is dispensable for formation of atrioventricular conduction tissue in zebrafish (Milan et al., 2006). Since Endothelin signaling is well-established as a shear/flow-responsive signaling system (Hall et al., 2004; Morita et al., 1993; Yoshizumi et al., 1989), it may be critical for birds to have evolved (or maintained) a signaling system capable of transducing mechanical information in response to hemodynamic forces from the endocardium and arterial vasculature to the myocardium, whereas this may be dispensable in mice.

Neuregulin signaling can also induce cardiomyocytes to express some conduction system markers and has been implicated in Purkinje fiber transdifferentiation. Neuregulin signaling is required in mice for formation of trabecular myocardium, the functional and structural precursor cells of Purkinje fibers (Gassmann et al., 1995; Hertig et al., 1999; Lee et al., 1995; Meyer and Birchmeier, 1995). Similarly, morpholino knock down studies suggested an

involvement of *neuregulin* for the development of atrioventricular conduction tissue in zebrafish (Milan et al., 2006). Rentschler et al. (2002) showed that Nrg-1 treatment of CCS-*lacZ* transgenic hearts explanted at E9.5 resulted in conversion of cardiomyocytes to a cardiac conduction cell phenotype. Other studies in which Nkx2-5+ cardiac progenitor cells were treated with Nrg-1 also found induction of cardiac conduction system markers (Patel and Kos, 2005). In contrast, embryonic stem cells differentiated in vitro into cardiomyocytes did not transdifferentiate in response to Nrg-1 treatment (Gassanov et al., 2004). Although we did not examine neuregulin signaling in the present study, our work demonstrates the dispensability of Endothelin signaling for murine cardiac conduction system specification and function, suggesting that another signaling system, perhaps neuregulin, may be essential for cardiac conduction system development in mice. A clearer understanding of neuregulin and other signaling pathways during early heart development is required to define the inductive mechanisms involved in mammalian cardiac conduction system specification and maturation.

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