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Lab Resource: Multiple Cell Lines

Murine transgenic embryonic stem cell lines for the investigation of sinoatrial node-related molecular pathways

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ABSTRACT

The elucidation of molecular mechanisms that restrict the potential of pluripotent stem cells and promote cardiac lineage differentiation is of crucial relevance, since embryonic stem cells (ESCs) hold great potential for cell based heart therapies. The homeodomain transcription factor Shox2 is essential for the development and proper function of the native cardiac pacemaker, the sinoatrial node. This prompted us to develop a cardiac differentiation model using ESC lines isolated from blastocysts of *Shox2*-deficient mice. The established cell model provides a fundamental basis for the investigation of molecular pathways under physiological and pathophysiological conditions for evaluating novel therapeutic approaches.

Resource Table

Unique stem cell lines identifier	N/A
Alternative names of stem cell lines	*Shox2*$^{+/+}$ ESCs *Shox2*$^{+/-}$ ESCs *Shox2*$^{-/-}$ ESCs
Institution	Department of Human Molecular Genetics, Institute of Human Genetics
Contact information of distributor	Sandra Hoffmann, sandra.hoffmann@med.uni-heidelberg.de
Type of cell lines	ESC
Origin	Mouse
Cell source	Mouse blastocysts
Method of reprogramming	N/A
Multiline rationale	Isolated from the same mouse
Gene modification	YES
Type of modification	Gene targeting
Associated disease	N/A
Gene/locus	*Shox2*, Chr3: 66971727-66981771
Method of modification	Homologous recombination
Name of transgene or resistance	Neomycin only in *Shox2*$^{+/-}$ and *Shox2*$^{-/-}$ ESC lines
Inducible/constitutive system	N/A
Date archived/stock date	2015
Cell line repository/bank	N/A
Ethical approval	N/A

Resource utility

The homeodomain transcription factor Shox2 is essential for the development and proper function of the native cardiac pacemaker, the sinoatrial node. This prompted us to develop a cardiac differentiation model using ESC lines isolated from blastocysts of *Shox2*-deficient mice.

Resource details

Isolation of pluripotent embryonic stem cells from Shox2-deficient mice

Shox2 controls the development and function of the cardiac pacemaker with a highly restricted expression pattern in the sinoatrial node (Blaschke et al., 2007; Puskaric et al., 2010; Hoffmann et al., 2013; Hoffmann et al., 2016). Three genotypically different ESC lines *Shox2*$^{+/+}$ (wild type; WT), *Shox2*$^{+/-}$ (heterozygous; HET) and *Shox2*$^{-/-}$ (knockout; KO) were established by isolating pluripotent stem cells from the inner cell mass (ICM) of *Shox2*-deficient mouse blastocysts at E3.5 (Fig. 1A). Blastocysts were placed on preseeded MitomycinC mitotically inactivated mouse embryonic fibroblasts (miMEFs) (Fig. 1Aa, 0d). After five days the ICM was separated and replated for ESC expansion (Fig. 1Aa, 5d). The genotype of each isolated ESC clone was determined by PCR using WT and KO specific primers (Fig. 1Ab, Table 2). In total, at least two different ESC lines per genotype could be obtained. For further characterization, one ESC clone per genotype was analyzed (Table 1).

