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Development of the cardiac conduction system in zebrafish

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ABSTRACT

The cardiac conduction system (CCS) propagates and coordinates the electrical excitation that originates from the pacemaker cells, throughout the heart, resulting in rhythmic heartbeat. Its defects result in life-threatening arrhythmias and sudden cardiac death. Understanding of the factors involved in the formation and function of the CCS remains incomplete. By transposon assisted transgenesis, we have developed enhancer trap (ET) lines of zebrafish that express fluorescent protein in the pacemaker cells at the sino-atrial node (SAN) and the atrio-ventricular region (AVR), termed CCS transgenics. This expression pattern begins at the stage when the heart undergoes looping morphogenesis at 36 h post fertilization (hpf) and is maintained into adulthood. Using the CCS transgenics, we investigated the effects of perturbation of cardiac function, as simulated by either the absence of endothelium or hemodynamic stimulation, on the cardiac conduction cells, which resulted in abnormal compaction of the SAN. To uncover the identity of the gene represented by the EGFP expression in the CCS transgenics, we mapped the transposon integration sites on the zebrafish genome to positions in close proximity to the gene encoding *fibroblast growth homologous factor 2a* (*fhf2a*). *Fhf2a* is represented by three transcripts, one of which is expressed in the developing heart. These transgenics are useful tools for studies of development of the CCS and cardiac disease.

1. Introduction

The CCS is the key to initiating, maintaining and coordinating the rhythmic contraction of the mammalian heart that drives blood circulation throughout the body (Wiese et al., 2009; Mikawa and Hurtado, 2007). Pacemaker cells located at the sinoatrial node (SAN) generate a spontaneous electrical impulse that rapidly radiates across the atrium and triggers its contraction (Christoffels et al., 2010). The further spread of the electrical impulse is checked at the atrioventricular (AV) canal, maintaining a relaxed ventricle necessary for filling with blood. After the AV conduction delay, the impulse propagates rapidly to, and throughout, the ventricles via the His-Purkinje system with a concomitant ventricular contraction, thereby completing a cycle of staggered contractions that constitute a heartbeat. The specialized regions of myocardium representing the pacemaker cells and other cardiac conduction cells at the SAN and AVC possess a molecular signature defined by the expression of specific transcription factor, ion channel and gap junction genes that differs from that of the working myocardium. A number of genes encoding transcription factors, including *tbx3* and *tbx18* (Wiese et al., 2009; Hoogaars et al., 2004), *shox2* (*short stature homeobox 2*) (Blaschke et al., 2007), *isl1* (*islet-1*) (Blaschke et al., 2007; Hoffmann et al., 2013; Liang et al., 2015; Sun et al., 2007) and *irx3, 5* (Gaborit et al., 2012) are implicated in development of cardiac pacemaker cells.

A functionally equivalent CCS is evident in the two-chambered heart of the zebrafish from simple observations of synchronized cardiac contraction and unidirectional blood flow. Mutant analysis in zebrafish has implicated several factors in development of the CCS. Studies of the *cloche* (*clo*) mutant lacking endocardium demonstrated that endocardial signaling is required for development of the AV conduction tissue (Gaborit et al., 2012; Chi et al.,

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2008; Stainier et al., 1995). Further, the *silent heart* (*sih*) mutant, harboring a mutation in the *cardiac troponin T* (*tnnt2*) suffers a non-beating heart and lacks blood flow, which ultimately impact on the development of the fast conduction cells (Sehnert et al., 2002; Hove et al., 2003).

Abnormal cell-to-cell electrical signal propagation in the heart resulting from malformation of conduction cells, and/or aberrant ion channel function, can lead to deadly cardiac arrhythmias, fetal bradyarrhythmias and sudden infant death syndrome (Knollmann and Roden, 2008; Napolitano et al., 2012; Jaeggi and Friedberg, 2008; Schwartz et al., 1998). Ion channel genes expressed in cardiac pacemaker cells, whose deficiency has been linked to abnormal cardiac rhythm, include *hcn4* (*hyperpolarisation activated cyclic nucleotide-gated potassium channel 4*) (Nof et al., 2007; Stieber et al., 2003) and L-type calcium channels (Splawski et al., 2005). In addition, several distinct forms of cardiac arrhythmia, *i.e.* long- and short-QT syndrome, Brugada syndrome, sick sinus syndrome, and cardiac conduction disease are associated with dominant mutations in cardiac sodium channels *SCN5A*, which encodes the α-subunit of the cardiac voltage-gated sodium channel $Na_v1.5$ (Tan et al., 2001; Wang et al., 2002; Amin et al., 2010; Abriel, 2010). The activity of SCN5a is partially regulated by its interaction with protein modulators such as members of the Fgf homologous factors family (FHF) (Wang et al., 2011). Fhfs are structurally similar to FGFs, but lacking the classical secretion signal sequence they are confined to the cytoplasm as partners and regulators of ion channels, etc (Goldfarb, 2005). Hence, it is reasonable to hypothesize that mutations affecting *Fhf2* can lead to cardiac arrhythmias (Wang et al., 2011; Wei et al., 2011).

