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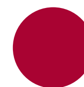

Generation of two iPSC lines with either a heterozygous V717I or a heterozygous KM670/671NL mutation in the APP gene

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Lab Resource: Multiple Cell Lines

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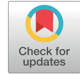

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A B S T R A C T

Alzheimer's disease (AD) is the most common form of dementia, affecting millions of people worldwide. Mutations in the genes *PSEN1*, *PSEN2* or *APP* are known to cause familial forms of AD with an early age of onset. In this study, specific pathogenic mutations in the *APP* gene were introduced into an iPSC line from a healthy individual by the use of CRISPR-Cas9. The study resulted in the generation of two new cell lines, one carrying the V717I *APP* mutation and one with the KM670/671NL *APP* mutation.

Resource table

Unique stem cell lines identifier	1. *BIONi010-C-37* 2. *BIONi010-C-38*
Alternative names of stem cell lines	1. *BIONi010-C London (V717I)* 2. *BIONi010-C Swedish (KM670/671NL)*
Institution	*Bioneer A/S Hørsholm Denmark and University of Copenhagen (UCPH) Copenhagen Denmark*
Contact information of distributor	*Contact at Bioneer: Benjamin Schmid, bsc@bioneer.dk–* *Contact at UCPH: Kristine Freude, kkf@sund.ku.dk*
Type of cell lines	*iPSCs*
Origin	*Human*
Cell Source	*Fibroblasts*
Clonality	*Clonal*
Method of reprogramming	*Episomal plasmids (Okita et al., 2011)*
Multiline rationale	*Mutated clones*
Gene modification	*YES*
Type of modification	*Induced point mutation*
Associated disease	*Alzheimer's disease (AD)*
Gene/locus	*1. APP/ Chr21:27264096 2. APP/ Chr21:27269939 and Chr21:27269938*
Method of modification	*CRISPR-Cas9*
Name of transgene or resistance	*N/A*
Inducible/constitutive system	*N/A*
Date archived/stock date	*February 2018*
Cell line repository/bank	*1. (https://hpscreg.eu/cell-line/BIONi010-C-37)* *2. (https://hpscreg.eu/cell-line/BIONi010-C-38)*
Ethical approval	*The study was approved by the Ethics Committee of the Capital Region of Denmark (H-4-2011-157), and written informed consent was obtained from the participant before enrolment*

Resource utility

The exact mechanisms leading to Alzheimer's disease (AD) remain unknown. In order to increase our understanding of disease development and underlying cellular pathological mechanisms, we have established two novel iPSC lines containing either a heterozygous V717I mutation or a heterozygous KM670/671NL mutation in the Amyloid precursor protein (APP) gene. These mutations are known to result in familial forms of AD.

Resource details

Mutations in one of the genes *PSEN1, PSEN2* and *APP* are known to result in familial forms of AD. The two mutations V717I (London) and KM670/671NL (Swedish) in the *APP* gene are both used in various mouse models, such as the common 5xFAD (Oakley et al., 2006). Due to their strong phenotype, these specific mutations are highly relevant for establishment of iPSC based disease models that carry a single pathogenic mutation. The iPSC model is supported by other models and patient phenotype information, which will facilitate validation of the *in vitro* model and provide further knowledge about the mutations and AD.

