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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.

Table 1
Detailed overview about the applied differentiation protocols.

Differentiation protocol	Reference	Cell number/drop	Volume/drop	Hanging drop duration	Suspension culture duration	Plating time point of EBs	Enrichment of SAN-cells	FCS amount in media	Harvesting time point
A	Chen et al., 2013	1000	30 μl	3d	2d	5d	5 + 9d-5 + 11d 5 μM AG1478 (Biomol)	20%	5 + 12d
B	Wiese et al., 2011	400	20 μl	2d	5d	7d	5d-7d 500 μM Suramin (Sigma-Aldrich)	10%	7 + 10d
C	Wobus et al., 2002 modified	600	20 μl	2d	5d	7d	5d-7d 500 μM Suramin (Sigma-Aldrich)	20%	7 + 10d
D	Hashem et al., 2013	500	20 μl	2d	3d	5d	no treatment	10%	5 + 12d
E	Scavone et al., 2013 modified	600	20 μl	2d	5d	7d	7 + 1d CD166 FACS (eBiosciences)	20%	7 + 1d

3. Results

3.1. Isolation and characterization of ESCs from Shox2-deficient mice

We have established three genotypically different ESC lines (*Shox2*$^{+/+}$, *Shox2*$^{+/-}$ and *Shox2*$^{-/-}$) by isolating pluripotent stem cells from the inner cell mass (ICM) of *Shox2*-deficient mouse blastocysts at stage E3.5. The ICM was separated from the blastocysts after 5d in co-culture on murine embryonic fibroblasts (MEFs). After expansion, the genotype of each isolated ESC clone was determined by PCR (data not shown). In total, at least two different ESC lines per genotype have been isolated. For further characterization, one ESC clone per genotype was analyzed, respectively (data for *Shox2*$^{+/-}$ ESC clone are not shown). Pluripotency was verified by immunofluorescent staining of the key markers Oct4 and SSEA-1. Complementing alkaline phosphatase activity analysis resulted in a distinct red staining of ESC colonies, while the surrounding MEFs remained unstained (Fig. 1A).

Our standard differentiation protocol includes embryoid body (EB) formation for 2d in hanging drops followed by 5d in suspension culture. Further differentiation was initiated by plating of EBs after 7d. Expression analysis of the pluripotency marker *Pou5f1* by qPCR revealed a strong decrease in differentiated ESCs after 7 + 12d compared to undifferentiated controls (d0) for both, wildtype (WT) and knockout (KO) (Fig. 1B). Lineage-specific marker gene qPCR analysis representing the three primary germ layers (mesoderm: *Des*; endoderm: *Sox17;* ectoderm: *Nes*) pointed to a spontaneous EB differentiation of all ESC clones (Fig. 1B). Morphological evaluation calculating the percentage of EBs with specific differentiated cell types confirmed the potential of all isolated ESC clones to differentiate into cardiomyocytes, skeletal muscle and neuronal cells (Fig. 1C). Further analysis of the differentiated ESC clones verified the expected Shox2 expression profile on transcript (Fig. 1D) and protein (Fig. 1E) levels. No Shox2 transcript or protein was detectable in the *Shox2* KO ESCs. Furthermore, the co-staining of differentiated ESCs with the sarcomere protein Titin confirmed the differentiation into cardiomyocytes (Fig. 1E).

3.2. Differentiation of ESCs into cardiomyocytes of the SAN subtype

Various protocols for the differentiation of ESCs into specialized cardiomyocytes have been established in the past. To evaluate the most efficient enrichment of SAN-like cells in vitro, we tested five differentiation protocols (Fig. 2A; Table 1) using the established and commercially available ESC line R1. We compared three previously published (A, B, D) and two specifically modified protocols (C, E) to assess the efficiency of SAN cell enrichment by marker gene expression analysis. All five protocols applied the hanging drop approach (Fig. 2A; Table 1). To investigate and compare the subtype specification of SAN-like cells during differentiation of mouse ESCs, comparative expression analysis of the SAN-specific marker genes *Shox2* and *Hcn4* was performed by qPCR (Fig. 2B). Most efficient enrichment of SAN-like cells was achieved with protocol E. To further evaluate this differentiation protocol on cell type specification, expression analysis of additional cell type-specific marker genes was carried out (Fig. 2C). We could show that the SAN genes *CD166*, *Shox2* and *Hcn4* and the general cardiac cell type-specific markers *Gata4* (core cardiac transcription factor), *Nppa* (working myocard), *Actc1* (pan cardiac marker) and *Ddr2* (cardiac fibroblasts) are significantly enriched in CD166^{+} cells compared to unsorted controls. In contrast, a clear reduction of markers such as *Sox17* (mesendoderm), *Hnf6α* (endoderm) and *CD105* (endothelium) was demonstrated, while *Tubb3* (neurons) and *Acta2* (smooth muscle) showed no difference in expression levels between unsorted controls and CD166^{+} ESCs (Fig. 2C).

