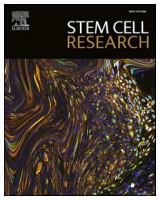
Stem Cell Research 67 (2023) 103023

Contents lists available at ScienceDirect

Stem Cell Research

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



Lab Resource: Single Cell Line

Generation of an induced pluripotent stem cell line CSSi015-A (9553), carrying a point mutation c.2915C > T in the human calcium sensing receptor (CasR) gene

Giovannina Rotundo [a], Elisa Maria Turco [a], Giorgia Ruotolo [a,b], Isabella Torrente [c], Ornella Candido [c], Gianluca Lopez [d], Daniela Ferrari [b], Caterina Caputi [e], Mario Mastrangelo [e], Francesco Pisani [e], Maurizio Gelati [f], Vito Guarnieri [c], Angelo Luigi Vescovi [a,b], Jessica Rosati [a,*]

[a] Cellular Reprogramming Unit, Fondazione I.R.C.C.S. Casa Sollievo della Sofferenza, Viale dei Cappuccini, San Giovanni Rotondo, Foggia 71013, Italy
[b] Biotechnology and Bioscience Department, Bicocca University, Piazza della Scienza 2, Milan 20126, Italy
[c] Medical Genetics Unit, Fondazione I.R.C.C.S. Casa Sollievo della Sofferenza, Viale dei Cappuccini, San Giovanni Rotondo, Foggia 71013, Italy
[d] Department of Clinical and Molecular Medicine, Sapienza University and Sant'Andrea Hospital, Rome, Italy
[e] Child Neurology and Psychiatry Unit Department of Human Neuroscience, Sapienza-University of Rome Via dei Sabelli, 00185, Italy
[f] UPTA Unit Fondazione IRCCS Casa Sollievo della Sofferenza, Viale dei Cappuccini, San Giovanni Rotondo, Foggia 71013, Italy

A B S T R A C T

Familial Hypocalciuric Hypercalcemia (FHH1) is a rare autosomal dominant disease with low penetrance, caused by inactivating mutations of the calcium-sensing receptor (*CaSR*) gene, characterized by significant hypercalcemia, inappropriately normal serum PTH levels and a low urinary calcium level. Human induced pluripotent stem cells (hiPSCs) from a patient carrying a previously identified heterozygous mutation, a p.T972M amino acid substitution in cytoplasmic tail of CasR, were produced using a virus, xeno-free and non-integrative protocol.

1. Resource table

Unique stem cell line identifier	CSSi015-A(9553)
Alternative name(s) of stem cell line	*FC 17 cl D1*
Institution	*Fondazione IRCCS Casa Sollievo della Sofferenza*
Contact information of distributor	*Jessica Rosati; j.rosati@css-mendel.it*
Type of cell line	*iPSC*
Origin	*human*
Additional origin info required for human ESC or iPSC	*Age:38* *Sex: Male* *Ethnicity if known: Caucasian/Italian*
Cell Source	*Dermal fibroblasts*
Clonality	*Clonal*
Method of reprogramming	*Non integrating episomal vectors*
Genetic Modification	*yes*
Type of Genetic Modification	*congenital*
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	*qRT-PCR*
Associated disease	*Familial benign Hypocalciuric Hypercalcemia (FHH)*
Gene/locus	*CaSR c.2915C > T*

(*continued on next column*)

(*continued*)

Unique stem cell line identifier	CSSi015-A(9553)
Date archived/stock date	*Luglio 2018*
Cell line repository/bank	*https://hpscreg.eu/user/cellline/edit/CSSi015-A*
Ethical approval	CE: Prot n 230/16, RIF CE 4047, *Università degli studi di Roma "La Sapienza", Dipartimento di Scienze Neurologiche, Psichiatriche e Riabilitative dell'età evolutiva.*

2. Resource utility

Familial Hypocalciuric Hypercalcemia (MIM#145980) is a rare genetic disorder caused by inactivating mutations of the Calcium Sensing receptor (CaSR) gene. Variable phenotypic expression, due to the high number of CaSR identified mutations, makes difficult a possible phenotype-genotype association. The derivation of iPSCs permits to study this disease *in vitro*. Table 1.