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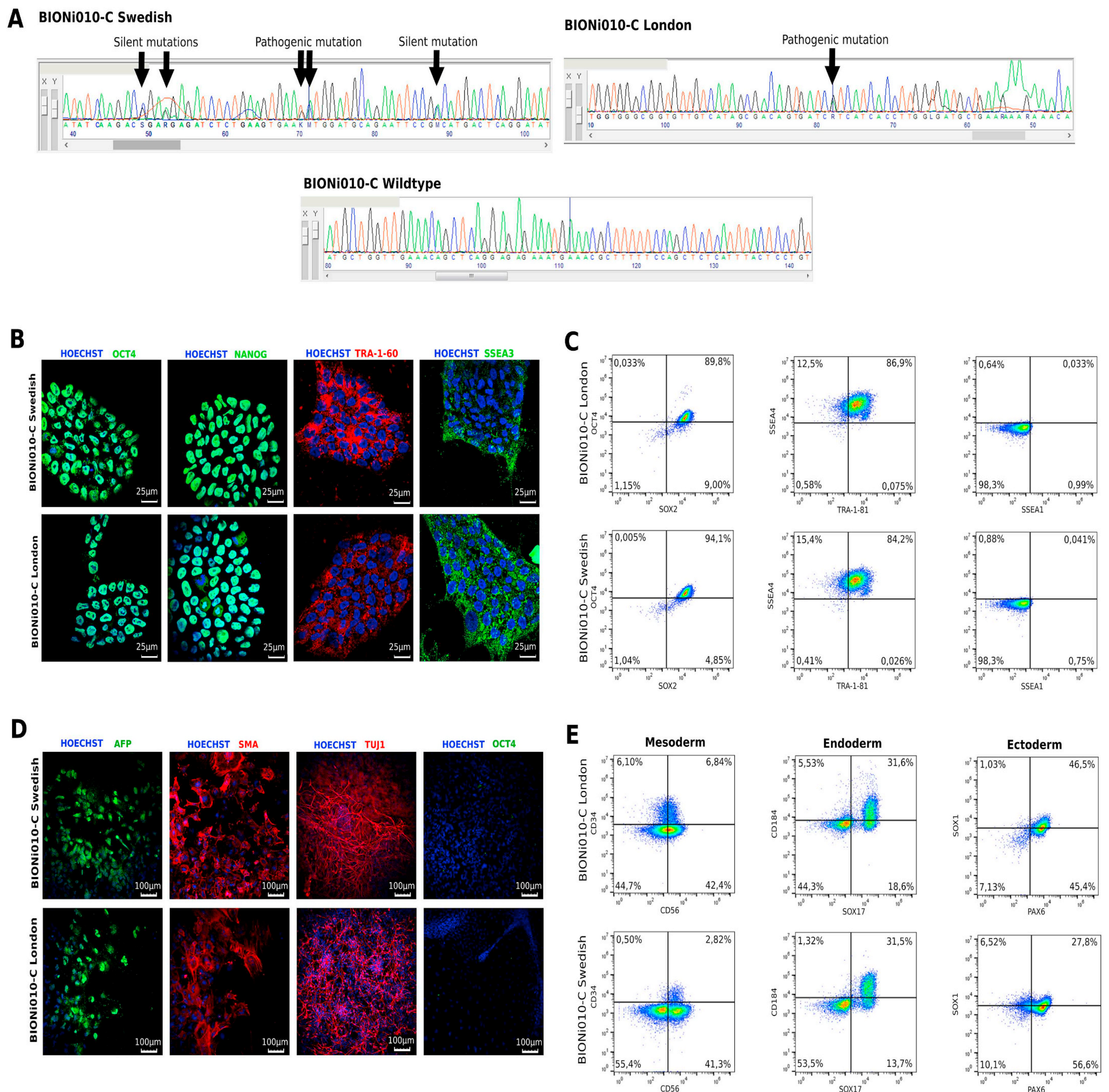

Fig. 1. Characterization of two iPSC lines with either a heterozygous V717I or a heterozygous KM670/671NL mutation in the APP gene.
(A) DNA sequencing for the heterozygous V717I (London), heterozygous KM670/671NL (Swedish) APP mutation and parental non gene edited iPSC line.
(B) ICC for pluripotency markers: OCT4 (green) nuclear labeling, NANOG (green) nuclear labeling, TRA1-60 (red) cytoplasmatic labeling and SSEA3 (green) cytoplasmatic labeling. Scale bar represents 25 μm.
(C) FACS analysis of pluripotency markers: OCT4 + SOX2 and TRA1-81 + SSEA4. SSEA1 served as a negative control for pluripotency. Upper right quadrant in the FACS plots represents double positive cells, thereby determined as pluripotent.
(D) ICC for differentiation markers representing the three different germ layers: AFP (green) for endoderm, SMA (red) for mesoderm and TUJ1 (red) for ectoderm. OCT4 labeling served as negative control for successful spontaneous differentiation.
(E) FACS analysis of differentiation markers: CD56 + CD34 (mesoderm), SOX17 + CD184 (endoderm) and PAX6 + SOX1 (ectoderm). Upper right quadrant in the FACS plots represents double positive cells, thereby determined as differentiated into the respective lineage.

