iPSCs characterization in terms of pluripotency

Pluripotency of iPSCs was determined by quantitative real-time PCR (qRT-PCR) to detect gene expression of the pluripotent transcription factors OCT-4, SOX-2 and NANOG, and also by immunofluorescence to detect the protein expression of the same transcrition factors, as well as of the cell surface epitope TRA-1-60.

qRT-PCR

For qRT-PCR, the expression of the pluripotency genes *OCT-4*, *SOX-2* and *NANOG* was determined in ML II iPSCs at passages 12 and 20, and in the same ML II Fibroblast line, at passage 6, that originated the iPSCs. TaqMan® Gene Expression assays labeled with FAM were used (OCT-4/Pou5F1: hs00999632_g1; SOX2: Hs01053049_s1; NANOG Hs02387400_g1;). The expression levels were normalized for the *GAPDH* housekeeping gene (TaqMan® assay hs02786624_g1). Briefly, RNA from iPSCs and fibroblastos was extracted with TRIzol™ Reagent (Invitrogen, ThermoScientific) followed by reverse transcription from 1 µg of RNA using the NZY M-MuLV First-Strand cDNA Synthesis Kit (MB17201, NZYTech). For the qPCR reaction, three independent experiments with two technical replicates were done using the NZYSupreme qPCR Probe Master Mix (MB41601, NZYTech). The reaction was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories). Data were processed using Bio-Rad CFX® Manager Software 3.1 (Bio-Rad Laboratories). Ct-values were normalized to the housekeeping gene *GAPDH* using the standard ΔCt method and relative quantification of the levels of expression in iPSCs compared to the fibroblasts was performed using the ΔΔCt-method. The mean and standard deviation (SD) of the relative expression ($2^{-\Delta\Delta Ct}$) were calculated.

Immunofluorescence

Briefly, ML II iPSCs were cultured for three to four days in 8-well chamber slide coated with vitronectin prior to the immunocytochemical protocol, which was done using the Fluorescent Human ES/iPS Cell Characterization Kit (SCR078, Merk Milipore) according to the manufacturer's instructions. The antibodies included in the kit [mouse anti-Oct-4 (POU5f1), clone 7F9.2, Alexa Fluor® 488 conjugate; mouse anti-Sox-2, clone 10H9.1, Cy3 conjugate; mouse anti-Nanog, clone 7F7.1, Alexa Fluor® 488 conjugate; mouse anti-TRA-1-60, clone TRA-1-60, Cy3 conjugate] were diluted 1:100 in the appropriate blocking buffer. The slides were mounted in VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories). Cells were analyzed on DM4000 M fluorescence microscope (Leica).