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Lab resource: Stem cell line

Generation of human iPS cell line ihFib3.2 from dermal fibroblasts

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ABSTRACT

The human ihFib3.2 iPS cell line was generated from dermal fibroblasts obtained from a healthy donor. Lentiviral particles were produced with the polycistronic hSTEMCCA vector with Oct4, Sox2, cMyc and Klf4 as reprogramming factors.

Name of stem cell construct	ihFib3.2
Institution	Federal University of Rio de Janeiro — UFRJ
Person who created resource	Fernanda Cristina Paccola Mesquita
Contact person and email	Adriana Bastos Carvalho, carvalhoab@biof.ufrj.br
Date archived/stock date	08/2013
Origin	Human dermal fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPS); generated by reprogramming
Sub-type	Cell line
Key transcription factors	Oct4, Sox2, cMyc, Klf4 (hSTEMCCA)
Authentication	Identity and purity of cell line confirmed (Figs. 1 and 2)
Link to related literature (direct URL links and full references)	Not available
Information in public databases	Not available

1. Resource details

Lentiviral particles were produced with hSTEMCCA, VSV-G, Gag/Pol, Tat and Rev and the first colonies emerged 12 days after transduction (Fig. 1A–C). The hSTEMCCA transgene was silenced in ihFib3.2 at passage 7 (Fig. 1G).

The expression of pluripotent stem cell markers was confirmed by RT-PCR (OCT4, SOX2, NANOG, REX1, KLF4, DNMT3B, TDGF, GDF3, LIN28 and NODAL) (Fig. 1H), flow cytometry (OCT4, NANOG, SOX2, SSEA4, TRA-1–60 and TRA-1–81) (Fig. 1I) and immunofluorescence (OCT4, NANOG, SOX2, cMYC, SSEA4, TRA-1–60 and LIN28) (Fig. 1J). Moreover, GTG banding revealed a normal karyotype (46, XX) (Fig. 1F).

To demonstrate differentiation into the three embryonic germ layers, ihFib3.2 were aggregated to form embryoid bodies and allowed to attach to tissue culture plates (Fig. 2A–B). Spontaneous differentiation induced the transcription of the following genes: NES and TUBB3 (ectoderm), T, BMP4 and MSX1 (mesoderm), and AFP, GATA6 and SOX17 (endoderm) (Fig. 2C). In addition, the formation of the three germ layers was confirmed at the protein level by immunofluorescence, which demonstrated the expression of Nestin, Brachyury (Fig. 2D) and α-fetoprotein (Fig. 2E).

2. Materials and methods

2.1. Human dermal fibroblast reprogramming

Tissue fragments were obtained from a skin biopsy after informed consent. Small fragments were directly plated in DMEM high glucose with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Life Technologies). Human fibroblasts (hFib) spontaneously migrated to tissue culture plates and were expanded at 37 °C in 5% CO_2 until the third passage. The experimental protocol was approved by the research ethics board of the National Institute of Cardiology under number 24138414.1.0000.5272.

Recombinant lentiviral particles were produced in HEK293FT packaging cell line (Life Techonologies) using FuGene 6 transfection reagent (Roche Life Sciences) and the following vectors: hSTEMCCA, VSV-G, Gag/Pol, Tat and Rev. hSTEMCCA was kindly donated to us by Dr. Gustavo Mostoslavsky from Boston University (Somers et al. 2010). The culture medium containing the lentiviral particles was collected for three days and viruses were concentrated by ultracentrifugation. Titration was performed by transducing HEK293FT cells and verifying OCT4 expression by flow cytometry after 72 h.