* Corresponding author.
E-mail address: j.rosati@css-mendel.it (J. Rosati).

https://doi.org/10.1016/j.scr.2023.103023
Received 8 October 2022; Received in revised form 12 December 2022; Accepted 5 January 2023
Available online 7 January 2023

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	*Normal*	Fig. 1*A*
Phenotype	Qualitative analysis *Immunocytochemistry*	*Staining of pluripotency markers: Oct4; Tra 1–60;*	Fig. 1*B*
	Quantitative analysis *RT-qPCR)*	*Expression of pluripotency markers: KLF4; LIN28; L-MYC; OCT4; SOX2;*	Fig. 1*D*
Genotype	Karyotype (G-banding) and resolution	*46XY Resolution 450–500*	Fig. 1*I*
Identity	STR analysis	*All the 19 sites tested matched*	*e.g.submitted in archive with journal*
Mutation analysis (IF APPLICABLE)	Sequencing	*CasR c.2915C > T*	Fig. 1*H*
Microbiology and virology	Mycoplasma	*E.g. Mycoplasma testing by N-Garde Mycoplasma PCR kit. Negative.*	*Supplementary Fig. 1*
Differentiation potential	Embryoid body formation Teratoma formation	*Genes expressed in embryoid bodies: GATA4; FOXA2; T; EOMES; NESTIN; PAX6; Proof of three germ layers formation*	Fig. 1*E and 1F* Fig. 1*G*
List of recommended germ layer markers	**Expression of these markers has to be demonstrated at mRNA (RT PCR) levels**	*Ectoderm: NESTIN; PAX6.* Endoderm: GATA4; FOXA2. Mesoderm: EOMES; T.	Fig. 1*F*
Donor screening (OPTIONAL)	HIV 1 + 2; Hepatitis B and C	*Not applicable*	
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	Not applicable *Not applicable*	

3. Resource details

CaSR gene (chr.3q13.3–21) encodes for a G-protein of 1078 amino acids, localized at the plasma membrane that acts as a sensitive receptor of extracellular calcium. CASR mutations lead to changes in calcium sensitivity, which requires a higher concentration in serum to reduce the release of PTH (Mastromatteo et al., 2014). These changes are connected to an increase of calcium and magnesium reabsorption in the proximal renal tubules, which leads to hypercalcemia and hypocalciuria. Fibroblast cells were isolated from a 38 years old patient, carrying a point mutation at codon 972, located within the cytoplasmic domain of the CaSR, that changes Threonine with Methionine. The patient showed no clinical disease's signs, but transmitted the genetic mutation to his female child, which instead displayed severe manifestations such as hypocalciuria, hypophosphaturia, hypocreatinuria, myoclonic epilepsy and borderline cognitive functioning. iPSCs were generated by a non-integrating episomal method through expression of the reprogramming factors oct4, sox2, klf4, l-myc, lin28 and p53 shRNA (Okita et al., 2011). The established iPSC colonies showed typical human stem cell-like morphology as judged by brightfield microscopy (Fig. 1A). Staining for endogenous markers TRA-1–60 and OCT-4 demonstrated the pluripotency of these iPSCs (Fig. 1B), that was confirmed through qRT-PCR (Fig. 1D). We also examined the presence of episomal DNA in the established iPSCs, using primers specific to a sequence that is common in all three reprogramming plasmids and we observed that after 10 passages the iPSCs were free of vector sequences as shown by qRT-qPCR, using, as positive control, the fibroblasts after one week from nucleofection, and, as negative control, an already published hiPSCs line named CSSi014-A 9407 (Casamassa et al., 2022)(Fig. 1C). The capacity of spontaneous differentiation was demonstrated by the formation of both embryoid bodies (EBs) *in vitro* (Fig. 1E) and teratoma formation *in vivo* (Fig. 1G). qRT-PCR analysis showed the endogenous expression of the three germ layer markers into EBs (Fig. 1F). The presence of the disease-related mutation CasR c. 2915C > T in fibroblasts was confirmed by Sanger sequencing (Fig. 1H) in the generated iPSCs. We confirmed the genomic stability of iPSCs through karyotype, which provided a normal diploid 46, XY chromosome arrangement without any detectable abnormalities (Fig. 1D). PCR-based detection tests confirmed the absence of Mycoplasma contamination at this stage (Supplementary Fig. 1). In addition, Short Tandem Repeat (STR) profiling confirmed the same genetic identity between the iPSC line and the donor's fibroblasts (Data available with the authors).

