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Generation of an induced pluripotent stem cell line from a patient with hereditary multiple endocrine neoplasia 2A (MEN2A) syndrome with *RET* mutation

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ABSTRACT

Multiple Endocrine Neoplasia Type 2A (MEN2A) is a cancer-predisposing syndrome that affects patients with germline RET mutations. The clinical spectrum of the syndrome includes medullary thyroid carcinoma (MTC), pheochromocytoma, hyperparathyroidism and cutaneous lichen amyloidosis (CLA) and/or Hirschsprung disease in some variants.

Currently, there is no satisfactory animal model recapitulating all the features of the disease especially at the level of stem cells. We generated induced pluripotent stem cells (iPSCs) from a patient with *RET* mutation at codon 634 who developed pheochromocytoma and MTC. RET^{C634Y}-mutated cells were reprogrammed by non-integrative viral transduction. These iPSCs had normal karyotype, harboured the RET^{C634Y} mutation and expressed pluripotency hallmarks as well as RET. A comprehensive pathological assessment of teratoma was performed after injection in immunodeficient mice.

Resource table

Name of stem cell line	PB48
Institution	INSERM U935 and INGESTEM, Université Paris Sud
Person who created resource	J. Hadoux, O·Feraud
Contact person and email	Ali G Turhan: turviv33@gmail.com, abenna@hotmail.fr
Date archived/stock date	Sept, 15, 2014
Origin	Human peripheral blood cells
Type of resource	Biological reagent: induced pluripotent stem cell (iPSC) generated by Sendai-virus mediated pluripotency gene transfer
Sub-type	Cell line
Key transcription factors	Oct4, Sox2, cMyc, Klf4
Authentication	Identity and purity of cell line, expressing the same RET mutation as the peripheral blood cells of the patient (figure 1)

Resource table (*continued*)

Name of stem cell line	PB48
Link to related literature	http://www.sciencedirect.com/science/article/pii/S0092867407014717 http://www.stembook.org/node/765.html http://stembook.org/node/723.html http://online.liebertpub.com/doi/abs/10.1089/thy.2014.0335?journalCode=thy
Information in public databases	None
Ethics	The project was approved by INSERM and Ethical Committee CPP (Date of approval: 17th January 2013, Ref: PP-13-001)

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1. Resource details

Multiple Endocrine Neoplasia Type 2 (MEN2) are autosomal dominant hereditary diseases, related to mutations of the *RET* (REarranged during Transfection) proto-oncogene (Donis-Keller et al., 1993). MEN2A is a rare syndrome which associates medullary thyroid cancer (MTC), pheochromocytoma, hyperparathyroidism, cutaneous lichen amyloidosis, and Hirschsprung disease in some patients (Wells et al., 2013). It affects therefore organs originating from endodermal and ectodermal layers. Mouse models have been unsuccessful in recapitulating the full clinical spectrum of MEN2A (Michiels et al., 1997). The most common mutation, accounting for over 80% of all mutations associated with the classic MEN2A, affects codon 634 (Wells et al., 2013). Mutations at codon 634 are classified as high risk, according to the American Thyroid Association (ATA) guidelines (Wells et al., 2015). In this work we sought to determine the feasibility of modelling the MTC from a patient with the germline RET mutation who developed this tumor in the context of familial cancer. This patient was a 28-year old man with MTC and pheochromocytoma. The mother of the patient had undergone surgery for MTC 20 years previously.

Cryopreseved peripheral blood mononuclear cells (PBMCs) from this patient were used for the generation of an induced pluripotent stem cell (iPSC) that harbour a "high risk" *RET* mutation according to ATA guidelines, i.e. a 1901G > A missense mutation leading to the most frequent C634Y amino acid substitution seen in Ret in MEN2A. iPSCs were generated by non-integrative transduction of Oct3/4, Sox2, Klf4, and cMyc (Takahashi et al., 2007). They expanded as typical iPSC colonies on either mouse embryonic fibroblast feeders (MEF) or in feeder-free conditions (Fig. 1A). Cytogenetics analysis was normal (Fig. 1B). These iPSCs exhibited typical markers of pluripotency with as expression of SSEA3, SSEA4 and TRA-1-60 (Fig. 1B). Cells also expressed Oct-4 at a high level (Fig. 1C). The 1901G > A missense mutation was confirmed at both genomic (Fig. 1D) and cDNA levels. RET expression on iPSC was demonstrated by FACS analysis using a phospho-RET antibody (Fig. 1E). A teratoma assay was performed to further confirm the pluripotent nature of the RET-mutated iPSC.

