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Supplemental Information

In Vitro Generation of Vascular Wall-Resident Multipotent Stem Cells of Mesenchymal Nature from Murine Induced Pluripotent Stem Cells

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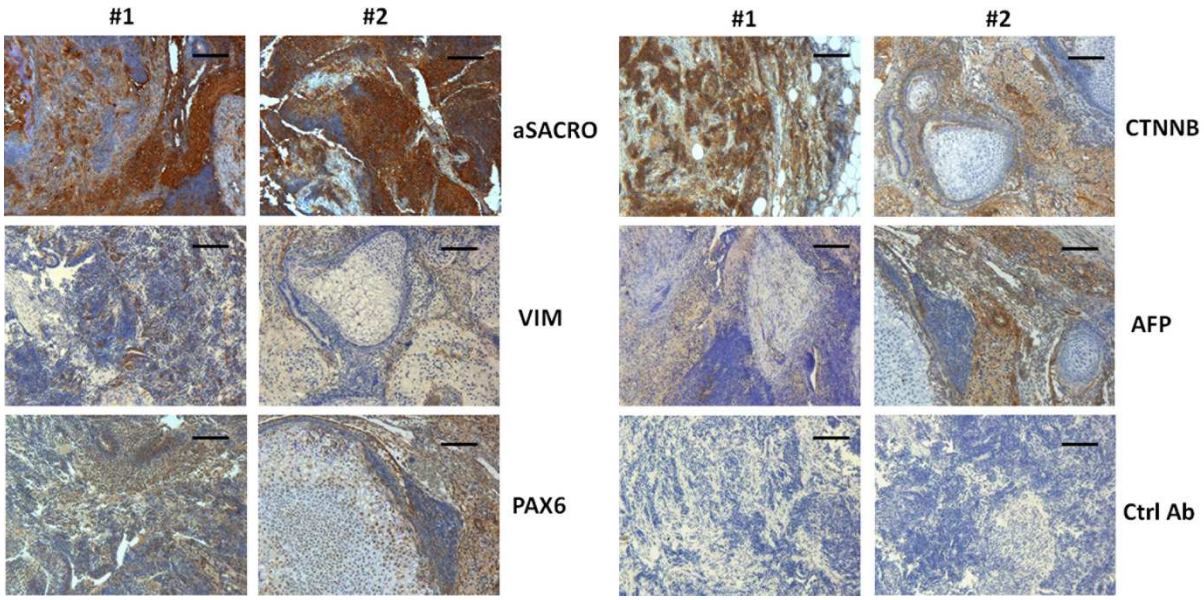
1. Supplemental Figures

