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Safety of GMP-compliant iPSC lines generated by Sendai virus transduction is dependent upon clone identity and sex of the donor

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Abstract

Human induced pluripotent stem cells (hiPSCs) are a potential source of somatic cells for cell therapies due to their ability to self-renew and differentiate into various cells of the body. To date, the clinical application of hiPSCs has been limited due to safety issues. The present study aims to standardize the safety procedure of the derivation of GMP-compliant induced pluripotent stem cell (iPSC) lines from human fibroblasts. The hiPSC lines were generated using the nonintegrative Sendai virus method to incorporate Yamanaka reprogramming factors (OCT3/4, SOX2, KLF4 and c-MYC) into cells. A constant temperature was maintained during the cell culture, including all stages of the culture after transduction with Sendai virus. Pluripotency was proved in six independently generated hiPSC lines from adult female (47 years old) and male (57 years old) donors' derived fibroblasts via alkaline phosphatase live (ALP) staining, qPCR, and immunocytochemistry. The hiPSC lines showed a gradual decrease in the presence of the virus with each subsequent passage, and this reduction was specific to the hiPSC line. The frequency and probability of chromosomal aberrations in hiPSCs were dependent on both the iPSC clone identity and sex of the donor. In summary, the generation of hiPSC for clinical applications requires safety standards application (biosafety protocol, quality control of hiPSC lines, viral and genetic integrity screening) from the first stages of the clonal selection of hiPSC from the same donor.

Key words: *human induced pluripotent stem cell (hiPSC), iPSC lines, Yamanaka factors, iPSC GMP-compliant, Sendai virus transduction, safety of iPSC lines, sex-specific hiPSCs.*

Introduction

The groundbreaking advancement in the field of stem cells by introducing somatic stem cell reprogramming procedure and derivation of human induced pluripotent stem cells (hiPSCs) offered new possibilities in cell therapies. Human iPSCs can serve as an autologous self-renewing source with the ability to differentiate towards a specific cell type under defined culture conditions. However, the development of therapies is not as advanced as expected, with only a few worldwide therapeutic clinical trials using autologous induced pluripotent stem cell (iPSC)-derived differentiated cells, so far [19].

Progress in the application of hiPSCs in treatments is mainly hampered by safety concerns. In particular, tumorigenicity, immunogenicity, and heterogeneity are the three main impediments to potential therapy with

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iPSCs [54]. However, the risk of these side effects associated with the use of hiPSCs can be reduced during the generation and selection phase of hiPSC lines since individual hiPSCs are heterogeneous in terms of different morphology, growth rate, genetic abnormalities, epigenetic changes, and altered differentiation potentials [54].

Reprogramming factors such as OCT3/4, SOX2, KLF4 and c-MYC, as well as vector delivery methods, are also risk factors that contribute to the possible adverse effects of potential hiPSC therapy. Of all the possibilities to deliver Yamanaka factors into the patient-derived cells, Sendai viruses are the most suitable for clinical applications as they are replicated in the transduced cell cytoplasm, do not integrate into the host genome and are non-pathogenic to humans [23]. Therefore, using the Sendai virus is considered a safe way to provide reprogramming factors to cells for clinical cell therapies [11]. However, prior to clinical use, Sendai virus vectors must be completely removed from transduced cells. It is possible to do this over time during cell proliferation. Nevertheless, it is noteworthy that Sendai virus vectors are sometimes eliminated after 10 passages [29,55], while in other cases, they can persist for more than 20 passages [13]. Furthermore, the method of generating hiPSC lines and their further differentiation into the desired cell type must meet strict requirements [44] and be easily handled and reproduced in clinical settings. In addition, it is also necessary to consider the use of cell culture reagents that meet the stringent requirements for clinical application, which means the use of reagents that comply with specific manufacturing quality requirements as defined under Good Manufacturing Practices (GMP).

The aim of this work was to standardize the safety procedure of the derivation of GMP-compliant iPSC lines from human fibroblasts in terms of viral depletion. Fibroblasts isolated from both sex donors were reprogrammed using the commercially available kit based on the Sendai virus delivery of Yamanaka factors. A constant temperature was maintained during the cell culture process, including the stage where the Sendai virus was removed from the cell. The pluripotent-positive status of hiPSC lines was confirmed for all selected colonies.

Material and methods

Isolation of fibroblasts

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethical Committee (63/PB/2013). The method to isolate fibroblasts from skin biopsy was performed based on a previously established protocol by Wiley *et al*. [50]. Skin fragments were harvested from healthy obese donors (female 47 years old and male 57 years old) during abdominal plastic surgery using a scalpel. A fragment of the skin from the abdomen was washed in DPBS with 1% antibiotic-antimycotic solution (Gibco). The fat tissue was removed and the skin fragment was cut into ~1 mm × 1 mm pieces. These fragments were transferred to a 6-well plate and left for 5 minutes for the edges to dry. Subsequently, UIX medium [MEM-α (Gibco), 10% KnockOut Serum Replacement XenoFree (Gibco), 20% human platelet lysate (Macopharma), 1 : 100 GlutaMax supplement (Gibco), 1 : 100 Antibiotic-Antimycotic solution (Gibco), 2 IU/ml of heparin (Sigma-Aldrich)] was added drop-by-drop on the skin pieces. The plate was then incubated overnight at 37°C, 21% O_2 and 5% CO_2. Fresh medium was added on the next day. The medium was changed every other day with UIX medium supplemented with 10% human platelet lysate. Fibroblasts were found to be migrating out of the tissue in the following week. The cells were passaged with TrypLe™ Select (Gibco) when they reached 80% confluency and transferred to a new plate containing UIX medium supplemented with 10% human platelet lysate at a density of 26000 cells/cm^2. The fresh medium was changed every 3 days.

Generation of hiPSCs

Fibroblasts were seeded at a density of 75000 cells/well in a 6-well plate in UIX medium (day 0). On the next day, UIX medium with 10% human platelet lysate was changed to a serum-free (SF) UIX medium consisting of MEM-α (Gibco), 10% KnockOut Serum Replacement XenoFree (Gibco), 1 : 100 GlutaMax supplement (Gibco). After 2 days of cell culture, the cells were transduced by the human KLF4 (MOI = 3), human c-MYC (MOI = 5) and KOS (MOI = 5, mix of three human factors: KLF4, OCT3/4 and SOX2) according to the manufacturer's instructions [CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Invitrogen)] in a total volume of 1 ml of SF UIX medium. The SF UIX medium was changed every other day for the next six days. On day 7, cells were detached using TrypLe™ and 25000 cells were transferred to the well of a 6-well plate in SF UIX supplemented with the 10 μM ROCK inhibitor Y-27632 (Selleckchem). On the next day, the medium was changed to a 1 : 1 composition of SF UIX and Essential 8 (E8, Gibco) with 10 ng/ml of bFGF (basic fibroblast growth factor; Gibco). From the following day, the E8 medium with bFGF was changed daily. The colonies were manually transferred to separate wells of a vitronectin-coated 12-well plate in E8 supplemented with bFGF and Y-27632 when they reached a diameter of around 1-2 mm. The cells were expanded to over 15 passages to establish the hiPSC phenotype. The hiPSC lines with the best morphology were selected for further analysis. Whenever the cells reached 80% confluency, they were detached using 0.5 mM

EDTA (Gibco) and split in a ratio varying between 1 : 6 and 1 : 24.

