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Lab Resource: Stem Cell Line

Generation of a human control iPSC line with a European mitochondrial haplogroup U background☆

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ABSTRACT

Human iPSC line N44SV.5 was generated from primary normal human dermal fibroblasts belonging to the European mitochondrial haplogroup U. For this purpose, reprogramming factors Oct3/4, Sox2, Klf4, and cMyc were delivered using a non-integrative methodology that involves the use of Sendai virus.

Name of stem cell line	N44SV.5
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Person who created resource	Teresa Galera
Contact person and email	M. Esther Gallardo, egallardo@iib.uam.es
Date archived/stock date	April 15, 2014
Origin	Fibroblasts isolated from normal human juvenile foreskin cells (Promocell; C12300)
Type of resource	Biological reagent: Induced pluripotent stem cells (iPSC) from normal human dermal fibroblasts belonging to the European mitochondrial haplogroup U
Sub-type	Cell line
Key transcription factors	Oct3/4, Sox2, cMyc, Klf4
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	None
Information in public databases	None

1. Resource details

To generate the human iPSC line, N44SV.5, non-integrative Sendai viruses containing the reprogramming factors Oct3/4, Sox2, cMyc, Klf4 have been employed (Takahashi et al., 2007). For this purpose, commercially available fibroblasts from a healthy juvenile donor were acquired in Promocell (C12300). Subsequently, mitochondrial haplogroup determination was carried out in the N44SV.5 line and in the original fibroblasts by PCR amplification, followed by restriction fragment length polymorphism analysis. The obtained restriction pattern analysis clearly showed that fibroblasts and N44SV.5 belong to the European mitochondrial haplogroup U (Fig 1A). N44SV.5 iPSC colonies displayed a typical ES-like colony morphology and growth

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behavior (Fig. 1B) and they stained positive for alkaline phosphatase activity (Fig. 1C). We confirmed the clearance of the vectors and the exogenous reprogramming factor genes by RT-PCR after eight culture passages (Fig 1D). The endogenous expression of the pluripotency-associated transcription factors OCT4, SOX2, KLF4, NANOG, CRIPTO, and REX1 was also evaluated by RT-PCR (Fig. 1E). Immunofluorescence analysis revealed expression of transcription factors OCT4, NANOG, SOX2 and surface markers SSEA3, SSEA4, TRA1-60, and TRA1-81 characteristics of pluripotent ES cells (Fig. 1F). Promoters of the pluripotency-associated genes, OCT4 and NANOG, heavily methylated in the original fibroblasts were almost demethylated in the N44SV.5 line suggesting an epigenetic reprogramming to pluripotency (Fig. 1G). The iPSC line has been adapted to feeder-free culture conditions and displays a normal karyotype (46, XY) after more than twenty culture passages (Fig. 1H). We also confirmed by DNA fingerprinting analysis that the line N44SV.5 was derived from the original fibroblasts (Fig. 1I). Finally, the capacity of the generated iPSC line to differentiate into the three germ layers (endoderm, mesoderm, and ectoderm) was tested in vitro using an embryoid body based assay (Fig. 1J).

2. Materials and methods

2.1. Non-integrative reprogramming of normal human dermal fibroblasts into iPSC

All the experimental protocols included in the present study were approved by the Institutional Ethical Committee of the Autonoma University of Madrid according to Spanish and European Union legislation. Commercially available normal human dermal fibroblasts isolated from the dermis of juvenile foreskin were purchased from Promocell (C12300). These fibroblasts were reprogrammed using the CytoTune-iPS 2.0 Sendai reprogramming kit following the instructions of the manufacturer. After seven passages of the iPSC line, silencing of the exogenous reprogramming factors genes and sendai virus genome was confirmed by RT-PCR following the manufacturer's instructions. N44SV.5 was maintained and expanded both on feeder layers and on feeder-free layers. In the first case, irradiated human fibroblasts feeders with ES medium containing Knockout DMEM (Life technologies), Knockout serum replacement 20% (Life technologies), MEM non-essential amino acids solution 1X (Life technologies), GlutaMAX 1X (Life technologies), β-mercaptoethanol (100 μM), penicillin/streptomycin 1X (Life technologies), and bFGF (4 ng/ml) (Miltenyi Biotec) were used. Subsequently, N44SV.5 was adapted and cultured in feeder-free conditions on matrigel (354277, Corning) with mTeSR1 medium (StemCell) following the recommendations of the manufacturer. For the propagation of the line, both enzymatic (dispase, collagenase IV, and accumax; Millipore) and mechanical procedures have been used.