2. Materials and methods

2.1. Human iPS cell generation and culture

4 days before reprogramming, peripheral blood mononuclear cells (PBMCs) were thawed, cultured and expanded in Myelocult™ medium (Stemcell Technologies) supplemented with 1% penicillin-streptomycin (Life technologies), hSCF 100 ng/mL, hFLT-3100 ng/mL, hIL-3 20 ng/mL, hIL-6 20 ng/mL, and hIL7 20 ng/mL (all of them from Peprotech). 2×10^5 PBMC were then transduced overnight with Sendai viruses containing Oct3/4, Sox2, Klf4, and cMyc (CytoTune®-iPS Sendai Reprogramming Kit, Life technologies) each of them at multiplicity of infection (MOI) of 15. The next 2 days, medium was changed daily and cells were resuspended in expansion medium after 5 min centrifugation at 200 ×*g*. The third day, cells were recovered by centrifugation and plated on Mitomycin-C-treated mouse embryonic fibroblasts (MEF, CD1 strain) in expansion medium for 2 additional days. At day 6, half of the medium was changed to human pluripotent stem cell medium (hPSC medium) based on DMEM/F12 supplemented with 20% Knock Out Serum Replacer, 1 mM L-glutamine, 1% penicillin/streptomycin, 100 μM 2-mercaptoethanol (all of them from Life technologies) and 12.5 ng/mL basic FGF (Miltenyi Biotech). Then, the medium was changed daily with hPSC medium. At day 26, fully reprogrammed colonies were manually picked and transferred to freshly Mitomycin-C-treated MEF for amplification.

iPSC culture was performed according to two different procedures: in the presence of feeders or in feeder-free conditions. The feeder cultures were performed on Mitomycin C-treated MEF layer as described above with passaging every 7 days using 1 mg/mL collagenase IV in DMEM/F12 (Life technologies)); the feeder-free culture was performed on Geltrex™ (Life technologies) in Essential 8 medium (Life technologies) and 1% penicillin/streptomycin with passaging every 3–4 days using in DPBS (Life technologies) supplemented with 0.5 mM EDTA (Life technologies) and 1.8 mg/L NaCl (Sigma).

2.2. Karyotyping and mutation analysis

For conventional cytogenetics, cells grown in 60 mm plates (Corning) on Mitomycin-C treated MEF for 5 days were cultured 2 h in hPSC medium supplemented with 100 ng/mL Colcemid™ (Life technologies). Cells were detached from the plate by 5 min treatment with 1 mL per plate of TrypLE™ Express (Life technologies) at 37 °C, 5% CO_2. After trituration with a P1000 micropipet, 9 mL of 0.075 M KCl (Life technologies) was added to the cell suspension followed by incubation at 37 °C during 20 min to obtain an osmotic shock. Cell suspension was then prefixed by the addition of 1 mL of fixative composed of Methanol/Acetic Acid (3:1) followed by 10 min centrifugation at 130 ×*g*. Cell pellet was then resuspended in 10 mL of fixative and centrifuged 10 min at 130 ×*g* two times before a final resuspension in 10 mL of fixative. Cells were then spread on slides and stained with Giemsa. A minimum of 10 metaphases were captured and analysed using an automated imaging system (Meta Systems).

