Stem Cell Research 46 (2020) 101874

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab resource: Stem Cell Line

Generation of a homozygous ARX nuclear CFP ($ARX^{nCFP/nCFP}$) reporter human iPSC line (HMGUi001-A-4)

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A B S T R A C T

The aristaless related homeobox (ARX) transcription factor plays a crucial role in glucagon-producing α-cell differentiation. Here, we generate an ARX reporter iPSC line by 3′ fusion of an intervening viral T2A sequence followed by a nuclear-localized histone 2B-cyan fluorescent protein (nCFP). The resulting cells have a normal karyotype and preserved pluripotency. *In vitro* differentiation of the $ARX^{nCFP/nCFP}$ reporter iPSCs towards the endocrine lineage confirmed the specific co-expression of the reporter protein in human glucagon$^+$ α-like cells. Thus, $ARX^{nCFP/nCFP}$ iPSC line will provide a powerful tool to monitor human α-cell progenitor differentiation as well as ARX$^+$ α-like cell function *in vitro*.

1. Resource table

Unique stem cell line identifier	HMGUi001-A-4
Alternative name(s) of stem cell line	hiPSC-ARX-T2A-H2B-CFP-Flag
Institution	Institute of Diabetes and Regeneration Research, Helmholtz Zentrum München, 85764 Neuherberg, Germany
Contact information of distributor	Heiko Lickert, heiko.lickert@helmholtz-muenchen.de
Type of cell line	iPSCs
Origin	Human, HMGUi001-A hiPSCs described in 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 insertion of fluorescent reporter
Associated disease	N/A
Gene/locus	ARX gene (ARX)/Xp21.3
Method of modification	CRISPR/Cas9
Name of transgene or resistance	Thosea asigna virus 2A (T2A)-histone 2B (H2B)-CFP-3x Flag
Inducible/constitutive system	N/A
Date archived/stock date	May 2019
Cell line repository/bank	N/A
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.

2. Resource utility

The newly generated ARX-H2B-nuclear CFP ($ARX^{nCFP/nCFP}$) reporter iPSC line offers a useful tool to study the formation and function of human ARX-expressing cell types, including pancreatic α-cells *in vitro* (Gécz et al., 2006). Furthermore, the nCFP reporter allows for monitoring and isolation of ARX-expressing cells that can be used for tissue engineering, gene functional testing, as well as developmental and disease modeling studies (Table 1).

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https://doi.org/10.1016/j.scr.2020.101874
Received 1 April 2020; Received in revised form 26 May 2020; Accepted 3 June 2020
Available online 09 June 2020

3. Resource details

To generate the fluorescent *ARX*^{nCFP} cell line, we used our recently established female iPSC line (HMGUi001-A) that has proven to be extremely efficient in pancreatic and endocrine differentiation (Wang et al., 2018). The *ARX-T2A-H2B-CFP-Flag* targeting vector was cloned (Fig. 1A; Material & Methods) and together with a Cas9-Venus fusion/gRNA-expressing vector (Yumlu et al., 2019) were transfected into HMGUi001-A iPSCs. 48 h after transfection, Cas9-Venus-expressing cells were flow sorted, plated at limited dilution and single-cell clones were isolated after 7 days. Because the ARX locus is located on the X chromosome, we selected only homozygous *ARX*$^{nCFP/nCFP}$ iPSC clones to avoid mosaic reporter expression due to X chromosome inactivation. The homologous recombination at the ARX locus was confirmed by 5′ and 3′ genomic PCR analysis spanning the homologous recombination border (Fig. 1B). We further confirmed in frame knock-in of the T2A-H2B-CFP sequencing (Supplementary Fig. 1A). Additionally, we detected no single gRNA off-targets through sequencing of three off target sites of the gRNA used with the highest specificity score located within genes (Supplementary Fig. 1B). The obtained clone had a normal karyotype (46, XX) (Fig. 1C) and was negative for mycoplasma (Supplementary Fig. 1C). Next, we tested and confirmed the pluripotency and multi-lineage differentiation potential of the *ARX*$^{nCFP/nCFP}$ iPSCs by tri-lineage differentiation followed by immunostaining and FACS analysis (Fig. 1D and E). The *ARX*$^{nCFP/nCFP}$ iPSCs line was able to differentiate into the three principal germ layers, namely endoderm, mesoderm, and ectoderm (Fig. 1F). Finally, live imaging of the *ARX*$^{nCFP/nCFP}$ iPSCs disclosed no phenotypically signs of differentiation and reporter gene expression at the pluripotent state (Fig. 1G). Altogether, these data demonstrate the successful generation of *ARX*$^{nCFP/nCFP}$ iPSCs line that exhibits pluripotency (Table 2).