For this study, the V717I mutation or the KM670/671NL mutation was inserted into the human iPSC line BIONi010-C, which had earlier been established from a skin biopsy obtained from a healthy male individual aged 18 (Rasmussen et al., 2014).

To generate a cell line containing the V717I mutation, the point mutation resulting in an amino acid change from valine (V) to isoleucine (I) was knocked into the *APP* gene by using the CRISPR-Cas9 system (Jinek et al., 2012). The CRISPR system was also used to insert the KM670/671NL double mutation resulting in an amino acid change from lysine (K) and methionine (M) to asparagine (N) and leucine (L)

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
BIONi010-C-37	*BIONi010-C London*	*Male*	*18*	*African*	*GA*	*AD*
BIONi010-C-38	*BIONi010-C Swedish*	*Male*	*18*	*African*	*GT/AC*	*AD*

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	*Normal*	*Supplementary Fig. 1 panel B*
Phenotype	Qualitative analysis by immunocytochemistry	Both cell lines are positive for OCT4, NANOG, SSEA3 and TRA-1-60	*Fig. 1 panel B*
	Quantitative analysis by flow cytometry	BIONi010-C APP London positive for: 89,8% double positive for OCT4 and *SOX2.* *86,9% are double positive for TRA-1-81 and SSEA4.* *1.02% are positive for SSEA1(control).* BIONi010-C APP Swedish positive for: *94.1% double positive for OCT4 and SOX2.* *84.2% double positive for SSEA4 and TRA1–81.* *0.79% SSEA1(control).*	*Fig. 1 panel C*
Genotype	Karyotype (G-banding), *Resolution 300–400*	*Both 46XY*	*Supplementary Fig. 1 panel A*
Identity	STR analysis	*DNA profiling performed* *16 sites tested, all match*	*Available with author*
Mutation analysis	Sequencing	*Successful integration of heterozygous mutation G > A causing V717I and heterozygous integration of the double mutation GA > TC causing KM670/671NL.*	*Fig. 1 panel A*
Microbiology and virology	Mycoplasma	*Mycoplasma testing by PCR Mycoplasma Test Kit*	*Supplementary Fig. 1C*
Differentiation potential	Directed differentiation	*Specific differentiation markers for all 3 germ layers present in both lines after differentiation*	*Fig. 1 panel E (control with iPSCs Supplementary Fig. 1 panel D)*
	Spontaneous differentiation	*Positive for: AFP, SMA and TUJ1. Negative for OCT4*	*Fig. 1 panel D*

into the *APP* gene.

For both mutations, the nucleotide substitution was confirmed by restriction digest (Supplementary Fig. 1E), followed by DNA sequencing. Sequencing analysis confirmed a successful G > A transition causing the V717I mutation on protein level, and a successful G > T and A > C transition causing the KM670/671NL mutation on protein level (Fig. 1A). The two lines are further referred to as BIONi010-C-London and BIONi010-C-Swedish, respectively.

Pluripotency of the gene-edited lines was confirmed by immunocytochemistry (ICC) (Fig. 1B) and quantified by flow analysis (Fig. 1C) showing expression of the pluripotency markers OCT4, NANOG, SSEA3, SSEA4, TRA-1-81, TRA-1-60 and SOX2. The differentiation potential of the iPSCs was tested *via* directed- and spontaneous differentiation into *endo*-, meso- and ectodermal cell types. The test showed that the cells were capable of differentiation into all three germlayers (Fig. 1D and E) and that iPSCs do not express any differentiation markers (Supplementary Fig. 1D). A karyotype analysis was carried out to confirm chromosomal integrity (Supplementary Fig. 1A), and a STR analysis was performed to confirm cell line identity (Available with author). The morphology of the cells was investigated by light microscopy and showed a normal morphology throughout the process of gene editing (Supplementary Fig. 1B). Finally, cells were tested negative for mycoplasma (Supplementary fig. 1C).

