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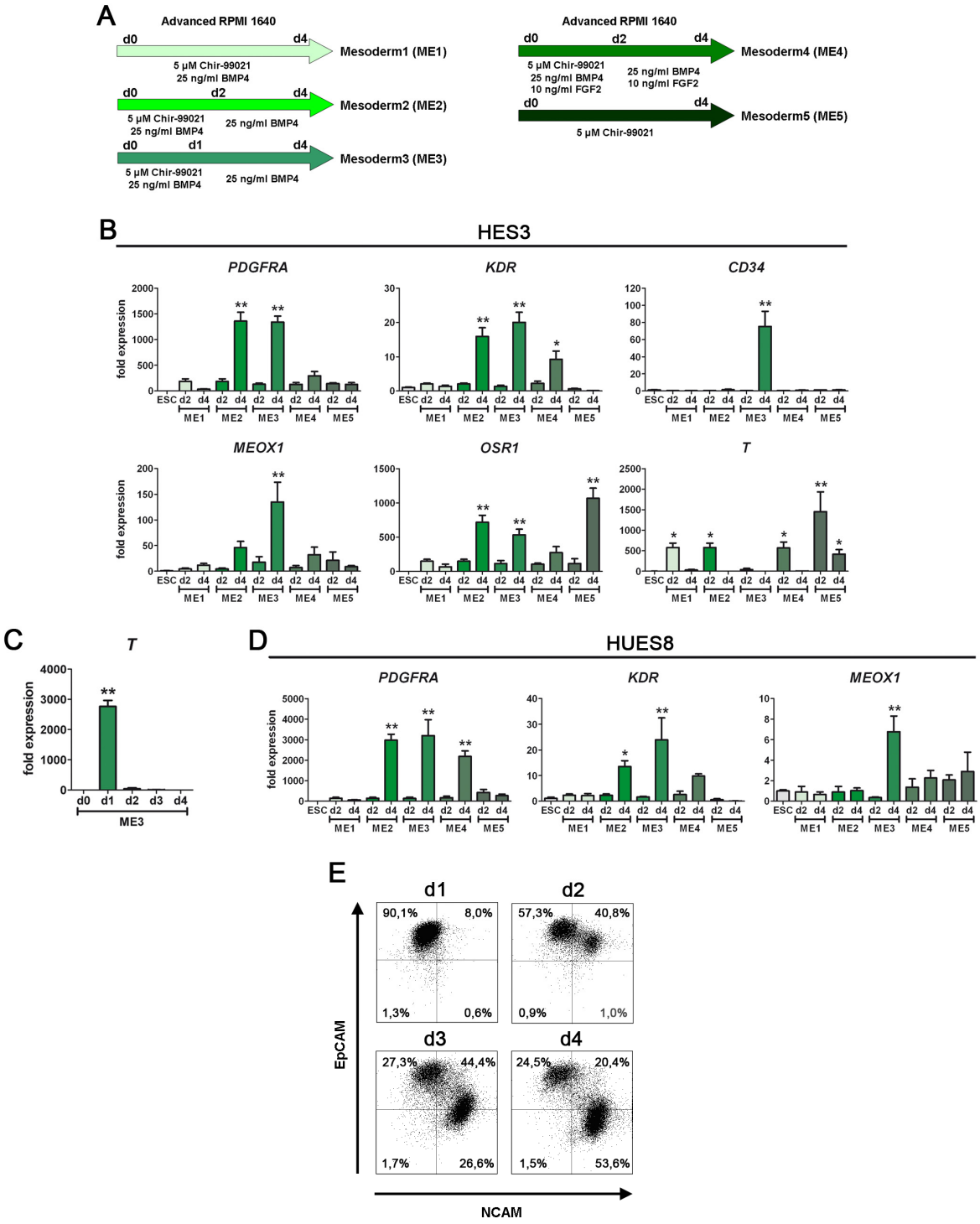
Supplemental Information