4. Materials and methods

4.1. Skin biopsy and fibroblast reprogramming

The skin biopsies were cultured in Dulbecco's Modified Eagle Medium (DMEM) High Glucose with 20 % FBS, 1 % L-Glutamine, Penicillin-Streptomicin and Non-essential AminoAcids (Sigma Aldrich), 37 °C and 5 % CO2. 1×10^5 fibroblasts (passage V) were nucleofected with 3 μg 1:1:1 mix of the episomes pCXLE-hUL (Addgene#27080), pCXLE-hSK (Addgene#27078) and pCXLE-hOCT4-shp53 (Addgene#27077) using Nucleofector4D (Lonza Amaxa) and FF113 as program. Every reaction was performed using a pMAX-GFP TM vector as DNA control to calculate the transfection efficiency. On day 7, the fibroblasts were counted, plated on Matrigel (1:100) (BD Biosciences) and cultured in Nutristem XF medium (Biological Industries). The iPSC colonies were picked and expanded. Absence of mycoplasma was verified using *N*-Garde Mycoplasma PCR kit (EuroClone).

4.2. Embryoid bodies formation

Mechanically detached iPSCs (XIII passage) were plated in floating conditions, 37 °C and 5 % CO2, in Petri dishes. The Nutristem XF medium was gradually substituted with DMEM/F12, 20 % KOSR (Gibco), 0,1mM NEAA, 0.1 mM β-mercaptoethanol, 1 % Pen/Strep. At day 14 EBs were collected.

4.3. Teratoma assay

3×10^5 dispase-treated iPSCs (XIII passage), in 100 μl Matrigel (Life Technology), were injected into NOD/SCID mice according ethical guidelines. After 9 weeks, teratoma was dissected, fixed in 10 % formalin (Sigma Aldrich), paraffin-embedded, sectioned and stained with hematoxylin/eosin.

4.4. Real-Time PCR analysis

Total RNAs were isolated using TRIzol reagent (Life Technologies) according to manufacturer's instructions.

Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), after digestion with DNAse I (Life Technologies). qRT-PCR was performed in a 7900HT Fast Real-Time PCR system (Applied Biosystem). For pluripotency gene expression we used Power SYBR Green PCR Master Mix (Applied Biosystem) while for differentiation marker expression TaqMan Universal PCR Master Mix (Applied Biosystem), Table 2. For each target gene, three biological replicates in duplicate were tested, β-ACTIN was used as normalizer, the expression ratio calculated with the 2-ΔΔCt method.

