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Inducible Gene Deletion in the Entire Cardiac Conduction System using Hcn4-CreERT2 BAC Transgenic Mice

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Abstract

Developmental defects and disruption of molecular pathways of the cardiac conduction system (CCS) can cause life-threatening cardiac arrhythmias. Despite decades of effort, knowledge about the development and molecular control of the CCS remains primitive. Mouse genetics, complementary to other approaches such as human genetics, has become a key tool for exploring the developmental processes of various organs and associated diseases. Genetic analysis using mouse models will likely provide great insights about the development of the CCS, which can facilitate the development of novel therapeutic strategies to treat arrhythmias. To enable genetic studies of the CCS, CCS-associated Cre mouse models are essential. However, existing mouse models with Cre activity reported in the CCS have various limitations such as Cre leak, haploinsufficiency, and inadequate specificity of the Cre activity. To circumvent those limitations, we successfully generated Hcn4-CreERT2 BAC transgenic mice using BAC recombineering in which Cre activity was specifically detected in the entire CCS after tamoxifen induction. Our Hcn4-CreERT2 BAC transgenic line will be an invaluable genetic tool with which to dissect the developmental control of CCS and arrhythmias.

Keywords

Cardiac conduction system; inducible Cre line; Hcn4-CreERT2; BAC

Heart growth and its proper function depend on the coordinated development of various cardiac cell types (Garg, 2006; McCulley and Black, 2012; Srivastava, 2006). The early heart tube contracts peristaltically to pump blood to support embryo growth. As it develops further, the heart undergoes growth and chamber septation and, eventually remodels into a four-chambered structure. During these developmental processes, a tiny pool of specialized cells, constituting the cardiac conduction system (CCS), orchestrates coordinated, sequential cardiac contraction (Christoffels et al., 2010; Mikawa and Hurtado, 2007). The CCS consists of the SAN (sinoatrial node), AVN (atrioventricular node), His bundle, bundle branches, and Purkinje fibers. It has been established that the His-Purkinje system has a myogenic origin, but how it develops and assembles into a fast CCS is debated (Christoffels and Moorman, 2009; Gourdie et al., 1995). Recent retrospective clonal analysis support the notion that common progenitors give rise to His-Purkinje cells and contractile cardiomyocytes, and these lineage-restricted cells then proliferate (Miquerol et al., 2010). Although exciting progress has been made, numerous questions remain to be answered. For example, the cell lineage of the SAN is not certain (Bressan et al., 2013; Liang et al., 2013).

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Coordinated cardiac contraction is vital for the heart to pump blood efficiently. In the adult heart, the SAN initiates the action potential, which travels through the atria to the AVN, where the electrical signal is delayed. The electrical signal then rapidly travels to the His bundle, bundle branches, and Purkinje fibers, eventually traveling throughout the ventricles to trigger ventricular contraction.

Cardiac arrhythmias can be life-threatening. Developmental abnormalities of CCS lineages may be associated with arrhythmogenic regions in adult hearts (Jongbloed et al., 2012). Disorders of the CCS include sick sinus syndrome, heart block, AV nodal reentry (Park and Fishman, 2011). Ventricular arrhythmias such as idiopathic fascicular tachycardia and arrhythmias in patients after myocardial infarction can arise in the ventricular conduction system (Scheinman, 2009). While it is well established that dysregulation of ion channels or their mutations can cause arrhythmias, how developmental defects and disruptions of molecular control of CCS cause arrhythmias is not clear.