Alkaline phosphatase activity

Alkaline phosphatase activity was evaluated using the Alkaline Phosphatase Live Stain Kit (Invitrogen). Human iPSCs were seeded in the vitronectin-coated 24-well plate. When the cells reached ~60% confluency, they were washed twice with PBS and the AP Live Stain working solution (AP Live Stain 500X stock diluted with DMEM 1 : 500) was added. After 30 minutes of incubation at 37°C, the cells were washed twice with FluoroBrite DMEM (Gibco) and images were acquired using the Axio Vert.A1 fluorescent microscope (Zeiss).

Immunocytochemistry

Cells were seeded in a 24-well plate on vitronectin-coated glass coverslips. Human iPSCs at ~60% confluency were fixed with 4% paraformaldehyde (Sigma Aldrich) for 15 minutes and washed with PBS. The cell membrane was permeabilized with 0.2% Triton X-100 (Sigma Aldrich) for 10 minutes, followed by blocking for 1 hour at room temperature using 10% normal goat serum (Sigma Aldrich). Primary antibodies targeting Ki67 (1 : 1000, Millipore), OCT3/4 (1 : 100, Santa Cruz), SOX2 (1 : 100, Invitrogen), NANOG (1 : 100, Cell Signaling Technology), hemagglutinin-neuraminidase (HN) of the Sendai virus (1 : 1000, Invitrogen), SSEA4 (1 : 200, Millipore), and CD73 (1 : 200, Santa Cruz) were added. After staying overnight at 4°C, cells were washed three times with PBS, and secondary antibodies were added and incubated for 1 hour at room temperature. The nuclei were stained with 5 µM Hoechst 33258 for 15 minutes at room temperature. After being rinsed with PBS, the coverslips were transferred to the glass slides with droplets of Fluorescent Mounting Medium (Dako) to seal the slides. Images were acquired using the LSM780 confocal system (Zeiss). All visualizations were performed in the Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Institute, Polish Academy of Sciences.

Real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the cells using the Total RNA Mini Kit (A&A Biotechnology) and digested with DNAase to remove genomic DNA contamination according to the manufacturer's instructions. The concentration and quality of the RNA were assessed spectrophotometrically [NanoDrop 2000 (Thermo Scientific)]. The RNA was subsequently reverse-transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's protocols. The primers for the RT-qPCR reaction are listed in Table I. The reaction was prepared by mixing 1 µl of cDNA, 4 µl of primer mix, and 5 µl of SYBR Green (Invitrogen). The plate with samples was sealed and centrifuged at 2000 rpm (~900 g) for 2 minutes. The reaction was carried out by preincubation: 1 cycle at 95°C for 5 minutes; amplification: 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s using the LightCycler® 480 System (Roche Diagnostics). The reaction was completed with a melting curve: 1 cycle of 95°C for 5 s, 65°C for 1 minute, and 97°C.

The expression of transgenes and Sendai virus vectors was analysed using predesigned TaqMan probes according to a standard RT-qPCR protocol. TaqMan qPCR was performed on 20 ng reverse-transcribed RNA in a final volume of 10 µl using the LightCycler® 480 System (Roche Diagnostics) following the manufacturer's protocol. The following predesigned TaqMan gene expression probes were used: SeV (Mr04269880_m1), KOS (Mr04421257_m1), Klf4 (Mr04421256_m1) and cMyc (Mr04269876_m1). RPLP0 (Hs00420895_gH) was chosen as the housekeeping gene.

The results were calculated as the fold change in expression compared to that in the positive control using the Livak $2^{-\Delta\Delta Ct}$ method of relative quantification [25].

Table I. Primers used in RT-qPCR

Gene	Primer	Sequence	NCBI reference	Gene ID
NANOG	Forward	F: 5'GAACCTCAGCTACAAACAGG3'	NM_024865.4	79923
	Reverse	R: 5'CGTCACACCATTGCTATTCT3'		
OCT3/4	Forward	F: 5'CAAGAACATGTGTAAGCTGC3'	NM_001159542.3	5462
	Reverse	R: 5'GGGTTTCTGCTTTGCATATC3'		
RPLP0	Forward	F: 5'AAAATCTCCAGGGGCACCATT3'	NM_001002.4	6175
	Reverse	R: 5'GCTCCCACTTTGTCTCCAGT3'		
SOX2	Forward	F: 5'GCGCCGAGTGGAAACTTTTG3'	NM_003106.4	6657
	Reverse	R: 5'CATGAGCGTCTTGGTTTTCCG3'		

Differentiation into the three germ layers

The STEMdiff Trilineage Differentiation Kit (STEMCELL Technologies) was used for differentiation into the three germ layers. The iPSCs dispersed into single cells were transferred to the 24-well plate with Cultrex® coated glass slips (Cultrex® Stem Cell Qualified Reduced Growth Factor Basement Membrane Extract, Biotechne) in E8 medium with 10 µM Y-27632. For mesoderm, cells were seeded at a density of 1×10^5 cells/well and the Mesoderm Medium was changed daily starting on the next day until Day 5, when they were fixed with 4% paraformaldehyde. The density for endoderm was 4×10^5 cells/well and the Endoderm Medium was changed daily until Day 5. iPSCs for ectoderm were seeded at 4×10^5 cells/well, kept in the Ectoderm Medium, and fixed on Day 7.

The ability to differentiate into the three germ layers was confirmed by immunocytochemistry using the Human Three Germ Layer 3-Color Immunocytochemistry Kit (R&D Systems). Fixated cells were permeabilized with 0.2% Triton-X for 10 minutes and blocked with 10% donkey serum for 1 h. Subsequently, conjugated antibodies targeting endoderm markers GATA4 and SOX17, mesoderm markers Brachyury and HAND1, and ectoderm markers SOX1 and OTX2 were diluted (1 : 50) and added to coverslips with cells. After 4 h of incubation at room temperature, the samples were rinsed three times with PBS and nuclei were stained with Hoechst. Slides were sealed using Fluorescent Mounting Medium (Dako). The slides were imaged on the LSM780 confocal system.

Genetic screening of the most common karyotypic abnormalities reported in hiPSCs

A quantitative PCR (qPCR)-based screen (STEMCELL Technologies) using double-quenched probes with a 5-carboxyfluorescein (5-FAM) dye was implemented to detect the critical minimal regions of the eight most commonly mutated regions in human induced pluripotent stem cells in 10% or more of all cells in the hiPSC populations. The amount of a target locus was compared to a reference locus on chromosome 4 within the same sample with a known diploid karyotype. A copy number was calculated using the $(2^{-\Delta\Delta Ct})x2$ method. A reference gene was on chromosome 4. A copy number > 2.2 indicated the probability of the presence of an abnormality within the culture.

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism 9.0.0 software. The Kolmogorov-Smirnov test was used as the normality assay. The statistical significance between examined hiPSC lines was obtained using a one-way ANOVA with Tukey's post hoc test. Results shown in the figures were obtained as mean with (±SD). *P*-values lower than 0.05 were considered statistically significant.

Results

Generation of hiPSC lines

Fibroblasts isolated from female (47 years old) and male (57 years old) donors were reprogrammed to generate hiPSCs using the Sendai virus carrying Yamanaka factors (Fig. 1A). All colonies were selected by manual transfer and cultivated. Three colonies with the hiPSC-specific morphology generated from human adult fibroblasts were chosen for further proceeding. The commercial hiPSC line generated from $CD34^+$ mononuclear cells was also used as a positive control in further analysis as the company can offer only phenotypically unaltered products. This was useful additional support for us during an assessment of cell culture quality.