After successful differentiation of the ESC line R1 into cardiomyocytes of the SAN subtype, protocol E was also applied to the isolated *Shox2*$^{+/+}$ ESCs. Expression analysis of different SAN marker genes (*CD166*, *Shox2*, *Hcn4*, *Tbx18*) by qPCR points to a highly significant enrichment of SAN-like cardiomyocytes in CD166^{+} cells compared to unsorted controls (Fig. 2D).

3.3. Comparative expression analysis of *Shox2*$^{+/+}$ vs *Shox2*$^{-/-}$ SAN-like cells by nCounter technology

For detailed investigation of differentiated CD166^{+} *Shox2*$^{+/+}$ vs *Shox2*$^{-/-}$ SAN-like cells, we performed a comparative expression analysis using the nCounter technology (Fig. 3). This RNA quantification technology allows the multiplexed target measurement from only 100 ng of input with high sensitivity and specificity. We examined the expression levels of cell type specific markers (Supplementary Table 2) including cardiac cell subtypes, pluripotency markers and non-cardiac cell markers (e.g. neuronal markers). While no differences or no expression was detected for the non-cardiac markers (data not shown), differential expression was observed for most of the analyzed cardiac genes. In total, six of nine investigated SAN-specific genes were significantly downregulated in *Shox2*-deficient cells including *Shox2*, *Hcn4*, *Tbx18*, *Tnni1*, *Cacna1h*, *Gjc1*, while *Cacna1g* and *Gjd3* showed the same trend but did not reach statistical significance (Fig. 3B). Consistent with previously described data, the myocardial-specific gene *Gja1* (Cx43) was upregulated, while *Acta1* and *Nppa* were not significantly differently expressed (Fig. 3C). Remarkably, the working myocard marker *Nppb* was significantly upregulated in *Shox2*$^{-/-}$ SAN-like cells. To validate the differential expression identified in ESCs, we also examined *Nppb* expression in right atrial tissue (including the SAN) of *Shox2*-KO embryos compared to WT. This analysis revealed a significant upregulation of *Nppb* and confirmed this gene as a putative target of Shox2 in ESCs and mouse atrial tissue (Fig. 3D). The expression of the pluripotency markers *Pou5f1* and *Nanog* was significantly increased in the *Shox2*$^{-/-}$ cells, suggesting a lower differentiation potential of these cells (Fig. 3E).