Although various components of the CCS were discovered more than 100 years, our understanding of the developmental and molecular control of the CCS remains limited (Christoffels and Moorman, 2009; Munshi, 2012). Several transcription factors such as Tbx3, Tbx5, Tbx18, Id2, Irx3, Nkx2.5, Shox2, and Notch have been identified as playing important roles in the CCS (Blaschke et al., 2007; Espinoza-Lewis et al., 2009; Frank et al., 2012; Hoogaars et al., 2007; Moskowitz et al., 2007; Moskowitz et al., 2004; Rentschler et al., 2011; Wiese et al., 2009; Zhang et al., 2011). A complete delineation of the developmental control of CCS relies on specific genetic manipulations such as loss-of-function or gain-of-function studies. Over the past several years, several Cre lines with Cre activity in the CCS have been reported (Arnolds and Moskowitz, 2011; Beyer et al., 2011; Hoesl et al., 2008; Liang et al., 2013; Sun et al., 2013). However, these lines have several limitations. First, in all of these lines except for the minK-CreERT2 line, a knock-in strategy was used. Given that *Hcn4*, *Cx40*, and *Shox2* play key roles in CCS, haploinsufficiency is a concern when using these knock-in mice to delete important genes in the CCS as noted previously (Liang et al., 2013). In this situation the haploinsufficiency can complicate the interpretation of genetic deletion in CCS. Second, Cre activity is not specifically in the CCS but in other cardiac cells as well. For example, *Cx40* encodes a gap junction protein responsible for the rapid propagation of cardiac action potential in the His-Purkinje system and for cell-cell communication in arterial endothelial cells. As such, Cre activity is seen in His-Purkinje system and arterial endothelial cells in Cx40-CreERT2 mice. Third, leakage of Cre activity limits the usefulness of the minK-CreERT2 line for certain applications such as lineage tracing. Finally, Cre activity in Cx40-CreERT2, minK-CreERT2, and Shox2-Cre is only seen in part of the CCS. A comparison between these Cre lines is summarized in the Table 1.

To elucidate developmental control of the CCS, we sought to generate a CCS-specific, inducible Cre line without those limitations. For this purpose, we chose *Hcn4* as a marker of the CCS. Hcn4 belongs to a family of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels that consists of Hcn1-4. *Hcn4* is specifically expressed in the entire CCS in all species examined so far. The voltage-gated ion channel Hcn4 mediates the inward sodium-potassium current and is responsible for initiating the diastolic depolarization of SAN cells (DiFrancesco, 2010). Human genetic studies showed that mutations in *HCN4* cause bradycardia and sick sinus syndrome (DiFrancesco, 2010; Herrmann et al., 2007). To avoid haploinsufficiency, we chose a transgenic strategy using a bacterial artificial chromosome (BAC) as BACs are likely to contain all of the regulatory elements necessary to confer endogenous expression *in vivo* (Gong et al., 2003). A CreERT2 cassette was selected to control Cre recombinase activity in a tempo-spatial fashion. *CreERT2* encodes a Cre recombinase fused to a mutant estrogen ligand-binding domain (ERT2). In the presence

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of the estrogen receptor antagonist tamoxifen, CreERT2 rapidly relocates into the nucleus to excise LoxP-flanked DNA regions.

To develop the Hcn4-CreERT2 BAC transgenic mice, a 200-kb BAC containing *Hcn4* locus (RP23-414K12) was chosen to direct the expression of CreERT2 (FIG. 1). This BAC does not contain other protein-coding genes or microRNAs, which excludes the over-expression of other genes in the resulting transgenic mice. A CreERT2 cassette was inserted into the *Hcn4* locus in the BAC by BAC recombineering (FIG. 1) (Warming et al., 2005). The successfully engineered BAC was purified and used for pronuclear injection of fertilized eggs. Two independent founders were generated from the injection. Both transgenic founders showed essentially identical Cre activity after tamoxifen induction so we selected one of the lines for the detailed analysis described below.

To evaluate the inducible properties of the transgenic Hcn4-CreERT2 allele, we crossed our line with the R26R reporter line (FIG. 2a). The R26R reporter mice have been widely used for testing Cre activity and lineage tracing, as deletion of a floxed stop cassette leads to constitutive expression of β-galactosidase (Soriano, 1999). In the Hcn4-CreERT2:R26R mice, no β-galactosidase activity was seen without tamoxifen induction at any stages (FIG. 2b & 2j). To examine the Cre activity of our Hcn4-CreERT2 transgenic line, tamoxifen was administrated by intraperitoneal (IP) injection at E12.5, and embryos were stained with X-gal at E15.5 (FIG. 2c–e). X-gal-stained cells were detected in the SAN, AVN, His bundle, bundle branches, and Purkinje fibers in the left ventricle. Only a few Purkinje fibers in the right ventricle were stained blue, consistent with the finding that the majority of right ventricular conduction system does not arise from *Hcn4* progenitor cells but rather from the second heart field (SHF) (Liang et al., 2013). In addition to the CCS, X-gal-stained cells were seen in the right superior vena cava and coronary sinus but not in other parts of the embryos (FIG. 2d and Supplemental FIG. 1).