Figure S1

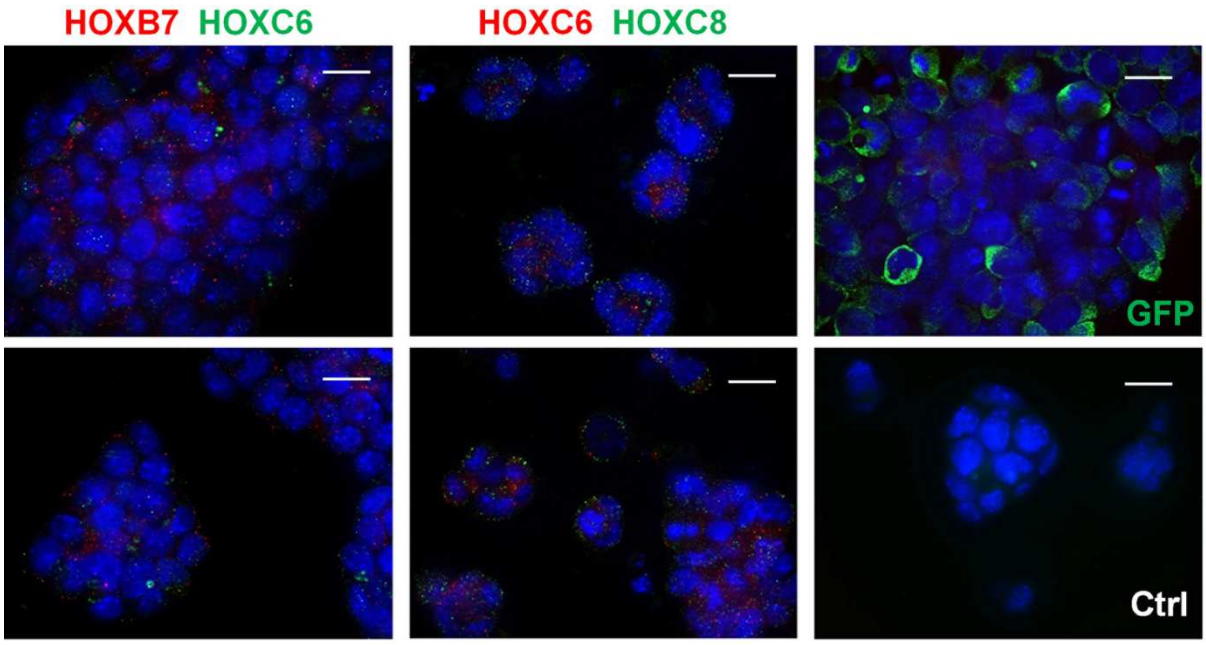


Supplemental Figure S1. Related to Figure 1.

Pluripotency analysis in vivo: teratoma formation of implanted NEST-iPSCs clones.

Immature teratomas derived from two different NEST-iPSCs clones (#1, #2) were explanted 4 weeks after subcutaneous cell injection and further subjected to immunohistochemistry. The presence of derivatives of all three germ layers was further confirmed using specific antibodies to alpha-sacromeric actin and vimentin (mesoderm), beta-catenin and alpha-fetoprotein (endoderm), and PAX6 (ectoderm) in combination with DAB staining. Mouse IgG1 isotype controls (Ctrl Ab) were used as staining controls. Representative photographs of n=6 independent experiments are shown Magnification 100x (scale bars 100 µm).