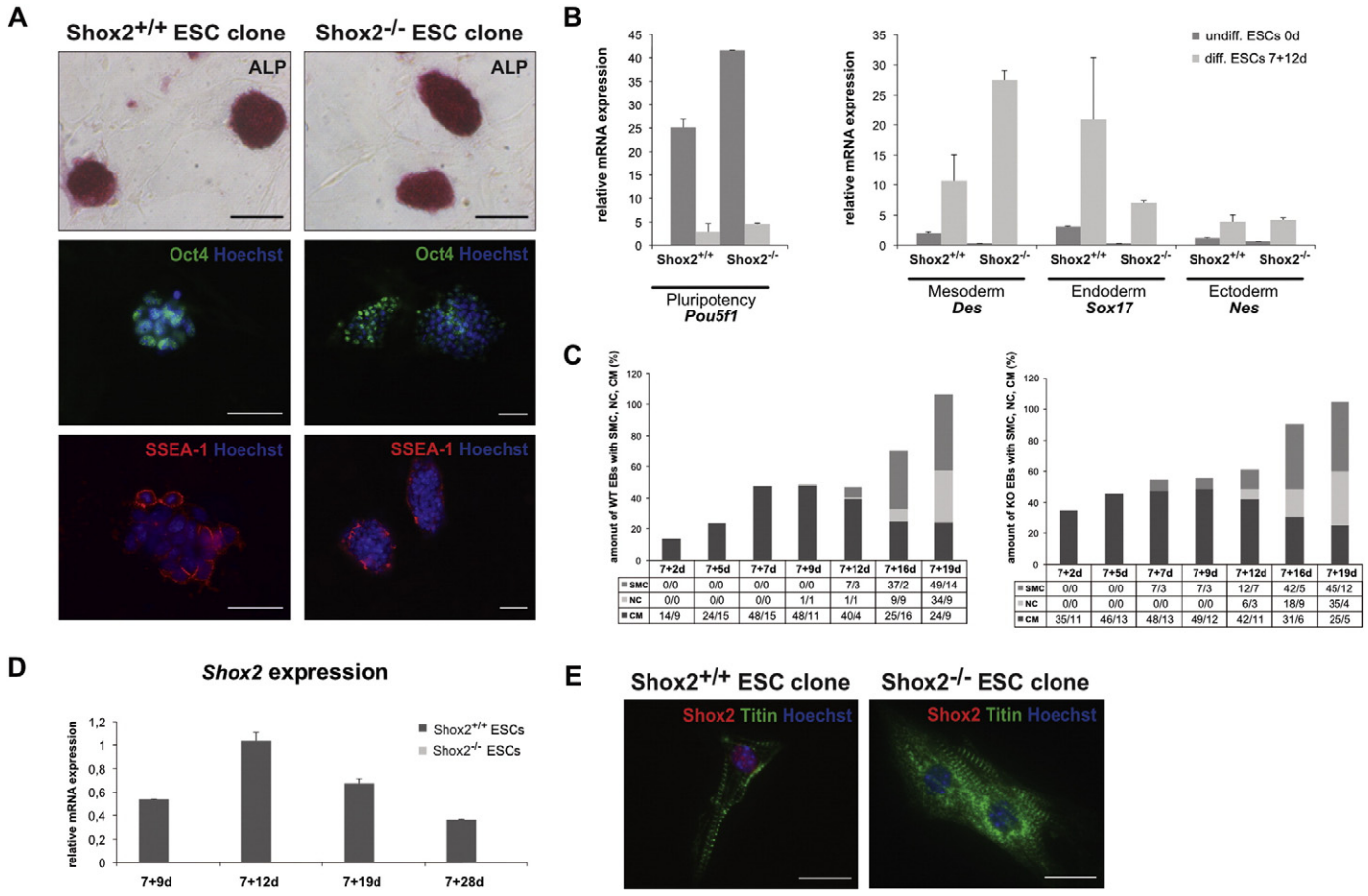

Fig. 1. Isolation and spontaneous differentiation of ESCs from *Shox2*-deficient mice. A) Characterization of undifferentiated ESCs by ALP staining (ESC colonies visualized in red) and immunofluorescent detection of Oct4 (green) and SSEA-1 (red). Nuclei were counterstained with Hoechst 33342 (blue). Scale bars: ALP 100 μm; IF 50 μm. B) Expression analyses of pluripotency and lineage-specific marker genes in differentiated $Shox2^{+/+}$ and $Shox2^{-/-}$ ESCs at 7 + 12d ($n = 3$) compared to undifferentiated ESCs d0 ($n = 2$). C) Morphological evaluation of $Shox2^{+/+}$ ($n = 4$) and $Shox2^{-/-}$ ($n = 5$) ESCs calculating the percentage of EBs with specific cell types at various time points of differentiation: cardiomyocytes (CM), neuronal cells (NC), skeletal muscle cells (SMC); data table (value/SEM). D) Relative mRNA expression of *Shox2* during differentiation of $Shox2^{+/+}$ and $Shox2^{-/-}$ ESC clones ($n = 3$). E) Immunofluorescent detection of the sarcomere protein Titin (green) and Shox2 (red) in isolated beating cardiomyocytes (7 + 12d) derived from $Shox2^{+/+}$ and $Shox2^{-/-}$ ESC clones. Nuclei were counterstained with Hoechst 33342 (blue). Scale bars: 20 μm.