To confirm that the blue cells stained by X-gal were CCS cells, we evaluated if the ones in the developing SAN at E15.5 co-expressed β-galactosidase and Hcn4. Double-label fluorescent immunohistochemistry was performed using antibodies against β-galactosidase and Hcn4 on frozen sections. As shown in FIG. 2f–I, β-galactosidase and Hcn4 were completely co-localized in the SAN, documenting that the β-galactosidase-expressing cells were *bona fide* CCS cells.

The CCS matures after birth, a process that is completed by the age of 1 month in mice (Moskowitz et al., 2004). Having shown specific induction of Cre activity in the developing heart at mid-gestation, we wanted to determine if this Cre line worked at the neonatal stage and in adults as well. Tamoxifen was administrated at E16.5, and X-gal staining was performed at P2 (FIG. 2j, k). After induction, X-gal-stained cells were detected in the SAN, AVN, His bundle, bundle branches, and Purkinje fibers in the left ventricle. A few Purkinje fibers in the right ventricle were also stained (FIG. 3l,m). These lineage tracing data are similar to those with induction at E12.5. In addition, X-gal-stained cells were seen in the right superior vena cava, pulmonary vein, coronary sinus, and atrioventricular ring bundles around mitral valve and tricuspid valve (FIG. 2l & m). These regions are closely related to CCS lineage development and are arrhythmogenic foci responsible for atrial and ventricular arrhythmia (Jongbloed et al., 2012; Liang et al., 2013; Yamamoto et al., 2006). No blue cells were seen in other parts of the heart except for a few cells in the right side of interatrial septum (Supplemental FIG. 2). These cells may represent the internodal conduction tracts that are related to the development of the left venous valves and septum spurium (Blom et al., 1999; Jongbloed et al., 2012). A detailed characterization of these blue cells using our Hcn4-CreERT2 model may provide novel insights on how these regions causes arrhythmia in various heart diseases. To evaluate the Cre activity in adult mice, tamoxifen was

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administrated by IP injection at 1-month and the Cre activity was analyzed. X-gal staining was specifically detected in the entire CCS (FIG. 2o–r). Similar to the observation in the embryos, no X-gal-stained cells were seen in other organs (Supplemental FIG. 3).

Recent studies have found that *Hcn4* is transiently expressed in the progenitors of first heart field (FHF), and that *Hcn4*$^+$ progenitor cells give rise to the left ventricle, part of both atria, and almost all components of the CCS (Liang et al., 2013; Spater et al., 2013). Tamoxifen induction at E7.5 in Hcn4-CreERT2 transgenic mice confirmed these findings (Supplemental FIG. 4), suggesting that our Hcn4-CreERT2 model can also be used to study development of FHF as well.

Because our model is likely to be widely used to conditionally inactivate genes in the CCS, it is important to explore if the transgenic line is normal. The Hcn4-CreERT2 mice heterozygous as well as homozygous for the transgene insertion were viable and fertile. We have not seen any difference in terms of life span or breeding properties between homozygous transgenic mice and wild-type controls (FIG.3a). Surface electrocardiogram (EKG) revealed no discernible difference between transgenic mice and wild-type littermate controls (FIG. 3b & c). In conclusion, these transgenic mice were indistinguishable from wild-type mice.

Taken together, we have generated a new transgenic mouse line, Hcn4-CreERT2, which exhibits Cre activity specifically in the entire CCS under tight control with tamoxifen.

This transgene can permit conditional gene deletion in the CCS during development and in adults, and it can also be harnessed as an important tool to model CCS diseases. As *Hcn4* is transiently expressed in the FHF, this line can be a novel genetic tool to study early heart development as well.

MATERIALS AND METHODS

Mouse Strains

R26R reporter mice were ordered from Jackson Laboratories. All experiments with animals were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai.