To monitor the expression of the nCFP reporter protein in ARX-expressing α-cell progenitors and the α-cell lineage, we differentiated the *ARX*$^{nCFP/nCFP}$ iPSCs towards pancreatic endocrine lineage. We adapted the previously established differentiation protocol, which recapitulates endocrine pancreas development (Fig. 1H) (Rezania et al., 2014). Compared to the original protocol, we differentiated iPSCs in suspension culture to obtain a large number of 3D clusters, ranging in size between 100 and 200 μm. Similar to the parental iPSC line, the newly generated *ARX*$^{nCFP/nCFP}$ iPSCs line was capable to efficiently differentiate towards the endocrine lineage. Live imaging of cell clusters at day 21 of endocrine differentiation at the α- and β-like cell stage (S6) revealed the presence of cells exhibiting a strong nCFP signal (Fig. 1I). To test whether the nCFP reporter-positive cells can be isolated from the clusters, we performed FACS analysis. We could successfully separate the nCFP^{+} and nCFP^{-} cells based on the fluorescence signal (Fig. 1J). To assess whether the nCFP reporter signal mirrors the endogenous ARX protein expression, we performed immunostaining using antibodies against CFP and ARX. Confocal imaging indicated the co-localization of both proteins, further confirming the co-expression of the reporter and ARX proteins in α-cell progenitors and the α-cell lineage (Fig. 1K). Next, we characterized nCFP reporter-expressing cells and differentiated *ARX*$^{nCFP/nCFP}$ iPSCs for 21 days to the stage in which hormone-positive endocrine cells appear. To assess which endocrine cells predominantly express the nCFP reporter protein, we co-stained with glucagon (GCG) for α-like cells and C-peptide (C-PEP; an equimolar cleavage product from insulin) for β-like cells. While all GCG^{+} cells expressed nCFP (Fig. 1L, white arrowheads), only a very small fraction of C-PEP^{+} cells expressed the reporter (Fig. 1L, yellow arrowhead). Of note, most of the nCFP^{+}/C-PEP^{+} cells expressed low levels of GCG, indicating their poly-hormonal nature. Additionally, we found a portion of nCFP reporter-positive cells, which did not express any hormone (Fig. 1L, pink arrowhead). These cells are hormone-negative ARX^{+} α-cell progenitors that require further differentiation to become GCG^{+} α-like cells. Indeed, FACS analysis at day 21 indicated two separate ARX^{+} populations (Fig. 1J) that are likely α-cell progenitors and GCG^{+} α-cells. Overall, our analysis demonstrates the specific expression of the nCFP protein in human α-like cells similar to the endogenous ARX protein (Bramswig and Kaestner, 2011). The cell line has been registered in hPSCreg and is publicly available under the link https://hpscreg.eu/cell-line/HMGUi001-A-4.

4. Materials and Methods

4.1. Generation of the *ARX*$^{nCFP/nCFP}$ iPSC reporter cell line

The Arx locus was targeted by homologous recombination and CRISPR/Cas9 technology using *Arx-2A-H2B-CFP-Flag* targeting vector: We cloned the genomic sequence of the ARX gene into a targeting vector and utilize the 737 bp upstream region and the 1076 bp downstream region of the translational stop codon as 5′ and 3′ homology arms (HA). Before the stop codon, we introduced a 2A sequence followed by a Histone 2B (H2B) fused to the cyan fluorescent protein (CFP) tailed by a Flag tag. Thereby the reporter line would translate equal amounts of ARX and H2B-CFP-Flag protein, separated by an autonomous intra-ribosomal self-processing of the 2A-peptide from thosea asigna virus. A gRNA, that introduced dsDNA breaks 3 bp upstream of the stop codon of the *ARX* was cloned into the pU6-(BbsI)sgRNA_CAG-

Table 1
Characterization and Validation.