Here we report the characterization of zebrafish transgenic lines that label the conduction cells in the SAN and the AVC as well as cell lineages in the central nervous system (CNS). The transgenics were instrumental to study the development of the conduction cells and the dependence of this process on functionally intact endocardium and hemodynamic flow. Genomic mapping of the transposon insertion sites in the CCS transgenics indicates that they represent the *fhf2a* reporter. These results demonstrate potential of using the CCS transgenics for studies of development of the CCS and its diseases in the zebrafish model.

2. Materials and methods

2.1. Zebrafish lines and care

Zebrafish were maintained according to established protocols (Westerfield, 2007) in agreement with Institutional Animal Care and Use Committee regulations (Biological Resource Center of Biopolis, license no. 120787) that approved the study and rules of the Institute of Molecular and Cell Biology zebrafish facility. All experiments involving zebrafish embryos/larvae were carried out in accordance with the IACUC rules. Breeding and animal husbandry at the Harefield Heart Science Center were according to the Animals (Scientific Procedures) Act 1986. Zebrafish transgenics used in this study were Tg(*gata1*:dsRed), Tg(*myl7*:dsRed), cloche mutant (clo^{s5}), ET(krt-GFP)^{sqet33mi28} (referred here to as ET33-mi28) and ET(krt-GFP)^{sqet33mi59B} (referred here to as ET33-mi59B).

2.2. RNA in situ hybridization and immunohistochemistry

Whole-mount *in situ* hybridization (WISH) and fluorescence immunohistochemistry was performed on whole-mount zebrafish embryos and on sections as previously described (Korzh et al., 1998). Both the *nppa* and *shox2* clones were purchased from Open Biosystems and the anti-sense RNA probes were transcribed from *Eco*RI-linearised clones using T7 RNA polymerase. The *bmp4* probe was transcribed from *Eco*RI-linearised cDNA using T7 RNA polymerase. To generate the *hcn4* and the different variation of *fhf2a* probes, these genes were cloned from 2 dpf zebrafish embryos into pGEMTeasy vector (Promega, Madison, WI), linearised, and transcribed accordingly. Photographs of WISH embryos were taken using a Leica dissecting photomicroscope (Leica, Wetzlar, Germany).

Fluorescence immunohistochemistry was performed on whole-mount zebrafish embryos and on sections of adult zebrafish heart. The antibodies used are: Acetylated tubulin (Developmental Studies Hybridoma Bank [(DSHB); 1:200], anti-alpha-actinin (Sigma, A7811, 1:500), MF20 (DSHB, 1:20), GFP (Abcam, 1:200) and secondary antibodies Alexa594-or Alexa488-coupled goat-anti-mouse (Molecular Probes, 1:500). Nuclei were counter-stained with DAPI. Whole embryos or adult hearts were fixed in 4% PFA/PBS, mounted in 1.5% agar for cryosectioning, and 15-mm sections were collected for immunohistochemistry.

2.3. Microscopy

All microscopy was done as described previously (Lee et al., 2011). Stained embryos and sections were imaged by confocal microscopy using LSM5 (Carl Zeiss) or Olympus Fluoview (Olympus, Japan). For *in vivo* imaging, embryos were dechorionated at the selected stages, anaesthetised with 0.2% tricaine, and oriented ventral to the imaging plane by embedding in 1% low-melting agarose (LMA) in embryo water on a confocal dish. Microscopic observations were done using a dissecting fluorescent microscope (SZX12; Olympus). To capture still confocal images, embryos were treated with 20 μM 2,3-butanedione monoxime (Sigma, USA) to arrest cardiac contraction before mounting in agarose for confocal scanning. For *in vivo* confocal imaging of heart contraction in real time, a Z-series of time-lapse movies at an interval of 2.5 mm were taken using a slit scanning confocal microscope (LSM5 LIVE; Carl Zeiss), and processed for 4-D reconstruction with the Imaris imaging software (Bitplane AG, Switzerland).

3. Results

3.1. Characterization of CCS transgenics

In an enhancer trap screen in zebrafish (Kondrychyn et al., 2009; Poon et al., 2010), we identified two transgenic lines (ET33-mi28 and ET33-mi59B) with their respective transposon insertion sites mapping very closely on chromosome 14 (see below), and which at low-resolution imaging exhibit similar EGFP expression in the heart and nervous system (Fig. 1; Table 1).

At high-resolution imaging the EGFP-positive cells of the embryonic heart tube were detected at 36 hpf at the SAR (Fig. 2A). When crossed to the Tg(*gata1*:dsRed) line with labeled red blood cells, a EGFP-positive cell area of 5–6 rows of cells was detected at the venous pole of embryonic heart, forming a ring next to the sinus venosus where blood enters the heart tube (Fig. 2B, movie 1). By 2 dpf, the ring of about 40 EGFP-positive cells became asymmetrical, which results in up to 8 rows of cells on the ventral side of the heart and 2 rows of cells on the opposing dorsal side (movie 2). By 3 dpf the ventral area of this domain is narrow too (Fig. 2C, D; movie 3). Thus, SAN development may involve an early asymmetric developmental reduction of a territory occupied by the EGFP-positive cells, first, on the dorsal side and, second, on the ventral side.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.gep.2016.08.003.

To determine the cell types labeled, we crossed the ET33-mi59B transgenics with the myocardium-specific Tg(*myl7*:dsRed) (Fig. 2C) and labeled ET33-mi59B embryos with both anti-GFP and MF20