Generation of the Hcn4-CreET2 BAC transgenic Line

A BAC was purchased from the BACPAC Resources Center at Children's Hospital Oakland Research Institute in Oakland, California. A 2.0-kb CreERT2 cassette was inserted immediately after the first ATG of the *Hcn4* gene in BAC using recombineering. The BAC ends and the flanking regions of CreERT2 in the *Hcn4* locus were confirmed by sequencing. BAC DNA was purified using NucleoBond BAC-100 (Clontech). The PCR primers used to genotype the Hcn4-CreERT2 transgenic mice amplifying a 401-bp sequence are:

CreERT2-F: GTGCCTGGCTAGAGATCCTG;

CreERT2-R: GATGTGGGAGAGGATGAGGA

We identified putative homozygous transgenic mice using quantitative PCR with primers that detect a 179-bp fragment from the CreERT2 cassette.

CreERT2-F: GACAGGAACCAGGGAAAATG

CreERT2-R: TCCAGAGACTTCAGGGTGCT

Putative homozygous transgenic mice were crossed with wild-type mice. If all of the pups born from these crosses were heterozygous, then we concluded that those transgenic mice

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were homozygous. The Hcn4-CreERT2 BAC transgenic line will be available to the research community on request.

Tamoxifen Administration

Tamoxifen (Sigma T5648) was dissolved in 100% ethanol at a concentration of 200 mg/ml, and then diluted in sesame oil (Sigma S3547) to a concentration of 10 mg/ml.

For tamoxifen induction in embryos, pregnant dams were injected with 2 mg/25 g body weight by intraperitoneal injection once. For tamoxifen induction in adults, adult mice were injected intraperitoneally at the amount of 1 mg tamoxifen/25 g body weight for 3 consecutive days and sacrificed three days after the final injection to analyze recombination.

X-Gal staining

For whole mount staining, the hearts were dissected out in ice-cold PBS and fixed with 4% paraformaldehyde in PBS at 4 °C for 1 h, then rinsed 3 × 30 min at room temperature in β-galactosidase rinse buffer (0.2 M sodium phosphate, pH7.3, 2 mM magnesium chloride, 0.02% NP40, 0.01% sodium deoxycholate). The hearts were stained in X-gal staining solution (4 mM ferrocyanide, 4 mM ferricyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal) at room temperature overnight and postfixed in 4% paraformaldehyde in PBS. To examine the X-gal-stained cells in sections, tissues were cryosectioned at a thickness of 10 μm, and fixed for 10 min at room temperature in 4% paraformaldehyde in PBS. The slides were rinsed in PBS and incubated in X-gal staining solutions at 37 °C overnight. After staining, the slides were rinsed briefly in PBS and then stained with Eosin Y.

Immunohistochemistry

Immunohistochemistry was performed on cryosections. The hearts were infused in 0.5 M sucrose for 2–3 h, and then in OCT frozen in dry ice. Samples were sectioned at the thickness of 10 μm. Immediately before performing immunohistochemistry, the sections were immersed in methanol at −20 °C for 5 min and washed three times with PBS for 5min. The sections were blocked with 1× Animal-Free Blocker (SP-5030, Vector lab) for 1 h. Hcn4 antibody (ab32675, Abcam, 1:200 dilution) and anti-β-galactosidase antibody (ab9361, Abcam, 1:200 dilution) were applied overnight at 4 °C. The slides were washed in PBS and then were incubated in secondary antibodies (Alexa Fluor® 488 Donkey anti-Chicken, Jackson Immuno, 1:3000 dilution, Alexa Fluor® 594 Donkey Anti-rat, Invitrogen, 1:3000) for 1 h at room temperature. The slides were mounted in VECTASHIELD Mounting Medium with DAPI.