4. Discussion

We have previously demonstrated that a gene regulatory network involving the homeodomain transcription factor Shox2 controls the development and function of the cardiac pacemaker with a highly restricted expression pattern in the SAN (Blaschke et al., 2007; Puskaric et al., 2010; Hoffmann et al., 2013). Shox2-deficiency is associated with impaired SAN function and increased susceptibility to atrial fibrillation (Blaschke et al., 2007; Hoffmann et al., 2016). The molecular mechanisms underlying Shox2 function in health and disease are barely understood. This prompted us to develop a murine ESC-based cardiac differentiation model using *Shox2*-deficient mice, which may contribute fundamentally to the current knowledge of the intrinsic genetic program. The enrichment of an ESC-derived SAN-like cell population is a prerequisite to specifically investigate signaling pathways, mandatory for differentiation of pacemaker cells. The differentiation of ESC-derived cardiomyocytes is influenced by many parameters, including the initial number of cells per EB, media composition and specific additives (Wobus et al., 2002). We applied and modified in total five differentiation protocols using pharmacological treatments or FACS-based selection (Wobus et al., 2002; Wiese et al., 2011; Chen et al., 2013; Hashem et al., 2013; Scavone et al., 2013) and compared the expression levels of SAN-specific marker genes (*Shox2* and *Hcn4*). Most efficient enrichment of SAN-like cells was observed with a previously described FACS-based differentiation protocol (Scavone et al., 2013). The media composition and the predetermined number of ESCs were adapted to our standard differentiation protocol. For FACS analysis the surface marker CD166 was applied, as it is specifically but transiently expressed in the prospective SAN during embryonic development (Hirata et al., 2006). FACS-based selection does not influence molecular mechanisms compared to chemical-based enrichment procedures, which is a major advantage of this protocol. However, a limitation of this methodology is the restricted amount of $CD166^{+}$ cells. Only 5% of the sorted cells were $CD166^{+}$, consequently an enormous number of differentiated ESCs are required to obtain a sufficient cell number for RNA isolation and subsequent expression analysis. To overcome this challenge, nCounter technology was used for comparative expression analysis, as very low amounts of input material (100 ng) are sufficient. We observed a significant downregulation of most SAN-specific genes (6/9 targets) in the $Shox2^{-/-}$ cells. This confirms the specificity of our established ESC-based cardiac differentiation model and recapitulates the data observed in the mouse model. The $Shox2^{-/-}$ SAN-like cells showed highly increased transcript levels for the pluripotency markers *Pou5f1* and *Nanog*, suggesting that *Shox2*-deficiency may influence the differentiation potential of ESCs and thereby explain the hypoplastic SAN phenotype observed in the KO mouse.

Most remarkably, we identified *Nppb* (Bnp), a well-studied marker in cardiovascular disease, as a novel putative Shox2 target gene. This