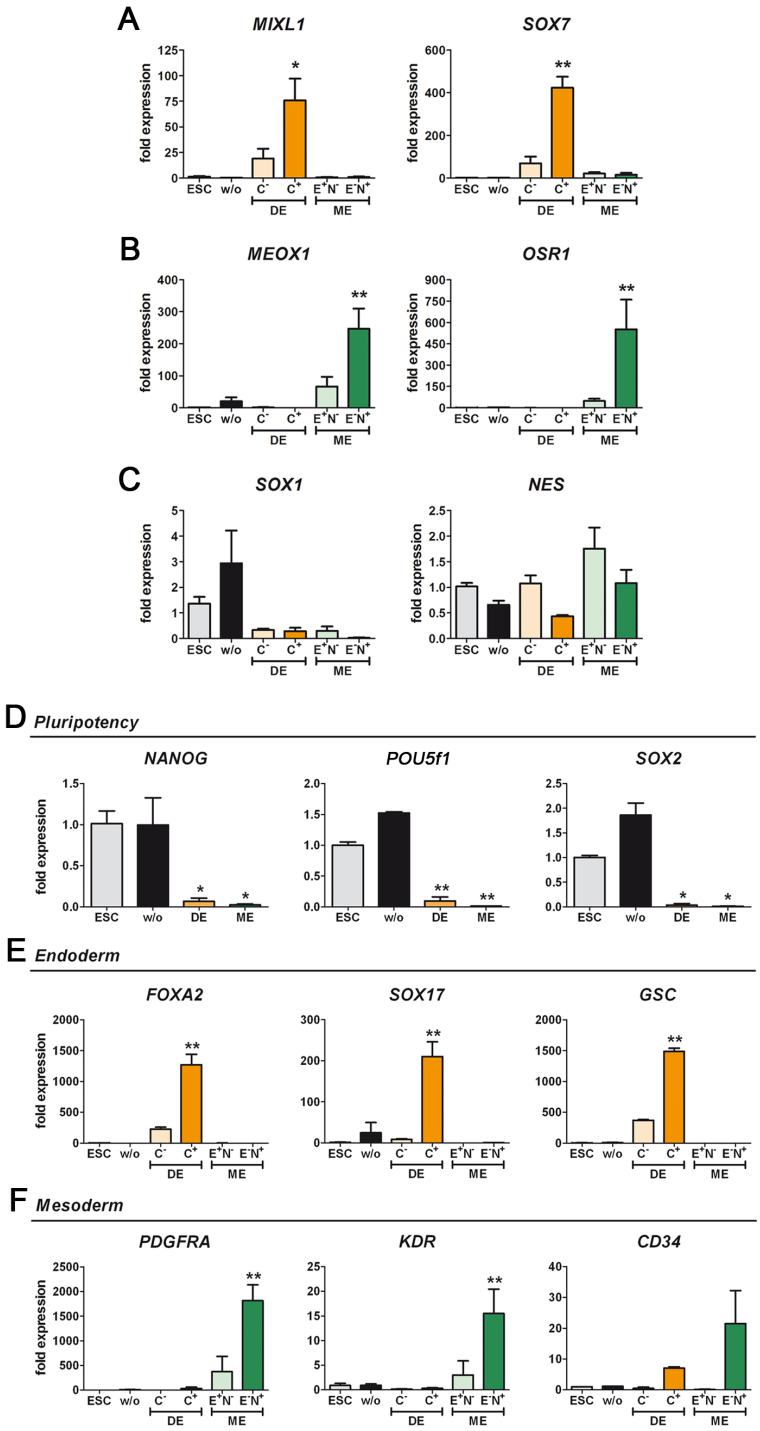
miRNome Profiling of Purified Endoderm and Mesoderm Differentiated from hESCs Reveals Functions of miR-483-3p and miR-1263 for Cell-Fate Decisions

Daichi Ishikawa, Ulf Diekmann, Jan Fiedler, Annette Just, Thomas Thum, Sigurd Lenzen, and Ortwin Naujok



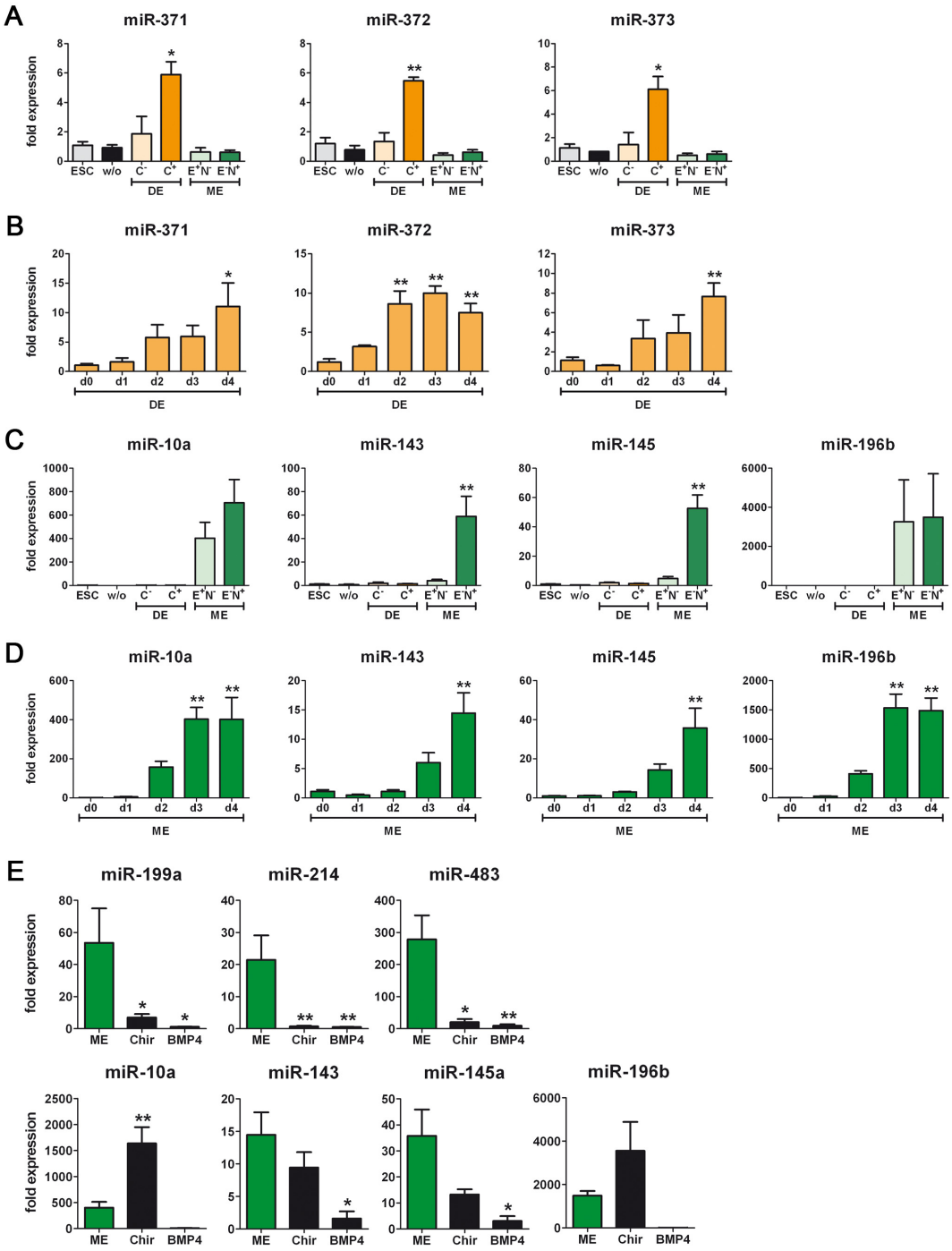
Supporting Information Figure S1. Protocols tested to induce mesodermal differentiation.