Materials and methods

Gene editing

Cells were grown on Matrigel coated plates in E8 media and incubated at 37 °C and 5% CO_2. Cells were passaged every 4 days using Accutase in a ratio of 1:3. When reaching 80% confluency, cells were detached by using Accutase for 5 min after which cells were nucleofected. Nucleofection was made by using the Lonza nucleofector, P3 solution and the CA167 pulse setting Table 1.

Gene editing was performed using the CRISPR-Cas9 system in combination with a specifically designed ssODN (single stranded oligo deoxynucleotide) as homologous template. The template contained either the London mutation or the Swedish mutation in combination with silent mutations (changing the DNA sequence but not the protein sequence) to avoid repeated cutting by the CRISPR-Cas9. The pathogenic mutation is also a recognition site for a specific restriction enzyme. For London, Bcl1 recognize the mutation and for Swedish, Tfi1 recognize the mutation. The 20 nt crRNA was designed using the software http://crispr.mit.edu/. crRNA, tracrRNA, ssODN, and Cas9 protein were all ordered from Integrated DNA Technologies (IDT) and the CRISPR system assembled *ex vivo*.

Genotyping and restriction enzyme

To screen for positive clones, DNA was extracted using the prepGEM kit (ZyGEM) followed by amplification of the gene by using PCR with the AmpliTaq GOLD DNA polymerase (Thermo Fisher) according to the manufacturer's instructions. Annealing temperature: 60^0C, elongation time 30 s, 40 cycles in a T100 thermocycler (BioRad). The primers SURV *APP London* FW/RV and SURV *APP Swedish* (IDT) used for PCR, were designed to cover the mutation at locus Chr21:27269939 + 27,269,938 or the mutation at locus Chr21:27264096. These give rise to a 512 bp product size for Swedish and 694 bp for London. (Table 3). The screening was carried out by making a restriction digest on the PCR product, using Tfi1 for 1 h at 65^0C (Swedish) or Bcl1 for 1 h at 50^0C (London) (New England Biolabs).

Sequencing

The PCR product from positive clones were sequenced by Sanger

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Marker	*Goat anti-OCT4*	*1:200*	Santa Cruz Cat: sc-8628 RRID:AB_653551
Pluripotency Marker	*Rabbit anti-NANOG*	*1:50*	Peprotech Cat: 500-P236 RRID:AB_1268805
Pluripotency Marker	*Rat anti-SSEA3*	*1:100*	Biolegend Cat: 330302 RRID: AB_1236554
Pluripotency Marker	*Mouse anti-SSEA4*	*1:100*	Biolegend Cat: 330402 RRID: AB_1089208
Pluripotency Marker	*Mouse anti-TRA-1-60*	*1.200*	Biolegend Cat: 330602 RRID: AB_1186144
Pluripotency Marker	*Mouse anti-TRA-1-81*	*1:200*	Biolegend Cat: 330702 RRID: AB_1089240
Pluripotency Marker	*OCT4 PE*	*1:25*	BD Pharmigen Cat: 560186 RRID:AB_1645331
Pluripotency Marker	*SOX2 AF647*	*1:50*	BD Pharmigen Cat: 560294 RRID:AB_1645324
Pluripotency Marker	*TRA-1-81 AF647*	*1:25*	BD Pharmigen Cat: 560124 RRID:AB_1645449
Pluripotency Marker	*SSEA4 PE*	*1:25*	BD Pharmigen Cat: 560128 RRID:AB_1645533
Differentiation Markers	*SSEA1 PE*	*1:25*	BD Pharmigen Cat: 560142 RRID:AB_1645246
Differentiation Markers	*Rabbit anti-Alpha-1-Fetoprotein*	*1:200*	*DAKO Cat: A0008 RRID:* AB_2650473
Differentiation Markers	*Chicken anti-TUJ1*	*1:500*	*Raybiotech Cat: 119–15,313* RRID:AB_2753341
Differentiation Markers	*Mouse anti-Smooth Muscle Actin*	*1:200*	*DAKO Cat: M0851* RRID:AB_2313736
Differentiation Markers	*PAX6 Cy5.5*	*1:50*	*BD Pharmigen Cat:* 562388 RRID:AB_2753343
Differentiation Markers	*SOX1 PE*	*1:50*	BD *Pharmigen* Cat: *561592 RRID:*AB_10714631
Differentiation Markers	*CD34 PE*	*1:25*	*BD Pharmigen Cat: 555822* RRID:AB_396151
Differentiation Markers	*CD56 APC*	*1:25*	*BD Pharmigen Cat: 555518* RRID:AB_398601
Differentiation Markers	*CD184 PE*	*1:25*	*BD Pharmigen Cat: 555974* RRID:AB_396267
Differentiation Markers	*SOX17 AF647*	*1:50*	*BD Pharmigen Cat: 562594* RRID:AB_2737670
Secondary antibodies	Donkey Anti-Mouse IgG Alexa fluor 488	1:200	Life technologies Cat#A21202 RRID: AB_141607
Secondary antibodies	Donkey Anti-Mouse IgG Alexa fluor 647	1:200	Invitrogen Cat# A31571 RRID: AB_162542
Secondary antibodies	Donkey Anti-Rat IgG Alexa fluor 594	1:200	Invitrogen Cat# A21209 RRID: AB_2535795
Secondary antibodies	Donkey Anti-Goat IgG Alexa fluor 488	1:200	Invitrogen Cat# A11055 RRID: AB_2534102
Secondary antibodies	Donkey Anti-Rabbit IgG Alexa fluor 488	1:200	Life technologies Cat# A21206 RRID: AB_2535792