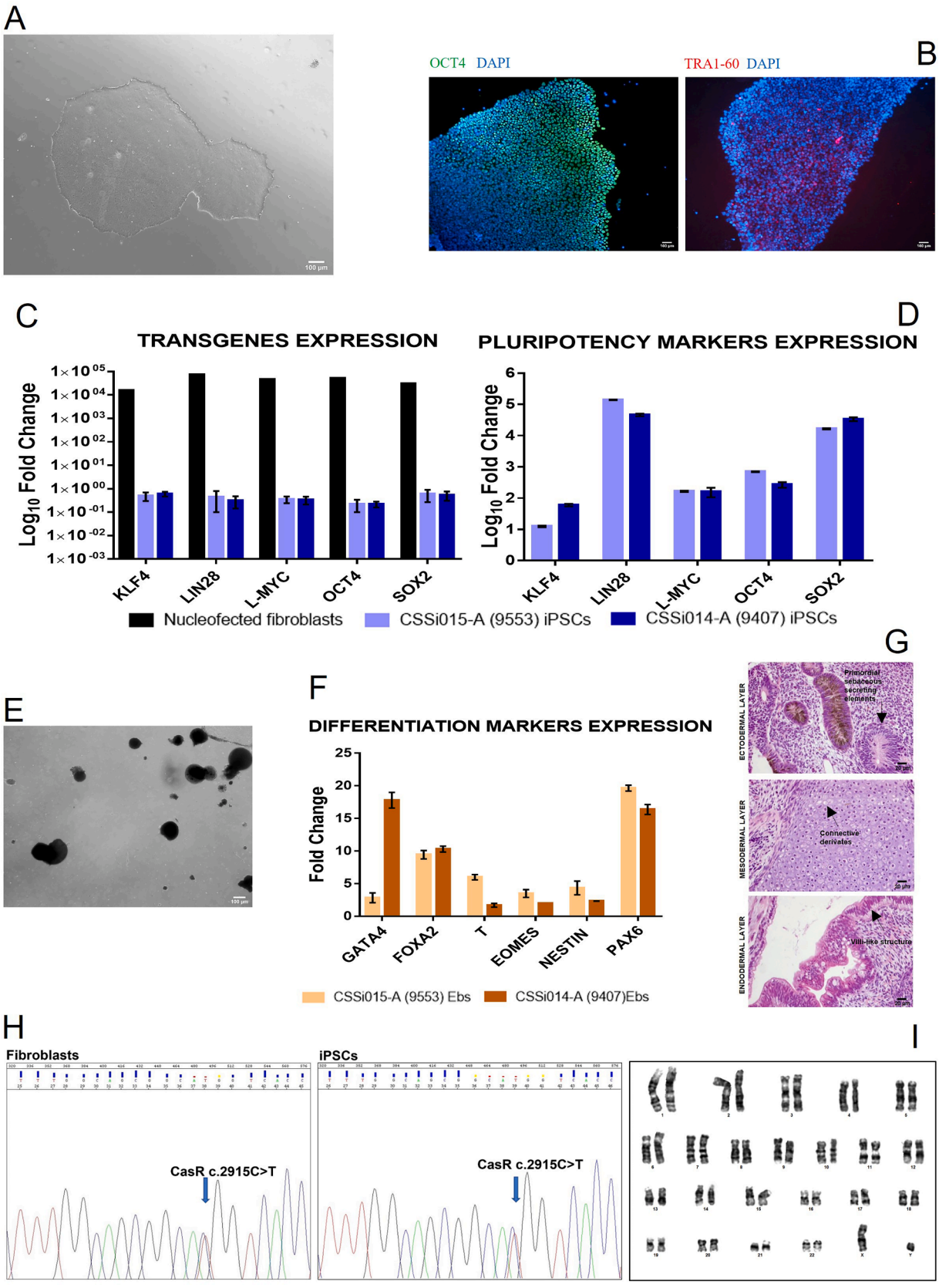

A
B
OCT4 DAPI
TRA1-60 DAPI
C
TRANSGENES EXPRESSION
Log10 Fold Change
KLF4
LIN28
L-MYC
OCT4
SOX2
Nucleofected fibroblasts
CSSi015-A (9553) iPSCs
CSSi014-A (9407) iPSCs
D
PLURIPOTENCY MARKERS EXPRESSION
E
F
DIFFERENTIATION MARKERS EXPRESSION
Fold Change
GATA4
FOXA2
T
EOMES
NESTIN
PAX6
CSSi015-A (9553) Ebs
CSSi014-A (9407)Ebs
G
ECTODERMAL LAYER
Primordial sebaceous secreting elements
MESODERMAL LAYER
Connective derivates
ENDODERMAL LAYER
Villi-like structure
H
Fibroblasts
iPSCs
CasR c.2915C>T
CasR c.2915C>T
I

Fig. 1.

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	*Rabbit anti-OCT4; Mouse anti TRA-1–60*	*1:100 1:100*	*Life technologies (A13998); Life technologies (411000)*	*RRID: AB_2534182; RRID: AB_2533494.*
Secondary antibodies	*anti-Rabbit AlexaFluor 488; anti-Mouse AlexaFluor 594*	*1:1000 1:1000*	*Invitrogen (A11034); Invitrogen (A21422)*	*RRID: AB_2576217; RRID: AB_2535844*
	Primers Target	Size of band	Forward/Reverse primer (5′-3′)	
SyBr green Primers used for qPCR Episomal genes	*eOCT4*	83 *bp*	*Fwd: CAT TCAAAC TGA GGTAAG GG*	
	eKLF4	112 *bp*	*Rev: TAG CGTAAA AGG AGCAAC ATA G*	
	eLIN28	205 *bp*	*Fwd: CCA CCTCGC CTT ACACAT GAA GA*	
	eL-MYC	80 *bp*	*Rev: TAG CGTAAA AGG AGCAAC ATA G*	
	eSOX2	66 *bp*	*Fwd: AGC CATATG GTA GCCTCA TGT CCG C*	
			Rev: TAG CGTAAA AGG AGCAAC ATA G	
			Fwd: GGC TGAGAA GAG GATGGC TAC	
			Rev: TTT GTTTGA CAG GAGCGA CAA T	
			Fwd: TTC ACATGT CCC AGCACT ACC AGA	
			Rev: TTT GTTTGA CAG GAGCGA CAA T	
SyBr green Primers used for qPCR Pluripotency Markers (qPCR)	*OCT4*	143 *bp*	*Fwd: CCC CAGGGC CCC ATTTTG GTA CC*	
	LIN28	129 *bp*	*Rev: ACC TCAGTT TGA ATGCAT GGG AGAGC*	
	L-MYC	142 *bp*	*Fwd:* AGC CAT ATG GTA GCC TCA TGT CCG C *Rev:* TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG	
	SOX2	80 *bp*	*Fwd: GCG AACCCA AGA CCCAGG CCT GCTCC*	
			Rev: CAG GGGTC TGC TCGCAC CGT GAT G	
			Fwd: TTC ACATGT CCC AGCACT ACC AGA	
			Rev: TCA CATGTG TGA GAGGGG CAG TGTGC	
House-Keeping Genes (qPCR)	*β-ACTIN*	203 *bp*	*Fwd: GGC ATCCTC ACC CTGAAG TA*	
			Rev: GGG GTGTTG AAG GTCTCA AA	
TaqMan primers used for qPCR Differentation markers	*NESTIN*	50 *bp*	*Hs04187831_g1*	
	PAX6	*76 bp*	*Hs00240871_m1*	
	T	132 *bp*	*Hs00610080_m1*	
	EOMES	81 *bp*	*Hs00172872_m1*	
	GATA4	*68 bp*	*Hs00171403_m1*	
	FOXA2	*66 bp*	*Hs00232764_m1*	
	β-ACTIN	*171 bp*	*Hs 99999903_m1*	
	CASR	*167 bp*		
e.g. Targeted mutation analysis/ sequencing			*Fwd: CACAGCAGCAACGATCTCAG Rev: GGG GTGTTG AAG GTCTCA AA*	