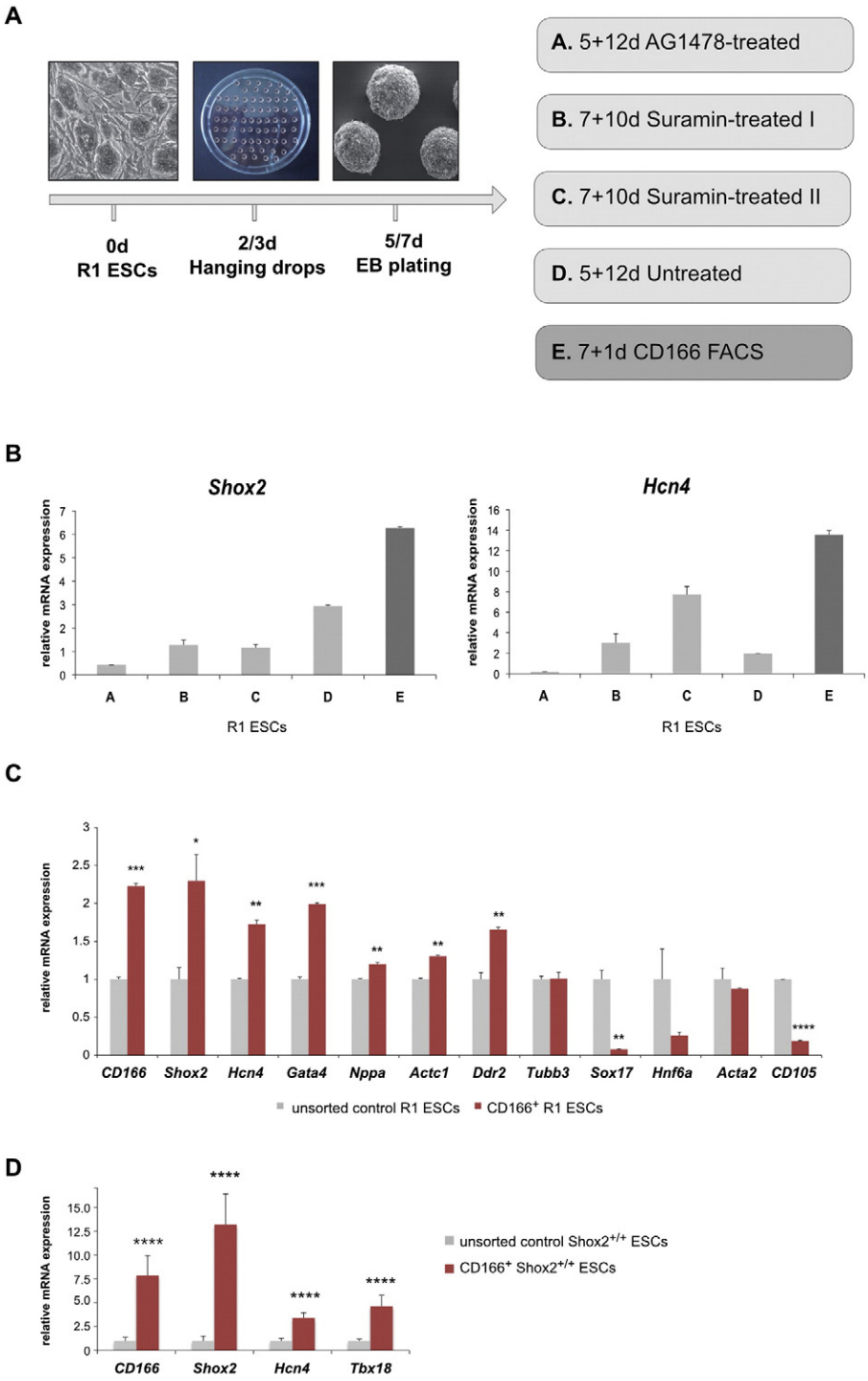

Fig. 2. Enrichment of ESC-derived SAN-like cells. A) Schematic representation of the differentiation protocols. B) Comparison of differentiation protocols (A–E) with R1 ESCs ($n = 2$) using *Shox2* and *Hcn4*. C) Expression analysis of cell type-specific marker genes using CD166 FACS-sorted (red bars) and unsorted (grey bars) R1 ESCs ($n = 2$) applied to method E (Scavone et al., 2013; modified). D) Expression analysis of CD166 and SAN marker genes using $CD166^{+}$ (red bars) and unsorted (grey bars) $Shox2^{+/+}$ ESCs ($n = 3$) applied to method E (Scavone et al., 2013; modified). *p*-values were determined by an unpaired *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$).

gene was analyzed as working myocard marker and turned out to be significantly upregulated in $Shox2^{-/-}$ SAN-like cells and in right atrial tissue of *Shox2*-KO embryos. Interestingly, *NPPB* (BNP) has been previously described as direct target of SHOX2 and SHOX (a highly homologous transcription factor) in skeletal development (Marchini et al., 2007; Aza-Carmona et al., 2014) suggesting this gene as reliable target also in heart development. Recently, we could link *SHOX2* mutations to atrial fibrillation, the most common arrhythmia in humans (Hoffmann et al., 2016). In turn, elevated BNP levels and higher *NPPB* mRNA expression have been detected in subjects with atrial fibrillation (Tuinenburg et al., 1999; Silvet et al., 2003) suggesting that our generated ESCs have the potential to serve as an excellent in vitro model system. Taken together, we established an ESC-based cardiac differentiation model and successfully purified $Shox2^{+/+}$ and $Shox2^{-/-}$ SAN-like cells. This provides a fundamental basis for the investigation of molecular pathways under physiological and pathophysiological conditions and may serve as drug testing system for evaluating novel therapeutic approaches.