sgRNA, ssODN and primers

	Target	Forward/Reverse primer (5′-3′)
Cr-RNA V717I	*APP*	GACAGTGATCGTCATCACCT
Cr-RNA KM670/671NL	*APP*	ACATGACTCAGGATATGAAG
ssODN V717I	*APP*	GTGCAATCATTGGACTCATGGTGGGCGGTGTTGTCATAGCGACAGTGATCATCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATTCATCAT
ssODN KM670/671NL	*APP*	CAGGTTCTGGGTTGACAAATATCAAGACCGAAGAGATCTCTGAAGTGAATCTGGATGCAGAATTCCGCCATGACTCAGGATATGAAGTTCATCATCAAAA
SURV *APP London* FW/RV (*Mutation analysis (PCR) V717I*)	*APP*	TGATCGTGCCTTTCCAGCTT/ AGAACAACTGTAACCCAAGCA
SURV *APP Swedish* FW/RV (*Mutation analysis (PCR) KM670/671NL*)	*APP*	GGCCTAGAAAGAAGTTTTGGGTAGG/CCACCACACCCAGCTAATCTTTTTT
SURV *APP London* seq (*Sequencing V717I*)	*APP*	Rv: AATTCCCACTTGGAAACATGC
SURV *APP Swedish seq* (*Sequencing KM670/671NL*)	*APP*	CACCCATTTACAAGTTTAGC

sequencing using the primers SURV *APP London seq* and SURV *APP Swedish* seq (Table 3).

Karyotyping and quality control

Quality control was performed after the lines had been banked and the quality controls are listed in Table 2. The general morphology of the cells was investigated daily by light microscopy. Karyotyping was made at passage 41 for cells carrying the *APP* London mutation (20 passages after nucleofection), and at passage 39 for cells carrying the *APP* Swedish mutation (18 passages after nucleofection). When cells were 70–85% confluent, karyotyping was initiated by treating cells with Colcemid (Gibco) for 1.5 h after which cells were detached with Accutase for 5 min. Next, cells were incubated with 0.075 M KCl for 30 min at 37^{0}C, and afterwards fixed by using a mixture consisting of 25% acidic acid and 75% methanol. Fixed cells were cooled overnight at -20 °C and then shipped for GTG-band karyotyping at Anand Diagnostic Laboratory, Bangalore where 20 metaphase spreads were counted. STR analysis was performed by extracting DNA with the DNeasy Blood and Tissue Kit (Qiagen) and subsequently analysing the DNA using the AmpFLSTR Identifier PCR Amplification kit (Applied Biosystem) according to manufacturer's instructions. Microbiological contamination was tested by growing 500 μl of the supernatant in LB medium for 2 days at 37^{0}C. Cells were tested for mycoplasma using the PCR mycoplasma test kit (PromoKine) according to manufacturer's instructions.