Surface EKG

Cardiac electrophysiological function was assessed with surface electrocardiography as previously described . Data analysis was performed using the Chart5Pro (v 5.4.2, AD Instruments).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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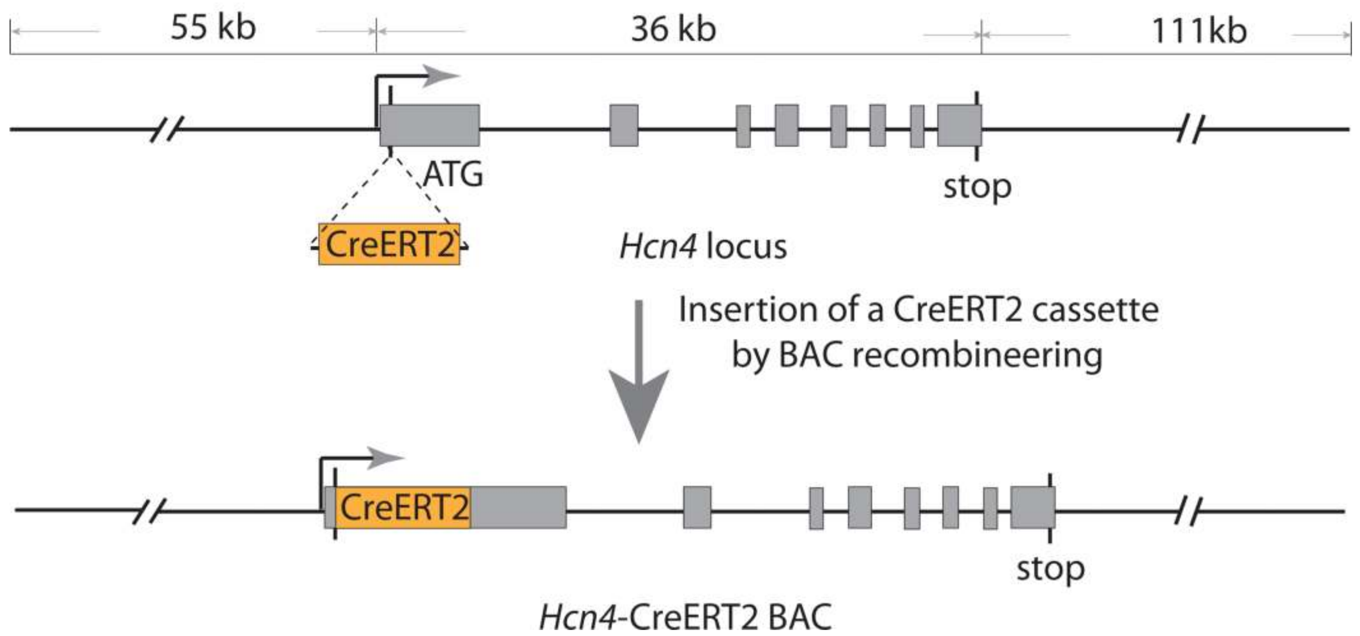

FIG. 1.

Generation of the Hcn4-CreERT2 BAC by recombineering. A CreERT2 cassette with a poly(A) tail was specifically inserted immediately after first ATG of *Hcn4* locus in BAC using an homologous recombination-based technique. The correct insertion and integrity of modified BAC was verified by Sanger sequencing before pro-nuclear injection.