Figure S2

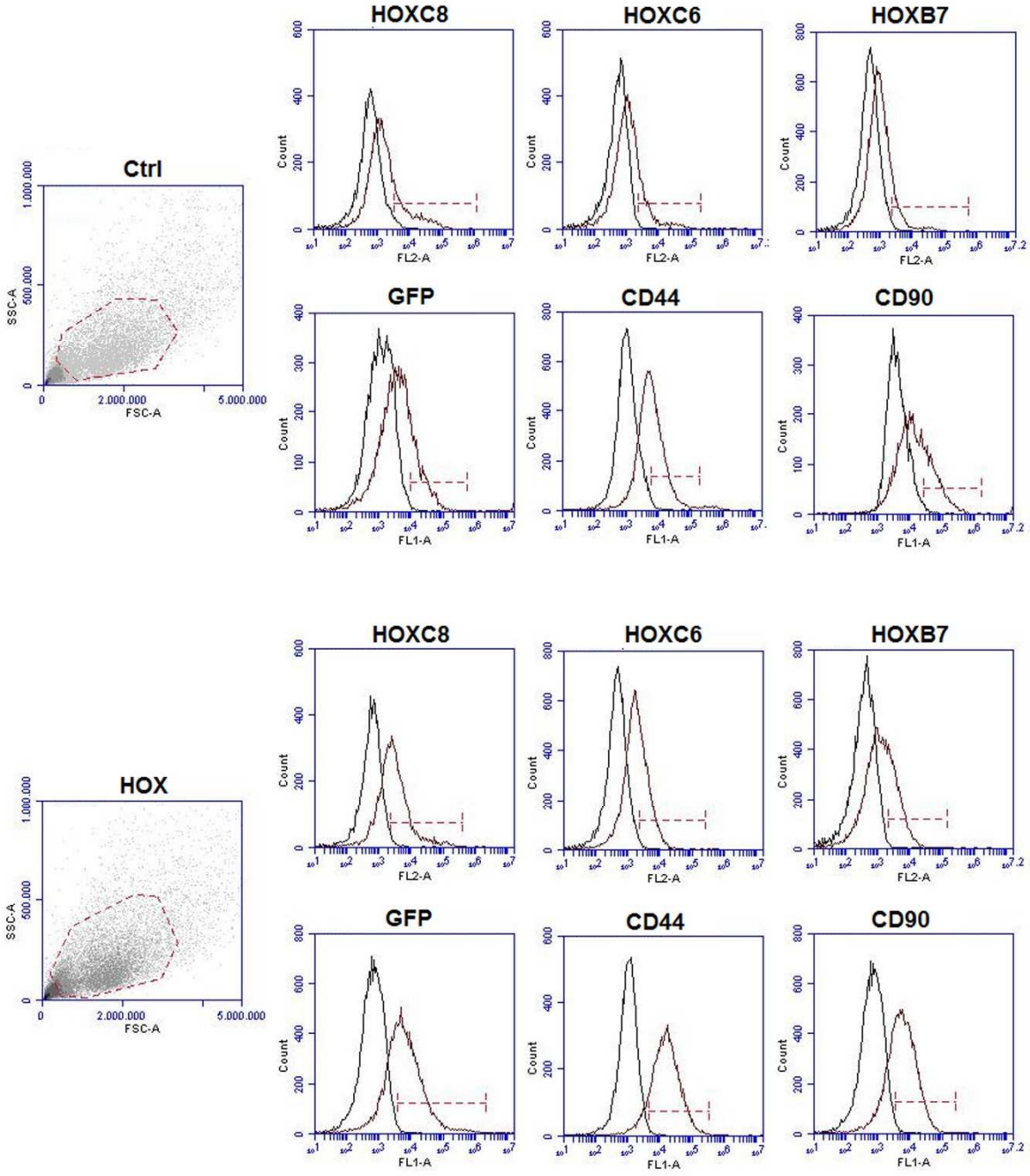


Supplemental Figure S2. Related to Figure 5.

Confocal microscopy of fluorescently labelled *HOX*-transduced NEST-iPSCs and subsequently differentiated EBs.

Six days after transduction, aggregated EBs were seeded on gelatine-coated cover-slips and immunofluorescence analysis for HOXB7, HOXC6 and HOXC8 as well as GFP expression was performed followed by confocal microscopy. Representative images of n=4 independent experiments are shown**.** Scale bar: 15 µm.

Figure S3

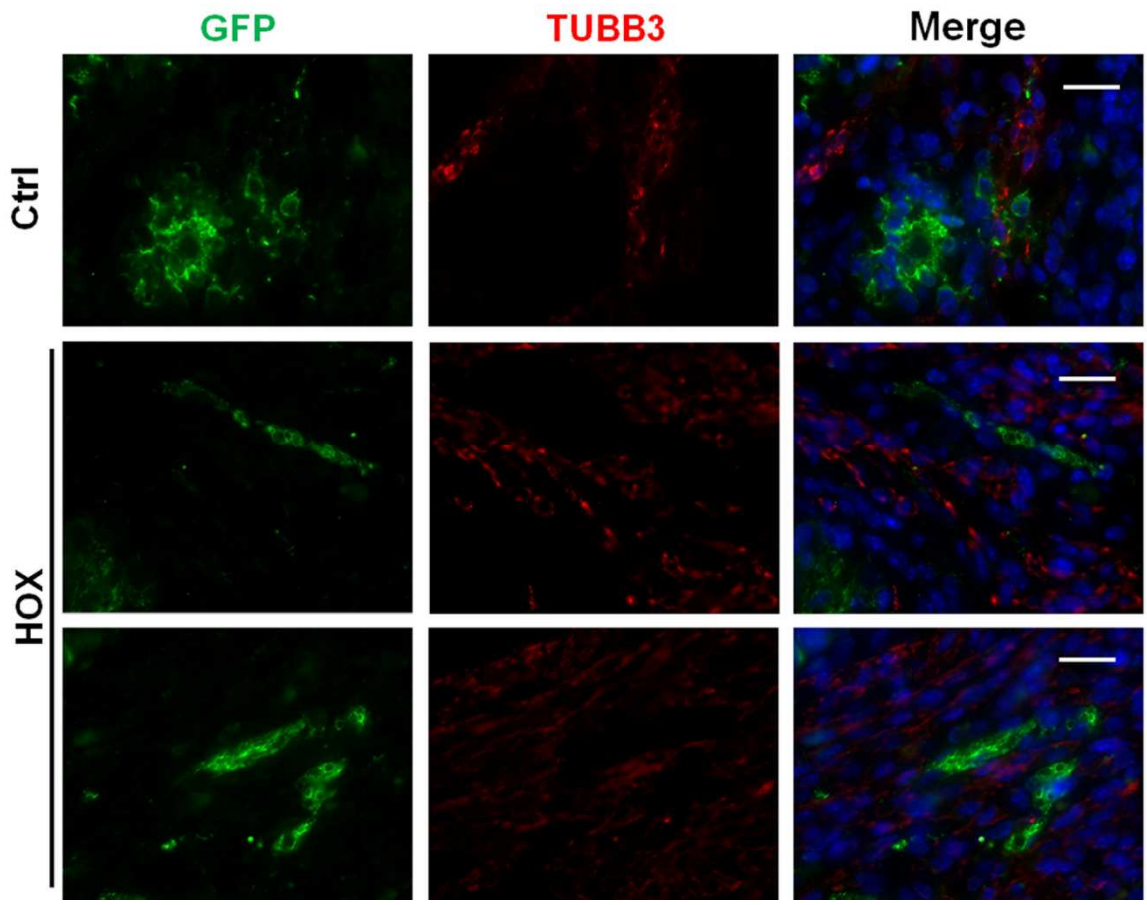


Supplemental Figure S3. Related to Figure 6.

