Developmental Biology 393 (2014) 245–254

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

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

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ARTICLE INFO

Article history:
Received 29 August 2013
Received in revised form
4 July 2014
Accepted 11 July 2014
Available online 19 July 2014

Keywords:
Endothelin
ET_A
ET_B
CCS-lacZ
Purkinje fibers
Cardiac conduction system
Mouse

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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http://dx.doi.org/10.1016/j.ydbio.2014.07.008
0012-1606/

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;