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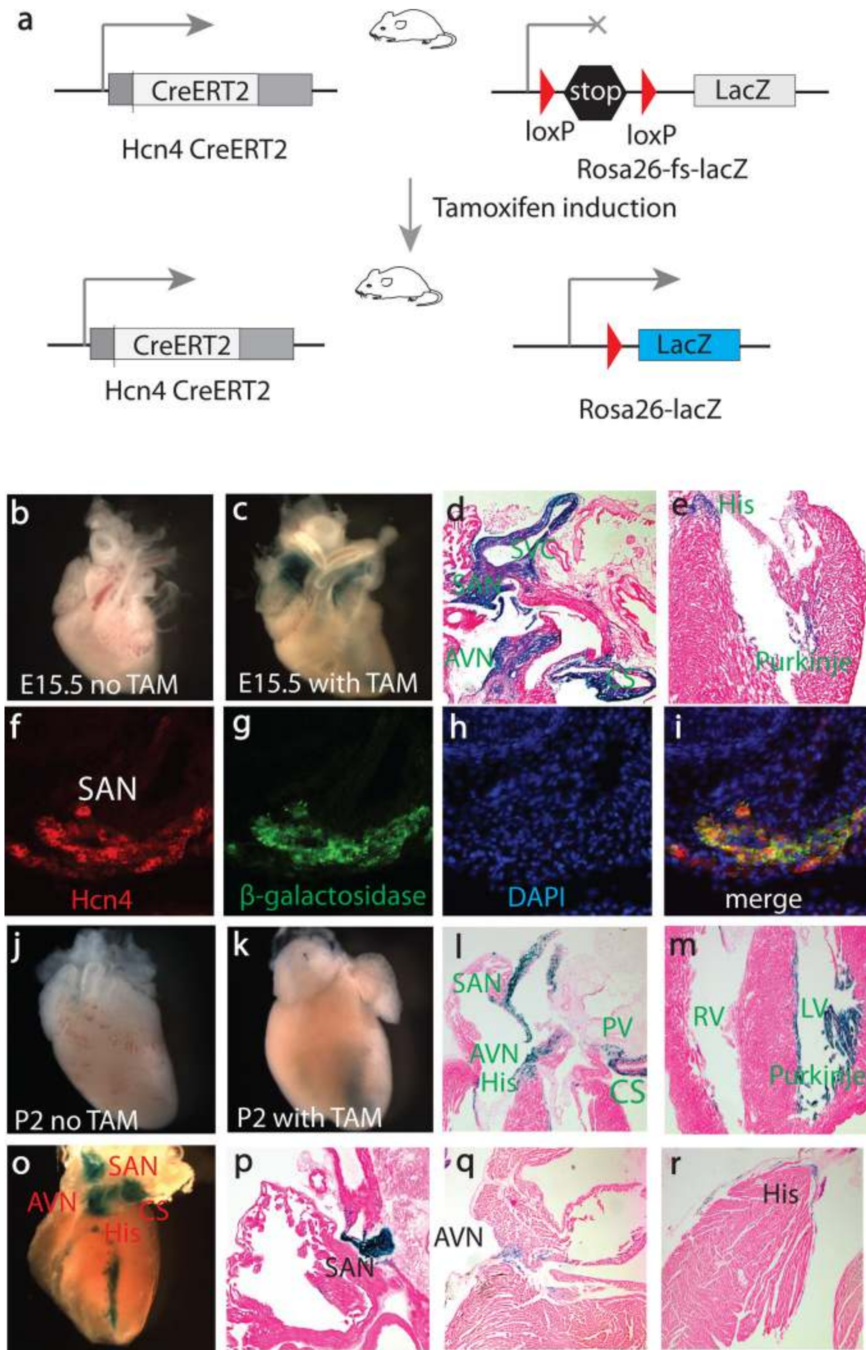

FIG. 2.

Tamoxifen-induced Cre activity in the entire CCS. (a) Breeding scheme to test functionality of the Hcn4-CreERT2 BAC transgenic line. (b–e) Cre activity after tamoxifen induction at E12.5. (f–i). Double-label fluorescent immnohistochemistry at E15.5 to show expression of Hcn4 and β-galactosidase. Hcn4 (f, red), β-galactosidase (g, green), DAPI (h, blue), Three imaged are merged in (i). (j–m) Cre activity after tamoxifen induction at perinatal stage. (o–r) Cre activity was detected in the entire CCS after tamoxifen induction in adults. TAM, tamoxifen; SVC, superior vena cava; SAN, sinoatrial node; AVN, atrioventricular node; PV, pulmonary vein; CS, coronary sinus, RV, right ventricle; LV, left ventricle.

