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Lab resource: Stem Cell Line

Generation of a human iPSC line harboring a biallelic large deletion at the *INK4* locus (HMGUi001-A-5)

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A B S T R A C T

The INK4 locus is considered as a hot-spot region for the complex genetic disorders, including cancer, type 2 diabetes (T2D) and coronary artery disease (CAD). By CRISPR/Cas9 gene editing, we generated a human induced pluripotent stem cell (hiPSC) line (HMGUi001-A-5) deleting an 8 kb genomic DNA encompassing six T2D-associated SNPs at the INK4 locus. The resulting hiPSC line revealed a normal karyotype, preserved pluripotency and was able to differentiate towards germ layers, endoderm, mesoderm and ectoderm. Thus, the HMGUi001-A-5 line could provide a valuable cellular model to explore the molecular mechanisms linking these SNPs to T2D and other genetic disorders.

1. Resource Table

Unique stem cell line identifier	HMGUi001-A-5
Alternative name(s) of stem cell line	*ΔINK4 T2D risk region* hiPSC
Institution	Institute of Diabetes and Regeneration Research, Helmholtz Zentrum München, 85,764 Neuherberg, Germany
Contact information of distributor	Heiko Lickert, heiko.lickert@helmholtz-muenchen.de
Type of cell line	iPSCs
Origin	Human, HMGUi001-A hiPSC line described by Wang et al. (2018)
Additional origin info	Age: N/A Sex: Female Ethnicity: Caucasian
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Nucleofection
Genetic Modification	Yes
Type of Modification	Homozygous deletion of a large non-coding genomic DNA at *INK4* locus
Associated disease	N/A
Gene/locus	*INK4* locus, 9P21.3
Method of modification	CRISPR/Cas9 genome engineering
Name of transgene or resistance	Lacking 8 kb T2D risk region of *INK4* locus
Inducible/constitutive system	N/A
Date archived/stock date	30th June 2020
Cell line repository/bank	https://hpscreg.eu/cell-line/HMGUi001-A-5
Ethical approval	The choice of appropriate human donors, the procedures for skin biopsy, isolation of dermal fibroblasts, generation of iPSCs and their use in further scientific investigations were performed under the positive votes of the Ethics Committee of the Medical Faculty of the Eberhard Karls University, Tübingen (file numbers 629/2012BO2 and 130/2018BO2) and of the Medical Faculty of the Technical University Munich (file number 219/20 S). The study design followed the principles of the Declaration of Helsinki. All study participants gave informed consent prior to entry into the study.