Classification	Test	Result	Data
Morphology	Photography	Normal morphology	Fig. 1 panel G Scale bar :200 μm
Phenotype	Immunocytochenistry	Expression of pluripotency markers positive for OCT4, SOX2	Fig. 1 panel D Scale bar :100 μm
	Flow Cytometry	Around 99% of *ARX*$^{nCFP/nCFP}$ iPSC reporter line are OCT4 positive.	Fig. 1 panel E
Genotype	Karyotype (G-banding) and resolution	46, xx Resolution 450–525 bands Passage 25	Fig. 1 panel C
Identity	Microsatellite PCR	Not performed	
	STR analysis	16 sites were tested, 100% matched	Submitted in journal archive
Mutation analysis	Sequencing	Homozygous insertion of T2A/H2B cassette, confirmed by Sanger sequencing, sgRNA putative off-target site analyzed by Sanger sequencing not performed	Supplementary Fig. 1 panel A, B
	Southern Blot or WGS	Not performed	
Microbiology and virology	Mycoplasma	Biochemical luminescence MycoAlert™ Plus Mycoplasma Detection Kit, Lonza, Negative	Supplementary Fig. 1 panel C
Differentiation potential	Direct differentiation	*ARX*$^{nCFP/nCFP}$ iPSC were differentiated into the three germ layers (StemMACS™ Trilineage Differentiation Kit, Miltenyi Biotec) and towards pancreatic lineage cells	Fig. 1 panel F Scale bar :100 μm Fig. 1, K, L Scale bar:50 μm