A) Depicted are the five different protocols tested for mesoderm (ME) differentiation and their abbreviations. **B**) Relative expression of the mesodermal marker genes (*PDGFRA*, *KDR*, *CD34*, *MEOX1*, *OSR1* and *T*) on day 2 and 4 of differentiation. **C**) Time course of the *T (Bry)* expression during differentiation with the ME3 protocol. **D**) Relative expression of the mesodermal marker genes (*PDGFRA*, *KDR*, *MEOX1*) on day 2 and 4 of differentiation with the indicated ME protocols using a second hESC line (HUES8). Gene expressions were normalized to three stably expressed housekeeping genes (*G6PD*, *TUB1A1*, *TBP*) and scaled to undifferentiated hESCs. Values are means ± SEM, n = 3-7. ANOVA plus *Bonferroni's post hoc test*, *p < 0.05, **p < 0.01 compared with hESCs. **E**) Representative flow cytometric dot plot diagrams of EpCAM/NCAM staining during differentiation with the ME3 protocol (HES3). Indicated numbers represent the percentages of cells in each quadrant.



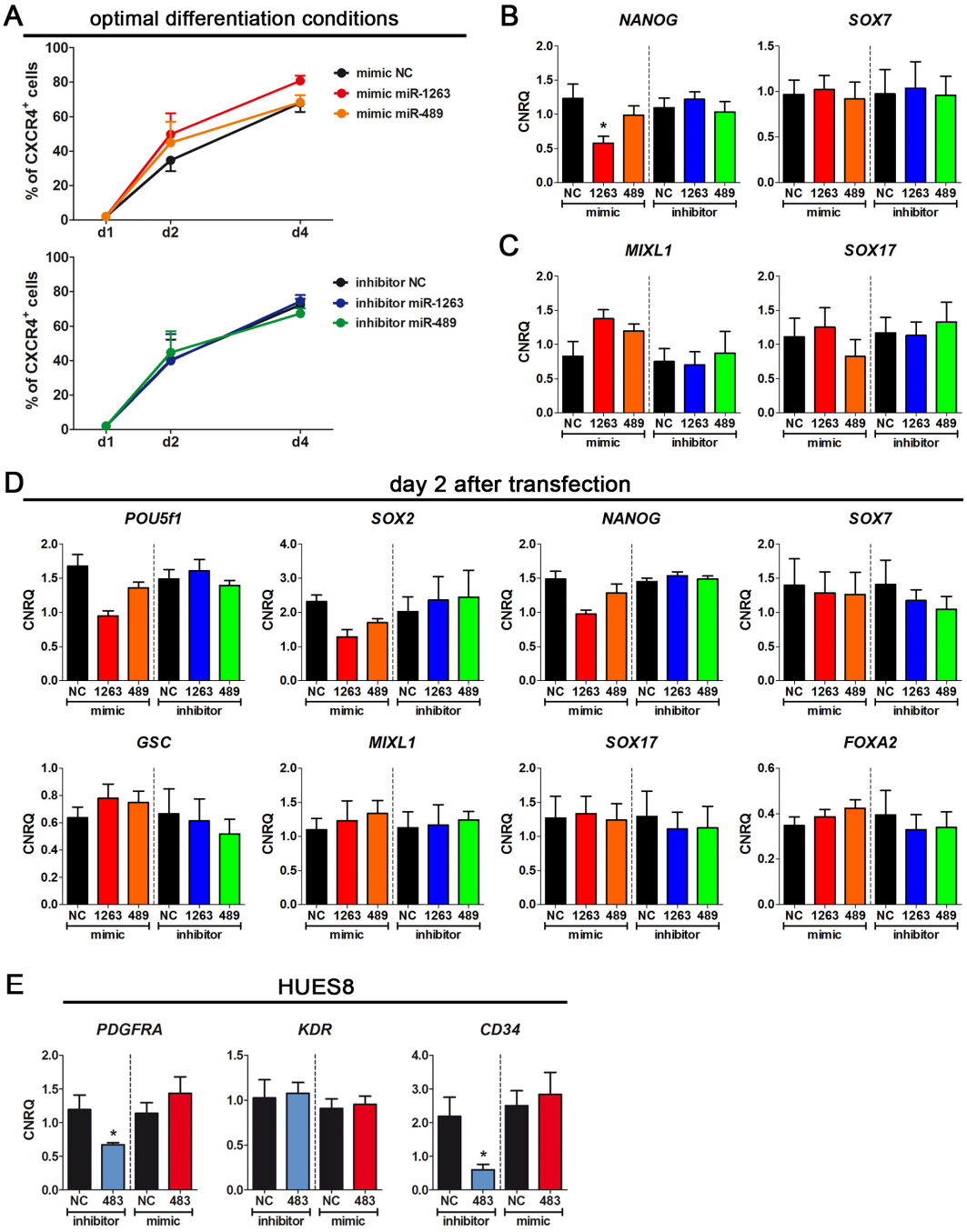
Supporting Information Figure S2. Gene expression analysis of additional lineage markers in different populations of HES3 and HUES8 cells.