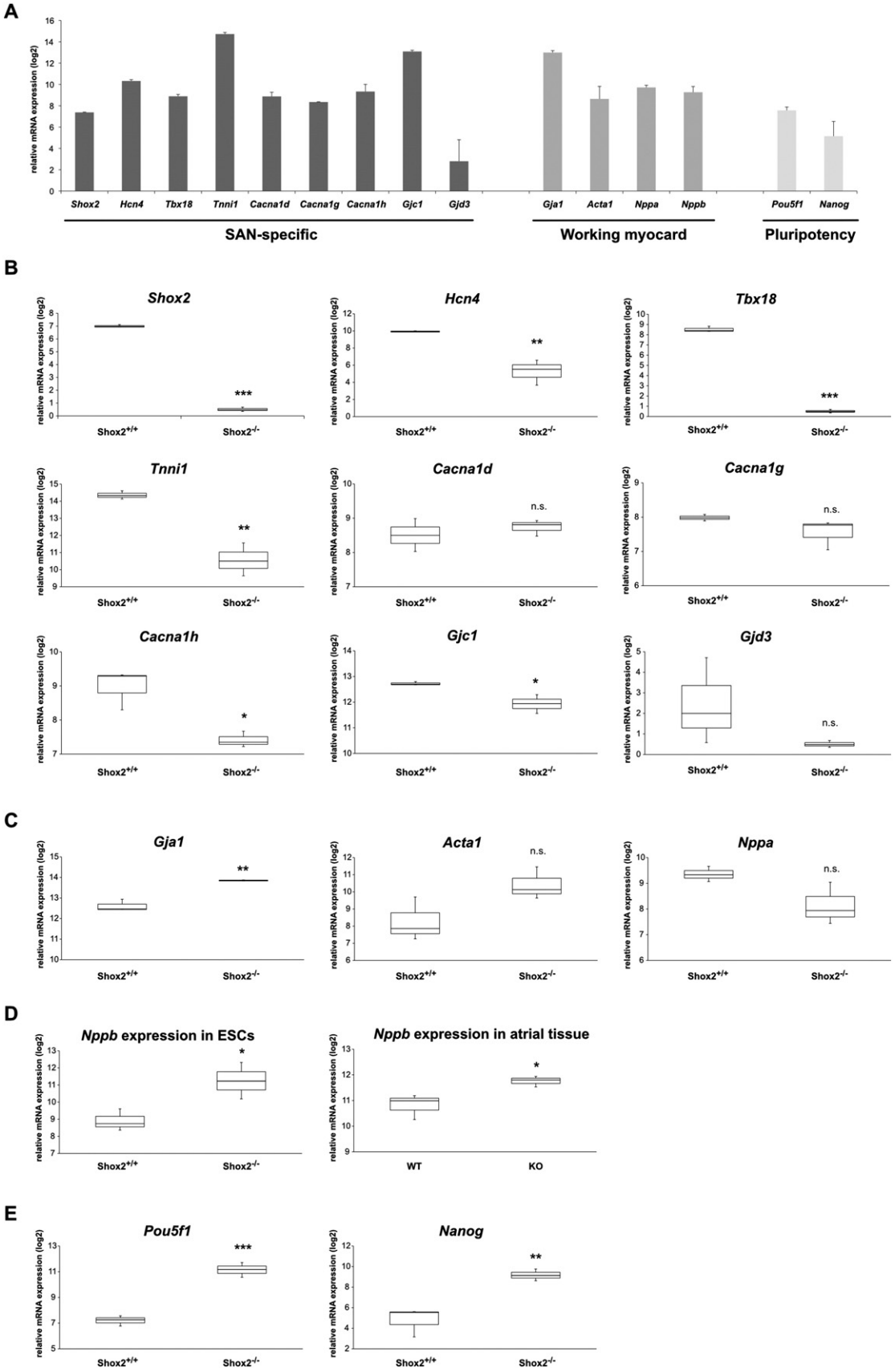

Fig. 3. Comparative expression analysis of CD166$^+$ *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ SAN-like cells by nCounter technology after 7 + 1d. A) Expression of SAN, working myocard and pluripotency markers was analyzed in CD166$^+$ *Shox2*$^{+/+}$ cardiomyocytes ($n = 3$). B) Expression differences of SAN markers between CD166$^+$ *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ SAN-like cells ($n = 3$). C) Expression differences of working myocard markers between CD166$^+$ *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ SAN-like cells ($n = 3$). D) nCounter analysis revealed differential expression of *Nppb* in CD166$^+$ *Shox2*$^{-/-}$ ESCs and confirmed *Nppb* as significantly differential expressed gene in right atria of E11.5 Shox2-KO embryos ($n = 3$). E) Expression differences of pluripotency markers between CD166$^+$ *Shox2*$^{+/+}$ and *Shox2*$^{-/-}$ SAN-like cells ($n = 3$). *p*-values were determined by an unpaired *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

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References

Aza-Carmona, M., Barca-Tierno, V., Hisado-Oliva, A., Belinchon, A., Gorbenko-del Blanco, D., Rodriguez, J.I., Benito-Sanz, S., Campos-Barros, A., Heath, K.E., 2014. NPPB and ACAN, two novel SHOX2 transcription targets implicated in skeletal development. PLoS One 9.

