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Short Report

Comparative expression analysis of *Shox2*-deficient embryonic stem cell-derived sinoatrial node-like cells

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ABSTRACT

The homeodomain transcription factor Shox2 controls the development and function of the native cardiac pacemaker, the sinoatrial node (SAN). Moreover, *SHOX2* mutations have been associated with cardiac arrhythmias in humans. For detailed examination of Shox2-dependent developmental mechanisms in SAN cells, we established a murine embryonic stem cell (ESC)-based model using *Shox2* as a molecular tool. *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ ESC clones were isolated and differentiated according to five different protocols in order to evaluate the most efficient enrichment of SAN-like cells. Expression analysis of cell subtype-specific marker genes revealed most efficient enrichment after CD166-based cell sorting. Comparative cardiac expression profiles of *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ ESCs were examined by nCounter technology. Among other genes, we identified *Nppb* as a novel putative Shox2 target during differentiation in ESCs. Differential expression of *Nppb* could be confirmed in heart tissue of *Shox2*$^{-/-}$ embryos. Taken together, we established an ESC-based cardiac differentiation model and successfully purified *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ SAN-like cells. This now provides an excellent basis for the investigation of molecular mechanisms under physiological and pathophysiological conditions for evaluating novel therapeutic approaches.

1. Introduction

Due to their potential to differentiate into all cell types of the three primary germ layers, embryonic stem cells (ESCs) are perfectly suited to investigate early developmental processes such as heart development (Wobus, 2001). The investigation of cardiac cell subtype specification in differentiating ESCs provides a promising source for cell-based heart therapies including biological pacemakers.

The rhythmic beating of the heart is initiated by a population of pacemaker cells located in the sinoatrial node (SAN) (Opthof, 1988). The development of the SAN involves a multistep differentiation process of specialized cells originating from multipotent stem cells (Christoffels et al., 2010). Functional impairment of these cells leads to arrhythmogenic heart diseases with increased mortality risk (Choudhury et al., 2015). The elucidation of molecular mechanisms in normal and impaired SAN development and function is therefore of crucial clinical relevance.

Previously, we and others have demonstrated that a gene regulatory network involving the homeodomain transcription factor Shox2 controls cardiac pacemaker development and specification in mouse and zebrafish (Blaschke et al., 2007; Espinoza-Lewis et al., 2009; Puskaric et al., 2010; Hoffmann et al., 2013; Ye et al., 2015). Homozygous *Shox2* deletion leads to early embryonic lethality due to developmental defects of the SAN, while heterozygous mice are viable and fertile (Blaschke et al., 2007). In zebrafish embryos, the loss of *Shox2* substantially impairs pacemaker function with severe bradycardia and irregular heartbeat (Blaschke et al., 2007; Hoffmann et al., 2013). In line with this, we recently showed for the first time that *SHOX2* mutations associate with atrial fibrillation, the most common cardiac arrhythmia in humans (Hoffmann et al., 2016).

Shox2 activates the SAN genetic program either by direct regulation of its target *Isl1*, which controls cardiac pacemaker subtype identity (Hoffmann et al., 2013; Dorn et al., 2015; Vedantham et al., 2015), or by antagonizing the transcriptional output of *Nkx2.5* (Espinoza-Lewis et al., 2009, 2011; Ye et al., 2015). Shox2-deficient mice used in the

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current study recapitulate this molecular pathway by diminished expression of SAN-specific genes and ectopic expression of chamber-specific genes (Blaschke et al., 2007; Puskaric et al., 2010; Hoffmann et al., 2013). Furthermore, the *SHOX2* promoter is suited for the isolation of mouse ESC-derived SAN-like cells and as lineage-specific promoter to drive the expression of a voltage-sensitive fluorescent protein in nodal-like human induced pluripotent stem cell-derived cardiomyocytes (Hashem and Claycomb, 2013; Hashem et al., 2013; Chen et al., 2017). In addition, *Shox2* overexpression during embryonic and mesenchymal stem cell differentiation strongly favours pacemaker cell specification (Ionta et al., 2015; Feng et al., 2016). Shox2 among other embryonic transcription factors such as Tbx3, Tbx18 or Isl1 (Bakker et al., 2012; Kapoor et al., 2013; Dorn et al., 2015), is able to direct pacemaker cell type determination. Taken together, Shox2 represents one of the major genes in the developing SAN and proper function is of crucial relevance regarding the origin of arrhythmogenic heart diseases. The elucidation of the underlying molecular mechanisms is therefore mandatory. To provide a cell-model for unravelling these mechanisms in health and disease, we established a stem cell-based cardiac differentiation model using Shox2 as a molecular tool.

2. Material and methods

2.1. Generation of ESC clones from Shox2-deficient mice

Superovulation was induced in viable, female *Shox2*$^{+/-}$ C57BL/6 mice (Blaschke et al., 2007), followed by mating. At embryonic stage E3.5 single blastocysts were collected and plated on mouse embryonic fibroblasts (MEFs). Preparation and MitomycinC (Sigma-Aldrich)-inactivation of MEFs were described earlier (Wobus et al., 2002). Collected blastocysts were cultivated for 5 days (d) in ESC proliferation medium (Wobus et al., 2002). Thereafter, the inner cell mass (ICM) of adherent blastocysts was mechanically picked, dissociated with trypsin/EDTA (Gibco) and replated onto MEFs in ESC proliferation medium. Cells were always cultivated at 37 °C with 5% CO_2 and 95% humidity. Generated ESC clones were genotyped as previously described (Blaschke et al., 2007).