2.2. Phosphatase alkaline analysis

The iPSC line N44SV.5 was seeded on a feeder layer plate. After 4 days, direct phosphatase alkaline activity was determined using the phosphatase alkaline blue membrane substrate solution kit (Sigma, AB0300) following the instructions of the manufacturer.

2.3. Mitochondrial haplogroup genotyping

Total DNA from fibroblasts and N44SV.5 was extracted using a standard phenol-chloroform protocol. The samples were haplogrouped by PCR amplification of short mtDNA fragments, followed by restriction fragment length polymorphism (RFLP) analysis to assess the mtDNA haplogroup, as previously described (Gallardo et al., 2012). Primers, PCR, and RFLP conditions are available upon request.

2.4. qPCR analyses.

Total mRNA was isolated using TRIZOL and 1 μg was used to synthesize cDNA using the Quantitect reverse transcription cDNA synthesis kit. One microliter of the reaction was used to quantify by qPCR the expression of the endogenous pluripotency-associated genes (OCT4, SOX2, KLF4, NANOG, CRIPTO, and REX1). Primers sequences were described by Aasen et al., 2008. All the expression values were normalized to the GAPDH housekeeping gene. Plots are representative of at least three independent experiments.

2.5. Bisulfite pyrosequencing

Bisulfite modification of genomic DNA was performed with the EZ DNA Methylation-Gold kit (Zymo Research) following the manufacturer's instructions. The set of primers for PCR amplification and sequencing of NANOG and OCT4 were designed using the software PyroMark Assay Design (version 2.0.01.15; Qiagen): Forward-NANOG (5′-TAT TGG GAT TAT AGG GGT GGG TTA-3′), Reverse-NANOG (5′-[Btn]-CCC AAC AAC AAA TAC TTC TAA ATT CAC-3′), and sequencing primer S-NANOG (5′-ATA GGG GTG GGT TAT-3′); Forward-OCT4_prox (5′-GGG GTT AGA GGT TAA GGT TAG TG-3′), Reverse-OCT4_prox (5′-[Btn]-ACC CCC CTA ACC CAT CAC-3′), and sequencing primer S-OCT4_prox (5′-GGG GTT GAG TAG TTT-3′); Forward-OCT4_dist (5′-TTT TTG TGG GGG ATT TGT ATT GA-3′), Reverse-OCT4_dist (5´-[Btn]-AAA CTA CTC AAC CCC TCT CT-3′), and sequencing primer S-OCT4_dist (5′-ATT TGT ATT GAG GTT TTG GA-3′). PCR was performed with primers biotinylated to convert the PCR product to single-stranded DNA templates, using the Vacuum Prep Tool. After PCR amplification, pyrosequencing reactions and methylation quantification were performed using PyroMark Q24 reagents, equipment, and software (version 2.0.6; Qiagen), according to the manufacturer's instructions.

2.6. Karyotype analysis

Karyotype analyses of the iPSC line were carried out using cells with more than twenty culture passages. These cells were processed using standard cytogenetic techniques. Briefly, cells were treated with 10μg/mL of Colcemid (Gibco) for 90 min at 37 °C, trypsinized, treated with 0.075 M hypotonic KCl solution, and fixed with Carnoy's fixative. Cells were then dropped on a microscope glass slide and dried. Metaphase cells were G banded using Wright staining. At least 20 metaphases were karyotyped.