During the reprogramming, the cells lost fibroblast-specific morphology and started forming colonies with the hiPSC-specific morphology, reaching 1-2 mm in diameter after around 30 days (Fig. 1B). Individual colonies with the hiPSC-specific morphology were manually picked up, transferred onto the 12-well plate for expansion, and live-stained for alkaline phosphatase (ALP) on day 30 after reprogramming to confirm the pluripotency of hiPSCs. All iPSC lines were ALP-positive (Fig. 1C).

The iPSC lines chosen for further analysis were morphologically indistinguishable from the iPSC line generated from the commercial human $CD34^+$ mononuclear cells (at day 30 after reprogramming) and consisted of well-defined, smooth edges, and prominent nuclei in square-shaped, tightly packed cells, which were maintained in time (Fig. 2).

Confirmation of the pluripotent phenotype

Further identification of the pluripotent phenotype was performed using immunocytochemical (ICC) and RT-PCR analyses to validate generated cell lines having characteristic features of hiPSCs (Fig. 3). All generated hiPSC lines were tested at the same passage 17.

The presence of OCT3/4, SOX2, and NANOG was confirmed by ICC (Fig. 3A) in all tested iPSC lines.

The relative gene expression analysis of pluripotency markers *OCT3/4* (officially known as *POU5F1*), *SOX2*, *NANOG* in six hiPSC lines generated from female and male donors (donor A, donor B, respectively) showed significant upregulation in *OCT3/4* expression in two inde-

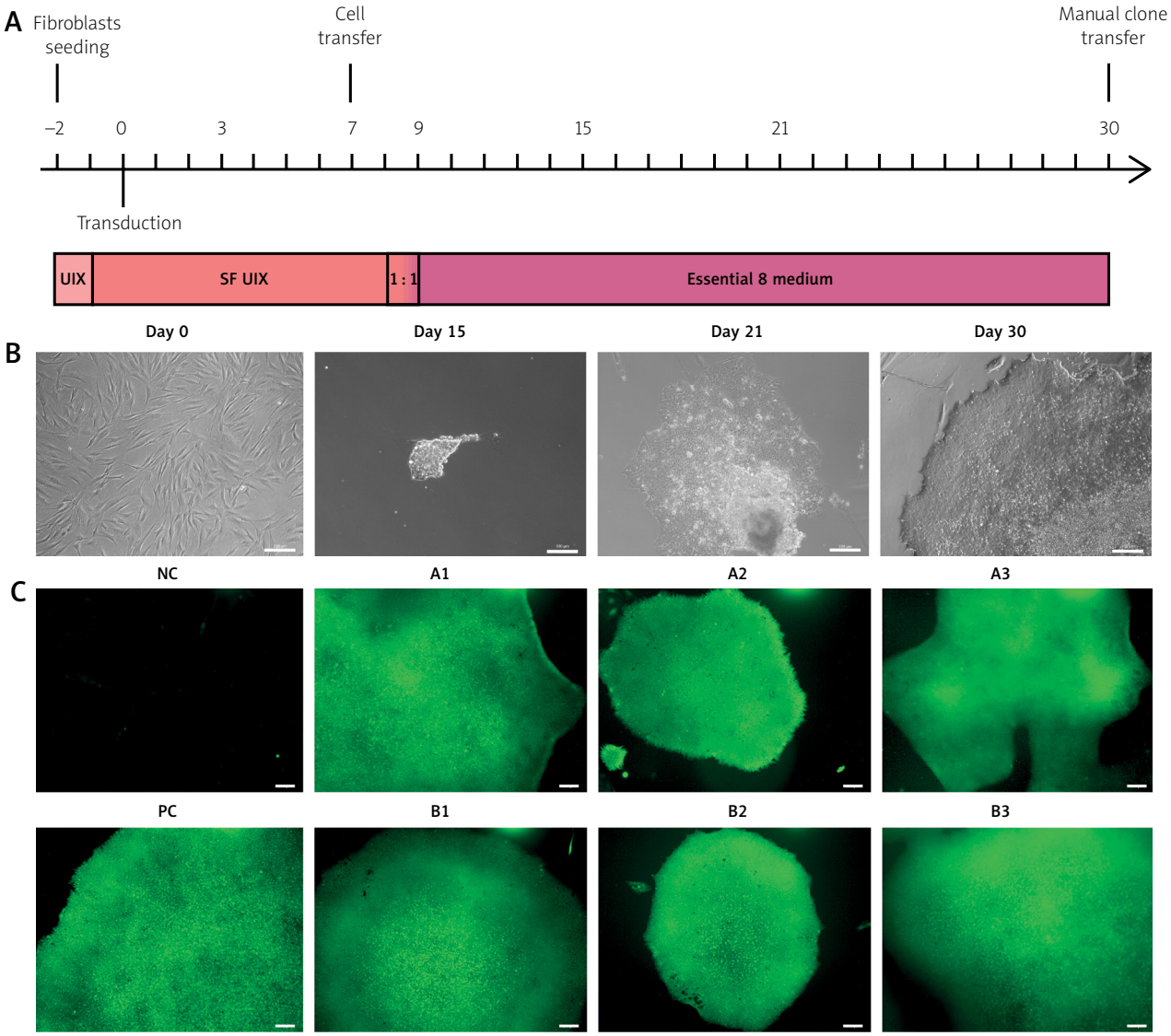


Fig. 1. A) Schematic representation of the protocol for the generation of human induced pluripotent stem cells from fibroblasts. **B**) Changes in a colony morphology throughout the reprogramming process. Scale bar: 100 µm. **C**) Live staining of alkaline phosphatase activity in 6 independent hiPSC lines generated from female fibroblasts (3 hiPSC lines: A1, A2, A3) and male fibroblasts (3 hiPSC lines: B1, B2, B3) compared to the control hiPSC line (PC). The human fibroblast line served as the negative control (NC). Scale bar: 100 µm.

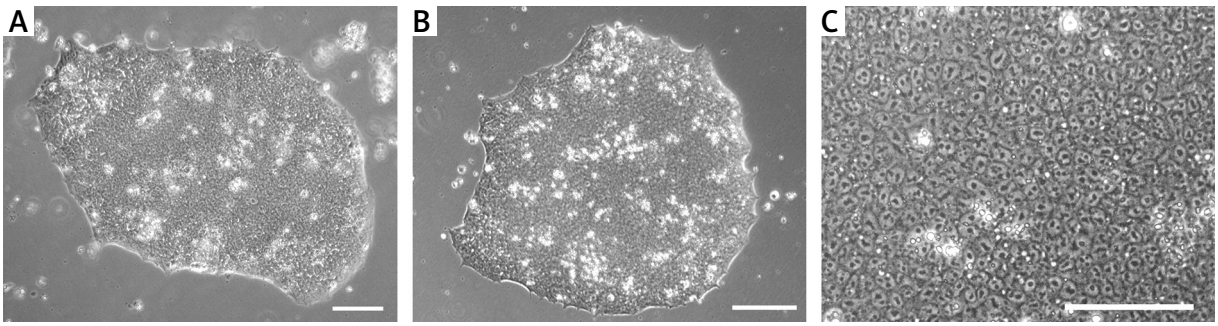


Fig. 2. Representative cell morphology of the control hiPSC line (**A**) and the hiPSC line B3 of the whole colony with defined, smooth edges at passage 1 (**B**). **C**) The morphology of the iPSC line (B3) with tightly packed, square-shaped cells with a high nuclei/cytoplasm ratio at passage 20. Scale bar: 100 µm.