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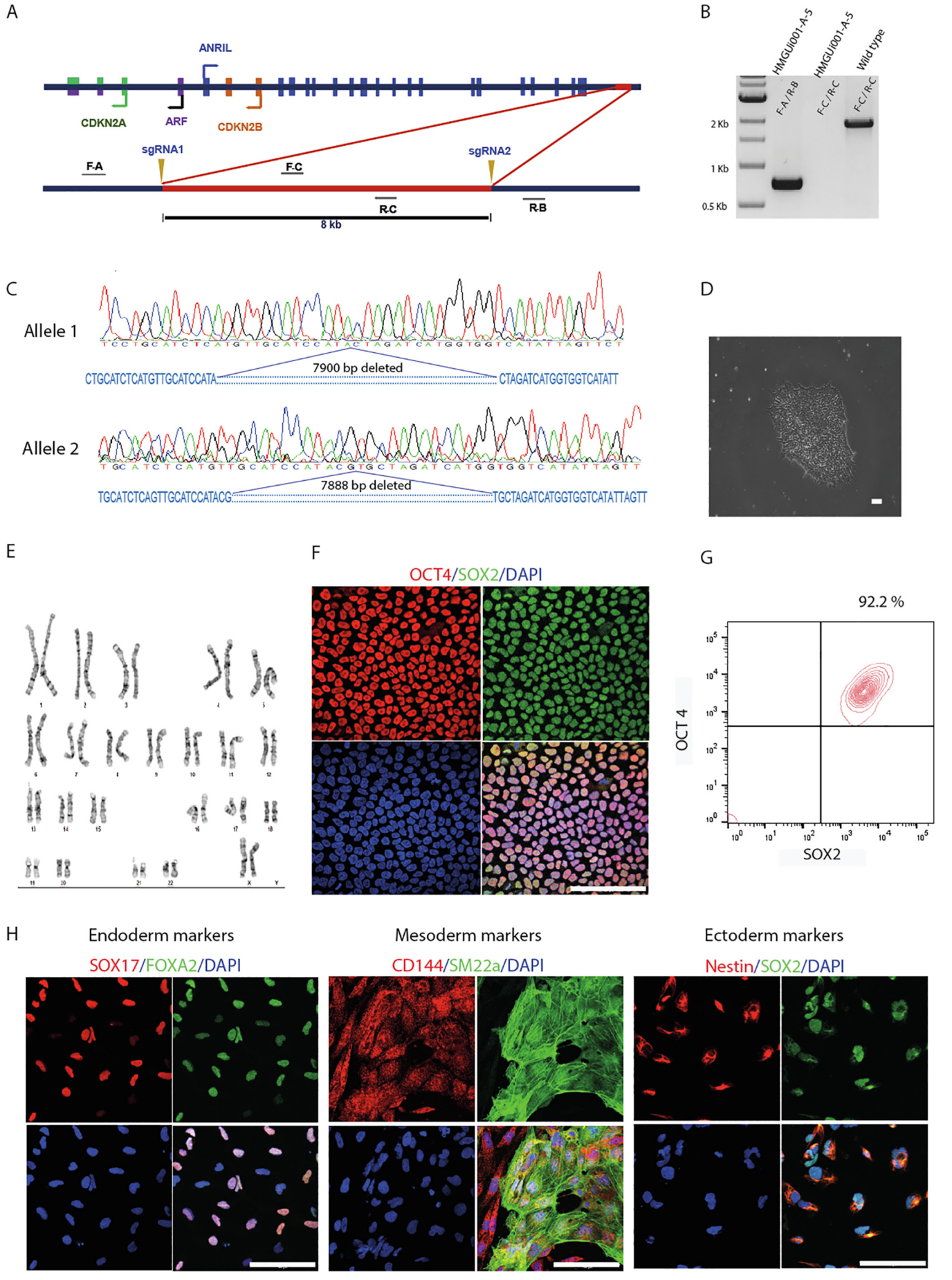

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Fig. 1. Generation and characterization of *ΔINK4 T2D risk region* hiPSC line. (A) Schematic representation of the human INK4 locus genes and the GWAS-T2D risk region of the locus. The candidate sgRNA pair for the genomic DNA deletion are shown as sgRNA1 and sgRNA2 at the border points of the target region. The primers used for sequencing and screening are indicated. F_A-R_B and F_C-R_C primer pairs were used to detect the deletion amplicons. (B) The 750 bp and 1910 bp PCR products are indicators for biallelic deletion and wild type respectively. (C) Sanger sequencing confirmed the joint of the two border points of the deletion DNA fragments. Deletion nucleotides are presented by dash marks. (D) Bright field image of the colony at pluripotency stage. Scale bar, 100 μm. (E) Chromosome karyotyping of the newly generated clone revealed normal karyotype (46, XX). (F) ICC staining shows SOX2 and OCT4 as pluripotency markers in the *ΔINK4 T2D risk region* hiPSC line at the maintenance stage. Scale bar, 100 μm. (G) Representative FACS plots of OCT4+ and SOX2+ cells in the generated cell line. (H) Multi-lineage potency assay of the cell line (endoderm, mesoderm and ectoderm lineages). Scale bars, 100 μm.

Table 1
Cell line characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal morphology	Fig. 1 panel D Scale bar: 100p~
Phenotype	Immunocytochenistry	Expression of pluripotency markers positive for OCT4 and SOX2	Fig. 1 panel F Scale bar: 100p~
	Flow cytometry	92.2% of *ΔINK4 T2D Risk Region* hiPSC line are OCT4 and SOX2 positive.	Fig. 1 panel G
Genotype	Karyotype (G-banding) and resolution	46, xx Resolution 450–525 bands	Fig. 1 panel E
Identity	Microsatellite PCR	Not performed	
	STR analysis	16 sites were tested, 16 site were identical (100% matched)	Submitted in journal archive
Mutation analysis	Sequencing	Homozygous deletion of *ΔINK4 T2D risk region* hiPSC line confirmed by Sanger sequencing/six sgRNA off-target sites analyzed by Sanger sequencing and revealed no mutation	Fig. 1 panel C, Supplementary Fig. S1 panel A
	Southern Blot or WGS	Not performed	
Microbiology and virology	Mycoplasma	Negative	Supplementary Fig. S1 panel B
Differentiation potential	Direct differentiation	*ΔINK4 T2D risk region* hiPSC line were differentiated into the three germ layers.	Fig. 1 panel H Scale bar: 100 μm