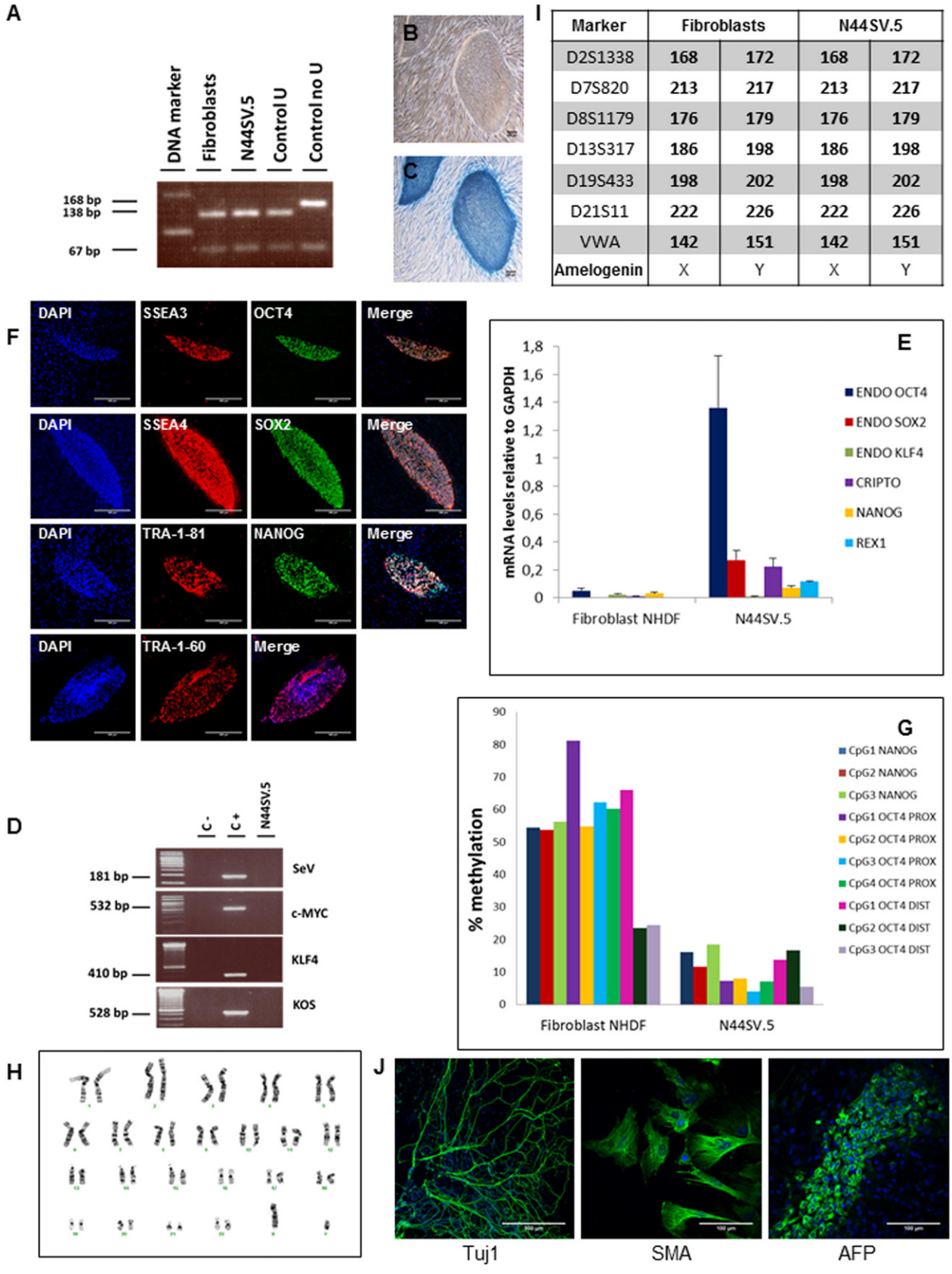
2.7. Immunofluorescence analysis

Cells were grown on 0.1% gelatin-coated P35 culture plates (81156, Ibidi) and fixed with 4% paraformaldehyde. The following antibodies for the staining were used: TRA-1-60 (Millipore; MAB4360; 1:150); TRA-1-81 (Millipore; MAB4381; 1:150); SOX2, (Thermo Scientific; PA1-16,968; 1:100); NANOG (R&D Systems; AF1997; 1:25); SSEA-4 (Millipore; MAB4304; 1:10); SSEA-3 (Millipore; MAB4303; 1:10); OCT4 (Santa Cruz Biotechnology; Sc-5279; 1:100); neuron-specific class III beta-tubulin (Tuj1) (Sigma, T8660, 1:300), α-fetoprotein (AFP) (Sigma, WH0000174M1, 1:300), smooth muscle alpha actin (SMA) (Sigma, A2547, 1:400). Secondary antibodies used were all from the Alexa Fluor Series (1:500) or from Jackson Immunoresearch (Cy2, 1:50; Cy3, 1:250). Images were taken using a Zeiss confocal microscope.