2.2. Cultivation and differentiation of ESC clones

Generated ESC clones and the mouse ESC line R1 (Nagy et al., 1993) were cultivated and expanded on MEFs in ESC proliferation medium. In order to investigate the differentiation properties of the generated *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ ESC clones, cells were differentiated according to a standard hanging drop protocol (600 cells/drop, plating of embryoid bodies (EBs) after 7d) and morphologically characterized as described earlier (Wobus et al., 2002). Briefly, 7d EBs were plated separately in 24-well plates (one EB/well) coated with 0.1% gelatin (Sigma-Aldrich). Every second or third day, the appearance of either beating cardiomyocytes, neurons or skeletal muscle cells per EB was assessed by bright field microscopy. The appearance of the respective cell type per EB was counted, not the total number of the respective cells. The morphological analysis was limited to ectodermal and mesodermal cell derivatives, as endodermal cells are difficult to characterize by morphology.

2.3. Alkaline phosphatase (ALP) staining

Detection of ALP in generated ESC clones was carried out using an ALP-Kit (Sigma-Aldrich) as recommended by the manufacturer.

2.4. Immunofluorescence (IF) staining

IF analyses were performed with generated ESC clones and isolated beating cardiac clusters. ESC clones were stained with rabbit anti-Oct4 (1:120; abcam) and mouse anti-SSEA-1 (1:50; abcam) and visualized by incubation with anti-rabbit Alexa Fluor (AF) 488 (1:200; Molecular Probes) and anti-mouse Cy3 (1:600; Dianova). Beating cardiac clusters isolated at differentiation stage 7 + 12d (Maltsev et al., 1993) from *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ ESC clones were incubated with rabbit anti-Shox2 (1:200; abcam) and mouse anti-Titin (1:200, provided by Prof. Fürst, University of Bonn, Germany) and visualized with anti-rabbit AF 488 and anti-mouse AF 568 (both 1:250; Molecular Probes). Counterstain was performed using Hoechst 33342 nuclear staining dye (ThermoFisher Scientific).

2.5. Quantitative real-time PCR (qPCR)

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using the SuperScript III First-Strand Synthesis Kit (Invitrogen). qPCR was conducted using the SYBR Green Lo-Rox Fast Mix (Bioline) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Each sample was analyzed in duplicates and relative mRNA levels were assessed using the Relative Standard Curve Method by normalization to the reference genes succinate dehydrogenase complex subunit A (*Sdha1*) and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*). Calculated values are means ± SD and presented as fold-change. Oligonucleotide sequences are given in Supplementary Table 1.

2.6. Enrichment of ESC-derived SAN-like cells

To enrich ESC-derived SAN-like cells, the R1 mouse ESC line was differentiated according to five different protocols (Fig. 2A, Table 1). In brief, protocol A utilizes AG1478 (Chen et al., 2013), whereas protocol B utilizes Suramin (Wiese et al., 2011) for the enrichment of ESC-derived SAN-like cells. Protocol C was established according to the conventional hanging drop protocol for cardiac differentiation (Wobus et al., 2002) with additional treatment by Suramin. Protocol D differs in the initial amount of cells used for EB formation, the length of suspension culture and media composition (Hashem et al., 2013). Scavone et al. established a FACS-based protocol to isolate SAN-like cardiomyocytes during murine ESC differentiation using the surface marker CD166 (Scavone et al., 2013). This protocol (E) was modified with regard to media composition and the predetermined number of ESCs in order to improve the efficiency of SAN cell isolation. Fluorescent activated cell sorting (FACS) in protocol E was performed by the FACS Core Facility of the Department of Medicine V in Heidelberg using a FACS Aria II SORP cell sorter. Results were evaluated by qPCR assessing the expression of *Shox2* and *Hcn4*. Cells that had undergone treatment according to the most efficient protocol E were further investigated by comparative expression analysis using cell type-specific marker genes (Supplementary Table 1). Thereafter, protocol E was also applied to the *Shox2*$^{+/+}$ ESC clone for subsequent comparative expression analysis (Supplementary Table 1).

2.7. nCounter expression analysis of *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ CD166^{+} SAN-like cells

The cardiac expression profile of CD166^{+} *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ SAN-like cells (7 + 1d) was investigated by nCounter expression analysis at the nCounter Core Facility Heidelberg using the nCounter Dx analysis system GEN1. Detailed probe design is given in Supplementary Table 2. The workflow is described at http://www.nanostring.com/elements/workflow. Background correction and normalization of data was performed using the nSolver Analysis Software 3.0 (NanoString Technologies). Most stable expressed genes were chosen for normalization based on the geNorm method. Log2 values are presented as ± SD.