Gene expression analysis in HES3 (**A**-**C**) and HUES8 cells (**D**-**F**). Depicted are early lineage markers (*MIXL1*, *SOX7*) (**A**), further mesoderm markers (*MEOX1*, *OSR1*) (**B**) and ectodermal markers (*SOX1*, *NES*) (**C**) in the specified populations (HES3). **D**-**F**) shows the relative expression of marker genes for pluripotency (*NANOG*, *OCT4*, *SOX2*) (**D**), endoderm (*FOXA2*, *SOX17*, *GSC*) (**E**) and mesoderm (*PDGFRA*, *KDR*, *CD34*) (**F**) in the different populations (HUES8). All gene expressions were normalized to three stably expressed housekeeping genes (*G6PD*, *TUB1A1*, *TBP*) and scaled to undifferentiated ESCs. Values represent means ± SEM, n = 3-7 (**A**-**C**), n = 3-5 (**D**-**F**). ANOVA plus Bonferroni's post hoc test, *$p < 0.05$, **$p < 0.01$ compared with hESCs in **D**, and compared with all other populations in **A**-**C** and **E**/**F**. Abbreviations: C^-, CXCR4$^-$ cells; C^+, CXCR4$^+$ cells; E^+N^-, EpCAM$^+$/NCAM$^-$ cells; E^-N^+, EpCAM$^{-/low}$/NCAM$^+$ cells; DE, definitive endoderm; ME, mesoderm; ESC, embryonic stem cells.



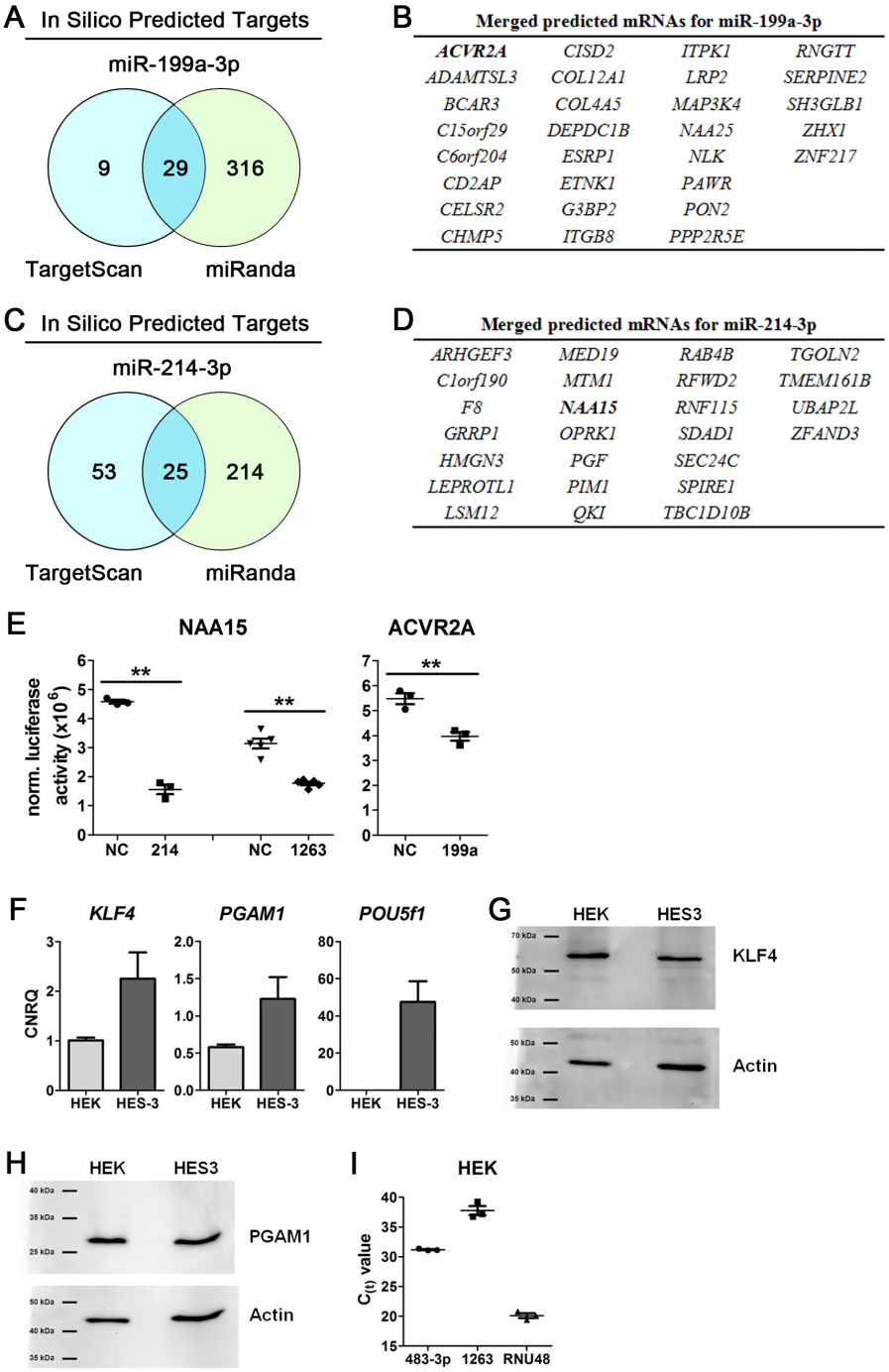
Supporting Information Figure S3. Validation of additional endoderm- or mesoderm-specific miRNAs.