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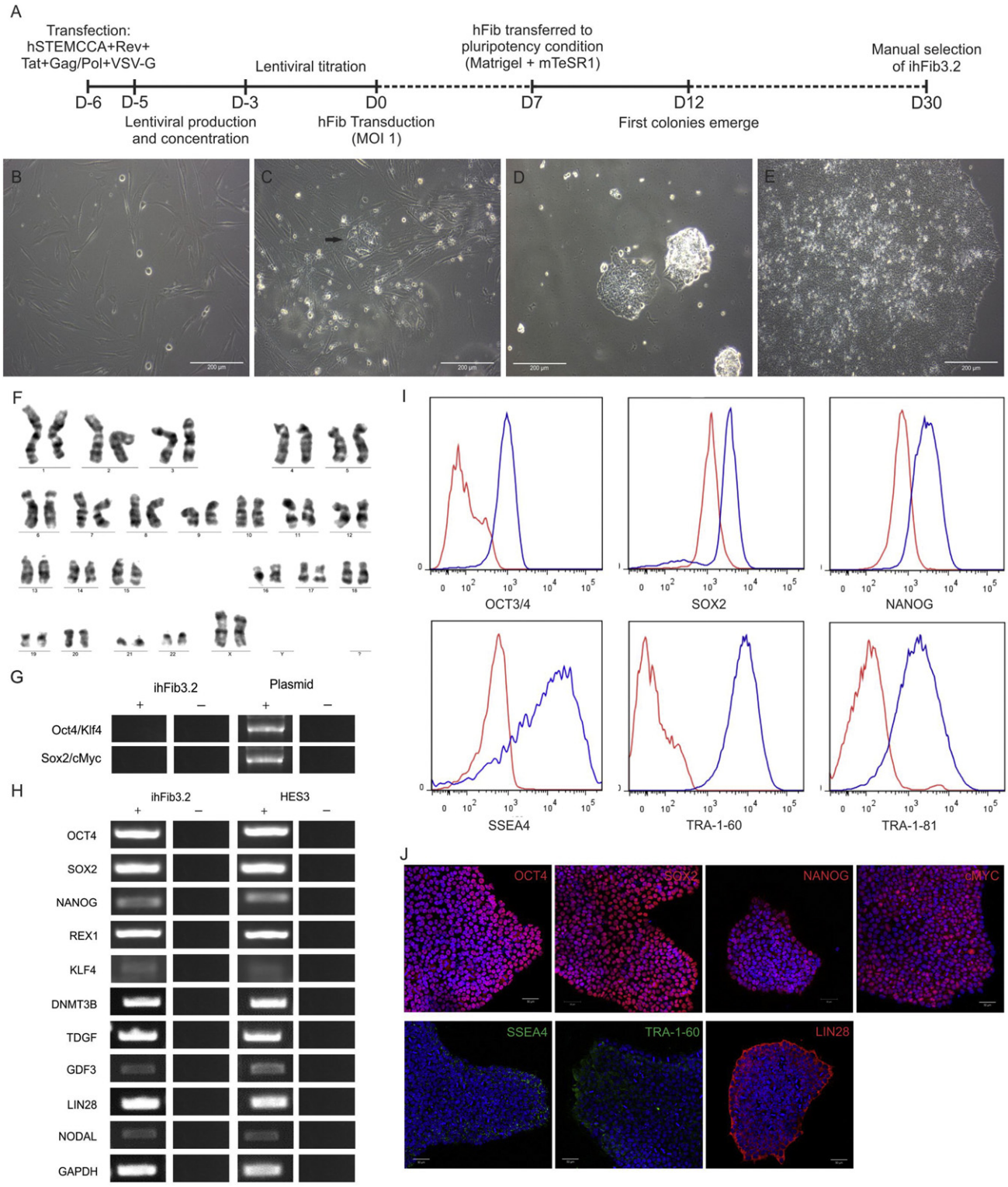

Fig. 1. Generation and characterization of ihFib3.2. (A) Schematic protocol of iPSC generation using hSTEMCAA vector. (B) hFibs 24 h after transduction. Colonies started to emerge after 12 days (C) and clones were selected (D). (E) ihFib3.2 after 6 passages. (F) Karyotype was normal (46, XX) at passage 6. (G) Transgenes were absent after 7 passages. The hSTEMCCA plasmid was used as positive control. (+) indicates samples that were reverse transcribed and (−) indicates non reverse transcription controls. (H) Expression of pluripotency-related molecules by RT-PCR. GAPDH was used as endogenous control and the human embryonic stem cell line HES3 was used as positive control. (I) Flow cytometry demonstrated the presence of high levels of OCT4 (90.7%), SOX2 (84.7%), NANOG (97.7%), SSEA4 (78.5%), TRA-1–60 (98.2%) and TRA-1–81 (79.2%). Red histograms represent isotype controls. (J) Expressions of pluripotency markers by immunofluorescence: OCT4, SOX2, NANOG, cMYC and LIN28 are shown in red, SSEA4 and TRA-1–60 are shown in green and nuclei are stained in blue.