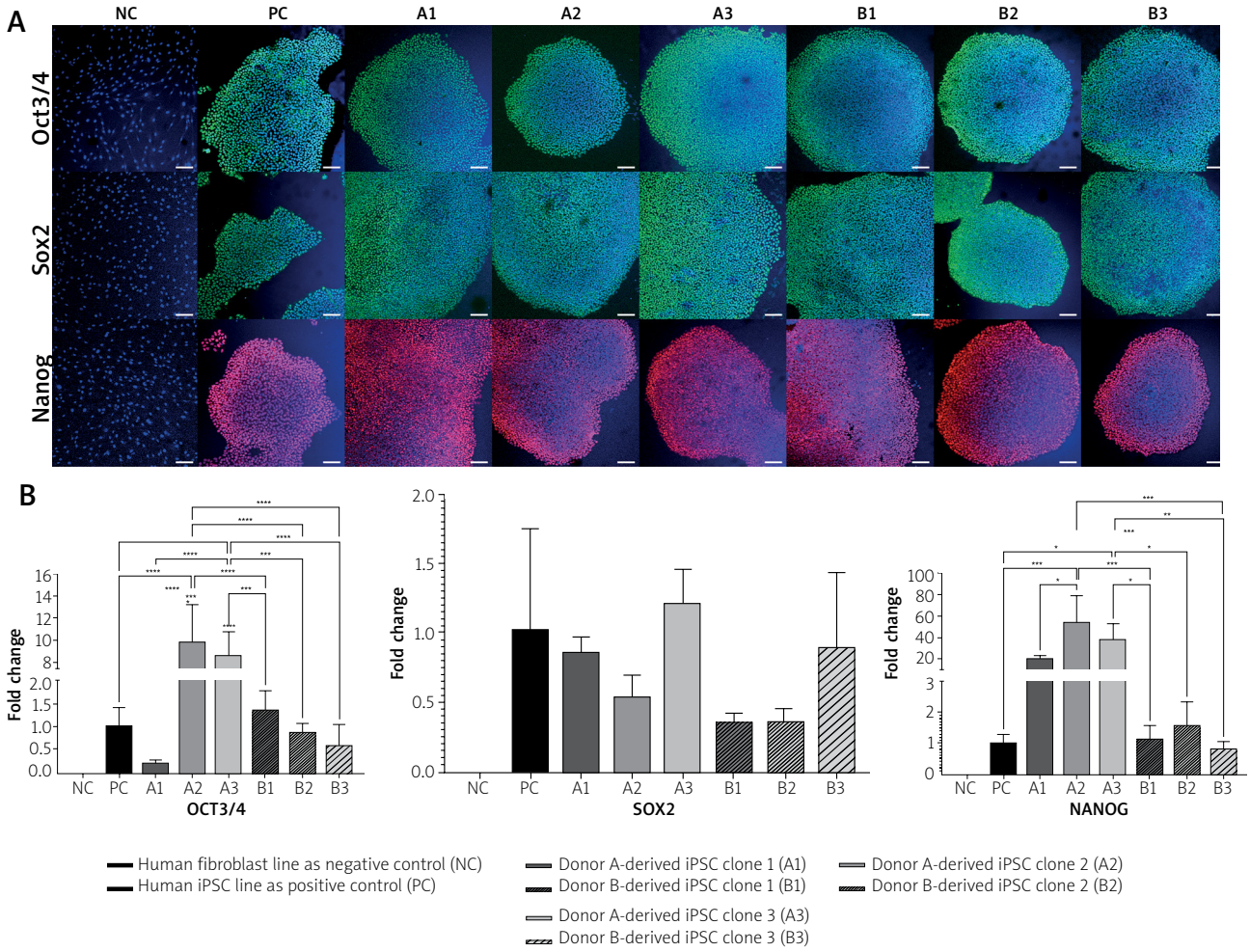


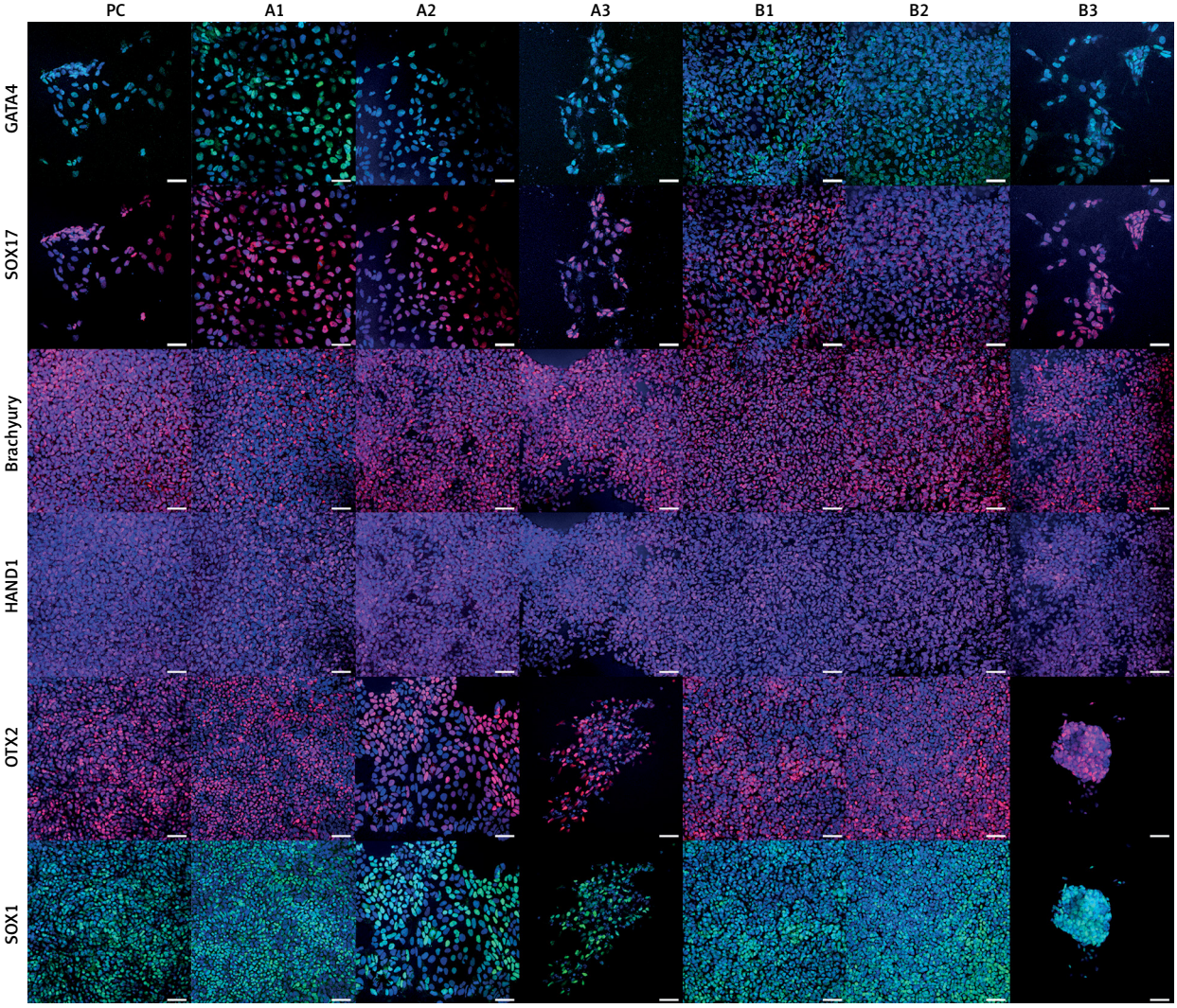
Fig. 3. Confirmation of the pluripotent phenotype of hiPSC lines generated from donor A (female) and donor B (male). **A**) Representative confocal fluorescence microscopic images of hiPSC lines independently generated from each donor showing positive OCT3/4, SOX2, and NANOG staining. Cell nuclei were counterstained with Hoechst 33258 (blue). Scale bar: 100 µm. **B**) Relative gene expression analysis of *OCT3/4* (*POU5F1*), *SOX2*, *NANOG* in six hiPSC lines independently generated from female and male donors (donor A, donor B, respectively) and in human fibroblast line as negative control (NC) compared to control hiPSC line (PC) calculated by 2–ΔΔCt method. Error bars represent mean ±SD. Asterisks show statistical significance between examined cell lines (one-way ANOVA, Tukey's post-test): *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$.