Representative dot plots and histograms from the flow cytometry analysis as quantified and summarized in Figure 6B.

Single cell suspensions generated from freshly ex vivo isolated teratoma tissue pieces grown from implanted control- and *HOX*-transduced NEST-iPSCs were analyzed by FACS with indicated antibodies. 20.000-30.000 cells were analysed per sample. Representative blots are shown (biological replicates: n=8-10 for each epitope and group). Isotype controls are shown by the left peak. The red horizontal lines (red lines) depict the markers.

Figure S4



Supplemental Figure S4. Related to Figure 7.

Differentiated NEST-GFP(+) cells do not express neuronal beta-III tubulin.

Subcutaneously grown tumors were analysed by immunofluorescence and confocal microscopy. Sections were stained for GFP and (green) and beta-III tubulin (TUBB3) (red) Representative images from n=4 independent experiments are shown. Magnification 63x (scale bars 20µm).

2. Supplemental Tables

Table S1. Reprogramming efficiencies. Related to Figure 1.

Fibroblasts	**Seeded**	**Transduction efficiency in %**	**Clones isolated**	**Reprogramming efficiency in %**
Mouse 1	10.000	39	412	1.08
Mouse 2	10.000	18	269	1.49
Mouse 3	10.000	16	488	3.05

Table S2. Antibodies used in this study. Related to Figure 1-7.

Antibody, Clone (host)	Supplier	Cat#	Dilution
Nestin, H85 (rabbit)	Santa Cruz Biotech	sc-20978	1:200
GFP, 15 (mouse)	Santa Cruz Biotech	sc-101525	1:500
GFP, G10362 (rabbit)	Invitrogen	991639	1:500
HOXB7, 747C4a (mouse)	Santa Cruz Biotech	sc-81292	1:200
HOXB7, Z25 (rabbit)	Santa Cruz Biotech	sc-133670	1:200
HOXC6, B7 (mouse)	Santa Cruz Biotech	sc-376330	1:200
HOXC6 (rabbit)	Acris Antibodies	AP06660PU-N	1:200
HOXC8 (rabbit)	Bioss Inc.	bs-0394R	1:200
CD44, IM7 (rat)	Biolegend	103002	1:200
CD90, 3H1751 (rat)	Novus Biologicals	NB-200-528	1:200
CD73, TY/23 (rat)	BD Bioscience	550741	1:100
CD31, SZ31 (rat)	Dianova	DIA-310	1:200
Actin, AC74 (mouse)	Sigma-Aldrich	A2228	1:5000
Vimentin, EPR3776 (rabbit)	Abcam	ab92547	1:500
Catenin beta, E5 (mouse)	Santa Cruz Biotech	sc-7963	1:200
Alpha fetoprotein (rabbit)	Dako	A000829-2	1:200
Beta III tubulin (mouse)	Life Technologies	PA-1-46430	1:200
GFAP (rabbit)	Abcam	ab7260	1:200
PAX6 (rabbit)	Thermo Fischer Scientific	42-6600	1:100
Actin alpha sacromeric, 5C5 (mouse)	Sigma-Aldrich	A2172	1:200
OCT3/4, 40 (mouse)	BD Bioscience	611203	1:100
SSEA1, 480 (mouse)	Santa Cruz Biotech	sc-21702	1:100
SOX2, Btjce (rat)	eBioscience	14-9811-82	1:100
SOX2, D17 (goat)	Santa Cruz Biotech	sc-17319	1:200

Table S3. Oligonucleotides used for qRT-PCR. Related to Figure 1, 2 and 4.