One hundred thousand hFib were plated in a 6-well plate for 16 h and transduced with lentiviral particles at multiplicity of infection (MOI) 1 with 8 μg/mL of polybrene (Merck Millipore). Viruses were removed after 24 h and the culture medium was changed every other day. After 7 days, cells were transferred to plates treated with hESC-qualified Matrigel™ (BD Biosciences) and cultured in mTeSR1 defined

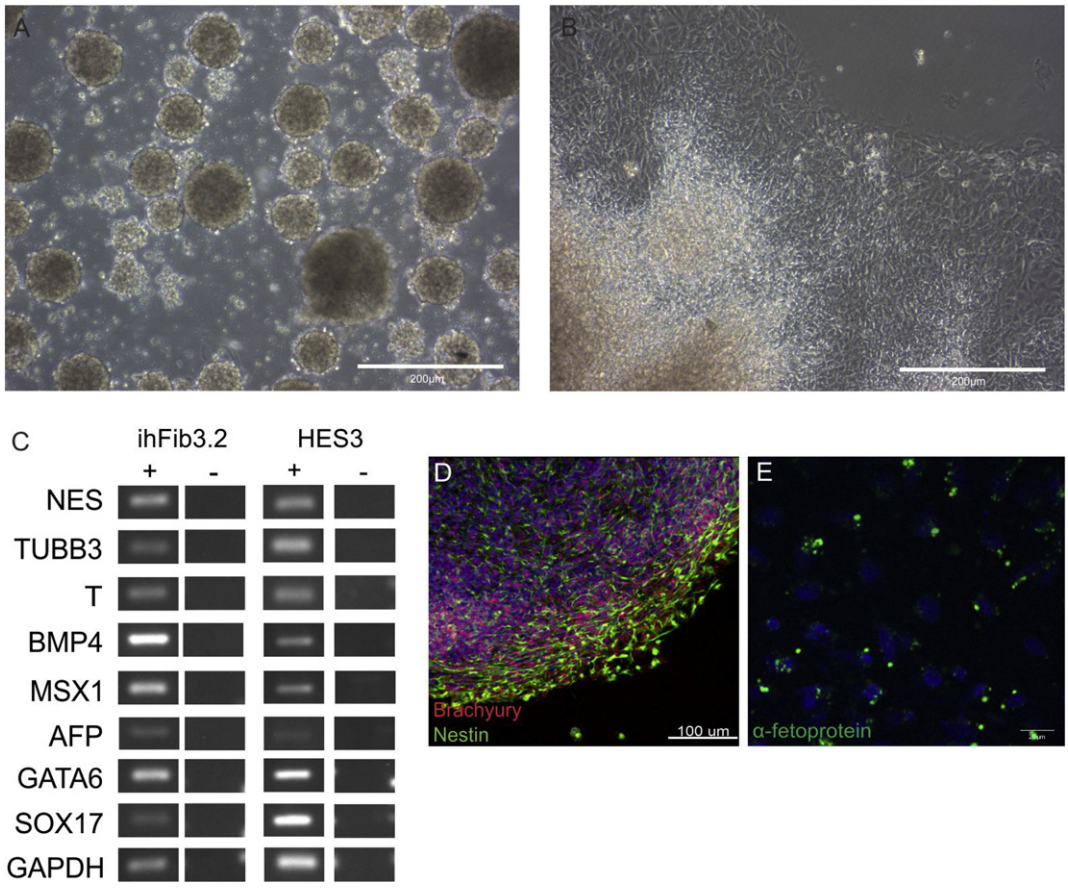

Fig. 2. *In vitro* differentiation of ihFib3.2. (A) Floating embryoid bodies 7 days after aggregation. (B) EBs were attached to plastic and adherent cells started to migrate. (C) Expression of molecules characteristic of the three embryonic germ layers by RT-PCR. GAPDH was used as endogenous control and the human embryonic stem cell line HES3 was used as positive control. (+) indicates samples that were reverse transcribed and (−) indicates non reverse transcription controls. Ectoderm, mesoderm and endoderm differentiation was confirmed by the expression of Nestin (D, green), Brachyury (D, red) and α-fetoprotein (E, green) respectively. Nuclei are shown in blue.

medium (STEMCELL Technologies). iPSC clones were manually selected based on morphology after 30 days of transduction. Clones were expanded in Matrigel™ and mTeSR1 for characterization.