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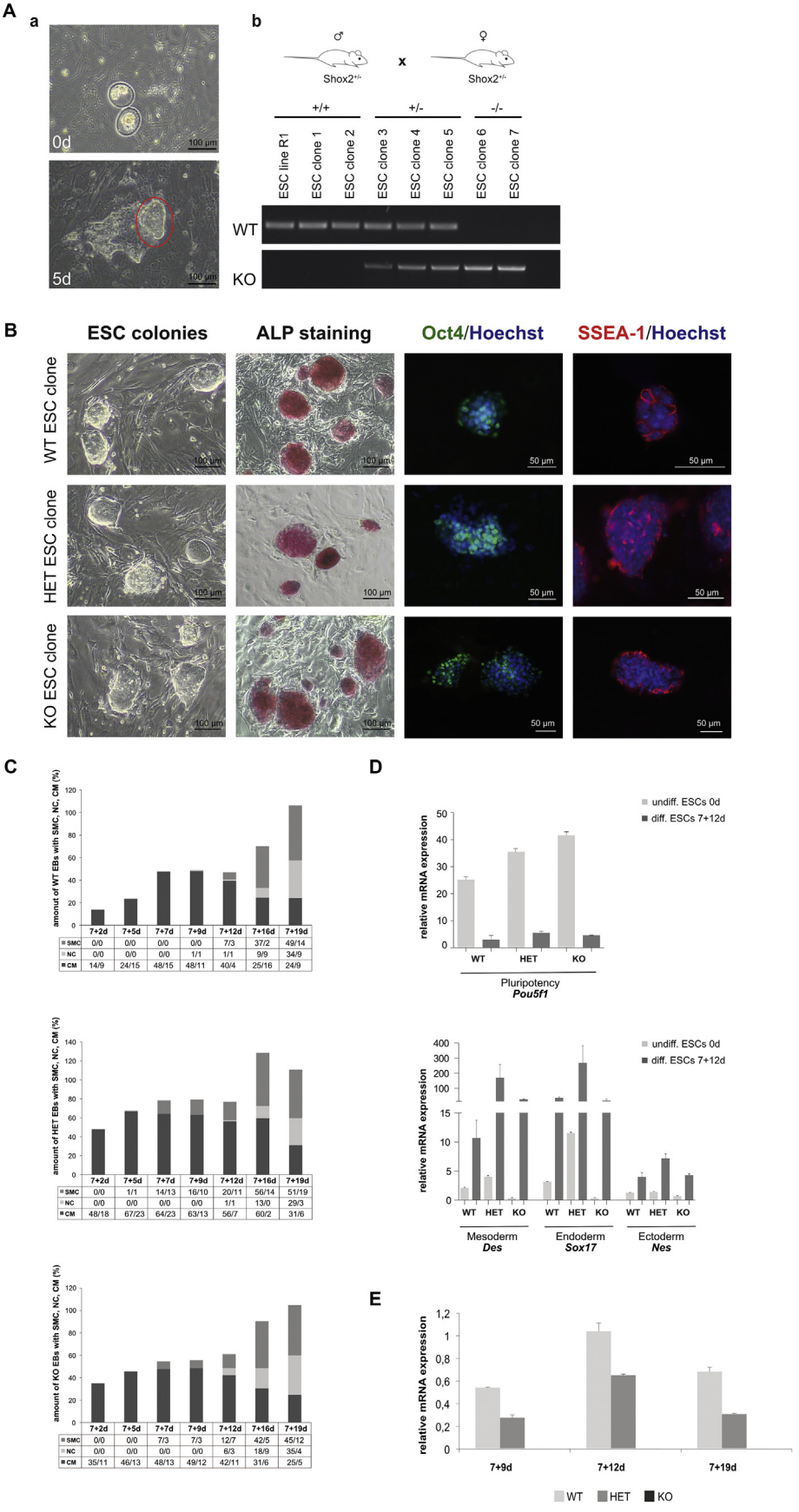

Fig. 1. Isolation and characterization of ESC lines from *Shox2*-deficient mice.

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Table 1
Summary of lines

ESC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
mouse *Shox2*$^{+/+}$ ESCs	WT ESCs	N/A	N/A	N/A	+/+	N/A
mouse *Shox2*$^{+/-}$ ESCs	HET ESCs	N/A	N/A	N/A	+/D	N/A
mouse *Shox2*$^{-/-}$ ESCs	KO ESCs	N/A	N/A	N/A	D/D	N/A

Characterization of Shox2 ESC lines

The pluripotency of undifferentiated *Shox2* ESC clones was confirmed by typical ESC morphology indicated by formation of colonies, ALP activity and immunocytochemical detection of Oct4 (green), SSEA-1 (red) and Hoechst (blue) (Fig. 1B, Table 2). M-FISH analysis revealed a complex karyotype of *Shox2* ESC clones (WT, HET, KO), however aberrations in chromosome 3 harboring the *Shox2* locus have never been found (Table 2, Supplementary Fig. 1, Supplementary Table 1). We confirmed the original source of all mouse ESC lines generated from C57BL/6 mice by STR analysis (Table 2, Supplementary Tables 2 and 3) and DNA barcoding (Table 2, Supplementary Data), while mycoplasma contamination was excluded by PCR (Table 2, Supplementary Fig. 2).