Immunocytochemistry

Expression of pluripotency markers, were investigated using a Leica DMRB-fluorescence microscope (Leica Microsystems Wetzlar, Germany). Cells were fixed in 4% PFA for 20 min at RT and labeled according to standard ICC procedures by permeabilising fixed cells with 0,2% Triton X-100 in PBS for 20 min at RT followed by 30 min of blocking with 3% BSA. All antibodies were diluted in 3% BSA in accordance with Table 3. The primary antibodies (OCT4, NANOG, SSEA4, SSEA3, Tra-1-60 and Tra-1-81) were incubated at 4 °C overnight and were visualised with the secondary antibodies Alexa488 or Alexa647 (Life Technologies) diluted 1:200 in BSA. Secondary antibodies were incubated for one hour at RT and in darkness. In all samples, Hoechst bisbenzimide 33,258 was used to stain the nuclei.

Trilineage differentiation

IPSCs were cultured for differentiation into mesoderm, ectoderm or endoderm following two different approaches. One method is by making directed differentiation for 5 days. For mesoderm differentiation, cells were cultured for 3 days in mesodermal induction medium (MIM) consisting of StemDiff™ APEL™ medium (STEMCELL Technologies) with 0,1% Pen/Strep (Lonza), 25 ng/ml Activin A (Cell Guidance Systems), 30 ng/ml BMP4 (Peprotech), 50 ng/ml VEGF (Peprotech) and 1,5 μM CHIR99021 (Selleckchem). For the last two days cells were cultured in vascular specification medium (VSM) consisting of StemDiff™ APEL™ medium with 0.1% Pen/strep, 50 ng/ml VEGF and 10 μM SB431542 (Selleckchem). For endoderm differentiation, cells were cultured for 5 days in MCDB131–1 medium (Sigma) containing glucose (Sigma), GlutaMAX (Gibco), Pen/Strep, NaHCO3 (Gibco), BSA (Sigma) and Activin A. For the first 24 h 3 μM CHIR99021 was added to the media. For ectoderm differentiation, cells were cultured in neural induction medium (NIM) consisting of 1:1 DMEM/F12 (Gibco) and Neurobasal medium (Gibco). The NIM contains 1% N2 (Gibco), B27 without Retinoic acid (Gibco), GlutaMAX (Gibco), Pen/Strep (Lonza) and the inhibitors SB431542 (Selleckchem) and LDN193189 (Selleckchem). After five days, expression of the markers, PAX6/SOX1 (ectoderm), CD34/CD54 (mesoderm) and CD184/SOX17 (endoderm) were analysed by Flow cytometry.

The other method, involves growing the iPSC as embryoid bodies. Embryoid bodies were formed by treating 80% confluent iPSCs with Accutase for 1 min and detaching them using a cell scraper. Afterwards cells were transferred to low attachment plates where they were left to grow in E8 media for 7 days with media change every second day. After 7 days, they were plated on Matrigel coated plates, and left to spontaneously differentiate for 14 days in fibroblast media (DMEM +10% FBS + 0.1% Pen/Strep). Afterwards, ICC was performed for smooth muscle-actin (mesoderm), alpha 1-fetoprotein (endoderm) and beta-3-tubulin (ectoderm) and evaluated using a Leica DMRB-fluorescence microscope.

Flow cytometry

Cells for flow cytometry were detached with Accutase for 5–10 min and 0.2×10^5 cells were fixed in a 0.5 ml solution consisting of 1 part Foxp3 fixation solution and 3 parts permeabilisation solution (R&D systems) and incubated at RT for 30 min. Cells were washed in 1 × permeabilisation buffer (R&D systems) after which antibodies diluted in 100 μl permeabilisation buffer, were added for 45 min at RT.

Cells were washed additionally three times and afterwards run at a calibrated BD Accuri C6 Flow Cytometer. 50.000 cells were analysed with the BD Accuri C6 Flow Cytometer system at high speed in 150 μl buffer. The data was analysed by using the FlowJo_V10 software.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.101368.

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