A) Expression of additionally selected endoderm-specific miRNAs in the different populations. **B**) Time courses of their expressions during endoderm differentiation. **C**) Expressions of additional mesoderm-related miRNAs in the analyzed population. **D**) Expression kinetics of the additional mesoderm-specific miRNAs during ME differentiation. **E**) Comparison of mesoderm-specific miRNAs upon ME differentiation or upon exposure either to CHIR alone or to BMP4 alone. All miRNA expressions were normalized to the stably expressed housekeeping miRNAs RNU48, U6 snRNA and miR-425-5p and scaled to undifferentiated ESCs. Values are means ± SEM, n = 4-6. ANOVA plus *Bonferroni's post hoc test,* *$p < 0.05$, **$p < 0.01$ compared with all other populations in **A**/**C,** with undifferentiated cells on day 0 in **B**/**D** or with cells differentiated with ME protocol (**E**). Abbreviations: C^-, $CXCR4^-$ cells; C^+, $CXCR4^+$ cells; E^+N^-, $EpCAM^+/NCAM^-$ cells; E^-N^+, $EpCAM^{-/low}/NCAM^+$ cells; DE, definitive endoderm; ME, mesoderm; ESC, embryonic stem cells.



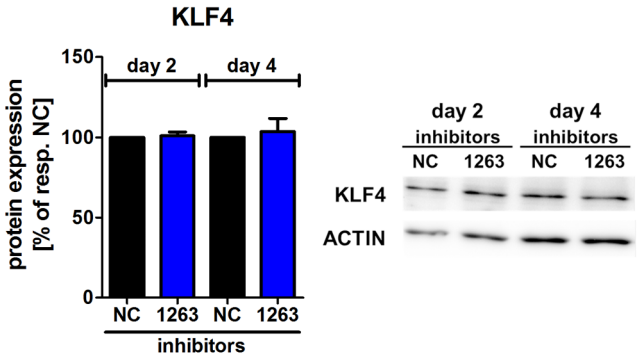
Supporting Information Figure S4. Gene and protein expression after transfection with endoderm- and mesoderm-specific miRNA mimics or inhibitors.

Two hESC lines were used in these experiments. In **A-D** are results shown using the HES3 line, while in **E** HUES8 cells were used. **A**) Time courses of the derivation of CXCR4$^+$ cells during optimal DE differentiation after transfection with mimics or inhibitors. Values are means ± SEM, n = 5-6. **B-C**) Normalized gene expression of markers for pluripotency (*NANOG*), extraembryonic endoderm (*SOX7*) (**B**) and primitive streak/DE (*MIXL1*, *SOX17*) (**C**) after four days of differentiation post transfection. Values are means ± SEM, n = 5-6. **D**) Normalized expression of pluripotency (*POU5F1*, *SOX2*, *NANOG*), extraembryonic endoderm (*SOX7*) and primitive streak/DE marker genes (*GSC*, *MIXL1*, *FOXA2*, *SOX17*) 2 days post transfection. Values are means ± SEM, n = 4-6. **E**) Normalized gene expression of mesodermal markers (*PDGFRA*, *KDR*, *CD34*) after transfection with miR-483-3p inhibitors/mimics using HUES8 cells. Values were scaled to undifferentiated ESCs and represent means ± SEM, n = 4-6. All expression values were normalized to the three stably expressed housekeeping genes. ANOVA plus *Bonferroni's post hoc test*, *$p < 0.05$, **$p < 0.01$ compared with cells transfected with miRNA inhibitor NC or mimic NC.



Supporting Information Figure S5. In silico predicted target genes for miR-199a-3p and miR-214-3p plus the analysis of KLF4 and PGAM1 expression in HEK293 and HES3 cells.

Predicted targets of miR-199a-3p (**A-B**) and miR-214-3p (**C-D**) calculated by the TargetScan or miRanda algorithm. The merge of both data sets for the specific miRNA resulted in an overlap of 29 or 25 targets, respectively (**B** and **D**). **E**) Luciferase activity normalized to the β-galactosidase activity for the sub-cloned 3´-UTRs of *NAA15* and *ACVR2A* in HEK293 cells upon transfection with mimics and reporter plasmids. Values are means ± SEM, n = 4-6. *Student's t-test*, **p < 0.01. **F**) Gene expressions of *KLF4*, *PGAM1* and the pluripotency marker gene *POU5f1* in HEK293 (HEK) or HES3 cells. All values were normalized to the stably expressed housekeeping genes (*G6PD*, *TUB1A1*, *TBP*) and represent means ± SEM, n = 4. Western Blot detection of KLF4 (**G**) and PGAM1 (**H**) in these two cell lines. **I**) MiRNA expression as raw $C_{(t)}$-values of the ME-enriched mir-483-3p, the DE-enriched mir-1263 and an expressed control (RNU48) in HEK293 cells. The late $C_{(t)}$-values of ~32 and >35 for mir-483-3p and mir-1263, respectively, indicated a nearly absent expression in these cells. The depicted values are means ± SEM, n = 3.



Supporting Information Figure S6. MiRNA inhibitor for miR-1263 during endodermal differentiation of HES3 cells.

Normalized protein expression of KLF4 after transfection with the indicated miRNA mimics and differentiation under suboptimal DE condition. Additionally, a representative western blot of KLF4 for this experimental setup is depicted on the right. Values are means ± SEM, n = 4.

Supporting Information Table S1: Standard TaqMan qRT-PCR assays (Thermo Fisher Scientific) and used primer pairs for gene expression analysis.