4.5. Immunofluorescence staining

Cells, at XII passage, were fixed using 4 % paraformaldehyde, incubated with Blocking Buffer (PBS containing 20 % Normal Goat Serum, 0.1 % Triton X-100) for 30 min at room temperature and then with primary antibodies, Table 2, O/N at 4 °C. After washing, Alexa Fluor 594- and/or Alexa Fluor 488-conjugated secondary antibodies were added 1 h at RT. Cellular nuclei were counterstained with DAPI. Microphotographs were taken using a Nikon *C*2 fluorescence microscope and NIS Elements 1.49 software.

4.6. Karyotype analysis

Pluripotent cells (VII passage) were cultured in T 25 flasks coated with Matrigel in Nutristem medium for 2–3 days. Cells were treated with a 0.1 μg/mL COLCEMID solution (Thermo Fisher Scientific) for 60 min at 37 °C, 30 mM KCl in 10 %FBS at 37 °C for 6 min and coldfresh-made 3:1 ethanol:acetic acid solution. Karyotype analysis was carried out on GTG-banded metaphases. Fifteen metaphases were counted.

4.7. Mutation analysis

Genomic DNA was extracted from iPSCs and fibroblasts (VI passage) using ReliaPrep™ Blood gDNA Miniprep System. CaSR exon 7 was amplified by PCR using Forward: 5- CACAGCAGCAACGATCTCAG-3, Reverse: 5-CGTATCGCTGCTTTTCTGGG −3′ primers. The amplicon (product size: 167 bp) was sequenced by BigDye terminator v.3.1 Cycle Sequencing kit on ABI 3130XL Genetic Analyzer.

4.8. STR analysis

PCR amplification of 16 distincts STRs (D3S1358, TH01, D21S11, D18S51,D10S1248,D1S1656, D2S1338, D16S539, D22S1045, vWA, D8S1179, FGA,D2S441, D12S391, D19S433,SE33) was performed using Power Plex ® ESX 17 Fast System (Promega), PCR products were separated on an ABI Prism 3130 DNA sequencer and analyzed by Gene Mapper IDX v3.2 (Applied Biosystems).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jessica Rosati reports financial support was provided by Ministry of Health.

Funding

This work was supported by the Italian Ministry of Health, Ricerca Corrente Program 2022-2024 and 5×1000 funds (to JR).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103023.

References

Casamassa, A., Zanetti, A., Ferrari, D., Lombardi, I., Galluzzi, G., D'Avanzo, F., Cipressa, G., Bertozzi, A., Torrente, I., Vescovi, A.L., Tomanin, R., Rosati, J., 2022. Generation of an induced pluripotent stem cells line, CSSi014-A 9407, carrying the variant c.479C>T in the human iduronate 2-sulfatase (hIDS) gene. Stem Cell Res. 63 https://doi.org/10.1016/j.scr.2022.102846. Epub 2022 Jun 21.PMID: 35759972.

Mastromatteo, Eugenio, Lamacchia, Olga, Campo, Michela Rosaria, Conserva, Antonella, Baorda, Filomena, Cinque, Luigia, Guarnieri, Vito, Scillitani, Alfredo, Cignarelli, Mauro, 2014. A novel mutation in calcium-sensing receptor gene associated to hypercalcemia and hypercalciuria. BMC Endocr Disord. 7 (14), 81. https://doi.org/10.1186/1472-6823-14-81.

Okita, K., Matsumura, Y., Sato, Y., Okada, A., Yamanaka, S., 2011. A more efficient method to generate integration-free human iPS cells. Nat. Methods. 8 (5), 409–412.