Bakker, M.L., Boink, G.J.J., Boukens, B.J., Verkerk, A.O., van den Boogaard, M., den Haan, A.D., Hoogaars, W.M.H., Buermans, H.P., de Bakker, J.M.T., Seppen, J., Tan, H.L., Moorman, A.F.M., 't Hoen, P.A.C., Christoffels, V.M., 2012. T-box transcription factor TBX3 reprogrammes mature cardiac myocytes into pacemaker-like cells. Cardiovasc. Res. 94.

Blaschke, R.J., Hahurij, N.D., Kuijper, S., Just, S., Wisse, L.J., Deissler, K., Maxelon, T., Anastassiadis, K., Spitzer, J., Hardt, S.E., Schöler, H., Feitsma, H., Rottbauer, W., Blum, M., Meijlink, F., Rappold, G., Gittenberger-de Groot, A.C., 2007. Targeted mutation reveals essential functions of the homeodomain transcription factor Shox2 in sinoatrial and pacemaking development. Circulation 115.

Chen, M., Bi, L.L., Wang, Z.Q., Zhao, F., Gan, X.D., Wang, Y.G., 2013. Time-dependent regulation of neuregulin-1beta/ErbB/ERK pathways in cardiac differentiation of mouse embryonic stem cells. Mol. Cell. Biochem. 380.

Chen, Z., Xian, W., Bellin, M., Dorn, T., Tian, Q., Goedel, A., Dreizehnter, L., Schneider, C.M., Ward-van Oostwaard, D., Ng, J.K., Hinkel, R., Pane, L.S., Mummery, C.L., Lipp, P., Moretti, A., Laugwitz, K.L., Sinnecker, D., 2017. Subtype-specific promoter-driven action potential imaging for precise disease modelling and drug testing in hiPSC-derived cardiomyocytes. Eur. Heart J. 38 (4), 292–301.

Choudhury, M., Boyett, M.R., Morris, G.M., 2015. Biology of the sinus node and its disease. Arrhythm. Electrophysiol. Rev. 4.

Christoffels, V.M., Smits, G.J., Kispert, A., Moorman, A.F., 2010. Development of the pacemaker tissues of the heart. Circ. Res. 106.

Dorn, T., Goedel, A., Lam, J.T., Haas, J., Tian, Q.H., Herrmann, F., Bundschu, K., Dobreva, G., Schiemann, M., Dirschinger, R., Guo, Y.C., Kuhl, S.J., Sinnecker, D., Lipp, P., Laugwitz, K.L., Kuhl, M., Moretti, A., 2015. Direct Nkx2-5 transcriptional repression of Isl1 controls cardiomyocyte subtype identity. Stem Cells 33.

Espinoza-Lewis, R.A., Liu, H., Sun, C., Chen, C., Jiao, K., Chen, Y., 2011. Ectopic expression of Nkx2.5 suppresses the formation of the sinoatrial node in mice. Dev. Biol. 356.

Espinoza-Lewis, R.A., Yu, L., He, F., Liu, H., Tang, R., Shi, J., Sun, X., Martin, J.F., Wang, D., Yang, J., Chen, Y., 2009. Shox2 is essential for the differentiation of cardiac pacemaker cells by repressing Nkx2-5. Dev. Biol. 327.

Feng, Y., Yang, P., Luo, S., Zhang, Z., Li, H., Zhu, P., Song, Z., 2016. Shox2 influences mesenchymal stem cell fate in a co-culture model in vitro. Mol. Med. Rep. 14.

Hashem, S.I., Claycomb, W.C., 2013. Genetic isolation of stem cell-derived pacemaker-nodal cardiac myocytes. Mol. Cell. Biochem. 383.

Hashem, S.I., Lam, M.L., Mihardja, S.S., White, S.M., Lee, R.J., Claycomb, W.C., 2013. Shox2 regulates the pacemaker gene program in embryoid bodies. Stem Cells Dev. 22.

Hirata, H., Murakami, Y., Miyamoto, Y., Tosaka, M., Inoue, K., Nagahashi, A., Jakt, L.M., Asahara, T., Iwata, H., Sawa, Y., Kawamata, S., 2006. ALCAM (CD166) is a surface marker for early murine cardiomyocytes. Cells Tissues Organs 184.

Hoffmann, S., Berger, I.M., Glaser, A., Bacon, C., Li, L., Gretz, N., Steinbeisser, H., Rottbauer, W., Just, S., Rappold, G., 2013. Islet1 is a direct transcriptional target of the homeodomain transcription factor Shox2 and rescues the Shox2-mediated bradycardia. Basic Res. Cardiol. 108.