Gene Symbol	Primer Sequence 5'-3'	Exon spanning	Accession #
CD34	TaqMan Assay: Hs02576480_m1		
FOXA2	Fw: gggagcggtgaagatgga Rev: tcatgttgctcacggaggagta	Yes	NM_153675.2
G6PD	Fw: aggccgtcaccaagaacattca Rev: cgatgatgcggttccagcctat	Yes	NM_000402
GSC	Fw: gaggagaaagtggaggtctggtt Rev: ctctgatgaggaccgcttctg	Yes	NM_173849.2
KDR	Fw: gacagtggtatggttcttgcc Rev: ctgcttcctcactggagtacac	Yes	NM_002253
MEOX1	TaqMan Assay: Hs00244943_m1		
MIXL1	Fw: ccgagtccaggatccaggta Rev: ctctgacgccgagacttgg	Yes	NM_031944.1
NANOG	Fw: ccgagggcagacatcatcc Rev: ccatccactgccacatcttct	Yes	NM_024864.2
NES	Fw: ctacagagccagatcgctcag Rev: ctagggaattgcagctccag	Yes	NM_006617
OSR1	Fw: ctacacctgtgacatctgccac Rev: cttcacctgtgagtgtagcgtc	Yes	NM_145260
PDGFRa	Fw: ccagagctatggggacttcc Rev: cttcactctccccaaagcatc	Yes	NM_006206
POU5F1	Fw: cttgctgcagaagtgggtggagg Rev: ctgcagtgtgggtttcgggca	Yes	NM_001173531.2
SOX2	Fw: agctacagcatgatgcagga Rev: ggtcatggagttgtactgca	Yes	NM_003106.3
SOX7 (v2)	Fw: gatgctgggaaagtcgtggaagg Rev: tgcgcggccggtacttgtag	Yes	NM_031439.3
SOX17	TaqMan Assay Hs00751752_s1		
SOX17_Sybr	Fw: gatgctgggcaagtcgtg Rev: ctgcatgtgctgcacgc	Yes	NM_022454.3
T	Fw: tgcttccctgagacccagtt Rev: gatcacttctttcctttgcatcaag	Yes	NM_003181.2
TBP	Fw: caacagcctgccaccttacgctc Rev: aggctgtggggtcagtccagtg	Yes	NM_003194
TUBA1A	Fw: ggcagtgtttgtagacttggaaccc Rev: tgtgataagttgctcagggtggaag	Yes	NM_006009

Supporting Information Table S2: Human TaqMan® miRNA qRT-PCR assays.

miRNA	Assay
hsa-mir-10a-5p	Assay-No.: 000387
hsa-miR-143-3p	Assay-No.: 000466
hsa-miR-145-5p	Assay-No.: 002278
hsa-miR-196b-5p	Assay-No.: 002215
hsa-miR-199a-3p	Assay-No.: 002304
hsa-miR-214-3p	Assay-No.: 002306
hsa-miR-371-3p	Assay-No.: 002124
hsa-miR-372-3p	Assay-No.: 000560
hsa-miR-373-3p	Assay-No.: 000561
hsa-mir-425-5p	Assay-No.: 001516
hsa-miR-483-3p	Assay-No.: 002339
hsa-mir-489	Assay-No.: 002358
hsa-mir-1243	Assay-No.: 002854
hsa-mir-1263	Assay-No.: 002784
RNU6B	Assay-No.: 001093
RNU48	Assay-No.: 001006
U6 snRNA	Assay-No.: 001973

Supplemental Experimental Procedures

Human ES cell culture

Cultivation of the hESC lines HUES8 and HES3 was performed under feeder-free conditions with minor modifications as described earlier (Diekmann et al., 2015; Diekmann and Naujok, 2016; Naujok et al., 2014). Briefly, hESCs were cultivated as colonies on tissue culture treated 6-well plates coated with hESC-qualified Matrigel (Corning, Amsterdam, Netherlands) in mTeSR™1 (Stemcell Technologies, Cologne, Germany) or StemMACS™ iPS-Brew XF (Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer's instructions. Passaging was performed every 5-7 days using an enzyme-free passaging solution (Nie et al., 2014) and the cells were re-seeded as small clusters in a ratio of 1:8-40 onto 6-well plates freshly coated with Matrigel in the respective cultivation medium.

Differentiation experiments

All differentiation experiments were initiated from dispersed single cells as described earlier (Diekmann et al., 2015). Briefly, hESC colonies were dissociated with enzyme-free Gentle Cell Dissociation Reagent (StemCell Technologies) or trypsin/EDTA (Thermo Fisher Scientific, Braunschweig, Germany), collected with Knockout-DMEM/F12 (Thermo Fisher Scientific), centrifuged for 3 min at 300x g and the cell pellet was resuspended in hESC cultivation medium containing 10 µM Y-27632 (Selleck Chemicals, Munich, Germany) prior to counting with the Cellometer Auto T4 (Nexcelom Bioscience, Massachusetts, USA). Subsequently, 95,000-100,000 cells/cm^2 (HUES8) or 65,000-75,000 cells/cm^2 (HES3) were seeded on dishes coated with Matrigel. Germ layer induction was initiated the next day after re-seeding. For induction of mesodermal (ME) and endodermal (DE) differentiation Advanced RPMI 1640 (Thermo Fisher Scientific) supplemented with 1% penicillin/streptomycin, 1% Glutamax (Thermo Fisher Scientific) and 0.2% FBS (PAA Laboratories, Germany, Cölbe) was used as base medium. Differentiation towards DE was performed by supplementation of the base medium with 50 ng/ml activin A (Peprotech, Hamburg, Germany) and 5 µM CHIR-99021 (Cayman Chemicals, Tallinn, Estonia) for 24 h followed by a 72 h treatment in the same medium without CHIR-99021. Suboptimal DE differentiation was performed accordingly to this protocol with the difference that the CHIR-99021 concentration was reduced to 2.5 µM (instead of 5 µM) for the first 24 h of DE differentiation. Induction of ME differentiation was performed in base medium supplemented with 25 ng/ml BMP4 (Peprotech) plus 5 µM CHIR-99021 for 24 h followed by cultivation in the same medium without CHIR-99021 for 72 h. Randomized differentiation was performed

applying solely the base medium. During differentiation the respective medium was changed every day.