pendent hiPSC lines generated from the female donor. One hiPSC line generated from a female donor and three hiPSC lines independently generated from a male donor showed no upregulation in *OCT3/4* expression. In all independently generated hiPSC lines tested, no upregulation of *SOX2* expression was observed compared to the positive control line. Furthermore, *NANOG* expression in three hiPSC lines independently generated from a female was upregulated compared to three hiPSC lines independently generated from a male and positive control iPSC line (Fig. 3B).

To confirm whether selected hiPSC cells can differentiate into three germ layers, the expression of markers characteristic for endoderm (GATA4 and SOX17), mesoderm (Brachyury and HAND1), and ectoderm (OTX2 and SOX1) was verified in all tested iPSC lines (Fig. 4).

Verification of transgene-free human induced pluripotent stem cells lines

To confirm the generation of transgene-free hiPSC lines obtained as a result of the reprogramming of adult fibroblasts, the analysis of Sendai virus expression and reprogramming vectors was conducted in six independent hiPSC lines by immunocytochemistry (ICC) and gene expression analysis (Fig. 5).



Scale bar: 100 µm.

Fig. 4. Confirmation of differentiation of human induced pluripotent stem cells (hiPSC) lines into three germ layers by immunocytochemistry. Immunofluorescence staining for SOX17 and GATA4 (endoderm), Brachyury and HAND1 (mesoderm), OTX2 and SOX1 (ectoderm) was performed on cells from six independent iPSC lines generated from human adult fibroblasts and compared to positive control iPSC line. Representative images present differentiated cultures performed on three iPSC lines independently generated from a female donor (A1, A2, A3) and three iPSC lines independently generated from a male donor (B1, B2, B3), as well from the control hiPSC line (PC). Cell nuclei were counterstained with Hoechst 33258 (blue).

ICC labelling showed that viral hemagglutinin-neuraminidase (HN), an envelope glycoprotein of Sendai virus that mediates the attachment of virions to host cell surface was present only in two hiPSC lines out of six (hiPSC A1 and hiPSC A3, Fig. 5A).

The TaqMan gene expression analysis of the Sendai virus and reprogramming vectors confirmed that two independent hiPSC lines from the female donor (A1 and A3) and one hiPSC lines from the male donor B (B1) were positive for the SeV and c-Myc vectors at passage 17 (Fig. 5B). The SeV and c-MYC vectors were not detected in one hiPSC line from the female donor (A2) and two iPSC lines from the male donor (B2 and B3) at passage 17 (Fig. 5B). The KOS vector was noted only in two iPSC lines from the female donor (A1 and A3) and not detected in all iPSC lines generated from the male donor at the 17th passage (Fig. 5B). All iPSC lines generated from adult fibroblast were negative for the KLF4 vector at passage 17 (data not shown). The following passages were continued to eliminate the remaining