Genomic DNA from iPSC was extracted with DNeasy blood & Tissue kit (Ref 69504, Qiagen) according to the manufacturer instruction. Human Genomic DNA was used as control (Roche). *RET* exon 11 and surrounding intronic regions were PCR amplified with high-fidelity PCR polymerase (Herculase II fusion DNA polymerase, Agilent) using the following cycling parameter: 2 min of denaturation at 95 °C followed by 30 cycles with 10 s at 95 °C, 20 s at 55 °C and 30 °C at 72 °C and a final 3 min elongation step at 72 °C. Primers forward 5′-GCCAGCTGGTG TAATGAGCA-3′ and reverse 5′-ACCGTTCACTCACACCCTTG-3′ were used, leading to a 1058 bp amplicon (NC_000010.11, 43114189–43115246). This PCR product was then purified with exo SAP mix (0.5 μL Exonuclease I, Eurobio and 2 μL Shrimp Alkaline Phosphatase, Ozyme) incubation (15 min at 37 °C and then 15 min at 80 °C) associated with BigDye Xterminator kit used according to the manufacturer instruction (Applied Biosystems). Purified product was then subjected to sequencing reaction with BidDye Terminator kit (Applied biosystem): denaturation 5 min at 95 °C followed by 25 cycles with 10 s at 95 °C, 5 s at 50 °C and 4 min at 60 °C using either RET634_seq forward 5′-CAGAGCATACGCAGCCTGTA-3′ *RET*_seq reverse 5′-CTCCGGAAGGTC ATCTCAGC-3′ primers.

2.3. Pluripotency markers and ret expression

For flow cytometry analysis, iPSC colonies were recovered from Mitomycin-C inactivated MEF after incubation for 2 h in 1 mg/mL collagenase IV in DMEM/F12. After two cycles of washing/sedimentation with 10 mL DMEM/F12, colonies were washed once in Ca^{2+}/Mg2 + free PBS (Life technologies) and then dissociated into a single cell suspension by 10 min incubation in Hank's balanced enzyme-free cell dissociation buffer (Life Technologies). For pluripotency marker analysis, 1×10^5 cells were incubated in 10 μL PBS containing 1 μL Phycoerythrin-conjugated rat monoclonal antibody anti-SSEA3, 1 μL V450-conjugated mouse monoclonal anti-SSEA-4 and 1 μL Alexa647-conjugated mouse monoclonal antibody anti-TRA1-60 (all of them from BD Biosciences) or conjugated isotype control. For Ret expression analysis, 1×10^5 cells were incubated in 10 μL PBS containing 1 μL APC-conjugated mouse monoclonal antibody anti-Ret (R&D) or conjugated isotype control. Cells were analysed with a MACSQuant flowcytometer using the MACSQuantify software.

Immunostaining were also performed on colonies cultured in 8 wells Lab-Tek™ chambers (Nunc). Briefly, cells were fixed 20 min at room temperature in 4% paraformaldehyde in PBS and rinsed twice times with PBS. Cells were then permeabilized 5 min in 0.25% Triton-