Gene	sense/antisense	Primer sequence
Oct4	s	5'–CGC–CCG–CAT–ACG–AGT–TCT–3'
	as	5'–GCA–CCA–GGG–TCT–CCG–ATT–T–3'
Klf4	s	5'–CCA–GGA–GAA–CCC–CAA–GAT–GC–3'
	as	5'–GGG–TGC–CCT–GCT–GCG–AGT–A–3'
HOXB7	s	5'–CCG–AGA–GTA–ACT–TCC–GGA–TCT–A–3'
	as	5'–TCT–TGA–TCT–GTC–TTT–CGG–TGA–A–3'
HOXC6	s	5'–ATG–CTC–TCA–AAC–TGC–AGA–CAA–A–3'
	as	5'–CGA–GTT–AGG–TAG–CGG–TTG–AAG-T–3'
HOXC8	s	5'–AAG–GAC–AAG–GCC–ACT–TAA–ATC–A–3'
	as	5'–CCT–CCT–CTT–TCT–CCT–CTT–CCT–C–3'
GFP	s	5'–ACG–TAA–ACG–GCC–ACA–AGT–TC–3'
	as	5'–AAG–TCG–TGC–TGC–TTC–ATG–TG3–3'
Sca-1	s	5′-ACT-GTG-CCT-GCA-ACC-TTG-TCT-GAG-A-3′
	as	5′-GTC-CAG-GTG-CTG-CCT-CCA-TT-3′
c-Kit	s	5′-GCC-CTA-ATG-TCG-GAA-CTG-AA-3′
	as	5′-TTG-CGG-ATC-TCC-TCT-TGT-CT-3′
Nestin	s	5′-TCA-AGG-GGA-GGC-CAG-GAA-GGA-3′
	as	5′-CTG-CAG-CCC-CAC-TCA-AGC-CAT-C-3′
Adiponectin	s	5′-AGC-CGC-TTA-TGT-GTA-TCG-CT-3′
	as	5′-GAG-TCC-CGG-AAT-GTT-GCA-GT-3′
Osteocalcin	s	5′-GCA-ATA-AGG-TAG-TGA-ACA-GAC-TCC-3′
	as	5′-GTT-TGT-AGG-CGG-TCT-TCA-AGC-3′
Aggrecan	s	5′-CCC-TCG-GGC-AGA-AGA-AAG-AT-3′
	as	5′-CGC-TTC-TGT-AGC-CTG-TGC-TTG-3′

Specific primers were designed with the program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) based on available NCBI nucleotide CDS sequences. Cross-reaction of primers was excluded by comparison of the sequence of interest with the NCBI database (Blast 2.2, U.S. National Centre for Biotechnology Information, Bethesda, MD) and all primers used in our study were intron-spanning. PCR products are 200-300 bp in size.

as

3. Supplemental Experimental Procedures

Tail-tip fibroblast isolation

An ~1 cm length of tail-tip from 8-week-old male nestin-GFP (NEST-GFP) transgenic donor mice was washed with 70% ethanol and PBS, the superficial dermis was peeled off, and the remaining tissue was cut into 1-mm pieces using a scalpel (1, 2). Five or six pieces were plated in one well of a 0.1% gelatin-coated six-well plate and cultured with 2 mL of Dulbecco's modified Eagle's medium (DMEM, high glucose) with 10% FBS, 1% Sodiumpyruvate and 50 units/mL penicillin/streptomycin (nTTF medium) for 5–7 days. Cells migrating out were trypsinized and expanded into T75 flasks (passage 1).

Reprogramming of mouse NEST-GFP tail-tip fibroblasts

We reprogrammed primary dermal tail tip fibroblasts from a transgenic mouse in which the gene encoding GFP is expressed under the regulatory control of the nestin promotor (NEST-GFP) (Klein et al., 2014). Transduction of fibroblasts was performed with a lentiviral vector co-expressing the four Yamanaka factor genes (*OCT4*, *KLF4*, *SOX2* and *MYC*) together with the coding sequence of the red fluorescent tdTomato protein (3). Expression plasmids encoding lentiviral gag-pol (pcDNA3.GP.CCC), HIV-rev (239_RSV_Rev) and the reprogramming cassette (pRRL.PPT.SF.OKSM.I.GFP.Pre) were transiently transfected into HEK293 cells together with pMDG_VSVG for pseudotyping (Stanurova et al., 2016). Culture supernatants were collected 24 and 48 hours post transfection. The produced virus was concentrated by ultracentrifugation of the supernatants at 27,000 x g, 1.5 h, 4 °C and titrated on HT1080 cells.