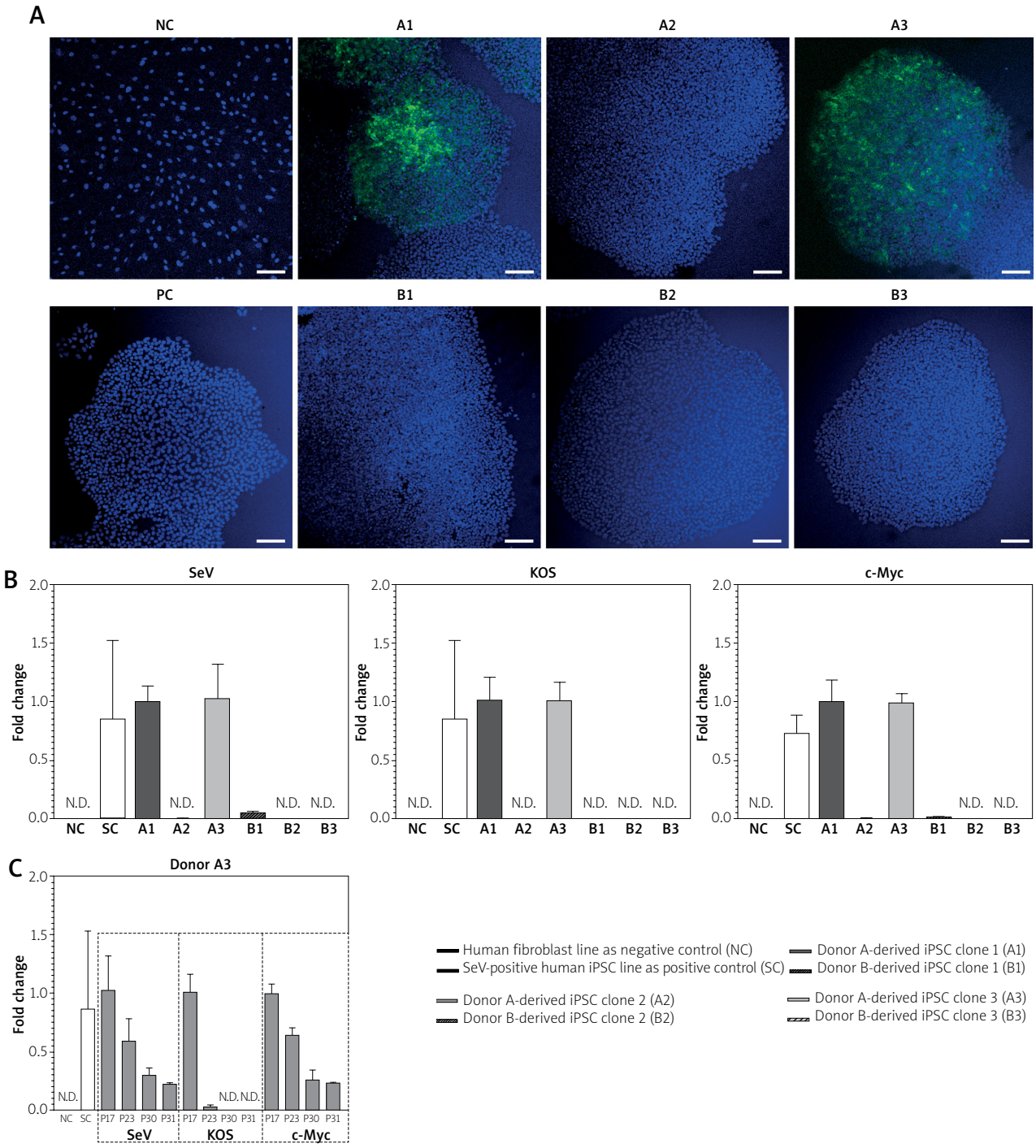


Fig. 5. A) Analysis of the presence of Sendai virus hemagglutinin-neuraminidase (HN, green) in the independent human induced pluripotent stem cells (hiPSC) lines generated from a female donor (A1, A2, A3), a male donor (B1, B2, B3) and control hiPSC line (PC) performed at passage 17. Cell nuclei are counterstained with Hoechst 33258 (blue). Fibroblast served as the negative control (NC). Scale bar: 100 µm. **B**) The TaqMan gene expression analysis of Sendai virus vector (*SeV*) and reprogramming vectors (*C-MYC* and *KOS*) in the independent hiPSC lines generated from a female donor (A1, A2, A3), a male donor (B1, B2, B3) at passage 17 and compared to the positive control hiPSC line (SC) obtained 30 days after Sendai virus transduction. The human fibroblast line before transduction was the negative control (NC). All data were calculated by the $2^{-\Delta\Delta Ct}$ method. The bars represent mean ± SD. **C**) The level of reprogramming factors after transduction with Sendai virus vectors during the following passage number of representative hiPSCs compared to positive control hiPSC line (SC) obtained 30 days after Sendai virus transduction. *P* means passage numbers. N.D. means not detected.

Sendai virus vectors if the hiPSC lines were still positive for the Sendai virus and reprogramming vectors after expansion for 17 passages. This allowed us to observe a gradual decline in the number of virus vectors in the next passages (Fig. 5C).

Probability of chromosomal aberrations

Events occurring before and during reprogramming and cell culturing may lead to different aberrations in hiPS cells. These aberrations can include chromosomal abnormalities, genetic mutations, changes in gene expression patterns or variations in cell line properties, which can affect the hiPS cells' functionality and stability. Therefore, the probability of chromosomal aberration was assessed in all selected hiPSC lines and compared to the commercial hiPSC line (Fig. 6).

For our analysis of genomic stability, we concentrated on iPSC lines from passages 17 to 31, at the conclusion of experiments, which deemed an optimal time window for such evaluation. This approach also aided in more effective capturing of sex differences.

The probability of appearance of chromosomally abnormal cells in the hiPSCs was iPSC line-dependent and indicated as a value > 2.2 (Fig. 6). Additionally, the hiPSC lines generated from female fibroblasts (A1, A2, A3) had a higher probability of chromosomal aberrations than the hiPSC lines generated from male fibroblasts (B1, B2, B3). Two karyotypically abnormal hiPSC lines from the female donor (A2, A3) were found to carry the chromosomal aberration associated with chromosome 8 that slightly exceeds the limits of the commercial iPSC line. All hiPSC lines generated from female fibroblasts (A1, A2, A3) and a commercial human female iPSC line (PC) showed the probability of chromosomal aberrations associated with chromosomes 12 and 20. The highest probability of chromosomal aberration was associated with chromosome 1 and was found in almost all hiPSC lines, including commercial hiPSCs.

Discussion

In the present study, using a simple protocol, we successfully obtained six independently generated human induced pluripotent stem cells lines from adult female (47 years old) and male (57 years old) donor-derived fibroblasts under GMP-compliant conditions that are free of animal-derived products. The reprogram-

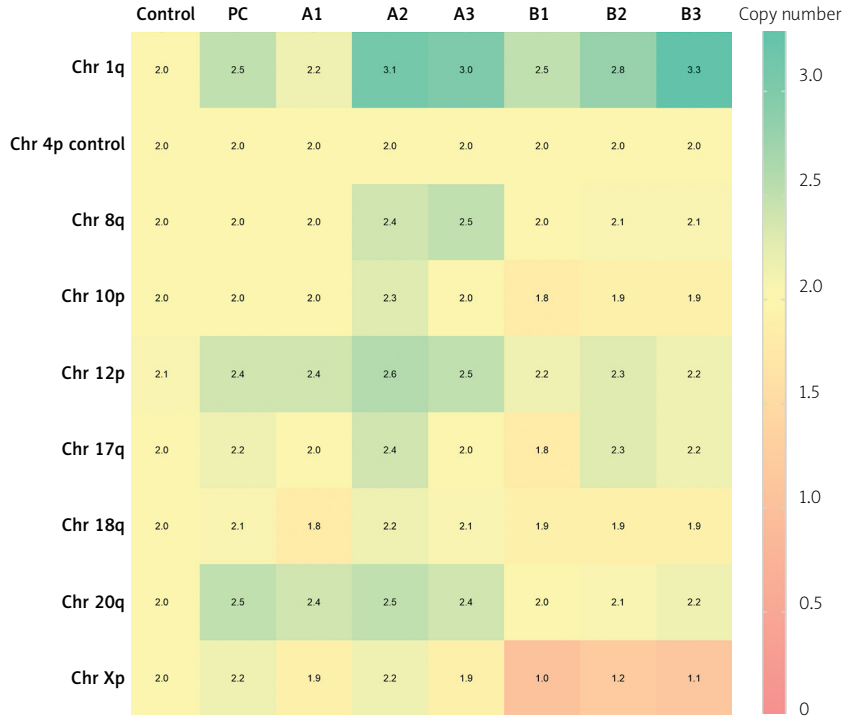


Fig. 6. Analysis of genomic stability in six independent human induced pluripotent stem cells (hiPSC) lines generated from adult fibroblasts: a female donor (A1, A2, A3) at passages from 17 to 23 and a male donor (B1, B2, B3) at passages from 26 to 31. The commercial control hiPSC line (PC) was generated from $CD34^+$ mononuclear female cells. A copy number was calculated using the $(2^{-\Delta\Delta Ct})x2$ method. A reference gene was on chromosome 4. A copy number > 2.2 (green) indicates the presence of an abnormality within the cell culture.

ming was performed employing a consistent protocol in the same laboratory. A constant temperature was maintained during the cell culture process, including the stage where the Sendai virus was spontaneously eliminated from the cell. All hiPSC lines acquired a pluripotent phenotype and revealed a proper morphology. It was shown that the elimination of the Sendai virus from the obtained cell line depended on the particular iPSC colonies rather than the donor. The hiPSC lines exhibited a progressive reduction of the virus presence with each successive passage. The frequency and probability of chromosomal aberrations in hiPSCs were dependent on both the iPSC line derived from the specific clone and the sex of the donor that the hiPSCs were generated; additionally it was higher in a woman. These conclusions were compared with existing literature data for both sexes, revealing a consistent trend [8,12,16-18,22,26,30-33,38,57].