2.8. In vitro differentiation assay

The in vitro pluripotency capacity of the iPSC line was tested by spontaneous embryoid body differentiation. For this purpose, iPSCs from a P100 plate with 80% of confluency were dissociated into a single cell suspension with accumax (SCR006,Millipore) and resuspended in 12 ml of mTeSR1 medium (Stemcell). Embryoid body formation was

induced by seeding 120 μl of the iPSC suspension in each well of 96-well v-bottom low attachment plates and centrifuging the plates at 800 g for 10 min to aggregate the cells. After 2–3 days, the embryoid bodies were transferred to an untreated P60 culture plate for 2–4 days. Subsequently, the embryoid bodies were transferred to 0.1% gelatin-coated P35 culture plates (81156, Ibidi) and cultured in differentiation medium (DMEM F12



supplemented with 20% fetal bovine serum, 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1X non-essential amino acids, and 1X penicillin-streptomycin, all from Invitrogen) for 2–3 weeks to allow spontaneous endoderm formation. For mesoderm differentiation, iPSCs were maintained for 2–3 weeks in differentiation medium supplemented with 100 μM ascorbic acid (A4403, Sigma–Aldrich). For ectoderm differentiation, embryoid bodies were transferred to matrigel-coated P35 culture plates and cultured in a special differentiation medium containing (50% DMEM F12, 50% neurobasal medium, 1X GlutaMAX, 1X penicillin/streptomycin, non-essential aminoacids, 0.1 mM 2-mercaptoethanol, 1X N2 supplement, and 1X B27 supplement, all from Invitrogen). In all the cases, the medium was changed every other day.

2.9. DNA fingerprinting analysis

For DNA fingerprinting analysis, highly polymorphic regions containing short tandem repeated sequences of DNA have been evaluated. For this purpose, the following markers (D13S317, D7S820, VWA, D8S1179, D21S11, D19S433, D2S1338, and amelogenin for sex determination) have been amplified by PCR and analyzed by ABI PRISM 3100 Genetic analyzer and Peak Scanner v3.5 (Applied Biosystems). Primer sequences and PCR conditions are available upon request.

Author disclosure statement

There are no competing financial interests in this study.

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Fig. 1. Molecular and functional characterization of the control N44SV.5 iPSC line. 1A. Mitochondrial haplogroup genotyping. PCR-amplified mtDNA fragments were digested with HinfI and separated by gel electrophoresis. Amplified fragments of 235 bp are cut into 30 bp, 67 bp, and 138 bp fragments, after digestion with HinfI, when sample belongs to haplogroup U. When sample belongs to a different haplogroup, two fragments are expected: 67 bp and 168 bp. Lanes: 1. DNA marker, 2. Original fibroblasts, 3. N44SV.5 iPSC line, 4. Haplogroup U control DNA sample, 5. Haplogroup no U control DNA sample. 1B. Typical ES-like colony morphology of the N44SV.5 line. 1C. Positive phosphatase alkaline staining of the N44SV.5 line. 1D. RT-PCR for detecting the clearance of the vectors and the exogenous reprogramming factor genes. 1E. QPCR showing the expression of the pluripotency associated markers NANOG, OCT4, SOX2, KLF4, CRIPTO, and REX1. 1F. Immunofluorescence analysis showing expression of typical pluripotent ES cell markers such as the transcription factors OCT4, NANOG, SOX2, and the surface markers SSEA3, SSEA4, Tra1-60, and Tra1-81; scale bars: 300 μm. 1G. Bisulfite pyrosequencing of the OCT4 and NANOG promoters. The promoters of the transcription factors, OCT4 and NANOG, were almost demethylated in the generated iPSC line. 1H. Karyotype analysis. N44SV.5 has a normal karyotype (46, XY). 1I. DNA fingerprinting analysis showing that N44SV.5 comes from the original fibroblasts. 1J. Embryoid body based in vitro differentiation assays. N44SV.5 differentiates into all three germ layers, demonstrated by positive AFP endoderm staining (I), positive Tuj1 ectoderm staining, and positive SMA mesoderm staining.