HOX gene expression vector and transduction

Generated NEST-GFP iPSCs were transduced using a lentiviral self-inactivating (SIN) vector (4, 5) co-expressing the coding sequences of *HOXB7*, *HOXC6* and *HOXC8* separated by *2A esterase* elements together with the gene encoding TURQUOISE2 (cyan) fluorescent protein (Figure 1C) (6).

Alkaline phosphatase staining

iPSC colonies were stained for alkaline phosphatase activity using the Alkaline Phosphatase Detection Kit from Millipore as recommended by the manufacturer. The staining reaction was stopped after 15 minutes by washing with PBS and cell colonies were evaluated by phase contrast microscopy.

Trilineage differentiation assay

Differentiation of cultivated MSCs into adipocytes, chondrocytes, and osteocytes was done using ready-to-use differentiation media from Lonza (hMSC Differentiation BulletKit–Adipogenic, PT-3004; –Chondrogenic, PT-3003; –Osteogenic, PT-3002) according to the manufactures instructions. Adipogenic differentiation was verified using Oil red staining, and osteogenic differentiation was verified using NBT/BCIP staining (Sigma) for alkaline phosphatase activity.

Cell proliferation assay

The number of living cells was determined upon staining of the cells with the vital dye trypan blue. For this, cells were harvested with Trypsin-EDTA, re-suspended in fresh medium, diluted with trypan blue, and counted at the indicated time points employing a Neubauer chamber.

RNA isolation and cDNA synthesis.

For RNA isolation the cells were lysed directly in the plastic Petri dishes as previously described and the RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Klein et al. 2013 and 2016a).

Flow cytometry

For FACS analysis of pluripotent cells, cells were treated with Trypsin/EDTA (Life Technologies) to generate a single-cell suspension, which was passed through a 40 µm cell strainer and counted. For FACS analysis of teratoma cells, freshly ex vivo isolated tissue pieces were mechanically minced and dissociated for 15-20 minutes at 37°C

in OptiMEM-I medium containing 0.2% type 2-collagenase (CLS2, ≥125 units per mg dry weight; Worthington, Lakewood, WA). Cells were washed twice in PBS/5% FCS and passed through a 40 µm cell strainer and counted. For each antibody staining, $1x10^5$ cells were used in a volume of 100-200 µl FACS buffer (10% FBS in PBS). Antibodies were used in a 1:100 dilution and incubated with the cell suspension for 20 min at 4 °C. For staining of nuclear proteins, cells were fixed prior staining using 4 % paraformaldehyde for 15 min at room temperature and permeabilized by incubation in 0.1 % (v v) Triton X-100 for 5 min at room temperature prior antibody staining. After addition of 1 ml FACS buffer and centrifugation (700 x g, 5 min), cells were resuspended in 200 µl FACS buffer and analyzed on a FACS Aria (Becton Dickinson) using the FACSDiva software (Becton Dickinson). Isotype controls were applied for every antibody. Data analysis was done using Kaluza® software (Beckman Coulter). Antibodies are listed in Table S2.

Real-time RT-PCR analysis.

Analysis was carried out using the oligonucleotide primers listed in Table S3. The PCR program consisted of initial denaturation at 95°C for 30 s, annealing at 58°C for 40 s and extension at 72°C for 30 s for 25-30 cycles. Specificity of all PCR reactions was tested by parallel reactions using water instead of cDNA. Real-time PCR analysis was performed using SYBR®Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) and standard conditions. The experiments were performed on ABI PRISM® 7000 sequence detection system (Applied Biosystems). Expression levels of analysed genes were normalized to the reference gene β-actin mRNA expression (relative quantification). Mean values ± SEM from at least three independent samples per group and gene were quantified, measured in duplicates each

Western blot

Whole cell lysates were generated by scraping cells into ice-cold RIPA-P buffer (150 mmol/L NaCl, 1% NP40, 0.5% sodium-desoxycholate, 0.1% sodium-dodecylsulfate, 50 mmol/L Tris/HCL pH 8, 10 mmol/L NaF, 1 mmol/L Na_3OV_4), supplemented with a complete Protease-Inhibitor-Cocktail (Roche) and performing 2-3 freeze-thaw cycles. Protein samples (50–100 µg total protein) were subjected to SDS-PAGE electrophoresis and Western blots were done as previously described using HOXB7, HOXC6, HOXC8, GFP and NEST (all 1/200) or β-Actin (1/5000) antibodies (Klein et al. 2013 and 2014). For quantification, blots were analyzed by densitometry and the respective signal was related to the reference protein beta-actin.

4. Supplemental References

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