The pluripotency of generated cells was primarily confirmed by the higher activity of alkaline phosphatase compared to fibroblasts. It is known that the high activity of alkaline phosphatase is considered a marker

Table II. Human induced pluripotent stem cells (hiPSCs) predisposition to karyotypic instability relative to hiPSCs at different passages

Stem cell line identifier	Parental somatic cells (healthy donors)	Gender/age at sampling	Normal karyotype	Abnormal karyotype passage number/ chromosome number	References
iPSCs	Human NSCs	Male (foetal)	For more than 30 passages	N/A	[30]
MRC5-iPS12	Human lung fibroblasts	Male (foetal)	At passage 19	N/A	[31,38]
hiPSC18	Human dermal fibroblasts	Male (neonatal)	At passage 48	Passage 58/trisomy 12	[8,26,33]
hiPSC1-8	Human dermal fibroblasts	Male (neonatal)	Up to 13 passage	Passage 14/trisomy12	[33,32]
iPS (foreskin)	Foreskin fibroblasts	Male (neonatal)	At passage 25	N/A	[31,57]
BJ1-iPS12	BJ1foreskin fibroblasts	Male (neonatal)	At passage 5	N/A	[38]
hFIB2-iPS4	Human dermal fibroblasts	Male (adult)	At passage 5	N/A	[38]
Tuba1-GFP iPSC line	Peripheral blood mononuclear cell	Male (34 years)	Up to 31 passage	Passage 32/trisomy12	[12]
KUMi003-A	APL-M3 patient bone marrow CD34+ cells	Male (55 years)	At passage 13	N/A	[16,17]
HDF51	Human dermal fibroblasts	Female (foetal)	Up to 5-8 passages	Passage 5-8/trisomy 12 passage 25-34/ trisomy 20	[22]
iPS IMR90	Human lung fibroblasts	Female (foetal)	At passage 5 (iPS IMR90-YZ1) At passage 24 (iPS IMR90-TZ1) At passage 28 (iPS IMR90-3,4) At passage 29 (iPS IMR90-2) At passage 35 (iPS IMR90-1)	Passage 24/trisomy12 (iPS IMR90-YZ1)	[31,57]
p-hiPS01 and p-hiPS02	The somatic cells (human newborn fibroblasts)	Female (neonatal)	N/A	Passage 0/trisomy1+9	[18]
hiPSC lines generated from HDFa	Human dermal fibroblasts	Female (36 years)	At early (passages 4-8 in eight hiPSC lines), middle (passages 10-20 in four hiPSC lines) and late (passage 34 and 64 in two hiPSC lines respectively) passages	Passage 5/inv(8) (q24.1q13), t(18;20)(q21.3;q11.2) in (HDFa-YK22 line) Passage31/t(6;16) (q15;q13), t(13;14)(q14.1;q24.3) in (HDFa-TK2)	[12]

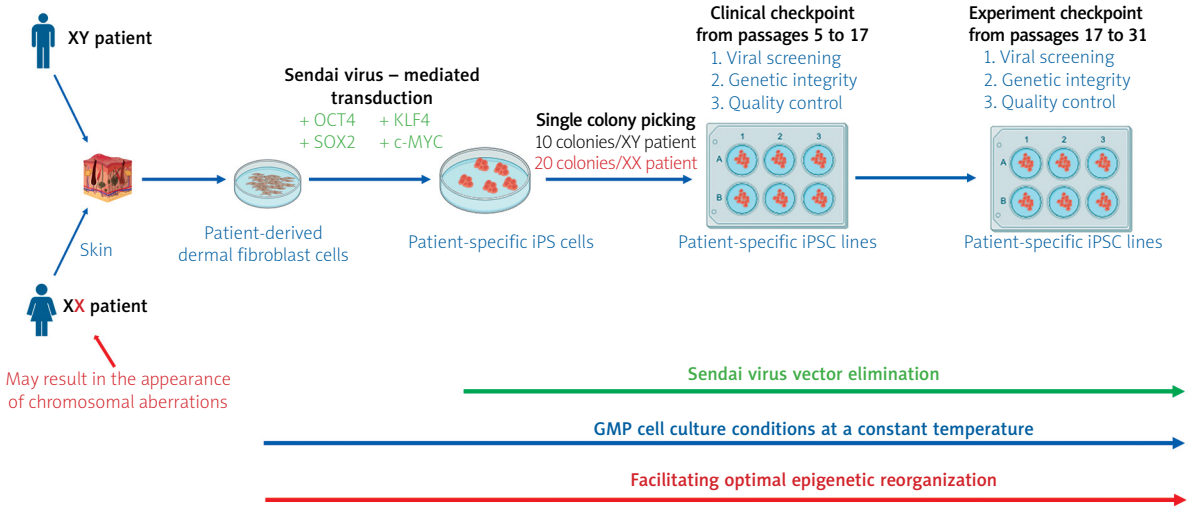


Fig. 7. The critical points in sex-specific human induced pluripotent stem cells (hiPSC) lines.

of pluripotent stem cells [45]. Subsequently, immunocytochemistry revealed the presence of pluripotent markers OCT3/4, SOX2, and NANOG at the protein level, whereas analysis at the gene expression level allowed us to capture statistically significant differences in the *OCT3/4* and *NANOG* expression between both sexes and hiPSC lines generated from the same donor. Similar relationships have been observed in another study [14]. Nevertheless, the differentiation potential of the hiPSCs depended on sex of the donor rather than clonal variation from the same donor [14,52]. Interestingly, it was shown that the single-colony picking method of hiPSC isolation might not be necessary to obtain a homogenous population of these cells, as no significant differences in gene expression and differentiation potential were detected between lines obtained with such method and generated in bulk culture [51]. However, we observed the differences in pluripotent gene expression between hiPSC lines independently generated from the same donor by single-colony picking of individual colonies with hiPSC-specific morphology. It is not surprising because reprogramming is associated with the removal of epigenetic signatures acquired during development or imposed by the environment. Moreover, it is possible that DNA methylations and histone modifications occur *de novo* even though the epigenetic memory inherited from the parent cells is lost [24,35]. Therefore, the different iPSC lines generated from the same fibroblast culture may differ in epigenome reorganization that contributes to altering gene expression without modifying the genomic DNA sequence.

Notably, it has been shown that the variation in hiPSC lines independently generated from adult fibroblasts manifests itself in the efficiency of differentiation [21,56]. It is known that overexpression of SOX2 and OCT3/4 is required to successfully generate hiPSC from somatic cells [47], and NANOG, although not necessary to trigger reprogramming, is compulsory to establish the pluripotent state [41]. Moreover, SOX2 and OCT3/4 are considered the gatekeepers of the pluripotent state and they are regulated by a positive-feedback loop [48,37]. Furthermore, they are engaged in cell fate commitment. It was observed that the increase and decrease in OCT3/4 expression differently affect the commitment of embryonic stem cells (ESCs) and prompt differentiation into germ layers [36,58]. Similarly, a slight increase in the expression level of SOX2 resulted in the differentiation of ESCs in ectoderm and mesoderm [20]. Moreover, maintaining the expression of OCT3/4 and SOX2 in the narrow interval is crucial to keep the self-renewal of ESCs [20], while the expression of NANOG has to be kept above some threshold [7,34,42]. The decrease in the NANOG expression level resulted in the commitment of ESC to endoderm lineage [34,42]. All these associations suggest that differences in the gene expression of *OCT3/4*, *SOX2*, and *NANOG* between iPSC lines could affect their self-renewal stability and differentiation ability into endoderm, mesoderm, and ectoderm. However, in our study, all iPSC lines were able to differentiate into the three germ layers as it is shown in Fig. 3, despite differences in *SOX2*, *OCT3/4*, and *NANOG* expression levels between iPSC lines. The capacity to eliminate reprogramming vectors within generated hiPSC lines is one of the main criteria used to evaluate hiPSCs' safety. Therefore, testing for the presence of the reprogramming vectors is considered mandatory during the generation of clinical-grade hiPSC lines [44]. As Sendai viruses replicate only in the