Under appropriate in vitro culture conditions in suspension, all isolated ESCs form 3D cell aggregates called embryoid bodies (EBs), which can spontaneously differentiate into all derivatives of the three primary germ layers. In our standard differentiation protocol, EB formation occurred for 7 days in suspension culture, following adherent culture for differentiation (7 + Xd). Morphological evaluation at various time points confirmed the potential of all isolated ESC clones (WT n = 4; HET n = 4; KO n = 5) to differentiate into different cell types. The amount of EBs with beating cardiomyocytes (CM), neuronal cells (NC) and skeletal muscle cells (SMC) was determined by calculating the percentage of EBs with the respective cell types using brightfield microscopy (Fig. 1C, Table 2). Data table comprises value and SEM (value/SEM).

Comparative expression analyses of lineage-specific markers representing the three primary germ layers (mesoderm: *Des*; endoderm: *Sox17*; ectoderm: *Nes*) revealed a spontaneous EB differentiation of all ESC clones (7 + 12d, n = 3), while the pluripotency marker *Pou5f1* showed diminished expression levels compared to undifferentiated ESCs (n = 2) of the respective clones (Fig. 1D, Table 2).

Further analysis of the differentiated ESC clones revealed the expected level of *Shox2* expression according to the respective genotype on mRNA (Fig. 1E, Table 2) as well as on protein level (data not shown). While the heterozygous ESC clone (HET) showed approximately 50% of *Shox2* expression, no *Shox2* transcript was detectable in the *Shox2* homozygous ESCs (KO) compared to the wild type *Shox2* clone (WT) (Fig. 1E).

Materials and methods

Isolation of ESC lines from Shox2-deficient mice

Superovulation was induced in previously generated *Shox2*$^{+/-}$ mice (Blaschke et al., 2007) followed by mating. At E3.5 blastocysts were transferred on preseeded miMEFs and cultivated in ESC proliferation medium (Wobus et al., 2002). After five days, the ICM of the adherent blastocysts was mechanically separated, enzymatically dissociated and replated onto preseeded miMEFs in ESC proliferation medium supplemented with 0.015 μg/ml LIF. Cells were always cultivated at 37 °C with 5% CO_2 and 95% humidity.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	ESC colony formation visualized by brightfield microscopy: normal	Fig. 1B very left panel
Phenotype	Immunocytochemistry	Assessment of pluripotency: Immunocytochemical detection of Oct4, SSEA-1 and ALP-staining: both positive	Fig. 1B
	Flow cytometry	Not performed	
Genotype	Genotype PCR	*Shox2* WT, HET and KO ESC clones were detected	Fig. 1 A b
	Karyotype (G-banding) and resolution	Most occurring chromosomal aberrations detected by M-FISH (resolution ≥ 10 Mb): WT: 42, XY, +1 [11/15], +8 [10/15]; HET: 39, X [15/15]; KO: 40, XY, der(14)t(14;14;1) [14/15]	Supplementary Fig. 1 Supplementary Table 1
Identity	COI barcoding	COI sequences revealed species-specificity (*Mus musculus*)	Supplementary Data
	STR analysis	STR profiling excluded contaminants of human cells; Matching STR strain profile of C57BL/6 mice was confirmed	Supplementary Table 2; Supplementary Table 3
Mutation analysis (IF APPLICABLE)	Sequencing	N/A	
	Southern Blot OR WGS	N/A	
Microbiology and virology	Mycoplasma	Mycoplasma detection by PCR. Negative	Supplementary Fig. 2
Differentiation potential	Embryoid body formation OR Teratoma formation OR Scorecard	Embryoid body formation was initiated by hanging drop culture; Subsequent morphological assessment confirmed differentiation properties by visual detection of cardiomyocytes, skeletal muscle and neuronal cells; Differentiation into all three germ layers of the generated ESC lines was approved by comparative qPCR (undifferentiated vs differentiated ESCs) analyzing the marker genes *Des* (mesoderm), *Sox17* (endoderm) and *Nes* (Ectoderm).	Fig. 1C, D, E
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

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Genotyping

Genomic DNA was purified using the Quick DNA Universal kit (Zymo Research) according to manufacturer's instruction. Genotyping was performed using the standard Taq DNA polymerase with ThermoPol buffer (NEB) according to manufacturer's instructions. Primer sequences are given in Table 3.

Cell line identification (DNA barcoding and STR profiling)

DNA barcoding of mitochondrial Cytochrome Oxidase subunit 1 (COI) and STR analysis of polymorphic locations of Short Tandem Repeats (STRs) were performed by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (www.dsmz.de) to provide species identification and interspecies contamination.

Mycoplasma detection

Mycoplasma detection was performed by conventional PCR using the Venor GeM Classic Kit (Minerva Biolabs) according to manufacturer's instruction.