2. Resource utility

The genetically engineered HMGUi001-A-5 line (also named *ΔINK4 T2D risk region* hiPSC) offers a useful resource to investigate the potential regulatory role(s) of the T2D-linked risk region of the *INK4* locus in pancreatic or endocrine progenitors, insulin-producing β-cells or any other iPSC-derived cell type.

3. Resource details

According to genome-wide association studies (GWAS) many genomic loci have an association with T2D prevalence. Among these, the *INK4* locus, which is embedded within the human chromosomal region of 9P21.3 encodes for a family of cyclin-dependent kinase inhibitors (known as $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$) inhibiting the cyclin-dependent kinases, CDK4 and 6. Furthermore, a non-coding RNA named *ANRIL* is located within the locus and is transcribed in opposite direction compared to the other three genes (Fig. 1A) (Cunnington et al., 2010; Popov and Gil, 2010). Importantly, the *INK4* locus harbors six T2D-linked single-nucleotide polymorphisms (SNPs) termed as *rs2383208*, *rs10965250*, *rs10811661*, *rs10757283*, *rs1333051* and *rs7018475* located in an 8 kb genomic block (*INK4* T2D risk region) downstream of the *ANRIL* gene (Fig. 1A) (Pasmant et al., 2010). Yet, if these SNPs are causally associated with T2D and how these regulate the *INK4* locus is not clear. In order to provide a tool enabling functional analysis of the T2D-linked SNPs of the *INK4* locus, we aimed to generate a hiPSC line lacking both alleles of this *INK4*-T2D risk region (homozygous or biallelic deletion). To achieve this, we used our recently established hiPSC line (HMGUi001-A-1) (Wang et al., 2018) for gene targeting with the CRISPR/Cas9 genome editing system. As a low conserved non-coding DNA was targeted, the CRISPR target sites upstream (A region) and downstream (B region) of the *INK4*-T2D risk region were initially sequenced. Then, two sets of forward and reverse primers (FA, RA; FB, RB) were designed for amplification of the border regions of sgRNA sites (two double strand breakages). Next, single guide (sg) RNAs (sgRNA1 and sgRNA2) with high specificity score were designed and cloned into the CRISPR expression vector. By Gibson assembly cloning we generated a dual-sgRNA CRISPR/Cas9-GFP vector, which simultaneously expresses two sgRNAs targeting the flanking borders of the 8 kb intervening DNA segment, and could successfully generate a biallelic deletion in the hiPSC clone. Furthermore, an additional pair of screening primers (FC, RC) was designed to screen for the deletion (Fig. 1A). Two days post-transfection, the GFP-expressing cells were sorted by fluorescence-activated cell sorting (FACS). Following plating and expansion, genomic DNA was extracted from the GFP+ hiPSC single-cell clones and screened for the 8 kb deletion by conventional PCR with the FA-RB and FC-RC primer pairs (Fig. 1B). Wild type and biallelic clones for the large deletion were identified by a 1910 bp wild type-specific PCR product and a 750 bp deletion-specific PCR product, respectively (Fig. 1B). To confirm the precise deletion of the target region, the PCR products were subjected to Sanger sequencing. The result confirmed joining of the deletion borders at both alleles (Fig. 1C). Furthermore, the sequencing result of three intergenic and/or intragenic loci with the highest off-side targeting score for each sgRNA demonstrated no mutations at these sites (Supplementary Fig. 1A). Brightfield imaging of the newly generated iPSC clone showed normal hiPSC morphology and no phenotypical signs of spontaneous differentiation (Fig. 1D). The obtained *ΔINK4 T2D risk region* hiPSC clone revealed a normal karyotype (46, XX) (Fig. 1E). Short tandem repeat (STR) analysis showed derivation from the parental hiPSC line HMGU-001-A. Moreover, the iPSC clone was negative for mycoplasma (Supplementary Fig. 1B). Next, the pluripotency markers OCT4 and SOX2 were evaluated and confirmed by immunostaining and FACS analysis (Fig. 1F and G). Finally, the hiPSC line was differentiated into the three germ layers, endoderm, mesoderm, and ectoderm (Fig. 1H) (Table 1). These data demonstrate the successful generation of the *ΔINK4 T2D risk region* hiPSC line that exhibit multi-lineage potency. Taken together, the newly generated hiPSC line will allow investigating the potential causal link of these six SNPs of the *INK4* locus with diabetes prevalence during endocrine lineage formation and allows to study other genetic diseases.