Hoffmann, S., Clauss, S., Berger, I.M., Weiss, B., Montalbano, A., Roth, R., Bucher, M., Klier, I., Wakili, R., Seitz, H., Schulze-Bahr, E., Katus, H.A., Flachsbart, F., Nebel, A., Guenther, S.P.W., Bagaev, E., Rottbauer, W., Kaab, S., Just, S., Rappold, G.A., 2016. Coding and non-coding variants in the SHOX2 gene in patients with early-onset atrial fibrillation. Basic Res. Cardiol. 111.

Ionta, V., Liang, W.B., Kim, E.H., Rafie, R., Giacomello, A., Marban, E., Cho, H.C., 2015. SHOX2 overexpression favors differentiation of embryonic stem cells into cardiac pacemaker cells, improving biological pacing ability. Stem Cell Rep. 4.

Kapoor, N., Liang, W.B., Marban, E., Cho, H.C., 2013. Direct conversion of quiescent cardiomyocytes to pacemaker cells by expression of Tbx18. Nat. Biotechnol. 31.

Maltsev, V.A., Rohwedel, J., Hescheler, J., Wobus, A.M., 1993. Embryonic stem-cells differentiate in-vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell-types. Mech. Dev. 44.

Marchini, A., Hacker, B., Marttila, T., Hesse, V., Emons, J., Weiss, B., Karperien, M., Rappold, G., 2007. BNP is a transcriptional target of the short stature homeobox gene SHOX. Hum. Mol. Genet. 16.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., Roder, J.C., 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 90.

Opthof, T., 1988. The mammalian sinoatrial node. Cardiovasc. Drugs Ther. 1.

Puskaric, S., Schmitteckert, S., Mori, A.D., Glaser, A., Schneider, K.U., Bruneau, B.G., Blaschke, R.J., Steinbeisser, H., Rappold, G., 2010. Shox2 mediates Tbx5 activity by regulating Bmp4 in the pacemaker region of the developing heart. Hum. Mol. Genet. 19.

Scavone, A., Capilupo, D., Mazzocchi, N., Crespi, A., Zoia, S., Campostrini, G., Bucchi, A., Milanesi, R., Baruscotti, M., Benedetti, S., Antonini, S., Messina, G., DiFrancesco, D., Barbuti, A., 2013. Embryonic stem cell-derived CD166+ precursors develop into fully functional sinoatrial-like cells. Circ. Res. 113.

Silvet, H., Young-Xu, Y.N., Walleigh, D., Ravid, S., 2003. Brain natriuretic peptide is elevated in outpatients with atrial fibrillation. Am. J. Cardiol. 92.

Tuinenburg, A.E., Brundel, B.J.J.M., Van Gelder, I.C., Henning, R.H., Van den Berg, M.P., Driessen, C., Grandjean, J.G., Van Gilst, W.H., Crijns, H.J.G.M., 1999. Gene expression of the natriuretic peptide system in atrial tissue of patients with paroxysmal and persistent atrial fibrillation. J. Cardiovasc. Electrophysiol. 10.

Vedantham, V., Galang, G., Evangelista, M., Deo, R.C., Srivastava, D., 2015. RNA sequencing of mouse sinoatrial node reveals an upstream regulatory role for islet-1 in cardiac pacemaker cells. Circ. Res. 116.

Wiese, C., Nikolova, T., Zahanich, I., Sulzbacher, S., Fuchs, J., Yamanaka, S., Graf, E., Ravens, U., Boheler, K.R., Wobus, A.M., 2011. Differentiation induction of mouse embryonic stem cells into sinus node-like cells by suramin. Int. J. Cardiol. 147.

Wobus, A.M., 2001. Potential of embryonic stem cells. Mol. Asp. Med. 22.

Wobus, A.M., Guan, K., Yang, H.T., Boheler, K.R., 2002. Embryonic stem cells as a model to study cardiac, skeletal muscle, and vascular smooth muscle cell differentiation. Methods Mol. Biol. 185.

Ye, W.D., Wang, J., Song, Y.N., Yu, D.K., Sun, C., Liu, C., Chen, F.D., Zhang, Y.D., Wang, F., Harvey, R.P., Schrader, L., Martin, J.F., Chen, Y.P., 2015. A common Shox2-Nkx2-5 antagonistic mechanism primes the pacemaker cell fate in the pulmonary vein myocardium and sinoatrial node. Development 142.