Flow cytometry and cell sorting

For flow cytometry and fluorescence activated cell sorting (FACS) the cells were washed twice with PBS, dissociated with trypsin/EDTA, cetrifugated (700x g for 3min), resuspended in PBS plus 2% FCS and counted. For routine flow cytometry 1-2×10^5 cells were washed once and stained with primary conjugated antibodies in 100 µl PBS plus 2% FCS for 20-45 min at 4°C in the fridge. Unstained cells were used to set appropriate gates. Subsequently the cells were washed once or twice with 500 µl PBS plus 2% FCS and measured using a CyFlow ML flow cytometer (Partec, Münster, Germany). The subsequent analysis was performed with the FlowJo software (Ashland, OR, USA). For cell sorting 5-20x10^6 cells were stained with the respective primary conjugated antibodies as described above and FACS was performed at the central facility of Hannover Medical School using a XDP (Beckman-Coulter) cell sorter.
The following conjugated antibodies were used: anti-human CXCR4-APC (130-098-357, Miltenyi Biotec), anti-human CXCR4-PE (FC15004, Neuromics, Edina, MN, USA), anti-human CD49e-FITC (328008, BioLegend, London, UK), anti-human CD56/NCAM-BV510 (318340, BD Bioscience, Heidelberg, Germany), anti-human CD140a/PDGFRA-PE, anti-human CD326/EpCAM-PE (135905, 324206, BioLegend), anti-human CD34-APC, anti-human CD309/VEGFR2/KDR-VioBright FITC and CD309/VEGFR2/KDR-APC (130-090-954, 130-105-261, 130-093-604, Miltenyi Biotec).

Gene expression analysis

Total RNA was isolated using the peqGOLD RNAPure kit (Peqlab, Erlangen, Germany) and total RNA plus small RNAs using the miRNeasy Kit (Qiagen, Hilden, Germany) following the manufacturer´s instructions. Synthesis of cDNA was performed with random hexamer primers (Thermo Fisher Scientific) from 1,000-2,000 ng total RNA using the RevertAid™ H Minus M-MuLV Reverse Transcriptase (Thermo Fisher Scientific). For each qPCR reaction 5-10 ng of the initial total RNA was used from the cDNA samples. Every sample was amplified as triplicate using specific primer pairs or TaqMan® assays (Table S1) with the GoTaq® master mix (Promega, Mannheim, Germany) or TaqMan® Gene Expression Master Mix (Fisher Thermo Scientific) according to the manufacturer's instructions. All reactions were performed on a ViiA7 Real-Time PCR system (Thermo Fisher Scientific) and a melting curve analysis was performed to verify the correct amplification for SybrGreen-based PCR reactions. Data normalization was performed with qBasePlus (Biogazelle, Zwijnaarde,

Belgium) against the geometric mean of the three housekeeping genes *G6PD*, *TBP* and *TUBA1A*. Their stable expression was verified using the geNorm algorithm (M-values < 0.5) in qBasePlus.

MiRNA expression analysis

Reverse transcription was carried out with the TaqMan® MicroRNA Reverse Transcription kit (Thermo Fisher Scientific) using samples isolated with the miRNeasy Kit (Qiagen). For single miRNA assays 25 ng of total RNA with the small RNAs was reverse transcribed with sequence-specific primers (Table S2), whereas 500 ng were reverse transcribed for the miRNA array cards (v3.0) with the respective Megaplex™ RT primer pools (Thermo Fisher Scientific). For miRNA array cards each analyzed population represents a pooled sample from four independent experiments using equal RNA amounts to obtain the pooled sample. All TaqMan® miRNA assays and array cards were measured on the ViiA 7 Real-Time PCR system (Thermo Fisher Scientific). Raw-data of miRNA array cards were normalized applying the modified algorithm of the global mean normalization strategy (D'Haene et al., 2012) in qBasePlus. Data normalization of single miRNA assays was performed with qBasePlus against the stably expressed controls RNU48, U6 snRNA and hsa-mir-425-5p (M-values < 0.5, geNorm algorithm).

MiRNA transfection experiments

For transfection of miRNA inhibitors or mimics (Thermo Fisher Scientific) Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific) was used as instructed by the manufacturer´s protocol. Briefly, 3 µl of Lipofectamine® 2000 was diluted in 100 µl of Opti-MEM medium (Thermo Fisher Scientific) and mixed with 30 pmol of the respective miRNA inhibitor or mimic. After incubation for 5 min at room temperature the miRNA-lipid complex was added dropwise to the culture medium of the cells. The medium was changed the next day (18-24 h). During DE differentiation the miRNA inhibitors or mimics were transfected on day 0 by changing to the first DE differentiation medium and subsequent transfection with the next 10-30 min. During ME differentiation transfection was performed on day 2 of differentiation. Therefore, one to three hours prior transfection differentiated cells were dissociated with trypsin/EDTA, counted and 7.5×10^4 cells/cm^2 seeded onto 12-well plates coated with Matrigel in mesoderm differentiation medium containing 10 µM Y-27632. This step was performed to reduce the high confluency on day 2 and thereby reaching acceptable transfection efficiencies. For each experiment the transfection efficiency was examined by transfecting a FAM-conjugated negative control that was analyzed one day post transfection

by flow cytometry. For further analysis only experiments with a chosen cut-off of 60% positive cells 24 hrs post transfection were used.