Table 2
Reagents details.

Antibodies used for Immunocytochemistry and Flow cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers (Immunofluorescence)	Goat anti-OCT4	(1:500)	Santa Cruz Cat # sc-8628, RRID: AB_653551
	Rabbit anti-SOX2	(1:500)	Cell Signaling Cat # 3579S, RRID: AB_2195767
	Goat anti-SOX17	(1:500)	Neuromics Cat # GT15094/100, RRID: AB_2195648
	Rabbit anti-FOXA2	(1:500)	Cell Signaling Cat # 8186S, RRID: AB_10891055
	Mouse anti-Nestin	(1:200)	Abcam Cat # ab 22035, RRID:AB_446723
	Mouse anti-CD144	(1:200)	Abcam Cat # ab 7047, RRID:AB_2077943
	Rabbit anti-SM22a	(1:200)	Abcam Cat # ab 14106, RRID:AB_443021
Pluripotency Markers (Flow cytometry)	Goat anti-OCT4	(1:100)(1:100)	Santa Cruz Cat # sc-8628, RRID: AB_653551Cell Signaling Cat # 3579S, RRID: AB_2195767
	Rabbit anti-SOX2		
Secondary antibodies (Immunofluorescence)	Donkey anti-mouse IgG (H + L) Alexa Fluor 555	(1:500)	Invitrogen Cat # A31570, RRID: AB_2536180
	Donkey anti-rabbit IgG (H + L) Alexa Fluor 488	(1:500)	Invitrogen Cat # A21206, RRID: AB_141708
	Donkey anti-goat IgG (H + L) Alexa Fluor 555	(1:500)	Invitrogen Cat # A21432, RRID: AB_141788
Secondary antibodies (Flow-cytometry)	Donkey anti-goat IgG (H + L) Alexa Fluor	(1:500)	Invitrogen Cat # A21432, RRID: AB_141788
	555Donkey anti-rabbit IgG (H + L)	(1:500)	Invitrogen Cat # A21206, RRID: AB_141708
	Alexa Fluor 488		

Oligos and primers		
sgRNA Oligo	Forward/Revere primers (5́–3́)	RT-PCR Products
sgRNA1-sense: caccgggATATGGCTAAATAGTCCGTA	Forward-A (F_A): CCAAATTGCCTCAGCCAATG	FA/RA – 740 bp
sgRNA1-antisense: aaacTACGGACTATTTAGCCATATccc	Reverse-A (R_A): CAAATGGCCTTAGCCAGAGC	FB/RB – 771 bp
sgRNA2-sense: caccgggCCACCATGATCTAGCACTAA	Forward-B (F_B): AAGCCACTTAGCTAGAGTAAGG	FC/RC – 1910 bp
sgRNA2-antisense: aaacTTAGTGCTAGATCATGGTGGccc	Reverse-B (R_B): CACCAGTCGTGTTGGATAAATG	(Wild type)
	Forward-C (F_C): GGAGCCATTCTATCGTGAACAG	FA/RB – 750 bp
	Reverse-C (R_C): AAGCATAGGTGGGTTTCACTTC	(Generated line)

4. Materials and methods

4.1. CRISPR/Cas9; design and dual-sgRNA expression plasmid construction

sgRNAs for targeted large genomic DNA deletion by CRISPR/Cas9 were designed using the web tool CRISPETa (crispeta.crg.eu) (the sequences of the oligos are listed in Table 2). Due to genome variation, sgRNAs DNA binding sites were initially sequenced. For efficient expression, the sequence of *CACCGGG* was added to the 5′ site of sense oligo, and the sequences of *AAAC* and *CCC* were added to 5′ and 3′ sites of anti-sense oligo, respectively. PU6-(BbsI) sgRNA_CAG-Cas9-GFP-bpA plasmid, Addgene ID86985, containing BbsI site was used for cloning single gRNAs. Gibson assembly technique, according to Yumlu et al. 2019, was used, to produce a dual sgRNA expression cassette in the plasmid. Following the cloning, the single and dual gRNAs expression plasmids were subjected to Sanger sequencing.