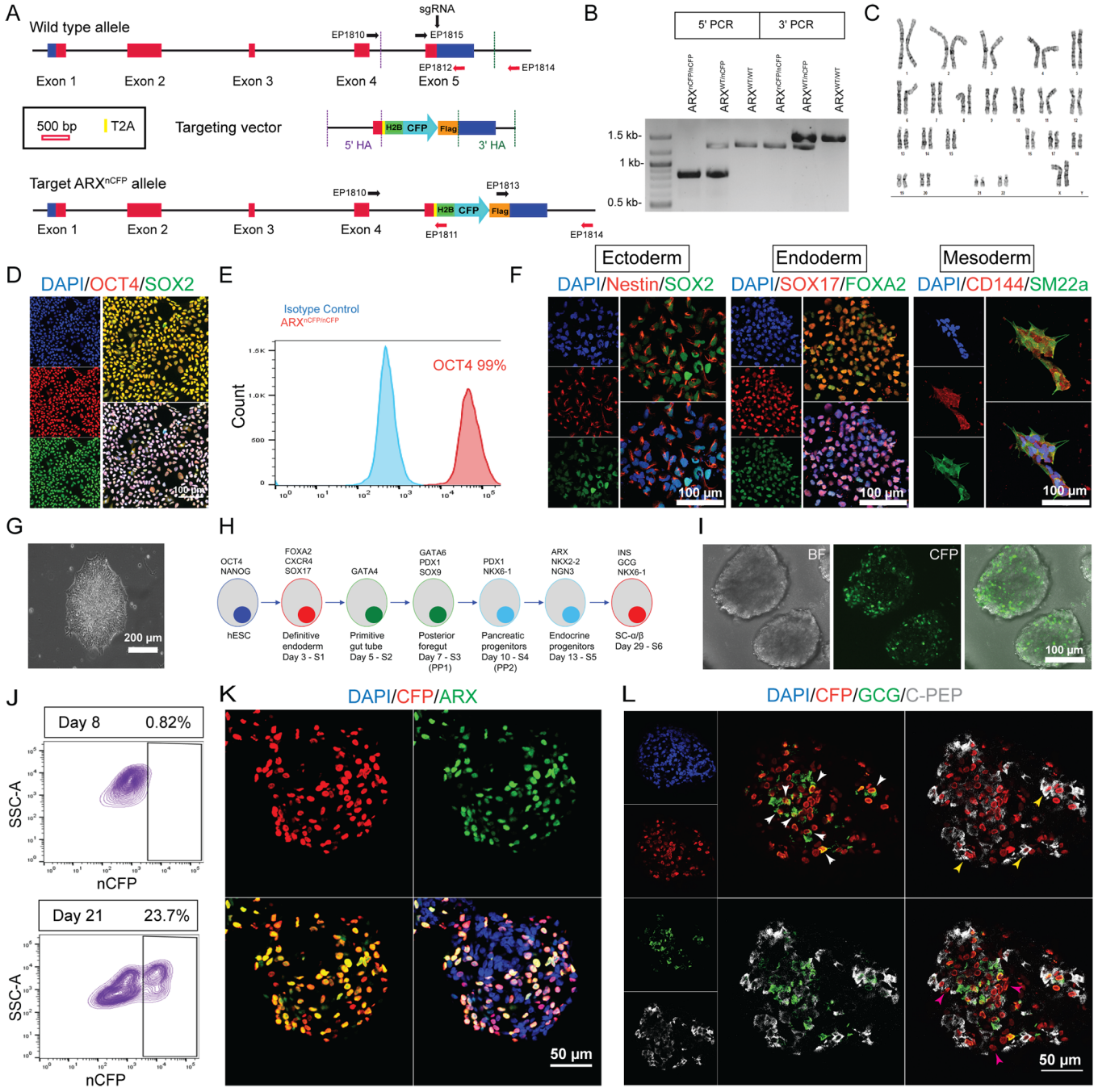

Fig. 1. Generation and characterization of *ARX*$^{nCFP/nCFP}$ hiPSC line. (A) Cloning strategy to generate *ARX*$^{nCFP/nCFP}$ reporter cells. HA; homology arm. (B) 5′ and 3′ PCR products confirm the generation of heterozygous and homozygous clones compared to wild type iPSCs. (C) Normal karyotype (46, XX) of the *ARX*$^{nCFP/nCFP}$ reporter clone. (D) Immunostaining showing SOX2 and OCT4 as pluripotent markers in the *ARX*$^{nCFP/nCFP}$ iPSC at pluripotency stage. Scale bar, 100 µm (E) Representative FACS plot of OCT4^{+} cells in the *ARX*$^{nCFP/nCFP}$ reporter cells. (F) Multi-lineage potency assay of *ARX*$^{nCFP/nCFP}$ iPSC line. Scale bars, 100 µm. (G) Phase contrast of *ARX*$^{nCFP/nCFP}$ reporter colony at pluripotent stage. Scale bar, 200 µm. (H) Schematic representation of the suspension endocrine lineage differentiation protocol. (I) Live image of *ARX*$^{nCFP/nCFP}$ reporter at day 21 of the differentiation. Scale bar, 100 µm. (J) FACS analysis of the *ARX*$^{nCFP/nCFP}$ iPSC line at days 8 and 21 showing the appearance of the nCFP^{+} cells as differentiation progresses. (K) Immunostaining of cell clusters at day 21, showing co-localization of ARX and nCFP reporter protein. Scale bar, 50 µm. (L) Immunostaining of cell clusters at day 21 showing co-localization of nCFP with GCG-positive cells. Scale bar, 50 µm.

Cas9-venus-bpA vector that allowed FACS sorting (Yumlu et al., 2019). The cocktail of all vectors was transfected into iPSCs using the standard Lipofectamine transfection protocols. Transfected cells were sorted by FACS, colonies were picked, expanded and screened by PCR to select the desired clones.

4.2. Characterization of *ARX*$^{nCFP/nCFP}$ iPSCs

Karyotyping of the *ARX*$^{nCFP/nCFP}$ iPSC line was performed during cell growth in a logarithmic phase. Cells at passage number 25 were incubated with colcemid for 2 h, trypsinized and treated with hypotonic

Table 2
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)	Santa Cruz Cat # sc-8628, RRID:AB_653551
Pancreatic differentiation markers	Sheep anti-ARX	(1:100)	R&D systems Cat # AF-7068, RRID:AB_10973178
	Rabbit anti-GFP	(1:300)	Invitrogen Cat # A-6455, RRID:AB_221570
	Goat anti-PDX1	(1:300)	R&D systems Cat # AF-2419, RRID:AB_355257
	Guinea Pig anti-C-Peptide	(1:300)	Abcam Cat # ab30477, RRID:AB_726924
	Mouse anti-GCG	(1:500)	Sigma-Aldrich Cat # G2654, RRID:AB_259852
Secondary antibodies (Immunofluorescence)	Donkey anti-mouse IgG(H + L) Alexa Fluor 555	(1:500)	Invitrogen Cat # A31570, RRID:AB_2536180
	Donkey anti-sheep IgG(H + L) Alexa Fluor 488	(1:500)	Jackson ImmunoResearch Labs Cat # 713-546-147,
	Donkey anti-rabbit IgG(H + L) Alexa Fluor 488	(1:500)	RRID:AB_23407
	Donkey anti-goat IgG(H + L) Alexa Fluor 555	(1:500)	Invitrogen Cat # A21206, RRID:AB_141708
	Donkey anti-guinea pig IgG(H + L) Alexa Fluor 488	(1:500)	Invitrogen Cat # A21432, RRID:AB_141788 Dianova Cat # 706-545-148, RRID:AB_2340472
Secondary antibodies (flow-cytometry)	Donkey anti-goat IgG(H + L) Alexa Fluor 555	(1:500)	Invitrogen Cat # A21432, RRID:AB_141788