2.2. Karyotyping

For karyotype analysis, iPSCs were treated with 0.05 µg/mL of KaryoMAX® Colcemid™ solution (Life Technologies) in mTeSR1 for 30 min. Cells were dissociated, submitted to a hypotonic treatment with 0.57 M potassium chloride and fixed in methanol and acetic acid. GTG banding was performed and slides were visualized and digitally captured in a Leica DM 2000 microscope. Cytogenetics software LUCIA-KARYO was used for analysis.

2.3. RT-PCR

RNA was extracted using RNeasy Mini kit (QIAGEN) and quantified by spectrophotometry with NanoDrop. Reverse transcription was performed using the High-Capacity Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. For PCR amplification, GoTaq® Flexi DNA Polymerase (Promega) and the primers described in Table 1 were used. PCR products were analyzed by electrophoresis in agarose gels stained with SYBR® Safe (Life Technologies).

2.4. Flow cytometry

Cells were dissociated, blocked with 0.5% of bovine serum albumin (BSA) in PBS and stained with the following antibodies for 20 min at 4 °C: TRA-1–60, TRA-1–81 and SSEA4 (BD Biosciences). For intracellular staining, cells were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.3% Triton X-100 in PBS for 40 min. The following antibodies were used: OCT4, SOX2 and NANOG (BD Biosciences). Isotypes were used as negative controls. Dead cells were excluded by DAPI staining and samples were analyzed using BD FACSAria IIu and Flow Jo v10 software.

2.5. Immunofluorescence

iPSCs were plated in glass slides pre-treated with Matrigel™ and cultured in mTeSR1. After 3 days, samples were fixed in 4% paraformaldehyde for 20 min at room temperature. The following antibodies were used after three 10-min permeabilizations with 0.3% Triton X-100 in PBS: OCT4, SOX2, NANOG and cMYC (cell signaling). The following antibodies were used after one 10-min permeabilization with 0.05% Triton X-100 in PBS: SSEA4, TRA-1–60, LIN28, Brachyury, AFP (Cell Signaling), and Nestin (Millipore). Primary antibodies were incubated at 4 °C for 12 h. Then, cells were washed and incubated for 2 h at room temperature with Cy3-AffiniPure Donkey Anti-Rabbit (Jackson Immunoresearch) and/or Alexa Fluor® 488 Goat Anti-Mouse (Life Technologies) secondary antibodies associated to TO-PRO®-3 (Life Technologies) for nuclei counterstaining. Slides were mounted in Vectashield® Mounting Medium (Vector Laboratories) and visualized in confocal microscope Zeiss LSM 510 Meta. Image processing was performed with ZEN 2009 software.

Table 1
List of primers used to amplify transgenes, pluripotency and *in vitro* differentiation markers by RT-PCR.