4.2. HiPSCs culture and CRISPR/Cas9 plasmid transfection

The HMGUi001-A-1 hiPSCs were maintained on Geltrex (Life Technologies)-coated plates in human StemMACS iPS-Brew XF medium (Miltenyi Biotec). Culture medium was renewed daily. 5 mM EDTA in PBS (AppliChem) was used to dissociate and passage the confluent hiPSCs under the condition of 37 °C for 3 min. To reduce cellular stress, 10 μM ROCK inhibitor (Y-27632, Santa Cruz Biotechnology) was added to the culture medium for the first 24 h after dissociation. The cells were incubated at 37 °C, 21% O_2 and 5% CO_2. 2×10^5 cells were seeded in 6-well plates one day before plasmid transfection. For each well, 2.5 μg dual-sgRNA vector was transfected into the hiPSCs using 5 μl Lipofectamine™ Stem Transfection Reagent (Fisher Scientific) and 200 μl OptiMEM medium (Fisher Scientific) according to manufacturer's instruction.

4.3. FACS enrichment of transfected hiPSCs

48 h after the transfection, cells were dissociated and collected for the FACS experiment. For sorting, the cell suspension was filtered and re-suspended in FACS buffer consisting of 2 mM EDTA and 1% BSA (Sigma) in PBS. Cells with high levels of GFP signal were sorted using FACSAria III (BD Bioscience). Two thousand cells were seeded in 10 cm dishes. One week later, single cell derived clones were picked, expanded and screened by PCR. Flow cytometry was also applied to quantify the pluripotency markers (OCT4 and SOX2) expression of the clone. Detailed information about primary and secondary antibodies is listed in Table 2. The data were analyzed using FlowJo software.

4.4. Screening of the clones for CRISPR/Cas9-mediated genomic deletion

The appropriate PCR primers for sequencing and clones screening were designed using Clone Manager Molecule software (Table 2). Routine Taq DNA Polymerase enzyme and LongAmp Taq DNA Polymerase enzyme (NEB) were used for PCR reactions for the short and long fragments, respectively. PCR product was directly cloned into a TA vector (NEB) and, then sequencing was performed to confirm the authenticity of the deletion at both alleles.

4.5. Immunofluorescence and imaging

For immunocytochemistry (ICC) staining, the cell were fixed with 2% paraformaldehyde for 20 min at room temperature, followed by three washes in PBS. Then, the cells were rehydrated and permeabilized with 0.1 M Glycine and 0.2% Triton for 30 min, followed by blocking with the blocking solution (3% serum donkey, 0.1% BSA and tween). Primary antibodies were added and incubated overnight at 4 °C in blocking solution. The following day, the cells were washed several times with 0.1% Tween20 in PBS (PBS-T). Then, the secondary antibodies were added and incubated for 4 h at room temperature. Nuclei were stained with DAPI. Finally, images were taken using a Leica SP5 microscope. Detailed data related primary and secondary antibodies used is listed in Table 2.

4.6. Characterization of the hiPSCs for karyotyping and STR analysis

Karyotyping was carried out in the logarithmic phase point during

the cellular growth of the positive clone (hiPSCs P32). Cells were treated with Colcemid chemical for 120 min, trypsinized, and then incubated with hypotonic solution (0.075 M KCl) for 20 min. Samples were fixed by methanol/acetic acid solution at a ratio of 3:1. Metaphase chromosomes were categorized using the standard G banding procedure. Around 50 counts were carried out at metaphase stage, and the average of 85% was considered as the final karyotype. Furthermore, AmpFℓSTR™Identifiler™ PCR Amplification Kit (appliedbiosystems, Cat# 4322288) was used to perform STR analysis according to manufacturer's instructions.

4.7. Three germ layers differentiation

The *HMGUi001-A-5* hiPSC line was directly differentiated towards three germ layers using StemMACS™ Trilineage Differentiation Kit (Miltenyi Biotec, Cat# 130-115-660) according to manufacturer's instructions. Then, the differentiated cells were stained for expression of endoderm, mesoderm and ectoderm markers.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2020.101927.

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