SiRNA transfection experiments

Transfection of siRNAs (Thermo Fisher Scientific) was performed with Lipofectamine® 2000 transfection reagent as instructed by the manufacturer. The siRNA transfection procedure was similar performed as described above for the miRNA mimic/inhibitor transfections. Briefly, transfection at day 0 of DE differentiation were performed with 5 µl Lipofectamine® 2000 and 5 µl siRNA (10 pmol) per 12-well diluted in 100 µl Opti-MEM medium. Subsequently, the complexed siRNA was added dropwise to the cells, which already had received the media for DE differentiation. For the transfection during ME differentiation the cells were re-seeded at day 2 as described above and transfected with 3 µl Lipofectamine® 2000 and 3 µl siRNA (10 pmol) per 12-well. A FAM-conjugated negative control was used to determine the transfection efficiency 24 h post transfection by flow cytometry. Also a second unlabeled negative control was used as an additional control to exclude an effect of the FAM-labeling of the siRNA negative control.

Luciferase Reporter Assay

Partial 3'UTRs of genes that harbor at least one potential binding site for the respective miRNA were individually cloned into the SpeI/HindIII cloning site of the pMIR-REPORT vector (Thermo Fisher Scientific) behind the sequence that encodes the luciferase gene. HEK293 cells seeded in 48-well-plates were co-transfected with 20 ng of the particular luciferase reporter construct, 20 ng β-galactosidase control plasmid (Promega, Mannheim, Germany) and 30 nM of either control miRNA or the respective miRNA mimic using Lipofectamine® 2000. The transfected cells were incubated 24 h prior to detection of the luciferase and the β-galactosidase activity by applying the different substrates (Promega). Data normalization was performed with the measured β-galactosidase activity of the respective sample.

Western Blot analysis

The cells were dissociated with trypsin/EDTA, centrifuged for 3 min at 700x g and resuspended in RIPA buffer (Thermo Fisher Scientific). These whole cell extracts were additionally sonified with 6 pulses of 60% amplitude and 0.8 cycle using a Labsonic M sonificator (Sartorius, Göttingen, Germany). Subsequently, complete protease inhibitor mixture (Roche Diagnostics, Manheim, Germany) was added to the extracts. The protein content was determined by BCA assay (Thermo Fisher Scientific) and 10-20 µg of total

protein was separated by a 12.5% SDS-PAGE followed by transferring the proteins to a PVDF (polyvinylidene fluoride) membrane via electro-blotting. Nonspecific binding was blocked for 1 h at room temperature with 2% or 5% nonfat dry milk in PBS plus 0.1% Tween 20. Thereafter, the membranes were incubated with primary antibodies overnight at 4 °C. After three washing steps (PBS, 0.1% Tween 20) they were incubated with peroxidase-labeled secondary antibodies (711-035-152, diluted 1:40,000 and 115-035-166, diluted 1:10,000, Dianova, Hamburg, Germany) at room temperature for 1 h. The bands were visualized by chemiluminescence using the ECL select or the ECL detection kit (GE Healthcare Europe, Freiburg, Germany) with an automated chemiluminescence imager (INTAS Science imaging, Göttingen, Germany). Densitometic analysis was performed with the Image Studio Lite software (LI-COR, Bad Homburg, Germany).

The following specific antibodies were used: anti-KLF4 (ab72543, diluted 1:1000, Abcam, Cambridge, UK), anti-PGAM1 (#12098, diluted 1:1000, Cell Signaling Technology, Leiden, Netherlands) and anti-ß-ACTIN (sc-47778, diluted 1:1000, Santa Cruz Biotechnology, Dallas, USA).

Statistics

Unless stated otherwise all data values represent means ± SEM. In each figure legend the respective number of independent experiments is stated (n). Statistical analyses were performed using the GraphPad Prism software (Graphpad, San Diego, CA, USA) using *Student's t-test* or ANOVA followed by *Bonferroni's* or *Dunnett's post hoc* test for multiple comparisons.

Supplemental References

D'Haene, B., Mestdagh, P., Hellemans, J. & Vandesompele, J. 2012. miRNA expression profiling: from reference genes to global mean normalization. *Methods Mol Biol,* 822**,** 261-72.

Diekmann, U., Lenzen, S. & Naujok, O. 2015. A reliable and efficient protocol for human pluripotent stem cell differentiation into the definitive endoderm based on dispersed single cells. *Stem Cells Dev,* 24**,** 190-204.

Diekmann, U. & Naujok, O. 2016. Generation and Purification of Definitive Endoderm Cells Generated from Pluripotent Stem Cells. *Methods Mol Biol,* 1341**,** 157-72.

Naujok, O., Diekmann, U. & Lenzen, S. 2014. The generation of definitive endoderm from human embryonic stem cells is initially independent from activin A but requires canonical Wnt-signaling. *Stem Cell Rev,* 10**,** 480-93.

Nie, Y., Walsh, P., Clarke, D. L., Rowley, J. A. & Fellner, T. 2014. Scalable passaging of adherent human pluripotent stem cells. *PLoS One,* 9**,** e88012.