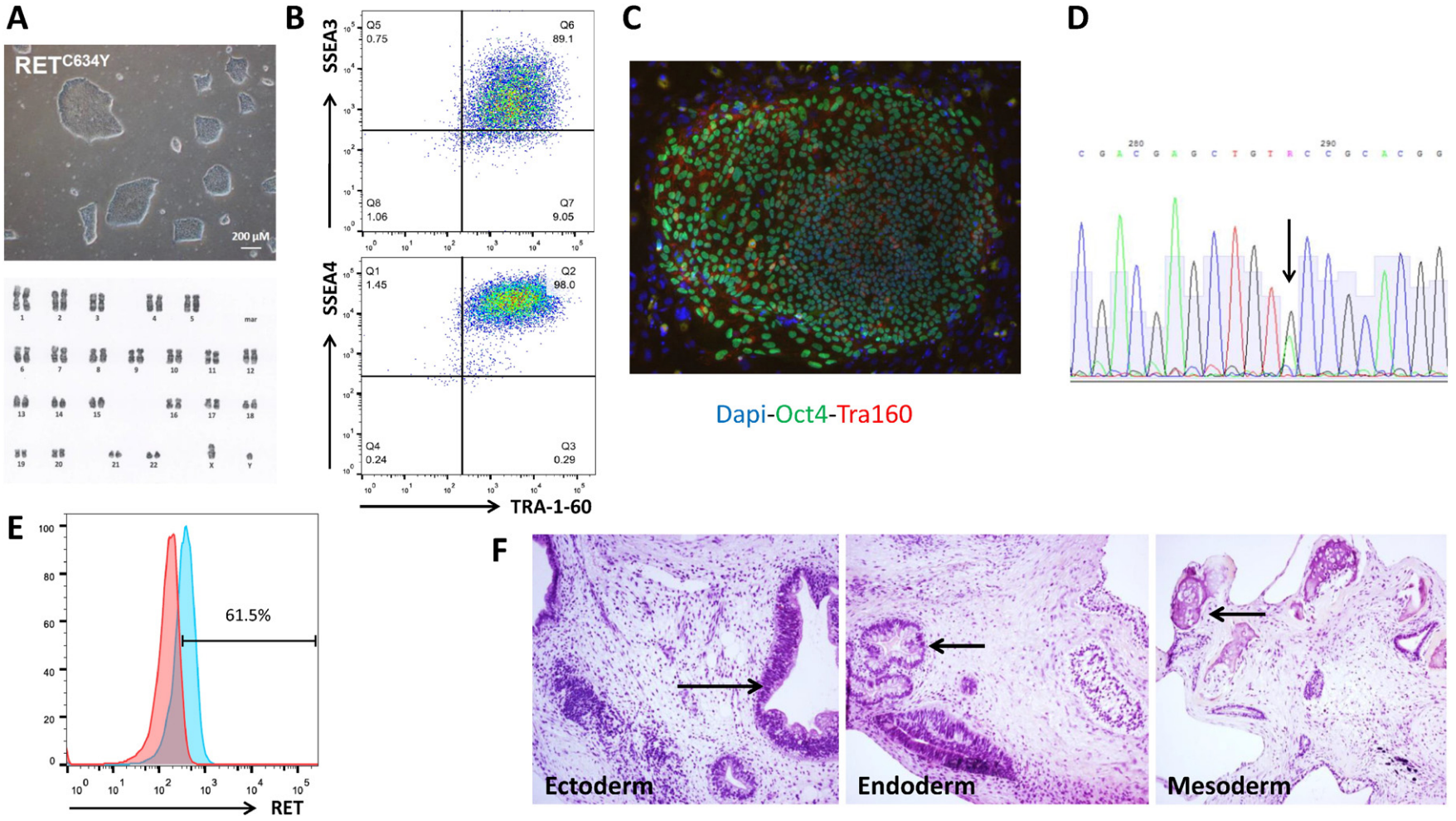

Fig. 1. Characterization of the *RET*^{C634Y} PB48 iPSC line A: Phase contrast imaging of PB48 colonies grown on matrigel (×4) and cytogenetics analysis of iPSC revealing a normal karyotype. B: FACS evaluation of pluripotency markers using SSEA3, SSEA4 and TRA-1-60 staining. C: Immunofluorescence analysis of Oct4 and TRA-1-60 expression (×20) D: C634Y mutation identified by Sanger sequencing in PB48 iPSC. E: RET expression in PB48 iPSC by flow cytometry using a phosphor-RET antibody. F: Pathological analysis of teratoma from PB48 iPS, showing a normal ectodermal, endodermal and mesodermal differentiation.

X100 (Sigma) in PBS and then rinsed twice in PBS. Wells were stained 45 min at room temperature in 100 μL PBS containing 1 μL rat monoclonal antibody anti-Tra160 (Millipore) and 1 μL rabbit polyclonal antibody anti-Oct-4 (Millipore) or 1 μL of each irrelevant isotype controls. After two washes in PBS, cells were stained 45 min at room temperature in 100 μL PBS containing 0.2 μL Alexa488 conjugated goat anti-rabbit antibody and 0.2 μL Alexa594 conjugated goat anti-mouse IgM antibody (both from Life technologies). Wells were washed twice in PBS and mounted in Dapi containing mounting medium (Vector Laboratories) before analysis on a Nikon 90i fluorescent microscope.

2.4. In vivo pluripotency analysis by teratoma formation

MEN2A-iPSCs were subjected to teratoma formation assay by intramuscular injection of NSG mice ($2 \cdot 10^6$ cells in 150 μL of Geltrex/DMEMEF12 1:1 dilution) and pathological analysis after 8 to 10 weeks of tumour growth. Teratoma formation assays were performed in triplicate in order to analyse the development of potentially malignant tumor tissue within teratomas. At +2 months after injections, RET-mutated iPSC gave rise to teratomas, thus confirming the pluripotency of these cells (Fig. 1D). Pathological analyses showed the presence of a normal endodermal, ectodermal and mesodermal differentiation with no malignant tissue identified in several teratomas analysed (Fig. 1F).

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