Primers	Target	Forward/Revere primers (5′-3′)
Episomal Plasmids of genome sequencing (RT-PCR)	EP 1810 – EP 1812 CFP/Left homology arm – 1291 bp	EP 1810 TTTCTTCAGGGCGCAGAAAGTC EP 1811 CGTCGCCGCATGTTAGTAAAGAG EP 1812
	EP 1815 – EP 1814 CFP/Right homology arm – 1482 bp	AGCTATCTTACAGGCTCGCATTTG EP 1813 CGGCATGGACGAGCTGTACAAGGAC
	EP 1813 – EP 1814 Right homology arm – 1292 bp	EP 1814 CAGGTGCGCGTGGAAAGTACCG EP 1815 GCACAGCTCCCGAGGCCATGAC

solution (0.075 M KCL) for 20 min. Follow by methanol/acetic acid (3:1) fixation. Chromosomes from $ARX^{nCFP/nCFP}$ iPSCs' methaphases were classified using the standard G banding technique. Around 20 methaphases were counted, and the final karyotype is the average of 85% of them. For mycoplasma testing we used the Lonza MycoAlert Mycoplasma Detection kit (Lonza, Cat. No. LT07-418), indicating that $ARX^{nCFP/nCFP}$ iPSCs were mycoplasma free.

4.3. Cell culture

The $ARX^{nCFP/nCFP}$ iPSC line at passage number 20 was maintained under a feeder-free system on Geltrex (Invitrogen) in StemMACS iPS-Brew XF, human (Miltenyi Biotec). We passaged cells at 75% confluence using StemPro Accutase Cell Dissociation Reagent (Thermo Fisher Scientific). After dissociation, cells were seeded in iPS-Brew XF media including Y-27632. To differentiate iPSCs towards the endocrine lineage we use a multistep differentiation procedure that has been reported previously (Rezania et al., 2014). Different to the original protocol, we started the differentiation in suspension. Two million cells were plated per well of a 6-well low-binding plate and placed in orbital shaker at a rotational speed of 90 rpm inside the incubator at 37 °C and 5% of CO_2.

4.4. Immunostaining and imaging

For immunofluorescence staining, the cell clusters were fixed with 2% paraformaldehyde for 20 min at room temperature, followed by multiple washes in PBS. Clusters were embedded in 2% agar (Sigma), followed by dehydration, paraffin embedding, and sectioning. Tissue sections were rehydrated and permeabilized with (0.1 M Glycine and 0.2% Triton) for 30 min and then were blocked with the blocking solution (3% serum donkey, 0.1% BSA and tween). Primary antibodies were incubated overnight at 4 °C in blocking buffer. The next day, sections were washed three times with 0.1% PBS-Tween (PBST) and incubated with appropriate secondary antibodies for 4 h at room temperature in blocking buffer. Slides were washed in PBST before mounting. Nuclei were visualized with DAPI. Images were acquired using a Leica SP5 microscope.

4.5. Cell sorting by flow cytometry

The cell clusters were collected, washed in PBS and dissociated by gentle pipetting after 12 to 15 min incubation in StemPro Accutase. For sorting, the cell suspension was filtered and re-suspended in FACS buffer consisting of PBS containing 2 mM EDTA (Ambion) and 1% BSA (Sigma). Cell sorting was performed on a FACSAria II (BD Bioscience) and the data were analyzed using FlowJo.

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.

Acknowledgements

We thank G. Lederer, G. Eckstein and T. Meitinger for the karyotyping and the STR analysis. We thank the donor of the fibroblasts for supporting research projects with human material, Prof. Andreas Fritsche and his team for taking the skin samples and Xianming Wang for generating the parental hiPSC line. We also thank Elke Schlüssel for her comments on the manuscript. We are grateful to Ralf Kühn for discussion and advice. This work was supported by the Helmholtz-Gemeinschaft (Helmholtz Portfolio Theme 'Metabolic Dysfunction and Common Disease) and Deutsches Zentrum für Diabetesforschung (DZD) both from Germany.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.101874.

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