Primer	Foward reverse
Oct4/Klf4 (hSTEMCCA)	5' - CAACGAGAGGATTTTGAGGCTGC - 3' 5' - ATCGTTGAACTCCTCGGTCTCTCT - 3′
Sox2/cMyc (hSTEMCCA)	5' - TTGGCTCCATGGGTTCGGTG - 3' 5' - AAGGGTGTGACCGCAACGTAGG - 3'
OCT4	5' - AGCCTGAGGGCGAAGCAGGA - 3' 5' - CCCCAGGGTGAGCCCCACAT - 3'
NANOG	5' - CAGCCCTGATTCTTCCACCAGTCCC - 3' 5' - TGGAAGGTTCCCAGTCGGGTTCACC - 3'
SOX2	5' - AGCTACAGCATGATGCAGGA - 3' 5' - GGTCATGGAGTTGTACTGCA - 3'
GDF3	5' - CTTATGCTACGTAAAGGAGCTGGG - 3' 5' - GTGCCAACCCAGGTCCCGGAAGTT - 3'
DNMT3B	5' - TGCTGCTCACAGGGCCCGATACTTC - 3' 5' - TCCTTTCGAGCTCAGTGCACCACAAAAC - 3'
KLF4	5' - TCTCAAGGCACACCTGCGAA - 3' 5' – TAGTGCCTGGTCAGTTCATC - 3'
DPPA4	5' - GGAGCCGCCTGCCCTGGAAAATTC - 3' 5' - TTTTTCCTGATATTCTATTCCCAT - 3'
REX1	5' - CAGATCCTAAACAGCTCGCAGAAT - 3' 5' - GCGTACGCAAATTAAAGTCCAGA - 3'
NODAL	5' - GGGCAAGAGGCACCGTCGACATCA - 3' 5' - GGGACTCGGTGGGGCTGGTAACGTTTC - 3'
LIN28	5' - CAAAAGGAAAGAGCATGCAGAA - 3' 5' - ATGATCTAGACCTCCAGAGTTGTAGC - 3'
TDGF	5' - GATATCTCAGCAAACAAGTTTGCCA - 3' 5' – GGCAGGTCACTCAGGTTATTGTTGC - 3'
NES	5' - CACCTCAAGATGTCCCTCAG - 3' 5 - AGCAAAGATCCAAGACGCC - 3'
TUBB3	5' - GCTCAGGGGCCTTTGGACATCTCTT - 3' 5' - TTTTCACACTCCTTCCGCACCACATC - 3'
MSX1	5' - CGAGAGGACCCCGTGGATGCAGAG - 3' 5' - GGCGGCCATCTTCAGCTTCTCCAG - 3'
T	5' - GCCCTCTCCCTCCCCTCCACGCACAG - 3' 5' - CGGCGCCGTTGCTCACAGACCACAGG - 3'
BMP4	5' - GCACTGGTCTTGAGTATCCTG - 3' 5' - TGCTGAGGTTAAAGAGGAAACG - 3'
GATA6	5' - CCAACTGTCACACCACAAC - 3' 5' - TGGGGGAAGTATTTTTGCTG - 3'
AFP	5' - GAATGCTGCAAACTGACCACGCTGGAAC - 3' 5' - TGGCATTCAAGAGGGTTTTCAGTCTGGA - 3'
Sox17	5' - GACGACCAGAGCCAGACC - 3' 5' - CGCCTCGCCCTTCACC - 3'
GAPDH	5' - ACCATGGGGAAGGTGAAGGT - 3′ 5' - CATGGGTGGAATCATATTGG - 3'

2.6. In vitro differentiation of iPSC

For formation of Embryoid Bodies (EBs), iPSCs were incubated with 1 mg/mL of collagenase I (Sigma-Aldrich) in PBS with Ca^{++} and Mg^{++} and 20% FBS for 20 min at 37 °C. Subsequently, cells were dissociated to form small aggregates of 10 to 20 cells by 0.05% Trypsin-EDTA (Life Technologies) digestion and mechanical scrapping. Aggregates were plated in ultra-low attachment plates (Corning) at 37 °C and 5% CO_2 with the following basal culture medium: StemPro®-34 SFM (Life Technologies), 1% glutamine, 1% penicillin–streptomycin, 150 μg/mL of transferrin (Roche Life Sciences), 0.039 μL/mL of monothioglycerol (Sigma-Aldrich), 50 μg/mL ascorbic acid (Sigma-Aldrich). In addition, only for this 24-h aggregation step, cells were cultivated with 1 μM of ROCK inhibitor Y-27,632 dihydrochloride (R&D Systems) (Kattman et al. 2011).

For spontaneous differentiation into the three embryonic germ layers, embryoid bodies (EBs) were cultured in suspension for 7 days in the basal culture medium described above. After this, EBs were transferred back to adherent plates and cultured for another 7 days (Kehat et al. 2001). Differentiated cells were analyzed by RT-PCR and immunofluorescence.

Acknowledgements

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