Table 3
Reagents details

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-Oct4	1:120	Abcam Cat# ab19857, RRID:AB_445175
Pluripotency Markers	Mouse anti-SSEA-1	1:50	Abcam Cat# ab16285, RRID:AB_870663
Secondary antibodies	Alexa488 Chicken Anti-Rabbit IgG	1:250	Molecular Probes Cat# A21441, RRID:AB_141735
Secondary antibodies	Cy3 Goat Anti-Mouse IgG	1:700	Jackson ImmunoResearch Cat# 715-165-150, RRID:AB_2340813

Primers		
	Target	Forward/Reverse primer (5′-3′)
Genotyping	*Shox2 intron1/2*	Forward: 5′-CAGGGTTAGGA GTCTCTAGCCT'-3′
Genotyping	*Shox2 exon2*	Reverse: 5′-TGCT TGATTTTGGTCTGGCCTTCGT-3′
Genotyping	Neomycin cassette	Forward: 5′-TGAGCGGGACTCTGGGGTTCGA-3′
Pluripotency Marker (qPCR)	*Pou5f1*	Forward: 5′-TGAAGAACAAGTGCCAAATAGC-3′ Reverse: 5′-GCGGCTATACAAAGTGGACAA-3′
Differentiation Marker (qPCR)	*Des*	Forward: 5′-GCGTGACAACCTGATAGACG-3′ Reverse: 5′-TGGATTTCCTCCTGTAGTTTGG-3′
Differentiation Marker (qPCR)	*Sox17*	Forward: 5′-CACAACGCAGAGCTAAGCAA-3′ Reverse: 5′-CGCTTCTCTGCCAAGGTC-3′
Differentiation Marker (qPCR)	*Nes*	Forward: 5′-GAGGTGGCCACGTACAGG-3′ Reverse: 5′-AAGCTGAGGGAAGTCTTGGA-3′
Target Gene (qPCR)	*Shox2*	Forward: 5′-ACCAATTTTACCCTGGAACAAC-3′ Reverse: 5′-TCGATTTTGAAACCAAACCTG-3′
Reference Gene (qPCR)	*Sdha*	Forward: 5′-CATGCCAGGGAAGATTACAAA-3′ Reverse: 5′-GTTCCCCAAACGGCTTCT-3′
Reference Gene (qPCR)	*Hprt1*	Forward: 5′-TCCTCCTCAGACCGCTTTT-3′ Reverse: 5′-CCTGGTTCATCATCGCTA ATC-3′

Alkaline phosphatase staining (ALP)

An ALP assay was performed using an ALP-Kit (Sigma-Aldrich) as recommended by the manufacturer. The staining results were examined by brightfield imaging.

Immunocytochemistry

ESC clones were fixed, permeabilized by 0.1% TritonX100 (Sigma-Aldrich) and stained with primary anti-Oct4 and anti-SSEA-1 as well as secondary AlexaFluor 488 and Cy3 antibodies. Details are given in Table 3.

Multiplex fluorescence in situ hybridization (M-FISH)

For M-FISH seven pools of flow-sorted whole chromosome mouse painting probes were amplified, directly labeled using seven different fluorochromes and hybridized to metaphases spreads of the respective cell lines. Images ($n = 15$ each) were recorded using a DM-RXA epifluorescence microscope, processed using the Leica MCK software and one representative image per clone is presented as multicolor karyogram.

Differentiation of ESC clones via embryoid body formation

A modified differentiation procedure was carried out according to a standard hanging drop protocol (Wobus et al., 2002). For embryoid body (EB) formation 600 cells/20 µl drop were plated as hanging drops in ESC differentiation medium. After two days in hanging drop culture EBs were transferred in suspension culture for five further days. On day seven, EBs were plated for differentiation.

Morphological analyses

7d single EBs were plated for morphological assessment. The appearance of either beating cardiomyocytes, neurons or skeletal muscle cells per EB was examined by brightfield microscopy (Wobus et al., 2002).

Quantitative Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The reverse transcription was performed using the SuperScript III First-Strand Synthesis Kit (Invitrogen). Quantitative Real-time PCR (qPCR) was conducted using the SYBR Green Lo-Rox Fast Mix (Bioline) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). All samples were measured in duplicates and the relative expression levels were evaluated using the Relative Standard Curve Method. Values were normalized to the reference genes *Sdha* and Hprt1. Primers are listed in Table 3.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.07.011.

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