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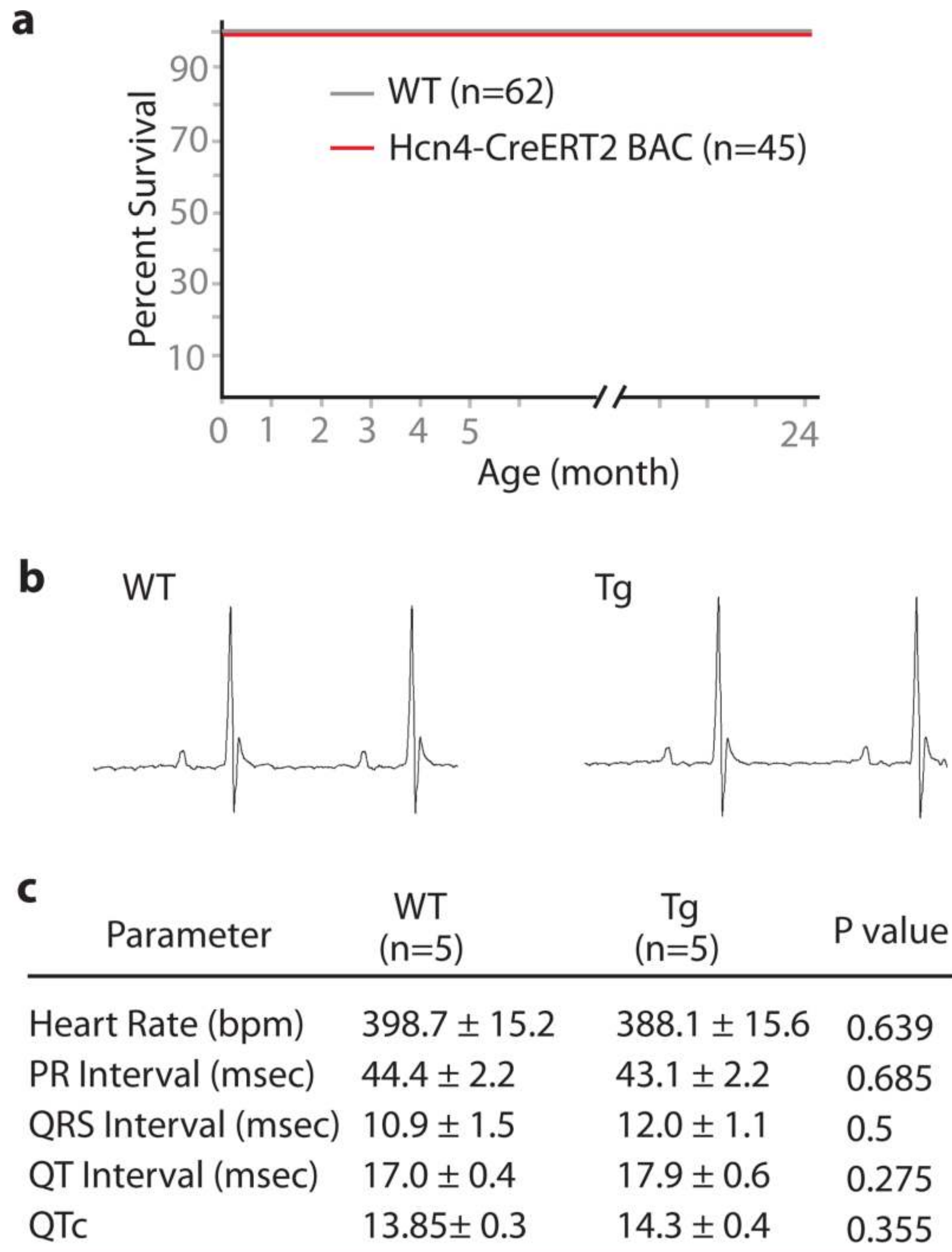

Parameter	WT (n=5)	Tg (n=5)	P value
Heart Rate (bpm)	398.7 ± 15.2	388.1 ± 15.6	0.639
PR Interval (msec)	44.4 ± 2.2	43.1 ± 2.2	0.685
QRS Interval (msec)	10.9 ± 1.5	12.0 ± 1.1	0.5
QT Interval (msec)	17.0 ± 0.4	17.9 ± 0.6	0.275
QTc	13.85± 0.3	14.3 ± 0.4	0.355

FIG. 3.

The Hcn4-CreERT2 animal homozygous for the transgene insertion showed normal life span (a) and normal EKG parameter (b & c). Representative traces of EKG in lead II was shown (b).

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Table 1

Comparison of various Cre and inducible Cre lines with reported Cre activity in the CCS.

Cre lines	Cre activity				KI	BAC	Leaky	other cardiac expression	reference
	SAN	AVN	His/BB	Purkinje					
Hcn4-CreERT2	+	+	+	+	yes	no	no	no	Hoesl et al., 2008
Hcn4-CreERT2	+	+	+	+	yes	no	no	no	Liang et al. 2013
Cx40-CreERT2	−	−	+	+	yes	no	no	yes	Beyer et al., 2011
minK-CreERT2	−	+	+	+	no	yes	yes	no	Arnolds et al., 2011
Shox2-Cre	+	n/a	+/−	−	yes	no	−	n/a	Sun et al., 2013
Hcn4-CreERT2	+	+	+	+	no	yes	no	no	This report

SAN: sinoatrial node; AVN: atrioventricular node; BB: bundle branches; KI: knockin