cytoplasm and do not integrate into the host genome [23], they have been used in clinical trials (e.g. https://clinicaltrials.gov/ct2/show/NCT00186927). Therefore, the non-integrative, DNA-free method of Yamanaka factors delivery using the Sendai virus, with an RNA-based reproductive cycle, was the most attractive method of fibroblast reprogramming to pluripotency in our study. However, steps in the protocol to erase the replicating virus from the reprogrammed cells are required. Especially given that the elimination rate of Sendai virus vectors after transduction depends on the cell line origin. In certain cases, transgenes can remain detectable for more than 20 passages, as seen in the 4BJ1 line generated from neonatal foreskin or even longer in cases like the HDF-iPSC line generated from the facial dermis of a 36-year-old female [13].

A negative selection with antibodies allows the separation of cells with the virus due to the heterologous expression of Sendai hemagglutinin-neuraminidase (HN) in colonies [13]. While the negative selection may decrease the proportion of HN-positive cells, it does not guarantee the complete removal of cells containing viral plasmids, as demonstrated in our study.

Another method of removing Sendai virus vectors is to increase the temperature. The involvement of pluripotency factors NANOG, OCT4, and KLF4 in the transcriptional response to the increased temperature was demonstrated in hESCs exposed to 43°C for 60 minutes [28]. Thus, the cellular reprogramming process is temperature sensitive and the increase in temperature affects chromatin remodelling. In fact, an increase of 1°C is in the physiological range, and an increase to 38-40°C is tolerable under stress conditions [9]. Moreover, incubation of iPSC lines at 38°C for 5 days [55] effectively removes viral vectors in a few passages [2,55]. Tolerance to temperature variations is possible due to the expression of heat shock proteins (HSPs), which are stimulated in response to an elevated temperature to maintain a proper proteasome [15]. The enhanced transcription and translation of HSPs following stress, such as heat shock, can cause alterations in the epigenetic landscape and influence the cellular reprogramming process. In fact, the viability and adhesion of hiPSCs were not affected after the temperature change performed to remove Sendai virus plasmid residues [2]. However, *in vitro* studies in the human respiratory epithelial cell line, A549, showed that an increase in temperature to 38.5°C was sufficient to activate the expression of HSP72, a member of the human HSP70 family, and the DNA-binding activity of heat shock factor 1 (HSF-1) [49] that binds the heat shock elements of the *HSP2A* gene and mediates transcription *in trans*. Similarly, exposure to the 37-39°C range increased the expression of HSP70 in peripheral blood mononuclear cells (PBMC) [34]. Moreover, the studies mentioned above showed the proportional temperature-dependent induction of these HSPs [27,44]. Since HSP70 is required for the ubiquitination of histone H2A [4,53], it can also indirectly alter the phenotypic plasticity of the chromatin structure and contribute to the reprogrammed cell state. According to this, the highly expressed histone variant TH2A in oocytes is enriched on the X chromosome during reprogramming, and its overexpression in somatic cells increases the sensitivity of chromatin to DNase I and can affect the fibroblast reprogramming process to hiPSCs [40]. On the other hand, in a study by Saretzki *et al*., the expression of HSP70 was found to decrease during the differentiation of hESCs [39]. These associations confirm the important role of HSP in maintaining pluripotency and controlling the differentiation process of pluripotent cells by regulating chromatin remodelling which influences on epigenetic stability of hiPSC cells. Therefore, we did not change the temperature in our study to remove the Sendai virus from the tested hiPSC lines independently generated from female and male fibroblasts to facilitate optimal epigenomic reorganisation. Since Sendai virus replication is independent of the nuclear function of the infected cell, successive passages without increasing the temperature are sufficient to purge the reprogrammed cell of the replicating virus and eliminate the remaining genes related to viral vectors. Our data confirmed that some iPSC lines independently generated from the same fibroblast donor were more resistant to spontaneous elimination by subsequent passages, therefore needed more time to became transgene-free and Sendai virus-free cells. Thus for clinical application, careful selection of purified colonies generated from the same donor is required before further processing. Residual transgenic expressions and Sendai viruses may alter the epigenetic landscape of the hiPSC, affecting the functionality of the hiPSC derivatives. Furthermore, as shown in our study, only gene expression analysis can definitively verify the lack of transgenes and Sendai virus vectors in a reprogrammed cell.

In the following step of our study, we compared the changes in the number of copies of chromosomes to reference sites of chromosome 4 in the same sample with a known diploid karyotype. Based on the literature, copy number changes of chromosome 4 are rare in hiPSCs [1]. Intriguingly in our study, the probability of chromosomally abnormal cells in hiPSC lines increased, with the aberrations occurring faster, when the donor was a female rather than a male, suggesting that the presence of two X chromosomes carried by a female provided a greater diversity of responses against the cell culture microenvironment during reprogramming or the cell proliferation stage. This observation is correlated with strong upregulation of the *NANOG* gene and a greater probability of chromosomal aberrations

associated with chromosome 12 in our experiments in all iPSC lines independently generated from female fibroblasts suggesting that double X chromosome in iPSC lines may play a supporting role in regulating pluripotency.

This explanation is provided by evidence from studies showing that overexpression of NANOG promotes self-renewal in human ESCs without feeder cell-secreted factors [10], the involvement of various X chromosome-encoded immune proteins [6], and the particularly increased expression of many autosomal genes (21%) due to erosion of X chromosome inactivation (XCI) [5]. Furthermore, the *NANOG* gene resides on human chromosome 12 and is overexpressed upon selection for trisomy 12 in the hiPSC culture [33], while trisomy 12 in the hiPSCs increases the proliferation of hiPSC lines [3]. Unfortunately, this phenomenon of super sensitivity of the cells with two X chromosomes to the cell culture microenvironment may be a double-edged sword that both stimulates the appearance of chromosomal aberrations in the cell and sustains self-renewal and proliferation of the cells.

Finally, it should be noted that trisomy 12 is the predominant abnormality in iPSC cells whereas trisomy 8 and 20 are less common [46]. All these chromosomal aberrations we observed in our female iPSC lines generated from adult fibroblasts. Since sex-specific mechanisms follow than precede the initiation of X chromosome inactivation [43], different female hiPSC lines generated from the same fibroblast culture can differ in which of the two X chromosomes they express and it will affect the epigenome reorganization.

All the above observations are consistent with the literature on the relationship between sex and dynamic changes in the copy number of chromosomes during reprogramming and time in culture (data review in Table II). Moreover, it seems much easier to obtain genetically stable male iPSC lines than female ones (Fig. 7).

To summarize, there are numerous limitations during the generation of clinically safe hiPSC cells simultaneously in all independent iPSC lines from the same donor. Henceforth, our future *in vitro* study using hiPSCs generated from both sexes will prioritize overcoming these barriers.

Ethics

Report from the Bioethical Committee 63/PB/2013.

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Disclosure

The authors report no conflict of interest.

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